USE OF CROP RESIDUES AS SUBSTRATES FOR THE CULTIVATION OF KING STROPHARIA (*STROPHARIA RUGOSOANNULATA*) MUSHROOMS

by

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ABSTRACT

King stropharia (*Stropharia rugosoannulata*) is a white rot fungus that produces nutritious edible mushrooms. The species is prized among backyard mushroom cultivators due to its ability to grow on various lignocellulosic substrates in a range of environmental conditions and compete with contaminant microorganisms. These characteristics make king stropharia a great potential tool for enhancing crop residue decomposition in northern environments while producing a valuable crop of mushrooms. However, the species is understudied and underutilized since its production parameters have never been optimized.

This study sought to determine 1) which readily available substrate (of alder chips, barley straw and hemp straw) produces the best yield of king stropharia mushrooms, 2) whether substrate impacts the nutritional content of king stropharia mushrooms, 3) how king stropharia chemically alters substrates, 4) how king stropharia alters substrate microbial communities, and 5) which spent substrate makes the best soil amendment for crop production.

A cultivation trial was conducted at a farm in Prince George, British Columbia, Canada, from June to October 2022. Eight 1 m by 1 m wooden frames of each substrate were prepared. Five frames each of alder chips and barley straw were inoculated with king stropharia spawn and the three remaining frames served as uninoculated controls. Six frames of hemp straw were inoculated, leaving two uninoculated controls.

Substrate samples were collected prior to inoculation and again after the cultivation period. The date, mass and count of mushrooms produced from each frame was recorded. Mushrooms samples were also collected for analysis. Mushroom and substrate samples were

analysed for content of carbon, nitrogen and a suite of other elements. Mushrooms were analysed for protein and lipid content. Substrate sample lignocellulosic biomass fractions (lignin, cellulose and hemicellulose) were quantified, and substrate pH and electrical conductivity were tested. Substrate fungal and bacterial DNA were extracted, amplified, sequenced and analyzed.

Hemp straw tended to be the fastest and highest yielding substrate in the cultivation trial. Hemp straw appears to have been the best performing substrate due to its water content, nutrient profile, lignin content and surface area to volume ratio compared to the other substrates. There also seemed to be a distinctive bacterial consortium associated with the successful cultivation of king stropharia in barley straw and hemp straw, with high relative abundance of the genera *Bacillus* and *Paenibacillus* in these samples. Further cultivation experiments using fresh spawn are necessary to properly assess king stropharia's yield and effect on substrates since the results of this study were impacted by spawn contamination.

All post-cultivation mushroom substrate types had beneficial properties for agricultural soil amendment, even though the substrates were not completely spent at the end of the cultivation trial. King stropharia presents a potential win-win scenario whereby farmers can produce nutritious mushrooms with their crop residues while enhancing crop residue decomposition and nutrient cycling with minimal technology and labour. Although further research is required to fully realize the potential of this species, this study demonstrates how king stropharia can contribute to sustainable agricultural practices.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GLOSSARY

Term	Meaning
AD	Air Dry
ASV	Amplicon Sequence Variant
BE	Biological Efficiency
CFE	Chloroform Fumigation Extraction
DM	Dry Matter
EC	Electrical Conductivity
FTIR	Fourier Transformed Infrared Radiation
FW	Fresh weight
GFIC	Guelph Food Innovation Centre
ICP-OES	Inductively Couple Plasma - Optical Emission Spectrometry
MPN	Most Probable Number
NALS	Northern Analytical Laboratory Service
NRAL	Natural Resources Analytical Laboratory
OD	Oven Dry
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
SMS	Spent Mushroom Substrate
WRF	White Rot Fungi/Fungus

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1 Introduction

Worldwide consumption of mushrooms has been rising steeply since the 1990s, both in overall and per capita consumption (De Cianni et al., 2023; Royse, 2014). This rise has been driven in part by increased consumer awareness of the health benefits of mushrooms as a low-calorie source of protein, nutrients and medicinal compounds. To keep pace with demand, mushroom production must be increased and diversified. There is increasing interest in the sustainable use of agricultural waste streams, such as crop residues, in the face of mounting pressure on global food systems from climate change, population growth and land degradation (Grimm & Wösten, 2018; Selvaraju et al., 2011). The use of crop residues as a substrate for mushroom production presents a potential win-win situation in which the decomposition and nutrient cycling of crop residues is enhanced while producing mushrooms as an additional food source (Grimm & Wösten, 2018; McKoy, 2016; Sheldrake, 2021; Stamets, 2000).

Saprophytic (decomposer) mushroom cultivation can convert low-quality lignocellulosic crop residues into high quality food products (Grimm & Wösten, 2018). The cultivation of white rot fungi (WRF) reduces the lignin content of crop residues, potentially making the spent mushroom substrate suitable for many applications such as a soil amendment, a peat substitute in horticulture, a substrate for further mushroom cultivation, a component of feed for ruminants, or as a bioenergy feedstock (Madadi & Abbas, 2017; Paula et al., 2017; Zied et al., 2020).

Roughly eighty-five percent of the world's mushroom production consists of just five genera of mushrooms: *Lentinula* (*L. edodes*, shiitake)*, Pleurotus* (*P. ostreatus,* oyster mushroom and

P. eryngii, king oyster)*, Agaricus* (*A. bisporus,* button mushrooms)*, Auricularia* (a genus of jelly fungi) and *Flammulina* (*F. velutipes,* enoki) (Royse, 2014; Singh et al., 2020). Higher crop species diversity can result in greater stability and resilience in food systems (Merlos & Hijmans, 2020). In consideration of climate change, population growth and supply chain challenges, creating more resilient food systems is an urgent priority. Therefore, it is important to explore the potential of underutilized mushroom species. One promising species and the focus of this thesis is king stropharia (*Stropharia rugosoannulata* Farl. ex. Murrill).

1.1 About King Stropharia

King stropharia, also known as wine cap mushroom or garden giant, is a nutritious, gourmet mushroom. It is reported to contain 22% protein on a dry matter basis, though the mushrooms are only 8% dry matter (Szudyga, 1978). King stropharia mushrooms have also been found to contain antioxidant polysaccharides and to be a source of niacin (vitamin B3) (Liu et al., 2020; Szudyga, 1978). The mushrooms have a mild, umami flavour and dense, white flesh (Stamets, 2000).

The species has a cosmopolitan distribution, having been reported growing wild in Europe, North and South America, Japan and Oceania (Gibson, 2020). It was first cultivated in the 1960s in Eastern Europe but remains underutilized (Bonenfant-Magné, 2000; Szudyga, 1978). The species has had some limited industrial applications as a biological agent for delignification to make cereal straws more digestible for animals, and to make paper pulp (Bonenfant-Magné, 2000).

King stropharia possesses characteristics that make it popular among amateur and hobby mushroom growers (Mercy, 2021; Szudyga, 1978). King stropharia is a white rot fungus (WRF) (Buta et al., 1989). WRF, along with a limited number of bacterial species, are the

only aerobic organisms able to degrade lignin; they are also able to degrade cellulose and hemicellulose (de Gonzalo et al., 2016; Rodríguez-Couto, 2017). Lignin is a bulky, complex aromatic heteropolymer that provides structure and protection in woody plant cell walls, and is resistant to decay by most microorganisms (Bugg et al., 2011; Zabel & Morrell, 2020). Lignin decomposition occurs through oxidative reactions that break C-C bonds or ether linkages and separate functional groups, aromatic rings and side chains from lignin macromolecules (Zabel & Morrell, 2020). WRF produce various extracellular enzymes involved in the degradation of lignin, including cellulases, laccases, and peroxidases (Bonenfant-Magné, 2000; Bugg et al., 2011).

King stropharia is considered to have high resistance to diseases, pests, and adverse environmental conditions (Szudyga, 1978). This fungus produces fruiting bodies at temperatures as low as 4.5°C and as high as 30°C (Sharma et al., 2007). Mycologist Paul Stamets calls king stropharia "the premier mushroom for outdoor bed culture by mycophiles in temperate climates" (Stamets, 2000). The ruggedness of this species allows for it to thrive in relatively inexpensive, low-tech cultivation systems.

King stropharia can be grown indoors or outdoors on hardwood chips, straw and other crop residues (Bonenfant-Magné, 2000; Sharma et al., 2007; Szudyga, 1978). While its hardiness lends it well to outdoor cultivation, there are practical limitations to the indoor production of king stropharia. Indoor commercial cultivation of this species is considered uneconomical due to the long period between inoculation and fruiting (6 to10 weeks for king stropharia, compared to 2 to 4 weeks for some *Pleurotus ostreatus* strains, for example) and inconsistent yields (Bonenfant-Magné, 2000; Bruhn et al., 2010; Stamets, 2000). Indoor mushroom cultivation is resource- and energy-intensive. Temperature, humidity, light levels, CO_2 levels

and contaminants are carefully monitored and controlled. King stropharia does not produce a consistent return on investment that would justify this level of intensive management. However, given the time and space, it has the potential to be fruitful outdoors with minimal effort, making it a good candidate for on-site inoculation of crop residues.

2 Literature review

2.1 Cultivation methods

Literature on the cultivation of king stropharia is sparse and scattered, and several key publications are now out of print (Domondon & Poppe, 2000; Szudyga, 1978). Below is a summary of information largely drawn from four papers: Szudyga (1978), Bonenfant-Magné (2000), Domondon and Poppe (2000) and Bruhn et al (2010), with supplemental information from Stamets (2000) and web sources.

2.1.1 Substrate selection

King stropharia can been grown on many different lignocellulosic materials, including various crop residues and hardwood chips. Table 1 summarizes Domondon and Poppe (2000)'s findings on yield from different substrates, resulting from a decade of experimentation. The researchers note that there was a correlation between mycelial growth and mushroom yield, i.e., if king stropharia mycelium grew vigorously in a substrate, a higher yield of mushrooms was likely to follow.

Table 1. Yield (g) and count of king stropharia mushrooms on 15 different substrates. Numbers are averaged from four replicates. All replicates contained 10 kg moistened substrates. Biological efficiency = (mass of fresh mushrooms produced)/ (mass of dry substrate) x 100%. Modified from Domondon and Poppe (2000).

Substrates	% of mycelium growth	Total on 10 kg substrate		Mean weight per mushroom (g)	Biological efficiency (%)
		No.	Wt. (g)		
Winter pruning wood	100%	71	1608	22.6	48.2
Sawdust	90%	37	818	22.1	24.5
Sunflower peels + sawdust	65%	36	668	18.5	20
Hammermilled wheat straw	90%	30	705	23.4	21.1
Winter pruning + sunflower peels	55%	28	746	26.6	22.3
Winter pruning + grass chaff	45%	25	520	20.8	15.6
Summer pruning w/ dry leaves	65%	22	378	17.8	11.3
Grass chaff + sawdust	70%	21	492	23.4	14.7
Grass chaff + black peat	55%	21	279	13.2	8.3
Summer pruning w/ green leaves	45%	18	340	18.8	10.2
Winter pruning + Agaricus compost	30%	17	474	27.8	14.2
Sunflower peels	50%	16	441	27.5	13.2
Grass chaff	35%	13	296	22.7	8.8
Coconut fibers	30%	10	134	13.4	4
Corn cobs + black peat	5%	0	0	0	0

The wood chips used in Domondon and Poppe (2000)'s experiments came from *Tilia* and *Populus* trees, but Stamets has reported good results with *Alnus* as well, albeit without accompanying yield data (Stamets, 2000). Pea plant tops have also been successfully used as a fruiting substrate (Bonenfant-Magné, 2000).

Substrates should be fresh and uncontaminated with other fungi (Szudyga, 1978). Substrates should not be supplemented with inorganic fertilizers, as this results in poor mycelium development. If substrates must be stored prior to inoculation, they should be stored in cool, dry conditions to prevent contamination.

2.1.1.1 Hemp: a new substrate for king stropharia mushrooms

Hemp straw has not previously been studied as a substrate for king stropharia in the academic literature. The area of hemp cultivated in Canada increased from 2,400 hectares when industrial hemp production was legalized in 1998 to a peak of 37,400 hectares in 2019 following the legalization of recreational cannabis in October 2018 (Health Canada, 2023). Some hemp producers believed that the legalization of cannabis would bring about a massive expansion in the market for products containing cannabidiol (CBD), a non-psychoactive cannabinoid in hemp that research suggests may help to treat epileptic seizures, anxiety, insomnia, and chronic pain (Arnason, 2024; Grinspoon, 2021). Unfortunately, the growth of the CBD market did not live up to expectations. The area of hemp cultivated in Canada declined to 13,700 hectares in 2022 (Arnason, 2024). While hemp production in Canada varies with market and other conditions, the crop is unlikely to return to its pre-legalization obscurity.

Worldwide sales of hemp products continue to grow rapidly (Kaur & Kander, 2023). Business and policy changes, infrastructure investments, and improved cultivation methods help to support the growth and sustainability of the hemp industry. Some hemp is cultivated to use its fiber for textiles, paper products and even building materials. Other varieties of hemp are produced for flower, grain, or seed, which results in hemp straw as a by-product (Health Canada, 2023). Inoculation with king stropharia is an opportunity to generate another revenue stream from the cultivation of hemp for flower, grain or seed while accelerating the decomposition and nutrient cycling of the crop residue.

2.1.2 Substrate preparation

Before inoculation, substrates should be moistened to 65-75% water content (wet weight basis) via soaking or showering (Domondon & Poppe, 2000; Szudyga, 1978). Adequate moistening of the substrate is one of the most important steps for successful cultivation. This is because, while king stropharia mycelium needs moisture for its growth, once the mycelium has established too much free water is detrimental to it.

Soaking for 2 h at 65°C has been found to enhance the leaching of soluble sugars and amino acids from substrates, resulting in lower rates of contamination (Table 2) (Bonenfant-Magné, 2000).

Table 2. Yield of king stropharia mushrooms (g/kg substrate) and rate of contamination (%) of trials with respect to substrate and soaking method. Adapted from Bonenfant-Magné (2000). Translated from French by the author. The results are the mean of several replicates.

	Aged straw		Fresh straw	Pea plant tops	Corn cobs
	17 h, 20°C	2 h, 65°C	2 h, 65°C	2 h, 65°C	17 h, 20°C
Yield (g/kg)	77	135	25-160	220	150
Contamination (%)	80	75	18	0	75

2.1.3 Bed preparation, inoculation, and myceliation

The best sites for cultivation are warm and sheltered from the wind (Szudyga, 1978). Total shade will greatly decrease fruiting body development, but partial shade (60-80%) is ideal. A suitable microclimate for king stropharia can be created by preparing beds inside of wooden garden frames covered with an opaque material (Szudyga, 1978). Partial shade can be achieved by propping the frames open. The frames' lids should be sloping to allow rain to

run off. Moist substrate is placed in frames and thoroughly compacted by treading. The beds are filled to a depth of 20 to 30 cm (Szudyga, 1978).

King stropharia can also be grown in rounded heaps under 70% shade from forest cover or another source (Bruhn et al., 2010; Domondon & Poppe, 2000). Eliminating the use of garden frames reduces the time and capital expenses required to start production but also results in inconsistent microclimate conditions compared to when garden frames are used. In their forest edge cultivation experiment, Bruhn, Albright, and Mihail (2010) reported significant differences in mushroom yield depending on plot location.

Substrates can be inoculated with 3-4 cm diameter pieces of spawn buried at even spacing to a depth of 5-8 cm (Szudyga, 1978). Alternatively, spawn can be crumbled and distributed uniformly over the surface of the bed and then covered with the last 5-8 cm of humid substrate. The substrate should then be covered with moist burlap or cardboard (Domondon & Poppe, 2000; Szudyga, 1978). This covering material should be kept humid, but care should be taken so that free water doesn't drip down into the substrate (Szudyga, 1978). Myceliation of the substrate, also known as spawn run, requires 3-5 weeks outdoors, depending on conditions. It should be noted that spawn run and the time to first yield are not equivalent due the additional time elapsed between the application of the casing layer and the growth of the first fruiting bodies. Spawn run occurs at temperatures between 20-29°C, with an optimum range of 25-28°C (Domondon & Poppe, 2000; Szudyga, 1978). Below 20°C, myceliation will be slow, while prolonged periods above 30°C will damage the mycelium. If the top layer of substrate becomes desiccated, it should be removed to a depth where mycelium appears (Szudyga, 1978).

2.1.4 Tending to beds and triggering fruiting

Once the mycelium has grown through the substrate and begun to penetrate the covering material, this material should be removed (Szudyga, 1978). The substrate is then covered in a casing layer of 50/50 (v/v) peat and humus-rich soil (plain mineral soil is not suitable) with a pH of 5.5-6.5 (Domondon & Poppe, 2000; Szudyga, 1978). The requirement for a casing layer is not unusual. Several commonly cultivated mushrooms also benefit from a casing layer, including *Agaricus bisporus* (button mushrooms) and *Pleurotus* spp. (oyster mushrooms) (Shields, 2018). The casing layer provides the conditions for the fusion of hyphae into knots from which the fruiting bodies are formed, and therefore greatly enhances the yield of mushrooms (Bruhn et al., 2010; Szudyga, 1978). However, unlike many other cultivated mushroom species, king stropharia will yield little to no fruiting bodies if the casing layer is sterilized. It is believed that soil microbes are necessary to initiate fruiting, but the relationship between king stropharia and its microbial associates is not understood (Shields, 2018; Stamets, 2000).

About 50 L of casing material is needed per 1 m^2 bed (Szudyga, 1978). Stamets (2000) recommended light pasteurization of the casing layer at 55-60°C for 30 minutes. The intent of light pasteurization is to eliminate potential pests and pathogens, without killing beneficial soil bacteria that enhances fruiting. The casing layer must be kept moist, but care must be taken not to excessively wet it. The beds should be aerated and exposed to partial light 10-14 days after casing by propping open the garden frame lids (Szudyga, 1978).

Domondon and Poppe (2000) also experimented with adding fruit peels (apple, banana, and citrus) to the casing layer to test whether hormones from the decomposing fruit would affect mushroom fruiting. They only made one replicate with each type of fruit peel. They found

that the beds with fruit peels fruited an average of four days earlier than the control, but total yield from the fruit peel beds was not significantly different. Domondon and Poppe also remarked that 10-20% of fruiting bodies grew between grasses and herbs at the edge of beds. Stropharia mycelia were observed growing around fine plant roots. The authors suggest that this could be peritrophic mycorrhization and that king stropharia could be using sugars and amino acids from the plant roots. This is a potential area for further study.

2.1.5 Harvesting

Fruiting has been reported to begin 44 to 58 days after inoculation in central Missouri and 56 to 70 days after inoculation in the pacific northwest (Bruhn et al., 2010; Stamets, 2000). Mushrooms reach full maturity 10-12 days after fruit body setting, also known as pin set. Successive crops will continue to occur at 10-12 days intervals, with the first and second crops producing the highest yields (Stamets, 2000; Szudyga, 1978). Under natural conditions in Eastern Europe, fruiting begins in early August and lasts until frost reaches the beds (Szudyga, 1978). The optimum temperature range for fruiting is 17-26°C. However, day temperatures of up to 32°C can be tolerated for fruiting if the nights are approximately 10°C cooler, and fruiting can also occur at temperatures below 15°C (Bruhn et al., 2010; Domondon & Poppe, 2000).

The optimal growth stage to harvest king stropharia mushrooms is immediately before the veil breaks from the cap (Stamets, 2000). Harvesting at this stage allows the mushrooms to reach a medium size, with firm flesh and a longer shelf life. If allowed to develop longer, the mushrooms can attain a larger size, but this is achieved at the cost of a shorter shelf life. To harvest, the mushrooms are twisted (rather than cut) out of the casing layer. The ends of the stipes are cleaned or cut off. The mushrooms can be stored at 2°C to 5°C for two to three

days. According to Szudyga (1978), yields can range from 2-33 kg/m^2. The unpredictability of yields and the lack of understanding of the factors that control fruiting are among the reasons that large-scale cultivation of this species has not yet taken off (Bonenfant-Magné, 2000; Szudyga, 1978).

2.2 Spent mushroom substrate applications

Spent mushroom substrate (SMS), also referred to as mushroom compost, is the leftover biomass remaining at the end of mushroom cultivation, i.e., post-cultivation substrates. Although SMS can be considered a "waste product," its nutrient content, microbial activity, and chemical and physical properties give it many potential uses. The documented applications of SMS include feed for livestock, bioenergy feedstock, fertilizer, peat substitute in horticulture, a material in wastewater treatment, a source of degradative enzymes and biopesticides and more (Grimm & Wösten, 2018; Mohd Hanafi et al., 2018; Paula et al., 2017; Stamets, 2000). SMS can also be treated and amended for further mushroom cultivation (Grimm & Wösten, 2018; Stamets, 2000; Zied et al., 2020).

2.2.1 SMS as a soil amendment for crop production

SMS can improve soil structure by increasing organic matter content, nutrient retention, water holding capacity and microbial activity, and by decreasing compaction (Grimm & Wösten, 2018). SMS can also provide nutrients for crop production, which can help offset the use of financially and environmentally costly synthetic fertilizers. There is a large body of literature on the use of SMS as a soil amendment to improve crop production. The bulk of this literature concerns itself with the most widely commercially cultivated mushrooms (*Agaricus bisporus*, *Pleurotus* spp. and *Lentinula edodes*) (Rinker, 2017). Since king stropharia is a white rot fungus like *Pleurotus* spp. and *L. edodes*, there will likely be some

similarities in the characteristics of the compost produced, but this remains to be confirmed. The quality of the compost produced also depends in large part on the mushroom cultivation substrate.

Composts are chemically complex, and they vary in their quality as soil amendments (Bernal et al., 2009; Ozores-Hampton, 2017). Table 3 provides a list of parameters compiled by Ozores-Hampton (2017) for assessing compost quality.

Table 3. Optimal compost physical, chemical, and biological properties for use in vegetable production and other production systems. Compiled information is from various sources, listed below. The composts studied to determine these parameters were made from a variety of feedstocks, including crop residues, manures, paper products and vegetable scraps. This table is from "Guidelines for Assessing Compost Quality for Safe and Effective Utilization in Vegetable Production" (https://journals.ashs.org/horttech/view/journals/horttech/27/2/article-p162.xml) by Monica Ozores-Hampton. This article is currently licensed under CC BY-NC 4.0 (https://creativecommons.org/licenses/by-nc/4.0/). © 2017 Monica Ozores-Hampton.

Parameter (units)[z]	Optimal range[x,y]	TMECC methods no.[x]
	Physical	
Bulk density (lb/yard^3 wet basis)	740–980	3.03
Moisture (%)	30 (dry) – 60 (wet)	—
Organic matter (%)	40–60	5.07-A
Particle size	98% pass through 3/4-inch screen or smaller than 1 inch	2.02-B
Physical contaminants (%)	<2%	3.08-A
	Chemical	
Ph	5.0–8.0	4.11-A
EC (mmho/cm)	<6	4.10-A
Stability [carbon dioxide (CO_2) evolution rate or oxygen consumption]	CO_2-carbon/unit volatile solid (VS) per day (<2 = very stable, 2–8 = stable, >8 = unstable). Oxygen (O_2) uptake O_2/VS per hour (<0.5 very stable, 0.5–1.5 = not stable, >1.5 = not stable)	5.08-B
Solvita maturity test (Woods End Research Laboratory, Mt Vernon, ME)	≥6	—
Carbon:nitrogen ratio	10–25	4.01 and 4.02
Nitrogen (%)	0.5–6.0	4.02
Phosphorous (%)	0.2–3.0	4.03
Potassium (%)	0.10–3.5	4.04
Heavy metals	Meet or exceed USEPA Class A standard, 40 CFR § 503.13 or DEP 62-709	
Arsenic [As (ppm)]	<41	4.06-As
Cadmium [Cd (ppm)]	<15 (DEP)	4.06-Cd
Copper [Cu (ppm)]	<450 (DEP)	4.06-Cu
Lead [Pb (ppm)]	<300	4.06-Pb
Mercury [Hg (ppm)]	<17	4.06-Hg
Molybdenum [Mo (ppm)]	<75	4.06-Mo
Nickel [Ni (ppm)]	<50 (DEP)	4.06-Ni
Selenium [Se (ppm)]	<100	4.06-Se
Zinc [Zn (ppm)]	<900 (DEP)	4.06-Zn
	Biological	
Maturity (seed emergence and seedling vigor)	>80% relative to positive control	5.05-A
Weed-free	No or very low weed seeds	—
Pathogen	Meet or exceed USEPA Class A standard, 40 CFR § 503.32(a)	
Fecal coliform (MPN/g total solids)	<1000	7.01
Salmonella (MPN/4 g)	<3	7.02

[z]1 lb/yard^3 = 0.5933 kg·m^{-3}, 1 inch = 2.54 cm, 1 mmho/cm = 1 mS·cm^{-1}, 1 ppm = 1 mg·kg^{-1}, MPN = most probably number, 1 MPN/g = 28.3495 MPN/oz, 1 MPN/4 g = 7.0874 MPN/oz.
[y]Cooperband, 2002; DEP, 2010; Marriott and Zabosrski, 2015; Rynk, 1992; University of Massachusetts, 2016; USCC, 1996; USEPA, 1994, 1995.
[x]Test methods for the examination of composting and compost.

The terms maturity and stability are sometimes used interchangeably, but they have different, albeit overlapping, meanings. Stability refers to an advanced degree of organic matter decomposition, with resistance to further decomposition. A mature compost is one that does

not cause adverse effects to crop plants when applied (i.e., phytotoxins, pathogens and weed seeds have been broken down by the heat generated from the composting process) (Ozores-Hampton, 2017; Rynk et al., 2021; Wichuk & McCartney, 2010).

Complete decomposition is important to produce good quality compost. If decomposition is incomplete, the high degree of microbial activity can cause dangerous levels of self-heating if the compost is stored in large heaps or windrows (Rynk et al., 2021; Wichuk & McCartney, 2010). Continued decomposition can also cause odours and disease vector attraction. Immature composts can be phytotoxic due to high levels of intermediate decomposition by-products such as ammonia and short-chain organic acids. SMS is not guaranteed to be stable or mature after mushroom cultivation and may require further decomposition before optimal use as a soil amendment (Paula et al., 2017). Fortunately, SMS is considered low risk for pathogens compared to some other compost feedstocks (e.g., manures, infected plant materials, biosolids).

One potential issue with SMS compost is high electrical conductivity (EC), which indicates high salt concentrations. Excessive salinity can negatively affect plant growth and development (Paula et al., 2017). In cases where EC is high, irrigation can help leach the excess salts from the SMS.

2.2.2 SMS as a peat substitute in horticulture

Peat is a spongy material formed by the partial decomposition of organic matter, often sphagnum moss, in wetlands (Kopp, 2024). It is widely used in horticulture due to its favourable physical characteristics, including high water availability, water buffering capacity and wettability. These properties of peat support the germination of seedlings and the growth of plants in containers and soilless mediums (Eudoxie & Alexander, 2011;

Michel, 2010). However, peat bogs are ecologically important for the unique plant and wildlife habitat they provide and the vast amounts of carbon they store (Alexander et al., 2008). Sphagnum moss is slow-growing, and the regeneration of peat falls woefully short of the pace of peat harvesting, leading to destructive environmental consequences (Keddy, 2010). This has created an imperative to find effective substitutes for peat in horticulture.

Several experiments have been conducted on the use of commercial SMS as a peat substitute (da Silva Alves et al., 2024; Eudoxie & Alexander, 2011; Gao et al., 2015; Paula et al., 2017; Prasad et al., 2021). The results of these experiments support the idea that SMS can at least partially, and sometimes completely, substitute peat in horticultural applications without sacrificing plant growth, yield, or quality.

The limiting factors of using SMS as a peat substitute include high EC and particle size. High EC results from high available K (da Silva Alves et al., 2024; Eudoxie & Alexander, 2011; Prasad et al., 2021). It is possible to use untreated post-mushroom crop SMS as a component up to 25% (v/v) of soilless growing media and seed starting mixes without negative effects on horticultural crops, depending on the properties of the SMS. Particle size is another limiting factor (Abad et al., 2001; Eudoxie & Alexander, 2011). Ideal seed germination substrates have a particle size range of 0.25-2.0 mm to provide even water holding capacity throughout the substrate, but most SMS contains a significant portion of larger particles.

Fortunately, both high EC and large particle size can be overcome through simple treatments. Composting and washing/leaching can be used to reduce the EC of SMS to appropriate levels such that it can completely replace peat as a growing medium while maintaining or even increasing horticultural outcomes (da Silva Alves et al., 2024; Eudoxie & Alexander, 2011;

Paula et al., 2017). Further composting has the added benefit of simultaneously reducing particle size.

Eudoxie & Alexander (2011) found that sieving sugarcane bagasse-based SMS through a 2 mm mesh significantly improved its performance as a medium for producing tomato seedlings compared to both un-sieved replicates of the same SMS and to peat-based Pro-Mix. SMS also provides significantly more nutrients to plants than peat (da Silva Alves et al., 2024; Eudoxie & Alexander, 2011). Therefore, if the appropriate treatments are used to reduce EC and particle size, SMS can outperform peat while reducing fertilizer requirements in horticulture.

2.3 Knowledge gaps

Literature on the cultivation of king stropharia is limited. Some documents are out of print or difficult to locate. Most of the material published on king stropharia from the 1980s onward cites Szudyga's chapter in *Biology and Cultivation of Edible Mushrooms* (1978). Despite these frequent references, I was unable to find a more recent study that has replicated Szudyga's garden frame cultivation method. Szudyga reports king stropharia mushroom yields ranging from 2-33 kg/m^2 but provides no explanation of how these numbers were obtained.

Outdoor cultivators report growing king stropharia in partially shaded patches or beds, but they either have not achieved the yields Szudyga reports (Bruhn et al., 2010), reported yields in a different format to which a direct comparison cannot be made (Bonenfant-Magné, 2000) or do not address yield quantitatively because the publications are non-commercial and non-academic (Stamets, 2000, and many resources for hobby growers e.g. Mercy, 2021b). There is no consensus on best practices for cultivating this mushroom outdoors. Although king

stropharia has a history of being cultivated at temperate latitudes, there is no academic literature on its cultivation in subboreal zones like that of the Prince George area. We know it is possible to grow the mushroom in this region since there is a producer (Michael Doyle from Ancient Forest Mushroom Farm) who grows king stropharia outside in Dome Creek, approximately 125 km east of Prince George.

There is scant data on how substrates are chemically altered by inoculation with king stropharia. There is one published paper on the topic (Buta et al., 1989), but the experiment only examined lignin content and was designed to simulate the conditions in a solid-state fermenter, not outdoor cultivation conditions. There is virtually no literature on the specific nutritional needs of king stropharia to optimize yield.

2.4 Research objectives

The objectives of this research were to determine:

1. Which readily available substrate (alder chips, barley straw or hemp straw) produces the best yield of king stropharia,
2. If substrate impacts the nutritional content of king stropharia,
3. How king stropharia chemically alters substrates,
4. How king stropharia alters microbial communities in substrates, and
5. Which spent substrate makes the best soil amendment for crop production.

3 Methods

3.1 Experimental design

Three lignocellulosic substrates, alder chips, barley straw and hemp straw were chosen for the cultivation of king stropharia mushrooms in 1 m x 1 m outdoor garden frames. Eight frames of each substrate were prepared, for a total of 24 frames. Five frames each of alder chips and barley straw were inoculated with king stropharia spawn and three frames were kept as uninoculated controls. It was decided that there should be more inoculated replicates than uninoculated replicates to generate more robust data with regards to king stropharia mushroom yield and nutrition. While a larger number of replicates would have desirable to increase statistical power and therefore be able to draw stronger conclusions, funding and time constraints limited the number of garden frames I could build and tend to. Six frames of hemp straw were inoculated (one extra frame was inoculated due to a labeling error), leaving two frames as uninoculated controls. Table 4 summarizes the experimental design.

Table 4: Summary of experimental design. The independent variable was the mushroom growing substrate, of which there were three types (alder chips, barley straw and hemp straw). There were 5 to 6 inoculated (treated) replicates of each substrate and 2 to 3 uninoculated (untreated/control) replicates of each substrate for a total of 8 each. The Dependent variables field lists the primary types of data collected from the experiment. The Constants field shows factors which were kept consistent regardless of substrate type or treatment.

Independent variable: substrate	**Alder chips**	**Barley straw**	**Hemp straw**
Inoculated (treated) replicates	5	5	6
Uninoculated (untreated/control) replicates	3	3	2
Dependent variables	Number and mass of fruiting bodies (yield)	Fungal and bacterial communities in substrates	Substrate chemical composition pre- and post-cultivation
	Micro and macronutrient	Micro and macronutrient content of substrates	Quality of spent substrate as a soil amendment

	content of fruiting bodies		
Constants	Garden frame design and materials	Preparation of substrates (relative age; pasteurization procedure)	Casing layer composition (NorGrow compost and peat)

3.2 Cultivation trial

3.2.1 Frame construction

Mushrooms were grown in 1m x 1m frames made with 2 x 4 SPF dimensional lumber and foundation wrap (a type of waterproof, corrugated plastic) as siding (Figure 1). The frames had inclined plywood roofs on hinges to allow for easy access to the frame contents and to shed precipitation. The bottoms of the frames were left open to allow for substrate-soil contact.

Figure 1. Photos of the garden frames used for the cultivation of king stropharia in this study. The first photo shows the 2x4 wooden structure used. The second photo shows a complete frame with foundation wrap siding and a hinged plywood roof. The frame in the second photo is filled with pasteurized hemp straw.

3.2.2 Study location

The mushroom cultivation trial was conducted at Three Seeds Farm, located at 1679 Foreman Road, Prince George, BC, from June to October 2022. The frames were laid out along a fence line running northeast to southwest in a hay and vegetable field that slopes gently (<5%) northeast towards the Fraser River.

3.2.3 Substrate sourcing and storage

All substrates were sourced in October 2021. The hemp (*Cannabis sativa* var. Finola®) straw and barley (*Hordeum vulgare*) straw were baled at Halltray Farm in Vanderhoof, BC. Finola® is a hemp variety developed for oil seed (Smeriglio et al., 2015). The hemp and barley were conventionally grown but were not sprayed with herbicides in the weeks prior to harvest to minimize herbicide residues on the crops. The straw bales were stored in a barn at Halltray Farm from October 2021 to May 2022. The wood chips were obtained from Sitka alder (*Alnus viridis* ssp. *sinuata*) branches harvested within 2 km of the Enhanced Forestry Lab at the University of Northern British Columbia and chipped using a 6" (15 cm) auto-feed Vermeer BC600X chipper. The chips were stored in tarped bins in the Enhanced Forestry Lab compound over the same period.

3.2.4 Substrate pasteurization

Substrates were pasteurized at ≥ 60 °C, with fluctuations up to 90 °C, for 2 h to reduce the content of soluble sugars and amino acids, based on methods described by Bonenfant-Magné (2000). This was achieved by heating the substrates in well water sourced from the study location using a 30-gallon food-grade metal drum over a propane burner while monitoring temperature using a probe thermometer. Two 30-gallon drums' worth of substrate were prepared for each garden frame. The contents of the drum took about one hour to reach 60°C,

resulting in a 3h total substrate soak time. After two hours at temperatures at or above 60°C, the drum was tipped over into a strainer box made of wooden pallets lined with hardware mesh to drain the water. The frames were then filled with substrate to a depth of 20–30 cm as described by Szudyga (1978). Substrates were then thoroughly compacted by walking on them.

3.2.5 Substrate sampling

Before fungal inoculation, ten subsamples of substrate were taken from each frame for a total fresh weight of approximately 500 g. I sampled in a three-dimensional spiral pattern through the frames so that material was taken from different areas and depths of the frames. The samples were packed into large Ziplock bags and stored at -18˚C until analysis. The same sampling procedure was used for the second round of substrate sampling, which was done after the mushrooms ceased to fruit for the season.

3.2.6 Inoculation and tending

King stropharia spawn was purchased from Mr. Mercy's Mushrooms, based in Nelson, BC. Spawn was kept in sealed bags in cold storage (4°C) for approximately eight months before inoculation. Over the course of the storage period, visible contamination of the spawn occurred, but was not discovered until inoculation time, at which point it was too late to order fresh spawn. I visually assessed the spawn for the growth of contaminants (e.g., *Trichoderma* spp. green mold). Only the cleanest spawn was used, and the rest was discarded. However, it is unlikely that sorting by eye resulted in the exclusion of contaminants from the inoculant used in the experiment. Also, even among the uncontaminated inoculant, mycelial vigour may have been reduced due to the prolonged storage period, although it was suggested that

spawn could be stored indefinitely in the bags when refrigerated (R. Mercy, personal communication, July 6, 2021).

Spawn was inoculated into substrates in 3 to 4 cm diameter chunks at even spacing to a depth of 5–8 cm at a rate of 250 g/m^2 (Mercy 2021b, 2021c, Szudyga 1978). Due to the time-consuming nature of pasteurizing the substrates, inoculation was done in three rounds, as outlined in Table 5 below.

Table 5: Timeline of inoculation and pasteurization. The garden frames were sequentially divided into three groups of eight. The pasteurization date range shows the timeframe during which the substrates for each group of eight frames was pasteurized. Once pasteurization was finished for a group, all the frames assigned a treatment within that group were inoculated on the same day.

Date of inoculation	Inoculation group number	Frames in group	Pasteurization date range
2022-06-15	1	1 to 8	2022-05-30 to 2022-06-15
2022-06-24	2	9 to 16	2022-06-15 to 2022-06-23
2022-07-02	3	17 to 24	2022-06-24 to 2022-07-02

Following inoculation, the contents of the frames were covered with a layer of moistened, unbleached cardboard to improve moisture retention. During spawn run (the period during which the mycelium colonizes the substrate), the lids of the frames were kept shut except to tend to and monitor the contents. Moisture content was assessed qualitatively by feel, and the substrates were moistened to approximately field capacity every 1-3 days.

3.2.7 Casing layer preparation and application

According to general mushroom cultivation principles, the casing layer is applied to a substrate once spawn run is complete (Stamets, 2000). In this experiment, to maintain consistency between treatments, when a frame full of inoculated substrate was ready to be

cased, I also applied the casing layer to the uninoculated control frames containing the same substrate which were pasteurized in the same batch.

The casing layer was made by thoroughly mixing a 27-gallon (100 L) tote of Norgrow compost sourced from the Foothills Boulevard Regional Landfill with one 3 cu.Ft. (85 L) bag of peat moss and gradually hydrating the mixture to field capacity. One batch of this recipe covered four frames. The packaged peat moss was assumed to have consistent composition between bags. The compost was thoroughly mixed before dividing it among batches. The mixture was pasteurized at ≥ 60˚C for 30 minutes using a propane burner and a stainless-steel canning pot bathed in hot water within the 30-gallon drum based on Stamets (2000). The casing mixture was set aside to cool to ambient temperature before spreading it on the substrate surface in a 5 cm-deep layer. The resulting pH of the mixture was approximately 6.5, measured with a garden pH probe.

3.2.8 Mushroom harvesting and sampling

During the fruiting period, I recorded the date, frame number, substrate type and mass to the nearest gram of each mushroom harvested. I harvested the mushrooms as near as possible to the developmental stage when the veil on the mushroom breaks from the cap. This is considered the best time to harvest to balance between optimizing the yield and the shelf life of the mushrooms (Stamets, 2000). Mushrooms were twisted out of the substrate at the stipe base and gently brushed with a soft-bristle brush to remove attached substrate and casing soil before weighing.

The first 200 to 220 g of fresh mushrooms harvested from each frame were set aside for sample processing and analysis. I kept the first 200 to 220 g of mushrooms instead of sampling throughout the fruiting period due to uncertainty about what the total yield would

be and how long the fruiting period would last, as well as based on the principle that the first flush of mushrooms from a substrate is usually the largest (McKoy, 2016; Stamets, 2000).

3.3 Laboratory analysis

3.3.1 Sample processing

3.3.1.1 Mushroom sample processing

Mushrooms collected for analysis were gently brushed to remove dirt, rinsed with tap water, and patted dry with clean towels. The mushrooms were cut into thin (~0.5 cm) slices and dehydrated at 71˚C for 48 h in a Hamilton Beach food dehydrator (model 32100C) based on the drying methods described in Kumar et al. (2013). After dehydration, the mushrooms were weighed again, and moisture content was calculated on a fresh weight basis, i.e.: Moisture content = (fresh weight – air-dry weight)/fresh weight x 100%.

The dried mushrooms were later ground into a fine powder using an A11 basic Analytical mill from IKA mills with a single beater. Dried and ground mushroom samples were used for the following analyses: carbon and nitrogen content analysis, ICP-OES elemental analysis, and lipid and protein analysis.

3.3.1.2 Substrate sample processing

After field collection, substrate samples were stored in large freezer bags at -18˚C until later use. A fresh weight of approximately 80 g was subsampled from each substrate sample bag. Material was taken from several different parts of the bag to create a more representative subsample. The subsamples were weighed into paper bags and dried in a kiln oven for 72 hours at 55˚C. Dry weights were recorded and used to calculate sample moisture content on a fresh weight basis, i.e.:

Moisture content = (fresh weight – air dry weight)/fresh weight x 100%.

The subsamples were then ground into a fine powder using a Wiley mill fitted with a #20 mesh screen, which corresponds to a particle size of 850 μm.

After drying and grinding, the samples were stored at room temperature in plastic containers until further use. Dried and ground substrate samples were used for the following analyses: carbon and nitrogen content analysis, ICP-OES elemental analysis, Van Soest fibre analysis, and pH and EC measurements.

3.3.1.2.1 Liquid nitrogen grinding

Substrate subsamples from which DNA was extracted were ground with liquid nitrogen to prevent heat-related DNA degradation. I wanted to have approximately 10 g dry mass equivalent to subsample from for the DNA extraction, so I calculated the fresh sample weight required for 10 g dry matter based on the moisture contents previously calculated.

Samples were ground using an A11 basic Analytical mill from IKA mills with a single beater. Based on the mill's user manual guidelines, I filled the grinding chamber with substrate, then poured in a sufficient volume of liquid nitrogen to submerge the substrate. Liquid nitrogen was allowed to boil off and then the samples were ground. After processing, the samples were stored in Falcon tubes in a freezer at -18˚C until ready for use.

3.3.2 Mushroom protein and lipid analysis

Two samples of 15 to 20 g of dried, ground mushrooms from each substrate were submitted to the Guelph Food Innovation Centre (GFIC) at the University of Guelph for protein and lipid analysis. Only two samples per substrate were submitted due to the mass of sample available and high laboratory fees. Barley straw and hemp straw-grown mushroom samples

were composited. Since only two alder chip frames produced mushrooms, the mushrooms from each of these frames were submitted as individual samples.

3.3.3 Total carbon and nitrogen content by combustion elemental analysis (mushrooms and substrates)

Total carbon and nitrogen in dried, ground fruiting body samples were measured on a Costech 4010 elemental combustion system by Northern Analytical Laboratory Services (NALS) at UNBC. Total carbon and nitrogen in dried, ground substrate samples were measured on a Thermo FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Bremen, Germany 2016) by the Natural Resources Analytical Laboratory (NRAL) at the University of Alberta.

The dry combustion method begins by dropping a known mass of sample in a tin or silver capsule into a combustion tube containing chromium (III) oxide and silvered cobaltous oxide catalysts. An aliquot of purified oxygen is added to the quartz tube to generate a flash combustion reaction. The carbon in the sample is converted to CO_2, and the nitrogen is converted to N_2 and NO_x. The combustion gases are carried through a reduction furnace, reducing NO_x species to N_2, then through sorbent traps to remove water. The resulting N_2 and CO_2 gases are separated on a 2m x 6mm OD stainless steel Porapak QS 80/100 mesh packed chromatographic column and detected quantitatively by a Thermal Conductivity Detector (TCD). The integrated TCD peak signal in the resulting chromatogram is directly proportional to the amount of C and N present in the sample which, along with the sample weight, is used to calculate %C and %N (w/w).

3.3.4 Elemental analysis with Inductively coupled plasma – optical emission spectroscopy (ICP-OES) (mushrooms and substrates)

ICP-OES was performed on dried, ground fruiting body samples at NALS (UNBC) and on dried, ground substrate samples at NRAL (University of Alberta). The fruiting bodies and substrates were tested for different standard suites of elements offered at the respective labs. The fruiting bodies were tested for Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Se, Sn, U, V and Zn. The substrates were tested for Al, B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, S and Zn.

For ease of statistical analysis and reporting results for the substrate samples, I divided the elements into three categories: macronutrients (Ca, K, Mg, P, S), micronutrients (B, Cu, Fe, Mn, Zn) and non-plant nutrient elements (Al and Na). Mo and Ni, which were below detectable limits in most or all samples, were omitted from analysis.

3.3.5 Substrate Van Soest Fiber Analysis

The Van Soest method of fiber analysis was selected to quantify lignin, cellulose and hemicellulose content in substrate samples (Van Soest et al., 1991; Van Soest & McQueen, 1973; Van Soest & Robertson, 1980). The method was originally developed to study the nutritional quality of animal feeds but is also useful in understanding fungal decomposition. I also considered Fourier-Transformed Infrared Spectroscopy (FTIR) as a method to analyze substrate lignin, cellulose and hemicellulose content, since FTIR is faster and more modern (Maceda et al., 2020). However, while the absorbance spectra curves generated by FTIR are effective in demonstrating the presence of organic compounds, it is difficult to convert this information into quantitative data. Conversely, the Van Soest method generates quantitative results which can be analysed with simple statistical methods.

Air-dry (A.D.), ground substrate samples were analyzed using an ANKOM fiber analyzer at the University of Alberta. A mass of 0.45 - 0.50 g of each air-dry substrate was weighed into an ANKOM bag. Initial sample weights were corrected to oven dry (O.D.) weights using the formula:

O.D. weight = A.D. weight * (100 – % A.D. moisture content)/100.

The samples then underwent a series of chemical digestions. After each digestion, the samples were rinsed in deionized water, oven-dried and reweighed. The samples were first digested in a neutral detergent, then in an acid detergent and finally in sulfuric acid. The lignin content of the samples was taken to be the sample mass remaining in the bag after sulfuric acid digestion. Cellulose content was calculated as acid detergent fiber minus lignin, and hemicellulose was calculated as neutral detergent residue minus acid detergent fiber. These values were then converted to percentages of the original O.D. sample weights.

3.3.6 Substrate pH and Electrical Conductivity

A mass of 5.0 g of each A.D. ground substrate was mixed with 50.0 mL deionized water in an Erlenmeyer flask on a VWR DS-500E orbital shaker at 150 rpm for twenty minutes. Mineral soil pH and EC are typically measured using a ratio of 1.0 g soil: 2.0 mL water (Weil & Brady, 2017). Due to the high water-holding capacity of the substrates compared to mineral soil, a ratio of 1.0 g substrate: 10 mL water was required to be able to produce sufficient filtrate to measure with the probe meter. After shaking, the flask contents were left to settle for one hour, then poured through Whatman No. 1 filter papers into falcon tubes. The pH and EC of the filtrates were measured using a freshly calibrated Hanna HI9813-61 portable pH/EC/TDS/temperature meter.

3.3.7 Genomics

3.3.7.1 Substrate fungal and bacterial DNA extraction

DNA in substrate samples was extracted using the DNEasy PowerSoil Pro Kit (Qiagen, USA). Based on the substrate moisture content data, the equivalent of approximately 0.125 g A.D. weight per sample was used to extract DNA following the steps outlined in the kit. Samples were then screened for a minimum DNA concentration of 10 ng DNA/ µL solution using the Nanodrop test. The quality of DNA was assessed via spectrophotometer using the A260/A280 ratio and A260/A230 ratio (Francioli et al., 2021).

The viability of replication of the DNA extracts was verified using polymerase chain reaction (PCR) and gel electrophoresis. Fungal DNA was amplified with primers ITS1-F-KYO1 5' -CTTGGTCATTTAGAGGAAGTAA-3' and ITS2-KYO1 5'-CTRYGTTCTTCATCGDT-3', which target the internal transcribed spacer region of the nuclear ribosomal repeat (Toju et al., 2012). This pair of primers has been used to examine soil fungal community composition (Bui et al., 2020).

Prokaryotic DNA was amplified with primers 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3' targeting the V4 regions of the 16S rRNA gene (Walters et al., 2011). This primer pair is recommended by the international scientific consortium Earth Microbiome Project (EMP) for the identification of bacteria and archaea from soils (Caporaso et al., 2023).

DNA extract samples were stored at -18˚C from the time of their extraction in April 2023 until shipment in January 2024. At this time, samples were thawed briefly at room temperature, vortexed and centrifuged to redistribute the extracted DNA, and then loaded

onto an Eppendorf full skirt 96-well plate. The plate was sealed, bagged, and packed in dry ice for shipment to Genome Quebec.

3.3.7.2 DNA Sequencing and Bioinformatics

At Genome Quebec, the extracted DNA was amplified, then sequenced with the NextSeq 2000 system (Illumina, San Diego, USA). Samples 4Ht-P, 20Bt-P and 14Bu-C failed to amplify with the ITS primers and therefore were not included in the fungal genomics analysis. Samples 9Ht-P, 10At-P, 11Bt-P and 12Ht-P failed to amplify with the 16S primers and therefore were not included in the bacterial genomics analysis. A Genome Quebec technician then cleaned and processed the reads through the following bioinformatics pipeline to prepare tables of counts of operational taxonomic units (OTU's).

3.3.7.3 Bioinformatics Analysis Pipeline

Raw reads were trimmed and clipped to remove technical sequences and low-quality regions using cutadapt v2.10. Low-quality regions and adapter clipping was performed with trimmomatic v0.36. Cleaned reads from the same R1/R2 pair were overlapped and merged with FLASH v1.2.11 to obtain the complete sequence of the targeted amplicon. Potentially chimeric amplicons were detected using usearch61 (via vsearch 1.11.1) with the ChimeraSlayer's "gold" database from Broad Microbiome Utilities. Chimeric amplicons were excluded from the analysis. The non-chimeric amplicons for every sample were compared to a reference database (greengenes v138 for the 16S pipeline and UNITE v1211 for the custom ITS pipeline). Amplicons presenting sequence homology higher than 97% with a reference sequence were considered to belong to the same taxon and were combined to form an OTU with qiime 1.9.1 pick_otus (usearch61 via vsearch 1.11.1). Each OTU's most

abundant sequence was selected as its representative sequence. This sequence was then compared to those of the reference databases. The top three hits were used to assign a taxonomic rank to the OTU. To be considered valid, a hit had to have a minimum of 90% sequence homology and cover 51% of the OTU's sequence.

3.3.7.4 Genomics Data Analysis and Visualization

The count data were cleaned in Microsoft Excel (e.g., removing OTUs that were not assigned a kingdom and removing kingdom Archaea entries from the bacterial data). The XLOOKUP function was used to map primary lifestyle data from the FungalTraits database onto the fungal genera identified in the count data (Põlme et al., 2020). Relative abundance graphs were generated to represent fungal and bacterial diversity in R Studio (R version 4.3.3) using the ggplot2 package (Wickham, 2016).

3.3.8 Microbial biomass

Substrate sample microbial biomass carbon was estimated based on a method developed for soil microbial carbon with several modifications (Vance et al., 1987). Because the method was developed for soils and not for lignocellulosic biomass substrates, an initial run of nine samples was conducted to verify that the method would work and that the extracted organic carbon would be in the detectable range. Samples were chosen to represent the different substrate, treatment, and cultivation stage combinations. A pre-cultivation sample, a treated post-cultivation sample and an untreated post-cultivation sample were run for each substrate type.

For each sample, two portions of approximately 40 mL of frozen substrate were measured into 100 mL beakers. The substrates were weighed, and an oven dry weight was calculated

based on prior moisture data. 1.0 mL of deionized water was added to each beaker. The samples were covered in aluminum foil to block light and left to thaw for 24 hours at room temperature.

After thawing, one of each pair of samples was placed in a vacuum desiccator with a beaker containing approximately 40 mL of chloroform ($CHCl_3$) to be fumigated. The desiccator was connected to a vacuum pump and a Schlenk line and was evacuated until the chloroform had boiled for over two minutes. At this time, the unfumigated samples were extracted following the same protocol as the fumigated samples described below. Meanwhile, the vacuum desiccator was sealed and left to incubate in the dark under a plastic tote for 24 hours at room temperature.

Following the incubation period, the desiccator was evacuated six times to remove most of the remaining chloroform gas. For the extraction, 0.5 M K_2SO_4 was added to the sample beakers at a rate of 4.0 mL solution per 1.0 g of oven dried substrate. The beakers were covered in parafilm and shaken on a VWR DS-500E orbital shaker at a speed of 150 rpm for 30 minutes. A shaking speed was not specified in the original method, so this speed was selected because it was the highest setting at which the beakers would not slide around on the shaker platform. The suspensions were filtered through Whatman no. 42 filter papers into Falcon tubes.

The resulting filtrate was submitted to NALS for total organic carbon (TOC) analysis. Due to an issue with the TOC analyser at NALS, the filtrate samples had to be forwarded to AGAT Laboratories in Calgary. Before sending, the samples were diluted by a factor of 30 by a NALS technician to reduce the K_2SO_4 concentration to appropriate levels for the TOC

analyser. The TOC values resulting from AGAT Laboratories' analysis were corrected for the dilution.

The data were received in mg C/L 0.5 M K_2SO_4 solution and were converted to µg C/g substrate dry matter by multiplying by 4.0 mL solution/1.0 g substrate dry matter and performing the appropriate unit conversions.

The carbon extractable by fumigation, E_c, was calculated as E_c = (organic C extracted by 0.5 M K_2SO_4 from a fumigated soil) – (organic C extracted by 0.5 M K_2SO_4 from a non-fumigated soil) (Powlson & Jenkinson, 1976). Biomass C was calculated as (2.64 ± 0.060)*E_c (Vance et al., 1987).

3.4 Statistical analysis

Substrate samples were grouped into four categories: pre-cultivation substrate samples designated for inoculation, pre-cultivation samples designated control, post-cultivation inoculated samples and post-cultivation control samples. Unless otherwise noted, there were no significant differences between the pre-cultivation samples that were and were not designated for inoculation. However, for statistical thoroughness, I still compared data between these two pre-cultivation groups for each analysis. The results can be found in the "Treatment comparisons" section of each Appendix.

Data were analysed statistically in Minitab 21 (Minitab LLC, USA) statistical software. Datasets were tested for normality quantitively using the Ryan-Joiner test (similar to Shapiro-Wilk) and visually by examining histograms. Many data sets were not normally distributed.

The Kruskal-Wallace test was used for unpaired data (i.e., comparing among substrates, and treatments) and the Wilcoxon signed rank confidence interval for paired data (i.e., comparing

before and after cultivation). For unpaired data where there were more than two groups to be compared (i.e., comparing between substrates), the Kruskal-Wallace multiple comparison test was run using the %KRUSMC macros in Minitab. The Kruskal-Wallace multiple comparison test performs Dunn's post-hoc test and uses a family alpha value of 0.2, a Bonferroni individual alpha of 0.067 and a Bonferroni 2-sided Z-value of 1.834. Differences between unpaired data were considered significant if $p < 0.05$ (denoted *** in data tables) and of borderline significance of $0.05<p<0.067$ (denoted * in data tables). For paired data, I looked at the upper and lower limits of the ~95% confidence interval to see if they indicated an increase, a decrease, or no trend of change over the cultivation period. Data visualization was later performed in R Studio (v4.3.3; R Core Team, 2024) using the ggplot2 package to generate colour-coded boxplots (Wickham, 2016).

4 Results

4.1 Mushroom harvest timeline

4.1.1 Days to first yield

Alder chips had the longest median period between inoculation and fruiting at 114 days (n = 2), while hemp straw had the shortest median period at 74 days (n = 6) (Figure 2). The median fruiting period for barley straw (n = 5) was 80 days.

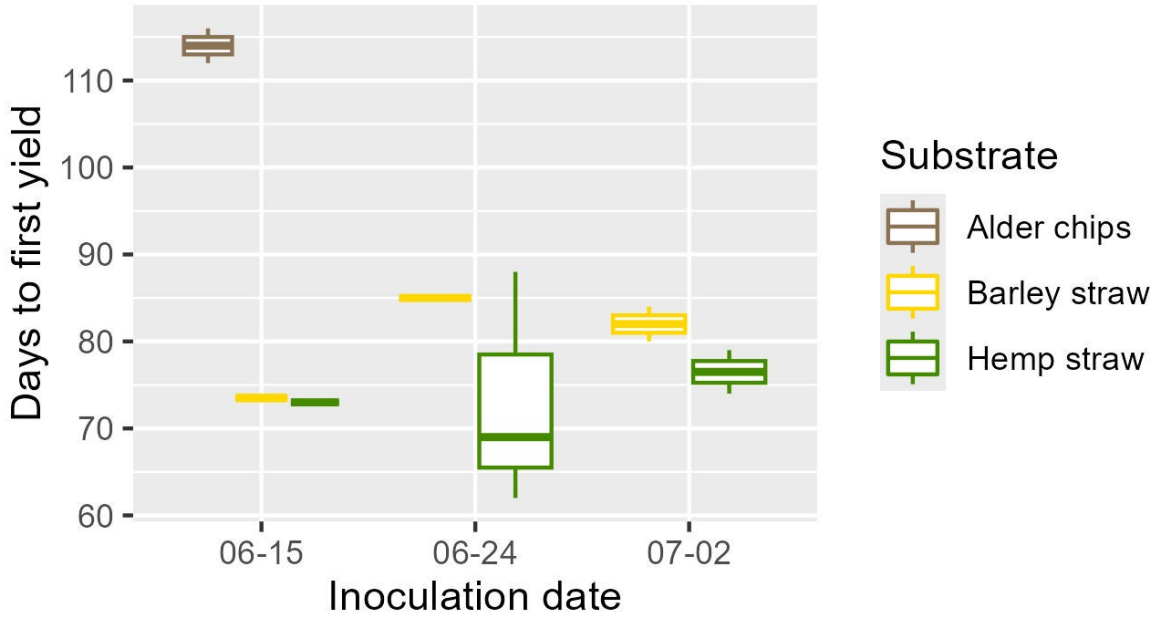


Figure 2. Boxplot of days to first mushroom yield by king stropharia inoculation date. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values.

4.1.2 Harvest period

Alder chips (n = 2) had the shortest median harvest period at 12 days, while hemp straw (n = 6) had the longest median harvest period at 36 days (Figure 3). The median harvest period of barley straw (n = 5) was 25 days.

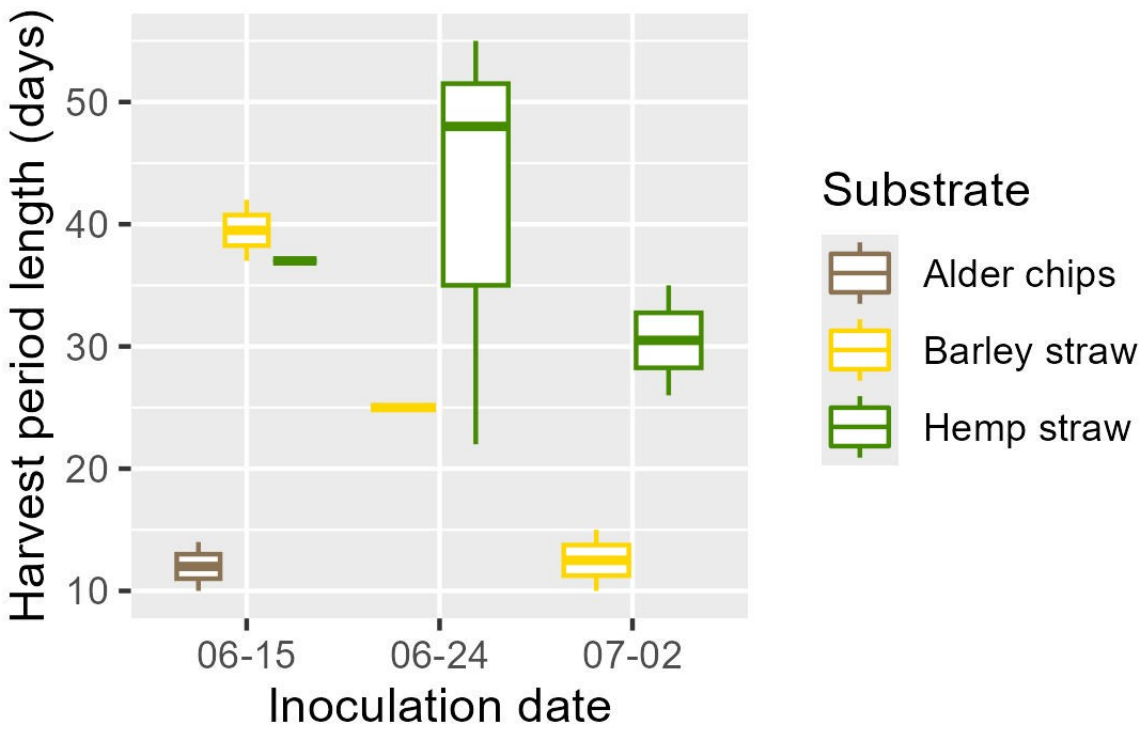


Figure 3. Boxplot of harvest period lengths grouped by substrate and inoculation date. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values.

4.2 Mushroom yield and count

Hemp straw (n = 6) produced the highest median mushroom yield at 4900 g/m^2, which was 2.7 times more than the median yield of barley straw (n = 5) at 1800 g/ m^2. Alder chips had a median value of 0 g/m^2 due to three out of five inoculated alder chip frames not producing any mushrooms (Figure 4). The difference in yields between hemp straw and alder chips was significant at $p < 0.05$.

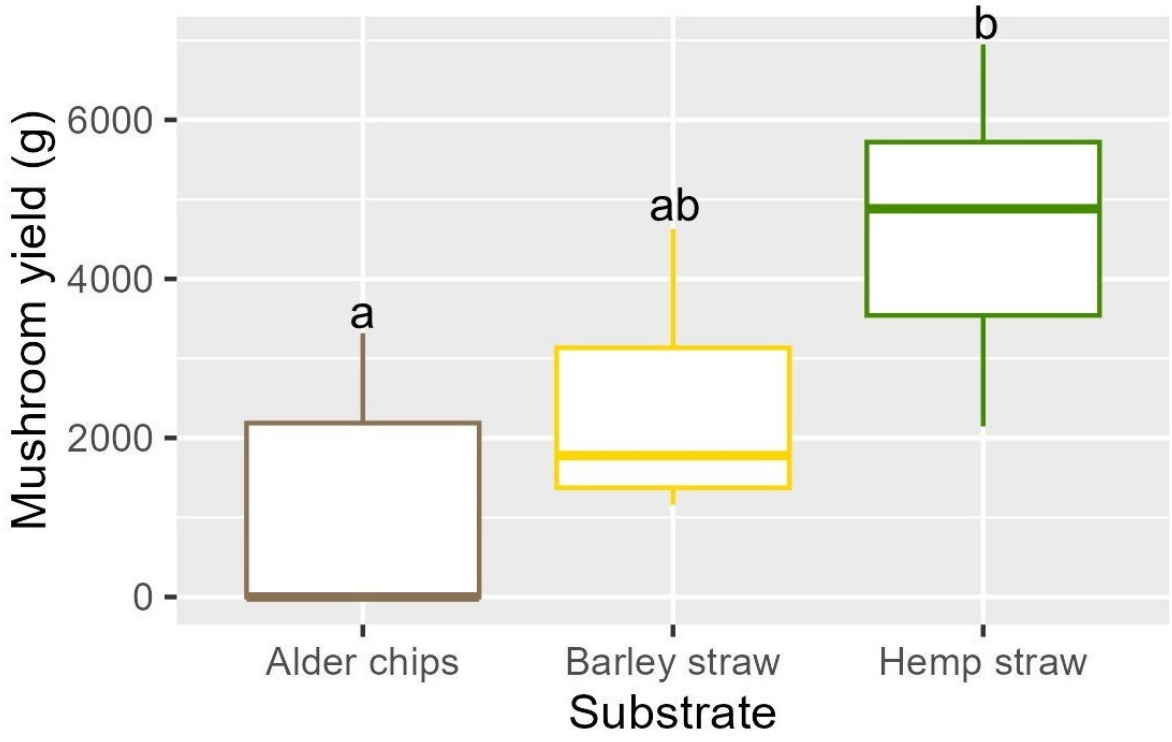


Figure 4. Boxplot of fresh mushroom yield (g) per m^2 by substrate. The horizontal bar in each box represents the median value. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values.

The trends in mushroom yield are paralleled by the trends in the mushroom count data. Hemp straw produced the highest median number of mushrooms per frame (119), followed by barley straw (44) and alder chips (0) (Figure 5). The difference in the median number of mushrooms harvested from alder chips versus hemp straw was significant at $p < 0.05$.

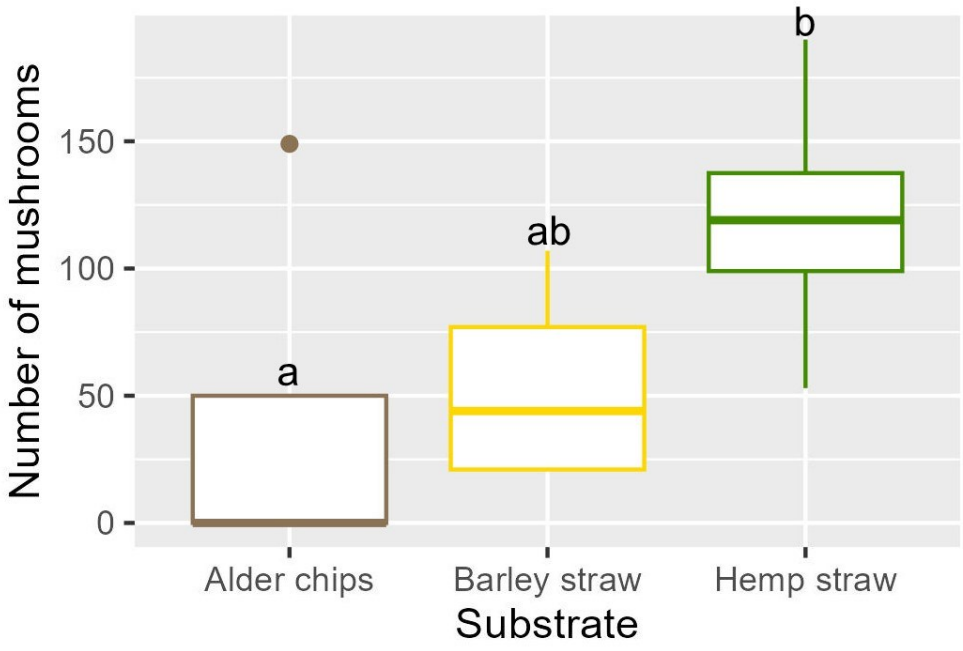


Figure 5: Boxplot of the number of mushrooms harvested per frame by substrate. The horizontal bar in each box represents the median value. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. The dot above the box for alder chips represents an outlier.

4.3 Mushroom composition

4.3.1 Mushroom moisture content

King stropharia mushrooms grown in alder chips had a slightly lower median moisture content (90%) than mushrooms grown in barley straw (93%) ($p < 0.05$). The median moisture content of mushrooms grown in hemp straw was 92%, which was not significantly different from the other substrates.

4.3.2 Mushroom carbon and nitrogen content

There were no significant differences in carbon or nitrogen content between fruiting bodies grown on different substrates (Appendix A). The median C content of fruiting bodies across all substrates was 43% (n = 13), and the median N content was 4.7 %, resulting a median C:N ratio of 9.2.

4.3.3 Mushroom elemental analysis

The following elements were below the detectable and/or quantifiable limits in most or all fruiting body samples and were omitted from statistical analysis: As, Co, Mo, Ni, Pb, Sb, Se, Sn, U and V. Fruiting bodies grown in different substrates did not differ significantly in their content of Al, Ba, Ca, Cu, K, Mg, Na, P, S or Zn. The median mushroom contents of these elements are compiled in Table 6.

Table 6: Median mushroom elemental contents for elements for which significant differences were not detected between mushrooms grown in different substrates (N = 13). All values are reported on a dry matter basis.

Element	Al	Ba	Ca	Cu	K	Mg	Na	P	S	Zn
Median content (mg/kg)	5	0.42	250	19	28000	990	270	7900	3800	51
Interquartile range	6	0.22	94	4	6800	250	160	3700	1000	15

Across all substrates, the mushrooms were richest in K (28 g/kg), P (7.9 g/kg) and S (3.8 g/kg), followed in descending order by Mg, Ca, Na, Fe, Zn and trace amounts of Cu, Mn, Al, B, Cd and Ba.

There were substrate-associated differences in the mushrooms' content of B, Cd, Fe and Mn (Appendix A). Mushrooms grown in hemp straw contained 5.5 times more B and Fe than those grown in the other substrates ($p < 0.05$) and 68% more Mn than those grown in alder

chips ($p < 0.05$). Mushrooms grown in alder chips contained 3.9 times more Cd than those grown in barley straw ($p < 0.05$).

4.3.4 Mushroom protein and fat content

Due to the high sample masses required for nutritional testing at GFIC, only two samples per substrate were submitted for protein and fat content analysis. Thus, no statistical analyses are reported. Barley straw-grown mushrooms had the highest average %DM protein content at 33% and alder chip-grown mushrooms had the lowest %DM protein content at 27% (Table 7). Hemp straw-grown mushrooms had the highest %DM fat content (3.6%), and alder chip and barley straw-grown mushrooms contained 2.7%.

However, when moisture content is accounted for, the protein and fat content rankings change. Alder chip-grown mushrooms have the highest %FW protein content (2.8%) and %FW fat content (0.27%) and barley straw-grown mushrooms the lowest (2.3 %FW protein, tied with alder chip-grown mushrooms, and 0.19 %FW fat).

Table 7: Summary of king stropharia mushroom protein and fat content analysis results. %DM values are the average of two replicates. %FW values were calculated based on mushroom moisture and dry matter content.

Substrate	Protein (% DM)	Fat (% DM)	% DM	Protein (% FW)	Fat (% FW)
Alder chips	27	2.7	10	2.8	0.27
Barley straw	33	2.7	6.8	2.3	0.19
Hemp straw	30	3.57	7.7	2.3	0.27

4.4 Substrate composition

4.4.1 Substrate moisture content

During the pasteurization process, substrates were soaked for three hours. Since the pre-cultivation substrate samples were taken soon after soaking, the samples' moisture content at this time can be regarded as a proxy of the samples' water-holding capacity.

Alder chips had the lowest median pre-cultivation moisture content (61%) of the samples designated for inoculation (Figure 6). Barley straw and hemp straw samples designated for inoculation did not differ significantly from each other in their pre-cultivation moisture content, with median values among the treated samples of 78% and 79% respectively. However, they were both significantly moister than alder chips ($p < 0.05$).

I did not perform the Wilcoxon signed rank interval test to compare pre- and post-cultivation substrate moisture content as I have done for other paired datasets because a valid comparison cannot be made between the two. The pre-cultivation substrate samples were taken after soaking, while the post-cultivation substrate samples were kept moist with light watering. These differences in substrate handling would confound the comparison.

Post-cultivation treated alder chip and hemp straw samples contained significantly less water than their untreated counterparts ($p < 0.05$, Figure 6). The median post-cultivation moisture content in inoculated alder chips was 54%, while in the corresponding controls it was 62% ($p < 0.05$). Inoculated hemp straw had a moisture content of 69% while untreated hemp straw had a moisture content of 79% ($p < 0.05$). The moisture content of post-cultivation barley straw did not differ significantly between treatments.

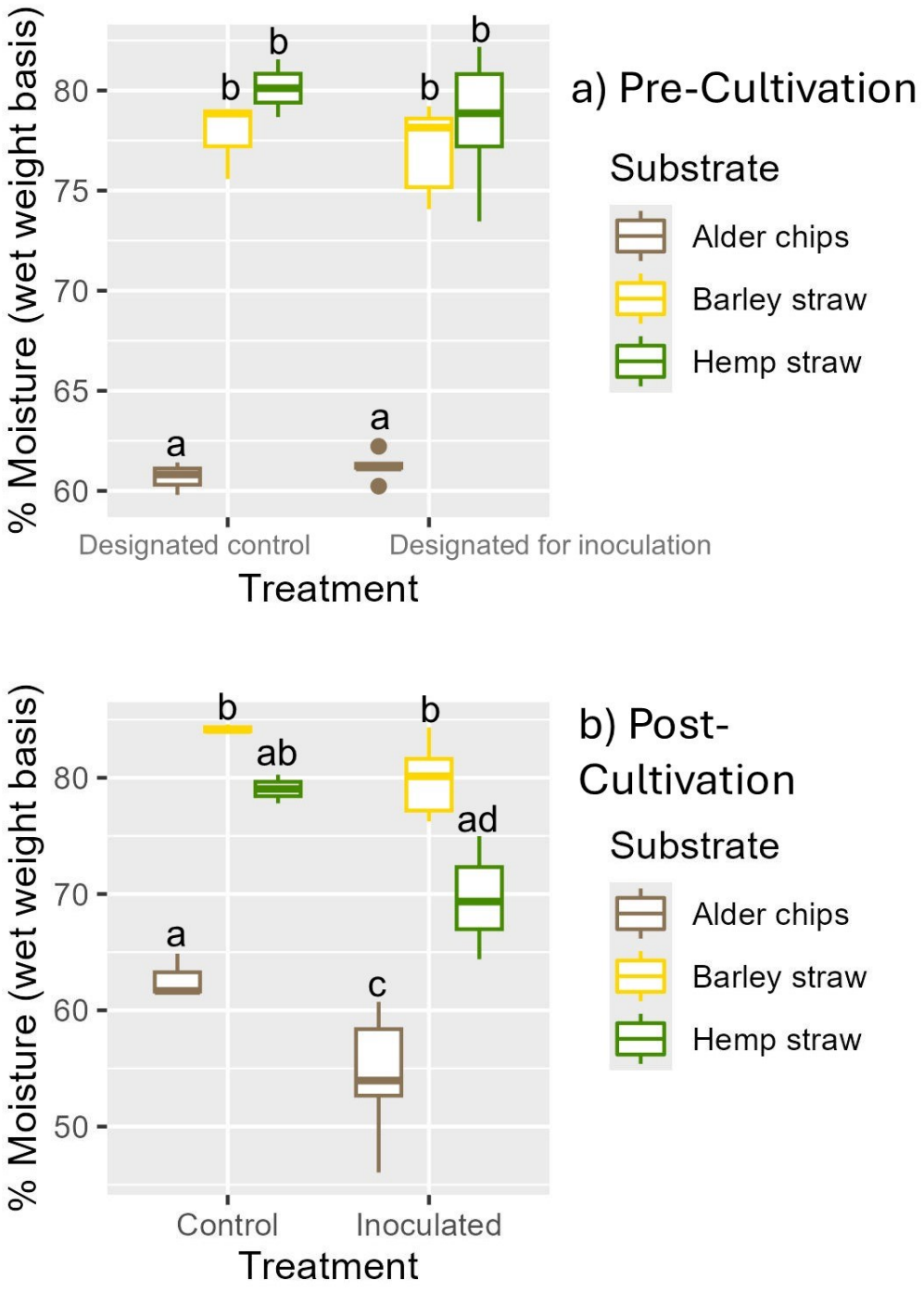


Figure 6: Boxplot of substrate sample moisture content. The horizontal bar in each box represents the median value. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The boxed area displays the interquartile range (Q1 – Q3), the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values and the dots represent outliers.

4.4.2 Substrate carbon and nitrogen content

4.4.2.1 Substrate comparisons

Pre-cultivation alder chips designated for inoculation contained 28% less N than their barley straw counterparts ($p < 0.05$, Figure 7 a). Alder chips also contained 8% more C than both barley straw hemp straw ($p < 0.01$, Figure 8 a). This resulted in alder chips having a significantly higher C:N ratio than the other substrates, with a median C:N ratio of 74 in alder chips compared to 51 for barley straw and 54 for hemp straw ($p < 0.05$, Figure 9 a).

Post-cultivation, alder chips contained 21% more C than hemp straw among the inoculated samples ($p < 0.01$, Figure 8 b) and 7% more C than barley straw among the control samples ($p < 0.05$, Figure 8 b). N levels were highly variable in the post-cultivation samples, and no significant differences were observed between substrates with regards to total N or C:N ratio in either the inoculated or control samples (Figures 7 b) and 9 b).

4.4.2.2 Treatment comparisons

Post-cultivation inoculated barley straw and hemp straw had 3% and 15% less C than their uninoculated counterparts, respectively ($p < 0.05$, Figure 7 b). Post-cultivation alder chip C content did not vary significantly between treatments. There were no significant post-cultivation treatment differences in N content or C:N ratio for any substrate tested.

4.4.2.3 Cultivation stage comparisons

Treated barley straw and treated hemp straw N content increased and C content decreased over the cultivation period. Therefore, these substrates had lower post-cultivation C:N ratios (Figure 9). The variation in N content increased after cultivation in all substrates, as shown in Figure 7.

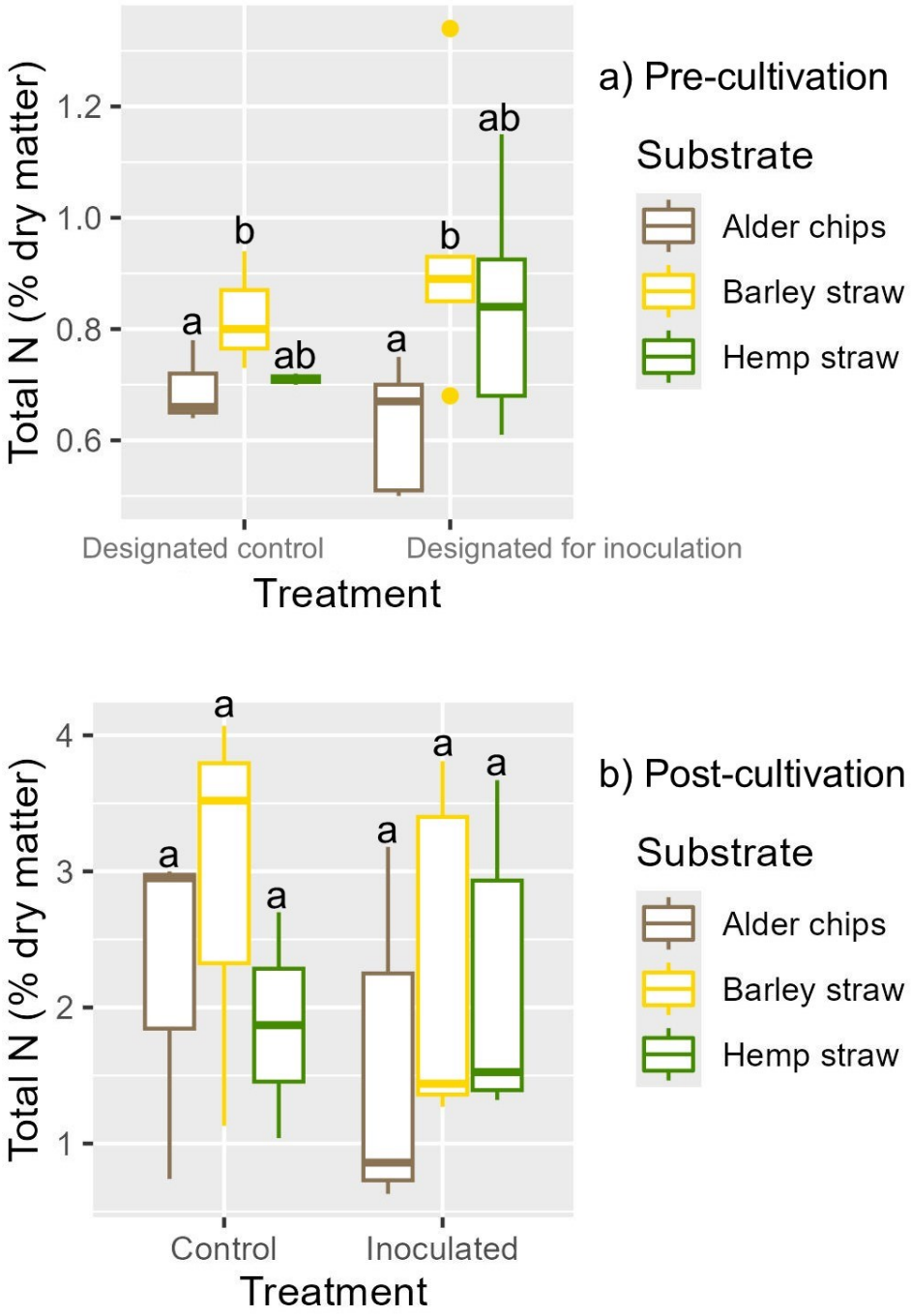


Figure 7: Boxplots of total N content of substrate samples. The horizontal bar in each box represents the median value. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The boxed area displays the interquartile range (Q1-Q3), the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values and the dots represent outliers.

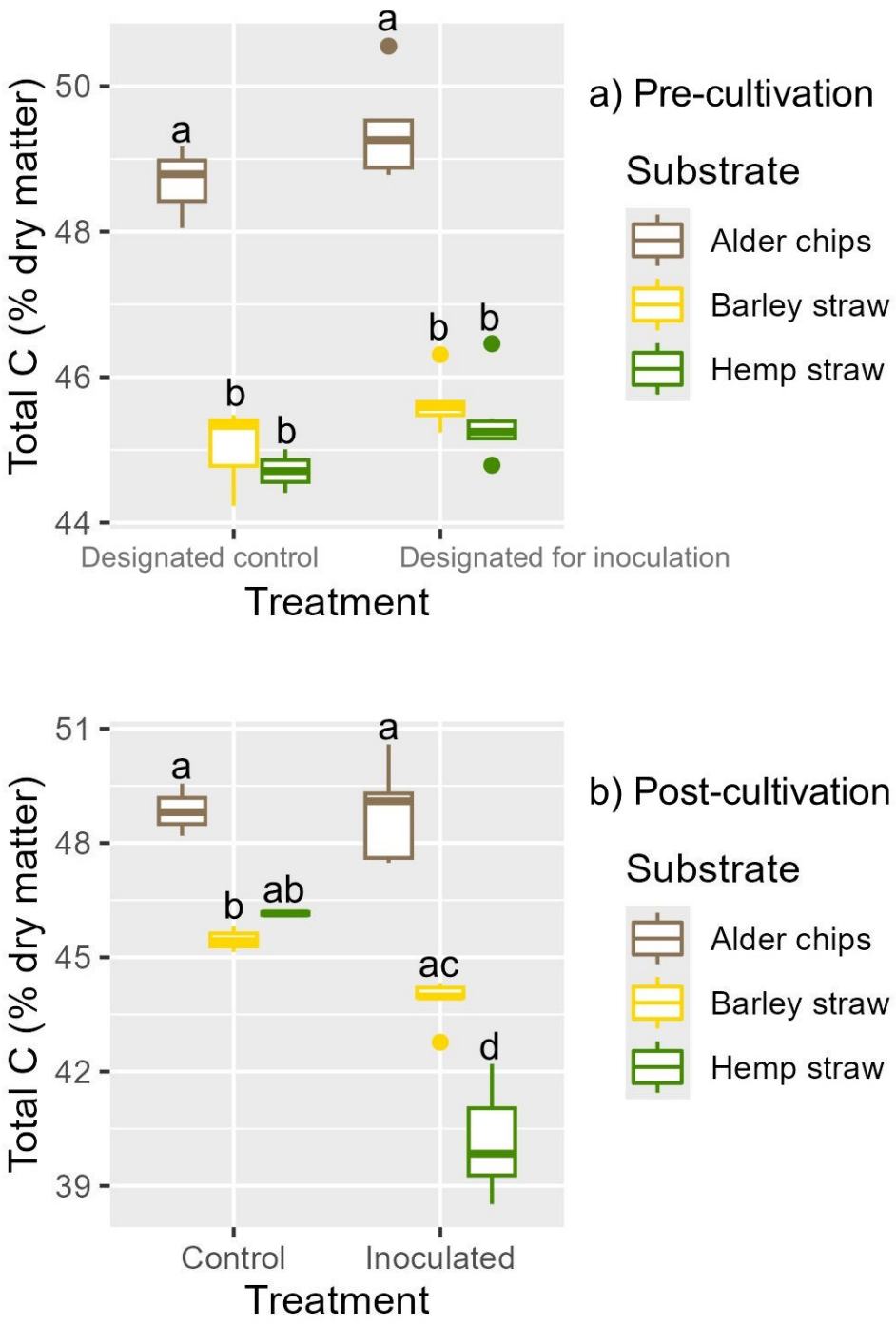


Figure 8: Boxplots of total C content of substrate samples. The horizontal bar in each box represents the median value. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The boxed area displays the interquartile range (Q1-Q3), the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values and the dots represent outliers.

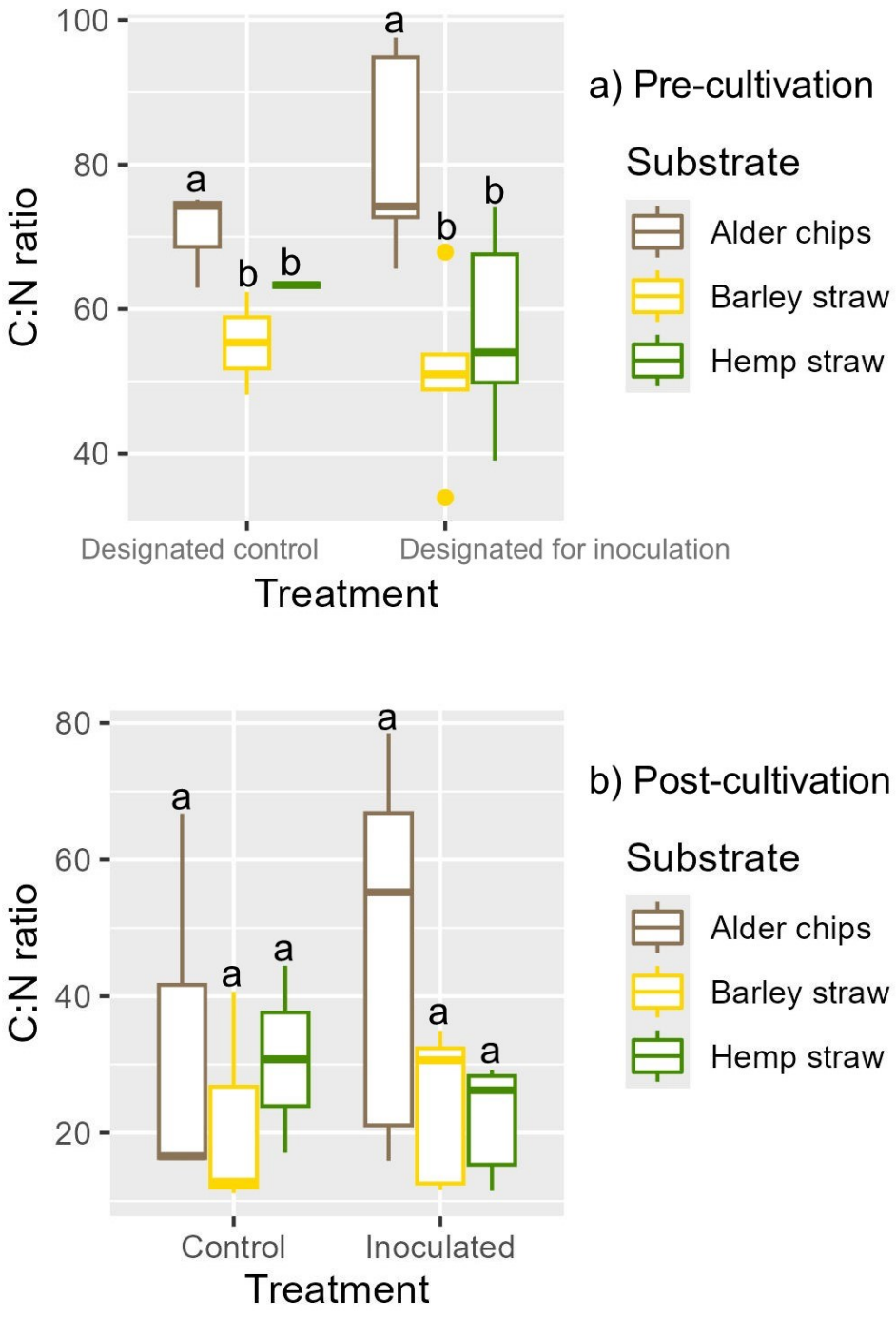


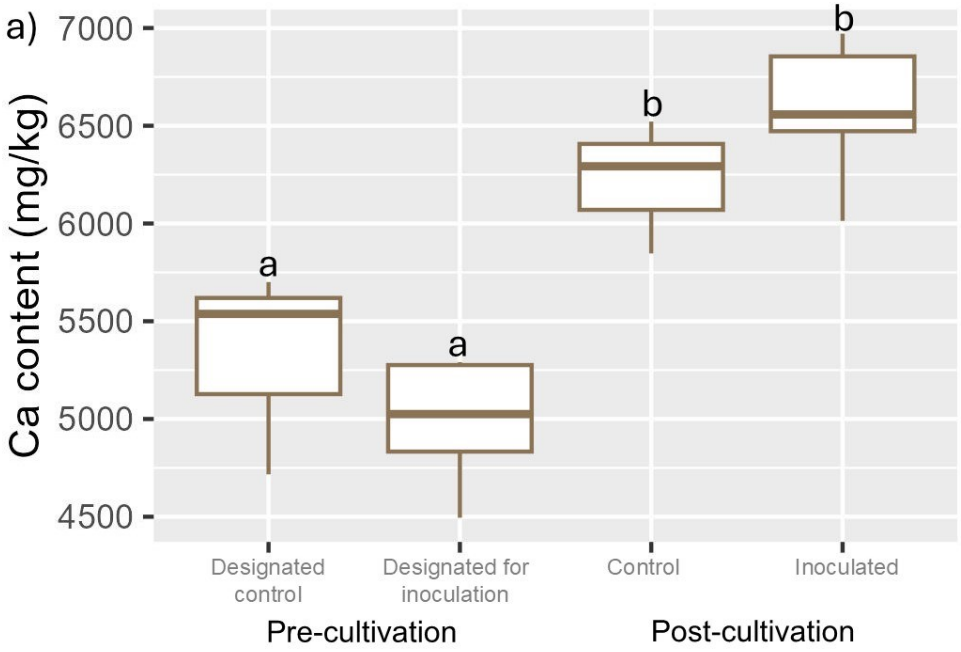
Figure 9: Boxplots of C:N ratio of substrate samples. The horizontal bar in each box represents the median value. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The boxed area displays the interquartile range (Q1-Q3), the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values and the dots represent outliers.

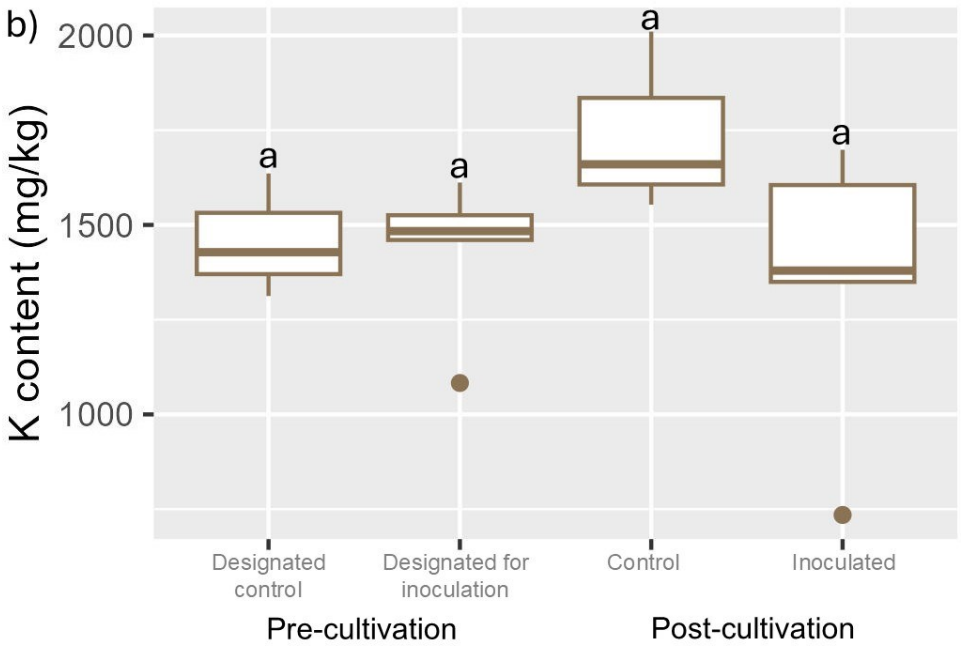
4.4.3 Substrate elemental analysis

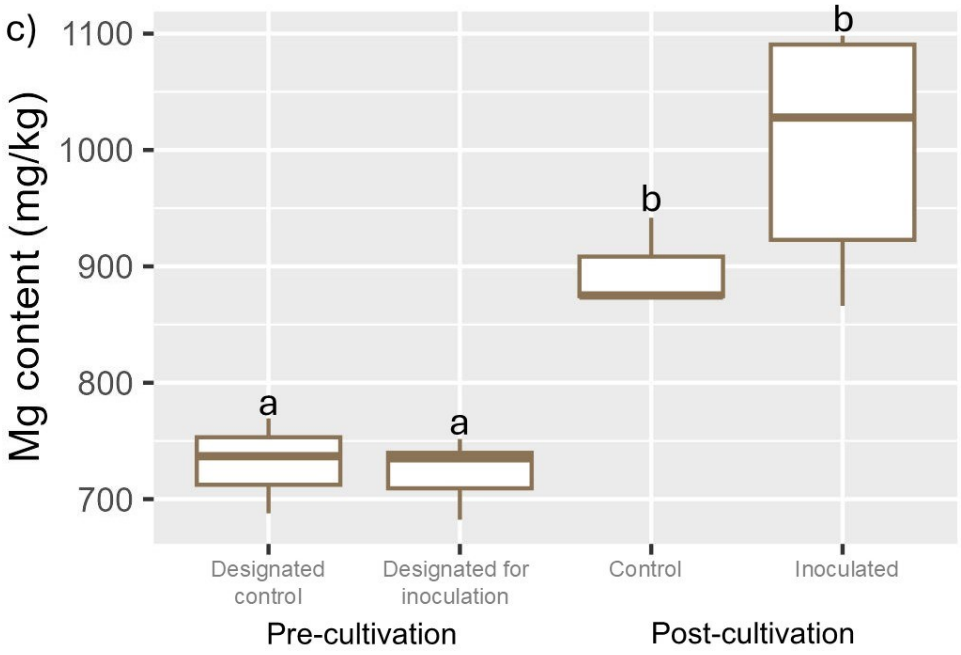
4.4.3.1 Macronutrients (Ca, K, Mg, P, S)

4.4.3.1.1 Alder chips

There were no significant treatment differences in the alder chips in any of the nutrients tested (Figure 10, a)-e). Ca, Mg and S content increased significantly over the cultivation period regardless of treatment (Figure 10 a), c), and e).




b)
K content (mg/kg)
2000
1500
1000
a
a
a
a
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation


c)
Mg content (mg/kg)
1100
1000
900
800
700
a
a
b
b
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation

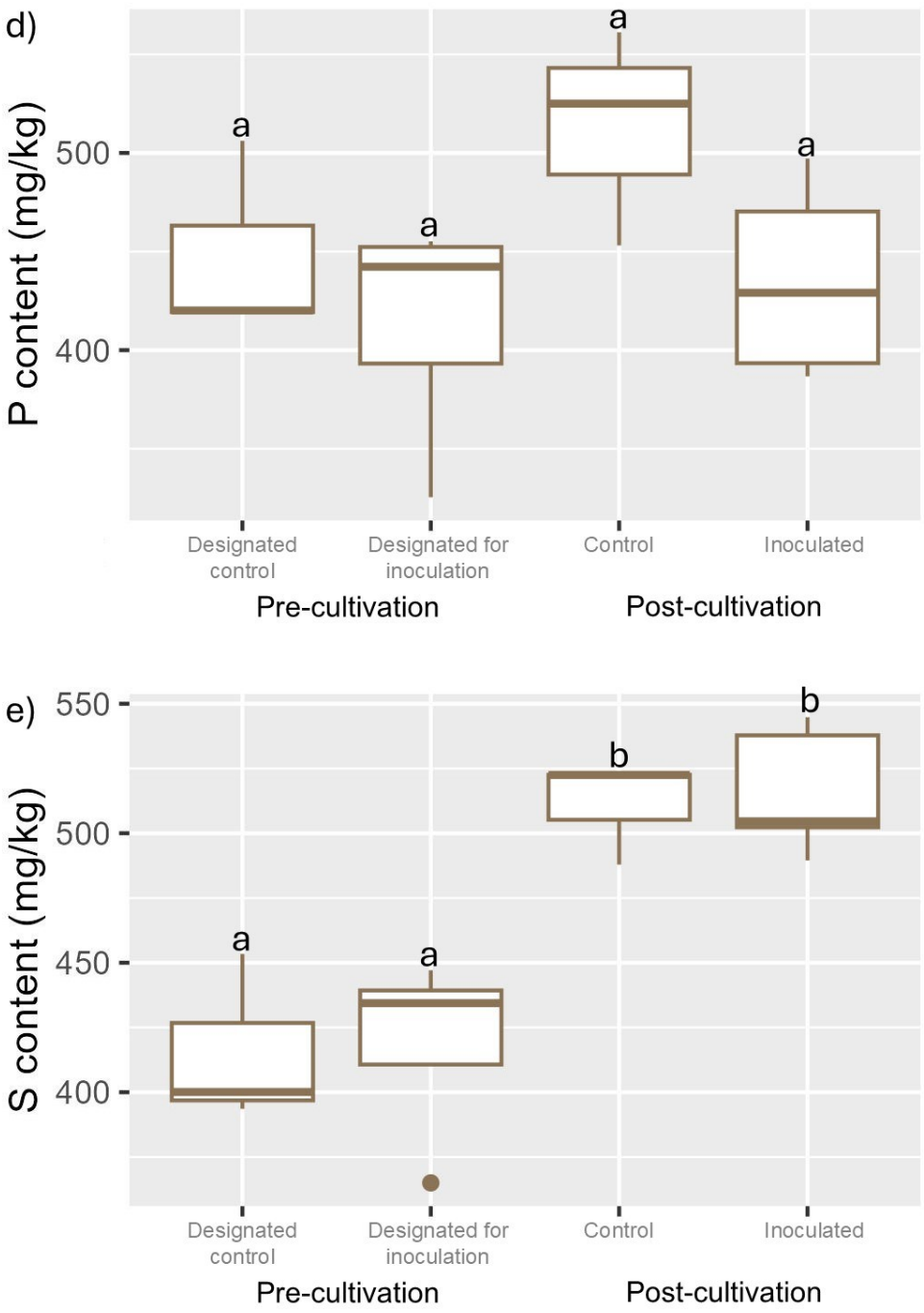
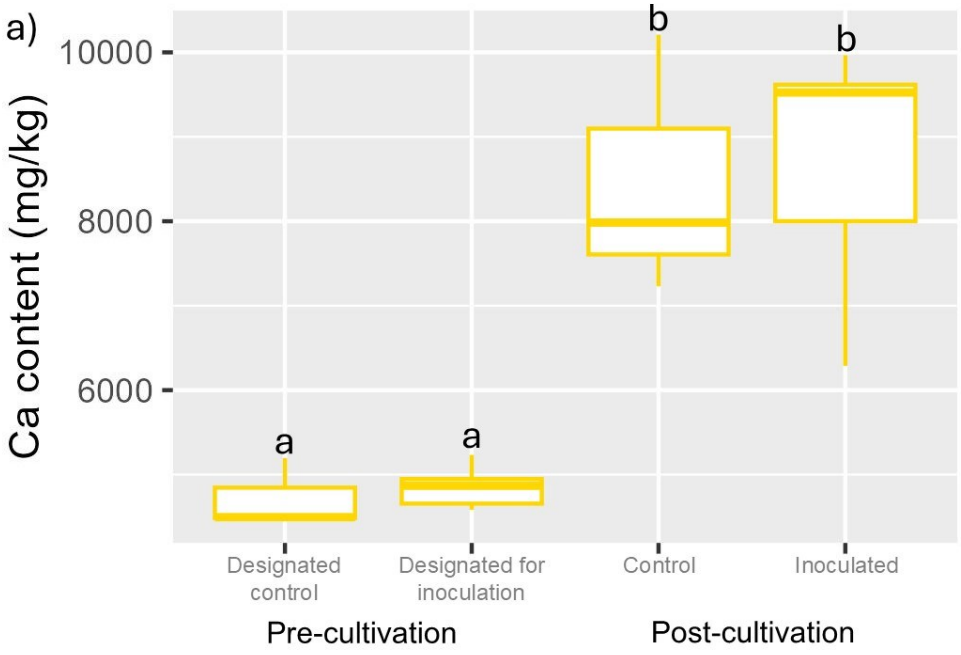
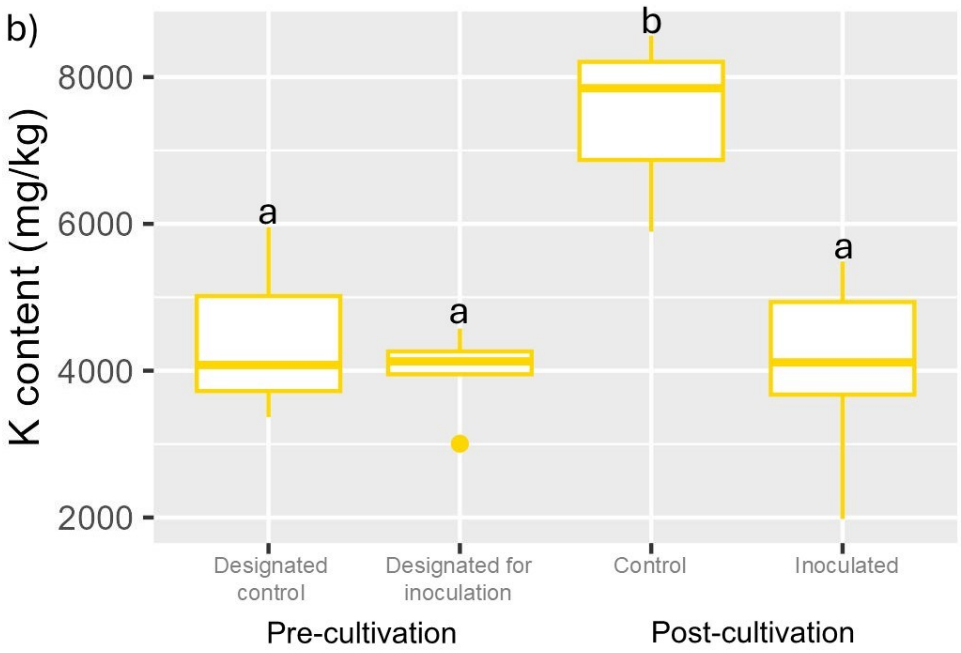


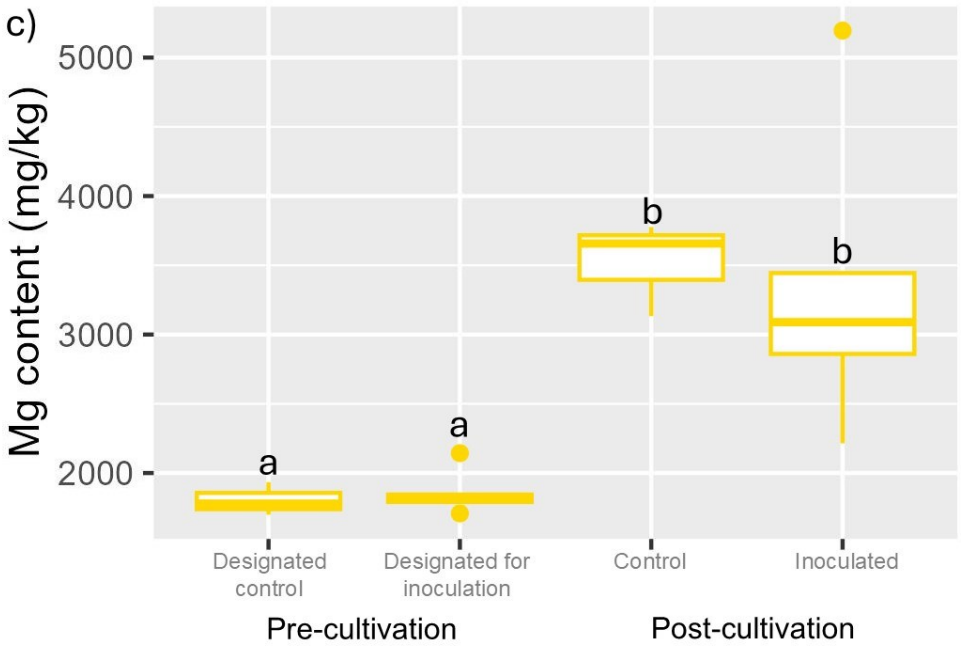
Figure 10 a)-e): Boxplots of alder chip dry matter content of a) Ca, b) K, c) Mg, d) P, and e) S. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

4.4.3.1.2 Barley straw

Post-cultivation control barley straw samples contained more K and P than their inoculated counterparts ($p < 0.05$). K and P did not increase significantly over the cultivation period in the inoculated samples (Figure 11 b) and d). Ca, Mg and S content in barley straw increased over the cultivation period regardless of treatment (Figure 11 a), c) and e).




b)
K content (mg/kg)
8000
6000
4000
2000
a
a
b
a
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation


c)
Mg content (mg/kg)
5000
4000
3000
2000
a
a
b
b
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation

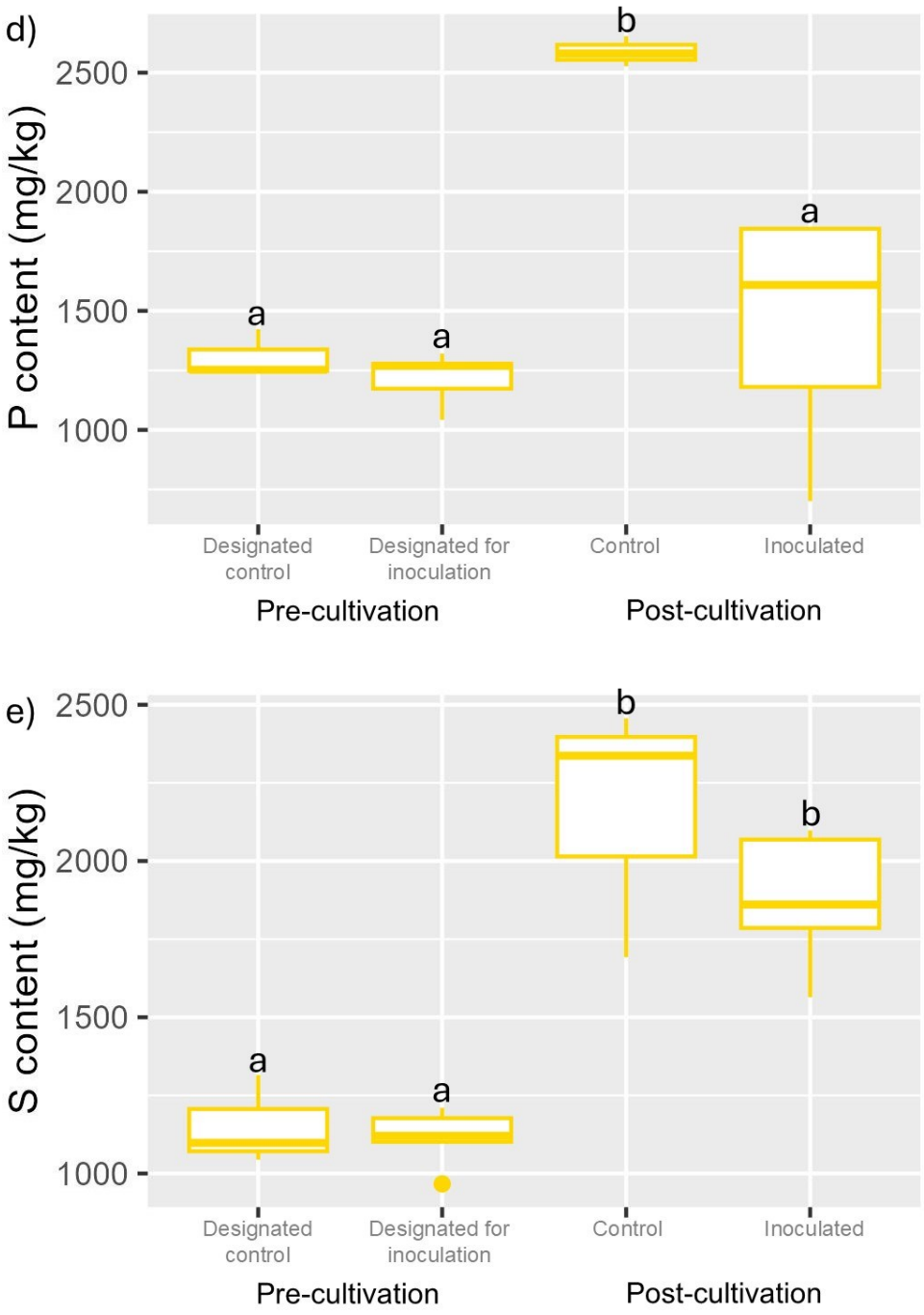
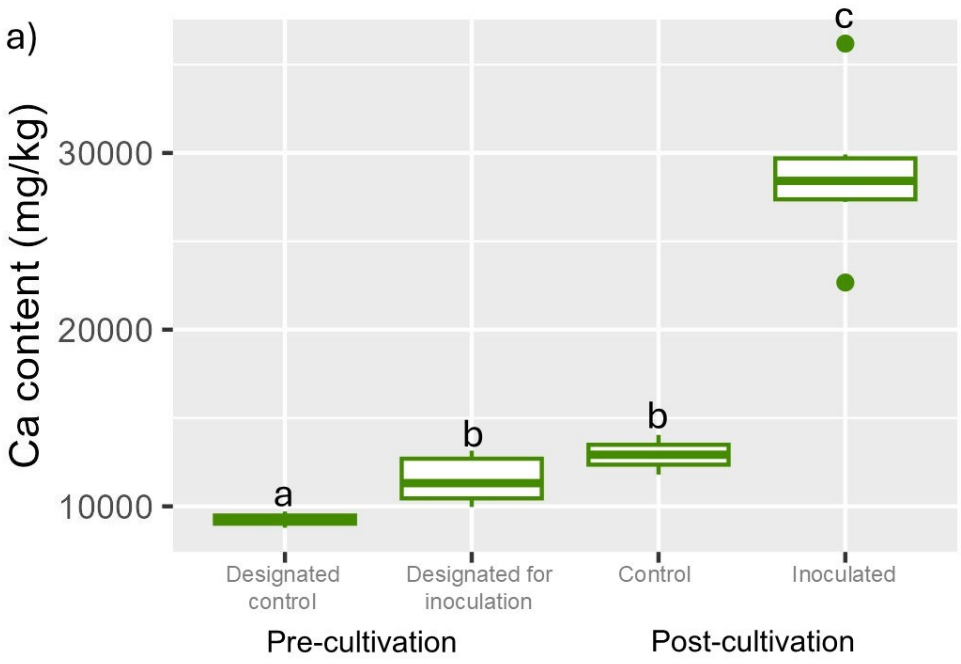
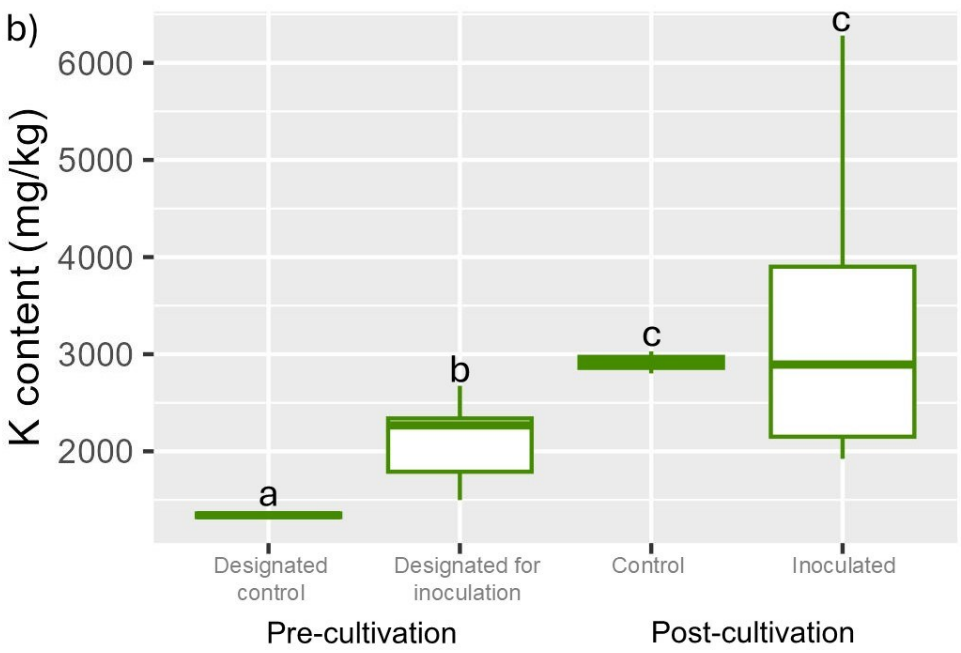


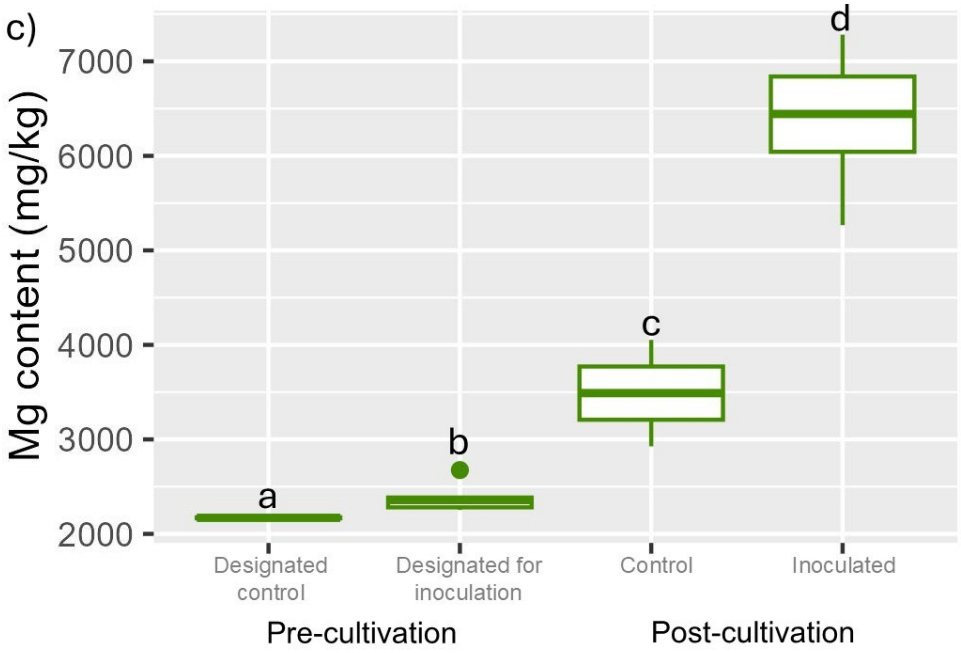
Figure 11 a)-e): Boxplots of barley straw dry matter content of a) Ca, b) K, c) Mg, d) P, and e) S. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

4.4.3.1.3 Hemp straw

There were significant differences in Ca, K and Mg between the pre-cultivation hemp straw samples that were designated for inoculation versus control. The samples designated for inoculation were higher in these three nutrients (Figure 12 a) to c). Post-cultivation treated hemp straw samples contained significantly more Ca, Mg and S than their untreated counterparts, and the content of these nutrients in the treated samples increased over the cultivation period (Figure 12 a), c) and e). K content increased regardless of treatment (Figure 12 b). There was no significant change in P content over the cultivation period (Figure 12 d).




b)
K content (mg/kg)
6000
5000
4000
3000
2000
a
b
c
c
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation


c)
Mg content (mg/kg)
7000
6000
5000
4000
3000
2000
a
b
c
d
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation

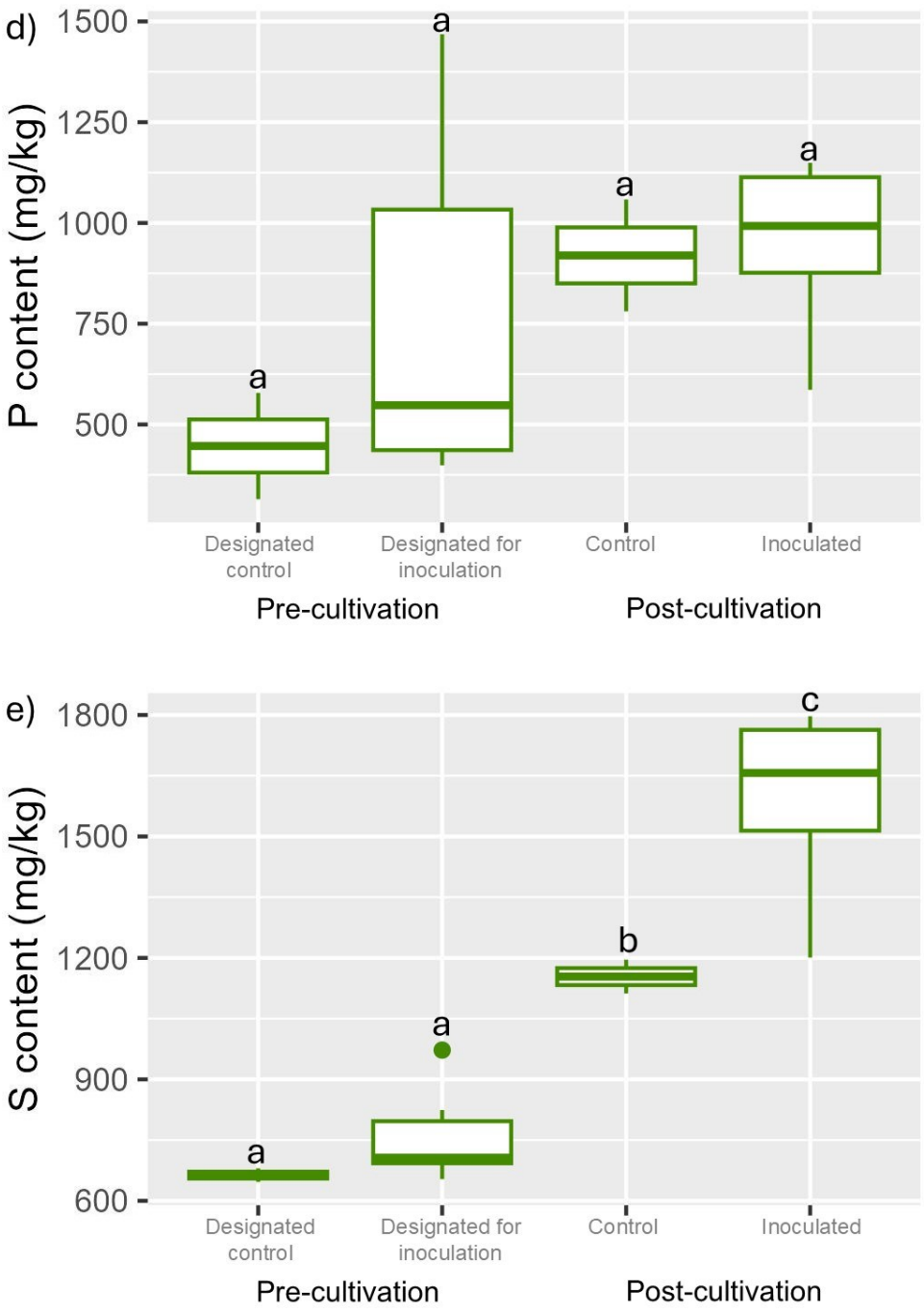
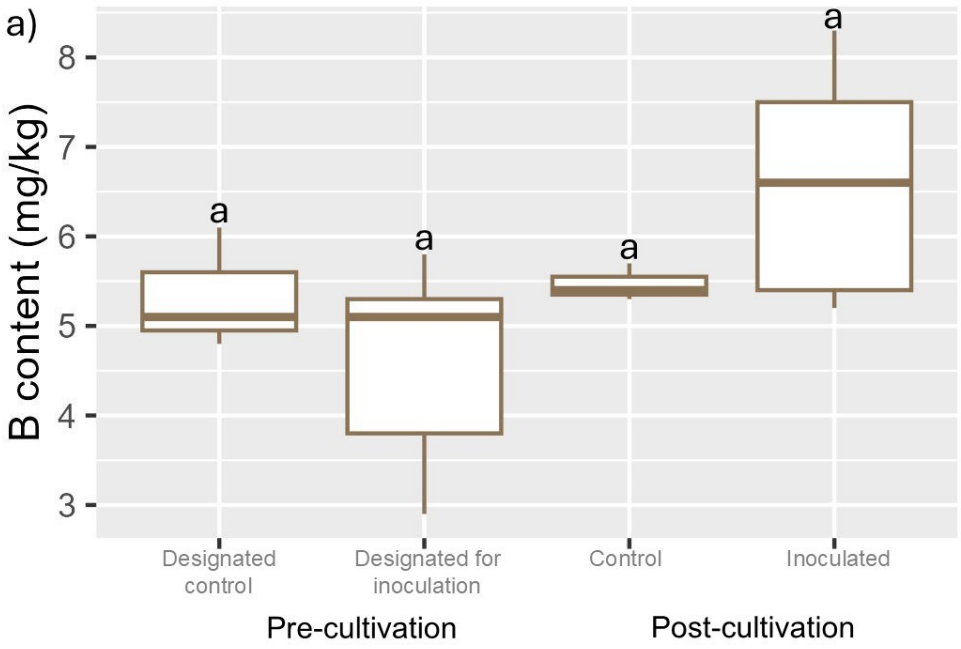


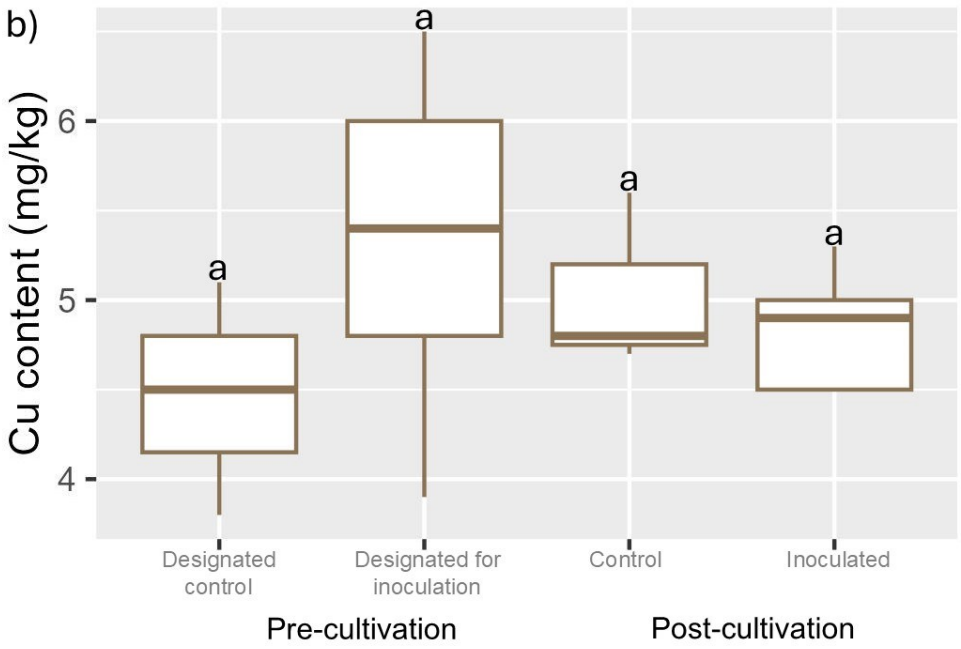
Figure 12 a)-e): Boxplots of hemp straw dry matter content of a) Ca, b) K, c) Mg, d) P, and e) S. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

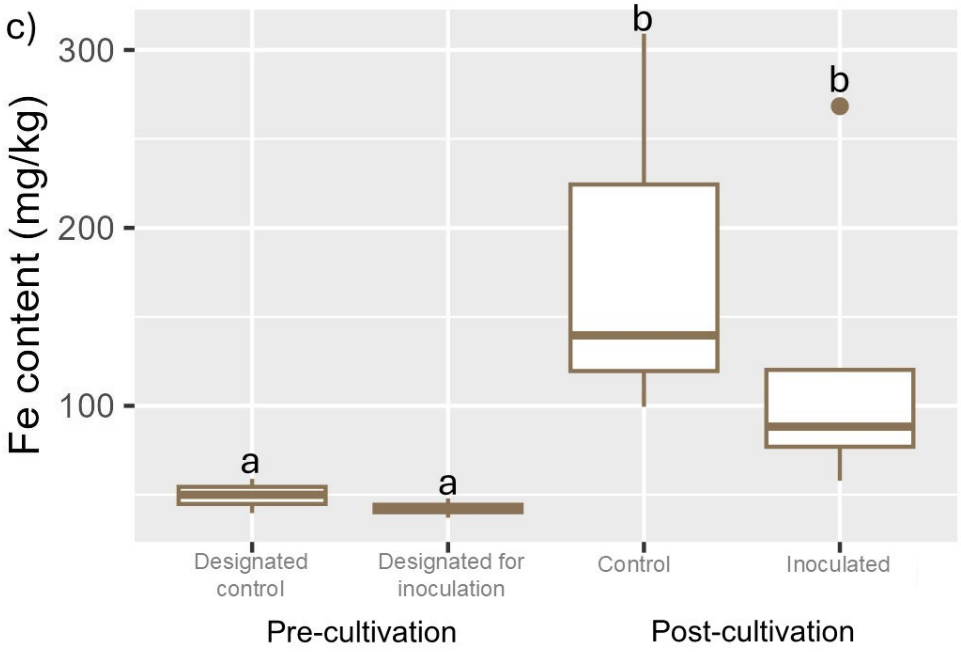
4.4.3.2 Micronutrients (B, Cu, Fe, Mn, Zn)

4.4.3.2.1 Alder chips

There were no micronutrient content treatment differences in the post-cultivation alder chip samples that met the $p < 0.05$ threshold for significance (Figure 13 a) to e). However, the inoculated samples contained more Mn and Zn than their corresponding controls at a borderline level of significance ($p = 0.053$ for both). Among the treated alder chip samples, Fe, Mn, and Zn content increased over the cultivation period, and B and Cu showed no trend.




b)
Cu content (mg/kg)
6
5
4
a
a
a
a
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation


c)
Fe content (mg/kg)
300
200
100
a
a
b
b
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation

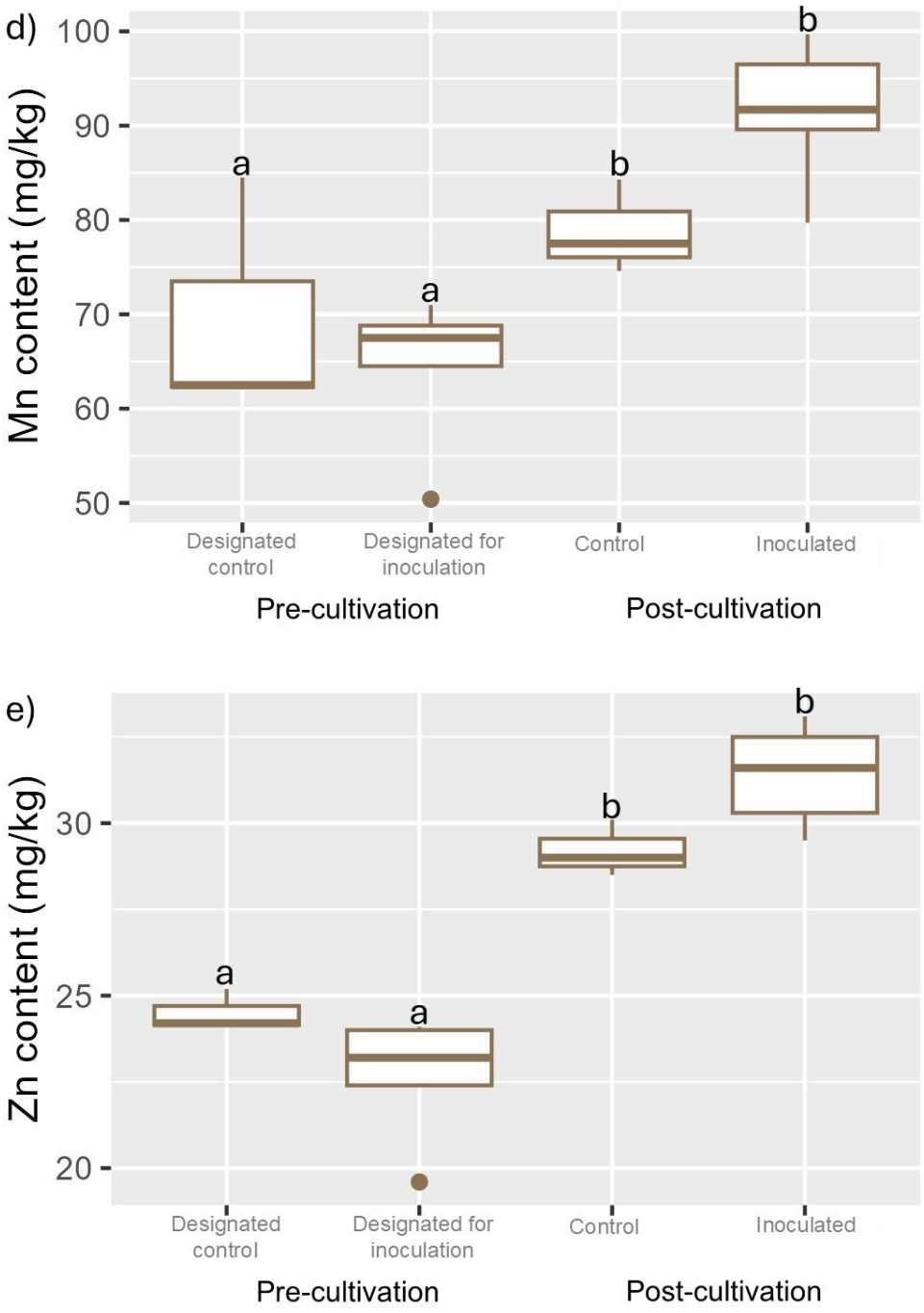
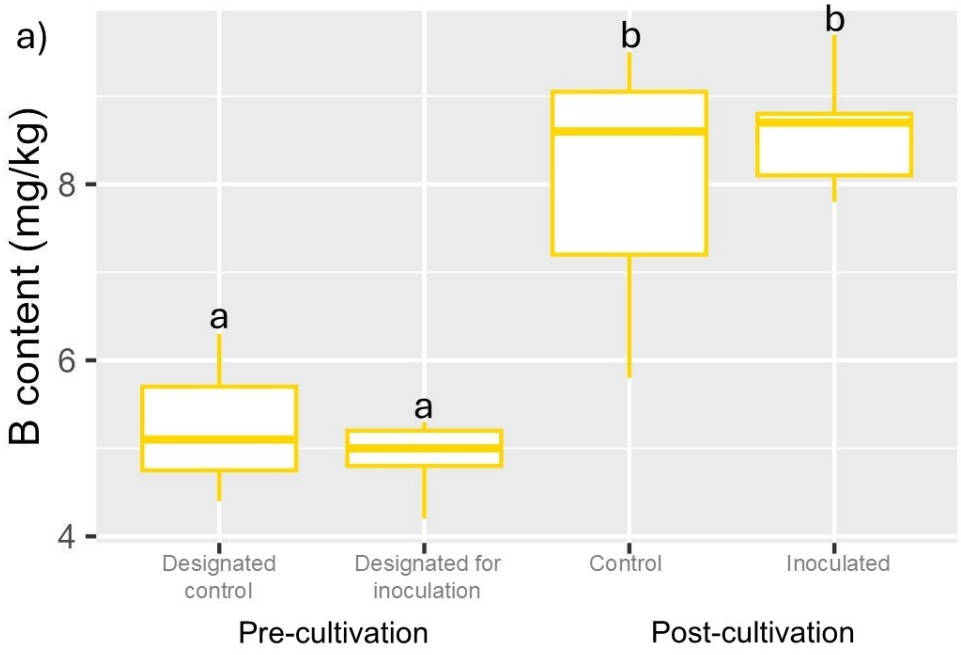
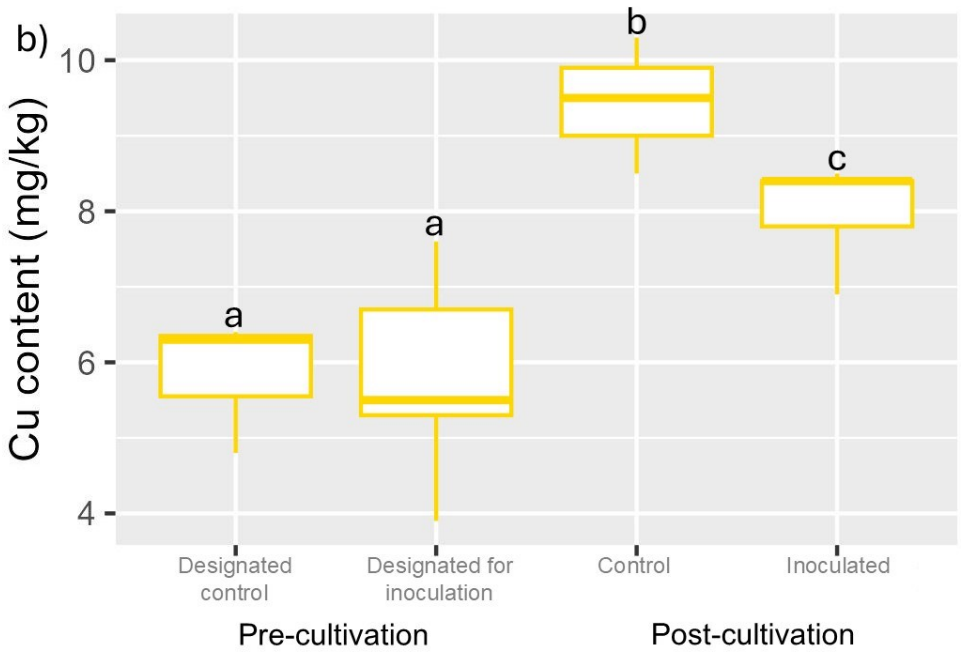


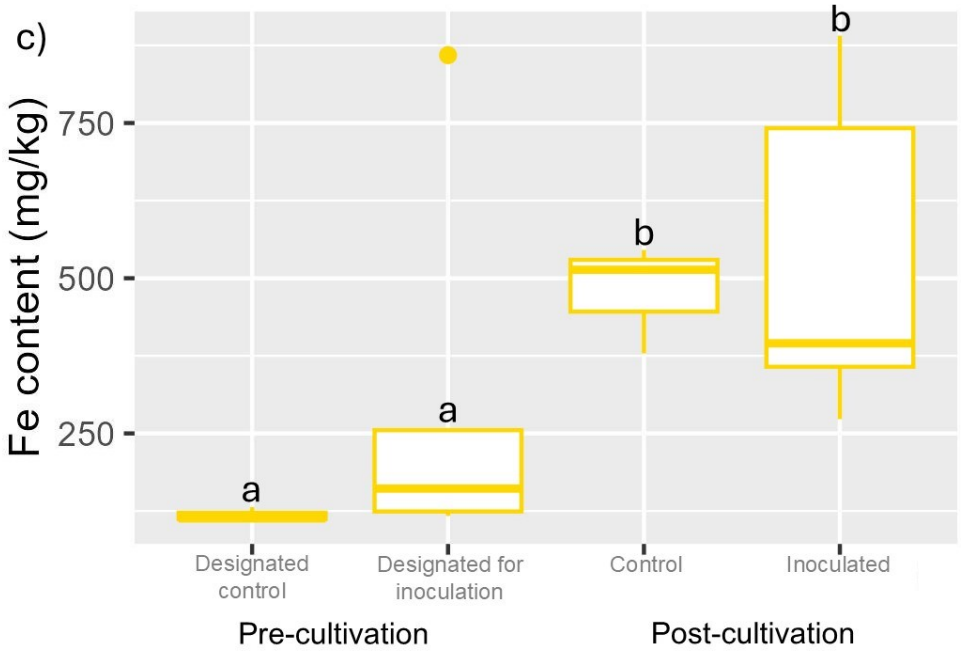
Figure 13 a)-e): Boxplots of alder chip dry matter content of a) B, b) Cu, c) Fe, d) Mn and e) Zn. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

4.4.3.2.2 Barley straw

Inoculated post-cultivation barley straw samples contained significantly less Cu than the corresponding controls ($p < 0.05$) (Figure 14 b). There were no post-cultivation treatment differences in B, Fe, Mn, or Zn content. The content of all micronutrients tested increased over the cultivation period (Figure 14 a) to e).




b)
Cu content (mg/kg)
10
8
6
4
a
a
b
c
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation


c)
Fe content (mg/kg)
750
500
250
a
a
b
b
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation

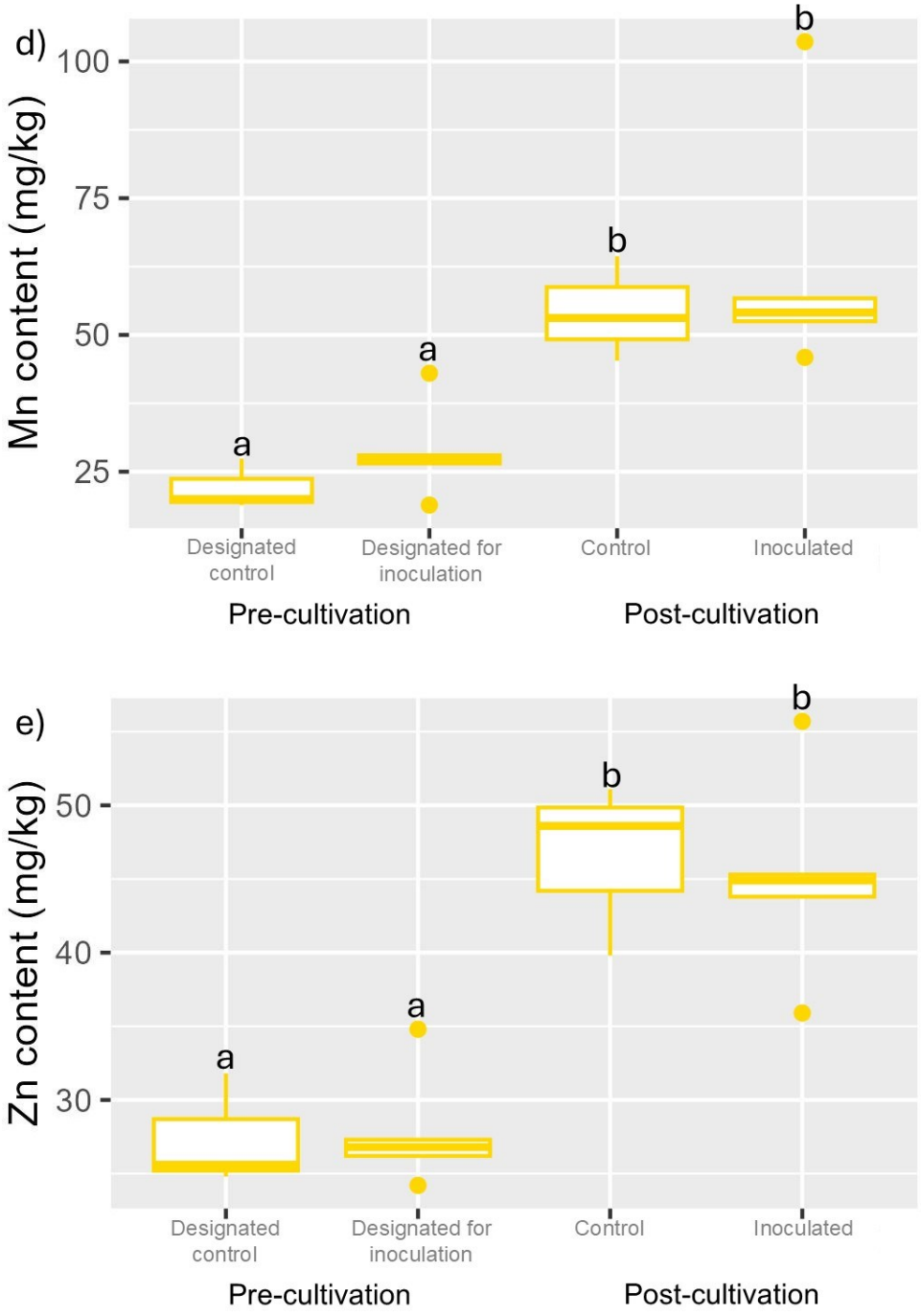
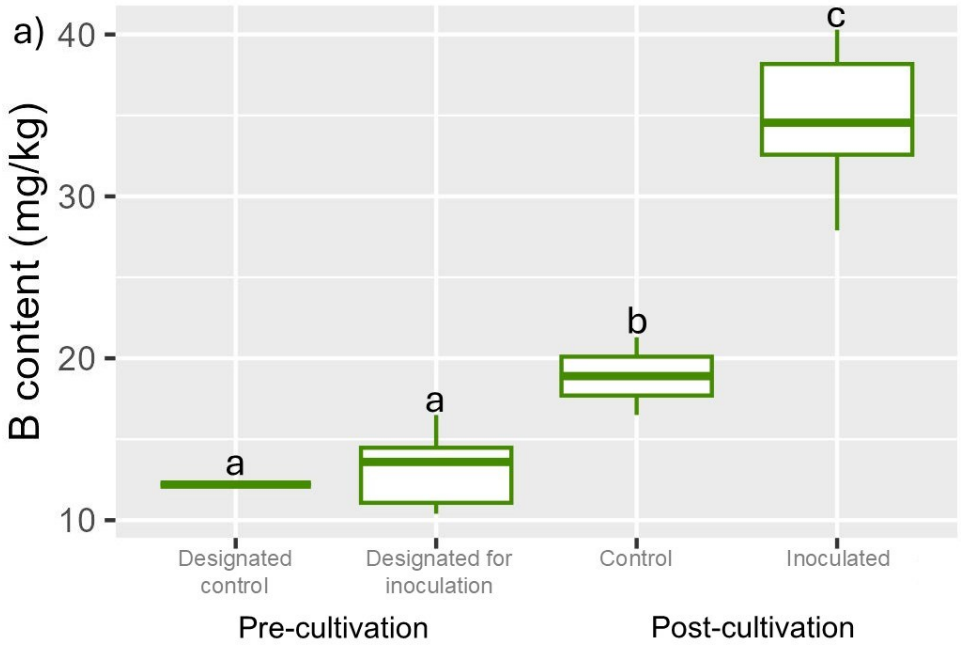
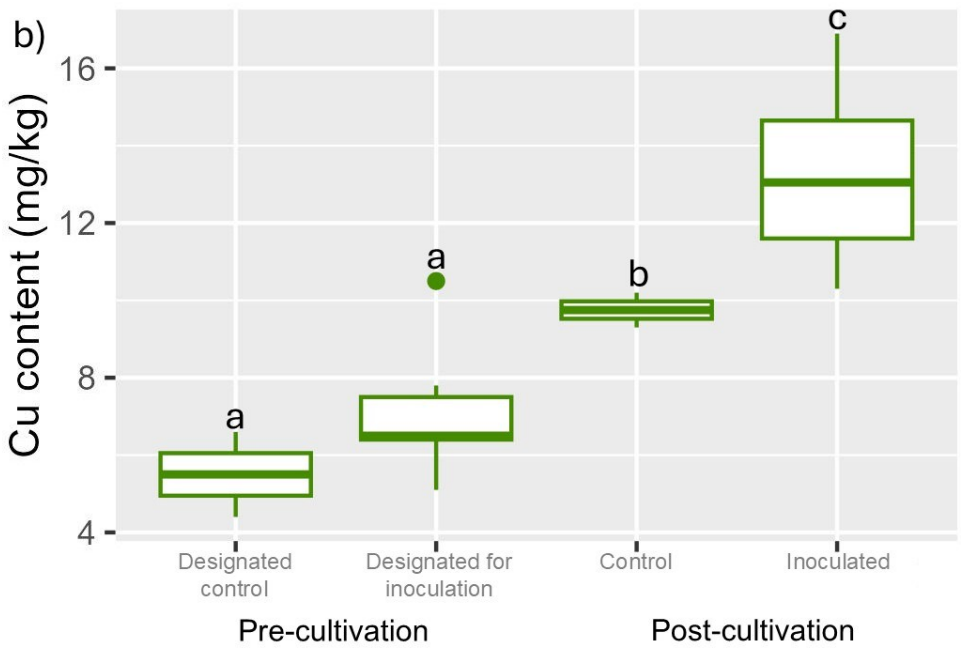


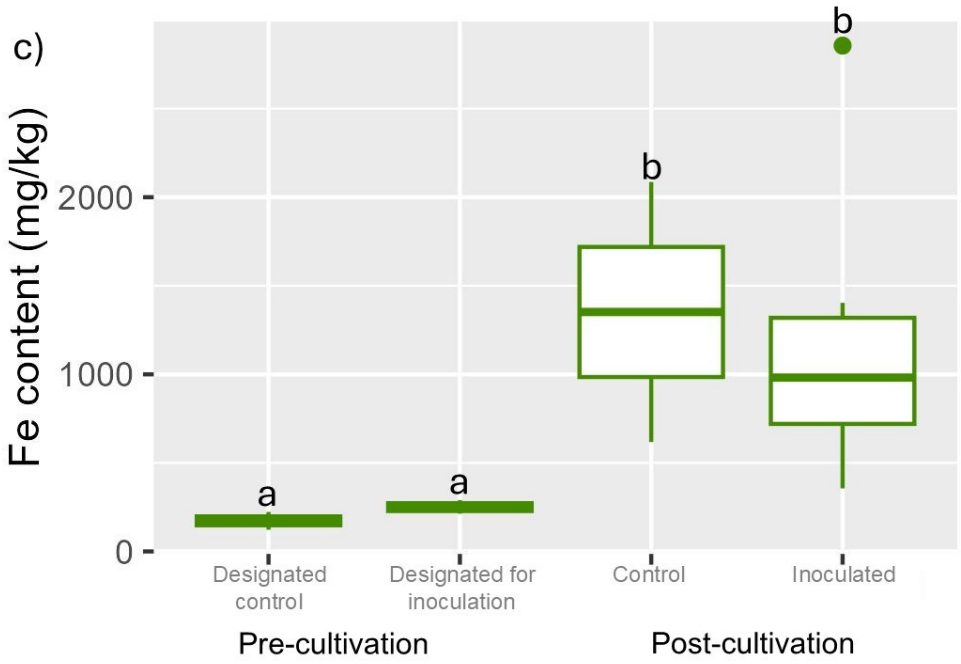
Figure 14 a)-e): Boxplots of barley straw dry matter content of a) B, b) Cu, c) Fe, d) Mn and e) Zn. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

4.4.3.2.3 Hemp straw

Pre-cultivation hemp straw samples designated for inoculation contained more Zn than their counterparts designated control ($p < 0.05$, Figure 15 e). Post-cultivation inoculated hemp samples contained significantly more B, Cu and Mn than the control samples ($p < 0.05$) (Figure 15 a), b) and d). Post-cultivation Fe and Zn content did not vary significantly between treatments (Figure 15 c) and e). Over the cultivation period, the content of all micronutrients tested increased in the hemp samples.




b)
Cu content (mg/kg)
16
12
8
4
a
a
b
c
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation


c)
Fe content (mg/kg)
2000
1000
0
a
a
b
b
Designated control
Designated for inoculation
Control
Inoculated
Pre-cultivation
Post-cultivation

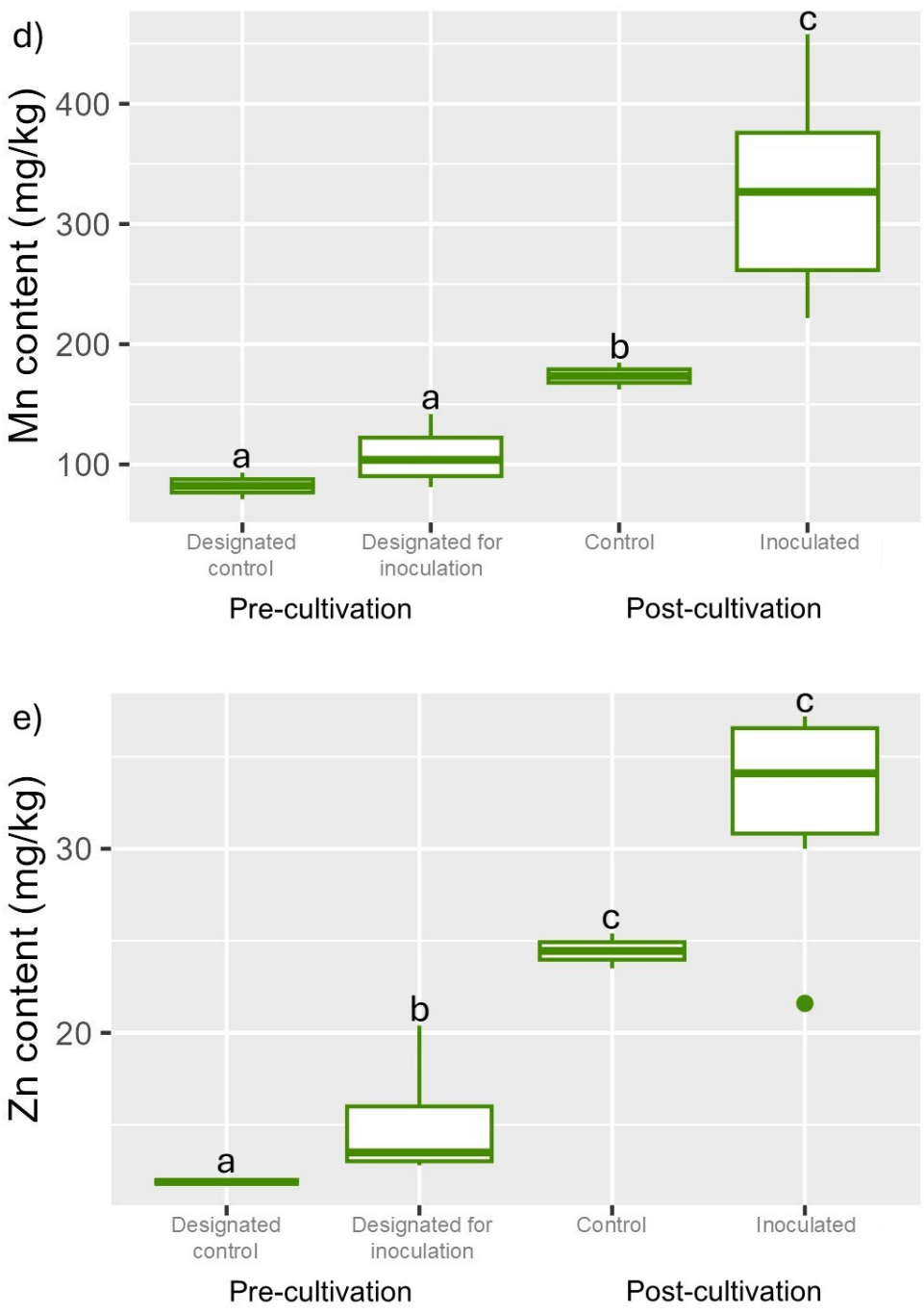
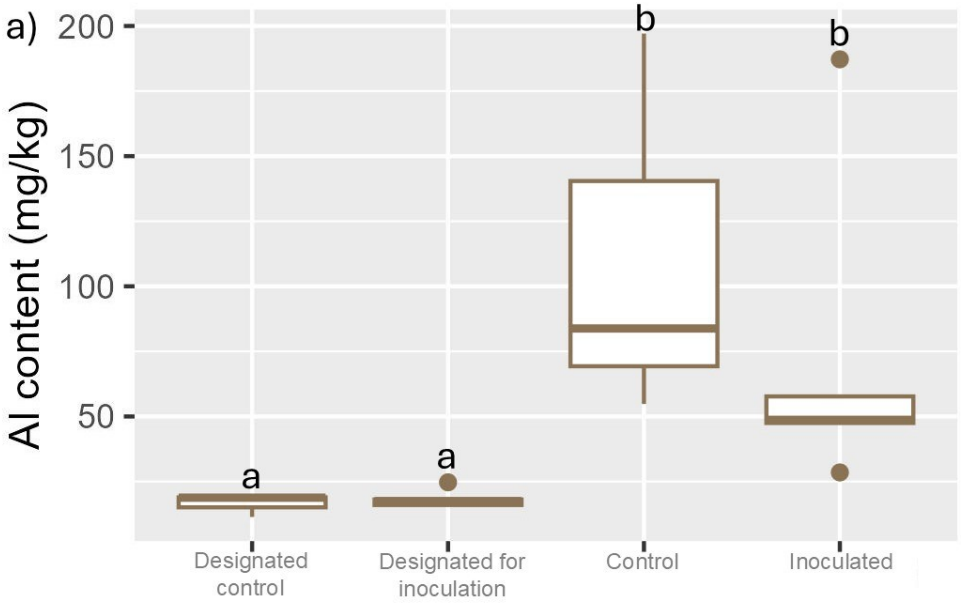


Figure 15 a)-e): Boxplots of hemp straw dry matter content of a) B, b) Cu, c) Fe, d) Mn and e) Zn. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

4.4.3.3 Sodium and aluminum

There were no significant treatment differences in Al or Na content for any substrate type. The trend over the cultivation period was for Al and Na content to increase in all substrates except alder chips, in which only Al content increased (Figures 16 to18).



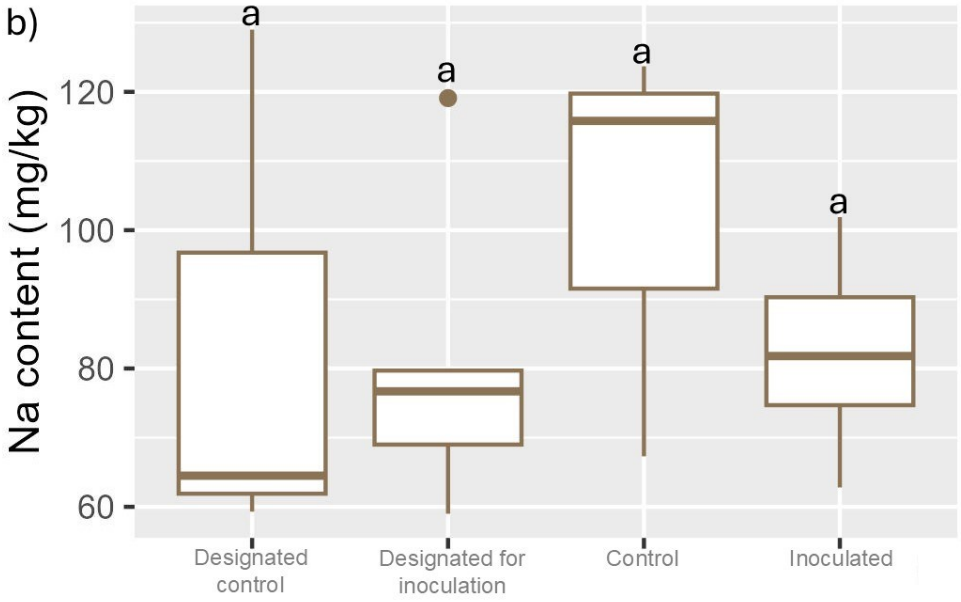


Figure 16: Boxplots of alder chip dry matter content of a) Al and b) Na. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

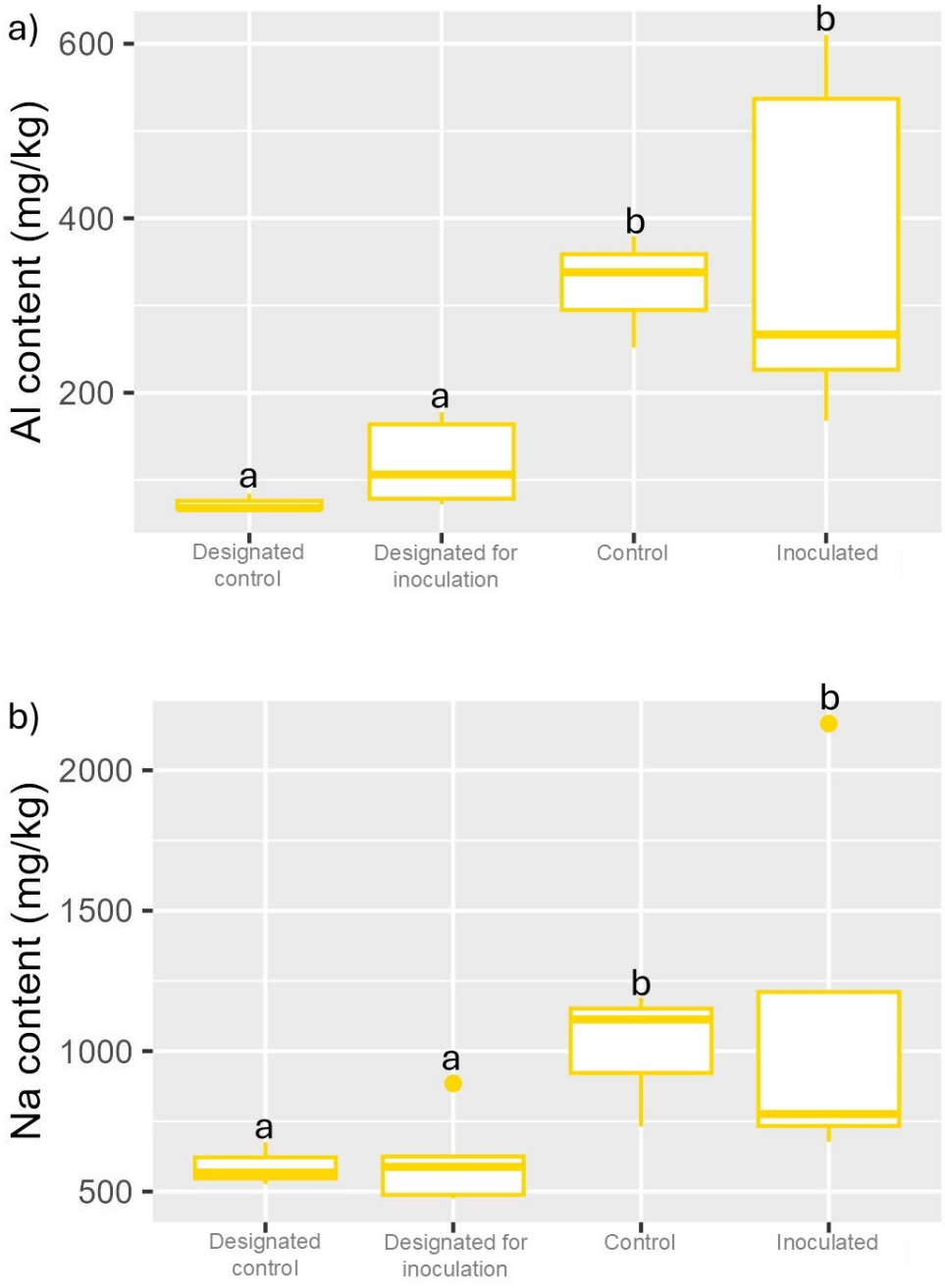


Figure 17: Boxplots of barley straw dry matter content of a) Al and b) Na. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile

range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

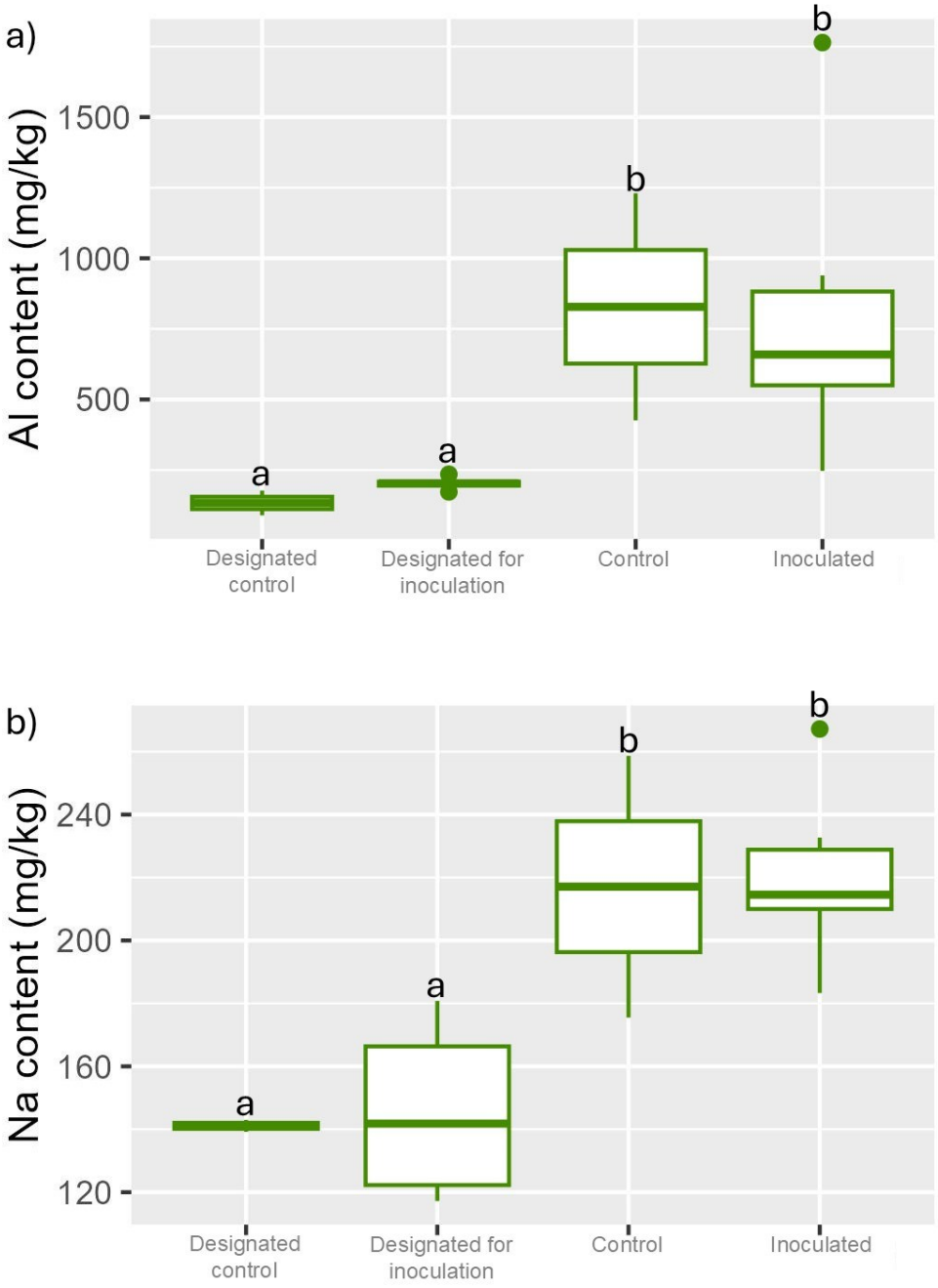
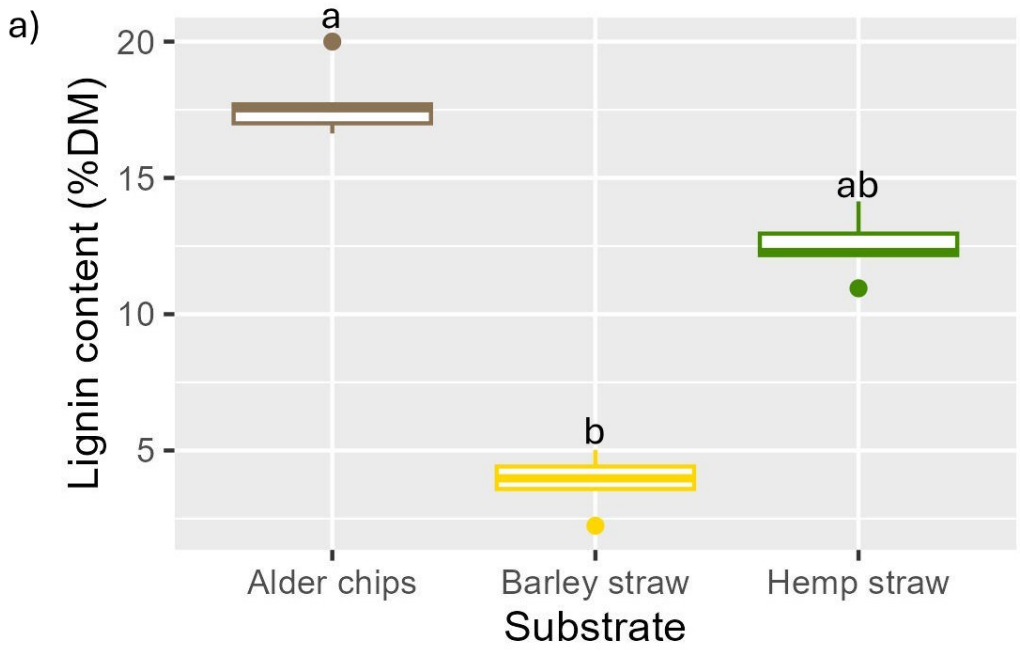


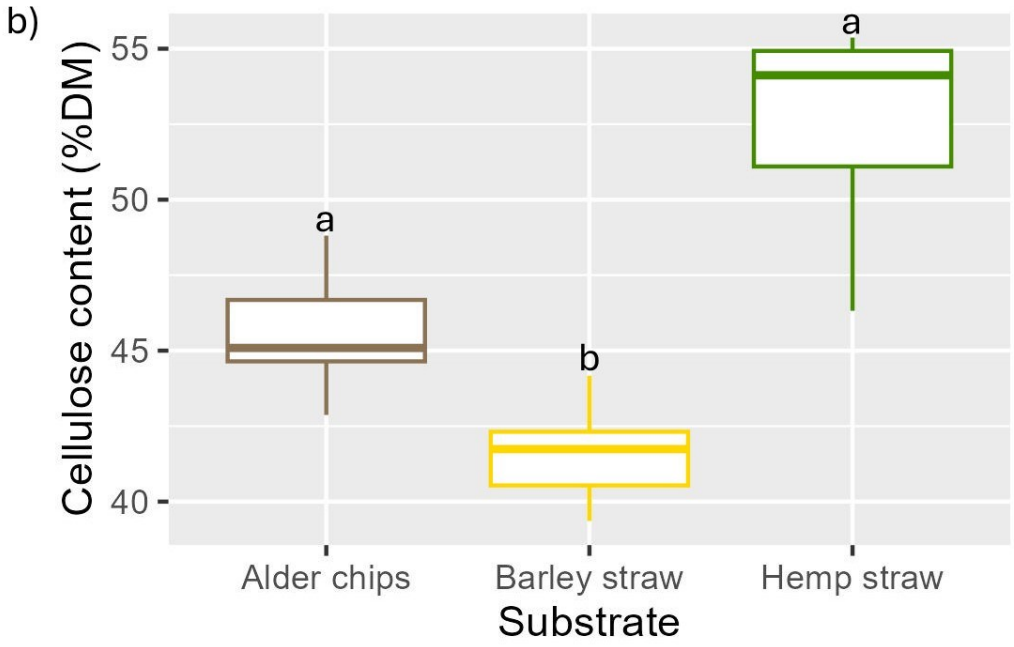
Figure 18: Boxplots of hemp straw dry matter content of a) Al and b) Na. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

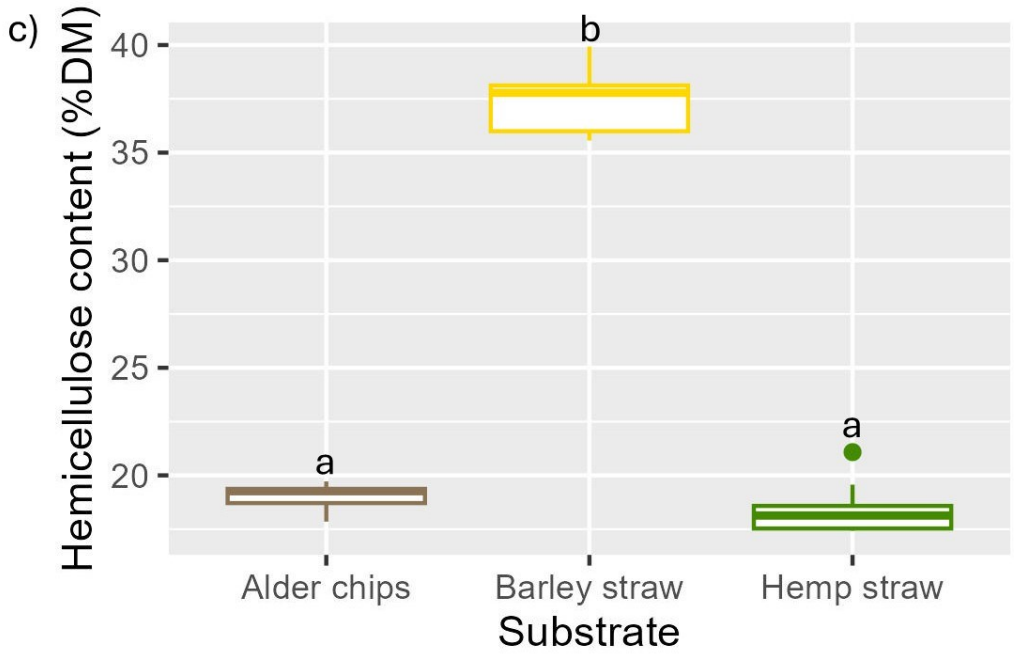
4.4.4 Substrate Van Soest fiber analysis

4.4.4.1 Pre-cultivation substrate comparisons

Substrates did not differ significantly in their pre-cultivation total lignocellulosic biomass content. Median lignocellulosic biomass content values ranged from 81 to 86% across substrates (Figure 19 d). However, proportions of lignin, cellulose and hemicellulose did vary. Among samples designated for inoculation, barley straw contained over four times less lignin than alder chips ($p < 0.01$) and three times less lignin than hemp straw ($p = 0.056$) (Figure 19 a). Barley straw contained almost twice as much hemicellulose as alder chips and more than twice as much hemicellulose as hemp straw ($p < 0.05$ and $p < 0.01$ respectively). Hemp straw and alder chips had similar proportions of lignin, cellulose, and hemicellulose.




b)
Cellulose content (%DM)
55
50
45
40
a
b
a
Alder chips
Barley straw
Hemp straw
Substrate


c)
Hemicellulose content (%DM)
40
35
30
25
20
b
a
a
Alder chips
Barley straw
Hemp straw
Substrate

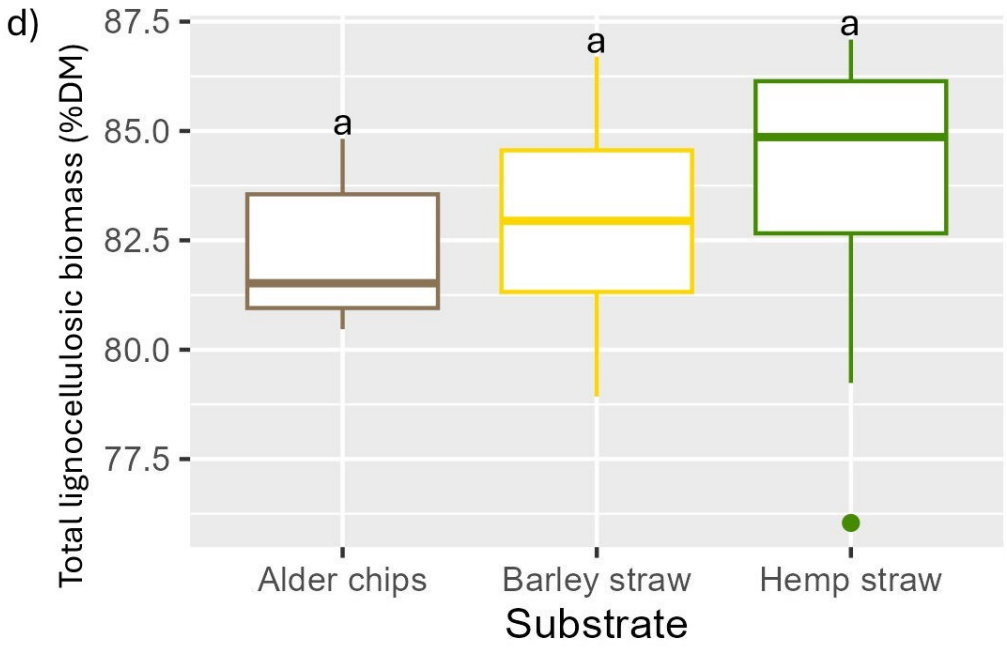


Figure 19: Boxplots of pre-cultivation substrate content of a) lignin, b) cellulose, c) hemicellulose and d) total lignocellulosic biomass (all expressed a percentage of dry matter). Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

4.4.4.2 Pre- and post-cultivation comparisons

4.4.4.2.1 Lignocellulosic biomass

The overall proportion of lignocellulosic biomass in all inoculated substrate types decreased over the cultivation period (compare Figures 19 d) and 20 d). This trend was strongest for hemp straw, which had a median decrease in lignocellulosic biomass content of 38%, followed by barley straw at -27% and alder chips at -9%. There was insufficient data to perform the Wilcoxon signed rank confidence interval on the change in the control samples, but the median decreases in the percentage of lignocellulosic biomass were smaller. For hemp straw, the median change in percentage was -0.8%, and for barley straw and alder chips it was -17% and +2.6%, respectively.

4.4.4.2.2 Lignin

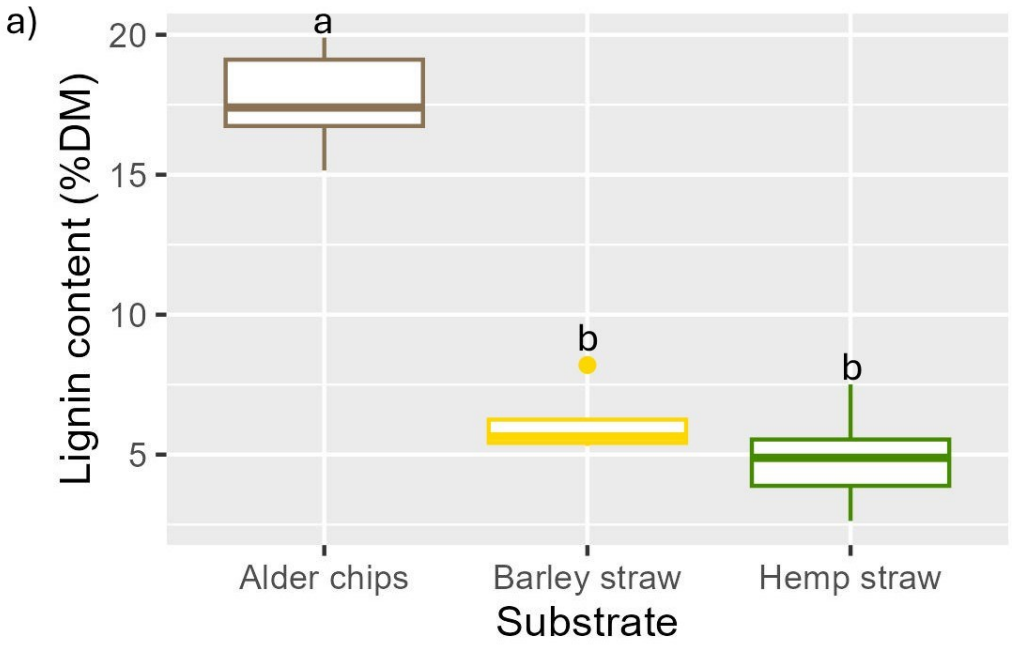
In all uninoculated substrate types, the median proportion of lignin increased slightly over the cultivation period. In the hemp straw control samples, the median increase in the percentage of lignin was 8.9% and in the barley straw and alder chip control samples, the increases in lignin percentage were 8.3% and 4.5% respectively.

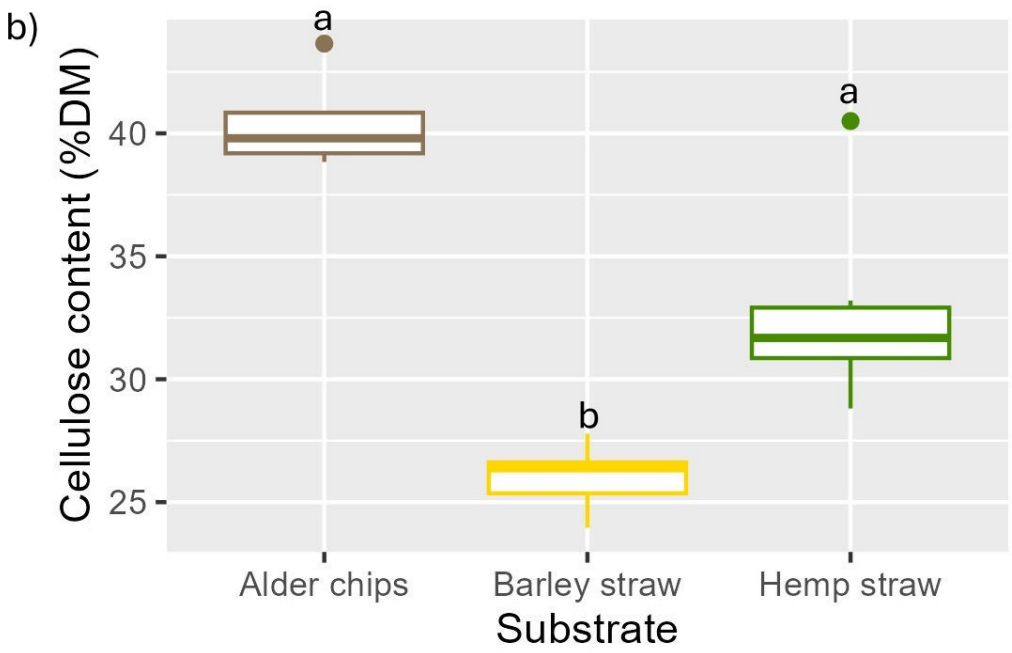
Lignin content remained relatively unchanged in inoculated barley straw and alder chips. Inoculated barley straw had a median increase in lignin percentage of 2.4% and treated alder chips had a median increase in lignin percentage of 0.22%. Hemp straw was the only substrate that showed a meaningful decrease in lignin content, with a median change in lignin percentage of -7.3%.

4.4.4.2.3 Cellulose and hemicellulose

Cellulose and hemicellulose content decreased in all inoculated substrates over the cultivation period, and these changes account for most of the overall change in lignocellulosic biomass before and after cultivation (compare Figures 19 and 20).

It was not possible to calculate a confidence interval for the changes in the control samples. However, based on the median percent change values, the trends for the untreated samples differed from those of the treated samples. In the alder chip control samples, the median change in cellulose content was +2.1% and the change in hemicellulose was -1.8%. In barley straw control samples, cellulose and hemicellulose content appeared to decrease (median changes of -15% and -7.9% respectively). In hemp straw control samples, cellulose content decreased 8.9%, but hemicellulose content did not differ greatly before and after cultivation.


a)
Lignin content (%DM)
20
15
10
5
a
b
b
Alder chips
Barley straw
Hemp straw
Substrate


b)
Cellulose content (%DM)
40
35
30
25
a
b
a
Alder chips
Barley straw
Hemp straw
Substrate

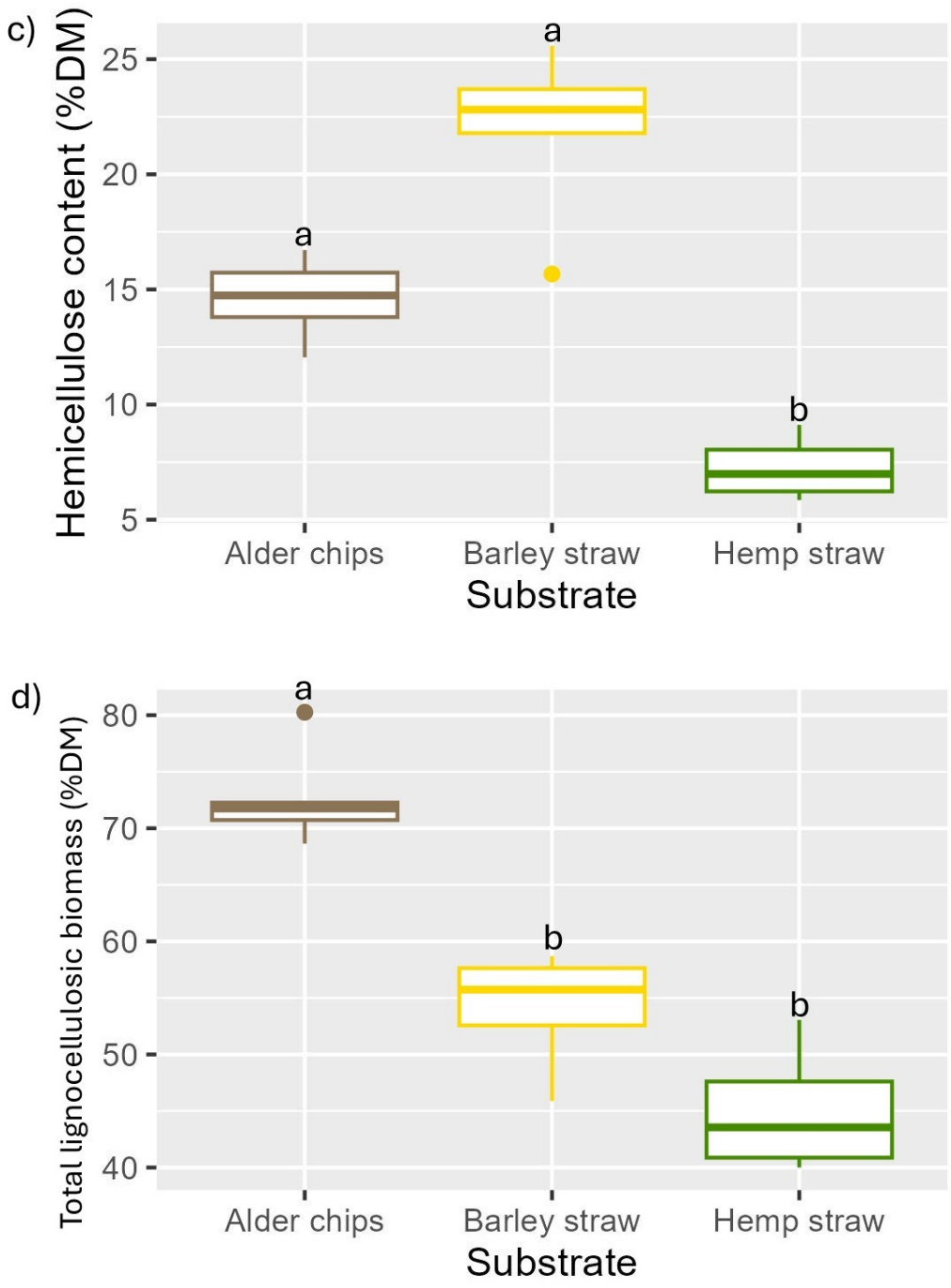
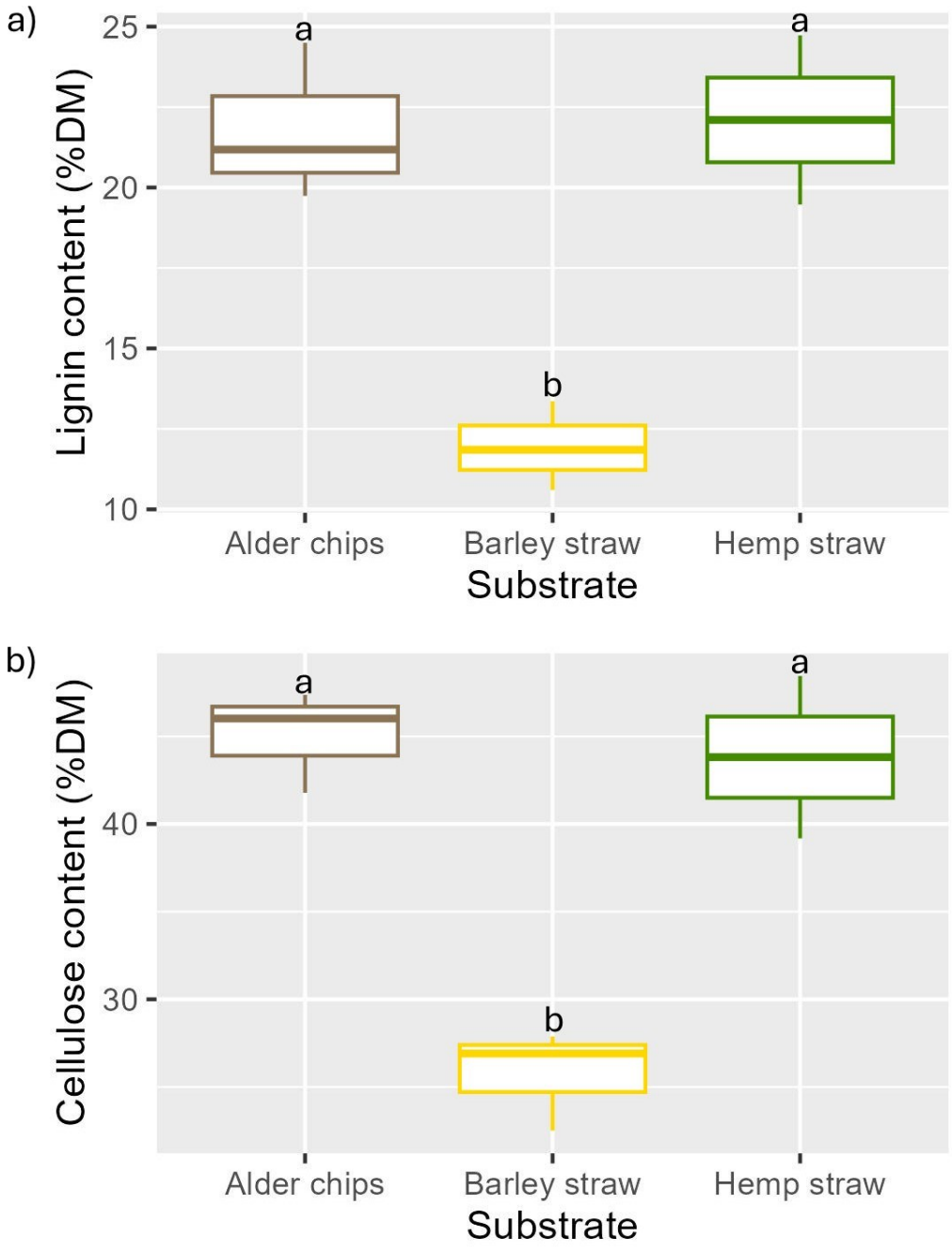


Figure 20: Boxplots of post-cultivation inoculated substrate content of a) lignin, b) cellulose, c) hemicellulose and d) total lignocellulosic biomass (all expressed a percentage of dry matter). Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.


a)
25
20
15
10
Lignin content (%DM)
a
b
a
Alder chips
Barley straw
Hemp straw
Substrate
b)
40
30
Cellulose content (%DM)
a
b
a
Alder chips
Barley straw
Hemp straw
Substrate

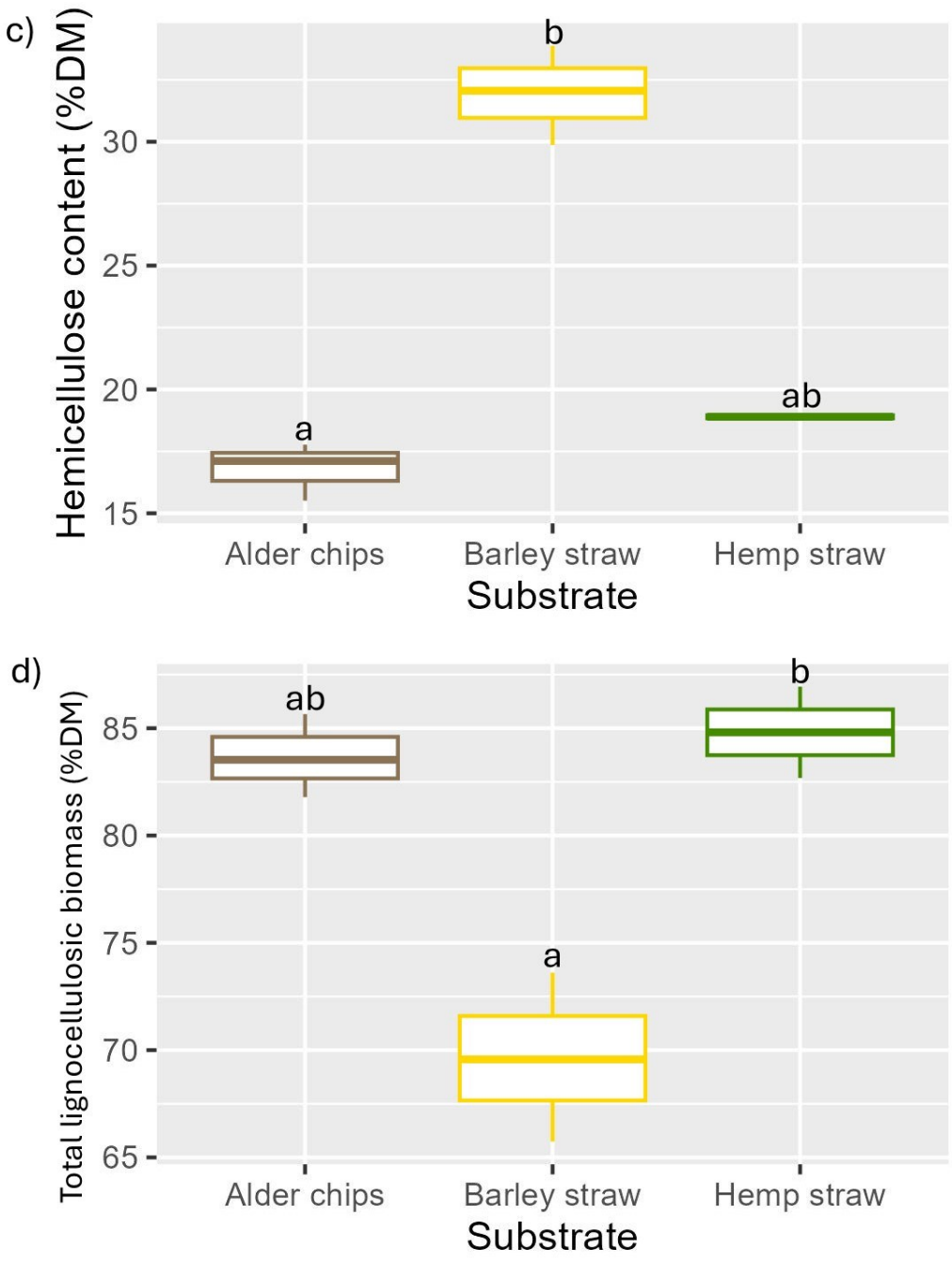


Figure 21: Boxplots of post-cultivation control substrate content of a) lignin, b) cellulose, c) hemicellulose and d) total lignocellulosic biomass (all expressed a percentage of dry matter). Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values.

4.4.4.3 Generalizability of results

It is worth considering the extent to which the results of this analysis can be generalized. The quantity and quality of plant fiber is affected by plant genotype, the location of the fiber within the plant, soil physical, chemical and biological properties, growing conditions such as light, temperature, precipitation and wind, and producer decisions such as planting and harvest time (Abdul Khalil et al., 2015; Tutt et al., 2013). Therefore, while the same plant parts (e.g. straw) from the same species (e.g. hemp) may contain similar portions of lignocellulosic biomass, there will also be differences in lignocellulosic biomass content resulting from variation in genotype, soil properties and growing conditions. The method of analysis used to quantify lignocellulosic compounds could also have an influence.

Table 8 compares the substrate samples designated for inoculation fibre analysis results with published values. There is substantial variation within the literature, and between the literature and my values. Therefore, caution must be exercised in drawing conclusions about the mushroom-growing suitability of the substrates tested in the present study based on their lignocellulosic biomass content. The same substrates produced under different conditions will differ in composition, and this would likely impact mushroom yield.

Table 8: Comparison of the lignocellulosic content of substrates (% dry matter) from literature data. Values from this study are for median treated, pre-cultivation substrate samples. N.d. = no data.

Substrate	Lignin	Cellulose	Hemicellulose	Total lignocellulosic	Publication
Alder (*Alnus viridis*) branchwood	17.65	46.53	19.3	83.44	This study
Alder (*A. glutinosa*)	21.81	50.94	n.d.	n.d.	(Španić et al., 2018)
Alder (*A. glutinosa*)	23.9	43.4	n.d.	n.d.	(Fengel & Wegener, 1984)

Alder (*A. sibirica*) branchwood	19.80	58.12	n.d.	n.d.	(X. Zhao et al., 2020)
Barley (*Hordeum vulgare*) straw	3.95	41.96	37.57	82.41	This study
Barley (*H. vulgare*) straw	14.6	34.8	27.9	77.3	(R. C. Sun & Tomkinson, 2000)
Barley (*H. vulgare*) straw	11	48	21	80	(Mahesh & Mohini, 2013)
Hemp (*Cannabis sativa*) straw	12.19	54.71	17.86	84.71	This study
Hemp (*C. sativa*) straw	8.76	53.86	10.60	73.22	(Raud et al., 2015)
Hemp (*C. sativa*) straw	7.43	37.08	10.46	54.97	(Tutt et al., 2013) (average values reported)

4.5 Substrate pH and Electrical Conductivity

4.5.1 pH

Alder chips had the lowest median pH in all treatment-cultivation stage combinations, significantly lower than barley straw, hemp straw or both in all cases (Figure 22). Barley straw and hemp straw pH values did not differ significantly within treatment-cultivation stage combinations. In the post-cultivation samples, all treated substrates were significantly more acidic than the controls.

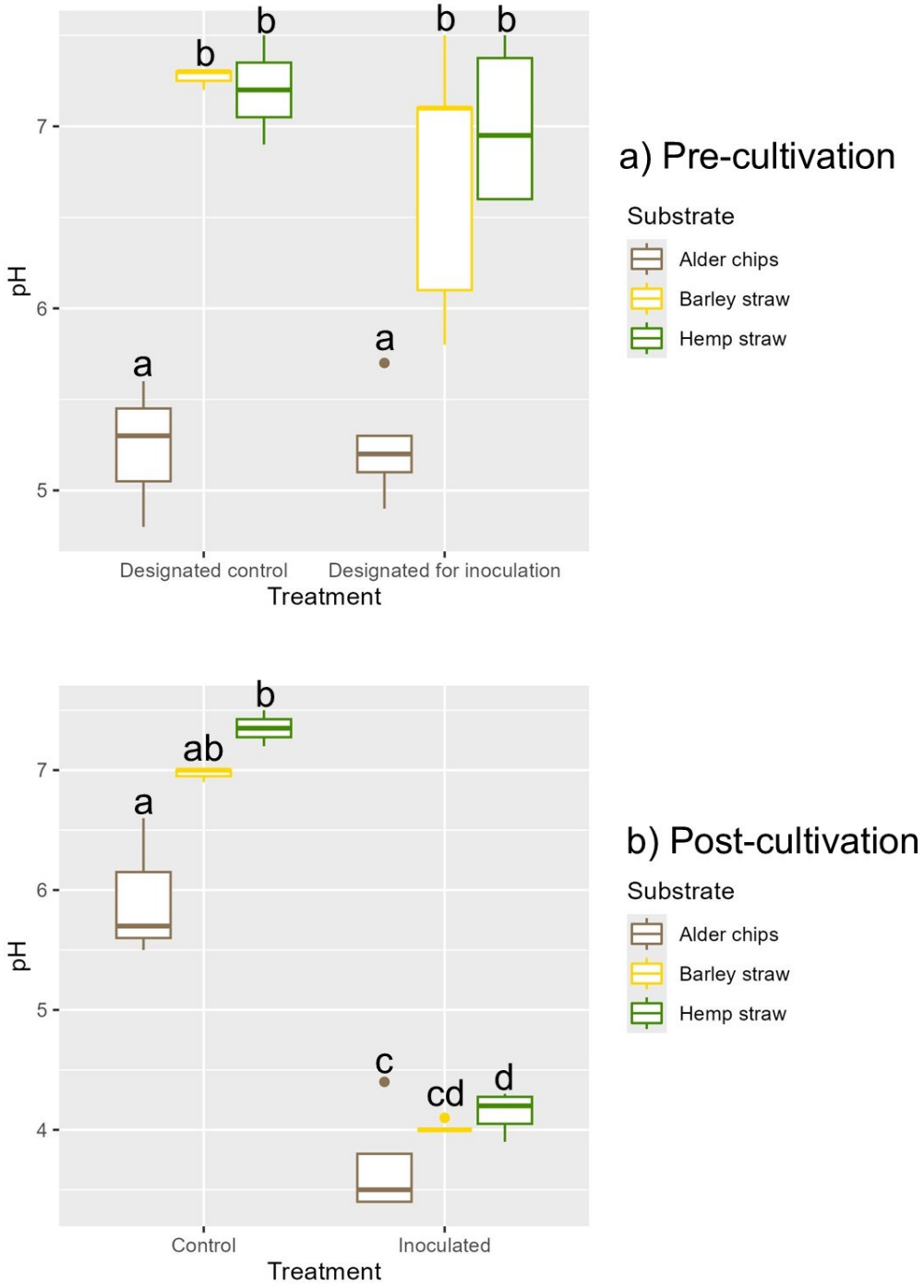
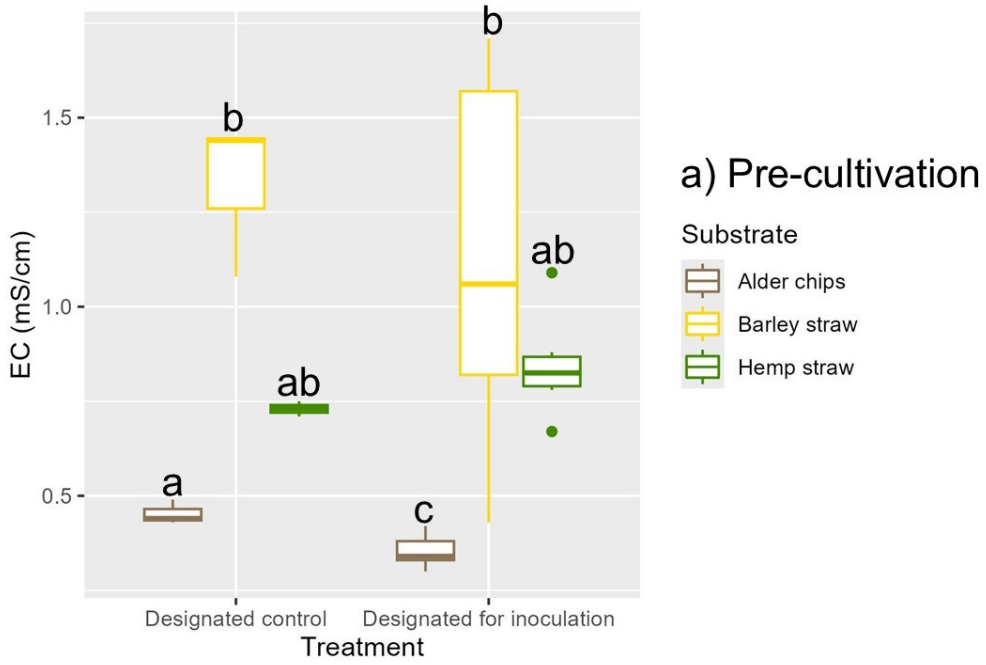


Figure 22: Boxplot of substrate pH a) pre-cultivation and b) post-cultivation. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

4.5.2 EC

Alder chips had the lowest median EC in all treatment-cultivation stage combinations, significantly lower than barley straw or than both barley straw and hemp straw (Figure 23). The EC of barley straw and hemp straw did not differ significantly within treatment-cultivation stage combinations.

In the pre-cultivation samples, the alder chip samples designated control had a higher median EC than those designated for inoculation ($p < 0.05$). In the post-cultivation samples, all inoculated substrates were found to have significantly higher EC values than their corresponding controls.



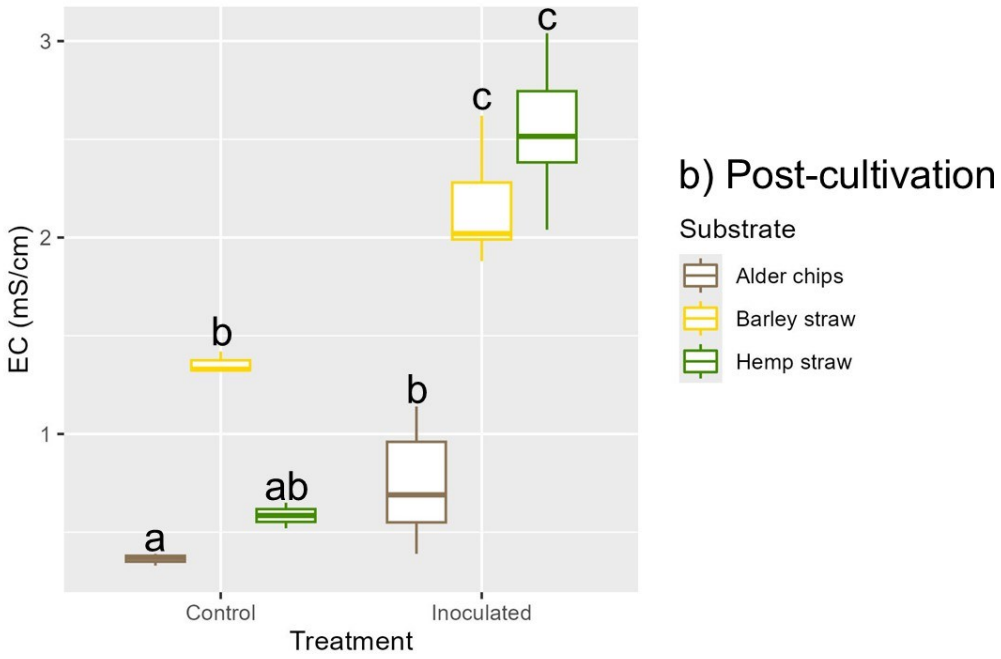


Figure 23: Boxplot of substrate electrical conductivity a) pre-cultivation and b) post-cultivation. Medians accompanied by a common letter are not significantly different by Dunn's test at the 0.05 level of significance. The horizontal bar in each box represents the median value. The boxed area displays the interquartile range (Q1 – Q3) and the whiskers show the minimum (Q1 – 1.5*IQR) and maximum (Q3 + 1.5*IQR) values. Dots represent outliers.

4.6 Genomics

4.6.1 Fungal diversity

4.6.1.1 Relative abundance by taxonomic order

The total number of fungal ASVs identified was 1472. At the phylum level, 23% of fungal ASVs were unassigned, while at the class and species levels, the percentage of unassigned fungal ASVs were 38% and 82%, respectively. There were no noteworthy trends in fungal abundance at the phylum or class level. The most abundant fungal orders in the pre-cultivation alder chip samples were *Hypocreales, Eurotiales, Filobasidiales* and ASVs with no order identified (Figure 24). Pre-cultivation barley straw samples also contained

Hypocreales and *Filobasidiales* components; however, the largest component in the pre-cultivation barley straw samples was unidentified ASVs and the abundance of *Eurotiales* was lower compared to alder chips (Figure 25). The largest component of the pre-cultivation hemp straw samples was unidentified ASVs, followed *Filobasidiales* (Figure 26).

The relative abundance of fungal orders in the post-cultivation control substrate samples are markedly different from both their pre-cultivation counterparts and from each other (compare control samples, Figures 24 to 26). The alder chip control samples contained a large percentage of *Coniochaetales*. By contrast, the barley straw and hemp straw control samples contain no such *Coniochaetales* component. The barley straw control samples are instead dominated by the orders *Agaricales* and *Sordariales*. The hemp straw control samples primarily contain unidentified ASVs, with components of *Hypocreales, Agaricales* and *Pleosporales*. The control samples did have a few commonalities between substrates. They shared a trend towards the shrinkage or disappearance of the *Filobasidiales* component compared to the pre-cultivation samples. They also contained a significant component of *Hypocreales* and unidentified ASVs.

The inoculated samples tended to be dominated by the order *Agaricales*, which includes the genus *Stropharia*. This trend was weaker for the treated alder chip samples, one of which (10At-C) contained a higher relative abundance of *Coniochaetales* than *Agaricales*.

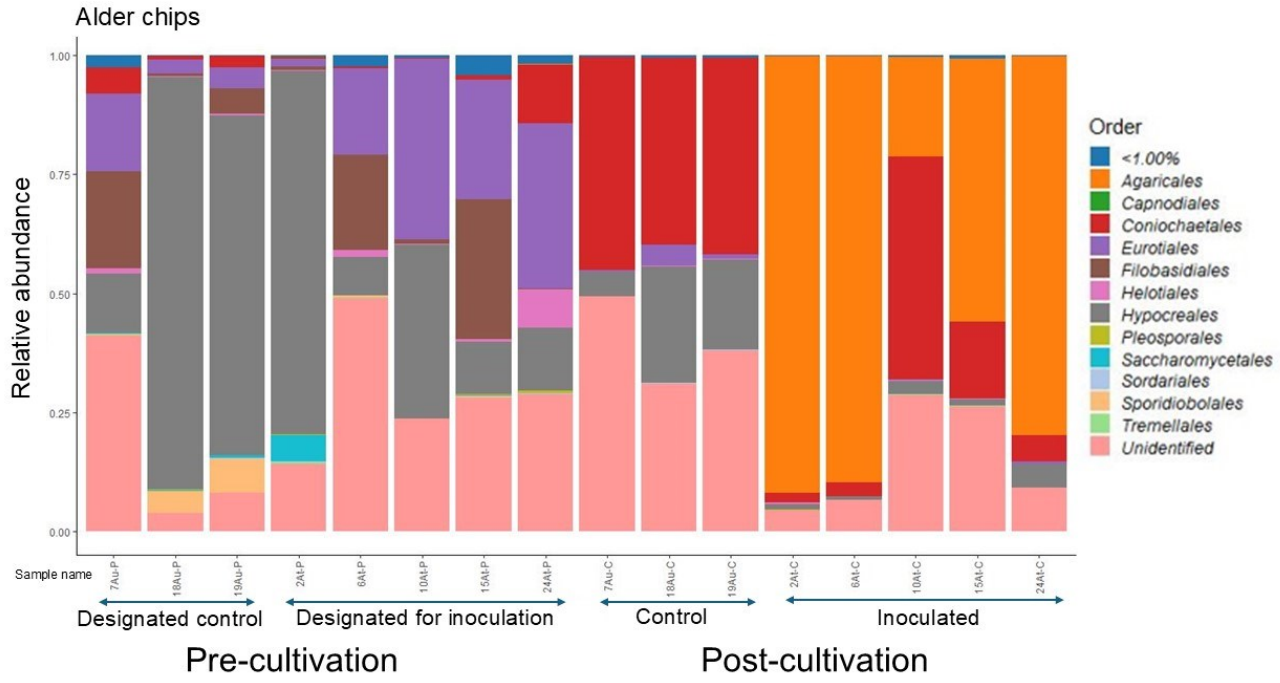


Figure 24: Relative abundance of fungal ASVs in alder chips by taxonomic order.

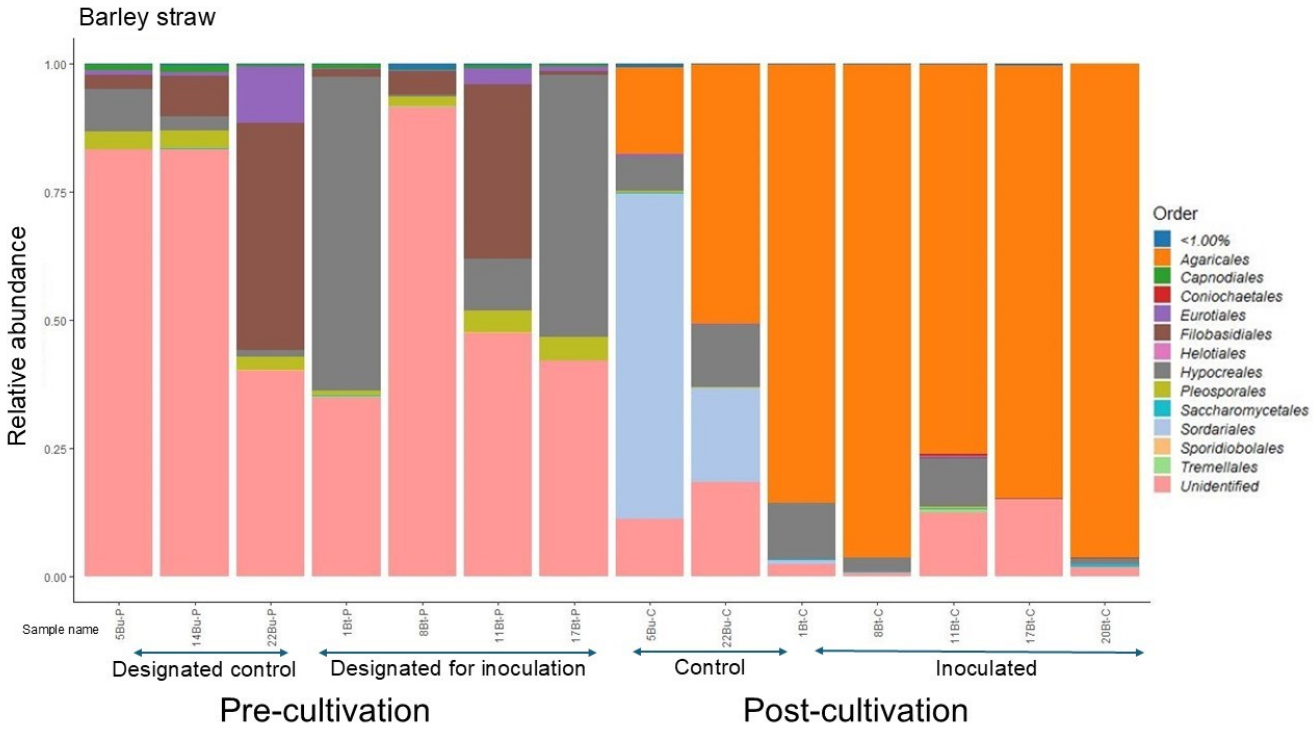


Figure 25: Relative abundance of fungal ASVs in barley straw by taxonomic order.

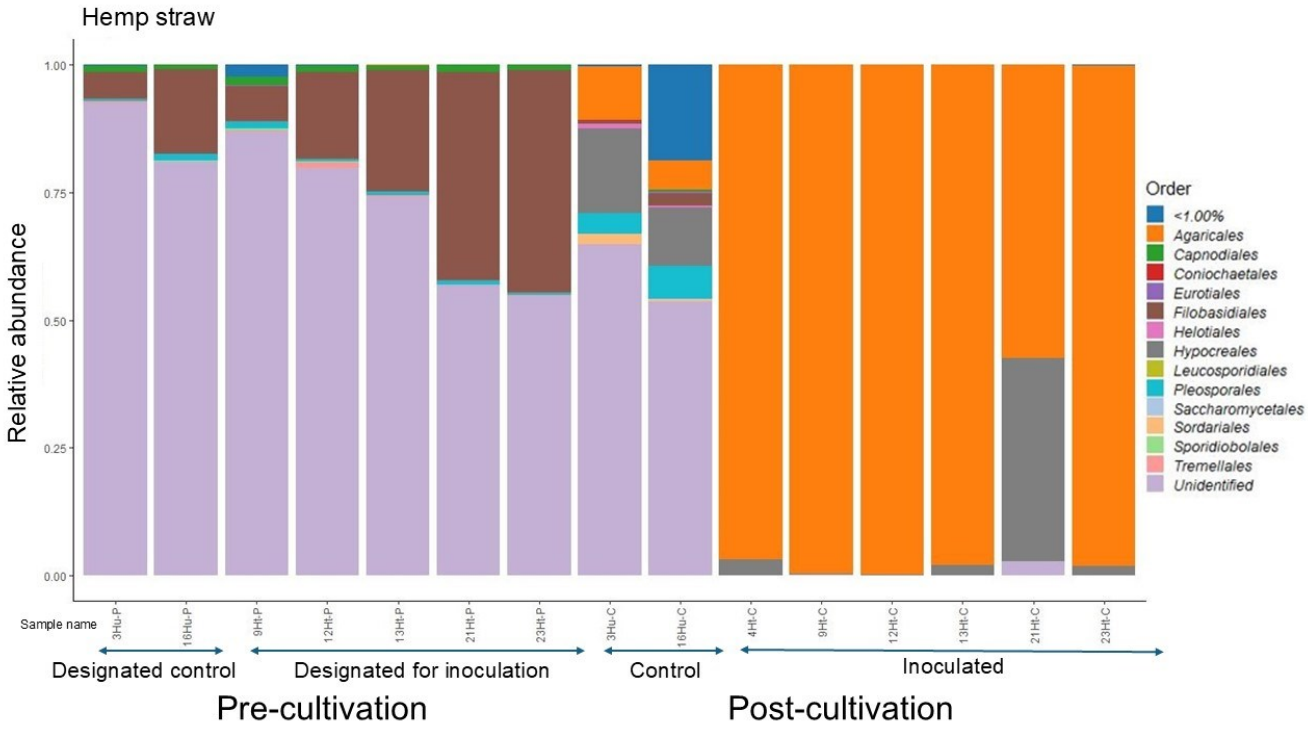


Figure 26: Relative abundance of fungal ASVs in hemp straw by taxonomic order.

4.6.1.2 Relative abundance by primary lifestyle

Sixty-two percent of fungal ASVs were not assigned a genus to which a primary lifestyle could be matched through FungalTraits. Therefore, there were large unidentified components in many substrate DNA samples. Despite this, there are observable trends in primarily lifestyle by substrate, treatment and cultivation stage (Figures 27 to 29). Pre-cultivation barley and hemp straw samples contained mostly unidentified ASVs, with a notable unspecified saprotroph component (Figures 28 and 29). Pre-cultivation alder chip samples also contained unidentified and unspecified saprotroph components, but in addition, they contained a large percentage of mycoparasites (Figure 27). In the read count data, over 99% of the pre-cultivation alder chip mycoparasite counts were for *Trichoderma*, a genus of green

moulds which are one of the most common contaminants in mushroom production (Ghimire et al., 2021).

Among the control samples, the alder chips showed a shift towards more unidentified ASVs after cultivation (Figures 27), the barley straw samples, showed an increase in the relative abundance of dung saprotrophs and soil saprotrophs (Figure 28), and the hemp straw samples showed an increase in plant pathogens and dung saprotrophs post-cultivation (Figure 29). The two dung saprotrophs that made up 52% and 47% of the count data in the barley control samples were from the genera *Cercophora* and *Schizothecium* respectively, which are both in the family *Lasiophaeriaceae*. *Coprinopsis* accounted for over 99% of the counts of soil saprotrophs in the barley straw control samples.

In inoculated samples, there was an increase in the relative abundance of litter saprotrophs in all post-cultivation substrates compared to the pre-cultivation samples. This trend appeared to be weaker in the alder chip samples compared to barley straw and hemp straw. In FungalTraits, *Stropharia* is classified as a litter saprotroph, and 99.9% of the total litter saprotroph reads in this dataset were for the genus *Stropharia*.

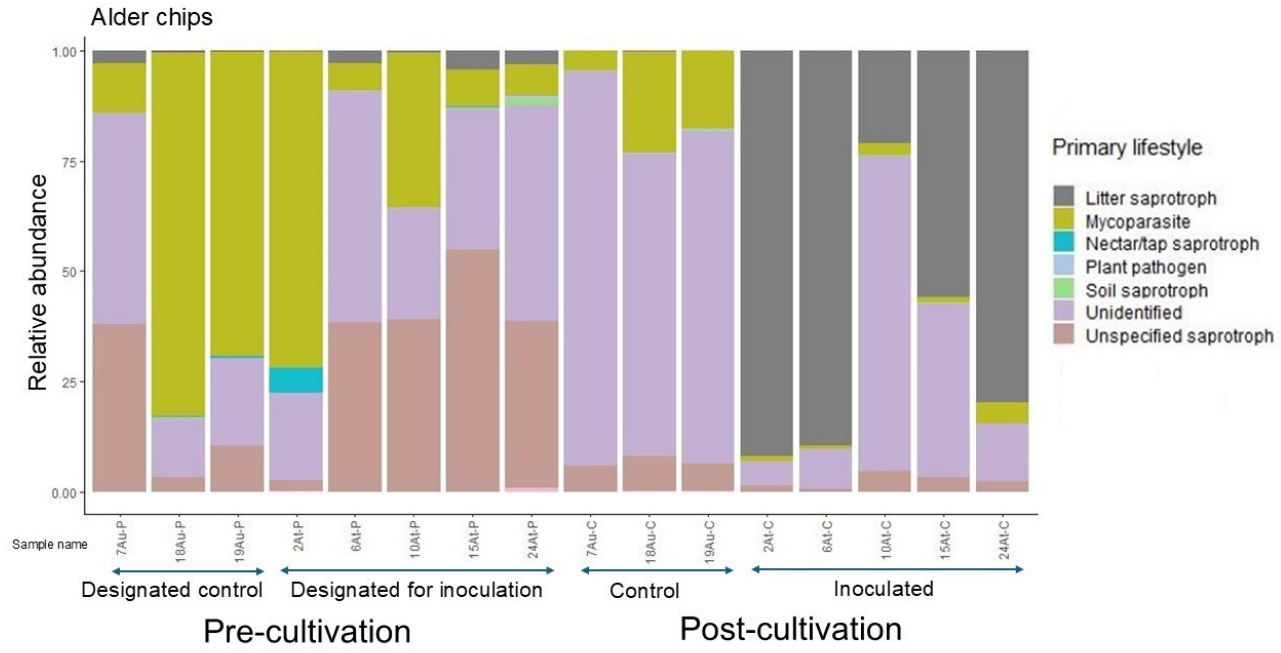


Figure 27: Relative abundance of fungal ASVs in alder chip samples, grouped by primary lifestyle at the genus level based on the FungalTraits database.

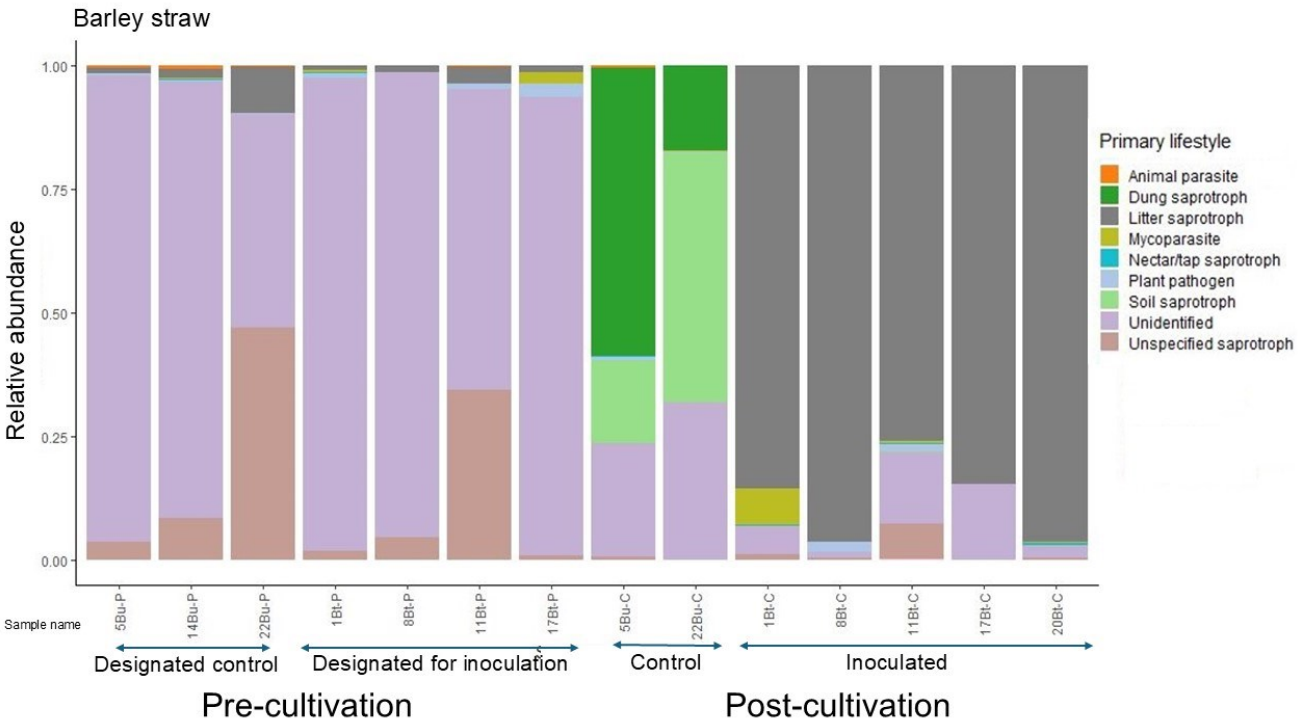


Figure 28: Relative abundance of fungal ASVs in barley straw samples, grouped by primary lifestyle at the genus level based on the FungalTraits database.

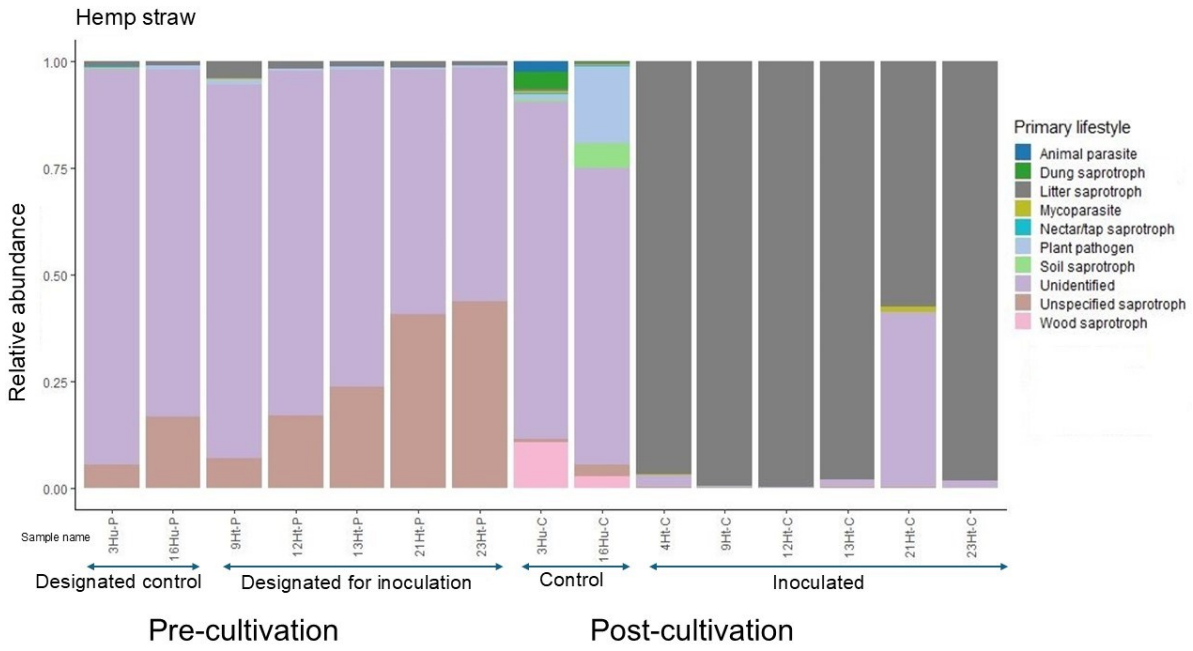


Figure 29: Relative abundance of fungal ASVs in hemp straw samples, grouped by primary lifestyle at the genus level based on the FungalTraits database.

4.6.2 Bacterial diversity

4.6.2.1 Relative abundance by bacterial phylum

The pre-cultivation samples appeared to have similar bacterial communities, across all substrates at the phylum level. These bacterial communities were dominated by *Proteobacteria*, followed in order of relative abundance by *Firmicutes* and then by *Actinobacteria* in the case of alder chips and barley straw and by *Bacteroidetes* in the case of hemp straw (Figures 30 to 32).

The post-cultivation control substrate samples had a greater diversity of phyla compared to the pre-cultivation samples. In addition to the phyla which were abundant in the pre-cultivation samples, the control samples also contained components of *Acidobacteria, Chloroflexi, Planctomycetes* and *Verrucomicrobia*. In the alder chip and hemp straw control

samples, the abundance of *Firmicutes* was markedly reduced compared to the pre-cultivation samples (Figures 30 and 32), whereas in the barley straw samples there does not appear to have been as much change in the abundance of *Firmicutes* (Figure 31).

The inoculated post-cultivation alder chip samples show the least differentiation from their pre-cultivation and untreated counterparts (Figure 30). The most easily observed differentiator of the inoculated alder chips samples compared to the pre-cultivation and untreated samples is an increase in the abundance of *Acidobacteria*, a trend which is not displayed in the inoculated barley straw and hemp straw samples. The phylum *Firmicutes* is also more abundant in the inoculated alder chip samples than in the controls but does not appear to differ greatly from the abundance of *Firmicutes* in the pre-cultivation samples.

The bacterial communities in the inoculated barley straw and hemp samples have a very high relative abundance of *Firmicutes*. The inoculated barley straw samples contained almost exclusively *Firmicutes* bacteria (Figure 31), while the treated hemp straw samples also contained noteworthy percentages of *Proteobacteria* and various other phyla (Figure 32).

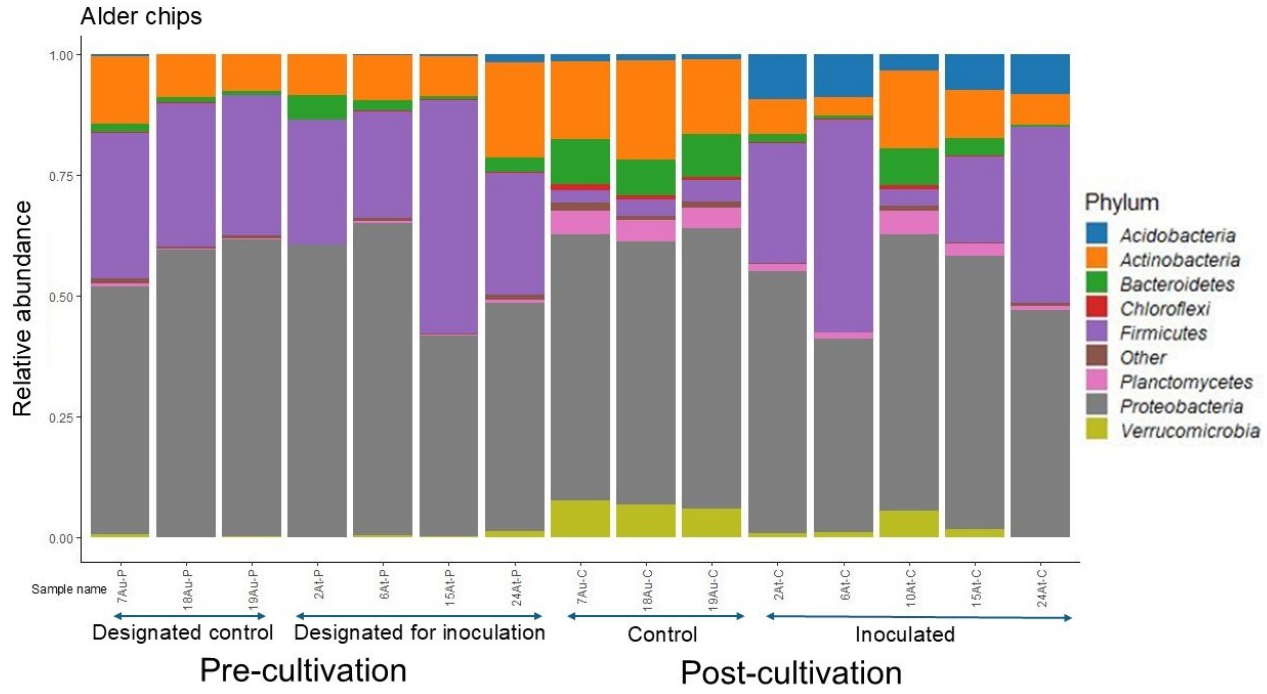


Figure 30: Relative abundance of bacterial ASVs in alder chip samples by phylum.

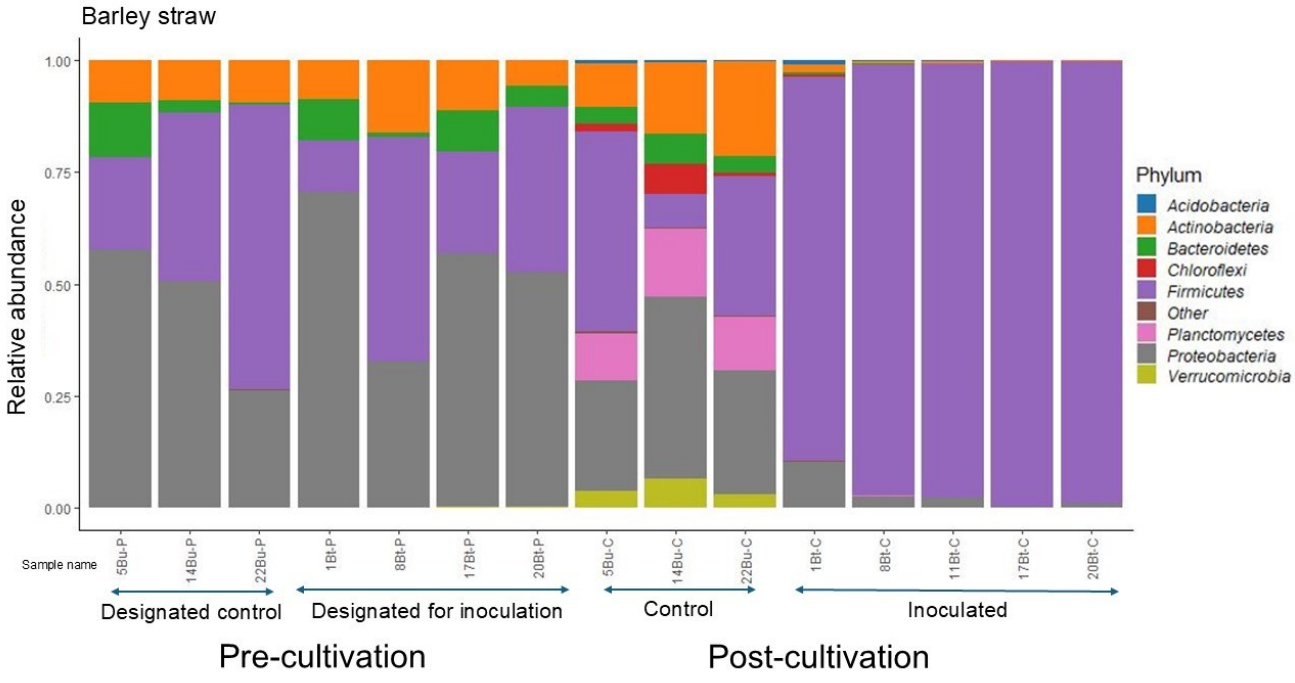


Figure 31: Relative abundance of bacterial ASVs in barley straw samples by phylum.

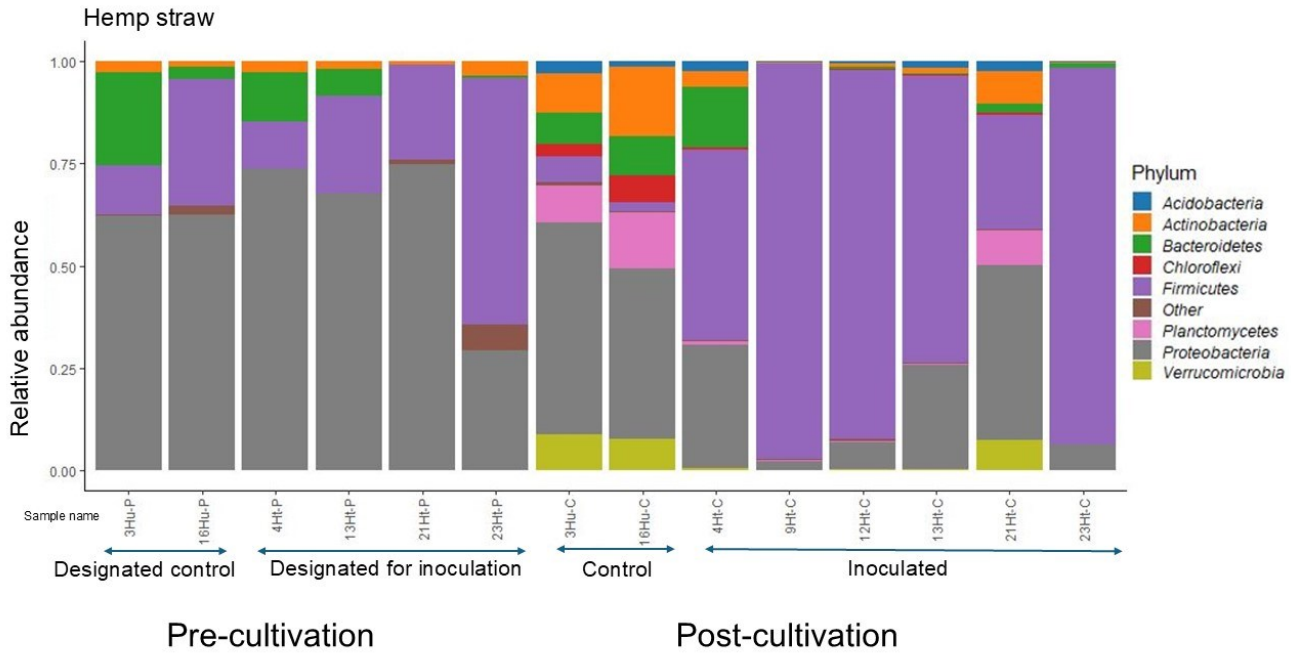


Figure 32: Relative abundance of bacterial ASVs in hemp straw samples by phylum.

4.6.2.2 Family and genus-level diversity within the phylum *Firmicutes* in post-cultivation treated samples

The obvious abundance of *Firmicutes* among the inoculated barley straw and hemp straw samples – the substrates which produced the highest yields of king stropharia mushrooms – prompted me to investigate the bacterial taxa contained within this phylum in more detail. In this section, the relative abundance of families and genera within *Firmicutes* in the inoculated substrate samples are presented (Figures 33 to 38). While the proportions vary between individual samples and between substrates, there are two families (*Bacillaceae* and *Paenibacillaceae*) and two genera within these families (*Bacillus* and *Paenibacillus*, respectively) that make up the majority of *Firmicutes* reads in all inoculated samples.

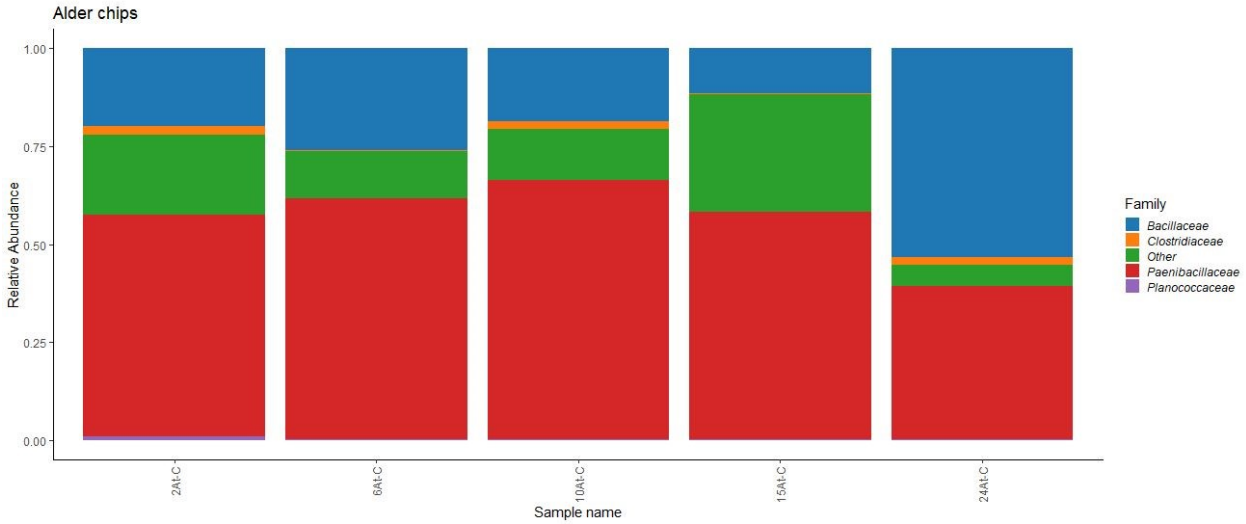


Figure 33: Relative abundance of bacterial ASVs in inoculated alder chip samples by family within the phylum *Firmicutes.*

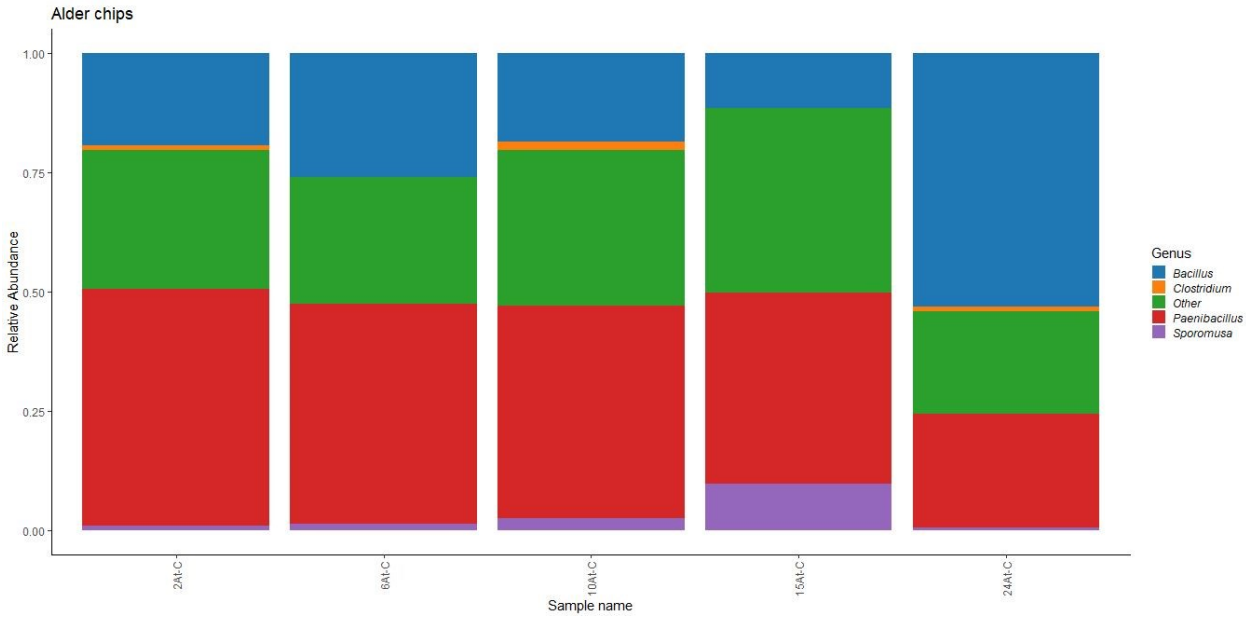


Figure 34: Relative abundance of bacterial ASVs in inoculated alder chip samples by genus within the phylum *Firmicutes.*

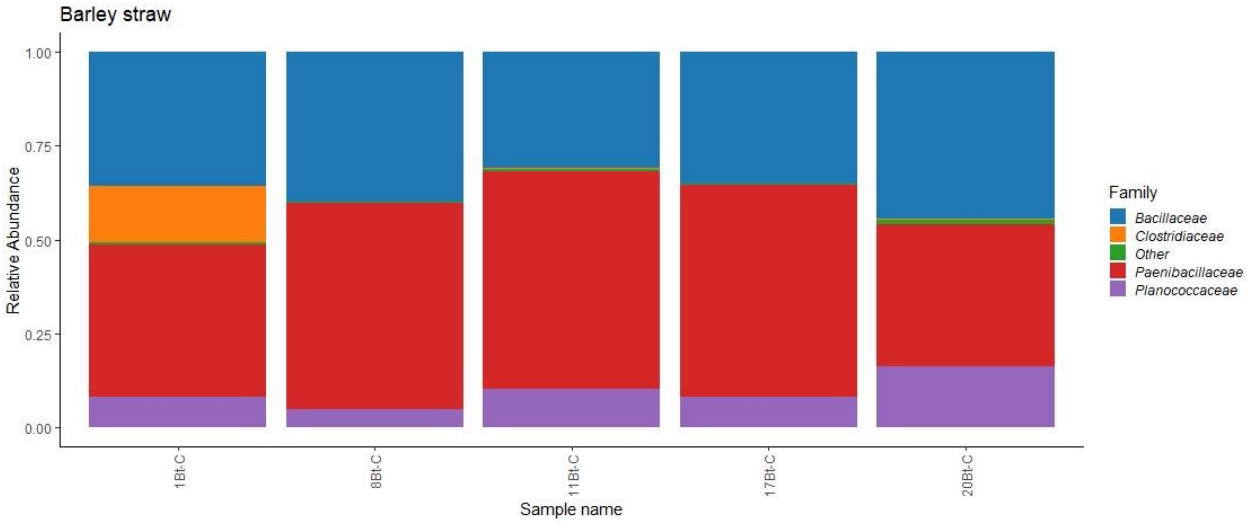


Figure 35: Relative abundance of bacterial ASVs in inoculated barley straw samples by family within the phylum *Firmicutes*.

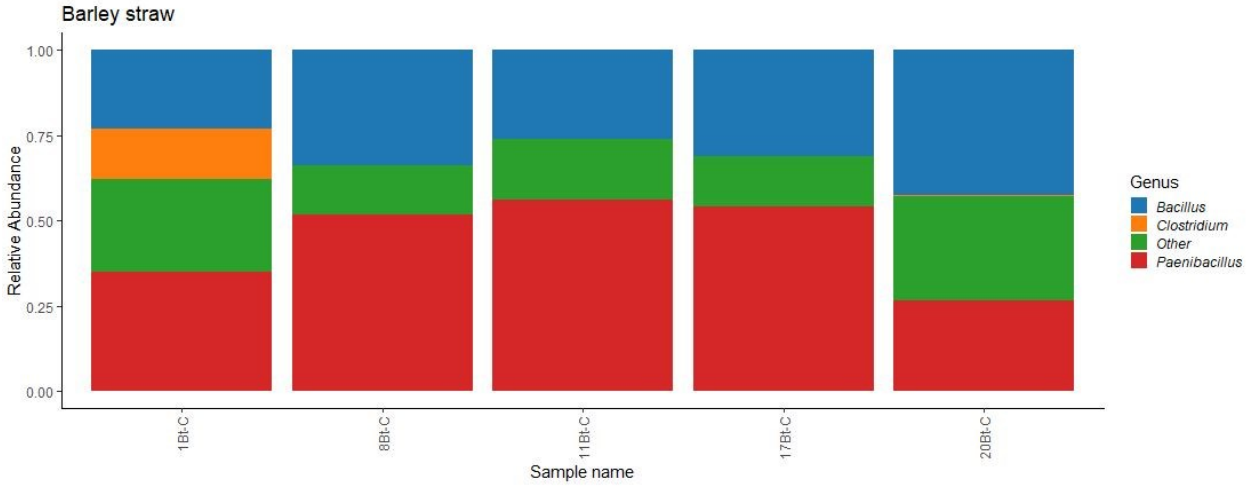


Figure 36: Relative abundance of bacterial ASVs in inoculated barley straw samples by genus within the phylum *Firmicutes*.

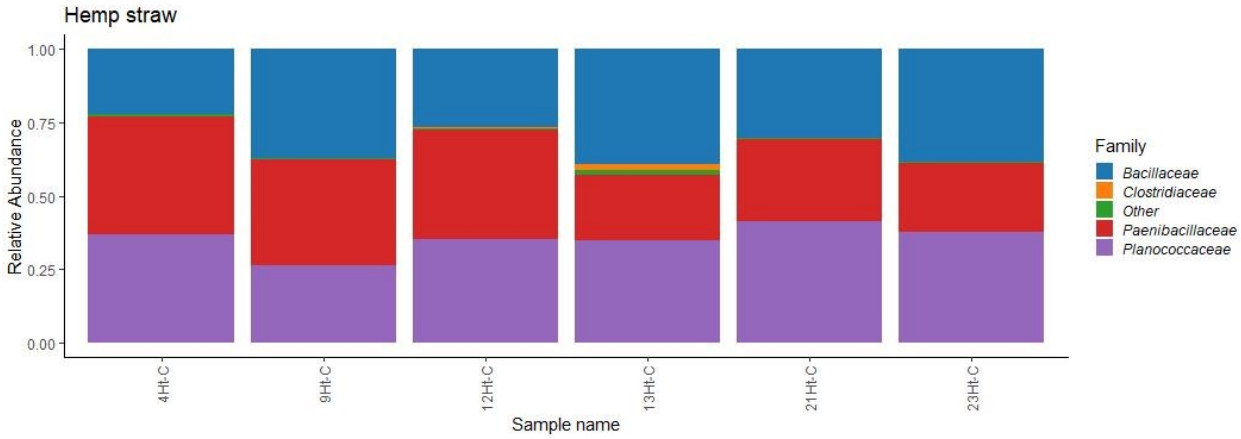


Figure 37: Relative abundance of bacterial ASVs in inoculated hemp straw samples by family within the phylum *Firmicutes*.

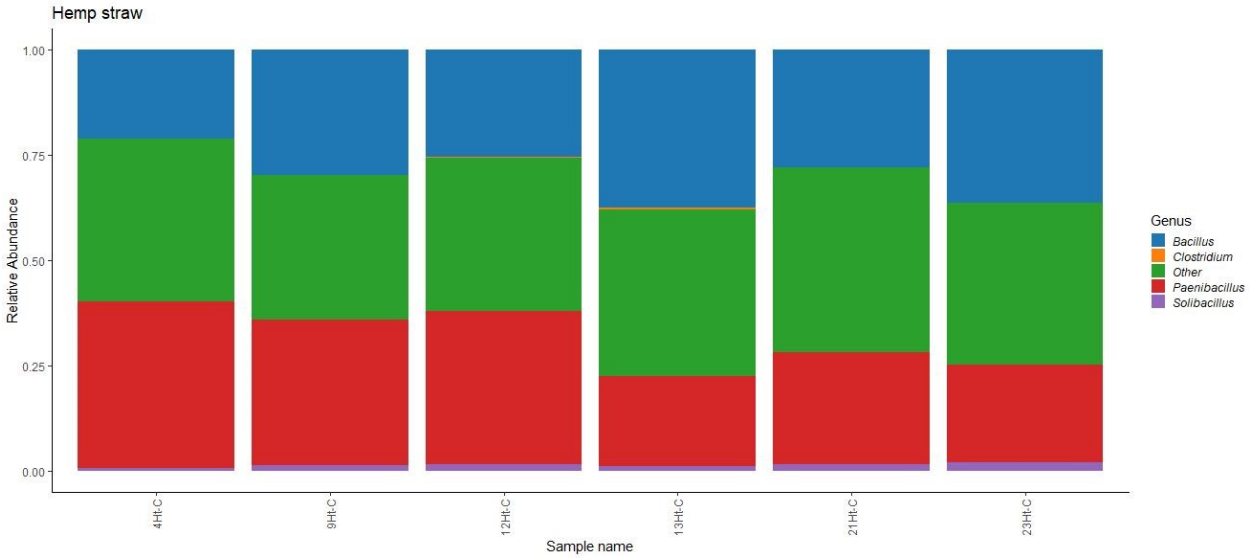


Figure 38: Relative abundance of bacterial ASVs in inoculated hemp straw samples by genus within the phylum *Firmicutes*.

4.7 Chloroform Fumigation Extraction for the determination of microbial biomass

Two of the nine pairs of samples analysed yielded negative results for microbial biomass C, which is a non-sensical result (Table 9). Due to the method seeming not to have produced credible results, time constraints and the laboratory costs associated with the method, this analysis was not performed on other samples. No statistical analyses were performed, and no Discussion section was written on the CFE data.

Table 9: Chloroform Fumigation Extraction data. Sample ID coding: Frame number (e.g. 10), Substrate code (A = alder chips, B = barley straw, H = hemp straw) Treatment (t = treated, u = untreated) – Cultivation stage (P = pre-cultivation, C = post-cultivation). EOC = extracted organic carbon. E_c = carbon rendered extractable by fumigation, calculated as (organic C extracted by 0.5 M K_2SO_4 from fumigated soil) – (organic C extracted by 0.5 M K_2SO_4 from non-fumigated soil).

Sample ID	Fumigated EOC (µg C/g substrate)	Unfumigated EOC (µg C/g substrate)	E_c (µg C/g substrate)	Biomass C (µg C/g substrate)	±
10 At-P	1116	768	348	918.72	55
11 Bt-P	11448	10824	624	1647.36	99
13 Ht-P	2736	1368	1368	3611.52	217
10 At-C	3060	3336	-276	-728.64	-44
11 Bt-C	38280	43800	-5520	-14572.8	-874
13 Ht-C	90960	67680	23280	61459.2	3688
7 Au-C	1080	336	744	1964.16	118
3 Hu-C	4644	1836	2808	7413.12	445
5 Hu-C	5772	3156	2616	6906.24	414

5 Discussion

5.1 Mushroom harvest timeline

5.1.1 Days to first yield

5.1.1.1 Comparison to outdoor trials in other locations

The time elapsed between inoculation and the beginning of fruiting in this study was between 4 and 70 days longer than that reported by growers in Missouri or in the American Pacific Northwest (Bruhn et al., 2010; Stamets, 2000).

I propose two contributing causes to the longer periods elapsed in this study before mushrooms were produced. The first factor is climate. Prince George, because of its northernly latitude, would not have been able to supply the same level of heat and humidity to king stropharia mycelium as Missouri, Oregon, or Washington. The average temperature in Prince George from June through October is 12˚C, compared with 22˚C over the same months in Columbia, Missouri and 17˚C and 16˚C in Portland, Oregon and Seattle, Washington, respectively (*Climate data for cities worldwide*, 2019).

The second factor is spawn vigour. As noted in Section 3.2.6 Inoculation and Tending, there were contaminant microorganisms in the king stropharia spawn used in this experiment. The long storage period and contaminants may have reduced the vigour of the mycelium, leading to a slower spawn run (McKoy, 2016).

5.1.1.2 Comparison between substrates

Alder chips likely took longer than barley straw and hemp straw to yield mushrooms because of the difference in substrate particle size and therefore surface area to volume ratio, which

has been documented as a factor in shiitake (*Lentinus edodes*) mushroom cultivation (McKoy, 2016; Stamets, 2000). Shiitake spawn run takes three months or less in nutrified sawdust blocks (high surface area: volume ratio) and five to twelve months in hardwood logs (low surface area: volume ratio) (Stamets, 2000). In this cultivation experiment, the larger average particle size of the alder chips compared to the straw substrates would have resulted in the king stropharia mycelium having to expend more energy and resources to access the same amount of feeding surface area. This could have resulted in a slower spawn run, and therefore delayed fruiting.

The effect of substrate chemical composition on yield timeline is unclear. Little is known about the specific nutritional needs of king stropharia. Higher substrate C and N contents have been found to suppress mushroom production in several cultivated saprophytic fungi (Sakamoto, 2018). This is because nutrient starvation signals to fungi that its current environment can no longer nourish it, and therefore it would be advantageous to produce spores to spread into other environments (Sakamoto, 2018). Of the substrates tested, alder chips had the highest post-cultivation C content. Post-cultivation N levels did not differ significantly between substrates. It is possible that the higher C content of alder chips contributed to the lower rates of fruiting that occurred in that substrate. However, mycelial starvation signals in the alder chips could also have been reduced due to the greater mass of substrate available in the alder chip-filled frames compared to the straw-filled frames. I filled the frames by volume, and the alder chips were denser than the straw substrates.

5.1.1.3 Another confounding factor: casing layer application

Since alder chips were slower to be myceliated by king stropharia, and because the casing layer is supposed to be applied when the substrate is fully myceliated, I never applied a

casing layer to three of the five inoculated alder chip frames (#'s 10, 15 and 24). One of the purposes of a casing layer is to retain moisture and create a consistently humid environment to aid with mushroom primordial formation, also known as pin set (Shields, 2018).

King stropharia tends to yield poorly if the casing layer is completely sterilized, which suggests a beneficial interaction between the mycelium and microorganisms in the casing layer (Stamets, 2000). *Agaricus bisporus* production has been shown to be enhanced by the presence of specific genera of bacteria (such as *Pseudomonas*) in the casing layer. The beneficial bacteria act in the degradation of volatile compounds that inhibit the formation of primordia (Dias et al., 2021). Such interactions have yet to be studied in king stropharia. However, in the present study, there is no clear indication in the relative abundance data that the microbial communities differ between the cased and uncased inoculated alder samples.

5.1.2 Fruiting period

Hemp straw had the longest fruiting period, followed by barley straw and alder chips. This mirrors the trends in days to first yield. King stropharia started producing mushrooms much earlier and therefore had a longer fruiting period before nighttime temperatures started to regularly drop below 0˚C in late October 2022. Conversely, the king stropharia in the alder chip bins did not begin to yield mushrooms until early October.

It is worth noting that none of the substrate types were completely exhausted by king stropharia during the first growing season. I was unable to collect a second season of data, but when I returned to the cultivation trial site in spring 2024, all substrate types were continuing to produce mushrooms.

5.2 Mushroom yield

Of the substrates tested in the cultivation trial, hemp straw produced the highest median yield of king stropharia mushrooms. This is noteworthy since hemp straw had not previously been documented as a substrate for king stropharia production. However, caution must be exercised with regards to this finding due to the high variability in yield within all substrates tested, and the lack of significant difference between hemp straw and the next highest yielding substrate, barley straw.

Literature on king stropharia yields is sparse and is often reported in terms of g mushrooms/ kg substrate (Bonenfant-Magné, 2000; Domondon & Poppe, 2000). Since the yield data in the present study is reported in g mushrooms/m^2 of substrate, it can only be compared to Szudyga (1978)'s report of yields from 2 to 33 kg/m^2. However, Szudyga does not describe how these yield data were obtained or analyzed. Szudyga's yield numbers have never been replicated or verified.

The contamination of the spawn used in the cultivation trial may have contributed to the variability in mushroom yields. Quality spawn is considered one of the key inputs for successful mushroom production. For many commercially cultivated mushroom species, spawn storage for over two months, even with refrigeration, can lead to decreased yields (Borah et al., 2019). Some of the common moulds that cause contamination of commercial spawn include *Aspergillus* sp., *Penicillium* sp., *Rhizopus stolonifera* and *Trichoderma* sp. However, these species were not prominent in the post-cultivation inoculated samples, indicating that king stropharia probably outcompeted these fungi over the cultivation period. The fungal relative abundance data show that *Trichoderma* spp. was a notable mycoparasite in the pre-cultivation alder chip samples, likely resulting from suboptimal storage conditions,

and not due to contaminated spawn. While the barley straw and hemp straw were kept dry inside a barn, the alder chips were stored in tarp-covered bins outside, where there were exposed to more moisture.

The genomic analysis did not capture the contaminants in the spawn. Based on Figures 27-29, there is no evidence of the presence of contamination in the post-cultivation samples. However, the post-cultivation samples were taken months after inoculation, therefore, it is possible that contaminant organisms, which were likely opportunistic fungi, would have run out of easily digestible substrate, and king stropharia and other fungi could have outcompeted them. At the time of post-cultivation sampling, king stropharia did not show signs of hindrance due to substrate contamination.

Our understanding of king stropharia production in these substrates would have been further enhanced if the yield data from a second cultivation season had been included in the study. The mushroom yield was lowest in alder chips during the experimental cultivation period, but it is possible that the alder chips would have continued to be productive over a longer period. This type of effect from surface area to volume ratio has been documented in shiitake (*Lentinus edodes*) mushroom cultivation. Shiitake spawn run takes three months or less in nutrified sawdust blocks (high surface area: volume ratio) and five to twelve months in hardwood logs such as oak, alder, poplar, and cottonwood (low surface area: volume ratio). However, the logs will continue producing mushrooms for three to six years depending on the wood type, whereas the nutrified sawdust blocks will only be productive for several months (Stamets, 2000).

The alder chips also had a lower initial moisture-holding capacity, lower pH and lower EC than the other substrates. While the initial EC of mushroom-growing substrates is generally

not considered, moisture content and pH have been shown to affect the production of decomposer mushroom species. The initial median moisture content of the alder chips fell below the 65-75% range recommended for king stropharia production (Domondon & Poppe, 2000; Szudyga, 1978). Oyster and shiitake mushrooms have been demonstrated to produce higher yields in substrates with initial pH values close to neutral (Khan et al., 2013; Odero, 2009; Philippoussis et al., 2003). King stropharia mushrooms may have similar requirements to produce optimal yields.

5.3 Mushroom composition

5.3.1 Mushroom moisture content

The lower moisture content of king stropharia mushrooms grown in alder chips corresponds with the lower moisture content of the alder chips themselves both before and after the cultivation trial (see Section 4.4.1 Substrate moisture content).

The small moisture content differences between the mushrooms grown in different substrates are not of a great enough magnitude to negate the differences in fresh mushroom yield between substrates. The median moisture values for the substrates are also close to the 8% dry matter content (and therefore 92% moisture content) that Szudyga (1978) reported.

5.3.2 Mushroom carbon and nitrogen content

There is no published data on the relationship between the C:N ratio of substrates and the C:N ratio of king stropharia mushrooms. In this experiment, there were significant differences in the initial C:N ratio of the substrates but no significant differences in the C:N ratio of the mushrooms grown in the different substrates. This suggests that the C:N ratio of king stropharia mushrooms may be relatively consistent regardless of substrate.

5.3.3 Mushroom elemental analysis

5.3.3.1 General elemental content

The elements for which no significant differences were observed between mushrooms grown in different substrates provide a sense of the general element composition of king stropharia mushrooms. Of the elements tested, the mushrooms were most abundant in K, P and S. There were no significant differences in K, P and S content between mushrooms grown in different substrates despite significant differences in the substrates' content of these elements. The order of abundance of elements in the fruiting bodies (K > P > S > Mg > Ca > Na > Fe > Zn > Cu, Mn > Al, B > Cd, Ba) also does not match the order of abundance of elements in the pre-cultivation substrates (Ca > K > Mg > S > P > Na > Fe > Al > Mn > Zn > Cu, B).

These results provide clues about king stropharia mushrooms' use of nutrients. When researchers compared the elemental content of the fruiting bodies of *Pleurotus eryngii*, *Flammulina velutipes*, and *Hypsizigus marmoreus* with that of their lignocellulosic growing substrates, they found a similar pattern: high Ca content in the substrates compared to the fruiting bodies, and high K content in the fruiting bodies compared to the substrates (Lee et al., 2009). Lee et al. (2009) suggest that Ca in the substrates may not be bioavailable to the fungi or that the mushrooms lack efficient Ca uptake channels, compared to their ability to take up K. High levels of K have also been reported in *Agaricus bisporus*, *Lentinus edodes* and *Pleurotus ostreatus* (Mattila et al., 2001). This suggests commonalities between king stropharia's nutrient uptake and that of other commonly cultivated mushrooms. Fruiting body formation causes the selective removal of nutrients from the substrate to meet the needs of the developing mushrooms (Zadražil, 1978). Therefore, K likely plays an important role in fruiting body formation in many fungal species.

It should be noted however, that mycelia and mushrooms differ in their nutritional composition, and mycelia make up the majority of fungal biomass (Ulziijargal & Mau, 2011). Therefore, elemental analyses of the isolated mycelia would be required for a deeper understanding of the nutrient uptake of this species.

5.3.3.2 Significant differences in elemental content by substrate

Mushrooms grown in hemp straw contained significantly more B and Fe than those grown in other substrates, and significantly more Mn than those grown in alder chips. With regards to human nutrition, B and Mn are extremely minor trace elements in the human body, but Fe is more abundant and plays an essential role in human health as a component of hemoglobin in red blood cells (Davey, 2021).

These differences in mushroom micronutrient content correspond with the differences in the initial concentration of these nutrients in the substrates. Pre-cultivation hemp straw contained the highest median levels of B, Fe and Mn. These results mirror the findings of a study that measured mineral content in *Pleurotus* ssp. mushrooms and the various substrates they were grown in (Hoa et al., 2015). According to Hoa et al. (2015), differences in oyster mushroom mineral content depend on the species of mushroom, the mineral concentration of the substrate and on the EC of the substrate. As it does in plants, high EC can inhibit the uptake of nutrients in fungi by increasing osmotic pressure outside mycelia (Hoa et al., 2015). The threshold at which this occurs in king stropharia is unknown. However, EC's effect on nutrient uptake could help explain why some but not all the substrate nutrient trends are reflected in the mushroom nutrient trends.

5.3.3.3 Cadmium concerns

Mushrooms grown in alder chips contained significantly more Cd than those grown in barley straw. Since substrate Cd content was not analysed, we do not know whether the mushrooms' elevated Cd content correlated with alder chip Cd content.

The question that follows is, are these Cd levels a concern for human health? The toxicological reference value for cadmium is 0.21-0.36 µg/kg bodyweight/day (Schaefer et al., 2023). Therefore, a 70-kg person could safely consume 92.5-158.6 g of alder chip-grown king stropharia mushrooms per day. Assuming king stropharia mushrooms have a similar density to common button mushrooms (*Agaricus bisporus*, approximately 70 g chopped raw mushrooms/cup), the safe consumption limit is well over what most people would consume in a typical serving (Cervonie, 2022). Therefore, moderation is advised, but toxicity is not of grave concern.

5.3.4 Mushroom protein and fat analysis

The differences in protein and fat content between mushrooms grown in different substrates are of unknown significance due to the small number of samples submitted. The rankings of the results differ based on whether they are considered on a dry or fresh weight basis due to significant differences in moisture content. Therefore, the relative nutritional merit of mushrooms grown in different substrates depends on whether they are consumed fresh or dried.

However, the results for protein and fat content between mushrooms grown in different substrates fell within a narrow enough range that it may not affect consumer choice. Consumers show a general preference for simplified nutrition information, and numbers on Nutrition Facts labels are often rounded to the nearest gram (Kiesel et al., 2011). Regardless

of substrate, fresh king stropharia mushrooms contained negligible fat (<1%) and modest protein content (2-3%). This, in addition to their elemental nutrient content, will place them into the "healthy food" category for most consumers.

The average %DM protein values for the substrates tested in this experiment, which ranged from 27.41-33.20%, were higher than the 22.0 %DM protein reported by Szudyga (1978) for king stropharia mushrooms grown on cereal straw or flax straw. Szudyga did not explain how the reported values were obtained. Reasons for the discrepancy may include a) the substrates tested in this experiment produce more protein-rich mushrooms than those Szudyga used, b) different growing conditions affected the protein content of the mushrooms, and/or c) different laboratory procedures were used to determine protein content.

5.4 Substrate composition

5.4.1 Substrate moisture content

The literature suggests that before inoculation, substrates should be moistened to 65-75% humidity (Domondon & Poppe, 2000; Szudyga, 1978). In this experiment, the median pre-inoculation moisture content of alder chips fell slightly below this range and that of barley straw and hemp straw fell slightly above it.

Post-cultivation inoculated alder chip and hemp straw samples contained less water than the uninoculated controls. Imaging technology could be used to compare the particle size distribution and pore sizes in the inoculated substrates and the controls (Lu et al., 2017). I hypothesise that as king stropharia mycelium consumes and replaces lignocellulosic biomass, there is a decrease in the substrates' proportion of water-holding pores.

5.4.2 Substrate carbon and nitrogen content

Alder chips had the highest pre-cultivation C:N ratio. The C:N ratio of a soil or substrate affects how much N is available to plants and microorganisms (Weil & Brady, 2017). Generally, the lower the C:N ratio, the greater the availability of N to plants and microorganisms such as fungi and bacteria. Materials with a high C:N ratio will tend to have a lower rate of decomposition due to the limited N available to decomposers (Gilmour et al., 1998; Pérez Harguindeguy et al., 2008).

In a cultivation study of WRF *Pleurotus ostreatus* and *P. cystidiosus* on a variety of substrates, Hoa et al. (2015) found a negative correlation between substrate C:N ratio and myceliation period, mushroom weight and yield, biological efficiency, and protein content of the mushrooms. This suggests that N availability can be a limiting factor in mushroom production from WRF. Lignocellulosic peroxidase production in *Bjerkandera* sp., a genus of WRF, was also found to be limited by substrate N availability (Kaal et al., 1993). The ideal nutritional profile of king stropharia is not known, but there could be similarities between its nutritional requirements and those of other WRF. A controlled cultivation experiment using a lignocellulosic substrate amended with known proportions of N could help to understand the relationship between C:N ratio and king stropharia mushroom yield.

Inoculated barley straw and hemp straw's N content increased, and C content decreased over the cultivation period, resulting in lower post-cultivation C:N ratios in these substrates. Inoculated barley straw and hemp straw also contained significantly less C than the uninoculated controls. This is likely because C was lost in the form of CO_2 due to cellular respiration, which occurred at a higher rate in the inoculated samples, resulting in a relative increase in the proportion of N (Sales-Campos et al., 2009).

Domondon and Poppe (2000) also suggested the possibility of periotrophic mycorrhizal associations between king stropharia and plants. Other WRF species such as *Ceriporia lacerata* have been found to increase biological N fixation of crop plants (Yin et al., 2022). The wooden frames used in this experiment were open bottomed to allow for soil contact, and king stropharia mycelium could have expanded into the soil to associate with plants in the surrounding field. King stropharia can obtain N by trapping and killing nematodes using cells with finger-like projections called acanthocytes (Luo et al., 2006). This may have contributed to higher N levels in the inoculated substrate samples, but the magnitude of the effect is unknown.

5.4.3 Substrate elemental analysis

5.4.3.1 Macronutrients (Ca, K, Mg, P, S)

5.4.3.1.1 Substrate comparisons

Pre-cultivation hemp straw had the highest Ca and Mg content, and barley straw had the highest K, P and S content. The alder chips were comparatively low in all nutrients. These initial differences in macronutrient content reflect the different management histories, nutrient uptake characteristics and growth habits of the plants the substrates are made of (Pourazari, 2016). The hemp straw and barley straw were both obtained from Halltray farm in Vanderhoof and were grown with conventional fertilizers, which increased the nutrient content of the plant tissues (Iványi & Izsáki, 2009; McKenzie et al., 2004). The alder chips came from wild-grown alder branches along a dirt road behind UNBC's Prince George campus and were not fertilized.

Alder is a perennial woody shrub, whereas barley is an annual grass, and hemp is an annual herb (Jacobs, 2016; MacKinnon et al., 1999; Pancaldi et al., 2025). These functional groups of plants have different life histories and strategies, which are connected to different biomass and nutrient allocation patterns (Pourazari, 2016). Annual grass and herb crops have been artificially selected for high resource allocation (including nutrient allocation) to reproductive parts and associated aboveground structures, likely at the expense of allocation to belowground parts (Van Tassel et al., 2010). In contrast, perennial plants tend to allocate more resources below ground. Metabolically active (e.g., photosynthesizing) tissues tend to contain more nutrients than woody structural tissues, which are usually carbon-rich and contain a lower relative proportion of other nutrients (Orji & Wali, 2021; Zhao et al., 2020).

Although the optimal nutrient profile for cultivating king stropharia mushrooms is not yet known, king stropharia growers should bear in mind the possible impact of substrate nutrient content on the yield of this mushroom species. It may be that annual crop plants are better able to meet king stropharia's nutritional needs than woody annual plants.

5.4.3.1.2 Treatment and pre- and post-cultivation comparisons

5.4.3.1.2.1 Alder chips

There were no significant differences in macronutrient content between inoculated and control alder chip samples. The low rate of myceliation of the alder chips may have resulted in a weak treatment effect. Regardless of treatment, Ca, Mg and S content in alder chips increased over the cultivation period, while P and K did not. Since there was no treatment effect, the difference in the behaviour of Ca, Mg and S versus P and K must have resulted from the differing mobility of these nutrients in alder chips.

A limitation of ICP-OES is that it does not detect specific nutrient-containing compounds and instead only measures elements. The ability of a nutrient to be leached from a substrate depends on its molecular species and its mobility within the substrate, which is in turn affected by pH (Lehmann & Schroth, 2003; Weil & Brady, 2017).

The inoculated alder chips were more acidic than the controls, but they were both still acidic. When pH is close to or below 5.0, P, K, S, Ca and Mg are all less mobile and less bioavailable (Hopkins & Hüner, 2009).

Ca and S are immobile in living plant tissues, whereas P, K and Mg are mobile. However, as plant matter decomposes, cell walls break down and the patterns of nutrient mobility in living plant tissues no longer apply (Hopkins & Hüner, 2009). Therefore, one possible explanation for the relative increase in Ca and S content over the cultivation period is that these nutrients were less mobile in the relatively undecomposed alder chip tissues. However, this still does not explain the relative increase in Mg content.

5.4.3.1.2.2 Barley straw

Ca, Mg and S content in barley straw increased over the cultivation period regardless of treatment. K and P increased in control samples but did not change significantly in inoculated samples. This difference could be partially explained by the acidification of the inoculated substrates by king stropharia mycelium's extracellular enzymes, which resulted in a median pH of 4.0 in the inoculated samples compared to 7.0 in the control samples. Although acidification tends to increase K^+ leaching, phosphate (PO_4^{3-}) leaching does not correlate linearly with decreasing pH (Deveau et al., 2018; Haynes & Swift, 1986). Instead, P behaviour depends partly on pH and partly on the presence of cations with which it can precipitate and substances that it can adsorb onto. In most mineral soils, P is most mobile at

pH 6.5 (Weil & Brady, 2017). At lower pH, it precipitates as Al/Fe-P minerals and/or absorbs to clays and Al/Fe oxides. At higher pH, P precipitates as Ca-P minerals and/or adsorbs to clays and $CaCO_3$. One might expect the relative concentration of P to be lower in the control samples, where the median pH of 7.0 was closer to P's peak mobility at pH 6.5, but this is not what happened.

The treatment differences may result partly from king stropharia mycelium's manipulation of nutrients through factors other than pH. At least one other WRF, *Ceriporia lacerata* GH2011, has been shown to mobilize P through several different biochemical mechanisms (Sui et al., 2022). This could also be the case for king stropharia. However, research also indicates that the effect of inoculation of biomass with WRF on nutrient concentration and cycling varies by species (Ostrofsky et al., 1997).

5.4.3.1.2.3 Hemp straw

There were unintended pre-cultivation treatment differences in the hemp straw samples. These differences may have been due to inadequate sample homogenization. Hemp straw samples designated for inoculation contained significantly more Ca, K and Mg than their counterparts designated as controls. This makes it more difficult to analyse the treatment differences in post-cultivation Ca and Mg content (the inoculated samples had higher levels of these nutrients).

The Wilcoxon signed rank interval test indicates increases over the cultivation period in Ca, Mg, K and S content in the treated hemp samples, with no trend in P content. Based on the boxplots, it appears that among the control samples, Ca and P content did not change meaningfully, while K and Mg content increased. It is unclear whether S increased in the

control samples. The difference in trends between the inoculated and control samples over the cultivation period indicates that there were treatment differences in Ca's behaviour.

Of the substrates tested, hemp straw had the highest initial Ca content. It is possible that less Ca was leached from the inoculated samples due to Ca's decreased mobility at a lower pH in the inoculated samples compared to the controls (Hopkins & Hüner, 2009). However, if pH were the explanation, we would expect a similar trend in barley straw, for which the inoculated samples were also more acidic than the controls. Yet we do not see the same trend for Ca in barley straw. More research is required to determine if the apparent behaviour of Ca in this study is a fluke of the data or if it is related to actual phenomena in the king stropharia cultivation process.

Hemp straw S content was higher in the inoculated samples than in the controls. Why S content would be higher in the inoculated hemp samples but not the inoculated barley samples is puzzling. The same can be said of P content, which displayed in different trends in hemp straw versus barley straw. Treated hemp straw and barley straw were both strongly myceliated and acidified by king stropharia, and S and P contents did not vary significantly between hemp straw and barley straw in the pre-cultivation samples. For reasons yet unknown, the nutrients did not behave the same way in these substrates.

5.4.3.2 Micronutrients (B, Cu, Fe, Mn, Zn)

5.4.3.2.1 Substrate comparisons

As discussed in Section 5.4.3.1, the initial differences in nutrient content between substrates reflect the different management histories, nutrient uptake characteristics and growth habits of the substrate plants.

5.4.3.2.2 Treatment and pre- and post-cultivation comparisons

The general trend in all substrates towards an increase in micronutrient content over the cultivation period likely results from the relative decrease in substrate carbon content due to microbial cellular respiration as the substrates decomposed.

The treatment differences in the post cultivation samples varied puzzlingly between substrates and could neither be explained by the initial nutrient content differences or by changes in pH. The micronutrient needs of king stropharia cannot be deduced from this small data set.

5.4.3.2.2.1 Alder chips

The post-cultivation alder chip samples contained more Mn and Zn that their untreated counterparts. This difference cannot be explained by the difference in pH between the inoculated and control samples (pH 3.5 and pH 5.7 respectively). The availability of Mn and Zn does not change greatly in this pH range (Hopkins & Hüner, 2009). Instead, this difference may result from microbial activity (M. Preston, personal communication, March 7, 2025).

5.4.3.2.2.2 Barley straw

The post-cultivation barley straw samples contained less Cu than their untreated counterparts. The inoculated samples were more acidic (pH 4.0) than the control samples (pH 7.0), but Cu is less mobile at lower pH values (Hopkins & Hüner, 2009). Therefore, we would expect the inoculated samples to contain more Cu due to reduced leaching. One could speculate that the explanation lies in the nutrient cycling effects of the other microorganisms present in the barley straw frames.

5.4.3.2.2.3 Hemp straw

Post-cultivation inoculated hemp samples contained significantly more B, Cu and Mn than their corresponding controls, whereas Fe and Zn did not vary significantly between treatments. The inoculated samples had a median pH of 4.2 and the control samples had a median pH of 7.4. B and Cu are less mobile at lower pH, so this could potentially explain why the content of these micronutrients was higher in the treated samples.

However, Mn is more mobile at a neutral pH than an acidic one. The concentration of Mn was also higher in the inoculated alder chip samples than the controls. Mn's increase in the inoculated samples of both substrates suggests that king stropharia uses Mn in its tissues and causes it to accumulate. However, the same trend was not observed in barley straw.

5.4.3.3 Aluminum and sodium

There were no significant treatment differences in Al and Na content in any substrate. Concentrations of these elements tended to increase over the cultivation period. This is in keeping with the idea that substrate C content decreased over the cultivation period because

of the cellular respiration of decomposer microbes, resulting in a relative increase in the concentration of other elements.

5.4.4 Substrate Van Soest fiber analysis

The proportion of lignocellulosic biomass in all inoculated substrates decreased over the cultivation period as substrate tissues were consumed and replaced with mycelium. This occurred because WRF like king stropharia degrade lignocellulosic compounds through the secretion of extracellular ligninolytic enzymes (Rodríguez-Couto, 2017).

The decrease in lignocellulosic biomass was strongest for hemp straw and weakest for alder chips. This mirrors the trend in king stropharia mushroom yield, implying that the more effectively the mycelium can consume the substrate, the greater the mushroom yield will be. This is supported previously observed correlation between mycelial growth and mushroom yield in this species (Domondon & Poppe, 2000).

The proportion of lignocellulosic biomass decreased over time to a lesser extent in the control samples. This is due to the presence of other decomposer microorganisms naturally present in the cultivation environment, including soil bacteria and fungi.

WRF have three main enzymatic systems: cellulases, polysaccharidases other than cellulases, and ligninases (Bonenfant-Magné, 2000). There are two modes of lignin degradation in WRF: selective and non-selective decay (Isroi et al., 2011). Selective decay targets lignin and hemicellulose while leaving the cellulose fraction relatively intact, whereas in the case of non-selective decay, all lignocellulose fractions are decayed. Only certain white rot fungal species are capable of selective decay, and this ability is affected by the lignocellulosic species, the cultivation time, and other factors. In all substrates tested in this study, the proportion of cellulose in the inoculated and control samples did not differ significantly. This suggests that king stropharia is capable of selective decay of lignin and hemicellulose, and that other microorganisms are responsible for the decrease in cellulose content.

The proportion of lignin increased over time relative to the other lignocellulosic fractions in the control samples due to lignin's natural resistance to decay. WRF are one of the only known groups of organisms that can decompose lignin, which is a recalcitrant and bulky heteropolymer (Rodríguez-Couto, 2017). There are anaerobic fungi capable of breaking down lignin, such as those present in the gut flora of ruminants (Lankiewicz et al., 2023). Some aerobic actinomycetes (filamentous soil bacteria) can also degrade lignin (Kirby, 2005; Wei et al., 2019). However, under aerobic conditions, lignin breakdown is primarily associated with WRF.

There were volunteer fungi growing in the mushroom frames during the cultivation period. The most common of these was a *Coprinopsis* species which produced mushrooms in several barley straw and hemp straw frames. *Coprinopsis* ssp. are saprotrophic, but without a definitive identification of the species we cannot know which compounds it was consuming (MacKinnon & Luther, 2021). However, the amount of mycelium the volunteer fungi produced and the amount of substrate they consumed was likely inconsequential compared to king stropharia, which was inoculated at a rate of 250 g spawn/m^2 and is a strong competitor with other fungi (Stamets, 2000; Szudyga, 1978).

The lignin content in inoculated hemp straw samples decreased significantly over the cultivation period. By contrast, lignin content in inoculated alder chips was relatively unchanged, and there was a slight increase in the lignin content of inoculated barley straw. Hemp straw and alder chips had similar starting proportions of lignin, cellulose, and hemicellulose, yet alder chips produced the lowest mushroom yield and hemp straw the highest yield. The greater surface area to volume ratio in straw versus chips may have resulted in higher decay rates (Fukasawa & Kaga, 2022; Stamets, 2000). It would have

improved the rigour of comparison between substrates in this study if the alder had been processed into smaller pieces. However, the alder chips were prepared using the most readily available equipment (a woodchipper), which makes the experimental results more applicable to real-world cultivation scenarios.

Barley straw had a more similar surface area to volume ratio to hemp straw and similar pre-cultivation water content and total lignocellulosic biomass content. However, barley straw did not contain as much lignin as hemp straw and produced a median mushroom yield less than half that of hemp straw (although the difference in yields between the substrates was not found to be significant due to high variability). This suggests a link between lignin content and king stropharia mushroom yield; however, more data is needed.

Post-cultivation, among the inoculated samples, hemp straw had the lowest lignocellulosic biomass content, while in the control samples, barley straw had the lowest content. This contrast illustrates the difference between the ease of decomposition in the presence of the study site's native decomposer microbes, which were more effective at decomposing barley straw, versus the ease of decomposition by king stropharia, a WRF. Overall, alder chips were the most decomposition resistant.

5.5 Substrate pH and Electrical Conductivity

The main trend in the pH data was that all inoculated post-cultivation substrate samples were found to be significantly more acidic than the corresponding controls. Many decomposer fungi can convert products of lignocellulosic biomass degradation, such as glucose, into organic acids, resulting in an increase in substrate acidity over the cultivation period (Liaud et al., 2014; Philippoussis et al., 2003). The results of the present study support the idea that king stropharia secretes enzymes that acidify substrates as it consumes them.

Likewise, all inoculated post-cultivation substrate samples had significantly higher EC than their corresponding controls. An increase in EC is typical in protected mushroom cultivation (Philippoussis et al., 2003; Zied et al., 2020). In the absence of precipitation to translocate salts deeper into the substrate, evaporation can cause salts accumulate in the surface layers, leading to an increase in EC (H. Sun et al., 2019). The increase in EC could be lessened if the mushrooms were cultivated in the shade of a forest edge rather than under plywood lids, as this would enhance the leaching of salts deeper into the soil profile.

High EC can be an issue for several applications of spent mushroom substrate (SMS), including its use as a soil amendment for crops, as a peat substitute in horticulture and as casing layer material for further mushroom cultivation (Ozores-Hampton, 2017; Paula et al., 2017; Zied et al., 2020). Fortunately, EC can be reduced through SMS washing, although there may be an accompanying loss of beneficial water-soluble nutrients in this process.

5.6 Genomics

5.6.1 Fungal diversity

5.6.1.1 Relative abundance by taxonomic order

The results demonstrate that there were qualitative differences in the fungal communities associated with each substrate in the pre-cultivation samples. Alder chips likely contained a higher initial diversity of fungal orders because of the plants' life history and substrate storage. As perennials growing on a forest edge, they would likely have been exposed to more fungi than conventionally grown annual crops in a farm field (Balami et al., 2020). As previously discussed, the alder chips' storage conditions were moister than those for barley straw and hemp straw, which led to higher levels of contaminants in the alder chips.

The post-cultivation control samples suggest that each substrate supports different assemblages of fungal volunteers from the study site environment. The differing physical and chemical characteristics of the substrates (e.g. particle size distribution, lignocellulosic biomass content, elemental composition etc.) provide slightly different niches to different fungi. However, the similarities in the change in fungal orders from pre-cultivation to post-cultivation among the control samples (namely the decrease in the abundance of *Filobasidiales*) is evidence of fungal succession from opportunistic species consuming easily degraded compounds towards wood-rotting fungi consuming more resistant compounds (Tian et al., 2014).

In the post-cultivation inoculated samples, the high abundance of the order *Agaricales*, which includes the genus *Stropharia* indicates successful colonization by the desired species. That this trend appeared to be weaker in alder chips correlates with the lower yield of mushrooms from this substrate.

5.6.1.2 Relative abundance by primary lifestyle

5.6.1.2.1 *Trichoderma* in pre-cultivation alder chip samples

Trichoderma spp. green mould, a common contaminant in mushroom production, was abundant in the pre-cultivation alder chips (Colavolpe et al., 2014). This contaminant had more favourable conditions to spread through the alder chips due to its moist storage conditions. Since the pre-cultivation substrate samples were collected after pasteurization, the presence of *Trichoderma* spp. also indicates that soaking for 2 hours at ≥60˚C was not effective in destroying this contaminant. However, the low abundance of *Trichoderma* spp.

and other mycoparasites in the inoculated post-cultivation alder samples suggests that king stropharia was able to outcompete it or create unfavourable conditions for it.

5.6.1.2.2 *Cercophora, Schizothecium* and *Coprinopsis* in post-cultivation barley straw control samples

FungalTraits classifies the primary lifestyle of the genera *Cercophora* and *Schizothecium* as dung saprotrophs. However, primary lifestyle is a broad designation that may not apply to every species within a genus (MacKinnon & Luther, 2021; Rodriguez & Redman, 1997). Individual species may also be capable of different lifestyles depending on environmental conditions (Sheldrake, 2021). *Cercophora* spp. are described as lignicolous (growing on wood) as well as coprohilous (growing on animal dung) (Bundhun et al., 2020). *Schizothecium* ASVs have been found to be enriched in wheat and maize straw (Zhang et al., 2023). Similarly, although FungalTraits lists the primary lifestyle of *Coprinopsis* as a soil saprotroph, species within the genus have also been documented living on lignocellulosic biomass and dung (Kombrink et al., 2019; Ragasa et al., 2016).

The question that remains is why these genera only successfully colonized uninoculated barley straw and not hemp straw and alder chips. This is difficult to ascertain without species-level fungal identification and a detailed knowledge of their needs. The physical and chemical conditions provided by barley straw (nutrient content, pH, EC, fibre content etc.) could have been better suited to these fungi than the other substrates.

5.6.1.2.3 Increase in the abundance of litter saprotrophs in treated post-cultivation samples

The sizeable increase in the abundance of litter saprotrophs in inoculated post-cultivation samples of all substrate types indicates the dominance of king stropharia in those samples since FungalTraits categorizes the genus *Stropharia* as a litter saprotroph.

5.6.2 Bacterial diversity

5.6.2.1 Relative abundance by bacterial phylum

At the phylum level, the pre-cultivation substrate samples appeared to have similar bacterial communities. However, at the phylum level, this apparent similarity may not be meaningful. Greater diversity and differences in abundance of phyla between substrates in the post-cultivation control samples result from bacteria in the environment colonizing the different substrates after pasteurization.

The very high abundance of the phylum *Firmicutes* among post-cultivation inoculated barley straw and hemp straw, which were the substrates most effectively colonized by king stropharia, suggests possible symbiotic relationships between bacteria in this phylum and king stropharia. Similar associations have been noted elsewhere. For example, *Firmicutes* enrichment has been observed in paper birch (*Betulina papyrifera*) rotted by WRF *Fomes fomentarius*, suggesting that wood decomposer fungi may exert selection effects on bacteria or vice versa (Haq et al., 2022).

5.6.2.2 Family and genus-level relative abundance within the phylum *Firmicutes* among post-cultivation inoculated samples

Two families (*Bacillaceae* and *Paenibacilaceae*) and two genera within these families (*Bacillus* and *Paenibacillus*, respectively) made up the majority of *Firmicutes* reads in all post-cultivation inoculated substrate samples. The two genera share several similarities. They have both been found to degrade lignin (Chandra et al., 2008). This could partially explain why they would occur in the same environment as WRF (they can use the same food source),

but it does not explain why they had higher relative abundance in the substrates that were successfully colonized by WRF, where lignin content was lower.

Bacillus and *Paenibacillus* are Gram-positive aerobic endospore-forming bacteria that are ubiquitous in agricultural soils (Govindasamy et al., 2011; McSpadden Gardener, 2004). They have been studied as potential plant-growth promoting rhizobacteria with sustainable agriculture applications thanks to a suite of benefits observed from these bacteria in crop plants, including atmospheric nitrogen fixation, solubilization of soil phosphorous, micronutrient uptake, and production of phytohormones and antimicrobial metabolites (Grady et al., 2016).

There is emerging research on how these genera interact with WRF, though not king stropharia specifically. *Bacillus* and *Paenibacillus* have been found to promote mycelial growth in *Pleurotus ostreatus* (Shamugam & Kertesz, 2023). Cultivation of *P. ostreatus* in the presence of these bacterial genera has been found to result in an increase in mycelial laccase activity, possibly as a defence response to the bacteria (Shamugam & Kertesz, 2023). *Bacillus* and *Paenibacillus* have also been found to inhibit the growth of competitor *Trichoderma* spp. fungi (Velázquez-Cedeño et al., 2008). It has also been suggested that, through their N-fixing ability, these bacterial genera could increase N availability to WRF in exchange for greater access to embedded carbohydrates (Haq et al., 2022).

5.6.3 Limitations of compositionality

Interpretation of the results of genetic sequencing are limited by their inherent compositionality. Compositional data is constrained to an arbitrary constant sum, which is this case is the total number of reads (Douglas & Langille, 2021). Because of the compositionality of the data, it is possible to compare relative proportions of different

taxonomic constituents, but the total population of these constituents cannot be known. This constrains our understanding of fungal-bacterial interactions because we do not know if bacterial biomass increased or decreased over the cultivation period, depending on substrate type and treatment. Knowing the change in bacterial biomass could be important because bacterial biomass can be a major nutrient source for WRF such as *P. ostreatus*. The growth of *P. ostreatus* corresponds with a 5 to 10-fold decrease in bacterial 16S rRNA levels, and this species has been shown to actively penetrate and lyse bacterial colonies, followed by profuse *P. ostreatus* hyphal growth (Bánfi et al., 2021). King stropharia may also use bacteria growing on the substrates and its own decomposition byproducts as a food source.

5.6.4 Limitations of timeline

The microbial community in the inoculated post-cultivation substrates at the time of analysis is unlikely to resemble the community that will be present later because of succession processes that occur throughout the decomposition process. Paula et al. (2017) found bacterial community structure to be a potential predictor of the stability of mushroom compost.

5.7 King stropharia post-cultivation substrate suitability as a soil amendment

I assessed the quality of king stropharia post-cultivation substrates by comparing their properties to the optimal ranges of the compost quality parameters compiled by Ozores-Hampton (2017) (Table 3). Since all inoculated substrate types continued to produce mushrooms for at least two years after the experimental cultivation period, they cannot be considered true “spent” mushroom substrates (SMS). Each post-cultivation substrate type was assessed separately (Tables 10, 11 and 12).

Not all the parameters listed in Table 3 were measured in this study, but there are a few un-measured parameters for which I can provide context. Based on handling the post-cultivation substrates, the alder chips would not meet the particle size criteria (98% of the material being able to pass through a ¾-inch screen). The straws contained narrower particles to begin with and were more substantially decomposed over the cultivation period. Most of the post-cultivation straw could be forced through a ¾-inch screen, but it is uncertain whether the 98% threshold would be met. Because the substrates were each prepared from one kind of single-origin plant biomass, they would have been virtually free of weed seeds.

The post-cultivation substrates met many parameters for optimal compost quality, but each substrate also failed to meet some parameters. All substrate types had pH values that were below the optimal range, C:N ratios above the optimal range and insufficient P content. Fortunately, values for these parameters can be improved through further maturation of the mushroom compost under aerobic conditions (Paula et al., 2017; Sundberg, 2005). Sufficient aeration is one of the most important factors determining the creation of high-quality compost (Guo et al., 2012). While these studies used ventilated in-vessel composters, compost aeration can be achieved simply by turning the piles of decomposing material (Rhoades, 2012). Further decomposition would increase C loss, which results in an increase in the relative concentration of nutrients such as N and P. Over time, the biochemistry of compost also tends to shift towards a decrease in the buildup of organic acids (Rynk et al., 2021; Sundberg, 2005).

Inoculated post-cultivation alder chip values were the furthest from meeting Ozores-Hampton's criteria. Alder chips had the largest particle size, lowest pH, and lowest nutrient content (Table 10). Hemp straw was the closest to meeting the criteria overall since it had the

highest pH, highest N content and its C:N ratio was almost in the optimal range (Table 12). However, barley straw had a higher mean P content than hemp straw (Table 11). All three substrate types could be made into suitable soil amendments to improve the physical, chemical, and biological properties of soil for crop production. However, the alder chips would require the longest period of maturation before achieving optimal quality.

Table 10: Compost quality assessment of inoculated post-cultivation alder chips.

Parameter (units)	Optimal range	Median value in **alder chips**	Within optimal range?	Comments
Moisture (%)	30 (dry) - 60 (wet)	54	Yes	
Organic matter (%)	40-60	>72	No	Based on total lignocellulosic biomass
Physical contaminants (%)	<2	<2	Yes	
pH	5.0-8.0	3.5	No	pH could be increased through further maturation of compost with aeration
EC (mmho/cm)	<6	0.69	Yes	
Carbon:nitrogen ratio	10-25	55	No	
Nitrogen (%)	0.5-6.0	0.86	Yes	
Phosphorous (%)	0.2-3.0	0.04	No	
Potassium (%)	0.10-3.5	0.14	Yes	
Copper (ppm)	<450	5	Yes	
Molybdenum (ppm)	<75	<3	Yes	Most values were below ICP-OES quantification limits
Nickel (ppm)	<50	<3	Yes	Most values were below ICP-OES quantification limits
Zinc (ppm)	<900	32	Yes	
Fecal coliform (MPN/g total solids)	<1000	NA	Very likely	Genera of fecal coliforms detected in 16S bacterial genome sequencing counts at extremely low levels

Salmonella (MPN/4 g)	<3	0	Yes	No reads of *Salmonella* detected in 16S bacterial genome sequencing

Table 11: Compost quality assessment of inoculated post-cultivation barley straw.

Parameter (units)	Optimal range	Median value in **barley straw**	Within optimal range?	Comments
Moisture (%)	30 (dry) - 60 (wet)	80	No	Easy to fix by allowing it to dry out
Organic matter (%)	40-60	>56	Likely	Based on total lignocellulosic biomass
Physical contaminants (%)	<2	<2	Yes	
pH	5.0-8.0	4.0	No	pH could be increased through further maturation of compost with aeration
EC (mmho/cm)	<6	2.0	Yes	
Carbon:nitrogen ratio	10-25	31	No	Ratio could be decreased through further maturation of compost
Nitrogen (%)	0.5-6.0	1.4	Yes	
Phosphorous (%)	0.2-3.0	0.16	Almost	P content could be increased through further maturation of compost
Potassium (%)	0.10-3.5	0.41	Yes	
Copper (ppm)	<450	8.4	Yes	
Molybdenum (ppm)	<75	<3	Yes	Most values were below ICP-OES quantification limits
Nickel (ppm)	<50	<3	Yes	Most values were below ICP-OES quantification limits
Zinc (ppm)	<900	44.9	Yes	
Fecal coliform (MPN/g total solids)	<1000	NA	Very likely	Genera of fecal coliforms detected in 16S bacterial genome sequencing counts at extremely low levels
Salmonella (MPN/4 g)	<3	0	Yes	No reads of *Salmonella* detected in 16S bacterial genome sequencing

Table 12: Compost quality assessment of inoculated post-cultivation hemp straw.

Parameter (units)	Optimal range	Median value in **hemp straw**	Within optimal range?	Comments
Moisture (%)	30 (dry) - 60 (wet)	69	No	Easy to fix by allowing it to dry out
Organic matter (%)	40-60	>44	Likely	Based on total lignocellulosic biomass
Physical contaminants (%)	<2	<2	Yes	
pH	5.0-8.0	4.2	No	pH could be increased through further maturation of compost with aeration
EC (mmho/cm)	<6	2.5	Yes	
Carbon:nitrogen ratio	10-25	26	Almost	Ratio could be decreased through further maturation of compost
Nitrogen (%)	0.5-6.0	1.5	Yes	
Phosphorous (%)	0.2-3.0	0.1	No	P content could be increased through further maturation of compost
Potassium (%)	0.10-3.5	0.29	Yes	
Copper (ppm)	<450	13	Yes	
Molybdenum (ppm)	<75	<3	Yes	Most values were below ICP-OES quantification limits
Nickel (ppm)	<50	<3	Yes	Most values were below ICP-OES quantification limits
Zinc (ppm)	<900	34	Yes	
Fecal coliform (MPN/g total solids)	<1000	NA	Very likely	Genera of fecal coliforms detected in 16S bacterial genome sequencing counts at extremely low levels
Salmonella (MPN/4 g)	<3	0	Yes	No reads of *Salmonella* detected in 16S bacterial genome sequencing

5.8 Areas for future research

5.8.1 More cultivation trials to improve yield data

The use of contaminated spawn in this experiment likely impacted mushroom yield. This was also a small-scale cultivation trial. More and larger trials with fresh, high-quality spawn are needed to assess the true yield potential of king stropharia.

5.8.2 Biological efficiency

Biological efficiency (BE) is a measure of how efficiently a fungus converts substrate biomass into mushroom biomass. It is calculated as:

BE % = total fresh weight of mushrooms (kg)/dry weight of substrate (kg) x 100 (Biswas & Layak, 2014).

BE is a common metric for assessing the suitability of different substrates and techniques for mushroom production (Cueva et al., 2017; Hoa et al., 2015; Onyeka et al., 2018). In future king stropharia cultivation experiments, the initial mass of the substrates should be recorded so that BE can be calculated. This data would help cultivators predict yields based on the type, preparation, and amount of substrate used.

It will take longer to collect BE data for king stropharia than it does for many other cultivated mushroom species due to king stropharia's slow growth rate. None of the substrates in the present study were completely spent at the end of one cultivation season, as evidenced by the presence of mushrooms in the garden frames the following two seasons. To know the total yield from a given substrate would require multiple seasons of data collection until mushroom production in the substrates tapered off. Long term yield data collection would also be helpful to cultivators wanting to know the longevity of their mushroom substrates.

5.8.3 Litterbag study

Analysis of pre- and post-cultivation substrate data (i.e., fiber analysis and nutrient content) in this study was limited by compositionality. Mass loss data would enhance our understanding substrate decomposition by king stropharia. The litterbag method is a commonly used technique for studying decomposition rates under field conditions (Bärlocher, 2020; Kurz-Besson et al., 2005; Xie, 2020). In this method, a known mass of substrate is enclosed in a mesh litterbag and laid on the substrate surface or buried in the substrate. Litterbags are then periodically collected and weighed, and decomposition rates are determined by mass loss modeling (Xie, 2020).

It would also be possible to approximate mass loss based on the ratio of non-volatile solids (NVS) or ash before and after cultivation, as is sometimes done for compost (Breitenbeck & Schellinger, 2004).

5.8.4 Cultivation structure comparison experiments

There is no published literature comparing the yield of king stropharia from different cultivation structures. This hardy, versatile mushroom has been intercropped with straw-mulched vegetables (Stamets, 2000), grown in wooden boxes (Szudyga, 1978) and produced in piles or beds of substrates in partially shaded areas (e.g., along a forest edge) (Bruhn et al., 2010; Stamets, 2000). I think king stropharia could also be grown in inexpensive tent structures to provide shade, and some ability to moderate temperature and humidity, as has been done successfully for the cultivation of mushrooms in developing countries (Oei & van Nieuwenhuijzen, 2005). Research is needed to compare different growing structures.

5.8.5 Nutrition experiments

More controlled experiments are needed to determine the ideal substrate nutrient profile for growing king stropharia mushrooms. The nutrient data in the present study are difficult to interpret because there are so many variables and confounding factors.

King stropharia could also be investigated for vitamin and medicinal compound content. Szudyga (1978) mentions that the niacin (vitamin B-3) content of king stropharia mushrooms is 3.41 mg. He does not write what mass of mushrooms this measurement corresponds to (Is it 100 g? or 1 kg?), nor whether king stropharia is rich in any other vitamins. Many mushrooms are rich in healthful compounds, but king stropharia has yet to be thoroughly studied in this regard (Stamets, 2000).

5.8.6 Further analysis of genomics results

Our understanding of the genomics results could be deepened through further analysis, such as compositional data analysis and calculation of alpha and beta diversity (Gloor & Reid, 2016; Martino et al., 2022; Willis, 2019). This would allow for a more detailed and quantitative understanding of the differences in microbial communities between cultivation stages and treatment types.

5.8.7 Post-cultivation substrate maturation and application experiments

The post-cultivation substrate data collected in the present study provide a basis for evaluating their use in applications such as a soil amendment. However, as previously noted, the substrates were not completely spent at the end of the experimental cultivation period. Re-evaluation after a second season of cultivation or longer would provide a better sense of the potential applications of the materials.

Experiments using the SMS in various potential applications would bring this area from theory into practice. Such experiments could include potted plant studies comparing the effects of king stropharia SMS to other growing mixes. Another area for experimentation is the use of king stropharia SMS for the cultivation of other mushroom species (Stamets, 2000; Zied et al., 2020).

5.8.8 Economic feasibility studies

For the cultivation of king stropharia mushrooms to become widespread, the economic feasibility of commercial production must be improved. This would involve reducing production costs, increasing yields through improved cultivation techniques, and generating additional value through SMS applications. For example, pasteurization must be achieved using more efficient methods than the small batch heating done in this study. The pursuit of the research areas identified above would result in a better understanding of how to achieve the greatest yields and improve the value of the SMS.

Because king stropharia mushrooms are relatively unknown to consumers, improving the economic potential of cultivating them would also involve market research about how to increase consumer awareness and appreciation of the species.

6 Conclusion

This study provides insights into the potential for cultivating king stropharia mushrooms in northern environments. Of alder chips, barley straw and hemp straw, hemp straw tended to be the fastest and highest yielding substrate in the cultivation trial but was not significantly different than barley straw in terms of yield. Hemp straw's good quality as a substrate for cultivating king stropharia mushrooms is likely due to its nutrient profile and lignin content

compared to the other substrates. There also appeared to be a distinctive bacterial consortium associated with the successful cultivation of king stropharia in barley straw and hemp straw, with high relative abundance of the genera *Bacillus* and *Paenibacillus* in these samples. Additional cultivation experiments using fresh spawn are necessary to properly assess king stropharia's yield potential and effects on substrates since the results of this study were impacted by spawn contamination. Furthermore, a longer-term trial is needed to assess the performance of alder chips as a substrate. This experiment showed a lower yield from the alder chip substrate, but compounding factors such as spawn run time and lack of casing layers applied due to a lack of full myceliation, likely affected the yield results. It is recommended that, for outdoor experimentation, the trial period begins as soon as possible in the growing season, and continues for 2 or 3 years to get a full picture of absolute yield differences between these three substrates.

All post-cultivation mushroom substrate types, and especially hemp straw, had beneficial properties for agricultural soil amendment, even though the substrates were not completely spent at the end of the cultivation trial. Further decomposition of the substrates from another season of mushroom cultivation likely would have further improved their soil amendment properties. These findings highlight king stropharia's potential for enhancing northern agricultural security and sustainability. King stropharia presents a win-win scenario whereby farmers can produce nutritious mushrooms on their crop residues while enhancing the crop residue decomposition and nutrient cycling with minimal technology and labour.

In conclusion, the cultivation of king stropharia mushrooms in northern environments, specifically using hemp straw, is a promising endeavor with several environmental and agricultural benefits. Although a lot more research is required to fully realize the potential of

this species, this study demonstrates how king stropharia can contribute to both local agriculture and sustainable waste management practices.

7 References

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Appendices

Appendix A: Mushroom composition data tables

Table A-1: Results of Kruskal-Wallace Multiple Comparison test on mushroom sample carbon and nitrogen content.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Total carbon (% dry matter)	Alder chips	2	43.25	7.8	0.3			
	Barley straw	5	42.7	4.8	-1.61			
	Hemp straw	6	43.3	8.6	1.36			
	Overall	13	43.2	7		2	2.71	0.258
Total nitrogen (% dry matter)	Alder chips	2	4.55	5.3	-0.69			
	Barley straw	5	5.6	9	1.46			
	Hemp straw	6	4.5	5.9	-0.93			
	Overall	13	4.7	7		2	2.26	0.323

Table A-2: Significant results of Kruskal-Wallace Multiple Comparisons test on mushroom ICP-OES elemental analysis data. P-values < 0.05 are indicated by *. All values are reported on a dry matter basis.

Element	Substrate	N	Median	Mean Rank	Z-value	DF	H value	P value
B	Alder chips	2	1	4	-1.18			
	Barley straw	5	1	4.9	-1.54			
	Hemp straw	6	5.5	9.8	2.36			
	Overall	13		7		2	6.68	0.035*
	Barley straw vs. Hemp straw				2.23946			0.0251*
	Alder chips vs. Hemp straw				1.96902			0.049*
Cd	Alder chips	2	1.55	12.5	2.17			
	Barley straw	5	0.4	4.9	-1.54			
	Hemp straw	6	0.45	6.9	-0.07			
	Overall	13				2	5.78	0.056
	Alder chips vs. Barley straw				2.40283			0.0163*
Fe	Alder chips	2	86.5	3.5	-1.38			
	Barley straw	5	109	4.8	-1.61			
	Hemp straw	6	145	10	2.57			
	Overall	13		7		2	6.79	0.034*

	Barley straw vs. Hemp straw				2.20811			0.0272*
	Alder chips vs. Hemp straw				2.04697			0.0407*
Mn	Alder chips	2	14	2.5	-1.78			
	Barley straw	5	20	5.7	-0.95			
	Hemp straw	6	23.5	9.6	2.21			
	Overall	13		7		2	6.07	0.048*
	Alder chips vs. Hemp straw				2.26526			0.0235*

Appendix B: Substrate moisture content data tables

Substrate comparisons

Table B-1: Results of Kruskal-Wallace multiple comparison test on the relationship between substrate type and substrate moisture content (fresh weight basis). Substrate: A = alder chips, B = barley straw, H = hemps straw. Treatment: t = treated (inoculated), u = untreated (uninoculated). Cultivation stage: P = pre-cultivation, C = post-cultivation. P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

Treatment	Cultivation stage	Substrate	N	Median % moisture content	Mean Rank	Z-Value	DF	H-value	P-value
t	P	A	5	61.2	3	-3.12			
		B	5	78.1	10.2	0.96			
		H	6	78.9	11.7	2.06			
		Overall	16		8.5		2	9.98	0.007***
		A vs. H				3.01			0.0026***
		A vs. B				2.39			0.0167***
u	P	A	3	60.8	2	-2.24			
		B	3	78.8	5.7	1.04			
		H	2	80.15	6.5	1.33			
		Overall	8		4.5		2	5.14	0.077
		A vs. H				2.01			0.0442***
t	C	A	5	54	3	-3.12			
		B	5	80.1	14	3.12			
		H	6	69.4	8.5	0			
		Overall	16		8.5		2	13.35	0.001***
		A vs. B				3.65			0.0003***
		A vs. H				1.91			0.0564*
		B vs. H				1.91			0.0564*
u	C	A	3	61.7	2	-2.24			
		B	3	84.1	7	2.24			
		H	2	79.05	4.5	0			
		Overall	8		4.5		2	6.25	0.044***
		A vs. B				2.5			0.0124***

Treatment comparisons

Table B-2: Results of Kruskal-Wallace test on the relationship between treatment (inoculation) and substrate moisture content (wet weight basis). Substrate: A = alder chips, B = barley straw, H = hemp straw. Cultivation stage: P = pre-cultivation, C = post-cultivation. Treatment: t = treated (inoculated), u = untreated (uninoculated).

Substrate	Cultivation stage	Treatment	N	Median % moisture content	Mean Rank	Z-Value	DF	H-value	P-value
A	P	t	5	61.2	5.1	0.89			
		u	3	60.8	3.5	-0.89			
		Overall	8		4.5		1	0.82	0.365
B	P	t	5	78.1	4	-0.75			
		u	3	78.8	5.3	0.75			
		Overall	8		4.5		1	0.56	0.456
H	P	t	6	78.9	4.2	-0.67			
		u	2	80.15	5.5	0.67			
		Overall	8		4.5		1	0.44	0.505
A	C	t	5	54	3	-2.24			
		u	3	61.7	7	2.24			
		Overall	8		4.5		1	5	0.025***
B	C	t	5	80.1	3.4	-1.64			
		u	3	84.1	6.3	1.64			
		Overall	8		4.5		1	2.69	0.101
H	C	t	6	69.4	3.5	-2			
		u	2	79.05	7.5	2			
		Overall	8		4.5		1	4	0.046***

Appendix C: Substrate carbon and nitrogen data tables

Substrate comparisons

Table C-1 (a) – (d). Results of Kruskal-Wallace Multiple Comparisons test on the relationship between substrate type and carbon, nitrogen (expressed as a percentage of oven dry sample mass) and C:N ratio. Substrates: A = alder chips, B = barley straw, H = hemp straw. P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

(a) Treated (inoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Total nitrogen	A	5	0.67	4.6	-2.21			
	B	5	0.89	11.4	1.64			
	H	6	0.84	9.3	0.54			
	Overall	16		8.5		2	5.39	0.067*
	A vs. B				2.26			0.0239***
Total carbon	A	5	49.26	14	3.12			
	B	5	45.59	7.6	-0.51			
	H	6	45.25	4.7	-2.49			
	Overall	16		8.5		2	10.74	0.005***
	A vs. H				3.24			0.0012***
	A vs. B				2.13			0.0335***
C:N	A	5	74.21	13.2	2.66			
	B	5	50.97	5.2	-1.87			
	H	6	54.015	7.3	-0.76			
	Overall	16		8.5		2	7.64	0.022***
	A vs. B				2.66			0.0079***
	A vs. H				2.03			0.0419***

(b) Untreated (uninoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Total nitrogen	A	3	0.66	3	-1.34			
	B	3	0.8	6.7	1.94			
	H	2	0.71	3.5	-0.67			
	Overall	8		4.5		2	3.81	0.149
Total carbon	A	3	48.79	7	2.24			
	B	3	45.33	3.3	-1.04			
	H	2	44.71	2.5	-1.33			
	Overall	8		4.5		2	5.14	0.077
	A vs. H				2.012			0.0442***

C:N	A	3	73.92	6.7	1.94			
	B	3	55.29	2	-2.24			
	H	2	62.98	5	0.33			
	Overall	8		4.5		2	5.56	0.062*
	A vs. B				2.33			0.0196***

(c) Treated, post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Total nitrogen	A	5	0.86	5.8	-1.53			
	B	5	1.44	9.8	0.74			
	H	6	1.525	9.7	0.76			
	Overall	16		8.5		2	2.34	0.31
Total carbon	A	5	49.1	14	3.12			
	B	5	43.97	9	0.28			
	H	6	39.845	3.5	-3.25			
	Overall	16		8.5		2	13.35	0.001***
	A vs. H				3.64			0.0003***
	B vs. H				1.91			0.0564*
C:N	A	5	55.36	11.2	1.53			
	B	5	30.53	8.4	-0.06			
	H	6	26.24	6.3	-1.41			
	Overall	16		8.5		2	2.85	0.24

(d) Untreated, post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Total nitrogen	A	3	2.95	4	-0.45			
	B	3	3.52	6	1.34			
	H	2	1.87	3	-1			
	Overall	8		4.5		2	2	0.368
Total carbon	A	3	48.81	7	2.24			
	B	3	45.43	2	-2.24			
	H	2	46.15	4.5	0			
	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***
C:N	A	3	16.55	5	0.45			
	B	3	12.82	3	-1.34			
	H	2	30.75	6	1			
	Overall	8		4.5		2	2	0.368

Treatment comparisons

Table C-2: Results of Kruskal-Wallace test on the relationship between treatment (inoculation) and carbon, nitrogen (expressed as a percentage of oven dry sample mass) and C:N. Treatment: t = treated (inoculated), u = untreated (uninoculated). P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

Cultivation stage	Substrate	Response variable	Treatment	N	Median	Mean Rank	Z-value	DF	H-value	P-value
Pre-cultivation	Alder chips	Total nitrogen	t	5	0.67	4.2	-0.45			
			u	3	0.66	5	0.45			
			Overall	8		4.5		1	0.2	0.655
		Total carbon	t	5	49.26	5.4	1.34			
			u	3	48.79	3	-1.34			
			Overall	8		4.5		1	1.8	0.18
		C:N	t	5	74.21	4.8	0.45			
			u	3	74.27	4	-0.45			
			Overall	8		4.5		1	0.2	0.655
	Barley straw	Total nitrogen	t	5	0.89	4.8	0.45			
			u	3	0.8	4	-0.45			
			Overall	8		4.5		1	0.2	0.655
		Total carbon	t	5	45.59	5.5	1.49			
			u	3	45.33	2.8	-1.49			
			Overall	8		4.5		1	2.25	0.134
		C:N	t	5	50.97	4.2	-0.45			
			u	3	55.37	5	0.45			
			Overall	8		4.5		1	0.2	0.655
	Hemp straw	Total nitrogen	t	6	0.84	4.8	0.67			
			u	2	0.71	3.5	-0.67			
			Overall	8		4.5		1	0.44	0.505
		Total carbon	t	6	45.25	5.3	1.67			
			u	2	44.71	2	-1.67			
			Overall	8		4.5		1	2.78	0.096
		C:N	t	6	54.015	4.2	-0.67			
			u	2	63.32	5.5	0.67			
			Overall	8		4.5		1	0.44	0.505
Post-cultivation	Alder chips	Total nitrogen	t	5	0.86	4	-0.75			
			u	3	2.95	5.3	0.75			
			Overall	8		4.5		1	0.56	0.456
		Total carbon	t	5	49.1	4.4	-0.15			

			u	3	48.81	4.7	0.15			
			Overall	8		4.5		1	0.02	0.881
		C:N	t	5	55.22	5	0.75			
			u	3	16.57	3.7	-0.75			
			Overall	8		4.5		1	0.56	0.456
	Barley straw	Total nitrogen	t	5	1.44	4.2	-0.45			
			u	3	3.52	5	0.45			
			Overall	8		4.5		1	0.2	0.655
		Total carbon	t	5	43.97	3	-2.24			
			u	3	45.43	7	2.24			
			Overall	8		4.5		1	5	0.025***
		C:N	t	5	30.63	4.6	0.15			
			u	3	12.82	4.3	-0.15			
			Overall	8		4.5		1	0.02	0.881
	Hemp straw	Total nitrogen	t	6	1.525	4.8	0.67			
			u	2	1.87	3.5	-0.67			
			Overall	8		4.5		1	0.44	0.505
		Total carbon	t	6	39.845	3.5	-2			
			u	2	46.15	7.5	2			
			Overall	8		4.5		1	4	0.046***
		C:N	t	6	26.225	4.2	-0.67			
			u	2	30.765	5.5	0.67			
			Overall	8		4.5		1	0.44	0.505

Pre- and post-cultivation comparisons

Table C-3. Results of Wilcoxon Signed Rank Confidence Interval test evaluating changes in pre- and post-cultivation substrate carbon, nitrogen (expressed as a percentage of oven dry sample mass) and C:N. P = pre-cultivation, C = post-cultivation. Note: Minitab statistical software will not perform the Wilcoxon test on sample sizes ≤3.

Substrate	Treatment	Response variable	N	Median change (P - C)	CI for η, lower limit	CI for η, upper limit	Achieved confidence %	Interpretation
Alder chips	Treated	Total nitrogen	5	0.9	-0.07	2.43	94.09	Probable increase
		Total carbon	5	-0.48	-2.05	1.33	94.09	No trend
		C:N	5	-38.84	-53.12	5.78	94.09	Probable decrease

Barley straw	Treated	Total nitrogen	5	1.465	0.09	2.96	94.09	Increase
		Total carbon	5	-1.75	-2.47	-1.46	94.09	Decrease
		C:N	5	-32.93	-42.12	-3.28	94.09	Decrease
Hemp straw	Treated	Total nitrogen	6	1.55	0.42	2.76	94.08	Increase
		Total carbon	6	-5.26	-6.97	-3.455	94.08	Decrease
		C:N	6	-34.055	-52.54	-17.3	94.08	Decrease
Alder chips	Untreated	Total nitrogen	3	2.16	Insufficient data to perform test			
		Total carbon	3	-0.36	Insufficient data to perform test			
		C:N	3	-46.38	Insufficient data to perform test			
Barley straw	Untreated	Total nitrogen	3	2.72	Insufficient data to perform test			
		Total carbon	3	0.49	Insufficient data to perform test			
		C:N	3	-42.55	Insufficient data to perform test			
Hemp straw	Untreated	Total nitrogen	2	1.16	Insufficient data to perform test			
		Total carbon	2	1.44	Insufficient data to perform test			
		C:N	2	-32.56	Insufficient data to perform test			

Appendix D: Substrate macronutrient ICP-OES analysis data tables

Substrate comparisons

Table D-1 (a) – (d). Results of Kruskal-Wallace Multiple Comparisons test on the relationship between substrate type and macronutrient (Ca, K, Mg, P, S) content (expressed in mg/kg of substrate dry matter). Substrates: A = alder chips, B = barley straw, H = hemp straw. P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

(a) Treated (inoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Ca	A	5	5025.2	6.2	-1.3			
	B	5	4867.7	4.8	-2.1			
	H	6	11314.1	13.5	3.25			
	Overall	16		8.5		2	10.8	0.005***
	B vs. H				3.02			0.0025***
	A vs. H				2.53			0.0113***
K	A	5	1483.7	3.4	-2.89			
	B	5	4127.1	14	3.12			
	H	6	2267.4	8.2	-0.22			
	Overall	16		8.5		2	12.44	0.002***
	A vs. B				3.52			0.0004***
	B vs. H				2.02			0.043***
Mg	A	5	735.1	3	-3.12			
	B	5	1807.2	8	-0.28			
	H	6	2358.5	13.5	3.25			
	Overall	16		8.5		2	13.35	0.001***
	A vs. H				3.64			0.0003***
	B vs. H				1.91			0.0564*
P	A	5	442.3	4.2	-2.44			
	B	5	1269.2	12.8	2.44			
	H	6	547.8	8.5	0			
	Overall	16		8.5		2	8.16	0.017***
	A vs. B				2.86			0.0043***

S	A	5	434.4	3	-3.12			
	B	5	1120.5	13.8	3			
	H	6	706.55	8.7	0.11			
	Overall	16		8.5		2	12.88	0.002***
	A vs. B				3.59			0.0003***
	A vs. H				1.97			0.0493***

(b) Untreated (uninoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Ca	A	3	5537.6	4.7	0.15			
	B	3	4495.6	2.3	-1.94			
	H	2	9249.95	7.5	2			
	Overall	8		4.5		2	5.36	0.069
	B vs. H				2.31			0.0209***
K	A	3	1428	3.7	-0.75			
	B	3	4077.5	7	2.24			
	H	2	1341.55	2	-1.67			
	Overall	8		4.5		2	5.56	0.062*
	B vs. H				2.24			0.0253***
Mg	A	3	736.9	2	-2.24			
	B	3	1778.9	5	0.45			
	H	2	2170.15	7.5	2			
	Overall	8		4.5		2	6.25	0.044***
	A vs. H				2.46			0.0139***
P	A	3	420.1	3	-1.34			
	B	3	1252.5	7	2.24			
	H	2	446.6	3	-1			
	Overall	8		4.5		2	5	0.082
	A vs. B				2			0.0455***
S	A	3	400.1	2	-2.24			
	B	3	1098	7	2.24			
	H	2	663.5	4.5	0			

	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***

(c) Treated (inoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Ca	A	5	6557.9	3.8	-2.66			
	B	5	9524.8	7.2	-0.74			
	H	6	28422	13.5	3.25			
	Overall	16		8.5		2	11.86	0.003***
	A vs. H				3.36			0.0008***
	B vs. H				2.19			0.0289***
K	A	5	1379.5	3	-3.12			
	B	5	4113.1	12	1.98			
	H	6	2893.4	10.2	1.08			
	Overall	16		8.5		2	10.11	0.006***
	A vs. B				2.99			0.0028***
	A vs. H				2.49			0.0129***
Mg	A	5	1027.9	3	-3.12			
	B	5	3089.4	8	-0.28			
	H	6	6443.6	13.5	3.25			
	Overall	16		8.5		2	13.35	0.001***
	A vs. H				3.64			0.0003***
	B vs. H				1.91			0.0564*
P	A	5	429.1	3	-3.12			
	B	5	1608.7	13	2.55			
	H	6	992.6	9.3	0.54			
	Overall	16		8.5		2	11.32	0.003***
	A vs. B				3.32			0.0009***
	A vs. H				2.2			0.028***
S	A	5	504.6	3	-3.12			
	B	5	1860.8	13	2.55			
	H	6	1656.95	9.3	0.54			

	Overall	16		8.5		2	11.32	0.003***
	A vs. B				3.32			0.0009***
	A vs. H				2.2			0.028***

(d) Untreated (uninoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Ca	A	3	6292.7	2	-2.24			
	B	3	7984	5	0.45			
	H	2	12921.6	7.5	2			
	Overall	8		4.5		2	6.25	0.044***
	A vs. H				2.46			0.0139***
K	A	3	1660	2	-2.24			
	B	3	7848.3	7	2.24			
	H	2	2915.85	4.5	0			
	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***
Mg	A	3	875	2	-2.24			
	B	3	3657.2	6	1.34			
	H	2	3489.65	6	1			
	Overall	8		4.5		2	5	0.082
	A vs. B				2			0.0455***
P	A	3	525	2	-2.24			
	B	3	2580.7	7	2.24			
	H	2	919.5	4.5	0			
	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***
S	A	3	522.5	2	-2.24			
	B	3	2337	7	2.24			
	H	2	1153.85	4.5	0			
	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***

Treatment comparisons

Table D-2: Results of Kruskal-Wallace test on the relationship between treatment (inoculation) and substrate macronutrient (Ca, K, Mg, P, S) content (expressed in mg/kg of substrate dry matter). Treatment: t = treated (inoculated), u = untreated (uninoculated). P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

Cultivation stage	Substrate	Response	Treatment	N	Median	Mean Rank	Z-Value	DF	H-value	P-value
Pre-cultivation	Alder chips	Ca	t	5	5025.2	3.8	-1.04			
			u	3	5537.6	5.7	1.04			
			Overall	8		4.5		1	1.09	0.297
		K	t	5	1483.7	4.6	0.15			
			u	3	1428	4.3	-0.15			
			Overall	8		4.5		1	0.02	0.881
		Mg	t	5	735.1	4.2	-0.45			
			u	3	736.9	5	0.45			
			Overall	8		4.5		1	0.20	0.655
		P	t	5	442.3	4.2	-0.45			
			u	3	420.1	5	0.45			
			Overall	8		4.5		1	0.20	0.655
		S	t	5	434.4	4.6	0.15			
			u	3	400.1	4.3	-0.15			
			Overall	8		4.5		1	0.02	0.881
	Barley straw	Ca	t	5	4867.7	5.2	1.04			
			u	3	4495.6	3.3	-1.04			
			Overall	8		4.5		1	1.09	0.297
		K	t	5	4127.1	4.4	-0.15			
			u	3	4077.5	4.7	0.15			
			Overall	8		4.5		1	0.02	0.881
		Mg	t	5	1807.2	5	0.75			
			u	3	1778.9	3.7	-0.75			
			Overall	8		4.5		1	0.56	0.456
		P	t	5	1269.2	4.2	-0.45			
			u	3	1252.5	5	0.45			

			Overall	8		4.5		1	0.20	0.655
		S	t	5	1120.5	4.6	0.15			
			u	3	1098	4.3	-0.15			
			Overall	8		4.5		1	0.20	0.881
	Hemp straw	Ca	t	6	11314.1	5.5	2			
			u	2	9250	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***
		K	t	6	2267.4	5.5	2			
			u	2	1341.55	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***
		Mg	t	6	2358.5	5.5	2			
			u	2	2170.15	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***
		P	t	6	547.8	5	1			
			u	2	446.6	3	-1			
			Overall	8		4.5		1	1.00	0.317
		S	t	6	706.55	5.3	1.67			
			u	2	663.5	2	-1.67			
			Overall	8		4.5		1	2.78	0.96
Post-cultivation	Alder chips	Ca	t	5	6557.9	5.4	1.34			
			u	3	6292.7	3	-1.34			
			Overall	8		4.5		1	1.80	0.180
		K	t	5	1379.5	3.6	-1.34			
			u	3	1660	6	1.34			
			Overall	8		4.5		1	1.80	0.180
		Mg	t	5	1027.9	5.2	1.04			
			u	3	875	3.3	-1.04			
			Overall	8		4.5		1	1.09	0.297
		P	t	5	429.1	3.4	-1.64			
			u	3	525	6.3	1.64			
			Overall	8		4.5		1	2.69	0.101
		S	t	5	504.6	4.8	0.45			
			u	3	522.5	4	-0.45			

			Overall	8		4.5		1	0.2	0.655
	Barley straw	Ca	t	5	9524.8	4.6	0.15			
			u	3	7984	4.3	-0.15			
			Overall	8		4.5		1	0.02	0.881
		K	t	5	4113.1	3	-2.24			
			u	3	7848.3	7	2.24			
			Overall	8		4.5		1	5.00	0.025***
		Mg	t	5	3089.4	3.8	-1.04			
			u	3	3657.2	5.7	1.04			
			Overall	8		4.5		1	1.09	0.297
		P	t	5	1608.7	3	-2.24			
			u	3	2580.7	7	2.24			
			Overall	8		4.5		1	5.00	0.025***
		S	t	5	1860.8	3.8	-1.04			
			u	3	2337	5.7	1.04			
			Overall	8		4.5		1	1.09	0.297
	Hemp straw	Ca	t	6	28422	5.5	2			
			u	2	12921.6	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***
		K	t	6	2893.4	4.5	0			
			u	2	2915.85	4.5	0			
			Overall	8		4.5		1	0.00	1.000
		Mg	t	6	6443.6	5.5	2			
			u	2	3489.65	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***
		P	t	6	992.6	4.8	0.67			
			u	2	919.5	3.5	-0.67			
			Overall	8		4.5		1	0.44	0.505
		S	t	6	1656.95	5.5	2			
			u	2	1153.85	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***

Pre- and post-cultivation comparisons

Table D-3. Results of Wilcoxon Signed Rank Confidence Interval test evaluating changes in pre- and post-cultivation substrate macronutrient (Ca, K, Mg, P, S) content (expressed in mg/kg of substrate dry matter). P = pre-cultivation, C = post-cultivation. Note: Minitab statistical software will not perform the Wilcoxon test on sample sizes ≤3.

Treatment	Substrate	Response	N	Median change (C-P)	CI for η, lower limit	CI for η, upper limit	Achieved confidence %	Interpretation
Treated (inoculated)	Alder chips	Ca	5	1585.85	1447	1724.7	94.09%	Increase
		K	5	-101.65	-725.6	522.3	94.09%	No trend
		Mg	5	276.2	126.3	415.9	94.09%	Increase
		P	5	-3.95	-48.9	171.8	94.09%	No trend
		S	5	95.8	50.2	172.8	94.09%	Increase
	Barley straw	Ca	5	4143.2	1420.2	5020.1	94.09%	Increase
		K	5	260.55	-1970.7	1361.3	94.09%	No trend
		Mg	5	1352.95	506.1	3051.6	94.09%	Increase
		P	5	216.9	-341.4	575.5	94.09%	No trend
		S	5	782.4	597.6	967.2	94.09%	Increase
	Hemp straw	Ca	6	15875.5	13582	22510.8	94.08%	Increase
		K	6	1024.2	280	2691.45	94.08%	Increase
		Mg	6	4119.4	3329.4	4563.2	94.08%	Increase
		P	6	242.3	-322.95	582.3	94.08%	No trend
		S	6	829.1	569.3	1048.45	94.08%	Increase
Untreated (ininoculated)	Alder chips	Ca	3	983.8	Insufficient data to perform test			
		K	3	241.2	Insufficient data to perform test			
		Mg	3	137.0	Insufficient data to perform test			
		P	3	34.7	Insufficient data to perform test			
		S	3	94.3	Insufficient data to perform test			
	Barley straw	Ca	3	3506.9	Insufficient data to perform test			
		K	3	3770.8	Insufficient data to perform test			
		Mg	3	1998.0	Insufficient data to perform test			
		P	3	1336.7	Insufficient data to perform test			
		S	3	1239.0	Insufficient data to perform test			

	Hemp straw	Ca	2	3671.7	Insufficient data to perform test
		K	2	1574.3	Insufficient data to perform test
		Mg	2	1319.5	Insufficient data to perform test
		P	2	472.9	Insufficient data to perform test
		S	2	490.4	Insufficient data to perform test

Appendix E: Substrate micronutrient ICP-OES analysis data tables

Substrate comparisons

Table E-1 (a) – (d). Results of Kruskal-Wallace Multiple Comparisons test on the relationship between substrate type and micronutrient (B, Cu, Fe, Mn, Zn) content (expressed in mg/kg of substrate dry matter). Substrates: A = alder chips, B = barley straw, H = hemp straw. P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

(a) Treated (inoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
B	A	5	5.1	5.5	-1.7			
	B	5	5	5.5	-1.7			
	H	6	13.6	13.5	3.25			
	Overall	16		8.5		2	10.60	0.005***
	A vs. H				2.78			0.0055***
	B vs. H				2.78			0.0055***
Cu	A	5	5.4	5.9	-1.47			
	B	5	5.5	8.1	-0.23			
	H	6	6.5	11	1.63			
	Overall	16		8.5		2	3.19	0.203
Fe	A	5	42.7	3	-3.12			
	B	5	161.2	9.8	0.74			
	H	6	251.95	12	2.28			
	Overall	16		8.5		2	10.29	0.006***
	A vs. H				3.12			0.0018***
	A vs. B				2.26			0.0239***
Mn	A	5	67.5	8	-0.28			
	B	5	27.3	3	-3.12			
	H	6	103.8	13.5	3.25			
	Overall	16		8.5		2	13.35	0.001***
	B vs. H				3.64			0.0003***
	A vs. H				1.91			0.0564*
Zn	A	5	23.2	8.8	0.17			

	B	5	26.8	14	3.12			
	H	6	13.5	3.7	-3.15			
	Overall	16		8.5		2	12.88	0.002***
	B vs. H				3.58			0.0003***

(b) Untreated (uninoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
B	A	3	5.1	3.5	-0.89			
	B	3	5.1	3.5	-0.89			
	H	2	12.2	7.5	2			
	Overall	8		4.5		2	4.10	0.129
Cu	A	3	4.5	3	-1.34			
	B	3	6.3	5.7	1.04			
	H	2	5.5	5	0.33			
	Overall	8		4.5		2	1.89	0.389
Fe	A	3	50	2	-2.24			
	B	3	113.2	5.3	0.75			
	H	2	172.9	7	1.67			
	Overall	8		4.5		2	5.56	0.062*
	A vs. H				2.24			0.0253***
Mn	A	3	62.5	5.3	0.75			
	B	3	20	2	-2.24			
	H	2	82.15	7	1.67			
	Overall	8		4.5		2	5.56	0.062*
	B vs. H				2.24			0.0253***
Zn	A	3	24.2	4.3	-0.15			
	B	3	25.6	6.7	1.94			
	H	2	11.9	1.5	-2			
	Overall	8		4.5		2	5.43	0.066*
	B vs. H				2.32			0.0201***

(c) Treated (inoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
B	A	5	6.6	3.4	-2.89			
	B	5	8.7	7.6	-0.51			
	H	6	34.55	13.5	3.25			
	Overall	16		8.5		2	12.53	0.002***
	A vs. H				3.50			0.0005***
	B vs. H				2.05			0.0407***
Cu	A	5	4.9	3	-3.12			
	B	5	8.4	8	-0.28			
	H	6	13.05	13.5	3.25			
	Overall	16		8.5		2	13.38	0.001***
	A vs. H				3.65			0.0003***
	B vs. H				1.91			0.0561*
Fe	A	5	88.3	3	-3.12			
	B	5	395.2	9.2	0.4			
	H	6	981.5	12.5	2.6			
	Overall	16		8.5		2	11.02	0.004***
	A vs H				3.30			0.001***
	A vs. B				2.06			0.0395***
Mn	A	5	91.7	7	-0.85			
	B	5	54.1	4	-2.55			
	H	6	326.75	13.5	3.25			
	Overall	16		8.5		2	11.58	0.003***
	B vs. H				3.30			0.001***
	A vs. H				2.25			0.0242***
Zn	A	5	31.6	4.8	-2.1			
	B	5	44.9	13.6	2.89			
	H	6	34.1	7.3	-0.76			
	Overall	16		8.5		2	9.12	0.01***
	A vs. B				2.92			0.0035***
	B vs. H				2.17			0.0297***

(d) Untreated (uninoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
B	A	3	5.4	2	-2.24			
	B	3	8.6	5	0.45			
	H	2	18.9	7.5	2			
	Overall	8		4.5		2	6.25	0.044***
	A vs. H				2.46			0.0139***
Cu	A	3	4.8	2	-2.24			
	B	3	9.5	6	1.34			
	H	2	9.75	6	1			
	Overall	8		4.5		2	5.00	0.082
Fe	A	3	139.6	2	-2.24			
	B	3	513.6	5	0.45			
	H	2	1352	7.5	2			
	Overall	8		4.5		2	6.25	0.044***
	A vs. H				2.46			0.0139***
Mn	A	3	77.5	5	0.45			
	B	3	53.1	2	-2.24			
	H	2	173.55	7.5	2			
	Overall	8		4.5		2	6.25	0.044***
	B vs. H				2.46			0.0139***
Zn	A	3	29	4	-0.45			
	B	3	48.6	7	2.24			
	H	2	24.45	1.5	-2			
	Overall	8		4.5		2	6.25	0.044***
	B vs. H				2.46			0.0139***

Treatment comparisons

Table E-2: Results of Kruskal-Wallace test on the relationship between treatment (inoculation) and substrate micronutrient (B, Cu, Fe, Mn, Zn) content (expressed in mg/kg of substrate dry matter). Treatment: t = treated (inoculated), u = untreated (uninoculated). P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

Cultivation stage	Substrate	Response	Treatment	N	Median	Mean Rank	Z-Value	DF	H-value	P-value

Pre cultivation	Alder chips	B	t	5	5.1	4.1	-0.6			
			u	3	5.1	5.2	0.6			
			Overall	8		4.5		1	0.36	0.549
		Cu	t	5	5.4	5.4	1.34			
			u	3	4.5	3	-1.34			
			Overall	8		4.5		1	1.80	0.180
		Fe	t	5	42.7	3.8	-1.04			
			u	3	50	5.7	1.04			
			Overall	8		4.5		1	1.09	0.297
		Mn	t	5	67.5	4.6	0.15			
			u	3	62.5	4.3	-0.15			
			Overall	8		4.5		1	0.02	0.881
		Zn	t	5	23.2	3.1	-2.09			
			u	3	24.2	6.8	2.09			
			Overall	8		4.5		1	4.41	0.036***
	Barley straw	B	t	5	5	4.2	-0.45			
			u	3	5.1	5	0.45			
			Overall	8		4.5		1	0.20	0.655
		Cu	t	5	5.5	4.6	0.15			
			u	3	6.3	4.3	-0.15			
			Overall	8		4.5		1	0.02	0.881
		Fe	t	5	161.2	5.6	1.64			
			u	3	113.2	2.7	-1.64			
			Overall	8		4.5		1	2.69	0.101
		Mn	t	5	27.3	5.1	0.89			
			u	3	20	3.5	-0.89			
			Overall	8		4.5		1	0.81	0.368
		Zn	t	5	26.8	4.8	0.45			
			u	3	25.6	4	-0.45			
			Overall	8		4.5		1	0.20	0.655
	Hemp straw	B	t	6	13.6	4.8	0.67			
			u	2	12.2	3.5	-0.67			
			Overall	8		4.5		1	0.45	0.502

		Cu	t	6	6.5	4.9	0.83			
			u	2	5.5	3.3	-0.83			
			Overall	8		4.5		1	0.71	0.399
		Fe	t	6	251.95	5.3	1.67			
			u	2	172.9	2	-1.67			
			Overall	8		4.5		1	2.78	0.096
		Mn	t	6	103.8	5.2	1.33			
			u	2	82.15	2.5	-1.33			
			Overall	8		4.5		1	1.78	0.182
		Zn	t	6	13.5	5.5	2			
			u	2	11.9	1.5	-2			
			Overall	8		4.5		1	4.05	0.044***
Post-cultivation	Alder chips	B	t	5	6.6	5.1	0.89			
			u	3	5.4	3.5	-0.89			
			Overall	8		4.5		1	0.81	0.368
		Cu	t	5	4.9	4.2	-0.45			
			u	3	4.8	5	0.45			
			Overall	8		4.5		1	0.20	0.653
		Fe	t	5	88.3	3.6	-1.34			
			u	3	139.6	6	1.34			
			Overall	8		4.5		1	1.80	0.180
		Mn	t	5	91.7	5.8	1.94			
			u	3	77.5	2.3	-1.94			
			Overall	8		4.5		1	3.76	0.053*
		Zn	t	5	31.6	5.8	1.94			
			u	3	29	2.3	-1.94			
			Overall	8		4.5		1	3.76	0.053*
	Barley straw	B	t	5	8.7	4.8	0.45			
			u	3	8.6	4	-0.45			
			Overall	8		4.5		1	0.20	0.655
		Cu	t	5	8.4	3.1	-2.09			
			u	3	9.5	6.8	2.09			
			Overall	8		4.5		1	4.46	0.035***

		Fe	t	5	395.2	4.4	-0.15			
			u	3	513.6	4.7	0.15			
			Overall	8		4.5		1	0.02	0.881
		Mn	t	5	54.1	4.8	0.45			
			u	3	53.1	4	-0.45			
			Overall	8		4.5		1	0.20	0.655
		Zn	t	5	44.9	4.2	-0.45			
			u	3	48.6	5	0.45			
			Overall	8		4.5		1	0.20	0.655
	Hemp straw	B	t	6	34.55	5.5	2			
			u	2	18.9	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***
		Cu	t	6	13.05	5.5	2			
			u	2	9.75	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***
		Fe	t	6	981.5	4.5	0			
			u	2	1352	4.5	0			
			Overall	8		4.5		1	0.00	1.000
		Mn	t	6	326.75	5.5	2			
			u	2	173.55	1.5	-2			
			Overall	8		4.5		1	4.00	0.046***
		Zn	t	6	34.1	5.2	1.33			
			u	2	24.45	2.5	-1.33			
			Overall	8		4.5		1	1.78	0.182

Pre- and post-cultivation comparisons

Table E-3. Results of Wilcoxon Signed Rank Confidence Interval test evaluating changes in pre- and post-cultivation substrate micronutrient (B, Cu, Fe, Mn, Zn) content (expressed in mg/kg of substrate dry matter). P = pre-cultivation, C = post-cultivation. Note: Minitab statistical software will not perform the Wilcoxon test on sample sizes ≤3.

Treatment	Substrate	Response	N	Median change (C-P)	CI for η, lower limit	CI for η, upper limit	Achieved confidence %	Interpretation

Treated (inoculated)	Alder chips	B	5	2.2	-0.4	5.4	94.09%	Probable increase
		Cu	5	-0.45	-2	1.4	94.09%	No trend
		Fe	5	56.3	20.8	220.4	94.09%	Increase
		Mn	5	26.9	12.2	46.1	94.09%	Increase
		Zn	5	8.8	5.6	12	94.09%	Increase
	Barley straw	B	5	3.65	2.6	4.7	94.09%	Increase
		Cu	5	2.3	0.2	4.5	94.09%	Increase
		Fe	5	240.4	31.5	486.4	94.09%	Increase
		Mn	5	27.9	25.2	60.6	94.09%	Increase
		Zn	5	18.6	9.1	20.9	94.09%	Increase
	Hemp straw	B	6	21.15	17.85	27.4	94.08%	Increase
		Cu	6	5.95	5.1	7.35	94.08%	Increase
		Fe	6	809	266.35	1868.02	94.08%	Increase
		Mn	6	219.65	141.95	298.7	94.08%	Increase
		Zn	6	17.15	10.85	22.7	94.08%	Increase
Untreated (uninoculated)	Alder chips	B	3	0.2	Insufficient data to perform test			
		Cu	3	0.2	Insufficient data to perform test			
		Fe	3	89.6	Insufficient data to perform test			
		Mn	3	15.4	Insufficient data to perform test			
		Zn	3	4.8	Insufficient data to perform test			
	Barley straw	B	3	2.4	Insufficient data to perform test			
		Cu	3	3.2	Insufficient data to perform test			
		Fe	3	382.3	Insufficient data to perform test			
		Mn	3	33.0	Insufficient data to perform test			
		Zn	3	23.7	Insufficient data to perform test			
	Hemp straw	B	2	6.7	Insufficient data to perform test			
		Cu	2	4.25	Insufficient data to perform test			
		Fe	2	1179.15	Insufficient data to perform test			
		Mn	2	91.4	Insufficient data to perform test			
		Zn	2	12.6	Insufficient data to perform test			

Appendix F: Substrate Al and Na ICP-OES analysis data tables

Substrate comparisons

Table F-1 (a) – (d). Results of Kruskal-Wallace Multiple Comparisons test on the relationship between substrate type and Al & Na content (expressed in mg/kg of substrate dry matter). Substrates: A = alder chips, B = barley straw, H = hemp straw. P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

(a) Treated (inoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Al	A	5	17.5	3	-3.12			
	B	5	106.3	8.2	-0.17			
	H	6	202.95	13.3	3.15			
	Overall	16		8.5		2	12.88	0.002***
	A vs. H				3.58			0.0003***
Na	A	5	76.7	3.4	-2.89			
	B	5	588.1	14	3.12			
	H	6	141.8	8.2	-0.22			
	Overall	16		8.5		2	12.44	0.002***
	A vs. B				3.52			0.0004***
	B vs. H				2.02			0.0430***

(b) Untreated (uninoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Al	A	3	18.8	2	-2.24			
	B	3	67.7	5	0.45			
	H	2	133.65	7.5	2			
	Overall	8		4.5		2	6.25	0.044***
	A vs. H				2.46			0.0139
Na	A	3	64.5	2	-2.24			
	B	3	567.3	7	2.24			
	H	2	141.1	4.5	0			
	Overall	8		4.5		2	6.25	0.044***

	A vs. B				2.5			0.0124***

(c) Treated (inoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Al	A	5	48.7	3.2	-3			
	B	5	266.7	9	0.28			
	H	6	659.25	12.5	2.6			
	Overall	16		8.5		2	10.49	0.005***
	A vs. H				3.22			0.0013***
	A vs. B				1.93			0.0541*
Na	A	5	81.8	3	-3.12			
	B	5	776	14	3.12			
	H	6	214.55	8.5	0			
	Overall	16		8.5		2	13.35	0.001***
	A vs. B				3.65			0.0003***
	A vs. H				1.91			0.0564*
	B vs. H				1.91			0.0564*

(d) Untreated (uninoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Al	A	3	83.8	2	-2.24			
	B	3	338.1	5	0.45			
	H	2	828.2	7.5	2			
	Overall	8		4.5		2	6.25	0.044***
	A vs. H				2.46			0.0139***
Na	A	3	115.8	2	-2.24			
	B	3	1112.5	7	2.24			
	H	2	217.1	4.5	0			
	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***

Treatment comparisons

Table F-2: Results of Kruskal-Wallace test on the relationship between treatment (inoculation) and substrate Al & Na content (expressed in mg/kg of substrate dry matter). Treatment: t = treated (inoculated), u = untreated (uninoculated). P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

Cultivation stage	Substrate	Response	Treatment	N	Median	Mean Rank	Z-Value	DF	H-value	P-value
Pre-cultivation	Alder chips	Al	t	5	17.5	4.4	-0.15			
			u	3	18.8	4.7	0.15			
			Overall	8		4.5		1	0.02	0.881
		Na	t	5	76.7	4.6	0.15			
			u	3	64.5	4.3	-0.15			
			Overall	8		4.5		1	0.02	0.881
	Barley straw	Al	t	5	106.3	5.6	1.64			
			u	3	67.7	2.7	-1.64			
			Overall	8		4.5		1	2.69	0.101
		Na	t	5	588.1	4.4	-0.15			
			u	3	567.3	4.7	0.15			
			Overall	8		4.5		1	0.02	0.881
	Hemp straw	Al	t	6	202.95	5.3	1.67			
			u	2	133.65	2	-1.67			
			Overall	8		4.5		1	2.78	0.096
		Na	t	6	141.8	4.5	0			
			u	2	141.1	4.5	0			
			Overall	8		4.5		1	0.00	1.000
Post-cultivation	Alder chips	Al	t	5	48.7	3.6	-1.34			
			u	3	83.8	6	1.34			
			Overall	8		4.5		1	1.80	0.180
		Na	t	5	81.8	3.8	-1.04			
			u	3	115.8	5.7	1.04			
			Overall	8		4.5		1	1.09	0.297
	Barley straw	Al	t	5	266.7	4.4	-0.15			
			u	3	338.1	4.7	0.15			
			Overall	8		4.5		1	0.02	0.881
		Na	t	5	776	4.6	0.15			

			u	3	1112.5	4.3	-0.15			
			Overall	8		4.5		1	0.02	0.881
	Hemp straw	Al	t	6	659.25	4.5	0			
			u	2	828.2	4.5	0			
			Overall	8		4.5		1	0.00	1.000
		Na	t	6	214.55	4.7	0.33			
			u	2	217.1	4	-0.33			
			Overall	8		4.5		1	0.11	0.739

Pre- and post-cultivation comparisons

Table F-3. Results of Wilcoxon Signed Rank Confidence Interval test evaluating changes in pre- and post-cultivation substrate Al & Na content (expressed in mg/kg of substrate dry matter). P = pre-cultivation, C = post-cultivation. Note: Minitab statistical software will not perform the Wilcoxon test on sample sizes ≤3.

Treatment	Substrate	Response	N	Median change (C-P)	CI for η, lower limit	CI for η, upper limit	Achieved confidence %	Interpretation
Treated	Alder chips	Al	5	35.4	12.5	162.5	94.09	Increase
		Na	5	2.8	-37.3	42.9	94.09	No trend
	Barley straw	Al	5	247	61.8	432.2	94.09	Increase
		Na	5	412.25	151.4	1281.5	94.09	Increase
	Hemp straw	Al	6	513.7	198.25	1136.9	94.08	Increase
		Na	6	73.95	38.4	112.55	94.08	Increase
Untreated	Alder chips	Al	3	64.8	Insufficient data to perform test			
		Na	3	51.3	Insufficient data to perform test			
	Barley straw	Al	3	270.3	Insufficient data to perform test			
		Na	3	586	Insufficient data to perform test			
	Hemp straw	Al	2	694.6	Insufficient data to perform test			
		Na	2	76	Insufficient data to perform test			

Appendix G: Van Soest fiber analysis data tables

Substrate comparisons

Table G-1 (a) – (d). Results of Kruskal-Wallace Multiple Comparisons test on the relationship between substrate type and lignin, cellulose, hemicellulose, and total lignocellulosic biomass content of substrate samples, expressed as a percentage of oven dry sample mass. Substrates: A = alder chips, B = barley straw, H = hemp straw. P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

(a) Treated (inoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Lignin (% of O.D. sample mass)	A	5	17.65	14	3.12			
	B	5	3.95	3	-3.12			
	H	6	12.185	8.5	0			
	Overall	16		8.5		2	13.35	0.001***
	A vs. B				3.65			0.0003***
	B vs. H				1.91			0.0564*
	A vs. H				1.91			0.0564*
Cellulose (% of O.D. sample mass)	A	5	46.53	8.8	0.17			
	B	5	41.96	3	-3.12			
	H	6	54.71	12.8	2.82			
	Overall	16		8.5		2	11.66	0.003***
	B vs. H				3.42			0.0006***
	A vs. B				1.93			0.0541*
Hemicellulose (% of O.D. sample mass)	A	5	19.3	7.6	-0.51			
	B	5	37.57	14	3.12			
	H	6	17.855	4.7	-2.49			
	Overall	16		8.5		2	10.74	0.005***
	B vs. H				3.24			0.0012***
	A vs. B				2.13			0.0335***
Total lignocellulosic biomass (% of O.D. sample mass)	A	5	83.44	8.2	-0.17			
	B	5	82.41	7.2	-0.74			
	H	6	84.71	9.8	0.87			
	Overall	16		8.5		2	0.86	0.649

(b) Untreated (uninoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Lignin (% of O.D. sample mass)	A	3	17.1	7	2.24			
	B	3	4.28	2	-2.24			
	H	2	13.25	4.5	0			
	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***
Cellulose (% of O.D. sample mass)	A	3	44.69	4.7	0.15			
	B	3	41.52	2.3	-1.94			
	H	2	52.75	7.5	2			
	Overall	8		4.5		2	5.36	0.069*
	B vs. H				2.31			0.0209***
Hemicellulose (% of O.D. sample mass)	A	3	18.94	2.7	-1.64			
	B	3	38.25	7	2.24			
	H	2	19.595	3.5	-0.67			
	Overall	8		4.5		2	5.14	0.077
	A vs. B				2.17			0.0303***
Total lignocellulosic biomass (% of O.D. sample mass)	A	3	80.97	3	-1.34			
	B	3	86.26	4.7	0.15			
	H	2	85.595	6.5	1.33			
	Overall	8		4.5		2	2.47	0.291

(c) Treated (inoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Lignin (% of O.D. sample mass)	A	5	17.4	14	3.12			
	B	5	5.65	7.8	-0.4			
	H	6	4.885	4.5	-2.6			
	Overall	16		8.5		2	11.02	0.004***
	A vs. H				3.3			0.001***
	A vs. B				2.06			0.0395***

Cellulose (% of O.D. sample mass)	A	5	39.8	13.4	2.78			
	B	5	26.38	3	-3.12			
	H	6	31.68	9	0.33			
	Overall	16		8.5		2	12.04	0.002***
	A vs. B				3.45			0.0006***
	B vs. H				2.08			0.0374***
Hemicellulose (% of O.D. sample mass)	A	5	14.74	9.4	0.51			
	B	5	22.81	13.6	2.89			
	H	6	6.985	3.5	-3.25			
	Overall	16		8.5		2	12.53	0.002***
	B vs. H				3.5			0.0005***
	A vs. H				2.05			0.0407***
Total lignocellulosic biomass (% of O.D. sample mass)	A	5	71.75	14	3.12			
	B	5	55.73	8.4	-0.06			
	H	6	43.555	4	-2.93			
	Overall	16		8.5		2	12.04	0.002***
	A vs. H							0.0005***
	A vs. B							0.0629*

(d) Untreated (uninoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
Lignin (% of O.D. sample mass)	A	3	21.18	6	1.34			
	B	3	11.85	2	-2.24			
	H	2	22.1	6	1			
	Overall	8		4.5		2	5	0.082
	A vs. B				2			0.0455***
Cellulose (% of O.D. sample mass)	A	3	46.02	6	1.34			
	B	3	26.91	2	-2.24			
	H	2	43.815	6	1			
	Overall	8		4.5		2	5	0.082
	A vs. B				2			0.0455***
	A	3	17.11	2	-2.24			

Hemicellulose (% of O.D. sample mass)	B	3	32.06	7	2.24			
	H	2	18.895	4.5	0			
	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***
Total lignocellulosic biomass (% of O.D. sample mass)	A	3	83.53	5.7	1.04			
	B	3	69.57	2	-2.24			
	H	2	84.81	6.5	1.33			
	Overall	8		4.5		2	5.14	0.077
	B vs. H				2.01			0.0442***

Treatment comparisons

Table G-2: Results of Kruskal-Wallace test on the relationship between treatment (inoculation) and lignin, cellulose, hemicellulose, and total lignocellulosic biomass content of substrate samples, expressed as a percentage of oven dry sample mass. Treatment: t = treated (inoculated), u = untreated (uninoculated). P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

Substrate	Cultivation stage	Response variable	Treatment	N	Median	Mean Rank	Z-value	DF	H-value	P-value
Alder chips	Pre-cultivation	Lignin (% of O.D. sample mass)	t	5	17.65	4.6	0.15			
			u	3	17.1	4.3	-0.15			
			overall	8		4.5		1	0.02	0.881
		Cellulose (% of O.D. sample mass)	t	5	46.53	5.4	1.34			
			u	3	44.69	3	-1.34			
			overall	8		4.5		1	1.8	0.18
		Hemicellulose (% of O.D. sample mass)	t	5	19.3	4.6	0.15			
			u	3	18.94	4.3	-0.15			
			overall	8		4.5		1	0.02	0.881
		Total lignocellulosic biomass (% of O.D. sample mass)	t	5	83.44	5.4	1.34			
			u	3	80.97	3	-1.34			
			overall	8		4.5		1	1.8	0.18
Barley straw	Pre-cultivation	Lignin (% of O.D. sample mass)	t	5	3.95	4.2	-0.45			
			u	3	4.28	5	0.45			
			overall	8		4.5		1	0.2	0.655

		Cellulose (% of O.D. sample mass)	t	5	41.96	4.4	-0.15			
			u	3	41.52	4.7	0.15			
			overall	8		4.5		1	0.02	0.881
		Hemicellulose (% of O.D. sample mass)	t	5	37.57	3.6	-1.34			
			u	3	38.25	6	1.34			
			overall	8		4.5		1	1.8	0.18
		Total lignocellulosic biomass (% of O.D. sample mass)	t	5	82.41	3.8	-1.04			
			u	3	86.26	5.7	1.04			
			overall	8		4.5		1	1.09	0.297
Hemp straw	Pre-cultivation	Lignin (% of O.D. sample mass)	t	6	12.185	3.8	-1.33			
			u	2	13.25	6.5	1.33			
			overall	8		4.5		1	1.78	0.182
		Cellulose (% of O.D. sample mass)	t	6	54.71	4.8	0.67			
			u	2	52.75	3.5	-0.67			
			overall	8		4.5		1	0.44	0.505
		Hemicellulose (% of O.D. sample mass)	t	6	17.855	4	-1			
			u	2	19.595	6	1			
			overall	8		4.5		1	1	0.317
		Total lignocellulosic biomass (% of O.D. sample mass)	t	6	84.71	4	-1			
			u	2	85.595	6	1			
			overall	8		4.5		1	1	0.317
Alder chips	Post-cultivation	Lignin (% of O.D. sample mass)	t	5	17.4	3.2	-1.94			
			u	3	21.18	6.7	1.94			
			overall	8		4.5		1	3.76	0.053*
		Cellulose (% of O.D. sample mass)	t	5	39.8	3.2	-1.94			
			u	3	46.02	6.7	1.94			
			overall	8		4.5		1	3.76	0.053*
		Hemicellulose (% of O.D. sample mass)	t	5	14.74	3.4	-1.64			
			u	3	17.11	6.3	1.64			
			overall	8		4.5		1	2.69	0.101
		Total lignocellulosic	t	5	71.75	3	-2.24			
			u	3	83.53	7	2.24			

		biomass (% of O.D. sample mass)	overall	8		4.5		1	5	0.025***
Barley straw	Post-cultivation	Lignin (% of O.D. sample mass)	t	5	5.65	3	-2.24			
			u	3	11.85	7	2.24			
			overall	8		4.5		1	5	0.025***
		Cellulose (% of O.D. sample mass)	t	5	26.38	4.2	-0.45			
			u	3	26.91	5	0.45			
			overall	8		4.5		1	0.2	0.655
		Hemicellulose (% of O.D. sample mass)	t	5	22.81	3	-2.24			
			u	3	32.06	7	2.24			
			overall	8		4.5		1	5	0.025***
		Total lignocellulosic biomass (% of O.D. sample mass)	t	5	55.73	3	-2.24			
			u	3	69.57	7	2.24			
			overall	8		4.5		1		0.025***
Hemp straw	Post-cultivation	Lignin (% of O.D. sample mass)	t	6	4.885	3.5	-2			
			u	2	22.1	7.5	2			
			overall	8		4.5		1	4	0.046***
		Cellulose (% of O.D. sample mass)	t	6	31.68	3.7	-1.67			
			u	2	43.815	7	1.67			
			overall	8		4.5		1	2.78	0.096
		Hemicellulose (% of O.D. sample mass)	t	6	6.985	3.5	-2			
			u	2	18.895	7.5	2			
			overall	8		4.5		1	4	0.046***
		Total lignocellulosic biomass (% of O.D. sample mass)	t	6	43.555	3.5	-2			
			u	2	84.81	7.5	2			
			overall	8		4.5		1	4	0.046***

Pre- and post-cultivation comparisons

Table G-3. Results of Wilcoxon Signed Rank Confidence Interval test evaluating changes in pre- and post-cultivation substrate sample content of lignin, cellulose, hemicellulose, and total lignocellulosic biomass, expressed as a percentage of oven dry sample mass. P = pre-cultivation, C = post-cultivation. Note: Minitab statistical software will not perform the Wilcoxon test on sample sizes ≤3.

Treatment	Substrate	Response variable	N	Median change (C – P)	CI for η, lower limit	CI for η, upper limit	Achieved confidence %
Treated (inoculated)	Alder chips	Lignin	5	0.22	-1.48	2.22	94.09
		Cellulose	5	-6.02	-7.97	-2.88	94.09
		Hemicellulose	5	-4.14	-7.25	-2.52	94.09
		Total lignocellulosic	5	-9.26	-15.25	-3.18	94.09
	Barley straw	Lignin	5	2.41	1.36	4.01	94.09
		Cellulose	5	-15.26	-18.22	-12.98	94.09
		Hemicellulose	5	-14.135	-22.32	-11.86	94.09
		Total lignocellulosic	5	-27.255	-36.53	-23.2	94.09
	Hemp straw	Lignin	6	-7.28	-9.085	-5.775	94.08
		Cellulose	6	-19.795	-25.565	-13.615	94.08
		Hemicellulose	6	-10.72	-12.2	-9.13	94.08
		Total lignocellulosic	6	-38.085	-44.805	-31.11	94.08
Untreated (uninoculated)	Alder chips	Lignin	3	4.47	Insufficient data to perform test		
		Cellulose	3	2.05	Insufficient data to perform test		
		Hemicellulose	3	-1.83	Insufficient data to perform test		
		Total lignocellulosic	3	2.63	Insufficient data to perform test		
	Barley straw	Lignin	3	8.27	Insufficient data to perform test		
		Cellulose	3	-14.61	Insufficient data to perform test		
		Hemicellulose	3	-7.87	Insufficient data to perform test		
		Total lignocellulosic	3	-16.69	Insufficient data to perform test		
	Hemp straw	Lignin	2	8.85	Insufficient data to perform test		
		Cellulose	2	-8.935	Insufficient data to perform test		
		Hemicellulose	2	-0.7	Insufficient data to perform test		
		Total lignocellulosic	2	-0.785	Insufficient data to perform test		

Appendix H: pH and EC data tables

Substrate comparisons

Table H-1 (a) – (d). Results of Kruskal-Wallace Multiple Comparisons test on the relationship between substrate type and pH and electrical conductivity (EC) of substrate samples. Substrates: B = barley straw, A = alder chips, H = hemp straw. P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

(a) Treated (inoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean Rank	Z-Value	DF	H-value	P-value
pH	A	5	5.2	3	-3.12			
	B	5	7.1	10.3	1.02			
	H	6	6.95	11.6	2.01			
	Overall	16		8.5		2	9.99	0.007***
	A vs. H				2.99			0.00028***
	A vs. B				2.44			0.0149***
EC (mS/cm)	A	5	0.34	3	-3.12			
	B	5	1.06	11.9	1.93			
	H	6	0.825	10.3	1.14			
	Overall	16		8.5		2	10.05	0.007***
	A vs. B				2.96			0.0031***
	A vs. H				2.52			0.0118***

(b) Untreated (uninoculated) pre-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean Rank	Z-Value	DF	H-value	P-value
pH	A	3	5.3	2	-2.24			
	B	3	7.3	6	1.34			
	H	2	7.2	6	1			
	Overall	8		4.5		2	5.06	0.08
	A vs. B				2.01			0.0442***
EC (mS/cm)	A	3	0.44	2	-2.24			
	B	3	1.44	7	2.24			
	H	2	0.73	4.5	0			

	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124

(c) Treated (inoculated) post-cultivation substrate samples.

Response variable	Substrate	N	Median	Mean rank	Z-value	DF	H-value	P-value
pH	A	5	3.5	5.2	-1.87			
	B	5	4	8.6	0.06			
	H	6	4.2	11.2	1.74			
	Overall	16		8.5		2	4.44	0.109
	A vs. H				2.12			0.0352***
EC (mS/cm)	A	5	0.69	3	-3.12			
	B	5	2.02	9	0.28			
	H	6	2.515	12.7	2.71			
	Overall	16		8.5		2	11.32	0.003***
	A vs. H				3.35			0.0008***
	A vs. B				1.99			0.0463***

(d) Untreated (uninoculated) post-cultivation substrate sample.

Response variable	Substrate	N	Median	Mean Rank	Z-Value	DF	H-value	P-value
pH	A	3	5.7	2	-2.24			
	B	3	7	5	0.45			
	H	2	7.35	7.5	2			
	Overall	8		4.5		2	6.33	0.042***
	A vs. H				2.47			0.0133***
EC (mS/cm)	A	3	0.37	2	-2.24			
	B	3	1.33	7	2.24			
	H	2	0.585	4.5	0			
	Overall	8		4.5		2	6.25	0.044***
	A vs. B				2.5			0.0124***

Treatment comparisons

Table H-2: Results of Kruskal-Wallace test on the relationship between treatment (inoculation) and pH and EC of substrate samples. Treatment: t = treated (inoculated), u = untreated (uninoculated). P-values < 0.05 are indicated by *** and P-values 0.05 < p < 0.067 are indicated by *.

Cultivation stage	Substrate	Response	Treatment	N	Median	Mean Rank	Z-Value	DF	H-value	P-value
Pre-cultivation	Alder chips	pH	t	5	5.2	4.5	0			
			u	3	5.3	4.5	0			
			Overall	8		4.5		1	0.00	1.000
		EC (mS/cm)	t	5	0.34	3	-2.24			
			u	3	0.44	7	2.24			
			Overall	8		4.5		1	5	0.025***
	Barley straw	pH	t	5	7.1	3.6	-1.34			
			u	3	7.3	6	1.34			
			Overall	8		4.5		1	1.84	0.174
		EC (mS/cm)	t	5	1.06	4.2	-0.45			
			u	3	1.44	5	0.45			
			Overall	8		4.5		1	0.2	0.655
	Hemp straw	pH	t	6	6.95	4.1	-0.83			
			u	2	7.2	5.8	0.83			
			Overall	8		4.5		1	0.74	0.39
		EC (mS/cm)	t	6	0.825	5.2	1.33			
			u	2	0.73	2.5	-1.33			
			Overall	8		4.5		1	1.78	0.182
Post-cultivation	Alder chips	pH	t	5	3.5	3	-2.24			
			u	3	5.7	7	2.24			
			Overall	8		4.5		1	5.06	0.024***
		EC	t	5	0.69	5.9	2.09			
			u	3	0.37	2.2	-2.09			
			Overall	8		4.5		1	4.41	0.036***
	Barley straw	pH	t	5	4.0	3	-2.24			
			u	3	7.0	7	2.24			
			Overall	8		4.5		1	5.75	0.016***
		EC	t	5	2.02	6	2.24			

			u	3	1.33	2	-2.24			
			Overall	8		4.5		1	5	0.025***
	Hemp straw	pH	t	6	4.2	3.5	-2			
			u	2	7.35	7.5	2			
			Overall	8		4.5		1	4.1	0.043***
		EC	t	6	2.515	5.5	2			
			u	2	0.585	1.5	-2			
			Overall	8		4.5		1	4	0.046***

Pre- and post-cultivation comparisons

Table H-3. Results of Wilcoxon Signed Rank Confidence Interval test evaluating changes in pre- and post-cultivation substrate pH and EC. P = pre-cultivation, C = post-cultivation. Note: Minitab statistical software will not perform the Wilcoxon test on sample sizes ≤3.

Substrate	Treatment	Response	N	Median change (C - P)	CI for η, lower limit	CI for η, upper limit	Achieved confidence %	Interpretation
Alder chips	Treated (inoculated)	pH	5	-1.8	-2.9	-1.1	94.09	decrease
		EC (mS/cm)	5	0.27	-0.53	0.81	94.09	no change
Barley straw		pH	5	-2.4	-3.5	-1.7	94.09	decrease
		EC (mS/cm)	5	1.335	0.45	2.28	94.09	increase
Hemp straw		pH	6	-2.95	-3.25	-2.55	94.08	decrease
		EC (mS/cm)	6	1.46	1.24	1.95	94.08	increase
Alder chips	Untreated (uninoculated)	pH	3	0.9	Insufficient data to perform test			
		EC (mS/cm)	3	-0.07	Insufficient data to perform test			
Barley straw		pH	3	-0.2	Insufficient data to perform test			
		EC (mS/cm)	3	-0.03	Insufficient data to perform test			
Hemp straw		pH	2	0.15	Insufficient data to perform test			
		EC (mS/cm)	2	-0.145	Insufficient data to perform test			