AGRICULTURAL DIVERSIFICATION: BENEFITS AND BARRIERS FOR SUSTAINABLE SOIL MANAGEMENT

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AGRICULTURAL DIVERSIFICATION: BENEFITS AND BARRIERS FOR SUSTAINABLE SOIL MANAGEMENT

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Editorial: Agricultural diversification: Benefits and barriers for sustainable soil management

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Editorial on the Research Topic
Agricultural diversification: Benefits and barriers for sustainable soil management

Opposite to agricultural intensification, agricultural diversification is a key management practice to improve crop productivity and deliver multiple ecosystem services by adopting crop rotation, multiple cropping or intercropping in arable crops, intercropping in orchards, and agroforestry, among other strategies. Agricultural diversification aims to reduce inputs of energy and agrochemicals in order to mitigate the negative impacts of intensive agriculture on soil quality, water pollution, and eutrophication, emissions of greenhouse gases, soil erosion, and biodiversity loss. If coupled with sustainable soil management strategies such as adopting cover crops, conservation agriculture, organic farming, and fertilization management, agricultural diversification could also contribute to stable yields, profitability, and make agroecosystems more resilient to climate change, environmental risks, and socio-economic shocks. Therefore, new research and policies must play a key role in supporting more sustainable practices for agri-food production while ensuring environmental and food security. Ultimately, the goal of agricultural diversification is to achieve a sustainable agroecosystem in terms of 1) enhancement of soil quality, fertility and structure, water availability and soil carbon sequestration, 2) reduction of soil erosion, greenhouse gas emissions and pollutants, and 3) economic profitability. The eleven papers in this Research Topic deal with the following topics related to agricultural diversification and sustainable soil management in different cropping systems.

Two contributions studied soil organic carbon (SOC) dynamics using simulation models. Oliveira et al. used the CQESTR model to study the effect of two Integrated Crop-Livestock Systems (ICLS) with pasture, and tillage management under tropical conditions in Brazil. ICLS included 1) corn + pasture and 2) soybean + rice + corn + pasture. ICLS increased soil C sequestration compared to simple grain cropping systems under both NT and CT. The ECOSSE model was modified, parameterized, and used to simulate diversified cropping systems by Begum et al. in four long-term experiments in Spain, Italy, and Finland. The addition of manure and cover crops and no-tillage management produced an increase in SOC and the loss of SOC was compensated when grass was introduced in the rotations.

Rahman et al. studied an Integrated Plant Nutrient System (IPNS) to restore soil fertility in degraded acidic and charland soils in Bangladesh to assess the effect of biochar and compost-based IPNS approaches. IPNS increased microbial biomass carbon (MBC) and basal respiration, with the largest increase in poultry manure biochar (PMB), and significantly improved SOC and particulate organic carbon.

Yang et al. addressed the problem of soil pollution by multiple metal(loid)s and their uptake in rice, studying the application of compounds containing iron (Fe) to remediate soil pollution. Using an Sb and Cd co-contaminated soil, soils were treated with a continued submergence condition plus $FeCl_3$, or with different water management. Results indicated that when $FeCl_3$ is used to remediate contaminated soils, dry farming for a short time is needed to avoid As accumulation, and intermittent irrigation is a potential choice to avoid the excessive accumulation of contaminants in the edible parts of rice plants.

Di Bene et al. made a survey of farmers and other stakeholders in Italy to find out their perceptions of barriers and opportunities for implementing crop diversification strategies. The profitability of agricultural production was considered the most important priority to be improved, followed by the need to improve biodiversity, soil structure and fertility, and to reduce energy consumption. Crop rotations were considered the most appropriate farming practices for progressing towards these priorities, though few farmers are experts in crop diversification. The findings also provide detailed and concrete suggestions for effective policy and competence building.

Torrus-Castillo et al. assessed the contribution of temporary spontaneous cover crops to atmospheric CO_2 fixation and nutrient retention in 46 commercial olive groves with different tree densities and cover crop layouts located in Southern Spain. They demonstrated the important role of temporary spontaneous cover crops in woody cropping systems for climate change mitigation through atmospheric CO_2 fixation as well as for nutrient retention, suggesting the adoption of these temporary spontaneous cover crops in the whole area between the tree rows in order to enhance these important agroecosystem services.

Franco-Luesma et al. assessed the potential of inter-cropping an irrigated conventionally managed maize system with legumes for mitigating soil N_2O emissions under Mediterranean conditions. Soil N_2O emissions were measured over 2 years in a maize monoculture and a pea-maize rotation under three different N fertilization levels. They observed that intermediate N fertilization levels showed lower yield-scaled N_2O emissions and N emissions factors than high N fertilization levels, demonstrating the potential of replacing the fallow period with a legume in combination with an adjusted N fertilization rate to mitigate soil N_2O emissions in high-yielding maize systems.

While diversification aims to improve numerous ecosystem services, there can be tradeoffs when it comes to soil greenhouse gas emissions. Hüppi et al. present field measurements of nitrous oxide and methane from two different diversification experiments in the Pannonian region. The results show that the addition of leguminous intercrops can increase nitrous oxide emissions whereas the addition of herbs to uncovered inter-rows significantly decreased nitrous oxide emissions.

Suproniene et al. addressed the important issue of improving soil health in three different cereal crops (spring wheat, triticale, and barley). The authors evaluated the effect of applying different types of animal waste-based digestates (pig, chicken, and cow manure) and synthetic mineral nitrogen on soil prokaryotic diversity and composition after 3 years through Illuminia MiSeq sequencing. They found that the richness and diversity of the soil prokaryotic community were not affected by digestate application, while other factors such as the yearly crop varieties, seasonal climate changes, and soil pH were the major contributors to shaping the prokaryotic community composition over time.

Chaudhary et al. considered the injudicious application of chemical fertilizers that affects soil quality and plant growth and studied the effect of an eco-friendly approach consisting of the application of different bioinoculants and agriusable nanocompounds to improve soil quality, using nanozeolite and nanochitosan along with two *Bacillus* spp., on rhizospheric microbial flora and indicator enzymes for maize. This novel research showed that nanocompounds with *Bacillus* spp. significantly enhanced total microbial count, NPK solubilizing bacteria, and the level of soil health indicator enzymes up to twofold over control plots.

Lu et al. highlighted the relevance of using plastic shed films to improve phthalate acid esters (PAEs) residues in ginseng cultivation and respective soils. These authors registered the status of a total of 19 PAEs in ginseng and soils, and plastic shed film samples from eight ginseng cultivation plots located in the Jilin Province (China). The main findings demonstrated that 6 PAEs are omnipresent contaminants in ginseng cultivation bases. They indicated that the use of plastic shed film could be possibly recognized as a source of PAEs in ginseng bases. The age of plantations also generated some differences in PAEs accumulation. Finally, noncancer and carcinogenic risks were detected for adult intake.

The outcomes of this Research Topic identify needs and directions for further research, such as assessing the effect of organic and biofertilizers on soil microbial diversity and functionality, developing remediation and management strategies to decrease soil pollution, boosting long-term experiments since they represent a unique platform to test any process taking place at an extremely slow rate, including cost-benefits analysis to assess profitability and finding effective agricultural management strategies locally adapted to mitigate greenhouse gas emissions.

Author contributions

RF coordinated the editorial writing, all authors contributed to writing and revision and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Risks for Using $FeCl_3$ Under a Submerged Condition, and Different Water Management to Reduce Uptake of Antimony and Cadmium in a Rice Plant

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Soil pollution by multiple metal(loid)s is a common problem, and it is not easy to synchronously reduce their uptake in crops. Compounds containing iron (Fe) are often used to efficiently remediate soil metal(loid) pollution; however, its associated risks did not receive much attention especially under unsuitable soil water conditions. Pot experiments were set up using an antimony (Sb) and cadmium (Cd) co-contaminated soil treated with a continued submergence condition plus 5, 10, or 20 mg kg^{-1} $FeCl_3$ (Experiment I), or treated with different water management including submergence, intermittent irrigation, and dry farming (Experiment II). Our results showed that the continued submergence resulted in excessive accumulation of arsenic (As) in different tissues of rice plants even if the soil As background concentration is low. High soil moisture content increased the available concentrations of Sb and As, but reduced that of Cd in rhizosphere soils, which was in line with their concentrations in different tissues of rice plants (Experiment II). Under a continued submergence condition, $FeCl_3$ significantly stimulated As concentration in the shoots, roots (excluded Fe20 treatment), and husks, but reduced it in the grains. $FeCl_3$ reduced Sb concentration only in the roots and grains, and reduced Cd concentration only in the husks, suggesting a limited efficiency of $FeCl_3$ to reduce Cd uptake under a submergence condition. In this study, the dynamic changes of As, Sb, and Cd concentrations in soil solution, their available concentrations in rhizosphere soils, their accumulation in root iron/manganese plaques, and the relationships among the above parameters were also discussed. We suggested that if $FeCl_3$ would be used to remediate the contaminated soils by Sb and Cd, dry farming for a short time is needed to avoid As accumulation, and intermittent irrigation is a potential choice to avoid the excessive accumulation of As, Sb, and Cd in the edible parts of rice plants.

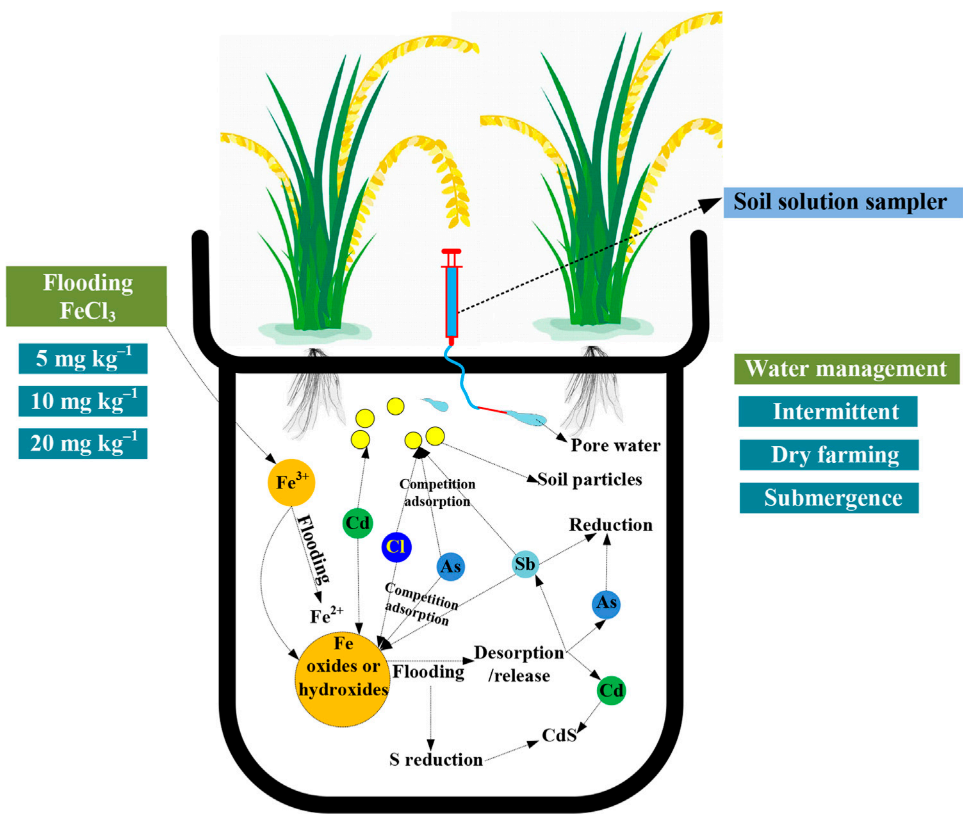
Keywords: dynamic change, arsenic, multiple metal(loid) contamination, soil solution, $FeCl_3$, water management

INTRODUCTION

Anthropogenic activities such as mining, smelting, and waste disposal usually release multiple metal(loid)s into the environment (Macgregor et al., 2015; Tabelin et al., 2018; Tabelin et al., 2021). Ever-increasing levels of arsenic (As), antimony (Sb), and cadmium (Cd) have been found in the environment in Canada (Fawcett et al., 2015) and China (Fu et al., 2010; Fu and Wei, 2013; Fu et al., 2016). Therefore, it is important to simultaneously immobilize toxic metal(loid)s in soils to reduce their accumulation in crops.

Few technologies are explored to repair the contaminated soils by multiple metal(loid)s, especially for Sb contamination. Materials containing Fe are often used to remediate the contaminated soils by As (Chou et al., 2016), Sb (Van Caneghem et al., 2016) and Cd (Huang et al., 2018) *via* some mechanisms, such as promoting Sb adsorption by iron (hydr) oxides (Van Caneghem et al., 2016) or forming a stable tripuhyite under strong acidic conditions (Leverett et al., 2012). In addition, Fe will stimulate the formation of Fe/ manganese (Mn) plaques on the surface of rice roots (Huang et al., 2012). However, the inhibition and stimulation of Sb uptake in plants resulting from the actions of root Fe/Mn plaques were simultaneously observed in many studies (Ren et al., 2014; Cui et al., 2015; Huang et al., 2012). The above contradictory results might suggest that there will be some factors controlling the outcomes of compounds containing Fe to restrict Sb uptake.

The normal cultivation pattern for rice plants needs a long-time submerged condition and a short-time dry farming for 5 to 10 days at the end of tillering stage or the beginning of jointing stage (Yao et al., 2012). This water cycle will increase the release risk of metal(loid) pollution in soils (Huyen et al., 2019). In China, there is plenty of rain within most of rice cultivation areas (mainly locate in the south provinces of China), where the situation of soil metal(loid) contamination is also heavy (Mao et al., 2019). Thus, it is possible that the time of dry farming for these contaminated paddy soils will not be enough. The submerged conditions will stimulate the release of metalloids into soil solution and result in their excessive accumulation in plants, such as As and Sb (Takahashi et al., 2004; Xu et al., 2008; Wan et al., 2013; Liao et al., 2016; Matsumoto et al., 2016). In contrast, high soil moisture will be beneficial to restrict Cd uptake in rice plants (Simmons et al., 2008; Liao et al., 2016) *via* combination of Cd with sulfur (S) to form an insoluble compound (CdS) under an anaerobic condition (Bingham et al., 1976). Some researchers have attempted to use the water management technology to control the availability of targeted



GRAPHICAL ABSTRACT | Schematic diagram of experimental design and processes of adsorption/desorption of elements and reduction of As, Sb and ferric iron in flooded soils.

metal(loid)s in soils and thus reduce their accumulation in crops (Hu et al., 2015). However, the optimal water management manner is not clear when using this technology to remediate the contaminated paddy soils by multiple metal(loid)s.

In addition, when using the compounds containing Fe or the technology of water management to reduce uptake of metal(loid)s in crops, few concerns were paid on their effects on quality of crop products. The fill-up of the above knowledge gaps will make people notice the risks when using $FeCl_3$ or water management to remediate Sb and Cd co-contaminated soils under an impeded drainage condition. Therefore, this study was conducted using $FeCl_3$ (under a continued submergence condition) and different water management patterns in potted experiments to investigate: (1) the efficiency and risks of $FeCl_3$ and water management to reduce uptake of Cd and Sb in a rice plant (Yangdao No.6); (2) the geochemical evolution of pore water with elongation of exposure time; (3) the formation of root Fe/Mn plaques and their roles in controlling uptake of elements; and (4) the uptake of essential elements and the yield of rice plants.

MATERIALS AND METHODS

Soil Preparation and Experimental Design

The tested soil was collected from a farmland in the vicinity of a mining area (Xikuangshan) in Lengshuijiang city, Hunan province, China. The pH value of the soil is 5.30, and the concentration of potassium (K), Fe, calcium (Ca), magnesium (Mg), Mn, and zinc (Zn) is 2.56 g kg^{-1}, 6.87 g kg^{-1}, 28.66 g kg^{-1}, 0.26 g kg^{-1}, 29.79 mg kg^{-1}, and 49.41 mg kg^{-1}, respectively. The concentration of soil organic matter, available phosphorus (P), available K, total nitrogen, and total P is 22.30 g kg^{-1}, 12.88 mg kg^{-1}, 0.09 g kg^{-1}, 2.81 g kg^{-1}, and 23.86 g kg^{-1}, respectively. The concentration of Sb, As, and Cd is 84.79, 10.89, and 0.43 mg kg^{-1}, respectively. The available concentration of Sb, Cd, and As is 0.37 g kg^{-1}, 0.47 mg kg^{-1}, and 0.12 mg kg^{-1}, respectively. According to the Environmental Quality Standards for soils in China (GB 15618–2008), the soil concentration of Cd and Sb is 1.72 and 8.48 times higher than their individual Quality Standard, respectively [pH $\leq$ 5.5 for a paddy field, 0.25 mg kg^{-1} for Cd, and 10 mg kg^{-1} for Sb (a suggested value)]. Although the soil As concentration was lower than its Quality Standard (pH $\leq$ 5.5 for a paddy field, 35 mg kg^{-1}), the unsuitable water management would lead to As accumulation in edible parts of crops (Liao et al., 2016). Therefore, in this study, we also monitored the As concentration. The collected soil was air dried, sieved through a 2-mm nylon screen, mixed thoroughly, and finally placed in darkness until use.

There were two experiments in this study. The first one was conducted to study the effects of $FeCl_3$ on the uptake of Sb, Cd, and As by rice plants. There were four treatments: control (CK), Fe5 (5 mg kg^{-1}), Fe10 (10 mg kg^{-1}), and Fe20 (20 mg kg^{-1}). The soil was submerged with a water depth of 3–4 cm above topsoil throughout the rice growing period (soil cultivation continued for 95 days).

The second experiment was designed to investigate the efficiency of different water management patterns to control the uptake of the above metal(loid)s by rice plants. There were three treatments as follows: (1) Flooding: The water management was the same as in Experiment I. (2) Intermittent irrigation: The soil was flooded first, and next naturally dried to make the soil moisture content be about 50% of field capacity (weight method), and then re-submerged. The above processes were repeated until harvest. (3) Dry farming: The soil moisture content was always maintained at 60%–80% of field capacity. Each treatment of the above two experiments had triple replications. The two experiments were performed synchronously.

Basal fertilizers were added to each pot as follows: 0.20 g $CO(NH)_2$, 0.12 g NaH_2PO_4, and 0.26 g KCl for both experiments. The soil was thoroughly mixed with the basal fertilizers (Experiment I and II) and the compound containing iron (Experiment I) as the designs, and then potted. Each pot was filled with 5 kg soil. To investigate the changes of Sb, Cd and As concentrations in soil pore water, a soil solution sampler (19.21.26F RHIZON FLEX, Agro Business Park Wageningen Netherlands) was buried into the soil of each pot (considered as a replication) to collect soil pore water. After that, the soil was flooded with de-ionized water to rebalance in a glass greenhouse for 2 weeks. Two weeks later, the rice seedlings were transplanted into the pots (two seedlings per pot).

Plant Culture and Management

Rice seeds (*Oryza sativa* L., Yangdao No.6) were disinfected by 30% H_2O_2 for 20 min, and then rinsed thoroughly with de-ionized water. These seeds were sown in a moist culture medium composed of vermiculite and perlite (1 : 1, v: v), germinated, and grew in a greenhouse for 3 weeks.

Three weeks later, the rice seedlings with a uniform size were transplanted into a half-strength Hoagland–Aron solution (Hoagland and Arnon., 1950) for their further development for 2 weeks. The nutrient solution was replaced every 3 days and its pH was adjusted to 5.5 using 0.1 mol L^{-1} NaOH and HNO_3. The 50% HN solution was composed of 2.5 mM KNO_3, 0.5 mM NH_4NO_3, 0.5 mM $NH_4H_2PO_4$, 2 mM $Ca(NO_3)_2{\bullet}4H_2O$, 1 mM $MgSO_4{\bullet}7\ H_2O$, 4.5 μM $MnCl_2{\bullet}4H_2O$, 23 μM H_3BO_3, 0.4 μM $ZnSO_4{\bullet}7H_2O$, 0.15 μM $CuSO_4{\bullet}5H_2O$, 0.05 μM H_2MoO_4, and 4.5 μM EDTA-Fe. The growth conditions of the greenhouse were 25/20°C (day/night) temperatures, a 60–70% relative humidity, and a 16-h photoperiod with a light intensity of 100 μmol $m^{-2}{\cdot}s^{-1}$.

Two weeks later, the rice seedlings were transplanted into plastic pots. During the soil cultivation process, the rice plants were placed in a glass greenhouse under a natural light at temperatures of 15–38°C.

Sampling and Analysis

Sampling of Soil Pore Water

The soil solution was collected by a soil solution sampler at the first, third, seventh, 10th, 20th, and 60th days after the transplanting of seedlings into soils. After being filtered through a 0.22-μM filter, diluted nitric acid solution (1%) was added into the soil solution to prevent the precipitation of ions, and then stored at 4°C until the determination of elemental concentrations using Inductively Coupled Plasma Mass

Spectrometry (ICP-MS, iCAP Qc ICP-MS, Thermo Fisher, United States).

Plant Harvest

The plant height and rice spike numbers were recorded before the seedlings were harvested (upon no visible water in the pots at the ninety-fifth day). The attached soil on the root surface was brushed off, collected, and assigned as the rhizosphere soils. After that, the seedlings were separated into the roots, shoots, and grains (with husks). The grains (with husks) were air dried, and the yield of each pot was recorded. Next, the grains (with husks) were de-hulled, and then the shelled grains and husks were separately ground using a micro-mill for the determination of elemental concentrations. The roots and shoots were rinsed with de-ionized water thoroughly, and the water adhering to the surface of samples was removed using filter papers. After that, the fresh weights of roots and shoots were recorded.

A part of fresh root samples was gathered to extract root Fe/Mn plaques using a modified dithionite–citrate–bicarbonate (DCB) method (Li et al., 2016). Briefly, approximately 1.00 g of fresh rice roots was weighed and incubated in a 30-ml DCB extract (0.03 mol L^{-1} sodium citrate, 0.125 mol L^{-1} $NaHCO_3$, and 0.5 g sodium hydrosulfite) for 1 h at 25°C. After that, the above rice root samples, the remaining root samples (excluding the root samples used for the extract of root plaques), and the shoot samples were oven-dried at 70°C for 48 h to a constant. The dry weights of the above oven-dried samples were recorded, and then pulverized for the determination of elemental concentrations.

Soil Sampling and Treatment Procedure

The air-dried rhizosphere soil was sieved through a 0.15-mm nylon screen and used to measure the pH value and the available concentrations of Sb, As, and Cd. The available concentrations of Sb, As, and Cd were measured according to the method of Liu et al. (2016). Briefly, approximately 5 g rhizosphere soil sample was weighted and transferred into a 50-ml centrifuge tube, and then 25 ml of 0.1 M HCl was added. The centrifuge tube was shaken at 180 r min^{-1} for 2 h at 25°C, and then the mixture was filtered into a volumetric flask, topped up to 100 ml, and stored at 4°C in a refrigerator until use.

Digestion Methods

An ED54 DigiBlock digestion system (Lab Tech, Inc., Hopkinton, MA, United States) was used to digest the plant and soil samples. The digestion method was described in the studies of Liao et al. (2016) and Wu et al. (2017). For the digestion of soil samples, 10 ml of HNO_3, 4 ml of HF, and 0.25 g of soil samples were mixed in a digestion tube and incubated overnight. Samples were heated first at 120°C for 1 h, and then at 150°C for 2 h. Next, the liquid in the tube was evaporated at 180°C until the liquid volume is approximately 1 ml. After that, the liquid volume was topped up to 25 ml using de-ionized water, filtered through a 0.22-μm filter, and then used to determine the elemental concentrations by ICP–MS.

The digestion method for plant samples was as follows: 15 ml of HNO_3 and 0.2 g of plant samples were mixed in a digestion tube and incubated overnight. The samples were first heated to 80°C for 1.5 h, and then at 120°C for 1.5 h, and at 150°C for 3 h. Finally, the liquid in the tube was evaporated to be approximately 1 ml under a temperature of 180°C. After that, the volume of the above liquid was topped up to 50 ml in a volumetric flask, and filtered through a 0.22-μm filter for the determination of elemental concentrations using ICP-MS.

The concentrations of Fe and Mn in the extraction solution were determined using a Flame atomic absorption spectrometer (Zeenit700P, Analytik jena, Germany) and the concentrations of other elements in the extraction solution were determined using ICP-MS.

Standard reference materials including green tea leaves (GBW10052), grains (GBW10045), and soils (GBW07452) were purchased from the Institute of Geophysical and Geochemical Exploration, China. When using ICP-MS to determine the elemental concentrations, the relative standard deviation (RSD) of each sample is less than 10% and the recovery rate is between 90% and 120%.

Data Analysis

The bioconcentration factors (BCFs) and translocation factors (1 and 2) of Sb, Cd, and As were calculated using **Eqs 1–3**, respectively (Takarina and Pin., 2017; Otones et al., 2011).

TABLE 1 | Plant growth and soil available concentrations of metal(loid)s.

Treatments	Plant height (cm)	Spike number (spike/pot)	Hundred-grain weight (g)	Shoot fresh weight (g)	Root fresh weight (g)	Soil pH	Available concentrations (mg kg^{-1})		
							Sb	As	Cd
Control (CK)	92 ± 0.6a[a]	7.0 ± 1.7a	2.0 ± 0.33a	141 ± 9.1b	137 ± 17b	6.81 ± 0.10a[a]	0.39 ± 0.014b	0.46 ± 0.057a	0.52 ± 0.013a
5 mg kg^{-1}Fe	93 ± 2.2a	8.0 ± 1.0a	2.3 ± 0.30a	137 ± 7.0b	126 ± 5.1b	6.60 ± 0.021b	0.52 ± 0.010a	0.40 ± 0.0010a	0.51 ± 0.011a
10 mg kg^{-1}Fe	91 ± 1.6a	7.0 ± 1.0a	2.0 ± 0.094a	141 ± 11b	112 ± 14b	6.62 ± 0.11b	0.51 ± 0.023a	0.39 ± 0.020a	0.50 ± 0.0070a
20 mg kg^{-1}Fe	85 ± 1.4b	8.0 ± 1.7a	2.3 ± 0.16a	164 ± 2.9a	167 ± 21a	6.77 ± 0.075ab	0.52 ± 0.027a	0.45 ± 0.023a	0.50 ± 0.0070a
Flooding	91 ± 3.2B[a]	5.3 ± 1.2B	2.0 ± 0.61A	147 ± 25A	117 ± 11.4A	6.66 ± 0.042A	0.47 ± 0.019A	0.31 ± 0.010A	0.53 ± 0.0030B
Intermittent irrigation	104 ± 1.4A	8.3 ± 0.58A	2.3 ± 0.18A	143 ± 15A	72 ± 5.1B	6.67 ± 0.095A	0.42 ± 0.00020B	0.16 ± 0.0070B	0.59 ± 0.011A
Dry farming	106 ± 5.0A	7.3 ± 1.2AB	2.3 ± 0.11A	127 ± 22B	36 ± 6.1C	6.84 ± 0.13A	0.32 ± 0.0041C	0.12 ± 0.0030C	0.59 ± 0.017A

[a]*Values are means ± SE (n = 3). The lowercases letters (Experiment I) and capital letters (Experiment II) in the same column indicate significant differences among different treatments (p ≤ 0.05).*

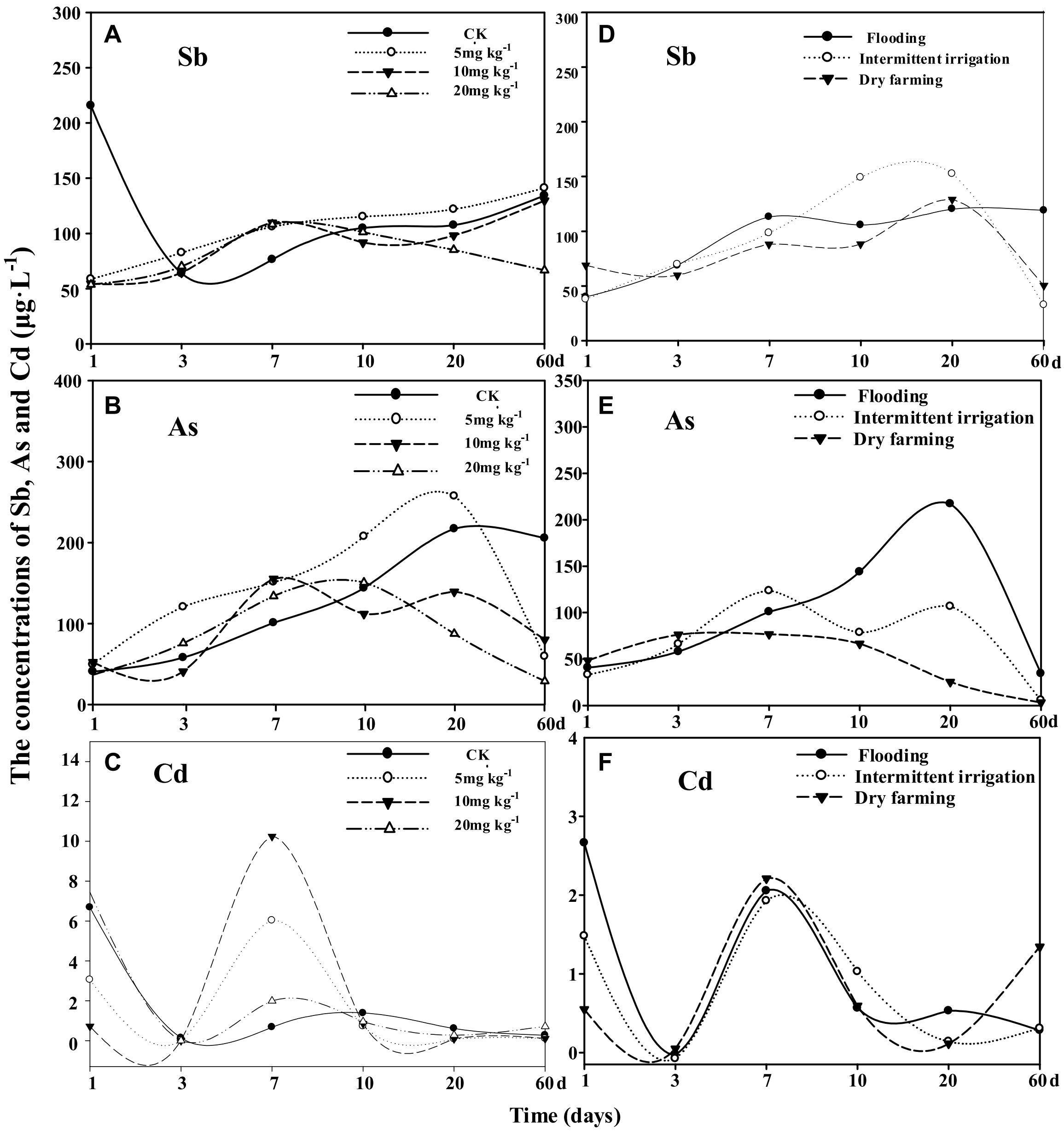


FIGURE 1 | Dynamic changes of Sb, As, and Cd concentrations in the soil solution with the elongation of time. Panels **(A–C)** indicates the changes of Sb, As, and Cd concentrations in the soil solution under different $FeCl_3$ treatments, respectively. Panels **(D–F)** indicate the changes of Sb, As and Cd concentrations in the soil solution under the flooding, intermittent irrigation, and dry farming treatments, respectively.

$$\text{Bioconcentrarion factors (BCF)} = C_{Sb/As/Cd\,(roots)} \Big/ C_{Sb/As/Cd\,(soil)} \tag{1}$$

$$\text{Translocation factor1 (TF1)} = C_{Sb/As/Cd\,(shoots)} \Big/ C_{Sb/As/Cd\,(roots)} \tag{2}$$

$$\text{Translocation factor2 (TF2)} = C_{Sb/As/Cd\,(grain)} \Big/ C_{Sb/As/Cd\,(roots)} \tag{3}$$

where $C_{Sb/As/Cd(roots)}$ denotes the concentration of Sb, As, and Cd in roots of rice plants, respectively; $C_{Sb/As/Cd(soil)}$ denotes the concentration of Sb, As, and Cd in soils, respectively; and $C_{Sb/As/Cd(grains)}$ indicates the concentration of Sb, As, or Cd in grains, respectively.

One-way ANOVA analysis combined with multiple comparisons (Tukey's test) was employed to compare significant differences between different treatments ($p \le 0.05$). Results were expressed as means with standard errors ($n = 3$). Data analysis was performed using the SPSS18.0 (SPSS Inc., Chicago, IL, United States) software and the figures were drawn using a SigmaPlot software14.0.

RESULTS AND DISCUSSION

Plant Growth and Yield

This study was conducted to investigate the efficiency and risks of using $FeCl_3$ or water management to control the uptake of Sb and Cd in a rice plant. The results showed that 20 mg kg^{-1} Fe significantly increased the fresh weights of shoots and roots but decreased the height of this plant (**Table 1**). However, this fresh weight stimulation was not accompanied with enhanced spike numbers and hundred-grain weight (**Table 1**). These results might indicate that the addition of Fe facilitated the vegetative growth but did not show beneficial effects on the reproductive growth of this rice plant under a continued submergence condition. The above results were not in line with the results of Huang et al. (2018), who reported that $FeSO_4 \bullet 7H_2O$ as a base fertilizer could significantly increase rice grain yield. This inconsistence might be partially due to the continued submergence condition. Because alternate wetting and drying farming regimes could increase grain yield and plant height in mature stages of rice plants (Maneepitak et al., 2019). In this study, similar stimulation on spike numbers and plant height were also demonstrated when compared to the flooding treatment (**Table 1**).

Dynamic Changes of Elements in Soil Solution and Their Available Concentrations in Rhizosphere Soils

Soil solution plays an important role in regulating dynamic balance of nutrient elements in soil (Li and Hou, 1998), which will affect their uptake in plants. On the first day, the addition of Fe resulted in obviously lower Sb concentration relative to the CK treatment (**Figure 1A** and **Supplementary Figure S1**), possibly indicating a co-precipitation of Fe and Sb since previous studies suggested that the Fe may precipitate with Sb in soils (Tandy et al., 2017). However, with the elongation of exposure time, the soil Eh under a submerged condition will decrease (Zheng et al., 2019) and result in the reduction of Fe^{3+} to Fe^{2+} (Frohne et al., 2011), As(V) to As(III) (Makino et al., 2016), and Sb(V) to Sb(III) (Wan et al., 2013). As(III) had a lower affinity to the soil solid phase than As(V) (Suriyagoda et al., 2018); however, Sb(III) showed a higher affinity with hydrous ferric oxide and goethite than Sb(V) (Zhu et al., 2020). Therefore, the reducing dissolution of added Fe led to the steady release of As and Sb with time from dissolved Fe oxides into the soil solution (**Figures 1A,B**), and the enhanced Sb available concentration in the rhizosphere soil (**Table 1**).

Reports have shown that the materials containing Fe can effectively immobilize As and Sb in soils (Ko et al., 2015; Yuan et al., 2017; Mitsunobu et al., 2006). However, there were no regular differences in the concentrations of As (third day to 20th day) and Sb (third day to 20th day) under Fe treatments relative to the CK treatment (**Figure 1** and **Supplementary Figure S1**). This might be due to the dynamic balance of plant uptake and the processes of biological as well as physicochemical occurring in soils. On the 60th day, the solution Sb concentration at 20 mg kg^{-1} Fe and the solution As concentration in all Fe treatments were obviously lower than that in the CK treatment (**Figure 1** and **Supplementary Figure S1**). The related reasons for these changes were discussed below.

1) Cl^- had a weak effect on the desorption of $[Sb(OH)_6]^-$ (Biver et al., 2011) and $H_2AsO_4^-$ (Ryden et al., 1987) for the same binding sites. Therefore, the supplement of Cl^- was not the reason for the decreased Sb and As concentrations in the soil solution on the 60th day.
2) The adsorption of As and Sb by soil Fe oxides was weakened, because of the reducing dissolution of Fe and thus the more release of adsorbed As (DeLemos et al., 2006) and Sb under a submerged condition (He et al., 2015). After the addition of $FeCl_3$, the unchanged As bioavailable concentration in the rhizosphere soils (**Table 1**) was not well in line with the unregular changes in As concentration in the soil solution (**Figure 1B**). The above results might suggest that $FeCl_3$ did not show a significant effect on the available concentration of As at harvest.
3) The decreased soil solution Sb and As concentrations on the 60th day seemed not to be due to the competitive adsorption between Sb(V) and As(III) or between As(III) and Sb(III). Because (1) although the flooding condition is supposed to facilitate the more transformation of Sb(V) to Sb(III) (Wan et al., 2013) and As(V) to As(III) (Makino et al., 2016), Sb(V) will be often detected in water under anaerobic conditions, which mainly exists in the water as $[Sb(OH)_6]^-$ (Filella et al., 2002). (2) As(III) is predominant under anaerobic conditions, as well as As(III) and Sb(III) mainly exist in the environment as neutral molecules (McFarlane et al., 2003).
4) It was speculated that the decreased soil solution Sb and As concentration is probably ascribed to the combined effects of (1) their adsorption/desorption in soil, (2) reducing dissolution of Fe accompanying with more release of As and Sb (Cui et al., 2015; Matsumoto et al., 2016; Karimian et al., 2019), (3) effects of soil microorganisms, and (4) the huge uptake of Sb and As at the stage of high nutritional demand in this rice plant.

Nevertheless, as compared to the CK treatment, the unchanged soil Cd available concentration in the rhizosphere soil (**Table 1**) matched well with the relatively constant concentrations of Cd in the soil solution on the 20th day to 60th day (**Figure 1** and **Supplementary Figure S1**). The above results might be related to the continued submergence condition, which resulted in the co-precipitation of Cd and S (Cornu et al., 2008).

The results of Experiment II further confirmed the important roles of soil moisture in regulating the concentrations of As, Sb, and Cd in soil and soil solution. We found that soil solution concentration (**Figure 1E**, on the 10th to 60th day) and available concentration (**Table 1**) of As were both higher under a high soil moisture than that under a

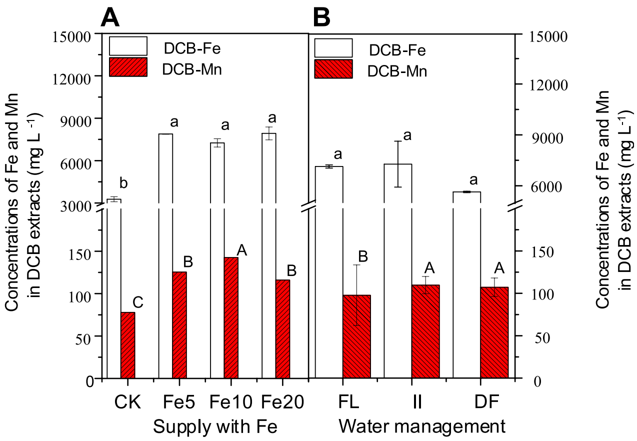


FIGURE 2 | Effects of $FeCl_3$ and water management on the formation of iron/manganese plaques. Bars are means and standard deviations for three replications. Different lowercase letters, capital letters, denote significant differences (at $p \le 0.05$) for Fe and Mn concentrations in the DCB extracts among different treatments, respectively.

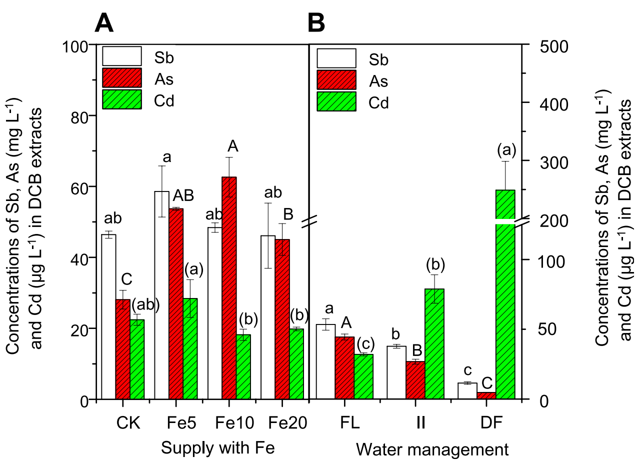


FIGURE 3 | Effects of $FeCl_3$ and water management on the concentrations of Sb, As, and Cd in the iron/manganese plaques. Bars are means and standard deviations for three replications. Different lowercase letters, capital letters, and parenthesized letters denote significant differences (at $p \le 0.05$) for Sb, As, and Cd concentrations in the DCB extracts among different treatments, respectively.

low soil moisture. On the 60th day, the Sb concentration in soil solution under a high soil moisture was significantly higher than that under a lower soil moisture content (**Figure 1D** and **Supplementary Figure S1**). In contrast, the available concentration of Cd was higher under a low soil moisture than that under high soil moisture (**Table 1**). All of the above results suggested that high soil moisture will facilitate the release of As and Sb but reduce Cd availability. Similar results were also reported in the studies of Li and Xu (2017).

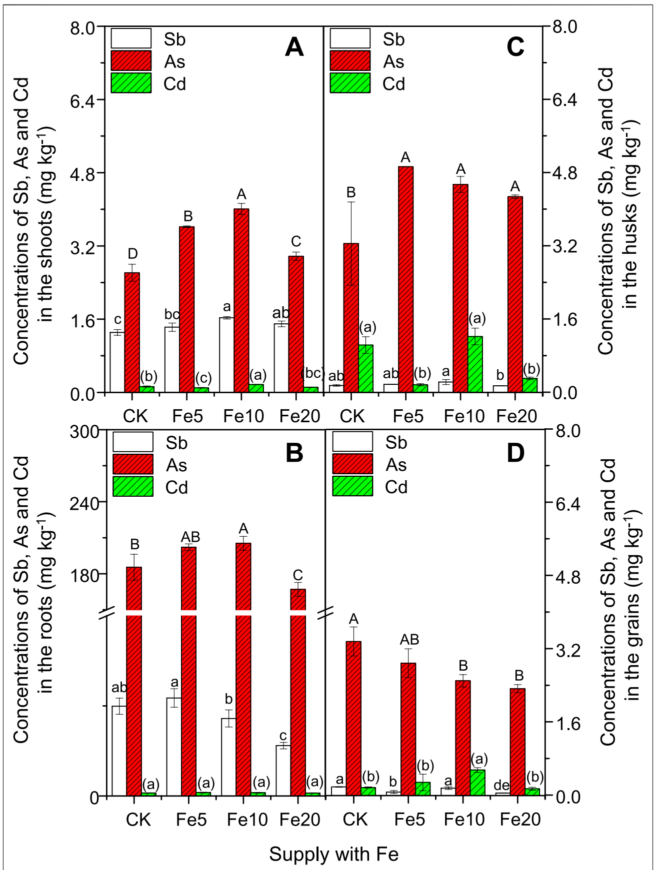


FIGURE 4 | Effects of $FeCl_3$ on the concentrations of Sb, As, and Cd in the shoots, roots, husks, and grains. Bars are means and standard deviations for three replications. Different lowercase letters, capital letters and parenthesized letters denote significant differences (at $p \le 0.05$) for Sb, As, and Cd concentrationsin the shoots, roots, husks and grains among different treatments, respectively.

Roles of Fe/Mn plaques in Constraining Sb, As, and Cd Might Be Ion-level-dependent in Soils

In this study, exogenous Fe stimulated the formation of Fe/Mn plaques (**Figure 2A**), which was well in line with the results of a previous study by Huang et al. (2012). Intermittent irrigation and dry farming treatments promoted the Mn plaque when compared to the flooding treatment (**Figure 2B**), suggesting that a low soil moisture will be beneficial for the formation of root Mn plaques. Reports suggest that the formation of Fe/Mn plaques needs an oxidation process of Fe/Mn (Pittman, 2005), and the stimulation of a low soil moisture on Mn plaque formation is thus expected.

Exogenous Fe significantly enhanced As concentration but did not significantly affect Sb and Cd concentrations in DCB extracts when compared to the control (**Figure 3A**). The enhanced As accumulation in root Fe plaque of rice plants was also observed in the study of Li et al. (2015). However, the enhanced accumulation of As in root Fe/Mn plaques did not result in less accumulation of As in most tissues of rice plants (**Figure 4**).

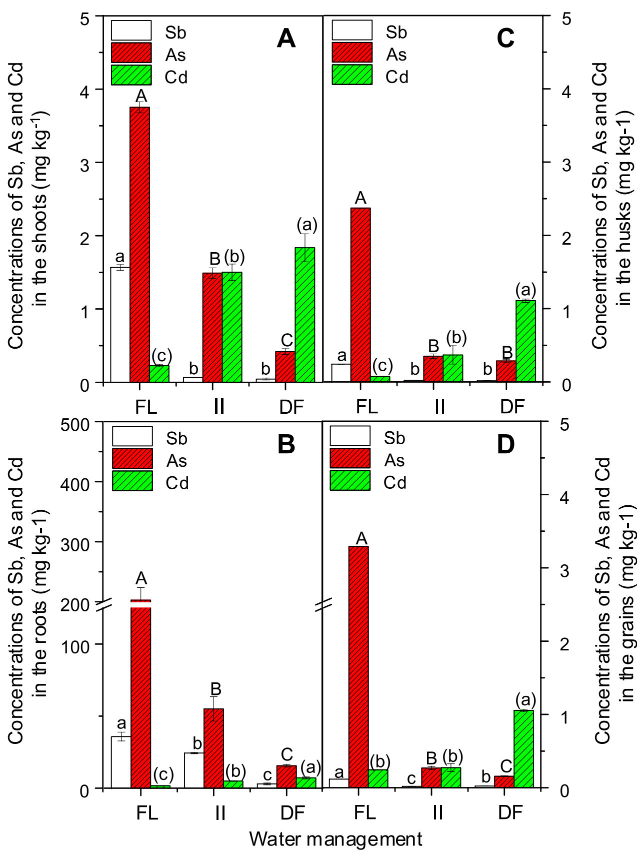


FIGURE 5 | Effect of different water management on the concentrations of Sb, As and Cdshoots, roots, husks and grains. Bars are means and standard deviations for three replications. Different lowercase letters, capital letters, and parenthesized letters denote significant differences (at $p \leq 0.05$) for Sb, As, and Cd concentrations in the shoots, roots, husks and grains among different treatments, respectively.

The above results further questioned the roles of Fe/Mn plaques acting as a barrier, and it was speculated that their roles acting as a barrier should be condition-dependent, such as dose-dependent (Ren et al., 2014; Cui et al., 2015) and speciation-dependent (Huang et al., 2012). Here, the Fe/Mn plaques will play a storage role for most elements, because (1) the unchanged As available concentration (**Table 1**) and unregular changes of As concentration in the soil solution (**Figure 1**) cannot explain the significantly enhanced As accumulation in the most tissues of this rice plant (**Figure 4**); (2) exogenous Fe generally enhanced concentrations of Mg, K, and Zn in root plaques, and K, Ca, Mn, and Zn in roots; and (3) the essential metal elements in the roots of this rice plant were much lower than that in the roots of normal growth rice plant in the study of Liu et al. (2019), especially for Mg, K, Ca, Mn, and Zn. Therefore, we speculated that when the concentrations of essential metal elements in soils are low, Fe/Mn plaques will accumulate these elements to support normal growth of plants (**Supplementary Table S1**). However, this process will result in synchronously the accumulation of toxic metal(loid)s because there are not enough cations to combine anions and thus keep anion–cation balance.

Unlike As, the addition of Fe had no significant effect on the DCB-Sb concentration (**Figure 3A**), but significantly enhanced the available concentration of Sb in the rhizosphere soils (**Table 1**). The unregular changes in Sb concentration in soil solution did not match well with the unaffected concentrations of Sb in Fe/Mn plaques and enhanced available concentration in the rhizosphere soil. Unexpectedly, the addition of Fe did not significantly affect the Cd concentration in DCB extracts (**Figure 3A**) and the available Cd concentration in the rhizosphere soils (**Table 1**). Liu et al. (2007) found that the addition of 50 mg L^{-1} Fe-EDTA significantly increased the concentration of Cd in root Fe plaque. However, root Mn plaque of cattail (*Typha latifolia* L.) was also found to loss its roles in inhibiting Cd uptake in a nutrient solution culture (Ye et al., 2003). In this study, the unaffected Sb and Cd concentrations in the root Fe/Mn plaques might be due to the scarce capacity of Fe/Mn plaques under a continued submergence condition to adsorb so many kinds of metal(loid)s. Because the addition of Fe also increased the concentrations of Mg, K, and Zn in the DCB extracts (**Supplementary Table S1**).

In Experiment II, we found that low soil moisture content facilitated the formation of Mn plaque (**Figure 2B**), which showed a negative effect on the accumulation of As and Sb, and a positive effect on the Cd accumulation in the root DCB extracts. The above results might suggest that under dry farming conditions, root Mn plaque might exert its functions on restraining Cd. A previous study showed that a large amount of As could be accumulated on Mn plaque of rice roots (Liu et al., 2005).

Exogenous Fe Stimulated as Accumulation in Most Tissues of This Rice Plant Under a Continued Flooding Condition and Thus Possessed Risks

Unexpectedly, the flooding condition results in excess accumulation of As in the shoots, roots, husks, and brown rice (**Figure 4**), even when the soil As concentration was as low as 10.89 mg kg^{-1}. In addition, exogenous Fe only significantly reduced the brown rice As concentration and in most cases increased As accumulation in the shoots, roots, and husks (**Figures 4A–C**). The unregular changes in soil solution As concentration and the unchanged available concentration of As in the rhizosphere soil (**Table 1**) did not match well with the enhanced As concentrations in the shoots, roots, and husks. Only the enhanced As concentration in DCB extracts (**Figure 3A**) was in line with the As accumulation in the above tissues, which might suggest the role of Fe/Mn plaques acting as a storage for As uptake at this moment. Although the $FeCl_3$ stimulated the As uptake in some tissues, it reduced the translocation of As from roots to shoots (decreased BCF value), and roots to grains (decreased TF2 value) (**Supplementary Table S2**).

Unlike As, the addition of $FeCl_3$ only significantly enhanced the shoot Sb concentration but reduced grain and root Sb concentration (**Figures 4B,D**). Similar to As, the addition of Fe reduced the translocation of Sb from roots to shoots (decreased BCF value), and roots to grains (decreased TF2 value) (**Supplementary Table S2**). The significant increases in soil available Sb concentration agreed with the enhanced shoot Sb concentration (5 and $10\,mg\,kg^{-1}$ Fe, **Figure 4A**). The unchanged Sb concentration in the DCB extracts (**Figure 3A**) indicated that root Fe/Mn plaques played a limited role in controlling Sb translocation at this moment. Similar results were obtained in other studies, where Fe plaques had no obvious effect on Sb uptake by wheat (*Triticum aestivum* L. Sella) (Ji et al., 2018).

It was also strange that the addition of $FeCl_3$ showed a dose-dependent effect on the Cd concentration in the shoots, husks, and grains. For example, $10\,mg\,kg^{-1}$ $FeCl_3$ significantly increased the grain and shoot Cd concentrations (**Figures 4A,D**), and the other treatments containing $FeCl_3$ either non-significantly affected or significantly reduced the Cd concentration in different tissues of this rice plants (**Figures 4A–D**). The above results suggested complicated physicochemical reactions plus biological effects of plant roots and microorganisms in soils. In this study, the addition of $FeCl_3$ did not have negative effects on the concentrations of Mg, K, Ca, Mn, and Zn in the grains (**Supplementary Table S3**).

A Moderate Soil Moisture is Needed for Rice Plants Growing in Sb and Cd Co-contaminated Soils

In the Experiment II, a low soil moisture resulted in low DCB-As and DCB-Sb concentrations (**Figure 3B**), available As and Sb concentrations (**Table 1**), and total concentrations of As and Sb in all tissues of this rice plant (**Figure 5**). However, a low soil moisture at the same time increased Cd concentration in all tissues of rice plants (**Figure 5**) and decreased the concentrations of Mg, K, Ca, Fe, Mn, and Zn in the grains (**Supplementary Table S4**). The above results indicated that when growing rice plants in Sb and Cd co-contaminated soils, a moderate field drying strategy is necessary to control the accumulation of Cd and Sb in tissues of rice plants, because (1) a high soil moisture will enhance the risks of As accumulation in plants despite a very low As concentration in soils, which was in line with the results reported by Carrijo et al. (2019); (2) a low soil moisture will increase the risks of Cd accumulation in plant tissues (**Figure 5**); and (3) a low soil moisture is also not beneficial for the accumulation of essential elements in the grains, just proven in this study (**Supplementary Table S4**) and the study of Li et al. (2009).

When using $FeCl_3$ to remediate contaminated soils by Sb and Cd in natural fields, some aspects should be taken into consideration. In actual field conditions, the growth conditions for crops are hard to be controlled, especially for excess rain, fertilization, and cultivation patterns. (1) Excess rain will result in more release of Sb and As (just mentioned above) and elevate the difficulty of using passivators to immobilize As and Sb in soils. (2) The fertilizers containing phosphorus (P) will stimulate the release of As and Sb *via* competition adsorption (Cao and Ma., 2004), but will reduce Cd availability *via* a co-precipitation reaction (Raicevic et al., 2005). Therefore, it is important to supply phosphorus fertilizers during the process of using passivators to remediate As- and/or Sb-contaminated soils. (3) In practice, a crop rotation measure is often used to improve soil physicochemical properties and soil microorganism communities (N'Dayegamiye et al., 2015). A rotation measure (like a rotation of paddy rice and wheat) will help soil re-organize its soil texture, and affect adsorption/desorption of elements and speciation transformation of As and Sb in soils (Datta and Sarkar., 2004; He et al., 2018). When cultivating a contaminated farmland using a rotation pattern, $FeCl_3$ can be used to reduce As, Sb, and Cd accumulation in grains of rice plants on the condition that the soil water will be well-controlled. Otherwise, an uncontrolled accumulation of As and Cd in grains of rice plants will very likely happen.

Conclusion

In this study, the dynamic changes in the concentrations of As, Sb, and Cd in the soil solution did not match well with their final available concentrations in the rhizosphere soils. After the addition of $FeCl_3$, the unchanged available concentration of As in the rhizosphere soils was not in line with the significantly enhanced As concentration in most tissues of the rice plants. However, the significantly enhanced available Sb concentration and unchanged available Cd concentration in the rhizosphere soils generally agreed with the enhanced shoot Sb concentration and unchanged root Cd concentration, respectively. The long-term submergence resulted in the excessive accumulation of As in all tissues of this rice plant. The addition of $FeCl_3$ could reduce the grain As, grain Sb, and husk Cd concentration in many cases. Different $FeCl_3$ treatments stimulated the formation of Fe/Mn plaques, and the Fe/Mn plaques accumulated a large amount of As. However, the above accumulation did not produce a less accumulation of As in many plant tissues. In addition, the accumulation of Sb and Cd in the Fe/Mn plaques did not differ within different Fe treatments. Low soil moisture content was found to be beneficial to the formation of Mn plaque, which accumulated more Cd but less As and Sb with the decreased soil moisture content. In line with the changes of these three element concentrations in the Fe/Mn plaques, the low soil moisture led to a higher accumulation of Cd but a lower accumulation of As and Sb in all tissues of rice plants. In summary, when using strategies to reduce the uptake of Cd and Sb in rice plants, adding $FeCl_3$ to the growth medium may pose risks when the plants are subjected to a continued submergence condition, including (1) the unexpected stimulation for As accumulation in rice plant tissues even when the As background concentration is low, and (2) decreased accumulation of many essential elements in the grains. In addition, the intermittent irrigation is the best way to simultaneously reduce the accumulation of As, Sb, and Cd in the grains of rice plants grown in the contaminated soils by Sb and Cd.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RF and ZF postulated and supervised the study. QW planned the experiment, obtained the data, and carried out the data analysis and interpretation. JY prepared the first draft, and RF thoroughly revised the manuscript and provided financial support. RF, ZF, JY, and QW read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.780961/full#supplementary-material

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Assessment of Soil Health Indicators Under the Influence of Nanocompounds and *Bacillus* spp. in Field Condition

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Agricultural yield of major crops is low due to the injudicious use of chemical fertilizers that affects soil fertility and biodiversity severely and thereby affecting plant growth. Soil health is regulated by various factors such as physicochemical properties of the soil, availability of micro/macronutrients, soil health indicator enzymes and microbial diversity which are essential for agriculture productivity. Thus, it is required to draw attention towards an eco-friendly approach that protects the beneficial microbial population of soil. Application of different bioinoculants and agriusable nanocompounds has been reported to enhance soil quality with increased nutrient status and beneficial bacterial population, but additive effects of combined treatments on soil microbial population are largely unknown. The present study investigated the impact of nanozeolite and nanochitosan along with two *Bacillus* spp. on rhizospheric microbial flora and indicator enzymes to signify soil health under field conditions on maize. Soil health was ascertained by evaluating physicochemical analysis; total bacterial counts including N, P, and K solubilizing bacteria; and soil health indicator enzymes like fluorescein diacetate hydrolysis, alkaline phosphatase, β-glucosidase, dehydrogenase, amylase, and arylesterase. Change in copy number of 16S rRNA as a marker gene was used to quantify the bacterial population using quantitative PCR (qPCR) in different treatments. Our study revealed that nanocompounds with *Bacillus* spp. significantly ($p < 0.05$) enhanced total microbial count (16.89%), NPK solubilizing bacteria (46%, 41.37%, and 57.14%), and the level of soil health indicator enzymes up to twofold over control after 20, 40, and 60 days of the experiment. qPCR analysis showed a higher copy number of the 16S rRNA gene in treated samples, which also indicates a positive impact on soil bacterial population. This study presents a valuable approach to improve soil quality in combined treatments of nanocompounds and bioinoculants which can be used as a good alternative to chemical fertilizers for sustainable agriculture.

Keywords: soil enzymes, *Bacillus* spp., nanocompounds, qPCR, soil health

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INTRODUCTION

Progression of life in all forms depends on the agriculture sector in most of the developing countries worldwide. Excessive and indiscriminate use of agrochemicals has inadvertently damaged soil health over time (Bunemann et al., 2018). Toxic chemicals have a detrimental effect on the key drivers of biogeochemical cycles and in the soil microbial community (Rousk and Bengtson, 2014; Kumar et al., 2021). It is therefore imperative to find safe and effective strategies contributing towards higher agronomic yield without jeopardizing the natural microflora of soil (Bargaz et al., 2018). Combined applications of plant growth promoting rhizobacteria (PGPR) and nanocompounds has potential to significantly improve the overall plant and soil health status. Use of microbes in the agricultural sector has a lengthy history, created through broad-scale inoculation of legumes in the 20th century (Desbrosses and Stougaard, 2011). Exploitation of beneficial PGPR such as *Azotobacter*, *Azospirillum*, *Bacillus*, and *Pseudomonas* in the form of biofertilizers can be an alternative to conventional chemical fertilizer (Vessey, 2003; Schütz et al., 2018). They promote plant growth by influencing plant hormone production, iron sequestration *via* siderophore, stress management *via* key enzymes such as 1-aminocyclopropane-1-carboxylate (ACC), and soil organic matter decomposition (Jahanian et al., 2012; Pandey and Gupta, 2019). Most importantly, they help to access macro/micro nutrients from the soil system and improve the plant growth (Beneduzi et al., 2012; Kour et al., 2020a). Microbial inoculants enhance nitrogen, phosphorus, and potassium fertilizer resource use efficiency, which is typically lost due to run-off and leaching in the atmosphere (Adesemoye and Kloepper, 2009). In particular, *Bacillus* and *Pseudomonas* species are best known to solubilize growth-limiting nutrients such as phosphate and potassium efficiently, which finally enhanced plant progress (Santoyo et al., 2012; Sharma et al., 2013; Chaudhary A. et al., 2021). *Acinetobacter calcoaceticus* is involved in phosphate solubilization and mitigated the drought toxic effects in foxtail (*Setaria italica*) (Kour et al., 2020b). More than 75% of globally marketed biofertilizers are associated with nitrogen fixing and P solubilizing/mobilizing property (Timmusk et al., 2017). High availability of NPK could extend survival rates of microorganisms in soil (Yang et al., 2011). Extracellular enzymes like dehydrogenase, fluorescein diacetate, alkaline phosphatase, and β-glucosidase produced by PGPR helps in functioning of soil ecosystem as well as nutrient cycling (Liu et al., 2017). Various reports support the positive impact of PGPR on seed germination, stimulation of root growth, and plant growth regulation though enzymatic activities (Vacheron et al., 2013). However, inconsistent behavior of biofertilizers under field conditions often limits their widespread adoption by farmers. Developing inoculant using beneficial microorganisms that have a longer shelf life and high efficacy is a major commercialization challenge (Backer et al., 2018).

The inclusion of nano-encapsulation knowledge might be used as a resourceful means to defend PGPR against environmental factors such as UV radiation and heat (Prasad et al., 2017). Enhancing their shelf life and allowing the controlled release of biofertilizers would allow their practical application worldwide (Vejan et al., 2016). Numerous studies have supported the possible application of nanocompounds in agricultural field to boost agricultural yield (Duhan et al., 2017). Foliar application of silver nanoparticles (AgNPs-40 mg L^{-1}) significantly improved agronomical parameters (shoot height, shoot weight, and number of leaves) of fenugreek (*Trigonella foenum-graecum*) by twofold (Sadak, 2019). Nanofertilizers can sustain slow release of nutrients due to higher surface tension than conventional surfaces (Ghormade et al., 2011). Out of the different agriusable nanocompounds, nanozeolites and nanochitosan have found their wide application in the agriculture sector due to their small size, high surface tension, chelation capacity, and biocompatibility, which are helpful in improving bacterial population and agronomic yield (Ming and Allen, 2001; Chaudhary and Sharma, 2019). High porosity of zeolites and their selectivity for cations make them useful to promote nutrient use efficiency (Ramesh and Reddy, 2011). Nanozeolite as a natural substrate can support microbial growth. Positive response of nanozeolite (50 mg L^{-1}) towards soil health indicator enzymes and thus microbial activity under *in vitro* conditions (Khati et al., 2018). A study by Yuvaraj and Subramanian (2018) suggested the possible application of nano-sized zeolites (90 nm) as Zn fertilizer carrier for slow release of zinc in soil. Another nontoxic polysaccharide-like chitosan being biodegradable and biocompatible is useful in the agricultural sector (Katiyar et al., 2015). It is known as a plant growth regulatory agent and suppresses the growth of fungal pathogens (Popova et al., 2016). According to Siddaiah et al. (2018), chitosan nanoparticles enhanced seed germination in pearl millet and protected from downy mildew. Nanocompounds (50 mg L^{-1}) and *Bacillus* spp. enhanced agronomical/biochemical attributes and maize productivity (Chaudhary A. et al., 2021). To study the beneficial effects of nanocompounds, it is important to focus on their impact over factors involved in soil health, which are critical for soil fertility and agricultural productivity. NPs in higher concentration not only affect the functional diversity of microorganism's enzyme activity in soil but indirectly pose risk to plant growth (Chavan and Nadanathangam, 2019). Soil microbial dynamics is a key factor for sustainable agricultural practice in the long term as a slight change in microbial population can severely deteriorate the soil quality (FAO, 2012; Jacoby et al., 2017). Microbial population, activities of soil enzymes, and availability of micro/macronutrients maintain soil health and its quality (Tahat et al., 2020). Physicochemical properties of the soil exhibit seasonal variation and are influenced by the nutrient content of the soil, which can modify the structure and composition of the bacterial community in the rhizosphere/bulk soil (Li et al., 2020). Among soil physicochemical properties, pH is known to affect bacterial community and enzymes, involved in solubilization of organic (C, N, P) and nutrient availability in soil/plant system (Lopez-Monejar et al., 2015; Ju et al., 2019).

Therefore, the main aim of this research was to investigate the role of nanocompounds along with *Bacillus* spp. on total bacterial count, nitrogen fixers (*Azotobacter*), potassium and phosphorus solubilizers, soil enzymes, and microbial community using advanced molecular techniques under field conditions on maize for the first time. Molecular methods provide distinctive insight into the composition, structure, and functioning of microbial population of an ecosystem (Griffiths et al., 2003). Relatively few studies have been conducted on the effect of agriusable nanocompound on soil health. The information obtained from these parameters provides the beneficial role of nanocompounds along with bioinoculants on soil, particularly focusing on soil management and the overall richness and diversity of bacterial population of maize rhizosphere.

MATERIALS AND METHODS

Bioinoculants and Growth Conditions

Bioinoculants *Bacillus* spp. (*Bacillus* sp. PS2 and PS10) with accession nos. KX650178 and KX650179 were isolated from the agricultural field of the University. Both the bacterial cultures had plant growth-promoting properties like phosphorus solubilization, indole acetic acid, and siderophore production (Khati et al., 2019a). Nanozeolite and nanochitosan used in this study have the following parameters: size < 80 nm; refractive index, 1.47; pH, 7–8 and 7–9; and 99.90% purity (Khati et al., 2019b).

Experimental Design

The field experiment was carried out in June to September 2017 at Govind Ballabh Pant University of Agriculture and Technology, Pantnagar (GBPUA&T). This site lies Southward of Shivalik Himalayas (79°E longitude and 29°N latitude). Summers are warm in this region with a maximum temperature of 35.5°C and a minimum temperature of 23°C, and a relative humidity of about 35% was recorded during the experiment. Maximum rainfall occurred in July. This study was carried out in randomized block design (RBD) with three replications for all treatment. A plot size of 14.70 m^2 was used for the experiment. Each plot has a length of 4.2 m and a width of 3.5 m, with a row-to-row space of 60 cm and a plant-to-plant space of 20 cm (**Supplementary Material S1**).

Seed Bacterization

Maize seed variety (DH296) was taken from the Crop Research Centre of GBPUAT, Pantnagar. Seeds were disinfected by ethanol (70%) and hydrogen peroxide (3%) followed by distilled water (Khati et al., 2017). Sterilized seeds were treated with bacterial cultures and nanocompounds (50 mg L^{-1}). There were a total of nine treatments used in the experiment: control (T1), PS2 (T2), PS10 (T3), nanozeolite (T4), PS2 + nanozeolite (T5), PS10 + nanozeolite (T6), nanochitosan (T7), PS2 + nanochitosan (T8), and PS10 + nanochitosan (T9). Different treatments received 2 × 10^6 cfu population per seed. After proper treatment, seeds were kept under an incubator shaker at 25°C for 15 min at 100 rpm. Treated seeds were further used for field trial (**Supplementary Material S2**).

Soil Sample Collection

Sampling was carried out after 20, 40, and 60D (days) of the experiment. Rhizospheric soil from a maize root depth of 15 cm was collected from each replicate randomly and mixed appropriately. Soil samples were passed through a 2-mm sieve and used for physicochemical analyses, total bacterial count. and soil enzyme activities (Chaudhary et al., 2021a) (**Figure 1**).

Physicochemical Analysis of Soil Samples

Soil pH was measured by making the solution of soil in distilled water (1:3) using a pH meter. Soil organic carbon was measured by using the method of Black (1965), while total nitrogen, available phosphorus, and potassium were detected by using the method of Jackson (1973) and Jackson, (1958).

Enumeration of Different Bacterial Population

Bacterial count was checked on diverse media such as nutrient agar, Ashby, Aleksandrow, and Pikovaskaya agar for total bacteria, nitrogen fixers (*Azotobacter*), potassium and phosphorus solubilizing bacterial count. Plates were incubated for 2–4 days at 30°C and bacterial colonies were counted. This analysis was performed in triplicate (Messer and Johnson 2000; Chai et al., 2015).

Soil Enzyme Activities

Fluorescein Diacetate Hydrolysis

One gram of soil, sodium phosphate buffer (50 ml, pH 7.6), and 0.5 ml of FDA solution were added in a flask and incubated for 1 h at 24°C. Acetone (2 ml) was added in the flask to stop the reaction, centrifuged for 5 min at 8,000 rpm, and filtered using Whatman filter paper. Enzyme activity was assessed at 490 nm and expressed as g fluorescein g^{-1} dry soil h^{-1} (Schnurer and Rosswall, 1982).

Dehydrogenase Activity

Triphenyl tetrazolium chloride (TTC) solution was used to estimate the dehydrogenase activity. Tris buffer (0.1 M, pH 7.4) and TTC solution (5 ml) were added in 5 g of soil and placed in an incubator for 8 h. Acetone (25 ml) was added in a reaction mixture to stop the reaction and centrifuged for 10 min at 4,000 rpm. Obtained supernatant was filtered and absorbance was measured at 485 nm (Casida et al. 1964).

Alkaline Phosphatase Activity

In a test tube, 1 g of soil, toluene (250 μl), modified universal buffer (MUB 4 ml), and p-nitrophenyl phosphate (1 ml, 25 mM) were added and incubated for 2 h at 37°C under shaking condition. Reaction was stopped using $CaCl_2$ and Tris buffer, centrifuged, and filtered using Whatman filter paper. Enzyme activity was measured by taking the absorbance at 400 nm (Tabatabai and Bremner, 1969).

β-Glucosidase Activity

One gram of soil was taken in a flask, and toluene (0.25 ml), p-nitrophenyl-D-glucoside (1 ml), and MUB (4 ml, pH 6) were

FIGURE 1 | Impact of nanocompounds and *Bacillus* spp. on soil health indicators under maize cultivation.

also added in the same flask. The mixture was incubated for 1 h at 37°C; tris buffer (4 ml) and $CaCl_2$ (1 ml) were added to terminate the reaction and centrifuged at 8,000 rpm for 10 min. The obtained supernatant was filtered and color intensity was measured at 415 nm (Tabatabai, 1994).

Amylase Activity

One gram of soil, 1 ml of starch, and phosphate buffer (2.5 ml, pH 6) were added in a flask. The flask was incubated for 6 h at 30°C and centrifuged for 10 min at 12,000 rpm. The obtained supernatant (1 ml) and 1 ml of dinitro salicylate (DNS) were added in a test tube and placed for 5 min in a water bath. Enzyme activity was calculated by taking the absorbance at 540 nm (Bernfeld, 1951).

Arylesterase Activity

One gram of soil, 2 ml of MUB, and p-nitrophenyl phosphate (0.5 ml, 200 mM) were added in a flask and kept for 1 h in a water bath. The mixture was centrifuged for 5 min at 6,500 rpm. The obtained supernatant (1 ml) and 2 ml of n-hexane were added in a test tube. Aqueous layer (0.5 ml) was taken; NaOH (0.5 ml) and 4 ml of distilled water were added. Enzyme activity was calculated by taking the absorbance at 400 nm (Nakamura et al. 1990).

Quantitative PCR Analysis of 16S rRNA

One gram of soil was used to isolate the DNA from different soil samples by using the DNA Purification Kit (HiMedia). Purity of DNA was checked at 260 and 280 nm. qPCR was performed in an iCycler iQ™ Multicolor instrument using universal primers (EUB 341F-5′CCTACGGGAGGCAGCAG 3′ and EUB 534R-5′ATTACCGCGGCTGCTGG 3′) to quantify the 16S rRNA gene (Muyzer et al. 1993). Total volume of reaction mixture was 25 µl containing both primers (0.5 µl), SYBR green supermix (12.5 µl), and soil DNA (1 µl).

Statistical Analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) using SPSS software 16.0. Significant differences were calculated using Duncan's test at $p < 0.05$. All analyses were made in triplicate.

RESULTS AND DISCUSSION

Physicochemical Analysis

Physiochemical analysis of the soil samples revealed significant variations in the chemical properties of treated soil samples. Soil pH showed variation in different treatments: T1 (7.2), T2 (7.4), T3 (7.5), T4 (7.44), T5 (7.6), T6 (7.9), T7 (7.65), T8 (7.7), and T9 (7.8). Different treatments showed enhanced level of total organic carbon, nitrogen, and phosphorus compared to control. Potassium was comparatively higher in T6 and T9 treatments (139.23 and 140.12 kg ha^{-1}) in comparison to other treatments (**Supplementary Material S3**). Variations in soil physiochemical properties can have a significant impact on the microbial population and, as a result, plant growth (Sui et al., 2021). Nanocompounds can improve nutrient mobilization, chelation, and release slowly, which could assist with nutrient utilization efficiency (Chaudhary et al., 2021c). A significant link between accessible macronutrients and soil microbial flora was found in this study, indicating that nanocompounds have a good impact on soil health. The treated soil had high levels of organic carbon, nitrogen, phosphate, and potassium, which could greatly boost the beneficial microbial population in maize rhizosphere soil.

Microbial Count on Different Media

Improved bacterial count over control was observed in nanocompound-treated soil at 50 mg L^{-1} concentration on nutrient agar. Level of bacterial counts (CFU g^{-1}) in different samples was 2.19×10^6 in T1, 2.42×10^6 in T2, 2.44×10^6 in T3, 2.43×10^6 in T4, 2.52×10^6 in T5, 2.56×10^6 in T6, 2.44×10^6 in T7, 2.52×10^6 in T8, and 2.53×10^6 in T9 after 60 days of sowing (**Table 1**). Nitrogen fixing bacterial count was higher in the combination of nanocompounds and bioinoculants while control had low N_2 fixing population. Counts of phosphate were significantly better in treated soil over control. The order for phosphate solubilizers was control having 8.75×10^5 (T1), 1.01×10^6 (T2), 1.04×10^6 (T3), 1.08×10^6 (T4), 1.23×10^6 (T5), 1.20×10^6 (T6), 1.07×10^6 (T7), 1.13×10^6 (T8), and 1.17×10^6 (T9) treatment, respectively (**Table 2**). Potassium solubilizing bacterial counts was highest in T9 (8.80×10^5) treatment followed by T6 (8.00×10^5), T5 (7.63×10^5), T8 (7.40×10^5), T4 (7.00×10^5), T7 (6.96×10^5), T3 (6.90×10^5), and T2 (6.80×10^5) respectively. Bacterial counts were significantly better in treated soil compared to control. Application of nanocompounds with test bacterial cultures enhanced bacterial counts in the rhizospheric soil of maize. Number of bacteria per gram soil was high in treated soil over control. Similarly, bacterial population involved in NPK recycling was high when maize was given a combined treatment of nanocompounds and *Bacillus* spp. Presence of these bacteria improves soil quality by providing essential nutrients to soil and then to the plants. Aziz et al. (2016)

TABLE 1 | Effect of nanocompounds and *Bacillus* spp. on total bacterial count and nitrogen fixers under maize cultivation.

Treatments	Total bacterial count			Nitrogen fixers (*Azotobacter*)		
	20D	40D	60D	20D	40D	60D
T1	$2.12 \times 10^6 \pm 3.00^a$	$2.23 \times 10^6 \pm 3.50^a$	$2.19 \times 10^6 \pm 4.80^a$	$5.50 \times 10^5 \pm 7.30^a$	$5.70 \times 10^5 \pm 5.12^a$	$5.91 \times 10^5 \pm 4.60^a$
T2	$2.32 \times 10^6 \pm 6.00^b$	$2.39 \times 10^6 \pm 3.00^b$	$2.42 \times 10^6 \pm 3.78^b$	$7.16 \times 10^5 \pm 7.63^b$	$7.33 \times 10^5 \pm 9.07^b$	$7.36 \times 10^5 \pm 9.60^b$
T3	$2.36 \times 10^6 \pm 5.68^c$	$2.44 \times 10^6 \pm 6.50^{cde}$	$2.44 \times 10^6 \pm 6.50^{cd}$	$7.23 \times 10^5 \pm 5.85^b$	$7.46 \times 10^5 \pm 6.80^b$	$7.60 \times 10^5 \pm 8.00^b$
T4	$2.40 \times 10^6 \pm 4.50^{cd}$	$2.43 \times 10^6 \pm 4.04^{cde}$	$2.43 \times 10^6 \pm 4.04^{cd}$	$7.50 \times 10^5 \pm 8.71^b$	$8.26 \times 10^5 \pm 5.68^b$	$7.63 \times 10^5 \pm 6.65^b$
T5	$2.50 \times 10^6 \pm 5.50^{de}$	$2.50 \times 10^6 \pm 6.02^{ef}$	$2.52 \times 10^6 \pm 7.00^{cde}$	$8.20 \times 10^5 \pm 9.16^b$	$8.36 \times 10^5 \pm 9.29^b$	$8.60 \times 10^5 \pm 8.54^b$
T6	$2.52 \times 10^6 \pm 7.02^e$	$2.53 \times 10^6 \pm 3.51^f$	$2.56 \times 10^6 \pm 6.02^e$	$8.16 \times 10^5 \pm 7.02^b$	$8.23 \times 10^5 \pm 6.50^b$	$8.63 \times 10^5 \pm 4.04^b$
T7	$2.34 \times 10^6 \pm 4.00^{bc}$	$2.41 \times 10^6 \pm 6.50^{cd}$	$2.44 \times 10^6 \pm 4.50^{cd}$	$7.00 \times 10^5 \pm 5.00^b$	$7.26 \times 10^5 \pm 4.04^b$	$7.50 \times 10^5 \pm 5.00^b$
T8	$2.48 \times 10^6 \pm 7.63^{de}$	$2.49 \times 10^6 \pm 5.00^{def}$	$2.52 \times 10^6 \pm 2.08^{cde}$	$7.33 \times 10^5 \pm 7.50^b$	$7.90 \times 10^5 \pm 7.54^b$	$7.96 \times 10^5 \pm 5.13^b$
T9	$2.47 \times 10^6 \pm 6.65^{de}$	$2.51 \times 10^6 \pm 5.00^{ef}$	$2.53 \times 10^6 \pm 5.56^{de}$	$8.03 \times 10^5 \pm 8.08^b$	$8.30 \times 10^5 \pm 9.53^b$	$8.56 \times 10^5 \pm 7.63^b$

Means in each column followed by the same letter were not significantly different ($p \leq 0.05$) as determined by two-way ANOVA and Duncan's Multiple Range Test (DMRT). Values were the means of three replications ± SD.

TABLE 2 | Effect of nanocompounds and *Bacillus* spp. on phosphate and potassium solubilizers under maize cultivation.

Treatments	Phosphate solubilizers			Potassium solubilizers		
	20D	40D	60D	20D	40D	60D
T1	$8.20 \times 10^5 \pm 4.24^a$	$8.59 \times 10^5 \pm 3.21^a$	$8.75 \times 10^5 \pm 3.60^a$	$5.28 \times 10^5 \pm 3.40^a$	$5.46 \times 10^5 \pm 3.50^a$	$5.60 \times 10^5 \pm 3.21^a$
T2	$9.70 \times 10^5 \pm 6.11^b$	$9.90 \times 10^5 \pm 5.56^b$	$1.01 \times 10^6 \pm 5.13^b$	$6.56 \times 10^5 \pm 4.50^{bc}$	$6.50 \times 10^5 \pm 5.56^{ab}$	$6.80 \times 10^5 \pm 6.00^{ab}$
T3	$1.01 \times 10^6 \pm 4.00^{bc}$	$1.00 \times 10^6 \pm 7.09^{bc}$	$1.04 \times 10^6 \pm 7.57^{bc}$	$6.60 \times 10^5 \pm 4.00^{bc}$	$6.76 \times 10^5 \pm 8.02^{bc}$	$6.90 \times 10^5 \pm 6.00^{bc}$
T4	$1.03 \times 10^6 \pm 7.00^{bc}$	$1.05 \times 10^6 \pm 9.60^{bcd}$	$1.08 \times 10^6 \pm 9.84^{bcd}$	$6.73 \times 10^5 \pm 1.52^{bcd}$	$6.93 \times 10^5 \pm 10.06^{bc}$	$7.00 \times 10^5 \pm 8.00^{bc}$
T5	$1.15 \times 10^6 \pm 5.00^d$	$1.20 \times 10^6 \pm 9.50^e$	$1.23 \times 10^6 \pm 5.50^e$	$7.20 \times 10^5 \pm 2.64^{cd}$	$7.46 \times 10^5 \pm 8.32^{bc}$	$7.63 \times 10^5 \pm 8.50^{bcd}$
T6	$1.18 \times 10^6 \pm 7.63^d$	$1.17 \times 10^6 \pm 9.84^{de}$	$1.20 \times 10^6 \pm 10.81^{de}$	$7.46 \times 10^5 \pm 2.51^{cd}$	$7.86 \times 10^5 \pm 3.21^c$	$8.00 \times 10^5 \pm 4.35^{cd}$
T7	$1.02 \times 10^6 \pm 7.21^{bc}$	$1.05 \times 10^6 \pm 5.00^{bcd}$	$1.07 \times 10^6 \pm 7.54^{bcd}$	$6.70 \times 10^5 \pm 10.44^{bcd}$	$6.93 \times 10^5 \pm 9.71^{bc}$	$6.96 \times 10^5 \pm 10.59^{bc}$
T8	$1.13 \times 10^6 \pm 7.57^{cd}$	$1.12 \times 10^6 \pm 8.88^{bcde}$	$1.13 \times 10^6 \pm 9.64^{bcde}$	$7.16 \times 10^5 \pm 6.65^{cd}$	$7.23 \times 10^5 \pm 5.50^{bc}$	$7.40 \times 10^5 \pm 5.00^{bc}$
T9	$1.15 \times 10^6 \pm 5.03^d$	$1.16 \times 10^6 \pm 4.72^{cde}$	$1.17 \times 10^6 \pm 4.35^{cde}$	$7.56 \times 10^5 \pm 3.51^d$	$8.03 \times 10^5 \pm 4.16^c$	$8.80 \times 10^5 \pm 8.54^d$

Means in each column followed by the same letter were not significantly different ($p \leq 0.05$) as determined by two-way ANOVA and Duncan's Multiple Range Test (DMRT). Values were the means of three replications ± SD.

reported that nano-formulations based on chitosan, zeolites, and clay, known to reduce the loss of nitrogen, had helped in enhancing nutrient uptake process in plant leaves. Pallavi et al. (2016) examined the impact of silver nanoparticle ($50\ mg\ L^{-1}$) concentration on total bacterial count, nitrogen fixers, and phosphorus solubilizers and found improved bacterial population in rhizospheric soil of *Brassica juncea* in India. Improved functional population of nitrogen fixers and potassium and phosphorus solubilizers was observed under the influence of SiO_2 in maize soil, but ZnO, TiO_2, and CeO_2 ($1\ mg\ g^{-1}$) decreased the microbial count through uptake of free ions released by nanoparticles in soil (Chai et al., 2015). Chaudhary et al. (2021b) reported that application of nanocompounds enhanced beneficial bacterial population of maize rhizosphere soil using metagenomics. Biochar along with *Bacillus megaterium* improved the soil urease activity and NPK concentration (Ren et al. 2019). Toxic impact of ZnO and TiO_2NPs ($1–2\ mg\ L^{-1}$) in microcosm on nitrogen fixing bacteria was observed using DNA-based fingerprinting (Ge et al., 2012). Total bacterial count and *Azotobacter* population were decreased when *Cambisols* treated with copper and zinc NPs (Kolesnikov et al., 2021). Bacterial consortium of *Bacillus* sp., *Agrobacterium tumefaciens*, and *Pseudomonas* sp. improved the NPK content in rhizosphere soil of wheat (*Triticum*) (Wang et al., 2020).

Soil Enzyme Activity

Soil of T5, T6, T8, and T9 treatments had highest activity of FDA hydrolysis, and the values of enzymes activity were 38.62, 40.58, 40.87, and 40.12 μg fluorescein $g^{-1}\ h^{-1}$ followed by 31.24, 30.79, 30.70, and 30.6 2 μg fluorescein $g^{-1}\ h^{-1}$ shown by T7, T3, T2, and T4 treatments, respectively, after 40 days of sowing. Minimum enzyme activity was observed in control (17.37 μg fluorescein $g^{-1}\ h^{-1}$). A gradual increase in FDA hydrolysis with time was observed after 20, 40, and 60 days of the experiment in all the treatments (**Figure 2**). Microbial population and activities of soil enzymes are important parameters to measure the quality of a soil. Activities of different enzymes act as an indicator to identify changes in soil quality, measurement of microbial diversity, and community structure (Yang et al., 2017; Chaudhary et al., 2021b). Nanoparticles may influence the activity and immovability of microbial enzymes. So, it is important to measure the specific enzyme activity, which can be used to identify changes in the soil environment, if any. FDA hydrolysis level was twofold higher in treated soil samples over control. It indicates that protease, lipase,

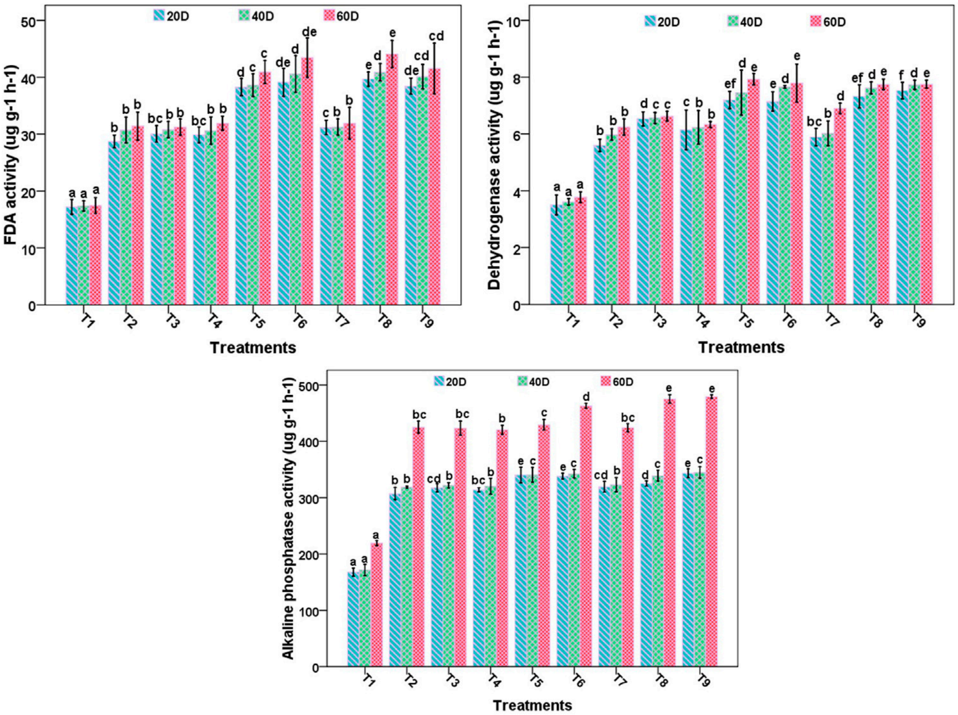

FIGURE 2 | Fluorescein diacetate hydrolysis, dehydrogenase, and alkaline phosphatase enzyme activities of soil in different treatments.

and esterase hydrolyzing bacterial population were enhanced by the application of nanocompounds and PGPR. The effect of nanochitosan, titanium oxide, and nanosilicon dioxide NPs was checked on soil enzymes, and a twofold increase in dehydrogenase and alkaline phosphatase was found (Kukreti et al., 2020; Kumari et al., 2020; Kumari et al., 2021). The presence of higher activity of FDA hydrolysis in treated soil might be correlated to availability of more substrate. The toxic effect of ZnO NPs on protease activity was due to dissolution of ions in treated soil when applied at the rate of 5 g in wheat soil (Du et al., 2011). Nanogypsum and *Pseudomonas taiwanensis* improved the soil enzyme activities by improving the nutrient status of soil reported by Chaudhary et al. (2021d).

Combined treatment of nanocompounds along with bioinoculants showed twofold increase in dehydrogenase activity over control. T5, T6, T8, and T9 treatments showed maximum dehydrogenase activity and were in the range of 7.45, 7.65, 7.62, and 7.73 μg $TPFg^{-1}\,h^{-1}$, followed by T3 (6.56), T4 (6.23), T7 (6.02), and T2 (5.97 μg $TPFg^{-1}\,h^{-1}$) after 40 days of sowing. Enzyme activity was consistent up to the end of the experiment. Minimum enzyme activity was found in control (3.61 μg $TPFg^{-1}\,h^{-1}$). Similarly, the highest phosphatase activity was observed in the treatment of nanocompounds with bioinoculants. The order of enzyme in the different treatments after 40 days was as follows: T1 (171.67), T2 (318.83), T3 (322), T4 (320.17), T5 (340.50), T6 (342.33), T7 (323), T8 (339), and T9 (344.83 μg pNP $g^{-1}\,h^{-1}$) (**Figure 2**). Treated soil showed up to 2-fold increases in enzyme activity compared to control. Our results showed a significant increase in dehydrogenase activity after different time intervals. Dehydrogenase is an intercellular enzyme, present only in viable cells and very sensitive to pollutants/heavy metals (Trevors, 1984). Increase in activity might be due to the increase in metabolic activities of bacterial population. Awet et al. (2018) observed a toxic effect of polystyrene nanoparticles (100–1,000 ng) on dehydrogenase activity due to the decrease in microbial biomass. An increase in dehydrogenase activity of up to twofold was found in a glass container by applying the nano CuO (Josko et al., 2019). The positive effect of Cu is due to the fact that it is used as a cofactor for enzyme activity. Alkaline phosphatase, a soil indicator enzyme, is involved in enhancement of soil fertility by mineralization of phosphorus. A positive correlation was observed between phosphorus solubilizing bacteria and alkaline phosphatase activity in soil. An increase in phosphatase activity may be due to the presence of more phosphate solubilizing bacteria in treated soil over control. Enhancement in dehydrogenase activity by 108.7% and alkaline phosphatase by 72% as compared to control was observed by Raliya et al. (2015) in mung bean (*Vigna radiata*) in the presence of TiO_2 (10 mg L^{-1}). Tarafdar et al. (2013) reported significant improvement of rhizospheric microbial population and activities of acid and alkaline phosphatase and phytase in cluster bean rhizosphere when treated with ZnONPs (10 mg L^{-1}). Application of nanophos, which consists of the phosphate solubilizing bacteria, also improved the different soil enzyme activities under maize cultivation (Chaudhary et al., 2021e). Silver NPs were not affected by the soil enzyme activities but decrease the

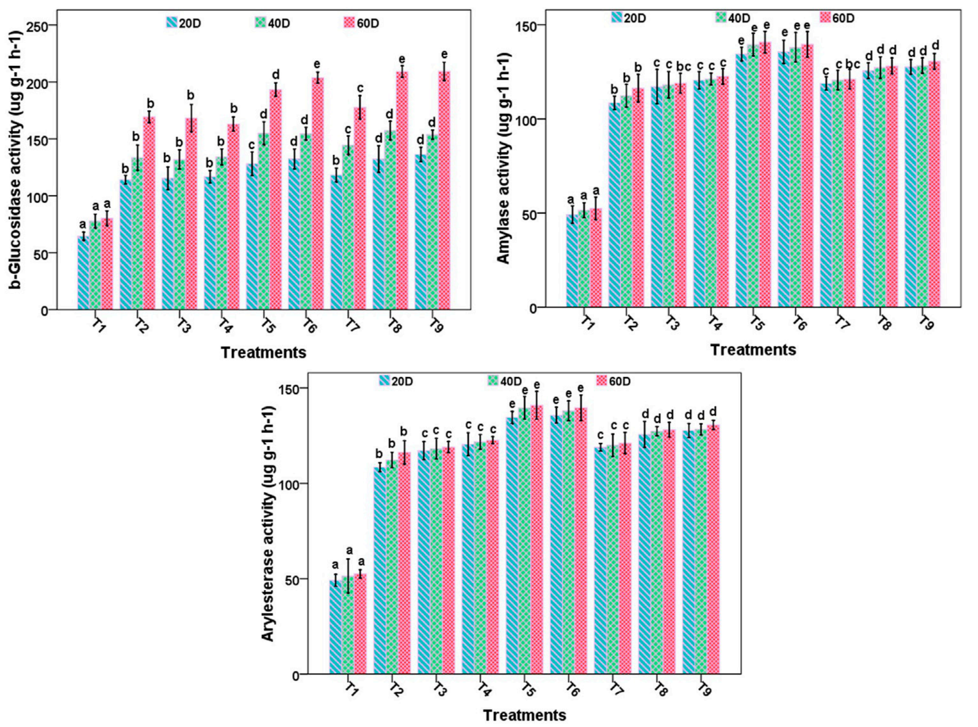

FIGURE 3 | β-glucosidase, amylase, and arylesterase enzyme activities of soil in different treatments.

actinobacterial population in tropical soil cultivated with *Coffea arabica* (Oca-Vasquez et al., 2020).

β-glucosidase activity was maximum in T8 and T9 treatments in the experimental soil throughout the experimental period. Twofold increases in glucosidase activity in all treated samples was observed over control (**Figure 3**). Amylase activity was highest (2.5 times than control) in T5 (140.90) and T6 (139.67 $\mu g\, h^{-1}$), respectively, followed by T9 (130.67), T8 (128.13), T4 (122.67), T7 (121.10), T3 (119), and T2 (116.23 $\mu g\, h^{-1}$). Least activity was observed in control (52.53 $\mu g\, h^{-1}$). Level of β-glucosidase was also high in treated soil in the present study. This enzyme takes part in the carbon cycle, which points out the existence of a higher population of microbes in treated soil. More enzyme activity indicated the presence of a high population of microbes involved in cellulose degradation in treated soil. Eivazi et al. (2018) reported inhibition of β-glucosidase activity in soil by nano silver NPs (3,200 $\mu g\, kg^{-1}$) over 1-month incubation. Li et al. (2017) reported that application of cerium oxide at different concentrations (100 and 500 $mg\, kg^{-1}$) increased phosphatase activity significantly due to the antioxidant property of NPs, which helps in the improvement of cell lifespan and strength in the soil grass microcosm system but observed a negative effect on β-glucosidase activity due to the accumulation of reactive oxygen species.

Amylase enzyme is involved in the conversion of starch to glucose and maltose. The level of this enzyme was higher in treated soil, which indicates that bacterial population responsible for carbon cycle was also high than control. The higher level of arylesterase activity in treated soil in comparison to untreated soil may be related to degradation of organophosphates and polymers in soil. Untreated soil (T1) had the lowest level (49.11–52.55 $\mu g\, h^{-1}$) of arylesterase activity in comparison to other treatments throughout the experiment. There was a twofold increase in enzyme activity from 20 days onwards in all the treatments (**Figure 3**). An increased level of enzyme activity is also a marker of action of microbes, that is, related to reprocessing of chemical elements by the enzymes (Tejada et al., 2006; Bastida et al., 2008). Our results revealed that nanocompounds along with bacterial culture did not have any toxic consequence on the soil enzyme activities. Cao et al. (2017) found that high concentration of iron oxide NPs (10 $mg\, kg^{-1}$) had a negative effect on bacterial abundance, but Arbuscular Mycorrhizal Fungi treatment altered the effect of nanoparticles and improved maize growth and bacterial abundance in test soil. He et al. (2011) did not find any significant increase in population size when soil was treated with Fe_2O_3 and Fe_3O_4 (1.26 $mg\, g^{-1}$). Mishra et al (2021) observed the toxic effect of silver NPs (100 $mg\, kg^{-1}$) on soil arylamidase and phenol oxidase enzyme activities.

qPCR Analysis of 16S rRNA Gene

A steady increase in copy number of 16S rRNA was observed in T6 and T9 treatments until the end of the experiment. Abundance of 16S rRNA was 2.57×10^7 and 1.98×10^7 in T6 and T9 treatments, respectively. After 60 days, the pattern of abundance of total bacterial gene in other treatments was: T7 > T5 > T2 > T8 > T3 > T4 > T1, which showed $5.87 \times 10^6 > 5.54 \times 10^6 > 4.29 \times$

TABLE 3 | Comparative 16S rRNA gene abundance at different sampling times as revealed by qPCR analysis.

Treatments	16S rRNA gene (per g of soil)		
	20D	40D	60D
T1	$4.70 \times 10^5 \pm 1.30 \times 10^2$	$4.31 \times 10^5 \pm 2.30 \times 10^2$	$4.22 \times 10^4 \pm 1.21 \times 10^2$
T2	$1.97 \times 10^6 \pm 2.20 \times 10^2$	$1.17 \times 10^6 \pm 1.16 \times 10^2$	$4.29 \times 10^6 \pm 1.67 \times 10^2$
T3	$5.30 \times 10^6 \pm 1.56 \times 10^2$	$4.82 \times 10^6 \pm 1.30 \times 10^3$	$1.95 \times 10^6 \pm 1.30 \times 10^2$
T4	$1.38 \times 10^6 \pm 1.42 \times 10^2$	$1.78 \times 10^6 \pm 1.29 \times 10^2$	$9.60 \times 10^5 \pm 1.34 \times 10^2$
T5	$1.74 \times 10^6 \pm 1.36 \times 10^2$	$3.57 \times 10^6 \pm 1.56 \times 10^2$	$5.54 \times 10^6 \pm 2.45 \times 10^2$
T6	$1.40 \times 10^6 \pm 1.16 \times 10^2$	$1.14 \times 10^7 \pm 1.29 \times 10^2$	$2.57 \times 10^7 \pm 2.28 \times 10^2$
T7	$7.21 \times 10^5 \pm 1.26 \times 10^2$	$6.02 \times 10^5 \pm 1.19 \times 10^2$	$5.87 \times 10^6 \pm 1.22 \times 10^2$
T8	$8.69 \times 10^5 \pm 1.06 \times 10^2$	$1.06 \times 10^6 \pm 1.12 \times 10^2$	$1.99 \times 10^6 \pm 1.29 \times 10^2$
T9	$6.91\times 10^6 \pm 1.18 \times 10^2$	$3.78 \times 10^6 \pm 1.20 \times 10^2$	$1.98 \times 10^7 \pm 1.19 \times 10^2$

Each value is the mean of three replicates. Values in ± indicate standard deviation of mean.

$10^6 > 1.99 \times 10^6 > 1.95 \times 10^6 > 9.60 \times 10^5 > 4.22 \times 10^4$, respectively, in terms of copy number (**Table 3**). Quantification of 16S rRNA showed high copy number in treated soil over control. This may be due to the positive effect of nanochitosan and nanozeolite on other bacterial populations, which helps in mobilization, chelation, and slow release of nutrients, improved the nutrient status of the soil, and enhanced plant growth (Alori et al., 2017; Agri et al., 2021). Titania nanoparticles and PGPR enhanced the valuable microorganism around roots and helped in the growth of wheat (Timmusk et al., 2018). Application of silver nanoparticles ($0.01\ \mathrm{mg\,kg^{-1}}$) significantly reduced the population of ammonia oxidizers (−17%) but have a positive impact on the population of *Bacteroidetes* and *Actinobacteria* (Grun et al., 2019). Overall observation determined that enhanced level of enzymes and bacterial population supported the biological efficiency of nanocompounds along with bioinoculants on maize.

CONCLUSION

The present study provides important implications of two nanocompounds on nutrient status, quality, and soil health if applied along with indigenous bioinoculants in maize. Application of combined treatments has potentially improved nutrient status, total microbial counts, nitrogen fixers, phosphorus, and potassium solubilizing bacteria in the experimental soil. Level of activities of signature soil enzymes was also improved in treated soil, which revealed that more nutrients are available to enhance the metabolic rate of soil bacteria. qPCR analysis also confirmed our observations as higher bacterial population in treated soil. The stimulation effect of nanocompounds was assumed to be increased due to better nutrient efficacy and survival of microbial population for longer duration by slow release of nutrients. The findings of the present study offer a possibility to use combined treatment of nanocompounds and bioinoculants in agricultural practices for better crop production as well as soil health.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PC: Conceptualization, wrote the manuscript, and participated in all the experiments; AC, PB, and GK: Visualization and editing the manuscript; HK and AR: Editing the manuscript; SK: Helped in qPCR analysis; AS: Experimental design and provided the laboratory facilities.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.769871/full#supplementary-material

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Biochar and Compost-Based Integrated Nutrient Management: Potential for Carbon and Microbial Enrichment in Degraded Acidic and Charland Soils

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Soil acidification and charland formation through alluvial sand deposition are emerging threats to food security in Bangladesh in that they endanger crop production in about 35% of its territory. The integrated plant nutrient system (IPNS) is a globally accepted nutrient management approach designed to revive the damaged soils' fertility level. Total organic carbon (TOC) in soil is a composite index of soil quality that has consequences for agricultural productivity and natural soil ecosystems. This study assesses the impacts of using biochar, compost, poultry litter, and vermicompost-based IPNS approaches on labile and TOC pools, TOC stocks, lability and management indices, and microbial populations under different cropping patterns after 2 years in acidic and charland soils. The application of IPNS treatments increased microbial biomass carbon (MBC) by 9.1–50.0% in acidic soil and 8.8–41.2% in charland soil compared to the untreated soil, with the largest increase in poultry manure biochar (PMB). Microbial biomass nitrogen (MBN) rose from 20 to 180% in charland soil compared to the control, although no effect was observed in acidic soil. Basal respiration (BR) rose by 43–429% in acidic soil and 16–189% in charland soil compared to the control, exhibiting the highest value in PMB. IPNS treatments significantly improved SOC and POC but did not affect POXc and bulk density in both soils. The PMB and organic fertilizer (OF, compost)-based IPNS wielded the greatest influence on the lability index of MBC in acidic soils and the management index of MBC in both soils. This is despite the fact that IPNS did not affect the lability and management indices of active carbon (AC). IPNS treatments increased the stocks of SOC and MBC in both the soils and POC stock in acidic soil. IPNS treatments significantly boosted the bacterial and fungal populations in both soils, despite having no effect on phosphorus-solubilizing bacteria (PSB). Thus, PMB and OF (compost)-based IPNS may be a better nutrient management practice in degraded acidic and charland soils. This is especially the case in terms of soil quality improvement, soil carbon sequestration, and microbial enrichment.

Keywords: poultry manure biochar, integrated nutrient management, microbial biomass, carbon pools, lability index, management index, microbial population

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INTRODUCTION

Acidic and charland soils currently constitute a major problem in soils comprising about 7.52 Mha of land and seriously affecting crop production in more than 36% of the territory in Bangladesh (Satter and Islam, 2010; FRG, 2018; Islam et al., 2021a; Uddin et al., 2021). Acidic soils have only small amounts of organic matter, low macronutrient availability, and micronutrient toxicity, thereby contributing to poor soil fertility (Kumar et al., 2019; Islam et al., 2021b). The poor fertility of charland soils is due to their coarse texture, low soil organic carbon (SOC) concentration, macronutrient content, and soil moisture retention capability (Ali et al., 2020; Rahman et al., 2021). Continuous and unchecked use of synthetic chemical fertilizers in these soils causes acute nutrient deficiencies and impairs soil physicochemical characteristics by increasing soil acidity, resulting in rapid degradation of soil health, productivity, stability, and sustainability (Selim and Al-Owied, 2017; Selim, 2018).

Conversely, the integrated plant nutrient system (IPNS), which combines chemical and organic fertilizers, is a widely accepted technology for sustaining soil health and its fertility, and supplying crops with their nutrient demands on a regular basis (Zhang et al., 2014a; Selim and Al-Owied, 2017; Selim, 2018; Jahangir et al., 2021a). Soil amendments such as poultry manure can significantly increase the SOC content, total exchangeable bases, pH, and microbial population (Kobierski et al., 2017; Islam et al., 2021a; Islam et al., 2021b). By contrast, biochar application in crop fields has attracted much research interest while its effects on soil quality and crop production are being debated. For example, applying biochar can improve plant growth by boosting soil microbial activity, soil moisture retention capacity, cation exchange capacity (CEC), and pH (Thomas and Gale, 2015; Gao et al., 2017; Sandhu et al., 2017; El-Naggar et al., 2018; Karimi et al., 2020). However, Deenik et al. (2010) and Dempster et al. (2012) observed diminished soil microbial biomass and activity when biochar was applied to soils. Likewise, Yu et al. (2018), Chen et al. (2018), and Zhou et al. (2020) reported that biochar has the potential to improve the population of bacteria, fungus, and phosphorus-solubilizing bacteria, as well as the total microbial diversity index. Very differently, Elzobair et al. (2016) discovered that biochar treatment (22 t ha^{-1}) wielded no great influence on the composition and abundance of microbes when compared to manure amendment. Lack of experimental evidence on biochar application as an IPNS approach in tropical nutrient-poor soils is hindering our understanding of the potential of biochar application for better soil health and crop productivity in these soils.

The SOC is a major driver of key soil ecosystem functions and subsequently, thus, quantitative and qualitative changes in SOC levels can exert a large effect on the functional components of terrestrial ecosystems (Li et al., 2018). It emerges as a vital component that shapes soil quality and crop growth performance (Kundu et al., 2007; Liu et al., 2014). In contrast, the amount of SOC is simply a balanced outcome of input and decomposition rates, but it cannot express variations in SOC attributes. It should be noted that because soils have a high C baseline and a high degree of heterogenicity, small or rapid changes in SOC are difficult to detect (Yang et al., 2005; Smith et al., 2020). Evaluation of the IPNS effects on carbon sequestration requires knowing not only the total amount of carbon but also the quality of carbon with regard to its pools and dynamics, which is not yet well reported. For example, labile organic carbon refers to fractions with a high activity level (Cheng et al., 2010), which has proven to be a useful metric for estimating soil quality and productivity (Sun et al., 2014; Yang et al., 2017) and an early indicator of changes in SOC due to management interventions.

Active carbon (AC) or permanganate oxidizable C (POXc), total microbial biomass C (MBC), potentially mineralizable C (PMC) and particulate organic C (POC) are the SOC pools deemed to be labile C (Islam and Weil, 2000; Vieira et al., 2007; Banger et al., 2010; Ghimire et al., 2012; Li et al., 2012; Yang et al., 2018; Zhang et al., 2020a). Furthermore, indices such as CPI, CLi, and CMI, derived from the SOC, SMB, BR, and POXc, respectively, have been proposed as sensitive and early indicators of SOC lability (Anderson and Domsch, 1993; Islam and Weil, 2000; Yang et al., 2018; Zhang et al., 2020a). Examining the changes in soil microbial diversity and SOC pools and indices as a function of organic amendments, including biochar, will provide new information about the management impacts on the sustenance of soil fertility.

At the location where the research took place, our understanding of SOC pools and soil microbial diversity as impacted by IPNS methods is largely limited. This research is the first attempt to investigate this issue in such soils. Our hypothesis is that using organic amendments, particularly biochar, in combination with chemical fertilizer as a means of IPNS will increase the concentration, stocks, and lability of TOC pools, as well as the soil microbial population. This is with reference to acidic and charland agroecosystems with a diverse cropping pattern. The objectives of our research were to: 1) measure the effects of different IPNS approaches on labile and TOC pools; 2) evaluate their impacts on TOC stocks, lability, and management indices; and 3) determine the microbial population under different IPNS approaches.

MATERIALS AND METHODS

Experimental Site and Soil Properties

The study focused on acidic soils in Madhupur, Tangail (24o59.82′ N, 90o03.99′ E) and charland soils in Islampur, Jamalpur (25o80.73′ N, 89o81.90′ E) in two farmers' fields with major cropping patterns at both sites. The climate in the region is subtropical monsoon, with an average annual temperature of 26°C, average annual rainfall of 1800 mm, and an average relative humidity of 85% (Local weather station). The general soil type of acidic soil in Madhupur, Tangail with a deep red to brown terrace soils (FAO and UNDP, 1988) is located in the Agro-ecological zone 28 (AEZ-28; Madhupur Tract), while charland soil in Islampur, Jamalpur with a Non-Calcareous Dark

TABLE 1 | Initial physical and chemical parameters of soil at Madhupur and Islampur site.

Soil properties	Madhupur site	Islampur site
Texture	Clay Loam	Sandy Loam
SOC (%)	0.99	0.53
TN (%)	0.09	0.05
Available P (ppm)	17.63	12.37
Available S (ppm)	14.95	10.53
Available K (meq. 100 g^{-1} soil)	0.12	0.12
pH	5.5	6.6
CEC (meq. 100 g^{-1} soil)	17.71	10.31
Soil moisture content (%)	21.69	17.28
Bulk density (g cm^{-3})	1.15	1.32

Grey Floodplain Soils (FAO and UNDP, 1988) is situated in Agro-ecological zone 9 (AEZ-9; Old Brahmaputra Floodplain). The initial physicochemical properties of soils in Madhupur and Islampur site are presented in **Table 1**.

Experimental Design and Management of Crop

At both locations, the experiment began in mid-October of 2018 and was completed by the end of November in 2020. The experiment was set up in a randomized complete block design, with the experimental area divided into four blocks representing the replications, in order to minimize the heterogenic effects of soil. The single plot size was 4 m × 5 m at both locations, with 0.75 m of inter-plot space, 1 m of inter-block space, and a total of twenty-four plots.

On a farmer's field at the Madhupur site, the treatments included poultry manure (PM), rice husk biochar (RHB), poultry manure biochar (PMB), and Dolomite whereas PM, PMB, compost (OF), and vermicompost (VC) served as treatments for a farmer's field on the charland soils at the Islampur site. At both locations, organic materials were applied at a rate of 3 t ha^{-1}, biochar at a rate of 2 t ha^{-1}, while the remaining nutrients were applied from chemical fertilizers derived from the IPNS approach on the basis of AEZ number and following the fertilizer recommendation guide-2018 (FRG-2018). The other two treatments at both sites were recommended dose (RD following FRG-2018), and no organic and inorganic fertilizer (control). Each manure had a moisture level of 15% when it was applied and the chemical compositions of the organic amendments are presented in **Table 2**.

At the Madhupur site, the cropping pattern was Mustard (*Brassica napus*)—Boro rice (Winter rice-*Oryza sativa L.*)–Transplanted Aman rice (monsoon rice–*Oryza sativa L.*), while the cropping pattern at Islampur site was Maize (*Zea mays*)—Jute (*Corchorus capsularis*)–T. Aman rice (monsoon rice). At both locations, three rice seedlings were transplanted per hill, with 20 cm × 20 cm spacing. Maize seed was sown in line at a rate of 20–25 kg ha^{-1} with a spacing of 60 cm × 20 cm. The seed rate of mustard was 6–7 kg ha^{-1} and seeds were subjected to line sowing with a distance of 25 cm between lines. Similarly, jute seed rate was 5–6 kg ha^{-1} and seeds were sown by line sowing with a 25 cm × 5 cm spacing. The crop varieties were BARI Sarisa-14 for mustard, BRRI dhan71 for T. Aman rice, BRRI dhan28 for boro rice, Kaveri 50 for maize and Tosha paat for jute, respectively.

Seeds of boro rice were sown in the seedbed between 15 and 29 November 2018 and transplanted to the main field from the last week of January to the first week of February 2019. Similarly, seeds of T. Aman rice were sown in the seedbed between 5 and 15 July 2019 and transplanted in mid-August 2019. Mustard seed was planted between mid-October and mid-November 2019 whereas jute and maize were planted in April, and between October and November 2019, respectively. The recommended fertilizer doses at the Madhupur site were: 90 kg N, 18 kg P, 40 kg K, 5 kg S, 1 kg B per ha for mustard; 144 kg N, 9 kg P, 60 kg K, 4 kg S, 1.5 kg Zn per ha for boro rice; and 90 kg N, 7 kg P, 50 kg K, 4 kg S, 1 kg Zn per ha for T. Aman rice. Likewise, the recommended fertilizer doses at the Islampur site were: 225 kg N, 40 kg P, 80 kg K, 30 kg S, 5 kg Mg, 2 kg Zn per ha for maize; 90 kg N, 8 kg P, 50 kg K, 8 kg S per ha for jute; and 90 kg N, 8 kg P, 50 kg K, 4 kg S per ha for T. Aman rice. The sources of N, P, K, S, Zn, and B were Urea, Triple superphosphate (TSP), Muriate of Potash (MoP), Gypsum, Zinc sulphate, and Boric acid, respectively.

During the land preparation, organic amendments, Dolomite, and all synthetic fertilizers except urea were applied. In the case of rice, urea was applied in three equal portions: 7–10 days after transplanting (DAT), 25–30 days after transplanting (DAT), and 5–7 days before panicle initiation (DAT). During the field preparation for maize, one-third of the urea and other fertilizers were applied. The rest of the urea was divided into two equal amounts. The 1st installment was applied at 8 to 10 leaf stage (30–35 days after sowing, DAS), while the 2nd one was applied at 60–65 DAS. Organic amendment, half of the urea, and all other nutrients were supplied during the land preparation in jute and mustard. The other half of N was applied at 40–45 days after seed sowing in jute and during the flowering stage in mustard.

In mustard, first irrigation was provided at 20–25 days after sowing (before flowering) and the second one within 55 days after seed sowing (during fruit setting). Depending on soil type and crop requirement, four to five irrigations were applied in maize and T. Aman whereas boro rice required eight irrigations during the cropping season. When the plants reached a height of 10–12 cm and had formed three to four leaves, jute was irrigated. Supplemental irrigation was applied to the crop as needed. The rice fields were irrigated a day prior to the land preparation and then as needed during the growing season to keep standing water at roughly 3 cm above the soil surface.

Three days before land preparation, a non-selective herbicide called Glyphosate (Round up®) was sprayed across the area at a rate of 1.85 kg ha^{-1}. Moreover, 7 days after transplanting rice seedlings, Pretilachlor (Superhit®, post-emergence herbicide) was applied at a dosage of 450 g ha^{-1}. To control rice insects, Brifer 5G and Cidial 5G (ACI Bangladesh Ltd.) were used as needed.

To control *Alternaria* leaf blight of mustard, seeds should be treated with Provax-200 before sowing and Ripcord 10 EC was

TABLE 2 | Chemical properties of organic amendments.

Organic amendments	OC%	TN%	pH	CEC (meq. 100 g^{-1} soil)	Available P (ppm)	Available K (meq. 100 g^{-1} soil)	Available S (ppm)
PM	8.56	2.05	8.3	12.29	839	6.34	1898
VC	7.57	1.08	7.7	11.83	1,020	4.99	377
OF	7.27	1.12	7.3	10.07	983	5.47	1,469
RHB	17.52	1.81	7.5	19.54	1,149	15.99	415
PMB	33.76	3.08	8.5	35.68	1,437	22.61	2094

PM , poultry manure; VC , vermicompost; OF , compost; RHB , rice husk biochar; PMB , poultry manure biochar.

sprayed to control cut worm. Diazinon 60 EC, Karate 2.5 EC and Dithane M-45 were sprayed to control major diseases and pests that can ruin jute. Contact insecticides like Ripcord 10 EC and DCC 100 EC were sprayed to control maize insects.

Soil Sampling and Analysis

Three soil samples were taken at 0–15 cm depth from geo-referenced sites in each replicated plot using a 10 cm internal diameter auger, composited, and kept in sealed plastic bags. At both the Madhupur and Islampur sites we collected a total of 24 soil samples. After sieving through a 2-mm mesh to remove visible organic residues and rock particles (if any), a portion of field-moist soil was treated and evaluated for MBC and associated biological characteristics. The remaining field-moist soil was air-dried for 2 weeks in the shade at room temperature (25°C) and then processed (2 mm sieved) for the purpose of physical and chemical analysis.

Soil Carbon Pools and Basal Respiration

The fumigation extraction method (Vance et al., 1987; Wang et al., 2020; Begum et al., 2021; Jahangir et al., 2021b) served to calculate microbial biomass carbon (MBC) from the following relationship:

$$MBC = F_c \times k_c,$$

Where, Fc is the difference between [(CO_2-C developed from fumigated soil, 0–10 days)–(CO_2-C evolved from non-fumigated soil)]. For 10-days incubations at 25°C, the proportion of microbial C evolved as CO_2 = 0.45. (Jenkinson and Ladd, 1981).

Brookes et al. (1985) described a method for determining microbial biomass N (MBN). In brief, fresh soil samples (10 g oven dry equivalent) were placed in tubes (six samples at a time), which were kept in a vacuumed desiccator containing 3 ml chloroform in another tube and incubated at 25°C for 72 h. Then the soil samples were mixed with 40 ml 0.5 M K_2SO_4, shaken at 300 rpm for 30 min and filtered to extract the MBC and MBN from the fumigated soil. Simultaneously, soil extraction was carried out on soils that had been incubated under the same conditions but without fumigation, these were referred to as non-fumigated samples. The wet oxidation method (Walkley and Black, 1934) was employed to examine MBC, while the semi-micro Kjeldahl method helped to analyze MBN (Bremner and Mulvaney, 1982). The anticipated differences between the fumigated and non-fumigated samples provided the MBC and MBN, respectively. The results for MBC and MBN were expressed as mg kg^{-1}.

Basal respiration (BR) was determined following the alkali absorption method for quantification of CO_2 evolution (Cheng et al., 2013; Diniz et al., 2020; Meena and Rao, 2021). In brief, a 20 g air-dried soil sample was adjusted to 60% of its water-holding capacity by adding water and pre-incubated at room temperature (25 °C) for 7 days. Then the pre-incubated soils were spread on the bottom of a 1-L glass jar in which a glass vial with 10 ml of NaOH (0.1 M) solution was hung. After 24 h of incubation at 25°C, 2 ml of 0.5 M $BaCl_2$ and two drops of phenolphthalein indicator were added to the glass vial, and then titrated with 0.1 M HCl. The controls were the jars that did not have any soil in them. The quantity of CO_2 evolution from soil microbes was calculated using the difference in consumed volume of HCl between the treatment and the control in titration; 1 ml of 0.1 M NaOH was equivalent to 2.2 mg of CO_2.

The SOC concentration was determined by following the standard wet oxidation method (Walkley and Black, 1934; Begum et al., 2021; Islam et al., 2021c). 1 g of ground, sieved, and oven-dried sample was placed into a 250 ml conical flask. Then 10 ml of 1 N $K_2Cr_2O_7$ solution was added to the soil and the flask was swirled gently to disperse the soil in the solution. After that, 20 ml of concentrated H_2SO_4 was added to the conical flask, and the flask was swirled until the soil and the reagent were mixed well. The conical flask was then placed in a hot water bath, with the stream directed into the suspension and heated until the temperature reached 135°C (about 12 min). After heating, the content in the conical flask was cooled for 300 min, and then 200 ml of deionized water and 10 ml of *ortho*-phosphoric acid were added. Following this, 2 ml of *o*-phenanthroline indicator were added to the content and titrated the solution with 0.5 M $(NH_4)_2$ Fe $(SO_4)_2.6H_2O$ until a dark green colour appeared. Particulate organic carbon (POC) was determined according to the method utilized by Cambardella and Elliott, (1992) and Begum et al. (2021). In short, 20 g of soil was dispersed in 100 ml of 5 g L^{-1} sodium hexametaphosphate solution by shaking for 15 h on an end-over-end shaker. The dispersed soil suspension was passed through a 53 mm sieve, and the material retained in the sieve was dried at 105°C after being rinsed several times with distilled water.

Organic carbon was measured in dried samples that were ground and analyzed. After mild oxidation of air-dried soil with a neutral $KMnO_4$ solution, the Permanganate oxidizable carbon (POXc) was quantified spectrophotometrically as a measure of chemically labile C pool (Weil et al., 2003; Begum et al., 2021). In brief, we used 20 ml of neutral $KMnO_4$ in water to react 1.0 g oven dry equivalent (ODE) of air-dried soil at concentrations of 0.005, 0.01, 0.0125, 0.025, 0.05, and 0.1 M. In screw-cap polycarbonate centrifuge tubes, the soil $KMnO_4$ suspensions were agitated at 200 rpm for 15 min at room temperature. To separate the dirt particles from the solution, the tubes were centrifuged at 3,000 rpm for 5 min after shaking. We then diluted 0.20 ml of the clear centrifuge with 10.0 ml of distilled water in a glass cuvette tube with a strong stream to ensure complete mixing. Using a Bosch and Lomb 2,500 spectrophotometer, we measured the absorbance of 565 nm light and compared the results to a standard curve made up of 0.20 ml of each unreacted KMnO4 solution and 10.0 ml pure water. The standard core method was used to determine the bulk density of the soil (McKenzie et al., 2004). The thermo-gravimetric method determined the antecedent soil moisture content (Black, 1965).

Soil Carbon Lability and Management Indices

Using the measured SOC, MBC, POC, and POX_C data, the CMI was calculated (Islam et al., 2021d; Begum et al., 2021; Franzluebbers, (2002)) as follows:

$$CMI = CPI \times CLI$$

where CPI is the C pool index and CL_I is the C lability index, which were calculated as:

CPI = TOC in the treatment soil/TOC in the control.

CL_I = CL in the treatment soil/CL in the control.where CL refers to the lability of C (CL = Labile C/Non-labile C).

The POXc pool consisted of the labile C pool, which was quantified as a percentage of SOC. By subtracting the POXc content from the TOC, the non-labile C pool was estimated. The calculated CMIs were normalized (CMI) by dividing the values with the highest CMI values in the database to a relative scale of >0 to ≤ 100. It was considered that higher CMI values are better indicators of SOC accumulation and lability in response to different treatments.

Soil Carbon Stocks

TOC, POC, POXc, and MBC stocks at 0–15 cm depth were estimated by multiplying their concentrations by 15 cm sample depth and measuring antecedent bulk density at the same time.

Microbial Population

The microbial population in soil was determined using the dilution spread plate technique as described in previous studies (Kakosová et al., 2017; Akande and Adekayode, 2019; Kumar et al., 2021). For bacteria and fungi, Nutrient Agar (NA) and Potato Dextrose agar (PDA) were the preferred growth media. To produce a dilution ratio of 10^{-6}, a 1 ml aliquot of the material was pipetted into a sterile test tube and serially diluted in six more test tubes, each holding 9 ml of sterile distilled water. 20 ml of cold (45°C) sterile molten agar medium was pipette separated aseptically into various sterile Petri dishes, spun gently for equal distribution of the inocula, allowed to set, and incubated at 30–37°C for 24 h (for bacteria), and at 25–27°C for 72 h (for fungi). At the end of the incubation time (24 h), bacteria colonies were counted and documented accurately, while fungus colonies were tallied and reported correctly at the end of the incubation period (72 h).

PSB was isolated and counted using a dilution plate method using hydroxy apatite media (Katzenelson and Bose, 1959; Narsian et al., 1994; Baliah et al., 2016; Ameena et al., 2020). The soil samples were serially diluted up to a 10^{-6} dilution, plated on Petri dishes, and incubated at 35 ± 2°C for 7 days to isolate PSB. At the end of the incubation period, PSB colonies were visually identified by the clear zone surrounding the bacterial colony. PSB was counted after incubation lasting 3–5 days by checking for a halo/solubilization zone around bacterial colonies that were surrounded by a turbid white background.

Statistical Analysis

A one-way analysis of variance (ANOVA) was done using different treatments as a random variable. Before using ANOVA, the data distribution was tested for normality. With the help of Statistix 10 software, the data were statistically evaluated to determine the significant differences in treatment effects. Using Tukey's multiple comparison test, a post-hoc test was used to find the differences between treatments. Unless otherwise stated, all statistical analyses were considered significant at $p \leq 0.05$.

RESULTS

The research work was conducted in two nutrient poor soil sites (i.e., acidic and charland soils) in Bangladesh to evaluate the impacts of different integrated plant nutrient systems (IPNS) on: firstly, soil labile and total organic carbon (TOC) pools; and secondly, indices and microbial populations under different cropping patterns. For this purpose, soil samples were collected after two consecutive years of field experiments to analyze soil chemical and biological properties following standard protocols. We hypothesized that integrated nutrient management would increase carbon sequestration and microbial populations in both the degraded soils.

Effects of Integrated Nutrient Management on MBC and MBN

At the Madhupur site, MBC was significantly influenced by different treatments ($p < 0.05$, **Figure 1A**) being higher in PMB than all other treatment combinations except the RHB and PM, where the latter two were also similar to each other. The MBC in the control was the lowest which was statistically identical to all the treatments except the PMB (ca. 275.0, 233.3, 225.0, 212.5, 200.0 and 183.3 mg kg^{-1} soil in PMB, PM, RHB, Dolomite + RD, RD and control, respectively) (**Figure 1A**). MBC increased by 9.1% in RD to 50.0% in PMB over the control.

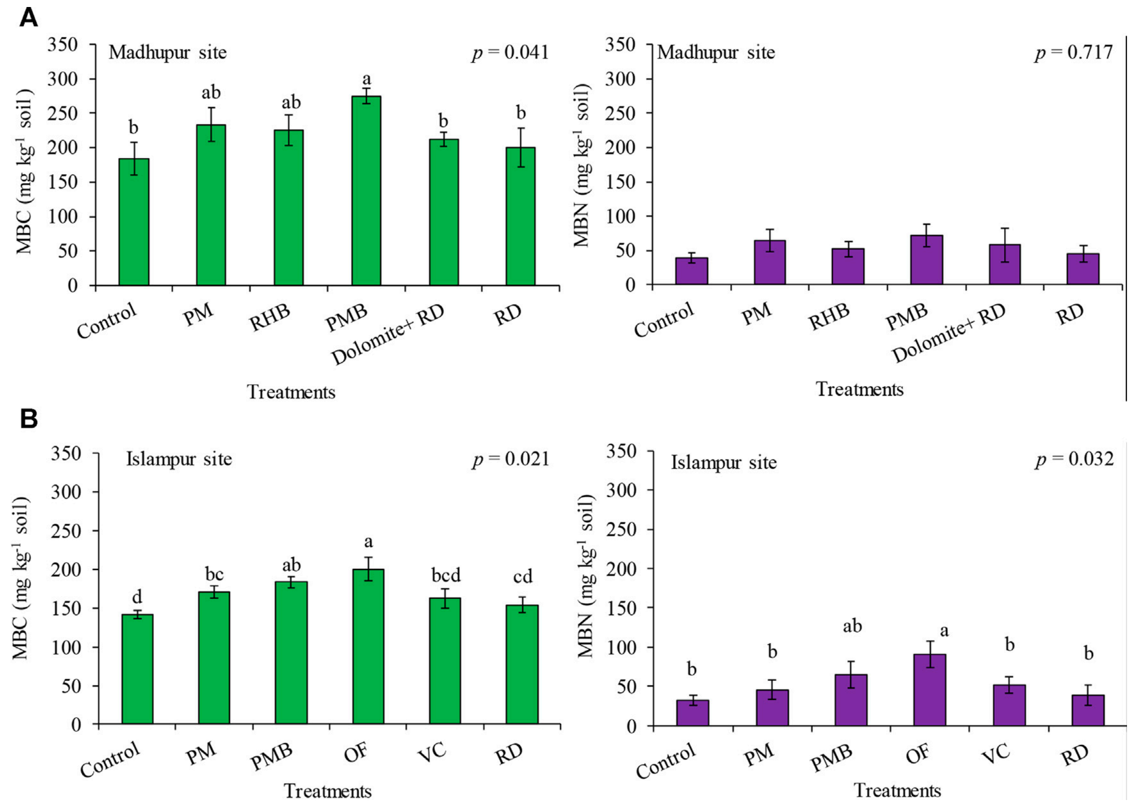

FIGURE 1 | Effects of different organic and inorganic treatments on MBC and MBN at Madhupur site **(A)** and Islampur site **(B)**. Data are average ± SE (n = 4); PM = poultry manure, RHB = rice husk biochar, PMB = poultry manure biochar, RD = recommended dose only from chemical fertilizer, OF = compost, VC = vermicompost.

In contrast, the effect of incorporating different organic and inorganic treatments to soil on MBN was not statistically significant (**Figure 1A**).

Likewise, at the Islampur site, different organic and inorganic treatments significantly influenced MBC and MBN (p < 0.05, **Figure 1B**). MBC in different treatments ranged from 141.7 to 200.0 mg kg^{-1} soil showing the highest value in OF (200.0 mg kg^{-1} soil), which was statistically similar to PMB (183.3 mg kg^{-1} soil) and the lowest in the control (141.7 mg kg^{-1} soil), being identical to VC (162.5 mg kg^{-1} soil) and RD (154.2 mg kg^{-1} soil). The MBC content in the soil increased from approximately 8.8–41.2% over the control. Similarly, MBN in different treatments ranged from 32.4 to 90.8 μg g^{-1} soil, exhibiting the highest value in OF (90.8 mg kg^{-1} soil), which was statistically similar to PMB (64.9 mg kg^{-1} soil), and the lowest in the control (32.4 mg kg^{-1} soil), which was identical to all the treatments except OF (**Figure 1B**). The MBN content in the soil increased by about 20% in RD to 180% in OF over the control.

Effects of Integrated Nutrient Management on Basal Respiration

Different treatments significantly influenced BR at both the Madhupur and Islampur sites (p < 0.05; **Figures 2A,B**). At the Madhupur site, the highest BR was observed in PMB and it was statistically similar to all the treatments except the control, whereas the lowest value was observed in the control. This was statistically identical to all the treatments except PMB. The mean BR was 154.17, 135.42, 72.92, 54.17, 41.67, and 29.17 mg CO_2-C h^{-1} kg^{-1} soil, in PMB, Dolomite + RD, RHB, RD, PM, and control, respectively (**Figure 2A**). The increase in BR due to the incorporation of different treatments varied from 43% in PM to 429% in PMB. Likewise, at the Islampur site, BR ranged from 39.58 to 114.58 mg CO_2-C h^{-1} kg^{-1} soil. The highest mean BR was observed in PMB and it was statistically similar to OF, while the lowest value was observed in the control, which was statistically similar to VC, RD, and PM (**Figure 2B**). The BR rose from 16% in PM to 189% in PMB over the control (**Figure 2B**).

Effects of Integrated Nutrient Management on Organic Carbon Pools

At the Madhupur site, application of biochar (PMB and RHB) and organic amendment (PM) based IPNS and inorganic fertilizer (Dolomite + RD and RD) had a significant impact on SOC content (p < 0.05, **Table 3**), being higher in Dolomite + RD than all other treatments except RHB, PMB, and RD, where the later three were also similar to each other. The SOC content in control was the lowest which was statistically similar to all the

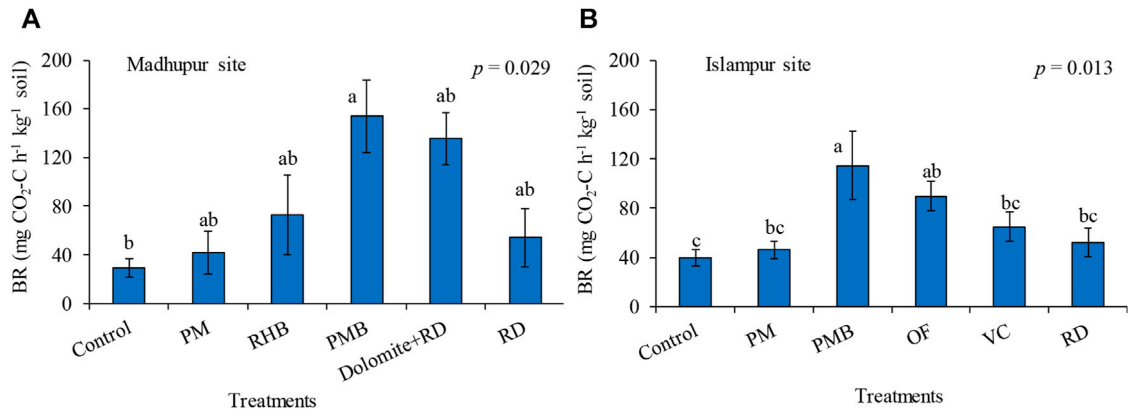

FIGURE 2 | Effects of different organic and inorganic treatments on basal respiration at Madhupur site **(A)** and Islampur site **(B)**. Data are average ± SE (n = 4); PM = poultry manure, RHB = rice husk biochar, PMB = poultry manure biochar, RD = recommended dose only from chemical fertilizer, OF = compost, VC = vermicompost.

treatment combinations except Dolomite + RD. The increase in SOC content in soil over the control ranged from 1.1% in PM to 23.6% in Dolomite + RD. Similarly, the POC content in soil was also significantly influenced by the organic and inorganic treatments ($p < 0.05$, **Table 3**), being higher in PMB which was statistically identical to all the treatments except in the control. The POC content in the control was the smallest which was identical to all the treatments except PMB and RD (**Table 3**). The increase in POC content in soil over the control ranged from 88.0% in PM to 176.2% in PMB. Unlike this, the effects of application of biochar (PMB and RHB) and organic amendment (PM) based IPNS and inorganic fertilizer (Dolomite + RD and RD) on POXc and soil bulk density were not significant (**Table 3**).

At the Islampur site, biochar (PMB and RHB) and organic amendment (PM) based IPNS and inorganic fertilizer (Dolomite + RD and RD) significantly influenced both SOC ($p < 0.05$) and POC ($p < 0.01$) content in soil (**Table 3**). The SOC content in soil varied from 4.9 to 6.8 g kg^{-1} soil in different treatments. The highest SOC content was determined in PMB which proved to be statistically similar to all the treatments except RD, whereas the lowest value was observed in RD. It was identical to all the treatments except PMB. SOC content in soil deminished over the control by about 0.9 and 11.8% when treated with PM and RD, respectively, whereas SOC content increased by 4.6, 9.1, and 20.9% in OF, VC, and PMB treated soil, respectively. Likewise, the POC content in soil varied from 642.8 to 1915.5 mg kg^{-1} soil in different treatments. The highest POC content in soil was observed in PMB, which was statistically similar to OF. Conversely, the lowest value was observed in the control, which was identical to all the treatments except PMB and OF. The increase in POC content in soil over the control ranged from 6.0% in RD to 198.0% in PMB. In contrast, application of biochar (PMB and RHB) and organic amendment (PM) based IPNS and inorganic fertilizer (Dolomite + RD and RD) did not affect POXc content and soil bulk density (**Table 3**).

Effects of Integrated Nutrient Management on Soil Carbon Lability and Management Indices

At the Madhupur site, the CL_I and CMI of AC were not significantly influenced by different organic and inorganic treatments (**Table 4**). In contrast, the CL_I and CMI of MBC significantly differed due to the application of different treatments ($p < 0.05$, **Table 4**). The CL_I of MBC ranged from 1.0 to 1.5 in different treatments. The highest value was observed in PMB, which was statistically similar to all the treatments except the control, whereas the lowest value was observed in the control; it was identical to all the treatments except PMB. Likewise, the highest value of CMI (1.8) was observed in PMB and it was identical to PM (1.6), and RHB (1.5), whereas the lowest value was noted in the control (1.1), which was identical to RD (1.3), Dolomite + RD (1.4), and RHB (1.5).

For the Islampur site, the CLI and CMI of AC were not significantly influenced by different treatments (**Table 2**). Similarly, the CL_I of MBC was not affected by different treatments (**Table 4**). In contrast, the CMI of MBC was significantly influenced by different treatments ($p < 0.05$, **Table 4**). The CMI of MBC ranged from 0.9 to 1.3 in different treatments, exhibiting the highest value in OF, which was statistically similar to PMB. In contrast, the lowest value was observed in RD, being identical to VC, control, and PM, respectively.

Effects of Integrated Nutrient Management on Soil Carbon Stocks

At the Madhupur site, TOC stock was significantly influenced by different organic and inorganic treatments ($p < 0.05$, **Table 5**), being higher in Dolomite + RD than all other treatments except PMB, RHB, and RD, where the latter three were statistically similar. The TOC stock in PM was the lowest, which was statistically similar to all the

TABLE 3 | Effects of different treatments on SOC, POC, POXc and bulk density at Madhupur and Islampur sites at a depth of 0–15 cm.

Madhupur site				
Treatment	**SOC (g kg^{-1} soil)**	**POC (mg kg^{-1} soil)**	**POXc (mg kg^{-1} soil)**	**Bulk density (g cm^{-3})**
Control	9.28 b	799.40 b	111.62	1.26
PM	9.38 b	1,502.80 ab	143.67	1.16
RHB	9.73 ab	1804.50 ab	120.62	1.22
PMB	10.77 ab	2,207.90 a	167.42	1.21
Dolomite + RD	11.47 a	1,574.90 ab	177.88	1.23
RD	10.48 ab	2061.70 a	165.38	1.24
SE	0.88	475.55	38.60	0.05
CV (%)	12.22	40.55	36.94	5.59
Level of significance	*	*	ns	ns
Islampur site				
Treatment	**SOC (g kg^{-1} soil)**	**POC (mg kg^{-1} soil)**	**POXc (mg kg^{-1} soil)**	**Bulk density (g cm^{-3})**
Control	5.60 ab	642.80 c	285.93	1.33
PM	5.55 ab	1,233.00 bc	287.88	1.32
PMB	6.77 a	1915.50 a	283.99	1.32
OF	5.86 ab	1,597.30 ab	270.38	1.29
VC	6.11 ab	1,179.50 bc	282.05	1.32
RD	4.94 b	681.40 c	270.38	1.35
SE	0.62	318.62	19.34	0.05
CV (%)	14.99	37.29	9.77	4.96
Level of significance	*	**	ns	Ns

SE, standard error of means, CV% = Co-efficient of variation, PM, poultry manure; RHB, rice husk biochar; PMB, poultry manure biochar; RD, recommended dose only from chemical fertilizer; OF, compost; VC, vermicompost. Data are mean ± SE (n = 4). Averaged values within a column, succeeded by different small letters (a, b, c), differ significantly between different treatments at p *< 0.05 significance level. * Significant at the 0.05 probability level. ** Significant at the 0.01 probability level. ns = non-significant.*

treatments except Dolomite + RD. The TOC stock fell by about 6.6% in PM compared to the control, while the highest increase (20.9%) in TOC stock was observed in Dolomite + RD (**Table 5**). Likewise, the POC stock was also significantly influenced by different treatments ($p < 0.05$, **Table 5**). The POC stock in soils treated with different treatments ranged from 1.5 to 3.9 Mg ha^{-1}. The PMB had the highest POC stocks, which was identical to all the treatments except the control, which exhibited the lowest POC stock, being identical to all the treatments except PMB. The increase in POC stock in different treatments over the control ranged from 74% in PM to 162% in PMB. In contrast, the impact of different treatments on POXc stock was not significant. On the other hand, different treatments significantly influenced MBC stock ($p < 0.05$, **Table 5**). The highest MBC stock was observed in PMB, which was statistically similar to PM, RHB, and Dolomite + RD, whereas the lowest value was observed in the control, which was identical to all the treatments except PMB. The increase in MBC stock in different treatments over the control ranged from 23.3% in RD to 66.7% in PMB.

At the Islampur site, different organic and inorganic treatments significantly influenced TOC stock in the soil ($p <$ 0.05, **Table 5**). The TOC stock in soils treated with different treatments ranged from 10.0 to 13.4 Mg ha^{-1}. The highest TOC stock was observed in PMB, which was statistically similar to all the treatments except RD, whereas the lowest value was observed in RD, which was identical to all the treatments except PMB (**Table 5**). The TOC stock in soil declined by 2.2 and 10.0% in PM and RD, respectively, compared to the control. Meanwhile, OF, VC, and PMB increased TOC stock over the control by 1.4, 8.9, and 20.3%, respectively. In contrast, POC and POXc stocks were not influenced by different organic and inorganic treatments (**Table 5**). Unlike, different treatments significantly influenced MBC stock ($p < 0.05$, **Table 5**). The highest MBC stock was observed in OF, which was statistically similar to PM and PMB, whereas the lowest value was observed in RD, being identical to the control, PM, and VC. MBC stock declined by 6.5% in RD compared to the control, whereas MBC stock increased by about 3.2, 9.7, 16.1, and 25.8% in VC, PM, PMB, and OF, respectively, over the control (**Table 5**).

Effects of Integrated Nutrient Management on Microbial Population

At the Madhupur site, the soil bacterial population was significantly influenced by different organic and inorganic treatments ($p < 0.05$, **Table 6**), being higher in Dolomite + RD than in all the treatments except RHB and PMB, where the later two were statistically similar. The bacterial population in the control was the lowest, which was statistically similar to all the treatments except Dolomite + RD (**Table 6**). Likewise, the soil fungal population was also significantly enhanced due to the addition of various organic and inorganic treatments ($p < 0.05$, **Table 6**). The highest fungal population (3.54×10^5 CFU g^{-1} soil) was observed in RHB, and the smallest number was observed in the control (2.68×10^3 CFU g^{-1} soil), which was statistically similar to all the treatments except RHB. In contrast, the addition of different treatments did not significantly influence the PSB population, while different treatments did significantly influence the B:F ratio in soil.

TABLE 4 | Effects of different treatments on carbon lability and management indices of active and microbial biomass at Madhupur and Islampur sites at a depth of 0–15 cm.

Madhupur site

Treatment	AC		MBC	
	CLI	CMI	CLI	CMI
Control	1.05	1.09	1.02 b	1.06 c
PM	1.40	1.54	1.48 ab	1.55 ab
RHB	1.03	1.14	1.41 ab	1.47 abc
PMB	1.41	1.67	1.53 a	1.83 a
Dolomite + RD	1.39	1.92	1.09 ab	1.40 bc
RD	1.31	1.52	1.16 ab	1.31 bc
SE	0.31	0.46	0.22	0.20
CV (%)	34.54	44.09	24.79	19.60
Level of significance	ns	ns	*	*

Islampur site

Treatment	AC		MBC	
	CLI	CMI	CLI	CMI
Control	1.02	1.02	0.97	0.97 bc
PM	1.04	1.01	1.17	1.05 bc
PMB	0.81	0.97	0.97	1.16 ab
OF	0.93	0.96	1.22	1.27 a
VC	0.85	0.97	0.85	0.98 bc
RD	1.05	0.94	1.02	0.90 c
SE	0.15	0.08	0.23	0.10
CV (%)	22.94	11.52	31.98	13.00
Level of significance	ns	ns	ns	*

*SE, standard error of means, CV% = Co-efficient of variation, PM, poultry manure; RHB, rice husk biochar; PMB, poultry manure biochar; RD, recommended dose only from chemical fertilizer; OF, compost; VC, vermicompost. Data are mean ± SE (n = 4). Averaged values within a column, succeeded by different small letters (a, b, c), differ significantly between different treatments at p < 0.05 significance level. * Significant at the 0.05 probability level. ns = non-significant.*

The highest B:F ratio was observed in PMB and it was identical to Dolomite + RD, while the lowest B:F ratio was observed in the control, which was statistically similar to PM, RHB, and RD (**Table 6**).

Referring to the Islampur site, different organic and inorganic treatments significantly influenced the soil bacterial population ($p < 0.05$, **Table 6**). The bacterial population in soil ranged from 1.13×10^7 to 2.96×10^7 CFU g^{-1} soil. The largest number of bacteria was recorded in soils treated with OF, which was statistically identical to all the treatments except PM, whereas the lowest number was observed in PM, which was identical to VC and RD. Similarly, the fungal population in soil was also significantly influenced by different organic and inorganic treatments ($p < 0.05$, **Table 6**). The highest fungal population was observed in soil treated with VC, which was statistically similar to OF, and the lowest one in RD. It proved to be identical to all the treatments except the control and VC. In contrast, the PSB population was not significantly influenced by the treatments. However, the B:F ratio in soil was statistically significant due to the incorporation of different treatments ($p < 0.05$, **Table 6**). The highest B:F ratio (7.75×10^3) was observed in PMB, which was statistically similar to RD (5.76×10^3) and OF (4.30×10^3), whereas the lowest ratio was noted in VC (2.07×10^3), which was identical to PM (2.24×10^3), control (3.04×10^3), and RD (5.76×10^3).

DISCUSSION

Effect of IPNS on Microbial Biomass Carbon and Nitrogen

Soil microbial biomass is an important biomarker for tracking rapid changes in soil quality due to human influences as well as agricultural management methods (Suman et al., 2006). MBC and MBN account for 3–7% of SOC and 1–5% of STN (Wu et al., 2007; Liddle et al., 2020). Our results demonstrated that IPNS treatments, especially biochar (PMB) and compost (OF)-based IPNS, significantly elevated MBC in both soils and MBN in charland soil. This is consistent with other research (Zhang et al., 2014b; Chaudhry et al., 2016; Liu et al., 2021). MBC and MBN increased by 8.8–50% and 20–180%, respectively, which was comparable with the recent work by Al-Suhaibani et al. (2020) who observed 20.0–47.5% and 32.2–65.2% increases in MBC and MBN, respectively, as a result of IPNS treatments. This is attributed to the decomposed organic manure and biochar itself being a carbon source, which provides an adequate energy supply in terms of C and N for various soil microbial biomass. The result is a heavy proliferation of soil microbial populations (Das et al., 2017; Li et al., 2017).

However, other studies discovered that: firstly, biochar application had no effect on soil MBC (Castaldi et al., 2011; Zavalloni et al., 2011); and secondly, MBC significantly diminished with biochar application while MBN remained undistorted in a coarse textured soil (Deenik et al., 2010; Castaldi et al., 2011; Zavalloni et al., 2011; Dempster et al., 2012). The build-up of organic compounds on the surface of the soil offers a substrate for increased microbial activity, which leads to greater MBC rates (Balota et al., 2004; Koković et al., 2021). The fastest mineralizable C and N in organic additions are the most important factors in biomass increase (Hao et al., 2008; Liu et al., 2010). A mixture of charcoal and inorganic fertilizer is more efficient for beneficial microbes in soil (Wardle et al., 2008; Brunn et al., 2012). The benefits flowing from IPNS treatments on MBN in charland soil are consistent with Kiem and Kandeler, (1997) findings that biological activity is greatest in coarse textured soils. This may be attributed to the more favorable habitat offered by the coarse textured soil for fungi in a microenvironment rich in bioavailable substrates formed by fresh unprotected organic manure. It results in the rapid mineralization of the substrate (Witzgall et al., 2021).

Effect of IPNS on Basal Respiration

Basal respiration is a measurement of fundamental recycling processes in soil that is assumed to represent the carbon available for microbial preservation (Dinesh et al., 2004; Vanhala et al., 2005; Li et al., 2019). Our findings strongly suggested that applying organic and inorganic fertilizers together boosted BR by 16–429% in acidic and charland soils, and this echoes what prior research has documented (Chang

TABLE 5 | Effects of different treatments on total organic carbon, particulate organic matter, particulate organic carbon, permanganate oxidizable carbon, microbial biomass carbon stocks at Madhupur and Islampur sites at a depth of 0–15 cm.

Madhupur site

Treatment	$Stock_{TOC}$ (Mg ha^{-1})	$Stock_{POC}$ (Mg ha^{-1})	$Stock_{POXc}$ (Mg ha^{-1})	$Stock_{MBC}$ (Mg ha^{-1})
Control	17.44 b	1.50 b	0.21	0.30 b
PM	16.28 b	2.61 ab	0.26	0.41 ab
RHB	17.82 ab	3.30 ab	0.22	0.41 ab
PMB	19.43 ab	3.93 a	0.30	0.50 a
Dolomite + RD	21.09 a	2.98 ab	0.33	0.39 ab
RD	19.49 ab	3.76 a	0.31	0.37 b
SE	1.62	0.85	0.07	0.05
CV (%)	12.32	40.05	37.48	18.72
Level of significance	*	*	ns	*

Islampur site

Treatment	$Stock_{TOC}$ (Mg ha^{-1})	$Stock_{POC}$ (Mg ha^{-1})	$Stock_{POXc}$ (Mg ha^{-1})	$Stock_{MBC}$ (Mg ha^{-1})
Control	11.16 ab	9.43	0.57	0.31 bc
PM	10.92 ab	9.34	0.57	0.34 abc
PMB	13.42 a	10.29	0.56	0.36 ab
OF	11.32 ab	9.33	0.53	0.39 a
VC	12.15 ab	8.94	0.56	0.32 bc
RD	10.04 b	8.68	0.55	0.29 c
SE	1.28	1.15	0.04	0.03
CV (%)	15.71	17.36	9.06	12.38
Level of significance	*	ns	ns	*

*SE, standard error of means, CV% = Co-efficient of variation, PM, poultry manure; RHB, rice husk biochar; PMB, poultry manure biochar; RD, recommended dose only from chemical fertilizer; OF, compost; VC, vermicompost. Data are mean ± SE (n = 4). Averaged values within a column, succeeded by different small letters (a, b, c), differ significantly between different treatments at $p < 0.05$ significance level. * Significant at the 0.05 probability level. ns = non-significant.*

TABLE 6 | Effects of different treatments on microbial population at Madhupur and Islampur sites at a depth of 0–15 cm.

Madhupur site

Treatments	Bacteria (CFU g^{-1} soil)	Fungi (CFU g^{-1} soil)	PSB (CFU g^{-1} soil)	B:F ratio
Control	3.75×10^6 b	2.68×10^3 b	3.71×10^5	1.63×10^3 b
PM	2.57×10^7 b	5.73×10^3 b	2.58×10^5	4.56×10^3 b
RHB	2.44×10^9 ab	3.54×10^5 a	2.76×10^5	1.52×10^4 b
PMB	1.77×10^9 ab	4.17×10^4 b	4.61×10^5	6.35×10^5 a
Dolomite + RD	3.07×10^9 a	4.63×10^3 b	4.65×10^4	6.23×10^5 a
RD	3.05×10^7 b	4.06×10^3 b	9.93×10^4	7.76×10^3 b
SE	1.24×10^9	1.41×10^5	2.20×10^5	2.20×10^5
CV (%)	142.95	291.09	123.29	144.88
Level of significance	*	*	ns	*

Islampur site

Treatment	Bacteria (CFU g^{-1} soil)	Fungi (CFU g^{-1} soil)	PSB (CFU g^{-1} soil)	B:F ratio
Control	2.95×10^7 a	1.00×10^4 ab	2.90×10^5	3.04×10^3 ab
PM	1.13×10^7 b	5.90×10^3 bc	1.87×10^5	2.24×10^3 b
PMB	2.39×10^7 ab	5.25×10^3 bc	2.07×10^5	7.75×10^3 a
OF	2.96×10^7 a	7.65×10^3 abc	3.26×10^5	4.30×10^3 ab
VC	2.28×10^7 ab	1.28×10^4 a	3.28×10^5	2.07×10^3 b
RD	1.69×10^7 ab	4.18×10^3 c	2.50×10^5	5.76×10^3 ab
SE	6.44×10^6	2.43×10^3	8.46×10^4	2.35×10^3
CV (%)	40.78	45.13	45.19	79.13
Level of significance	*	*	ns	*

*SE, standard error of means, CV% = Co-efficient of variation, PM, poultry manure; RHB, rice husk biochar; PMB, poultry manure biochar; RD, recommended dose only from chemical fertilizer; OF, compost; VC, vermicompost. Data are mean ± SE (n = 4). Averaged values within a column, succeeded by different small letters (a, b, c), differ significantly between different treatments at $p < 0.05$ significance level. * Significant at the 0.05 probability level. ns = non-significant.*

et al., 2008; Salehi et al., 2017; Li et al., 2018; Li et al., 2019). The increase in BR due to the addition of organic and inorganic fertilizers could be due to the increased amount of labile C in soils and microbial activities accelerated by inorganic phosphorus fertilizer addition, which will cause different impacts on soil microbial community and enzyme activities with N and P addition synchronously (Liu et al., 2019; Zhang et al., 2021). The positive effect of simultaneous application of inorganic N and P fertilizers on BR in forest ecosystems is also well documented in other recent studies (Zhang et al., 2020b; Xiao et al., 2020). According to Shrestha et al. (2013), cow manure had the highest BR, followed by compost, bare fallow, and cover crop treatments, yet Chang et al. (2008) detected no influence of soybean meal addition on BR. Besides, the addition of organic manures increased the quantity of organic matter available for soil respiration, resulting in increased BR. It also has the ability to greatly enhance both the number and activity of microorganisms, impacting both SOC transformation and CO_2 release and subsequently raising the microbial respiration rate (Zang et al., 2015). As a result, the treatments with organic manure in our study considerably enhanced soil CO_2 emissions and soil respiration rates.

Effect of IPNS on SOC, Carbon Pools and Bulk Density

The incorporation of manure amendment along with synthetic fertilizer enhanced SOC concentration by 0.9–23.6% in acidic and charland soils. Our finding is consistent with other analyses reporting an increase of about 2.9–46.7% in SOC content due to the application of both organic and inorganic nutrition sources (Libra et al., 2011; Roy and Kashem, 2014; Al-Suhaibani et al., 2020). Increased SOC supply from crop root exudation and high carbon content in readily biodegradable organic manures have resulted in an increase in SOC content (Yanardağ et al., 2014). The POC fraction is a labile SOC pool primarily composed of partially degraded crop residues not connected to soil minerals (Six et al., 2002). We observed a 6.0–198% increase in soil POC content and this is in line with previous research (Bongiorno et al., 2019; Zhang et al., 2020a). According to Liu et al. (2013) and Rudrappa et al. (2006), adding manure to the soil increased POC build-up. Increased POC is mostly due to increased subterranean biomass production, root exudation, and readily biodegradable organic input components (Purakayastha et al., 2008). (Zhang et al., 2021) reported that the POXc level of the soil had risen after a decade of wheat cultivation using synthetic fertilizer and residue application. Previous research has documented similar results (Rudrappa et al., 2006; Purakayastha et al., 2008; Cheng et al., 2010). Nevertheless, after 2 years of field trials, our findings revealed that IPNS had no effect on the build-up of POXc in soil. It may be due to the duration of the field experiment indicating that further long-term investigation is required.

Many studies have shown that including organic material decreases the bulk density of soil (Yazdanpanah et al., 2016; Ramos, 2017; Oladele et al., 2019). Surprisingly, after 2 years, we found no effect of IPNS on soil bulk density in either soil. This may be attributable to the balance in soil bulk density due to the combined application of organic and inorganic fertilizer.

Effect of IPNS on Carbon Lability and Management Indices and Carbon Stock

The carbon management index, which includes both the organic C pool and the C lability of the soil, may be used to assess the potential of management approaches to improve soil health. Incorporation of IPNS treatments into the soils had no impact on the lability and management indices of POXc (active carbon). After a short-term (2-years) trial, the IPNS treatments seemed to have no influence on POXc content in the soil, as expected. In previous studies, researchers reported an increased CLI and CMI after a long-term (11–32 years) combined fertilizer application (Benbi et al., 2015; Kumara et al., 2017; Tang et al., 2018). In our experiment, the CLI and CMI of MBC improved due to the use of IPNS treatments, which was comparable with other work (Benbi et al., 2015; Kumara et al., 2017; Tang et al., 2018). Such an outcome might be explained by soil microorganisms gaining access to organic carbon and other necessary nutrients (Kumara et al., 2017). Our results demonstrated that SOC, POC, and MBC stock increased significantly in both acidic and charland soils as a result of organic and inorganic inputs, which agrees with previous research (Maillard and Angers, 2014; Sun et al., 2014). This is due to the large C input through organic sources resulting in increased SOC, POC, and MBC content in the soil (Tang et al., 2018).

Effect of IPNS on Microbial Population

Soil microbes are crucial for OM decomposition, nutrient recycling, soil structure maintenance, insect and disease control, and the production of plant growth stimulants (Kirchman, 2018). Kumar et al. (2012) found that organic manures with or without chemical fertilizer had a beneficial impact on both the bacterial and fungal populations in soil. This could be due to the extra nutrients (N, P, K, and micronutrients) provided by the additives, which the bacteria break down for the plants to use. More exudates and plant compounds are released as a result of this breakdown product, which the rhizosphere bacteria can utilize. As a result, the bacterial biomass in the rhizosphere has increased (Richard and Ogunjobi, 2016).

Our results revealed that biochar-based IPNS increased the bacterial and fungal population in acidic soil, which was similar to what Narendrula-Kotha and Nkongolo (2017) and Alkharabsheh et al. (2021) reported. It might be due to its important properties such as pore space, surface area, porosity, minerals, surface volatile organic compounds, functional groups, free radicals, and high pH, which encourage bacterial development (Narendrula and Nkongolo, 2015; Gao et al., 2017; Zhu et al., 2017). Besides, biochar contains macronutrients like K^+, Mg^{2+}, Na^+, N, P, and other nutrients that can benefit microbial growth in the long-term upon release into the soil solution (Rodríguez-Vila et al., 2016). Some bacteria and fungus with pore sizes smaller than the pore size of particular biochars can inhabit these pores to shield themselves from soil predators. Furthermore, soluble compounds contained in the mesopores and micropores of biochar, such as sugars, alcohols, acids, ketones, and water molecules, can enhance microbial activity and affect microbial abundance and composition in soil (Seleiman et al., 2019; Adnan et al., 2020).

It should be noted that the microbiome remained statistically similar or smaller compared to the control in charland soil, which is a mystery that requires further investigation. In both soils, our findings revealed that the bacterial community population was frequently larger than the fungal community population in all treatments, as previously mentioned by Kamaa et al. (2011). It has been demonstrated in previous research that organic and chemical fertilizers help PSB (Richard and Ogunjobi, 2016). In both acidic and charland soils, however, we found that IPNS or chemical fertilizer exerted on influence on PSB. This may be attributed to: firstly, the smaller amount of PSB population present in the organic amendments; and secondly, the inhibitory effect of inorganic phosphate applied via chemical fertilizer on the growth of PSB (Kaur et al., 2017).

CONCLUSION

This research applied different organic and inorganic fertilizers for two consecutive years in two contrasting soil sites in Bangladesh, and evaluated soil microbial diversity, C pools and indices. Our findings suggest that combining various soil additions with chemical fertilizer had varying impacts on soil microbial biomass, basal respiration, carbon pools, carbon stocks, lability index, management index, and microbial population. Treatments with IPNS, especially PMB and OF (compost)-based IPNS, significantly enhanced MBC (29.4–50.0% compared to the untreated soil) and MBN (100–180% compared to the untreated soil) in both the soils, except MBN in charland soil. Likewise, IPNS treatments improved SOC and POC but did not affect POXc. The PMB and OF (compost)-based IPNS had the highest impact on the lability index of MBC in acidic soil and the management index of MBC in both soils, indicating increased C pools and stocks in both soil sites. The IPNS treatments significantly boosted the bacterial and fungal populations in both soils, although no effect was observed in the PSB. Based on our findings, PMB and OF (compost)-based IPNS can be recommended as the best nutrient management practices in degraded acidic and charland soils, especially in terms of soil quality improvement or restoration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MMR: Methodology, formal analysis, writing-original draft; MI: Conceptualization, methodology, funding acquisition, supervision, writing-review and editing; SU: Methodology, data analysis, writing-review and editing; MoR: Writing-review and editing. AG: Funding acquisition, writing-review and editing; AA: writing-reviewing and editing; MJ: Conceptualization, methodology, funding acquisition, supervision, writing-review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.798729/full#supplementary-material

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Predicting Soil Organic Carbon Dynamics of Integrated Crop-Livestock System in Brazil Using the CQESTR Model

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Land degradation and reduction in productivity have resulted in losses of soil organic carbon (SOC) in agricultural areas in Brazil. Our objectives were to 1) evaluate the predictive performance of CQESTR model for a tropical savannah; and 2) examine the effect of integrated management systems, including Integrated Crop-Livestock System (ICLS) scenarios on SOC stocks. Two long-term paddocks, under similar edaphic and climate conditions were used in this study. In Paddock 4 (P4) the rotation was corn (*Zea mays* L.) and 3.5/4.5 years pasture (*Urochloa ruziziensis*), while rotations in Paddock 5 (P5) included 2.5 years of soybean (*Glycine* max L.), dryland rice (*Oryza sativa* L.), and corn followed by 2.5/3.5 years pasture (*U. brizantha*). Measured and CQESTR simulated values were significantly (0.0001) correlated ($r = 0.94$) with a mean square deviation (MSD) of 7.55, indicating that the model captured spatial-temporal dynamics of SOC. Predicted SOC increased by 18.0 and 12.04 Mg ha^{-1} at the rate of 0.90 and 0.60 Mg ha^{-1} year^{-1} under current ICLS management for P4 and P5, respectively, by 2039. ICLS increased soil C sequestration compared to simple grain cropping systems under both NT and CT due to high biomass input into the production system.

Keywords: corn, cerrado biome, pasture, soil organic matter, soybean, neotropical savannah, no-tillage, dryland rice

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INTRODUCTION

Land-use changes or agricultural management practices can change soil organic carbon (SOC) stocks. Furthermore, the soil is an important carbon (C) pool and can be a source or sink of atmospheric CO_2 depending on the agriculture management adopted (Carvalho et al., 2010). In Brazil, unsustainable soil management has resulted in land degradation, loss of productive capacity of agricultural areas, loss of SOC, resulting in CO_2 emissions. It is estimated that 60–70% of Brazilian pastures show signs of degradation (Soares et al., 2020; Lapig, 2021), and up to 80% of the degraded pastures are in Central Brazil (Balbino et al., 2012). In general, pastures are considered degraded when they support very low stocking rates (<0.5 AU), show low plant cover, are invaded by non-palatable plant species, and are often densely populated with termite mounds (Boddey et al., 2004). Cardoso et al. (2016) report that the area of pasture required to produce 1 kg of a carcass (dead weight of animal) on a degraded pasture is approximately 320 m^2 but this falls to 71 m^2 on managed pastures, like mixed grass/legume.

Integrated Crop-Livestock Systems (ICLS) have emerged as an economically feasible management to restore degraded pastures (Cortner et al., 2019; Reis et al., 2019). Reis et al. (2019) report that the payback period of ICLS is shorter than continuous livestock or continuous cropping, and Cortner et al. (2019) showed that ranchers perceived ICLS as a necessity to maintain their livelihood amidst declining profits. In Brazil, integrated systems or mixed farming systems have received considerable attention from farmers and policy makers. They were part of the Low Carbon Emission Agriculture Plan (ABC Plan, known in Brazil as "Plano ABC", Brasil, 2012), which is the contribution of the agriculture sector to the National Climate Change Program, also launched as part of Brazil's Nationally Appropriate Mitigation Actions (NAMAs) in 2009, at the 15th UNFCCC-Conference of the Parties (COP 15). The ABC Plan consisted of six selected agricultural practices, one of which was integrated systems, supported by specific credit lines at low-interest rates for the period 2010–2020 (Mozzer, 2011; Brasil, 2012). Despite difficulties in measuring the effectiveness of positive incentive measures in agriculture such as the ABC Plan (Carauta et al., 2021), following the Paris Agreement during the 21st UNFCCC-Conference of the Parties, Brazil has presented a new edition of the ABC Plan for 2020–2030 (Plano ABC+, Brasil, 2021a), part of which is also included in the country's Nationally Determined Contributions (NDC, Brasil, 2020; Brasil, 2021b). The new edition of the ABC Plan aims at the additional adoption of 10 million hectares under ICLS and other integrated systems by farmers.

Well-designed ICLS both spatially and temporally allow better regulation of biogeochemical cycles and showing synergies between crop and livestock systems in system-wide evaluations of production and environmental quality (Franzluebbers et al., 2014; Lemaire et al., 2014). Long-term ICLS enables constant and efficient nutrient cycling because the animal, pasture, and crop residues release nutrients at different rates (Assmann et al., 2017). When grasses are intercropped, especially in the form of consortium (e.g., palisade grass planted during the same cycle as a grain crop), there is a higher biomass input and consequently higher nutrient recycling (i.e., N, P, K, and Mg) in the production system (Pariz et al., 2017). Measured nitrous oxide emissions are reduced in both ICLS (Sato et al., 2017) and ICLF (integrated crop-livestock-forestry), the most complex version of integrated systems with tree components (Franzluebbers et al., 2016; Carvalho et al., 2017). Integrated crop-livestock systems are also shown to improve nutrient cycling by re-coupling nitrogen (N) and C cycles (Ryschawy et al., 2017). Sant-Anna et al. (2017) investigated changes in SOC of pastures, crop production systems, and ICLS in the Brazilian Cerrado and found the highest SOC stocks under ICLS; however, not all ICLS increased SOC even under zero tillage. Oliveira et al. (2018) reported that soil N deficiency negatively affected SOC accumulation in ICLF and concluded that N management is a key to increasing SOC accumulation in integrated production systems. Damian et al. (2021) evaluated SOC changes in a poorly managed pasture with more intensively fertilized and diversified pasture systems (FP) and ICLS. They concluded that fertilization every year (FP) and the implementation of a cropping phase alternating with pasture (ICLS) resulted in the highest SOC stocks. When the pasture is recovered by inserting a crop (usually a grain crop), the residual fertilizer after cropping enhances pasture productivity. In this case, the cost of fertilizer application is covered by the income received for the crop (Kluthcouski and Yokoyama, 2003; Macedo, 2009). Meanwhile, perennial grasses such as brachiaria grass (*Urochloa* spp.) with large above-ground and root biomass contribute to SOC and increase in grain yields more than in a production system specialized for crop production only, particularly in years with poor rainfall distribution (Salton et al., 2014; Bieluczyk et al., 2020). Moraes Sá et al. (2014) reported a close correlation between SOC stocks and grain yield of soybean and corn from a long-term experiment in an Oxisol in southern Brazil under a subtropical humid climate. The added plant residues in ICLS differ in quantity and quality, being more recalcitrant compared with continuous pasture. Therefore, ICLS have been suggested as promising agricultural management contributing to the decarbonization of Brazilian agriculture (Tadini et al., 2021).

Assessing SOC dynamics in agroecosystems is challenging (Tornquist et al., 2009) mainly because of the complex interactions among components such as soil, vegetation, grazing animals, and humans (Godde et al., 2020). These changes often occur gradually and are difficult to detect in the short-term against the larger background (McGill et al., 1986; Ghani et al., 1996; Bolinder et al., 1999). Since the ICLS consists of a mixture of different grain crops often in rotation with several forage species (e.g., grass or legume), with varying grazing intensity and livestock densities, it can be difficult to determine whether the agricultural system is a C source or sink. Detecting small management-induced changes in SOC during short periods of time is difficult because of large differences in spatial and temporal SOC stocks (Kravchenko and Robertson, 2011). Long-term experiments, with historical datasets, have been used to assess impacts of past agricultural management practices on SOC dynamics. Process-based C models are potential research tools to predict the SOC changes; however, they need validation for the edaphoclimatic conditions of each region or country. Additionally, process-based C models are useful tools to analyze soil management options and to compare impacts of different management scenarios on SOC stocks. Thus, these models can be used to supplement field experiments to study SOC dynamics and to estimate the distribution of C in soil (Al-Adamat et al., 2007; Gollany et al., 2012). Furthermore, C models are useful in predicting the effects of potential management changes on the soil C stocks (Gollany et al., 2021). This makes it possible to test different scenarios and seek strategies to mitigate the negative impacts of such changes.

Carbon models have been used extensively for ecosystems and soil types under temperate conditions, while their evaluation under tropical conditions is less common (Kamoni et al., 2007; Tornquist et al., 2009; Damian et al., 2021). In these regions soil organic matter cycling is very different from that observed in temperate regions, because of the predominance of highly weathered acidic soils with low cation exchange capacity, high temperatures and high annual precipitation, where processes, including SOC decomposition rate, can be tenfold faster (Moreira and Siqueira, 2006). Given the importance of Brazil for global agriculture, it is necessary to validate C models and use them to investigate management options to increase SOC stocks in complex and diversified agriculture systems like integrated crop-livestock production.

Because of its relative simplicity, the readily available model inputs, and the possibility to compute SOC in a soil up to 5 layers, the CQESTR model was selected to study how different agricultural management practices affect soil C dynamics under tropical climate over time in two agroecosystems under ICLS. The CQESTR model was used previously to predict soil C dynamics in two tillage systems under tropical soils (Ultisol and Oxisol) in southeastern and northeastern Brazil (Leite et al., 2009); however, never used to predict SOC stocks under ICLS in Brazilian Cerrado. Due to the paucity of measured long-term SOC stocks data for the intensified and diversified pastures in Brazil, we hypothesize that process-based models can be an efficient and cost-effective tool to predict soil C stocks under several management practices and to project SOC stocks change for several ICLS scenarios. Therefore, the objectives of this study were to: 1) validate the CQESTR model for a tropical savanna (Cerrado) and predict the effect of several agricultural management systems and practices, including ICLS and no-tillage (NT); and 2) simulate the effect of conventional tillage and NT production scenarios on SOC dynamics in diversified ICLS.

MATERIALS AND METHODS

Study Site

The study was conducted at the "Capivara Research Farm" of the National Rice and Bean Research Center of the Brazilian Agricultural Research Corporation (Embrapa Rice and Beans), located in the Cerrado biome, in Santo Antônio de Goiás (16°28″ S, 49°17″ W; elevation 803 m. a.s.l), Goiás State, Brazil. The native vegetation is a semideciduous forest (cerradão) composed of about 30 tree species at a density of 2,800 individual plants ha^{-1}, of which seven (*Hirtella glandulosa* Spreng; *Hirtella gracilipes* (Hook.f.) Prance; *Protium heptaphyllum* (Aubl.) March.; *Tapirira guianensis* Aubl.; *Emmotum nitens* (Benth.) Miers.; *Copaifera langsdorfi* Desf; *Pterodon emarginatus* Vog.) are the most common (Silveira, 2010). The soil is a Typic Acrustox (clay, kaolinitic, thermic Typic Acrustox) or a Rhodic Ferralsol (WRB/FAO) or (Latossolo Vermelho Acriférrico Típico (Brazilian Soil Classification System)) with an average content of 524 g kg^{-1} clay and 349 g kg^{-1} sand. According to Köppen's classification, the climate is tropical megathermic savanna (Aw), where two well-defined seasons occur, a dry season from May to September and a rainy season, extending from October to April (IBGE, 2002). The average annual rainfall over the last 35 years (1983–2020) was 1,479 mm (Agritempo, 2014) of which about 90% was concentrated between October and April. The mean annual air temperature is 23.7°C.

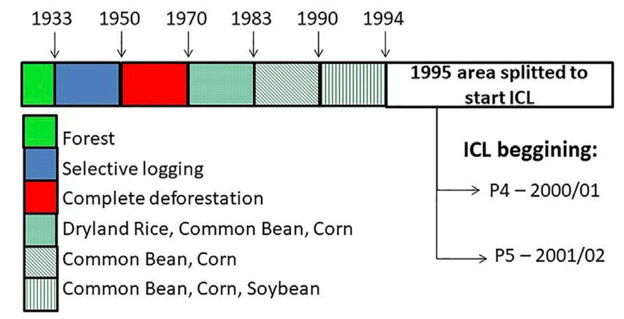

FIGURE 1 | Capivara Farm, State of Goiás, Brazil, site management history.

Crop-Livestock Management

Two areas under ICLS and a nearby area under native vegetation (reference site) were studied. The areas under ICLS are part of a Technological and Research Reference Unit (TRRU) which is part of the ILPF Network Association (Rede ILPF, 2021) that aims to accelerate the widespread adoption of crop-livestock-forestry integration technologies by rural producers as part of an effort aimed at the sustainable intensification of Brazilian agriculture. This TRRU is composed of six areas with sizes ranging from 5.1 to 9.2 ha.

The study area was covered by natural vegetation until 1933 when selective logging began and lasted until 1950 (**Figure 1**). Deforestation was completed in the 1970s when agriculture started. Common bean, dryland rice and corn were planted as main crops, including the areas currently under ICLS. In 1983 rice cultivation stopped and crop rotations changed to common bean and corn. In 1994, soybean was cultivated for the first time in the area, and 5 years later, soybean entered the rotation. Finally, in 1995, the area was divided into 6 paddocks as ICLS was gradually implemented between 2000–2005. Few variations in the rotation have occurred since 2000. The introduction of the pasture to the system occurred by planting corn in consortium with brachiaria grass. Two of the six long-term paddocks (**Figure 2A**) were selected for this simulation study, P4 (7.5 ha) and P5 (8.1 ha), because these two paddocks are under a long-term observation study including greenhouse gas (CO_2, water vapor and CH_4), soil, weather, and radiation sensors; these paddocks also have long-term soil data, including SOC. Initially, corn was the common crop used in the ICLS rotation, alternated with several years of pasture. The crop phase of the rotation became more diversified by introducing soybean, aerobic rice and common bean to the crop phase starting in 2007/2008, first in P5, then in P4 during 2013/2014. From 2014, there is a more pronounced presence of pasture in P5 compared to P4. The current rotation is based on the alternation of crops with a pasture phase (**Table 1**). The rotation in P4 includes 3 years of summer crops (rainy season) with pasture during the dry season and 2.5 years of pure pasture. In P5 the rotation consists of 3 years of summer crops with pasture in the dry season, and 3.5 years of continuous pasture phase. The whole system is conducted under no-tillage and pasture is always reintroduced by sowing corn in

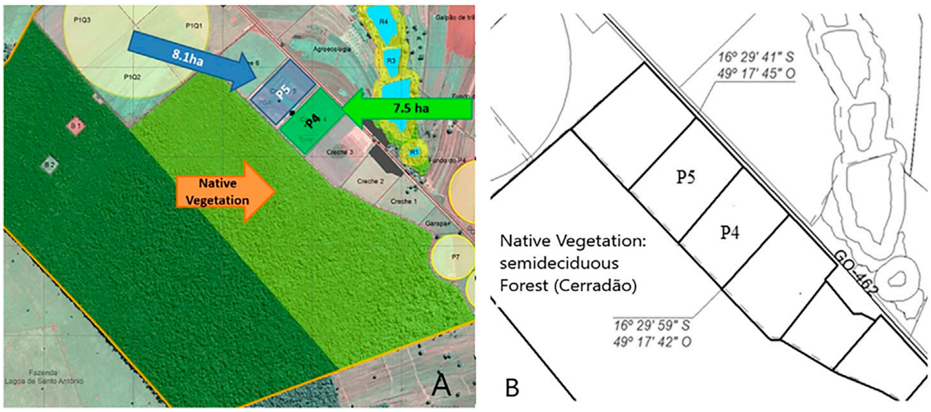

FIGURE 2 | Paddock 4 (P4) and Paddock 5 (P5) and native vegetation at Capivara Experimental Farm, Embrapa Rice and Bean in Santo Antônio de Goiás, GO, Brazil.

consortium with brachiaria grass. From 2020 on, another option for reintroducing pasture after the crop phase is a dryland rice-brachiaria grass consortium. The grass remaining after corn or rice harvest initiates the pasture phase. In 2020/2021, the summer crop was substituted by pasture in P4 because of the COVID-19 pandemic. Soil preparation until 1995 included tillage with a conventional disc plow (~10-cm depth) and leveling harrow (first operation at ~7-cm and a second at ~3-cm depth). Since then, exclusively direct seeding (no-tillage, NT) was used.

Fertilizers were applied according to soil fertility analysis and crop need at seeding or as top-dressing. Nitrogen fertilizer application in P4 varied from 0 to 288 kg ha^{-1} (averaged 49.6 kg N ha^{-1}): phosphorus 38–116 kg ha^{-1} (average 73.7 kg P_2O_5 ha^{-1}); and potassium ranged from 38 to 159 kg ha^{-1} (average 60.4 kg K_2O ha^{-1}); while application rates in P5 varied from 0 to 120 kg ha^{-1} (average 28.6 kg N ha^{-1}); phosphorous 26–183 kg ha^{-1} (average 86.2 kg P_2O_5 ha^{-1}); and potassium 31–129 kg ha^{-1} (average 65.2 kg K_2O ha^{-1}). Fungicides and insecticides were used when needed according to manufacturer's recommendation and control level. Glyphosate was used to control weeds and brachiaria grass growing to allow seeding in NT.

Soil Sampling and Analysis

In 2010, 2015 and 2020 samples were collected from the paddocks (P4 and P5) to 100 cm depth at 10-cm increments. Four main soil profiles were used in 4 quadrants of each studied site, and the samples were taken to 30-cm depth from the soil profile, and from 4 satellite points at a 5-m distance from each profile. A composite sample for each profile and depth was prepared from these sub-samples. In the other years, 30 individual samples were collected at 10 or 30 cm (0–10 cm in 1999 and 2013; 0–10, 10–30 cm in 2007) in a zigzag pattern. In this study, SOC stocks at 0–10 and 10–30 cm depths were used for each sampling year. In the case of quadrant sampling, each composite sample of 5 sub-samples represented 1.87 ha in P4 and 2.02 ha in P5; however, if considering sub-samples as individuals, the sampling density was 2.66 and 2.47 samples per ha for P4 and P5, respectively. In the case of zigzag sampling, each individual sample represented 0.25 and 0.27 ha, at the sampling density of 4.00 and 3.70 samples per ha, respectively. Machado et al. (2009) studied the spatial variability of SOC for 0–5, 5–10, and 10–20 cm in a 12.5 ha soybean field under NT in a clay (555 g kg^{-1}) Rhodic Ferralsol (Latosssolo Vermelho distroférrico, in the Brazilian Soil Classification System), comparable to our study area. They found that the recommended sampling density was a minimum of 0.64 samples per ha considering sampling depth between 0 and 20-cm depth. Before soil chemical analysis, plant tissues and other non-soil material was removed by hand and sieving. Air-dried soil samples were sieved in a 2 mm sieve. Soil samples were collected for SOC analysis using hand auger. The SOC was determined by the Walkley-Black method (Nelson and Sommers, 1996) without external heating, using sulfuric acid to generate internal heat for the reaction, and a correction factor of 1.3 to calculate SOC. The Walkley-Black method was used because the Dumas method (elemental analysis) was not available before 2010. We opted to use the same method for the sake of comparability of the SOC measurements.

The hydrometer method was used to determine clay, sand, and silt contents, with a standard hydrometer with Bouyoucos scale (Gee et al., 2002). Soil bulk density was determined with the soil core method using Kopecky rings (Grossman et al., 2002). Soil pH was determined in water (Thomas, 1996). Exchangeable calcium, magnesium and extractable potassium were extracted as described by Kuo (1996), then Ca and Mg were determined by atomic absorption spectroscopy and K by flame emission spectrometry (Wright and Stuczynski, 1996). Potential acidity was determined according to Silva (2009). Aluminum was extracted according to Bertsch and Bloom (1996) as modified by Silva (2009). Selected soil properties of the soil surface (0–10 cm depth) are shown in **Table 2**.

The CQESTR Model Data Inputs

Most data inputs required by CQESTR were provided by Embrapa and other required information was obtained from literature. Weather information, such as average daily air

TABLE 1 | Cropping sequences and tillage history of the two paddocks (4 and 5) under integrated crop-livestock systems at Santo Antônio de Goiás, Goiás State, Brazil (1990–2022).

Season Year	Paddock 4		Paddock 5	
	Summer	Winter	Summer	Winter
1990/91	Co. bean[a] CT[b]	Fallow	Co. bean CT	Fallow
1991/92	Corn CT	Fallow	Corn CT	Fallow
1992/93	Corn CT	Fallow	Corn CT	Fallow
1993/94	Soybean NT	Fallow	Soybean NT	Fallow
1994/95	Fallow	Co. bean CT	Fallow	Fallow
1995/96	Corn NT	Fallow	Corn NT	Fallow
1996/97	Corn NT	Fallow	Corn NT	Fallow
1997/98	Corn NT	Fallow	Corn NT	Co. bean NT
1998/99	Soybean NT	Fallow	Rice NT	Fallow
1999/00	Corn NT	Fallow	Corn NT	Fallow
2000/01	Corn + U (C)[c] NT	U (C)[d]	Soybean NT	Millet NT
2001/02	Soybean NT	Millet NT	Corn + U(C) NT	U(C)
2002/03	Corn + U (C) NT	U (C)	Soybean NT	Co. bean NT
2003/04	Rice CT	Fallow	Corn + U (P)[e] NT	U (P)[f]
2004/05	Corn + U (P) NT	U (P)	U (P)	U (P)
2005/06	U (P)	U (P)	U (P)	U (P)
2006/07	U (P)	U (P)	U (P)	U (P)
2007/08	U (P)	U (P)	Soybean CT	Co. bean NT
2008/09	U (P)	U (P)	Rice NT	Fallow
2009/10	Corn + U (P) NT	U (P)	Soybean NT	U (P)
2010/11	U (P)	U (P)	U (P)	U (P)
2011/12	U (P)	U (P)	Corn + U (P) NT	U (P)
2012/13	U (P)	U (P)	U (P)	U (P)
2013/14	Soybean NT	Fallow	U (P)	U (P)
2014/15	Rice NT	Sorgh + U(C)[g] NT	U (P)	U (P)
2015/16	Corn + U (P) NT	U (P)	Soybean (NT)	Fallow
2016/17	U (P)	U (P)	Rice NT	Millet NT
2017/18	U (P)	U (P)	Corn + U (P) NT	U (P)
2018/19	Soybean NT	Fallow	U (P)	U (P)
2019/20	Co. bean/Rice + U (P) NT	U (P)	U (P)	U (P)
2020/21	U (P)	U (P)	U (P)	U (P)
2021/22	Co. bean/Rice + U (P) NT	U (P)	U (P)	U (P)

[a]*Co. Bean, common bean.*
[b]*CT, conventional tillage; NT, no-till.*
[c]*Corn + U (C), corn with Urochloa spp. cultivated as a cover crop.*
[d]*U(C), Urochloa spp. cultivated as a cover crop.*
[e]*Corn + U (P), corn with Urochloa spp. cultivated for pasture.*
[f]*U (P), Urochloa spp. cultivated as a pasture.*
[g]*Sorgh + U (C), sorghum with Urochloa spp. cultivated as a cover crop.*

temperature and monthly rainfall, was provided by the weather station at Embrapa. Paddock 4 received 9.5 Animal Unit (AU) ha^{-1} and P5 received 8.8 AU ha^{-1}. The grazing rotations were 10 grazing days and 56 rest days per cycle. The amount of manure each paddock received was estimated at ~13 and ~12 Mg ha^{-1} $year^{-1}$ for P4 and P5, respectively. Embrapa provided information on above-ground biomass for *Urochloa* grass production, while below-ground biomass was estimated based on similar grass species root:shoot ratios (Bolinder et al., 2002). These grass species root:shoot estimates were successfully used ($r = 0.987$) in previous CQESTR simulations of reed canary grass and switchgrass pastures (Dell et al., 2018). For crop yield, we used an extensive dataset from the National Food Supply Company ["Companhia Nacional de Abastecimento"] (Conab, 2021). This information and harvest index were used to calculate the amount of above-ground crop biomass after harvest which was used to develop crop-specific yield files for each vegetation type. Average yields were calculated for different time spans where factors, such as weather and variety improvements, created obvious delineations in the yields for each crop individually. Corn yields of 2,225; 4,481; 4,939 and 7,620 kg ha^{-1}, dryland rice yields of 965 and 2,069 kg ha^{-1}, soybean yields of 1,845 and 2,801 kg ha^{-1}, and common bean yields of 522; 1,718 and 2,738 kg ha^{-1} were used in these simulations (Conab, 2021). Weeds and volunteer crop growth during fallow periods was estimated at 1,800 kg ha^{-1} (Pacheco et al., 2011). A 4,266 kg ha^{-1} residue yield was used for any millet crops; and yields for *Urochloa brizantha* and *U. ruziziensis* ranging from 4,850–12,736 and 3,100–15,095 kg ha^{-1}, respectively, were used for the simulation by duration of plant growth ranging from 0.5 to 4 years, based on data provided by Embrapa. A single pass no-till disc planter was used for seeding at 5-cm depth, in all years except summers of 2003 and 2007 for P4 and P5, respectively (**Table 1**). Prior to seeding of soybean in

TABLE 2 | Mean selected soil chemical properties and soil particle size distributions for the surface 10 cm for two paddocks under integrated crop-livestock systems and an adjacent forest at Santo Antônio de Goiás, Goiás State, Brazil.

Area	pH	Ca	Mg	Al	H + Al	K	Clay	Silt	Sand
	H2O	----- mmolc dm^{-3} -----				mg dm^{-3}	----- g kg^{-1} -----		
Paddock 4	6.3	14.6	8.5	0.0	22.0	296.5	534	144	321
Paddock 5	5.8	12.3	6.6	0.5	27.7	133.7	514	109	376
Forest	5.2	0.8	1.0	8.0	62.8	36.3	449	114	436

TABLE 3 | Soil bulk density and soil organic carbon content of each soil depth at Santo Antônio de Goiás, Goiás State, Brazil.

Soil depth	Year							Bulk density (g cm^{-3})
	1970	1999	2007	2010	2013	2015	2020	
	Soil organic carbon (g kg^{-1})							
Paddock 4								
0–10 cm	41.00	11.02	14.16	15.95	19.92	—	22.10	1.34
10–30 cm	21.83	—	12.53	12.18	—	—	14.56	1.36
Paddock 5								
0–10 cm	41.00	11.02	13.68	16.39	18.79	18.78	18.33	1.34
10–30 cm	21.83	—	10.60	11.75	—	11.50	13.81	1.35

those years, two passes were made with harrows at 3- and 9-cm depth.

Other information required by CQESTR: below ground biomass, nitrogen content of residue at decomposition initiation, fraction of pre-tillage residue weight remaining on the soil surface after each tillage, were based on literature (Santos et al., 2007; Leite et al., 2009; Pacheco et al., 2011; Mauad et al., 2012). Number and thickness of soil depths, SOC content and soil bulk density of each soil depth were provided by Embrapa (**Table 3**). The CQESTR input values used for the initial SOC in the 0–10 and 10–30 cm depths were 15 and 25 g kg^{-1}, and corresponding soil bulk densities were 1.34 and 1.35 g cm^{-3}, respectively, which were used for the model spin-up period dating back to native vegetation conversion to agricultural management in the 1970s as a starting point. Concentration of SOC (g C kg^{-1}) was converted to Mg C ha^{-1} using bulk density measured for each depth (**Table 3**) to determine soil mass per depth by area.

CQESTR Validation

The exact rotation (as shown in **Table 1**) was used in the simulations for each of the paddocks. Corn, soybean, and dryland rice were grown in annual rotations from 1990 until 2003 except for an entire fallow crop year in 1994 for P4 and during 1994/1995 for P5. From 2003 to 2039, a 5-years ICLS cycle was used starting with a crop in 2004/2005 for P4 and a 6-years ICLS cycle was used starting with a crop in 2003/2004 for P5, respectively, typified by a first year of a corn intercropped with pasture followed by 3- or 4-years of pasture only. For 3 years following pasture, soybean, dryland rice and common bean were grown in different phases before restarting the cycle. The yield file used for each year during the simulation was crop-specific, as described above, and a soil operation file, created specifically for each management operation, as well as estimated animal manure, were used in the simulations. The SOC dynamics in the 0–10 and 10–30 cm depths were simulated for 50 years (1990–2039), and the simulation results were compared to observed SOC stocks in 1999, 2007, 2010, 2013, 2015 (P5 only) and 2020 for the 0–10 cm depth, and in 2007, 2010, 2015 (P5 only), and 2020 for the 10–30 cm depth.

CQESTR Simulation of Crop Rotation Change Scenarios

At the end of the validation period (1990–2020), SOC was simulated for additional management scenarios to represent the adoption of cropping systems and the transition to ICLS with different crop rotations for nearly two additional decades (2021–2039). Two crop-livestock rotation simulation scenarios were prepared for the two paddocks. Paddock 4 had a 5-years rotation, which included corn as a summer crop followed by 4.5 years pasture. The rotation in P5 was 6 years and included soybean followed by fallow in the first year, dryland rice and fallow in the second year and corn followed by 3.5 years pasture. Actual crop biomass yields estimated pasture residue inputs and animal manure were used in the simulations.

Additionally, four scenarios were prepared including soybean and corn in rotation. In the first scenario, the same crop sequence as P4/P5 was used, but tillage was changed from NT to conventional tillage (CT). The second scenario included soybean as a summer crop followed by corn in a second harvest in the same crop year under no-till (Soy-CS(NT)); in the third scenario, soybean and corn alternated each year as summer crops under no-till (Soy/Corn(NT)) followed by a fallow period; and finally in the fourth scenario, soybean and corn alternated each year as summer crops under conventional

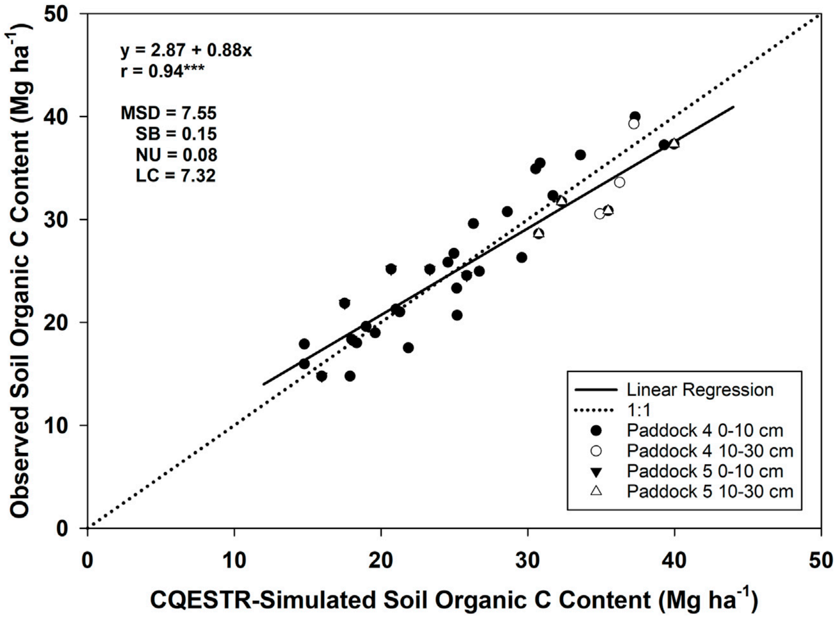

FIGURE 3 | Observed and simulated soil organic carbon (SOC) stocks at 0–10 and 10–30 cm depths for Paddock 4 and Paddock 5 in a Typic Acrustox soil under integrated crop-livestock system at Santo Antônio de Goiás, GO, Brazil. Dotted line refers to 1:1 correspondence of measured and simulated values. Observed and simulated data were significantly correlated (p < 0.0001) with a Pearson correlation (r) of 0.94. MSD, mean squared deviation; SB, squared bias; NU, nonunity slope; LC, lack of correlation.

tillage (Soy/Corn(CT)). Conventional tillage operations in the simulation consisted of tilling with a conventional disc plow (10-cm depth) and leveling harrow (first operation at 7-cm and a second at 3-cm depth).

CQESTR Model Evaluation

Model performance was evaluated as described by Liang et al. (2009) using regression analysis and mean square deviation (MSD) statistics. The three components of MSD are squared bias (SB), nonunity slope (NU) and lack of correlation (LC), which are entirely independent and related to terms of the linear regression equation (Y = a + bX) and the regression coefficient (r^2) (Gauch et al., 2003).

RESULTS

Evaluation of CQESTR Model Performance

The linear fit of simulated vs. observed SOC explained 94% of the variation (**Figure 3**). Regression analysis of 18 pairs of observed and simulated SOC values for Paddock 4 (P4) and Paddock 5 (P5) was significantly ($p < 0.0001$) correlated ($r = 0.94$), with a slope of 0.84 not significantly different from 1.0 for soil sampling depths of 0–10 and 10–30 cm (**Figure 3**). The total mean square deviation (MSD) was 7.55. It indicates that CQESTR can accurately predict measured SOC stocks at the two soil sampling depths at both ICLS sites without calibration. The MSD was partitioned into its components: lack of correlation (LC), square bias (SB), and non-unity slope (NU), and were 7.55, 0.15, and 0.08 Mg SOC ha^{-1}, respectively. The lack of correlation (LC), the highest contributing component of MSD accounted for 97% of the total MSD, which indicated that prediction errors were associated with data scattering and high standard deviation of observed SOC data. The square bias (SB) accounted for 2%, and non-unity slope accounted for 1% of the total MSD.

CQESTR Simulated Rotation Scenarios

CQESTR predicted the measured SOC values for P4 at both depths very well (r = 0.94), except underestimating the SOC values by 3.30 Mg SOC ha^{-1} in the top 10-cm depth in 2020 (**Figures 3**, **4**). CQESTR underestimated the measured SOC values for P5 by 4.14 and 4.33 Mg SOC ha^{-1} in the top 10-cm depth during 2010 and 2013 sampling periods and overestimated measured SOC values by 4.94 Mg SOC ha^{-1} in 10–30 cm during the 2015 sampling period. Measured and simulated SOC stocks increased in the topsoil for P4 more with time than for P5 (**Figure 3**). The measured SOC values for P4 (**Figure 4**) had a similar pattern to that in P5 (**Figure 5**), although the measured and simulated SOC stocks increased faster in P4 than in P5.

CQESTR Projected Soil Organic Carbon Stock and Rotation Scenarios

A comparison of simulated SOC stocks changes over 40 years for Paddock 4 (P4) and Paddock 5 (P5) indicates more SOC increase in P4 than in P5 (**Table 4**). CQESTR predicted SOC increase of 18.00 Mg ha^{-1} at a rate of 0.90 Mg ha^{-1} year^{-1} for P4, while the second largest SOC stocks increase of 16.36 Mg ha^{-1} predicted for P4 in the top 0–30 cm soil depth under CT with the same crop rotation as under NT. This increase exceeds the SOC increase of

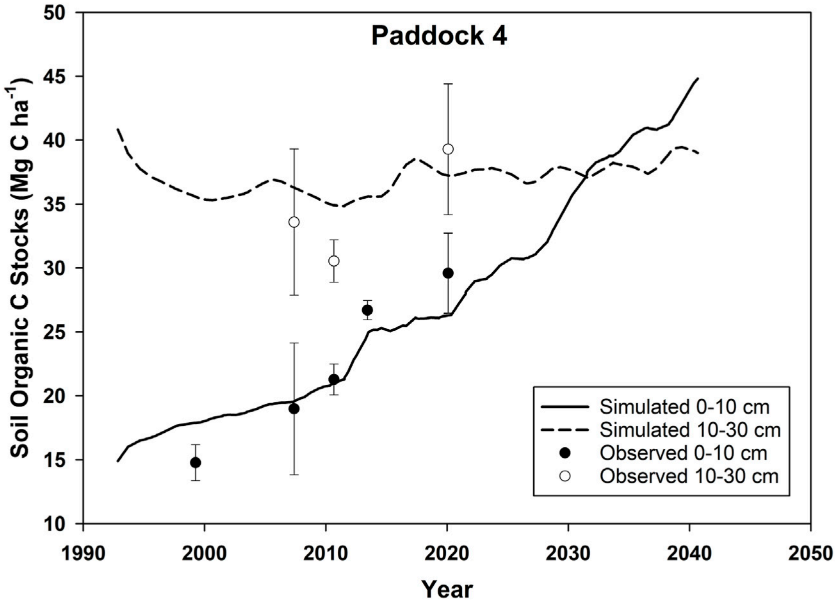

FIGURE 4 | Simulated and observed soil organic carbon (SOC) stocks at 0–10 and 10–30 cm depths for Paddock 4 area, in a Typic Acrustox soil under integrated crop-livestock system at Santo Antônio de Goiás, GO, Brazil.

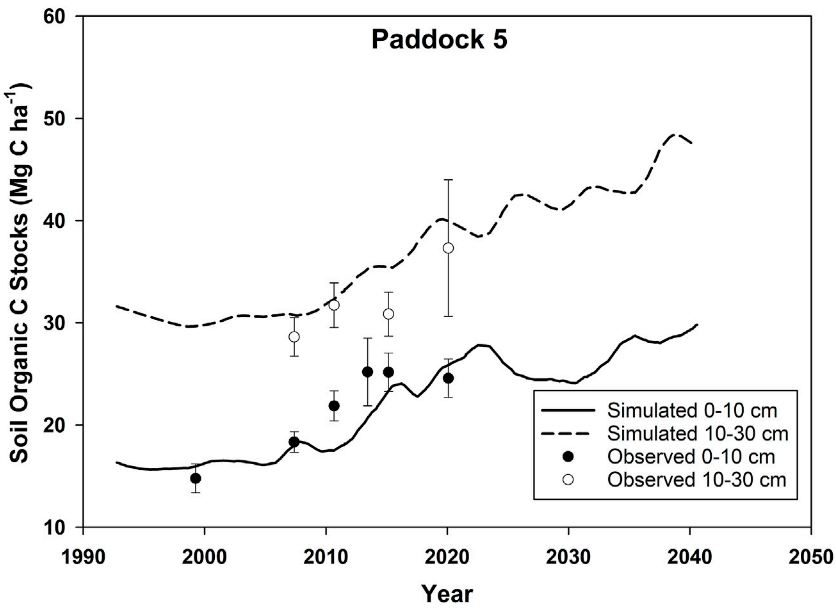

FIGURE 5 | Simulated and observed soil organic carbon (SOC) stocks at 0–10 and 10–30 cm depths for Paddock 5 area, in a Typic Acrustox soil under integrated crop-livestock system at Santo Antônio de Goiás, GO, Brazil.

12.04 Mg ha^{-1} at a rate of 0.60 Mg ha^{-1} year^{-1} predicted for P5 in the 0–30 cm depth by 2039. CQESTR also predicted a SOC increase of 11.46 Mg ha^{-1} at a rate of 0.57 Mg ha^{-1} year^{-1} for P5 under the CT scenario. An increase of SOC at 2.25 Mg ha^{-1} in the 0–30 cm soil depth for the Soy-CS(NT) in P5 is about half of 4.25 Mg ha^{-1} in P4 under the CT management scenario. Predicted SOC increase of 2.42 Mg ha^{-1} in P4 and 1.89 Mg ha^{-1} for P5 in the 0–30 cm soil depth are predicted under NT with Soy/Corn(NT), while decreases of 2.78 Mg ha^{-1} and 3.34 Mg ha^{-1} in the 0–30 cm soil depth predicted for P4 and P5 under Soy/Corn(CT), respectively.

TABLE 4 | Simulated soil organic carbon (SOC) stocks and rate of C changes for Paddock 4 and Paddock 5 between 2019–2039 under five management scenarios in a Typic Acrustox soil (Santo Antônio de Goiás, GO, Brazil). Bold values indicate the sum of total SOC content in the top 30-cm soil.

Scenario	Depth	SOC stocks			
	-- cm --	2019	2039	Δ	Rate
		--- Mg ha^{-1} ---			Mg ha^{-1} year^{-1}
Paddock 4					
ICLS4 (NT)[a]	0–10	26.10	42.58	16.48	0.82
ICLS4 (NT)	10–30	37.63	39.15	1.52	0.08
ICLS4 (NT)	**0–30**	**63.74**	**81.74**	**18.00**	**0.90**
ICLS4 (CT)[b]	0–10	26.10	37.04	10.94	0.55
ICLS4 (CT)	10–30	37.63	43.06	5.43	0.27
ICLS4 (CT)	**0–30**	**63.74**	**80.10**	**16.36**	**0.82**
Soy-CS (NT)[c]	0–10	26.10	26.15	0.05	0.00
Soy-CS (NT)	10–30	37.63	41.84	4.20	0.21
Soy-CS (NT)	**0–30**	**63.74**	**67.99**	**4.25**	**0.21**
Soy/Corn (NT)[d]	0–10	26.10	23.76	−2.34	−0.12
Soy/Corn (NT)	10–30	37.63	42.40	4.76	0.24
Soy/Corn (NT)	**0–30**	**63.74**	**66.15**	**2.42**	**0.12**
Soy/Corn (CT)[e]	0–10	26.10	20.73	−5.37	−0.27
Soy/Corn (CT)	10–30	37.63	40.22	2.59	0.13
Soy/Corn (CT)	**0–30**	**63.74**	**60.95**	**−2.78**	**−0.14**
Paddock 5					
ICLS5 (NT)	0–10	24.90	28.76	3.86	0.19
ICLS5 (NT)	10–30	39.83	48.01	8.18	0.41
ICLS5 (NT)	**0–30**	**64.73**	**76.77**	**12.04**	**0.60**
ICLS5 (CT)	0–10	24.90	28.69	3.79	0.19
ICLS5 (CT)	10–30	39.83	47.50	7.66	0.38
ICLS5 (CT)	**0–30**	**64.73**	**76.19**	**11.46**	**0.57**
Soy-CS (NT)	0–10	24.90	25.81	0.91	0.05
Soy-CS (NT)	10–30	39.83	41.17	1.34	0.07
Soy-CS (NT)	**0–30**	**64.73**	**66.98**	**2.25**	**0.11**
Soy/Corn (NT)	0–10	24.90	24.03	−0.87	−0.04
Soy/Corn (NT)	10–30	39.83	42.59	2.76	0.14
Soy/Corn (NT)	**0–30**	**64.73**	**66.62**	**1.89**	**0.09**
Soy/Corn (CT)	0–10	24.90	20.97	−3.93	−0.20
Soy/Corn (CT)	10–30	39.83	40.42	0.59	0.03
Soy/Corn (CT)	**0–30**	**64.73**	**61.39**	**−3.34**	**−0.17**

[a]*ICLS (NT); Integrated Crop Livestock System under no-tillage.*
[b]*ICLS (CT); Integrated Crop Livestock System under conventional tillage.*
[c]*Soy-CS (NT); Annual soybean winter crop followed by corn summer crop under no-tillage.*
[d]*Soy/Corn (NT); alternating year corn or soybean winter crop with fallow summer under no-tillage.*
[e]*Soy/Corn (CT); alternating year corn or soybean winter crop with fallow summer under conventional tillage.*

DISCUSSION

CQESTR Model Performance

The regression results illustrate that CQESTR accurately simulates long-term SOC dynamics under ICLS management for the tropical savannah conditions (**Figure 3**). The linear fit of simulated vs. observed SOC explained 94% of the variation with the MSD of 7.55, indicating that the model captured spatial-temporal dynamics of SOC in the topsoil well despite limited SOC data. Regression analysis for the long-term data from across North America (Liang et al., 2009), however, resulted in a higher *r* (0.98), feasibly due to a large number of pairs of predicted and measured data analyzed (306) and for the much wider range of SOM examined (7.3–57.9 g kg^{-1}). The relatively small (7.32 Mg ha^{-1}) lack of correlation (LC) between the observed and simulated values indicated that CQESTR prediction errors were mainly associated with data scattering. The factors such as the variability of SOC in the field, especially under paddock conditions, and sensitivity limits of the precision of SOC measurement related to sample collection, processing, and analysis. Leite et al. (2009) reported that square bias (SB) was the highest component of the MSD when simulating SOC under several tillage management systems under tropical conditions. CQESTR simulated SOC dynamics under different management systems in some soils better than others under tropical conditions (Leite et al., 2009).

Measured and simulated SOC stocks in the topsoil increased faster in P4 (**Figure 4**) than in P5. This increase in SOC stocks is most likely because *Urochloa* spp. is cultivated in P4 as a cover crop in the rotation for a longer duration than in P5. The measured SOC values in P4 (**Figure 4**) were similar to that for P5 (**Figure 5**). The rapid increase of SOC stocks in P4 and P5 is most likely due to the high capacity of these soils to retain SOC because of their high clay content (**Table 2**) and the presence of iron and aluminum oxides, which results in C stabilization (Leite et al., 2009; Bayer et al., 2011). Also, it could be due to the insufficient removal of plant and dung residues from the samples during soil preparation as evident from the large standard deviation of the samples (Gollany et al., 2013). However, the large standard deviation could also occur because the studies areas were production sites and not small plot experiments with replication.

Higher SOC values in the top 10 cm for P4 in 2010 and 2015 were observed (**Figure 3**), although during the ICLS, P5 had the more complex crop rotation cycle in that period. Both areas were under similar edaphic conditions; however, P4 had more time in the pasture phase than P5 (**Table 1**) (4.5 vs. 3.5 years pasture). Another reason could be the soil disturbance at seeding events, even under NT, which occurred more often in P5 at the beginning of the ICLS. Crop intensification started in P4 after 2013/14. In 2019/20, common bean and rice + *Urochloa* were planted during the summer season, which added more crop residues to P4. The rice + *Urochloa* consortium is a modification in the rotation of P4 to increase biomass and C input into the system.

Additionally, P4 is smaller (7.5 ha) than P5 (8.1 ha) but has received the same total amount of manure. Therefore, it is expected to reach the pre-agricultural SOC stocks earlier than P5 (**Figures 3**, **4**). Also, the influence of grazing cattle stimulates root growth and exudate production, which can modify the ratio of root and above-ground biomass and the quality of the C added to the soil (Bayer et al., 2011) and consequently influence C stocks and soil organic matter decomposition in the soil profile.

Paddock 5 received more legumes, typically soybean, than P4 and this could be another reason for the difference in SOC stocks of the two paddocks. Soybean with high biological N fixation efficiency is usually cultivated without N fertilizer, using only inoculation with bacteria of the genus *Rhizobium* spp. Most of this N is exported in grains, and the negative or null net N balance in cropping systems reduces biomass yields and C accumulation

TABLE 5 | Annualized biomass inputs for Paddock 4 (P4), Paddock 5 (P5) and future rotation scenarios.

Rotation	Tillage	Rotation cycle	Dates	Annualized biomass input			Annual aboveground
				Aboveground	Root	Total	Biomass N input
		---- years ----		---- Mg ha^{-1} year^{-1} ----			--- kg ha^{-1} year^{-1} ---
Historic							
Paddock 4	CT		1990–2021	14.3	8.5	22.8	169
Paddock 5	CT		1990–2021	15.0	8.7	23.8	177
Scenarios							
P4 ICLS[a]	NT	5	2022–2039	24.7	17.1	41.8	309
P5 ICLS	NT	6	2022–2039	19.3	11.9	31.2	230
P4 ICLS	CT	5	2022–2039	24.7	17.1	41.8	309
P5 ICLS	CT	6	2022–2039	19.3	11.9	31.2	230
Soy-CS[b]	NT	1	2022–2039	13.3	3.9	17.2	145
Soy/Corn[c]	NT	2	2022–2039	9.5	3.1	12.6	107
Soy/Corn	CT	2	2022–2039	9.5	3.1	12.6	107

[a]*ICLS; Integrated Crop Livestock System under no-tillage.*
[b]*Soy-CS; Annual soybean winter crop followed by corn summer crop under no-tillage.*
[c]*Soy/Corn; alternating year corn or soybean winter crop with fallow summer under no-tillage.*

(Sisti et al., 2004; Urquiaga et al., 2006; Bayer et al., 2011). These authors recommend the introduction of legumes as green manure in the rotation to promote soil C sequestration. According to Sisti et al. (2004) and Souza et al. (2009), N from legumes that biologically fix N can promote more C accumulation than N from mineral sources. These studies, however, were carried out in long-term experiments of grain crop systems. Our understanding of the C and N cycles in complex systems such as ICLS is still limited (Soussana and Lemaire, 2014).

CQESTR Simulated Scenarios

A comparison among the simulated scenarios by 2039 indicate differences in SOC stocks at the 0–10 cm depth (**Table 4**). Paddock 4 under NT had the largest SOC stocks increase of 42.58 Mg ha^{-1} (28%) at a rate of 0.82 Mg ha^{-1} year^{-1}, exceeding SOC stocks increase of 28.76 Mg ha^{-1} (19%) at a rate of 0.19 Mg ha^{-1} year^{-1} for P5, by 2039. Relative to P4 under NT, 5.55 Mg ha^{-1} less SOC was predicted at the 0–10 cm soil depth for P4 under CT at an annual biomass input of 24.7 Mg ha^{-1} year^{-1} (**Table 5**), relative to P4 under NT. This is likely because of less soil disturbance and residue decomposition, consequently reduced mineralization rate and less C losses under NT compared to CT. The rate of SOC stocks changes in the 0–30 cm soil depth for ICLS(NT) and ICLS(CT) in P4 were 0.90 and 0.82 Mg ha^{-1} year^{-1}, respectively (**Table 4**). This is consistent with other soil tillage studies. No-till promotes slower crop residue turnover and mineralization than CT (Sherrod et al., 2003), and less SOC loss (West and Post, 2002). In P4 under ICLS, especially under NT, most of the simulated SOC increase occurred in the 0–10 cm soil depth, while little changes in SOC were predicted for the 10–30 cm soil depth SOC, leading to more SOC in the top 10 cm than in the underlying 20-cm layer by 2039. The changes in SOC were due to the difference in the SOC accumulation rates of 0.82 vs. 0.08 Mg ha^{-1} year^{-1} for the 0–10 cm and 10–30 cm depths, respectively. Whereas in P5, larger SOC increases predicted at 10–30 cm depth in all the simulations. One possible explanation can be the deposition of large amounts of stubble in the ICLS, which under NT stays on the surface and is slowly incorporated into the soil. The stratification of SOC in NT is a widely reported characteristic of this practice (Mrabet, 2002; Sá and Lal, 2009). The proportionally longer participation of the pasture phase in P5 compared to P4 in the 2012–2020 period, that allowed better grass root development, is likely the reason for the higher SOC accumulation rate at the 10-30 cm depth in P5 (0.41 Mg ha^{-1} year^{-1}), compared to P4 (0.08 Mg ha^{-1} year^{-1}). The higher SOC accumulation rate in the lower soil layer under CT can be explained by the incorporation of the surface litter into the lower soil layer.

The role of roots in SOC accumulation or decomposition is still not completely understood (Dijkstra et al., 2020). On one hand, rhizodeposition is a great contributor to SOC stabilization, mainly through stimulating microorganisms. Mineral associated organic matter (MAOM) is protected from further microbial decomposition, and according to Cotrufo et al. (2013) MAOM is predominantly formed from microbial products. On the other hand, plant roots are suggested to be responsible for the destabilization of SOC in a process called rhizosphere priming effect, that is, rhizodeposition is used as substrate by a group of microbes, enhancing SOC decomposition (Huo et al., 2017). Nitrogen uptake by plants can increase competition with microbes that can further stimulate SOC decomposition by microorganisms. In fact, the contribution of the root system to SOC is the result of a balance between its SOC stabilizing and reactivating/ priming effect.

Organic matter formation and persistence in soils depend on environmental conditions, soil microbiota, the quality of soil minerals, and soil chemical and physical properties, especially soil structure (Hunt et al., 2020). Soil C stabilization and storage can occur by chemical stabilization, biochemical resistance, and physical protection. The adoption of NT increases water retention and lowers soil temperature, which favors microbial activity. Furthermore, fewer soil perturbations, favor build-up of larger soil aggregates (Madari et al., 2005), conferring more physical protection (Barreto et al., 2009).

The amount of crop residue is of key importance in accumulating soil C, particularly in the Cerrado biome where high temperatures and humidity during the rainy season do not favor crop residue maintenance over the soil surface. Soil organic C stocks were predicted to increase only by 2.42 and 1.89 Mg ha^{-1} at 0–30 cm between 2019 and 2039 for Paddock 4 and 5, respectively, under Soy/Corn(NT), which has alternating years of soybean and corn crops (**Table 4**). Introducing corn as a second harvest in the same year (Soy-CS (NT)) which resulted in somewhat higher annual biomass input due to corn residues (13.3 Mg ha^{-1} $year^{-1}$ vs. 9.5 in Soy-CS and Soy/Corn, respectively), did not increase SOC stocks substantially (4.25 and 2.25 Mg ha^{-1} in P4 and P5, respectively), especially compared to ICLS (**Tables 4** and **5**). This indicates that providing N through grain legumes without large increase in crop biomass will not result in substantial soil C accrual. Also, as mentioned before, in the case of grain legumes, like soybean, most of the N is removed with the grain, which results in no positive N balance in the system (Sisti et al., 2004). It is well known that positive N balance in the soil is necessary to achieve C accumulation due to a narrow range (10–14) of soil C:N ratio in most soils.

Therefore, even under NT, low disturbance systems such as Soy/CS(NT) with relatively low residue inputs to the soil are less likely to improve SOC as in ICLS with high residue and manure inputs. Soil organic C stocks loss are predicted for scenarios that have annual alternating corn and soybean crop with fallow under conventional tillage (Soy/Corn(CT)). Decreases in SOC stocks of 2.78 and 3.34 Mg ha^{-1} at rates of −0.14 and −0.17 Mg ha^{-1} $year^{-1}$ in the 0–30 cm soil depth under the Soy/Corn(CT) scenario; respectively, for P4 and P5 are predicted. Stockmann et al. (2013) reported that SOC negatively correlated with tillage. The above discussion shows that single promising conservative management practice adoption will not necessarily result in soil C sequestration; therefore, interactions of all the components need to be considered when managing ICLS (Valkama et al., 2020).

In summary, the CQESTR model predicted an increase in SOC stocks for both the NT and CT scenario under ICLS. Predicted SOC increased by 18.0 (28%) and 12.04 Mg ha^{-1} (19%) at the rate of 0.90 and 0.60 Mg ha^{-1} $year^{-1}$ under current ICLS management for Paddock 4 and Paddock 5, respectively, by 2039. In single crop rotations under NT (i.e., Soy-CS and Soy/Corn) SOC accumulation at 0–30 cm was still predicted (between 6.67 and 2.92%), but in Soy/Corn under NT, at the 0-10 cm depth, SOC loss was predicted. Clearly, SOC accumulation in ICLS was favored by the pasture phase, and by introducing brachiaria grass in the rotation.

CONCLUSION

The CQESTR model was validated for the edaphoclimatic conditions of the Cerrado biome in Brazil, and successfully predicted the effect of several agricultural management practices in Integrated Crop-Livestock Systems (ICLS). The model captured spatial-temporal dynamics of SOC very well. The CQESTR predicted SOC increases by 18.0 (28%) and 12.04 Mg ha^{-1} (19%) for Paddock 4 (with the long pasture phase of *Urochloa* spp.), and Paddock 5 (with the shorter pasture phase of *Urochloa* spp.), respectively, by 2039. The use of the extended pasture phase without the crop phase was found to be the best management to increase carbon stocks and could assist Brazilian national initiatives aimed at restoring degraded pasture areas (i.e., "ABC Plan"), as well as reducing carbon dioxide emission from the soil.

Because of the limited measured long-term data for the ICLS under tropical climate, process-based models can be a cost-effective tool to predict soil C stocks change under several ICLS scenarios, and to analyze soil management options and compare impacts of each scenario on SOC stocks.

The grass root biomass and root distribution under tropical savanna conditions may diverge widely from the characteristics of temperate grasses. CQESTR's estimation of SOC stocks could be improved with site-specific below-ground biomass and root distribution data. More long-term studies, SOC data, and root biomass for tropical grasses from diverse tropical biomes are needed to improve SOC stocks prediction. Furthermore, in comparing different management with complex rotations such as ICLS, soil samples must be taken during the same phases of the ICLS rotation. Careful sampling and sample cleaning can reduce plant and animal residue and reduce particulate organic matter or light fraction C incorrectly quantified as part of the stable SOC pool of the sample and improve long-term SOC stocks prediction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JO: sampling collection and analysis, data collection and curation, investigating, methodology, validation, visualization, formal analysis, writing, review and editing. HG: conceptualization, data curation, CQESTR software, formal analysis, funding, investigating, methodology, project administration, resources, supervision, validation, visualization, formal analysis, writing—original draft, writing—review and editing. RP, data curation, visualization, writing—review and editing. LL, writing—review and editing. BM: data curation, formal analysis, funding, investigating, methodology, project administration, resources, supervision, writing—review and editing. MC: data curation, formal analysis, funding, methodology, project administration, resources, supervision, writing—review and editing. PM: data curation, formal analysis, funding, investigating, methodology, project administration, resources, supervision, writing—review and editing.

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Soil Nitrous Oxide Emission and Methane Exchange From Diversified Cropping Systems in Pannonian Region

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Diversified farming systems are promoted to improve ecosystem services in agriculture while maintaining productivity. Intercropping could improve soil quality, the stability of yields and climate resilience. Whether direct emissions of greenhouse gases from soil are reduced as well, depends on the specific measures of diversification. Here, we determined the greenhouse gas emissions from soils of two diversification experiments in the Pannonian climate of Hungary. Firstly, in an asparagus field, oat and field pea was introduced as intercrop between the asparagus berms. Secondly, grass and aromatic herbs were intercropped in a vineyard between the grape rows. The results show that especially for nitrous oxide, average treatment emissions can increase with additional legumes (+252% with intercropped field peas) but decrease with aromatic herbs (–66%). No significant changes were found for methane exchange. This shows that, while other ecosystem services can be increased by intercropping, changes in soil greenhouse gas emissions by intercropping are highly context dependent.

Keywords: diversification in farming systems, intercropping, nitrous oxide emissions, non-linear gas fluxes, vineyard, asparagus field, soil

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1 INTRODUCTION

The intensification of agricultural practices has resulted in soil degradation, reduced biodiversity and increased economic risk for European farmers (Griffiths et al., 2016). Intense mechanization, excessive use of fertilizer (Zhang et al., 2021) and pesticides are the dominant features of modern monocultures; both in time and space the same species of plant is grown on arable fields to improve labor efficiency. In view of the socioeconomic and environmental problems arising from high-input systems, there is now a growing emphasis on crop diversification for an optimized use of resources (Wezel et al., 2014; Beillouin et al., 2021). A number of EU Horizon 2020 projects are addressing the challenge to reverse the dominance of monocultures by reintroducing diversity to agricultural systems: the crop diversity cluster (https://www.cropdiversification.eu/). One of these projects, DiverFarming, is focusing on stakeholder engagement to promote rural development while scientifically assessing measures taken by farmers. Practical diversification strategies are tested on their effects on productivity and ecosystem services such as carbon sequestration, reduced greenhouse gas (GHG) emissions, soil quality and fertility, erosion and contamination prevention. Diversification aims for an improvement of all ecosystem services, but there can be trade-offs that may hamper the provision of a certain service for the benefit of others (Tamburini et al., 2020). Especially changes in nitrous oxide (N_2O) and methane (CH_4) emissions from soil can be indirect effects of diversified management regimes. These effects are difficult to anticipate in advance and its measurement efforts are very high (Decock et al., 2015). N_2O production in soil can be changed by reduced nitrogen availability in soil when intercropping increases the plant covered soil surface (Davidson et al., 2000). Increased plant cover in time or space could also lead to changes in CH_4 oxidation due to root gas transport (Watson et al., 1997) and/or root exudates (Waldo et al., 2019). Additional plant growth is also expected to decrease soil moisture, increase soil organic carbon and increase water holding capacity, all of which have the potential to enhance methanotrophs in soils (Tiwari et al., 2018). An added value of diversification can be an increase of legumes in the field by inter-, cover- or mixed cropping that increases natural nitrogen fixation and soil fertility in general (Stagnari et al., 2017). This additional nitrogen, on the other hand, could increase losses in the form of N_2O at certain points in time (Pappa et al., 2011). In addition, increased plant-derived dissolved organic matter can increase N_2O emissions from soils (Qiu et al., 2015). However, there is evidence that the addition of legumes can mitigate N_2O emission from permanent grasslands (Fuchs et al., 2018). Process based models seem to disagree on the magnitude of the effect (Fuchs et al., 2020). Especially in complex diversification treatments, there is a need to measure the emissions in the field that can be used to verify model output.

In this paper, we report on the greenhouse gas emissions from two case studies in Hungary, where innovative diversification treatments are applied on an asparagus field and a vineyard. Hungarian agriculture is especially in need of adapting diversified farming practices. In our first case study, the asparagus field is challenged by high erosion losses of its sandy soil. Diversification is a key strategy to reduce the loss of soil fertility and organic carbon through wind erosion (Elliott and Chevalier, 1996). Such soil losses are especially pronounced with crops that need an intensive soil tillage treatment, like when asparagus ridges are created. The second case study (CS11) tackles water erosion on vineyards (Rodrigo Comino et al., 2016) by increaseing soil cover (Dittrich et al., 2021). Alternatively to bare soil between grape rows, the winegrower in Villany grew an aromatic herb (*Achillea millefolium*) and mixed grass vegetation. These two distinct examples of diversification will add to the general discussion of how the effects on GHG emissions from soil can be assessed and what mechanisms lead to positive or negative leaking that adds to the overall environmental impact of crop diversification.

2 MATERIALS AND METHODS

2.1 Field Sites

Diverfarming cooperates with farmers to implement diversification on farm experiments to measure the effects on ecosystem services including productivity and greenhouse gas emissions. In the two case studies within the Pannonian climate zone test diversifications that have been designed by the farmer and researchers together. We measured greenhouse gas emissions, mainly N_2O and CH_4, to better understand the consequences of the changed management for diversification. The pannonian case studies consist of two different cropping systems that were diversified according to the goals of Diverfarming and the needs of the local farmer. At both sites, data on meteorological parameters, yields, soil and greenhouse gases were collected for three cropping seasons.

2.1.1 Case Study 10-Jakabszállás

The Diverfarming experimental site CS10 (coordinates: 46°44′52.6″N; 19°34′25.7″E) lies in Central Hungary, in the vicinity of the village Jakabszállás. The soil is a Humic Arenosol on a Pleistocene alluvial fan of the Danube formed by wind blown sand, which has a uniform particle size distribution (median at 198 μm) (**Figure 1**) and is poor in nutrients (N_{total}: 0.3 $g\ kg^{-1}$, $P_{available}$: 110 $mg\ kg^{-1}$). The climate is continental, close to semiarid with mean annual temperature of 10.2°C and average annual precipitation of about 550 mm with frequent extremes. Annual potential evaporation amounts to 848 mm (meteo data from the Hungarian Meteorological Service, OMSz).

The asparagus field is used for intercropping in the 1.8 m wide space between the asparagus ridges (**Figure 2**). Pea and oat are chosen as intercrops to contrast the conventional bare soil area. Both intercrops are primarily used for green manure and partially as catch crops for animal feed. These species were chosen because they are widely grown in this pedoclimatic region and they have distinct agricultural features; field pea is a leguminous plant and has a high P and K content, whereas oat is also nutrient-rich but in contrast very tolerant to bad weather conditions and low soil fertility. The experimental design includes three replicates for each diversification treatment and the control in three blocks (alternating block design, **Figure 2**). Two chambers for GHG sampling were placed between the asparagus ridges on each replicate at the distance of 50 and 100 m from the field border. More information and results on the experiment including first soil quality results are provided in Rezsek et al. (2019).

The field was intensively managed with several chemical plant protection substances (i.e., herbicide metribuzin and fungicides: difenokonasol, azoxistobin, tebukonazol and metriram). Fertilization was done (kg-N/ha in brackets) in 2018 on June 7th (69) and August 31st (72), 2019 on June 13th (63), October 7th (63) and in 2020 on June 15th (72), September 17th (5) and 24th (33). In total this was 340 kg-N/ha during the three growing seasons and the time of GHG measurements. Also the soil management was intensive, forming the ridges for the asparagus, covering them with plastic foil or disking for weed management.

2.1.2 Case Study 11—Villány

Case study number 11 lies in the Villány Wine District, Baranya County, Southwest-Hungary (coordinates: 45°51′47.8″N, 18°26′39.6″E, **Figure 3**). Mean annual temperature is 10.7°C (1981–2010) and average annual precipitation is 680 mm. The plantation has been cultivated for several centuries on southern slopes of 15–20° inclination with loamy, slightly calcareous and

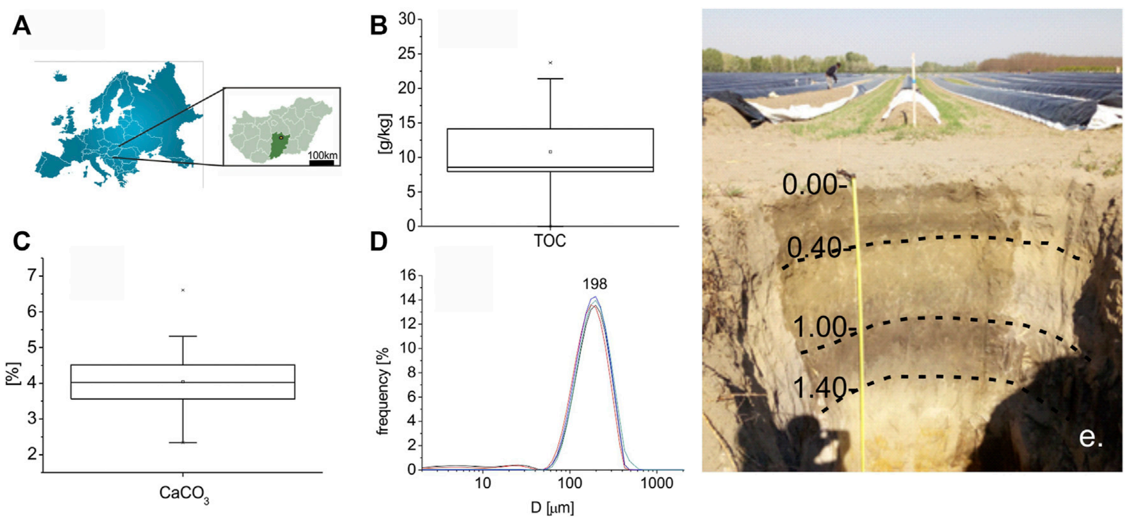

FIGURE 1 | Asparagus field with soil profile and topsoil properties. **(A)**, location; **(B)**, total organic carbon; **(C)**, carbonate content; **(D)**, particle size distribution of the top layer; **(E)**, soil profile (0–0.40 m: cultivated, 0.4–1.00 arenic, 1.00–1.40 buried organic, > 1.40 plinthic).

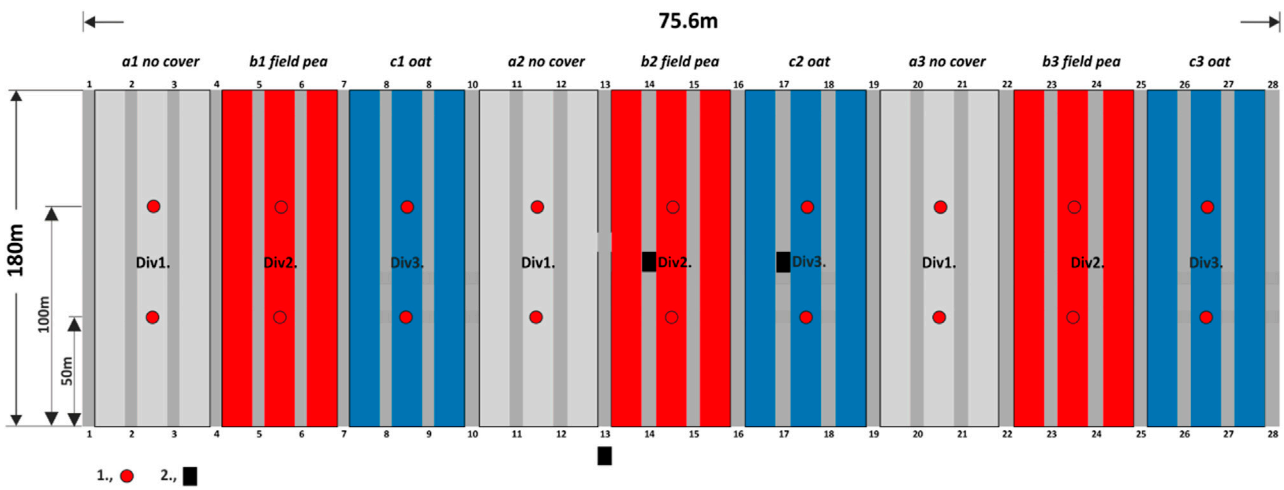

FIGURE 2 | Experimental plot design of CS10 1: ,chambers, 2, in situ loggers for environmental parameters.

compacted Ramann's brown forest soil (WRB: Chromic Cambisol). At the experimental plot, soil depth is 1.7–2.0 m. The average soil carbon content is 3.36% (SD 0.36), total carbon content is 25.96 $g\ kg^{-1}$ and C/N ratio is 19 (**Figure 4**). The site has a high susceptibility for water erosion due to the hill slopes.

The alleys between the rows of grapes were diversified with firstly the aromatic herb *Achillea millefolium* (Yarrow) and the originally growing mixture of grasses. The control treatment without soil cover was treated with disks and cultivators twice in the beginning of each year, when stems were also mulched. The diversified treatments were mowed and left on the field in the alley 2–4 times during the year depending on plant growth. **Figure 5** shows the experimental design of the vineyard with its two alleys between the grapes of 40 m length (north-south) per replicate where the diversification was applied. Hence, each replicate consists of a grape row that has the same diversification on both sides with a row of grapes in between the different treatments. Each treatment was replicated three times including 18 alleys of total width of 44 m (east-west).The three alternating replicates of each treatment form one block, that will also be used to analyse block effects in the field (aligned from left to right). Two chambers for GHG sampling per replicate were installed, one in the grape row between the plants and one in the alley between the grape rows (i.e., vines vs. tractor row). These two positions were analysed as additional fixed factor in the statistical model.

FIGURE 3 | Villány, location of CS11 area **(A,B)**, a part of treatments and place of the chambers, **(C)** setting chambers **(D)**.

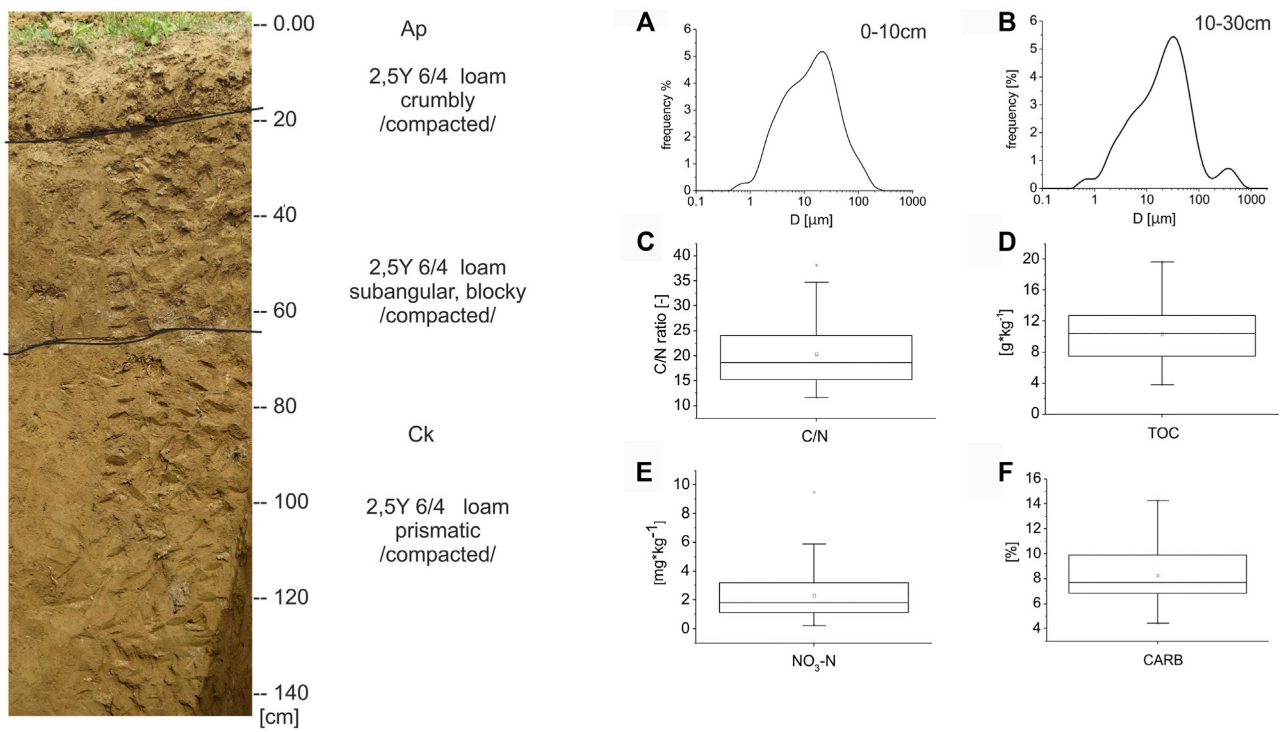

FIGURE 4 | Villány, general soil profile and main important properties of the soil (data from last experimental year (2020) **(A)**. characteristic PSD distribution of 0–10 cm and **(B)** 10–30 cm layer; **(C)**:, C/N ratio; d: TOC (*g* kg^{-1}); **(E)**: NO_3-*N* (*mg* kg^{-1}); **(F)**: carbonate content (%).

Numerous organic farming approved substances are used to protect the grapes from diseases: Copernico, Nectaflor P, Wetcit, Kendal TE, Fitokondi, Mélius, Kumulus S, Sergomil-L 60, Organit, Polyversum, Vitisan, and FlavoPlant. There was no specific fertilizer applied but the mulching of the stems and plant material from the alleys can be considered as recycling input of nutrients.

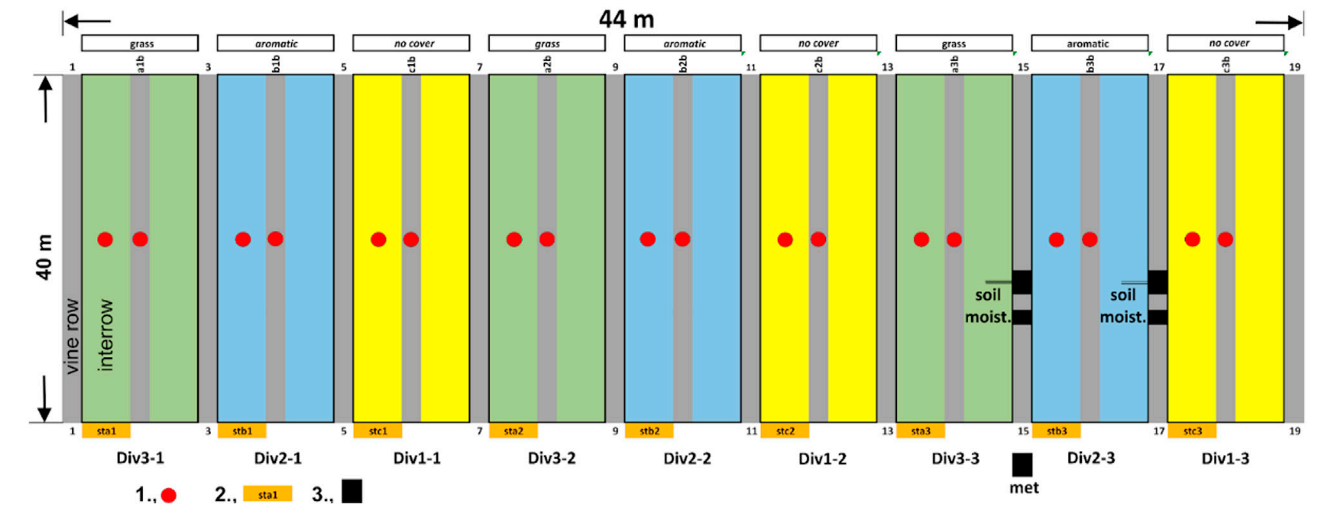

FIGURE 5 | Villány CS11 diversifications: green: grass; blue: aromatic herb (*Achillea millefolium*); yellow: uncovered; 1: chambers; 2 sediment traps; 3: stations for environmental parameters (d).

2.2 Greenhouse Gas Sampling and Analysis

The three greenhouse gases carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) were collected from static dark chambers of 25 cm diameter and height of 5 cm. The rims were permanently inserted into the soil to a depth that the headspace of the chamber is 5 cm high, leading to a chamber volume of 2.45 L. For the sampling, a lid with a vent tube and a sampling port was put on top. Four gas samples of 20 ml were taken at time 0, 10, 20, and 30 min into a pre-evacuated 12 ml Labco vial capped by a rubber septa. The glass vials were shipped to Zurich and measured on an Scion 456-GC (Bruker, Germany) gas chromatograph equipped with a thermal conductivity detector for CO_2, an electron capture detector for N_2O and a flame ionization detector for CH_4. Gas fluxes were calculated by the (Fuß, 2017) using a combination of linear and non-linear flux estimates based on the measurement uncertainty and the flux size (Hüppi et al., 2018). From the total 3′363 N_2O fluxes, 2′467 were evaluated by the robust-linear regression, 17 by standard linear regression (only 3 valid concentration measurements per chamber) and in 878 cases the non-linear HMR regression was applied. This shows that the chamber volume was appropriate since saturation effects were rarely observed from the low ratio of non-linear vs. linear cases (in roughly 1/4 of cases). By allowing for non-linear flux estimates, the average flux of the whole dataset was increased by 14.5%. Respecting the precision of the gas chromatograph and the performance of the chamber system the minimum detectable flux results in 2.49 μg N_2O $m^{-2}h^{-1}$ and 8.16 μg CH_4 $m^{-2}h^{-1}$(shiny app "minflxlim" 2020).

The sampling interval on the field was adjusted to the expected emissions during the growing seasons. In winter, the sampling frequency was reduced (winter break 1–2 months) from the normal bi-weekly sampling, whereas after fertilisation and soil management, the sampling was increased up to daily measurements. Aggregated emissions were calculated using linear interpolation of the daily fluxes (Fuß, 2017).

2.3 Statistical Analysis

For the analysis of N_2O flux data on the significance of the treatment effect, the data was first log transformed to improve normal distribution. A linear mixed effect model was used to test the effect on N_2O emissions and CH_4 exchange from diversification treatment and block as fixed effect and the chamber number as random effect. The chamber number as random effect reflects the repeated measures during the sampling period at the same site (chamber) in the field. For CS11 position was an additional fixed effect controlling for within or between grape rows. The model was controled to have a variance inflation factor below three. The aggregated emission boxplot compare the treatments in a pairwise t-test and indicate significance at * ($p < 0.05$), ** ($p < 0.01$) or N.S (not significant, $p > 0.05$). The pairwise t-test was used in the figures on aggregated emissions per treatment.

3 RESULTS

3.1 Case Study 10

3.1.1 Nitrous Oxide

The major N_2O emission peaks each year in June are well resolved (**Figure 6**) due to a higher sampling frequency whereas outside the growing season the sampling was reduced. The large peaks from the rows in between the asparagus berm, coincide with fertilisation of 70 kg N ha^{-1} mid May in 2020, early June in 2018 and mid June in 2019. Smaller peaks could be observed in May and September, which could be due to soil management and fertilization respectively.

The control plots show the lowest N_2O emissions in the asparagus field (**Figure 7**). There is a significant reduction of cumulated N_2O emissions in the bare soil treatment, especially in

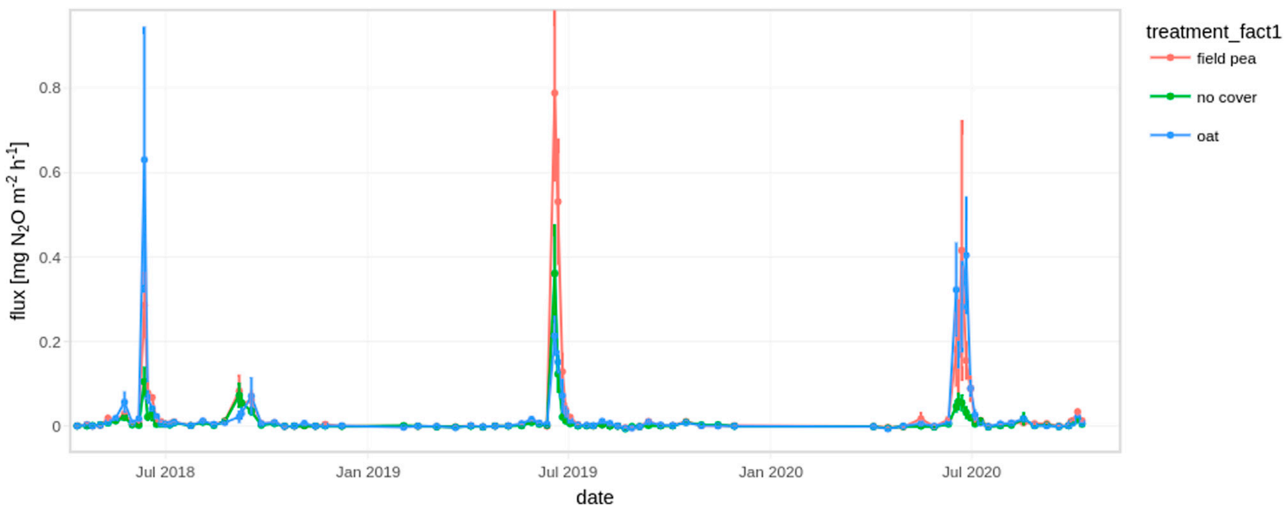

FIGURE 6 | N_2O emissions of CS10 during the three growing seasons (2018–2020) averaged by the diversification treatment (field pea, oat and no cover as control) with error bars indicating 1 standard error.

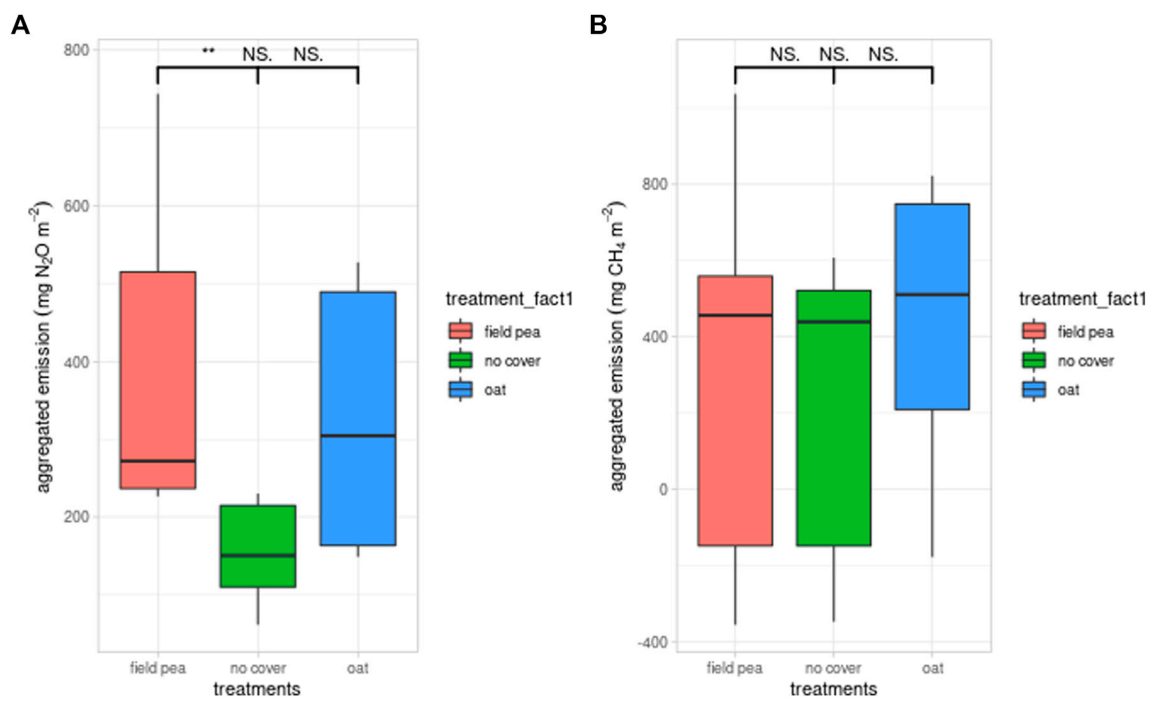

FIGURE 7 | Boxplots of the aggregated N_2O **(A)** and CH_4 **(B)** exchange from CS10 by the diversification treatment; field pea, no cover(control) and oats.

contrast to field pea in the asparagus field. The linear mixed effect model indicates a significant effect of the diversification treatment (p = 0.007). Hence, field pea increased N_2O emissions in comparison to bare soil to 252% over the 3 years of data. The oat treatment shows higher emissions than the control as well (211%) but without statistical significance. The average emission factor of the applied 340 kg $N\ ha^{-1}$ is 0.54% (hence 1.8 kg $N_2O-N\ ha^{-1}$).

3.1.2 Methane

Most methane measurements show an uptake by the soil (**Figure 8**). Only on September 27 in 2020 there was a large emission event in all treatments that dominated the aggregated methane balance over the whole measurement period. The average emission per treatment on this day was 6.2, 5.0, and 4.3 $mg\ CH_4\ m^{-2}h^{-1}$ in oat, field pea and no cover respectively (**Figure 7**). Including this special event in the aggregated emissions, turns them into an overall emission of about

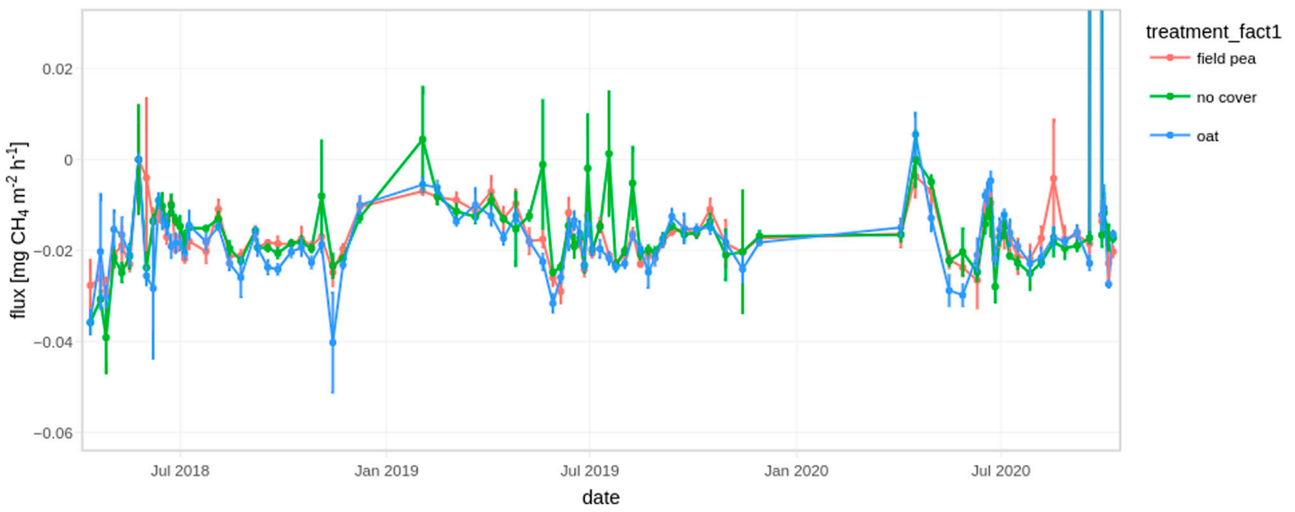

FIGURE 8 | CH_4 emissions of CS10 during the three growing seasons (2018–2020) averaged by the diversification treatment (field pea, oat and no cover as control) with error bars indicating 1 standard error.

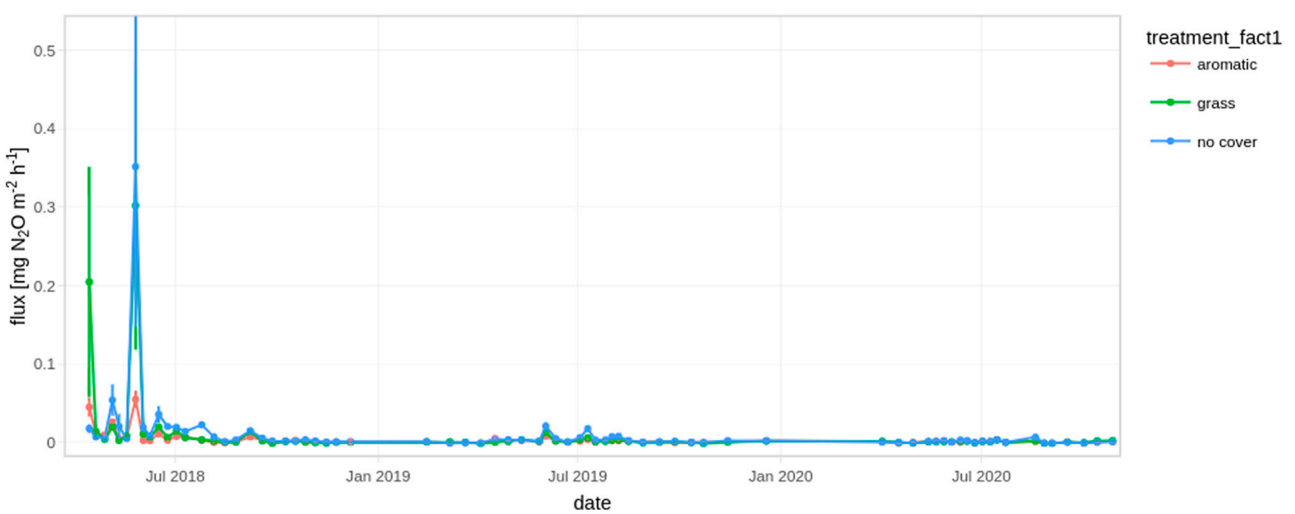

FIGURE 9 | N_2O emissions of CS11 during the three growing seasons (2018–2020) averaged by the diversification treatment (aromatic herbs, grass and no cover as control) with error bars indicating 1 standard error.

500 $mg\ CH_4\, m^{-2}$ without significant difference between the treatments. If this extraordinary event is omitted from the cumulative flux calculation, there is an uptake of roughly 360 $mg\ CH_4\, m^{-2}$ with a significant effect of the diversification treatment (p = 0.007). In this case the oats had an increased CH_4 uptake by roughly 9%, however the high emission event then masked this effect in the overall dataset.

3.2 Case Study 11

3.2.1 Nitrous Oxide

N_2O measurements in CS11 show rather small fluxes, mostly below 0.1 $mg\ N_2O\ m^{-2}h^{-1}$, with larger emission events only in the beginning until July 2018 (2 events 0.1 $mg\ N_2O\ m^{-2}h^{-1}$) followed by culivator activity and mulching of the stems in early spring (**Figure 9**). The diversification treatment shows a significant difference in the linear mixed model (p = 0.0022) over the whole measurement period, but also after the initial large emissions in 2018 (p = 0.065). Aggregated emissions show a significant reduction by aromatic herbs compared to bare soil of 66% over the 3 years of data (**Figure 11**). In addition to the diversification treatment also the block (p = 0.009) and the position (p = 0.0012) of the chamber within the replicate (i.e., vines vs. tractor row) had a significant effect on N_2O emissions.

3.2.2 Methane

In general, methane was taken up by the soil with minor seasonal variations (**Figure 10**). There are no differences

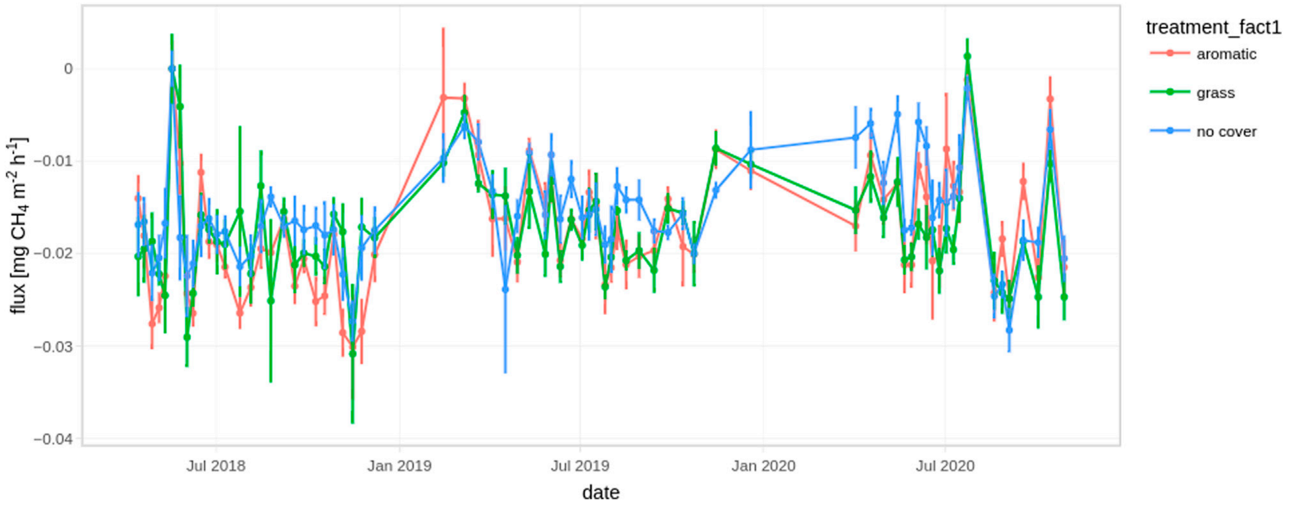

FIGURE 10 | CH_4 emissions of CS11 during the three growing seasons (2018–2020) averaged by the diversification treatment (aromatic herbs, grass and no cover as control) with error bars indicating 1 standard error.

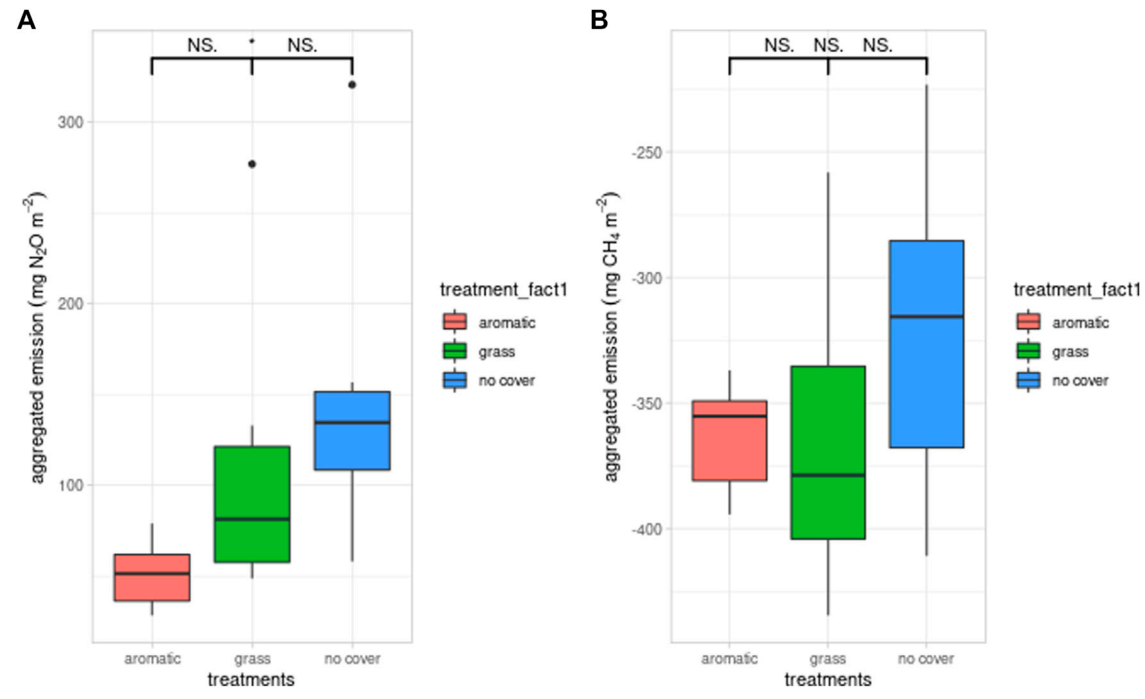

FIGURE 11 | Boxplots of the aggregated N_2O **(A)** and CH_4 **(B)** exchange from CS11 by the diversification treatment; aromatic herbs, grass and no cover (control).

between the treatments but just a possible minor tendency for reduced methane oxidation by the uncovered treatment (**Figure 11**).

3.3 Soil Moisture and Temperature Data

Soil moisture and temperature in the soil show differences between the 3 years of data (**Figures 12, 13**). Whereas the summer in 2018 and 2020 was rather dry, 2019 had lots of precipitation. The sensor setup did not allow for differentiation between the treatments.

4 DISCUSSION

With the large variety of diversifications applicable in European agriculture, the impact on GHG emissions is as diverse. There is

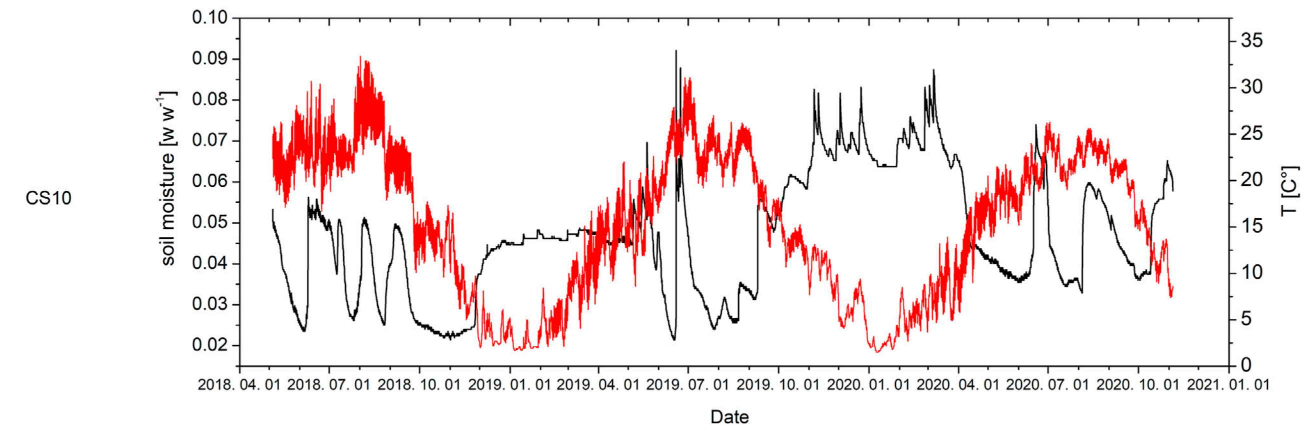

FIGURE 12 | Temperature (red line, right axis in °C) and soil moisture (black line, left axis in VWC m3/m3) of CS 10 during the three growing seasons (%).

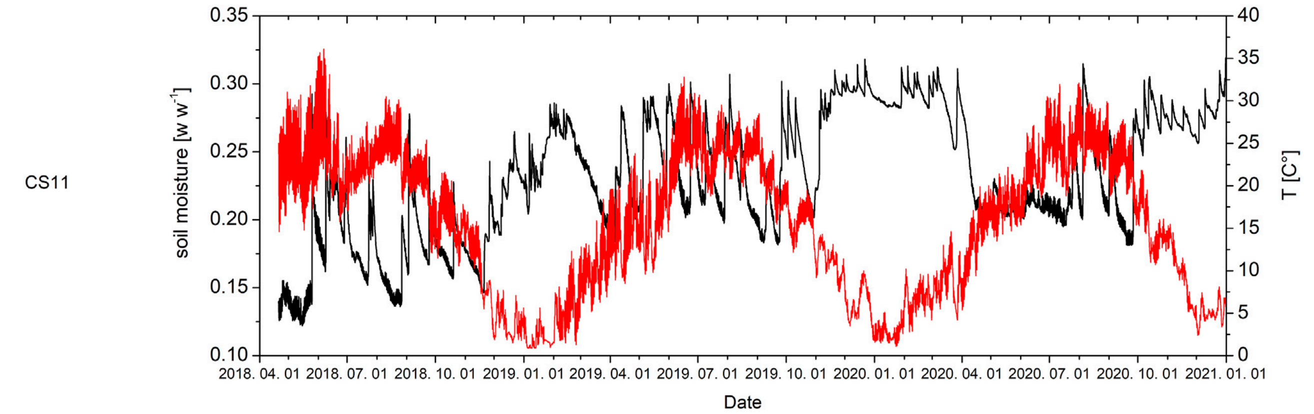

FIGURE 13 | Temperature (red line, right axis in °C) and soil moisture (black line, left axis in VWC m3/m3) of CS 11 during the three growing seasons (%).

no general hypothesis that with increased diversity of plants on the field, GHG emissions are lower. But there are opportunities for reduced N_2O emissions especially, with the appropriate rate and timing of organic input management (Saha et al., 2021). However, a meta analysis about the effects of crop diversification on ecosystem services, showed that there is a trend for increased GHG emissions (Beillouin et al., 2021). They state that climate impacts of crop diversification are variable and context-dependent.

With respect to N_2O emissions, decreased emissions can be expected due to reduced availability of mineral nitrogen from the increased plant cover (Davidson et al., 2000) and more balanced nitrogen supply with added legumes especially in grasslands (Fuchs et al., 2020). Cover cropping is a more specific example that according to a recent review publication decreases nitrogen leaching and increases carbon sequestration, but does not have a significant effect on N_2O emissions over the whole reviewed data set (Abdalla et al., 2019). Our data supports reduced emissions from increased soil cover in CS11, where no additional fertilizer was applied in the inter row section of the vineyard. Although there is no significant difference in the pairwise comparison, the grass reduced N_2O emissions less than herbs, possibly because in the grass mixture also some leguminous clovers were estabilshed. Alternatively, aromatic plants are known to produce substances that inhibit nitrification in soil, hence this could improve nitrogen efficiency and reduce N_2O emissions (Kiran and Patra, 2003). Another study supports our finding by showing a 60% decrease in N_2O emissions between cover cropping and tillage in a mediterranean vineyard (Marques et al., 2018). A similar vineyard field experiment also within Diverfarming showed that nitrate content did not change with aromatic herbs but ammonium was reduced, indicating that these plants impact nitrogen transformation in soil (Dittrich et al., 2021). Garland et al. (2011) looked into the effect of different management practices on N_2O emissions from a Mediterranean vineyard and found fertilization and cover cropping had a larger impact than till or no-till soil management. They also highlighted the importance of measuring GHG emissions at different functional locations within a vineyard (i.e., vines and tractor row) because these different sites have distinct emission patterns. In CS11 we also differentiated between below vine row and interrows, but although significant, the effect of the position did not contrast the diversification effect.

In contrast, CS10 shows significantly increased emissions from leguminous field peas and just a tendency for increased emissions with oat compared to the uncovered soil. Although an increase in N_2O emissions can be expected from increased nitrogen inputs by legumes, our results are not in agreement with studies that actually showed decreased N_2O emissions from leguminous intercrops (Pappa et al., 2011; Senbayram et al., 2016). Carter and Ambus (2006) showed that biologically fixed N_2 is a minor source for N_2O in a grass-clover mixture by ^{15}N tracers. Compared to the mulched annual field pea in our case, the perennial clover may not release as much nitrogen since the resulting N input from avoided root decomposition is less. Still our results of increased emissions in contrast to bare soil are not fully explained by just increased N content from field peas. Especially since oats also have a tendency for increased emissions. In our case an increase in plant-derived dissolved organic matter by the additional plants growing may have increased N_2O emissions from soil (Qiu et al., 2015). Nevertheless, another study did show increased N_2O emissions from non-legume cover crops in the absence of the growing season (Thomas et al., 2017). Although our measurements are increased mainly during the growing season and especially at the leguminous treatment, the suggested increase in N_2O fluxes may still be related to increased substrate concentration for denitrification due to higher amounts of root-associated soil with cover crops. Nevertheless, the main emissions occur after the fertilization events in CS10. Mulching of the intercrops could lead to high N_2O emissions and create a contrast to the bare soil situation where no plant material is decomposed (Garland et al., 2011; Saha et al., 2021). A meta analysis on the effect of cover crops on N_2O emissions supports the finding that leguminous cover crops and incorporation thereof into soil tend to increase emissions (Basche et al., 2014).

Methane emissions did hardly change in diversification treatments. While there is a lot of research on how vegetation affects timing and location of methane emissions from wetlands, there is very little known on plant interactions with oxic, mineral soils. An increase in plant cover and root exudation would however rather increase methane emissions from soils (Waldo et al., 2019). In contrast we found that in the asparagus field (CS10), the plants tend to increase methane oxidation, especially oats significantly increased methane uptake compared to the bare soil. A reason for this might be the increased oxidation by methanotrophic bacteria in the upper aerated soil layer, leading to higher uptake of methane by the sandy soil. Nitrogen limitation is known to reduce the growth of methanotrophic bacteria in soils (Bodelier and Laanbroek, 2004). The intercrops could probably help retain nitrogen in soil that was then still available for methane oxidation in CS10. Methanotroph bacteria abundance is also enhanced with increased soil moisture, organic carbon content and water holding capacity (Tiwari et al., 2018). When considering intercropping, especially in contrast to bare soil, these soil properties are improved, which would lead to an increased methane oxidation potential. Such a positive side effect of increasing plant cover in diversified cropping systems can lead to considerable improvements of the GHG balance.

5 CONCLUSION

This study shows the importance of monitoring GHG emissions in complex diversification experiments. While mostly ecosystem services are improved by increasing diversity in farming systems, non-CO_2 GHG emissions from soil can be both increased and decreased. However the extent of change is not necessarily high and it can be very cost and time consuming to monitor. There are general mechanisms that help to understand the impact of diversification *via* change in plant cover and consequtive effets on nutrient availability or soil moisture. The addition of leguminous intercrops can increase nitrous oxide emissions whereas the addition of herbs to uncovered interrows significantly decrease N_2O emissions. Methane on the other hand is less affected by the diversification measures but its oxidation is influenced by an increased plant cover due to increased soil aeration and change in nitrogen availability.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://zenodo.org/record/6369860

AUTHOR CONTRIBUTIONS

RH, JD, and JS contributed to conception and design of the study. RH and JS organized the data evaluation. RH performed the statistical analysis. RH wrote the first draft of the manuscript. RH and JS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Modeling Soil Carbon Under Diverse Cropping Systems and Farming Management in Contrasting Climatic Regions in Europe

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Sustainable agriculture has been identified as key to achieving the 2030 Agenda for the Sustainable Development Goals, which aims to end poverty and hunger and address climate change while maintaining natural resources. Soil organic carbon (SOC) sequestration is a key soil function for ecosystem services, and storing carbon (C) in soil by changing traditional management practices can represent an important step toward the development of more sustainable agricultural systems in Europe. Within the European project *Diverfarming,* the process-based ecosystem model ECOSSE was modified and evaluated in four long-term experiments (>8 years) to assess the impact of crop diversification and agricultural management in SOC dynamics. ECOSSE was able to simulate SOC under dry conditions in Mediterranean regions in Spain and Italy. In the site of Murcia, Spain, the addition of manure and cover crop in the diversified systems produced an increase of SOC in 9 years, when compared with the conventional management (16% measured increase, 32% simulated increase). The effect of tillage management on SOC stock in dry soil, in Foggia, Italy and Huesca, Spain, was also modeled, and a positive impact on SOC was predicted when no tillage was practiced. Finally, ECOSSE was used to understand the impact of diversifications in Boreal regions, Finland, where different proportions of legumes and grass were considered in a 4-year crop rotation compared with conventional cereal rotations. Experiments and modeling showed that the loss of SOC in conventional cereal was compensated when grass was introduced in the rotations. A good agreement (NRMSE <10%) and a nonsignificant bias were observed between model and experimental data for all sites. Mitigation scenarios considered in the modeling analysis for the test site Huesca showed that an integrated management of no tillage and manure is the best strategy to increase SOC, ~51% over 20 years, compared with the baseline scenario (current farmers practice). This study demonstrated the ability of the modified version of ECOSSE to simulate SOC dynamics in diversified cropping systems, with various soil management practices and different climatic conditions.

Keywords: soil organic carbon, sustainable agriculture, modeling, ECOSSE, mediterranean, boreal

INTRODUCTION

For many years intensive agricultural systems, e.g., large use of fertilizers, pesticides, intense mechanization, and monocultures, have been practised to increase crop productivity, and to meet the demand of a growing population and food security challenges. However, such simplified farming systems had detrimental effects not only on the cropping systems themselves, but also on the environment with respect to resilience, adaptation to climate change, groundwater pollution, biodiversity loss, reduction of ecosystem functions, soil erosion (Lichtenberg et al., 2017; Francaviglia et al., 2020), and economic loss (Pretty, 2018). Consequently, in the last few years greater attention has been paid to sustainability and to a use of resources that would minimize the costs and maximize the benefits, while enhancing resilience and increasing productivity. Sustainable agriculture has been identified as an important strategy to achieve the 2030 agenda for Sustainable Development Goals (SDGs). As addressed by Nhemachena et al. (2018), the sustainability challenge faced by the agriculture sector is to be able to provide enough food for the growing population without increasing the use of primary resources such as water and farmland, and without reducing biodiversity. The implementation of crop diversification strategies under low-input management in place of traditional farming systems is gaining attention in agriculture, not only to meet the demand of food for an ever-growing population, but also with respect to environmental resilience and sustainability.

Increasing depleting Soil Organic Carbon (SOC) stocks can represent an important step toward the development of more sustainable agricultural systems. The reservoir capacity of SOC in soil carbon (C) pool was estimated at 1,550 Gigaton (Gt), which is nearly twice as large as the atmospheric pool, and three times larger than the C present in the biotic pool (Lal, 2016). Soil organic carbon is considered one of the most important indicators of soil quality and agronomic sustainability because of its impact on other physical, chemical, and biological properties of the soil (Reeves, 1997; Lal, 2013; Yigini and Panagos 2016; Sanz-Cobena et al., 2017). Soil productivity and SOC storage are largely affected by the agricultural managements, e.g., crop cover, residue, and tillage management (Paustian et al., 2016). Approximately 90% of total greenhouse gas (GHG) mitigation potential could be derived from SOC sequestration (Smith et al., 2007; Begum et al., 2017), and the SOC sequestration potential is mainly derived from agricultural land management (Minasny et al., 2017). However, there are also studies suggesting that SOC sequestration potential is a limited tool to mitigate climate change (Schlesinger and Amundson, 2019). Using the "4 per mille" approach, the SOC stock from the global agricultural land is estimated to be 2 to 3 Gt C yr^{-1} from the top 1 m, which effectively offset 20–35% of global anthropogenic GHG emissions (Minasny et al., 2017). Improving agricultural management can contribute to prevent the global warming potential from exceeding between 1.5°C and 2°C, as mentioned in the Intergovernmental Panel on Climate Change (IPCC) sixth report. Since a small change in SOC can impact the global C cycle (Smith, 2004) and soil ecosystems, it is crucial to store SOC in the soil for longer periods (Smith, 2004; Rodeghiero et al., 2011).

In Europe with the exception of the Netherlands, other countries, e.g., Belgium, France, Finland, England, Wales, etc, found a decreasing trend in SOC stock in cropland (Heikkinen et al., 2013) and overall a decline of ~28% has been observed in the last 30 years (FAOSTAT, 2021). A modeling analysis by Smith et al. (2005) predicted a loss of 4–6 Gt from the cropland SOC stock under different climate change scenarios by 2080, which is a 39–54% decrease compared with the 1990 level. Loss of SOC has been identified as one of the major soil threats in the European Union's Thematic Strategy for Soil Protection (EC, 2006). Increasing SOC stock by agricultural means has also been emphasized in the Kyoto Protocol and Paris Climate Agreement (Begum et al., 2017). In the 21st conference of the parties to the United Nations (UN) Framework Convention on Climate Change (COP21), soil C and agriculture were first presented in the agenda, and a voluntary initiative called "4 per mille" was proposed, to increase the global SOC stocks annually by 0.4% at 40 cm depth (Lal, 2016; Begum et al., 2018a).

In this context, the H2020 Diverfarming project (www.diverfarming.eu) aims to increase SOC stock by crop diversification and reduction of external inputs across six European pedoclimatic regions. In this study we focus on the Mediterranean and Boreal regions, which occupy 11% and 14% of the EU 28 cropland area, respectively (EUROSTAT, 2015). Generally, in the Mediterranean climatic regions the SOC content is low (0.5–1%). Climate, e.g., high temperature, evapotranspiration, and low precipitation, crop and soil management, e.g., bare fallow practised for long periods of time (up to 1.5 years), crop residue removal, intensive tillage, and soil erosion favor the SOC decline (Hernanz et al., 2009; Alvaro-Fuentes and Paustian, 2011; Rodeghiero et al., 2011; Aguilera et al., 2018). Low crop growth rates, difficult land recovery after degradation, and potential high mineralization rates are also common phenomena in Mediterranean ecosystems (Almagro et al., 2017). Dry soils are significantly affected by agricultural management practices. However, there is potential to improve and increase SOC stock by adoption of innovative management practices, e.g., no tillage (NT), use of cover crops as green manure (GM) in the rotation, incorporation of residue, and C input by exogenous source (Mazzoncini et al., 2008; Álvaro Fuentes et al., 2009; Rodeghiero et al., 2011; Aguilera et al., 2018). A data-analysis carried out by Francaviglia et al. (2017) found that SOC in dry regions increases by 11% under diversified crop managements and by 25% under diversified fertilization managements in comparison with humid and subhumid regions. In European Boreal ecosystems the SOC content depends on the type of soil. A study on Finnish cropland (11% of total area covered with organic soils) found a decrease in SOC of 0.4% yr^{-1} and 0.2–0.3% yr^{-1} for mineral and organic soils, respectively, due to climate change, land use, and management practices. Moreover, a high decomposition rate due to peatland drainage increases CO_2 emissions and reduces SOC gain (Heikkinen et al., 2013).

The analysis of experimental data is necessary to appreciate changes in SOC (if any) due to diversification. However, since changes in SOC are slow, long-term field experiments are of key importance to get insights into the SOC dynamics in soil-atmosphere-plant interactions. Furthermore, due to diverse climatic pattern, the detailed studies on SOC dynamics across Mediterranean ecosystems are limited (Rodeghiero et al., 2011). The aid of process-based models gives the opportunity to extend the information obtained experimentally to future scenarios. This adds a new level of relevance to the research, and provides key information to farmers and stakeholders when they consider diversified cropping systems as a potential improvement to their own agroecosystems. In the last few years, the process-based model ECOSSE (Model to Estimate Carbon in Organic Soils-Sequestration and Emissions; Smith et al., 2010) was extensively modified and used to assess GHG emissions from different soils and cropping systems in Europe (Dondini et al., 2015). New land uses such as those for the production of bioenergy-woody crops were introduced (Dondini et al., 2015, 2016). Soil respiration was simulated in the European peatland sites (Abdalla et al., 2014) and in Ireland arable soil (Flattery et al., 2018), and Bell et al. (2012) simulated soil nitrogen (N) and nitrous oxide (N_2O) emissions in cropland sites in Europe. However, the use of ECOSSE to model SOC sequestration potential in arable cropland with diversified crops (e.g., multiple cropping, crop rotation) and agricultural managements (e.g., tillage, irrigation, fertilization) in the Mediterranean pedoclimatic regions is limited. SOC dynamics was modeled in barley cropping systems in association with different tillage practices, considering current (Álvaro Fuentes et al., 2009) and future climate scenarios (Alvaro-Fuentes and Paustian, 2011). Aguilera et al. (2018) analyzed SOC in the cropland under different managements. Both studies however were limited to Spain. Modeling work on SOC dynamics was also done in Mediterranean Italian sites (Farina et al., 2013, 2018) and at a regional level (Farina et al., 2017) by adapting the process-based model RothC (Jenkinson et al., 1999) to Mediterranean conditions.

The main aim of this study was to assess how SOC dynamics change in relation to diversified farming and soil management. We show how the model ECOSSE was modified, parameterized, and used to simulate C dynamics in the European Mediterranean and Boreal ecosystems. Through the numerical simulations of all considered management practices we could explore which factors in diversified managements have the largest impact on SOC in contrast to conventional agricultural systems, gaining insights into the effect of diversification and sustainable soil management on SOC. Simulations of long-term rotations in Mediterranean and Boreal croplands allowed us to compare the SOC sequestration potential in different European pedoclimatic regions and to attain a deeper understanding of SOC dynamics in contrasting regions. Finally, different mitigation scenarios were considered for the test site of Huesca (Spain) to estimate how different managements affect SOC dynamics in Mediterranean ecosystems.

MATERIALS AND METHODS

Site Description

The SOC data used in this study are from four long-term (>8 years) experimental sites, three in the Mediterranean regions in Murcia, Spain, in Foggia, Italy, and in Huesca, Spain; and one in the boreal region in Toholampi, Finland. The top layer soil properties (0–30 cm depth) are presented in **Table 1** for each of these sites. Soils in Mediterranean regions are generally basic (~pH 8) whereas Finnish soil is acidic (~pH 6). The average clay content is between 15% and 35% in Mediterranean regions and 6% circa in the Boreal region. In each region several tailored diversification systems and soil managements are considered as alternatives to conventional management. All sites and agricultural management practices are described below. Diversification codes with the main management features, including yearly amounts of C input, can be found in **Supplementary Table S1**.

LT1 Diversified Horticulture in Spain

In Murcia, Spain, the long-term effect of multiple cropping and rotations on SOC dynamics with the addition of organic amendment was evaluated. The experimental period is 2010–2018 with melon (*Cucumis melo*), pepper (*Capsicum annum*), and pumpkin (*Cucurbita moschata*) as rotated summer crops and cabbage (*Brassica oleracea var. sabellica*), celery (*Apium graveolens*), lettuce (*Lactuca sativa*), broccoli (*Brasica oleracea var. italica*), and fennel (*Foeniculum vulgare*) as rotated winter crops. Three managements were practised on this site: 1) conventional, 2) organic, and 3) biodynamic, with the same multiple cropping and rotation pattern. All of them included conventional tillage (CT) to a depth of 30 cm and fertigation. The conventional management received yearly sheep manure applications with inorganic mineral N fertilizer under fertigation and application of pesticides (LT1DV0). The organic management included sheep manure, soluble organic fertilizers for fertigation, and biological control of pests/diseases (LT1DV1). The biodynamic management included sheep compost, soluble organic fertilizers and compost tea for fertigation, biological control of pests/diseases, and cover crops (LT1DV2). Manure and compost were applied before the winter cycle, around September. Inorganic/organic fertilizers and compost tea were applied by fertigation according to crop needs. Cover crops consisted of a mixture of oats (*Avena sativa*) and vetch (*Vicia sativa*), grown in between the two main crops and incorporated in the field in April/May before summer crop was established. The residues from all treatments were incorporated in the field with CT, up to 30 cm depth.

1) Conventional (LT1DV0): 12–15 t ha^{-1} sheep manure (% C 22.5, %N 0.98%) + inorganic fertilizer containing 6.18 kg N ha^{-1} in fertigation.
2) Organic (LT1DV1): 15 t ha^{-1} sheep manure (% C 22.5, %N 0.98%), organic fertilizer containing 1.23 kg N ha^{-1} in fertigation.

TABLE 1 | Site description, soil, and crop management in studied area for model simulations.

Site name	LT1	LT2	LT4	LT7
Location (country, province, city)	Spain (Murcia, San Javier)	Italy (Foggia, Foggia)	Spain (Huesca, Senes)	Finland (Ostrobothnia, Toholampi)
Pedoclimatic regions	Mediterranean south	Mediterranean south	Mediterranean south	Boreal
Latitude/longitude	37°48′18.5″N, 0°51′49.2″W	41°47″N, 15°50″W	41°54′12″N, 0°30′15″W	63° 49′ 15.9″ N, 24° 09′ 37.6″ E
Elevation (m)	120	80	395	102
Duration of experiment (year)	9	9	9	22
Experimental area (ha)	0.2	1.2	1.0	2.6
Soil type	Haplic Calcisol (loamic, hypercalcic)	Typic Calcixerept	Typic calcixerept	Gleyic Podzol
Clay (%)	15.48	20.80	35.34	5.78
pH	8.44	7.98	8.12	6.16
Bulk density (g cm^{-3})	1.32	0.94	1.44	1.17
Initial SOC (kg/ha^{-1})	28,600	52,160	34,777	150,060
Cropping systems	Multiple cropping and rotations	Crop rotation	Monocropping	Four-year crop rotations
Irrigation (Hm^3 ha^{-1} yr^{-1})	0.0024	Not applied	Not applied	Not applied
SWW (cm^3/cm^{-3})	0.11	0.27	0.16	0.14
SWF (cm^3/cm^{-3})	0.23	0.39	0.27	0.39

SWW, soil water at wilting point; SWF, soil water at field capacity.

3) Biodynamic (LT1DV2): 10 t ha^{-1} sheep compost (% C 20, % N 1.3), organic fertilizer containing 1.23 kg N ha^{-1}, compost tea (140 mgL^{-1} C and 21 mgL^{-1} N) + cover crop. Carbon and nitrogen input by compost tea are 140 mg C/L and 21 mg N/L. Fertigation varies on a yearly basis and the simulations showed that between 0.9 and 1.7 t ha^{-1} C are added through fertigation in LT1DV2.

LT2 Durum Wheat in Italy

The impact of tillage and crop rotation on the soil physicochemical properties of the Italian site in Foggia has been analyzed. Two field experiments were established in 1995 to observe the soil physico-chemical properties in monocropping wheat (*Triticum aestivum*) under CT, tillage depth 35–40 cm), and NT. From 2008 two other management practices were included, wheat in rotation with tick bean (*Vicia faba*) under CT and NT systems. No tillage was associated with direct seeding to the field. The tick bean grown in the rotation systems were incorporated in the field as GM. Inorganic fertilizer containing 36 kg ha^{-1} N was applied during the wheat growing season. In conclusion, the four management practices for this site were the following: Conventional (LT2DV0), that is Durum wheat monocrop with CT; Diversification 1 (LT2DV1), Durum wheat monocrop with NT; Diversification 2 (LT2DV2), rotation tick bean-durum wheat with CT; and Diversification 3 (LT2DV3), rotation tick bean-durum wheat with NT.

LT4 Tillage in Rainfed Systems in Spain

The experiment designed to study the long-term impact of tillage on SOC dynamics in the Mediterranean semiarid regions started in 2010 in Huesca (Spain) and run for 9 years. Two tillage managements, reduced tillage (RT, 15 cm depth with disk ploughing and cultivator) and NT, were practised in barley (*Hordeum vulgare L.*) rainfed cropping systems.

The site was mostly dominated by barley monocropping, except in 2014 and 2016 when pea (*Pisum sativum*) and wheat were introduced. About 75 kg N ha^{-1} yr^{-1} was applied as an inorganic fertilizer in both management practices. All crop residues were left in the field. In summary, the two management practices considered in this site were as follows: Conventional (LT4DV0), with Barley monocrop under RT, and Diversification 1 (LT4DV1), with Barley monocrop under NT. The impact of tillage on SOC was assessed on a 0–30 cm depth as this was the considered sampling depth, and it is the minimum depth recommended by IPCC to estimate carbon stock with a minimum error. Also, many studies analyze the effect of tillage on SOC at a 0–30 cm depth making possible a comparison between their and our results (Alvarez et al., 1995; Álvaro Fuentes et al., 2009; Meurer et al., 2018).

LT7 Cereals and Forage Crop Rotations in Finland

The common rotation in Finnish croplands includes cereal, leys, and legumes (Hakala et al., 2016; Palosuo et al., 2016). However, cereal monoculture was observed quite commonly on crop production farms in southern Finland. It was practised on 20.1% of field parcels in 2007–2011 (Peltonen-Sainio et al., 2017).

In a sandy soil experimental field in Toholampi Ostrobothnia, western Finland, four crop rotations were practised from 1997 to study erosion and nutrient leaching, crop yield, and soil properties in crop rotations of organic and conventional farming. First 4-year crop rotations (1997–2000) included one conventional and three organic forage crop rotations. Cultivated plants and tillage of crop rotations were kept as similar as possible, the main difference was in fertilization (different nitrogen sources and fertilization intensities). In 2001, the unfertilized organic crop rotation was changed from organic cereal crop rotation to conventional cereal rotation. Organic and conventional crop rotations, fertilized with composted or un-composted farmyard manure, continued similarly, but farmyard manure was changed to cow slurry. The idea and implementation of crop rotations in 2001–2018 is described more precisely in Peltoniemi et al. (2021).

To summarize, the managements for this site are the following:

1) Conventional (LT7DV0): Conventional cereal [barley-barley-rye (*Secale cereale* L.)-oats (*Avena sativa* L.)], synthetic fertilizers (2001–2018). In 1997–2000: [barley-timothy (*Phleum pratense* L.) + clover (*Trifolium*) ley-timothy + clover ley-mixture of oats and common vetch (*Vicia sativa* L.)], fertilization with composted fox manure.
2) Diversification 1 (LT7DV1): Organic cereal [barley-timothy + red clover (*Trifolium pratense* L.) ley -rye-oats] fertilized with cow slurry (2001–2018). In 1997–2000 (barley-timothy + clover ley-timothy + clover ley-mixture of oats and common vetch), no fertilization.
3) Diversification 2 (LT7DV2): Conventional forage [barley-timothy + meadow fescue (*Festuca pratensis* Huds.) ley-timothy + meadow fescue ley-barley) fertilized with cow slurry + synthetic fertilizers in 2001–2008. In 1997–2000 fertilized with cow farmyard manure + urine.
4) Diversification 3 (LT7DV3): Organic forage (barley-timothy + red clover ley-timothy + clover ley-mixture of oats and common vetch) fertilized with cow slurry in 2001–2018 and composted cow farmyard manure + urine in 1997–2000.

The conventional management (LT7DV0, 2001–2018) in the 4-year rotation included the following cereals: barley (twice), rye (*Secale cereale* L.), and oats. Sowing and harvesting times for spring cereals, oats, and common vetch were in the beginning of June and in late August or early September, respectively. Rye was sown at the end of August or beginning of September and harvested in August of the next year.

In the diversified systems LT7DV1, LT7DV3, barley was under sown with grass and clover seeds and in diversified system LT7DV2 with grass seed. After the establishment year, ley was grown for 2 years and cut twice per year. In diversified systems LT7DV2 and LT7DV3 ley yields of both cuts were removed. In diversified cereal rotation (LT7DV1) the establishment year was followed by one ley year. Ley was cut twice, the first cut was removed and the second cut was measured and left on the soil surface as GM, except in 2006 when both cuts were removed.

The straw was removed from all crop rotations in the first year of each 4-year crop rotation (1997, 2001, 2005, 2009, 2013, and 2017). Otherwise, straw was left on the field and incorporated into soil during ploughing. Legumes and leys in crop rotation, tillage, and different types of manure (fox manure, farmyard manure, cow slurry) were practised on this site. The amount of manure varied over the study period.

SOC, Soil Properties and Yield Data Used for Simulations

All information required by ECOSSE to run the simulations in the long-term experiments, e.g., SOC, soil properties, and yield data, were collected by the Diverfarming project partners. The supplied SOC concentration in the four arable cropland sites was expressed in $g\,kg^{-1}$. To get the SOC stock in $t\,ha^{-1}$ at 0–30 cm depth, as required for simulations with ECOSSE, the value was converted into %, and calculated based on the following equations as reported by Nayak et al. (2015):

$$\text{SOC stock}\left(t\ ha^{-1}\right) = \sum_{i=0}^{n} Cc\% \times BD\left(g\ cm^{-3}\right) \times D\left(cm\right) \tag{1}$$

n: number of soil layers; Cc: carbon concentration; BD: bulk density; D: sampling depth.

In 2018 data were measured separately at 0–10 cm and 10–30 cm depth. Data for previous years were available at 0–30 cm depth without layering (LT1, LT2, LT4, LT7). In 2010 SOC in LT4 was measured at 0–5, 5–10, and 10–25 cm depth. In the absence of further information on SOC depth gradients in that year, SOC concentration at 25–30 cm depth was assumed to be the same as at 15–25 cm depth. In 1997, 2001, and 2009 the SOC concentration in LT7 was measured at 0–23 and 23–35 depth. The SOC concentration of layer 23–35 cm was assumed to represent the concentration of the 23–30 cm soil layer. The available soil water at field capacity and at saturation, and the yield data were supplied by the project partners. Three or more replicated values were available for each of the studied sites.

Weather Characteristics

The weather of three of the test sites Murcia (Spain), Foggia (Italy), and Huesca (Spain), located in the Mediterranean regions, are characterized by warm, dry summers, and wet winters. The annual precipitation averages are between 280 and 529 mm, and occur mostly between October and December and from March to April. The annual temperature averages are between 13°C and 17°C. July and August are the driest months (~30°C). The mean annual potential evapotranspiration (PET) at these sites reaches 730–1,200 mm maximum, which occurs in July and August. The test site in Finland has colder winters and summers than the other sites. The mean annual temperature in the Finland boreal regions Toholampi is 3°C with annual precipitation of 600 mm and PET of 480 mm. The temperature in July-August reaches between 12 and 20°C. The weather characteristics of the four sites are presented in **Figure 1**.

The ECOSSE Model

ECOSSE was developed in 2007 to examine the impact of changes in land-use and climate on thin organo-mineral soils with <50 cm surface organic horizon, which tend to undergo more land-use changes than the deeper peat soils and are more accessible for agriculture (Smith et al., 2010). In particular, Smith et al. (2010) aimed to simulate how land-use and climate change affect SOC and GHG emissions from organo-mineral, mineral, and peat soils. ECOSSE simulates the major below-ground C and N turnover in mineral and highly organic soils using concepts derived from two well-established models, RothC (Colman and Jenkinson, 1996) and SUNDIAL (Bradbury et al., 1993). In ECOSSE soil organic matter (SOM) is described as five pools: active pools of humus (HUM), biomass (BIO), resistant plant material (RPM) and decomposable plant material (DPM), and an inert organic matter (IOM) pool. The DPM/RPM ratio determines the decay of plant material added to the soil, this ratio being derived from standard values for each land use type or modified for new land-uses. Material in each pool decomposes at a specific rate depending on soil temperature, soil

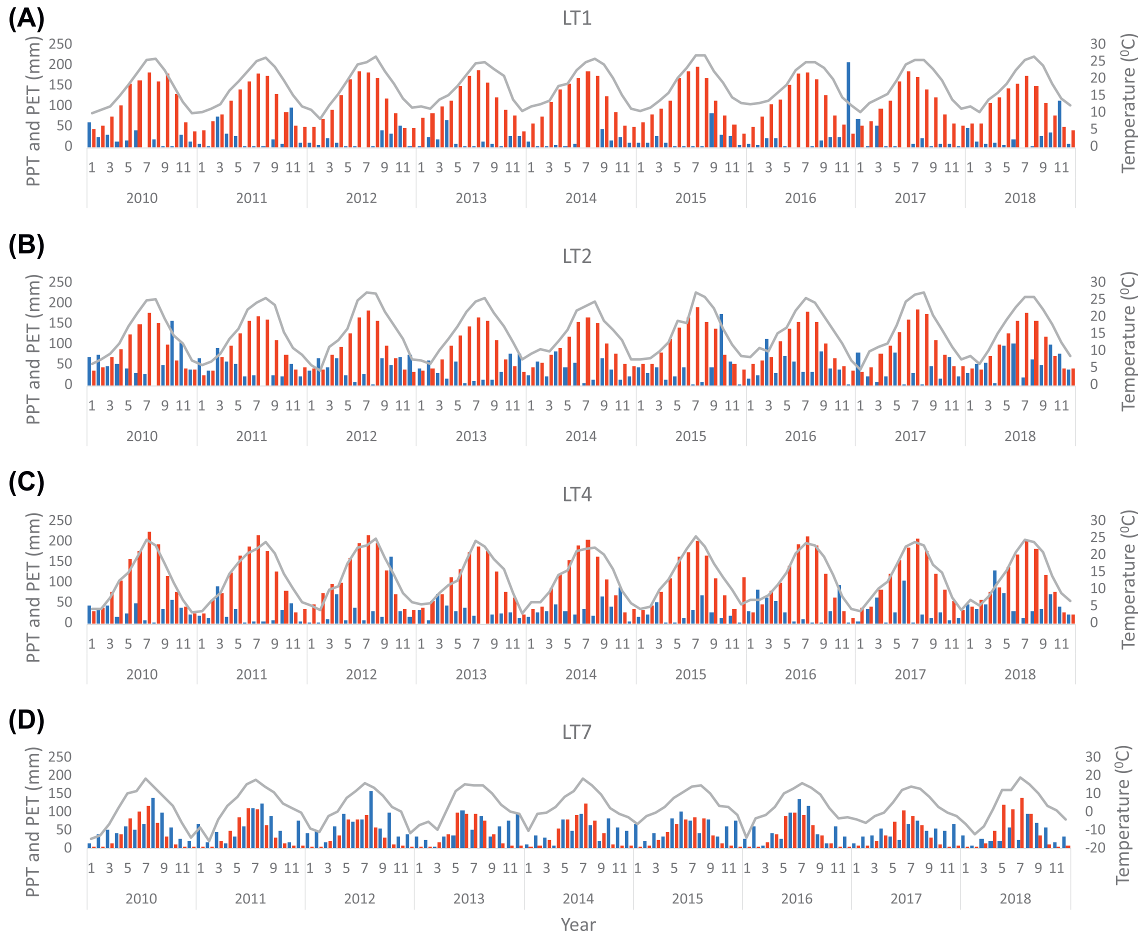

FIGURE 1 | Monthly climatic characteristics of European cropland during the 2010–2018 period. **(A)** LT1 Murcia Spain, **(B)** LT2 Foggia Italy, **(C)** LT3 Huesca Spain and **(D)** LT7 Toholampi, Finland [PPT, Precipitation mm, (blue bars); PET, Potential evapotranspiration (mm, red bars), Temperature, Average temperature (°C, gray line)].

moisture, pH, and cover crop. The C in the IOM pool is not active in the decomposition process. ECOSSE works with different levels of input detail, namely in *limited data* and *site-specific* mode. In the limited data mode, the only inputs are the commonly available meteorological data, such as monthly air temperature, precipitation, and PET; soil data such as soil pH, soil clay content, initial total SOC content, and soil texture class; and land-use (or management data) such as vegetation type, cultivation/planting schedules, and amount and timing of nutrient amendments. With these drivers the model can simulate daily N-gas flux (N_2O, N_2, and NH_3), C-gas flux (CH_4 and CO_2), dissolved organic C, dissolved organic N, and leached nitrate N, and therefore to predict how land-use and climate change impact C and N dynamics in organic and mineral soils. A complete and more detailed description of the structure and formulation of C and N stocks dynamics in ECOSSE is given Smith et al., 2010. The limited data mode is well suited for simulations at both national and field scales, thus allowing us to use results to directly inform policy decisions. In limited mode, the plant input is estimated from the provided SOC or estimated using the MIAMI model (Smith et al., 2010). In site-specific mode, along with the above-mentioned input, the user needs to provide detailed management data describing planting times, cultivation, fertilizer applications, manure, and crop type. Also, the user can decide to either provide detailed soil parameters, plant input and soil water parameters, or to have them calculated by the program as in limited mode. Simulations in site-specific mode allow us to get a better approximation of the contribution of factors that determine the activity of the SOM, and of the plant inputs needed to calculate the changes in SOC. The site-specific version has been usually applied to arable land use (Smith et al., 2010). Therefore, also in this study ECOSSE was run in site-specific mode, and all data used for the simulations were obtained from field measurements. ECOSSE was parameterized for 35 arable crops and one perennial crop Miscanthus. In the site version, C input from plant is obtained from the yield, using five empirical parameters as in the following equation (Smith et al., 2010):

$$C_{in,tot} = C_{a0,1}\left(C_{a0,3} + C_{a0,2}\left(C_{a0,5} - exp\left(C_{a,03} \times W_{yield}\right)\right)\right) \quad (2)$$

$C_{in,tot}$: C from plant input.
$C_{a0,1}, C_{a0,2}, C_{a0,3}, C_{a0,4}, C_{a0,5}$: empirical parameters set to each crop.
W_{yield}: yield of crop (t ha^{-1}).

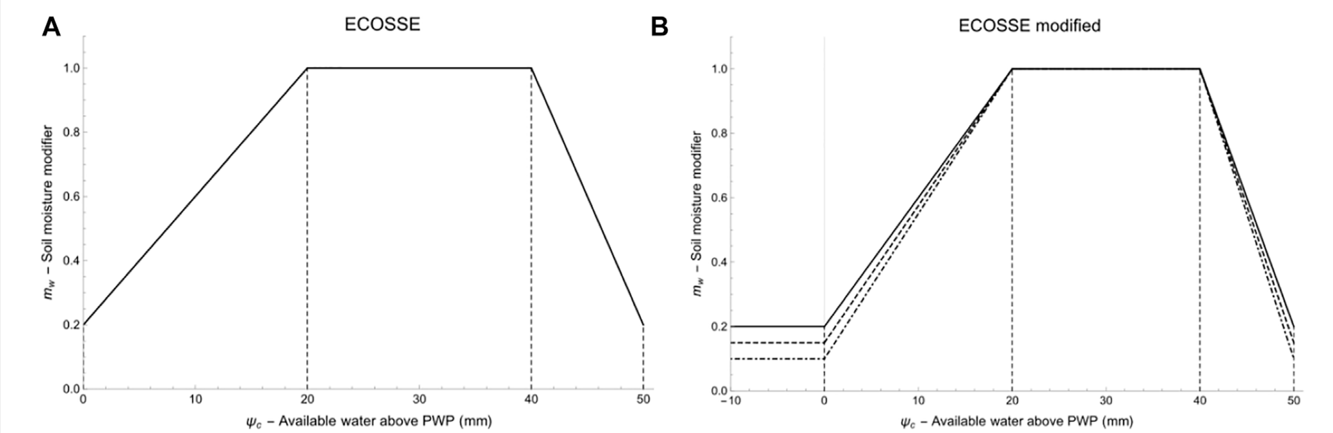

FIGURE 2 | (A) Soil moisture rate modifier for aerobic decomposition as a function of the available water above permanent wilting point (PWP) in ECOSSE; **(B)** and in its modified version–the continuous line refers to normal soil, dashed line to dry soil, and dot-dashed line to semiarid soil.

Plant residues can be either incorporated or removed. Unlike other C models, e.g., RothC, ECOSSE considers the effect of tillage in the different C and N pools. Based on the depth, tillage is classified as CT, RT, minimum tillage (MT), and NT. In ECOSSE CT represents a cultivation depth of 20–30cm, RT refers to 15–20 cm, and MT to 5–10 cm depth (Smith et al., 2011). Less tillage results in lower or no soil disturbance down to the cultivation depth which enhances SOC stabilization processes (Bell et al., 2012). The model can run with daily/weekly or monthly time steps. In this study the model was run in site mode with a monthly time step.

Model Modifications

To adapt the ECOSSE model to all European arable land uses, the object of this study, the following modifications were made.

Moisture Modifier

The original version of ECOSSE does not consider the dynamics of SOC below the permanent wilting point. Under aerobic conditions, the decomposition of soil organic matter is modified in response to soil temperature, moisture, pH, and crop cover. The soil moisture rate modifier for aerobic decomposition follows the equation proposed by Bradbury et al. (1993) for the SUNDIAL model and varies between 0.2 and 1.0. The aerobic decomposition proceeds at a maximum rate of 1.0 as the soil dries from field capacity (ψ_f) to the amount of water held at −1 bar (ψ_i, corresponding to the water deficit in the topsoil of 23 mm at Rothamsted), but then decomposition is inhibited below −1 bar until the soil is at its permanent wilting point ($\psi_c = 0$) where the rate modifier reaches its minimum value (m_{w0}).

$$m_w = \begin{cases} 1 - \dfrac{(1-m_{w0})\left(\psi_f - \psi_c - \psi_i\right)}{\psi_f - \psi_c} & if\ 0 < \psi_c < \psi_i \\ 1 & if\ \psi_i < \psi_c < \psi_f \end{cases} \tag{3}$$

The above field capacity, m_w follows a linear decline to its minimum value m_{w0} (**Figure 2**):

$$m_w = 1 - \frac{(1-m_{w0})\left(\psi_c - \psi_f\right)}{\psi_s - \psi_f}\ if\ \ \psi_c > \psi_f \tag{4}$$

Following the method used for RothC by Farina et al. (2013), the ECOSSE model was modified to account for dry and semiarid soil conditions, both in limited and site-specific mode. In the modified ECOSSE, when no crop is present (bare fallow) the soil can dry below wilting point, and the soil decomposition rate is assumed to be lower than 0.2 in dry and semiarid soils (**Figure 2**). The user can now choose between three types of dryness categories: normal (m_{w0} = 0.2), dry (m_{w0} = 0.15), and semiarid (m_{w0} = 0.1). The simulations were run considering normal soil for LT7, dry soil for LT1, and semiarid and bare dry fallow soil for LT2 and LT4.

Multiple Cropping, Manure application, and Irrigation

To be able to consider the managements practised in LT1 and LT2, ECOSSE was modified to allow simulations of multiple cropping systems, i.e., two crops growing one after the other in the same year. The crop melon was added to the arable crop list, and it was parameterized following the crop code for cauliflower as suggested by Bell et al. (2012). Although different types of manure were already included in ECOSSE, several other types have been used within Diverfarming and were added to the new version. These are sheep manure, sheep compost, compost tea, farmyard manure, cow slurry, and GM. ECOSSE computes C input from manure using C and N content in kg t^{-1} fresh manure. These values were provided for each manure type. Finally, the possibility of adding irrigation was implemented in the model.

Cover Crop

As previously mentioned, cover crops were sown in the LT1 and LT2 sites and all biomass was incorporated into the soil as GM, rather than harvested. In the simulations for LT1, the application of 2.8 t ha^{-1} yr^{-1} GM (41.9%C, 2.24%N, Almagro et al., 2021) was considered to simulate the effect of fully incorporated cover crops on soil properties. For LT2, plant C input from each crop was available for the entire period. The yield of tick bean necessary to obtain the equivalent (measured) amount of carbon in the soil (Farina et al., 2017) was calculated using the information provided, and simulations were run accordingly. An additional *in silico* experiment was run by adding 2.4 t ha^{-1} yr^{-1} GM instead of a growing crop after wheat, which reproduced a similar impact on SOC simulations.

Grass Ley

Like other process-based models (DayCent, DNDC), in site-specific mode ECOSSE is not able to simulate C dynamics in intercropping managements. Also, arable crops are assumed to have one sowing and one harvest date; therefore, grass with multiple mowing dates cannot be simulated. However, based on yield values it was possible to evaluate the C input for each crop and rotation in LT7. Carbon input was calculated according to Palosuo et al. (2016). A C input of 1 t ha^{-1} yr^{-1} was estimated from the grass incorporated in the soil as GM, which amounts to 2.5 t ha^{-1} yr^{-1} of GM in the field for the simulations. The already parameterized crop "oats" was used to model the combined oats + vetch crop (Smith et al., 2011). All other plots were run without further modifications.

Statistical Analysis

For each case study, simulated and measured SOC values were compared. The model performance was evaluated with MODEVAL (Smith and Smith, 2007; Smith et al., 1997a), which allows determining coincidence and association between measured and modeled SOC. MODEVAL calculates the normalized root mean square error (NRMSE), which indicates the total difference between the observed and predicted values (**Eq. 5**). The significance of NRMSE was assessed by comparing it to the value $NRMSE_{95\%}$ obtained assuming a deviation corresponding to the 95% confidence interval of the measurements **Eq. 6**. An NRMSE value that is less than $NRMSE_{95\%}$ indicates that the simulated values fall within the 95% confidence interval of the measurements. The degree of association between the modeled and measured values is determined using the correlation coefficient r. The value of r ranges from −1 to +1. Values close to −1 indicate a negative correlation between simulations and measured values, 0 indicates no correlation, and values close to +1 indicate a positive correlation (Smith et al., 1996). The significance of the association between simulations and measurements is assigned using a Student's t-test as outlined in Smith and Smith (2007). The mean difference between observation and simulation (M, **Eq. 8**) is calculated to assess bias in the modeled values and is expressed in the same unit as the analyzed data (Dondini et al., 2015). The bias is expressed as a percentage of the normalized error, E_N (**Eq. 9**). The significance of E_N was determined by comparing it to the value $E_{N95\%}$ obtained assuming a deviation corresponding to the 95% confidence interval of the measurements (**eq. 10**). An E_N value greater than $E_{N95\%}$ indicates that the bias in the simulation is greater than the 95% confidence interval of the measurements

$$NRMSE = \frac{100}{\bar{O}}\sqrt{\frac{\sum_{i=1}^{n}(P_i - O_i)^2}{n}} \tag{5}$$

$$NRMSE_{95\%} = \frac{100}{\bar{O}}\sqrt{\frac{\sum_{i=1}^{n}\left(t_{(n-2)95\%} \times S_e(i)\right)^2}{n}} \tag{6}$$

$$r = \frac{\sum_{i=1}^{n}(O_i - \bar{O})(P_i - \bar{P})}{\sqrt{\left[\sum_{i=1}^{n}(O_i - \bar{O})^2\right]}\sqrt{\left[\sum_{i=1}^{n}(P_i - \bar{P})^2\right]}} \tag{7}$$

$$M = \frac{\sum_{i=1}^{n}(O_i - P_i)}{n} \tag{8}$$

$$E_N = \frac{100}{\bar{O}}\frac{\sum_{i=1}^{n}(\mathrm{Oi} - \mathrm{P_i})}{\mathrm{n}} \tag{9}$$

$$E_{N95\%} = \frac{100}{n}\sqrt{\frac{\sum_{i=1}^{n} t_{(n-2)95\%} \times S_e(i)}{O_i}} \tag{10}$$

where $\bar{O}$ and $\bar{P}$ are the mean values of observed and predicted data, respectively, O_i and P_i indicate the observed and predicted values, and n is the number of samples. $t_{(n-2)95\%}$ is the critical value of the Student's t distribution with n-2 degrees of freedom and a two-tailed P-value of 0.05. $S_e(i)$ is the standard errors of the measurements. The significance of r and M is tested using an F-test (at probability levels of p = 0.05, 0.01, and 0.001), and a Student's two-tailed t-test (critical at 2.5%), respectively.

Simulations With Mitigation Scenarios

To understand the most suitable mitigation options in the Mediterranean ecosystem, ECOSSE was used to model the mitigation potential of different agricultural management scenarios (SCN) for SOC in the test site LT4. The model was run in this site for the period 2010–2018 using current weather and then for 21 more years using future weather data. The future weather data (denoted as METO-HC-HadRM3Q0-HadCM3Q0) were obtained from the UK Met Office Hadley Centre for Climate Prediction and Research (Christensen et al., 2010). This selected simulated scenario represents the warmest forecasted temperature. Eight management scenarios were evaluated to observe the impact of alternative management practices in the SOC dynamics compared with the baseline management. Here, the baseline management refers to the conventional management of LT4, i.e., LT4DV0. The alternative management scenarios are presented in **Table 2**. The cropping systems, inorganic N fertilizer management, residue management, and cropping seasons of all scenarios were considered the same as in the baseline management. Yields under the management scenarios associated with RT were obtained as average yield values from LT4DV0 and the management scenarios associated with NT were obtained from LT4DV1. Since different types of diversified managements were already practised in the long-term case

TABLE 2 | Management scenarios considered for the Mediterranean region of Spain (Huesca) for the period 2010–2039.

Management scenario	Description of the management
SCN 1	NT
SCN 2	Barley-fallow
SCN 3	Compost 10 t ha^{-1} yr^{-1}
SCN 4	GM 10 t ha^{-1} yr^{-1}
SCN 5	Barley-pea-wheat rotation
SCN 6	Integrated management coupled with sheep compost 5 t ha^{-1} yr^{-1}, GM 3 t ha^{-1} yr^{-1}, RT
SCN 7	Integrated management coupled with sheep compost 5 t ha^{-1} yr^{-1}, GM 3 t ha^{-1} yr^{-1}, NT
SCN 8	Barley-ley rotation

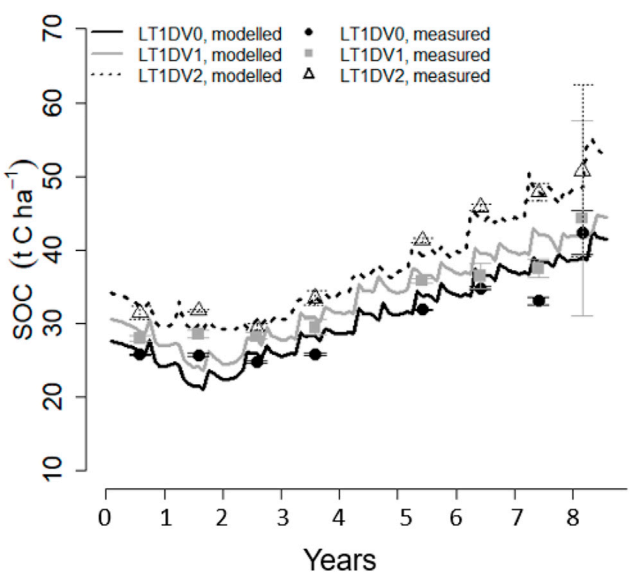

FIGURE 3 | Measured (point) and modeled (line) SOC in Spain (Murcia) Horticulture site, LT1, under conventional (LT1DV0), organic (LT1DV1), and diversified management (LT1DV2) for the period 2010–2018 at 0–30 cm depth. The error bar indicates standard deviation of the measured SOC.

studies LT1, LT2, and LT7, no other practices were considered. However, long-term simulations were run for these sites using the same weather scenarios used for LT4 (**Supplementary Figures S1–S3**).

RESULTS

Modeled and Measured SOC

LT1 Diversified Horticulture in Spain

Each year the application of organic amendment under all managements led to a ~60% increase in SOC over the experimental period 2010–2018 as shown in **Figure 3**. The highest increase in SOC stock at 0–30 cm depth, from 31 t ha^{-1} in 2010 to 51 t ha^{-1}, was measured in 2018 under LT1DV2. This represents a 16% gain when compared with the conventional management (LT1DV0). The simulated SOC also showed a similar increasing trend over the 9-year period with the highest gain (32% as compared with LT1DV0) under LT1DV2. The measured changes in SOC under the three managements were 16.56, 16.26, and 19.23 t ha^{-1} respectively, whereas the predicted SOC changes were 12.83, 13.22, and 16.95 t ha^{-1} respectively. The simulations are highly significantly correlated ($p < 0.001$, NRMSE <10%) with the measured data under the three management practices without any bias (**Table 3**).

LT2 Durum Wheat in Italy

Long-term impact of tillage in wheat monocropping was observed in the Italian dry soil. When the experiment started in 1995, the measured SOC was 49 t ha^{-1} and it increased to 62 t ha^{-1} in 2018 under CT (LT2DV0). Practising NT instead of CT led to a further 21% difference in the increase of SOC (LT2DV1) by the end of the experimental period (**Figure 4**). The impact of rotation was also observed under both tillage systems between 2010 and 2018. The introduction of tick bean in rotation with wheat led to a measured increase in SOC of 25% under CT (LT2DV2) and 21% under NT (LT2DV3). As in the observations, ECOSSE predicted that under monocropping wheat the long-term NT practice (LT2DV1) will lead to a larger SOC value, nearly 19%, than that simulated in the CT system (LT2DV0). Similarly, when wheat tick bean rotations were considered, the model demonstrated a ~24% increase in both LT2DV2 and LT2DV3 sites. The increase in measured SOC under LT2DV0 and LT2DV1 for the period 1996–2018 was of 13 and 15 t ha^{-1} respectively, whereas ECOSSE predicted an increase of 19 and 23 t ha^{-1} respectively. The increase in SOC stock for the period 2010–2018 under LT2DV2 and LT2DV3 was 13 and 11 t ha^{-1} respectively, while the model predicted 13 t ha^{-1} for both managements. A good agreement between the observed and predicted values (NRMSE <10%) was found for all of these sites under the four different treatments. A significant positive association between measured and modeled SOC was attained under all managements ($p < 0.05$) except LT2DV3. However, a highly significant association between modeled and measured SOC was observed when all values were compared under the three managements. In addition, a nonsignificant bias was found between modeled and measured SOC (**Table 3**).

LT4 Tillage in Rainfed Systems in Spain

Both modeled and measured SOC showed a similar increasing trend over the studied period under the conventional management associated with RT in cereal rotations (LT4DV0, **Figure 5**). SOC

TABLE 3 | Statistics for the evaluation of the performance of the ECOSSE model on SOC simulations in diversified cropping systems in the European agriculture at 0–30 cm soil depth.

LT	Management	r	NRMSE (%)	$NRMSE_{95\%}$	M ($t\ ha^{-1}\ yr^{-1}$)	E	$E_{95\%}$
LT1	All management (*n* = 24, rp = 3)	0.95^{a}	6.82	81.51	-0.18^{ns}	−0.60	42.48
LT2	All management (*n* = 20, rp = 5)	0.92^{a}	4.45	50.10	0.14^{ns}	0.24	24.52
LT4	All management (*n* = 4, rp = 6)	1.00^{a}	3.23	13.16	-0.56^{ns}	−1.52	13.02
LT7	All management (*n* = 16, rp = 4)	0.46^{ns}	6.86	30.39	0.99^{ns}	0.64	28.80

[a]*Significant correlation (r) between modeled and measured SOC, at* $p < 0.001$.
ns, nonsignificant; n, number of samples; rp, number of replications.

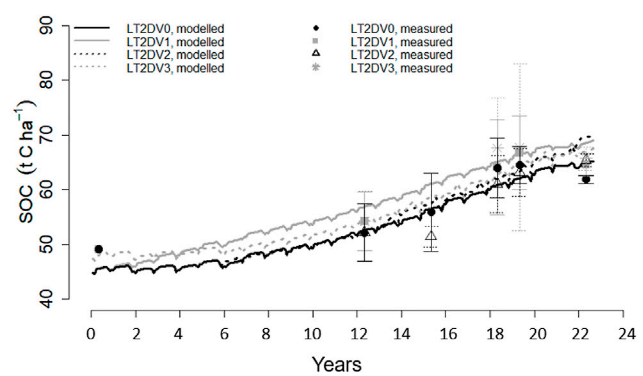

FIGURE 4 | Measured (point) and modeled (line) SOC in Foggia (Italy) arable cropland site, LT2, under conventional (LT2DV0), diversification 1 (LT2DV1), diversification 2 (LT2DV2), and diversification 3 (LT2DV3) for the period 1996–2018 at 0–30 cm depth (The field experimental period of LT2DV2 and LT2DV3 is 2010–2018). The error bar indicates standard deviation of the measured SOC. Please note that the simulated and experimental values of LT2DV2 slightly overlap with those of LT2DV0.

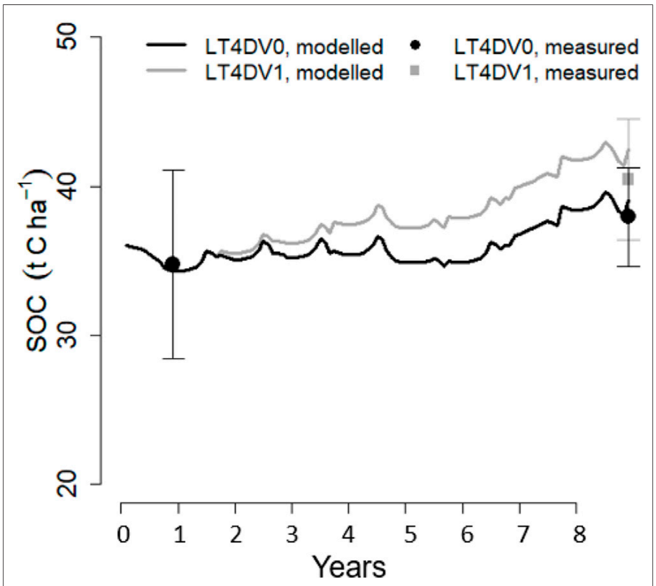

FIGURE 5 | Measured (point) and modeled (line) SOC in Huesca (Spain) cereal cropland site, LT4, under conventional (LT4DV0), and diversified management (LT4DV1) for the period 2010–2018 at 0–30 cm depth. The error bar indicates standard deviation of the measured SOC.

increased from 35 $t\ ha^{-1}$ in 2010 to ~38 $t\ ha^{-1}$ in 2018 as revealed in both the simulations and observations with this management. The increasing trend was also observed when RT was replaced with NT (LT4DV1). The field data showed 6% SOC increase, while the model predicted 8% increase in SOC under NT compared to RT in 9 years. A highly significant positive association between modeled and measured SOC values without bias was observed in this site. Moreover, the $NRMSE_{95\%}$ value was greater than the NRMSE values indicating that the simulated values fall within the 95% confidence interval of the measurements.

LT7 Cereals and forage crop rotations in Finland.

In the Boreal site of Toholampi, Finland, the long-term impact of 4-year cereal and forage rotations under diverse tillage, residue, and manure management practices was observed over 20 years, from 1997 to 2018. In all considered managements, the highest SOC value was observed in 2001, after 4 years from the start of the experiment and then followed a declining trend until the end of the experiment (**Figure 6**). The maximum SOC loss (nearly 23 $t\ ha^{-1}$) was observed under the conventional cereal rotation (LT7DV0) where the value decreased to 127 $t\ ha^{-1}$ during 2018 from 150 $t\ ha^{-1}$ in 1997. The SOC loss however was comparatively lower under the management with grass in the rotations. Besides, application of manure, tillage, and residue management led to a loss of 10 $t\ ha^{-1}$ in LT7DV1 and 5 $t\ ha^{-1}$ in LT7DV3, respectively. A SOC gain of nearly 4 $t\ ha^{-1}$ was observed under the management of conventional grass (LT7DV2), where an increase in SOC stock was observed from 151 $t\ ha^{-1}$ in 1997 to 155 $t\ ha^{-1}$ in 2018. As in the measured data, the model also predicted highest SOC gain under the management LT7DV2 amounting to 10 $t\ ha^{-1}$ SOC stock increase over the 23 years of simulations. An SOC gain was also observed by the model under the clover-grass management (LT7DV3), predicting an increase of 6 $t\ ha^{-1}$. As in observations the maximum decline in SOC was predicted under LT7DV0, with a value of 36 $t\ ha^{-1}$ at the end of the simulated period. A positive but nonsignificant association between the modeled and measured SOC values was found for this site, considering all measured data points. However, no bias between simulations and observations was found in the simulations for any management. Also, the $NRMSE_{95\%}$ value was greater than the NRMSE values of 7%.

Mitigation Scenarios

Eight management scenarios were modeled in the Spanish dry soil for the period 2010–2039 to observe the mitigation potential for SOC

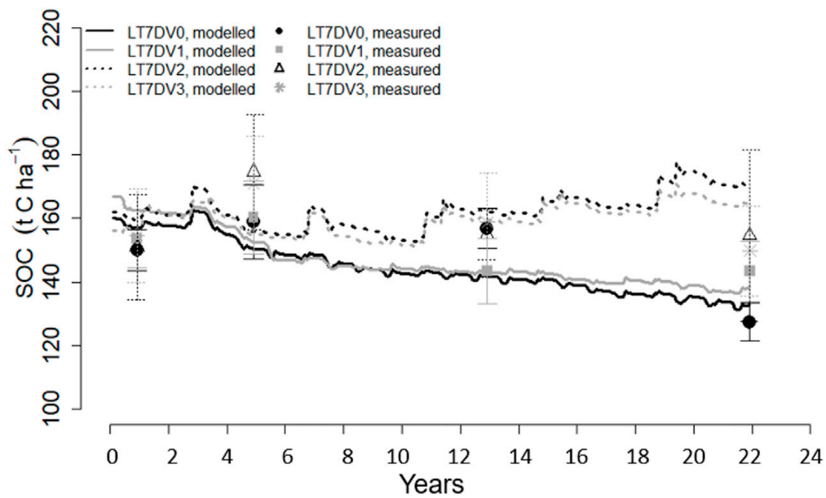

FIGURE 6 | Measured (point) and modeled (line) SOC under conventional (LT7DV0), diversification 1 (LT7DV1), diversification 2 (LT7DV2), and diversification 3 (LT7DV3) management in Toholampi (Finland) fodder cropland site, LT7, for the period 2010–2018 at 0–30 cm depth. The error bar indicates standard deviation of the measured SOC.

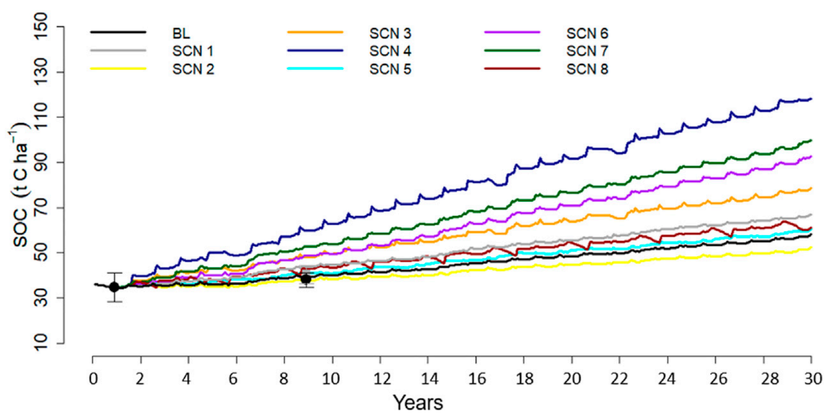

FIGURE 7 | The mitigation potential for SOC under different scenarios in the long-term test sites Huesca (Spain) for the period of 2010–2039 at 0–30 cm depth using ECOSSE modeling. BL: Baseline associated with barley cereal rotations with RT. SCN 1: NT, SCN 2: Barley-fallow rotations, SCN 3: sheep compost 10 t ha^{-1} yr^{-1}, SCN 4: GM 10 t ha^{-1} yr^{-1}, SCN 5: Barley-pea-wheat crop cycle, SCN 6: Integrated management of sheep compost 5 t ha^{-1} yr^{-1}, coupled with 3 t ha^{-1} yr-1 GM and RT, SCN 7: Integrated management of sheep compost 5 t ha^{-1} yr^{-1}, coupled with 3 t ha^{-1} yr^{-1} GM and NT and SCN 8: barley-ley rotations.

dynamics at 0–30 cm depth in comparison with baseline scenario which is associated with barley-cereal rotations under RT (LT4DV0) (**Figure 7**). All scenarios considered in the modeling analysis tend to increase SOC, except SCN 2. Implementing barley-fallow rotations in this scenario leads to a 10% decrease in SOC. No changes, or a slight increase (~4%), was observed in the barley-pea-wheat rotation (SCN 5), and barley-ley rotation (SCN 8) over 30 years, whereas NT in place of RT led to a 16% increase in SOC (SCN 1). The use of 10 t ha^{-1} yr^{-1} of sheep compost produced an increase in SOC of 36% (SCN 3), whereas the same amount of GM produced an increase of 106% (SCN 4), which is the highest gain among all scenarios. An integrated management coupling 5 t ha^{-1} yr^{-1} sheep compost and 3 t ha^{-1} yr^{-1} GM produced an increase of 59% under RT (SCN 6) and 71% under NT (SCN 7), respectively. The modeld SOC sequestration rate over the 30 years of simulations in this site suggested that an average increase of 1.28 t C ha^{-1} yr^{-1} SOC can be obtained by adopting an integrated management. Modifications of the tillage system from RT to NT generated an increase in SOC of 0.30 t C ha^{-1} yr^{-1}. The SOC sequestration rate predicted by the model for sheep compost was 0.69 t C ha^{-1} yr^{-1}, whereas with GM applications the simulated SOC sequestration rate was 2.02 t C ha^{-1} yr^{-1}.

DISCUSSION

LT1

Organic amendments are an important exogenous source of C for the soil, and the use of manure/compost can improve soil quality and enhance ecosystem functioning. High SOC values under the organic amendment are often associated with the presence of microbial abundance. A study conducted in Ravenna, Italy, found that compost applications (municipal solid waste, ~10 t C ha^{-1} yr^{-1}) can produce an increase of SOC at 0–30 cm depth, which is twice as much as the SOC measured in plots that receive only mineral fertilizers (70–130 kg N C ha^{-1} yr^{-1}). The microbial abundance did also increase by 50% compared to N fertilized plots (Baldi et al., 2018). An increase in SOC under organic amendment was also reported in the long-term wheat cropland in Broadbalk (Begum et al., 2017) and the barley cropland in Bavaria, Germany (Li et al., 2005). A similar pattern has been observed in all sites object of this work. As can be seen in **Figure 3**, over the 9-year field experiments in the semiarid Mediterranean region of Murcia, Spain (LT1), the addition of organic amendments led to a clear increase of SOC. An equivalent gain of SOC was observed in both LT1DV0 and LT1DV1, which might be due to the similar rate of sheep manure applied in both sites over the studied period. Among the three managements, the increase in SOC was relatively higher under LT1DV2, which received sheep compost and composted tea over the studied period. Compost is a source of stabilized organic matter, more prone to be incorporated in the soil under organic matter compared to direct applications of fresh manure, which rapidly mineralize (Farina et al., 2018). This site also received additional biomass from a cover crop which was then incorporated in the soil as GM and contributed to a further addition of C to the soil. The organic amendments used in the LT1DV2 produced an increase in SOC of 2.17 t C ha^{-1} yr^{-1}.

A good agreement between modeled and measured SOC was observed in this experimental site. The measured increasing trend was well matched by the model. The SOC increased sharply in the LT1DV2 site compared to LT1DV0. The simulations in LT1DV2 were run replacing the cover crop with applications of GM equivalent to a plant input of 1 t C ha^{-1} yr^{-1}. An initial underestimation of the SOC ~3 t ha^{-1} suggested the need to change how ECOSSE represents the effect of soil moisture on the decomposition rate in the case of dry soil. ECOSSE was therefore modified following the adaptation to dry soils of the process-based model RothC proposed by Farina et al. (2013) to adjust the unrealistic high C input values for simulations in dry and bare fallow soils, when the water reaches values below wilting point.

Overall, observed and simulated results for this site demonstrated that in contrast to traditional manure, composted manure and application of GM contribute to a significant (p < 0.001) increase of SOC in dry ecosystems. This could depend on the ratio of applied amounts. In LT1DV2 composted manure and GM were combined in one treatment, while in LT1DV0 and LT1DV1 only sheep manure was applied. Reducing the minimum value of the moisture modifier from 0.2 to 0.15 slowed down the decomposition rate in the case of dry conditions. Such adaptation of the model has produced an increase of 0.4 t C ha^{-1} yr^{-1} that resulted into matching the measured SOC output by the end of the simulated period without any further addition of C input.

LT2

Adoption of NT practices proved to be an important diversified management in the semiarid region of Foggia, Italy. Compared to intense cultivations this practice promotes the accumulation of SOC in the topsoil. Previous studies showed that great benefits derive from practicing NT instead of CT in the Mediterranean cropland, including an increase in SOC (Aguilera et al., 2013; Sanz-Cobena et al., 2017), and that the reduced disturbance of soil, associated with NT, leads to a smaller decomposition rate therefore increasing SOC (Álvaro Fuentes et al., 2009; Begum et al., 2018a). A gain of about 13–20% was observed in NT compared to CT in different regions of Spain at 0–30 cm depth (Alvaro-Fuentes and Paustian, 2011) which agrees well with our findings of a 20% increase under NT with respect to CT in monocropping wheat. Over the 22 years in which the experiment ran, the measured gain of SOC under wheat monocropping was 0.58 and 0.70 t C ha^{-1} yr^{-1} for CT (LT2DV0) and NT (LT2DV1), respectively. The simulated SOC under these two managements was 0.84 and 1.00 t C ha^{-1} yr^{-1}, respectively. Using the model Century to simulate monocropping barley under CT and NT in the semiarid Mediterranean region of Zaragoza, Spain, for 16 years, Álvaro Fuentes et al. (2009) found an increase in the rate of SOC of 0.21 (0.18, measured) and 0.37 (0.46, measured) t C ha^{-1} yr^{-1} under CT and NT, respectively. The results clearly demonstrated a greater increase in SOC under NT. The relatively higher SOC gain under monocropping wheat (compared to monocropping barley) could be due to a greater plant C input, as the measured annual average of above-ground plant C input from wheat monocropping is ~2.50 t C ha^{-1} yr^{-1} which is nearly twice that estimated in Spain under barley monocropping systems (Álvaro Fuentes et al., 2009). A slight decrease in SOC was found under NT in the rotations systems compared to CT; however, the difference in C gain is not significant (p > 0.05). Overall in this site the rotation with tick bean showed a much higher impact on SOC compared to different tillage management practices. Since ECOSSE does not consider root growth we assume that higher increase in SOC is due to N fixation by the tick beans and the increased amount of C input.

The higher $NRMSE_{95}$ and E_{95} values, in all sites, compared to NRMSE and E_N respectively (**Table 3**) indicate that the total error in the simulations is less than the total error in the measurements at 95% confidence interval (Smith et al., 1997a; Bell et al., 2012). Also, no systematic bias was observed between simulations and observations with a nonsignificant t-statistics, and the observed NRMSE <6% is comparable to that in similar studies (Álvaro Fuentes et al., 2009) indicating an acceptable agreement between model output and experimental measurements.

LT4

The temporal dynamics of SOC was observed in the Mediterranean dry region Huesca (Spain) under two tillage regimes for the period 2010–2018. Both the simulations and observations demonstrated a higher SOC increase under NT compared to RT. In this long-term experiment, it was observed that in addition to the lower soil disturbance, the measured crop production, and therefore the C input returned to the soil, was significantly higher under NT than under RT

(Plaza-Bonilla et al., 2017). In semiarid Mediterranean conditions, it has been demonstrated that conservation tillage systems, and particularly NT systems, promote soil water conservation and higher crop biomass production (Cantero-Martínez et al., 2007; Lampurlanés et al., 2016). Under NT, the crop residue layer in the soil surface reduces soil water losses by direct evaporation and also increases the infiltration to runoff ratio (Lampurlanés et al., 2016). These changes in soil water conservation result in a higher water use efficiency in NT compared to RT, and in the consequent increase in crop residue production.

Interestingly, even the RT system led to an increase in SOC levels. This is an opposite finding to what has been observed in other long-term tillage experiments located in NE Spain in which tillage systems reduced SOC stocks (Álvaro Fuentes et al., 2008). The increase in SOC stocks under RT can be explained by the change in tillage implements occurred in these areas during the last few decades. About 30 years ago, farmers adopted less intensive tillage systems, moving from moldboard ploughing to less intensive chisel or disk ploughing. This change in tillage systems resulted in an increase in SOC stocks which is still noticeable to date.

In simulations performed in a different long-term experiment located also in rainfed NE Spain, the RothC model predicted reasonably well the higher SOC accumulation rates observed under NT (Álvaro-Fuentes et al., 2012). In this last study, the higher C inputs measured under NT led to the increase in SOC accumulation rates. Similarly, in our study, the ECOSSE model was also able to capture the different SOC stock change rates observed between the two tillage systems and driven by the different C inputs in NT and RT.

LT7

Perennial crops in rotation represent an important conservation farming system, which disturbs the soil less, reduces erosion, conserves soil moisture, and improves soil health overall (Smith et al., 2007; Di Bene et al., 2011; Sanz-Cobena et al., 2017). The three diversified managements in the Boreal region of Toholampi, Finland, included legume and/or perennial grass in rotation with cereals. Initial observations showed that after 4 years from the beginning of the experiment, in 2001, a nonsignificant increase ($p > 0.05$) in SOC was observed. This was the case in all four treated plots, including LT7DV1 where no organic amendments were applied. After 2001, it was possible to observe a slightly declining trend for LT7DV0 and LT7DV1, slightly increasing trend for LT7DV2 and a more stable one for LT7DV3. The possible reason for the difference in SOC dynamics between the first 4 years and the following ones might be due to the practice of having clover-grass ley in the rotation barley-grass-grass-oats + vetch, which was then changed with the cereal rotation barley-barley-rye-oats (LT7DV0), after 2001. Moreover, the LT7DV0 received 14 t ha^{-1} fox manure in 1997 whereas only chemical N fertilizers were applied for the rest of the study period. Numerical simulations suggest that intense cultivations without any organic amendments in the cereal rotations lead to increased respiration and consequently decreased SOC (not shown). Grass ley in LT7DV2 and clover-grass ley in LT7DV3 was included for two successive years. For the LT7DV2 plot, the modeled average annual C input through root rhizodeposition during the grass growing seasons was 1.8 t C ha^{-1} yr^{-1}, which is nearly three times higher than that in the annual crops (LT7DV0). This supports the observation by Smith et al. (2007) that perennial crops accumulate more C in below ground biomass than annual crops. Despite the addition of 48 t ha^{-1} composted farmyard manure in 1997 and greater amount of cow slurry during most of the remaining period, nearly 28% more than that of the other manured plot, LT7DV3 had lower SOC stocks compared to LT7DV2 ($p > 0.05$). In the simulations, in agreement with the measurements, the model predicted a slight gain in SOC under LT7DV2 and LT7DV3. However, in both sites this increasing trend led to a negative correlation between modeled and measured values, with a nonsignificant r value of −0.55 and −0.77, respectively. The estimation of the uncertainty (NRMSE) for the four managements was found to be 7%, which falls within the values found by Falloon and Smith (2003) for the two process-based models Century and RothC applied to different management practices in European ecosystems (NRMSE between 1.8% and 16.4% for Century and 1.7%–29.2% for RothC). From this long-term study, it is clear that the factors with the largest impact on SOC are the type of crop, tillage, residue, and manure management/type. An additional benefit observed under LT7DV3 is associated with the fact that leguminous crops fix the atmospheric N so that the use of inorganic fertilizer can be reduced.

Modeling Mitigation Scenarios

Different mitigation scenarios have been modeled in Huesca to identify the most suitable managements to increase SOC sequestrations in Mediterranean ecosystems compared to a baseline scenario with RT and incorporation of crop residues (already considered in LT4). The simulation results, which could provide a guideline to policy makers on selecting best agricultural practices for this geographic region, showed that a few promising agricultural managements could be adopted to improve agricultural sustainability, soil resilience, SOC sequestration, and to mitigate the impact of climate change. The simulated changes in SOC were compared to the changes suggested by the IPCC after 20 years under different agricultural activities and categorized depending on climatic regions (Lasco et al., 2006). IPCC estimated an annualized stock change factor (SCF) of 1.10 for temperate dry climatic regions when changing from full tillage to NT, which indicates a 10% increase of SOC under such modification in arable cropland over 20 years. In our simulation a change of 13% (SCF, 1.13) over 20 years was obtained, which compares well with the IPCC value. Also, the 28%–74% range of predicted changes in SOC values under different management practices (SCN 3, SCN 4, SCN 6, and SCN 7) contains the value of 37% change in C stock reported by the IPCC under high nutrient inputs in temperate dry soil over the same period. According to the IPCC, low and medium input are classified as: low residue return, long time soil bare fallow, N fixing crop in rotations. The simulated annual variation in SOC values under these categories (SCN 2, SCN 5, and SCN 8) was between −0.19 and 0.12 t C ha^{-1} yr^{-1} compared to the reported SCF values of −0.05–−0.04 t C ha^{-1} yr^{-1}. The difference of 0.30 C ha^{-1} yr^{-1} found in this analysis between RT (BL) and NT (SCN 1) is within the range reported by the previous literature, for changes from 0.25–0.5 t C ha^{-1} yr^{-1} under temperate dry climate (Lal., 2008) and of 0.09–0.71 t C ha^{-1} yr^{-1} in European continental Mediterranean semi-arid regions in Spain (Álvaro Fuentes et al., 2009; Gonzalez-Sanchez et al., 2012). Also, our results compare well to those reported in the meta-analysis by Aguilera et al. (2013) on SOC changes in Mediterranean cropland. The authors found an 11.3% increase under NT compared to CT and an increase in SOC of 1.31–5.29 t C ha^{-1} yr^{-1} under external organic inputs, which corresponds to 23.8%–98% increase, based on the type and amount of organic amendment. However, these estimations refer to the global Mediterranean climatic regions including arable land and woody cropland. An increase in SOC of 0.72 t C ha^{-1} yr^{-1} was also found under organic amendments by a meta-analysis carried out by Francaviglia et al. (2017) across European Mediterranean arable cropland, which compares well with the increase of 0.69–2.02 t C ha^{-1} yr^{-1} in simulated SOC found in our tested sites (SCN 3, SCN 4, SCN 6, and SCN 7).

The model results under SCN 3 suggest avoiding periods of bare fallow, as such practice leads to SOC loss. A long-term field experiment (16 years) in Mediterranean regions in Spain under RT management found an increase of SOC of 0.24 t C ha^{-1} yr^{-1} in continuous barley cropping systems, whereas the value reduced to 0.003 t C ha^{-1} yr^{-1} under barley-fallow cropping systems (Álvaro Fuentes et al., 2009). The maximum gain in SOC appeared to be under SCN 4 with GM applied at a rate of 10 t C ha^{-1} yr^{-1}. However, this scenario might not be realistic as such high amount of GM is not practised in the European continent. Therefore, the best management scenarios considered in the modeling analysis appeared to be the integrated nutrient managements SCN 6 and SCN 7, followed by the NT scenario (SCN 1). The modeling analysis demonstrated that keeping the same soil management and cropping systems as in the baseline, there is scope to increase SOC by applying external organic C input, with compost being preferred to manure, as manure can lead to an increase in GHG emissions (Smith et al., 2007). In SCN 6 and SCN 7, in addition to the 5 t C ha^{-1} yr^{-1} compost, a 3 t C ha^{-1} yr^{-1} GM has been applied as a representative of cover crop. The adoption of the SCN 6 management could lead to a SOC increase of nearly 1.14 t C ha^{-1} yr^{-1} over 30 years.

When considering the 11.8 M ha of Spanish arable land (FAOSTAT, 2021), it is clear that the potential increase in SOC can be expected to be 3.4 Tg C yr^{-1} under NT and 13.3 Tg C yr^{-1} from diversified nutrient management practices, which correspond to a reduction in CO_2 emission of 12.5 and 48.9 Tg CO_2 yr^{-1} respectively. Applying the process-based model Century the mitigation potential for SOC in Spain was predicted to be 17.8 Tg CO_2 yr^{-1} (Álvaro Fuentes et al., 2009). Our modeled results demonstrate that there is scope to increase SOC stock by adopting diversified management practices including no tillage, diverse cropping systems, and exogenous organic input. Practising an integrated management can contribute to mitigate nearly 14% of the total GHG emissions occurring in Spain (Álvaro Fuentes et al., 2009). Our study suggests that the 4 “per mille” change in SOC can be achieved within 10 years changing tillage practices and within 4 years using an integrated nutrient management.

It is clear that there is great uncertainty in estimating mitigation effects at national level based on one site only. However, this can be deemed as an interim set of estimates obtained by keeping soil, climate, management, and crop growth constant. The estimated figures compare well with other modeling studies in Spain (Álvaro Fuentes et al., 2009), and provide information on SOC sequestration potential under different tillage management, manure management, and cropping systems in Spain.

CONCLUSION

To achieve the SDGs, the current research on European agroecosystems is focusing on the adoption of diversified strategies that would promote soil resilience and agricultural sustainability. This study presents how the process-based ecosystem model ECOSSE was modified and used to simulate data from four European long-term experiments, to investigate changes in SOC under diversified soil and crop managements in comparison with traditional management practices. ECOSSE can now account for different levels of soil dryness, including semiarid regions and vertisols, and it is able to simulate multiple cropping systems and cover crops. ECOSSE was used to assess the mitigation potential for SOC of different management scenarios in semiarid regions in Spain.

The modified ECOSSE well simulated trend and significant associations (if enough data points were provided, >2) between the modeled and observed values of SOC over the study period in both the Mediterranean and Boreal regions. Both simulations and measurements revealed that with the considered diversifications the gain in SOC is higher in the test sites located in the Mediterranean regions than in the Boreal ecosystems. On the other hand, the initial SOC stock in the Boreal regions was much higher, 150 t C ha^{-1}, than the one in the Mediterranean regions, 25 to 50 t C ha^{-1}, therefore allowing for a lower SOC sequestration potential. The declining SOC stocks are also attributed to the fact that following relative recent land use changes, the SOC has not reached an equilibrium state yet (Kätterer et al., 2013). A study by Kätterer et al. (2013) on the Northern European agriculture found decreasing SOC stocks in ley arable rotations compared to cereal based rotations due to higher initial SOC stocks, which they associated with the previous land use history and management. In Finland decreasing trend in soil C stock has also been identified (0.4%/per year) by Heikkinen et al. (2013). Our results suggest that further loss of SOC could be avoided by introducing grass/legumes in the conventional cereal rotations and by adding manure. The higher SOC sequestration potential with lower initial SOC contents that was observed in the Mediterranean soils is consistent with previous findings (Smith et al., 1997a; Minasny et al., 2017; Begum et al., 2018b). The long-term simulations obtained with future weather scenarios for LT1, LT2, and LT7 (**Supplementary Figures S1–S3**) and the mitigation scenarios considered for LT4 confirm these findings suggesting that among all sites the highest SOC sequestration potential can be achieved in LT2 and LT4.

IPCC identified SOC sequestration as an important strategy for C mitigation (Smith et al., 1997b). The modeling exercise in the semiarid arable lands in Spain demonstrates the feasibility of different agricultural management options on sequestering SOC in Mediterranean ecosystems. Our modeled results suggested that an integrated approach considering manure application, cover crop, and NT in the barley cropping systems would be an effective management option. The simulations indicated that crop type, tillage management, and type of manure are the factors with the largest impact on SOC. As suggested by Smith et al. (1997b) other management options could be feasible in European cropland, e.g., afforestation of surplus land, which has greater C mitigation potential but was not covered in our analysis. The applicability of the modified ECOSSE model to European agro-ecosystems at site scale is a first step toward a more thorough analysis of SOC sequestration potential at regional scale, which is necessary to provide effective and clear guidelines for policy makers and stakeholders to mitigate the impact of climate change at the national and international levels.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KB: Writing-original draft, Model calibration and validation. RZ: Project Co-ordinator, Funding acquisition, Data collection, Writing-review and editing. RF, RL, and JÁ-F: Data collection, Writing-review and editing. MC: Study supervision, Model coding and parameterization, Writing-review and editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.819162/full#supplementary-material

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Aboveground Carbon Fixation and Nutrient Retention in Temporary Spontaneous Cover Crops in Olive Groves of Andalusia

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In Southern Spain, olive trees have traditionally been cultivated in marginal areas with relatively shallow and bare soils under rainfed conditions, resulting in heavy soil losses and soil degradation. The implementation of temporary spontaneous cover crops in the inter-rows of olive groves, has proven to be a suitable diversification strategy to reduce soil erosion but it can also contribute to climate change mitigation and the boosting of internal nutrient recycling. However, information on the contribution of cover crops to atmospheric CO_2 fixation and on nutrient retention in olive groves is scarce, which is a major drawback when it comes to modelling on larger spatial scales. In this study, we aimed to assess the potential effects of temporary spontaneous cover crops in olive groves on CO_2 fixation and nutrient retention. The aerial biomass of cover crops ($0.25m^2$ frames) and contents of carbon and nitrogen (CNHS analyser), phosphorus and potassium (IPC-MS) were analyzed in 46 commercial olive groves with different tree densities and cover crop layouts; the whole farm (WCC), the whole farm except the area below the tree canopy (CCC) or in bands of a given width in the inter-row area (BCC). Cover crops of 56% of the olive groves were under BCC whereas only 17% were under WCC. The annual net primary production of cover crops under WCC (1,707.4 kg DM ha^{-1} y^{-1}) was significantly higher than that of CCC (769.5 kg DM ha^{-1} y^{-1}) and with intermediate values for BCC (1,186.4 kg DM ha^{-1} y^{-1}). Similarly, the annual rate of C-CO_2 fixation in the annual net primary production of olive groves with WCC (642.1 kg C ha^{-1} y^{-1}) was 1.35 and 2.1 times higher than the olive groves with BCC and CCC, respectively. On average, 19.5 kg N ha^{-1} y^{-1}, 2.48 kg P ha^{-1} y^{-1} and 24.30 kg K ha^{-1} y^{-1} was accumulated in the biomass of the cover crops. This study demonstrates that cover crops contribute significantly to CO_2 reduction and the retention of significant amounts of tree-unused nutrients. In addition, the higher the area covered by cover crops, the higher the contribution to these ecosystem services.

Keywords: Olive orchards, Cover crops, CO_2 fixation, nutrient retention, Diversification

Abbreviations: ANPP, annual net primary production; BCC, TSCC in bands of a given width in the inter-row area; CCC, TSCC in the whole farm except the area below the tree canopy; DM, dry matter; SOC, soil organic carbon; TSCC, temporary spontaneous cover crops; WCC, TSCC in the whole farm.

1 INTRODUCTION

Olea europaea L. is the most important perennial crop grown in the Mediterranean basin. It occupies about 10 million ha and it shapes the socio-economic and cultural life of many villages (Loumou and Giourga, 2003). Furthermore, olive orchards configure the rural natural landscapes of many areas in Spain, Italy, Portugal and Greece. Andalusia, the southernmost region of Spain, is the largest producer of olive oil in the world, and about 47% of its arable land is devoted to olive groves (MAPA, 2020a). Therefore, current and subsequent changes in management practices have a great impact on climate change mitigation and adaption, biodiversity and provision of ecosystem services on a regional scale (López-Vicente et al., 2021).

In the pre-industrial period, olive-groves often fulfilled multiple functions in rural communities, but they gradually shifted to intensive systems to maximize commercial profitability (Infante-Amate and de Molina, 2013). This entailed several changes in management from 1970 onwards, such as an increase in the tree density, the widespread application of synthetic agrochemicals and an intensive use of tillage, irrigation and fossil fuels (Beaufoy, 2001). In recent decades, olive farming has also become a paradigmatic example of territorial expansion and concentration with a clear trend towards monoculture (Ortega et al., 2020). This has in turn resulted in simplified landscapes with a relatively low capacity for providing ecosystem services other than the production of olives fruit.

Olive cultivation is strongly associated with soil and water losses by erosion and runoff mainly due to: 1) its location on marginal soils and steeply sloping soils (Gómez et al., 2003), 2) the relatively intensive and short rainfall episodes during autumn and late winter, characteristics of Mediterranean climate (Morugán-Coronado et al., 2020), 3) the sparse ground cover provided by the tree canopy (Fraga et al., 2021), 4) the soil management based on the systematic removal of the unwanted herbaceous vegetation to avoid competition for water, often by tillage (Gómez, 2017). As a result, erosion has been a historical source of environmental degradation in olive groves, which has been exacerbated by the intensification process (Vanwalleghem et al., 2011). Soil erosion not only means the loss of the inorganic fraction of the soil, but also the loss of soil organic matter, which is an important reservoir of organic carbon, nutrients and valuable soil biota (Zuazo and Pleguezuelo, 2008). Therefore, soil erosion is a great threat to the economical sustainability of the olive grove, since it impacts negatively on the soil fertility (Liu et al., 2021).

In order to mitigate soil losses by erosion, the regional authorities of Spain implemented a regulation that enforced the establishment of temporary herbaceous covers of a minimum of 1 m width in the inter-row area of olive groves with a mean slope greater than 15% (MAGRAMA, 2014). Moreover, it is likely that these conditions will be changed to a minimum of 2 m width on a mean slope higher than 10% in the new eco-schemes of the CAP 2023—2027 (European Commission, 2021).

Cover crops are a recognized agricultural diversification tool (Rosa-Schleich et al., 2019). In woody crops orchards, they usually consist of a native or seeded herbaceous cover intercropped in the inter-rows, which is mowed or removed at a given time to avoid competition with the trees for water and nutrients (Junta de Andalucía, 2007; Francaviglia and Vicente-Vicente, 2021). It has been demonstrated that cover crops provide a wide variety of soil ecosystem services such as reduction of soil and water loss by erosion and runoff (Sastre et al., 2017; Gómez et al., 2018; Repullo-Ruibérriz de Torres et al., 2018). They have also been shown to increase the stock of soil organic carbon (Almagro and Martínez-Mena, 2014; López-Vicente et al., 2021). When implemented between the harvest and the sowing of annual crops, cover crops have demonstrated to decrease nutrient leaching (Abdalla et al., 2019). In perennial woody crops, such as olive groves, temporary spontaneous cover crops (TSCC) reduced the nutrient losses during rainfall events by reducing runoff yield and soil loss (Rodríguez-Lizana et al., 2007; Gómez et al., 2009). Some studies have suggested that cover crops might retain nutrients when the nutrient demand of the tree is low (García-Ruiz et al., 2011) and then release them through mineralization when the olive tree is active (Gómez-Muñoz et al., 2014). Furthermore, cover crops may also reduce the downward movement of nitrate and potassium, retrieve N, P and K from superficial and deep soil layers, and fix atmospheric N_2 if the TSCC community includes a significant proportion of legumes. Therefore, one of the roles of TSCC in olive orchards is to boost internal nutrient recycling by transforming inorganic nutrients into organic nutrients embedded in the TSCC biomass. However, there is little information on the nutrient retention capacity of cover crops in tree crops and olive groves (Gómez-Muñoz et al., 2014; Rodríguez-Liazana et al., 2020; Repullo-Ruibérriz de Torres et al., 2021a). Most of the studies on the effects of cover crops on olive groves usually focus on particular species to be used as seeded cover crops, although they often include spontaneous cover crops for comparative purposes. On the other hand, these studies are not based on extensive sampling, but are performed on a single or few experimental plots. This is a major drawback in extrapolating the results on to a larger spatial extent (Gómez, 2017), which is necessary to model the beneficial effects of diversification in olive groves on climate change mitigation and nutrient retention.

Boosted by cross-compliance measures attached to agrarian subsidies and by scientific dissemination, the implementation of cover crops is becoming popular in olive-groves in Andalusia (Junta de Andalucía, 2015b). According to the last national survey on soil management (MAPA, 2020b), 37% of the olive grove areas in the region are managed by cover crops, of which about 99% are temporary spontaneous cover crops. However, this survey does not provide any information on the percentage of soil covered by cover crops, different cover crop design strategies, the number of months taken to maintain the cover crops, the control methods or the amount of biomass produced. Recent studies have used GIS and remote sensing methodologies to estimate the degree of soil cover (Peña-Barragán et al., 2004; Cruz-Ramírez et al., 2012; Lima-Cueto et al., 2019) or the biomass production of temporary spontaneous cover crops through satellite or aerial images (Blázquez et al., 2021). Although these methodologies are

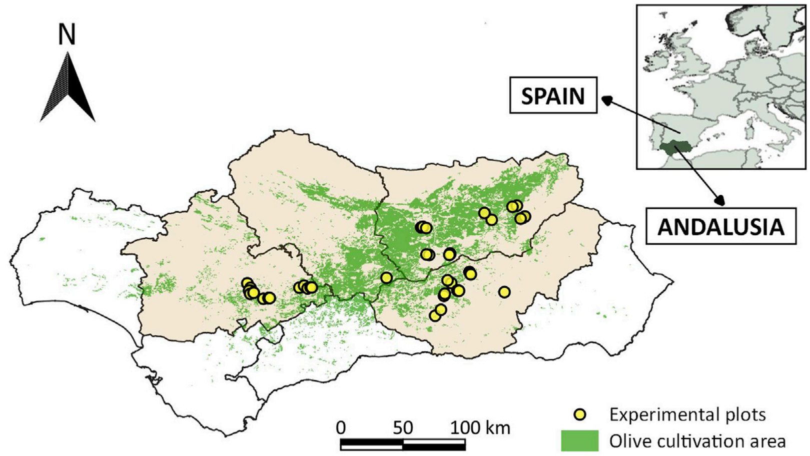

FIGURE 1 | Location of the selected olive groves in Andalusia.

suitable to estimate the covered area, they are still not sufficiently precise to estimate the biomass.

The capacity of temporary spontaneous cover crops as a diversification strategy to provide ecosystem services is ultimately determined by the fraction of soil covered (Zuazo and Pleguezuelo, 2008; Laflen et al., 1985; Unger et al., 1991; Sastre et al., 2017) and the biomass produced (Finney et al., 2016; Gómez, 2017). For instance, Gómez et al. (2009) found that by applying the RUSLE model in olive groves, the higher the area covered by TSCC (WCC and CCC), the lower the soil erosion. Therefore, proper characterization of the temporary spontaneous cover crops in many real and representative olive groves of Andalusia needs to be accomplished. We hypothesize that TSCC might significantly reinforce the role of olive groves in mitigating climate change and in retaining nutrients within the orchard, and that the magnitude of these will differ according to the TSCC layout models. This study aims to assess the aboveground net primary production, the nutrient retention potential and the carbon fixation of TSSC in 46 commercial olive groves of Andalusia.

2 MATERIALS AND METHODS

2.1 Olive Groves Selection

46 olive groves with spontaneous cover crops before 2015 were randomly selected using Google Earth Pro images from the provinces of Jaén, Córdoba, Granada and Seville (**Figure 1**; **Supplementary Table S1**). These are the areas with the highest olive oil production in Andalusia, accounting for 72% of the Spanish and 27% of the total world production (Vilar and Pereira, 2018; MAPA, 2020a).

Sampling was carried out on the selected olive groves from mid-March to mid-April 2021. In each olive grove, the canopy area of five trees was measured using Google Earth Pro and the total canopy area per hectare was obtained from the mean tree canopy area and the tree density. Sampled olive groves included 30 traditional (<150 trees per hectare), 13 intensive (150—400 trees per hectare), and three super-intensive orchards (>400 trees per hectare), with a tree canopy area ranging between 534.9 and 4,833.5 $m^2\ ha^{-1}$ (**Supplementary Table S1**). The annual rainfall during 2021 and over the previous 10 years (2011–2020) was obtained from the closest weather stations (Agroclimatic Information Network of Andalusia, RIA). The 10-years (2010–2020) mean annual rainfall ranged between 332 and 615 mm, whereas that of 2021 was on average, 25% lower (between 204–459) than that (RIA, 2021).

The soil types of the selected plots were obtained from the REDIAM WMS Soil Map of Andalusia (Junta de Andalucía, 2012), prepared by the Ministry of the Environment using as reference base the Landsat-TM satellite orthoimage. The soils appear in cartographic units characterized by associations grouped at the second order level of the FAO classification criteria (FAO-UNESCO, 1974) and the Soil Map of the European Union (CEC, 1985).

The main soil types in the selected olive groves are calcareous regosols and calcic cambisols with lithosols, calcareous fluvisols and rendsins (37% of the olive groves), calcium cambisols with calcareous regosols (13%) and calcareous regosols and calcium cambisols with calcium luvisols and calcareous fluvisols (11%).

2.2 Sampling of Temporary Spontaneous Cover Crop and Aboveground Net Primary Production Estimates

The aboveground biomass was estimated by the Ravindranath and Ostwald harvest method (2008). Olive groves were visited between mid-March 2021 to the end of April 2021 and the aboveground biomass of the temporary spontaneous cover crops was harvested a few days before the farmers controlled them. Mid-March to Mid-April is the recommended period to control the cover crop to avoid competition for water in Andalusia, especially in rainfed olive groves (Junta de Andalucía, 2007). In each of the olive groves, five 0.5 m × 0.5 m frames were randomly set in the area covered by temporary spontaneous vegetation and the aboveground biomass was manually cut to ground level with grass shears and stored in plastic bags

(Gómez-Muñoz et al., 2014). In each of the olive groves where the spontaneous cover crops occupied a strip in the inter-row tree area, the width of at least five strips was recorded.

Samples were dried in an oven at 50°C for 4 days. After being dried, the harvested biomass was weighed. The harvested aboveground biomass was assumed to be the aboveground net annual primary production as it was produced between April of 2020 to mid-April 2021, when sampling was carried out.

The area covered by temporary spontaneous cover crops (TSCC) in 1 ha was estimated by photointerpretation of Google Earth Pro images depending on the cover crop layout. For olives groves where the whole ground was covered by the TSCC (hereinafter referred to as WCC), an occupation of 100% of the area was assumed. For olives groves where the cover crop was absent under the tree canopy but occupied all the inter-tree rows (hereinafter referred to as CCC), the cleared area under the tree canopy of five trees was measured using Google Earth Pro and the mean was extrapolated to 1 ha by considering the tree density. This cleared area was subtracted from 1 ha to calculate the area covered by the TSCC. When the TSCC of the olive groves occupied a strip of the inter-tree row area (hereinafter referred to as BCC) of a given width, the number of inter-tree rows in 100 m and the mean width area were taken into account to calculate the area in 1 ha occupied by temporary cover crops.

The annual aboveground net primary production (kg DM ha^{-1} y^{-1}) was calculated by taking into account the harvested biomass in the frames (g DM in 0.25 m^2) and the area covered by the cover crop in 1 ha.

2.3 Carbon, Nitrogen, Phosphorus and Potassium Analyses

Once the biomass was weighed, an aliquot of between 20 and 100 g was completely ground to powder with a hammer mill with a 1 mm sieve. Carbon and nitrogen were analyzed in a CHN elemental analyzer (Leco TruSpect Micro). In addition, an aliquot of the milled biomass was subjected to perchloric-nitric (3:5 v/v) digestion and the total phosphorus (P) and potassium (K) were analyzed in an ICP-MS mass spectrometer (Agilent 7900). The contents of total carbon, nitrogen, phosphorus and potassium of the aboveground biomass of the temporary spontaneous cover crops were expressed as percentages on dry weight basis.

The accumulation of carbon, nitrogen, phosphorus and potassium in the TSCC (kg element ha^{-1} y^{-1}) was calculated by taking into account the harvested biomass in the frames (g DM in 0.25 m^2), the area covered by the cover crop and the C, N, P and K contents of the aerial biomass.

The atmospheric CO_2 captured annually (kg CO_2 ha^{-1} y^{-1}) by the TSCC was calculated from the C accumulated in the aerial biomass (kg C ha^{-1} y^{-1}), using a molecular weight ratio (1.0 g carbon = 3.66 g CO_2) and by assuming that the C taken up by plants comes exclusively from the atmospheric CO_2.

2.4 Statistical Analysis

Statistical analyses were performed using the statistical software STATISTICA v.10 (StatSoft Inc.). The effects of the typologies of the TSCC layout (WCC, CCC, and BCC) and the categories of tree densities (traditional, intensive and superintensive) on the studied variables were tested using the non-parametric Kruskal-Wallis test ($p < 0.05$) and differences between factor groups were tested by pairs with the Mann-Whitney *U* test ($p < 0.05$).

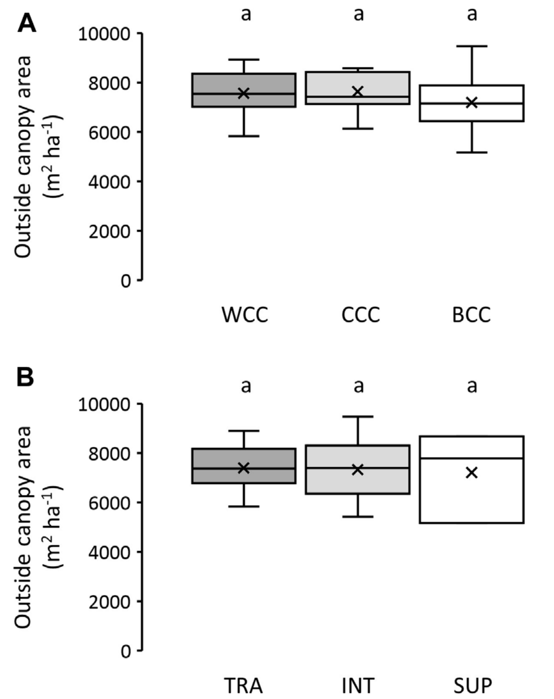

FIGURE 2 | Box and whiskers plot of the inter-row area of the olive groves for each of the cover crop layouts **(A)** and tree density categories **(B)**. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences ($p < 0.05$). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively. TRA, INT and SUP stand for olive grove with a traditional, intensive and superintensive tree density.

3 RESULTS

The statistical results of Kruskal Wallis and Mann-Whitney tests are presented in the **Supplementary Tables S2, S3, S4, and S5**.

3.1 Spontaneous Cover Crop Layout and Area Covered

Three main cover crop layouts were identified. In eight of the 46 olive farms, TSCC covered the whole olive grove (WCC), whereas in 12, TSCC was absent in the area under tree canopy (CCC). Finally, in 26 of the olive groves, the TSCC was distributed in strips of a given width in the inter-rows area (BCC). The mean width of the strips with TSCC was 3.2 m. Besides that, 69 % and 100% of the olive groves under intensive and super-intensive tree densities, respectively had a TSCC layout of BCC. The mean tree densities of the olive groves managed with WCC, CCC and BCC were 167, 93, and 260 trees per hectare, respectively.

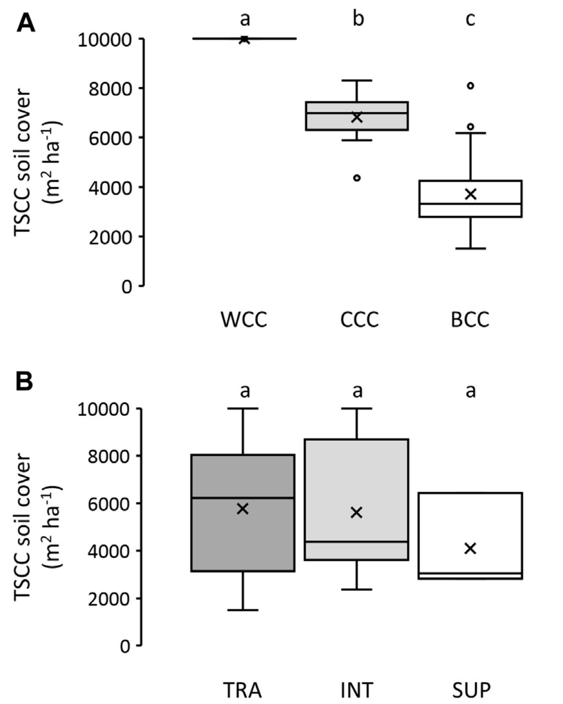

FIGURE 3 | Box and whiskers plot of soil covered by TSCC for each of the cover crop layout **(A)** and tree density **(B)**. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences (*p* < 0.05). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively. TRA, INT and SUP stand for olive grove with a traditional, intensive and superintensive tree density.

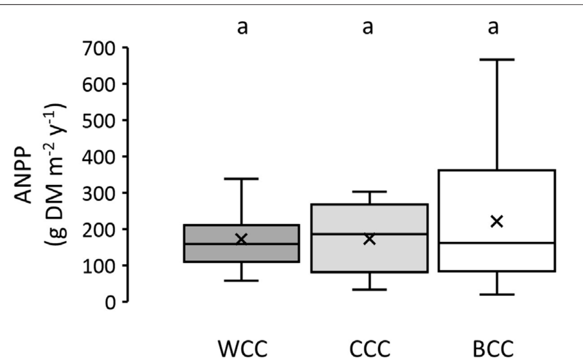

FIGURE 4 | Box and whiskers plot of ANPP for each of the cover crop layout. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences (*p* < 0.05). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively.

Overall, the area outside the tree canopy projection averaged 7,365.8 m^2 ha^{-1} and there were no significant differences between TSCC layouts or tree densities (**Figure 2**).

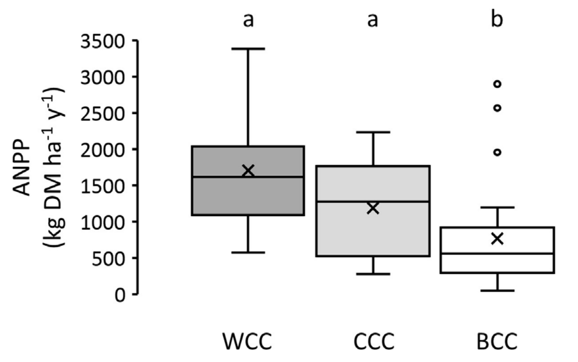

FIGURE 5 | Box and whiskers plot of ANPP for each of the cover crop layout. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences (*p* < 0.05). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively.

On average, the area covered by TSCC was 5,615.8 m^2 ha^{-1} with significant differences among the TSCC layouts (**Figure 3**). Under CCC, the mean area covered was 6,820.9 m^2 ha^{-1} which was significantly higher than the 3,710.6 m^2 ha^{-1} covered in BCC olive groves (**Figure 3**). No significant differences were found in the area covered by the TSCC among olive groves with different categories of tree densities, which was not unexpected as BCC was the predominant TSCC layout for the three categories of tree densities.

3.2 Aboveground Net Primary Production of TSCC

Supplementary Figure S1 shows the frequency distribution of the aboveground net primary production (ANPP) per m^2 of the TSCC. On average, for the whole set of olive groves, the ANPP in the covered area averaged 200.5 g DM m^{-2} y^{-1} with a very high variability (min 20.3 g DM m^{-2} y^{-1}; max 666.6 g DM m^{-2} y^{-1}). No significant differences among TSCC layouts were found (**Figure 4**), although differences were found due to tree densities. The ANPP in super-intensive olive groves (436.1 g DM m^{-2} y^{-1}) was significantly higher than that in the traditional olive groves (193.3 g DM m^{-2} y^{-1}).

Differences in the ANPP per ha were found between TSCC layouts. The average ANPP per ha in TSCC under WCC (1,707.4 kg DM ha^{-1} y^{-1} on average) and under CCC (1,186.4 kg DM ha^{-1} y^{-1}) were both significantly higher (122 % and 54% higher, respectively) than that of BCC (769.5 kg DM ha^{-1} y^{-1}) (**Figure 5**). Despite the average ANPP for CCC (1,186.4 kg DM ha^{-1} y^{-1}) being 30.5% lower than that of WCC, no significant differences were found (**Figure 5**). ANPP per ha showed no difference between tree densities.

3.3 Carbon Fixation and Nutrient Retention

The carbon content of the aboveground biomass of the TSCC averaged 40.4% with a very low coefficient of variation (4.4%) (**Supplementary Figure S2**). No differences were found between TSCC layout types.

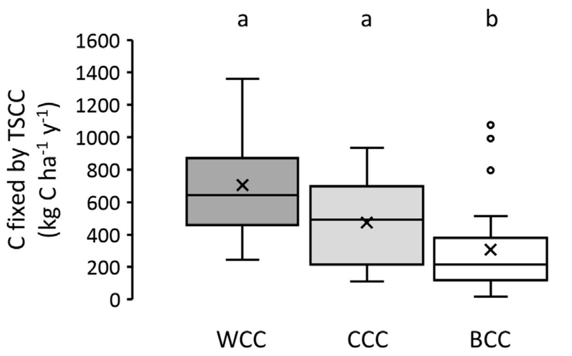

FIGURE 6 | Box and whiskers plot of carbon content of TSCC for each of the cover crop layout. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences (p < 0.05). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively.

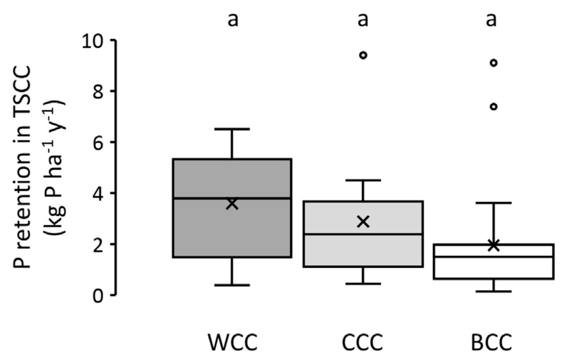

FIGURE 8 | Box and whiskers plot of phosphorus retention in TSCC for each of the cover crop layouts. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences (p < 0.05). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively.

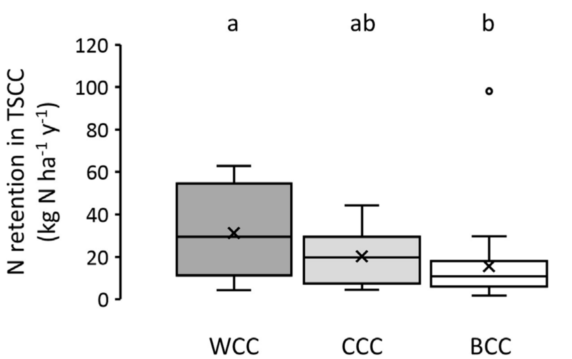

FIGURE 7 | Box and whiskers plot of nitrogen retention in the TSCC for each of the cover crop layouts. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences (p < 0.05). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively.

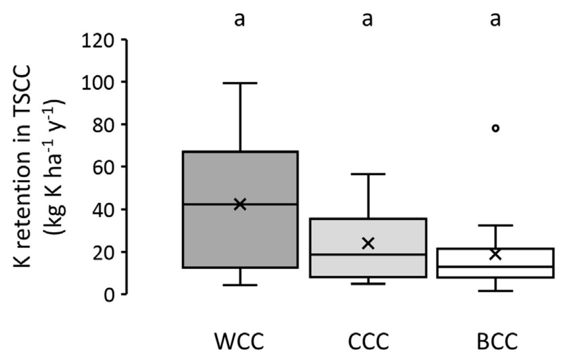

FIGURE 9 | Box and whiskers plot of potassium retention in TSCC for each of the cover crop layouts. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences (p < 0.05). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively.

The amount of C fixed by TSCC on the whole set of olive groves averaged 419.4 kg C ha^{-1} y^{-1} (or 1,537.8 kg CO_2 ha^{-1} y^{-1}) (**Figure 6**). As for the ANPP, the fixed carbon of the TSCC under WCC (704.9 kg C ha^{-1} y^{-1} or 2,584.6 kg CO_2 ha^{-1} y^{-1}) and under CCC (475.3 kg C ha^{-1} y^{-1} or 1,742.8 kg CO_2 ha^{-1} y^{-1}) were significantly higher than that under BCC (305.7 kg C ha^{-1} y^{-1} or 1,120.9 kg CO_2 ha^{-1} y^{-1}). No differences were found between CCC and WCC (**Figure 6**).

For the whole set of olive groves, the nitrogen content of the aboveground biomass of the TSCC was 1.96% with a relatively high variability (coefficient of variation of 47.5%) (**Supplementary Figure S2**). As expected, differences between tree densities or TSCC layouts were not significant but averages were higher for BCC (2.13%) than for WCC and CCC (1.71 % and 1.76%, respectively).

The amount of N in the aboveground biomass of TSCC averaged 19.5 kg N ha^{-1} y^{-1} but there were olive groves with values as low as 1.7 kg N ha^{-1} y^{-1} and as high as 98.0 kg N ha^{-1} y^{-1}. There were no significant differences among tree densities. However, results were significantly higher for WCC (31.1 kg N ha^{-1} y^{-1}) than for BCC (16.0 kg N ha^{-1} y^{-1}). No differences were found between CCC (20.3 kg N ha^{-1} y^{-1}) and the other treatments (**Figure 7**). Phosphorus content of the aboveground biomass of TSCC of the 46 olive groves averaged 0.24% but values as low as 0.05% and as high as 0.61% were found (**Supplementary Figure S2**). In this case, there were no significant differences between TSCC layouts or tree densities. Annual P accumulated in the aboveground biomass of TSCC averaged 2.48 kg P ha^{-1} y^{-1} with a high variability (89% coefficient of variation). Annual P accumulation in the WCC,

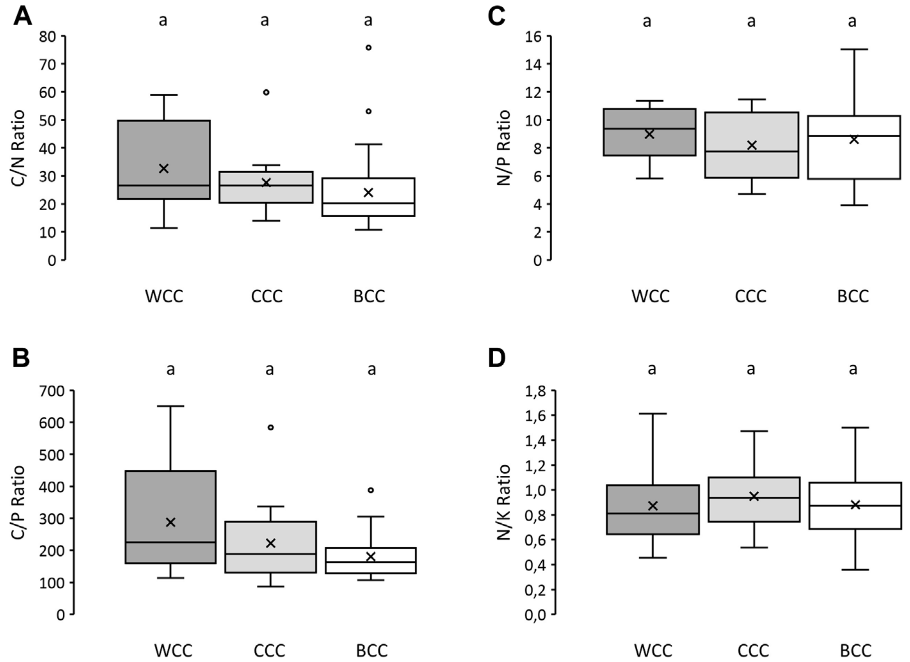

FIGURE 10 | Box and whiskers plots of C/N **(A)**, C/P **(B)**, N/P **(C)** and N/K **(D)** ratios of the of TSCC for each of the cover crop layouts. Horizontal black line, x-shaped cross, box borders and whiskers stand for the median, the mean, quartiles and outliers. Different letters over the box-plot indicate significant differences (p < 0.05). WCC, CCC and BCC stand for olives groves where TSCC occupied the whole area, was absent under tree canopy or only occupied a strip of the inter-row area, respectively.

CCC and BCC olive groves averaged 3.60, 2.89 and 1.95 kg P ha^{-1} y^{-1}, but differences were not significant (**Figure 8**).

The average potassium content of the dry biomass of the aboveground TSCC was 2.28% with a coefficient of variation of 38% (**Supplementary Figure S2**). No significant differences in the K content of the TSCC biomass was found due to TSCC layouts or tree densities. The annual rate of K accumulation in the aboveground biomass of TSCC was 24.3 kg K ha^{-1} y^{-1}. Values for WCC (42.4 kg K ha^{-1} y^{-1}) were 76.6 % and 123.2% higher than those of CCC (24.0 kg K ha^{-1} y^{-1}) and BCC (19.0 kg K ha^{-1} y^{-1}) olive groves, respectively, but the differences were not significant (**Figure 9**).

Mean C/N, C/P, N/P and N/K ratios of the aboveground biomass of the TSCC averaged 26.5, 210.2, 8.6 and 0.9, respectively (**Figure 10**). Mean C/N and C/P ratios tended to be higher under WCC and lower under BCC with intermediate values for CCC, although differences were not significant in any of the ratios. No significant differences were found between tree densities except for the N/P ratio. In this case, the biomass of the TSCC showed significantly higher values of the N/P ratio in super-intensive olive groves (12.2 on average) than in traditional (8.0 on average) and intensive olive groves (9.0 on average).

4 DISCUSSION

4.1 TSCC Layout and Soil Cover

TSCC in most of the olive groves of this study followed the BCC layout, and mainly in olive groves with a tree density higher than 150 trees ha^{-1}. This TSCC layout is the result of controlling the spontaneous vegetation under the tree rows by mechanical or chemical means. The strip width of the studied olive groves averaged 3.2 m, and was well above the 1 m width imposed by the normative, suggesting that the minimum width could be increased, at least for olive groves with a high mean slope.

In 26% of the olive groves, TSCC showed a CCC layout, mainly to facilitate olive fruit harvest and to avoid direct water and nutrient competition with the trees. Under this TSCC layout, typical of olive groves with relatively low tree density, cover crops are controlled manually and on a tree-by-tree basis mainly by using pre and post-emergency backpack herbicide sprayers or a brush cutter. Lastly, the number of olive groves in which TSCC showed a WCC layout was low and was typical of low tree density olive groves or those with higher tree densities but with irrigation.

4.2 Aboveground Net Primary Production of Temporary Spontaneous Cover Crops

Variability of the aboveground net primary productivity (ANPP) of TSCC (kg DM m^2 y^{-1}) was as high as one order of magnitude. Spatial variability of ANPP among olive groves was not unexpected as it is driven by a network of interrelated factors which include differences in: 1) pedoclimatic and landscape conditions (Taguas et al., 2017), 2) size and diversity of the seedbank, 3) current and previous management of soil and TSCC (Gómez, 2017; Vicente-Vicente, 2017), 4) main dominant species of the TSCC communities (Cruz-Ramírez et al., 2012), and 5) herbivory pressure level (Guerrero-Casado et al., 2015). Together with the variability amongst the olive groves, the intra-farm variability, expressed as a coefficient of variation, averaged 42% for the whole set of olive groves.

In addition to the high spatial variability found in this study, a relatively high inter-annual variability in the level of soil covered and ANPP are also a feature of the TSCC. For instance, in two different 3-year studies, the inter-annual variability of ANPP per hectare was about 3-fold (Repullo-Ruibérriz de Torres et al., 2012) and 20-fold (Castro et al., 2008), and mainly driven by the high inter-annual variability in precipitation which characterizes Mediterranean regions. Temporal variability of the degree of soil covered by TSCC has also been proved (Taguas et al., 2017).

The mean annual ANPP of TSCC of 1,041 kg DM ha^{-1} y^{-1} from our study is within the range of 0.65—2.53 Mg DM ha^{-1} y^{-1} obtained by Vicente-Vicente (2017) in 10 olive groves with mature TSCC. Similarly, the mean for BCC (769.5 kg DM ha^{-1} y^{-1}) and WCC (1,707.4 kg DM ha^{-1} y^{-1}) are consistent with results obtained by Blázquez-Carrasco et al. (2021) in three and four olive groves managed with strip and full-cover TSCC layout, respectively. Other field studies performed on only one olive grove reported generally higher figures. For instance, Repullo-Ruibérriz de Torres et al. (2012), in an olive orchard covered with spontaneous vegetation in Córdoba (Spain), found an ANPP of 2.1–6.7 Mg DM ha^{-1} y^{-1} during a study period of three agricultural years, and Rodríguez-Lizana et al. (2018) found an even wider range: 0.18—9.56 Mg DM ha^{-1} y^{-1}.

The mean olive fruit production of Andalusia olive groves in the period between 2014–2019 was of about 3,558 kg fresh weight ha^{-1} y^{-1} (Junta de Andalucía, 2014; 2015a, 2016, 2017, 2018, 2019), or 1,423 kg DM ha^{-1} y^{-1} considering 40% of dry matter in olives (Zeleke et al., 2012). Therefore, the mean dry biomass production of olive groves with cover crops could increase the productivity by 73%, taking into account only the olive fruit production. If the 1.66 million hectares devoted to olive groves in Andalusia (Junta de Andalucía, 2019) allowed the development of TSCC, about 1.73 million tons of dry biomass could be produced, a figure which should be higher if the belowground biomass of the TSCC had been taken into account. This estimate, which has many uncertainties attached to it, highlights the significance of the implementation of this technically and economically viable diversification practice on many ecosystem services, at least on a regional scale.

The amount of dry biomass produced by the TSCC is a crucial factor in the C sequestration (Peregrina et al., 2014), soil properties and nutrient dynamics (Ovalle et al., 2007; Repullo-Ruibérriz de Torres et al., 2021b). The area covered and the biomass produced by TSCC in BCC olive groves were significantly smaller than that of WCC and CCC, despite the area outside the tree canopy which is potentially covered by TSCC in olive groves being very similar. Therefore, there is a significant potential to increase the ANPP of olive groves under BCC. In spite of the BCC layout strategy fulfilling the requisite to acquire the economic subsidy, narrow strips could significantly limit the potential role of TSCC in providing and boosting agroecosystem services.

4.3 Carbon Fixation by Temporary Spontaneous Cover Crops

The content of carbon in the aboveground biomass of the TSCC showed little variability. It was independent of the tree densities, cover crop layouts and pedoclimatic conditions, and the values were similar to those from other studies (Gómez-Muñoz et al., 2014; Rodríguez-Lizana et al., 2020).

On average, for the 46 olive oil orchards, the organic carbon input to the soil in 1 year through the aboveground cover crop residues was 0.42 Mg C ha^{-1}. This average is within the range of 0.2–0.7 Mg C $ha^{-1}y^{-1}$ estimated by several researchers (Freibauer et al., 2004; Hutchinson et al., 2007) as the potential for C sequestration under scenarios of crop residue application. However, the extent to which the input of organic carbon derived from plant cover increases the soil C stock of olive orchards will ultimately depend on the decomposition rate of that organic carbon. The decomposition rate depends on many factors including plant biomass quality (e.g., C-to-N ratio and lignin and polyphenol contents), edaphic and environmental conditions and aboveground plant residue management (e.g., cover crop control method and residue displacement) (Kumar and Goh, 2000). Gómez-Muñoz et al. (2014) found that the mean percentage of the aboveground TSCC, with a C: N ratio of 30.3, remaining after 1 year of incubation on top of the soil in field conditions using the litterbag approach was 29.1%, whereas Repullo-Ruibérriz de Torres et al. (2021b), found that 55% of the carbon from TSCC remains on the field after a 1-year period. By applying the most conservative percentage (29.1%) to the mean of 0.42 Mg C ha^{-1} y^{-1}, about 0.122 Mg C ha^{-1} y^{-1} of TSCC-derived organic carbon would remain in the soil after 1 year, or 202,520 Mg C (or 741,223.2 Mg of CO_2) if the entire 1.66 M ha cultivated with olive groves in Andalusia permitted the development of TSCC. By 2030, the regional authorities have a commitment to reduce the annual CO_2 emissions of 2005 (62,070,000 Mg CO_2) by 41%, which means that by 2030, annual CO_2 emissions should have reduced by 25,448,700 Mg CO_2. The yearly amount of CO_2 fixed by the TSCC as organic carbon, after discounting that emitted as CO_2 during the TSCC residue decomposition, highlights the enormous contribution of TSCC in olive groves and other orchard type crops to climate change mitigation (Kaye and Quemada, 2019).

There is a lot of evidence regarding the increase in the stocks of soil organic carbon (SOC) due to the presence of TSCC in olive groves. For instance, in four out of the five paired TSCC versus non-TSCC comparisons, between 9.0 and 16.1 more Mg C ha^{-1} was stored in the top 15 cm of the soils of the TSCC olive groves after many years (>8 years) with the same management (Vicente-Vicente et al., 2017). These values were similar to the 8.4–15.0 Mg C ha^{-1} more SOC storage in the top 15 cm of an olive oil orchard under TSCC compared to a TSCC-free plot (Castro et al., 2008). In a short period of 3 years, different seeded cover crops, including TSCC, fixed on average 1.5 Mg C ha^{-1} of organic carbon (Repullo-Ruibérriz de Torres et al., 2012).

However, the increased SOC stock in soils under the cover crops treatment might not only be due to the annual inputs of organic carbon from the plant residues, but also due to a decrease in SOC losses through soil erosion (Gómez et al., 2009). In addition, the diversity of wild spontaneous plant cover might have an important impact on SOC accrual by improving the ability of soil microbial communities to rapidly process plant residues and protect them into aggregates, and by also introducing a greater diversity of organic carbon compounds into the soil, some of which may be more resistant to decomposition (Tiemann et al., 2015).

4.4 Role of Temporary Spontaneous Cover Crops in Nutrient Retention

The 19.6 kg N ha^{-1} y^{-1} that on average was, mainly, taken up from the soil by the aboveground biomass of TSCC and transformed into organic N might positively impact the N cycling of olive groves. Annual herbaceous vegetation of TSCC is likely efficient in the acquisition of nutrients when they are provided in pulses, such as peaks in soil organic matter mineralization during early spring when soil water content and soil temperature are optimal for organic matter decomposition. The highest annual growth rates, and thus nutrient uptake, of TSCC or seeded cover crops in olive groves of Mediterranean areas are usually found from mid-February to late April (Ordoñez-Fernández et al., 2018), when optimal soil water levels and daily temperatures coincide. Rainfall during late winter-early spring accounts for a relatively high proportion (about 24%) of the annual rainfall in Andalusia (RIA, 2021), and therefore the N taken up by TSCC might play an important role in reducing N losses by leaching in the olive groves. In a meta-analysis of 106 studies of 372 sites covering different countries, climatic zones and management, Abdalla et al. (2019) demonstrated that cover crops significantly decreased N leaching, although site-specific studies on olive groves are needed.

The extent to which the N uptake by the TSCC can compete with the tree demand for N, depends mainly on the magnitude and timing of the tree nutrients uptake. There are several indications that suggest that TSCC do not compete significantly with olive trees for nutrients. Typically, fertilization in olive trees is applied to the soil below the tree canopy to increase nutrient use efficiency. Therefore, it is expected that little of the soil available nutrients of the inter-row are used by trees. On the other hand, the highest tree demand for N and P takes place in late April–June and in October (Cadahía, 2005; García-Ruiz et al., 2011), several weeks after the highest TSCC demands for nutrients. Furthermore, the N balances in two olive groves of Fernández-Escobar et al. (2012) revealed that the usual N application rate of 1 kg N $tree^{-1}$ y^{-1} (as urea), split in two applications in the early spring, resulted in a very positive N balance. This, hence, highlighted that the rate of N fertilizer application was much higher than that demanded by the tree, and therefore cover crops are expected to have little competition for N.

On the other hand, it is rather likely that the main sources of available N and P taken up by TSCC are the N and P which are mineralized from the soil organic matter. Fernández-Escobar et al. (2012) estimated that 44.8 and 69.9 kg N ha^{-1} y^{-1} were available during the decomposition of soil organic matter in two olive groves, whereas Gómez-Muñoz et al. (2015) found values between 80 and 220 kg N ha^{-1} y^{-1} for the top 20 cm of soil in two olive groves with soil organic matter levels of 3%. These values are more than double the mean nitrogen taken up by TSCC in this study. Lastly, the main indications that TSCC do not compete with the olive trees for nutrients come from the assessment of the olive fruit production and olive oil quality in mature olive groves with and without cover crops. Zuazo et al. (2020) demonstrated during four consecutive years, that the olive yield did not differ significantly between olive groves without cover crops and comparable olive groves with TSCC or seeded cover crops layout in strips or in the entire orchard. In this regard, Sastre et al. (2016) found in an olive plantation of the drought-resistant cultivar Cornicabra that cover crops had not reduced fruit or oil yield, neither in heavy nor in low yield years. However, in a very high density olive grove (Gucci et al., 2012) and in an intensive young olive tree grove (Caruso et al., 2011), a significant decrease in the fruit yield under cover crops has been reported.

The fact that TSCC in 50% of the studied olive groves had a C/N ratio lower than 22.2 indicates that in general, TSCC grow up with little N limitation. Xu et al. (2020) found in a meta-analysis with 2236 paired observations from 123 published studies to investigate the responses of foliar C/N ratio to experimental N addition that the foliar C:N ratio declined with N availability, and Rodríguez et al. (2013) found that the aboveground biomass of the spontaneous cover crops in unfertilized and fertilized (60 kg N $ha^{-1}y^{-1}$) soils of the inter-row area of an olive grove in Portugal had a C/N ratio of 52.1 and 25.9, respectively. The relatively low C:N ratio of the aboveground biomass of the TSCC of our study indicates that the environmental conditions were suitable for plant growth and N was not limited, and therefore plants compete more for light (and thus C), exhibiting low C/N ratios (Zhang et al., 2019). According to Trinsoutrot et al. (2000), C/N ratios above 24, a value similar to that of our study, imply a net N immobilization. This could slightly affect the availability of N for olive trees.

TSCC in 75% of the examined olive groves had a N/P ratio lower than 10.6. There are many factors responsible for a given N/P ratio. Optimal N/P ratios depend on species, growth rate, plant age and plant parts. At vegetation level, N/P ratios <10 often correspond to a growth lacking P limitation (Güsewell, 2004), as shown by short-term fertilization experiments. The generalized low C/N and N/P ratios of the aboveground biomass of the TSCC for most of the olive groves of this study suggest no N or P limitation during their growth.

In summary, there are numerous indications that the 19.6 kg N ha^{-1} y^{-1}, 2.5 kg P ha^{-1} y^{-1} and 24.3 kg K ha^{-1} y^{-1} accumulated on average in the aboveground biomass of the TSCC of the 46 olive groves in this study, show that the TSCC do not compete significantly with the olive trees for nutrients, but might instead play an important role in reducing the N, P and K that are prone to be lost by leaching, soil erosion and runoff. The rapid growth rate typical of the herbaceous vegetation of the TSCC permits the interception and storage of highly mobile nutrients in its biomass that will then be progressively released at rates which are more in accordance to those required for acquisition by olive trees, contributing to more efficient nutrient cycling.

Assuming that annually, 19.6 kg N ha^{-1}, 2.5 kg P ha^{-1} and 24.3 kg K ha^{-1} are retained in the olive groves due to the TSCC, and TSCC are maintained in the 1.6 million hectares of olive groves of Andalusia, then about 29,400, 3,750 and 36,450 Mg of N, P and K could be saved, avoiding further environmental and economic costs. For instance, this amount of N is the equivalent of 29 million euros in N fertilizers (assuming an average price of 1 € kg^{-1} N). This estimate, which has many uncertainties, highlights the significance of the implementation of this technically and economically viable diversification practice on nutrient retention at a regional scale. In addition, the N taken up by the TSCC might help to alleviate the nutritional problems in olive-growing as a result of the overuse of N fertilizers, as has been reported for other fruit crops (Weibaum et al., 1992).

The extent to which TSCC have a significant role in retaining nutrients within the olive groves greatly depends on the area covered, since the annual rates of N, P and K taken up by TSCC growing in strips were almost half of that taken up in olive groves with TSCC growing in the entire area.

5 CONCLUSION

The variability of the aboveground net annual primary production of temporary spontaneous cover crops was as high as one order of magnitude. Hence, to model the beneficial effects of diversification in olive groves on climate change mitigation and nutrient retention on large spatial scales, extensive sampling that presents a high variability should be undertaken, as has been done in this study.

The 1.53 Mg of CO_2 ha^{-1} y^{-1} that was fixed annually on average by the aboveground biomass of temporary spontaneous cover crops could contribute significantly to the regional commitments of CO_2 reduction if all the areas devoted to olive groves implement this diversification strategy. Through the amounts of N, P and K accumulated into the aboveground biomass, the temporary spontaneous cover crops were also an adequate strategy in reducing the nutrient loss prone to occur in the olive orchards and in boosting the soil-plant internal nutrient loop, thus enhancing the nutrient retention within the olive groves.

This study proves that temporary spontaneous cover crops in olive orchards are a sound diversification strategy with the potential to contribute significantly to the increase in productivity in olive groves, to climate change mitigation and to nutrient retention. The level of contribution was highly dependent on the cover crop layout model and was lower when cover crops were distributed in strips in the inter-rows area. Therefore, by increasing the area that the inter-row covered, the potential contribution of temporary spontaneous cover crops could be enhanced further.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization, RG-R; Sampling and analyses, MT-C, PD, JH, and JC; formal analysis and visualization: PD; writing—original draft preparation, MT-C, PD, and RG-R; writing—review and editing, MT-C, JC, PD, and RG-R; funding acquisition, RGR. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Characterization of the Soil Prokaryotic Community With Respect to Time and Fertilization With Animal Waste–Based Digestate in a Humid Continental Climate

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There is a renewed global awareness to improve soil health through the intensification and management of organic inputs such as the application of animal waste–based digestate and other types of organic fertilizers to the soil. The objective of this study was to evaluate the influence of different types of animal waste–based digestate application on soil prokaryotic diversity and composition in an agricultural cropping system over a period of 3 years, cultivated with three different annual cereal crops (spring wheat, triticale, and barley). Treatments were laid out in a randomized design with five conditions (three replicates per condition): fertilizer treatments included three different types of digestate (pig manure, chicken manure, and cow manure digestates), synthetic mineral nitrogen, and unfertilized control. Prokaryotic soil communities were characterized by Illumina MiSeq sequencing. The three most abundant phyla identified were Actinobacteria, Acidobacteria, and Proteobacteria, which accounted for over 55% of the total prokaryotic community. Other phylogenetic groups such as Verrucomicrobia and Bacteroidetes were also identified as part of the native soil microbiota. It was observed that the period of digestate application did not significantly influence the prokaryotic diversity in the soil. On the contrary, sampling time was a major factor in driving β-diversity. A correlation with soil pH was also observed for several taxonomic groups, indicating its importance in shaping prokaryotic community composition. Our study showed that the richness and diversity of the soil prokaryotic community were not affected by digestate application, while other factors such as the yearly crop varieties and seasonal/climate changes were the major contributors to differentiating the prokaryotic community composition over time.

Keywords: microbiota, sampling time, crops, soil, digestate

INTRODUCTION

The consumption of animal-derived products is constantly increasing (Salter, 2017), and in the future years it is expected to rise; therefore, it is extremely important to develop sustainable systems for animal-waste product management. Biogas systems produce clean energy using organic waste such as animal byproducts and discarded food which are converted into methane and carbon dioxide (Aydin, 2017). Digestates are the end-products generated from the anerobic digestion of these organic substrates (Crolla et al., 2013; Nkoa, 2014). Digestates could be further used as fertilizer because of their high content of nutrients such as nitrogen (N), potassium (K), phosphorus (P), and organic matter, being a sustainable alternative to reduce the utilization of inorganic fertilizers (Bachmann et al., 2014; Lee et al., 2021). Moreover, digestates may contain beneficial bacteria as nitrogen fixers and phosphate solubilizers (Fernandez-Bayo et al., 2020; Raymond et al., 2020), conferring an additional value as biofertilizers (Crolla et al., 2013; Insam et al., 2015). The bacterial composition of the digestate is different with respect to the microbiota present in the primary feedstocks owing to anerobic treatments they have undergone (Fernandez-Bayo et al., 2020). This will also affect the persistence in the soil of the bacteria present in the digestate which is lower in comparison to other organic fertilizers such as woodchip compost (Akari and Uchida, 2021; Dincă et al., 2022). Organic fertilization contributes to increasing nutrient availability to plants (Chu et al., 2007), enhances soil microorganism activity (Makdi et al., 2012; Nkoa, 2014), and in turn improves crop yield (Šimon et al., 2015). Soil type and the kind of organic material applied are two critical factors influencing soil microbial activities such as respiration rate and soil microbial biomass (Li et al., 2018; Chen et al., 2019). Specifically, several studies indicated increased microbial biomass due to digestate application (Fuchs et al., 2008; Alburquerque et al., 2012; Nkoa, 2014). In terms of the time frame of continuous digestate application, there have been varying reports on the influence of digestate/organic fertilization on soil microbes from short- to long-term experiments based on the primary feedstocks and the mode of experiments (Luo et al., 2015; Möller, 2015; Nielsen et al., 2020). For instance, Möller (2015) reported that digestate with a high degradability of organic matter such as clover-grass has a stronger effect on the short-term soil microbial activity. In addition, Nielsen et al. (2020) reported in a previous review that higher effects of digestate application on some soil microbial activity define parameters such as metabolic content and basal respiration than those of their individual feedstock in the short-term involving specific experimental setup. In contrast, Luo et al. (2015) reported a shift in microbial community structure in a long-term study involving 33 years of fertilization, which was also affirmed in other studies with a different long-term period (Ruppel and Makswitat, 1999; Chu et al., 2007; Guo et al., 2019). Also, climatic fluctuations over a cultivation year result in soil bacterial communities being constantly exposed to changes and adaptations to environmental conditions such as moisture, resource availability, and temperature (Bardgett and Caruso, 2020).

The bacterial community may then be significantly altered in response to the individual components of the added waste and with respect to time. Therefore, it is important to understand the major factors in shaping the soil prokaryotic community to pave the way for improving soil quality and carrying out proper fertilization using alternative byproducts such as digestate (Peacock et al., 2001).

In previous studies, we analyzed the effects of three different types of digestates (from pig, chicken, and cow manure) on soil features and plant yield. Repeated digestate applications over 3 years of treatment lead to a slight decrease in nitrogen and carbon soil content, while a considerable increase was observed for potassium (K_2O), in particular for soils treated with cow and chicken manure digestates. Another difference was related to P content which increased in all treatments (including unfertilized plots and plots fertilized with synthetic nitrogen), with the exception of the plots treated with chicken manure digestates. There were no differences observed in relation to soil pH (Doyeni et al., 2021b).

In terms of plant quality and productivity, the effects of fertilization differed depending on the tested crop. Higher grain density was observed for spring wheat and spring barley treated with digestates or synthetic nitrogen fertilizers with respect to the control. The grain protein percentage was generally higher in all the fertilized plants comparable values were observed for pig manure digestate and synthetic fertilizer in spring wheat; in triticale, synthetic fertilizer outperformed with respect to digestates, while in spring barley, chicken and cow manure digestates gave better results (Doyeni et al., 2021b). Moreover, in pot experiments, where a similar soil was used, a general increase of the soil microbial biomass with all the three types of digestates was observed (Doyeni et al., 2021a). The aim of this study was to evaluate the composite effect of fertilization with these different sources of animal waste–based digestate and seasonal/annual variation on the soil prokaryotic community diversity and composition over 3 years.

MATERIALS AND METHODS

Experimental Design and Soil Sampling

The experimental field was located at the Lithuanian Research Centre for Agriculture and Forestry (55.40 N, 23.87 E), which is characterized by a humid continental climate (Belda et al., 2014). The soil in the experimental area is loamy (Endocalcaric Epigleyic Cambisol) (Baxter, 2007), and the soil chemical composition exhibited suitable parameters for cereal cultivation: pH (7.03), organic carbon content (1.3%), and nitrogen content (0.14%). For a complete characterization of physico-chemical properties, the details are shown in (Doyeni et al., 2021b). A complete randomized block design with five treatments was used to evaluate the effects of fertilization and time on soil microbiota. The complete randomized design was characterized by 15 plots (five fertilizer treatments × three replicates). Each treatment plot was 30 m^2 (3 m × 10 m). Fertilization conditions were as follows: 1) no fertilizers (Control; C), 2) synthetic nitrogen fertilizer ([NH_4NO_3]; SN), and three different organic fertilizers obtained from anerobic digestion of animal manure, 3) pig manure digestate (PM), 4) chicken manure digestate (ChM), and 5) cow manure digestate (CoM); for a complete characterization of the digestates used, see (Doyeni et al., 2021b). The experiment was carried on for 3 years (1–3) from April 2018 to August 2020. The samples were collected each year before fertilization (BF; April-May) and after harvest (AH; August). At the beginning of the field experiment, soil samples were randomly collected from five different spots at a depth of 0–20 cm. The samples were thoroughly mixed to form a composite, and soil specimens were immediately stored at a temperature of -80 °C. The samples were named according to fertilization conditions and sampling time. Before the beginning of the experiment, the field was cultivated with winter wheat (*Triticum aestivum*)-cultivar "Skagen" (Nordic seed A/S, Denmark). In the first, second, and third year, plots were cultivated with spring wheat (*Triticum aestivum*) cultivar "Collada" (Einbeck, Germany), in the second year with spring triticale, a hybrid between wheat and rye [cultivar "Milkaro" (Koscian, Poland)], and in the third year, spring barley (*Hordeum vulgare L.*) cultivar "Ema DS" (Akademija, Lithuania). The sowing rate was 270 kg ha^{-1} (spring wheat), 250 kg ha^{-1} (spring triticale), and 220 kg ha^{-1} (spring barley). The seeds were sown on 19 April 2018, 16 April 2019, and 16 April 2020. The field was fertilized in all years of the 3-year experiment. In all years (year 1–3), samples before fertilization (start of the cultivation year) were subdivided into two groups: control (no fertilization) and control-treated (samples fertilized the precedent year), while samples after harvest (end of the cultivation year-month of August of each year) were further subdivided with respect to the fertilization treatment used. Analysis was conducted considering two different variables: 1) the fertilization treatment used, and 2) the sampling time, a composite parameter influenced by multiple factors.

Total DNA Extraction From Soils

Total DNA was extracted using the FastDNA spin kit for soil (MP Biomedicals, California, United States). Briefly, around 0.5 g of soil was weighed, homogenized by bead beating in the FastPrep®-24 instrument (MP Biomedical) at 6 m/s for 40 s, and DNA was purified with the aforementioned column-based kit according to the manufacturer's instructions. Extracted DNA was checked by agarose gel electrophoresis. The DNA purity and quantity were measured using an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, United States) and standardized to a concentration of 10 ng μL^{-1}.

Sequencing and Data Processing

For each sample, the V3–V4 region of the 16S rRNA gene was amplified using primers Pro341f and Pro805R (Takahashi et al., 2014), which allow the amplification of both Bacteria and Archaea domains, and barcodes were added to the forward primer. Amplicons for each library were purified and mixed in equal proportions. Illumina MiSeq v3 chemistry 300 base paired-end (PE) amplification and sequencing were performed at BMR genomics (Padova, Italy). Briefly, PCR reactions were prepared using 0.2 U of Platinum *Taq* DNA Polymerase HiFi (Thermofisher, Massachusetts, United States), 10 μM of each primer, 10 mM dNTPs mix, 1X buffer, 50 mM of $MgSO_4$, and 50 ng of genomic DNA in a final volume of 25 μL. Amplification conditions were 94°C for 1 min, 25 cycles with 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s, and a final elongation step at 68°C for 7 min. Two samples (C2AH1 and CoM1AH3) were excluded from further analysis due to sequencing failure. The primer sequences were removed using Cutadapt (Martin, 2011). Read quality was evaluated using DADA2 (Callahan et al., 2016), and reads (R1 and R2) were then trimmed and filtered using the following parameters: truncLen = *c* (265,220), maxN = 0, maxEE = *c* (2,2), and truncQ = 2. The reads were merged with an overlap of at least 12 bases, identical to each other in the overlap region. Chimeras were removed and amplicon sequence variants (ASVs) were classified against the Silva database v138 (Yilmaz et al., 2014), using the function assignTaxonomy in the DADA2 package, version 1.18.0 (Callahan et al., 2016) with "R" version 4.0.3 (R Core Team, 2020). ASVs matching with chloroplast and mitochondria sequences were removed from the ASV table.

Statistical Analysis

The α-diversity measures (number of observed ASVs, Chao1 value, and Shannon index) were calculated using the vegan package, version 2.5–6 (Oksanen et al., 2020) in "R" version 4.0.3 (R Core Team, 2020). Pielou's evenness index was calculated as $J = H'/\ln(S)$, where H' is Shannon Weiner diversity and S is the total number of species (ASVs) (Pielou, 1966). A nonmetric multidimensional scaling (NMDS) and a permutational multivariate analysis of variance (PERMANOVA) based on Hellinger-transformed ASV abundance data were performed using the metaMDS and the adonis2 functions, respectively. Both the NMDS and the PERMANOVA were performed with the Bray–Curtis dissimilarity index. The taxa with different relative abundances between sampling times and treatments were identified by using a negative binomial mixed model (method = nb) using the function mms {y, fixed = ~ Sampling Time + Treatment + offset [log(N)], random = ~ 1 | plots, min. $p = 0.2$, method = "nb"}, where y is the matrix with the number of sequences for each taxonomic group (genus or phylum), sampling times and treatments were considered fixed variables, and plots as a random variable; only taxa with a proportion of nonzero values >0.2 (min p) were included in the analysis, and differences were considered significant for $p < 0.05$ (Zhang and Yi, 2020). Shapiro–Wilk and Levene tests were performed to check normality and homogeneity of variance, respectively, depending on results of the ANOVA or Kruskal–Wallis group test with false discovery rate ("fdr") *p*-value adjustment followed by Tukey's HSD or Dunn's post hoc test, respectively, were used. All tests were conducted in "R" version 4.0.3 (R Core Team, 2020). Pearson's correlations among different taxa (at phylum and genus level) and soil chemical features such as N, C, K_2O, P_2O_5, pH, and humus (previously measured in Doyeni et al., 2021b) were calculated in "R" using the package Hmisc (R core Team, 2020); *p*-values were adjusted using the Benjamini–Hochberg false discovery rate procedure (**Supplementary Datasheet S1**).

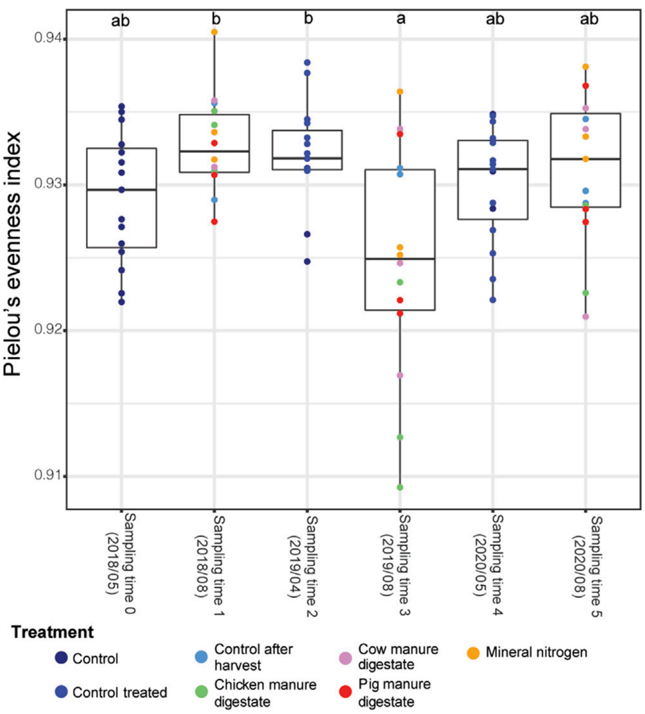

FIGURE 1 | Pielou's evenness index. On the *X*-axis are indicated sampling times, dots are differently colored with respect to treatments: control (before fertilization, no fertilization), control-treated (before fertilization, fertilized the precedent year), control after harvest (after harvest, no fertilization), mineral nitrogen (after harvest), chicken manure digestate (after harvest), cow manure digestate (after harvest), and pig manure digestate (after harvest).

RESULTS

Soil Prokaryotic Diversity

Illumina MiSeq v3 sequencing was performed on the variable V3–V4 region of the 16S rDNA gene, producing a total of 17,989,800 sequences (ranging from 59,530 to 208,305 sequences *per* library). Rarefaction curves showed sequencing coverage for all samples (**Supplementary Figure S2**), allowing the identification of 12,730 amplicon sequence variants (ASVs), with a range from 1,024 to 3,137 ASVs *per* sample (**Supplementary Figure S2**).

The α-diversity was calculated for the number of ASVs observed, Chao1 value, Shannon diversity, and Pielou's evenness indexes. We considered variations in soil prokaryotic relative abundance, namely: 1) treatment used and 2) sampling time. The sampling time was indicative as a composite parameter influenced by multiple variables: 1) different crops/varieties grown each year, 2) weathering conditions, and 3) the agricultural techniques used in each segment such as tillage and split fertilization. Species richness and Shannon diversity were not significantly different throughout the experiment in terms of treatment used or sampling time (**Supplementary Figure S3**). In contrast, a significant difference related to the species evenness (Pielou's evenness index) was observed for sampling time (**Figure 1**). Higher evenness values were found for sampling times 1 and 2, while sampling time 3 showed the lowest value (Kruskal–Wallis and Dunn test, $p < 0.05$). No differences were detected with respect to the different fertilizing treatments used.

Sampling time was also the major factor in driving the β-diversity as observed with the PCoA and PERMANOVA analysis ($p < 0.01$), with all the sampling times differing from each other (**Figure 2B**; **Supplementary Table S1**). In relation to the fertilization treatment, significant differences were observed between the control group (no fertilization, before fertilization) and all the groups "after harvest" (control after harvest, mineral nitrogen, chicken manure digestate, cow manure digestate, and pig manure digestate; PERMANOVA, $p < 0.01$), reflecting differences observed in relation to sampling time (**Figure 2A**). A significant difference was also found between the groups control and control-treated (PERMANOVA, $p < 0.01$, **Supplementary Table S2**).

Soil Prokaryotic Composition

99.81% of the ASVs were identified at least at the phylum level. ASVs were classified into 47 phyla, 113 classes, 256 orders, 324 families, and 591 genera. The overall prokaryotic community composition was similar in all the conditions tested (**Figure 3A**). The three most abundant phyla in all the treatments were Actinobacteria, Acidobacteria, and Proteobacteria, whose relative abundance was similar within samples: Actinobacteria (18.8 ± 1.9%), Proteobacteria (19.5 ± 1.5%), and Acidobacteria (19.8 ± 1.5%) (**Figure 3**). Together, these three phyla accounted for 55.7 to 60.9% of the total prokaryotic community. Other phyla whose relative abundance was relatively high (>5%) were Bacteroidetes (13.1 ± 1.6%), Verrucomicrobia (8 ± 0.9%), and Chloroflexi (6.4 ± 0.7%). The 10 most representative genera were the group 41 (2.04 ± 0.05%; Acidobacteria); *Nitrospira* (1.71 ± 0.02%; Nitrospira); *Candidatus Udaeobacter*, *Candidatus Xiphinematobacter*, and *Chthoniobacter* (1.61 ± 0.04%, 1.05 ± 0.02%, and 0.99 ± 0.02%, respectively; Verrucomicrobia); *Gaiella*, *Pseudoarthrobacter*, and *Nocardiodes* (1.55 ± 0.02%, 1.52 ± 0.05%, and 1.16 ± 0.02%, respectively; Actinobacteria); *Sphingomonas* (1.29 ± 0.05%; Proteobacteria), and *Chryseolinea* (1.03 ± 0.02%; Bacteroidetes). However, for many sequences, it was not possible identifying at the genus rank (54.7 ± 0.3% of the sequences); in particular within the phylum Acidobacteria, we observed the higher number of unassigned ASVs (16% of the total number of sequences), with only the 19.16% of the sequences falling in the phylum Acidobacteria assigned at the genus rank. Soil chemical features (nitrogen (N), carbon (C), potassium oxide (K_2O), phosphorus pentoxide (P_2O_5), pH, and humus content were previously measured at two time points: the beginning (sampling time 0) and the end of the trial (sampling time 5) (Doyeni et al., 2021b). Correlation analyses were performed between the different taxonomic groups identified (at phylum and genus level) and soil composition. No significant correlation was observed at the phylum level, while at the genus rank, 75 groups (out of 463 analyzed) showed a significant correlation with pH (*p*-value adjusted <0.05); among them only two genera, *Haliangium* (Mixococcota) and group TM7a (Patescibacteria), showed a negative correlation. Most of the genera correlating with pH belonged to Proteobacteria (30 genera) and Firmicutes (15 genera). The five most represented groups showing a significant correlation with pH were *Sphingomonas* (Proteobacteria), *Haliangium* (Mixococcota),

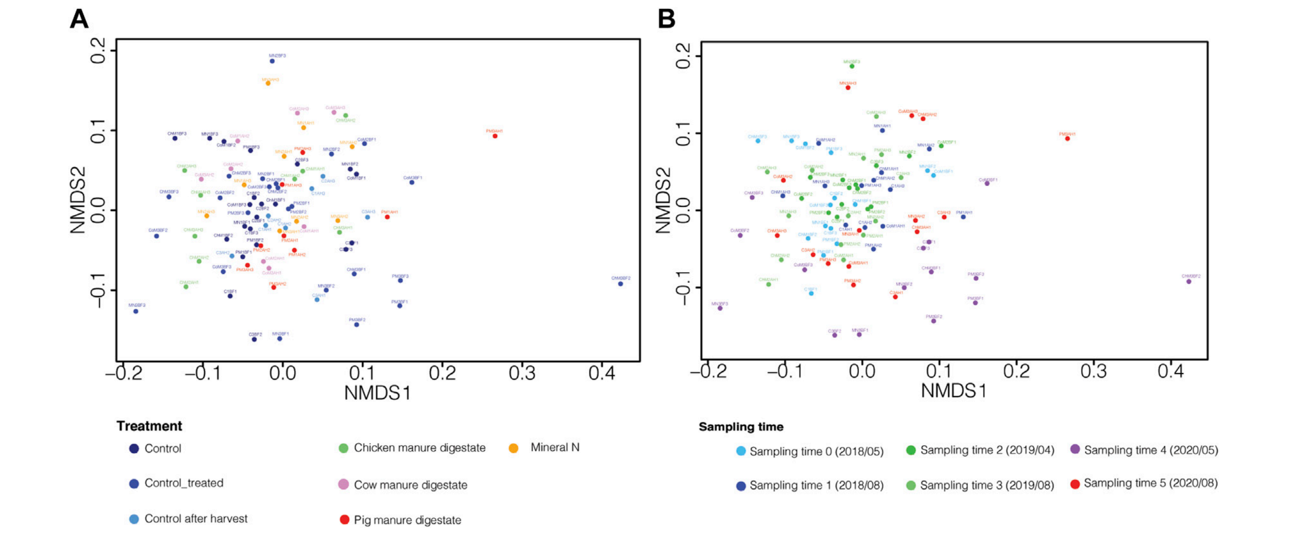

FIGURE 2 | Non-metric multidimensional scaling (nMDS) plot. nMDS plot based on the Bray–Curtis index. **(A)** nMDS plot with samples colored with respect to the treatment used: control (before fertilization, no fertilization), control-treated (before fertilization, fertilized the precedent year), control after harvest (after harvest, no fertilization), mineral nitrogen (after harvest), chicken manure digestate (after harvest), cow manure digestate (after harvest), and pig manure digestate (after harvest). **(B)** nMDS plot with samples colored with respect to sampling time.

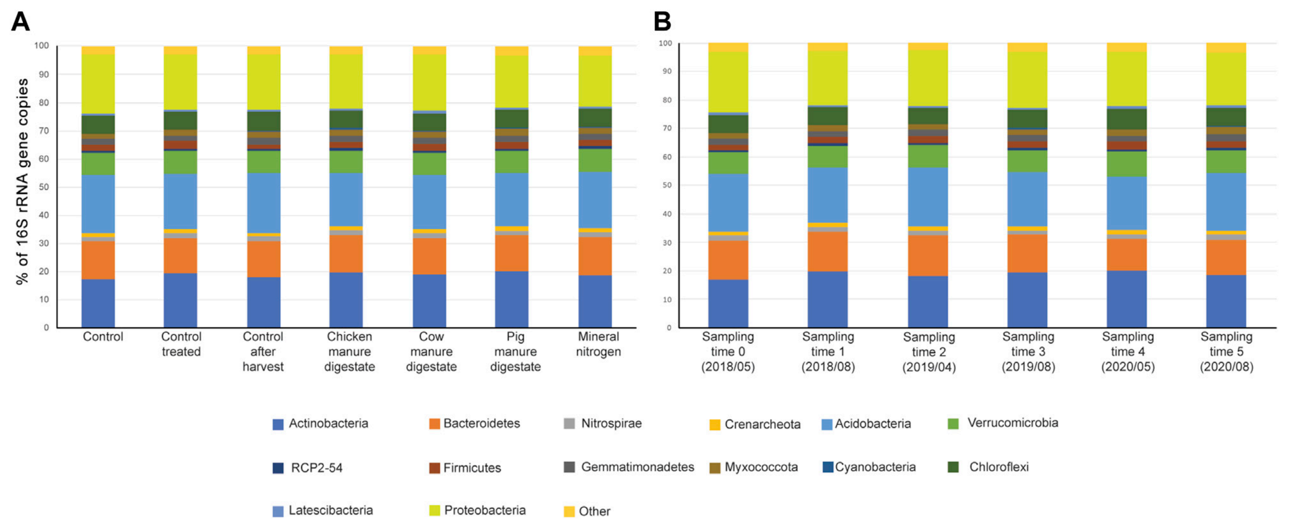

FIGURE 3 | Soil prokaryotic composition. Soil prokaryotic community composition with respect to **(A)** the treatment used or **(B)** sampling time. The fifteen major groups are reported at the phylum taxonomic level. Other phyla are collapsed within the group "other".

Massilia (Proteobacteria), *Puia* (Bacteroidetes), and *Arenimonas* (Proteobacteria) (Supplemental Dataset 1).

Effect of Different Treatments and Sampling Time on Soil Prokaryotic Composition

The PERMANOVA analysis at the phylum rank showed significant differences for both variants analyzed, fertilizing treatments, and sampling time ($p < 0.001$). For sampling time, differences were detected for all the six groups considered. Regarding fertilizing treatments, there were significant differences between control groups before fertilization (control and control-treated) and groups after fertilization, reflecting differences observed in relation to sampling time. The group control-treated showed no significant difference between cow manure digestate and chicken manure digestate groups.

Considering groups sampled after fertilization, significant differences (at phylum rank) were found between the control group and fertilized groups, while within fertilized groups were observed differences only among plots fertilized with mineral

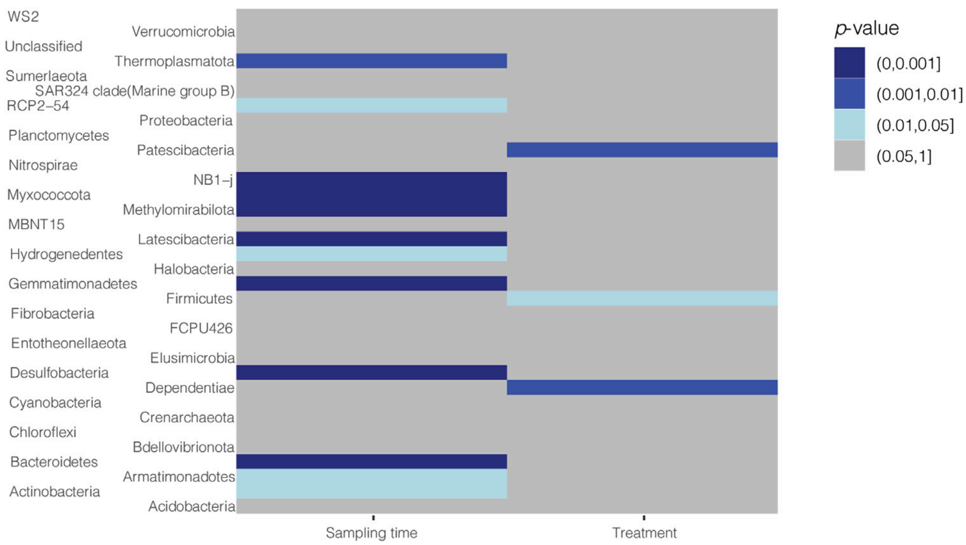

FIGURE 4 | Heatmap for the effects of sampling time and fertilization treatments at the phylum level. Differences in *p*-values at the phylum taxonomic rank for sampling time or treatment used in samples collected after harvest are indicated in gray (no differences) or different shades of blue (*p*-value ranging from 0.05 to 0).

nitrogen and pig manure digestate (PERMANOVA, $p < 0.05$). A negative binomial mixed model was applied to infer differences related to sampling time and/or treatment used (**Figure 4**). Most of the differences (12 phyla) were related to sampling time (**Figure 4**), while only three phyla were differently abundant in relation to the treatment used: Firmicutes, Patescibacteria, and Dependentiae (**Figures 4**, **5**), which accounted for the 2.18, 0.3, and 0.04% of the soil community, respectively. Significant differences in Firmicutes relative abundance were observed during the first year (sampling time 1) among control plots and plots treated with cow or chicken manure digestates (**Figure 5A**). A higher amount of sequences belonging to the phylum Patescibacteria was detected in plots treated with pig manure digestate in the second year (**Figure 5B**), while in the third year, there was a significant difference for Dependentiae phylum between the control group and plots treated with mineral nitrogen (**Figure 5C**).

Differences at the genus rank reflected what was observed at the phylum rank with most of the differences (113 genera) associated with sampling time and only a few genera (18) varying in relation to the different treatments used (**Supplementary Figure S5**). The 18 genera belong to eight different phyla, with six genera affiliated with Proteobacteria, five to Actinobacteria, and two to Firmicutes. We also analyzed the relative abundance of these groups in the three different years and for seven of them, we found significant differences ($p < 0.05$). In the first year, we observed significant differences for the genera *Streptomyces*, *Paenisporosarcina*, the subgroup 10 of the phylum Acidobacteria and *Opitutus*, and the group TM7a of the phylum Patescibacteria (**Figure 6**; Kruskal–Wallis or ANOVA, $p < 0.05$). The subgroup 10 relative abundance was higher in the control group with respect to the plots treated with mineral nitrogen and cow manure digestate. For the other genera, we observed a lower amount in the control group with respect to one or more fertilized groups, in particular *Streptomyces* relative abundance was higher in all the manure digestate groups (**Figure 6B**). For the second year, only *Acinetobacter* showed significant differences with a higher presence in plots treated with cow manure digestate with respect to control and chicken manure digestate groups (**Figure 6D**; Kruskal–Wallis, $p < 0.05$). In the third year, two genera, *Gaiella* and *Paenisporosarcina*, were characterized by higher levels of pig and cow manure digestate (respectively) versus the control group (**Figures 6A,C**; ANOVA, $p < 0.05$). *Paenisporosarcina* was the only genus showing a marked increase in treated groups in two different years (first and third, **Figure 6C**).

DISCUSSION

The overall community composition was quite similar for all the treatments used, and the major phyla detected at the initial sampling time in the year 2018 were well-known soil dominant phyla (Proteobacteria, Acidobacteria, and Actinobacteria) which are commonly found in this type of soil, and they kept their predominance throughout the duration of the project (Mhete et al., 2020; Wu et al., 2020; Li et al., 2021). These phyla accounted for over 55% of the soil's prokaryotic composition. Proteobacteria is one the most diverse and abundant phyla present in the soil; within this phylum, many different microbes can thrive and adapt to different soil conditions and influence plant growth either as plant growth–promoting rhizobacteria or as pathogens (Spain et al., 2009). Actinobacteria are typically dominant soil microbes partaking in the biogeochemical cycling of carbon, nitrogen, phosphorus, potassium, and several other elements in the soil. Furthermore, within Actinobacteria, there are aerobic saprophytes capable of producing extracellular hydrolytic enzymes that can degrade complex compounds (Ranjani et al., 2016). Actinobacteria presence helps in sustainably improving soil health and providing an effective

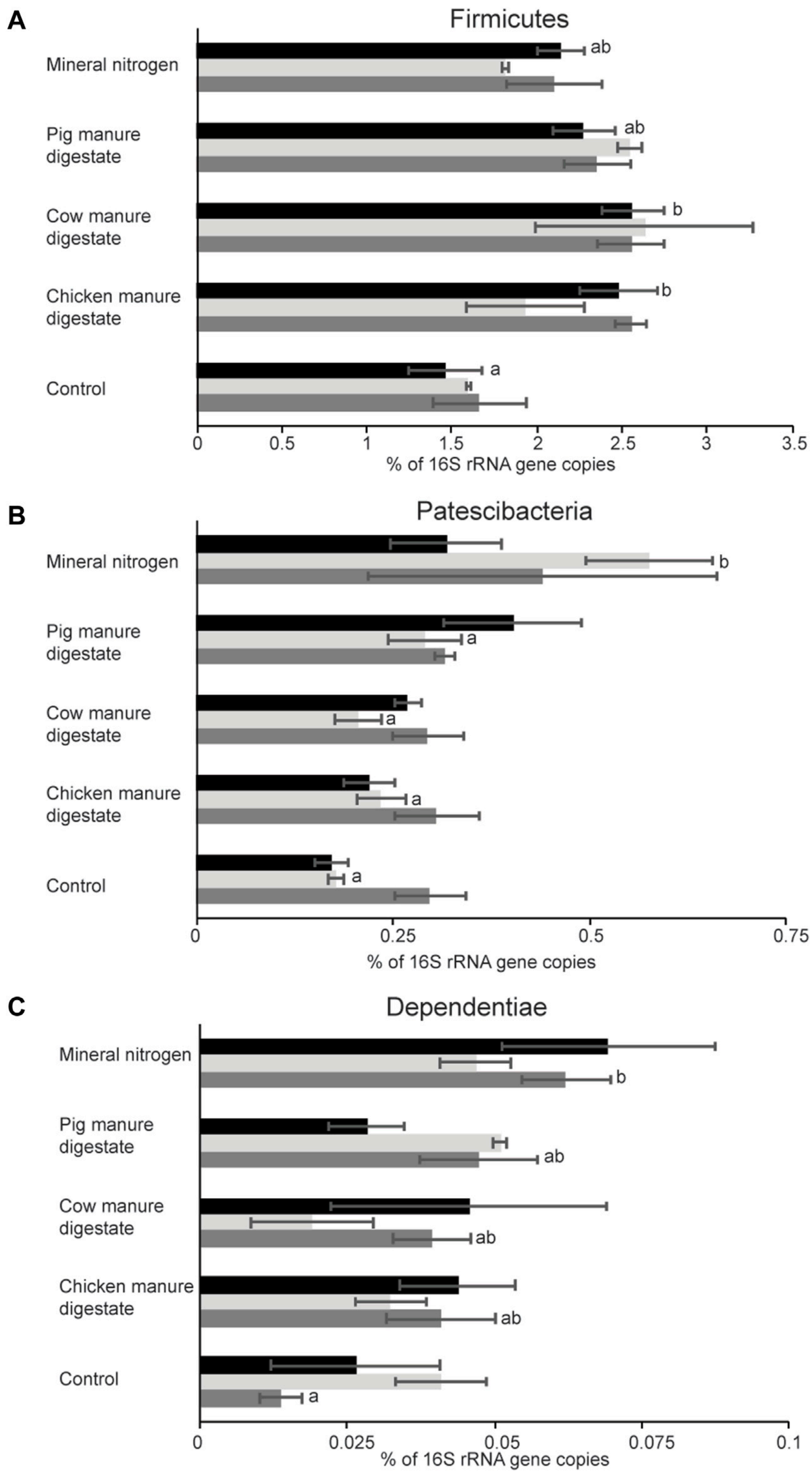

FIGURE 5 | Effect of the different fertilizers used at the phylum level after harvest. Each bar is labeled respective to the treatment applied and colored respective to the year of treatment: black (2018, first year), light gray (2019, second year), and dark gray (2020, third year). **(A)** Firmicutes, **(B)** Patescibacteria, and **(C)** Dependentiae. Means sharing the same letter within the same year are not significantly different (post hoc Tukey's HSD test or Dunn's test).

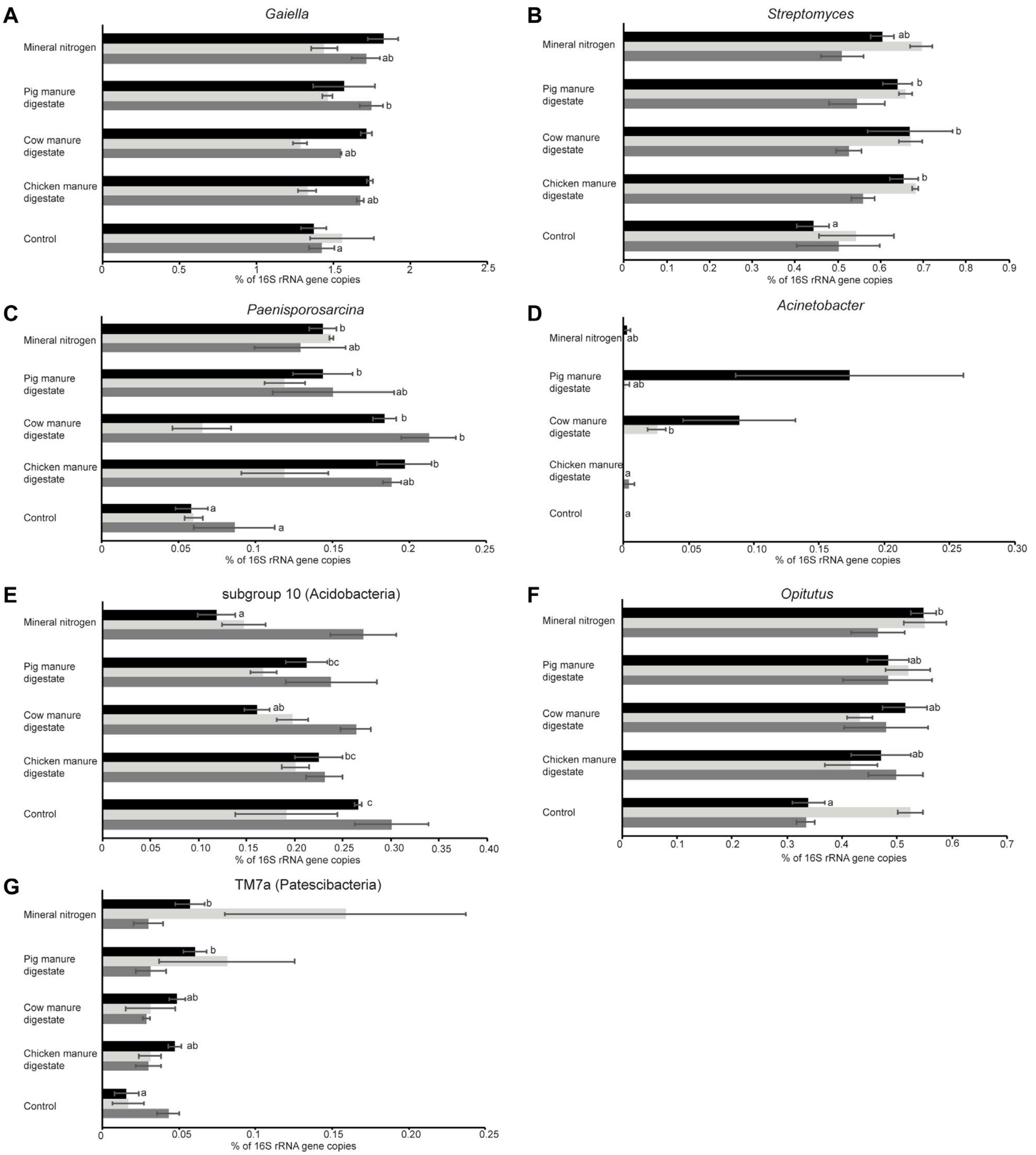

FIGURE 6 | Effect of the different fertilizers used at the genus level after harvest. Each bar is labeled respective to the treatment applied and colored respective to the year of treatment: black (2018, first year), light gray (2019, second year), and dark gray (2020, third year). **(A)** *Gaiella*, **(B)** *Streptomyces*, **(C)** *Paenisporosarcina*, **(D)** *Acinetobacter*, **(E)** Acidobacteria subgroup 10, **(F)** *Opitutus*, and **(G)** TM7a (Patescibacteria). Means sharing the same letter within the same year are not significantly different (post hoc Tukey's HSD test or Dunn's test).

pathway for nutrient cycling (Bhatti et al., 2017). Acidobacteria is considered one of the most abundant soil phyla with their relative abundances ranging from ca. 20–40% in temperate soils such as forests, grasslands, and pasture soils (Janssen, 2006). Here, we observed a relative abundance ranging from 16 to 23%, the relatively low amount of Acidobacteria found could be possibly linked to the pH (7.3) of the receiving soil used as most Acidobacteria prefer lower pH (3.0–6.5) (Kalam et al., 2020). The addition of manure digestates did not affect soil pH (Doyeni et el., 2021b) and Acidobacteria relative abundance was found similar in the 3-years analysis with no difference with respect to the treatments used. Indeed, in other similar studies (Xu et al., 2016; Zhang et al., 2021), the application of the digestate does not have negative effects on their availability. However, the correlation analysis showed that several genera (75) were affected by soil pH as a slight decrease (unrelated to soil treatments) was observed between the beginning and the end of the trial (from 7.03 to ~6.5) (Doyeni et el., 2021b). Noticeably, the decrease in pH we observed could be linked to seasonality (Wolińska et al., 2022). We did not find significant correlations between Acidobacteria and pH at phylum or genus ranks; however, this could be biased as it was not possible to assign most of the sequences falling in the Acidobacteria phylum at low taxonomic ranks (i.e., genus). Soil pH is a major driver of bacterial selection and abundance as for bacteria the pH range for optimal growth is quite narrow (Rousk et al., 2010; Tian et al., 2021). Most of the taxa influenced by acidification belonged to the Proteobacteria and Firmicutes phyla and showed a positive correlation with pH. The most represented genera showing a positive correlation with pH were *Puia* (Bacteroidetes) and three proteobacterial taxa (*Sphingomonas*, *Massilia,* and *Arenimonas*) which decreased when compared with the first year (before fertilization) and the third year (after fertilization). *Massilia* genus has been found to colonize root surfaces and is relatively abundant in the rhizosphere (Ofek et al., 2012; Wolińska et al., 2022). In contrast, *Haliangium* (Mixococcota) relative abundance increased with a lower pH, and members of this genus are commonly found in soil [e.g., in the rhizosphere of melon plants (Ling et al., 2014)] and may have different effects on soil microbiota: it has been observed that they are capable of predating on Gram-positive bacteria (Zhang and Lueders, 2017) and also have the potential to inhibit the growth of a wide spectrum of fungi (Fudou et al., 2001; Ling et al., 2014). Variations in *Massilia* and *Haliangium* relative abundance have been previously observed in relation to pH and seasonality in a trial using an intercrop mixture and a maize monoculture (Wolińska et al., 2022).

Soils are complex environments, and perturbations of their homeostasis may alter the microbial community composition. Therefore, digestate application may enrich soil phyla already present in soil and/or influence their relative abundance. Previous analysis showed a beneficial effect of digestate application on plant growth (Doyeni et al., 2021b), which was possibly due to an increase in microbial biomass (Doyeni et al., 2021a); however, the lack of direct measurement of these samples could not directly confirm this hypothesis. Moreover, it was not clear if this was also due to alterations in the prokaryotic community and/or to the presence of novel microorganisms present in the digestates. The samples were then collected at two different time points, a medium one few months after fertilization (3–4 months after digestate application, after harvest) and a long term before the fertilization of the following year. However, no significant differences were observed between the treatments. In contrast, the PERMANOVA analysis showed that most of the differences observed were related to sampling time, indicating that multiple factors related to this parameter had a major influencing role on soil bacterial community composition. Indeed, this parameter was associated to the period of sensitivity in weathering seasonal changes, cultivation years, and agricultural practices. The applied agricultural techniques such as annual tillage before the start of the cultivation season (before fertilization) and the harvesting activity (after harvest) could have impacted significant changes in microbial composition (Longepierre et al., 2021). Also, environmental factors are known to play a fundamental role in shaping microbial composition and diversity (Zhang et al., 2019).

Considering only the effects of a few months after fertilization (after harvest), the overall prokaryotic composition was similar for all treatments, with few differences observed at phylum and genus levels. For instance, for samples collected in the first year (after harvest), Firmicutes relative abundance was different in control with respect to the other treatments, but no differences were observed between the digestates and mineral nitrogen fertilizers, indicating an effect of fertilization on this group. Furthermore, Patescibacteria and Dependetiae are enriched in mineral nitrogen–treated soil in comparison to the control in the second and third years, respectively. Patescibacteria are ultra-small bacteria mostly uncultivated with reduced genomes and often found in groundwater environments (Tian et al., 2020); however, their presence has also been observed in endophytic communities (Wemheuer et al., 2019). Similarly, the phylum Dependentiae (formerly known as TM6) is a group of microorganisms widespread in different environments (mats, sediments, sulfur springs, and sinks) whose current knowledge comes from metagenomic data only (Yeoh et al., 2016). The comparative genomic analysis showed parasitism as a common feature within this group (Yeoh et al., 2016), suggesting that it could potentially affect plant growth; however, its presence was significantly higher in mineral nitrogen–treated plots only.

Regarding the differences among treatments at the genus level, similar trends (differences among control samples and mineral nitrogen and/or samples of plots treated with digestates) were noticeable. Taxa belonging to the genera *Gaiella*, *Streptomyces*, *Acinetobacter*, *Opitutus*, Acidobacteria (subgroup 10), and *Streptomycetes* showed differences among treatments in 1 year only, while *Paenisporosarcina* showed significant differences in the first and third years. The *Paenisporosarcina* genus has been characterized by mostly psychrophilic species (Reddy et al., 2013); however, it has been found also in soils where it may have a beneficial effect on plant growth (i.e., rice), inhibiting potential pathogens such as *Rhizoctonia solani* owing to VOC production (Wang et al., 2021). Also, members of the *Gaiellales* have been found associated to cereals in the root system of rice (Hernández et al., 2015). Microbes belonging to the genus *Streptomycetes* are often detected inside plant roots and can be beneficial (Olanrewaju and Babalola, 2019) while sometimes can act as plant pathogens (Seipke et al., 2012); however, the crop yield and quality of crops were not compromised in the period of digestate application (Doyeni et al., 2021b).

The identity of the host plant has a significant influence on the identity of its microbiome (Dastogeer et al., 2020), and promoting a soil microbiome for high plant production requires management of microbial abundance and activity, community composition, and specific functions (Lehmann et al., 2020). In essence, the different cereal-based crop plants cultivated in the 3 years may have played key roles in the prokaryotic relative abundance as each plant has unique requirements in terms of needs, uptake, and competitiveness with the soil microbes. These factors together with other environmental/agricultural factors were then the major drivers in influencing microbiota composition rather than digestate application.

CONCLUSION

All the three types of digestate tested gave similar results with the native prokaryotic community composition not significantly affected in a medium/long term response over the 3 years of application. The major effect on community composition was due to the sampling time possibly related to the changing environmental conditions and other agricultural management techniques factors such as the tillage before each year's cultivation, harvesting during summer, and different cereal crops grown each year. A pH decrease was observed between the beginning and the end of the trial, and this was unrelated to the treatment used and probably linked with seasonality. However, soil pH probably played a major role in microbiota relative abundance (positively) correlating with many taxa at genus rank.

Digestate application showed then a positive effect as the short- to long-term aim was to prevent the introduction/ increase of potential pathogens in the soil and avoid a perturbation of the native soil prokaryotic community.

DATA AVAILABILITY STATEMENT

The 16S rRNA gene amplicon sequence data are available at the National Centre for Biotechnology Information Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) with SRA accession from SAMN24344854 to SAMN24344941.

AUTHOR CONTRIBUTIONS

SS and VT conceived the experiment. MD and SS performed soil sampling and soil DNA extraction. MD, CV and FP analyzed data. All authors contributed to data interpretation, drafted the manuscript, agreed with its final version, and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.852241/full#supplementary-material

Supplementary Table S1 | P-values of the PERMANOVA test show differences among sampling times.

Supplementary Table S2 | P-values of the PERMANOVA test show differences among treatments.

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Phthalate Acid Esters in Soil, Plastic Shed Film, and Ginseng Tissues of Different Ages From Farmland: Concentration, Distribution, and Risk Assessment

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Plastic shed film used in ginseng cultivation could increase phthalate acid esters (PAEs) residues in ginseng and its planting soil. This study recorded the situation of 19 PAEs in ginseng, soil, and plastic shed film samples from eight ginseng cultivation bases in Jilin Province, China. The results showed that 6 PAEs are omnipresent contaminants in ginseng cultivation bases. The Σ19 PAEs residue ranged from 0.69 to 3.30 mg kg^{-1} in the soil and from 32.40 to 96.20 mg kg^{-1} in the plastic shed film. Plastic shed film is possibly one source of PAEs in ginseng bases. In addition, PAEs concentrations in different ginseng tissues (roots, stems, and leaves) showed significant differences (p < 0.05). The residues of Σ19 PAEs in ginseng roots and stems were 0.71–2.10 mg kg^{-1} [dry weight (dw)] and 1.56–4.69 mg kg^{-1} (dw), respectively, lower than 2.37–6.82 mg kg^{-1} (dw) in leaves (p < 0.05). PAEs more readily accumulated in ginseng leaves than in roots and stems. Cultivation age also influenced PAEs accumulation in ginseng. PAEs residues in 3-year-old ginseng plants were higher than those in annual and biennial ginseng (p < 0.05). The noncancer and carcinogenic risk assessments of the target PAEs in ginseng indicated acceptable levels for adult intake. This study provides data for understanding the concentration, distribution, and potential risk of PAEs in ginseng and its cultivated soil.

Keywords: phthalate acid esters, soil, ginseng tissue, cultivation age, risk assessment

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INTRODUCTION

Phthalate acid esters (PAEs), a kind of common plasticizer, are widely added to chemical plastics and agricultural films to increase the plasticity and toughness of these plastic products (Zolfaghari et al., 2014; Steinmetz et al., 2016). The application of plastic film in facility agriculture rose to 2.60 million tons in 2006 (Zhou et al., 2020), and some thick films (over 50 mm polypropylene) are used in facility-based agriculture, especially plastic greenhouses, playing an important role in the adjustment of environmental conditions. PAEs are still easily released into the environment due to sunlight, rain erosion, and other natural phenomena because PAEs are bonded to polymer chains by hydrogen bonding and van der Waals forces (Arfaeinia et al., 2019). PAEs have potential endocrine-disrupting effects in humans (Lu et al., 2020). Moreover, PAEs are known as "reproductive arsenic" in Europe. Dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate

(BBP), di-(2-ethylhexyl) phthalate (DEHP), and di-n-octyl phthalate (DnOP) were defined as priority control pollutants by the United States Environmental Protection Agency (Niu et al., 2014).

PAEs residues in many types of agricultural soils and various crops across China are among the most concerning organic pollutants in agricultural fields (Ma et al., 2013; Wang et al., 2015; Du et al., 2020; Li et al., 2020). Previous studies have provided information on PAEs occurrence in plants in greenhouse facilities affected by varieties, different temperatures, and cultivation patterns (Cai et al., 2015; Li et al., 2016; Cheng et al., 2020; Li et al., 2020; Zhao et al., 2022). However, few studies have characterized the PAEs residues and sources in the Chinese herbal medicine ginseng according to different cultivation ages and tissues.

In China, ginseng, as a perennial herb (mostly requiring 3–5 years to harvest), is planted in greenhouses with plastic shed films to avoid strong light and modify the microclimate. However, plastic shed films easily release PAEs into the air and soil, which results in higher PAEs residues in greenhouse soil than in open fields (Zhou et al., 2021). Plastic film is believed to be one of the important sources of PAEs in farmland (Wang et al., 2015). Some reports also show that PAEs can accumulate in plants at concentrations even higher than those in the corresponding soils. Leaf vegetables contain more PAEs than root vegetables (Zeng et al., 2020; Zhao et al., 2022). Ginseng requires multiple years of cultivation, which means there is a long time to accumulate pollution in soil. Ginseng plants have roots, stems, and leaves. Roots are grown underground. Stems and leaves are grown aboveground. Analysis of whole ginseng plants (roots, stems, and leaves) can help to clarify the distribution and potential uptake sources of PAEs. Most importantly, compared with vegetables grown for several months, the perennial herb ginseng can be especially well suited to research pollution accumulation.

Jilin, located in northeast China, is China's largest ginseng suppling province, contributing to 85% of the ginseng production in the country (Wang et al., 2018). With the continuous expansion of ginseng demand, wild ginseng is not enough to meet supply requirements, and the level of agricultural productivity must meet the demands of capital development. To increase ginseng output, Jilin Province has used farmland as the main means to cultivate ginseng. After several years of rapid promotion, a large-scale farmland cultivation base has been devoted to ginseng cultivation. However, limited data have been reported about PAEs pollution in ginseng cultivation bases. This study aimed to explore PAEs pollution characteristics in a ginseng cultivation environment and evaluate the noncarcinogenic and carcinogenic risks to adults via dietary consumption of ginseng.

MATERIALS AND METHODS

Sample Collection

Based on geographic location, eight large-scale ginseng cultivation bases ranging from 25 to 500 ha were selected for research in Jilin Province, China. These bases were located in the farmland of four areas, namely, Baishan city, Dunhua city, Jilin city, and Tonghua city (**Figure 1**).

The fresh ginseng, soil (0–20 cm), and shed films were combined from the eight ginseng cultivation bases in July–August 2021. The detailed collection process can be found in Li et al. (2016). Fifteen soil samples from farmland used to cultivate garden ginseng were collected (**Supplementary Table S1**). Every composite soil sample was collected from five points using a stainless steel auger and sealed in brown paper bags before being transported back to the lab. Then, the soil samples were freeze-dried, ground, sifted through an 80-mesh stainless steel sieve, and stored in glass bottles.

A total of 23 ginseng samples (including roots, stems, and leaves) under different cultivation ages (annual, biennial, 3-year-old, 4-year-old, and 5-year-old) were cultivated in greenhouses covered by plastic shed films. They were separated into roots, stems, and leaves and rinsed clean with deionized water. Then, the samples were freeze-dried, ground, and sieved through a 60-mesh stainless steel sieve. Regarding the ginseng sampling strategy, we mainly considered the ginseng planting years of different bases. A total of 1–5 ginseng samples were collected from each ginseng cultivation base (**Supplementary Table S1**). Each composite ginseng sample was randomly collected from five different sites in the same bases and homogenized as one sample.

An inserted map displays the location of Jilin Province, including the plastic films and nets, and these materials were also collected for potential source analysis. A total of 1–3 film samples were collected from each ginseng cultivation base. In total, 15 plastic shed film samples were collected from eight cultivation bases (**Supplementary Table S1**). These film samples were washed with deionized water, air-dried, cut into 4 mm × 4 mm fragments, mixed and kept in sealed kraft paper.

Standard and Reagents

A mixed standard solution (1,000 mg L^{-1}) of 19 target PAEs was purchased from Alta Scientific Co., Ltd. (Tianjin, China). Dimethyl phthalate (DMP), diethyl phthalate (DEP), diisopropyl phthalate (DIPrP), diallyl phthalate (DAP), dipropyl phthalate (DPrP), diisobutyl phthalate (DIBP), dibutyl phthalate (DBP), bis (2-Methoxyethyl) phthalate (DMEP), bis (4-Methyl-2-pentyl) phthalate (BMPP), di (2-ethoxyethyl) phthalate (DEEP), diamyl phthalate (DPP), dihexyl phthalate (DHXP), butyl benzyl phthalate (BBP), bis(2-n-butoxyethyl) phthalate (DBEP), dicyclohexyl phthalate (DCHP), di-(2-ethylhexyl)phthalate (DEHP), dipentyl phthalate (DPhP), di-n-octyl phthalate (DnOP), and dinonyl phthalate (DNP). Acetone, acetonitrile, n-hexane, and other chemicals were all HPLC grade and purchased from Fisher (Pittsburgh, United States).

Sample Processing

PAEs were extracted following a QuEChERS method with slight modification (Zhao et al., 2022). A total of 2.5 g ginseng root, stem or leaf powder in a 50 ml glass centrifuge tube with 2 g sodium chloride was mixed with 10 ml acetonitrile for ginseng sample extraction. The polypropylene cap was screwed onto the tube with aluminum foil, and the glass tube was swirled and subjected to ultrasound treatment for 30 min, followed by centrifugation at 5,000 rpm for 5 min. Five milliliters of the extracts was transferred to a new glass column

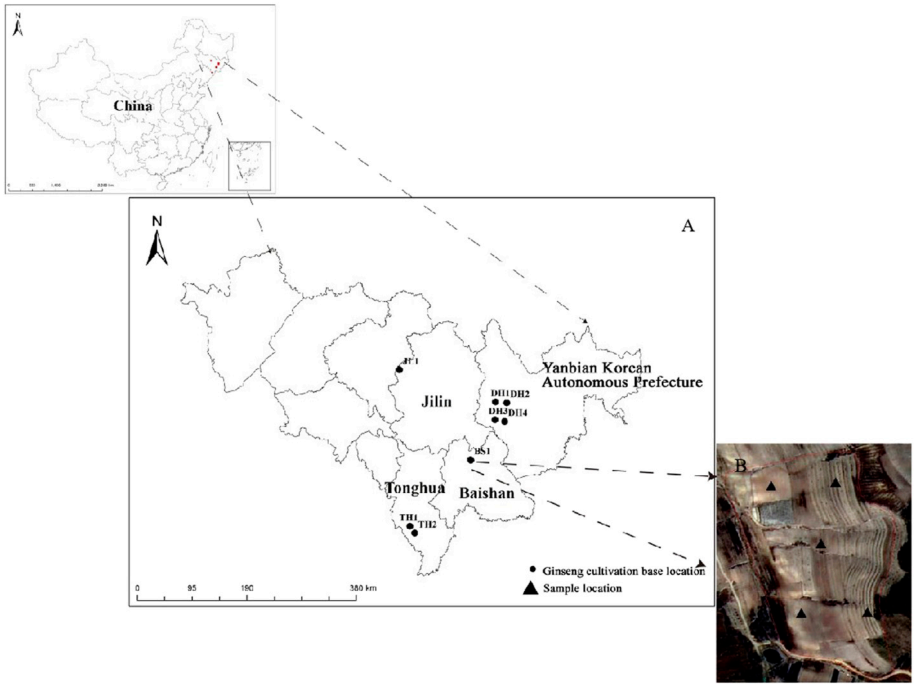

FIGURE 1 | Map showing the eight ginseng cultivation bases of Jilin Province, China. **(A)** Research areas within Jilin Province. **(B)** Satellite imagery showing the ginseng sampling layout at the BS1 site as an example. The ginseng cultivation base names were BS1, DH1, DH2, DH3, DH4, JL1, TH1, and TH2 (BS, Baishan city; DH, Dunhua city; JL, Jilin city; TH, Tonghua city).

containing 30 mg of GCB and 100 mg of PSA for the clean-up procedure. This extraction process was repeated twice. The eluate was concentrated to nearly dry, and 1.0 ml hexane was added and redissolved for GC–MS analysis.

The PAEs extraction method in the soil and shed film was performed according to a previous study with some modifications (Li et al., 2021). A total of 2.5 g of soil or 0.1 g of plastic shed film in a glass centrifuge tube was mixed with 40 ml hexane and acetone (V: V = 1:1) for 24 h. The samples were vortexed and treated ultrasonically for 15 min, followed by centrifugation at 5,000 rpm for 5 min. The supernatant was separated through centrifugation. The extraction procedure was repeated twice, and the supernatant was combined. The total extracts were transferred to a new glass column containing 2 g of anhydrous magnesium sulfate, 6 g of neutral alumina, and 12 g of silica gel for the clean-up procedure. Next, 15 ml n-hexane and 15 ml n-hexane:acetone (V:V = 4:1) were used to prewash the column in turn. The extract was eluted with 40 ml of n-hexane and acetone (V: V = 4:1). Then, the leachate was concentrated to nearly dry and diluted to a final volume of 1.0 ml with n-hexane. All the final extracts were tested with external standards by GC-MS.

GC-MS Analysis

The determination of PAEs was done using an ISQ gas chromatography triple quadrupole mass spectrometry (GC–MS) system (Thermo Fisher, United States) and separation by an HP-5MS capillary column (30 m × 0.25 mm×0.25 μm) with selected ion monitoring (SIM) mode for quantitative determination. The MS/MS transitions and parameters for the compounds used in this study are shown in **Supplementary Table S2**. The carrier gas helium was supplied with a flow rate of 1.0 ml min^{-1}. The column temperature program was initiated at 60°C for 1.0 min, ramped up to 220°C at a rate of 20°C·min^{-1}, held for 1.0 min, further increased to 290°C at 5°C·min^{-1}, and held for 2 min. Then, the temperature was held at 285°C for 2 min. The ion source temperature and transfer line in GC–MS were all set at 280°C. The injector temperature was set at 260°C. One microliter of extract was injected by splitless injection mode in GC-MS.

Quality Control and Analysis

Plastic materials were not used during sampling and processing to avoid PAE contamination. All glassware was baked at 450°C for 8 h to remove all organic matter. The linearity range and equation, determination coefficient (R^2), limit of detection (LOD), and limit of quantification (LOQ) of the 19 PAEs are shown in **Supplementary Table S3**. Five-point standard calibration curves were employed with R^2 values ranging from 0.9939 to 0.9999. The LODs of the 19 PAEs ranged from 0.125 to 1.150 μg L^{-1}, with a signal-to-noise ratio of 3. The matrix-spiked method was used to ensure the validity of the method by calculating the recoveries of target PAEs accuracies in soil, ginseng, and shed film matrix-spiked samples. The spiked levels were set according to the residues of PAEs in the samples. The results shown in **Supplementary Tables S4, S5** indicate that three spiked levels, that is, low (0.02 mg kg^{-1}), medium (0.10 mg kg^{-1}), and high (0.50 mg kg^{-1}), were added for recovery of each compound of the 19 PAEs in ginseng and soil: 73.0%–109.1% and 70.2%–113.0%, respectively. The recoveries of 19 PAEs in the shed film samples ranged from 70.5 to 114.5% as shown in **Supplementary Table S6**. Solvent blank samples, duplicates, and matrix-spiked samples were used to ensure the stability and accuracy of the instrument during the injection process. Because it is difficult to find blank ginseng free of PAEs, the slope comparison method was used to evaluate the ginseng matrix effect. The slope ratios (slope matrix/slope solvent) for each compound were obtained considering a signal enhancement or suppression effect as acceptable if the slope ratio ranged from 80 to 120%. Only small amounts of DMP, DIBP, and DHXP were detected in the solvent blank from 0.0002 to 0.006 mg kg^{-1}, and the final results of all samples were blank corrected.

Health Risk Assessment

The risk of PAEs to consumer health caused by ingestion of garden ginseng was determined through methods recommended by the US EPA (2013). Among the detected PAEs in ginseng root, DBP, DEP, DMP, and DEHP are recognized as noncancerous pollution compounds for human health, while DEHP is carcinogenic (Kluwe et al., 1982). The average daily doses (ADDs) were calculated following **Eq. 1** via the ginseng intake route. Noncancer risk was calculated by the hazard index (HI) *via* the intake of garden ginseng. The HI was used to calculate the sum of hazard coefficients of exposure to various chemicals having the same toxic mechanism. HI was defined by the daily maximum allowable contaminant level (RfD) and the ratio of average daily dietary intake doses (ADD) as shown in **Eq. 2**. If the HI is above 1, it is believed that noncancer risk cannot be ignored.

$$ADD = \frac{C \times I \times EF \times ED,}{BW \times AT \times 10^{-6}} \tag{1}$$

where C is the concentration of PAEs detected in the ginseng sample (mg·kg^{-1}), I is the daily ingestion of ginseng root (mg·$person^{-1}day^{-1}$), EF is the exposure frequency (days·$year^{-1}$), ED is the exposure cycle (year), BW is the body weight (kg), and AT is the average lifetime exposure (days).

$$HI = \Sigma\frac{ADD,}{RfD} \tag{2}$$

where RfD is the daily maximum permissible level of contaminants (mg·kg^{-1}·day $^{-1}$).

$$CR = \Sigma(ADD \times CFS), \tag{3}$$

where CFS is the slope factor of carcinogenic (kg·day·mg^{-1}).

Carcinogenic risks (Girkin et al., 2018) were calculated using the ADD multiplied by the CSF, as shown in **Eq. 3**. The CFS values for DEHP are 0.014 mg kg^{-1} day^{-1} (Kluwe et al., 1982). These values of the parameters for the above formula are shown in **Supplementary Table S7**. Carcinogenic risks were regarded as acceptable if the CR < 10^{-6} (USEPA, 2013).

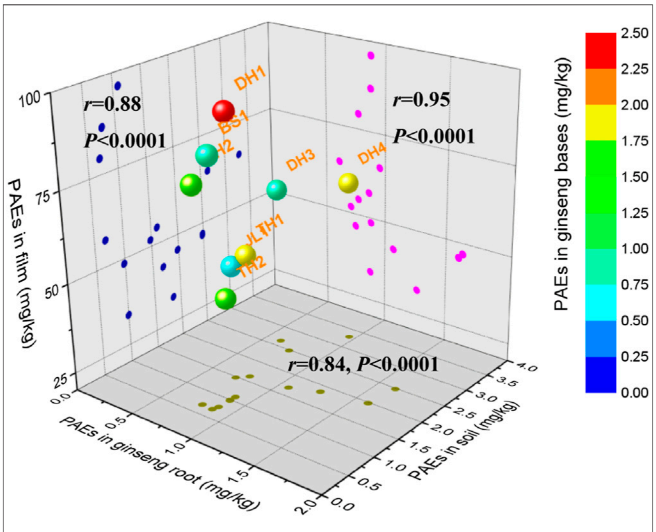

FIGURE 2 | PAEs distributions and correlations among soil, shed film, and ginseng root. The color ball represents the total concentration of 19 PAEs in soils from eight ginseng cultivation bases (mg·kg^{-1}); *r*: Pearson correlation coefficient, *p*: *p* value.

RESULTS AND DISCUSSION

Occurrence of Phthalate Acid Esters in Soils From Ginseng Cultivation Bases

The concentrations and detection frequencies of PAEs in soils, films and ginseng roots are listed in **Supplementary Table S9** and **Figure 2**. PAEs were detected in all the soil samples from eight cultivation bases. The total 19 PAEs concentrations (Σ19PAEs) in soils ranged from 0.69 to 3.30 mg kg^{-1} (mean 1.65 mg kg^{-1}). These results were similar to those of other studies, in which the average Σ16PAEs (DMP, DEP, DiBP, DnBP, DOP, DMEP, BMPP, DEEP, DPP, DHXP, BBP, DBEP, DCHP, DEHP, DPhP, and DnOP) in plastic greenhouse soil ranged from 0.12 to 5.76 mg kg^{-1} (mean: 1.86 mg kg^{-1}) (Zeng et al., 2020) and Σ15PAEs (DMP, DEP, DiBP, DnBP, DMEP, BMPP, DEEP, DAP, DHXP, BBP, DBEP, DCHP, DEHP, DPhP, and DnOP) ranged from 0.14 to 2.13 mg kg^{-1} (mean 0.99 mg kg^{-1}) (Li et al., 2016). Among the 19 PAEs, 7 PAEs compounds, including DMP, DEP, DIBP, DBP, DHXP, DEHP, and DNOP, were detectable. DMP, DIBP, DBP, DHXP, and DEHP occurred in all samples, accounting for approximately 94% of Σ19PAEs. PAEs detected from eight bases showed the order of DBP > DIBP > DEHP > DnOP > DMP = DNP > DEP. This distribution was different from that found for vegetable agricultural bases in China (Wang et al., 2014; Ma et al., 2015).

According to New York State Department of Environmental Conservation (2010) guidelines about soil cleanup in the United States, six PAEs, including DMP, DEP, DBP, BBP, DnOP, and DEHP, should be limited to 0.020, 0.071, 0.081, 1.125, 1.200, and 4.350 mg kg^{-1}, respectively. In the eight ginseng cultivated bases, the DEP, BBP, DnOP, and DEHP residue levels in the soil samples met the standards. However, DBP was over the recommended allowable values, ranging from

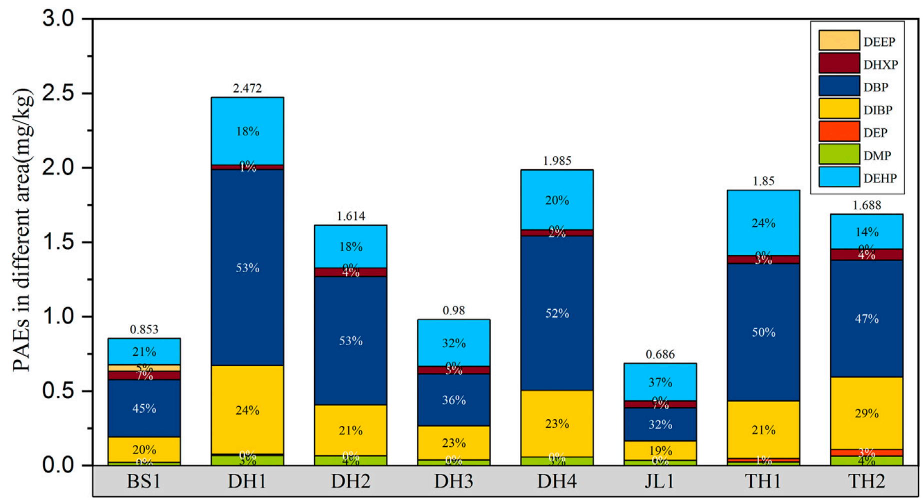

FIGURE 3 | Concentrations and distribution of seven PAEs detected in soils from eight ginseng cultivation bases.

0.22 to 1.86 mg kg^{-1}. The concentrations of DMP in a vast majority of ginseng base soil samples (93%) except those of BS1 surpassed the recommended allowable values. Therefore, DBP and DMP in farmland soil in ginseng cultivation bases should receive attention.

However, the concentrations of PAEs in the soil varied significantly among the different ginseng cultivation bases (**Figure 3**). The residue of Σ7PAEs in the DH1 ginseng cultivation bases was higher than those in the other bases. The Σ7PAEs detected in soils was 2.47 mg kg^{-1}. Lower PAEs residues were found in JL1 located in Zuojia town, Jilin city, with a mean concentration of 0.69 mg kg^{-1}. JL1 is an experimental farmland, and compared with other bases, the farming intensities and agricultural activities were relatively low in this area. This might be the reason why PAEs residues were accordingly lower.

In recent years, soil organic matter (SOM) and pH have been reported to affect PAEs fate in soil (Xu et al., 2008; Chang et al., 2009; Ren et al., 2016). SOM content crucially influenced the adsorption process of PAEs, and the soil solution pH and its concentration of dissolved organic matter (DOM) affected biodegradation by providing nutrients, which supported the growth of bacteria and improved overall enzyme activity (Tang et al., 2020). In fact, a slight positive correlation between PAEs residues and pH was confirmed in agricultural soils (Katsoyiannis, 2006; Zeng et al., 2008), and no correlations between PAEs and total organic carbon were detected in green vegetable production base soils (Chai et al., 2014). In this study, the SOM content ranged from 30.2 to 119.0 g kg^{-1} and the pH ranged from 3.70 to 5.80 in soil from eight large ginseng cultivation bases. However, the correlation of SOM and pH with PAEs concentration in soil was evaluated. SOM was negatively correlated with the concentrations of 19 PAEs in soils (r = −0.63, p = 0.022), and no clear correlation was found with pH (r = 0.08, p = 0.79), as shown in **Supplementary Table S8** and **Supplementary Figure S1**. Niu et al. (2014) also reported that pH has no clear correlation with the level of PAEs residues in Chinese soil, and a positive correlation was shown with DMP and SOM.

Phthalate Acid Esters Concentrations in Plastic Shed Films From Ginseng Cultivation Bases

Plastic shed films in ginseng cultivation bases are mainly composed of shading nets made by high-density polyethylene (HDPE) and plastic films made by polypropylene, which have different colors to absorb different kinds of light. To determine the level of PAEs in shading films used in the cultivation bases of ginseng, 15 plastic film samples were detected, as shown in **Supplementary Table S9** and **Figure 2**. Σ19 PAEs were detected in shed plastic film (thick film) from 32.4 to 96.2 mg kg^{-1} (mean 56.80 mg kg^{-1}), which are higher than those in mulching films (thin films) from 3.66 to 30.8 mg kg^{-1} (mean 12.1 mg kg^{-1}) (Li et al., 2016). In addition, the results showed that DEHP, DIBP, and DBP were the most omnipresent congeners, accounting for N88% of Σ19PAEs, and DMP, DEP, and DHXP remained at 12%, having a similar content ratio in the PAEs components detected in corresponding farmland soils. PAEs components in plastic polymers can easily enter the environment because PAEs rely on hydrogen bonding and van der Waals forces to bind with the chemical structure of the plastic (Wang et al., 2014; Zolfaghari et al., 2014; He et al., 2015). The PAEs levels in the plastic shed films were 10–30 times higher than those in the soils. To better understand the potential sources of PAEs residues in ginseng cultivated soil, Pearson's correlation coefficients were used to calculate the correlations of residual levels of PAEs between soil and shed films. The correlation coefficient was 0.88, between 0.8 and 1.0, indicating a strong correlation. This result indicates that PAEs concentrations in farmland soil were significantly correlated with the type and

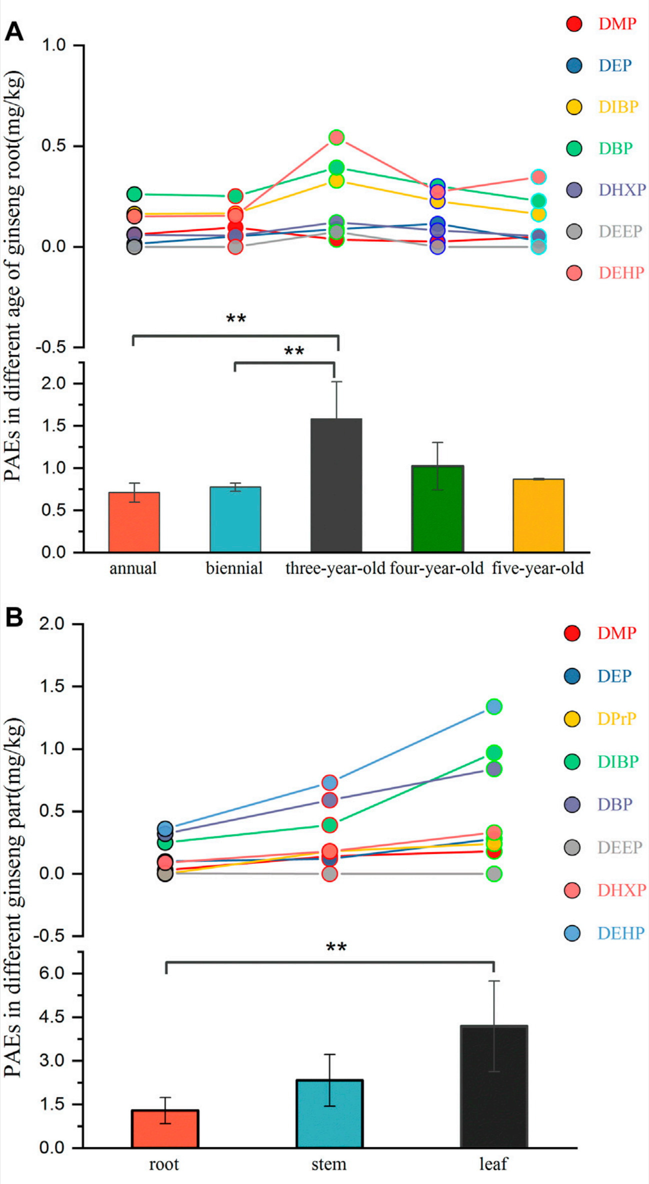

FIGURE 4 | Concentrations and distribution of PAEs in ginseng roots under five cultivation ages (annual, biennial, 3-year-old, 4-year-old, and 5-year-old) **(A)** and ginseng tissues (root, stem, and leaf) **(B)**. ** in the picture indicates significant differences ($p < 0.05$).

amount of plasticizer in the shed film. Therefore, plastic shed films may result in enhanced PAEs contamination in the environment.

Distribution of Phthalate Acid Esters in Ginseng Roots Under Different Cultivation Ages

The application of PAEs additives could cause plants, such as vegetables and crops, to absorb PAEs from the environment (Cai et al., 2015). However, there are few studies on the accumulation of PAEs in plants with longer growing periods (more than 1 year). In this study, 23 ginseng roots with five different cultivation ages (annual, biennial, 3-year-old, 4-year-old, and 5-year-old) were collected from eight large cultivation bases, and the levels of 19 PAEs were determined. The contents of individual PAEs in ginseng samples are shown in **Supplementary Table S10** and **Figure 4A**. Seven PAEs were found in ginseng samples, ranging from 0.71 to 2.10 mg·kg^{-1} (1.16 mg kg^{-1} on average). The annual, biennial, 3-year-old, 4-year-old, and 5-year-old averages of Σ19PAEs in ginseng roots were 0.71, 0.78, 1.58, 1.02, and 0.87 mg kg^{-1}, respectively. Lower PAEs residues in ginseng samples were observed in the annual ginseng and biennial ginseng, which showed a significant difference ($p < 0.05$) from those in 3-year-old ginseng. With the growth of ginseng, the 4-year-old and 5-year-old ginseng groups showed a decreasing trend for total PAEs residues. Plant root exudates and their associated microbiota have an important impact on the behavior of soil contaminants (Rodríguez-Garrido et al., 2020). Ginseng has rich root exudates that can provide carbon and energy sources for soil microbes (Girkin et al., 2018). These microbes could promote the degradation or dissipation of organic contaminants (Turkovskaya and Muratova, 2019). The ginseng endophyte *Paenibacillus polymyxa* was reported to degrade pesticide residues (fluazinam, benzene hexachloride, pentachloronitrobenzene, chlorpyrifos, and dichloro-diphenyl-trichloroethane) (Zhang et al., 2019). Ginseng root exudates and the associated genera of microbes became diverse with the growth of ginseng (4year-old and 5-year-old), which probably enhances the degradation and bioremediation of phthalate acid esters. Similar results have been reported in rice, whose root exudates enhance the desorption of PAEs in soil (Du et al., 2020).

The most abundant PAEs were DIBP, DBP, and DEHP in ginseng, which were also observed from soil and shed films. The ginseng, soil, and shed film samples had nearly the same PAEs, but the PAEs contents in the shed films were greater than those in the soil and ginseng samples. More importantly, the coefficient of determination showed that the level of PAEs residues in ginseng was strongly correlated with the detected PAEs in the soil and film ($r = 0.84$ and 0.95, respectively; $p < 0.01$). Compared with other potential sources of PAEs pollution, such as water, air or fertilizers, total concentrations of the main PAEs have been reported to be up to 4.04 μg L^{-1} in surface water (He et al., 2013), 4,393 ng/m^3 in air (∑16 PAEs) (Zeng et al., 2020) and 0.25 mg kg^{-1} in 22 fertilizers (∑6 PAEs) (Mo et al., 2008). These reported values were much lower than those of PAEs from plastic shed films (average 56.8 mg kg^{-1}). In addition, PAEs can be absorbed by air, water, and soil, especially in plastic greenhouses, and this could threaten crops (Zhou et al., 2021). Therefore, plastic films may be the primary source of PAEs in ginseng samples and their cultivated soils.

Distribution of Phthalate Acid Esters in Ginseng Roots, Stems, and Leaves

Concentrations of the eight detected PAEs in ginseng (root, stem, and leaf) showed significant differences ($p < 0.05$). The Σ19PAEs in ginseng leaves were 2.37–6.82 mg kg^{-1} (dw) and 1.56–4.69 mg kg^{-1} (dw) in ginseng stems and

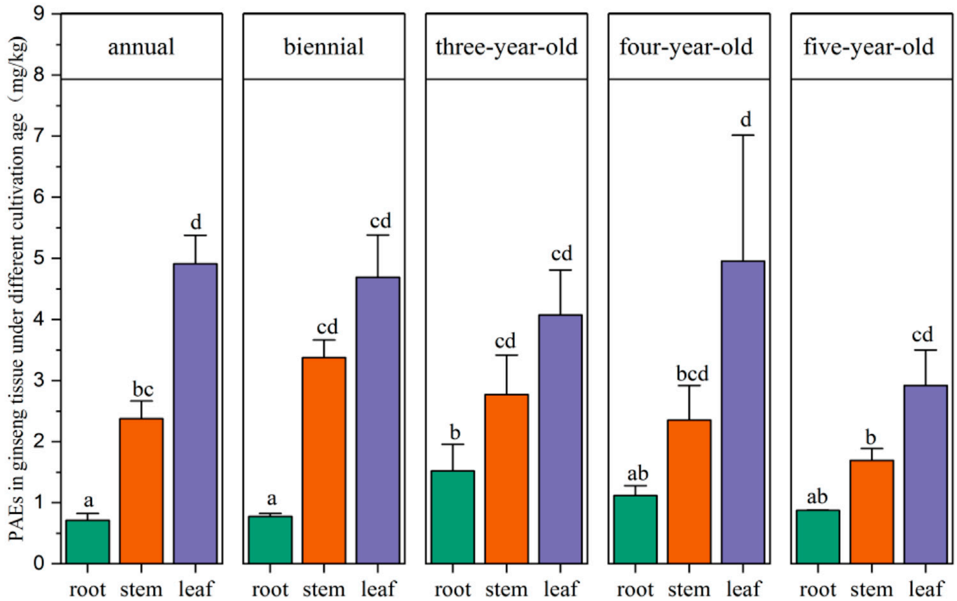

FIGURE 5 | Concentrations of PAEs in ginseng roots, stems, and leaves according to cultivation age (annual, biennial, 3-year-old, 4-year-old, and 5-year-old). Labels containing the same letters indicate no significant difference (p > 0.05). Labels in different letters indicate significant difference (p < 0.05).

0.71–2.10 mg kg^{-1} (dw) roots. **Supplementary Table S11** and **Figure 4B** indicate that the concentrations of DMP, DEP, DPrP, DIBP, DBP, DHXP, and DEHP in ginseng leaves were higher than those in stems and roots. DEEP was only found in individual ginseng roots. A similar distribution of PAEs in celery tissues was reported by Zhao et al. (2022), and the total content of 16 PAEs (DiBP, DBP, DEHP, DEP, DMP, DMEP, BMPP, DPP, DHXP, DEEP, BBP, DCHP, DBEP, DnOP, DNP, and DPhP) was 0.089–1.13 mg kg^{-1} (dw) in celery stems and 0.16–2.73 mg kg^{-1} (dw) in celery leaves. Wang et al. (2014) also reported that stem vegetables (celery and lettuce) and leaf vegetables (spinach, cabbage, and pot herb mustard) accumulated more PAEs than root vegetables (radish). Among other environmental pollutants, brominated flame retardants (BFRs) and organophosphate flame retardants (OPFRs) were reported to be taken up by plants through partitioning to root lipids and through gaseous and particle-bound deposition to the leaves (Zhang et al., 2021). This result shows that discrepancies in plant tissue distribution indicated different absorption pathways of PAEs pollutants.

To research the uptake pathways in different tissues of ginseng, the distribution of the concentration of PAEs in ginseng roots, stems, and leaves at different ages is presented in **Figure 5** and **Supplementary Table S10**. Ginseng roots can bioaccumulate PAEs with the years of cultivation, but PAEs residues in ginseng stems and leaves exhibited higher levels in the first year and second year. This result indicated that the accumulation of PAEs in ginseng aboveground tissue caused by root or soil migration is difficult. Most PAEs are hydrophobic compounds with low translocation factors, and DEHP and its metabolite MEHP were proven to have a poor translocation ability from roots to aboveground tissues with a translocation factor below 1 (Cheng et al., 2020). A similar result was found in stem and leaf vegetables, which accumulated more PAEs (Wang et al., 2015; Li et al., 2020). Therefore, PAEs released by plastic shed film were transferred to soil and leaves through atmospheric deposition, which may be the main exposure route by which aboveground ginseng tissues are contaminated. The PAEs contamination of ginseng roots was mainly from the soil. There was a strong correlation between the type and content of PAEs contaminants and ginseng root growth under the ground. Different ginseng tissues have different pollutant exposure pathways and accumulation behavior, but the mechanisms underlying transport pathways require further investigation. Plastic shed films are still one main source of

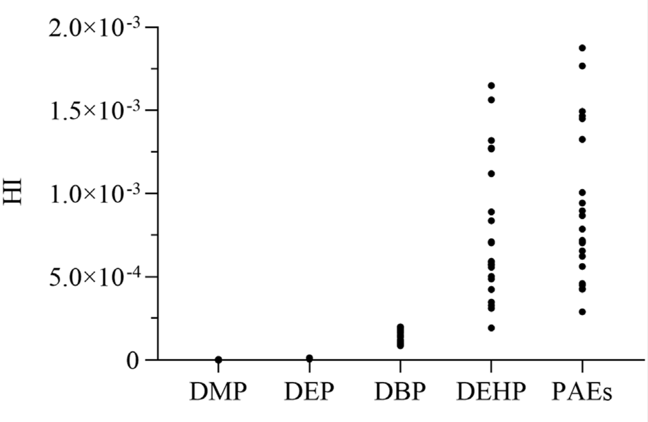

FIGURE 6 | The hazard index (HI) was calculated to assess the noncancer risk posed by the intake of phthalate acid esters (PAEs) in ginseng roots. Black dots represent the HI value of the target PAEs per ginseng sample.

PAEs in cultivation environments. In addition, humans may accumulate PAEs through the food chain. Therefore, it is necessary to assess the health risk of potential hazards from PAEs in edible cultivated ginseng.

Human Health Risk Assessments for Ingested Ginseng

Since 2012, the Ministry of Health has issued "the Announcement on the Approval of Ginseng (Artificially Cultivated) as a New Resource Food" Restrictions on ginseng use will be lifted, and adults can eat no more than 3 g of ginseng per day. According to US EPA 2013 guidelines, DMP, DEP, DBP, and DEHP may result in noncarcinogenic risk through the maximal reference doses as recommended, while DEHP was related to cancer risks. The calculated HIs of the target PAEs from the collected cultivated ginseng samples (n = 23) are shown in **Figure 6**. This result showed that the noncarcinogenic risk of PAEs to consumer health was slight for the ginseng cultivation bases in Jilin Province, and DEHP was the main phthalate posing noncarcinogenic risk. The CR of DEHP through ginseng ingestion ranged from 1.80×10^{-8} to 1.58×10^{-7}, as shown in **Supplementary Table S12**, and values below 1×10^{-6} are negligible for humans. In total, the results of PAEs risk assessments for ginseng indicated that cultivated ginseng from the Jilin Province farmland cultivation bases was acceptable.

CONCLUSION

This study explored the occurrence of PAEs residues in soils, shed films, and ginseng tissues of plants of different ages from the ginseng cultivation bases in Jilin Province. The predominant PAEs were DEHP, DIBP, and DBP, with over 88%, 94%, and 80% of Σ19PAEs in soil, plastic shed film, and ginseng roots, respectively. As for soils, the concentration of DBP (0.83 mg kg^{-1} on average) and DMP (0.05 mg kg^{-1} on average) reveals a serious situation of PAEs pollution in soil from ginseng cultivation bases in Jilin Province. The PAEs concentration in ginseng roots exhibited a closer relationship with those in soil and plastic films, indicating that the widespread use of plastic shed films is possibly the main potential source of PAEs in ginseng cultivation bases. In addition, the accumulation of PAEs in ginseng roots was easily affected by cultivation years. Three-year-old ginsengs had higher PAEs residues than annual and biennial ginsengs ($p < 0.05$). In addition, PAEs were more readily accumulated in ginseng leaves than in roots and stems ($p < 0.05$), which indicated different uptake pathways of PAEs contaminants in different ginseng tissues. Risk assessment showed that the noncarcinogenic risk was mainly due to DEHP. Carcinogenic and noncarcinogenic risks *via* ginseng dietary routes were within acceptable levels. Overall, this work could be useful for understanding the characteristics and distribution of PAEs pollution in ginseng cultivation areas.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Y-SL: investigation, validation, formal analysis, methodology, and writing-original draft. Y-YX: methodology, writing-review, and editing. Y-TZ: formal analysis and methodology. Z-BL: methodology. WL: resources. Y-SS: conceptualization, supervision, and resources.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.917508/full#supplementary-material

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Barriers and Opportunities for Sustainable Farming Practices and Crop Diversification Strategies in Mediterranean Cereal-Based Systems

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Agricultural intensification negatively affects the environment through soil degradation, loss of agrobiodiversity, greenhouse gas emissions, and nutrient leaching. Thus, the introduction of crop diversification strategies and alternative management practices is crucial to re-design agricultural intensification systems. To better understand the contribution of crop diversification to more sustainable agricultural systems, an accurate evaluation of synergies and trade-offs is needed. In this context, the 5-year Horizon 2020 DIVERFARMING project aims to define sustainable, diversified cropping systems with low-input farming practices, adopting a multi-disciplinary approach. The overall objective of this study was to improve the understanding of the stakeholders' perceptions of barriers and opportunities for implementing farming practices and crop diversification strategies in intensive rainfed and irrigated cereal-based cropping systems in Italy. Fifty stakeholders, grouped in farmers and technical agricultural advisors, field technical officers from public agricultural administrations, technical experts from NGOs with experience on farming practices, and researchers in agriculture, were engaged by public consultations to capture their practical knowledge of current farming practices for promoting suitable diversified cropping system, as alternative to agricultural intensification systems. The analysis of the stakeholders' perceptions of barriers and opportunities to the transition of cropping systems towards diversification was done using a multi-criteria decision analysis The most important agro-environmental problem identified by the stakeholders in both the cropping systems was the loss of profitability, associated with the risk of farm abandonment, while minimum tillage, maintenance of vegetation covers, application of organic matter/manure and use of green manure, integrated pest management, and change of rotations were identified as the most adequate and effective practices to be adopted in the case study areas. Crop rotation and legumes were the most adequate diversification strategies selected for the intensive rainfed cereal-based cropping systems, while crop rotations with processing tomato and multiple cropping with short cycle maize and wheat were selected as the most appropriate alternatives for irrigated cereal-based production. Our findings highlight relevant strengths and drawbacks for the implementation of diversified cropping systems under low-input agricultural practices. An important strength is that the crop alternatives selected for the diversification are already cultivated as monocultures and are adapted to the local pedoclimatic conditions, while a major weakness is that few farmers are experts in crop diversification. These results can provide insights to support the planning of agricultural policies at different levels.

Keywords: crop rotation, intercropping, low-input agricultural practices, multi-criteria decision process, multiple cropping, soil challenges, survey, stakeholder perception

1 INTRODUCTION

Agricultural intensification aims at maximising crop productivity in space and time by adopting new technologies and modernisation of production techniques (Pancino et al., 2019). It is based on specialised agri-food production in either crop or livestock systems, associated with low genetic and landscape diversity (Hufnagel et al., 2020). Agricultural intensification is characterised by high use of external inputs, especially energy and agrochemicals that negatively affects the environment (Messéan et al., 2021) through soil degradation, progressive depletion of soil organic matter (SOM), decline of soil quality and agrobiodiversity, greenhouse gas emissions (Kirschenmann, 2010; Bommarco et al., 2013; Wezel et al., 2018), and nutrient losses from agricultural soils that cause water pollution and eutrophication (Garnett et al., 2013; Hunter et al., 2017).

Scientists agree that agricultural sustainability still needs crucial changes to balance an economically viable and socially fair food production with environmental goals (Rockstrom et al., 2017; Rodriguez et al., 2021). This is particularly evident in the Mediterranean Basin, where the highly specialised agricultural systems are mostly oriented on cereal-based intensive cropping systems under rainfed or irrigated conditions as monoculture, or short-rotations such as wheat-summer irrigated crops, or mixed succession with bare fallow (Di Bene et al., 2016), leading to high incidences of pests and diseases, loss of soil fertility and biodiversity among other things.

Therefore, the introduction of appropriate crop diversification strategies and alternative management practices in typical intensive systems is crucial for promoting the redesign of agricultural systems (Kremer and Miles., 2012; Iocola et al., 2020). This transition is an important path to reach the goals of ensuring the availability of resources (e.g., nutrients, water, and land) by increasing the dependence on ecosystem services that minimise the use of external inputs (Bonnet et al., 2021) and promote healthy agroecosystems (Rodriguez et al., 2021).

Enhancing temporal and spatial crop diversity in arable cropping systems can improve crop productivity and resource use efficiency (Tamburini et al., 2020) by delivering multiple ecosystem services (Kremen and Miles, 2012; Beillouin et al., 2019; Iocola et al., 2020) through crop rotations, integration of cover crops as agro-ecological service crops (Diacono et al., 2019), green manure, and species mixtures such as multiple cropping and/or intercropping (Francaviglia et al., 2019, 2020) that can include legumes (Pelzer et al., 2017; Stagnari et al., 2017), leys, grassland, and minor crops of local interest (Hufnagel et al., 2020), with overall socio-economic benefits (Feliciano, 2019; Rosa-Schleich et al., 2019). Coupling agricultural diversification (AD) with more diverse management strategies by adopting cover crops for green manure or fodder, conservation agriculture (i.e., tillage, crop diversification and residue management), organic farming, and fertilisation management, also contributes to increase crop yields, profitability and cropping system resilience in the long-term (Rosa-Schleich et al., 2019; Hufnagel et al., 2020). However, the economic costs in the short-term can offset the environmental and ecological benefits, thus some financial instruments might be needed to increase the adoption of combined AD strategies rather than single crop diversification systems (Rosa-Schleich et al., 2019).

Despite the large scientific consensus on the potential agro-ecological and socio-economic benefits of crop diversification, the agronomic solutions for crop diversification strategies are often hampered and not always affordable by various technical, organisational, and institutional barriers, linked to the overall functioning of the dominant agro-food chains (Kleijn et al., 2019; Iocola et al., 2020). In this context, new crops could be out of market, or they can be affected by technical knowledge gaps and lack of skills for production, especially in the initial implementation phases.

The awareness on the benefits of crop rotations and the costs of machinery or new labour organization are still scarce, while market uncertainty is high. These and other simplification forces affect farmers choices in the use of their agricultural land and entrepreneur resources, indicating a specialisation scenario for several agricultural products in different regions (Mortensen and Smith, 2020). Therefore, research and policy play a key role in supporting more sustainable practices for agri-food production while ensuring environmental improvements (Rodriguez et al., 2021). At EU level, the launch of the Farm to Fork and Biodiversity Strategies within the Green Deal aimed at encouraging a more sustainable and resilient form of food production systems, with a neutral or positive environmental impact (European Commission, 2019; European Commission, 2020a,b). In this context, food legumes and legume-inclusive production systems can play a crucial role by delivering multiple services in accordance with sustainability principles (Stagnari et al., 2017).

To better understand the contribution of AD to more sustainable agricultural systems there is a need to accurately evaluate synergies and trade-offs resulting from the implementation of crop diversification strategies of the elements composing the diversified cropping systems to avoid introducing new problems into the agri-food system (Kremer and Miles., 2012; Iocola et al., 2020).

In this context, the 5-year Horizon 2020 DIVERFARMING project (www.diverfarming.eu) aims to define sustainable, diversified cropping systems with low-input farming practices, adopting a multi-disciplinary approach across Europe. There is a gap on the specific local knowledge of soil and land management to support transitions towards diversified cropping systems by involving local stakeholders and actors of agri-food systems from the beginning of research activities with participatory methods (Bampa et al., 2019).

Thus, to fill this gap, the overall objective of this study was to improve the understanding of the perceptions of different stakeholders on the barriers and opportunities for implementing farming practices and crop diversification strategies in cereal-based cropping systems Italy. At first, this study engaged stakeholders by public consultations to capture their practical knowledge of current farming practices for promoting suitable diversified cropping systems both in irrigated and rainfed areas, as alternatives to the intensive ones.

The analysis of the stakeholders' perceptions of barriers and opportunities to AD transition was done using a multi-criteria decision process (Calatrava et al., 2021). The consultations also aimed to investigate the interest of stakeholders on potential crop associations and alternative low-input farming strategies for decreasing external inputs and minimising agri-environmental and socio-economic problems. The consultations were guided by the following research questions:

1) What are the most important agro-environmental problems and the priorities for action in the case study areas?
2) What are the most adequate farming practices and their effectiveness for each cropping system and case study area?
3) How do stakeholders' perceptions of barriers and opportunities to crop diversification relate to the characteristic cropping systems adopted in the case study areas?

2 MATERIALS AND METHODS

2.1 Agricultural Context and Case Study Areas

In 2019, utilised agricultural area (UAA) in the 27 Countries of the European Union (EU-27) covered 1,629,058.10 km^2, corresponding to 36.8% of the total land area. The UAA is mainly based on arable land (61.4%), permanent grassland (31.2%), and permanent crops (7.4%). In recent decades, European agriculture has specialised on the production of few crop species named majors crops, with the aim to increase the economic efficiency of agri-food systems (Messéan et al., 2021). In the last 10-year, cereal production in Europe covered about 85% of the total production (Eurostat, 2020; https://ec.europa.eu/eurostat/databrowser/view/tag00025/default/table?lang=en).

Within EU member states, Italy is one of the most important in terms of UAA and agri-food products of high quality (Dal Ferro and Borin, 2017). Similarly to EU-27, in Italy more than half of the UAA is occupied by arable land (52.8%), which include both rainfed and irrigated crops. Pastures and meadows cover 28.8% and are quite common in the Northern region (mainly Aosta Valley and Trentino-South Tyrol regions), while permanent crops (e.g., vineyards, olive groves, etc.) cover 18.4%. In Italy, agricultural systems are highly variable, depending on orographic layout, latitude extension from north to south, and heterogeneous pedoclimatic conditions, that influence the development of diversified landscapes with specific local and highly specialised agri-food value chains.

In this context, agricultural areas are dominated by cropping systems mostly oriented on winter and summer cereals, in monocropping or short-rotation with other rainfed or summer irrigated crops such as processing tomato (*Solanum lycopersicum* L.), forage-based systems, or other mixed succession also including bare fallow (Di Bene et al., 2016). In 2019, the cereal-based cropping systems cover an area of 3, 086, 163.00 ha, representing 45.9% of the national UAA. In the last 10-year (2010–2019), the UAA decreased, but the area cultivated with winter cereals such as durum wheat (*Triticum durum* Desf.), winter wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.) increased by 3.4%, 0.9%, and 1.0%, respectively (ISTAT, 2021; http://dati.istat.it/?lang=en&SubSessionId=a0433fd1-878a-4fd3-853d-aeb580487429). This trend showed different features looking at crops' shift among the areas of Northern and Southern Italy. In the North, the reduction of maize (*Zea mais* L.) cultivation (up to 38% in Lombardy) has favoured the increase of wheat cultivation areas. Although maize still represents the first national cereal crop in terms of production and level of yield per hectare, the sector has progressively lost competitiveness due to a series of converging critical issues: the drop in prices, the high fixed costs, and the increased risk from pathogens to which these crops are exposed which also affects the variable component of costs. Conversely, in the South there has been an increase of about 6% of the agricultural land cultivated with cereals, mainly due to the expansion of durum wheat cultivation, which can be traced back to the increase in prices due to the scarcity of supply compared to demand, both nationally and globally (ISTAT, 2021; http://dati.istat.it/?lang=en&SubSessionId=a0433fd1-878a-4fd3-853d-aeb580487429).

Therefore, considering the above-mentioned context, the DIVERFARMING case studies were selected to represent the most widespread irrigated and rainfed cereal-based cropping systems in both the Northern and Southern Italian areas (**Figure 1**). The cropping system investigated as common farming baseline in both study areas are generally specialised in rainfed and irrigated cereal-based cropping systems for food production, adopting a 2-year cash crops rotation, based on processing tomato followed by cereals.

FIGURE 1 | Selected case study areas: Po Valley in Northern Italy (green colour) and Capitanata Plain in Southern Italy (orange colour).

In the Northern part such as the Po Valley, the primary sector is mainly characterised by well-structured professional farms, including a variety of specialised intensive production systems such as arable cereal-based and horticulture cropping systems (no livestock), animal farming systems (all crops used as on-farm livestock feed), mixed farming systems (crops for selling and for on-farm livestock feed), because of the presence of many agri-food companies such as Barilla Group and Casalasco cooperative for industrial tomato production (Pancino et al., 2019). On the other hand, in the Southern areas such as the Capitanata Plain in Apulia Region, the primary sector is less specialised compared to the Northern systems and it is generally oriented to the production of rainfed winter cereals, mainly durum wheat, and irrigated summer horticultural crops, mostly processing tomato (Blasi et al., 2015; Diotallevi et al., 2015; Di Bene et al., 2016; Farina et al., 2017).

2.2 Survey Questionnaire

Five different categories of stakeholders were surveyed: 1) Farmers; 2) private farm advisory services; 3) public agricultural technical officers; 4) agricultural researchers; and 5) experts from non-governmental organizations (NGOs) with experience on farming. Between 20 and 30 stakeholders were intended to be consulted with the following distribution: 1) Farmers and technical agricultural advisors (n = 12–15); 2) field technical officers from public agricultural administrations (n = 3–5); 3) technical experts from NGOs with experience on farming practices (n = 2–5); and 4) researchers in agriculture (n = 3–5). The study did not intend to survey a representative sample of stakeholders but to gather the opinions of selected stakeholders that were experts on Italian cereal cropping systems. They were contacted, not only to answer the survey questionnaire, but also to participate in other participatory assessment activities carried out within the Horizon 2020 DIVERFARMING project. The stakeholders were selected with the involvement of Italian farmers associations and agricultural cooperatives and companies, that proposed potential participating stakeholders based on their knowledge and experience on the corresponding cereal-based cropping systems in the areas of study, and therefore do not constitute a representative sample. A total of 50 stakeholders were finally selected and directly invited to fill in the questionnaire.

Perceptions of relevant stakeholders on the most adequate farming practices and diversification strategies to increase cropping systems sustainability were collected using a common survey questionnaire developed within the Horizon 2020 DIVERFARMING project. For the Italian case studies, the questionnaire was specifically tailored and adapted to the peculiarities of the cropping systems and pedoclimatic conditions of the investigated case study areas based on an explicit literature review, focused on diversified strategies and sustainable farming practices for rainfed and irrigated cereal-based cropping systems (Francaviglia and Di Bene, 2019; Francaviglia et al., 2019, 2020).

The survey questionnaire was implemented online using the Survey Monkey platform and was organised in two parts. The first part was focused on the identification and qualitative assessment of agro-environmental problems, priorities for action, and effectiveness of farming practices. It was composed by four blocks of questions:

1) Stakeholder's general information (e.g., name, gender, type of stakeholder, affiliation, etc.);
2) identification and qualitative assessment of the most relevant agro-environmental and socio-economic problems of each cropping system considered in the case study areas (choice made from an open list of options), and assessment of the priority for possible actions and measures that could be selected to address the previously identified problems (choice made from an open list of options);
3) identification of farming practices considered by the stakeholders most appropriate for the implementation in the specific cropping system and the considered study areas (choice made from a list of options with open options for additional potentially implementable farming practices that can be proposed by the stakeholders). Moreover, for those practices not considered suitable, the stakeholders were asked to indicate the main reasons for not selecting them;
4) qualitative assessment of the effectiveness of the farming practices to face the agro-environmental and socio-economic problems previously identified by the stakeholders.

The second part of the survey was focused on the identification of the best crop diversification strategies for both the rainfed and irrigated cereal-based cropping systems in the Po Valley and Capitanata Plain case study areas. More precisely, the surveyed stakeholders were asked to identify from a list of options which type of diversification could be the most appropriate to be adopted in the specific cropping system and case study area. The list of crop diversification options followed the results of the literature review as reported in Francaviglia et al. (2019, 2020). Three major crop diversification options were proposed as follows:

1) Intercropping: complementary crops from different families with different nutritional requirements that are grown simultaneously on the same field within a single growing season. The practice of growing two or more crops together at the same time in a beneficial manner aimed at increasing land productivity, crop quality, and ecosystem services. Row intercropping refers to structured arrangements of different species planted in alternate rows. Strip intercropping is a more industrialised version with rows of individual crops wide enough to be harvested with machinery. Mixed intercropping means randomly arranged plants of different species bunched together with no separation in rows or strips. Relay intercropping indicates the interplanting of two or more crop species before the main crop reaches maturity and they simultaneously grow during part of the crop cycle.
2) crop rotation: complementary crops from different families with different nutritional requirements and different crop cycles that are grown on the same field in sequence for a period of two or more years (alternating crops in different years);
3) multiple cropping: complementary crops from different families with different nutritional requirements and different crop cycles that are grown on the same field in seasonally succession (a second crop is planted after the first crop has reached maturity, within the same year) to preserve the productive capacity of the soil.

After the selection of the preferred type of crop diversification, the stakeholders were asked to identify two crops from an open list of several combinations that they considered most adequate for the diversification of the cropping system in the Po Valley and Capitanata Plain in Foggia Province case study areas. Anyhow, they could also indicate alternative diversification crops if those proposed in the list were not exhaustive.

2.3 Data Analysis

2.3.1 Statistical Analysis

The assessment of the severity of the agro-environmental problems, the priority for action, and the effectiveness of farming practices were measured using a six-level categorical ordered scale ranging from "Very low/null" to "Very high." The use of this scale was justified by the non-existence of possible neutral positions in the assessment of the aforementioned aspects as well as by the need to prevent the consulted stakeholders without an opinion from using the middle point as a "save-the-face response" instead of using the "do not know/do not answer" option (Sturgis et al., 2012). Then, the qualitative answers were converted to numerical correlated values, representing the correspondent quantitative assessment using a 0 to 5 numerical scale, referred to "Very low/null" and "Very high," respectively.

Stakeholders' answers related to the agro-environmental problems and the priority for action were statistically analysed using STATA/SE 15 software (Software for statistics and data science; https://www.stata.com/). Generally, a univariate descriptive analysis of stakeholders' answers was presented. In detail, when variables measured the proportion of stakeholders selecting a given answer, only the number of stakeholders or the proportion of answers per each stakeholder category was shown. Conversely, when variables were continuous, both average and standard deviation values were shown. Finally, for the severity of agro-environmental problems and the assessment of the priority for action due to the categorical nature of the variables, the median was also reported. In the analysis of stakeholders' choice related to the diversification alternatives, the answers were discriminated per type of stakeholder. The Fisher's exact probability test was used to analyse the statistical significance of the differences in the choice of the diversification alternatives among the types of stakeholders. This non-parametric statistical test that exactly measured the association between two categorical values is commonly used as a substitute of Chi-Square test for small samples.

2.3.2 Multi-Criteria Assessment

The analysis of the stakeholders' responses regarding the assessment of the effectiveness of farming practices was done by establishing a ranking of their effectiveness using multi-criteria decision analysis (MCDA).

The use of MCDA requires converting the stakeholder's qualitative assessment into a quantitative assessment for its numerical treatment. The conversion was done using standardised ordinal linguistic labels that have a numerical correlation to collect stakeholder's assessments in the questionnaire. More detail is reported in Calatrava et al. (2021).

The main purpose of MCDA is to evaluate and choose alternatives based on multi-criteria using systematic analyses that overcome the limitations of unstructured decision problems. Several methodologies have been developed to rank alternatives regarding different types of information (Bampa et al., 2019). Multi-criteria alternative selection systems are defined by the following elements (Munda et al., 1993):

$$\{C, D, r, I, \prec\}$$

Where: $C = \{C1, ..., Cj, \ldots Cm\}$ are the m criteria used to compare alternatives. In this analysis, the criteria (C) considered are the preferences of the decision makers, i.e., the stakeholders answering the survey.$D = \{D1, \ldots, Di, ..., Dn\}$ are the n alternatives considered as feasible in the survey (farming practices) that the decision-maker must assess qualitatively. Unlike more complex decision systems, C and D are finite sets that allow avoiding problems of convergence, integrability and measurability.r: $D \times C \rightarrow r$ is a function that generates a matrix in which each term corresponds to a reality associated with each alternative Di and criterion Cj:

$$(Di, Cj) \rightarrow r(Di, Cj) = rij$$

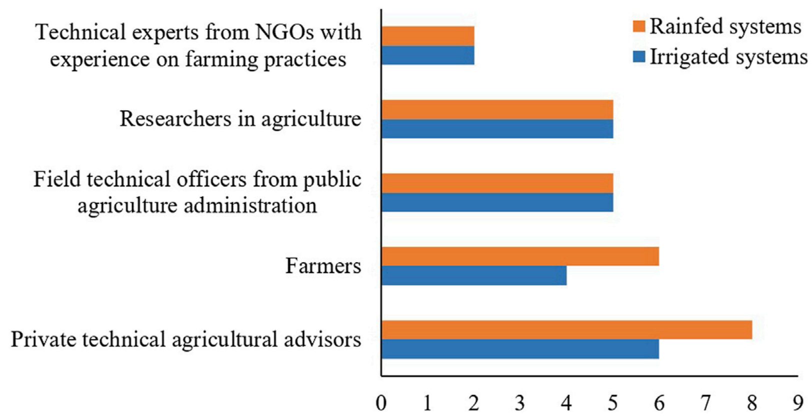

FIGURE 2 | Number of respondents grouped per type of stakeholders for both rainfed and irrigated cereal-based cropping systems.

I: is the set of linguistic labels used by the decision makers to assess each alternative (*Di*) for each criterion (*Cj*).$\prec$: are the preferences of the decision-makers with respect to the different alternatives (*D*) considered in the decision.

The effectiveness of each alternative (*Di*) was assessed for each criterion, i.e., each decision maker (*Cj*) qualitatively evaluated each alternative using linguistic terms (*I*) to express the effectiveness (*rij* values). The use of linguistic labels allows the decision maker to express his/her perception of the goodness of each alternative. The *rij* values obtained configure the decision-making matrix that represent the preferences ($\prec$) of the decision-makers with respect to the different alternatives. It is assumed that the decision-maker is rational, in the sense of acting coherently with his/her preferences and objectives and his/her previous knowledge, expressing them through these linguistic labels. More precisely, as previously commented, the importance of the criteria was obtained through direct assignment using a valuation scale with six levels (labels) that ranges between "Very low/null" and "Very high." The question template used was "According to your criterion, the effectiveness of practice Y for addressing agro-environmental problems in rainfed/irrigated cereal production is Very low, Low, Medium low, Medium high, High, and Very high." These standardised ordinal linguistic labels used to collect the stakeholder's qualitative assessments were converted to numerical correlated labels using a 0 to 5 numerical scale, which represent the corresponding quantitative assessment from "Very low/null" to "Very high," respectively.

In this study, the definition of the MCDA was based on a group of agricultural practices representing the alternatives to be selected, the stakeholders' point of view that are based on their prior knowledge and preferences (criteria), and the corresponding assessment of the effectiveness of each alternative. The selection and assessment of alternatives was made in independent decision processes for each cropping system and stakeholder. The aggregation of the different types of stakeholders allowed us to obtain a single result of the priority ranking of farming practices for each cropping system (group decision). In this case, each type of stakeholder was equally weighted in the group's decision. The mathematical calculation to obtain the preferences ranking of farming practices was carried out using the Order Preference Technique for Similarity with the Ideal Solution (TOPSIS) methodology developed by Hwang and Yoon (1981), Zeleny (1982) and Lai et al. (1994). The TOPSIS methodology is based on the calculation of the geometric distance to the ideal solution. The ranking of alternatives is built by prioritizing the alternatives that are closer to the positive ideal solution (PIS) and further from the negative ideal solution (NIS). A relative closeness index, ranging from 0 to 1, is calculated for each alternative as a combination of both the distance to the PIS and to the NIS (Shih et al., 2007). The greater the index the more effective the alternative is. A priority ranking of alternatives (i.e., farming practices) is established based on these relative closeness indices. Those practices with the highest ranking were selected as the most effective ones.

3 RESULTS AND DISCUSSION

3.1 Main Characteristics of Surveyed Stakeholders

A total of forty-eight anonymised stakeholders participated in the survey process for both the rainfed and irrigated cereal-based cropping systems, covering all stakeholder groups (**Figure 2**). Among them, twenty-six (54.17%) were consulted as experts of the rainfed cereal-based cropping systems (average age of stakeholders was 45.15 with ±10.81 as standard deviation), while twenty-two (45.83%) were consulted as experts of the irrigated cereal-based cropping systems (average age of stakeholders was 47.13 with ±10.34 as standard deviation). For both the rainfed and irrigated cereal-based cropping systems, the largest group was represented by private technical agricultural advisors, which covered 30.77% and 27.27% of the total stakeholders involved in the survey process of the rainfed and irrigated cereal-based cropping systems, respectively. Conversely, the technical experts from NGOs with experience on farming practices represented the smallest group, covering 7.69% and

9.09% of the total stakeholders involved in the survey process of the rainfed and irrigated cereal-based cropping systems, respectively.

3.2 Assessment of Agro-Environmental Problems and Priorities for Action

The surveyed stakeholders were asked to qualitatively assess and rating the severity of several agro-environmental problems for both rainfed and irrigated cereal-based cropping systems. The stakeholders' subjective answers on perception of the severity of the agro-environmental problems were converted to a 0 to 5 scale and the main statistics (i.e., average, median, and standard deviation values) are shown in **Tables 1**, **2**, for the rainfed and irrigated cereal-based cropping systems, respectively.

Generally, the stakeholders' perception of the severity of the agro-environmental problems was very similar in both the rainfed and irrigated cereal-based cropping systems and was consistent with the type of cropping systems analysed. The three agro-environmental problems perceived by the stakeholders as the most severe in both the rainfed and irrigated cereal-based cropping systems were loss of profitability and associated risk of farm abandonment, that is clearly linked to a socio-economic concern, followed by loss of SOM content, and soil degradation by erosion. In the case study areas, the decrease of SOM content is mainly caused by the high mineralisation rate due to conventional tillage highly used for seedbed preparation and for weed control. Moreover, other agro-environmental problems assessed as important were the excessive use of plant protection products and use of fertilisers, which also entail high costs for farmers, landscape degradation, and loss of biodiversity (**Tables 1**, **2**). Conversely, soil pollution and waterlogging soil were barely assessed as a serious problem for both the cereal-based cropping systems in the Po Valley and Capitanata Plain case study areas.

After assessing the severity of the agro-environmental problems, the stakeholders were asked to qualitatively evaluate the priority for action to be considered for tackling such problems. The answers related to the qualitative assessment were also converted to a 0 to 5 scale. The average, median, and standard deviation values of such actions were reported in **Tables 3**, **4**, for the rainfed and irrigated cereal-based cropping systems, respectively.

Generally, the stakeholders' qualitative assessment was consistent with the relevant agro-environmental problems as identified in **Tables 1**, **2**. In both the Po Valley and Capitanata Plain case study areas, the surveyed stakeholders assigned the highest priority for action to the increase of the farm profitability for both the rainfed (a mean value of 4.08; **Table 3**) and irrigated cereal-based cropping systems (a mean value of 4.00; **Table 4**). For the rainfed cereal-based cropping systems, other important priorities identified by the stakeholders were represented by the improvement of soil conditions (e.g., increase soil biodiversity and fertility, improve soil structure, and reduce soil erosion) and the reduction of energy consumption aiming at decreasing the costs for farmers (a mean value ranging from 3.77 to 3.68). Conversely, for the irrigated cereal-based cropping systems, the second priority identified by the stakeholders was again the reduction of energy consumption (a mean value of 3.81), followed by the improvement of soil conditions (e.g., increase soil biodiversity and fertility, improve soil structure, and reduce soil erosion) and the modernization of agriculture (a mean value ranging from 3.77 to 3.59, respectively) that improve farmer's incomes. Interestingly, for both rainfed and irrigated cereal-based the cropping systems, the actions related to the recovery of traditional crops, reduction of flooding in fields, and increase of crop yields were assigned the lowest priority by the stakeholders with a mean value of 2.89 for both the cropping systems

TABLE 1 | Stakeholders' qualitative subjective assessment and rating of the severity of agro-environmental problems in rainfed cereal-based cropping systems, measured on a 0 to 5 scale (i.e., 0 = Very low/null, 5 = Very high), listed in a decreasing order.

Problem assessed	Rainfed cereals		
	Average	Median	Standard deviation
Loss of profitability / farm abandonment	4.08	5.00	1.35
Loss of soil organic matter (SOM)	3.73	4.00	1.08
Soil degradation by erosion	3.50	4.00	1.27
Excessive use of plant protection products	3.40	4.00	1.19
Excessive use of fertilisers	3.31	3.00	1.12
Landscape degradation	3.31	3.00	1.26
Loss of biodiversity	3.19	3.00	1.36
Excessive use of machinery	2.77	3.00	1.48
Water pollution	2.64	3.00	1.11
Soil pollution	2.50	3.00	1.06
Waterlogged soils	2.08	2.00	1.20
Excessive use of irrigation water	–	–	–

TABLE 2 | Stakeholders' qualitative subjective assessment and rating of the severity of agro-environmental problems in irrigated cereal-based cropping systems, measured on a 0 to 5 scale (i.e., 0 = Very low/null, 5 = Very high), listed in a decreasing order.

Problem assessed	Irrigated cereals		
	Average	Median	Standard deviation
Loss of profitability / farm abandonment	4.09	4.50	1.27
Loss of soil organic matter (SOM)	3.77	4.00	1.07
Soil degradation by erosion	3.64	4.00	1.26
Excessive use of plant protection products	3.43	4.00	1.25
Excessive use of fertilisers	3.23	3.00	1.19
Landscape degradation	3.09	3.00	1.23
Loss of biodiversity	3.09	3.00	1.41
Excessive use of irrigation water	3.05	3.00	1.33
Water pollution	2.76	3.00	1.00
Excessive use of machinery	2.64	3.00	1.56
Soil pollution	2.60	3.00	1.10
Waterlogged soils	2.14	2.00	1.21

TABLE 3 | Stakeholders' qualitative subjective assessment of the priority for action in rainfed cereal-based cropping systems, measured on a 0 to 5 scale (i.e., 0 = Very low/null, 5 = Very high) and listed in a decreasing order.

Actions	Rainfed cereals		
	Average	Median	Standard Deviation
Increase farm profitability	4.08	4.00	1.15
Increase biodiversity	3.77	4.00	1.34
Increase soil fertility	3.73	4.00	1.19
Improve soil structure	3.73	4.00	1.19
Reduce energy consumption	3.68	4.00	1.22
Reduce soil erosion	3.60	4.00	1.26
Modernisation of agriculture	3.54	3.50	1.10
Increase carbon sequestration in soil and arboreal biomass	3.27	3.00	1.54
Conserve traditional landscapes	3.25	3.50	1.29
Recover traditional crops	3.08	3.00	1.38
Reduce flooding in fields	2.96	3.00	1.62
Increase crop yields	2.64	3.00	1.35

TABLE 4 | Stakeholders' qualitative subjective assessment of the priority for action in irrigated cereal-based cropping systems, measured on a 0 to 5 scale (i.e., 0 = Very low/null, 5 = Very high) and listed in a decreasing order.

Actions	Irrigated cereals		
	Average	Median	Standard Deviation
Increase farm profitability	4.00	4.00	1.18
Reduce energy consumption	3.81	4.00	1.08
Increase soil fertility	3.77	4.00	1.19
Improve soil structure	3.73	4.00	1.16
Increase biodiversity	3.68	4.00	1.43
Modernisation of agriculture	3.59	3.50	1.14
Reduce soil erosion	3.57	4.00	1.33
Increase carbon sequestration in soil and arboreal biomass	3.41	3.50	1.56
Conserve traditional landscapes	3.10	3.50	1.29
Reduce flooding in fields	2.95	3.00	1.56
Recover traditional crops	2.90	3.00	1.37
Increase crop yields	2.81	3.00	1.29

(**Tables 3**, **4**). It is well-known that the choice of a farming practice depends on markets, pedo-climatic conditions, crop rotation aspects, availability of genetic varieties, governmental subsidies, and farmer's preferences. These agronomic practices can have positive or negative effects on soil quality and the wider environment, depending on crop type, rotation, management, and agro-environmental conditions. Choices made on these factors can influence profitability as well as sustainability of crop production systems (Reckling et al., 2016).

3.3 Identification of Adequate Farming Practices and Assessment of Their Effectiveness

From a list of potentially implementable farming practices (i.e., tillage, soil cover, erosion control, fertilisation, plant protection, and farm design), the surveyed stakeholders were asked to identify the most appropriate practices to be implemented in both the rainfed and irrigated cereal-based cropping systems, considering the characteristics of the Po Valley and Capitanata Plain case study areas. The survey also asked to qualitatively assess the effectiveness of those farming practices identified suitable by the stakeholders to face the agro-environmental problems for both the rainfed and irrigated cereal-based cropping system in the case study areas. Conversely, for those practices not considered adequate for both the rainfed and irrigated cropping systems, the stakeholders selected the most important reasons. **Tables 5**, **6** show the percentage of the stakeholders that identified each farming practice as adequate for both the rainfed and irrigated cereal-based cropping systems, respectively. **Supplementary Tables S1, S2** show the rating of preferences for the farming practices resulting from the TOPSIS MCDA relative closeness index. The closeness index ranged from 0 to 1. A higher ranking means that the corresponding farming practice is considered more effective by the surveyed stakeholders. The results from the MCDA allowed us to select the most effective farming practices alternatives. As expected, the ranking of the farming practices provided by the stakeholders (**Supplementary Tables S1, S2**) was similar to the results shown in **Tables 5**, **6** because the most selected adequate farming practices were also the most effective.

Regarding tillage, most stakeholders choose minimum tillage as the most effective alternative farming practice to address the most important agro-environmental problems for both the rainfed and irrigated cropping systems (TOPSIS MCDA ranking score equal to 0.61 and 0.54, respectively; **Supplementary Tables S1, S2**) compared to tillage without heavy implements and no tillage options. Particularly, the percentage of stakeholders that consider this practice as the most adequate and effective for the cereal-based production in the study areas was higher for the irrigated system compared to the rainfed cereal-based production (86.36% vs. 65.38%, respectively). Conversely, a very small number of stakeholders considered conservation tillage with grazing an adequate farming practice for both the rainfed (19.23%) and irrigated cropping systems (13.64%).

Soil cover refers to the fraction of the land covered by crops. This is important for preventing loss of nutrients and pesticides by runoff and reducing the risk of soil erosion, especially during the winter season. In this context, most of the stakeholders identified the maintenance of vegetation covers the most effective and selected practice for both the rainfed and irrigated cereal-based cropping systems. As shown in **Tables 5**, **6**, the percentage of stakeholders that considered the maintenance of vegetation covers as adequate was greater for the irrigated cereal-based cropping systems compared to the rainfed production (72.73% vs. 65.38%, respectively). Nevertheless, the TOPSIS MCDA ranking score for the effectiveness of these practices in the case study areas was similar for both the rainfed and irrigated cropping systems (0.55 and 0.53, respectively; **Supplementary Tables S1, S2**). Among the soil cover, the maintenance of vegetation strips between crop lines was the less selected practice by the stakeholders for the rainfed and

TABLE 5 | Identification of adequate farming practices for rainfed cereal-based cropping systems, listed in a decreasing order. For each practice, values refer to the percentage of stakeholders that identified it suitable.

Farming practice in rainfed systems	Percentage (%)
Tillage	
Minimum tillage	65.38
Tillage without heavy implements	42.31
No-tillage with mechanical weed control (brush cutter)	34.62
No-tillage with chemical weed control	19.23
Conservation tillage with grazing	19.23
Tillage following contour lines	15.38
Soil cover	
Maintain vegetation covers (natural or cover crops)	65.38
Mulching (with crushed pruning offcuts, reeds, etc.)	46.15
Maintain strips of vegetation between crop lines	38.46
Erosion control	
Maintain the natural vegetation on the edges of the farm plots	46.15
Installing hedges on the edges of the plots	46.15
Construction of erosion barriers or margins without vegetation	19.23
Construction of erosion barriers or margins with vegetation	19.23
Fertilisation	
Contribution of organic matter/manure	88.46
Use of green manure	88.46
Precision agriculture to optimise fertilisation (variable rate)	53.85
Combination of mineral and organic fertilisers	50.00
Use of biostimulants and biofertilisers	34.62
Plant protection	
Integrated pest control	61.54
Farm design	
Changing crop rotations	76.92

TABLE 6 | Identification of adequate farming practices for irrigated cereal-based cropping system, listed in a decreasing order. For each practice, values refer to the percentage of stakeholders that identified it suitable.

Farming practice in irrigated systems	Percentage (%)
Tillage	
Minimum tillage	86.36
Tillage without heavy implements	59.09
No-tillage with chemical weed control	27.27
No-tillage with mechanical weed control (brush cutter)	27.27
Tillage following contour lines	18.18
Conservation tillage with grazing	13.64
Soil cover	
Maintain vegetation covers (natural or cover crops)	72.73
Mulching (with crushed pruning offcuts, reeds, etc.)	45.45
Maintain strips of vegetation between crop lines	18.18
Erosion control	
Maintain the natural vegetation on the edges of the farm plots	40.91
Installing hedges on the edges of the plots	36.36
Construction of erosion barriers or margins with vegetation	27.27
Construction of erosion barriers or margins without vegetation	13.64
Fertilisation	
Contribution of organic matter/manure	81.82
Use of green manure	72.73
Precision agriculture to optimise fertilisation (variable rate)	59.09
Combination of mineral and organic fertilisers	54.55
Use of biostimulants and biofertilisers	45.45
Plant protection	
Integrated pest control	68.18
Farm design	
Changing crop rotations	77.27

irrigated cropping systems. Nevertheless, the percentage of stakeholders that considered this practice as adequate was twice in rainfed cereal-based systems compared to the irrigated cereal-based production (38.46% vs. 18.18%, respectively). The rationale is likely to be related to the fact that cover crops are more used and better known by farmers compared to vegetation strips practice.

Regarding erosion control, all the alternatives were not considered a priority for the Po Valley and Capitanata Plain case study areas due to the limited effectiveness for the pedoclimatic conditions. However, other stakeholders identified the maintenance of natural vegetation on the edges of farm plots and the installation of hedges as the most important options to limit water erosion by runoff for both the rainfed and irrigated cereal-based systems (46.15% and 40.91%, respectively) in the Po Valley and Capitanata Plain case study areas (**Tables 5, 6**). Although in the rainfed cereal-based systems, the two farming practices were considered adequate by the same percentage of stakeholders (46.15%), the TOPSIS MCDA ranking score identified the installation of hedges the most effective alternative for the case study areas, while the maintenance of natural vegetation on the edges of farm plots was considered the second practice most effective (0.40 vs. 0.25; **Supplementary Table S1**). Conversely, in the irrigated cereal-based systems all the alternatives are perceived to have a low effectiveness with a similar TOPSIS MCDA ranking score, ranging from 0.25 to 0.26, for the construction of erosion barriers or margins with vegetation and the three other alternative practices, respectively (**Supplementary Table S2**).

For the fertilisation practices, the stakeholders agreed to choose the application of organic matter/manure and the use of green manure as the most adequate practices for both the cropping systems in the case study areas. Such preferences were higher in the rainfed cereal-based cropping systems compared to the irrigated production (a mean value of 88.46% vs. 77.28%, respectively). Moreover, the adoption of precision agricultural to optimise fertilisation using variable rate and the combined use of mineral and organic fertilisers were considered adequate farming practices by more than half of the stakeholders in both the rainfed and irrigated cereal-based cropping systems (a mean value of 51.93% and 56.82%, respectively). Similarly to the erosion control, in the rainfed cereal-based systems the contribution of organic matter/manure was considered the most effective practice, while the green manure practice was considered the second most effective practice (0.78 vs. 0.68; **Supplementary Table S1**). In the irrigated cereal-based systems, the use of precision agriculture techniques was identified the most effective fertilisation alternative (TOPSIS MCDA ranking score 0.76) compared to the other alternatives (**Supplementary Table S2**) such as the contribution of organic matter/manure (TOPSIS MCDA ranking score 0.62) and the use of green manure (TOPSIS MCDA ranking score 0.56), which were the two alternative farming practices considered most adequate by the stakeholders (**Table 6**). For both the rainfed and irrigated cereal-based cropping systems, the use of biostimulants and biofertilisers was identified as the least effective fertilisation alternative (TOSPIS MCDA ranking score 0.19 and 0.30, respectively; **Supplementary Tables S1, S2**).

TABLE 7 | Reasons given by the stakeholders for not selecting a farming practice as adequate for rainfed cereal-based cropping systems, expressed as percentage of responses, listed in a decreasing order.

Farming practice	Reason*									No. of responses
	A	B	C	D	E	F	G	H	I	
Tillage										
Tillage following contour lines	4.5	50.0	27.3	9.1	4.5	–	–	–	4.5	22
Conservation tillage with grazing	19.0	23.8	14.3	4.8	4.8	19.0	4.8	–	9.5	21
No tillage with chemical weed control	9.5	9.5	14.3	9.5	9.5	28.6	4.8	–	14.3	21
No tillage with mechanical weed control (brush cutter)	11.8	–	11.8	11.8	17.6	5.9	–	29.4	11.8	17
Tillage without heavy implements	20.0	20.0	26.7	–	6.7	–	6.7	–	20.0	15
Minimum tillage	22.2	11.1	–	11.1	33.3	11.1	–	–	11.1	9
Soil cover										
Maintain strips of vegetation between crop lines	12.5	18.8	31.3	12.5	12.5	–	6.3		6.3	16
Mulching (with crushed pruning offcuts, reeds, etc.)	7.1	14.3	35.7	14.3	14.3	–	7.1		7.1	14
Maintain vegetation covers (natural or cover crops)	11.1	11.1	33.3	22.2	11.1	–	–		11.1	9
Erosion control										
Construction of erosion barriers or margins without vegetation	23.8	33.3	9.5	4.8	9.5	–	–	9.5	9.5	21
Construction of erosion barriers or margins with vegetation	14.3	38.1	14.3	4.8	4.8	–	4.8	9.5	9.5	21
Maintain the natural vegetation on the edges of the plots	28.6	28.6	7.1	21.4	–	–	–	7.1	7.1	14
Installing hedges on the edges of the plots	14.3	35.7	14.3	7.1	–	–	7.1	21.4	–	14
Fertilisation										
Use of biostimulants and biofertilisers	11.8	–	11.8	11.8	17.6	–	5.9	11.8	29.4	17
Combination of mineral and organic fertilisers	–	15.4	15.4	15.4	7.7	15.4	7.7	15.4	7.7	13
Precision agriculture to optimise fertilisation (variable rate)	16.7	16.7	8.3	–	25.0	–	–	16.7	16.7	12
Contribution of organic matter/manure	–	–	33.3	–	–	33.3	–	33.3	–	3
Use of green manure	–	–	33.3	33.3	–	–	–	–	33.3	3
Plant protection										
Integrated pest control	10.0	10.0	10.0	20.0	10.0	20.0	10.0	–	10.0	10
Farm design										
Changing crop rotations	33.3	–	33.3	33.3	–	–	–	–	–	6

**A-I reasons refer to: Limited effectiveness (A), practice is not adequate for the characteristics of the area (B), it is not a traditional practice in the area (C), it is complex/difficult to implement without technical advice (D), it is complex/difficult to carry out even with technical advice (E), this practice is not compatible with other farming practices (F), this practice requires a high investment cost (G), the cost of carrying out this practice is high (H), the benefits of this practice do not outweigh its costs (I).*

Integrated pest management is a crucial EU agro-climate-environmental measure included in the second pillar of common agricultural policy (CAP) under the rural development programs. Therefore, more than 60% of the stakeholders considered the use of integrated pest control an adequate plant protection practice for both the rainfed and irrigated cereal-based systems in the case study areas (**Tables 5**, **6**). Nevertheless, about 40% of the stakeholders did not identify this practice as a priority because of the complexity of its adoption linked to the higher cost of implementation and technical advice. As expected, the TOPSIS MCDA ranking score for the effectiveness of this practice in the case study areas was higher in the irrigated cereal-based systems compared to rainfed cropping systems (0.55 vs. 0.49, respectively; **Supplementary Tables S1, S2**).

Most stakeholders agreed to identify the change of current crop rotations an efficient farm design strategy to improve the sustainability of both the rainfed and irrigated cereal-based cropping systems (76.92% and 77.27%, respectively) by increasing crop diversification and plant protection and reducing the use of fertilisers and pesticides (**Tables 5**, **6**). The TOPSIS MCDA ranking score for the effectiveness of this practice in the case study areas was higher in the rainfed cereal-based systems compared to irrigated cropping systems (0.62 vs. 0.55, respectively; **Supplementary Tables S1, S2**).

The results of the most important reasons given by the stakeholders for those farming practices not considered adequate for both the rainfed and irrigated cropping systems in the Po Valley and Capitanata Plain case study areas, are presented in **Tables 7**, **8**. Although some relevant similarities among farming practices and reasons exist, the main reasons for not choosing a specific farming practice generally varied between the rainfed and irrigated cereal-based cropping systems.

Regarding tillage practices, most stakeholders identified conservation tillage with grazing, tillage following contour lines, and no tillage with chemical or mechanical (brush cutter) weed control as not much adequate for both the rainfed and irrigated cereal-based cropping systems. The most frequent reasons identified by the stakeholders for not choosing the different tillage options in both the cropping systems and case study areas were mainly related to their inadequacy for the characteristics of the area (i.e., conservation tillage with grazing and tillage following contour lines), the limited compatibility with other farming practices (no tillage with chemical weed control) and the high cost of carrying out the practice (no tillage with mechanical weed control). In detail, the relatively flat topography of the case study areas might explain the low number of stakeholders that considered tillage following contour lines as an adequate alternative. Moreover, the inadequacy for the characteristics of the area linked to soil texture that could affect water retention and availability for crops, especially in rainfed cereal-based systems (Francaviglia et al., 2020). Other important reasons considered by the stakeholders for

TABLE 8 | Reasons given by the stakeholders for not selecting a farming practice as adequate for irrigated cereal-based cropping systems, expressed as percentage of responses, listed in a decreasing order.

Farming practice	Reason*									No. of responses
	A	B	C	D	E	F	G	H	I	
Tillage										
Conservation tillage with grazing	5.3	42.1	15.8	15.8	–	15.8	–	–	5.3	19
Tillage following contour lines	16.7	44.4	27.8	5.6	–	5.6	–	–	–	18
No tillage with chemical weed control	18.8	12.5	18.8	12.5	12.5	18.8	–	–	6.3	16
No tillage with mechanical weed control (brush cutter)	6.3	18.8	12.5	18.8	12.5	6.3	–	18.8	6.3	16
Minimum tillage	10.0	30.0	20.0	10.0	–	10.0	–	–	20.0	10
Tillage without heavy implements	44.4	–	33.3	–	–	11.1	11.1	–	–	9
Soil cover										
Maintain strips of vegetation between crop lines	16.7	33.3	22.2	5.6	5.6	5.6	–	–	11.1	18
Mulching (with crushed pruning offcuts, reeds, etc.)	16.7	16.7	33.3	8.3	8.3	–	–	–	16.7	12
Maintain vegetation covers (natural or cover crops)	33.3	33.3	16.7	–	16.7	–	–	–	–	6
Erosion control										
Construction of erosion barriers or margins without vegetation	21.1	36.8	15.8	10.5	–	5.3	5.3	5.3	–	19
Construction of erosion barriers or margins with vegetation	25.0	43.8	12.5	6.3	–	–	6.3	6.3	–	16
Installing hedges on the edges of the plots	7.1	28.6	21.4	7.1	–	–	7.1	7.1	21.4	14
Maintain the natural vegetation on the edges of the plots	30.8	23.1	23.1	–	–	7.7	7.7	–	7.7	13
Fertilisation										
Use of biostimulants and biofertilisers	16.7	16.7	–	8.3	8.3	8.3	–	25.0	16.7	12
Combination of mineral and organic fertilisers	10.0	20.0	10.0	20.0	10.0	30.0	–	–	–	10
Precision agriculture to optimise fertilisation (variable rate)	11.1	22.2	11.1	–	33.3	–	–	22.2	–	9
Use of green manure	–	50.0	16.7	33.3	–	–	–	–	–	6
Contribution of organic matter/manure	25.0	25.0	–	25.0	25.0	–	–	–	–	4
Plant protection										
Integrated pest control	14.3	42.9	–	–	–	14.3	–	28.6	–	7
Farm design										
Changing crop rotations	20.0	20.0	40.0	–	–	20.0	–	–	–	5

**A-I reasons refer to: Limited effectiveness (A), practice is not adequate for the characteristics of the area (B), it is not a traditional practice in the area (C), it is complex/difficult to implement without technical advice (D), it is complex/difficult to carry out even with technical advice (E),this practice is not compatible with other farming practices (F), this practice requires a high investment cost (G), the cost of carrying out this practice is high (H), the benefits of this practice do not outweigh its costs (I).*

not selecting these farming practices as adequate for both the rainfed and irrigated cereal-based cropping systems were the limited effectiveness, not being a traditional practice in the area, and the complexity/difficulty to implement the practice without technical advice (**Tables 7**, **8**). In the case of minimum tillage, which was the most chosen alternative tillage practice for both the rainfed and irrigated cereal-based cropping systems, the stakeholders identified the limited effectiveness and the inadequacy of the practice for the characteristics of the area as the most frequent reasons for not adopting this practice in the rainfed and irrigated systems, respectively.

Regarding soil cover, the stakeholders identified the maintenance of vegetation strips between crop lines and mulching as not very adequate for both the rainfed and irrigated cereal-based cropping systems. The most frequent reasons reported by the stakeholders for not choosing these practices in both the cropping systems and case study areas were related to their inadequacy for the characteristics of the area and to not being a traditional practice in the area (**Tables 7**, **8**). For the maintenance of vegetation cover, which was the most chosen alternative practice for both the rainfed and irrigated cereal-based cropping systems, the stakeholders identified not being a traditional practice in the area, its limited effectiveness, and its inadequacy for the characteristics of the area as the most frequent reasons for not adopting the practice in both the rainfed and irrigated systems.

For the erosion control practices, most stakeholders agreed that these practices were not adequate for the characteristics of the case study areas for both the rainfed and irrigated cereal-based cropping systems (**Tables 7**, **8**).

Regarding fertilisation, most stakeholders considered the use of biostimulants and biofertilisers, the combination of mineral and organic fertilisers, and the precision agriculture options to optimise fertilisation using variable rate as difficult practices to be adopted in both the rainfed and irrigated cereal-based cropping systems. It is well-known that biostimulants and biofertilisers can reduce the application of chemical fertilisers due to the role of microorganisms included in the products that solubilise soil nutrients and enhance crop yield and quality (Pellegrino and Bedini, 2014; Rodrigues et al., 2018; Calatrava et al., 2021). Nevertheless, most stakeholders agreed to not use them in both the rainfed and irrigated cereal-based systems in the case study areas mainly because this practice is still not widely used due to higher costs compared to chemical fertilisers (Ramakrishna et al., 2019). Moreover, in the short-term, the economic and environmental benefits of this practice do not outweigh the costs. Other reasons were linked to the complexity of the practice, the lack of profitability, and the need for technical advice (**Tables 7**, **8**). For the mineral and organic fertilisers, most stakeholders pointed out the incompatibility of the combined use with other farming practices as an important reason in both the rainfed and irrigated cereal-based systems in the case study areas.

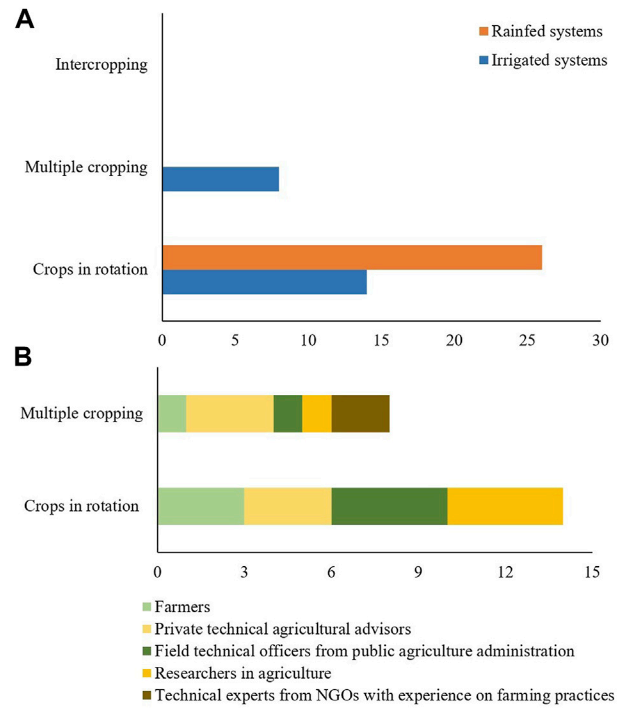

FIGURE 3 | In the Po Valley and Capitanata Plain case study areas, number of consulted stakeholders that identified each type of crop diversification strategy as most adequate for both the rainfed and irrigated systems **(A)**. Number of respondents grouped per type of stakeholders for the irrigated cereal-based cropping systems **(B)**.

Other reasons were linked to the inadequacy of the practice for the characteristics of the area, not being a traditional practice in the area, the complexity/difficulty to implement the practice without technical advice, and the high cost of the practice (**Tables 7**, **8**). For precision agriculture, several challenges (socio-economical, agronomical, and technological) could still limit the use of this practice because not many farmers are really familiar with farming innovation and digitalisation tools. In this context, the main drawbacks identified by the stakeholders for not adopting this practice in both the rainfed and irrigated cereal-based cropping systems were mainly linked to the high costs and to the complexity/difficulty to implement the use of variable rate without technical advice. The responses of this survey were in line with those reported by Kernecker et al. (2020), who highlighted that the most important barriers identified by farmers were the investment costs and the lack of perception of the benefits of precision farming technologies. These findings confirmed previous studies (Diacono et al., 2013; Calatrava et al., 2021) that pointed out additional costs for precision agriculture due to the significant investments in equipment/materials and the need of training for using more precise technologies (**Tables 7**, **8**). For the use of green manure and the contribution of organic matter/manure only few stakeholders did not consider these practices adequate, and the main reasons changed between the rainfed and irrigated cereal-based cropping systems. In the rainfed cereal-based cropping systems, the reasons for not adopting the green manure practice were related to not being a traditional practice in the area, the complexity/difficulty to implement the practice without technical advice, and the belief that the benefits of this practice in the short-term do not outweigh the costs. Moreover, for the contribution of organic matter/manure the reasons were mainly related to the incompatibility of the practice with other farming practices and to the related high cost (**Table 7**). In the irrigated cereal-based cropping systems the most important reason identified by the stakeholders for not adopting the green manure was mainly related to the inadequacy of the practice for the characteristics of the area, while for the contribution of organic matter/manure the main reasons were mainly related to the limited effectiveness and the complexity/difficulty to implement this practice with or without technical advice (**Table 8**). Similarly to the use of green manure and the contribution of organic matter/manure, the main reasons for not adopting the integrated pest management and the change of crop rotation changed between the rainfed and irrigated cereal-based cropping systems, as shown in **Tables 7**, **8**.

3.4 Identification of the Preferred Crop Diversification Strategies

In the second part of the survey, the stakeholders identified the best crop diversification strategies for both the rainfed and irrigated cereal-based cropping systems in the Po Valley and Capitanata Plain case study areas. For the rainfed cereal-based production, all the consulted stakeholders ($n = 26$) identified crop rotation as the most adequate diversification strategy to be adopted in the intensive cropping systems of the Po Valley and Capitanata Plain case study areas. For the irrigated cereal-based production, two thirds of the stakeholders (66%) selected crop rotations as the most appropriate crop diversification strategy, while one third (33%) selected multiple cropping. Interestingly, for both the cropping systems, the consulted stakeholders did not identify intercropping as an adequate crop diversification strategy for the cereal-based production in the Po Valley and Capitanata Plain case study areas (**Figure 3A**). Regarding the irrigated cereal-based cropping systems, the number of respondents that selected crop rotation or multiple cropping as alternative diversification strategies were grouped per type of stakeholders. Although the results of Fisher's exact probability test combining the choice of crop diversification strategy with the type of stakeholders were not statistically significant ($p = 0.348$), 75% of the farmers and 80% of the public technical officers and researchers selected crop rotations as the most adequate option for crop diversification strategy. Conversely, private technical advisors equally chose crop rotation and multiple cropping (50%), while 100% of the technical experts from NGOs considered multiple cropping the most adequate crop diversification option (**Figure 3B**).

The results for crop rotation confirmed the findings of the literature review by Francaviglia et al. (2019, 2020), indicating that this practice is unambiguously considered as the most adequate alternative for crop diversification of the cereal-based production in Italy, while intercropping was not selected because it is a practice mainly adopted in other agro-environmental zones such as the humid conditions of the Atlantic and Boreal regions.

As stated by Francaviglia et al. (2020) longer crop rotations (more than 3-year) resulted in higher crop productivity compared to monoculture for both the rainfed and irrigated cereal-based cropping systems. In this context, Bonciarelli et al. (2016) observed an average yield increase of 18% in a long-term crop rotation of winter and summer cereals in rainfed conditions of Central Italy, while in the semiarid conditions of Southern Italy Martiniello et al. (2012) showed that crop rotations with legumes increased crop productivity both in the rainfed (48%) and irrigated conditions (37%) compared to wheat monoculture. The increase of crop yields due to longer crop rotations (3–5 years) also favours positive changes in SOC content. As reported in the data analysis carried out by Francaviglia et al. (2019), in Southern Italy, SOC changes in longer crop rotation can be 24.9% higher compared to the 2-year rotation or monoculture.

Once the preferred type of crop diversification was identified (i.e., crop rotation and multiple cropping), the surveyed stakeholders selected the most adequate crops (two crops as maximum) for the chosen type of diversification. **Table 9** shows the number of stakeholders that selected each type of diversification and diversification crop for both the rainfed and irrigated cereal-based cropping systems.

For the rainfed cereal-based cropping systems, most stakeholders agreed to introduce legumes in rotation. In detail, grass-clover mixture for fodder use was the most selected crop diversification alternative (n = 19), followed by rotation with faba beans, and alfalfa, with a similar number of responses (n = 13 and 12, respectively). Although the choice of crop and diversification strategy was not significantly related to the type of stakeholder (Fisher's exact probability test p = 0.732), grass-clover mixture for fodder use was mainly selected by farmers, private technical advisors, and public technical officers, while private advisors mainly chose faba beans and alfalfa (**Supplementary Table S3**).

For the irrigated cereal-based systems, significant differences were found between crop rotation and multiple cropping. Particularly, the results for the Fisher's exact probability test show a statistically significant relation between the crops and the preferred type of diversification (i.e., crop rotation and multiple cropping) identified by the stakeholder. In the case of crop rotation, processing tomato was the most selected rotation alternative (n = 12), followed by maize (n = 6), and wheat (n = 3). A full range of minority options was identified such as sunflower (n = 2) and horticultural crops (n = 1). For multiple cropping, the most chosen alternatives for crop diversification were short cycle maize (n = 6) and wheat (n = 5). Similarly to the rainfed cereal-based cropping systems, the choice of crop and diversification strategy was not significantly related to the type of stakeholder (Fisher's exact probability test p = 0.618; **Supplementary Table S4**). However, within crop rotation processing tomato was mainly selected by farmers and public technical officers, while researchers mainly chose maize and wheat. For multiple cropping diversification, short-cycle maize and wheat were mainly selected by representatives of NGOs and private technical advisors, respectively.

These findings confirmed some solutions on crop diversification strategies that are supported by the CAP in order to achieve the national sustainability targets of cropping systems at European level (Stoate et al., 2009; Passeri et al., 2016). Moreover, the most adequate farming practices that were selected by the stakeholders are consistent with the strategies proposed in the Agri-Environmental Schemes of several EU countries, the CAP and Rural Development Programs (RDPs), since the 1990s (Matthews, 2013; Turpin et al., 2016). In Italy, the last two programs of the European Agricultural Fund for Rural Development (EAFRD), funded through the regional RDPs, have often paid subsidies to farmers who have voluntarily committed themselves to the introduction of practices such as minimum tillage, green cover and cover crops, green manure, crop rotation, creation of buffer strips against erosion and leaching of nutrients (European Parliament and the Council, 2013).

TABLE 9 | List of the most adequate crops selected for each crop diversification strategy for both the rainfed and irrigated cereal-based cropping systems by the stakeholders (number of respondents).

Diversification crop	Crop diversification strategy*		No of responses
	Crops in rotation	Multiple cropping	
Rainfed cereal-based cropping systems			
Grass-clover mixture	19	–	19
Faba bean	13	–	13
Alfalfa	12	–	12
Wheat	6	–	6
Protein pea	1	–	1
Spring-summer crop	1	–	1
Total answers	**52**	–	**52**
Irrigated cereal-based cropping systems			
Processing tomato	12	–	12
Wheat	3	5	8
Maize	6	–	6
Short-cycle maize	–	6	6
Hemp	–	2	2
Horticultural crops	1	1	2
Soybean	1	1	2
Sunflower	2	–	2
Barley	1	–	1
Barley, rapeseed or pea	–	1	1
Green manure mixed with legumes, cereals and brassicaceae	1	–	1
Sorghum	1	–	1
Total answers	**28**	**16**	**44**

Each stakeholder chose two possible crops for the type of diversification selected as more adequate. Fisher's exact probability test used for irrigated cereal-based cropping systems (p=0.000; *).*

4 CONCLUSION

Findings allowed to identify relevant strengths and drawbacks for the implementation of diversified cropping systems under low-input agricultural practices. A major strength is that the crop alternatives selected for the diversification are already cultivated as monocultures and are adapted to the local pedoclimatic conditions. Thus, farmers just need to learn how to use them in combination as rotations, multiple cropping, or intercropping.

In this context, RDPs have provided payments per hectare of agricultural area as financial support to favour the adoption of crop diversification strategies by farmers. Other financial support provided by RDPs are connected to the renewal of the machinery and tools, also when this option is useful for the implementation of low impact techniques especially for arable land preparation and sowing. The purchase of tools useful for the implementation of soil protection practices was encouraged through appropriate selection criteria, which rewarded farmers' projects aimed to improve farm environmental performances.

On the other hand, a major weakness is that few farmers are experts in crop diversification. Thus, providing adequate training for public officers and agricultural technical advisors is crucial for successfully implementing diversified cropping systems among farmers. Additionally, the identified low-input farming practices are easy to implement, are not costly, do not require major investments in new machinery nor great farming skills to learn them. This suggests a further significant potential for their implementation at the technical level.

These results can provide insights to support the planning of agricultural policies for sustain crop diversification in order to develop long-term strategies for the agri-food system at different scales.

In the RDPs definition, arable land diversification practices are included into the eco-schemes by the CAP National Strategic Plans. In this context, the stakeholder consultation and territorial features on agricultural needs should be considered more than in the past to tailor local-based solutions for crop diversification.

More in-depth analysis based on this method could support policy makers to distinguish easy to apply practices related to arable land farms from more complex ones that involve structural changes to the entire farm and cropping systems. This demarcation would allow to increase the rate of implementation of eco-schemes, considering production context features, and similarly to design voluntary measures for rural development suitable tailored for ambitious farmers that could lead further agro-ecological transitions towards sustainable and diversified agri-food systems in the production areas.

This step could be enriched by a more widespread field research activity, where long-term trials can allow technical and sociological new practices evaluation. In this context, building a network of field experiments within real farms should be supported. Looking at the new opportunities of the Horizon Europe program, specific funds should be dedicated to the creation of a research infrastructure based on a wide network of living-labs of crop diversification. The goal is to create opportunities for experiential and multidisciplinary dialogue between researchers, farmers' associations, citizens and agri-food chains operators.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. The datasets generated for this study are available at https://zenodo.org/communities/diverfarming. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study was conducted according to the ethical guidelines of the DIVERFARMING H2020 project (grant agreement 728003), which were approved by the Ethics Committee of the Universidad Politécnica de Cartagena and the European Commission (funder of DIVERFARMING). Informed consent was obtained from all subjects involved in the study.

AUTHOR CONTRIBUTIONS

Funding acquisition and supervision for Italy: RFa; WP leader of Selection of sustainable diversified cropping systems: MDG-L; task leader of data mining: RFr; task leader of mathematical calculation of the aptitude of each proposed alternative: JC; conceptualization: MDG-L, JC, CDB, and RFr; research design, methodology: MDG-L, JC, and DM-G; literature review: CDB, RFr, and EB; investigation: JC, MDG-L, DM-G, CDB, RFa, and RFr; survey adaptation and translation: CDB and RFr; data curation: DM-G; statistical analysis: JC and DM-G; multi-criteria analysis MDG-L; socio-economic and policy evaluation: EB; original draft writing: CDB, MDG-L, RFr, DM-G, and JC; writing review and editing: CDB, RFr, EB, and RFa. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Maize diversification and nitrogen fertilization effects on soil nitrous oxide emissions in irrigated mediterranean conditions

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Maize is a major irrigated crop in Mediterranean areas and its typical intensive management may impact soil nitrous oxide (N_2O) emissions. In these irrigated continuous maize systems, the legumes incorporation as well as adjusted nitrogen (N) fertilization might be interesting strategies to reduce soil N_2O emissions. The objective of this study was to assess the impact of cropping diversification and different N rates on soil N_2O emissions in flooded irrigated maize under Mediterranean conditions. To achieve this, two cropping systems (maize monoculture system, MC; and pea -maize rotation, MP) and 3N rates (unfertilized, 0N; medium rate, MN; and high rate, HN) were evaluated in a field experiment established in NE Spain during 2 years (2019; 2020). During the studied period, the N rate had a significant effect on soil N_2O emissions, with a non-linear positive response of cumulative soil N_2O emissions to N rates. In both systems, quick and high increases of soil N_2O fluxes were observed immediately after the N application reaching 55 and 100 mg N_2O-N m^{-2} day^{-1} in MC and MP, respectively. Both years, the pea phase of the MP rotation showed greater cumulative N_2O emissions than the fallow of MC. However, N_2O losses in the maize phase were similar (2019) or even higher (2020) in MC than in MP. Moreover, in both seasons, the MN treatments showed lower yield-scaled N_2O emissions and N emission factor than the HN treatments, being this last lower than 1% in all cases. The results obtained showed that in irrigated Mediterranean conditions the replacement of a fallow by a legume, together with an adjusted N fertilization are favourable strategies to mitigate soil N_2O emissions in high-yielding maize systems.

KEYWORDS

soil N_2O emissions, cropping diversification, nitrogen fertilization, irrigated systems, maize monoculture

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Abbreviations: N_2O, nitrous oxide; CIR, crop irrigation requirement; ETo, Reference evapotranspiration; ETc, Crop evapotranspiration; WFPS, Water-filled pore space.

Introduction

Semiarid Mediterranean areas are characterized by high solar radiation conditions and long frost-free periods that, combined with irrigated systems, lead to high crop productivity (Cavero et al., 2003). Although these conditions would allow to increase crop diversification, one of the most common systems in these areas is the maize monoculture, which is normally produced under high-intensive conditions (Berenguer et al., 2008; Cavero et al., 2018). The large amount of fertilizer applied in these intensive agricultural systems is behind great N losses and, indeed, these irrigated agricultural areas are particularly susceptible to groundwater pollution (Quemada et al., 2013). Moreover, N fertilization is the main source of soil N_2O emissions in agriculture, a potent greenhouse gas (GHG) (Syakila and Kroeze, 2011; IPCC, 2014). Besides, the changes in the soil water content and soil structure resulting from these intensive practices may influence the bacterial processes of nitrification and denitrification that control N_2O production in the soil (Butterbach-Bahl et al., 2013).

Over the last decade, crop diversification, including legumes, is becoming a common practice to reduce the external N supplies and the environmental impacts associated with the highly intensive agriculture systems (Lin, 2011; Sanz-Cobena et al., 2014). The inclusion of legume crops in the rotation is an interesting strategy to provide environmental benefits and increase the sustainability of farming (Preissel et al., 2015; Lötjönen and Ollikainen, 2017). Due to the legume crops' ability to fix N and their low C/N ratio, they are an adequate management practice to increase soil fertility, making crop rotations less dependent on N inputs (Drinkwater et al., 1998; Sant'Anna et al., 2018; De Antoni Migliorati et al., 2021).

Under Mediterranean irrigated conditions, Cela et al. (2011) and Salmerón et al. (2010, 2014) carried out different studies to assess the impact of legume crop rotations on the N availability for the subsequent crop, and observed that legumes allowed a reduction in the N fertilizer rate of the following maize crop by maintaining the grain yields. Regarding the impact on soil N_2O emissions, different authors observed higher N_2O emissions in crop rotations including legumes (Drury et al., 2008). Adviento-Borbé et al. (2010) observed a higher N_2O emission in a maize-alfalfa rotation compared to a maize monoculture under temperate field conditions. In agreement with the previous studies, Davis et al. (2019) found an increase in soil N_2O emissions for the legume cover crops compared to bare soil. Likewise, (Saha et al., 2021) reported greater emissions during the maize phase when the precedent crop was a legume. Besides, higher soil N_2O fluxes during the growing period of a legume cover crop compared to a fallow were also reported in Mediterranean conditions (Sanz-Cobena et al., 2014; Guardia et al., 2016). In contrast to the previous studies, (Olivera et al., 2021) did not observe differences or, even, lower N_2O losses in cereal phases after legumes compared to cereal monocultures under Mediterranean conditions. However, if the N supplied by the legume is considered when adjusting the N fertilizer rate of the following crop in the rotation, this negative-side effect can be partially offset and no differences would be observed in the cumulative N_2O emission of the whole cropping period (Lemke et al., 2007; Guardia et al., 2016; Hansen et al., 2019).

In Mediterranean agroecosystems, the optimization of N fertilization has a GHG mitigation potential ranging from 30% to 50% compared to a non-adjusted practice (Sanz-Cobena et al., 2017). Different studies have shown that high N fertilizer rates lead to greater soil N_2O emissions in maize monocultures. For example, (Pareja-Sánchez et al., 2019, Pareja-Sánchez et al., 2020) observed an increase in soil N_2O emissions by increasing the amount of N applied in a maize monoculture. Likewise, (Cayuela et al., 2017), in a meta-analysis study for different crops including maize in the Mediterranean region, reported an increment in the emission factor (EF) by increasing the N application. Nevertheless, these studies did not include crop rotation systems with legumes.

Therefore, the combination of the inclusion of legume crops in the rotations and different N fertilizer rates can increase sustainability in irrigated maize systems under semiarid Mediterranean conditions while maintaining their high crop productivity. More studies combining both practices would allow to quantify their impact on N_2O emissions and contribute to design more sustainable agricultural practices in these areas. Accordingly, the general objective of this study was to assess the impact of different N fertilization rates on the soil N_2O emissions in different cropping systems (maize monoculture and pea-maize rotation) under irrigated Mediterranean conditions during 2 years.

Material and methods

Site description

A 2-years field study (November 2018 to November 2020) was carried out at the research farm of the Aula Dei Experimental Station (EEAD-CSIC) in the province of Zaragoza, Spain (41º42′N, 0º49′W, 225 m altitude), covering two complete growing seasons of a maize monoculture system and a pea-maize rotation. The soil is a Typic Xerofluvent (Survey Staff, 2015) with a silty loam texture, characterized by a basic pH and low contents of carbon (C) and nitrogen (N) and with a carbonate ($CaCO_3$) content higher than 30% (Table 1). The area is characterized by a Mediterranean semiarid climate with a mean annual air temperature of 14.1°C, mean annual precipitation of 298 mm and mean annual reference evapotranspiration (ETo) of 1,243 mm. Daily mean air temperature, daily precipitation, daily ETo and MP and MC irrigation for the given experimental period are presented in Figure 1.

TABLE 1 Soil characteristics at the field site.

Depth (m)	pH	EC (dS m^{-1})	C (%)	N (%)	$CaCO_3$ (%)	Sand (%)	Silt (%)	Clay (%)	FC (m^3 m^{-3})	WP (m^3 m^{-3})
0.0–0.1	7.90	0.33	1.08	0.14	33.26	19.62	61.63	18.75	26.84	14.40
0.1–0.3	8.05	0.25	0.92	0.14	33.11	19.41	61.59	19.00	26.23	15.91

Note. FC, field capacity (−0.033 MPa). WP, permanent wilting point (−1.5 MPa).

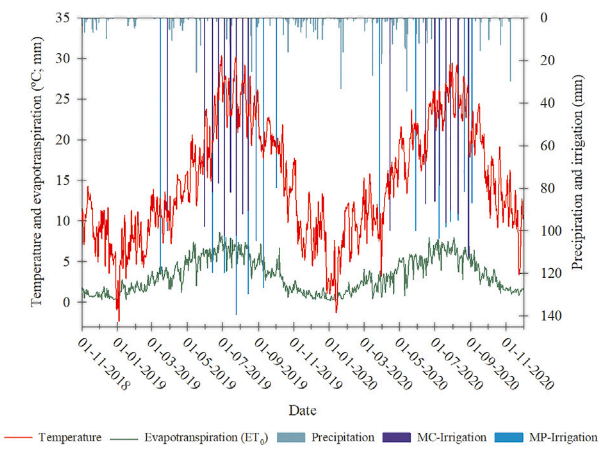

FIGURE 1
Daily mean air temperature (red continuous line), reference evapotranspiration (ET_O) (green continuous line), precipitation (green vertical bars), maize monoculture (MC) system irrigation (purple vertical bars) and pea-maize (MP) cropping system irrigation (blue vertical bars) and throughout the entire experimental period.

Experimental design and management practices

The experimental layout consisted of a split-block design with three replications and a plot size of 6 × 25 m. The historical management of the field consisted of alternating different cereal crops, mainly winter wheat (*Triticum aestivum* L.) and maize under conventional tillage and flood irrigation. In August 2018, a field of 0.6 ha was divided into two parts. In one part, a maize monoculture (MC) was established while in the other part a pea-maize rotation (MP) was set up. Besides, in both cropping systems, three different N rates were tested (i.e., control or unfertilized, 0N; medium rate, MN; and high rate, HN).

Nitrogen application rates were selected based on the typical amounts of N applied for irrigated maize cropping systems in the region, which are between 300 and 350 kg N ha^{-1} (Sisquella et al., 2004). Likewise, based on previous studies carried out in the same region, a reduction of 50 kg N ha^{-1} was established for the maize crop after the pea (Salmerón et al., 2011).

Under the MC cropping system, the N application rates selected were 0, 200 and 400 kg N ha^{-1} for 0N, MN, and HN, respectively. The total amount of N in each treatment was split into two applications, a quarter of the total N at pre-sowing application and the rest at one top-dressing application at the V8-V10 maize growth stage, when maize plant present 8 to 10 developed leaves. In 2019, the pre-sowing application under the MC cropping system consisted of an NPK 8-15-15 fertilizer compound at 0, 625 and 1,250 kg ha^{-1} application rates for 0N, MN, and HN treatments, respectively. In 2020, the pre-sowing application consisted of 0, 185, and 370 kg ha^{-1} of calcium ammonium nitrate (CAN 27%) for 0N, MN, and HN treatments, respectively, together with 1,000 kg ha^{-1} of an 8–10 PK fertilizer compound in all treatments. In both growing seasons, the top-dressing consisted of CAN 27% application up to the N rate established (Supplementary Table S1).

Fertilization for the MP cropping system was different between cropping phases. For the pea phase, one application of a 10–12 PK fertilizer compound at 400 kg ha^{-1} was done before pea sowing in the 2019 growing season. Pea did not receive any N fertilization. During the maize phase, N applications were 0, 150, and 350 kg N ha^{-1} for 0N, MN, and HN, respectively. The total amount of N fertilizer was split into two applications, a third of the total N before maize sowing and the rest at one top-dressing application at the V8-V10 maize growth stage in the form of CAN 27%.

Field management practices and their timing varied between cropping systems (Supplementary Table 1S). In the MC, cropping system tillage operations consisted of one pass of a disk harrow and one pass of a subsoiler to 0.30 m depth, followed by one pass of a rotary tiller for preparing the maize seeding bed. In the MP cropping system, tillage operations for both pea seasons consisted of one pass of a disk harrow followed by one pass of a rotary tiller before sowing. In the 2019 growing season, maize after pea was sown under no-tillage conditions, while in 2020 one pass of a disk harrow and one pass of a rotary tiller were performed to ameliorate soil compaction after the second pea season. In both growing seasons, maize cv. Pioneer P1921 (FAO 700) was sown in rows 75 cm apart at a planting density of 89,500 plants ha^{-1} under the MC cropping system. Under the MP cropping system, pea cv. Cartouche and cv. Furious were sown at 350 and 325 kg of seed ha^{-1} in the 2019 and 2020 growing seasons, respectively, and maize cv. DKC5032YG (FAO 400) was sown in rows 75 cm apart at a planting density of 89,500 plants ha^{-1}. Maize harvest was done with a commercial combine and then the maize stover was chopped. From harvest to the subsequent maize sowing (fallow period), the soil was left unvegetated and only covered by the stover under the MC cropping system. In both growing seasons, pea residues were chopped after harvest. Differences in the maize growing cycle between MP and MC modified the timing of the different management practices in each cropping system (Supplementary Table 1S).

Both cropping systems were irrigated under flood conditions. Maize crop irrigation requirement (CIR) was determined weekly by subtracting the effective precipitation, 75% of the total weekly precipitation (Dastane, 1978), to the weekly crop evapotrantspiration (ETc), considering an irrigation efficiency of 75%. Maize daily ETc was obtained by multiplying the reference evapotranspiration (ETo), estimated using the FAO Penman–Monteith method (Allen et al., 1998), and the maize crop coefficient (Kc). Maize crop coefficient was determined using a thermal time function developed on the same experimental farm (Kiniry, 1991; Martínez-Cob et al., 2008). Pea irrigation was based on crop development coupled with the soil water content. The total amount of irrigation water applied in the MP cropping system was 1,027 and 861 mm in 2019 and 2020, respectively, distributed in nine different irrigation events. Under the MC cropping system, 826 and 743 mm of irrigation water were applied in different irrigation events for the 2019 and the 2020 growing seasons, respectively (Figure 1). Weed control was carried out by applying a pre-emergence and post-emergence herbicide for the maize, while only a pre-emergence application was done for the pea. No pesticides were applied to the maize or the pea crops.

Soil, plant and gas sampling and analyses

Soil temperature at 5 cm depth and soil moisture at 0–5 cm depth were measured on every gas sampling date using a Crison TM 65 probe (Carpi, Italy) and GS3 soil moisture probes (Decagon Devices, Pullman, WA, United States), respectively. Soil water-filled pore space (WFPS) was calculated using bulk density and assuming a soil particle density of 2.65 Mg m^{-3} (Danielson and Sutherland, 1986). Soil mineral N content (ammonium plus nitrate) from the 0–5 cm soil depth was determined by extracting 20 g of fresh soil with 100 ml of 2 M KCl. The extracts were frozen and later analysed by spectrophotometry.

Soil N_2O emissions were measured with the closed chamber technique (Hutchinson and Mosier, 1981) using polyvinyl chloride (PVC) chambers (20 cm height and 31.5 cm internal diameter) yielding an internal volume of 15.5 L and coated with white thermal paint (Imperlux Termic Outdoor; Arelux, Zaragoza, Spain) to diminish internal increases in temperature. In November 2018, a ring was inserted 5 cm into the soil in each plot, in the inter-row space, removing it only for tillage, sowing and harvesting operations.

During the pea phase of the MP rotation and the fallow phase of the MC system, gas sampling frequency consisted of one sampling every 21 days. However, during the maize phase of both cropping systems, the gas sampling frequency was increased. Thus, weekly measurements were performed from sowing to maize tasselling growth stage (VT) and every 2 weeks from VT stage until harvest. Moreover, gas sampling frequency was increased during fertilization events, taking gas samples 24 h before and 24, 48, 72, 96, 144, and 192 h after fertilization. Likewise, gas sampling frequency was increased after each irrigation event. In each gas sampling 20 ml of sample were taken in each chamber and transfer to a 12 ml Exetainer ® borosilicate glass vial (model 038W, Labco, High Wycombe, United kingdom). Gas sampling was performed between 06:00 to 07:00 GMT and between 07:00 and 08:00 GMT during summer and wintertime, respectively.

Gas samples were analysed by gas chromatography using an Agilent 7890B (Agilent, Santa Clara, CA, United States) equipped with an autosampler (PAL3 autosampler, Zwingen, Switzerland). N_2O concentration was determined with an electron capture detector (ECD). The system was calibrated using ultra-high purity N_2O standards (Carburos Metálicos, Barcelona, Spain). Emission rates were calculated from the linear increase in the gas concentration within the chamber during the sampling time (0, 20, and 40 min after chamber enclosure) and correcting for the air temperature inside the chamber. The goodness of the N_2O concentration data for fluxes estimation was checked per chamber and sampling date by assuming an $R^2 > 0.90$. When R^2 values were lower than this threshold, data were inspected to discard the possible existence of outliers based on the CO_2 concentration to discard chamber leakages during the enclosure time. Cumulative soil N_2O emissions were quantified on a mass basis (i.e., kg N ha^{-1}) using the trapezoid rule (Levy et al., 2017).

Manual harvest was done for pea and both maize crops to determine yield components before the whole field harvest. Pea grain and biomass yield were determined by harvesting two subsamples of 0.25 m^2 per plot, while maize grain and biomass yield were determined by harvesting two three-metre maize rows per plot. After that, grain was separated from the aboveground biomass and dried separately at 60°C for 48 h to determine grain and biomass yield. Grain and biomass subsamples were ground and analysed to determine the N content by dry combustion (TruSpec CN, LECO, St Joseph, MI).

For 2018, 2019 and 2019, 2020 growing seasons, which include the bare fallow phase and maize phase, and pea phase and maize phase for MC and MP cropping systems, respectively, the EF was calculated from the next expression:

$$EF\ (\%) = \frac{E_i - E_0}{Nrate_i} x\ 100$$

where Ei are the cumulative soil N_2O emissions from the i treatment (kg N_2O-N ha^{-1}), E0 are the cumulative soil N_2O emissions (kg N_2O-N ha^{-1}) from the control treatment, unfertilized, and Nratei is the amount of N fertilizer applied in the i treatment including the N surplus derived from the legume stover (kg N ha^{-1}).

Data analysis

Normality assumptions of all the data obtained were checked in the residuals by a Shapiro-Wilk test. Moreover, when it was necessary, data were transformed to comply with normality assumptions. Squared root and logarithm transformations were performed for grain yield and daily soil N_2O fluxes, respectively. Different repeated measures analysis of variance (ANOVA) were performed independently for MC and MP cropping systems and each measurement period with N rate, date of sampling and their interaction as fixed effects and block and their interactions as random effects. In addition, ANOVA analyses were performed for cumulative N_2O emissions, grain yield, N_2O yield-scaled emissions and N_2O emission factor, with the cropping system, N rate and their interactions as fixed effects and block and their interactions as random effects. Simple regression analysis was used to determine the relationships between cumulative soil N_2O emissions and N rate. The significance of the model between cumulative soil N_2O emissions and N rate was tested by cumulative soil N_2O emissions logarithm transformation to comply normality assumption. All statistical analyses were performed with the JMP 10 statistical package (Institute Inc., 2012).

Results

Soil temperature, soil water-filled pore space and soil available nitrogen content

In both cropping systems, the sampling date affected soil temperature. Besides, in the MC system, soil temperature also showed a significant interaction between sampling date and N rate (Table 2). Both cropping systems showed a similar temporal pattern with an increase in soil temperatures during the spring months (March-June), reaching the maximum values during the summer period, and a decrease during autumn and winter months (Figure 2).

In both cropping systems, the soil WFPS was only affected by the sampling date (Table 2). During the first 24 h after each flood irrigation event, a rapid increase in soil WFPS was observed in both cropping systems reaching up to 100% WFPS (Figure 2). Thereafter, WFPS tended to decrease rapidly and within the first 5 days after irrigation soil WFPS reached 30% (Figure 2).

Soil nitrate and ammonium content under the MC cropping system were affected by the sampling date and the N rate in both cropping systems. Besides, the interaction between them was significant except for the soil nitrate content under the MC cropping system (Table 2; Figure 2). In both cropping systems, following each N fertilizer application, a significant increase in the soil mineral N content was observed. On the other hand, soil nitrate and ammonium content presented the lowest values during the pea/fallow season with average values that did not exceed 25 and 2 kg N ha^{-1} of nitrate and ammonium, respectively. Only soil nitrate content showed values greater than 100 kg N ha^{-1} in the first fallow period for the MC cropping system (Figure 2).

Soil N_2O fluxes and cumulative emission

In both cropping systems, soil N_2O fluxes were significantly affected by the sampling date, the N fertilization rate and the interaction between both (Table 2). Soil N_2O fluxes presented a temporal emission pattern characterized by low flux values during most of the experimental period followed by a sharply and quick increase of the soil N_2O flux immediately after the application of N fertilizer. This behaviour resulted in several N_2O emission peaks over the 2 years coinciding with the fertilization events, being these peaks higher during the N top-dressing applications than during the pre-sowing N applications.

Differences in soil N_2O emissions among N rates were mainly observed during the maize growing seasons and, in particular, following the N top-dressing application, observing the greatest N_2O peaks under HN treatment in both cropping systems. However, the magnitude of peaks differed between cropping systems and maize seasons. Under the MC cropping system, the greatest daily flux values were 55 mg N_2O-N m^{-2} day^{-1}, whereas under the MP cropping system the maximum fluxes values were 100 and 50 mg N_2O-N m^{-2} day^{-1} during the 2019 and 2020 maize season, respectively (Figure 3). Conversely, over both pea/fallow periods and both cropping systems, soil N_2O emissions presented the lowest fluxes values, alternating positive and negative fluxes that ranged between −0.5 and 0.5 mg N_2O-N m^{-2} day^{-1} (Figure 3).

The interaction between the cropping system and N application rate was significant only for the 2019 maize growing season, observing the greatest emissions in the MP-HN treatment followed by the MC-HN, while the lowest cumulative emissions were observed in the two 0N treatments (MC-0N and MP-0N) (Table 3; Figure 4A). In both pea/fallow seasons, the MP cropping system showed the highest cumulative soil N_2O emissions. Oppositely, in the 2020 maize season, greater cumulative N_2O emissions were observed in the MC system than in the MP cropping system (Table 3). On the other hand, the N application rate presented a significant impact on the cumulative soil N2O emissions for all considered periods except during the first pea/fallow period. In all cases, cumulative soil N_2O emissions increased with the N application rate. Thus, the greatest and the lowest cumulative N_2O emissions were observed in the

TABLE 2 Analysis of variance (*p-values*) of daily soil temperature, soil water-filled pore space (WFPS), soil nitrate (NO_3^--N), soil ammonium (NH_4^+-N) and soil N_2O fluxes for maize monoculture (MC) and pea-maize cropping system (MP) as affected by sampling date (Date), N fertilization rate (N rate) and their interaction.

Cropping system	ANOVA table	Soil temperature	Soil WFPS	Soil NO_3^--N	Soil NH_4^+-N	Soil N_2O fluxes
MC	Date	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	N rate	n.s.*	n.s.	< 0.01	< 0.01	< 0.01
	Date x Nrate	< 0.01	n.s.	n.s.	< 0.001	< 0.001
MP	Date	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	N rate	n.s.	n.s.	< 0.01	< 0.05	< 0.01
	Date x Nrate	n.s.	n.s.	< 0.01	< 0.05	< 0.001

Note. *n.s., not significant at the 0.05 probability level.

0N and HN levels, respectively (Table 3). Besides, in both fertilized treatments, the MP cropping system showed a reduction in the cumulative soil N_2O emissions of 60% and 55% between seasons for the pea and maize cropping seasons, respectively (Table 3). Additionally, over both maize growing seasons, the cumulative soil N_2O emissions showed a significant positive exponential response to the increase of N fertilization rate with large N_2O emissions when N rate exceeded 200 kg N ha^{-1} (Figure 5).

Grain yield, N_2O yield-scaled emissions and nitrogen emission factor, EF

Grain yield was significantly affected by the N application rate but neither the cropping system nor the interaction between both had an impact on the grain yields. In both growing seasons and for the whole experimental time, the unfertilized treatment (0N) always showed the lowest grain yield. Regarding fertilized treatments, there were no significant differences between the HN and the MN treatments in both growing seasons or the whole experimental period (Table 4).

In both growing seasons and considering also the entire experimental period, the grain yield-scaled emissions were significantly affected by the Nrate. In all cases, the greatest grain yield-scaled emissions were observed under the HN treatment, while the lowest values were observed under the 0N treatment. On the other hand, the cropping system only showed significant differences for the 2018–2019 season, reporting greater yield-scaled emission in the MP system than in the MC system (Table 4).

The nitrogen emission factor only showed significant differences between cropping systems during 2019–2020 growing season, presenting MP lower values compared to MC cropping system. Conversely, the Nrate affected the EF in both growing season, reporting the HN rate the greatest EF values in both seasons. Moreover, in both seasons and for all treatments, the EF always showed values below 1% (Table 4).

Discussion

In this study, in irrigated Mediterranean systems, the change from maize monoculture to a more diversified system with a double annual rotation with pea and maize and the adjustment in N fertilization rates had a significant impact on soil N_2O emissions. In general, the N rate positively affected daily soil N_2O fluxes, but significant differences among N rates were observed after fertilizer applications. In both years and cropping systems, significant N_2O emission peaks were measured right after the application of N fertilizer as observed in other maize experiments in irrigated Mediterranean conditions (Franco-Luesma et al., 2019; Pareja-Sánchez et al., 2020). The soil N_2O flux peaks observed during top-dressing applications were higher than the peaks measured in the pre-sowing applications. Maize top-dressing applications are performed during the warmest months of the year and, in turn, when irrigated events are concentrated over the year. Therefore, the increase in available soil mineral N after fertilization together with high soil moisture levels (70%–80% WFPS) and high soil temperatures boosted high soil N_2O peaks (Franco-Luesma et al., 2020). The combined effect of these three factors (high moisture, temperature, and soil mineral N) resulted in favourable conditions for the production of soil N_2O by denitrification (Sánchez-Martín et al., 2010; Butterbach-Bahl et al., 2013).

Soil N_2O emissions during the legume growth season tended to be lower than during fertilized crops (Jeuffroy et al., 2013; Lemke et al., 2018). However, in our case, cumulative soil N_2O emissions in the pea phase of the MP rotation were greater than in the fallow phase of the MC system, similar to the results reported by Davis et al. (2019) in a study that compared different cropping systems such as bare fallow-maize, cereal-maize and hairy vetch-maize. Likewise, other authors (Rochette et al., 2004; Jensen et al., 2012; Schwenke et al., 2015; Hansen et al., 2019) have also observed an increase in soil N_2O emissions associated with the growth of legumes, concluding that possible increases in N_2O during the growth of legumes may be the consequence of higher soil labile N availability by the mineralization of legume roots and fallen leaves. In our experiment, the difference in cumulative soil N_2O emissions between the pea and fallow phases varied between years, a fact that would be explained by the differences between the systems, legume crop against a bare soil fallow, as well as

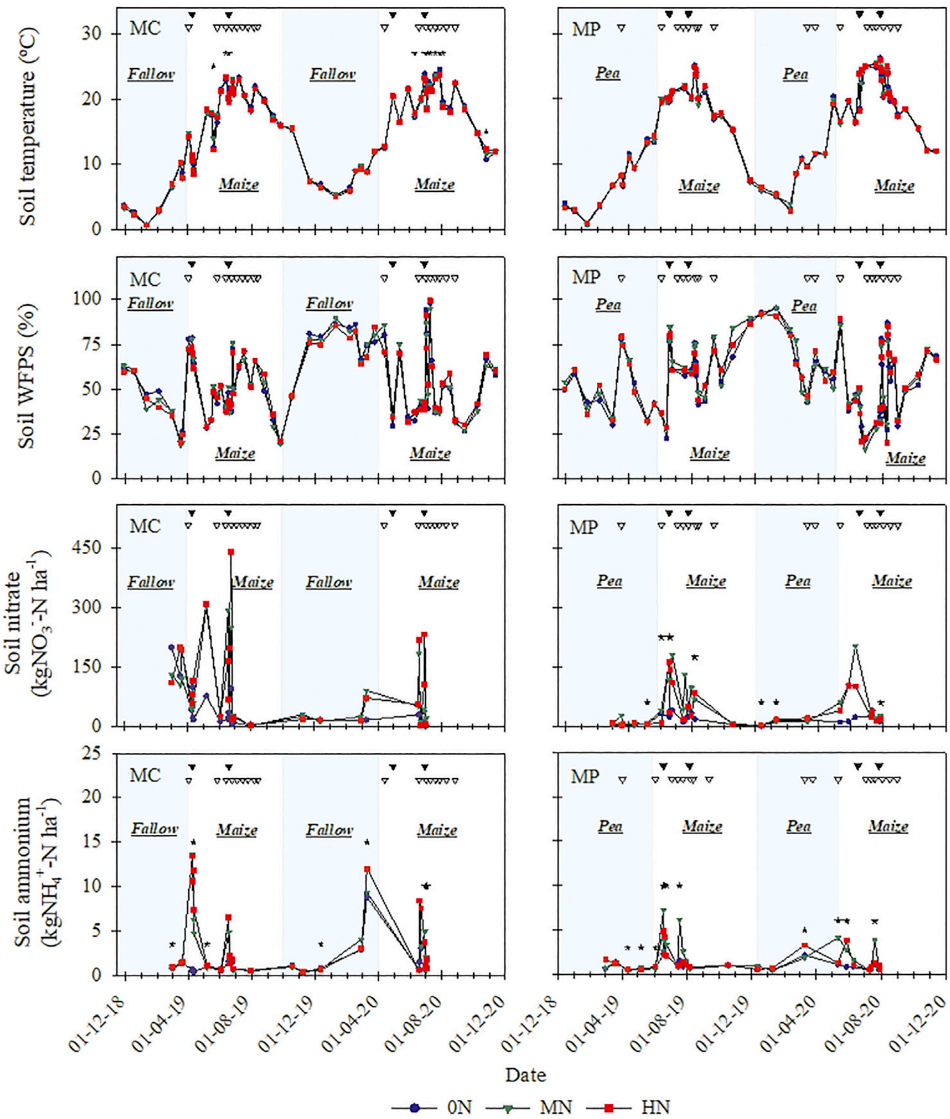

FIGURE 2
Soil temperature at 5 cm depth, soil water-filled pore space (WFPS) and soil mineral N content for the 0–5 cm soil depth in the maize monoculture (MC) system and the pea-maize (MP) rotation as affected by soil N rate: 0N (unfertilized), MN (medium N rate) and HN (high N rate). *Indicates significant differences between treatments within a date at $p < 0.05$. Black triangles indicate fertilizer applications. White triangles indicate flood irrigation events.

the different pea crop performance between seasons rather than the biological N_2 fixation process associated to the legume crops (Zhong et al., 2009). During the first year (2019), cumulative soil N_2O emissions in the pea phase were about tenfold higher than in the fallow phase but in the second year (2020) this difference was only twofold. One possible explanation for this difference would be the different pea growth observed between the two growing seasons. In 2019, the pea crop showed an exceptional growth reaching grain yield values up to 3,400 ± 502 kg ha^{-1} (data not shown). However, in the following year, pea yield values did not exceed 820 ± 147 kg ha^{-1} (data not shown). Then, the higher pea biomass production in 2019 led to higher N release from mineralized aboveground biomass and roots, resulting in an N supply at the end of the season of 257 and 57 kg N ha^{-1} for 2019 and 2020 pea seasons, respectively. This fact explained the greatest soil N_2O emissions found in the pea phase compared to the soil bare fallow due to mineralization of the pea dead biomass during the pea growing season and also the highest soil N_2O emissions measured during the 2019 pea/fallow season compared to the 2020 pea/fallow season. Likewise, this difference in N release from the legume biomass between

TABLE 3 Analysis of variance of cumulative soil N_2O emissions for pea or fallow phases (Pea/fallow) and maize season (Maize) during 2019 and 2020 and the entire study duration (Total) as affected by cropping system (maize monoculture, MC; pea-maize rotation, MP), N fertilization rate (control, 0N; medium rate, MN, and high rate, HN) and their interaction.

Treatments	Cumulative soil N_2O emissions (kg N_2O-N ha^{-1})				
	Pea/fallow 2019	Maize 2019	Pea/fallow 2020	Maize 2020	Total
Cropping system (Crop)					
MC	0.05 b	1.92	0.12 b	2.01 a	3.79
MP	0.56 a	2.98	0.22 a	1.33 b	5.09
N fertilization rate (N rate)					
0N	0.20	0.38 c	0.10 c	0.30 c	1.07 c
MN	0.30	1.85 b	0.18 b	1.46 b	3.89 b
HN	0.40	5.12 a	0.25 a	3.36 a	9.29 a
ANOVA (*p*-values)					
Crop	< 0.05	n.s.*	< 0.05	< 0.05	n.s.
N rate	n.s.	< 0.001	< 0.05	< 0.001	< 0.001
Crop x N rate	n.s.	< 0.05	n.s.	n.s.	n.s.

Note. For each variable, measurement period and effect, values followed by different letters are significantly different according to Tukey test at 0.05 level. *n.s., non-significant.

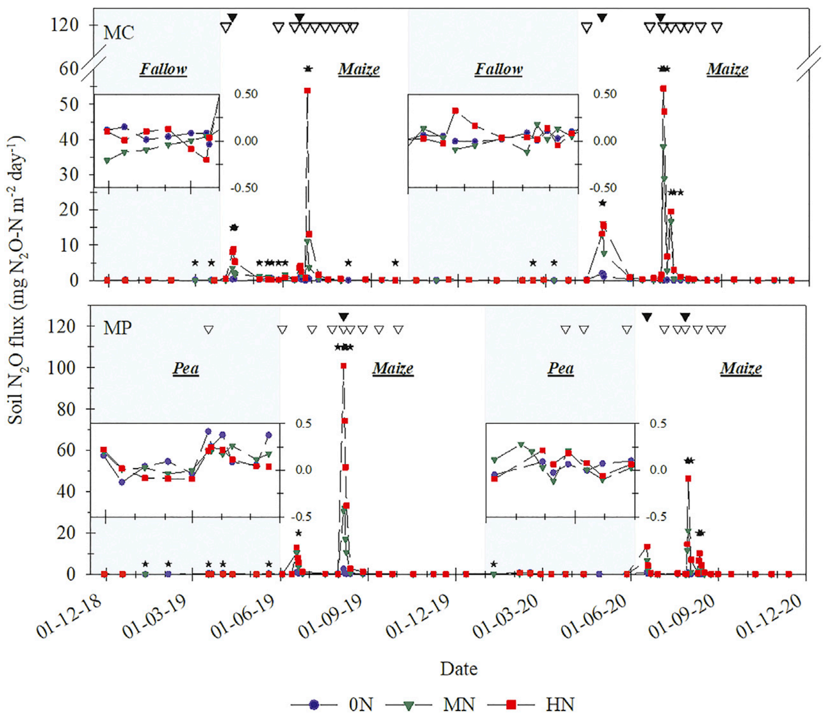

FIGURE 3
Soil N_2O fluxes in the maize monoculture (MC) system and the pea-maize (MP) rotation as affected by soil N rate: 0N (unfertilized), MN (medium N rate) and HN (high N rate). * Indicates significant differences between treatments within a date at $p < 0.05$. Black triangles indicate fertilizer applications. White triangles indicate flood irrigation events.

both pea seasons would explain the greater soil N_2O emissions found in the maize phase during the 2019 growing season under the MP system compared to the MC cropping system, as well as the greater soil N_2O emissions observed under the MP cropping system in the 2019 growing season compared to 2020 growing season.

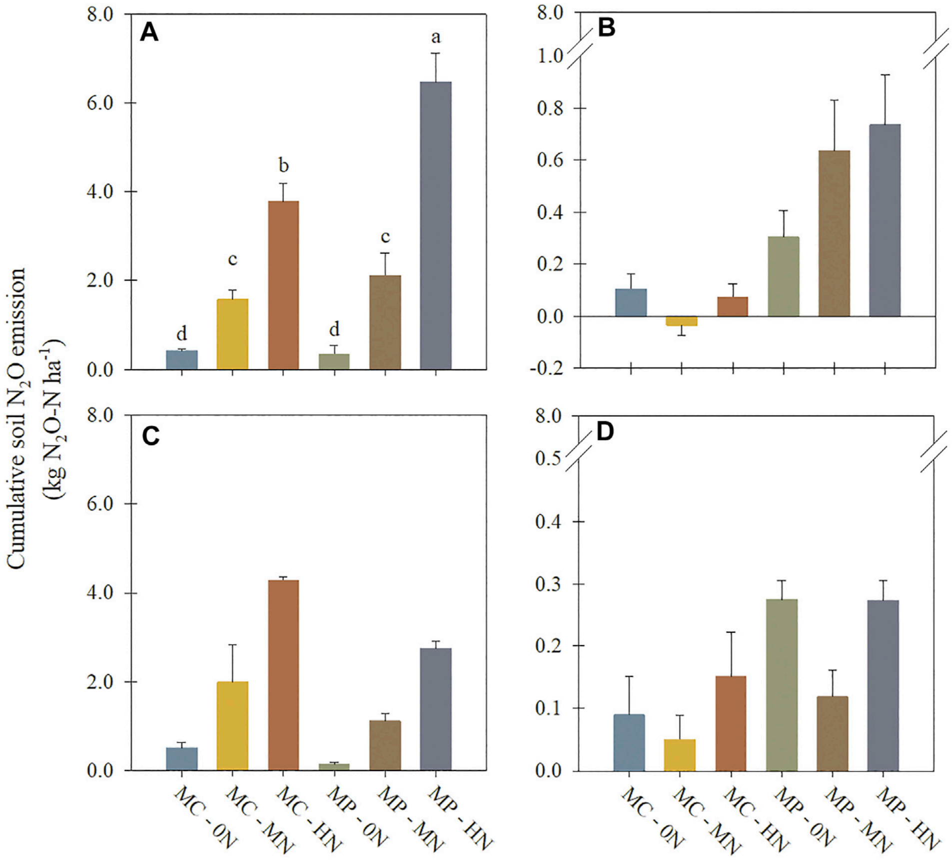

FIGURE 4
Cumulative soil N_2O emissions for **(A)** 2019 maize growing season, **(B)** 2019 pea/fallow season, **(C)** 2020 maize growing season, **(D)** 2020 pea/fallow season as affected by maize monoculture (MC) and pea-maize rotation (MP) and by soil N application rate: 0N (unfertilized), MN (medium N application rate), HN (High N application rate). Different letters are significantly different according to Tukey test at p = 0.05. Error bars represent standard error.

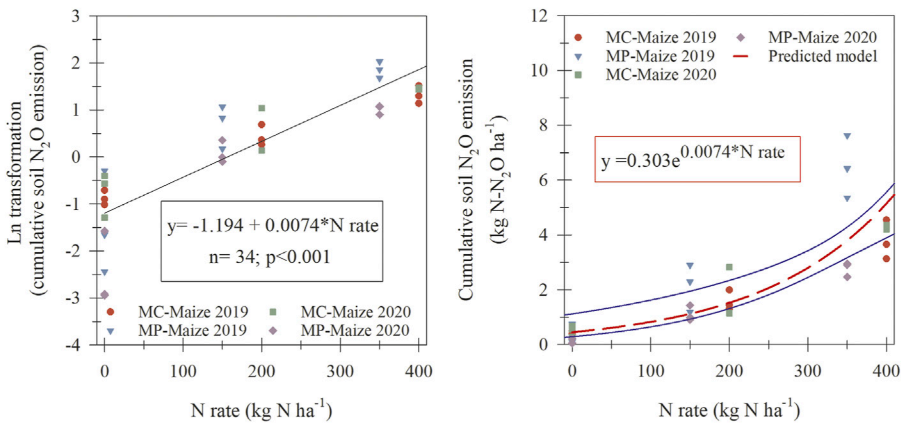

FIGURE 5
Regression analysis between cumulative soil N_2O emissions and N application rate for both cropping systems (maize monoculture system, MC; pea-maize cropping system, MP) and both maize growing seasons (maize growing season 2019, Maize 2019; maize growing season 2020, Maize 2020. Black solid lines represent the 95% confidence interval.

TABLE 4 Dry grain yield, yield-scaled emissions and nitrogen emission factor for the growing season periods of 2018, 2019 and 2019, 2020 and the total experimental period as affected by cropping system (Crop) (maize monoculture, MC; pea maize cropping system, MP), nitrogen application rate (Nrate) (Control, 0N; Medium rate, MN, and High rate, HN) and their interactions.

Effects and levels†	Dry grain yield† (Mg grain ha^{-1})			Yield-scaled emissions (kg N_2O-N Mg grain ha^{-1})			Emission factor (%)	
	Season			Season			Season	
	2018–2019	2019–2020	Total	2018–2019	2019–2020	Total	2018–2019	2019–2020
Crop	n.s.*	n.s.	n.s.	**< 0.05**	n.s.	**< 0.05**	n.s.	**< 0.05**
MC	11.44 (1.23)	6.82 (1.28)	16.74 (2.45)	159 (30) b	274 (59)	198 (43) b	0.69	0.87 b
MP	8.97 (0.70)	5.44 (0.97)	13.42 (1.38)	411 (121) a	209 (519)	335 (80) a	0.72	0.55 a
Nrate	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.05**	**< 0.01**	**< 0.05**	**< 0.05**
0N	7.84 (0.92) b	2.62 (0.49) c	8.72 (1.21) b	78 (12) c	104 (45) c	102 (18) c		
MN	10.71 (1.16) a	5.57 (0.68) a,b	15.35 (1.74) a	235 (61) b	213 (45) b	264 (53) b	0.51 b	0.59 b
HN	11.88 (1.33) a	9.29 (0.86) a	21.18 (1.90) a	542 (143) a	377 (35) a	488 (83) a	0.89 a	0.76 a
CropxNrate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MC-0N	7.22 (1.66)	2.08 (0.56)	8.61 (2.15)	80 (16)	198 (14)	115 (32)		
MC-MN	12.53 (0.65)	6.61 (0.92)	16.93 (1.33)	131 (20)	145 (17)	123 (79)	0.55	0.78
MC-HN	14.57 (0.78)	10.12 (0.24)	24.69 (0.66)	266 (30)	415 (16)	318 (22)	0.83	0.97
MP-0N	8.76 (1.17)	2.98 (0.67)	8.82 (1.27)	16 (22)	40 (22)	92 (24)		
MP-MN	8.89 (1.74	4.88 (0.44)	13.77 (2.16)	340 (86)	236 (55)	310 (71)	0.48	0.45
MP-HN	9.20 (1.03)	8.46 (1.72)	17.66 (2.29)	818 (159)	351 (57)	600 (83)	0.96	0.65

For each variable, measurement period and effect, values followed by different letters are significantly different according to Tukey test at $p = 0.05$ level. *n.s. non-significant. † For the pea maize cropping system, the dry grain is the sum of the pea and maize grain dry yield. †Values in brackets represent the standard error.Bold highlighting the p-values.

During the second year (2020), the maize phase of the MC system presented greater emissions than the maize phase of the MP rotation. However, in 2019, no significant differences were observed between MC and MP. As commented previously, there was a great difference in the pea production obtained in both years. During the 2020 maize growing season, the lower N inputs from pea residues together with the reduction of N fertilizer in the fertilized treatments (50 kg N ha^{-1} less N fertilizer than that in the MC plots) resulted in lower soil N_2O emissions in the MP rotation compared with the MC system. However, oppositely, in 2019, when greater pea production was obtained, the maize after pea with the highest N rate (MP-HN) achieved the greatest cumulative soil N_2O emissions, being greater than the maize monoculture at high N rates (MC-HN). This finding is in agreement with the results finding by other authors (Drury et al., 2008; Adviento-Borbé et al., 2010; Saha et al., 2021) who reported greater soil N2O emissions during the maize phase of the maize-legume cropping system compared to systems that did not include legume.

In the MP rotation, the build-up of soil N levels, as a result of the noteworthy amount of pea stover left after the 2019 pea season (average N supply from aboveground pea biomass of 257 kg N ha^{-1}, data not shown) together with the significant amount of N applied in the HN rate (350 kg N ha^{-1}) boosted soil N_2O emissions. Interestingly, this behaviour, however, was not observed when the maize was fertilized with adjusted N rates (MN) considering that in MN rates, independently of the precedent phase (pea or fallow), soil N_2O emissions during maize were similar.

In Mediterranean systems, intensive irrigated crops involve large N additions (Cela et al., 2011). In our experiment, soil N_2O losses positively responded to the amount of N applied, particularly, during the maize phase (Bouwman et al., 2002; Cayuela et al., 2017). Cumulative soil N_2O emissions showed a non-linear response to the N application rates as has been observed in several studies (McSwiney and Robertson, 2005; Hoben et al., 2011; Kim et al., 2013; Shcherbak et al., 2014). This non-linear response of the soil N_2O emissions to the soil N content was emphasised when the N application rate exceeded the 200 kg N ha^{-1}, N dose that coincided with the upper range of the optimal N rate, 160 and 200 kg N ha^{-1}, for irrigated maize conditions in NE Spain (Berenguer et al., 2008; Pareja-Sánchez et al., 2020) and reached the maximum emission at 400 kg N ha^{-1}. This behaviour of the soil N_2O emissions was explained by the N surplus which remained available in the soil for the production of N_2O by soil microorganisms. Therefore, the high N rates considered in our experiment consisting of 400 and 350 kg N ha^{-1} rates (MC and MP, respectively) would have greatly surpassed the N needs by the maize crop and thus favour soil N_2O emission.

The increase in the N application rate did not have a significant impact on the grain yield. In none of the growing seasons nor the entire experimental period. This finding is in concordance with other studies (Binder et al., 2000); Pareja-Sánchez et al. (2020) that reported similar maize grain yields when they applied medium and high nitrogen application rates, showing the existence of an optimal N application rate between 150–200 kg N ha^{-1} (Berenguer et al., 2008). Conversely to the grain yield, the grain yield-scaled emissions always showed significant differences between fertilized treatments, with the lowest yield-scaled emissions in the Medium treatment. The non-linear response of the cumulative soil N_2O emissions to the N application rate, which resulted in the highest cumulative soil N_2O emissions under the HN treatment could not be compensated by the differences in grain yield between the High and the Medium N rates.

Emission factor values reported in this study presented an EF value lower than the 1% established by the Intergovernmental Panel on Climate Change (IPCC, 2014). The mean EF obtained (0.71%) is in agreement with the EF reported by Cayuela et al. (2017) for irrigated-maize cropping with N application rates between 100 and 400 kg N ha^{-1} in a meta-analysis study for the Mediterranean region. Besides, and in agreement with Kim et al. (2013), Shcherbak et al. (2014) and Cayuela et al. (2017), the application of a nitrogen dose higher than the optimal N rate leads to an increase in the EF values, observing always the greatest EF values under the highest N application rates.

Conclusion

The results presented in this work showed that cropping diversification and N fertilization affected soil N_2O emissions. The impact of introducing a pea crop in maize monocultures depends on the quantity of residues produced by the pea, particularly when maize is fertilized at high N rates. However, at optimal N rates, soil N_2O emissions during the maize crop are similar independently of the precedent phase (pea or fallow). Likewise, the application of N at optimal rates led to a reduction of yield-scaled emissions and N emission factor which showed values lower than the 1% nitrogen emission factor proposed by the IPCC. Our study only comprised 2 years of results, therefore conclusions should be taken with caution since further research is needed to address the mid-/long-term impact of legume incorporation and N fertilizer rates on maize soil N_2O emissions. Nonetheless, the results obtained in this work pointed out the importance of considering the N release from legumes and the adjustment of N rates to crop requirements to reduce soil N_2O emissions in irrigated maize systems of Mediterranean areas.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

SF-L: Conceptualization, data acquisition, writing—original draft, data curation, methodology, formal analysis, investigation. VL: Data acquisition, formal analysis. MA-A: Writing—review; editing. AB: Data acquisition, formal analysis. IK-S: Data acquisition, formal analysis. JA: Writing—review; editing. JÃ-F: Conceptualization, methodology, investigation, writing—review; editing, supervision, project administration, resources, funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.914851/full#supplementary-material

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