Università Politecnica delle Marche
Department of Agricultural, Food and Environmental Sciences
Scientific field: AGR/02 - Agronomy and field crops

Ph.D. IN AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES
XXXIII (19°) EDITION (2017 -2020)

Innovations to enhance Ecosystem Services in Mediterranean forage-cereal system

Ph.D. Thesis of :

Nora Baldoni

Tutor Dott. Marco Toderi

Cotutor Dott. Paride D'Ottavio

Cotutor Prof. Stefania Cocco

PhD School Director:
Bruno Mezzetti

ACADEMIC YEAR 2019/20

Università Politecnica delle Marche
Department of Agricultural, Food and Environmental Sciences
Via Brecce Bianche — 60131 - Ancona, Italy

Università Politecnica delle Marche
Department of Agricultural, Food and Environmental Sciences
Scientific field: AGR/02 - Agronomy and field crops

Ph.D. IN AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES
XXXIII (19°) EDITION (2017 -2020)

Innovations to enhance Ecosystem Services in Mediterranean forage-cereal system

Ph.D. Thesis of :

Nora Baldoni

Cotutor Dott. Paride D'Ottavio

Tutor Dott. Marco Toderi

Cotutor Prof. Stefania Cocco

PhD School Director:
Bruno Mezzetti

ACADEMIC YEAR 2019/20

Acknowledgements

I would like to express my highest appreciation and gratitude to my supervisors, Dr. Marco Toderi, Dr. Paride D'Ottavio, Prof. Stefania Cocco, and Prof. Giuseppe Corti for supporting and guiding me during the whole PhD period.
All wonderful employees of ASSAM (Jesi) for their precious help.
My colleagues Matteo Francioni and Laura Trozzo, who have strongly helped me with my research so many times, supported me as friends, and without whom this PhD studies would not have been accomplished

Thank you
Nora Baldoni

''Per aspera ad astra''

Abstract

In rainfed areas of Central Italy a conventional crop rotation includes a long-lasting meadow interrupted by 2-3 years of cereal crops. The decision when to interrupt the meadow and to start the cereal cropping influences the ecosystem services (ES) that are provided by this forage-crop system. The present research wanted to investigate which practices can be adopted to manage this transition from a point of view of climate change mitigation and other ES.

Three field experiments were carried out with this scope:

The 1st experiment wanted to evaluate whether alfalfa meadow interruption in Mediterranean climate is convenient compared to its prolongation over the 6th year in terms of ES. I wanted to find out if its ES benefits can be replaced by the addition of a high rate of biochar addition before the growing of 2 successive wheat crops.
Twelve ES services were examined. Alfalfa prolongation appeared to be the best choice to provide the highest number of ES. Biochar addition after alfalfa interruption didn't show any significant increase of the ES, nor on plant biodiversity, which always resulted higher in alfalfa crop.

The 2nd experiment compared N2O soil emission at the end of alfalfa by tillage followed by wheat growing with the continuation of a long-term alfalfa.
The results indicated that tillage provoked a greater N2O emission (0.37 g N-N2O ha-1h-1) than that from undisturbed alfalfa (0.14 g N-N2O ha-1h-1). But this greater emission didn't last long; after one year the emissions of the 2 options were similar. In conclusion, to reduce the GHGs emission linked to alfalfa termination it is recommended to delay the tillage under of alfalfa vegetation in the autumn, soon before wheat sowing.

The 3rd experiment was carried out to determine if biochar addition can reduce the detrimental effects of alfalfa termination linked both to GHG emission and to pedological properties. For this scope I studied how a high rate of biochar (60 Mg ha^{-1}) influences the soil characteristics and the behavior of 12 soil enzymes linked to the main element cycles in the soil. The comparison was made in the 2 wheat crops which were grown subsequently after alfalfa interruption. The results showed a slight tendency of biochar to promote soil aggregate stability and an increase of soil organic matter. Biochar incorporation, in the short term, had no effect on soil pH, Total N, available P and wheat yields. Biochar didn't influence the enzymes, perhaps because of its alkalinity. Conversely tillage for wheat sowing and biochar incorporation resulted in an increased enzyme activity, mainly of those linked to carbon cycle.

As a conclusion, the results confirm the beneficial effects of rainfed alfalfa meadow, which appeared the best crop even if it is kept for more than 6 years. The forage production might decrease, but the ES that it provides, are always higher than those of a cereal-based system.
To terminate alfalfa (because of a drastic yield reduction of forage quantity), it's better to delay the ploughing for wheat late in the season, to reduce the N2O emission. Biochar addition might replace some of the benefits of alfalfa, but this research, didn't reveal any significant effect of biochar on the enzyme dynamic, probably because it was a woody, alkaline, high temperature biochar spread on an alkaline soil. Instead, it showed an influence on some physical chemical soil characteristics (aggregate stability and soil organic matter build up) that, if confirmed in the long term, can be of paramount importance on the hills of Mediterranean area.

Contents

1 Introduction

1.1 Preface

In central Italy, the crop rotation systems include the sequence of long-term (4-5 years) alfalfa-crop and rainfed cereal crops (e.g., durum wheat) (Pecetti et al., 2008, Monaci et al., 2017).
Alfalfa crop offers many environmental and economic benefits. The literature reports that long-term alfalfa meadows result in improved soil structure (Meek et al., 1989, Martin et al., 2020) and in a drastic reduction of soil erosion (Bronick and Lal, 2005; Ferreira et al, 2015). Moreover, when they are interrupted, they leave in the soil great amounts of residues which are rich in nitrogen, whose benefits can last even in two following crops (Ballestra and Lloveras, 2010). Also, the long-term alfalfa crop can significantly increase the soil organic matter content (Börjesson et al., 2018).

In Mediterranean regions, however, alfalfa meadows cannot be indefinitely maintained (Pecetti et al., 2008), like occurring in China (Xia et al., 2013). It is interrupted when its production becomes too low (Julier et al., 2017). For alfalfa termination, tillage with incorporation residues (i.e., by ploughing at 40 cm depth in the middle of June) is commonly used. In Central Italy, this practice is generally performed in the 4th cropping year, although it might be maintained longer.

The change from an alfalfa ley to annual crops is a crucial passage, with potential generation of trade-offs between ecosystem services. For example, the alfalfa termination by tillage would likely favours mineralization of the soil organic matter accumulated under the ley (Monaci et al., 2017; Francioni et al., 2020). Many papers report that ploughing under alfalfa causes the emission of greenhouse gases (GHG) (especially CO2 and N2O) into the atmosphere (e.g., Navaz et al., 2016; Krauss et al., 2017), with impact on climate change. Some other main issues linked to alfalfa termination in summer are related to the long-lasting bare soil condition until the autumn, before wheat sowing, with potential effects on soil erosion in hilly areas a higher risk of water runoff (Bronick and Lal, 2005) and nitrogen leaching (Kunrath et al., 2015; Wang and Li, 2019). In this perspective, alfalfa termination postponed in autumn might short the time window between N release by alfalfa residues mineralisation and N uptake from subsequent crop.
Under a climate change perspective, another mitigation option could be identified in the use of biochar that can potentially bring several benefits in similar systems (Lehmann et al., 2006). Biochar is the by-product of biomass pyrolysis for energy production. It is mainly made up of aromatic carbon (C) molecules that are recalcitrant to microbe degradation and has a porous structure (Verheijen et al., 2009). It is widely used as a soil amendment in agriculture and, especially when it comes from wood sources and is pyrolyzed at high temperatures, it is a recalcitrant and stable material (Lehman et al., 2015). Its physical and chemical properties can promote soil structure and SOM build-up, contributing to climate change mitigation (Vaccari et al., 2011; Han et al., 2020), but its effects on the soil microbial biomass and activity are still scarcely known (Lehmann et al., 2011).
Even if the effects of the termination of long-term forage crops in terms of carbon cycle and soil fertility (i.e., GHG, SOM, etc) have been deeply studied (Ballestra and Lloveras, 2010, Navaz et al., 2016, Börjesson et al., 2018), the research dealing with the effects of this common practice on other ecosystem services are still few. The production of food and materials for the human wellbeing is the agriculture's main scope. Indeed, this activity provides a wide array of ecosystem services, which are defined as the benefits that people obtain from the ecosystem (Millennium Ecosystem Assessment, 2005). The provision of these ecosystem services depends on the different management practices (e.g., fertilization, tillage) that human applies in the different agro-ecosystems (Palma e al., 2014). The Millennium Ecosystem Assessment (2005), a major assessment of the human impact on the environment, distinguishes four types of ecosystem services:

(i) Supporting: services that support the ecosystem functions and operate in the long-term. For example: maintaining soil fertility, through the improvement of physiochemical characteristics of soils (e.g., ground cover in the rainy months to prevent soil erosion, nutrients availability, soil enzyme activity, soil organic matter content).

(ii) Provisioning: services identified as products, such as food. From this point of view, the forage production for livestock systems is also included in this group.

(iii) Regulating: services obtained from the regulation of ecosystem processes in agriculture systems, such as climate and water regulation/purification. Considering the wide area that is interested by farming systems, the regulation can act at different spatial (local, landscape, global) and temporal (short, medium, long-term) scales.

(iv) Cultural: agriculture represents an important part of the history and tradition of all peoples in the World. The primary sector, thus, provides non-material benefits to humans, such as spiritual enrichment, cognitive development, reflection, and aesthetic experiences.

On the above-mentioned perspectives, the aim of the present research was to assess the effect innovative practices (i.e., postponed tillage for alfalfa termination and biochar soil amendment) in the alfalfa-wheat system in terms of soil GHGs emissions and maintenance of soil fertility. For this scope, (i) the amount of soil N2O emissions after alfalfa termination that was postponed in the autumn and performed by spading and (ii) the effects of biochar soil amendment on soil enzyme activities, regulating soil fertility, were assessed.

To fully evaluate two alternatives 'climate friendly' management options to the wheat-after-wheat crops after alfalfa termination (i.e., postponing alfalfa termination by keeping alfalfa for an even longer time; add biochar to the wheat-after-wheat grain to improve carbon sequestration), an ecosystem service multisectoral approach is adopted using a basket of 13 indicators estimated to compare each option.

The thesis contains three chapters based on the research activity performed during the PhD. Each chapter was written as a standalone manuscript. The body of the thesis is the following:
(i) A research paper entitled: Ecosystem service basket as affected by management options in a Mediterranean forage/cereal-based copping system under climate change perspective.

(ii) A research paper entitled: Soil N2O emissions after perennial legume termination in an alfalfa-wheat crop rotation system under Mediterranean conditions

(iii) A research paper entitled: Effect of high temperature pyrolyzed biochar on physicochemical properties and enzyme activities of sub-alkaline soil, and on wheat production.

Literature

Ballesta A., Lloveras J., 2010. Nitrogen replacement value of alfalfa to corn and wheat under irrigated Mediterranean conditions. Spanish Journal of Agricultural Research, 8 (1): 159- 169. https://doi.org/10.5424/sjar/2010081-1155

Börjesson G., Bolinder M.A., Kirchmann H., Kätterer T., 2018. Organic carbon stocks in topsoil and subsoil in long-term ley and cereal monoculture rotations. Biology and Fertility of Soils, 54: 549–558. https://doi.org/10.1007/s00374-018-1281-x

Bronick C.J., Lal R., 2005. Soil structure and management: a review. Geoderma, 124: 3–22. https://doi.org/10.1016/j.geoderma.2004.03.005

Ferreira V., Panagopulos T., Cakula A., Andrade R., Arvela A., 2015. Predicting soil erosion after land use changes for irrigating agriculture in a large reservoir of southern Portugal. Agriculture and Agricultural Science Procedia, 4: 40-49. https://doi.org/10.1016/j.aaspro.2015.03.006.

Francioni M, Lai R, D'Ottavio P, Trozzo L, Kishimoto-Mo AW, Budimir K, Baldoni N, Toderi M, 2020. Soil respiration dynamics in forage-based and cereal-based cropping systems in central Italy. Sci. Agr. 77, e20180096.

Han L., Sun K., Yang Y., Xia X., Li F., Yang Z., Xing B., 2020. Biochar's stability and effect on the content, composition and turnover of soil organic carbon. Geoderma, 364: 114-184. https://doi.org/10.1016/j.geoderma.2020.114184

Julier B, Gastal F, Louarn G et al (2017) Lucerne (alfalfa) in European cropping systems. In: Legumes in cropping systems. CAB International, Wallingford, pp 168–192.

Krauss M, Ruser R, Müller T, Hansen S, Mäder P, Gattinger A, 2017. Impact of reduced tillage on greenhouse gas emissions and soil carbon stocks in an organic grass-clover ley - winter wheat cropping sequence. Agr. Ecosyst. Environ. 239:324-33.

Kunrath T.R., Berrangerc C. de, Charrier X., Gastal F., César P., Carvalho de F., Lemaire G., Emile J.C., Durand J.L., 2015. How much do sod based rotations reduce nitrate leaching in a cereal cropping system? Agric. Water Management, 150: 46–56. https://doi.org/10.1016/j.agwat.2014.11.015

Lehmann J., Rillig M.C., Thies J., Masiello C.A., Hockaday W.C., Crowley D., 2011. Biochar effects on soil biota–A review. Soil Biology & Biochemistry, 43 (9), 1812–1836. https://doi.org/10.1016/j.soilbio.2011.04.022

Martin G., Durand J.-L., Duru M., Gastal F., Julier B., Litrico I., Louarn G., Médiène S., Moreau D., Valentin-Morison M., Novak S., Parnaudeau V., Paschalidou F., Vertès F., Voisin A.S., Cellier P., Jeuffroy M.H., 2020. Role of ley pastures in tomorrow's cropping systems. A review. Agronomy for Sustainable Development volume 40, Article number: 17. https://doi.org/10.1007/s13593-020-00620-9

Meek B.D., Rechel E.A., Carter L.M., DeTar W.R. 1989. Changes in infiltration under alfalfa as influenced by time and wheel traffic. Soil Sci. Soc. of America Journal, 53 (1): 238-241. https://doi.org/10.2136/sssaj1989.03615995005300010042x.

MEA (Millenium Ecosystem Assessment) (2005) Ecosystems and human well-being: a framework for assessment. Island Press, Washington

Monaci, E., Polverigiani, S., Neri, D., Bianchelli, M., Santilocchi, R., Toderi, M., Ottavio, P.D., Vischetti, C., 2017. Effect of contrasting crop rotation systems on soil chemical and biochemical properties and plant root growth in organic farming: first results. Ital. J. Agron. 12, 364– 374. https://doi.org/10.4081/ija.2017.831

Navaz A., Lal R., Shrestha R.K., Farook M., 2016. Mulching affects soil properties and greenhouse gas emissions under long-term no-till and plough till systems in Alfisol of central Ohio. Land Degradation and Development, 28 (2), 673-681. https://doi.org/10.1002/ldr.2553

Palma C., Blanco-Canquib H., DeClerckc F., Gaterea L, Graceda P., 2014. Conservation agriculture and ecosystem services: An overview. Agriculture, Ecosystems and Environment 187: 87–105. http://dx.doi.org/10.1016/j.agee.2013.10.010

Pecetti L., Carroni A.M., Annicchiarico P., Manunza P., Longu A., Congiu G. 2008. Adaptation, summer survival and autumn dormancy of lucerne cultivars in a South European Mediterranean region (Sardinia). In “Sustainable Mediterranean grasslands and their multifunctions”. Porqueddu C., Tavares de Sousa M.M. (eds.). Zaragoza CIHEAM / FAO / ENMP / SPPF, 2008. p. 471-474 (Options Méditerranéennes: Série A. Séminaires Méditerranéens; n. 79). ISBN: 2-85352-378-0

Trozzo L., Francioni M., Kishimoto-Mo A.W., Foresi L., Bianchelli M., Baldoni N., D’Ottavio P., Toderi M., 2020. Soil N2O emissions after perennial legume termination in an alfalfa-wheat crop rotation system under Mediterranean conditions. Italian Journal of Agronomy; 15 (3): 229- 238. http://dx.doi.org/10.4081/ija.2020.1613

Vaccari F.P., Baronti S., Lugato E., Genesio L., Castaldi S., Fornasier F., Miglietta F., 2011. Biochar as a strategy to sequester carbon and increase yield in durum wheat. European Journal of Agronomy, 231-238. https://doi.org/10.1016/j.eja.2011.01.006

Vereijen F.G.A., Jeffrey S., Bastos A.C., Velde van der, Diafas I., 2009. Biochar application to soils. A critical review of effects on soil properties, processess and functions. EUR 24099 EN Office for the official publications of the European Communities. Luxembourg: 149 p. https://doi.org/10.2788/472

Wang Z-H., Li S-X., 2019. Nitrate N loss by leaching and surface runoff in agricultural land: A global issue (a review). Advances in Agronomy. DOI: 10.1016/bs.agron.2019.01.007

Xia L., Hoermann G., Ma L., Yang L., 2013. Reducing nitrogen and phosphorus losses from arable slope land with contour hedgerows and perennial alfalfa mulching in Three Gorges Area, China. Catena 110: 86-94. http://dx.doi.org/10.1016/j.catena.2013.05.009

2 Chapter 1

2.1 Ecosystem service basket as affected by management options in a Mediterranean forage/cereal-based copping system under climate change perspective

2.1.1 Abstract

The paper wanted to determine the best practices to manage a six-year-old alafalfa meadow in Mediterranean climate from the point of view of ecosystem services (ES). The evaluated possibilities where: to prolong the meadow or to interrupt it by growing wheat crops in the two successive years. To reduce the inevitable soil organic matter reduction and consequent GHGs emission that alfalfa ploughing under implies, the addition of 60 Mg ha-1 of woody, high-temperature biochar was compared with not-amended wheat (W0) at alfalfa termination. These options were compared based on 13 ES indicators. Results indicate that continuing alfalfa crop can offer a wider basket of ES with respect to its interruption to start a cereal cropping system. Alfalfa, even if old, could provide the best provision services. The negative effect of alfalfa termination, mainly linked to the necessary ploughing, can be partially counterbalanced by biochar addition during soil tillage. It offered supporting services that were like those of alfalfa and provided even better regulating services. Biochar influence on biodiversity and other ES categories were not significant. However, further research is needed to ascertain how long alfalfa can be profitably kept in Mediterranean climate and posible biochar pedological effects in the long term.

2.1.2 Introduction

Agriculture is considered a key-actor in the issue of climate change because it increases the release of greenhouse gases from the soil (IPCC, 2014). Among the management practices, soil tillage is recognized for favoring soil organic matter mineralization (Lescourret, 2015), and, when coupled with fertilization, for increasing greenhouse gas emission (GHG) into the atmosphere (Mitchell et al., 2016), nitrate leaching (Kunrath et al., 2015), soil erosion (Franzluebbers et al., 2014), and other detrimental effects. Currently, cropping systems are increasingly based on intensified techniques that are applied to simpler rotations (e.g., monocultures or successions limited to two crops). While crop yields are generally stagnating (Moore and Lobell, 2015), they appear to be more sensitive to climate change, particularly due to increased water stress (Meng et al., 2016) and higher air temperature (Zaho et al., 2017).

Ecosystems services (ES) are defined as the benefits that people obtain from the ecosystems and, according to the framework proposed by the Millennium Ecosystem Assessment (MEA, 2005), they can be divided in four categories: provisioning services (e.g., production of food, wood and fuel), supporting services (e.g., nutrient cycling, soil formation, habitat provision), regulating services (e.g. climate regulation, reduction of water runoff and soil erosion), and cultural services (e.g., spiritual, aesthetic, educational services). Farming systems can provide a wide array of ecosystem services to human being (Dale and Polasky, 2007). In general, the provisioning of ES varies as a function of agronomic practices (Palma et al., 2014).

Mediterranean climate is characterized by mild summers nd two wet seasons, spring and autumn. In this area, the high provisioning services of annual crops succession often come at the expenses of increased greenhouse gas emission (Montanella, 2007) and soil erosion (Bronick and Lal, 2005). In these systems the inclusion in rotation of perennial legumes such as alfalfa (*Medicago sativa* L.) can offer great ecological improvements, including the reduction of GHG emissions.

Alfalfa, which is the most important ley crop in the world, provides elevated yields of a forage with high protein content (Cavero et al., 2017), also under rainfed conditions in temperate areas (Rogers et al., 2016). Its cropping is highly energy efficient (Mobtaker et al., 2010) because it does not require nitrogen fertilization and pesticides inputs, that instead are particularly energy demanding (Ballesta and Lloveras, 2010). Moreover, ley crops such as alfalfa provides for other ecosystem services including the enhancement of soil structure due to soil tillage suspension during its copping years (Meek et al., 1989). The capacity of alfalfa ley to accumulate organic matter in the soil is much higher than in cereals, particularly in deep soil layers (Börjesson et al., 2018). For example, a study conducted in Mediterranean climate reported a higher soil organic matter, coupled with a more intense soil respiration in alfalfa-based system with respect to cereal-based system, suggesting an increased soil microbial activity and thus of fertility due to longer alfalfa persistence (Francioni et al., 2018). Most of the alfalfa ES increase with the ley duration (Kelner et al., 1997).

In Mediterranean areas, under rainfed conditions, alfalfa leys normally last up to five years, mainly because of excessive hot and dry summers (Pecetti et al., 2008). When hay yields become economically unsatisfactory, alfalfa is generally terminated by ploughing or herbicide, and generally followed by winter cereals, mainly wheat (*Triticum durum* Desf.) (Malhi, 2010). Cereal crops after a prolonged alfalfa can result in a good production also without fertilizers input because of a significant nitrogen residue in the soil (Vertès et al., 2015). In general, annual crops such durum wheat is usually more attractive for farmers than forage leys from the economic point of view (Montella, 2007). In some other cases, landowners might choice to not terminate the alfalfa field for various reasons including cost-effectiveness to start a new annual crop.

The change from an alfalfa ley to annual crops is a crucial passage, with potential generation of trade-offs between ecosystem services. For example, the alfalfa termination by tillage would likely favours mineralization of the soil organic matter accumulated under the ley (Monaci et al., 2017). This implies the release into the atmosphere of CO_2 (Francioni et al., 2020) and a higher risk of water runoff (Bronick and Lal, 2005) and nitrogen leaching (Kunrath et al., 2015). Some possible solutions have recently been proposed to limit these trade-offs. For example, termination of alfalfa by tillage in autumn might slow the mineralization process (Trozzo et al., 2020) or how the use of biochar can potentially bring several benefits in similar systems (Lehmann et al., 2006).

Biochar is produced by pyrolysis of biomass in the absence of oxygen, is mainly made up of aromatic carbon molecules that are recalcitrant to microbe degradation and presents a porous structure (Verheijen et al., 2010). Generally, wood-derived biochars degrade very slowly in the soil and result in long persistence of organic matter in the soil, contributing to climate change mitigation (Han et al., 2020) and to increase the soil organic matter content (Lehmann et al., 2006). Biochar supply to agricultural land can also provide many other ecosystem services in terms of physical, chemical, and microbiological soil characteristics (Baiamonte et al., 2019). Beside carbon sequestration, biochar can effectively amend soil physical conditions (Kavitha et al., 2018), correct pH (Liu and Zhang, 2012) and decontaminate polluted soils (Paz-Ferreiro et al., 2012). The porous structure of biochar can also increase the water holding capacity (Fischer et al., 2019) and cation exchange capacity of soils (Tan et al., 2017). Moreover, biochar can greatly influence soil microbial activity and diversity. For example, the use of biochar resulted in an enhanced diversity in the soil microbe population (Lehmann et al., 2011). All these properties of biochar can result in higher yields of annual crops (e.g., wheat, Olmo et al., 2014) at a low environmental impact (Yu et al., 2019). In some studies, the use of biochar has been associated to a diverse spontaneous flora, and thus to an increase of biodiversity in the agroecosystem (Major et al., 2005), thus improving its resilience (Smuckler et al., 2010). The use of biochar might also reduce water runoff risks providing for a plant ground cover during wet seasons (Major et al., 2005), which are particularly important in hilly areas that are prone to water erosion (Li et al., 2019). Some negative effects of biochar have been reported (Hilber et al., 2017). Mainly linked to its high content of polycyclic aromatic hydrocarbons (PAH) and heavy metals. It can reduce the earthworm's population and result toxic to the plants Many of them are linked to their content of PAH (Polyciclic Aromatic Hydrocarbons) (Freddo et al., 2012, Wang et al., 2017) that can result toxic to plants and earthworms (Gomez-Eyles et al., 2011), and high concentration of toxic heavy metals and metalloids (Koppoluet al. 2003).

Thus, on a multisectoral perspective as proposed by the Millennium Ecosystem Assessment (MEA, 2005), it is not enough to evaluate only the effect of soil carbon sequestration provided by biochar because trade-offs between ecosystem services are likely to occur. At the same time, it is not clear which synergies or trade-offs between ecosystem services might arise from a long-lasting alfalfa crop. To clearly address different possible management options for these systems here is the need to adopt a multisectoral approach to investigate a basket of ecosystem services. The aim of this study is to evaluate two alternative "climate friendly" management options to the wheat-after-wheat crops after alfalfa termination: i) postponing alfalfa termination by keeping alfalfa for an even longer time; ii) add biochar to the wheat-after-wheat grain to improve carbon sequestration.

The hypothesis under this study is that the biochar addition to the wheat-after-wheat would result in a soil carbon sequestration enhancement without generate significant trade-offs with other ecosystem services. At the same time, keeping alfalfa for an even longer period should guarantee a higher level of biodiversity than the wheat-after-wheat options (with and without biochar addition). To investigate the negative and positive aspects of these two alternative options an ecosystem service multisectoral approach is proposed and a basket of 13 indicators has been estimated for each option.

2.1.3 Materials and methods

2.1.3.1 Study area

The experiment was carried out on a hilly area of Marche region, central Italy (43° 33' N, 13° 25' E; 100 m a.s.l.; 23% slope SW exposure). In this site rainfed alfalfa-wheat rotation is one of the most common cropping systems (Monaci et al., 2017; Trozzo et al., 2020). Within this cropping system, alfalfa is usually mowed from two to three times per year from May to September. Usually, the alfalfa ley duration is up to 5-6 years and after its termination, at least two-three crops are usually grown (mainly wheat, sunflower, and corn, before starting a new alfalfa ley.

The climate of the study area is classified as a variant of the temperate oceanic sub-Mediterranean climate (Agnelli et al., 2008) and it is characterized by a mean annual precipitation of about 800 mm and a mean annual temperature of 14.6 °C. The meteorological data during the study period was recorded by a weather station located 0.3 km from the experimental field and are reported in Figure 1.

The soil of the study area is classified as Inceptisol according to the United States Department of Agriculture soil taxonomy system (Smith, 2014) with a pH of 8.13, a silt-clay texture (36.06% of sand and 25.60% of clay content), a 1.52% of soil organic matter, a field capacity of 24.55% and a wilting point of 17.81% (Trozzo et al., 2020).

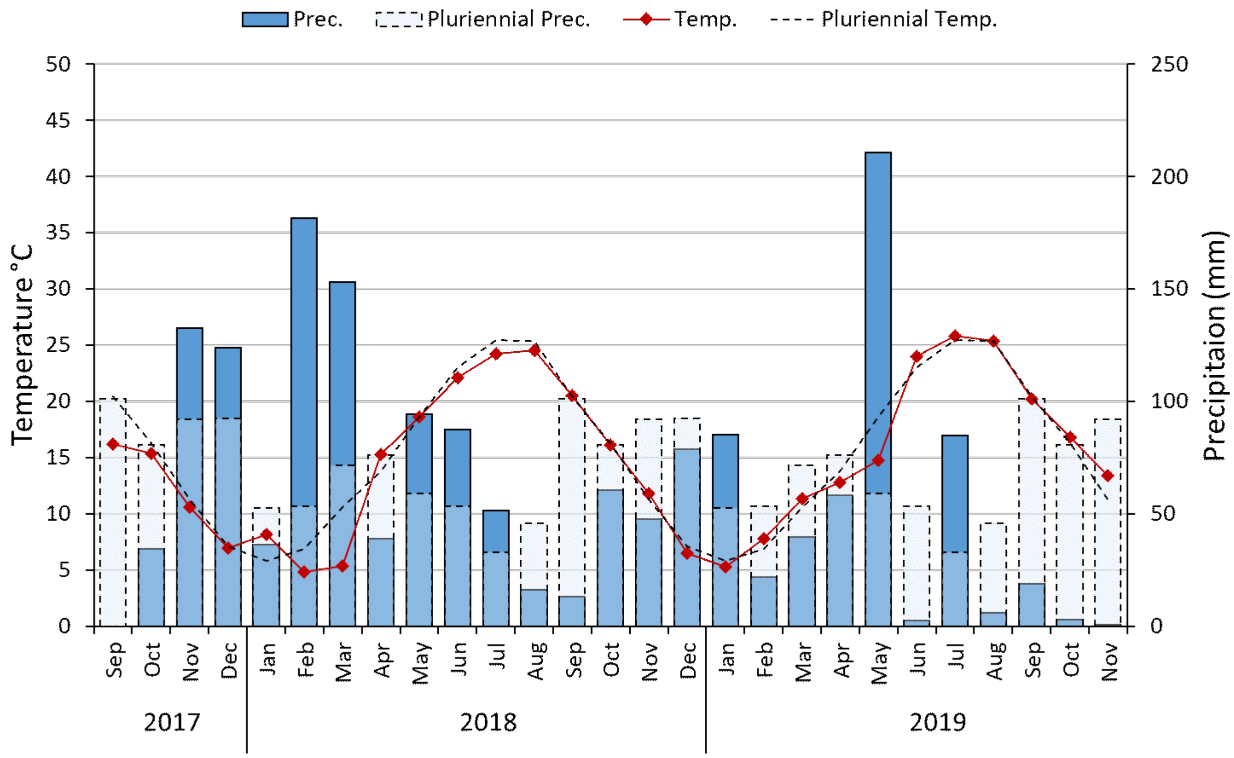


Figure 1. Monthly mean precipitation and air temperature during the experimental period (September 2017 – September 2019) and the long-term period (1998 – 2012) in the study area.

2.1.3.2 Experimental design and management practices

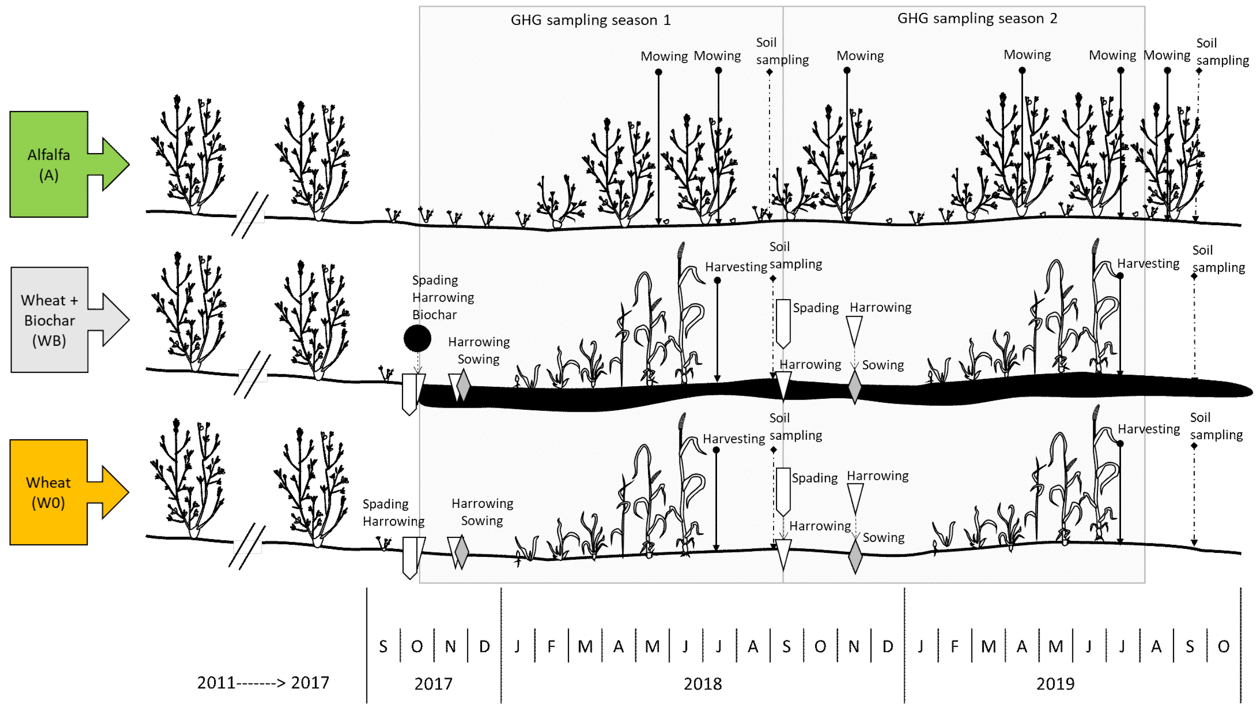
a) Experimental design

The field trial lasted from October 2017 to September 2019. In the Summer 2017, an area that was uniform for soil, crop vegetation and topographic conditions was identified within a 6-year-old alfalfa ley and was fenced to prevent any disturbance. In this area, three experimental treatments were allocated according to a complete randomised block design with three replicates, with individual plots of 2.5 m × 10.0 m. The compared treatments were a 6-year-old alfalfa ley continued during all the experiment (continuous alfalfa), wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.)) sowed after the termination of the 6-year alfalfa (W_0) and the same wheat with a soil addition of 60 t ha^{-1} of biochar (W_B). The used biochar originated from a mix of beech (*Fagus sylvatica* L.), pine (*Pinus pinea* L.), and fir (*Abies alba* Mill.) wood and was pyrolyzed at about 850 °C in an industrial plant system.

b) Management practices

For the continuous alfalfa (A), three mowing per year were performed with a bar mower set at a cutting height of 50 mm from the ground. In both years, the first and second mowing were performed at the beginning of May and July, the third mowing between beginning of September (2018) and at the end of August (2019). Soon after this operation all the mowed vegetation was removed from each plot. The treatment A did not require any fertilizers, amendments, pesticides, or irrigation.

For both W_0 and W_B, the termination of the 6-year-old alfalfa filed was performed on October 11th of 2017, using a spading machine to a depth of 0.2 m. All alfalfa residues were incorporated into the soil and accounted approximately for 2.70 ± 0.23 t ha^{-1} of dry matter. In three plots, 60 Mg ha^{-1} of biochar was applied on the soil surface in form of powder on October 16th, 2017. For both wheat-after-wheat (with and without biochar), a subsequent tillage was performed with a rotary harrowing to 0.15 m depth in the same date of biochar spreading. This also allowed the biochar to be incorporated in the soil in W_B. The biochar was applied only once during all the monitoring periods. For both W_0 and W_B a second tillage with rotary harrowing to 0.15 m depth was performed on November 21st. For both W_0 and W_B, wheat (cv. 'Antalis') was sowed on 23rd November 2017, at a sowing rate of 400 seeds m^{-2} and at a depth of 30 mm. Wheat was harvested on July 4th of 2018 in both wheat-after-wheat (with and without biochar). In 2019 the tillage operations were the same of the previous year (spading to 0.3 m depth, followed by two rotatory harrow passages to 0.15 m depth performed respectively on the beginning of September and half of November). On 16th November 2019 wheat was sown in both W_0 and W_B with the same procedure and rates described for 2018. Wheat was harvested on July 4th of 2019 in bothW_0 and W_B. For all the treatments no fertilizers, amendments and pesticides were applied throughout the whole monitoring period.



"

Figure 2. Management practices applied to the different treatments during the study period (October 2017-October 2019).

2.1.3.3 Sampling

a) Biomass sampling

For all the treatments, biomass was sampled in the central part of the plot and it was immediately transported to the laboratory for further analysis. For the continuous alfalfa, the sample area was of 1 m^2 (1.0 m x 1.0 m) while for both W_0 and W_B it was 2 m^2 (1.0 m x 2.0 m). For the continuous alfalfa, the sampling date correspond to the date of mowing (figure 2) and it was performed tree-times per year. For both W_0 and W_B the biomass sampling was performed at the harvest dates, thus once per year (figure 2). For the continuous alfalfa, all the above-ground vegetation was removed (i.e., alfalfa and other species), while for both W_0 and W_B only wheat plants were cut at the base and removed.

b) Soil sampling

For each treatment, soil was sampled on 5th September 2018 and 25th September 2019 (Figure 2). On each date, the soil was collected in three points within each plot by a manual 0.5-diameter auger from one depth layer of 0-0.4 m. The soils collected from the same plot was mixed to obtain a single sample. Soils samples were taken immediately to the laboratory and sieved through a 0.02 mm mesh sieve and stored in open plastic bags at ambient temperature for subsequent analyses.

c) Greenhouse gas sampling

Soil N_2O, CO_2 and CH_4 emission were monitored during the whole monitoring period using closed static PVC chambers following the method described by Trozzo et al. (2020). Briefly, the chambers were permanently installed in the soil (depth, 0.1 m) and only removed for soil tillage, after which they were immediately reinstalled. Gas samples were collected between 9:00 a.m. and 12:00 a.m. (standard time), approximately every 15 days except for the period between tillage and sowing when they were intensified to 3-4 days. Before each sampling, the above-ground parts of the plants inside the chambers were clipped off to avoid disturbance to the soil gas emissions. The chambers were placed in position for 45 min, during that time four gas samples were withdrawn from the headspace of each chamber (30 mL each, at 15 min intervals). The gas samples were injected into 30 mL glass pre-evacuated vials sealed with a butyl rubber septum (Parkin and Venterea, 2010). In all collected air samples, the following gases concentrations were determined: N_2O and CH_4, by gas chromatography (GC8A; Shimadzu Corporation, Kyoto, Japan) with an electron capture detector; CO_2 flux was measured by a Li-Cor 7000 system (LI-COR, Lincoln, NE, USA). Soil temperature was measured in each date of GHG sampling at a depth of 0.10 m with a soil thermometer (Model: 620-0909, VWR International, Italy). The GHG sampling was divided in two seasons of equal length (323 days each), the first started and ended with the spading performed in W_0 and W_B plots while the second started the day after the spading 2019 and ended exactly 323 days after (figure 2).

d) Floristic diversity and soil vegetation cover

Floristic diversity was estimated three times per year in the A plots (i.e., in the same date of alfalfa mowing), after the wheat harvesting in the W_0 and W_B plots. Soil vegetation cover was estimated throughout the monitoring period for each treatment (A, W_0 and W_B) (figure 2).

2.1.3.4 Estimation of ecosystem service proxies

A variety of different ground-based indicators were elaborated starting from the samplings described above. These were selected to assess the provision of a well-balanced basket of ecosystem services. Dry matter production (DM) and Crop energy output (NRG) have been considered proxies of Provisioning ecosystem services; Global warming potential (GWP), Greenhouse gas intensity (GHGI), Total organic Carbon (TOC) and temperature sensitivity to soil respiration (Q_{10}) have been considered proxies of Regulating ecosystem services; Soil vegetation cover (SVC), pH, Soil total Nitrogen (N-tot), Soil Carbon-Nitrogen ration (C/N) and three biodiversity indexes (i.e., Species richness, Shannon–Wiener diversity and Evenness indices) were considered proxies of Supporting ecosystem services.
A final integrated ecosystem services index was developed following the method described by Papanastasis et al. (2015).

a) Provisioning services
For the continuous alfalfa, the forage dry matter content was estimated by sample oven drying at 65 °C for 72 hours. For both W_0 and W_B, the harvested plants were threshed using a Wintersteiger Delta combine to determine the wheat grain production.
To estimate the production of gross energy per unit of surface, the different dry matter content per unit surface (alfalfa aboveground grass, wheat grain) was multiplied for their respective energetic contents according to Heuzé et al. (2016). They were 18.1 and 18.2 MJ kg DM^{-1} for alfalfa and durum wheat grain, respectively.

b) Regulating services
For each treatment, the relationship between soil CO_2 emission and soil temperature was determined using the following exponential equation:

$$y = a\, e^{bx} \qquad (1)$$

Where y is the measured soil CO_2 emission, b is the soil temperature at 0-10 cm depth, and x is the fitting parameter. Subsequently, the b parameter from Eq. 1 was used to calculate Q_{10} (Davidson et al., 1998) by the following equation:

$$Q_{10} = e^{10b} \qquad (2)$$

The global warming potential (GWP) for each treatment was calculated according to the IPCC guidelines (IPCC, 2014) using the following equation:

$$GWP = 28 \times CH_4 + 265.28 \times N_2O + CO_2 \qquad (3)$$

The greenhouse gas intensity (GHGI) was calculated dividing the GWP by the crop yield (Shang et al., 2011) using the following equation:

$$GHGI = GWP / \text{grain yield} \qquad (4)$$

The total carbon content was determined for each treatment by the dry combustion Springer-Klee method.

c) Supporting services
For each treatment, the soil pH was measured in water (1:2.5 weight/volume) by a combined glass-calomel electrode potentiometric method, the soil total nitrogen was determined by the Kjeldahl method, and the soil carbon/nitrogen ratio was obtained dividing the total organic carbon by total nitrogen.

d) Biodiversity indicators
Plant diversity was estimated in terms of plant species richness, Shannon–Wiener diversity and evenness indices. The Shannon–Wiener and evenness indices were based on the cover data collected from botanical survey. Species richness was estimated as the mean number of species per survey.

e) Integrated Provision of Ecosystem Services and data analysis
To integrate the information provided by the various indicators assessed into an overall assessment of ecosystem services provision according to Papanastasis et al. (2015), the indicator values for each treatment were standardized and then used to yield a composite value for each treatment: for plant biodiversity (averaging Species richness and Shannon–Wiener diversity and Evenness indices) and for each type of ecosystem service category: Provisioning (averaging dry matter

production and Crop Energy output), regulating (averaging global warming potential, greenhouse gas intensity, total organic carbon, and temperature sensitivity to soil respiration), and supporting (averaging soil vegetation cover and soil pH, total nitrogen, and carbon/nitrogen).
A global value for ecosystem services provision was estimated for each treatment as the average of the integrated standardized values for each ecosystem service category, including plant biodiversity. GWP, GHGI and Q10, as provinding an inverse contribution to the global value for ecosystem services, were considered disservices and for this reason subctracted to this global value.
All the estimated indicators were compared among treatments within the same year of monitoring using a one-way analysis of variance, followed by a Tukey test as *post hoc* analysis. All data were box-cox transformed to meet the assumption of normality and homoscedasticity.

2.1.4 Results

2.1.4.1 Provisioning services

Both dry matter production and crop energy output, considered indicators for the provisioning services, clearly show that the continuous alfalfa resulted in higher production compared to W_B and W_0 in each of the two years with more marked differences in 2019. In neither of the two years of monitoring the addition of 60 t ha^{-1} of biochar resulted in any production differences compared to W_0.

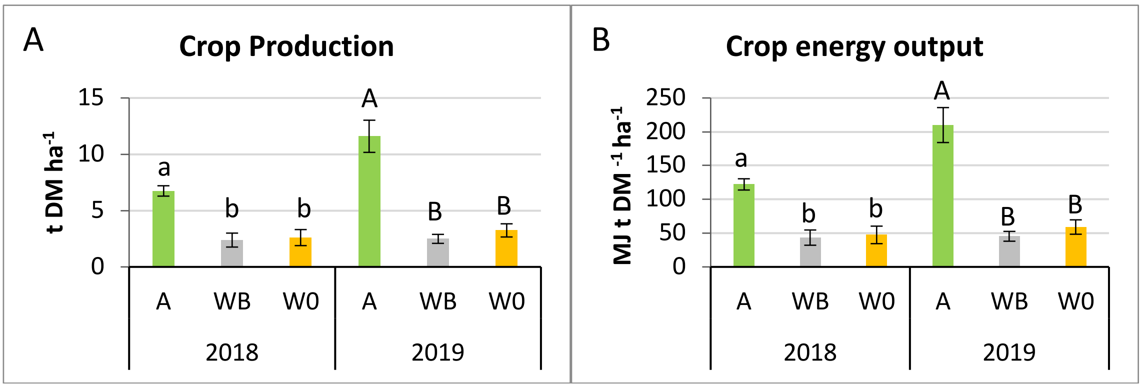


Figure 3. Provisioning Ecosystem Services (dry matter production and crop energy output) of the compared treatments in the two years. Different letters indicate significant differences ($p < 0.05$ = small letters and $p < 0.01$ = capital letters) between crops, within the same year. The vertical lines in each bar indicate the standard error of the means at $p< 0.05$.
A= continuous alfalfa; W_B = wheat + biochar; W_0 = wheat.

2.1.4.2 Regulating services

The global warming potential (i.e., the soil greenhouse gas emissions, expressed as t of equivalents CO_2 ha^{-1}) was not significantly different for any of the treatments in any of the monitoring years. However, in 2019 a contraction of soil greenhouse gas emissions was observed for all the treatments compared to the previous year (Figure 4A). No significant differences in sensitivity to soil respiration were observed among the treatments in any of the year of monitoring nor between the years (Figure 4B). Greenhouse gas index was higher for W_0 and W_B compared to the continuous alfalfa despite significant differences emerged only in 2019 only between the continuous alfalfa and W_B. In the second year of monitoring smaller values were observed for all the treatments compared to the 2018 cropping season (Figure 4C). As expected, the soil total organic carbon measured at 0-40cm depth was always higher for W_B in both the monitoring years compared to the other treatments. In 2019 soil total organic carbon was lowest in W_0 with significant differences also with the continuous alfalfa (Figure 4 D).

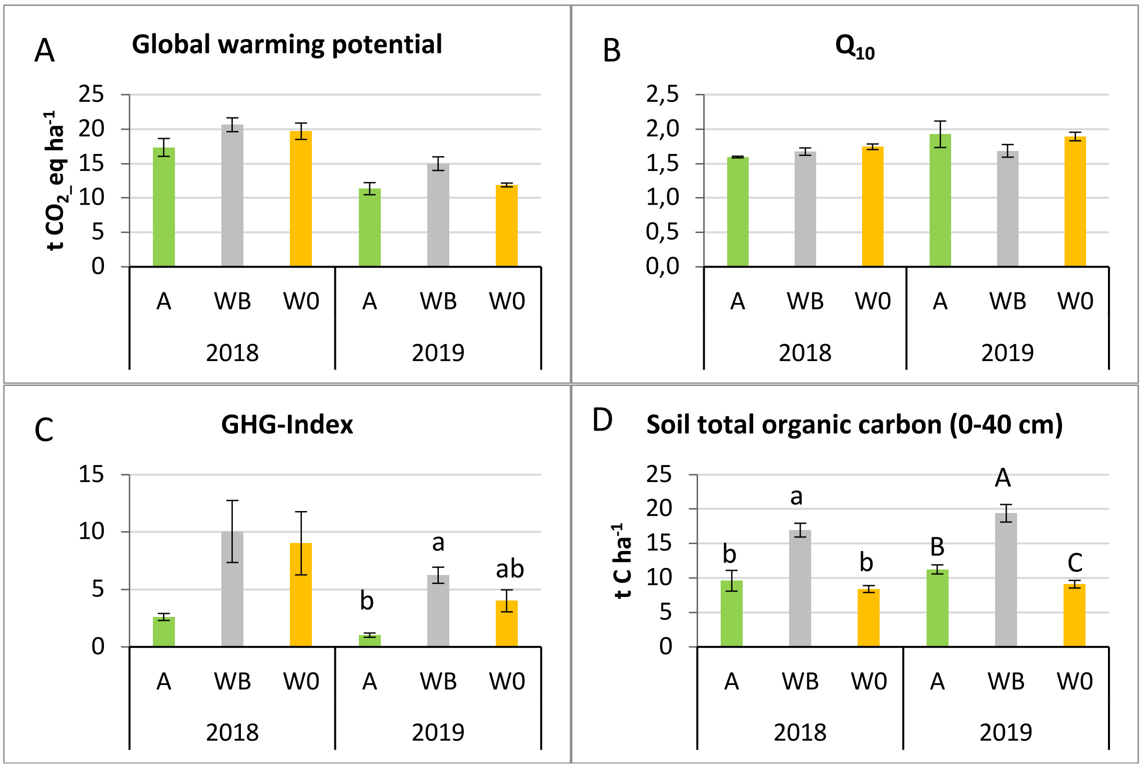


Figure 4. Regulating Ecosystem Services. Different letters indicate significant differences ($p < 0.05$ = small letters and $p < 0.01$ = capital letters) between crops, within the same year. Vertical bars represent the standard errors. Data are means of three replicates per crop. A= continuous alfalfa; WB = wheat + biochar; W0 = wheat.

2.1.4.3 Supporting services

Soil pH was found to be significantly higher for W_0 and W_B in the second year of monitoring (Figure 5A). The soil carbon-nitrogen ratio was significantly higher than the other two treatments in each of the monitoring years with differences more marked in 2019 (Figure 5B). Significant differences in terms of soil total nitrogen emerged only in 2019 when it was found to be higher for the continuous alfalfa (figure 5C). Soil vegetation cover was found to be always higher for the continuous alfalfa throughout all the monitoring period (Figure 5C and 6).

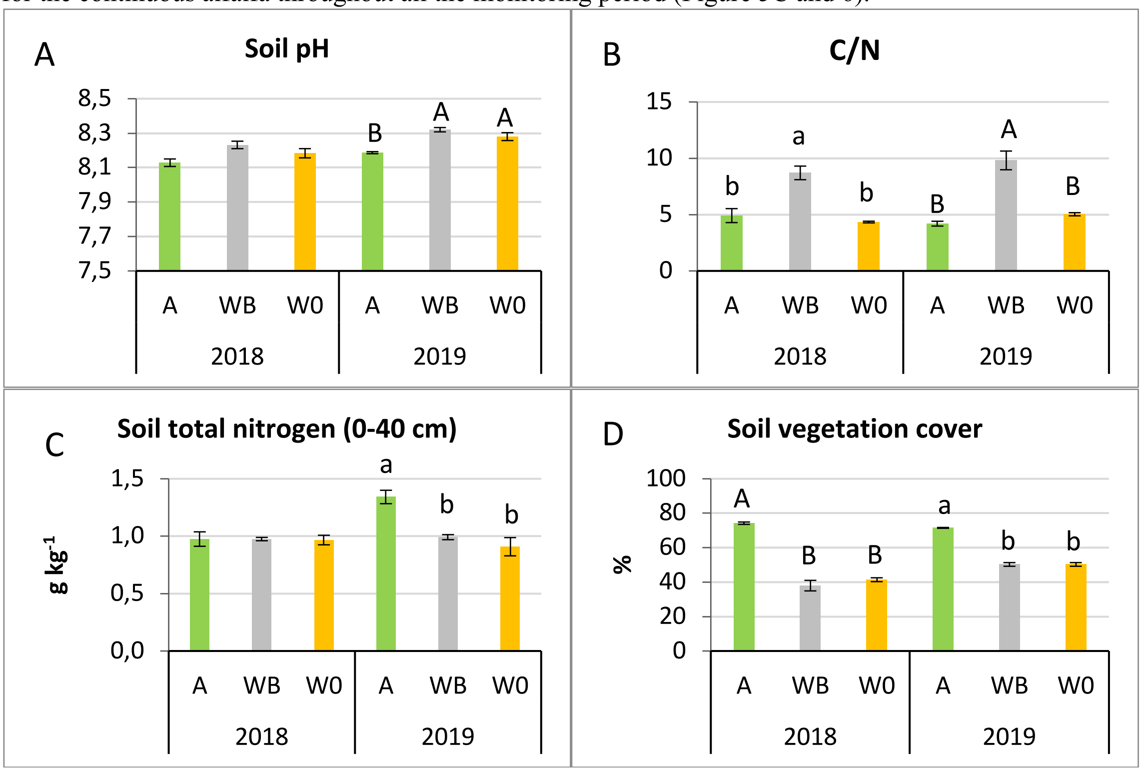


Figure 5. Supporting Ecosystem Services. Different letters indicate significant differences ($p < 0.05$ = small letters and $p < 0.01$ = capital letters) between crops, within the same year. Vertical bars represent the standard errors. Data are means of three replicates per crop. A= continuous alfalfa; WB = wheat + biochar; W0 = wheat.

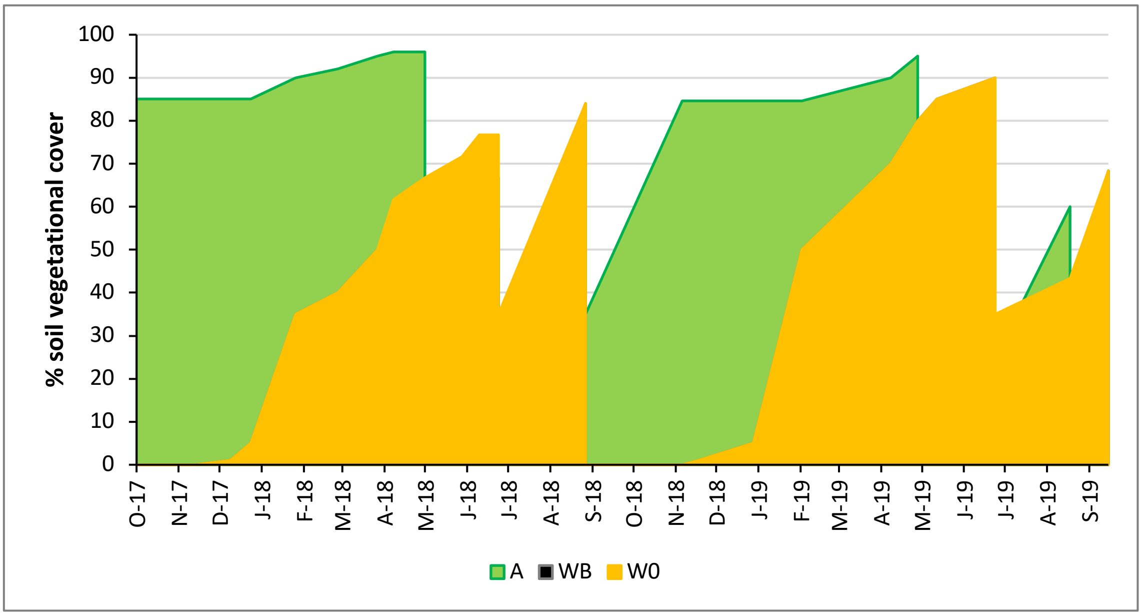


Figure 6. Soil vegetational cover dynamics. A= continuous alfalfa; W_B = wheat + biochar; W_0 = wheat.

2.1.4.4 Plant biodiversity

The estimated plant biodiversity showed marked differences between all the treatments. In the first year of monitoring, both Shannon and Evenness indicators showed significant lower values for W_0 compared to the continuous alfalfa and W_B (Figure 7A and C). In contrast, the number of species (Richness index) was found higher for W_0 compared to the continuous alfalfa in the first year of monitoring (Figure 7B). In the second year of monitoring, no differences emerged between the different treatments according to the Shannon and Evenness indexes (Figure 7A and C), while Richness indexes was found to be higher for W_0 compared to W_B (Figure 7B). Contrasting trends were observed between the years for each treatment. Indeed, for W_0 a general higher plant biodiversity was observed for the second year of monitoring while for the continuous alfalfa and W_B such clear trend did not emerge as Shannon and Evenness indexes showed contrasting trend in respect to the Richness index (Figure 7).

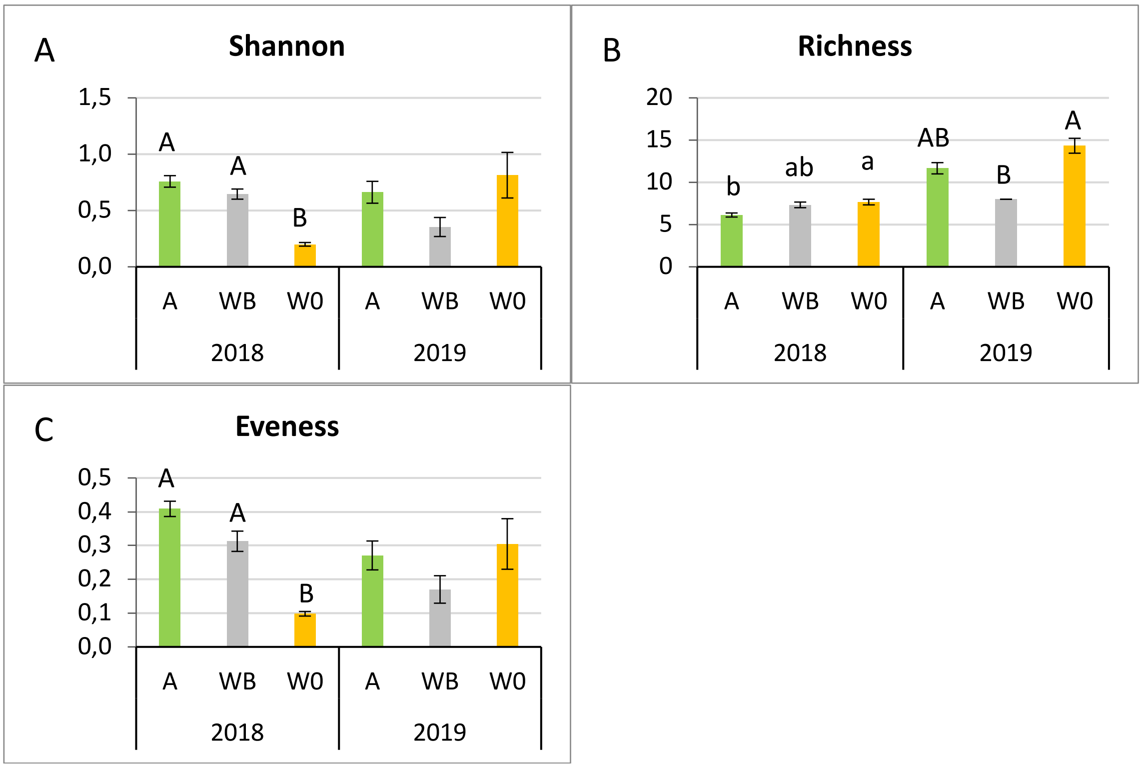


Figure 7. Biodiversity indicators. Different letters indicate significant differences ($p < 0.05$ = small letters and $p < 0.01$ = capital letters) between crops, within the same year. Vertical bars represent the standard errors. Data are means of three replicates per crop. A= continuous alfalfa; WB = wheat + biochar; W0 = wheat.

2.1.4.5 Integrated Provision of Ecosystem Services

Figure 8A shows a synthetic picture of the variation among treatments in the provision of the three analyzed categories of ecosystem services and biodiversity. While provisioning services resulted clearly higher for the continuous alfalfa, supporting services appear to be lowest for W_0. As expected, the biodiversity appears to be highest for the continuous alfalfa and comparable between W_0 and W_B. When all ecosystem services and biodiversity were pooled together, the overall provision of ecosystem services were much lower for W_0 compared to the other two treatments (Figure 8B).

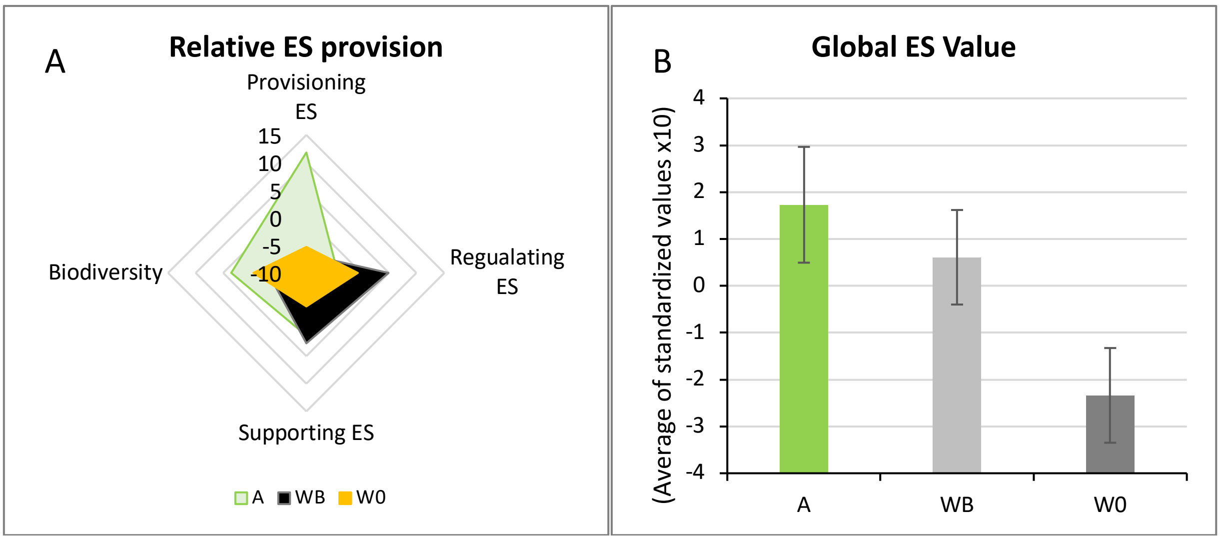


Figure 8. A = Relative contribution of each category of ecosystem services and plant biodiversity. Contribution data are based on the averages of standardized values for the various indicators considered. B= Relative overall provision of ecosystem services for each crop. Vertical bars represent the standard errors.

2.1.5 *Discussion*

2.1.5.1 Enhancing the provisioning services

In the W_0 management option, where a long-lasting alfalfa crop is terminated and wheat is subsequently cultivated without the use of any fertilizer, a relatively low grain yield is expected especially in the first year. The positive effects of alfalfa crops on subsequent wheat crop are well known from decades and progressive lower yield is expected if fertilization is not provided (Hoyt, 1971) and this is reflected in less energy transferred to the kernels/grains (Pierre et al., 2008, Tabak et al., 2020). According to ISTAT (2021), in 2020 durum wheat production in Italy averaged 3.2 t ha-1, but its yields can be even lower if subjected to water and stress (Basso et al., 2012). The low dry matter production of W_0 observed in both the monitoring years was probably due to the absence of spring rains, the drought, and hot conditions in the last stages of the crop cycle (Figure 1), which reduced the filling of the cereal kernels (Basso et al., 2012). Probably, the fasciculate roots of the wheat did not allow it to draw water from the deeper layers of the soil. In fact, wheat productions in 2018 (the least rainy year) were slightly lower than those of the following year (the wettest year).
Recent studies suggest that the use of soil amendments such as biochar to the previous system could have potentially overcome this problem since it can favor greater water retention by decreasing soil compaction (Olmo et al. 2014). However, this effect depends on the quantity and type of soil and the type of biochar and on its feedstock and pyrolysation temperature (Lehman et al., 2015). For example, Vaccari et al. (2011) observed positive effects on biomass and yield of wheat amended with 30 and 60 t ha^{-1} of wood-derived biochar. Olmo et al. (2014) did not observe improvement in wheat grain quality or nutrient content adding 40 Mg ha^{-1} of wood-derived biochar slowly pyrolyzed at 450 °C. In the present experiment, the absence of any effect in the W_B management option could be explained by the very recalcitrant and therefore inactive material distributed from the nutrient point of view (Lehmann et al., 2015). Furthermore, the changes in soil water retention may not have been sufficient to modify the availability of water.
The highest production yields and therefore energy productions were found in the continuous alfalfa (Figure 3A-B). Previous study under Mediterranean conditions showed that rainfed alfalfa crops (4 years old) can be mowed up to four time per year, resulting in about 9 Mg ha^{-1} per mowing and progressive decreasing production in the years (Testa et al., 2011). Despite being considered not any more cost-effective, an alfalfa crop lasting more than 5 years can still provide enough production to be mowed up to three times per year (Porqueddu et al., 2016). This however comes at forage quality and quantity cost because significantly lower yields are in comparison with short-term alfalfa crop (Testa et al., 2011) in a Mediterranean context, and mainly due to the dominant participation of grasses.

2.1.5.2 Enhancing the regulating services

In Mediterranean cropping systems, the main contributor to soil GHG emission is expected to be CO2 and N2O with the latter having a remarkable effect only if fertilization is provided (Sanz-Cobena 2017).
Indeed, Tellez-Rio et al. (2017) found that in Mediterranean climate low cumulative N2O fluxes and yield-scaled N2O losses constituted an important advantage of rainfed cereal/legume agro-ecosystems.The analysis of other GHG sources and potential sinks through NetGWP calculations indicated that No Tillage was the most useful strategy to reduce CO2-equivalent fluxes, mainly as a result of C sequestration and lower fuel consumption. The non-fertilized legume crop was also recommended for reducing Net GWP (in spite of higher N2O emissions under Conventional tillage), mainly through the reduction of inputs (N fertilizers and herbicides). Therefore, the use of Conservation Agriculture practices such as NT and crop rotation including legumes, as opposed to continuous cropping of winter cereals, could be considered as a good strategy in semiarid agro-ecosystems for decreasing Net GWP without affecting crop yield
Moreover. Cayuela et al. (2017), found that the average overall emission factor of the N input from fertilizers relative to N2O in Mediterranean agriculture is 0.5%, which is substantially lower than the IPCC default value of 1%. Also Guardia et al. (2017) report a reduction of 32-40% of N2O emissions without N fertilization in Mediterranean climate.The GHG emissions observed in the present study are in line with expectations because it is known that fertilization influences GHG emissions, especially N_2O emission (Wei *et al.*, 2010; Aguilera *et al.*, 2013; Liu *et al.*, 2015).
Also, the soil organic carbon for the continuous alfalfa and W_0 were in the range of similar soils subjected to tillage, and this might also explain the low GHG emissions because low mineralization is expected when soils are poor in terms of soil organic matter (Francioni et al., 2020). However, in W_0-alike systems, the crop rotation is relatively short and alfalfa crop is planted again after few years and will be likely terminated again within 4 to 5 years (Porqueddu et al., 2016; Trozzo et al., 2020). Despite being widely considered conservative systems, the organic matter that alfalfa accumulates in this soil is low and recent studies suggest that will be lost very quickly (Monaci et al., 2017). This cropping system highlights a criticality of the alfalfa-wheat succession under organic farming that deserves to be better addressed. Some advantages might be provided using alternative practices to the conventional tillage such as the adoption direct sowing with the use of desiccants. However, this is the case of conventional systems as desiccants are not allowed in organic farming and many uncertainties on the effect of such desiccants in respect to soil microbial biomass and respiration are still present with effect that seems to be more marked for non-acidic soils (Nguyen et al., 2016).
The addition of 60 t ha^{-1} of biochar (W_B) did not result in marked enhancements or decrement in the provision of regulating ecosystem services, except for the expected increase of soil organic carbon (Figure 4D). This immediate input of organic

matter did not result in significant increment of soil GHG emission (Figure 4A), also suggesting the absence of a priming effect. Other studies reported a lack of priming effect after biochar incorporation (e.g., Zavalloni et al., 2011). The lack of priming effect in the present study could be explained by both wood feedstock source and the high temperature of pyrolysation. Previous study using the wood biochar in similar soils observed also a no significant alteration of soil moisture or temperature, known drivers of source of soil respiration and N_2O emissions.
The CO_2_eq (i.e., the sum of CO_2, N_2O and CH_4) appears lower than the range reported by Francioni et al. (2020) for similar cropping system, although the different weather conditions might have played a key role in the rates of soil GHG emissions.
Also, the soil organic carbon was found to be relatively low. Lower than the range that are reported for cereal-cropped inceptisols which didn't receive any fertilization for long periods of time (1,9-3,3 g kg-1 in the first 60 cm of depth)(Gosh et al., 2018) In the second year of continuous alfalfa, the soil organic carbon was however higher than the one of W0, supporting the findings of Monaci et al. (2017) which reported a quick (90%) decrement of the soil organic matter during wheat following alfalfa termination.

Enhancing the supporting services and biodiversity
In cropping systems like W0, low nitrogen in the soil is usually present (in an inceptsoil less than 1%, on average, Mulvaney et al., 2009) even if a supply of residual-N is expected from previous leguminous crop in inceptisol (Kumar et al., 2018) that can reach 76 N kg ha-1 in the instance of two-years alfalfa meadow (Ballesta & Loveras J., 2010).
However, in the case of long-lasting alfalfa this residual-N in the soil was probably lower than the one provided by a shorter duration alfalfa crop (3-4 years) (Ning et al., 2020). This lower soil residual-N could be explained by three factors: i) the presence of Medicago sativa is strongly reduced in terms of number of plants per surface unit (Coruh & Tan, 2008) and thus the crop fixes much less N than a shorter duration alfalfa crop, also because of a major weed infestation (Coruh & Tan, 2008); ii) the studied continuous alfalfa was mowed once a year before the starting of the trial. In this case only one mowing per year; but Burnett et al., (2020) found no relation between cutting frequency and alfalfa persistence; iii) perhaps there is a loss in the long-term alfalfa albeit limited, linked to N-leaching (Basso & Ritchie, 2005).
Moreover, a potential detrimental effect is expected after wheat harvest, due to the soil uncovered for a long time (Figure 6) in periods when conditions are favorable for N-leaching (Kunrath 2015) and for soil erosion (Porqueddu 2016), under climate change scenario when intensification of precipitation is expected (Ergon 2018, Moore 2015). A solution to this might be represented by the addition of biochar since it has been suggested that it can improve soil structure (Li et al 2019). The soil cover of W_B practically is equal to the one observed for W_0, while the continuous alfalfa ensured a prolonged and constant cover that goes down only immediately after mowing and is quite quick in recovering.
Biochar (W_B) did not increase the soil pH, and this could be explained by the original alkalinity of the studied soil as previous studies showed that biochar ability in correction pH is general successful in acid soils (Lehman et al., 2015). A long stand of alfalfa is supposed to maintain longer the soil fertility (Ning et al., 2020). Even soil nitrogen resulted higher than the other treatments in the second year after alfalfa termination.

The continuous alfalfa management option can act an erosion prevention as studies shows that prolonged legume crops enhance the soil structure, porosity, (Meek et al., 1989). and soil biodiversity (Lemanceau et al., 2015).
The continuous alfalfa (at the 7 and 8 years) did not reach elevated levels of biodiversity. This low level of biodiversity could be in some ways, from the production point of view, an advantage if the dominance of the legumes over the grasses were maintained. The mowing performed in the spring show that the productivity found for the continuous alfalfa was linked to the absolute dominance of grasses (e.g., *Bromus sterilis, Avena sterilis*). However, the dominance of the legumes returns in the following mowings and especially in the last one when the main legume species contributes together with many others of medium-low size, both grasses (e.g., *Setaria viridis*) and plants of other families (eg, *Convolvulus arvensis*, *Rumex crispus*, *Polygunum aviculare*, *Sonchus oleraceus*, *Conyza canadiensis*) and resulted in a prolonged and likely effective and prolonged soil vegetation cover (Figure 6).

2.1.5.3 Synergies and trade-offs between the management options

The greater contribution of provisioning services in alfalfa was linked to the greater production of biomass and consequently greater energy. This was to some extent surprising because suggests that alfalfa might be kept longer that the theoretical "efficiency" limit of 5-6 years (Porqueddu et al 2016).

This came at a cost of forage quality (Brink & Marten, 1989) but leaves many windows opened in terms of soil carbon accumulation, soil erosion prevention and, indirectly, food provisioning. While it is obvious that wheat is intended for human consumption and that alfalfa provides forage for sedentary systems, it is important to report that the latter also offers basic winter pasture for sheep transhumant systems (Budimir et al., 2020). This in turn as well as providing lambs twice a year and cheese in addition to a wide array of other ecosystem services not limited only to meat, cheese but that include cultural services. For example, the provisioning of landscape, recreational services in the mountain areas together with biodiversity conservation of mountain permanent grasslands could be mostly attributed to the presence of transhumant farming systems in lowlands of Central Italy (Toderi et al., 2018).

As expected, W_B was the greatest contributor in the provision of regulating services. This was mainly due to the soil total organic carbon enhancement provided by 60 t ha^{-1} of wood-derived biochar. Some recent studies seem to agree that soil amended with high quality biochar can benefit from a great carbon sequestration, but future studies should address the effect of biochar aging (Wang et al. 2020). In the effect of biochar in terms of soil structure stability may be crucial for cropping systems in hilly territories where slopes can easily reach 30%. Concerning the soil GHG emissions, it seems that the meteorological conditions of each year affect more than the different management options. Indeed, in 2018 it was observed an overall higher emission than in 2019 in terms of CO_2_eq (Figure 4A). In contrast the sensitivity to soil respiration 'Q_{10}' proved to be stable in the different years and reported values fall within the ranges reported in the reviews/metanalysis (D'Ottavio et al., 2018). However, it appears that there was a significant decrease in total organic carbon for W_0 compared to alfalfa. This however became evident only at the end of monitoring period (Figure 4D).

In both the tilled treatments (W0 and WB), the increase the pH of the soil could have been attributed to the more aerobic environment. In line with other studies (Verheijen et al., 2010) the already basic pH of the studied soil did not show any remarkable increase after the biochar incorporation.However, in terms of supporting services a balance between continuous alfalfa and W_B clearly emerged. This derived respectively from a high coverage value for the continuous alfalfa and a high soil carbon for W_B, leaving W_0 as the least desirable options for enhancing supporting services.
In terms of biodiversity (i.e., as base for the provision of all the ecosystem services), a general higher value was observed for the continuous alfalfa. This was however likely linked to the age of the stand which is certainly not positive in terms of quality of the forage. The positive aspects in terms of biodiversity enhancement registered for W_0 and W_B might be however only apparent as in such cereal-based systems the management of weeds (i.e., the reason of higher biodiversity) is still problematic. Future studies might investigate the role of such weeds and the connection of biodiversity (Marshall, 2007)

2.1.6 Conclusions

This study aimed at estimating a basket of ecosystem services derived by three management options for a 6-year-old alfalfa stand. Two alternative management practices to the common wheat-after-wheat management, customary within the organic farming systems of central Italy were evaluated: i) adopting a conservative option and avoid alfalfa termination by keeping alfalfa for an even longer time; ii) add biochar to the wheat-after-wheat grain to improve carbon sequestration. The trade-offs and synergies of the two alternative management options were evaluated with a set of 13 indicators in respect to the W_0, as control.
Results suggest that the W_0 options would result in the narrower basket of ecosystem services while the continuous alfalfa was the option offering a wider basket of ecosystem services, this can be attributed to the provisioning services. The negative effect of W_0 might be partially counterbalanced by the addition of biochar that resulted in a similar provision supporting services compared to the continuous alfalfa and in the highest in terms of regulating services. Biochar did not result in any positive nor detrimental effect on the biodiversity and other ecosystem services category. However future studies might investigate the effect of biochar again in terms of synergies and trade-offs with other ecosystem services.
Biodiversity which is the base of all the ecosystem services results higher for the continuous alfalfa, suggesting that future studies should investigate more in depth the potential wide array of ecosystem services that the long-lasting alfalfa can provide.

2.1.7 Literature

Aguilera E., Guzmán G., Alonso A., 2015, Greenhouse gas emissions from conventional and organic cropping systems in Spain. I. Herbaceous crops. Agron. Sustain. Dev. (2015) 35:713–724. https://doi.org/10.1007/s13593-014-0267-9

Baiamonte G., Crescimanno G., Parrino F., De Pasquale C., 2019. Effects of biochar on the physical and structural properties of a sandy soil. Catena: 294-303. https://doi.org/10.1016/j.catena.2018.12.019

Ballesta A., Lloveras J., 2010. Nitrogen replacement value of alfalfa to corn and wheat under irrigated Mediterranean conditions. Spanish Journal of agricultural research, 8 (1): 159-169. https://doi.org/10.5424/sjar/2010081-1155

Ballesta A., Lloveras J., 2010. Nitrogen replacement value of alfalfa to corn and wheat under irrigated Mediterranean conditions. Spanish Journal of Agricultural Research, 8 (1): 159-169. https://doi.org/10.5424/sjar/2010081-1155

Basso B., Fiorentino C., Cammarano D., Cafiero G., Dardaniello J., 2012. Analysis of rainfall distribution on spatial and temporal patterns of wheat yield in Mediterranean environment. Europ. J. Agronomy 41 (2012) 52– 65

Basso B., Ritchie J.T., 2005. Impact of compost, manure and inorganic fertilizer on nitrate leaching and yield for a 6-year maize–alfalfa rotation in Michigan. Agriculture, Ecosystems and Environment 108: 329–341. https://doi.org/10.1016/j.agee.2005.01.011

Basso B., Ritchie J.T., 2005. Impact of compost, manure and inorganic fertilizer on nitrate leaching and yield for a 6-year maize–alfalfa rotation in Michigan. Agriculture, Ecosystems and Environment 108: 329–341. https://doi.org/10.1016/j.agee.2005.01.011

Börjesson G., Bolinder M.A., Kirchmann H., Kätterer T., 2018. Organic carbon stocks in topsoil and subsoil in long-term ley and cereal monoculture rotations. Biology and Fertility of Soils, 54: 549–558 https://doi.org/10.1007/s00374-018-1281-x

Brink G.E., Marten G.C., 1989. Harvest management of alfalfa - nutrient, yield vs. forage quality and relationship to persistence. Journal of Production Agriculture, 12: https://doi.org/10.2134/jpa1989.0032

Bronick C.J., Lal R., 2005. Soil structure and management: a review. Geoderma, 124: 3–22. https://doi.org/10.1016/j.geoderma.2004.03.005

Burnett, V.F.; Butler, K.L.; Hirth, J.R.; Mitchell, M.L.; Clark, S.G.; Nie, Z. 2020. Lucerne (Medicago sativa L.) Persistence Remains Unchanged under Variable Cutting Regimes. Agronomy, 10, 844. https://doi.org/10.3390/agronomy10060844

Cavero J., Faci J.M., Medina E.T., Martínez-Cob A., 2017. Alfalfa forage production under solid set sprinkler irrigation in a semiarid climate. Agricultural Water Management, 191: 184–192. https://doi.org/10.1016/j.agwat.2017.06.018

Cayuela, M.L., Aguilera, E., Sanz-Cobena, A., Adams, D.C., Abalos, D., Barton, L., Ryals, R., Silver, W.L., Alfaro, M.A., Pappa, V.A., Smith, P., Garnier, J., Billen, G., Bouwman, L., Bondeau, A., Lassaletta, L., 2017. Direct nitrous oxide emissions in Mediterranean climate cropping systems: emission factors based on a meta-analysis of available measurement data. Agric. Ecosyst. Environ. 238, 25–35. https://doi.org/10.1016/j.agee.2016.10.006

Coruh I., Tan M., 2008. Lucerne persistence, yield and quality as influenced by stand aging, , 51:1, 39-43. https://doi.org/10.1080/00288230809510433

D'Ottavio, P.; Francioni, M.; Trozzo, L.; Sedić, E.; Budimir, K.; Avanzolini, P.; Trombetta, M.F.; Porqueddu, C.; Santilocchi, R.; Toderi, M. 2018. Trends and approaches in the analysis of ecosystem services provided by grazing systems: A review. Grass Forage Sci., 73, 15–25. https://doi.org/10.1111/gfs.12299

Dale V.H., Polasky S., 2007. Measure of the effects of agricultural practices on ecosystem services. Ecological Economics, 64: 286–296. https://doi.org/10.1016/j.ecolecon.2007.05.009

Ergon A, Seddaiu G, Korhonen P et al (2018) How can forage production in Nordic and Mediterranean Europe adapt to the challenges and opportunities arising from climate change? Eur J Agron 92:97–106. https://doi.org/10.1016/j.eja.2017.09.016

Fischer B.M.C., Manzoni S., Morillas L., Garcia M., Johnson M.S., Lyon S.W., 2019. Improving agricultural water use efficiency with biochar – A synthesis of biochar effects on water storage and fluxes across scales. Science of the Total Environment, 657, 853–862. https://doi.org/10.1016/j.scitotenv.2018.11.312

Francioni M., Lai R., D'Ottavio P., Trozzo L., Kishimoto-Mo A.W., Budimir K., Baldoni N., Marco Toderi M., 2018. Soil respiration dynamics in forage-based and cereal-based cropping systems in central Italy. Sci. Agric., 77 (3), e20180096. http://dx.doi.org/10.1590/1678-992X-2018-0096

Franzluebbers A.J., Sawchick J., Taboada M.A., 2014. Agronomic and environmental impacts of pasture-crop rotations in temperate North and South America. Agric. Ecosyst. Environ., 190: 18-26. https://doi.org/10.1016/j.agee.2013.09.017

Freddo A, Cai C., Reid B.J., 2012. Environmental contextualisation of potential toxic elements and polycyclic aromatic hydrocarbons in biochar. Environmental Pollution, 171: 18-24. http://dx.doi.org/10.1016/j.envpol.2012.07.009

Ghosh A., Bhattacharyya R., Meena M.C., Dwivedi B.S., Singh G., Agnihotrid R., Sharma C. 2018. Long-term fertilization effects on soil organic carbon sequestration in an Inceptisol. Soil & Tillage Research, 177: 134–144. https://doi.org/10.1016/j.still.2017.12.006

Gomez-Eyles J.L., Sizmur T., Collins, C.D., Hodson M.E., 2011. Effects of biochar and the earthworm Eisenia fetida on the bioavailability of polycyclic aromatic hydrocarbons and potentially toxic elements. Environmental Pollution, 159, 616-62.http://dx.doi.org/10.1016/j.envpol.2010.09.037

Guardia, G., Cangani, M.T., Sanz-Cobena, A., Junior, J.L., Vallejo, A., 2017. Management of pig manure to mitigate NO and yield-scaled N2O emissions in an irrigated Mediterranean crop. Agric. Ecosyst. Environ. 238, 55–66. http://dx.doi.org/10.1016/j.agee.2016.09.022

Han L., Sun K., Yang Y., Xia X., Li F., Yang Z., Xing B., 2020. Biochar's stability and effect on the content, composition and turnover of soil organic carbon. Geoderma, 364: 114-184. https://doi.org/10.1016/j.geoderma.2020.114184

Hilber I., Bastos A.C., Loureiro S., Soja G., Marsz A., Cornelissen G., Bucheli T.D., 2017. The different faces of biochar: contamination versus remediation tool. Journ. Of Environm. Engineering and landscape management, 25 (2): 86-104. https://doi.org/10.3846/16486897.2016.1254089

ISTAT Agricoltura 2021 http://dati.istat.it/Index.aspx?DataSetCode=DCSP_COLTIVAZIONI

Kavitha B., Reddy P.V.L., Kim B., Lee S.S., Pandey S.K., Kim K.-H., 2018. Benefits and limitations of biochar amendment in agricultural soils: a review. Journal of Environmental Management, 227: 146-154 https://doi.org/10.1016/j.jenvman.2018.08.082

Kelner D.J., Vessey J.K., Entz N.K., 1997. The nitrogen dynamics of 1-, 2- and 3-year stands of alfalfa in a cropping system. Agriculture, Ecosystems and Environment, 64: 1-10 https://doi.org/10.1016/S0167-8809(97)00019-4

Koppolu, L., Agblevor, F.A., Clements, L.D., 2003. Pyrolysis as a technique for separating heavy metals from hyperaccumulators. Part II: lab-scale pyrolysis of synthetic hyperaccumulator biomass. Biomass and Bioenergy 25, 651-663. http://dx.doi.org/10.1016/j.biombioe.2003.08.010

Kumar M., Kundu D.K., Ghorai A.K., Mitra S., Singh S.R., 2018. Carbon and nitrogen mineralization kinetics as influenced by diversified cropping systems and residue incorporation in Inceptisols of eastern Indo-Gangetic Plain. Soil and Tillage research, 178: 108-177. https://doi.org/10.1016/j.still.2017.12.025

Kunrath T.R., Berrangerc C. de, Charrier X., Gastal F., César P., Carvalho de F., Lemaire G., Emile J.C., Durand J.L., 2015. How much do sod based rotations reduce nitrate leaching in a cereal cropping system? Agric. Water Management, 150: 46–56. https://doi.org/10.1016/j.agwat.2014.11.015

Lehmann J., Gaunt J., Rondon M., 2006. Biochar sequestration in terrestrial ecosystems – a review. Mitigation and adaptation strategies for global change, 11: 403-427. https://doi.org/10.1007/s11027-005-9006-5

Lehmann J., Rillig M.C., Thies J., Masiello C.A., Hockaday W.C., Crowley D., 2011. Biochar effects on soil biota–A review. Soil Biology & Biochemistry, 43 (9), 1812–1836. https://doi.org/10.1016/j.soilbio.2011.04.022

Lemanceau P., Maron P.A., Mazurier S., Mougel C., Pivato B., et al. 2015. Understanding and managing soil biodiversity: a major challenge in agroecology. Agronomy for Sus- tainable Development, Springer Verlag/EDP Sciences/INRA, 35 (1), pp.67-81. https://doi.org/10.1007/s13593-014-0247-0

Lescourret F., Magda D., Richard G., Adam-Blondon A.F., Baudry J., Bardy M., Doussan I, Dumont B., Lefevre F., Litrico I., Martin-Clouaire R., Montuelle B., Pellerin S., Plantegenest M., Tancoigne E., Thomas A., Guyomard H., Soussana J.F., 2015. A social–ecological approach to managing multiple agro-ecosystem services. Current Opinion in Environmental Sustainability, 14: 68–75. https://doi.org/10.1016/j.cosust.2015.04.001

Li L Peng, Liu Y ying, Luo S guo, Peng X long, 2012. Effects of nitrogen management on the yield of winter wheat in cold area of northeastern China. J. Integr. Agr. 11:1020-5.

Li Y., Zhang F., Yang M., Zhang J., Xie Y., 2019. Impacts of biochar application rates and particle sizes on runoff and soil loss in small cultivated loess plots under simulated rainfalls. Science of the Total Environment, 649, 1403-1413. https://doi.org/10.1016/j.scitotenv.2018.08.415

Liu X.H., Zhang X.C., 2012. Effect of biochar on pH of alkaline soils in the loess plateau: results from incubation experiments. Int. Journ. Agric. Biol., 14: 745–750.

Major J., Steiner C., Ditommaso A., Falcão N.P.S., Lehmann J., 2005. Weed composition and cover after three years of soil fertility management in the central Brazilian Amazon: compost, fertilizer, manure and charcoal applications. Weed Biology and management, 5: 69-76. https://doi.org/10.1111/j.1445-6664.2005.00159.x

Marshall E.J.P., 2007. Weeds and biodiversity. In book: Weed Management Handbook, Ninth Edition Marshall Agroecology, 2 Nut Tree Cottages, Barton, Winscombe, Somerset BS25 1DU, UK 10.1002/9780470751039.ch5

MEA (Millenium Ecosystem Assessment) (2005) Ecosystems and human well-being: a framework for assessment. Island Press, Washington

Meek B.D., Rechel E.A., Carter L.M., DeTar W.R. 1989. Changes in infiltration under alfalfa as influenced by time and wheel traffic. Soil Sci. Soc. of America Journal, 53 (1): 238-241. https://doi.org/10.2136/sssaj1989.03615995005300010042x

Meng Q., Chen X., Lobell D.B., Cui Z., Zhang Y., Yang H., Zhang F., 2016. Growing sensitivity of maize to water scarcity under climate change. Sci. Rep. 6: 19605–19607. https://doi.org/10.1038/srep19605

Mitchell M., Scheer C., Rowlings D.W., Contant R.T., Cotrufo M.F., Deiden L. van, Grace P.R., 2016. The influence of above-ground residue input and incorporation on GHG fluxes and stable SOM formation in a sandy soil. Soil Biol. & Biochem., 101, 104-113. https://doi.org/10.1016/j.soilbio.2016.07.008

Mobtaker H.G., Akram A., Keyhani A., 2010. Investigation of energy consumption of perennial alfalfa production: Case study of Hamedan province. Journal of Food, Agriculture & Environment, 8 (2): 379-381. https://doi.org/10.1234/4.2010.1691

Monaci, E., Polverigiani, S., Neri, D., Bianchelli, M., Santilocchi, R., Toderi, M., Ottavio, P.D., Vischetti, C., 2017. Effect of contrasting crop rotation systems on soil chemical and biochemical properties and plant root growth in organic farming: first results. Ital. J. Agron. 12, 364–374. https://doi.org/10.4081/ija.2017.831

Moore F.C. and Lobell D.B., 2015. The fingerprint of climate trends on European crop yields. Proc. Natl. Acad. Sci. U S A 112: 2670–2675. https://doi.org/10.1073/pnas.1409606112

Mulvaney R.L., Khan S.A., Ellsworth T.R., 2009. Synthetic Nitrogen Fertilizers Deplete Soil Nitrogen: A Global Dilemma for Sustainable Cereal Production. J. Environ. Qual. 38:2295–2314. https://doi.org/10.2134/jeq2008.0527

Ning J., He X.Z., Hou F., et al., 2020. Optimizing alfalfa productivity and persistence versus greenhouse gases fluxes in a continental arid region. PeerJ., 8: 8738. Published 2020 Mar 10. https://doi.org/10.7717/peerj.8738

Olmo M., Albuquerque J.A., Barrón V., Campillo M.C., Gallardo A., Fuentes M., Villar R. 2014 Wheat growth and yield responses to biochar addition under Mediterranean climate conditions. Biol. Fertil. Soils, 50: 1177-1187. https://doi.org/10.1007/s00374-014-0959-y

Palma C., Blanco-Canquib H., DeClerckc F., Gaterea L, Graceda P., 2014. Conservation agriculture and ecosystem services: An overview. Agriculture, Ecosystems and Environment 187: 87–105. http://dx.doi.org/10.1016/j.agee.2013.10.010

Pecetti L., Carroni A.M., Annicchiarico P., Manunza P., Longu A., Congiu G. 2008. Adaptation, summer survival and autumn dormancy of lucerne cultivars in a south European Mediterranean region (Sardinia). In: Porqueddu C. (ed.), Tavares de Sousa M.M. (ed.). Sustainable Mediterranean grasslands and their multi-functions. Zaragoza : CIHEAM / FAO / ENMP / SPPF, 2008. p. 471-474 (Options Méditerranéennes: Série A. Séminaires Méditerranéens; n. 79)

Pierre C.S., James Peterson C., Ross, A.S., Ohm J.-B., Verhoeven M.C., Larson M., Hoefer B., 2008. Agronomy J., 100 (2), 414-420.

Porqueddu C., Ates S., Louhaichi M. et al (2016) Grasslands in 'Old World' and 'New World' Mediterranean-climate zones: past trends, current status and future research priorities. Grass Forage Sci 71(1): 1–35. https://doi.org/10.1111/gfs.12212

Sanz-Cobena A., Lassaletta L., Garnier J., Smith P., 2017. EditorialMitigation and quantification of greenhouse gas emissions inMediterranean cropping systems.Agriculture, Ecosystems & Environment, 238: 1-4 https://doi.org/10.1016/j.agee.2016.12.032

Smuckler S.M., Sánchez-Moreno S., Fonte S.J., Ferris H., Klonsky K., O'Green A.T., Scow K.M., Steenwerth K.L., Jackson L.E., Biodiversity and multiple ecosystem functions in an organic farmscape. Agriculture, Ecosystems and Environment, 139: 80-97. https://doi.org/10.1016/j.agee.2010.07.004

Tabak M., Lepiarczyk A., Filipek-Mazur B., Lisowska A., 2020. Eciency of Nitrogen Fertilization of Winter Wheat Depending on Sulfur Fertilization. Agronomy, 10, 1304. https://doi.org/10.3390/agronomy10091304

Tan Z., Lin C.S.K., Ji X., Rainey T.J., 2017. Returning biochar to fields: A review. Applied Soil Ecology, 116, 1-11. https://doi.org/10.1016/j.apsoil.2017.03.017

Tellez-Rio A., Vallejo A., García-Marco S., Martin-Lammerding D., Tenorio J.L., Rees M.R., Guardia G., 2017. Conservation Agriculture practices reduce the global warming potential of rainfed low N input semi-arid agriculture, European Journal of Agronomy, 84: 95-104. https://doi.org/10.1016/j.eja.2016.12.013

Testa G., Gresta F. and Cosentino S.L. (2011) Dry matter and qualitative characteristics of alfalfa as affected by harvest times and soil water content. European Journal of Agronomy, 34, 144–152

Trozzo L., Francioni M., Kishimoto-Mo A.W., Foresi L., Bianchelli M., Baldoni N., D'Ottavio P., Toderi M., 2020. Soil N2O emissions after perennial legume termination in an alfalfa-wheat crop rotation system under Mediterranean conditions. Italian Journal of Agronomy; 15: 1613. http://dx.doi.org/10.4081/ija.2020.1613

Vaccari F.P., Baronti S., Lugato E., Genesio L., Castaldi S., Fornasier F., Miglietta F., 2011. Biochar as a strategy to sequester carbon and increase yield in durum wheat. European Journal of Agronomy, 231-238. https://doi.org/10.1016/j.eja.2011.01.006

Verheijen F., Jeffery S., Bastos A.C., van der Velde M., Diafas I., 2010. Biochar Application to Soils A Critical Scientific Review of Effects on Soil Properties, Processes and Functions J.R.C. Scientific Report. European Commission, Luxembourg.

Vertès F., Jeuffroy M.H., Louarn G., Voisin A.S., Justes, E., 2015. Legume use in temporary pastures: supplying nitrogen in crop-rotation systems. Fourrages, 223: 221–232.

Wang C., Wanga Y., Herath H.M.S.K, 2017. Polycyclic aromatic hydrocarbons (PAHs) in biochar – Their formation, occurrence and analysis: A review. Organic Geochemistry, 114: 1-11.https://doi.org/10.1016/j.orggeochem.2017.09.001

Wang L., O'Connor D., Rinklebe J., Ok Y. S., Tsang D.C.W., Shen Z., Hou D., 2020. Biochar Aging: Mechanisms, Physicochemical Changes, Assessment, And Implications for Field Applications. Environ. Sci. Technol., 54 (23) 14797–14814. https://doi.org/10.1021/acs.est.0c04033

Wei S., Bai Z.H., Chadwick D., Hou Y., Qin W., Zhao Z.Q., Jiang R.F., Ma L., 2010. Greenhouse gas and ammonia emissions and mitigation options from livestock production in peri-urban agriculture: Beijing – A case study, Journal of Cleaner Production, 178, 2018,: 515-525. https://doi.org/10.1016/j.jclepro.2017.12.257

Zhao C., Liub B., Piaoa S., et al., 2017. Temperature increase reduces global yields of major crops in four independent estimates. PNAS, 114 (35), 9326-9331. www.pnas.org/cgi/doi/10.1073/pnas.1701762114

3 Chapter 2

3.1 Soil N_2O emissions after perennial legume termination in an alfalfa–wheat crop rotation system under Mediterranean condition *

3.1.1 Abstract

Agricultural activities are potential sources of greenhouse gas (GHG) emissions, and nitrous oxide (N_2O) is one of the most important non-carbon-dioxide GHGs. Perennial legumes such as alfalfa (*Medicago sativa* L.) have potential roles for reduction of soil GHG emissions as part of crop rotation systems. However, the implications of perennial legume termination by tillage and subsequent soil incorporation of the residues for reduced GHG emissions have been poorly examined in Mediterranean environments. With the aim to assess the magnitude of soil N_2O emissions (important for the definition of mitigation strategies) after perennial legume termination in alfalfa–wheat crop rotation systems in a Mediterranean environment, we defined the hypothesis that alfalfa termination by tillage with incorporation of the crop residues will increase soil N_2O emissions during the subsequent wheat season. To test this hypothesis, closed static chambers were used in a field–plot experiment, using a complete randomised block design with three replicates. Soil N_2O emissions were monitored across 33 sampling dates from October 2017 to July 2018, as a comparison between an original 6-year-old alfalfa field ('continuous alfalfa') and alfalfa termination followed by wheat ('alfalfa+wheat'). The soil N_2O emission fluxes varied markedly across the treatments and throughout the monitoring period (from -0.02 ±0.01 to 0.53 ±0.14 g N-N_2O ha^{-1} h^{-1}, and from 0.02 ±0.07 to 0.37 ±0.11 g N-N_2O ha^{-1} h^{-1} for continuous alfalfa and alfalfa+wheat, respectively), generally following the changes in soil temperature. Several soil N_2O emission peaks were recorded for both treatments, which mainly coincided with rainfall and with increased soil water content. In the 2 months following alfalfa termination, alfalfa+wheat showed higher cumulative weekly soil N_2O emissions compared to continuous alfalfa. Following alfalfa termination for alfalfa+wheat, the increased cumulative weekly soil N_2O emissions appeared to be due to asynchrony between nitrogen (N) released into the soil from mineralisation of the alfalfa residues and N uptake by the wheat. Despite these initial high soil N_2O emissions for alfalfa+wheat, the seasonal cumulative soil N_2O emissions were not significantly different (0.77 ±0.09 *vs*. 0.85 ±0.18 kg N-N_2O ha^{-1} for continuous alfalfa and alfalfa+wheat, respectively). These data suggest that legume perennial crop termination in alfalfa–wheat rotation systems does not lead to significant loss of N_2O from the soil. The alfalfa termination by tillage performed in autumn might, on the one hand, have slowed the mineralisation process, and might, on the other hand, have synchronised the N release by the mineralised crop residues, with the N uptake by the wheat reducing the soil N_2O emissions.

*Trozzo L., Francioni M., Kishimoto A. W., Foresi L., Bianchelli M., Baldoni N., D'Ottavio P., Toderi M. 2020. Soil N2O emissions after perennial legume termination in an alfalfa-wheat crop rotation system under Mediterranean conditions. Italian Journal of Agronomy, Vol. 15 No. 3. https://doi.org/10.4081/ija.2020.1613

3.1.2 Introduction

Many recent studies have indicated that increased greenhouse gas (GHG) emissions into the atmosphere are linked to human activities and to land use and management (Stehfest and Bouwman, 2006; Wang and Fang, 2009; Reay *et al.*, 2012; Smith *et al.*, 2014; Cayuela *et al.*, 2017; Francioni *et al.*, 2019). Nitrous oxide (N_2O) is one of the most relevant non-carbon-dioxide GHGs (Forster *et al.*, 2007), with a global warming potential 265-fold that of carbon dioxide (CO_2) over a 100-year time horizon (Smith *et al.*, 2014). Evaluation of the magnitude of the agricultural N_2O emissions and definition of the possible mitigation strategies are important, as the soil is the largest natural source of N_2O (Stehfest and Bouwman, 2006; Van Groenigen *et al.*, 2010) and agriculture is responsible for around 60% of N_2O emissions (Syakila and Kroeze, 2011; Reay *et al.*, 2012).

Apart from climate conditions, many other factors have key roles in the nitrogen (N) cycle, and consequently on soil N_2O emissions from agriculture, such as N fertiliser type and application rate, crop type, crop residue type and timing of incorporation, tillage type (Signor and Cerri, 2013), cropping system (Signor and Cerri, 2013; Autret *et al.*, 2019) and soil physicochemical properties, which include its organic carbon content, pH and texture (Stehfest and Bouwman, 2006). Soil N_2O emissions generally increase with higher clay content of the soil (Lesschen *et al.*, 2011), compared to sandy soil, due to higher levels of anaerobic microsites (Signor and Cerri, 2013). As N_2O is one of the by-products of microbial nitrification and denitrification processes, the fertiliser type and application rate affect the soil N_2O (Malhi *et al.*, 2010; Sanz-Cobena *et al.*, 2017; Volpi *et al.*, 2018; Tenuta *et al.*, 2019), to increase the emissions, especially at N input rates higher than the crop requirements (Kim *et al.*, 2013). The type of crop residues and the C:N ratio (which is low in alfalfa crop residues) also affect soil N_2O emissions (Gomes *et al.*, 2009; Lin *et al.*, 2013). For example, Toma and Hatano (2007) reported that the incorporation of crop residues with low C:N ratio in a Grey Lowland soil in Hokkaido (Japan) resulted in high soil N_2O emissions, due to rapid mineralisation of residues, and to the resulting suitable conditions for nitrification and denitrification processes (Huang *et al.*, 2004). According to these data, Signor and Cerri (2013) reported a close relationship between low C:N ratio of residues and production of N_2O, due to reduced N immobilisation and N increase into the soil. The crop residue type is not the only aspect that affects the soil N_2O emission, but also the way these residues are returned to the soil, although there remain some uncertainties about their effects on N_2O emissions that should be investigated (Shan and Yan, 2013). In the literature, the effects of tillage practices have been widely reported in terms of the soil organic N mineralisation, nitrification and denitrification processes, and consequently the N_2O soil emissions. As reported by Abalos *et al.* (2016), by improving soil aeration and reducing soil aggregation, soil tillage can enhance crop residue mineralisation and increase N availability for the nitrification and denitrification processes, with N_2O soil emissions increasing some 10-fold after soil tillage. Similar data were reported by Estavillo *et al.* (2002), who showed that soil tillage promotes mineralisation of soil organic N, with the subsequent release of N_2O. This process and the consequent soil N_2O emissions can be affected by the tillage timing and depth. Higher soil N_2O emissions appear to be linked to summer tillage, compared to autumn tillage (Ball *et al.*, 2007; Krauss *et al.*, 2017), and also to deeper tillage (Forte *et al.*, 2017).

Legumes are widely used as an alternative to chemical fertilisers because of their N fixation, which provides a N_2O emissions mitigation role for perennial systems (Abalos *et al.*, 2016). This also results from reduction of other direct and indirect N_2O emissions that come, for example, from chemical fertiliser production and transport (Aguilera *et al.*, 2013). Adoption of the best agronomic practices that take into account all of the factors that can affect N_2O emissions, combined with the use of specific mitigation strategies, can thus lead to reduced soil N_2O emissions.

Alfalfa (*Medicago sativa* L.) is one of the most important forage crops worldwide (Tesfaye *et al.*, 2006). It represents one of the most used perennial legumes in organic farming systems, with continually increasing areas of cultivation throughout the world (Willer and Lernoud, 2017). However, very few studies have investigated the effects of perennial legume termination on soil N_2O emissions (e.g., Westphal *et al.*, 2018; Tenuta *et al.*, 2019), while crop residues (Jensen *et al.*, 2012) might also have a key role in such emissions (Basche et al., 2014; Autret et al., 2019).

Considering the mitigation of soil N_2O emissions by perennial legumes through N fixation, the detrimental effects of soil tillage in this respect and the GHG impact on cropping systems (Autret *et al.*, 2019), we hypothesise that alfalfa termination by tillage will increase soil N_2O emissions, which would cancel out the positive effects of the perennial legume in terms of mitigation of N_2O soil emissions. With this regard, the aim of the present study was to assess the magnitude of soil N_2O emissions after perennial legume termination in alfalfa–wheat crop rotation systems in a Mediterranean environment.

3.1.3 Materials and Method

3.1.3.1 Site description

The site was in a hilly area of the Marche region (Ancona Province, central Italy) (43° 33' N, 13° 25' E; 100 m a.s.l.; SW exposure; 23% slope) where alfalfa–wheat rotation is one of the most typical crop rotation systems (Monaci *et al.,* 2017; Francioni *et al.,* 2020). In this territory, long-term alfalfa fields are mainly grazed during the winter by the transhumant flocks of sheep, which are generally moved to permanent mountain grasslands during the summer (Budimir *et al.*, 2018).

The bioclimate was the temperate oceanic sub-Mediterranean variant (Agnelli *et al.*, 2008), with mean annual precipitation of 788 mm and mean annual temperature of 14.6 °C (Figure 1). The meteorological data recorded by a weather station located 0.3 km from the study site indicated cumulative rainfall of 890 mm and mean air temperature of 13.5 °C over the experimental period (October 2017-July 2018).

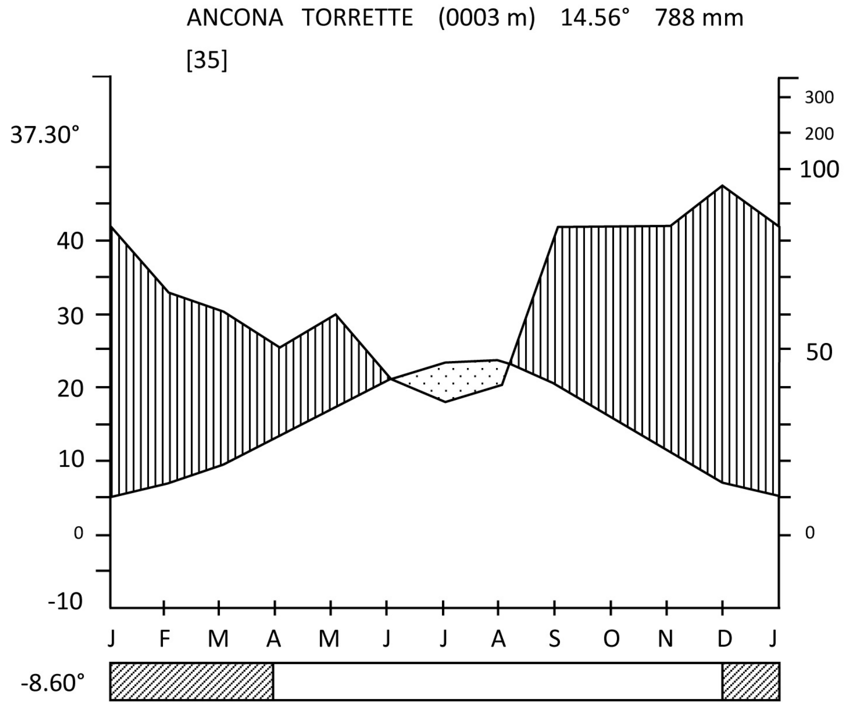


Figure 1. Walter-Lieth climate diagram of the study area (Walter and Lieth, 1960).

The soil of the study site was classified as Inceptisol according to the United States Department of Agriculture soil taxonomy system (Smith, 2014). Soil ancillary measurements were carried out at the beginning of the trial (i.e., October 2017), following the guidelines of the Italian Ministry of Agriculture and Forestry (DM 13/09/99 GU 248), which is the Italian official reference for soil chemical analyses. Soil samples were collected at a depth of 0.1 and 0.4 m (Table 1), following the non-systematic 'W' pattern described by Paetz and Wilke (2005).

Sample depth (m)	pH	C/N ratio	Soil Texture ($g\ kg^{-1}$)			Soil organic matter ($g\ kg^{-1}$)	Total organic C ($g\ kg^{-1}$)	Total N ($g\ kg^{-1}$)	Humic & fulvic acids ($g\ kg^{-1}$)	C.E.C. [meq $(100\ g)^{-1}$]	Field capacity (%)	Wilting point (%)
			Sand	Silt	Clay							
0-0.1	8.11	8.40	363	383	254	15,0	8.60	1.03	5.17	22.53	24.60	17.93
0.1-0.4	8.14	8.60	358	384	258	15.5	8.80	1.03	4.77	22.63	24.45	17.69

Table 1. Basic properties of the soil at the 0-0.1 and 0.1-0.4 m sampling depths.

3.1.3.2 Experimental design

The study was conducted from October 2017 to July 2018. In October 2017, an area that was homogeneous for soil, crop vegetation and topographic conditions was identified in a 6-year-old alfalfa field. The experimental area was fenced off to prevent any disturbance. A complete randomised block design with three replicates and individual plots of 25 m^2 (2.5 m × 10 m) was applied to measure the soil N_2O emissions under the following defined treatments (Table 2):

- Continuous 6-year-old alfalfa ('continuous alfalfa'). The original alfalfa field was mowed at the initial flowering stage (i.e., 11 May, 4 July 2018) using a bar mower (cutting height, 5 cm), and a standard rake to collect and remove the cut herbage immediately after mowing.
- Alfalfa termination followed by durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.) ('alfalfa+wheat'). The alfalfa was terminated at the beginning of October 2017 using a spading machine (i.e., 11 October) followed

by two uses of a rotary harrow (i.e., 16 October, 21 November 2017), with the alfalfa residues (mean dry matter content, 2.70 ±0.23 t ha^{-1}) incorporated into the soil (0.20 m depth). The durum wheat plots were sown at the end of November 2017 (i.e., 23 November) in rows (sowing rate, 400 seeds m^{-2}) and were manually harvested at the beginning of July 2018 (i.e., 4 July), in a central plot area of 2 m^2. Manual weeding was performed twice in the second half of May 2018 (i.e., 17, 24 May), with *Convolvulus arvensis* L. and *Papaver rhoeas* L. as the main species removed.

As the effects of N fertilisation on soil N_2O emissions are well known and have been demonstrated in many studies (Wei *et al.*, 2010; Aguilera *et al.*, 2013; Liu *et al.*, 2015), N fertilisation was not applied. This allowed isolation of the effects of only the alfalfa termination on the soil N_2O emissions.

Year	Management practice		Time of treatment (Julian day)	
	Type	Soil depth (m)	Continuous alfalfa	Alfalfa + wheat
2017	Spading	0.20	na	284
	Harrowing 1	0.15	na	289
	Harrowing 2	0.15	na	325
	Sowing	0.03	na	327
2018	Mowing/ raking 1	na	131	na
	Weeding 1	na	na	137
	Weeding 2	na	na	144
	Harvesting	na	na	185
	Mowing/ raking 2	na	185	na

Table 2. Management practices performed to the different treatments during the study period. (na, not applicable)

3.1.3.3 Soil temperature and water content measurements

In each experimental unit, soil temperature and water content were measured from October 2017 to July 2018, for a total of 33 recordings for each variable. At each N_2O sampling, soil temperature was determined using hand-held digital thermometers equipped with a stainless-steel probe (Model: 620-0909, VWR International, Italy) inserted to a depth of 0.1 m. Soil samples were taken with a manual auger at 0.1 m depth, and used to determine the soil water content (SWC) using the oven-dried method (105 °C, to constant weight).

3.1.3.4 Nitrous oxide sampling, analysis, and calculation

Nitrous oxide was measured using closed static chambers, as described by Parkin and Venterea (2010). The chambers were made of polyvinyl chloride (height, 0.15 m; diameter, 0.25 m) and were equipped with a thermometer to measure the variations in the internal temperature during the sampling period. Two polyvinyl chloride base rings (pseudo-replicates) per plot (n = 6 chambers per treatment) were permanently installed in the soil (depth, 0.1 m), to explore spatial heterogeneity (Krauss *et al.*, 2017); these were only removed for soil tillage, after which they were immediately re-installed (Ghimire *et al.*, 2017).

Gas samples were collected between 9:00 am and 12:00 am (standard time) (Krauss *et al.*, 2017) every 3 or 4 days, from tillage (11 October 2017) to sowing (23 November 2017), and after any rain, and later at about every 15 days (Volpi *et al.*, 2018). Before each sampling, the above-ground parts of the plants inside the chambers were clipped off (Westphal *et al.*, 2018), to avoid disturbance to the soil N_2O emissions. The chambers were placed in position for 45 min, during which time four gas samples were withdrawn from the headspace of each chamber (30 mL each, at 15 min intervals). The gas samples were injected into 30 mL glass pre-evacuated vials sealed with a butyl rubber septum (Parkin and Venterea, 2010).

In a following step, the N_2O concentrations were determined using gas chromatography (GC8A; Shimadzu Corporation, Kyoto, Japan) with an electron capture detector.

N_2O fluxes were calculated starting from the change in chamber headspace N_2O concentration (concentration *vs* time), using linear regression analysis (Vitale *et al.*, 2018). The linearity of the headspace concentration of N_2O was previously checked over the adopted closure period (45 min) (all fluxes were screened for potential nonlinearity). According to Gelfand *et al.* (2016) and Koga *et al.* (2017), N_2O fluxes were calculated as:

$$F = M\ V_0\ P\ P_0\ 273 + T_0 273+T\ h\ dCdt \qquad (1)$$

where T_0, P_0 and V_0 are the absolute air temperature, atmospheric pressure, and molar volume under standard conditions, respectively, M is the molecular weight of gas X, P is the pressure outside the chamber, dCdt is the slope of the curve of gas X concentration variation with time (ppm h^{-1}), and h is the height of the chamber from the base ring to the top.
The cumulative weekly soil N_2O emissions were calculated by linear interpolation between the successive measurements (Ball *et al.*, 2007), and determined by summing the daily fluxes over periods of 7 days.
The seasonal cumulative soil N_2O emissions were calculated by linear interpolation (Gelfand *et al.*, 2016), assuming a linear flux change between sampling days (Abalos *et al.*, 2016; Volpi *et al.*, 2016, 2018; Westphal *et al.*, 2018), and summing over the whole experimental period.

3.1.3.5 Statistical analysis

According to Krauss *et al.* (2017), the soil N_2O emissions of the two pseudo-replicates per plot underwent arithmetic averaging before the statistical analysis was performed. The soil N_2O emissions are presented as fluxes (N-N_2O g ha^{-1} h^{-1}), cumulative weekly emissions (N-N_2O kg ha^{-1}) and seasonal cumulative emissions (N-N_2O kg ha^{-1}).
Prior to any analysis, all of the data were tested for normal distributions (Shapiro-Wilk tests) and homogeneous variance (Levene's tests), and when required for sphericity (Mauchly's tests). Where assumptions were met, repeated measures ANOVA was carried out to determine the effects of time (within factor), treatment (between factors) and their interactions (time × treatment), with one-way ANOVA carried out to determine the differences within each sampling date. Conversely, where assumptions were not met, Wilcoxon signed-rank tests were used instead of repeated measures ANOVA, and Kruskal-Wallis ANOVA instead of one-way ANOVA. Significance was assumed for all of the tests at the limiting value of $p < 0.05$, unless otherwise indicated. Both the parametric and non-parametric tests were carried out using SPSS Statistics, version 25.0 (SPSS Inc., IBM, Chicago, IL, USA).

3.1.4 Results

3.1.4.1 Nitrous oxide fluxes

The soil N_2O fluxes varied markedly across the treatments and throughout the monitoring period. These ranged from -0.02 ± 0.01 to 0.53 ± 0.14 g N-N_2O ha^{-1} h^{-1} for continuous alfalfa, and from 0.02 ± 0.07 to 0.37 ± 0.11 g N-N_2O ha^{-1} h^{-1} for alfalfa+wheat (Figure 2c). According to the Wilcoxon signed-rank tests, the difference scores in terms of the daily soil N_2O emissions between continuous alfalfa and alfalfa+wheat were approximately symmetrically distributed, as assessed using a histogram with the superimposed normal curve. This test did not highlight any statistically significant median increase in soil N_2O fluxes for alfalfa+wheat (0.13 g N_2O-N ha^{-1} h^{-1}) compared to continuous alfalfa (0.09 g N_2O-N ha^{-1} h^{-1}; $z = 1.97$; $p = 0.053$).
During the study period, out of the 33 sampling dates, alfalfa+wheat had higher soil N_2O emissions for 22 of the sampling dates, and continuous alfalfa for 11. One-way ANOVA highlighted differences between the treatments for only two dates: 21 December 2017, about 2 months after alfalfa termination for alfalfa+wheat, and 5 July 2018, the day after the wheat harvest (alfalfa+wheat) and the second alfalfa mowing (continuous alfalfa) (Figure 2c). On the sampling dates for which the homogeneity of variance of the data was not met, Kruskal-Wallis H tests highlighted that for 1 December 2017 and 20 June 2018, the soil N_2O emissions were different between the two treatments, as assessed by visual inspection of the boxplot. In particular, on 1 December 2017, the distributions of the soil N_2O emission levels were significantly higher for alfalfa+wheat compared to continuous alfalfa ($H(1) = 3.857$, $p = 0.05$); while for 20 June 2018, the distributions of the soil N_2O emission levels were not significantly different between these treatments ($H(1) = 0.048$, $p = 0.827$).
Both treatments showed fluctuations in their fluxes during the whole study period (Figure 2c). Several soil N_2O emission peaks were recorded for both treatments, mainly from October to December 2017 for alfalfa+wheat, and from the end of May to the end of June 2018 for continuous alfalfa.
During the first period (i.e., October-December 2017), the alfalfa+wheat treatment was characterized by a peak on the first sampling day (0.28 ±0.07 g N_2O-N ha^{-1} h^{-1}), and then after a strong drop, another peak at the end of October 2017 (0.37 ±0.11 g N_2O-N ha^{-1} h^{-1}; on 24 October). Then, another peak was recorded on 17 November 2017 (0.24 ±0.05 g N_2O-N ha^{-1} h^{-1}), which was followed by a marked drop before a peak on 5 December 2017 (0.16 ±0.04 g N_2O-N ha^{-1} h^{-1}). Soil N_2O emissions peaks recorded during this period coincided with important rainfall events that occurred immediately before the N_2O emission pulses (Figure 2a) and with increasing soil water content (Figure 2b). During this first monitoring period, the soil N_2O fluxes followed the soil temperature dynamics (Figure 2b), except for the sampling dates on which rainfall and N_2O pulses occurred.
Between January and the end of February 2018, both treatments showed fluctuations and similar soil N_2O emission fluxes, except for 3 January 2018, when a peak of soil N_2O emission rate was recorded for alfalfa+wheat (0.15 ±0.03 g N_2O-N ha^{-1} h^{-1}), and for 16 January 2018, when an increase in the soil N_2O emission fluxes occurred for continuous alfalfa (0.13 ±0.07 g N_2O-N ha^{-1} h^{-1}), as compared to a sharper decrease for alfalfa+wheat (0.02 ±0.07 g N_2O-N ha^{-1} h^{-1}). During this time the decrease in the soil N_2O emissions followed the reduction in the soil temperature compared to the previous period, while a general increase in soil water content was observed.

From March to the end of April 2018, the soil N_2O emissions were comparable to the previous period, and both treatments showed similar fluxes. In this period an increase in soil temperatures occurred in conjunction with a reduction in soil moisture and a slow increase in soil N_2O emissions.

Between the beginning of May and early July 2018, continuous alfalfa was characterized by three relevant N_2O peaks that occurred at the end of May 2018 (0.34 ±0.11, 0.53 ±0.14, 0.42 ±0.17 g N_2O-N ha^{-1} h^{-1}, for 22, 24, 29 May 2018, respectively), soon after rainfall. Compared to the previous period, the alfalfa+wheat treatment showed an increase in the soil N_2O emissions fluxes in line with the increasing soil temperature and decreasing soil water content, except in the second half of May, when rainfall and N_2O pulses occurred. In conclusion, compared to continuous alfalfa, alfalfa+wheat showed similar soil N_2O emission trends, except for the last three sampling dates when it showed a constant decrease.

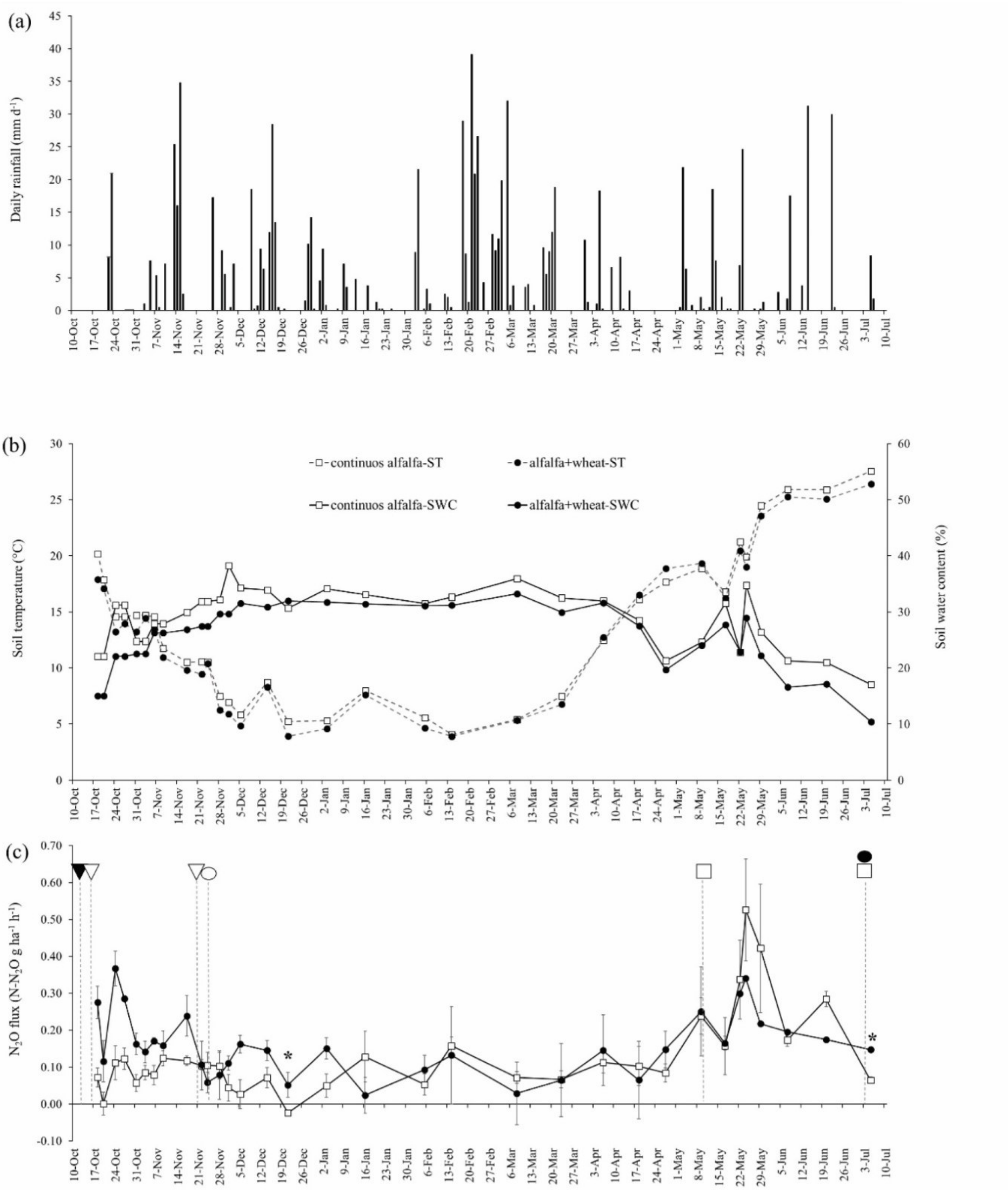


Figure 2. (a) Monitored rainfall during the study period. (b) Seasonal variations of the soil water content (SWC) and the soil temperature (ST), as indicated. (c) Soil nitrous oxide fluxes for ‘continuous alfalfa’ (open squares) and ‘alfalfa+wheat’ (closed circles). Filled upside-down triangle, date of spading for alfalfa+wheat; empty upside-down

triangles, dates of harrowing for alfalfa+wheat; open circle, date of wheat sowing for alfalfa+wheat; open squares, date of the mowing for continuous alfalfa; black circle, date of wheat harvesting for alfalfa+wheat. Data are means ±standard errors (n = 3). *, p <0.05, continuous alfalfa *versus* alfalfa+wheat.

3.1.4.2 Cumulative weekly and seasonal soil nitrous oxide emissions

The cumulative weekly soil N_2O emissions increased over the study period for both treatments, as 0.00 ±0.00 to 0.77 ±0.09 N-N_2O kg ha^{-1} for continuous alfalfa, and 0.01 ±0.00 to 0.85 ±0.18 N-N_2O kg ha^{-1} for alfalfa+wheat. The cumulative weekly soil N_2O emissions showed higher values for alfalfa+wheat compared to continuous alfalfa throughout the monitored period (Figure 3). No significant time×treatment interactions were seen, while the treatment had a significant effect on the cumulative weekly soil N_2O emissions over the monitored period.

Immediately after alfalfa termination for alfalfa+wheat there were higher soil N_2O emissions compared to continuous alfalfa. Indeed, from October 2017 to the first week of February 2018, the cumulative weekly soil N_2O emissions were almost always significantly greater for alfalfa+wheat, compared to continuous alfalfa (i.e., from 25 October to 29 November 2017; from 27 December 2017 to 7 February 2018).

From the second decade of February 2018 until the beginning of May 2018, no significant differences between the treatments emerged and a slight increasing trend was observed for both treatments. Then from early May 2018 until the end of the study period, the absence of significant differences in soil N_2O emissions between treatments was confirmed, although a more pronounced increasing trend was recorded compared to the previous period.

In general, the seasonal cumulative soil N_2O emissions for continuous alfalfa and alfalfa+wheat (i.e., last cumulative weekly point of Figure 3) did not differ significantly between the two treatments over the monitored period: 0.77 ±0.09, 0.85 ±0.18 kg N-N_2O ha^{-1}, respectively.

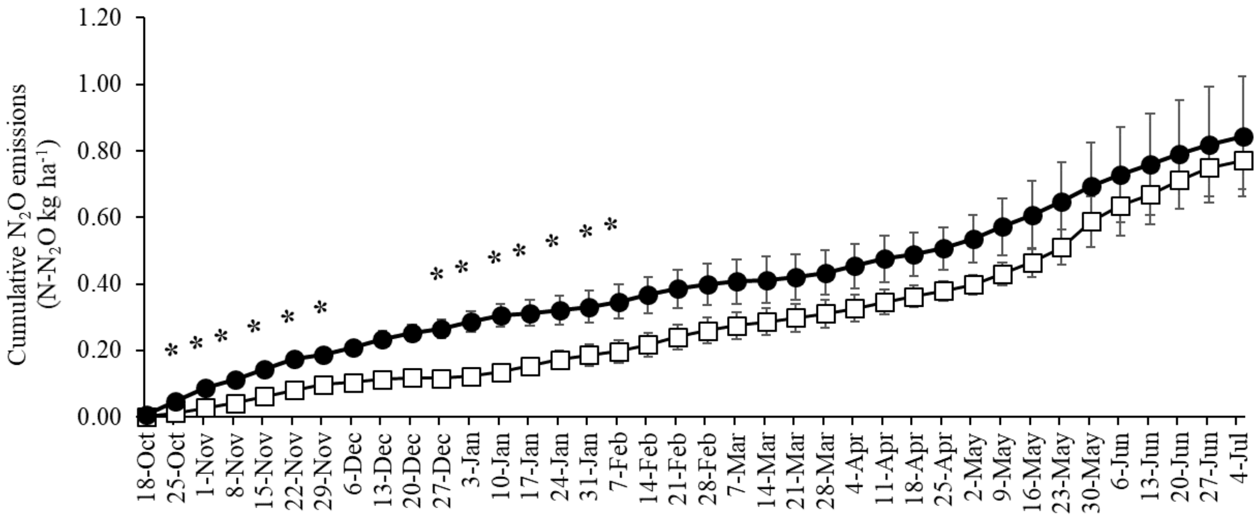


Figure 3. Cumulative weekly soil N_2O emissions over the study period for continuous alfalfa (open squares) and alfalfa+wheat (closed circles). Data are means ± standard error (n = 3). *, p <0.05, continuous alfalfa *versus* alfalfa+wheat. The last cumulative weekly point represents the cumulative seasonal soil N_2O emission.

3.1.5 Discussion

Soil N_2O emissions are related to pedo-climatic conditions (e.g., rainfall, soil temperature) and management factors (e.g., crop residue type and incorporation depth, tillage type and timing), which thus represent the main drivers of soil variations in N_2O emissions (Estavillo *et al.*, 2002; Ball *et al.*, 2007; Butterbach-Bahl *et al.*, 2013; Krauss *et al.*, 2017; Luo *et al.*, 2013). In addition to these factors, some others might also affect soil N_2O emissions. These include crop type and behaviour, in relation to the phenological stages, and overall, the cropping system used (Liu *et al.*, 2015). The dynamics of the soil N_2O emission were therefore examined over the study period in relation to these main factors.

Unlike some studies that were carried out under similar climatic conditions (Cayuela *et al.*, 2017; Volpi *et al.*, 2018), in the present study, no relationships were found between soil N_2O emissions, soil temperature and soil water content (data not shown). Although the soil water content is one of the major drivers of soil N_2O emissions and regulation of oxygen availability (Butterbach-Bahl *et al.*, 2013), in the present field study the soil N_2O fluxes varied markedly throughout the study period following the dynamics of the soil temperature, which is another important climatic factor that induces variations in soil N_2O fluxes (Luo *et al.*, 2013). Soil N_2O emission pulses are typical of Mediterranean climates, and these usually occur after rainfall and rewetting after dry periods (Aguilera *et al.*, 2013). As also seen by Aguilera *et al.* (2013), in the present study the soil N_2O emissions increased sharply after rainfall, and the consequent increase in soil water content.

As expected, an increase in soil N_2O emissions occurred soon after alfalfa termination under alfalfa+wheat, compared to continuous alfalfa, which was probably due to soil aeration by the tillage and the quality of the crop residues incorporated (low C:N ratio) (Lin *et al.*, 2013; Basche *et al.*, 2014). Both these factors might have promoted easier mineralisation of the residues (Toma and Hatano, 2007), to increase the substrate for the nitrification and denitrification processes (Estavillo *et al.*, 2002). Nitrification and denitrification, which are particularly active for the 0-0.1 m soil layer, were identified as the processes that contribute most to soil N_2O emissions over the short term after termination of permanent grassland by tillage (Estavillo *et al.*, 2002). In the present study, the soil conditions were mainly favorable to nitrification processes, especially soon after the alfalfa termination, although denitrification processes might also have occurred. Indeed, a study by Huang *et al.* (2004) with conditions favorable to nitrification showed a negative correlation between soil N_2O emission and residue C:N ratio. This was attributed to both ease of mineralization of this type of crop residue, with the consequent increase in N availability (Basche *et al.*, 2014), and stimulation of microbial activity, which promoted oxygen consumption (Lesschen *et al.*, 2011), and which might have created temporary anaerobic microsites that would then have enhanced N_2O production via denitrification processes (Huang *et al.*, 2004; Mutegi *et al.*, 2010; Jensen *et al.*, 2012). Similarly, in the present study, the soil tillage might have promoted oxygen diffusion into the soil, with the consequent mineralization and nitrification processes, which might have also caused a temporary anoxic environment through increased microbial activity and respiration; this would have favored denitrification processes. Furthermore, as indicated by Álvaro-Fuentes *et al.* (2008), soil tillage for alfalfa+wheat might have led to break-up of the soil aggregates, with the consequent release of N_2O from their core, which is under anoxic conditions (Borer *et al.*, 2018), and which would promote denitrification processes.

The data obtained in the present study are in line with Abalos *et al.* (2016), who reported an important increase in soil N_2O emission soon after termination of a perennial grass–legume mixture in September, with ploughing to a depth of 0.20 m. The emissions recorded in the present study soon after alfalfa termination were probably lower than would be expected after summer alfalfa termination (Krauss *et al.*, 2017), as also observed by Ball *et al.* (2007), who carried out second-year grass–clover termination by ploughing (depth, 0.25 m) and recorded soil N_2O emissions that were almost double after the summer tillage (i.e., in July), compared to autumn tillage (i.e., in October). Indeed, although soil moisture has the greatest effect on soil N_2O emission, denitrification is particularly sensitive to increased and increasing temperatures. These conditions increase oxygen consumption by microorganism respiration and the consequent soil anaerobiosis, with this anaerobiosis is a precursor and major driver of soil N_2O emissions (Butterbach-Bahl *et al.*, 2013). However, in addition to the paucity of information on the effects of perennial legume termination in terms of soil N_2O emissions (Jensen *et al.*, 2012), the correct interpretation of the data available is uncertain, as they are mainly context dependent. Indeed, some studies have reported that high soil N_2O emissions after alfalfa termination by ploughing in late summer is mainly due to the environmental conditions, such as high autumn soil moisture and spring thawing of the soil, as for a glacio-lacustrine clay floodplain (Westphal *et al.*, 2018; Tenuta *et al.*, 2019). Alternatively, in a field experiment in Grey Luvisol with loam texture, another study reported that the timing of alfalfa termination (i.e., spring, summer, late summer) and the method used (i.e., tillage, herbicide, both) had no influence on soil N_2O emissions for 7-year-old alfalfa (Malhi *et al.*, 2010).

In the present study, crop type and rotation also had fundamental roles in the regulation of the magnitude of the soil N_2O emissions. From October 2017 to the end of December 2017, during the wheat seedling stage, the alfalfa+wheat soil N_2O emissions accounted for 32.6% of the total emissions, which was double that for continuous alfalfa (15.5%). Indeed, during the initial wheat stage, when the wheat N uptake is expected to be low compared to the later stages (Delogu *et al.*, 1998; Li *et al.*, 2012), high N levels from the mineralized legume residues after the tillage should be available in the soil for alfalfa+wheat. On this basis, the N released into the soil after alfalfa termination (on 11 October 2017) cannot be used immediately by the wheat that was sown about a month and a half later (on 23 November 2017), and this therefore might have increased the nitrification and denitrification substrate for N_2O production. Between January and the end of February 2018, during the wheat tillering–double ridge stage, the alfalfa+wheat soil N_2O emissions accounted for 14.4% of its total emissions, with similar amounts for continuous alfalfa (18.3%). In this second period, the soil N_2O emissions were reduced for alfalfa+wheat, which was probably due to the greater use of N by the wheat in the sowing–greening stages, in line with that reported by Liu et al. (2015) under a wheat crop cycle. From March to the end of April 2018, during the wheat double ridge–jointing stage, the alfalfa+wheat soil N_2O emissions were like the previous period (15.0%) and similar to continuous alfalfa (16.9%). As in the previous wheat stage, during this period the low level of soil N_2O emission might have been due to nitrate subtraction in potential nitrification and denitrification processes, due to the high N uptake by the wheat crop at this stage (Delogu *et al.*, 1998). Between the beginning of May and early July 2018, during the wheat booting–maturity stage, the alfalfa+wheat soil N_2O emissions accounted for 37.7% of its total emissions, which was lower than for continuous alfalfa (49.4%). During this last monitoring period a general increase in soil N_2O emission might also have been due to the increase in soil temperature and rainfall that occurred in May (Aguilera *et al.*, 2013). For continuous alfalfa, the increase in the soil N_2O emission fluxes might also have been due to the mowing that was performed in early May 2018, which might have reduced the N uptake from the root system (Erice *et al.*, 2011). This might be related to the removal of the photosynthetic tissues, with the consequent change in the alfalfa N metabolism. In particular, herbage cutting might have led to reduction in the uptake of the mineral N forms from the soil, and to a general decrease in nodule activity (Erice *et al.*, 2011). For alfalfa+wheat, the decreasing trend of the last sampling period can be explained by the higher N uptake that occurred from the heading to the maturity stage (Delogu *et al.*, 1998; Li *et al.*, 2012).

Despite the variations in the soil N_2O emissions highlighted through the study period, especially for the alfalfa+wheat treatment, the cumulative seasonal emissions did not show significant differences. This might be linked to the autumn alfalfa termination which might have shortened the time window between N release and N uptake, which will have reduced the substrate for nitrification and denitrification processes, except soon after the incorporation of the crop residues. The cumulative seasonal soil N_2O emissions in this study are consistent with those reported in other studies under similar climatic conditions. For example, Volpi *et al.* (2018) reported a cumulative soil N_2O emission of 0.87 kg N-N_2O ha^{-1} for durum wheat seeded after clover, which included minimum tillage (i.e., disk harrow, at the beginning of September; depth, 0.10 m) without N fertilisation, in a Mediterranean environment. These data contribute to the definition of mitigation strategies for GHG emissions (Purwanto and Alam, 2020) that can be used for this crop rotation in a Mediterranean environment. Moreover, to increase the impact and effectiveness of the mitigation practices, these should be included in site-specific agro-environmental climate measures at the landscape scale (Toderi *et al*., 2017).

3.1.6 Conclusions

This study helps to fill an important knowledge gap concerning the effects on soil N_2O emissions relating to perennial legume termination in Mediterranean crop rotation systems. To identify GHG mitigation options, the present study analysed soil N_2O emissions with termination of the perennial legume in an alfalfa–wheat rotation in a Mediterranean environment.

Perennial legume termination in early autumn appears to have provided less favorable conditions for the mineralization process compared to hypothetical termination in summer, with higher temperatures. The initial higher soil N_2O emissions for alfalfa+wheat that emerged from the cumulative weekly analysis appeared to be due to the alfalfa mineralization process after the tillage, and to the unavoidable asynchrony between the N released following alfalfa termination and the low N uptake by the following wheat. Reducing time-window between alfalfa termination and wheat N uptake can contain the N_2O emissions**,** except after an initial inevitable increase in the soil N_2O emissions. However, this initial higher soil N_2O emission for alfalfa+wheat did not affect the seasonal cumulative soil N_2O emissions, compared to continuous alfalfa.

In conclusion, under the rotation system analyzed here, the mitigation effects of the perennial legume on the soil N_2O emissions were not lost after its termination by tillage.

In this production context, further studies are needed to confirm these effects of perennial legume termination by tillage on soil N_2O emissions, including the need to compare this to other alfalfa termination methods (e.g., using a desiccant and subsequent cereal sod-seeding). To contribute further to the identification of mitigation strategies for GHG emissions in crop rotation systems under Mediterranean conditions, studies are also needed that focus on N_2O and other important GHGs (e.g., CO_2, CH_4) in this and other relevant production contexts. These should include: i) soils where the main tillage performed in autumn is more difficult due to the rainfall regime and the soil requirements; and ii) other cereal-based crop rotation systems with short-lived perennial legumes, such as sulla (*Hedysarum coronarium* L.) and common sainfoin (*Onobrychis viciifolia* Scop.).

3.1.7 Literature

Abalos D, Brown SE, Vanderzaag AC, Gordon RJ, Dunfield KE, Wagner-Riddle C, 2016. Micrometeorological measurements over 3 years reveal differences in N2O emissions between annual and perennial crops. Glob. Chang. Biol. 22:1244-55.

Agnelli A, Allegrezza M, Biondi E, Cocco S, Corti G, Pirchio F, 2008. Pedogenesi e paesaggio vegetale: il ruolo dell'esposizione. Fitosociologia 45:23-8.

Aguilera E, Lassaletta L, Sanz-Cobena A, Garnier J, Vallejo A, 2013. The potential of organic fertilizers and water management to reduce N2O emissions in Mediterranean climate cropping systems. A review. Agr. Ecosyst. Environ. 164:32-52.

Álvaro-Fuentes J, Arrúe JL, Cantero-Martínez C, López MV, 2008. Aggregate breakdown during tillage in a Mediterranean loamy soil. Soil Till. Res. 101:62-8.

Autret B, Beaudoin N, Rakotovololona L, Bertrand M, Grandeau G, Gréhan E, Ferchaud F, Mary B, 2019. Can alternative cropping systems mitigate nitrogen losses and improve GHG balance? Results from a 19-year experiment in northern France. Geoderma 342:20-33.

Ball BC, Watson CA, Crichton I, 2007. Nitrous oxide emissions, cereal growth, N recovery and soil nitrogen status after ploughing organically managed grass/clover swards. Soil Use Manage. 23:145-55.

Basche AD, Miguez FE, Kaspar TC, Castellano MJ, 2014. Do cover crops increase or decrease nitrous oxide emissions? A meta-analysis. J. Soil Water Conserv. 69:471-82.

Borer B, Tecon R, Or D, 2018. Spatial organization of bacterial populations in response to oxygen and carbon counter-gradients in pore networks. Nat. Commun. 9:1-11.

Budimir K, Trombetta MF, Francioni M, Toderi M, D'Ottavio P, 2018. Slaughter performance and carcass and meat quality of Bergamasca light lambs according to slaughter age. Small Ruminant Res. 164:1-7.

Butterbach-Bahl K, Baggs EM, Dannenmann M, Kiese R, Zechmeister-Boltenstern S, 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philos. T. R. Soc. B 368.

Cayuela ML, Aguilera E, Sanz-Cobena A, Adams DC, Abalos D, Barton L, Ryals R, Silver WL, Alfaro MA, Pappa VA, Smith P, Garnier J, Billen G, Bouwman L, Bondeau A, Lassaletta L, 2017. Direct nitrous oxide emissions in Mediterranean climate cropping systems: emission factors based on a meta-analysis of available measurement data. Agr. Ecosyst. Environ. 238:25-35.

Delogu G, Cattivelli L, Pecchioni N, De Falcis D, Maggiore T, Stanca AM, 1998. Uptake and agronomic efficiency of nitrogen in winter barley and winter wheat. Eur. J. Agron. 9:11-20.

Erice G, Sanz-Sáez A, Aranjuelo I, Irigoyen JJ, Aguirreolea J, Avice J-C, Sánchez-Díaz M, 2011. Photosynthesis, N2 fixation and taproot reserves during the cutting regrowth cycle of alfalfa under elevated CO2 and temperature. J. Plant Physiol. 168:2007-14.

Estavillo JM, Merino P, Pinto M, Yamuli S, Gebauer G, Sapek A, Corré A, 2002. Short term effect of ploughing a permanent pasture on N2O production from nitrification and denitrification. Plant Soil 239:253-65.

Forster P, Ramaswamy V, Artaxo P, Berntsen T, Betts R, Fahey DW, Haywood J, Lean J, Lowe DC, Myhre G, Nganga J, Prinn R, Raga G, Schulz M, Van Dorland R, 2007: Changes in Atmospheric Constituents and in Radiative Forcing. In: Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL (eds.)]. Cambridge University Press, Cambridge, UK and New York, USA, pp 129-234.

Forte A, Fiorentino N, Fagnano M, Fierro A, 2017. Mitigation impact of minimum tillage on CO_2 and N_2O emissions from a Mediterranean maize cropped soil under low-water input management. Soil Till. Res. 166:167-78.

Francioni M, D'Ottavio P, Lai R., Trozzo L, Budimir K, Foresi L, Kishimoto-Mo AW, Baldoni N, Allegrezza M, Tesei G, Toderi M, 2019. Seasonal soil respiration dynamics and carbon-stock variations in mountain permanent grasslands compared to arable lands. Agriculture 9:165.

Francioni M, Lai R, D'Ottavio P, Trozzo L, Kishimoto-Mo AW, Budimir K, Baldoni N, Toderi M, 2020. Soil respiration dynamics in forage-based and cereal-based cropping systems in central Italy. Sci. Agr. 77, e20180096.

Gelfand I, Shcherbak I, Millar N, Kravchenko AN, Robertson GP, 2016. Long-term nitrous oxide fluxes in annual and perennial agricultural and unmanaged ecosystems in the upper Midwest USA. Glob. Change Biol. 22:3594-607.

Ghimire R, Norton U, Bista P, Obour AK, Norton JB, 2017. Soil organic matter, greenhouse gases and net global warming potential of irrigated conventional, reduced tillage and organic cropping systems. Nutr. Cycl. Agroecosys. 107:49-62.

Gomes J, Bayer C, de Souza Costa F, de Cássia Piccolo M, Zanatta JA, Vieira FCB, Six J, 2009. Soil nitrous oxide emissions in long-term cover crops-based rotations under subtropical climate. Soil Till. Res. 106:36-44.

Huang Y, Zou J, Zheng X, Wang Y, Xu X, 2004. Nitrous oxide emissions as influenced by amendment of plant residues with different C:N ratios. Soil Biol. Biochem. 36:973-81.

Jensen ES, Peoples MB, Boddey RM, Gresshoff PM, Hauggard-Nielsen H, Alves BJR, Morrison MJ, 2012. Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. Agron. Sustain. Dev. 32:329-364

Kim DG, Hernandez-Ramirez G, Giltrap D, 2013. Linear and nonlinear dependency of direct nitrous oxide emissions on fertilizer nitrogen input: a meta-analysis. Agr. Ecosyst. Environ. 168:53-65.

Koga N, Shimoda S, Iwata Y, 2017. Biochar impacts on crop productivity and greenhouse gas emissions from an Andosol. J. Environ. Qual. 46:27-35.

Krauss M, Ruser R, Müller T, Hansen S, Mäder P, Gattinger A, 2017. Impact of reduced tillage on greenhouse gas emissions and soil carbon stocks in an organic grass-clover ley - winter wheat cropping sequence. Agr. Ecosyst. Environ. 239:324-33.

Lesschen JP, Velthof GL, De Vries W, Kros J, 2011. Differentiation of nitrous oxide emission factors for agricultural soils. Environ. Pollut. 159:3215-22.

Li L peng, Liu Y ying, Luo S guo, Peng X long, 2012. Effects of nitrogen management on the yield of winter wheat in cold area of northeastern China. J. Integr. Agr. 11:1020-5.

Lin S, Iqbal J, Hu R, 2013. Nitrous oxide emissions from yellow brown soil as affected by incorporation of crop residues with different carbon-to-nitrogen ratios: a case study in central China. Arch. Environ. Con. Tox. 65:183-92.

Liu YN, Li YC, Peng ZP, Wang YQ, Ma SY, Guo LP, Lin E Da, Han X, 2015. Effects of different nitrogen fertilizer management practices on wheat yields and N2O emissions from wheat fields in north China. J. Integr. Agr. 14:1184-91.

Luo GJ, Kiese R, Wolf B, Butterbach-Bahl K, 2013. Effects of soil temperature and moisture on methane uptake and nitrous oxide emissions across three different ecosystem types. Biogeosciences 10:3205-19.

Malhi SS, Lemke R, Schoenau JJ, 2010. Influence of time and method of alfalfa stand termination on yield, seed quality, N uptake, soil properties and greenhouse gas emissions under different N fertility regimes. Nutr. Cycl. Agroecosys. 86:17-38.

Monaci E, Polverigiani S, Neri D, Bianchelli M, Santilocchi R, Toderi M, D'Ottavio P, Vischetti C, 2017. Effect of contrasting crop rotation systems on soil chemical and biochemical properties and plant root growth in organic farming: first results. Ital. J. Agron. 12:364-74.

Mutegi JK, Munkholm LJ, Petersen BM, Hansen EM, Petersen SO, 2010. Nitrous oxide emissions and controls as influenced by tillage and crop residue management strategy. Soil Biol. Biochem. 42:1701-11.

Paetz A, Wilke BM, 2005. Soil sampling and storage. In: Margesin R, Schinner F (ed.) Manual of soil analysis: monitoring and assessing soil bioremediation. Springer-Verlag, Berlin, DE, pp 1-45.

Parkin TB, Venterea RT, 2010. Chapter 3. Chamber-based trace gas flux measurements. Sampling protocols. In: USDA-ARS GRACEnet project protocols, Beltsville, MD, pp 1-39.

Purwanto BH, Alam S, 2020. Impact of intensive agricultural management on carbon and nitrogen dynamics in the humid tropics. Soil Sci. Plant. Nutr. 66:50-9.

Reay DS, Davidson EA, Smith KA, Smith P, Melillo JM, Dentener F, Crutzen PJ, 2012. Global agriculture and nitrous oxide emissions. Nat. Clim. Chang. 2:410-6.

Sanz-Cobena A, Lassaletta L, Aguilera E, del Prado A, Garnier J, Billen G, Iglesias A, Sánchez B, Guardia G, Abalos D, Plaza-Bonilla D, Puigdueta-Bartolomé I, Moral R, Galán E, Arriaga H, Merino P, Infante-Amate J, Meijide A, Pardo G, Álvaro-Fuentes J, Gilsanz C, Báez D, Doltra J, González-Ubierna S, Cayuela ML, Menéndez S, Díaz-Pinés E, Le-Noë J, Quemada M, Estellés F, Calvet S, van Grinsven HJM, Westhoek H, Sanz MJ, Gimeno BS, Vallejo A, Smith P, 2017. Strategies for greenhouse gas emissions mitigation in Mediterranean agriculture: a review. Agr. Ecosyst. Environ. 238:5-24.

Shan J, Yan X, 2013. Effects of crop residue returning on nitrous oxide emissions in agricultural soils. Atmos. Environ. 71:170-5.

Signor D, Cerri CEP, 2013. Nitrous oxide emissions in agricultural soils: a review. Pesqui. Agropecuária Trop. 43:322-38.

Smith DW, 2014. Soil survey staff: keys to soil taxonomy. 12th ed. Natural Resources Conservation Service, Washington, DC, USA.

Smith P, Bustamante M, Ahammad H, Clark H, Dong H, Elsiddig EA, Haberl H, Harper R, House J, Jafari M, Masera O, Mbow C, Ravindranath NH, Rice CW, Robledo Abad C, Romanovskaya A, Sperling F, Tubiello F, 2014. Agriculture, Forestry and Other Land Use (AFOLU) In: Climate Change 2014: Mitigation of Climate Change Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Edenhofer O, Pichs-Madruga R, Sokona Y, Farahani E, Kadner S, Seyboth K, Adler A, Baum I, Brunner S, Eickemeier P, Kriemann B, Savolainen J, Schlömer S, von Stechow C, Zwickel T, Minx JC (eds)]. Cambridge University Press, Cambridge, UK and New York, USA.

Stehfest E, Bouwman L, 2006. N_2O and NO emission from agricultural fields and soils under natural vegetation: summarizing available measurement data and modeling of global annual emissions. Nutr. Cycl. Agroecosys. 74:207-28.

Syakila A, Kroeze C, 2011. The global nitrous oxide budget revisited. Greenh. Gas Meas. Manag. 1:17-26.

Tenuta M, Amiro BD, Gao X, Wagner-Riddle C, Gervais M, 2019. Agricultural management practices and environmental drivers of nitrous oxide emissions over a decade for an annual and an annual-perennial crop rotation. Agr. Forest. Meteorol. 276:107636.

Tesfaye M, Silverstein KAT, Bucciarelli B, Samac DA, Vance CP, 2006. The Affymetrix Medicago GeneChip array is applicable for transcript analysis of alfalfa (*Medicago sativa*). Funct. Plant Biol. 33:783-8.

Toderi M, Francioni M, Seddaiu G, Roggero PP, Trozzo L, D'Ottavio P, 2017. Bottom-up design process of agri-environmental measures at a landscape scale: evidence from case studies on biodiversity conservation and water protection. Land Use Policy 68:295-305.

Marks E. A. N., 2013. Biochar Effects on Soil Quality as Evaluated by Physical, Chemical, and Biological Parameters. Centre de recerca Ecològica i Aplicacions Forestale (CREAF). Universitat Autònoma de Barcelona.

Toma Y, Hatano R, 2007. Effect of crop residue C:N ratio on N2O emissions from gray lowland soil in Mikasa, Hokkaido, Japan. Soil Sci. Plant Nutr. 53:198-205.

Van Groenigen JW, Velthof GL, Oenema O, Van Groenigen KJ, Van Kessel C, 2010. Towards an agronomic assessment of N_2O emissions: a case study for arable crops. Eur. J. Soil Sci. 61:903-13.

Vitale L, Tedeschi A, Polimeno F, Ottaiano L, Maglione G, Arena C, De Marco A, Magliulo V, 2018. Water regime affects soil N_2O emission and tomato yield grown under different types of fertilisers. Ital. J. Agron. 13:74-9.

Volpi I, Bosco S, Nassi o Di Nasso N, Triana F, Roncucci N, Laville P, Neri S, Virgili G, Bonari E, 2016. Nitrous oxide emissions from clover in the Mediterranean environment. Ital. J. Agron. 11:133-6.

Volpi I, Laville P, Bonari E, Nassi o Di Nasso N, Bosco S, 2018. Nitrous oxide mitigation potential of reduced tillage and N input in durum wheat in the Mediterranean. Nutr. Cycl. Agroecosys. 111:189-201.

Walter H, Lieth H, 1960. Klimadiagramm weltatlas. Jena. Gustav Fischer, Stuttgart.

Wang W, Fang J, 2009. Soil respiration and human effects on global grasslands. Global. Planet. Change 67:20-8.

Wei XR, Hao MD, Xue XH, Shi P, Horton R, Wang A, Zang YF, 2010. Nitrous oxide emission from highland winter wheat field after long-term fertilization. Biogeosciences 7:3301-10.

Westphal M, Tenuta M, Entz MH, 2018. Nitrous oxide emissions with organic crop production depends on fall soil moisture. Agr. Ecosyst. Environ. 254:41-9.

Willer H, Lernoud J, 2017. The World of Organic Agriculture. Statistics and Emerging Trends 2017. Research Institute of Organic Agriculture (FiBL), Frick, CH and IFOAM - Organics International, Bonn, DE. Version 1.3 of February 20, 2017.

4 Chapter 3

4.1 Effect of high-temperature pyrolyzed biochar on physicochemical properties and enzyme activities of sub-alkaline soil, and on wheat production.

4.1.1 Abstract

Biochar is the product of heating biomass through pyrolysis which is intended for use as a soil amendment. Biochar might have several other positive and-or negative effects on soil nutrient cycle, crop yields and greenhouse gas emissions. These effects tend to be however strongly linked to both biochar original source and soil characteristics. Enzymes are considered reliable indicators of soil organic matter decompositions and they might be affected by agricultural practices, including the addition of biochar. The influence of biochar addition on enzyme activities can occur through both biochar incorporation and biochar persistence in the soil. This can occur either from a long or a short-term perspective. The aim of this study was to assess the short-term effects of the incorporation of a high dose of wood-derived biochar (60 t ha^{-1}) produced at high-temperature into a sub-alkaline soil. The study included the analysis of the eeffects of biochar incorporation on soil fertility (physio-chemical properties) and the monitoring of set of 12 enzyme activities involved in the carbon, nitrogen, phosphorous and sulphur nutrient cycles. Effects of biochar incorporation on grain yield were also assessed. The study was carried out from October 2018 to September 2019 in a representative hilly area of Marche region where winter cereals are put in rotation with forage crops. The studied biochar results intended to increase aggregate stability and to build up SOM, but results were not significant. The biochar incorporation and its short-time presence had no effect on soil pH, TN, available P and on wheat production. Results suggest that the activity of the analysed enzymes were not affected by the presence of wood-derived biochar, in the short term. Conversely, the tillage required to incorporate biochar in the soil resulted in an increase of the activity of the enzymes mainly linked to the carbon-cycle, but this emerged regardless of biochar incorporation.

4.1.2 Introduction

Biochar is the product of biomass pyrolysis and is often produced to be used as soil amendment (Burns et al., 2013; Lehmann and Joseph, 2015). In the last two decades biochar has received much attention due to its beneficial effects on soil fertility and quality (Han et al., 2020; Rafael et al., 2019b), greenhouse gas mitigation (Kavitha et al., 2018), and crop yields (Macdonald et al., 2014; Subedi et al., 2017). In fact, because of its high surface area and porosity (Anawar *et al*., 2015; Blackwell *et al*., 2015), biochar is considered a valuable soil conditioner as it can increase soil nutrient retention (Uchimiya *et al*., 2011; Anawar *et al*., 2015), improve soil biological properties (Bamminger *et al*., 2014; Chen *et al*., 2015; Rafael et al., 2019), and increase soil pH of acid soils (Atkinson *et al*., 2010; Anderson *et al*., 2011). However, biochar might have also detrimental effects, being responsible for limiting the development of roots, earthworms, and fungi, but also for enhancing weed growth (Kavitha et al., 2018; Ouyang et al., 2014).

Biochar is an organic carbon rich material (from 40 to 80%) obtained from thermochemical conversion of biomass at temperatures above 250 ºC in the absence of or with limited air. Biochar is roughly made of recalcitrant (aromatic), and labile (amorphous) carbon fractions, and ashes which ratios vary depending on its original biomass source and its production temperature. While for the biomass source, generally, a wood-based material has a more recalcitrant carbon in dotation than a material such as straw (Lehmann et al., 2011), for the temperature of pyrolysation, the higher the temperature, the higher will be the prevalence of recalcitrant carbon compared to the labile one (Lehmann et al., 2011; Schmidt and Noack, 2000).

Biochar has an estimated persistence in the soil and a positive carbon balance that, for the most recalcitrant fraction, accounts for decades or centuries (Wang et al., 2016). Because of this, biochar has been proposed as a carbon-offsetting tool, if it is intimately sequestered in the soil (De Gryze et al., 2010). However, biochar properties like retention or release of carbon and nutrients (e.g., nitrogen, phosphorus) and persistence in soil mainly depend on pyrolysis temperature and the original biomass source (Atkinson *et al*., 2010; Karer et al., 2013; Anawar *et al*., 2015; Molnár *et al*., 2015; Subedi et al., 2017; El-Naggar et al., 2019; Rafael et al., 2019), while its effects depend on soil properties and biochar granulometry (Anawar *et al*., 2015; Solaiman and Anawar, 2015). Therefore, the original biomass source, pyrolysis temperature, and particle size are key features when the main purpose is the use of biochar in agriculture to reduce the inorganic fertilizer rate and/or improve crop yield (Solaiman and Anawar, 2015; Kavitha et al., 2018; Subedi et al., 2017; Wang et al., 2016). However, the effects of biochar incorporation also depends on soil type and climatic conditions (Agegnehu et al., 2016; Han et al., 2020; Kavitha et al., 2018; Macdonald et al., 2014; Subedi et al., 2017; Wang et al., 2016).

When biochar is applied to acid and sub-acid soils, the effects of the addition are maximized mainly because of the increased soil pH, with the consequent increased availability of nutrients and activity of enzymes involved in the carbon (C), nitrogen (N), phosphorus (P), and sulphur (S) cycles (Ouyang et al., 2014; Rafael et al., 2019a; van Zwieten et al., 2010). Instead, in alkaline and sub-alkaline (calcareous) soils, even for a high application rate of biochar, a poor effet is expected because of its alkalinity reaction (Macdonald et al., 2014). In these cases, a possible indicator of biochar application effect can be the activity of a wide pool of enzymes. In fact, enzyme activities are frequently used as indicators of soil functional changes (Allen et al., 2011; Schloter et al., 2003), although the use the enzyme activities only as indicators of soil perturbations might be problematic since enzymatic assays determine potential and not real enzyme activities (Nannipieri et al., 2012; Rao et al., 2014). Many authors evidenced that biochar could improve soil physicochemical properties like soil aeration, specific surface area, and soil water holding capacity, with tangible enhancement of soil enzyme activities (Lehmann et al., 2011, Ouyang et al., 2014; Gul et al., 2015). Other authors, in both long and short-term experiments conducted under field or laboratory conditions, reported that biochar application can increase the activity of soil extracellular enzymes involved in C, N, and P cycles (Masto et al., 2013; Paz-Ferreiro et al., 2012; Demisie et al., 2014; Gascó et al., 2016), and of intracellular enzymes involved in microbial processes such as dehydrogenase and catalase (Kumar et al 2013.). The effect of biochar addition on enzyme activities is controlled by two main factors: i) biochar incorporation (e.g., tillage type, depth, and timing), and ii) biochar persistence in the soil. In summary, the direct and indirect effects of biochar application on soil enzyme activities in both long and short term appear to be due to biochar type and soil characteristics (Palansooriya et al., 2019).

With the aim to assess both direct and indirect effects of a recalcitrant biochar on soil properties and wheat production, we conduct a field experiment by applying a high dose (60 Mg ha^{-1}) of a biochar obtained with the pyrolysis at high temperature (850°C) of a mix woody biomass (beech, pine, and fir) to a sub-alkaline soil under a Mediterranean type of climate like that of central Italy. In doing this we hypothesized that the recalcitrant biochar applied to a sub-alkaline soil could scarcely affect soil functional activity and, consequently, wheat production. The hypothesis was tested by monitoring the effect of this recalcitrant biochar on physicochemical soil properties and on a pool of 12 enzyme activities involved in the C, N, P, and S cycles during a wheat-growing season. The effect on grain yield was also evaluated.

4.1.3 Materials and Methods

4.1.3.1 Study site

The experiment was performed on a hilly area of Marche region, central Italy, where winter cereals (mainly wheat) are often put in rotation with alfalfa meadows that can last up to ten years and can be grazed by transhumant sheep flocks during winter (Francioni et al., 2020; Monaci et al., 2017). The experimental field site is located at about 100 m above sea level, on a SW exposure, with 23% slope, on soil developed from thinly layered marine sediments, had a mesic temperature regime, an ustic moisture regime, and was poorly drained. The content of sand, silt and clay were 28.81, 39.29 and 31.9%, respectively. The soil before the experimentation hosted a forage cultivation (*Medicago* sativa L.). The experimental field was installed in the upper portion of the slope, since in the bottom part the soil manifested a relatively larger morphological diversity due to sedimentation of the upslope eroded material.

The Ap horizons are those made by soil tillage. In the two field areas, the different depth reached by the Ap horizons was attributed to a combination of tillage and erosion. The deepest reworked horizon (Ap4) was probably made in the past when deeper tillage was commonly performed and now abandoned in favor of shallower depth of tillage (≈30 cm). Therefore, in the upper field area, where erosion is higher, the depth of the Ap4, reached with the past tillage, has been reduced, and the recent tillage removed part of the old-tilled soil. Instead, in the intermediate part of the field erosion rate is lower and the depth of the old tillage was maintained or even increased by accumulation of the upper-eroded soil material. This made the intermediate part of the field more fertile than the upper part, and this was probably the reason for the formation of an Oi horizon and for the higher root content. All this considering, to better evaluate the role of biochar in improving soil fertility, the experimental plots were installed in the upper soil portion. However, the ubiquitous presence of mesofauna witnessed a generalized good soil quality.

The climate of the area is a sub-Mediterranean variant of the temperate oceanic climate (Agnelli *et al.*, 2008), which is characterized by a mean annual precipitation of 788 mm and a mean annual air temperature of 14.6 °C, with July as the warmest month (23.3 °C) and January as the coldest one (5.4 °C). Over the study period (August 2018-September 2019), both precipitation and air temperature were monitored by a weather station located about 0.3 km away from the experimental site. The decadal mean of temperature and precipitation during the study period and in the last 15 years are reported in Figure 1.

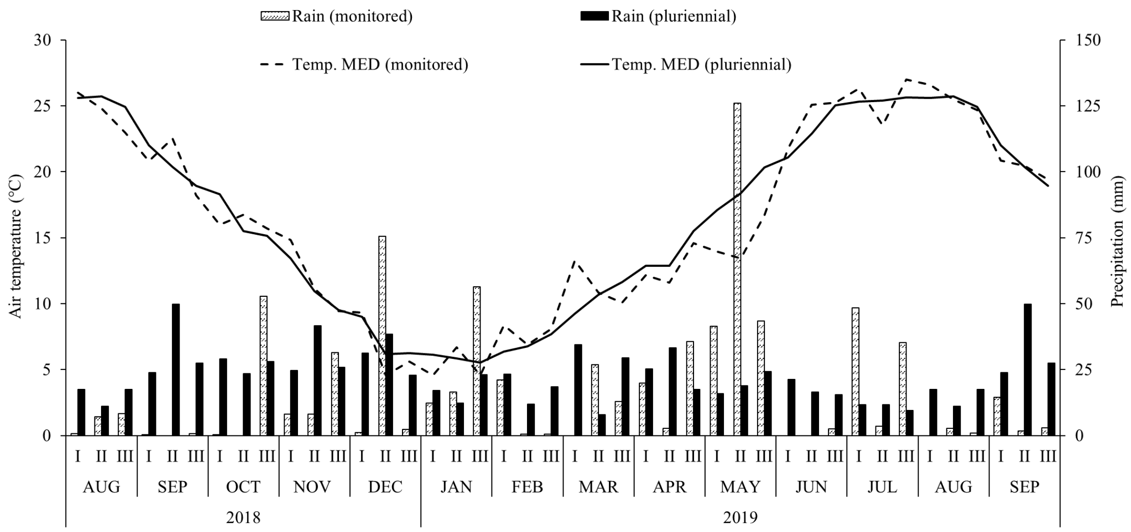


Figure 1. Decadal mean precipitation and air temperature during the experimental period (September 2018 – September 2019) and the long-term period (1998 – 2012) in the study area.

4.1.3.2 Experimental design and management practices

The trial was conducted from September 2018 to September 2019. The experimental field was a six-year-old alfalfa (*Medicago sativa* L.) stand, which was tilled in 2017 and subsequently sowed with durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] in 2017-2018 and 2018-2019 cropping years. The present work was carried out during the second year of durum wheat cultivation. The sowing of wheat for one or two cropping seasons after alfalfa termination is a common practice in semi-arid and rainfed regions, both in Italy and other countries with a Mediterranean type of climate. In our experimentation, the crops were rainfed and no fertilisation was applied in the two wheat cropping seasons to isolate the effect of the biochar application.

A complete randomized block design with three replicates and individual plot of 4.0 m^2 (2.0 m × 2.0 m) was established in 2018. The treatments were: *i*) durum wheat (W_0) and *ii*) durum wheat with biochar added (W_B). The biochar was manually distributed in September 2018 (before the wheat sowing) at a rate of 60 Mg ha^{-1} and was buried with a rotary harrow to 0.20 m depth, following the method reported by Castaldi et al. (2011).
The biochar used was a commercial product from BioDea originated from a mix of beech, pine, and fir biomass pyrolyzed at 850 °C by an industrial system. The final product was mechanically sieved to eliminate the excess of ash to maximize the effect of the pyrolyzed wood. Then, the biochar was ground to less than 0.5 mm to increase its reactivity and facilitate soil incorporation. At Point 1 of Supplementary Materials and in Table S1 are reported the methodologies adopted for biochar analysis and the characteristics of the final product, respectively.
The six-year-old alfalfa field was terminated in October 2017 using a spading machine followed by two passages of a rotary harrow in October and November 2017 before sowing wheat. In the following year, the same plots were subjected to the same soil tillages before the sowing of a subsequent wheat crop. These practices accounted on average for 2.53 ±0.14 Mg ha-1 of alfalfa (in 2017) and 3.27 ±0.50 Mg ha-1 wheat straw (in 2018) dry matter incorporated into the soil. In both crop years, the durum wheat (with the organic cv. 'Antalis') plots were sown in rows (sowing rate, 400 seeds m–2) at the end of November and harvested at the beginning of July. The management practices adopted from 2017 to the end of the second cropping year are illustrated in Figure 2.

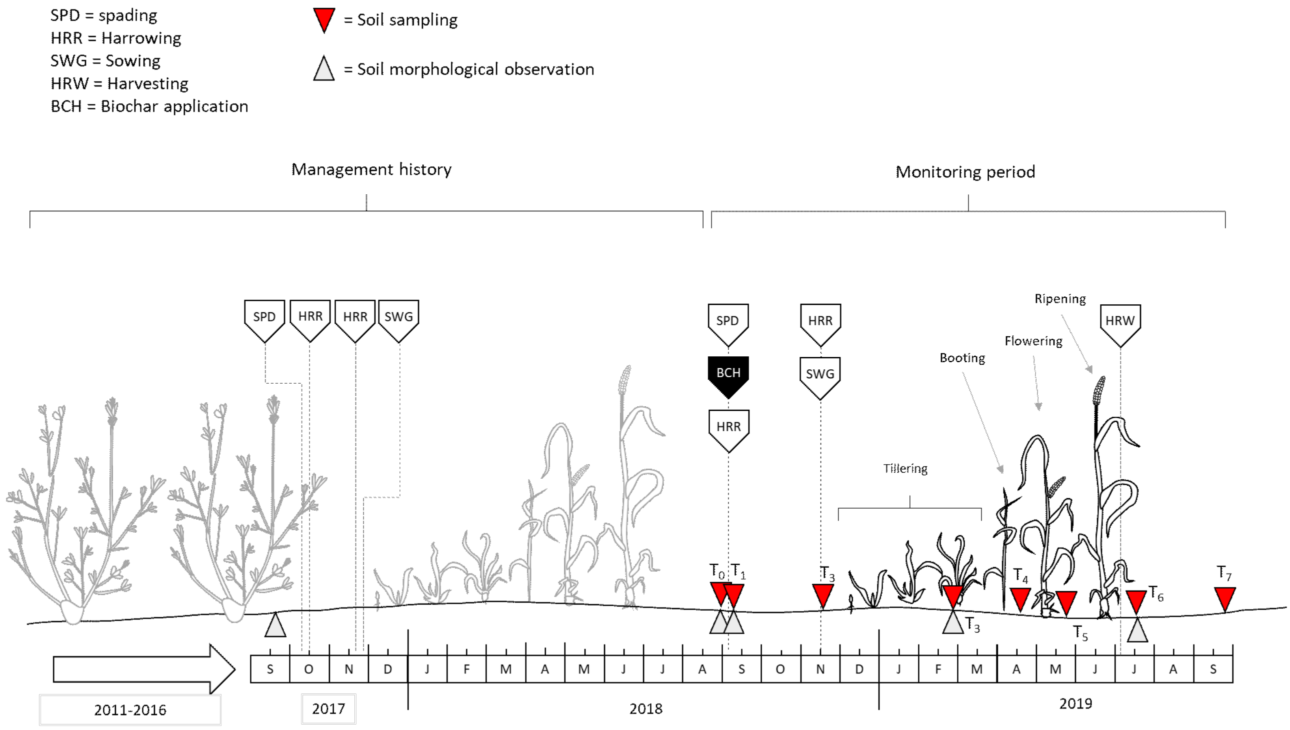


Figure 2. Crop succession and management practices adopted from 2011 to 2019.

4.1.3.3 Soil morphology and soil sampling

In September 2017, soil morphological observations (Schoeneberger et al., 2012) were made before alfalfa termination in the area where the randomized block design would have been established. This survey indicated that the soil was slightly affected by erosion, with small rills running along the maximum gradient. In Table S2 of Supplementary Materials, Profile 1 was representative of the relatively upper and more eroded area, while the Profile 2 was representative of the relatively lower and less eroded belt. The horizons generated by past soil tillage (Ap) reached the depth of ≈35 cm in the upper belt, while in the lower belt they reached the depth of 55 cm. Underneath these depths, there was a Bw horizon never interested by soil tillage. In both belts, the soil showed a moderately to strongly developed structure and scarce skeleton content (≈ 5%) made of fragments with < 1 cm in diameter mainly made of travertine and majolica. Mesofauna was ubiquitous, with earthworms mainly present in the upper field belt.
Soil morphological observations according to Schoeneberger et al. (2012) were also made in August and September 2018, before and after soil tillage (T_0 and T_1, respectively) and in February and July 2019 (T_3 and T_6, respectively) (see Figure 2) in the six plots with wheat. These morphological observations were restricted to the Ap horizons generated by the soil tillage made since the beginning of the field experiment.

By knowing soil morphology, soil samples were collected from the six plots with wheat simultaneously with each soil enzyme sampling (T0 to T7) (Figure 2) by a 5-cm diameter manual auger or the depth 0-20 cm, so to collect Ap1 and Ap2 horizons together. The total amount of samples collected was 48: 8 sampling dates (T_0-T_7) × 2 treatments (with and without biochar) × 3 replicates. Soil samples were maintained in a refrigerated bag and in the dark during the field activities and, once in the laboratory, they were subdivided into two aliquots: one was maintained at a temperature of -20°C until the analyses of enzyme activity, the other could air-dry. The dry samples were sieved through a 2-mm sieve to be submitted to physicochemical analyses.

4.1.3.4 Soil analyses

a) Soil characterization

Particle-size distribution was determined by the pipette method (Day, 1965) after the soil samples were maintained submerged in ≈2% Na-esametaphosphate solution with a solid:liquid ratio (w:v) of 1:2.5 for 24 h at room temperature. Sand was recovered by sieving at 0.05 mm, while silt was separated from clay by sedimentation maintaining the columns at 19-20 °C. Soil pH was determined in water (1:2.5 weight/volume) using a combined glass-calomel electrode. On specimens' ground to less than 0.5 mm, the total organic C (TOC) content was estimated by the Walkley-Black method without the application of heat (McLeod, 1975), while the total N (TN) content was determined by the semi-micro Kjeldahl method. Available P was determined according to Olsen et al. (1954).

b) Soil enzyme activities

The activity of 12 hydrolytic soil enzymes involved in the principal nutrient cycles was determined following the method illustrated by Cowie et al. (2013), which consists in the desorption of enzymes by heteromolecular exchange using lysozyme as desorbing protein. The main activity of each enzyme and its relative nutrient cycle are summarised in Table S4.

c) Wheat yield components

At the beginning of July 2019, ten wheat plants were manually harvested in each plot to assess yield components: number of ears per spike, number of caryopses per ear, weight of the straw, and weight of the chaff and of the caryopses (Monaci et al., 2017).

d) Data handling and statistical analysis

All data were tested for normality distribution (Shapiro-Wilk's test), homogeneous variances (Levene's test) and, when necessary, for sphericity (Mauchly's test) prior to analysis. When data were not normal distributed and/or not homoscedastic, each numerical variable was transformed by the Box and Cox procedure (Osborne, 2010). A paired-sample T-Test was used to determine the differences within sampling dates. The effect of time, treatments, and their interactions were analysed through a repeated measure ANOVA. When assumptions were not met, Wilcoxon signed-rank tests were used instead of repeated measures ANOVA. In all the tests, the differences were considered significant at $P < 0.05$. All analyses were performed with IBM SPSS Statistics version 25.0 (SPSS Inc., Chicago, IL).

4.1.4 Results

4.1.4.1 Soil morphology

In the wheat plots, from three Ap horizons present at T_0 and T_1 (Ap1, Ap2, and Ap3), they became two (Ap1 and Ap2) at T_3 and T_6. At T_0 the state of aggregation of Ap_1, Ap_2 and Ap_3 horizons under alfalfa was slightly more developed than one year before, now of the first survey, and tended to maintain until T_6.

The soil color was similar in all the plots at T_0 and assumed darker tinges in both Ap1 and Ap2 horizons in the plots treated with biochar. For these horizons, the general color was the result of the presence of particles with the color typical of the horizon and dark particles due to biochar varnishing; this is the reason of the reported "salt and pepper" effect. The state of aggregation under wheat was similar or slightly less developed than in alfalfa at T_0, while in the plots treated with biochar, the state of aggregation increased. Worthy to note is that aggregate consistence was hard under alfalfa at T0 became friable at T_1 and T_3 to change back to a firm consistence at T_6. Under wheat the aggregate consistence was friable at T_0, T_1, and T_3, to become firm at T_6; in contrast, after the addition of biochar the aggregates became very friable. Ants, worms, and mesofauna were present in many different plots (Table S3).

4.1.4.2 Effect of biochar incorporation on the soil chemical properties

As expected, the addition of biochar increased the soil TOC significantly immediately after the biochar incorporation (10th September 2018, T_1) which increased more than double. The biochar addition did not result in significant changes on soil TN which remained substantially constant over the whole monitoring period for both W_0 and W_B. Soil available P decreased constantly during the whole monitoring period for both W_0 and W_B with a marked decrease soon after the main tillage (i.e., between T_0 and T_1). Biochar incorporation having no effect on soil available P, except at the end of monitoring period (T_7, bare soil undisturbed since the wheat harvest) when W_B recorded higher values compared to W_0. Biochar incorporation did not result in significant alteration of soil pH in any of the measurements dates (Table 1)

Table 1. Soil properties during the monitored period for soil under wheat (W_0) and soil under wheat amended with biochar (W_B). Different letters denote differences at $P < 0.05$ (Two tails paired T-Test). TOC = Total organic carbon, TN = Total nitrogen.

	Treatment	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7
TOC ($g\ kg^{-1}$)	W_0	9.45±0.12	9.45±0.09 b	9.73±0.12	9.47±0.08	10.15±0.12	10.47±0.09	10.18±0.13	9.37±0.07
	W_B	8.50±0.06	23.87±0.38 a	24.85±0.56 a	24.20±0.36 a	32.83±0.89 a	37.43±0.59 a	27.33±0.65 a	31.93±0.78 a
TN tot ($g\ kg^{-1}$)	W_0	1.03±0.01	1.02±0.01	1.08±0.01	1.07±0.00	1.08±0.01	1.12±0.01	1.15±0.01	1.03±0.0.0
	W_B	0.95±0.01	1.07±0.01	1.11±0.00	1.13±0.01	1.08±0.00	1.28±0.01	1.13±0.01	1.1±0.01
Available P ($mg\ kg^{-1}$)	W_0	16.53±0.07	9.60±0.04	8.65±0.05	7.70±0.05	6.07±0.1	5.70±0.06	3.37±0.05	2.37±0.08
	W_B	14.17±0.05	11.63±0.26	9.22±0.45	9.23±0.47	7.50±0.9	7.47±0.36	4.5±0.39	4.77±0.5 a
pH	W_0	8.20±0.01	8.21±0.01	8.4±0.05	8.42±0.04	8.40±0.05	8.35±0.02	8.45±0.03	8.51±0.03
	W_B	8.30±0.02	8.21±0.07	8.42±0.07	8.33±0.07	8.47±0.17	8.46±0.12	8.41±0.09	8.48±0.06

4.1.4.3 Effect of biochar on enzyme activity

All the enzymes linked to the C-cycle (Nonanoate-esterase, Xylosidase, β -glucuronidase, β-galactosidase, and β-glucosidase) showed a rapid increase of activity immediately after the main tillage (first decade of September 2018) and this happened for both W_0 and W_B (Figure 3). In general, after these increases, all the enzymes examined did not show a significant different activity between treatments. However, during the monitoring period (August 2018-Spetmerber 2019), some divergent trends were observed for some enzymes related to the C-cycle: an increasing trend in the case of nonanoate-esterase, a decreasing trend in the case of β-glucuronidase and fluctuating trends in the case of xylosidase, β-galactosidase and β-glucosidase which reached all a notable low peak in late February 2019 (T_3, end of wheat tillering phase) (Figure 3).
Similar results were observed for the activity of chitinase, that anyway varied more markedly for W_0 compared to W_B. The same enzyme was found to have a higher activity in W_B compared to W_0 in the last decade of May (T_5, during wheat flowering phase) (Figure 3).
The activity of leucine increased remarkably for both W_0 and W_B from the end of February (T3, at the end of the wheat tillering phase). The activity of this enzyme peaked at the end of July (T_6, after wheat maturity) for W_0, while in W_B it reached a plateau for March 2019 onwards (T_3-T_7, from the end of the tillering to the end of the monitoring period) (Figure 3).
The enzyme linked to phosphorus-cycle showed a general less pronounced time-variation compared to the ones linked to the C- or N-cycle, more evident for alkaline phosphomonoesterase and phosphodiesterase compared to the Pyrophosphatase-phosphodiesterase and Acid phosphomonoesterase. For this latter enzyme, a sharp decrease in activity was observed between November 2018 and February 2019 (i.e., between T_2, wheat sowing and T_3, wheat tillering phase), and when W_B registered a higher activity of this enzyme compared to W_0. A similar decrease was observed also for pyrophosphatase-phosphodiesterase although it was less pronounced and without any significant difference (Figure 3).
A general steady increasing trend was observed for arylsulfatase, with a slight decrease occurred in April 2019 (T_4, end of wheat elongation phase) for W_B (Figure 3).

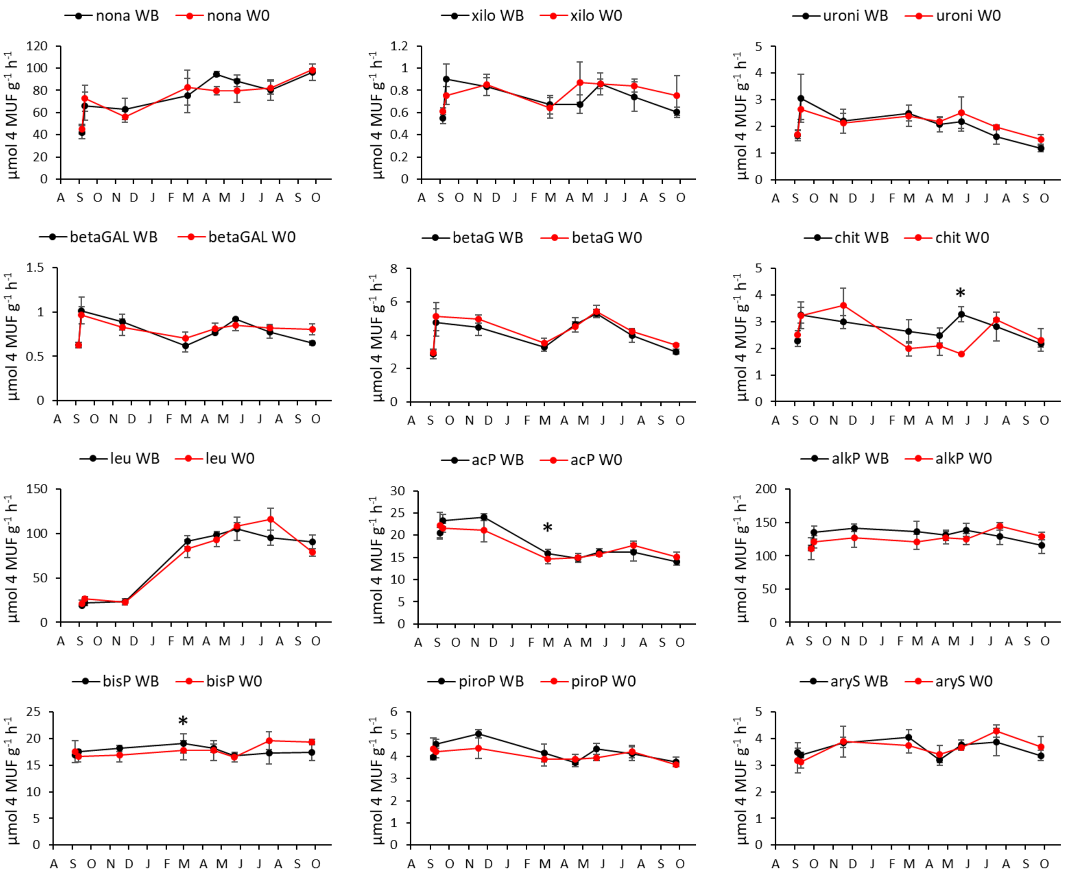


Figure 3. Enzyme's activity in the treatments over the monitoring period. *= difference significance at $P < 0.05$ (Two tails paired T-Test). W_B= with the addition of Biochar, W_0= without the addition of biochar. Abbreviations for enzymatic activities: nona= nonanoate-esterase, xylo = xylosidase, uroni = β-glucuronidase, betaGAL= β-galactosidase, betaG = β-glucosidase, chit = chitinase, leu = leucine-aminopeptidase, acP = acid phosphomonoesterase, alkP = alkaline phosphomonoesterase, bisP = phosphodiesterase, pyroP = pyrophosphatase-phosphodiesterase, aryS = arylsulfatase.

4.1.4.4 Effect of biochar on soil properties and enzyme activities over the time

No interaction between time and biochar application emerged for the main soil characteristics analyzed (Table 2). As expected, the effect of biochar application emerged for the total organic carbon which was found higher for W_B. A significant decrement of available phosphorus over time occurred for both W_B and W (Table 2). However, despite the lack of significance found in each date of sampling (Table 2), the biochar application resulted in a higher soil phosphorous availability for W_B compared to W_0 over all the monitoring period. No significant variation in soil nitrogen or soil pH was observed over the time or as affected by biochar application.

No significant interaction between time and biochar application emerged during the whole monitoring period for all the analyzed enzymes (Table 2). Overall, the effect of biochar application was never significant in any of the 12 enzymes analyzed. Some enzymes showed significant changes over time (i.e., nonanoate-esterase, β-glucuronidase, β-glucosidase leucine-aminopeptidase and acid phosphomonoesterase).

Table 2. Analysis of the different enzyme activity and soil properties over time.

Cycle	Soil property and Enzyme	Time		Treatment		Time × Treatment	
		F	P	F	P	F	P
C	Total Organic Carbon (g kg^{-1})	6.02	0.08	240.78	0.01	5.52	0.09
N	Total Nitrogen (g kg^{-1})	4.89	0.10	1.50	0.35	1.67	0.30
P	Available Phosphorous (mg kg^{-1})	46.15	0.01	20.32	0.05	1.99	0.26
-	pH	5.09	0.11	0.25	0.67	0.01	0.99
C	Nonanoate-esterase (nona)	20.54	0.01	1.02	0.42	1.22	0.39
C	Xylosidase (xylo)	3.90	0.15	2.87	0.23	0.76	0.50
C	β-glucuronidase (uroni)	15.51	0.02	1.29	0.37	1.28	0.37
C	β-galactosidase (betaGAL)	5.73	0.12	0.89	0.46	0.64	0.53
C	β-glucosidase (betaG)	12.23	0.03	3.04	0.22	0.16	0.82
C/N	Chitinase (chit)	5.23	0.08	3.44	0.21	3.22	0.15
N	Leucine-aminopeptidase (Leu)	159.78	0.00	0.02	0.87	1.95	0.26
P	Acid P. monoesterase (acP)	21.57	0.02	0.01	0.93	1.13	0.41
P	Alkaline P. monoesterase (alkP)	1.50	0.33	0.93	0.44	0.96	0.46
P	Phosphodiesterase (bisP)	0.90	0.47	0.01	0.97	1.26	0.34
P	Pyrophosphatase P.diesterase (piroP)	5.01	0.10	1.16	0.40	0.86	0.45
S	Arylsulfatase (aryS)	4.24	0.13	0.01	0.98	0.95	0.50

Abbreviations for enzymatic activities: nona= nonanoate-esterase, xylo = xylosidase, uroni = β-glucuronidase, betaGAL= β-galactosidase, betaG = β-glucosidase, chit = chitinase, leu = leucine-aminopeptidase, acP = acid phosphomonoesterase, alkP = alkaline phosphomonoesterase, bisP = phosphodiesterase, pyroP = pyrophosphatase-phosphodiesterase, aryS = arylsulfatase.

4.1.4.5 Effect of biochar on wheat yield

As reported in table 3, biochar addition had no effect on wheat yield with main parameters substantially equal for W_0 and W_B.

Table 3. Main parameters of wheat yield ± standard error of the mean.

Treatment	N of spikelets/ spike	N of caryopses/ spike	Straw DM/ plant (g)	Chaff DM/ plant (g)	Caryopses weight at 14% moisture/ plant (g)	Caryopses Unit weight (mg)	Harvest Index (%)
W_B	13.07±0.84	20.43±2.73	0.82 ±0.14	0.34 ±0.05	0.38 ±0.04	18.6	22.1
W_0	12.08±0.24	21.17±1.72	0.81±0.04	0.32 ±0.02	0.39 ±0.04	18.4	23.1

W_B= with the addition of Biochar, W_0= without the addition of biochar

4.1.5 Discussion

4.1.5.1 Effect of biochar incorporation on soil morphology

In the wheat plots, the reduction from three to two Ap horizons in the first 25-30 cm soil was mainly due to the obliteration of the Ap1 and Ap2 horizons, which roughly fused acquiring similar morphological properties. Evidently, due the absence of tillage from November to February, the ecological pressure was able to homogenize these two horizons in terms of color, state of aggregation, and root abundance. The "salt and pepper" effect of the color of the biochar treated plots was ascribed to the fact that biochar was not fully incorporated within the aggregates, but it was mainly adsorbed at their surface behaving like a varnish. The lack of incorporation of biochar into the aggregates even after six months from the distribution is an indication of the recalcitrance of this biochar. As Guo and Chen (2014) observed, the aromatic components and recalcitrance of the biochars increased with increasing pyrolysis temperatures, so our biochar which has been obtained after 800°C temperature treatment, probably represent an example of this thesis. However, morphological field observations, as shown in Supplemnetary, showed a good soil aggregation in biochar treated parcels, like the others (under wheat and under alfa-alfa). These observations allow us to recognize in treated soil, good physisical properties but only few presences of mesofauna. As Domene et al. (2015) observed, biochar also caused contrasting effects on the behaviour of mesofauna species. In Gallignano, soils treated parcels showed a decreased faunal activity than the others, probably indicating a negative effect as also been occasionally observed by Marks (2013), in an alkaline soil cropped to barley and amended at high addition rates (50 Mg ha-1) with a gasification pine wood biochar. These authors observed a strict relationship between mesofauna and microorganisms after biochar treatemnts.

In the present study, the addition of biochar induced even more friability to the aggregates probably because of the higher soil moisture which can reduce consistence in aggregates mostly cemented by clay minerals as observed during soil manipulation in field. However, the absence of a proper incorporation of the biochar within the aggregates improve porosity and water infiltration, but it also increases the erosion risk especially on steep slopes because of a too rapid saturation of the upper horizons as observed on the experimental field.

4.1.5.2 Effect of biochar incorporation on the soil chemical properties

a) Soil pH

In the present experiment a slight but not significant increase of pH was observed through time and this increase was similar for both W_0 and W_B. This suggest that biochar had no effect on soil pH in the studied soil, at least over the short-term. The results appears to be partially in contrast with the ones reported by Macdonald et al. (2014) who reported that soil pH was significantly influenced by biochar additions in both acid soil (i.e., arenosol and ferralsol) and alkaline calcisol, although in the latter only on a limited extent. No variation of soil pH was observed by (Castaldi et al., 2011) after 14 months of biochar incorporation (3 and 6 kg m^{-2}) in an silty-loam soil (pH = 5.4) cultivated with wheat, although, in the short term (3 months after biochar incorporation) biochar-amended soil showed an higher pH. Studies that report a not significant effect of biochar on soil pH have been reported by Foster et al. (2016) in maize cropping system using pine-wood biochar at 30 t ha^{-1} rate and by Ventura et al. (2014) in an apple orchard using 10 t ha^{-1} of wood branches biochar. In the metanalysis carried out by (Lehmann and Joseph, 2015), the greatest positive response of biochar emerged in soils with pH ranging from value <4 to 5.5, while no significant effect was seen in neutral to slightly alkaline soils (i.e., pH ranges 7.1-7.5 or 7.6-8.0), but significant increase was observed also for soils in the pH range of 8.1-8.5, thus similar to the one reported in the present study.

b) Total Organic Carbon

Despite being considered a recalcitrant and stable material, some studies suggest that the inclusion of biochar into the soil might actually alter the mineralization of soil organic matter (Lehmann and Joseph, 2015). Therefore, it is important to look beyond the mere incorporation of stable carbon, but attention must be given to the relation of biochar and the already existing soil organic matter. Indeed, the incorporation of biochar into the soil might generate a priming effect which might alter the mineralization rate of organic matter previously present into the soil and this might occur even over the very short term (Lehmann et al., 2011; Lehmann and Joseph, 2015; Purakayastha et al., 2016; Zavalloni et al., 2011). In the present study, es expected, an increment of TOC was observed which remain unaltered throughout the whole monitoring period. This suggest that the biochar form distributed was very recalcitrant (Table 1 and 2) and did not generate any priming effect.

c) Soil Nitrogen

The biochar pyrolysis temperature, the feedstock type and the surface properties have a strong influence on biochar characteristics and on soil nutrient cycling (Nguyen et al., 2017)(El-Naggar et al., 2019).

Indeed, soil biochar amendment could affect the NO_3^- availability through the adsorption of NH_4^+ and consequent changes in soil N-cycling (Lehmann and Joseph, 2015). The results of the present study indicate that total nitrogen (TN)concentration remained overall unaffected throughout the monitored period and that might be due to the woody source and high C/N ratio of the biochar. Indeed, crop derived biochar characterized by high level of labile carbon (Wang et al., 2016) can stimulate microbial activity increasing N immobilisation (Nguyen et al., 2017). On the contrary, our results are in line with (Wang et al., 2016) that found wood biochar has a low level of biochar carbon degradability and therefore a lower ability to stimulate microbial activity and to immobilize soil N. Our biochar biomass source made by wood materials might explain the null effect of the biochar addition on the soil TN concentration level in our study.
Also, the pyrolysis temperature can affect the soil N concentration level. Indeed, Wang et al. (2016), in their meta-analysis found that biochar produced at high pyrolysis temperature has a slower decomposition rate. This could lead to a little stimulation of microbial activity and therefore to low nitrate immobilization. Contrary to our results, Kameyama et al. (2016) found that wood-derived biochar produced at high pyrolysis temperature (i.e., 800 °C) had the greatest capability to adsorb the nitrate-nitrogen attributing this to the formation of surface basic functional groups during high temperature pyrolysis.

d) Soil Phosphorus

The addition of biochar into the soil can affect the soil available phosphorous. Gao et al. (2019) showed in a recent meta-analysis that the application of wood-derived biochar, and biochars produced at > 600°C, did not affect the soil available phosphorus in general. In contrast, the result of the present study suggests a positive effect of biochar on soil available phosphorus (Table 4). Positive effect of biochar application on soil P were observed by (Sun and Lu, 2014) in clay vertisol with different types of biochar including wood derived and was linked to the presence of P in the biochar itself.

4.1.5.3 Soil enzyme activities

No improvements were found with biochar application.
The recalcitrance of this material seems to reduce every enzyme activity:

a) Carbon cycle enzyme activities

Lehmann et al. (2011a) reported that in general, biochar addition reduces the soil enzymatic activities but also that all the interactions between biochar and soil enzymes related to carbon mineralization are not fully understood. A recent and extensive metanalysis on the effects of biochar incorporation on extracellular enzymes (Pokharel et al., 2020) reported that C-cycle enzymes (including β-glucuronidase, β-galactosidase, urease and Xylosidase) are generally influenced by biochar addition. The results obtained in the present study seems to be aligned with these findings as only the activity of Nonanoate-esterase, β-glucuronidase and β-glucosidase changed over time, but the short-time effect of biochar presence did not emerge over the monitoring period (Table 2).
The lack of divergent dynamics for the enzymes linked to the C-cycle for both W_B and W_0 after the 60t ha^{-1} biochar incorporation (T_1, first decade of September 2018) might be attributed to the high quality and stability of the biochar itself since this was obtained from wood and produced at high temperature (Table 1).
These results are in line with other studies dealing with wood-derived biochars carried out under controlled laboratory or open field conditions. For example Zimmerman et al. (2011), in an incubation experiment found that lower temperatures (i.e., 250 and 400 °C) generated biochar which degraded at faster rates than higher temperature-biochars (i.e., 525 and 650 °C) and that biochar made from grasses generally degraded faster than those from hard woods. Similarly, Zavalloni et al. (2011), in an incubation experiment did not found any effect of microbial carbon after the addition of wood derived-biochar obtained at 500 °C. The results of the present study appear to be in line with the ones of Luo et al. (2017) that reported null effects with biochar rates up to 50 t ha^{-1} applied in silt loam soil with a pH of 7.9.
Increases in the activity of C-cycle enzymes (β-glucosidase and chitinase) were observed by Awad et al. (2012) in an incubation experiment with commercial biochar (67% carbon) and plant residues in sandy and sandy loam-soils. Foster et al. (2016) reported that the addition of 30 t ha^{-1} of a pine-wood biochar to a Fort Collins loam (51% sand, 20% silt and 29% clay) during a maize-crop negatively affected soil C-cycle enzymes including β-glucosidase. The same authors proposed that this effect might be attributed to a decrease on potential sorption of enzymes or substrate on biochar surfaces due to enzyme alteration and malfunction. This results indeed support the finding of Lammirato et al. (2011) who showed how chestnut-wood biochar might regulates the availability of substrates by adsorption and thus limit the β-glucosidase activity. Increasing activity of β-glucosidase after biochar incorporation have been reported by Li et al. (2019) when apple-branch biochar was added to a silt-clay soil in an incubation experiment. However, this occurred only when the soil N-content was enhanced by adding 0.2 g kg^{-1} of urea.

b) Nitrogen cycle enzyme activities

The two enzymes analyzed in the present study involved in the cycle of nitrogen were chitinase and Leucine-aminopeptidase. Chitinases are mainly produced by fungi and hydrolyses the glycosidic bonds of chitin releasing a smaller organic compound containing N. If in one hand chitinase production by the microorganisms probably does not regulate N-supply, on the other hand chitinase activity is controlled by N supply (Olander and Vitousek, 2000). The leucine-

aminopeptidase is an important enzyme which hydrolyses peptide bonds in proteins. Both the activity of chitinase and leucine-aminopeptidase are correlated with the N availability within soil organic matter pools (Chen et al., 2018; Jian et al., 2016).
In the present study the chitinase activity substantially did not vary after the incorporation of biochar. In W_0 chitinase activity showed a fluctuating trend reaching the minimum in the last decade of May (T_5, during wheat flowering phase), when W_B reached its peak (Figure 3).
If in the present study, leucine-aminopeptidase showed a very similar pattern for both W_0 and W_B, other studies observed an alteration of this enzyme after biochar addition. For example Bailey et al. (2011) found a reduction of leucine-aminopeptidase after switchgrass-biochar produced by a fast pyrolysis and linked this reduction to the substrate adsorption by the biochar itself. On the contrary, other studies observed an increase of the leucine-aminopeptidase when smaller rate of wood-derived biochar (10 t ha^{-1}) were applied to a haplic calcisol soil (Ventura et al., 2014).
Chen et al. (2017) suggest that the increase of N-cycle enzymes is linked to an high level of soil C:N that promote and stimulate nitrogen mineralization by the soil microorganisms. In the present study, regardless of biochar addition, the leucine-aminopeptidase showed a significant increase during the whole wheat cycle with a remarkable increase between T_2 (wheat sowing) and T_3 (wheat tillering phase) (Figure 3). This activity enhancement can be correlated with the rise in the air temperature, as it has been showed how soil enzyme activities is doubled for every 10 °C increase in temperature (Wallenstein et al., 2011). Indeed, as shown in Figure 1, by the end of February the mean air temperature raised above 8 °C and constantly increased until the first part of March reaching a mean daily temperature of 13 °C.

c) Phosphorous cycle enzyme activities

None of the enzymes involved in the phosphorous cycle was significantly affected by the biochar application except for one sampling date in which acid phosphomonoesterase and phosphodiesterase were higher in W_B compared in W_0 (Figure 3). At first, the biochar incorporation seems to slightly enhance the activity of the enzymes linked to phosphorus cycle until the end of May. Later, a reverse but not significant trend was observed and the activity of acid phosphomonoesterase, alkaline phosphomonoesterase and phosphodiesterase were slightly higher for W_0 (Figure 3). The recalcitrance of this biochar seems to block every activity.

The incorporation of biochar into the soil might sorb a wide range of inorganic and organic molecules thus inhibiting enzymatic activity (Lehmann et al., 2011; Xu et al., 2014). Results tends to be again context dependent and contrasting. For example, Li et al. (2019) observed a significant increase of alkaline phosphatase after the addition of apple branch-biochar while (Elzobair et al., 2016) did not observed effects on phosphatase enzyme activity after wood-biochar incorporation. This absence of differences might be attributable to reduced intake of phosphorous that was not able to stimulate the enzyme activity. The activities of acid phosphomonoesterase was the only P-cycle enzyme which showed a significant little change over time regardless of biochar addition. In contrast with our findings, Du et al. (2014) observed a significat interaction effect of biochar application, date of sampling on alkaline phosphatase and reported that the higher activity was in spring, during the wheat growing season. However, according to the recent metanalysis carried out by Pokharel et al. (2020), increases of alkaline phosphatase activities may be detected only in long-term studies.

d) Sulphur cycle enzyme activities

No significant changes were detected for arylsulfatase (aryS) overall the monitoring period and in respect to the biochar addition. These results seems to be in line with the findings from an incubation study reported by Paz-Ferreiro et al. (2012) and also with what reported by Sun *et al.* (2014) in a field experiment using biochar from birch wood.

4.1.5.4 Effect of biochar addition to wheat yield

No effect of biochar on the yield of wheat emerged in the present study. The effect of biochar on crop-yield remains context dependent and their interactions not fully understood (Lehmann and Joseph, 2015). For example, Macdonald et al. (2014) in a study involving different soil types (i.e., acidic aerosol, acidic ferralsol, neutral vertisol and alkaline calcisol) observed a different wheat response to different biochar rates (i.e., 1, 5 and 10 t ha^{-1}) and biochar type (poultry litter with 38.3% of organic carbon and wheat straw with 53.1% of organic carbon). The same author found that the same rate and type of biochar might either suppress or enhance wheat production depending on the soil type. Karer et al. (2013) in a cambisol observed a non-variation on wheat crop yield at different wood-based biochar rates (0, 24, 72 t ha^{-1}, 80% carbon) if 120 kg ha^{-1} of N-fertilization was provided. However, in the same study a relevant decrease in wheat yield was observed when biochar was provided at 72 t ha^{-1} when no fertilization was applied. Similarly, in the present study no fertilization was applied because the N provided by the incorporation of alfalfa was considered a sufficient nitrogen-based fertilizer. This could partially explain the lack of difference that were observed in this study.
Karer et al. (2013) from a field experiment report a reduction of nutrient release and uptake with the use of high rate of wood-based biochar (i.e., 72 t ha^{-1}) and that this was linked to the immobilization of macro- and micro-elements under neutral soil pH. In the metanalysis included in (Lehmann and Joseph, 2015) it is suggested that biochar application below 5 t ha^{-1} might not be sufficient to generate a crop yield increase while an application rate around 50 t ha^{-1} should increase the wheat yields of 17%. However, the results of this metanalysis gives an idea of the general expectation of wheat and

other crops at a global scale, without specifying soil conditions nor biochar types and thus interpretation of such a result must be undertaken with caution.

4.1.6 Conclusions

Effects of high-temperature pyrolized biochar on physicochemical properties and enzyme activities of subalkaline soil during a wheat-growing season have been monitored:
After alfa-alfa two different treatments (W0 and WB) has been compared to study biochar effects
-Specific characteristics of this recalcitrant biochar generally favored a good soil aggregation but a lack of biochar incorporation
-a slight but not significant similar increase of pH was observed for both W0 and WB through the time
-as expected, was observed an increment of TOC after biochar incorporation which remain unaltered throughout the whole monitoring period
-A significant decrement of available phosphorus over time occurred for both WB and W0 and the biochar application resulted in a higher soil phosphorous availability for WB compared to W0 over all the monitoring periods.
-No significant variation in soil nitrogen was observed over the time or as affected by biochar application.
-No significant interaction between time and biochar application emerged during the whole monitoring period for all the analyzed enzymes. Overall, the effect of biochar application was never significant in any of the 12 enzymes analyzed. Some enzymes showed significant changes over time (i.e., nonanoate-esterase, β-glucuronidase, β-glucosidase leucine-aminopeptidase and acid phosphomonoesterase).
Minimal differences between two different soil treatments have been attributed to the recalcitrance of the high-temperature pyrolized biochar
As aspected, biochar addition had no effect on wheat yield with main parameters substantially equal for W0 and WB.

4.1.7 Literature references

Agegnehu, G., Bass, A.M., Nelson, P.N., Bird, M.I., 2016. Benefits of biochar, compost and biochar-compost for soil quality, maize yield and greenhouse gas emissions in a tropical agricultural soil. Sci. Total Environ. 543, 295–306. https://doi.org/10.1016/j.scitotenv.2015.11.054

Agnelli A, Allegrezza M, Biondi E, Cocco S, Corti G, Pirchio F, 2008. Pedogenesi e paesaggio vegetale: il ruolo dell'esposizione. Fitosociologia 45:23-8.

Allen D.E., Bhupinder P.S., Dalla R.C., 2011. Soil Health Indicators Under Climate Change:A Review of Current Knowledge . In "Soil health and Climate change", Singh B.P., Des., Soil Biology 29. Springer Verga Berlin, https://doi.org/10.1007/978-3-642-20256-8_2

Anawar, H.M., Akter, F., Solaiman, Z.M., Strezov, V., 2015. Biochar: An Emerging Panacea for Remediation of Soil Contaminants from Mining, Industry and Sewage Wastes. Pedosphere 25, 654–665. https://doi.org/10.1016/S1002-0160(15)30046-1

Anderson, C.R., Condron, L.M., Clough, T.J., Fiers, M., Stewart, A., Hill, R. a., Sherlock, R.R., 2011. Biochar induced soil microbial community change: Implications for biogeochemical cycling of carbon, nitrogen and phosphorus. Pedobiologia (Jena). 54, 309–320. https://doi.org/10.1016/j.pedobi.2011.07.005

Atkinson, C.J., Fitzgerald, J.D., Hipps, N. a., 2010. Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: A review. Plant Soil 337, 1–18. https://doi.org/10.1007/s11104-010-0464-5

Awad T. M., Blagodatskaya E., Blagodatskaya, Ok Y. S., Kuzyakov Y. 2012. Effects of Polyacrylamide, Biopolymer, and Biochar on Decomposition of Soil Organic Matter and Plant Residues as Determined by 14C and Enzyme Activities. European Journal of Soil Biology 48:1-10 DOI: 10.1016/j.ejsobi.2011.09.005

Bailey, V.L., Fansler, S.J., Smith, J.L., Bolton, H., 2011. Reconciling apparent variability in effects of biochar amendment on soil enzyme activities by assay optimization. Soil Biol. Biochem. 43, 296–301. https://doi.org/10.1016/j.soilbio.2010.10.014

Bamminger, C., Poll, C., Marhan, S., 2014. Effects of biochar and elevated soil temperature on soil microbial activity and abundance in an agricultural system 16, 11874. https://doi.org/10.1007/s00374-014-0968-x

Blackwell, P., Joseph, S., Munroe, P., Anawar, H.M., Storer, P., Gilkes, R.J., Solaiman, Z.M., 2015. Influences of Biochar and Biochar-Mineral Complex on Mycorrhizal Colonisation and Nutrition of Wheat and Sorghum. Pedosphere 25, 686–695. https://doi.org/10.1016/S1002-0160(15)30049-7

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biol. Biochem. 58, 216–234. https://doi.org/10.1016/j.soilbio.2012.11.009

Castaldi, S., Riondino, M., Baronti, S., Esposito, F.R., Marzaioli, R., Rutigliano, F.A., Vaccari, F.P., Miglietta, F., 2011. Impact of biochar application to a Mediterranean wheat crop on soil microbial activity and greenhouse gas fluxes. Chemosphere 85, 1464–1471. https://doi.org/10.1016/j.chemosphere.2011.08.031

Chen, H., Li, D., Zhao, J., Xiao, K., Wang, K., 2018. Effects of nitrogen addition on activities of soil nitrogen acquisition enzymes：A meta-analysis. Agric. Ecosyst. Environ. 252, 126–131. https://doi.org/10.1016/j.agee.2017.09.032

Chen, J., Li, S., Liang, C., Xu, Q., Li, Y., Qin, H., Fuhrmann, J.J., 2017. Response of microbial community structure and function to short-term biochar amendment in an intensively managed bamboo (Phyllostachys praecox) plantation soil: Effect of particle size and addition rate. Sci. Total Environ. 574, 24–33. https://doi.org/10.1016/j.scitotenv.2016.08.190

Chen, J., Liu, X., Li, L., Zheng, Jinwei, Qu, J., Zheng, Jufeng, Zhang, X., Pan, G., 2015. Consistent increase in abundance and diversity but variable change in community composition of bacteria in topsoil of rice paddy under short term biochar treatment across three sites from South China. Appl. Soil Ecol. 91, 68–79. https://doi.org/10.1016/j.apsoil.2015.02.012

Cowie, A.L., Lonergan, V.E., Rabbi, S.M.F., Fornasier, F., MacDonald, C., Harden, S., Kawasaki, A., Singh, B.K., 2013. Impact of carbon farming practices on soil carbon in northern New South Wales. Soil Res. 51, 707–718. https://doi.org/10.1071/SR13043

Day P.R., 1965. Particle Fractionation and Particle-Size Analysis. Agronomy Monographs. Chapter 43. https://doi.org/10.2134/agronmonogr9.1.c43

De Gryze S., Cullen M., Durschinger D., 2010. Evaluation of the Opportunities for Generating Carbon Offsets from Soil Sequestration of Biochar. Terra Global Capital, San Francisco, USA. ISBN: 415-830-6061

Domene X., Hanley K., Enders A., Lehmann J., 2015. Short-term mesofauna responses to soil additions of corn stover biochar and the role of microbial biomass. Applied Soil Ecology 89 (2015) 10–17. http://dx.doi.org/10.1016/j.apsoil.2014.12.005

Du, Z., Wang, Y., Huang, J., Lu, N., Liu, X., Lou, Y., Zhang, Q., 2014. Consecutive biochar application alters soil enzyme activities in the winter wheat-growing season. Soil Sci. 179, 75–83. https://doi.org/10.1097/SS.0000000000000050

El-Naggar, A., El-Naggar, A.H., Shaheen, S.M., Sarkar, B., Chang, S.X., Tsang, D.C.W., Rinklebe, J., Ok, Y.S., 2019. Biochar composition-dependent impacts on soil nutrient release, carbon mineralization, and potential environmental risk: A review. J. Environ. Manage. 241, 458–467. https://doi.org/10.1016/j.jenvman.2019.02.044

Elzobair, K.A., Stromberger, M.E., Ippolito, J.A., Lentz, R.D., 2016. Contrasting effects of biochar versus manure on soil microbial communities and enzyme activities in an Aridisol. Chemosphere 142, 145–152. https://doi.org/10.1016/j.chemosphere.2015.06.044

Foster, E.J., Hansen, N., Wallenstein, M., Cotrufo, M.F., 2016. Biochar and manure amendments impact soil nutrients and microbial enzymatic activities in a semi-arid irrigated maize cropping system. Agric. Ecosyst. Environ. 233, 404–414. https://doi.org/10.1016/j.agee.2016.09.029

Francioni, M., Roberto, L., D'Ottavio, P., Trozzo, L., Kishimoto-Mo, A.W., Budimir, K., Baldoni, N., Toderi, M., 2020. Soil respiration dynamics in forage-based and cereal-based cropping systems in central Italy. Sci. Agric. 77, 10. https://doi.org/10.1007/s11431-

Gao, S., DeLuca, T.H., Cleveland, C.C., 2019. Biochar additions alter phosphorus and nitrogen availability in agricultural ecosystems: A meta-analysis. Sci. Total Environ. 654, 463–472. https://doi.org/10.1016/j.scitotenv.2018.11.124

Gascó G., Paz-Ferreiro J., Cely P., Plaza C., Méndez A., Influence of pig manure and its biochar on soil CO2 emissions and soil enzymes. Ecological Engineering, 95: 19-24. https://doi.org/10.1016/j.ecoleng.2016.06.039

Gul, S., Whalen, J.K., Thomas, B.W., Sachdeva, V., Deng, H., 2015. Physico-chemical properties and microbial responses in biochar-amended soils: Mechanisms and future directions. Agric. Ecosyst. Environ. 206, 46–59. https://doi.org/10.1016/j.agee.2015.03.015

Guo J. and Chen B., 2014. Insights on the Molecular Mechanism for the Recalcitrance of Biochars: Interactive Effects of Carbon and Silicon Components. Environmental Science & Technology 2014 48 (16), 9103-9112. DOI: 10.1021/es405647e

Han, L., Sun, K., Yang, Y., Xia, X., Li, F., Yang, Z., Xing, B., 2020. Biochar's stability and effect on the content, composition and turnover of soil organic carbon. Geoderma 364, 114184. https://doi.org/10.1016/j.geoderma.2020.114184

Jian, S., Li, J., Chen, J., Wang, G., Mayes, M.A., Dzantor, K.E., Hui, D., Luo, Y., 2016. Soil extracellular enzyme activities, soil carbon and nitrogen storage under nitrogen fertilization: A meta-analysis. Soil Biol. Biochem. 101, 32–43. https://doi.org/10.1016/j.soilbio.2016.07.003

Kameyama, K., Miyamoto, T., Iwata, Y., Shiono, T., 2016. Influences of feedstock and pyrolysis temperature on the nitrate adsorption of biochar. Soil Sci. Plant Nutr. 62, 180–184. https://doi.org/10.1080/00380768.2015.1136553

Karer, J., Wimmer, B., Zehetner, F., Kloss, S., Feichtmair, S., Kitzler, B., 2013. Biochar application to temperate soils - effects on soil fertility and crop yield. Agric. Food Sci. 22, 390–403.

Kavitha, B., Reddy, P.V.L., Kim, B., Lee, S.S., Pandey, S.K., Kim, K.H., 2018. Benefits and limitations of biochar amendment in agricultural soils: A review. J. Environ. Manage. 227, 146–154. https://doi.org/10.1016/j.jenvman.2018.08.082

Kumar S., Chaudhuri S., Maiti S.K., 2013. Soil Dehydrogenase Enzyme Activity in Natural and Mine Soil - A Review. Middle-East Journal of Scientific Research 13 (7): 898-906. https://doi.org/10.5829/idosi.mejsr.2013.13.7.2801

Lammirato, C., Miltner, A., Kaestner, M., 2011. Effects of wood char and activated carbon on the hydrolysis of cellobiose by β-glucosidase from Aspergillus niger. Soil Biol. Biochem. 43, 1936–1942. https://doi.org/10.1016/j.soilbio.2011.05.021

Lehmann, J., Joseph, S., 2015. Biochar for Environmental Management, Biochar for Environmental Management. https://doi.org/10.4324/9780203762264

Lehmann, J., Rillig, M.C., Thies, J., Masiello, C.A., Hockaday, W.C., Crowley, D., 2011. Biochar effects on soil biota - A review. Soil Biol. Biochem. 43, 1812–1836. https://doi.org/10.1016/j.soilbio.2011.04.022

Li, S., Liang, C., Shangguan, Z., 2017. Effects of apple branch biochar on soil C mineralization and nutrient cycling under two levels of N. Sci. Total Environ. 607–608, 109–119. https://doi.org/10.1016/j.scitotenv.2017.06.275

Luo, S., Wang, S., Tian, L., Li, S., Li, X., Shen, Y., Tian, C., 2017. Long-term biochar application influences soil microbial community and its potential roles in semiarid farmland. Appl. Soil Ecol. 117–118, 10–15. https://doi.org/10.1016/j.apsoil.2017.04.024

Macdonald, L.M., Farrell, M., Zwieten, L. Van, Krull, E.S., 2014. Plant growth responses to biochar addition: an Australian soils perspective. Biol. Fertil. Soils 50. https://doi.org/10.1007/s00374-014-0921-z

Masto R.E., Kumar S., Rout T.K., Sarkar P., George J., Ram L.C., 2013. Biochar from water hyacinth (Eichornia crassipes) and its impact on soil biological activity. Catena, 111: 64-71. https://doi.org/10.1016/j.catena.2013.06.025

McLeod, S., 1975. Studies on wet oxidation procedure for the determination of organic C in soil. Notes on Soil Techniques. CSIRO Division of Soil, Adelaide, Australia, pp. 73–79

Molnár, M., Vaszita, E., Farkas, É., Ujaczki, É., Fekete-Kertész, I., Tolner, M., Klebercz, O., Kirchkeszner, C., Gruiz, K., Uzinger, N., Feigl, V., 2015. Acidic sandy soil improvement with biochar - A microcosm study. Sci. Total Environ. 563–564, 855–865. https://doi.org/10.1016/j.scitotenv.2016.01.091

Monaci, E., Polverigiani, S., Neri, D., Bianchelli, M., Santilocchi, R., Toderi, M., Ottavio, P.D., Vischetti, C., 2017. Effect of contrasting crop rotation systems on soil chemical and biochemical properties and plant root growth in organic farming : First results. Ital. J. Agron. 12, 364–374. https://doi.org/10.4081/ija.2017.831

Nannipieri P., Giagnoni L., Renella G., Puglisi E., Ceccanti B., Masciandaro G., Fornasier F., Moscatelli M.C., Marinari S., 2012. Soil enzymology: classical and molecular approaches. Biology and Fertility of soils, 48: 743-762 https://doi.org/10.1007/s00374-012-0723-0

Nguyen, T.T.N., Xu, C.Y., Tahmasbian, I., Che, R., Xu, Z., Zhou, X., Wallace, H.M., Bai, S.H., 2017. Effects of biochar on soil available inorganic nitrogen: A review and meta-analysis. Geoderma 288, 79–96. https://doi.org/10.1016/j.geoderma.2016.11.004

Olander, L.P., Vitousek, P.M., 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. Biogeochemistry 49, 175–190. https://doi.org/10.1023/A:1006316117817

Olsen SR, Cole CV, Watanabe FS, Dean LA (1954) Estimation of available phosphorus in soils by extraction with sodium bicarbonate. US Dept. Agric. Circ. 939, Washington

Osborne, J.W., 2010. Improving your data transformations: Applying the Box-Cox transformation. Pract. Assessment, Res. Eval. 15.

Ouyang, L., Tang, Q., Yu, L., Zhang, R., 2014. Effects of amendment of different biochars on soil enzyme activities related to carbon mineralisation. Soil Res. 52, 706–716. https://doi.org/10.1071/SR14075

Palansooriya, K.N., Ok, Y.S., Awad, Y.M., Lee, S.S., Sung, J.K., Koutsospyros, A., Moon, D.H., 2019. Impacts of biochar application on upland agriculture: A review. J. Environ. Manage. 234, 52–64. https://doi.org/10.1016/j.jenvman.2018.12.085

Paz-Ferreiro, J., Gascó, G., Gutiérrez, B., Méndez, A., 2012. Soil biochemical activities and the geometric mean of enzyme activities after application of sewage sludge and sewage sludge biochar to soil. Biol. Fertil. Soils 48, 511–517. https://doi.org/10.1007/s00374-011-0644-3

Pokharel, P., Ma, Z., Chang, S.X., 2020. Biochar increases soil microbial biomass with changes in extra- and intracellular enzyme activities: a global meta-analysis. Biochar 2, 65–79. https://doi.org/10.1007/s42773-020-00039-1

Purakayastha, T.J., Das, K.C., Gaskin, J., Harris, K., Smith, J.L., Kumari, S., 2016. Effect of pyrolysis temperatures on stability and priming effects of C3 and C4 biochars applied to two different soils. Soil Tillage Res. 155, 107–115. https://doi.org/10.1016/j.still.2015.07.011

Rafael, R.B.A., Fernandez-Marco, M.L., Cocco, S., Ruello, M.L., Fornasier, F., Corti, G., Fernandez-Marcos, M.L., Cocco, S., Ruello, M.L., Fornasier, F., Corti, G., 2019. Benefits of Biochars and NPK Fertilizers for Soil Quality and Growth of Cowpea (Vigna unguiculata L. Walp.) in an Acid Arenosol. Pedosphere 29, 311–333. https://doi.org/10.1016/S1002-0160(19)60805-2

Rao M.A., Scelza R., Acevedo F., Diez M.C., .Gianfreda L., 2014. Enzymes as useful tools for environmental purposes. Chemosphere, 107: 145-162. https://doi.org/10.1016/j.chemosphere.2013.12.059

Schloter M., Dilly O., Munch. 2003. Indicators for evaluating soil quality. Agriculture, Ecosystems and Environment 98: 255–262. https://doi.org/10.1016/S0167-8809(03)00085-9

Schmidt, M.W.I., Noack, A.G., 2000. Analysis , distribution , implications , and current challenges. Global Biogeochem. Cycles 14, 777–793. https://doi.org/10.1029/1999GB001208

Schoeneberger, P.J., Wysocki D.A., Benham E.C., and Soil Survey Staff. 2012. Field book for describing and sampling soils, Version 3.0. Natural Resources Conservation Service, National Soil Survey Center, Lincoln, NE, USA.

Solaiman, Z.M., Anawar, H.M., 2015. Application of Biochars for Soil Constraints: Challenges and Solutions. Pedosphere. https://doi.org/10.1016/S1002-0160(15)30044-8

Subedi, R., Bertora, C., Zavattaro, L., Grignani, C., 2017. Crop response to soils amended with biochar: Expected benefits and unintended risks. Ital. J. Agron. 12, 161–173. https://doi.org/10.4081/ija.2017.794

Sun, F., Lu, S., 2014. Biochars improve aggregate stability, water retention, and pore-space properties of clayey soil. J. Plant Nutr. Soil Sci. 177, 26–33. https://doi.org/10.1002/jpln.201200639

Sun, Z., Bruun, E.W., Arthur, E., de Jonge, L.W., Moldrup, P., Hauggaard-Nielsen, H., Elsgaard, L., 2014. Effect of biochar on aerobic processes, enzyme activity, and crop yields in two sandy loam soils. Biol. Fertil. Soils 50, 1087–1097. https://doi.org/10.1007/s00374-014-0928-5

Uchimiya, M., Klasson, K.T., Wartelle, L.H., Lima, I.M., 2011. Influence of soil properties on heavy metal sequestration by biochar amendment: 1. Copper sorption isotherms and the release of cations. Chemosphere 82, 1431–1437. https://doi.org/10.1016/j.chemosphere.2010.11.050

Van Zwieten, L., Kimber, S., Morris, S., Chan, K.Y., Downie, A., Rust, J., Joseph, S., Cowie, A., 2010. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant Soil 327, 235–246. https://doi.org/10.1007/s11104-009-0050-x

Ventura, M., Zhang, C., Baldi, E., Fornasier, F., Sorrenti, G., Panzacchi, P., Tonon, G., 2014. Effect of biochar addition on soil respiration partitioning and root dynamics in an apple orchard. Eur. J. Soil Sci. 65, 186–195. https://doi.org/10.1111/ejss.12095

Wallenstein, M., Allison, S.D., Ernakovich, J., Steinweg, J.M., Sinsabaugh, R., 2011. Controls on the Temperature Sensitivity of Soil Enzymes: A Key Driver of In Situ Enzyme Activity Rates, in: Shukla, G., Varma, A. (Eds.), Soil Enzymology. Springer-Verlag, Berlin Heidelberg, pp. 275–285. https://doi.org/10.1007/978-3-642-14225-3

Wang, J., Xiong, Z., Kuzyakov, Y., 2016. Biochar stability in soil: Meta-analysis of decomposition and priming effects. GCB Bioenergy 8, 512–523. https://doi.org/10.1111/gcbb.12266

Xu, G., Sun, J., Shao, H., Chang, S.X., 2014. Biochar had effects on phosphorus sorption and desorption in three soils with differing acidity. Ecol. Eng. 62, 54–60.

Zavalloni, C., Alberti, G., Biasiol, S., Vedove, G.D., Fornasier, F., Liu, J., Peressotti, A., 2011. Microbial mineralization of biochar and wheat straw mixture in soil: A short-term study. Appl. Soil Ecol. 50, 45–51. https://doi.org/10.1016/j.apsoil.2011.07.012

Zimmerman, A.R., Gao, B., Ahn, M.Y., 2011. Positive and negative carbon mineralization priming effects among a variety of biochar-amended soils. Soil Biol. Biochem. 43, 1169–1179. https://doi.org/10.1016/j.soilbio.2011.02.005

4.2 SUPPLEMENTARY MATERIALS

4.2.1 Biochar analysis

For the biochar, the particle-size distribution was determined by dry and water sieving at 0.25 and 0.10 mm. pH was determined using a combined glass-calomel electrode in water after the suspension (1:100 weight/volume) was heated in a water bath to 90 °C, stirred for 20 min to allow dissolution of the soluble components, and cooled to ≈ 25±2.5 (Ahmedna *et al*., 1997). The content of carbonate C was quantified by dissolution in 2M HCl solution and successive titration of the evolved CO_2 (Bundy and Bremner, 1972). Biochar volatile matter was determined by weight loss after heating (Jindo *et al*., 2014; Zhao *et al*., 2015; Rafael et al., 2019); the muffle furnace was set to 950 ºC, and the sample containing crucible was heated for 2 min on the outer edge of the furnace with the door open (≈300 ºC), and then for 3 min on the edge of the furnace with the door closed (≈500 ºC). Thus the crucible was left in the muffle for the night at 750°C and the ash content of the biochar was determined as the remaining weight. Total organic carbon (TOC) after the specimens were treated with a 2 M HCl solution, total nitrogen (TN), and total sulphur (TS) were determined using a CHNS-O analyzer (EA1110, Carlo Erba Instruments, Italy), following the protocol reported by Laberge *et al.* (2010) and Calvelo Pereira *et al.* (2011). The easily oxidizable organic carbon (EOOC) was estimated by the Walkley-Black method (Pansu and Gautheyrou, 2006). The total P (TP) was extracted by heating 0.5000 g of sample at 500 ºC in a muffle furnace for 16 h and dissolving the ashes in a 5 mol L^{-1} HCl solution (Lambert, 1976). Water-extractable P was obtained by forming a suspension of 0.1000 g of sample and 30 mL of water; the suspension was shaken at 120 r m^{-1} for 48 h in 50 mL centrifuge tubes and then centrifuged (3000 *g*, 15 min) and the supernatant filtered using Whatman No. 42 filter paper prior to colorimetric determination (Zhang *et al*., 2016). A simple colorimetric method based on ascorbic acid reduction of the ammonium phosphomolybdate complex was used to measure P in the solutions for both TP and water-extractable P (Kuo, 1996). The cation exchange capacity (CEC) was determined as the summation of the exchangeable cations displaced by a 0.2 $BaCl_2$ solution (solid/liquid ratio of 1:10) and shaken for about 10 min (Corti et al., 1997). The mixture was left to rest for a while and then gently shaken for other 10 min and then centrifuged. The extracted solution was filtered through Whatman 42 filter paper, and analysed for Ca, Mg, K, and Na by atomic absorption with a Shimadzu AA-6300 (Tokyo, Japan) spectrophotometer.

All the determinations were run in triplicate.

Table S1. Main physicochemical characteristics of the biochar obtained from wood sources (34% beech, 33% pine, and 33% fir) used in the field experiment.

Parameter	
0.50-0.25 mm (g kg^{-1})	78
0.25-0.10 mm (g kg^{-1})	579
< 0.10 mm (g kg^{-1})	343
pH_{water}	8.85 (0.07)
Moisture (%)	14.2 (1.2)
Volatile matter (%)	84.6 (1.3)
Ash (%)	11.36 (2.08)
EC (dS m^{-1})	2.42 (0.11)
TOC (g kg^{-1})	795.4 (22.5)
EOOC (g kg^{-1})	29.15 (4.11)
Carbonates-C (g kg^{-1})	1.65 (0.17)
TN (g kg^{-1})	0.38 (0.05)
TS (g kg^{-1})	10.62 (1.27)
TP (g kg^{-1})	1.74 (0.31)
Water-P (mg kg^{-1})	35.3 (2.5)
Exchangeable Ca ($cmol_c$ kg^{-1})	1.65 (0.14)
Exchangeable Mg ($cmol_c$ kg^{-1})	1.94 (0.21)
Exchangeable K ($cmol_c$ kg^{-1})	1.33 (0.13)
Exchangeable Na ($cmol_c$ kg^{-1})	0.26 (0.05)
Exchangeable Al ($cmol_c$ kg^{-1})	0.00 (0.00)
CEC ($cmol_c$ kg^{-1})	5.18 (0.09)
Base saturation (%)	100.0 (0.0)

Table S2. General features and morphology of the soil from Gallignano field experimental plot. For symbols see legend.

Altitude: 98-102 m; Parent rock: thinly layered marine sediments (Plio-Pleistocene); General slope: ≈15%; Exposure: SW. Drainage class: poorly drained; Soil use: six-year-old alfalfa (*Medicago sativa* L.) forage crop.
Weeds: *Convolvulus arvensis* L., *Rumex crispus* L., *Cirsium arvense* L., *Anagallis arvensis* L., *Avena* sp. Mean annual precipitation: 788 mm. Mean annual air temperature: 14.6°C; Warmest month: July (23.3°C); Coldest month: January (5.4°C).
Soil temperature regime: mesic; soil moisture regime: ustic.
Soil: fine-loamy, mixed, mesic Typic Haplustept (Soil Survey Staff, 2014).

Profile 1, relatively upper field belt (100-102 m above sea level)

Horizon	Depth	Thickness	Boundary[a]	Color[b]	Structure[c]	Consistence[d]	Rock fragments	Other observations
	cm	cm					%, by sight	
Ap1	0-5	2-6	CW	10YR 4/3	2 f,m abk-sbk	fr-fi	5	mesofauna, worm channels
Ap2	5-16	8-13	CW	10YR 5/4	2-3 f,m sbk	fr-fi	5	worm channels
Ap3	16-28	10-13	CW	10YR 5/4	2 f,m sbk	fr-fi	5	worm channels
Ap4	28-36	5-10	CW	10YR 5/4	3 f,m sbk	fr-fi	5	worm channels
Bw	36-52+	-	-	10YR 6/4	1 m sbk	fr-fi	5	

Profile 2, relatively lower field belt (98-100 m above sea level)

Horizon	Depth	Thickness	Boundary[a]	Color[b]	Structure[c]	Consistence[d]	Rock fragments	Other observations[f]
	cm	cm					%, by sight	
Oi	2-0							
Ap1	0-4	1-5	CW	10YR 4/3	1 f gr,sbk,abk	fr-fi	5	mesofauna, brick fragments
Ap2	4-22	15-20	CW	10YR 4/3	2-3 f,m abk-sbk	fr-fi	5	brick fragments
Ap3	22-36	12-20	CW	10YR 4/3	2-3 m abk	fr-fi	5	black-coated roots channels
Ap4	36-54	15-19	CW	10YR 4/3	2 f,m abk	fr-fi	5	brick fragments
Bw	54-62+	-	-	10YR 5/3	3-2 f sbk	fr-fi	5	

[a] C=clear, W=wavy

[b] moist and crushed, according to the Munsell Soil Color Charts.

[c] 1=weak, 2=moderate, 3=strong; f=fine, m=medium, c=coarse; gr=granular, abk=angular blocky, sbk=sub-angular blocky.

[d] fr=friable; fi=firm.

[e] 0=absent, 1= few, 2=common, 3=many; mi=micro, vf=very fine, f=fine, m=medium, co=coarse.

Table S3. Soil general features and morphology for the 9 plots on Aug. 2018, September 2018, February 2019, and July 2019 at the Gallignano experimental field. For symbols see legend.

	Horizon	Depth				Color[a]				Structure[b]				Consistence[c]				Other observations			
		cm																			
Plot 1 - wheat		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
	Ap1	0-4	0-5	0-13	0-12	10YR 4/4	10YR 4/3	10YR 4/3	10YR 4/3	3f,m,c sbk	3f,m,c sbk	3f,m,c sbk	3f,m,c sbk	fr	fr	fr	fi			crust	
	Ap2	4-19	5-14	13-25	12-26	10YR 5/4	10YR 4/3	10YR 4/3	10YR 4/3	3f,m,c sbk	2m,c abk 3f,m,c sbk	2m,c sbk	2m,c sbk	fr	fr	fr	fi			worm casts	worm casts
	Ap3	19-26	14-25	-	-	10YR 5/4	10YR 4/3	-	-	2m,c abk	3f,m,c sbk	-	-	fr	fr	-	-	worm casts	worm casts	-	-
	Horizon	Depth				Color[a]				Structure[b]				Consistence[c]				Other observations			
		cm																			
Plot 2 - wheat+ biochar		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
	Ap1	0-8	0-7	0-16	0-14	10YR 4/3	10YR 4/3 10YR 3/1	10YR 3/1 10YR 4/3	10YR 3/2 10YR 4/3	3f,m sbk abk	3f,m,c sbk	3m,c sbk	3m,c sbk	fr	vfr	vfr	vfr		Salt and pepper color	Salt and pepper color, ants	Salt and pepper color, ants
	Ap2	8-20	7-18	16-25	14-26	10YR 5/4	10YR 2/1 10YR 4/3	10YR 2/1 10YR 4/3	10YR 2/1 10YR 4/3	3f,m,c abk sbk	3f,m,c sbk	2m,c sbk	2m,c sbk	fr	vfr	vfr	vfr	charcoal fragments	Salt and pepper color	Salt and pepper color, worm casts	Salt and pepper color, worm casts
	Ap3	20-27	18-26	-	-	10YR 5/4	10YR 4/4	-	-	3f,m,c sbk	2f,m abk-sbk	-	-	fr	fr	-	-	worm casts	worm casts	-	-
	Horizon	Depth				Color[a]				Structure[b]				Consistence[c]				Other observations			
		cm																			
Plot 3 - alfa-alfa		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
	Oi	1-0	1-0	1-0	1-0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ap1	0-3	0-3	0-4	0-3	10YR 4/4	10YR 4/4	10YR 4/4	10YR 4/4	3f,m sbk	3f,m sbk	3m,c sbk	3m,c sbk	vfi	fr	fr	vfi			mesofauna	mesofauna
	Ap2	3-17	3-16	4-16	3-16	10YR 5/4	10YR 4/4	10YR 4/3	10YR 4/4	3f,m abk	3m abk sbk	2m,c abk sbk	3m,c abk sbk	vfi	fr	fr	vfi				
	Ap3	17-27	16-25	16-26	16-26	10YR 5/6	10YR 4/3	10YR 4/4	10YR 4/3	3m,c abk sbk	2f,m abk	2f,m abk	3f,m,c abk	fi	fr	fr	vfi				
	Horizon	Depth				Color[a]				Structure[b]				Consistence[c]				Other observations			
		cm																			

Plot	Horizon	Depth				Color[a]				Structure[b]				Consistence[c]				Other observations			
Plot 4 - wheat+ biochar		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
	Ap1	0-8	0-11	0-17	0-15	10YR 4/3	10YR 2/1 10YR 4/3	10YR 4/3 10YR 2/1	10YR 4/3 10YR 2/1	3f,m,c abk sbk	2-3f,m sbk gr	3f,m,c sbk	3f,m,c sbk	fr	vfr	vfr	vfr	worm casts	Salt and pepper color, worm casts	Salt and pepper color, worm casts	Salt and pepper color, worm casts
	Ap2	8-19	11-19	17-26	15-26	10YR 5/4	10YR 4/3	10YR 4/3	10YR 4/2	3f,m,c abk sbk	2-3m,c abk sbk	2m,c abk sbk	2m,c abk sbk	fr	vfr	vfr	vfr	charcoal fragments, worm casts	Salt and pepper color, worm casts	Salt and pepper color, worm casts	Salt and pepper color, worm casts
	Ap3	19-27	19-26	-	-	10YR 5/4	10YR 4/3	-	-	3f,m,c abk sbk	2f,m abk sbk	-	-	fr	fr	-	-	worm casts	worm casts	-	-
	Horizon	Depth				Color[a]				Structure[b]				Consistence[c]				Other observations			
		cm																			
Plot 5 - wheat		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
	Ap1	0-4	0-5	0-15	0-14	10YR 4/4	10YR 4/3	10YR 4/3	10YR 4/3	3f,m,c abk sbk	3f gr abk sbk	3f gr abk sbk	3f abk sbk	fr	fr	fr	fi	worm casts	worm casts	worm casts	worm casts
	Ap2	4-20	5-15	15-26	14-26	10YR 5/4	10YR 4/3	10YR 4/3	10YR 4/4	3f,m,c abk sbk	3f,m abk sbk	3f,m abk sbk	3f,m abk sbk	fr	fr	fr	fi	worm casts	worm casts	worm casts	worm casts
	Ap3	20-27	15-26	-	-	10YR 5/6	10YR 4/4	-	-	3m,c abk sbk	3m,c abk sbk	-	-	fr	fr	-	-	worm casts	worm casts	-	-
	Horizon	Depth				Color[a]				Structure[b]				Consistence[c]				Other observations			
		cm																			
Plot 6 - alfa-alfa		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
	Oi	1-0	1-0	1-0	1-0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ap1	0-3	0-4	0-4	0-4	10YR 4/4	10YR 4/4	10YR 4/4	10YR 4/4	3f,m,c abk sbk	3m abk sbk	3m,c sbk	3m,c sbk	vfi	fr-fi	fr	vfi	very hard		mesofauna	mesofauna
	Ap2	3-19	4-20	4-19	4-19	10YR 5/4	10YR 4/4	10YR 4/3	10YR 4/3	3m,c abk sbk	2m abk sbk	2m,c abk sbk	2m,c abk sbk	vfi	fr	fr	vfi	very hard			
	Ap3	19-28	20-27	19-26	19-26	10YR 5/4	10YR 4/3	10YR 4/4	10YR 4/3	3m,c abk sbk	2-3m,c abk sbk	2m,c abk sbk	2-3m,c abk sbk	vfi	fr	fr	vfi				
	Horizon	Depth				Color[a]				Structure[b]				Consistence[c]				Other observations			
		cm																			
Plot 7 - wheat+ biochar		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
	Ap1	0-9	0-11	0-14	0-13	10YR 4/3	10YR 2/1	10YR 2/1	10YR 2/1	3f,m sbk	3f,m gr sbk	3m,c sbk	3m,c sbk	fr	vfr	vfr	vfr	worm casts	Salt and pepper color,	Salt and pepper color,	Salt and pepper color,

Horizon	Depth (cm)				Color[a]				Structure[b]				Consistence[c]				Other observations			
						10YR 3/4	10YR 3/4	10YR 3/3										ants, worm casts	ants, worm casts	ants, worm casts
Ap2	9-18	11-18	14-26	13-25	10YR 5/4	10YR 4/3 10YR 2/1	10YR 4/3 10YR 2/1	10YR 4/2 10YR 2/1	3m,c,vc abk sbk	2-3f,m, abk sbk	2m,c abk sbk	2m,c abk sbk	fr	vfr	vfr	vfr	worm casts	Salt and pepper color, ants, worm casts	Salt and pepper color, ants, worm casts	Salt and pepper color, ants, worm casts
Ap3	18-27	18-27	-	-	10YR 5/6	10YR 4/3	-	-	3m,c abk sbk	2m abk sbk	-	-	fr	fr	-	-	worm casts	worm casts	-	-

	Horizon	Depth (cm)				Color[a]				Structure[b]				Consistence[c]				Other observations			
		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
Plot 8 - wheat	Ap1	0-3	0-4	0-13	0-13	10YR 4/3	10YR 4/3	10YR 4/4	10YR 4/4	3f,m sbk abk	3f,m sbk abk	3m,c sbk	3m,c sbk	fr	fr	fr	fi			charcoal fragments	
	Ap2	3-21	4-20	13-26	13-26	10YR 5/4	10YR 5/4	10YR 4/6	10YR 4/4	3f,m,c sbk abk	2-3m sbk abk	2m,c abk sbk	2m,c abk sbk	fr	fr	fr	fi			worm casts	worm casts
	Ap3	21-28	20-27	-	-	10YR 4/6	10YR 4/6	-	-	3f,m,c sbk abk	2m sbk abk	-	-	fr	fr	-	-	worm casts	worm casts	-	-

	Horizon	Depth (cm)				Color[a]				Structure[b]				Consistence[c]				Other observations			
		Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019	Aug. 2018	Sept. 2018	Febr. 2019	July 2019
Plot 9 - alfa-alfa	Oi	1-0	1-0	1-0	1-0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ap1	0-4	0-4	0-4	0-4	10YR 4/4	10YR 4/4	10YR 4/4	10YR 4/4	3f,m,c sbk abk	3f,m sbk abk gr	3m,c sbk	3m,c sbk	vfi	fr-fi	fr	vfi	mesofauna			mesofauna
	Ap2	4-20	4-12	4-15	4-15	10YR 5/4	10YR 4/4	10YR 5/3	10YR 5/4	3m,c,vc sbk abk	3m,c sbk abk	2m,c abk sbk	2m,c abk sbk	vfi	fr	fr	vfi				
	Ap3	20-27	12-27	15-26	15-26	10YR 5/4	10YR 5/3	10YR 5/3	10YR 5/3	3m,c,vc abk	2-3m,c,vc abk	2m,c abk	2-3m,c abk	vfi	fr	fr	vfi				

[a] moist and crushed, according to the Munsell Soil Color Charts.

[b] 1=weak, 2=moderate, 3=strong; vf= very fine, f=fine, m=medium, c=coarse, vc=very coarse; gr=granular, abk=angular blocky, sbk=sub-angular blocky.

[c] vfr=very friable; fr=friable; fi=firm; vfi= very firm.

[d] 0=absent, 1=very few, moderately few, 2=common, 3=many; mi=micro, vf=very fine, f=fine, m=medium, co=coarse.

4.2.2 Determination of enzyme activities

250 mg of each soil samples were placed in 2-mL Eppendorf tubes together with glass and ceramic beads and 1.4 mL of 3% lysozyme at pH 6. The tube was subjected to bead-beating for 3 min at 30 strokes s^{-1} using a Retsch MM400 mill (Haan, Germnay), and then centrifuged for 3 min at 20,000 *g*. Enzyme activity was assayed fluorometrically in microplates using 4- methylumbelliferyl and L-Leucine-7-amino-4-methylcoumarin derivatives. The activities of arylsulfatase, chitinase, β-glucuronidase, β-galactosidase, β-glucosidase, xylosidase, and acid phosphomonoesterase were determined in 200 mmol L^{-1} 2-(N-morpholino) ethanesulfonic acid buffer solution (pH 5.8), while the activities of phosphodiesterase, pyrophosphatase/phosphodiesterase, leucine aminopeptidase, and nonanoate-esterase were determined in a 200 mmol L^{-1} tris-HCl buffer solution at pH 7.5; alkaline phosphomonoesterase activity was determined in a 200 mmol L^{-1} tris-HCl buffer solution at pH 9.0. The main activity of each enzyme and its relative nutrient cycle are summarized in Table S3.

Table S3. Main natural cycle and role of the assayed enzymes.

Cycle	**Enzyme**	**Activity**
Carbon	Nonanoate-esterase	Nonanoate-esterase activity reflects the contribution of multiple enzymes acting on ester bonds, including true esterases as well as proteases and others (Mattarozzi et al., 2020)
Carbon	Xylosidase	Hydrolysis of hemicellulose (Jian et al., 2016)
	β -glucuronidase	Hydrolysis of hemicelluloses (Sun and Cheng, 2002)
	β-galactosidase	The galactosidase have a key role in releasing low molecular weight sugars that are important as energy sources for microorganisms (Bandick and Dick, 1999)
	β-glucosidase	This enzyme breaks down cellobiose, which is the final step in the utilization of cellulose (Zang et al., 2018) in (Mattarozzi et al., 2020)
Carbon and nitrogen	Chitinase	A class of enzymes involved in the main pathway of chitin degradation. They hydrolyze the glucosidic bonds of chitin releasing smaller N containing organic compounds (Olander and Vitousek, 2000)
Nitrogen	Leucine-aminopeptidase	Cleaving of peptide bonds in proteins (Jian et al., 2016)
Phosphorus	Acid phosphomonoesterase	Hydrolysis of phosphate esters, prevailing in acid soils (Nannipieri et al., 2011)
	Alkaline phosphomonoesterase	Hydrolysis of phosphate esters, prevailing in alkaline soils (Nannipieri et al., 2011)
	Phosphodiesterase	Hydrolysis of P compounds with diester bonds such as phospholipids and nucleic acids. The hydrolysis of a phosphodiester is initiated by phosphodiesterase to release a phosphomonoester, which is then hydrolyzed by phosphomonoesterase to release free phosphate that can be taken up by plants (Nannipieri et al., 2011)
	Pyrophosphatase-phosphodiesterase	Hydrolysis of organic pyrophosphates (which are byproducts of nucleic acids, carbohydrates, proteins, and fatty acid degradation) in orthophosphates (Hernández-Domíguez et al., 2012)
Sulphur	Arylsulfatase	This enzyme is important in nutrient cycling because it releases plant available SO_4. Also, it may be an indirect indicator of fungi as only fungi (not bacteria) contain ester sulfate, the substrate of arylsulfatase (Bandick and Dick, 1999)

4.2.3 Literature

Ahmedna, M., Johns, M.M., Clarke, S.J., Marshall, W.E., Rao, R.M., 1997. Potential of agricultural by-product-based activated carbons for use in raw sugar decolourisation. J Sci Food Agric. 75, 117-124

Bandick, A.K., Dick, R.P., 1999. Field management effects on soil enzyme activities. Soil Biol. Biochem. 31, 1471–1479. https://doi.org/10.1007/BF02854228

Bundy, L.G., Bremner, J.M., 1972. A simple titrimetric method for determination of inorganic carbon in soils. Soil Sci Soc Am Proc 36, 273–275.

Calvelo Pereira, R., Kaal, J., Camp Arbestain, M., Pardo Lorenzo, R., Aitkenhead, W., Hedley, M., Macías, F., Hindmarsh, J., Maciá-Agulló, J.A., 2011. Contribution to characterisation of biochar to estimate the labile fraction of carbon. Organic Geochem. 42, 1331-1342.

Corti, G., Agnelli, A., Ugolini, F.C., 1997. Release of Al by hydroxy-interlayered vermiculite and hydroxy-interlayered smectite during determination of cation exchange capacity in fine earth and rock fragments fractions. European Journal of Soil Science 48, 249-262. doi: 10.1111/j.1365-2389.1997.tb00545.x

Hernández-Domíguez, E.E., Valencia-Turcotte, L.G., Rodríguez-Sotres, R., 2012. Changes in expression of soluble inorganic pyrophosphatases of *Phaseolus vulgaris* under phosphate starvation. Plant Sci. 187, 39–48. https://doi.org/10.1016/j.plantsci.2012.01.009

Jian, S., Li, J., Chen, J., Wang, G., Mayes, M.A., Dzantor, K.E., Hui, D., Luo, Y., 2016. Soil extracellular enzyme activities, soil carbon and nitrogen storage under nitrogen fertilization: A meta-analysis. Soil Biol. Biochem. 101, 32–43. https://doi.org/10.1016/j.soilbio.2016.07.003

Jindo, K., Mizumoto, H., Sawada, Y., Sanchez-Monedero, M.A., Sonoki, T., 2014. Physical and chemical characterization of biochars derived from different agricultural residues. Biogeosciences 11, 6613-6621.

Kuo, S., 1996. Phosphorus. In Sparks D L, Page A L, Helmke P A, Leoppert R H, Soltanpour P N (Eds.) Methods of Soil Analysis Part 3---Chemical Methods. Soil Science Society of America, American Society of Agronomy, Madison. pp. 869-919.

Laberge, G., Haussmann, B.I.G., Ambus, P., Høgh-Jensen, H., 2011. Cowpea N rhizodeposition and its below-ground transfer to a co-existing and to a subsequent millet crop on a sandy soil of the Sudano-Sahelian eco-zone. Plant Soil 340, 369-382.

Lambert, M.J., 1976. Preparation of Plant Material for Estimating a Wide Range of Elements. National Library of Australia, Sydney.

Mattarozzi, M., Di Zinno, J., Montanini, B., Manfredi, M., Marengo, E., Fornasier, F., Ferrarini, A., Careri, M., Visioli, G., 2020. Biostimulants applied to maize seeds modulate the enzymatic activity and metaproteome of the rhizosphere. Appl. Soil Ecol. 148, 103480. https://doi.org/10.1016/j.apsoil.2019.103480

Nannipieri, P., Giagnoni, L., Landi, L., Renella, G., 2011. Phosphorus in Action 26, 215–243. https://doi.org/10.1007/978-3-642-15271-9

Olander, L.P., Vitousek, P.M., 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. Biogeochemistry 49, 175–190. https://doi.org/10.1023/A:1006316117817

Pansu, M., Gautheyrou, J., 2006. Handbook of Soil Analysis: Mineralogical, Organic and Inorganic Methods. Springer-Verlag, Berlin, Heidelberg.

Rafael, R.B.A., Fernandez-Marcos, M.L., Cocco, S., Ruello, M.L., Fornasier, F., Corti, G., 2019. Benefits of biochars and NPK fertilizers for soil quality and growth of Cowpea (*Vigna unguiculata* L. Walp.) in an acid Arenosol. Pedosphere 29, 311–333. https://doi.org/10.1016/S1002-0160(19)60805-2

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour. Technol.

83, 1–11. https://doi.org/10.1016/S0960-8524(01)00212-7

Zhang, H.Z., Chen, C.R., Gray, E.M., Boyd, S.E., Yang, H., Zhang, D.K., 2016. Roles of biochar in improving phosphorus availability in soils: A phosphate adsorbent and a source of available phosphorus. Geoderma. 276, 1-6.

Zang, X., Liu, M., Fan, Y., Xu, J., Xu, X., Li, H., 2018. The structural and functional contributions of β-glucosidase-producing microbial communities to cellulose degradation in composting. Biotechnol. Biofuels 11, 1–13. https://doi.org/10.1186/s13068-018-1045-8

Zhao, R.D., Coles, N., Kong, Z., Wu, J.P., 2015. Effects of aged and fresh biochars on soil acidity under different incubation conditions. Soil Till Res. 146, 133-138.

5 Concluding remarks

From the first experiment, aimed at estimating a basket of ecosystem services emerging from alternative management options for a 6-year-old alfalfa stand, suggests that the wheat-after-wheat option would result in the narrower basket of ecosystem services while the continuous alfalfa was the options that offers a wider basket of ecosystem services, this can be attributed to the provisioning services. The negative effect of wheat-after-wheat might be partially counterbalanced by the addition of biochar that resulted in a similar provision supporting services compared to the continuous alfalfa and also on in the highest in terms of regulating services. Biochar did not result in any positive nor detrimental effect on the biodiversity and other ecosystem services category. However future studies might investigate the effect of biochar again in terms of synergies and trade-offs with other ecosystem services. Biodiversity which is the base of all the ecosystem services results higher for the continuous alfalfa, suggesting that future studies should investigate more in depth the potential wide array of ecosystem services that the long-lasting alfalfa can provide.

The second experiment, perennial legume termination in early autumn emerged to have provided less favourable conditions for the mineralization process compared to hypothetical termination in summer, with higher temperatures. The initial higher soil N2O emissions for alfalfa with the addition of wheat that emerged from the cumulative weekly analysis appeared to be due to the alfalfa mineralisation process after the tillage, and to the unavoidable asynchrony between the N released following alfalfa termination and the low N uptake by the following wheat. Reducing time-lapse between alfalfa termination and wheat N uptake can contain the N2O emissions, except after an initial increase in the soil N2O emissions. However, this initial higher soil N2O emission for alfalfa with the addition of wheat did not affect the seasonal cumulative soil N2O emissions, compared to continuous alfalfa. Finally, the mitigation effect of the perennial legume on the soil N2O emissions was not lost after its termination by tillage.

The last study confirmed the importance of the feedstock source used for biochar production, the pyrolysation temperature and the soil characteristics as critical factors to be taken into consideration in the evaluation of the effects on the soil physicochemical properties and enzyme activities. Indeed, the studied enzymatic activities involved in the C, N, P and S soil nutrient cycles, were not affected by the present wood-derived biochar applied at field rates of 60 t ha-1. On the other hand, no consistent effect on soil N, phosphorous and pH could be related to the biochar at this application rate and therefore, as conceivable, no effect emerged in terms of wheat production. The results obtained from the C cycle enzyme activity and TOC analysis confirmed that wood-derived biochar does not stimulate C mineralization on the short term, while possessing the ability for soil C sequestration. This build-up can be very important for the structure of a soil with poor SOM content.