Can repeated soil amendment with biogas digestates increase soil suppressiveness toward non-specific soil-borne pathogens in agricultural lands?

Research Paper

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Abstract

Soil suppressiveness which is the natural ability of soil to support optimal plant growth and health is the resultant of multiple soil microbial components; which implies many difficulties when estimating this soil condition. Microbial benefits for plant health from repeated digestate applications were assessed in three experimental sites surrounding anaerobic biogas plants in an intensively cultivated area of northern Italy. A 2-yr trial was performed in 2017 and 2018 by performing an in-pot plant growth assay, using soil samples taken from two fields for each experimental site, of which one had been repeatedly amended with anaerobic biogas digestate and the other had not. These fields were similar in management and crop sequences (maize was the recurrent crop) for the last 10 yr. Plant growth response in the bioassay was expressed as plant biomass production, root colonization frequency by soil-borne fungi were estimated to evaluate the impact of soil-borne pathogens on plant growth, abundance of *Pseudomonas* and actinomycetes populations in rhizosphere were estimated as beneficial soil microbial indicators. Repeated soil amendment with digestate increased significantly soil capacity to support plant biomass production as compared to unamended control in both the years. Findings supported evidence that this increase was principally attributable to a higher natural ability of digestate-amended soils to reduce root infection by saprophytic soil-borne pathogens whose inoculum was increased by the recurrent maize cultivation. *Pseudomonas* and actinomycetes were always more abundant in digestate-amended soils suggesting that both these large bacterial groups were involved in the increase of their natural capacity to control soil-borne pathogens (soil suppressiveness).

Introduction

In response to The European Renewable Energy Directive of 2009 (2009/28/EC) along with the Italian public policy to support biogas, the number of production plants has led to an increase of more than 200 anaerobic digestion plants since 2002, with a logarithmic increase from 2008 to 2013 reaching a total of 1300 plants. In 2015, the total capacity of these plants has been about 2 billion m^3 of methane per year (Carrosio, 2011; Molinari and Donati, 2015). This growth trend is expected to remain constant until 2030 when about 8.5 billion m^3 of methane per year is expected to be produced (Consorzio Italiano Biogas, 2017). Most plants for anaerobic biogas production are associated with intensive agricultural and livestock activity in northern Italy. They represented a solution to the limits imposed by the European Nitrate Directive (91/676/CEE), and further EU updates aiming at protecting water from nitrate contamination through intensive use of fertilizers and intensive livestock farming (Monteny, 2001; Musacchio *et al.*, 2020). With the aim of curbing nitrate pollution, some areas within the regions were classified as nitrate vulnerable zone either because they belonged to the watersheds of river basins or due to their inherently drainable soils which make them prone to nitrate leaching on underlying water tables (Monteny, 2001).

The fast increase of anaerobic digestion plants for biogas production in Northern Italy provided an opportunity for sustainable disposal of manure and drastic reduction of the great environmental risk deriving from that high nitrogen surplus (Carrosio, 2011). In the meantime, digestates from anaerobic digestion biogas plants associated with agricultural activities, which currently cover 75% of the total national biogas production in Italy, were used as organic amendment and fertilizer in cropland surrounding biogas production in compliance with the limits imposed by the European Nitrate Directive. For years, silage maize (*Zea mays* L.), livestock manure and energy crops have been the predominant feedstock for biogas anaerobic digesters. As a consequence, maize has been widely cultivated in the Po Valley (northern Italy) until 2018. More recently, in response to the European Renewable Energy Directives and the EU Directive of 2018 (2018/2001) on the promotion of use of energy from renewable

sources, Italy promoted a reduction of the use of silage maize as feedstocks for anaerobic digestion by incentivizing the use of crop residues, biomass from cover crops and food-processing waste. Owing to the widespread diffusion of anaerobic digestion plants in the Po Valley, there is significant scope for investigating the effects of digestate on maize, which is the crop predominantly cultivated in these areas.

Although digestates from anaerobic digestion have several known agricultural benefits such as soil amendment for high fertilization properties and the ability to enrich soil with carbon (Tambone *et al.*, 2010; Nkoa, 2014; Chiew *et al.*, 2015; Barzee *et al.*, 2019), the effect of digestates on soil suppressiveness has yet to be investigated sufficiently. Soil suppressiveness is the natural ability of soil to support optimal plant growth and health. The main basis of this soil state is the microbial component together, of course, with the organic matter content (Broadbent and Baker, 1974; Mazzola, 2002; De Corato, 2020). Soil microbiome has a primary role in controlling soil-borne pathogens through a number of mechanisms such as competition for nutrients, antibiosis and induction of host resistance, which do not permit a pathogen to become established or to persist or for the pathogen to become progressively less severe even though it survives in the soil (Cook and Baker, 1983). The short-term in-pot experiments performed to date to investigate the effects of digestate and sewage sludge application on soil suppressiveness have not always show clear positive effects due to the variability of material of origin, doses, methodology applied and different time intervals between incorporation and soil sampling (Ghini *et al.*, 2007; Franke-Whittle *et al.*, 2020). Nevertheless, little has been investigated until now on microbial changes that can increase soil suppressiveness with repeated field digestate applications.

The majority of agricultural soils in the Po Valley are characterized by low soil organic matter content (SOM <2%) and they are subjected to further SOM decline due to climate change and intensive agricultural practices . SOM and biodiversity decline are linked to crop yield decline worldwide in intensive growing regions (Oldfield *et al.*, 2019). Crop yield decline is an overall reduction of plant vigor and resistance to abiotic stress, due to the loss of soil resilience and microbial diversity, which affect plant production in intensively cultivated areas such as the Po Valley (Manici and Caputo, 2010). This phenomenon is mainly due to a reduction of rooting ability in the crops caused by a reduced soil microbial activity and diversity with a consequent gradual increase of opportunistic soil-borne pathogens.

In this context, microbial benefits from the incorporation of digestate from anaerobic biogas production were assessed in digestate-amended fields located near three anaerobic biogas production plants associated with agricultural and livestock activity in the Po Valley. The final aims of this study were: (i) evaluating whether and to what extent repeat soil amended with digestates can improve soil suppressiveness and (ii) identifying soil microbial components responsible for soil suppressiveness increase under this experimental set.

Materials and methods

A 2-yr study was performed to compare three digestate-amended soils to the relevant non-amended soils. Microbial benefits (soil suppressiveness) were evaluated as plant growth response with a greenhouse plant growth assays using maize, on which quali-quantitative analysis of root fungal endophytes was performed, whereas in the rhizosphere quantitative changes of actinomycetes

and *Pseudomonas* spp., taken as bacterial indicator of soil suppressiveness, were evaluated.

Experimental fields and soil sampling

The impact of digestate amendment was assessed using soil sampled in three sites located near plants for anaerobic production of biogas which were selected in three representative growing areas across the Po Valley (Fig. 1). They were in the following provinces: Cremona (CR), Modena (MO) and Forlì (FL) located, respectively, in the central-west, central and east Po Valley (Fig. 1, Table 1). Two close experimental fields were selected in each of the three sites where soil samples were collected in the consecutive years 2017 and 2018. Each couple of experimental fields was similar for management and crop sequences (maize was the recurrent crop in the last five-teen years, with fewest periodical breaks with triticale or wheat) and both were cultivated with maize at sampling time. However, one field (Digestate) had a history of soil amendment with digestate from a near anaerobic digestion plant, the other (Control) did not (Table 1). The amount of digestate in each site had been applied by not exceeding the annual limits of nitrogen per hectare based on EU-Nitrate Directives for the Nitrate Vulnerable Zones (Monteny, 2001) which were identified in 2006 by the two regions where the experimental fields were located (Lombardy and Emilia Romagna) (Table 1). In the site of Cremona province (CR), located in a nitrate vulnerable zone of the Lombardy region (ERSAF, 2013), separated solid fraction of digestate at a dose estimated to apply a nitrogen rate to not exceeding the annual amount per year, accounting for $170 \, \text{kg N ha}^{-1} \, \text{yr}^{-1}$, was incorporated into the soil three times from fall 2006 to fall 2016 according to the regional directives (Capponi and Barbanti, 2010). In the sites of Modena (MO) and Forlì (FL) provinces, where the experimental sites were out of nitrate vulnerable zone (Emilia Romagna, 2013), separate digestate fraction and whole digestate fraction were applied at doses estimated to apply a nitrogen rate not exceeding the limits (maximum $340 \, \text{kg N ha}^{-1} \, \text{yr}^{-1}$) and according to the rules for application as imposed by the regional regulation in compliance with the nitrates directive (Capponi and Barbanti, 2010).

Soil sampling was performed in the same fields in late May 2017 and 2018 at V6–V10 (six leaves to ten leaves) maize developmental stage (Ciampitti, 2016). Five soil subsamples in the 0–25 cm topsoil after having removed the 2 cm superficial layer were collected in five points along the diagonal of each field to obtain a total of 10 kg of soil per field. Subsamples were mixed manually and partially dried for 6 days at room temperature (20–22°C). In 2017, 300 g-sample was taken for physico-chemical analysis.

Soil texture was estimated with the standard pipette method and clay (Cl), silt (Si) and sand (Sa) percentages of USDA classification were determined using the Soil Triangle Hydraulic Properties Calculator (Saxton *et al.*, 1986). Total organic carbon (TOC) content was quantified through dry combustion using a TOC Vario Select analyzer (Elementar, Germany), which conducts a catalytic combustion by high temperatures in an air environment (Vitti *et al.*, 2016). TOC of each sample (6) was the mean of three analyzed subsamples.

Weather data

Daily values of air temperatures (°C) and precipitation (mm) for the period from 1st January to 30th May of the years 2017 and
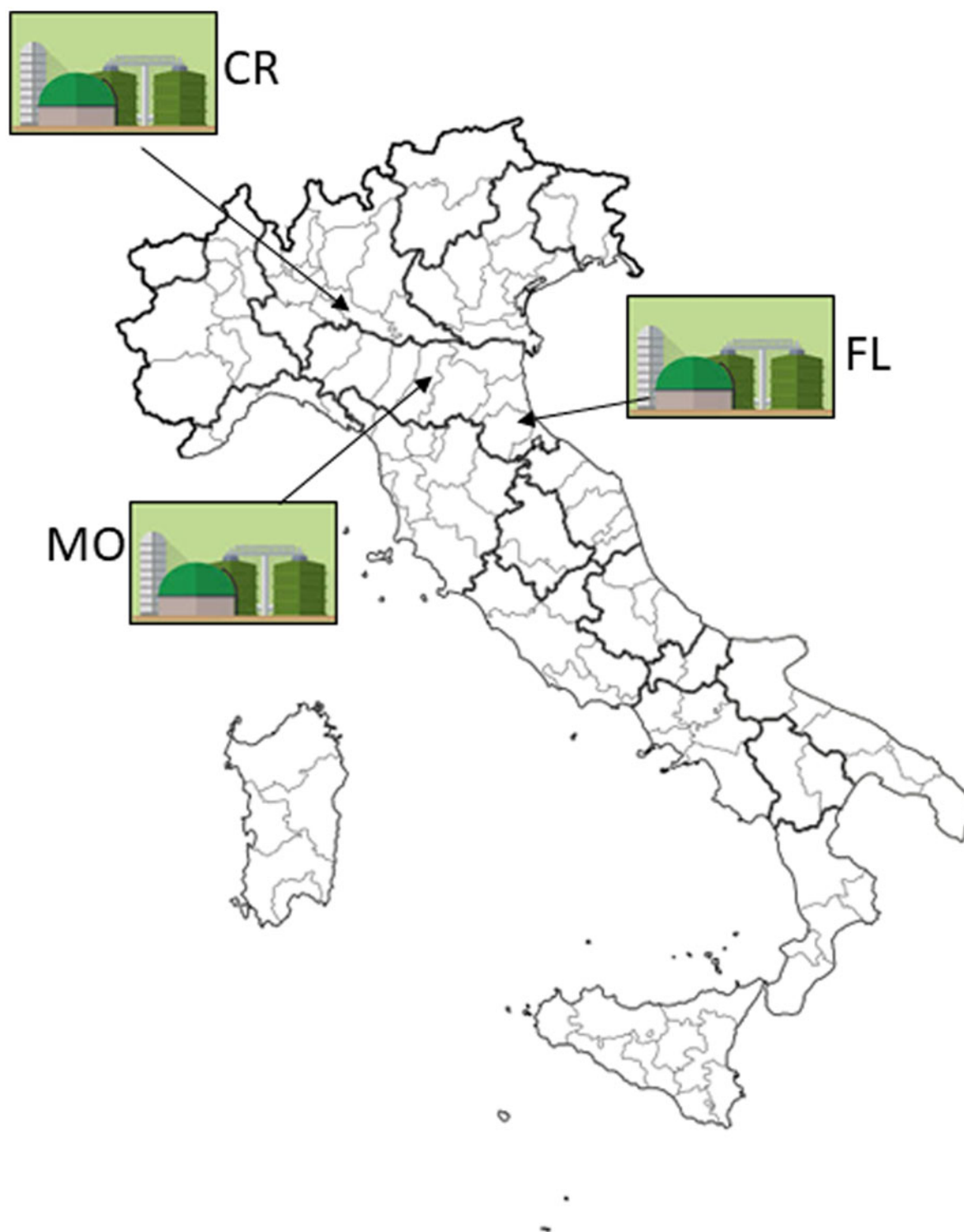
Fig. 1. Map of the plants for anaerobic biogas production selected for this study in northern Italy.

2018 were retrieved by weather stations maintained by the Regional Agencies for Environmental Protection (https://www.arpalombardia.it/; https://www.arpae.it/). Data were recorded electronically every 15 min for temperature and every hour for precipitation, automatically validated and harmonized according to national guidelines for hydro-meteorological data (Barbero *et al.*, 2017) and then aggregated to daily values. Such weather stations are located at distances ranging from 3.3 to 8.1 km from the sampled fields. According to Launay and Guérif (2003), a 14 km-threshold can be regarded as the 'maximum distance between

Table 1. Main features of sampling sites and main organic sources of the relevant anaerobic digestion plants for biogas production.in all cases previous

Province	Treatment	Digestate origin	Digestate form	Current crop[a]	Soil texture[b]	pH	SOM[c]	NVZ
Cremona (CR)	Amended	Animal manure[d] Agricultural waste[e]	Separated solid fraction of digestate	Maize	Loam	7.30 ± 0.30[f]	1.31 ± 0.04[f]	Yes[g]
Cremona (CR)	Control	–	–	Maize	Loam	7.32 ± 0.25	1.26 ± 0.02	Yes
Modena (MO)	Amended	Food waste feedstocks[d] Dairy manure[e]	Separated solid fraction of digestate	Maize	Silty loam	7.86 ± 0.15	1.35 ± 0.06	No
Modena (MO)	Control	–	–	Maize	Silty loam	7.82 ± 0.21	1.13 ± 0.05	No
Forlì (FL)	Amended	Energy crops[d] Animal manure[e]	Whole digestate fraction	Maize	Silty clay loam	7.20 ± 0.30	1.52 ± 0.03	No
Forlì (FL)	Control	–	–	Maize	Silty clay loam	7.25 ± 0.15	0.98 ± 0.04	No

[a]Maize was the recurrent crop in all sampling fields, in all cases they were subjected to periodical (3–4 yr) rotation with sugar beet or wheat or legume crop (alfalfa?).
[b]Soil texture classified using the Soil Triangle Hydraulic Properties Calculator (Saxton *et al.*, 1986).
[c]Soil organic matter (%).
[d,e]Main and secondary waste sources respectively for anaerobic digestion in the close plant.
[f]Standard deviation near to mean of three replicates.
[g]Nitrate vulnerable zones (Monteny, 2001).

fields and stations beyond which it was no longer possible to consider the weather described by the station as being sufficiently representative of that of the field'.

The climate in the three sites is warm temperate with hot summer (*Cfa*) according to the Köppen-Giger taxonomy (Peel *et al.*, 2007). Average annual air temperature is about 12.8–13.4°C, ranging from 0 to 6°C in winter months, to peaks over 30°C in summer. Total annual precipitation is in the range of 690–810 mm, with two main rainy periods in spring and fall, and a minimum in July and August.

Plant growth assay

Soil samples from each of six sampled fields were arranged in 18 pots (500 g of soil per pot) to obtain three replicates of six pots each per field; then they were arranged in a greenhouse according to a randomized block design. Each pot was sown with one untreated maize seed and grown in the greenhouse at 24 ± 2°C under natural light for 30 days. Water was regularly supplied to avoid water limitation to plant growth. Being plant growth with the in-pot assay a way to estimate the weight of the soil microbial components on crop health (namely, way to compare soil suppressive ability) (Neiendam Nielsen *et al.*, 2002), pots were fertilized with ammonium sulfate (1 g per pot, co-responding to a rate of 120 kg N ha^{-1}). This aimed at reducing the effect of different N supply for maize plants in digestate-amended (Digestate) and unamended (Control) soils. At the end of the 30-day period, plants were harvested, carefully separated from soil clod. Then rhizosphere soil adhering to roots was sampled after having gently shaken the roots of each replicate of six plants to remove soil which was not tightly adhering to the roots. In that way, three replicates of rhizosphere soil sample from each of the six treatments were obtained. A subsample of 25 g of soil was taken, air dried at room temperature for 12 h and stored in 50 ml sterile vials at −80°C. Finally, biomass of individual plants was then divided into below and above ground part. Six above ground parts of each replicate were grouped and dried until constant weight at 65°C to determine dry matter production; whereas six roots of each replicate were grouped and processed to analyze root colonization by fungal endophytes.

Root-colonization by fungal endophytes and pathogenicity of test

Roots of each replicate of six plants were washed under running water for 10 min, disinfected for 2 min in 1.5% sodium hypochlorite, rinsed twice with sterile water, dried under sterile air flow for 15 min; then 18 (three per plant) root explants of 0.2–0.3 mm per replicate, for a total of 54 explants per each of six treatments, were processed as described in Manici *et al.* (2018) to estimate colonization frequency and relative species composition of root endophytic fungal communities using culture-based methodology and the most common taxonomy manuals for fungal species identification. Instead, *Setophoma terrestris* was identified using molecular tools by comparing with BLAST internal transcribed spacer (ITS) the sequences generate according to the method described by Manici and Bonora (2007) to the corresponding nucleotide sequences in the GenBank of the National Centre for Biotechnology Information (NCBI), U.S. National Library of Medicine, available on the web at: https://www.ncbi.nlm.nih.gov/.

In July 2018, one isolate of *S. terrestris*, the root endophyte most abundant in both 2017 and 2018, was subjected to pathogenicity test on maize to estimate its impact on dry matter losses. Fifteen-day-old pure culture grown on potato sucrose agar of the *S. terrestris* isolate, whose ITS sequence reference number in the GenBank was MG944392, was inoculated on sterile sand–maize meal media into two 800 ml flasks containing 270 g sand, 30 g corn meal and 60 ml water. Two flasks were inoculated with three 6 mm diameter colony disks each and incubated for 28 days at 24°C. A total of 7.2 kg of topsoil taken from the experimental field of Forlì was equally divided. One part was amended with the two flasks of maize meal inoculum of *S. terrestris* (1:4 v/v) and divided into nine pots. Similarly, the other part was amended at the same v/v rate with sterile maize-meal substrate and divided into nine pots to obtain the un-infested treatment. Pathogenicity test was arranged according to a totally randomized design with three replicates of three pots each. Plants were grown for 1 month in July in a shady greenhouse and were regularly irrigated to avoid water stress. Plants were then processed as described for previous in-pot test. Data of above ground part were expressed as dry matter (g) per plant.

Table 2. Description of primer pairs

Target	Primers	16S rDNA region	Amplicon (bp)	References	Sequences 5′–3′
Pseudomonas spp.	F968 Ps-r	V6–V7	314	Nübel *et al.* (1996); Chen *et al.* (2017)	AACGCGAAGAACCTTAC TTAGCTCCACCTCGCGGC
Actinomycetes	F243 518r	V2–V3	289	Heuer *et al.* (1997); Muyzer *et al.* (1993)	GGATGAGCCCGCGGCCTA ATTACCGCGGCTGCTGG

Table 3. Two-way ANOVA for the variables plant biomass (dry matter) and root fungal infection frequency recorded in two subsequent years with a bioassay with maize grown in-pot on soil samples taken from (A) digestate-amended and non-amended fields, in (B) three sites of the Po Valley

Plant biomass		2017	2018
Main effects	df	*P* value	*P* value
A treatment (2)	1	***	**
B site (3)	2	***	***
Interactions			
A × B	2	**	ns
Root infection frequency		2017	2018
Main effects	df	*P* value	*P* value
A treatment (2)	1	***	ns
B site (3)	2	***	*
Interactions			
A treatment × B site	2	*	ns
Pearson correlation		2017	2018
Plant biomass *vs* root infection		−0.88[a]***	−0.2[ns]

The analysis of root infection frequency was performed on arcsin-transformed data.
***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; [ns]not significant.
[a]r value, 18 counts.

Quantification of actinomycetes and Pseudomonas

Total genomic DNA was extracted from 0.25 g of rhizosphere soil (dry weight) using the PowerSoil DNA Isolation kit according to the manufacturer's instructions (MoBio Laboratories, Carlsbad, CA, USA). Quantification and quality control of DNA were performed using Infinite 200 NanoQuant (Trading AG, Switzerland) and DNA was stored at −20°C until use. Three DNA extractions per sample were performed for quantitative polymerase chain reaction (qPCR) and PCR-denaturing gradient gel electrophoresis (DGGE).

Quantification of rhizosphere bacterial DNA was performed by qPCR quantification assays using the primer pairs shown in Table 2. *Arthrobacter sialophilus* (DSMZ 7306) and *Pseudomonas chlororaphis* (DSMZ 6508) strains from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) were inserted as reference of Actinomyces and *Pseudomonas*, respectively. The DNA of the two aforementioned DSMZ bacterial strains was amplified as described in Saccà *et al.* (2019). Resulting amplicons were purified using the PureLink Quick PCR Purification kit (Invitrogen) and quantified by Infinite 200 NanoQuant (Trading AG, Switzerland). The gene copy number calculation was obtained using the formula: gene copy/μl = DNA $[ng/\mu l] \times 6.02 \times 10^{23}$/base pairs $\times 660 \times 10^9$. Purified amplicons were serially diluted tenfold

and four replicates were used for standard curve generation for quantification of unknown samples (Bustin *et al.*, 2009). The slope of the standard curves was used to calculate qPCR reaction efficiency.

qPCR assays were carried out using the Rotor-Gene SYBR® Green PCR kit (Qiagen, Hilden, Germany) on a Rotor-Gene 6000 (Corbett Research), according to the manufacturer's instructions. Two technical replicates were performed for two identical independent runs, to assess reproducibility of the assays. Briefly, 1× Rotor-Gene SYBR® Green PCR Master mix was used in a final reaction volume of 25 μl, with a final primer concentration of 1 μM and 2.5 μl of template. After an initial PCR activation step at 95°C for 5 min, cycling conditions consisted of 5 s denaturation at 95°C, and 40 cycles of combined annealing extension at 65°C for 10 s. Post-amplification melting curve analysis was performed to verify specificity and identity of qPCR products, with a ramp from 55 to 99°C, rising by 1°C each step. Results were analyzed with the Rotor-Gene 6000 series software 1.7 program. Sterile water was used as no-template control in each run. The resulting CT values, namely the value inverse to the amount of target nucleic acid in soil samples, were subjected to statistical analysis.

DGGE fingerprinting of actinomycetes and Pseudomonas

Qualitative analysis of actinomycetes and *Pseudomonas* populations was performed with DGGE analysis using a nested PCR approach and the primer pairs quoted in Table 2 as already described by Saccà *et al.* (2019). Two replicates of PCR products per each treatment were loaded on DGGE gel and when analyzing the electrophoretic pattern, a relative density score to each band based on a 1–5 scale (maximum band size 5 = 100%; minimum band size 1 = 20%) was assigned. In that way, taxa-abundance matrices were obtained by making possible comparing alpha diversity.

Data analysis

Data were analyzed with Statgraphics centurion software (2005 STATPOINT Inc., Virginia, USA). Quantitative, or parametric, data were subjected to two-way analysis of variance (ANOVA) and mean separation test using Fisher's least significant difference procedure. All percentage data were $\ln(x + 1)$ transformed before running statistical analysis. Multiple Pearson's correlation was used to estimate the relationship between plant growth and all the other estimated variables. Species/abundance data were handled with nonparametric multivariate analysis of variance (NPMANOVA) with Bray–Curtis distance using PAST version 3.24, software for analysis in paleoecology (Hammer *et al.*, 2001). Shannon (H) and Simpson (1-Dominance) biodiversity indices were calculated from the species/abundance of PCR-DGGE matrices using the above-mentioned PAST version.
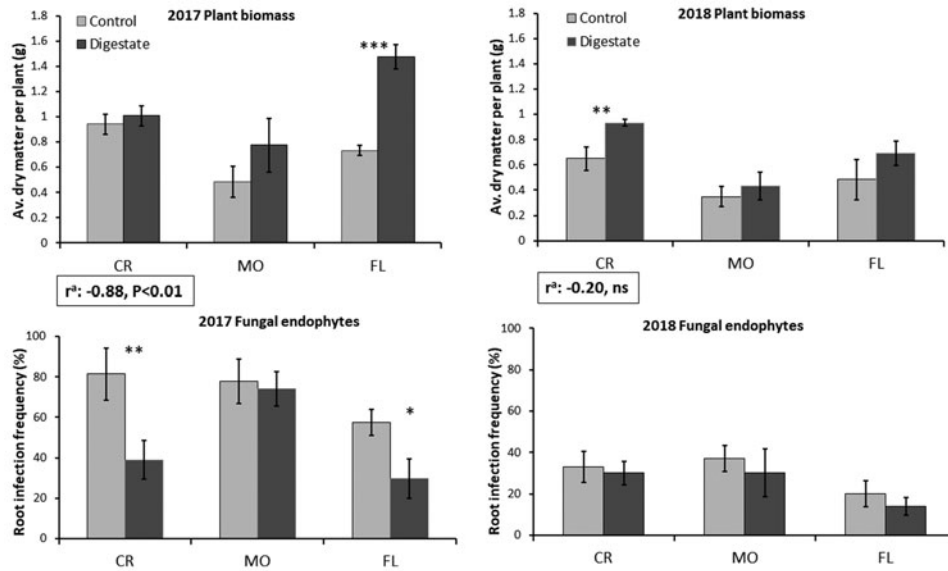
Fig. 2. Plant biomass and root colonization frequency recorded at end of in-pot growth assay with maize in digestate-amended and unamended control soils taken from the experimental field in three sites (located in the provinces of CR: Cremona; MO: Modena; FL: Forlì). [a]Pearson correlation (18 counts) between plant biomass and root colonization frequency.
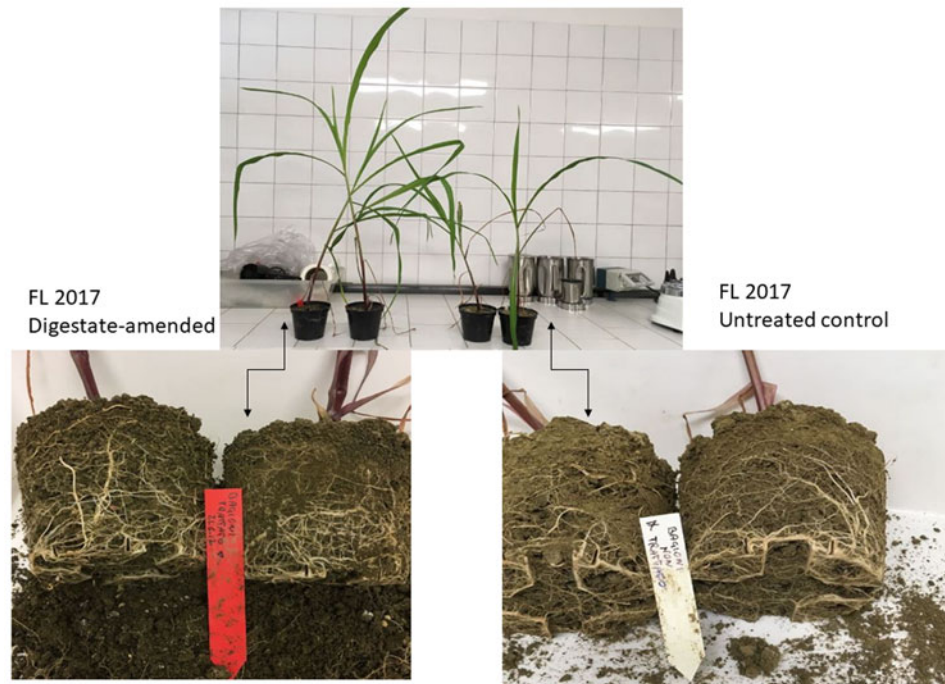

Fig. 3. Higher root development of maize grown in pot on digestate-amended soil as compared to unamended control. Soil samples from FL site that in 2017 showed a significant higher biomass development and a lower root fungal infection frequency in digestate-amended as compared to unamended control.

Biodiversity indices were compared with nonparametric Kruskal–Wallis test using the Statgraphics program.

Results

Plant growth response and root fungal colonization

The growth response of maize significantly differed both for site and for treatment in 2017 and 2018 (Table 3).

Digestate-amended soil (Digestate) gave an increase in plant biomass accounting for 34% in 2017 and 28% in 2018, respectively, as compared to unamended soil (Control). As shown by the significant interaction site × treatment, in 2017 plant response to amended and unamended soils varied according to the soil origin (Table 3). Plant response to digestate-amended and to corresponding untreated soil (Control) in each site is shown in Figure 2 in which plant response to amended and unamended
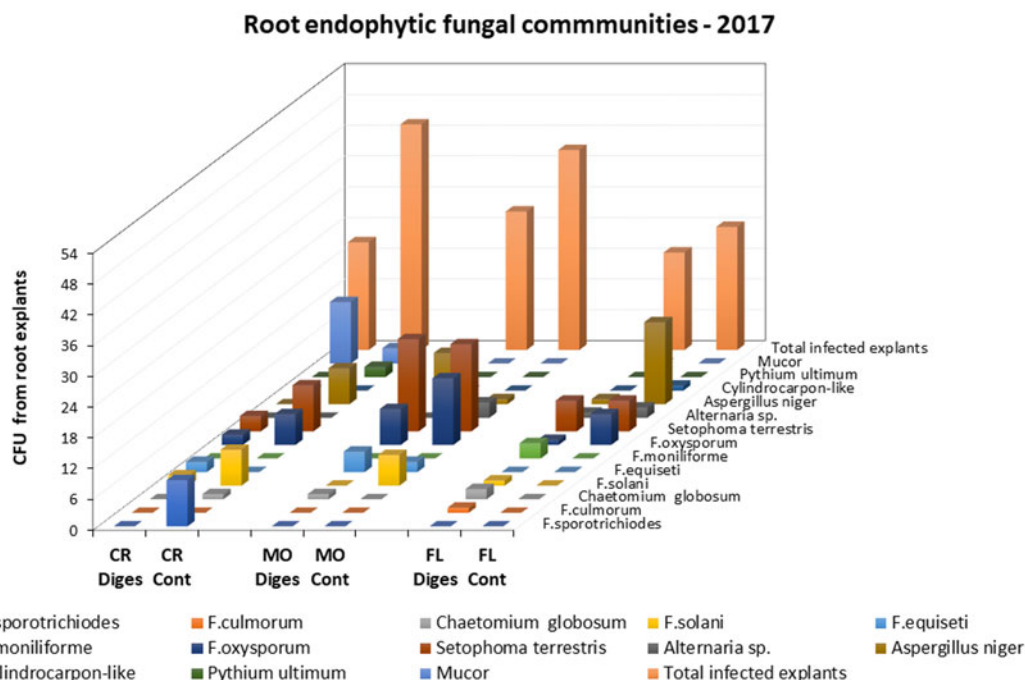
Fig. 4. 2017. Composition of root endophytic fungal communities of maize grown in soil samples from the couple of fields (digestate-amended and unamended control) of each site (located in the provinces of CR: Cremona; MO: Modena; FL: Forli).

soils was analyzed within each site. The latter showed that plant biomass in digestate-amended soil largely exceeded the control soil only in the easternmost experimental site (FL) ($P < 0.001$) in 2017 and in the north western (CR) ($P < 0.01$) in 2018 (Fig. 2).

Fungal root colonization frequency was in all cases higher in digestate-amended soils as compared to the corresponding unamended control soils in both 2017 and 2018 (Fig. 2). Nevertheless, root infection frequency by fungal endophytes significantly differed for soil treatment only in 2017 (Table 3). Fungal root colonization frequency always negatively correlated with plant biomass (Table 3); nevertheless, Pearson correlation between those parameters was high and significant (r: −0.88, $P > 0.01$) in 2017, but not in 2018 (Table 3). The negative correlation between plant biomass production and root colonization frequency indicated that root colonizing fungi acted as root pathogens and digestate-amended soils were able to reduce the fungal root infection responsible of root development reduction (Fig. 3).

Among the fungal species isolated from maize roots at the end of in-pot test, *Fusarium oxysporum* and *S. terrestris* were the two most abundant and widespread fungal species occurring in both 2017 and 2018 (Figs. 4 and 5). *F. oxysporum* is the most spread soil inhabiting *Fusarium* sp. worldwide which can vary from neutral to weakly pathogenic on maize like many fusariums; conversely, *S. terrestris*, which had been already reported as responsible for yield losses in maize in other counties (Chambers, 1987; Newby *et al.*, 1997; Lević *et al.*, 2011), had not been so far reported as important maize root pathogen in Italy; therefore, it was subjected to a pathogenicity test. Quite all the other root colonizing fungi such as *Fusarium* spp., *Helminthosporium turcicum*, *Macrophomina phaseolina* and others (Figs. 4 and 5) which were isolated with lower frequency from maize roots, were all reported as potential root pathogens of maize (Munkvold and White, 2015).

Pathogenicity test indicated *S. terrestris* as responsible for biomass loss of 69 and 64% in dry and fresh biomass, respectively, and this fungus was re-isolated only from roots of inoculated plants and not from the uninoculated control ones. This finding suggested that *S. terrestris* isolated from maize in this study acted as root pathogen and was responsible of plant growth reduction.

Weather

The two years considered in this study showed very contrasting values of precipitation, but not of temperature. In 2017, cumulated precipitation was respectively 167 mm in CR, 148 mm in MO and 164 mm in FL in the period January–May, showing a dry trend compared to the seasonal averages of the Po Valley. Year 2018 was more rainy and cumulative precipitation increases ranged from +189% in CR (315 mm), +207% in MO (306 mm) and +248% in FL (406 mm) in the same period (Fig. 6). Temperature at sowing time (day 9, Fig. 6) was above 10°C in 2017 and around 5°C in 2018, without marked differences among the experimental sites. During the period from sowing to V6–V10 maize developmental stage average temperature increased up to 15.6°C in MO, 15.8°C in FL and 18.3°C in CR, with reduced variability between years.

Quantitative analysis of *Pseudomonas* and actinomycetes

Actinomycetes resulted significantly higher in digestate-treated soil in both the years (Table 4). However, as suggested by the significant ($P < 0.01$) interaction between the two factors of variability (site × treatment), they showed different response to soil treatment in the three experimental sites. Actinomycetes quantity did not correlate with plant biomass production or with root infection (Table 4).
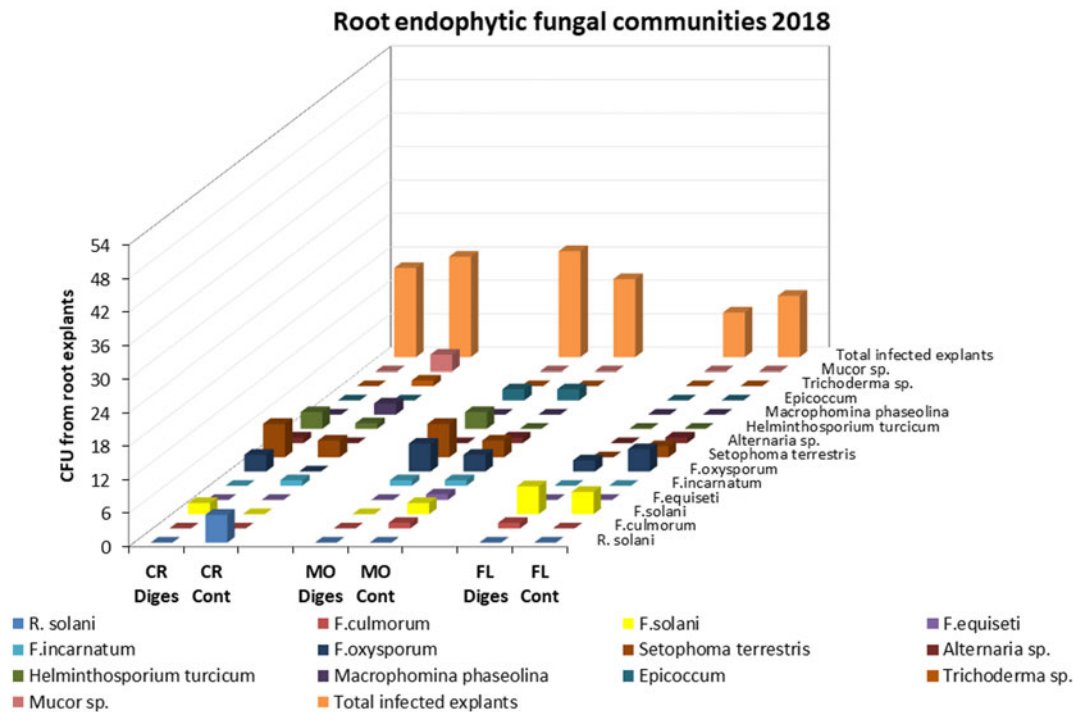
Root endophytic fungal communities 2018

Fig. 5. 2018. Composition of root endophytic fungal communities of maize grown in soil samples from the couple of fields (digestate-amended and unamended control) of each site (located in the provinces of CR: Cremona; MO: Modena; FL: Forlì).
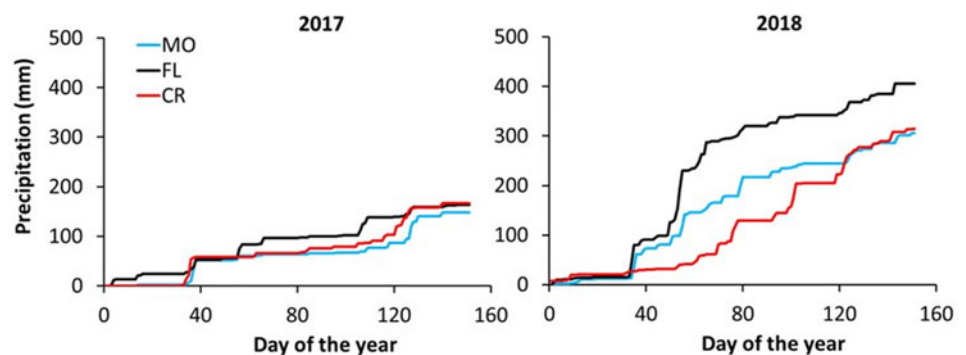

Fig. 6. Cumulated precipitation in the experimental sites in the Cremona (CR), Modena (MO) and Forlì (FL) provinces during the 5 months before soil sampling for the in-pot assay to test soil suppressiveness in 2017 and 2018.

Pseudomonas overall showed higher abundance in digestate-amended compared to unamended control soils; however, they significantly differed only for site in 2017. The FL site showed a higher occurrence of native *Pseudomonas* being the only in which digestate-amended soil showed higher abundance than control soil in both 2017 and 2018 (Supplementary material 1). *Pseudomonas* abundance was highly correlated with root infection (Table 4); as the *CT* value of qPCR is a value inverse to the amount of target nucleic, we can conclude that there was negative correlation between *Pseudomonas* abundance and root colonization by soil-borne pathogens. This finding indicates that this bacterial group was related to a highest natural soil ability to counteract root infection by fungal pathogens.

Biodiversity and difference in community composition

Actinomycetes and *Pseudomonas* composition highly differed for site and treatment in both the years showing also a high

significant interaction site × treatment ($P < 0.01$) (Table 5). Shannon (H) and Simpson (1-Dominance) biodiversity indices of *Pseudomonas* and actinomycetes did not significantly differ between digestate-amended and unamended control soils neither within each site nor as a whole; conversely, they differ significantly between sites ($P < 0.001$) in both 2017 and 2018.

Discussion

This study highlighted benefits of anaerobic digestates for improving soil health in an intensively cultivated region. The main results of this study are the improvement of plant growth in periodically digestate-amended soils and a negative correlation between plant growth and root fungal infection. This suggested that periodical digestate amendments can increase the natural capacity of soils to counteract root colonization by soil-borne pathogens, that is soil suppressiveness. Furthermore, the higher abundance of actinomycetes and *Pseudomonas* in digestate-amended soils indicated

Table 4. Two-way ANOVA of nucleic acid amount of actinomycetes and *Pseudomonas* recorded with qPCR

qPCR	Actinomycetes		*Pseudomonas*	
	2017	2018	2017	2018
A site	***	ns	*	ns
B treatment	**	*	ns	ns
A site × B treatment	**	*	ns	ns
	CT	CT	CT	CT
CR	24.7c	26.5	19.6a	19.1
MO	25.0b	27.0	19.7a	19.2
FO	25.7a	26.7	18.7b	19.5
Digestate-amended	24.9b	26.5b	19.2	19.2
Control	25.3a	27.0a	19.5	19.2
Pearson correlation				
CT vs root infection	ns	ns	*	ns
			(r 0.88)	

Data were expressed as CT values, which is the value inverse to the amount of target nucleic, therefore lower CT vales indicate higher amount.
***P < 0.001; **P < 0.01; *P < 0.05; ns not significant.

Table 5. Two-way NPMANOVA of species/abundance data of PCR-DGGE analysis of actinomycetes and *Pseudomonas*

PCR-DGGE	Actinomycetes		*Pseudomonas*	
A site	**	**	***	***
B treatment	**	**	****	***
A site × B treatment	***	**	***	***

****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05; ns not significant.

Pseudomonas genus (Chen et al., 2017) and qualitative (PCR-DGGE) analysis showed a large variability in the composition of *Pseudomonas* populations, no specific antagonistic mechanisms were identified.

Actinomycetes is a group of Gram-positive bacteria (order *Actinomycetales*) producing various bioactive compounds including antibiotics that are active in biological control of soil-borne pathogens (Crawford et al., 1993; Mendes et al., 2011; Latz et al., 2016). Actinomycetes in this study did not directly correlate with plant growth or with root fungal infection frequency, but their abundance in digestate-amended soil was always higher than that in unamended ones. The latter finding suggested an overall beneficial action of actinomycetes, which is likely to have resulted from the sum of different functional groups that, according to the high variability of the community composition observed with the PCR-DGGE analysis, occurred in a very variable way across the experimental sites. Therefore, unlike what was observed for *Pseudomonas*, we cannot hypothesize a direct control effect for actinomycetes toward root pathogens, but we can hypothesize the involvement of a series of mechanisms capable of directly or indirectly promoting plant growth and health. Indeed, besides biocontrol (El-Tarabily and Sivasithamparam, 2006), actinomycetes can provide a series of other functions such as: (i) ability to make insoluble organic and inorganic phosphorus forms suitable for plant uptake, which was reported to vary widely according to climatic conditions (Ghorbani-Nasrabadi et al., 2013) and (ii) an active action in breaking down plant residues which contributes to maintaining the biotic equilibrium of soil by cooperating with nutrient cycling (Bhatti et al., 2017).

Communities of root fungal endophytes among which typical maize root pathogens such as *H. turcicum* was only occasionally recovered (Figs. 4 and 5), were rather composed of some secondary weak pathogens such as *Fusarium* spp. and *M. phaseolina* (Munkvold and White, 2015) which however varied largely according to the sampling site and year. *S. terrestris* was the only soil-borne fungus occurring everywhere and present in both years of study. *S. terrestris* (past names *Pyrenochaeta terrestris* and *Phoma terrestris*), whose pathogenicity toward maize was confirmed by an ad hoc test in this study, is known for a certain specialization toward maize worldwide, but particularly in the USA where it has been reported to cause yield losses in continuous maize cropping systems either alone or associated with other soil fungi (Newby et al., 1997; Koenning et al., 2007; Lević et al., 2011). This soil-borne fungus, which has been already reported in Italy as the agent of pink root rot on canola (Yang et al., 2017), seem to be selected by the return of maize on the same agricultural soils which is a well-known effect of monoculture (Shipton, 1977). Therefore, according to finding of this study, *S. terrestris* can be considered an additional indicator of maize monoculture effect in intensively cultivated areas such as the Po Valley.

that those bacterial groups could be involved in the observed plant growth improvement. Moreover, our findings provide evidence of high year-to-year variability of root fungal infection in response to precipitation patterns and inherent crop growth response.

The in-pot plant growth assay, which is one of the most common tests for soil suppressiveness (Neiendam Nielsen and Winding, 2002), associated with nitrogen fertilization, which was applied to reduce differences of N availability between the sampling sites of origin, permitted reducing a series of environmental variability factors thus allowing the identification of the main microbial components responsible of root development reduction as well as the mechanism of root defence. Indeed, though microorganisms are recognized as the major component of soil suppressiveness (Schlatter et al., 2017), when plant growth reduction is caused by nonspecific root rot fungal agents such as in the case of this study, identifying major antagonists and biological control mechanisms is much more difficult.

Pseudomonas is a widespread bacterial genus, which includes a wide range of functional groups, such as plant growth promoters and antagonists toward many soil-borne pathogens, whose activity is mediated by releasing of a number of antibiotics and biologically active compounds (Patten and Glick, 2002; Ganeshan and Kumar, 2005; Mazurier et al., 2009; Novik et al., 2015; Chen et al., 2017). Although, quantitative analysis (qPCR) of *Pseudomonas* spp. showed an overall higher abundance in repeatedly digestate-amended soils compared to unamended ones, a clear correlation between *Pseudomonas* spp. abundance and soil suppressiveness was only observed in 2017, when they significantly correlated with root infection frequency thus suggesting *Pseudomonas* involvement in control action toward saprophytic soil-borne pathogens. This finding was in line with what was observed by Dignam et al. (2019) who reported that long-term soil enrichment through plant residue incorporation into the soil increased activity of indigenous plant-beneficial bacterial taxa among which *Pseudomonas* was one of the most represented. As the V6/V7 region was amplified with primers targeting

Root fungal infection of maize at the end of in-pot testing, negatively correlated with plant biomass showing that root fungal endophytes impacted plant growth as pathogens thus resulting an indicator of agricultural soil health in this study. However, this correlation was high in 2017 and poor in 2018, when, respectively, a high and low average root infection frequency was recorded. A different plant response in soil samples taken from the same fields was in part explained by the different weather patterns in the previous months. Indeed, it was a favorable occurrence that the two subsequent years in which the soil was sampled were characterized by contrasting patterns of cumulated precipitation. We postulate that this had determined water stress in the early growth stages of maize in the first year (2017), but not in the second (2018), when optimal water availability was assured for maize in field up to six to ten leaves, the vegetative stage at which soil samples for in-pot testing were collected. As water stress can increase the release of root exudates into the soil in several plant species, including maize (Song *et al.*, 2012; Karst *et al.*, 2017), this increase was hypothesized as being responsible for the increase of the inoculum of soil-borne pathogens of maize under water stress, by favoring the further root colonization, as observed in other plant species (Manici and Caputo, 2020). Therefore, different weather patterns during the early maize growing stages were probably responsible for a different soil inoculum of root pathogens at soil sampling time for the in-pot trials in 2017 and 2018.

As bacterial composition is deeply affected by the environment (Fierer and Jackson, 2006; Manici *et al.*, 2017), differences in actinomycetes and *Pseudomonas* composition between sites were expected. Indeed, such environmental variability overcomes differences in bacterial composition between digestate-amended and unamended fields across the three different agro-environments considered in this study. The latter observation was consistent with high resilience of the resident microbiota in response to digestate and compost amendment in microcosm experiments (Podmirseg *et al.*, 2019). Therefore, the overall increase of *Pseudomonas* and actinomycetes in digestate-amendment soils is likely to concern indigenous bacterial populations rather than allochthonous microorganisms introduced with digestate.

This study shows that repeated application of digestate to soils can improve its natural ability to control root pathogen. That ability appeared to be linked primarily to microbial factors implemented by repeated digestate amendment. Among these microbial factors we have not mentioned microbial biomass (Bongiorno *et al.*, 2019). However, the increase of basal respiration after digestate incorporation into the soil has been observed to totally decrease in 1–3 months after incorporation (Podmirseg *et al.*, 2019); whereas in this case, the last digestate amendment was applied to the soil more than 12 months earlier. Moreover, as soil fungi are the main component of microbial biomass in volume (Bailey *et al.*, 2002), no difference in total fungi DNA content between digestate-amended and unamended soils in each experimental site was found (data not shown).

In conclusion, this study based on multiple indicators for soil health indicates that further environmental and economic benefits may come from biogas production increased through the soil amendment with digestates. Although this type of waste-derived materials have well-known agronomic benefits (Tambone *et al.*, 2010; Barzee *et al.*, 2019), in Italy they did not classified as by-products until 2016 and were, as such, considered for agronomic use according to the same rules as animal manure (MIPAAF, 2016). The improvement of soil suppressiveness is a

so far disregarded effect that throws light on the additional benefits of soil amendment with digestates and indicates the advantages of their long-term application to agricultural soils. This improvement seems due to multiple and variable microbial functionalities; however, based on the findings of this study, it is possible to state that actinomycetes and *Pseudomonas* are two bacterial groups involved and they can be considered useful indicators of benefits from medium term soil amendment with biogas digestates. Finally, maize is commonly considered a crop that can be repeatedly cultivated in the same soil with no major disadvantages; nevertheless, findings of this study indicate that monocropping of maize for at least 15 yr reduces crop health. This result is consistent with those of Borrelli *et al.* (2014) who reported higher yield and yield stability for maize cultivated in a 3-yr rotation compared to maize monocrop in a 30-yr experiment conducted in the irrigated lowland of Lombardy (the Po Valley). This suggests that the current European rules, which discourages the use of maize as a feedstock for anaerobic digestion for biogas production, while encouraging the use of crop residues and no-food cover crops (European Parliament and the Council of the European Union, 2015), are likely to determine gains from the standpoints of biodiversity and soil suppressiveness and crop health.

Author contributions. L.M. Manici: conceptualization; ideas, methodology, formal analysis and writing original draft. F. Caputo: investigation, resources and methodology. G.A. Cappelli: curation and analysis of weather data. E. Ceotto: supervision, writing, review and editing, support to conceptualization and formal analysis.

References

Bailey VL, Smith JL and Bolton H (2002) Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. *Soil Biology and Biochemistry* **34**, 997–1007.

Barbero S, Zaccagnino SM, Mariani S, Lastoria B, Braca G, Bussettini M, Casaioli M, Marsico L, Rotundo R, Pavan V, Ricciardi G, Zenoni E, Cicogna A, Micheletti S, Cazzuli O, Di Priolo S, Ranci M, Rondanini C, Bianco G, Egiatti G, Montanini P, Saccardo I, Campione E, Pupillo S, Locca F, Lazzeri M, Tedeschini M, Marzano V, Schena P, Licciardello A, Manzella F, Brunier F and Ratto S (2017) Linee guida per il controllo di validità dei dati idro-meteorologici. (Guidelines for the quality check of hydrometeorological data). Available at Website https://www.certifico.com/component/attachments/download/5512.

Barzee TJ, Edalati A, El-Mashad H, Wang D, Scow K and Zhang R (2019) Digestate biofertilizers support similar or higher tomato yields and quality than mineral fertilizer in a subsurface drip fertigation system. *Frontiers in Sustainable Food Systems* **3**, 58.

Bhatti AA, Haq S and Bhat RA (2017) Actinomycetes benefaction role in soil and plant health. *Microbial Pathogenesis* **111**, 458–467.

Bongiorno G, Postma J, Bünemann EK, Brussaard L, de Goede RGM, Mäder P, Tamm L and Thuerig B (2019) Soil suppressiveness to *Pythium ultimum* in ten European long-term field experiments and its relation with soil parameters. *Soil Biology and Biochemistry* **133**, 174–187.

Borrelli L, Castelli F, Ceotto E, Cabassi G and Tomasoni C (2014) Maize grain and silage yield and yield stability in a long-term cropping system experiment in northern Italy. *European Journal of Agronomy* **55**, 12–19.

Broadbent P and Baker KF (1974) Behaviour of *Phytophthora cinnamomi* in soils suppressive and conducive to root rot. *Australian Journal of Agricultural Research* **25**, 121–137.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J and Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**, 611–622.

Capponi S and Barbanti L (2010) Utilizzo agronomico del digestato. *Normative regionali a confronto.Terra e Vita* **25**, Supplemento Fertilizzare Oggi.

Carrosio G (2011) La produzione di energia da biogas nelle campagne italiane: un'analisi neo-istituzionale. *Agriregionieuropa* **7**, 54.

Chambers KR (1987) Epidemiology of maize root rot in South Africa. *Journal of Phytopathology* **118**, 84–93.

Chen J, Wu L, Xiao Z, Wu Y, Wu H, Qin X, Wang J, Wei X, Khan MU, Lin S and Lin W (2017) Assessment of the diversity of *Pseudomonas* spp. and *Fusarium* spp. in *Radix pseudostellariae* rhizosphere under monoculture by combining DGGE and quantitative PCR. *Frontiers in Microbiology* **8**, 1748.

Chiew YL, Spångberg J, Baky A, Hansson PA and Jönsson H (2015) Environmental impact of recycling digested food waste as a fertilizer in agriculture—a case study. *Resources, Conservation and Recycling* **95**, 1–14.

Ciampitti I (2016) *New corn growth and development poster from K-State.* Agronomy eUpdate - Kansas State University. Manhattan, KS, USA. Available at Website https://webapp.agron.ksu.edu/agr_social/m_eu_article.throck?article_id=1010.

Consorzio Italiano Biogas (2017) Lo sviluppo del biometano: un'opzione sostenibile per l'economia e per l'ambinete. [Internet]. Available at Website https://www.consorziobiogas.it/wp-content/uploads/2017/03/LA-BIOGAS-REFINERY-NELLA-TRANSIZIONE-ENERGETICA-ITALIANA_SINTESI-marzo-2017.pdf.

Cook RJ and Baker KF (1983) The nature and practice of biological control of plant pathogens. *American Phytopathological Society (APS),* St. Paul, MN, USA. pp. 539.

Crawford DL, Lynch JM, Whipps JM and Ousley MA (1993) Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Applied and Environmental Microbiology* **59**, 3899–3905.

De Corato U (2020) Disease-suppressive compost enhances natural soil suppressiveness against soil-borne plant pathogens: a critical review. *Rhizosphere* **13**, 100192.

Dignam BEA, O'Callaghan M, Condron LM, Raaijmakers JM, Kowalchuk GA and Wakelin SA (2019) Impacts of long-term plant residue management on soil organic matter quality, *Pseudomonas* community structure and disease suppressiveness. *Soil Biology and Biochemistry* **135**, 396–406.

El-Tarabily KA and Sivasithamparam K (2006) Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Soil Biology and Biochemistry* **38**, 1505–1520.

Emilia Romagna R (2013) Le zone vulnerabili ai nitrati (ZVN) in Emilia-Romagna—agricoltura, caccia e pesca. Available at Website https://agricoltura.regione.emilia-romagna.it/produzioni-agroalimentari/temi/bio-agro-climambiente/effluenti-e-nitrati-1/le-zone-vulnerabili-ai-nitrati-zvn-in-emilia-romagna.

ERSAF (2013) Le Zone Vulnerabili ai Nitrati. Carta delle Zone Vulnerabili ai Nitrati. Avaialble at Website https://www.ersaf.lombardia.it/it/servizi-al-territorio/nitrati/normativa/regione-lombardia.

European Parliament and the Council of the European Union (2015) Directive (EU) 2015/1513 of the European Parliament and of the Council of 9 September 2015 amending Directive 98/70/EC relating to the quality of petrol and diesel fuels and amending Directive 2009/28/EC on the promotion of the use of energy from renewable sources. *Official Journal of The European Union* L239, September 2015, 1-29.

Fierer N and Jackson RB (2006) The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 626–631.

Franke-Whittle IH, Juárez MFD, Insam H, Schweizer S, Naef A, Topp AR, Kelderer M, Rühmer T, Baab G, Henfrey J and Manici LM (2020) Performance evaluation of locally available composts to reduce replant disease in apple orchards of central Europe. *Renewable Agriculture and Food Systems* **34**, 543–557.

Ganeshan G and Kumar AM (2005) Pseudomonas fluorescens, a potential bacterial antagonist to control plant diseases. *Journal of Plant Interaction* **1**, 123–134.

Ghini R, Patrício FRA, Bettiol W, de Almeida IMG, de H A and Maia N (2007) Effect of sewage sludge on suppressiveness to soil-borne plant pathogens. *Soil Biology and Biochemistry* **39**, 2797–2805.

Ghorbani-Nasrabadi R, Greiner R, Alikhani AH, Hamedi J and Yakhchali B (2013) Distribution of actinomycetes in different soil ecosystems and effect of media composition on extracellular phosphatase activity. *Journal of Soil Science and Plant Nutrition* **13**, 223–236.

Hammer Ø, Harper DAT and Ryan PD (2001) Past: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**, 9.

Heuer H, Krsek M, Baker P, Smalla K and Wellington EM (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied Environmental Microbiology* **63**, 3233–3241.

Karst J, Gaster J, Wiley E and Landhäusser SM (2017) Stress differentially causes roots of tree seedlings to exude carbon. *Tree Physiology* **37**, 154–164.

Koenning SR, Frye JW, Pataky JK, Gibbs M and Cotton D (2007) First report of Phoma terrestris causing red root rot on sweet corn (*Zea mays*) in north Carolina. *Plant Disease* **91**, 1054–1054.

Latz E, Eisenhauer N, Rall BC, Scheu S and Jousset A (2016) Unravelling linkages between plant community composition and the pathogen-suppressive potential of soils. *Scientific Reports* **6**, 1–10.

Launay M and Guérif M (2003) Ability for a model to predict crop production variability at the regional scale: an evaluation for sugar beet. *Agronomie* **23**, 135–146.

Lević J, Petrović T, Stanković S, Krnjaja V and Stanković G (2011) Frequency and incidence of *Pyrenochaeta terrestris* in root internodes of different maize hybrids. *Journal of Phytopathology* **159**, 424–428.

Manici LM and Bonora P (2007) Molecular genetic variability of Italian binucleate *Rhizoctonia* spp. Isolates from strawberry. *European Journal of Plant Pathology* **118**, 31–42.

Manici LM and Caputo F (2010) Soil fungal communities as indicators for replanting new peach orchards in intensively cultivated areas. *European Journal of Agronomy* **33**, 188–196.

Manici LM and Caputo F (2020) Growth promotion of apple plants is the net effect of binucleate *Rhizoctonia* sp. as rhizosphere-colonizing fungus. *Rhizosphere* **13**, 100185.

Manici LM, Saccà ML, Caputo F, Zanzotto A, Gardiman M and Fila G (2017) Long-term grapevine cultivation and agro-environment affect rhizosphere microbiome rather than plant age. *Applied Soil Ecology* **119**, 214–225.

Manici LM, Caputo F, Nicoletti F, Leteo F and Campanelli G (2018) The impact of legume and cereal cover crops on rhizosphere microbial communities of subsequent vegetable crops for contrasting crop decline. *Biological Control* **120**, 17–25.

Mazurier S, Corberand T, Lemanceau P and Raaijmakers JM (2009) Phenazine antibiotics produced by fluorescent pseudomonads contribute to natural soil suppressiveness to Fusarium wilt. *ISME Journal* **3**, 977–991.

Mazzola M (2002) Mechanisms of natural soil suppressiveness to soilborne diseases. *Antonie van Leeuwenhoek* **81**, 557–564.

Mendes R, Kruijt M, De Bruijn I, Dekkers E, Van Der Voort M, Schneider JHM, Piceno YM, DeSantis TZ, Andersen GL, Bakker PAHM and Raaijmakers JM (2011) Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science (New York, N.Y.)* **332**, 1097–1100.

MIPAAF (2016) Criteri e norme tecniche generali per la disciplina regionale dell'utilizzazione agronomica degli effluenti di allevamento e delle acque reflue, nonche' per la produzione e l'utilizzazione agronomica del digestato. Gazzetta Ufficiale. (GU Serie Generale no. 90 del 18-04-2016 - Suppl. Ordinario no. 9) [Internet]. Available at website https://www.gazzettaufficiale.it/eli/id/2016/04/18/16A02762/sg.

Molinari F and Donati F (2015) Valutazione del potenziale energetico ed economico dei residui colturali in Italia. *Agriregionieuropa* **11**, 41.

Monteny GJ (2001) The EU nitrates directive: a European approach to combat water pollution from agriculture. *The Scientific World Journal* **1**, 927–935.

Munkvold GP and White DG (2015) Infectious diseases. In: Munkvold GP and White DG (ed.), *Compendium of Corn Diseases Fourth Edi.* St. Paul, MN, USA: American Phytopathology Society. p. 7–130.

Musacchio A, Re V, Mas-Pla J and Sacchi E (2020) EU Nitrates directive, from theory to practice: environmental effectiveness and influence of regional governance on its performance. *Ambio* **49**, 504–516.

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

Neiendam Nielsen A and Winding M (2002) Microorganisms as indicators of soil health. NERI Technical Report No. 388.

Neiendam Nielsen M, Winding A, Svend B, Hansen BM, Hendriksen NB and Kroer N (2002) Microorganisms as indicators of soil health. NERI Technical Report No. 388. National Environmental Research Institute, Denmark.

Newby DL, Carroll RB and Whittington DP (1997) Incidence of *Phoma terrestris*, causal agent of red root rot, in corn roots and soil in Delaware and on the eastern shore of Maryland. Plant Disease 81, 232.

Nkoa R (2014) Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: a review. *Agronomy for Sustainable Development* **34**, 473–492.

Novik G, Savich V and Kiselev E (2015) An insight into beneficial *Pseudomonas* bacteria. In *Microbiology in Agriculture and Human Health InTech*. London, UK: IntechOpen, pp. 132.

Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W and Backhaus H (1996) Sequence heterogeneities of genes encoding 16SrRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. *Journal of Bacteriology* **178**, 5636–5643.

Oldfield EE, Bradford MA and Wood SA (2019) Global meta-analysis of the relationship between soil organic matter and crop yields. *Soil* **5**, 15–32.

Patten CL and Glick BR (2002) Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology* **68**, 3795–3801.

Peel MC, Finlayson BL and McMahon TA (2007) Updated world map of the Köppen-Geiger climate classification. *Hydrology and Earth System Sciences* **11**, 1633–1644.

Podmirseg SM, Waldhuber S, Knapp BA, Insam H and Goberna M (2019) Robustness of the autochthonous microbial soil community after amendment of cattle manure or its digestate. *Biology and Fertility of Soils* **55**, 565–576.

Saccà ML, Manici LM, Caputo F and Frisullo S (2019) Changes in rhizosphere bacterial communities associated with tree decline: Grapevine esca syndrome case study. *Canadian Journal of Microbiology* **65**, 930–943.

Saxton KE, Rawls WJ, Romberger JS and Papendick RI (1986) Estimating generalized soil-water characteristics from texture. *Soil Science Society of America Journal* **50**, 1031–1036.

Schlatter D, Kinkel L, Thomashow L, Weller D and Paulitz T (2017) Disease suppressive soils: new insights from the soil microbiome. *Phytopathology* **107**, 1284–1297.

Shipton PJ (1977) Monoculture and soilborne plant pathogens. *Annual Review of Phytopathology* **15**, 387–407.

Song F, Han X, Zhu X and Herbert SJ (2012) Response to water stress of soil enzymes and root exudates from drought and non-drought tolerant corn hybrids at different growth stages. *Canadian Journal of Soil Science* **92**, 501–507.

Tambone F, Scaglia B, D'Imporzano G, Schievano A, Orzi V, Salati S and Adani F (2010) Assessing amendment and fertilizing properties of digestates from anaerobic digestion through a comparative study with digested sludge and compost. *Chemosphere* **81**, 577–583.

Vitti C, Stellacci AM, Leogrande R, Mastrangelo M, Cazzato E and Ventrella D (2016) Assessment of organic carbon in soils: a comparison between the Springer–Klee wet digestion and the dry combustion methods in Mediterranean soils (Southern Italy). *CATENA* **137**, 113–119.

Yang Y, Zuzak K, Harding M, Neilson E, Feindel D and Feng J (2017) First report of pink root rot caused by *Setophoma* (*Pyrenochaeta*) *terrestris* on canola. *Canadian Journal of Plant Pathology* **39**, 354–360.