OPTIONS FOR TRANSITION OF LAND TOWARDS INTENSIVE AND SUSTAINABLE AGRICULTURAL SYSTEMS

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OPTIONS FOR TRANSITION OF LAND TOWARDS INTENSIVE AND SUSTAINABLE AGRICULTURAL SYSTEMS

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The INTENSE (FACCE SURPLUS) project had field experiments in most participating countries. This is from Martlhof near Tegernsee in Bavaria, Germany. Image by Peter Schröder

Climate and environment of Gaia, mother Earth, are under multiple significant stresses. The increase in world population demands large increases in food production, but this must be reached by use of sustainable methods. Emission of climate gasses needs to be dramatically decreased, overall ecological footprints have to be diminished, and socioeconomy of rural areas has to be boosted. These aims are not easy to combine. However, the bio-economy and green solutions may provide mankind with tools of great value both to mitigate pollution and climate change and to adapt to future changes.

It is clear that all forms of agriculture cause changes in balances and fluxes of pre-existing ecosystems, thereby limiting resiliency functions. Intensive agriculture in regions that are influenced by industrial pollution, with strong reduction of landscape structures and vast decoupling of energy and matter cycles, has caused stress and degradation of the production base; massive influence has also been exerted on neighbouring compartments. Average yields are probably close to 50 % of maximum yield many places, due to mismanagement of the crops during the production phase, or due to the inappropriate use of key resources. This relationship often leads to a mis-match between input of resources and process outputs, and creates pollution and unbalance in the landscape. Fertilizer runoff and salt accumulation occurs if water supply is in surplus or deficiency, due to soil compaction after use of large machines, and pollinating insects are suffering in regions with large monocultures and high pesticide inputs. These few examples show some of the dilemmas of using input factors in a way that does not fit with the overall conditions.

Hence it will be as important as ever to develop new agricultural systems exploiting seasonal growth cycles through intercropping and the integration of mixed perennial crops to ensure permanent availability of plant fractions to be delivered to end users. The problem of degrading soils threatened by overuse, compaction, pollution and loss of biology can only be tackled by a cross disciplinary research approach addressing the entire spectrum of agricultural, environmental and socioeconomic functions of our agricultural systems. While efforts to demonstrate the benefit of site-specific management are relatively recent and have taken various approaches, they specifically refer to variable-rate applications of single inputs, e.g. seeds, fertilizers, chemicals. It is high time to deploy principles of precision agriculture for integrated crop management through combined variable inputs of irrigation water, fertilizers, composts and crop density to improve degrading land and on the other side produce valuable raw products for biorefineries and biobased industries

In order to implement such novel production systems, for food and non-food products, the demonstration of land use changes, for biodiversity, for sufficient food and biomass production is essential, with emphasis on the diversity of species and varieties grown, harvested and converted to valuable products. Therefore this Research Topic combines studies demonstrating improved use of soil amendments, nutrients, as well as improved soil fertility for higher resilience against climate stress and recuperation of abandoned or contaminated soils for cropping and animal husbandry. Mixed cropping for high biomass production to create higher added value through the production and transformation of green biomass into novel products is presented as one of the solutions.

Applied research for a sustainable and ecologically compatible land use aimed at sufficient food production is as important as ever. Adequate management plans have to be developed from modeling and implemented to increase soil life at the level of the local farm and the region. Growing biomass plants for biorefinery processes should lower production costs, avoid pollution of surface and groundwater, reduce pesticide residues, reduce a farmer's overall risk, and increase both short- and long-term farm profitability. Such production systems are established amongst the authors of this Research Topic and will allow to obtain an integrated picture of the role of closed cycling loops for N, P and K, and water in an agricultural ecosystem. The next step will be to support decision-making using sustainability indicators and toolboxes as they have been developed for different agricultural systems. The availability of stable research networks of study sites across Europe will help to develop decision support systems applicable across a variety of domains for integrated food and non-food production in the EU, in regards to socio-economy, sustainability and ecology.

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EDITORIAL
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Editorial: Options for Transition of Land Towards Intensive and Sustainable Agricultural Systems

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Keywords: sustainable intensification, food production, biomass, soil amendments, fertilizers

Editorial on the Research Topic

Options for Transition of Land Towards Intensive and Sustainable Agricultural Systems

Development of agricultural practices must be in accordance with the "Great Challenges" for the twenty-first century, for which soil and water protection are key factors. There is no single solution to these enormous challenges but efforts need to be made on all frontiers to feed our growing population and provide it with bio-based raw materials. Modern agriculture must adapt to fulfill these demands in a sustainable way. The main factors governing agroecosystem productivity are soil, water, balanced nutrients input, and a correct land management. In this context, adaptations to climate change must be addressed. Low water availability in Mediterranean arid and semi-arid conditions is one of the main threats for cropland. On the other hand, soils that today may be rather marginal because of cool summer climates, may become important for future food, feed, or industrial crops production if water is not limiting the production. The present research topic focuses on marginal land that can be considered for environmentally sound production of biomass or other marginal goods avoiding the use of good soil for this purpose.

Soils in many places are degraded to a stage where it takes long time to recuperate these resources, especially in contaminated areas. Thousands of sites in Europe have been declared as polluted with trace elements, and excluded from agricultural production. As a possible solution, Kidd et al. reports the adequate fertilization regimes; plant cropping patterns and plant microbial and fungal interactions and the biomass processed for Ni recovery. Mench et al. report a successful sunflower—tobacco crop rotation for copper removal, and Thijs et al. contribute results on the slow removal of Cd, Zn, and Pb from diffusely polluted soils by phyto-management using energy plants in a short rotation strategy.

It is a paradox that nutrients that may be very scarce in the near future, as P and N, are dispersed in far too large quantities, especially in areas with livestock production causing severe environmental problems. Resource depletion may also lead to price tensions with an impact on food security. Furthermore, some conventional agriculture practices represent a threat to sustainability of the long-term productivity. New methods, in line with principles of bioeconomy and circular economy models, spur the use of organic wastes as raw material for fertilizer and soil amendments. Ghaley et al. simulated soil organic carbon effects on winter wheat under different N-fertilizer amounts, using long term data from an experiment in Denmark. They concluded that agronomic productivity was enhanced by soil organic carbon build up when N-rates were in the range of 0–100 kg N ha^{-1}, but not when N-fertilization was higher. Reichel et al. scrutinized the role of carbon source for the microbial storage or release of N compounds from arable land. They found that N immobilization by incorporated straw and sawdust may be more important for storage of excess N than microbial N immobilization over a growth period whereas pure lignin did not stimulate microbial N immobilization.

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Regarding biochar production from feedstocks, it is necessary to establish critical factors for their application as soil amendments. Marmiroli et al. found that the feedstock type determined biochar microstructure and elemental composition, which were linked to toxicity: biochar from animal origin were phytotoxic at lower concentrations than those from plant feedstock.

Synthetic fertilizers are annually 11.4 million tons of nitrogen and 1.1 million tons of phosphorus in EU-28. Meanwhile, between 118 and 138 million tons of biowaste are produced annually, but only about 25% is effectively recycled into compost and digestate. Fortunately, there is an increasing awareness that recycling of nutrients and organic matter is essential for creating sustainable food and non-food value chains. Energy crops and residues may be one option for more efficient use of biomass. Weiß and Glasner addressed how to sort out fractions that have negative impact on incinerators, by decreasing the chlorine content of the biomass of wheat chaff.

To prevent environmental pollution and ensure safety related to the uncontrolled application of inadequate amendments for agricultural purposes, updating of the regulations are being enforced on international level. In this context, a study investigating the effects of N sources and tillage practices on NH_3 volatilization, grain yield and nutrient use efficiency (NUE) from paddy fields in central China is of high interest (Liu et al.). Furthermore, van Duijnen et al., found that N fertilizers affected wheat yield most but also pre-crops were important, and plants with N-fixing bacteria had a better effect on barley yield than mycorrhiza associated pre-crops. Improving fertility of marginal soils for the sustainable production of biomass is a strategy for reducing land use conflicts between food and energy crops as it is shown by Nabel et al. They demonstrate that the intercropping of legumes, can stimulate the yield of *S. hermaphrodita* on marginal soils for sustainable plant biomass production. Further on, Nabel et al. scrutinized the role of depot fertilization. This technique promoted a deep reaching root system of *S. hermaphrodita* seedlings with a dense root cluster around the depot-fertilized zone, resulting in 5-fold increased biomass yield.

A big problem is fodder, water and resource use for cattle growth. When forage production, feed intake, and animal performance of abandoned grassland before and after the common practice of rangeland grazing were compared, it became clear that extended spring grazing on abandoned grassland would improve lamb performance. Quantifying these aspects of reintroducing abandoned grassland into sheep farming gives both sheep farmers and land owners a knowledge basis for valuing the area in monetary terms and for decision-making (Steinshamn et al.).

New methods and technologies are needed, implementing remote sensing tools and drones, the use of precise methods and unmanned vehicles and transfer to smaller and smarter machines that are precisely guided. We must avoid destruction of soil structure, which encompasses a multitude of soil organisms important for soil functioning. Furthermore, crop rotation, intercropping, improved soil amendments, cultivar selection and irrigation methods need to be adapted for each specific situation. Finally, agricultural wastes must be investigated for their usability. Current research shows that we have many options and solutions that must be followed up and further developed to technology readiness (Schröder et al., 2018). In this context, poor, contaminated and dry soils may have to be re-activated in order to increase soil quality and soil potential for food, feed, and other biomass production (Mench et al.; Marmiroli et al.).

The current Frontiers research topic gives input to mobilization for increased food supply and shows that a multitude of actions are needed, and research and development is an important part. By showing that marginal and abandoned crop or grasslands have a value, and that techniques for sound production on such sites exist, such areas can give crucial contributions to biomass production. Future land use must embrace efficient production and utilization of biomass for improved economic, environmental, and social outcomes. New options open up a wide range of novel products and services across farming communities. A holistic approach is needed to identify common traits and at the same time enable the development and dissemination of production chains for sustainable intensification, which are adapted to the environmental and socioeconomic diversity situations.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Potential of Wheat Straw, Spruce Sawdust, and Lignin as High Organic Carbon Soil Amendments to Improve Agricultural Nitrogen Retention Capacity: An Incubation Study

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Plants like winter wheat are known for their insufficient N uptake between sowing and the following growing season. Especially after N-rich crops like oilseed rape or field bean, nitrogen retention of the available soil N can be poor, and the risk of contamination of the hydrosphere with nitrate (NO_3^-) and the atmosphere with nitrous oxide (N_2O) is high. Therefore, novel strategies are needed to preserve these unused N resources for subsequent agricultural production. High organic carbon soil amendments (HCA) like wheat straw promote microbial N immobilization by stimulating microbes to take up N from soil. In order to test the suitability of different HCA for immobilization of excess N, we conducted a laboratory incubation experiment with soil columns, each containing 8 kg of sandy loam of an agricultural Ap horizon. We created a scenario with high soil mineral N content by adding 150 kg NH_4^+-N ha^{-1} to soil that received either wheat straw, spruce sawdust or lignin at a rate of 4.5 t C ha^{-1}, or no HCA as control. Wheat straw turned out to be suitable for fast immobilization of excess N in the form of microbial biomass N (up to 42 kg N ha^{-1}), followed by sawdust. However, under the experimental conditions this effect weakened over a few weeks, finally ranging between 8 and 15 kg N ha^{-1} immobilized in microbial biomass in the spruce sawdust and wheat straw treatment, respectively. Pure lignin did not stimulate microbial N immobilization. We also revealed that N immobilization by the remaining straw and sawdust HCA material in the soil had a greater importance for storage of excess N (on average 24 kg N ha^{-1}) than microbial N immobilization over the 4 months. N fertilization and HCA influenced the abundance of ammonia oxidizing bacteria and archaea as the key players for nitrification, as well as the abundance of denitrifiers. Soil with spruce sawdust emitted more N_2O compared to soil with wheat straw, which in relation released more CO_2, resulting in a comparable overall global warming potential. However, this was counterbalanced by advantages like N immobilization and mitigation of potential NO_3^- losses.

Keywords: greenhouse gases, high organic carbon amendment, isotope labeling, microbial decomposition, nitrogen immobilization, nitrogen cycle, nitrogen fertilizer

INTRODUCTION

Global demands for nitrogen (N) in crop production have been 110 million tons in 2015. N demands are expected to increase to almost 119 million tons in 2020 (Lu and Tian, 2017). About one third of this amount is not directly assimilated by plants and, if not stored in the pedosphere, contaminates the hydrosphere with nitrate (NO_3^-) and the atmosphere with nitrous oxide (N_2O) and nitric oxide (NO) (Zhu and Chen, 2002). N retention is of particular importance when residues of N-rich crops, such as field bean (*Vicia faba* L.), oilseed rape (*Brassica napus* L.), sugar beet (*Beta vulgaris* L.), and potato (*Solanum tuberosum* L.) are mineralized in soil. Such residues are substantial sources of mineral N, supplying between 20 and 60 kg N ha^{-1} to the soil, depending on the leaf and straw yield (Döhler, 2009). Crop residues with a C:N ratio below 20–25 are supposed to be mineralized more quickly than those with higher C:N ratios (Mooshammer et al., 2014). Sugar beet residues, for instance, with a narrow C:N ratio of 11 can be mineralized by up to 75% within the first 10 weeks after incorporation (Whitmore and Groot, 1997).

In German crop rotations, oilseed rape is harvested already in July, often followed by winter wheat (*Triticum aestivum* L.), which is sown in September/October, but grows only very slowly until the following spring, associated with a correspondingly low N uptake of up to 30 kg N ha^{-1} during this period of time (Sieling et al., 1999). In contrast, soil mineral N content after oilseed rape harvest sometimes exceeds 100 kg N ha^{-1} (Henke et al., 2008). As a result, about 50% of mineral N derived from decomposition of N-rich crops like oilseed rape can be lost from soil, particularly in sandy soil (Döhler, 2009). Sieling and Kage (2006) reported annual soil mineral N losses via nitrate leaching of 44 or 73 kg N ha^{-1} after oilseed rape harvest and subsequent winter barley (*Hordeum vulgare* L.) or winter wheat cultivation from Germany. Annual N losses by N_2O from long-term field experiments with oilseed rape in Germany ranged between 1.4 and 4.0 kg N ha^{-1}, of which 53 to 81% occurred during the winter season (Kaiser and Ruser, 2000). Thus, managing soil mineral N after harvest during times without sufficient winter crop N uptake is of great importance for reducing N losses and improving the agricultural N use efficiency (NUE) by achieving a similar quantity of N in the harvested crop by less N input (Zhang et al., 2015). Although this fact has been known for a long time, the nitrogen surplus is a persistent and pressing problem. Thus research efforts are needed to manage the fate of unused fertilizer N in crop production (Liu et al., 2010). Closing the post-harvest time gaps in crop rotations without N uptake by plants may help to retain N in soil for subsequent crop production.

It is known since long that N-poor crop residues such as wheat straw can cause a strong immobilization (or "lock") of N, which means a reduction of plant-available N. It has been shown that available N in soil is immobilized after application of decomposable, C-rich organic residues with large C:N ratios, such as wheat straw (Richards and Norman, 1931; Cheshire et al., 1999), which might lower crop yield in the next season. Malhi et al. (2011), however, proposed that well managed soils will benefit in the long term from retaining straw residues in the soil, thereby improving crop yield and plant N uptake. Nonetheless, if N immobilization interferes with plant growth, the probability is high that microorganisms are more competitive for nutrients than plants if plant and microbial N uptake occur simultaneously in the same soil volume (Hodge et al., 2000).

Organic substrates with large C:N ratios, such as wheat straw (C:N 50–100), are required to promote microbial N immobilization, forcing microbes to take up N from soil, in order to maintain their C:N ratio at least ten times lower (Cleveland and Liptzin, 2007; Scheller and Joergensen, 2008). However, large C:N ratios alone do not warrant intensive microbial growth and thus N immobilization, but serve as a rough indicator for its N immobilization potential. Spruce sawdust with a C:N ratio of 100 to 400 contains more recalcitrant compounds, leading to lower degradation rates and prolonged times of decay (Kostov et al., 1991). Thus, in order to immobilize N, two major requirements have to be fulfilled to stimulate growth of microbial decomposers: (1) sufficient available C as energy source; (2) sufficient available N and P to fulfill stoichiometric requirements and to avoid nutrient mining of original soil organic matter (Fontaine et al., 2004).

High organic carbon amendments (HCA) consist of very different quantities and qualities of fast, moderately, and slowly degradable organic sub-fractions. Microorganisms respond very differently to these organic fractions, and microbial N immobilization can be expected to increase with increasing size and C:N ratio of the fast degradable fraction, which usually is degraded within a few days (Plante and Parton, 2007). The fast degradable fraction contains easily soluble sugars, amino acids, and nucleic acids (Müller et al., 1998). Such compounds are favorable for a rapid growth of zymogenous (fermentative) microorganisms (Kuzyakov and Blagodatskaya, 2015) with short half-lives of a few days (Cochran et al., 1988). Despite this fast turnover, C and N released by dead microbial biomass serve as additional substrates for microbes that grow more slowly on more complex organic compounds (Fontaine et al., 2004).

More complex, polymeric components such as cellulose and hemicellulose are the dominating fraction of wheat straw. Investment in depolymerizing enzymes may reduce the microbial growth efficiency, but the large C:N ratio of this fraction and its accessibility to many soil microbes account for its N retention capacity (Plante and Parton, 2007). Woody sawdust contains more recalcitrant compounds than wheat straw, mainly in the form of lignin. Specialists like white-rot fungi are needed to break up the lignified structures of wood (van Kuijk et al., 2017). However, this process is too slow to support significant microbial growth and, hence, fast microbial N immobilization. In addition to biotic N immobilization and N adsorption in soil, also less prominent ways of abiotic N immobilization were reported (Miyajima, 2015), e.g., the reaction of nitrite with HCA-derived lignin or its derivatives (Wei et al., 2017).

The aim of the present work was to test the potential of different HCA for efficiently managing a post-harvest excess of soil mineral N in arable soil without sufficient plant N uptake. To this end, a static laboratory incubation study was conducted to answer the following research questions: (1) Can HCA with large C:N ratio such as wheat straw, spruce sawdust, and lignin help to manage temporal N excess in agricultural soil by inducing microbial growth at a relevant magnitude? (2) Can such HCA application strategies help improve the nitrogen retention capacity of the soil by reducing an unintended N loss in form of nitrate and greenhouse gases?

MATERIALS AND METHODS

Soil and HCA

For the incubation study, substrate of a Cambic Luvisol soil type with sandy loam soil texture, containing 21% clay and 35% silt in the dry matter (dm) was used, sampled in summer of 2016 from the Ap horizon at the Hohenschulen experimental site, Achterwehr field (54°19′05″N, 9°58′38″E), Kiel, Germany. In 2015, 1 year before sampling, last crops were a mixture of catch crops like red clover (*Trifolium pretense* L.) and alfalfa (*Medicago sativa* L.) without any fertilization. In 2014, 2 years before sampling, maize (*Zea mays* L.) was grown, with typical slurry and triple superphosphate fertilization.

Results were recalculated on a kilogram per hectare basis using the following input parameters of the field site: 10,000 $m^2 \times 0.2$ m soil depth × bulk density of 1,500 kg $m^3 = 3{\cdot}10^6$ kg dry soil ha^{-1}. The air-dried, sieved (Ø 2 mm), and homogenized soil had the following basic characteristics: pH ($CaCl_2$) = 6.0 ± 0.1, organic carbon (C_{org}) = 1.3 ± 0.1%, total N = 0.15 ± 0.01%.

Wheat straw was also obtained from the Hohenschulen experimental site (Kiel, Germany) with the following characteristics: C = 44.4 ± 0.1%, N = 0.28 ± 0.02%. Spruce sawdust was obtained in 2015 from Holz Ruser (Bornhöved, Germany): C = 45.8 ± 0.2%, total N = 0.06 ± 0.01%. Alkali lignin was obtained from VWR (Germany) C = 61.6 ± 0.1%, N = 0.43 ± 0.01%, suspended in water and sieved to obtain particulate pure lignin. Organic substrates were applied at a rate of 1.5 g C kg^{-1} soil, equivalent to a field application of 4.5 t C ha^{-1}. In total, wheat straw, spruce sawdust, and lignin treatments received 3.4, 3.3, and 2.4 g dm kg^{-1}. At the beginning of the pre-incubation period, 90% of the HCA were mixed homogeneously with the soil and 10% was buried horizontally at a soil depth of 2 cm in litter bags made of 0.2 μm nylon meshes, a soil contact area of 36 cm^2, and an average thickness of 0.25 cm after filling (Supplementary Figure S1). The particle size of the HCA applied to soil and litter bags ranged around 1 mm.

Experimental Setup

A combined application of N fertilizer and organic application was used for the wheat straw (SWF = <u>S</u>oil + <u>W</u>heat straw + <u>F</u>ertilizer), spruce sawdust (SSF = <u>S</u>oil + <u>S</u>pruce sawdust + <u>F</u>ertilizer), and lignin (SLF = <u>S</u>oil + <u>L</u>ignin + <u>F</u>ertilizer) treatments. The control treatment (S = <u>S</u>oil) did not receive any fertilizer or HCA, the fertilizer control treatment (SF = <u>S</u>oil + <u>F</u>ertilizer) received only N fertilizer, but no HCA. Each treatment consisted of three independent replicates. Custom-made stainless steel incubation columns with a height of 30 cm, a diameter of 20 cm, and a detachable, unsegmented headspace unit were constructed by the workshop of Forschungszentrum Jülich, Germany (Supplementary Figure S1). On average, 8,000 cm^3 of headspace volume were achieved after connecting the headspace unit to the column, considering individual differences of each soil column, e.g., slightly deviating soil levels. Airtightness was achieved by a silicone rubber O-seal, embedded in cut grooves of the headspace and incubation column before screwed in place. Gas-tightness of the system was checked before the experiment, using helium and a helium detector.

Each soil column contained six equally spaced sections made of polyvinyl chloride (PVC) (Supplementary Figure S1), allowing sampling in time intervals without substantial disturbance of the soil structure. Void spaces after sampling where filled with solid PVC spacers to keep the gas sampling headspace at constant volume. 8 kg of soil was mixed with or without HCA before filling equal amounts of soil in each column section. Bulk density of the soil was adjusted to the field value of 1.5 g cm^{-3} by using a special triangle-shaped tool that exactly fit into the column section. During the first 12 h of the pre-incubation period of 7 days before fertilizer application (DBF), soil was rewetted to a water volume equivalent of 40% of the water holding capacity measured in soil without HCA (WHC, 35 g H_2O 100 g^{-1} dm). Deionized H_2O was dripped onto the soil surface in steps of maximal 50 ml to re-activate soil microorganisms before adding fertilizer N. To simulate an excess of soil mineral N, ammonium sulfate $(NH_4)_2SO_4$ was applied as fertilizer with a ^{15}N content of 2.65 atom-% (corresponding to a $\delta^{15}N$ of about 6,400‰; VWR, Germany). The N fertilizer, equivalent to 50 mg N kg^{-1} soil and to 150 kg N ha^{-1}, was dissolved in 100 ml deionized H_2O to prepare a stock solution. After further dilution with deionized H_2O, the N solution was poured stepwise onto the soil surface of the fertilized treatment, finally adjusting the water content to 60% WHC. Soil substrates of the control treatment (S) only received deionized H_2O instead of N solution. Soil columns were incubated over a total of 120 days at room temperature from 11th November 2016 to 16th March 2017. Average temperatures during incubation ranged from 20.3 to 22.3°C. Soil moisture loss was monitored between the sampling dates by weighing the soil column, and gravimetrically after each sampling event. Soil moisture was kept in a range of 50 to 60% WHC by dripping deionized H_2O equivalent to 7.5 ml d^{-1} onto the soil surface of each sampling segment. As indicated in **Figure 1A**, average water contents were at a comparable level of ± 1%-dm in all treatments after application of H_2O with and without N at 7 DBF and 7 days after fertilizer application (DAF), despite anticipated effects of organic amendments on the WHC.

Soil Sampling and Analyses

Spatulas and spoons with prolonged shafts were used to sample soil equivalent to 1.3 kg dm from each column section without disturbance of the remaining ones. Seven days prior to N fertilizer application, the first soil column section was sampled as reference. All other soil samplings were conducted at 7, 21, 49, 77, and 113 DAF. Soils for chemical analyses were immediately frozen, freeze-dried and stored in plastic bags at room temperature. Litter bags with HCA were treated the same way, but additionally were cleaned by gentle brushing, before HCA decomposition was calculated by determining the weight loss between applied and recovered HCA dry matter.

Fresh soil was directly stored after sampling at −20°C for later determination of microbial biomass, and at −80°C for PCR analysis of the major N cycling genes.

Microbial biomass carbon (C_{mic}) was determined after chloroform fumigation and extraction with 0.01 M $CaCl_2$ solution as described in Reichel et al. (2017). C_{mic} was calculated using a fraction of 0.45 as extractable part of microbial biomass C (kEC). We did not apply pre-extraction of fresh soil with 0.5 M K_2SO_4 to remove the large inorganic N background as recommended by Widmer et al. (1989) or Wachendorf and Joergensen (2011), which may have confounded measurements of N retained in the microbial biomass (N_{mic}). We then decided to calculate the N_{mic}, using a calculated average C_{mic}:N_{mic} ratio of 7, which was reported for comparable agricultural soils with organic C amendments by Scheller and Joergensen (2008).

Abundance of the ammonia oxidizing bacteria (AOB) and archaea (AOA), as well as of various denitrifiers, was calculated based on marker genes quantified by real-time quantitative polymerase chain reaction (qPCR) on selected samples taken at 7, 49, and 113 DAF. For ammonia oxidizers the *amoA* gene coding for an ammonia monooxygenase was used as marker; for denitrifiers nitrate reductase (*narG*), nitrite reductases (*nirK* and *nirS*), and nitrous oxide reductase (*nosZ*) genes were determined. In short, DNA was extracted from 0.5 g of fresh soil using the NucleoSpin Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. Dilutions (1:16 v/v) of the raw extracts were used for quantification by (qPCR) with the 2X Takyon for SYBR Assay master mix (Kaneka Eurogentec S.A., Seraing, Belgium). To reduce the inhibitory effect of polyphenolic compounds co-extracted from soil, we added bovine serum albumin (BSA) to a final concentration of 0.06% to each reaction and 2.5% dimethyl sulfoxide (DMSO) to reactions involving the *nir* genes. All reactions were run in triplicate on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). The PCR program involved an initial activation step at 95°C for 3 min, followed by 39 cycles at 95°C for 10 s, primer melting temperature (T_M) for 20 s, 72°C for 45 s. T_M corresponded to 55°C for *AOA*, 60°C for *AOB*, 63°C for *narG* and *nirK*, 57°C for *nirS* and 65°C for *nosZ*. Reaction specificity was checked using a melting curve analysis. The copy number was calculated from a standard curve of serial 10-fold dilutions of plasmids containing the target gene in known concentrations (Ollivier et al., 2010). More details about the gene primer sequences of AOB-*amoA, AOA-amoA, nirS, nirK*, and *nosZ* are available in Zhang et al. (2013), and of *narG* in Bru et al. (2007). Gene copies per gram soil were normalized to C_{mic} (gene copies per μg C_{mic}).

Soil pH was determined with a pH meter (multi 340i, WTW GmbH, Weilheim, Germany) according to the ISO 10390 method (ISO, 2005) using 1 M KCl solution at a soil:solution ratio of 1:5 (w/v) and mixed for 2 h.

High organic carbon soil amendments residues of litter bags were extracted three times with 1 M KCl solution at a ratio of 1:5 (w/v), before freeze-dried HCA residues were analyzed for N content and ^{15}N isotope signature, using an isotope-ratio mass spectrometer (IRMS, Delta V plus, Thermo Fisher Scientific, Bremen, Germany). Soil NH_4^+ and NO_3^- content was determined by applying sequential micro-diffusion and liquid-liquid extraction techniques (Mulvaney et al., 1997; Huber et al., 2012): 80 ml 1 M KCl solution (the solid KCl had been pre-treated by heating for 16 h at 550°C to minimize background NH_4^+ prior to preparation of the KCl solution) was mixed with 8–9 g soil, shaken at 200 rpm for 1 h, centrifuged for 20 min at 3000 rpm, and filtrated through Whatman no. 42 filter paper with 2–3 μm pore size. 60 ml of the KCl extract was transferred to 100-ml polypropylene (PP) bottles, and the pH was adjusted to about 12, using 1 M NaOH solution, to convert NH_4^+ to NH_3. NH_3 was allowed to volatilize at room temperature for 7 days and was collected with 2 × 15 μl of saturated oxalic acid pipetted onto quartz glass filter disks. Afterward, the disks were transferred to a desiccator and dried over silica gel for 24 h. Then, the dry filter disks were packed in tin (Sn) capsules and analyzed using an elemental analyzer coupled to an IRMS (EA-IRMS, Flash EA 2000 and Delta V Plus; Thermo Fisher Scientific, Bremen, Germany). The remaining KCl extract was dried at 65°C, re-dissolved in 3 ml of 1 M NaOH, and mixed with 37 ml acetone for 30 s. After centrifugation at 3,000 rpm for 20 min, the acetone supernatant containing most of the NO_3^- was transferred into glass beakers, dried at 30–40°C, re-dissolved in 5 ml of deionized water, freeze-dried, and finally transferred into Sn capsules for EA-IRMS analysis (Zhu et al., 2013). Nitrite was monitored as secondary parameter, using an improved colorimetric method according to Colman (2010) and Homyak et al. (2015). A DU-800 spectrophotometer (Beckman Coulter, Fullerton, United States) at a wavelength of 560 nm was used for measurement.

Gas Sampling and Analysis

CO_2 and N_2O emission was measured on average two to three times a week, using PVC chambers 20 cm in height and 20 cm in diameter, with a gas sample port with septum and a 100 cm long vent tube with 0.5 cm in diameter, equipped with an air-tight clamp. PVC headspace units were installed gas-tight on top of the column before gas sampling (Supplementary Figure S1). After mounting the headspace unit, a gas volume of 35 ml was sampled with a syringe through the septum of the sampling port at 0, 20, 40, and 60 min between 7 DBF and 49 DAF, increasing the interval to a maximum of 240 min at 113 DAF. The clamp on the inlet tube was opened to allow pressure equilibration during each gas sampling. These intrusions of ambient air were considered before calculating the gas emission (Equation 1). The gas samples were transferred completely to 22.5-ml pre-evacuated gas chromatography vials, thereby creating an overpressure for a proper processing by the GC autosampler during analysis. N_2O and CO_2 analyses were performed with a gas chromatograph, equipped with an electron capture detector and a flame ionization detector (GC-ECD/FID, Clarus 580, PerkinElmer, Rodgau, Germany). CO_2 and N_2O emission was calculated from the increase of corrected gas concentrations as follows:

$$F = \frac{slope\,(C_1 : C_4, t_1 : t_4) \times V \times K \times M}{m \times (K + T) \times Vm} \qquad (1)$$

F: gas emission flux; *slope*: change of gas concentration per unit time, gas concentrations one to four (C_1:C_4) in ppmv for CO_2 and ppbv for N_2O at sampling time interval one to four (t_1:t_4) in hours; *V*: headspace volume in liter; *m*: amount of soil in gram dry weight; *Vm*: molar volume of ideal gases (22.414 l at 0°C and 101.325 kPa), corrected for the gas sample temperature using *K* (273.15 Kelvin) and *T* (air temperature in °C); *M*: molar mass of N in N_2O or C in CO_2, respectively.

Total global warming potential (GWP) was calculated as the sum of direct CO_2-C emissions and CO_2-C equivalents of N_2O emissions, using a factor of 265 for the conversion of N_2O in CO_2 equivalents (IPCC, 2014), divided by 28 (atomic mass of the two nitrogen atoms of N_2O) and multiplied by 12 (atomic mass of one carbon atom) to convert 1 kg N_2O-N into 1 kg CO_2-C equivalents.

Statistical Analysis

We used three independent replicates, i.e., separate incubation columns, for each of the treatments (S, SF, SSF, SWF, and SLF) (Supplementary Figure S1). Furthermore, each soil column contained six equally spaced, independent sections, which allowed separate soil samplings from each column at incubation time 7 DBF and 7, 21, 49, 77, and 113 DAF (Supplementary Figure S1). Since $n = 3$ is low to reliably test normal distribution and inhomogeneity of variance, we decided to test for significant differences between treatments at certain sampling times by using an analysis of variance (ANOVA) with the Tukey-B or Games-Howell *post hoc* test, which is less susceptible to inhomogeneous variances and non-normality (Janssen and Laatz, 2010). The significance threshold value for the comparisons was set at $p = 0.05$. Statistical software used was Origin 2015 (Originlab Corporation, Wellesley Hills, MA, United States) and SPSS Statistics 20.0 (IBM Deutschland GmbH, Ehningen, Germany).

RESULTS

Soil Physical Parameters

Water Content

Watering caused fluctuations of the gravimetric soil moisture within a range of ± 2.5% (**Figure 1A**). The lignin treatment (SLF) tended to lower H_2O content in relation to all other treatments. There was only one significant difference between the SLF and SWF treatment at 113 DAF.

pH

Relative to the control treatment, the pH of other treatments slightly, but significantly decreased after fertilization with NH_4^+ (**Figure 1B**). In soil with wheat straw (SWF), the decrease in pH after mineral N fertilization was less pronounced compared to all other treatments.

High Carbon Soil Amendment Decomposition and Fate of N

HCA Decomposition

Overall, only HCA of the SSF and SWF treatments showed a clear decomposition trend (**Figure 2A**). The rate of wheat straw decomposition was characterized by three phases: 0.9% dry mass loss d^{-1} (0–21 DAF), 0.3% dm loss d^{-1} (21–77 DAF), and 0.04% dm loss d^{-1} for the remaining incubation period. At the end of the experiment, 70% of the initial wheat straw dry mass was decomposed. In contrast, no clear decomposition trend occurred in the SSF treatment until 49 DAF. While the lignin treatment showed no change in degradation, the spruce sawdust decomposition after 49 DAF proceeded linearly at a rate of 0.3% d^{-1}. Spruce sawdust showed a total decomposition around 30% dm in the end.

N Immobilization by HCA Residues

Only N that was not extractable by a combination of 1 M KCl solution and two H_2O washing steps was considered. The application of wheat straw added 28 kg N ha^{-1} to the soil. The remaining wheat straw residues became further enriched in N during the incubation period after mineral N fertilization, particularly during the period with the fastest decomposition until 21 DAF. N in wheat straw residues increased to 53 kg N ha^{-1}, retaining an extra of 25 kg N ha^{-1} (**Figure 2B**).

In contrast, only 7 kg N ha^{-1} were added with the spruce sawdust to the soil, thus about four times less compared to wheat straw. The N content associated with spruce sawdust residues increased the most between 21 and 77 DAF to 30 kg N ha^{-1}, reaching a plateau afterward. Despite a slower and 40% lower degree of decomposition compared to wheat straw, N enrichment of the spruce sawdust residues reached comparable levels, retaining an extra amount of 23 kg N ha^{-1} at 77 DAF and likely also at 113 DAF, but variability impeded a significant differentiation from the SLF treatment at this incubation time. Hence, spruce sawdust and wheat straw both helped to immobilize similar N amounts (on average 24 kg N ha^{-1}). Due to the higher N content of the technically processed lignin, application initially added 15 kg N ha^{-1} to the soil, containing more N in HCA compared to the SSF treatment at 7 DBF and 7 DAF. N enrichment of lignin was low and not significant over the experimental period, ranging at 1 kg N ha^{-1}.

δ^{15}N of HCA

δ^{15}N values were determined in the same washed HCA-fractions (**Figure 2C**). The original δ^{15}N values of HCA were 8.0‰ vs. air-N_2 for the synthetic lignin, 3.2‰ for spruce sawdust, and 3.1‰ for wheat straw. The δ^{15}N values of the wheat straw and the spruce sawdust increased to over 2,000‰ until 21 DAF. The δ^{15}N of lignin, which showed only weak decomposition and N dynamics, was only enriched to maximum values of about 300‰ over the course of the incubation experiment.

HCA-Induced Changes in the Soil Microbiome

N Immobilization by the Microbial Biomass

Microbial N retention per hectare and microbial biomass values (kg C_{mic} ha^{-1}) are shown in **Figures 1E**, **2D**, respectively. Amending soil with wheat straw (SWF) instantaneously increased the microbial biomass by 309 kg C_{mic} ha^{-1} (corresponding to 42 kg N_{mic} ha^{-1}) relative to the S treatment without mineral N or HCA at 7 DBF. Generally, adding

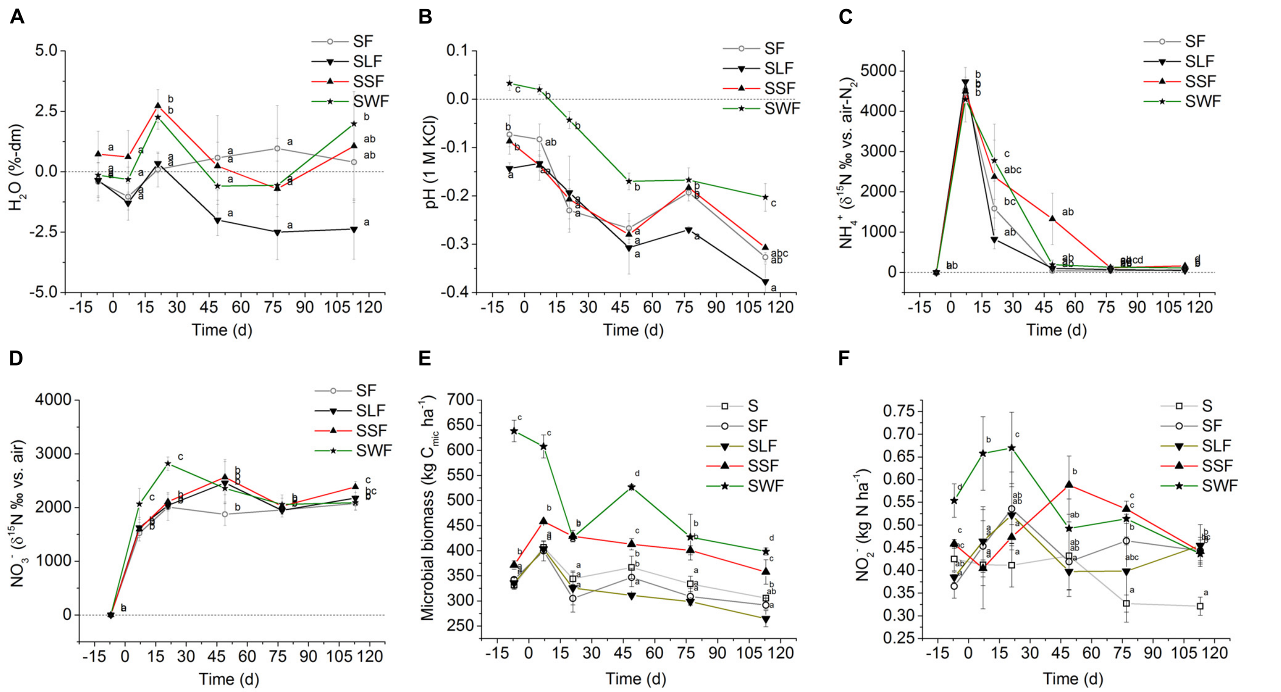

FIGURE 1 | (A) H_2O dynamics (%-dm, dry soil), **(B)** pH development (1 M KCl soil extract), **(C)** $^{15}NH_4^+$ ($\delta^{15}N$ ‰ vs. air-N_2), and **(D)** $^{15}NO_3^-$ ($\delta^{15}N$ ‰ vs. air-N_2) in soil of the fertilizer control treatment (SF, open circle) with mineral N fertilizer, and the HCA treatments with mineral N fertilizer plus lignin (SLF, solid triangle downward), spruce sawdust (SSF, solid triangle upward), or wheat straw (SWF, solid star symbol) relative to the control treatment (S, $y = 0$) without any fertilizer or HCA. **(E)** microbial biomass C (kg C_{mic} ha^{-1}) and **(F)** nitrite concentration (NO_2^- μg N ha^{-1}) in soil of the control treatment (S, open square) without any fertilizer or HCA, the fertilizer control treatment (SF, open circle) with mineral N fertilizer, and the HCA treatments with mineral N fertilizer plus lignin (SLF, solid triangle downward), spruce sawdust (SSF, solid triangle upward), or wheat straw (SWF, solid star symbol). Incubation time $d = 0$ divides the experiment into a period before (–7 DBF) and after (7, 21, 49, 77, and 113 DAF) mineral N fertilization. Standard deviations of mean values ($n = 3$) are displayed. Statistically significant differences between treatments at a certain incubation time are depicted by different lowercase letters next to the symbols ($p < 0.05$).

H_2O without or with mineral N significantly increased most C_{mic} values, except in soil with wheat straw. C_{mic} values of all treatments decreased over the course of the incubation experiment (**Figure 1E**). In the end, C_{mic} values of the SSF and SWF treatment still remained larger than in all other treatments. Starting from 7 DAF, the rapid increase in C_{mic} after wheat straw application was followed by a decrease. Between 7 DBF and 21 DAF, microbially immobilized N in the SWF treatment was released at a rate of 1.6 kg N ha^{-1} d^{-1}. Afterward, microbial N immobilization showed a second maximum at 49 DAF with an immobilization of 26 kg N ha^{-1} relative to the SF treatment (**Figure 2D**). This again was followed by a release of N from the microbial biomass at a rate of 0.5 kg N ha^{-1} d^{-1}. Relative to the SF treatment, microbially immobilized N in the SWF treatment finally amounted to 15 kg N ha^{-1} (113 DAF). About 8 kg N ha^{-1} were immobilized by the microbial biomass of the SSF treatment relative to the SF treatment at 7 DAF. Afterward, microbial N release also occurred in the SSF treatment, but at a low rate of 0.3 kg N ha^{-1} d^{-1} compared to the SWF treatment (**Figure 2D**). After 4 months of incubation, spruce sawdust still had immobilized about 9 kg N ha^{-1} in the HCA-derived microbial biomass relative to the SF treatment, which was significantly lower than in the SWF treatment, but clearly larger compared to the lignin (SLF) treatment (**Figure 2D**).

Influence of HCA on the Abundance of Nitrifiers and Denitrifiers

N application to soil without (SF) or with litter (SSF, SWF, and SLF) induced specific changes in the abundances of AOA and AOB as well as of denitrifiers (*narG*, *nirK/S*, and *nosZ*) relative to C_{mic} (**Figure 3**). N application to soil without HCA (SF) and with spruce sawdust (SSF) significantly increased the abundance of *AOA* and *AOB* at 7 DAF. Wheat straw of the SWF treatment significantly lowered the abundance of *AOA* and *AOB* at 7 and 49 DAF, relative to the unfertilized control (S). To some extent, the SF, SSF, and SLF treatment also lowered the *AOA* abundances, but only at incubation time 49 DAF. Also the abundance of denitrifers harboring the nirS gene was significantly lower in the SWF compared to the SF treatment. Wheat straw and lignin significantly lowered the abundance of denitrifiers harboring the *nosZ* genes compared to the SF and especially to the SSF treatment at 49 DAF. Trends on the abundance of nitrifiers and denitrifiers were identified also in soil of the SF and SLF treatment, but showed no statistical significance compared to the S treatment. All analyzed functional groups returned to the level of the S treatment until 113 DAF, with the exception of the SLF treatment (**Figure 3**, 113 DAF). Water content and pH are strong drivers of C_{mic} development, which likely decreased the functional gene abundances in the SLF treatment less than the

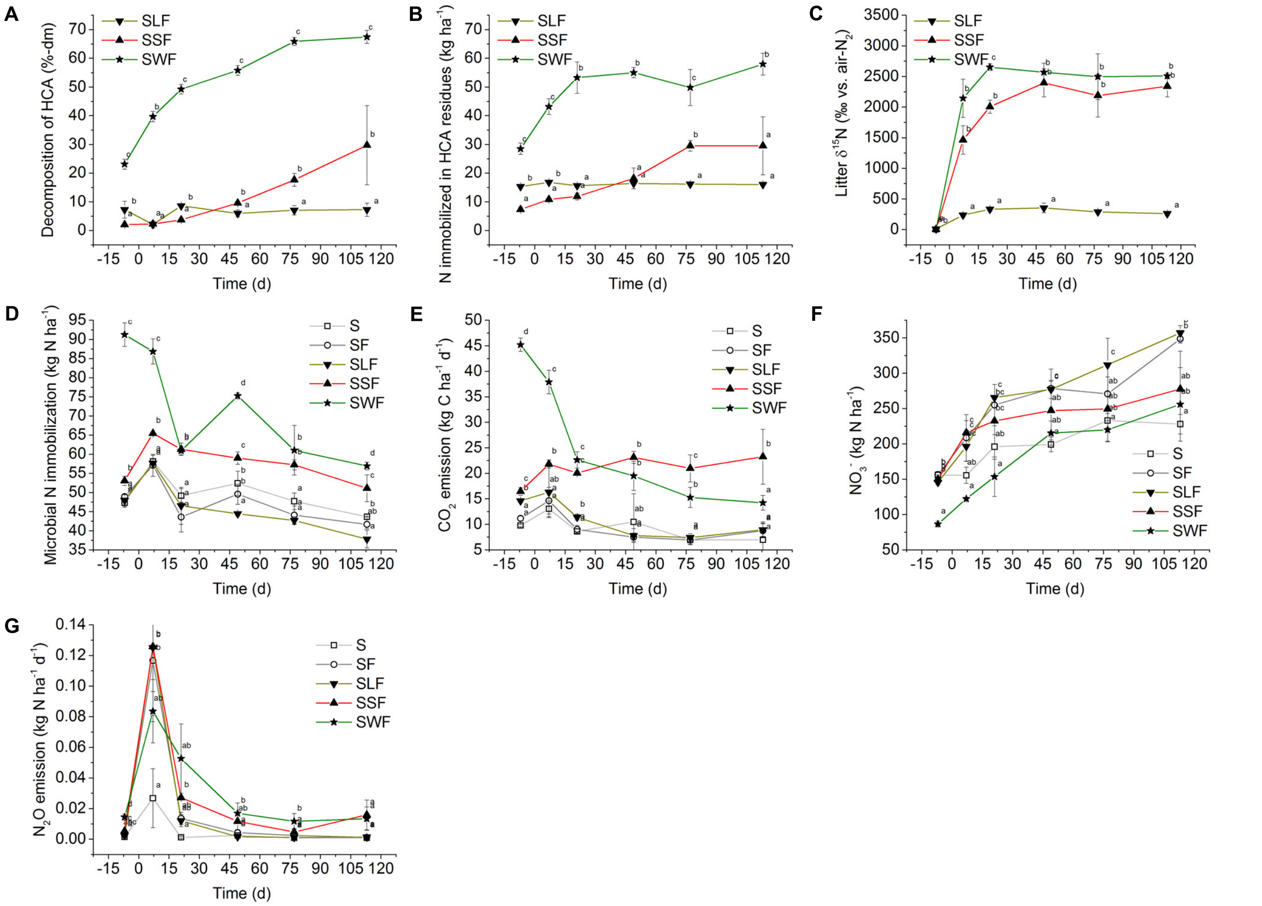

FIGURE 2 | (A) HCA decomposition (% loss of initially applied HCA dry matter), **(B)** physical and/or chemical N immobilization by the remaining HCA residues in kg N ha^{-1} and **(C)** δ^{15}N values in ‰ vs. air-N_2 in soil treated with mineral N and lignin (SLF, solid triangle downward), spruce sawdust (SSF, solid triangle upward), and wheat straw (SWF, solid star symbol). **(D)** Microbial N immobilization (kg N ha^{-1}), **(E)** CO_2 emission 24 h before soil sampling in kg C ha^{-1} d^{-1}, **(F)** NO_3^- concentration in kg N ha^{-1}, and **(G)** N_2O emission in kg N ha^{-1} d^{-1} in soil of the control treatment (S, open square) without any fertilizer or HCA, the fertilizer control treatment (SF, open circle) with mineral N fertilizer, and the HCA treatments with mineral N fertilizer plus lignin (SLF, solid triangle downward), spruce sawdust (SSF, solid triangle upward), or wheat straw (SWF, solid star symbol). Incubation time $d = 0$ divides the experiment into a period before (–7 DBF) and after (7, 21, 49, 77, and 113 DAF) mineral N fertilization. Standard deviations of mean values ($n = 3$) are displayed. Statistically significant differences between treatments at a certain incubation time are depicted by different lowercase letters next to the symbols ($p < 0.05$).

abundance (C_{mic}) of other microorganisms without those genes (**Figures 1A,B,E**).

Effect of HCA on CO_2 and N_2O Emissions and on Soil NO_3^-

CO_2 Emissions

CO_2 emissions were calculated for a time interval of 24 h before soil sampling (**Figure 2E** and Supplementary Figure S2). Additionally, the cumulative CO_2 emissions were calculated by integration of the area below the curve within the time interval between 7 DBF and 113 DAF (Supplementary Figure S2). During the phase with the largest wheat straw decomposition rates (**Figure 2A**), significantly larger CO_2 emissions were found in the SWF treatment compared to all other treatments. After N application, CO_2 emissions decreased until 113 DAF, which was in accordance with the microbial biomass development of the SWF treatment (**Figure 1E**). CO_2 emissions from the SSF soil showed a delayed increase at 7 DAF and then remained at a comparable level over the entire incubation period. This is in contrast to the SWF treatment, which later significantly decreased to a lower CO_2 emission rate comparable to the S treatment at 113 DAF. CO_2 emitted from N-fertilized soil without HCA and with lignin did not differ from the unfertilized control treatment (S), reflecting a low microbial activity in these treatments. Consequently, cumulative CO_2 emissions were significantly larger in the SSF and SWF treatment compared to the SF and SLF treatment. Soil of the SF treatment showed a slightly negative CO_2 budget of –35 kg CO_2-C ha^{-1} compared to the S treatment. Cumulative CO_2 emissions of the SLF treatment amounted to 202 kg CO_2-C ha^{-1}, in the SSF treatment to 1,613 kg CO_2-C ha^{-1}, and in the SWF treatment to 1,822 kg CO_2-C ha^{-1} relative to the S treatment.

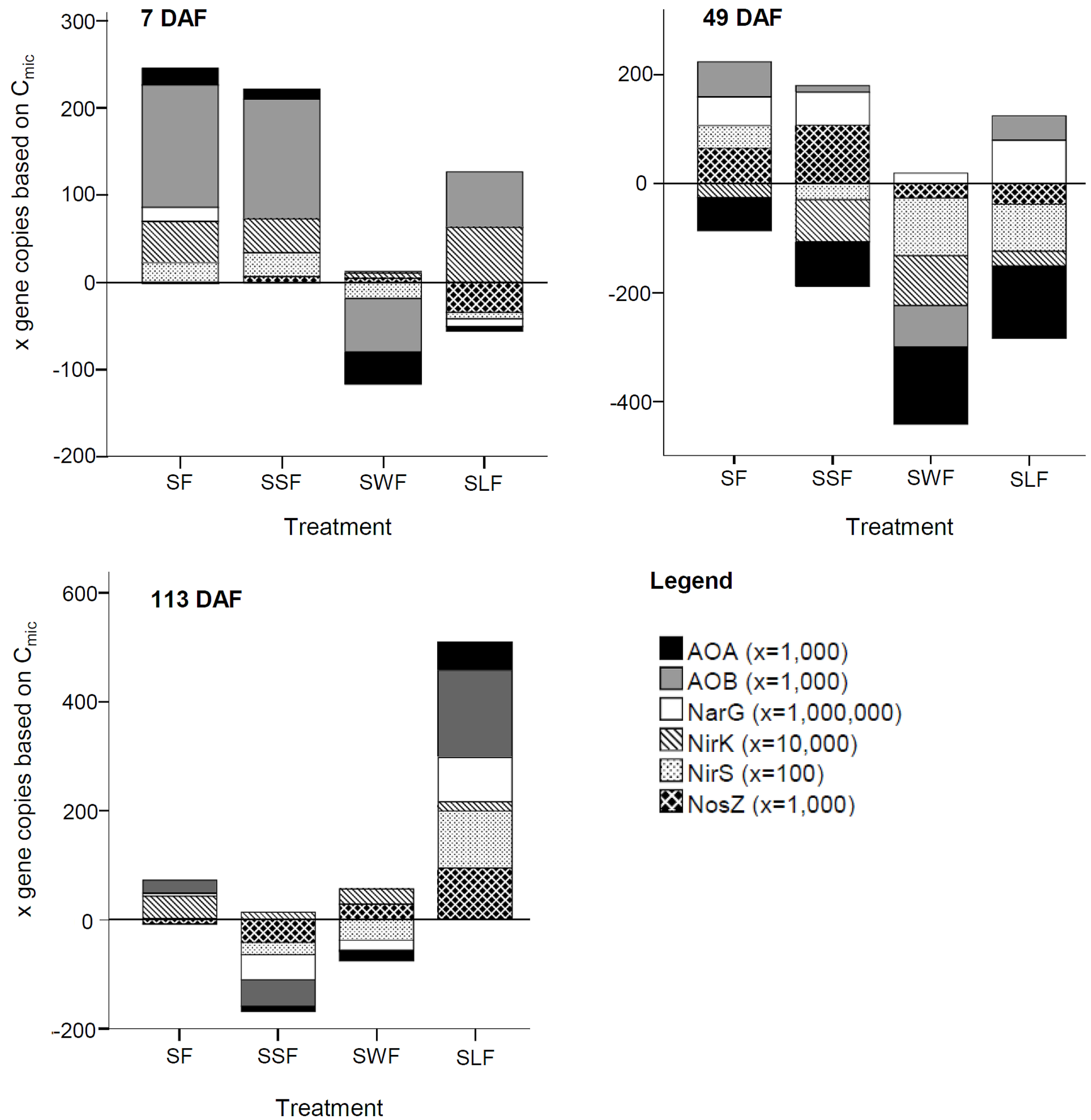

FIGURE 3 | Influence of HCA on the abundance of the ammonia oxidizing bacteria (AOB) and archaea (AOA), as well as of various denitrifiers (NarG, nirK, nirS, and NosZ) at 7, 49, and 113 days after mineral N fertilization (DAF) in soil with only mineral N fertilizer (SF), with N fertilizer plus lignin (SLF), spruce sawdust (SSF), or wheat straw (SWF) relative to the control treatment S ($y = 0$) without any N fertilization or HCA application. Gene copies per gram soil were normalized to C_{mic} (gene copies per µg C_{mic}). Multiplication factors of gene copies per µg C_{mic} are available in the according brackets next to the gene symbol and name. Statistical significances are described in the Section "HCA-Induced Changes in the Soil Microbiome" of the manuscript.

NO_3^- Concentrations

Nitrification was the main source of NO_3^- after application of ^{15}N-fertilizer as indicated by a transfer of ^{15}N signal from NH_4^+ to NO_3^- (**Figures 1C,D**). Agricultural soil obtained from the experimental site had already initially a large NO_3^--N background of 156 kg N ha^{-1} at 7 DBF (S treatment, **Figure 2F**). Application of wheat straw instantaneously and significantly reduced the soil NO_3^- content by 69 kg N ha^{-1} at 7 DBF compared to the S treatment. Even after addition of extra mineral N of 150 kg N ha^{-1}, NO_3^- content in soil with wheat straw remained at a level of the S treatment without additional HCA or mineral N fertilization. Also spruce sawdust application tended to lower the NO_3^- content in soil between 21 and 113 DAF, but to a lower extent compared to wheat straw. Lignin application did not reduce the NO_3^- content and ranged around the same level as the SF treatment. During incubation, NO_3^- content tended to increase in all treatments at the following rates: 0.7 (S), 1.3 (SF), 1.6 (SLF), 0.8 (SSF), 1.4 kg N ha^{-1} d^{-1} (SWF).

N_2O Emissions

Like CO_2 emissions, also N_2O emissions were calculated for a 24-h time interval before soil sampling (**Figure 2G** and Supplementary Figure S2). Large N_2O emissions occurred in all treatments after addition of NH_4^+-N as indicated at 7 DAF

(**Figure 2D**). N_2O emission tended to be lower in the SWF treatment at this time point compared to the SF, SLF and SSF treatments, which is in line with the rapid initial microbial N immobilization, especially in the SWF treatment. N_2O emission patterns seemed to be influenced by the type of HCA, with a slight tendency to higher N_2O emissions in the SSF and SWF treatment from 21 DAF onward. Nonetheless, N_2O emissions dropped steeply at 21 DAF in most N-fertilized treatments, but with a delay in the SWF treatment. Cumulative N lost via N_2O emissions from N-fertilized soil (SF) was 1.5 kg N ha^{-1} higher compared to the S treatment. The SWF and SLF treatment had both a cumulative N_2O loss of 3.0 kg N ha^{-1}, while the SSF treatment lost 4.0 kg N ha^{-1} in the form of N_2O compared to the S treatment. In relation to the S treatment, the overall GWP (CO_2+N_2O) of soils with N fertilization (SF) or with additional HCA increased in the following order: 140 (SF) $<$ 545 (SLF) $<<$ 2,060 (SSF) $<$ 2,162 (SWF) kg CO_2-C equivalents ha^{-1}.

DISCUSSION

HCA-Derived N Immobilization

In our experiment, wheat straw, applied at a rate of 4.5 t C ha^{-1}, increased N uptake by the microbial biomass up to 42 kg N ha^{-1} during a short period of time, before releasing two thirds of this amount again within the following 4 months of incubation at room temperature (**Figure 2D**). Such rapid N immobilization can also occur under field conditions after incorporation of organic C-rich crop residues (Congreves et al., 2013). Hence, HCA might be suitable to retain mineral N quickly in times of maximum mineral N availability, with a potential of dosed N release to germinating crops applied together with HCA. Some plants, such as cereal rye (*Secale cereale* L.), root down to a soil depth of 15 cm within 4 weeks after sowing, effectively reducing the mineral N loss (Brinsfield and Staver, 1991; Staver and Brinsfield, 1998) after its release from the microbial biomass. In contrast, winter wheat N uptake (30 kg N ha^{-1}) is rather low during the winter season (Henke et al., 2008), but might be large enough to buffer the release of approximately 27 kg N ha^{-1} from the wheat straw-derived microbial biomass during 4 months and under conditions optimal for microbial activity.

Our data also indicates that different types of HCA, such as wheat straw and spruce sawdust, have the potential to retain 15 and 9 kg N ha^{-1} for more than 4 months, which, together with the N uptake of winter crops, might compensate N surpluses typically found in post-harvest fields (Döhler, 2009). In the medium term, N immobilized by microbial biomass may reduce N fertilizer demand in the following growing season, increasing the NUE of crop production. However, this requires that the N retained by the HCA is also released at the appropriate time and rate in order to transfer N to the next crop, which might be not easy to achieve under realistic field conditions as indicated by reduced yield and N uptake in the subsequent crop season and consequently lower profit margins (Thomsen and Christensen, 1998; Chaves et al., 2007, Congreves et al., 2013). Similar to the recommendations for appropriate mineral N application, choosing the right composition, amount, placement, timing (Bindraban et al., 2015), and the right particle size (Angers and Recous, 1997) may improve the applicability of HCA to retain N in soil until the next growth season. Hence, the N immobilization-release dynamics of this study might differ from field conditions in view of the microbially favorable conditions applied in this study (60% WHC, room temperature), but might be at least partly reached in the field by using a comparably small HCA particle size (Angers and Recous, 1997) like in our study, or by decreasing the depth of HCA incorporation into the soil (Christensen, 1986) in order to enhance microbial activities.

A fast N release from the wheat straw-derived microbial biomass might be related to the zymogenous microbial biomass as pioneers of the microbial wheat straw decomposer succession (Bastian et al., 2009) with rather short half-life of a few days (Cochran et al., 1988), and a rapid decay after exhaustion of easily accessible HCA components. This decay appears to have initiated a second microbial N immobilization maximum at 49 DAF (**Figure 2D**). In contrast, no such N immobilization-release dynamics were observed after application of spruce sawdust, which showed a more stable microbial N immobilization at 7 DAF and afterward (**Figure 2D**). Hence, microbial N release in soil with wheat straw could be combined with the spruce sawdust-derived microbial N immobilization. A combination of HCA with similar organic composition such as wheat straw and spruce sawdust might help to mitigate the fast microbial N re-mineralization after wheat straw application. Furthermore, adding HCA with more recalcitrant organic composition such as spruce sawdust and lignin potentially improves global agricultural C sequestration in soil.

In our experiment, microbial N immobilization-release dynamics (**Figure 2D**) were also paralleled by physical and / or chemical sequestration of about 24 kg N ha^{-1} in the remaining fraction of the wheat straw and spruce (**Figure 2B**). Also lignin was reported as matrix of chemical N fixation (Wei et al., 2017), particularly if reactive N forms like nitrite are present as demonstrated also in our study (**Figure 1F**). However, no significant N sequestration was found in soil with pure lignin application (**Figure 2B**). We assume that chemical N fixation by lignin is coupled to HCA-derived microbial dynamics. Miltner et al. (2012) provide strong evidence that disintegrating microbial populations are a relevant source of non-living soil organic matter. In accordance, we found indicators that a fast initial microbial growth based on easily available carbon derived from wheat straw was followed by such a rapid dieback of at least part of the microbial population. N derived from dead microorganisms, which does not directly serve as substrate for other microorganisms in the decomposer succession (Bastian et al., 2009), might be further transformed and fixed to recalcitrant organic molecules (Miyajima, 2015; Wei et al., 2017). Hence, N retained physically and / or chemically in the HCA matrix might be less available in the medium term, but incorporation of straw can improve crop yield and plant N uptake in the long term (Malhi et al., 2011). Overall, wheat straw (39 kg N ha^{-1}) and spruce sawdust (33 kg N ha^{-1}) induced microbial, physical and / or chemical N immobilization at field-relevant timescale and quantity, with clear potential to improve agricultural NUE and N retention capacity.

Impact of HCA on Greenhouse Gas Release and Nitrate

Decomposition of HCA applied in our experiment, as prerequisite for microbial N immobilization, increased CO_2 emission from soil over the runtime of the experiment (**Figure 2E**). Inversely to the HCA-derived CO_2 emission, temporarily more C remained sequestered in soil with increasing recalcitrance of HCA to microbial decomposition. This makes spruce sawdust and lignin attractive to implement the recommendation of the French Ministry of Agriculture (2015), which aims to increase soil carbon stocks yearly by 4‰, helping to mitigate the adverse climatic influences of anthropogenic CO_2 emissions. Mixed applications of HCA types such as wheat straw and spruce sawdust may be of benefit in order improve the temporal C sequestration and in parallel also the NUE. In 2010, global agricultural application on average reached a NUE of 0.42, but an increase by more than 60% (NUE of 0.68) is needed to secure the same food status for 9.1 billion people as assumed for the year 2050 (Zhang et al., 2015). Managing NO_3^- seems particularly promising to improve NUE in crop production and N retention capacity, particularly in crop rotations with large potential N leaching losses of 44–73 kg N ha^{-1}, such as winter crops following oilseed rape (Sieling and Kage, 2006). Our experiment showed a promising potential of wheat straw to instantaneously reduce the NO_3^- concentration by 69 kg N ha^{-1} in soil with high mineral N background (**Figure 2F**), providing sufficient availability of C and N as a requirement of microbial growth and N immobilization (Fontaine et al., 2004). Application of HCA also showed a potential to mitigate NO_3^- losses in cropping systems (Congreves et al., 2013), however, previously retained N appears to be lost at a later stage, e.g., in the following post-harvest season, from sandy loam soil with annual average precipitation and temperature of 862 mm and 7.7°C, respectively (Christensen, 1996; Thomsen and Christensen, 1998).

Despite continuous NO_3^- release of re-mineralized microbial N during the entire incubation, wheat straw, and less pronounced and delayed also spruce sawdust, tended to reduce soil NO_3^- content at a field-relevant time scale. The observed NO_3^- mitigation might be a result of outcompeting nitrifiers and denitrifiers by zymogenous N immobilizers (Burger and Jackson, 2003), as indicated by our data (**Figure 3**). Hence, HCA such as wheat straw and spruce sawdust with high C content have the potential to mitigate NO_3^- losses if applied to soil in the right amount and under conditions suitable for large microbial competition for mineral N.

Another major source of critical N losses in agriculture with negative impact on NUE and climate change is the emission of N_2O (Smith, 2017). In our experiment, spruce sawdust and wheat straw, as promising substrates for N immobilization, did not decrease N_2O emission (**Figure 2G**). Most of the N_2O emission was associated with the mineral NH_4^+ excess directly after fertilization. Ammonia oxidation, as part of nitrification, likely was the main source of N_2O emissions in our well-aerated soil (Mathieu et al., 2006). Transfer of the ^{15}N signal from the fraction of NH_4^+ to NO_3^- (**Figures 1C,D**) also underlines the importance of nitrification for the observed N_2O emissions after fertilization with NH_4^+, which as an autotrophic process is fairly independent from the type of organic carbon amendment. Also nitrification after mineralization of N-rich crops such as lettuce can cause large N_2O emissions of 1.1 kg N ha^{-1} within a short period of time (Baggs et al., 2000). NO_3^- as potential substrate for denitrification was not limited during the entire incubation period and obviously had no relevant influence on N_2O emissions from our well-aerated soil. Nonetheless, remaining NO_3^- might be problematic in the winter season under field conditions (Kaiser and Ruser, 2000), particularly in almost water-saturated soils after fertilization, after incorporation of crop residues, but also after weather-induced pulses of N mineralization (Liang et al., 2016).

Microbial N turnover occurs in soils particularly during times of microbial biomass decay, providing mineralized N to nearby nitrifiers and denitrifiers. Thus, only HCA with high C availability, such as wheat straw, temporarily tend to reduce nitrification-derived N_2O emissions (**Figure 2G**) by increasing the microbial competition of N_2O-producers with fast growing microorganisms with large N immobilization rates (Burger and Jackson, 2003). Nonetheless, our data shows that this only leads to an increase of N_2O emission at a later stage, when part of the HCA-derived microbial biomass becomes mineralized again.

A complete denitrification of N_2O to N_2 might be another option to reduce problematic N_2O emissions (Zhang et al., 2013). In the mid-term, soil with spruce sawdust induced a larger abundance of denitrifiers harboring the *nosZ* gene, which codes the transformation of N_2O to N_2, however, not early enough to mitigate the initial N_2O peak. Hence, soil amended with spruce sawdust emitted more N in form of N_2O compared to all other soil treatments in our study, resulting in an overall GWP of CO_2 and N_2O emissions from soils with spruce sawdust comparable to wheat straw, which in relation released more CO_2, but less N_2O. We assume that HCA, such as wheat straw and sawdust, do not have a great potential to mitigate N_2O emission at a field-relevant scale, which is in line with previous findings (Frimpong and Baggs, 2010).

CONCLUSION

We hypothesized that HCA such as wheat straw, spruce sawdust, and lignin have the potential to manage temporal N excess by inducing microbial growth at a field-relevant scale. We conclude that HCA based on wheat straw or similar material can be particularly effective in buffering an excess of N at a field-relevant scale in a short period of time (days), as often reported for post-harvest sugar beet and oilseed rape fields. N immobilization benefits might be optimized by combining HCA with properties similar to wheat straw and spruce sawdust. In our experiment, wheat straw and spruce sawdust were capable of retaining N long enough within the microbial and HCA residue pool to serve as additional N supply for the following crop growth next spring. A prerequisite of N immobilization is microbial activation by HCA, which cannot be induced by applying very recalcitrant HCA types such as lignin. Furthermore, we hypothesize that HCA mitigates N losses in form of NO_3^-. Application of HCA such as wheat straw and spruce sawdust to mineral N-rich soil temporarily increases CO_2 and N_2O emissions, which were, however, counterbalanced by advantages such as N immobilization and mitigation of the NO_3^- loss potential. We conclude that HCA applications can be part of a whole set of novel agricultural cultivation strategies to improve NUE of agricultural production, without losing agricultural productivity and sustainability. However, further research is needed to optimize HCA amount, composition, and particle size for particular crop rotations and N fertilization regimes to ensure appropriate immobilization and re-release of N at the right time and at the required rate.

AUTHOR CONTRIBUTIONS

RR and NB wrote the manuscript. RR, JW, and NB designed the study. RR, MI, and JW performed the sampling. MI installed and maintained the experiment. RR provided information and performed the microbial biomass measurement. RR, JW, and NB calculated and reviewed the greenhouse gas data. HW performed the N liquid-liquid extractions and the $\delta^{15}N$ measurements. CS performed the quantitative real time PCR on the abundance of nitrifiers and denitrifiers. CS, PS, and MS reviewed the real time PCR results and the manuscript. RR, JW, MI, CS, HW, PS, MS, and NB revised the manuscript.

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Precrop Functional Group Identity Affects Yield of Winter Barley but Less so High Carbon Amendments in a Mesocosm Experiment

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Nitrate leaching is a pressing environmental problem in intensive agriculture. Especially after the crop harvest, leaching risk is greatest due to decomposing plant residues, and low plant nutrient uptake and evapotranspiration. The specific crop also matters: grain legumes and canola commonly result in more leftover N than the following winter crop can take up before spring. Addition of a high carbon amendment (HCA) could potentially immobilize N after harvest. We set up a 2-year mesocosm experiment to test the effects of N fertilization (40 or 160 kg N/ha), HCA addition (no HCA, wheat straw, or sawdust), and precrop plant functional group identity on winter barley yield and soil C/N ratio. Four spring precrops were sown before winter barley (white lupine, faba bean, spring canola, spring barley), which were selected based on a functional group approach (colonization by arbuscular mycorrhizal fungi [AMF] and/or N_2-fixing bacteria). We also measured a subset of faba bean and spring barley for leaching over winter after harvest. As expected, N fertilization had the largest effect on winter barley yield, but precrop functional identity also significantly affected the outcome. The non-AMF precrops white lupine and canola had on average a positive effect on yield compared to the AMF precrops spring barley and faba bean under high N (23% increase). Under low N, we found only a small precrop effect. Sawdust significantly reduced the yield compared to the control or wheat straw under either N level. HCAs reduced nitrate leaching over winter, but only when faba bean was sown as a precrop. In our setup, short-term immobilization of N by HCA addition after harvest seems difficult to achieve. However, other effects such as an increase in SOM or nutrient retention could play a positive role in the long term. Contrary to the commonly found positive effect of AMF colonization, winter barley showed a greater yield when it followed a non-AMF precrop under high fertilization. This could be due to shifts of the agricultural AMF community toward parasitism.

Keywords: crop rotation, arbuscular mycorrhizal fungi, rhizobia, barley, high carbon amendment, immobilization, plant functional group, nitrate leaching

INTRODUCTION

An ever-increasing yield is necessary to feed the growing world population, but this is coupled with high fertilizer use and associated environmental problems (Matson, 1997). Nitrate leaching is one of these problems, especially in intensive agriculture (Nixon and European Environment Agency, 2003), leading to multiple negative effects such as eutrophication of surface waters, or pollution of drinking water with consequences to human health (Di and Cameron, 2002). In temperate agroecosystems, the most crucial time point for leaching to occur is in the fall and winter, when crop residues decompose, and plant nitrogen uptake and evapotranspiration is low (Di and Cameron, 2002). Certain management practices to avoid nitrate leaching at this time point have been tested, such as addition of a substrate with a high C/N ratio (high carbon amendment; HCA) in an attempt to immobilize the nitrate microbially. The rationale behind this is that microbes in soils are commonly C-limited (Kallenbach and Grandy, 2011; Farrell et al., 2014), and by adding easily available carbon the microbes will take up the excess carbon and simultaneously immobilize excess mineral nitrogen. The advantage of this concept has been tested various times already with mixed success in mechanistic incubation studies (Zavalloni et al., 2011; Congreves et al., 2013a) and field studies (Thomsen and Christensen, 1998; Vidal and López, 2005; Burke et al., 2013; Congreves et al., 2013b; Török et al., 2014), for both agricultural and restoration purposes. However, due to the complexity of soil nitrogen dynamics, it is not clear whether remineralization of the immobilized N takes place the following spring, thus bridging the high leaching risk period in fall/winter and providing nitrogen when plant uptake is high (Chaves et al., 2007).

The amount of nitrogen susceptible to leaching in fall also depends on the previous crop (from now on referred to as 'precrop'). This can largely be affected by the crop type. Cereals like wheat have relatively low leftover N and risk of N leaching (Maidl et al., 1991; Francis et al., 1994), whereas N-intensive crops with a deep rooting system, such as canola (*Brassica napus*), or vegetable crop residues typically have very high leftover N (Henke et al., 2008; Agneessens et al., 2014). Similarly, grain legumes can increase leaching risk due to easily decomposable high-N plant residues (Chalk, 1998; Plaza-Bonilla et al., 2015). However, in the case of legumes as a precrop, of which benefits commonly have been attributed to a more positive N balance due to atmospheric N fixation (Maidl et al., 1996; Herridge et al., 2008), the overall effects of legumes in crop rotations cannot solely be attributed to increased N benefits. Reduced soil-borne pathogens, reduced soil water usage, and deep tap root systems loosening the soil can also positively affect the next crop (Peoples et al., 2009). This generally results in a yield increase compared to non-legumes in cereal cropping systems (Chalk, 1998; Angus et al., 2015).

Besides the positive effect of legumes in crop rotation, precrops that form a symbiosis with arbuscular mycorrhizal fungi (AMF), thus providing a host, typically increase the AMF spores in the soil and colonization of the next (mycorrhizal) crop. Although the benefits of AMF are usually linked to increased phosphorus and water uptake, there might also be benefits to N uptake, although this matter is still open to question (Smith and Smith, 2011; Thirkell et al., 2016), and disease resistance (Cameron et al., 2013). However, most studies neglect the possible role of AMF in affecting yields, in contrast to the well-studied effects of management practices, such as fertilizer addition and tillage, on subsequent crop yields. One reason for this might be that both fertilizer levels (especially P), tilling depth and the extent of fallow periods generally negatively affect AMF performance (Lekberg and Koide, 2005; Fester and Sawers, 2011). Moreover, these intensive agricultural practices could indirectly select for AMF strains which do not provide the benefits to the host species, but instead are closer to the parasitic end of the spectrum, investing more in their own reproduction and maximizing carbon acquisition from the host plants (Ryan and Graham, 2002; Verbruggen and Kiers, 2010). However, it is not clear what effect non-mycorrhizal crop species (the most common ones belong to the *Brassicaceae)* in crop rotations have on AMF community and structure both during the cropping with the non-mycorrhizal plant species and for the subsequent mycorrhizal crop (Kirkegaard et al., 2008; Verbruggen and Kiers, 2010). We know little about the extent to which having a mycorrhizal vs. a non-mycorrhizal precrop affects the yield and performance of the subsequent crop.

The effect and applicability of HCAs to counter nitrate leaching might depend on the specific precrop. To this end, we combine precrops with an ecologically based plant functional group approach based on two common plant-microbe symbioses: colonization by AMF and/or N_2-fixing bacteria. We explore the role of the symbiotic status of the precrop by combining all possible combinations of these two symbioses in the precrop, e.g., from rhizobial and mycorrhizal to non-rhizobial and non-mycorrhizal species (see **Table 1**). We include high and low N fertilization to disentangle effects of precrop functional groups and HCAs and their interactions. To our knowledge, this is the first study that explicitly tests the role of such plant functional groups (based on symbiosis) with a full factorial design within an agricultural experiment. Therefore, our study incorporates an ecological concept within a mainly agricultural experimental setup.

We experimentally investigated the role of HCA, precrop functional group and nitrogen fertilization on winter barley yield in an outside mesocosm experiment. We measured a subset, consisting of faba bean and spring barley as precrops, for the effect of these precrops and HCA on nitrate leaching over winter. We asked the following questions:

(1) Does the previous crop identity affect winter barley yield and do precrops forming root symbioses (rhizobia/AMF) show a bigger positive effect under low N?
(2) Is the effect of HCA on winter barley yield modulated by N fertilization level?
(3) Is the effect of HCA affected by the precrop identity? More specifically, does nitrate leaching increase after harvest of a legume precrop compared to a non-legume precrop and is this reduced by HCAs?

Overall, we hypothesized that an AMF-colonizing legume precrop amended with wheat straw under high N conditions

TABLE 1 | Sowing and harvest dates, fertilizer amount and symbioses with AMF and/or rhizobia of the crops used in this study.

	Crop	Sowing date	Harvest date	Fertilizer addition (kg/ha)					AMF	Rhizobial
				N	K_2O	P_2O_5	MgO	S		
Precrops	Spring barley	26/05/16	26/08/16	75	130	40	35	98	X	
	Spring canola	26/05/16	22/09/16	100	140	70	50	90		
	Faba bean	26/05/16	07/09/16	0	50	115	35	60	X	X
	White lupine	26/05/16	28/09/16	0	50	60	35	65		X
Focal crop	Winter barley	07/10/16	10/07/17	160/40	100	70	50	86	X	

Precrop complete fertilizer was added at precrop sowing date, while complete fertilizer addition to winter barley occurred at 23/03/17, except for two more N additions at 01/05/17 and 15/05/17 in the high N treatment.

results in the highest winter barley yield. Specifically, we hypothesized that:

(1a) Legume precrops have a positive effect on winter barley yield (especially under low N fertilization), since they introduce extra N into the system.
(1b) AMF crops have a positive effect compared to non-AMF crops on winter barley yield; we expect the highest yield increase with faba bean (both rhizobial and AMF).
(2) HCA has no effect under optimal N conditions, but could either decrease or increase winter barley yield under low N conditions by continuous N-immobilization or N-immobilization followed by remineralization in the spring, respectively.
(3a) Precrop identity modifies HCA effects on winter barley yield, since we expect more leftover mineral N after harvest of the legumes and canola. This would lead to potentially higher N immobilization over winter.
(3b) Nitrate leaching after precrop harvest is higher for a legume precrop compared to a non-legume precrop (faba bean vs. spring barley) and decreases for both with HCA addition.

MATERIALS AND METHODS

Experimental Site and Conditions

Our mesocosm experiment was conducted outside in an experimental garden of the University of Lüneburg (Lüneburg, Germany, 53°14′23.8′′N 10°24′45.5′′E). Mean annual temperature and rainfall is 9.2°C and 718 mm respectively. For detailed meteorological measurements during the experiment see Supplementary Figure S1, data was taken from the nearby weather station of the Deutsche Wetterdienst, Wendisch Evern (53°12′49.0′′N 10°28′13.1′′E).

Experimental Design

We applied a mesocosm experiment to quantify treatment effects on winter barley yield. We used relatively square mesocosms with an edge length of 37.5 and 26.5 cm at the top and the bottom, respectively, and a height of 37 cm; the resulting volume was 38 L. We used a surface area of 0.16 m^2 when converting to g m^{-2} and calculating fertilizer and HCA rates from kg ha^{-1}. Mesocosms were subject to three experimental factors (**Figure 1**): HCA (three levels; no HCA, wheat straw, sawdust), precrop identity (four levels; spring barley, spring canola, faba bean, white lupine), and N fertilization (2 levels; high: 160 kg/ha, low: 40 kg/ha). We applied a full factorial design with 5 replicates ($n = 5$) for each treatment combination, resulting in 120 mesocosms. Mesocosms were placed randomly (with 25 cm distance between mesocosms) in the experimental garden. Mesocosms were filled to a bulk soil density of ∼1.1 g cm^{-3} in the top 10 cm with soil passed through a 1 cm sieve. The soil originated from the top 0–30 cm of the experimental farm Hohenschulen of the Christian-Albrechts-University in Kiel (54°19′05.6′′N 9°58′38.8′′E). The soil is a sandy loam (Cambic Luvisol) and has a history of agricultural practice. In the growing season before the experimental start, a mixture of catch crops (such as clover and lupine) had been grown without fertilization, while the season before that maize had been cultivated and fertilized with 40 m^3 slurry (∼3% N, ∼1.8% P) and 100 kg/ha triple superphosphate (20% P). The soil had a total of 1.26% C, 0.14% N, a C/N ratio of 9.2 and a pH of 6.0 at the start of the experiment. We constructed a setup to measure nitrate concentrations in the leachate after the precrop harvest until N fertilization of winter barley at 23/03/2017 (see subsection leachate measurements). After the precrop harvest, mesocosms were reorganized and six out of ten replicates of the precrops faba bean and barley, and all HCAs were randomly selected for leachate measurements. At this time point, mesocosms were also isolated with air cushion foil (Luftpolsterfolie 3S, Hermann Meyer KG) and covered with white plastic sheets to avoid extreme temperature fluctuations within the mesocosm.

Study Species and Crop Husbandry

In May 2016, all mesocosms were sown with the precrops, which were chosen according to their ability to either be colonized by AMF or rhizobia (i.e., *Fabaceae*). The chosen precrops were spring barley (*Hordeum vulgare* cv. Barke, Saatzucht Breun), spring canola (*Brassica napus* cv. Medicus, NPZ), faba bean (*Vicia faba* cv. Tiffany, NPZ) and white lupine (*Lupinus albus* cv. Energy, Feldsaaten Freudenberger). Winter barley (*Hordeum vulgare*, cv. Antonella, Nordsaat Saatzucht), the focal crop, was sown the season after the precrops. The planting density (seeds/m^2) and row distance (cm, if applicable) was the following: spring barley 300, 9 cm; canola 120, 19 cm; faba bean 45; white lupine; 70, winter barley; 240, 13 cm. Mesocosms were fertilized according to their crop and standard agricultural practice in Germany (for exact values

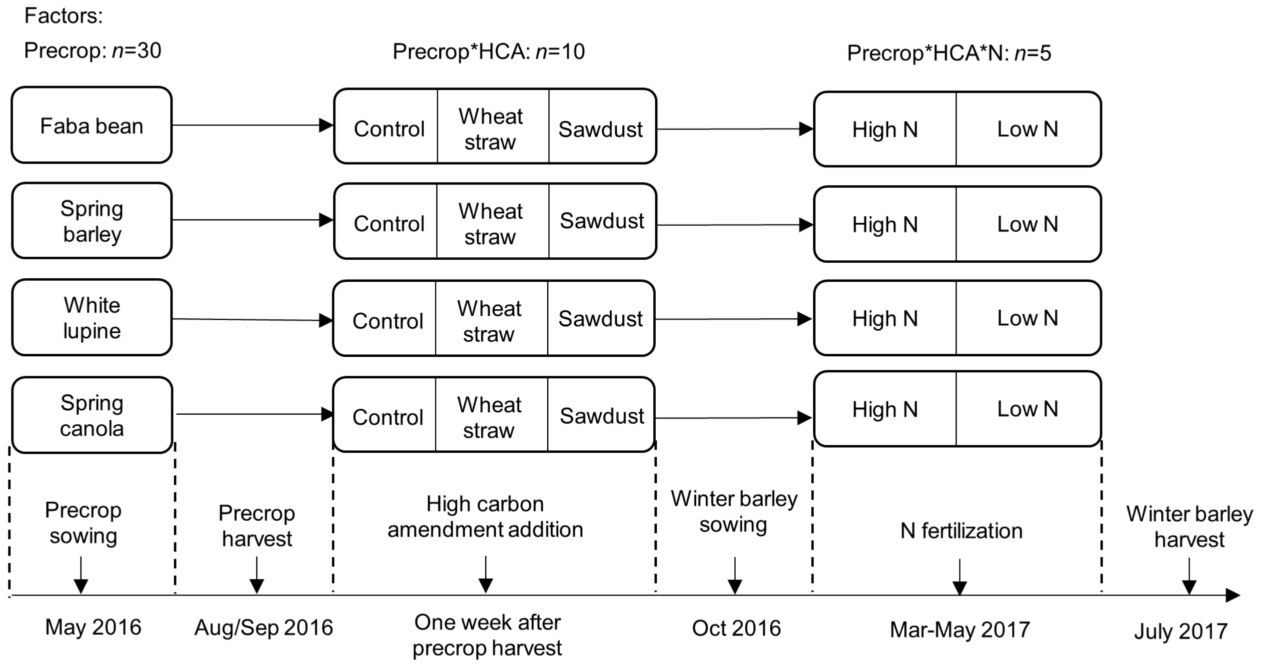

FIGURE 1 | Experimental design of the study with the main factors, precrops, HCA and winter barley N fertilization, added at different time points. For exact sowing and harvest time points see **Table 1**.

see **Table 1**) at either the sowing date (all precrops) or on 23/03/17 (winter barley). N Fertilization of winter barley for the high N treatment was spread over three time points, 60 kg N/ha at 23/03/2017 and 01/05/2017, and 40 kg N/ha at 15/05/2017. Nitrogen was added in the form of calcium ammonium nitrate, phosphate as superphosphate, potassium oxide as Korn-Kali, magnesium oxide as Korn-Kali and Epsom salt, and sulfur was contained in superphosphate, Epsom salt and Korn-Kali.

All mesocosms received 0.8 g Schneckenkorn (9.9 g/kg iron(III)-phosphate; Neudorff GmbH) on 14/06/2016 to counter plant damage by slugs. Furthermore, all mesocosms were sprayed with roughly 200 ml diluted Spruzit Schädlingsfrei per pot (45.9 mg/L pyrethrin; Neudorff GmbH) on 01/07/2016 due to an aphid infestation. No pesticides or herbicides were necessary during winter barley cultivation. Weeding was done by hand when necessary on multiple occasions. Mesocosms were watered during dry and warm spring/summer days when deemed necessary, but never during fall/winter, as to not affect the leachate amount.

High Carbon Amendments

High carbon amendments were added within 1 week after the precrop harvest. HCAs were air-dry wheat straw or spruce sawdust at a rate of 8.6 t/ha (137.6 g/mesocosm). The C/N ratios were 71 and 539, total C 46 and 51%, and total N 0.7 and 0.1%, respectively. The wheat straw had a particle size of 5–10 cm, whereas the sawdust contained particles of 1–2 cm. The HCAs were mixed in the top 10 cm of the soil and afterwards watered slightly to promote incorporation. The top 10 cm soil in the control treatment was also mixed, but without any amendment added.

Leachate Measurements

A leachate setup was built after the precrop harvest to collect water flowing through the mesocosms, which was subsequently analyzed for nitrate concentration. Mesocosm pots were put into slightly smaller mesocosms (30 × 30 × 32.5 cm) on top of two stacks of pallets. The drainage holes of the smaller mesocosms were sealed with cement and coated with a nitrogen-free resin at a slight angle so all water would flow to an attached drain. The drain was connected to a 5 L canister stored under the pallets and covered with white plastic sheet. Drainage holes of the large mesocosm were covered with nitrogen-free drainage fleece (Drainage-Geotextilvlies, Haga-Welt), to prevent contamination by soil particles or root growth into the smaller mesocosms. From 01/09/16 until 23/03/17, every 3–4 weeks (when enough water for analysis was leached through) the total leachate volume was recorded and a subsample of 50 ml was taken. The subsample was stored at −20°C before analysis for nitrate content. Samples were filtered before analysis using 0.45 μm filters (CHROMAFIL Xtra RC 45/25 membrane, Macherey-Nagel, Germany). The first two time points were analyzed with a direct UV measurement (VWR UV-3100PC, Denmark) at 220 nm and subtraction of interference at 275 nm according to (Goldman and Jacobs, 1961). However, at the third time point (28/11/16) the interference at 275 nm was too high (>10%) and we measured this and subsequent time points with an ion chromatograph (Dionex DX-120, AS14 column, United States). We correlated the UV and ion chromatograph for the third time point and the measured values showed good agreement (R^2 = 0.961; Supplementary Figure S2), but the UV method underestimated nitrate content due to the high interference at 275 nm. Three samples were excluded from the analysis due to broken tubes.

Plant and Soil Analyses

All precrops were harvested at maturity in September/August 2016 (for exact dates see **Table 1**), winter barley at 10/07/17, and separated into seeds and other aboveground biomass tissues, i.e., stems and leaves. Stems were cut off at 3 cm above the ground and leftover stubble and roots remained in the mesocosm. Furthermore, the seeds, depending on the crop, were manually separated from the spike (spring and winter barley) or the pods (lupine, canola and faba bean) to get the final cleaned seed mass. Dry mass of each plant component was measured after drying for at least 48 h at 70°C to constant weight. Winter barley seeds free from spikes were milled (MM 400, Retsch, Germany), dried at 105°C overnight and analyzed for C and N content (Vario EL, Elementar, Germany). Soils were sampled for roots at precrop harvest for screening for AMF colonization to see if the potential for symbiosis actually resulted in a symbiotic interaction in the experiment. Pooled composite samples of 6 soil cores (0–10 cm depth, 1 cm diameter) per mesocosm were sieved at 0.5 mm and precrop root fragments were sampled and stored at −80°C until analyses. A subset of precrop root fragments were then stained with Trypan blue and screened for AMF structures.

Soils were sampled for C/N analysis on two separate occasions. From 13 till 15 March 2017, before nitrogen fertilization, a composite sample of 6 cores (0–10 cm depth, 1 cm diameter) per mesocosm was taken. Afterward, autoclaved soil of the start of the experiment was used to fill the holes, as to not interfere with the leachate setup. Sampled areas were marked with a wooden toothpick to avoid resampling the same position later on. After the winter barley harvest on 10 July 2017, a composite sample of again 6 cores (0–10 cm depth, 2 cm diameter) was taken. Samples were air-dried before sieving (2 mm), milling (MM 400, Retsch, Germany), drying 24 h at 105°C and subsequent C/N analysis (Vario EL, Elementar, Germany).

Statistical Analysis

We first fitted three-way ANOVA models testing the effect of HCA, precrop and N fertilization as fixed factors on grain yield, straw biomass, C/N ratio and total N uptake of the seeds. The factor levels were as following; Precrop: spring barley, faba bean, white lupine, canola; HCA: control, wheat straw, sawdust, N: high, low. We included all interactions, because we were mainly interested in the two-way interactions between N fertilizer and either precrop species or HCA, i.e., whether the response to these factors differs between low and high N conditions. Moreover, we tested for the interaction between precrop and HCA, in case the HCA response was dependent on the precrop. We started with the full model and also checked for significance of the three-way interaction. If this was not significant and if dropping this improved the model, which was so in all cases, the three-way interaction was dropped. In case of no interaction, we averaged over the other factors for the factor of interest when plotting means or describing effect sizes.

In most cases heteroscedasticity was observed due to the choice of extremely contrasting N levels (i.e., high and low). Thus, for all data involving N as a factor, we used a generalized least square model with the "weights" function to allow different error terms for high and low N and correct for this heteroscedasticity [varIdent (form = ∼1|N)] using the nlme package (Pinheiro et al., 2017). The same approach was applied to soil C/N analysis, but replacing N levels with HCA levels within the weights function. Multiple comparisons between groups were tested for significance by using generalized linear hypotheses with Tukey's HSD adjusted *p*-values using the lsmeans (Lenth, 2016) and multcomp (Hothorn et al., 2008) packages. The leachate data was analyzed with a two-way ANOVA testing the effect of HCA (control, wheat straw, sawdust) and precrop (faba bean, spring barley) on total nitrate leached from the precrop harvest in August/September 2016 until N fertilization in March 2017. Multiple comparisons were tested for significance using Tukey's HSD adjusted *p*-values using the multcomp package (Hothorn et al., 2008). All statistical analyses were performed using R 3.4.2 (R Core Team, 2017).

RESULTS

N Fertilization

N fertilization was the main factor affecting the yield of winter barley with an average increase of 75% at high N compared with the low N level. This main factor effect was expected and we were mainly interested in the interactions. We found strong interaction effects with N levels and precrop species for the yield parameters grain yield, straw biomass, seed C/N ratio and total seed N uptake (**Table 2**).

Precrop Species Affect Yield Under High N

The precrop had a pronounced effect on the winter barley yield, but mostly under high N conditions only (**Figure 2A**, precrop*N: $F_{3,102}$ = 7.56, $p < 0.001$). Non-AMF precrops lupine and canola resulted in an on average 23% increase in yield compared to the AMF precrops spring barley and faba bean under high N. Although under low N we found significantly higher yields in winter barley when grown after lupine compared to spring barley, this effect was not as pronounced compared to the stimulating effect of having a non-mycorrhizal precrop found in the high N treatment (**Figure 2A**). For the winter barley straw yield, although roughly half the biomass of the yield in all treatments, the same pattern was found for high N but no significant difference found under low N (**Figure 2B**). Interestingly, the C/N ratio of the seeds showed the reverse pattern: Non-AMF precrops resulted in a lower seed C/N ratio than AMF precrops, but only under low N conditions (precrop*N: $F_{3,102}$ = 12.36, $p < 0.001$, **Figure 2C**). Total grain N uptake, however, which is the N concentration of the seeds multiplied by grain yield,

TABLE 2 | Results of the GLS ANOVA of N fertilizer, precrop and HCA on different yield parameters.

Factor	df	Grain yield		Straw biomass		C/N seeds		Total N yield	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
N	1, 102	490.58	**<0.0001**	935.99	**<0.0001**	1017.73	**<0.0001**	1749.64	**<0.0001**
Precrop	3, 102	8.12	**0.0001**	9.37	**<0.0001**	0.31	0.8179	16.96	**<0.0001**
HCA	2, 102	11.05	**<0.0001**	10.69	**0.0001**	2.18	0.1186	8.55	**0.0004**
N*precrop	3, 102	7.58	**0.0001**	6.60	**0.0004**	12.36	**<0.0001**	7.07	**0.0002**
N*HCA	2, 102	1.43	0.2433	2.13	0.1239	0.37	0.6919	0.84	0.4355
Precrop*HCA	6, 102	2.11	0.0589	2.70	**0.0179**	1.56	0.1655	1.33	0.2503

ANOVA p-values are in bold when p < 0.05.

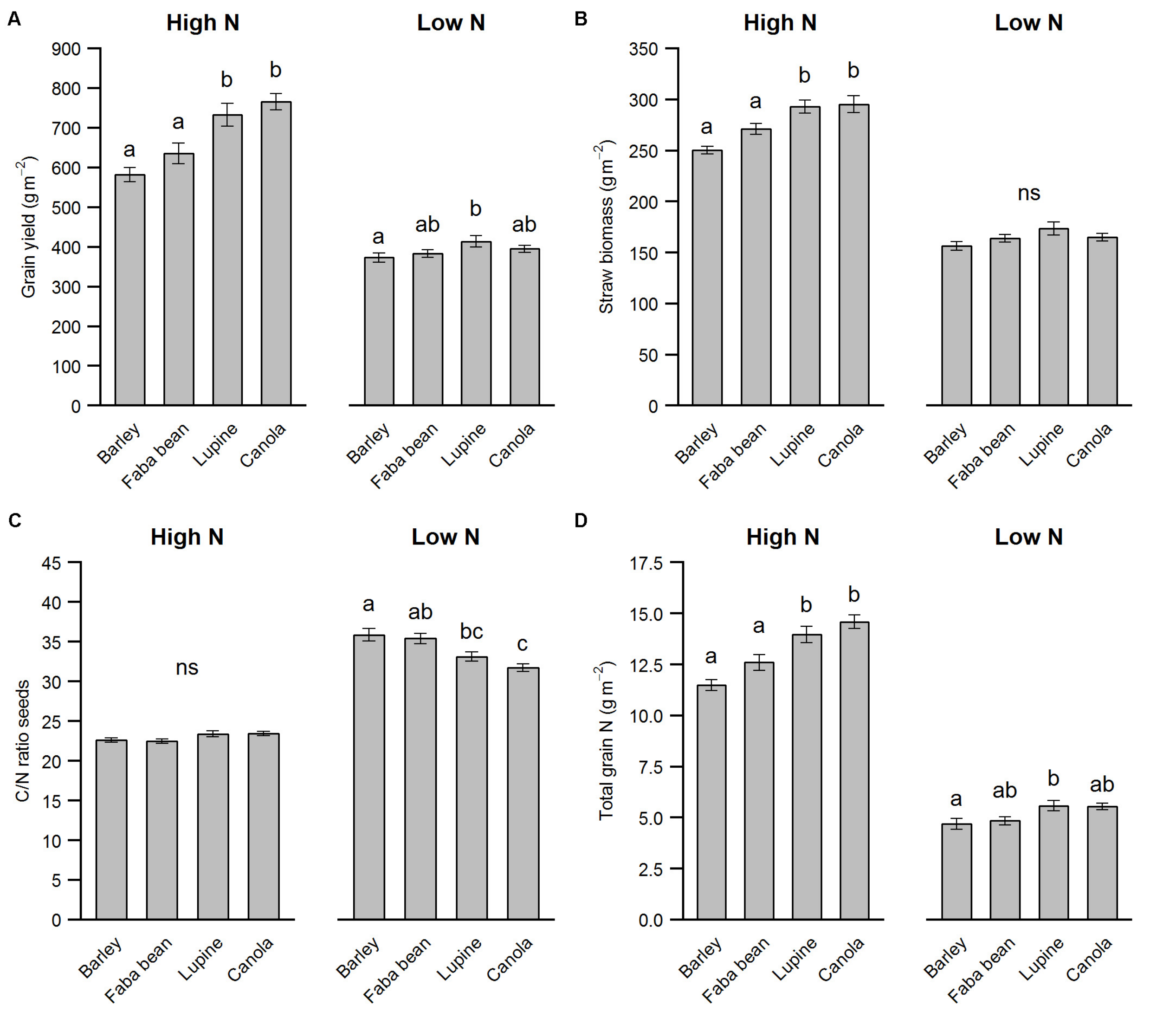

FIGURE 2 | Grain yield parameters of winter barley as affected by nitrogen fertilization and precrop species. The parameters are grain yield **(A)**, straw biomass **(B)**, C/N ratio of the seeds **(C)** total grain N **(D)**. The values are means per factor combination averaged over HCA (n = 15) ± SE. Different letters indicate significant differences between groups within N levels (p < 0.05).

showed the same pattern as the grain yield, indicating that the C/N ratio of the seeds is a less important indicator for total N uptake (**Figure 2D**). Although we had two legumes in the crop rotation, we did not see a clear legume effect on the yield, but instead observed a consistent effect of non-AMF vs. AMF precrops.

Sawdust Decreases Yield, but Increases Soil C/N Ratio

Overall, HCA application did not result in a winter barley yield increase compared to the control treatment, irrespective of N fertilization (HCA: $F_{2,102} = 11.05$, $p < 0.001$; N*HCA: $F_{2,102} = 1.43$, $p = 0.243$). However, sawdust application consistently decreased grain yield (sawdust: −6.3%, wheat straw: +3.8% compared to control, **Figure 3A**). Although seed C/N ratios were not affected by HCA (**Table 2**), total N uptake was lower in the sawdust treatment due to decreased yield (**Figure 3B**). Furthermore, we found a marginally significant HCA and precrop interaction on grain yield (precrop*HCA: $F_{6,102} = 2.11$, $P = 0.059$). This was mostly seen in spring barley and faba bean causing a slightly increased yield and white lupine and canola slightly decreased yield when wheat straw was applied.

Top soil (0–10 cm) C/N ratios increased with HCA application, more so with sawdust than wheat straw (**Table 3**). Total C and corresponding C/N ratios increased with higher addition of carbon, but no change in total N was observed before N fertilization in March 2017. However, while wheat straw resulted in an increase of soil C/N ratios in March, no lasting effect compared to the control was found by the time of the winter barley harvest in July 2017. There is a trend that C/N and total C due to sawdust addition remained high even over the main growing season of winter barley (**Table 3**), although in our experimental setup this was statistically not testable due to addition of N over the sampling period. Lastly, we did not find an effect of any precrops on the measured soil parameters.

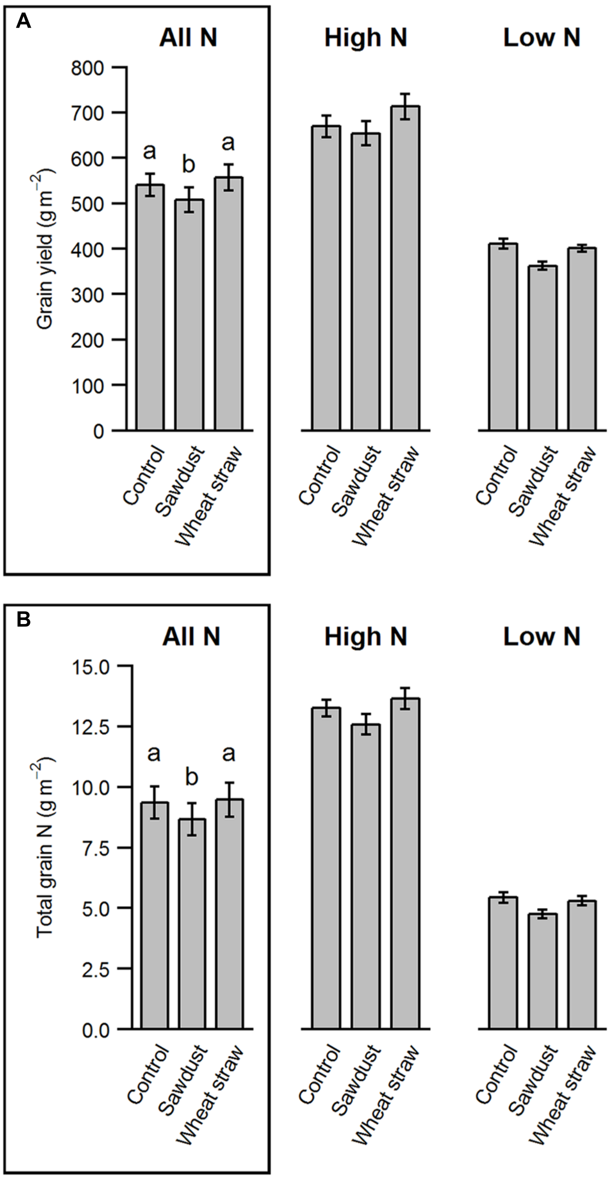

FIGURE 3 | Grain yield parameters of barley as affected by HCA. The parameters are grain yield **(A)** and total grain N **(B)**. The values are means ± SE per factor combination averaged over N and precrop (n = 40) in the boxed panel and separately plotted for high and low N to show variability between N levels (n = 20). Different letters indicate significant differences between groups ($p < 0.05$).

Leachate

Measurement of nitrate leaching showed a clear trend of increased leaching when faba bean was grown as a precrop compared to barley (**Figures 4**, **5**). The first sampling time point was 25/10/16, although we had the leachate sample setup ready 01/09/16, due to a very dry and warm September month (Supplementary Figure S1). Wheat straw showed a pattern of decreasing nitrate leaching for both precrops (**Figure 4**). For the cumulative nitrate leaching over fall and winter, faba bean, being a legume, had significant higher nitrate leaching in the control group (precrop*HCA: $F_{2,27} = 10.22$, $P < 0.001$; **Figure 5**), but wheat straw addition reduced nitrate leaching by 43% compared to the control. Although wheat straw addition lowered faba bean leaching to values similar to barley as a precrop, there was no significant overall decrease in barley due to HCAs.

AMF Colonization of Precrops

We did a screening of the roots of all four precrops to see if they showed signs of AMF or other fungal structures. Some fungal structures in these roots are shown in Supplementary Figure S4. We found that faba bean had vesicles, hyphae and spores resembling AMF structures, whereas we did not find clear signs of AMF colonization in spring barley. As expected, the non-AMF precrops canola and lupine showed no signs of AMF colonization.

DISCUSSION

We determined the response of winter barley yield to the previous crop and HCA under low or higher N conditions. We used a plant functional group approach based on two important plant–microbe symbioses (AMF and rhizobia) to disentangle their temporal effect on crop yield. Contrary to our hypotheses, we did not see a large effect of either plant functional group under N-limiting conditions. However, under high N fertilizer conditions non-AMF precrops significantly increased the yield

TABLE 3 | Effect of HCA and N fertilization on soil C/N ratio, total C (%) and total N (%).

Sampling date	HCA	N	*n*	C/N	Total C (%)	Total N (%)
13/03/17	C		20	9.68 (0.0640)a	1.29 (0.009)a	0.134 (0.0005)ns
	W		20	10.08 (0.0628)b	1.36 (0.009)b	0.135 (0.0008)ns
	S		20	11.87 (0.2445)c	1.59 (0.033)c	0.134 (0.0007)ns
10/07/17	C	High	20	9.52 (0.071)a	1.29 (0.017)a	0.135 (0.0018)a
		Low	20	9.51 (0.046)a	1.26 (0.014)a	0.132 (0.0014)a
	W	High	20	9.70 (0.057)a	1.36 (0.015)b	0.141 (0.0018)b
		Low	20	9.60 (0.062)a	1.33 (0.014)b	0.139 (0.0011)b
	S	High	20	11.48 (0.164)b	1.60 (0.022)c	0.141 (0.0018)b
		Low	20	11.27 (0.203)b	1.53 (0.035)c	0.138 (0.0015)b
N effect				ns	*	*

C: Control, W: Wheat straw, S: Sawdust. Sampling of March 2017 did not have the N treatment yet, thus a random subsample of low and high N factors was taken. Values are means ± SE averaged over precrops. Different letters indicate significant main effects of HCA (Tukey HSD; $p < 0.05$). Asterisks indicate a significant N effect (only applicable for sampling date 10/07/17).

compared to AMF precrops. Whereas HCA did not have a strong effect on the yield, it resulted in an increase in total soil C and N, indicating possible longer term positive effects on nutrient retention. HCA also directly reduced nitrate leaching in the top soil, but only for faba bean compared to spring barley as a precrop.

Effect of Precrop and Its Type of Symbiosis on Winter Barley Yield

We hypothesized that precrops that are leguminous and/or have a symbiosis with AMF would positively affect winter barley yield, especially under low N conditions. Instead, we hardly found an effect under low N conditions and a positive effect of non-AMF precrops on barley yield under high N (**Figure 2A**). The legumes were clearly fixing atmospheric nitrogen, since (a) we found large numbers of nodules on the main tap roots when mixing in the HCA after precrop harvest, and (b) we found no signs of N stress, and legume yield comparable to canola and spring barley without any N fertilization (**Table 1** and Supplementary Figure S3). Nevertheless, our results suggest that the legume precrop effect was not the dominant driver for winter barley yield, but more that the AMF-symbiosis of the precrop played a key role, since winter barley yield after canola (non-AMF and non-rhizobial) was just as high as that after lupine (non-AMF, rhizobial). This result is surprising because of the many studies showing a positive effect of legumes on the subsequent crop in crop rotations (Chalk, 1998; Angus et al., 2015).

This lack of strong legume facilitation on the subsequent crop might be explained by our crop husbandry and experimental setup. First of all, we grew grain legumes until maturity and removed all of the aboveground biomass, (both stems and seeds) which may complicate a direct comparison to typical leguminous cover crops where the goal is to increase nutrient retention and add biologically fixed nitrogen in the system (Tonitto et al., 2006). However, just the legume grain alone can contribute to 45–75% of the total aboveground biomass N (Van Kessel and Hartley, 2000), thus normally the majority of N is taken off in grain legumes. In our study, any carry over N facilitation effect would have to be mediated via decomposition of roots or direct exudation of compounds. However, belowground N contributions to the N budget are often ignored or vary widely in their estimates (Herridge et al., 2008), especially in the case of rhizodeposition (Wichern et al., 2008).

Secondly, our mesocosms were only 37 cm deep, which limits the extrapolation to field conditions, since the roots of our species could not grow as deep as in field conditions. Canola and barley, and grain legumes similar to the species in our study such as narrow leaf lupine (*Lupinus angustifolius)* and soybean (*Glycine max)* are known to have roots as deep as 1.6, 1.7, 2.5, and 1.8 m, respectively (Canadell et al., 1996; Fan et al., 2016). Thus, one would expect such roots under field conditions to be able to take up more of the excess N before being lost out of the system as leachate. After harvest in winter, nitrate will leach down to lower soil depths (Pedersen et al., 2009), some of which may then be taken up by the next crop in spring, some of which will be lost as leachate. In our study, however, we measured leachate derived from a 37 cm deep mesocosm, such that one could not know whether the N would be lost in the same way as leachate under field conditions. Field experiments and models show large amounts of nitrate leaching into deeper soil layers (Pedersen et al., 2009). Leachate measurement in our study nevertheless allowed us to compare differences between a legume and non-legume precrop. We know that cropping systems with legume species tend to have a larger leachate problem than non-leguminous crops since legumes tend to not rely on soil N as much as other crops, and leave low residues with low C/N ratios (Francis et al., 1994; Hauggaard-Nielsen et al., 2009). Our direct finding that faba bean had higher nitrate leaching compared to spring barley as a precrop (**Figure 5**) confirms this.

In addition to a positive legume precrop effect, we expected a positive effect of AMF precrops on the winter barley yield. This was not the case in the high N treatment (**Figure 2A**) and a surprising finding, because, assuming a positive effect on mycorrhizal colonization when the previous crop is a host to AMF compared to a non-host, a higher AMF colonization is associated with a higher yield (Lekberg and Koide, 2005). However, due to inclusion of low and high N, we can rule out a significant N carry-over of the previous crop, because

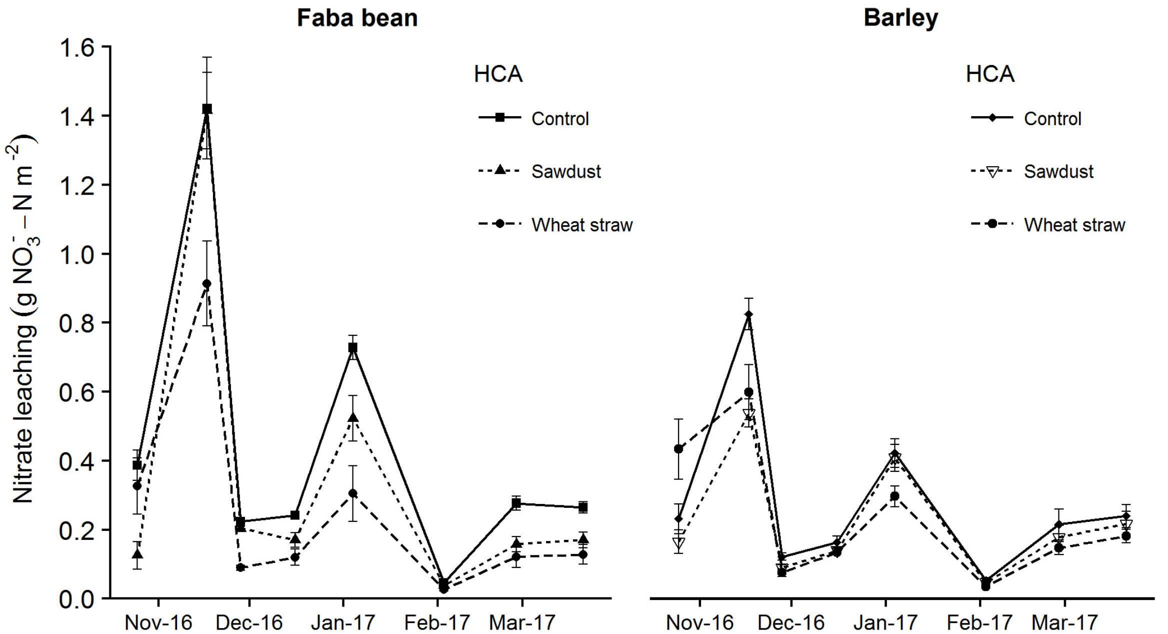

FIGURE 4 | Nitrate leaching after precrop harvest until first N fertilizer application as affected for the precrops faba bean and spring barley, and HCA. Nitrate leaching is calculated as the concentration of a subsample times the volume of water leached through the mesocosm. The values are means ± SE (*n* = 4–6).

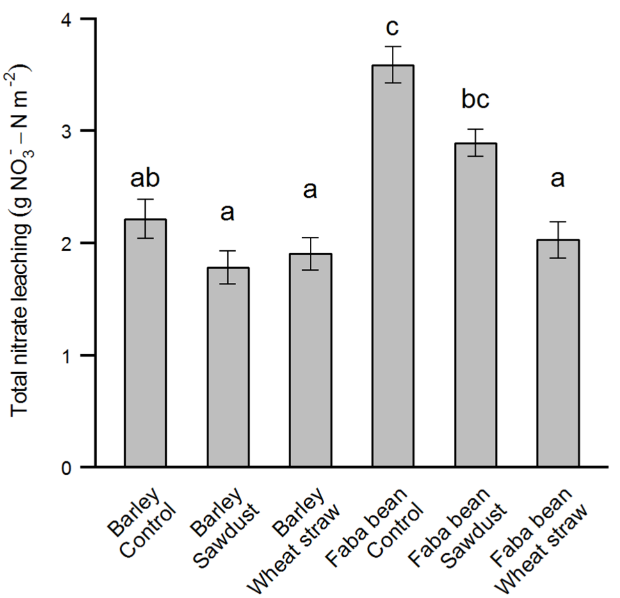

FIGURE 5 | Total amount of nitrate leached between 01/09/16 and 21/03/17 as affected by the precrops faba bean and barley and HCA. Total nitrate leaching is calculated as the concentration of a subsample times the volume of water leached through the mesocosm summed over all time points between 25/10/16 and 21/03/17. The values are means ± SE (*n* = 4–6). Different letters indicate significant differences between groups ($p < 0.05$).

we found no legume-exclusive effect compared to non-legumes (spring barley or canola; **Figure 2A**). Thus, the non-AMF precrop effect under high N might be attributed to other factors, such as reduced AMF colonization or a reduction in soil-borne pathogens by bio-fumigation of canola (Matthiessen and Kirkegaard, 2006). However, a bio-fumigation effect would not explain the similar positive effect of lupine. Therefore, a decrease in winter barley yield due to AMF precrops might be the most plausible explanation. Root staining showed AMF colonization in faba bean roots, but no clear colonization in spring barley (in comparison, in canola or lupine we found other fungal structures, but no colonization by AMF; Supplementary Figure S4). We can therefore not say with certainty whether the negative precrop effect of spring barley on winter barley yield compared to canola or lupine is directly related to AMF performance.

Explicit comparisons, other factors being equal, between non-mycorrhizal and mycorrhizal crops in crop rotations are limited (Ryan and Graham, 2002; Lekberg and Koide, 2005). Although rather controversial, Ryan and Graham (2002) and Ryan and Kirkegaard (2012) question the function of AMF and their contribution to crop yields in intensive agriculture. Similarly in our experiment, nutrient conditions were standard for German agriculture, which is generally regarded as very high (de Vries et al., 2011; MacDonald et al., 2011). High fertilizer rate/soil nutrients, especially soluble P, is known to negatively affect AMF colonization (Mäder et al., 2000; Treseder, 2004), but could also change the functioning of the AMF community toward more parasitism (Verbruggen and Kiers, 2010). If the AMF community represented a typical agricultural community (due to the history of our soil), this could explain the positive effect of non-AMF precrops, with the AMF precrops possibly introducing rather parasitic AMF to the system that may have contributed to the lower yield in winter barley after these crops in our study.

HCA Effects on Winter Barley and Soil Parameters

Addition of HCAs to reduce nitrogen leaching specifically after harvest has been attempted multiple times, with mixed results (Thomsen and Christensen, 1998; Vidal and López, 2005; Chaves et al., 2007; Congreves et al., 2013b). HCAs can have effects on a number of parameters. Some studies found effects on the soil chemistry (which is often the main focus), whereas effects on the subsequent crop performance are much rarer (Congreves et al., 2013b). This is surprising, since the preferred outcome of HCA N immobilization over winter would be to retain more N in the topsoil, thus making it more available to the next crop and reducing the N fertilizer needs of the subsequent crop.

In our study, we did not find strong evidence of remineralization of immobilized N due to HCA (**Table 3**). On the contrary, sawdust application had a negative effect on winter barley yield, potentially caused by strong N immobilization under either N fertilizer levels. Wheat straw application resulted in a positive trend of winter barley yield under higher N conditions (**Figure 3A**), which could be either caused by remineralization of immobilized N during the growing season, but also due to decomposition and subsequent N release contained in the wheat straw itself (Di and Cameron, 2002). We found no difference in total N content in soils in March 2017, which indicates a lack of N transfer over fall/winter, although at winter barley harvest we did find a significant increase of total N in the wheat straw and sawdust treatment compared to the control (**Table 3**). This increase might be mainly due to fertilizer added during the winter barley growing season being immobilized in the soil rather than an N carry-over effect from the precrop. It is worthwhile noting that because of the small particle size of sawdust some particles were not sieved out with a 2 mm sieve before milling, while pieces of wheat straw were, which could inflate the soil C measurement. However, the results were consistent and wheat straw also showed a higher total C (%) than the control (**Table 3**).

We found a strong reduction in nitrate leaching when wheat straw was applied to faba bean as a precrop (**Figure 5**). Other studies on HCAs and nitrate leaching show mixed results. Chaves et al. (2007) found a reduction in N leaching of 56–68% due to wheat straw or sawdust after high N vegetable crop residues, which might be comparable to the increased leftover N in legumes. On the other hand, little to no reduction in nitrate leaching due to straw incorporation was found by Thomsen and Christensen (1998) when cereal crops or sugar beet were grown beforehand, similar to our findings for spring barley as a precrop. A common finding in both these studies is that remineralization in the next spring does not seem to occur in considerable amounts. Paradoxically, HCAs could increase N leaching when immobilized N is being mineralized next fall instead. Finally, we did not find a precrop species effect on soil C/N ratios or total soil C or N contents in either March before N fertilization or at winter barley harvest, despite the clear reduction in nitrate leaching after faba bean amended with wheat straw. This could be because the mineral N pool is relatively small compared to the total N pool, and, coupled with the hypothesis of long-term immobilization due to HCAs, might explain the lack of a positive HCA effect, especially under low N conditions.

CONCLUSION

Using a semi-natural setup our experiment bridged the gap between short-term artificial greenhouse experiments and the heterogeneity of field studies, allowing for relatively realistic weather conditions and temporal scale whilst reducing spatial heterogeneity, in order to improve our understanding of carry-over effects of precrops. We found evidence that AMF precrops had possibly parasitic effects on the subsequent winter barley when large amounts of fertilizer were added to the system, whereas there was no clear legume precrop effect. In our setup, short-term immobilization of N by HCA addition after harvest was not generally achieved, despite a slight positive effect of wheat straw on winter barley yield. HCAs do show potential to counter nitrate leaching of high-risk leaching crops such as grain legumes. Furthermore, other effects such as an increase in SOM or nutrient retention could play a positive role in the long term, since we found higher soil total C and total N nearly a year after application of HCAs.

AUTHOR CONTRIBUTIONS

VT and RvD designed the experiments. RvD and JR collected the data. RvD analyzed the data. RvD and VT led the writing. WH reviewed the manuscript. All authors contributed to critical revisions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00912/full#supplementary-material

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Coming Late for Dinner: Localized Digestate Depot Fertilization for Extensive Cultivation of Marginal Soil With *Sida hermaphrodita*

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Improving fertility of marginal soils for the sustainable production of biomass is a strategy for reducing land use conflicts between food and energy crops. Digestates can be used as fertilizer and for soil amelioration. In order to promote plant growth and reduce potential adverse effects on roots because of broadcast digestate fertilization, we propose to apply local digestate depots placed into the rhizosphere. We grew *Sida hermaphrodita* in large mesocosms outdoors for three growing seasons and in rhizotrons in the greenhouse for 3 months both filled with marginal substrate, including multiple sampling dates. We compared digestate broadcast application with digestate depot fertilization and a mineral fertilizer control. We show that depot fertilization promotes a deep reaching root system of *S. hermaphrodita* seedlings followed by the formation of a dense root cluster around the depot-fertilized zone, resulting in a fivefold increased biomass yield. Temporal adverse effects on root growth were linked to high initial concentrations of ammonium and nitrite in the rhizosphere in either fertilizer application, followed by a high biomass increase after its microbial conversion to nitrate. We conclude that digestate depot fertilization can contribute to an improved cultivation of perennial energy-crops on marginal soils.

Keywords: digestate fertilization, localized fertilizer placement, marginal substrate, perennial plants, rhizotron, root plasticity

INTRODUCTION

Biomass is the primary source of energy in Europe's renewable energy mix, contributing 67% to the total (Eurostat, 2017). However, if biomass (e.g., energy maize) for bioenergy purposes is produced on agricultural soils, it is competing with food crops for the same land resources, resulting in land use conflicts (FAO, 2009; Fritsche et al., 2010). The cultivation of energy crops on marginal soils is currently discussed as a more sustainable alternative, minimizing potential conflicts with food production (Schröder et al., 2008; Graham-Rowe, 2011). A sustainable use of marginal soils to produce biomass for bioenergy purposes requires adapted cropping strategies that give consideration to the specific soil conditions of marginal substrates and the environment with special attention to the biodiversity of plants and animals (Fang et al., 2007; Blanco-Canqui, 2010; Brüll, 2015).

Following the idea of a closed nutrient loop, organic residues such as biogas digestates are applied to the fields as organic fertilizers after the biomass conversion to bioenergy (Arthurson, 2009; Vaneeckhaute et al., 2013) thereby reducing the need for synthetically produced fertilizers (Haraldsen et al., 2011; Walsh et al., 2012). Further, the carbon content of such organic fertilizers can play an important role as soil amendment increasing the soil fertility of marginal substrates in the long-term (Beare et al., 1994; Tiessen et al., 1994; Reeves, 1997; Nabel et al., 2017). Combinations of perennial energy crops with organic fertilization have been discussed as a possible cropping scenario on marginal sites (Blanco-Canqui, 2010; Nabel et al., 2016). Perennial energy crops are of special interest for the cultivation of marginal soils, as their extensive and perennial root system increases their ability to access the limited water and nutrient resources, often characteristic for marginal soils (Voigt et al., 2012).

However, there are still obstacles that hold farmers off from bringing cropping systems based on organic fertilization, perennial plants and the use of marginal soils into agricultural practice. As first obstacle, physical soil properties like the low water-holding capacity of sandy marginal substrates may increase by surface application of organic fertilization such as digestates. On sandy soils, surface application of digestates strongly decreases the wettability of the substrate by increasing the water repellency of the sand via CH-groups, coating the sand grains, resulting in increased surface water runoff (Voelkner et al., 2015a,b). A second obstacle is the fact that digestates contain a high concentration of NH_4^+ (Möller and Müller, 2012), which can have a negative impact on the establishment of an extensive root system (Forde and Lorenzo, 2001; Nabel et al., 2014). As a third obstacle, sandy substrates with low water holding capacity (WHC) can be very prone to nitrogen leaching as nutrients are rapidly washed out of the rhizosphere (Di and Cameron, 2002; Nabel et al., 2016). Further risks for nitrogen losses are linked to the fact that digestates cannot be plowed into an established stand of perennial energy crops, because that would cause severe damage of the perennial root systems of the crops. Furthermore, the risk of nitrogen losses via gaseous NH_3 and N_2O emissions is high, irrespective of soil surface or subsurface application (Möller and Stinner, 2009; Rochette et al., 2009). In addition, perennial energy crops need special attention in the year of crop establishment, before they establish their extensive root system and become competitive against weeds (Borkowska et al., 2009). For the above-mentioned reasons broadcast digestate fertilization could counteract a fast and successful establishment of a vigorous and competitive perennial energy crop canopy.

In order to overcome these problems and allow for an efficient and sustainable use of digestates on sandy marginal substrates for plant biomass production, we aim to adapt the digestate fertilization to the Controlled Uptake Long Term Ammonium Nutrition (CULTAN) method proposed by Sommer (2003). According to this method developed for mineral NH_4^+-rich fertilizers, fertilizers are not applied broadcast but injected locally directly into the rhizosphere (Sommer, 2005). As the plant roots adjust to the heterogeneous distribution of nutrients in the rhizosphere, they can increase the rooting density and share of fine roots in the fertilized zone (Nkebiwe et al., 2016).

In recent years, the method has been adapted to organic fertilizers that are rich in NH_4^+ like sludge, manure slurries and digestates (Dell et al., 2000; Pote et al., 2011; Bittman et al., 2012). In various studies on agricultural soils planted with conventional agricultural crops or grasslands, a positive impact of a localized application of organic fertilizers on drought (Garwood and Williams, 1967; Ma et al., 2009), nitrate leaching (Baker, 2001), root growth and nutrient use efficiency (Hodge, 2004; Weligama et al., 2008) as well as NH_3 volatilization (Nyord et al., 2008) was shown. All these positive effects of localized organic fertilizer placement sum up in increased biomass yields (Cooke, 1954; Jing et al., 2012). For more information about the underlying processes of the methodology we refer to the review by Nkebiwe et al. (2016), who also focused on localized placement of organic fertilizers into the rhizosphere.

The fertilizer placement technology, already well established on conventional agricultural sites, is however, not yet used for the application of digestates to perennial energy crop cultures grown on marginal sandy field sites. Still, the technology of controlled fertilizer placement tackles major problems of digestate fertilization on sandy marginal sites and is therefore of interest for investigation. In this study, we tested digestate fertilization, applied as localized nutrient depots in the rhizosphere of the perennial energy crop *Sida hermaphrodita*, grown on a sandy marginal substrate. We compared localized to a broadcast digestate fertilization and mineral fertilization using a conventional NPK-fertilizer and an unfertilized control. *S. hermaphrodita* is a prairie forb species from North America that grows well on sandy or rocky soils with low organic matter content, producing relatively high biomass yields even with low nutrient levels in the soil (Spooner et al., 1985; Borkowska et al., 2009). In order to follow the structural adaptation of the root system of *S. hermaphrodita* to the different fertilizer applications a rhizotron experiment was performed under controlled greenhouse conditions. To verify our results under conditions that resemble more the field conditions, we also conducted a 3-year outdoor mesocosm experiment where root proliferation of depot-fertilized plants were followed by using mini-rhizotrons.

The study was designed to answer the following research questions and hypotheses:

Question 1: What causes the reduced root growth on *S. hermaphrodita* seedlings following digestate application as fertilizer on marginal soil and how does it develop over time?

Hypothesis 1: The root growth is affected by the nitrogen turnover of the fertilizer. High concentrations of ammonium and nitrite following the fertilizer application instantly are toxic to plant roots. The mineralization of ammonium to nitrate will allow enhanced root growth at a later stage.

Question 2: What could be an effective measure to apply digestate fertilization and promote root growth at the same time?

Hypothesis 2: The application of localized patches of digestate (digestate depot) into the rhizosphere will promote root growth, as the major part of the rhizosphere remains unaffected by the fertilization and therefore by initial adverse effects on root growth.

MATERIALS AND METHODS

Study Site and Plant Cultivation

A glasshouse rhizotron study and an outdoor mesocosm experiment were performed at Forschungszentrum Jülich GmbH, IBG-2: Plant Sciences, Germany (50°54′34′′N 6°24′47′′E). For the glasshouse experiment, 80 *Sida hermaphrodita* seedlings of BBCH stage 12-13 (Jablonowski et al., 2017) were transplanted in February 2016 to a rhizotron (inner dimensions: height: 75 cm; width: 36 cm; depth: 2.6 cm) filled with a sandy substrate (RBS GmbH, Inden, Germany; particle size: ≤1 mm; pH 6.6, no detectable amounts of N, P, K, and C), accounting for 80 rhizotrons in total. Rhizotrons were irrigated manually three times a week to 60–70% of substrate WHC. The detailed climate data for temperature and exposure to light over the 3 months experimental time are presented in **Table 1**.

An outdoor mesocosm experiment was conducted using 21 containers, each filled with 250 L of the same sandy substrate as used in the rhizotrons. Big outdoor containers were chosen to keep growing conditions of *S. hermaphrodita* as close to field conditions as possible (Poorter et al., 2012a, 2016). Seedlings of *S. hermaphrodita* of BBCH stage 13–14 were transplanted into the mesocosms in May 2014 (Jablonowski et al., 2017). The detailed establishment of *S. hermaphrodita* plants into the mesocosms was described earlier (Nabel et al., 2016). The climate data over the 3-year experimental time can be found in **Table 1**.

Treatments and Fertilization

Rhizotrons and mesocosms received digestate fertilization, applied either homogeneously incorporated (here after referred to as "broadcast fertilization") or as a localized depot (here after referred to as "depot fertilization") in the rhizosphere, broadcast mineral fertilization or no fertilizer supplement. The digestate was obtained from an operating biogas plant using maize silage as feedstock (digestate dry matter content: 7.2%; N_{total}: 0.53%; NH_4^+: 0.32%; P: 0.14%; K: 0.68%; Mg 0.037%; Ca: 0.16%; S: 0.03%; organic matter: 5.3%, C:N ratio: 6; pH 8.2; all values referring to fresh weight; ADRW Naturpower GmbH and Co. Kg, Titz-Ameln, Germany). An NPK-fertilizer with an N:P:K-ratio similar to the digestate and a high share of ammonium was chosen to allow a comparison between the mineral and the organic digestate fertilization (NPK-fertilizer composition: N: 15% [1% nitrate; 9.5% ammonium; 4.5% isobutylidenediurea]; P: 5%; K: 8%; Mg: 3%; Compo Rasendünger, Compo GmbH, Münster, Germany). Both fertilizers were calculated to simulate a total N application of 40 t digestate ha^{-1}, which was identified earlier as the optimum dose of digestate fertilization for *S. hermaphrodita* grown on sandy substrate (Nabel et al., 2014). In mesocosms fertilization treatments were applied in May 2014–2016 to the soil surface and immediately incorporated to minimize possible losses of nitrogen via NH_3 (Hayashi et al., 2009). In rhizotrons, broadcast fertilization treatments were applied by homogeneously mixing fertilizers into the top 20 cm of the rhizotrons. For the localized digestate depot fertilization, the same amount of digestate was applied locally as a patch of 4.6 cm diameter. In mesocosms, the digestate depot was injected at a 20 cm distance from the shoot of *S. hermaphrodita,* at 30 cm depth. In rhizotrons, digestate depots were placed in 15 cm depth and 6 cm distance to the rhizotron wall (**Figure 1**). In rhizotrons each fertilization treatment was applied with 24 replicates (8 replicates × 3 harvests), the unfertilized control treatment was applied in eight replicates (only one harvest). In mesocosms all treatments were applied in six replicates (one harvest after each growing season).

Measurements

Rhizotrons with *S. hermaphrodita* plants were placed into a novel phenotyping platform enabling simultaneous and non-destructive measurements of root and shoot growth of rhizotrons-grown plants (*GrowScreen-Rhizo2*, developed at Forschungszentrum Jülich GmbH, Germany). The design of the new phenotyping system *GrowScreen-Rhizo2* is based on the experiences with the *GrowScreen-Rhizo1* platform published in 2012 (Nagel et al., 2012). However, the *GrowScreen-Rhizo1* concept of having fixed positions for rhizotrons and a moving cabinet for imaging the rhizotrons has been changed to enable higher flexibility and modularity. In the new platform, rhizotrons are moved in trays (five rhizotrons per tray) on a conveyor system and transported to an imaging station which has a fixed position. The inclination angle of the rhizotrons is adjusted always to 45° with the transparent plate of the rhizotrons facing downward to force the roots to grow toward the transparent plate. Inside the imaging station individual rhizotrons are taken out of the tray and positioned automatically in front of a high resolution camera (AV GT6600, Allied Vision Technologies, Germany; resolution 142 μm per pixel) which is taking one image of the whole transparent rhizotron surface (**Figure 1C**). During image acquisition, the door of the imaging station is closed automatically with rolling cutter gates to prevent undesired light reflections on the Plexiglas plate of the rhizotrons and the roots are illuminated using two LED panels (30 W 40 mm × 40 mm × 940 mm, color temperature 4000 K, item Industrietechnik GmbH, Germany). After imaging, the gate is opened and the rhizotron is placed back to into the tray completing the routine. Pictures were taken automatically once a week. In addition, plants were harvested at 30, 60, and 90 days after transplanting seedlings to determine leaf, stem, and root biomass. For harvest, rhizotrons were opened and the sandy substrate was carefully washed out with tap water and roots were cleaned thoroughly. Leaves, stems, and roots were cut and separately dried at 70°C to constant weight to determine the biomass dry weight. The pictures of the roots, acquired by *GrowScreen-Rhizo2* during growth were evaluated for total visible root length and root distribution by using the software *GROWSCREEN-Root* in combination with a graphic tablet with pens (Wacom Cintiq 21UX, 31 CANCOM Deutschland GmbH, Düsseldorf, Germany) (Nagel et al., 2009). In order to visualize the spatial distribution of roots for any given treatment, we wrote an additional software to combine the data from all root images

TABLE 1 | Climate data for the greenhouse rhizotron experiment and the outdoor mesocosm experiment: mean temperature, precipitation and daily light integral (DLI) values during the experimental time from 2014 to 2016 at the Forschungszentrum Jülich GmbH (50°53′47′′ north and 6°25′32′′ east; 80 m a.s.l.).

Year	Mean air temperature (°C)		DLI (mol m^{-2} day^{-1})	Precipitation (mm)
	Day	Night		
Greenhouse rhizotron experiment				
2016	22.0	16.0	7.4	–
Outdoor mesocosm experiment				
2014	15.2		37.0	801.4
2015	15.1		39.9	678.1
2016	15.8		37.7	651.3

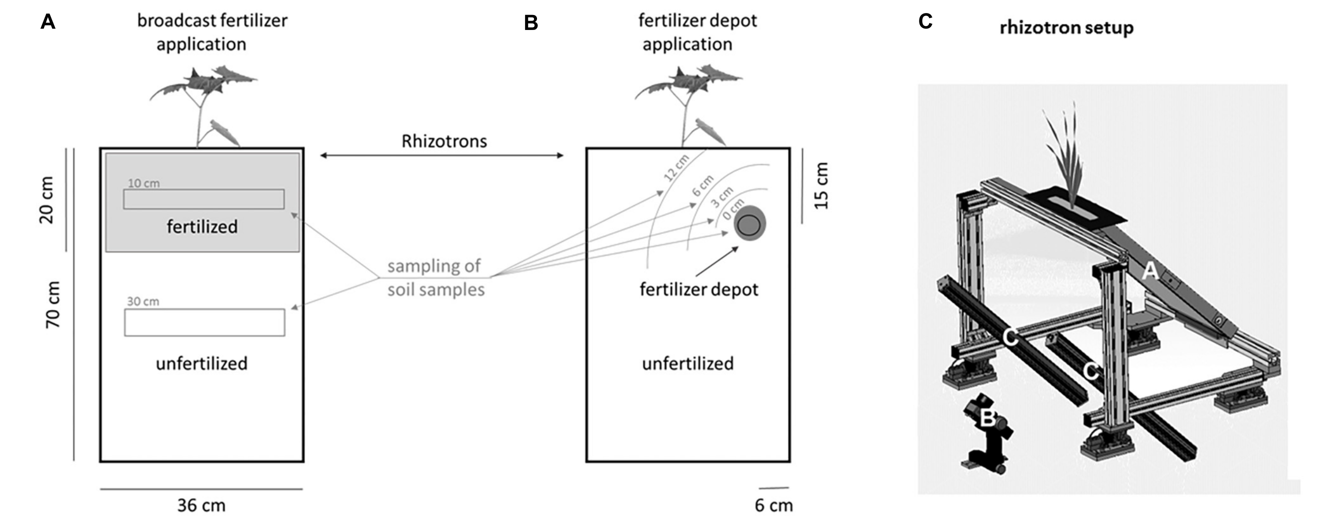

FIGURE 1 | Illustration of the fertilizer application as broadcast (**A**: homogeneous digestate/NPK application) or depot (**B**: localized digestate application) fertilization in rhizotrons and soil sampling schema. Control rhizotrons did not receive any fertilization and are therefore not included in the illustration. **(C)** Schematic drawing of the imaging station of the automated phenotyping platform, *GrowScreen-Rhizo2*. The inclination angle of the rhizotron **(A)** is fixed to 45° with the transparent surface of the rhizotron facing downward. The image of the root system is taken by a high-resolution camera **(B)**, positioned at a distance of 1 m to the rhizotron surface. The positions and angles of the two light panels **(C)** are adjusted to allow homogeneous illumination and prevent reflections in the images.

of each treatment in one image, representing root structure and distribution for any given treatment. To create these images, the area of a given root picture, as acquired by the *GrowScreen-Rhizo2* system, was subdivided into a grid of cells, sized 25 × 25 pixels each. Each cell was initialized with zero. Every time, any part of the root structure was found in a given cell, the value of this particular cell increased by one. Further limiting the maximum increase per pixel per plant to one, allowed us to visualize root distribution in relation (%) to the number of plants per treatment. The generated images were converted from gray scale single channel images to three channel false-color images (Ware, 1988). This conversion was done using Opencv's build-in function applyColorMap using the JET colormap (Bradski, 2000).

In mesocosms, the above-ground biomass was harvested at the end of the growth season in 2014, 2015, and 2016, and dried at 70°C to constant weight to determine the biomass dry mass. In 2016, the root biomass of four replicates for each treatment was determined by washing out the substrate, cleaning roots thoroughly and drying them at 70°C to constant weight. Root growth of plants grown in mesocosms was monitored non-invasively by mini-rhizotrons, which are Plexiglas tubes horizontally installed in 30 and 60 cm depth of the mesocosms. Pictures of root systems were taken after 6, 18, and 30 months after planting, using the CI In-Situ Root Imager (CID Bio-Science Inc., Camas, WA, United States). The pictures were than further processed as described above by using the software *GROWSCREEN-Root* to determine root length and distribution.

Daily light integral values (DLI) were directly measured at place on the rhizotron and mesocosm facility, employing a LI-COR: Li-190 device (LI-COR Environmental – GmbH, Bad Homburg, Germany).

Soil samples were taken at the date of destructive biomass harvests in each rhizotron. In unfertilized-, mineral-fertilized and broadcast digestate-fertilized rhizotrons, one mixed sample was taken in a depth from 0 to 20 cm and a second in the depth of 25–50 cm. In rhizotrons with digestate depot fertilization, one sample was taken in a radius of 3, 6, and 12 cm around the digestate patch, in each rhizotron (**Figure 1**). Soil samples were stored at 4°C until determination of NH_4^+, NO_2^-, NO_3^-, electric conductivity and pH were performed. NH_4^+, NO_2^-, and

NO_3^- were measured via ion chromatography (Dionex DX-500, AS23; eluent: 0.8 mM sodium bicarbonate and 4.5 mM sodium carbonate) in 0.1 M KCl extraction. Soil pH was determined using standard electrodes (Hanna Instruments pH 209 pH-meter, Vöhringen, Germany), using 0.01 M $CaCl_2$ solution at 20°C. Nitrogen content of *S. hermaphrodita* leaves was determined by elemental analysis (VarioELcube, Elementar Analysensysteme GmbH, Langenselbold, Germany) after milling dried leaves for 60 sec. in a ball mill at 30 Hz (Retsch Mixer Mill MM 400, Retsch GmbH, Haan, Germany).

Statistical Analysis

The rhizotron experiment consisted of four treatments (digestate broadcast fertilization, localized digestate depot fertilization, mineral broadcast fertilization, and unfertilized control) with three sampling dates (30, 60, and 90 days), with eight repetitions per treatment. The mesocosm experiment had the same treatments in six repetitions of which the above ground biomass was harvested at the end of the vegetation period in 2014, 2015, and 2016. The below-ground biomass of the mesocosms was harvested ($n = 4$) for each treatment in October 2016. Statistical analysis was performed with analysis of variance (ANOVA) with an *a posteriori* test in R 3.0.3 (The R Foundation for Statistical Computing, 2014) using the work package "Agricolae" (de Mendiburu, 2014).

RESULTS

Soil Analysis in Rhizotrons

Thirty days after transplanting the seedlings, all fertilization treatments showed a rapid conversion from ammonium to nitrate (**Table 2**). However, for the two digestate fertilized variants (broadcast and depot) also the intermediate product of mineralization, nitrite, was found in high concentrations, especially in a 3–6 cm radius around the digestate depot. In digestate broadcast fertilized rhizotrons, 85% of the measured nitrogen was found in the form of nitrate. In digestate depot fertilized rhizotrons, 20% of the measured nitrogen remained as ammonium and 33% was found as nitrite. Sixty days after planting, nitrite was not detected, while nitrate concentrations reached their maximum levels in the digestate-fertilized horizon of broadcast fertilized digestate rhizotrons as well as around the depot fertilized zones. Nitrogen in the form of ammonium was only detected in a 6–12 cm radius around the digestate depots. In digestate broadcast and NPK fertilized rhizotrons, only minor amounts of ammonium were detected in the fertilized horizon. Ninety days after planting, almost all nitrogen in all treatments was present in the form of nitrate, mainly located in the fertilized horizons of digestate broadcast -, NPK fertilization and a 3–6 cm radius around the digestate depots.

After 30 days, nitrogen fertilization, regardless of application technique, lowered the pH to 6.4. Contrastingly, the pH of the sandy substrate outside the 12 cm zone that was affected by depot fertilization had values of pH 7.0. Only within the radius closest to the digestate depots (<3 cm) pH 8.5 was reached. In the following 2 months, pH values generally increased and leveled off between pH 7 and 7.5. Lower pH values (6.6) were only found within a 6 cm radius around the digestate depots and the NPK fertilized horizon of mineral fertilized rhizotrons.

Root Growth and Root System Architecture

Rhizotrons

Thirty and sixty days after planting of *S. hermaphrodita* into rhizotrons, the form and application technique of the different fertilizers had a strong influence on the distribution of roots (**Figure 2**). However, root mass did not differ significantly between treatments until day 60 but differed strongly in their foraging behavior in the rhizotron experiment (**Figure 3**). Unfertilized control and digestate depot fertilized plants accessed the maximum depth of the rhizotrons within 60 days. Digestate broadcast and NPK fertilized plants only reached a rooting depth of 40 cm with 99% of the measured root length located in the fertilized horizon for digestate broadcast and 60 % for NPK fertilization, respectively. Digestate depot fertilization resulted in a strongly heterogeneous distribution of roots over the rhizotrons. Even though after 30 and 60 days when roots accessed deeper areas of the rhizotrons, no roots were present within a 6 cm radius around the digestate depots. After 60 days, only 1% of the total root length was located within this radius. After 90 days, the root growth pattern changed dramatically and 45% of the root biomass of digestate depot fertilized plants were found within this 6 cm radius around the digestate depots resulting in the formation of a dense root cluster around the digestate depot. Even after 90 days, roots in NPK and digestate broadcast fertilized rhizotrons did not reach the maximum depth of the rhizotrons and major part of their roots remained located in the fertilized horizon (60% for NPK and 80% for digestate broadcast fertilization).

Mesocosms

After 1 year of growth, *S. hermaphrodita* in mesocosms located 60% of the measured root length in a depth of 30 cm when fertilized with broadcast digestate or NPK-fertilizer. When fertilized with localized digestate depot, a root cluster was visible at 30 cm depth, accumulating 90% of the measured root length in 30 cm depth, while unfertilized control plants had 70% of the measured root length in the same depth. After 3 years root distribution generally shifted more toward the lower soil levels with 60% of the measured root length at 60 cm depth for digestate broadcast fertilized plants and 55% for NPK fertilized plants. Digestate depot fertilized plants as well as control plants still had the higher share of the measured root length in 30 cm depth with 55 and 60%, respectively.

Biomass and Mass Fraction

Rhizotrons

In rhizotrons biomass increased on average by 40% from 0.7 g after 1 month to 1 g after 2 months, with no significant difference between treatments (**Figure 3**). However, after 90 days the biomass of digestate depot fertilized plants had increased by a factor 5–6.5 g. Broadcast fertilization with digestate or NPK did

TABLE 2 | Soil and leaf analysis of *S. hermaphrodita* grown in rhizotrons.

		Soil				Plant
	Depth/ Radius (cm)	Ammonium ppm	Nitrite ppm	Nitrate ppm	pH	Leaf nitrogen %
Day 30						
Digestate broadcast (depth)	10	14.2 ± 3.5	1.5 ± 1.0	96.7 ± 33.1	6.4 ± 0.2	4.3 ± 0.6
	30	0 ± 0	0 ± 0	79.1 ± 31.0	7.0 ± 0.1	
Digestate depot (radius)	3	54.7 ± 10	92.5 ± 32.2	132.0 ± 45.0	8.5 ± 0.2	2.9 ± 0.3
	6	59.9 ± 7.1	86.4 ± 25.2	21.0 ± 11.7	6.4 ± 0.1	
	12	0 ± 0	5.4 ± 5.0	100.0 ± 51.8	7.1 ± 0.1	
NPK (depth)	10	37.3 ± 3.0	0 ± 0	138.6 ± 48.6	6.4 ± 0.1	4.3 ± 0.7
	30	0 ± 0	0 ± 0	58.6 ± 21.0	7.1 ± 0.1	
Day 60						
Digestate broadcast (depth)	10	5.1 ± 3.5	0 ± 0	250.4 ± 35.0	7.2 ± 0.0	4.9 ± 0.5
	30	0.1 ± 0.1	0 ± 0	3.1 ± 2.9	7.3 ± 0.0	
Digestate depot (radius)	3	0 ± 0	0 ± 0	187.9 ± 28.0	7.5 ± 0.1	4.1 ± 0.7
	6	91.8 ± 75.6	0 ± 0	161.0 ± 26.4	6.8 ± 0.0	
	12	78.3 ± 73.2	0 ± 0	54.4 ± 21.1	7.3 ± 0.1	
NPK (depth)	10	5.7 ± 1.9	0 ± 0	89.7 ± 15.4	6.8 ± 0.1	4.7 ± 0.4
	30	0 ± 0	0 ± 0	0 ± 0	7.3 ± 0.0	
Day 90						
Control (depth)	10	0 ± 0	0 ± 0	0 ± 0	7.3 ± 0.1	1.7 ± 0.2
	30	0 ± 0	0 ± 0	0 ± 0	7.3 ± 0.2	
Digestate broadcast (depth)	10	0 ± 0	1.8 ± 0.5	130.6 ± 34.1	7.0 ± 0.1	4.1 ± 0.4
	30	0.3 ± 0.2	0 ± 0	1.1 ± 1.0	7.2 ± 0.1	
Digestate depot (radius)	3	0 ± 0	0 ± 0	124.4 ± 18.2	7.1 ± 0.2	4.4 ± 0.5
	6	3.9 ± 1.4	0 ± 0	137.6 ± 16.9	6.6 ± 0.1	
	12	0 ± 0	1.3 ± 0.8	54.8 ± 14.8	7.0 ± 0.0	
NPK (depth)	10	0.1 ± 0.1	0 ± 0	110.4 ± 25.0	6.5 ± 0.1	4.1 ± 1.1
	30	0.2 ± 0.2	0.8 ± 0.5	0.0 ± 14.7	7.5 ± 0.1	

Toxic nitrite concentrations occur especially after 30 days in a close radius to the digestate depot. All fertilization treatments were adjusted to a digestate application of 40 t ha^{-1}. Digestate broadcast: homogeneously incorporated in the top 20 cm; Digestate depot: localized digestate application; NPK: homogeneously incorporated in the top 20 cm; Control: no fertilization. Rhizotrons n = 8 replicates for each treatment and sampling date; ± indicates the standard error.

not result in a significant increase in biomass compared to the unfertilized control. At that time, unfertilized control plants as well as NPK and digestate depot fertilized plants accumulated approx. 50% of their total biomass in roots. Further, for digestate depot fertilized plants, half of the root biomass was located within a 6 cm radius around the digestate depot (**Figure 4**). Plants that received digestate broadcast fertilization had a 40% smaller root mass fraction than unfertilized control plants while localized digestate depot fertilization and mineral NPK fertilization did not show a significant difference to unfertilized control plants.

Mesocosms

After three growing seasons in mesocosms, no significant difference between total biomass of plants fertilized with biogas digestate broadcast or depot fertilization was found (**Figure 3**). Depot fertilized plants developed a 25% larger root system than broadcast fertilized plants. NPK fertilized plants produced 20% less biomass than digestate-fertilized plants but six times more than the unfertilized control plants. Digestate depot fertilization resulted in nine times more biomass compared to the unfertilized control and broadcast digestate fertilization seven times, respectively.

Leaf Nitrogen Content in Rhizotrons

In rhizotrons, the nitrogen concentration of the leaves for digestate broadcast and NPK fertilized plants was between 4 and 5% throughout the experimental time of 3 months (**Table 2**). After 30 days, digestate depot fertilized rhizotron plants only contained 2.9% of nitrogen in their leaves. After 60 days, nitrogen concentration increased to 4.1% and after 90 days it was in the same range as measured for digestate broadcast and NPK fertilized plants. Unfertilized control plants only contained 1.7% of nitrogen.

DISCUSSION

Nitrogen Turnover in Rhizotrons

In accordance with the processes described of the CULTAN Method – proposed by Sommer (2003) for mineral ammonium fertilizers, the main nitrogen form of the digestate was

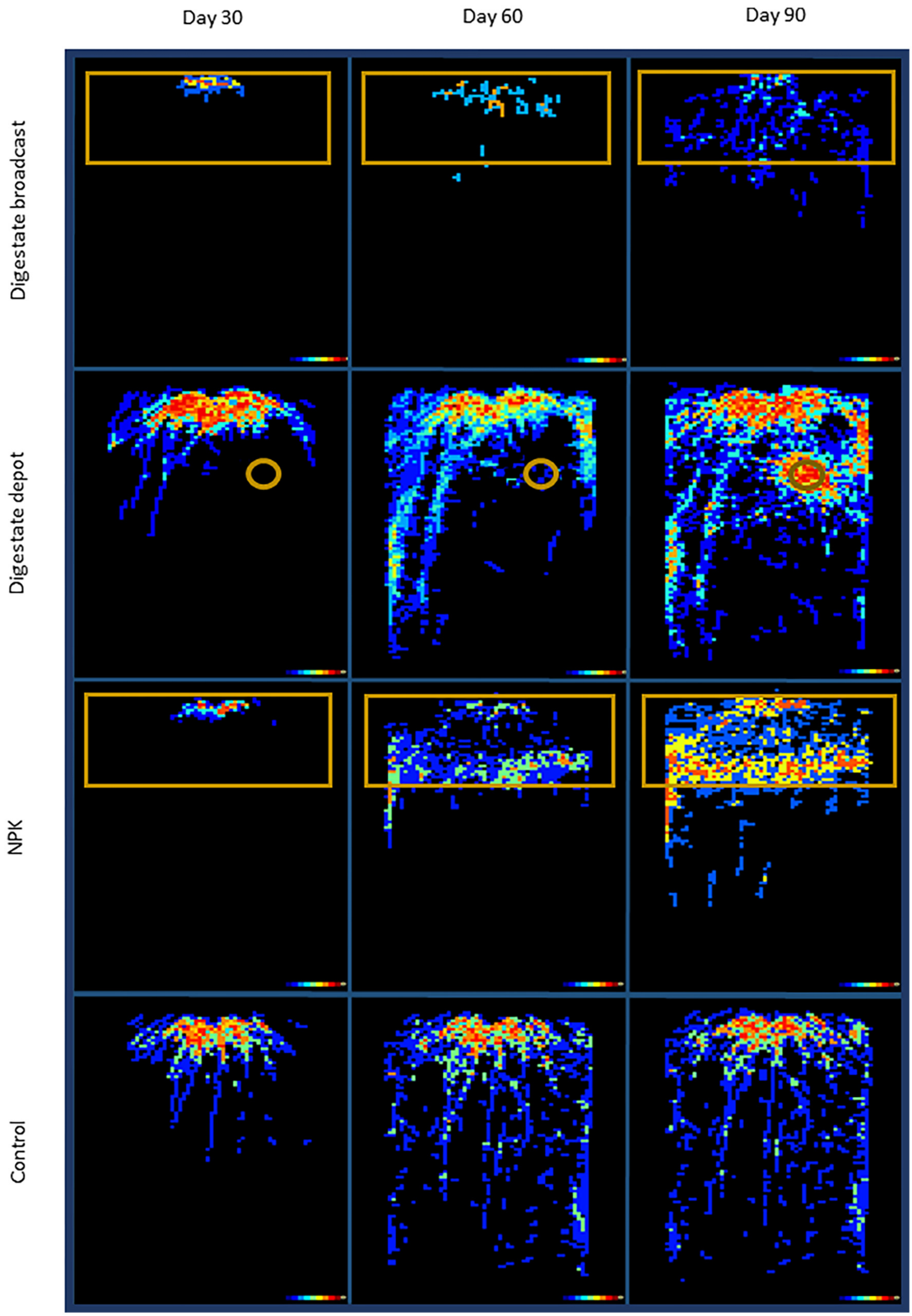

FIGURE 2 | Hit-Map of rhizotrons. In digestate depot fertilized rhizotrons roots of *S. hermaphrodita* avoid the digestate depot zone for 60 days but form a dense root cluster in the depot zone after 90 days. Colors indicate the number of replicates that grew roots at the specific pixel (black: none; blue: 1–2; green 3–4; orange 5–6; red 7–8 replicates). Orange frames illustrate the fertilized zones of the rhizotrons. All fertilization treatments were adjusted to a digestate application of 40 t ha^{-1}: Control: no fertilization; Digestate broadcast: homogeneously incorporated in the top 20 cm; Digestate depot: localized digestate application; NPK homogeneously incorporated in the top 20 cm; n = 8 replicates for each treatment and time of harvest.

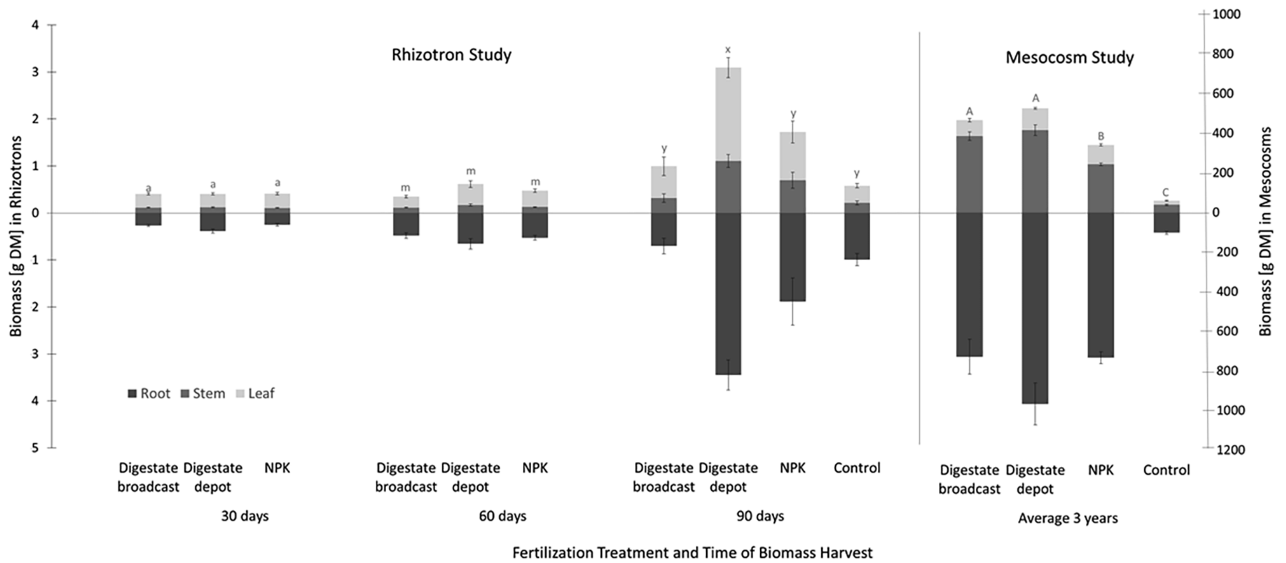

FIGURE 3 | Plant biomass yield of *S. hermaphrodita* in rhizotrons and mesocosms. All fertilization treatments were adjusted to a digestate application of 40 t ha^{-1}. Digestate broadcast: homogeneously incorporated in the top 20 cm; Digestate depot: localized digestate application; NPK: homogeneously incorporated in the top 20 cm; Control: no fertilization. Rhizotrons: $n = 8$ replicates for each treatment and time of harvest; Mesocosms: $n = 7$ replicates for each treatment harvested in 2014, 2015, and 2016. Root biomass was only measured in 2016. Values labeled with the same letter are not significantly different at $p < 0.05$; error bars indicate the standard error.

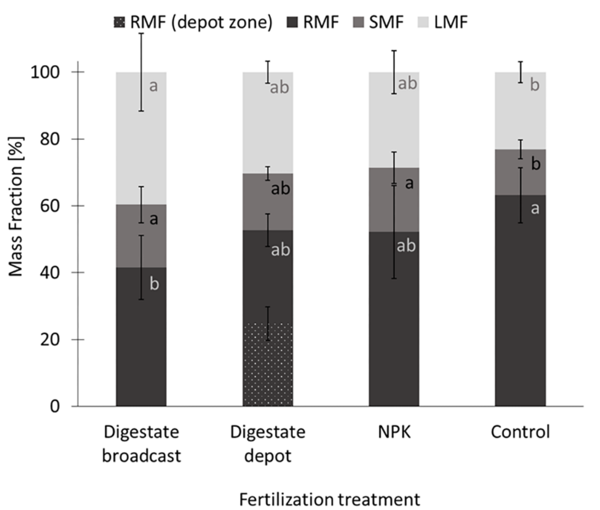

FIGURE 4 | Mass fractions of *S. hermaphrodita* grown in rhizotrons for 90 days. RMF, root mass fraction; depot zone, zone in a 6 cm radius around the digestate depot; SMF, stem mass fraction; LMF, leaf mass fraction. All fertilization treatments were adjusted to a digestate application of 40 t ha^{-1}. Digestate broadcast: homogeneously incorporated in the top 20 cm; Digestate depot: localized digestate application; NPK: homogeneously incorporated in the top 20 cm; Control: no fertilization; $n = 8$ replicates for each treatment. Values labeled with the same letter are not significantly different at $p < 0.05$. Error bars indicate the standard error.

mineralized from ammonium to nitrate. For digestate broadcast and NPK fertilization 80–90% of the measured nitrogen was available in the form of nitrate as early as 30 days after fertilization. Nitrate, compared to ammonium, is very mobile in the soil. Accordingly, nitrate was also the form of nitrogen that was found in the non-fertilized lower horizon of the rhizotrons. Since to the lower horizon, no nutrients were added, they must have arrived there by leaching (Baker, 2001). In rhizotrons receiving digestate depot fertilization, conversion from ammonium to nitrite was faster than the conversion from nitrite to nitrate. Accordingly, nitrite accumulated in a radius of 6 cm around the digestate depot. In digestate depot fertilized rhizotrons, digestate was applied locally in very high amounts and thus contained highest NH_4^+ concentrations. The combination of a regular watering of the rhizotrons with high concentration of organic material with a C/N-ratio of approx. 6 in the depot fertilized zone may have allowed for high microbial activity and created an environment with partially anoxic conditions that favors the formation of nitrite (Nelson and Bremner, 1969; Van Cleemput and Samater, 1995; Möller and Müller, 2012). In addition, the alkaline pH near the digestate fertilized zone favors the formation of nitrite in sandy soils (Greco et al., 2012). Once nitrite starts to accumulate in the soil, it can inhibit microbial activity. In a feedback loop, this may have further favored the formation of even more nitrite as transformation from ammonium to nitrite is not as much effected as the succeeding transformation from nitrite to nitrate (Grant et al., 1979). Nitrate, the mobile form of nitrogen was found from 12 cm distance to the digestate depot (Baker, 2001). Ammonium was only found up to a distance of 6 cm from the digestate depot, as it is not very mobile in soils (Clarke and Barley, 1968; Pang et al., 1973). In our study, 60 days after fertilization, ammonium was almost completely mineralized to nitrate, leaving only traces of ammonium and nitrite in digestate broadcast and NPK fertilized rhizotrons. In digestate depot fertilized rhizotrons, also after 60 days, high concentrations of ammonium were present, indicating a delayed conversion from ammonium to nitrate compared to broadcast fertilization (Grant et al., 1979; Sommer, 2005). Even though the high pH of >8 in the near depot zone was found to be very favorable for the mineralization of organic N-compounds from corn residues, like digestate (Deng and Tabatabai, 2000). The pH in the depot zone dropped to a range of pH 6.8–7.5 until day 60 after fertilization partially due to diffusion and partially due to the release of protons in the mineralization process (Giusquiani et al., 1995; Barak et al., 1997). Yet, also in digestate depot fertilized rhizotrons almost all ammonium was mineralized to nitrate within 90 days leaving only traces of nitrite and ammonium near the digestate depot.

Nutrient Status and Root-Growth

Rhizotrons

The root growth of *S. hermaphrodita* plants responded to the different fertilizer distributions and consequently different available forms of nitrogen over time. Even though there were no significant differences between fertilizer treatments in terms of biomass, clear differences in root distribution were observed (**Figures 2**, **3**). The broadcast application of digestate and NPK fertilizer, combined with a regular irrigation resulted in optimal supply of nutrients for seedlings of *S. hermaphrodita* (also indicated by the leaf nitrogen content >4%), reducing the necessity of seedlings to invest into root-growth (Forde and Lorenzo, 2001). Contrastingly, no nutrients were available in unfertilized control plants, while nutrients for digestate depot fertilized plants were not available in the rhizosphere (observable via the low leaf nitrogen content <3%). Consequently, plants invested into a deep and far-reaching root system to gain access to nutrients. These findings match the theory behind the CULTAN-method proposed by Sommer (2003) and also correspond well with results reviewed by Nkebiwe et al. (2016), focusing on controlled placement of fertilizers rich in ammonium. Additionally, the roots of plants growing in digestate depot fertilized rhizotrons, avoided to colonize a zone of approximately 6 cm radius from the digestate depot. This growing pattern was even more pronounced after 60 days. Here, roots in zones of the rhizotrons, not effected by fertilization, reached already the bottom of rhizotron while still very little root growth was observed in the 6 cm radius zone around the digestate depot. Given the high concentrations of ammonium and nitrite found within the 6 cm radius zone around the digestate depot in combination with root damages observed at root tips that contacted the area (**Figure 5**), we conclude that nitrite and ammonium toxicity are the main causes for the avoidance of this zone by the roots within the first 60 days after transplanting (Pan et al., 2016). Again, this temporarily zone of avoidance in a localized depot has been described earlier by Sommer (2003). Oke (1966) found that on sandy soil already nitrite concentrations >40 ppm can be toxic, especially to plant seedlings. In this experiment, we measured nitrite concentrations of <90 ppm in the depot zone. Further, ammonium toxicity was already studied intensively and observed when organic fertilizers, based on biogenic residues after anaerobic digestion were applied (Shaviv, 1988; Salminen and Rintala, 2002; Nkebiwe et al., 2016; Pan et al., 2016). Overall, results show consistently that high ammonium doses from digestate negatively affect seedling growth, mainly due to hampered root development.

After 60 days, the root growth pattern in depot-fertilized rhizotrons changed dramatically and 90 days after planting a dense root cluster of *S. hermaphrodita* around the nutrient rich digestate depot was observed. Root proliferation in nutrient rich patches was not observed yet for *S. hermaphrodita*, but for several herbaceous plant species (Rajaniemi and Reynolds, 2004). Plants can increase their rooting density in nutrient rich patches to forage for these nutrients, increasing the nutrient uptake efficiency and thus confer a competitive advantage toward weeds without access to nutrient patches (Robinson et al., 1999; Hodge, 2004; Rajaniemi, 2007). The increased access to nutrients is also shown by a parallel increase in leaf nitrogen content from only 2.9% at 60 days after transplanting to 4.4% measured in leaves of digestate depot fertilized *S. hermaphrodita* plants 90 days after transplanting. The massive increase of root biomass in the depot zone between day 60 and day 90 after planting, contributing to 50% of the total root mass of digestate depot fertilized *S. hermaphrodita* plants, resulted in the highest root mass across the four treatments (**Figure 1**). Also in our mesocosm study, the formation of dense root clusters in the digestate depot fertilized zone was observable throughout the 3-year experimental time under outdoor conditions (**Figure 6**).

In rhizotrons, after 90 days, NPK and digestate broadcast fertilization did not result in maximum possible rooting depth (i.e., depth of the rhizotron), and the major share of the root systems were located in the fertilized horizon. As plants were watered throughout the experiment, and roots had already sufficient access to the nutrients (indicated by the leaf nitrogen content >4%) plants did not invest into the formation of a deep reaching root system (Poorter et al., 2012b). Overall, the root systems that developed following digestate broadcast treatment remained smaller than the root systems of NPK fertilized plants. Phytotoxic effects of digestates, particularly affecting root growth of plants at early developmental stages, have been reported earlier and have besides ammonium toxicity also been explained by high concentrations of organic acids in fresh digestates (Salminen and Rintala, 2002; Abdullahi et al., 2008; Drennan and DiStefano, 2014). Control plants that had no access to nitrogen in the sandy substrate, resulting in a low leaf nitrogen content <2% and high investment into a deep reaching root-system, resulted in the highest root mass fraction of all variants (**Figure 4**) and thereby corresponded well to findings of previous studies (Poorter et al., 2012b).

Mesocosms

In mesocosms, the root mass fraction of *S. hermaphrodita* was generally higher after three growing seasons than found in rhizotrons. We explain this by the fact, that *S. hermaphrodita* is a perennial species, of which the shoots die off at the end of each vegetation period, while the major part of the root system stays intact and also serves as reservoir for a successful regrowth

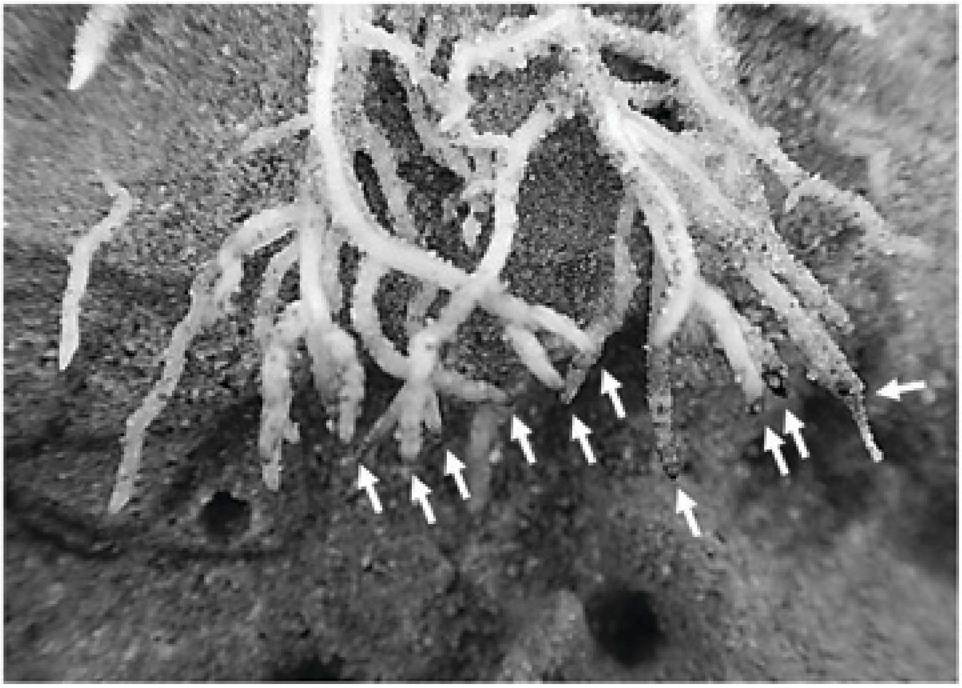

FIGURE 5 | Brown and rotten root tips of *S. hermaphrodita* grown in digestate depot fertilized rhizotrons occurred in a 6 cm radius around the digestate depot 60 days after planting. Digestate depot fertilization was calculated to simulate a digestate application of 40 t ha^{-1}. Arrows indicate the damaged root tips.

in the following growing period (Borkowska et al., 2009). As we only measured the root mass after three growing periods of mesocosms grown plants, this has a clear effect in the root mass fraction when compared to root mass measured after 90 days in rhizotrons.

Biomass Yield

Rhizotrons

In rhizotrons, the above ground biomass increased gradually until day 60 of the experiment as plants invested mainly in root-growth. Accordingly, no significant differences in biomass of *S. hermaphrodita* were observed at day 60 throughout all treatments. This changed drastically when roots in digestate depot fertilized rhizotrons started to access the nutrient rich depot zone. The intense foraging for nutrients resulted in a faster increase in stem and leaf biomass as observed in all other variants. In his meta-analysis over 39 studies, Nkebiwe et al. (2016) also found a positive effect of localized fertilizer placement on biomass yield, especially for early plant development stages. Specifically for organic fertilizers like poultry litter (Pote et al., 2011), separated dairy sludge (Bittman et al., 2012) and manure slurries (Dell et al., 2000), the localized application had a positive effect on the total biomass.

Broadcast application of digestate yielded less biomass than NPK fertilization, even though leaf and soil analysis showed an adequate supply with nutrients and water for both variants. However, the root system of digestate broadcast fertilized plants was smaller and less outstretched as the root system of NPK fertilized plants as discussed earlier.

Mesocosms

After three vegetation periods of *S. hermaphrodita* in mesocosms under outdoor conditions, the different fertilization treatments showed different responses with respect to biomass than the same treatments in the rhizotrons. No significant biomass yield difference between digestate depot and digestate broadcast fertilization was observed. As discussed earlier, the localized placement of fertilizers has beneficial effects, especially in the early development stages (Nkebiwe et al., 2016). Further, the phytotoxic effects of the digestate, especially observed on the root growth of digestate broadcast fertilized plants in the rhizotrons, also relates to the early development stages of plants (Möller and Müller, 2012). Accordingly, it cannot be expected that effects last over this 3-year experimental time, especially as *S. hermaphrodita* is a perennial plant that keeps its extensive root system over the years (Borkowska et al., 2009).

The fact that digestate fertilization, independently from the type of application, produced higher biomass yields than mineral NPK fertilization, can be related to an increase of soil fertility over the years and has been discussed in a separate publication (Nabel et al., 2017).

General Discussion

Localized application of biogas digestate as digestate depot in the rhizosphere of *S. hermaphrodita* seedlings fostered the successful establishment of *S. hermaphrodita* on marginal soils. Digestate depot fertilization strongly increased the rooting depth of seedlings, allowing improved access to water which could make them less susceptible to drought stress (Ma et al., 2009;

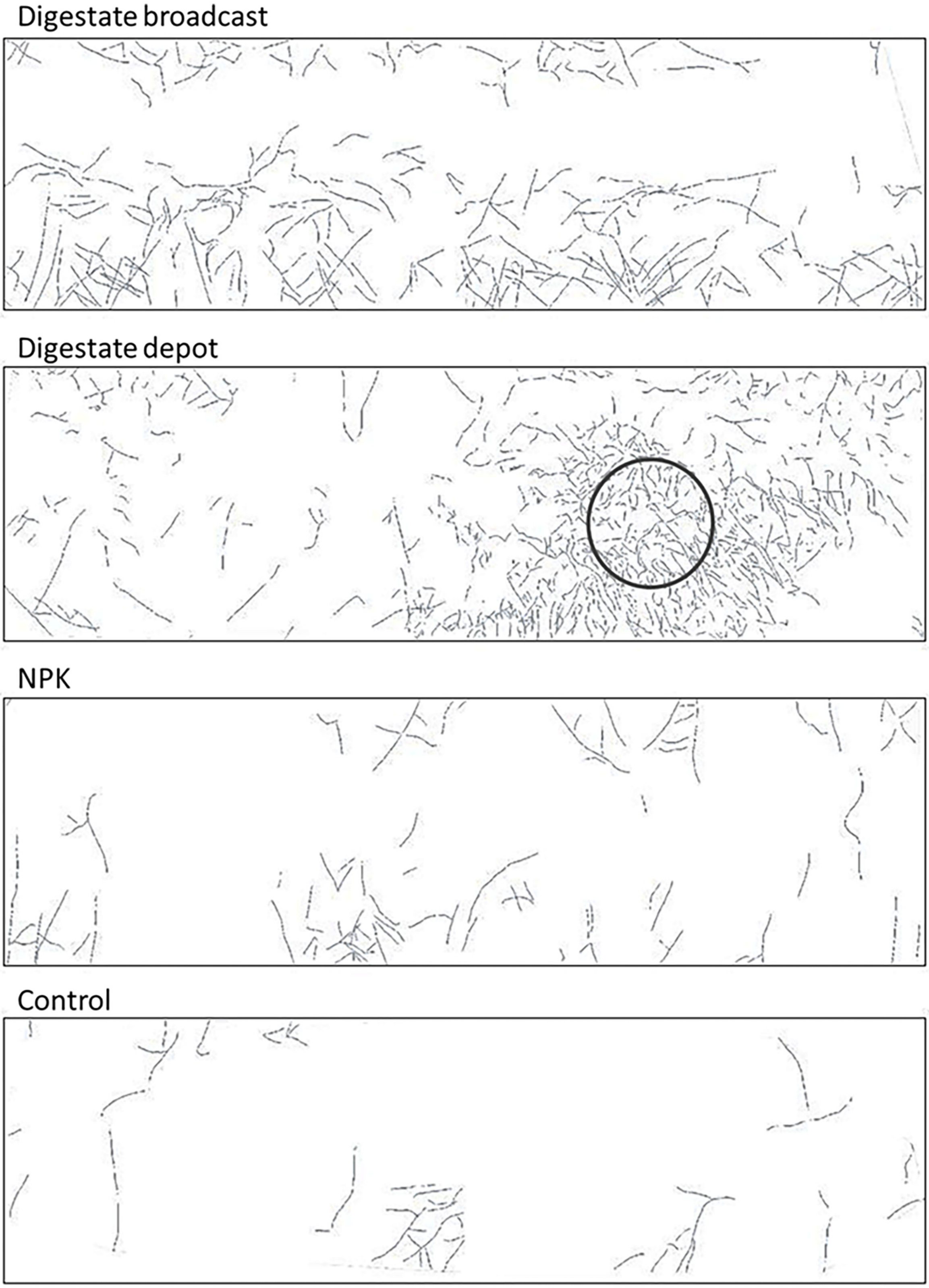

FIGURE 6 | Root distribution of *S. hermaphrodita* cultivated in mesocosms measured by mini-rhizotrons after 2 years in 30 cm depth: roots form a dense root cluster in the depot-fertilized zone. Control: no fertilization; Digestate broadcast: homogeneous surface application; Digestate depot: localized digestate application; NPK homogeneous surface application. Pictures show a representative example of $n = 4$ replicates for each treatment and time of harvest.

Su et al., 2015). Drought can be the main growth-limiting factor, especially on marginal sandy soils with low WHC, making this deep rooting side effect of depot fertilization a positive one for adapting to drought regimes (Borkowska et al., 2009). The root-cluster formation around the digestate depot zone allowed the *S. hermaphrodita* seedling good access to the nutrients, resulting in rapid increase of biomass. Earlier studies showed that this can be an efficient way to make plants more competitive against weeds and thus lower the need for additional weed control (Blackshaw et al., 2002; Melander et al., 2005). Even though in the presented mesocosms study digestate depot fertilization did not show an increased biomass yield compared to digestate broadcast fertilization after three growing seasons, we still see a high potential in the application of digestate in the form of localized depots in the rhizosphere in marginal soils. As *S. hermaphrodita* is a perennial crop with an extensive root system, any form of soil cultivation in order to incorporate the digestate after a broad surface application via, e.g., plowing could do severe harm to the plants. In addition, a lack of digestate incorporation could cause high volatile losses of ammonia, with high environmental costs (Rochette et al., 2009; Ma et al., 2010). A controlled placement of digestate depots in the rhizosphere, e.g., via spike-weal injection, would minimize the impact on soil and also minimize volatile losses of ammonia (Kozlovský et al., 2009). As this application technique might also contribute to an increased competitiveness of *S. hermaphrodita* over weeds and thus reduce the need to weed control, it would further strengthen the concept of extensive biomass production on marginal soils.

CONCLUSION

The application of digestate as a fertilizer in local depots into the rhizosphere of *S. hermaphrodita* grown in a marginal sandy substrate in rhizotrons and mesocosms resulted in a dense root formation around the depot-fertilized zone. After 3 months, half of the root biomass of depot-fertilized plants grown in rhizotrons was associated with the depot zone as a dense root-cluster; in contrast, root growth in the digestate broadcast fertilization treatment was strongly reduced. Overall, digestate depot fertilization resulted in a fivefold increase of total biomass compared to digestate broadcast fertilization in rhizotrons. Under outdoor conditions in a 3-year mesocosm experiment the increase in plant biomass was not significant, however. We conclude that digestate depot fertilization can contribute to an improved cultivation of *S. hermaphrodita* as a perennial energy-crop on marginal soils, especially for a successful establishment of seedlings, but its potential growth stimulation effect now needs more research under field conditions.

AUTHOR CONTRIBUTIONS

MN, SDS, HP, RK, and NDJ conceived the study. MN performed the main experiments and conducted the research under the supervision of SDS, HP, RK, and NDJ. MN wrote the manuscript. KN planned and designed *GrowScreen-Rhizo2*. CD and CB processed images and analyzed data. VT helped with study design and data evaluation. All authors discussed the results, assisted in the manuscript preparation, and contributed to revisions.

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Simulation of Soil Organic Carbon Effects on Long-Term Winter Wheat (*Triticum aestivum*) Production Under Varying Fertilizer Inputs

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Soil organic carbon (SOC) has a vital role to enhance agricultural productivity and for mitigation of climate change. To quantify SOC effects on productivity, process models serve as a robust tool to keep track of multiple plant and soil factors and their interactions affecting SOC dynamics. We used soil-plant-atmospheric model viz. DAISY, to assess effects of SOC on nitrogen (N) supply and plant available water (PAW) under varying N fertilizer rates in winter wheat (*Triticum aestivum*) in Denmark. The study objective was assessment of SOC effects on winter wheat grain and aboveground biomass accumulation at three SOC levels (low: 0.7% SOC; reference: 1.3% SOC; and high: 2% SOC) with five nitrogen rates (0–200 kg N ha^{-1}) and PAW at low, reference, and high SOC levels. The three SOC levels had significant effects on grain yields and aboveground biomass accumulation at only 0–100 kg N ha^{-1} and the SOC effects decreased with increasing N rates until no effects at 150–200 kg N ha^{-1}. PAW had significant positive correlation with SOC content, with high SOC retaining higher PAW compared to low and reference SOC. The mean PAW and SOC correlation was given by PAW% = 1.0073 × SOC% + 15.641. For the 0.7–2% SOC range, the PAW increase was small with no significant effects on grain yields and aboveground biomass accumulation. The higher winter wheat grain and aboveground biomass was attributed to higher N supply in N deficient wheat production system. Our study suggested that building SOC enhances agronomic productivity at only 0–100 kg N ha^{-1}. Maintenance of SOC stock will require regular replenishment of SOC, to compensate for the mineralization process degrading SOC over time. Hence, management can maximize realization of SOC benefits by building up SOC and maintaining N rates in the range 0–100 kg N ha^{-1}, to reduce the off-farm N losses depending on the environmental zones, land use and the production system.

Keywords: grain yield, DAISY model, nitrogen, plant available water, pedotransfer functions, long-term experiment, crop productivity

INTRODUCTION

Soil organic carbon (SOC) supports multiple soil functions determining soil physical, chemical and biological quality parameters (Reeves, 1997; Pan et al., 2009) contributing to the productive capacity of soils for food, fodder, and energy production (Lal, 2004). A number of factors influence SOC stocks and flows, spatially and temporally, in an ecosystem due to climate, land use, soil management, and cropping systems (Canadell et al., 2007). Building up SOC stock through agricultural measures (e.g., cover cropping, residue incorporation, reduced tillage) can affect soil properties, soil water retention and nutrient storage, affecting the productive capacity of soils (Ingram et al., 2016; Paustian et al., 2016). Decomposition of SOC releases mainly N, which can increase crop yields where crop N supply is limited (Palmer et al., 2017). Maintenance or build-up of SOC will require regular inputs of organic matter (OM) into the soil as the mineralization process will continually deplete the SOC over time, especially in environmental zones, where soil moisture and temperature are conducive for the mineralization process. The other effects of increased SOC content are decrease in the bulk density (Chen et al., 2017; Palmer et al., 2017; Minasny and McBratney, 2018) and small increase in volumetric water holding capacity (Rawls et al., 2003). Due to these multiple effects, there is a great interest to quantify SOC effects in agro-ecosystems. SOC increase can have positive and negative effects (Palmer et al., 2017; Minasny and McBratney, 2018). Among the multiple SOC effects, crop productivity and soil water retention are the priorities of the farmers to maintain sustainable agro-ecosystems. As European arable cropping systems are estimated to lose 300 Tg C (10^{12}) year^{-1} (Janssens et al., 2003), it is necessary to segregate the SOC effects on crop yields and soil water retention and their combined synergistic benefits on crop productivity. Hence, quantification of SOC effects on N supply, soil water retention and crop productivity under varying fertility production system provides a science-based evidence of SOC benefits for making management decisions by farmers.

Winter wheat is one of the most widely cultivated arable crops, and the assessment of SOC-productivity relationship can generate insights into wheat crop management at field scale (Hansen et al., 2000; Christensen et al., 2009). An earlier study assessed SOC effects in winter wheat agro-ecosystem in seven sites representing diverse soil types, SOC content, management and climate including Netherlands (Palmer et al., 2017) found that SOC benefits are tangible in N deficient wheat production systems, whereas the benefits disappear in wheat agro-ecosystems, with surplus N. To add to this body of knowledge, this study provided insights into SOC effects under a context-specific set of soil type, SOC content, management and climate regimes in Denmark. Further, this study provided additional value to the findings of Palmer et al. (2017) because the range of SOC used in our study (0.7–2% SOC) is different than the SOC considered in Netherlands (2.8% and 4.3% SOC). SMARTSOIL[1] consortium had access to the SOC and agronomic data on winter wheat from a long-term field trial in Askov from 1929 to 2008 and the field data provided us a unique opportunity to carry out the calibration and validation of DAISY model, to assess the productivity and SOC dynamics under winter wheat cultivation over 80 years. Hence, the study objective was to determine winter wheat productivity at three SOC ranges viz. low: 0.7% SOC; reference: 1.3% SOC; and high: 2% SOC with five nitrogen rates (0–200 kg N ha^{-1}) and plant available water (PAW) at the low, reference, and high SOC levels.

[1]http://smartsoil.eu/

MATERIALS AND METHODS

Long-Term Field Trial in Askov

The long-term trial site in Askov (LTE-Askov; 55°28′N, 09°06′E) was established in 1923 and cropping system was 4-year crop rotation cycle of winter wheat, root crop, spring cereal and grass-clover from 1929 to 2008. In the 0–0.20 m plow layer, SOC was 1.3% and sand, silt and clay contents were 76%, 13%, and 11%, respectively, and the bulk density at plow layer was 1.5 g cm^{-3} (Christensen et al., 2006). LTE-Askov treatments consisted of two treatments, viz: Askov_0N and Askov_1.5NPK, implemented in a 4-year crop rotation cycle. Askov_0N treatment received no input of farmyard manure, nitrogen (N), phosphorus (P), and potassium (K) and crop residues were removed and Askov_1.5NPK treatment received 150 kg N, 28.5 kg P and 131.4 kg K ha^{-1}. The measured field data from Askov_0N and Askov_1.5NPK winter wheat plots for the period 1929–2008, were split into calibration dataset (1929–1969) and validation dataset (1970–2008).

DAISY Model Initialization and SOC Simulations

To assess long-term SOC dynamics in arable production systems, process models serve as a robust tool to keep track of multiple plant and soil factors and their interactions affecting SOC dynamics. The soil-plant-atmospheric model, DAISY, was implemented, due to its robustness for simulation of SOC dynamics and crop productivity in diverse climatic and cropping systems (Abrahamsen and Hansen, 2000). DAISY is a dynamic and deterministic soil-plant-atmosphere system model with separate sub-models for crop growth, C and N dynamics, heat, soil water and fate of pesticide use (Abrahamsen and Hansen, 2000). In the model, OM is constituted by added organic matter (AOM), soil microbial biomass (SMB), and soil organic matter (SOM) pool. AOM and SMB constitute relatively fast and slow turnover pools, whereas SOM is split into three pools; inert (SOM3), fast (SOM2), and slow turnover pools (SOM1), characterized by fixed C:N ratios and first-order decomposition rate coefficients (Hansen et al., 1991). AOM constitutes plant residues, added organic fertilizer or compost, etc.; the SMB pool is driving the biodegradation process and SOM is the recalcitrant humus fraction. Soil C and N dynamics were modeled by assuming constant C:N ratios in each pool (Bruun et al., 2003). The SOM pool, at the start of the simulation period was initialized to a steady state by simulating the pre-experimental period for 10 years before the onset of the experiment (Bruun and Jensen, 2002).

Daisy model was implemented in two steps viz. calibration and validation steps. For calibration step, DAISY model inputs were soil, weather, and winter wheat management data from LTE-Askov. The soil data on sand%, silt%, and clay%, bulk density and 1.3% SOC (hereafter called the "reference") was provided to the model. The weather data was retrieved from the weather database, a common database created by the SMARTSOIL project (see footnote 1) to share and store information on long-term trial sites in SMARTSOIL consortium. Where the weather data was missing, the missing data was generated by the LARS-WG 5 weather generator (Semenov and Barrow, 2002; Semenov and Stratonovitch, 2010) based on statistical characteristics of actual sample of available measured weather data from LTE-Askov. The winter wheat management data included land preparation, sowing, fertilization and harvesting dates and application timing of 0, 50, 100, 150, and 200 kg N ha^{-1}. Every year, the winter wheat was sown on 20th September and harvested on 20th August in Askov_0N and Askov_1.5NPK plots. The same planting and harvesting schedule was followed during the simulation period to reduce the yield variability due to these two factors. The N rates of 0, 50, and 100 kg N ha^{-1} was applied on 15th March and the N rates of 150 and 200 kg N ha^{-1} was split into two equal doses. The two equal dose consisted of basal dose on 15th March (50% of application rate) and second dose on 25th April (50% of application rate) to coincide with the critical growth stages of winter wheat for maximum uptake of N. In order to accommodate the residual nitrogen effect after the preceding glass-clover in the 4-year crop rotation, the model was provided with nitrogen dose of 40 kg N ha^{-1} (Høgh-Jensen and Schjoerring, 1996) in Askov_0N and Askov_1.5NPK plots.

In order to assess SOC effects on winter wheat productivity, the validated DAISY model was run with 0.7% SOC (low) and 2% SOC (high) in addition to model run with 1.3% SOC during the calibration and validation steps. The low, reference, and high SOC levels reflected the spectrum of SOC levels in Danish soils from sandy to loamy soils and the N rate reflected the standard N application rate in winter wheat production in Denmark. Each SOC level (low, reference, and high) was simulated under five different N rates. Each simulation run sequence consisted of an initial 10 years of the pre-experimental period followed by simulation of low, reference, and high SOC content under five N rates (0–200 kg N ha^{-1}) for the period 1929–2008. The initial 10-year run was included in every simulation run to stabilize the treatment effect to a steady state. Each simulation was carried out with low, medium, and high SOC and the same SOC was used for the entire simulation period (1970–2008), which provided the SOC trends over the years during the simulation period. However, after each year of simulation, the same management practice was reset into the model with same dates for land preparation, sowing, fertilization, and harvest dates in each year, during the entire simulation period. Each cycle of wheat production starts with land preparation on 01 September, consisting of plowing the field, followed by seedbed preparation, sowing, fertilizer application, and harvesting.

Pedotransfer Functions (PTF) for Determination of Plant Available Water (PAW)

To assess the SOC effects on PAW (m^3 m^{-3}), the correlation between the PAW and SOC was derived by regressing PAW contents at the low, reference, and high SOC contents. LTE-Askov soil data on clay%, silt%, OM%, and bulk density were used in PTF functions to derive saturated moisture content (θ_s), residual moisture content (θ_r), van Genuchten curve-fitting parameter α (1/cm = α) and van Genuchten curve-fitting parameter n and $m = 1 - 1/n$ (Wösten et al., 2001). Four different PTF functions calculated the PAW to compare the differences and improve the reliability in estimation of hydraulic properties. The PTF functions were (a) HYPRES (Wösten et al., 1999), (b) hydraulic properties calculator (HPC) (Saxton and Rawls, 2006), (c) Rosetta model (Schaap et al., 1998), and (d) Danish PTF (Borgesen and Schaap, 2005). HYPRES PTF functions were developed based on 5,521 soil horizon profiles from different countries in Europe (Wösten et al., 1998) whereas HPC was developed with data from 1,722 United States soil samples (Saxton et al., 1986). The Rosetta model was built on the United States soil database whereas the Danish PTF is based on 3,226 soil samples from Denmark (Borgesen and Schaap, 2005). Subsequently, soil water content was calculated at different soil water potentials (kPa) by van Genuchten–Mualem model (VGM) (Vereecken et al., 2010), and PAW was considered as the difference in soil water content between the wilting point and the field capacity. We defined field capacity at 10 kPa and wilting point at 1,500 kPa and the difference of soil water content between the field capacity and the wilting point was taken as PAW.

Model Calibration, Validation, and Statistics

The model validation was carried out with MODEVAL 2.0 (Smith et al., 1997) by comparative plotting of measured and simulated SOC content in 0–0.20 m soil profile over 1970–2008 in winter wheat plots in Askov_0N (**Figure 1A**, RMSE = 4.05%) and Askov_1.5NPK treatments (**Figure 1C**, RMSE = 7.9%) and grain yields in Askov_0N (**Figure 1B**, RMSE = 5.83%). Measured SOC and grain yields were available every 4 years (4-year crop rotation) and so, 10 measured values were available for 1970–2008 period and the corresponding simulated values from the same 1970–2008 period were used for validation (**Figures 1A–C**). ANOVA tests were run on to assess effect of SOC, N and SOC × N on winter wheat grain and aboveground biomass yields at low, reference, and high SOC under 0–200 kg N ha^{-1}. The standard error and $LSD_{0.05}$ of the simulated values were calculated in MS excel using the data analysis tool pack and significant effects are denoted as $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, ns, non-significant.

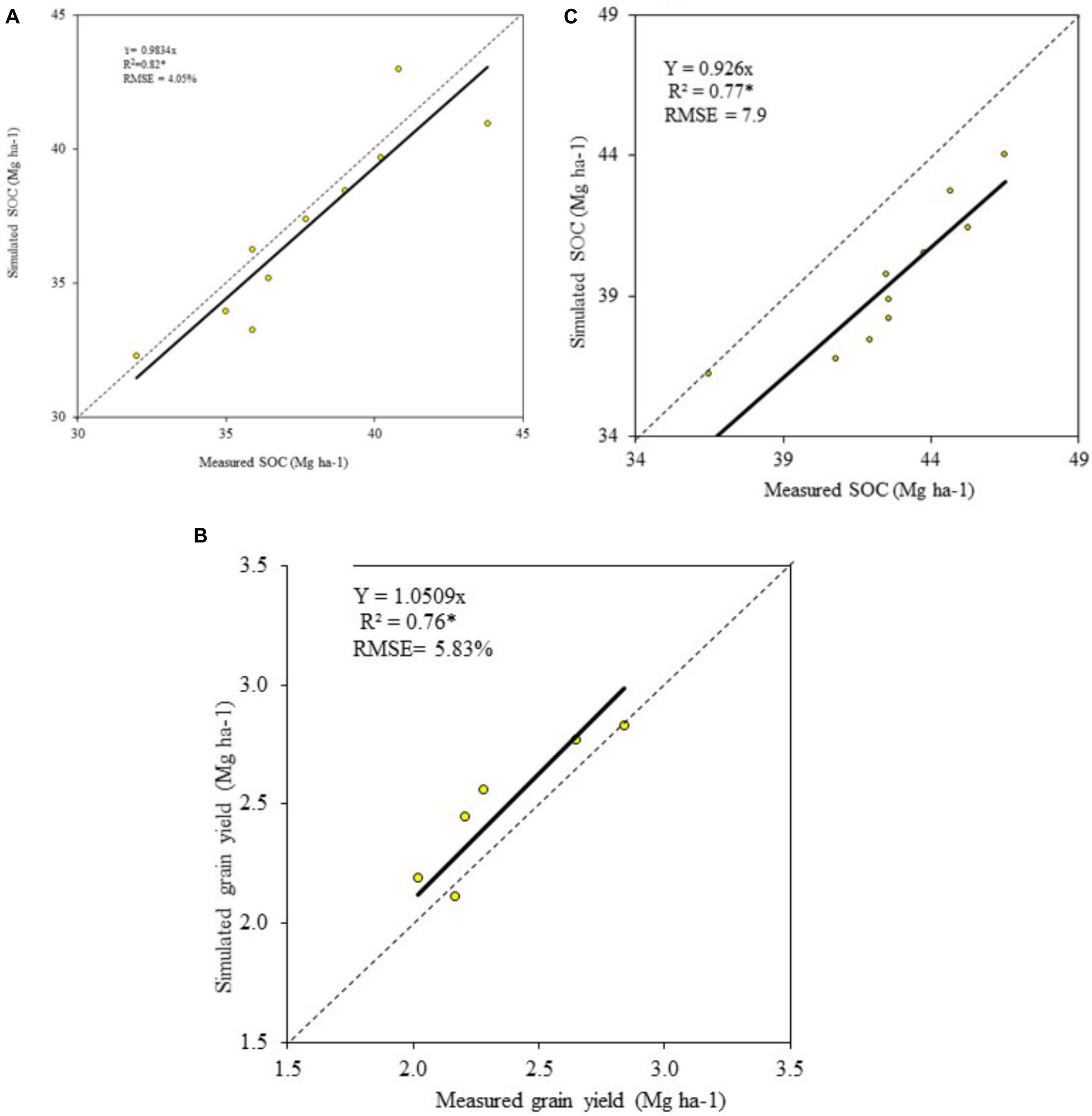

FIGURE 1 | Validation of **(A)** SOC in Askov_0N, **(B)** winter wheat grain yield in Askov_0N, and **(C)** SOC in Askov_1.5NPK plots.

RESULTS

Validation of SOC Dynamics, Winter Wheat Productivity, and PAW

The modeled and the measured values of SOC in 0–0.20 m soil profile in Askov_0N (**Figure 1A**, R^2 = 0.82* and Askov_1.5NPK treatments (**Figure 1C**, R^2 = 0.77*), had significant positive correlation coefficient. Similarly, significant positive correlation coefficient was obtained for winter wheat grain yields in Askov_0N (**Figure 1B**, R^2 = 0.76*). The validation on SOC dynamics to 0.20 m soil depth under fertilized (Askov_1.5NPK, RMSE = 7.9%) and non-fertilized treatments (Askov_0N, RMSE = 4.05%) demonstrated that DAISY was robust in simulation of winter wheat productivity at the tested SOC range under 0–200 kg N ha^{-1} treatments. DAISY model has been used in Denmark to quantify soil water balance (Salazar et al., 2013) and SOC (Bruun et al., 2003). This provided the scientific rationale for using DAISY for simulation of grain yields and aboveground biomass (grain + straw) accumulation at low, reference, and high SOC content under 0–200 kg N ha^{-1} treatments in this study.

The long-term change dynamics of SOC, presents challenges to simulate the SOC dynamics over time due to unavailability of data for calibration and validation of models. In this regard, we had unique access to LTE-Askov data and simulation window of 80 years (1929–2008) to assess the long-term change dynamics of SOC and triangulate the field data with simulated data and its effects on agronomic productivity. We chose DAISY, due to its robustness to keep track of the SOC flows and stocks in the soil, taking account of the plant and the management factors. We validated the DAISY model SOC and grain yield outputs with measured data from Askov_0N and Askov_1.5NPK. The model considers only N as the limiting factor and the grain yields and aboveground biomass are not affected by P and K inputs. In similarity to our study, DAISY model had been used for simulation of crop grain yield and aboveground biomass accumulation in several model comparison exercises (Dewilligen, 1991; Vereecken et al., 1991; Diekkrüger et al., 1995) and validation of crop yield in winter wheat in three sites in the Netherlands (Hansen et al., 1991). In a comparison of nine SOM models to assess management effects (land use, fertilizer, manure, and rotation treatments) on SOC dynamics in seven LTEs in diverse climatic gradients, DAISY outputs were comparable, with a similar margin of error among other models (DNDC, RothC, CENTURY, CANDY, NCSOIL) and even better than the SOMM, ITE, and Verberne models (Smith et al., 1997). This provides a scientific rationale for use of DAISY model to assess SOC dynamics.

Trend Comparisons of Measured and Modeled SOC Data

The measurement of the SOC at the experimental site started in 1923 in Askov_0N and in 1929 in Askov_NPK plot. In 1923, Askov_0N plot had 1.6% SOC content, which decreased to 1.4% by 1969 and to 1.1% by 2008. Similarly, the Askov_1.5NPK had 1.8% SOC in 1929 and it reduced to 1.5% by 1969 and to 1.2% by 2008. Bulk density measurements remained the same throughout the measurement period and so the changes in SOC was due to continuous removal of the crop residues and decomposition of the available SOC in the soil. The measured and the modeled SOC values during the calibration period (1969–2008) showed a similar trend for Askov_0N and Askov_1.5NPK (**Figure 2**) and the correlation between the measured and modeled SOC and grain yield values are provided in **Figure 1A** (SOC), **Figure 1B** (grain yield), and **Figure 1C** (SOC). The measured SOC value provided from the experimental site showed that the SOC range used for simulation is achievable in the soil and climate conditions at the experimental site.

SOC and N Effects on Winter Wheat Grain Yield and Aboveground Biomass (Grain + Straw) Accumulation

Soil organic carbon levels and N rates had significant effects ($P < 0.001$) on winter wheat grain yields and aboveground biomass accumulation. In similarity, SOC × N effects were significant ($P < 0.01$) for grain yields and aboveground biomass accumulation. The SOC × N interactions implied that the SOC level effects differed at varying N application rates from 0 to 200 kg N ha^{-1}.

With 0 kg N ha^{-1}, the winter wheat grain yield increased significantly by 0.28 Mg ha^{-1} from low to reference SOC content and by 0.30 Mg ha^{-1} from reference to high SOC content (**Table 1**). Hence, the increase in grain yields from low to high SOC content was 0.58 Mg ha^{-1}, 31% increase in grain yield, which was a significant improvement in grain yields over the low SOC content. Similarly, at 0 kg N ha^{-1}, the aboveground biomass (straw + grain) increased significantly by 0.59 and 0.63 Mg ha^{-1} from low to reference and reference to high SOC levels, respectively.

At 50 kg N ha^{-1}, the winter wheat grain yield increase was significant, with increase of 0.86 and 0.85 Mg ha^{-1} from low to reference and reference to high SOC, respectively (**Table 1**), whereas aboveground biomass increase was significant only from low to reference SOC. Similarly, at 100 kg N ha^{-1}, the grain yield and aboveground biomass increase was significant by 1.17 Mg ha^{-1} and 1.32 Mg ha^{-1}, respectively, from low to reference SOC. At 150 kg N ha^{-1} and 200 kg N ha^{-1}, there was no significant increase in grain yield and aboveground biomass between low, reference, and high SOC contents.

In summary, there was relatively higher SOC effects on both grain yields and aboveground biomass at 0–100 kg N ha^{-1} and the effects decreased with increasing N rates until there was no SOC effects at 150–200 kg N ha^{-1}. With higher SOC content, lower N rate is required to attain a locally relevant yield 'plateau' compared to the soils with lower SOC content and in contrast, higher N rate will be required to attain the same yield 'plateau' with lower SOC content.

SOC Effects on Plant Available Water (PAW)

A highly significant positive correlation between PAW and SOC was obtained with Danish PTF, given by $Y = 1.3094x + 21.319$ ($R^2 = 0.99^{***}$) (Y = PAW and X = SOC) (**Figure 3**). The measured PAW at LTE-Askov was 21%, in close proximity to calculated value of 23%, demonstrating the robustness and reliability of the Danish PTF to predict PAW, validating the highly positive correlation between SOC and PAW.

Soil organic carbon and calculated PAW content had significant positive correlation ($R^2 = 0.99–1^{***}$), and higher SOC content retained correspondingly higher PAW in LTE-Askov soils (**Figure 3**). The PAW calculated by VGM, based on generated hydraulic parameters by the four PTFs, demonstrated a similar trend of significant positive correlation between SOC and PAW. PAW based on Danish PTF (Borgesen and Schaap, 2005) resulted in highest calculated PAW content compared to the three other PTFs (HPC, Rosetta, and HYPRES). HYPRES PTF calculated the second highest PAW content followed by HPC and Rosetta PTFs. When the mean of PAW was averaged across the four PTFs at different SOC contents, there was positive correlation between PAW and SOC given by the linear relationship PAW (%) = 1.007 × SOC (%) + 15.641.

The regression relationship showed that the increase in PAW within the tested SOC range (0.7–2% SOC) was only 1.4%, which is not large enough change to affect yields. The effect of such a small change in PAW is difficult to verify in the field and is unlikely to have any significant change in yields and biomass accumulation. Hence, PAW did not have any significant role in yield and aboveground biomass accumulation.

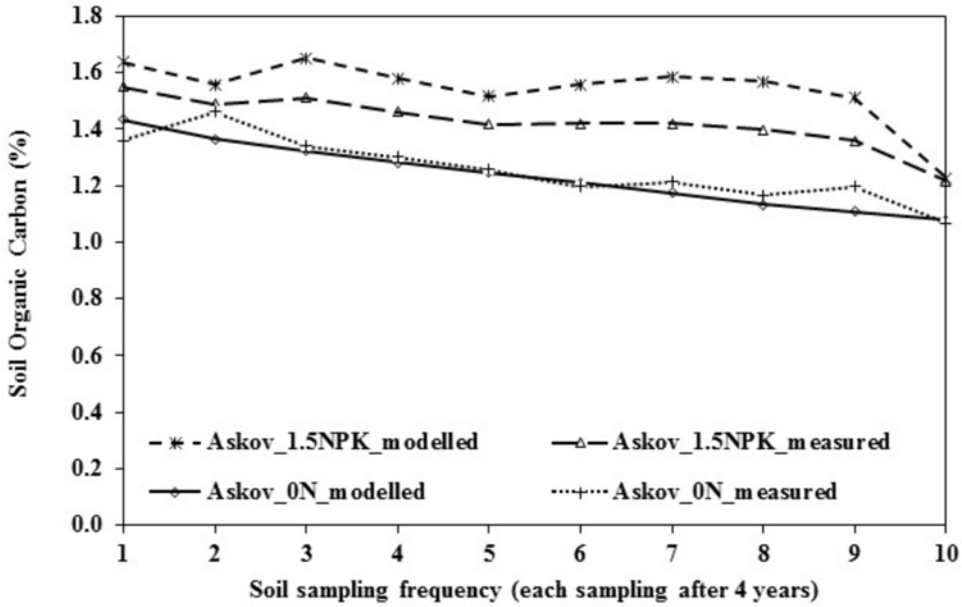

FIGURE 2 | Comparison of modeled and measured SOC values in Askov_0N and Askov_1.5NPK plots. Sampling frequency is 4 years and the timeline of sampling period is 40 years.

DISCUSSION

SOC and Provision of Ecosystem Services

Soil organic carbon affects multiple ecosystem services, and SOC build-up and management can pose different challenges depending on the environmental zones and the context-specific production systems (Palm et al., 2014). SOC maintenance is a challenge, as the mineralization processes continuously degrade SOC over time. Where the arable farming systems are integrated with livestock, the manure from livestock are good sources of OM to build up SOC whereas it can be a challenge in other arable production systems unless dedicated practices like cover crops, no-till or mulch farming are practiced to replenish the SOC (Lehtinen et al., 2014). SOC can have both positive and negative effects, and the management have huge influences on the benefits from SOC. Under N non-limiting wheat production systems, nutrients released through the decomposition of the SOC especially mobile N can leach beyond the root zone and pollute the groundwater, contaminating the water supply for human consumption (Palmer et al., 2017). The losses of N downstream can induce algal bloom and eutrophication, which can have devastating impacts on the aquatic and other fish species. In addition, some of the nitrogen forms can be lost as nitrous oxides, which have global warming potential of 300 times more than the carbon dioxide (Burgin et al., 2013). In contrast, under N deficient wheat production systems, building and maintaining SOC can provide wider benefits with provision of multiple ecosystem services like supply of macro and micronutrients, carbon sequestration, food and fodder production, mitigation of soil erosion and support habitat for biodiversity (Ghaley et al., 2014). Hence, the benefits accrued from SOC increase is evident only in N deficient wheat production system, an important management decision for the wheat producing farmers.

SOC Effects on Grain Yield and Aboveground Biomass

The range of SOC values used for the simulation at the LTE-Askov is within the ranges reported for the trial site, as evident from the measured SOC values in Askov_0N and Askov_1.5NPK plots (LTE-Askov, **Figure 2**). Similar SOC range of 1.2–1.7% was reported from another study at the same trial site (Thomsen and Christensen, 2004). The reduction of SOC content during the experimental period was attributed to decomposition of the SOC releasing N and other macro and micronutrients, and the SOC effects are only transient if efforts are not put into replenishment of OM to maintain the SOC stock.

Our study demonstrated that, at N application rates of 0–100 kg N ha^{-1}, SOC had benefits in terms of enhancing winter wheat productivity (**Table 1**). Similar positive correlations in grain yield-SOC relationships were reported in several field studies (Thomsen and Christensen, 2004; Persson et al., 2008; Seremesic et al., 2011; Yang et al., 2011; Mikanova et al., 2012) and one simulation study across seven sites and pedo-climatic zones (Palmer et al., 2017). In our simulations, winter wheat grain and straw yields increased with increases in SOC (low, reference, and high), which is supported by findings from another field experiment at LTE-Askov, where increased SOC increased spring barley yields (Christensen et al., 2009). Spring barley grain and straw yields increased with increase in SOC indicating a positive relationship between SOC and yield (Christensen

TABLE 1 | Winter wheat grain and aboveground biomass (grain + straw) yields (mean ± standard error) at low, reference, and high SOC at 0–200 kg N ha^{-1}.

N rate/SOC	Low SOC (0.7%)		Reference SOC (1.3%)		High SOC (2%)		$LSD_{0.05}$	
Kg N ha^{-1}	Grain Mg ha^{-1}	Grain + straw Mg ha^{-1}	Grain Mg ha^{-1}	Grain + straw Mg ha^{-1}	Grain Mg ha^{-1}	Grain + straw Mg ha^{-1}	Grain	Grain + straw
0	1.88 ± 0.06	4.18 ± 0.11	2.16 ± 0.06	4.77 ± 0.12	2.46 ± 0.07	5.40 ± 0.14	0.18	0.36
50	2.27 ± 0.28	6.55 ± 0.62	3.13 ± 0.26	8.26 ± 0.35	3.98 ± 0.25	9.64 ± 0.17	0.82	1.47
100	4.13 ± 0.45	10.03 ± 0.49	5.30 ± 0.32	11.35 ± 0.21	6.10 ± 0.15	12.20 ± 0.14	1.14	1.11
150	6.06 ± 0.14	12.16 ± 0.36	6.67 ± 0.25	12.80 ± 0.42	6.67 ± 0.25	12.80 ± 0.42	0.78	1.39
200	6.67 ± 0.25	12.80 ± 0.42	6.67 ± 0.25	12.80 ± 0.42	6.67 ± 0.25	12.80 ± 0.42	0.89	1.46

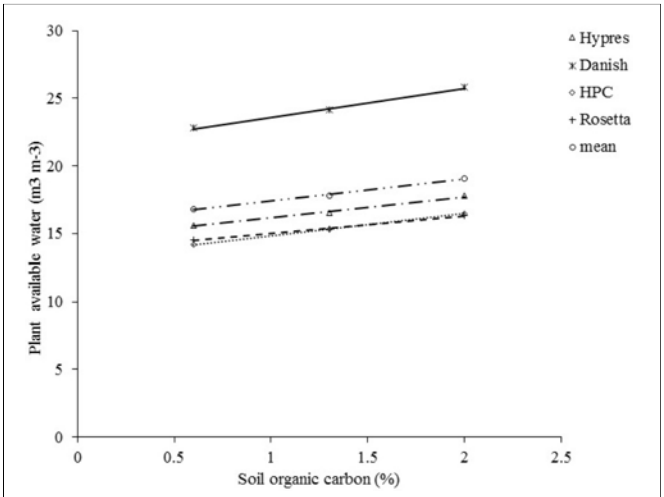

FIGURE 3 | Relationship between plant available water content and SOC at 0.7%, 1.3%, and 2% SOC at LTE-Askov in Denmark. All four PTF functions exhibited linear relationship given by $Y = 0.945x + 14.52$, $R^2 = 0.99$*** (Hypres), $Y = 1.309x + 21.319$, $R^2 = 0.99$*** (Danish), $Y = 0.991x + 13.109$, $R^2 = 1$*** (HPC), and $Y = 0.783x + 13.617$, $R^2 = 0.99$*** (Rosetta).

et al., 2009) in conformity to our study. However, application of more than 90 kg N ha^{-1} nullified the SOC effects on grain and straw yield (Christensen et al., 2009) which conforms to our decreasing SOC effects on winter wheat with increasing N fertilizer with significant effect only up to 100 kg N ha^{-1} (**Table 1**). The benefits of SOC on spring wheat grain yields was reported from a 24 years trial at Jyndevad in Denmark, where an N substitution rate of 15–27 kg N ha^{-1} was attained with long term catch crops building up higher SOC in the soil (Hansen et al., 2000). This increase in yield is similar to the winter wheat yields in our study, where significant increase in grain and biomass yields were obtained with increasing SOC content at 0–100 kg N ha^{-1} (**Table 1**), providing evidence of SOC × N effects on winter wheat productivity. Higher SOC content had a significant influence on grain yield and aboveground biomass increase only at 0–100 kg N ha^{-1}, which demonstrated the benefits of building up SOC to compensate for the fertilizer N inputs. This indicated that the maximum SOC benefits can be realized only at 0–100 kg N ha^{-1} input under the Danish wheat production agro-ecosystems and the benefits were non-existent as the N rates are increased to more than 100 kg N ha^{-1} due to N losses into groundwater, eutrophication and algal blooms downstream and nitrous oxide losses as greenhouse gas. Hence, SOC benefits are contextual and multiple benefits are only realized in N deficient wheat production systems.

SOC Effects on PAW (m^3 m^{-3})

Our study demonstrated that the four PTFs are robust enough to predict PAW based on the minimum soil parameters collected in the field trials, which can provide insights into water availability (m^3 m^{-3}) in the soil. In line with our study, significant positive correlations (**Figure 3**) between SOC and PAW, have been reported in other studies in volumetric (Rawls et al., 2003) and gravimetric basis (Emerson, 1995) under diverse environments (Bationo et al., 2013) including a study (gravimetric) on 41 Danish soils (Resurreccion et al., 2011). A study in North Dakota in sandy, medium and fine textured soils demonstrated that soils with higher SOC retained more soil water (gravimetric) irrespective of the soil types (Bauer and Black, 1992) supporting the outcome of this study that SOC has positive effects on soil water retention. An exhaustive investigation of soil type-PAW correlation, based on the soil samples collected from across United States (Hudson, 1994), demonstrated a significant positive SOM-PAW correlation (volumetric) across three soil types (sandy, silty clay, and silty loamy clay), in line with our findings. Some recent studies (Palmer et al., 2017; Minasny and McBratney, 2018) also reported SOC positive effects on PAW in line with our study. Hence, our study supports the positive relationship between SOC and PAW. However, the PAW increase was too small to affect the crop yields and aboveground biomass accumulation. Moreover, the underlying mechanisms of SOC-PAW relationship need to be further explored.

CONCLUSION

The benefits of SOC can be positive and negative and maintenance of SOC will require regular inputs of OM into the soil. The efforts to maintain the SOC and reap the benefits, are contextual depending on the land use, environmental zones, and management practices. In our study, increasing SOC content had significant positive effects on winter wheat grain yield and aboveground biomass at only 0–100 kg N ha^{-1} and the SOC effects were non-significant with increasing N inputs at 150–200 kg N ha^{-1}. SOC and PAW were positively correlated but the increase in PAW was minimal with no significant effects on grain yields and aboveground biomass accumulation. Our study findings were similar to other studies (Minasny and McBratney, 2018) carried out in diverse environments (Palmer et al., 2017), which lends credence to this study in confirming that the earlier results from Netherlands and other six sites were equally applicable in Denmark and other relevant environments. In order to improve our analysis, future investigations should include quantification of dis-benefits viz. N leaching, N loss downstream and nitrous oxide loss, to provide additional insights into the extent of dis-benefits with increasing N input. Hence, benefits and dis-benefits parameters need to be measured in future studies in order to generate a complete analysis of SOC effects for improved management decision by farmers, agricultural advisors and policy makers.

AUTHOR CONTRIBUTIONS

BG wrote the first draft and made the subsequent revisions of the paper with data inputs from HW, JO, JY, and PS. KS, SB, PK, J-PL, and JP reviewed the document and provided inputs to improve the scientific content of the manuscript. RF and MB helped with cleaning the weather data and CB and YK helped with setting up the DAISY model for carrying out simulations.

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Structural and Functional Features of Chars From Different Biomasses as Potential Plant Amendments

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Biochars result from the pyrolysis of biomass waste of plant and animal origin. The interest in these materials stems from their potential for improving soil quality due to increased microporosity, carbon pool, water retention, and their active capacity for metal adsorption from soil and irrigation water. Applications in agriculture have been studied under different conditions, but the overall results are still unclear. Char structure, which varies widely according to the pyrolysis process and the nature of feedstock, is thought to be a major factor in the interaction of chars with soil and their metal ion adsorption/chelation properties. Furthermore, biochar nutrients and their elemental content can modify soil fertility. Therefore, the use of biochars in agricultural settings should be examined carefully by conducting experimental trials. Three key problems encountered in the use of biochar involve (i) optimizing pyrolysis for biomass conversion into energy and biochar, (ii) physicochemically characterizing biochar, and (iii) identifying the best possible conditions for biochar use in soil improvement. To investigate these issues, two types of wood pellets, plus digestate and poultry litter, were separately converted into biochar using different technologies: pyrolysis/pyrogasification or catalytic (thermo)reforming. The following physicochemical features for the different biochar batches were measured: pH, conductivity, bulk density, humidity and ash content, particle size, total organic substances, and trace element concentrations. Fine porous structure analysis and total elemental analysis were performed using environmental scanning electron microscopy along with energy-dispersive X-ray spectrometry (EDX). Phytotoxicity tests were performed for each biochar. Finally, we were able to (i) differentiate the biochars according to their physicochemical properties, microstructure, elemental contents, and original raw biomass; (ii) correlate the whole biochar features with their respective optimal concentrations when used as plant fertilizers or soil improvers; and (iii) show that biochars from animal origin were phytotoxic at lower concentrations than those from plant feedstock.

Keywords: biochar, low-vacuum SEM/EDX, phytotoxicity, principal component analysis, pyrolysis, soil improvers, trace elements

INTRODUCTION

According to the International Biochar Initiative (IBI) guidelines, "biochar is a solid material obtained from the thermochemical conversion of biomass in an oxygen-limited environment" (International Biochar Initiative [IBI], 2012). Charcoal is a carbon-rich solid product prepared via biomass pyrolysis and is used as a fuel source for producing energy. Charcoal and biochar constitute part of the black carbon by-products resulting from the combustion of organic matter (OM) that includes coal, soot, and graphite (Crombie et al., 2013). Biochar is currently a by-product of the transformation of biomass into bioenergy in thermochemical processes (Qambrani et al., 2017). The physicochemical properties of biochar are mainly connected to its large surface area, ranging from 200 to 400 m^2 g^{-1}. The thermal decomposition of organic material leads to loss of volatile compounds, producing a network of carbon chains present as porous structures with a large inner surface. In turn, this affects the retention of water, the capacity for binding metals and other elements and hosting microbial communities, and changes in soil structure such as porosity and density (Beesley et al., 2013; Joseph et al., 2013; Fellet et al., 2014). Biochar therefore possesses intrinsic capabilities for stimulating plant growth and increasing tolerance to abiotic stresses, by improving the water holding capacity of soils and nutrient retention because of its chemical and electrical properties (Jeffery et al., 2011).

A recent (2015) Italian law allows the use of biochar from plant biomass as a soil amendment and should favor the market introduction of biochars because of their greater efficiency and sustainability when compared to fossil carbon-based solutions. The application of biochar in agriculture would increase carbon storage in soils, provide cobenefits in saving water, avoiding emissions of greenhouse gasses from soils, substituting for fossil fuels used in the production of other soil-improving agents, and providing additional ecosystem services (Woolf et al., 2010). A detailed analysis of biochar benefits is reported by Schröder et al. (2018), in comparison with other types of soil-amending agents.

However, the benefits derived from biochar are strictly dependent on the original biomass (feedstock), on the production process, especially temperature, and on the characteristics affecting its performance as a soil improver (Mimmo et al., 2014; Agegnehu et al., 2017). Converting biomass into biofuels through thermochemical processes is one of the methods more accepted by both legislation and industry to reduce waste and obtain valuable products (Dodds and Gross, 2007). Catalytic (thermo)reforming and pyrolysis processes generate energy, bio-oil, gas, and biochar. In the former, a thermochemical process, catalyzed by specific elements/compounds transforms organic biomass into energy, gas, bio-oil, and a minimal amount of biochar. In this type of process, biochar can also be used as catalyst (Shen, 2015; Ahmad et al., 2018). Pyrolysis is defined as the thermal biomass decomposition under limited O_2 and at an intermediate temperature to obtain energy, gasses, bio-oil, and biochar (Liu W.J. et al., 2017). The quality and elemental content of the biochar obtained during the two processes depends on the operating parameters, temperature, heating rate, type of catalyst, and on the physicochemical features of the feedstock.

New approaches to biochar production are under study in several research projects aiming at developing technologies where small quantities of pyrogases can provide heat to the process, and the remainder is available to supply further renewable heat for agricultural or industrial uses, improving the final energy efficiency and economy.

It has been calculated that globally 140 billion tons of biomass waste from agriculture are produced annually (United Nations Environment Program[1], accessed March, 2018). In the European Union, feedstocks available from renewable sources consist mostly of residues from forestry and agriculture. Forestry residues can be primary, from the field, secondary, from the wood industry, or tertiary, from post-consumer wood. Recent data on biomass availability estimate a total of about 9 and 85 Mt of dry matter from forestry and agriculture respectively, not used for other purposes (Searle and Malins, 2016), that could be available for transformation into biochar applicable to agriculture. According to the EU Waste Framework Directive, the approach to waste management in EU Member States should be based on three main principles, listed in order of priority: (i) preventing waste, (ii) recycling and reusing, and (iii) improving final disposal and monitoring. The use of forestry and other plant maintenance residues to produce biochar is a positive step in activities aiming at producing environmental and economic advantages by upgrading waste material.

In this work, we compare biochars derived from different feedstocks (animal and plant) as sustainable products from waste management, with the aim to establish critical factors that affect the usefulness of biochars as soil improvers and amendments in agriculture, according to international guidelines. Some of the biochars' chemical and physical characteristics were measured; phytotoxicity tests were carried out to establish for the different biochars the germination index using *Lepidium sativum* L. For two biochars, growth inhibition tests were performed using *Hordeum vulgare* L. The main findings were that the feedstock type determined the biochar microstructure and elemental composition, which were linked to the toxicity: biochar from animal origin were phytotoxic at lower concentrations than those from plant feedstock.

MATERIALS AND METHODS

Biochar Origin and Production

Five different biochars were used in the study. Chars A1, A2, and A3 were obtained as by-products of catalytic (thermo)reforming in a prototype developed in the framework of the research project TERMOREF (Regione Emilia-Romagna); temperature was between 400 and 500°C for 2–3 h. Char A1 was derived from anaerobic digestate, char A2 from poultry litter, and char A3 from wood pellets. Char A4 was derived from wood

[1] www.unep.org

pellets in a prototype pyrogasification system <50 kWe in the framework of the research project TERMOREF; temperature was between 500 and 700°C for 1–2 h. Char E1 was acquired from commercial sources and derived from forest wood and brushwood waste through pyrogasification (Borgo Val di Taro, Italy).

Physicochemical Analyses

Sample Pretreatment

Biochar samples were sieved and prepared according to the protocol given in EN European Standards (2008).

pH Evaluation

The pH values of the biochar samples were determined following the guidelines of EN European Standards (1999a) protocol for growing media and soil improvers.

Briefly, samples were passed through a 20 mm sieve and extracted with deionized water in a ratio of 1:5 (v/v). To a sample weight equivalent of 60 mL, 300 mL of deionized water were added in 500 mL glass jars, and samples were shaken for 1 h on an orbital shaker (Model Unimax 2010, Heidolph Instruments, Schwabach, Germany). The pH of each suspension was measured in triplicate using a Model S213 Seven Compact Duo meter (Mettler Toledo, Columbus, OH, United States).

Electrical Conductivity Evaluation

Electrical conductivity (EC) values of the biochar sample extracts were determined following the EN European Standards (1999b) protocol for growing media and soil improvers.

Briefly, samples were treated as for the pH evaluation; after shaking, suspensions were filtered through Whatman filters (N°1, Whatman, Maidstone, United Kingdom) discarding the first 10 mL. The conductivities of the suspensions were measured, in triplicate, within an hour after extraction, using a Model S213 Seven Compact Duo meter (Mettler Toledo, Columbus, OH, United States), and expressed in mS cm^{-1}.

Bulk Density Evaluation

The bulk density of the biochar samples were evaluated following the EN European Standards (2008) protocol, with minor modifications.

A rigid cylinder with a capacity of 1,000 mL and diameter of 100 mm was used to perform the experiment. The cylinder was filled with each sample and a plunger of 650 g with the same diameter of the cylinder was placed on the top for 3 min. After the compaction time, the plunger was removed and the sample weight and volume measured. Results were expressed in g L^{-1}.

Particles' Size Distribution Evaluation

The size distribution of the particles of the biochar samples was assessed following the EN European Standards (2007) protocol, with minor modifications.

Briefly, dried samples of biochar were passed through five sieves with different mesh sizes (20, 10, 5, 2, and 1 mm) positioned in tiers (Endecotts, Ltd., London, United Kingdom) from the largest to the smallest. A known mass of sample was placed on the upper sieve and the column was gently shaken by hand for 5 min. The biochar fractions retained on each sieve were then carefully collected and weighed, and the percentage fraction distributions calculated.

Dry Matter and Moisture Content Measurements

Dry matter and moisture content of the biochar samples were determined following the EN European Standards (2008) protocol.

Briefly, samples of known weight were oven-dried at 105°C (M710 Thermostatic Oven, F.lli Galli, Milan, Italy) until the difference between two successive weightings did not exceed 0.1 g. The final weight of the samples represents the dry matter content, and the weight loss represents the moisture content; moisture content is expressed as a percentage of the starting weight.

OM and Ash Content Evaluation

Organic matter and ash content of the biochar samples were determined according to the EN European Standards (2011) protocol.

Fresh samples were oven-dried at 105°C (M710 Thermostatic Oven, F.lli Galli, Milan, Italy) to a constant weight, and a known weight of the sample was incinerated at 500°C in a muffle furnace (Model A022, Matest S.p.A., Bergamo, Italy) for 14 h. After incineration, the residues (ash) were weighed and OM was calculated as the difference between the fresh and final incinerated weights. Ash content and OM content were expressed as a percentage of the total initial weight.

Measurement of Trace Metal Concentrations: Cu, Fe, Ni, Zn, Pb, and Cd

The concentration of six trace metals (Cu, Fe, Zn, Pb, Ni, and Cd) in the biochar samples were determined following the UNI Ente Italiano di Normazione (1998) protocol, with minor modifications.

Samples were oven-dried at 105°C (M710 Thermostatic Oven, F.lli Galli, Milan, Italy) up to a constant weight, and a known weight of the sample was incinerated at 500°C in a muffle furnace (Model A022, Matest S.p.A., Bergamo, Italy) inside ceramic crucibles with lids for 14 h. For each biochar, the ash was retrieved from the crucible and solubilized by wet digestion with HNO_3 65% (Carlo Erba, Milan, Italy) at 165°C for 30 min and 230°C for 30 min in a heated digester thermoblock (DK20, Velp Scientifica, Usmate Velate, MB, Italy). Digested solutions were diluted with deionized water to 30% (v/v) acid concentration. The concentration of each metal was measured using flame atomic absorption spectrometry (AA240FS, Agilent Technologies, Santa Clara, CA, United States) at the following wavelengths: λ Cu: 324.7 nm; λ Fe: 248.3 nm; λ Zn: 213.9 nm; λ Pb: 217.0 nm; λ Ni: 232.0 nm; λ Cd: 228.8 nm. Calibration curves for each metal were prepared using 1,000 ppm certified standard solutions (Agilent Technologies, Santa Clara, CA, United States). Three instrumental replicates were performed on each biological replicate (three for each sample). The metal concentrations were expressed in mg kg^{-1} of biochar.

Low-Vacuum Scanning Electron Microscope With X-Ray Microanalysis (Lv SEM/EDX)

To overcome the problems with high vacuum, an environmental low-vacuum (Lo-vac, 60 Pa) environmental scanning electron microscopy (ESEM/EDX) was used. Finely-powdered biochar (between 10 and 100 μm grain size) was analyzed with no fixation or staining, after a careful positioning and adhesion on 2 cm diameter stainless-steel sample holders covered with adhesive carbon tape. A scanning microscope ESEM FEG2500 FEI (FEI Europe, Eindhoven, Netherlands), operating in low-vacuum (70 Pa) with a large-field detector allowed optimal secondary electron imaging, while the cone pressure-limiting aperture set at 500 μm improved the signal available to the Bruker XFlash®6 | 30 X-ray detector equipped with a high efficiency 30 mm^2 silicon drift detector for nanoanalysis and high-count-rate spectral imaging (Bruker Nano GmbH, Berlin, Germany). Secondary electron imaging was performed at 5 or 10 keV with a beam size of 2.5 μm, and EDX analysis at 20 keV acceleration voltage and beam size of 4 μm. The working distance was approximately 10 mm, and the scanning time 1–3 μs. The xT Microscope Control, xT Microscope Server, and FEI User Management software were used for imaging; the Esprit 1.9 package was used for X-ray spectra acquisition and analysis during acquisition in either point analysis or line-scan mode. X-ray spectra deconvolution and elemental standardless quantification were performed using the P/B-ZAF interactive method [Peak/Background evaluation matrix with atomic number (Z), absorption (A), and secondary fluorescence (F) correction] supported by the "Quantify Method Editor" option of Esprit 1.9 (Goldstein et al., 2003).

Effects on Growth and Germination

Germination Test

The germination index of the biochar matrices was determined following the UNICHIM (2003) protocol.

Biochar samples were sieved to pass a 2 mm mesh. Aliquots of 10, 7.5, 5, 2.5, 1, 0.5, or 0.1 g of dried and sieved biochar were placed in Petri dishes (Ø 90 mm, Sarstedt, Germany) and saturated with deionized water. The suspension was homogenized and covered with a Whatman N°41 filter (Whatman, Maidstone, United Kingdom). Control dishes were prepared containing only deionized water and filter paper. Ten seeds of *Lepidium sativum* L. (Sementi Dotto Spa, Udine, Italy) were sown in every Petri dish; three biological replicates were performed for each concentration. Sealed dishes were placed in a growth chamber (MIR-554-PE, Panasonic, Osaka, Japan) at 25°C in the dark. After 72 h, the germinated seeds were counted, and their root lengths were measured to calculate the germination index (GI %).

GI % = (Gt * Lt/Gc * Lc) * 100.

where Gc = germinated seeds in the control; Gt = germinated seeds in the treatments; Lc = main root length in the control; Lt = main root length in the treatments.

Germination Rate and Growth Inhibition Tests

Germination and growth inhibition tests were performed following the EN European Standards (2012) protocol.

The growing substrates were prepared according to the protocol by mixing char at doses of 1, 3, or 5% (w/v) with sphagnum peat (Lithuanian peat, UAB Presto Durpes, Vilnius, Lithuania) limed at pH 5.5–6 with $CaCO_3$ (Sigma-Aldrich, St. Louis, MO, United States), using commercial garden soil as a control (Ecomix, Vialca S.r.l., Pistoia, Italy). Peat had an EC of 400 μS cm^{-1} and a starting pH of 5.1, and concentration of all metals were below detection limits. Approximately 700 mL of the different mixtures of peat and biochar were distributed in 1,500 mL glass jars and fertilized initially with half-strength Hoagland solution (J.T. Baker, Deventer, Holland) at the rate of 40 mL per kilogram of substrate; each treatment was repeated in triplicate. Control pots were prepared without biochar. Twenty seeds of *Hordeum vulgare* L. (provided by CIEMAT, Madrid, Spain) were sown in each jar. Jars were kept in a greenhouse under controlled conditions: average temperature of 27°C during day-time, 16°C during night-time, with natural light supplemented by metal halide lamps to maintain a minimum light intensity of 300 μmol m^{-2} s^{-1} and a photoperiod of 14 h. Jars were watered daily with 10 mL of deionized water. After 5 days, the number of germinated seeds was recorded to calculate the germination rate percentage:

Germination rate % = (number of germinated seeds/number of starting seeds) * 100.

After 11–14 days, when the second true leaf was clearly visible in 50% of the plants in control condition, the above-ground parts of all plants were collected, thoroughly washed, and weighed. The material was then oven-dried at 75°C to constant weight. The relative growth index (RGI) for each biochar type and growing medium concentration was calculated for both fresh and dry plant biomass:

(RGI) F/D = (Wt/Wc)F/D

where Wt = fresh or dry weight of treated plants; Wc = fresh or dry weight of control plants.

Statistical Analyses

All statistics was calculated using IBM-SPSS v25[2]. After verifying the normal distribution and the homogeneity of variance with Levene's test, an analysis of variance (ANOVA) was carried out on the data, followed by Tukey's *post hoc* test. Dimension reduction calculations were applied to the physicochemical analyses: principal component analysis (PCA) extraction with the Kaiser criterion ($\lambda > 1$) and canonical discriminant functions analysis. For testing phytotoxicity, Student's t-test with the Bonferroni correction was used.

[2]https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software

TABLE 1 | Physicochemical characterization of chars from thermocatalytic reforming of digestate (A1), poultry litter (A2), and wood pellet (A3) and from pyrogasification of wood pellet (A4) and brushwood (E1).

Parameter	A1	A2	A3	A4	E1
pH	**10.23** (0.01) a	**10.25** (0.04) a	9.84 (0.03) b	8.11 (0.04) c	9.93 (0.04) b
Electrical conductivity (mS cm^{-1})	18.6 (0.2) a	14.5 (0.2) b	2.4 (0.1) d	1.4 (0.1) e	7.9 (0.1) c
Bulk density (g cm^{-3})	0.54 (0.01) b	0.62 (0.02) a	0.45 (0.02)c	0.37 (0.01) d	0.44 (0.01) c
Moisture content (% fresh weight)	2.91 (0.02) b	48.88 (0.64) a	3.73 (0.40) b	6.29 (0.30) b	52.46 (5.11) a
Organic matter (% dry weight)	50.57 (0.25) d	49.42 (0.32) d	81.89 (0.32) b	95.63 (0.12) a	62.32 (0.33) c
Ash (% dry weight)	49.4 (0.3) a	50.6 (0.3) a	18.1 (0.3) c	4.4 (0.1) d	37.7 (0.3) b

Means of three biological replicates are reported with standard errors in parentheses. Numbers in bold type represent values that exceed the prescriptions of international guidelines (REFERTIL Consortium, http://www.refertil.info/) on soil improvers. Different lower case letters in rows indicate the significant difference at $p < 0.05$ (Tukey HSD test).

TABLE 2 | Particle size distribution of chars from thermocatalytic reforming of digestate (A1), poultry litter (A2), and wood pellet (A3) and from pyrogasification of wood pellet (A4) and brushwood (E1).

Particle size	A1	A2	A3	A4	E1
10 > x > 5	58.7	3.7	3.0	8.4	0
5 > x > 2	40.3	10.4	36.3	61.7	4.0
2 > x > 1	0.3	15.0	11.7	10.4	10.0
<1	0.7	**70.8**	**49.0**	**19.5**	**86.0**

Data are expressed as the percentage of the total weight of three replicates. Numbers in bold type represent values that exceed the prescriptions of international guidelines (REFERTIL Consortium, http://www.refertil.info/) on soil improvers.

RESULTS

Comparison of Physicochemical Features in Different Chars

The applicability in agriculture of chars for soil amendment is strongly affected by their characteristics including structure, chemical composition, and levels of contaminants. Several studies on the standardization of measurements and the choice of relevant parameters are underway (Bachmann et al., 2016). The chars analyzed in the present study share the same production family of origin because they are all surplus agricultural waste; their reuse follows the principles of zero residues within the framework of a circular economy approach (Riding et al., 2015). Three chars (A3, A4, E1) were produced from wood, either pelleted or chipped, whereas two chars (A1, A2) were produced from non-plant sources, digestate and poultry litter respectively. The processes used in producing chars were different, using prototypes for (thermo)reforming and pyrogasification (A1-A2-A3 and A4-E1 respectively). In view of these differences, their physicochemical parameters (**Tables 1–3**) were analyzed to provide information about possible correlations between biomass of origin, production process, and char features (**Figures 1**, **2**).

pH and Electrical Conductivity

All chars showed high pH values greater than 8 (**Table 1**). There were significant differences, and the highest values were found in the chars of non-plant origin (**Figure 1**). Compared to (thermo)reforming, pyrogasification produced chars with significantly lower pH (**Figure 1**).

Electrical conductivity values were extremely variable (**Table 1**), with highly significant differences linked to the origin of the biomass (**Figure 1**); chars derived from wood showed low values of conductivity compared to chars of non-plant origin, with fourfold differences.

Density and Particle Size Distribution

Densities of the chars ranged around 0.5 g L^{-1} (**Table 1**), with the highest values found in chars of non-plant origin produced via (thermo)reforming (**Figure 1**). The distribution of particle size, determined through sequential sieving, did not correlate strictly with density (**Table 2**). Char E1, from chipped brushwood, had the highest proportion of very fine particles of less than 1 mm diameter. A similar size distribution was found in char A2 from poultry litter. Char A1 from digestate had the highest proportion

TABLE 3 | Total concentration of trace elements in chars from thermocatalytic reforming of digestate (A1), poultry litter (A2), and wood pellet (A3) and from pyrogasification of wood pellet (A4) and brushwood (E1).

Trace element	A1	A2	A3	A4	E1
Cd	0.09 (0.02) c	**1.35** (0.06) a	<0.001	<0.001	0.42 (0.11) b
Cu	73.91 (1.12) b	**262.07** (8.36) a	14.40 (0.92) c	2.91 (0.04)c	56.74 (1.39) b
Fe	5735 (126) a	2060 (5) b	505 (18) c	340 (13) c	1839 (151) b
Ni	13.47 (1.20) bc	16.20 (1.23) b	10.35 (0.60) c	2.23 (0.03) d	28.75 (0.67) a
Pb	12.4 (5.2) ab	18.3 (0.8) a	7.2 (3.4) ab	1.6 (0.1) b	19.6 (4.3) a
Zn	435.3 (7.2) b	**1128.7** (11.0) a	18.2 (1.6) d	4.5 (0.2) d	260.6 (9.2) c

Means in mg kg^{-1} dry weight of three biological replicates are reported with standard errors in parentheses. Numbers in bold type represent values that exceed the prescriptions of international guidelines (REFERTIL Consortium, http://www.refertil.info/) on soil improvers. Different lower case letters in rows indicate the significant difference at $p < 0.05$ (Tukey HSD test).

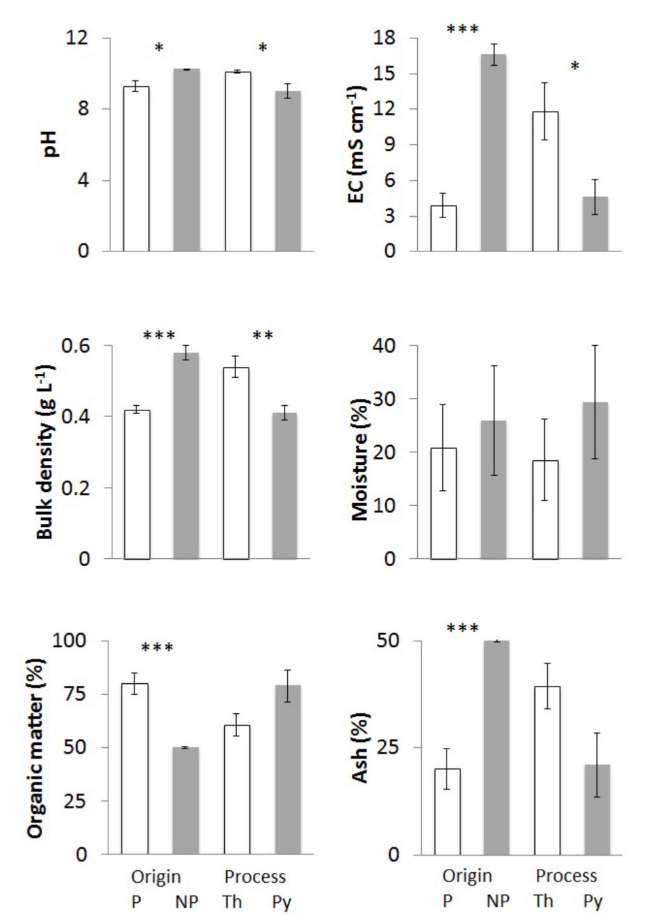

FIGURE 1 | Physicochemical parameters of chars from different feedstock and production process: pH, electrical conductivity, bulk density, moisture, organic matter, and ash content. Bars "P" indicate chars of plant origin (A3, A4, E1 – white) and bars "NP" chars of non-plant origin (A1, A2 – gray). Bars "Th" indicate chars from (thermo)reforming (A1, A2, A3 – white), and "Py" chars from pyrolysis (A4, E1 – gray). Means of at least three biological replicates per char are reported, with standard error of the mean. Asterisks show the significance of pairwise comparisons (one-way ANOVA) between chars derived from different feedstocks (P/NP) or from different processes (Th/Py): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

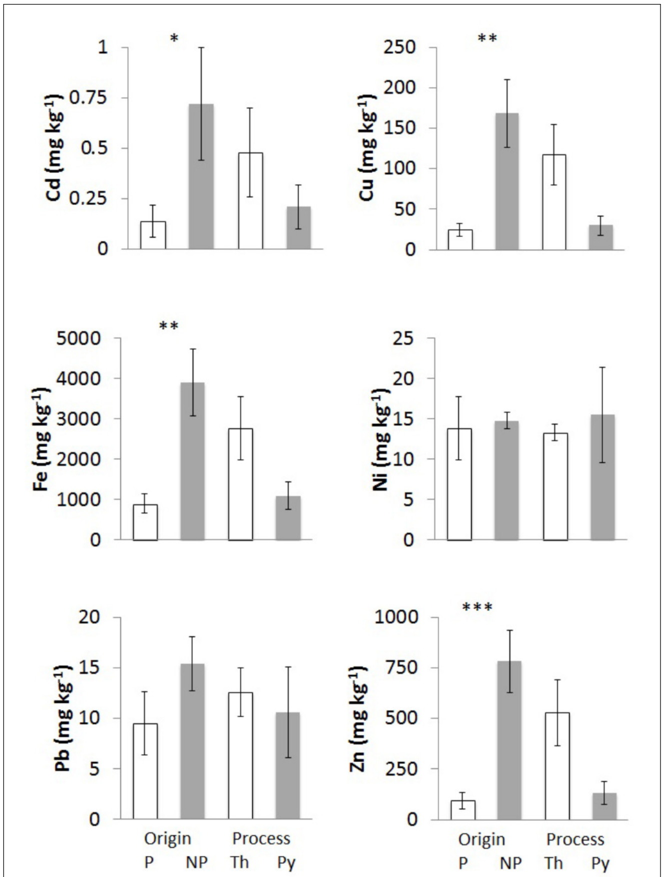

FIGURE 2 | Trace element concentrations of chars from different feedstocks and production processes: Cd, Cu, Fe, Ni, Pb, and Zn. Bars "P" indicate chars of plant origin (A3, A4, E – white) and bars "NP" chars of non-plant origin (A1, A2 – gray). Bars "Th" indicate chars from (thermo)reforming (A1, A2, A3 – white), and "Py" chars from pyrolysis (A4, E1 – gray). Means of at least three biological replicates per char are reported, with standard error of the mean. Asterisks show the significance of pairwise comparisons (one-way ANOVA) between chars derived from different feedstocks (P/NP) or from different processes (Th/Py): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

of large particles, mostly of size greater than 5 mm. In char A3, produced by pyrogasification from wood pellet, most particles were of size between 2 and 5 mm. The size of particles is a critical factor in their distribution and applicability in agricultural conditions, since fine char particles cannot be distributed easily except after transformation into sludge.

Moisture Content, Organic Matter, and Ash

After the production process, char might contain considerable amounts of water. Moisture content of the original biomass is one of the most critical features for the feed-in process and the efficacy of combustion. The proportion of moisture in fresh chars, which is complementary to the dry weight of the char, is shown in **Table 1**. There is no correlation with the type of biomass or the type of process (**Figure 1**), and the highest values for moisture are found in poultry litter char A2 and in brushwood char E1.

The dry matter in char can be further divided into OM and residual ash after incineration at 500°C. **Table 1** reports both values that together equal 100% of dry weight. Significant differences are evident, with chars from wood having significantly higher OM content and less ash (**Figure 1**). No effect can be linked to the production process.

Content of Trace Metals

One of the potential hazardous features of chars is the possible increase in concentrations of metals and other contaminants due to the reduction of volume and water content from the original biomass. It is expected that all non-volatile elements become more concentrated in char at increasing temperatures. The concentrations on a dry weight basis of six main trace elements were determined and results are reported in **Table 3**.

The elements Cu, Fe, and Zn, which are micronutrients, could originate from the biomass of origin, being essential constituents of cells, whereas Cd, Ni, and Pb should be present only as contaminants.

Iron is present in the chars at the level of g kg^{-1} dry weight (**Table 3**), with the highest values in chars of non-plant origin (**Figure 2**). Char E1 is an exception since its Fe content is much higher than in chars derived from wood pellets; a possible explanation lies in the origin of the wood as waste in forests and undergrowth, possibly mixed with small amounts of soil and of materials of diverse origin. Chars from pure wood, A3 and A4, have the lowest Fe levels.

Zinc and Cu also reach high levels in chars of non-plant origin (**Table 3**), with highly significant differences (**Figure 2**). Differences in concentrations of Cu and Zn of >100-fold are evident comparing poultry litter char A2 and wood char A4.

Nickel and Pb concentrations do not show any significant differences among chars of different origin or production process (**Figure 2**), but the values were highly variable.

Cadmium was below the detection limit in chars from pure wood pellet, independent of the production process (**Table 3**), while values were significantly higher in chars of non-plant origin (**Figure 2**).

Classification of Chars Based on Physicochemical Properties

The complete set of data from all replicates and for all parameters was subjected to PCA. In this analysis, OM was retained instead of ash content, and moisture content was retained instead of dry matter; bulk density and particle size distribution were not considered. **Figure 3A** shows the trends of the different parameters as vectors in three dimensions accounting for 93.6% of variance (PC1 = 62.4%, PC2 = 18.6%, PC3 = 12.6%). In the first two dimensions (**Supplementary Figure 1a**), OM content is clearly separated from all other parameters; Cu, Cd, and Zn group together; and pH parallels EC. The second and third dimensions discriminate pH from conductivity and Fe from Ni. Considering the vector lengths, which indicate their relevance in explaining the total variance, and their loading coefficients along the principal components (**Supplementary Figures 1b–d**), we can deduce the best variables for discriminating among chars of different origins and processes. Among the metals the best variables are Cu (PC1), Fe (PC2), and Ni (PC3), and among the other parameters EC (PC2) and OM (PC1 and PC3). Using a 3D representation of the datasets (**Figure 3B**), it can be seen that chars A3, A4, and E1 were grouped together while A1 and A2 were separate but independent of one another.

Through the canonical discriminant function analysis, it was possible to divide the whole variance between only two canonical (orthogonal) functions. This technique, which is a simplified PCA, allowed grouping the different chars in a 2D graph, as shown in **Figure 4**. Chars from wood—A3, A4, and E1—group together and quite distinctly from the chars of non-plant origin—A1 and A2—that do not cluster together. There is no clustering associated with the production process.

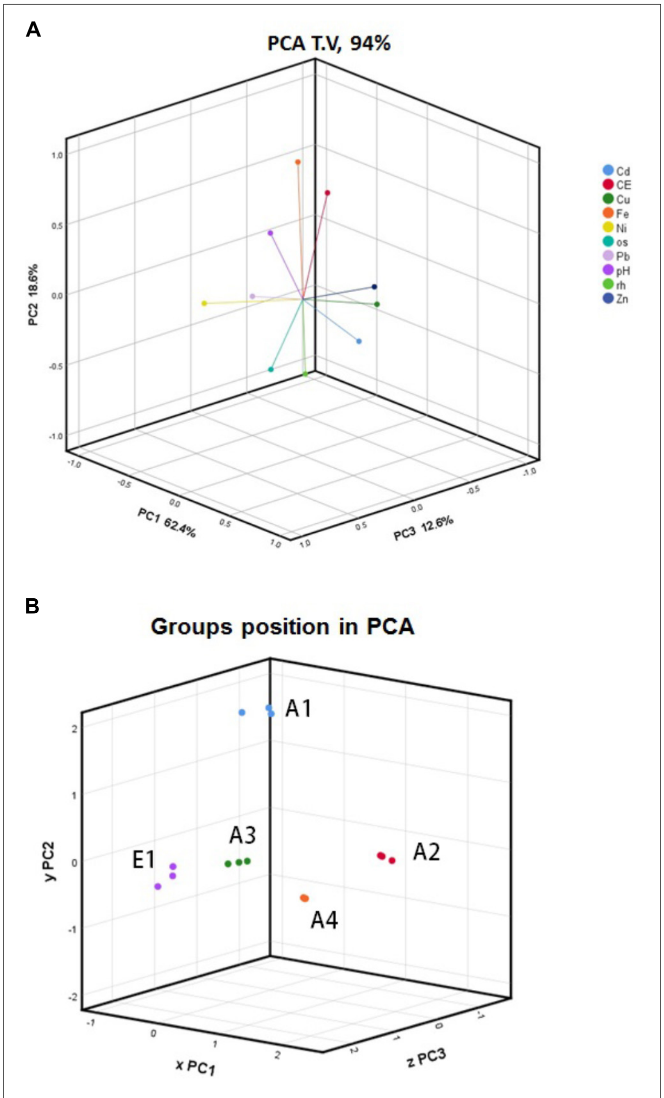

FIGURE 3 | Principal component analysis (PCA) of **(A)** physicochemical parameters of chars and **(B)** char data sets. All data gathered from all biological replicates of five chars, A1, A2, A3, A4, and E1, were analyzed using PCA. The legend indicates the color codes of the different parameters: CE, electrical conductivity; os, organic matter; rh, moisture content.

Microstructural Features of Chars

SEM images obtained for chars A1, A2, and E1 demonstrate the presence of plant and non-plant matrices (**Figure 5**). Chars A1 and A2, from digestate and poultry litter respectively, showed mostly animal substrates that appeared as lumps encrusted with fine and coarse powder (**Supplementary Figure 2**). In the case of char E1, we observed a prevalence of highly porous matrix, which was interspersed with lumpy structures covered with micrometer-sized powder (**Figure 5** and **Supplementary Figure 3**). Exclusively porous substrates were seen in chars A3 and A4 (**Figure 6**). In particular, in A3 we observed pores of different sizes, between 5–20 μm and larger than 20 μm, while in A4 the pores were almost exclusively between 5 and 20 μm. Both the

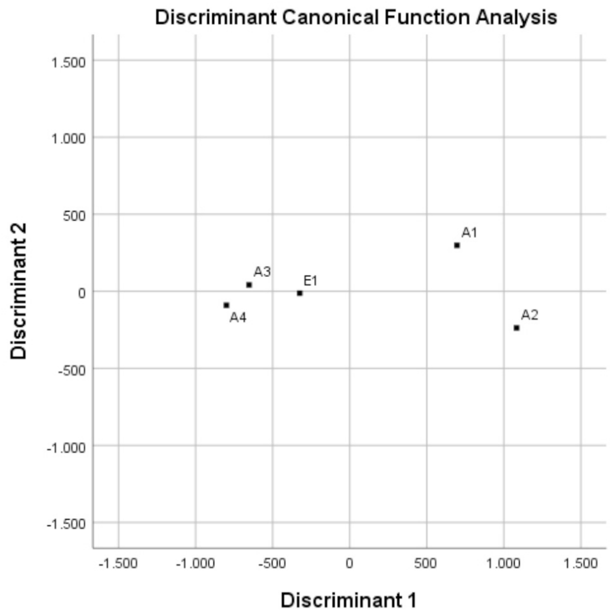

FIGURE 4 | Discriminant canonical function analysis of data sets for chars A1, A2, A3, A4, and E1, derived from different feedstocks and processes.

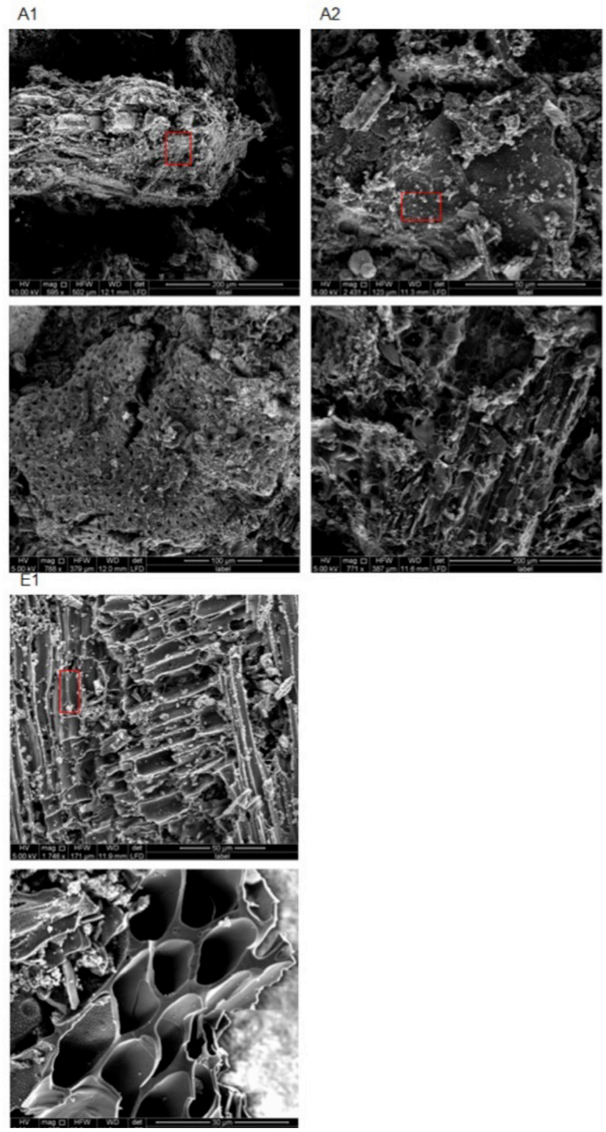

FIGURE 5 | Environmental scanning electron microscopy (ESEM) images of biochars A1 (2 top left), A2 (2 top right), and E1 (2 bottom left) at different magnifications (see figure bottom and white bar bottom right). All images were acquired with electron beam at 5 KV and a working distance between 11.9 and 12 mm. Red rectangles indicate the areas from which the EDX spectra in **Figure 7** were acquired.

porous matrices appeared disorganized with pores of different sizes distributed randomly (**Figure 6** and **Supplementary Figure 4**).

Representative examples of electron-induced emitted X-ray spectra for the different char types are reported in **Figures 7**, **8**. On average, 25 X-ray spectra were collected for each biochar to gather a reliable set of measurements of their elemental content. For all chars, the X-ray analysis confirmed the presence of Fe, Cu, Zn, Ni, and Pb, but other metals were also found, for example Ti and Mn. Concentrations of Cd were below the detection limit for all samples. Spectra for chars A1 and E1 confirm the high content of Fe. Chars differed in their contents of light elements as well. Chars A1, A2, and E1 were rich in Si, K, and Ca while in particular the chars of non-plant origin were rich in S, Na, Cl, P, and Mg (**Figures 7**, **8**). Chars A3 and A4 from pure wood pellets showed lower amounts of all light elements and metals in comparison to the other biochars (**Figure 8**). The contents of light elements were comparable in A3 and A4, but K and Ca were lower in A4 than in A3 (**Figure 8**).

Effect of Chars on Germination and Growth of Plants

Germination Index

A basic phytotoxicity test was performed by using germinating seeds of *Lepidium sativum* on Whatman paper in the presence of increasing quantities of char in water. The effect is measured by taking into account the number of seeds that germinate and the growth of roots after 3 days of incubation, compared to the control with water (**Figure 9** and **Supplementary Figure 5**). Chars A3 and A4 from pure wood pellets, irrespective of the production process, stimulated plant germination and root growth at the lowest dose, 0.1 g per plate. Char A4 was significantly less toxic than A3 at the higher doses, with a germination index greater than 20%. On the contrary, chars A1, A2, and E1 were strongly inhibitory, and stopped growth altogether at doses between 1 and 3 g per plate. Considering only the germinated seeds, **Figure 7** shows the differences in root growth at different doses, demonstrating the positive effects of chars A3 and A4 and the inhibitory effects of chars A1, A2, and E1.

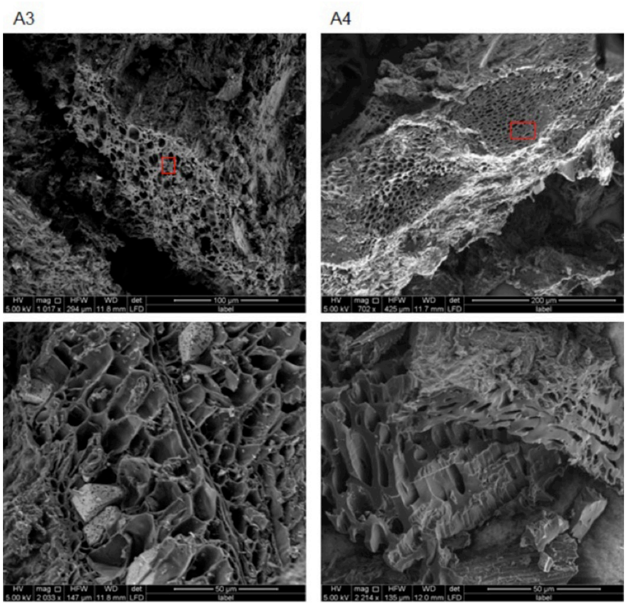

FIGURE 6 | Environmental scanning electron microscopy images of biochars A3 (2 left) and A4 (2 right) at different magnifications (see figure bottom and white bar bottom right). All images were acquired with electron beam at 5 KV and a working distance between 11.9 and 12 mm. Red rectangles indicate the areas from which the EDX spectra in **Figure 8** were acquired.

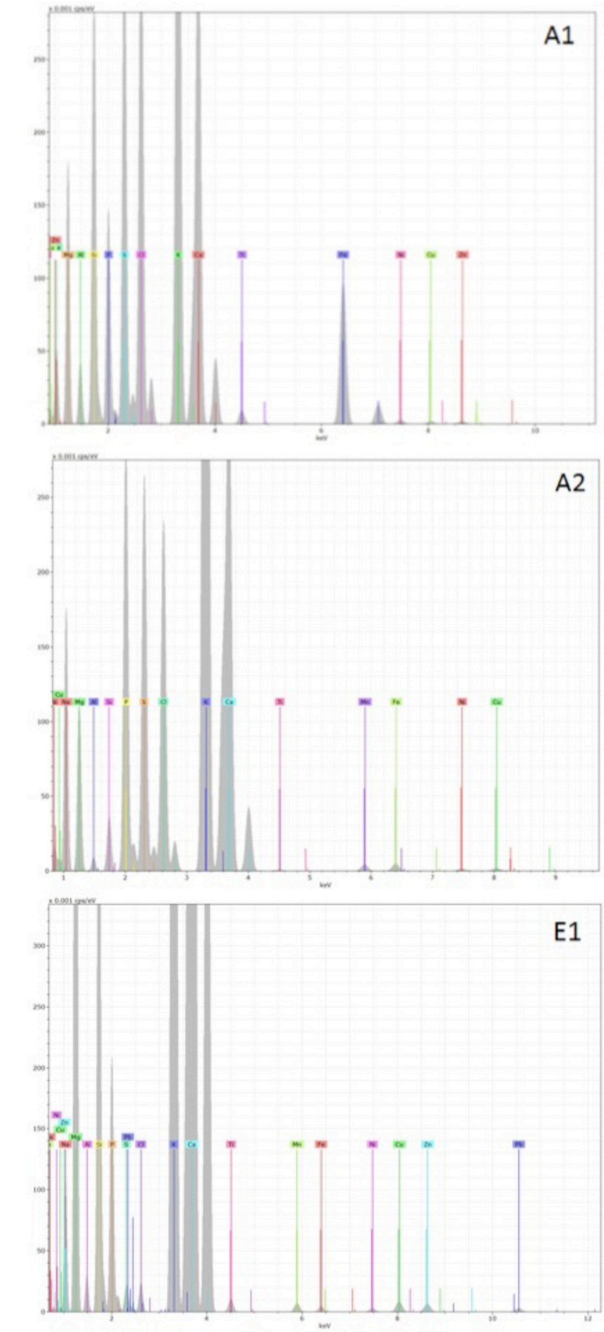

FIGURE 7 | Deconvoluted X-ray emission spectra acquired from biochars A1, A2, and E1. The electron beam energy was set at 20 KV and the acquisition live time at 60 s. In each spectrum, the X axis represents the X-ray energy in eV, and the Y axis represents the peak intensity.

Relative Growth Index

Evaluation of the effects of chars on plant growth can also be assessed in pot experiments, by measuring a RGI at different concentrations of char mixed into the growth substrate (soil or peat). The test was performed on *Hordeum vulgare*, comparing the best- (A4) and worst- (E1) performing chars according to the germination index (**Figure 10**). Char A4 from pyrolysis of pure wood pellets significantly enhanced plant growth at the concentrations of 1, 3, and 5%. Unexpectedly, char E1 also enhanced the growth of plants in comparison to control, with stimulating effects at 3%. In this test on barley, chars E1 and A4 were found to be non-phytotoxic and promising as soil amendments. The dose of 1% in pots corresponds to a distribution of about 45 t ha^{-1} of char in the field, assuming dispersal in the first 30 cm of topsoil.

DISCUSSION

The choice of feedstock in biochar production has a significant effect on the properties of the product, its applicability, the benefits in terms of reduction in greenhouse gas emissions, and economic advantages (Galinato et al., 2011; Field et al., 2013). In addition, using biochar as a way of disposing several types of agricultural and forestry wastes complies with the circular economy approach, advocated in several policies and strategies in the European Union and in other countries (Monlau et al., 2016). However, the evaluation of the benefits in adding biochar to agricultural soils has been so far inconclusive (Jeffery et al., 2011; Agegnehu et al., 2017), even though several studies suggest that biochar addition could provide considerable advantages, but also has a few drawbacks (Tan et al., 2017; Schröder et al., 2018). Characterization of the main properties of chars derived from different feedstocks and different production systems is therefore a necessity to identify the main parameters that can guide the

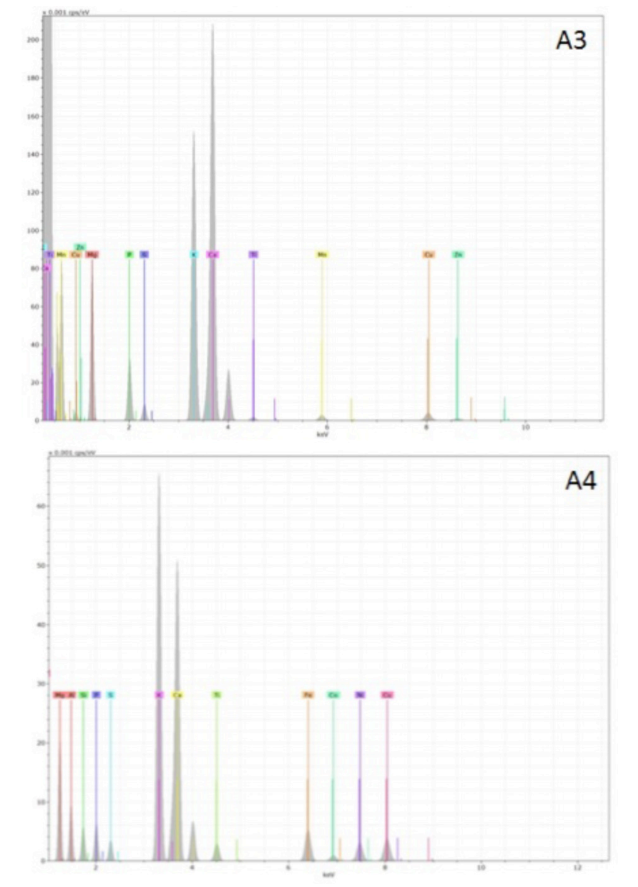

FIGURE 8 | Deconvoluted X-ray emission spectra acquired from biochars A3 and A4. The electron beam energy was set at 20 KV, and the acquisition live time at 60 s. In each spectrum, the X axis represents the X-ray energy in eV, and the Y axis represents the peak intensity.

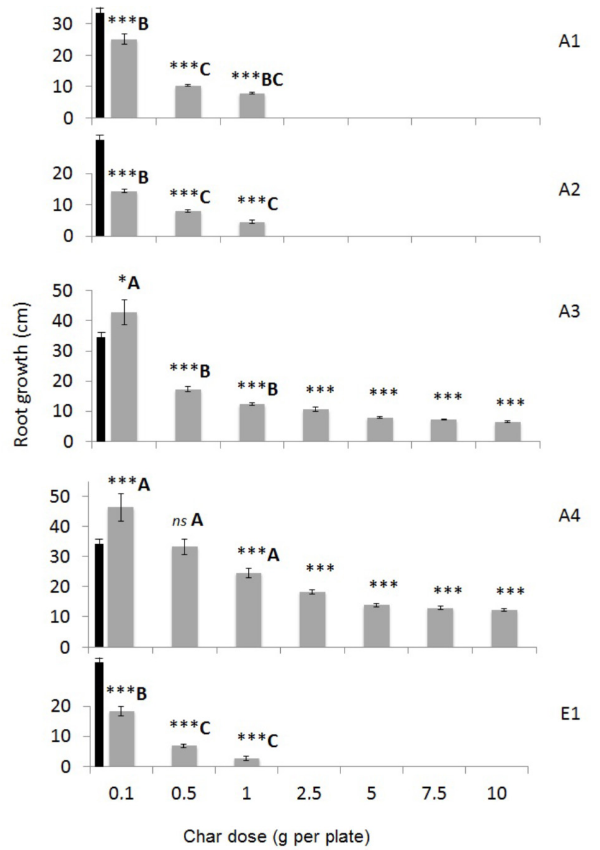

FIGURE 9 | Root growth of *Lepidium sativum* seedlings germinated for 72 h in the presence of chars from different feedstocks and production processes at increasing concentrations (0.1 to 10 g per plate). Means of 30 seeds are reported with standard errors. Growth in distilled water (control) was 34.29 ± 1.31 cm and is shown as a black bar in all graphs. Asterisks indicate significant differences in the growth of treated samples with respect to control: *$p < 0.05$; ***$p < 0.001$. Capital letters indicate significant differences assessed with one-way ANOVA at $p < 0.05$ between chars tested at the same concentration (in the conditions 0.1, 0.5, and 1 g per plate).

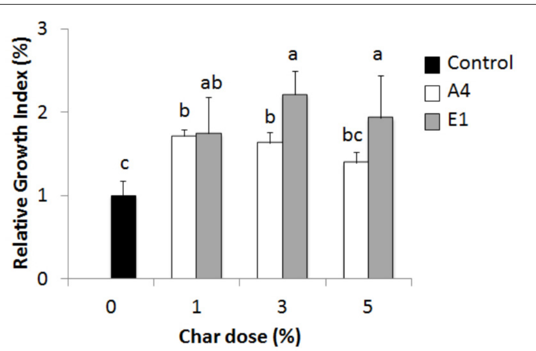

FIGURE 10 | Relative growth index (RGI) of *Hordeum vulgare* seedlings germinated for 72 h in the presence of chars from pyrolysis of different feedstocks at increasing concentrations (1 to 5%). White bars, char A4; gray bars, char E1. Means of 30 seeds relative to growth of the control in pure soil (black bar) are reported with standard errors. Letters indicate significant differences assessed with one-way ANOVA at $p < 0.05$.

decision process before a full-fledged application of biochar to agricultural crops (Tan et al., 2017).

The chars considered in our analysis were obtained from wastes from agriculture and forestry, and they were produced using two different processes at similar temperatures (around 500°C), (thermo)reforming and pyrolysis/pyrogasification. The properties of the chars were studied to understand the main factors in their application as soil improvers in agriculture.

The distribution of particle size is a relevant factor, since the finest particles can be released to the atmosphere during handling, consequently leading to an undesirable negative impact, thus counteracting the positive effects in terms of climate-altering emissions. In addition, fine particles in the soil decrease the water holding capacity compared to coarser particles (Liu Z. et al., 2017). Pelletizing chars could improve the handling procedures but on the other hand might decrease the large surface and microporosity, which are some of the main positive properties of char (Erlich et al., 2006; Karkania et al., 2012). Wetting biochar is probably a better solution to control small particles (Maienza et al., 2017), as is the case with some of the chars studied here.

The highest ash content in our study was found in chars derived from digestate and poultry litter, in line with the data on char from organic waste obtained through slow pyrolysis and other processes (Field et al., 2013; Qambrani et al., 2017). On the contrary, chars from wood have ash content <10%. Products with an ash content >50% are not compatible with the definition of biochar and are classified as pyrogenic carbonaceous material (European Biochar Certificate [EBC], 2015).

Microscopic examination of the different chars provided evidence of how the animal feedstocks, litter, and digestate became biochar matrix devoid of pores; conversely, plant substrates became porous biochar (Beesley and Marmiroli, 2011; Lu et al., 2012; Song et al., 2014). Biochar E1 showed a combination of characteristic wood-derived micro-, meso-, and macro-pores, covered with fine dust and interspersed with lumps of non-plant origin (**Supplementary Figures 3c,d**). In A3 and A4, the porous matrix appeared disorganized with pores of different sizes distributed at random. These structures could be ascribed to the wood pelleting process that brings together wood fragments with diverse structures (Karkania et al., 2012; Xue et al., 2013). The lower level of porosity of animal-derived chars, observed by other authors (Lu et al., 2012; Song et al., 2014), makes them less effective in retaining water in soil (Liu Z. et al., 2017).

Metals and trace elements were analyzed after wet digestion, and with X-ray analysis accompanying SEM. The data from EDX analysis were semi-quantitative, while those from the chemical analysis were quantitative. For many elements, such as Fe, Ni, Zn, and Cu, both techniques confirmed the elemental presence in all chars. EDX identified additional elements such as titanium and manganese, which might be derived from stainless steel components in the processing equipment (Fellet et al., 2014). The presence of heavy metals in high concentrations could be a factor in phytotoxicity (Jones and Quilliam, 2014), particularly evident in chars of non-plant origin analyzed in the present study. The possible release of toxic metals, present in the original feedstock and concentrated into the chars, has been considered a general hazard in the use of chars derived from animal waste, such as sludge and manure (Garlapalli et al., 2016; Wu et al., 2016). However, the high phytotoxicity of chars A1 and A2 could be explained in terms not only of metals, but also of excessive levels of S, Na, and Cl. For example, poultry litter feedstocks are normally rich in S, Na, and Cl and so are the chars obtained from them (Cantrell et al., 2012; Novak et al., 2013). In the case of sewage sludge, there is a wide variation in element content; however, S and Na are abundant and concentrated in derived biochars, as has been reported in other studies (Lu et al., 2012; Song et al., 2014; Mierzwa-Hersztek et al., 2017).

The toxicity of char E1 might be explained partly by its high Ni and Pb content, accompanied by a low pH (**Figure 7** and **Table 1**). A low pH is commonly associated with an increased concentration of aluminum (Al), which is highly toxic to plant roots (Gruba and Mulder, 2008). It is however interesting that while char E1 was toxic in germination tests on *Lepidium sativum*, it was highly phytostimulating in growth tests with *Hordeum vulgare*. Similar differences in the response of dicots and monocots have been demonstrated by Knox et al. (2018) using chars from wood pellets. It appears that the phytotoxic or phytostimulating activities of the biochar could be related not only to their metal content, but also to their macro- or micronutrient (K, Ca, S, P, Cl) content because it contributes to the salinity of the growth substrates (Farifteh et al., 2008). It has been shown that the high K content in many chars increases soil K availability and reduces K leaching (Laird et al., 2010). Phosphorus is often tightly bound in soils rich in Fe and Al oxides, but biochar addition increases soil pH, making P more available to plants and microorganisms (Asai et al., 2009; Hale et al., 2013).

It is known that carbonization processes can lead to phytotoxic products, such as PAHs or phenols, and other contaminants might be present depending on the input feedstocks used in the production of the chars (Bachmann et al., 2016). Organic compounds have not been analyzed in this study. Knox et al. (2018) attribute part of the phytotoxic effects to volatile compounds emitted by the biochar, and such emissions could be particularly important when tests are performed in closed containers, such as Petri dishes.

Restoring carbon in soils with biochar fits into the objectives of climate change priorities in Europe and in the world (Barrow, 2012). Biochar has also been proposed as a peat substitute, after the Commission Decisions of 2006 for soil improvers and for growing media specifying that the EU Ecolabel scheme for these products should apply only to those containing neither peat nor OM in their formulations. However, the application of biochar in real agricultural conditions requires simultaneous deployment of other improvers, such as OM, or standard fertilizers (Agegnehu et al., 2017; Bonanomi et al., 2017). It has been demonstrated recently that a coating of OM on the surface of biochar particles after co-composting increases the benefits to soil (Hagemann et al., 2017). In a recent experiment on arid and acidic Nepalese soils, wood biochar addition at 2% (v/v), implemented with NPK fertilization, significantly increased soil water retention capacity, plant-available water, increased soil EC, pH, exchangeable K^+, Mg^{2+} and Ca^{2+}, plant-available P, and organic C, giving rise to significantly improved maize biomass production in comparison to NPK fertilization (Pandit et al., 2018). However, the increase in soil EC corresponds to increase in salinity, which can lead to soil infertility (Farifteh et al., 2008). Therefore, careful land management should be followed according to the type of soil on which the biochars are applied: clay soils have a greater salinity buffer potential than sandy soils, and accurate balance between irrigation and drainage avoids the build-up of ions in the root zone, thus reducing exposition of plants to salt stress (Payen et al., 2016). Another study evaluated the application in a weathered tropical soil of biochar derived from sewage sludge, in combination with mineral fertilizer; after 2 years, only P availability from soil was increased by biochar, but overall the plant production rate increased (Monteiro Faria et al., 2017). These two experiments demonstrate that the direct effects of biochar on soil can be diverse, depending on the feedstock, on the overall biochar features, and on the soil type. However, accompanied by sensible land management, a predominantly stimulating effect on plant growth and yield was observed in both cases.

In this work, we were able to differentiate the biochars according to their physicochemical properties, microstructure, elemental contents, and the raw original biomass and to correlate these features with their respective optimal concentrations when used as plant fertilizers. In particular, biochars of animal origin were more phytotoxic at low concentrations than those of plant origin. In conclusion, it appears that additional experiments are needed to determine other characteristics of biochars to facilitate the use of appropriate feedstock and processing technologies to produce biochars that can be used as safe soil improvers.

AUTHOR CONTRIBUTIONS

UB, DI, GL, and FM were responsible for chemical analyses and tests with plants. NM, EM, and MM designed the experiments, collected bibliography, and performed the statistical analyses. All authors contributed to data analysis and writing of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01119/full#supplementary-material

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Effects of N Fertilizer Sources and Tillage Practices on NH_3 Volatilization, Grain Yield, and N Use Efficiency of Rice Fields in Central China

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Tillage practices and nitrogen (N) sources are important factors affecting rice production. Few studies, however, have examined the interactions between tillage practices and N fertilizer sources on NH_3 volatilization, nitrogen use efficiency (NUE), and rice grain yield. This study aimed to investigate the effects of N fertilizer sources (no N fertilizer, inorganic N fertilizer, organic N fertilizer alone, organic N fertilizer plus inorganic N fertilizer, and slow-release N fertilizer plus inorganic N fertilizer) and tillage practices (no-tillage [NT] and conventional intensive tillage [CT]) on NH_3 flux, grain yield, and NUE in the rice field of central China. N sources significantly affected NH_3 volatilization, as the cumulative volatilization from the treatments of inorganic N fertilizer, organic N fertilizer, organic N fertilizer plus inorganic N fertilizer, slow-release N fertilizer plus inorganic N fertilizer was 4.19, 2.13, 3.42, and 2.23 folds in 2013, and 2.49, 1.68, 2.08, and 1.85 folds in 2014 compared with that under no N fertilizer treatment, respectively. The organic N fertilizer treatment had the lowest grain yield and NUE among all N fertilizer treatments, while slow-release N fertilizer plus inorganic N fertilizer treatment led to relatively higher grain yield and the greatest N use efficiency. Moreover, NT only markedly increased NH_3 volatilization from basal fertilizer by 10–14% in average compared with CT, but had no obvious effects on total volatilization during the whole seasons. Tillage practices had no significant effects on grain yield and NUE. Our study suggested that the combination of slow-release N fertilizer plus inorganic N fertilizer and NT might be a sustainable method for mitigating greenhouse gas and NH_3 emissions and improving grain yield and NUE in paddy fields of central China.

Keywords: N recovery efficiency, NH_3 flux, no-tillage, organic N fertilizer, slow-release N fertilizer

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INTRODUCTION

Nitrogen (N) is one of the most important nutrients for agricultural systems, and thus N fertilizers are frequently used with the aim to achieve high yields of crop. As the most important cereal crop in China, rice accounts for 18.2% of total cultivated land area in China, and inorganic N fertilizers account for 36.2% of the total chemical N fertilizers used in rice production in the world (Department of Rural Social and Economic Investigation of the National Bureau of Statistics, 2017).

However, nitrogen use efficiency (NUE) of the N fertilizers applied in rice production usually falls within the range of 20–40% in China (Xu et al., 2013; Zhang and Zhang, 2013), which not only leads to low rice yields but also causes threat to the environment and human health. The NUE may be ascribed to nitrification, denitrification, NH_3 volatilization, runoff, and leaching in rice fields (Peng et al., 2006). Therefore, it is highly necessary to optimize the use of N fertilizers to reduce N losses and increase NUE in rice fields in China.

NH_3 volatilization is an important pathway of N fertilizer loss in paddy fields in China (Xu et al., 2013), which can usually account for 9–40% of the used N fertilizers (Fan et al., 2006). N management involves using an application source, rate, placement and timing that affect NH_3 emissions (Huang et al., 2016; Zheng et al., 2016). Effective N management, such as using controlled-release N fertilizer and mixture of organic and inorganic N fertilizers or deep N placement (Qi et al., 2012; Chen et al., 2015; Geng et al., 2015; Liu et al., 2015), can more closely match crop N uptake and lower NH_3 volatilization (Huang et al., 2016), which ensures an adequate amount of N required by the crop to maximize crop yields and NUE. Therefore, great efforts have been made to reduce NH_3 volatilization and increase NUE through using slow-release N or organic N fertilizers to partly or totally substitute inorganic N fertilizers in paddy fields (Singh et al., 2009; Xu et al., 2013; Huang et al., 2016; Ke et al., 2017; Li et al., 2017). There is growing evidence showing that full or partial substitution of inorganic N fertilizers with slow-release of organic N fertilizers could mitigate NH_3 emissions and thus increase NUE and rice yields (Chen et al., 2010; Huang et al., 2016; Li et al., 2017). However, other researchers found that crop yields and NUE could be significantly decreased when more inorganic N fertilizers was replaced by slow-release N fertilizers or organic N fertilizers (Bayu et al., 2006; Golden et al., 2009; Yang et al., 2015). Therefore, it is highly necessary to investigate effects of different N fertilizers on NH_3 emission, NUE, and yields in paddy fields.

As one of conservation tillage practices, no-tillage (NT) has been adopted worldwide due to its advantages in conserving water and soil, reducing input costs, increasing soil organic carbon, and improving crop productivity (Zhang et al., 2014; Pittelkow et al., 2015a,b). In recent years, the NT has been widely implemented in paddy fields in China (Derpsch et al., 2010; Huang et al., 2011; Liang et al., 2016). There is consensus on the effects of NT on NH_3 volatilization compared with conventional intensive tillage (CT) (Rochette et al., 2009; Zhang et al., 2011; Afshar et al., 2018). It has been well demonstrated that NT could promote NH_3 volatilization compared with CT due to the improvement of soil urease activity and the presence of crop residues under NT as well as the penetration of a fraction of the fertilizer N into soil shallow cracks under CT (Mkhabela et al., 2008; Rochette et al., 2009). However, the effect of NT on rice grains yields varies considerably (Pittelkow et al., 2015a,b). For example, Gao et al. (2004) reported that the rice grain yield under NT was higher than that under CT in eastern China, possibly due to the improvement of soil physical and chemical properties. Mishra and Singh (2012) also reported similar results, and found that NT resulted in significantly higher yields of rice, wheat and rice–wheat system relative to CT of a dry seeded rice–wheat system on a Vertisol in central India. Panday et al. (2008) observed similar rice grain yields between NT and CT in the northwestern Himalayan region and Zhang et al. (2015) reported that soil tillage did not affect both rice and wheat grain yields on a rice-wheat cropping system of Taihu region in China. Some researchers reported lower rice grain yields under NT relative to under CT (Gathala et al., 2011; Liang et al., 2016). The variations regarding the effects of NT on rice yield may be attributed to the differences in soil properties and field management practices (Gathala et al., 2011; Huang M. et al., 2012; Zhang et al., 2015). Hence, more research is needed to determine the influence of tillage practices on rice grain yields. The interaction between tillage practices and N fertilizer sources is important from the perspective of crop production (Balkcom and Burmester, 2015). However, little is known about the interactions between tillage practices and N fertilizer sources on NH_3 volatilization, NUE, and rice grain yield. Hence, this study was aimed to investigate the effects of N sources and tillage practices on the above-mentioned parameters in the paddy fields of central China. We hypothesized that N sources significantly affected NH_3 volatilization, grain yields and NUE, in which inorganic N fertilizers replaced by slow-release N fertilizers or organic N fertilizers could mitigate NH_3 volatilization and increase grain yields and NUE. We also hypothesized that NT could improve NH_3 volatilization, and increase grain yields and NUE compared with CT.

MATERIALS AND METHODS

Site Description

The experimental field (29°51$'$0N, 115°33$'$0E) is situated in Wuxue City, Hubei Province, China. The climate of this region is a humid mid-subtropical monsoon climate as described in detail by Zhang et al. (2016). The paddy soil, a type of sandy loam soil, is classified as Gleysol (FAO classification). The rice (*Oryza sativa*, LYP9) and oilseed rape (*Brassica napus*, HS3) varieties were planted. The mean monthly air temperature and rainfall of the experimental site are shown in **Table 1**. The soil properties were described in our previous study (Zhang et al., 2016).

Experimental Design

The field study was conducted from 2013 to 2014. The study included five N fertilizer treatments [no N fertilizer (N0),

TABLE 1 | Mean monthly air temperature (°C) and rainfall (mm) of the experimental site.

Month	2013		2014	
	Mean air temperature	Rainfall	Mean air temperature	Rainfall
June	25.80	226.80	25.72	124.70
July	29.85	30.10	27.46	218.90
August	29.76	65.70	26.20	51.70
September	23.50	64.40	24.34	22.30

inorganic N fertilizer (IF), organic N fertilizer (OF), organic N fertilizer + inorganic N fertilizer (OFIF), and slow-release N fertilizer + inorganic N fertilizer (SRIF)] and two tillage treatments (NT and CT) using a split-plot randomized complete block design with three replications. The N fertilizer sources were used as the main plots and tillage practices as the sub-plots. Each plot was 40 m^2 (5 m × 8 m) in area. Plastic films were inserted into 40 cm depth covered ridges (40 cm wide and 40 cm high) between the plots for preventing the movement of water and fertilizer. To further prevent the transferring of water and fertilizer, border rows with width of 1 m were planted between treatments.

Middle-season rice was direct-seeded on June 3rd in each year, and the harvest was conducted in early October. Throughout the whole rice season, fertilizers were applied at the rates of 180 kg N ha^{-1}, 90 kg P_2O_5 ha^{-1}, and 180 kg K_2O ha^{-1} for N fertilizer treatments. Both P (single super-phosphate) and K (potassium chloride) fertilizers were used as basal fertilizers only at the seedling stage, and the N fertilizers were applied in four split doses: at 50% as basal fertilizer, 20% as tillering fertilizer, 12% as jointing fertilizer and 18% as earing fertilizer under IF, OFIF and SRIF treatments. Conventional urea (46%) was used as the topdressing N. For OF treatment, 3082 kg ha^{-1} rape seed cakes (equal to 180 kg N ha^{-1}) were used in a single dose at only the seedling stage. For OFIF and SRIF treatments, 925 kg ha^{-1} rape seed cakes (equal to 54 kg N ha^{-1}) and 616 kg ha^{-1} slow release fertilizers (equal to 90 kg N ha^{-1}) were applied as basal fertilizer, respectively. The details of fertilizer management were described by Zhang et al. (2016).

The fertilizers were spread under NT subplots in which the soil was not disturbed. For CT subplots, the basal fertilizers were applied on the soil surface, and then the soil was plowed to 20 cm depth with a spade and harrowed by a multi-passes of chisel rake subsequently. The water depth of the plots was maintained at the depth of 8 cm during the rice growing season except for the tillering and maturing stages. Herbicides (36% glyphosate at 3 L ha^{-1}) and pesticides (20% chlorantraniliprole at 150 mL ha^{-1} and 3% emamectin benzoate at 450 mL ha^{-1}) were used to control weeds and pests during rice growing seasons when needed.

Measurement of NH_3 Volatilization

The NH_3 volatilization was measured by the ventilation method immediately after the mid-season rice was directly seeded (Wang et al., 2004; Jantalia et al., 2012). The detailed measurement was described by Liu et al. (2015). During the rice growing seasons of 2013 and 2014, the NH_3 flux was measured 22 times in each year. Cumulative NH_3 loss in each plot throughout the whole season was computed according to Liu et al. (2015).

Rice Plant Sampling and Analysis

To measure the rice grain yield, three frames (1 m × 1 m) were harvested in each plot. The grains were adjusted to the moisture content of 14%. Yield components were investigated from 12 hills sampled from the three harvested frames. The detailed measurement of productive panicle number per m^2, grain number per panicle, grain filling percentage, and 1000-grain weight was as described by Liu et al. (2015). Moreover, 10 hills in every plot were divided to panicle and straw, oven-dried at 80°C and weighed. The dried tissues were ground to determine the N concentrations by FIAstar5000 continuous flow injection analysis. N uptake was calculated as the product of N concentration and dry matter.

Data Analysis

The methods as described by Deng et al. (2014) and Liu et al. (2015) were used to compute N recovery efficiency (NRE), N agronomic efficiency (NAE), and N partial factor productivity (NFP). N loss rate through NH_3 emission was calculated as the ratio of cumulative NH_3 volatilization to applied amount of N.

Two-way ANOVA with SPSS 12.0 analytical software package was used to determine the effects of N sources and tillage practices on NH_3 flux, NUE, and grain yield. The least significant difference (LSD) test at the 0.05 or 0.01 probability level was conducted to compare the difference in the means between treatments.

RESULTS

NH_3 Fluxes

Seasonal changes of NH_3 fluxes under different treatments are shown in **Figures 1**, **2**. N fertilization significantly increased NH_3 fluxes, and peaks were observed 1–3 days after each N fertilization. The fluxes under tillage treatments ranged from 0.10 mg m^{-2} h^{-1} to 6.28 mg m^{-2} h^{-1} in 2013, and from 0.003 mg m^{-2} h^{-1} to 5.58 mg m^{-2} h^{-1} in 2014. Moreover, the fluxes under N fertilizer treatments fell within the range of 0.03 mg m^{-2} h^{-1} – 10.89 mg m^{-2} h^{-1} in 2013, and of 0.03 mg m^{-2} h^{-1} – 10.09 mg m^{-2} h^{-1} in 2014.

N fertilizer sources significantly affected cumulative NH_3 volatilization (**Table 2**). N fertilization remarkably increased the volatilization compared with N0. The volatilization under IF, OF, OFIF, and SRIF treatments were 4.19, 2.13, 3.42, and 2.23 fold in 2013, and 2.49, 1.68, 2.08, and 1.85 fold in 2014 relative to that in N0 treatment, respectively. Tillage practices had no effect on the volatilization throughout the whole seasons. The volatilization from basal fertilizer was obviously different between NT and CT, where NT significantly increased the volatilization by 10–14% in both years compared with CT. Moreover, the volatilization from basal fertilizer accounted for 50–69% in 2013 and 53–76% in 2014 of total volatilization under N fertilizer treatments. Significant interactive effects of N source and tillage practice on the volatilization was only observed in 2013.

Grain Yields and Yield Components

N fertilization significantly enhanced the grain yield due to the increase of productive panicle number, grain number per panicle, and grain filling percentage (**Table 3**). The grain yield under IF, OF, OFIF, SRIF treatments was 1.23, 1.15, 1.30, and 1.42 fold in 2013, and 1.17, 1.10, 1.30, and 1.28 fold in 2014 compared with that under N0 treatment, respectively. Moreover, tillage practices did not affect rice grain yield and its components. No significant

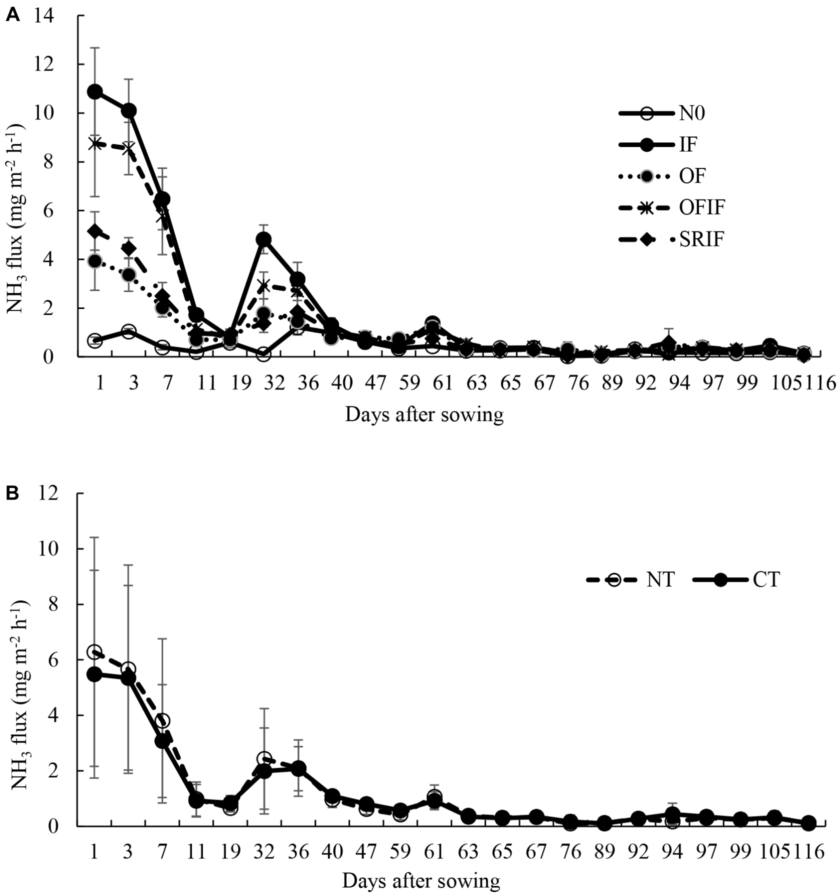

FIGURE 1 | Changes in NH_3 fluxes from different N fertilizer **(A)** and tillage practice **(B)** treatments during 2013 rice growing season. The arrows indicate N fertilization. N0, no N fertilizer; IF, inorganic N fertilizer; OF, organic N fertilizer; SRIF, slow-release N fertilizer combined with inorganic N fertilizer; OFIF, organic N fertilizer combined with inorganic N fertilizer; NT, no-tillage; CT, conventional intensive tillage.

interactive effects of N sources and tillage practices on grain yield were observed in each year, while there were significantly interactive effects of N sources and tillage practices on grain number per panicle and grain filling percentage in 2013 and on grain filling percentage in 2014.

NUE

N sources had obvious influence on NUE (**Table 4**). In general, OF treatment resulted in the lowest NRE, NAE and NFP among all N fertilizer treatments, while SRIF and OFIF treatments led to higher NRE, NAE and NFP than IF treatment. OFIF treatments increased the NRE, NAE and NFP by 11–42%, 31–75%, and 6–11%, and SRIF treatments increased the NRE, NAE and NFP by 58–77%, 59–84%, and 9–16%, compared with IF treatments, respectively. No interactive effects of N sources and tillage practices on NUE were observed.

DISCUSSION

This study investigated the effects of N sources and tillage practices on NH_3 volatilization, grain yield and NUE from paddy fields in central China. The results part supported our hypotheses that N sources had significant effects on NH_3 volatilization, grain yield and NUE, and SRIF treatment had the second-lowest NH_3 volatilization and the highest grain yield and NUE among N fertilizer treatments. However, tillage practices only influenced NH_3 volatilization at the early stage of rice under N fertilized conditions, but did not affected grain yield and NUE.

NH_3 Volatilization

NH_3 flux peaks observed 1–3 days after each N fertilizer treatment (**Figures 1**, **2**) may be attributed to the enzymatic hydrolysis of the applied N (Zhang et al., 2011; Shang et al., 2014). Enhancement of NH_3 volatilization caused by N fertilization has been reported in numerous studies (Zhang et al., 2011; Liu et al., 2015; Huang et al., 2016).

In the present study, the cumulative NH_3 volatilization under IF treatment was estimated to be 41.4–51.6 kg ha^{-1}, which is similar to the results reported by Liu et al. (2015) in this region. The cumulative NH_3 volatilization accounting for 50–76% of total NH_3 volatilization occurred in basal fertilizer under N fertilizer treatments in both years (**Table 2**). Similar results were observed by Xu et al. (2012), who reported that

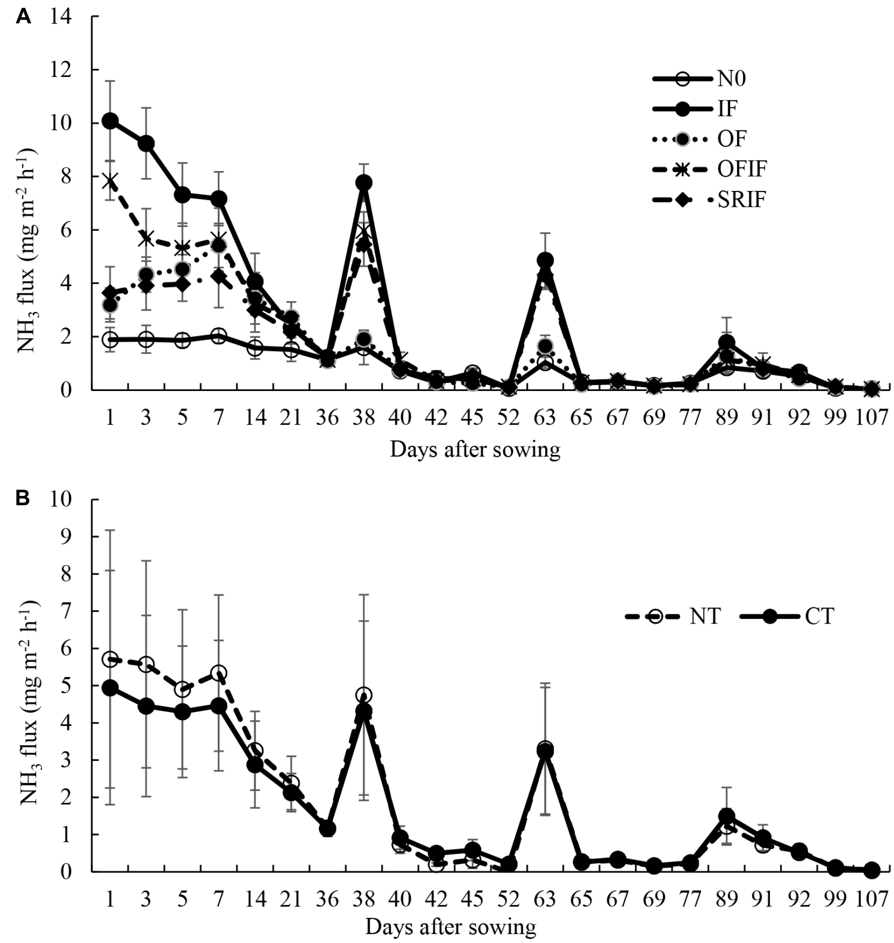

FIGURE 2 | Changes in NH_3 fluxes from different N fertilizer **(A)** and tillage practice **(B)** treatments during 2014 rice growing season. The arrows indicate N fertilization. N0, no N fertilizer; IF, inorganic N fertilizer; OF, organic N fertilizer; SRIF, slow-release N fertilizer combined with inorganic N fertilizer; OFIF, organic N fertilizer combined with inorganic N fertilizer; NT, no-tillage; CT, conventional intensive tillage.

TABLE 2 | Cumulative NH_3 volatilization (g m^{-2}) at different stages of N application under different treatments.

Treatments	2013					2014				
	Basal	Tillering	Jointing	Earing	Total	Basal	Tillering	Jointing	Earing	Total
N0	0.33 ± 0.05	0.45 ± 0.09	0.11 ± 0.01	0.10 ± 0.01	0.99 ± 0.10	1.32 ± 0.15	0.38 ± 0.06	0.25 ± 0.02	0.13 ± 0.02	2.08 ± 0.15
IF	2.81 ± 0.28	0.92 ± 0.12	0.22 ± 0.07	0.19 ± 0.06	4.14 ± 0.33	3.34 ± 0.30	1.18 ± 0.13	0.48 ± 0.05	0.17 ± 0.04	5.16 ± 0.32
OF	1.09 ± 0.10	0.62 ± 0.03	0.25 ± 0.09	0.15 ± 0.05	2.11 ± 0.15	2.58 ± 0.38	0.45 ± 0.08	0.32 ± 0.09	0.14 ± 0.02	3.49 ± 0.33
NFIF	2.19 ± 0.13	0.79 ± 0.07	0.22 ± 0.06	0.17 ± 0.04	3.38 ± 0.18	2.74 ± 0.29	1.06 ± 0.06	0.37 ± 0.04	0.16 ± 0.01	4.33 ± 0.22
SRIF	1.27 ± 0.07	0.62 ± 0.13	0.15 ± 0.02	0.16 ± 0.05	2.20 ± 0.13	2.23 ± 0.31	1.00 ± 0.11	0.46 ± 0.14	0.16 ± 0.04	3.86 ± 0.32
NT	1.61 ± 0.97	0.65 ± 0.23	0.19 ± 0.06	0.37 ± 0.06	0.15 ± 1.24	2.60 ± 0.77	0.78 ± 0.36	0.36 ± 0.09	0.15 ± 0.03	3.89 ± 1.16
CT	1.47 ± 0.85	0.71 ± 0.14	0.19 ± 0.09	0.41 ± 0.05	0.16 ± 1.05	2.28 ± 0.67	0.85 ± 0.34	0.40 ± 0.13	0.16 ± 0.03	3.69 ± 0.32
F-value										
N source	446.55**	28.98**	4.76**	3.23*	355.26**	58.34**	97.10**	7.87**	2.26^{ns}	112.22**
Tillage practice	12.25**	3.62^{ns}	0.10^{ns}	0.70^{ns}	1.31^{ns}	14.04**	4.76*	1.81^{ns}	1.78^{ns}	4.30^{ns}
N source × Tillage practice	3.18*	2.59^{ns}	0.40^{ns}	0.83^{ns}	4.03*	0.95^{ns}	0.31^{ns}	0.12^{ns}	0.70^{ns}	0.91^{ns}

Different letters between N fertilizer treatments under the same tillage practice indicate significant differences at the 5% level. N0, no N fertilizer; IF, inorganic N fertilizer; OF, organic N fertilizer; SRIF, slow-release N fertilizer combined with inorganic N fertilizer; OFIF, organic N fertilizer combined with inorganic N fertilizer; NT, no-tillage; CT, conventional intensive tillage. "" and "**" mean $P < 0.05$ and $P < 0.01$, respectively; ns, not significant.*

TABLE 3 | Grain yields and yield components under different treatments.

Treatments	2013					2014				
	Productive Panicle(10^{-2})	Grain number per panicle	Grain filling percen tage (%)	1000-grain weight (g)	Grain yield (kg ha^{-1})	Productive panicle (10^{-2})	Grain number per panicle	Grain filling percentage (%)	1000-grain weight (g)	Grain yield (kg ha^{-1})
N0	217.12 ± 10.31	152.85 ± 7.23	0.72 ± 0.01	26.05 ± 0.60	6474.35 ± 168.64	213.99 ± 9.58	174.77 ± 7.76	74.40 ± 1.14	25.19 ± 0.51	7491.41 ± 135.50
IF	261.94 ± 9.99	175.52 ± 6.17	0.81 ± 0.02	27.60 ± 0.65	7957.60 ± 274.83	232.92 ± 6.75	206.28 ± 14.09	77.59 ± 2.26	24.93 ± 0.16	8798.62 ± 177.88
OF	261.12 ± 15.65	171.99 ± 10.85	0.74 ± 0.01	25.52 ± 0.23	7440.32 ± 255.85	237.24 ± 12.50	193.93 ± 14.72	72.61 ± 6.99	24.77 ± 0.34	8261.00 ± 124.66
OFIF	274.34 ± 13.35	167.42 ± 3.35	0.80 ± 0.01	27.11 ± 0.71	8417.22 ± 172.88	245.47 ± 11.83	202.74 ± 7.82	79.55 ± 4.03	25.28 ± 0.36	9766.00 ± 630.58
SRIF	288.23 ± 15.17	181.62 ± 13.37	0.76 ± 0.01	26.52 ± 0.80	9208.55 ± 473.41	293.83 ± 17.81	194.73 ± 7.73	82.38 ± 1.99	25.73 ± 0.32	9569.00 ± 243.08
NT	259.69 ± 29.33	170.44 ± 10.31	0.77 ± 0.03	26.70 ± 1.01	7959.04 ± 980.43	247.33 ± 30.10	194.13 ± 16.08	77.17 ± 6.60	25.30 ± 0.52	8776.18 ± 966.56
CT	261.40 ± 25.77	169.31 ± 15.39	0.77 ± 0.05	26.42 ± 0.90	7840.17 ± 996.97	242.06 ± 29.48	194.85 ± 14.48	77.44 ± 3.10	25.06 ± 0.32	8778.23 ± 873.46
F-value										
N source	21.13**	21.39**	137.98**	10.41**	65.90**	37.23**	6.51**	9.08**	6.58**	43.07**
Tillage practice	0.11^{ns}	0.29^{ns}	0.38^{ns}	1.42^{ns}	1.10^{ns}	1.45^{ns}	0.03^{ns}	0.06^{ns}	3.75^{ns}	0.00^{ns}
N source × Tillage practice	0.29^{ns}	10.01**	21.47**	0.90^{ns}	0.23^{ns}	1.19^{ns}	0.42^{ns}	4.30*	0.54^{ns}	0.32^{ns}

Different letters between N fertilizer treatments under the same tillage practice indicate significant differences at the 5% level. N0, no N fertilizer; IF, inorganic N fertilizer; OF, organic N fertilizer; SRIF, slow-release N fertilizer combined with inorganic N fertilizer; OFIF, organic N fertilizer combined with inorganic N fertilizer; NT, no-tillage; CT, conventional intensive tillage. "" and "**" mean $P < 0.05$ and $P < 0.01$, respectively; ns, not significant.*

the cumulative NH_3 volatilization from basal and tillering fertilizer (about 1 month) accounted for more than 80% of the total NH_3 volatilization from rice fields in the Tai-lake region of China. The high volatilization in this stage may be due to the application of relatively more N fertilizers and high temperatures in this stage (**Table 1**). Moreover, a previous study indicated that dense canopy may act as a sink of NH_3 in more vigorous stages (Bash et al., 2010). Thus, the climate conditions in the sparse canopy at the early stages of rice growth may facilitate NH_3 emission (Cao and Yin, 2015; Zhao et al., 2015).

In this study, compared with IF treatment, the other three N fertilizer treatments significantly decreased NH_3 volatilization (**Table 2**), suggesting that the application of organic N or slow-release N fertilizers is an effective strategy for mitigating NH_3 emission from paddy fields. The results are consistent with those reported by Xu et al. (2013), Ye et al. (2013), Huang et al. (2016), and Ke et al. (2017). Moreover, the OF treatment resulted in the lowest NH_3 volatilization among all N fertilizer treatments, which may be due to the relatively low availability of N from the decomposition of rape seed cake (Bayu et al., 2006). NH_4^+ released from the mineralization of the rape seed cake can be partly immobilized by microbes and the soil (Heal et al., 1997; Yang et al., 2015), which thereby reduces NH_4^+ concentration in soil and then decreases the NH_3 volatilization. Compared with OFIF treatment, SRIF treatment decreased the NH_3 volatilization (**Table 2**) possibly due to a synchronization of N release with rice requirement (Ke et al., 2017). The prolonged release of fertilizer N matches the requirement of rice, and reduces soil NH_4^+ concentrations and NH_3 volatilization subsequently (**Figures 1**, **2** and **Table 2**).

In this study, higher NH_3 volatilization under N fertilized conditions from NT at the early stage of rice growth was higher than that from CT (**Table 2**). Similar results were reported by Zhang et al. (2011). Greater urease activities in soil surface under NT than CT (Zhang et al., 2011) may result in higher concentration of NH_4^+ in the soil and floodwater from hydrolyzed fertilizer N, which thereby promotes NH_3 emission under NT. Moreover, the contact of fertilizer particles and the soil may be reduced by the residues retained in NT soil surface, which means reduced adsorption of NH_4^+ from hydrolyzed fertilizer N by soil particles under NT. However, dense canopy may act as a sink of NH_3 in the middle and later stages of rice growth (Bash et al., 2010). Therefore, promoting effects of NT on NH_3 volatilization was only recorded in the early stage of rice growth in this study (**Table 2**). As Rochette et al. (2009) reported, fraction of fertilizer N may diffuse into the shallow cracks under CT, which may result in lower NH_3 volatilization at the early stage of crop growth under CT than under NT.

Grain Yield

The lowest yield was recorded under OF treatment, while the highest was observed under OFIF and SRIF treatments (**Table 3**). It has been reported that the application of organic matter alone may not be enough to sustain crop yield due to its relatively low nutrient supply (Bayu et al., 2006), which is in agreement with the results reported by Yang et al. (2015), Wei et al. (2016),

TABLE 4 | Nitrogen use efficiency from different treatments.

Treatments	2013			2014		
	NRE (%)	NAE (kgkg^{-1})	NFP (kgkg^{-1})	NRE (%)	NAE (kgkg^{-1})	NFP (kgkg^{-1})
N0	–	–	–	–	–	–
IF	26.63 ± 2.84	8.24 ± 1.61	44.21 ± 1.53	37.45 ± 4.34	7.26 ± 1.29	48.88 ± 0.99
OF	22.81 ± 2.80	5.37 ± 0.88	41.34 ± 1.42	23.51 ± 2.57	4.28 ± 1.15	45.89 ± 0.69
OFIF	37.76 ± 3.48	10.79 ± 0.65	46.76 ± 0.96	41.57 ± 4.17	12.64 ± 3.95	54.26 ± 3.50
SRIF	47.10 ± 2.43	15.19 ± 2.83	51.76 ± 2.63	59.24 ± 3.93	11.54 ± 1.19	53.16 ± 1.35
NT	32.80 ± 10.19	10.03 ± 3.88	46.22 ± 3.95	39.19 ± 12.84	9.31 ± 4.30	50.62 ± 4.21
CT	34.35 ± 10.44	9.77 ± 4.32	45.51 ± 4.25	41.69 ± 14.60	8.55 ± 3.85	50.48 ± 3.73
F-value						
N source	83.44**	29.76**	30.14**	100.39**	15.57**	19.59**
Tillage practice	1.64^{ns}	0.110^{ns}	0.88^{ns}	2.90^{ns}	0.60^{ns}	0.03^{ns}
N source × Tillage practice	0.60^{ns}	0.25^{ns}	0.25^{ns}	1.20^{ns}	0.24^{ns}	0.30^{ns}

*Different letters between N fertilizer treatments under the same tillage practice indicate significant differences at the 5% level. N0, no N fertilizer; IF, inorganic N fertilizer; OF, organic N fertilizer; SRIF, slow-release N fertilizer combined with inorganic N fertilizer; OFIF, organic N fertilizer combined with inorganic N fertilizer; CT, conventional intensive tillage; NRE, N recovery efficiency; NAE, N agronomic efficiency; NFP, N partial factor productivity; NT, no-tillage; CT, conventional intensive tillage. "**" means $P < 0.01$; ns, not significant.*

and Zhang et al. (2017). These results demonstrate that it is impossible to increase the grain yield to meet the food demand in the world through establishing a rice production system that depends exclusively on organic matter (Seufert et al., 2012). However, it was noted that the use of organic fertilizer alone can substantially increase the yield if sufficiently large quantities are applied (Wei et al., 2016). For example, Lu et al. (2012) found that the application of 270 kg N ha^{-1} slurry can bring about a rice grain yield similar to that results from the application of 270 kg N ha^{-1} urea. We found that the application of organic N fertilizer + inorganic N fertilizer (OFIF) resulted in a higher grain yield than the application of inorganic N fertilizer only (**Table 3**), which may be due to the improvement of nutrient efficiency and organic matter impacts (Han et al., 2004; Wei et al., 2016). Wei et al. (2016) performed a comprehensive review based on 32 long-term experiments in China, and reported the positive effects of the combination of organic and inorganic fertilizers on rice grain yield. However, Zhang et al. (2017) found that amending inorganic fertilizer with anaerobically digested pig slurry had no significant effects on rice grain yield. This discrepancy may be attributed to different types of organic fertilizers and the ratio of organic and inorganic fertilizers used (Wei et al., 2016). Slow-release N fertilizer can release N into the soil that can closely match the N demand in different growing stages of crop, which has been widely implemented in China to improve crop production and mitigate environmental problems caused by the application of inorganic fertilizers (Yang et al., 2015; Zheng et al., 2016; Ke et al., 2017). In this study, the substitution of half of inorganic N fertilizer by slow-release N fertilizer resulted in a higher grain yield relative to inorganic N fertilizer (**Table 3**). The N released from slow-release N fertilizer at the early and middle stages of rice growth is relatively low, which may result in N supply deficiency at the stages (Ke et al., 2017); thus, topdressing N at the tillering and jointing stages may better satisfy the N demand at different growing stages of rice in this study. Chen et al. (2010) and Ke et al. (2017) also reported that a mixture of inorganic and slow-release N fertilizers could increase grain yield.

In the present study, the grain yield was increased under N fertilization due to the increase of productive panicle number, grain number per panicle, and grain filling percentage (**Table 3**), which is basically consistent with the previously reported results (Li et al., 2017).

Yield is an important indicator to assess the response of crop to tillage practices. In this study, no significant effects of tillage practices on grain yield were observed (**Table 3**). The effects of NT practice on rice grain yield can be promoting (Gao et al., 2004), decreasing (Gathala et al., 2011), and no effect (Zhang et al., 2011, 2016) compared with CT practice. For example, Gao et al. (2004) reported that NT significantly increased rice yield in eastern China compared with CT because of the improvement of paddy soil physical and chemical properties. Sharma et al. (2005) reported the reduction of rice yields in rice-based systems under NT in northern India. In the northwestern Himalayan region, NT did not affect rice yield compared with CT (Panday et al., 2008). The variables might be related to soil properties (e.g., texture and pH), climates (e.g., temperature and light) and field management practices (e.g., N application rate, planting method, crop rotation, residue management, and the duration of NT use) (Xie et al., 2007; Gathala et al., 2011; Huang M. et al., 2012). Moreover, the yields varied between the 2 years in this study (**Table 3**), which might result from the year-specific climate (**Table 1**).

NUE

N source significantly affected NUE of rice, and OF treatment resulted in the lowest NUE among four N fertilizer treatments (**Table 4**). Bayu et al. (2006) proposed that the application of organic materials alone may not be enough to maintain crop production due to the limited availability and relatively low nutrient content of organic materials. Moreover, it is commonly believed that the combination of organic and chemical N fertilizers can reduce N losses by converting inorganic N into organic forms, and thus can enhance the efficiency of the fertilizers compared with the application of inorganic N fertilizer alone (Yang et al., 2015). The combination could improve the nutrient uptake efficiency of crops (Han et al., 2004). Thus, we found higher NUE under OFIF than under IF in this study (**Table 4**). Slow-release N fertilizer has been reported to decrease N losses through denitrification, NH_3 volatilization (**Table 2**), N leaching and N runoff because N release of the fertilizer can closely match the N demand at the later stages of rice growing (Timilsena et al., 2015; Ke et al., 2017). Therefore, although half of inorganic N fertilizer was replaced by slow-release N fertilizer in this study, SRIF treatment resulted in higher NUE compared with IF treatment. Similar result was reported by Ke et al. (2017), who observed that the combination of organic and inorganic N fertilizers (83.3%:16.7%) resulted in higher NUE than the application of inorganic N alone due to the relatively uniform N release from slow-release N fertilizer and the synchronization of the N release with the N requirement of rice.

Although it has been reported that NT promotes N losses through NH_3 volatilization, N leaching, and N runoff (Zhang et al., 2011; Liang et al., 2016), the combination of NT with retained residues could help to reduce the negative effects of NT on the N losses in paddy fields due to the improvement of properties, fertility and microbial activities in the soil, which can provide rice with sufficient N sources (Huang J. et al., 2012). In the present study, the previous crop resides were retained in the field, and thus no significant effect of tillage practices on NUE was observed (**Table 4**). The result was inconsistent with the result based on meta-analysis (Liang et al., 2016) that NT overall decreased N uptake and NUE. The discrepancy may be attributed to the differences in agricultural management practices, climate and soil property and the duration of NT (Liang et al., 2016).

Our previous study has reported that SRIF plus NT showed the lowest global warming potential and greenhouse gas intensity among all treatments (Zhang et al., 2016). Therefore, from this study, the SRIF plus NT treatment may be recommended as a sustainable strategy to reduce greenhouse gas and NH_3 emissions, and increase grain yield and NUE in central China.

CONCLUSION

N sources remarkably affected NH_3 volatilization, NUE, and grain yield; while tillage practices had significant effects on NH_3 volatilization, but had no effects on grain yield and NUE. SRIF treatment resulted in relatively low NH_3 volatilization and high grain yield and NUE. Our results suggest that the combination of SRIF and NT is an economic and environmental strategy for mitigating greenhouse gas and NH_3 emissions, improving NUE, and increasing rice yields in central China.

AUTHOR CONTRIBUTIONS

CL and CC designed the research. TL, JH, and KC performed the experiments. TL analyzed the data and wrote the manuscript. All of the authors read and approved the final manuscript.

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Developing Sustainable Agromining Systems in Agricultural Ultramafic Soils for Nickel Recovery

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Ultramafic soils are typically enriched in nickel (Ni), chromium (Cr), and cobalt (Co) and deficient in essential nutrients, making them unattractive for traditional agriculture. Implementing agromining systems in ultramafic agricultural soils represent an ecological option for the sustainable management and re-valorisation of these low-productivity landscapes. These novel agroecosystems cultivate Ni-hyperaccumulating plants which are able to bioaccumulate this metal in their aerial plant parts; harvested biomass can be incinerated to produce Ni-enriched ash or "bio-ore" from which Ni metal, Ni ecocatalysts or pure Ni salts can be recovered. Nickel hyperaccumulation has been documented in ∼450 species, and in temperate latitudes these mainly belong to the family Brassicaceae and particularly to the genus *Odontarrhena* (syn. *Alyssum pro parte*). Agromining allows for sustainable metal recovery without causing the environmental impacts associated with conventional mining activities, and at the same time, can improve soil fertility and quality and provide essential ecosystem services. Parallel reductions in Ni phytotoxicity over time would also permit cultivation of conventional agricultural crops. Field studies in Europe have been restricted to Mediterranean areas and these only evaluated the Ni-hyperaccumulator *Odontarrhena muralis s.l*. Two recent EU projects (Agronickel and LIFE-Agromine) have established a network of agromining field sites in ultramafic regions with different edapho-climatic characteristics across Albania, Austria, Greece and Spain. Soil and crop management practices are being developed so as to optimize the Ni agromining process; field studies are evaluating the potential benefits of fertilization regimes, crop selection and cropping patterns, and bioaugmentation with plant-associated microorganisms. Hydrometallurgical processes are being up-scaled to produce nickel compounds and energy from hyperaccumulator biomass. Exploratory techno-economic assessment of Ni metal recovery by pyrometallurgical conversion of *O. muralis s.l.* shows promising results under the condition that heat released during incineration can be valorized in the vicinity of the processing facility.

Keywords: *Alyssum s.l.*, hydrometallurgy, *Leptoplax emarginata*, *Odontarrhena*, phytomining, serpentine soils

INTRODUCTION

Ultramafic Soils in Europe

Ultramafic (serpentine) soils are natural metalliferous soils derived from the weathering of ultramafic rocks which comprise at least 70% ferromagnesian (or mafic) minerals (particularly within the olivine and pyroxene groups) and <45% silica (SiO_2) (Kruckeberg, 2002). Ultramafic outcrops are found worldwide but with a patchy distribution, covering ~1% of the terrestrial surface (Echevarria, 2018). Major outcrops can be found in temperate (e.g., Alps, Balkans, Turkey, California) and tropical regions (e.g., New Caledonia, Cuba, Brazil, Malaysia, Indonesia).

Ultramafic soils typically present a series of geochemical peculiarities, which include an elevated concentration of magnesium (Mg) and iron (Fe), a low calcium (Ca):Mg ratio and elevated concentrations of trace elements such as nickel (Ni), chromium (Cr), and cobalt (Co). Total Ni concentrations in soils globally range between 2 and 750 mg kg^{-1} but can reach 3,600 mg kg^{-1} or more in soils developed over ultramafic rocks (Sparks, 2002). They are also known for their deficiency in macronutrients such as nitrogen (N), potassium (K), or phosphorus (P) and micronutrients such as molybdenum (Mo) or boron (B) (Nkrumah et al., 2016). Since many ultramafic outcrops are steep and rocky their associated soils are often skeletal, with limited organic matter and low water holding capacity (Brooks, 1987). In temperate climates, the most common soil types include high pH Regosols/Leptosols with cambic properties and cation exchange complex dominated by Mg over Ca, to Cambisols with neutral to slightly acidic pH (Echevarria, 2018). This unusual geochemical composition is commonly referred to as the "serpentine syndrome" and creates an inhospitable environment for plant growth. As a result, ultramafic soils often support plant communities with high rates of endemism which have evolved both morphological and physiological adaptations differentiating them from the flora of neighboring areas (Brooks, 1987; Bergmeier et al., 2009). For example, hyperaccumulator plants accumulate extreme amounts of metals within their living tissues. There are ~500 taxa of plants that are known to hyperaccumulate one or more metal(loid)s, and around 90% of these accumulate Ni (van der Ent et al., 2013a; Pollard et al., 2014). Ni-hyperaccumulators accumulate >1,000 mg Ni kg^{-1} DW matter in their shoots when growing in such metal(loid)-enriched substrates, concentrations that would be toxic to most other plant species (Brooks, 1987). The distribution and taxonomy of European Ni-hyperaccumulators is described in more detail below.

The Concept of Agromining

The European economy is highly dependent on imports of many raw materials including metals, metalloids, and minerals, with applications in several high-tech industries (ICMM, 2012). Many of these are threatened by supply shortage, due to political instability or rapidly declining ore reserves, and the EU prioritizes research on the extraction, recovery, or recycling of metals from unconventional mineral resources which are not accessible using traditional mining techniques (http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:52011DC0021&from=EN). Agromining cultivates plants that accumulate trace metal(loid)s from metal-rich soils or substrates in their shoots, which at the end of the growth period can be harvested and burnt to produce metal(loid)-enriched ash or "bio-ore." It is considered a commercially viable technique in the case of high-value elements such as Ni, Co, or Au (Chaney et al., 2018). The European Innovation Partnership (EIP) classified Ni as a raw material with high economic importance and Ni agromining was proven to be efficient in the 2000's and became a real market opportunity in 2007 (Chaney et al., 2007; Tang et al., 2012; Bani et al., 2015a). Nickel has been successfully recovered from bio-ores in pure form, as a mineral salt (ANSH, ammonium nickel sulfate hexahydrate), or as eco-catalysts (Simonnot et al., 2018). Nickel-hyperaccumulator plants are ideal candidates for Ni agromining due to their unusual ability to bioconcentrate and purify Ni from ultramafic soils. However, the only large-scale European field trial carried out to date was done in Albania using *Odontarrhena muralis sensu lato* (syn. *Alyssum murale s.l.*) (Bani et al., 2007, 2015a,b). In addition to metal extraction, agromining agrosystems can also be managed to provide multiple ecosystem services, such as C sequestration, enhanced soil biodiversity, renewable biomass production, improved agricultural crop productivity (safe edible and non-edible crops) and land restoration (Echevarria et al., 2015). Nickel agromining could also stimulate the rural development of ultramafic regions.

The growing interest in this emerging technology is reflected through the recent funding of two EU projects: Agronickel (*Developing Ni agromining on ultramafic land in Europe*; funded through the FACCE Surplus–Cofund) and LIFE-Agromine (*Cropping hyperaccumulator plants on nickel-rich soils and wastes for the green synthesis of pure nickel compounds*; funded by the LIFE Environment and Resources

Programme, http://life-agromine.com). These two projects aim to implement agromining at large scale and follow on from the "Agromine" project funded by the ANR (*Agence Nationale de la Recherche*) in France. Agronickel is dedicated to the selection of adequate nickel crops, optimisation of their biomass productivity and Ni extraction capacity using resource-friendly agriculture (so-called "agroecological" agriculture), and the development of the metal recovery process and synthesis of new Ni-products. The optimal plant cropping pattern and the potential benefits of using plant associated-microbial inoculants or phytohormones will be assessed. On the other hand, through the LIFE-Agromine project the opportunity arose to establish a European network of agromining field-scale demonstrations across distinct geographical areas and under different edapho-climatic conditions. The LIFE-Agromine project aims to demonstrate at a pilot-scale the provision of ecosystem services through agroecological agromining cropping systems, the recovery of valuable Ni-products, as well as the environmental and socio-economic viability of the agromining cycle, including professional training of potential stakeholders. Studies evaluating the environmental, technical, and economic viability of agromining at all stages of the cycle are rare. LIFE-Agromine will carry out a full-cycle assessment of the potential benefits of cropping hyperaccumulator plants on these metalliferous soils for the green synthesis of nickel compounds and provision of ecosystem services.

A SYNTHETIC OVERVIEW OF NI-HYPERACCUMULATORS IN EUROPE

Nickel-hyperaccumulators native to Europe are mostly distributed in the southern parts of the continent, where ultramafic outcrops occur at various altitudinal belts and bioclimatic regions. Their diversity follows a west-to-east gradient, with the Iberian Peninsula and Corsica hosting only one or two native species, vs. ca. eight in CS Europe-C Mediterranean and some 30 taxa in SE Europe. Due to the massive distribution of ultramafic soils, the Balkan Peninsula is an outstanding hot-spot of metallophytic flora and serpentine endemism of both paleo- and neo-endemic type (Shallari et al., 1997; Stefanović et al., 2003). Albania and Greece are especially rich in Ni-accumulators, most of them endemics, showing the role of the large ultramafic bodies of the so-called "Dinaric-Hellenid" belt (Bortolotti et al., 2013) as centers of speciation and glacial refugia (Médail and Diadema, 2009). However, the systematics of Ni-accumulators, especially from E Europe is still not completely known, preventing an exact estimation of their diversity to be safely presented.

Undoubtedly, their taxonomic and phylogenetic distribution is highly uneven. All species known so far belong to the Brassicaceae family (crucifers), with a few exceptions reported from the Asteraceae genus *Centaurea* L. Uneven distribution also occurs within the Crucifers, since all species belong to only two of the ca. 23 tribes of the family, e.g., the Coluteocarpeae and the Alysseae. The former group includes the large genus *Noccaea* Moench, separated from *Thlaspi* L. based on phylogenetic evidence (Koch and Al-Shehbaz, 2004; Al-Shehbaz, 2014 and references therein). This includes 11 species that can grow on ultramafic or metal-contaminated soils of Europe and accumulate metals such as Ni and Zn (**Table 1**). Most of these taxa, however, are of limited interest for agromining applications because of their small biomass production or high ecological specificity (alpine habitats). With four genera and 21 species recognized to date (**Table 1**), tribe Alysseae is the largest group of Ni-hyperaccumulators in the continent and includes the most promising candidates for Ni-phytoextraction. These are all perennial herbs or small shrubs belonging to three genera, *Odontarrhena* C.A.Mey., *Alyssoides* Mill., and *Bornmuellera* Hausskn. Based on phylogenetic evidence (Cecchi et al., 2010; Rešetnik et al., 2013) the latter is very close to genus *Leptoplax* O.E. Schulz with the only species *L. emarginata*, a strict endemic to the serpentines of N Greece and Euboea. In spite of distinctive characters in habit and fruit compared to typical taxa of *Bornmuellera* (Hartvig, 2002), the occurrence of intergeneric F1 hybrids with partially sympatric *B. tymphaea* and *B. baldaccii* led Rešetnik et al. (2013) to include *Leptoplax* in *Bornmuellera*, and this was recently accepted in the Alybase treatment (Španiel et al., 2015). Three features convey to *B. emarginata* a remarkable potential for Ni-phytoextraction, e.g., high accumulation ability (Bani et al., 2010), biomass production (Chardot et al., 2005), and adaptive capacity to different habitats and climates, from low to high altitudes (ca. 300–2100 m a.s.l). Drought tolerance is a fourth feature that makes its use possible also in Mediterranean xeric conditions. The four other taxa of *Bornmuellera* are also powerful Ni-accumulators restricted to serpentine soils of N Greece, Albania, and Kosovo (Bani et al., 2009), but their smaller stature, biomass production and restriction to alpine habitats (above 1,000 m and up to 2,600 m a.s.l.) represent limiting factors to their use for field applications.

Odontarrhena has long been considered a section of the genus *Alyssum* L., but there is morphological and molecular evidence showing that it represents a distinct lineage without direct relationship to *Alyssum* (Warwick et al., 2008; Cecchi et al., 2010, 2013; Rešetnik et al., 2013). While Ni-accumulation is unknown among the "true" Alyssums, nearly all species and populations of *Odontarrhena* that grow on ultramafic soil possess this ability. This is the case for a high proportion of the nearly 90 taxa of this genus that range in S Europe, Mediterranean and W Asia (Španiel et al., 2015). *Odontarrhena* is taxonomically difficult because of the high phenotypic plasticity, the strong incidence of polyploidy and the presence of hybridization processes within at least given groups. Examples of critical species complexes are the two facultative serpentinophytes *O. serpyllifolia* and *O. muralis* in the W and E Mediterranean, respectively, in which several names have been adopted to separate the serpentine populations. Both are effective Ni-hyperaccumulators (Bani et al., 2010, 2013; Tumi et al., 2012; Konstantinou and Tsiripidis, 2015; Morais et al., 2015), and the complex of *O. muralis s.l.* is currently one of the most promising candidates taxa for Ni-agromining because of its biomass production and growth capacity in various field conditions (Bani et al., 2015a,b). Nickel concentrations recorded in field-collected and herbarium material over the last 30 years in the Balkan Peninsula found this species to accumulate the

TABLE 1 | Nickel-accumulator species in tribes Alysseae and Coluteocarpeae (Brassicaceae) native to Europe (including Cyprus); nomenclature of *Noccaea* and Alysseae follows respectively Al-Shehbaz (2014) and Spaniel et al. (2015; see also Alybase; http://www.alysseae.sav.sk/checklists/search).

Taxon	Distribution
TRIBE ALYSSEAE	
Alyssoides utriculata (L.) Medik.	France, Italy, former Yugloslavian countries, Albania, Bulgaria, Greece
Bornmuellera baldaccii (Degen) Heywood subsp. *baldaccii*	Greece
B. baldaccii subsp. *rechingeri* Greuter	Albania
B. dieckii Degen	Kosovo
B. emarginata (Boiss.) Rešetnik (syn.: *Leptoplax emarginata* (Boiss.) O.E. Schulz)	Greece
B. tymphaea (Hausskn.) Hausskn.	Greece
Odontarrhena akamasica (B.L.Burtt) & al.	Cyprus
O. argentea (All.) Ledeb.	Italy
O. diffusa Jord. & Fourr.	Greece
O. fallacina (Hausskn.) Španiel & al.	Greece
O. bertolonii (Desv.) Jord. & Fourr.	Italy
O. chalcidica (Janka) Španiel & al.	Greece, Albania, FYROM, Kosovo
O. cyprica (Nyár.) Španiel & al.	Cyprus
O. euboea (Halácsy) Španiel & al.	Greece
O. heldreichii (Hausskn.) Španiel & al.	Greece
O. lesbiaca P.Candargy	Greece
O. muralis (Waldst. & Kit.) Endl.	Bulgaria, Greece, Romania, Serbia
O. robertiana (Bernard ex Gren. & Godr.) Španiel & al.	France (Corsica)
O. serpyllifolia (Desf.) Jord. & Fourr.	Iberian Peninsula, France
O. smolikana (Nyár.) Španiel & al.	Greece, Albania
O. troodi (Boiss.) Španiel & al.	Cyprus
TRIBE COLUTEOCARPEAE	
N. alpestris (Jacq.) Kerguélen	Austria, France, Italy, Switzerland
N. boeotica F.K.Mey (Spruner)	Greece
N. brachypetala (Jord.) F.K.Mey.	Austria, Czech Rep. Finland, France, Italy, Slovakia, Spain, Sweden, Switzerland
N. bulbosa (Spruner) Al-Shehbaz	Greece
N. caerulescens (J.Presl & C.Presl) F.K.Mey.	C, W, N Europe
N. cepaeifolia (Wulfen) Rchb.	France, Austria, Italy
N. epirota (Halácsy) F.K.Mey.	Greece
N. goesingensis (Halácsy) F.K.Mey.	Austria, Bosnia and Herzegovina, Bulgaria, Serbia
N. graeca (Jord.) F.K.Mey,	Greece
N. rotundifolia (L.) Moench	Austria, France, Germany, Italy, Slovenia, Switzerland
N. tymphaea (Hausskn.) F.K.Mey.	Albania, Bosnia and Herzegovina, Greece, FYROM

Odontarrhena corsica (Duby) Španiel & al., also a Ni-hyperaccumulator naturalized in Corsica but native to Turkey.

highest Ni concentrations (Bani et al., 2010, 2013). Furthermore, *O. muralis s.l.* is crucial for the structure of the serpentine vegetation in the Balkans (Bergmeier et al., 2009). While the taxa from Greece are better understood, some of those from Albania, the former Yugoslav Republic of Macedonia (FYROM), and Bulgaria are still poorly known, preventing a correct estimation of the diversity of accumulators in the Balkans to be presented. A recent systematic revision of the genus in Albania (Cecchi et al., 2018) points to the existence of seven taxa, of which six Ni-hyperaccumulators are restricted to ultramafic soils (except for *O. chalcidica*, facultative serpentinophyte). A polyploid species of likely hybrid origin between *O. chalcidica* and *O. smolikana*, originally described from Mt. Smolikas (Greece) as *Alyssum decipiens* Nyár. and widely distributed on the Albanian ultramafics, could be a promising candidate for phytoextraction because of the high Ni levels, the robust habit and the ability to grow in a wide range of habitats, including anthropogenic ones, from low to high altitudes.

SUITABLE AGROECOSYSTEMS FOR NI-HYPERACCUMULATORS

Agromining aims to extract metals from the soil through the implementation of hyperaccumulator cropping systems, to improve soil quality and ecological functions, and to provide complementary income to farmers and local populations. However, climatic conditions, soil properties, and metal bioavailability can limit the production of plant biomass and metal accumulation, and consequently the effectiveness of agromining (van der Ent et al., 2015). Several studies have focused on different agronomic aspects to optimize the metal extraction process.

Adequate Fertilization Regimes

Due to nutrient deficiencies of ultramafic soils, mineral fertilization has a positive effect on the biomass production of Ni hyperaccumulators such as *Odontarrhena* (syn. *Alyssum*) spp. (Bani et al., 2015a; Kidd et al., 2015; Álvarez-López et al., 2016). The application of mineral fertilizers was also found to improve Ni yields (Li et al., 2003a). NPK amendments, coupled with herbicide applications and irrigation management, allowed the production of at least 20 t ha^{-1} of dry biomass of *O. muralis s.l.* and 400 kg Ni ha^{-1} (Chaney et al., 2007). Similarly, after 5 years of field experiments, biomass and Ni yields of a spontaneous cover of *O. muralis s.l.* in Albania were improved from 0.3 to 9.0 t DW biomass ha^{-1} and from 1.7 to 105 kg Ni ha^{-1} after the addition of 120 kg N and 100 kg P, K ha^{-1} (Bani et al., 2015a). Phosphorus has some effect on biomass yield and Ni uptake by hyperaccumulators (Shallari et al., 2001; Chaney et al., 2008), including the stimulation of flowering of the plants. Additionally, the fractional application of N is advisable to minimize excessive leaching of N to groundwater (Li et al., 2003a; Bani et al., 2015a) and to compensate soil nutrient absorption by the hyperaccumulator plants and maintain biomass production over several years (Kidd et al., 2015; Nkrumah et al., 2016).

The addition of organic amendments can improve soil quality and structure, reduce compaction and erosion, supply essential nutrients, and indirectly stimulate biological activity. Amendment with composted sewage sludges during the cultivation of the Ni-hyperaccumulators *O. serpyllifolia* and *O. bertolonii* in ultramafic soil led to a 24- and 62-fold increase, respectively, in biomass production compared to untreated plant covers (Álvarez-López et al., 2016). Similarly, Ghasemi et al. (2018) showed that three Ni-hyperacumulating *Odontarrhena* spp. produced significantly higher DW yields, improved nutritive status, and increased total Ni phytoextracted when grown in ultramafic soils amended with cow manure compared to NPK-fertilized soils. Alternatively, Saad R. F. et al. (2018b) incorporated green manure from legumes into the soil and found Ni yields were significantly increased following a stimulation of biomass production. The increase in biomass production could be favored by the improvement in soil physical properties, such as structure, porosity, and water retention capacity, but also by the stimulation of microbial activity. Thus, it appears that organic amendments are good candidates to improve agromining, but this strategy needs to be further demonstrated in large-scale field trials (Nkrumah et al., 2016).

Plant Cropping Patterns

Studies focusing on agricultural crop systems or on grasslands have shown that increased plant diversity enhances soil microbial biomass, activity, and diversity (Benizri and Amiaud, 2005). Plant species coexistence enhances interactions between micro- and macro-organisms in soils. Diversified organic compounds (i.e. rhizodeposits) released from different plants growing together in multi-species vegetation covers, can change the rhizosphere conditions and affect the abundance, functions and diversity of associated microorganisms (Berg and Smalla, 2009). Mixed cropping patterns also increase the diversity of plant litter returned to the soil, increasing diversity of litter-degrading organisms (Brussaard, 2012). Significantly higher values of many biological indicators have been found under multi-species vegetation (Gao et al., 2010).

However, few studies have examined the effect of cultivating metal hyperaccumulators together with other plant species. Some studies focused on the associations of *Sedum alfredii* + *Zea mays* (Wei et al., 2011), *Sedum alfredii* + *Alocasia macrorrhiza* (Wu et al., 2007), *Noccaea caerulescens* + *Lolium perenne* (Jiang et al., 2010), and *Noccaea caerulescens* + *Hordeum vulgare* + *Lepidium heterophyllum* (Gove et al., 2002). Most of these showed that co-cropping with non-metal hyperaccumulator plants can improve the growth of the hyperaccumulator and increase metal extraction. Additionally, co-culture enhanced the size of the microbial community and stimulated certain microbial functional groups (Jiang et al., 2010). The efficiency of nickel phytoextraction in mixed hyperaccumulator covers has also been studied (Lucisine et al., 2014; Rue et al., 2015). Co-cropping *Odontarrhena muralis s.l.*, *Noccaea tymphaea*, *Bornmuellera emarginata* and *Bornmuellera tymphaea* increased biomass (by 21%) and Ni phytoextraction (by 47%) of *B. tymphaea* in mixed cover compared to its mono-culture (Rue et al., 2015). Moreover, Lucisine et al. (2014) found that the coexistence of different hyperaccumulating plants promoted soil metal bioavailability and modified the genetic and phenotypic structures of the rhizosphere bacterial communities.

Legumes are widely used in conventional agriculture due to their ability to fix atmospheric nitrogen; through the presence of symbiotic N_2-fixing *Rhizobium* bacteria which can convert it into a plant-assimilated form (De Antoni Migliorati et al., 2015). Co-cropping hyperaccumulating plants with legumes could also improve plant growth and consequently metal phytoextraction. Moreover, part of the N fixed is usually transferred to companion plants (Rodrigues et al., 2015). Agromining could benefit from this strategy provided the legume tolerates the metal concentrations present in ultramafic soils (Saad et al., 2016). In a mixed legume-crop cover the rhizosphere microbial communities of this association also differ from those found in mono-cultures and can positively influence nutrient availability and plant uptake (St. Luce et al., 2015; Saad et al., 2017). As a result, improvements in soil quality, structure and biological activity are expected (Stevenson and van Kessel, 1996; Evans et al., 2003). During legume decomposition in co-cropping systems, the N-rich residues constituted a significant quantity of carbon returned to the soil, thus increasing the soil carbon stock by up to 0.6 t ha^{-1} per year (Kuo et al., 1997). In agromining cropping systems, the presence of legumes also improved soil physical properties, increasing soil porosity and aggregate stability (Saad et al., 2018a). The introduction of legumes into these systems also replaces the need for high inputs of mineral fertilizers, thus promoting agromining as an advanced ecological 'green' system.

Plant-Microbial Associations

Microbial-assisted phytoextraction approaches for the remediation of metal-contaminated or metal-rich soils are relatively recent concepts. Nickel-hyperaccumulators, as all plants, are inhabited by symbiotic microorganisms (Cardinale, 2014). These protect the host from pathogens, stimulate growth, nutrient uptake and transport, attenuate stress (Lugtenberg and Kamilova, 2009; Yang et al., 2009; Rashid et al., 2016) and moderate phenotypic and epigenetic plasticity (Partida-Martínez and Heil, 2011). Extensive research has been dedicated to the identification of microbial inoculants which can be incorporated into phytoextraction systems, for overcoming limitations in these processes due to reduced plant biomass or limited soil metal availability (Khan et al., 2009; Zaidi et al., 2011; Thijs et al., 2016; Kidd et al., 2017a; Benizri and Kidd, 2018).

Plant-Bacterial Interactions

Plant associated-bacteria are well known to play an important role in plant growth, fitness and nutrient supply. Growth promotion is generally associated with the ability to increase nutrient availability, such as nitrogen (N_2-fixing organisms), phosphorus (by solubilization or mineralization through release of organic acids and/or phosphatases), or iron (by releasing Fe(III)-specific chelating agents or siderophores), or via the production of plant hormones or reduction in stress ethylene levels (Khan et al., 2009; Glick, 2010; Benizri and Kidd, 2018). Nickel hyperaccumulating plants have been shown to select for Ni-tolerant bacterial strains in their rhizosphere (Mengoni et al., 2001; Abou-Shanab et al., 2003; Álvarez-López et al., 2016), and these rhizobacteria have recently been linked to the metal hyperaccumulation process itself (Becerra-Castro et al., 2013; Muehe et al., 2015). Bacterial metal solubilization has been attributed to mechanisms such as proton extrusion or the production of complexing agents, e.g., organic acids (Gadd, 2010). Their capacity to enhance soil metal availability and/or plant metal yields offers the possibility to improve agromining efficiency. The results in terms of soil metal removal when using such plant-bacterial associations are promising: numerous examples of enhanced hyperaccumulator plant growth and metal uptake and accumulation can be found after soil, seed or plant inoculation (Kidd et al., 2017a; Benizri and Kidd, 2018). Increases in phytoextracted metal yields have been attributed to either plant growth promotion or the ability of isolates to mobilize metals from less labile soil fractions. Some inoculants have been selected for their ability to enhance Ni yields when applied to more than one plant species and/or soil type (Cabello-Conejo et al., 2014; Durand et al., 2016; Ghasemi et al., 2018).

Overall, inoculation with plant growth-promoting or metal-mobilizing microorganisms is considered a promising approach for increasing metal phytoavailability and the growth and health of "metal crops". However, the vast majority of these studies have been carried out on a bench-scale and there is still a need for field evaluations of such plant-bacterial associations. Aspects such as the degree of plant species-specificity, the compatibility of the selected host species and inoculant, or the performance of the bacterial strains and their ability to proliferate in the soil, need to be studied under natural field conditions. As part of the Agronickel project, field trials were established in an ultramafic outcrop in NW Spain to test the potential use of three rhizobacterial strains (all originally isolated from rhizosphere of Ni-hyperaccumulating plant species) for improving the establishment and yield of *O. muralis s.l.* Plant coverage and aerial-biomass (**Figure 1**), and plant Ni yield (but not shoot Ni concentration), were significantly increased after inoculation with *Paenarthrobacter nitroguajacolicus* strain LA44 and *Pseudoarthrobacter oxydans* strain SBA82 (Pardo et al., 2017).

Plant-Fungal Interactions

Our knowledge of the role of symbiotic fungi in plant adaptation to ultramafic soils is scarce. Available reports indicate that fungal symbionts facilitate plant growth in these environments by affecting Ni uptake and distribution within the plant. The best described group of fungi which has been shown to positively affect plant growth in Ni-enriched soils is the arbuscular mycorrhizal fungi (AMF). Interestingly, there is no unequivocal "mode" of action of fungi (mycorrhiza and others), in terms of their effect on metal uptake and distribution; some species of fungi protect the plant by decreasing Ni uptake, while others increase uptake and translocation from root to shoot (Cao et al., 2008; Rozpądek et al., 2018). Additionally, there is no consensus concerning the mechanism(s) by which fungi alleviate Ni phytotoxicity. It remains open for discussion whether symbiotic fungi improve plant growth under metal toxicity by facilitating water and nutrient acquisition and thus indirectly fine-tuning the host's metal tolerance or by directly upregulating specific metal tolerance mechanisms.

Hyperaccumulators were believed to be unable to form mycorrhiza, until the discovery of AMF in roots of *Berkheya coddii*, *Senecio coronatus* and, later, in other Ni-hyperaccumulating species (Turnau and Mesjasz-Przybylowicz, 2003). AMF supported *B. coddii* by increasing nutrient (K, Fe, Zn, Mn, P, Ca) acquisition and distribution (Orłowska et al., 2013). Co and Ni uptake was lower in mycorrhizal plants. Nevertheless the significantly higher biomass production of mycorrhizal plants resulted in a 20-fold increase in Ni yield per plant. The role of mycorrhizal symbiosis has not yet been studied in European hyperaccumulators, even though there are some candidates from the Asteraceae family that could become a promising tool in phytoextraction. So far, however, the importance of AMF and ectomycorrhiza in ultramafic soils is mainly considered to be the driver of ecosystem diversity, playing an important role in stimulating plant yield in these environments which are rich in Ni and poor in nutrients (such as N, P, or K) but host a unique and rich vegetation.

The non-mycorrhizal Brassicaceae, a family particularly rich in metal-tolerant species, were found to be abundantly inhabited by highly metal-tolerant fungal endophytes (García et al., 2013; Zhang et al., 2014), shown to affect plant metal homeostasis and facilitate vegetation in metalliferous soils (Rozpądek et al., 2018). Their low host specificity and ease

FIGURE 1 | Aerial view of field plots (4 m^2) with non-inoculated **(A)** and inoculated **(B)** *Odontarrhena muralis s.l*. Plants were inoculated with *Paenarthrobacter nitroguajacolicus* strain LA44 (Pardo et al., 2017).

in cultivation make them promising candidates for improving the efficiency of phytomining. Ni uptake and most importantly extraction efficiency were positively affected in *Brassica juncea* inoculated with *Trichoderma atroviride* strain F6. Additionally, inoculated *B. juncea* exhibited higher tolerance to metal toxicity (Cao et al., 2008). The mycobiome of Ni-hyperaccumulators from the *Odontarrhena* and *Noccaea* genera have been recently studied within the Agronickel project (Turnau et al., 2017). The benefits of available molecular resources for some of these species will allow for studying the role of symbiotic fungi in Ni hyperaccumulation and tolerance more thoroughly. According to preliminary results, plants of both the abovementioned taxa from serpentine soils harbor numerous fungal endophytes. Ninety percent of isolated fungi belong to two classes Dothideomycetes and Sordariomycetes (Class 3 endophytes according to the classification of Rodriguez et al., 2009), the former being most commonly isolated from leaves and the latter from roots (Turnau et al., 2017). Some taxa inhabited both plant roots and shoots. Dark pigmented mycelium of these fungi was visible in cross sections of shoots of hyperaccumulating plants collected in the field; within air spaces of green and healthy leaf tissues and the stem central cylinder. Many of the fungi developed brown/black pigmentation similar to dark septate endophytes (DSE), and considered as Class 4 endophytes (Rodriguez et al., 2009). Members of DSE show extraordinary tolerance to metal toxicity and an ability to accumulate up to 20% DW Pb (Zhang et al., 2008). The DSE *Phialocephala fortinii* was commonly isolated from roots of Ni-hyperaccumulators, however, it had little phenotypic effect on the host plants (Rozpądek et al., 2017; Ważny et al., 2017).

One of the most commonly encountered fungal endophytes in *Noccaea* species was *Embelisia thlaspsis* (Dothideomycetes; Rozpądek et al., 2017). A Ni-adapted strain of this fungus alleviated metal toxicity: the plants accumulated less stress protective anthocyanins when exposed to elevated Ni concentrations in the soil (**Figure 2A**), even though Ni acquisition by symbiotic plants was significantly higher (**Figure 2B**). Additionally, the root system of inoculated plants was more developed; showing that co-cultivation with *E. thlaspis* resulted in significant lateral root elongation (**Figure 2C**). The possible utilization of fungal endophytes in improving phytoextraction efficiency of Ni-hyperaccumulating Brassicaceae is currently under investigation. Preliminary results indicate that these microorganisms may indeed be used to support plant vegetation and Ni accumulation.

EUROPEAN NETWORK OF AGROMINING FIELD SITES

The Agronickel and Life-Agromine projects established a network of pilot-scale field sites in ultramafic regions across western, central and southern Europe in order to cover a range in climatic and edaphic conditions (**Figure 3** and **Table 2**; Echevarria et al., 2017). Experimental field plots have been set-up to demonstrate the benefits of incorporating organic amendments, legumes or bioinoculants into agromining agrosystems with the aim to maximize plant yields and Ni removal at the same time as improving soil quality and functioning.

Ultramafic areas in Albania occupy ∼11% of the total surface area; geologically they vary from partly serpentinized peridotite (harzburgite) to serpentinite (Bani et al., 2014; Estrade et al., 2015). Long-term agromining studies have been carried out intermittently over the period 2007–2017 in the Pogradec district (Pojskë), E Albania (**Figure 3** and **Table 2**; Bani et al., 2007, 2015a,b). The current land use in the area is low-productivity agriculture (pasture or arable land) into which agromining could be successfully incorporated. These studies optimized the agronomic aspects of the agromining chain for these Balkan agricultural landscapes. *In-situ* agromining experiments using the native *O. muralis s.l.* were conducted from 2005 to 2009 and from 2012 to 2014, and continued as part of the Life-Agromine project in 2016–2017. The effects of fertilization, herbicide application, soil tillage, crop establishment and plant density (natural cover vs. sown crop), and competition

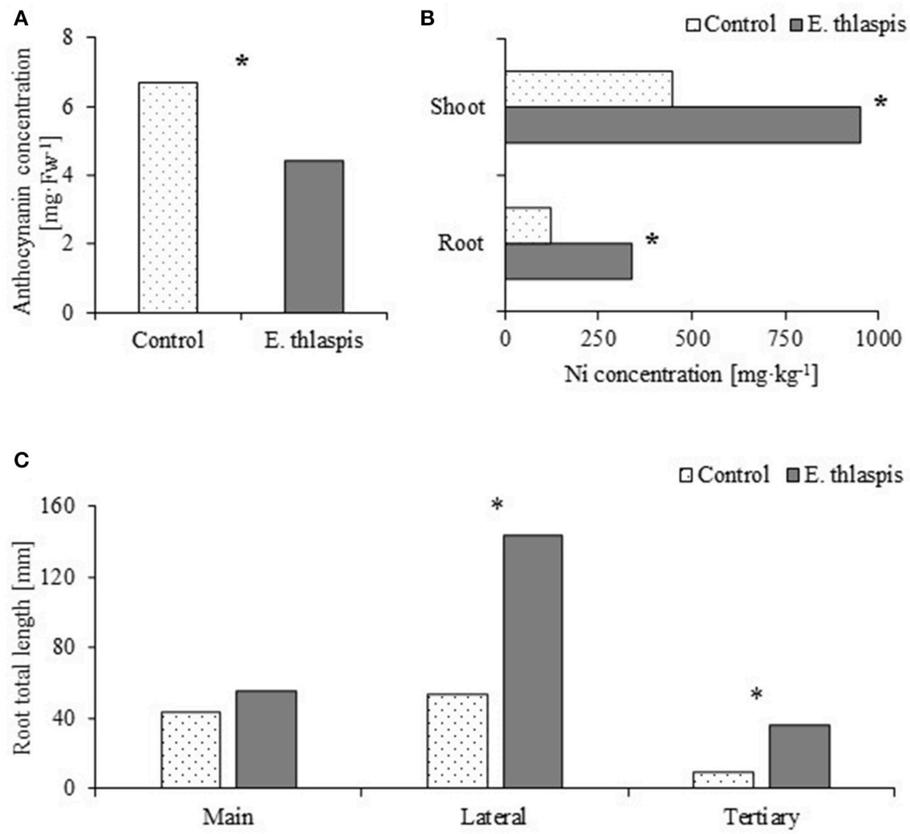

FIGURE 2 | The beneficial effect of endophytic fungus *Embellisia thlaspis* on *Noccaea caerulescens*. **(A)** Anthocyanin concentration [analyzed according to Fukumoto and Mazza (2000)]. **(B)** Ni accumulation in plant root and shoot (analyzed by Atomic Absorption Spectrometry). **(C)** Changes in root architecture. Statistical significances were evaluated with the *t*-test, $P \leq 0.0.05$, $N = 5$ (in **A**,**C**) and $N = 50$ (in **B**). Plants were grown in sand mixed with perlite (1:1; v:v) for 50 days in a vegetation chamber with a 16 h photoperiod and 21/17°C day/night temperature. Ten day-old seedlings were inoculated with 3 ml of mycelium suspended in water ($OD_{600} = 0.15$). Fourteen days after inoculation the substrate was supplemented with 150 μM of $NiSO_4$ in Hoagland solution. Plants were irrigated with Hoagland solution twice per week. *Indicates statistical significance between inoculated and control. Data based on Rozpądek et al. (2017).

between plant species, have been assessed. Over the period 2005 to 2009, the optimal cropping system, with split-N fertilization, irrigation and post-emergent herbicide (FocusTM ultra) treatment, progressively achieved a biomass production of 9.0 t ha^{-1} and Ni phytoextraction yield of 105 kg Ni ha^{-1} (Bani et al., 2007, 2015a,b; **Figure 4**). Maximum biomass was obtained with a density of 4 plants m^{-2} (10.2 t ha^{-1} in 2013 and 8.8 t ha^{-1} in 2014); the drop in biomass observed in 2014 was probably due to the competition between planted *O. muralis s.l.* and its own spontaneous recruits. The highest Ni yields were achieved in 2013 with 112 kg ha^{-1} (**Figure 4**). New plots evaluate the application of organic amendments (sheep and pig manure): biomass production in 2017 was 9.96 t ha^{-1} in fertilized plots and Ni yield reached its maximum of 139.3 kg ha^{-1}. Phenological studies have shown that, in this region, Ni bioaccumulation is maximal during the mid-flowering stage which was then set as the recommended harvesting time (mid-June) (Bani et al., 2015a; Estrade et al., 2015).

New agromining field trials have been established in May 2017 in the Pindus Mountain Range in Greece, which runs along the borders of Thessaly, Epirus, and W Macedonia, and hosts a large number of serpentine outcrops and many serpentinophytes (see section A Synthetic Overview of Ni-Hyperaccumulators in Europe). Experimental plots are located in Koutsoufliani, near the village Panagia at the borders of the above-mentioned regions (NW Greece) on an abandoned field with a mean total Ni concentration of 2,347 mg kg^{-1} (**Table 2** and **Figure 3**; Echevarria et al., 2017). *Odontarrhena muralis s.l.* is a constant member of the herbaceous vegetation. The ongoing field assessments are evaluating the Ni agromining capacity of three Ni-hyperaccumulators (*O. muralis s.l.*, *B. tymphaea,* and *B. emarginata*) in monoculture plots (three replicate 50 m^2 plots/species). Additional plots assess the effects of inorganic fertilization or goat manure addition on the growth and Ni bioaccumulation of *O. muralis s.l.* and *B. emarginata*, as well as the benefits of co-cultivating or rotating these hyperaccumulators with the legume *Medicago sativa*. Harvesting is programmed for June 2018. Results so far indicate maximum plant survival in the organic-amended plots (95–97%) compared to either the inorganic

TABLE 2 | Soil physicochemical and climate characteristics of the field sites included in the Life-Agromine network (mean values ± SE).

Country	Pojskë	Bernstein	Koutsoufliani	Eidián
Site	**Albania**	**Austria**	**Greece**	**Spain**
Coordinates	40°59′N 20°38′E	47°24′45.2″N 16°16′04.7″E	39°51′37.8″N 21°18′50.2″E	42°49′54.2″N 8°00′13.4″W
Altitude (m)	700	620	930	430
SOIL GENERAL PROPERTIES				
Texture	**Clay**	**Sandy loam**	**Silty clay**	**Sandy loam**
pH_{H2O}	7.5 ± 0.2	6.1 ± 0.1	7.2 ± 0.0	5.8 ± 0.1
CEC ($cmol_c$ kg^{-1})	38.9 ± 1.1	15.9 ± 2.0	–	4.9 ± 1.0
TOC (g kg^{-1})	28.4 ± 4.0	22.4 ± 0.5	–	52.6 ± 8.0
TN (g kg^{-1})	2.3 ± 0.3	2.2 ± 0.5	–	3.0 ± 0.4
P Olsen (mg kg^{-1})	<0.05	9.0 ± 1.5	4.6 ± 0.4	4.5 ± 0.3
Ca-Total (g kg^{-1})	3.9 ± 0.4	6.7 ± 0.2	7.0 ± 0.3	7.6 ± 0.6
Mg-Total (g kg^{-1})	60.0 ± 2.5	103.0 ± 8.6	138.0 ± 3.0	45.1 ± 2.3
K-Total (mg kg^{-1})	4500 ± 250	905 ± 70	1863 ± 34	438 ± 25
Fe-Total (g kg^{-1})	98.0 ± 0.2	64.0 ± 1.2	–	71.0 ± 10.7
Ni-Total (mg kg^{-1})	3140 ± 60	1450 ± 180	2347 ± 37	967 ± 13
Cr-Total (mg kg^{-1})	1600 ± 160	1840 ± 140	–	1263 ± 65
Co-Total (mg kg^{-1})	207.0 ± 12.0	113.0 ± 4.2	–	77.5 ± 3.3
Soil Ni availability				
Ni-DTPA (mg kg^{-1})	124.0 ± 6.0	38.4 ± 4.6	71.1 ± 2.3	36.8 ± 13.8
Ni-$Sr(NO_3)_2$ (mg kg^{-1})	–	0.53 ± 0.07	0.80 ± 0.05	1.20 ± 0.27
CLIMATE CHARACTERISTICS				
Climate type	**Sub-mediterranean continental**	**Warm-summer humid continental**	**Sub-mediterranean continental**	**Humid temperate**
Mean annual precipitation (mm)	700	718	666	1200
Mean annual temperature (°C)	10.6	8.3	12.4	12.1

CEC, cation exchange capacity; TOC, total organic C; TN, total N.

NPK-fertilized plots (15–20%) or co-culture/rotation treatments (3–38%) (Echevarria et al., 2017). The high plant mortality was attributed to elevated temperatures during transplantation and prolonged drought (exacerbated by the slope) during the summer period of 2017 (despite periodic irrigation). The addition of organic amendment appears to have a positive effect on plant resistance to drought and general adverse conditions.

The Austrian field site is located in the province of Burgenland, coinciding with one of the main ultramafic outcrops in this country (Wenzel and Jockwer, 1999). Experimental plots were set up in autumn 2016 on an arable field with a mean total Ni concentration of 1,450 mg kg^{-1} (**Table 2** and **Figure 3**; Ridard et al., 2017). The first experimental period lasted from October 2016 to September 2017. *Odontarrhena muralis s.l.*, the main agromining crop in the Life-Agromine project, was compared with the indigenous *Noccaea goesingensis*. The effects of different plant cropping patterns (co-culture with *Lotus corniculatus*), planting densities or soil amendments (addition of elemental sulfur) on plant yields and Ni bioaccumulation are currently being assessed. The pre-harvest average shoot Ni concentration of *O. muralis s.l.* was 12,400 mg kg^{-1} with average shoot DW yields of 3.77 t ha^{-1}. In contrast, *N. goesingensis* clearly showed a lower accumulation and estimated biomass production (7,900 mg Ni kg^{-1} and 2.90 t ha^{-1}, respectively; Ridard et al., 2017). The second vegetation period in 2018 will only cultivate *O. muralis s.l.* due to the temperate climate and shorter growing season in the Bernstein area the vegetation period for *O. muralis s.l.* was adapted; i.e. planting in spring and harvesting in autumn. Interestingly, flowering was already observed a few weeks before harvesting, i.e., ∼5 months after planting. Further treatments (testing different plant densities and fertilization regimes) for optimizing the yield and thus the total content of phytoextracted Ni are planned.

In Spain, the experimental site is located in the Melide ultramafic complex close to the village of Eidián in Galicia (NW Spain; **Table 2** and **Figure 3**), one of the principal periodite outcrops in the Iberian Peninsula together with Andalusia (S Spain) and Trás-os-Montes (NE Portugal). Agricultural crops growing in the ultramafic soils of this region have been shown to accumulate considerable amounts of Ni and Cr (Fernandez et al., 1999). The Ni-hyperaccumulator *O. serpyllifolia* is a feature of fallow fields at this site. Field trials are evaluating the viability of *O. muralis s.l.*, *O. serpyllifolia* and *B. emarginata* for agromining purposes. Monoculture plots (50 m^2) were established in 2015 to initially test the two Mediterranean hyperaccumulators, *O.*

FIGURE 3 | European network of nickel agromining field sites.

muralis s.l. and *B. emarginata* (Pardo et al., 2018). Soil was fertilized with gypsum (1,000 kg ha^{-1}) and inorganic NPK fertilizers (120:120:150 kg ha^{-1}) and both species planted at a density of 4 plants m^{-2} following Bani et al. (2015b). The elevated precipitation which is characteristic of this region (European humid-temperate climate) led to waterlogging which, together with competition from weeds (mainly *Poaceae*), significantly affected plant survival: after one growth season (June 2015–May 2016) plant mortality of up to 50–60% was recorded in some plots. Nonetheless, all surviving plants showed good growth and a healthy aspect. The final plant DW yields (1.0 ± 0.3 and 0.7 ± 0.2 t DW ha^{-1} for *O. muralis s.l.* and *B. emarginata*, respectively) were significantly lower than those obtained by Bani et al. (2015b) for *O. muralis s.l.* after 8-years of cultivation in Albania but of a similar magnitude to the yields obtained during the first years after implementing agromining at this site (Bani et al., 2007). *Odontarrhena muralis s.l.* (4.2 kg Ni ha^{-1}) produced a slightly higher Ni yield than *B. emarginata* (3.0 kg Ni ha^{-1}). As observed by Bani and colleagues, Ni bioaccumulation was maximal at the mid-flowering stage and this was the case for both species, confirming that this is the optimal stage for harvesting, not only for *O. muralis s.l.*, but also for *B. emarginata*. Moreover, Ni accumulation was strongly compartmentalized with the main contribution to Ni yields coming from the plant leaves, especially the leaves of flowering stems, but the contribution from flowers and fruits was also significant in the case of *B. emarginata*. Implementing the agromining system increased soil nutrient availability, and modified microbial community structure and metabolic activity (Pardo et al., 2018). Bacterial community structure and diversity are being monitored over time and compared with surrounding soils where agromining was not implemented. The soil bacterial communities are dominated by Proteobacteria, Actinobacteria, Acidobacteria, and Chloroflexi, and after one growth season the agromining crops modified the relative abundance of some phyla (increasing Proteobacteria, Bacteroidetes, and Nitrospirae and reducing Acidobacteria and Planctomycetes) (Pardo et al., 2018).

Implementing a drainage system and weed control during the second year of cultivation (2016–2017) had a dramatic effect on plant survival and biomass production: plant yields were up to 10-fold higher in the case of *O. muralis s.l.* (reaching close to 10 t ha^{-1} in some plots) and 7-fold higher for *B. emarginata* (close to 5 t ha^{-1}) (Kidd et al., 2017b). In 2017 the endemic *O. serpyllifolia* was also cultivated but showed a significantly lower biomass yield than *O. muralis s.l.*, but a similar magnitude to that of *B. emarginata* (1.0–5.9 t ha^{-1}) (Kidd et al., 2017b). These preliminary results suggest that *O. muralis s.l.* has more potential for agromining on this site. However, crop management practices (e.g., plant density) could be optimized so as to improve biomass yields of *B. emarginata* or *O. serpyllifolia*, since both species presented similar shoot Ni concentrations to *O. muralis s.l.* At the same site, ongoing field experiments are evaluating the benefits of co-cropping systems and the application of cow manure instead of NPK fertilizers. After 1 year of cultivation, co-cropping of *O. muralis s.l.* with the legume *Vicia sativa* had positive effects on plant growth and Ni removal: hyperaccumulator biomass and Ni yields were increased by 417% (0.82 t ha^{-1}) and 493% (7.93 kg ha^{-1}), respectively, compared to non-fertilized mono-cropping

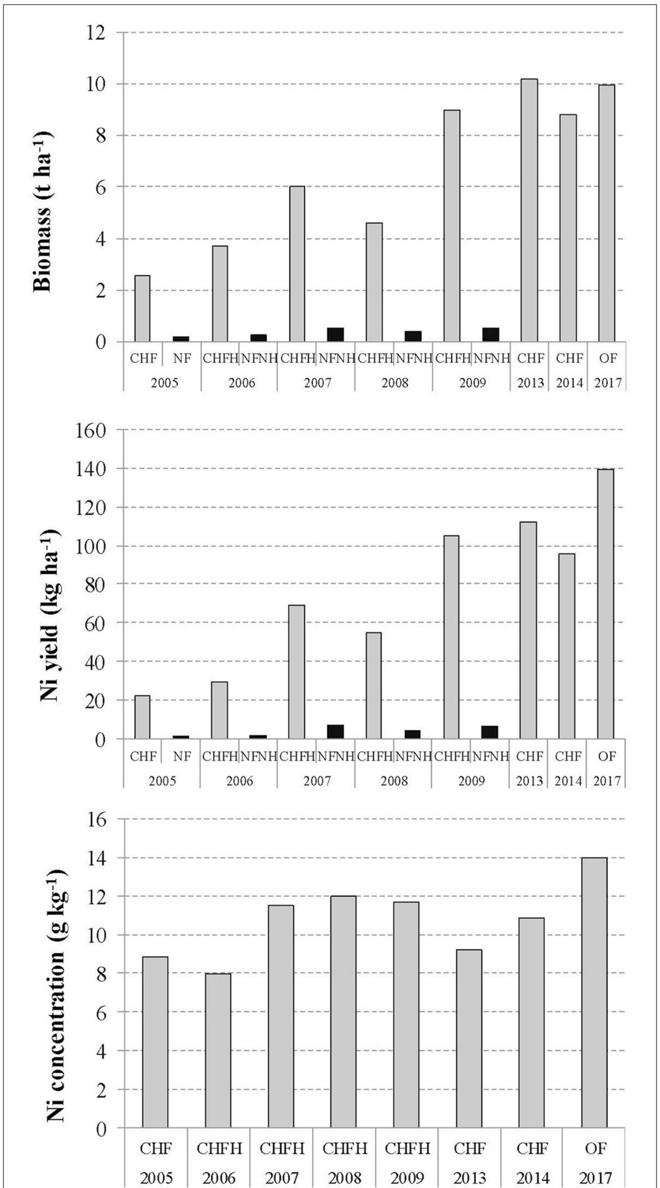

FIGURE 4 | Evolution of biomass production, Ni phytoextracted yield and shoot Ni concentration of *Odontarrhena muralis s.l.* during 8 years of a field study in Pojska, Pogradec (Albania). CHF-chemical fertilizer, NF-no fertilizer, H-herbicide, OF-organic fertilizer. Modified with permission from Echevarria (2018); Bani and Echevarria (in press) and Bani et al. (2007, 2015a).

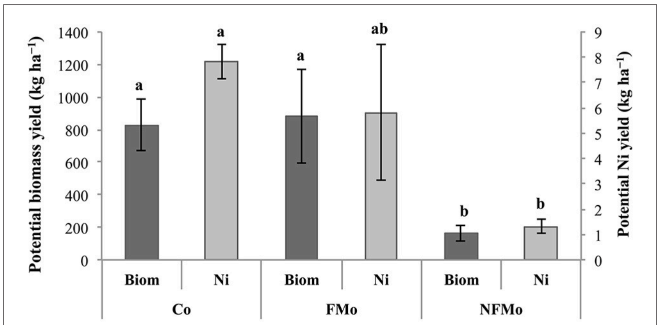

FIGURE 5 | Potential biomass (Biom) and Ni yields (Ni) of *Odontarrhena muralis s.l.* shoots (kg ha^{-1}). "Co", "FMo" and "NFMo" correspond to co-cropping with the legume, fertilized mono-cropping, and non-fertilized mono-cropping, respectively. Means ± standard error followed by different letters are significantly different at $p < 0.05$ (Duncan's multiple range test).

(0.16 t ha^{-1} and 1.32 kg ha^{-1}) (**Figure 5**; Saad et al., 2017). However, no significant differences were found between co-cropping and fertilized (NH_4NO_3) mono-cropping treatments. After a second year of cultivation, yields were doubled in all treatments but plant biomass production and phytoextracted Ni was highest in the co-cropping treatment (2.01 t ha^{-1} and 13.6 kg ha^{-1}, Saad et al., 2017). On the other hand, parallel experiments showed that after one growth season the addition of cow manure did not significantly modify plant yields of either *O. muralis s.l.* or *B. emarginata* compared to NPK fertilization (Kidd et al., 2017b). The Ni yield obtained using either fertilization regime is currently being determined, as well as any differences in soil fertility or microbial community diversity and activity.

PROCESSING BIOMASS FROM NI-HYPERACCUMULATOR PLANTS

The whole nickel (Ni) agromining chain consists in two stages: (1) the cultivation of hyperaccumulator plants to obtain sufficient aerial biomass with a high Ni concentration, and (2) the transformation of the biomass to obtain valuable end products. In step 1, the dry plants are burnt to remove organic matter and concentrate Ni by a factor of around 12. The resulting ash is a real bio-ore, containing up to 20 wt % of Ni. In step 2, it has been proven possible to obtain a bunch of Ni compounds (e.g., Ni metal, Ni-based catalysts, Ni salts or oxides) by hydrometallurgical processes (Simonnot et al., 2018). Energy is potentially another end product of agromining. The Agronickel aims to investigate new routes for optimizing the valorization of the hyperaccumulator biomass in terms of energy and metal recovery. The Life-Agromine project aims to up-scale the process, to produce nickel compounds and energy from hyperaccumulator biomass, especially *O. muralis s.l.* at demonstration levels.

Nickel Recovery and Reactor Design

The up-scaled process is the patented synthesis of ANSH (ammonium nickel sulfate hexahydrate) from the biomass of *O. muralis s.l.* (Barbaroux et al., 2012). The process consists of (1) washing the ashes with water to remove soluble K (around 80% of the total K content), (2) transferring Ni into the aqueous phase by sulfuric acid leaching and (3) precipitating and purifying ANSH (**Figure 6**). Each step has been thoroughly investigated to assess the influence of the process parameters (e.g., stirring speed or reaction time) on process efficacy and salt purity (Zhang et al., 2016; Houzelot et al., 2017a,b). However, up-scaling also requires technical adjustments and process intensification. The process has been designed through batch reactor experiments, but its implementation at a larger scale requires a different strategy. The main options are (1) moving from discontinuous batchwise to continuous process or (2) increasing the treatment capacity by using reactors in parallel. This second option is considered here and applied to ash washing and ash leaching.

The investigation of the batchwise process has shown that:

- The efficacy of ash washing only depends on the solid/liquid (S/L) weight ratio and ash wettability. S/L ratio must not overcome 20% to allow stirring, above 20% suspension viscosity is too high.
- During the leaching step (2 M sulfuric acid), a part of the protons is involved in ash neutralization (initial pH around 13) and another part in Ni leaching. Around 30% of initial H^+ have not reacted (final pH around 0.6), but they are essential to increase nickel extraction yield. The leachate has to be neutralized before ANSH precipitation, which leads to the consumption of alkali.

To increase the treatment capacity while saving chemical reagents (and limiting proton loss during leaching), it is proposed to develop a discontinuous process using reactors in parallel.

Theoretical Aspects for the Design of Parallel Batch Reactors

The proposed configuration is composed of J units in parallel (**Figure 7**). Unit #i ($1 \le i \le J$) is made up of two batch reactors in series, the first one (W_i) for ash washing and the second one (L_i) for ash leaching. A mass m of ash and a volume V_w of water are introduced into reactor W_1; after a washing time of *ca* 15 min, ash (without K) is transferred into reactor L_1, a part of the solution (V_w') is introduced into W_2 and the remaining part (V_w") is removed. To simplify, we assume that the amount of water transferred from W_1 to L_1 with the ash is negligible (ash is dry). Leaching is run in reactor L_1 by adding a volume V_L of sulphuric acid at the concentration C_1. At the end of the leaching (2 h at 90 °C), we obtain the ash (Ash_1, without K and Ni) and a volume V_L" of leachate rich in Ni. Reactor W_2 is fed by the same mass m of ash, the volume V_w' of solution exiting from reactor W_1, and the volume V_w" of water and so on. The same method is applied to the leaching reactors, the concentration of inlet acid being adjusted. The proposed configuration enables us to increase the concentration of K from reactor W_1 to W_2 and so on, and the concentration of Ni from L_1 to L_2 and so on, until reaching a limit value. In this way, the concentrations of K and Ni increase without changing the S/L ratio. The main advantage of this configuration is to lead to identical solutions exiting the reactors when the limit value is reached. At the exit of the complete system, we obtain a volume (V_w'+J V_w") of solution rich in K and a volume (V_L'+J V_L") of leachate rich in Ni. The solution rich in K is an effluent, which could be used for other purposes, and the leachate rich in Ni is treated to obtain the ANSH salt. The problem to be solved is to determine how many units are needed to reach the limit value of the concentration.

Application to ANSH Preparation

Previous experiments have shown that K and Ni cannot be concentrated more than twice compared to the concentrations obtained after ash washing and leaching. Otherwise they would precipitate in the ashes, mainly in the sulfate form.

In the proposed configuration, choosing V_w' = V_w" = 0.5 V_w and V_L' = V_L" = 0.5 V_L, mass balances show that at least 6 units in parallel are needed to reach the limit value. If we compare this with the case where V_w' = V_L' = 0, half of the water and 30% of the sulphuric acid are saved. As a consequence, the amount of alkaline solution required for leachate neutralization decreases as well.

To conclude, this section has shown how ash washing and leaching can be up-scaled to intensify the process, reach a high efficacy and save chemicals.

Energy Recovery

The calorific values of hyperaccumulators from the *Brassicaceae* family have been monitored to assess the interest in energy recovery during agromining. HHVs (higher heating values) obtained using a calorimetric bomb with dried ground biomass of *O. muralis s.l.* and *B. emarginata* were 16.7 and 17.4 MJ kg^{-1}, respectively (Hazotte et al., 2017). These values are comparable with HHV of wood pellets (20 MJ kg^{-1}; Rollinson and Williams, 2016).

Pellet manufacturing may improve combustion efficiency and facilitate plant handling and storage. However, this will be a challenge when using biomass from this hyperaccumulator family. First tests have shown that, unlike wood, the low content of lignin leads to a low pellet stability. The addition of a binder may limit this effect, but inevitably, it produces Ni dilution in ashes, as well as an increase in operating costs.

Biomass combustion upscaling requires mandatory gas control, especially for SOx and NOx emissions. However, it is also necessary to take care of Ni emissions, especially in fly ash. In France, this element is grouped with Sb, Cr, Co, Cu, Sn, Mn, V, and Zn, and the total flow rate must not exceed 25 g h^{-1}, with <5 mg m^{-3}. A 20 kWh boiler has been set up, with a combustion capacity of 7 kg biomass per hour (**Figure 6**). Its functioning is being validated and emissions analyses will be performed under standard guidelines NF EN 14385. Other parameters which should be taken into account during agromining development include the biomass chloride content, which might be a criterium for choosing hyperaccumulators. Hydrochloric acid formation during combustion may significantly cause corrosion of boilers. For example, *O. muralis s.l.* dried biomass contains around 0.25% Cl, which is more than wood (0.01–0.03%) but less than straw and grass (0.4–0.8%). This value is close to that of *Miscanthus* (Obernberger et al., 2006).

ECONOMIC AND ENVIRONMENTAL ASPECTS OF AGROMINING

Economics of Agromining

Until today, research on the economics of the agromining chain focuses on costs and benefits from a business perspective. Most of the literature considers Ni as the extraction target, although

FIGURE 6 | Process description for the preparation of ANSH from *Odontarrhena muralis s.l.* at the pilot scale.

other elements such as Ge, Re and Au have also attracted attention (Harris et al., 2009; Novo et al., 2015; Rentsch et al., 2016). Aspects being discussed can be grouped as follows: (i) characteristics of the metal market; (ii) process flow diagrams; (iii) inventory of costs, and revenues; and (iv) sensitivity analyses. These topics correspond to the elements of a techno-economic analysis as proposed by Van Dael et al. (2015), and will be discussed below.

Since 1989 Ni prices have fluctuated between 2 and 13 USD lb^{-1} (4 and 23 EUR kg^{-1}) with a sharp peak of 23 USD lb^{-1} (40 EUR kg^{-1}) right before the start of the financial crisis in the summer of 2007 (InfoMine, 2018). According to Li et al. (2003b), Ni cannot be agromined economically at the lower Ni prices. Therefore, the production of Ni compounds with much higher value, such as ANSH could be a better alternative. Current prices of ANSH depend on the purity and the amount: 97.50 EUR for 500 g with 98% purity, and 134 EUR for 25 g with 99.999% purity (Sigma-Aldrich, 2018).

Ideally the released energy during biomass incineration should be valorized; however, this depends on the vicinity of energy demand to the location of the processing facility. Hence, small-scale mobile units should be compared to large-scale central processing. The former requires measures for quality control of the produced bio-ores when it is mixed with another feedstock by local smallholders, whereas the latter needs carefully optimized storage facilities. As an alternative to the pyrometallurgical thermo-chemical treatment for the production of Ni, the hyperaccumulators can be treated hydrometallurgically (as discussed above) for the production of the higher value Ni compounds.

So far, costs have not been reported in detail. Harris et al. (2009) mention the cost of producing the Ni-hyperaccumulator *Berkheya codii*, but not for *O. muralis s.l.* Bani et al. (2015a) estimated land rental and production costs for agromining with *O. muralis s.l.* to be 150 USD ha^{-1} yr^{-1} and 390 USD ha^{-1} yr^{-1}, respectively. Nkrumah et al. (2016) compare intensive agromining systems with extensive systems and assume its production costs in 2016 to be 1074 USD ha^{-1} yr^{-1} and 600 USD ha^{-1} yr^{-1}, respectively. The latter is comparable to the cost of 500 USD ha^{-1} yr^{-1} mentioned by van der Ent et al. (2013b).

Moreover, both Bani et al. (2015a) and Nkrumah et al. (2016) expect 20 % of the Ni value to cover the cost of the metal recovery process, which would result in a profit of ca. 990 USD ha^{-1} yr^{-1}. Although the production costs in van der Ent et al. (2013b) are comparable, they expect higher net economic gains of 1900 USD ha^{-1} yr^{-1}. This difference can be explained by higher expected Ni uptake: 150 kg ha^{-1} yr^{-1} in Indonesia compared to 105 kg ha^{-1} yr^{-1} in Albania. Li et al. (2003b) mention that 25% of the Ni value would cover the cost of metal recovery, and license and royalty fees, resulting in a net profit of ca. 1800 USD ha^{-1} yr^{-1}. In fact, the estimate of Li et al. (2003b) corresponds to a Ni recovery cost of 1.75 USD kg^{-1}, whereas the one of Bani et al. (2015a) corresponds to 3.4 USD kg^{-1}. This indicates that the cost of the processing part of the process flow still needs thorough investigation as one would actually expect the cost to decrease over time due to the learning effect.

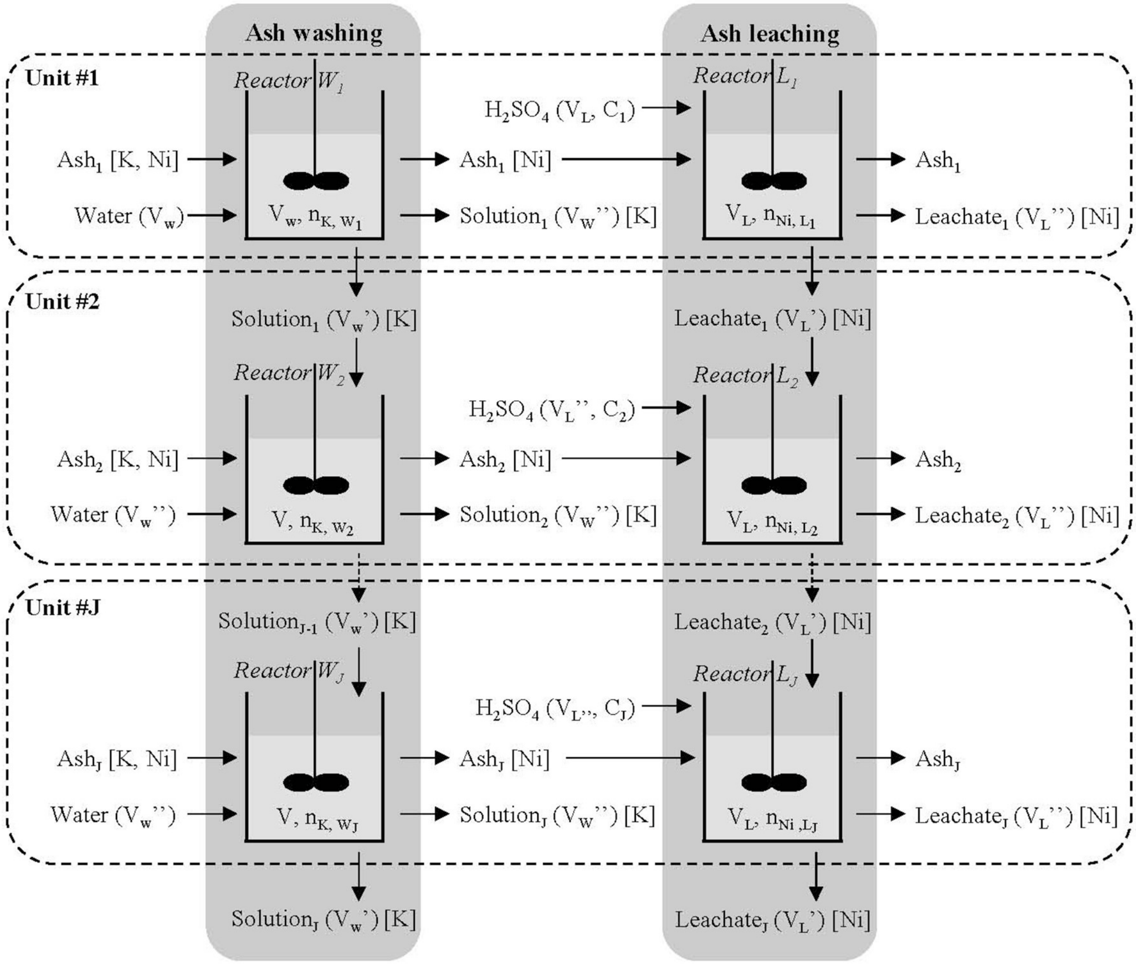

FIGURE 7 | Parallel batch configuration for *Odontarrhena muralis s.l.* ash washing and leaching. Liquid properties are indicated in brackets and targeted elements in square brackets.

To conclude, a techno-economic model for the agromining chain should consist of two stages: (i) cultivation and (ii) conversion. To determine the cultivation costs, one should include land rental, land preparation, seeds, fertilizers, chemicals, irrigation, etc. The Ni metal or Ni compound extraction cost is determined by the cost of the subsequent pyro- or hydro-metallurgical process. To date, no literature exists containing detailed costing of the conversion stage; nevertheless, supporting information can be found in Kuppens (2012) for the incineration plant, in Crundwell et al. (2011) for Ni smelting, and in Rodrigues et al. (2016) and Zhang et al. (2016) for the Ni compounds. Finally, the techno-economic model should be customized to country-specific data reflecting differences in Ni concentrations in the soils, hyperaccumulator yields and prices. Preliminary calculations for the pyrometallurgical process for Ni agromining show promising results under the condition that heat released during incineration can be valorized close to the processing facility.

Environmental Assessment

Although agromining is focused on the use of plants to recover valuable metals out of natural or man-polluted soils, it remains an anthropogenic process, at the intersection between agriculture and soil remediation: it needs to be assessed in terms of its sustainability and global environmental impact. Life Cycle Assessment (LCA) is the most recognized method for this type of assessment. LCA is a systematic tool which obeys the rules specified by International Standards (ISO, 2006a,b) and has seen many applications in the field of agriculture (for food crops Keyes et al., 2015; Ingrao et al., 2018; etc. and energy crops Arodudu et al., 2017; Hoekman and Broch, 2018; etc.) or remediation of contaminated soil (Suer and Andersson-Sköld, 2011; Lemming et al., 2012). This type of analyses was used to assess the combination of phytoremediation of contaminated soils with energy production by Witters et al. (2012) and Kuppens et al. (2015) for willow, rapeseed and maize, and by Nsanganwimana et al. (2014) for *Miscanthus x giganteus*. Vigil et al. (2015) confirmed that biomass valorization was necessary to make this soil remediation technique sustainable. In the case of Ni-agromining, studies applying LCA to assess the environmental impact of this process are rare. To the best of our knowledge, only one study (Rodrigues et al., 2016) can be found in the literature. Rodrigues et al. (2016) emphasized that energy from *O. muralis s.l.* ashing should be recovered to make the process really environmentally beneficial.

The basic impacts which are generally considered in LCA are related to natural resources (depletion of water, abiotic and renewable resources for irrigation, fertilizers production, energy, etc.), natural environment (land use, climate change, eutrophication, acidification, ecotoxicity, etc.) and human health. In the case of soil remediation it has been proposed to combine LCA with a specific local health assessment for decontamination operators to guide the selection of sustainable remediation processes (Hou et al., 2017). According to Rodrigues et al. (2016), cultivation techniques in agromining should be selected to limit soil erosion, either due to rainfall or wind, as erosion favors the transport and dispersion of metal-laden particles in the environment.

In spite of LCA normalization, there are still questions arising regarding the way to deal with land use (LU) and its change (LUC). This is a critical issue in the study of any system in which an agricultural process is developed. Land surface and functions should be taken into account, but it is difficult to reach a consensus on the descriptors that should be used: there is a growing awareness of the need to improve the cause-effect chain models related to ecosystem services provision in life cycle inventories (Othoniel et al., 2016). Morais and Delerue-Matos (2010) pointed out the difficulty of spatial and temporal differentiation of local impacts assessment, especially when taking into account the prediction of long-term toxicity effects.

Generally speaking any land use is seen as negative (Koellner et al., 2013). However in the case of soil remediation the problem is more complex as the ultimate goal is to improve the soil properties and functions. In the case of agromining of ultramafic soils the initial land state is natural. The contamination stage is outwith the scope of the agromining process if this is carried out on soil polluted due to human activity.

Soil carbon sequestration is one of the descriptors used to assess land use change: the development of a clear soil carbon accounting method is still under discussion for agricultural processes (Goglio et al., 2015). In particular the timing of soil CO_2 emissions during long-term agromining should be taken into account, together with agricultural management practices (fertilization, tillage, crop rotation, etc.; Levasseur et al., 2010). Experiments are undertaken to monitor C status in agromining experimental fields at the four locations as well as greenhouse gas emissions at two of the four sites (AT and ES). The data obtained from these experiments will help a better estimation of these issues in the LCA. Another descriptor largely used to assess land use is biodiversity. Gabel et al. (2016) reviewed twenty-two different biodiversity impact assessment methods susceptible to be used in LCA of agricultural processes. Most of them were not initially developed for these processes and questions still arise as to which biodiversity aspects should be considered, which indicators should be used and how references should be selected. A conceptual model of effect of land intervention (occupation, transformation) has been proposed by Curran et al. (2016): the effect on ecosystem quality is assessed through indicators of biodiversity damage potential, at the local and regional scales. In agromining the introduction of non-endemic species will be detrimental to local biodiversity: local plants have developed at least tolerance to metals, if not metal accumulation properties.

Since most metal hyperaccumulators discussed so far are angiosperms, another ecosystem function is also relevant: pollination. Although necessary for maintaining endemic species, pollen transport may favor dissemination of non-endemic species. Furthermore high-metal contents in flowers could affect the behavior of honey bees (Di et al., 2016; Nikolić et al., 2016), with potential incorporation of metals in honey and propolis (Matin et al., 2016). Meindl and Ashman (2017) discussed the effect of soil chemistry on pollen germination in a Ni-hyperaccumulator, *Streptanthus polygaloides*. However, *O. muralis s.l.* has always been a widespread weed ("*Pulë verdhë*" in Albanian) in agricultural ultramafic areas of the Pogradec district: wild and domesticated honey bees have dealt with it for at least several millennia since Antiquity. Opening the landscape by clearing the native woodland vegetation has probably provoked a boost in the populations of *O. muralis s.l.*, which is not very common in the native maquis (Bani et al., 2014). More knowledge about the potential incorporation of agromined metals in the human food chain is desirable (Herrero-Latorre et al., 2017). However, there might be a positive effect on the pollination service since *O. muralis s.l.* is a strong nectar producer and stimulates pollinating insects), and also because there is absolutely no need to use insecticides with this crop (insecticides may have a negative impact on pollinating insects populations).

The application of LCA to agromining processes is not yet fully developed. The methodology already applied to agroprocesses as well as to soil remediation serve as starting points, but certain aspects related to land use and land use changes in a context of using local/non-local species in their native/non-native habitat will require specific adjustments and research efforts.

FINAL REMARKS

Agromining offers new possibilities for the recovery of strategic metals from natural resources. This review focused on natural metalliferous soils, but agromining can also be applied to secondary resources derived from industrial activities. The exceptional capacity of hyperaccumulator plants to bioaccumulate metals in their harvestable tissues at commercial ore grade levels makes these techniques viable. The EU initiatives summarized here demonstrate at field scale the potential for Ni agromining on ultramafic soils across a range in climatic and edaphic conditions. Nickel agromining in these areas represent a new form of agriculture which can generate income from low-productivity agricultural land derived from ultramafic bedrock. Agromining efficiency can be optimized using appropriate agronomic practices, with positive effects on soil health and quality, as well as the generation of wider services (e.g., biodiversity conservation, C sequestration, etc.).

AUTHOR CONTRIBUTIONS

PK, ÁP-F, BR-G, RS, and EB carried out the experimental work in the agromining field site in Spain. AB, J-LM, and GE have been involved in the development of agromining in the Albanian field site. CR, TR, and MP carried out experimental work in the Austrian field site, and MK and DK established the agromining experimental plots in Greece. PK coordinated the overall preparation of the manuscript, and all authors contributed to the writing of the manuscript. GE coordinates the LIFE-Agromine and Agronickel projects.

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Phytomanagement and Remediation of Cu-Contaminated Soils by High Yielding Crops at a Former Wood Preservation Site: Sunflower Biomass and Ionome

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This long-term field trial aimed at remediating a Cu-contaminated soil to promote crop production and soil functions at a former wood preservation site. Twenty-eight field plots with total topsoil Cu in the 198–1,169 mg kg^{-1} range were assessed. Twenty-four plots (OMDL) were amended in 2008 with a compost (made of pine bark chips and poultry manure, OM, 5% w/w) and dolomitic limestone (DL, 0.2%), and thereafter annually phytomanaged with a sunflower—tobacco crop rotation. In 2013, one untreated plot (UNT) was amended with a green waste compost (GW, 5%) whereas 12 former OMDL plots received a second compost dressing using this green waste compost (OM2DL, 5%). In 2011, one plot was amended with the Carmeuse basic slag (CAR, 1%) and another plot with a P-spiked Linz-Donawitz basic slag (PLD, 1%). Thus six soil treatments, i.e., UNT, OMDL, OM2DL, GW, CAR, and PLD, were cultivated in 2016 with sunflower (*Helianthus annuus* L. cv Ethic). Shoots were harvested and their ionome analyzed. At high soil Cu contamination, the 1M NH_4NO_3-extractable vs. total soil Cu ratio ranked in decreasing order: Unt (2.35) > CAR (1.02), PLD (0.83) > GW (0.58), OMDL (0.44), OM2DL (0.37), indicating a lower Cu extractability in the compost-amended plots. All amendments improved the soil nutrient status and the soil pH, which was slightly acidic in the UNT soil. Total organic C and N and extractable P contents peaked in the OM2DL soils. Both OMDL and OM2DL treatments led to higher shoot DW yields and Cu removals than the GW, CAR, and PLD treatments. Shoot DW yields decreased as total topsoil Cu rose in the OMDL plots, on the contrary to the OM2DL plots, demonstrating the benefits to repeat compost application after 5 years. Shoot Cu concentrations notably of OMDL and OM2DL plants fitted into their common range and can be used by biomass processing technologies and oilseeds as well. In overall, there is a net gain in soil physico-chemical properties and underlying soil functions.

HIGHLIGHTS

- Compost incorporated into Cu-contaminated soils improves the sunflower growth.
- Soil organic matter increases in compost-amended soils.
- Extractable soil Cu decreases in compost-amended soils.
- Shoot Cu removal by sunflower reaches 26–88 g Cu ha^{-1} $year^{-1}$.

Keywords: basic slag, compost, carbon sequestration, *Helianthus annuus* L., marginal land, organic matter, phytoextraction, phytoremediation

INTRODUCTION

An estimate of local, anthropogenic soil contamination to the whole of Europe has totaled 2.5 million of potentially contaminated sites, a considerable fraction having real or perceived contamination problems (Panagos et al., 2013; Science Communication Unit University of the West of England, 2013). With an estimated area of 2 ha per site and knowing that 37.3% of the total contamination is caused by metal(loid)s, roughly 1.86 million ha would be contaminated by these ones (Evangelou et al., 2012; Van Liedekerke et al., 2014). Three hundred forty thousand contaminated sites would require a remediation (Van Liedekerke et al., 2014). Similarly, the US EPA tracks nearly 9 million ha of possibly contaminated land (USEPA, 2013) and 1,438 abandoned, worst hazardous waste sites on its National Priority List (USEPA, 2016). Contaminated soils in China would reach 10 million ha and 10–17% of the farmland (more than 20 million ha) would be metal(loid)-contaminated based on food survey (Yao et al., 2012; Zhang et al., 2015). Many impacted sites have been remediated to productive use, but numerous large sites remain derelict or underutilized because their remediation is uneconomic or unsustainable using conventional methods (Le Corfec, 2011; ADEME, 2014; Van Liedekerke et al., 2014; JRC, 2015; Cundy et al., 2016). The 7th Environment Action Programme of the EU however aims that by 2020 "soil is adequately protected and the remediation of contaminated sites is well underway" (Official Journal of the European Union, 2013).

The long-term combination of gentle remediation options (GRO) with profitable crop production and/or green technologies, i.e., phytomanagement, can be applied as part of integrated, mixed, site risk management solutions for the return of low-level risk sites to productive usage and can gradually provide a range of economic and other benefits (Mench et al., 2010; Cundy et al., 2015, 2016; Kidd et al., 2015). Demonstrating the wider benefits of undertaking soil remediation is crucial for several questions, notably: how long it will take for the solutions to reduce pollutant linkages, are the solutions sustainable, and how much it will cost? (Bardos et al., 2016; Cundy et al., 2016; Gerhardt et al., 2017).

The harvested biomass can be used by various biomass processing technologies and sectors, and appropriate biomass cultivation can improve soil functions and underlying ecosystem services, e.g., storage and supply of nutrients, plant and microbe biodiversity, regulation of water supply and quality, erosion control, recycling of raw materials, reduced greenhouse gas emissions and waste generation, landscaping medium, etc. (Carrier et al., 2011; Delplanque et al., 2013; Bourgeois et al., 2015; Evangelou et al., 2015; Strezov and Evans, 2015; Cundy et al., 2016; Gonsalvesh et al., 2016; Asad et al., 2017; Bauddh et al., 2017; Bert et al., 2017a; Ciadamidaro et al., 2017; Clifton-Brown et al., 2017; Šimek et al., 2017; Schröder et al., 2018).

A number of perceived or actual barriers or impediments related to technical issues and stakeholder perceptions is limiting on site phytomanagement application (Cundy et al., 2015; Bert et al., 2017b; Montpetit and Lachapelle, 2017). For overcoming such barriers, several sets of field trials have been either implemented or developed in Europe, notably for metal(loid)-contaminated sites with funding from European projects, i.e., Greenland, PhytoSUDOE, Intense, and Miscomar, and national environment agencies, e.g., Ademe in France (Mench et al., 2010; Kidd et al., 2015; Nsanganwimana et al., 2016; Bert et al., 2017a,b; Friesl-Hanl et al., 2017; Krzyzak et al., 2017; Quintela-Sabarís et al., 2017; ADEME, 2018). Here the purpose was to assess the long-term efficiency and limits of phytomanagement options at a wood preservation site with sandy Cu-contaminated soils.

Copper ranks 5th out of the 10 most frequent contaminants detected on French contaminated sites (singly and in combination; 6% in term of occurrence of soil and water contamination; potentially present at 1,413 sites) after hydrocarbons, Pb, Cr, and polycyclic aromatic hydrocarbons (BASOL, 2017), notably due to smelting, metallurgy and wood preservation, without accounting for vineyard, orchard and horticultural soils contaminated by Cu-based fungicides (at least 1 million ha) and historically sludge-amended soils (Godin, 1983; Hedde et al., 2013). The European wood preserving industry produce around 6.5 million m^3 $year^{-1}$ of pressure treated wood, 71% of this wood being treated with water-borne products (Salminen et al., 2014).

Copper excess in soils at wood preservation sites, often associated with As, Cr(VI), B, Hg, and xenobiotics such as creosote-derived PAH, contributes to impact soil ecological functions, e.g., microbial communities, biogeochemical cycles of organic matter (OM) (U. S. Congress, 1995; Dumestre et al., 1999; Buchireddy et al., 2009; Lagomarsino et al., 2011; OECD, 2013). Soil structure and texture are also altered, notably through the OM cycle, resulting in low water and nutrient retention (Mench and Bes, 2009; Asensio et al., 2013) and in less plant resilience to drought and low fertility (Wong, 2003).

Here, the remediation solution and soil amendments were selected after risk assessment and option appraisal (Bes and Mench, 2008; Mench and Bes, 2009; Negim et al., 2012). As soil pH, OM and Al, Fe, and Mn oxyhydroxides are key-players mutually driving Cu precipitation, reactions with soil fractions, and operational mobility, plots amended with either a combination of compost and dolomitic limestone or basic slags were implemented on site (Kolbas et al., 2011; Le Forestier et al., 2017).

Out of potential Cu-tolerant crops (e.g., willows, poplars, pines, Miscanthus, vetiver, tobacco, sorghum, etc.), sunflower is an annual high yielding plant species and a secondary metal accumulator in shoots, relatively tolerant to metal(loid) excess and suitable for cultivation on derelict areas (Marchiol et al., 2007; Fässler et al., 2010; Mench et al., 2010). It can be used to phytoextract bioavailable metal (Cd, Zn, and Cu) fraction in contaminated soils and provide financial returns from the biomass processing (Mench et al., 2010; Kolbas et al., 2011; Herzig et al., 2014; Kidd et al., 2015). Due to its high biomass, sugar, protein and oil production, sunflower shoots and oilseed are relevant raw feedstock for several biomass processing technologies and various sectors: e.g., insulation material, hydrothermal processing which converts raw materials such as lignocellulosic materials into bioenergy and high added-value chemicals (Ruiz et al., 2013), fatty acids for supporting microbes, oil, production of bioethanol, fermentation and biogas (Alaru et al., 2013; Camargo and Sene, 2014; Hesami et al., 2015), fibers to reinforce plastic products (Malkapuram et al., 2009; Strezov and Evans, 2015; Mati-Baouche et al., 2016; Liu et al., 2017), etc. As water supply and its distribution during the crop cycle is one limiting factor for crop production in SW France, sunflower ability to resist to more frequent heatwaves and long droughts due to the climate change is an advantage (Kidd et al., 2015). A crop rotation however is mandatory in France to avoid fungi diseases related to sunflower cultivation (CETIOM, 2011).

This field trial aimed at assessing the long-term efficiency of phytomanagement options based on various soil amendments and a crop rotation with high yielding plants relatively Cu-tolerant (sunflower and tobacco) to remediate a Cu-contaminated soil at a former wood preservation site. The hypotheses were (1) to initially reduce the phytoavailable soil Cu, through soil amendment, for allowing a better crop production, usable by biomass processing technologies and the bioeconomy, and then (2) to annually strip a part of the phytoavailable soil Cu corresponding to shoot Cu removal, leading progressively to ameliorate soil functions. Regarding soil amendments, we compared (1) a single dressing of compost combined with dolomitic limestone, (2) a compost dressing renewed 5 years after the first incorporation of compost and dolomitic limestone into the soil, and (3) a single addition of basic slags.

Potential processes behind these soil amendments were: (1) Cu sorption by the compost-derived OM, liming effect for enhancing Cu sorption on soil bearing phases, promotion of soil microbial communities, and nutrient (N, P, K, Ca, and Mg) supply for biomass production and better cellular homeostasis; (2) liming effect and Cu reaction with Fe/Mn oxides, carbonates and phosphates (Bes and Mench, 2008; Kumpiene et al., 2008). Changes in soil physico-chemical properties, shoot dry weight (DW) yield, ionome, and Cu removal of sunflower plants grown in year 9 were reported. Changes in available soil Cu and other soil physico-chemical parameters in line with sunflower parameters were discussed and potential biomass processing as well.

MATERIALS AND METHODS

The field trial was set up in 2008 at a wood preservation site (10 ha) located in Saint-Médard-d'Eyrans, Gironde, SW France (44°43.353′N, 000°30.938′ W—France, Kolbas et al., 2011). The soil developed on an alluvial soil in terrace containing alluvial materials from the Garonne River combined with wind deposits (Fluvisol—Eutric Gleysols, World Reference Base for soil resources) (Mench and Bes, 2009). Its texture is sandy, i.e., 85.8% sand, 5.9% clay, 8.3% silt, 1.6% OM, C/N 17.2, and neutral pH (7.1 $\pm$ 0.3), with a low CEC (3.5 $cmol^+ \ kg^{-1}$).

Industrial activity dates back to 1846 with the construction of a railway line and a facility for wood preservation (Mench and Bes, 2009). Copper sulfate (from 1913 to 1980), chromated copper arsenate type C (from 1980 to 2006), Cu hydroxycarbonates (17.3%) with benzylalkonium chloride (4.8%) and Tanalith E (Cu carbonate 16.4%, Tebuconazole 0.18%, and propiconazole 0.18 % w/w) were successively used. No preserved wood was stored on the field trial area since at least 2003. Soil contamination was mainly due to wood washing, Cu being the main contaminant (**Table 3**) and total soil Cu decreasing rapidly in the soil profile. Plant communities were previously characterized (Bes et al., 2010). Soil quality and risks were assessed on site, revealing topsoil ecotoxicity with diffuse contamination generating pollutant linkages (Mench and Bes, 2009; Kolbas et al., 2011; Marchand et al., 2011).

Field Trial

Originally, the site was divided into 15 sub-sites according to past and present activities (Bes et al., 2010). The field trial is located at the P1-3 sub-site (10 m $\times$ 11 m) and started in March 2008 (Kolbas et al., 2011). It consists in four blocks (2 m $\times$ 10 m) of 10 plots (1 m $\times$ 2 m), defined according to total soil Cu (**Figure 1**), i.e., block B1: plots #1 to #10 (198–381 mg Cu kg^{-1}), block B2: plots #11 to #20 (257–556 mg Cu kg^{-1}), block B3: plots #21 to #30 and block B4: plots #UNT, #CAR, #PLD, #GW (719–1,169 mg Cu kg^{-1}) (Kolbas et al., 2011). Six soil treatments were tested on site: OMDL, OM2DL, UNT, GW, CAR, and PLD (**Table 1**). Soil amendments were carefully mixed in the topsoil (0–0.25 m) with a stainless-steel spade. The amendment composition is detailed in **Table 2**. Hereafter plots from the same block and with the same soil treatments were labeled as followed: (block:treatment) (e.g., B2: OMDL). Since 2008, a crop rotation has been annually carried out with sunflower and tobacco (Kolbas et al., 2011; Kolbas, 2012).

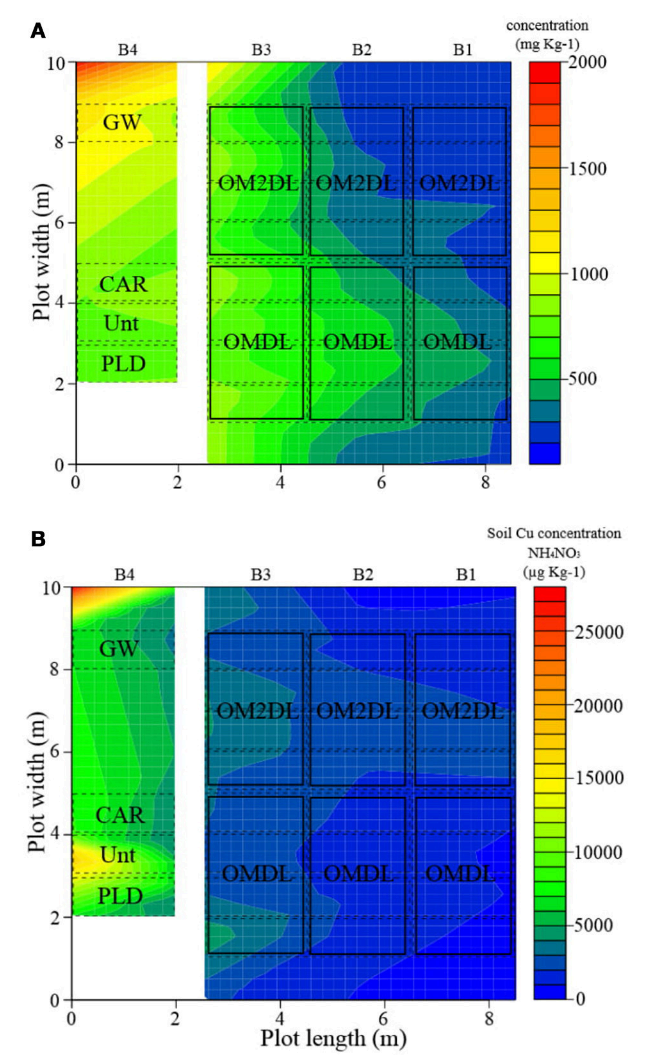

FIGURE 1 | Spatial variability of **(A)** total topsoil Cu (mg Cu kg^{-1} DW) and **(B)** 1M NH_4NO_3-extractable topsoil Cu (µg Cu kg^{-1} DW) (0–0.11 m depth layer) within the field trial. B1: block #1, B2: block #2, B3: block #3, and B4: block #4.

Soil Sampling

In February 2017 (year 10), four soil samples per plot were collected in the topsoil layer (0–11 cm) with a sampling cylinder (ø 3.6 cm × 11.5 cm – 0.39 L) and in the subsoil layer (11–30 cm) with a soil auger. In overall, 28 plots × 4 replicates × 2 soil layers were collected. Fresh topsoil replicates were weighed to determine their bulk density (mean value = 0.9 $g.cm^{-3}$). Thereafter all fresh soil samples were sieved at 4 mm, paying attention to aggregates and to avoid any loss of compost particles. Soil samples were then weighed, air-dried and weighed again to determine their moisture content. Replicates were further combined to make a composite soil sample (∼ 1 kg soil fresh weight), sieved at 2 mm (nylon mesh) and manually homogenized. After pooling replicates from one plot, we had 28 samples per layer including the 6 treatments. Soil texture and physico-chemical parameters were determined with standard methods and a quality scheme by INRA LAS (2014), Arras, France, e.g inductively coupled plasma/atomic emission spectroscopy (ICP-AES) for metals after wet digestion in HF and $HClO_4$, and hydride-generation for As after wet digestion in H_2SO_4/HNO_3 (2/1) with V_2O_5 at 100°C (3 h). Two certified reference materials, i.e., BCR No. 141 (calcareous loam soil) and BCR No. 142 (light sandy soil) from the Bureau Communautaire de Référence, were used by INRA LAS in the quality scheme. To avoid making this paper too cumbersome, only the topsoil data are presented here and the subsoil data are included in the (**Table S1**).

Sunflower Cultivation and Analysis

In April 2016 (year 9), face to a severe spring drought, sunflower seeds (cv. LG545010 ES Ethic) were firstly sowed in plastic pots (6.5 cm × 6.5 cm × 6.5 cm) filled with a plant growth substrate (compost 33%, soil 33%, and perlite 33%) and then placed in a greenhouse during 1 month. In early May, plantlets were transplanted in field plots in three rows (0.33 m between rows, 0.28 m between plants, 21 plants per plot, and 105,000 plants ha^{-1}). The soils were fertilized twice, i.e., before and 2 months after transplantation, with NPK fertilizer [Blaukorn classic, 12-8-16 (3–25)] at 40 kg N (58% NH_4, 42% NO_3), 26.7 kg P_2O_5, 53 kg K_2O, 10 kg MgO, 83 kg SO_3, 0.06 kg B, 0.2 kg Fe, and 0.04 kg Zn per ha. Plant shoots were harvested in September 2016. Stem bottoms were carefully brushed to remove soil particles, shoots cut and placed in paper bags, and oven-dried at 50°C until constant weight. The shoot dry weight (DW) yields were determined (including stem, leaves and flower heads). Flower heads were separated and shoots ground in an universal cutting mill (<1.0 mm particle size, Fritsch Pulverisette 19). Weighed aliquots (0.5 g DW) were wet-digested under microwaves (CEM Marsxpress 1200 W) with 5 mL suprapure 14 M HNO_3 and 2 mL 30% (v/v) H_2O_2 not stabilized by phosphates and 1 mL MilliQ water. Certified reference material (BIPEA maize V463) and blank reagents were included in all series. Mineral composition (As, Ca, Cu, Cr, Fe, K, Mg, Mn, Na, Ni, P, and Zn) in digests was determined by ICP-MS (Thermo X series 200, INRA USRAVE laboratory, Villenave d'Ornon, France). All elements were recovered (>95%) according to the standard values and standard deviation for replicates was <5%. All element concentrations in plant parts are expressed in mg or g DW kg^{-1}. The shoot Cu removal was calculated as follows: Cu (µg $plant^{-1}$) = shoot DW yield (g $plant^{-1}$) x shoot Cu concentration (µg g^{-1}), assuming similar Cu concentration in shoots and flower heads as reported by Kolbas (2012).

Statistical Analyses

Influence of soil treatments in the B3 and B4 plots on shoot DW yields, shoot ionome and Cu removal of sunflower plants were tested using one-way analyses of variance (ANOVA). When significant differences occurred between treatments, multiple comparisons of mean values were made using *post-hoc* Tukey HSD tests. When assumptions were not met, Wilcoxon pairwise tests adjusted with a Bonferroni correction were used (i.e., shoot Mg concentration). Differences between OMDL and OM2DL, in the B1 and B2 plots for these same plant parameters were tested

TABLE 1 | Soil treatments.

Soil treatments	Block: plots	Set-up	Amendments	References
Unt	B4: #31	March 2008	None (untreated)	Kolbas et al., 2011 Kumpiene et al., 2011 Quintela-Sabarís et al., 2017
OMDL	B1: #2 to 5 B2: #12 to 15 B3: #22 to 25	March 2008	Single dressing of a compost of pine bark chips and poultry manure (OM, 5% w/w) and dolomitic limestone (DL, 0.2% w/w),	Kolbas et al., 2011 Kumpiene et al., 2011 Quintela-Sabarís et al., 2017
OM2DL	B1: #6 to 9 B2: #16 to 19 B3: #26 to 29	March 2008	2008: one dressing of compost and dolomitic limestone as for OMDL; 2013: one dressing of green waste compost (GW, 5% w/w)	Jones et al., 2016 Oustriere et al., 2016
GW	B4: #GW	March 2013	One dressing of green waste compost (GW, 5% w/w)	Jones et al., 2016 Oustriere et al., 2016
CAR	B4: #CAR	March 2011	One single dressing of Carmeuse basic slag (1% w/w)	Le Forestier et al., 2017
PLD	B4: #PLD	March 2011	One single dressing of P-spiked Linz-Donawitz basic slag (1% w/w)	Negim et al., 2012; Le Forestier et al., 2017

using a Student test (T-test). Normality and homoscedasticity of residuals were met for all tests. Differences were considered statistically significant at $p < 0.05$. Changes in shoot Cu concentration, Cu removal and shoot DW yield of sunflower plants depending on soil treatments (i.e., OMDL and OM2DL), total topsoil Cu and their interaction were analyzed using an ANCOVA for the B1, B2, and B3 plots. The mapping of total topsoil Cu and organic C was carried out using the surface trends analysis technic, with the Lattice and Akima packages of the R software (**Figure 1** and **Figure S1**). All statistical analyses were performed using R software (version 3.0.3, Foundation for Statistical computing, Vienna, Austria).

RESULTS

Soil Physico-Chemical Parameters (Topsoil, 0–11 cm, Table 3)

Total topsoil Cu (mg Cu kg^{-1}) in year 10 varied from 237 (B1: OM2DL) to 1169 (GW) and exceeded its pedogeochemical background and screening values (**Table 3**). Mapping of total soil Cu showed its higher values in the B3 and B4 plots (**Figure 1A**). Total topsoil Cu was similar for the OMDL and OM2DL treatments in the B1 and B3 plots. The corresponding mean values for the B2 block differed, however, being significantly higher in the OMDL plots than in the OM2DL ones. For total topsoil As, Cd, Cr, Co, Ni, Pb, and Zn, values were generally at the background levels (**Table 3**). Total topsoil Zn increased in compost-amended plots, notably in the OM2DL and GW ones, as compared to the untreated and basic slag-amended soils. As expected, total topsoil Fe, Mn, and in a lesser extent Cr were enhanced in the basic slag-amended plots. Across all plots, extractable topsoil Cu ranged from 0.71 (B1:OMDL) to 17.9 (Unt) mg Cu kg^{-1} soil (mean values varied between 0.89 and 17.9, **Table 3**; **Figure 1B**). The values were normalized based on total topsoil Cu, and the ratio (R_L) varied between 0.24 (B1:OMDL) and 2.35 (Unt) (**Figure 2A**). This ratio was significantly (p-value 1.63e^{-07***}) lower in the OMDL soils, i.e., 0.31 $\pm$ 0.10 and 0.32 $\pm$ 0.07, than in the OM2DL soils, i.e., 0.8 $\pm$ 0.08 and 0.65 $\pm$ 0.05, for the B1 and B2 plots, respectively. This indicated a higher NH_4NO_3-extractable Cu fraction in the OM2DL topsoils of these plots. In contrast, the extractable vs. total soil Cu ratio was similar in the OMDL and OM2DL soils for the B3 plots. In this last one, this ratio ranked in decreasing order: Unt (2.35) > CAR (1.02), PLD (0.83) > GW (0.58), OMDL (0.44 $\pm$ 0.09), OM2DL (0.37 $\pm$ 0.10), indicating a lower Cu extractability in the compost-amended plots. Considering all OM2DL plots, the extractable soil Cu fraction decreased as total topsoil Cu rose, and fitted well a quadratic function (**Figure 2B**). For the OMDL plots, this Cu fraction matched less with a quadratic function, with an opposite trend as total topsoil Cu decreased.

The UNT topsoil pH was slightly acidic (6.3) and rose in all amended soils from 7.0 (OMDL in B1 and B2) to 7.7 (PLD and CAR in B4) (**Table 3**). The topsoil CEC increased in all amended soils in the 4–15.5 cmol^{+} kg^{-1} range as compared to the UNT soil (3.1 cmol^{+} kg^{-1}), and notably peaked in all plots amended with the green waste compost in year 6. For comparison, its value was 16 cmol^{+} kg^{-1} for an uncontaminated soil of the same soil series (**Table 3**). The soil moisture, and total soil organic N and C were higher in the compost-amended soils than in the UNT and basic slag-amended soils. The soil organic matter (SOM) peaked in the topsoils amended with the green waste in year 6, but the C/N ratio (in the 14–15 range) was similar in all plots and matched with its value for the uncontaminated soil, slightly exceeding that reported for French sandy soils (10). Soil available P (Olsen method) ranged (mg kg^{-1}) from 73 (UNT) to 96 (B2:OM2DL), being higher in all soils amended with the green waste, albeit differences were significant between OMDL and OM2DL treatments in the B1 and B2 plots. Soil $CaCO_3$ concentration was low, in relation with neutral pH, and ranged (g kg^{-1}) from $\leq$1 (UNT and PLD in B4) to 4 (GW). Total topsoil K, Mg, Na and P were similar in all plots, ranging from 7 to 8 g K kg^{-1}, 0.8 to 1 g Mg kg^{-1}, 1.9 to 2.1 g Na kg^{-1}, and 0.7 to 1 g P kg^{-1}, respectively. Total soil Fe and Mn increased in the PLD- and CAR-amended soils as compared to the UNT soil. Total soil Ca (g kg^{-1}) was higher in all amended soils than in the UNT soil (0.9), raising from 1.3 (B1:OMDL) to 6.1 (GW).

TABLE 2 | Composition and main physico-chemical properties of soil amendments.

	OM	GW	DL	CAR	PLD
pH	6.94 ± 0.08	7.53	–	12.7	11.6
EC (μS cm^{-1})	–	–	–	10,700	2,100
SiO_2 (g kg^{-1})	–	–	–	111	146
Al_2O_3 (g kg^{-1})	–	–	–	14	56
Fe_2O_3 (g kg^{-1})	–	–	–	366	214
TiO_2 (g kg^{-1})					10.9
CaO (g kg^{-1})	47.1	–	300	448	307
MgO (g kg^{-1})	4.7	–	200	64	95
K_2O (g kg^{-1})	10.9	–	–	–	–
Na_2O (g kg^{-1})	1.4	–	–	2	–
MnO (g kg^{-1})	–	–	–	42	25
P_2O_5 (g kg^{-1})	17.7	–	–	12	140
SO_3 (g kg^{-1})	4.9	–	–	–	–
CEC (cmol kg^{-1})	–	26.7	–	–	–
Major elements					
Total *N* (% DW)	–	0.69	–	–	–
Organic C (g kg^{-1})	321	109	–	–	–
C/N	19.4	15.8	–	–	–
Nutrients (g kg^{-1})					
Ca	–	22.5	–	–	–
K	–	5.4	–	–	–
Mg	–	1.9	–	–	–
Na	–	–	–	–	–
P	–	0.374	–	–	–
Trace elements (mg kg^{-1})					
Al	–	12,700	–	–	
As	0.8	4.47	–	–	<5
Cd	0.5	<0.5	–	–	–
Cr	<0.5	21	–	–	–
Cu	32.1	85.8	–	139	<5
Mn	–	–	–	–	11,300
Fe	–	6,830	–	–	–
Hg	0.2	<0.1	–	–	–
Ni	1.8	7.98	–	–	<10
Pb	9	51.5	–	–	<20
Zn	131	174	–	–	24

OM, compost of poultry manure and pine bark chips; GW, compost of green wastes; DL, dolomitic limestone; CAR, Carmeuse basic slag; PLD, P-spiked Linz-Donawitz slag.

Shoot DW Yield of Sunflower Plants (Table 4)

In year 9, sunflower plants did not display visible phytotoxicity symptoms on their shoots for all plots. Survival rate was roughly 100% in all compost-amended plots. In the UNT and basic slag-amended plots, with high Cu exposure, plants had a lower maximum stem length, more brittle, leading to lower shoot DW yield (**Table 4**). At high total soil Cu (B3 and B4 plots), the shoot DW yield in year 9 was significantly higher for plants grown in all compost and dolomitic limestone-amended soils than in the UNT soil and peaked for the OM2DL plants. In contrast no significant difference occurred between plants from the basic slag- and GW-amended plots and the UNT plot. At intermediate soil Cu contamination (B1 and B2 plots), the shoot DW yields of OMDL and OM2DL plants did not differ (g DW $plant^{-1}$: 48 ± 20 and 43 ± 16 in B1 and 45 ± 17 and 51 ± 22 in B2, respectively). These values were similar to those for the B3:OM2DL plants, but higher than for the B3:OMDL ones. Based on ANCOVA analyses, the shoot DW yield decreased in the OMDL plots as total and extractable soil Cu increased whereas it remained steady in the OM2DL plots (**Figure 3**; **Table S2**; **Figure S2**), underlining the slight benefit to repeat the compost addition in year 6.

Shoot Ionome and Shoot Cu Removal of Sunflower Plants (Table 4)

The shoot Cu concentration decreased significantly for all plants from the amended plots as compared to the UNT one (i.e., 48 ± 10 mg Cu kg^{-1}). In the amended soils, it varied between 10 ± 2 (B1 and B2:OM2DL) and 31 ± 4 (PLD) mg Cu kg^{-1}, this upper value slightly exceeding common values for sunflower. In the B1 and B2 plots, shoot Cu concentration was higher in the OMDL plants than in the OM2DL ones, despite a higher extractable Cu fraction in the OM2DL soils (**Tables 3**, **4**; **Figure 4**). Shoot Cu concentration increased more in the OMDL shoots than in OM2DL ones with total soil Cu, and significantly differed between both treatments at high total soil Cu (B3 plots) (**Table 4**; **Figure S3**). Shoot Cu concentration also increased with extractable soil Cu for both OMDL and OM2DL plants and, on the whole extractable Cu range, Cu concentration was higher in the OMDL shoots than in the OM2DL ones (**Figure 4**). Based on ANCOVA analysis, shoot Cu concentration in OMDL sunflower plants can exceed the upper critical threshold value (20 mg Cu kg^{-1} DW) at NH_4NO_3-extractable soil Cu over 2.7 mg Cu kg^{-1} soil (**Figure 4**, **Table S2**).

Shoot Cu removal (g Cu ha^{-1}) increased significantly for plants grown in amended soils from 42 ± 26 (PLD) to 88 ± 55 (B3:OM2DL) as compared to the UNT plants (19 ± 8), except for the GW and CAR plants (respectively, 26 ± 14 and 39 ± 25). Shoot Cu removal significantly differed between OMDL and OM2DL plants in the B1 and B2 plots but this difference was bridged as total and extractable soil Cu increased (**Table 4**, **Table S2**; **Figure 5** and **Figure S4**). Compared to the extractable topsoil Cu, annual shoot Cu removal corresponded respectively for the OMDL and OM2DL treatments to 9 ± 3.9% and 2.8 ± 1.4% in the B1 plots, 5.4 ± 3.4% and 3.0 ± 1.5% in the B2 plots, and 2.6 ± 1% and 2.8 ± 1.7% in the B3 plots.

Shoot Ca, K, and P concentrations were significantly higher for plants grown in all amended soils than for the UNT plants, whereas the shoot Zn and Mg concentrations only significantly increased for plants grown in compost-amended soils (**Table 4**). Changes in NH_4NO_3-extractable soil Zn from 0.023 to 1.2 mg kg^{-1} did not induce clear changes in shoot Zn concentration, which varied between 41 and 89 mg kg^{-1} under the influences of soil pH and total soil Zn and Cu. Shoot Mn concentration was significantly higher for the OMDL plants than for the UNT ones. In contrast, the shoot Fe concentration (mg Fe kg^{-1} DW) decreased for all amended soils from 239 ± 63 (UNT) to the 64 ± 22 (B3:OM2DL) −109 ± 25 (B1:OMDL) range as the shoot DW yield rose (exponential relationship, R^2: 0.51). For the B1 and B2 plots, shoot Ca, P, and Na concentrations did not differ between the OMDL and OM2DL plants. Shoot Fe and Mn concentrations

TABLE 3 | Main soil properties and physico-chemical parameters in the 0–10 cm soil layer.

Treatments	Block 3 and 4						Block 2		Block 1		Year 1[c]	Background levels		
	UNT	GW	OMDL	OM2DL	PLD	CAR	OMDL	OM2DL	OMDL	OM2DL		French sandy soils[a]	Control soil (same soil series)[b]	French agricultural soils[a]
pH KCl	5.5	7.1	6.5 ± 0.3	6.6 ± 0.3	7.1	7.2	6.4 ± 0.1	6.6 ± 0.1	6.4 ± 0.1	6.8 ± 0.1			7–8	
pH water	6.3	7.5	7.4 ± 0.2	7.1 ± 0.2	7.7	7.7	7.0 ± 0.1	7.1 ± 0.1	7.0 ± 0.03	7.2 ± 0.1	5.9 – 7.4			
Moisture (g kg^{-1})	3.8	12	13 ± 7	17 ± 8.1	3.9	4.1	7.3 ± 0.3	8.4 ± 0.5	6.6 ± 0.9	10 ± 0.6				
CEC ($cmol^{+}$ kg^{-1})	3.1	15.5	7.0 ± 3.3b	11 ± 2.3a	4.5	5.1	6.4 ± 0.2b	11 ± 0.5a	6.0 ± 1.1b	10 ± 1.1a	3.5		16.1	
P-Olsen (mg kg^{-1})	73	89	78 ± 10ab	85 ± 4.9a	82	79	72 ± 6b	96 ± 10a	75 ± 4b	92 ± 7a				
$CaCO_3$ (g kg^{-1})	<1	4.0	1.5 ± 0.7	1.7 ± 0.6	<1	1.0	1 ± 0.0	1.5 ± 0.6	1 ± 0.0	2.5 ± 0.6				
OM (g kg^{-1})	17	72	33 ± 14ab	43 ± 12.5a	17	16	25 ± 1.2b	42 ± 3a	24 ± 2.9b	40 ± 2a	16		69.9	
Organic C (g kg^{-1})	10	2	19 ± 8ab	25 ± 7.2a	10	9	15 ± 0.7b	24 ± 1.5a	14 ± 1.7b	23 ± 1.2a		14.5	40.4	
Total N (g kg^{-1})	0.7	2.9	1.4 ± 0.6ab	1.8 ± 0.5a	0.7	0.7	1.1 ± 0.02b	1.7 ± 0.1a	1.0 ± 0.1b	1.6 ± 0.1a			2.94	
C/N	14	15	14 ± 0.3	14 ± 0.1	15	14	14 ± 0.4	14 ± 0.5	14 ± 0.4	14 ± 0.3	17.2	10.0	13.8	
Texture (g kg^{-1})														
Sand	837	821	844 ± 13	839 ± 14	832	846	838 ± 3	833 ± 5	836 ± 5	829 ± 5		≥650	665	
Silt	109	107	96 ± 8	96 ± 7	107	97	99 ± 2	97 ± 3	101 ± 3	101 ± 5		≤350	155	
Clay	54	72	60 ± 6	66 ± 8	1	57	63 ± 0.8	70 ± 4	64 ± 4	70 ± 3		≤180	180	
Elements (mg kg^{-1})														
As	6.2	5.4	4.6 ± 0.5	3.8 ± 0.2	5.6	5.7	5.2 ± 0.3	5.0 ± 0.7	5.1 ± 0.2	4.5 ± 0.7		1–25	3.6	
Cd	0.12													
Cu	**760**	**1169**	**842 ± 69c**	**807 ± 81c**	**794**	**796**	**514 ± 48b**	**316 ± 52a**	**307 ± 60a**	**237 ± 41a**	163–1,170	3.2–8.4	21.5	7.1–28
Cr	14	16	11 ± 1.4	11 ± 1.5	19	22	12 ± 1	14 ± 2	13 ± 0.7	13 ± 1.5		14–40	17.9	16.7–69.4
Co	1.73	1.94	1.75 ± 0.11	1.77 ± 0.17	1.64	1.66	1.73 ± 0.05	1.89 ± 0.07	1.81 ± 0.01	1.82 ± 0.03				
Mn	173	179	171 ± 12	173 ± 9	359	412	191 ± 6	191 ± 4	184 ± 3.3	192 ± 6		72–376	189	
Pb	27													
Ni	5.0	6.3	5.3 ± 0.4	5.5 ± 0.9	4.9	4.7	5.0 ± 0.2	5.7 ± 0.2	5.1 ± 0.2	6.8 ± 2.6		4.2–14.5	7.46	9.1–41.8
Zn	29	**93**	45 ± 18ab	**59 ± 18ab**	30	31	43 ± 9b	**58 ± 5a**	**53 ± 12ab**	**68 ± 2a**	35–98	17–48	50.9	31.1–102
Extractable Cu[d]	17.9	6.78	3.40 ± 0.88a	3.53 ± 0.68a	6.65	8.17	1.54 ± 0.21*b*	2.02 ± 0.27*b*	0.89 ± 0.16*c*	1.90 ± 0.32b				
Nutrients (g kg^{-1})														
Ca	0.9	6.1	2.3 ± 1.7ab	3.4 ± 1.4a	1.9	1.9	1.6 ± 0.1*b*	3.3 ± 0.2a	1.3 ± 0.2*b*	3.3 ± 0.4a			1.04 ± 0.05	
Fe	6.6	6.5	6.4 ± 0.3	6.3 ± 0.2	7.8	7.7	6.5 ± 0.3	6.4 ± 0.1	6.3 ± 0.02	6.4 ± 0.1		6–14.3	6.55	
K	7.9	7.0	7.6 ± 0.3	7.4 ± 0.2	7.6	7.8	7.8 ± 0.1	7.5 ± 0.2	8.0 ± 0.03	7.4 ± 0.1			1.9 ± 0.2	
Mg	0.8	0.9	0.9 ± 0.1	0.9 ± 0.03	1.0	1.0	0.9 ± 0.03	0.9 ± 0.03	0.8 ± 0.02	0.8 ± 0.05			0.012 ± 0.01	
Na	2.0	1.9	1.9 ± 0.05	2.0 ± 0.09	1.9	2.0	2.1 ± 0.03	1.9 ± 0.09	2.1 ± 0.03	2.0 ± 0.05				
P	0.8	1.1	0.9 ± 0.04	0.9 ± 0.1	1.0	0.8	1.0 ± 0.06	0.7 ± 0.4	0.9 ± 0.04	0.9 ± 0.1				

Mean values ± standard deviation ($n = 1$ for UNT, CAR, GW, and PLD; $n = 4$ for OMDL and OM2DL in each block); mean values followed by the same letter did not differ; no letter in a row indicates no significant difference. <1 = below detection limit. Numbers in bold exceed background values in French sandy soils. OM, organic matter.

[a] *Frequent values in French sandy topsoils (Baize, 2009; Mench and Bes, 2009; Saby et al., 2009).*

[b] *Uncontaminated soil Eutric gleysol from a kitchen garden nearby (12 km) the site, Gironde, France.*

[c] *Kolbas et al. (2011).*

[d] *1M NH_4NO_3-extractable soil Cu.*

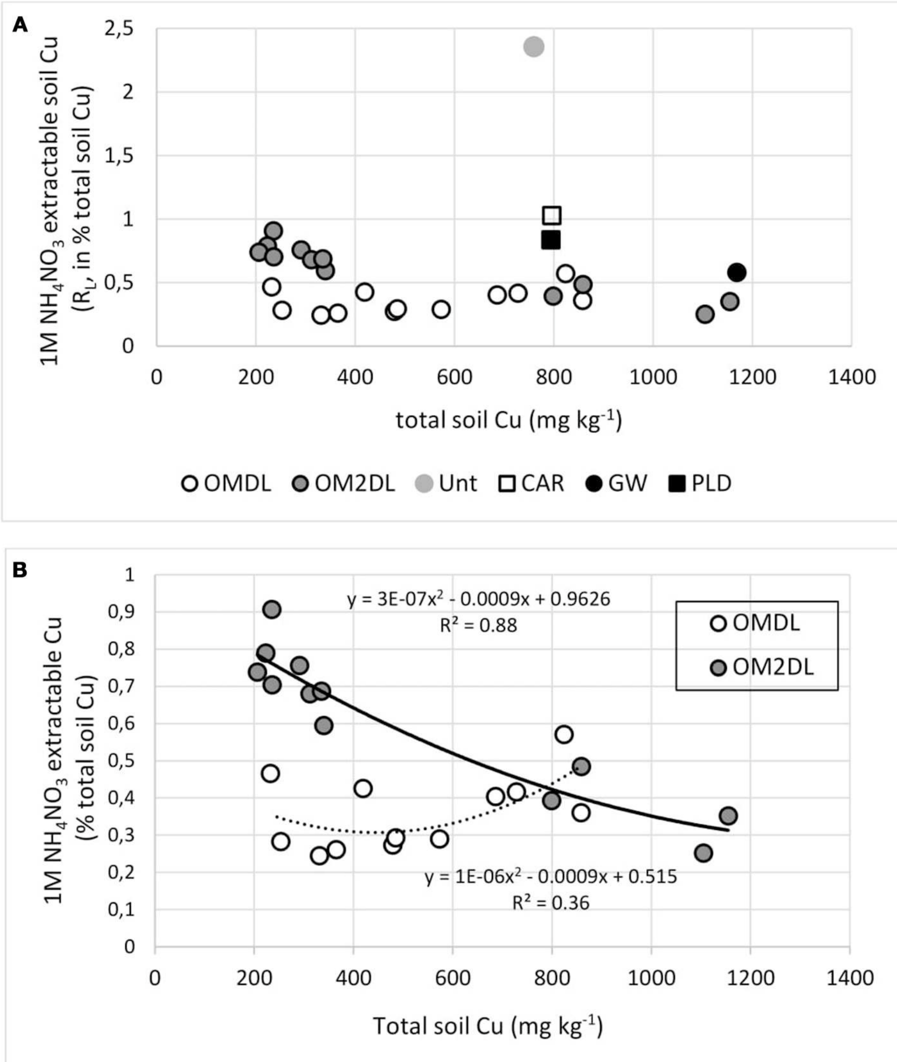

FIGURE 2 | (A) 1M NH_4NO_3-extractable soil Cu (expressed in % of total soil Cu, R_L) depending on total soil Cu in the topsoil (0–0.11 m) and soil treatments; **(B)** modeling for the OMDL and OM2DL topsoils.

in the B1 plots and shoot Mg concentration in the B2 plots peaked for the OMDL plants, whereas shoot K and Zn concentrations were higher for the B2:OM2DL plants.

In all plots, shoot Ca and Mg concentrations were similar to common values for sunflowers (respectively 19–24 and 3–6.5 mg kg^{-1}), but with a lowest value for the UNT plants. For all amended plots, shoot Fe, Mn, and Zn concentrations were slightly higher than common values for sunflowers but remained in the common ranges for aboveground plant parts. Shoot P and K concentrations were lower than their common values for sunflowers.

DISCUSSION

Compared to year 1 (Kolbas et al., 2011), mean values of total topsoil Cu in year 10 (**Table 1**) slightly decreased in the B1 and B3 plots (i.e., by 7–21 and 13%, respectively) but these decreases were not significant as well as in the B2 plots (**Figure S5**). The potential cumulative effects of annual shoot Cu removal and Cu leaching for decreasing total topsoil Cu were not statistically evidenced in year 10. Total topsoil Cu still exceeded the pedogeochemical Cu background and screening values, notably for French sandy soil (**Table 3**; Saby et al., 2009). Spatial variability of total topsoil Cu, such as the "hot spot" at the edge of the GW plot, was mainly attributed to variability in cumulative wood washings resulting from long-term storage of preserved wood. Based on its composition (**Table 2**; Oustriere et al., 2016) and addition rate, Cu inputs by the green waste compost was negligible (4.3 mg Cu kg^{-1} soil, 1.9 g Cu plot^{-1}) regarding total topsoil Cu (0.4%). The ratio of total topsoil Cu vs. total subsoil Cu was in the 0.99–1.09 range for all plots, except for the B3:OM2DL plots where its value decreased to 0.82. This raises

TABLE 4 | Shoot DW yield, ionome, and Cu removal of sunflowers.

	Shoot DW yield	Copper			Shoot nutrient concentrations							
		Shoot Cu concentration	Shoot Cu removal		Ca	Mg	K	P	Fe	Mn	Na	Zn
	g plant^{-1}	mg kg^{-1}	mg plant^{-1}	g ha^{-1}	g kg^{-1}				mg kg^{-1}			
Block 3 and 4 (Total Soil Cu: 719–1,169 mg Cu kg^{-1})												
UNT	4 ± 1c	48 ± 10a	0.2 ± 0.1c	19 ± 8c	13 ± 2c	2.5 ± 0.2c	4.4 ± 0.9c	0.8 ± 0.1b	239 ± 63a	41 ± 11bc	155 ± 24c	44 ± 5b
GW	10 ± 4c	24 ± 7**b**	0.3 ± 0.1bc	26 ± 14bc	23 ± 3**b**	3.8 ± 0.6bc	14.7 ± 3.8**a**	1.7 ± 0.4**a**	89 ± 26**bc**	38 ± 6bc	252 ± 15**a**	80 ± 9**a**
OMDL	30 ± 10**b**	26 ± 5**b**	0.7 ± 0.2**a**	77 ± 23**a**	21 ± 2**b**	5.5 ± 0.8**a**	11.4 ± 3.4b	1.7 ± 0.7**a**	91 ± 21b	37 ± 6c	159 ± 35c	70 ± 13**a**
OM2DL	50 ± 21**a**	16 ± 5c	0.8 ± 0.5**a**	88 ± 55**a**	21 ± 3b	5.2 ± 1.5**ab**	10.8 ± 2.4b	1.6 ± 0.7**a**	64 ± 22c	45 ± 14bc	178 ± 33c	82 ± 16**a**
PLD	14 ± 10c	31 ± 4b	0.4 ± 0.2**ab**	42 ± 26**ab**	29 ± 2**a**	3.6 ± 0.2abc	12.4 ± 0.8**ab**	1.7 ± 0.4**a**	99 ± 17b	64 ± 3**a**	180 ± 4bc	41 ± 1b
CAR	14 ± 9c	27 ± 2b	0.4 ± 0.2bc	39 ± 25bc	23 ± 3b	2.6 ± 0.4c	13.3 ± 1.4**ab**	1.7 ± 0.4**a**	93 ± 30b	51 ± 11b	223 ± 57**ab**	43 ± 7b
Block 2 (Total Soil Cu: 257–556 mg Cu kg^{-1})												
OMDL	45 ± 17a	15 ± 6**a**	0.7 ± 0.4**a**	73 ± 42**a**	19 ± 3a	4.9 ± 0.9**a**	7.4 ± 2.3**a**	1.5 ± 0.5a	88 ± 30a	72 ± 24a	164 ± 46a	67 ± 13a
OM2DL	51 ± 22a	10 ± 2b	0.5 ± 0.3b	56 ± 29b	20 ± 3a	3.8 ± 1.1b	13.7 ± 3.9b	1.5 ± 0.6a	73 ± 20a	67 ± 26a	178 ± 38a	78 ± 17b
Block 1 (Total Soil Cu: 198–381 mg Cu kg^{-1})												
OMDL	48 ± 20a	14 ± 4**a**	0.7 ± 0.3**a**	70 ± 31**a**	21 ± 4a	4.2 ± 0.8a	12.5 ± 3.3a	1.7 ± 0.5a	109 ± 25**a**	59 ± 17**a**	190 ± 25a	89 ± 21a
OM2DL	43 ± 16a	10 ± 2b	0.5 ± 0.2b	47 ± 20b	19 ± 3a	4.5 ± 0.8a	11.1 ± 3.6a	1.9 ± 0.4a	72 ± 16b	47 ± 11b	196 ± 44a	80 ± 16a
Common values for sunflower (non-contaminated soils)												
(Kolbas et al., 2014)[a]	–	6	–	–	19.68	3.43	37.84	3	48	22	–	24
Unpublished data	–	10	–	–	24.2	6.5	29.5	4.3	57	37	–	38
(Blum et al., 2012)[b]	–	3–12	–	–	–	–	–	–	50–200	20–400	–	20–100
(De Maria and Rivelli, 2013)[c]	60–64	10 ± 0.5	–	–	–	–	–	–	–	–	–	23 ± 2

Mean value ± SD for each treatment ($n = 5$ for UNT, CAR, and GW; $n = 2$ for PLD; $n = 20$ for OMDL and OM2DL in each block). Block 3 and 4: values with different letters in a column differ significantly (one way ANOVA. p-value <0.05); Mean values followed by letters in bold are significantly higher or lower as compared to the UNT plants. Block 1 and 2: values with different letters in a column differ significantly (t-test, p-value <0.05).

[a]*Values from sunflowers grown in an uncontaminated soil.*

[b]*Common values in aboveground plant parts.*

[c]*Shoot DW yield and foliar Cu and Zn concentrations of sunflowers grown in an uncontaminated soil.*

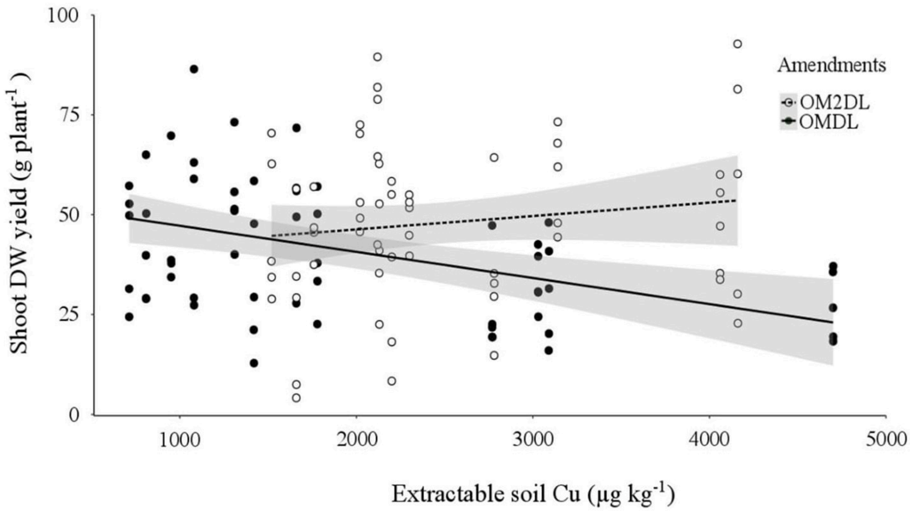

FIGURE 3 | ANCOVA analysis for shoot DW yield (g DW plant^{-1}) of sunflower plants depending on 1M NH_4NO_3-extractable soil Cu (mg Cu kg^{-1}) and soil treatments: OMDL (• and black lines) and OM2DL (○ and dotted lines) (n = 20 plants).

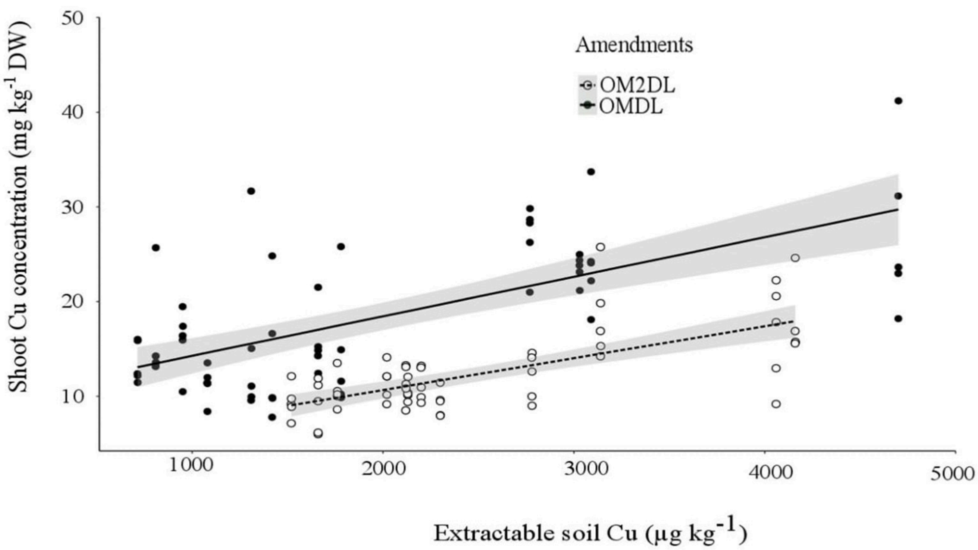

FIGURE 4 | ANCOVA analysis for shoot Cu concentration (mg kg^{-1}) of sunflower plants depending on 1M NH_4NO_3-extractable soil Cu (mg Cu kg^{-1}) and soil treatments: OMDL (• and black lines) and OM2DL (○ and dotted lines) (n=20 plants).

a question concerning a possible downward Cu migration with the organic matter, whose content was higher in the B3:OM2DL subsoils (**Table S1**). This may mark the green waste inputs in year 6 as total subsoil Zn was higher in all OM2DL and GW plots than in the OMDL plots (**Table S1**).

Total topsoil concentrations of other assessed metal(loid)s did not exceed their background levels (**Table 3**). Total topsoil Zn in year 10 was higher in all compost-amended plots as compared to the Unt and basic slag-amended plots, in line with the compost composition (**Table 2**), e.g., Zn inputs by the green waste compost in year 6 corresponding to 9% of total topsoil Zn. This was mirrored by increased shoot Zn concentration in plants from all compost-amended plots (**Table 4**), and notably in the B3 plots in contrast with plants from the Unt, CAR and PLD plots, likely also due to better root development and less Zn/Cu antagonism. Nevertheless, in year 1, total topsoil Zn already varied in the 35.2–98.4 mg Cu kg^{-1} range (Kolbas et al., 2011).

The NH_4NO_3-extractable Cu fraction for years 4, 6, and 10 did not vary in the B3:Unt plot, remaining in the 15–24 mg Cu kg^{-1} soil (**Figure S6**). This fraction was already lower in the B3:OMDL plots in year 4 and remained significantly lower in years 6 and 10 for both the B3:OMDL and OM2DL plots, with no influence of the number of compost dressing. For the B1 and B2 plots, the higher extractable Cu fraction in the OM2DL plots

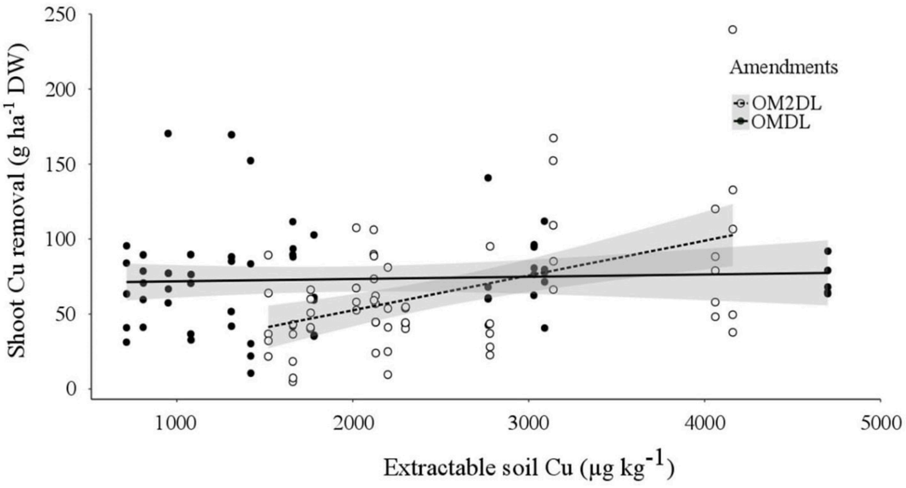

FIGURE 5 | ANCOVA analysis for shoot Cu removal (mg kg^{-1}) of sunflower plants depending on 1M NH_4NO_3-extractable soil Cu (mg Cu kg^{-1}) and soil treatments: OMDL (• and black lines) and OM2DL (○ and dotted lines) (n=20 plants).

than in the OMDL ones may be related to the dissolved organic matter (DOM) derived from the second compost dressing and the buffer effect of a higher total soil organic C (**Table 3**), whereas soil pH increase was similar in compost-amended soils, in line with compost pH and application of dolomitic limestone in year 1 (**Table 2**). In the B1 and B2 plots, the extractable vs. total soil Cu ratio was merely driven by the SOM. This might promote downward Cu migration in the OM2DL plots. Higher spatial variability in SOM and higher total soil Cu led to similar extractable Cu fractions in both OMDL and OM2DL topsoils of B3 plots. Increase in the SOM influenced less the extractable vs. total topsoil Cu ratio in the B3 plots (**Table S1**; **Figure 2** and **Figure S6**). Strong complexation of Cu with SOM and DOM is widely claimed (Ruttens et al., 2006; Kumpiene et al., 2008; Beesley et al., 2010; 2011; Karami et al., 2011; Park et al., 2011).

NH_4NO_3-extractable topsoil Cu in total data set (without accounting for soil treatments) was weakly correlated to total topsoil Cu ($R^2 = 0.5$, **Figure 2B**). Based on Hattab-Hambli et al. (2016), total dissolved Cu concentrations ($\mu g\ L^{-1}$) in the soil pore water of these contaminated soils increased with total soil Cu and available Cu concentration was generally low as compared to total soil Cu, i.e., <0.01% in year 3 and <0.007% in year 4. Accounting for spatial variability of total topsoil Cu, for the B1 and B2 plots, NH_4NO_3-extractable Cu was positively correlated to a cluster of soil parameters related to the SOM, i.e., total soil organic C, N, and Ca, clay content, soil CEC and pH, and available P (**Figure S7**); in contrast, at high total topsoil Cu (B3 and B4 plots), it was negatively correlated to total soil organic C, N, and Ca, clay content and available-P and positively to total soil Mn, Fe, and soil pH, showing the buffer effect of the SOM and the influence of basic slags (Le Forestier et al., 2017). In overall, this confirmed SOM, soil pH, and Fe/Mn oxyhydroxides as key players on Cu availability (Kumpiene et al., 2008, 2011; Bolan et al., 2014). The second compost dressing has improved other soil parameters that may enhance plant production, i.e., SOM, total organic N, available P, and soil CEC (**Table 3** and **Table S1**). Compost incorporation into the soil can enhance Cu sorption, microbial biomass, OM cycle, soil CEC, and water holding capacity (Clemente et al., 2005; Chiu et al., 2006; Asensio et al., 2013; Touceda-González et al., 2017).

Regarding C sequestration in the context of climate change, increases in total topsoil organic C varied from 40% (B1:OMDL) up to 150% (B3:OM2DL), respectively 10 and 5 years after the last compost dressing. To repeat compost dressing was beneficial as mentioned in Kidd et al. (2015). Based on soil organic C in years 1 and 10, compost composition and inputs (**Tables 2**, **3**, Kolbas et al., 2011), the apparent remaining rate of C inputs (including the contribution of annual root residues for all plots and winter crops for the OM2DL treatment) was 22% for the OMDL treatment and 55% for the OM2DL one, agreeing with the number of compost dressing. Increase in SOM had a positive effect on soil humidity (+28 to +132%, **Table 3**) and likely the soil structure, which was visually ameliorated in all OM2DL plots. The water holding capacity was positively correlated with the cluster of soil parameters related to SOM (i.e., soil CEC, total soil organic C, and N, etc.) and clay content (**Figure S7**). This is beneficial to face the more frequent heat waves and drought in Aquitaine, France, to promote the soil microbial community, sorption of exchangeable cations (notably in these sandy soils), and to limit soil erosion (Roy et al., 2016). According to Chenu et al. (2000), SOM increase from 1.7 to 4.3% (**Table 3**) would enhance the structural stability from instable to stable. Thus the combination of compost incorporation into the topsoil, winter cropping with clover and crop rotation with sunflower and tobacco can ameliorate the quality of these Cu-contaminated soils and potentially the crop production. Necrosis and chlorosis on leaves and reduced shoot DW yields reported for the B3:OMDL sunflower plants in year 1 (Kolbas et al., 2011) did not occur in year 9.

Basic slags are alkaline by-products of steel mills consisting of Ca, Al, Fe, Mn, and other metal oxides, which are used for soil liming, P fertilization and *in situ* metal immobilization (Mench et al., 1994; Bert et al., 2012; Negim et al., 2012; Le Forestier et al., 2017). The UNT soil was slightly acidic, while the PLD- and CAR-amended soils had higher pH values (**Table 3**) due to the liming effect (Le Forestier et al., 2017). At neutral pH, Cu tends to precipitate with carbonates and hydroxides dissolved from slags rather than adsorbing on the slag surface (Kim et al., 2008). Liming would also increase Cu sorption on native soil compounds. Consequently, the ratio of extractable vs. total topsoil Cu (R_L) decreased by 56% for CAR and 64% for PLD, but less than in the compost-amended soils (**Figure 2A**). This 1% (w/w) addition rate of PLD and CAR slags in the same Cu-contaminated soils increased Cu concentration in the residual fraction, reduced free ionic Cu concentration in the soil pore water and labile Cu pool measured by diffuse gradient in thin film (DGT), and lowered the Cu bioavailability (Le Forestier et al., 2017). In outdoor lysimeters, Cu leaching was lower in the PLD soils than in the OMDL and Unt soils (Marchand et al., 2011). As expected soil CEC and extractable P-Olsen were slightly higher in the PLD and CAR soils than in the UNT soil (**Table 3**), agreeing with previous findings (Bert et al., 2012). Total topsoil K, Mg, Na, and P were similar in all plots (**Table 3**), so amendment influence on those nutrients was not detected, may be due to the annual mineral fertilization. Phosphates might be less phytoavailable due to liming and sorption by Ca, Fe, and Al oxides in basic slag-amended soils, but in fact extractable P (Olsen) remained at least steady in these plots and its values were similar to those for the compost-amended plots (**Table 3**).

Morphological parameters of sunflower depend on several ecological factors including Cu exposure (Kolbas et al., 2014). Mineral and organic amendments, e.g., compost, alkaline materials and phosphate minerals, alone and in combination, can increase plant yields, and reduce plant exposure to Cu and Zn in metal-contaminated soils (Brallier et al., 1996; Su and Wong, 2004; Bes and Mench, 2008; Beesley et al., 2010). The Ethic cultivar used here was selected based on its high oleic acid content and Cu tolerance (Mench et al., 2013). In year 1, the shoot DW yield was in the 0.2–6.6 t ha^{-1} range depending on plots and commercial cultivars, and 4.5 t ha^{-1} in an uncontaminated soil of the same soil series (Kolbas et al., 2011). Here, in year 9, it varied between 0.42 and 5.35 t ha^{-1}, and peaked in compost-amended plots, except the GW one (**Table 4**). This increase ranged from 7- to 12-fold as compared to the Unt plot. Shoot DW yield reached its common ranges for sunflower in both OMDL and OM2DL treatments, except for the B3:OMDL plots (**Table 4**). In addition to nutrient supply, composts improve many soil characteristics, including soil structure and water retention, which can promote crop yields (Andersson-Sköld et al., 2014; Huang et al., 2016; Wiszniewska et al., 2016; Sharma and Nagpal, 2018). Plants grown in the OMDL and OM2DL soils of the B1 and B2 plots were less exposed to Cu than those in the B3 and B4 plots, reflecting both a lower total soil Cu and long-term influence of compost and liming. This confirmed compost amendments improved sunflower growth (**Table 4**; **Figure 3**), mirroring previous findings in pot experiments (Beesley et al., 2010; Jones et al., 2016). Liming influenced positively yields of sunflower biomass in other studies (Barman et al., 2014; Hajduk et al., 2017).

Lower shoot DW yield of sunflower plants in the GW plot (**Table 4**) may be explained by (1) a higher total topsoil and subsoil Cu than in the OMDL and OM2DL plots (**Table 3** and **Table S1**) and (2) a higher SOM and soil CEC that may buffer and resupply Cu in the soil solution, whereas the extractable soil Cu was similar in these plots (**Figure 2A**). In the B3 and B4 plots, shoot DW yield was significantly higher for the OM2DL plants than for the OMDL ones, demonstrating the benefits to repeat compost dressing in year 6 and of the cultivation of white clover as winter crop (**Table 4**). Shoot DW yield of OM2DL plants remained steady as extractable soil Cu increased, whereas it decreased for OMDL plants, suggesting that a regular compost supply would be suitable to produce a high shoot DW yield at high total soil Cu (**Figure 3**). Similar findings were obtained with lettuce cultivated in potted soils sampled in the plots in year 6 (Quintela-Sabarís et al., 2017).

Shoot DW yields of CAR and PLD plants were similar to that of the UNT plants (**Table 4**). In potted soils collected in year 5, dwarf bean growth was higher in the CAR and PLD soils than in the UNT soil (Le Forestier et al., 2017) confirming other findings (Negim et al., 2012). However, Linz-Donawitz slag incorporation into contaminated soils can reduce the mobility and phytoavailability of Cd, Zn, and Pb without increasing the plant growth (Mench et al., 1994), and total subsoil organic C and N were lower in the CAR and PLD plots than in the compost-amended ones (**Table S1**). Roots in field plots can also colonize the unamended subsoil. Moreover, unlike field trials, potted soils were managed at an optimal soil humidity for the root development, avoiding water stress and making a difference. One additional option to optimize crop production would be to irrigate, although it is somewhat in contradiction with one phytomanagement objective, which is saving resources for a sustainable land management.

Upper critical threshold values of shoot Cu concentration for most plants are (mg Cu kg^{-1}) 15–30 (MacNicol and Beckett, 1985) and 25–40 (Chaney, 1989), while common values for sunflower shoots are in the 6–12 range (**Table 4**). Here, shoot Cu concentrations peaked for the UNT plants (48 ± 10 mg kg^{-1}) and exceeded these upper critical threshold values (**Table 4**) but remained far lower than values required to produce Cu-ecocatalysts (i.e., 1,000 mg kg^{-1}) (Clavé et al., 2016). To detoxify and sequester high metal (Cu) amounts, plants need to spend energy, leaving less resources for growth, reproduction, and other processes (Audet and Charest, 2008; Maestri et al., 2010; Printz et al., 2016). For the UNT sunflower plants, decrease in shoot DW yield would indicate an increase in plant maintenance cost, but also a less developed root system. Nutrient deficiencies (i.e., Ca, Fe, K, Mg, and P) cannot explain this decrease in shoot DW yield as their concentrations in sunflower shoots were within the common ranges (**Table 4**; **Figure S8**), although some differences between the soil amendments were observed. Root and shoot DW yields of 1 month-old sunflower plants grown in our soil series decreased by 10% as total soil Cu respectively reached 252 and 323 mg kg^{-1} (Kolbas et al., 2014), which already occurred in the B1 and B2 plots (**Table 3**). These values were even lower using the fading technique, i.e., 74 and 166 mg Cu kg^{-1} soil (Kolbas et al., 2018).

Shoot Cu concentration generally mirrors root Cu exposure, but it depends on plant species and cultivars (Poschenrieder et al., 2001). All tested amendments significantly reduced shoot Cu concentrations to the common range for sunflower or slightly above (i.e., mg Cu kg^{-1}) from 10 for the OM2DL plants in the B1 and B2 plots to 31 for the PLD plants (**Table 4**). In year 1, shoot Cu concentrations varied from 5 to 68 mg kg^{-1} for the OMDL plants (Kolbas et al., 2011). Lowest shoot Cu concentrations of the OM2DL plants could be related to (1) soil factors, e.g., low Cu availability, high soil CEC, and higher total N, Ca, organic matter and water contents in the OM2DL soils (**Figure 4**, **Table 3**), and (2) plant factors, i.e., Cu dilution into the shoot biomass as for Fe (**Table 4**) and high shoot K, Zn, and Mn concentrations helping likely to regulate ion cellular homeostasis (**Table 4**; **Figure S8**, Malachowska-Jutsz and Gnida, 2015; Printz et al., 2016). For lettuce in year 6, shoot Cu concentration was also lower in the OM2DL plants than in the OMDL and Unt ones (Quintela-Sabarís et al., 2017).

Shoot Cu removal in year 9 (26–88 g Cu ha^{-1}) was higher in all amended plots than in the UNT plot (19 ± 8 g Cu ha^{-1}) due to the lower shoot DW yield of UNT plants (**Table 4**). In year 1, shoot Cu removal varied from 20 to 116 g Cu ha^{-1} (Kolbas et al., 2011). Shoot Cu removal by sunflower in year 9 was similar for both basic slag-amended soils, i.e., PLD 42 ± 26 and CAR 39 ± 25 g Cu ha^{-1} (**Table 4**). In contrast, for dwarf beans cultivated in potted soils collected in year 5, the PLD plants had a shoot Cu removal twice higher than the CAR plants due to their higher leaf biomass (Le Forestier et al., 2017). At high total topsoil Cu, shoot Cu removal peaked in both OMDL and OM2DL plots, as the OM2DL plants had a higher shoot biomass than the OMDL ones, but it was the reverse for shoot Cu concentration (**Table 4**). This reflected a Cu dilution in the sunflower shoots. As both agronomic options were relevant for progressively stripping bioavailable topsoil Cu, the remediation strategy will depend on site manager aims, i.e., to produce biomass and/or to remove Cu from the topsoil without the cost of compost amendment. However a regular compost supply promote many soil processes and underlying soil services.

To phytomanage the Cu-contaminated soils of this wood preservation site, organic matter such as compost should be regularly supplied to ensure the crop production taking advantages of nutrient supply and SOM properties including Cu complexation, amelioration of soil structure and water holding capacity. Even though Cu leaching was reduced after a single compost dressing (Marchand et al., 2011) attention should be paid to the following ones. Investigations on structural and functional diversity of soil microbial communities, micro- and mesofauna are ongoing. In the OMDL soils of the B3 plots collected in year 5, soil microbial biomass and respiration, and enzyme activities were consistently higher than in the Unt soils, with shifts in the bacterial community structure at both the total community and functional group levels (Touceda-González et al., 2017). Other soil functions, e.g., xenobiotic biodegradation, stability of soil aggregates, regulation of water run-off and soil erosion, can be investigated.

By 2020, 20% share of energy resources should be supplied by renewable energy sources to achieve the objectives set by the European Union (Directive, 2009/28/EC). This feedstock may essentially be provided by biomass production through energy crops and by using agricultural by-products and forest logging residues. Faced with the need for arable land to produce food resources, energy crop cultivation on marginal land is an option (Schröder et al., 2018). The biomass processing is a pivotal pillar of the phytomanagement concept. Here, even at high soil Cu exposure, shoot Cu concentrations of sunflower plants did not reach the values required for producing Cu-ecocatalysts used by the biosourced fine organic chemistry (>1,000 mg Cu kg^{-1}, Clavé et al., 2016). Use of mutant lines and plant inoculation with endophytic bacteria were however assessed to promote Cu tolerance and shoot Cu removal by sunflower, and root Cu concentrations of 1-month-old sunflower plants reached up to 2,000 mg Cu kg^{-1}, being suitable for producing Cu-ecocatalysts (Kolbas et al., 2015). Copper did not end up in oil and kernel Cu concentrations of sunflower plants grown in our field trial were in the range of permitted concentrations to feed cattle, so sunflower oil cake can be produced from our crops (Madejón et al., 2003; Kolbas et al., 2011).

Shoots and seed hulls can be merged with other lots and used by various sectors processing non-food crops such as (1) biorefineries for bio-oil (Casoni et al., 2015), biofuel (Ziebell et al., 2013; Zhao et al., 2016) and bioethanol (Dhiman et al., 2017) via the production of fermentable sugars (Ruiz et al., 2013; Liguori et al., 2016; Tavares et al., 2016), (2) bioconversion into branched-chain fatty acids (Dulermo et al., 2016) (3) solid fuel production (Alaru et al., 2013), (4) syngas and biogas production (Zabaniotou et al., 2010; Graß et al., 2013; Hesami et al., 2015), (5) energy production by co-firing with coal (Kułazynski et al., in press), (6) organic fertilizers for marginal land and Cu-deficient soils as compost or biochar amendment (Evangelou et al., 2015; Colantoni et al., 2016; Saleh et al., 2016); and (7) insulation eco-material and biocomposites (Mati-Baouche et al., 2016; Liu et al., 2017; Brouard et al., 2018).

Crop rotation is mandatory for sunflower cultivation in France (CETIOM, 2011). Here, the phytomanagement is based on a sunflower-tobacco crop rotation. Data for tobacco will be reported in a companion paper. Other crop rotations were assessed including energy and biomass sorghum, but both plant species were too sensitive to water stress and Cu excess (Kolbas, 2012).

AUTHOR CONTRIBUTIONS

MM mainly drafted the paper, made the hypotheses, implemented and managed the field trial, and took the measurements. CB contributed to drafting the paper and implementing and managing the field trial. NO carried out the statistical analysis, assisted in taking measurements, and contributed to drafting the paper, implementing and managing the field trial. LM contributed to drafting the paper, the analysis of statistics, the implementation and management of the field trial, and took the measurements. AK and MD contributed to drafting the paper, implementing and managing the field trial, and worked on taking the measurements. PL contributed to drafting the paper and producing the P-spiked basic slags.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00123/full#supplementary-material

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Tobacco, Sunflower and High Biomass SRC Clones Show Potential for Trace Metal Phytoextraction on a Moderately Contaminated Field Site in Belgium

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Phytoextraction could be a potential management option for diffusely Cd-Zn-Pb-polluted agricultural land in Northeast Belgium. The use of high yielding crops with a sufficiently high metal accumulation is preferred as these are expected to both gradually decontaminate the soil while generating an income through biomass valorization. To find out which high biomass crop possessed the highest and most constant (in time) phytoextraction potential on these soils, different plant species and different mutants or clones of each species, were evaluated during consecutive years. Biomass production and metal accumulation of pre-selected tobacco somaclonal variants (*Nicotiana tabacum* L.) and pre-selected sunflower mutants (*Helianthus annuus* L.) were investigated for two productivity years, while the phytoextraction potential of experimental poplar (*Populus*) and willow (*Salix*) in short rotation coppice (SRC) was assessed at the end of the second cutting cycle (after two times four growing seasons). The tobacco clones and the sunflower mutants showed efficient extraction of, respectively, Cd and Zn, while the highest simultaneous extractions of Cd and Zn were gained with some SRC clones. Variation in biomass production and metal accumulation were high for all crops over the years. The highest biomass production was observed for the experimental poplar clone of the crossing type *Populus deltoides* (*P. maximowiczii x P. trichocarpa*) with 9.9 ton DW per ha per year. The remediation period to reach legal threshold values for the pseudo-total content of Cd in this specific soil was estimated to be at least 60 years. Combining estimated phytoextraction potential and economic and environmental aspects, the SRC option is proposed as the most suitable crop for implementing metal phytoextraction in the investigated area.

Keywords: phytoextraction, metal, cadmium, zinc, short rotation coppice, sunflower, tobacco

INTRODUCTION

In the northeast of Belgium, an area of about 280 km^2 is historically polluted by mainly cadmium (Cd), zinc (Zn) and lead (Pb) (Vangronsveld et al., 1995; Hogervorst et al., 2007). The negative impacts on inhabitants and the environment in general as well as economic losses in the agricultural sector urged regional policy makers to endorse remediation of the metal-polluted soils. Given the vastness of the area and the diffuseness, moderation and shallowness of the pollution, phytoextraction, using plants to extract metals from the soil and accumulate them in harvestable biomass, is proposed as a suitable remediation option (Ruttens et al., 2011). More specifically, cultivating non-food high biomass crops with moderate metal accumulation capacity is promising for this area as these crops result in both a gradual soil depollution by extracting metals as well as in an alternative income for the farmers.

Woody plants such as willow and poplar have been the topic of research over the past years for soil trace metal remediation (Mench et al., 2010; Courchesne et al., 2016). They are fast growing tree species often grown in short rotation coppice (SRC) for bioenergy production (Mola-Yudego et al., 2015). These species can also accumulate high concentrations of available metal(loid)s (Dickinson et al., 2009; Ruttens et al., 2011). In a number of European countries, phytoextraction of soil trace metals by willow and poplar was investigated during phytoremediation of metal-polluted soils, e.g., in Sweden (Perttu and Kowalik, 1997; Klang-Westin and Eriksson, 2003), Poland (Landberg and Greger, 1996; Perttu and Kowalik, 1997), France (Robinson et al., 2000), Denmark (Jensen et al., 2009), Switzerland (Hammer et al., 2003; Rosselli et al., 2003), the Czech Republic (Fischerová et al., 2006; Zárubová et al., 2015) and the United Kingdom (Dickinson and Pulford, 2005; French et al., 2006; Maxted et al., 2007). Although, overly the studies point to high variation in metal accumulation between the genera, and also at species and cultivar level, indicating that selection for metal accumulation and resistance traits is an important strategy to make differences in phytoextraction efficiency in the long term, though more data are needed to support this. Besides plant genetics, environmental factors such as soil pH, nutrient level, pollution concentrations, and soil microbiota differ widely between field sites, which makes a direct site-to-site comparison difficult. To account for these site-specific effects and to discriminate which are some of the most influential factors, knowledge can only be gained through more field studies.

In contrast to SRC, a fewer number of studies have looked into the potential of tobacco or sunflowers for metal extraction potential, though the studies that are there show promising results. For example, the capacity of *Nicotiana tabacum* L. to extract Cd from soil was reported in the early onsets of phytoextraction field experiments, by Mench et al. (1989). In this experiment soil Cd enrichment (5.4 mg Cd kg^{-1}) invariably increased the Cd concentrations in plant parts, which varied from 10.1 to 164 mg kg^{-1} dry weight (DW). Moreover, they showed that between 75 and 81% of total Cd taken up by the plant was transported to the leaves, irrespective of the Cd level in the soil. Guadagnini (2000) also mentioned excellent Cd accumulation properties and a high biomass productivity for tobacco. Its metal extraction potential was further investigated at field scale in different countries (Vangronsveld et al., 2009; Fässler et al., 2010; Herzig et al., 2014). Sunflower (*Helianthus annuus* L.) is a bioenergy plant able to accumulate high amounts of several metals in its aerial tissues, gaining growing interest for phytoremediation purposes (De Maria and Rivelli, 2013; Kötschau et al., 2014). Phytoextraction of metal-polluted soils using sunflower was investigated before in pot trials (De Maria and Rivelli, 2013; Rivelli et al., 2014; Zalewska and Nogalska, 2014) and at field scale (Nehnevajova et al., 2007, 2009; Fässler et al., 2010; Herzig et al., 2014; Kötschau et al., 2014).

There still exist many reservations concerning the longer-term effectiveness of phytoextraction (Dickinson et al., 2009). Extrapolations of phytoremediation efficiency based on hydroponic and pot experiments are often unrealistic (Vangronsveld et al., 2009) and long lasting experiments at field scale are scarce (Dickinson et al., 2009). A large-scale field experiment in the polluted Campine region, in the Northeast of Belgium, dates back to 2006 (Ruttens et al., 2011; Van Slycken et al., 2013b, 2015) and offers a unique opportunity to investigate some aspects of this concern. Evaluation of high biomass crops on the metal-polluted field in Belgium was also part of the Greenland EU project (FP7-KBBE-266124)[1].

This manuscript evaluates the phytoextraction potential of the above-mentioned high biomass crops based on longer-term field data, in the Campine region in Belgium. More specifically this paper addresses differences in biomass production and metal accumulation between pre-selected tobacco clones, sunflower mutants, and experimental poplar and willow clones, as well as variations throughout different years where possible. Annual crops (tobacco and sunflower) were cultivated for subsequent years, and results are reported over the years 2012–2014, while woody crops were examined after four growing seasons in 2011. Our data, despite the large variation in biomass and accumulation for all crops, show significant differences between plant genera, within mutants and clones, as well as over the years.

MATERIALS AND METHODS

Site Description

The Cd-Zn-Pb-polluted experimental field is located in Lommel, Belgium (51°12′41″ N; 5°14′32″ E). The site is a former maize field, taken out of production since 1999 and situated 500 m NE of a Zn smelter. The soil is a sandy soil (88% sand, 8% silt, 4% clay) with a pH of 4.6–5 (Meers et al., 2007b). The zinc smelter had its major environmental polluting production processes until the 1970s. During this period, metal slags were dumped in the soil around the factory and in the wider neighborhood. This study is part of a larger phytoremediaton experiment (10 ha) set up in 2006 as a collaboration between Hasselt University, Ghent University and the Research Institute for Nature and Forest (INBO) in Belgium (Van Slycken et al., 2013b). The field was subdivided into zones, and blocks for the different plantations,

[1] http://www.greenland-project.eu/

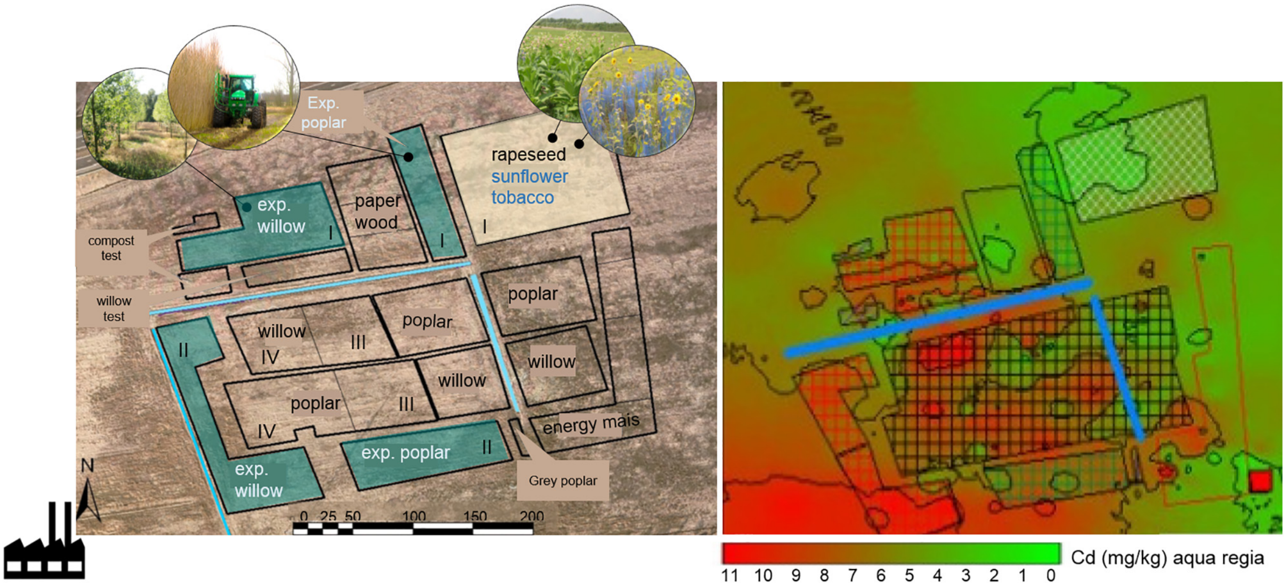

FIGURE 1 | Scheme of the experimental field site in Lommel, and overview of the total Cd concentrations. The location of the sunflower and tobacco plot (2012–2014) analyzed in this study is highlighted in yellow (block I). The installed experimental willow and poplar plots (blocks I, II) are indicated in blue, the commercial willow and poplar plots were located in blocks III and IV (Van Slycken et al., 2015). Total Cd concentration map is based on an aqua regia destruction of subsurface (0–30 cm) collected soils (*n* = 47, year = 2006) (Van Slycken et al., 2015).

TABLE 1 | Average pH-H_2O, total Cd, total Zn, and $CaCl_2$ extractable Cd and Zn concentrations in the topsoil (0–30 cm) of the field site.

Block	pH-H_2O	Aqua regia		$CaCl_2$ extractable conc.	
		Cd mg kg^{-1}	Zn mg kg^{-1}	Cd mg kg^{-1}	Zn mg kg^{-1}
Low (I)	6.78 ± 0.13	4.05 ± 0.83	234.3 ± 37.9	0.21 ± 0.06	11.15 ± 5.12
Low (II)	6.3 ± 0.3	6.1 ± 2.2	344 ± 128	0.46 ± 0.18	3.9 ± 5.6
Mod (III)	6.5 ± 0.2	8.0 ± 1.6	445 ± 93	0.50 ± 0.12	2.2 ± 1.3
Mod-high (IV)	6.4 ± 0.2	8.4 ± 1.9	488 ± 97	0.59 ± 0.12	3.0 ± 2.5

Values are mean ± SD of eight independent replicates.

2 ha for annual crops, and the remaining 4 ha was reserved for SRC (**Figure 1**).

Total metal concentrations throughout the field are very heterogeneous with hot spots of pollution dispersed across the site (**Figure 1** and **Table 1**). The highest pollution concentrations are in the SW direction away from the former Zn smelter. The plantation plots are spread across the field (**Figure 1**).

The sunflower and tobacco experimental field plot is located in the NE, block I (marked in yellow, **Figure 1**). The measured pseudo-total Cd concentrations ranged from 3.9 to 4.56 mg kg^{-1} DW soil and $CaCl_2$ extractable Cd concentrations ranged from 0.2 to 0.34 mg kg^{-1} (**Table 1**). The cation exchange capacity (CEC) of this plot was on average 7.1 ± 0.7 meq per 100 g soil and the electrical conductivity (EC) was 54.24 ± 5.86 μS cm^{-1}. Average Pb concentrations were 141.75 ± 16.3 mg kg^{-1} with $CaCl_2$-extractable concentrations of 0.15 ± 0.04 mg kg^{-1}. For Zn and Cd concentrations see **Table 1**. For this field plot, the pseudo-total Zn and Pb concentrations in the soil were lower than the remediation thresholds in Flanders (respectively, 282 and 200 mg kg^{-1} DW soil). The pseudo-total Cd-values exceeded the threshold value for an agricultural soil in Flanders (2 mg Cd kg^{-1}).

The experimental willow and poplar clones were planted in 2006, and they were distributed over blocks I and II (**Figure 1**). Total Cd and Zn concentrations were higher for block II, closer to the Zn smelter than block I (**Table 1**). The pH-H_2O for these areas is between 5.6 and 6.7, and pH-KCl of 5.5–6.3 (Ruttens et al., 2011).

Climate Data and Field Maintenance

Climatological data for the cultivation period of tobacco and sunflower (June-July-August) for the years 2012 until 2014 are given in **Supplementary Table S1**. Compared to the normal values, i.e., mean climatological values for the 30-year period 1981–2010, some deviations were found for the year 2013. Relative air humidity, total rainfall and total days of rain were lower than normal (respectively, 7, 25 and 36% lower) while total hours of sunshine was higher (13%). Furthermore, the mean wind direction (NNE) was different from normal (SW) and less common in general in Belgium. For 2012 and 2014, differences compared to normal values were observed for total rainfall and total days of rain, which were higher than normal. The exceptional dry summer also had an effect on the plants, mainly the tobacco clones showed a significantly lower yield in 2013 and suffered the most from the drought, so we left this year out of the analyses.

Field Plantation

Tobacco and Sunflower Set Up and Harvest

The plantations of tobacco and sunflower were started in 2011 and were followed up till 2014. Seeds of pre-selected *in vitro* bred tobacco clones (*N. tabacum* L. sp.) and mutant lines of sunflower (*H. annuus* L. sp.) were provided by Phytotech Foundation (PT-F) in Bern (Switzerland). Two tobacco somaclonal variant lines were tested: mother clone BAG (*Badischer Geudertheimer*) and its promising descendants NBCu104 and NBCu108 selected for higher metal accumulation and tolerance. The other tobacco line was the mother clone FOP (*Forchheim Pereg*) and derivatives NFCu715 and NFCu719. Second and third generation descendants of each of the selected clones were tested in subsequent years. Sunflower mutants belonged to three mutant line families (15-35-190-04, 86-35-190-04 and 14-185-04), all resulting of chemical mutagenesis for metal tolerance, of inbred line IBL04. From the fifth (M5) up to the eighth (M8) generation of different sunflower mutants were evaluated.

Seeds of the plants were germinated in the greenhouse under controlled conditions (day temperature 22°C, night temperature 18°C, air humidity 60%, photoperiod 15 h). This was done mainly to avoid the seed loss due to foraging rabbits or birds. Three week old seedlings were transferred to pots, acclimatized outside in the shade, before planting in the field.

Initially at the start of the field experiment, the soil was rototilled. For tobacco, a planting distance of 80 cm was chosen in 2012 (15,625 plants ha^{-1}) while this was 60 cm in 2013 and 2014 (27,778 plants ha^{-1}). 20–45 replicates were planted per clone. For sunflower, a planting distance of 25 cm in the row and 40 cm between the rows was followed (respectively, 166,667 plants ha^{-1} or 100,000 plants ha^{-1}). 20–100 replicates per mutant were planted. In total the non-overlapping plots of sunflower and tobacco were 1,500 m^2 each (**Figure 1**).

For maintenance, yearly, "Champions Blend" (Kooter B.V, Netherlands) was applied between the plans at a dose of 5 m^3 per 100 m^2 and mixed with the topsoil layer, to improve overall soil texture and water retention capacity. In 2014, the plot was treated with a glyphosate-based herbicide to remove all weeds and grasses.

Aboveground fresh weight (FW) production and height (H) of all tobacco and sunflower clones was determined on the field directly after harvest. A group of 10 plants per clone/mutant was chosen with a representative FW compared to the overall clone/mutant FW. All selected sunflower and tobacco plants were chipped individually using a garden chipper and chips were air-dried until constant weight (about 2 months). Thereafter, aboveground DW production was determined. DW production of the other, non-selected plants was estimated based on the regression equation expressing the FW-DW relationship of selected plants. In 2013, mass of produced sunflower seeds was also measured.

SRC Set Up and Harvest

In total 100 experimental poplar clones from 42 different families, and 160 experimental willow clones from 11 families were selected, produced by INBO, Belgium. The experimental poplar clones can be divided in three groups: *Populus trichocarpa* {T} clones, intraspecific crossings of *P. trichocarpa* × *P. trichocarpa* {T × T} and crossings of *P. trichocarpa* × *P. maximowiczii* {T × M} including two backcrossings to *P. deltoides* {D (T × M)}. The experimental willow clones can also be summarized in three groups: a *Salix alba* {A} group with purebred *S. alba* and intraspecific crossings of *S. alba* with *S. alba/S. rubens/S. fragilis*, a *S. viminalis* {V} group and a third group comprising crossings of *S. viminalis* × *S. viminalis* {V × V} derived from the second group. In April 2006, cuttings (20 cm) of all clones were planted on the experimental field in a twin row design with a row distance of 0.75 m between twins and 1.5 m between twin rows. For each tested experimental poplar and willow clone, 25–50 trees were planted in blocks in duplex repetition with planting distances of 90 (poplar) and 60 (willow) cm.

Plants were harvested in 2014 after a second 4-year growing season using the harvester "Stemster" from the Danish firm Nordic Biomass. It cuts the stems of twin rows close to the ground using two circular saws. For each clone biomass, 10 replicates were sampled and samples were collected from the stem, bark, and leaves. In the lab, stem and leaf tissue were separated and dried at 105°C to calculate DW of each of the fractions. In between the years also non-destructive plant biomass recordings were performed (Van Slycken et al., 2015). Because of the size of the harvesting machine, from each poplar and willow clone only one plot was harvested. Prior to harvest, height and diameter was determined of 20 representative plants per clone. The DW biomass data were used to calculate the biomass production at ha level, and expressed as productivity per tree according to Van Slycken et al. (2013b).

To collect samples for metal extractions, a mixed sample was used collected from six surrounding trees (mixture sample of ±200 g FW per clone), around each sampling location. In total, we chose five sampling locations for each clone dispersed over the field. The plants were defoliated, and divided into shoot, leaves and wood (stem, bark). On all plant fractions destructive analyses were performed to determine concentrations of Cd and Zn. Also, at the same moment soil samples were collected (0–25 cm) using a soil corer and analyzed for pH, and pseudo-total and $CaCl_2$ extractable concentrations, as described above.

Plant Metal Extractions and ICP-OES

All chipped and dried plant material was individually hammer-milled (Retsch SM100) to obtain a fine powder. To determine total Cd, Zn and Pb concentrations in the biomass, this powder was wet-digested in Pyrex tubes in a heating block. The digestion consisted of three cycles in 1 mL HNO_3 (70%) and one cycle in 1 mL HCl (37%) at 120°C for 4 h. Samples were thereafter dissolved in HCl (37%) and diluted to a final volume of 5 mL (2% HCl) with Millipore water.

The extracts were subsequently analyzed with inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies 700 Series). All samples were examined at least in triplicate. Blanks and certified reference material® (trace elements in spinach, Standard Reference Material 1570a, National Institute of Standards and Technology, USA Department of Commerce) were included for quality control of the data. For pseudo-total metal concentrations, a reference soil (CRM 143 R Sewage Sludge Amended Soil, Community Bureau of Reference—BCR N° 230) was included for confirmation of the analysis.

Soil Analyses

Soil samples were analyzed for physico-chemical parameters and total metals. Soil was oven-dried (60°C) and sieved (<2 mm). pH-H_2O and pH-KCl were determined after 1 h of equilibration (120 rpm) with, respectively, deionized H_2O and 1 M KCl in a 1:5 (w:v) solution. EC of the soil was determined using a conductivity meter (WTW LF340) and measured after 1 h of equilibration (120 rpm) with deionized H_2O in a 1:5 (w:v) ratio. The effective CEC_e was calculated as the sum of cations (Ca/20+Mg/12+K/39+Al/9, cations in mg L^{-1}) extracted by 1 M NH_4Cl (Gillman and Sumpter, 1986). A 1:10 (w:v) extraction solution was shaken for 2 h (120 rpm) and cations present in the extract were measured using ICP-OES. Pseudo-total metal (Cd, Zn and Pb) concentrations of the soil samples was estimated by *aqua regia* digestion (Van Ranst et al., 1999). For this, 0.5 g of oven-dried soil was microwave digested in a HNO_3-HCl solution (1:3 v:v) at 160°C (25 min ramp time, 10 min ventilation). $CaCl_2$-extractable concentrations were determined in a 1:5 (w:v) extraction ratio as described previously (Van Ranst et al., 1999).

Calculation of Metal Extraction Potential

The extraction potential was defined as the amount of metals removed from a soil and calculated based on the amount of metals accumulated in harvestable plant parts per unit of area and time. For tobacco and sunflower, the extraction potential was estimated by multiplying the mean above-ground DW production of the clone/mutant (kg ha^{-1} $year^{-1}$) with the mean Cd, Zn and Pb concentrations in the evaluated plants (mg kg^{-1}). For every poplar and willow clone, the extraction potential was calculated by multiplying yearly stem production with the concentration of metals in the woody biomass and expressed in g ha^{-1} $year^{-1}$. As independent variables, also the effect of the block, crossing type, and Cd-concentrations in the soil was determined.

Bioconcentration Factor and Hypothetical Remediation Time

For comparing phytoextraction efficiencies of tobacco and sunflower, bioconcentration factors (BCFs) were calculated. The BCF, defined as the ratio of metal concentration in above-ground biomass to (local) total soil metal content in the soil, allows to compare extraction efficiencies of crops even on different pollution levels. Results of the same clones/mutants (disregarding the generation) were averaged over the tested years to obtain more representative means.

For the best performing clones/mutants of the investigated species, hypothetical remediation periods were calculated to reduce pseudo-total Cd, Zn and Pb concentrations measured at the location of the tobacco and sunflower plots of 2012–2014, and in the middle of the field where poplar and willow was growing, respectively, referred to as moderate pollution levels, to pseudo-total remediation thresholds. For Cd, the time required to decrease the $CaCl_2$-extractable (bioavailable) fraction to an assumed reference value for Flanders (based on values obtained for non-polluted soils) was also calculated.

Statistical Analyses

Statistical analyses were performed in R 3.1.3 (R Development Core Team, 2013). The effect of tobacco clone/sunflower mutant and year on the biomass DW production per plant and Cd, Zn, and Pb concentrations in the biomass was analyzed using ANOVA. The QQ-plots were used to examine normality of the residuals. In case of non-normality, transformations of the outcome (logarithmic, inverse, square root, exponential) were performed. When an indication of non-normality was present for all these transformations, a Box-Cox was used. All decisions about the transformations of the outcomes were taken *a priori*. Model-robust SEs were used in all analyses due to potential differences in the variance of the outcome for different clones/mutants and years. Since interaction between clone/mutant and year was present in all analyses, the differences between clones/mutants for each year and the differences between years for each clone/mutant were analyzed separately. Two-by-two comparisons were conducted using Tukey correction for multiple testing.

RESULTS

Tobacco: Biomass Production, Metal Accumulation and Extraction Potential

Above-ground DW biomass production per plant differed significantly between the years (**Figure 2**). For example, mean above-ground DW production of a BAG plant varied from 150.68 ± 47.87 g in 2014 to 336.04 ± 75.13 g in 2012. Within 1 year, e.g., 2012, the highest mean above-ground biomass was observed for NBCu108 (328 ± 76) and its mother clone BAG (336 ± 75) which performed significantly better than FOP (238 ± 77) and its descendent clone NFCu715 (200 ± 75). In 2014, a different pattern was observed, with the highest biomass producing clone FOP, followed by NFCu719, and then the BAG clone with its derived clones NBCu108 and NBCu104.

When comparing metal concentrations per plants over the years, a variation in the best performing clones could be observed. For 2012, the plants with the highest metal concentrations of Zn and Pb were the clones with the lowest biomass. This is not so clear for 2014, as the highest biomass plant, FOP, also had the highest Pb concentration in the tissues. In 2014, NBCu108 had higher Zn and Cd concentrations in the aboveground tissues than its mother clone BAG, whereas this was not significant in 2012. FOP and its derived NFCu719 performed similar in

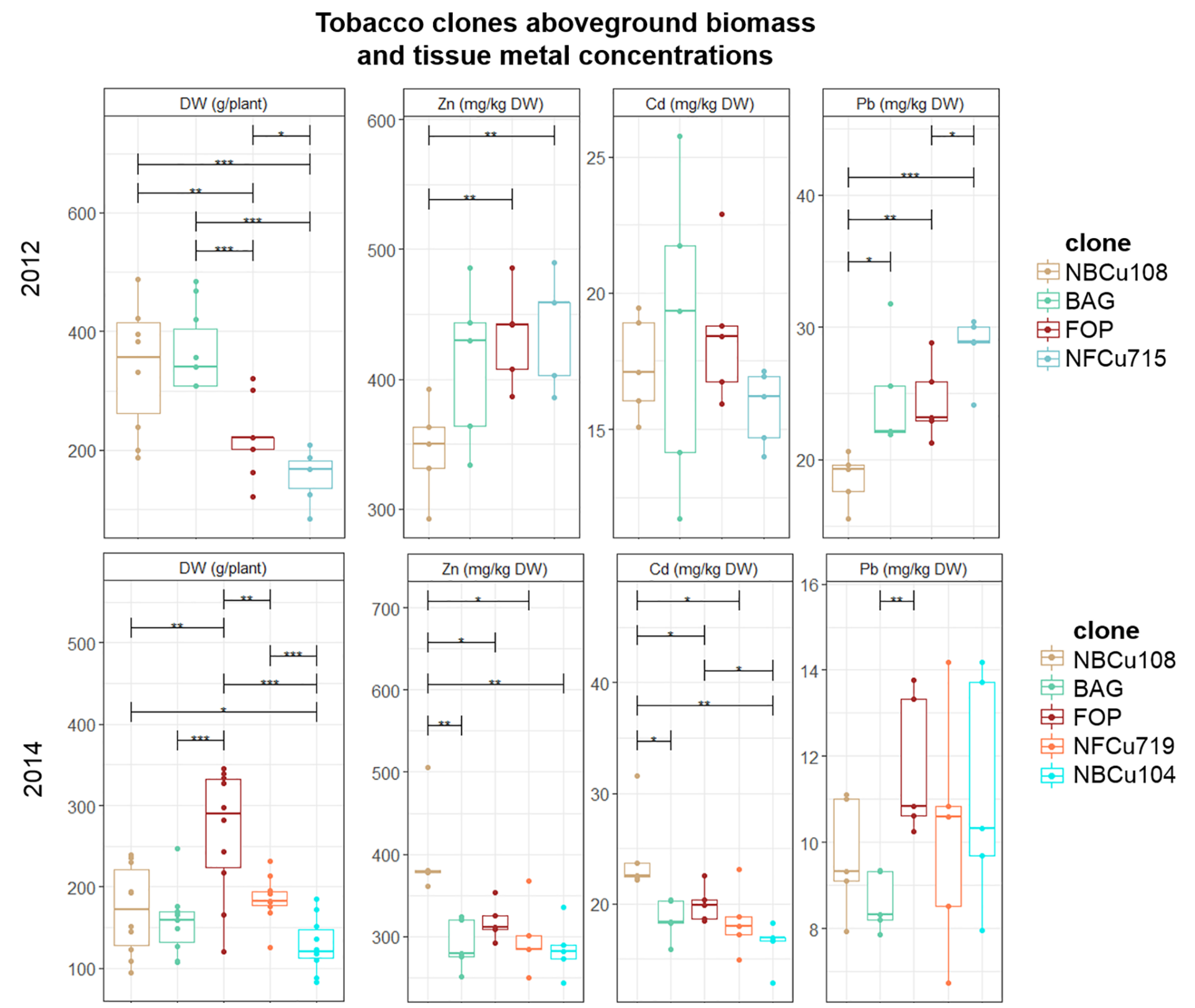

FIGURE 2 | Mean aboveground DW production per plant (g) of *in vitro* bred tobacco clones tested in 2012 and 2014. Error bars are SEs (n = 10). Different colors represent the different clone, ranked according to highest mean above-ground biomass per year. BAG is the mother clone of NBCu clones while FOP is the mother clone of NFCu clones. Stars represent significant differences at the level of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) between clones within a year (ANOVA, Tukey-HSD). No significant differences were detected between the years for each clone (*t*-test).

Zn, Cd and Pb extraction in 2014. Only in 2012, NFCu715 extracted significantly more Pb than FOP. BAG and NBCu104 also performed similar in Zn, Cd and Pb extraction, not different from FOP and NFCu719 in 2014.

Sunflower: Biomass Production, Metal Accumulation and Extraction Potential

Since rabbits consumed all sunflowers planted in 2012, no results were available for that year. Of all tested sunflower mutants grown, mean height varied between 106 cm (2014: 15-35-190-04 M8) and 147 cm (2013: 14-185-04 M5). Above-ground DW production per plant was similar in 2013 compared to 2014, but within each year statistical differences between the mutants could be observed. For example, mutant 15-35-190-M8 showed significantly lower production compared to 14-185-04-M5 and IBL04, but in 2014 they were not statistically different from each other. Here, 15-35-190-M8 showed higher productivity compared to 86-35-190-04-M8 and 15-35-190-04-M7. Across both years, the mutant showing the highest biomass was 14-185-04-M5 with on average 116 ± 51 mg kg^{-1} DW and 82.7 ± 33 mg kg^{-1} DW.

Cadmium and Zn concentrations in above-ground biomass were significantly higher in 2014 compared to 2013 for the mother line IBL04, mutant 14-185-04 M5 and 86-35-190-04 M8 (**Figure 3**). For the mutant line family 15-35-190-04, similar shoot concentrations of Cd and Zn were found. For most of the mutants, shoot Pb concentrations did not differ considerably

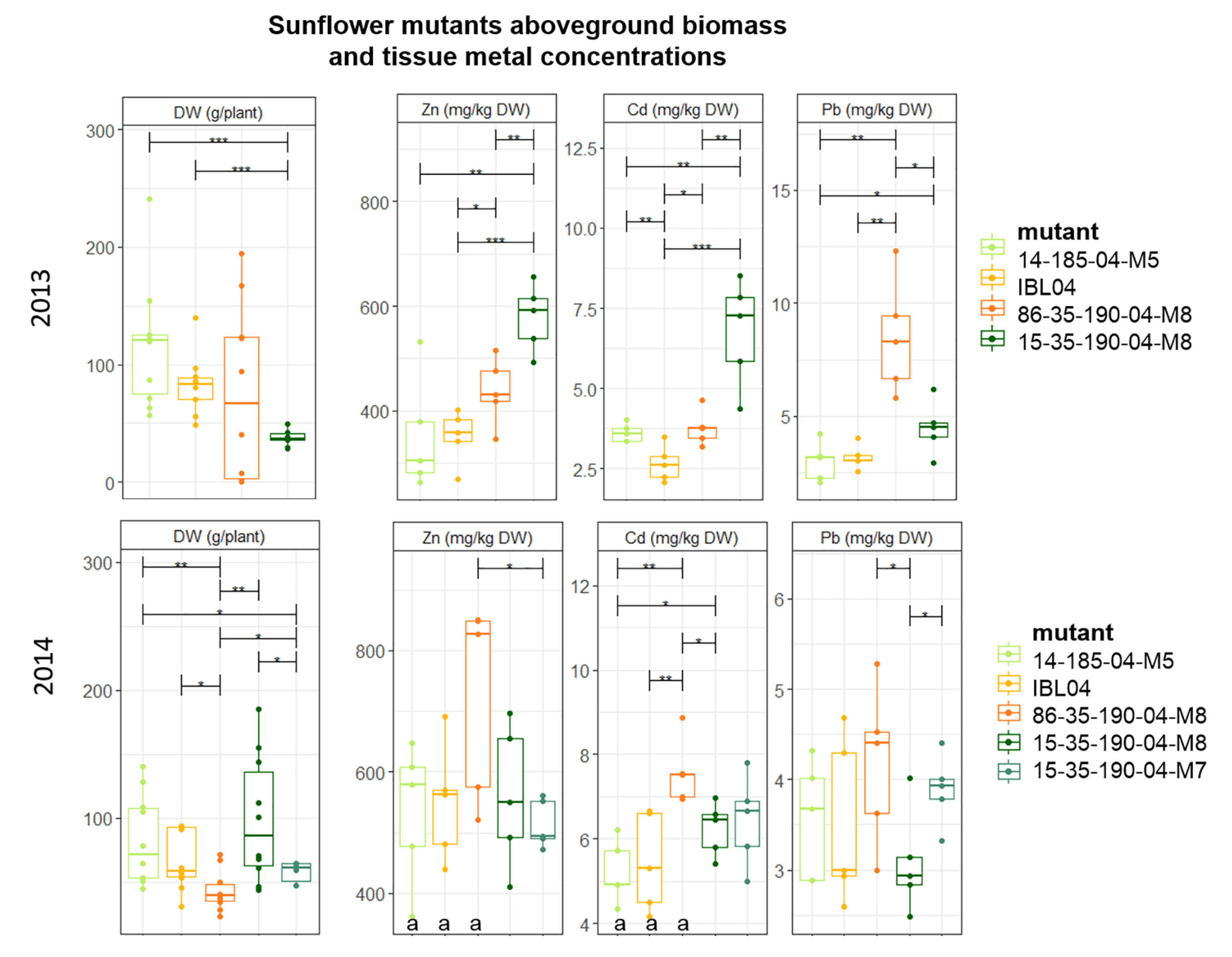

FIGURE 3 | Mean aboveground DW production per plant (g) of sunflower mutants tested in 2013 and 2014. Error bars are SEs (n = 10). IBL04 is the mother line of all mutants. Stars represent significant differences at the level of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) between clones within a year (ANOVA, Tukey-HSD). Significant differences between the years for each clone are denoted with the letter a ($p < 0.01$, t-test).

between 2013 and 2014. In 2013, the mutant 15-35-190-04 M8 showed significantly higher concentrations of Cd (6.76 ± 1.4) and Zn (578 ± 57) in above-ground biomass compared to IBL 04 (2.65 ± 0.5 mg kg^{-1} Cd and 350.9 ± 45.6 mg kg^{-1} Zn) and other derivatives. However, in 2014, this result was not repeated.

In addition to the metal concentrations measured in the above-ground leafy and stem tissue, we measured mean seed yield for the year 2013, next to Cd, Zn and Pb content in one mixed seed sample per mother line or mutant. In all cases, Pb values were below the detection limit. The average Cd and Zn concentrations measured ranged from 0.96 to 1.95 mg Cd kg^{-1}, with the highest for 86-35-190-04 M8 (1.95) followed by 15-35-190-04 (1.42), IBL04 (1.34), and the lowest for 14-185-04 (0.96). Zn concentrations in seeds ranged from 64.28 to 111.16 mg Zn kg^{-1} with the highest Zn concentrations in IBL04, followed by 14-185-04 (87.25) and the other two mutants had on average 65.31 mg Zn kg^{-1}.

Metal BCF of Tobacco and Sunflower

The BCF of tobacco and sunflower was based on the local soil pseudo-total metal contents. From **Table 2**, it can be observed that for Cd and Zn, BCFs were almost always >1, while this was not the case for Pb. The BCF of Cd was the highest for tobacco, while the BCF of Zn was highest for sunflower. Overall, the BCF of Pb for tobacco and sunflower was very low (≤0.10).

SRC: Biomass Production, Metal Accumulation and Extraction Potential

Biomass production and Cd and Zn concentrations in the stems of poplars and willows varied to a great extent from clone to clone and no clear distinction was observed between poplar and willow (**Figure 4**). Because of heterogeneity of Cd and Zn concentrations in the field, results were analyzed for the clones growing within one block, harvested in their fourth growth year. For block I, highest productivity was recorded for poplar crossing

TABLE 2 | Mean BCF of Cd, Zn and Pb for tobacco clones and sunflower mutants.

Species	Clone/mutant	Cd (min-max)	Zn (min-max)	Pb (min-max)
Tobacco	BAG	2.8–6.3	1.1–2.1	0.06–0.2
	NBCu108	3.7–7.9	1.2–2.2	0.06–0.1
	FOP	3.9–4.5	1.2–1.8	0.07–0.2
	NBCu104	2.1–3.8	0.8–1.2	0.05–0.07
	NFCu719	3.6–5.7	1.06–1.5	0.04–0.2
Sunflower	IBL 04	0.5–0.9	1.14–2.3	0.01–0.03
	15-35-190-04	1.0–2.1	2.1–2.8	0.02–0.03
	86-35-190-04	0.9–1.9	1.5–2.4	0.04–0.08
	14-185-04	0.8–0.9	1.1–2.3	0.01–0.02

BCF values are based on in planta metal concentrations expressed in ranges (min-max) for identical clones/mutants over two tested years, versus the mean pseudo-total metal concentrations in the soil.

type *P. trichocarpa* (T) (6.3 ± 0.1 ton DW ha^{-1} $year^{-1}$) and for intraspecific crossings of the *S. alba* group *S. alba/S. rubens/S. fragilis*, (a × a) with 6.2 ± 3.2 ton DW ha^{-1} $year^{-1}$. In block II, poplar (T) showed the highest biomass productivity, not statistically different from block I. Though there was a trend in higher metal concentrations for all plants growing in block II compared to block I. For block I and II, the most promising metal extractor is the *S. viminalis* (v) willow group, with on average 20.6 ± 3.5 mg Cd kg^{-1} DW, and 548 ± 126 mg Zn kg^{-1} DW. The metal concentrations in *S. viminalis* (v) were almost two times higher compared to poplar clone T and willow (a × a), for both blocks. The second most promising clone is poplar (T × T), with on average 7.8 ± 3.2 mg Cd kg^{-1} DW, and 293 ± 57 mg Zn kg^{-1} DW in the stems.

Metal concentrations were analyzed in the leaves as well (data not shown). The highest leaf Zn cadmium concentrations were detected for *S. viminalis* × *S. viminalis* (v × v) with an average of 2,000 mg Zn kg^{-1} DW leaves, followed by *S. viminalis* (v) with an average of 1,600 mg Zn kg^{-1} DW, and *S. alba* 1,000 mg Zn kg^{-1} DW. Poplar clones T × M and T × T contained on average 1,600 mg Zn kg^{-1} DW leaves, and the Cd concentration was on average 35 mg kg^{-1} for both. Poplar (T) contained the lowest Zn (1,200) and Cd (17) concentrations in the leaves. Cd concentrations in the leaves were similar for willows compared to poplar, with on average 32 mg Cd for *S. viminalis* group (v) and (v × v), while *S. alba* group (a) contained 8 mg Cd.

Biomass Productivity and Metal Extraction Efficiency of the Best Performing Tobacco, Sunflower and SRC Clones

Table 3 summarizes the biomass productivity, Cd and Zn extraction potentials of all evaluated species on the experimental field. The plants are organized per block, with the commercial SRCs growing in the blocks with highest pseudo-total metal concentrations (Van Slycken et al., 2013b, 2015). For each plant, the three best performing plants are shown. **Figure 5** gives a graphical representation of the Zn and Cd extraction potential of all tested genera.

The tobacco clone NBCu-108 and sunflower mutant line 15-35-190-04 demonstrated highest average extraction of Cd and Zn in their groups. Sunflowers are the best Zn extractors, followed by some of the commercial willows. Poplar clones produced between 2.6 tons of stem biomass ha^{-1} $year^{-1}$ [experimental clone *P. trichocarpa* × *trichocarpa* (T × T)] and 14.7 tons of stem biomass ha^{-1} $year^{-1}$ [experimental clone (T × T)] while willow stem production varied between 2.7 (experimental clone v × v) and 15.6 [experimental clone of crossing type *S. alba* (a × a)] ton ha^{-1} $year^{-1}$. Cadmium and Zn concentrations in stems ranged between, respectively, 35.5–142 g ha^{-1} $year^{-1}$ and 1.1–4.4 kg Zn ha^{-1} $year^{-1}$ for experimental poplars and between 30–152 and 1.1–4.1 kg^{-1} Zn ha^{-1} $year^{-1}$ for willow. Highest average Cd and Zn extraction potentials within the experimental poplars were observed for a clone of the (T × M) and one of crossing type T (2.7 kg Zn ha^{-1} $year^{-1}$). For willow, the commercial clone Zwarte Driebast showed the highest extraction potentials for Cd and Zn, respectively, 185 g Cd ha^{-1} $year^{-1}$ and 5.1 kg ha^{-1} $year^{-1}$.

When comparing the evaluated species, sunflower mutants clustered together at high Zn concentrations and low Cd extraction concentrations (**Figure 5**). The tobacco clones exhibited rather low Zn extraction and showed moderate amounts of Cd removal. The experimental poplar and willow clones covered a large range of Cd and Zn extraction and indicated a rather linear trend in combined Cd and Zn removal. While poplar crossings seemed to cluster in one group with a broad range of Cd extraction, willow experimental clones fell into two groups, the *S. alba* group *S. alba/S. rubens/S. fragilis*, (a × a) and the *S. viminalis* (v) and *S. viminalis x S. viminalis* (v × v). Between these groups, willow v showed the highest potential for Cd extraction, while the *S. alba* group in contrary had very low Cd extraction potential and a low to moderate Zn extraction. The best commercial willow clone (Zwarte Driebast) and most promising commercial poplar crossing type (Vesten) were plotted on top (mean ± SD, abstracted from Van Slycken et al., 2013b, 2015). Taking into account that these commercial clones were growing in soils with higher total metal concentrations (block III and IV), the data suggest that Zwarte Driebast holds most promise for Cd extraction and to some extend Zn, and performs better than the experimental clones. Vesten is also a good Cd extractor but less than Zwarte Driebast, and much less for Zn. Calculated extraction potentials ranged between 18 and 80 g Cd and 1.7 and 7.5 kg Zn ha^{-1} $year^{-1}$ (**Table 3**). Over the 2 years, the three best performing sunflower mutants were 15-35-190-04 M7, 14-185-04 and 86-35-190-04 M8. Their average biomass productivity ranged from 3.7 to 10.1 ton DW ha^{-1} $year^{-1}$.

Calculated Hypothetical Remediation Times

Calculated hypothetical remediation times for these best performing clones/mutants revealed that the shortest depollution periods for Cd as well as Zn concentrations on the experimental field would most likely be obtained with Zwarte Driebast (**Table 4**). It would last a time span of 60 ± 36 years to decrease pseudo-total concentrations of the moderately

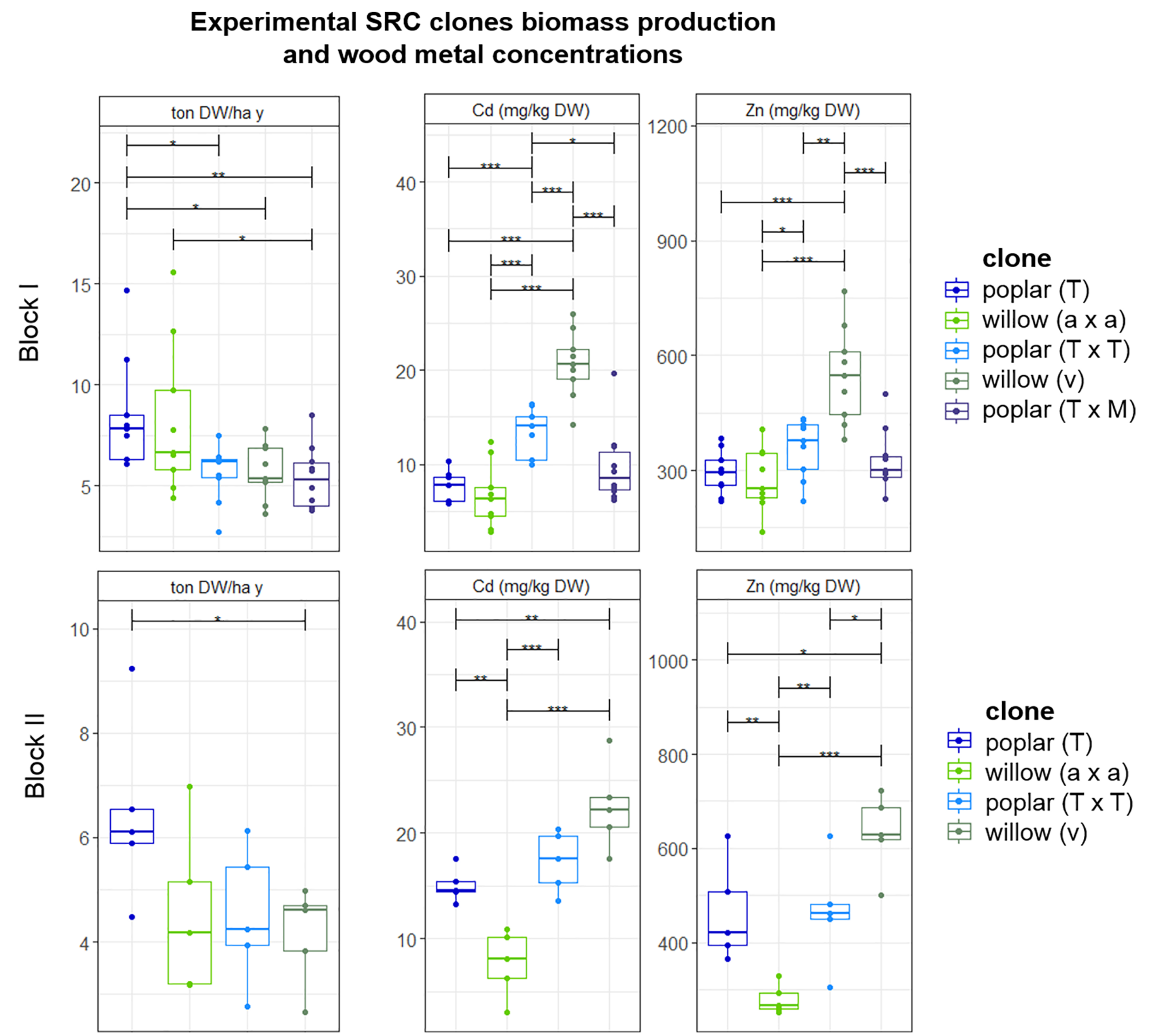

FIGURE 4 | Mean DW stem biomass production per plant (ton) ha^{-1} year^{-1} and the metal concentrations in stem tissues of experimental willow and poplar clones after a second 4-year growth cycle, organized per block and crossing type. Error bars are SEs (n = 5–10). Stars represent significant differences at the level of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) between clones within a year (ANOVA, Tukey-HSD). No significant differences for each crossing type between the blocks were detected (t-test).

polluted soil to pseudo-total remediation thresholds. When calculating the remediation time using $CaCl_2$-extractable Cd concentrations, much shorter time spans (e.g., 5 ± 3 years for Zwarte Driebast) were estimated to reach $CaCl_2$-exchangeable Cd concentrations that were found in unpolluted soils in Flanders (Meers et al., 2007a). Phytoextraction of Pb from the polluted soil revealed to be highly unrealistic when using the tested species/clones/mutants.

In Flanders, remediation criteria are site-specific as they are a function of destination type, clay, organic matter content and pH (Vlarebo, 2008). For the area under investigation, calculated pseudo-total remediation threshold values for soil were 2 mg Cd, 282 mg Zn and 200 mg Pb kg^{-1} DW soil.

DISCUSSION

Biomass Production Tobacco Sunflower and SRC

Tobacco and sunflowers plant height and DW production per plant differed significantly among the years. Since both

TABLE 3 | Biomass production (ton DW ha^{-1} $year^{-1}$), Cd and Zn extraction potential (g ha^{-1} $year^{-1}$) of the three most promising tobacco clones, sunflower mutants, and experimental and commercial SRCs Tobacco and sunflower data were averaged over 2 years.

Block	Type	Biomass production (stem for SRC) (ton DW ha^{-1} y^{-1})		Cd extract. potential (g ha^{-1} $year^{-1}$)		Zn extract. potential (kg ha^{-1} $year^{-1}$)	
Species (#plants/ha)	**Mutants, clones (# SRC clones)**	**Range (min max)**	**Mean (SD)**	**Range (min max)**	**Mean (SD)**	**Range (min max)**	**Mean (SD)**
Tobacco I (15 625)	**NBCu108**	**4.3–5.2**	**4.8 (0.2)**	**67.8–143.8**	**105 (30)**	**1.9–2.6**	**1.9 (0.5)**
	FOP	3.07–5.0	4.9 (0.2)	66.1–194.8	95.2 (31)	1.2–2.7	1.7 (0.5)
	NFCu719	3.9–5.3	5.1 (0.7)	79.2–111.4	93.1 (10)	1.1–2.5	1.5 (0.3)
Sunflower I (27 778)	**15-35-190-04**	**5.6–7.1**	**6.3 (2.3)**	**31.5–76.2**	**42.5 (21)**	**1.9–7.5**	**5.6 (3.2)**
	14-185-04	5.7–10.1	8.6 (0.1)	26.5–80.3	35.6 (30)	1.7–6.7	4.2 (2.1)
	86-35-190-04	3.7–7.2	5.4 (2.4)	18.1–41.5	30.2 (2)	2.1–4.6	3.5 (2.1)
Poplar I (10 000)	**{D(T × M)} (1)**	**–**	**9.9**	**–**	**87.2**	**–**	**2.3**
	{T} (2)	6.2–6.4	6.3 (0.2)	61.5–105.1	83.3 (30)	2.5–2.7	2.6 (0.2)
	{T × T} (8)	6.1–14.7	8.5 (3.0)	35.5–125.9	67.4 (31)	1.3–4.4	1.5 (1.2)
Willow I (10 000)	**{v} (9)**	**3.6–7.8**	**5.7 (1.4)**	**83.3–152.0**	**115 (28)**	**2.9–4.1**	**3.1 (0.9)**
	{v × v} (1)	–	3.0	–	54.0	–	2.4
	{a × a} (14)	3.6–15.6	7.1 (3.5)	30.0–109	41.9 (23)	0.8–3.4	2.0 (1.2)
Poplar II (15 000)	**{T × M} (1)**	**–**	**5.1**	**–**	**99.0**	**–**	**1.5**
	{T} (3)	4.5–9.2	6.5 (2.4)	65.0–142.1	95.1 (41)	1.7–3.3	2.7 (0.3)
	{T × T} (15)	2.6–7.5	4.6 (1.6)	43.3–102.6	69.2 (22)	1.1–2.9	1.7 (0.4)
Willow II (15 000)	**{v × v} (5)**	**2.7–5.0**	**4.2 (0.9)**	**62–110**	**91.7 (18)**	**1.6–3.2**	**2.6 (0.6)**
	{a × a} (7)	3.2–7.0	4.5 (1.4)	16.1–56.7	29.9 (13)	1.1–3.2	1.1 (0.3)
Poplar III (10 000)	**Vesten (D × N)**	**–**	**6.2 (0.7)**	**–**	**143.5 (9)**	**–**	**2.2 (0.2)**
	Koster (D × N)	–	5.2 (1.4)	–	90.1 (41)	–	1.5 (0.6)
	GrimmingeD (T × D)	–	5.1 (0.1)	–	77.0 (7.1)	–	1.3 (0.1)
Willow III (15 000)	**Z. Driebast (Tr)**	**–**	**12.3 (3)**	**–**	**185 (49)**		**5.1 (0.5)**
	Loden (Da)	–	3.2 (0.7)	–	119 (18)		3.2 (0.8)
	Tora (S × V)	–	3.3 (0.4)	–	71.2 (14)		1.6 (0.2)

D, Populus deltoides; M, Populus maximowiczii; N, Populus nigra; T, Populus trichocarpa; A, Salix alba; Da, Salix dasyclados; F, Salix fragilis; S, Salix schwerinii; Tr, Salix triandra; V, Salix viminalis; {}, collection of all experimental clones with this crossing type (# clones). Values are min-max and mean ± SD. "-" means data not available. Stem biomass production and Cd and Zn concentrations in the stems of commercial poplar clones were derived from Van Slycken et al. (2015), those of commercial willow clones from Van Slycken et al. (2013b). Extraction potentials of these commercial clones were calculated based on the abstracted data.
SRC data were collected from the first four growing seasons of most promising experimental clones and commercial clones planted on the field in 2006. The data from the commercial poplar clones were abstracted from Van Slycken et al. (2015), and the data from the commercial willow clones from Van Slycken et al. (2013b). The values in bold indicate the most promising clone within its genus.

crops were grown next to each other on the same plots, this might suggest that they both are quite susceptible to yearly variations in climatological conditions, field preparation and maintenance actions and/or that the quality of the seeds might differ substantially between years. For example, the abnormal low tobacco biomass production in 2013 (data not shown), could be related to (a combination of) three factors: (1) plantlets were quite old (about 11 weeks) when the field was ready for planting. A growth spurt might already have taken place when the plants were still in pots, hampering their biomass production; (2) because no weed control was performed before and after planting, a considerable amount of weeds (mainly *Polygonum* sp.) between the tobacco plants was competing for nutrients and water; (3) in comparison with the other years and normal values, the quantity of rain was lower and there was more sun which might have caused drought stress to some extent. Above-ground yields of tobacco not only varied considerably over the years (**Figure 2**) but also did not reach yield values like reported by Kayser et al. (2000) (10–12.5 t ha^{-1} $year^{-1}$), Fässler et al. (2010) (8.5–10.5 t ha^{-1} $year^{-1}$) and Herzig et al. (2014) (24.7–37.5 t ha^{-1} $year^{-1}$) obtained in phytoremediation field experiments in Switzerland.

Above-ground production of an IBL 04 sunflower plant on the metal-polluted soil in Lommel was similar to biomass productions of this inbred line found on the metal-polluted site in Rafz, Switzerland (93.7, 68 ± 17 and 78 ± 8.6 g) (Nehnevajova et al., 2007, 2009). Above-ground yields per hectare and year are rather low in comparison with above-ground yields reported for sunflowers cultivated on other metal-polluted soils in Switzerland, ranging from 7.5 up to 29 t ha^{-1} $year^{-1}$ (Kayser et al., 2000; Fässler et al., 2010; Herzig et al., 2014) (**Figure 3** and **Table 3**).

After four growing seasons of poplar and willow clones on the field in Lommel, biomass productivity differed considerably between clones but was in general quite low (mostly <6 t ha^{-1} $year^{-1}$; **Table 3**). Biomass productivity levels of SRC depend on site-specific conditions, clonal selection, climatic conditions, plant spacing and management. For willow SRC,

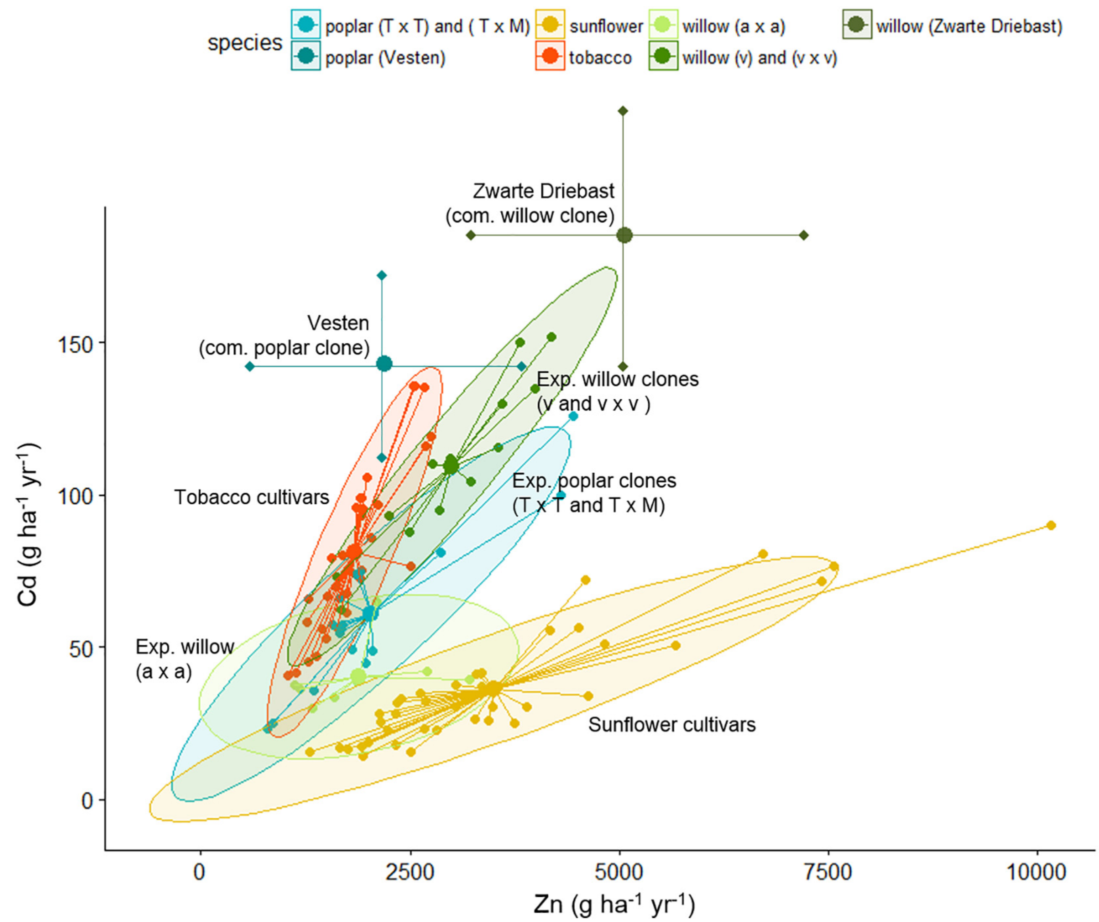

FIGURE 5 | Cadmium and Zn extraction potential (g ha^{-1} year^{-1}) of tobacco clones, sunflower mutants and poplar, willow clones evaluated on the experimental field. In case of tobacco and sunflower, extraction potential of identical clones/mutants were averaged over 2 years. Each clone is shown as a dot, connected with the spider-lines, and the overall average per group is indicated with the thick dot. Groups are colored, based on 90% ellipses. In case of commercial poplar and willow clones, results reflect data from **Table 3**.

TABLE 4 | Hypothetical remediation times (years) of best performing clones/mutant of tested species to reduce 1 mg of Cd, Zn and Pb kg^{-1} DW soil for high or moderate field Cd, Zn and Pb concentrations to remediation thresholds (2 mg Cd, 282 mg Zn and 200 mg Pb kg^{-1} DW soil).

Hypothetical remediation times (years)		Contamination level:			
		High			Moderate
Species	**Clone/mutant**	**7 mg Cd kg^{-1} DW**	**429 mg Zn kg^{-1} DW**	**217 mg Pb kg^{-1} DW**	**4 mg Cd kg^{-1} DW**
Poplar	D × (T × M)	336	320	n.d.	134
Willow	Zwarte Driebast	150 ± 90	181 ± 107	n.d.	60 ± 36
Tobacco	NBCu-10-8	401 ± 211	657 ± 345	1,864 ± 1,145	160 ± 84
Sunflower	15-35-190-04	744 ± 407	250 ± 152	4,250 ± 2,380	298 ± 163

Assumptions made were: (i) species' extraction potentials are independent of soil metal concentrations, (ii) total soil metal content decreases linearly due to a constant yearly extraction, (iii) contamination and rooting depth are 0.5 m, and (iv) soil density is 1,250 kg m^{-3}.

expected biomass productivity is between 6 and 10 t ha^{-1} year^{-1} in Sweden (Dimitriou et al., 2006) while higher values (10–20 t ha^{-1} year^{-1}) were considered common by Maxted et al. (2007). Annual yields reported for poplars in SRC are between 10 and 15 t ha^{-1} in less intensive conditions (Laureysens et al., 2004a). Zegada-Lizarazu et al. (2010) mentioned an average biomass yield between 10 and 12 t ha^{-1} year^{-1} for poplar and willow in temperate climates. The lower productivity levels on the Lommel field site can be attributed to the nutrient poor, sandy characteristics of the soil (Van Slycken et al., 2015). Given the absence of fertilization and irrigation, the productivity of willow and poplar clones can be expected to be less in comparison with

SRC cultures on more fertile soils. Furthermore, yields obtained after the first growing seasons tend to be lower than yields from later cutting cycles (Aronsson et al., 2014; Van Slycken et al., 2015) since, during the 1st years, a plant will allocate a considerable amount of its energy for the establishment of its root system. Also for Cd and Zn concentrations in the stem, obvious differences exist between clones (**Table 3**). Differences in metal uptake between cultivars were also reported by Landberg and Greger (1996, 2002), Granel et al. (2002); Mleczek et al. (2010), Ruttens et al. (2011) and Van Slycken et al. (2013b) (for willow) and Laureysens et al. (2004b) (for poplar). The high variability in stem biomass production and stem Cd and Zn concentrations for a commercial clone can partly be attributed to the heterogeneity of the field given that the same clone is planted (and measured) in different plots on different locations of the field. In case of the experimental groups, the large ranges are due to clonal differences as explained above.

Metal Phytoextraction Potential of Tobacco, Sunflower and SRC

The significant differences in concentrations of Cd, Zn and Pb for tobacco clones or sunflower mutants throughout the years cannot be due to differences in soil metal content since all experiments were conducted in a restricted part of the field with similar soil characteristics (**Figure 1** and **Table 2**). Also Fässler et al. (2010) reported considerable year-to-year variations in metal accumulation of tobacco and sunflower in field trials. It is speculative which (combination of) factors (climate conditions, seed quality/generation, field preparation and management, planting distance. . .) account for these differences between years.

The tobacco and sunflower cultivation at the Lommel field provided some information concerning potentially stable improvements after selection based on somaclonal variation and conventional *in vitro* breeding of tobacco and chemical mutagenesis of sunflower. Over the years, mean Cd and Zn removals of tobacco clone NBCu-10-8 were higher than mean values of BAG (**Figure 2**) which might indicate an improved tobacco clone for metal phytoextraction, although more research is required for confirmation. Regarding the sunflowers, averaging extraction potentials over the years 2013 and 2014 revealed a slight extraction improvement for mutant lines 15-35-190-04 and 14-185-04 in comparison with the IBL 04 control for Cd (**Figure 3**). The large biomass increments of these mutants compared to control, the "giant mutants" reported by Nehnevajova et al. (2007, 2009), were not observed in this case. The vegetative propagation of selected clones of willow and poplar is an important advantage to this concern. Vegetative propagation helps to maintain the improved characteristics of a certain genotype/cultivar/clone/variant (Zegada-Lizarazu et al., 2010). Furthermore, using stem cuttings to establish clonal plantations is expected to reduce variability between plants compared to plants raised from seeds (Dickinson et al., 2009).

Variability of extraction potentials of all evaluated clones/mutants is high (**Figure 4**), as a result of the heterogeneity of the field (for the SRC clones) and yearly variations in many factors (for tobacco and sunflower). In general, however, extraction potentials (**Figure 2**) together with BCFs (**Table 4**) suggest sunflower as a highly efficient Zn extractor and tobacco as a more prominent Cd extractor, confirming previous findings (Kayser et al., 2000; Fässler et al., 2010). BCFs $\geq$1 furthermore confirm efficient extraction (accumulation of metals in the crops relative to the soil) (Dickinson and Pulford, 2005; Kötschau et al., 2014) of Cd and Zn by sunflowers, and tobacco. Most evaluated poplar and willow clones showed phytoextraction potentials between that of tobacco and sunflower. However, the large range of (combined) Cd and Zn extraction covered by commercial willow clones and group means of experimental poplars and willows lead to optimism concerning clone selection (construing experimental groups) and/or conventional breeding approaches that may provide clones with a higher combined extraction of Cd and Zn. Furthermore, high combined metal extraction potentials for the commercial willow clone Zwarte Driebast indicates that this clone possess high efficiencies regarding Cd and Zn extraction in comparison to tobacco and sunflower (**Figure 5**). In addition, harvesting the leaves of SRC trees (which was performed in this research but not the main focus) and yield increments expected with increased age of the SRC plantations (see above) might even considerably increase metal extraction potentials of SRC clones (in later cutting cycles).

Phytoextraction of Pb using the species tested in Lommel is utopia (**Figures 2**, **3**). The BCFs indicate a generally very low translocation of Pb to the above-ground biomass. Moreover, its low bioavailability in the soil, and even increased inactivation by a vegetation cover (Chaney et al., 1997), makes that soil Pb concentrations, even when exceeding remediation thresholds, rarely cause problems for agriculture (plant Pb uptake from soil) or the environment in general (spreading risks).

Caution needs to be taken with the calculated remediation times (based on pseudo-total or $CaCl_2$-extractable fractions) (**Table 4**). Firstly, all tested plant species are assumed to possess a steady extraction potential, independent of soil metal concentrations. However, it was not evaluated to which extent extraction potentials of sunflower, tobacco, willow or poplar depend on soil pollution levels. Secondly, the yearly linear decrease in total soil metal concentration due to phytoextraction most likely is a simplistic approximation compared to the real situation. It assumes that a species' biomass production, its metal accumulation (or at least the product of both) and the bioavailability of metals in the soil does not change over time. Several authors (Robinson et al., 2003, 2006; Koopmans et al., 2007; Van Nevel et al., 2007; Manzoni et al., 2011) proposed decay models incorporating, to some extent, soil chemistry (with all kinds of sorption, retention and leaching processes to describe evolutions in the "bioavailable" metal pool), changes in plant metal accumulation and biomass production over time. However, involving more factors increases uncertainty and since no model is acknowledged to be valid in all cases, the simplest approach is used here. The calculated remediation times of the investigated crops differ enough to conclude that willow clone Zwarte Driebast would need the shortest time to decrease the metal contents in this specific soil to the legislative threshold/adopted reference value(s). Furthermore, a more profound study of the individual clones in the experimental INBO crossing types might unravel other clones suitable for phytoextraction purposes. The tobacco clones and sunflower mutants used resulted from, respectively, *in vitro* breeding and mutagenesis followed by continuous breeding and selection for improved phytoextraction efficiencies. Therefore, further enhancement of the remediation potential in these groups is not very likely.

Whether to rely on remediation times based on pseudo-total ("total") or $CaCl_2$-exchangeable ("bioavailable") metal concentrations is disputable. In many countries "total" metal concentrations are used in legislation. However, very promising prognosis of short depollution times of only a few years, can be made for the phytoextraction of the "bioavailable" metal pollution in soils that is adopted in, e.g., Switzerland (Vangronsveld et al., 2009; Herzig et al., 2014). This "bioavailable" pool of metals in soil is justifiably regarded as the main risk for pollution of both, food chains and groundwater (Karlaganis, 2001). Application of the "bioavailability" concept in risk assessment and management of polluted sites is increasing (Onwubuya et al., 2009; Kumpiene et al., 2014), considering that the risks for human health and ecosystems in metal-polluted soils are often poorly predicted by the total metal concentrations (McLaughlin et al., 2000). Several authors described a replenishment of the "bioavailable" metal pool (Zhang et al., 1998; Whiting et al., 2001; Hammer and Keller, 2002; Keller and Hammer, 2004; Fischerová et al., 2006), whereas Herzig et al. (2014) showed the relative stability of labile Zn topsoil concentrations ($NaNO_3$-extracts) 1–3 years after stopping a 5 years phytoextraction treatment in Switzerland. In any case, this major consideration definitely demands for further investigation as also mentioned by Van Nevel et al. (2007). In addition, the requirements and the protocols of assessing "bioavailability" still differ considerably between European countries. Moreover, in Flanders no "bioavailable" remediation thresholds are acknowledged. Therefore, in this study, further considerations were based on remediation times calculated using pseudo-total metal concentrations.

These estimated remediation periods are long and generally considered too long for the implementation of phytoextraction as a stand-alone remediation technology [e.g., Blaylock and Huang (2000) suggested a period of about 10 years as threshold to render the technology economically feasible in itself]. Therefore, this research (as well as almost all evaluations of this matter in literature) emphasizes the necessity to combine phytoextraction with other opportunities. Synergies between social, economic as well as environmental agendas seem indispensible for the justification, advancement and eventual implementation of metal phytoextraction (Dickinson et al., 2009). Firstly, phytoextraction crops may generate economic revenues, for example, through conversion of produced biomass. Secondly, the growth of high biomass crops may restore ecosystem services (e.g., CO_2 abatement, improving quality of soil, water, air. . .). Finally, social benefits (recreation, educational value, visual and aesthetical power) might arise from "green" remediation technologies and public acceptance is considered high (Kennen and Kirkwood, 2015). These external and indirect advantages of growing phytoextraction crops might not only compensate for long remediation times but, together with the remediation potentials, also determine the overall sustainability and effectiveness of a metal phytoextraction treatment. The way of thinking about metal phytoextraction as a larger concept of sustainable and risk mitigating land use is illustrated in this manuscript by means of a case study in Belgium.

Given the extraction potentials of tobacco, sunflower and SRC of willow and poplar in a case study in Belgium and available information on economics (biomass conversion) and environmental benefits of cultivating these crops so far, it is concluded that SRC would be the most interesting crop for metal phytoextraction in the investigated area. Besides this, it was an interesting finding that sunflower mutants are more suited for Zn extraction while tobacco plants are the better choice for Cd, and some of the most performing new mutants are identified for further studies. Finally, the optimal combination of the properties of a clone with high metal uptake capacities combined with high biomass productivity could lead to the formation of groups of clones showing high potential for trace metal phytoextraction. These results can then be incorporated into future breeding programs, research and rotation coppices.

In future, more elaborate investigations should also be dedicated to optimizing metal-enriched crop conversion in order to become sustainable and economically profitable. For the conversion of biomass in this case study, a prominent role seems to be reserved for pyrolysis, and the generation of metal-enriched activated carbon for filter medium purposes especially deserves further attention. Furthermore, finding a way to reward for the restoration of ecosystem services (including CO_2 abatement) when growing phytoextraction crops will be crucial for the eventual implementation. In addition, also benefits related to social topics (e.g., recreation, education, design. . .) should be recognized and compensated in some way. Finally, conflicts between the factors determining the overall sustainability of a phytoextraction plantation might arise and case-to-case evaluations impose themselves to obtain intelligently designed phytoextraction concepts.

CONCLUSION

The shortest estimated remediation time for simultaneous Cd and Zn clean-up was obtained with the commercial willow clone Zwarte Driebast. The best tobacco clone identified in this study for Cd phytoextraction was NBCu-108, and the most promising sunflower mutant line for Zn extraction was 15-35-190-04. The experimental willow and poplar clones show a large range of combined Cd and Zn extraction providing a substantial basis for optimism that clone selection and/or conventional breeding approaches may produce additional clones with high combined extraction of Cd and Zn.

A drawback still of metal phytoextraction using the evaluated high biomass crops, is the long period of time (>60 years) that would be needed to decrease "total" metal concentrations in the soil to legal threshold values. Although much shorter times are estimated when adopting "bioavailable" metal concentrations, these outcomes are still not generally accepted due to the uncertainty regarding equilibria between the various metal species in the soil and the eventual replenishment of the "bioavailable" metal pool on the longer term. Economic revenues through biomass conversion and a rewarding for environmental benefits of a phytoextraction crop plantation are crucial for large-scale, commercial implementation of metal phytoextraction.

AUTHOR CONTRIBUTIONS

JV, NWe, NWi, and AR conceived the study design. JV, NWe, and NWi coordinated the execution of the project by JJ and ST. ST and NWi wrote this manuscript in collaboration with JV and NWe. JV is promoter of the study. All authors contributed to the elaboration of the study design and took part in reviewing the methods, each member contributed specifically to the parts of the study corresponding with their own expertise. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01879/full#supplementary-material

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Legume Intercropping With the Bioenergy Crop *Sida hermaphrodita* on Marginal Soil

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The cultivation of perennial biomass plants on marginal soils can serve as a sustainable alternative to conventional biomass production via annual cultures on fertile soils. *Sida hermaphrodita* is a promising species to be cultivated in an extensive cropping system on marginal soils in combination with organic fertilization using biogas digestates. In order to enrich this cropping system with nitrogen (N) and to increase overall soil fertility of the production system, we tested the potential of intercropping with leguminous species. In a 3-year outdoor mesocosm study, we intercropped established *S. hermaphrodita* plants with the perennial legume species *Trifolium pratense, T. repens, Melilotus albus,* and *Medicago sativa* individually to study their effects on plant biomass yields, soil N, and above ground biomass N. As a control for intercropping, we used a commercial grass mixture without N_2-fixing species as well as a no-intercropping treatment. Results indicate that intercropping in all intercropping treatments increased the total biomass yield, however, grass species competed with *S. hermaphrodita* for N more strongly than legumes. Legumes enriched the cropping system with fixed atmospheric nitrogen (N_2) and legume facilitation effects varied between the legume species. *T. pratense* increased the biomass yield of *S. hermaphrodita* and increased the total biomass yield per mesocosm by 300%. Further, the total above ground biomass of *S. hermaphrodita* and *T. pratense* contained seven times more N compared to the mono-cropped *S. hermaphrodita*. *T. repens* also contributed highly to N facilitation. We conclude that intercropping of legumes, especially *T. pratense* and *T. repens* can stimulate the yield of *S. hermaphrodita* on marginal soils for sustainable plant biomass production.

Keywords: perennial energy crop, *Sida hermaphrodita*, marginal soil, legume intercropping, biomass production, bioenergy, facilitation

INTRODUCTION

Cultivation of biomass crops on marginal soils requires careful design, establishment and maintenance of suitable cropping systems (Spiertz, 2013; Solinas et al., 2015). Marginal soils lack the nutrient resources necessary to provide a certain minimal productivity for plant biomass production and thus require a more costly input than what can be recovered by the output (European Environmental Agency, 2015). Physically, such soils may be sandy, rocky, shallow, low in plant-available nutrients and water and they need to be adequately prepared and maintained to support successful plant growth (Schröder et al., 2008). Thus, a suitable cropping system for particular local abiotic conditions needs to be implemented with regard to selecting the most appropriate plant species and cropping approaches.

Naturally occurring soils that are not suited for high-output agricultural production nevertheless harbor a distinct and well-adapted plant community of which perennial plants are an important part (e.g., grasslands on sandy soils; Loebel et al., 2015). Such plants often produce a deep- reaching root system, which enables access to water and nutrient reservoirs that are otherwise unreachable to roots of shallow-rooting plants. In addition, due to their long-lasting nature, roots of perennials store energy and N during dormant periods and then re-sprout early in the following growing season, allowing for high nutrient use-efficiency and recycling of N over time (Millard and Grelet, 2010; Voigt et al., 2012). Such N remobilization traits would be attractive options during the selection of plant species for biomass production on marginal soils.

A current prominent example of a perennial woody biomass plant is *Sida hermaphrodita* (Spooner et al., 1985); a species that has become a model plant for sustainable biomass production on marginal substrates in recent years especially in Poland (Borkowska et al., 2003; Barbosa et al., 2014; Nabel et al., 2016, 2017; Jablonowski et al., 2017). Following the establishment of the root system during the first growing season, *S. hermaphrodita* shoots emerge early in the next growing season from underground rhizomes which contain stored energy reserves (Borkowska and Molas, 2012). Early shoot emergence and the increasing amount of tillers with progressing cultivation time result in dense canopies reducing the necessity of weed and pest control and mechanical soil disturbance (Beare et al., 1994; Borkowska and Molas, 2012). Once established, the deep-reaching root system allows efficient exploitation of large volumes of soil for nutrients and water. Particularly on marginal soils, such root systems are beneficial for building soil structure (Bronick and Lal, 2005). However, in order to successfully cultivate *S. hermaphrodita* on a marginal substrate with the aim to produce ample biomass, adequate fertilization needs to be supplied to achieve the desired above-ground biomass production.

Compared to mineral fertilizers, organic fertilizers contain a high carbon concentration in addition to organically bound essential plant nutrients (Möller and Müller, 2012), which reduces the probability of nutrient leaching due to reduced mobility of the organically bound nutrients (Alburquerque et al., 2012a). Since marginal substrates are *per se* low in carbon content they would benefit from continuously applied organic fertilization which would gradually increase soil carbon content (West and Post, 2002). Digestate, a by-product of anaerobic fermentation of plant biomass for biogas production has been shown in our previous work to be a valuable organic fertilizer for *S. hermaphrodita* on marginal substrates (Barbosa et al., 2014; Nabel et al., 2014, 2017). The relative biomass increment of *S. hermaphrodita* fertilized with digestate continuously increased over three consecutive years compared to mineral fertilization, presumably due to the introduction of organic compounds and their additive effects on soil fertility (Nabel et al., 2017). Additionally, the re-introduction to the soil of nutrients that were removed with plant biomass during harvest and converted during biogas production, enables a cropping system that is independent of mineral fertilizers and allows closed nutrient loops, an essential concept of sustainable plant biomass production (Alburquerque et al., 2012b).

To stably provide N to such a cropping system, legume intercropping is commonly implemented in low-yield farming systems (Stagnari et al., 2017). The ability of legumes to fix atmospheric nitrogen (N_2) is generally considered the reason for the experimental evidence that intercropping with legumes results in increased above-ground biomass production compared to mono crop cultures (Temperton et al., 2007; Roscher et al., 2012). With respect to biomass production on marginal soils, legume intercropping thus seems an obvious choice to create a more sustainable cropping system. Intercropping has further been described to produce earlier canopy closure, thus reducing the need for weed and pest control measures and also increases biodiversity, an important factor in every cropping system (Liebman and Dyck, 2009). Backing this up, in biodiversity ecosystem functioning experiments in grasslands, faster and more effective canopy closure occurs when more than one species is grown in a plot (Spehn et al., 2000). Most importantly, reduced need for mineral fertilizer renders the cropping system more economically viable since the main factor that determines the profitability is the cost for fertilization, in particular with N (Boehmel et al., 2008).

Interestingly, in our previous work we intercropped *S. hermaphrodita* with the highly efficient, deep-rooted N_2-fixing legume *Medicago sativa* (with both species sown at the same time) and observed that this combination negatively affected *S. hermaphrodita* biomass compared to mono-cropping, even though total biomass of *S. hermaphrodita* combined with *M. sativa* was strongly stimulated (Nabel et al., 2016). Depending on the species combination, such an outcome is not uncommon since legumes, although bringing extra N into a system, are often strong competitors being fast growing (Malézieux et al., 2009). One of the mechanisms underlying improved biomass accumulation in intercropping is assumed to be complementary spatial distribution of roots within soil in more diverse mixtures resulting in differences in resource acquisition in either time or, more commonly, in space (von Felten and Schmid, 2008; Kahmen et al., 2012). Thus, using promising species combinations with complementary rooting depths (one shallow, one deeper- rooting species) could result in a vertical root orientation that circumvents direct competition for water and nutrients (Berendse, 1979). Thus, the careful selection of plant species regarding their root system traits and their composition with the aim of plant-specific complementarity could theoretically result in the identification of combinations of species with the potential to outyield compared to mono-cropping (Hoekstra et al., 2015; Hernandez and Picon-Cochard, 2016).

In the presented work, we aimed at an optimized *S. hermaphrodita* biomass production by intercropping with four different legume species (*M. sativa, Melilotus albus, Trifolium repens, T. pratense*), as well as a grass mixture as a control treatment. The aim of this study was to identify (i) if legume species selection and their respective N_2-fixing abilities can result in outyielding the non-N_2-fixing *S. hermaphrodita*, and (ii) to analyze whether this is reflected in total N content in the above-ground plant biomass and in the marginal substrate.

TABLE 1 | Climate data for the outdoor mesocosm experiment: annual mean temperature and yearly precipitation values during the experimental time from 2015 to 2017 at the Forschungszentrum Jülich GmbH (50°53′47′′ North and 6°25′32′′ East; 80 m a.s.l.).

Year	Mean temperature (°C)	Precipitation (mm year^{-1})
2015	14.3	678.1
2016	14.9	666.3
2017	14.9	658.0

MATERIALS AND METHODS

Study Site and Plant Cultivation

A 3-year outdoor mesocosm experiment was established at the Forschungszentrum Jülich GmbH (50°53′47′′ North and 6°25′32′′ East; 80 m a.s.l.) using 48 containers (40 cm × 40 cm × 40 cm), each filled with a sandy substrate (0/1 fine aggregate sand, RBS GmbH, Inden, Germany; TOC: 0 g kg^{-1}; TN: 0 g kg^{-1}; Ca: 0.3 g kg^{-1}; K: 0.2 g kg^{-1}; Mg: 0.8 g kg^{-1}; P: 0.1 g kg^{-1}), used as a model marginal substrate. The climate data during the experimental time from 2015 to 2017 are presented in **Table 1**. Besides the natural precipitation, mesocosms received additional irrigation via an automated drip irrigation system to prevent plants from severe drought stress (0.1 – 0.5 L day^{-1}). Single seedlings of *Sida hermaphrodita* of BBCH stage 13–14 (Jablonowski et al., 2017) were transplanted into the mesocosms in April 2015. Legumes (alfalfa – *Medicago sativa;* white sweet clover – *Melilotus albus;* red clover – *Trifolium pratense;* and white clover – *Trifolium repens*; all purchased from Feldsaaten Freudenberger GmbH & Co. KG, Krefeld, Germany) as well as a commercially available grass mixture, not containing legumes (composed of 10% perennial ryegrass – *Lolium perenne;* 50% red fescue - *Festuca rubra;* 40% blue grass - *Poa pratensis; WB* 130 Mulchmischung III - Weinbergdauerbegrünung I; Feldsaaten Freudenberger GmbH & Co. KG, Krefeld, Germany) were sown (0.7 g mesocosm^{-1}) in April 2016 into mesocosms containing the one year old *S. hermaphrodita* plants (n = 8). Additionally, eight mesocosms with *S. hermaphrodita* without intercropping were used as a control.

In May 2015, 2016, and 2017 all mesocosms received a fertilization using biogas digestate in a dose equal to a total N application of 160 kg ha^{-1}. We chose this fertilization dose as it resulted in optimal plant growth in a previously published dose-response experiment for digestate fertilization of *S. hermaphrodita*, grown on the same marginal substrate used in this study (Nabel et al., 2014). The digestate was obtained from a commercially operating biogas plant using maize silage as feedstock (digestate dry matter mass fraction: 7.2%; N_{total}: 0.53%; NH_4^+: 0.32%; P: 0.14%; K: 0.68%; Mg: 0.037%; Ca: 0.16%; S: 0.03%; organic matter: 5.3%, C:N ratio: 6; pH 8.2; all values refer to fresh weight; ADRW Naturpower GmbH & Co. KG, Titz-Ameln, Germany).

Measurements

At the end of the growing season 2017, the aboveground biomass of *Sida hermaphrodita* and the intercropped species was harvested separately. Dry plant biomass was determined after drying at 70°C to constant weight. Additionally, soil samples were taken at 0–15 cm depth at the time of the biomass harvest and dried to constant weight at 30°C. Carbon (C) and nitrogen (N) content of the soil and the nitrogen concentration in the total aboveground plant biomass samples were determined by elemental analysis (VarioELcube, Elementar).

Estimation of Effectiveness of Biological Nitrogen Fixation

In order to estimate the N_2-fixation potential of the intercropped legume species on the marginal substrate, we invasively assessed nodulation of all legume species at the time of overall biomass harvest in October 2017 by extracting roots and assessing levels and quality of nodulation, following an ordinal scale-based field protocol of the British Columbia Ministry of Forestry, Canada (British Columbia Ministry of Forests, 1991). Soil cores of 20 cm depth and 7 cm diameter were taken to extract roots with nodules. The score took into account aboveground plant vigor (based on greenness of leaves and lack of wilting) and the number of nodules as well as nodule position, color, and appearance. The final score was then separated into three different possible categories that allow a swift assessment of nodulation efficiency: (1) effective nodulation (score: 20–25), (2) less effective nodulation (score: 15–20) or (3) not effective nodulation (score: 0–15), thus providing a rough indication of biological N_2 fixation. This is a rough field method, but it allows to swiftly assess the effectiveness of nodulation (Nabel et al., 2016).

Statistical Analysis

The experiment has a one factorial design with the intercropping factor separated into six levels: *Sida hermaphrodita* intercropped with one legume species each time (*Medicago sativa, Melilotus albus, Trifolium pratense,* or *T. repens,* respectively) or with grasses, as well as *S. hermaphrodita* grown alone as a control. Eight biological replicates were used for each treatment level. The collected soil samples were analyzed for C and N and plant samples were analyzed for N in four replicates. Statistical analysis was performed with analysis of variance (ANOVA) after trimming the data in R 3.0.3 (The R Foundation for Statistical Computing 2014) using the work package "Agricolae" (de Mendiburu, 2014).

RESULTS

Biomass

In their third year of growth, the *S. hermaphrodita* plants in the control treatment developed an average plant biomass of 50 g dry matter per mesocosm (**Figure 1**). Intercropping with the legumes *M. sativa* and *M. albus* did not result in changes of the *S. hermaphrodita* biomass, but increased significantly the total biomass yields per mesocosm by 100–200%. Intercropping of *S. hermaphrodita* with *T. pratense* and *T. repens* increased significantly the biomass yield of *S. hermaphrodita* by 8-15% compared with *S. hermaphrodita* mono-cropping, but *T. repens* also delivered the least additional biomass of all intercropped species. Grass produced the highest plant biomass dry matter yield of all intercropped species with 150 g mesocosm^{-1}, while *S. hermaphrodita* biomass yield was significantly reduced compared with intercropping with *M. sativa, T. pratense,* and *T. repens* (**Figure 1**).

Nodulation Assessment

All intercropped legumes had active nodulation with nodulation scores between 17 and 21.5 (**Table 2**). Both *T. pratense* and *T. repens* had scores in the range of "effective nodulation" while *M. sativa* and *M. albus* both showed scores in the range of "less effective nodulation" (British Columbia Ministry of Forests, 1991).

Nitrogen in the Plant Material

Trifolium pratense and *T. repens* showed N contents in their above ground biomass of 2.7 and 3.2%, respectively (**Table 2**). *M. sativa* and *M. albus* contained between 2.3 and 2.5% N while the grass mixture only contained 1.1% of N in the above ground biomass. The above ground biomass of *S. hermaphrodita* in the control treatment contained 1% of N. Intercropping with legumes increased N contents of *S. hermaphrodita* by 20–30%. Intercropping of *S. hermaphrodita* with the grass mixture resulted in 60% lower N content of the *S. hermaphrodita* above ground biomass.

Mesocosms of the control treatment without any intercropping contained the lowest content of total N in the above ground *S. hermaphrodita* biomass (0.4 g N mesocosm^{-1}; **Figure 2**). Grass intercropping increased the total N in the above ground biomass fourfold compared to the control treatment, but this N was mainly found in the grass biomass. Treatments with legume intercropping showed the highest total N content in the above ground biomass per mesocosm with the highest value of 3.6 g N mesocosm^{-1} for *S. hermaphrodita* intercropped with *T. pratense*.

Soil Analysis

After three growing periods, control-treated mesocosms, receiving digestate fertilization but no intercropping treatment, had a soil carbon concentration of 0.8% and a total N concentration of 0.7‰ (**Table 2**). No statistically significant difference in soil C or N between legume and grass intercropping treatments was found. The highest soil C (2.1%) and N (1.4‰) concentrations were found in mesocosms with *S. hermaphrodita* intercropped with *M. sativa*.

DISCUSSION

Intercropping of *S. hermaphrodita* with legumes generally increased the biomass yield per mesocosm, without reducing

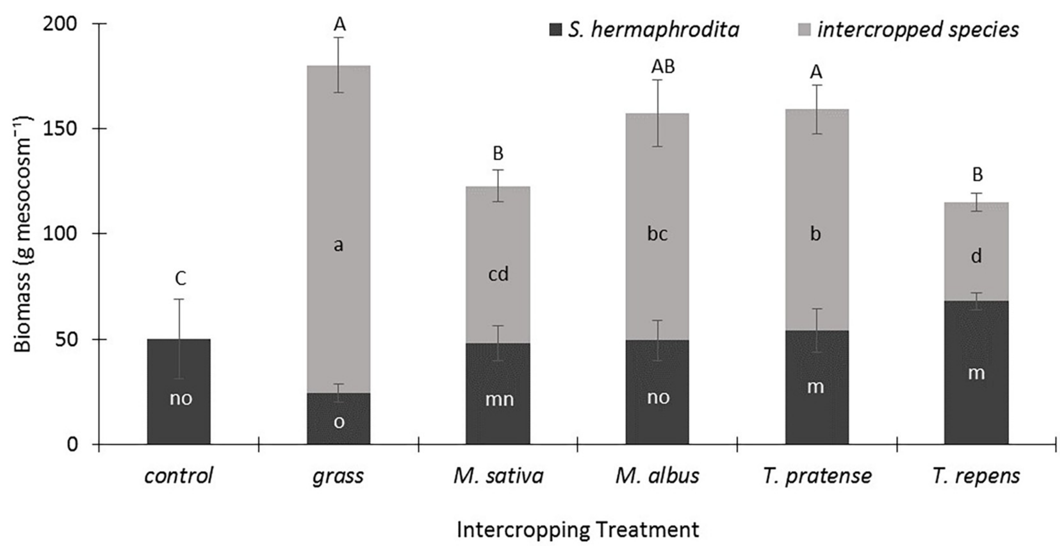

FIGURE 1 | Total aboveground biomass is strongly increased by intercropping *S. hermaphrodita* with grass or legume species in outdoor mesocosms filled with sandy substrate. Control: Digestate fertilization of 40 t ha^{-1}. Bars indicate the standard error (n = 8). Variants with the same letter are not significantly different (0.05 level). Capital letters indicate differences in significance of the total-above ground biomass. Small letters indicate differences in significance of the biomass of the individual species.

TABLE 2 | Biomass and soil analysis of *Sida hermaphrodita* and intercropped species grown in outdoor mesocosms.

Intercropping Treatment	Intercropped species		*S. hermaphrodita*	Soil	
	Nodulation Score	**Nitrogen (%)**	**Nitrogen (%)**	**Carbon (%)**	**Nitrogen (‰)**
Dig (con)			1.0±0.1 a	0.8±0.1 b	0.7±0.0 a
grass mixture		1.1±0.0 c	0.4±0.0 b	2.1±0.1 ab	1.3±0.1 a
M. sativa	18.8±1.5 a	2.5±0.1 b	1.3±0.1 a	2.1±0.5 a	1.4±0.2 a
M. albus	17.3±1.0 a	2.3±0.1 b	1.2±0.1 a	1.4±0.2 ab	0.9±0.1 a
T. pratense	20.4±0.5 a	2.7±0.0 ab	1.3±0. a	1.7±0.4 ab	1.3±0.3 a
T. repens	21.5±0.4 a	3.2±0.1 a	1.2±0.1 a	1.2±0.1 ab	0.9±0.1 a

The nodulation of legumes follows the "Field Guide to Nodulation and Nitrogen Fixation Assessment" of the British Columbia Ministry of Forests (1991). Score 0–14: no effective nodulation. Score 15–20: less effective nodulation. Score 20–25: effective nodulation. Dig (con): Digestate fertilization of 40 t ha^{-1} without intercropping. n = 8 replicates for each treatment. ±Indicates the standard error. Different letters indicate statistically significant differences (p ≤ 0.05)

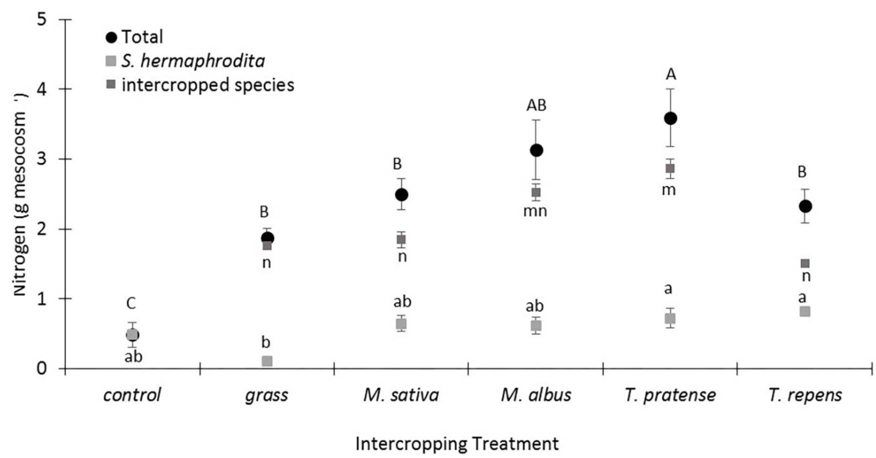

FIGURE 2 | The total nitrogen content per mesocosm increased especially by intercropping *S. hermaphrodita* with *T. pratense*. Outdoor mesocosms filled with sandy substrate. Control: Digestate fertilization of 40 t ha^{-1}. Bars indicate the standard error (*n* = 8). Variants with the same letter are not significantly different (0.05 level). Capital letters indicate differences in significance of the total nitrogen content per mesocosm. Small letters indicate differences in significance of the nitrogen content in the individual species.

the yield of *S. hermaphrodita* compared to *S. hermaphrodita* mono-cropping, although *S. hermaphrodita* grown with the grasses showed the lowest aboveground biomass (**Figure 1**). In the concept of intercropping, a reduced yield of the individual species, but a higher total biomass yield would be expected (Malézieux et al., 2009). In our earlier study, we already tested the intercropping of *S. hermaphrodita* with *M. sativa* (Nabel et al., 2016). However, in this previous experiment we planted both species in the same year. This resulted in a clear advantage of *M. sativa* and a strongly reduced biomass of *S. hermaphrodita*. For the present study, we therefore created a priority effect by establishing *S. hermaphrodita* one year earlier than the intercropped species (see von Gillhaussen et al., 2014; Weidlich et al., 2017). This allowed *S. hermaphrodita* to be already more competitive against the intercropped species, promoting asymmetric competition in favor of the focus crop. Thus, the intercropped species did not negatively influence *S. hermaphrodita* yield compared to the mono-cropping of *S. hermaphrodita* (**Figure 1**). These findings agree with those of Weidlich et al. (2018) where maize grown with a legume neighbor grew as well as one maize plant growing without any neighbor, but where growth next to another grass species (wheat) reduced maize biomass significantly. Our results show no biomass reduction of *S. hermaphrodita* in the legume-intercropped treatments compared to mono-cropped *S. hermaphrodita* (**Figure 1**). This can possibly partly be explained by the fact that our experiments were performed in mesocosms, receiving fertilization and irrigation, reducing the yield-limiting factor of the resources water, light and nutrients. Due to the strong priority effect we found in our earlier study (Nabel et al., 2016), we consider the positive effect of adding the strongly N-facilitating intercrop after the establishment of the target crop *S. hermaphrodita* a promising approach, and indeed, it is a standard procedure in many intercropping systems.

Evidence for N facilitation usually includes either higher leaf N of intercropped species or higher aboveground biomass (Temperton et al., 2007). Technically speaking, since biomass is usually correlated with fitness and reproduction, one could argue that N facilitation has only occurred when biomass is stimulated. Overall, in our study intercropping with the four different legume species positively affected different traits of the *S. hermaphrodita* plants, but only the *Trifolium* species stimulated *S. hermaphrodita* biomass significantly (**Figure 1**). A finding that is also supported by the best nodulation status in the *Trifolium* species (**Table 2**). Seen through the strict facilitation lens, therefore, our study suggests using these two *Trifolium* species (i.e., *T. pratense* and *T. repens*) for *S. hermaphrodita* intercropping (Roscher et al., 2011). If one focuses on above ground biomass N, however, then the winning legume species for intercropping are *M. albus* and *T. pratense*. The species that most increases soil C and N on the other hand is *M. sativa*. This highlights the multifunctional roles different species play in a range of environments and the necessity to decide which ecosystem service one is most interested in fostering in a cropping system, e.g., biomass, carbon storage, shoot nutrient concentrations, or soil fertility. Overall though, it seems that *T. pratense* is an excellent all-rounder for intercropping with *S. hermaphrodita*, growing vigorously and fixing N at high rates. *T. repens* is also a good candidate since it shows high rates of N fixation with non-legume neighbors. However, its competitive ability is not as high as for *T. pratense* such that it would compete less with *S. hermaphrodita*. The higher yields for *S. hermaphrodita* growing with *T. repens* vs. *T. pratense*, not statistically significant though underline this finding.

During intercropping, the individual biomass of *S. hermaphrodita* increased when intercropped with either of the *Trifolium* species compared to the control treatment (**Figure 1**). These two legume species have a long history of successful use in extensive agriculture and are known to often transfer large amounts of their atmospherically fixed N_2 to neighbors or subsequent crops, and have high potential for N_2 fixation in mixtures (Roscher et al., 2011). Carlsson et al. (2009) found that *T. hybridum* and *T. repens* growing with grass neighbors in more diverse grassland communities increased the proportion of N derived from N_2-fixation per biomass of plant compared to growing without grasses. This shows that competitive interactions can push the legume species to rely less on soil N and increase its N_2-fixation reliance. Our study seems to find a similar effect, since the two legume *Trifolium* species that facilitated *S. hermaphrodita* during intercropping the most had the best nodulation scores.

In a similar experiment to our study, using the perennial energy crop switchgrass (*Panicum virgatum* L.) and the intercropping of different legume species including *T. pratense* and *T. repens*, Ashworth et al. (2015) also found a positive effect on biomass yield of the switchgrass. They explain this positive effect on yield by the legume-driven biological N_2 fixation delivering additional N into the system.

The intercropped legume species all showed nodulation and *T. repens* and *T. pratense* were in the range of effective nodulation (British Columbia Ministry of Forests, 1991). These results are backed up by the results of the plant biomass N concentration, where *T. repens* and *T. pratense* both showed highest values of 3.2 and 2.7%, respectively (**Table 2**). In contrast, grass biomass only had a shoot N concentration of 1.1%. *T. repens* and *T. pratense* have been identified earlier for their high potential for N_2 fixation in a 6-year grassland experiment, analyzing 12 different legume species (Roscher et al., 2011). Earlier studies indicated that neighboring species can also benefit from the biological N_2 fixation of the intercropped legume and that one of the possible mechanisms is N sparing, whereby the reliance of the legume on N_2 from the atmosphere leaves soil N ("spare N") for the non-N_2-fixing neighbors (Temperton et al., 2007). Especially for *Trifolium* species a high potential for this N-sharing and N-sparing was found (Frankow-Lindberg and Dahlin, 2013). Results of our study indicate the same, as *S. hermaphrodita* biomass showed a 20–30% increased N concentration when intercropped with the *Trifolium* species compared to the control treatment without legume intercropping. In contrast, grass intercropping reduced the N concentration of the above ground biomass by 60% compared to the control, indicating a strong competition for N (**Table 2**). Grasses like *Lolium perenne, Festuca rubra,* and *Poa pratensis* are known to be very strong competitors that effectively take up soil N (Ravenek et al., 2014).

The calculated total N in the above ground biomass illustrates these differences even more clearly as it combines the data of N content in the biomass of the individual species with the total biomass yield per mesocosm (**Figure 2**). Here we can show a clear difference between the total N content in the mono-cropped *S. hermaphrodita* control treatment and all intercropping treatments, no matter if a legume or grass was intercropped. We suggest that this effect can be explained by nitrate leaching out of the mesocosms, before *S. hermaphrodita* plants were able to take it up. Leaching of nitrate in the used model marginal substrate is a high risk as we could show in an earlier mesocosm experiment with *S. hermaphrodita* cultivated on sandy substrate (Nabel et al., 2016). Intercropping systems can be more effective in taking up N before it leaches out of the rhizosphere (Malézieux et al., 2009). Further, mesocosms in which *S. hermaphrodita* was intercropped with *T. pratense* contained more than 75% higher total N than mesocosms in which *S. hermaphrodita* was intercropped with grass. We relate this difference to the biological N_2 fixation in *T. pratense*, while grass depends on the available N in the soil and rhizosphere. N derived from the atmosphere by biological N_2 fixation can also be susceptible to leaching (Böhm et al., 2009; Warwick et al., 2016). Promising further steps in research would be to explicitly combine species in intercropping systems that have complementary root architecture (Malézieux et al., 2009). In the present study, the best performing intercropping species for *S. hermaphrodita* were *T. repens* and *T. pratense*, both of which have relatively shallow root systems, while *S. hermaphrodita* has a very deep reaching root system (Borkowska et al., 2009). In contrast, *M. sativa* and *M. albus* both have deeper reaching root systems like *S. hermaphrodita* and are therefore less suited for the intercropping with *S. hermaphrodita* since both may compete for the same local resources, especially in pots, limiting the maximum rooting depth. However, since plant biomass was analyzed after a total growth period of 3 years a reliable assessment of spatial root distribution was not feasible in the mesocosms we used with a maximum depth of 0.4 m.

Therefore, field trials under agricultural conditions on marginal soils analyzing the dynamics of spatial root distribution of the used species combinations are now required to test whether our findings really were mainly driven by different root architectures and rooting depths.

An additional aspect of the more densely rooted soil is the potential for short term carbon storage in the soil (Steinbeiss et al., 2008a,b). We discussed the potential of an increased soil carbon content of marginal sandy substrate and the associated beneficial effects, like increased water holding capacity and soil respiration on soil fertility in an earlier publication (Nabel et al., 2017). Besides, the dense colonization of the top soil can reduce the high risk of erosion of the light sandy substrate (Liebman and Dyck, 2009).

SUMMARY AND CONCLUSION

Intercropping of *S. hermaphrodita* with legume species can be an efficient way to increase the biomass output per unit area. We found no negative influence of legume intercropping on the biomass yield of *S. hermaphrodita* compared to mono-cropping. Legumes performed biological N_2 fixation and thus enriched the cropping system with this essential nutrient. *S. hermaphrodita* benefited from the intercropping particularly when grown with *T. repens* or *T. pratense*. Highest biomass as well as highest total N content were reached when *S. hermaphrodita* was intercropped with *T. pratense.* Intercropping with *M. sativa* and *M. albus* still increased the total biomass yield but less effectively than intercropping with the *Trifolium* species. Further experiments could elucidate if these findings are mainly driven by differences in root architecture, with deeper rooting *M. sativa* and *M. albus*, being less complementary to the deep-reaching root system of *S. hermaphrodita.* When *S. hermaphrodita* was intercropped with grass, the latter caused strong competition for N. However, intercropping generally increased the N uptake, presumably reducing the risk of nitrate leaching in the light substrate.

The presented results were obtained from a model marginal substrate in mesocosms and are therefore not directly transferable into agricultural practice but indicate a promising possible direction for larger scale tests of such intercropping systems. Site-specific case studies in the field are now needed to test suitable combinations, such as those used in this study and others, to particular sites and management regimes (McWhinney, 2001). Results from such experiments could support the idea that efficient identification of growth traits of species can enable an optimal promotion of facilitation and niche complementarity resulting in better yields as well as more resilient systems (Hernandez and Picon-Cochard, 2016). We conclude that legume intercropping into perennial *S. hermaphrodita* energy crop cultures is an efficient way to increase the total biomass yield, and decrease the dependency on additional N fertilization whilst allowing organic C to enrich the soils, allowing for an extensive cultivation of marginal soils; specific legume species with not so deep rooting systems may be the best option for this when intercropping with *S. hermaphrodita*.

AUTHOR CONTRIBUTIONS

MN, SDS, VT, and NDJ designed the study. MN performed the main experiments and conducted the research under the supervision of SDS and NDJ. LH helped with experimental work and data acquisition. MN and SDS wrote the manuscript. All authors discussed the results, assisted in the manuscript preparation, and contributed to revisions.

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Evaluation of the Process Steps of Pretreatment, Pellet Production and Combustion for an Energetic Utilization of Wheat Chaff

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This paper investigates the potential energetic utilization of wheat chaff via combustion and necessary process steps such as pretreatment and pellet production as an example of stramineous biomass. Chaff is one out of three main fractions during harvesting and remains usually on the field. Vice versa, it is a potentially so far unexploited biomass resource, which could serve as renewable energy source. Simultaneously, exploiting wheat chaff could intensify the economic benefit of agricultural land use. The combustion application requires choosing an applicable firing system adjusted to the properties of chaff. The economic feasibility of the combustion application depends strongly on the technical effort, which depends on the fuel. Chaff consists mainly of husks and straw. Due to the low ash melting point and the high chlorine content of straw, it can be advantageous for the combustion to remove short straw from the chaff by sieving. To evaluate the combustion properties of presorted and original chaff the elemental composition, the net calorific value, the volatiles and ash content and the ash melting behavior were determined. The efficient logistic of chaff is decisive for its economic exploitation. This is in conflict with the low bulk density of chaff and the resulting high storage volume. Compressing chaff into pellets optimizes its handling. This vital step for an economic exploitation of chaff was therefore investigated by this study. Parameters such as water content of the feed, addition of binders and the geometry of the pressing die bores influence the pelletizing success significantly. Thus, these parameters were investigated in this study. The resulting pellets were characterized with standardized methods and compared to the requirements of EnPlus standards in terms of mechanical durability, amount of fines, bulk density and final moisture content. Finally, combustion experiments in a Large-scale Oven for Kinetics Investigation characterized the burnout behavior of the produced pellets. This was compared to pine wood pellets as conventional fuel. The performed investigations show that the pellet production and the subsequent combustion of wheat chaff pellets is a feasible approach for an energetic utilization.

Keywords: wheat chaff, stramineous biomass, harvest residues, energetic utilization, weed reduction concept, pellet production, densification, combustion behavior

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INTRODUCTION

Since the EU Directive on the promotion of electricity produced from renewable energy sources has become effective in 2001, exploiting renewable energy resources is at the center of ecopolitical attention. To achieve the goal of covering 20% of the final energy demand in the EU from renewable sources by 2020, the energetic usage of biomass residuals and the mobilization of new biomass potentials are of vital importance (Directive 2009/28/EC, 2009). Using biomass residuals have the essential advantages of not requiring an additional acreage and of not competing with the food industry. Wheat chaff is such a biomass residual with unexploited energetic potential. It is a side product of annual harvesting and remains usually on the field. There, it serves as humus and nutrient supplier for the soil. Depending on farming practices and local conditions, such as climate and soil type, crop residuals can be collected with a different rate without affecting the soil balance (Cherubini and Ulgiati, 2010; Monteleone et al., 2015). Weiser et al. (2014) estimate a removal rate of 0.33 in order to provide a stable soil balance. The total harvested material consists approximately of 50 %(w/w) corn, 25 %(w/w) straw and 25 %(w/w) chaff (Stern, 2010). With an annual harvest of 152 Mt wheat and spelt in the EU-28, chaff represents a biomass potential of 38 Mt/a (EUROSTAT, 2017). The net calorific value of spelt chaff varies in literature between 15.1 and 16.8 MJ/kg (Kiš et al., 2017; Wiwart et al., 2017). Considering the lowest calorific value of 15.1 MJ/kg and the removal rate of 0.33, wheat and spelt chaff have a theoretical potential of 191 PJ/a in the EU.

However, collecting chaff comes with more advantages than making an unexploited energy source available. Agricultural production is increasingly fighting with weed infestation of herbicide resistant weeds (Jacobs and Kingwell, 2016; Peterson et al., 2017). According to the international survey of herbicide resistant weeds, the number of resistant weeds doubled from 1998 until 2017 globally (Heap, 2018). During harvesting most of the harvested weed seeds end up in the chaff fraction and thus the collection of chaff prevents weed seeds of entering the soil bank (Walsh and Newmann, 2007; Douglas et al., 2013; Chauhan, and Mahajan, 2014). Collecting chaff can enhance the crop yield by lowering weed pollution in the following years. Simultaneously a new biomass potential is created. From an ecological point of view, the energetic use of chaff contributes to a CO_2-neutral and sustainable energy supply. Furthermore, it generates additional economic benefit of agricultural fields and intensifies the agricultural land use.

Chaff is a heterogeneous mixture, consisting mainly of husks and straw. While the combustion and energetic use of straw is already well investigated and applied, for chaff it is so far not sufficiently studied. Chaff is expected to be a beneficial fuel for combustion processes due to a low chlorine content and a high ash melting point (Stern, 2010; Heidecke et al., 2014). The low ash melting temperature, the high ash content and the high content of Cl, N and alkali metals increase the technical effort for the combustion application of straw (Salzmann and Nussbaumer, 2001; Kaltschmitt et al., 2009; Wang et al., 2014). Pre-sorting chaff to sort out straw can therefore be beneficial for the combustion properties. It leads potentially to a lower technical and monetary effort for the combustion application. Untreated chaff has a low bulk density of around 56 kg/m^3 (McCarntey et al., 2006). Its usage is therefore connected with high transport and logistic costs. Pelletizing chaff can optimize the logistics by reducing the transport and storage volume. To evaluate the pelletizability of chaff, optimized parameters need to be found.

This work provides a first comprehensive study about the feasibility of exploiting chaff energetically via combustion. First investigations and insights of the effect of pre-sorting, the pelletizability of chaff and the combustion behavior were done.

MATERIALS AND METHODS

Wheat Chaff

The examined material is winter wheat chaff from a harvest in Germany in 2015. The chaff was harvested by the company Claas Selbstfahrende Erntemaschinen GmbH and stored sealed since then. At the time of investigation the material had a water content of 7.5 %(w/w). The original chaff fraction consists mainly of husks with a length of 0.8 cm and straw with a length of 1–20 cm. The bulk density is 34 g/L.

Used Devices

Pellet Press

The used pelletizing device is the press 14-175 by Amandus Kahl. It is a flat die press (**Figure 1**). Dies with different l/d-ratio can be applied. The dies differ in the length of the bores (l) and have a bore diameter (d) of 6 mm. A thermocouple allows checking the temperature at the outer side of the die. The press is fed manually from the top. The produced pellets fall onto a vibrating sieve with a mesh size of 4 mm and a total length of 1.3 m.

Large-Scale Oven for Kinetic Investigations (LOKI)

The combustion experiments were carried out in a Large-scale Oven for Kinetics Investigation (LOKI). **Figure 2** shows the schematic layout of the oven. The reactor itself is a tube out of quartz glass with a diameter of 198 mm and a length of 1610 mm. A gas-permeable frit separates the tube into two oven zones (OZ1 and OZ2). The oven allows heating up to 900°C with a heating rate of maximum 40°C/min. The heating elements of the two oven zones are regulated separately. The reactor setup provides a defined atmosphere inside the oven zones. The lower oven zone (OZ1) can be supplied by technical air, nitrogen or a combination of both. The upper oven zone (OZ2) can be supplied by technical air and serves as secondary combustion chamber for the released gases. A flow regulator can set the volume flow of the respective gases. The reactor setup provides kinetic information about the sample mass and the sample temperature under heat exposure.

The sample mass is measured by a scale system. Therefore, a wide-meshed glass fiber basket is mounted on a sample suspension, which is connected to the scale. The sample is placed inside the basket. To prevent an impact on the scale, the scale system is purged continuously by nitrogen during the experimentation. The core temperature of the sample is measured by sticking the sample on a thermocouple type k (thermocouple 1). The thermocouple is fixed at a flange, which

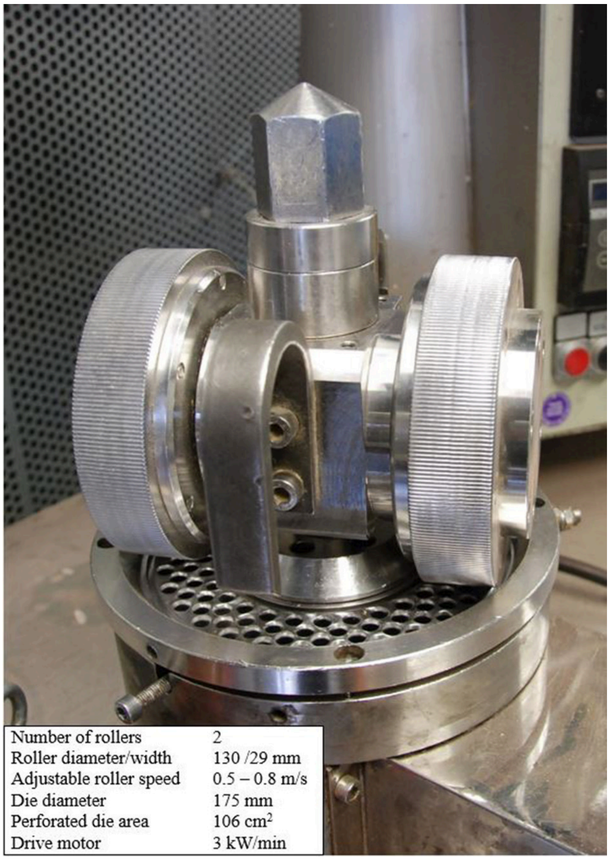

FIGURE 1 | Used flat die press 14-175 by Amandus Kahl.

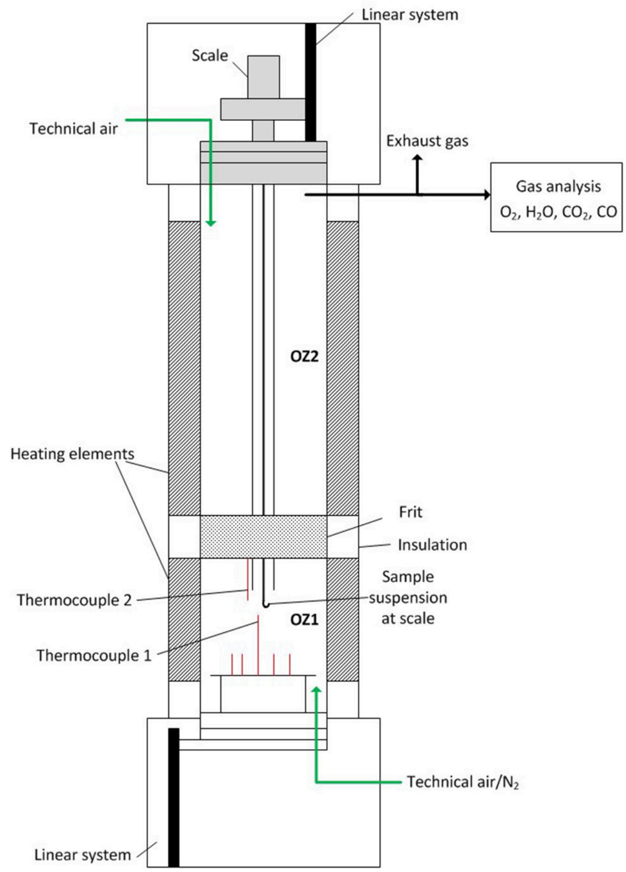

FIGURE 2 | Schematic layout of the used Large-scale Oven for Kinetic Investigations (LOKI).

closes the lower part of the reactor. The sample suspension and the flange are moved by pneumatic linear systems. At the beginning of the investigation the sample is moved up into the middle of the OZ1. Moving up takes 3 s. Due to the movement the signal of the scale is disturbed. After reaching the end position the mass loss can be recorded without interruptions.

A gas-analysis unit allows the measurement of O_2, H_2O, CO_2, and CO in the exhaust gas. H_2O, CO_2, and CO are measured by nondispersive infrared technology (NDIR). O_2 is measured electrochemically. The gas pipe is heated to prevent condensation of the exhaust gases.

The scale works on the vibrating wire principle. A tensioned wire vibrates at its natural frequency after being electromagnetically induced. The mass of the sample changes the tension of the wire leading to a change of its natural frequency. This is converted into a mass.

Experimental Procedures

Pretreatment

As pre-treatment, wheat chaff was sieved using a drum screen in order to sort out straw. The sieve holes of the first half of the drum screen are circular with a diameter of 8 mm. The sieve holes of the second half of the drum screen are elongated with a length of 21 mm and a width of 8 mm. By pre-sieving, 20 %(w/w) of the original chaff fraction was sorted out as coarse fraction consisting mainly of straw. The fine fraction consists mainly of husks and small thin straw of up to 5 cm. The bulk density of the fine fraction is 50 g/L. The pelletizing and combustion experiments were performed with the original chaff and the fine fraction.

Pelletizing

Each pelletizing trial was performed with a feedstock of 3 kg. Initially the raw material was dried in an oven at 80°C to ensure a defined water content for the experiments. The coarse chaff fraction f was milled with a cutting mill to a maximum size of 8 mm to ensure a smooth feeding of the pellet press. The fine chaff fraction did not require milling. The dry material and potential binders were mixed with tap water to adjust the intended initial water content for the pelletizing experiments. The mixing was done in a conventional cement mixer for at least 20 min or until homogeneity of the mixture.

Prior to each test, the die was preheated until the thermocouple on the outer side of the die showed >50°C, because the glass transition temperature of straw is between 53 and 63°C (Whittaker and Shield, 2017). The die was preheated by adding pre-grinded initial material to the running pellet press until the desired temperature was reached due to friction. Once the temperature was reached, the feed was switched to the desired feedstock. The produced pellets were collected after 5–10 min of pelletizing to ensure that the initial material was flushed out completely. The feeding was done manually and continuously. The speed of the rollers was adjusted depending on the pelletizing flow. The pellets cooled down at room temperature and were stored in a sealed box.

Combustion

The combustion experiments were performed with pellets produced with an initial feedstock moisture content of 20 %(w/w). The water content of fine fraction chaff pellets was 9.3 %(w/w) and 9.7 %(w/w) for original chaff pellets respectively. The combustion behavior was compared to the combustion behavior of commercial pinewood pellets produced by EcoPowerPlant Sp. Z o. o. with a water content of 6.3 %(w/w) (product code: 6BFM15W100). The chaff as well as pinewood pellets have a diameter of 6 mm and a length of 16–17 mm. The weight of a single pellet differed between 0.57 and 0.61 g.

The combustion experiments included experiments to measure the core temperature and the mass loss of a single pellet as well as of a bulk of seven pellets. The combustion was performed in technical air atmosphere with 21 vol.% O_2 with a sudden sample supply into the hot oven. The volume flow in OZ1 was set to 10 L/min and in OZ2 to 5 L/min. OZ2 was heated up to a temperature of 1,000°C. OZ1 was heated up to 900°C.

In order to stick the pellet on the thermocouple a hole of 1 mm width was drilled into the pellet so that the top of the thermocouple is located at the core of the pellet. After 10 min when steady conditions prevailed, the sample was moved upwards into the hot oven. The combustion experiments of a single pellet were switched off after 600 s and for 7 pellets after 1,400 s respectively. Thereafter no further mass loss and volatile release were recorded. All experiments were performed twice.

Evaluation Methods

Characterization of Chaff Fractions

The fine and the coarse chaff fraction were characterized with regard to their elemental composition and the relevant combustion properties.

The parameters were determined according to following norms:

Net calorific value: DIN 51900 (comparable to DIN EN ISO 18125)
Ash content: DIN EN ISO 18122
Volatiles: DIN 51720 (comparable to DIN EN ISO 18123)
H_2O content: DIN 51718 (comparable to DIN EN ISO 18134)
C, H, N: DIN 51732 (comparable to DIN EN ISO 16948)
S, Cl: DIN 51724-1 and DIN 51727 (comparable to DIN EN ISO 16994)
Heavy metals, nutrients: following DIN 51729-11 (comparable to DIN EN ISO 16967)
Ash melting behavior: DIN CEN/TS 15370-1

The calculation of the net calorific value on a wet basis ($q_{net;wb}$) into the net calorific value on a dry basis ($q_{net;db}$) was calculated according to

$$q_{net,db} = \frac{q_{net,wb} \times 100 + 2.443 \times w}{100 - w} \quad [MJ/kg] \tag{1}$$

$$w = \text{water content}[\%(\text{w/w})]$$

following Kaltschmitt et al. (2009).

Evaluation of the Pelletizing Success

The moisture content, the pellet durability Index (PDI), the bulk density (BD) and the amount of fines classify pellets. The pellet formation rate (PFR) is relevant to assess the pelletizing process.

Moisture content

To determine the moisture content, a sample of 300 g (m_w) dried in an oven at a temperature of 80°C. After 24 h the sample weight was measured and the sample was placed back to the oven. After 48 h the sample was taken out of the oven and the weight (m_d) was measured again. If there has been no further mass loss, the moisture content (x_m) was calculated according to

$$x_m = \frac{m_w - m_d}{m_w} \times 100 \ [\%(w/w)] \tag{2}$$

Pellet durability index (PDI)

To evaluate the mechanical stability of the pellets, the durability of the produced pellets was determined according to DIN EN ISO 17831-1. 500 g of pellets (m_0) were placed in a rotating box containing an impact crusher. The box rotated 50 times per minute for 10 min. Afterwards the sample was sieved with a hole size of 3.15 mm. The final weight of the coarse fraction (m_f) was measured and the PDI was calculated according to

$$PDI = \frac{m_f}{m_0} \times 100 \ [\%] \tag{3}$$

Bulk density (BD)

The bulk density of pellets was determined by using a test volume of 1 L. The mass, which filled a volume of 1 L after slight shaking, was measured. This relation is the bulk density in g/L.

Amount of fines

To quantify the abrasion of the pellets the amount of fines (x_f) was determined following DIN EN ISO 18846. Approximately 500 g of pellets (m_0) were sieved manually. The used sieve had a hole size of 3.15 mm. The sieve was filled so that the surface was covered with one layer of pellets. The amount of fines was calculated according to

$$x_f = \frac{m_{fines}}{m_0} \times 100 \ [\%] \tag{4}$$

Pellet formation rate (PFR)

The PFR was evaluated by measuring the mass (m_P), which was collected behind the vibrating sieve as pellets and the mass (m_u), which trickled through the vibrating sieve as uncompressed material. The PFR is an orientation to quantify the pelletizing success and is given as

$$PFR = \frac{m_P}{m_P + m_u} \times 100\ [\%] \tag{5}$$

RESULTS

Characterization of Chaff Fractions

Table 1 shows the elemental composition of the fine and the coarse chaff fraction as well as the weighted average of both. 80% fine and 20% coarse fraction represent the original chaff. Pre-sorting chaff decreases the Cl content significantly. In addition, the content of the alkali metals K and Na are lower in the fine fraction compared to the original chaff. The Si content in contrast is increased. The N content of all chaff fractions is with 0.508 to 0.668 %(w/w) high in comparison to conventional biomass fuel such as wood. **Table 2** shows further determined properties relevant for the combustion. The fine fraction has a 7% higher ash content compared to original chaff. The slightly lower calorific value of the fine fraction corresponds to the higher ash content.

Figure 3 shows the measured characteristic temperatures of the ash melting behavior. The sintering starts for all fractions below 800°C. While the coarse fraction starts to deform at temperatures below 1,000°C, the fine fraction and the original chaff have a significantly higher deformation temperature (DT) of >1,300°C. One determined DT of the fine fraction is with 1,095°C significantly lower than the DT of the other measurement with 1,377°C. This can be explained by the determination procedure. For the determination a software evaluates the shape of the sample and characterizes it using a form factor. The software defines the DT as the temperature at which the form factor of the sample is 15% different to the form factor of the original sample. The change of the form factor does not necessarily need to be caused by rounding of the edges due to melting. Therefore, the determination of especially the DT is subject to errors. Thus, this outlier is likely due to the software evaluation. The hemisphere and the flow temperature of pre-sieved chaff and the original chaff are above 1,500°C. These values are comparable with the flow temperature of wood (Kaltschmitt et al., 2009). For the coarse fraction in contrary, the flow temperature is only slightly above 1,200°C. An effect of sorting cannot be noted by the determined values. The high ash melting temperatures of pre-sieved chaff and the original chaff can be explained by the higher Si and the lower K content in the fine fraction compared to the coarse fraction (see **Table 1**; Stern, 2010).

Pelletizing Experiments

The pelletizing success strongly depends on the used die and the moisture content of the feedstock. Furthermore, binders can be applied to improve the pelletizing success. The influence of the l/d ratio of the die, the moisture content of the feed and the addition of starch as a potential binder are discussed in the following.

Effect of l/d

Changing the die in an industrial pellet press requires high technical effort. It is therefore preferable to choose a die, which is suitable for a wide range of feedstock parameters. To decide which die to use for further experimentation, the fine fraction was pelletized using a die with a l/d-ratio of 4 (= die 4) and of 5 (= die 5) at initial moisture contents of 12, 16 and 20 %(w/w).

During pelletizing it was observed that a higher l/d-ratio leads to increased evaporation and therefore to drier material. This is confirmed by the final moisture contents of the produced pellets. All determined pellet characteristics are summarized in **Table 3**. In case of dry material with an initial feedstock moisture content of ≤ 16 %(w/w), a higher l/d-ratio decreased the pellet formation and impeded the pelletizing process. At a moisture content of 12% the pelletizing process was difficult with both dies. The pellet press often got jammed and dust trickled through the die along with marginal pellet formation. With die 5 the process was terminated as the press got jammed too often. At a moisture content of 16% similar problems occurred with die 5. The pelletizing process became difficult when the material becomes too dry due to evaporation. With die 4 the problems diminished and the pelletizing was smooth. At a moisture content of 20 mass-% the pelletizing was smooth with both dies. The pellets produced with die 5 exhibited better durability, a higher bulk density and a lower final pellet moisture content (see **Table 3**).

The experiments with different dies showed that pelletizing at a moisture content of 16% with die 4 and at a moisture content of 20% with die 5 exhibited good pellets in combination with an easy pelletizing. Overall, the pellets produced at 20% with die 5 showed the best characteristics of all produced pellets. Considering that die 4 only exhibited acceptable results at a

TABLE 1 | Elemental composition of chaff fractions after sieving in %(w/w) on a dry basis (averaged values with standard deviation).

	C	H	N	S	Cl	K	Ca	Mg	Na	Si
Fine fraction	44.4 (±0.2)	5.430 (±0.08)	0.668 (±0.164)	0.070 (±0.009)	0.053 (±0.005)	0.526 (±0.025)	0.103 (±0.025)	0.043 (±0.010)	0.008 (±0.001)	3.450 (±0.19)
Coarse fraction	46.0 (±0.6)	5.519 (±0.096)	0.508 (±0.052)	0.095 (±0.008)	0.155 (±0.003)	0.852 (±0.05)	0.139 (±0.016)	0.044 (±0.003)	0.023 (±0.005)	1.788 (±0.213)
Chaff (Weighted average*)	44.7	5.448	0.636	0.075	0.073	0.591	0.112	0.043	0.011	3.117
Wood**	50.1	6.1	0.090	0.010	0.010	0.044	0.09	0.017	0.004	0.038
$\Delta \frac{\text{Fine fraction}}{\text{Chaff}}$ [%]	−0.7	−0.3	+5.0	−6.7	−27.4	−11.0	−8.00	0	−27.3	+10.7

**80% fine chaff fraction and 20% coarse chaff fraction. **values taken from Amand et al. and referred to the dry material incl. ash (Amand et al., 2006).*
C, carbon; H, hydrogen; N, nitrogen; S, sulfur; Cl, chlorine; K, potassium; Ca, calcium; Mg, magnesium; Na, sodium; Si, silicon.

TABLE 2 | Determined parameters on a dry basis (averaged value with standard deviation).

	Net calorific value [MJ/kg]	Ash content [%(w/w)]	Volatiles [%(w/w)]
Fine fraction	16.14 (±0.09)	8.36 (±0.31)	85.10 (±1.36)
Coarse fraction	16.83 (±0.30)	5.55 (±0.42)	86.89 (±0.63)
Chaff (Weighted average)	16.28	7.80	85.46
Wood*	18.6	0.5	81.49

*values taken from Amand et al. and referred to the dry material incl. ash (Amand et al., 2006).

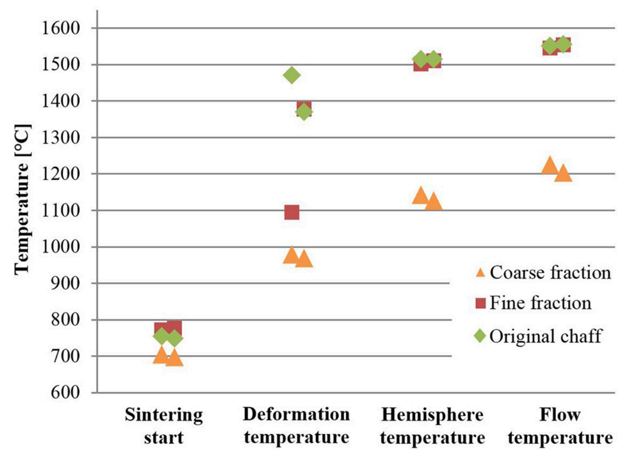

FIGURE 3 | Characteristic temperatures of the ash melting behavior of different fractions (double determination).

TABLE 3 | Characteristics of produced pellets with different dies and from feedstocks with different initial moisture content.

Water content	12%		16%		20%	
l/d	4	5*	4	5	4	5
Final moisture content of pellets [%(w/w)]	8.3	n/a	10	6.3	13.3	9.3
Bulk density [g/L]	631.0	n/a	624.7	700	434.4	677.5
PDI [%]	87.6	n/a	97.6	89.8	96.4	98.6
Amount of fines [%]	0.8	n/a	≤0.2	0.6	≤0.2	≤0.2

*Experiment aborted as the press got jammed too often.

moisture content of 16%, die 5 was chosen to examine a wider range of initial moisture contents for the pelletizing process. Furthermore, the difference of pelletizing the original chaff and the fine fraction was examined using die 5.

Effect of Initial Moisture Content

To analyze the effect of the initial moisture content of the feedstock only the experiments performed with the same die (die 5) are compared. The effect was investigated for the fine fraction as well as for the original chaff by varying the moisture content from 8 to 28 %(w/w) in 4 absolute %(w/w) steps. The performed experiments present a screening of the pelletizability of chaff at different initial moisture contents. To evaluate the reproducibility, the experiments at a moisture content of 20 and of 24% were repeated.

The experiments showed that the pelletizing process becomes easier with an increasing moisture content until a certain level. Exceeding this level decreases the stability of the produced pellets rapidly. If the material is too dry, the press often gets jammed and the pellets crumble inside the bores to dust. This shows the binding as well as the lubricating function of water during pelletizing. If the initial moisture content is too high, the densification is low and the final pellet moisture content is too high. This results in an insufficient shelf life of the pellets. At a moisture content of ≤16 %(w/w) for fine fraction chaff and ≤12 %(w/w) for original chaff, the produced pellets are bright and their lengths vary from 3 mm up to 15 mm. At a moisture content of 16 %(w/w) uniform pellets with a length of 14 to 16 mm were produced from original chaff. At moisture contents of 20 %(w/w) the pellets have a characteristic length of around 16 to 18 mm, are darker and have an external gloss. At a moisture content of 24%, the pellets of the different trials vary a lot. The pellets of one trial look the same as the ones produced at 20%. The pellets of the other trial are shorter, have no external gloss and are partly crumbly. One possible reason for such variation is insufficient mixing of water and dry feed during conditioning. Another explanation is a variation in evaporation during pressing due to varying temperatures inside the press. At a moisture content of 28 %(w/w) the pellets are short, crumbly and fall easily apart. **Table 4** summarizes the determined parameters of the produced pellets. The low bulk density confirms the insufficient densification at too high moisture contents. The final pellet moisture content is depending on the moisture content of the feed as well as the evaporation during the pressing process. This explains the varying moisture content of the produced pellets. Pellets produced with a feed moisture content of 28 and 24% for the second trial exceed the limit of 10 %(w/w) final moisture in the end product clearly.

Successful pelletizing needs to offer a high PFR in combination with durable pellets. **Figure 4** shows the PFR as well as the durability of the produced pellets. When pelletizing the original chaff, a moisture content of 16 and 20% showed smooth pelletizing in combination with a durability exceeding the EnPlus standards. For the fine fraction this applies only for pellets produced at 20%. The characteristics of the pellets produced at 24% vary strongly between the trials.

The different pelletizing results at 24% show the difficulty to ensure a uniform process. Already small differences of the conditions can influence the pelletizing process significantly. Pelletizing at a moisture content of 20% showed a good reproducibility. The produced pellets of both trials showed the best results for the fine fraction as well as for original chaff and are in good accordance with the EnPlus standards.

Effect of Binding Agent

To investigate the effect of a binding agent, 1 and 2 %(w/w) starch was added on a dry basis at initial feedstock moisture contents of 16 and 28 %(w/w). To examine if adding binder

TABLE 4 | Characteristics of produced pellets from feedstocks with different initial moisture contents (l/d = 5).

Feed	Initial moisture content [%(w/w)]	Final moisture content of pellets [%]	Amount of fines [%]	Bulk density [kg/m^3]
Fine fraction	12*	n/a	n/a	n/a
	16	6.3	0.6	700
	20	9.6 (±0.3)	≤0.2 (±0.0)	733.8 (±56.3)
	24	12 (± 2.0)	0.7 (±0.5)	561.5 (±129.8)
	28	14.4	1.6	297.7
Original chaff	8	6.3	4.7	746.7
	12	8.7	0.2	670.6
	16	10.3	≤0.2	647.5
	20	9.8 (±0.1)	≤0.2 (±0.0)	705.5 (±6.3)
	24	13.6 (±2.9)	0.6 (±0.4)	589.85 (±68.25)
	28	15.04	1.7	388.75
EnPlus standards		≤10	≤1	600 ≤ BD ≤750

Experiment aborted as the press got jammed too often.

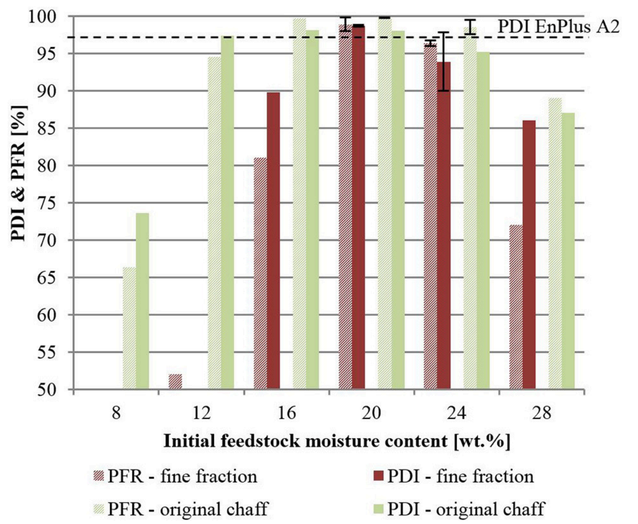

FIGURE 4 | Pellet durability index (PDI) and pellet formation rate (PFR) for wheat chaff pellets of fine fraction chaff and original chaff at different moisture contents (l/d = 5).

allows using a die with a smaller l/d-ratio while keeping the pellet quality it was added to the fine fraction at a moisture content of 16 %(w/w) using die 4. The pellets produced at 16 %(w/w) water content showed no improved characteristics. To investigate if starch requires a higher water content in order to develop its binding function, it was also added at a moisture content of 28 %(w/w). Here, also starch could not influence the pellet properties positively as the same problems of a too high moisture content occurred as well.

Combustion Behavior

During all combustion experiments the pre-sieved and the original chaff pellets kept their form and only shrank in size. The size of the ash pellets after the combustion was measured in case they did not break after removing them from the oven. The shrinkage of the pellets was around 20% in length and 30% in diameter. Unlike wood ash, the ash of chaff was dimensionally stable (see **Figure 5**). This observation fits to the determined sintering temperature of <800°C. The black color of the chaff ash can be a sign that the pellet was not completely oxidized. The TOC of the ash was determined as 0.67% (±0.12). The values were at the determination limit due to the small sample weight. This shows that already small amounts of oxidizable elements can be responsible for the black color. Between the residuals at 800 and 900°C combustion temperature no difference was notable. The residuals after 1,400 s had partly white spots and were slightly brighter than the ones after 600 s. The color change may indicate that the longer dwell time in the oven allowed a further burnout. Measuring the core temperature revealed that the pellets of wood and chaff heat up with the same speed (**Figure 6**). While the pellet heats up pyrolysis and gasification reactions take place and determine the heating speed of the pellet. The sudden drop of the core temperature of wood pellets after around 160 s indicates that the wood pellet lost its form and fell from the thermocouple. In contrast, the chaff pellet stays on the thermocouple during the whole experiment. This is shown by the slow temperature decrease until the core temperature has declined to ambient temperature. It stays constant after 260–270 s. A constant temperature indicates that there is no further oxidation.

During the devolatilisation in the first 45 s the mass loss of chaff and of pinewood happens with the same speed. The devolatilisation phase for chaff pellets is 5–10 s shorter than for wood pellets due to its lower volatile content. The speed of the char combustion is similar for both, chaff and wood. After around 330 s no further mass loss is registered. The higher ash content of chaff compared to wood is clearly visible by the residual curves. Between the burnout of the original chaff pellet

FIGURE 5 | Pellets before **(left)** and after **(right)** combustion at 900°C; t = 1,400 s; above: chaff pellets of fine fraction; below: pinewood pellets.

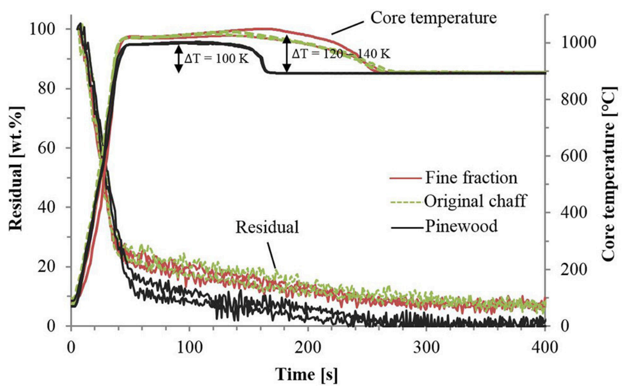

FIGURE 6 | Core temperature and residual of one pellet during the combustion at 900°C with $\dot{V}_{tech.air}$ = 10 L/min.

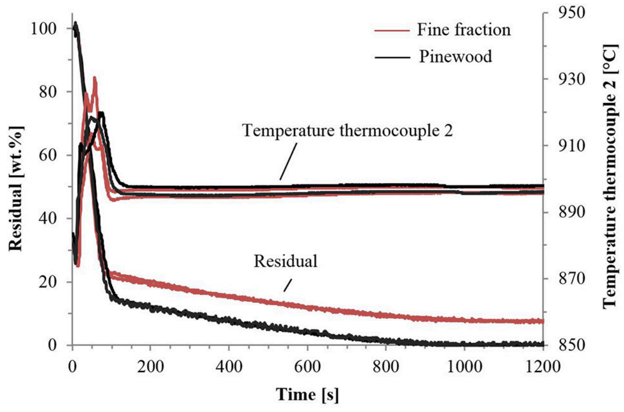

FIGURE 7 | Temperature above the sample and residual of seven pellets during the combustion at 900°C with $\dot{V}_{tech.air}$ = 10 L/min.

and the pre-sieved chaff pellet, no differences are observed. The graph for the residual matches for all experiments. This shows the repeatability of the experiments and allows evaluating chaff pellets as potential fuel.

When combusting seven pellets as a bulk the different phases during the combustion are clearly visible (**Figure 7**). The endotherm release of free and bound water causes the temperature drop above the sample during the first 6–9 s. Afterwards the temperature increases. The temperature peak indicates the oxidation of released gases outside of the pellet. The temperature above the sample stabilizes after devolatilisation and decreases to ambient temperature. The temperature stabilizes earlier for chaff than for wood. This confirms the shorter devolatilisation time of chaff due to a lower percentage of volatiles. The end of the devolatilisation phase is 15 to 20 s earlier compared to wood. During the char combustion the oscillation of the residual measurements is lower due to the higher residual mass. After around 950 s there is no further mass loss for chaff and for wood pellets.

DISCUSSION

In order to achieve a comprehensive study about the feasibility of exploiting chaff energetically via combustion, this work investigated pre-sorting, pelletizing and the combustion of wheat chaff. The following evaluates the process steps pre-treatment, pelletizing and combustion based on the performed investigations. The combustion properties of chaff pellets are compared with pine wood pellets in order to derive the applicability of chaff for existing firing systems.

Pre-treatment

As potential pre-treatment, pre-sorting chaff and its effect on the physical and chemical properties of the chaff mixture was investigated. Sorting out straw increased the bulk density and eased the handling. This effect is advantageous for the pelletizing process. The pelletizing experiments showed that the small and homogeneous particle sizes of pre-sieved chaff allow feeding the pellet press without prior milling. Original chaff in contrary requires milling prior pelletizing. Pre-sieved chaff has a 7% higher ash content compared to original chaff. The ash content of pre-sieved chaff as well as original chaff is with around 8% high compared to wood. Such a high ash content requires special measures for the combustion application of pre-sieved as well as original chaff. The higher ash content after pre-sieving has therefore no direct effect on the required technical effort. Furthermore, pre-sieved chaff has a 27% lower chlorine content. The chlorine decrease combined with the decrease of the alkali metals potassium and sodium lowers the risk of high temperature chlorine corrosion in furnaces (Kaltschmitt et al., 2009; Ma et al., 2017). High temperature chlorine corrosion has a direct influence on investment and maintenance costs of a firing system. Although sieving as pre-treatment means additional effort, it is expected that it has a positive cost-benefit ratio due to the lower chlorine content together with the improved handling of pre-sieved chaff.

Pelletizing

Pelletizing chaff exhibits a homogeneous fuel with an energy density of 9.7 MJ/m^3. The storage and transport volume is decreased by around 93% compared to uncompressed chaff. As chaff accrues annually it needs to be stored throughout the year. Considering the dimensions of storing, it is expected that compressing is beneficial in order to improve the logistics even though it requires an additional process step. The pelletizing experiments showed that pre-sieved as well as original chaff can be sufficiently pelletized at a moisture content of 20%. The received pellets fulfill the ENPlus standards. Furthermore, the pelletizing experiments showed that the success of the pelletizing process requires uniform feed parameters regarding the moisture content. Therefore, the moisture content of chaff needs to be determined and maybe adjusted during processing at industrial scale.

Combustion

The reaction kinetics of the chaff pellet combustion revealed that the application of chaff as fuel in combustion plants is in principle possible. The speed of the mass loss during devolatilisation was the same for chaff as for pinewood. Nevertheless, the devolatilisation time of chaff was overall shorter due to a lower volatile content. The mass loss during char combustion was similar for wood and for chaff. The type of applicable combustion technology as well as the economy of the combustion application depends strongly on the technical effort required by the fuel. Chaff has with 0.61 %(w/w) a significantly higher nitrogen content than wood (Amand et al., 2006). This makes primary measures such as air or fuel staging necessary to reduce the formation of NO_x and to obey the legal limits (Salzmann and Nussbaumer, 2001). The hemisphere and flow temperature of chaff were determined as >1,500°C and are comparable with the characteristic temperatures of wood (Kaltschmitt et al., 2009). Such high deformation and flow temperatures can significantly lower the technical effort for the combustion application. Nevertheless, the determined sintering temperature of chaff was below 800°C and the combustion experiments showed the dimensional stability of the ash pellets after combustion. Such dimensional stability caused by sintering can lead to problems like inhomogeneous air distribution and difficult ash discharge in furnaces. This needs to be counteracted by stoking the material bed and by an optimized ash discharge (Wang et al., 2014). Furthermore, the ash content of pre-sieved and original chaff is with 8.4 and 7.6% significantly higher than for wood. Due to the high ash content, the technical application requires an optimized ash removal and dedusting.

Due to the low sintering temperature and the high ash content, it is expected that the application of a moving grate is necessary for the chaff pellet combustion. Moving grates are available for furnaces with a nominal heat output (NHO) of >150 kW (Kaltschmitt et al., 2009). Besides grate firing systems also bubbling fluidized bed furnaces (BFB) are applicable for biomass combustion. Compared to grate firing systems they require higher technical effort. The combustion of wheat chaff in BFB was investigated by the Fraunhofer Institute for Factory Operation and Automation IFF (Appelt and Brith, 2016). During the combustion, problems occurred due to slagging of the ash, which is why they recommend using additives to improve the ash melting behavior. Due to the technical effort and required auxiliary energy, BFB are economically feasible for plants with a NHO of >5 MW (Van Loo and Koppejan, 2008; Kaltschmitt et al., 2009). The decisions for a firing system, therefore depends on the aimed plant size.

CONCLUSION

To evaluate the energetic use of wheat chaff via combustion, a comprehensive characterization of chaff was done in this research. Furthermore, potential treatment such as sorting and pelletizing was investigated and evaluated according to its effects. Finally, the combustion behavior was analyzed in order to derive requirements for the technical firing system. Such comprehensive investigations allowed assessing the feasibility of the energetic utilization of chaff. This study showed that the combustion of chaff, with pre-sorting and pelletizing as pretreatment, is a feasible approach for the energetic use of chaff, which is worth to pursue.

The pelletizing experiments revealed ideal pelletizing parameters. Furthermore, they showed that the pelletizing success is very sensitive to the water content of the feed. This makes precise conditioning of the feed necessary. Although the experiments present a broad screening of parameters, experiments at industrial scale are necessary to evaluate if pelletizing chaff can be a continuous process without interruptions. Analyzing pre-sorted and original chaff revealed that pre-sieving is advantageous to improve the handling as well the combustion properties. To make a final judgement about the implementation of pre-sorting and pelletizing, an extended case study is necessary which takes required energy, technical, transportation and storage effort into account.

The investigations showed that chaff is a valuable fuel for energetic utilization. Because of the combustion properties and the observations during the experiments, it is concluded that the combustion of chaff requires special measures such as a moving grate and an optimized ash removal. Considering these special measures, the combustion of chaff is expected to be economical for furnaces with a nominal heat output of >150 kW. To be able to evaluate the technical requirements for an optimized burnout and ash discharge of the sintered ash, the combustion in a small-scale oven under realistic conditions is necessary. Furthermore, experiments measuring the composition of the exhaust gases need to be done in order to evaluate, if special measures are necessary to stay within legal limits.

AUTHOR CONTRIBUTIONS

BW and CG conceived the procedure to evaluate chaff as potential fuel. BW performed the experiments. BW and CG analyzed the data. CG drafted the structure and content of the paper, BW wrote the first draft of the paper. All authors reviewed the paper.

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frontiers in Environmental Science

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Effects of Grazing Abandoned Grassland on Herbage Production and Utilization, and Sheep Preference and Performance

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Large areas of farmland are abandoned in Norway, which for various reasons are regarded as undesirable. Loss of farmland may have negative implications for biodiversity and ecosystem function and food production potential. The objectives of this study were to assess forage mass production and utilization, botanical composition, lamb performance, and grazing distribution pattern when reintroducing livestock grazing to an abandoned grassland. The study area was located in Central Norway, unmanaged for 12 years. Sheep grazed the area for 10 weeks in 2013 and 4 weeks in spring and autumn, respectively, in 2014 and 2015. During the summer of 2014 and 2015, the area was subjected to the following replicated treatments: (1) No grazing, (2) grazing with heifers, and (3) grazing with ewes and their offspring. The stocking rate was similar in the grazed treatments. Forage biomass production and animal intake were estimated using grazing exclosure cages and botanical composition by visual assessment. Effect on lamb performance was evaluated by live weight gain and slaughter traits in sheep subjected to three treatments: (1) Common farm procedure with summer range pasturing, (2) spring grazing period extended by 1 month on the abandoned grassland before summer range pasturing, and (3) spring and summer grazing on the abandoned grassland. Grazing distribution patterns were studied using GPS position collars on ewes. Total annual biomass production was on average 72% higher with summer grazing than without. Annual consumption and utilization was on average 218 g DM/m^2 and 70% when summer grazed, and 25 g DM/m^2 and 18% without grazing, respectively. Botanical composition did not differ between treatments. Live weight gain was higher in lambs subjected to an extended spring grazing period (255 g/d) compared to common farm practice (228 g/d) and spring and summer grazing on the abandoned grassland (203 g/d), and carcass value was 14% higher in lambs on extended spring grazing compared to common farm practice. In autumn, sheep preferred to graze areas grazed by sheep during summer. Re-introduction of grazing stimulated forage production, and extended spring grazing improved performance in lambs. This study has quantified the value of abandoned grassland as a feed resource.

Keywords: abandoned grassland, grazing, herbage production, herbage utilization, sheep performance, grazing pattern, carcass value, weight gain

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INTRODUCTION

Large areas of farmland have been abandoned during the last decade in Europe (Estel et al., 2015). In the western and northern counties of Norway, 12% (26,986 ha) of the total farmland was abandoned between 2000 and 2016 (Statistics Norway, 2018a). About 92% of this abandoned farmland was used as grassland for cutting, grazing, or combined cutting and grazing. The drivers for farmland abandonment in Europe are many and includes low income, increasing aging of farmers, and low farm size, and population density (Terres et al., 2015). In Norway, many farmers have left agriculture, farming has rationalized into larger farm units, and there is an increase in the proportion of agricultural land rented (Forbord et al., 2014). At the same time, farming has intensified with an increase in production per animal head, particularly in dairy cattle production (Steinshamn et al., 2016). High quality land is commonly preferred at the expense of more marginal, remote, and less suitable areas for intensive farming. The consequences of agricultural abandonment are many, which may both be positive and negative (van der Zanden et al., 2017). Positive effects are, for example, that abandonment may increase carbon sequestration and give room for large mammals (Navarro and Pereira, 2012). It is, however, also suggested that shrub expansion may decrease total ecosystem carbon pools (Sørensen et al., 2017). Species richness declines with increasing time after abandonment, and the area will over time be encroached by shrub and trees (Staaland et al., 1999; Pykälä et al., 2005; Wehn et al., 2017). In the Norwegian context, this is regarded as undesirable since grasslands are important cultural landscape elements and an import asset for the tourism industry (Daugstad, 2008). It is also regarded as important for food security reasons to maintain the production potential of agricultural goods, and agricultural activity is important for the rural economy in Norway as well as many parts of the EU (Terres et al., 2015).

Grassland is the main asset for sheep farmers, and availability of grasslands is decisive for performance of production on sheep farms. Common sheep husbandry practice in Norway is to keep sheep indoors during winter and free-ranging on unfenced mountain or forest land during summer before slaughter in autumn (Ross et al., 2016). Access to grassland, particularly for spring and autumn grazing, is a limiting factor in Norwegian sheep farming (Vatn, 2009). Most lambs are born indoors during spring and kept for a few weeks on cultivated grasslands close to the farm at the foot of the mother before being turned out on summer range pasture (Vatn, 2009). Grazing in spring is at the expense of the yield of winter feed. The sheep farmers therefore try to limit the period of spring and autumn grazing by leading the flock to rangeland as soon as possible in spring and to slaughter the lambs in autumn just after collection from rangeland. Lamb performance is, however, affected by their weight when turned out on range pasture, i.e., performance on rangeland improves with increasing spring weight (Steinheim et al., 2008). Thus, extending the spring grazing period on cultivated pasture prior to the free-ranging period may improve the lamb performance. To our knowledge, the effect of extending the spring grazing period on lamb performance has not been tested.

Sheep farming in Norway is generally found in marginal agricultural areas, also where the land abandonment rate is high. Grasslands that are abandoned are typically owned by landowners that have quit farming. Grassland that is abandoned, or in danger of being abandoned, is therefore a potential additional grazing resource both in spring and in autumn. Including such land in existing sheep farming systems could reduce the pressure on grassland used for silage or hay production, and thereby increase the winter feed supply. Furthermore, a general change from small to larger sheep flocks (Statistics Norway, 2018b) implies that there is a need for access to more farmland, particularly for spring and autumn grazing, for those that invest in future sheep farming.

Knowledge on how to manage such grasslands during summer, between an extended spring grazing period and before autumn grazing is needed, particularly for grasslands that are not suitable for efficient mechanical harvesting. If grasslands are only used for grazing in spring and autumn, with no cutting or grazing during summer, it is assumed that the pasture feed quality for autumn grazing is poor. This is because forage plants reach maturity and have a high proportion of leaf senescence. Grazing with sheep or cattle during summer will likely maintain the sward at a more leafy stage with higher nutritional quality than without any grazing. Grazing also leaves patches of feces and herbage with higher level of nutrients that may be attractive for herbivores (Haynes and Williams, 1993). On the other hand, sheep may also avoid areas of fecal contamination (Cooper et al., 2000).

The objective of this study was to assess the grazing value, i.e., forage production, feed intake, and animal performance, of abandoned grassland as spring and autumn pastures for sheep before and after the common practice of rangeland grazing during the summer. The hypothesis was that extended spring grazing on an abandoned grassland would improve lamb performance. Moreover, it was an objective to evaluate if grazing of the abandoned grassland during the summer period, with heifers or sheep, had an impact on grassland productivity and grazing value. It was assumed that only spring grazing, without summer grazing, would lead to inferior feed quality later in the season. Quantifying these aspects of reintroducing abandoned grassland into sheep farming gives both sheep farmers and landowners a knowledge basis for valuing the area in monetary terms and for decision-making.

MATERIALS AND METHODS

Experimental Site

A 22 ha grassland that has been unmanaged since 2001 was used in the study. The soil is of morainic origin with high content of organic matter (ignition loss 12.1%), with moderate pH (5.4). The phosphorous and potassium values were 7.8 and 5.8 mg/100 g, respectively, according to the extraction method of Egnér et al. (1960). The grassland is located in the municipality of Sunndal, Møre og Romsdal County (62.85°N and 8.40°E), ranging from 200 to 270 m above sea level. Before abandonment, the area was used as pasture for dairy cows. The prevailing plant species prior to onset of the experiment, assessed in the autumn 2013, included species that most likely were seeded when the grassland was used for dairy cows, such as common bent [*Agrostis capillaris* L., 37% of dry matter (DM) yield] and smooth meadow-grass (*Poa pratensis* L., 12%), and naturally occurring species such as tufted hair-grass [*Deschampsia cespitosa* (L.) P. Beauv., 18%] and meadow buttercup (*Ranunculus acris* L., 7%). Reed canary grass (*Phalaris arundinacea* L.) constituted dense stands in small patches. In 2013, the area was fenced and grazed with sheep for about 10 weeks and horses for ∼2 weeks in order to reduce the amount of dead material the following spring. The experiment was run for two consecutive years, in 2014 and 2015. Except for fencing and grazing, no other management measures were taken.

The total precipitation during the growing season, from May 1 to November 1, was 564 and 520 mm in 2014 and 2015, which were 3 and 11% lower than the average of the recent 30-year period, respectively. The mean daily temperature was 13.0°C in 2014 and 11.5°C in 2015, which was warmer compared to the recent average of 10.7°C.

Experimental Design

Grazing Treatments and Measurements

Ewes with offspring grazed the entire area for ∼1 month in spring (**Figure 1**, Period 1: from May 23/20 to June 20/19 in 2014/2015) and ewes with lambs for replacement for ∼1 month in autumn (Period 4: from September 17/14 to October 21/14 in 2014/2015). The stocking rate of ewes was similar in both years and on average 0.4 livestock units (LU)/ha in period 1 and 0.65 LU/ha in period 4. An area of 15.3 ha of the total 22 ha was divided into three blocks with three fields, averaging 1.7 ha within each block. Three treatments were assigned randomly to the three fields within each block. The treatments were applied during the summer (Period 2): G_0, Control with no grazing; G_H, Heifers grazing; and G_S, Ewes grazing with offspring. The stocking rate was 1.8 LU/ha, in both G_H and G_S, for a duration of ∼6 weeks (from June 20/19 to August 12/3 in 2014/2015). The area was left resting for about a month (Period 3: from August 12/3 to September 17/14 in 2014/2015) after grazing treatment Period 2 and before autumn sheep grazing (Period 4). The sheep and heifers were removed from the trial during Period 3. The stocking rates used were low to be sure that the animals' requirement were covered. In Period 2, the stocking rate was similar to the one used in a Norwegian model study of the costs of keeping sheep on enclosed pastures (Kjuus et al., 2003).

Temporary movable exclosure cages, made of iron nets (height 1 m, diameter 1.59 m, and area 1.99 m^2), were used to protect the herbage from grazing, enabling pasture production assessment. Five cages were allocated at random in each field. Intake was estimated by comparing herbage growth inside and outside the cage. Sickles were used to cut one 0.5 × 0.5 m square of grass to ground level outside the cage and one inside the cage at four occasions, i.e., the end of each of the four periods (**Figure 1**). Before cutting, the sward heights were measured within each square with a rising plate meter (30 cm in diameter which applied a force of 3.5 kg/m^2 at rest). Subsequent to each sampling, the exclosure cages were moved to previously grazed locations to account for the herbage growth. The herbage samples were sorted into forage species (grazed by cattle and sheep) and non-forage species, i.e., species that are avoided, such as meadow buttercup (*R. acris* L.) and marsh thistle (*Cirsium palustre* L.). Dead or senescent plant material was included in the non-forage fraction. The sorted material was force dried at 65°C for 48 h and weighed. Intake, net production and utilization of total herbage (sum of forage and non-forage species) and of forage species only were calculated according to the following equations:

$$\begin{aligned}
HI_{gn}[gDM/m^2] &= (Wu_n - Wg_n) \\
HP_n[gDM/m^2] &= (Wu_n - Wg_{n-1}) \\
HPU_n[g/g] &= HIg_n/HPg_n \\
HGP_n[g/g] &= HPg_n/HPt_n
\end{aligned}$$

where HIg_n is herbage dry matter intake, HP_n is herbage production (total and forage), HPUn is herbage intake as the proportion of the forage production, and HGP_n is forage proportion of total biomass production in period n (n = 1–4). Wu_n = dried herbage mass inside the cage, ungrazed, in g/m^2 in period n (n = 1–4); Wg_n = dried herbage mass outside the cage, grazed, in g/m^2 in period n (n = 1–4); $Wg_{n-1=}$ dried herbage mass outside the cage, grazed, in g/m^2 in the previous period n-1 (n = 1–4). Biomass production and intakes were calculated for each exclosure cage and averaged for each paddock. Annual herbage production and intake were calculated as the sum of HP and HI in the four periods, respectively.

For herbage feed quality analysis, the forage samples taken from the grazed sward outside the cages were ground to 1 mm by a UDY Cyclone Sample Mill (UDY Corporation, Fort Collins,

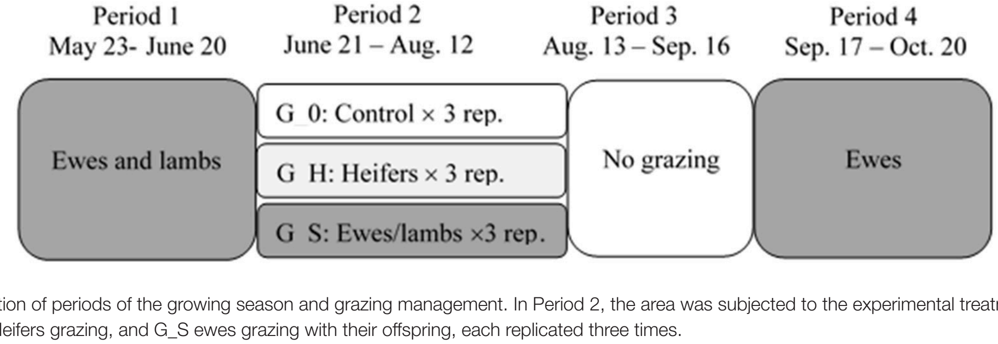

FIGURE 1 | Illustration of periods of the growing season and grazing management. In Period 2, the area was subjected to the experimental treatments G_0 Control (no grazing), G_H Heifers grazing, and G_S ewes grazing with their offspring, each replicated three times.

Colorado). Samples were analyzed at Dairy One Laboratory (Ithaca, NY) using wet chemistry analysis. The analysis included determinations of DM (AOAC 930.15), crude protein by the AOAC method (990.03), acid detergent fiber (ADF by Ankom A200 Filter Bag Technique; Ankom Technology, Macedon, NY), and neutral detergent fiber (aNDF by Ankom A200 Filter Bag Technique with amylase).

Botanical Composition

Herbage botanical composition was estimated by using the dry-weight-rank method and by estimating plant ground cover for individual species in each field in autumn (2013, 2014, and 2015). The dry-weight-rank method is based on visual evaluation of dry matter yields of the predominant plant species in a large number of squares per field (Jones and Hargreaves, 1979). Based on the scores, the proportion of individual plant species on a dry-matter basis can be calculated. In the present study ~50 squares of 40 × 40 cm were evenly distributed over each field. The GPS-log of the observation in autumn 2013 was subsequently used so that the observations were done on the same spots in order to reduce spatial variation. In addition to the ranking of the predominant species, a list of all observed species was recorded. For the recording of ground cover of each species, four permanent plots of 1 × 1 m per field were established in autumn 2013. For items covering the ground with <11%, the cover was estimated at 1% intervals and for items with >10% ground cover, 10% intervals were used. All plant species were determined to species level except for the taxa of *Taraxacum*, *Polypodiopsida*, and *Bryophyta*. In addition to plant species, observations of open soil, stones, manure, and dead plant material were recorded.

Animal Recordings

An official ethical review process was not needed for this animal study according our institute's, The Norwegian Institute of Bioeconomy Research, guidelines and the national laws and regulations authorized by the Norwegian Food Safety Authority. The reasoning is expressed by The Norwegian Food Safety Authorities (Brumundal, Norway) as follows: "In the experiment the animals were treated according to normal husbandry practices and no practices likely to cause pain, suffering, and distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice were executed. Therefore, the Norwegian Regulation concerning the use of animals for scientific purposes (based on the EU directive 2010/63/EU) does not apply for this study." The normal husbandry practices conducted in this study were transport, weighing, and preventive treatment for parasites including catching and holding sheep. Handling was conducted by experienced workers to keep handling stress at a minimum. The sheep handlers were not certified veterinarians, but experienced sheep handlers. One of us, EB, holds the certificate for conducting animal experiments. Any sheep with signs of health problems were immediately checked by a veterinarian.

We used a commercial sheep flock of Norwegian white spæl breed with 83 (88) ewes (lambs) in 2014 and 77 (106) ewes (lambs) in 2015. The sheep farm is situated in Tingvoll municipality in Møre og Romsdal County (63.03°N and 8.15°E, 25 masl). Each year, the sheep were assigned, in a stratified random manner according to age of ewe and number of lambs born per ewe, into three treatments: S_Common, common farm procedure with short spring grazing period before summer grazing on range pasture; S_Spring, spring grazing period extended by 1 month of grazing (Period 1, **Figure 1**) on the abandoned grassland before summer range pasturing when joining the S_Common group; S_Summer, spring and summer grazing on the abandoned grassland [Period 1 and 2 (**Figure 1**), treatment "G_S" (see section Experimental design)]. In Period 3, the resting period of the experimental grassland, the animals in the S_Summer treatment were removed from the trial area and grazed an area with similar quality.

The summer range pasture, where the flock grazed free-range during the summer, is located close to the farm and is about 3,461 ha. The summer range consists of 45% forest with patches of grasses, herbs and shrubs between the trees (mainly *Betula* spp.), 48% is sub alpine vegetation with dwarf shrub heath, and 3% is bog (Ahlstrøm et al., 2014). The range is within a humid region with a quite heterogeneous topography. Calcareous rocks within the range contribute to areas of rich fens and tall herb-rich deciduous forests. Most of the forest is sub alpine birch forest dominated by blueberries (*Vaccinium myrtillus)* and in drier areas berries (*Ericaceae* spp.) in general.

Lambs were weighed at birth, at start of spring-extended pasturing (Start Period 1, **Figure 1**) after the spring-extended pasturing (Start Period 2, **Figure 1**), and in autumn (Start Period 4, **Figure 1**). Lambs were 27 (*SD* 6.7) and 25 (*SD* 4.3) days old at the start of spring-extended pasturing (period 1) in 2014 and 2015, respectively. Ewes and lambs in treatment S_Spring and treatment S_Summer were moved to the abandoned cultivated grassland by animal transport, a distance of 55 km, after recording spring weight. The animals were inspected regularly, ~3 times per week. Ewes and lambs in treatment S_Spring were returned to the common farm summer grazing system on range pasture at the start of Period 2, **Figure 1**.

Slaughter weight, carcass conformation, carcass fatness and carcass value, in NOK, for slaughtered lambs were obtained from the abattoir. This information was obtained from 47 to 77 of the lambs in 2014 and 2015, respectively. Ewes and lambs were monitored regularly for internal parasites. All lambs were treated with tick repellent at the beginning of Period 1 (**Figure 1**).

All ewes were fitted with Global Position System (GPS) collars (Telespor AS, Tromsø, Norway). The GPS collars log and transmit position at regular intervals and the interval settings can be changed from a computer. During Period 4, the GPS tracking frequency was set to log position every 30 min.

Statistical Analysis

Botanical Composition

Herbage botanical composition estimated by the dry-weight-rank method was analyzed using the mixed procedure in SAS (SAS Institute Inc., 2011). Grazing treatment (G_0, G_H, G_S), year (2014, 2015) and their interaction were considered as fixed effects. Block (1, 2, 3) and block interaction with grazing treatment were considered as random effects.

Plant ground cover for individual species was analyzed using the mixed procedure in SAS (SAS Institute Inc., 2011). Grazing treatment (G_0, G_H, G_S), year (2014, 2015), and their interactions were considered as fixed effects. Block, square (1–4 within field) and their interactions with grazing treatment were considered as random effects. In both models, observations from the 2 years taken on the same field were treated as repeated observations. Observations from autumn 2013 were used as covariate in the statistical analysis if $P < 0.05$. Differences between least squares means were estimated with the Tukey-Kramer test.

Grassland Production

Sward height, herbage production, intake, proportion of forage biomass of total biomass produced, and proportion of forage biomass consumed of total forage biomass produced were analyzed using the mixed procedure in SAS to discern significant effects of treatments (SAS Institute Inc., 2011). Year (2014, 2015), grazing period (1–4), and grazing treatment (G_0, G_H, G_S) and their interactions were considered as fixed effects. Block and block by treatment interactions were regarded as random effects. The “Repeated” statement was used to account for correlation among measurements taken on the same plot across time (period). Year and interaction with year were for most variables not statistically significant. For variables where the residuals were not normally distributed, the data were transformed. Normal data are presented as least square mean (LSM) and transformed data are presented as the back transformed LSM. The optimal covariance structure was assessed for each parameter with attention to Schwarz's Bayesian criterion as explained by Littell et al. (2006). Tukey's *post-hoc* means test was used for comparisons of means.

Animal Performance

Lamb live weight gain, slaughter weight, carcass conformation, carcass fatness, and carcass value were analyzed using the mixed procedure in SAS (SAS Institute Inc., 2011) to discern significant effects of treatments. Treatment (S_Common, S_Spring, S_Summer), sex (1, 2), parity (1, 2, 3), and day of birth were regarded as fixed effect and year (2014, 2015) and mother were regarded as random effects. The initial model included the fixed effects treatment, sex, parity, day of birth, and their interactions, and the random effects year, mother and their interaction. There were no significant effects of the interactions of the fixed effects, so all interactions were omitted from the final analysis. The interaction of year and mother was significant and included in the model. The initial model also included year and mother by year interactions as random effects. There was no significant effect of year, and year was therefore omitted from the final analysis.

Sheep Grazing Distribution Pattern

A Chi-square Goodness-of-fit test (FREQ procedure in SAS Institute Inc. 2011) was used to compare the distribution of GPS observations of the ewes in each of the three treatments (G_0, G_H or G_S) within each spring and autumn periods and years.

RESULTS

Botanical Composition

The total number of species were 48 and 37 as assessed by the dry weight-rank method and the ground cover method, respectively (**Tables 1**, **2**). Some species disappeared while new appeared, but the total number of species declined from 2014 to 2015 as assessed by the dry weight method (**Table 1**). The species that were observed in 2013 but not in 2015 were *Alopecurus geniculatuse* L., *Poa annua* L., *Carex echinata* Murr., *Tussilago farfara* L., *Achillea millefolia* L., *Potentilla erecta* L. Raeusch, *Rumex longifolius* D.C., and *Thelypteridaceae*, while *Alopecurus pratensis* L., *Poa trivialis* L., *Taraxacum officinale* G. H. Weber ex Wigg. appeared in 2015 but not in 2013.

The botanical composition was not affected by the grazing treatments during the 2-year experiment as measured by both assessment methods (**Tables 1**, **2**). On average, the grass species that most likely have been cultivated earlier on the land (*A. capillaris*, *P. pratensis*, *F. pratensis*, *P. arundinacea*, and *P. pratense*) had a ground cover of 74% and made up 63% of the dry matter yields. Open soil, rocks and manure accounted for only small proportions of the ground cover. The yield proportion of *D. cespitosa* and *R. repens* were higher in 2015 than 2014 for G_S (**Table 1**). This was not confirmed by the ground cover assessment, but ground cover of *R. repens* was higher in 2015 than 2014 on G_0. The ground cover of dead plant material was on average 44%. In 2014, dead plant material covered a larger ($P = 0.03$) proportion of the G_S plots than G_H, but in 2015 the effect was the opposite.

Forage Production, Intake, and Utilization

The sward height was, as expected, higher in the control plot (G_0) than in the grazed plots (G_S and G_H) at the end of grazing in Period 2 (**Table 3**). The effect of grazing during Period 2 on sward height was also present at the end of Period 3, when all plots were left resting. During the spring period (Period 1), the accumulated total (HP_t), and forage (HP_g) DM yields were on average 196 and 135 g/m^2, which corresponded to a daily growth rate of 7.0 and 4.9 g DM/m^2, respectively. The sheep consumed (HI_g), on average 26 g/m^2 during this period. In the summer (Period 2), when the area was subjected to the different grazing treatments, the sheep and heifers consumed on average 160 g DM/m^2 (**Table 3**) and utilized about 67% (figures not shown) of the forage biomass. The forage production and growth rate was negative in late summer and autumn (**Table 3**). There were no effects of animal species on herbage production, consumption, and utilization. Grazing with heifers and sheep during the summer (Period 2) resulted in on an annual basis 72% more total biomass production (HP_t, 527 vs. 306 g DM/m^2), 159% more forage production (HP_g, 323 vs. 125 g DM/m^2), 9 times more forage consumed (HI_g, 218 vs. 25 g DM/m^2), and 3.2 times higher utilization of total forage produced than in the control plots (HPU, 0.70 vs. 0.22).

Summer grazing resulted in lower ($P < 0.05$) forage ADF and aNDF concentrations in the autumn (Period 4) compared to no grazing (**Table 3**). The concentration of net energy of

TABLE 1 | Effect of grazing treatment [control without grazing (G_0), grazing with heifers (G_H) or grazing with sheep (G_S)] and year on botanical composition in autumn (Period 4) of 2014 and 2015 estimated using the dry-weight-rank method (Jones and Hargreaves, 1979).

	2014			2015			SEM[1]	P-value[2]			
	G_0	G_H	G_S	G_0	G_H	G_S		Covariate	T	Y	T×Y
n[3]	3	3	3	3	3	3					
POACEAE, %											
Agrostis capillaris L.	48.3	50.9	62.0	48.2	50.7	45.8	7.42	0.07	0.71	0.14	0.18
Deschampsia cespitosa (L.) P. Beauv.	18.1[ab]	18.9[ab]	10.0[b]	17.9[ab]	19.0[ab]	22.6[a]	3.75	0.01	0.89	0.01	0.01
Poa pratensis L.	11.8	11.1	9.1	8.7	12.5	14.7	2.49	0.01	0.56	0.63	0.41
Festuca pratensis L.	5.7	2.9	5.9	2.4	2.4	5.0	2.65	NS	0.59	0.24	0.59
Phalaris arundinacea L.	0.4	1.1	1.6	1.9	0.6	1.6	1.14	0.001	0.80	0.75	0.66
Phleum pratense L.	0.4	0.0	0.5	1.7	0.2	0.8	0.48	NS	0.27	0.14	0.34
Other Poaceae[4]	1.9	1.2	0.8	2.2	1.5	0.8	1.07	NS	0.70	0.55	0.92
DICOTYLEDONES, %											
Ranunculus acris L.	1.8	3.7	4.8	1.2	1.6	1.4	1.08	NS	0.11	0.08	0.58
Cirsium palustre (L.) Scop.	4.4	2.8	2.9	6.1	3.8	5.5	2.30	NS	0.61	0.35	0.94
Ranunculus repens L.	1.6	3.4	−1.5	2.5	4.3	1.1	0.96	0.05	0.17	0.02	0.31
Rubus idaeus L.	0.3	0.8	0.8	0.6	0.1	0.1	0.46	NS	1.00	0.29	0.42
Urtica dioica L.	0.2	0.3	0.4	1.4	0.6	0.2	0.52	NS	0.68	0.32	0.42
Other dicotyledones[5]	1.4	2.1	1.0	1.6	1.1	0.6	0.54	NS	0.35	0.46	0.58
Bryophyta and Pteridophyta[6]	2.4	1.4	1.9	2.5	3.3	−0.2	1.55	NS	0.66	0.99	0.32
Number of plant species[7]	19.1	16.3	15.5	16.6	14.3	13.0	1.41	NS	0.26	0.02	0.95

[1]Standard error of means for the interaction of treatment by year (T×Y).
[2]The observation from autumn 2013 was used as a covariate if P < 0.05. The fixed effects were treatment (T) and year (Y).
[3]The botanical composition of each replicated field was based on an evaluating of 50 squares per field.
[4]Other Poaceae in descending prevalence: Carex nigra (L.) Reichard, Juncus conglomeratus L., Festuca rubra L., Dactylis glomerata L., Carex rostrata Stokes, Holcus mollis L., Carex echinata Murray, Festuca ovina L., Poa trivialis L., Poa annua L., Juncus filiformis L., Alopecurus pratensis L., Alopecurus geniculatus L., Luzula multiflora (Ehrh.) Lej.
[5]Other Dicotyledones in descending prevalence: Stellaria graminea L., Trifolium repens L., Galeopsis bifida Boenn., Rumex acetosa L., Viola epipsila Ledeb., Stellaria media (L.) Vill., Salix spec. L., Epilobium hornemannii Reichb., Rumex longifolius DC., Betula pubescens Ehrh., Galium odoratum (L.), Tussilago farfara L., Vaccinium myrtillus L., Cerastium fontanum Baumg., Taraxacum spec. F. H. Wigg., Veronica chamaedrys L., Achillea millefolium L., Potentilla erecta (L.) Räusch.
[6]Bryophyta species were not determined. Pteridophyta in descending prevalence: Equisetum palustre L., Dryopteris expansa (C. Presl) Fraser-Jenk. & Jermy, Phegopteris connectilis (Michx.) Watt, Dryopteris filix-mas (L.) Schott, Gymnocarpium dryopteris (L.) Newman.
[7]Bryophyta, Salix spec. and Taraxacum spec. were counted with one count each.
[a,b]Values followed by different letters were statistically different (P < 0.05).

lactation tended ($P < 0.1$) to be higher in Period 4 and the CP concentration was higher ($P < 0.05$) in Period 3 on the grazed swards than in the control. The effect of period on forage feed quality differed between years, but the treatment effect was similar among the years (figures not shown).

Animal Performance

Weight gain was significantly ($P < 0.05$) higher in lambs assigned to S_Spring compared to S_Control and S_Summer (**Table 4**). Weight gain, slaughter weight, carcass confirmation, carcass fatness, and carcass value were all significantly ($p < 0.05$) higher in lambs assigned to S_Spring compared to S_Summer (**Table 4**).

Sheep Grazing Distribution Pattern

The GPS observations of ewes deviated from even distribution among treatments in all periods (**Figure 2**). There was a clear trend, particularly in the year 2014, that ewes preferred to graze where sheep grazed during the Period 2 (G_S) and to avoid the area that was not grazed (G_0).

DISCUSSION

Botanical Composition

Many of the plant species present at the onset of the current study are species that are adapted to herbivory, and the observed high proportion of *D. cespitosa* is a characteristical stage in succession after abandonment of a grassland (Jensen et al., 2001; Rosef et al., 2007). The increase in the dry weight proportion of *D. cespitosa* from 2014 to 2015 in the plots summer grazed by sheep is in accordance with another study (Krahulec et al., 2001), and may be due to sheep preference for other species than the less palatable *D. cespitosa*. The botanical assessment methods were consistent in ranking the prevailing species, being the grass species *A. capillaris*, *D. cespitosa*, and *P. pratensis*. The methods were also concurrent in that the botanical composition was not affected by grazing treatments.

The decline in number of species with year observed in the current study is in contrast with other findings. Abandonment of grazing causes a decline in plant species richness because grazing-tolerant species have a reduced ability to compete for light and space (Smith and Rushton, 1994; Wehn et al., 2017),

TABLE 2 | Effect of grazing treatment [(control without grazing (G_0), grazing with heifers (G_H) or grazing with sheep (G_S)] and year on vegetation ground cover estimated in autumn (Period 4) of 2014 and 2015 in permanent 1 × 1 m squares.

	2014			2015			SEM[1]	*P*-value[2]			
	G_0	G_H	G_S	G_0	G_H	G_S		Covariate	T	Y	T×Y
n[3]	3	3	3	3	3	3					
POACEAE, %											
Agrostis capillaris L.	59.4	75.8	66.9	68.3	66.0	65.7	7.56	<0.001	0.60	0.97	0.12
Deschampsia cespitosa (L.) P. Beauv.	9.6	10.3	11.7	9.2	9.2	9.9	2.67	<0.001	0.95	0.94	0.50
Poa pratensis L.	12.7	5.7	6.4	11.8	8.2	8.5	4.26	<0.001	0.97	0.95	0.17
Holcus mollis L.	7.9	4.0	6.2	4.0	4.0	4.0	2.92	<0.001	0.73	0.15	0.13
Festuca pratensis L.	1.2	1.6	0.4	0.8	0.6	1.1	0.62	0.08	0.87	0.005	0.87
Other poaceae[4]	0.3	0.2	1.5	1.0	1.0	0.8	0.38	0.006	0.74	0.005	0.28
DICOTYLEDONS, %											
Ranunculus acris L.	1.7	1.6	1.1	2.0	2.1	1.3	0.52	0.41	0.76	0.57	0.32
Ranunculus repens L.	0.7[b]	1.4[b]	2.6[ab]	6.6[a]	3.8[ab]	1.6[b]	1.10	<0.001	0.19	<0.001	0.21
Cirsium palustre (L.) Scop.	1.5	1.2	1.4	2.2	0.9	1.5	0.59	<0.001	0.71	0.83	0.22
Other dicotyledons[5]	2.4	2.3	3.5	1.9	2.3	2.7	0.68	0.001	0.58	0.86	0.31
Bryophyta and *Pteridophyta*[6]	7.7	6.7	3.5	7.9	8.4	7.2	1.57	<0.001	0.42	0.61	0.11
Number of species[7]	7.0	6.8	7.3	7.7	7.9	7.7	0.45	<0.001	0.46	0.14	0.72
OTHER STRUCTURES, %											
Open soil	0.0	0.2	0.0	0.0	0.2	0.2	0.10	0.72	0.28	0.49	0.62
Stones	0.0	0.2	0.1	0.0	0.1	0.0	0.08	0.01	0.85	1.00	0.24
Dead plant material	47.5[ab]	27.1[bc]	64.2[a]	58.3[a]	47.5[ab]	17.1[c]	4.88	0.09	0.03	<0.001	0.17

[1] *Standard error of means for the interaction of treatment by year (T×Y).*
[2] *The observation from autumn 2013 was used as a covariate if $P < 0.05$. The fixed effects were treatment (T) and year (Y).*
[3] *The botanical composition of each replicated field was based on an evaluating of 4 squares per field.*
[4] *Other Poaceae in descending prevalence: Poa trivialis L., Phalaris arundinacea L., Phleum pratense L., Festuca rubra L., Carex nigra (L.) Reichard, Juncus conglomeratus L., Festuca ovina L., Luzula multiflora (Ehrh.) Lej., Dactylis glomerata L.*
[5] *Other dicotyledons in descending prevalence: Stellaria graminea L., Viola epipsila Ledeb., Trifolium repens L., Rubus idaeus L., Stellaria media (L.) Vill., Galeopsis bifida Boenn., Taraxacum spec. F. H. Wigg., Epilobium hornemannii Reichb., Urtica dioica L., Potentilla erecta (L.) Räusch., Veronica chamaedrys L., Cerastium fontanum Baumg., Betula pubescens Ehrh., Veronica officinalis L., Trientalis europaea L., Rumex acetosa L.*
[6] *Bryophyta and Monilophyta species were not determined.*
[7] *Bryophyta and Taraxacum spec. were counted with one count each.*
[a,b,c] *Values followed by different letters were statistically different ($P < 0.05$).*

and reintroducing grazing usually increases the number of plant species (Pavlu et al., 2007). However, the effect of grazing on species richness depends on factors like grazing intensity and the time of year grazing is conducted (Bullock et al., 2001; Pavlu et al., 2007). Most of the species found in 2013 but not in 2015, and the new ones appearing during the same time, are species associated with grassland. They contributed, however, very little to the total biomass, and the observed changes may have been caused by grazing, difference in weather conditions, and their interaction. Discrepancy in the number of species observed between the two assessment methods, is likely due to the fact that with the dry weight-rank method 50 observations were taken per field at each assessment, covering an area 8m^2, while with the ground cover method four observations on fixed squares were done with a total area of 4 m^2.

Grazing the entire area with sheep in spring and autumn, short grazing period during summer and variation of plant communities between fields likely contributed to veil potential differences between treatments. The duration of the experiment may also have been too short for revealing any difference among treatments. In addition, the amount of time since the area was abandoned, and not grazed by livestock, may have been too short for substantial change in the plant community.

Forage Production, Intake and Utilization

The positive effect of summer grazing on the primary productivity in the current study is in line with other studies with light or moderate grazing (McNaughton, 1979; Patton et al., 2007). Grazing may both enhance and reduce subsequent growth rate of plants, depending on many factors like availability of leaf area, meristems, nutrients, and frequency and intensity of grazing (Noy-Meir, 1993).

McNaughton (1979) summarizes mechanism that stimulate or compensate plant productivity with grazing, of which the following were likely important in our study: "increased photosynthetic rates in residual tissue, reallocation of carbohydrates from other plant parts, removal of older tissues, increased light intensities upon more active underlying tissues, reduction of the rate of leaf senescence, and nutrient recycling from dung and urine."

The observed negative growth rate in late summer and autumn is likely due to photoperiodic (short-day) depression of

TABLE 3 | Effect of grazing treatment, Control without grazing (G_0), grazing with heifers (G_H) or grazing with sheep (G_S), during the summer period on total (HPt) and forage (HPg) dry matter (DM) production (g/m^2 and g/m^2 and day), estimated DM intake of forage (HIg) DM (g/m^2 and g/m^2 and day), forage proportion of total biomass produced (HGP, g/g), the proportion of consumed biomass of forage biomass produced (HPU, g/g) and feed quality ($n = 6$).

	[1]Period (P)	Treatment (T)				*P*-value		
		G_0	G_H	G_S	SEM	P	T	P×T
Sward height (cm)	1	10.5	11.3	10.5	0.87	<0.001	0.014	<0.001
	2	18.8^a	7.8^b	8.7^b	1.14			
	3	14.6^a	11.3^b	12.2^b	1.54			
	4	7.5	6.5	7.3	0.98			
HPt (g DM/m^2)	1	144	247	197	16.7	<0.001	0.013	0.364
	2	198	222	266	17.8			
	3	−53	43	11	58.7			
	4	17	14	53	48.0			
	Annual	306^b	526^a	527^a	48.4	–	0.010	–
Daily HPt (g DM/m^2 d)	1	5.1	8.8	7.0	0.60	<0.001	0.070	0.589
	2	4.1	4.6	5.5	0.37			
	3	−1.2	1.1	0.4	1.44			
	4	0.6	0.4	1.8	1.74			
HPg (g DM/m^2)	1	100	171	143	15.0	<0.001	0.009	0.461
	2	191	187	220	17.6			
	3	−119^b	−3^a	−31^a	52.1			
	4	−47	−35	−4	34.1			
	Annual	125^b	320^a	327^a	41.8	–	0.023	–
Daily HPg (g DM/m^2 d)	1	3.6	6.1	5.1	0.53	<0.001	0.007	0.252
	2	4.0	3.9	4.6	0.36			
	3	−3.1^b	−0.2^a	−0.8^a	1.30			
	4	−1.6	−1.2	−0.2	1.23			
HIg (g DM/m^2)	1	18	45	31	6.4	0.002	0.002	0.029
	2	6^b	165^a	153^a	25.0			
	3	–	–	–				
	4	1	27	16	25.4			
	Annual	25^b	236^a	200^a	40.8	–	0.002	–
HGP = HPg/HPt (g/g)	Annual	0.40	0.60	0.62	0.076	–	0.104	–
HPU = HIg/HPg (g/g)	Annual	0.22^b	0.75^a	0.65^a	0.112	–	0.032	–
NEL (MJ/kg DM)[2]	1	5.04	5.05	5.12	0.058	<0.001	0.089	0.111
	2	4.49	4.54	4.60	0.075			
	3	4.80	4.97	4.94	0.068			
	4	4.65	5.03	5.06	0.083			
CP (g/kg DM)[3]	1	145	145	143	4.2	<0.001	0.020	0.069
	2	108	119	130	7.1			
	3	133^b	163^a	168^a	7.4			
	4	121	134	141	5.9			
aNDF (g/kg DM)[4]	1	561	560	552	6.6	<0.001	0.042	0.190
	2	634	630	620	9.4			
	3	597	575	576	7.4			
	4	612^a	565^b	561^b	11.3			
ADF (g/kg DM)[5]	1	315	305	298	7.4	<0.001	0.033	0.014
	2	366	357	353	7.4			
	3	352	335	335	7.4			
	4	346^a	311^b	318^b	7.4			

[1]Period 1, 2, 3, and 4 is spring, summer, late summer and autumn in ***Figure 1****. Period is not included in the statistical model for the annual sums.*
[2]NEL is net energy of lactation.
[3]CP is crude protein.
[4]aNDF is ash free neutral detergent fiber.
[5]ADF is acid detergent fiber.
[a,b,c]Values followed by different letters within rows were statistically different ($P < 0.05$).

TABLE 4 | Effect of spring grazing treatment on lamb live weight gain, slaughter weight, carcass conformation, fatness and value (LSMeans with standard error of mean in brackets).

	Treatment[1]			*P*-value
	S_Common	**S_Spring**	**S_Summer**	
LIVE WEIGHT GAIN (G/DAY)				
n	75	54	64	
Birth—start Period 1[2]	288 (12.0)[a]	278 (13.1)[a]	280 (12.8)[a]	0.795
Start period1—Slaughter date[3]	228 (6.1)[a]	255 (6.6)[b]	203 (6.5)[c]	0.001
Period 1[4]		327 (10.8)[a]	321 (10.6)[a]	0.674
Start Period 2—Slaughter date[5]		229 (8.6)[a]	164 (8.6)[b]	0.001
SLAUGHTER PARAMETERS				
n	48	33	43	
Slaughter weight (kg)[6]	14.3 (0.45)[ab]	15.7 (0.52)[a]	13.2 (0.48)[b]	0.002
Carcass conformation[7]	5.80 (0.228)[a]	6.24 (0.264)[a]	5.06 (0.243)[b]	0.003
Carcass fatness[8]	1.68 (0.102)[ab]	1.87 (0.117)[a]	1.50 (0.108)[b]	0.059
Carcass value (NOK)	614 (28.2)[ab]	700 (32.6)[a]	542 (30.0)[b]	0.002

[1] *S_Common;, common farm procedure with short spring grazing period before summer grazing on range pasture; S_ Spring; ∼4 weeks extended spring grazing period on the abandoned grassland before summer grazing on range pasture as in S_Common; S_Summer; grazing on the abandoned grassland all summer).*
[2] *Live weight gain from birth to start Period 1 (See **Figure 1**).*
[3] *Live weight gain from start Period 1 to autumn.*
[4] *Live weight gain during Period 1, ∼4 weeks extended spring grazing period (See **Figure 1**). Lambs in S_Common on Rangeland were not weighed.*
[5] *Live weight gain from end of Period 1 to autumn (Period 2 and 3). Lambs in S_Common on Rangeland were not weighed.*
[6] *Slaughter weight.*
[7] *EUROP system: P– = 1, P = 2, P+ = 3, O– = 4, O = 5, O+ = 6, R– = 7, R = 8, R+ = 9.*
[8] *EUROP system: 1– = 1, 1 = 2, 1+ = 3, 2– = 4, 2 = 5, 2+ = 6, 3– = 7, 3 = 8. … 5+ =15.*
[a,b,c] *Values followed by different letters were statistically different ($P < 0.05$).*

growth, in order to cold-harden the plants. This is common in plants originating from higher latitudes (Hay, 1990).

The improved nutritive value of the forage by grazing is in line with findings in other studies (Pontes et al., 2007; Schönbach et al., 2012). Grazing reduces the number of stems and tissues that reach mature stage, and the age of tissue is generally lower than under no grazing (Schönbach et al., 2012).

Sheep Grazing Distribution Pattern

As the swards in the plots that were grazed during the summer had shorter plant height with higher nutritional quality at the end of Period 3, it was expected that ewes in the autumn (Period 4) preferred to graze more in these plots than in the control plots. Sheep are more generalist than specialist herbivores as they do not change foraging behavior and become more specialist when food is abundant (Arnold, 1987). As long as forage availability is sufficient, we would not expect difference in grazing distribution pattern. However, herbivores generally select short, leafy swards that contain relatively high concentrations of nutrients (McNaughton, 1984). Sheep avoid grazing areas infected by intestinal parasites (Cooper et al., 2000), and their feces aversion outweighs their attraction toward high forage

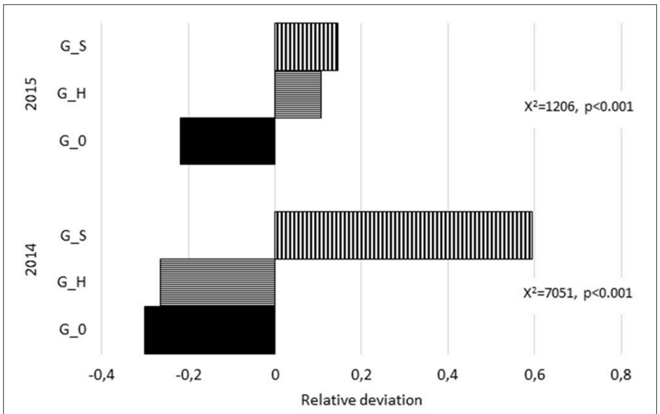

FIGURE 2 | Relative deviation of the GPS observations of ewes from hypothesized (i.e., random) GPS distribution in autumn (Period 4) after the summer grazing in 2014 and 2015, respectively, in each of the three summer grazing treatments [G_0 Control (no grazing), G_H Heifers grazing, and G_S ewes grazing with their offspring]. In Period 4, the ewes could graze the entire area freely. Relative deviation is calculated as the difference between the observed and hypothesized percentage (33%) divided by the hypothesized percentage. Positive deviation indicates preferred area by the sheep while negative deviation indicates avoided area. The distributions are compared by a X^2 Goodness-of-fit test.

quality sward (Hutchings et al., 1999). We have no record of parasite infection status of the studied grassland, but as it had not been used for a long period of time the parasitic pressure was expected to be low. However, the difference between the 2 years, with higher preference for the area grazed by sheep in autumn of the first than the second year, may be due to increased parasitic pressure.

Animal Performance

The higher weight gain, slaughter weight, carcass confirmation, carcass fatness, and carcass value observed in lambs kept for an extended 1 month period on spring pastures (S_Spring) are likely due to both animal and pasture related factors. As weight gain correlates with slaughter characteristics this is as expected (Sents et al., 1982). Animal factors such as age, weight, and condition of lambs when turned out on summer range pasture is known to affect performance of lambs (Warren and Mysterud, 1995; Dwyer, 2009).

The common farm practice in Norway is to keep the spring pasturing period as short as possible, turning ewes and lambs as early as possible on summer range pastures. Extending the spring grazing period implies that lambs are older and heavier when turned out on summer range pastures, explaining their better weight gain in this study. Further, spring pastures are commonly cultivated grasslands close to the farm building and at lower altitudes than range summer pastures. In spring, grass growth starts earlier at lower altitudes. Extending the spring grazing period on lowland, cultivated pastures may have provided access to high quantities of easy available, high quality grass for a longer period in spring explaining the improved performance in lambs. However, during the summer the forage quality of the enclosed pasture declines. Keeping ewes and lambs on the same enclosed cultivated grasslands for the entire grazing season (S_summer) gave the lowest average weight gain in lambs of all treatment groups. This can be explained by the forage maturation hypothesis (Albon and Langvatn, 1992), i.e., the forage quality declines with the maturity but the rate depends on altitude. Sheep on rangeland may move to higher altitude and have access to plants in a young stage of development for a longer period of time than those kept on enclosed pastures in the lowland.

The study farm was situated in the county of Møre and Romsdal, which is considered to have medium quality rangeland pastures with an average lamb weight gain of 252 g/day compared to national average of 261 g/day in 2015 according to the Norwegian Sheep Recording System (Ringdal et al., 2016). This indicates that there is genetic potential for increased weight gain. The observed weight gains in our study of 255g/day in the treatment with the highest (S_spring) and 228 g/day in the group with the poorest weight gain (S_summer) further shows that including abandoned cultivated grasslands has the potential to exploit the genetic potential for weight gain in lambs in this region.

Ensuring animal health and welfare in farming systems receives increased attention and new policies and legislations are implemented (Main and Mullan, 2017). Grazing unfenced mountain and forest rangelands provides an opportunity for the animals to perform natural behaviors, and therefore has the potential to provide a high level of animal welfare. Sheep on rangeland are, however, also associated with risk factors such as undetected disease and predators causing suffering as well as lamb losses. Providing ewes and lambs with an extended spring grazing period, and thus a farming system where older and heavier lambs are released on range pasture, has the potential to improve performance, condition, and thus animal welfare in sheep farming (Warren and Mysterud, 1995; Dwyer, 2009). There is abundant range summer pastures available for domestic herbivores in Norway (Rekdal, 2013), and the Norwegian authorities state that food production should be increased and that livestock production should be based on domestic feed resources (Landbruks- og matdepartementet., 2016). Our results suggest that access to abandoned cultivated grasslands, commonly located close to arable farmland, may allow sheep farmers to increase flock size and ensure healthy, robust lambs for rangeland pasturing. This will also increase the stocking rate on rangeland areas, and thus to increased food production on abundantly available feed resources.

Abandoned farmland, or land in danger of being abandoned in Norway, is owned by landowners that for various reason have quit farming. The areas are often marginal, steep sloping with high content of stones in the soil, and difficult or impossible to till with tractors. Putting a value on such land is often difficult, and even if the Norwegian legislation states that landowners are obliged to maintain farmland, it is not necessarily done for marginal land, as it is less attractive to rent. However, it is likely that social and juridical factors as much as technical and management factors account for why these areas are not rented out to active farmers (Flemsæter et al., 2011; Sang et al., 2014). Social and juridical constraints were beyond the objectives of the current study, but our findings may serve as a basis for sheep farmers and landowners to value the land in monetary terms.

CONCLUSIONS

The global need for food with an increasing world population and a national responsibility to ensure food security implies that available feed resources should be used for food production. By showing that marginal and abandoned grassland has a value, we provide both the landowner and the potential farmer of the land with information that may be important for decision-making and rental agreements. The abandoned grassland used in this study showed that the productivity and forage quality improved when it was grazed, and that the performance of lambs improved when using it for extending the spring grazing period. Including such grassland in existing sheep farming therefore shows potential for improving animal welfare, performance, and economy.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available in the Mendeley data repository doi: 10.17632/gb7822ngty.1.

AUTHOR CONTRIBUTIONS

HS, LG, UL, and EB conceptualized the study. SA: did the botanical assessment. HS analyzed the data from the herbage production. LG data from the sheep performance. HS, LG, and SA wrote the manuscript and all authors were involved in reviewing, revision and final approval of the manuscript.

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