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Department of Arctic and Marine Biology

Growth responses of seedlings along encroachment gradients of the allelopathic plant *Empetrum nigrum*

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Abstract

Empetrum nigrum is a native evergreen shrub distributed in alpine and circumpolar areas that is currently encroaching into new habitats. This is an allelopathic plant that releases secondary metabolites, which has a negative impact on seed germination and seedling growth. The species has been recorded to expand in altitude and latitude parallel to a warmer climate and is encroaching into new habitats. This study aims to test if recent encroachment by *E. nigrum* is causing environmental change through allelopathy using a space for time experiment. Soil samples were collected along *E. nigrum* encroachment gradients from 15 sites in northern Norway, with each gradient being sampled in a meadow at four locations; where the *E. nigrum* had been present for ~15 years, at the clone edge, and at one and two meters into the meadow. The allelopathy was assessed using a bioassay-approach. Seedlings of three local plants, a grass, a forb and a legume species were grown in the soil for 5 weeks under stable conditions in a phytotron. After five weeks, length and dry biomass of the species were measured and nodules on the roots of the legume were counted. The results showed that the three species had different growth responses in the *E. nigrum* encroachment gradient. The length and dry biomass growth of *Phleum alpinum* were negatively impacted where *E. nigrum* had been present for ~15 years, compared to the meadow near. *Solidago virgaurea* and *Trifolium pratense* had no growth trends related to the encroachment gradient. However, the length and dry biomass growth of these species was significantly correlated to the nitrogen content in the soils. The growth of *T. pratense* would also increase when nodules were present, and by the number of nodules present at each individual plant. These results indicate that *E. nigrum* needs to be present for more than ~15 years to influence the soil growth conditions for herbs and legumes. Grasses on the other hand are more sensitive to toxic compounds released by *E. nigrum* early in its encroachment. Other mechanisms of *E. nigrum* are therefore important for the species when it is successfully encroaching into new habitats. Management of the species as a tool to avoid biodiversity loss, would therefore be most efficient in the early phase of encroachment before the soil is strongly influenced by *E. nigrum´s* allelopathy.

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1 Introduction

The arctic tundra of northern Norway is located in an area where climate changes occur faster than in many other places (IPCC, 2019). Abiotic factors such as nutrient availability, temperature, and precipitation are changing, which influences plant performance and distribution in the area (Chaudhry & Sidhu, 2021; Rosa et al., 2019). Methods to measure such factors are well developed, which makes the changes easy to track (Lambers et al., 2008). Meanwhile, interactions between living organisms and how these regulate ecosystem composition are more challenging to understand (Lambers et al., 2008). Knowledge of how plant-plant interactions are influencing species distribution patterns is therefore important for further understanding how the arctic tundra might be influenced by climate change.

Empetrum nigrum is an allelopathic plant that has been found to increase recently along with a warmer climate (Tuomi et al., 2022). The encroachment of this native species leads to a change from important forage species to a dominance of *E. nigrum*, threatening the biodiversity of the area (Bråthen et al., 2024; Tuomi et al., 2022). The encroachment can therefore have a negative cascade effect on the ecosystem.

1.1 Vegetation- and climate change

Climate change is an ongoing threat to the world's species diversity (IPCC, 2023), as a rise in average global temperature and more frequent extreme weather events are expected (IPCC, 2023). At higher latitudes these changes are expected to occur faster than the global average (IPCC, 2019). The species living in these areas are specialized to a life in an extreme environment, with low temperatures, limited nutrients and a lack of daylight in the winter. The species are therefore very vulnerable to changes in their surrounding environment (Blix, 2016; Descamps et al., 2016). Plants have a certain temperature range where they are able to grow and reproduce. This means that their distribution is likely to change as the temperature increases (Chen et al., 2011; Klanderud & Birks, 2003; Lenoir et al., 2008). A change from cold-adapted vegetation to species adapted to warmer climate might change the tundra flora into a more species rich community, as the temperature increases at higher altitudes and latitudes (Elmendorf et al., 2015; Gottfried et al., 2012; Kullman, 2010). Another study indicates a different outcome, anticipating that more than half of the tundra areas will change into shrublands by the end of the 21st century, if the temperature increases with 2-10°C by that time (Maliniemi et al., 2018; Pearson et al., 2013). Changes in vegetation are expected to occur in the circumpolar areas, however, any notable rise in species richness over the last three decades have yet to be observed (Elmendorf et al., 2012b). The height and abundance of shrubs, graminoids and forbs on the other hand, have been recorded to increase (Elmendorf et al., 2012a).

The common occurrence of shrubs is already an important characterization of tundra ecosystems (Walker et al., 2005). The response of this shrub encroachment on other species is uncertain and depends on the ecological context (Bråthen et al., 2018). The species richness of vascular plants has been found to decrease as a response to a higher distribution of shrubs (Klein et al., 2004; Wilson & Nilsson, 2009), but other studies have found responses of either stable (Pajunen et al., 2011) or increasing species richness (Bråthen & Lortie, 2016). *Empetrum nigrum* is a native shrub species in northern Norway that has encroached into new habitats, parallel to the increasement of the average temperature in the area (Tuomi et al., 2022). This species is also expected to tolerate changes in the environment better than other coexisting species in tundra ecosystems and may therefore have a large impact on how climate change will impact the vegetation in the Arctic (Bråthen et al., 2018; Buizer et al., 2012; Kaarlejärvi et al., 2012; Tybirk et al., 2000; Wada et al., 2002). Furthermore, *E. nigrum* is an allelopathic plant, which makes it complex to understand the factors that drive the encroachment of this shrub.

1.2 *Empetrum nigrum*

Empetrum nigrum, known as crowberry, is an evergreen shrub species which is common in alpine and circumpolar areas (Bell & Tallis, 1973; González et al., 2015), including coastal and mountain areas of Arctic Norway. This is a slow growing, much-branched species, with a strong primary root and shallow lateral shoots (Bell & Tallis, 1973; Gonzáles, 2018). The shoots are covered by many small leaves, which are, when withered, easily distributed by abiotic factors such as rain, wind and snow melt (Lid & Lid, 2005; Pilsbacher et al., 2021). As the shrub develops, horizontal creeping shoots with shallow lateral roots attached, will grow out from a central point of the plant (Gonzáles, 2018), with new shoots on each branch of the clone every year. *E. nigrum* can become more than 140 years old, meaning that it has a large potential to encroach into a wide area from the central primary root (Bell & Tallis, 1973), and potentially into new habitats. *E. nigrum* is intolerant to shade and is mainly found in open spaces like treeless heathlands, potentially creating monospecific vegetation with other woody shrubs, bryophytes and lichen (Gonzáles, 2018). Due to the shallow roots of the species, *E. nigrum* is sensitive to disturbances such as trampling and fires (Gonzáles, 2018: Tybirk et al., 2000). The berries of *E. nigrum* are eaten by herbivores, but the other parts of the plant are not edible, and heathlands mainly covered by *E. nigrum* are commonly avoided by grazing animals (Ims et al., 2007; Iversen et al., 2014). This means that disturbance from trampling is mostly close to reindeer fences (Moen & Danell, 2003).

Former studies of *E. nigrum´s* growth pattern under different environmental conditions propose that the species is sensitive to the impact of climate change (Büntgen et al., 2015; Myers-Smith et al., 2015). Experimental studies also indicate that the species may be vulnerable to certain extreme weather events during winter (Bokhorst et al., 2008), while at the same time being tolerate to other events such as ice encasement (Preece et al., 2012; Preece & Phoenix, 2014). Observations over time have provided evidence of *E. nigrum* expanding both in altitude and latitude in the arctic tundra, parallel with a warming climate (Felde et al., 2012; Klanderud & Birks, 2003; Wilson & Nilsson, 2009). These findings suggest that an encroachment of *E. nigrum* is expected as extreme winter warming events, average temperature and growth season is increasing.

The leaves of *E. nigrum* are not palatable to herbivores, as they contain batatasin III and other secondary metabolites that can be toxic to digest (Gallet et al., 1999; Odén et al., 1992; Tybirk et al., 2000). These compounds are released from superficial glands in the leaves (Wardle et al., 1998; Tybirk et al., 2000) to the surrounding environment through rain, thaw, and snowmelt (Gallet et al., 1999; Odén et al., 1992). When batatasin III and phenolic acids from *E. nigrum* are mixed with soil they create recalcitrant complexes with soil organic matter and become very resilient (Wardle et al., 1998). These toxins are commonly found in areas dominated by *E. nigrum*, but as the leaves are spread by the wind and snowmelt, the toxic compounds can be distributed to a wider area. This means that the impact of *E. nigrum* can be found in other areas that are not necessarily dominated by the species (Pilsbacher et al., 2021).

1.3 Niche construction

Organisms that interact both with each other and the environment are the foundation of ecosystems (Wardle et al., 1998). Plant material is produced from sun energy through photosynthesis, where the energy is accessible for other consumers. Plants are therefore at the bottom of the food-chain in ecosystems. Every terrestrial ecosystem relies on plants as primary producers, which further means that the plants regulate the functioning of other components in the system (Oksanen, 1990; Vitousek & Walker, 1989; Wardle et al., 1998) (Figure 1). An understanding of how plant communities' function and are regulated is therefore fundamental knowledge for further understanding the whole ecosystem (Oksanen, 1990; Vitousek & Walker, 1989; Wardle et al., 1998). The different components of an ecosystem are interconnected, meaning that changes in one of the components is likely to have a cascade effect on other components (Abrams et al., 1996).

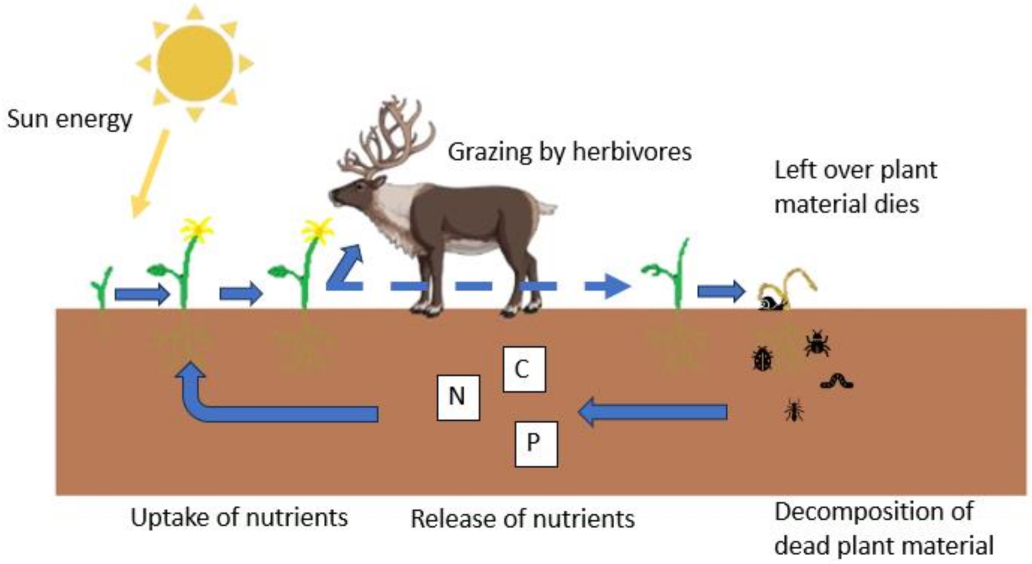

Figure 1: Regular, undisturbed life cycle of a plant in the arctic tundra. Sun energy and soil nutrients are required for plant growth, and plants are providing herbivores with nutrients and energy. Leftover plant material will wither and enter the soil system, together with waste (faces and urine) from herbivores, which return digested plant nutrients back to the soil system. In the soil, macro- and microorganisms will decompose the dead plant material, making the nutrients available for new plants.

Some species have a higher capacity to modify their own and/or other organisms' niches (Odling-Smee et al., 2003). This is called niche construction, when organisms and their interaction with the environment, both biotic, abiotic, directly, or indirectly elements, can modify their surrounding environment (González et al., 2021; Odling-Smee et al., 2013). Examples of niche construction are nest building by birds or alteration of nutrient cycling by plants (Laland et al., 2016). Niche construction can be both long-term legacy from the presence of a species and a result of current processes from specific organisms (Bråthen & Ravolainen, 2015; Odling-Smee et al., 2013). The main purpose behind this concept is that an organism significantly modifies environmental conditions, and that these modifications are influencing selection pressure on a recipient organism (Matthews et al., 2014).

1.4 Allelopathy

Allelopathy is a mechanism found among plants that release chemicals to the surrounding environment, to cope with stressful conditions (Yang et al., 2018). These chemicals can have both positive and negative impact on the neighboring environment, but most common is a negative effect on the associated plants (Wardle et al., 1998). These chemicals are mainly secondary metabolites, that are organic compounds which are not essential for growth and reproduction but have a specific ecologic function (Yang et al., 2018). These specific ecological functions will provide some type of advantage when competing for resources or space in the plant community (Yang et al., 2018). Allelopathic plants are strongly influencing their surroundings, which further can have a wide-range influence on ecosystem level (Wardle et al., 1998). The ecosystem function and the nature of the plant community would therefore be partly regulated through the allelopathic effect of some specific plant species (Wardle et al., 1998). The primary production of the ecosystem will therefore be largely affected by the secondary metabolites (Figure 2). Allelopathic plants are therefore often referred to as niche constructures (González et al., 2021; Wardle et al., 1998).

Herbivores depend on plants for nutrition and energy, and the herbivores populations are strongly correlated with the biomass of plants available to feed on (Hobbs, 2024). Secondary metabolites are therefore impacting the feeding activity of the herbivores, which again impact other strong processes in the ecosystem (Lason, 2005). Common for many of the allelopathic plant species is their low palatability. This is to some degree because of the secondary metabolites, but also due to a low nitrogen status and structural complexity (Attia-Ismail, 2015; Pastor et al., 1993). The fact that these plants are less palatable because of their low nutrient structure is a benefit in areas with heavy grazing and an advantage when competing with other species. The food selection of herbivores will therefore impact the plant community in such situations, potentially resulting in a dominance of these allelopathic plants in the ecosystem (Erfanian et al., 2021).

Most of the plants in an ecosystem are not consumed by herbivores, but will with time wither and become plant litter, which will enter the soil system. This material will be broken down to simpler molecules, including nutrients, by macro- and microorganisms in the soil (Aerts, 1997). The leftovers from the dead plant material will end up as soil organic matter. The quality of the soil is one of the factors that is regulating plant growth and community composition, meaning that the quality of the plant litter itself can influence a whole ecosystem (Giweta, 2020; Figure 2). The allelopathic compounds are major determinants of litter quality (Chomel et al., 2016). This highlights how big of an impact the concentration of plant secondary metabolites and vegetation type have on soil microbial communities (Chomel et al., 2016). The feedback effect of such an impact will be important for the nutrient mineralization, which will impact the plant growth and therefore also the plant community structure (Wardle et al., 1998). If a plant has such an impact on one biotic component of the ecosystem, there is a high possibility that the plants are impacting other biotic components as well (Wardle et al., 1998).

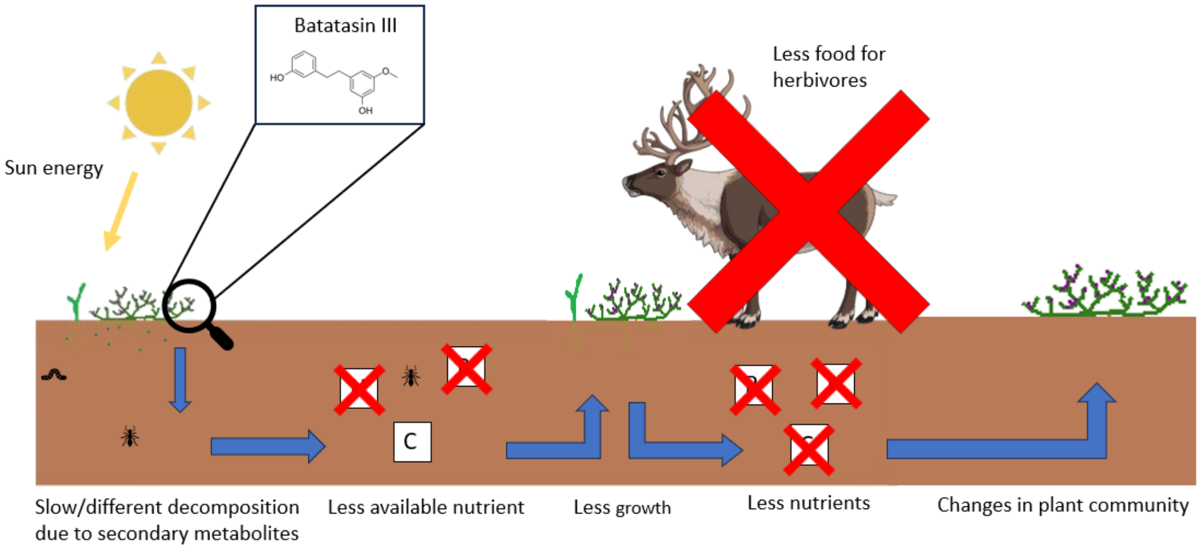

*Figure 2: Demonstration of how an allelopathic plant (*Empetrum nigrum*) can influence the whole ecosystem by releasing secondary metabolites to the environment. The leakage of secondary metabolites (batatasin III) from* E. nigrum *leaves into the soil influences the macro- and microorganisms in the soil system (Widden, 1986). This makes nutrients less available for other plants and limits their growth, meaning that there is less food for herbivores (Wardle et al., 1998). Less growth means less nutrients in the soil, which in the end will make it difficult for the plants to grow, and the allelopathic plant can increase its biomass.*

1.5 The ecological impact of *Empetrum nigrum*

The impact of the toxic compounds of *E. nigrum* to other species has been well documented. The recalcitrant complexes can reduce nitrogen availability in the soil, making it difficult for other plants to take up this nutrient (Wardle et al., 1998). Nitrogen is one of the most important nutrients for plant growth and development (Shrivastav et al., 2020). Limited nitrogen in the soils due to *E. nigrum´s* allelopathic properties can therefore negatively influence the growth of other plant species. It is known that seed germination and seedling growth for several graminoids and tree species is highly reduced by the presence of *E. nigrum* leaves (Bråthen et al., 2010; González et al., 2015; Nilsson et al., 1998), as well as the growth of aquatic fauna (Brännäs et al., 2004). The humus impacted by *E. nigrum* creates challenging conditions for microbial activity, which again impacts decomposition rates and nutrient cycles (Wardle & Lavelle, 1997). Mycorrhizal symbiosis of coniferous trees is also negatively impacted by the toxins (Nilsson et al., 1993).

Some plant families are more specialized to tolerate environments with a low soil nitrogen accessibility. Legumes, found in the family of *Fabaceae,* can make symbiotic relationships with rhizobia bacteria, creating new organs on the roots called nodules that fixate atmospheric nitrogen (Andrews et al., 2013; Raven, 2010). The bacteria are enclosed into the roots by the plant cell membrane, where it differentiates into bacteroids (Wang, 2019). These are polymorphic cells with modified cell walls, which on the outside appear as small, round outgrowths on the roots (Wang, 2019). This mechanism makes it possible for the plants in areas with low soil nitrogen to fixate nitrogen from the atmosphere, which is an advantage in challenging environments (Andrews et al., 2013; Raven, 2010). This could mean that legume plants found in *E. nigrum* shrubland where there is low nitrogen availability might have a benefit when competing for aboveground space with other plants in the same area. *Astragalus, Trifolium* and *Vicia* are plant genera of the *Fabaceae* family that are commonly distributed in sub-Arctic areas, such as northern Norway (Artsdatabanken, 2024).

The presence of *E. nigrum* has a large impact on the surroundings with the ability to modify ecosystem processes and is therefore referred to as a niche constructor (Bråthen & Ravolainen, 2015). This can be a very important factor considering climate change, and the understanding of how climate change will affect the tundra vegetation. The species has a negative impact on the seedling stage of other plant species (González et al., 2015), especially forbs and grasses (Mod et al., 2014; Pellissier et al., 2010; Wilson & Nilsson, 2009). However, species with higher dry matter content seem to thrive in the environments where *E. nigrum* is dominating (Mod et al., 2014; Pellissier et al., 2010). The environment where *E. nigrum* is dominating is influenced by a slow nutrient process due to the properties of its leaves, making the habitat favorable for species who thrive in such environments (Wilson et al., 1999). Ericaceous shrubs such as the genera *Cassiope* and *Vaccinium* are common to find in *E. nigrum* shrublands in arctic and sub-arctic areas (Fremstad, 1992; Haapasaari, 1988).

A species' capacity to modify ecosystems is important to consider when evaluating how climate change is impacting the tundra vegetation (Bråthen et al., 2018). As the temperature in the Arctic is rising, plants that tolerate higher temperatures would be expected to increase in abundance and biomass in the region (Kullmann, 2010). However, the presence of *E. nigrum* seems to modify these changes, as the biomass of herbaceous plants are reduced in the proximity of the species due to its allelopathic characteristics (Bråthen et al., 2018). The secondary metabolites are changing the soil properties, making nutrients less accessible to other plants and microbes (Wardle et al., 1998). The toxic leaves can be spread to the surroundings through abiotic factors, where secondary metabolites continue to leak into the soil and change the soil properties over time (Pilsbacher et al., 2021). Other plant species becomes vulnerable under such conditions, which allows *E. nigrum* to encroach into new habitats (Figure 3). Some functional groups of plants such as grasses and forbs, are more sensitive to the impact of *E. nigrum* (Bråthen et al., 2010; Mod et al., 2014; Pellissier et al., 2010; Wilson & Nilsson, 2009). These plants are common to find in meadows, which makes this habitat more vulnerable to *E. nigrum* compared to other vegetation types such as heath and wetlands. Meadows are one of the few vegetation types with a high species diversity. This means that meadows are important forage areas for herbivores. Loss of meadows due to *E. nigrum* encroachment can therefore have a large impact on the whole tundra ecosystem. Establishment of seedlings and soil microorganisms are directly impacted by this niche constructor, which in turn have a negative effect on species diversity and herbivores in the ecosystem (Aerts, 2010; Bråthen et al., 2010; Pellissier et al., 2010; Tuomi et al., 2022;

Wardle et al., 1998).

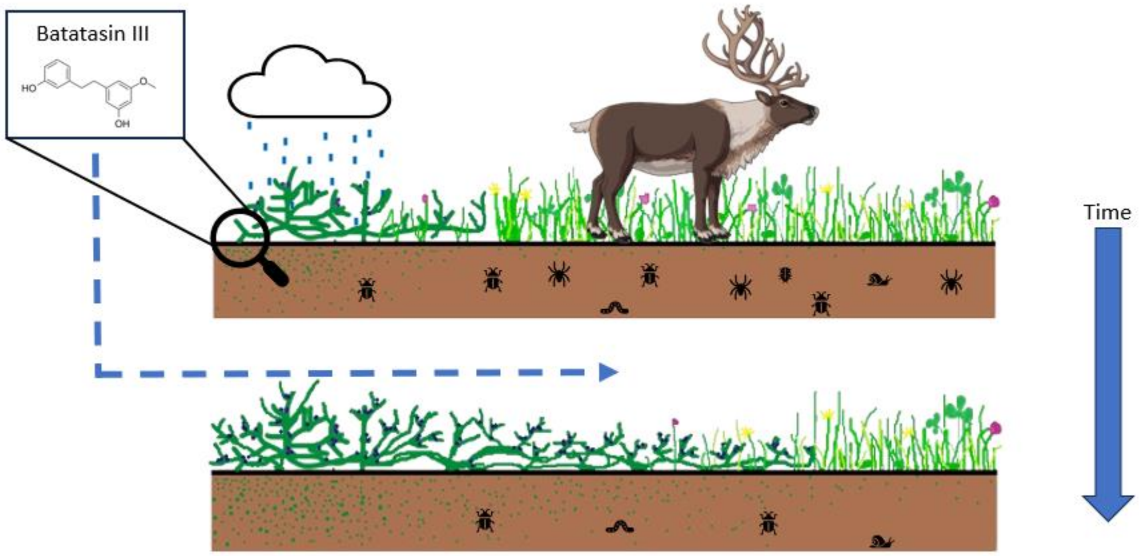

Figure 3; E. nigrum *as a niche constructor. The allelopathic compounds of the shrub leak from the leaves to the environment through rain. The effect over time is an encroachment of* E. nigrum *that increase in biomass and distribution, less biodiversity, fewer soil microbes and less herbivores as there are fewer edible plants to graze on.*

E. nigrum has been found to encroach into important forage areas for grazing animals in Northern Norway, leading to a shift from important forage species to a dominance of *E. nigrum* (Tuomi et al., 2022). The encroachment of *E. nigrum* is likely to threaten the distribution of biodiversity of meadows in the arctic tundra (Bråthen et al., 2024), which potentially can have large consequences and cause a domino effect to the surrounding areas (Figure 3). To minimize the encroachment of *E. nigrum*, knowledge about the mechanisms behind its horizontal growth would be the first step to learn how to handle this challenge. An understanding of how long *E. nigrum* needs to be present before the species have a negative effect on the soil properties and plant growth will widen the knowledge that already exists about the allelopathy of this species. This information will be crucial when further evaluating management solutions to minimize the encroachment of *E. nigrum* in meadow habitats.

1.6 Aim and hypotheses

It is well known that the toxins have a negative impact on seedling establishment and growth, but little is known about the time it takes for *E. nigrum´s* presence to have an impact on the growth conditions in the soil. The allelopathic effect of *E. nigrum* was therefore studied through a space for time experiment to assess the soil-residing allelopathic effect of *E.*

nigrum. Growth of meadow plant seedlings were used to assess the allopathic effect and determine its relative importance in the encroachment process. Soil collected in an *E. nigrum* encroachment gradient was therefore used in a bioassay. Seedlings of three local plant species were planted in the soil to compare the growth condition in the *E. nigrum* encroachment gradient. The gradient had four sampling points; one where the species had been present for ~15 years, at the clone edge, and at one and two meters into the meadow. Based on the results of this data, this study will answer the following research question:

Does *E. nigrum* have an allelopathic effect on soil after approximately 15 years of presence?

To widen the understanding of potential growth results from the bioassay, this study will also investigate the soil nitrogen content and the production of nodules by one legume species in the *E. nigrum* encroachment gradient. The production of nodules could be a potential coping mechanism in soils impacted by *E. nigrum.* The local seedbank in the gradient soils will also improve knowledge about soils capacity to germinate seeds of local seedlings.

To answer the question, the following hypothesises were tested:

H1. The negative effect on seedling growth and survival in soils influenced by *E. nigrum´s* secondary metabolites is most noticeable where the abundancy of the species is highest, with a decreasing effect as the distance to *E. nigrum* is increasing in an encroachment.

H2.1. *Trifolium pratense* has more nodules where the abundancy of *E. nigrum* is highest, with a decreasing number as the distance to *E. nigrum* is increasing in an encroachment.

H2.2. The number of active nodules of *Trifolium pratense* is highest where the abundancy of *E. nigrum* is highest, with a decreasing number as the distance to *E. nigrum* is increasing in an encroachment.

H.3. Soil nutrient content is lowest where the abundancy of *E. nigrum* is highest, with increasing values as the distance to the species is increasing in an encroachment.

H.4. The number of germinating seedlings from the local seedbank is the lowest where the abundancy of *E. nigrum* is the highest, with an increasing number as the distance to the species increases in an encroachment.

2 Material and methods

2.1 Study area

The data sampling was conducted in Troms and Finnmark county, in northern Norway between latitudes 69°4 and 70°5 N and longitudes 21°3 to 29° (Figure 4). The climate in the area is influenced by the North Atlantic Current, which makes a steep climatic gradient between the western coastal areas, the inland and the eastern part of the county, as the current declines from the west to the east (Johannessen, 1970). The coastal areas have typically mild winters and cooler summers, but this is less prominent further east along the shore (Norsk klimaservicesenter, 2022). The average precipitation of the area varies greatly from 600-1000mm along the west coast, with a decline towards the north shore where the annual average is from 600mm to 800mm (Meteorologisk institutt, 2022). However, the average yearly precipitation of the whole study area is between 300-500mm (Meteorologisk institutt, 2022), but the study sites are mainly located along the coast.

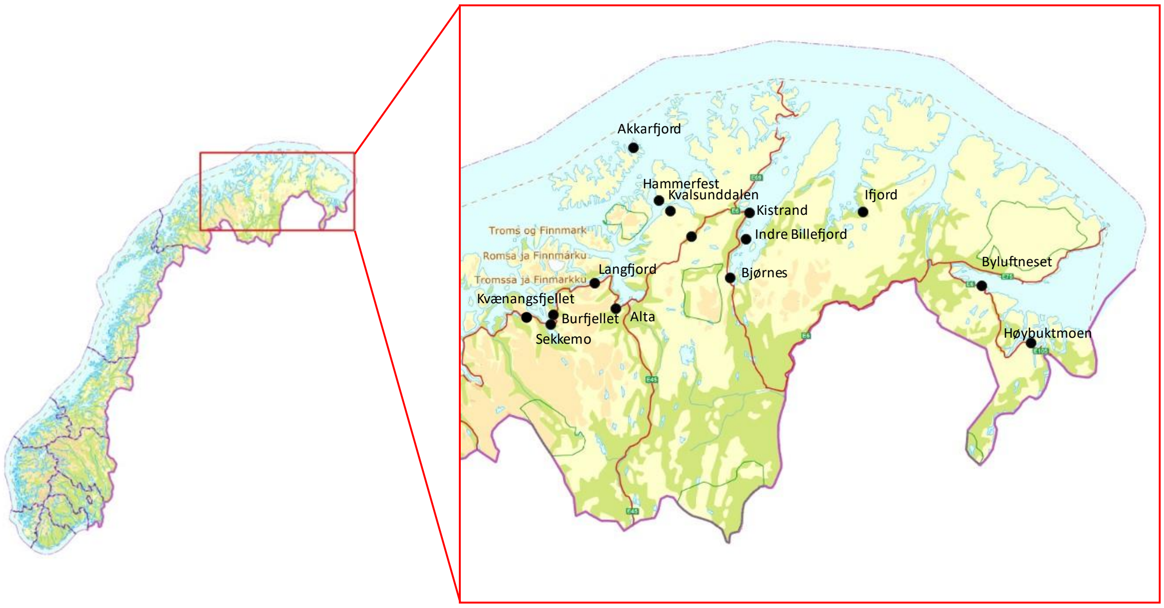

Figure 4: Map of the study area in Northern Norway. Data was collected at each of the 15 locations marked in this map.

The area is mainly characterized as a coastal alpine tundra (Bråthen et al., 2018), where the most common vegetation types are heath, meadows and marches (Bråthen et al., 2018). Dwarf-shrub tundra is the most frequent ecosystem, mainly dominated by *E. nigrum*, but also different *Vaccinium* species and *Betula nana* is evenly distributed in the region (Bråthen et al., 2018). Forbs such as *Bistorita vivpara, Achillea millefolium, Viola biflora,* grasses as *Agrostis sp.,* and *Festuca sp.,* and the sedge *Carex sp.* were common species to find in the meadows of the study area.

2.2 Study design

To get an understanding of the allelopathic effect on seedling growth where *E. nigrum* is encroaching into a new habitat, a bioassay with soil samples was conducted. Soil samples for the bioassay were collected along 45 separate *E. nigrum* encroachment gradients. There were in total 15 study sites, with three transects of the encroachment gradient at each site. The *E. nigrum* encroachment gradient had four sampling points, one where *E. nigrum* had been present for ~15 years, at the clone edge, and one and two meters into a meadow. Three plant species of different functional groups native to the study area were planted in pots that contained the soil samples.

2.2.1 Sample stage

Soil samples for the bioassay were collected at 15 sites in Troms and Finnmark, in August 2023 (Figure 4). Soil was collected in an *E. nigrum* encroachment gradient, from where *E. nigrum* was dominating the vegetation, at the end of the *E. nigrum* encroachment, in addition to one- and two-meters distance from the encroachment, into a new habitat (Figure 5). The fieldwork was conducted in August 2023, from east to west.

In all sites, there was a clear encroachment of *E. nigrum* into a new meadow habitat dominated by herbaceous plants. The new habitat would always have approximately 90% cover or more of herbaceous plants, determined by eye. The flat landscape makes it less likely for the leaves from the *E. nigrum* to be moved through rain and snowmelt into new areas, as there is no incline for the water to naturally transport the leaves from the clone into a new habitat. Therefore, hills and slopes were avoided in the study site when possible. However, the natural landscapes of the arctic tundra are rarely flat, and the slope for the gradient varies from 0° to 12°.

At each site, three *E. nigrum* branches from three distinct clones that were encroaching into a new habitat were selected, with at least five meters distance from each other. These branches represented three transects at each site. The GPS coordinates were recorded, and a simple map of the sites was drawn. For each site, the inclination and aspect were recorded. Each *E. nigrum* branch was carefully dug out, starting from an individual shoot above ground that was at the end of the encroachment. The end of the shoot represented the current year and every shoot represented a year of growth. This was an assumption, based on former descriptions of the species development and growth. The length of the branch was measured, and the age of the branch was determined by counting the shoots from the main branch. Branches of 15 years were used as a standard number for each transect. If the branch was less than 15 years old, the actual age of the branch was divided by the length of the branch and multiplied by 15.

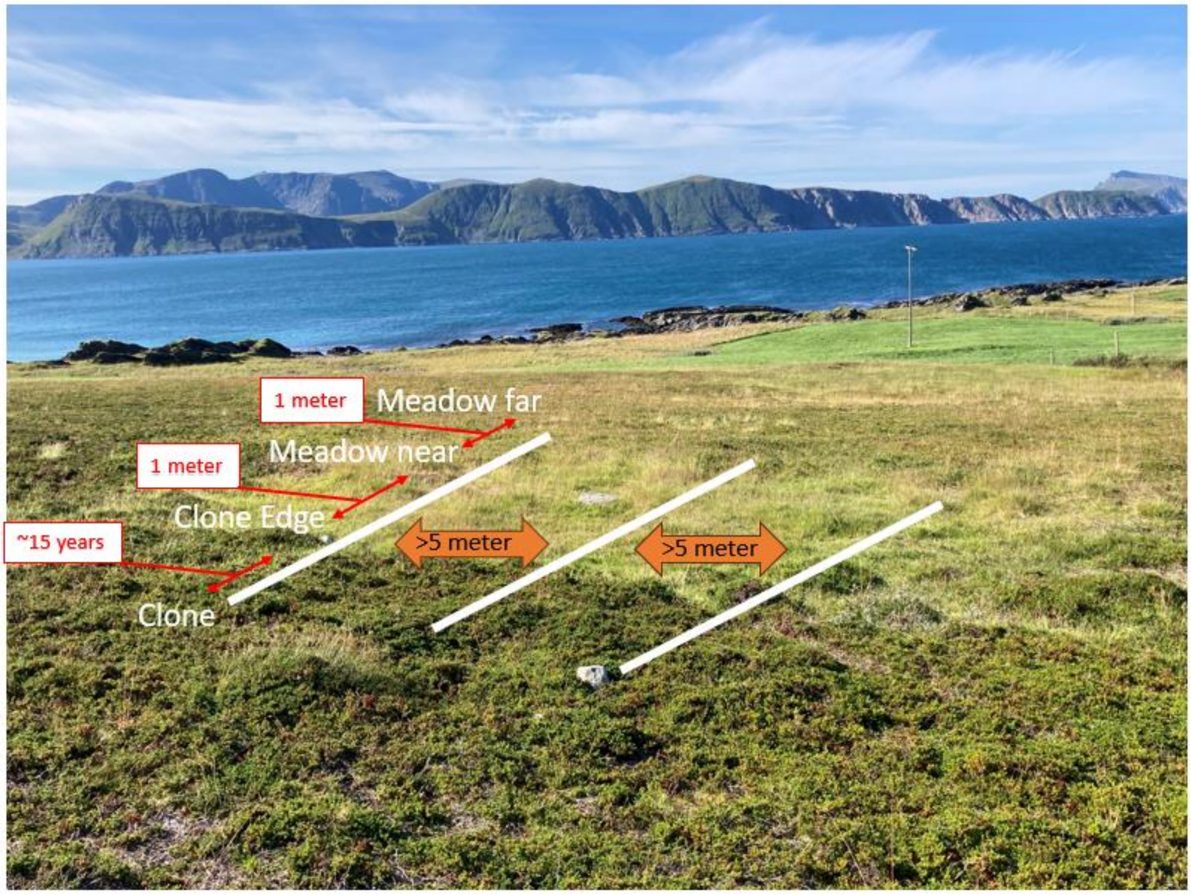

Figure 5: Study design from each of the sites. Two soil samples were collected in each of the sampling points (clone core, clone edge, meadow near and meadow far) at three transects at each site. Picture from Akkarfjord, Sørøya (site 11). Picture taken by Liina Soonvald.

The first sampling point of each branch was where the branch was determined to be ~15 years old, at the most encroached *E. nigrum* site in the gradient (clone core). The next sampling point was at the edge of the encroachment (clone edge). The third sampling point was one meter away from the encroachment edge into a meadow (meadow near) and the last was located two meters away from the encroachment edge (meadow far) (Figure 5).

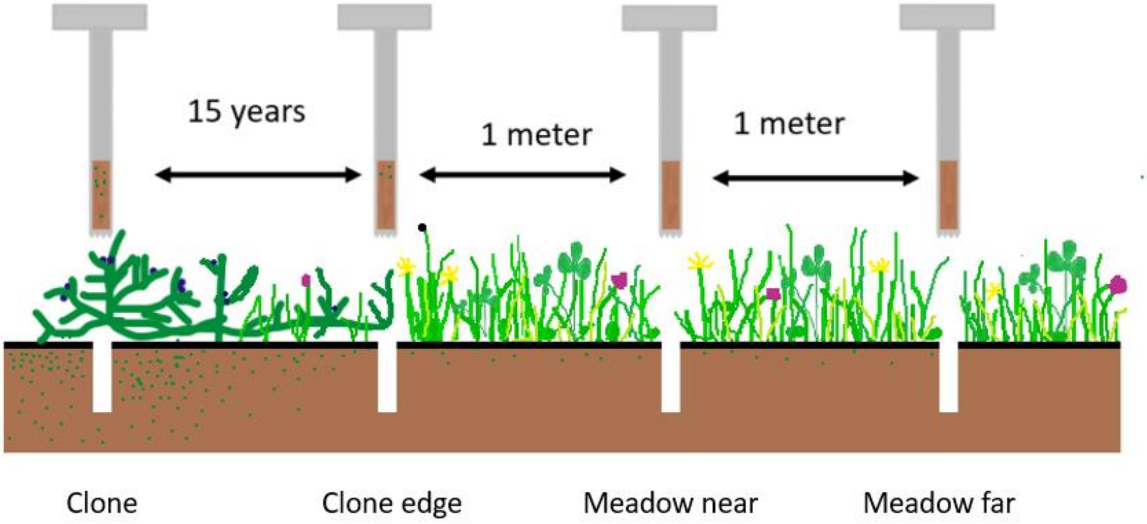

Figure 6: Sampling points for soil and vegetation cover in the E. nigrum *encroachment gradient.*

Two soil samples were collected from the sampling points with a clean soil corer (3 cm diam. to 5 cm depth) (Figure 6). One of the samples was used in the bioassay, the second sample was analyzed for nitrogen content. This was repeated for each of the three transects, meaning that there were six subsamples from each of the sampling points at each site. The soil for the bioassay was pooled per sampling point per site and stored and dried in paper bags during the fieldwork. After the fieldwork samples were kept at 19°C in a regulated temperature room until further use.

2.2.1.1 Soil nitrogen content by Near-infrared spectroscopy analysis

Measurements of the nitrogen content in the soil samples were conducted with the near infrared spectroscopy method. Soil samples were first dried at 60°C for 48 hours, and sieved through a 2 mm mesh size sieve. The equipment was cleaned between each sample to avoid contamination from the other samples. Soil samples were again dried at 60°C for 12 hours and stored in a desiccator until they were analyzed. A Fieldspec3 and a MugLight was applied for the NIRS scan. Before starting the analysis, a fiber optic check was conducted to assure that all the fibers worked correctly. The program Indico Pro was used to scan samples.

Subsamples of the soil were placed in a Petri dish, and covered the whole bottom so no light was visible through the soil samples. The Petri dish was then placed on top of the MugLight and scanned five times. Between the scans, samples were rotated, and the soil was mixed at least once. A baseline was taken on a white surface before the first sample was scanned, and every 20-30 minutes during the analysis. The nitrogen content of the soils (%) was predicted from every scan by using established prediction models (Petit Bon et al., 2020).

2.2.2 Bioassay stage

The growth conditions for vascular plants in the soil from the different sampling points were tested at the phytotron at Holt, Tromsø.

2.2.2.1 Soil and seedling preparations

The soil collected from the encroachment sites was kept dry until further treatment. Large roots and rocks were removed from the samples and discarded. Conical, square-topped plastic pots, with pot size 5,5cm x 5,5cm and 6cm depth, with a total volume of 80ml were used for the bioassay. Some of the soil samples contained less than 160 ml of soil after the removal of roots and rocks. Therefore, 15 ml of commercial sand was used in the bottom of the pots to fill out some of the volume. 60 ml of the collected soil from the gradient was added to the pots on top of the sand. The collected volume of soil was enough to make two replicates from each sampling point in the gradient at each study site (Figure 7). In total there were 30 pots of each sampling point, and 120 pots altogether in the bioassay.

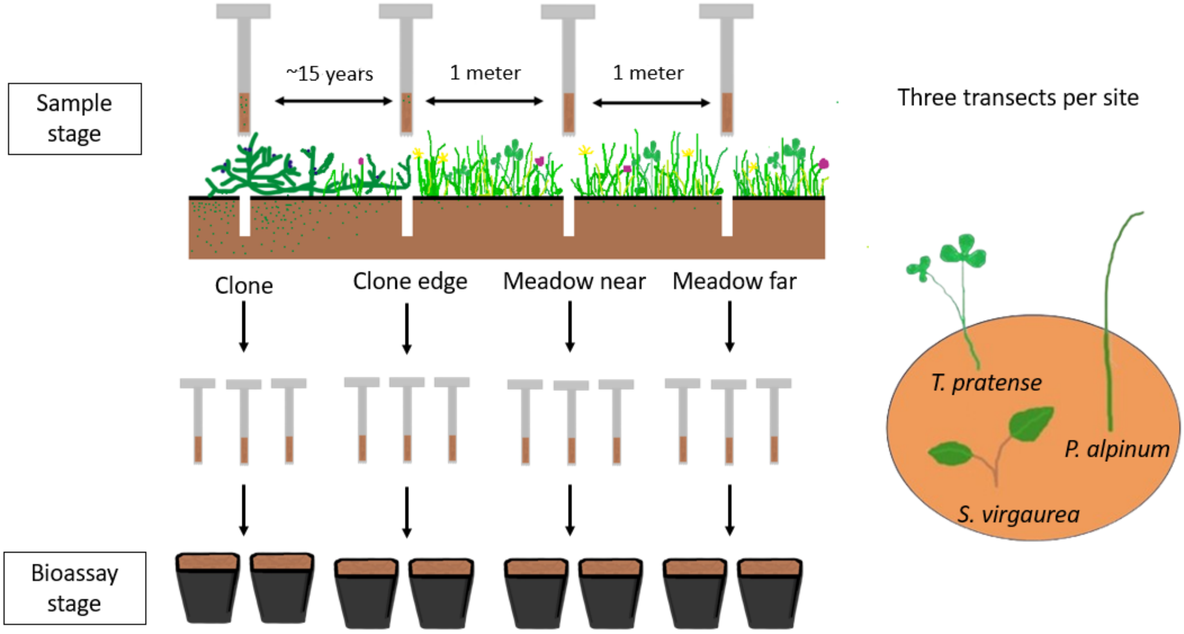

Figure 7: From sampling to bioassay: soil collected from each of the sampling points was moved into pots after removal of rocks and roots. Each sampling point at each site had two replicates of pots. One seedling of Trifolium pratense, Solidago virgaurea *and* Phleum alpinum *was planted in each of the pots.*

2.2.2.2 Species selection and germination

Seedlings of *Solidago virgaurea, Phleum alpinum* and *Trifolium pratense* were used in the bioassay (Figure 7). The species chosen were all native to the study area**.** The germination of the seedlings took place in the Biology building at UiT.

S. virgaurea seeds were collected in the area around Charlottenlund, Tromsø, in the summer of 2021. After the collection the seeds were kept at -20°C until further use. Seeds of *T. pratense* and *P. alpinum* were collected outside of the Biology Building at UiT, Tromsøya, Norway, on the 20th of September 2023. After collection, the seeds were dried for two days, and then stored in a freezer at -20°C for five days. Before the germination phase, *T. pratense* seeds were cleaned in room temperature water for 30 minutes. All the seeds were set to germinate in trays with moist sand covered in plastic with small holes. *S. virgaurea* and *T. pratense* were set to germinate in a 19°C without light for the first six days. After these six days, seedlings of *T. pratense* had germinated, and were therefore moved to an 8°C room without light, to slow down the growth of the seedlings. The lights were from the sixth day on both day and night in the germination room, and the seeds of *P. alpinum* were set to germinate in the same room as *S. virgaurea*. Seeds of *P. alpinum* had germinated within 11 days, while *S. virgaurea* germinated within 14 days.

Figure 8: Seedlings at the start of the bioassay.

Randomly selected seedlings were carefully removed from the germination tray with tweezers, without disturbing the roots. Water was used to remove sand from the roots of the seedlings. The length of 50 seedlings for each of the species was measured with laminated mm paper and the use of imageJ (Rasband, 1997-2020). After the measurement, the seedlings were gently placed with the roots into pots with the sampled soil. The seedlings of *T. pratense* were repotted one day before *P. alpinum* and *S. virgaurea*. One seedling of each species was placed in all the pots (Figure 7).

2.2.3 Growth and measurements

The repotted seedlings were placed in an 18°C room with 24 hours of daylight and 75% relative humidity for five weeks. The seedlings were watered between two and three times a week through the whole experiment. Seedlings that died within the first week were replaced with a new seedling of the same species, assuming that this was due to the damage from the transplantation, whereas seedlings that died after the first week were assumed to die because of the soil conditions, and their death was recorded. During the first three weeks, some seeds from the local seedbank germinated. These seedlings were moved into pots containing moist 70% peat and 30% fertilizer throughout the experiment, to avoid competition between these seedlings and the planted seedling. This was repeated a couple of times during the bioassay. The seedlings from the seedbank were placed in the same room as where the bioassay took place, with the same conditions and equal amount of water. The seedlings were recorded to the closest taxa possible after eight to ten weeks.

Figure 9: Results of the bioassay from site 7, 8, 9 and 10.

Plants from the bioassay were harvested after five weeks. The plants were carefully removed from the soil. Water was used to remove soil from the roots. *T. pratense* is in the legume family, and nodules were observed on some of the roots of this species. When nodules were present, the total number of nodules were recorded, and they were categorized as active or not active, depending on the color of the nodules. The nodules with a red/pink color were assumed active (Van de Velde et al., 2006). It was difficult to assure that the whole root still was attached to the plant when it was removed from the soil. Therefore, other measurements including the root were discarded, and the root was removed from the aboveground plant by using a scissor. The aboveground length of each plant, from the bottom of the stem to the tip of the longest leaf was measured to the closest mm by using a laminated mm paper. Each plant was carefully stored in separate tea bags after the length measurements. Dry biomass was measured after the plants had been dried at 60°C for 24 hours, with measurements conducted to the precisions of 0,00001g.

2.3 Data analysis

Data from the bioassay was treated and analyzed in R-studio version 4.3.3 (2024-02-29) (R core team, 2024). The data was tested with normal distribution and transformed when needed. The measured length of the plants from the bioassay was tested in a linear fixed effect model (lmer), with length as dependent variable, gradient as the predictor and site and replicate as random effects. Emmeans was then used to calculate the estimated marginal means from the fitted linear fixed effect model, with length as the response variable and gradient as the predictor variable. This was done for all the four levels of the gradient, by using pairwise~gradient in the model. The same method was used for analyzing the dry biomass of the plants from the bioassay, with dry biomass as a dependent variable.

The relation between the gradient and number of plants with nodules, in addition to the number of plants with active nodules were tested with the generalized linear fixed effect model. A linear mixed effect model was used for the number of nodules. Estimated marginal means were estimated for all the three of these models.

The correlation between the presence of nodules and the growth of *T. pratense* was analyzed by a linear mixed effect model, with length or dry biomass as dependent variable and the presence of nodules as the predictor. The same analysis was conducted with number as the predictor variable.

A linear fixed effect model was used to analyze the difference in nitrogen content of the soil samples, with the gradient at predictor, nitrogen values as dependent variables and site and replication as random effects. The result from this model was further investigated by calculating the estimated marginal mean for all the levels in the gradient with emmeans.

A linear regression model was used to test the correlation between nitrogen and plant growth. Length and dry biomass were the dependent variable, and nitrogen content as the predictor variable.

Several packages from R-studio were used to analyze and visualize the data. To analyze the linear fixed effect model of the data with the lmer function, the lme4 package was used (Bates et al., 2015). The emmeans function from the emmeans package (Lenth, 2024) was used to calculate the estimated marginal means from the linear fixed effect model. Variations of the ggplot function in the ggplot2 package (Wickham, 2016) was used to visualize the data in all of the plots. Different functions from the tidyverse package (Wickham et al., 2019) were used for data treatment and sorting.

3 Results

3.1 Plants that withered under the bioassay

The number of plants that died during the bioassay were counted at the end of the experiment. At the clone core, three *T. pratense* withered. Two *P. alpinum* and five *T. pratense* died at the clone edge, while one *P. alpinum* and four *T. pratense* did not survive in the meadow near sampling point. The only sampling point in the gradient where *S. virgaurea* withered throughout the bioassay was in the meadow far, where three died, in addition to four *T. pratense*. In total, three, seven, five and seven plants died in the clone core, clone edge, meadow near and meadow far respectively.

3.2 Length and dry biomass measurements

P. alpinum had an average growth through the bioassay across all sites and sampling points of 23,0 cm, from 1,97 (±0,4 SD) as a seedling to 24,97 (±5,25 SD) at the end of the experiment. *S. virgaurea* grew from 1,0008 cm as a seedling (±0,32 SD) to 5,84 cm (±2,36 SD) at the end of the experiment. The average growth from seedling to grown plant was 4,84 cm. *T. pratense* had the tallest seedlings at 3,24 cm (±1,06 SD). The species had an average grown length of 7,39 cm (±3,18 SD), with the average growth of 4,15 cm. These are numbers from the raw data.

3.2.1 Phleum alpinum

The length measurements of *P. alpinum* at the end of the experiment increase from the clone core to the meadow near, followed with a decrease to meadow far. (Figure 10). *P. alpinum* had the best growth in the soil sampled in the meadow near sampling point of the gradient, whereas the length was the shortest in the clone core. The difference between these sampling points was significant (p=0,007). Furthermore, the difference of length between meadow near and meadow far was significant (p=0,02). No other comparisons between the sampling points were found to be significant. The dry biomass followed a similar pattern as the one for *P. alpinum's* length values. The values for *P. alpinum's* average dry biomass increased from the clone core to the meadow near, followed by a decrease to the meadow far (Figure 10). The contrast between the clone core and meadow near was significantly different (p=0,0066).

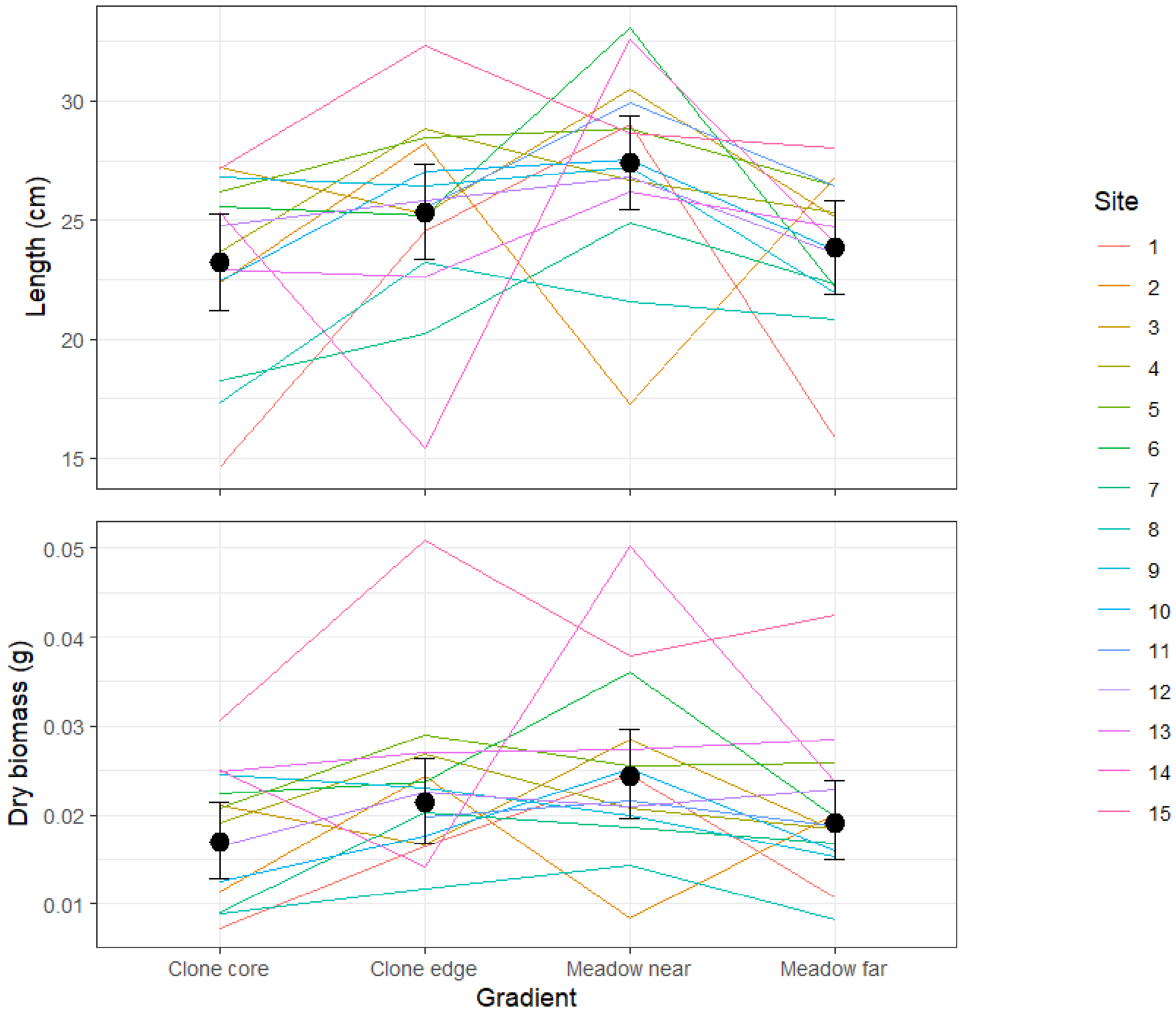

Figure 10: Length (cm) and dry biomass (g) measured at the end of the bioassay for P. alpinum in the gradient sampling points. The black points show the estimated marginal mean for the fixed effect values, with a 95% confidence level. The colored lines are the average length from the replicates at each site and sampling point.

3.2.2 Solidago virgaurea

S. virgaurea had the highest measured length at the meadow far. The length decreased for each sampling point from the meadow far towards the clone core, with the lowest average height measured in the clone core (Figure 11). Even though there was an increase of the length from the clone core to meadow far, there are no significance differences between any of the sampling points. The dry biomass increases from the clone core to meadow near, then the value decreased at meadow far (Figure 11). There are no significant values between any of the sampling points and the dry biomass of *S. virgaurea*.

There is a large variety between the sites considering the length of *S. virgaurea* for each sampling point. The average results for length at each sampling point is close to consistent between the sampling points. At the same time, most of the sites have non-linear trends for both length and dry biomass.

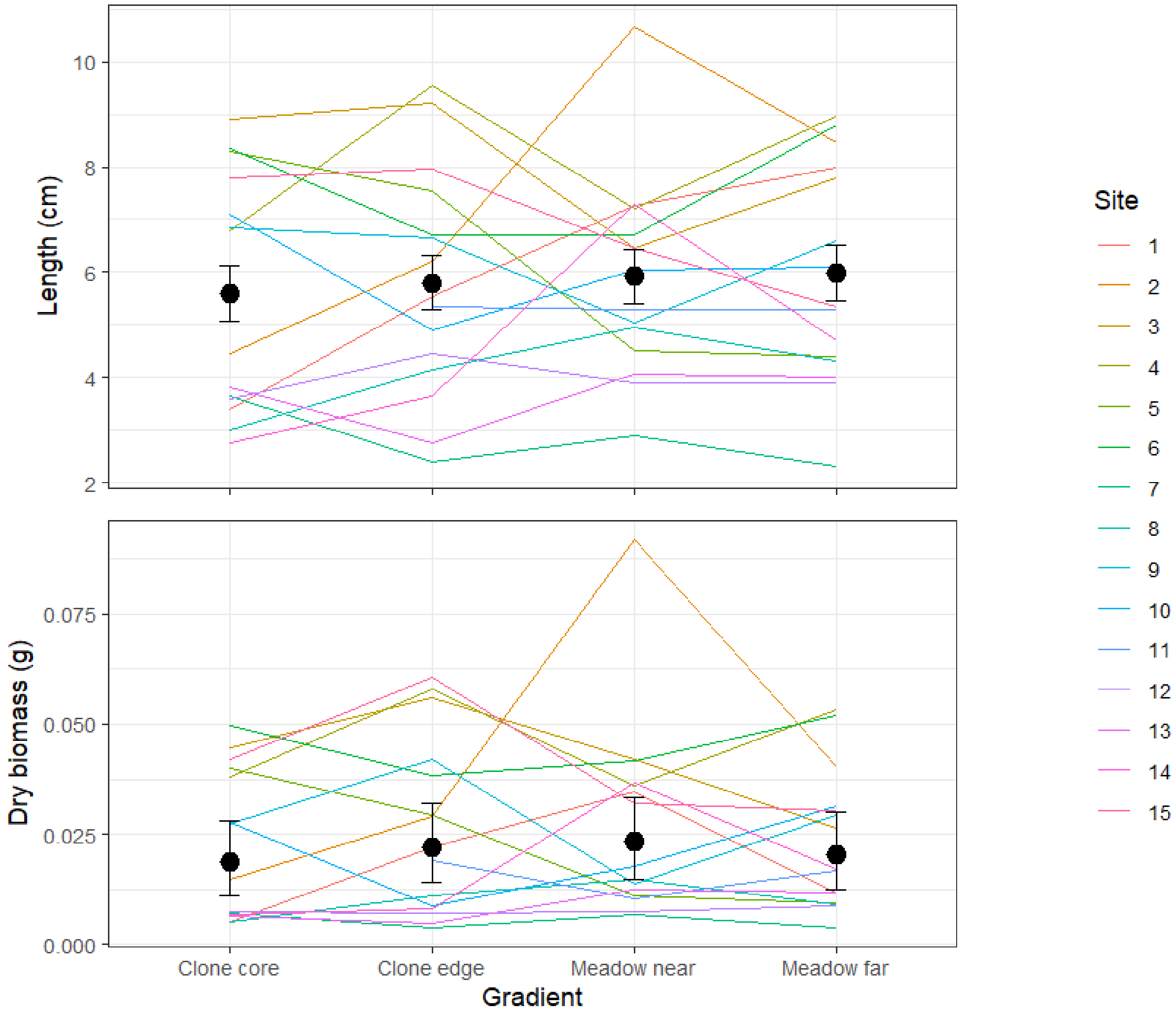

Figure 11: Length (cm) and dry biomass (g) measured at the end of the bioassay for S. virgaurea *in the gradient sampling points. The black points show the estimated marginal mean for the fixed effect values, with a 95% confidence level. The colored lines are the average length from the replicates at each site and sampling point.*

3.2.3 *Trifolium pratense*

T. pratense had a large variety of length measured in the *E. nigrum* encroachment gradient. The length decreased from the clone core to the clone edge, increased in meadow near, followed by a new decrease to meadow far (Figure 12). There were no significant differences for *T. pratense's* length between any of the sampling points in the gradient. The estimated dry biomass for *T. pratense* had the same pattern as the result for length measurements (Figure 12). There was a significant difference between the clone core and clone edge (p=0,0477) and the clone edge and meadow near (p=0,0154).

There was a large variation of *T. pratense's* length at each sampling point (Figure 12). Site patterns were mainly non-linear, which is reflected in the estimated growth for each of the sampling points. The raw data from the dry biomass of *T. pratense* is closer distributed compared to the length measurement, except from a few sites with higher values. However, the overall pattern is similar.

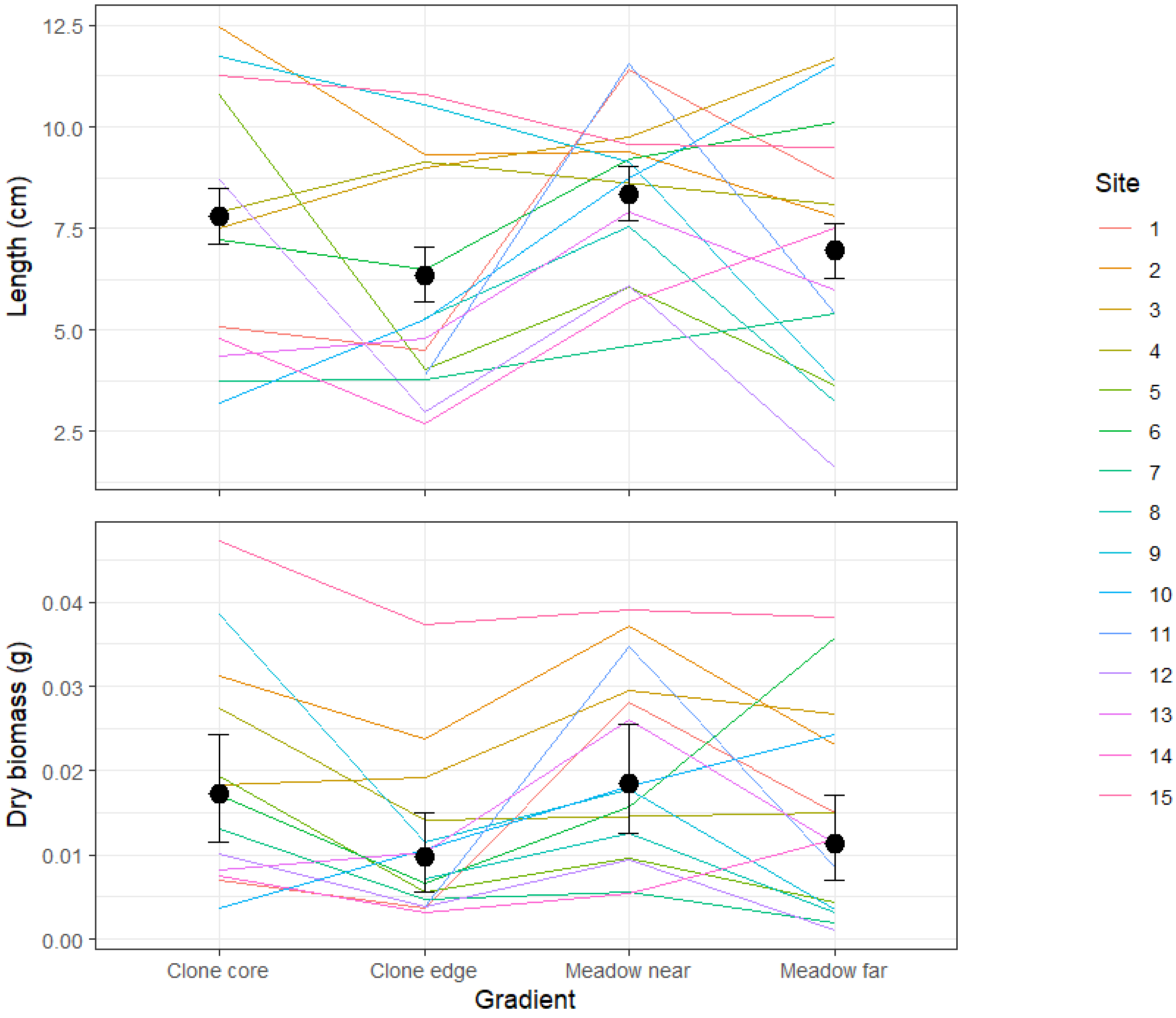

Figure 12: Length (cm) and dry biomass (g) measured at the end of the bioassay for T. pratense *in the gradient sampling points. The black points show the estimated marginal mean for the fixed effect values, with a 95% confidence level. The colored lines are the average length from the replicates at each site and sampling point.*

3.2.4 The average growth for the three species together

The average estimated length of all the species after the bioassay increased slightly from the clone core to meadow near, followed by a decrease in meadow far. No clear trend could be found between the sampling points in the encroachment gradient, and there was no significant differences for the average length of the species. The same growth trend was found for the dry biomass as well. The contrast between meadow near and meadow far was the only significant contrast for dry biomass for all the three species together (p=0,0362).

3.3 Nodules

There were in total 101 *T. pratense* individuals that survived the bioassay and were investigated for nodules. 60 *T. pratense* had not developed nodules, while 41 individuals had. Out of these 41 individuals, 15 plants had nodules that were active (Figure 13). The distribution of plants with nodules is demonstrated in figure 13. The sampling point with the highest number of plants with nodules was the clone core, where 50% of the plants had developed nodules. Out of these 12 plants, 6 had nodules that were active. The clone core was therefore the sampling point with the highest number of plants with nodules, in addition to the highest number of active nodules. The number of plants without nodules was highest in meadow far. Meanwhile, samples from the clone edge had fewest plant with active nodules.

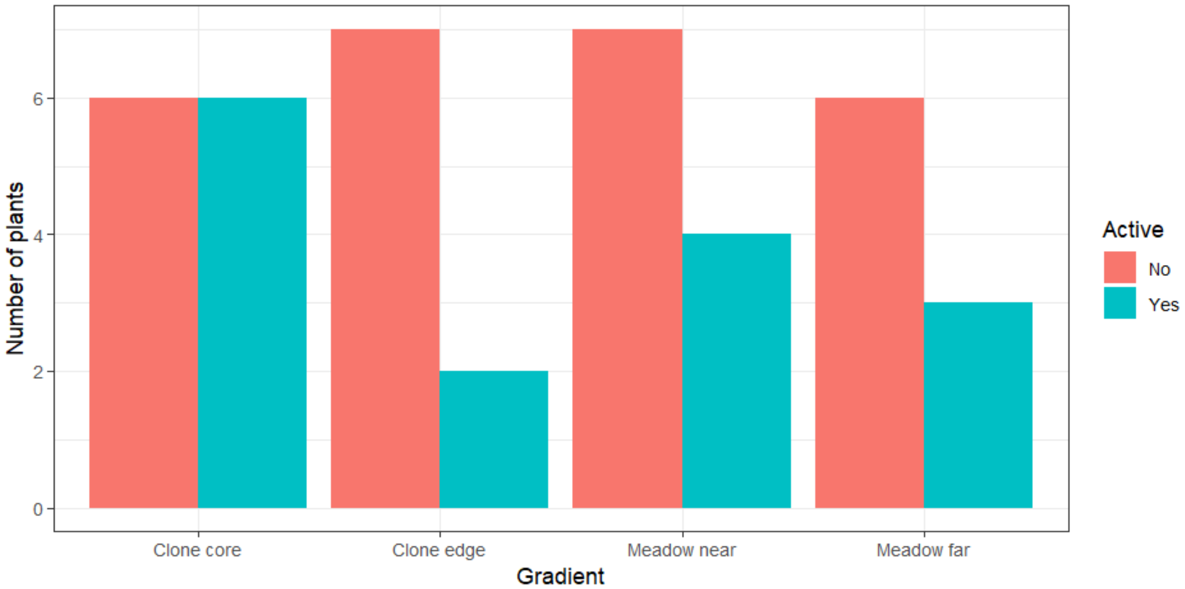

Figure 13: Distribution of T. pratense individuals with active and not active nodules across the gradient.

There were in total 134 nodules across the gradient, with the highest number of 47 in meadow near (Figure 14). The lowest number was in the clone edge with 27 nodules, followed by 29 nodules in meadow far and 31 nodules in the clone core. The percentage of active nodules were the highest in the clone core (46,16%), followed by meadow near (38,3%) and meadow far (31,03%). The lowest percentage of active nodules were found in the clone edge (14,8%).

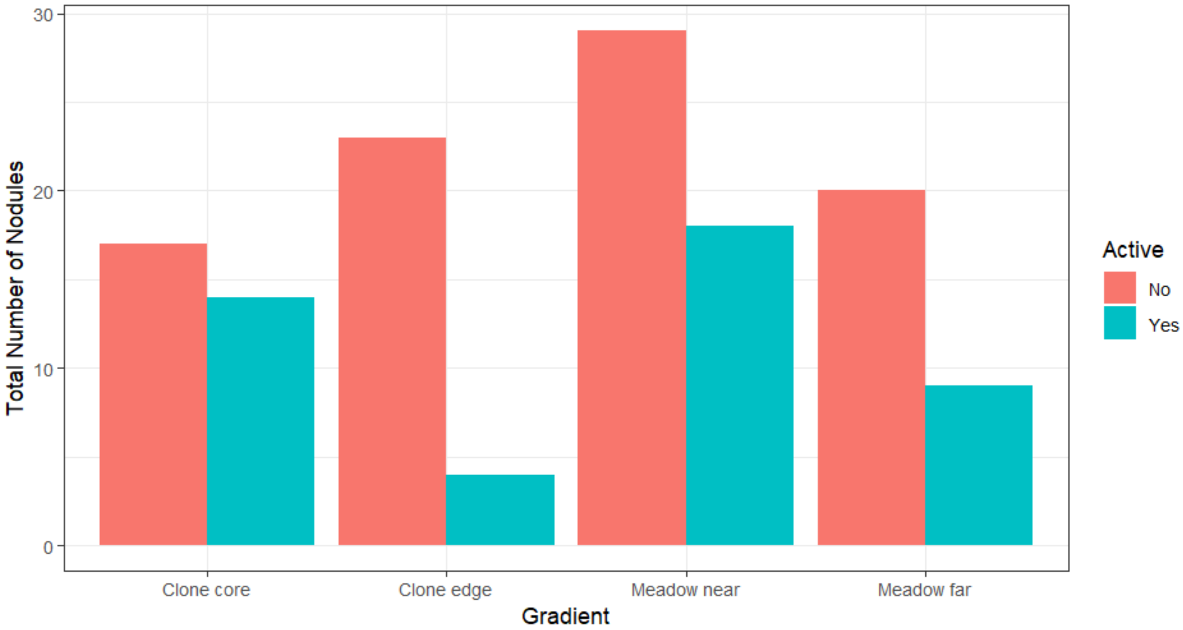

Figure 14: The distribution of active and not active noduels across the gradient.

The length of *T. pratense* increased by 1,64 cm when nodules were present (p=0,00964). The length also increased by 0,4 cm for each number of nodules present at the roots of the plant (p=0,0012). The dry biomass of the species also increased by 0,001933 g for every number of present nodule (p=0,005). However, there were no significant correlations between the dry biomass and presence of nodules on the roots. There were neither any significant correlations between active nodules and plant growth.

3.4 Soil nitrogen content

The content of nitrogen in the soil varied between the sites. Site 2 had the overall highest values measured for nitrogen across the gradient. The sites with the overall lowest values for nitrogen content had values between 0,1% and 0,5%. The mean values for nitrogen content across the sampling points were all between 1% and 1,25% (Figure 15). There were no significant differences between any of the sampling points.

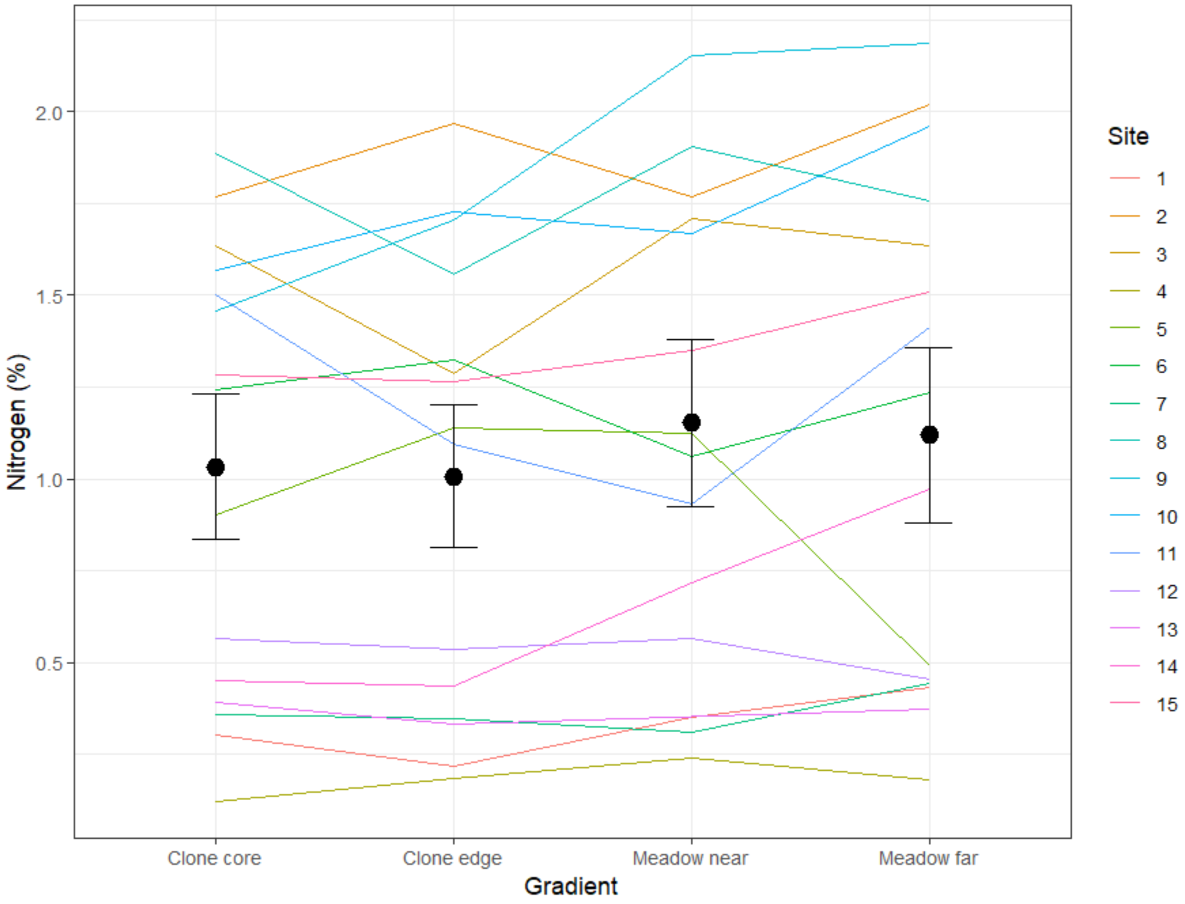

Figure 15: Nitrogen content at each sampling point in the encroachment gradient. The black points show the mean for each sampling point, with a 95% confidence level. The colored lines are the average nitrogen content from the replicates at each site and sampling point.

There was a significant positive correlation between length of *S. virgaurea* and the nitrogen content of the soils (p=0,0221). The same relationship was found for the dry biomass of *S. virgaurea* (p=0,0109). Similar response was found for *T. pratense* for both length (p=0,00242) and dry biomass (p=0,0117). This means that the growth of these two species increased when the nitrogen content of the soils increased.

3.5 Local seedbank

There were in total 230 seeds that germinated from the local seedbank of the soils. The clone core had the lowest number of 31 germinated seedlings, followed by 53 at the clone edge, 74 in meadow near and 72 in meadow far (Figure 16). Grasses were the most abundant group in the local seedbank, followed by forbs which had six fewer germinated seeds across the encroachment gradient. The number of grass seedlings were lowest in the clone core and accounted for only 19% of the germinating seeds in this sampling point. The lowest values of germinating forbs were at the clone edge. Only 18,8% of all the germinating seedlings at this sampling points were forbs.

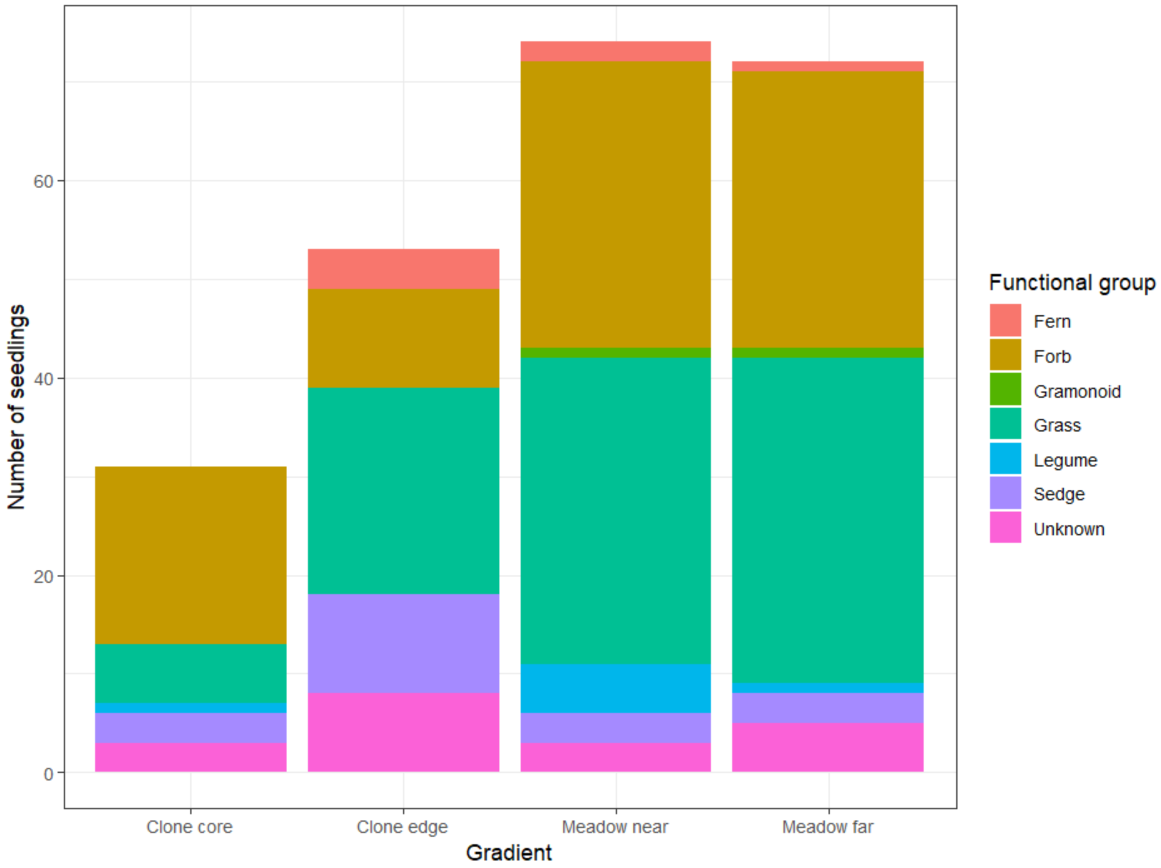

Figure 16: Total number of seedlings from the local seedbank and their functional group across the gradient.

4 Discussion

4.1 *E. nigrum's* allelopathic effect on survival and growth

In this study, *E. nigrum's* impact on soil growth condition in an *E. nigrum* encroachment gradient was investigated using a bioassay. There were no overall patterns that indicated any connection between the impact of *E. nigrum* on the soil, and the survival rate of the plants. Contrary to the expectations, the lowest number of dead plants was found in the clone core, where the abundance of *E. nigrum* was the highest. This contradicts the hypothesis, whereas there was no overall trend of plants that died connected to the *E. nigrum* encroachment gradient. Meanwhile, the clone core was the sampling point with the overall lowest values for growth. The number of seedlings that germinated from the local seedbanks were also the lowest for this sampling point. Less plant biomass in each of the pots causes less competition between the species for nutrients and space (Aschehoug et al., 2016). This could have influenced the number of dead plants in the clone core. The overall trends do not correspond to what was expected considering the allelopathic capacity of *E. nigrum.*

T. pratense was the only species that withered in all the sampling points. Meanwhile, the number of dead plants was the lowest in the clone core. The number of *T. pratense* with nodules and active nodules were the highest in the clone core, which might have contributed to a higher survival rate of the species in this sampling point, compared to the other three. Seeds of *T. pratense* germinated faster than the two other species and were therefore more developed when the bioassay experiment started. It is possible that the fast germination had an impact on the result, including the number of dead *T. pratense*.

T. pratense is a legume and had a different growth pattern in the gradient compared to the two other species. The growth trend was unexpected and non-linear. Even though there were significant differences between some of the sampling points, these cannot be directly connected to the allelopathic influence of *E. nigrum* in the gradient. The result of *T. pratense's* growth pattern was influenced by other factors such as nodulation and nitrogen availability.

P. alpinum is the only species which had significant differences between the clone core and meadow near for both the dry biomass and the length. There was an increase of growth from the clone core to meadow near. Earlier studies have found grasses and forbs to be most sensitive to the impact of *E. nigrum* in the seedling stage (Bråthen et al., 2010; Mod et al., 2014; Pellissier et al., 2010; Wilson & Nilsson, 2009). A study which investigated the direct influence of batatasin III on species germination found that *Phleum pratense* were among the most sensitive grass species, were the smallest concentrations of batatasin III inhibited more than 50% of germination (Gonzáles et al., 2015). This contributes to the understanding of why *P. alpinum* is more sensitive to *E. nigrum´s* allelopathic capacity early in its encroachment, compared to the other species used in the bioassay. The results from the bioassay shows that the allelopathic capacity also have a negative effect on the growth of the seedlings, in addition to the germination phase. At the same time, this does not explain why there was a decrease from meadow near to meadow far for this species. This is an unexpected result, whereas these values were expected to be even higher than the values found in meadow near. Meanwhile, the decrease of growth between these sampling points were common for the three measured species. It is challenging to find an explanation to this result, whereas there are no comparable studies similar to this project. However, there are measurements that were not conducted that potentially could contribute to understanding these results. Competition for space between roots (Cahill, 1999; Schenk, 2006), different texture and compactness of soils (Dexter, 2004), or changes in pH (Neina, 2019) between the sampling points are factors that were not investigated and can influence the plant growth. The differences for the sampling points with only one meter distance between each other can potentially be explained by microtopography in the gradient, which can create differences of growth conditions in the soils (Falco et al., 2019). Another explanation could be the edge effect, which refers to the variation of ecological processes in the transition zones between different ecosystems (Liding et al., 2004). A study on the edge effect of shrub patches in different types of grasslands found that the peak values of soil indexes were within one meter distance to the edge (Wang et al., 2022). The edge effect could therefore be an important factor which potentially contributed to the high growth results in meadow near.

S. virgaurea is a forb and has a small increase of growth from the clone core to meadow far for the length, and to meadow near for the dry biomass. This indicates that the species is to a small degree sensitive to the impact of *E. nigrum's* proximity during an encroachment. Earlier studies of the impact of batatasin III on *S. virgaurea* suggests that the growth of this species is being inhibit by the humus impacted by *E. nigrum*, and few survive the seedling stage in *E. nigrum* shrublands (Bråthen et al., 2010; Gonzáles et al., 2015). However, the results from *S. virgaurea's* growth in the bioassay were not significant, and the growth results were not necessarily impacted specifically by the presence of *E. nigrum* during its encroachment. Three individual plants of *S. virgaurea* died in the encroachment gradient, and all of them were in meadow far, where the impact of *E. nigrum* is expected to be the smallest. The near horizontal results for the growth for *S. virgaurea*, together with the individuals that withered, could explain the lack of *E. nigrum's* impact on the soil early in its encroachment, as it is already known that *S. virgaurea* struggle with surviving in soil that is highly influenced by *E. nigrum* (Gonzáles et al., 2015). *E. nigrum* must be present for a longer time than ~15 years for this allelopathic effect to be significant on the growth of *S. virgaurea*.

The overall result of the average growth and dry biomass for the three species together, show a significant decrease in dry biomass from meadow near to meadow far. This was the only significant difference between the sampling points in the encroachment gradient. However, these were the two sampling points that was expected to be the least influenced by *E. nigrum*, and the growth was expected to increase from meadow near to meadow far. The allelopathic effect has been found to be more species specific, meaning that it has varying impacts on different plant species rather than having the same negative impact on every plant species (Bråthen et al., 2010; Brännäs et al., 2004; González et al., 2015; Nilsson et al., 1998). The values for the three species together would therefore not provide knowledge of a connection between *E. nigrum's* influence on the soil growth conditions. At the same time, the three species belong to functional groups that are negatively influenced by *E. nigrum´s* allelopathy (González et al., 2015). Meanwhile, none of the growth results for the three species separately had a significant positive trend from the clone core to meadow far, as this study expected. The only species that partly met the expectations of growth in the *E. nigrum* encroachment gradient was *P. alpinum*. The allelopathic capacity of *E. nigrum* cannot alone be the main mechanism behind the encroachment.

Soil samples were collected from 15 different sites across Troms and Finmark county. The different sites were chosen to investigate if there were an overall influence of *E. nigrum* during its encroachment on the soil growth conditions, independent of site factors. However, the growth results had a large variety between the sites. There were site specific differences across the studies variables. This highlights the importance of how environmental site-specific factors influence plant growth, and has a larger influence on the results than the proximity to *E. nigrum* does during an encroachment. The different growth results between the sites can be interpreted as a result of the soil properties at each location. The fate of allelochemicals is determined by abiotic factors such as soil bulk density, retention, transport and transformation after the allelochemicals are released from a plant (Cheng, 1995; Parepa & Bossdorf 2016). The amount and species composition of soil microbes are also influencing the fate of the allelochemicals in the soils (Inderjit, 2005; Mishra et al., 2013). This means that the allelopathic capacity of *E. nigrum* potentially is determined by the soil conditions, which is likely to vary between the sites. The complexity of how these factors influences *E. nigrum´s* role in different ecosystems could therefore vary, which also can explain the diversity between the sites for the growth results.

4.2 The effect of *E. nigrum* encroachment on nodule development

The highest number of plants with nodules were found in the clone core, followed by meadow near. These were the two sampling points that also had the highest number of nodules, in addition to active nodules. The highest values for *T. pratense's* growth were also in these two sampling points. Studies have found that secondary metabolites released from *E. nigrum* limits soil nitrogen availability to plants (Wardle et al., 1998). It was therefore expected that the number of nodules, and active nodules would be highest at the clone core and decreasing towards meadow far. The result partly supports this hypothesis; however, the trend is not linear across the gradient. The high number of nodules and active nodules in meadow near were contradictory to the expectations. Root nodules is created by a symbiotic relationship between the plant and nitrogen-fixing soil rhizobia (Andrews et al., 2013; Raven, 2010). The presence of these bacteria in the soil can differ between the sampling points and sites, which could be the reason for the high number of nodules in meadow near.

The presence of nodules and number of nodules had a positive effect on the growth, except for the correlation between presence of nodules and dry biomass. It is therefore likely that the growth pattern found in the encroachment gradient can be due to the development of nodules, independent of the effect of *E. nigrum* in the encroachment gradient. Former studies of different legume species suggest that high values of soil nitrogen results in depressed nodulation (Abdel-Wahab & Abd-Alla, 1996; Xia et al., 2017). Nitrogen fixation in the atmosphere has a higher energy cost compared to roots, which absorbs nitrogen directly from the soil (Voisin et al., 2002). When enough organic nitrogen is accessible in the soil, nitrogen fixation in the nodules would be unnecessary use of energy. However, small amounts of additional nitrogen have been found to increase nodulation (Abdel-Wahab & Abd-Alla, 1996; Xia et al., 2017). Studies of another legume species suggest that seedlings are less influenced by nitrogen content in soils, when developing nodules (Alon et al., 2021). The correlation between the number of nodules and *T. pratense's* growth might therefore be partly explained by the soil nitrogen content at each site. However, there were no correlation between the presence of nodules, number of nodules or active nodules and nitrogen content in the soil. Meanwhile, the number of plants with nodules and active nodules in the clone core could also be a result of *E. nigrum's* negative impact on nitrogen accessible for plants.

4.3 Soil nitrogen content under encroachment of *E. nigrum* and nitrogen's influence on plant growth

Soil nitrogen is essential for plant metabolism and is crucial in protein and nucleic acid synthesis which forms the living materials (Shrivastav et al., 2020). Nitrogen is therefore among the most important minerals for plant growth and development, along with phosphorus and potassium (Shrivastav et al., 2020). Arctic- and alpine tundra ecosystems are characterized by low productivity, mainly due to low temperatures and short growing seasons that constrains the development and growth of plants (Ernakovich et al., 2014). The low amount of accessible nutrients in the soil, especially nitrogen, might however be the major limitation of plant growth (Chapin et al., 1995). Phenolics that are released by *E. nigrum* to the soil system slows down soil processes and limit the nutrient content in the soil (Hättenschwiler & Vitousek, 2000). Organic nitrogen can also be bound to protein-phenolic complexes, directly decreasing the available nitrogen to plants (Northup et al., 1995). The soil collected for this study had no differences of nitrogen content in the soil across the gradient. This can indicate that *E. nigrum* would have to be present for more than 15 years, to influence the decomposition rate of nutrients in the soils, and further the nitrogen content and plant growth. However, the method used to analyze nitrogen content in this study displays the total soil nitrogen content, where nitrogen might be bound to protein-phenolic complexes or other organic molecules and were unavailable to plants. It is therefore not possible to know if *E. nigrum* is influencing the available nitrogen for plants when the species has been present for approximately 15 years, based on the method used in this study.

The values for length and dry biomass for *S. virgaurea* and *T. pratense* were significantly connected to nitrogen content in the soils. The growth increased, as the nitrogen content of the soil increased. These two species are therefore more influenced by the nitrogen content in the soils, than the presence of *E. nigrum* for ~15 years considering their growth. Meanwhile, *P. alpinum* did not have a significant correlation between growth and nutrient content. A comparison of nutrients influence on growth at each site also demonstrates that *P. alpinum's* growth varies independent of the nitrogen content.

Low nitrogen in leaves and roots have earlier been found for grasses, including *P. alpinum* (Makarov et al., 2020; Salpagarova et al., 2014). Low leaf nitrogen content is connected to resource conservation and stress tolerance, which are associated with specialized species

(Garnier et al., 2016; Denelle et al., 2020). This species is distributed in mountain areas around the world but is also found by the coast at latitudes above 60° (Stewart et al., 2011). *P. alpinum* is therefore adapted to grow and survive in challenging conditions such as alpine and arctic areas (Körner, 1995), where the soil nitrogen content is low (Chapin et al., 1995). *S. virgaurea* and *T. pratense* have a wider distribution in temperate and northern regions of Europe, Asia and North Africa, where they occupy diverse environments (Hultén & Fries, 1986; Kiełtyk & Mirek, 2014; Semerikov et al., 2002). Both species have been found to contain higher nitrogen content in roots compared to *P. alpinum* (Salpagarova et al., 2014). Nitrogen content in roots is positively correlated with nitrogen content in leaves, which indicates that the species has a higher leaf nitrogen content than *P. alpinum* (Salpagarova et al., 2014; Tjoelker et al., 2005). This suggests that *S. virgaurea* and *T. pratense* have a different nutrient allocation compared to *P. alpinum*¸ and together with the wide distribution of these species, they can be described as generalist plant species. This differs the species from *P. alpinum,* which is specialized to live in challenging environments and soil with low nitrogen content. The dependency of soil nitrogen for growth of *S. virgaurea* and *T. pratense* in this study can therefore be explained by their functional traits and nutrient allocation, which differ them from the specialist *P. alpinum* (Pérez-Harguindeguy et al., 2016).

4.4 *E. nigrum´s* influence on the local seedbank

The local seedbank of the soil samples can be used as an indicator for the capacity of the soil to germinate seeds when *E. nigrum* is not physically present. The clone core had the lowest number of germinating seeds, with less than half of the seeds that germinated in meadow near and far. Furthermore, this sampling point had fewer types of functional groups that germinated, compared to other sampling points. The number of germinating seedlings from the local seedbank was increasing from the clone core to meadow near, with similar numbers of germinating seedlings in meadow near and far. The local seedbank was therefore not influenced by *E. nigrum´s* presence when there was one meter distance to the clone edge. Meanwhile, the soils where *E. nigrum* has been present for ~15 years are still fertile enough for seeds to germinate. It is likely the physical absence of *E. nigrum* in the bioassay contributes to better conditions for seeds to germinate.

Grasses are among the most sensitive functional groups to the allelopathic influence of *E. nigrum* (Gonzáles et al., 2015). This group had the fewest germinating seeds in the clone core compared to the other sampling points. Forbs however, had similar amounts of germinating seeds in the clone core and the other sampling points. The results contribute to the assumptions of *E. nigrum* having a higher influence on grasses than other functional groups early in the encroachment. The diversity index of the vegetation cover differs between the sampling points, with the lowest values in the clone core (Bråthen et al., 2023). It is likely that the number of germinating seeds is related to the low diversity index. However, this provides knowledge of how the species composition above ground influenced the number of seeds that germinate at the clone core, in addition to what type of functional groups that are capable to germinate in soils influenced by *E. nigrum.*

4.5 Potential mechanisms behind *E. nigrum's* encroachment

There were not many significant differences for the growth of the three species in the encroachment gradient. This indicates that *E. nigrum* must be present for more than 15 years to have a consistent negative effect on the growth conditions in the soil for other species. However, grasses like *P. alpinum* might be more sensitive to the early phase of the encroachment compared to other functional groups. This is supported by another study of batatasin-III impact on germination on different species (González et al., 2015). This study showed that *Phleum pratense* was more sensitive during germination than *S. virgaurea,* which was unaffected by the highest concentration of batatasin-III used in the study (4mM) (González et al., 2015). Our study indicates that species have different responses to *E. nigrum's* influence on soil growth conditions during its encroachment, as well as germination. Hence, the release of secondary metabolites from *E. nigrum* leaves cannot alone explain the increasement of the species.

The allelopathic impact has earlier been described as one of the main factors for the successful encroachment of *E. nigrum* (Bråthen et al., 2010; Nilsson & Wardle 2005; Gonzáles, 2018). However, this is not evident in the findings from this study. Soil influenced by *E. nigrum* for ~15 years had little or no impact on growth of three species from three different functional groups. The species would have to be present for a longer period to influence the soil growth conditions for other species, which eventually would contribute to the species' encroachment. Meanwhile, the allelopathic effect and the negative impact this has on other species' germination, establishment and growth is necessary for *E. nigrum* to maintain its dominance over larger areas.

The vegetation cover in the encroachment gradient has been investigated in a separate study (Bråthen et al., 2023). The diversity index from the clone edge and meadow near had a mean close to 0,7, while the mean at the clone core was about 0,3 (Bråthen et al., 2023). This means that only a few species dominate the vegetation in the clone core, while the distribution of species is more even at the clone edge and the meadow. The difference between the clone core and clone edge highlights *E. nigrum* success in encroaching into new habitats. Over ~15 years, there is a large decrease in the evenness of species diversity. The differences in growth conditions across the gradient in the bioassay indicate that only grasses, more specifically *P. alpinum,* is influenced by the allelopathic effect of *E. nigrum* early in its encroachment. However, the large changes in the above ground species composition over ~15 years contribute to the assumption that *E. nigrum* is successfully competing with plants above ground before it is significantly influencing the growth properties of the soil.

Other characteristics of *E. nigrum* than the allelopathic properties must be pivotal for the species successful encroachment. Evergreen shrubs, such as *E. nigrum* do not shed their leaves when the growth season is ending (Tomlinson et al., 2013). This means that these plants don't have to use energy on producing new leaves as the growth season is starting, and therefore have a longer period of photosynthesis at the beginning and the end of the growth season (Baldocchi et al., 2010). Hence, evergreen shrubs have more time to gain nutrients, compared to other species. This is an advantage over deciduous plants that produces new leaves at the beginning of the growth season and shed their leaves as the growth season is coming to an end. *E. nigrum* is also one of the species that flowers first after snowmelt in tundra areas (Bell & Tallis, 1973), which contributes to the dispersion of the species before many other plants have the possibility. The growth of *E. nigrum* is compact, which creates shadow and less accessible sunlight for other plants to grow (Bråthen et al., 2023). Only the berries are eaten by herbivores, and the rest of the plant is left undisturbed (Ims et al., 2007; Iversen et al., 2014). Other plants preferred by herbivores is therefore more exposed to grazing in areas where *E. nigrum* is present (Hanley & Taber, 1980: Pastor et al., 1988). These are all advantages for *E. nigrum* when the species is competing with other plants, which is likely to contribute to a successful encroachment of the species into new areas. However, it is not known if these advantages are the main reasons for the success of *E. nigrum´s* encroachment. Other mechanisms than the allelopathic capacity must therefore be investigated to further understand how this species continues to increase its abundancy in the arctic tundra, especially during the encroachment process.

4.6 Suggestions for *E. nigrum* management

The encroachment of *E. nigrum* into new habitats can lead to a loss of important forage areas for grazing animals (Tuomi et al., 2022). Local reindeer herders, sheep farmers and scientist in Northern Norway have since 2020 tested different methods, where the aim was to transform small patches of *E. nigrum* shrubland into more species rich areas (Bråthen et al., 2023). The findings from these experiments will be used to investigate potential management methods for minimizing the encroachment of *E. nigrum* and increasing the biological diversity. Growth results from the *E. nigrum* encroachment gradients indicates that the species would have to be present for more than 15 years to perceptibly change the growth conditions in the soil for other plants species. These results can be valuable when possibilities for management of *E. nigrum* are evaluated. It often takes years for other plant species to reestablish in soil were *E. nigrum* has been removed, after influencing the soil for a long time (Bråthen et al., 2023). This is due to the quantity of secondary compounds released by *E. nigrum* for a long period, which makes the soil unfertile for many native plant species (Bråthen et al., 2023; González et al., 2015; Wardle et al., 1998). Removal of *E. nigrum* early in its encroachment would likely lead to better results for reestablishment of meadow species, compared to areas that has been influenced by *E. nigrum* for a long time. If removal of the species is considered as a management method, it would be beneficial to focus on the edges of *E. nigrum* clones. Meanwhile, this study does not provide any precise numbers for the exact time it takes for *E. nigrum* to negatively influence the soil growth conditions. Further investigation of soil from areas where the species has been present for more than 15 years would therefore contribute to more precise numbers. This will provide information of how far from the clone edge towards the core of the *E. nigrum* shrubland the soil is sustained fertile. Furthermore, this will give more precise numbers of how much *E. nigrum* that can be removed from the clone edge into the *E. nigrum* shrubland, without spending extra capacity on soil improvements for plant species to reestablish. In a management perspective, this can slow down or stop the encroachment of the species, in addition to increase the biological diversity, as this soil is more fertile compared to soil that has been influenced by *E. nigrum* for a longer time.

5 Conclusion

The experimental bioassay with soils collected in an *E. nigrum* encroachment gradient confirms that the growth of grasses is more sensitive to the early allelopathic impact of the species during an encroachment, compared to the forb *S. virgaurea* and the legume *T. pratense.* The growth of these two species were directly connected to the nitrogen content of the soils. Presence of nodules and number of nodules also had a positive correlation with the growth of *T. pratense*. The growth results indicates that *E. nigrum* would have to be present for more than 15 years to have a negative influence on the soil's growth condition for other plants. The allelopathic capacity of *E. nigrum* is important for the species to retain its dominance in tundra vegetation. However, other mechanisms above ground are likely to have a crucial role during the encroachment of the species, which must be investigated further to understand why *E. nigrum* continues to increase in the arctic tundra.

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Appendix

Table 1: The site number and their location name.

Site 1	Høybuktmoen	Site 6	Alta	Site 11	Akkarfjord
Site 2	Byluftneset	Site 7	Kvænangsfjellet	Site 12	Kvalsunddalen
Site 3	Ifjord	Site 8	Indre Billefjord	Site 13	Langfjord
Site 4	Bjørnes	Site 9	Kistrand	Site 14	Burfjellet
Site 5	Reppafjordalen	Site 10	Hammerfest	Site 15	Sekkemo

Table 2: Total number of individual plants that were measured for each species at each sampling points in the encroachment gradient.

	Clone core	Clone edge	Meadow near	Meadow far
P. alpinum	28	28	28	30
S. virgaurea	28	30	30	27
T. pratense	24	25	26	26

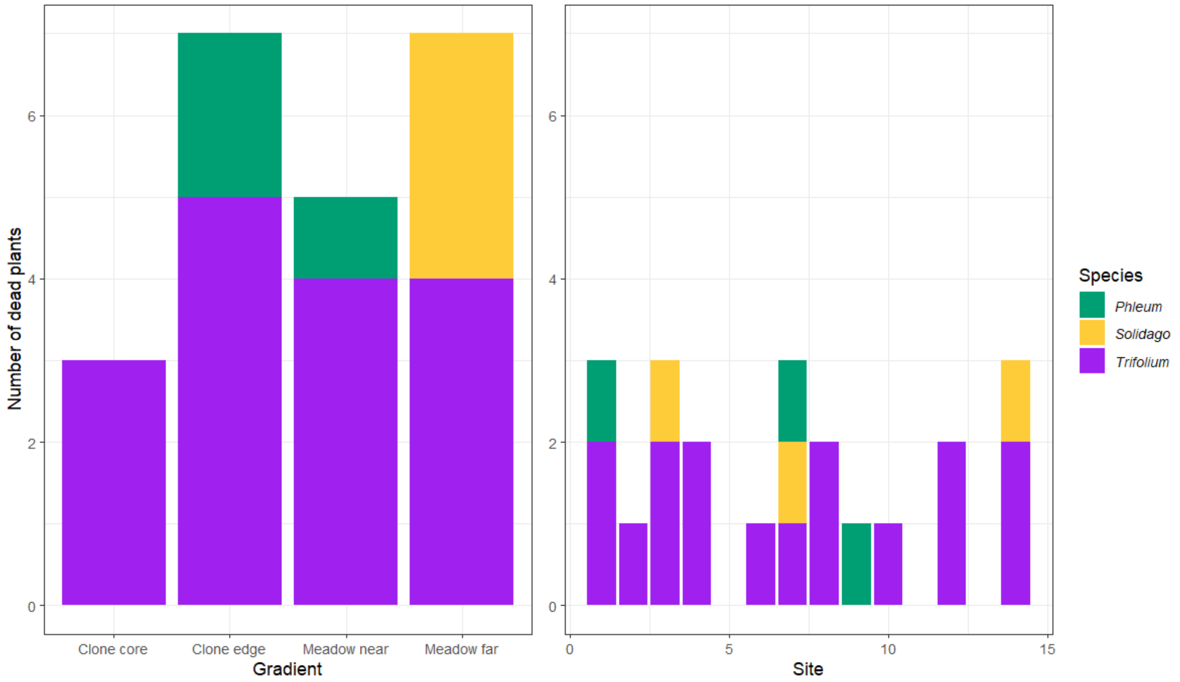

Figure 17: Total plants of each species that died during the experimental bioassay in the encroachment gradient and at each sampling site..

Table 3: Average length (cm) measured of seedlings before potting, at the end of the experiment and their total average growth through the bioassay experiment for the three species. ± standard deviation for seedling height and grown plant height. The numbers are from the collected raw data.

	Seedling height (±SD)	Grown plant height (±SD)	Average total growth
P. alpinum	1,9727 (±0,3957)	24,9704 (±5,2498)	22,9977
S. virgaurea	1,0008 (±0,3206)	5,8435 (±2,3628)	4,8427
T. pratense	3,2368 (±1,058)	7,3901 (±3,1752)	4,1533

Table 4: Average total length for each species at each sampling point, based on untreated data. ± Standard deviation.

	Clone core	Clone edge	Meadow near	Meadow far
P. alpinum	23,1893 (±5,1221)	25,4786 (±5,5437)	27,3862 (±4,7448)	23,8233 (±4,8016)
S. virgaurea	5,625 (±2,481)	5,8 (±2,3715)	5,9133 (±2,0693)	6,0407 (±2,6341)
T. pratense	7,8833 (±3,4481)	6,38 (±3,2427)	8,3154 (±2,3777)	6,9808 (±3,365)

Table 5: Results of P. alpinum´s *estimated marginal mean length at the end of the bioassay. SE = standard error, Df = degree of freedom, cl = confidence level.*

	Fixed values average length (cm)	SE	Df	Lower cl	Upper cl
Clone core	23,2	1,012	19,8	21,1	25,4
Clone edge	25,3	1,009	19,8	23,2	27,4
Meadow near	27,4	0,994	18,7	25,3	29,5
Meadow far	23,8	0,980	17,8	21,8	25,9

Table 6: Results of P. alpinum’s *estimated marginal mean dry biomass at the end of the bioassay. SE = standard error, Df = degree of freedom, cl = confidence level.*

	Fixed values average dry biomass (g)	SE	Df	Lower cl	Upper cl
Clone core	0,0168	0,00204	23,4	0,0130	0,0214
Clone edge	0,0213	0,00229	23,2	0,0169	0,0263
Meadow near	0,0244	0,00242	22,4	0,0196	0,0296
Meadow far	0,0192	0,00213	21,7	0,0150	0,0239

Table 7: Results of S. virgaurea's *estimated marginal mean length. SE = standard error, Df = degree of freedom, cl = confidence level.*

	Fixed values average length (cm)	SE	Df	Lower cl	Upper cl
Clone core	5,59	0,535	22,4	4,48	6,70
Clone edge	5,8	0,525	20,9	4,71	6,89
Meadow near	5,91	0,525	20,9	4,82	7,00
Meadow far	5,98	0,538	22,9	4,87	7,10

Table 8: Results of S. virgaurea's *estimated marginal mean dry biomass. SE = standard error, Df = degree of freedom, cl = confidence level.*

	Fixed values average dry biomass (g)	SE	Df	Lower cl	Upper cl
Clone core	0,0186	0,00404	21,6	0,0112	0,0280
Clone edge	0,0221	0,00434	20,5	0,0140	0,0321
Meadow near	0,0233	0,00444	20,5	0,0149	0,0334
Meadow far	0,0204	0,00425	22,1	0,0126	0,0302

Table 9: Results of T. pratense's *estimated marginal mean length. SE = standard error, Df = degree of freedom, cl = confidence level.*

	Fixed values average length (cm)	SE	Df	Lower cl	Upper cl
Clone core	7,79	0,694	23,1	6,35	9,22
Clone edge	6,36	0,680	21,6	4,95	7,77
Meadow near	8,35	0,672	20,8	6,95	9,74
Meadow far	6,96	0,67	20,6	5,56	8,35

Table 10: Results of T. pratense´s *estimated marginal mean dry biomass. SE = standard error, Df = degree of freedom, cl = confidence level.*

Sampling point	Fixed values average dry biomass (g)	SE	Df	Lower cl	Upper cl
Clone core	0,01734	0,00309	23,9	0,01155	0,0243
Clone edge	0,00973	0,00227	22,5	0,00559	0,0150
Meadow near	0,01846	0,00310	21,7	0,01258	0,0254
Meadow far	0,01144	0,00243	21,6	0,00695	0,0170

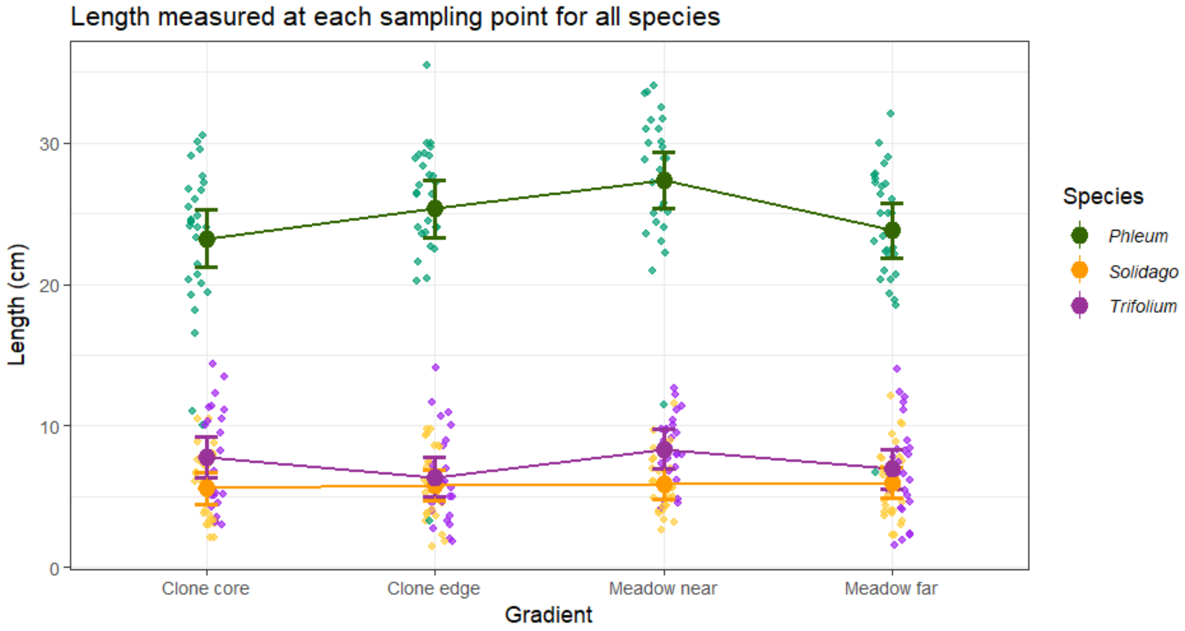

Figure 18: Average fixed effect values of the length for P. alpinum, S. virgaurea *and* T. pratense*, with the overall trendline for each species. The colored points in green, yellow and purple show the raw data of length measured for each individual plant for the respectively species. The points with a darker shows the estimated marginal mean for the fixed effect values for the three different species, with a 95% confidence level.*

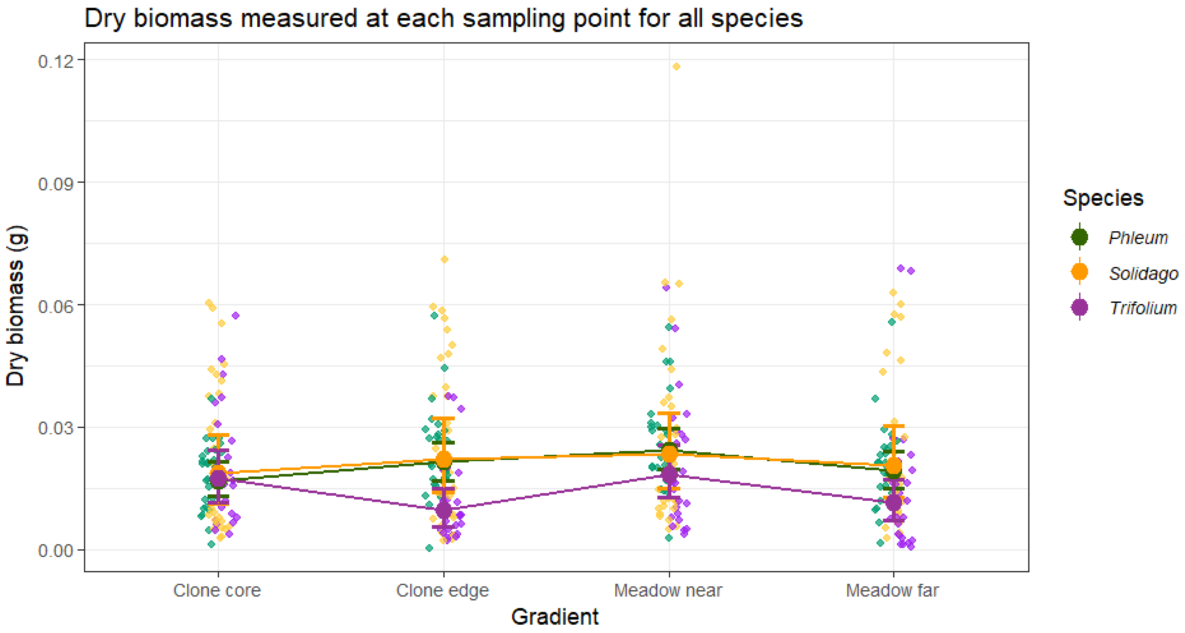

Figure 19: Average fixed effect values of the dry biomass for P. alpinum, S. virgaurea *and* T. pratense*, with the overall trendline for each species. The colored points in green, yellow and purple show the raw data of length measured for each individual plant for the respectively species. The points with an darker color shows the estimated marginal mean for the fixed effect values for the three species, with a 95% confidence level.*

Table 11: Results for the estimated marginal mean of all the species length. SE = standard error, Df = degree of freedom, cl = confidence level.

	Fixed values average length (cm)	SE	Df	Lower cl	Upper cl
Clone core	12,4	1,08	15,6	10,2	14,7
Clone edge	12,6	1,06	14,6	10,4	14,9
Meadow near	14,0	1,04	14,0	11,7	16,2
Meadow far	12,8	1,06	14,6	10,5	15,0

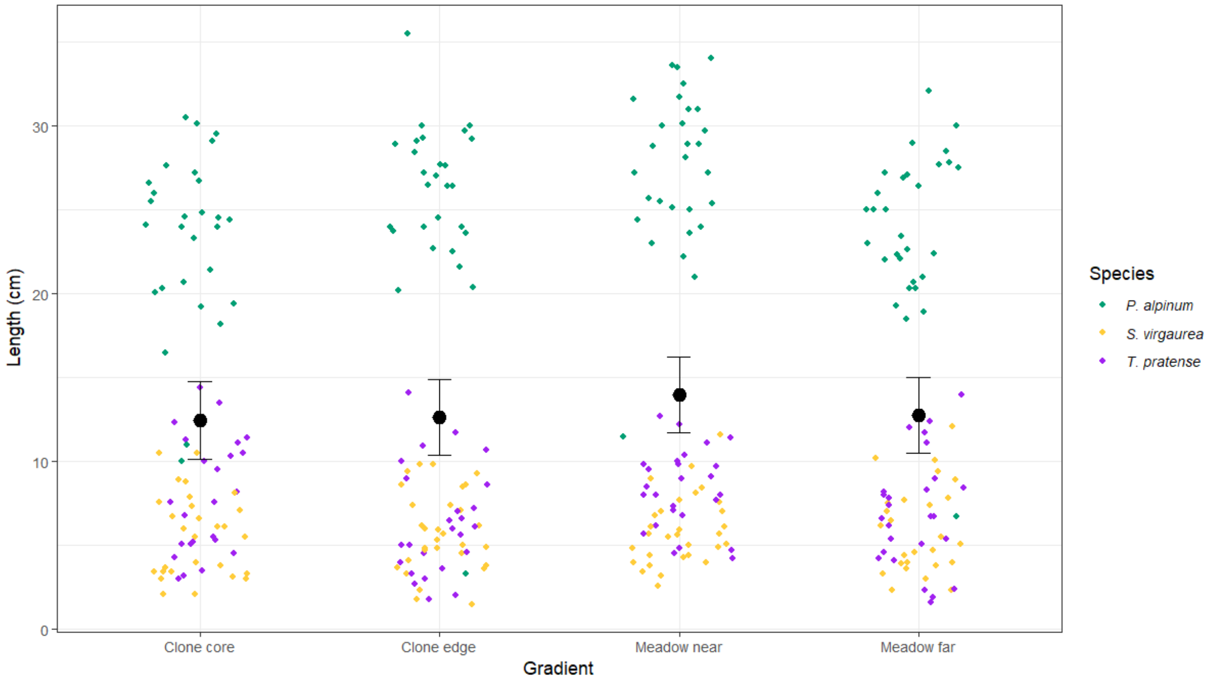

Figure 20: The overall average length measured for the three species in the encroachment gradient. The colored points are the raw data for each individual of P. alpinum, S. virgaurea and T. pratense. *The Black point shows the estimated marginal mean for the fixed effect values, with a 95% confidence level.*

Table 12: Results for the estimated marginal mean for all the species dry biomass. SE = standard error, Df = degree of freedom, cl = confidence level.

	Fixed values average dry biomass (g)	SE	Df	Lower cl	Upper cl
Clone core	0,0174	0,00239	21,4	0,0128	0,0228
Clone edge	0,0177	0,00239	20,7	0,0131	0,0230
Meadow near	0,0222	0,00267	20,4	0,0170	0,0281
Meadow far	0,0169	0,00234	20,7	0,0124	0,0221

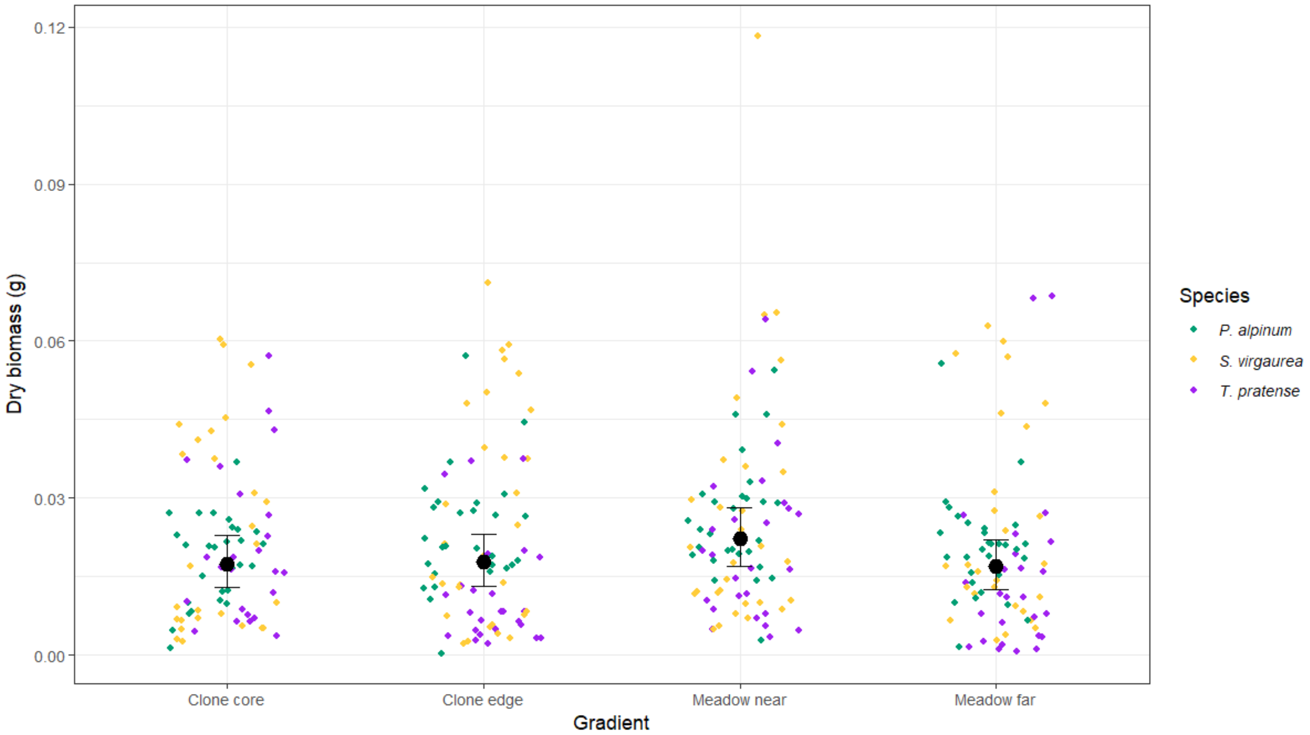

Figure 21: The overall average dry biomass measured for the three species in the encroachment gradient. The colored points are the raw data for each individual of P. alpinum, S. virgaurea and T. pratense*. The Black point shows the estimated marginal mean for the fixed effect values, with a 95% confidence level.*

Table 13: The number of T. pratense *with or without nodules at each sampling point in the encroachment gradient.*

Number of plants/gradient	Clone core	Clone edge	Meadow near	Meadow far
Without nodules	12	16	15	17
With nodules	12	9	11	9
% with nodules	50%	36%	42%	34%
Active nodules	6	2	4	3

Table 14: Total number of nodules and active nodules in the encroachment gradient.

	Clone core	Clone edge	Meadow near	Meadow far
Number of nodules	31	27	47	29
Active nodules	14	4	18	9
% active nodules	45,16	14,8	38,30	31,03

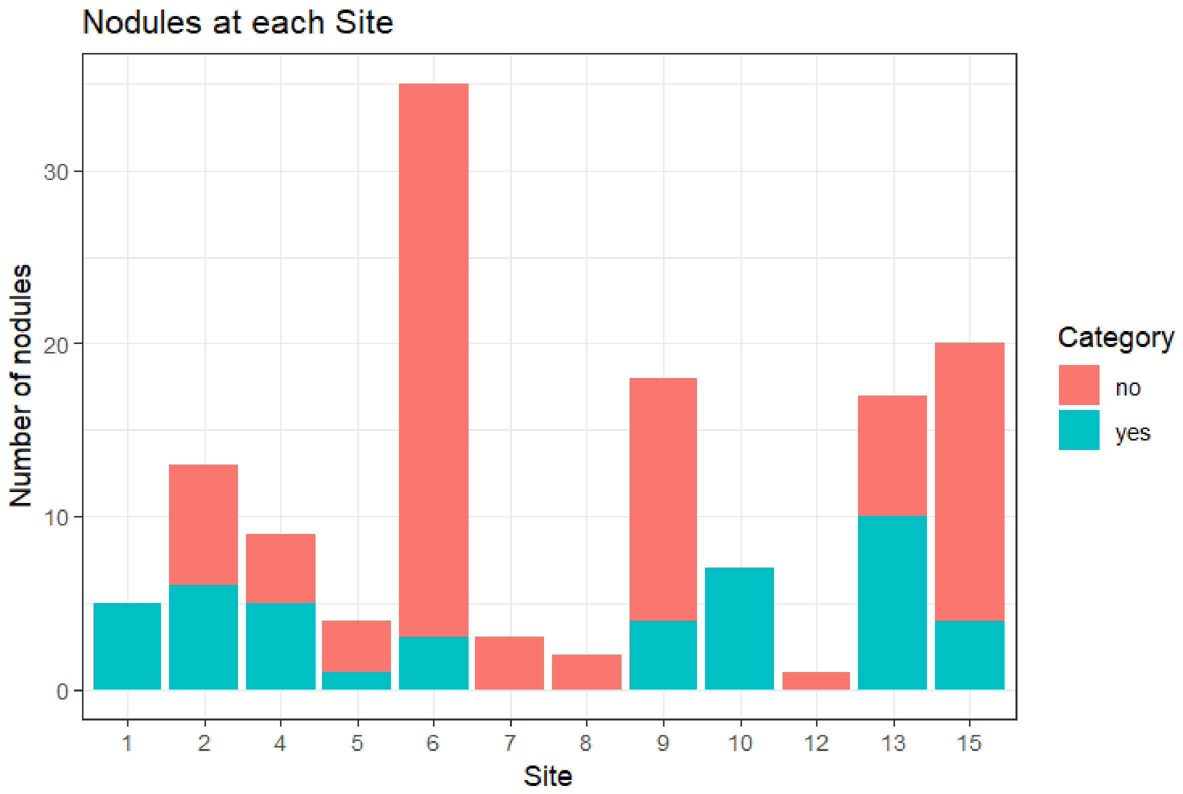

Figure 22: Number of nodules and if they were active or not at each sampling sitet.

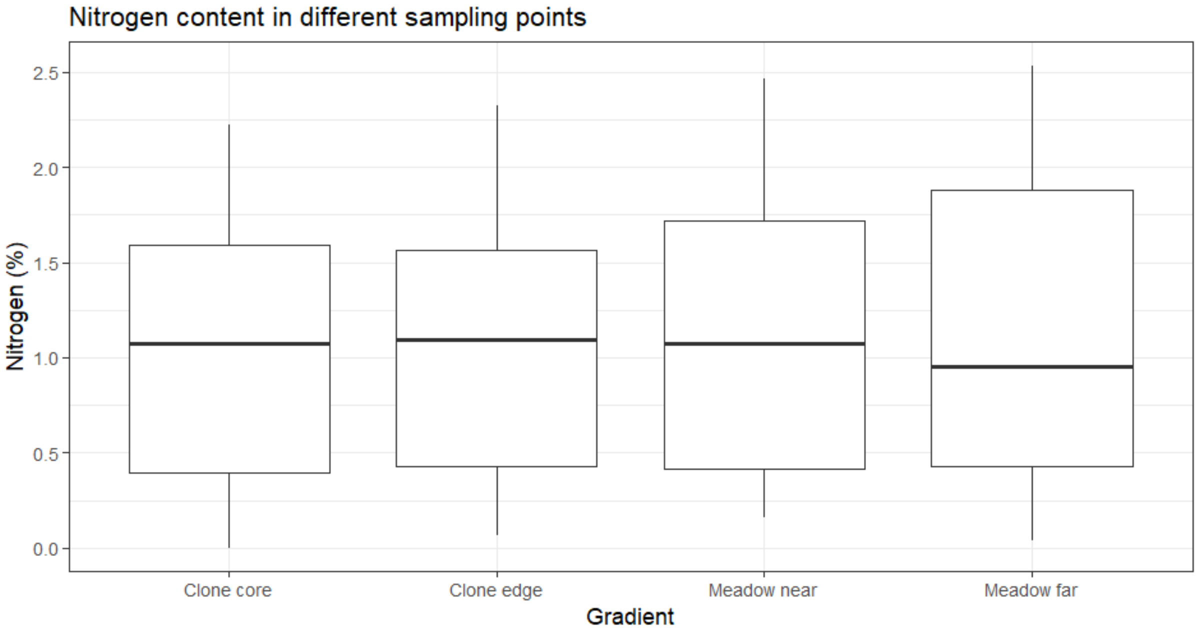

Figure 17: Nitrogen content across the different sampling points in the encroachment gradient for all sites. Boxes represent the interquartile range (IQR) of the soil sampled in the three transect at each sampling point for all sites. The thick line in the box represents the mean. The whiskers represent the minimum and maximum values of IQR times 1.5.

Table 15: The species collected from the local seedbank and their functional group.

Functional group	**Species**
Fern	Unknown species

Forb	*Cerastium sp., Campanula sp., Rumex sp., Achillea millefolium., Solidago sp, Potentilla sp., Silena sp., Ranuculus sp., Sagina saginoides,* unknown
Graminoid	*Luzula sp.,*
Grass	*Agrostis, sp., Poa sp., Avenella sp., Deschampsia sp., Festuca sp.,* unknown
Legume	*Trifolium sp.*
Sedge	*Carex sp.*
Unknown	Unknown functional group and species