PhD thesis

Wood ash and Biochar as soil amendments:

To what extent can they help forests to adapt to climate change?

Egur errautsa eta biocharra lurzoruetako ongarri gisa: zenbateraino dira basoek larrialdi klimatikoari aurrea hartzeko tresna baliagarriak?

Lur Moragues Saitua

eman ta zabal zazu

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SUMMARY (EN)

Forest biomass is considered an alternative to fossil fuels in energy *production*, as part of global strategies for climate change mitigation. Application of by-products such as wood ash (WA) and biochar (BC) to soil could replace the nutrients removed by tree harvesting, increase soil carbon stocks, and therefore improve soil properties in order to face up to climate change. However, the extent to which these amendments can provide benefits depends on how the by-products interact with the system. We studied the short-term responses of WA and BC application in two different temperate forests: ES-K: a Typic Udorthent with mature *Pinus radiata*; ES-O: a Typic Dystrudept with young *Quercus pyrenaica*, to test whether the addition of WA or BC to the forest floor could be a tool to adapt forest (by the forest soil) to Climate Change. In order to address the stated objectives, we established a field trial consisting of two experimental sites, and developed a laboratory trial based on incubations. In the field, BC was applied at rates of 3.5, 10 and 20 Mg ha^{-1} and WA at rates of 1.5, 4.5 and 9 Mg ha^{-1} (calcium equivalent). A nitrogen enriched treatment was applied for the intermediate doses, to test for possible N-limitation. Incubations were performed with the ES-K soil, with the intermediate dosage of BC (with and without N). Our results were not conclusive to confirm that BC or WA are good amendments to protect Atlantic forests against climate change. Indeed, our findings confirm that the effects of addition of biochar and wood ash to temperate humid forest soils vary between different sites, depending on the soil type and system, as well as on application rates and whether nitrogen was also added.

LABURPENA (EU)

Mundu mailan, klima larrialdiari aurrea hartzeko zehazten ari diren estrategien baitan, erregai fosilen ordezko aukeratzat hartzen ari da baso-biomasa. Energia eraldatzeko prozesuetan sortutako albo-produktuak, bai errautsa (WA) bai biocharra (BC), lurzoruan aplikatzeak ustiaketa-prozesuan biomasaren baitan erauzitako mantenugaiak ordezka ditzake eta lurzoruko karbono kantitatea handitu, eta, beraz, klima-aldaketari aurre egiteko ezinbesteko diren lurzoruaren propietateak hobetu. Hala ere, emendakinok eragin dezaketen onura, sistemarekin emango diren elkarrekintzen araberakoa izango da. Tesi honetan WAa eta BCa aplikatzeak epe laburrean duen eragina aztertu dugu, bi baso epeletako lurzoru desberdinetan : ES-K: *Pinus radiata* landaketa heldua duen *Typic Udorthent* bat, eta ES-O: *Quercus pyrenaica* landaketa gaztea duen *Typic Dystrudept* bat. Helburua WAa edota BCa aldaketa klimatikoari (CC) aurrea hartzeko eta berari egokitzeko ongarri izan daitezkeen frogatzea izan da. Helburuari heltzeko, landa-saiakuntza bat ezarri genuen, bi ikerketa-eremuk osatua, eta laborategiko saiakuntza bat garatu genuen, inkubazioetan oinarritua. Landa-saiakuntzan, BCa 3,5, 10 eta 20 Mg ha^{-1} dosietan aplikatu genuen, eta WAa 1,5, 4,5 eta 9 Mg ha^{-1} dosietan, BCaren dosi bakoitza WAaren dosi bakoitzaren baliokide izanik kaltzio kantitatean. Dosi ertainekin nitrogenoz osatutako tratamendu gehigarri bat aplikatu genuen, N-a mugatzaile ote zen frogatzeko. Inkubazioak ES-Kko lurrarekin egin genituen, BCaren tarteko dosiak baliatuta (N-arekin eta gabe). Ez dugu BCa edota WAa ongarri egokia den baieztatzeko moduko emaitza sendorik izan, klima-aldaketari aurre egiteko baso atlantikoen gaitasuna hobetzeari dagokionean. Gure aurkikuntzek berresten dute baso heze eta epeletako lurzoruei BCa eta WAa gehitzearen eragina aldagai askoren araberakoa dela. Hau da, eragina aldatu egiten dela leku batetik bestera, lurzoru-motaren eta sistemaren arabera, baita aplikatutako dosiaren arabera eta nitrogenoa ere gehitu baldin bazen ala ez.

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Appendix II: **“Assessment of improvement of soil physical properties following organic amendments”**. Draft for submission as short communication in Geoderma

Apendix III: Article “**Towards meaningful quantification of glomalin-related soil protein (GRSP), taking account of interference with the Coomassie Blue (Bradford) assay**” published in the European Journal of soil science in 2018.

Appendix IV: “**Effect of Biochar in water stable aggregate formation**” . Communication published in the The CICS2014 congress book *Retos y oportunidades en la ciencia del suelo* ISBN: 978-84-8408-769-4.

Appendix V: **Effects of Biochar and Wood ash amendments in the soil-water-plant environment of two temperate forest plantations**” article accepted for publication in *Frontiers in Forests and global change in 2023.*

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1. SARRERA (EU)

Mundua larrialdi klimatikoaren aferari aurre egin beharrean aurkitzen da. Hortaz, aldaketa klimatikoa mitigatzea[1] eta lekuan leku aurreikusten diren aldaketak ahal den heinean txikitu, arindu eta aurrea hartzea ezinbestekoa da etorkizun bizigarri bat bermatzeko. IPCCak (Intergovernmental Panel on Climate Change) abisua eman zigun 2021eko udan: berotze globala[2] 1,5 gradutan mantentzeko ezinbestekoa dela karbono neutraltasuna 2050erako lortzea. Izan ere, giza jarduerek eragin dute industrializazio aurreko mailaren gaineko 1 °C inguruko (0,8–1,2 °C tartean) berotze globala. Berotegi-efektuko gasen iturri antropogenikoen artean hiru nagusitzen dira: 1) erregai fosilen errekuntza, 2) deforestazio tropikala eta lurzoruaren erabileraren aldaketak, eta 3) lurzoruaren higadura azeleratua (IPCC, 2018).

Europako Batzordeak proposamen batzuk egin zituen Europar Batasunak (EB) klimaren, energiaren, garraioaren eta fiskalitatearen arloan dituen politikak egokitzeko, berotegi-efektuko gasen emisio garbiak gutxienez % 55 murrizteko hemendik 2030era (European Commission, 2019). Bide honetan, Euskal Autonomi Erkideagoko (EAE) Basque Green Deal-ek emisioak murrizteko eta energia berriztagarria sortzeko helburuak jasotzen ditu, eta energia estrategiak biomasa bidezko energia erabilera handitzea aurreikusten du, besteak beste. Itun Berdeak ez du hala ere lurzoruak karbonoaren zikloan duen papera aintzat hartzen, adierazle gisa hartzera mugatzen da (European Commission, 2019).

2050erako berotegi-efektua eragiten duten gasen emisioak murrizteko helburua lortzeko, lurzoruak aintzat hartu eta hobeto kudeatzea ezinbestekoa da. Zentzu honetan, 2021eko azaroaren 17an, Europako Batzordeak 2030erako EBren Lurzorurako Estrategia argitaratu zuen.

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[1] Mitigazioa (Aldaketa klimatikoarena), berotegi-efektuko gasen emisioak murrizteko edo gordailuak hobetzeko giza esku-hartzea da. (IPCC AR6 WGIII, 2022)

2Berotze globala: gizakiaren ekintzaren ondorioz emandako lurrazaleko tenperatura globalaren pixkanakako igoera da. Bai behatutako igoera, bai eta aurreikusia ere bai.

Estrategia honek, lurzoruak babesteko, kaltetuta daudenak biziberritzeko eta modu iraunkorrean jarduteko neurri zehatzak ezartzen ditu. Era berean, borondatezkoak eta juridikoki lotesleak diren neurriak proposatzen ditu, eta helburuak lortzeko funtsezko ekintzen artean, lurzoruaren ikerketa eta ezagutza handitzeko beharra oraindik ere handia dela aitortzen du (European Commission, 2021a). Eusko Jaurlaritzak ere 2030eko Lurzorua Babesteko lehen estrategia onartu berri du (Eusko Jaurlaritza, 2022). Estrategia honekin, orain arteko lurzoru kutsatuen ikuspegi tradizionala gainditu eta lurzoruei buruzko ikuspegi integrala landuz, 2050erako lurzoruak modu jasangarrian kudeatuta egotea bilatzen da.

Lurzorua lehorreko bizitzaren eta bizidunen oinarrian dago. Gizakiok ere bai, izaki lehortarrak garen heinean, lehengaiak hornitzeko bitarteko nagusia daukagu eta gure jarduerak garatzeko funtsezko baliabidea izanik, gure ondarearen iturria eta espazioa da. Fenomeno fisiko, kimiko eta biologikoek elkar-eragiten duten dimentsio anitzeko espazioak dira lurzoruak eta ondorioz, biodibertsitate handikoak. Lurzoru osasuntsuak ere planetako lurreko karbono-erreserba handiena dira. Ezaugarri honek, ura xurgatzeko eta uholde eta lehorteen arriskua murrizteko funtzioarekin batera, lurzorua ezinbesteko aliatu bihurtzen du larrialdi klimatikoari aurrea hartzeko garaian.

Lurzoruaren egoera hobetzeko asmoz, ongarriak gehitu ohi dizkiogu. Biomasa bidezko energia ekoizpenak azpi produktu ezberdinak sor ditzake erabilitako erreketa prozesuaren arabera (pirolisia edo erreketa osoa), eta azpi produktu hauek, biocharra[3] (BC) pirolisiaren ondorioz eta errautsa (WA, Ingelesezko Wood Ash) erreketa osoaren ondorioz, basora bueltatu daitezke zikloa itxi eta biomasaren erauzketan sistematik ateratako mantenugaiak bueltatzeko. Errautsak historian maiz erabilitako ongarriak dira. Erreketaren bitartez kontzentratutako mantenugaiak

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[3] Biocharra: *Biochar is produced by thermal decomposition of organic material under limited supply of oxygen (*O_2*), and at relatively low temperatures (<700°CBC). It is produced to be used as a soil amendment with the intention to improve soil functions and to reduce emissions. (European biochar initiative)*

lurzorura gehitzeaz gain, pHa igo egiten dutelako. Errautsek askotan guztiz erre gabe geratutako ikatz zatiak ere badauzkate, BCaren antzeko eragina izan dezaketenak. Pirolisiaren bidez sortutako BC askok bestalde, errautsek bezala pHa igotzeko gaitasuna daukate. Materia beraren izaeragatik ere, BCa aldaketa klimatikoari begirako ongarri ona izan daitekeela aipatzen da askotan, duen porositateak eta azalera espezifiko handiak lurzoruaren estruktura hobetu dezakeelako eta lurzoru-agregatuen baitan gordeta geratu.

Hala, tesi honen helburu nagusia WAa eta BCa basora gehituta, aldaketa klimatikoari aurrea hartzeko eta berari egokitzeko ongarri izan daitezkeen frogatzea da. Horretarako, bi ikerketa eremutan (bi baso landaketa) ezarritako landa entsegu bat eta inkubazioetan oinarritutako laborategiko entsegu bat baliatu ditugu. Errautsak eta BCak, lurzoru-ur-landare sisteman (V. Eranskina), lurzoru-agregatuen dinamiketan (IV. Eranskina) eta egonkortasunean (II. Eranskina), eta lurzoruko propietate hidraulikoetan (I. Eranskina) duten eragina aztertu dugu. III. Eranskinean aldiz, Glomalinarekin lotutako lurzoru-proteina (GRSP) kantitatea estimatzeko metodologiaren optimizaziorako pausu bat gehiago eman genuen. Izan ere, GRSPek, agregatuen egonkortasunarekin loturarik ba ote duten aztertzea izan da gure helburuetako bat, aktibitate fungikoaren paperaren indikatzaile bezala. Baita GRSP kopurua aplikatutako tratamenduaren ondorioz aldatu egiten den edo ez aztertzea.

Tesi hau CHARFOR proiektuaren baitan kokatuta egon da (RTA2012-00048-00-00). Honen helburu nagusia BCak eta WAak lurzoruan duen eragina konparatzea zen, lurzoruen aldaketa klimatikorako mitigazio-gaitasunaren handipenari dagokionez eta egokitzapenari begira. Ikerketa-eremuetako bat bestalde, Reinfforce proiektuaren baitan ezarri zen (REsource INFrastructures for monitoring, adapting and protecting European Atlantic FORests under Changing climate, DS-19), aldaketa klimatikoari adaptatzeko demostrazio gune bezala.

Lan hau ingelesez eta euskaraz idatzita dago. Hori dela eta, atal batzuk bikoiztuta daude, hala nola SARRERA, HIPOTESIAK ETA HELBURUAK, EZTABIADA OROKORRA eta ONDORIOAK. ESKER ONA atala hizkuntzen mosaiko bat eginez agertzen da, oso pertsonalizatuta idatzita dagoelako. METODOLOGIA atalaren kasuan euskaraz idatzita dago, eta honen hasieran, laburtuta ingelesez, metodologia bakoitza zein eranskinetan ageri den adierazi da. Izan ere, eranskin gisa atxikitako artikuluetan deskribatuta ageri dira denak. MARKO TEORIKOA ETA EMAITZAK ingelesez baino ez daude. Ingelesaren nagusitasuna tesia ebaluatuko duen epaimahaiaren nolakotasunagatik da. Zenbait kontzepturen definizioak oin-ohar gisa agertuko dira, kontzeptu bakoitza lan honetan ulertzen deneko modua argi egon dadin.

1. INTRODUCTION (EN)

The world is facing the climate emergency. Therefore, mitigating climate change and reducing as much as possible the impacts, and anticipating on-site changes is essential to ensure a sustainable future. The IPCC (Intergovernmental Panel on Climate Change) alerted us in the summer of 2021 that maintaining global warming at 1.5 degrees requires achieving carbon neutrality by 2050. In fact, human activities have led to global warming of about 1°C (range 0.8–1.2°C) above the pre-industrial level. Anthropogenic sources of greenhouse gases include three: (1) fossil fuel combustion, (2) tropical deforestation and land use changes, and (3) accelerated soil erosion. (IPCC, 2018).

The European Commission adopted a set of proposals to make the EU's climate, energy, transport and taxation policies fit for reducing net greenhouse gas emissions by at least 55% by 2030, compared to 1990 levels (European Commission, 2019). Similarly, the Basque Green Deal of the Basque Autonomous Community (CAPV) covers the objectives of reducing emissions and generating renewable energy, and the energy strategy foresees, among others, increasing the use of biomass-based energy. The Green Deal, however, does not consider the role of soil in the carbon cycle, but merely considers it an indicator (European Commission, 2019).

Improving soil management is essential to achieve the greenhouse gas emission reduction target for 2050. On 17 November 2021, the European Commission published the EU Soil Strategy for 2030. This strategy sets out concrete measures for soil protection, the revitalization of those affected and sustainable action. It also proposes voluntary and legally binding measures and recognises the need to increase soil research and knowledge as one of the key facts for achieving the objectives (European Commission, 2021a). The Basque Government has just adopted the first 2030 Strategy for Soil Protection (Eusko Jaurlaritza, 2022). This strategy seeks sustainable soil management by 2050, overcoming the traditional view of contaminated soils and addressing an integrated view of soils.

The soil is at the basis of terrestrial life. Human beings, as terrestrial beings, also have soil as our main means of supplying raw materials and, it is a fundamental resource for the development of our lives. It is the source and focus of our heritage. Soils are multidimensional spaces where physical, chemical and biological phenomena interact, and therefore harbour high biodiversity. Healthy soils are also the largest land C sink on the planet. This characteristic, coupled with the function of absorbing water thus reducing the risk of floods and droughts, makes the soil an essential ally for facing up to climate emergency.

Fertilizers are usually added to improve soil condition. Biomass energy production can produce different by-products depending on the combustion process used (pyrolysis or complete combustion), and these by-products, biochar (BC) via pyrolysis and wood ash (WA) via complete combustion of wood, can be returned to the forest to close the cycle and return the nutrients extracted from the system due to the biomass harvesting. Ash is a fertilizer that has widely been used in history, because apart from adding to the soil the nutrients concentrated by burning, they increase soil pH. The ashes also contain charred carbon fragments that often have not been completely burned and may have a similar effect to BC. Many BCs produced by pyrolysis can increase pH, as is the case for ashes. It is also reported, on many occasions, that BC can be a good fertilizer to mitigate climate change, as its porosity and its large specific surface area can improve the soil structure and be stored within soil aggregates.

The main purpose of this thesis is to demonstrate the potential of additions of WA and BC to the forest to prevent and adapt to climate change. For this purpose, we performed a field trial established in two experimental sites (two forest plantations) and a laboratory trial based on incubations. We analysed the effects of WA and BC in the soil-water-plant system (Annex V), in soil aggregate dynamics (Annex IV) and stability (Annex II), and on the hydraulic properties of soil (Annex I). Annex III proposes another step in optimizing the methodology for estimating the amount of Glomaline-related soil proteins (GRSP). In fact, one of our objectives has been to analyse whether GRSPs are related to aggregate stability as indicators of fungal activity. We also analysed whether the number of GRSP varies with the amendments applied.

This thesis was framed within the CHARFOR project (RTA2012-00048-00-00). Its main objective was to compare the influence of BC and WA on soil in terms of increasing adaptability and mitigating capacity of soil for climate change. One of the experimental sites was established within the Reinfforce project (REsource INFrastructures for monitoring, adapting and protecting European Atlantic FORests under Changing climate, SD -19) as a demonstration site for strategies to adapt to climate change.

This thesis is written in English (EN) and Basque (EU). For this reason, some sections are duplicated, such as the *INTRODUCTION*, the OBJECTIVE AND HYPOTHESIS, the GENERAL DISCUSSION and CONCLUSIONS. In the case of the METHODOLOGY section, written in Basque, it starts with an English summary of the annexes in which each methodology is already described in English. Indeed, they are all described in the articles attached as annexes. The THEORETICAL FRAMEWORK and the RESULTS are in English only. The predominance of English is due to the nature of the tribunal that will evaluate the thesis. Definitions of some concepts will appear as footnotes to make it clear how each concept is understood in this work.

2. THEORETICAL FRAMEWORK (EN)

2.1. Climate change: strategies, governance, and commitments

The Paris Agreement marked a global temperature target of below 2 °C and encouraged efforts to limit increase to 1.5 °C above pre-industrial levels. To limit warming to 1.5 °C (and 2 °C), countries will need to plan for a more rapid transformation of their national energy, industry, transport and land-use sectors (Roe et al., 2019). Integrated assessment models aim to model the future state of energy systems and technologies by generating energy scenarios. Based on integrated assessment model projections, the Intergovernmental Panel for Climate Change (IPCC) emphasized that the more delay in the peak of global CO_2-emissions, the more the world will need to rely on Carbon Dioxide Removal (CDR) technologies [or Negative Emission Technologies (NETs)] to achieve climate goals (Terlouw et al., 2021).

Therefore, to prevent global warming, an urgent threat to many lives on Earth, achieving carbon neutrality has become an important goal that is pursued by various organizations across the world, with the ultimate aim of measuring and controlling direct and indirect emissions of greenhouse gasses (Smith and Fletcher, 2020). In short, neutralizing the emissions of greenhouse gases caused by human activities [“the root cause of global warming” (IPCC, 2018)] requires both robust scientific understanding of the impacts of global warming on both natural and human systems and collective action on strengthening and implementing the global response (Zhang et al., 2021). Currently, the deployment of CDR technologies at the gigatonne scale (e.g. Biochar (BC) production and application to soil for carbon sequestration[4]) is still debatable. Relying mitigation of climate change (CC) on CDR technologies could result in an ethical hazard, since policy-makers could use CDR technologies as a safeguard to postpone climate mitigation measures. The multidisciplinary nature of carbon neutrality goes beyond

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[4] Biochar was among the IPCC’s short-list of Negative Emission Technologies (NETs) that could provide a significant sequestration impact. For example, Cameroon plans to establish some fifty biochar production plants by 2030 (Bussines in Cameroon, 2021/11/11)

natural/earth sciences and technology and involves issues such as policies, investment, communication, behaviour change and adaption, and international relations (Hawken, 2017; Farnworth, 2018). Since among others, may involve several important industrial bodies and various human daily activities and land use competition, which may also lead to new conflicts and struggles (Zhang et al., 2021). From a life-cycle perspective, CDR technologies could result in infeasible climate goals if the environmental benefits are less than expected. Still, real demonstration projects are scarce (Terlouw et al., 2021).

At European level, the aim of achieving net-zero greenhouse gas emissions by 2050 relies, among others, on carbon removals through the restoration and better management of forests and soils. On July 14, 2021 the European Commission published the new forest strategy for 2030, one of the flagship initiatives of the European Green Deal. It builds on the EU Biodiversity strategy for 2030. The strategy will contribute to achieving the EU's biodiversity objectives as well as greenhouse gas emission reduction target and climate neutrality by 2050. It recognises the central and multifunctional role of forests, and the contribution of foresters and the entire forest-based value chain for achieving a sustainable and climate neutral economy by 2050 and preserving lively and prosperous rural areas (European Commission, 2021b). In the same vein, EU Soil Strategy for 2030 was published on November 17, 2021, where a framework and voluntary or legally binding actions that the Commission intends to undertake to protect, restore and sustainably use European soils is outlined. The strategy translates some of the objectives of the European Green Deal too and integrates a series of strategies and policies already decided by the European Commission: from Farm to Fork to the Zero Pollution Action Plan, from the Biodiversity Strategy to the new CAP. Given the lack of EU soil policy so far, the communication has the ambition to address this gap and proposes an overall strategy on all aspects of a healthy soil having an impact on CC, biodiversity, circular economy, food security and water protection and all policies to be adopted to overcome its degradation (European commission, 2021a)

The Basque Government has just approved (June 2022) the first Soil Protection Strategy 2030 that comprehensively addresses soil management, overcoming the traditional approach on contaminated soils, based on the law for the prevention and correction of soil contamination 4/2015 adopted by the Basque government in 2015. In addition, the Basque Country's Regional CC Strategy to 2050, named KLIMA 2050, aims to reduce greenhouse gas emissions by 40% by 2030, and by 80% by 2050 based on 2005 levels; to supply 20% of final energy consumption by renewable energy by 2030 and 40% by 2050 (Eusko Jaurlaritza, 2015). The twelfth line of action of the Euskadi 2050 CC Strategy foresees an increase in the capacity of the Basque region as a C sink. Mitigation strategies are based on increasing the carbon retention capacity of soils and ecosystems and, in general, on reducing human carbon emissions. Within the Basque Energy Strategy 2030 (known as 3E-2030), in order to reduce GHG emissions and contribute to the targets of KLIMA 2050, it aims to achieve 40% contribution of renewable energy to meeting final consumption by 2050, which mostly involves harnessing the resources with greatest potential in the Basque Country, such as biomass and wind, which account for 90% of planned use of renewables (EVE, 2017).

2.1.1. Climate change projections for the Atlantic Basque Country

Activities coordinated by the IPCC include the creation and monitoring of scenarios in the global space environment. In the fifth assessment report (AR5) four scenarios were established: RCP2.6, RCP4.5, RCP6.0 and RCP8.5, characterised by the approximate calculation of total radiation forcing in 2100 compared to 1750, i.e., depending on assumptions about how the energy balance of the Earth system will vary (IPCC, 2014).

For climate projections at local level, data available from regional projections on Europe, given by the EURO-CORDEX project have been used, which provides a set of 12 alternative simulations produced with various combinations of 5 global models and 4 regional climate models on the same grid (with 0.11 horizontal resolution, equivalent to about 12 km).

The Klimatek project (IHOBE, 2019) developed a high-resolution climate atlas (~1km) of climate trends for the Autonomous Community of the Basque Country, made through the General Circulation Models (GCM-General Circulation Models) and calibrated with observational data representative of the study area (Gutiérrez et al., 2019). Models represent the dynamics of the climate system and its components (atmosphere, hydrosphere, cryosphere, lithosphere and biosphere), subjected them to fictitious scenarios (at least two, e.g. RCP4.5 and RCP8.5 from Fifth Assessment Report of the IPCC [AR5], to obtain a range of results) in which the future evolution of anthropogenic factors affecting the climate system is attempted, such as the emission of greenhouse gases, the main source of uncertainty in the second half of the century.

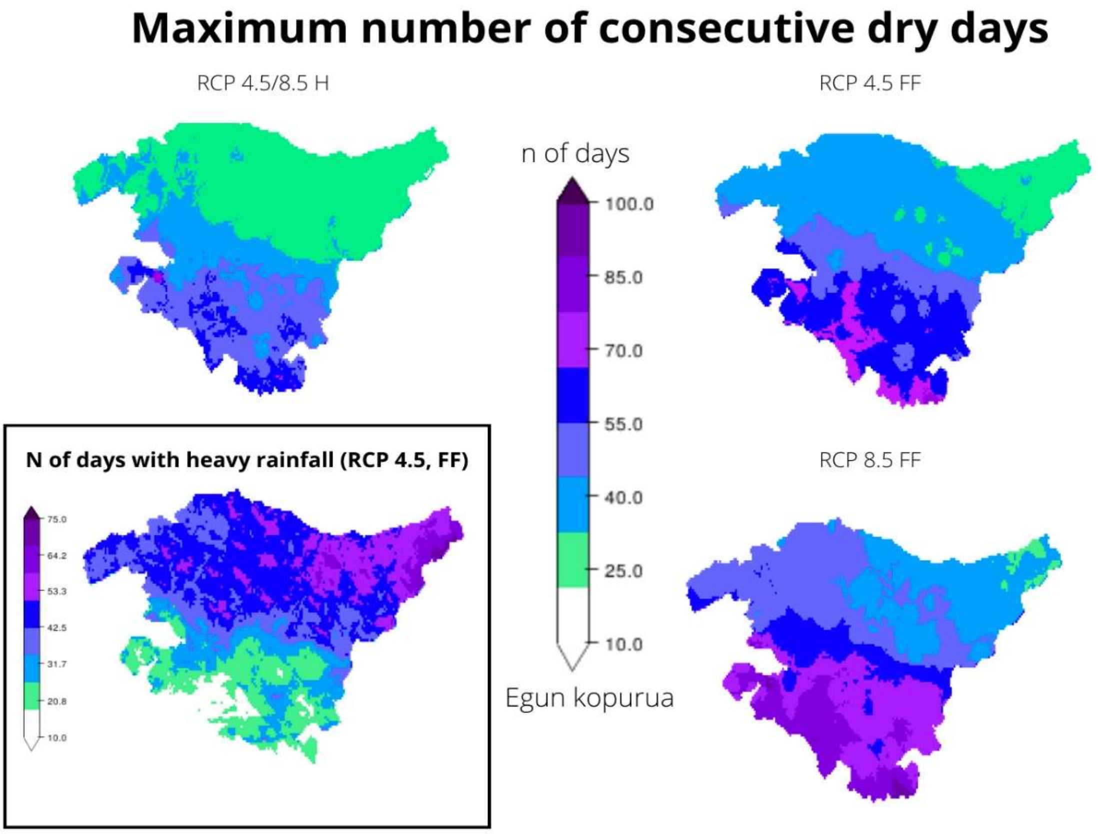

Figure 2.1 Maximum number of consecutive dry days (Pr<1mm) and Number of days with heavy rainfall (Pr>10mm), previewed for the Basque autonomous community, by different scenarios (RCP 4.5 and RCP 8.5 from Fifth Assessment Report of the IPCC [AR5]) and Periods: H=historical and FF= Far Future (from yrs 2075-2100). Source: http://escenariosklima.ihobe.eus (accessed 2022/08/20)

In our region the temperature trend is upward and the frequency of heat waves is expected to increase. This increase, according to scenario and model, ranges from 1.5ºC and 5ºC, and it becomes more remarkable by the end of the century and for CPR 8.5 scenario. The model of change is very homogeneous in the CAPV as a whole, although the increase is expected to be more moderate on the coast (north) than inland (south). Rainfall is expected to follow a negative trend. It will be reduced with annual precipitation expected to be reduced by an average of 15% by the end of the century (for scenario RCP8.5). Although the mean rainfall variation is initially expected to be less than 5%, it is expected to rise from 10-15% by the end of the century, depending on the scenario. The latter modification will not occur in the same way throughout the territory. A lighter change is expected in the north-east of the territory. Overall, the number of rainfall days will be reduced and long periods of drought will be foreseen, although rainfall will be higher per day (Figure 1.1). In other words, gentle rains will be scarcer (Ihobe & Eusko Jaurlaritza, 2019). In short, in the Basque region (CAPV+Navarra), it is expected a Mediterranealization of the Atlantic area and a desertification of the Mediterranean area. All this, together with the expected variation of wind speed and the increase of extreme phenomena (Sanz and Galan, 2020). Healthy ecosystems and soils are therefore needed to be more resilient and to reduce vulnerability to CC (European Commission, 2021a).

2.2. The role of forests in climate change mitigation and adaption

2.2.1. Forests as carbon sink and fossil fuel substitutes

Forests can act as either carbon sources or carbon sinks. A forest is considered to be a carbon source if it releases more carbon than it absorbs, and the forest carbon sink is quantified by measuring the net annual accumulation of carbon (carbon sequestration) by living and death biomass, aboveground and belowground within soils. Harris et al. (2021) found that the world's forests sequestered about twice as much carbon dioxide as they emitted between 2001 and 2019. Forest management activities can promote C sequestration, but calculations of the GHG mitigation potential of forestry are highly sensitive to the assumptions of the analysis and vary markedly among forest types, complexity, species composition, sites and management systems (Fahey et al., 2010).

The understanding of carbon cycle mechanisms is still not fully understood, and it is still difficult to determine the exact C stocks and fluxes (Zhao et al., 2020). Fahey et al. (2010) reported that in most forests, the largest C pools are aboveground live biomass and mineral soil organic matter (SOM), with lesser amounts in roots and surface detritus. However, few studies have attempted to quantify fungal biomass in forest C budgets (Ouimette et al., 2020). Fahey et al. (2005) estimated a rhizosphere flux to mycorrhizal fungi and root exudates of 80 g C m^{-2} yr^{-1}, suggesting that the biomass of mycorrhizal fungi can represent a substantial proportion of forest Net Primary Production (NPP, around 30%), especially in low-N, conifer-dominated stands, as also reported by other authors (Gill and Finzi, 2016). However, this proportion varied from about 4.16% in nearly pure deciduous broadleaf stands up to about 58.33% in conifer-dominated stands (Ouimette et al., 2020).

There are overall estimations of global fluxes and stocks. Harris et al. (2021) estimated that global forests were a net carbon sink of -7.6 ± 49 PgCO2e yr^{-1}, reflecting a balance between gross carbon removals (-15.6 ± 49 GtCO2e yr^{-1}) and gross emissions from deforestation and other disturbances (8.1 ± 2.5 GtCO2e yr^{-1}). The annual rate of deforestation (2015-2020) was estimated at 10 million ha (FAO, 2020). Globally, 72% of gross removals were concentrated in older (>20 yr) secondary natural and seminatural forests, 12% in tropical primary forests, 10% in plantations, 3.5% in young (<20 yr) forest regrowth, 1.3% in mangroves and 0.34% in boreal and temperate intact forest landscapes (Harris et al., 2021). Adams et al (2019) reported that in temperate forests annual C sequestration via biomass growth peak in stands between 11 and 30 years of age, although net storage (biomass and soil) is greatest in older stands, which can contain up to 2 or 3 times more C than younger stands (Adams et al., 2019). However, as

Anderson-Teixeira et al. (2021) concluded in their review, it is important to consider climate mitigation potential of every type forest in order to avoiding catastrophic CC (IPCC 2018).

Vayreda et al. (2015), comparing living biomass and its growth between the two periods of the Spanish National Forest Inventory (IFN), (IFN2 [1986-1997] and IFN3 [1997-2007]), observed that some forests increased their biomass, notably the Atlantic-northern watershed where forests presented a greater increase, in particular the ones situated in Galicia, Cantabria, Pyrenees and the Basque Country. Overall, net productivity in temperate forests is increasing worldwide, largely through "re-wilding" following abandonment of agricultural lands, or through the establishment of forest plantations that are usually intensively managed (FAO, 2015), so they are currently significant C sinks. They accumulate 0.2 to 0.4 Pg C annually, 37% of the global C deposit (Adams et al., 2019).

The ability of forests to sequester carbon also depends on its structure (Gough et al., 2019), since ecosystems with diverse plant assemblages often have higher NPP because functional complementarity optimizes whole-ecosystem light acquisition and light-use efficiency (Williams et al. 2017), as well as in species composition. Ruiz-Peinado et al. (2017) hypothesized that the carbon stock in living biomass in mixed stands of complementary species will also be higher than in monocultures. In addition, tree species richness has an impact on soil carbon stocks through litter quality, nitrogen fixation and rooting pattern, as well as on the water balance, soil microclimate and nutrient availability (Böttcher & Lindner, 2010). Diaz-Pines et al. (2011) observed that forest floor in Mediterranean mixed stands of Scots pine and Pyrenean oak (Quercus pyrenaica Willd.) presented intermediate soil carbon stocks between pure pine (highest) and pure oak stands (lowest).

The intrinsic climate mitigation potential of forests in conjunction with other sustainability aspects of using wood as raw materials to replace GHG-intensive materials and of using wood for energy are attracting increasing attention in scientific and policy discussions (Grassi et al., 2021). Several studies (Rüter et al., 2016; Nabuurs et al., 2017) assess the climate benefits of the substitution effect of wood-based materials in the EU, with values ranging from -18 Mt CO2eyr^{-1} to -43 Mt CO2e yr^{-1}.

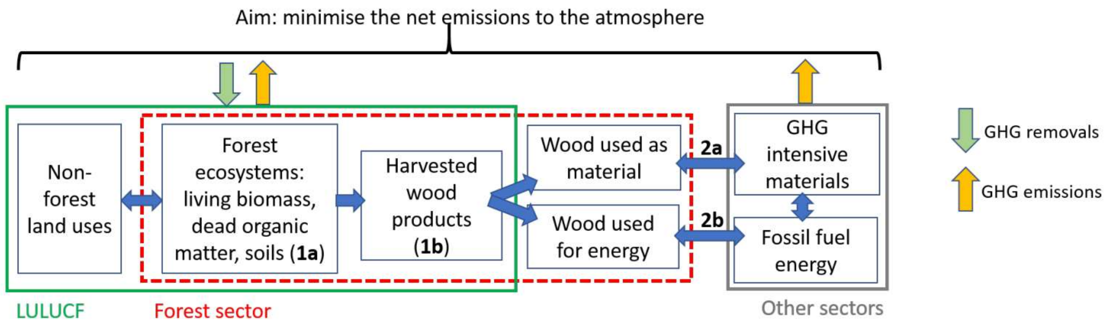

Figure 2.2.- A system perspective of the role of the forest-based bioeconomy in climate change mitigation includes sectors beyond the Land Use, Land-Use Change and Forestry (LULUCF), and covers different mitigation options: increasing carbon stocks (1a and 1b) and substation effects (2a and 2b). Source: IPCC, 2007.

However, if the greater use of wood material is associated with an increase in harvesting, this will likely negatively impact the forest carbon sink in the short to medium term (2030-2050). In this regard, as mentioned in the European Forest strategy 2030, wood of high ecological value should not be used, and wood-based bioeconomy should remain within sustainability limits and be compatible with the EU climate and biodiversity targets for 2030 and 2050.

2.2.2. Forests to strengthen societal adaptation to climate change

In conjunction with a rising demand for wood and other forest products, there is an urgent need and a complex challenge to reconcile forest uses in order to sustain the delivery of ecosystem services by preserving the ecological complexity and inherent resilience of forest ecosystems (Royer-Tardif et al., 2021). Therefore, managing forests to sustain ecosystem services in the face of CC is perhaps the biggest challenge of present-day forestry. Forests provide a multitude of benefits to humans in terms of climate regulation, health, water supply and regulation, timber, energy, habitat for biodiversity, clean air, erosion control and immense recreational, aesthetic, and spiritual benefits for millions of people (Jenkins and Schaap, 2018).

Forest microclimates and the myriad of microhabitats available within forests (e.g. root caverns, tree holes, fallen trunks) enable organisms to avoid extreme heat and drought (De Frenne et a., 2021). The physiological and ecological importance of forest microclimates has long been recognized (DeFrenne et a., 2021). Below forest canopies, direct sunlight and wind speed are strongly reduced, leading to a dampening of temperature and humidity variations (De Frenne et al., 2019). However, the biophysical conditions that promote and maintain microclimatic buffering and its stability through time are largely unresolved. Forest microclimatic buffering is sensitive to local canopy cover and water balance, which in turn is expected to change with CC, and rapid losses in forest microclimatic buffering may amplify CC impacts where forest canopies are lost (Davis et al., 2019).

Human exposure to forests has also shown to be strongly correlated with stress regulation (Hansen et al., 2017a; Morita et al., 2007). Some studies have highlighted the shift in forest priorities from production and conservation to recreation and promotion of health (Shin et al., 2017; Kim et al., 2015). however, few studies have unveiled the mechanisms and pathways by which forests interact with human health (Cho et al., 2017) and there is a need for further research to understand the specific mechanisms and pathways by which forests' variables can affect human health (Bach Pagès et al., 2020).

Forests regulates the provision, filtration and flow of water, along with stream ecosystem support and water-related hazards control, e.g., soil protection from erosion and runoff (Bredemeier, 2011). In this sense, the rate of increase in the area of forest allocated for this purpose has grown since 1990, but especially in the last ten years. At present, an estimated 399 million ha of forest in the world is designated primarily for the protection of soil and water (FAO, 2020). Forest ecosystems interactions with water and energy cycles have been highlighted as the foundations for carbon storage, water resources distribution and terrestrial temperature balancing. Forest management may thus play a key role to meet CC mitigation goals.

2.2.3. Forests at risk

Changes in temperature and precipitation due to CC are expected to result in greater impacts on terrestrial ecosystems (Sanz and Galan, 2020), affecting organisms (phenology, physiology), their populations (demographic, geographic distribution), their communities (structure and dynamics, biotic relationships) and, in general, ecosystems and their functions. As expected by many models, current CC poses a serious threat to biodiversity and ecosystem functions. Therefore, it is important to understand the vulnerability of these ecosystems to CC (Pecl et al., 2017).

The Basque Government announced that a Mediterranization of forest ecosystems is expected (Eusko Jaurlaritza, 2019). The impact is expected to be significant in three characteristic species of the forested areas: *Quercus robur* L., *Fagus sylvatica* L., and *Pinus radiata* (Eusko Jaurlaritza, 2019). It is expected that almost all of their niches will disappear by 2080 and a migration to the north of the communities that compose them (Eusko Jaurlaritza, 2019). The modification of the dominant tree species involves, inter alia, the modification of the main mycorrhizal associations (Brzostek et al., 2017). So, the vulnerability of terrestrial ecosystems related to CC is not only caused by climate factors, the interactions between living beings can also significantly affect ecosystems. (Herrero and Zavala, 2015). For example, fungi such as *Mycosphaerella dearnessii* and *M. pini* that produce the brown spot and Dothistroma needle blight, respectively of *Pinus radiata* plantations have increased in the region and have caused high mortality rates in radiata pine plantations (Ortíz de Urbina et al., 2017). This is attributable to the climate variable and its change, as rainier and warmer weather improve the reproductive conditions of these fungi (Pinkard et al., 2017; Mesanza et al., 2021)

It is estimated that the increase in extreme episodes (storms, bandages, explosive cyclogenesis, etc.) following drought periods, will significantly increase runoff and water erosion, causing SOM loss and soil degradation (Sanz and Galan, 2020). This may constitute a danger, especially in soils with high use intensity (e.g. forest clear cutting, intensive agriculture) and steep slopes.

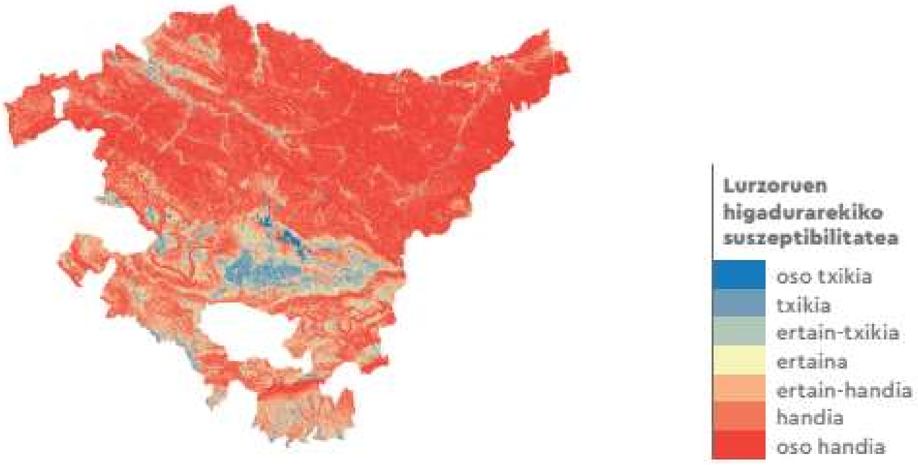

Figure 2.3.- Map of the Basque autonomous community's soil's susceptibility to erosion. Red represents very high susceptibility and blue very low susceptibility. Source: Eusko Jaurlaritza, 2022.

The projected increase in the worldwide demand for forest products over the next few decades, such as biomass for power and heat (e.g. 3E-2020), may lead to more intensive forest management (Anderson and Lockaby, 2011; Stolte et al., 2016), including extension of planted forests (Baral et al., 2016), increased mechanization (McEwan et al., 2020) and greater pressure to extract timber from sensitive zones such as steep slopes (Najafi et al., 2009). At present, the Atlantic watershed of the Basque Country is characterized by monospecific, even- aged regularly spaced plantations that are managed by clearcutting and systematic thinning. It is mainly driven by short (eucalyptus, 12-15yr) and mid rotation (radiata pine, 35-40yr) forestry. *Pinus radiata* D.Don is still the most important commercial species in the Basque Country, accounting for 51.42% of the planted forests, even though within the last decade (data from 2005-2020) its extension has been reduced in a 24% (Eusko Jaurlaritza, 2021), mainly due to the brown spot needle blight. Eucalyptus spp., in contrast, is increasing its area year after year, a 90% of increase from 2005 to 2020 (Eusko Jaurlaritza, 2021).

This type of management can result in soil physical degradation, SOM lose and nutrient depletion (Virto et al., 2015; Achat et al., 2015;). These loses are mainly due to the frequency, weight and size of forest machinery employed and the biomass removal. Forest hydrology may be altered for up to 15 years after site preparation resulting in higher run-off rates and soil loss (Gartzia-Bengoetxea et al., 2021). Harvesting reduces soil C, on average, by 11.2% even though there is substantial variation between responses at different soil depths, with greatest losses occurring in the O horizon (James and Harrison, 2016), effects that can last even 16 years after mechanized operations (Gartzia-Bengoetxea et al., 2021). Merino et al. (2004) reported that stem-only harvesting in *Eucalyptus* sp. stands was found to involve the export, every 15 years, of more than 80% of the nutrients available in weathered, acidic soils. Stem-only harvesting of *P. radiata* also involved high exports of K, Mg, P and Ca, leading to losses of 60%–100% of the available soil stores (Merino et al., 2004). In a vicious cycle, forest soil degradation drastically curtails biomass quantity and quality returned to the soil, which, in turn, negatively affects the organic carbon pools in soil (Lal, 2004), and SOM losses in eroded soil inhibit the formation and stabilization of soil aggregates, thus fuelling the cycle of soil loss. (Lehmann et al., 2017).

2.3. Increasing resistance and resilience of forest soils

Forest vegetation has profound influences on the genesis of forest soils through their canopy, biomass, litters, and roots. Forest soils vary as widely as the vegetation that covers them; they may be shallow, deep, rich or poor. Trees are widely known to impact the ecosystem hydrological cycle and resultant water availability and quality (Rodrigues et al., 2020). The canopy cover reduces de energy of heavy rainfall on impact, and through their dense root network and their ability to enhance the activity of soil meso-fauna, trees are able to improve soil structure and associated properties such as infiltration rate, hydraulic conductivity, porosity and bulk density (Eldridge and Freudenberger, 2005; Price et al., 2010). This increases the amount of water that flows into the soil and reduces surface runoff which is a major source of flooding (O'Connor and Costa, 2004; Archer et al., 2010, Marapara et al., 2021). Mycorrhizae increases the effective surface area of tree roots, enhances the absorption of nutrients, and their exudates encourage the formation of stable macroaggregates, thus enhancing the hydraulic conductivity of soil and other rhizosphere effects (Miller and Jastrow, 2000; Phillips and Fahey, 2006; Lehto and Zwiazek, 2011). Leake and Reed (2017) estimated that globally, mycorrhizal hyphae in the top 10 cm of soils to be of approximately 4.5×10^{17}km. In undisturbed forest floors there may be literally thousands of kilometres of hyphal filaments per gram of leaf litter and several kilometres of fungal hyphae even in the mineral fraction of forest soils (Jeffery et al., 2010). The extraradical mycorrhizal mycelium in the forest floor annually produces an absorptive area of 70–112 $m^2 \cdot (m^2$ of forest floor$)^{-1}$,

Soils and forest vegetation are mutually interdependent variables. It is difficult to disentangle the influence of particular tree species or forest types on the development of specific soil properties from other factors affecting soil formation, including climate, parent material, and topographic influences (Binkley and Fisher, 2013). However, tree species can influence soil properties through variation in the amount and chemical composition of the organic material produced that could alter soil pH and rate of organic matter decomposition, the depth of rooting and allocation differences to above-ground and below-ground pools, and available water content through differences in water use and canopy structure that could alter mineralization and nutrient availability. The majority of temperate forest soils fall into the Alfisols and Inceptisols categories (37.3% of the total areal extent), and about 18% of all soils are Lithic subgroups, meaning the soils are shallow with hard rock near the surface (Adams et al., 2019) and reflecting the space to which forests have been relegated as consequence of the development of agriculture or industry, at least in the Atlantic watershed of the Basque Country (Michel and Gil, 2013).

Differences in soil properties may also arise as a result of management practices, as mentioned in the previous section. In general, forest soils are characterised by low and infrequent levels of disturbance, particularly under continuous cover management systems; also act to promote soil stability, as the complex networks of tree roots present in a healthy forest act to hold soil in place, even on steep hillsides or during heavy rainfall when soil would otherwise erode away. When sustainably managed, forests can play a central role in preventing soil degradation (FAO, 2010).

2.3.1. Enhancing soil properties for climate change

Soil aggregation and C stabilization processes are interlinked because the water-stable aggregates protect against SOC decomposition and pave the way for C stabilization (Six et al., 2002; Totsche et al., 2018).

Soil is the basis of terrestrial trophic webs, where the transfer of energy and matter begins (Mataix-Solera et al., 2010) and it also regulates the movement of water and elements. Soil is therefore an essential factor in ecosystem functioning, productivity and resilience (Lal and Shukla, 2004). Management activities can increase the adaptive capacity of ecosystems and minimize adverse impacts.

Protecting, restoring, and sustaining SOC stock, soil fertility, water holding capacity, and reducing risk of erosion (Lal, 2004) is critical because of its numerous ecosystem services. It is essential that soil’s self-regulating capacity or resiliency is protected and enhanced to strengthen the provisioning of goods and services. Table 2.1 describes some key soil properties, whose threshold limits must be defined so that these are not to be breached.

2.1. Taula / Table 2.1
(EN) Example of key soil properties
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Soil parameters	Critical properties
Physical	Bulk density, aggregation, pore size distribution, available water capacity, effective rooting depth, infiltrability, heat capacity, erodibility, soil erosion rate, penetration resistance.
Chemical	pH, electrical conductivity, cation exchange capacity, nutrient reserves, carbonates/bicarbonates
Biological	SOC, microbial biomass carbon, enzyme activity, soil respiration quotient
Ecological	Potential erosion risk, gaseous flux,

Source: Lal, 2019

Successful adaptation to CC implies strong understanding of processes and properties of soils and the related natural resources. (Lal, 2012)

Forest fertilisation (mainly N and P), widely used in northern countries' forestry, can significantly increase biomass production and connected benefits. However, such fertilization implies larger economic and environmental risks, along with a lower profitability for the landowner, which must be balanced against these benefits (Hedwall, 2014). There is a growing emphasis on identifying and implementing natural solutions (Griscom et al. 2017) toward adaptation and mitigation of CC. Usually, all sources of organic matter in forest soils are native. Forest vegetation continuously cast litters from the above- and belowground parts. However, organic and C-rich amendments have gained increasing attention as they can enhance organic carbon (OC) stocks in soil, improve soil quality, and help restore the soil C lost and thus soil ecosystem functioning (Lehmann and Joseph, 2009; Bruckmann and Pumpanen 2019).

Soil application of by-products derived from bioenergy production, such as wood ash (WA) and biochar (BC), has been proposed as a means of enhancing soil quality within the circular economy framework, as well as a solution for disposing of the residues (Insam and Knapp, 2011). These waste products can replace the nutrients removed by tree harvesting, as well as increase soil carbon stocks, particularly BC (e.g. Sohi, 2012), and influence soil structure and biogeochemical processes. However, the extent to which soil amendments can provide benefits to soil properties and plant nutritional status depends on the interactions between the added products and the soil-water-plant system.

2.3.1.1. Wood Ash application

Wood ash (WA) is the inorganic incombustible part of the biomass that is left after total ignition of wood.

Countries are increasingly incorporating bioenergy into local energy strategies (Hannan et al., 2018). Historically, biomass have been a common source of energy for the timber and pulp and paper industries and, more recently, biomass thermal plants are being established explicitly for electricity and heat production. It represents 8–15% of the world energy supplies as electricity, heat and fuels for transportation and would contribute up to 33–50% of the world's current primary energy consumption by 2050. Therefore, the amount of WA produced will increase in the future. Some 476 million Mg of ash are generated yearly assuming 6.8% mean ash yield on biomass dry basis. In total, about 95–97% of the world’s bioenergy is produced by direct combustion of biomass. Hence, approximately 476 million Mg of biomass ash may be generated worldwide annually if the burned biomass is assumed to be 7 billion tonnes with 6.8% mean ash yield on dry basis (Vassilev et al., 2013).

Ash contains the bulk of the mineral fraction of the original biomass. Most of the inorganic elements assimilated by plants during growth remain in the ash after burning (Pasquali et al., 2018). The chemical composition of ashes obtained from biomass combustion depends on i) the raw material itself: the type of biomass, plant species or plant parts, growth processes and conditions, harvest time and techniques, processing methods, as well as on ii) the process of combustion itself: fuel preparation, combustion technology, and combustion conditions plus other technical conditions. These factors can enrich or reduce the content of elements in the ash (Zagac et al., 2018). Two types of WA are produced in biomass combustion plants: fly ash and bottom ash (Tlustoš et al., 2012). Fly ash is captured from boiler emissions, in cyclone separators, and contains large amounts of nutrients (except N), which are present as dissolvable salts and are rapidly released. In contrast, bottom or boiler ash, usually referred to as mixed WA, which is produced in wood fired furnaces, is less reactive and contains lower amounts of heavy metals than fly ash (Santalla et al., 2011; Omil et al., 2013).

Ashes generated from biomass combustion are classified as a solid waste according to the European List of Wastes (European Commission, 2000) (codes 100101 and 100103 for bottom and fly ashes respectively), and are commonly derived to landfill disposal. The European Landfill Directive (European Council, 1999) attempted to restrict this practice by increasing costs and penalties (Tarelho et al., 2015) in order to encourage companies to seek alternative management options (European Environment Agency, 2009).

There are several examples of use of WA as fertilising material in agriculture and forestry (Schiemenz and Eichler-Lobermann, 2010; Omil et al., 2011), since its application to soil allows recycling of essential nutrients (e.g., potassium, phosphorous) (Pels et al., 2005; Okmanis et al., 2015; Vassilev et al., 2013), reduce offsite deposition of waste material, and is thought to improve long-term forest productivity (Vance, 1996). The high temperature of the biomass combustion process leads to mineralization of biomass inorganics (ash) and the basic cations are further transformed into oxides, which are slowly hydrated and subsequently carbonated under atmospheric conditions (Demeyer et al., 2001). Ca, Mg and K are usually present in the form of carbonates. WA is typically alkaline, and so it can increase the pH (Adotey et al., 2018) of soils which increases microbial activity and nutrient availability (Rosenberg et al., 2010). In some cases, WA amendment can increase soil carbon pools as well as increase tree growth (López et al., 2018). There are additional possible downstream benefits that should also be considered, such as the alleviation of calcium limitation in watersheds that have been affected by acid rain (Azan et al., 2019) and, some studies have recorded increases to amphibian and earthworm assemblages (Gorgolewski et al., 2016; McTavish et al., 2020). However, the use of WA in forestry has been questioned, since biomass ash use is limited by the heavy metal enrichment (e.g. Perkiömäki and Fritze, 2005), as well as by the pronounced changes provoked in soil pH due to its alkaline substances that may also have an undesirable effect, leading to increased leaching of pollutants and nutrients (Pasquali et al,. 2018). WA application guidelines, which are essential components of Biomass Harvesting Guidelines, were developed in many countries to account for the increasing accumulation of WA from bioenergy production (Bruckman et al., 2018) and its use on forestry and agriculture is regulated in some European countries, such as Denmark, Finland, Lithuania, Sweden, Austria and Germany (Hannam et al., 2018).

A review by Aronsson and Ekelund (2004) concluded that the biological effects of WA application on forest soil and aquatic ecosystems were ambiguous. Since then, experiments have matured and a considerable body of new results has been produced regarding ash fertilisation impacts on, e.g., heavy metal concentrations in berries and mushrooms (Moilanen et al., 2006), ground vegetation (Huotari et al., 2015), soil microbial processes (Rosenberg et al., 2010), greenhouse gas emissions (Klemedtsson et al., 2010) and watercourses (Piirainen et al., 2013).

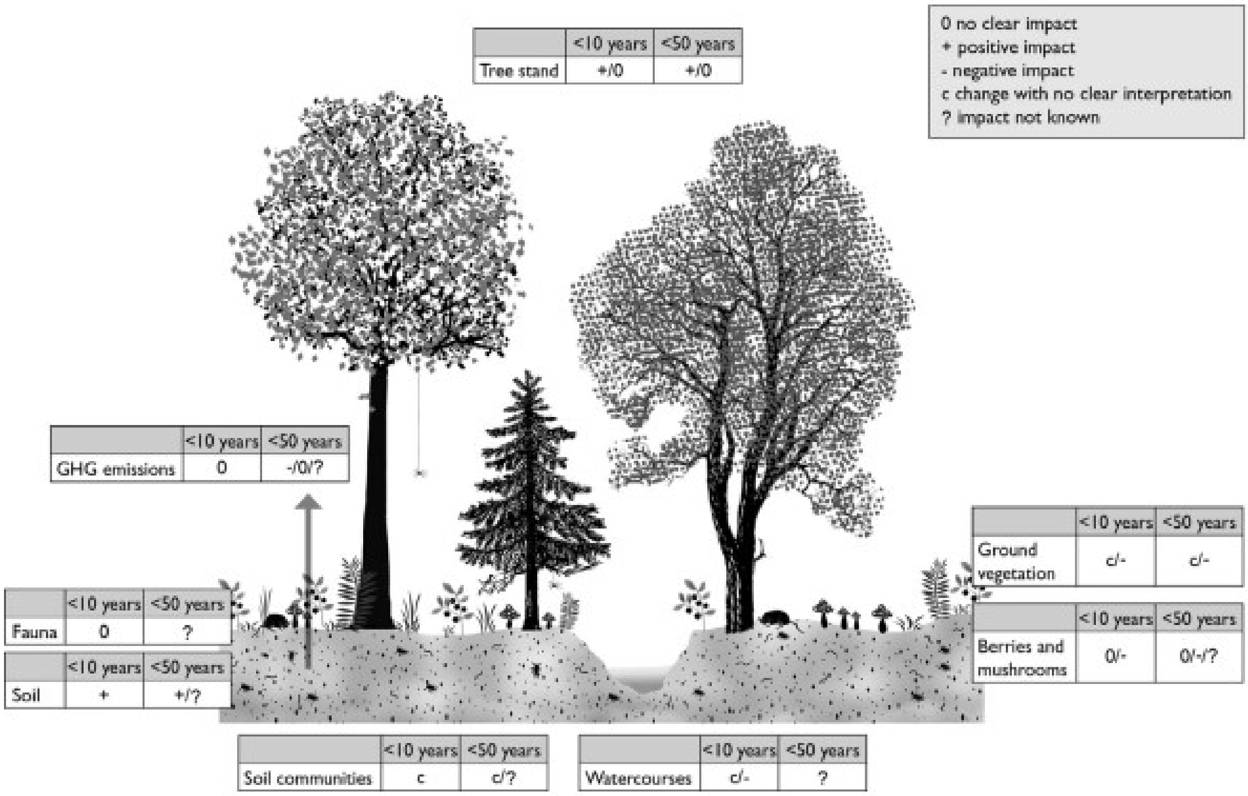


Figure 2.4 Summary of the ecosystem effects of ash fertilisation on two time scales. <10 yrs and <50 yrs. Source: Huotari et al., 2015

Huotari et al. (2015) concluded in their review that in well-targeted sites, ash application may increase tree production, and/or reduce the acidity of forest soils while increasing base cation reserves, with few harmful side-effects, but still, (i) the responses may depend on the time scale (e.g., GHG emissions), and (ii) no results extending beyond 50 years currently exist. Further, (iii) several crucial questions still remain unanswered, and it is thus not warranted to stop monitoring different ecosystem components.

2.3.1.2. Biochar application

Biochar is a solid material obtained from the carbonization, thermochemical conversion, of biomass in an oxygen-limited environment. In more technical terms, BC is produced by thermal decomposition of organic material (biomass such as wood, manure or leaves) under limited supply of oxygen (O_2), and at relatively low temperatures (<700°CBC). It can

> *be distinguished from charcoal—used mainly as a fuel—in that a primary application is use as a soil amendment with the intention to improve soil functions and to reduce emissions from biomass that would otherwise naturally degrade to greenhouse gases.* (International Biochar initiative)

The production process, together with the feedstock defines the nature of the BC. (Lehmann and Joseph, 2009). BC contains several mineral nutrients that are not oxidized during pyrolysis (e.g., phosphorus (P), potassium (K) and calcium (Ca)). These are constituents of the ash phase and the remaining quantity of nutrients depends on the feedstock material, pyrolysis conditions, and the amount of ash produced. The moisture, as well as lignin and cellulose content in biomass have considerable influence on BC formation.

Moura Chagas et al. (2022) reported in their meta-analysis that application rate was the factor with most influence on soil C stocks , and no differences in soil C contents were observed for raw materials, pyrolysis temperature or the C content of the BC applied. The increase in SOC achieved by BC may be up to 6 times more than other alternatives, such as the use of cover crops and the adoption of conservation tillage systems (Wang et al., 2016). However, BC induces a positive short-term priming effect, while providing long-term C sequestration benefits by promoting physical protection of soil organic matter (Maestrini et al., 2015). The balance of short- and long-term effects will determine whether BC could be a successful land management approach to increasing long-term soil C pools given its resistance to microbial decomposition (Ouyang et al., 2014). BC's aromatic C ring structure poses an obstacle for microbial decomposition (Leng et al., 2019), as well as does its ability to form organo-mineral compounds (Weng et al., 2017). The interaction between BC, soil, microbes, animals and plant roots are known to occur after application to the soil and also varies from soil to soil (Lehmann and Joseph, 2009). Types and rates of these complex interactions depend on various factors: (i) composition of feedstock materials (ii) pyrolysis conditions; (iii) physio-chemical properties of BC and (iv) soil characteristics and local environmental conditions (Gogoi et al., 2019)

BC is among the IPCC's short-list of Negative Emission Technologies (NETs) that could provide a significant sequestration impact and it's being considered one of the most affordable negative emission technologies (NET) at hand for future large-scale deployment of carbon dioxide removal (CR), via carbon sequestration in soil (Bruckman and Pumpanen, 2019). BC's potential for GHG reduction and CC mitigation has been reported to be as high 35Gt CO_2 yr^{-1} by 2050 (de Coninck et al., 2018). It has already become part of the carbon markets. In 2019 BC was listed for the first time on a voluntary carbon marketplace in Finland (www.Puro.earth) and in 2020 a second voluntary marketplace, CarbonFuture began to list carbon removal credits for BC (www.carbonfuture.earth). However, if assumed limited available feedstock for BC production the potential range for C sequestration is reduced to values between 0.6 and 11.9 Gt CO_2 yr^{-1} (Fuss et al., 2018).

Apart from the advantages of BC addition in the soil, some studies showed insignificant or sometimes negative effects (Jeffery et al., 2017) of large-scale and long-term soil application, such as BC induced SOM loss (Gogoi et al., 2019). The size and scale when employing BC also affect the cost and economic feasibility of it. In addition, hazardous heavy metals, such as cadmium (Cd), chromium (Cr), zinc (Zn) and nickel (Ni), are also concentrated in BC, as well as a wide variety of organic chemicals, including volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs) and dioxins, which are formed during charring processes, adsorbed onto the porous structure of BC and are potentially toxic or carcinogenic (Zheng et al., 2019). Thus, legislation is being developed by regional and state agencies to regulate different types of amendments. The European BC certificate (EBC, 2012) was created to ensure that BC can be safely applied to soil. However, even when certified products are used, chemical analysis alone is not sufficient to estimate the risk associated with the use of these materials as soil fertilisers (Malara and Oleszczuk, 2013). Interactions between the soil and products applied may make it difficult to predict how these compounds will behave in the ecosystem (Oleszczuk et al., 2013). Long-term field studies are required to understand the interaction of biochar where various natural dimensions are active

BC amendments could likely improve soil physical and hydrological properties (Omondi et al., 2016) through varying with BC and soil conditions. Use of BC thus could offer a viable option to improve moisture storage and water use efficiency for soils poor in organic carbon in arid/semiarid zones. But still, more studies on dynamics of soil hydrological behaviours following BC amendment should be developed in field conditions in order to understand BC's potential, covering specific soil types, climatic zones and different types of ecosystems. Since other authors reported no consistent direct improvement derived from BC application (de Melo et al., 2014), or simply no effect (Jeffery et al., 2015).

Results from agriculture should be carefully interpreted in a forestry context. Research on the application of BC in forests is limited and much work has been done under controlled conditions (McElligott et al., 2011). In a field scale study in a managed temperate hardwood forest most important BC effect was increased supply of limited plant nutrients, especially P and Ca (Sackett et al., 2015). Higher site productivity after BC addition in forests is attributed to increased microbial activity, nutrient retention, and water storage. In a meta-analysis conducted by Thomas and Gale (2015), positive tree growth responses were noted, especially in the early growth stages. These growth increases are larger than those observed in agricultural crops. However, impacts were more pronounced in tropical and boreal systems than in temperate forests, which is confirmed by recent findings by Sherman et al. (2018), who did not find any stand benefits to BC application in a temperate forest. From a management perspective, technical solutions are available to spread BC on the forest soil surface (Anderson et al., 2016). Due to the low bulk density of BC, relatively thick layers may form on top of the forest floor, which may persist for more than one season (Bruckman and Pumpanen, 2019). However, there are evidences that part of the BC is already moved from the forest floor through the mineral soil within one year (Fernández-Ugalde et al. 2017; Bruckman and Pumpanen, 2019).

3. OBJECTIVE AND HYPOTHESES (EN)

The main objective of this work was to test whether the addition of wood ash (WA) or biochar (BC) to the forest floor could be a tool to adapt forest (by the forest soil) to Climate Change.

More specifically, we wanted to

- Evaluate the effect of these amendments in some soil physical, chemical and biological characteristics from a climate change mitigation, adaptation and resilience point of view, of two existing temperate forests.
- Test if the effect differs when soil properties and environmental conditions change
- Test if the effect differs when dosage of applied BC and WA changes
- See if the effect of BC and WA was limited by N supply and so tested the additional effect of addition of mineral nitrogen.

SPECIFIC HYPOTHESES

1) **Application of WA and BC will improve soil structure, and therefore, improve soil hydraulic properties**
2) The addition of WA and BC with and without N will enhance aggregate stability.
3) Incorporation of biochar will promote the formation of macroaggregates, as a result of enhanced activity of decomposer microorganisms, following the hierarchical model of aggregate formation and stabilization.
4) **Application of WA and BC enhances soil nutrition for healthy vegetation** by increasing plant nutrient uptake.
5) Vegetation biomass growth might increase due to increased nutrient uptake by plant.
6) **Application of WA and BC may increase SOC stocks.**

7) **Application of WA and BC could induce toxicity** in the soil-water-plant system through direct supply of hazardous compounds or by changing soil conditions affecting the bioavailability of them.
8) Glomalin related soil proteins (GRSP) are an interesting indicator on the relationship between biological activity and soil structure.

Hypothesis 1 was developed in the article "**Effects of biochar and wood ash on soil hydraulic properties: A field experiment involving contrasting temperate soils**" (Appendix I) published in the journal Geoderma in 2017. https://doi.org/10.1016/j.geoderma.2017.05.041

Hypotheses 2 and 8 were developed in the short communication titled **"Assessment of improvement of soil physical properties following organic amendments"**/ (Appendix II) Draft for submission as short communication in Geoderma. Linked with this objective, identification of best methodological approach to the quantification of GRSP has been developed. To approach this objective, we performed the work described in the article "**Towards meaningful quantification of glomalin-related soil protein (GRSP), taking account of interference with the Coomassie Blue (Bradford) assay**" (Appendix III) published in the European Journal of soil science in 2018. doi: 10.1111/ejss.12698

Hypothesis 3 was developed in the communication "**Effect of Biochar in water stable aggregate formation**" (Appendix IV) published in the The CICS2014 congress book *Retos y oportunidades en la ciencia del suelo* ISBN: 978-84-8408-769-4. https://www.secs.com.es/wp-content/uploads/2014/07/Retos-y-Oportunidades-en-las-Ciencias-del-Suelo.pdf

Hypotheses 4, 5, 6 and 7 were developed in the article "**Effects of Biochar and Wood ash amendments in the soil-water-plant environment of two temperate forest plantations**" (Appendix V) accepted by the reviewers in the journal *Frontiers in Forests and global change*. doi: 10.3389/ffgc.2022.878217

3. HELBURUAK ETA HIPOTESIAK (EU)

Tesi honen helburu nagusia errautsa (WA) zein biocharra (BC) lurzoruari gehituta, aldaketa klimatikoari (CC) aurrea hartzeko eta berari egokitzeko ongarri izan daitezkeen probatzea da, bi baso epel Atlantiarretan.

Zentzu horretan,

- Emendakinok lurzoruko zenbait ezaugarri fisiko, kimiko eta biologikoetan positiboki eragiten duten aztertu nahi dugu, aldaketa klimatikoarekiko mitigazioa, erresilientzia eta adaptazioari dagokionez.
- Lurzoru-ezaugarriak eta ingurumen baldintza ezberdinetan emendakinek modu bereizian eragiten duten aztertu nahi dugu.
- Aplikatutako BCaren eta WAaren dosiak eragina duen aztertu nahi dugu.
- Aplikatutako BCk eta WAk nitrogeno mineralarekin batera gehitzeak berdin eragiten duen aztertu nahi dugu.

HIPOTESI ZEHATZAK

1) **WA eta BC aplikatzeak lurzoruaren egitura hobetuko du, eta, beraz, lurzoruaren propietate hidraulikoak hobetuko ditu.**
2) BC eta WAak lurzoruko agregatuen estabilitatea handituko dute.
3) BC gehitzeak lurzoruko makroagregatuak eratzea eragingo du, mikroorganismo deskonposatzaileen jarduera aktibatzearen ondorioz, lurzoru-agregatuak eratzeko eta egonkortzeko eredu hierarkikoari jarraituz.
4) **WA eta BC aplikatzeak landaredi osasuntsu baterako beharrezkoa den lurzoruaren nutrizioa hobetu egiten du**.

5) Landare biomasaren hazkuntza bultzatuko dute, landareek mantenugai gehiago xurgatu dituztelako.
6) **BC eta WA gehitzeak lurzoruko karbono kantitatea handituko du.**
7) **BC eta WA gehitzeak lurzoru-ur-landare sisteman toxikotasuna eragin dezake** kaltegarriak izan daitezkeen konposatuen ekarpen zuzen batengatik edota lurzoru baldintzen aldaketaren ondorioz hauen bio-eskuragarritasuna handitu daitekeelako.
8) Glomalinarekin erlazionatutako lurzoru-proteinak (GRSP) lurzoruaren egituraren eta jarduera biologikoaren loturaren adierazle interesgarriak dira.

1. hipotesia 2017an *Geoderman* publikatutako "**Effects of biochar and wood ash on soil hydraulic properties: A field experiment involving contrasting temperate soils**" artikuluan garatu dugu (I. Eranskina). https://doi.org/10.1016/j.geoderma.2017.05.041

2. eta 8. hipotesien lanketa "**Assessment of improvement of soil physical properties following organic amendments**"/ Geoderma aldizkarian komunikazio labur bezala aurkezteko zirriborroa (II. Eranskina) azaltzen da. Helburu honekin lotuta GRSPak estimatzeko metodologiarik onena identifikatzeko ahalegina egin zen. Emaitza 2018an *European Journal of Soil Science*n argitaratutako "**Towards meaningful quantification of glomalin-related soil protein (GRSP), taking account of interference with the Coomassie Blue (Bradford) assay**" artikuluan (III. Eranskina) deskribatzen da . doi: 10.1111/ejss.12698

3. hipotesia CICS2014 kongresuan publikatutako *Retos y oportunidades en la ciencia del suelo* liburuan (ISBN: 978-84-8408-769-4) deskribatu ditugu "**Effect of Biochar in water stable aggregate formation**" komunikazioaren bidez (IV. Eranskina). https://www.secs.com.es/wp-content/uploads/2014/07/Retos-y-Oportunidades-en-las-Ciencias-del-Suelo.pdf

1., 2. 3. eta 4. Hipotesiak "$**Effects of Biochar and Wood amendments in the soil-water-plant environment of two temperate forest plantations**" artikuluan landu ditugu (V. Eranskina). *Frontiers in Forests and global change* aldizkarian onartua. doi: 10.3389/ffgc.2022.878217

4. METHODOLOGY (EN) / BALIABIDE METODOLOGIKOAK (EU)

In order to address the stated objectives, we established a field trial consisting of two experimental sites, and developed a laboratory trial based on incubations. The experimental design of the field trial is explained in detail in Appendix V. Maps of the Experimental sites are attached in Apendix VI. The exact samplings, methods, measurements and calculations performed are explained in the communications already published (Apendix I, III and IV) or drafts for publication (Apendix II and V) and synthesized in Figure 4.1.

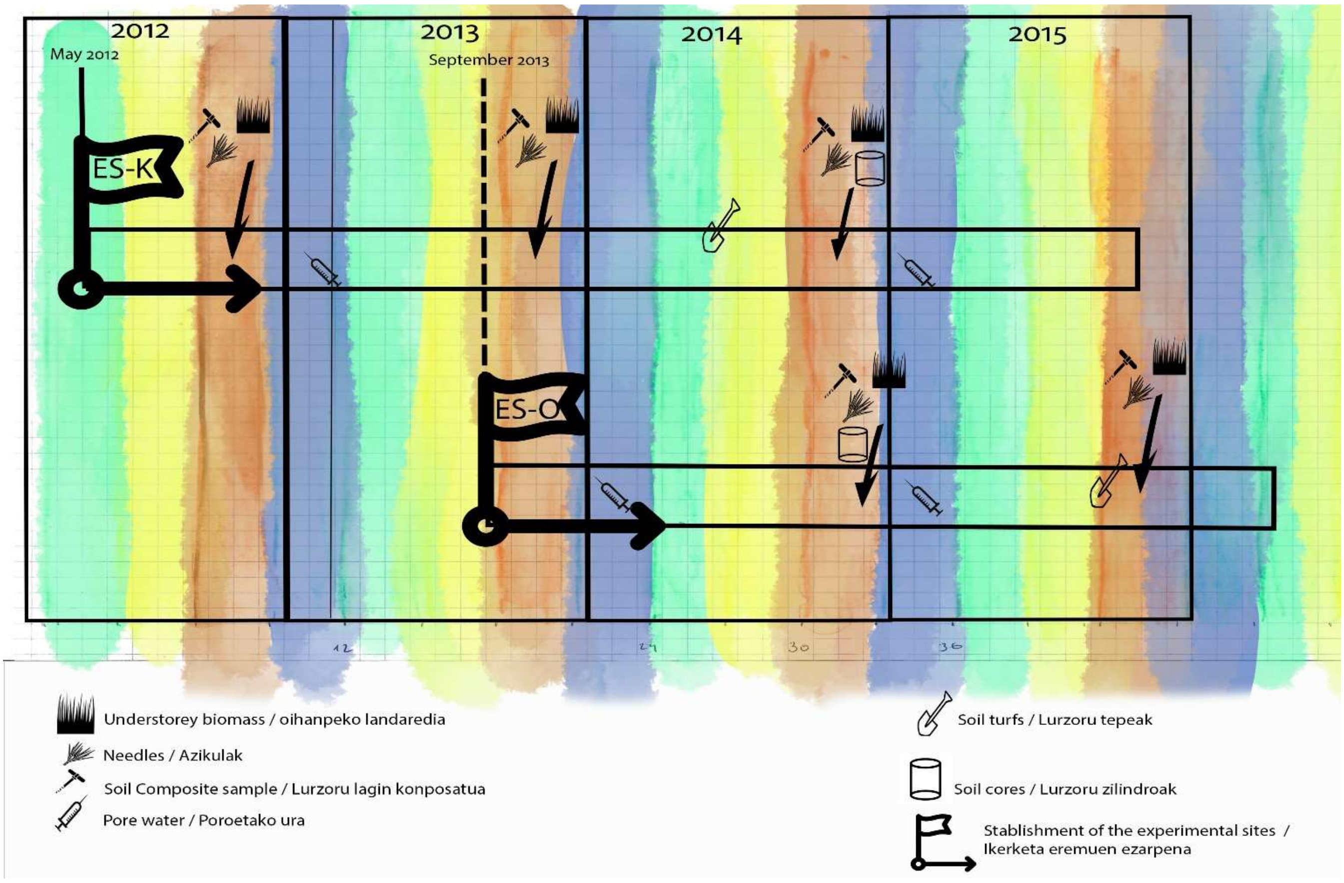


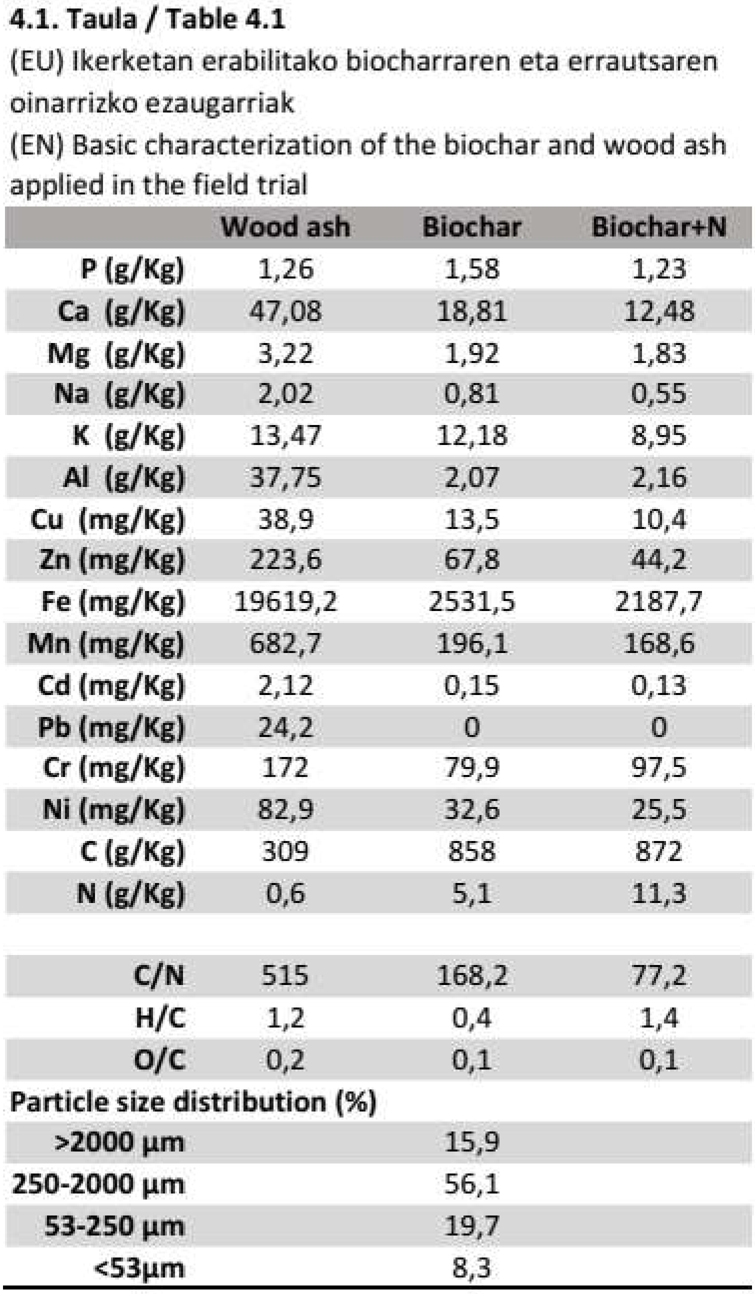
Figure 4.1.- *Schematic explanation of the samplings at each experimental site [Experimental site of Karrantza (ES-K), at the top and Experimental site of Oiz (ES-O) at the bottom], during the time (Years in blocks, months in vertical lines). Colors indicate seasons of the year (Green: spring; yellow: summer; brown: autum; blue: winter). Understory biomass, needles, soil composite samples and pore water samples were taken to test the hypotheses 1,2,3 and 4 (Appendix II), Soil turfs were taken to test hypotheses 6, 7 and 8 (Appendixes III, IV and V), and soil cores were taken to test hypothesis 8 (Appendix V).*

Planteatutako helburuei heltzeko, landa-saiakuntza bat ezarri genuen, bi ikerketa-eremuk osatua, eta laborategiko saiakuntza bat garatu genuen, inkubazioetan oinarritua.

4.1.Biocharra eta errautsa

Lan honetan erabilitako biocharra C4 metabolismodun *Myscanthus* sp landarea Pyreg® labe batetan 450ºC-tan pirolisatuta ekoiztu zen.

Errautsa biomasarako galdara komertzial baten ekoiztu zen, C3 metabolismodunak diren *Pinus radiata* D. Don landaketetako hondakinak erreta.



4.1. Taula / Table 4.1
(EU) Ikerketan erabilitako biocharraren eta errautsaren oinarrizko ezaugarriak
(EN) Basic characterization of the biochar and wood ash applied in the field trial

	Wood ash	Biochar	Biochar+N
P (g/Kg)	1,26	1,58	1,23
Ca (g/Kg)	47,08	18,81	12,48
Mg (g/Kg)	3,22	1,92	1,83
Na (g/Kg)	2,02	0,81	0,55
K (g/Kg)	13,47	12,18	8,95
Al (g/Kg)	37,75	2,07	2,16
Cu (mg/Kg)	38,9	13,5	10,4
Zn (mg/Kg)	223,6	67,8	44,2
Fe (mg/Kg)	19619,2	2531,5	2187,7
Mn (mg/Kg)	682,7	196,1	168,6
Cd (mg/Kg)	2,12	0,15	0,13
Pb (mg/Kg)	24,2	0	0
Cr (mg/Kg)	172	79,9	97,5
Ni (mg/Kg)	82,9	32,6	25,5
C (g/Kg)	309	858	872
N (g/Kg)	0,6	5,1	11,3
C/N	515	168,2	77,2
H/C	1,2	0,4	1,4
O/C	0,2	0,1	0,1
Particle size distribution (%)			
>2000 µm		15,9	
250-2000 µm		56,1	
53-250 µm		19,7	
<53µm		8,3	

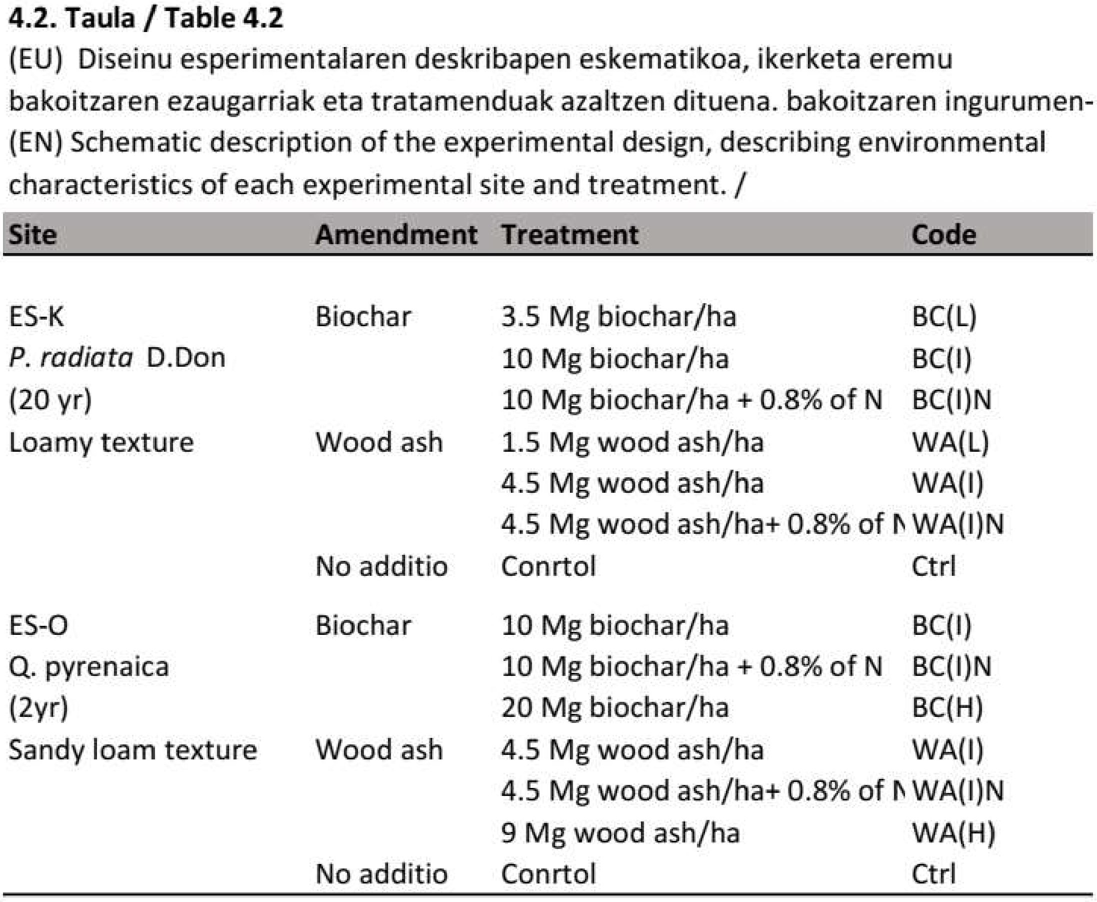
4.2. LANDA ENTSEGUA: Oiz eta Karrantzako ikerketa eremuak

Helburu nagusiei erantzuteko landa-saiakera bat abiatu genuen Euskal Herriko isurialde atlantiarrean bi ikerketa-eremu ezarriz (Ikerketa eremu bakoitzaren mapak VI. eranskinean ikus daitezke). Ikerketa-eremu biak klima epelean eta latitude berdintsuan ezarri genituen. Urteko batezbesteko tenperatura 10.5ºCkoa izanik, urtean 1200mm inguruko prezipitazioekin (EUSKALMET, 2012-2014 urteak), baina altueran, lurzoruan eta komunitatean besteak beste, bereizten diren adin ezberdineko bi zuhaitz landaketa desberdinetan.

Figure 4.2.- *Pictures of the two experimental sites. At the left, the experimental site of Karrantza (ES-K), and at the right, the experimental site of Oiz (ES-O)*

Biocharra eta errautsa lurraren gainazalera botaz gehitu genuen, emendakinak lurzoruan barrena naturalki inkorpora zitezen baimenduz.

Emendakin-mota bakoitzarekin, hau da BC-rekin zein WA-rekin, kaltzio kantitatean baliokideak ziren lau tratamendu ezarri genituen, eta 4.2 taulan ikus daitekeen bezala, kopuru ezberdinetan eta nitrogeno gehigarriaren presentzia/ausentzian oinarritzen dira. Emendakin-mota bakoitzaren kantitate ertainarekin bi tratamendu desberdin sortu genituen, bata nitrogenoduna eta bestea nitrogenorik gabea. Emendakinik gabeko tratamendua, kontrol (Ctrl) bezala agertuko dena, erreferentziazko tratamendu gisa hartu dugu ikerketa osoan zehar. Tratamendu guztietatik lau, ikerketa eremu bietan errepikatzen dira: WA(I), WA(I)N, BC(I) eta BC(I)N. Modu berdintsuan eragiten ote duten aztertu ahal izateko.



4.2. Taula / Table 4.2

(EU) Diseinu esperimentalaren deskribapen eskematikoa, ikerketa eremu bakoitzaren ezaugarriak eta tratamenduak azaltzen dituena. bakoitzaren ingurumen-(EN) Schematic description of the experimental design, describing environmental characteristics of each experimental site and treatment. /

Site	Amendment	Treatment	Code
ES-K	Biochar	3.5 Mg biochar/ha	BC(L)
P. radiata D.Don		10 Mg biochar/ha	BC(I)
(20 yr)		10 Mg biochar/ha + 0.8% of N	BC(I)N
Loamy texture	Wood ash	1.5 Mg wood ash/ha	WA(L)
		4.5 Mg wood ash/ha	WA(I)
		4.5 Mg wood ash/ha+ 0.8% of N	WA(I)N
	No additio	Conrtol	Ctrl
ES-O	Biochar	10 Mg biochar/ha	BC(I)
Q. pyrenaica		10 Mg biochar/ha + 0.8% of N	BC(I)N
(2yr)		20 Mg biochar/ha	BC(H)
Sandy loam texture	Wood ash	4.5 Mg wood ash/ha	WA(I)
		4.5 Mg wood ash/ha+ 0.8% of N	WA(I)N
		9 Mg wood ash/ha	WA(H)
	No additio	Conrtol	Ctrl

Karrantzako ikerketa-eremua (ES-K) 2012. urteko Maiatzean ezarri genuen, Karrantza haraneko ekialdean, La Moradillan (30N ETR89 475 081, 4 786 389). Itsas mailatik 270m-tara kokatuta dagoen eta % 13.1 eko malda duen Bizkaiko Foru Aldundiaren pinu landaketa batetan. Bertako landaredi potentziala harizti kantauriarra bada ere, (https://www.geo.euskadi.eus/) 1996an ezarri zen gutxienez bigarren errotaziokoa den (https://www.geo.euskadi.eus/) hobekuntza genetikorako *Pinus radiata* D. Don hazitegia. Helburu bereizgarri hau dela eta, ezohikoa den 6 m x 6 m-ko landaketa-markoa daukan pinu landaketa da. Lurzorua *Typic Udorthent* motaren baitan sailkatuta dago (Soil Survey Staff, 2014), eta 4.3. Taulan adierazi ditugu lurzoru honen ezaugarri fisiko-kimiko nagusiak. Esperimentua blokeko diseinua oso batean oinarritzen da, maldarekiko perpendikularki dauden hiru mailatan zazpi tratamenduak hiruna bloketan zoriz ezarriz: BC(L), BC (I), BC (I)N, WA(L), WA (I), WA(I)N eta Ctrl (VI. eranskina) Ikerketa eremu honetan ez genituen dosi altuenak testatu: BC (h); WA (h). Tratamenduak 6 x 6 m-ko partzelatan aplikatu ziren gutxienez 10 m-ko tarte bat utziz partzelen artean.

Bigarren ikerketa eremua, ES-O, Oiz mendiaren hego magalean ezarri genuen 2013ko Irailean, Berrizko Sarria auzoko Txara inguruan (30N ETR89 532 673 4 785 572). Itsas mailatik 760 m-tara dagoen eta % 37.6ko malda duen txilardi-otadi-iratzediaren baitan 2012an landatutako *Quercus pyrenaica* Willd amezti gazte batetan. Bertako landaredi potentziala harizti kantauriarra bada ere (https://www.geo.euskadi.eus/), mendeak daramatza baso izan barik. Gutxienez Aro modernotik garaikidera bitartean errotarri harrobiak izandako lurrak dira (Castro, 2018) eta behi eta behor haziendak ibili dira txilardi-otadi-iratzedi landarediak estalia izan den eremu honetan. Lurzorua *Typic Dystrudept*-a da (Soil Survey Staff, 2014), azalekoa, hareatsua (4.3. Taula) eta materia organiko kantitate altuak metatzen dituena. Tratamenduak 3 m x 3 m-ko partzelatan aplikatu genituen, zoriz, bloke-diseinuan. Guztira zazpi tratamendu hauen lau erreplika ezarriz, eta partzelen artean gutxienez 1m-ko tartea utziz: BC (I), BC (I), BA (I), WA (I), WA (I), WA (I), WA (H) eta Ctrl. Ikerketa eremu honetan ez genituen dosi baxuak (BC (L) eta WA (L)) gehitu.

4.1 Irudian laginketa eskema ikus daiteke. Hipotesi zehatzei erantzuteko, ikerketa-eremu bakoitzean egindako laginketak zeintzuk izan ziren eta noiz egin ziren azaltzen da.

4.2.1. Oinarrizko elementu eta mantenugaien azterketa

Lurzoruetako oinarrizko elementuak aztertzeko 0-10cm-tako lurzoru laginak hartu genituen laztabin batekin (2 cm ϕ) ikerketa eremua ezarri aurretik eta 6, 18 eta 30 hilabetera ES-Kn eta 0, 15 eta 26 hilabetera ES-On (4.1 Irudia). Partzela bakoitzean zoriz aukeratutako hamar puntutan zulatuz lagin konposatua osatu genuen, giro tenperaturan lehortzen utzi eta 2 mm-ko bahetik pasa zedin txikitu genuen. Laginak gela lehor batetan mantendu genituen analitikak egin arte.

Landarediaren hazkuntza eta oinarrizko elementuen azterketa egiteko ES-Kn azikulak eta oihanpeko landaredi laginak hartu genituen 6, 18 eta 30 hilabetera, eta ES-On oihanpeko laginak hartu genituen 15 eta 26 hilabetera (4.1 Irudia). Oizen ez zen zuhaitz hostorik hartu zuhaitzak gazteegiak zirelako. Karrantzako pinu azikulak lagintzeko zoriz aukeratutako pinu adarretatik hazi berriak ziren ehun azikula hartu genituen. Oihanpeko landaredia berriz 0.25 m^2ko karratuak baliatuta zoriz karratuaren baitan geratutako lurgaineko begetazioa arrasetik moztuta lagindu genuen. Bai azikulak baita moztutako begetazioa ere labe bidez lehortu genuen 70 ºCtan, txikitu 0.50 mm-tik behera eta gela lehor baten gorde genituen analitikak egin bitartean. Lurgaineko biomasa begetala hauen pisu lehorrak erregistratu eta ekoizpen-unitate bihurtu genituen: $Kg \cdot ha^{-1}$ oihanpeko landaredirako eta $Kg \cdot 100$ $azikula^{-1}$ pinu orratzetarako.

Lurreko poroetako urak bi aldiz jaso genituen oinarrizko elementuen azterketa egiteko eta test ekotoxikologikoak gauzatzeko. Lehenengo laginketa tratamenduak gehitu eta lehenengo euri-jasa jarraian zehar egin genuen ikerketa-eremu bietan, urtarrilean biak. Bigarren laginketa tratamenduak gehitu eta 32 hilabetera egin genuen ES-Kn, eta 17 hilabetera ES-On, hauek ere denbora hori bete eta lehenengo euri-jasa jarraituan zehar. Poroetako urak MacroRhizon^{Tm} tresnak (Rhizogue Research Products, Wageningen, Herbehereak) 30 cm-ko sakoneran sartuta eta 50 ml-ko xiringetara konektatua erauzi genituen. Laginak izoztuta mantendu genituen analisiak egin arte.

4.3.- Irudia:- *MicroRhizonaren ageriko zatia. Hutsa eginez poroetako ura erauzteko erabili ohi den xiringa lotzen zaioneko giltza. Ikusgai ez dagoen zatia lur azpian dago eta 30 cm-ko sakonerararte iristen da.*

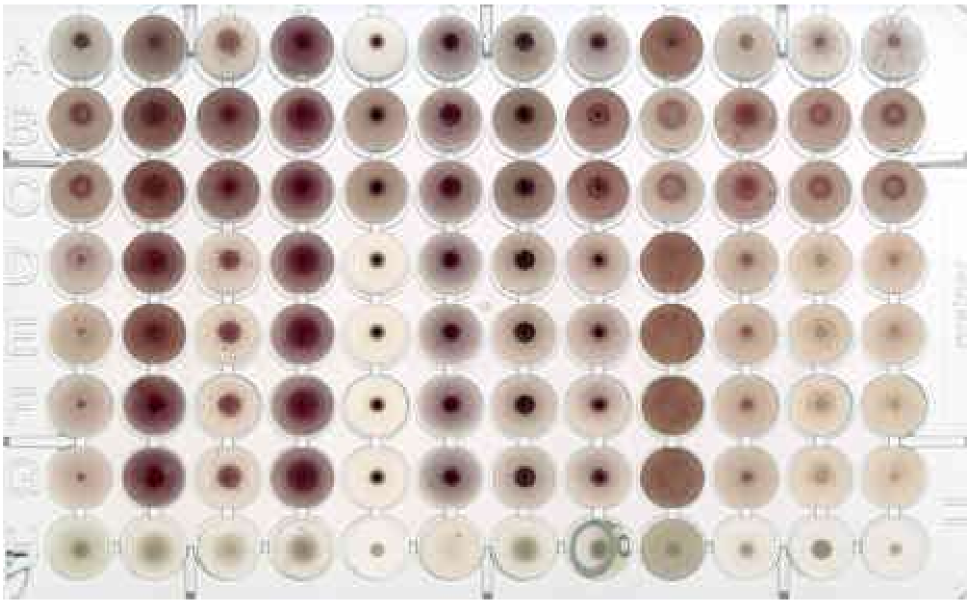
Lurzoruaren pHa (1:2.5) uretan neurtu genuen. Lurzoru eta biomasa laginen N eta C totala LECO TruSPEC® CHN-S analizatzaile elemental baten bidez neurtu genuen. Neurtutako C totalaren balioak lurzoruko karbono organiko (SOC) edukien baliokideak dira, ikerketa honetan aztertutako lurzoruetan karbonatorik ez dagoelako. Lurzoru, biomasa eta ur laginen gainerako oinarrizko elementuen kopuru pseudo-totalak [Fosforoa (P), potasa (K), Magnesioa (Mg), Kaltzioa (Ca), Nikela (Ni), Kadmioa (Cd), Kromoa (Cr) eta Beruna (Pb)] induktiboki akoplatutako plasmaren emisio optikoko espektrometria (ICP-AES) bidez neurtu genituen. Lagin lehorren kasuan, hau da, lurzoru eta landare-biomasa laginak, aurrez azidotan (nitriko-perklorokioaren nahasketa, % 85) digeritu genituen (Croisé et al., 1999) eta ur-laginak azido nitrikoarekin prestatu genituen.

Elementuen kantitateak balio absolutuetara pasa genituen: g·ha^{-1}. Lurzoruaren kasuan balio absolutuetara pasatzeko lurzoru-dentsitatea hartu genuen aintzat. 4.2.1.3.A atalean azalduta agertzen da lurzoru-dentsitatea neurtzeko erabilitako metodoa.

4.2.2. Azterketa ekotoxikologikoak

Arrisku ekotoxikologikoak aztertzeko bi test komertzial erabili genituen: MARA, Arriskua ebaluatzeko entsegu mikrobiologikoa (Microbial Asssay for Risk Assessment) eta luminiszentzian oinarritutako entsegua den LumiMARA. Testean 4.2.1.1. atalean azaldutako poroetako uren laginak erabili genituen. Lehenengo laginketan hartutako poro-urak MARArekin txekeatu genituen eta bigarren laginketan hartutakoak LumiMARArekin txekeatu genituen.

MARA hazkuntza entsegua da, eta hamar bakteria andui eta legamia baten hazkuntza-pikorren garapenean laginak duen inhibizio-efektua neurtzean datza. Ur laginak Wadhiak eta lankideek (2007) deskribatu bezala prestatu genituen, aldez aurretik pHa ajustatuta. Hazkuntza plaketan urak diluitu barik inokulatu genituen, Gabrielsonek eta lankideek (2003) egin bezala.



__4.4 Irudia__. Test ekotoxikologikoaren mikroplaka baten irudia, poro-urak gehitu ondoren, bakterioen hamar andui eta legamia baten hazkuntza duena.

Hazkuntza pikorrak kolorimetria bidez aztertu genituen. Mikroplaka irakurle baten bitartez (Zenyth 3100 Anthos Labtec Instruments GmbH, Salzburg. Austria.) pikor bakoitzaren absorbantzia 620nm-tara neurtuta. Hazkuntzaren inhibizioa andui bakoitzaren erreakzioari banan-banan zein entsegua osatzen zuten andui guztien batezbestekoari erreparatuta ebaluatu genuen.

LumiMARAren bidezko testa egiteko ere poroetako diluitu gabeko ur laginak erabili genituen. Hamaika bakteria andui luminiszente (itsasoko 9 eta ur gezako 2) laginekin kontaktuan ipini genituen fabrikatzaileak ingurumeneko laginetarako duen protokoloari jarraituz (Jung et al., 2015). Toxizitatea bakterioek igortzen duten luminiszentziaren txikitzearen bitartez aztertu genuen. Luminiszentzia mikroplaka irakurle bat (Zenyth 3100. Anthos Labtec Instruments GmbH, Salzburg. Austria) erabilita kuantifikatu genuen eta eskuratutako datuak LumiMARAren software-arekin (NCIMB Ltd., Bucksburn, Aberdeen. UK) prozesatu genituen.

4.2.3. Ezaugarri fisiko eta hidraulikoen azterketa

Ikerketa eremu bakoitzean horizonte organikoa alboratu eta lurzoru-profileko lehen 5 cm-tan, i) asaldatu gabeko 28 zilindro hartu genituen (D = 53 mm, h = 50 mm), 30 hilabetera ES-Kn eta 15 hilabetera ES-On (4.1 irudia), ageriko dentsitatea, ur-kondiktibitatea (Ks), porositatea eta ur atxikimendurako kurbak (SWRC) aztertzeko, eta ii) 10x10cm-ko Tepeak[5] hartu genituen tratamendua bota eta 24 hilabetera ikerketa-eremu bietan (4.1 Irudia), lurzoru agregatuen tamainaren banaketa eta estabilitatea aztertzeko.

4.5 Irudia.- Lurzoru-tepeen langinketaren irudia.

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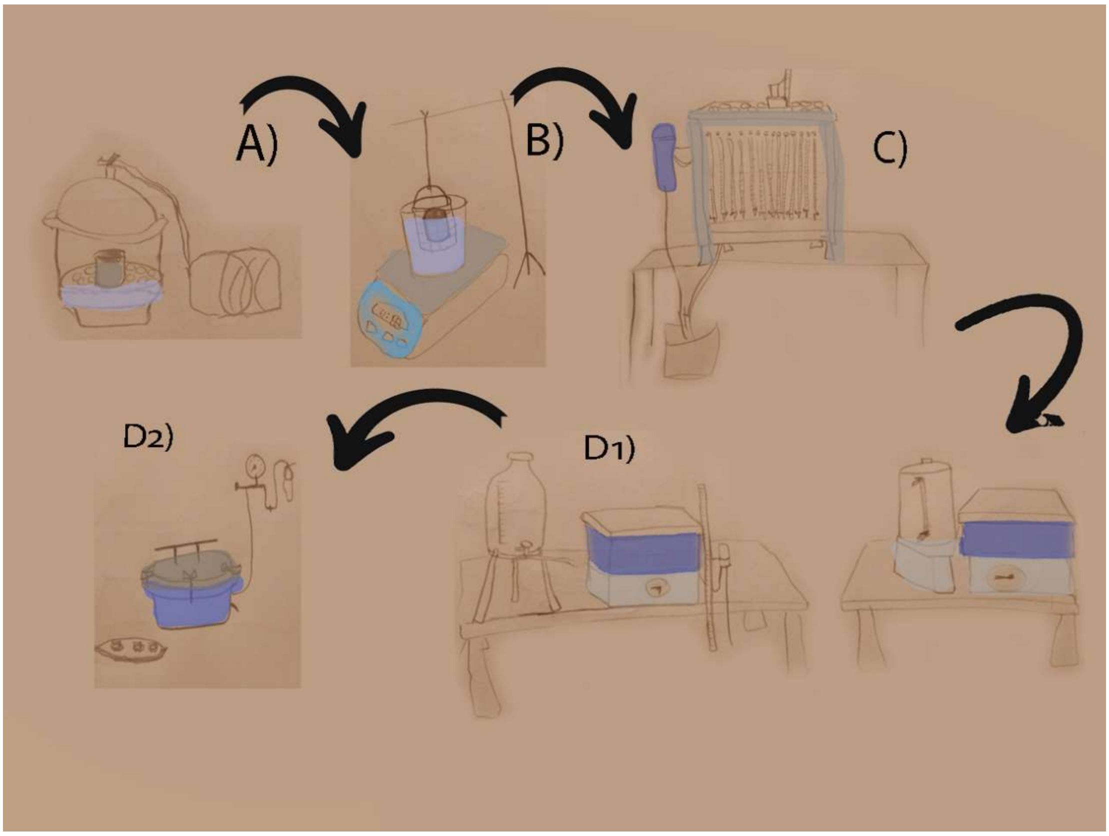
[5] Tepeak: kasu honetan 10 x 10 cm-ko azalerako karratuak dira, lurzoru-landaredi zatiak hartzeko lagin-mora.

ASALDATU GABEKO ZILINDROEKIN

Asaldatu gabeko zilindroak 4ºCtan mantendu genituen aurretik aipatu ditugun neurketak egin arte. Neurketak egiteko, zilindroak urez asetu genituen Flint eta Flintek (2002) deskribatu bezala. Urez betetako ontzi baten baitan zilindroak azalera porotsu baten gainean ipini genituen eta hutsa eraginez, poro guztiak kapilaritatez bete zitezen baimendu genuen (4.6. Irudia, A), eta horrela lurzoruaren egitura asalda zedin ekidin. Urez asetutako laginak pisatu genituen lehenik eta behin, saturazio egoerako ur edukiera grabitazionala (GWC: Gravitational water content) neurtzeko, eta gainontzeko propietate hidraulikoak metodo segida batekin aztertu genituen ondoren:



4.6. irudia.- Asaldatu gabeko lurzoru laginekin (zilindroekin) egindako neurketak egiteko jarraitutako metodoen sekuentzia azaltzen duen irudia

B. **Ageriko dentsitatea**ren estimazioa Arkimedesen desplazamendu metodoaren bitartez (Flint & Flint, 2002) neurtu genuen, zilindroak ur-desplazamendurako gailu batean ipinita (4.6. Irudia). Pisu lehorra propietate hidraulikoak neurtzeko metodo segida guztia amaitutakoan neurtu genuen, izan ere, ageriko dentsitatea neurtzeko ezinbestekoa izango da datu hau.

C. **Konduktibitate hidrauliko saturatua (Ks)** neurtzeko urez asetutako zilindroak ur-maila konstantedun permeometro batean ipini genituen (Eijelkamp Agrisearch equipment, the Netherlands). Ur-mailen arteko diferentzia neurtuta eta emari konstantedun drainatze-urek denbora zehatz batetan betetako bolumena bureta batetan josoz.

D. **Lurzoruaren ur-atxikimendu kurbak** (**SWRC**-soil water retention curves-) osatzeko tresna ezberdinak baliatu genituen, saturatutako laginek saturaziotik lehorreranzko sekuentzian sei potentzial matriko aplikatuta, bakoitzarekin galdutako ura neurtuz horrela. Lehortze prozesu honetan zehar potentzial matriko bakoitzarekin oreka lortutakoan ur edukiera grabimetrikoa erregistratuz joan ginen. Hau da, presio-matriko bakoitzarekin ur edukiera egonkortutakoan laginen masa balioak erregistratuz joan ginen. Harezko eta Hare eta kaolinezko kutxak (Eijkelkamp Agrisearch, Herbehereak) tentsio altueneko tarterako (-1, -5 eta -10KPa) erabili genituen, eta oreka 6-14 egunetara lortu zen.

Hurrengo urratsean, zilindroetako laginak leunki atera genituen eta lurzoruaren norabide bertikalari jarraituta moztu genituen bi azpi-lagin ateratzeko (1/3 eta 2/3). Heren bat kontu handiz birrindu genuen presio-plaken aparailuan sartzeko (Soilmoisture Equipment Corp., USA) eta horrela tentsio negatiboenetan (-33, -278 eta -1500KPa) ura atxikitzeko gaitasuna neurtzeko. Lehortze-oreka 6-14 egunetara lortu genuen tentsio baxuenetan eta 11 egunetara 33 kP-tan.

TEPEEKIN

Lurzoru-tepeak 20 mm-ko bahetik pasa zitezen pitzadura naturalak jarraituta desegin genituen, kontu handiz. Ondoren, laginak giro tenperaturan lehortzen utzi genituen eta gela lehor batetan mantendu genituen azterketak egin arte.

Laginaren erdia **landa-agregatuen tamainaren banaketa** neurtzeko erabili genuen. Lehorreko baheketa bat egin genuen Gartzia-Bengoetxea eta lankideek (2009) deskribatu bezala, tamaina ezberdinetako agregatuak banatuaraziz: 20–10, 10–5, 5–2 mm; 2.0–0.25 mm; 0.25–0.053 mm; and < 0.053 mm, tamaina ezberdinetako bahe dorre bat dardaragailu baten gainean ipinita (Retsch AS200 Control: Retsch Technology, Düsseldorf, Alemania)

Laginaren beste erdia 3 eta 5 mm artean bahetu genuen tamaina horretako **agregatuen egonkortasuna** eta **Glomalinarekin lotutako lurzoru-proteinak** (GRSP, Glomealin related soil proteins) neurtzeko. Bestalde, 3 eta 5mm arteko azpilagin hauetan GRSP edukia estimatzeko laginaren parte bat almaiz baten bidez txikitu genuen <200 µm-ko tamaiana izan zedin.

4.7 irudia.- Agregatuen egonkortasuna neurtzeko egindako bapateko murgilketaren testaren (LeBissonnais, 1996$) argazkia.

Agregatuen Egonkortasuna bapateko murgilketaren (fast wetting) metodoa erabilita ISO/CD 10930 (Le Bissonnais, 1996) neurtu genuen. Bapateko murgilketarekin jasotoko >50 µm frakzioa lehortu eta eskuz, sei tamainetako (2000, 1000, 500, 200, 100 eta 50 µm) bahe-dorre batean lagina bahetu genuen.

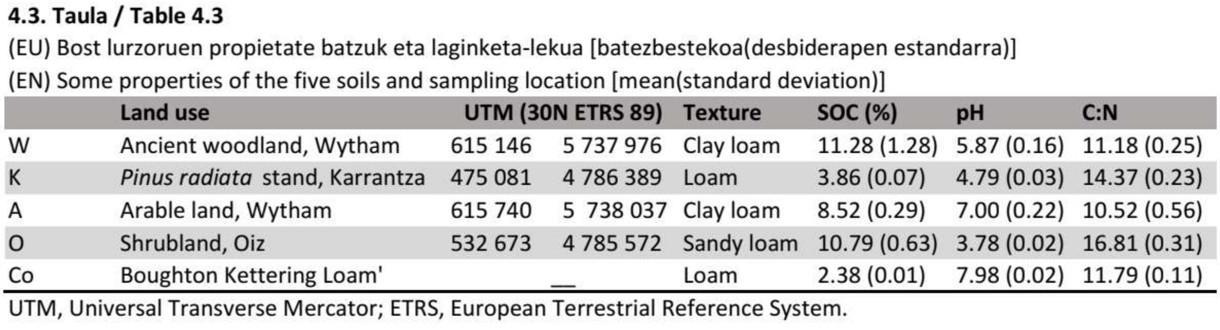
Agregatuen batez besteko diametroa (MWD, Mean weight diameter ingelesez) Kemper eta Rosenauk (1986) proposatutako adierazpenaren arabera kalkulatu genuen, bai Landa-agre**gatu lehorren tamainaren banaketa**ren datuekin (**MWD_f**), bai eta **agregatuen egonkortasuna**ren testaren agregatuen banaketaren datuekin (**MWD _s**)

$$MWD = \sum_{i=1}^{n} xi \cdot wi$$

xi Bahearen batez besteko irekiera (mm), eta wi banantzearen ondoren bahe bakoitzean geratzen den lurzoruaren masaren proportzioa izanik.

4.2.4. Glomalinarekin erlazionatutako lurzoru proteinen (GRSP) kantitatearen estimazioa

Operazionalki definitutako GRSP kantitatea estimatzerakoan metodologia optimizatzeko beharra ikusi genuen. Horretarako jatorri eta propietate ezberdinetako (textura/ehundura, mineralogia eta C organikoa) lurzoruak erabili genituen, CHARFOR eta FIXSOIL proiektuetan aztergai geneuzkanak. Lau toki eta lurzoru-erabilera ezberdinetakoak dira eta azken bat komertziala da: (a) ‘W’ baso zahar batekoa (Wytham Woods, Oxfordshire, UK), (b) ‘A’ Nekazal lur batekoa (Oxfordeko nekazal lursailsail unibertsitariokoa, Wytham, Oxfordshire, UK, farmed by FAI farms), (c) ‘K’ *Pinus radiata* D. Don hazitegi batekoa (Karrantza, Bizkaia), (d) ‘O’ Txilardi-otadi batetik hartutakoa (Oiz Mendia, Bizkaia) eta (e) ‘Co,’ baldintza kontrolatuetan 6 hilez landareak izandako lurzoru komertziala.



4.3. Taula / Table 4.3

(EU) Bost lurzoruen propietate batzuk eta laginketa-lekua [batezbestekoa(desbiderapen estandarra)]

(EN) Some properties of the five soils and sampling location [mean(standard deviation)]

	Land use	UTM (30N ETRS 89)		Texture	SOC (%)	pH	C:N
W	Ancient woodland, Wytham	615 146	5 737 976	Clay loam	11.28 (1.28)	5.87 (0.16)	11.18 (0.25)
K	*Pinus radiata* stand, Karrantza	475 081	4 786 389	Loam	3.86 (0.07)	4.79 (0.03)	14.37 (0.23)
A	Arable land, Wytham	615 740	5 738 037	Clay loam	8.52 (0.29)	7.00 (0.22)	10.52 (0.56)
O	Shrubland, Oiz	532 673	4 785 572	Sandy loam	10.79 (0.63)	3.78 (0.02)	16.81 (0.31)
Co	Boughton Kettering Loam'		__	Loam	2.38 (0.01)	7.98 (0.02)	11.79 (0.11)

UTM, Universal Transverse Mercator; ETRS, European Terrestrial Reference System.

Laginketa-modua Agregatuen egonkortasuna aztertzeko berbera izan zen. Izan ere, 5 lurzoru mota hauen agregatuen estabilitatea ere neurtu genuen.

Operazionalki definitutako **GRSP frakzioak erauzteko** Wrightek eta Upadhyayak (1996) proposatutako erauzketa-metodoak erabili genituen:, ondoren azalduko dugun bezala:

- Errez erauzitako lurzoru proteina ($GRSP_{EE}$): 20 mM-eko sodio zitrato (pH 7) soluzio batekin nahastuta, autoklabetik 30minutuko ziklo baten bitartez erauzi genuen.
- Erauzitako lurzoru proteina totala GRSP ($GRSP_T$): 60 minutuko bi autoklabe ziklotan erauzi genuen. 50 mM sodio zitratoarekin (pH 8ra) nahastuta. Bi ziklotan erauzi genuenez, GRSPen frakzio kuasitotala erauzi genuela esan daiteke. Izan ere, beste azterketa batzuetan zortzi autoklabe ziklo egitera iristen dira, estraktua koloregabea izan arte.

  Kasu bietan ($GRSP_{EE}$ eta $GRSP_T$), lurzoru : soluzio ratioa 1:8 izan zen, eta faseak zentrifugazio bidez (15 000 g-tan 15 minutuz) bereizi genituen, behin hoztuta. $GRSP_T$ren erauzketarako, autoklabearen lehen zikloaren ondoren, soluzioa erretiratu eta gorde genuen eta zitrato-soluzioaren bolumen bera berriro gehitu. Lurzoru pelleta zitrato soluzioa nahastu eta vortexa baten laguntzaz astindu genuen tutua, bersuspenditzeko asmoz. Autoklabe-ziklo bakoitzetik bereizitako soluzioak batu genituen $GRSP_T$ren lagina sortzeko. Hau da, $GRSP_T$ frakzioa gainjalkin bien soluzio konposatua da. Soluzioak izoztu egin genituen, neurketak egin arte. Desizoztu ondoren, laginak berriro zentrifugatu genituen 15 000 g-tan 15 minutuz, sor zitekeen edozein hauspeakin kentzeko.

Erauzitako **proteina kantitatea estimatzeko** Bradford teknika erabili genuen, BioRad Laboratorioko Bradford QuickStart kitarekin (Hercules, CA, AEB). Hainbat kalibrazio metodo maneiatu genituen, behi gazuraren Albumina (BSA) soluzioa baliatuta, ematen ziren interferentziak identifikatzeko asmoz. Kolorimetria neurketak hirukoiztuta prestatu genituen, mikroplaka berean, lagin bakoitzeko hiru putzu desberdin erabiliz. Laginen (diluituak izan ala ez) eta Bradfordeko tintura-erreaktiboen bolumenak 20 eta 230 µL-koak izan ziren, hurrenez hurren.

- Laginak hainbat diluziotan aztertu genituen, BSA gehituta eta gehitu gabe, diluitutako laginek 80 mg dm^{-3} kontzentrazioa lortu asmoz. BSA bi modutan gehitu genien laginei: 1) lagina diluitu aurretik (B serie bezala aipatuko duguna) edo 2) lagina 1:2 diluitu eta gero gehituta (A seriea).
- Absorbantzia-espektroak (400-800nm) neurtu genituen: diluzio guztienak, erreaktiboaren zurienak (Bradfordeko erreaktiboa, zitratoa erauzteko disoluzioarekin bakarrik) eta laginaren zurienak (lagin diluitua, zitratoa erauzteko disoluzioarekin eta pH-a 1era doituta).
- Amaierako diluzioak hauek izan ziren: 1:1, 1:2, 1:5 eta 1:10 A, Co, W eta K lurzoruetarako, eta 1:20 eta 1:40 O lurzorurako.
- Proteina-kontzentrazioa (BSAren baliokidea) modu hauetan kalkulatu genuen:
    - Laginaren kolorearen (laginaren zuriak) zuzenketarekin edo zuzenketarik gabe
    - Funtzio lineal eta ez-linealak doituz kalibrazio kurbetan
    - 595 nm-ko Absorbantziaren kalibrazio-kurbak sortuz eta 595nm-ko eta 465 nm-ko Absorbantzia ratioaren kalibrazio kurbak sortuz.

Erauzitako proteina-kantitatea modu hauetan estimatu genuen:

- Estandarraren gehiketaren puntuen erregresioa x jatorri-ordenatura estrapolatuz. Horrela disoluzioan dagoen proteina-kontzentrazioaren balio absolutua lortzen da.
- Zuzeneko estimazioaren bidez, kalkuluak egin genituen.Laginaren zuriaren (diluzio eta pH berean) zuzenketarekin edo zuzenketarik gabe,

595 nm-ko Absorbantziaren kalibrazio-kurbak sortuz eta 595nm-ko eta 465 nm-ko Absorbantzia ratioaren kalibrazio kurbak sortuz.

- Diluzio-kurben gradienteak erabilita Zor eta Selinger-ek (1996) proposatu bezala. Laginaren diluzio ezberdinen absorbantziak, 595 nm-tara, osatutako kurbaren malda kalibrazio-kurbak sortutakorekin zatituta.

Ekosistema mailako entsegu orokor hau osatzeko, inkubazioetan oinarritutako laborategiko entsegu bat ere egin genuen.

3.3. LABORATEGIKO ENTSEGUA: Epe laburreko lurzoru inkubazioa

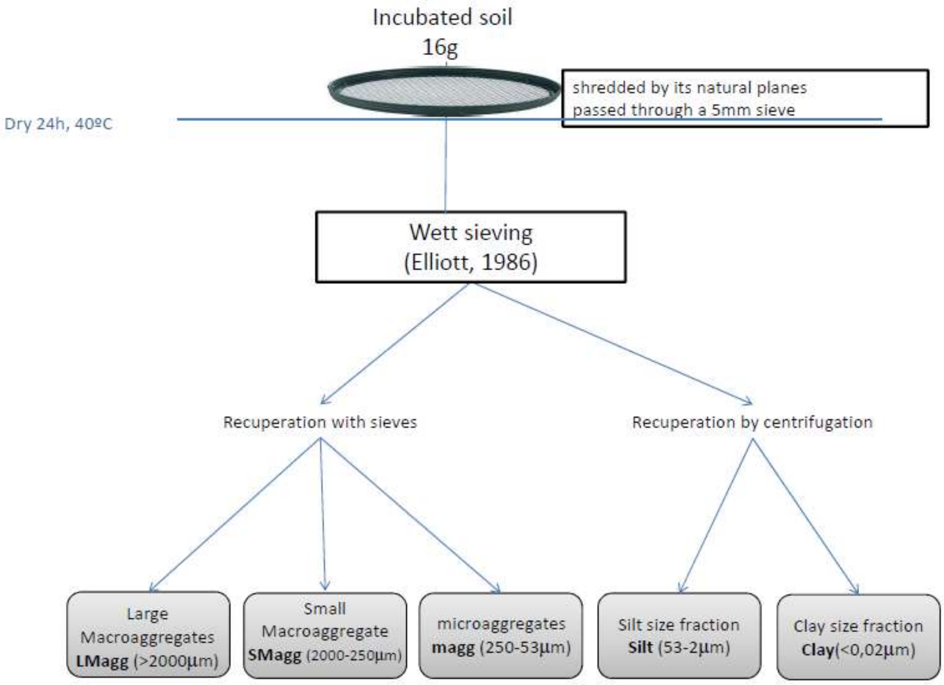
Inkubazio-esperimentua ES-Kko lurzoruarekin eta BCaren eragina aztertzeko baino ez genuen burutu. Lurzorua zoriz bildu genuen, lehenengo 10 cm-etatik eta ikerketa eremua ezarri aurretik. Airez lehortutako laginak lur-errota baten laguntzaz, 2 mm-tik bahetu genituen eta tamaina handiagoko harriak baztertu. Ondoren, 250 µm-ko bahetik pasatu genuen, Magg guztien desegitea ziurtatzeko eta 250 µm-tik gorako frakzioa, hareak eta SOMa, 105 °C-tan berotu genuen 24 orduz, inkubazioan haziek ernetzea saihesteko. Labetik ateratakoan gainontzeko laginarekin nahastu genuen, <250 µm-ko frakzioarekin, lagin homogeneo bat lortzeko. Biocharra almaiz batekin txikitu genuen hurrengo partikulen tamaina-banaketa lortuz: >2mm:1.5%; 2000-250 µm 24.9%; 250-53 µm: 63.3% and <53 µm:10%.

Inkubazioetarako, altzairu herdoilgaitzezko zilindroak (diametroa = 5 cm, altuera = 2,5 cm) lurrez bete genituen, oinarria 53 µm-ko kalibreko nylonezko sare batekin itxi ondoren. 30 g lagin lehor sartu genituen, ageriko dentsitatea 1.2 mg m^{-3}-koa izan zedin. Ikerketa-eremuaren dentsitatea berdinduta, alegia. Tratamenduak landa entseguaren baliokideak izan zitezen, zilindroetako batzuk hutsean inkubatu genituen, Ctrl tratamedua, eta beste batzuk BCa eta lurra nahasiz inkubatu genituen, BC(I) eta BC(I)N tratamenduen BC kopuru proportzionala gehituz. Guztira 48 zilindro prestatu genituen: 3 tratamendu × 6 laginketa-denbora × 3 erreplika. Cosentinok eta lankideek (2006) eta Fernandez-Ugaldek eta lankideek (2011) deskribatutako metodologiari jarraiki, zilindroak eremu-edukieraraino umeldu genituen ur desionizatua erabiliz. Zilindro bakoitza 0,5 L-ko beirazko pote hermetiko baten barruan sartu genuen, suspentsioan, eta potearen ondoan ur desionizatua gehituta, lehortzea ekiditeko asmoz (Cosentino et al., 2006). 28 egunez inkubatu genituen, 25 ºC-tan, baldintza aerobikoetan eta iluntasunean.

Inkubazioaren 0., 1., 3., 7., 14. eta 28. egunean, tratamendu bakoitzeko hiru erreplika hartu genituen aggregatuen frakzionamendu hezea (Elliot, 1986), C totala eta ^{13}C isotopia aztertzeko (Balesdent eta Mariotti, 1987) eta lurzoruaren arnasketa neurtzeko. Inkubazio-denbora bakoitzean, zilindro bakoitzaren erdia gutxi gorabehera, agregatuen frakzionamendu hezea eta isotopia neurketak egiteko erabiltzen genuen, beste laurdena ur edukia zehazteko 50 ºC-tan lehortzen genuen eta gainerakoa izozten genuen biltegiratzeko.

4.8. Irudia.- Inkubazioen prestaketaren irudiak. Ezkerrean, zilindroak betetzeko tratamenduak prest. Eskuman goian, zilindro guztiak umeldura. Eskuman behean, inkubazio-potea eta zilindroa barruan, suspentsioan kokatuta dagoelarik.

- **Baheketa hezearen bidezko agregatuen frakzionamendua** (Elliot, 1986): Agregatuen frakzionamendu hezea egiteko lurzoru-laginak bere apurketa plano naturaletatik desegin genituen, 5 mm-ko bahe batetik kontu handiz pasa arazi eta 40 ºC-tan lehortu genituen 24 orduz. Agregatu lehorren laginak uretan murgildu genituen, sakabanatze fisikoa eraginez, eta agregatuak tamainaka bahetzen joan ginen, Fernández-Ugalde eta lankideek (2013) deskribatu bezala, bahetzeko ekipo bat erabilita (wet sieving apparatus, Eijkelkamp©). 3 min ± 5 s-ko eta 1,3 cm-ko gora beherako ibilbidean egindako zikloak egin genituen bahe tamaina bakoitzarekin (2000µm, 250 µm eta 53 µm) bahe bakoitzak eutsitako agregatuak labera eraman genituen, 24 orduz 50°C-tan lehortu zitezen, eta iragazitakoa hurrengo tamainako bahearekin baheketa aparatuaren zikloa egin genuen. Lohi eta buztin tamainako agregatuak, hau da, 53 µm-tik beherako frakzioak, zentrifugazio bidez bereizi genituen. Sekuentzialki zentrifugatu genituen laginak, lehenengo 50 g-tan 5.5 minutuan (5.59 minutuz, 1000 rpm-ren ekibalentea, rotoredun makinan) lohiak jasotzeko, eta suspentsioan gelditu zena beste ontzi batera



4.9. Irudia.- Baheketa hezearen azalpen eskematikoa.

pasatu genuen eta 200g 12 minututan (73.92 minutuz 11000 rpm-ren ekibalentea). Zentrifugatu beharreko denbora Stokesen Legearen bidez kalkulatu genuen.

- **Lurzoruaren arnasketa**: Inkubazio denbora bakoitzean lurzoruak arnastutakoaren estimazioa egin genuen ISO 16072 arauari (2002) jarraituta. 10 ml-ko ontzietan NaOH (1N) sartu genuen. Askatutako CO_2-ak NaOHrekin erreakzionatzen duenez, HCl eta fenolftaleinarekin titulatuz posiblea da jakitea ontziko zenbat NaOHk erreakzionatu duen zenbat CO_2-rekin, eta beraz, lurzoru horrek askatu duen CO_2-a.
- **Karbono organiko totalaren eta C-aren isotopia analisiak**: Inkubatutako lurzoru zilindro bakoitzeko frakzio guztietan C organiko totala aztertu genuen, 4.2.1.1 atalean deskribatu den moduan.

  Frakzionamendua egin eta geroko tamainaren araberako agregatuetan ^{13}C analisia burutu genuen. Denbora eta frakzio ezberdinetako marka isotopikoa ($\delta^{13}C$) ^{13}C-a, baita BCrena eta lurzoruarena (Ctrl) ere. Horretarako, IRMS fluxu jarraituko VG Isochrom (Isoprime Inc., Manchester, UK) bati akoplatutako Carlo Erba NA 1500 (Milano, IT)

  Biocharretik eratorritako C proportzioa (BC-C) ekuazio honekin kalkulatu genuen (Balesdent eta Mariotti, 1996):

$$f = \frac{\boldsymbol{\delta}\ \mathrm{sample} - \boldsymbol{\delta}\ \mathrm{Ctrl}}{\boldsymbol{\delta}\ \mathrm{BC} - \boldsymbol{\delta}\ \mathrm{Ctrl}}$$

Non δ_{sample} lagin-frakzioaren $\delta^{13}C$ den, δ_{Ctrl} ES-Kko lurzoruaren $\delta^{13}C$, eta δ_{BC} Biocharraren $\delta^{13}C$ (-16,38 ± 0,1).

5. RESULTS (EN)

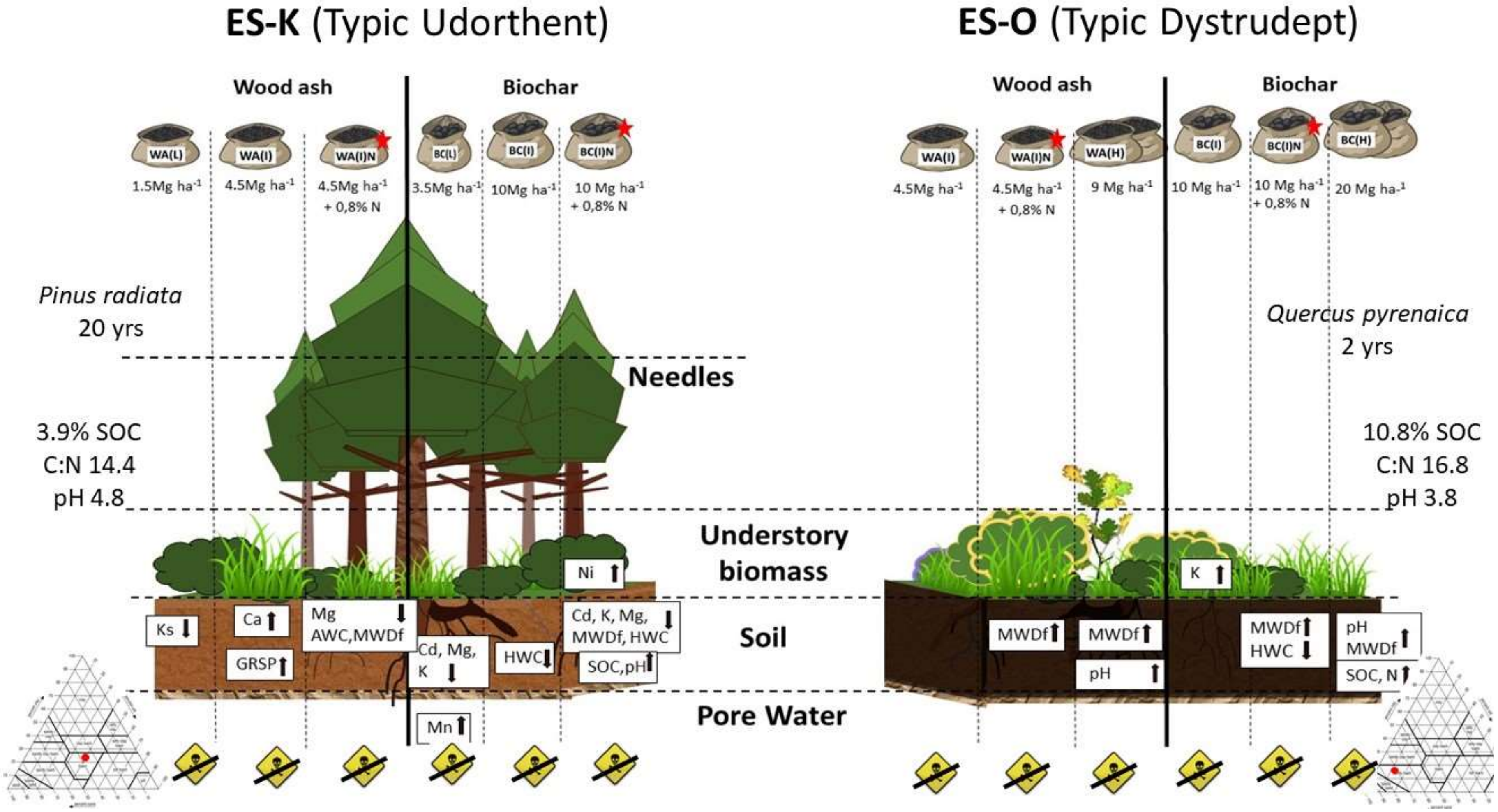


Figure 5.1.- Graphical abstract of the field experiment. On the left side is the research area of Karrantza (ES_K) and on the right side is the research area of Oiz (ES-O) whith their respective characteristics. The effects of the treatments (as columns, wood ash (WA) on the left and Biochar (BC) on the right. High dose (H), intermediate dose (I) and low dose (L); 0.8% of N (N, always with intermediate rate) in the different phases of the ecosystem studied (rows, needles, understorey biomass, mineral soil and pore waters) are represented as a table

5.1. Plant biomass growth

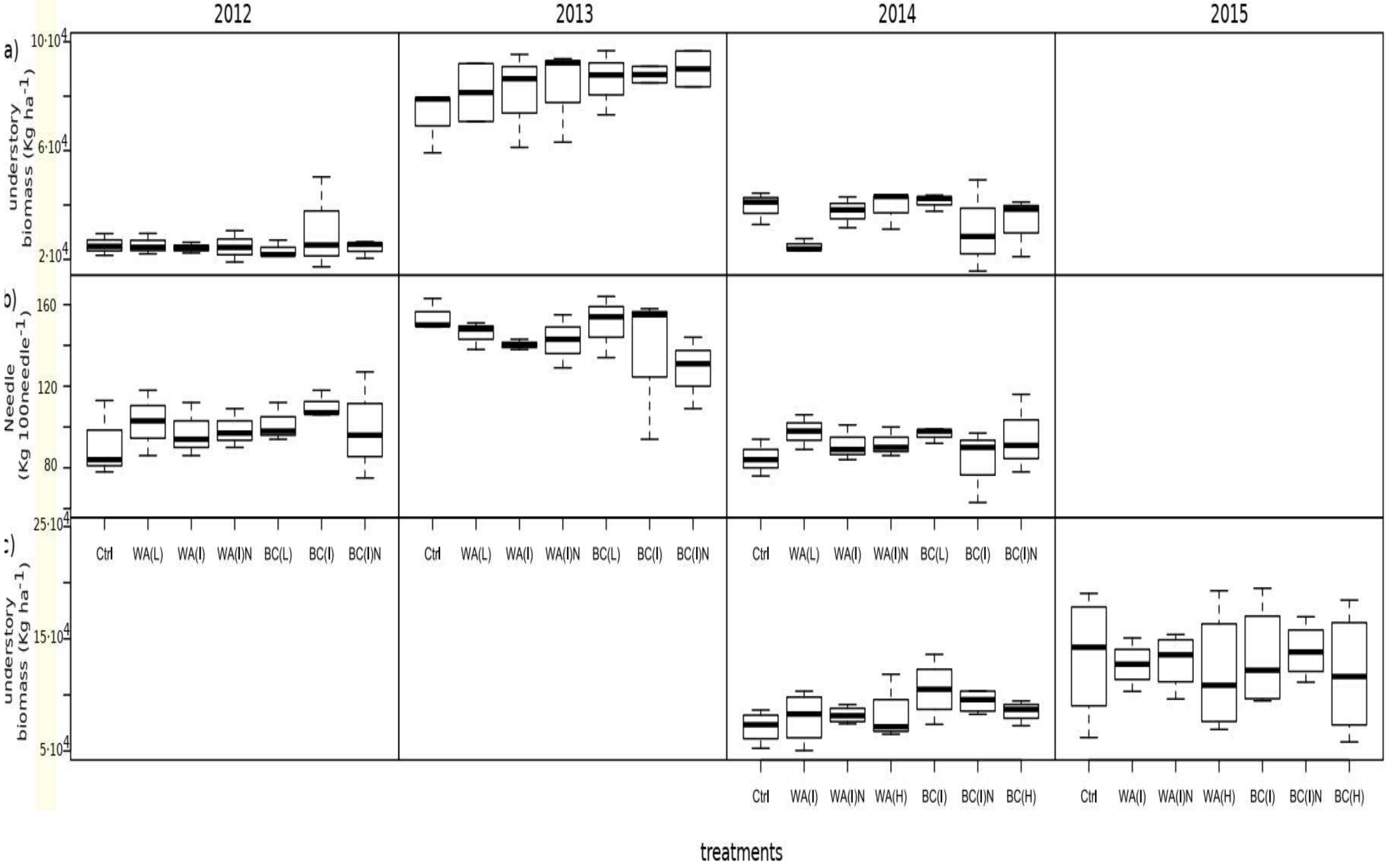


***Fig. 5.2**.- Boxplots presenting biomass production (a and b present understory biomass, and b needle biomass production) relative to each treatment (Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N) in each experimental site: ES-K (a and b) and ES-O (c) in each sampling time. Boxes show the density data within the first to the third quartile of the dataset for each treatment. The horizontal line inside the box represents the median value. The upper and lower whiskers extend from the higher to the lower production data*

The understorey biomass and needles produced over the 30 month study period are shown in Figure 5.2. No significant differences were observed in understorey biomass or needle production in relation to WA and BC application.

5.2. Soil pH and soil organic carbon

Figure 5.3 shows the pH in soil of the two experimental sites: ES-K and ES-O. A treatment effect is observed (F=9.13, df=6, p<0.001). Soil pH increased in ES-K only when treated with BC(I)N, although pH values equated at the end of the research period (Figure 5.3c). In ES-O application of high doses of WA and BC [WA(H) and BC(H)], increased soil pH (Figure 1; F=2.86, df=6, p=0.02).

In ES-K, BC affected SOC content (F=3.03, df=6, p=0.02). BC(I)N presented a significant increase of %34 over control (12.1±0.77 Mg·ha^{-1}). In ES-O, only the highest dose of BC increased in a 42% SOC content over control (38.3±2.95 Mg·ha^{-1}; F=3.4, df=6, p=0.01).

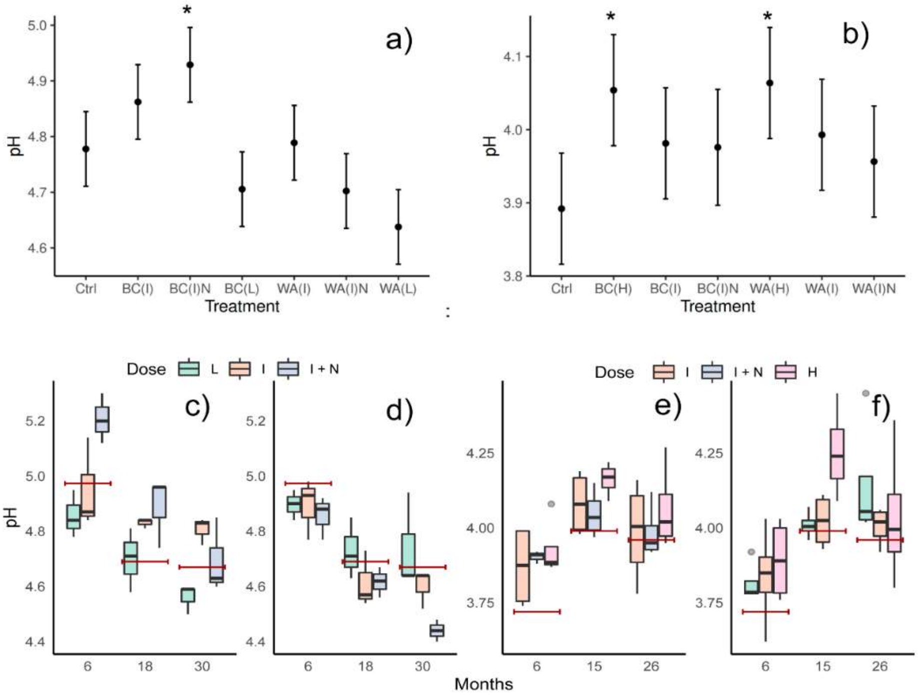


__Figure 5.3.-__ Estimated Marginal Means (EMM) of pH (top) by treatment and experimental site: Experimental site of Karrantza (ES-K, a) and Experimental site of Oiz (ES-O, b), and boxplots showing pH values of each experimental site (bottom): ES-K (c and d) and ES-O(e and f) during time (X axis). Values for biochar (BC) treatments are represented by boxplots c and e, and wood ash (WA) treatments are represented by boxplots d and f. EMM of control is represented in the boxplots by red lines, to provide a visual comparison. high dose (H, purple), intermediate dose (I, pink) and low dose (L, green); 0.8% of N (always with intermediate rate), (N, blue). Boxes show the density data within the first to the third quartile of the dataset for each treatment. The horizontal line inside the box represents the median value. The upper and lower whiskers extend from the higher to the lower pH data. Means which differ significantly from control are in bold and mark with asterisks (p<0,05)*

5.3. Nutrients in soil-water-plant system

Table 5.1 summarizes treatment effects, estimated marginal means of nutrient content in soil (control), and the differences of each treatment.

Table 5.1.- Treatment effects (third row) reported by F-values of the ANOVAs (df=6). Estimated marginal means of control (forth row) ± SE, and comparisons of each treatment against control. Positive values mean increase over control and negative values mean decrease. Significant effects and means which differ from control are in bold and mark with asterisks (* p<0,05; ** p>0,01; ***p>0,001)

	N (Kg·ha^{-1})		**P (Kg·ha^{-1})**		**K (Kg·ha^{-1})**		**Mg (Kg·ha^{-1})**		**Ca (Kg·ha^{-1})**	
	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O
ANOVA (df=6)	F=0.72	**F=3.08***	F=1.17	F=1.97	**F=4.85*****	F=0.72	**F=5.7*****	F=0.63	**F=3.86****	F=1.81
Ctrl	**831±69.8**	**2120±144**	**134±8.53**	**104±7.66**	**5123±214**	**781±53**	**894±34.5**	**262±19**	**289±32.8**	**495±67.8**
WA(L)	+117.6	_	-6.09	_	-199.1	_	-71.31	_	-12.26	_
WA(I)	+19.9	+322.3	+0.28	-1.17	-549.2	-0.36	-94.62	+1.33	**+133.47***	+117.2
WA(I)N	+148.7	+446	+4.67	+2.06	-510.3	-0.54	**-111.48***	-33.12	+23.08	-156.4
WA(H)	_	+408.7	_	+4.72	_	-0.18	_	-9.89	_	+29.6
BC(L)	+67.4	_	-15.59	_	**-805****	_	**-155.08****	_	-15.17	_
BC(I)	+43.5	+243	+8.73	+9.87	-198.5	-0.01	-29.17	-27.4	+82.94	+14.1
BC(I)N	+93.0	+405.7	-4.12	+4.53	**-725.8****	+0.00	**-149.16****	-7.26	+72.49	-108.8
BC(H)	_	**+815.6****	_	+24.29	_	-0.34	_	-20.62	_	-43.3

BC(I) treated soils in ES-O present significantly superior values of K content in understorey biomass, if compared to control (Figure 5.4; t-ratio=-3.085, df=39, p=0.05), although no evidence of treatment effect has been found. The rest of the treatments did not affect understorey biomass nutrient content in none of the experimental sites. Nutrient content in needles was not affected either, by none of the treatments.

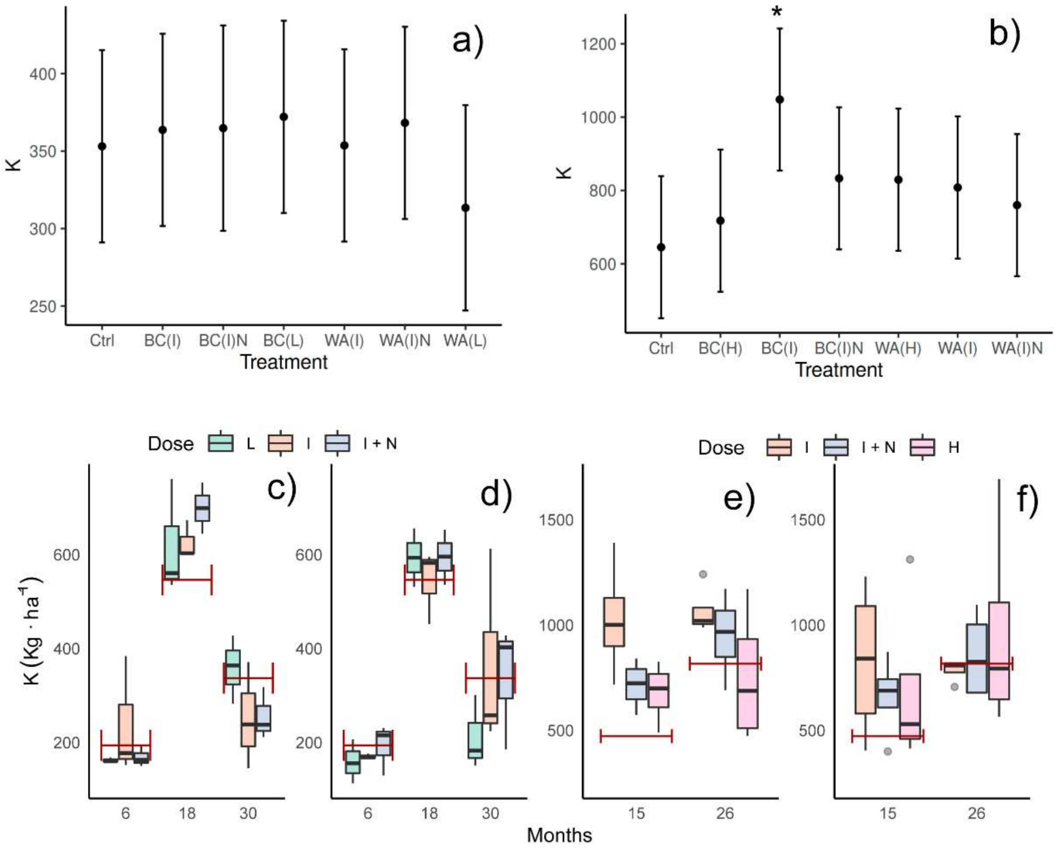


__Figure 5.4__.- Representation of Estimated Marginal Means (EMM) of K content (Kg·ha-1) in understorey biomass (top) by treatment and experimental site: Experimental site of Karrantza (ES-K, a) and Experimental site of Oiz (ES-O, b), and boxplots showing K values of each experimental site: ES-K (c and d) and ES-O (e and f) during the time (X axis). Values for biochar (BC) treatments are represented by boxplots c and e, and wood ash (WA) treatments are represented by boxplots d and f. EMM of control is represented in the boxplots by red lines, to provide a visual comparison. High dose (H, purple), intermediate dose (I, pink) and low dose (L, green); 0.8% of N (always with intermediate rate), (N, blue). Boxes show the density data within the first to the third quartile of the dataset for each treatment. The horizontal line inside the box represents the median value. The upper and lower whiskers extend from the higher to the lower K content data. Means which differ significantly from control are in bold and mark with asterisks (p<0,05*

Pore waters did not contain differences in macronutrient content between treated and not treated samples, in none of the experimental sites.

5.4. Heavy metals in soil-water-plant system

Figure 5.5 shows the Cd in soil of the ES-K and the ES-O, and changes observed in BC treated plots in ES-K. No more changes in heavy metal content were observed in none of the soils after application of any treatment.

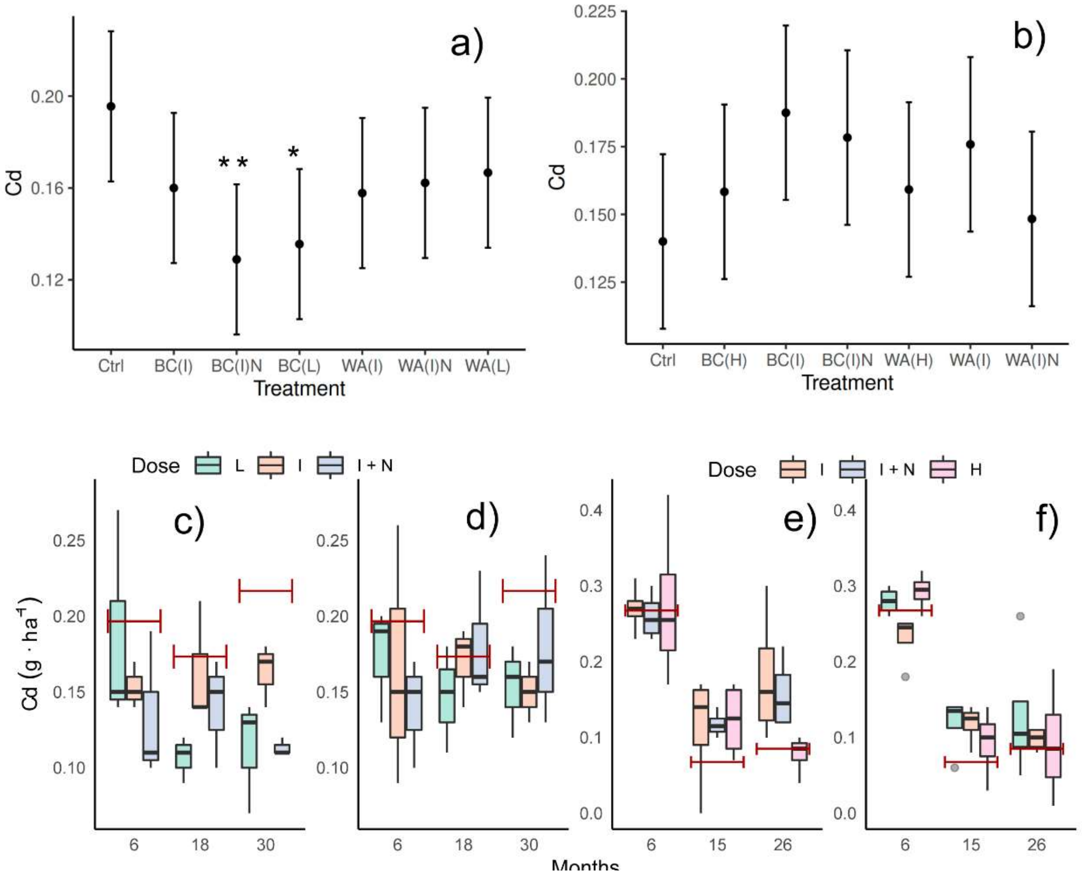


__Figure 5.5.__- Estimated Marginal Means (EMM) of Cd content (g·ha-1) in soil (top) in ES-K soil (a) and in ES-O (b), and boxplots showing Cd content in soil at each experimental site: ES-K (c and d) and ES-O (e and f) during the time (X axis). Values for biochar (BC) treatments are represented by boxplots c and e, and wood ash (WA) treatments are represented by boxplots d and f. EMM of control is represented in the boxplots by red lines, to provide a visual comparison. High dose (H, purple), intermediate dose (I, pink) and low dose (L, green); 0.8% of N (always with intermediate rate), (N, blue). Boxes show the density data within the first to the third quartile of the dataset for each treatment. The upper and lower whiskers extend from the higher to the lower Cd content data. Means which differ significantly from control are in bold and mark with asterisks (p<0,05; ** p>0,01)*

Understorey biomass from ES-K increased 1.5 times Ni content after BC(I)N application (F=16.01, df=6, p<0.001) if compared with Crtl (259±41.4 g·ha^{-1}). No more effects were observed in aboveground biomass heavy metal content.

Pore waters collected from BC(L) treated plots in ES-K, presented mean values of 0.06±0.01 mg Mn cm^{-3}, 6 times higher than Ctrl (0.01mg·ha^{-1}). No more effects were observed in pore waters after treatment application.

5.5. Ecotoxicity testing

WA and BC did not induce toxic effects in the pore waters (Figure 5.6). The MARA indicated that application of WA and BC caused very low growth inhibitions rates that were not different from those in the control treatments. The LumiMARA showed that application of WA to ES-K yielded significantly greater reduction of luminescence in plots amended with WA(L) than in control plots. However, the overall mean inhibition of luminescence and growth did not exceed the 40% in either of the experimental sites.

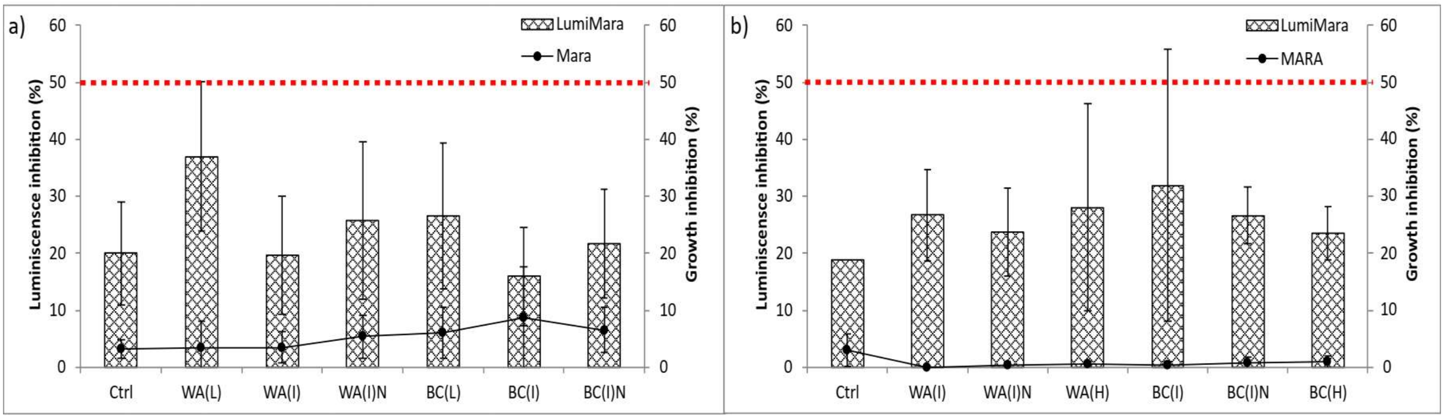


Figure 5.6.- Effect of the treatments (Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N= 0.8% of N) on bioluminescence inhibition (left y axis) measured with LumiMARA (bars) and pellet mean growth inhibition represented by dots (right y axis) determined by the MARA bioassay. Treatment effect was analysed for both experimental sites: ES-K (a) on the left side, and ES-O (b) soil on the right side. Red flashing line (dotted red line) represents the 50% of growth and luminescence inhibition threshold for being toxic)

5.6. Hydraulic properties

The application of WA(L) in ES-K led to a significant decrease in the saturated hydraulic conductivity, to $3.06 \cdot 10^{-7}$ ms-1 (IQR: $2.2 \cdot 10^{-7}$–$8.47 \cdot 10^{-7}$ ms-1), while the rest of the treated plots remained similar to the control: $6.16 \cdot 10^{-6}$ m·s-1 (IQR: $3.66 \cdot 10^{-6}$–$8.66 \cdot 10^{-6}$ ms-1) (Figure 5.7a). In the ES-O, the median values of K_s were similar in treated and control plots ($4.05 \cdot 10^{-6}$ ms-1, IQR: $3.06 \cdot 10^{-6}$-$8.66 \cdot 10^{-6}$ ms-1) (Figure 5.7b).

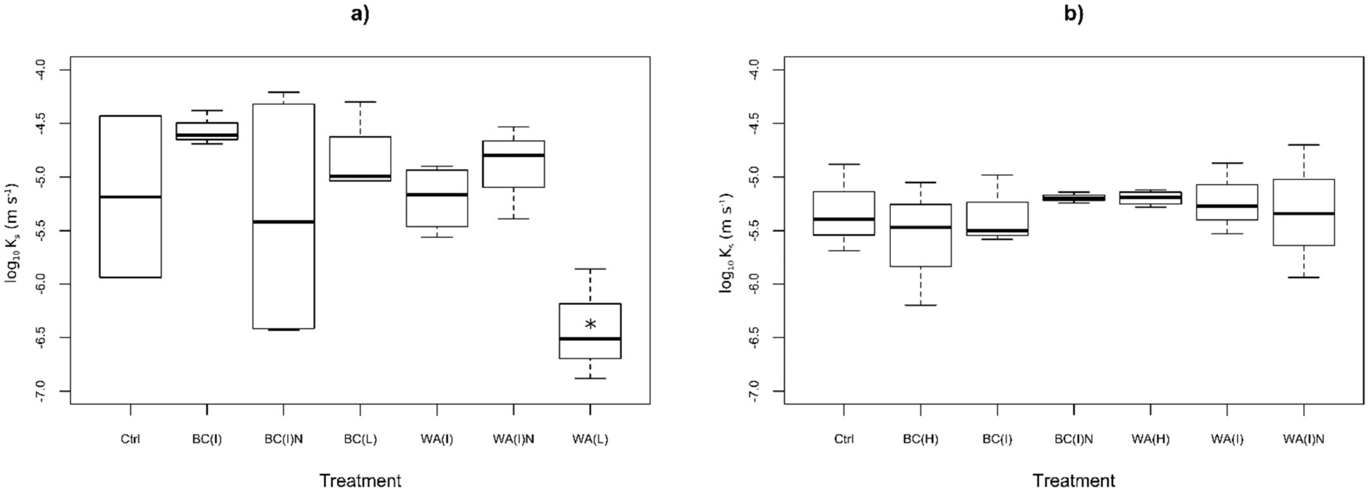


Figure 5.7.- *Saturated hydraulic conductivity for each treatment (Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N) at each experimental site: a) ES-K (loam), and b) ES-O (sandy loam). Significant differences ($p < 0.1$) relative to the control are based on mixed effects models (Appendix A) and indicated by an asterisk. The horizontal line inside the box represents the median value.*

The soil water retention curves (SWRC) are shown in Figure 5.8. Both BC and WA enriched with N (BC(I)N and WA(I)N) significantly altered the shape of the SWRC in ES-K (Fig. 4.8.a and b), reducing water holding capacity at all pressures applied, relative to the control. Application of WA(I)N led to a significant decrease in the available water content (Table 5), of 11.5% relative to non-amended soils in the same site. Application of BC(I) and BC(I)N in ES-K yielded significant reductions in the hygroscopic water content (Table 5.2). In ES-O, the only significant effect of biochar application (a reduction in hygroscopic water content) was observed when BC(I)N was applied. The SWRCs were affected by WA(I) and WA(H) application, which promoted a decrease in water holding capacity relative to the control (Figure 5.8c and d). Nevertheless, this change did not affect the potential water storage. On the other hand, application of WA(I)N increased the gravimetric water content (Table 5.2) in this site.

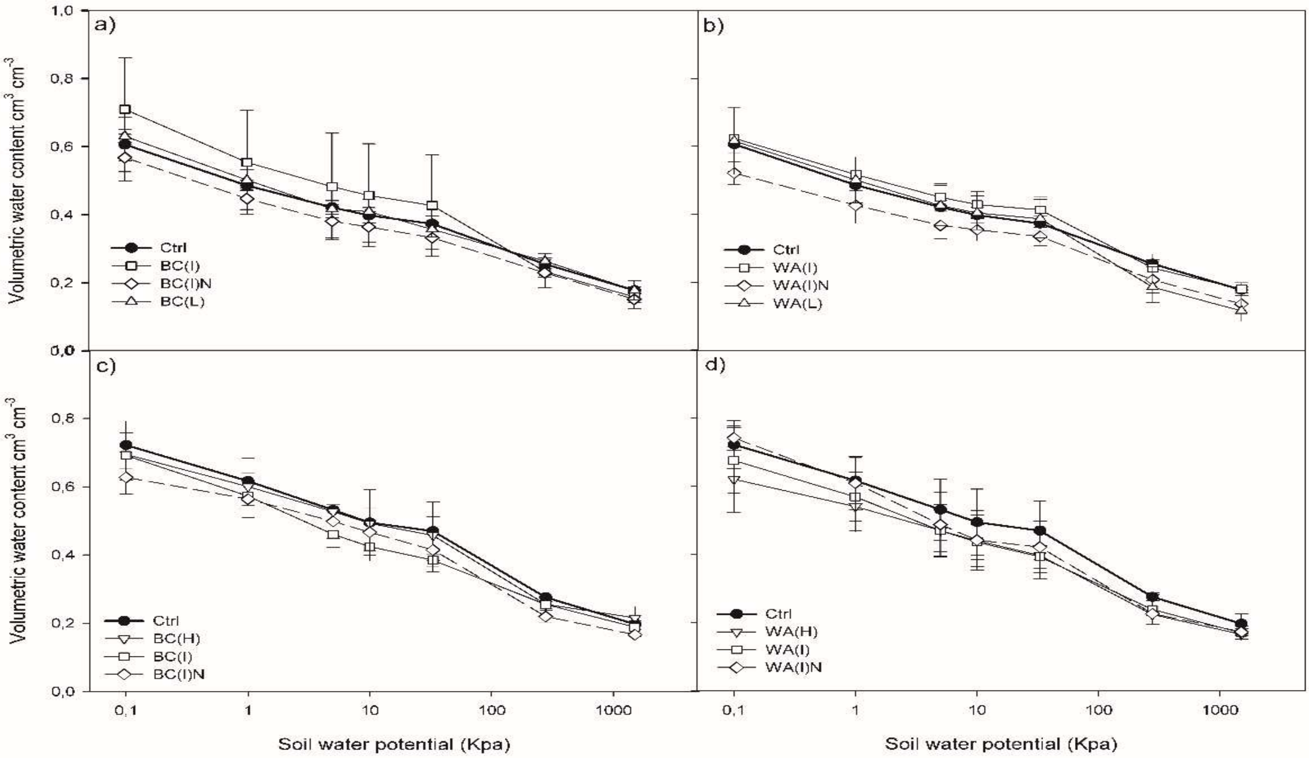


Figure 5.8.- *Soil water retention curves (SWRC) based on volumetric water content measured by desorption at 1 kPa, 5 kPa, 10 kPa, 33 kPa, 278 kPa, 1500 kPa. a) biochar application to ES-K, b) wood-ash application to ES-K, c) biochar application to ES-O and d) wood-ash application to ES-O. Ctrl: Control, BC: Biochar, WA: Wood ash;(H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N. Symbols represent mean values and error bars indicate standard deviation.*

Table 5.2

The potential volumetric soil water storage (in cm^3. median (IQR); $n = 3/4$) of AWC, TWC, GWC and HWC by treatment at each experimental site: ES-K and ES-O. Minimum significant differences ($p < 0.1$) over control are based in mixed effects models (Appendix), and indicated by an asterisk and in bold.

Treatment	AWC		TWC		GWC		HWC	
	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O
Ctrl	0.392 (0.014)	0.438 (0.092)	0.604 (0.096)	0.701 (0.062)	0.218 (0.133)	0.202 (0.055)	0.018 (0.004)	0.016 (0.003)
BC(H)	–	0.478 (0.064)	–	0.687 (0.073)	–	0.157 (0.063)	–	0.015 (0.003)
BC(I)	0.395 (0.138)	0.416 (0.050)	0.689 (0.161)	0.663 (0.049)	0.259 (0.082)	0.243 (0.100)	**0.015 (0.003)***	0.017 (0.001)
BC(I)N	0.358 (0.058)	0.430 (0.019)	0.537 (0.051)	0.593 (0.046)	0.159 (0.082)	0.167 (0.061)	**0.013 (0.000)***	**0.014 (0.004)***
BC(L)	0.394 (0.041)	–	0.622 (0.015)	–	0.215 (0.064)	–	0.013 (0.003)	–
WA(H)	–	0.433 (0.117)	–	0.633 (0.118)	–	0.189 (0.057)	–	0.016 (0.002)
WA(I)	0.420 (0.049)	0.468 (0.048)	0.634 (0.044)	0.663 (0.093)	0.184 (0.025)	0.179 (0.043)	0.017 (0.005)	0.014 (0.002)
WA(I)N	**0.347 (0.028)***	0.347 (0.079)	0.519 (0.035)	0.741 (0.032)	0.155 (0.039)	**0.326 (0.078)***	0.019 (0.002)	0.013 (0.002)
WA(L)	0.380 (0.041)	–	0.610 (0.151)	–	0.195 (0.089)	–	0.017 (0.003)	–

AWC: Available water content; TWC: Total water content; GWC: Gravitational water content; HWC: hygroscopic water content BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose, (L): low dose; N: nitrogen addition (0.8% of N).

5.7. Soil structure

Microporosity in ES-K was significantly reduced in soils amended with BC(I) and BC(I)N (to respectively 1.50 cm^3, IQR:1.33–1.63 and 1.36 cm^3, IQR: 1.34–1.40) relative to the control (1.92 cm^3, IQR: 1.65–2.16). However, no significant differences were observed in either mean pore diameter (MPD) (80.5 µm, IQR: 55.8–100.4) or total porosity (64.2 cm^3, IQR: 61.1–67.6) 30 months after treatment application. Addition of WA(I)N to ES-O significantly reduced the mesoporosity, from 45.4 cm^3 (IQR: 74.0–53.3) to 33.6 cm^3 (IQR:. 32.0–39.7), while MPD and total porosity of all amended plots were not significantly different from control (67.9 µm, IQR: 55.2–72.2 and 72.6 cm^3, IQR: 69.9–75.5, respectively) fifteen months after application of the treatments.

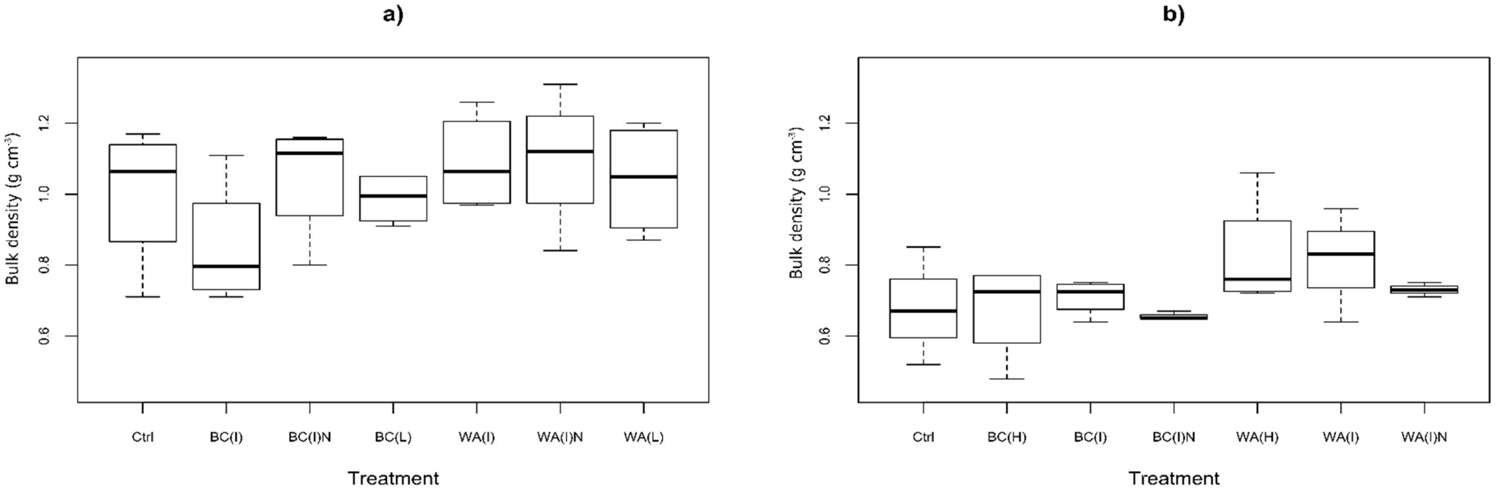


Figure 5.9.- *Bulk density at each experimental site: a) ES-K (loam) and b) ES-O (sandy loam). Treatments: Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N. The horizontal line inside the box represents the median value.*

No significant differences in bulk density were observed in relation to BC or WA application after either thirty months for site ES-K (Figure 5.9a) or fifteen months for site ES-O (Figure 5.9b).

Aggregate size distribution of field samples twenty-four months after treatment applications in both experimental sites is shown in Fig. 5.10. Application of BC(I)N and WA(I)N in ES-K, led to a significant reduction in the MWD_f of dry soil aggregates relative to the control (Figure 5.10). In ES-O, the MWD_f of soil aggregates increased significantly twenty-four months after the addition of the BC(H), relative to control. Increase in aggregate MWD$_f$ was also yielded by WA(H) application in this site.

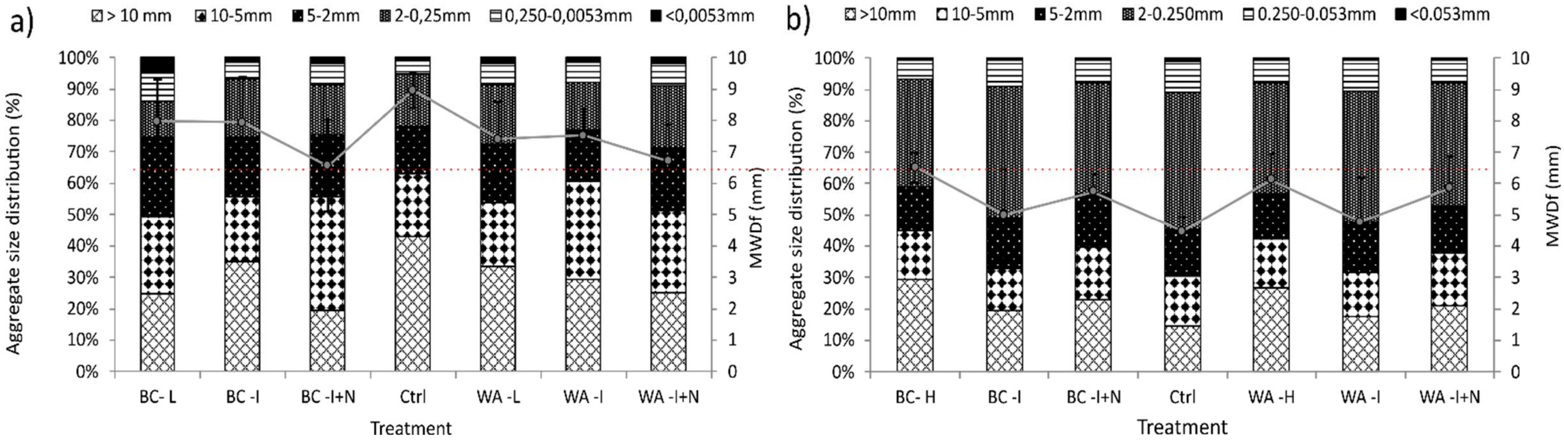


Figure 5.10.- *Aggregate size distribution represented by bars (left of y axis) and mean weight diameter (MWDf) represented by dots (right y axis) for both experimental sites: a) ES-K (loam), and b) ES-O (sandy loam). Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N = 0.8% of N.*

Nitrogen enriched treatments, BC(I)N and WA(I)N, led to increases in the MWD in ES-O, but no significant changes of a certain aggregate size fraction were observed.

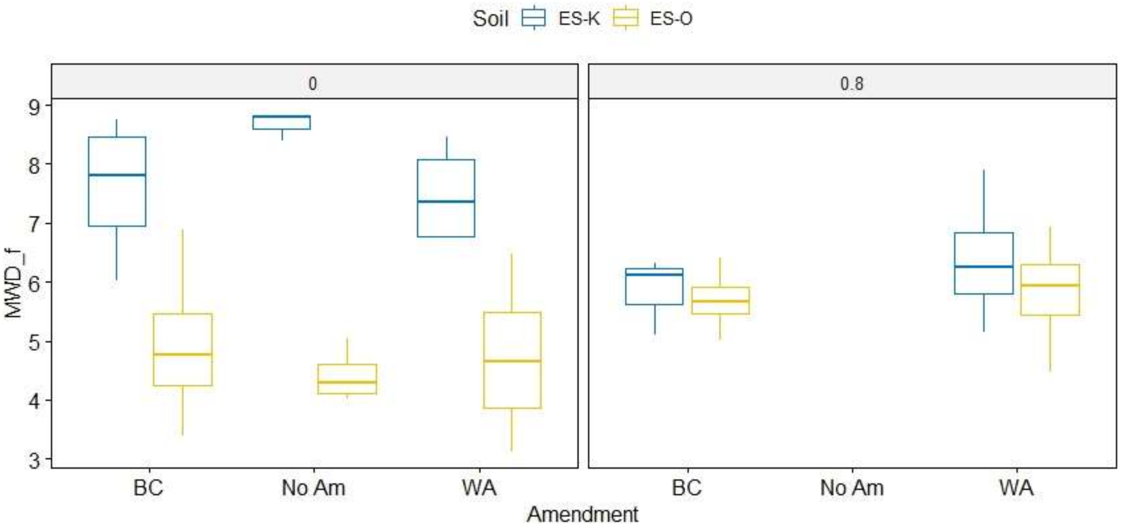


Figure 5.11 *Boxplots representing MWD of field samples samples repeated in both experimental sites: ES-K (loam, blue) and ES-O (sandy loam, yellow). Treatments: Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N.*

Figure 5.11 represents MWD of field aggregates (MWDf) for treatments repeated in both experimental sites. A significant difference was found in the former MWDf between the two forest soils (F=39.45, df=1, 28, p<0.01). No amended ES-K soils presented MWDf over 8 mm whilst median MWDf values of no amended soils in ES-O are of 4.30 mm (IQR=0.48 mm). Notably, these differences between soils dissapeared with N application (signifficant interaction of N addition and forest soil on MWDf (F=10.22, df=1,28, p<0.01). MWDf values of soils treated with N, despite the type of amendment and ES, are between 5-8 mm.

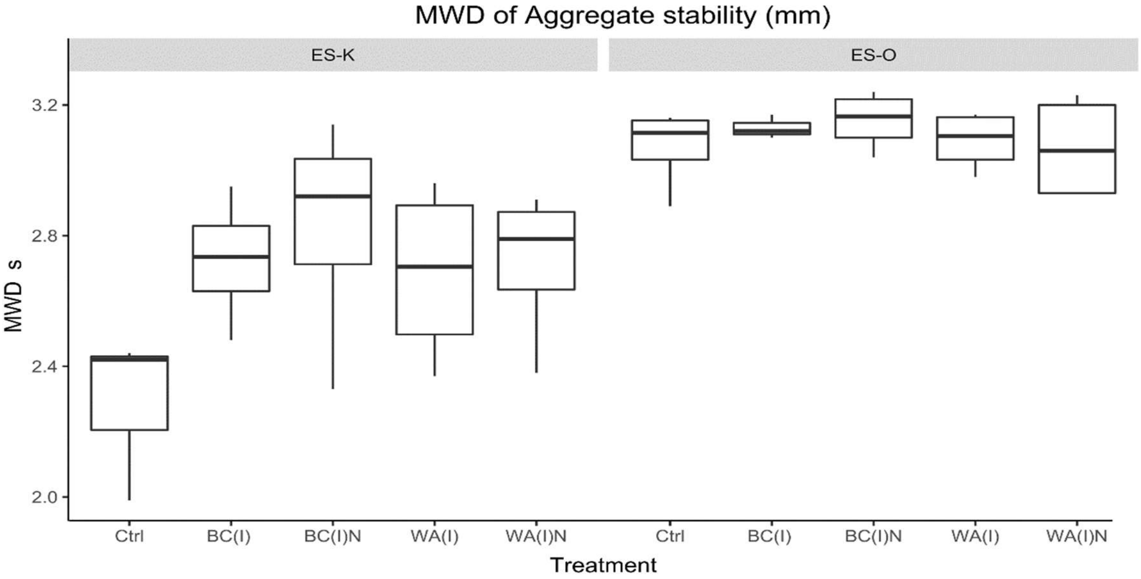


Figure 5.12 *Boxplots representing MWD of samples at each experimental site: ES-K (loam) and) ES-O (sandy loam). subjected to fast wetting stability testing. Treatments: Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N.*

Samples subjected to fast wetting test had MWD (MWD_s) values of 2mm in the two ESs (Figure 5.12), even though MWDs differ from one soil to the other, (F=73.778, df=1,28, p<0.01). MWDs were not signifficantly affected by any of the treatments.

WA addition increased Glomalin related soil proteins (GRSP) (H(2)=5.64, p=0.05). No correlation between GRSP and MWD_s nor MWD_f has been observed, as shown in Figure 4.12.

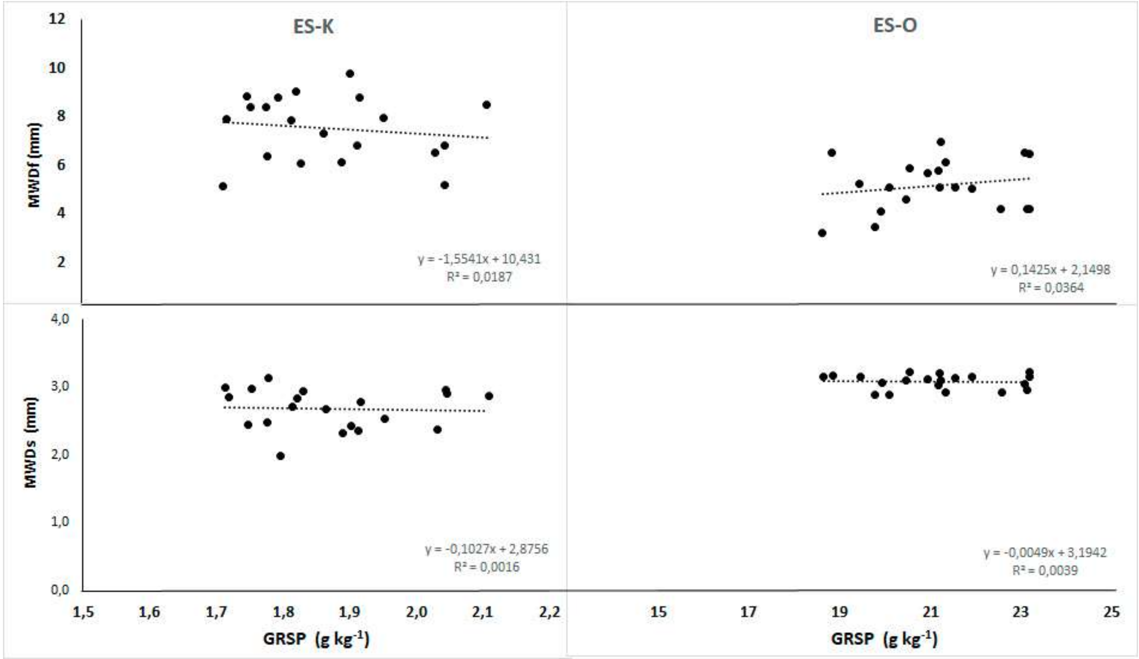


Figure 5.13 *Scatter plots with regression lines to check for any the correlation between Glomalin related soil proteins (GRSP ; X axis) and MWD of aggregates subjected to fast wetting test (MWD_s ; Y axis, bottom) and MWD of field aggregates (MWD_f, Y axis, top)*

5.8. Water stable aggregate formation

Addition of BC to the loamy *Typic Udorthent* resulted in a change in aggregate dynamics during an incubation (Figure 5.13). During the first days of incubation, soils amended with biochar (with and without N) formed no LMagg. In Ctrl soils, LMagg and magg turnover were correlated (R=-0.89, p<0.01). In contrast, for soils incubated with BC, magg turnover was correlated with SMagg (R=-0.89, p<0.01).

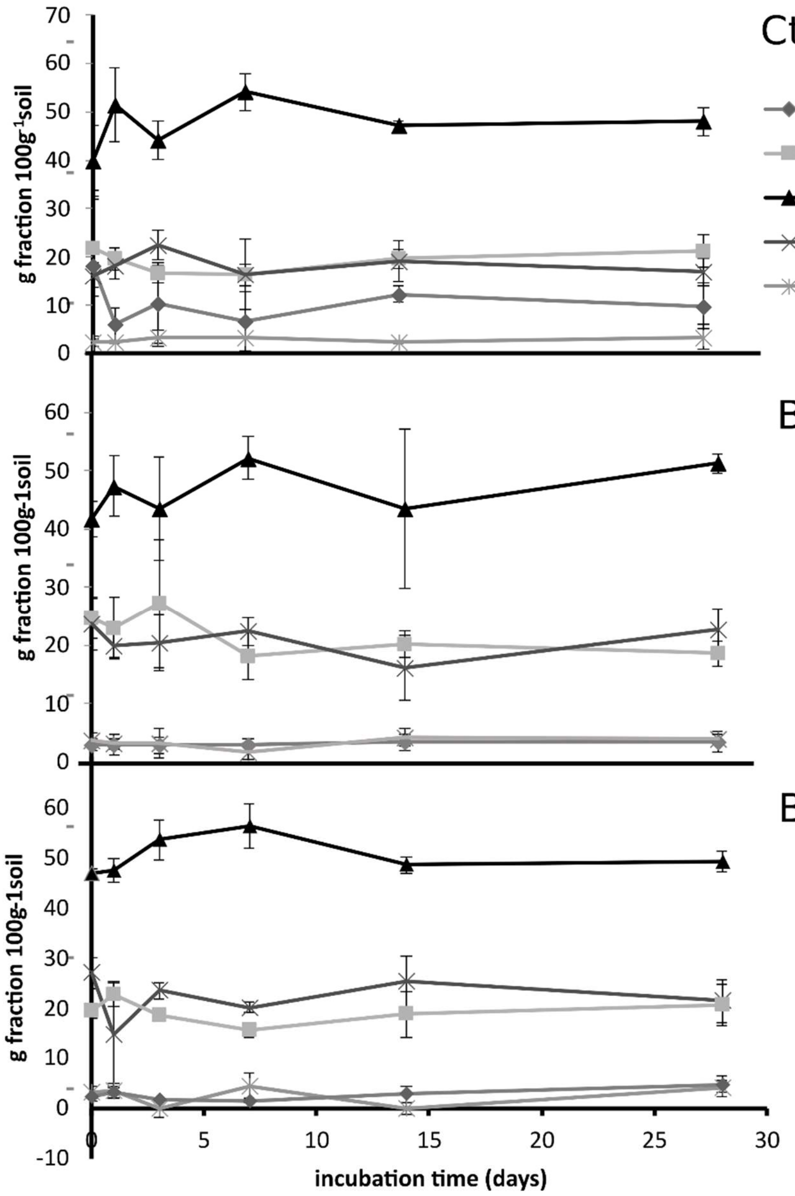


Figure 5.14 Graphs showing aggregate dynamics per each treatment: a) Ctrl, b) BC(I) and c) BC(I)N.. Proportion of each water stable fraction represented in Y axis and incubation time in the X axis.

Soils incubated with BC(I)N show no correlation between fractions. Isotopic analysis presented an enrichment of ^{13}C from day 1 to 28 within microaggregates formed in the typic Udorthent (F=5.59, p=0.02, df=2) For the two BC treatments applied in the incubation: BC(I) and BC(I)N.

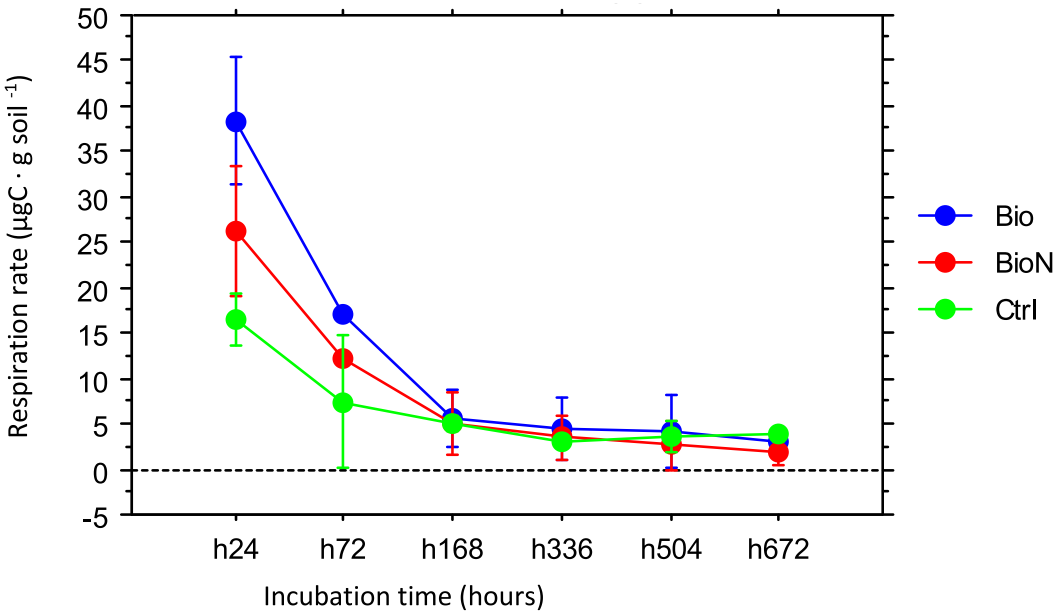


Figure 5.15 Interaction line plot for soil respiration rates (in Y axis) for each treatment (Control [Ctrl] in green, BC(I) [Bio] in blue and BC(I)N [BioN] in red), during the time (X axis, hours).

The addition of BC(I) also resulted in a significant increase in soil microbial respiration (F=17.7, p=0.02, df=2) of the loamy soil (from ES-K). In the case of N enriched samples microbial respiration was 10 times lower than in the BC samples (Figure 5.15).

6. GENERAL DISCUSSION (EN)

6.1. The capacity of biochar as soil enhancer to face climate change seems limited

Overall, biochar affected soil properties depending on the biochar dose and site properties, as observed in many other studies (e.g. Cybulak et al., 2016).

The expected increase in temperature, longer drought periods and heavy rainfalls with the projected scenarios (Section 2.1.1) may induce soil erosion and likely influence the availability of water in terrestrial ecosystems, and therefore in forests. Thus, a well-structured soil will reduce erodibility (Le Bissonnais, 1996) and contribute to improve hydraulic properties by increased water holding capacity and regulate hydraulic conductivity, reducing water and soil loss via runoff (Ellison, 1945).

In this study we did not observe significant effects of BC on soil aggregate stability at neither of the experimental sites. Soils from both sites already were very stable before any amendment. Neither of the initial soils is considered vulnerable to erosion or crustability, since they show MWD_s greater than 2 mm (Le Bissonnais, 1996). Indeed, the greater stability of soil aggregates against slacking observed in ES-O, despite its high sand content, highlights the importance of SOM on aggregate stability, and thus, on soil structure. The soil in ES-O is very rich in Glomalin related soil proteins (GRSP), which have been described as soil gluing agents and enhancers of aggregate stability (Gao et al., 2019) due to the Fe-rich flocs they form with clay particles (Wang et al., 2021). In the same vein, organic materials are directly responsible for the formation of macroaggregates also through the actions of fungal hyphae and microbial extracellular polysaccharides (Lehmann et al., 2017) and they become especially important in coarse textured soils, where sand content negatively correlates with MWD (Ćirić et al., 2012). So, the maintenance of aggregate stability after amendment addition is still positive in order to face up CC by applying BC for other purposes, e.g. for C sequestration (Lehmann et al., 2021). Since some studies reported a reduction in aggregate stability after Biochar addition (Teutscherova et al., 2020; Sun et al., 2021) even though some others reported an increase (Sun et al., 2021). This is because the effect of biochar on soil aggregation largely depends on pyrolysis process, pyrolysis temperature, feedstock, particle size, biochar morphology, experimental condition, soil type, and site characteristics (Chagas et al., 2022).

Even though macroaggregates remained stable against slacking, BC application changed field aggregate size distribution. Since the largest amendment was not applied in both ESs, it is impossible to determine if this change in soil structure was due to the dosage of BC applied and subsequent increase in SOC, or to the increase or changes in physicochemical properties and therefore SOC dynamics. The combination of both, BC and N, may have affected SOM dynamics. In fact, BC(I)N application affected aggregation and microporosity of both experimental sites: ES-K and ES-O. Although the effect on aggregation was contrary in each area, the MWD after the application of BC(I)N became very similar (Figure 5.11). Erktan et al. (2020) observed in their incubation experiment that non-aggregated sieved soils attained median aggregation values of >4 mm highlighting a positive correlation of soil aggregate formation with fungal biomass and a negative correlation with bacterial biomass. They also reported that changes in the activity of microbiota, notably the release of extracellular compounds in response to predation by protists, were responsible for changes in soil aggregation as side effects. The idea of the repulsive biogenic organic matter coating reported by Guhra (2019) fits our results, since these coatings could act as bridging or separating agents to varying extent, according to the environmental conditions in soil (Guhra et al., 2022). So, it could explain somehow, the similar but contradictory effects of each soil with the addition of the same treatment: BC(I)N. Which in turn would reinforce the idea that changes in MWD_f could be related to the soil functioning. Since as mentioned before, addition of BC(I)N leads MWD_f of both soils to equalize, despite their different former MWD_f.

Increase in hydrophobicity could also explain the changes obtained in porosity reducing the amount of water adsorbed to soil surface. As the porosity was calculated from SWRCs (Moragues-Saitua et al., 2017) the reduction of HWC observed could have happened without really affecting microporosity. Microtomography analysis or other image studies could help to understand the real processes happening. One of the possible reasons would be soil compaction caused by root growth (Whalley et al., 2004).

Cybulak et al. (2016) observed a decrease in Hygroscopic water content (HWC) after biochar application in the grasslands investigated, depending on the dose applied, something we also observed. This suggests that the effect of BC application is ambiguous. Probably, more time and research are needed to see if the effects on HWC have any negative impact, since there's little information about the implication that these small, micro-scale changes have in the ecosystems. Even less, about the implication of HWC, since it has been understood as a fraction of water adsorbed to the soil not usable by plants, even though there is who rejects this assertion too, as there are plants capable of absorbing soil water at < -1500 kPa, even at -6000 kPa (Asgarzadeh et al., 2010). However, the dry range of the SWRC (matric potential < -1500 kPa) has been under researched (Zhou et al., 2020). Aquatic and semi-aquatic microorganisms, such as protists and nematodes, are restricted by the size and connectivity of the aqueous habitats they live in. As large pores drain first, small pores, water-films and pendular water in grain contact become the primary habitat for these organisms under drying conditions (Erktan et al., 2020), and drying out of surface layers or a reduction of small pore content could cause a strong reduction in microbial activity (Quijano et al., 2013).

The rest of the hydraulic properties measured in this study were not affected by BC, coinciding with other studies reporting no effects of BC on hydraulic conductivity in loamy soils amended with biochar (Jeffery et al., 2015). Studies using repacked soil columns, such as that performed by Herath et al. (2013), often show a biochar-induced increase in soil hydraulic properties, while studies with undisturbed samples from field experiments have produced rather inconclusive results (Tammeorg et al., 2014). Burrell et al. (2016) observed that the water holding capacity of sandy acidic soils with low organic carbon content (1.64 % OC) increased in response to biochar addition. The sandy loam Typic Dystrudept (ES-O) was characterized by high organic carbon content (10.8 % OC). Our findings are consistent with those of Abel et al. (2013) who observed that addition of biochar to a loamy sand soil with high organic matter content (5.3 % OC) did not increase the AWC. Moreover, Barnes et al. (2014), reported that native soil organic matter played an important role in soil dynamics after addition of 10% of mesquite-derived wood biochar (400 °C) to an organic-rich (37.9% OC) sandy loam soil.

The activity of soil biota is an essential driver of pedogenesis, structure dynamics, and nutrient and organic matter turnover in soils (Guhra et al., 2022). These small-scale processes are key to understanding how organisms affect soil structure and infiltration processes (Stoltze et all., 2022), which are important feedback mechanisms that structure and sustain vegetation and communities in water-limited ecosystems and long drought periods (Vereecken et al., 2022). The small-scale processes are therefore key for soil biodiversity and nutrient cycling (Erktan et al., 2020).

The increase in K uptake by plants observed in ES-O after BC application suggests a fertilization effect of BC, as reported by other authors (Buss et al., 2019; Joseph et al., 2021). The increased K uptake by plants observed after BC application was not proportional to the dose applied, contrary to the observations of many authors (Clapham and Zibilske, 1992; Demeyer et al., 2001; Park et al., 2012). This suggests that the highest doses of BC applied to the Typic Dystrudept may have resulted in formation of a thick amendment layer on top of the soil, as reported in some studies after forest fires (Bodí et al., 2014) or may have prolonged changed the physicochemical properties of the soil (Brais et al., 2015) thus affecting the processes taking place in the soil, as a different dose-depended response was observed in soil too, in ES-O. Indeed, the system in ES-

O is probably adapted to make use of K more efficiently (Cornut et al., 2021), as K was efficiently taken up by plants and prevented its loss via leaching, despite the low energy of adsorption of K (Brais et al., 2015). This is consistent with some studies analysing fertilizing effects of wood ash, were increase in exchangeable K concentration in the forest floor was reported and revealed its fertilizing potential in relation to stand growth in K deficient forests (Augusto et al., 2008; Brais et al., 2015; Solla-Gullón et al., 2008). Indeed, studies performed in tropical K limited forests reported specific adaptations (Cornut et al., 2021), among others, skeweding fine root distribution of trees towards upper soil layers, compared with richer soils and forming fine root mats above the mineral soil active to catch the flux of K and other nutrients.

In ES-K, the application of BC seems to mobilize Cd, K and Mg in soil, since less content of this nutrients have been observed after applying the amendments. The fate of these elements remains unknown as the concentrations did not increase in any of the fractions analysed (needles, understorey o pore waters). Some of these nutrients could have accumulated in other parts of the plants, such as roots. However, it is possible that they could have been translocated deeper into the soil adsorbed to DOC (Loganathan et al., 2012) or to the negatively charged small fractions of BC and then translocated deeper into the soil, since Cao et al. (2021) observed that the transport of Cd^{2+} is facilitated in the cotransport of BC.

6.2. Role of biochar on carbon sequestration

Enhancement of the SOC content, improves among others soil structure, increases soil biodiversity by providing food and habitat for soil biota, increases soils resistance to erosion, increases nutrient retention and long-term availability, and regulates infiltration rates (Bot and Benites 2005).

BC increased SOC stocks in both experimental sites, although the effect varied according to the site and treatment. In the case of ES-K, the increase in SOC was observed when BC(I)N was applied, while in ES-O the same treatment had no effect and the increase in SOC in the highest doses was accompanied by an increase in nitrogen content. This suggests different organic matter dynamics in both sites. Depending on soil properties and characteristics of the site, the biotic community present varies and together determine the pathway that SOM decomposition and transformation follows on each site (Prescott and Vesterdal, 2021). Zhang et al. (2017) concluded that BC addition increases leaf litter and deadwood decomposition at the soil surface, enhancing soil microbial biomass and activity, and thus, increasing the mean concentration of DOC (Gömöryová et al., 2016) that would be translocated through the soil profile. On the other hand, in ES-K, the increase in SOC may be due to the contribution of C through direct incorporation of the biochar in the mineral layer. Fernández-Ugalde et al. (2017) investigated the incorporation of BC-derived C (C4 type plant feedstock) into various particle-size fractions and examined the effect of BC on the storage of total OC in the particle-size fractions in the Karrantza experimental site (ES-K, dominated by C3 type *Pinus radiata*). These authors observed that in the first year, part of the BC spread on the field was degraded and incorporated into the finest fraction of the mineral soil.

The increase in SOC observed could contribute to mitigate climate change via sequestration of SOC if the incorporation and stabilization rate of SOC in the mineral soil layers is higher than the decomposition rate occurring in the organic layer of the soil. Therefore, longer-term studies incorporating the humus layers would be interesting to confirm these hypotheses. However, many authors confer to BC the capacity to increase soil C sequestration due to its persistence in soil, explained by an increased proportion of fused aromatic structures (Schmidt and Noack, 2000; Kanaly and Harayama, 2000; Shing et al., 2012) that remain undecomposed for years. Indeed, if considered the proportion of carbon persistence of BCs H/Corg <0.5 reported by Lehmann et al. (2021), at least 4.29Mg C·ha^{-1} of the BC applied (with the BC(I) treatment) will persist wherever in the soil profile of both experimental sites after 100 yrs. The incubation experiment performed with the soil from the ES-K and the ^{13}C tracing allowed us to observe that BC derived C tends to accumulate within the microaggregates over time, suggesting that the ES-

K soil has the ability to physically protect BC within its microaggregates. Thus, it is possible that, the amount of BC-derived C will exceed 4.29Mg·ha^{-1}. However, dynamics under controlled conditions, such as incubations, are very different to real interactions and dynamics taking place in the field.

Carbon can be sequestered both in soil and in aboveground biomass. We did not observe any increase in aboveground biomass after BC application, despite the observed increase in K uptake by understorey biomass. A review on the effects of BC application in forest ecosystems (Li et al., 2018) concluded that BC addition alone is not sufficient to meet the nutrient needs for tree growth and productivity. In fact, in an analysis of *P radiata* D. Don stands in the Basque Country, most of the soils were found to be deficient in P, and fertilization with between 20 and 150 Kg P ha^{-1} was recommended (Martínez de Arano, 1999). Considering that in our field experiment the largest dose applied was 31.6 Kg P ha^{-1} (treatment BC(H) in ES-O) pine growth was probably limited by lack of P. It is therefore important to know which are the limiting nutrients in each soil, as well as to identify the needs of the system itself.

6.3. Addition of wood ash does not improve soil ability to face climate change in the short term

The overall effect of WA application observed during the three-year field trial does not show any clear positive effect at any of the experimental sites in order to face up climate change.

Regarding the soil structure, WA affected both soils in the same way as BC did. We did not observe significant effects of WA on aggregate stability at any of the experimental sites, which remained very stable, with MDW$_s$ greater than 2mm, as before WA application. There is thus no risk for crustability or soil erosion (Le Bissonnais, 1996) is promoted by WA application.

WA addition increased significantly GRSP content in ES-K, 24 months after its application, but no significant effect has been observed in aggregate formation nor stability. So, our results showed that in this case, GRSP is not an appropriate biochemical proxy for aggregate stability, since no correlation between GRSP and MWD_s nor MWD_f was observed.

WA(I)N promoted changes in field aggregate size distribution twenty-four months after being applied (as did BC(I)N), reaching similar MWD_f values in both sites (Figure 5.11), ES-O and ES-K, differentiating from the respective controls. We also saw a similar effect on ES-O at higher applications. These changes in soil structure coincided with changes in AWC, but only in the ES-K. While the application of nitrogen-enriched wood ash treatment led to a reduction in the slope of the SWRCs for ES-K, in the sandy loam soil (ES-O) no effect on hydraulic properties was observed with the same treatment nor with the highest dosage of WA applied, even though MWD_f of the field aggregates were changed. The reduction in the slope of the SWRCs reflects a reduction in AWC thirty months after the application of WA(I)N in ES-K. The available water content of the soil mainly depends on the soil structure, such as larger pore sizes and soil aggregates (Hillel, 1982), and Ouyang et al. (2013) attributed a negative change in SWRCs to the increasing amount of microaggregates and the smaller pore sizes.

From an ecosystem resilience point of view, a decrease in water holding capacity does not help to maintain soil moisture content during drought periods. The distribution of rainfall will be increasingly variable and it is possible that there will no longer be a linear relation between rainfall and net primary production, as response of complex interactions (e.g., Felton et al., 2021). Indeed, in a study performed in southern Germany and northern Switzerland during 2012 and 2013, Chakraborty et al. (2021) reported that trees have higher resilience and recovery capacity in less-dry plots (AWC>60mm) compared to the trees in dry plots (AWC<60mm), even though resistance was higher in the trees from dry plots.

Despite the differences in soil structure, none of the WA treatments modified the moderately high saturated hydraulic conductivity of the Typic Dystrudept (ES-O). This is consistent with the findings of Adriano and Weber (2001), who did not observe any effect on soil hydraulic conductivity in coarse texture soils to which wood ash was applied at rates as high as 1120 Mg ha^{-1}. The lowest dose of WA (1.5 Mg ha-1) yielded a reduction in saturated hydraulic conductivity from moderately high to moderately low of the Typic Udorthent (ES-K), thirty months after application. This may be a sign of a pore clogging process promoted by the small grain size of the wood ash applied (Gartzia-Bengoetxea and Arias-González, 2013). Post-wildfire studies have also demonstrated that ash caused pore clogging in some cases (Bodí et al., 2014), depending on the soil type and ash behaviour, which depends on, amongst other factors, on burned matter and burning processes. The higher dose of WA (4.5 Mg ha-1) did not result in lower Ks, because thick ash layers might increase the number of active micropores limiting the effects of pore clogging (Woods and Balfour, 2010). Nevertheless, we did not observe any significant change in porosity in response to WA(L) application. Indeed, pore shape, tortuosity and connectivity have been suggested to be more important than total porosity for determining saturated hydraulic conductivity (Bodner et al., 2014; Deb and Shukla, 2012).

The release of compounds from amendments in the field is subjected to many variables on a site-to-site basis, with time since application also playing an important role (Reid and Watmough, 2014). The increase in Ca content observed in ES-K in soil revealed that some of the nutrients were directly supplied through WA. The benefits of Ca inputs for forest health are perhaps the most evident in a series of WA addition studies conducted at the Hubbard Brook Experimental Forest in northern New Hampshire (Kim et al., 2022). Calcium additions led to numerous benefits for the forest, such as increase in soil pH, foliar chlorophyll, leaf and fine-root Ca concentrations, photosynthesis, transpiration, crown health of mature trees, seed production, seedling germination, and mycorrhizal colonization of seedling roots (Juice et al., 2006). In the studied period we did not see any clear benefit derived from the increased Ca content. However, it could be related to the abovementioned increase in GRSP content, and for example to the increase in fungal biomass. More research is needed, because its structure and origin remain unclear, even though it was thought that GRSP was related to fungal activity

(Wright and Upadhyaya, 1996). Still, GRSP is an interesting fraction of soil organic matter and possibly an indicator of changing organic matter dynamics (Xie et al 2015), so it could be indicating some changes in the system that could be important to face up CC.

Studies have shown that WA fertilisation has the highest positive effect on productivity on organic soils (e.g. Pitman, 2006). On the mineral soils that are mainly present in the north of Sweden, WA fertilisation has not been shown to have particularly strong effect on productivity (Jacobson, 2003; Ekvall et al., 2014). In ES-O the application of WA did not increase K uptake by plants, in contrast to observations with BC, even though the total content of K was greater in WA than in BC (Table 4.1). These differences in nutrient release from WA and BC might be complex, due to chemistry of the product (Verheijen et al., 2009) and its interaction with soil biota (e.g., Anderson et al., 2011) or organo-mineral associations (Fernández-Ugalde et al., 2017). Buss et al. (2019) suggested an improved fertilising effect of biochar, derived from a moderate supply of K instead of instant leaching of K from WA. Potassium in wood ashes forms highly soluble salts and oxides (Steenari et al., 1999). It is therefore quickly released following precipitation events (Nieminen et al., 2005).

A recent study examining the effects of wood ash application (up to 20 t·ha-1) on various soil quality metrics at several sites across Canada, found either slight improvements in soil quality or no effects (Joseph et al. 2022). The biggest doses of WA increased soil pH in ES-O, but we did not see any derived effect on vegetation growth or nutritional status. Even considering WA simply as liming treatment for temperate forest soils (Augusto et al., 2008), large quantities of WA must be applied in order to detect any alkalinization in the mineral layers of the Typic Dystrudept. This places in doubt the overall effectiveness of WA for liming temperate forest soils in areas with high precipitation rates. However, WA application affects mainly the pH of the topsoil, and the depth of the effect depends largely on the dosage (Jacobson et al., 2004; Hansen et al., 2017b). Further research observing effects on the organic layers are also needed to complement this type of studies, since these are the horizons that are in direct contact with the amendment and the local changes can lead to effects on the overall ecosystem functioning (Hansen et al., 2017b). Both organic matter mineralization and leaching of dissolved organic carbon (DOC) are mainly controlled by soil pH.

6.4. Role of wood ash on carbon sequestration

Wood ash application did not increase SOC stocks even though it contained a big proportion of charcoal (31% C). The proportion of H/Corg of the carbon present in the ashes was higher than 0.5, unlike BC, making it less persistent and more vulnerable to being used by decomposing organisms (Lehmann et al., 2021). Decomposition of charcoal within the ash mostly may have happened in the organic horizon, which we did not analyse, since the layers closest to the surface where wood ash is applied show the greatest change in response to WA application (Joseph et al., 2022). Joseph et al. (2022) in their study from eight experiments across a broad geographic area in Canada, did observed no consistent effects of WA application in SOM content and nature, for any of the horizons analysed (organic and mineral), suggesting that there was not a generalized response to wood ash application. However, Augusto et al. (2022) observed in their 11yrs experiment, which was performed in a close pine plantation (same climatological conditions as our experimental sites), a decrease of the topsoil organic matter content, with negative consequences on organic carbon and total nitrogen contents. Even though the overall C content did not change in ES-K, GRSP content increased with the intermediate dose applied, suggesting a change in SOM dynamics. GRSP is considered a thermostable SOM fraction, so it could contribute to SOC sequestration due to an increased residence time in soil. The recalcitrance indices for GRSP (the ratio of the sum of alkyl and aromatic C, over the totality of O-alkyl C and carboxyl C) are approximately 2 times higher than SOM (Zhang et al., 2017). Agnihotri et al. (2022) mentioned that its residence time is about of 50-60 years. However, as it contains 2-4% N, it tends to mineralization by lowering the soil N limitation (Treseder and Turner, 2007) and shows contradictory trends in incubation experiments (Agnihotri et al., 2022). In addition, and contrary to many reports (e.g. Agnihotri et al., 2022), we did not observed increased soil aggregate formation nor stability related to GRSP content, so we cannot confirm any physical protection of SOM via GRSP induced soil aggregation. So, still more research is needed to revel its function in soil, if had, since it's still and interesting SOM fraction and an indicator of changes in SOM dynamics.

Regarding vegetation growth, we observed no changes in nutritional status nor in biomass growth. Reid and Watmough (2014) reported in their meta-analysis that about 33% of trials indicated positive tree growth only. They attributed the low mean response in growth to limited number of long-term studies available in the literature, highlighted in their quantitative analysis as well as by other authors (Vance 1996; Pitman 2006). It has been also reported that the growth response of trees is species dependent (Kim et al., 2022). To date, most of the studies examining tree growth responses to ash addition have been conducted on conifers. Thus, while species like jack pine may be well suited to treatment with wood ash, others like black spruce may not be, as the latter exhibited a negative growth response to wood ash as application rates increased (Emilson et al. 2020). Augusto et al. (2022) observed in the oligotrophic forested areas they performed the experiments, that trees treated with WA tended to grow fastest. However, as they also discussed, the positive repercussion on tree growth observed by wood ash application is not compensated by the decrease of the topsoil organic matter content, in terms of C sequestration.

Although nutrition and toxicity are key factors determining plant growth, the yearly fluctuations in biomass growth suggest that water availability is a more constraining factor than nutrients in these ESs, as the variations in needle and understorey biomass production in ES-K (Fig 5.2) clearly coincide with the variations in annual rainfall: 978 mm in 2012, 1413 mm in 2013, and 945 mm in 2014 (data obtained by the closest meteorological stations ([<10km], EUSKALMET 2012, 2013 and 2014). A positive net primary productivity-precipitation linear relationship has been widely recognized across many ecosystem types (Felton et al., 2021; Sala et al., 2012). Evidence also suggests that the global extent of water limitation is increasing (Babst et al., 2019), driven by rising temperatures, which will also increase the magnitude of droughts via increases in atmospheric evaporative demand (Allen et al., 2015; Williams et al., 2020). Thus, water availability is projected to exert an increasingly dominant influence on terrestrial ecosystem functioning, and especially net primary production, throughout the 21st century (Green et al., 2019).

6.5. Potential Risks and further research

6.5.1. Poptential toxicity

The amendments applied do not seem to be toxic or harmful in the two types of forest plantations under study, as revealed by MARA and LumiMARA testing-with pore water samples, although an increase in Mn in pore waters was observed with lowest dose of BC [BC(L)] applied in the ES-K.

The observed increase in Ni uptake by understorey plants in ES-K when BC(I)N was applied is another example of the complexity of the interactions that occur after the soil amendments have been applied. Even though BC(I)N contains less Ni than WA(I) and BC(I), it is the only treatment in ES-K that directly contributes to Ni uptake by understorey biomass. This could be due to the increase in soil pH observed. Ni absorption usually increases with increasing pH up to 5 (Pandaa et al., 2007). It could be also linked to the decrease in Mg, Cd and K content observed in soil and subsequent reduction of their inhibitory effect on the absorption and translocation of Ni^{2+} from roots to shoots (Temp, 1991), or probably, it could be due to the interaction of the mentioned reasons above. Yusuf et all. (2011) suggested that Ni is a high mobile trace metal that tends to accumulate in newly formed plant parts as well to seeds. Ni uptake and accumulation in plants depends on plant species, soil pH, quantity, and form of Ni present in the soil (Hassan et al.,2019). Still more research is needed to understand all processes, since Ni, in contrast to other toxic heavy metals like cadmium, lead, mercury, copper and chromium has received little attention from plant scientists due to its dual character (essential-toxic) and complex electronic chemistry which is a major hurdle in disclosing its toxicity mechanism in plants (Yusuf et al., 2011).

6.5.2. Further research

Few, but negative effects on soil hydraulic properties observed 30 months after wood ash application alert us about the complexity of the interactions occurring with amendment application, which among others, depend on the site, as many authors have stated, as well as on the dose applied and whether nitrogen was added.

Considering that different dose-depended responses were observed, and coinciding with many other authors (Emilson et al., 2020; Clapham and Zibilske, 1992; Demeyer et al., 2001; Park et al., 2012), longer-term further research is needed to know which are the implications and links with the promoted changes in the physicochemical properties of the soil and the biological community.

Further research observing effects on the organic layers are needed to complement this type of studies, since these are the horizons that are in direct contact with the amendment and the local changes can lead to effects on the overall ecosystem functioning (Hansen et al., 2017b).

Soil aggregates, basic units of the soil structure, are the result and limiting agents of the different processes and interactions occurring in the soil. They determine the flows of water, gases and nutrients, among others, and they limit the interactions occurring between underground living and determines the food web. The trends we observed in field aggregate size distribution suggests that MWD_f may be related to soil functioning. Integration of complex analyses of different aggregation analysis with microscale soil and biota interactions are needed in order to understand different interactions driving soil structure. How soil biota engineers the soil and interfere on aggregation and soil hydraulic properties is a multifaceted task that includes trophic links and span complex food webs (Brose and Scheu, 2014).

6. EZTABAIDA OROKORRA (EU)

6.1. Aldaketa klimatikoari aurrea hartzeko biocharrak ongarri gisa duen gaitasuna txikia da

Oro har, BCak lurzoru-propietateetan duen eragina gehitutako dosiaren eta tokiko ezaugarrien arabera desberdina da, beste ikerketa askotan ikusi duten bezala (e.g. Cybulak et al., 2016).

Aurreikusitako tenperaturaren igoerak, lehorte-aldi luzeek eta eurite bortitzek (2.1.1 atala) lurzoruaren higadura eragin dezakete, eta, ziur aski, lurreko ekosistemetako eta, beraz, basoetako uraren eskuragarritasuna mugatu dezakete. Hori dela eta, ondo egituratutako lurzoru bat higaduratik babestuago egongo da (Le Bissonnais, 1996), eta propietate hidrauliko hobeak izango ditu, ura atxikitzeko gaitasuna handituz eta konduktibitate hidraulikoa erregulatuz, eta, hala, uraren eta lurzoruaren gainazal bidezko galera murriztuko du (Ellison, 1945).

Ikerketa honetan ez dugu BCaren eraginik ikusi agregatuen egonkortasunean, ikerketa eremuetako baten ere. Le Bissonnais-ek (1996) proposatutako sailkapenaren arabera, bi tokietako lurzoruak edozein tratamendu gehitu aurretik jada oso egonkorrak ziren. Bietako lurzoru bat bera ere ez da higadurarekiko edo azal gogortuak sortzeko kaltebera, 2 mm-tik gorako MWDs-ak dituzte-eta (Le Bissonnais, 1996). ES-On ikusi dugun egonkortasun mailak, nahiz eta lur hareatsua izan, SOMak agregatuen egonkortasunean eta, beraz, lurzoruaren egituran duen garrantzia agerian uzten du. ES-Oko lurzoruak Glomalinarekin lotutako lurzoru-proteinetan (GRSP) oso aberatsa da eta lurzoruaren agente aglutinatzailetzat eta agregatuen egonkortasunaren bultzatzailetzat hartzen dira (Gao Et al., 2019), buztin-partikulekin Fe-an aberatsak diren multzoak eratzen dituztelako (Wang et al., 2021). Materia organikoak ere, orokorrean, onddo-hifak hedatzea eta mikroorganismoek molekula erreaktiboak kanporatzea eragiten du, besteak beste, eta ondorioz, makroagregatuen eraketa (Lehmann et al., 2017). Materia organikoa bereziki garrantzitsua bihurtzen da testura hareatsuko lurzoruetan; harearen edukia MWD-arekin negatiboki korrelazioan baitago (Ćirić et al., 2012). Hala, BCa bota eta gero egonkortasunari eustea oraindik ere positiboa da, CCari aurre egiteko helburuetarako erabili nahi bada, adibidez, C-a bahitzeko (Lehmann et al., 2021). Ikerketa batzuetan ikusi da BCak agregatuen egonkortasuna murriztu duela (Teutscherova et al., 2020; Sun et al., 2021), nahiz eta badira BCak agregatuen egonkortasuna handitu izanaz informatu dutenak ere bai (Sun et al., 2021). Izan ere, biomasak lurzoruaren agregazioan duen eragina, neurri handi batean, honako aldagai hauen menpe dago: pirolisi-prozesua, pirolisi-tenperatura, lehengaia, partikulen tamaina, BCaren morfologia, baldintza esperimentalak, lurzoru-mota eta lekuaren ezaugarriak (Chagas et al., 2022).

Makroagregatuak bapateko murgilketarekin egonkor mantendu baziren ere, BCaren aplikazioak landa-agregatuen tamainaren banaketa aldatu egin zuen. Tratamendurik altuena ez zenez ES-Kn aplikatu, ez dakigu lurzoruaren egituran ikusitako aldaketa horiek aplikatutako BC dosiaren ondorio diren, hau da, lurzoruan inkorporatutako BCaren ondorio zuzena izan den, ala propietate fisiko-kimikoak eta SOCaren dinamikak aldatu izanaren ondorio. Bien konbinazioak, BCa eta N-a, eragina izan dezake SOMaren dinamiketan. Izan ere, BC(I)Naren aplikazioak bi gune esperimentalen (ES-K eta ES-O) lurzoru-agregazioari eta mikroporotasunari eragin zien. MWDf-ean ikusitako eragina ikerketa-eremu bakoitzean kontrajarria izan bazen ere, hau da handitzea vs txikitzea, BC(I)N aplikatu ondoren MWDf-aren balio oso antzekoak ikusi genituen ikerketa eremu bakoitzeko kontroletan (Figure 5.11). Erktanek et al. (2020) haien esperimentuan ikusi zuten agregatu gabeko lurzoruek inkubazio prozesuaren ondoren 4 mm-tik gorako MWDf-ak lortu zituztela. Ikerketa horretatik nabarmentzekoa da lurzoru-agregatuen eraketak lotura positiboa duela biomasa fungikoarekin, eta korrelazio negatiboa duela bakterio-biomasarekin. Halaber, aipatu zuten mikrobiotaren jardueran emandako aldaketak, batez ere harrapari protistei erantzunez bakterioek askatutako zelulaz kanpoko konposatuak, lurzoruaren agregazioa aldatzearen erantzule zirela. Guhrak (2019) aipatutako materia organiko biogenikoaren estalduraren ideia bat dator gure emaitzekin, estaldura horiek zubi-eragile edo banatzaile gisa jardun baitezakete, lurzoruaren ingurumen-baldintzen arabera (Guhra et al., 2022). Beraz, lurzoru bakoitzak tratamendu bera [BC(I)N] gehitzearen antzeko baina kontrako emaitzak azal ditzake, nolabait. Horrek, era berean, MWDf-ean ikusitako aldaketak lurzoruaren funtzionamenduarekin lotuta egon daitezkeenaren ideia sendotuko luke. Izan ere, lehenago aipatu dugun bezala, BC(I)N gehitzeak bi lurzoruak MWDf tamainan berdintzera eraman ditu, hasierako MWDf-ak esanguratsuki desberdinak zirela kontuan izanda.

Hidrofobizitatea handitzeak ere porositateari egotzitako aldaketak azal ditzake, lurzoruaren azalera adsorbatutako uraren kantitatea murriztuz. Izan ere, porositatea SWRCtik abiatuta kalkulatu genuen (Moragues-Saitua et al., 2017), beraz, behatutako Ur eduki higroskopikoaren (HWC) murrizketa mikroporositateari eragin gabe gerta zitekeen. Hala ere, zuzenean sustraien hazkundeak eragindako lurzoruaren trinkotzearen ondorio izan zitekeen (Whalley et al., 2004). Horretarako, irudi bidezko azterketek, hala nola mikrotomografian oinarritutakoek, ematen ari diren prozesuak hobeto ulertzen lagun dezakete.

Cybulakek eta lankideek (2016)BCak zelaietan duen eragina ikertu zuten eta aplikatutako dosiaren arabera HWCa murriztu egiten zuela ikusi zuten, gure antzera. Honek, beste behin, adierazten du BCa aplikatzearen eragina anbiguoa dela. Segur aski, denbora eta ikerketa gehiago beharko dira HWCaren gaineko ondorioek eragin negatiborik ote duten ikusteko, informazio gutxi baitago eskala txikiko aldaketa txiki horiek ekosistemetan duten eraginari buruz. Are gutxiago, HWCren inplikazioari dagokionean, lurzorura adsorbatutako eta landareek erabil ezin dezaketen ur-frakzio gisa ulertu baita luzaro; hala ere, zenbaitek uko egiten dio baieztapen horri; izan ere, badaude lurretik ura -1.500 kPa-etik behera, baita -6.000 kPa-ra ere, xurgatzeko gai diren landareak (Asgarzadeh et al., 2010). Hala ere, SWRCaren tarte lehorra (-1.500 kPa> potentzial matrikoa) ez da oso ikertua izan (Zhou et al., 2020). Mikroorganismo urtarrak eta semi-urtarrak, hala nola protistak eta nematodoak, habitat hezeen tamainak eta konektagarritasunak mugatuta bizi dira. Poro handiak lehortu ahala, poro txikiak, ur-geruzak eta pikorrak ukitzen duen ur pendularra organismo horien habitat primario bihurtzen dira lehortebaldintzetan (Erktan et al., 2020), eta gainazaleko geruzak arin lehortzeak edo poro txikienen edukia murrizteak jarduera mikrobianoa nabarmen murriztea eragin dezake (Quijano et al., 2013).

Azterketa honetan neurtutako gainerako propietate hidraulikoetan BCk ez zuen eraginik izan, bat eginez BCak eraginik ez zuela adierazten duten beste azterketa batzuekin (Jeffery et al., 2015). Ontziratutako lurzoru-zutabeekin egindako azterketek sarritan, hala nola Herath eta lankideenak (2013), BCaren eragin positiboa erakusten dute lurzoruaren propietate hidraulikoetan. Landa-entseguetan berriz, asaldatu gabeko laginekin egindakoetan, nahiko emaitza kontrajarriak ikusten dira (Tammeorg et al., 2014). Burrellek eta lankideek (2016) ikusi zuten BCak karbono organiko gutxi zuten lurzoru hareatsu azidoen ura atxikitzeko ahalmena (% 1,64 OC) handitu egin zuela. Guk aztertutako Typic Disstrudept-arekin (ESo-O) ikusitakoa, lurzoru hareatsua baina karbono organiko askoduna (% 10,8 OC), bat dator Abelek eta bestek (2013) egindako aurkikuntzekin, zeinetan ikusi zuten materia organiko asko zuen lurzoru hareatsu bati BCa gehitzeak (% 5,3 OC) ez zuela AWC handitu. Gainera, Barnesek eta lankideek (2014) ikusi zuten BCa materia organikoan aberatsa zen lurzoru hareatsu bati (OC% 37,9) gehitzean (%10, 400ºC-tan ekoiztua) lurzoruko jatorrizko materia organikoak paper garrantzitsua jokatzen zuela lurzoru-dinamiketan.

Lurzoruko biotaren jarduera funtsezko motorra da pedogenesian, lurzoruaren egitura dinamiketan, eta mantenugaien eta materia organikoaren eraldaketetan (Guhra et al., 2022). Eskala txikiko prozesu hauek funtsezkoak dira organismoek lurzoru-egituran eta infiltrazio-prozesuetan nola eragiten duten ulertzeko (Stoltze et all., 2022); izan ere, ura eta lehorte-aldi luzeetan ekosistemak eta landare komunitateak egituratzen eta mantentzen dituzten elkarrekintza-mekanismo garrantzitsuak dira (Vereecken et al., 2022). Beraz, eskala txikiko prozesuak funtsezkoak dira lurzoruaren biodibertsitaterako eta elikagaien ziklorako (Erktan et al., 2020).

BCa aplikatu ondoren ES-On landareek K gehiago xurgatzen dutela ikusteak BCaren ongarritze-efektua iradokitzen du, beste autore batzuk ere adierazi bezala (Buss et al., 2019; Joseph et al., 2021). Hala ere, autore askok aipatutakoa ez bezala (Clapham eta Zibilske, 1992; Demeyer et al., 2001; Park et al., 2012), behatutako K xurgapena ez zen aplikatutako dosiarekiko proportzionala izan. Honek aditzera ematen du aplikatutako BCren dosirik altuenek Typic Dystrudept-ean aldaketaren bat eragin duela; izan geruza berri bat eratu izana lurzoruaren gainazalean, suteen ondorengo zenbait azterlanetan ikusi bezala (Bodí et al., 2014), edo izan lurzoruaren ezaugarri fisiko-kimikoak aldatu izana (Brais et., 2015), eta ondorioz, lurzoruetan gertatzen diren prozesuetan eragin. Izan ere, Lurzoru mineralean ere dosiaren araberako emaitza ezberdinak ikusi dira ES-On. Badirudi ES-Oko sistema K-a modu eraginkorrean erabiltzeko adaptatua dagoela (Cornut et al., 2021). Landareek K gehiago hartzen baitute, garbitu dadin galaraziz nahiz eta adsortzio-gaitasun oso txikiko molekula den (Brais et al., 2015). Hau bat dator Braisek eta lankideek (2015), Augustok eta lankideek (2008) eta Solla-Gullónek eta lankideek (2008) adierazitakoarekin, errautsak K eskuragarria handitu egiten zuela orbel-geruzan eta fertilizazio-gaitasuna agerikoa zela K eskasia zuten basoetan. Are gehiago, Cornutek eta lankideek (2021) K eskasia zuten baso tropikaletan berau ustiatzeko moldaera zehatzak ikusi zituzten. Zuhaitzek sustrai finak azaleko geruzetan garatzeko joera erakusten zuten, K eskasiarik ez zegoen basoekin alderatuta, eta lurzoru mineralaren gainean sustrai finez osatutako sareak sortzen zituztela ikusi zuten, K-a eta bestelako mantenugaiak modu aktiboan harrapatzeko, besteak beste.

ES-Kn, badirudi BCak Cd-a, K-a eta Mg-a mobilizatzen dituela, lurzoruan mantenugai hauen gutxitzea ikusten delako, behin BCa botata. Elementu hauen helmuga ezezaguna da, ez baita elementu hauen gehitze bat ikusi neurtutako frakzio baten ere (azikulak, oihanpea, poroetako urak). Elementuotako batzuk neurtu gabeko landareen beste atal batzuetan meta zitezkeen, sustraietan adibidez. Hala ere, balizkoa da lurzoruan barrena garraitu izana, DOCari adsorbatuta (Loganathan et al., 2012), edota negatiboki kargatutako BC zatietara lotuta. Izan ere, Caok eta lankideek (2021) ikusi zuten Cd^{2+}-aren garraioan BCak laguntzen zuela.

6.2. Biocharraren rola karbonoaren bahiketan

SOCaren emendioak lurzoru-egitura hobetzen du, biodibertsitatea handitzen du lurzoruko biotarentzako bazka eta habitata eskainiz, higadurarekiko erresistentzia handitzen du, mantenugaien erretentzioa eta epe-luzeko eskuragarritasuna handitzen du eta infiltrazio-tasa erregulatzen du, besteak beste (Bot eta Benites 2005).BCak SOC kantitatea handitu du ikerketa eremu bietan, baina efektua tokiaren eta tratamenduaren arabera desberdina da. ES-Karen kasuan, SOCaren igoera BC(I)N aplikatutako partzeletan ikusi da; ES-Oaren kasuan, berriz, tratamendu berak ez du eraginik izan, eta dosirik altuenekin SOCaren emendioa, nitrogeno-edukiarenarekin batera eman da. Horrek materia organikoaren dinamika desberdinak iradokitzen ditu ikerketa eremu bakoitzean. Tokiko lurzoruaren propietateen eta ezaugarrien arabera, komunitate biotikoa aldatu egiten da, eta beraz, SOMaren deskonposatzeko eta eraldatzeko bideak (Prescott eta Vesterdal, 2021). Zhangek eta lankideek (2017) ondorioztatu zuten BCa gehitzeak lurzoruko biomasa mikrobianoa eta honen jarduera handitu egiten zuela, eta, beraz, orbelaren eta hildako egurraren deskonposizioa handitu, hala, DOCen batez besteko kontzentrazioa handituz (Gömöryová et al., 2016) eta lurzoruan barrena garraiatuaz. ES-Kren kasuan, bestalde, SOCaren igoera C-aren ekarpen zuzen baten ondorio izan daiteke, BCa zuzenean geruza mineralean sartzen dela. Fernández-Ugaldek eta lankideek (2017), BCarekin egindako ikerketa baten, ikusi zuten lehen urtean BCaren zati bat behintzat degradatu eta lurzoru mineraleko frakziorik finenari batu zitzaiola. Karrantzako ikerketa-eremuan (ES-K, C3 metabolismo motakoa den *Pinus radiata* nagusi) BCetik (C4 motako landarea jatorriz) eratorritako C partikulen inkorporazioa aztertu zuten lurzoruko mineraletan, tamainaren araberako lurzoru-partikulen frakzionamendua eta isotopia baliatuta.

Behatutako SOCaren emendioak klima-aldaketa mitigatzen lagun lezake SOCa metatzearen ondorioz, baldin eta lurzoru mineraleko geruzetako SOCaren sartze- eta finkatze-tasa lurzoruaren geruza organikoan gertatzen den deskonposizio-tasa baino handiagoa bada. Beraz, interesgarria litzateke orbel-geruzak ere aztertzea mota honetako hipotesiak berresteko. Hala ere, autore askok lurzoruan C-a gordetzeko gaitasuna ematen diote BCari, lurrean luzaro irauten omen duelako. Izan ere, egitura aromatiko fusionatu gehiago dauzkate (Schmidt eta Noack, 2000; Kanaly eta Harayama, 2000; Shing et al., 2012), eta ondorioz urteak irauten dituzte deskonposatu gabe. Lehmannek eta lankideek (2021) H/Corg<0.5-eko proportzioan oinarrituta proposatutako BCaren C iraunkortasunaren estimazioa aintzat hartuta, guk aplikatutako BCetik [BC(I) tratamenduarekin] 4,29 Mg C·ha^{-1} gutxienez, 100 urte igaro ondoren ere deskonposatu gabe jarraituko lukete, ikerketa-eremua eta lurzoru-horizontea edozein dela ere. ES-K-ko lurzoruarekin eta ^{13}C trazadurarekin egindako inkubazio-esperimentuaren bidez ikusi ahal izan genuen, bestalde, BCetik eratorritako C-ak mikroagregatuen baitan metatzeko joera duela, ES-Kko lurzoruak BCa mikroagregatuen baitan fisikoki babesteko gaitasuna duela iradokiz. Beraz, lurzoru mineralean gordeta geratuko den BCetik eratorritako C kantitatea 4,29 Mg·ha^{-1} baino handiagoa izan liteke. Hala ere, baldintza kontrolatuetako dinamikak, inkubazioetakoak kasu, basoan ematen diren elkarrekintzen eta dinamiken oso desberdinak dira.

Karbonoa lurrazpian zein lurgaineko biomasan gorde daiteke. Ikerketa honek iraun duen denbora-tartean, BCa aplikatu ondoren, ez dugu lurgaineko landare-biomasaren hazkuntza areagotu denik ikusi, biomasan K-aren edukia handitu bazen ere. Basoetako ekosistemetan BCa aplikatzeak dituen ondorioei buruzko review batek (Li et al., 2018) ondorioztatu zuen BCa gehitzea berez ez dela nahikoa zuhaitzen hazkunde eta produktibitaterako elikagai-beharrak asetzeko. Izan ere, Euskal Herrian kokatutako *P radiata* landaketetan egindako azterketa batean, Martínez de Aranok (1999) ikusi zuen lurzoru gehienek P-ren urritasuna zutela, eta 20 eta 150 kg P ha^{-1} artean ongarritzea gomendatzen zuen. Kontuan hartuta gure landa-esperimentuan aplikatutako dosirik handiena 31,6 kg P ha^{-1}-koa izan zela [ES-On BC(H) tratamendua], pinuaren hazkundea P ezagatik mugatu zen ziurrenik.

6.3. Errautsa gehitzeak ez du aldaketa klimatikoari aurre egiteko lurzorua hobetzen epe laburrean

Landa-ikerketak iraun zituen hiru urteetan ez genuen WAren eragin positiborik ikusi, ez behintzat CCari aurrea hartzeko garrantzitsuak diren ezaugarrietan.

Lurzoruaren egiturari dagokionez, WAk BCaren eragin berdina erakusten du lurzoru bietan. WAak ez dauka eragin adierazgarririk agregatuen egonkortasunean, ikerketa eremua edozein dela ere. Izan ere, lurzoru bietako agregatuak oso egonkorrak dira, 2 mm-tik gorako MWDak dauzkate, WAa bota bariko partzeletan bezala. Beraz, WA aplikatzeak ez du inolako arriskurik eragiten lurzoruaren higadurarako, ez eta azalera gogortzeko ere (Le Bissonnais, 1996).

WAa gehitzeak nabarmen handitu zuen GRSParen edukia ES-Kn aplikatu eta 24 hilabetera, baina ez da efektu adierazgarririk hauteman agregatuen eraketan, ez eta egonkortasunean ere. Beraz, gure emaitzek erakusten dute kasu honetan GRSPa ez dela agregatuen egonkortasunerako adierazle biokimiko egokia, ez baitzen korrelaziorik hauteman GRSPa eta MWDf edo MWDs-aren artean.

WA(I)N-k aldaketak eragin zituen landa-agregatuen tamainaren banaketan, aplikatu eta hogeita lau hilabetera [BC(I)N-k eragin bezala], tratamendu berbera jasotako laginen MWDf-ak berdintzeko joera erakutsiz, eta elkarren artean hain desberdinak ziren ikerketa-eremu bakoitzeko kontroletatik bereiziz (ES-k eta ES-O; 5.11 irudia). Antzeko efektua ere ikusi genuen ES-On aplikatutako dosi handienekin. Lurzoruko ur erabilgarriaren edukia lurzoruaren egiturak baldintzatua da nagusiki, lurzoruko poro-tamaina handiagoa izateak eta agregazioak besteak beste (Hillel, 1982). N-rekin aberastutako WA tratamenduarekin ES-Kko SWRCen malda murriztu egin zen; aldiz, hareatsuagoa den lurzoruan (ES-O) ez zen inolako eraginik hauteman tratamendu berarekin ezta aplikatutako WAaren dosirik altuenarekin ere, nahiz eta landa-agregatuen MWDf-an aldaketak ikusi ditugun. ES-Kn WA(I)Nrekin ikusitako SWRCaren malda apaltzeak AWCaren murrizketa islatzen du, tratamendua bota eta hogeita hamar hilabetera. Ouyangek eta lankideek (2013) SWRCean ikusitako eragin negatiboa mikroagregatuen kopuru gero eta handiagoari eta poro-tamaina txikiagoei egotzi zieten. Ekosistemaren erresilientziari dagokionez, ura atxikitzeko ahalmena murrizteak ez du lagunduko lehorte garaietan lurzoruko hezetasuna mantentzen. Prezipitazioen banaketa gero eta aldakorragoa izatea espero da, eta elkarrekintza konplexuen ondorioz, prezipitazioa eta produkzio primario garbiaren artean gaur egun ematen den erlazio lineala desagertzeko arriskua aurreikusi da (e.g., Felton et al., 2020). Honekin lotuta, Alemaniako hegoaldean eta Suitzako iparraldean 2012an eta 2013an egindako azterlan batean, Chakrabortyk eta lankideek (2021) jakinarazi zuten zuhaitzek erresilientzia eta berreskuratze-ahalmen handiagoa zutela hainbeste lehortu ez ziren lursailetan (AWC>60 mm), lursail lehorretako zuhaitzekin alderatuta (AWC<60 mm), nahiz eta erresistentzia handiagoa izan lursail lehorreko zuhaitzek.

Lurzoruaren egituran desberdintasunak egon arren, WA tratamendu bakar batek ere ez zuen aldatu *Tipic Dystrudept*aren (ES-O) konduktibitate hidraulikoa. Hori bat dator Adriano eta Weberren (2001) aurkikuntzekin, testura hareatsuko lurzoruetan ez baitzuten inolako eraginik hauteman lurzoruaren konduktibitate hidraulikoan, nahiz eta 1120 Mg ha^{-1} arteko egur errautsa dosiak aplikatu zituzten. WAaren dosirik baxuenak (1,5 Mg ha^{-1}) berriz, saturatutako konduktibitate hidraulikoa murriztu zuen, *Typic, Udorthent*ak kontrolean erakusten zuen konduktibitate nahiko altua izatetik, nahiko baxua izatera pasaz WA dosirik baxuena aplikatu eta hogeita hamar hilabetera. Hau poroa itxi izanaren adierazle izan daiteke, aplikatuko errautsaren pikor-tamaina txikiak bultzatuta adibidez (Gartzia-Bengoetxea eta Arias-González, 2013). Suteen ondorengo azterketek ere adierazi dute errautsek poroak itxi izana kasu batzuetan (Bodí et al., 2014), lurzoru-motaren eta errautsen portaeraren arabera, azken hau lehengaiaren eta erredura-prozesuen araberakoa izanik, besteak beste. WAaren dosirik altuenak (4,5 Mg ha^{-1}) ez zuen konduktibitate hidraulikoa txikitu, errauts-geruza lodiek mikroporo aktiboen kopurua handitu baitezakete poroen itxieraren ondorioak arbuiatuz (Woods eta Balfour, 2010). Hala ere, WA(L) aplikazioaren ondoren ez dugu porositatean aldaketa nabarmenik ikusi. Izan ere, poroforma, bihurritasuna eta konektibitatea, poro kantitatea bera baino garrantzitsuagoak dira konduktibitate hidraulikoa baldintzatzeko (Bodner et al., 2014; Deb eta Shukla, 2012).

Emendakinetatik mantenugaiak askatzea aldagai askoren menpe dago, eta hein handi batean tokiaren eta aplikaziotik igaro den denboraren araberakoa da (Reid eta Watmough, 2014). ES-Kko Lurzoruan behatutako Ca edukiaren hazkundeak erakusten du WAak baduela elikagai batzuk zuzenean aportatzeko gaitasuna. New Hampshire-ko iparraldean, Hubbard Brook baso esperimentalean ikusi zuten Ca-aren ekarpena dela WAaren emendioak basoaren osasunean duen onurarik nabarmenena (Kim et al., 2022). Kaltzioa gehitzeak onura ugari ekarri zizkion basoari, hala nola lurzoruaren pH-a igotzea, hosto-klorofila areagotzea, Ca kontzentrazioak handitzea hostoetan eta sustrai finetan, fotosintesi eta transpirazio-tasak areagotzea, zuhaitz helduen koroaren osasuna hobetzea, hazien ekoizpena, plantulen ernamuintzea eta plantulen sustraien kolonizazio mikorrizikoa bultzatzea (Juice et al., 2006). Guk aztertutako denbora-tartean ez genuen inolako onura argirik ikusi Ca edukia handitzearen ondorioz. Hala ere, GRSParen edukian ikusitako emendioarekin lotuta egon liteke, Ca-k bultzatutako biomasa fungikoaren handitzearen ondorio. Hala ere, hau baieztatzeko gehiago ikerketu behar da, GRSPen egitura eta jatorria ez dagoelako argi oraindik, nahiz eta jarduera fungikoarekin lotuta zegoela uste izan den (Wright eta Upadhyaya, 1996). Hala ere, GRSPak lurzoruaren materia organikoaren frakzio interesgarri bat da, eta, seguru asko, materia organikoaren dinamika aldaketen adierazle bat (Xie et al 2015). Beraz, CCari aurre egiteko garrantzitsua izan daitekeen aldaketaren bat adierazten ibil zitekeen

Zenbait azterlanek (Pitman, 2006) frogatu dute WAak lurzoru organikoetan duela fertilizazio-gaitasunik handiena. Suediako iparraldean dauden lurzoru mineralak WAarekin ongarritzeak ez du landareen hazkuntzan eragin nabarmenik izan (Jacobson, 2003; Ekvall et al., 2014). Bestalde, WAak ez zuen handitu landareek hartutako K kantitatea ES-On, BCrekin gertatu ez bezala, nahiz eta K-ren eduki orokorra handiagoa izan WAean BCean baino (4.1 taula). WAaren eta BCaren artean egon daitezkeen desberdintasunak, mantenugaien askatzeari dagokionean, konplexuak dira, produktu bakoitzaren izaera kimikoa (Verheijen et al., 2009), eta lurzorua eta biotaren artean ematen diren elkarreraginak direla eta (adib., Anderson et al., 2011), baita sortu daitezkeen konplexu organo-mineralak direla medio (Fernández-Ugalde et al., 2017). Bussek eta lankideek (2019) iradoki zuten BCa WAa baino ongarri hobea zela, BCetik Ka neurriz hornitzen baitzen eta WAetik K-a berehala lixibiatzen baitzen. Potasioak errautsetan gatz eta oxido oso disolbagarriak eratzen ditu (Steenari et al., 1999), beraz, euriarekin berehala garbitzen dira (Nieminen et al., 2005).

Josephek eta lankideek (2022) egindako ikerketa berri batetan, non Kanadan zeharreko hainbat tokitan lurzoru-kalitateko zenbait aldagaitan WAa aplikatzeak (20 $t \cdot ha^{-1}$ arte) zuen eragina aztertu zuten, hobekuntza txiki batzuk edo efekturik ez zegoela ikusi zuten. Errauts dosirik handienak ES-Oko lurzoruaren pHa handitu zuen, baina ez genuen landarediaren hazkuntzan edo nutrizio-egoeran inolako eraginik ikusi. Errautsa baso epeletako lurzoruak kareztatzeko substantzia soil gisa hartu arren (Augusto et al., 2008), WA kantitate handiak aplikatu behar dira *Typic Dystrudept*a bezalako lurzoruetako geruza mineraletan edozein pH aldaketa detektatzeko. Honek zalantzan jartzen du euri asko egiten duen baso epeletarako WAaren eraginkortasuna kareztatzaile gisa. Hala ere, WAaren aplikazioak, batez ere azaleko geruzetan du pHan eragina, eta sakonerazko efektua dosiaren menpekoa da (Jacobson et al., 2004; Hansen et al., 2017b). Emendakinen eragina geruza organikoetan aztertuko duten ikerketa gehiago behar dira; izan ere, horizonte hau da aplikatutako substantziarekin kontaktu zuzenean dagoena, eta aldaketa oso lokalizatuek ekosistemaren funtzionamendu orokorrean izan dezakete eragina (Hansen et al., 2017b). Materia organikoaren mineralizazioa eta DOCaren lixibiazioa, besteak beste, lurzoruaren pH-aren bidez kontrolatzen dira-eta.

6.4.Errautsaren rola karbonoaren bahiketan

WAaren aplikazioak ez zuen SOC kopurua handitu, nahiz eta ikatz-proportzioa handia izan (%31 C). Errauts honen H/Corg proportzioa 0,5 baino handiagoa zen, BCarena ez bezala. Horrek esan nahi du ez dela hain iraunkorra eta errazagoa dela organismo deskonposatzaileek erabiltzea (Lehmann et al., 2021). Beraz, posiblea da deskonposaketa WAren baitako ikatzaren deskonposaketa horizonte organikoan eman izana; izan ere, gainazaleko geruza hori da errautsarekin kontaktu zuzenean egon dena eta beste ikerketa batzuetan errautsarekiko erantzun nabarmenenak erakutsi dituen geruza (Joseph et al., 2022). Josephek eta lankideek (2022), Kanadako eremu geografikoan zehar burututako zortzi azterketetan, ez zuten ikusi WAa aplikatzeak SOM edukian eta izaeran ondorio sendorik, aztertutako horizonte bakar baten ere (organikoak zein mineralak); WAaren aplikazioaren ondoriozko erantzun orokorrik eman ez zela agerian utziz. Hala ere, Augustok eta lankideek (2022) gure ikerketa eremutik gertu (baldintza klimatologiko berdintsuak) burututako 11 urteko esperimentuan ikusi zuten pinu landaketa bateko orbel-geruzako materia organikoaren edukia murriztu zela WAren aplikazioaren ondorioz. ES-Kn errautsaren ondorioz C-aren eduki totala aldatu ez bada ere, GRSP edukia handitu egin da WAaren tarteko dosia aplikatuta, SOMaren dinamikan aldaketak iradokiz. GRSPa SOMaren frakzio termoegonkortzat hartzen da, eta beraz, izan daiteke horrela SOCa lurzoruan luzaro mantentzea, lurzoruan denbora gehiago ematen duelako. GRSParen rekaltzitrantzia-indizea (karbono-alkiloaren eta aromatikoaren baturaren proportzioa, karbono-O-alkilo eta -karboxilo guztiekiko) gutxi gorabehera, SOMaren bikoitza da (Zhang et al., 2017). Agnihotrik eta lankideek (2022) aipatu zuten GRSP iraupena gutxi gorabehera 50-60 urtekoa dela. Hala ere, % 2-4a N duenez, mineralizazioa jasateko joera du lurzoruko N-aren muga murrizten duelako (Treseder eta Turner, 2007), hala ere, inkubazio-esperimentuetan emaitza kontraesankorrak erakusten ditu (Agnihotri et al., 2022).

Gainera, eta ikerketa askoren kontrara (adib. Agnihotri et al., 2022), ez dugu ikusi lurzoru-agregatuen eraketa handitzen denik, ez eta GRSP edukiarekin lotutako egonkortasunaren handipenik ere; beraz, ezin dugu GRSPek bultzatutako SOMaren babes fisikorik baieztatu. Ikerketa gehiago behar dira lurzoruan duten funtzioa azaltzeko, baleukate; izan ere, SOMen frakzio interesgarria izaten jarraitzen du eta SOMaren dinamikan izandako aldaketen adierazle bada.

Landarediaren hazkundeari dagokionez, ez dugu aldaketarik hauteman nutrizio-egoeran, ez eta biomasaren hazkundean ere. Reidek eta watmough-ek (2014) egindako meta-analisian jakinarazi zuten, saiakuntzen %33ak bakarrik, gutxi gorabehera, adierazi zutela hazkunde positiboa. Azken hauek, hazkundean ikusitako erantzun mugatua literaturan dauden epe-luzeko ikerketa kopuru mugatuari egotzi zioten, besteak beste, beste zenbait ikerlariekin bat eginez (Vance 1996; Pitman 2006). Halaber, zuhaitzen hazkundearen erantzuna espezieen araberakoa dela adierazi zuten Kimek eta lankideek (2022). Orain arte, WAa gehitzearen eragina koniferoen hazkuntzan aztertu da, gehien bat. Hala, *Pinus banksiana* bezalako espezieak WArekin tratatzeko oso egokiak izan badaitezke ere, beste batzuk, izei beltza kasu, ez dira. Azken honek hazkunde negatiboko erantzuna eman zion WAaren aplikazio-tasak handitzeari (Emilson et al., 2020). Augustok eta lankideek (2022) landaketa oligotrofikoetan egindako esperimentuan ikusi zuten WArekin tratatutako zuhaitzak tratatu gabekoak baino azkarrago hazi zirela. Hala ere, C-a gordetzeari dagokionez, eztabaidatu zuten bezala, WAa aplikatzeak zuhaitzen hazkundean izandako eragin positiboa ez zen orbel-geruzan eman zen materia organikoaren gutxitzearekin konpentsatzen.

Nahiz eta nutrizioa eta toxikotasuna funtsezko faktoreak diren landareen hazkuntza mugatzeko, biomasaren hazkundean urte batetik bestera emandako gorabeherek iradokitzen dute gure ikerketa-eremuetan ur-eskuragarritasuna mantenugaiak baino mugatzaileagoa dela; izan ere, ES-Kko biomasan (bai hostoetan bai eta oihanpean ere) ikusitako hazkuntza (5.2. irudia) urterik urte, bat dator, argi eta garbi, urteko prezipitazioekin eta hauen gorabeherekin: 978 mm 2012an, 1413 mm 2013an, eta 945 mm 2014 ([<10km], EUSKALMET 2012, 2013 and 2014).

Onartua da, ekosistema-mota askotan prezipitazioaren eta ekoizpen primario garbiaren arteko erlazio lineal bat existitzen dela (Felton et al.,ç 2021; Sala et al., 2012). Ebidentziak iradokitzen du, halaber, uraren mugaren magnitude orokorra handitzen ari dela (Babst et al., 2019), tenperaturen igoerak bultzatuta, zeinak ere lehorteen magnitudea handitu egingo duen lurruntze atmosferikoaren eskariaren handitzea dela-eta (Allen et l., 2015; Williams et al., 2020). Hala, aurreikusten da uraren eskuragarritasunak gero eta eragin handiagoa izango duela, XXI. mendean zehar, lurreko ekosistemen funtzionamenduan, eta bereziki, ekoizpen primario garbian (Green et al., 2019).

6.5.Balizko arriskuak eta aurrera begirako ikerketak

6.5.1.Balizko toxizitatea

Aztertutako bi baso-landaketetan eta aztertutako denboran, ez dirudi gehitutako emendakinak toxikoak edota kaltegarriak direnik. Hala adierazten dute poro-uren laginekin egindako MARA eta LumiMARA probek, nahiz eta *Typic udorthent*ean BC dosirik txikienak [BC(L) tratamenduak] poro-uretan Mn maila handitu zuen.

Lurzoruan emendakinak bota ondoren gertatzen diren elkarrekintzen konplexutasunaren beste adibide bat da ES-Kko oihanpeko biomasak xurgatutako Ni kantitatea BC(I)N aplikatutako partzeletan. Tratamenduen artean, BC(I)Nk, WA(I)k eta BC(I)k baino Ni gutxiago baldin badu ere, ES-Kn BC(I)N da Ni-a kopuru handiagotan xurgatzen laguntzen duen tratamendu bakarra. Behatutako lurzoruaren pH-aren igoeragatik izan daiteke hori. Ni-aren xurgapena pH-a 5era igotzean handitzen baita (Pandaa et al., 2007). Lurzoruan ikusitako Mg, Cd eta K-aren edukiaren gutxitzearekin ere lotuta egon daiteke; izan ere hauek Ni^{2+}-aren xurgatzean duten inhibizio-efektua desagertuko litzateke, horrela, Ni-a sustraietatik kimuetara translokatzea baimenduz (Temp, 1991); edo, agian, arestian aipatutako arrazoien elkarrekintzaren ondorio izan daiteke. Ikerketa sakonagoak behar dira Ni bidezko toxikotasuna ekiditeko, izan ere, Ni-a mugikortasun handiko metal bat da (Yusuf et al., 2011), sortu berri diren landare-ataletan eta hazietan metatzeko joera duena. Landareek Ni-a xurgatzeko eta metatzeko duten gaitasuna landare-espezieen, lurzoruaren pHaren, eta lurzoruan dagoen Ni kopuruaren eta formaren araberakoa da (Hassan et al., 2019). Oraindik ere ikerketa sakonagoa behar da ematen diren prozesu guztiak ulertzeko; izan ere, zenbait metal astuni ez bezala (adib. Cd, Pb, Hg, Cu eta Cr), Ni-ari arreta gutxi jarri diote zientzialariek, elementu duala baita, hau da, esentziala eta toxikoa, eta kimika elektroniko konplexua dauka, eta horrek zailtzen du landareetan duen toxikotasun-mekanismoa ulertzea (Yusuf et al., 2011).

6.5.2.Aurrera begirako ikerketak

Errautsa aplikatu eta 30 hilabetera, lurzoruko propietate hidraulikoetan ikusitako ondorio negatiboek, txikiak badira ere, ohartarazten gaituzte, besteak beste, lekuaren, aplikatutako dosiaren eta nitrogenoa gehitzearen mende dauden interakzioen konplexutasunaz. Dosiaren arabera erantzun desberdinak eman direla kontuan hartuta, eta beste autore askorekin bat eginez (Emilson et al., 2020; Clapham eta Zibilske, 1992; Demeyer et al., 2001; Park et al., 2012), epe-luzeko ikerketa berriak egin behar dira, lurzoruaren eta komunitate biologikoaren propietate fisiko-kimikoetan ematen diren aldaketak eta elkarreraginak ezagutzeko.

Geruza organikoetan ere fokoa ipintzea beharrezkoa da, hau bezalako azterketak osatzeko; izan ere, orbel-geruzak dira emendakinekin kontaktu zuzenean egoten diren horizonteak, eta aldaketa lokalizatu eta txikiek eragina izan dezakete ekosistemaren funtzionamendu orokorrean (Hansen et al., 2017 b).

Lurzoru-agregatuak, lurzoruaren egituraren oinarrizko unitateak, lurzoruan gertatzen diren prozesu eta elkarreraginen emaitza dira, bai eta hauen eragile mugatzaileak ere. Ur-, gas- eta mantenugai-fluxuak mugatzen dituzte, besteak beste, bai eta lurpeko bizidunen arteko harremanak ere: sare trofikoa zehazten dute. Landa-agregatuen tamainaren banaketan ikusitako joerek MWDf-a lurzoruaren funtzionamenduarekin lotuta egon daitekeela iradokitzen dute. Lurzoruaren egituraketa zehazten duten elkarrekintzak ulertzeko, eskala mikroan ematen diren lurzoruaren eta biotaren arteko elkarreragina eta konplexutasuna integratzen dituzten agregazio-analisiak dira beharrezkoak dira. Lurzoru-biotak lurzoruaren formazioan eta agregazioan, bai eta lurzoruaren propietate hidraulikoetan nola eragiten duen jakitea lan multifazetikoa da, eta lotura trofikoak eta elikadura-sare konplexuak barne bildu behar ditu ezinbestean (Brose eta Scheu, 2014)

7. ONDORIOAK (EU)

- Egur errautsa edota biocharra aplikatzeak ez du sistematikoki eragin positiborik. Gure aurkikuntzek berresten dute baso heze eta epeletako lurzoruei biocharra eta errautsa aplikatzeak hainbat modutan eragiten duela, lurzoru-motaren eta sistemaren arabera, baita aplikatutako dosiaren arabera eta nitrogenoa ere gehitu baldin bazen ala ez.
- Errautsak lurzoruaren propietate hidraulikoetan izandako eragin negatiboa, eragin apala bada ere, zalantzan jartzen du errautsek klima-aldaketari egokitzeko ongarri gisa izan dezakeen baliagarritasuna. Segurtasunez aplikatu ahal izateko, inolako aplikaziorik egin aurretik, lekuaren berariazko ikerketaren beharra dagoela adierazten du.
- Biocharra ongarri interesgarria izan liteke K eskasia duten basoetan, baina dosi-efektuak tentua eskatzen du. Izan ere, gure emaitzek ez dute erakusten landareek, dosia handitu ahala, K gehiago xurgatzen dutenik . Biochar dosirik altuenaren aplikazioak *Typic Distrudept*aren sisteman aldaketak eragin dituela ematen du; lurzoruaren gainazalean geruza berri bat eratuz edo lurzoruaren propietate fisiko kimikoetan luzarorako aldaketak eraginez, adibidez. Badirudi K modu eraginkorragoan erabiltzera egokitutako sistema dela ES-Okoa. Sistema naturaletan ematen diren elkarreraginak konplexuak dira, eta lurzoruaren biotak paper erabakigarria du.
- Biocharra aplikatzeak klima-aldaketaren mitigazioan lagun lezake lurzoruan karbono organikoa gordetzen lagunduz. Beti ere, lurzoru mineralaren geruzetan emandako karbono organikoaren inkorporazio- eta egonkortze-tasa lurzoruaren geruza organikoan gertatzen den deskonposizio-tasa baino handiagoa bada.

Beraz, humus-geruzak aintzat hartzen dituzten azterketak beharrezkoak dira azken hipotesi hau baieztatzeko. Hala ere, *Typic Distrudept*eko karbono organiko erreserbak handitu dituen biochar-tasak ez dira errentagarriak praktikan Euskal Autonomia Erkidegoko Atlantiar isurialdeko baso landaketetan; izan ere, landaketetarainoko garraioa eta eskuz botatzea garestiegia izan daiteke.

- Aztertutako bi baso-landaketetan eta aztertutako denboran, ez dirudi gehitutako emendakinak toxikoak edota kaltegarriak direnik. Hala adierazten dute poro-uren laginekin egindako MARA eta LumiMARA probek, nahiz eta *Typic Udorthent*-ean nitrogenodun Biochar tratamenduak oihanpeko landareek xurgatutako Ni kopurua handitu zuen, eta biochar dosirik txikienak poro-uretan Mn maila handitu zuen. Honek agerian uzten du kontuz ibili behar dela eta tokian tokiko ikerketa luzeagoak beharrezkoak direla.
- Hiru urteko esperimentu hau ez da behar bezain luzea egiturazko aldaketarik eman den ikusteko. Hala nola, lurzoruko karbono-erreserbaren emendioak bere horretan jarraituko duen ikusteko, edo pH-aren, mantenugaien, lurzoruaren karbono organikoaren eta lurzoru-egituraren aldaketek klima-aldaketan ondorio positiboak izango ote dituzten ikusteko. Garrantzitsua da landa-azterketa luzeak eta egokiak egitea, errautsak zein biocharrak ekosistema osoan kalterik eragiten ez dutela ziurtatzeko, eta hortaz, klima-aldaketara egokitzeko estrategietan, modu seguruan, erabil daitezkeen zehazteko.

7. CONCLUSIONS (EN)

- Wood ash or biochar amendments do not systematically produce positive effects. Our findings confirm that the addition of biochar and wood ash to temperate humid forest soils affects different sites in different ways, depending on the soil type and system, as well as on application rates and whether nitrogen was also added.
- The small negative effects observed after wood ash application on soil hydraulic properties raises concern about its usefulness as a soil enhancer to adapt to climate change, and indicates the requirement for site specific research before safe application.
- Biochar could be an interesting fertilizing agent in K deficient forests, but dosage effect requires attention. Our findings did not show a proportional increase in K uptake by plants to increasing dose. The highest doses of biochar applied to the *Typic Dystrudept* may have resulted in formation of a thick amendment layer on top of the soil, or may have prolonged changed the physicochemical properties of the soil, affecting the system in ES-O, which seems to be adapted to make use of K more efficiently. Interactions in the field are complex, and soil biota plays a crucial and determining role.
- Biochar application could contribute to mitigate climate change via sequestration of soil organic carbon, the incorporation and stabilization rate of soil organic carbon observed in the mineral soil layers is higher than the decomposition rate occurring in the organic layer of the soil. Therefore, longer-term studies incorporating the humus layers are required to confirm these hypotheses. However, the biochar rates that increased soil organic carbon stocks

of the *Typic Dystrudept* are not cost effective in practice, at least in the Atlantic forest plantations in the Basque Country, since transportation costs to the stands and application by hand might be expensive.

- The amendments applied do not seem to be toxic or harmful in the two types of forest plantations under study, as revealed by MARA and LumiMARA testing with pore water samples, although biochar in combination with nitrogen application increased Ni uptake by understorey plants and the increased Mn level in pore waters of the *Typic Udorthent* treated with the lowest dose of biochar suggest that care should be taken and needs for longer-term and site-specific research.
- This three-year experiment is not long enough to see any structural change. For example, to see if the augmented soil carbon stock will persist, or if the changes in pH, nutrients, soil organic carbon and soil structure will derive in positive effects facing climate change. It is important to conduct appropriate long-term field experiments to determine whether by-products can be used in climate change adaptation strategies, as well as to ensure that substances of this nature are not harmful to the entire ecosystems, as this is essential for the safe application of biochar or wood ash to soil.

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ABBREVIATIONS and Acronyms

AS	Aggregate stability
AWC	Available water content
BC	Biochar
C	Carbon
CC	Climate Change
CDR	Carbon Dioxide Removal
DOC	Dissolved Organic Carbon
ES	Experimental site
ES-K	Experimental site of Karrantza
ES-O	Experimental site of Oiz
GRSP	Glomalin related soil proteins
	GRSP_{EE} Easily extracted soil protein fraction
	GRSP_{T} “Totally” extracted soil protein fraction
GWC	Gravitational Water Content
HWC	Hygroscopic water content
Ks	Saturated hydraulic conductivity
Magg	Macroaggregates
	LMagg Large Macroaggregates
	SMagg Small macroaggregates
magg	Microaggregates
MARA	Microbial Asssay for Risk Assessment
MWD	Mean Weight Diameter
	MWDf, ‘f’ refers to field samples
	MWDs, ‘s’ refers to stability test samples
NET	Negative Emission Technologies
SOC	Soil organic carbon
SOM	Soil organic matter
SWRC	Soil water retention curves
WA	Wood Ash

LABURDURAK, siglak eta akronimoak

AE	Agregatuen egonkortasuna
AWC	Ur eduki eskuragarria
BC	Biocharra
C	Karbonoa
CC	Aldaketa klimatikoa
CDR	Karbono dioxidoaren erauztea
DOC	Disolbatutako Karbono Organikoa
ES	Ikerketa-eremua
ES-K	Karrantzako Ikerketa eremua
ES-O	Oizeko ikerketa eremua
GRSP	Glomalinarekin lotutako lurzoru-proteinak
	GRSP$_{EE}$ Errez erauzitako lurzoru-proteinak
	GRSPT Erauzitako proteina kopuru totala
GWC	Ur eduki askea edo grabitazionala
HWC	Ur eduki higroskopikoa edota erresiduala
Ks	Saturatutako laginen konduktibitate hidraulkikoa$
LMagg	Makroagregatu handiak
Magg	Makroagregatuak
magg	mikroagregatuak
MARA	Arriskua ebaluatzeko entsegu mikrobiologikoa
MWD	Agregatuen batezbesteko tamaina
	MWDf, ‘f’- k zuzenean hartutako laginei egiten die erreferentzia “field”
	MWDs ‘s’-k estabilitate testa aplikatu ondorengo agregatuen banaketari
NET	Emisio negatiboetarako teknologiak
SMagg	Makroagregatu txikiak
SOC	Lurzoruko karbono organikoa
SOM	Lurzoruko materia organikoa
SWRC	Lurzoruko uraren atxikimendu kurbak
WA	Errautsa

Appendix I

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Effects of biochar and wood ash on soil hydraulic properties: A field experiment involving contrasting temperate soils

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ABSTRACT

The application to soils of energy co-products derived from forest biomass (biochar [BC] and wood ash [WA]) with the aim of regulating soil hydraulic conductivity and water availability, thereby reducing soil erosion and increasing resilience to drought, has been suggested as a strategy for climate change mitigation and adaptation. The main objective of this study was to investigate the effects of BC and WA application on the hydraulic properties of contrasting afforested soils in the Atlantic region of the Iberian Peninsula. Two experimental sites were established on acidic soils: site ES-K was established on a loamy soil (SOC% 3.9; pH: 4.8) and site ES-O on a sandy loam soil (SOC% 10.8; pH: 3.8). Biochar derived from *Miscanthus* sp. (pyrolysed at 450 °C: containing 87% C) was applied at rates of 0, 3.5 and 10 Mg ha^{-1} to soil in ES-K and at rates of 0, 10 and 20 Mg ha^{-1} to soil in ES-O. Pine WA (30% C) was applied at rates of 0, 1.5 and 4.5 Mg ha^{-1} to ES-K, and at rates of 0, 4.5 and 9 Mg ha^{-1} to ES-O. Nitrogen- enriched (0.8% N) BC and WA were also applied at rates of respectively 10 Mg ha^{-1} and 4.5 Mg ha^{-1} in both experimental sites. Bulk density, saturated hydraulic conductivity (K_s), porosity and aggregate size distribution were determined and soil water retention curves (SWRCs) constructed. In ES-K, application of N-enriched WA (4.5 Mg ha^{-1}) led to alterations in the SWRCs and reduced the available water capacity (AWC) by 11.5%; the lowest dose of WA (1.5 Mg ha^{-1}) reduced K_s due to pore-clogging. In ES-O, changes were observed in the soil structure after application of BC (20 Mg ha^{-1}) and WA (9 Mg ha^{-1}) as well as after application of the N-enriched materials. However, no effects on available water content or saturated hydraulic conductivity were observed fifteen months after the treatments. Further field research is required to determine the soil specific, long-lasting effects of BC and WA on soil structure and soil hydraulic properties.

1. Introduction

Climate Change (CC) projections remain quite uncertain. However, southern Europe is expected to be affected by more irregular rainfall and longer dry periods in summer (IPCC, 2014). Such events would magnify the risk of drought and soil erosion, thus limiting forest growth and many other ecosystem services provided by forests. New management strategies are therefore needed to increase the resistance and adaptation of forest systems to CC and to maintain ecosystem functions such as water storage and biomass production.

In this context, the application to soil of energy co-products derived from forest biomass has been suggested as a CC mitigation and adaptation strategy (Lehmann and Joseph, 2009; Omil et al., 2011) that may also enable the targets of the Europe 2020 growth strategy (European Commission, 2010) to be met in relation to CC and energy sustainability. The energy stored in biomass can be released through combustion and used as a substitute for fossil fuels to generate energy; the wood ash produced during the process can also be returned to the soil to replace the nutrients exported by harvesting (Demeyer et al., 2001; Pitman, 2006). Biomass can also be pyrolysed for use in this circular economy framework. Pyrolysis of biomass yields a carbon-rich product called biochar that can increase carbon sequestration in soils and improve soil properties (Lehmann and Joseph, 2009; Sohi, 2012). The incorporation of wood-ash or biochar into soil may increase the resistance of soils to flooding and drought in forest ecosystems.

Soil water retention in combination with saturated hydraulic conductivity governs the rate of water flow through soils and determines the vulnerability of soil surface run-off. Several authors have reported that the application of ash to soils has positive effects on soil hydraulic properties, such as increased water holding capacity in coarse textured ash-amended soils (Pathan et al., 2003; Stoof et al., 2010), as well as improved water infiltration and root growth (Yunusa et al., 2006). Negative effects have also been observed, especially in wildfire-related studies, in which e.g. entrapped ash has been reported to cause pore

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clogging (Pitman, 2006; Woods and Balfour, 2010). Although studies of the effects of wood ash amendment on soil physical and hydraulic properties are scarce, Bodí et al. (2014) reviewed several studies conducted on burned ecosystems. Most research concerning wood ash application to forest soils have focused on soil chemical and nutritional status (Augusto et al., 2008; Demeyer et al., 2001; Pitman, 2006). The use of biochar to modify soil hydraulic properties has also been investigated (Ajayi et al., 2016; Jeffery et al., 2015). Some authors conclude that biochar may cause clay-rich soils to drain more rapidly and sandy soils to drain more slowly (Barnes et al., 2014; Hardie et al., 2014). Other authors concluded that biochar application improves soil water retention (Basso et al., 2013), especially in tropical soils (i.e. highly weathered, coarse-textured, acidic soils; Glaser et al., 2002). The effect is mainly attributed to higher porosity, surface area and sorption capacity relative to other types of soil organic matter (SOM) (Downie et al., 2009; Glaser et al., 2002). Yet other authors reported no consistent direct improvement derived from biochar application (Carvalho et al., 2014), or simply no effect (Jeffery et al., 2015). The above-mentioned findings clearly indicate that the effects of biochar or wood-ash on soil hydraulic properties depend on the type of soil. Soil hydraulic properties are important in determining the partitioning of precipitation between infiltration and overland flow, which affects water storage in the subsurface soil and thus plant available water. Understanding how both biochar and wood-ash influence the hydraulic properties of different soil types is essential. However, the use of sieved repacked soils in the study of soil physical characteristics is of particular concern. In trials with repacked soils, the soil structure, pore architecture and pore size distribution (and therefore field capacity, available water content, infiltration, hydraulic conductivity and drainable porosity values) are artefacts of the sieving and repacking processes and bear little resemblance to in situ soil properties. Studies conducted under field conditions are therefore needed in order to improve our understanding of this topic. Nonetheless, the findings may be highly variable, particularly in forest soils (Hendrayanto et al., 1999), which contain rock fragments, root systems and living organisms, as well as organic matter at different stages of decomposition (Ilek and Kucza, 2014).

If intense rainfall occurs in the future, soils with high hydraulic conductivity will be more capable of reducing run-off, erosion and field waterlogging. Moreover, if water availability is reduced in the future, soils with high water holding capacity will be more resilient. Considering these possibilities, the main objective of this study was to determine how the addition of a biochar or wood ash affected the hydraulic properties of two contrasting afforested soils.

2. Materials and methods

2.1. Study sites and experimental design

This study was conducted in experimental sites established in two forest plantations in the Atlantic region of the Iberian Peninsula. The sites mainly differed in relation to soil type (Table 1). The Karrantza experimental site (ES-K) was established in May 2012 in the eastern side of the Karrantza valley (UTM 30N ETRS 89_ 475081, 4786389) at 280 m a.s.l. This site comprises a *Pinus radiata* D. Don stand seed orchard established in 1996 with a planting distance of 6 × 6 m. The mean annual temperature was around 12 °C and mean annual precipitation around 1200 mm. Four replicates of each treatment (Table 2) were applied in individual plots (8 × 8 m) each including at least three pine trees. The second field trial was established one year later, in August 2013, in the Oiz experimental site (ES-O), on the southern slope of the Oiz Mountains (UTM 30N ETRS 89_ 532673, 4785,572) at 760 m a.s.l. The trial was established in a restored tree plantation of *Quercus pyrenaica* Willd. planted in 2012. The mean annual temperature was around 10 °C and mean annual precipitation around 1100 mm (Euskalmet, 2013). The wood ash and biochar were also applied at higher rates in ES-O (Table 2). Four replicates of each treatment were applied in 3 × 3 m plots, with a minimum buffer distance of 1 m between each. The experimental set-up is summarised in Table 2. Biochar and wood-ash were applied by top-dressing, i.e. by spreading the product on to the soil surface. It was assumed that the products would then be incorporated into the topsoil by natural processes.

Table 1
General characterization of the soil in each experimental site (ES-K and ES-O). Data show means and standard deviation in parenthesis, of different parameters at each experimental site.

	ES-K	ES-O
Soil class (Soil Survey Staff, 2014)	Typic Udorthent	Typic Dystrudept
Texture	Loam	Sandy loam
Sand (%)	36.8 (0.5)	67.9 (4.6)
Silt (%)	39.7 (1.2)	23.8 (1.9)
Clay (%)	23.1 (0.3)	8.3 (1.0)
SOC (%)	3.9 (0.4)	10.8 (3.3)
C:N	14.4 (1.2)	16.8 (1.6)
pH-H_2O	4.8 (0.2)	3.8 (0.01)

SOC: Soil organic carbon; C:N carbon and nitrogen ratio. Texture was measured by laser diffractometry, and total carbon and total nitrogen by a LECO TruSPEC® CHN-S elemental analyser. The TC provided a measure of SOC, considering TC equal to total organic carbon by the absence of carbonates in both soils.

Table 2
Schematic description of the experimental design. Environmental characteristics of each experimental site and treatments (type of amendment, treatment details and code).

Site	Amendment	Treatment	Code
	No addition	Control	Ctrl
ES-K			
P. radiata D. Don.	Biochar	10 Mg biochar ha^{-1} + 0.8% of N	BC(I)N
(20 yr)		10 Mg biochar ha^{-1}	BC(I)
Loamy texture		3.5 Mg biochar ha^{-1} + 0.8% of N	BC(L)
	Wood ash	4.5 Mg wood ash ha^{-1} + 0.8% of N	WA(I)N
		4.5 Mg wood ash ha^{-1}	WA(I)
		1.5 Mg wood ash ha^{-1}	WA(L)
ES-O			
Q. pyrenaica Willd.	Biochar	10 Mg biochar ha^{-1} + 0.8% of N	BC(I)N
(2 yr)		20 Mg biochar ha^{-1}	BC(H)
Sandy loam texture		10 Mg biochar ha^{-1}	BC(I)
	Wood Ash	4.5 Mg wood ash ha^{-1} + 0.8% of N	WA(I)N
		9 Mg wood ash ha^{-1}	WA(H)
		4.5 Mg wood ash ha^{-1}	WA(I)

BC: Biochar, WA: Wood ash; (L): Low dose, (I): intermediate dose, (H): high dose; N: nitrogen addition.

Biochar (BC) was produced by pyrolysis of *Miscanthus* sp. at 450 °C in a Pyreg® pyrolysis unit. The wood ash (WA) was produced by combustion of *Pinus radiata* D. Don harvest residues in a commercial boiler. The amounts of biochar and wood ash applied to the soil were equivalent in terms of the Calcium (Ca) content, considering a ratio of the Ca in WA to that in BC of 2.5. The calcium content of biochar and wood ash was balanced in order to generate similar liming effects as well as to equalize particle binding by Ca bridges and the clay flocculating potential (Wuddivira and Camps-Roach, 2007). The nitrogen (N) content of the enriched treatment for both co-products was the amount of ammonium nitrate relative to the 0.8% of the biochar applied. The main properties of the wood ash and biochar used in this study are shown in Table 3.

2.2. Soil sampling and processing

In each experimental site, 28 undisturbed soil cores (D = 53 mm, h = 50 mm) were sampled from the first 5 cm of soil profile (after

Appendix I

Table 3
Chemical composition of the amendments.

	P ($g\ kg^{-1}$)	Ca ($g\ kg^{-1}$)	Mg ($g\ kg^{-1}$)	K ($g\ kg^{-1}$)	Al ($g\ kg^{-1}$)	Cu ($mg\ kg^{-1}$)	Zn ($mg\ kg^{-1}$)	Fe ($g\ kg^{-1}$)	C (%)	C:N	H:C	pH-H_2O (1:5)
Biochar	1.58	18.81	1.92	12.18	2.02	13.5	67.8	2531.5	85.8	168.2	0.35	9.8
Wood ash	1.26	47.08	3.22	13.47	37.75	38.9	223.6	19,619.2	30.9	515	1.17	10.6

removal of the forest floor) in order to determine saturated hydraulic conductivity (K_s), bulk density and porosity for constructing SWRCs. The mean weight aggregate diameter (MWD) was also determined in undisturbed soil samples (10 × 10 cm turfs) taken from the top layer (0-5 cm). Core sampling was carried out thirty months after application of the treatments in ES-K and fifteen months after application in ES-O. Turf sampling was carried out twenty-four months later in both experimental sites.

Undisturbed field moist soil cores were stored at 4 °C. Turfs were gently crumbled by hand following the natural fissures, and the soil was then passed through a 20 mm sieve, air-dried and stored until analysis.

2.3. Soil hydraulic properties

Undisturbed field moist soil cores were prepared for saturation as described by Flint and Flint (2002) and placed in a bucket with a porous bottom, so that all pores were filled by capillary action without affecting the soil structure. The samples were weighed to determine the saturated gravimetric water content. Hydraulic properties were then determined following a sequential method, as follows:

2.3.1 Samples were placed in a water displacement device to determine the bulk density by the Archimedes' displacement method (Flint and Flint, 2002). The dry weight (needed to calculate bulk density) was determined after completion of the SWRC measurements.

2.3.2 The still saturated samples were then transferred to a laboratory constant head permeameter (Eijkelkamp Agrisearch equipment, Netherlands) to determine the saturated hydraulic conductivity (K_s) by measuring the difference in water levels and collecting the drained water in a burette during a fixed period of time when the water was flowing constantly.

2.3.3 Once the K_s was measured, the samples were transferred to a combination of different types of de-watering equipment to establish a wet to dry sequence with six soil-matric potentials for construction of SWRC. During the entire de-watering process, water content data was scored on a mass basis (gravimetric water content), by weighing the samples after equilibrium was reached at each matric potential. Sand and sand/kaolin boxes (Eijkelkamp Agrisearch equipment, Netherlands) were used for low-tension ranges (−1, −5 and −10 kPa), and pressure plate apparatus (Soilmoisture Equipment Corp., USA) was used for high-tension ranges (−33, −278 and −1500 kPa). Equilibrium was reached within 6–14 days for the low-tension range. The undisturbed soil cores were placed in pressure plate extractors for 11 days at 33 kPa. In the next step, the samples were gently extracted from the cores and cut from a vertical plane (following the vertical direction of the soil) into two disturbed subsamples (1/3 and 2/3 portions). The 1/3 portion was weighed and placed in an oven at 105 °C for 48 h. The 2/3 portion was also weighed and gently crumbled and homogenized to enable measurement of the lowest matrix potentials in pressure plate extractors. The disturbed subsamples were placed in rubber cores of length 1.5 cm, saturated by capillary action and pressure at −278 and −1500 kPa. Pressure was applied until no more water was extracted from the chambers (four days). The samples were then weighed and oven dried for 48 h at 105 °C.

The gravimetric water content at each matric potential was converted to volumetric water content on the basis of the volumes and bulk densities measured in each core. The potential water storage was calculated on the basis of different tension ranges: 0 to −10 kPa for gravitational water (GWC), −10 to −1500 kPa for available water content (AWC), and water content beyond −1500 kPa tensions for unavailable or hygroscopic water content (HWC). The total water content (TWC) was determined as the water content measured between 0 kPa and dryness at 105 °C.

2.4. Porosity and soil aggregate size distribution

2.4.1. Porosity

Total porosity was calculated using the data obtained by the bulk density measurement (Section 2.3.1), as described by Flint and Flint (2002), and expressed as absolute values. Pore size was estimated from the data on water desorption at different water potentials, according to the Young-Laplace equation (Tuller and Or, 2005).

The water content at each matric potential was used to estimate the absolute volume of water content in the different pore size classes and also the mean pore diameter (MPD). Pore size classes were established on a size basis, where macropores are defined as pores > 30 µm equivalent cylindrical diameter (ECD), mesopores as pores between 30 and 0.2 µm ECD, and micropores as pores < 0.2 µm ECD (Kay, 1990). The MPD is the sum of the proportional water content ($g{\cdot}g_{saturation}^{-1}$) remaining in the soil at each pressure applied, multiplied by the mean relative pore diameter (µm): $\Sigma(W_j . d_{pore(j)}/W_s)$, where j is the pressure application point, W_j is the water content remaining in the sample (g), $d_{pore\ (j)}$ is the mean diameter of the pore estimated from the radius in the above equation (µm), and W_s is the maximum water content that the soil can accumulate, at saturation (g).

2.4.2. Aggregate size distribution

The soil was fractionated into aggregates by a dry-sieving method. Briefly, 100 g of soil was crumbled gently by hand and placed in nested sieves mounted on a sieve shaker (Retsch AS200 Control: Retsch Technology, Düsseldorf, Germany). The soil was then processed as described by Gartzia-Bengoetxea et al. (2009) to separate it into the following field aggregate-size classes: 20–10, 10–5, 5–2 mm (mega-aggregates); 2.0–0.25 mm (macroaggregates); 0.25–0.053 mm (microaggregates); and < 0.053 mm (silt & clay size fraction). The test was carried out in triplicate and the values obtained were used to calculate the mean weight diameter (MWD) of the soil aggregates. The MWD of aggregates was calculated as an index that characterizes the structure of the bulk soil.

2.5. Data processing and statistical analysis

Four of the K_s values were excluded as they were considered very high ($> 36 cm\ h^{-1} = 1 \cdot 10^{-4}\ ms^{-1}$) (Soil Survey Division Staff, 1993), especially relative to preferential flows (Allaire et al., 2009).

The data were subjected to logarithmic transformation to correct for heteroscedasticity (see Supplementary data, Appendix A).

Linear mixed-effects models (LMMs) were fitted to investigate the effects of treatments (fixed effect) on each soil parameter, and each experimental site was analysed separately.

For each of the parameters, a specific LMM was constructed in R, by using the lme procedure in the 'nlme' package (Pinheiro et al., 2016), version 3.3.1. For model fitting, the parameters were estimated using the Restricted or Residual maximum likelihood (REML) estimator, although fixed effects were previously adjusted using the maximum

Table 4
Matrix synthetizing the results. Increment (+), reduction (−) and non-significant ($p > 0.1$) effects (ns) of treatments relative to control for each of the parameters analysed, in the two experimental sites: ES-K and ES-O.

	ES-K						ES-O					
	Biochar			Wood ash			Biochar			Wood ash		
	BC(I)	BC(I)N	BC(L)	WA(I)	WA(I)N	WA(L)	BC(H)	BC(I)	BC(I)N	WA(H)	WA(I)	WA(I)N
BD	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
K_s	ns	ns	ns	ns	ns	−	ns	ns	ns	ns	ns	ns
SWRC	ns	−	ns	ns	−	ns	ns	ns	ns	−	−	ns
AWC	ns	ns	ns	ns	−	ns	ns	ns	ns	ns	ns	ns
TWC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
GWC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	+
HWC	−	−	ns	ns	ns	ns	ns	ns	−	ns	ns	ns
Total porosity	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MPD	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Macrop	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Mesop	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	−
Microp	−	−	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MWD	ns	−	ns	ns	−	ns	+	ns	+	+	ns	+
Megagg	ns	ns	ns	ns	ns	ns	+	ns	ns	+	ns	ns
Magg	ns	ns	ns	ns	ns	ns	−	ns	ns	−	ns	ns
magg	ns	ns	ns	ns	ns	ns	−	ns	ns	ns	ns	ns
S + C	ns	ns	ns	ns	ns	ns	−	ns	ns	ns	ns	ns

BD: bulk density; Ks: saturated hydraulic conductivity; SWRC: Soil water retention curves; AWC: available water content; TWC: total water content; GWC: gravitational water content; RWC: residual water content; MPD: mean pore diameter; MWD: aggregate mean weight diameter; BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose, (L): low dose; N: nitrogen addition (0.8% of N).

likelihood (ML) estimator. Low AIC values (Akaike criterion) and the Bayesian information criterion were included as selection criteria. Details of the models are given in Appendix A.

Residual analysis was carried out to validate the model with a normal Q–Q plot of standardized residuals and with plots of the standardized residuals against predicted values.

Fisher's least significant difference (LSD) tests and contrast *t*-tests were performed to identify significant treatment effects and differences between treatments and controls (Appendix A). Differences were considered significant at $p < 0.1$, due to the high variability inherent in characterizing field experiments (Kuhn et al., 2014).

3. Results

The overall effects produced by the application of BC and WA in ES-K and in ES-O are summarised in Table 4. Significant increment (+), significant reduction (−) and non-significant effects (ns) relative to control for bulk density, hydraulic conductivity, soil water retention curves, the potential water storage, porosity and aggregation are shown.

3.1. Bulk density

No significant differences in bulk density were observed in relation to BC or WA application after either thirty months for site ES-K (Fig. 1a) or fifteen months for site ES-O (Fig. 1b).

3.2. Saturated hydraulic conductivity

The application of WA(L) in ES-K led to a significant decrease in the saturated hydraulic conductivity, to $3.06 \cdot 10^{-7}\,\mathrm{ms}^{-1}$ (IQR: $2.2 \cdot 10^{-7}$–$8.47 \cdot 10^{-7}\,\mathrm{ms}^{-1}$), while the rest of the treated plots remained similar to the control: $6.16 \cdot 10^{-6}\,\mathrm{m{\cdot}s}^{-1}$ (IQR: $3.66 \cdot 10^{-6}$–$8.66 \cdot 10^{-6}\,\mathrm{ms}^{-1}$) (Fig. 2a). In site ES-O, the median values of K_s were similar in treated and control plots ($4.05 \cdot 10^{-6}\,\mathrm{ms}^{-1}$, IQR: $3.06 \cdot 10^{-6}$-$8.66 \cdot 10^{-6}\,\mathrm{ms}^{-1}$) (Fig. 2b).

3.3. Soil water holding capacity

The SWRCs are shown in Fig. 3. Both biochar and wood-ash enriched with nitrogen (BC(I)N and WA(I)N) significantly altered the shape of the SWRC in ES-K (Fig. 3a and b), reducing water holding capacity at all pressures applied, relative to the control.

Application of WA(I)N led to a significant decrease in the available water content, of 11.5% relative to non-amended soils (Table 5) in the same site.

Application of BC(I) and BC(I)N in ES-K yielded significant reductions in the hygroscopic water content (Table 5).

In ES-O, the only significant effect of biochar application (a reduction in hygroscopic water content) was observed when BC(I)N was applied (Table 5). The SWRCs were affected by WA(I) and WA(H) application, which promoted a decrease in water holding capacity relative to the control (Fig. 3c and d). Nevertheless, this change did not affect the potential water storage. On the other hand, application of WA(I)N increased the gravimetric water content (Table 5) in this site.

3.4. Porosity

Microporosity in ES-K was significantly reduced in soils amended with BC(I) and BC(I)N (to respectively $1.50\,\mathrm{cm}^3$, IQR:1.33–1.63 and $1.36\,\mathrm{cm}^3$, IQR: 1.34–1.40) relative to the control ($1.92\,\mathrm{cm}^3$, IQR: 1.65–2.16). However, no significant differences were observed in either mean pore diameter (MPD) (80.5 μm, IQR: 55.8–100.4) or total porosity ($64.2\,\mathrm{cm}^3$, IQR: 61.1–67.6) 30 months after treatment application.

Addition of WA(I)N to ES-O significantly reduced the mesoporosity, from $45.4\,\mathrm{cm}^3$ (IQR: 74.0–53.3) to $33.6\,\mathrm{cm}^3$ (IQR:. 32.0–39.7), while MPD and total porosity of all amended plots were not significantly different from control (67.9 μm, IQR: 55.2–72.2 and $72.6\,\mathrm{cm}^3$, IQR: 69.9–75.5, respectively) fifteen months after application of the treatments.

3.5. Aggregate size distribution

The aggregate size distribution twenty-four months after treatment applications in both experimental sites is shown in Fig. 4. Application of BC(I)N and WA(I)N in ES-K, led to a significant reduction in the MWD

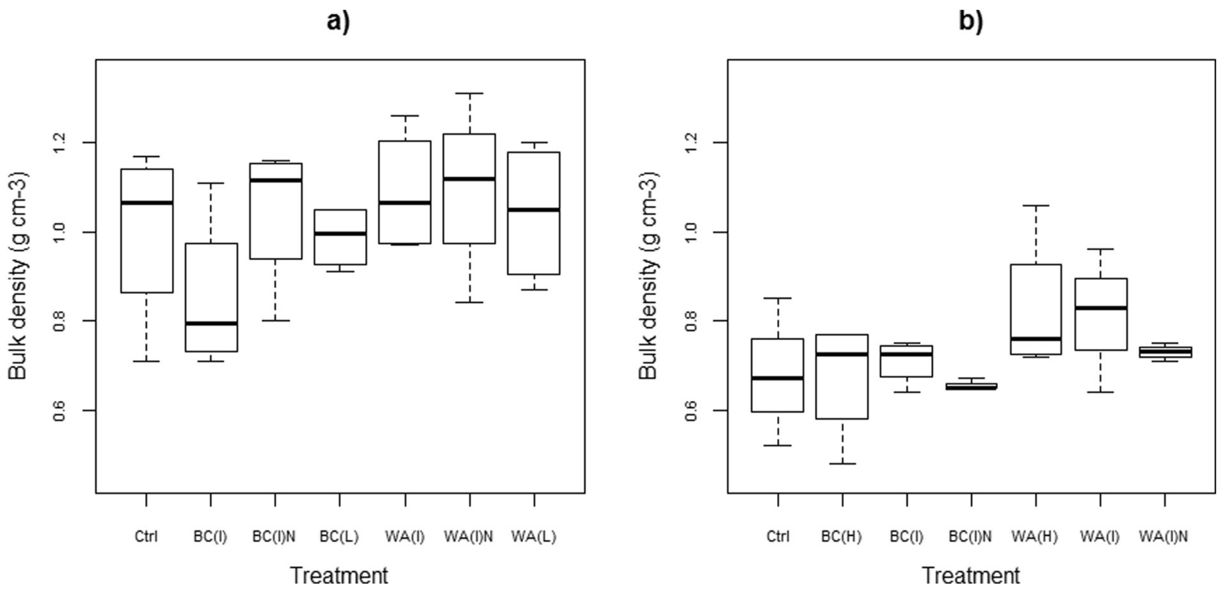

Fig. 1. Bulk density at each experimental site: a) ES-K (loam) and b) ES-O (sandy loam). Treatments: Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N. The horizontal line inside the box represents the median value.

of soil aggregates (to respectively 5.5, IQR: 0.9 and to 5.7 mm, IQR: 1.0) relative to the control (6.1 mm, IQR: 0.27) (Fig. 4a).

In site ES-O, the MWD of soil aggregates had increased significantly (to 5.03 mm, IQR: 0.14) twenty-four months after the addition of the BC(H), relative to control (3.56 mm, IQR: 0.345), mainly due to a significant increase in mega-aggregate formation, and a decrease in macroaggregates, microaggregates and silt-clay size aggregates formation (Fig.4b).

Increase in aggregate MWD was also yielded by WA(H) application (4.89 mm, IQR: 0.43), mainly due to a significant increase in mega-aggregates and a decrease in macroaggregates (Fig.4) in this site.

Nitrogen enriched treatments, BC(I)N and WA(I)N, led to increases in the MWD in ES-O (to respectively 4.53 mm, IQR: 0.34 and 4.87 mm, IQR:0.45), but no significant change in aggregate size distribution was observed.

4. Discussion

4.1. Effect of biochar on hydraulic properties

Improvements in soil physical properties of variable magnitude have commonly been observed in biochar amended soils (Abel et al., 2013; Ajayi et al., 2016; Asai et al., 2009; Case et al., 2012; Herath et al., 2013; Ibrahim et al., 2013; Laird et al., 2010). However, most studies highlighting the improvement brought about by biochar application were conducted using sieved repacked soils under controlled laboratory conditions. Our findings indicate that the effects of biochar application to soils in a temperate humid climate under field conditions depend on the soil type.

The changes in soil hydraulic properties thirty months after the application of biochar to the loamy Typic Udorthent (ES-K) depended on the application rate and whether or not nitrogen was added.

Application of biochar (10 Mg ha^{-1}) produced by pyrolysis of *Miscanthus* spp. at 450 °C and enriched with 0.8% nitrogen altered the shape of the SWRCs in this loamy soil, reducing water holding capacity

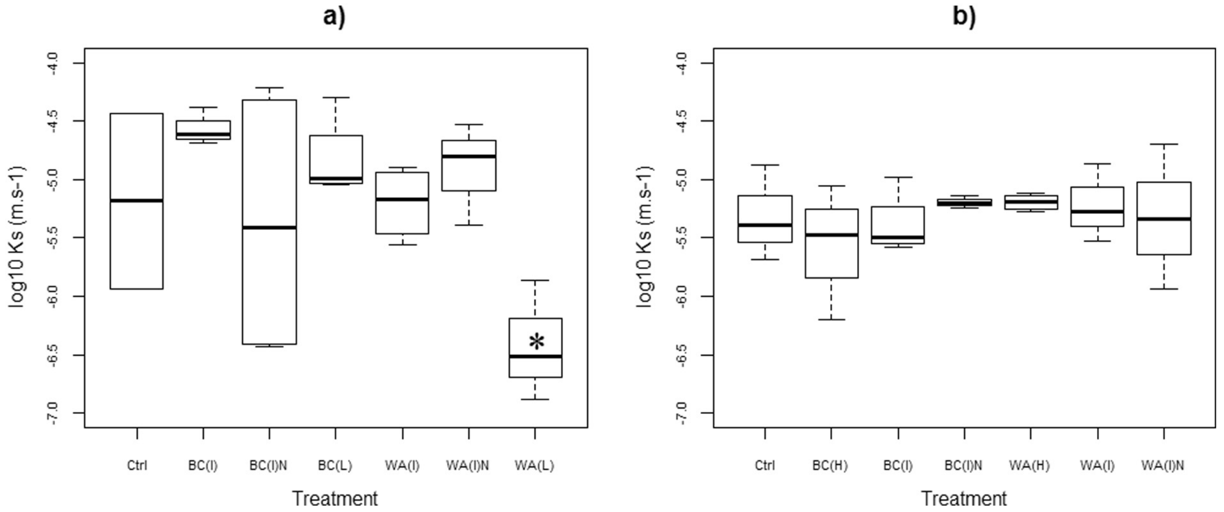

Fig. 2. Saturated hydraulic conductivity for each treatment (Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N) at each experimental site: a) ES-K (loam), and b) ES-O (sandy loam). Significant differences ($p < 0.1$) relative to the control are based on mixed effects models (Appendix A) and indicated by an asterisk. The horizontal line inside the box represents the median value.

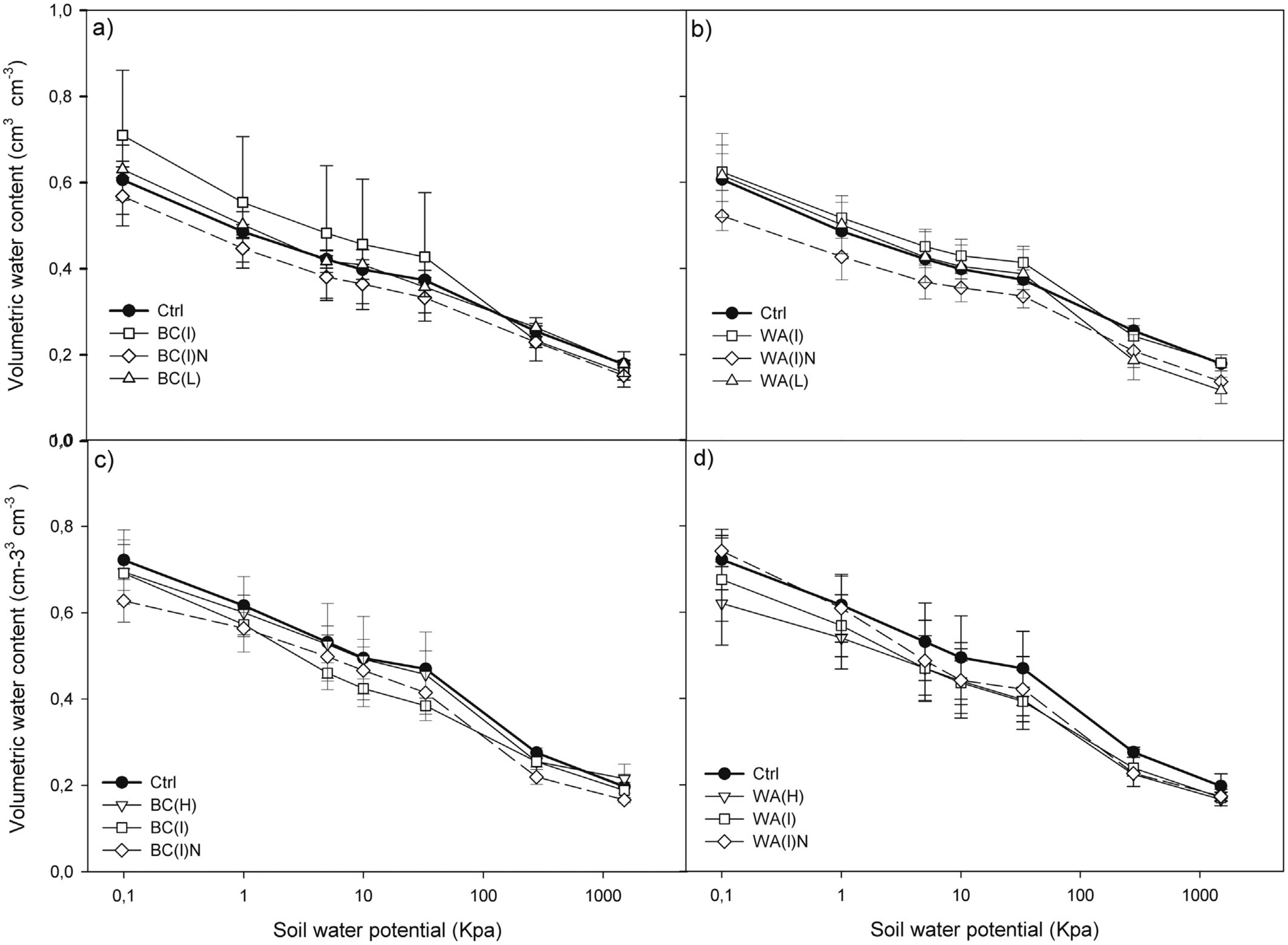

Fig. 3. Soil water retention curves (SWRC) based on volumetric water content measured by desorption at 1 kPa, 5 kPa, 10 kPa, 33 kPa, 278 kPa, 1500 kPa. a) biochar application to ES-K, b) wood-ash application to ES-K, c) biochar application to ES-O and d) wood-ash application to ES-O. Ctrl: Control, BC: Biochar, WA: Wood ash;(H): high dose, (I): intermediate dose and (L): low dose; N: 0.8% of N. Symbols represent mean values and error bars indicate standard deviation.

over the entire tension range. Verheijen et al. (2010) suggested that soil water retention is determined by the distribution and connectivity of pores in the soil medium, which is largely regulated by soil particle size (texture), in combination with structural characteristics (aggregation) and SOM content. Indeed, BC(I)N produced significant changes in soil structural characteristics, reducing the MWD of soil aggregates and the microporosity. The changes in soil structure may have led to a reduction in the slope of the SWRC. Previous laboratory-based studies have shown that addition of N-enriched biochar resulted in a change in the aggregate dynamics that did not allow the formation of large water stable macroaggregates in the short term, resulting in a lower MWD, probably due to the adsorption capacity of biochar (Moragues-Saitua et al., 2014). Dil et al. (2014) also observed in a pot study that a biochar pre-conditioned with urea ammonium nitrate increased maize crop production and suggested that the concentrated N solution may promote the mineralization of C-rich materials such as biochar and thus

Table 5
The potential volumetric soil water storage (in cm^3. median (IQR); $n = 3/4$) of AWC, TWC, GWC and HWC by treatment at each experimental site: ES-K and ES-O. Minimum significant differences ($p < 0.1$) over control are based in mixed effects models (Appendix), and indicated by an asterisk and in bold.

Treatment	AWC		TWC		GWC		HWC	
	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O
Ctrl	0.392 (0.014)	0.438 (0.092)	0.604 (0.096)	0.701 (0.062)	0.218 (0.133)	0.202 (0.055)	0.018 (0.004)	0.016 (0.003)
BC(H)	–	0.478 (0.064)	–	0.687 (0.073)	–	0.157 (0.063)	–	0.015 (0.003)
BC(I)	0.395 (0.138)	0.416 (0.050)	0.689 (0.161)	0.663 (0.049)	0.259 (0.082)	0.243 (0.100)	**0.015 (0.003)***	0.017 (0.001)
BC(I)N	0.358 (0.058)	0.430 (0.019)	0.537 (0.051)	0.593 (0.046)	0.159 (0.082)	0.167 (0.061)	**0.013 (0.000)***	**0.014 (0.004)***
BC(L)	0.394 (0.041)	–	0.622 (0.015)	–	0.215 (0.064)	–	0.013 (0.003)	–
WA(H)	–	0.433 (0.117)	–	0.633 (0.118)	–	0.189 (0.057)	–	0.016 (0.002)
WA(I)	0.420 (0.049)	0.468 (0.048)	0.634 (0.044)	0.663 (0.093)	0.184 (0.025)	0.179 (0.043)	0.017 (0.005)	0.014 (0.002)
WA(I)N	**0.347 (0.028)***	0.347 (0.079)	0.519 (0.035)	0.741 (0.032)	0.155 (0.039)	**0.326 (0.078)***	0.019 (0.002)	0.013 (0.002)
WA(L)	0.380 (0.041)	–	0.610 (0.151)	–	0.195 (0.089)	–	0.017 (0.003)	–

AWC: Available water content; TWC: Total water content; GWC: Gravitational water content; HWC: hygroscopic water content BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose, (L): low dose; N: nitrogen addition (0.8% of N).

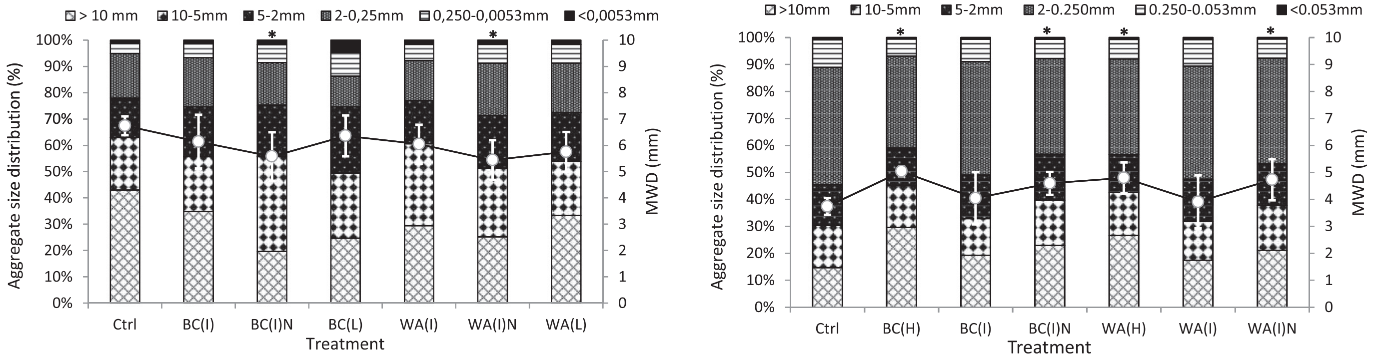

Fig. 4. Aggregate size distribution represented by bars (left of y axis) and mean weight diameter (MWD) represented by dots (right y axis) for both experimental sites: a) ES-K (loam), and b) ES-O (sandy loam). Ctrl: Control, BC: Biochar, WA: Wood ash; (H): high dose, (I): intermediate dose and (L): low dose; N = 0.8% of N. Significant differences ($p < 0.1$) for MWD relative to the control are based on mixed effects models (Appendix A) and indicated by an asterisk.

enhance plant nutrient availability and uptake. Greater nutrient availability may also alter the function and activity of the soil microbial community (Paul and Clark, 1996). Fungi and bacteria play well-known roles in soil aggregation (Martin and Waksman, 1940; Lynch and Bragg, 1985) and the quality of the organic matter added may influence their respective involvement. Fungi are considered to be the main decomposers of recalcitrant organic matter such as biochar with high C/N, whereas bacteria are thought to be more competitive regarding readily available organic matter with low C/N (De Boer et al., 2005). However, the interactive effects under field conditions of the biochar adsorption capacity, mineral N, activity and structure of soil microorganisms and soil physical properties such as aggregation, porosity and soil water retention remain unclear.

However, these structural changes were not reflected in soil hydraulic conductivity 30 months after treatment application. In contrast to the present findings, Herath et al. (2013) observed an increase in saturated hydraulic conductivity in a repacked soil column assay with poorly drained Typic Fragiaqualf soil to which biochar produced from the pyrolysis of corn stover feedstock was added. The differences in findings may be explained by the following: i) the saturated hydraulic conductivity in the Typic Udorthent (ES-K) was already moderately high (Soil Survey Division Staff, 1993), and ii) studies using repacked soil columns, such as that performed by Herath et al. (2013), often show a biochar-induced increase in soil hydraulic properties, while studies with undisturbed samples from field experiments have produced rather inconclusive results (Tammeorg et al., 2014). However, our findings coincide with other reports of no effect on hydraulic conductivity in loamy soils amended with biochar (Jeffery et al., 2015).

Amendment rates below 10 Mg ha^{-1} of biochar did not have a significant impact on soil hydraulic properties in the loamy soil (ES-K). A number of studies indicate that, at least in some soils, the addition of sufficiently high doses of various types of biochar may improve soil physical properties (e.g. Yu et al., 2013); however, in large scale systems such as forest plantations or agricultural plots, biochar application at such high rates (mostly above 40 Mg ha^{-1}) is not feasible. Liu et al. (2014) investigated the effects of different rates of biochar application (0, 2.5, 5, 10, 20, 30 and 40 Mg ha^{-1}) on aggregate stability, concluding that doses higher than 10 Mg ha^{-1} were needed to induce a significant change in MWD in a red soil in China.

Twenty-four months after application of biochar to the sandy loam Typic Dystrudept soil (ES-O), the highest doses of biochar (20 Mg ha^{-1}) induced a significant increase in aggregate MWD and a significant reduction in macroaggregates, microaggregates and silt/clay-sized aggregates. Addition of biochar to soils influences soil structure owing to the high surface area and porosity of the material (Downie et al., 2009; Major et al., 2010). It is therefore possible that macroaggregates, microaggregates and silt/clay-sized aggregates bound to biochar in ES-O thus forming mega-aggregates and increasing MWD of the soil aggregates. This is consistent with the conceptual model of soil aggregation proposed by Tisdall and Oades (1982) and Oades (1984). However, the change in aggregate size distribution did not lead to any significant changes in soil hydraulic properties. By contrast, Burrell et al. (2016) observed that the water holding capacity of sandy acidic soils with low organic carbon content (1.64% OC) increased in response to biochar addition. However, ES-O was characterized by high organic carbon content (10.8% OC). Our findings are consistent with those of Abel et al. (2013) who observed that addition of biochar to a loamy sand soil with high organic matter content (5.3% OC) did not increase the AWC. Moreover, Barnes et al. (2014), reported that native soil organic matter played an important role in soil dynamics after addition of 10% of mesquite-derived wood biochar (400 °C) to an organic-rich (37.9% OC) sandy loam soil. Therefore, the lack of any improvement in water holding capacity or K_s reinforces the idea that soil hydraulic properties are not only governed by soil texture.

4.2. *Effects of wood ash on hydraulic properties*

Numerous field experiments have been conducted over the last few decades to study the effects of wood ash application on soil chemical properties (Naylor and Schmidt, 1986), ground vegetation and stand growth on drained peatland (Lukkala, 1951) and forest mineral soil (Jacobson, 2003). However, the effects of the application of wood ash on the physical and hydraulic properties of soil have not been clearly established (Demeyer et al., 2001).

Some effects on soil hydraulic properties of the loamy Typic Udorthent (ES-K) were observed 30 months after wood ash application, depending on the dose applied and whether nitrogen was added. The lowest dose of WA (1.5 Mg ha^{-1}) yielded a reduction in saturated hydraulic conductivity from moderately high to moderately low. This may be a sign of a pore clogging process promoted by the small grain size of the wood ash applied (Gartzia-Bengoetxea and Arias-González, 2013). Post-wildfire studies have also demonstrated that ash caused pore clogging in some cases (Bodí et al., 2014), depending on the soil type and ash behaviour, which depends on, amongst other factors, burned matter and burning processes. The higher dose of WA (4.5 Mg ha^{-1}) did not result in lower K_s, because thick ash layers might increase the number of active micropores limiting the effects of pore clogging (Woods and Balfour, 2010). Nevertheless, we did not observe any significant change in porosity in response to WA(L) application. Indeed, pore shape, tortuosity and connectivity have been suggested to be more important than total porosity for determining saturated hydraulic conductivity (Bodner et al., 2014; Deb and Shukla, 2012).

The slope of the SWRCs and AWC were significantly lower thirty months after the application of nitrogen-enriched wood ash (4.5 Mg ha^{-1}) to ES-K. The available water content of the soil mainly depends on the type of soil structure, such as larger pore sizes and soil aggregates (Hillel, 1982). We found that application of the N-enriched WA promoted changes in soil structure, as reflected in the MWD of aggregates. Addition of nitrogen to soil is known to promote biological activity (Brais et al., 2015), which may lead to changes in soil structure (Bronick and Lal, 2005). Saarsalmi et al. (2012) observed that thirty years after application of wood ash plus urea (46% N) to a boreal Scots pine stand, the microbial biomass C and N, C mineralization and dissolved organic carbon (DOC) were still higher than in unamended forest soils. An increase in microbial activity due to WA fertilization has previously been suggested to explain an increase in DOC concentration (Jokinen et al., 2006). The increase in DOC may favour the formation of organic Al and Fe complexes in acidic forest soils (Rasmussen et al., 2006) and also affect the distribution of aggregate size fraction and MWD.

While the application of nitrogen-enriched wood ash (4.5 Mg ha^{-1}) led to a reduction in the slope of the SWRCs for ES-K, the same was observed for the sandy loam soil (ES-O) with the application of WA without nitrogen (Fig. 3). The WA(I) and WA(H) treatments led to alterations in the shape of the SWRC by reducing water content within the low-tension range (-1 KPa and -33 Kpa). This may be due to a higher nitrogen retention capacity of the sandy loam soil resulting from the high amount of organic matter with high complexation capacity and the consequently different effects on nutrient availability and soil dynamics (Parvage et al., 2015). The highest rates of WA application led to an increase in the MWD of the aggregates and to a reduction in the amount of soil macroaggregates. This would probably result in a reduction in the slope of the SWRC. Ouyang et al. (2013) attributed a negative change in SWRCs to the increasing amount of microaggregates and the smaller pore sizes. Despite these differences in soil structure, none of the WA treatments modified the moderately high saturated hydraulic conductivity in ES-O. This is consistent with the findings of Adriano and Weber (2001), who did not observe any effect on soil hydraulic conductivity in coarse texture soils to which wood ash was applied at rates as high as 1120 Mg ha^{-1}.

5. Conclusions

The study findings show that effects of adding biochar and wood ash to temperate humid soils depend on the soil type, application rate and whether or not nitrogen was also added.

Application of biochar alone did not increase water holding capacity at any dose or in any site; however, the application of 10 Mg ha^{-1} of biochar + 0.8% N led to reductions in the MWD and microporosity in the loamy Typic Udorthent, resulting in a lower soil water holding capacity and reduced hygroscopic water content relative to the control. In the sandy loam Typic Dystrudept, changes in soil structure but not in soil hydraulic properties were also observed after the addition of 10 Mg ha^{-1} of biochar + 0.8% N.

Equivalent hydraulic conductivity values were obtained for biochar-amended and unamended soils in both sites.

Application of wood ash decreased the soil water holding capacity in both sites. In the loamy soil, this change was observed after application of N-enriched WA, while in the sandy loam soil the effect was observed in treatments without nitrogen. This highlights the different role of nitrogen in promoting changes in soil hydraulic properties that may result from changes in soil structure. Future research should use ^{15}N stable isotope techniques to determine the relative contribution of N from soil or from biochar or wood ash amended soil. This will help to determine the role of nitrogen in promoting microbially-mediated soil structural changes that may be reflected in soil hydraulic properties. Further research efforts should also focus on the interactive effects under field conditions of the biochar adsorption capacity, mineral N, soil microorganism activity and structure and soil hydraulic properties.

Considering the importance of soil hydraulic properties in increasing the resilience of soil to drought and preventing soil erosion, long-term field research is needed to investigate the root-soil interactions, changes in microbial community structure and soil hydraulic properties in biochar and wood ash amended soils, with a view to developing appropriate landscape management strategies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.geoderma.2017.05.041.

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Title: Effects of biochar and wood ash amendments on field aggregate size distribution and stability and GPRS content: a case study in two temperate forest with contrasting soil properties

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HIGHLIGHTS:

- Wood ash and Biochar don't affect well-structured forest soils' stability.
- Amendments enriched with N reduced field aggregates' MWD of the loamy soil
- GRSP can't be considered a simple, universal indicator of soil physical stability

KEYWORDS: Wood ash, Biochar, Soil aggregates, MWD, aggregate stability, GRSP

Soil structure is an important property determining many biological, hydraulic and chemical processes in soil, and hence influencing many ecosystem services including energy and food supplies. Soil degradation is a serious problem in the Atlantic watershed of the Basque Country, characterized by intensively managed tree plantations established on hills (Gartzia-Bengoetxea et al., 2009). It is likely that runoff and erosion will be accentuated by climate change (CC). Combining bioenergy production with carbon sequestration via soil amendments, including biochar (BC) and wood ash (WA) may contribute to mitigate CC and can be used to improve soil physical properties. Their effects may be also indirect by stimulating microbial activity and growth of plant fine roots and fungal hyphae. Various markers or proxies of soil physical stability can be used to determine the effects of amendments on soil properties. These markers can include hydraulic properties such as pore size and soil aggregate size distribution and stability. An earlier study of the addition of BC and WA in two experimental field-sites (Moragues-Saitua et al., 2017), showed that after 15 months of amendment a slight change in hydraulic properties was observed. The objective of the present study is to assess changes in field aggregate size distribution and stability and its relationships with changes in glomalin-related soil protein (GRSP) content, as early indicators of the improvement of soil physical properties. Field aggregate size distribution can inform us about the complexity of a soil, apart from giving us an idea of resistance to abrasion and wind erosion (USDA, 2008) whilst soil aggregate stability is used to analyse the soil physical behaviour against different forces. Thus, both properties can be used as indicators of soil quality or degradation, often expressed as mean weight diameter (MWD), as an index of soil structural quality. Besides, GRSP, an operationally defined category of soil proteins, is thought to be related to fungal activity and to soil physical stability (Wright and Upadhyaya, 1996).

A detailed explanation of the field experimental design can be found in (Moragues-Saitua et al., 2017). Briefly, a complete block design was stablished with the addition to soil of either BC (10 Mg ha^{-1}) or WA (4.5 Mg ha^{-1}), and with or without additional nitrogen (0.8 % N), with four replicates in two forest plantations in the Basque Country: a loamy *Typic Udorthent* (Soil Survey Staff, 2014) under pine (*Pinus radiata* D.Don) at Karrantza (hereafter K); and a sandy loam *Typic Dystrudept* (TD) under Pyrenean oak (*Quercus pyrenaica* Willd) at Oiz (hereafter O). Nitrogen addition treatment was included to avoid N-limitation of the mineralisation of added organic amendment. Soils were sampled 24 months after treatment, gently crumbled, passed through a 20 mm sieve and air-dried. Half of the sample was sieved using nested sieves mounted on a sieve shaker (Retsch AS200 Control: Retsch Technology, Düsseldorf, Germany) to obtain field aggregate-size classes: 20–10, 10–5, 5–2 mm; 2.0–0.25 mm; 0.25–0.053 mm; and < 0.053 mm (see Gartzia-Bengoetxea et al. (2009) for more details). The other half of the sample was sieved between 3 and 5 mm for measurement of aggregate stability by the fast-wetting standard method, ISO/CD 10930 (Le Bissonnais, 1996) and GRSP content measurements. Citrate extracts were diluted (2-fold for K and 20-fold for O) and protein quantified using the Bradford assay with correction for sample colour as described by (Moragues-Saitua et al., 2019). The >50 µm fraction collected after the aggregate stability test was oven-dried and subjected to the size separation to obtain field aggregate-size fractions: 5-2mm, 2-1mm, 1-0.5mm, 0.5-0.2mm, 0.2-0.1mm and 0.1-0.05mm. A MWD index was calculated for the aggregate size distribution of field aggregates (MWD_f) and for aggregates obtained after the stability test (MWD_s). Statistical analyses were performed in R, version 4.0.4 Three-way ANOVAs were performed and when not fulfilled norrmality or homoscedasticity assumptions, non parametric Kruskal-Wallis tests were performed with the rstatix package (Kassambara, 2020).

Figure 1 represents MWD of field aggregates (MWD_f) and aggregates subjected to fast wetting stability test (MWD_s). A significant difference was found in MWD_f between the two forest soils (F=39.45, df=1, 28, p<0.01). No amended ES-K soils presented MWD_f over 8 mm whilst median MWD_f values of no amended soils in ES-O are of 4.30 mm (IQR=0.48 mm). Notably, these differences between soils dissapeared whith N application (signifficant interaction of N addition and forest soil on MWD_f (F=10.22, df=1,28, p<0.01). MWD_f values of soils treated with N, despite the type of amendment and ES, are between 5-8 mm. There was a reduction in mega-aggregate fraction in ES-K (10-20mm) with the combined application of N and both BC and WA amendments (p<0.01, df=18, F=3.03). A similar effect was found by Miller et al. (2012) after 11 years of amendments, where they found a decrease in the proportion of the large (12.7 mm) aggregates, and decreased the GMD relative to the unamended controls by a yearly manure application. These authors attributed this reduction to an increase in organic matter content in the soil, which makes the aggregates more friable and susceptible to breakdown by long-term tillage. However, there is yet scarce literature reporting implications of changes on field aggregate size distribution and wether this reduction is negative or reflective of functional biological traits, e.g. root and hyphal growth in non-tilled systems and more research is needed (Lehmann et al, 2017). The combination of N with organic amendments may have act as a nucleus of aggregation, by increasing the input of labile components and acting as a microbial C source (Wang et al., 2016) affecting nutrient and aggregate dynamics. However, it still remains unclear, and more research is needed to detect if the effect is derived only from N application or wether it is provoked by the combination of the two amendments. It would also be interesting to see if the relationship of each aggregate fraction with different types of microorganisms or root traits is affected by N-rich treatments.

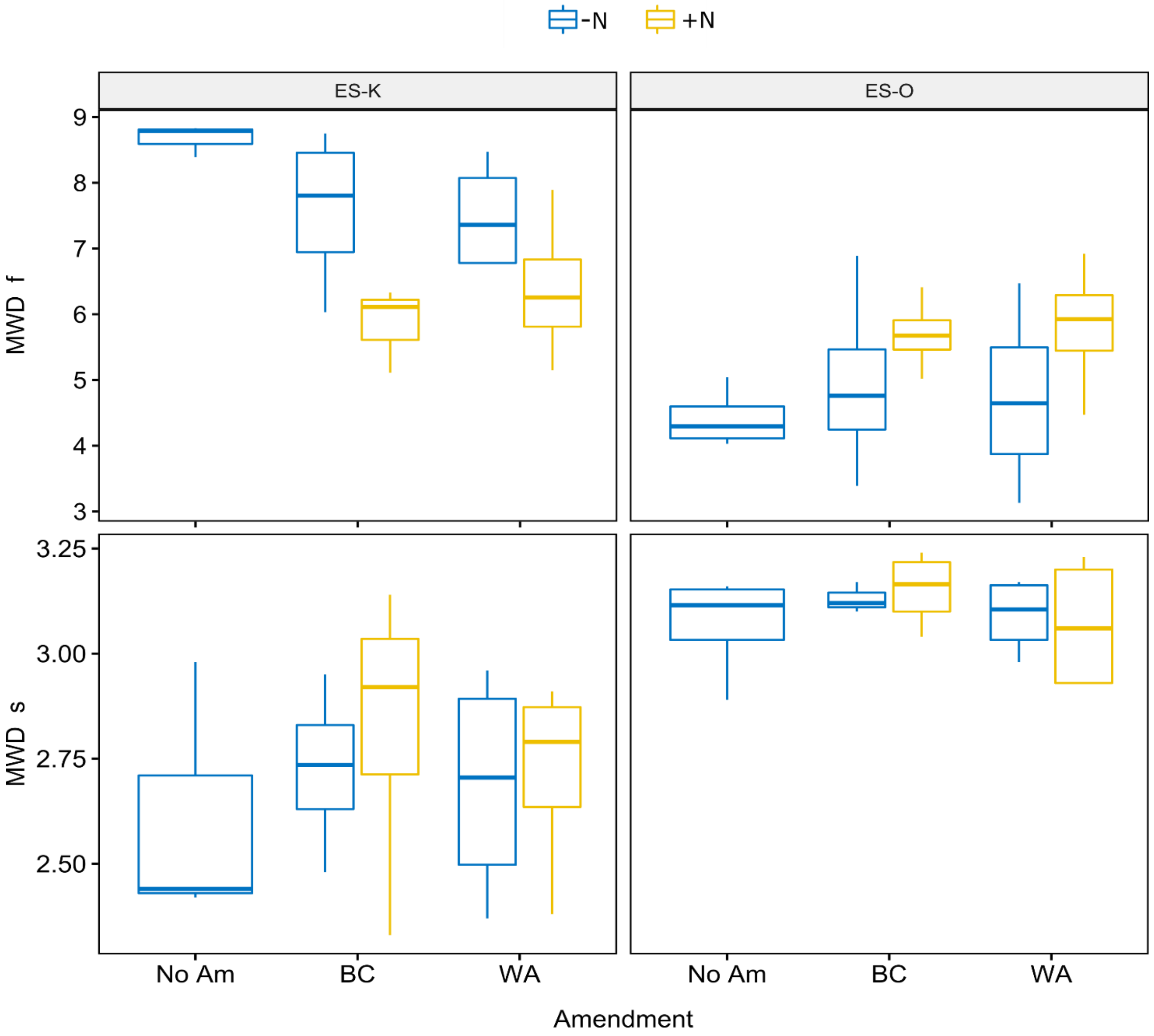

Figure 1 Boxplots representing MWD of field aggregate size distribution (at the top, MWDf) and of aggregates subjected to fast wetting (at the bottom, MWDs. Each colour represent samples of each experimental site : in blue samples from the experimental site (ES) of Karrantza (ES-K, loamy Typic Udorthent) and in yellow samples from the ES of Oiz (ES-O, sandy loam Typic Dystrudept). X axis represents type of amendment: No Amendment (No AM), Biochar (BC) and wood ash (WA), without nitrogen (left block), and with 0.8% N (right block)

MWD$_s$ values of both soils are over 2 mm, meaning that these forest soils were already very stable, with a very low risk of erosion and crustability (Le Bissonnais,1996). However, MWD$_s$ differed between forest soils, with the coarser textured soil (ES-O) being more stable (F=73.778, df=1,28, p<0.01). This higher soil aggregate stability despite the higher sand content remarks the importance of SOM on aggregate stability, an thus, on soil structure. Organic materials are directly responsible for the formation of macroaggregates through the actions of fungal hyphae and microbial extracellular polysaccharides (Lehmann et al., 2017) and they become especially important in coarse textured soils, where sand content negatively correlates with MWD (Ćirić et al., 2012). Our study showed no effects (remarkably not negative effects) of the amendments at none of the experimental sites. This is particularly important in the forest plantation with coarse texture soils, since they are more vulnerable to erosion and degradation once SOM is depleted, and the mantainance of aggregate stability after amendment addition is still positive in order to face up CC by applying amendments of this nature.

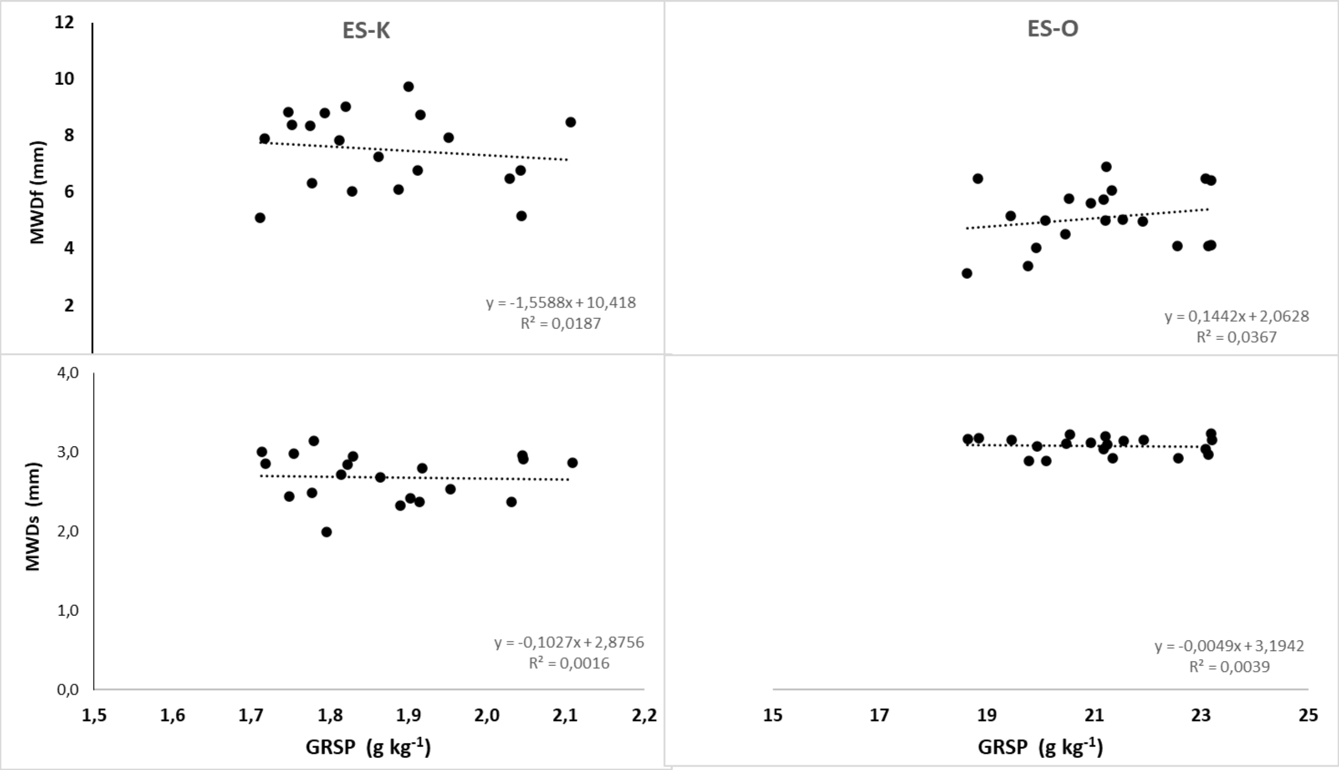

Figure 2. Scatter plots with regression lines to check for any the correlation between Glomalin related soil proteins (GRSP ; X axis) and MWD of aggregates subjected to fast wetting test (MWD_s ; Y axis, bottom) and MWD of field aggregates (MWD_f, Y axis, top)

GRSP, are considered soil gluing agents and enhancers of aggregate stability (Gao Et al., 2019) due to the Fe-rich flocs they form with clay particles (Wang et al., 2021). WA addition increased GRSP [H(2)=5.64, p=0.05], but no significant effect has been observed in aggregation.. Even though we did not find a correlation between SOC and GRSP (Gao et al., 2019), some authors have reported a linear correlation between these two factors (Wu et al., 2014). We observed that in ES-O, the soil containing higher ammounts of SOC and more stable to slacking, presented the highest values of GRSP. However, our results showed that in this case, GRSP is not an appropiate biochemical proxy for aggregate stability, since no correlation between GRSP and MWD$_s$ nor MWD$_f$ was observed (Figure 2).

CONCLUSIONS

- N application affects field aggregate size distribution. Mega-aggregate fraction in the loamy Typic Udorthent is reduced after N application (combined with Biochar or wood ash), and decreased the field aggregate MWD relative to the unamended control. Still more research is needed in order to understand its meaning, since a reduction in MWD does not always mean a negative effect.
- Soil amendments can be used in structured forest soils since no negative effects on soil aggregate distribution and stability were found.
- Relations between GRSP and aggregate stability differs between soils, so it can't be considered a simple, universal indicator of soil physical stability.

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APPENDIX-III

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Special issue article

Towards meaningful quantification of glomalin-related soil protein (GRSP), taking account of interference with the Coomassie Blue (Bradford) assay

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Summary

Glomalin-related soil protein (GRSP), an operationally defined fraction of soil organic matter containing protein and various other components, is usually quantified using the colorimetric non-specific Bradford method. This method is limited by a short working range, a non-linear response and interference from co-extracted compounds. These limitations hinder the exact quantification of the protein component. The aim of this study was to investigate the source of interference in the Bradford quantification of GRSP and propose several methodological improvements based on identified interferences. The easily extractable and total GRSP in five topsoils with contrasting texture, organic carbon content and land use were compared. Results showed that: (i) the extent of interference varied between different soils, (ii) the standard addition method overestimated the extent of inhibition, (iii) absorbance should be corrected for colour, (iv) use of the ratio of absorbances at 595 and 465 nm, A_{595}/A_{465}, is not recommended because it is sensitive to pH and dilution-dependent absorbance at 465 nm, (v) although a quadratic fit to the protein calibration curve was better than the linear fit, it was not possible for the dilution method and (vi) estimation of protein content from the dilution curve of the soil extract appeared to be suitable as it integrates the often observed, and hitherto unexplained, effect of dilution on the calculated protein content of soil extracts and avoids artefacts because of the choice of protein spike and dilution.

Highlights

- Soil protein colorimetric quantification is hampered by co-extracted compounds
- Variants of Bradford assay of glomalin-related soil protein are tested for five contrasting soils
- Direct assay underestimates soil protein, but standard addition may overestimate
- Controlled sample dilution with colour correction might give the best estimate of soil protein

Introduction

Glomalin, reputed to be a protein of arbuscular mycorrhizal origin and a putative homolog of heat shock protein 60, was first reported in 1996 and has since been widely studied (Wright & Upadhyaya, 1996; Gadkar & Rillig, 2006). It is now evident that it is more appropriate to refer to this operationally defined fraction of soil organic matter obtained by autoclaving soil in neutral or alkaline citrate solution as glomalin-related soil protein (GRSP, Rillig, 2004) because it is not purely protein and is not solely of fungal origin (Whiffen *et al.*, 2007; Gillespie *et al.*, 2011). The often excessive claims about the properties it confers on soil (Nichols & Wright, 2005; Nichols & Wright, 2006) are also debated; however, it might be an interesting fraction of soil organic matter and possibly an indicator of stable organic matter or of changing organic matter dynamics.

Glomalin-related soil protein is usually quantified by the colorimetric non-specific Bradford method based on protein complexation with the Coomassie Blue dye at acid pH (Bradford, 1976).

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The Coomassie brilliant blue protein assay is widely used because it is commercially available, and because of its ease of performance, rapidity and relative sensitivity. Nevertheless, quantification is limited by the short working range of this method, the non-linear response and interference, both positive and negative, from co-extracted compounds. These effects result in dilution effects that complicate the exact quantification of the protein component (Redmile-Gordon *et al.*, 2013; Reyna & Wall, 2014; Jorge-Araújo *et al.*, 2015).

To overcome the various interferences with and limitations of this colorimetric protein assay, different strategies may be adopted to minimize artefacts. The apparent increase in GRSP detection with dilution has been proved to be unrelated to the disaggregation of glomalin protein complexes as the addition of disaggregating detergents or chelating agents does not modify the results of the Bradford assay (Reyna & Wall, 2014). Therefore, this dilution effect might arise in part because dilution also dilutes any interfering or enhancing components that may be present in the protein extract.

Another important feature of the Bradford method is that the calibration curve is non-linear (Bradford, 1976; Splittgerber & Sohl, 1989). A more accurate estimation of protein content of samples may be obtained using a non-linear calibration method. Many spectrometers have software that allows a non-linear, quadratic calibration to be used, but extrapolation is inaccurate, so care is required in the choice of the sample dilution and range of the calibration curve. Another approach often recommended for protein assay by the Bradford method is to consider the absorbance of both the protein reagent complex at 595 nm and one of the peaks of the reagent at 465 nm that decreases as the blue-coloured protein reagent complex is formed (Bearden, 1978). Protein analytical chemists have recommended calibration using the ratio of absorbances at 590 and 450 nm, A_{590}/A_{450} (there is some variation in the exact wavelengths chosen for these peaks, 590–600 nm and 450–465 nm), because there is little overlap between these peaks (Bradford, 1976). Zor & Selinger (1996) demonstrated an improvement in sensitivity of the assay by one order of magnitude by protein calibration using A_{590}/A_{450}. This linearization, however, might not solve all the interference from coloured samples such as soil extracts. The soil extracts are coloured and absorb light strongly, especially at shorter wavelengths. Therefore, sample absorbance at each wavelength should be subtracted before calculating the ratio. It should be considered that absorptivity of the sample extract blanks might be both dilution and pH dependent, making measurement of the appropriate sample blank non-trivial. Splittgerber & Sohl (1989) suggested that a better linear fit to the calibration curve may be obtained using the decrease in absorbance at 465 nm with increasing protein concentration, but for soil extracts, which absorb strongly at this wavelength, this would not be appropriate.

When samples are thought to contain interfering, or enhancing, components and it is impossible to reproduce the composition of the matrix in the calibration standards, the solution often adopted in analytical chemistry is to make a controlled addition of the compound to be quantified. In the case of protein quantification, it is usual to add bovine serum albumin (BSA), the same protein that is usually used to obtain calibration curves, despite the fact that the sensitivity of BSA is greater than for many other proteins because of the greater number of arginine residues (and the specificity of the Bradford assay for arginine residues; Compton & Jones, 1985). In a previous report, controlled additions of BSA were used to assess the underestimation of GRSP in forest soils and the interference was attributed to humic substances (Jorge-Araújo *et al.*, 2015). Humic substances may interfere by forming complexes with either protein or dye, thereby reducing the formation of the protein–dye complex that absorbs strongly at 595 nm (Nichols & Wright, 2005; Schindler *et al.*, 2007).

Ideally protein solutions should be purified to remove interfering components, including humic substances, prior to quantification (Nichols & Wright, 2005). Attempts to do this have been largely unsuccessful, with very small yields; however, yields are rarely given in published reports. Jorge-Araújo *et al.* (2015) postulated that irreversible, possibly co-valent, bonds are formed between soil proteins and polyphenols during the high-pressure, high-temperature extraction. If this is the case, soil protein and added BSA might not experience the same interference. Soil protein might be irreversibly complexed with humic substances and so unable to form complexes with Coomassie Blue dye, as indicated by sequential extractions of humic substances and glomalin (Nichols & Wright, 2005).

Another experimental approach recommended for the quantification of unknown proteins is to compare the gradients of the calibration curve and the absorbance–dilution curve of the sample with Coomassie Blue dye (Zor & Selinger, 1996). The ratio of these gradients is a measure of the unknown protein concentration at the smallest dilution (or no dilution). Unlike the addition of a protein, this gives an assessment of the formation of a dye complex with soil protein in the presence of interfering substances. Finally, another source of underestimation of protein concentration could be a shift in the peak of the protein–dye complex because of the matrix or complexation of the protein with other solubilized soil components.

The aim of this study was to investigate the sources of interference in the Bradford quantification of soil protein extracted by the GRSP citrate-autoclave technique. We chose to use the Bradford method because most studies on the GRSP fraction have used this assay, although recent studies have suggested that interference might be less with an alternative protein assay, a modified Lowry method (Redmile-Gordon *et al.*, 2013; Reyna & Wall, 2014). The easily extractable and quasi-total GRSPs in five topsoils with contrasting texture, organic carbon content and land use were compared. Protein was assayed by Bio-Rad Quick-Start™ (BioRad Laboratories, Hercules, CA, USA) with various methods of calibration. The objective was to identify the best methodological approach to the quantification of GRSP. The scope of this study does not include any reflection on the reality of the nature of the protein or fungal origin of GRSP. Despite ongoing controversy about GRSP, some trends, such as the conservation of GRSP as soil organic matter decreases with land-use change, suggest that GRSP may nevertheless be an interesting fraction of soil organic matter and possibly a convenient marker of changes in soil organic matter dynamics.

Table 1 Some properties of the five soils and sampling location (mean (standard deviation))

	Land use	UTM (30 N ETRS 89)		Texture	SOC/%	pH	C:N
W	Ancient woodland, Wytham	615 146	5 737 976	Clay loam	11.28 (1.28)	5.87 (0.16)	11.18 (0.25)
K	*Pinus radiata* stand, Karrantza	475 081	4 786 389	Loam	3.86 (0.07)	4.79 (0.03)	14.37 (0.23)
A	Arable land, Wytham	615 740	5 738 037	Clay loam	8.52 (0.29)	7.00 (0.22)	10.52 (0.56)
O	Shrubland, Oiz	532 673	4 785 572	Sandy loam	10.79 (0.63)	3.78 (0.02)	16.81 (0.31)
Co	'Boughton Kettering Loam'	–		Loam	2.38 (0.01)	7.98 (0.02)	11.79 (0.11)

Materials and methods

Soil samples

Samples of soil with contrasting origin and properties (texture, mineralogy and organic carbon) that are currently under study in two European Projects (CHARFOR and FIXSOIL) were selected for this research. They are from four different locations with different land uses and texture and one commercial soil: (i) 'W' collected from an ancient woodland (Wytham Woods, Oxfordshire, UK), (ii) 'A' collected from a plot of arable land (Oxford University farmland at Wytham, Oxfordshire, UK, farmed by FAI farms), (iii) 'K' collected from a *Pinus radiata* D. Don seed orchard (Karrantza Valley, Bizkaia, Spain), (iv) 'O' collected from a mountainous shrubland (Oiz Mountain, Bizkaia, Spain) and (v) 'Co', which was a commercial soil exposed to controlled environmental conditions and plant growth for 6 months. Samples were collected from the topsoil (0–10 cm). They were air-dried for 10 days and then sieved between 3 and 5 mm. These aggregates were gently crushed with a mortar and pestle and sieved to <200 µm, and were stored until required for GRSP extraction. Some soil physicochemical properties of the five soils are given in Table 1.

Extraction

Operationally defined GRSP fractions were obtained with the extraction methods proposed by Wright & Upadhyaya (1996) as follows. Easily extractable soil protein ($GRSP_{EE}$) was obtained by autoclaving soil in a solution of 20 mM sodium citrate at pH 7 for 30 minutes. Total GRSP ($GRSP_T$) was obtained by two successive autoclave extractions of soil in 50 mM sodium citrate at pH 8, each for 60 minutes. It should be noted that only two extractions were used to obtain the quasi-total fraction, in contrast to other studies where a variable number, up to eight, are often performed until the extract is colourless. This criterion is based on the unverified assumption that the colour is caused by protein and not humic substances. In each case, the soil:solution ratio was 1:8 and phases were separated after cooling by centrifugation at 15 000 ***g*** for 15 minutes. For $GRSP_T$, after the first autoclave cycle, the solution was removed and replaced by the same volume of a new addition of citrate solution, and the mixture was vortexed to resuspend the soil. The solutions were combined to form the total extract. Solutions were frozen until required for analysis. After thawing, samples were centrifuged again at 15 000 ***g*** for 15 minutes to remove any precipitate that may have formed.

Assessment of GRSP and calibration methods for GRSP interference

Protein was assayed with Bio-Rad Quick-Start™ and calibrated by various methods against solutions of bovine serum albumin (BSA). All colorimetric measurements were prepared in triplicate by using three different wells in the same microplate. Sample (diluted or not) and Bradford dye reagent volumes were 20 µl and 230 µl, respectively. Similarly, sample blank absorbances were measured using 20 µl of diluted soil extract and 230 µl of citrate solution (pH 7 or 8 or adjusted to 1). Absorbance at 465 and 595 nm and spectra (obtained in the range 400–800 nm) were measured with a ThermoScientific Multiskan GO spectrometer (Waltham, MA, USA). Samples were analysed at various dilutions, with and without a single addition of BSA to obtain an added concentration in the diluted sample of 80 mg dm^{-3}. The BSA spike was added either as the first step of the dilution (series denoted B) or as the final 1:2 dilution (series denoted A). The absorbance spectra of reagent blanks (Bradford reagent with citrate extraction solution) and sample blanks (diluted sample with citrate extraction solution and pH adjusted to 1) were also measured.

The final dilutions were 1:1, 1:2, 1:5 and 1:10 for soils A, Co, W and K and in addition 1:20 and 1:40 for soil O. Protein concentration (equivalent BSA) was calculated, with or without sample blank colour correction, by fitting linear and non-linear functions to the calibration curves of the absorbance at 595 nm and of the ratio of absorbances at 595 and 465 nm.

Standard additions were performed by a single addition of BSA before (B) or after (A) dilution in the extraction solution to assess reversibility of the formation of such complexes. Extrapolation of the linear plots of the standard addition to the x-intercept gives the absolute value of the protein concentration in solution, and therefore an estimate of the amount of protein extracted. As for direct measurement, calculations were made with or without correction for sample blank colour (at the same dilution and pH) and calibration of absorbance at 595 nm and of the ratio of absorbances at 595 and 465 nm.

Protein concentration was also calculated by the gradient of the dilution curves, proposed by Zor & Selinger (1996), to measure absorbance at 595 nm after various dilutions as above, against the gradient of the BSA calibration curve. Extractable soil protein was calculated from the apparent concentration in the extract and the soil : solution ratio.

Results and discussion

The extracts of each soil were coloured to various degrees, and absorbance decreased continuously with increasing wavelength and followed the Beer–Lambert law. Colour intensity at any given dilution and wavelength followed the order O≫>W≫A>K>Co. Absorbance decreased with increasing dilution, but not always as a linear function of dilution factor, leading to dilution-dependent calculated protein content, which will be discussed later. The woodland soil, W, is used to illustrate some of the common trends.

A better fit to the calibration curve of absorbance at 595 nm was obtained with a non-linear (quadratic) function, as shown in Figure 1(a). More importantly, the non-linear function also avoids underestimation of protein concentration at the smallest and largest absorbances and overestimation in the middle of the calibration range. The use of the non-linear calibration curve should avoid some of the apparent dilution effect sometimes observed. However, the non-linear calibration does not enable any extrapolation. An alternative, more commonly used before the advent of advanced spectrometer software, was to use straight lines to interpolate between points on the calibration curve. Figure 1(a) also shows the absorbances of solutions after 1:2 or 1:5 dilutions with or without addition of a BSA spike to obtain a final concentration of 80 mg dm^{-3}. The gradient of the lines joining absorbances with and without BSA standard addition are visibly smaller than the gradient of the calibration curve, which is evidence of negative interference in the protein assay.

Figure 1(b) shows the calibration using the ratios of absorbances at 595 nm (complexed protein–dye peak) and 465 nm (one of the uncomplexed dye peaks). The ratios of absorbances for the EE extract of soil W are also shown after 1:2 or 1:5 dilution with and without addition of a BSA spike to give a concentration of 80 mg dm^{-3}. Some curvature remains in the calibration curve, and more importantly for the context of this study, the slope of the calibration curve is visibly greater than the slopes of either of the two-point standard addition curves for soil W at 1:2 and 1:5 dilutions. Calibration with the ratio might extend the linear range of the calibration curve, but it does not resolve the problems posed by interference with the assay by soluble soil components.

Figure 1(c) shows the dilution curves of the EE extracts of soil W with (A) and without (S) standard additions of BSA. The gradient of the line fitted to S data depends on the number of points included, with the point for the 1:1 dilution showing a clear change in slope, although the slopes of lines fitted to dilutions 1:2–1:10 are close for both S and A.

Figure 2 shows the various estimates of GRSP$_{EE}$ for soil W. There is a clear dilution effect for direct measurement using absorbance at 595 nm and a linear calibration, whereas the dilution effect is much smaller if the non-linear calibration is used. The same trend of estimated protein increasing with increasing dilution was observed for soil O, the shrubland soil with a large organic C content, and similar to W. However, there was an increase followed by a decrease for the other three soils. Reyna & Wall (2014) reported an increase in estimated protein content with increasing dilution (up to a factor of 1/32) using the Bradford assay in the EE fractions of four soils with different textures, pH and organic carbon contents. The relative increase varied between soils but was not reported to be related to any measured soil property or the GRSP content. In this study, the dilution effect was generally smallest between dilutions 1:5 and 1:10. Therefore, we could infer that an average of the calculated soil protein contents at these two dilutions would be a

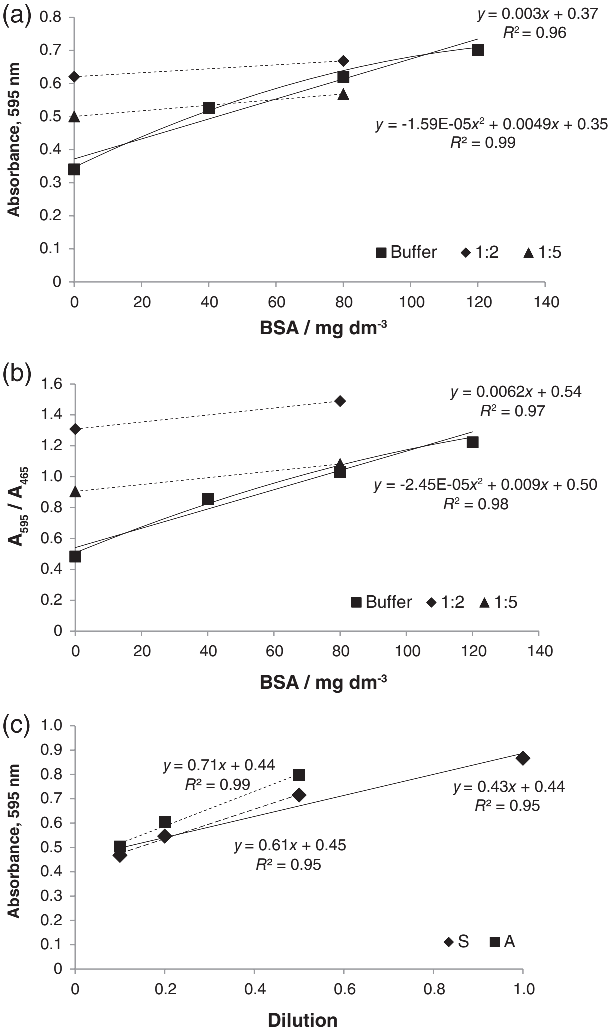

Figure 1 Calibration curves and comparison of methods for calculating protein concentration. (a) Absorbance at 595 nm as a function of bovine serum albumin (BSA) showing the calibration curve with linear and quadratic functions fitted and data for easily extractable (EE) glomalin-related soil protein (GRSP) of woodland soil W, after 1:2 and 1:5 dilutions with and without controlled addition of a BSA spike to give a concentration of 80 mg dm^{-3}. (b) Non-linear calibration using the ratios of absorbances at 595 nm (complexed protein–dye peak) and 465 nm (one of the uncomplexed dye peaks). Also shown are the ratios of absorbances for the EE extract of soil W, after 1:2 or 1:5 dilution with and without addition of a BSA spike to give a concentration of 80 mg dm^{-3}. (c) Absorbance at 595 nm as a function of dilution for EE GRSP of soil W with (A) and without (S) controlled addition of BSA.

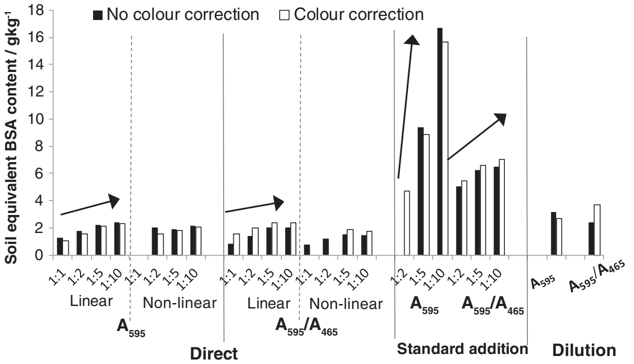

Figure 2 Calculated easily extractable glomalin-related soil protein (GRSP) content of soil W by the various methods and calibrations described and illustrated above.

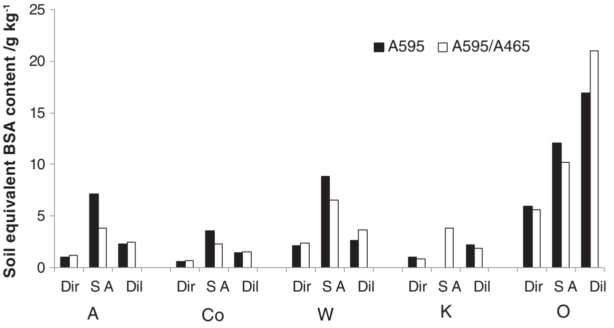

Figure 3 Calculated easily extractable glomalin-related soil protein ($GRSP_{EE}$) of all soils using different methods. All data were obtained with a linear calibration and after dilution to obtain blank sample absorbance of about 0.1 at 465 nm and were corrected for sample colour. Dir, direct measurement; SA, standard addition; Dil, dilution.

good estimate of the true value. It is probable that many studies use this strategy without mentioning it explicitly. In this study soil O, which had the largest estimated protein content and most intense colour, was a marked exception; the dilution effect was smallest between dilutions 20 and 40. An alternative strategy would be to recommend that the appropriate dilution is that where the sample blank absorption at 595 nm is close to the background value. In this study, this corresponded to an absorbance at 465 nm of about 0.1 (as adopted in Figure 3 below).

Previous studies do not mention colour correction prior to calculation of protein content. Correction for sample colour leads to a small decrease in the estimated protein content, which can be seen by comparing the black and white bars in Figure 2. In contrast, sample colour correction had a larger effect when protein content was calculated with the ratio of absorbances. This results from the much larger sample absorbance at smaller wavelength, but over-correction in the most concentrated samples. This was possibly caused by a small pH dependence of sample colour that prevented calculation of protein concentration. As is the case for the direct measurement, there was a notable dilution effect; the amount of GRSP calculated increased with increasing dilution and levelled off between dilution 1:5 and 1:10. The difference in calculated GRSP obtained with the linear and non-linear calibration was small.

A striking feature of the data in Figure 2 is that the method of standard addition yields much larger calculated values of GRSP regardless of the dilution. The assay of BSA added was greatly inhibited by the matrix of soil extract and was similar to results obtained by Rosier *et al.* (2006) in another BSA standard addition experiment. Redmile-Gordon *et al.* (2013) also reported a marked decrease in the Bradford assay calibration curve of BSA in a soil extract compared with a citrate buffer solution and decreases in gradient on addition of polyphenol to either buffer solution of the soil extract. Although they did not use the standard addition curves to make an alternative assessment of protein content by extrapolation of the calibration curves, their data indicated that standard addition would give a larger estimate of protein content than direct calculation. It should be noted that BSA was added after autoclaving and so was not subjected to the same conditions as the extracted soil protein. This was unavoidable because when BSA is added prior to extraction, it would be adsorbed and an unknown and soil-dependent proportion of added BSA would be recovered.

The increase in estimated GRSP by the standard addition approach was much greater with the direct method at A_{595} than the ratio method with A_{595}/A_{465}. This indicates that interaction between added BSA and soil components, assumed to be polyphenols, had more effect on the spectrum at wavelength 595 nm than at 465 nm. The much greater estimate of GRSP with the standard addition accords with a previous study of forest soil where we reported a two- to six-fold increase in estimated GRSP with the standard addition method (Jorge-Araújo *et al.*, 2015). Furthermore, there was no tendency for the estimated GRSP to level off at the greatest dilution for the direct assay. The dilution effect was less marked with the A_{595}/A_{465} assay and there was little difference between dilutions 1:5 and 1:10, suggesting again that the average of these values might be representative of a true value of GRSP. It should be noted that the standard addition method requires the use of a linear function fitted to the calibration curve, although a non-linear curve would give a better fit. Finally, Figure 2 also shows the GRSP estimated from the linear portion of the dilution curve (Figure 1c). This method gives an estimate of GRSP that is intermediate between the direct measurement and standard addition. A clear advantage of this method is, in addition to the simplicity of its execution, that there is no arbitrary choice of dilution or of the amount of model protein to be added.

Figure 3 shows a summary of the estimates of $GRSP_{EE}$ for each of the other soils obtained by direct measurement, standard addition or dilution. Data are shown for direct measurement and standard addition at the dilution that gave an absorbance of the sample blank at 465 nm of about 0.1, corresponding to a 1:5 dilution of soil W, 1:2 dilution of soils Co, A and K and 1:20 dilution of O. There were similar differences for the total fraction. Although the non-linear calibration gave a better fit, linear calibration is required for the standard addition and dilution techniques; therefore, all data were obtained from the linear calibration for this comparison. All

Table 2 GRSP assessment obtained with the different methods in the five soils under study (A, Co, W, K and O).

	Equivalent BSA in soil/g kg^{-1}				
	Soil				
Easily extractable	A	Co	W	K	O
[a] Direct, linear calibration	1.01	0.58	2.00	0.91	7.36
[a] Direct, linear calibration, blank correction	0.94	0.56	2.19	0.78	6.22
Direct, linear calibration, blank correction (A400 ≈ 0.15)	1.04	0.58	2.11	0.99	5.92
[a] Direct, non-linear calibration	0.91	0.51	1.97	0.86	6.57
[a] Direct, A_{595}/A_{465}, linear calibration	1.04	0.59	2.00	0.69	4.83
[a] Direct, A_{595}/A_{465}, non-linear calibration	0.79	0.46	1.49	0.55	3.47
[a] Direct, A_{595}/A_{465}, blank correction, linear calibration	1.17	0.67	2.35	0.82	5.78
[a] Direct, A_{595}/A_{465}, blank correction, non-linear calibration	0.91	0.54	1.78	0.64	4.19
Standard addition, 1:2	7.43	3.61	5.35		
Standard addition, 1:2, colour correction	7.13	3.57	4.72		
Standard addition, 1:5	5.22	1.28	9.37	2.91	
Standard addition, 1:5, colour correction	5.17	1.28	8.87	2.91	
Standard addition, 1:10	2.26		16.68	3.45	17.55
Standard addition, 1:10, colour correction	2.13		15.67	3.45	13.42
Standard addition, 1:20					14.74
Standard addition, 1:20, colour correction					12.07
Standard addition, 1:40					13.88
Standard addition, 1:40, colour correction					12.07
Dilution curve (linear portion)	2.37	1.49	3.13	2.27	25.13
Dilution curve (linear portion), colour correction	2.27	1.47	2.66	2.19	16.95
Total					
[a] Direct, linear calibration	1.97	1.16	2.55	1.25	12.87
[a] Direct, linear calibration, blank correction	1.77	1.09	3.05	0.95	10.64
[a] Direct, non-linear calibration	1.75	1.02	2.25	0.65	11.50
A_{595}/A_{465}, linear calibration	1.74	1.07	2.86	1.02	9.10
[a] A_{595}/A_{465}, non-linear calibration	1.33	0.78	2.08	0.84	6.55
[a] A_{595}/A_{465}, blank correction, linear calibration	2.04	1.24	3.39	1.32	10.71
[a] A_{595}/A_{465}, blank correction, non-linear calibration	1.57	1.00	2.51	1.11	7.79
Standard addition, 1:2	7.86	3.85	11.63	2.25	
Standard addition, 1:5	4.85	1.78	10.44	1.37	
Standard addition, 1:10	4.91	1.37	11.18	1.23	31.09
Standard addition, 1:20					30.30
Standard addition, 1:40					24.14
Dilution curve (linear portion)	3.89	2.71	3.68	2.73	42.14

[a] Average of data obtained at two or three dilutions, differing by about 10% or less. A, arable land; Co, 'Boughton Kettering Loam' commercial soil; W, Wytham Ancient Woodland, Wytham; K, *Pinus radiata* stand, Karrantza; O, shrubland, Oiz.

data were corrected for sample colour for these calculations. A common feature for all soils was that the absorbance at 595 nm and the ratio A_{595}/A_{465} provided similar estimates of GRSP by direct measurement. In contrast, for the standard addition, the calculation of GRSP with A_{595} always gave a larger estimate of GRSP than calculation with A_{595}/A_{465}. Given the rather small working range of the Bradford method, it was not possible to calculate protein concentration for each soil at each dilution. Furthermore, the comparison differed with dilution. Although the GRSP calculated for soil W increased with increasing dilution, for calculations with A_{595} and to a lesser extent for A_{595}/A_{465} (Figure 2), the dilution effect was less marked for K, and was reversed for the other soils. The absence of a coherent trend is a serious drawback for the use of standard additions to assess the extent of interference and to obtain a reliable estimate of GRSP. The dilution method gives estimates of GRSP that are up to four times larger than those obtained by the direct method, and usually larger using the ratio of absorbances rather than A_{595}.

Table 2 shows that the soil protein content estimated from the dilution curves greatly exceeds the direct measurement, and is similar to that of the standard addition estimates for four of the soils, but exceeds that estimated for soil O. The estimate obtained by the dilution method, however, did exceed that of the standard addition for soil O only. This might be because there was less difference between the estimates of GRSP by the direct and the standard addition methods. Because of the intense sample colour

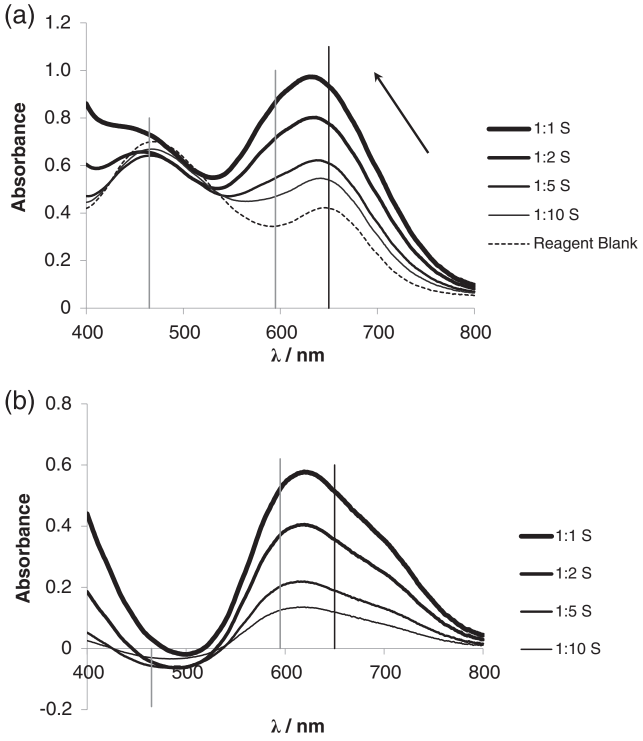

Figure 4 (a) Spectra of extractable glomalin-related soil protein ($\mathrm{GRSP_{EE}}$) of soil W at different dilutions, without and with addition of bovine serum albumin (BSA) and (b) difference spectra (subtraction of spectrum of Coomassie blue reagent) as functions of wavelength.

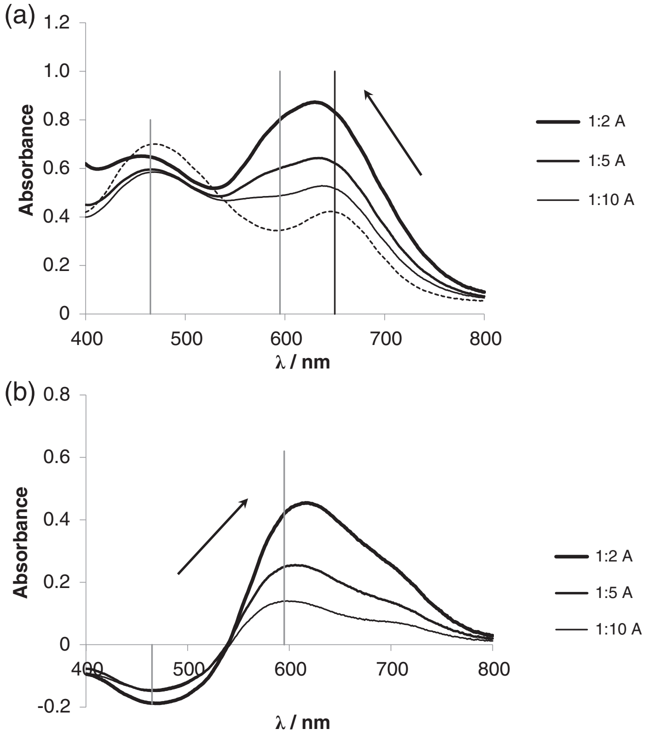

Figure 5 (a) Spectra of extractable glomalin-related soil protein ($\mathrm{GRSP_{EE}}$) of soil W with addition of a bovine serum albumin (BSA) spike at different dilutions. (b) Difference spectra (subtraction of spectra of Coomassie blue reagent and sample blank) as functions of wavelength, λ.

of soil O, the standard addition was carried out on a sample diluted 20-fold and the effect of dilution was smaller than that observed for other soils.

To elucidate the origin of the dilution effects and differences between estimates of GRSP by the various methods, it is useful to examine the spectra of the samples. Figure 4(a) shows the spectra of the reagent blank and extract of soil W at different dilutions. The increase in absorbance at the shortest wavelength at smallest dilution, namely the most concentrated samples, is due to soil colour. The absorbance of the blank sample made little contribution to absorbance at 595 nm or 650 nm. The peak of the protein–dye complex at 595 nm is visible only as a shoulder in the most concentrated samples. There appears to be a hypsochromic shift (to shorter wavelength) of the 650 nm dye peak as the soil solution extract increases from zero (reagent blank) to the most concentrated soil extracts. However, the magnitude of the absorbance increases with decreasing dilution, so this apparent shift could be due to increasing absorbance of the protein dye complex and not a true shift in the dye peak. The difference spectra (Figure 4b) show a constant shift of the peak of the protein–dye complex from 595 nm to 615 nm. There is a strong linear correlation between maximum peak height and the dilution factor that fitted better than those obtained with A_{595} and gradients that are larger. The shoulder at a wavelength of about 740 nm suggests that the dye peak had also been influenced by the soil matrix and so the simple subtraction of the dye spectrum to obtain the difference spectra was imperfect. Each of the soils showed these shifts in the protein–dye complex peak and the shifts increased in the order A≈Co≈W<K<O (465 nm).

Similarly, for added BSA, there is an apparent hypsochromic shift of the dye peak (Figure 5a) that is (largely) due to a bathochromic shift in the protein–dye complex peak as seen in the difference spectra (Figure 5b). In these cases, the apparent shift increases with increasing extract concentration as the proportion of soil protein increases with respect to BSA. The BSA–dye complex is thus less affected by the soil matrix than is soil-extracted protein. This strongly suggests that the underlying mechanism is a strong irreversible association between soil protein and other soil components formed during the high temperature and pressure extraction. This is a strong indication that added non-soil protein does not reproduce the complex formation of soil protein. In addition, the difference in behaviour of soil-extracted protein and added protein, together with the observation that the order of addition of the protein spike is of minor importance, substantiates the suggestion that soil protein and other soil components are irreversibly associated during the extraction process, making efficient purification impossible.

Conclusions

Co-extracted soil components, probably humic substances, cause negative interference in the Bradford colorimetric assay, leading to underestimation of the extractable protein content of soil. The extent of interference varies between soils and depends on sample dilution. We recommend correction of absorbance for colour and the blank absorbance measured at the same dilution and pH as the assay. We strongly recommend that authors explain their choice of dilution factor and either verify that estimated protein content does not vary with dilution or dilute samples to a given intensity of sample colour. The use of the ratio of absorbances of the protein–dye and dye peaks, A_{595}/A_{465}, is not recommended, in particular for intensely coloured extracts because the ratio is sensitive to absorbance at 465 nm. The standard addition method probably overestimates the extent of inhibition. Although a quadratic function gave a better fit to the protein calibration curve than a simple linear fit, this was not possible for the dilution method. The estimation of protein content from the dilution curve of the soil extract takes account of the difference in sensitivity of soil proteins to the Coomassie Blue dye and appears to be suitable for protein assay. It fully integrates the observed and hitherto unexplained effect of dilution on the calculated protein content of soil extracts. This method also avoids artefacts resulting from a choice of protein spike and dilution.

Acknowledgements

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Appendix IV

FELIPE MACÍAS
MONTSERRAT DÍAZ-RAVIÑA
MARÍA TERESA BARRAL
(eds.)

RETOS Y OPORTUNIDADES EN LA CIENCIA DEL SUELO

VI CONGRESO IBÉRICO DE LA CIENCIA DEL SUELO
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Effect of Biochar in water stable aggregate formation

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Abstract

There exists limited information about how biochar affect soil aggregation. Soil aggregation is a key parameter in soil structure and therefore in soil quality. The aim of this study was to evaluate the effect of biochar addition with and without mineral nitrogen in organic matter dynamics and microbial activity in a clayey loam acidic forest soil, in the Atlantic coast of the Iberian Peninsula. A control soil treatment without amendment and two treatments of a C4 plant derived biochar were analysed: 9 Mg C-biochar ha^{-1} and 9 Mg C-biochar ha^{-1} with 0.8% of ammonium nitrate. Soil cores were incubated and fractionated by wet sieving at days: 0, 1, 3, 7, 14 and 28. Soil respiration was also determined at each incubation date; and organic carbon (OC) and ^{13}C were analysed in each aggregate fraction. The results confirmed that the addition of biochar followed the hierarchical model of aggregation: the isotopic analysis reflected an enrichment in the ^{13}C content within the micro-aggregates (magg), from the day 1 to the day 28, but resulting in a modification on the formation of water stable macro-aggregates (Magg). The addition of biochar didn't allow the formation of water stable Large Magg (LMagg) at short term. The interactive effect of biochar, mineral N, soil microorganism's activity and soil aggregation remain still unknown and complex.

Introduction

Biochar application to soils is being considered as a strategy to sequester carbon (C) from a climate change mitigation perspective. In addition, biochar has higher surface area and greater porosity relative to other types of soil organic matter (SOM) and can therefore, improve soil aggregation and soil structure through interactions with native SOM, minerals, microorganism (Six et al., 2004). Understanding the process of how aggregates are formed and stabilized help us to understand and predict the retention and movement of gases, water and solutes in soil; the growth, vigour and productivity of plants; and the activity, growth and movement of soil organisms (Six et al., 2004). Hence, to gain an insight in the effect of biochar amendment in soil aggregation has a potential to understand a win-win strategy for ecosystem service delivery.

Data are scarce on soil aggregation of biochar amended soils. Furthermore, the existing information is still conflicting. Glaser et al. (2002) showed that a large proportion of biochar in Terra Preta was present in unprotected fractions. However, Brodowski et al. (2006) found that biochar was associated mainly with the magg (<250um) soil fraction and Liang et al. (2008) also demonstrated that biochar was predominately present in small soil aggregates, rather than as free organic matter. Brodowski et al. (2006) also observed that the small proportion of biochar particles in soil occurred in the LMagg fractions (> 2 mm) and biochar might act as a binding agent for organic matter in aggregate formation and then protect against degradation (Brodowski et al., 2006).

Biochar might be incorporated combined with nitrogen to prevent the absorption and fixing of available nutrients and soil moisture in the biochar due to its high adsorption capacity and increasing cation exchange capacity (CEC). In addition, soil mineral N availability has been shown to influence the decomposition of OM and therefore the formation of Magg (Le Guillou et al., 2011). In this context, we hypothesized that the addition of biochar with and without N will promote both the formation of Magg and magg but still following the hierarchical model of aggregation. The main objective was to study the formation at short term of water stable aggregates (WSA) in a clayey loam forest soil, with addition of biochar with and without N.

Materials and methods

1. Biochar characteristics

Myscanthus sp., a C4 plant, was pyrolysed at 450ºC in a Pyreg® pyrolisis unit. The pH (1:5 H_2O) was 9.8, it contained a 87% of C, and a C:N ratio of 168. A subsample of biochar was also charged with 0.8% N in NH_4NO_3 form, resulting in a C:N ratio of 77. The dry biochar was grounded in a mortar before applied in the assay and the particle size distribution was >2mm:1.5%; 2000-250 µm: 24.9%; 250-53 µm: 63.3% and <53 µm:10%

2. Soil collection and experimental design

The soil was collected from a pine (*Pinus radiata* D.don) plantation where the annual mean temperature is 11.7ºC and the annual precipitation is 1204mm, in the Karrantza valley, in Bizkaia (UTM 30N ETRS89, 475081, 4786389). Samples were collected randomly, for the first 10cm. The soil was clayey loam texture, with a pH of 4, and 2 % of organic carbon (OC) content.

The soil was air-dried and passed through a 250µm sieve to ensure that all Magg were disrupted. The light OM and sand fraction >250 µm were heated at 105ºC for 24h to avoid seed germination during incubation and added to <250 µm fraction (Fernández-Ugalde et al., 2011).

30 g of air-dried soil was mixed with biochar as follows: 9 Mg C-biochar ha^{-1} (Bio), 9 Mg C-biochar ha^{-1} with 0.8% of N (BioN), and the control (Ctrl). The samples were then packed into stainless steel cores (diameter = 5 cm, height = 2.5 cm), resulting in a bulk density of 1.2 Mg m^{-3}, and closed at the bottom by a nylon mesh of 53 µm. The cores were wetted up to field capacity with deionized water. Each core was suspended inside a sealed 0.5L glass jar with deionized water in a beaker minimize desiccation (Cosentino et al., 2006) and incubated in aerobic conditions at 25 ºC in the dark for 28 days. In total, 48 cores (3 treatments × 6 sampling dates × 3 replicates).

3. Soil sampling and analysis

At days 0, 1, 3, 7, 14 and 28 of the incubation, three replicates per treatment were processed for aggregate-size fractionation, total organic C and 13C isotope analyses. In addition, soil respiration was determined each sampling date by alkali tramps (NaOH) and titration with HCl to phenolphtalein end point.

For the fractionation, the soil sample was crumbled by its natural failures to pass through a 5mm sieve, and dried at 40ºC for 24h. The dry aggregates were submerged in deionized water within a 2mm sieve for 5min, and then sieved in a wet sieving apparatus for 3min with an amplitude of 1.3 cm and 35 times min^{-1}. The fractions retained in each sieve were recuperated and dried at 40ºC. The rest was sieve again, as in the figure 1. Silt and clay fraction aggregates were separated by centrifugation.

The OC and N were determined for each fraction using a LECO TruSPEC® CHN-S elemental analyser, and 13C analysis by Carlo Erba NA 1500 (Milano, IT) elemental analyzer coupled to a VG Isochrom continuous flow IRMS (Isoprime Inc., Manchester, UK).

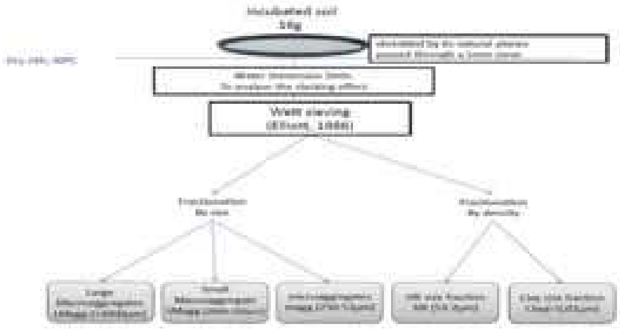

Figure 1.- Aggregate-size fractionation protocol for incubated samples

Results and discussion

In this study, the tested clayey loam soil (the control treatment) followed the hierarchical model of aggregation according to the observations suggested by Elliot (1986). When increasing aggregate size class increased OC proportion (Figure 2), and when a dispersive force was applied (i.e. slaking and wet sieving) Magg gradually broke down into magg (Table1). Therefore, soil organic matter might be the major factor influencing soil water-stable aggregation as stated by Tisdall and Oades, 1982. But the addition of biochar (with and without N) resulted in a change in the aggregate dynamics that didn't allow the formation of water stable LMagg at short term as it occurred in Ctrl (Figure 3), probably due to the biochar properties and its adsorption capacity, and its interaction with the binding agents of it stage. Biochar amendment resulted in a SMagg broke down into magg (table 1) as revealed by the data obtained in the isotopic analysis, which presented an enrichment (Fernández-Ugalde et al., 2011;) (F=5.59, p=0.02, df=2) in the ^{13}C content within the magg of Bio samples, from the day 1 to the day 28. These results confirmed a transfer of added OM from Magg to magg, an observation established in the hierarchical model of aggregation (Six et al., 2004).

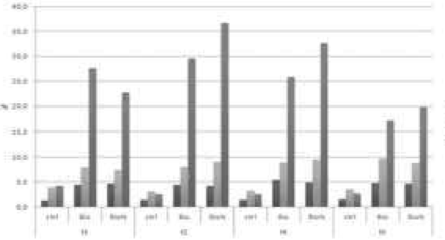

Figure 2.- Mass balance (%) of larer fractions obtained at different fractionation time (days1,3,14 and 28), for different treatments

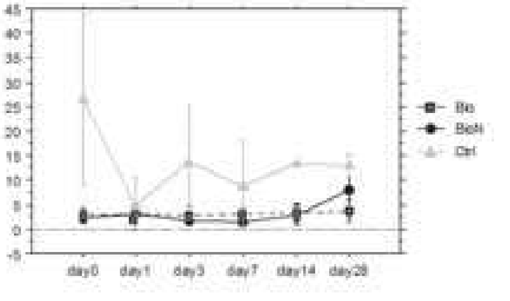

Figure 3.- LMagg (>2mm) proportion (%) dynamic for each treatment, during the time.

The addition of biochar also resulted in a significant increase in soil microbial respiration (F=17.7, p=0.02, df=2). The activity of the microorganisms produces binding agents that promote the formation of Magg (Six et al., 2004). This response could be related to the decomposition of readily decomposable compounds and to the oxidative activity (Le Guillou et al 2011). However, in our case, the higher soil respiration rate did not influence the formation of Magg. In addition, and probably due to suppression by the addition of mineral N, the respiration rates on N charged biochar containing soils were 10 times lower than in the biochar amended soils, but still with no influence on Magg formation.

Besides, the addition of biochar charged with N did affect the hierarchical model of aggregation as observed by the weak correlations between Magg and magg (Table 1), and the results didn't fulfil clearly what is established by the model, although showed a similar pattern. This coincides with the conclusions obtained by Le Guillou et al. (2011), who confirmed the hypothesis that an addition of mineral N in combination to high C:N added OM to soil microbial communities adapted to low soil mineral N content depresses soil microbial activity. So differences might be explained by the effect on the microbial community response and its activity, but more research is needed to explain it. Aggregation and whether another substrate, plant-roots, mycorrhizal fungi or active-C source might be needed to increase WSA in biochar-amended soils.

Bio Bio N	LMagg	SMagg	magg	silt	clay	Ctrl	LMagg	SMagg	magg	silt	clay
LMagg	1	0,52	-0,53	-0,15	0,01	LMagg					
SMagg	0,69*	1	**-0,89*****	-0,39	0	SMagg	0,13				
magg	-0,52	-0,51	1	0,03	0,02	magg	**-0,89*****	-0,21			
silt	-0,40	-0,36	-0,38	1	-0,36	silt	-0,43	-0,47	-0,43		
clay	0,14	0	0,03	-0,36	1	clay	0,22	0	-0,1753	-0,24	

Table1. - Correlations (Adjusted p-values by Holm's method: *<0.1 and ***<0.0001) between the mass balance values of each fraction during the time. The values are presented for each treatment in a different corner: BioN in the right down side corner, Bio in the middle up corner and the control in the left down corner.

In conclusion, when biochar is added, the soil still follows the hierarchical model of aggregation, where gradually, presented a stabilization within the magg and consequently within Smagg. The interactive effect of biochar, mineral N, soil microorganisms activity and soil aggregation remain still unknow and complex.

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Appendix V

Effects of biochar and wood ash amendments in the soil-water-plant environment of two temperate forest plantations

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Forest biomass is considered an alternative to fossil fuels in energy production, as part of global strategies for climate change mitigation. Application of by-products such as wood ash (WA) and biochar (BC) to soil could replace the nutrients removed by tree harvesting and could also increase soil carbon stocks. However, the extent to which these amendments can provide benefits depends on how the by-products interact with the soil-water-plant system. We studied the short-term responses of WA and BC application in two different mineral soil-water-plant systems in temperate forests: A. Typic Udorthent (TU) with mature *Pinus radiata*; B. Typic Dystrudept (TD) with young *Quercus pyrenaica*, to test the following hypotheses: (1) the application of WA and BC will increase nutrient uptake by plants, but (2) these products could induce toxicity in the soil-water-plant system, and (3) in case of no toxicity, plant biomass growth in these temperate forest soils will increase due to increased plant nutrient uptake. Biochar was applied at rates of 3.5, 10, and 20 Mg ha^{-1} and WA at rates of 1.5, 4.5, and 9 Mg ha^{-1} (calcium equivalent). A nitrogen enriched treatment was applied with the intermediate doses. Ecotoxicity testing indicated that WA and BC were not toxic, although Ni uptake increased in biomass of the TU after BC + N application. BC increased SOC stocks of both sites, depending on treatment. In TD BC increased K uptake by plants, but did not increase biomass. In summary, this study shows that the application of BC and WA had different effects on the soil - water-plant system in two different forest soils. This difference was attributed to (i) the soil characteristics, (ii) the application rates and (iii) whether or not nitrogen was applied. Long-term field experiments are required to test the performance and potential toxicity of these by-products as soil enhancers.

KEYWORDS

biochar, wood ash, fertilization, toxicity, SOC, soil pH, pore water

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1. Introduction

Forest tree biomass has become a substantial source of biomass-based energy in the northern countries over last decades (Pugliese et al., 2014). Increased production energy based on forest biomass is envisaged in either international or national climate and energy strategies (e.g., EVE, 2017), as an alternative to the use of fossil fuels and as part of global climate change mitigation strategies, such as the target of carbon neutrality by 2050 proposed by the European Union within the "Green Deal." Biomass production, the raw material for fulfilling these targets, are mainly determined by climate and soil.

Application to soil of products derived from bioenergy production, such as wood ash (WA) and biochar (BC), has been proposed as a means of enhancing soil quality within the circular economy framework, as well as a solution for disposing of the residues (Insam and Knapp, 2011). These products can replace the nutrients removed by tree harvesting, as well as increase soil carbon stocks, particularly BC (e.g., Sohi, 2012). However, the extent to which soil amendments can provide benefits to soil fertility and plant nutritional status depends on the interactions between the added products and the soil-water-plant environment.

Wood ash has been used in both the USA and Europe to replace nutrients in forest soils (e.g., Pitman, 2006). Calcium (Ca) is the most abundant element in WA, followed by potassium (K), magnesium (Mg), and phosphorus (P) (Reid and Watmough, 2014), as well as increasing pH directly through the reaction with water of the anion bound to the Ca. Although WA affects soil physical properties (Moragues-Saitua et al., 2017), it is mainly used to modify the chemical properties of soil (Demeyer et al., 2001).

Many governments have become interested in the production and use of BC, especially because of its potentiality in climate change mitigation, mainly by reducing greenhouse gas emissions in energy production, as well as increasing C persistence within the soil (Lehmann et al., 2021). Biochar is derived from a pyrolysis process and could be used to increase organic matter stocks in soils while increasing soil quality (Lehmann and Joseph, 2009). Biochar has the potential to increase the cation exchange capacity of soil (Liang et al., 2013) and to enhance nutrient retention (Biederman and Harpole, 2013) because of its characteristic properties, such as porous structure, large surface area and negative surface charge. Biochar can also supply nutrients and liming compounds, depending on the ash content and how fresh the product is (Verheijen et al., 2009).

In addition to nutrients, hazardous heavy metals, such as cadmium (Cd), chromium (Cr), zinc (Zn), and nickel (Ni), are also concentrated in WA and BC (e.g., Reimann et al., 2008). WA application guidelines were developed in many countries, some as essential components of biomass harvesting guidelines (Titus et al., 2021) and its use on forestry and agriculture is regulated in some European countries, such as Denmark, Finland, Lithuania, Sweden, Austria, and Germany (Hannam et al., 2018). However, chemical analysis alone is not sufficient to estimate the risk associated with the use of these materials as soil fertilizers (Malara and Oleszczuk, 2013). Interactions between the soil and products applied may make it difficult to predict how these compounds will behave in the ecosystem (Oleszczuk et al., 2013). In this respect, biological assays may complement chemical analysis and provide a wider overview to increase knowledge about possible interactions between various contaminants and demonstrate the existence or absence of toxic effects in a wide range of organisms (Oleszczuk et al., 2013).

Some studies have examined the effects of WA on heavy metal contents in soil, water and forest biota (Olsson et al., 2017). By contrast, studies reporting toxic or beneficial effects of the application of BC to forest soils remain scarce (Gogoi et al., 2019). There is also a lack of research examining both toxicological and nutritional effects simultaneously in the soil-water plant environment of temperate forests where WA or BC have been applied. The existing meta-analysis based on studies of the application WA and BC in the field could provide an overview of the significant effects. For example, Jeffery et al. (2011) reported an overall small, but positive effect of BC application on crop production, suggesting liming and increased water holding capacity of the soil as the main underlying mechanisms. On the other hand, Augusto et al. (2008) reported that WA application only increased stand production in organic boreal soils, and that it should primarily be considered a liming treatment. However, most authors agree that the effects BC or WA are not universally beneficial (Augusto et al., 2008; Novak et al., 2010; Jeffery et al., 2011; Reid and Watmough, 2014). Therefore, whether WA and BC act as sources or sinks for nutrients and toxic compounds depends on, as pointed out by Clough et al. (2013) and Malara and Oleszczuk (2013), (i) feedstock material, (ii) burning processes, (iii) soil characteristics such as pH, surface area, porosity, cation exchange capacity, (iv) transfer of nutrients into the amended soil, (v) vegetation (Schiemenz et al., 2011), and (vi) climate.

In the present study, we examined the short-term response (during the first 3 years after application) of the soil-water-plant environment in two forests, to address the following objectives: (1) evaluate if application of WA and BC increase plant nutrient uptake from forest soils improving the nutritional status of the vegetation, (2) evaluate if these products, mainly at high doses, induce toxicity in the soil-water-plant system, and (3) evaluate if WA and BC increase the SOC stocks in temperate forests.

2. Materials and methods

2.1. Biochar and wood ash production

Biochar was produced by pyrolysis (450°C) of *Myscanthus* sp. in a Pyreg® pyrolysis unit. WA was produced by combustion of *Pinus radiata* D. Don harvest residues in a commercial biomass boiler. The type of WA used in this study was bottom ash. The chemical characteristics of the WA and BC used are summarized in **Table 1**.

2.2. Experimental design and study sites

This study was conducted in two experimental sites located on the Atlantic side of the Basque country (northern Iberian Peninsula). The sites are both - characterized by a mean annual temperature of 10.5°C and a mean annual precipitation of around 1,200 mm (EUSKALMET, 2012–2014).

The treatments consisted of spreading different doses of BC and WA (calcium equivalent) on the soil surface, with and without nitrogen. The product was spread on the topsoil. Biochar doses of 3.5, 10, and 20 Mg ha^{-1} corresponded to low BC(L), intermediate BC(I), and high BC(H) treatments, respectively, although these treatments

TABLE 1 Chemical characterization of the biochar and wood ash applied in the field trial.

	Wood ash	Biochar	Biochar + N
P (g/kg)	1.26	1.58	1.23
Ca (g/kg)	47.1	18.8	12.5
Mg (g/kg)	3.22	1.92	1.83
Na (g/kg)	2.02	0.81	0.55
K (g/kg)	13.5	12.18	8.95
Al (g/kg)	37.7	2.07	2.16
Cu (mg/kg)	38.9	13.48	10.4
Zn (mg/kg)	223.6	67.8	44.2
Fe (mg/kg)	19619.2	2531.5	2187.7
Mn (mg/kg)	682.7	196.1	168.6
Cd (mg/kg)	2.12	0.15	0.13
Pb (mg/kg)	24.2	0.01	0.04
Cr (mg/kg)	172	79.9	97.5
Ni (mg/kg)	82.9	32.6	25.5
C (g/kg)	309	858	872
N (g/kg)	0.6	5.1	11.3
C/N	515	168.2	77.2
H/C	1.2	0.4	1.4

were not applied at both sites (see below). Wood ash doses of 1.5, 4.5, and 9 Mg ha^{-1} of WA corresponded to treatments WA(L), WA(I), and WA(H). Nitrogen [115 Kg ha^{-1} of nitrogen (N) in the form of ammonium nitrate] was applied with the intermediate doses [BC(I) and WA(I)] to produce treatments BC(I)N and WA(I)N. Control (Ctrl) treatment, with no addition of any compound, was also considered.

Two experimental sites (ES) were established in different soil types, and four treatments WA(I), WA(I)N, BC(I) and BC(I)N plus the control were common in both. The Karrantza experimental site (ES-K) was established in May 2012, in a 25 years old *Pinus radiata* D. Don stand, in the Karrantza valley (at 280 m a. s. l, 30N ETRS89 475081, 4786389). The soil was characterized as a Typic Udorthent (Soil Survey Staff, 2014) (**Table 2**), with a 3.9% SOC content, C:N ratio of 14.4 and pH 4.8. Two additional treatments, BC(L) and WA(L), were applied in this site. The high dose treatments BC(H) and WA(H) were not applied. A complete randomized block design with three blocks running perpendicular to the slope (25%) was established. In each block there were three plots per treatment. The size of each plot was 8 × 8 m. A buffer distance of at least 10 m was left between plots. Therefore, in each block there were 21 plots.

The second experimental site (ES-O) was established in September 2013 on the southern slope of the Oiz Mountain (760 m a. s. l. 30N ETRS89 532673, 4785572) in a 2 years old *Quercus pyrenaica* Willd. plantation mainly covered by shrubs and grass. The soil was characterized as a Typic Dystrudept (Soil Survey Staff, 2014; **Table 2**). It contains a 10.8% of SOC characterized by a C:N ratio of 16.8 and a pH 3.8. Two additional treatments, BC(H) and WA(H), were applied in this site. The low dose treatments BC(L) and WA(L) were not applied. Four plots per treatment were established in a complete randomized design. The size of the plots in ES-O was 3 × 3 m, leaving a buffer distance of at least 1 m between plots.

TABLE 2 General characterization of the soil in each experimental site of Karrantza and Oiz (ES-K and ES-O).

	ES-K	ES-O
Soil class (Soil Survey Staff, 2014)	*Typic Udorthent*	*Typic Dystrudept*
Texture	Loam	Sandy loam
Sand (%)	36.8 (0.5)	67.9 (4.6)
Silt (%)	39.7 (1.2)	23.8 (1.9)
Clay (%)	23.1 (0.3)	8.3 (1.0)
Bulk density (g cm^{-3})	1.00 (0.2)	0.68 (0.2)

Texture was measured by laser diffractometry. Bulk density was measured by the Archimedes' displacement method (Flint and Flint, 2002). Data show means and standard deviation in parenthesis of different parameters.

2.3. Sampling, processing, and analysis

2.3.1. Soil sampling, processing, and analysis

In ES-K, the soil was sampled 6, 18, and 30 months after treatment application, always in November. In ES-O the first soil sampling was performed 6 months after treatment application, and the following two sampling periods 15 and 26 after treatment application, in November too. Samples of soil (upper 10 cm after separated the forest floor layer by hand) were collected at ten randomly selected points in each plot, with a gouge auger (D = 2 cm). The soil samples were air dried, sieved (2 mm) and stored dry at room temperature until analysis. In the case of the ES-K, samples of the three plots per block were compiled, resulting in three composite samples per treatment.

Soil pH was determined in water (1:2.5). Total carbon, hydrogen and total nitrogen were determined in a LECO TruSPEC® CHN-S elemental analyzer. The total carbon was considered equivalent to the soil organic carbon (SOC) content, due to the absence of carbonates in both soils. The chemical composition (pseudo-total amounts of P, K, Mg, Ca, Ni, Cd, Cr, and Pb) was measured by ICP-AES with CCD (Varian, Inc., California, CA, USA). The samples were previously digested by acidic digestion (mixture of nitric-perchloric acid, 85–15%) (Croisé et al., 1999). Nutrient and metal contents in soil were converted to absolute values (kg ha^{-1}) considering the bulk density of each sampling unit. Bulk density was measured by the Archimedes' displacement method (Flint and Flint, 2002).

2.3.2. Vegetation sampling, processing, and analysis

Aboveground part of the vegetation present in the studied sites was sampled to determine biomass production and available nutrient content *via* plant uptake. In ES-K, the understorey vegetation and tree foliage were sampled 6, 18, and 30 months after treatment application. In ES-O only aboveground biomass of understorey vegetation was sampled (trees in ES-O were too young for foliage sampling) 15 and 26 months after treatment application.

Foliage samples were collected from the trees in each experimental plot in ES-K. One hundred needles were randomly collected from randomly selected branches bearing current-year needles. In both sites, samples of understorey vegetation were collected from a randomly selected 50 × 50 cm quadrat in each plot. Before analysis, the foliar and vegetation samples were oven-dried (70°C) to a constant weight, milled (0.50 mm) and stored dry at

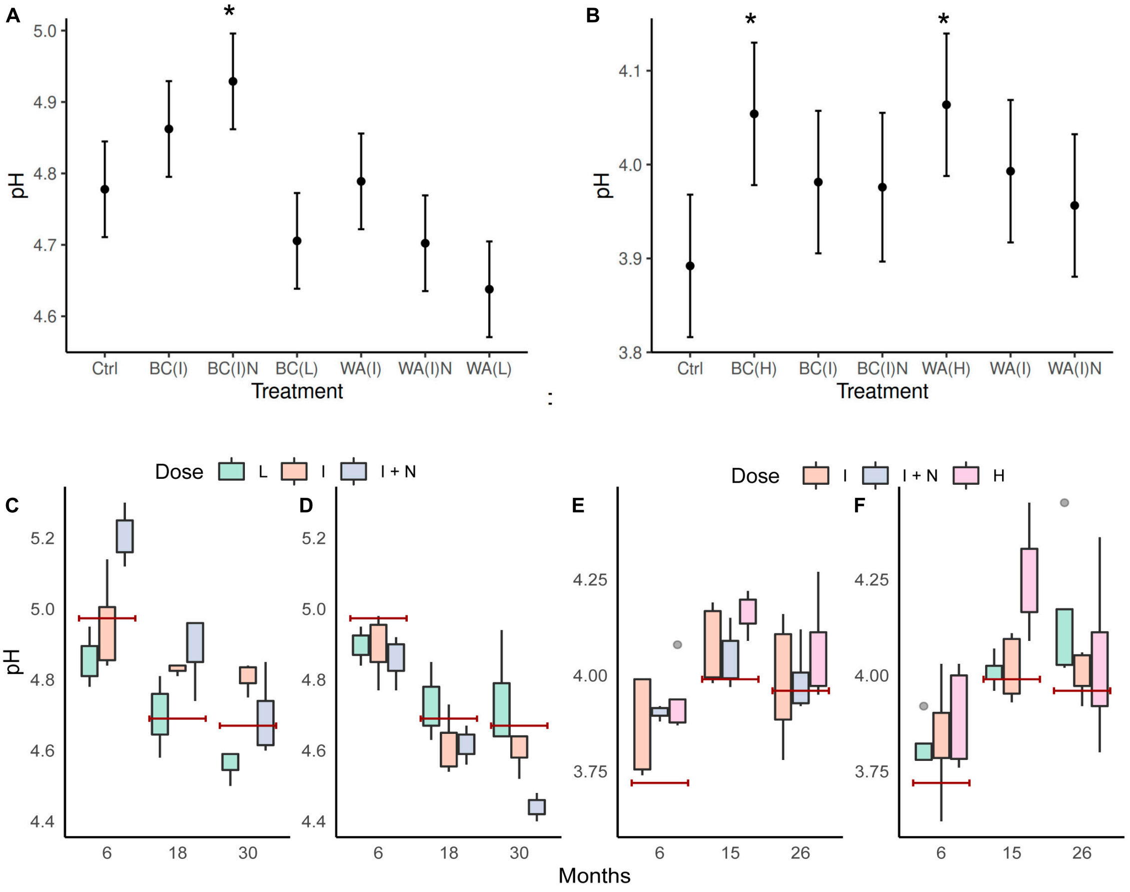

FIGURE 1

Representation of estimated Marginal Means (EMM) of soil pH (top) by treatment and experimental site: Experimental site of Karrantza [ES-K, **(A)** and Experimental site of Oiz (ES-O, **(B)**], and boxplots showing soil pH values of each experimental site (bottom): ES-K **(C,D)** and ES-O **(E,F)** during time (X-axis). Values for biochar (BC) treatments are represented by boxplots c and e, and wood ash (WA) treatments are represented by boxplots **(D,F)**. EMM of control is represented in the boxplots by red lines, to provide a visual comparison. High dose (H, purple), intermediate dose (I, pink) and low dose (L, green); 0.8% of N (always with intermediate rate), (I + N, blue). Boxes show the density data within the first to the third quartile of the dataset for each treatment. The horizontal line inside the box represents the median value. The upper and lower whiskers extend from the higher to the lower pH data. Means which differ significantly from control are in bold and mark with asterisks (*$p < 0.05$).

room temperature for nutrient analysis. In the case of the ES-K, as performed with soil samples, samples of the three replicate plots (same treatment) within the same block were mixed to perform composite samples per block, resulting in three composite samples per treatment.

Dry weights of oven dried understorey biomass and needle samples were recorded and converted to production units: kg ha^{-1} in the case of understorey biomass, and kg 100 needle^{-1} for pine tree foliage. Nutrient and metal content in aboveground biomass (tree foliage and understorey biomass, P, K, Mg, Ca, Ni, Cd, Cr, and Pb) were determined by ICP-AES with CCD (Varian, Inc., California, CA, USA). The samples were previously digested by acidic digestion (mixture of nitric-perchloric acid, 85–15%) (Croisé et al., 1999). Nutrient and metal contents in soil were converted to absolute values (kg ha^{-1}, for understorey biomass) considering the production units.

2.3.3. Pore water sampling, processing, and analysis

Water from soil pores was sampled twice after treatment application. The first sampling was performed after the first prolonged winter rainfall after treatment application, after 8 months in ES-K and after 4 months in Es-O, and the second sampling was performed 32 months after treatment application in ES-K, and 17 months after in ES-O. Pore water was extracted using MacroRhizon^{TM} samplers (Rhizosphere Research Products, Wageningen, Netherlands) inserted at 30 cm depth and connected *via* 50 ml disposable syringes. The samples were frozen until analysis, 30 ml per sample.

The pore water samples were subjected to eco-toxicity screening. Two commercial microbial bioassays were used: a growth assay (MARA, Microbial Assay for Risk Assessment) with the first pore water samples collected and a bioluminescence assay (LumiMARA) with the samples of the second pore waters collection event.

In the MARA, toxicity was evaluated by measuring inhibition of the growth of pellets formed by ten bacterial strains and one yeast inoculated with undiluted soil pore water samples, as described by Gabrielson et al. (2003). The pH of the pore water was previously adjusted and the samples prepared for inoculation as described by Wadhia et al. (2007). We examined pellet formation by colorimetry. Absorbance at 620 nm was measured in a microplate reader (Zenyth 3100. Anthos Labtec Instruments GmbH, Salzburg. Austria). Inhibition of pellet growth was assessed for each individual species, by examining the mean values of all constituent species in the test.

TABLE 3 Effects of the treatments (Third row) in soil nutrients, reported by F-values of the ANOVAs (df = 6).

	N (Kg ha^{-1})		P (Kg ha^{-1})		K (Kg ha^{-1})		Mg (Kg ha^{-1})		Ca (Kg ha^{-1})	
	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O	ES-K	ES-O
Treatment effect	F = 0.72	F = 3.08*	F = 1.17	F = 1.97	F = 4.85***	F = 0.72	F = 5.7***	F = 0.63	F = 3.86**	F = 1.81
Ctrl	831 ± 69.8	2,120 ± 144	134 ± 8.53	104 ± 7.66	5,123 ± 214	781 ± 53	894 ± 34.5	262 ± 19	289 ± 32.8	495 ± 67.8
WA(L)	+117.6	–	−6.09	–	−199.1	–	−71.31	–	−12.26	–
WA(I)	+19.9	+322.3	+0.28	−1.17	−549.2	−0.36	−94.62	+1.33	+133.47*	+117.2
WA(I)N	+148.7	+446	+4.67	+2.06	−510.3	−0.54	−111.48*	−33.12	+23.08	−156.4
WA(H)	–	+408.7	–	+4.72	–	−0.18	–	−9.89	–	+29.6
BC(L)	+67.4	–	−15.59	–	−805**	–	−155.08**	–	−15.17	–
BC(I)	+43.5	+243	+8.73	+9.87	−198.5	−0.01	−29.17	−27.4	+82.94	+14.1
BC(I)N	+93.0	+405.7	−4.12	+4.53	−725.8**	+0.00	−149.16**	−7.26	+72.49	−108.8
BC(H)	–	+815.6**	–	+24.29	–	−0.34	–	−20.62	–	−43.3

Treatments Listed in the first column. Ctrl, control; WA, wood ash; BC, biochar; (H), high dose, (I), intermediate dose, (L), low dose; N, nitrogen addition (0.8% of N). Estimated marginal means of control (Fourth row, in bold) ± SE, and comparisons of each treatment against control. Positive values mean increase over control and negative values mean decrease. Significant effects and means which differ from control are in bold and mark with asterisks (*$p < 0.05$; ** $p > 0.01$; *** $p > 0.001$).

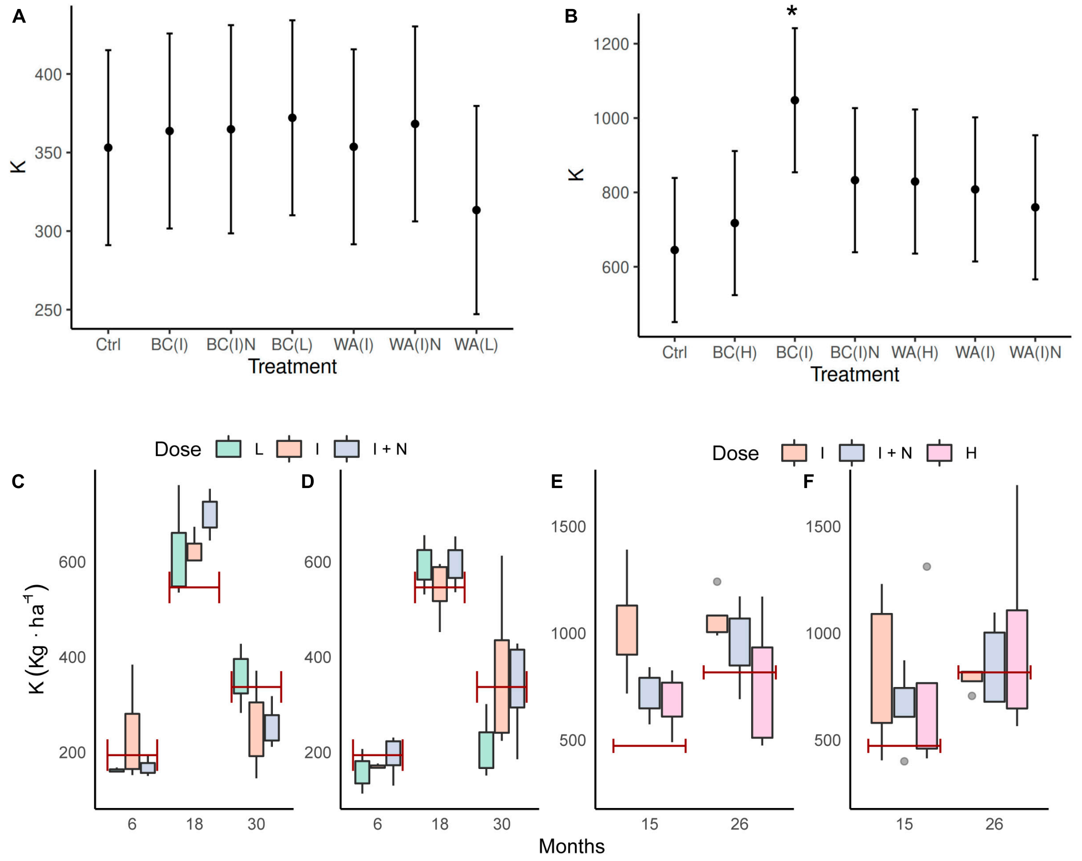

FIGURE 2

Representation of Estimated Marginal Means (EMM) of K content (Kg ha^{-1}) in understorey biomass (top) by treatment and experimental site: Experimental site of Karrantza [ES-K, **(A)**] and Experimental site of Oiz [ES-O, **(B)**], and boxplots showing K content (Kg ha^{-1}) of understorey biomass at each experimental site: ES-K **(C,D)** and ES-O **(E,F)** during the time (X-axis). Values for biochar (BC) treatments are represented by boxplots **(C,E)**, and wood ash (WA) treatments are represented by boxplots **(D,F)**. EMM of control is represented in the boxplots by red lines, to provide a visual comparison. High dose (H, purple), intermediate dose (I, pink) and low dose (L, green); 0.8% of N (always with intermediate rate), (I + N, blue). Boxes show the density data within the first to the third quartile of the dataset for each treatment. The horizontal line inside the box represents the median value. The upper and lower whiskers extend from the higher to the lower K content data. Means which differ significantly from control are in bold and mark with asterisks (*$p < 0.05$).

The LumiMARA was also performed with undiluted soil pore water samples, for toxicity screening. Eleven bioluminescent bacterial strains (nine marine and two freshwater bacteria) were incubated with pore water samples, following the manufacturer's protocol for environmental samples (Jung et al., 2015). The toxicity was indicated by the reduction in luminescence. Light from the luminescent bacteria was measured in a microplate reader (Zenyth 3100. Anthos Labtec Instruments GmbH, Salzburg. Austria) and data were processed with the LumiMARA software (NCIMB Ltd., Bucksburn, Aberdeen. UK).

The pore water samples were treated with nitric acid prior to chemical analysis. The chemical composition (P, K, Mg, Ca, Ni, Cd, Cr, and Pb concentrations) of pore water in the first sampling was determined by simultaneous inductively coupled plasma atomic emission spectrometry (ICP-AES) with charge coupled devices (CCD) (Varian, Inc., California, CA, USA).

2.4. Statistical analysis

Linear mixed-effects models (LMMs) were used through REML (Restricted Maximum Likelihood) using lme4 v.1.1-30 (Bates et al., 2015) and lmerTest v.3.1-3 (Kuznetsova et al., 2017) packages, to investigate the effects of treatments (fixed effect) on each soil parameter, and each experimental site was analyzed separately.

Multiple models have been adjusted for each of the data sets (soil, needles, understorey biomass) in which treatment, time and interaction have been included as fixed factors, and block as random factor. On the other hand, multiple models have been adjusted for the pore water dataset where treatment and block have been included as a fixed factor.

For each of the models, the overall effects of each factor have been analyzed using a type III ANOVA. A post-hoc pairwise comparison of the estimated marginal means (EMM) with the emmeans package v.1.7.5 (Lenth et al., 2022) has been performed to identify significant differences between each treatment and its respective control. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Soil pH and soil organic carbon

Figure 1 shows the pH in soil of the two experimental sites: ES-K and ES-O. A treatment effect is observed (F = 9.13, df = 6, $p < 0.001$). Soil pH increased in ES-K only when treated with BC(I)N (median values of 5.2 and 4.96, in month 6 and 18, respectively), over Ctrl (medians of 4.94 and 4.64, months 6 and 18) although pH values equated at the end of the research period (**Figure 1C**). In ES-O application of high doses of WA and BC [WA(H) and BC(H)], increased soil pH (**Figure 1**; F = 2.86, df = 6, $p = 0.02$). Being the medians of Ctrl of 3.72 and 3.96 in the first two sampling times and of 3.89 and 4.24 for WA(H) and of 3.88 and 4.17 for BC(H) (months 5 and 15, respectively).In ES-K, BC affected SOC content (F = 3.03, df = 6, $p = 0.02$). BC(I)N presented a significant increase of 34% over control [estimated marginal mean (emm) of 12.1 ± 0.77 Mg·ha^{-1}]. In ES-O, only the highest dose of BC increased in a 42% SOC content over control (emm of 38.3 ± 2.95 Mg·ha^{-1}; F = 3.4, df = 6, $p = 0.01$).

3.2. Nutrients in the soil-water-plant system

Table 3 summarizes treatment effects, estimated marginal means of nutrient content in soil (control), and the differences of each treatment.

Biochar(I) treated soils in ES-O present significantly superior values of K content in understorey biomass (medians of 431 and 841 Kg ha^{-1}, for months 15 and 26, respectively), if compared to control (1,001 and 1,020 Kg ha^{-1} months 15 and 26; **Figure 2**; t-ratio = –3.085, df = 39, $p = 0.05$), although no evidence of treatment effect has been found (**Supplementary Table 2**). The rest of the treatments did not affect understorey biomass nutrient content in none of the experimental sites (**Supplementary Table 2**). Nutrient content in needles was not affected either, by none of the treatments (**Supplementary Table 3**).

Pore waters did not contain differences in macronutrient content between treated and not treated samples, in none of the experimental sites (**supplementary Table 4**).

3.3. Heavy metals in the soil-water-plant system

Figure 3 shows the Cd in soil of the ES-K and the ES-O, and changes observed in BC treated plots in ES-K, e.g., medians of 0.13, 0.17, and 0.11 g·ha^{-1} in month 30 [BC(L), BC(I), BC(I)N, respectively], significantly lower (F = 3.29, df = 6, $p = 0.01$) than control (e.g., 0.2 g·ha^{-1}, month 30). No more changes in heavy metal content were observed in none of the soils after application of any treatment (**Supplementary Table 1**).

Understorey biomass from ES-K increased 1.5 times Ni content after BC(I)N application (F = 16.01, df = 6, $p < 0.001$) if compared with Crtl (emm of 259 ± 41.4 g·ha^{-1}). No more effects were observed in aboveground biomass heavy metal content (**Supplementary Tables 2**, **3**).

Pore waters collected from BC(L) treated plots in ES-K, presented an estimated marginal mean of 0.06 ± 0.01 mg Mn cm^{-3}, 6 times higher than Ctrl (0.01 mg·ha^{-1}). No more effects were observed in pore waters after treatment application (**Supplementary Table 4**).

3.4. Ecotoxicity testing

WA and BC did not induce toxic effects in the pore waters (**Figure 4**). The MARA indicated that application of WA and BC caused very low growth inhibitions rates that were not different from those in the control treatments. The LumiMARA showed that application of WA to ES-K yielded significantly greater reduction of luminescence in plots amended with WA(L) than in control plots. However, the overall mean inhibition of luminescence and growth did not exceed the 40% in either of the experimental sites.

3.5. Plant biomass growth

No significant differences were observed in understorey biomass or needle production in relation to WA and BC application (**Supplementary Figure 1**).

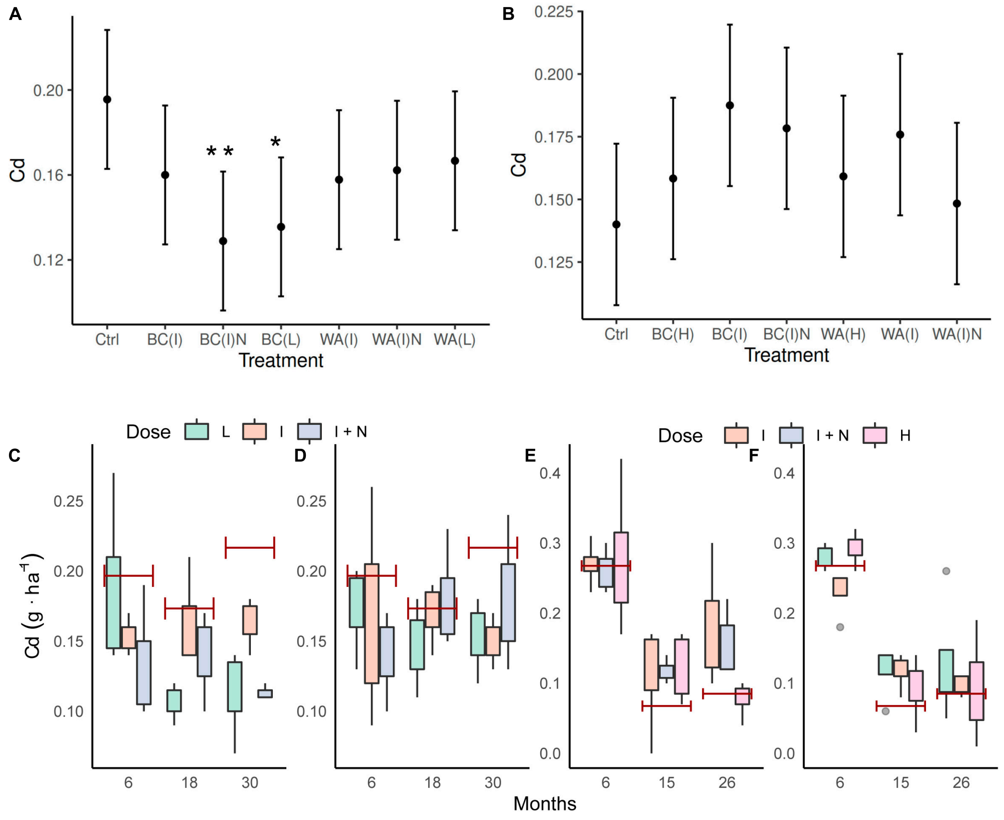

FIGURE 3

Estimated Marginal Means (EMM) of Cd content (g·ha^{-1}) in soil (top) in ES-K soil **(A)** and in ES-O **(B)**, and boxplots showing Cd content in soil at each experimental site: ES-K **(C,D)** and ES-O **(E,F)** during the time (X-axis). Values for biochar (BC) treatments are represented by boxplots c and e, and wood ash (WA) treatments are represented by boxplots **(D,F)**. EMM of control is represented in the boxplots by red lines, to provide a visual comparison. High dose (H, purple), intermediate dose (I, pink) and low dose (L, green); 0.8% of N (always with intermediate rate), (N, blue). Boxes show the density data within the first to the third quartile of the dataset for each treatment. The upper and lower whiskers extend from the higher to the lower Cd content data. Means which differ significantly from control are in bold and mark with asterisks (*$p < 0.05$; **$p > 0.01$).

4. Discussion

4.1. Vegetation response to soil amendments

The increase in K uptake by plants observed in ES-O after BC application suggests a fertilization effect of BC, as reported by other authors (e.g., Ingerslev et al., 2014). Contrary, when WA was applied to ES-O, even though the total content of K was greater in WA than in BC (**Table 1**), no increased K uptake by plants was observed after application of WA to the ES-O. These differences in nutrient release from WA and BC might be complex, due to chemistry of the product (Verheijen et al., 2009) and its interaction with soil biota (e.g., Anderson et al., 2011) or organo-mineral associations (Fernández-Ugalde et al., 2017). Buss et al. (2019) suggested an improved fertilizing effect of biochar, derived from a moderate supply of K instead of instant leaching of K from WA. The increased K uptake by plants observed after BC application was not proportional to the dose applied, contrary to what observed by many authors (Clapham and Zibilske, 1992; Demeyer et al., 2001; Park et al., 2012). This suggests that the highest doses of both WA and BC applied to the Typic Dystrudept may have resulted in formation of a thick amendment layer on top of the soil, as reported in some studies after forest fires (Bodí et al., 2014) or may have prolonged changed the physicochemical properties of the soil (Brais et al., 2015) thus affecting the processes taking place in the soil, as a different dose-depended response was observed in soil too, in ES-O. Indeed, the system in ES-O is probably adapted to make use of K more efficiently (Cornut et al., 2021), as K was efficiently taken up by plants and prevented its loss *via* leaching, despite the low energy of adsorption of K (Brais et al., 2015). This is consistent with some studies analyzing the effects of wood ash, were increase in exchangeable K concentration in the forest floor was reported, and revealed its fertilizing potential in relation to stand growth in K deficient forests (Augusto et al., 2008; Solla-Gullón et al., 2008; Brais et al., 2015).

However, we did not observe biomass growth after the increased nutrient content in biomass. A review on the effects of BC application in forest ecosystems (Li et al., 2018) concluded that BC addition alone is not sufficient to meet the nutrient needs for tree growth and productivity. In fact, in an analysis of *P radiata* D. Don stands in the Basque Country, most of the soils were found to be deficient in P, and fertilization with between 20 and 150 Kg P ha^{-1} was recommended (Martínez de Arano, 1999). Considering that in our field experiment the largest dose applied was 31.6 Kg P ha^{-1} (treatment BC(H) in ES-O) and that Martínez de Arano (1999) recommended 50–100 Kg P ha^{-1} for this type of soil, pine growth was probably limited by lack of P. It is therefore important to know which are the limiting nutrients in each soil, as well as to identify the needs of the system itself.

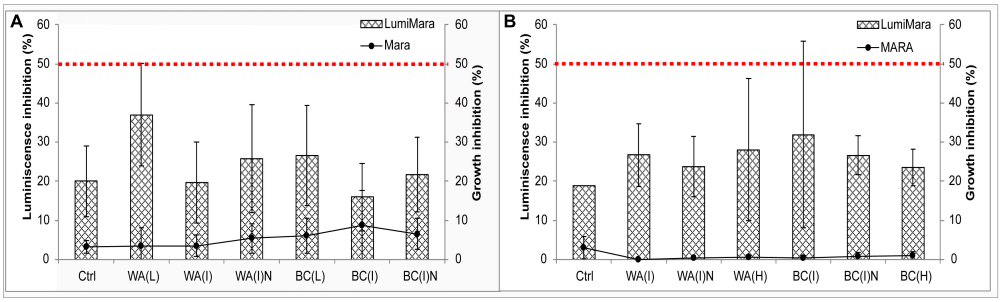

FIGURE 4
Effect of the treatments (Ctrl, control; BC, biochar; WA, wood ash; (H), high dose; (I), intermediate dose; (L), low dose; *N* = 0.8% of *N*) on bioluminescence inhibition (left y axis) measured with LumiMARA (bars) and pellet mean growth inhibition represented by dots (right y axis) determined by the MARA bioassay. Treatment effect was analyzed for both experimental sites: ES-K **(A)** on the left side, and ES-O **(B)** soil on the right side. Red flashing line (dotted red line) represents the 50% of growth and luminescence inhibition threshold for being toxic).

The observed increase in Ni uptake by understorey plants in ES-K when BC(I)N was applied is another example of the complexity of the interactions that occur after the soil amendments have been applied. Even though BC(I)N contains less Ni than WA(I) and BC(I), it is the only treatment in ES-K that directly contributes to Ni uptake by understorey biomass in ES-K. This could be due to the increase in soil pH observed. Ni absorption usually increases with increasing pH up to five (Pandaa et al., 2007). It could be also linked to the decrease in Mg, Cd, and K content observed in soil and subsequent reduction of their inhibitory effect on the absorption and translocation of Ni^{2+} from roots to shoots (Temp, 1991), or probably, it could be due to the interaction of the mentioned reasons above. However, more research and time is needed in order to prevent toxicity, since Yusuf et al. (2011) suggested that Ni is a high mobile trace metal that tends to accumulate in newly formed plant parts as well to seeds. Ni uptake and accumulation in plants depend on plant species, soil pH, quantity, and form of Ni present in the soil (Hassan et al., 2019). Still more research is needed to understand all processes, since Ni, in contrast to other toxic heavy metals like cadmium, lead, mercury, copper and chromium has received little attention from plant scientists due to its dual character (essential-toxic) and complex electronic chemistry which is a major hurdle in disclosing its toxicity mechanism in plants (Yusuf et al., 2011).

Although nutrition and toxicity are key factors determining plant growth, the yearly fluctuations in biomass growth suggest that water availability is a more constraining factor than nutrients in these ESs, as the variations in needle and understorey biomass production in ES-K (**Supplementary Figure 1**). Table clearly coincide with the variations in annual rainfall: 1,413 mm in 2013, 978 mm in 2012 and 945 mm in 2014 (data obtained by the closest meteorological stations [(<10 km), EUSKALMET, 2012–2014]. A positive net primary productivity-precipitation linear relationship has been widely recognized across many ecosystem types (Sala et al., 2012; Felton et al., 2021). The distribution of rainfall will be increasingly variable and possibilities for the disappearance of this linearity are expected as response of complex interactions (e.g., Felton et al., 2021). It is therefore important, from an ecosystem resilience point of view, to increase water holding capacity if needed, in order to maintain as much as possible the soil moisture during drought periods. BC has widely considered enhancer of the hydraulic properties due to its porosity and soil structuring capacity. However, Moragues-Saitua et al. (2017) did not observe any increase in water holding capacity after 1–2 years in the same experiment.

4.2. Soil response to amendments

The increase in soil pH observed after WA and BC application may just have been too small to increase nutrient uptake by plants. Even considering WA or BC simply as liming treatments for temperate forest soils (Augusto et al., 2008), our findings show that these by-products cannot be generally applied, at least in the short term. As many authors (e.g., Augusto et al., 2008; Godlewska et al., 2021) have mentioned, the effects of amendments are soil and dosage dependent (Hansen et al., 2017), and for example large quantities of BC must be applied in order to detect any increase in pH in the mineral layers of the Typic Dystrudept. This places in doubt the overall effectiveness of BC or WA for liming temperate forest soils in areas with high precipitation rates. Some authors have reported that WA application affects mainly the pH of the topsoil, being the depth of the effect largely dosage dependent (Jacobson et al., 2004; Hansen et al., 2017). Further research observing effects on the organic layers are also needed to complement this type of studies, since these are the horizons that are in direct contact with the amendment and the local changes can lead to effects on the overall ecosystem functioning (Hansen et al., 2017).

Biochar increased SOC stocks in both experimental sites, although the effect varied according to the site and treatment. In the case of ES-K, the increase in SOC was observed when BC(I)N was applied, while in ES-O the same treatment had no effect and the increase in SOC in the highest doses was accompanied by an increase in nitrogen content. This suggests different organic matter dynamics in both sites. Depending on soil properties and characteristics of the site, the biotic community present varies and together determine the pathway that SOM decomposition and transformation follows on each site (Prescott and Vesterdal, 2021). The release of compounds from WA and BC in the field is subjected to many variables on a site-to-site basis, with time since application also playing an important role (Reid and Watmough, 2014). Zhang et al. (2017), Knoblauch et al. (2010), Wardle et al. (2008) concluded that BC or WA addition increases leaf litter and deadwood decomposition at the soil surface, enhancing soil microbial biomass and activity, and thus, increasing the mean concentration of DOC (Gömöryová et al., 2016) that would be translocated through the soil profile. On the other hand, in ES-K, the increase in SOC may be due to the contribution of C through direct incorporation of the biochar in the mineral layer. Fernández-Ugalde et al. (2017) investigated the incorporation of BC-derived C (C4 type plant feedstock) into various particle-size fractions and examined the effect of BC on the storage of total OC in the particle-size fractions in the Karrantza experimental site (ES-K, dominated by C3 type *Pinus radiata*). These authors observed that in the first year, part of the BC spread on the field was degraded and incorporated into the mineral soil. Although increased SOC did not affect plant responses during this field experiment, it may have a long-term fertilization effect (Bot and Benites, 2005) and could contribute to mitigating climate change *via* sequestration of SOC if the incorporation and stabilization rate of SOC in the mineral soil layers is higher than the decomposition rate occurring in the organic layer of the soil. Addition of biochar could also contribute to soil health, soil structure (Six et al., 2004), water holding capacity and other processes (Bronick and Lal, 2005; Moragues-Saitua et al., 2017) that are important for enabling ecosystems to adapt to climate change.

In ES-K, the application of both BC and WA seem to mobilize Cd, K, and Mg in soil, since less content of this nutrients have been observed after applying the amendments. The fate of these elements remains unknown as the concentrations did not increase in any of the fractions analyzed (needles, understorey o pore waters) Some of these nutrients could have accumulated in other parts of the plants, such as roots. However, it is possible that they could have been translocated deeper into the soil adsorbed to DOC (Loganathan et al., 2012) or to the negatively charged small fractions of BC and then translocated deeper into the soil, since Cao et al. (2021) observed that the transport of Cd^{2+} is facilitated in the cotransport of BC.

4.3. Water responses to soil amendments

Pore water analysis and ecotoxicological tests did not provide any evidence of toxicological effects at either of the experimental sites. However, some studies have reported leaching and increased bioavailability of hazardous trace elements after application of BC (Kloss et al., 2015) or WA (Williams, 1997; Ingerslev et al., 2014). Since we observed increased Mn content in pore waters of plots treated with BC(L) in ES-K, this could indicate a potential availability of this toxic element, it is important to conduct long-term field experiments to ensure that substances of this nature are not harmful to water and soil resources or to the entire ecosystem. This is essential for the safe application of BC or WA to soil.

In summary, this research provides further information regarding the effectiveness of using by-products of biomass-based energy production as soil amendments in temperate forests.

The amendments applied do not seem to be toxic or harmful in the two types of forest plantations under study, as revealed by MARA and LumiMARA testing with pore water samples. Concerns about the risks of increase in Ni mobility and availability derived from BC(I)N application are highlighted, although this did not limit the growth of understorey biomass.

These findings show that the addition of biochar and wood ash to temperate humid forest soils affects different sites in different ways depending on the soil type and system, as well as on application rates and whether nitrogen was added. Biochar addition increased K uptake by plants only in ES-O and with the intermediate rate. The biggest rates increase SOC content and pH of the Typic Dystrudept, and similar effects were observed in the Typic Udorthent, but with the intermediate dose combined with N. Application of WA contributed to increase soil pH in the Typic Dystrudept, but not of the Typic Udorthent, and only when high doses were applied. Widespread use of these by-products in forestry plantations on steep slopes in remote locations may be limited because the high cost of transporting and applying large amounts of the products that would reduce the cost-effectiveness of the treatments. On the other hand, WA or BC could be used as liming treatments, or applied to soil as a carbon sink, the effects cannot be generalized and have been shown to be depend on soil type.

It is important to conduct appropriate long-term field experiments to determine whether by-products can be used in climate change adaptation strategies, as well as to ensure that substances of this nature are not harmful to water, soil or the entire ecosystems, as this is essential for the safe application of BC or WA to soil.

Data availability statement

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

Author contributions

LM-S, NG-B, and AA-G contributed to conception and design of the study. GB-C and FB optimized and performed the ecotoxicology tests. LM-S organized the database and performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Appendix V

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2022.878217/full#supplementary-material

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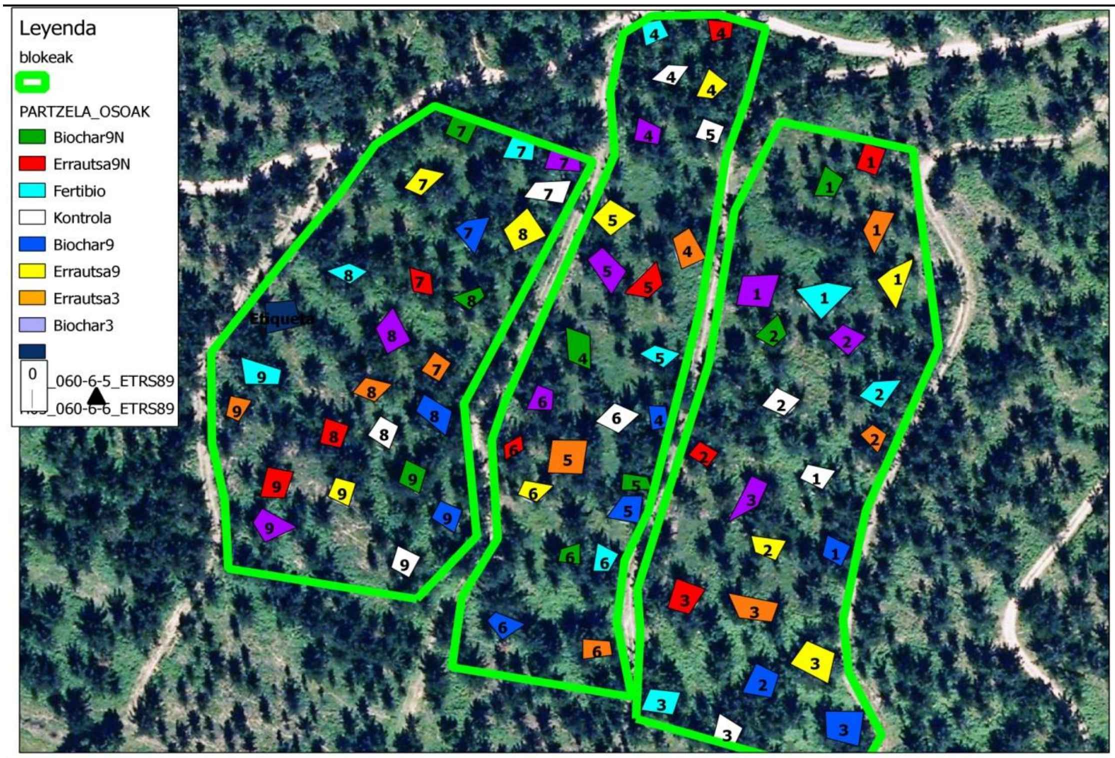

Map.- 1 Map of the study area of Karrantza on orthophoto. Treatment plots appear in polygons, each colour corresponds to a treatment (see legend, in basque) and each number corresponds to the number of replicates. The three blocks arranged along the altitude are represented by the green line. being the right (east) highest and the left (west) lowest.

Map 2.- Experimental site of Oiz. Treatment plots appear in polygons, each colour corresponds to a treatment (see legend, in basque) and each number corresponds to the number of replicates. the slope goes north (highest) to south (lowest)