Ploidy and Ecophysiology analysis of the *Arenaria ciliata* L. complex (Caryophyllaceae) in Europe

A thesis submitted to the National University of Ireland, Maynooth for the degree of doctor of philosophy

By

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Declaration

This thesis has not been submitted in whole or in any part to this or any other university for any other degree and is except where otherwise stated the original work of the author.

Signed ——————————————————————

Date ——————————————————————

List of Publications and Presentations

- Abukrees, F., Kozlowski, G., and C. Meade (2018). Characterization of diverse ploidy in the arctic-alpine *Arenaria ciliata* species complex (Caryophyllaceae) using shoot meristem staining and flow cytometry analysis of archived frozen tissue. Plant Species Biology 33:144-152. doi: 10.1111/1442-1984.12200.
- Abukrees, F., Jansen, M., and C. Meade *(manuscript in preparation).* Local adaptation in temperature stress responses among ecotypes of the *A. ciliata* complex (Caryophyllaceae).
- Ploidy variation in climate-sensitive Arctic-Alpine plants. 1st Ecology and Evolution Ireland Conference November 2016 Institute of Technology Sligo. Poster presentation.
- Ploidy analysis in climate-sensitive Alpine plants. Maynooth University, Biology Department. Annual Research day 2017. Poster presentation.
- Characterization of diverse ploidy in the arctic-alpine *Arenaria ciliata* species complex. Maynooth University, Biology research day 2018. Poster presentation.
- Local climate adaptation in ecotypes of the *Arenaria ciliata* species complex. IPSAM 2019, Carlow Institute of Technology, 24-26 June 2019. Oral presentation (presented by CM).

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APPENDICES

Abbreviations

µ - micro

°C - degree Celsius

Ac BB - *Arenaria ciliata* from Ben Bulben, Ireland

ALP - Alpine habitat

An Bu - *Arenaria norvegica* from Burren, Ireland

An N - *Arenaria norvegica* from Tromso, Norway

ARC - Arctic habitat

CFIM - Chlorophyll fluorescence imaging

cm - Centimetre

CV - Coefficient of variance

DAPI - 4′,6-diamidino-2-phenylindole (fluorescent stain)

DDH_2O - doubled distilled water

DNA - Deoxyribonucleic acid

dNTPs - Deoxyribonucleotide triphosphate

gm - gram

GPS - Global Positioning system

h - hours

M - Mole

min - minutes

ml - millilitre

mm - millimetre

MOPS - 3-(N-Morpholino) propane sulfonic acid

MS - Salt Murashinge and Skoog

nm - Nanometre

PAM - Pulse amplitude-modulation

PAR - Photosynthetic active radiation

PCR - Polymerase Chain Reaction

PS II - Photosystem II

RNase A - Bovine pancreatic ribonuclease A

TEMP M - Temperate mountain habitat

TEMP SL - Temperate sea level habitat

UV - Ultraviolet

Y(II) - Quantum yield of absorbed energy converted to chemical energy in photosystem II (PSII).

Y(NO) - Quantum yield of non-regulated heat and fluorescence (non-photochemical energy) dissipation in PS II.

Y(NPQ) - Quantum yield of regulated thermal energy (non-photochemical energy) dissipation in PS II

Abstract

The *Arenaria ciliata* species complex (Caryophyllaceae) comprises a group of 3 species with overlapping morphological and ploidy identities. The group has a wide but fragmented distribution across Europe, extending from high habitats in the mountains of central Europe and Iberia to sea-level habitats in the arctic. Because of their cold habitat distribution, they are vulnerable to climate change. This distribution also indicates there are likely to be many local ecotypes across the fragmented distribution. The aim of this project was to better understand the biology of *Arenaria ciliata* species complex, in terms of whether and how these species respond to various environments, measured for 5 ecotypes from Ireland, the Swiss Alps, Northern Norway, and Svalbard in the Arctic. This study demonstrated wide variation in ploidy and ecophysiology among ecotypes of *A. ciliata* species complex. Ploidy analysis reveals that among analysed taxa various ploidy levels were recorded. Distribution of ploidy variation of *A. ciliata* species across Europe in the present study indicates that polyploidisation has occurred on multiple separate occasions in isolated populations, but only in Alpine habitats. Biometric analysis showed that local Irish ecotypes of both *A. ciliata* and *A. norvegica* retain the ability to grow and survive through freezing habitat conditions. However, *A. norvegica* shows clear divergence from *A. ciliata* in terms of growth and phenology. Chlorophyll fluorescence analysis illustrated that cold stress tolerance capacity in each ecotype is more closely associated with the climate of the home population site than with evolutionary lineage, showing that cold stress tolerance capacity is greatest in the ecotypes of *A. bernensis* (Swiss Alps) and *A. norvegica* (Norway). These analyses showed also there is a clear improvement in cold and heat stress tolerance between juvenile and adult plants of *A. ciliata* and *A. norvegica* from Ireland. Finally, with regard to likely future climate change stresses, adult plants of all analysed ecotypes displayed very strong heat stress tolerance.

Summary of Key Findings

The *Arenaria ciliata* complex is a group of perennial herb species from the family *Caryophyllaceae* comprising 3 core species, *A. ciliata*, *A. norvegica* and *A. gothica*, and two additional species associated with *A. ciliata* (*A. ciliata* subsp. *pseudofrigida* and *A. ciliata* subsp. *bernensis*) distributed in mountainous and arctic areas north of the Mediterranean basin. The group has a likely origin in the mid- to late-Pleistocene era, and modern genetic identities seem to have diverged between 50-200,000 years bp. Since that time, and especially in the period since the last ice age began 30k years bp, many populations have become isolated and genetically distinct throughout the range of the complex due to postglacial habitat fragmentation. The *A. ciliata* complex is considered to be sensitive to global warming in terms of both geographical distribution and genetic diversity because they are limited to disjunct habitats characterized by mean low temperatures.

This project had two principal aims: (i) to understand the relationship between ploidy and genetic diversity in the *A. ciliata* complex, and (ii) to analyse how ecophysiological adaptation varies between different ecotypes of the complex, especially with respect to local climates across the biogeographical range of the species.

Following assembly of a seed and living plant collection, an updated phylogeny for the group was completed using phylogenetics methods (Chapter 2). Next, in order to understand ploidy variation in the complex, a novel composite staining protocol, modified from several sources, was developed, evaluated, and subsequent chromosome counting completed using confocal microscopy and flow cytometry (Chapter 3). Observed ploidy levels revealed a clear evaluation of ploidy state variation across European populations of *Arenaria ciliata* complex group. When analysed alongside the phylogeny, this data reveals that polyploidisation has occurred many times,

independently, across the range of the species complex. Northern and northwestern ranges are associated with low ploidy levels, while high ploidy levels are only recorded from southern high-altitude Alpine populations.

Analysis of ecophysiological variation involved the cultivation and analysis of five distinct ecotypes collected from across the range of the *A. ciliata* complex in Europe and the Arctic. The first phase of analysis focused on biometric evaluation of growth responses in *A. ciliata* (from Ireland) and *A. norvegica* (from Ireland) when grown over a single 4 season cycle in 4 distinct habitat settings that reflect idealised habitats from across the range of the species complex: Temperate Sea Level; Temperate Montane; Alpine; and Arctic (Chapter 4). These analyses revealed many differences in growth and development between the two species, with more rapid growth, earlier flowering, higher chlorophyll content and greater biomass accumulation in *A. norvegica* compared to *A. ciliata*.

The second phase of this work focused on stress tolerance analysis in 5 ecotypes grown in the same seasonal cycle and habitat treatments, *A. ciliata* and *A. norvegica*, as above, plus *A. ciliata* subsp. *pseudofrigida* (from Svalbard), *A. ciliata* subsp. *bernensis* (from Alpine Switzerland) and *A. norvegica* (from Arctic mainland Norway). Analysis of stress responses to changing temperature was completed using photosynthesis fluorometry, where respective levels of utilised light energy [Y(II)], managed excess energy [Y(NPQ)] and unmanaged excess energy [Y(NO)] were recorded at the end of each season in each habitat to determine stress levels and biological function in sample plant cohorts.

In general ecotypes showed maximal Y(II) and minimal Y(NO) levels in their home compared to any of the other habitats, indicating local adaptation. *A. ciliata* subsp. *pseudofrigida* performed least well among all cohorts in the milder seasonal settings, however it displayed significantly better Y(II) performance in freezing conditions. *A.*

ciliata subsp. *bernensis* and *A. norvegica* (from Norway) showed greatest stress tolerance for cold and heat, with elevated Y(NPQ) and reduced Y(NO) evident in all treatments, compared to the other ecotypes. In contrast, *A. ciliata* and *A. norvegica* from Ireland both responded poorly at the beginning of autumn freezing, with relatively lower Y(NPQ) and higher Y(NO) readings, indicating a loss of tolerance to freezing stress, compared to the other ecotypes.

Separately, it was established that the age individual plants influences stress tolerance capacity; in the case of both *A. ciliata* and *A. norvegica*, mature plants displayed enhanced tolerances compared to juvenile seedlings. Additional investigations showed that in freezing conditions, leaves covered by snow displayed significantly greater Y(NPQ) capacity compared to leaves exposed to freezing air. Additional heat-stress tests revealed that all ecotypes retain the ability to photosynthesise are near maximal rates at 30°C, indicating that capacity for cold stress tolerance does not inhibit capacity for heat stress tolerance. Overall the data show clear adaptation to prevailing climate conditions among biogeographic ecotypes, suggesting that risk levels to ongoing climate change are variable, depending on local changes.

Chapter 1

1 General introduction.

1.1 Plant growth and development.

The growth of plants is controlled by many factors, some of these factors are external such as environmental and climate while others are internal and affect the ability of plant to respond to and exploit external conditions. Among these light, temperature, and nutrient availability are the most important environmental factors that regulate plant growth and development.

Plant cells have the ability to sense and respond to a variety of external and internal signals (Felle, 2001; Trewavas, 2016). In plants, cells are in continuous communication with one another. Plant cells communicate to organise their activities in response to the changing conditions of light intensity, dark, day length and temperature in the external environment. These communications guide the plant's cycle of germination, growth, development, flowering, and fruiting. Plant cells also signal to one another to coordinate activities in the roots, stems and leaves. In relation to light sensing, set of light-sensitive proteins (photoproteins), which are all structurally related and occur throughout the plant tissue, monitor the direction, duration, quality and quantity of light. Different photoproteins are dedicated to different wavelengths of the light spectrum, and interactions between these receptors determine the activity of signal-transduction pathways and plant responses. For example, phytochromes respond to red light and cryptochromes and phototropin respond to blue light (Alberts *et al*., 2002; Trewavas, 2016; van Gelderen *et al*., 2018) Absorption of blue light controls phototrophic growth of stems toward the light and the opening of stomata, whereas red light determines seed germination time, shoot and root growth and flowering phenology.

Plant hormones are also involved in response to external environmental stimuli, and direct plant growth and development in response to the outside environment, such as light, temperature, daylength, wind or to herbivore and pathogen activity (Nabors, 2004).

Hormones play a key role in a plant's communication system, both internally and externally. Hormones and photoreceptors function to provide plants with information about the environment, and facilitate changes in response to external stimuli or internal needs. They are therefore key players in regulation of plant responses throughout development from seed into maturity. They also regulate plant responses to seasonal variation in daylength, light and temperature (Campbell *et al*., 2008)

The sensing of biotic and abiotic stresses begins with several complex signalling pathways in plant tissue. Some of the early signalling occurrences involve alteration of intracellular Ca^{2+} concentration, associated with production of secondary signalling molecules such as reactive oxygen species (ROS) and inositol phosphate in addition activation of Kinase cascades (Verma *et al*., 2016). Intracellular Ca^{2+} levels increase in response to harsh environmental conditions and these changes in abundance are detected by calcium-binding proteins that act as Ca^{2+} sensors. These sensors can bind to cis-elements of major stress-responsive genes or can interact with DNA-binding proteins and control these genes, resulting in activation or deactivation of specific responses. Moreover, the increase in intracellular Ca^{2+} can activate calcium/calmodulin-dependent proteins, calcium-dependent protein kinases or phosphatases which in turn can phosphorylate or dephosphorylate specific transcription factors. In this way, regulation of gene expression for stress responsive genes can be controlled, and plants maintain a dynamic ability to respond to the environment (Campbell *et al*., 2008; Verma *et al*., 2016)

Light impacts many aspects of plant function. Light intensity impacts on photosynthesis rate and efficiency; day length regulates some of the vital phenological processes such as flowering, seed germination, dormancy and leaf loss. Temperature has a controlling effect on the speed and efficiency of enzymatic reactions. Higher temperature, to a certain extent, is better for plant growth, however some plants need low temperatures to complete the flowering cycle (Hatfield and Prueger, 2015).

There are many stimuli that impact on vegetation development, including climatic, edaphic and cultural conditions. Variations in local climate therefore have a direct impact on plants development and distribution (Mather and Yoshioka, 1968; Mather and Brewer, 2005). For example, in a study of North American vegetation, measured values of Thornthwaite moisture index and annual potential evapotranspiration were found to be the principal climatic limits for vegetation formation classes within each observed biome, in addition to differentiating among the mid-latitude grassland, desert and forest habitats (Mather and Yoshioka, 1968).

Plants exhibit a variety of responses (physiological, biochemical and molecular processes) to biotic and abiotic stresses that enable them to resist or adapt to negative conditions. For instance, when plants are exposed to certain stress this leads to activation of related genes to play important roles in the stress tolerance and adaptation. The most rapid plant response to climate change is likely to be in phenotype (Franks *et al*., 2014).

1.2 Climate change and plant response: migration, adaptation, or extinction.

Many fluctuations occurred in the global climate during the Quaternary glaciations, when ice sheets expanded southward and retreated northward, affecting most living organisms

in terms of both geographical distribution and genetic diversity. In terms of genetic diversity, climatic oscillations and the accompanying changes may influence the genetic structure of populations through adaptations and reorganization processes that could alter the genome structure considerably (Hewitt, 2000).

Earth history includes many gradual and abrupt climate fluctuations that resulted in biological impacts evident on regional, ecosystem and habitat levels (Dansgaard *et al.*, 1993; Zachos *et al*., 2001). Palaeoecological evidence indicates the biota often react to these changes through migration, where species disperse into spatially mobile optimum habitats (Hewitt, 2000; Petit *et al*., 2002)

During the Quaternary period, which began approximately two million years ago, many climatic changes happened as a result of shifts in the Earth's orbit (Taberlet and Cheddadi, 2002). All these changes caused many redistribution events for plant and animal species, to areas and habitats where they were better able to survive. Over the last 20,000 years, since the end of the Pleistocene (the final era of the Quaternary), there has been a period of transition from a glacial to an interglacial environment, with global temperature increase resulting in widespread melting and shrinking of ice sheets. This change contributed to the migration of many populations northwards to occupy new suitable territory. Rapid colonization of limited space often led to a reduction in genetic diversity in populations. On the other hand, a warmer climate on the southern margin of their distribution will reduce the ability of species to survive in these areas (Hewitt, 1996; Ibrahim *et al*., 1996). At lower altitudinal and equatorial areas, increased temperature and water availability and competitive exclusion may have led to many species being replaced (Woodward, 1987).

Rapid climate change may act as a strong selective force in a natural population and its potential interaction with recent habitat fragmentation, as a result of human activities, is leading to increasing the risk of extinction. This scenario can happen if the rate of climate change is occurring faster than the maximum gene flow rate between populations (Jump and Penuelas, 2005), (Figure 1.1).

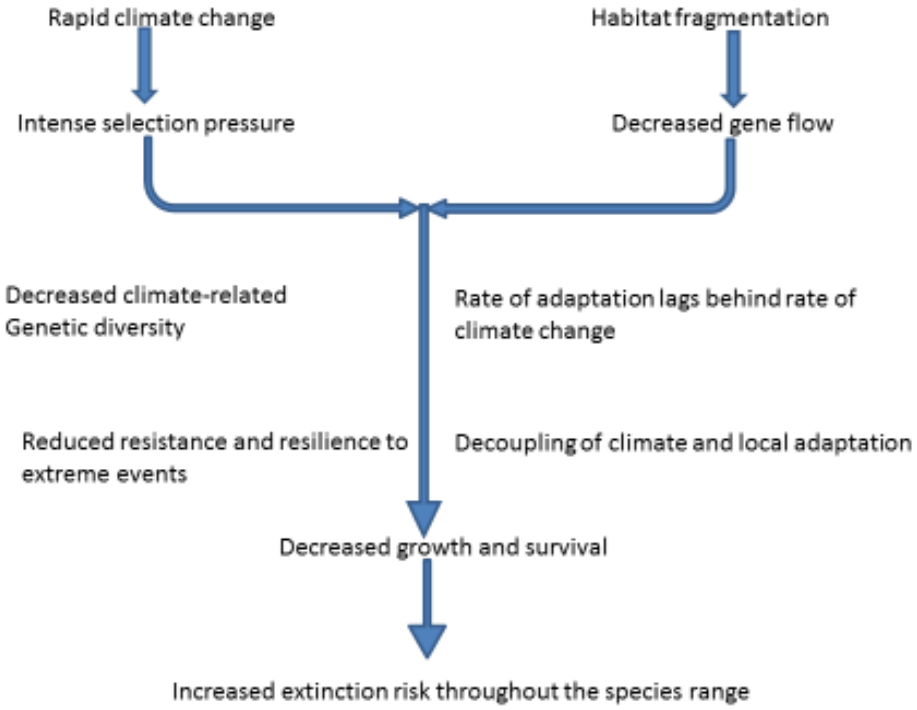

Figure 1.1: The interaction between rapid climate change and habitat fragmentation and their role in the reduction of genetic diversity and increase extinction risk. Adapted from Jump and Penuelas 2005.

1.3 Ongoing impacts of global warming on plant growth, development and productivity.

The average global atmospheric temperature in the 20th century has increased by approximately 0.6 ± 0.2 ˚C and that the rate of temperature increase is accelerating (McCarthy *et al*., 2001,Walther *et al*., 2002; Ciupertea *et al*., 2017). Data from long-term monitoring studies show evidence that the climate of the past three decades is anomalous compared with previous recorded climate variation (Hughes, 2000; Walther *et al*., 2002). These recent temperature and atmospheric trends are already affecting organisms in terms of species distribution, phenology and physiology (Edwards and Richardson, 2004; Hughes, 2000).

Predictions also indicate that temperature is likely to further exceed the observed rate of change in the future with an increase between 0.1˚C and 0.4 ˚C per decade across Europe (McCarthy *et al*., 2001; Gosling and Arnell, 2016). The average changes in temperature over the next 30-50 years are predicted to be within a range of 2-3 °C (Meehl *et al*., 2007; Moritz and Agudo, 2013) (Figure 1.2). In addition, extreme temperature events or heat waves are predicted to be more frequent and last longer compared with recent years (Meehl *et al*., 2007), with increases in temperature of 5 ˚C or more above the historic averages (Hatfield and Prueger, 2015).

Extreme fluctuations in temperature during hot summers can have profound impacts on vegetation causing damage to the molecular and physiological processes that plants depend on for proper growth, thus impairing plant development and plant productivity (Bita and Gerats, 2013; Hatfield and Prueger, 2015). Heat stress induces changes in leaf structure that affects photosynthetic membranes. Moreover, the detrimental impacts of heat on chlorophyll and the photosynthetic system are also correlated with production of

damaging reactive oxygen species (ROS), which leads to oxidative stress (Camejo *et al*., 2006; Hasanuzzaman *et al*., 2013), (Figure 1.3). These structural alterations are often accompanied by ion-leakage from leaf cells exposed to heat stress and changes in energy movement and allocation within the photosystem (Allakhverdiev *et al*., 2008).

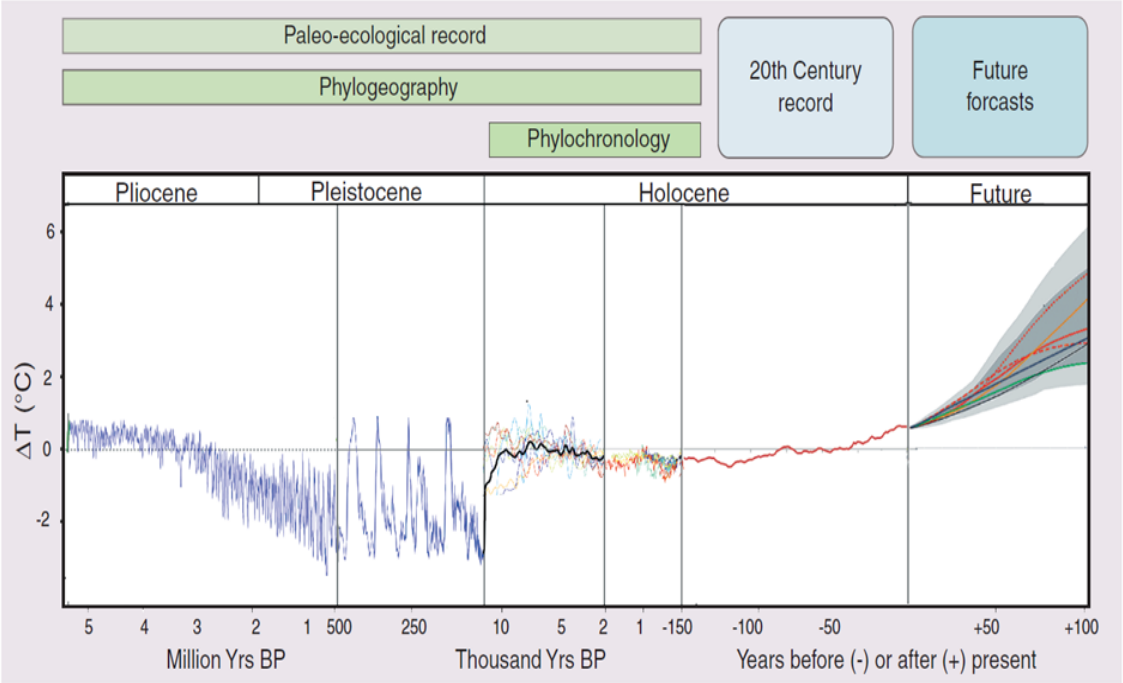

Figure 1.2: Variation in global mean atmospheric temperature compiled from historical records, contemporary observations and from climate model predictions for the near future adapted from Craig Moritz and Rosa Agudo 2013. Data sources are indicated above the plot.

Some of the most direct effects of alterations to ambient climate are in the phenology of animals and plants. For instance, germination and bud burst timing in many plants are determined by spring temperature and these temperatures also influence the timing of growth and reproductive activities in animals (Walther, 2010; Van der Putten *et al*., 2010). Furthermore, changes in temperature occur more immediately in air compared to soil

which can cause delay in responses between aboveground and belowground subsystems (Gehrig-Fasel *et al*., 2008). These time lags in responses vary from year to year and lead to annual fluctuations in species fitness, physiological performance and population abundance (Figure 1.3). However, when changes in temperature begin to progress consistently in one direction, it is likely to affect the interactions of species permanently. If phenotype flexibility is not sufficient, and the rate of adaptive evolutionary changes are too slow to keep pace with alterations in climatic variables, the linked phenology of species at multiple trophic levels will begin to break down (Van der Putten *et al*., 2010).

By adjusting the spatial distribution of ideal habitat conditions for example, northwards in latitude or upslope in a mountain range climate change can alter the position of range limits, leading to range expansion, or range shrinkage. Many of these range shifts have been documented over the last few decades and this process is assumed to continue (Parmesan and Yohe, 2003;McLachlan *et al*., 2005; Cannone *et al.,* 2007; Holzinger *et al*., 2008). Through the last century, climate fluctuation forced many species to alter their range limits and phenotypes (Moritz and Agudo, 2013). Habitat shifts may result in divergence between species, which can cause a break in trophic interactions by introducing differences in dispersal rates and a reduction in geographical overlap between interacting species, and these and other factors may affect species evolution and selection pressure (Menéndez *et al*., 2008).

These changes in temperature are expected to increase the 'push and pull' effect in favour of the migration of plant species, where species are both unable to cope with local temperature rises, and successfully disperse to new areas where temperatures are suitable. As a result species can be expected to move to higher elevations and latitudes as their typical habitat shifts to these locations. The migration rates will differ greatly between

plant species, and thus we might see a new plant formation in response to climate changes (Jump and Penuelas, 2005;Crawford, 2008a, 2008b).

There is ample evidence indicating that climate has already been a key influence determining the plant species range and distributions and in driving changes in organism phenology and genetic identity within populations (Van der Putten *et al.*, 2010; Walther *et al.*, 2002). Repeated climate change events are understood to lead to the accumulation of genome differences and adaptations through the creation of isolated population groups and protected regions from mixing by hybrid zones. The most likely population response to changes in climate is an attempted migration to track the ideal habitats to which they are adapted, resulting in formation of small habitats, which may reduce the genetic variation by reducing gene flow between adjacent populations.

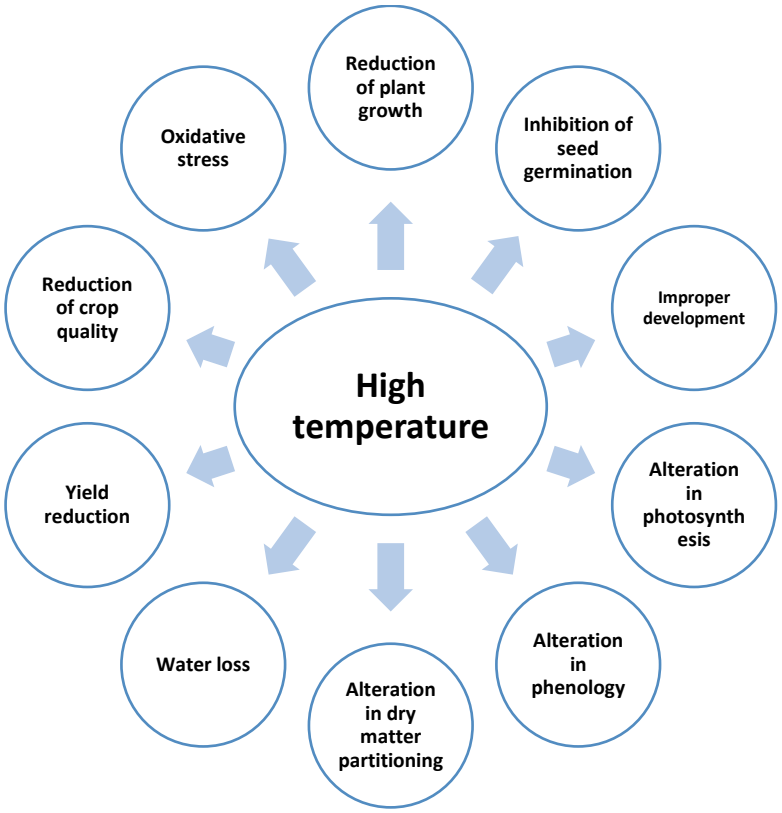

Figure 1.3: Adverse effects of high temperature on plant growth, development and physiological processes. Modified from Hasanuzzaman *et al.*, (2013).

1.4 Cold-climate species response to past climate changes.

In the recent decades, high latitude and high altitude regions have experienced warmer temperatures, and are predicted to continue to warm in the future (Myers-Smith *et al.*, 2011). This poses a major challenge for numerous cold-adapted arctic and montane species, placing many many populations in danger of being displaced from their optimal habitats (Thuiller *et al*., 2005; Sinervo *et al*., 2010; Pachauri *et al*., 2014). The present distribution of global biota (including plants, animals and ecosystems) differs markedly from those evident at the end of the Pleistocene (Hewitt, 2000). Fossil evidence and geographic patterns of DNA diversity have yielded valuable insights into how flora and fauna responded to shifts in global warming events in the past including responses to the rapid change in temperatures at the Pleistocene-Holocene transition (For time period see Figure 1.2) (Moritz and Agudo, 2013).

Data gathered from fossil evidence of the past climate fluctuations, including the Pleistocene-Holocene transition, indicate there was no elevated extinction rate through periods of rapid change (Blois and Hadly, 2009; Willis and MacDonald, 2011). However, this does not mean that the biota was static through past shifts in global temperature. The dominant response was individual shifts in geographic range (Williams *et al*., 2004; Graham *et al*., 2006) with associated changes of community composition. Comparative phylogeographic studies identify another type of response by identifying regions in which species persisted through past climate change, and these are known as evolutionary refugia (Figure 1.4). Studies have also indicated variation in species responses (Taberlet *et al*., 1998), with some clearly persisting in many regions and others in a few major refugia (Hewitt, 2000; Provan and Bennett, 2008; Howard-Williams, 2013).

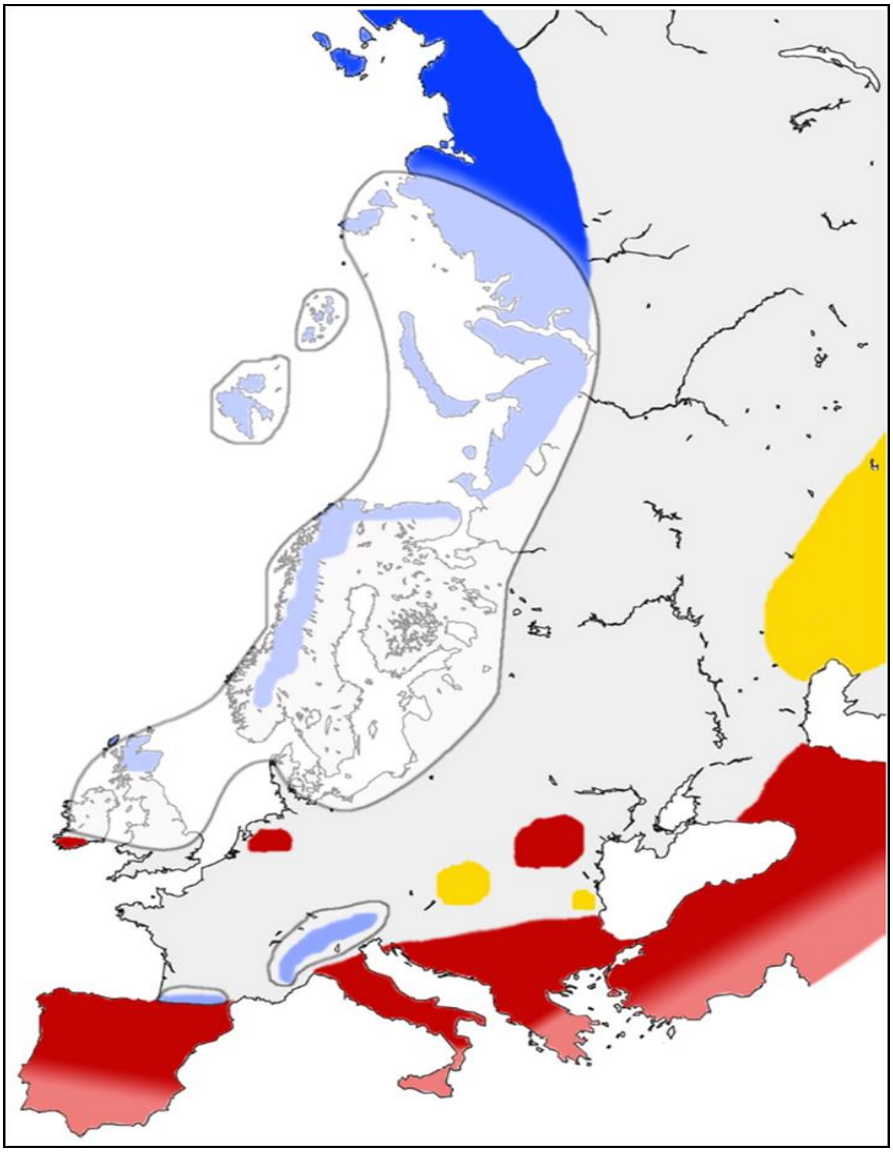

Figure 1.4: Map of Europe and western Asia adapted from Stewart *et al*. (2010), showing types of refugia. Interglacial refugia for cold-adapted species are indicated by blue areas, glacial refugia for temperate species shown in red, long-term refugia, indicated by darker colours (blue/red) areas that are inhabited throughout at least one glacial/interglacial cycle where the yellow areas indicate interglacial refugia along the oceanic/continental gradient in the east and cryptic refugia further west. The areas surrounded by the grey line present the extent of the ice-sheet during the Last Glacial Maximum.

1.4.1 Consequences of quaternary glacial cycles on the genetic structure and distribution of Arctic-alpine plants.

Arctic plants are found in arctic and/or subarctic regions but rarely in temperate regions whereas alpine species usually occur in high altitudes above the tree line but are rare or absent in lowland areas (Webb, 1983). Alpine plants usually inhabit mountainous areas at or above c.2000 meters above sea level in the temperate region such as the Alps and Pyrenees in southern Europe. However in northern regions such as in Ireland these conditions are found on Benbulben mountaintop above c. 400 meters. Some species have distributions in both regions and these are known as Arctic-Alpine species. Because of the topographical diversity and wide geographic area involved, alpine habitats are currently very fragmented across Europe. Arctic habitats are more extensive, but in the European Arctic, are fragmented into several island and mainland areas, including Iceland, Svalbard, Greenland, Scandinavia and Scotland, and in other isolated regions, notably in Ireland (Webb, 1983).

Alpine-arctic plants, in the above distributions, are considered to be affected by global warming in terms of both geographical distribution and genetic diversity because they are limited to habitats with low temperature. Moreover, they are likely to be susceptible to invasion by other species when it becomes warmer in their local areas (Pauchard *et al*., 2009). Arctic-alpine organisms were able to survive past climatic oscillations throughout the Quaternary during which time the ice sheets expanded southward and retreated northward in cycles of approximately 100,000 years. There has been much interest in how the flora and fauna survived during climate change in the last Pleistocene glaciation cycle up until 18000 years before present, and in particular migration routes when the ice sheets were receding in the postglacial period (Taberlet *et al*., 1998). It believed that major

potential refugia regions in southern Europe played a crucial role to protect organisms during the last glacial maximum approximately 25000 to 18000 years ago. For example, Balkans and Iberian Peninsula, Italy, where Pyrenees and Alps mountains served as natural barriers between ice sheets and tundra in the north side and temperate regions in the south (Hewitt, 2000).

Much research has been completed on the genetic outcome of postglacial migration in temperate species, and different lineages seem to have met in several zones of central Europe allowing them formed mixed populations, with sometimes a wide variety of haplotype identities found in the same population (Figure 1.5), (Taberlet *et al*., 1998; Petit *et al*., 2003). Because cold-adapted species like arctic-alpine plants were already widely distributed in Northern Europe during the cold phases of the ice age, their distribution has actually contracted since the end of the Pleistocene. The current fragmented distribution of organisms in Europe and the Arctic could lead to reduced diversity in isolated populations, allowing intraspecific divergence to occur between populations where inter-population gene flow is limited by geographical isolation. This isolation would occur at the same time as potential stress from interglacial climate change processes.

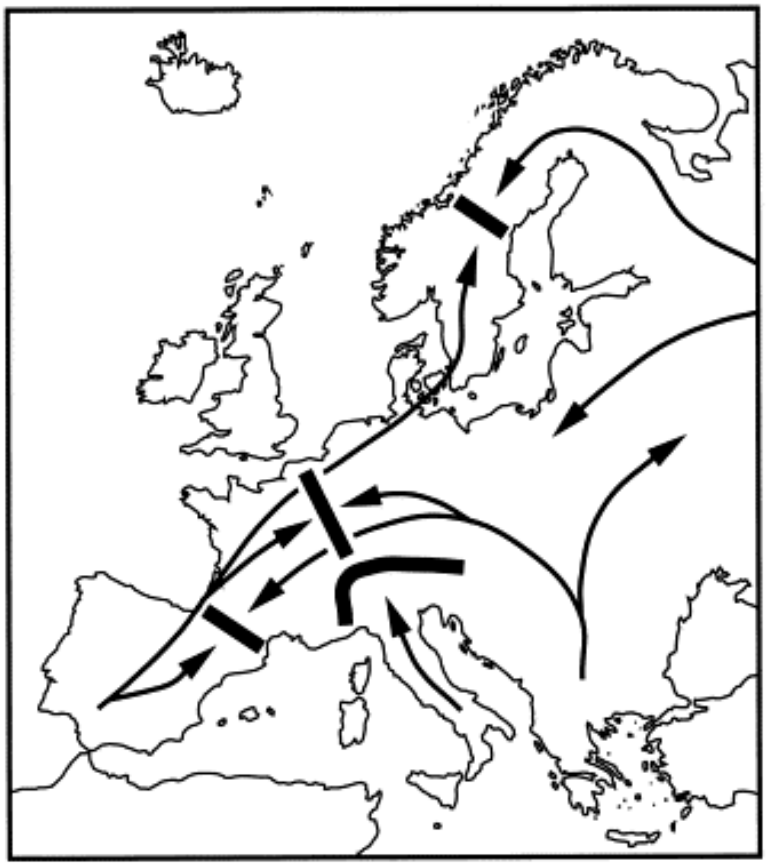

Figure 1.5: Map of Europe showing the main postglacial colonization routes for lowland temperate species. Arrows show immigration routs and thick lines indicate suture zones (from Taberlet *et al*. 1998).

1.5 Impact of Temperature on Plant Ecophysiology

1.5.1 Plant ecophysiology.

Plant ecophysiology is the study of complex interactions between plants and their environment, how plants respond to changes that can be happen, dealing with the effect of biotic and abiotic factors on plant functionalities and physiological adaptation to the surrounding environment (White and Hammond, 2008). In other words, it is the exploration of functional interactions and relationships of plant or plant population responses to environmental change.

Plant physiology is the science that deals with plant developmental change, growth, metabolism and reproduction. Plant physiology focuses on the whole process of plant growth, how it reacts to environmental change, relations with water, seasonal effects, temporal changes in plant functioning, how changes occurs in plants in different seasons. Plant physiology explains the life aspects of the plants. The three main functions covered by plant physiology include: (i) plant nutrition and metabolism; (ii) plant growth, development and reproduction; and (iii) how plants reacts in unusual environmental conditions (Taiz *et al.*, 2015).

1.5.2 Climate, habitat and phenology in Arctic and Alpine plant communities

Low light, cold temperature, variable soil water conditions, low nutrient availability and short growing seasons have up to now limited the productivity of Arctic plants distributed in the Arctic zone. Human-mediated climate warming has great potential to indirectly effect the growth of arctic plants by altering nutrient and water cycles and season length and severity, and this is potentially a more significant factor than the direct effects of warmer air temperatures on photosynthesis (Shi *et al*., 2014; May *et al.*, 2017).

The effects and impact of temperature on plant physiology at high latitudes and altitudes have been extensively studied. Plants in these regions often spend more than their half life cycle overwintering, utilising a range of ecophysiological strategies to limit the impact of low temperatures, low incident radiation and in freezing conditions low water availability (Bjerke *et al*., 2015). Because arctic and alpine plant species are adapted to this ecological strategy and niche, they are competitively vulnerable under rapid changes in climate, especially warming temperatures during the traditionally cold winter period.

To fully understand the rapid changes in the composition and responses of Arctic vegetation to these changes, extensive long-term Arctic flora monitoring is required (Sturm *et al*., 2001; Tape *et al*., 2006), however it is already evident that plant phenology and ecosystem processes have been affected by recent climate warming, particularly in Arctic areas (May *et al.*, 2017). The Tibetan plateau region is considered as one of the most sensitive ecosystems that has already been effected by climate change than other areas thus, rapid changes of the prominent vegetation in that area would be expected (Shi *et al*., 2014).

One of the essential factors for successful adaptation to Alpine and Arctic environments is the evolution of a metabolic system that enable plants to capture, store and use energy at low temperatures and over short periods of time. The optimum temperature for photosynthesis ranges between 10 and 35˚C, depending on the species, however exposure to higher or lower temperature ranges than normal can potentially cause severe damage to the photosynthetic system of stress-sensitive plants (Berry and Bjorkman, 1980).

At high latitudes and altitudes, Arctic and Alpine plants are relatively insensitive to reduction of temperatures below the optimum, as they have enzymatic and metabolic adaption towards lower temperatures, and thus photosynthetic activity can occur whenever the temperature exceeds 0˚C, or even, for some species, below freezing conditions (Billings, 1987; Good, 2007). Importantly, the photosynthetic capacity of Arctic-Alpine plants at higher temperatures is similar to that of lower altitude and temperate plant species (Good, 2007). As a result the overall chlorophyll content in Arctic and Alpine vegetation is not greatly different from herbaceous communities growing in temperate regions (Billings and Mooney, 1968).

Most physiological processes of Arctic-alpine plants are often limited by factors other than ambient temperature of the shoot tissue, such as nutrient and water availability (Chapin, 1983; Jaikumar *et al*., 2016). Temperature variation in the arctic or alpine environment can thus affect arctic-alpine plants in several ways, either directly or indirectly, which may in turn limit the level of resource availability and rate of plant development (Onwuka and Mang, 2018). For example, temperature can indirectly impact nutrient movement, soil processes and wider ecosystem functioning, affecting rates of growth and competitive interactions between species (Sardans and Peñuelas, 2012). However temperature alone is not a constraining factor that limits plant growth and development in Arctic and Alpine regions (Billings, 1987; Chapin, 1983). Light intensity, wind exposure, nutrient availability and soil water status all impact on growth and productivity. Soil temperature may cause a major effect on plant growth and development by influencing root and shoots nutrient transport and water uptake (Toselli *et al.*, 1999). For instance, low soil temperature decreases water uptake through increase viscosity and reduced water rate absorption. This results in decrease the rate of photosynthesis (Weih and Karlsson, 1999). Moreover, soil temperature has a great effect on micro-organisms and root cells metabolic activities, such these activities are responsible for the availability of nutrients for plants. Soil with low temperature leads to reduce microbial activities and tissue nutrient concentrations, causing reduce root growth (Puhe, 2003). In addition, in case of freezing conditions, most biological processes (seed germination, leaf appearance, seedling and root growth and microbial activities) cease and soil macro-organisms may not survive in freezing temperature (Allison, 2005; Onwuka and Mang, 2018). Vegetation in Arctic-alpine habitats is dominated by dwarf and prostrate shrubs and perennial herbs, and together these plants create a mosaic of micro-habitats in response to the overall constraints of the abiotic environment (Gehrke *et al*., 2015).

1.5.3 Impact of temperature seasonal changes on plant phenology.

Phenology refers to the study of changes in the timing in relation to biological events in animals and plants such as leafing, budburst, flowering, migration, dormancy, hibernation and reproduction. In plant phenology, some phenological responses are due to temperature whereas others are responsive to day length (Polgar and Primack, 2011).

Advancing phenology is one of the major impacts of global warming on plant communities generally, however many differences in phenological responses have been observed between species, with some species displaying more rapid responses to observed temperature changes than others (Prevéy *et al*., 2018). Rising temperatures in Arctic and Alpine regions is expected to drive major changes to the phenology of Arctic-alpine plants, affecting ecosystem processes such as pollination, germination, food webs and trace gas fluxes (Oberbauer *et al*., 2013).

Climate warming prolongs the Arctic growth season with potentially significant impacts on plant phenology and ultimate productivity (May *et al*., 2017; Shi *et al*., 2014). The changes in phenology are interlinked with the ecosystem productivity and functionality. Increasing the length of the growing season will lead to more days that are available for carbon fixation and biomass growth (Richardson *et al*., 2010).

As the temperature of high-latitude and Alpine regions continues to increase in response to climate change, the growing season is expected to lengthen as snowmelt will onset earlier in the spring. Long-term phenological studies, satellite images, plant specimen notes and field observations that have been used to generate historical phenological records can help to predict future plant phenological sensitivity to these likely changes (Panchen and Gorelick, 2017). In addition, variation in the relative change of seasonal temperatures is also an important factor, for example flowering and fruiting time is mostly

affected by temperature changes impacting the month or two prior flowering or fruiting, whereas germination is impacted by changes to temperature in early spring (Panchen *et al*., 2012). In the Arctic region there is a great variety in plant flowering time sensitivity to climate change, suggesting temperature impacts for Arctic environmental communities will probably include competition, pollinator interactions and change ecosystem composition (Panchen and Gorelick, 2017). Because of the many ways that temperature will impact phenology, the overall plant phenology transition in response to changes in seasonal temperature and season length are uncertain, however recent remote sensing studies have identified earlier and longer green periods, greater vegetation and reduced seasonality in Arctic and Alpine environments, compared to historical trends (Cleland *et al*., 2007; Oberbauer *et al*., 2013).

Currently the Arctic is experiencing the unprecedented change in temperature and climate, and the observed rise in temperature can possibly be doubled in the coming century (Aerts *et al*., 2006); thus, it is essential to explore arctic plant phenological responses to changes in climate. This can be achieved by examining several scenarios of winter and summer climate changes in experimental temperature change studies, based on the future climate projections at regional scale to assess the impacts of warmer temperature on natural ecosystems (Aerts *et al*., 2006).

1.6 Ecology, taxonomy and description of *Arenaria ciliata* and *A. norvegica* in Ireland.

Background.

Two closely related arctic-alpine species, *Arenaria ciliata* L. and *A. norvegica* Gunnerus, co-exist in Ireland, however they occupy distinct habitats in the Dartry mountains, Co. Sligo and the Burren, Co. Clare, respectively (Webb, 1983). Among Ireland's flora, they are representative of the c.30 species that are truly Arctic-Alpine in their physiology and distribution, and are both red-data list species.

They are not interfertile, and outside Ireland they have very different biogeographic distributions in fragmented and geographically isolated populations (Howard-Williams, 2013). *Arenaria norvegica* is associated with the southern Arctic Fringe of Northern Europe, occurring in Iceland, Norway, Sweden, Britain and Ireland. *Arenaria ciliata* is much more widespread, occurring across the high alpine mountains of central and southern Europe, with an outlying population in Ireland. The island of Ireland is the only region where the two species co-occur.

Both species belong the polyploid *A. ciliata* complex, a taxonomic group that includes several different species and subspecies, each with characteristic taxonomic and ploidy traits. *A. ciliata* is perennial, has a base ploidy of 2N=40 and typically occupies high mountain habitats, whereas *A. norvegica* is typically annual, has a base ploidy of 2N=80, and is most frequent in lowland areas across its distribution.

Earlier work has established the two species lineages separated some time after 50,000 years before present, and in the intervening period have experienced distinct phylogeographic histories (C. Meade, pers. comm).

Figure 2.6: Photograph of *Arenaria norvegica* from May 2016, growing at Black Head, the Burren, Co. Clare, Ireland. This image illustrates the short stems with opposite leaves, and open flowers with 5 sepals, 5 petals, 3 carpels and 10 fertile stamens, that are typical of the *A. ciliata* complex. The plants in this population were fertile at the time of the photograph, indicated here by the dehiscing anthers (Photo: Conor Meade NUIM, 2016).

1.6.2 Ecology, taxonomy and description of *Arenaria ciliata* and *A. norvegica* in Europe.

According to the description of Wyse Jackson and Parnell (1987), the white-flowered herbs *A. ciliata* and *A. norvegica* are closely relative species within the *A. ciliata* L complex (Caryophyllaceae), which includes the three core species *A. ciliata senso lato*, *A. gothica* Fries and *A. norvegica*.

The perennial *A. ciliata* occurs in open shallow-soil habitats across arctic, alpine and temperate mountain areas of Europe (Tutin *et al*., 1976; Jackson and Parnell, 1987). The perennial *A. gothica* has similar habitat preferences to *A. ciliata*, but is confined to the mountains of continental Europe. *Arenaria norvegica* is an annual or less often perennial species found predominantly in the arctic region, occurring in open habitats on basic soils, similar to *A. ciliata*. However, *A. norvegica* is more confined to exposed and shallower soils, frequently in lowlands, as compared to *A. ciliata* and *A. gothica*, which usually occur on scree slopes, cliffs and upland grasslands alongside with other flora (Curtis and McGough, 1988).

There is some overlap in the taxonomic descriptions of species in the complex (Tutin *et al*, 1993). This occurs because of the variation that exists within the *Arenaria ciliata* complex, where a variety of subspecies are identified, and there is a large of variation and overlap in taxon morphology, chromosome number, and geographic distribution across Europe (Jackson and Parnell, 1987). *Arenaria ciliata* L. subspecies include *A. ciliata* subsp. *ciliata*, *A. ciliata* subsp. *pseudofrigida* Ostenf. & Dahl and *A. ciliata* subsp. *bernensis* Favarger (Jalas and Suominen 1983; Tutin *et al.*, 1993). Two subspecies are recorded for *A. gothica* Fries, including A. *gothica* subsp. *gothica* and subsp. *moehringioides* (Murr.) Murr., and also two subspecies in *Arenaria norvegica* that

include *A. norvegica* subsp. *norvegica* and *A. norvegica* subsp. *anglica* Halliday (Tutin *et al*., 1993).

A comprehensive morphometric analysis of the *Arenaria ciliata* complex was completed by Wyse Jackson and Parnell (1987), which included 25 morphological characters to analyse similarities and differences among and between taxa this complex using biometric analysis. The researchers showed that in terms of morphology no one species or subspecies in the *A. ciliata* complex was clearly differentiated from the others, however consideration of multiple traits identified three principal taxonomic identities centred on *A. ciliata* L, *A. gothica* and *A. norvegica*, respectively. Recent molecular phylogenetic analysis has resolved the biogeographic structure of the complex, and established a principal division between *A. norvegica*, the *A. ciliata* genotypes in Ireland and Svalbard, and the remainder of the *A. ciliata* complex in continental Europe (Dang, 2012; Dang *et al*., 2012; Howard-Williams, 2013; Howard-Walker *et al*., 2013).

1.6.3 Distribution of *Arenaria ciliata* and *A. norvegica* in Europe.

According to the Flora Europea (Tutin *et al*., 1964), *Arenaria ciliata* is arctic-alpine plant widely found in high latitude or mountainous areas across Europe ranging from Southern and Central Spain to North of Italy. In addition, *A. ciliata* is widely distributed in the central and Eastern Alps, the Pyrenees, and in an isolated population in northwest Ireland. *A. ciliata* subsp. *psuedofrigida* occurs in Northern Finland, Norway and in Svalbard. *Arenaria gothica* subsp. *moehringioides* has been recorded in Central and northern Spain, the Pyrenees, Alps and north Apennines (Jalas and Suominen, 1988).

Arenaria norvegica is generally a quasi-arctic species, *A. norvegica* subsp. *norvegica* occurs in disjunct and scattered localities in northern England, west and north Scotland, western Ireland, northern Sweden, in the mountains of Norway, Iceland and small area of Finnish Lapland (Jonsell, 2001;Walker *et al*., 2013). The dubious subspecies *Arenaria norvegica* subsp. *anglica*, was identified by Lister Rotheray in 1889 amongst limestone fragments with thin soils in the upper Ribblesdale, Mid-west Yorkshire (Walker, 2000; Walker *et al*., 2013).

1.6.4 Distribution and ecology of *Arenaria ciliata* and *A. norvegica* in Ireland.

Arenaria ciliata and *A. norvegica* are both red-data species in Ireland, and are legally protected under the Flora Act of 1987. As Arctic-alpine plants they are considered to be sensitive to global warming because they are limited to disjunct habitats with low temperature and they are susceptible to invasive other species once their local area becomes warmer (Pauchard *et al.*, 2009).

Found only in one location in Ireland, *Arenaria ciliata* occurs on the summit of Ben Bulben Mountain in County Sligo, the westernmost extension of the Dartry Mountains system. There are several sources of evidence to suggest that Ben Bulben mountain may have acted as a refugium for cold-tolerant plants through the last glacial maximum approximately 20,000 years before present, with the small population of *A. ciliata* on Ben Bulben displaying high endemic genetic diversity that is typical of refugial populations in southern Europe (Synge, 1969; Dang, 2012). *A. ciliata* occurs in open habitats on the exposed limestone rocks, cliffs and grasslands across the Ben Bulben plateau, where the closest population occurs 1350 kilometres away in the Swiss Alps. The diverse topography and geological composition of the Ben Bulben plateau supports one of the

botanically richest regions in Ireland and gives a good example of Arctic-alpine and Alpine vegetation (Howard-Williams, 2013).

The core distribution area of *A. ciliata* on Ben Bulben is protected by Special Area of Conservation (SAC) status, as laid out under the EU Habitats Directive (transposed into Irish law as SI no. 477 of 2011).

The first discovery of *Arenaria norvegica* in Ireland was in 1961 by John Heslop Harrison during field trip to Burren, Co. Clare. The habitat of *Arenaria norvegica* was described as shallow crevices on an area of limestone pavement on the Gleninagh Mountain overlooking Caher Lower, near Black Head on the south shore of Galway Bay. Since that time until 2008, it had not been reported despite repeated searches. In 2008, two small populations of *A. norvegica* subspecies *norvegica* were rediscovered a few Kilometres from the Harrison site description, on the lower slopes of Carnesfin Mountain (Walker *et al*., 2009) . This site is located in the middle of a Green road between Murroogh and Black head. The plant occurrences were limited to shallow sandy soils between and on limestone pavement. This area of the Burren was covered with ice during previous glacial maxima, and is of international scientific interest as the bare limestone pavement and calcareous grasslands are rich with species and rare species that worthy of research and study (Howard-Williams, 2013). The Burren landscape is covered by multiple legal protections, including SAC designations, National Park regulations and Nature Reserves.

This project presents an analysis of genetic identity and ecophysiological characteristics in the *A. ciliata* complex, to help determine if and how regional ecotypes differ from one another in relation to responses to climatic variation and stress, both in terms of current ranges in climate variation, and different conditions that may arise in the future as a result of global climate change.

1.7 Objectives

With the overall aim of improving our understanding of recent climate adaptation in the *A. ciliata* complex, there were 3 principal objectives for this project.

(i) To determine ploidy variation across all taxa in the species complex and how this relates to genetic identity.

(ii) To evaluate seasonal growth responses in *A. ciliata* and *A. norvegica* ecotypes in four representative ecogeographic habitats.

(iii) To analyse ecophysiological responses in terms of photosynthesis performance in five ecotypes of the *A. ciliata* complex relative to seasonal environmental variation in four representative ecogeographic habitats, and to selected environmental stress scenarios.

Chapter 2

2 Assembly and preparation of sample and tissue collections from target populations of the *A. ciliata* complex, and validation of phylogenetic and taxonomic identities in sample group.

2.1 Introduction

Species of the *Arenaria ciliata* complex occur in mountainous regions north of the Mediterranean basin, extending down to sea level in the Arctic, in open grassland areas and in fissures and hollows on bare rock, where they thrive in lime-rich soil (Figure 2.1 and 2.2) (Tutin *et al*. 1964; Wyse Jackson and Parnell 1987). In the sub-arctic populations can be extensive and contiguous, however elsewhere populations are small, covering sometimes as little as 100 m^2, and usually isolated.

Based on the analysis of Dang (2012), Dang *et al* (2012), Howard-Williams (2013), and Walker *et al* (2015), and on the earlier analysis of Wyse-Jackson and Parnell (1987), three core species are recognised in the *A. ciliata* complex: *A. ciliata senso lato, A. gothica* and *A. norvegica*. Because of the complex methodologies involved in this project, particularly for ecophysiological analysis, understanding the biology of species in this complex requires a number of preparation steps in order to facilitate later analyses, and the work presented in this chapter has the objective of assembling a representative collection of populations, ecotypes and species, as well as completion of these key steps. With respect to the classifications above, the included samples need to be representative of the taxonomic, phylogenetic and habitat variation in the species complex. For this reason sample collection focussed on five ecotypes from across the group; *A. ciliata senso stricto* (occurring in Ireland, Italy, Austria and Switzerland), *A. ciliata* subsp. *pseudofrigida* (Svalbard), *A. ciliata* subsp. *bernensis* (Switzerland), *A. gothica* subsp. *moerhingioides* (Iberia) and *A. norvegica* subsp. *norvegica* (Ireland, Scotland and Norway) (Table 2.1).

Living material is necessary for ecophysiological analysis, but also baseline calibration for ploidy analysis through provision of living cells where direct chromosome counting is possible. Dried frozen tissue can be used for PCR amplification and gene sequencing

for phylogenetic data analysis, but also, following calibration with living cell chromosome counts, for ploidy estimation using flow cytometry (Galbraith *et al*., 1983; Suda *et al*., 2007) .Seed material can be used for both of the above applications, with the advantage that new plant material can be continually produced as necessary. In this way, completion of the project objectives outlined in the introduction required assembly of 3 kinds of specimen collection: (i) a dried tissue collection (from new field collections and frozen archival specimens), to facilitate genetic and ploidy analysis; (ii) a collection of living plants from the field, and (iii) a collection of plants grown from field-collected seed – the latter two comprising the living plant collection.

This chapter describes how these collections were assembled, in preparation for ploidy analysis, and analysed to establish a phylogenetic structure for the sampled populations.

The objectives for the work described in this chapter, are as follows:

(i) Assembly of *A. ciliata* complex accessions to facilitate analysis of ploidy levels, biometric variation and ecophysiology.

(ii) Develop a protocol for propagation of living tissue for the purposes of harvesting active meristems for ploidy analysis

(iii) Validate the phylogenetic identity of sample accessions so that relative biological differences observed between accessions can be correlated with their evolutionary relationship.

After the sample collections were done, two phases of work were undertaken. Firstly, micropropagation from individual plants in the living plant collection was used to generate growing meristem tissue for ploidy analysis. Secondly, in order to analyse the

target sample populations and ecotypes within the context of the evolution of *Arenaria ciliata* complex, phylogenetic identity of sample accessions was confirmed.

2.2 Materials and Methods

All the chemical used in this project were purchased from Sigma unless otherwise stated

2.2.1 Sample Collection

Material was assembled from three different sources:

(i) Assembly of archival frozen tissue from *A. ciliata* complex species

(ii) Assembly of field-collected *Arenaria ciliata* spp *ciliata* mature plant collection from resident Irish populations

(iii) Assembly of seed collections from field populations of *A. ciliata* complex species.

In each case, samples were further treated and/ or analysed according to the needs and objectives of the project. For the analysis of ploidy and genetic identity presented in Chapter 3, sample material was drawn from all three sources, with the majority coming from archival material, and additional samples generated using micropropagation from living plants. For the biometric analysis of ecotype behaviour in Chapter 4, and the evaluation environmental stress responses in Chapter 5, only living plants were used. Here follows, firstly, a description of how these collections were assembled, in the following order: (1) archival samples, (2) field-collection of living adult plants, and (3) field collection of seed and germination of seedlings, and then secondly, a section

describing the development of micropropagation protocols for harvesting tissue of plants from the assembled living collection. Table 2.1 describes all of the collection used in the project.

2.2.1.1 Assembly of archival frozen tissue from *A. ciliata* complex species

Green tissue material of *Arenaria ciliata* subsp. *ciliata, A. gothica* subsp. *moerhingioiges* and *A. norvegica* were collected as a part of two previous studies (Dang 2012, Howard-Williams, 2013), desiccated with silica gel, and frozen at -80 °C in Molecular Ecology lab at Maynooth University in the years 2008 to 2012. The list of samples from these collections that were used in the current study are detailed in Table 2.1. A distribution map showing where these *A. ciliata* complex populations occur across Europe is presented in Figure 2.1.

The sampled populations covered the core geographic distribution of the species complex in Europe from Svalbard to Iberia and from Iceland to Austria. Two isolated population groups were not included in the sampling, from Greenland, in the extreme northwest and the Carpathian Mountains to the southeast of Europe.

Figure 2.1: Map showing the distribution of populations sampled for *Arenaria ciliata* ssp. *ciliata,* (red circles), *A. gothica* subsp. *moerhingioiges* (red circles), *A. norvegica* subsp. *norvegica* (green circles) and *A. ciliata* subsp. *pseudofrigida* (black circle) from frozen archival material at Maynooth University. Site descriptions are detailed in Table 2.

Table 2.1: Population locations and taxonomic identity of plant tissue samples from the *Arenaria ciliata* complex used in flow cytometry analysis and molecular biology analysis. Taxonomic identities follow Wyse-Jackson and Parnell (1987).

Taxon identity	Population code	Country	Site location of sampled population	Tissue type			Latitude	Longitude	Elevation (m)
				living	seed	desiccated			
A. ciliata spp. *ciliata*	Ac1	Ierland	Glencarbury Mine, Ben Bulben, Co. Sligo	√	√		N54° 21.549'	W8° 24.044'	475
	Ac2	Ireland	Gowlaun Valley, Ben Bulben, Co. Sligo	√	√		N54° 21.353'	W8° 27.290'	475
	Ac3	Ireland	Kings Mountain, Ben Bulben, Co. Sligo			√	N54° 20.672'	W8° 27.373'	490
	Ac6	Italy (W. Alps)	Refugio Mongioe, Ligurische Alpen, Piemonte			√	N44° 09.864'	E07° 47.201'	2057
	Ac8	Italy (W. Alps)	Colle delle´Angelo, Piemonte			√	N44° 40.709'	E06° 59.484'	2475
	Ac16	Austria (E. Alps)	Niedere Tauren, Wolzer Tauren, Steiermark			√	N47° 16.270'	E14° 21.210'	2135
	Ac17	Austria (E. Alps)	Karawanken. Karten			√	N46° 30.200'	E14° 29.120'	2100
	Ac19	Switzerland (W. Alps)	Daubensee, Bernese Alps			√	N46 25.165	E07° 37.478'	2257
	AC20	Austria (E. Alps)	Steiermark, Nordostliche, Kalkalpen		√		N47° 41.570'	E15° 36.36'	1720
A. ciliata spp. *bernensis*	Ab1	Switzerland (W. Alps)	Les Galeres, Canton Fribourg		√		N46° 53.630'	E07° 15.363'	2175
	Ab2	Switzerland (W. Alps)	Dent de Brenleire, Canton Fribourg		√		N46° 55.263'	E07° 17.358'	2100
A. ciliata spp. *pseudofrigida*	Ap1	Norway	Svalbard		√	√	N78° 54.480'	E12° 04.70'	200
	Ap3	Norway	Svalbard		√		N78° 53.410'	E16° 21.50'	100
A. gothica ssp. *moehringioides*	Ac10	Spain (Iberia)	Valle de Benasque, Aragon			√	N42° 40.957'	E00° 36.010'	1804
	Ac13	Spain (Iberia)	Hospital de Benasque, Huesca, Pyrenees			√	N42° 41.408'	E00° 36.950'	2834
	Ac14	Spain (Iberia)	Cabana Veronica, Picos de Europa, Cantabria			√	N43° 10.644'	W04° 49.967'	2325
	Ac15	Spain (Iberia)	Hacendida de Corarrobres, Picos de Europa, Cantabria			√	N43° 09.374'	W04° 48.213'	1933
A. norvegica	An1	Ireland	Black Head, Co. Clare		√		N53° 08.243'	W09° 16.048'	60
	An9	Norway	Tromso, Troms		√		N69° 38.570'	E18° 57.190'	14
	An5	Scotland	Cnoc Eilid Mathain, Inchnadamph			√	N58° 07.493´	W04° 55.374´	380
	An8	Iceland	Fjallsjokull Glacier, Hofn			√	N64° 00.431´	W16° 22.200´	20

2.2.1.2 Assembly of field-collected *Arenaria ciliata* spp *ciliata* mature plant collection from resident Irish populations

Collection of living *Arenaria ciliata* (Ac) material was carried out at the end of May 2015. 12 mature plants of *A. ciliata s.s* (Ac) was collected from two sites, Glencarbury Mine (Ac1) and Gowlaun Valley (Ac2) at summit of Ben Bulben Mountain, Co. Sligo, Ireland (Figure 2.4). *Arenaria ciliata* at the BenBullben sites generally occurs on open mountain grassland, in shallow loamy soil on steep-sloping beds of limestone scree, with frequent pockets of organic tufa in weathered rock crevices, especially on exposed bedrock and on larger limestone boulders (Figure 2.2 & 2.3). Extensive populations of *Arenaria cilitata* are also present on the limestone gravel paths associated with the abandoned Glencarbury Mine complex. Surrounding areas of impermeable shale bedrock on the Ben Bulben massif, which are less prone to weathering, have a flat aspect and are overlain by acidic mountain blanket bog at depths of up to 1-5m. *Arenaria ciliata* and associated arctic-alpine plant communities are entirely absent from these peaty areas. For sampling, individual mature plants were isolated within the soil at intervals of at least 50m, using a sharp trowel and lifted. For each individual plant, site details were recorded and then it was placed in a small marked bag together with intact roots and soil. Following transport to Maynooth, these individual plants were transferred to 12cm pots containing pH-neutral John Innes No. 2 potting compost, and then maintained under controlled growing conditions (Temperature 15^{o}C in, 16 hours light and, 8 hours dark) (Figure 2.5). A detailed site of each population included in this study was carried out incorporating GPS coordinates (Table 2.1)

Figure 2.2: *Arenaria ciliata* plant growing on limestone bedrock on Ben Bulben, Co. Sligo, Ireland at 490m altitude.

Figure 2.3: Typical habitats and landforms where *Arenaria ciliata* (Ac2) is found on BenBulben mountain, here pictured looking west over the upper Gowlaun Valley. In the foreground on the steep slope the substrate comprises well-drained neutral loamy soil and limestone scree, with pockets of neutral tufa organic material in rock crevices, in the distance where the topography has a flatter aspect, mountain blanket bog covers shale and bedrock

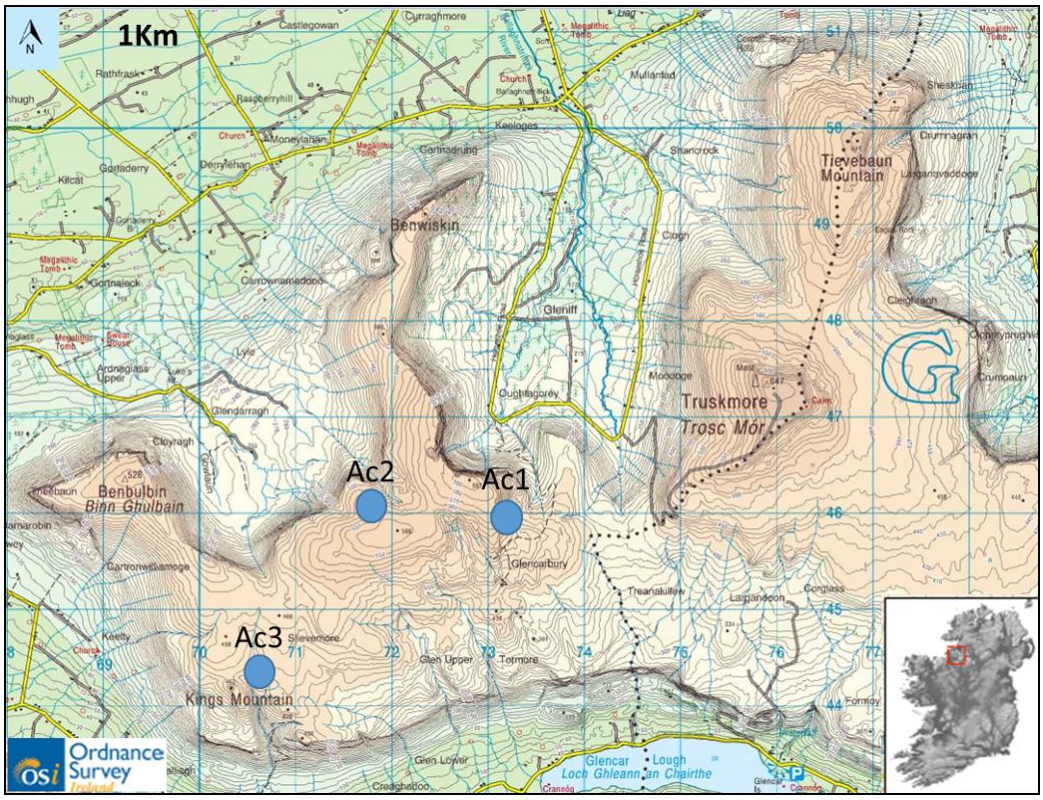

Figure 2.4: Ordnance Survey map of the mountain range of BenBulben in Co. Sligo. The blue sircles shows the sites of the Irish populations sampled of *Arenaria ciliata*.

Figure 2.5: Individual plants of *A. ciliata* spp *ciliata* collected from Ben Bulben mountain Co. Sligo, Ireland, grown on in John Innes No. 2 potting compost in 11cm pots at Maynooth University plant growth facilities.

2.2.1.3 Assembly of seed collections from field populations

Arenaria ciliata (Ireland).

Arenaria ciliata spp. *ciliata* seeds were collected at the end of August 2016 from Ben Bulben Mountain, Co. Sligo, Ireland, from the sample sampling sites where living plants were collected. Detailed site location is shown in Table 2.1. The objective was to gather sufficient seeds to allow biometric analysis of ecotype behaviour, as presented in Chapter 4. In total approximately 1000 seeds were collected.

Arenaria norvegica (Ireland).

Arenaria norvegica (An), was until recently believed to be extinct in Ireland. In 2008 *A. norvegica* was rediscovered in two small populations on the lower slopes of Carnsefin Mountain in Black Head Burren Co. Clare (Figure 2.6) (Walker *et al*., 2013). In a site survey carried out in May 2015 for the present project *A. norvegica* was found in typical limestone pavement habitat with shallow soil in the crevices (Figure 2.7, 2.8). Only mature, senesced adult individuals were recorded, <50 in total in this survey, and for this reason it was not possible to collect living *A. norvegica* accessions. With a return visit in August 2015, seed-production was observed throughout the sampled population, and so seed collection was carried out by removing only 1 seed pod from each mature plant, and only in cases where >5 seed pods were present. Where less than 5 pods were present, no seeds were collected (Table 2.1). One more visit was made to the same location at the end of August 2016, when less than 200 seeds were collected, using the same sampling protocol. In all cases, a cohort of seeds were retained for assembly of an *ex-situ* living

collection, to support on-site conservation of the *A. norvegica* population in the Burren. The purpose of this collection is to investigate the impact of different environmental conditions on the plant growth (Chapter 4).

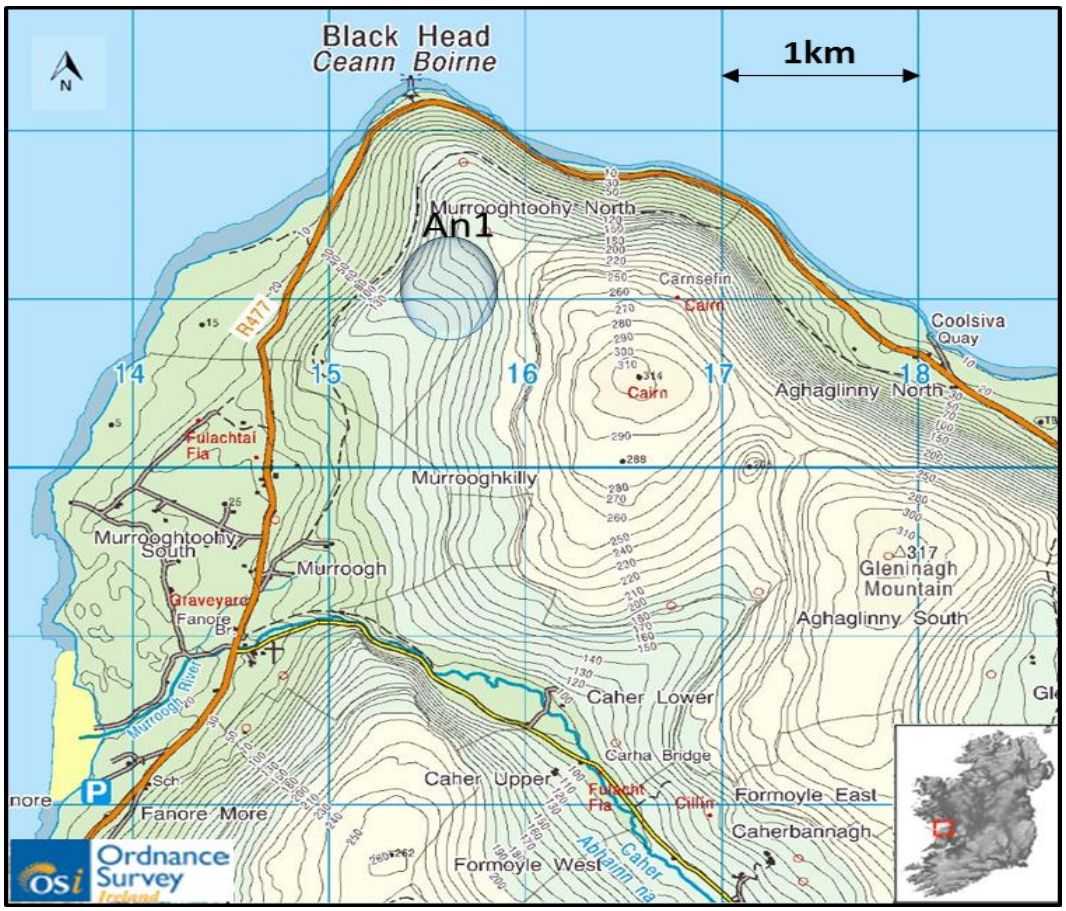

Figure 2.6: Ordnance Survey map of Black Head, The Burrn, Co. Clare. The blue area shows where the Irish population of *Arenaria norvegica* collected.

Figure 2.7: *Arenaria norvegica* growing in their typical habitat in the Black Head, Burren, Co. Clare, with shallow soils in the limestone pavement (Photo: Conor Meade NUIM).

Figure 2.8: Site of *Arenaria norvegica* typical habitats where this species is found in Black Head, Ireland. An1 population was sampled from this location (Photo: Emma Howard- Williams NUIM)**.**

Arenaria ciliata ssp bernensis (Switzerland), A. norvegica (Norway), A. ciliata senso stricto (Austria) and A. ciliata subsp. *pseudofrigida (Svalbard, Norway).*

A number of colleagues assisted in collecting seed material from the field for this project. Gregor Kozlowski, from the University of Fribourg, collected multiple seed accessions of *A. ciliata* subsp. *bernensis* in the Swiss Alps. Arve Elvebakk and colleagues from the University of Tromso, Norway, collected multiple seed accessions of *A. norvegica* from Northern Norway, and seeds of *A. ciliata* subsp. *pseudofrigida*, growing *ex-situ* at the University of Tromso Botanic Garden, but originally sourced from Svalbard.

The geographical distribution of these ecotypes is shown in Figure 2.9. Trial evaluation of chlorine gas sterilization, prior to planting, resulted in seed mortality and was abandoned. Germination was initiated in the following way: the seeds were placed between filter paper layers in 9-cm Petri dishes and the wrapped in aluminium foil and put in a refrigerator at 4 ^{o}C for 8 weeks. After this period of cold-incubation (Garcia-Fernandes *et al.* 2011), batches of three seeds were put in a new Petri dishes between two layers of wet filter paper for germination at room temperature. Germinated seeds were transferred to the soil in a growth room under controlled conditions (15 ^{o}C, 8h dark, 16h day light) at Maynooth University plant growth facilities (Figure 2.10 and 2.11).

The seeds of *A. ciliata* and *A. norvegica* from Ireland were planted directly into the soil without the cooling step at the same previous mentioned conditions (Figure 2.10 and 2.11). The location details of the sampled populations are provided in (Table 2.1 and Figure 2.9). The collection of mature plant and seedlings were used as a supplier of stem cutting for micropropagation experiment and molecular biology work.

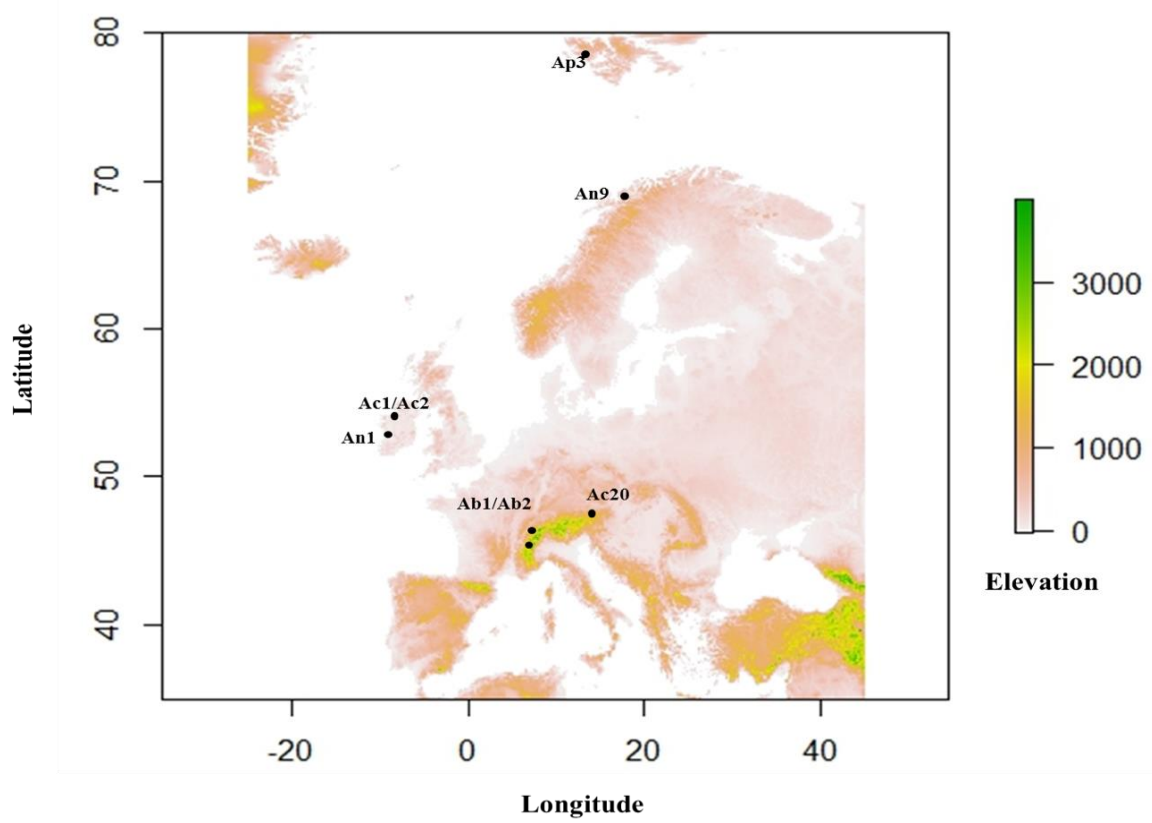

Figure 2.9: Geographic distribution of the sampled seed populations of *Arenaria ciliata* ssp. *ciliata*, (Ac), *A. ciliata* subsp. *bernensis* (Ab), *A. ciliata* subsp. *pseudofrigida* (Ap) and *A. norvegica* (An). Source of fresh green tissue.

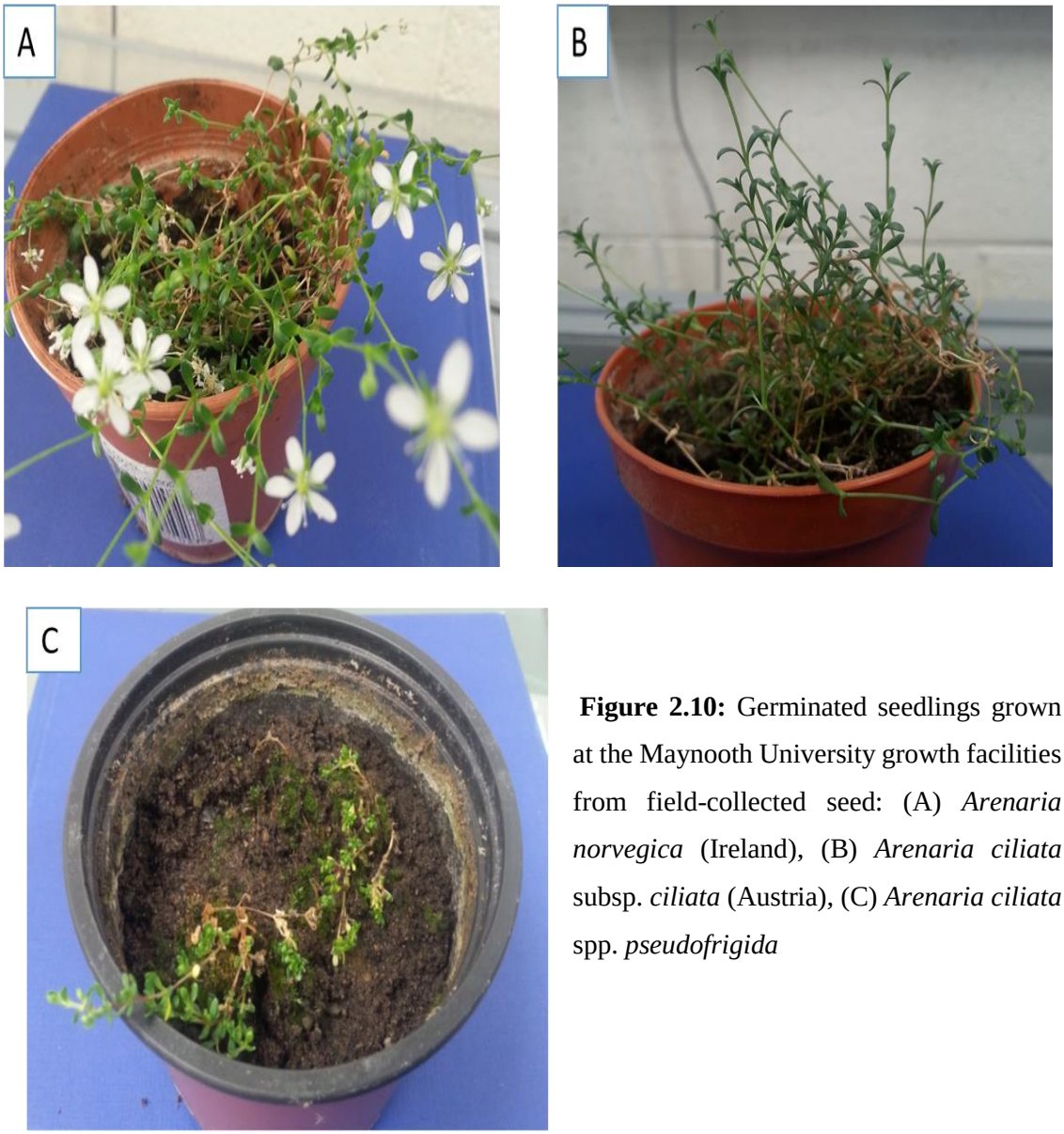

Figure 2.10: Germinated seedlings grown at the Maynooth University growth facilities from field-collected seed: (A) *Arenaria norvegica* (Ireland), (B) *Arenaria ciliata* subsp. *ciliata* (Austria), (C) *Arenaria ciliata* spp. *pseudofrigida*

Figure 2.11: Germinated seedlings grown at the Maynooth University growth facilities from field-collected seed: **(A)** *Arenaria ciliata* spp *ciliata* from Ireland, **(B)** *A. norvegica* from Norway and **(C)** *A. bernensis* from Switzerland.

2.3 Micropropagation of *Arenaria ciliata* species from stem cuttings containing nodes and axillary buds.

In order to develop a micropropagation protocol for stem cuttings of *Arenaria ciliata* and related species, two propagation methods were explored: growing cuttings in growth media and growing cuttings in sterile water to generate new shoot and root tips with (active meristem cell clusters) which can be used for the investigation of ploidy levels.

2.3.1 Materials and Methods

2.3.1.1 Micropropagation of plant cuttings using growth medium.

Sample material.

Tissue material was sampled from nodal and shoot tip cuttings from *Arenaria ciliata* mature plants collected from two sites: Ac1 and Ac2 at Kings Mountain and Glencarbury Valley, respectively, from the summit of Ben Bulben Mountain, Co. Sligo. Each plant was maintained in separate pots of John Innes No. 2 potting compost in Maynooth University Growth facilities. Approximately 3 cm of shoot tip containing 6-9 nodes were used for plant micropropagation.

Growth media preparation.

The method of growth medium preparation was carried out according to the protocol of (Markovic *et al*., 2013). One litre of growth medium was prepared by dissolving 4.4g of MS (Duchefa Biochemie) and 500 µl of NAA [0.5 mg/l] in one litre of DDH_2O. The pH was then neutralised to 5.7- 5.8 with titration of 0.1M KOH. The medium was divided

into two 500ml volumes in separate flasks. Subsequently, 2 gm Phytagel (Duchefa Biochemie) was added to each. Finally, 5 g of Sucrose was added to one flask, and then both were placed in the autoclave and heated to 120^{o}C for 20 minutes. When the two media flasks were cool enough to handle the media was distributed between fifteen sterile plastic growth cups in a laminar flow cabinet and allowed to cool prior to sealing with parafilm, giving [8 + sucrose and 7–sucrose media].

Planting of sample cuttings.

Approximately 3cm of shoots containing nodal, axillary and terminal buds were cut between 10 and 12 a.m. Shoots were placed in separate Petri dishes with distilled water. Samples were divided to two groups, the first set of cuttings were washed in sterile distilled water and planted directly into the 7 sugar-free media cups under the laminar flow hood. The second set of cuttings were sterilized by 70% Ethanol for few seconds, followed by 10 minutes in c.10% bleach solution (NaOCl Sodium hypochlorite) and three serial washes in distilled water before being placed in the +sugar culture medium. All 15 media cups (Sterile 9cm plastic cups with lids) were re-sealed with parafilm and transferred to a growth room set at 15^{o}C and a 16:8 day: night lighting regime (Figure 2.12). Sample treatments and planting details are shown in Table 2.2.

The micropropagation experiment was repeated with a second set of cuttings, this time without additional sugar, but with the addition of stronger concentrations of NAA to promote root growth. The same plants and same number of shoots were used (Table 2.2), however to reduce the likelihood of contamination, the time of incubation in the bleach was increased to 20 minutes instead of 10 minutes. 5mg/l of NAA was added into 7/15 cups while 1mg/l NAA was added to the remaining 8/15 cups.

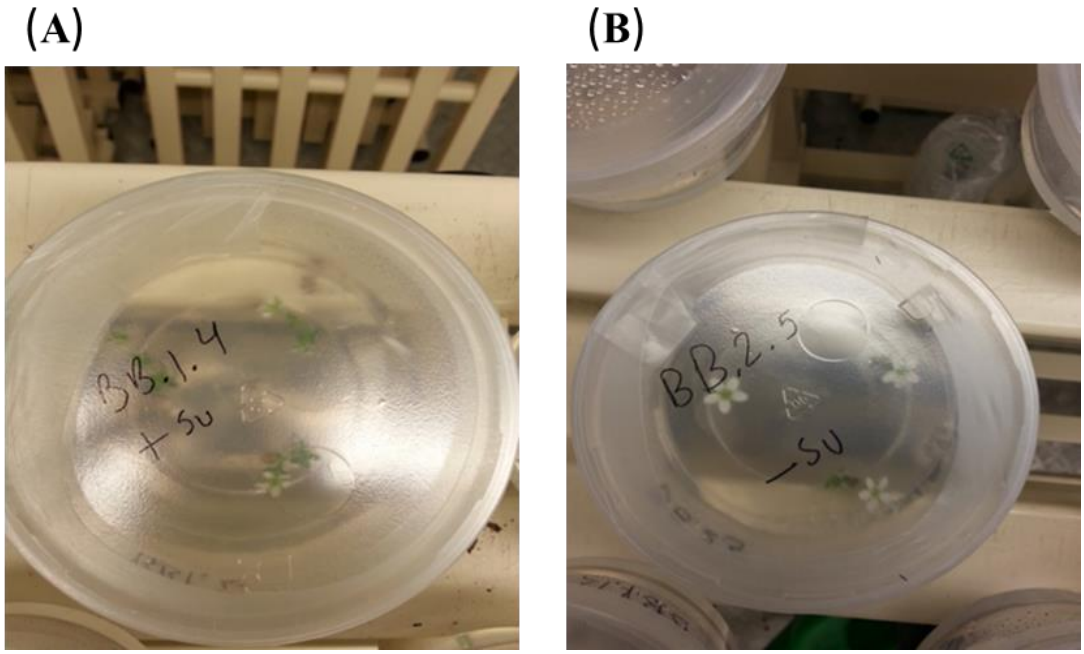

Figure 2.12: Micropropagation sample cuttings in growth cups. (A) *A. ciliata* 2.5 shoot cuttings placed in non-sucrose non-sterilized media. (B) *A. ciliata* 1.4 sample cutting placed in sucrose-sterilized media.

Table 2.2: Micropropagation treatments for sample cuttings of *Arenaria ciliata*. Sample i.d. numbers refer to the site of origin Ac1/ Ac2 on Beb Bulben Mountain, Co. Sligo, Ireland.

Sample Identity	Number of Cuttings	Sterilization	Medium + Sucrose	Medium - Sucrose
Ac 1.12	4	×	×	√
Ac 1.11	3	×	×	√
Ac 1.9 a	3	×	×	√
Ac 2.5	3	×	×	√
Ac 2.2	4	×	×	√
Ac 1.7 a	3	×	×	√
Ac 1.4 a	3	×	×	√
Ac 1.9 b	3	√	√	×
Ac 1.9 c	3	√	√	×
Ac 1.9 d	4	√	√	×
Ac 1.7 b	3	√	√	×
Ac 1.7 c	3	√	√	×
Ac 1.4 b	3	√	√	×
Ac 1.4 c	3	√	√	×
Ac 1.4 d	3	√	√	×
Total	48			

2.3.1.2 Micropropagation of plant cuttings using immersion in water

Materials and Methods

This protocol for accession propagation was developed following observation of the positive growth response of duplicate cuttings that were being stored in sterile water. Excised shoots of different mature and seedlings of *A. ciliata* spp. *ciliata* from Ben Bulben Co. Sligo, Ireland, *Arenaria norvegica* Tromso, Norway and *A. ciliata subsp. bernensis* Caton Fribourg, Switzerland (Table 2.1), growing in controlled conditions (15° C 16 hours light, 8 hours dark) at Maynooth University growth room facilities, were taken at 9 am (Table 2.3) and placed in sterilised cold water at 4°C for 24 hours. Subsequently, they were left in the sterile water at room temperature (Figure 2.13). To maintain clean water and prevent the growth of fungi, every two days water was changed. At 18 days, some of these cuttings started to form roots. 5 to 8 mm of root tips were cut from those, which had appropriate length.

The excised active meristem were used to perform ploidy analysis. A selection of cuttings which formed roots were transferred into separate pots of John Innes No. 2 potting compost, and grown on. The remainder were harvested for meristem analysis.

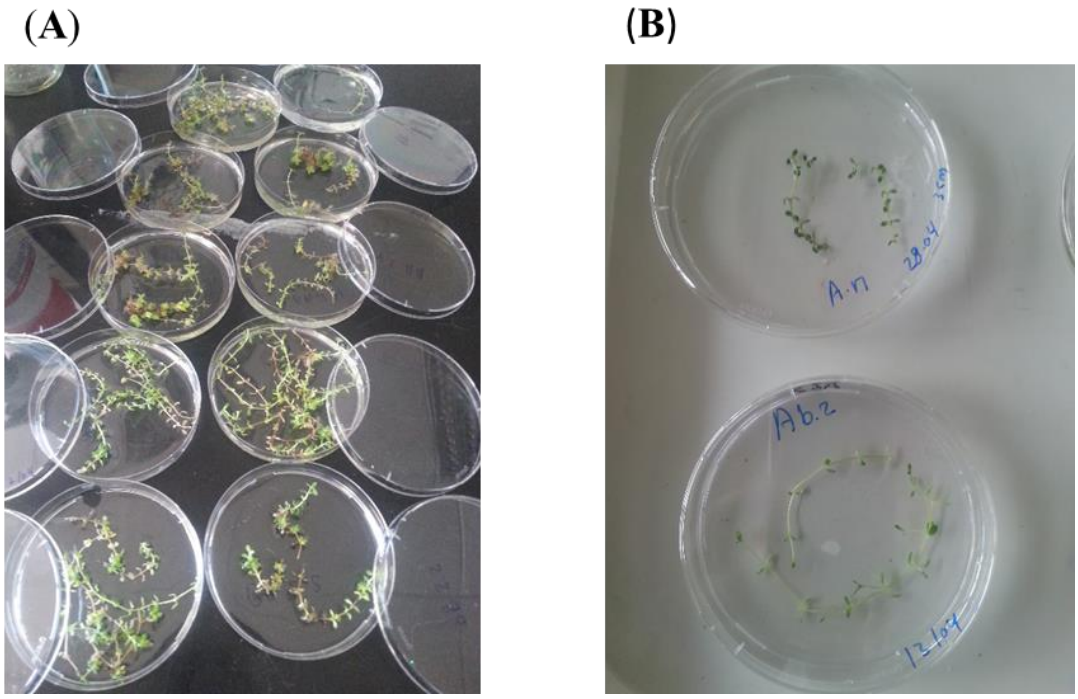

Figure 2.13: (A) *Arenaria ciliata* shoot cuttings **(B)** *A. norvegica* and *A. berenesis* cuttings are placed in Petri dishes with water at room temperature.

Table 2.3: Micropropagation of *Arenaria ciliata* species using sterilized water. Source of newly roots and leaves that were used for Confocal microscopy analysis.

Sample Identity	Cutting Length in cm (Day 1)	No: Nodes	Cutting Length in cm (Day 10)	Stem extension ratio %	Root emergence time (Days after cutting)
Ac 1.7	4.5	9	6	33.3	12
Ac 1.7	4	7	5.5	37.5	20
Ac 1.7	3.5	7	4.5	28.6	22
Ac 1.8	3	7	4.1	36.7	-
Ac 1.8	3	8	4.5	50	-
Ac 1.8	3.5	8	4.5	28.6	-
Ac 1.8	4	9	5.7	42.5	22
Ac 1.6	3	7	4.5	50	18
Ac 1.6	3	6	4	33.3	18
Ac 1.6	3	7	4.8	60	16
Ac 1.9	4.5	8	5.7	26.6	16
Ac 1.9	4.5	8	6.5	44.4	22
Ac 1.9	4	7	5.5	37.5	22
Ac 1.5	4	9	5.7	42.5	24
Ac 1.5	3.5	7	5	42.8	24
Ac 1.5	3.5	6	4.8	37.1	28
Ac 2.2	3	7	4.5	50	24
Ac 2.2	3.5	8	4.7	34.3	28
Ac 2.3	3.5	7	4.5	28.6	22
Ac 2.3	3	8	4	33.3	-
Ac 2.3	4.5	9	6	33.3	-
Ac 2.4	4.5	8	5.8	28.9	24
Ac 2.4	3.5	7	5	42.8	24
Ac 2.5	3	7	5	66.6	12
Ac 2.5	4	8	5.5	37.5	16
An 4	3	7	5	66.6	-
An 4	4	8	5.8	45	24
An 4	4	8	6	50	18
An 4	4	6	5.5	37.5	18
Ab 2	4	7	6	50	12
Ab 2	4	6	6	50	12
Ab 2.1	5	8	7	40	-
Ab 2.1	4.5	7	6	33.3	16
Ab 2.1	5	8	7	40	18
Mean				41.15	

2.4 Phylogenetic Analysis of *A. ciliata* complex accessions

In order to place the analysis results of the target sample populations and ecotypes within the context of the evolution of the *Arenaria ciliata* complex, and confirm that the target populations were correctly identified, it was necessary to first confirm their genetic identity. Previous work at the Molecular Ecology Laboratory generated an extensive database of DNA sequences from *A. ciliata* complex populations across Europe. Among these, the combination of chloroplast loci *rps*16 and *trn*TL (Shaw *et al*., 2007) yielded the most informative and variable non-coding sites of the 12 loci available (Dang, 2012; Dang *et al*., 2012; Howard-Williams, 2013) and these were selected as target regions for identification of the ecotype populations used in this study. The objective was to generate *rps*16 and *trn*TL sequences from one or two representatives of each ecotype population, depending on their size.

2.4.1 DNA extraction from target species for the study

Silica-dried leaf tissue was used for extraction of total genomic DNA using the DNeasy Plant Mini Kit from QIAGEN. Fresh tissue was placed in a sealed bag with silica gel beads and allowed to desiccate for 72 hours. For each sample, approximately 20 mg of dried leaf tissue was then placed in a 2 ml Eppendorf tube with two tungsten ball bearings, and disrupted for 1 minute at 60 Hz on a mixer mill (Retsch Mixer Mill 300). Ground leaf tissue powder transferred into new 1.5 Eppendorf tubes, 400 µl of Qiagen Buffer AP1 was added to each sample, and the solution was vortexed for 20 seconds. 4 µl RNase A solution was added, mixed and incubated at 65 °C for 10 minutes, during which time tubes were inverted 2-3 times. 130 µl Qiagen Buffer P3 was added, followed by vortex and incubation for 5 minutes on ice then centrifuged at 20000 × g (14000 RPM) for 5

minutes. The samples were then loaded into a QIAshredder spin column placed in 2 ml collection tubes and centrifuged for 2 minutes at 20000 × g. The flow-through was collected in new tubes and the QIAshredder spin columns were discarded. 1.5 volumes of Qiagen Buffer AW1 were added, and the solution was mixed by pipetting. 650 µl of the mixture from each sample was loaded into a Qiagen DNeasy Mini spin column in a new 2 ml collection tube and centrifuged for 1 minute at 6000 × g (8000 RPM). The flow-through discarded and each spin column was placed into a new 2 ml collection tube, taking care the column did not come into contact with the flow-through. 500 ml Qiagen Buffer AW2 (wash buffer) was added to each column and centrifuged for 1 minutes at 6000 × g, with the flow-through discarded from each tube. Another 500 µl Buffer AW2 was added to each sample and centrifuged at 20000 × g for 2 minutes, with flow through discarded. The spin columns were placed into new 1.5 ml microcentrifuge tubes, 100 µl Qiagen Buffer AE (Elution buffer) was added onto the column membrane and incubated for 5 minutes at room temperature and then the spin column was centrifuged at 6000 × g for 1 minute. Subsequently, another 100 µl Buffer AE was added, followed by incubation for 5 minutes at room temperature and centrifuge for 1 minute at 6000 × g. The DNA flow-through (c. 200 µl) was collected using the same microcentrifuge tube.

2.4.2 Amplification of *rps*16 and *trn*TL chloroplast DNA loci using PCR

2.4.2.1 PCR conditions for extracted DNA

The PCR reagent and sample mix was made up to a volume of 50 µl (Table 2.6 and 2.7) in 200 µl PCR tubes. 1 µl of DNA template (ranging in concentration from 20 to 100 ng DNA) was add to 49 µl of primer-specific reagent master mix (Table 2.6 and 2.7), and following vortexing and a brief centrifugal spin, placed in a Peltier Thermal cycler (PTC-200, Bio-Science Dublin) for the PCR cycle (Table 2.5). To optimize the PCR amplification of *rps*16 and *trn*TL amplicons (Table 2.4), primer concentration and $MgCl_2$ volume was varied in PCR reactions (Table 2.6 & 2.7). Primer sequences (Eurofins Genomics) for the target loci were as used by (Dong *et al*., 2012) based in part on original description by Shaw *et al*. (2005; 2007).

Table 2.4: Information of DNA loci and primers sequence used for the amplification of the target locus in chloroplast genome of *Arenaria ciliata* group.

Amplicon name	Sequence	Annealing Temp.	Aligned size (bp)
rps16		52 °C	~ 750
rps16 F	AAACGATGTGGTARAAAGCAAC		
rps16 R	AACATCWATTGCAASGATTCGATA		
trnT-trnL		56 °C	~ 640
trnT-trnL F	CATTACAAATGCGATGCTCT		
trnT-trnL R	TCTACCGATTTCGCCATATC		

Table 2.5: PCR program for amplification of the rps16 and trnT-trnL regions.

Step	Phase	Temperature (°C)	Duration (mins)
1	Denature	95	1
2	Anneal	refer to Table 1	1
3	Extension	72	1
4	Go to 1 repeat 40 times	_	_
5	Final extension	72	7
6	Forever	4	forever

Table 2.6: Reagents used for PCR amplification of rps16 amplicon.

Reagent	Manufacturer	Concentration	Volume
DDH_20			31.75µl
Forward primer	Eurofins Genomics	40pmol/µl	1µl
Reverse primer	Eurofins Genomics	40pmol/µl	1µl
$MgCl_2$	Promega	25mM	3µl
Taq Buffer	Promega (5x Green Go*Taq* Flexi Buffer)	5X	10µl
dNTP's	Sigma Life Science	10 mM	1µl
DNA	Extracted in Molecular Ecology lab	20-100 ng/µl	1µl
Betaine	Sigma Life Science	5M	1µl
Taq Polymerase	Promega (GoTaq G2 Flexi DNA Polymerase)	5µl/µl	0.25µl

Table 2.7: Reagents used for PCR amplification of trnT-trnL amplicon.

Reagent	Manufacturer	Concentration	Volume
DDH_20			28.75
Forward primer	Eurofins Genomics	10pmol/µl	1µl
Reverse primer	Eurofins Genomics	10pmol/µl	1µl
$MgCl_2$	Promega	25mM	6µl
Taq Buffer	Promega (5x Green GoTaq Flexi Buffer)	5X	10µl
dNTP's	Sigma Life Science	10 mM	1µl
DNA	Extracted in Molecular Ecology lab	20-100 ng/µl	1µl
Betaine	Sigma Life Science	5M	1µl
Taq Polymerase	Promega (GoTaq G2 Flexi DNA Polymerase)	5µl/µl	0.25µl

2.4.2.2 Agarose gel Preparation

For validation of PCR products, gel electrophoresis was used to separate DNA based on their molecular size and charge. The gels comprised either 0.75% or 1.5% agarose gel (equal to 1.5 gm and 3 gm agarose (Invitrogen), respectively, dissolved in 200 ml of TAE buffer [Tris-Acetate-EDTA]). The solution was heated for approximately three minutes until the agarose dissolved completely. 60 ml of gel was transferred to a Durand mixing bottle where 10 µl of SYBR Safe DNA gel stain (Product No: S33102) was added to allow DNA visualisation under UV transillumination. After gentle mixing the gel was poured onto a gel tray, a well comb was placed onto the tray, and the gel allowed to cool

at room temperature until solid. The gel tray was placed in an electrophoresis rig (TermoEC CSSU911) and inundated with TAE buffer. 5 µl of each PCR product was loaded into separate wells. In addition, 5 µl of wide-range DNA ladder [50 – 10,000bp] (D-7058, Sigma Ireland) was used as a size standard at the uppermost and lowermost wells on the gel. Gels were run between 30 and 60 minutes at 80 - 120 volts, depending on the gel concentration and the target locus in each run. Gels were photographed using the Gel-Doc digital transilluminator camera.

2.4.2.3 PCR purification for sequencing.

Purification of confirmed PCR products was carried out using the QIAquick PCR purification kit supplied by QIAGEN.

Five volumes of buffer PB were added to one volume of the PCR product and mixed. QIAquick column was placed in a 2ml collection tube and then sample was loaded into the column and centrifuged for 1 minute, (All centrifugation steps were carried out at 13000 rpm). The flow-through was discarded and the QIAquick column was placed back in the same tube. Then 750µl buffer PE was added to the sample and centrifuged for 1 minute. The flow-through was discarded and the column was centrifuged again for 1minute and the column was placed in a new 1.5 ml microcentrifuge tube. Finally, 50 µl of buffer EB was added to the centre of the QIAquick membrane and allowed to stand for 1 minute before centrifuging for 1 minute. Finally, 5µl of the purified DNA from each sample were placed in a new microcentrifuge tube and labelled then sent to GATC Biotech for sequencing, along with 5 µl of primers used (diluted to 5 pmol/µl).

2.4.2.4 Phylogenetic Analysis.

When sequence data was returned from GATC, PCR samples with good quality reads were assembled. Forward and reverse DNA reads for the target loci rps16 and trnTL loci were trimmed and aligned using Geneious 9.1 (© Biomatters, 2016), and then the consensus sequences for both loci were concatenated using the master rps16-trnTL template RTC01 (Dang, 2012), using the Geneious 'map to reference sequence' facility. Concatenated sequences were then imported to the pre-existing matrix of *A. ciliata* haplotypes already generated at the Molecular Ecology Laboratory, and the newly expanded matrix was exported in .meg file format for use in MEGA 7.0 (Kumar *et al*., 2016). Within the MEGA application, Maximum likelihood phylogenetic analysis (Tamura and Nei, 1993) was completed on the entire matrix of chloroplast haplotypes, including 1000 bootstrap repetitions to confirm branch support.

2.5 Results

2.5.1 Propagation of Cuttings in Growth Medium with and without Added Sucrose

After five days of planting, it was noted that three cutting samples in the sucrose-sterilised media acquired fungal infection. Two weeks later, all samples were infected by fungus. Some cuttings died quickly, but all were dead by 8 weeks Three of forty-eight shoot cuttings from the second set of samples (sucrose-free media) survived (Ac 2.5, Ac 2.2 and Ac1.7a) and roots emerged from these samples.

It was notable that fungal growth began from the base of shoots, and because of this the experiment was duplicated once more. All the shoot samples were sterilised and incubation time in the bleach increased. Despite the precautions taken, nearly all shoots again acquired fungal infection. Two samples (Ac 1.4c and 1.7b), planted in non- sucrose sterilised media, survived and were rooted.

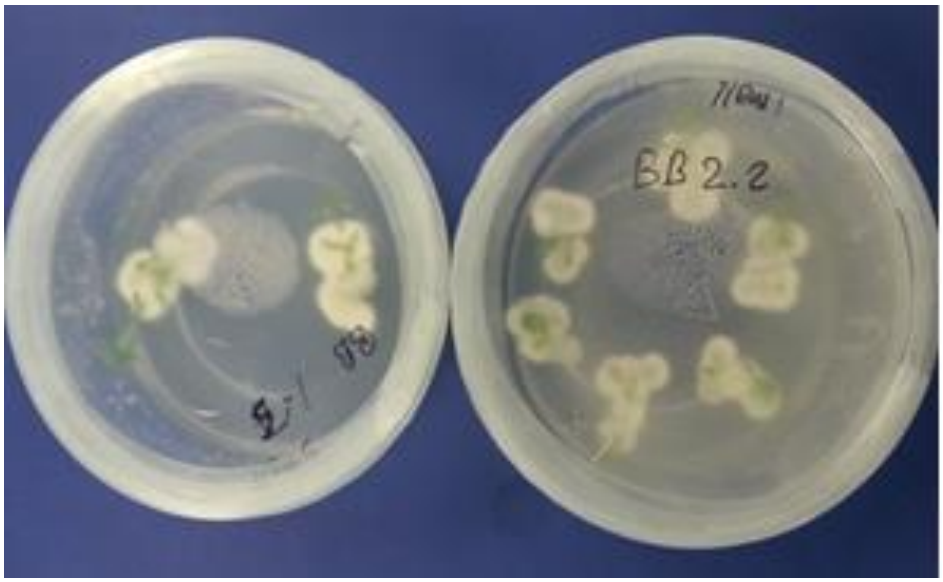

Figure 2.14: Photograph of *Arenaria ciliata* shoot cuttings planted in sterilised media that were infected by fungus.

2.5.1.1 Propagation of cuttings in Sterile Water

Initial efforts using growth media failed to generate acceptable results due to shoot fungal infection. At the end of this phase it was discovered during trial and error efforts that a

previously unpublished method of using immersion in sterilised water generated superior propagation results.

The *in vitro* micropropagation of *Arenaria ciliata* complex species was successfully established using this water treatment protocol. Shoot growth rate was observed to be between 1 to 2 cm ten days after immersion in water, equal to a mean 41.15 % extension of initial root length (Figure 2.15). Roots began emerging 18 days after shoots were placed in water and root emerging time ranging from 18 to 28 days (Table 5).

The rooting of shoot cuttings was most successful using the water treatment method. The rooting percentage was 79.4 % while the percentage of rooted shoots using growth medium lower than 6 %. The water treatment protocol enables successful *in vitro* propagation of *A. ciliata* complex group, and was selected for the project as a standard procedure.

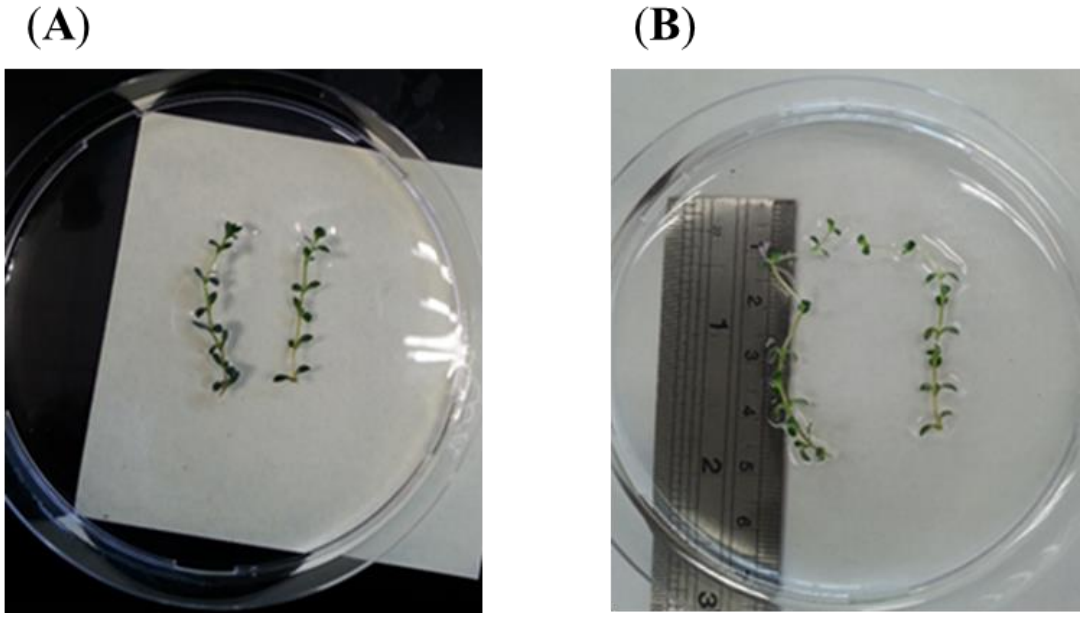

Figure 2.15: *A. norvegica* (Norway) shoot cuttings were placed in water. (**A**) Shoot length approx. 3 cm in the first day of planting (**B**) shoot length approx. 5 cm after 10 days of planting.

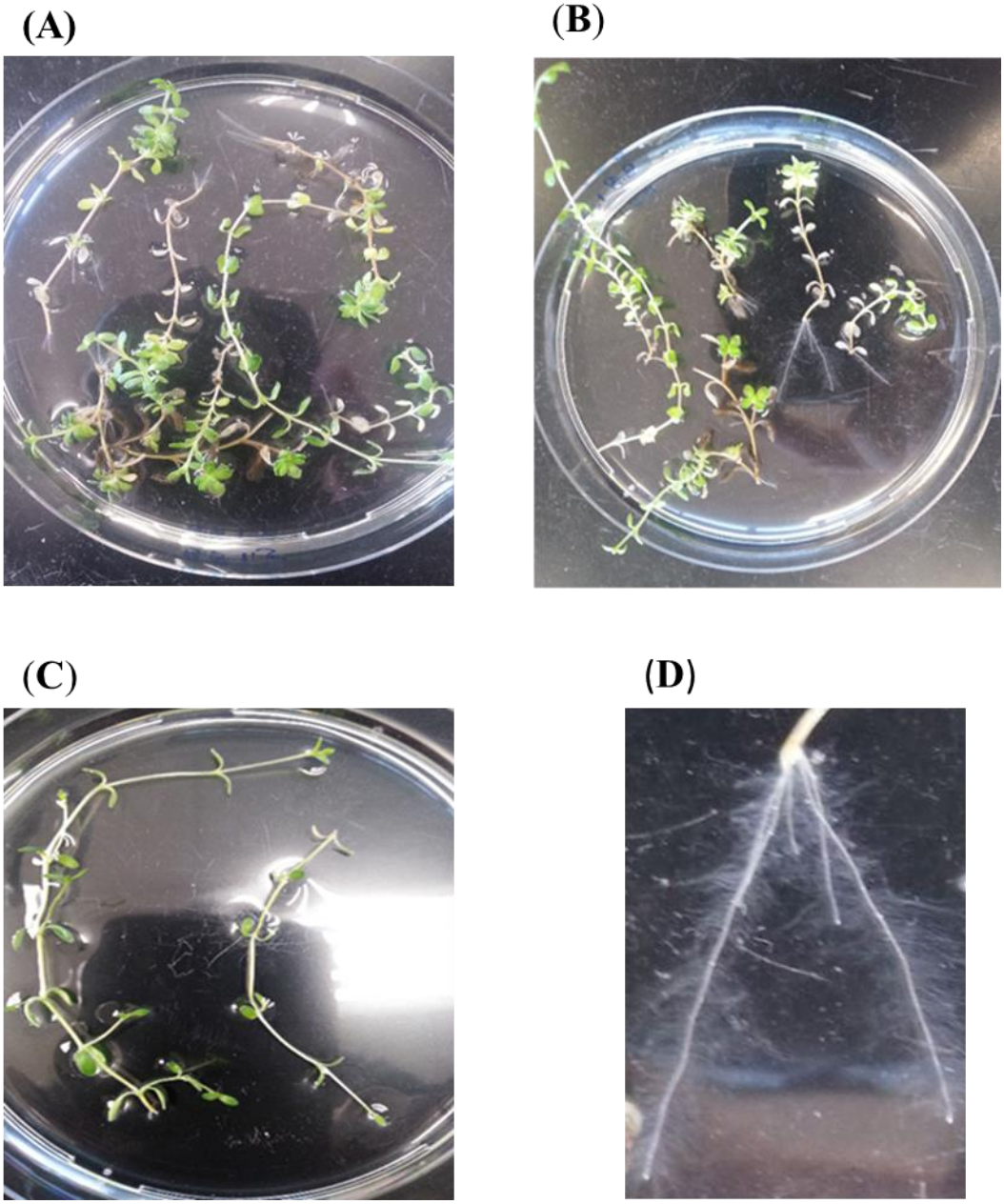

Figure 2.16: Photograph showing rooting shoots of *Arenaria ciliata* species. (A) *A. norvegica*, (B) *A. ciliata*, (C) *A. bernensis*, and (D) Magnified (x5) image of new roots emerging from *A. ciliata* shoot cutting.

2.5.2 Phylogenetic identity confirmation among the accessions being used for experimental analysis.

The combined *A. ciliata* haplotype dataset from Dang *et al.* (2012) plus the new samples from this study totaled 44 haplotype accessions, with an aligned matrix length of 1792 bp. The inferred Maximum-Likelihood tree topology for this matrix, with 4 outgroup taxa from the family Caryophyllaceae is shown in Figure 2.17. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The resulting of ML phylogenetic tree is shown in Figure 2.17. The Maximum Likelihood phylogenetic tree revealed that all analysed ecotypes, that being used in this study, belong to the five previously identified haplotype lineages (I-V) of the *Arenaria ciliata* complex.

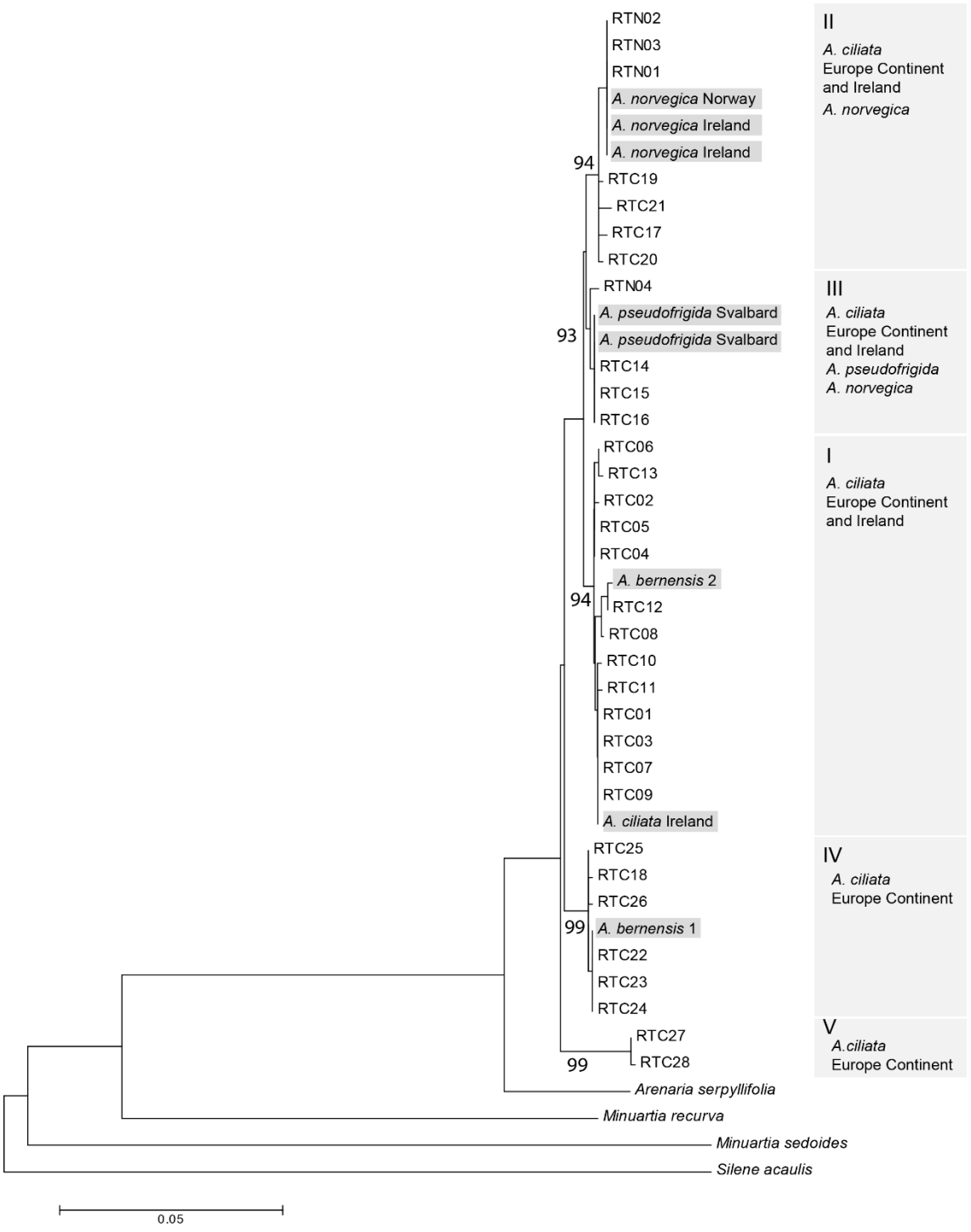

Figure 2.17: Evolutionary relationships of ecotypes in the present study (grey) within the total *Arenaria ciliata* complex as inferred using Maximum-Likelihood analysis of the DNA sequence data of Dang *et al*. (2012), and new sequences generated in this study. DNA sequence identity includes the combined rps16 and trnTL chloroplast spacer regions from all samples. For each of the 5 principal haplotype clusters identified in Dang *et al*. (2012) with >90% bootstrap support (I-V, support values indicated alongside respective branches), the taxonomic identity, population location and geographic range of source plant samples is indicated to the right.

2.6 Discussion

The work detailed in this chapter involved a considerable amount of trial and error evaluation of propagation protocols. The micropropagation of *Arenaria ciliata* species using growth media yielded very poor results. Despite of precautions taken, shoot cuttings of *A. ciliata* ecotypes consistently acquired fungus infection (Figure 2.14). It was observed that fungal growth began from the base of planted shoot cuttings, suggesting that fungal mycelia may have been emerging from within the plant tissue, rather than on the exterior or in the growth media. Time was not available to evaluate this hypothesis. In using this method, it was impossible to get clean active root meristems that could be used for chromosome counting experiments.

The *in vitro* rooting of *A. ciliata* species shoots was successfully obtained using a newly discovered and a previously unpublished method of immersion in sterilized water (Figure 2.15). The sterilised water treatment protocol was the most successful *in vitro* propagation of *A. ciliata* complex group (Figure 2.16), showing higher rooting percentage (at 79.4 %) compared to less than 6 % rooted shoots using growth media. Taken together, the obtained results suggest that micropropagation of the *A. ciliata* group species should be performed using water treatment method.

One of the objectives of this chapter was to determine the evolutionary identity and position of each of the ecotypes being used in the study. The evolutionary tree (Figure 2.17) generated using maximum likelihood analysis of the combined rps16 and trnTL sequences of an existing data matrix (Dang et a., 2012) plus the ecotypes from this study, identified that all studied populations identify within the *A. ciliata* group, as members of four of the five previously identified haplotype lineages (I-V) of the *Arenaria ciliata*

complex (Dang, 2012). *Arenaria ciliata spp. bernensis* is characterised by two distinct haplotypes, both previously unknown, belonging to Clades I and IV of the *A. ciliata* haplotype phylogeny. Multiple haplotype identities within a single population and taxonomic identity is a common feature of populations within the *A. ciliata* complex, and also among many other arctic-alpine plant species (Howard-Williams, 2013), and occurs as a result of of long histories of immigration and hybridisation among distinct but interfertile population groups (Brochmann et al., 2004). Clade I includes identities that are placed throughout the range of the *A. ciliata* complex in Europe, while Clade IV identities are restricted to the European Alps. The sampled *A. ciliata* spp. *pseudofrigida* ecotypes display just one haplotype, the previously identified RTC14, which falls within Clade III, associated with *A. ciliata* subsp. *pseudofrigida* in the Arctic and also *A. ciliata* in Ireland. The *A. ciliata* spp. *ciliata* Ireland ecotypes used in this study display the previously identified haplotype RTC01, which falls within Clade I. The two *A. norvegica* ecotypes display the same chloroplast haplotype, previously identified as RTN01, which falls among two other known *A. norvegica* haplotype identities in Clade II.

Based on the analysis of Dang (2012), Clade IV represents one of the earliest branching lineages among *A. ciliata* haplotypes from the mid-Pleistocene era, and is restricted to populations in the Alpine region, while clade I is of more recent origin, widespread across Iberia, central and Northwestern Europe. This combination of identities is similar for other alpine populations of the *A. ciliata* complex found in Southern Switzerland and Austria. For *A. norvegica* in Norway and Ireland, and for *A. ciliata* subsp. *pseudofrigida*, the recorded haplotype identities are the same for populations already recorded for these sites. For the *A. ciliata* Ireland ecotypes, all the sampled individuals display haplotype RTC01, one of the two common haplotypes among the 5 recorded in the Ben Bulben population. These data align with previous analyses for this group (Dang, 2012; Dang *et*

al., 2013; Howard-Williams, 2013) and reflect the commonly observed complexity among arctic-alpine plant populations in Europe (Brochmann *et al*., 2004; Birks and Willis, 2008; Eidesen *et al*., 2013) For the purposes of the later analyses presented in this project, this phylogenetic analysis confirms that the identity of the sampled ecotypes falls within the *A. ciliata* complex, and that they are representative of several different lineages with distinct biogeographic histories.

Chapter 3

3 Determination of ploidy levels within the *Arenaria ciliata* complex populations.

3.1 Introduction

Arenaria is a genus of about 160 species, placed in the sub-family Alsinoideae of the Caryophyllaceae, which occurs mainly across the arctic and temperate areas of the northern hemisphere (Jackson and Parnell, 1987). These species comprise a group of herbaceous taxa that show a range of overlapping morphological and ploidy identities, which make ambiguities in distinctions among them (Tutin *et al*.,1993; Dang *et al*., 2012; Berthouzoz *et al*., 2013; Howard-Williams, 2013; Walker *et al*., 2013).

The *Arenaria ciliata* complex has wide but very disjunct distribution across Europe, extending from the high mountains of central Europe and Iberia to sea level habitats in the arctic. While the taxonomy of the group has historically been seen as difficult, morphometric analysis suggests that there exists just three intergrading taxa: *A. ciliata* L., *A. gothica* Fries and *A. norvegica Gunnerus* Wyse-Jackson & Parell (1987). To accommodate the diversity of identities known within the group, this classification comprises multiple subspecific identities such as *A. ciliata* subsp. *pseudofrigida*, *A. cliata* subsp. *bernensis* Favarger and *A. gothica* subsp. *moehringioides* J. Murr (Jackson and Parnell, 1987; Jalas and Suominen, 1988). Across this range of identities there are also multiple ploidy states observed (Tutin *et al*., 1993), however it is unclear how these relate to variation in taxonomic and ecotype identity.

Polyploidy is a key aspect of cold-climate plant distribution and may be instrumental in allowing plants to adapt to and cope with harsh conditions of alpine and arctic regions (Stebbins, 1985; te Beest *et al*., 2011). In certain species, polyploid genotypes have shown elevated tolerance to biotic and abiotic stress factors and this resistance enables them to have greater adaptability to new ecological conditions (Brochmann *et al*., 2004; García-Fernández *et al*., 2012). This ability to cope with harsh conditions could be

attributable to increased gene expression associated with increased chromosome copy number, causing an increase in the content of certain of secondary metabolites and chemicals that enable the polyploid plants to tolerate biotic (pathogens and pests) and abiotic (cold and drought etc.) stresses. This may be the reason that polyploids are recorded from such a wide range of habitats (Hussain *et al*., 1997; Allario *et al*., 2011; Beyaz *et al*., 2013; Xue *et al*., 2017).

Arctic-alpine species show highly variable levels of ploidy among and between their populations, with a higher portion of polyploids compared with other ecogeographic plant categories (Bliss, 1971;Brochmann *et al*., 2004). One explanation for this difference is the impact of glacial cycles. It is thought that polyploidisation occurs as a consequence of selective pressure during postglacial recolonization, where polyploid identities are seen to facilitate adaptation and dispersal into novel habitats (Bliss, 1971; Paule *et al*., 2015), and recently there is some empirical support for this (Schinkel *et al*., 2016).

Ploidy variation within *A. ciliata* complex species was noted by Tutin *et al* (1993) and they report intergrading ploidy level ranges of 2N = 40, 80, 100, 120, 160, 200 and 240. According to paleoecological records, populations of *A. ciliata* complex taxa survived in Europe through the Pleistocene glaciations, and local haplotype identity evolution may have resulted from geographical isolation and the formation of cryptic refugia, where morphologically similar populations developed different population genetic identities. Molecular analysis of chloroplast haplotype identities in *A. ciliata* complex taxa across Europe revealed that they share a complex matrilineal history (Dang *et al*., 2012). Because the genetic affinity between populations does not easily map to their geographic proximity, it is possible that other non-spatial factors, like ploidy, may be contributing to reproductive isolation of different genotypes and populations, even when they exist in close proximity.

For this reason, validated ploidy estimates will reveal a clearer resolution of biogeographic history in the *A. ciliata* complex group, allowing us to determine the extent to which ploidy changes are associated with different lineages in the group. Achieving an accurate, inclusive estimate of ploidy variation across the *A. ciliata* complex is the goal of the work presented in the current chapter.

Direct chromosome counting is achieved using confocal microscopy (used to visual count chromosome numbers in multiple accessions). Multiple different treatments and various chemical combinations/ exposure time periods have been evaluated by multiple workers in the preparation of plant cells for chromosome counting (Ahloowalia,1965; Schwarzacher and Leitch, 1994; Ma *et al*., 1996).

Preparing cells for chromosome counting can be very challenging for some species partly because the processes which cause the spread of chromosomes is still not clearly understood (Kirov *et al*., 2014). While it is important to obtain accurate chromosome counts, counting an exact number of chromosomes is often achieved with great difficulty, especially in polyploid species where the spreading of large numbers of chromosomes into a single viewing plane presents many difficulties, often requiring an expert with cytological skills (Ahloowalia, 1965; te Beest *et al*., 2011). Also, capturing cells at the appropriate phase of cell division can be problematic, as can ensuring DNA stain penetrates into the dividing cells. For these reasons, counting chromosomes can be tedious, time consuming and a very slow process, involving multiple repeat experiments to achieve an optimal chromosome counts (te Beest *et al*., 2011).

Initial efforts in this project using root tissue failed to generate acceptable results, due to the hardness of root tissue and the very low metaphase index observed compared to young leaves (Kitajima *et al*., 2001;Anamthawat-Jónsson, 2004). Subsequently leaf meristem

tissue was evaluated on a trial basis and generated superior results, using excised axillary and terminal buds. Using an appropriate 4 °C pre-treatment, the metaphase index was elevated and cells spread very cleanly when pressed on slides using a standard squash technique (Hepler and Gunning, 1998). Building on this initial progress, this chapter presents a new composite method for staining and visually counting chromosomes in herbaceous Caryophyllaceae species.

While direct chromosome counting is essential for initial ploidy validation in any given species, once this is achieved, other methods such as flow cytometry can be used to efficiently measure ploidy in multiple unknown samples, using samples with microscope-counted ploidy for calibration. Flow cytometry methodology has greatly facilitated the acquisition of ploidy data, using standard samples of known ploidy for calibration genome size in fresh and unknown desiccated samples according to metaphase stages G_1 and G_2 (Galbraith *et al*., 1983; Suda *et al*., 2007).

In this chapter, a flow cytometry protocol has been optimized to allow rapid analysis of ploidy in new fresh samples and in freeze-dried samples drawn from -80°C storage. As described, the work presented comprises 2 distinct phases of analysis. In Section 1, the objective was to develop a chromosome staining and counting protocol using confocal microscopy, with the aim of determining ploidy level (or nuclear DNA amount) in a selection of species from the *Arenaria ciliata* complex group that represent the putative upper, middle and lower ploidy count levels for the group, to be used for baseline calibration of high-throughput flow cytometry analysis of samples with unknown ploidy.

Section 2 details flow cytometry analysis of ploidy diversity in a range of fresh and frozen tissue samples. The sequencing of steps in the methods described is novel and presents a new protocol for ploidy analysis in plant species.

3.2 Materials and Methods

3.2.1 Chromosome Counting using Confocal Microscopy

3.2.1.1 Root meristem tissue preparation procedure – Root Squash Technique

Sampling from *A. ciliata* complex species comprised two fresh tissue sources: (i) collected wild plants and (ii) germinated seedlings growing at Maynooth University plant growth facilities (Figure 2.9 and 2.10), as described in Chapter 2. To begin, 3-5 cm lengths of growing apical shoots were harvested from living plants of *A. ciliata* (Ac) Ireland, *A. norvegica* (An) Norway and *A. ciliata* subsp. *bernensis* (Ab) Switzerland (Table 2.1). These terminal shoots were propagated (propagation procedure has been described in chapter 2) to facilitate the development of new roots, from which the root tip, containing root meristem cells, could be isolated (Table 2.2 and 2.4) (Figure 2.15 and 2.16).

Using a scalpel blade, actively growing root tips of Ac, An and Ab plants that had been kept in the dark at 4 °C for 24-48 hours were severed into sections approximately 8 mm long (Table 2). The root tips were placed in a 2 ml an Eppendorf tube containing 0.002M 8-hydroxyquinoline solution (Sigma Aldrich) for 3-5 hours at room temperature. The root tips were then washed with DDH_2O, and using sterile forceps transferred to a 3.5 cm Petri dish containing a mixture of absolute ethanol and glacial acetic acid 2:1 for 20 minutes at 4 °C. The root tips were washed with DDH_2O, divided into two sets, and then placed in new 2ml Eppendorf tubes. The first set was incubated with an enzyme digest solution consisting of Cellulase and Pectinase for 3 hours at 37 °C. The second set was treated with a solution of 1N HCL at 60 °C for 5-10 min. Both sets of root tissue were then washed with DDH_2O twice for 20 minutes each time. The two sets of root tips were then

incubated in 50% Schiffs reagent (Fuelgen) for 20 minutes at room temperature, and then placed in 45 % acetic acid in a 3.5 cm Petri dish for 10 minutes. The root tissue was removed to a clean slide and covered with 45 % acetic acid to prevent desiccation. Under a dissecting microscope, the cells were separated out as much as possible and then covered with a cover slip. A piece of filter paper was placed over the cover slip and then pressed firmly with thumb to flatten cells and remove excess acetic acid. Finally, the Fuelgen-stained cells were screened using an Olympus FV1000 confocal microscope.

3.2.1.2 Shoot meristem tissue preparation procedure - axillary and apical bud squash technique

Leaf fresh tissue material of *A. ciliata* complex populations was obtained from two sources: collected wild mature plants and germinated wild seedlings growing in controlled conditions at Maynooth University plant growth facilities (figure 2.5, 2.9 and 2.10).

Newly emerged shoots from each sample plant were cut between 10.00 and 11.00 in the morning and transferred to sterile water in a Petri dish and stored for 24 hours in the refrigerator at 4 °C. Axillary and Apical buds were cut and placed in Eppendorf tubes containing 0.002 M 8-hydroxyquinoline solution for 3 to 4 hours at 20 °C (pre-treatment). Fixation was then carried out in a mixture of absolute ethanol and glacial acetic acid (3: 1) [Carnoy's solution]. Buds were put in Eppendorf tubes with the fixative solution for at least 1 hour at 4 °C. Buds were then washed with distilled water for 5 minutes. Subsequently, bud hydrolysis was completed with 1N hydrochloric acid (Sigma-Aldrich) at 60 °C for 5 minutes, and then buds were rinsed in water for 2 minutes. Buds were incubated in Schiff's Reagent (Feulgen stain) (VWR, Chemicals, Leuven, Belgium) for

20 minutes at room temperature, and then washed with 45% acetic acid three times for 5 minutes each. The buds were then transferred to a clean slide and covered with 45% acetic acid to keep the buds from drying. One or two buds were placed on a new glass slide and were covered with a small drop of acetic acid. Under a dissecting microscope, the tissue epidermis was removed by using a forceps and scalpel blade carefully and then cells were separated out as much as possible, with the goal of finishing with one layer of cells so that when the cells are squashed, they are not on top of each other. Buds were covered with a cover slip and piece of filter paper was placed over the cover slip and then pressed firmly with thumb to flatten cells and remove excess acetic acid (Figure 3.1). Using an Olympus FV1000 confocal microscope, Fuelgen-stained chromosomes were counted by viewing layered three-dimensional cell-section images.

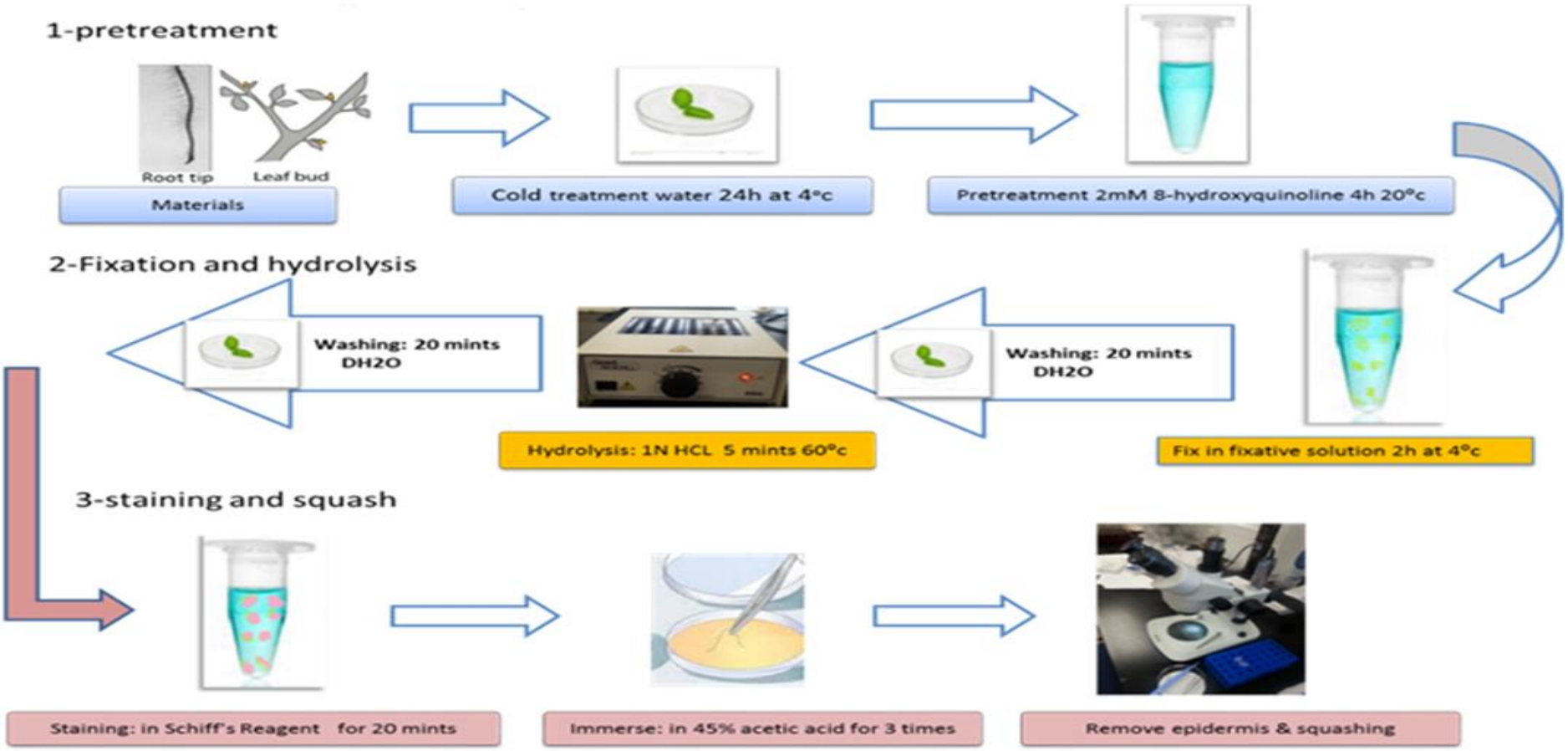

Figure 3.1: New protocol for *Arenaria ciliata* complex group chromosome counting based on modified shoot-squash technique.

3.2.2 Flow Cytometry analysis of ploidy variation

3.2.2.1 Sampling

Ploidy levels were estimated using flow cytometry of fresh tissue and freeze-dried specimens, collected in previous studies (2008 – 2012) and stored at - 80 ^{o}C (Table 2.1). Five ecotypes of the *Arenaria ciliata* complex were included in this study. Leaf tissue were collected from 52 individual plants of 21 population of *Arenaria ciliata* from (Ireland, Austria and Italy), *Arenaria norvegica* from (Norway, Ireland, Scotland and Iceland), *A. ciliata* subsp. *bernensis* from Switzerland, *A. ciliata* spp. *psevdofrigida* from Norway and *Arenaria gothica* subsp. *moehringioides* from Spain (Table 2.1).

3.2.2.2 Preparing and staining nuclei suspensions from fresh and frozen leaf tissue.

Following the protocol of Galbraith *et al*. (2001), 10 mg fresh leaf tissue or 0.3 mg dried tissue from each sample is cut and placed in plastic Petri dishes. 0.4 ml of ice homogenization buffer ('Galbraith's buffer'; Galbraith *et al*., 2001) is added to every 10 mg wet or 0.3 mg dried tissue, and tissue is homogenized using a new single-edged razor blade to release cellular nuclei. The buffer containing the suspension of nuclei is transferred to a fresh 1.5 ml Eppendorf tube. After the suspensions have been filtered through a 20 µm nylon mesh, RNase A is added to remove RNA (which can be stained by Propidium Iodide). After five minutes, Propidium Iodide is added and then samples are left standing for 10 min at room temperature prior to flow cytometric analysis.

Arenaria ciliata (2N= 40) from Ireland, *Arenaria norvagica* (2N=80) from Norway and *Arenaria bernensis* (2N=200) from Switzerland, (with ploidy levels already validated

using confocal microscopy) were used as an internal standard to estimate the ploidy level of freeze-dried and fresh specimens of *Arenaria ciliata* complex. The same wet weight amount of standard and sample tissues was prepared and analysed together. To maximize accuracy in the analysis of archive frozen samples, internal standard calibration samples were desiccated with silica gel and frozen prior to use in the analysis of these samples. Moreover, for all tested samples, in order to clarify the identification of G_1 and G_2 peaks, in each preparation batch the internal standard samples and unknown tested samples were first run separately before the pooled sample was run.

3.3 Results

3.3.1 Visual counting of chromosome numbers in control samples using confocal microscopy

3.3.1.1 Root meristem tissue

Typically, root tips are the preferred source of the ideal cells at appropriate stages in the cell-division cycle (Ahloowalia, 1965) however the root tips of *A. ciliata* treated with enzymatic digestion preparations (Schwarzacher and Leitch, 1994) did not yield any usable results as the nucleus failed to stain. When the root tip was viewed under a light microscope, it was obvious that the cell wall still completely intact as seen in figure (3.2). As the root tips of the target taxa are hard and durable, it proved to be very difficult to disrupt these tips without excessive damage to cellular integrity. Due to these difficulties, the enzymatic digestion technique failed to produce chromosome staining (Figure 3.2). However, root tip tissue disruption and nucleus staining was achieved using the hydrochloric acid preparation detailed above (Ma *et al.*, 1996). Although this method facilitated cell staining, chromosome counting was not reachable due to two reasons: the durable hardness of root tissue cells, which meant the cells did not spread well, and a low observed metaphase index in all exposed and stained nuclei, despite the use of cold-water pre-treatment to ensure an optimal cell division cycle (Figure 3.3).

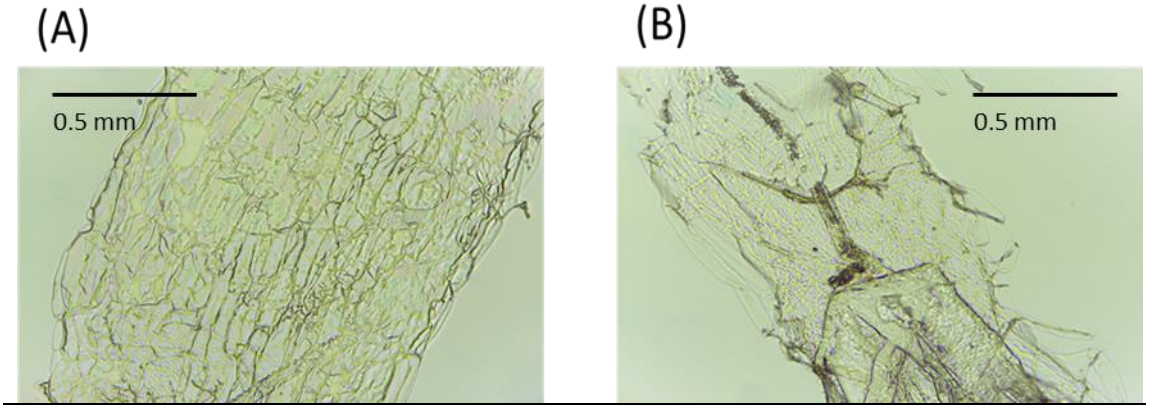

Figure 3.2: Light microscope images of enzymatic-treated root meristem tissue**. (A)** *Arenaria ciliata* and **(B)** *A. norvegica.*

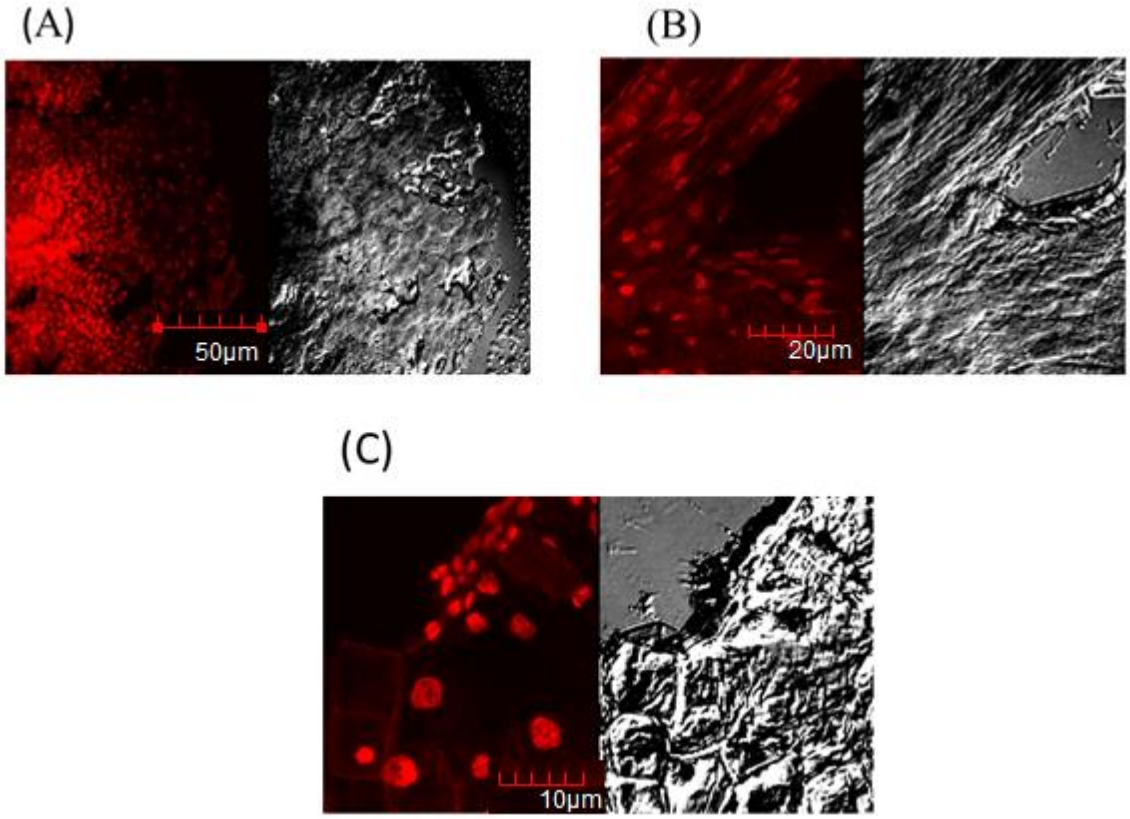

Figure 3.3: Confocal microscopy images of Feulgen-stained nuclei of root meristem cells. **(A)** *A. ciliata* 1.7 and **(B)** *A. ciliata* 1.5 root cells spread are not well. **(c)** *A. norvegica* nuclei show low metaphase index.

3.3.1.2 Fresh leaf meristem tissue

In the case of the sampled *A. ciliata* complex plants, as root meristems did yield any results, shoot meristems were used as an alternative source of dividing cells. Metaphase index in shoot tips (leaf bud) meristem is very high and leaf chromosomes tend to be longer or less condensed compared with root tip chromosomes (Kitajima *et al*., 2001; Anamthawat-Jónsson, 2004).

Using HCL-mediated tissue digestion of bud meristems, chromosome counts for control samples have been accomplished. This protocol yielded superior results compared with multiple alternative treatments that have been applied based on the literature, including modified HCL and enzymatic digestion preparations. Immersing and incubating excised buds for 4-24h in cold-water 4°C prior to pre-treatment allowed for arresting the cell cycle in the metaphase stage in the meristem (aiding chromosome staining). The maximum gain evident was achieved after 24 hours of water cold treatment.

Chromosome staining in mid-late metaphase shoot meristem cells was optimal at 9-12 am in the spring growth period (February to May), before broad tissue bud extension. Schiff's reagent (Feulgen stain) (VWR, Chemicals, Leuven, Belgium) caused the chromosomes to fluoresce under the confocal microscopy, generating high light emission and clear image contrast in the 555-655 nm wavelength. DAPI staining was also attempted, but generated very poor staining profiles compared with Schiff's reagent stain.

Using this staining protocol, direct counting of chromosome numbers in standard control samples was achieved. The fluorescent chromosome counts were confirmed on the Olympus FV-1000 confocal microscope by reviewing the cells layer by layer, generating a three-dimensional image with clear demarcation of individual chromosomes.

Numerous chromosome images were generated, but only the clearest images in which the chromosomes were adequately spread out were utilized for counting. Clear staining and visualization was completed for the control samples; *A. ciliata* (Ireland, five samples), *A. norvegica* (Norway, one sample) *A. ciliata* subsp. *bernensis* (Switzerland, one sample).

Ploidy analysis of *Arenaria ciliata* species revealed that; *A. ciliata* subsp. *bernensis* presents a ploidy level of 2N=200, *A. norvegica* has a ploidy level of 2N=80, (as shown in Figure 3.5 A and B respectively), while *A. ciliata* shows a ploidy level of 2N=40 as seen in Figure 3.4 A-F - confirming that the *A. ciliata* group of species is a polyploid complex. The obtained results yielded validated chromosome counts, which covered a range of anticipated ploidy levels across the *A. ciliata* complex as referred to in the literature (Tutin *et al*., 1993; Berthouzoz *et al*., 2013).

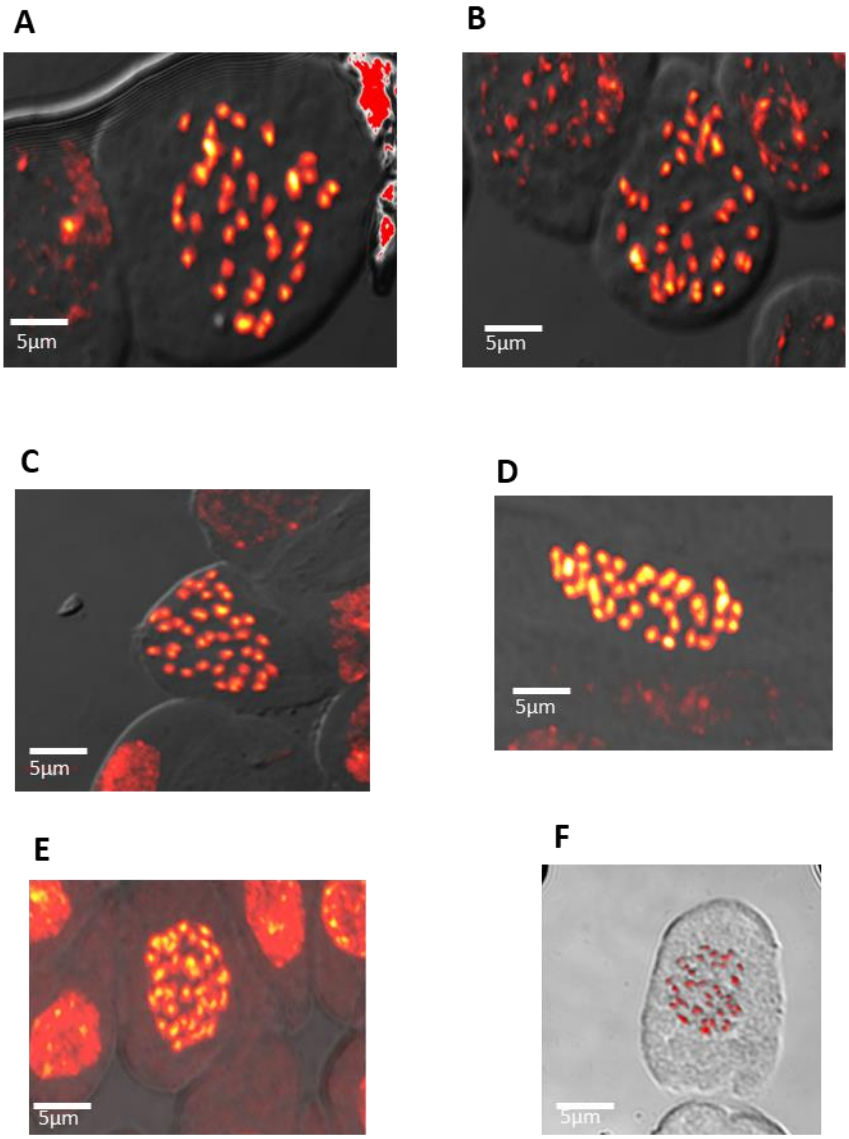

Figure 3.4: Confocal microscopy image of fuelgen-stained late metaphase chromosome in *Arenaria ciliata* ssp. *ciliata* growing in BenBulben Co. Sligo, Ireland. **(A)** Ac 1.5, **(B)** Ac 1.8, **(C)** Ac 1.7, **(D)** Ac 2.3, **(E)** Ac 2.2 and **(F)** Shows the single Cell border of Ac 1.7. Image scale 1000x, generated on Olympus FV1000 confocal microscope suite at 543nm laser excitation wavelength.

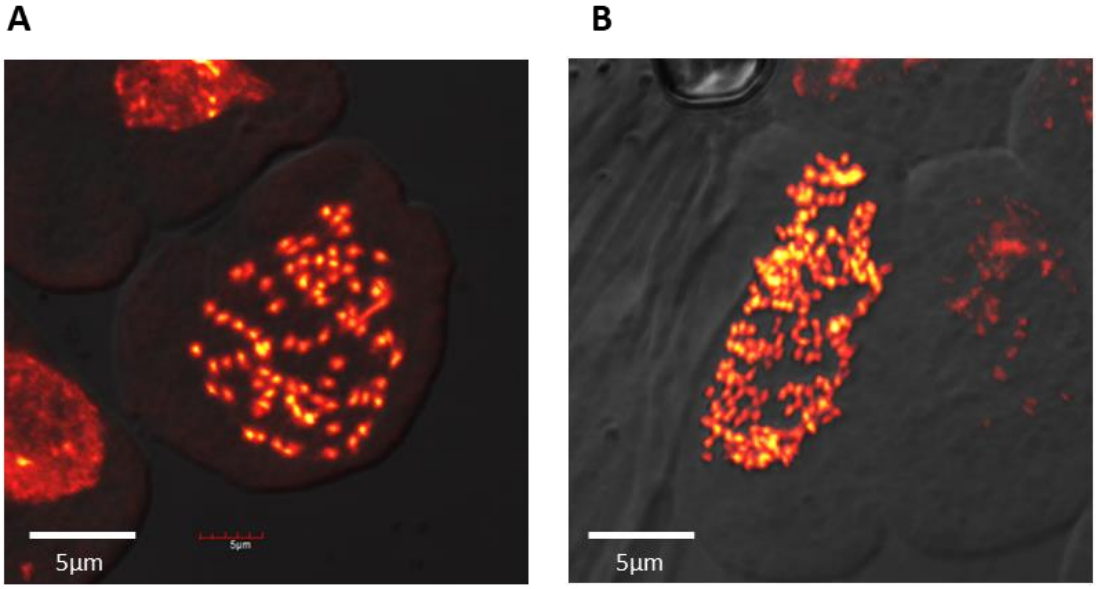

Figure 3.5: Confocal microscopy image of fuelgen-stained late metaphase chromosome in **(A)** *Arenaria norvegica* (Tromso, Norway) and **(B)** *Arenaria ciliata* subsp. bernensis (Alps, Switzerland). Image scale 1000x, generated on Olympus FV1000 confocal microscope suite at 543nm laser excitation wavelength.

3.3.2 Estimation of ploidy level in fresh and frozen leaf tissue across the *A. ciliata* group using flow cytometry

Ploidy levels were analyzed in five ecotypes belonging to *A. ciliata* complex using flow cytometry, covering fifty-two samples in total from twenty-one populations (Table 2.1). Eight populations were sampled from fresh leaf tissue or as a combination between fresh and frozen tissue. The remaining thirteen populations were sampled from the archived frozen tissue as shown in Table 3.1. Conformation of ploidy levels in three *A. ciliata* species using confocal microscopy counts (for use as internal size standard samples) allowed calibration of all flow cytometry reads, and facilitated high-throughput analysis of ploidy levels in the unknown samples. Using one or two standard samples in each run for each unknown test samples, combined with analyzing the internal standard samples and unknown samples separately and as combined samples in the same run yielded clear identification of G_1 and G_2 peak position of each sample. Frozen and fresh tissue showed near-identical position of G_1 and G_2 peaks for all tested samples as seen in Figure (3.6), with Coefficient of variance (CV mean) values of 3.70 for fresh samples and 2.81 for frozen samples.

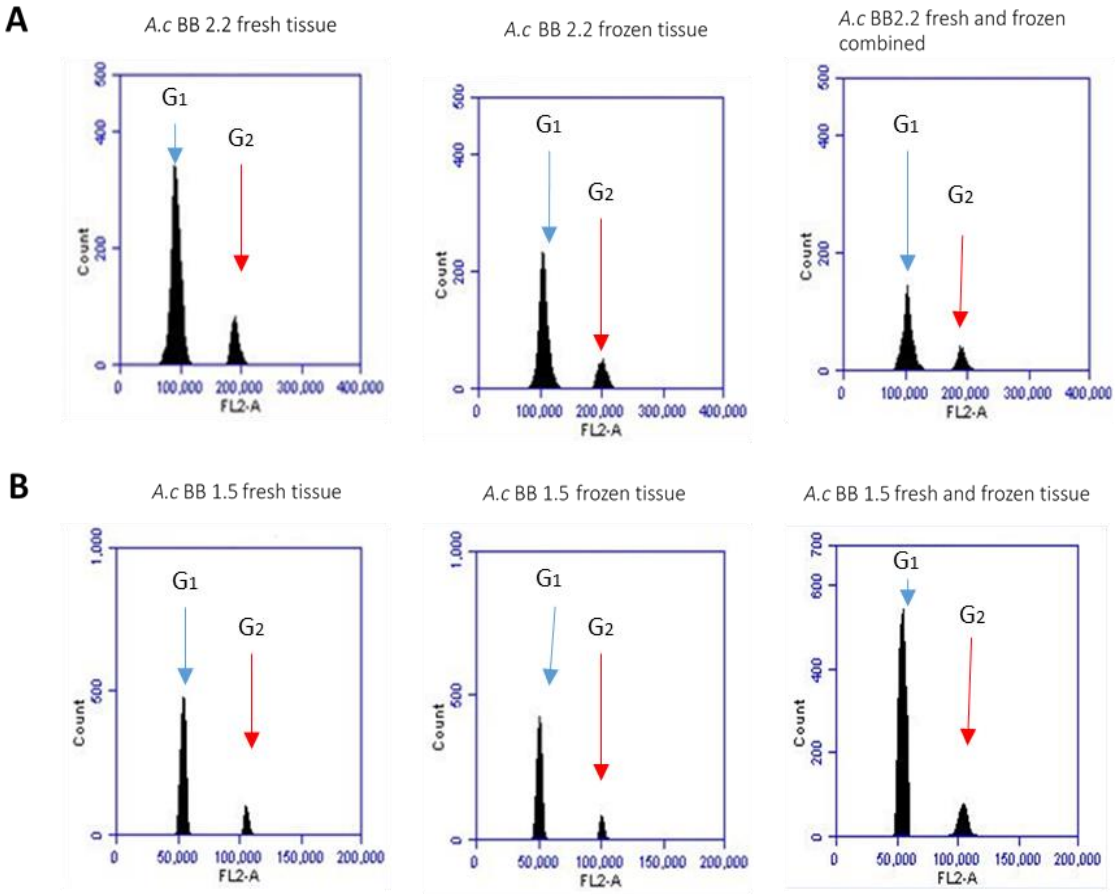

Figure 3.6: Fluorescence histograms of DNA ploidy level in PI-stained cells from dried-frozen material and fresh tissue of *Arenaria ciliata* ssp. *ciliata*, where G_1 and G_2 metaphase peaks are identified at a known ploidy level of 2N=40. (**A**) Fresh and frozen tissue from *A. ciliata* spp. *ciliata* A. c BB 2.2. (**B**) Fresh and frozen tissue from *A. ciliata* spp. *ciliata* A. c BB 1.5. Plot axes justified for scale is each panel.

In some individual tested samples, some shifts in peak amplitude emissions for metaphase peaks (G_1 and G_2) were noted between repeat runs. In each case the peak shift was consistent for control and unknown samples in the same batch run, and it was determined that the observed shifts were due mainly to variance in the final analyzed sample concentration (Figure 3.7).

Internal calibration using the confocal-microscopy confirmed baseline ploidy counts has facilitated ploidy estimation in all examined samples. In some samples that differ markedly in terms of ploidy levels from internal size standard samples, additional calibration was required in a second run, with the inclusion of two internal samples. In each instance, this approach facilitated ploidy estimation for those samples that have equivocal metaphase peaks as shown in Figure (3.9 A).

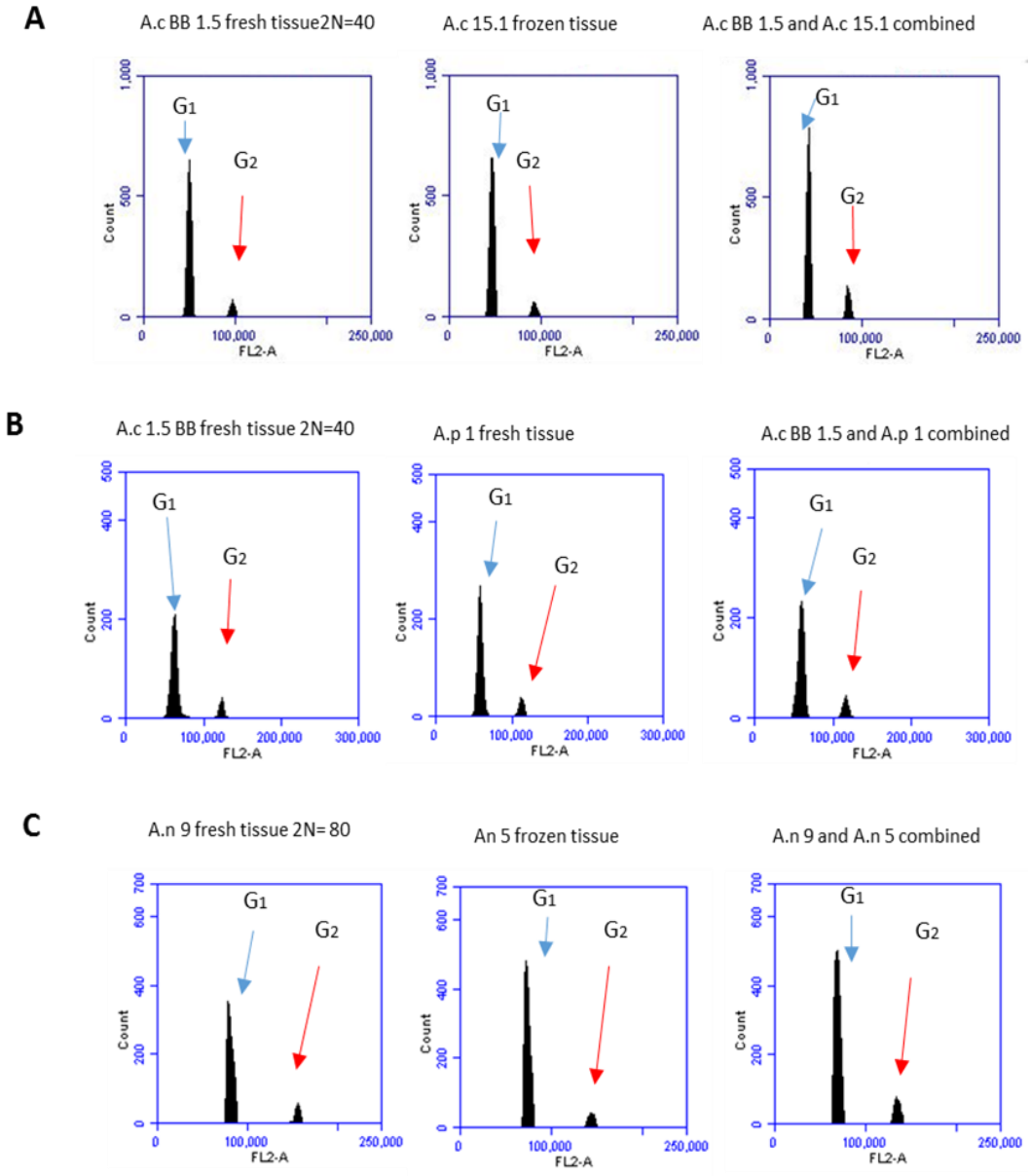

Figure 3.7: Fluorescence histograms in the FL2-A spectrum of G_1 and G_2 metaphase peaks in *A. ciliata* complex species with unknown ploidy levels, calibrated with size standards. (**A**) Fresh tissue standard from *A. ciliata* subsp. *ciliata* A.c BB 1.5 (Ireland) and frozen tissue *A. ciliata* subsp. *ciliata* A.c. 15.1 (Spain) both, 2N= 40. (**B**) Fresh tissue standard A. c BB1.5 (Ireland) and fresh tissue sample *A. ciliata* spp. *pseudofrigida* A. p1 (Norway) 2N= 40. (**C**) Fresh and frozen tissue from *Arenaria norvegica*, A.n 9 (Norway) fresh tissue standard and A.n 5 (Scotland) frozen tissue both sample shows 2N= 80.

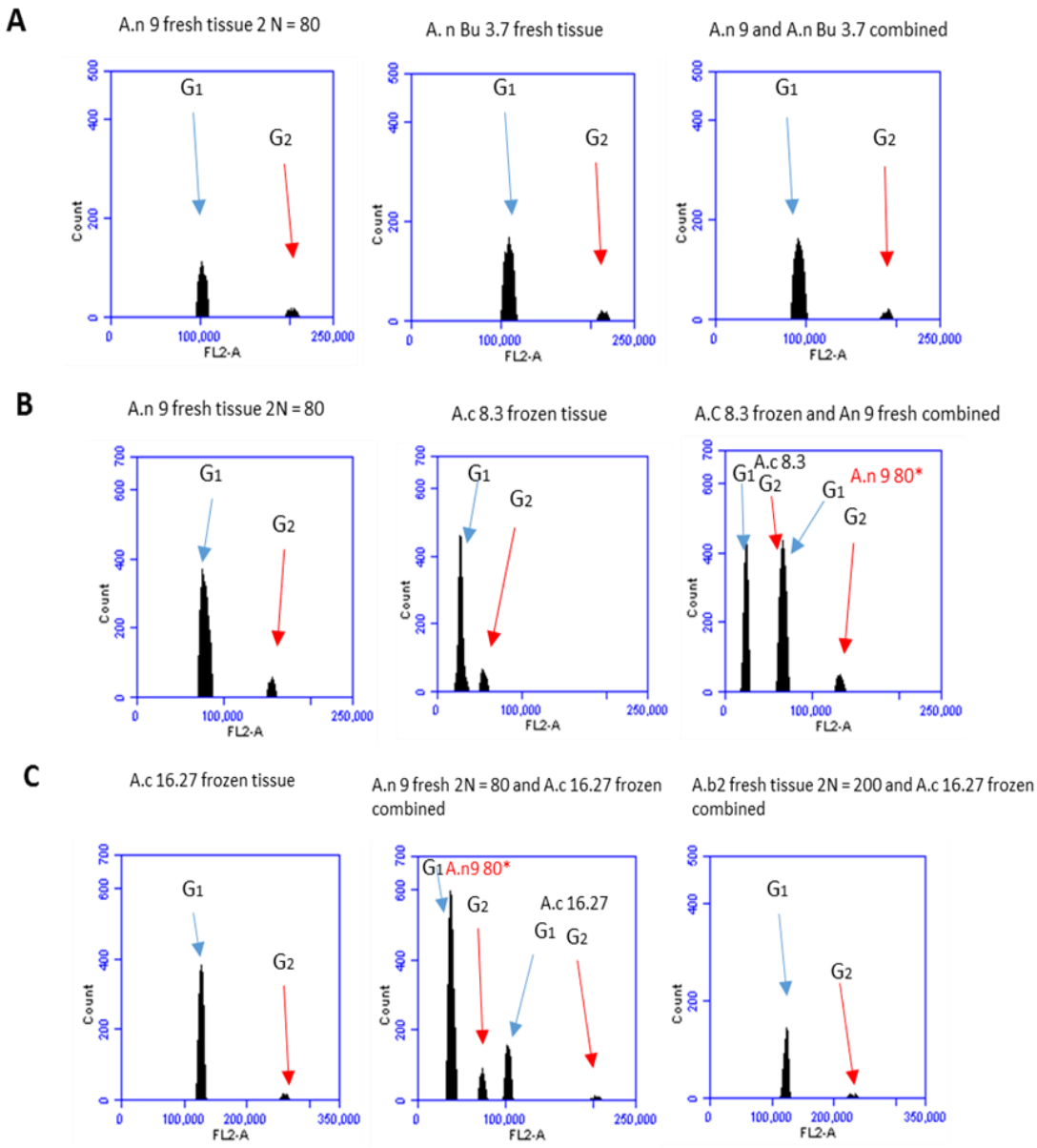

Figure 3.8: Fluorescence histograms in the FL2-A spectrum of G_1 and G_2 metaphase peaks in *A. ciliata* complex species with unknown ploidy levels, calibrated with size standards. (**A**) Fresh tissue from *Arenaria norvegica*, A.n 9 (Norway) standard sample and A.n 3.7 (Ireland) sample both showing 2N =80. (**B**) A.n 9 standard sample and A.c 8.3 (Italy) frozen sample, 2N=80 and 40 respectively. (**C**) Fresh tissue standard *A. norvegica* A.n 9 (Norway) and *A. bernensis* A.b 2 (Switzerland) 2N= 80 and 200 respectively and frozen sample *A. ciliata* subsp. *ciliata* Ac16.27 (Austria), 2N=200. Plot axes justified for scale is each panel.

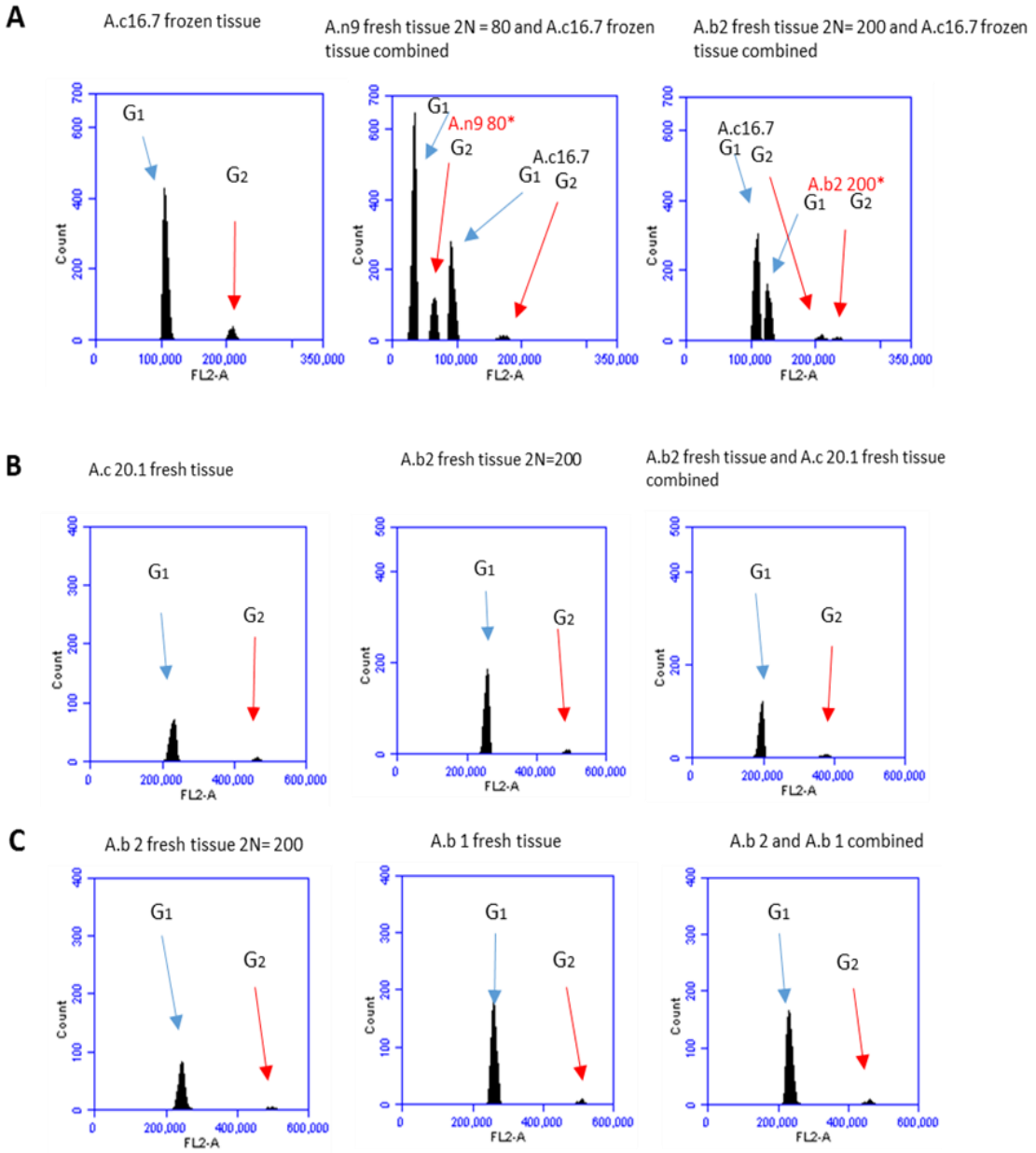

Figure 3.9: Fluorescence histograms in the FL2-A spectrum of G_1 and G_2 metaphase peaks in *A. ciliata* complex species with unknown ploidy levels, calibrated with size standards. (**A**) Fresh tissue standard *A. norvegica* A.n 9 (Norway), fresh tissue standard *A. ciliata* subsp. *bernensis* A.b2 (Switzerland) 2N= 80 & 200 respectively and frozen sample *A. ciliata* subsp. *ciliata* A.c16.7 (Austria) 2N=160. (**B**) Fresh tissue standard Ab2 and fresh tissue sample *A. ciliata* subsp. *ciliata* Ac20.1 (Austria), both 2N=200. (**C**) Fresh tissue standard Ab2 and fresh tissue sample *A. ciliata* subsp. *bernensis* Ab1 (Switzerland), both 2N=200. Plot axes justified for scale is each panel.

Table 3.1: Analysed populations using confocal microscopy and flow cytometry show variance of ploidy levels in *Arenaria ciliata* group.

Taxon identity	Cited ploidy*	Population	n	Ploidy analysis**			Recorded ploidy level						
				Microscopy Source tissue	Flow cytometry Source tissue	Internal control	40	60	80	120	160	200	
A. ciliata subsp. ciliata	40, 80, 120, 160	Ac1	2		frz	Ac3 [40]	2						
		Ac2	4		frz	Ac3 [40]	4						
		Ac3	7	f x 5	f/ frz	An [80], Ab1 [200]	7						
		Ac6	4		frz	Ac3 [40]	3		1				
		Ac8	1		frz	An9 [80]	1						
		Ac16	3		frz	Ab2 [200]					1	2	
		Ac17	3		frz	Ab2 [200]				1	2		
		Ac19	2		frz	Ac3 [40], Ab1 [200]	2						
		Ac20	1		f	Ab2 [200]						1	
A. ciliata subsp. bernensis	200, 240	Ab1	1	f	f/ frz	Ac3 [40], An9 [80]						1	
		Ab2	2		f/ frz	Ab1 [200]						2	
A. ciliata subsp. pseudofrigida	40	Ap1	2		f/ frz	Ac3 [40], An9 [80]	2						
		Ap3	1		f	Ac3 [40]	1						
A. gothica subsp. moehringioids		Ac10	3		frz	Ac3 [40], An9 [80]	2		1				
		Ac13	2		frz	Ac3 [40], An9 [80]	2						
		Ac14	2		frz	Ac3 [40], An9 [80]	2						
		Ac15	4		frz	Ac3 [40], An9 [80]	2		2				
A. norvegica	80	An1	3		f/ frz	An [80]			3				
		An5	1		frz	An1 [80], An9 [80]			1				
		An8	1		frz	Ac3 [40], Ab1 [200]		1					
		An9	4	f	f/ frz	Ac3 [40], Ab1 [200]			4				
Total							30	1	12	1	3	6	52

* Tutin *et al*. (1993). ** Tissue sample preparation: fresh (f), frozen (frz).

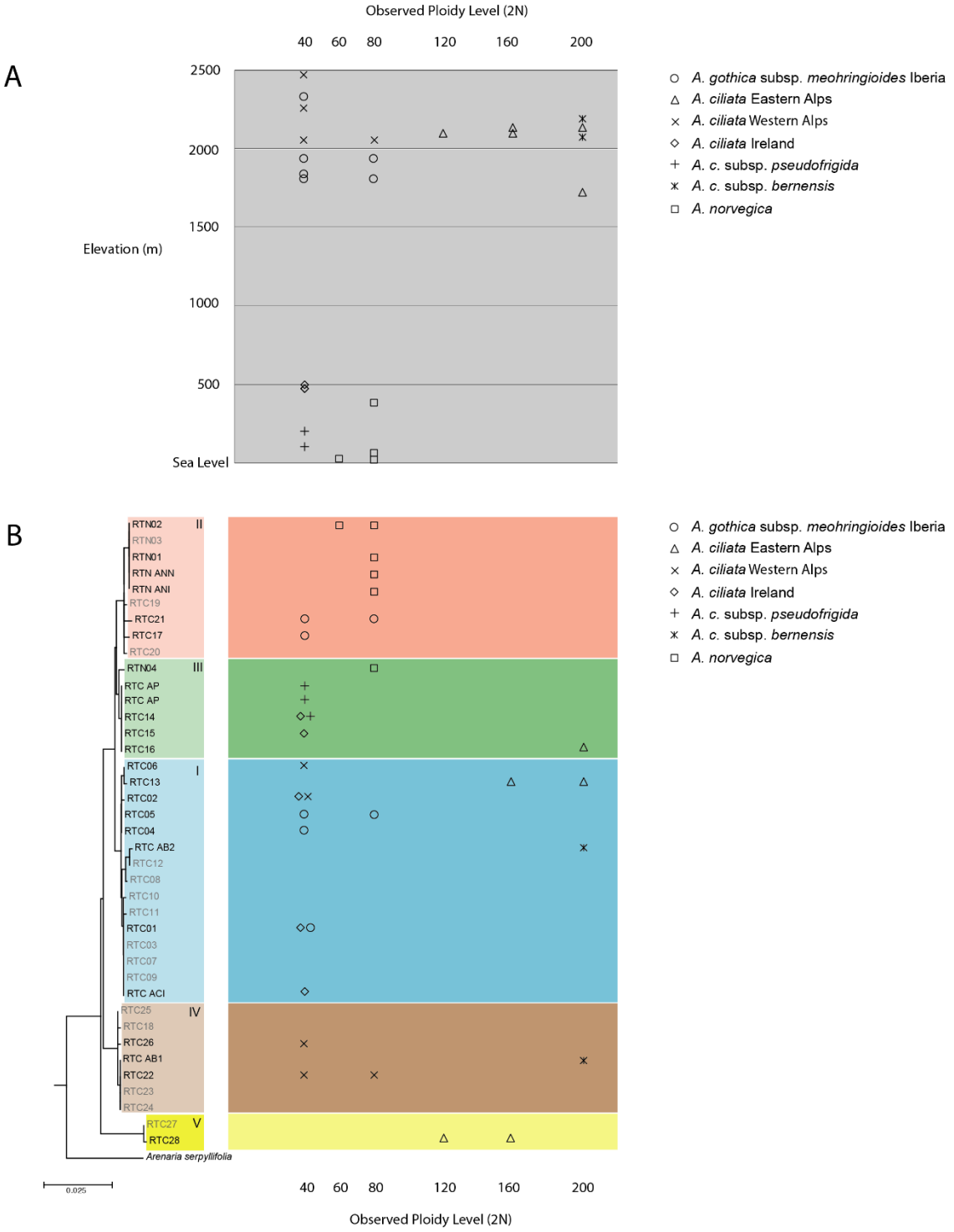

Figure 3.10: A Ploidy levels of analysed populations and taxa of the *Arenaria ciliata* complex relative to elevation of the sampled population. **B.** Distribution of observed ploidy levels relative to the haplotype identity of sampled individuals within these populations. The phylogenetic tree is a Maximum Likelihood analysis of *A. ciliata* haplotypes based on the combined DNA sequence identity of the rps16 and trnTL chloroplast loci (see chapter 2 for discussion of phylogenetic analysis).

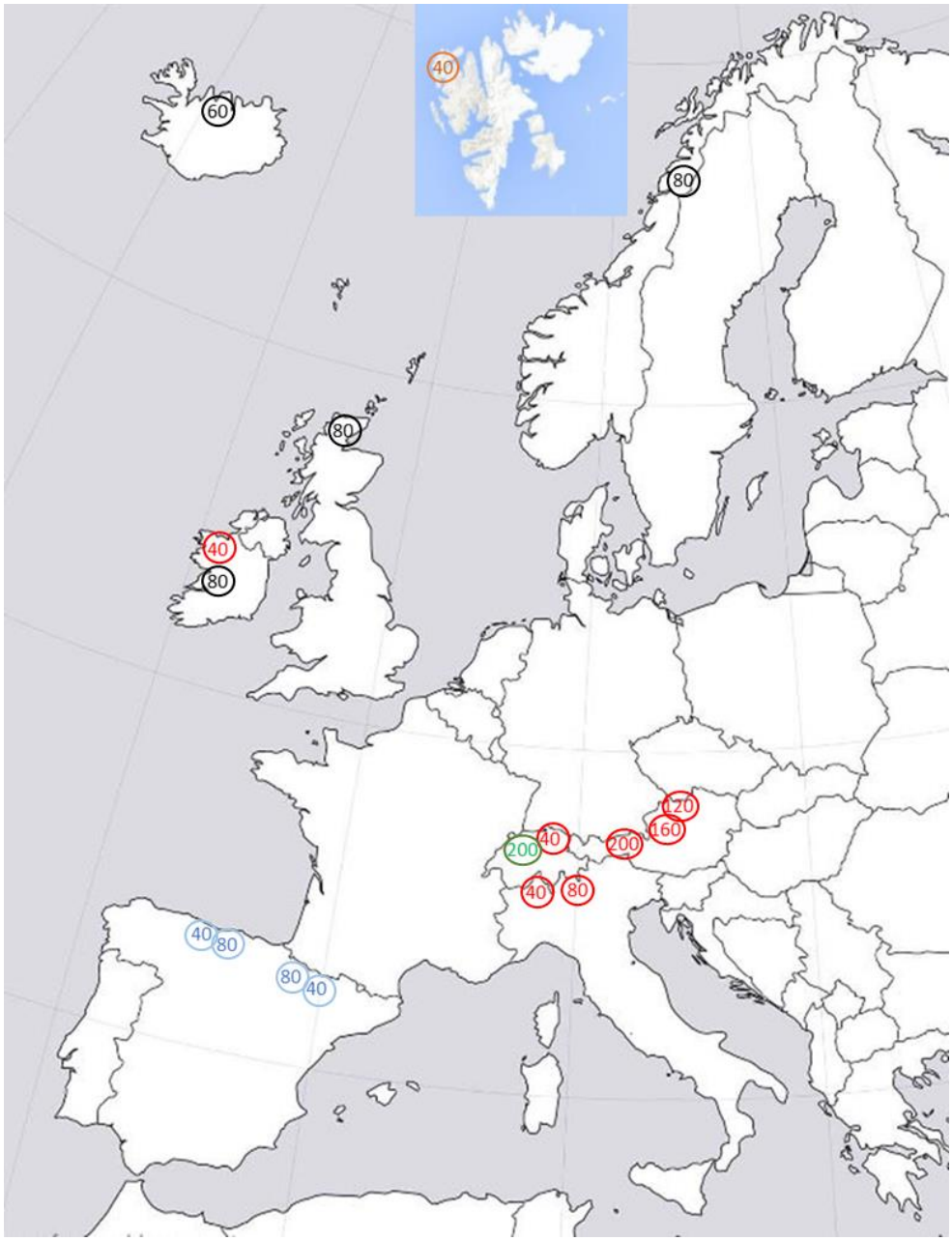

Figure 3.11: Distribution map of ploidy levels in sampled populations of the *A. ciliata* complex for *A. ciliata* spp. *ciliata*, (in red), *A. ciliata* subsp. *bernensis* (in green), *A. ciliata* subsp. *pseudofrigida* (in brown), *A. gothica* subsp. *moehringioides* (in blue) and *A. norvegica* (in black).

3.4 Discussion

The protocol developed for this project using confocal microscopy and flow cytometry provided a clear evaluation of ploidy state variation across European populations of the *A. ciliata* complex. Observed ploidy levels differed across the disjunct populations of the complex, ranging from 2N=40 to 2N=200, with the majority of populations showing 2N= 40-80. In general, higher levels of chromosomes were found at higher altitudes compared to lower altitudes, as has been documented for other alpine plant species (Mráz *et al*., 2007; García-Fernández *et al*., 2012). Populations occurring in high elevation collectively showed the full range of ploidy levels, whereas at low altitude observed ploidy levels ranged only between 2N=40 to 2N= 80 (Figure 3.10). Detailed observed ploidy distribution shown in Figure (3.11) and Table (3.1).

Among the analyzed taxa various ploidy levels were recorded, *A. ciliata* subsp. *pseudofrigida* and *A. ciliata* subsp. *bernensis* were invariant at 2N = 40 and 200 respectively, whereas *A. norvegica* and *A. gothica* subsp. *moehringioides* both showed two ploidy levels (2N = 60, 80 and 40, 80 respectively). Observed ploidy in *A. ciliata* subsp. *ciliata* was more variable, showing five ploidy states (2N = 40, 80, 120, 160 & 200) (Table 3.1). Among populations, only five of twenty-one sampled populations showed two ploidy states while the remaining populations showed one ploidy state (Table 3.1, Figure 3.10). Populations with the highest observed ploidy occur in the eastern Alps, where *A. ciliata* individuals ranged in ploidy between 2N= 120 and 200 (Figure 3.10).

Distribution of ploidy variation of *A. ciliata* species across Europe in the present study indicates that polyploidisation has been a key feature of recent evolution in the group, with especially high ploidy variation among high altitude populations (Figure 3.10A). In terms of the evolutionary history of ploidy changes, when observed ploidy states for

sampled individuals are superimposed onto the chloroplast haplotype phylogeny presented in Chapter 2, it is clear that high polyploid states have appeared separately on multiple different chloroplast lineages (Figure 3.10B). It is also clear that low ploidy states, characteristic of northern populations in the *A. cilaita* complex, are associated with haplotype identities that are also present in high ploidy individuals in southern populations. These are relevant findings, as they indicate that (i) local ploidy changes have occurred after the separation of distinct genetic lineages in the *A. ciliata* complex, and (ii) that polyploidisation in these lineages only occurs within populations that occur at high altitude.

These ploidy changes may have neutral selective impact, and may simply be artefacts of chance genetic events. It is also possible that these changes may have some selective role in the survival of each population. As suggested in the literature, the recorded variation in ploidy may have played an important role in enhancing plant tolerance and responses to abiotic and biotic stress. It is notable that ploidy variation at lower elevations is very limited (Figure 3.10). Based on the analyses of Dang (2012), Dang *et al* (2013) and Howard-Williams (2013), these lower elevation populations are mostly likely to be of more recent origin than the higher elevation populations to the south.

Higher ploidy diversity may simply be a function of population age. However in this scenario, only low ploidy identities are seen to have migrated across low elevation landscapes in Northern Europe, even though multiple ploidy levels existed in the likely centres of origin. This pattern suggests some adaptive or selective role for ploidy in high elevation versus low elevation habitats (Figure 3.11, Figure 3.10A &B).

Chapter 4.

4 Biometric analysis of *A. ciliata* (Ireland) and *A. norvegica* (Ireland) population phenotype behaviour in 4 biogeographic habitat scenarios.

4.1 Introduction.

Arctic and Alpine habitats present many abiotic challenges for growing plants, most importantly temperature stress, and this is associated with widespread evolutionary adaptation to cold stress among arctic and alpine plant lineages (Grime, 1977). These adaptations involve elevated oxidative stress tolerance and management mechanisms at a cellular level, as well as physical and life-history adaptations that lessen the probability and impact of major stress events on plant tissues (Billings, 1987). In cold habitats, these adaptations allow arctic and alpine plants to prosper where other ecological strategies fail, giving rise to the typical cold zone vegetation.

Populations of arctic-alpine plant species growing outside of these habitats in more temperate habitats are subjected to reduced temperature stress. Both *Arenaria ciliata* and *A. norvegica* in Ireland occur in typically warmer and less extreme growing conditions than any other populations of either species across Europe and the Arctic. Based on phylogenetic analysis (Dang *et al*., 2012, Dang, 2012, Howard-Williams, 2013) these local Irish populations have distinct genetic identities that date to the end of the Pleistocene, c.20,000 years before present - *A. ciliata* having an arctic affiliation, and *A. norvegica* deriving from Iberia. A key question is whether the reduced stress experienced by these populations in Irish climate regimes in the time since has resulted in adaptive change, and/or a reduction of native ability for cold-stress tolerance.

Ambient air temperature has a major effect on the rate of the plant growth and each individual plant species has specific optimal, minimum and maximum temperature thresholds. For the majority of plant species, temperature extreme events, such as frost or heat, can have dramatic impacts on plant vegetative development and productivity (Kumudini *et al*., 2014). For example, a review by Barlow *et al*. (2015) on the impact of

extreme temperature events in wheat revealed that freezing conditions caused sterility and termination of formed grains while extreme heat reduced total grain yield and caused reduction in duration of the grain-filling period. Stress-tolerating species possess a range of coping mechanisms to limit these kinds of stress impacts, and they form an important sub-group in most vegetation types, becoming the dominant species in arid and freezing habitats (Osmond *et al*., 1987). Within species, stress-coping ability is known to vary both over the lifetime of an individual plant, and to vary along environmental gradients across a species distribution, with ecotypes from more stressful habitats displaying elevated tolerance for these stresses compared to ecotypes from less challenging habitats, as well as unused stress-coping ability being a feature among populations that establish in less challenging habitats (Zhen and Ungerer, 2008).

In terms of temperature stress, cold extremes are a feature of both Arctic and Alpine habitats, and the extent of temperature change, especially in the transition from winter into spring and summer, is more extreme in Arctic and Alpine habitats compared to other biomes (Good and Millward 2007).

Plant phenology is considered to be one of the most easily observable and sensitive natural indicator of plant responses to temperature change (Shen *et al.*, 2015). In general, the rate of plant growth increases as temperatures rise to within the optimum range for the species, and slows when temperature is outside this optimum. Progression of specific vegetative development stages (germination, leaf and branches appearance rate etc.) is also controlled by temperature (Schlenker and Roberts, 2009), and in many plant species reproductive development usually has a lower optimum temperature than vegetative development (Hatfield *et al*., 2011). Ambient temperature ranges above or below specific thresholds during critical phases of development can have detrimental effects on phenology and productivity. For example, flower formation, sexual maturation,

pollination and fertilization are some of the most sensitive phenological stages to temperature, and during these developmental stages extreme temperature events are known to greatly affect viable seed production (Hatfield and Prueger, 2015). In addition, temperature effects are more severe where there is also a water deficit. Photoperiod may interact with temperature, especially light sensitive plants, causing a disruption in phonological responses (Hatfield and Prueger, 2015). In Arctic and Alpine habitats, changing day length and ambient temperature in spring triggers the rapid onset of growth and reproductive phases, often over a very short period of time (Good & Millward, 2007).

Adverse environmental conditions may lead to various physiological responses during growth and developmental stages (Lichtenthaler, 2009). Change in temperature and day light during the growing seasons affects tissue pigment composition and abundance. Chlorophyll and carotenoids are responsible not only for light absorption for photosynthesis, but also play a role in protecting plants from excessive radiation (Devi *et al*., 2015). Chlorophyll concentration is regulated by plants in response to environmental changes, and this is considered an adaptative response for plants that experience temperature fluctuations during growing seasons under environmental stress (Close *et al*., 2006). Seasonal variations in abiotic conditions lead to changes in the concentration and abundance of photosynthetic pigments, which can lead to disruption in photosynthesis performance. For this reason, chlorophyll content per unit area has become an important index measure of photosynthetic capacity and productivity for plants experiencing stress (Devi *et al*., 2015).

One of the key questions regarding ongoing climate change is the extent to which local ecotypes and species variants differ in their ability to respond to abiotic stress, especially temperature. Long-established populations that display strong adaptation to local

environmental conditions may be limited in their ability to cope with warmer temperatures, or freezing events.

This chapter presents an investigation of the growth performance of two Caryophyllaceae species, *Arenaria ciliata* and *A. norvegica*, exposed to varying environmental conditions and heat stresses during seasonal changes in four different habitats. These conditions included summer, autumn and winter and spring in representative Temperate Montane and Temperate Sea level habitats (home habitats for *A. ciliata* and *A. norvegica*, respectively, in Ireland) as well as representative Arctic and Alpine habitats, typical of the range of both species outside Ireland.

Objective

The overall aim of this work was to establish the level of divergence between *A. ciliata* and *A. norvegica* in terms of their growth and development characteristics, and to determine whether adaptation to Irish climate conditions has impacted the ability of *A. ciliata* and *A. norvegica* to grow in more stressful environments that occur elsewhere in the distribution of the two species, and also conditions that may arise as a result of climate change in Ireland. The objective was to analyse the phenotypic response of two ecotypes (*Arenaria ciliata* from Co. Sligo, Ireland [diploid plant, 2N=40] and *A. norvegica* from Co. Clare Ireland [tetraploid plant, 2N=80]) when grown over 4 consecutive seasons in 4 different habitat settings that reflect idealised biogeographic climate differences across the distribution of the *A. ciliata* species complex to which both species belong. In order to capture whole-plant responses to the environmental treatments, measurements were made of (i) conventional plant biometric traits and (ii) chlorophyll content at specific time intervals over the course of the growing seasons. These observations were designed to

capture variance in the phenology of key developmental and life-history traits, firstly to evaluate relative morphological and developmental differences between the two species, and secondly to compare within and between-species responses to environmental conditions across the range of idealised habitats.

4.2 Materials and Methods.

4.2.1 Seed planting for habitat growth experiments

Collection and site details for the *Arenaria* species seed lots used in these experiments is described in Chapter 2.11cm plastic seedling pots were filled to 2cm below the rim with John Innes Number 2 potting mix. Seeds were planted 5mm below the surface and watered until the soil column was saturated. Following planting, seed lots were assigned to one of four different habitat treatment cohorts, each comprising a number of germinating seedlings of each species, giving a starting population of 195 seedlings in total. The habitat treatments were designed to replicate idealised growing conditions over a 12 week period, for each season, in four biogeographic habitats; (i) a Temperate sea-level habitat [TEMP SL]; (ii) a Temperate montane habitat [TEMP M]; (iii) an Arctic habitat [ARC]; and (iv) an Alpine habitat [ALP] (Temperature and light conditions for each habitat are detailed in Table 4.1). In addition to *A. ciliata* subsp. *ciliata* (Ireland) and *A. norvegica* (Ireland), a reduced number of individuals of *A. ciliata* subsp. *bernensis* (population origin Switzerland, Alpine habitat, see Table 2.1 in Chapter 2), *A. ciliata* subsp. *pseudofrigida* (origin Svalbard, Arctic habitat) and *A. norvegica* (origin Norway, Arctic habitat) were grown for comparative purposes. Due to lower than expected survival rates, seedling data for these seedlings was not included in the statistical comparisons completed for the growth experiments.

4.2.2 Biometric measurement of growth parameters

In order to capture the relative growth and maturation of individual plants in the analysed species over the course of the annual growth cycle, the following biometric and life history events were recorded for each treatment (Causton and Venus, 1981): (a) time of germination; (b) time of first leaf appearance; (c) time of first branch; (d) time of flowering; (e) mean root and shoot biomass at the end of Spring. In addition, ongoing weekly measurements were also recorded for: (f) mean internode length, (g) length of the plant main stem; (h) number of branches emerging on the main stem and (i) number of branches overall on the plant.

Measurement of length and timing was completed by direct non-destructive sampling. Root and shoot biomass weights were calculated according to the following protocol. Above ground shoot tissue of each individual plant was clipped 1mm above ground level and placed in a labelled paper bag. Soil containing roots was placed in a sieve and carefully washed with tap water to remove the soil and clean the roots, while minimizing damage to the intact roots. The roots were placed on plain paper to dry out at room temperature for three hours and then they were placed in a labelled paper bags. All labelled bags of root and shoot biomass were placed into a drying oven at 60 ^{o}C for 48 hours. After this the dry weight of the plant material was recorded for each sample. Three days later, dry biomass was weighed again to ensure all moisture content was evaporated form plant tissues and the measurement of dry biomass was accurate.

Table 4.1: Light and temperature settings over four consecutive seasons for the four treatment habitats used in biometric experiments for *A. ciliata* and *A. norvegica*.

Growth season		**Summer**			**Autumn**			**Winter**			**Spring**		
Month		**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**
Treatment habitat	**Treatment Conditions**												
Arctic (ARC)													
	Treatment Temperature ^{0}C	9	9	9	4	2	-5	-5	-5	-5	-5	-5	-5
	Average Daylight Hours/Day	14.00	14.00	14.00	04:30	02:30	1.00	00:50	00:50	00:50	1.00	02:30	07:30
Alpine (ALP)													
	Treatment Temperature ^{0}C	20	17	17	10	4	4	-3	-3	-3	-2	0	3
	Average Daylight Hours/Day	16:00	16:00	16:00	14:00	12:30	11:00	09:30	09:00	09:00	10:00	11:00	12:30
Temperate Mountain (TEMP M)													
	Treatment Temperature ^{0}C	17	17	17	13	11	10	5	5	5	5	5	5
	Average Daylight Hours/Day	16:00	16:00	16:00	15:00	12:30	10:30	08:30	07:30	08:00	09:40	11:50	14:00
Temperate Sea Level (TEMP SL)													
	Treatment Temperature ^{0}C	17	17	17	15	13	11	8	8	8	8	8	8
	Average Daylight Hours/Day	16:00	16:00	16:00	15:00	12:30	10:30	08:30	07:30	08:00	09:40	11:50	14:00

4.2.3 Chlorophyll extraction.

Chlorophyll pigments are hydrophobic compounds that can be extracted from living plant tissue by using organic solvents such as acetone, ethanol or methanol into which chlorophyll can readily dissolve once plant tissue has been disrupted. Chlorophyll was extracted using 80% acetone (Fisher Chemicals) and measured by spectrophotometry according to the protocol of Jeffrey and Humphrey (1975).

At the end of each of the three growing seasons (summer, autumn and winter), chlorophyll content was measured in 5 individual plants of each species in each habitat treatment for both *A. ciliata* and *A. norvegica*. To prepare each sample, between two to three green leaf samples were weighed to get 5 mg fresh leaf tissue per sample. These were placed in a mortar with 2 ml aqueous acetone and the tissue was ground using a pestle. The extracted liquid mixture was transferred to 15 ml Falcon tube using a plastic pipette. Following the addition of further 1.5 ml acetone to the mortar the remaining leaf material was ground again, and the supernatant transferred to the Falcon collection tube. This additional step was repeated three times in total. Subsequently, the extracted solution containing chlorophyll was centrifuged at 2500 rpm for 10 minutes and the supernatant transferred to a new 15 ml Falcon tube. Then 1 ml aqueous acetone was added into the sediments in each tube and vortexed. Then samples were centrifuged at 2500 rpm for 10 minutes, and the supernatant transferred to a new Falcon collection tube. Finally, 80% acetone was added to bring the final volume to 8 ml in each Falcon tube. One ml from each sample was taken and placed into a Cuvette to allow quantification of the chlorophyll content by UV-VIS spectrophotometry on Beckman DU640 spectrophotometer. Light absorbance was measured at wavelengths between 645 nm and 663 nm. Total chlorophyll content was calculated using the following formula.

Total chlorophyll content = (20.2* A645) + (8.02* A663) = µ/mL

Where: A645 = absorbance at 645nm

A663 = absorbance at 663nm.

4.2.4 Statistical Analysis of Data

Prior to statistical analysis of biometric and chlorophyll data, all datasets were tested for normality and parametric distribution. In cases where sample sizes were too small ($n < 7$), [chlorophyll data] pairwise Mann-Whitney (U-test) comparison were completed. In cases sample where sample size was ≥ 8, D'Agostino and Pearson normality test was completed and where datasets were parametric, One-way ANOVA analysis was used for multiple comparison and students T-test for pairwise comparison. Kruskal-Wallis test were reserved for non-parametric datasets, however none were identified in these studies. All pairwise and multiple comparisons between cohort datasets were applied in GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA.

4.3 Results

4.3.1 Biometric measurement of growth parameters.

4.3.1.1 Germination and growth of *Arenaria ciliata* and *A. norvegica* seedlings cohorts in four different habitat treatments.

Germination and survival data for the seedling cohorts are given in tables 4.2 and 4.3, with statistical comparisons between observed data in tables 4.4 and 4.5. Germination rates for *A. ciliata* in the summer climatic conditions of the TEMP M, TEMP SL and ARC habitats was approximately 72%, 75% and 75%, respectively (Table 4.2, 4.4). Successful germination in the alpine habitat was lower at 47%. Overall more than 75% of seedlings survived in all habitats. Seedling survival following germination was highest in the ARC habitat, at 92.6%, while the lowest survival percentage (76.7%) was seen in TEMP SL habitat (Table 4.2, 4.4).

Overall *A. norvegica* showed lower germination ratios as compared to *A. ciliata* across all habitats, at 43.3% (Table 4.2). The highest germination rates of 58.5% was seen in the TEMP SL habitat, followed by TEMP M, at 49% and ALP, at 44%. The lowest germination of 22.2% was observed in ARC habitat. In the ARC habitat, the germination rate was very low with only 8 seeds germinated out of 36 seeds grown, however all the germinated seeds survived in this habitat. The lowest survived seedlings of 37.5% in all habitats for both species was observed in ALP region for *A. norvegica* (Table 4.2).

Table 4.2: Germination and growth of *A. ciliata* and *A. norvegica* seedling cohorts in 4 experimental habitats.

	Treatment Habitats	No. Seeds planted	No. Seeds germinated	Germination Ratio %	No. Seedlings Surviving *	Seedlings surviving Ratio %
A. ciliata	**Temperate Mountain**	43	31	72.09	27	87.09
	Temperate See level	40	30	75	23	76.6
	Arctic	36	27	75	25	92.6
	Alpine	55	26	47.3	23	88.5
A. norvegica	**Temperate Mountain**	53	26	49	16	61.5
	Temperate See level	41	24	58.5	19	79.2
	Arctic	36	8	22.2	8	100
	Alpine	55	24	43.6	9	37.5

* Number of seedlings surviving for use in experimental analysis.

Mean germination time after planting for *A. ciliata* seeds varied between 9 and 9.7 days in the TEMP SL, TEMP M and ALP habitats, however in the ARC habitat mean germination time was 17.52 days (Table 4.3, 4.5). *A. ciliata* seedlings in the former three habitats demonstrated similar trends for first leaf appearance, averaging between 18.47 and 18.85 days. However, in the ARC habitat first leaf appearance occurred after an average of 36.72 days. In terms of appearance of the first stem branch, this occurred after an average of 40.5 days in ALP habitat, while it took around 43.5 days in TEMP M and TEMP SL habitats. As for the other biometric measures, first branch appearance occurred latest in the seedlings in the ARC habitat at a mean of 50.24 days. Similarly, at the end of the Summer growing season (Table 4.3, 4.5) mean internode length of plant was smallest (at 2.32 mm) in seedlings in the ARC habitat, however in all other habitats, seedlings showed very similar internode mean lengths ranging between 6.56-6.92 mm.

Table 4.3: Growth milestones in mean number of days after planting for *A. ciliata* and *A. norvegica* seedlings growing in the four different experimental habitats

	Treatment Habitats	Germination time[1]	Appearance of 1st leaf [1]	Appearance of 1st stem branch[1]	Internode mean length mm[2]
A. ciliata	**Temperate Mountain**	9 .66 ± 0 .85	18.85 ± 0.56	43.25 ± 0.97	6.92 ± 0.14
	Temperate See level	9 .13 ± 0 .93	18.78 ± 0.93	43.65 ± 1.16	6.56 ± 0.22
	Arctic	17.52 ± 1 .11	36.72 ± 2.72	50.24 ± 4.68	2.32 ± 0.14
	Alpine	9 .65 ± 0 .87	18.47 ± 0.66	40.50 ± 0.86	6.69 ± 0.23
A. norvegica	**Temperate Mountain**	6. 8 ± 0.59	16.00 ± 0.57	33.88 ± 0.89	11.75 ± 0.30
	Temperate See level	7.73 ± 0.45	17.68 ± 0.53	37.73 ± 1.50	12.21 ± 0.28
	Arctic	19.42 ± 1.42	38.57 ± 3.53	58.28 ± 3.80	4.85 ± 0.26
	Alpine	7.66 ± 0.92	17.33 ± 1.10	34.88 ± 0.91	11.88 ± 0.45

[1]Number of days after planting. [2]At the end of summer season (12 weeks after planting).

All values are the mean ± SE

Table 4.4: Comparison of key growth milestones among *Arenaria ciliata* and *A. norvegica* seedlings in four experimental habitats at the end of the summer experimental growing season. Multiple comparisons were completed using the ANOVA test implemented in GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

	Arenaria ciliata.				*Arenaria norvegica*			
A. Germination time								
	TEMP SL	**ARC**	**ALP**		**TEMP SL**	**ARC**	**ALP**
TEMP M	ns	p ***	ns	**TEMP M**	ns	p ***	ns
TEMP SL		p ***	ns	**TEMP SL**		p ***	ns
ARC			p ***	**ARC**			p ***
B. Timing of first leaf appearance								
	TEMP SL	**ARC**	**ALP**		**TEMP SL**	**ARC**	**ALP**
TEMP M	ns	p ***	ns	**TEMP M**	ns	p ***	ns
TEMP SL		p ***	ns	**TEMP SL**		p ***	ns
ARC			p ***	**ARC**			p ***
C. Timing of first branch appearance								
	TEMP SL	**ARC**	**ALP**		**TEMP SL**	**ARC**	**ALP**
TEMP M	ns	p *	ns	**TEMP M**	ns	p ***	ns
TEMP SL		p *	ns	**TEMP SL**		p ***	ns
ARC			p *	**ARC**			p ***
D. Average internode length, 12 weeks after planting								
	TEMP SL	**ARC**	**ALP**		**TEMP SL**	**ARC**	**ALP**
TEMP M	ns	p ***	ns	**TEMP M**	ns	p ***	ns
TEMP SL		p ***	ns	**TEMP SL**		p ***	ns
ARC			p ***	**ARC**			p ***

Table 4.5: Comparison of growth milestones between *A. ciliata* and *A. norvegica* seedlings in four experimental growth habitats. Pairwise comparisons were performed using T-test implemented in GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Growth habitats	TEMP M	TEMP SL	ARC	ALP
A. Germination time	p *	ns	ns	ns
B. Timing of first leaf appearance	p **	ns	ns	ns
C. Timing of First branch appearance	p ***	p **	ns	p ***
D. Average Internode length	p ***	P ***	p ***	p ***

4.3.1.2 Time of first flowering in *A. ciliata* and *A. norvegica* grown in four different habitats.

In the experimental treatments, clear differences were evident in the flowering timing of *A. ciliata* and *A. norvegica* (Fig. 4.1). During the observation period, neither species germinated in the ARC habitat, and *A. ciliata* also failed to flower in the ALP habitat.

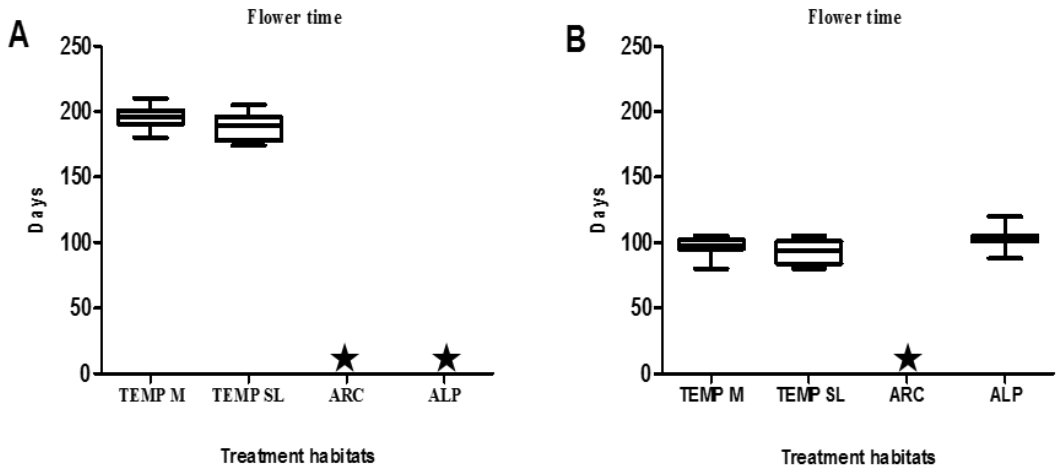

Figure 4.1: Time of first flower appearance of *A. ciliata* plants growing in the four different experimental habitats. **A.** *A. ciliata* and **B.** *A. norvegica.* Black stars indicate habitats that plants did not produce flowers (see table 5.4).

The mean flowering time of *A. norvegica* was similar in TEMP M, TEMP SL and ALP habitats (96.6, 92.2 and 103.5 days respectively). *A. ciliata* flowered only in the TEMP M and TEMP SL habitats, with similar dates for appearance of the first flowers (ranging from 189 to 195 days) However, the number of days to flowering was almost double that of *A. norvegica* in both habitats.

Where flowering occurred in the cohort, the percentage of plants flowering varied between treatments and between the two species (Table 4.6). For *A. ciliata*, the highest percentage of flowering (69.56%) was seen in TEMP SL habitat, while a comparatively lower percentage of 56.52% was seen in TEMP M habitat. On the other hand *A. norvegica*, showed 100% flowering in both TEMP M and TEMP SL habitats, while in ALP habitat 88.8% of plants in the cohort flowered (Figure 4.1 and Table 4.6).

Table 4.6: Shows number of plants and flowering percentage of *Arenaria ciliata* and *A. norvegica* in four different growth habitats.

Species	Treatment Habitats	No. Living plants	No. Flowered plants	Flowering percentage %
A. ciliata	Temperate Mountain	23	13	56.52
	Temperate See level	23	16	69.56
	Arctic	24	0	0
	Alpine	23	0	0
A. norvegica	Temperate Mountain	16	16	100
	Temperate See level	19	19	100
	Arctic	8	0	0
	Alpine	9	8	88.8

4.3.1.3 Growth and development measurements *Arenaria ciliata* species.

4.3.1.3.1 Development of the plant main stem of *A. ciliata* and *A. norvegica*.

Main stem length of A. ciliata cohorts up to 12 weeks.

The main stem growth development of *A. ciliata* plant in 4 different habitats is given in figure 4.2. The main stem showed a continuous growth up to 12 weeks in all the habitats, however the lowest growth was observed in ARC habitat, whereby the plant showed the lowest main stem length at all times. Up to 12 weeks, the highest main stem growth was noted in plant grown in TEMP SL habitat. This was followed by ALP, TEMP M and ARC habitats, where the plant main stem showed comparatively lower growth. Under ARC conditions, the main stem growth varied significantly throughout all weeks compared to other treatment habitats (Figure 4.2 and Table 4.7).

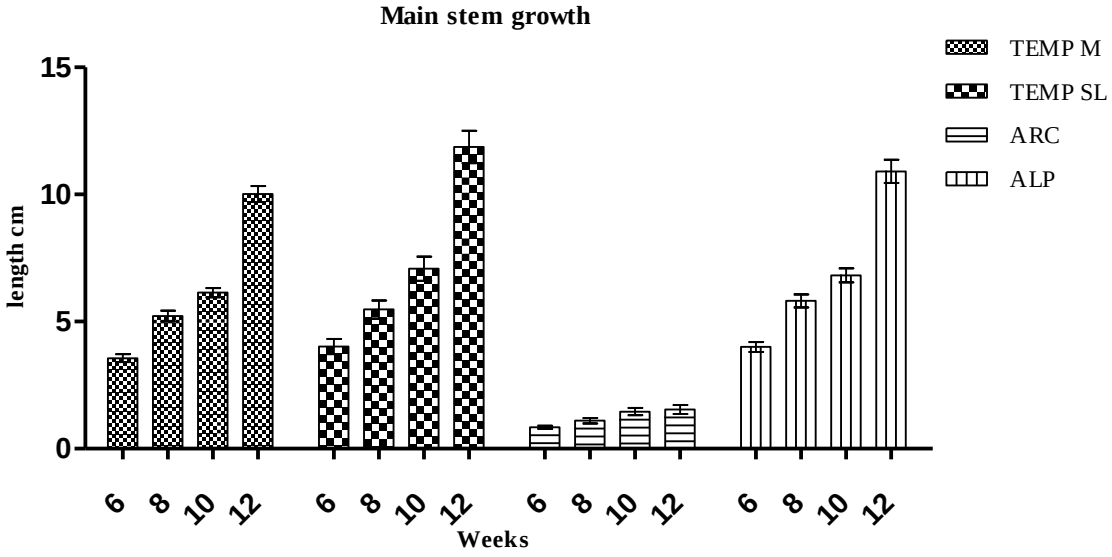

Figure 4.2: *A. ciliata* main stem growth up to 12 weeks in four treatment habitat cohorts.

Table 4.7: Statistical analysis of multiple differences in main stem growth between treatment cohorts of *A. ciliata*. Analysis of differences were performed by One-way ANOVA using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Weeks	**6**			**8**			**10**			**12**		
Habitats	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP
TEMP M	ns	P ***	ns	ns	P ***	ns	ns	P ***	ns	P *	P ***	ns
TEMP SL		P ***	ns		P ***	ns		P ***	ns		P ***	ns
ARC			P ***			P ***			P ***			P ***

Main stem length of A. norvegica cohorts up to 12 weeks.

Figure 4.3 shows the main stem growth development of *A. norvegica* plant in 4 different habitats. As observed for *A. ciliata*, the main stem of *A. norvegica* also showed a continuous growth up to 12 weeks in all the habitats, however the lowest growth of main stem was observed in Arctic habitat, whereby the plant showed the lowest main stem length at all times. This also shows that the *A. norvegica* is also least adopted to ARC habitat. Up to 10 weeks, the highest main stem length of the plant was observed in TEMP M habitat, however, after 12 weeks, the main stem length of plant grown in TEMP SL habitat became slightly higher than the plant grown in TEMP M habitat. The main stem growth under ARC treatment habitat was significantly lower than other treatment habitats throughout all growth weeks (Table 4.8).

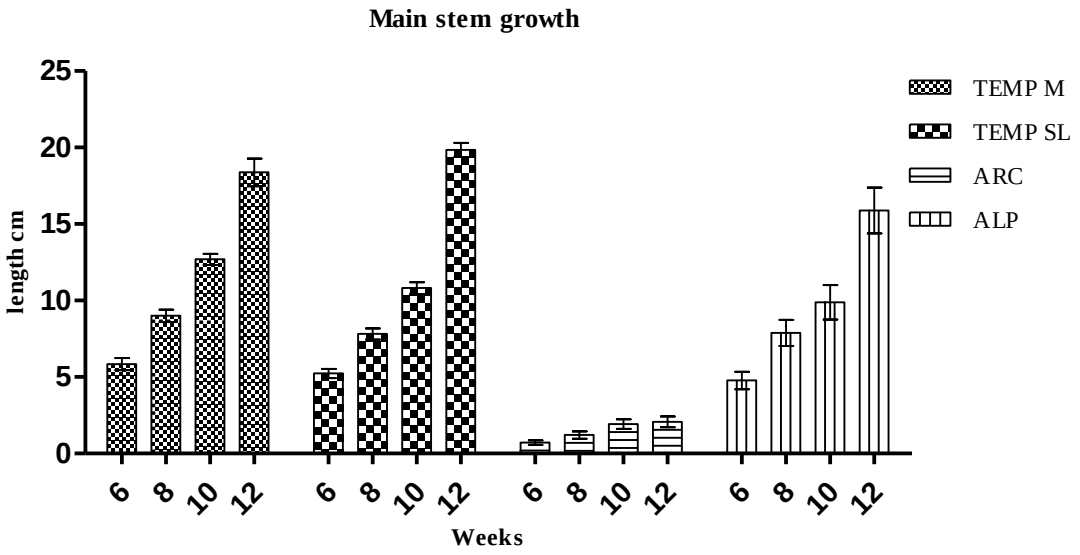

Figure 4.3: *A. norvegica* main stem growth up to 12 weeks in different habitat treatments.

Table 4.8: Statistical analysis of multiple differences in main stem growth between treatment cohorts of *A. norvegica*. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Weeks	**6**			**8**			**10**			**12**		
Habitats	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP
TEMP M	ns	P ***	ns	ns	P ***	ns	P *	P ***	ns	ns	P ***	ns
TEMP SL		P ***	ns		P ***	ns		P ***	ns		P ***	P *
ARC			P ***			P ***			P ***			P ***

Comparative main stem length up to 12 weeks between Arenaria ciliata and A. norvegica in four different habitats.

For comparison of two species, the same data as described in figure 4.2 and 4.3 was represented in figure 4.4 in a different format. This was done to compare the main stem growth performance of two species in different treatment habitats.

The main stem growth performance in all the habitats showed the same trend, whereby a continuous growth was observed for both plants up to 12 weeks. Much higher growth of *A. norvegica* was observed as compared to *A. ciliata* in TEMP M, TEMP SL and ALP (Figure 4.4 A, B and D). However, they showed a similar rate of growth in ARC habitat (Figure 4.4 C).

A. norvegica cohorts in TEMP M, TEMP SL and ALP habitats showed significantly higher main stem growth and development at all times compared to *A. ciliata* plants (Table 4.9). While main stem growth showed insignificant difference between the two species under ARC habitat treatment (Table 4.9).

In general. *A. norvegica* has much better growth performance in all the habitats as compared to *A. ciliata*.

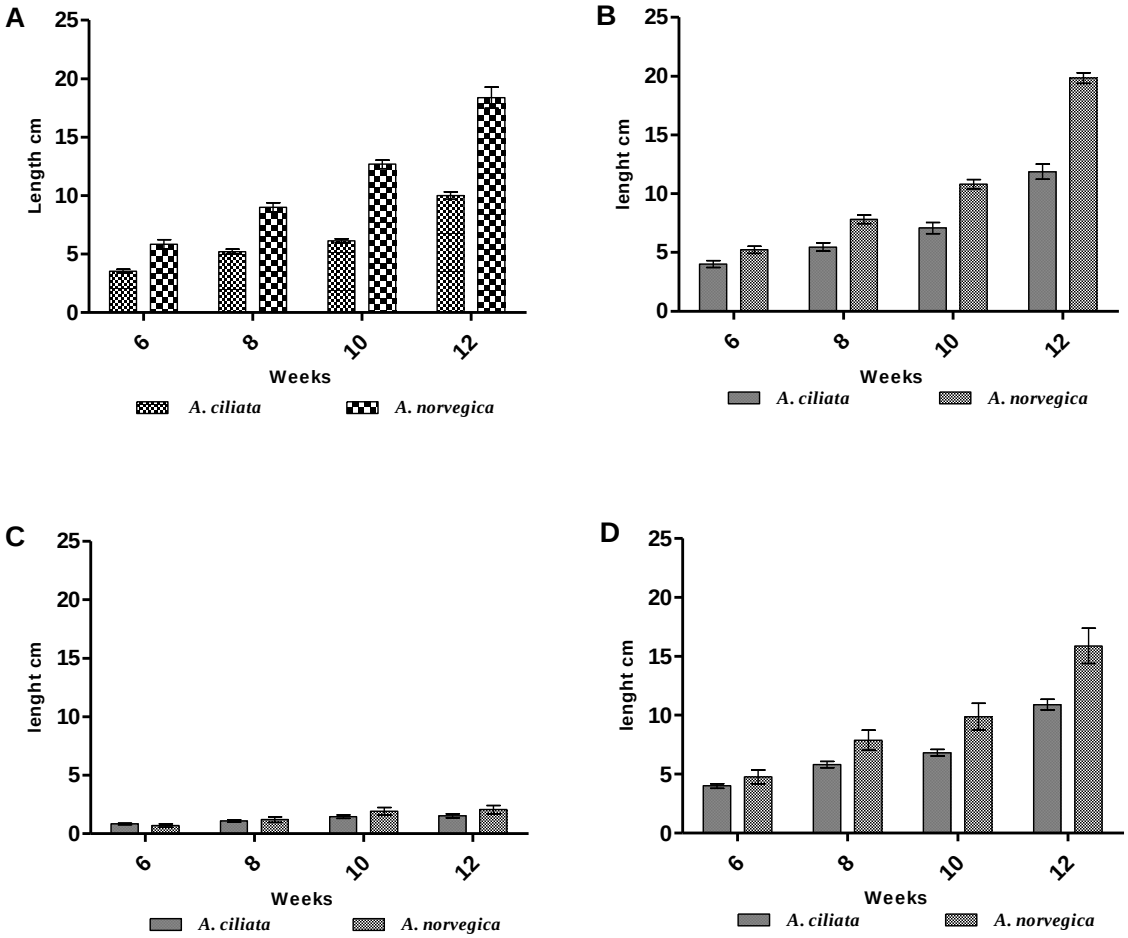

Figure 4.4: Main stem growth performance of *A. ciliata* and *A. norvegica* in different habitats up to 12 weeks. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Arctic habitat (ARC) **D.** Alpine habitat (ALP).

Table 4.9: Statistical analysis of pairwise differences in main stem growth between treatment cohorts of *A. ciliata* and *A. norvegica*. Analysis of differences were performed by T-test using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns:P > 0.05) (**: P < 0.01) (***: P < 0.001).

Weeks	6	8	10	12
TEMP M	P ***	P ***	P ***	P ***
TEMP SL	P **	P ***	P ***	P ***
ARC	ns	ns	ns	ns
ALP	ns	P **	P ***	P ***

4.3.1.3.2 Branch number emerging on the *A. ciliata* and *A. norvegica* main stem.

In order to further assess the growth performance of the two-targeted species in different treatment growth habitats, the number of stems emerging from main stem for each plant was counted under different habitats.

Number of main stem branches of A. ciliata cohorts up to 12 weeks.

Figure 4.5 shows that the number of stem branches from main stem of *A. ciliata* under different growth habitats were continuously increased up to 12 weeks. Figure further shows that the lowest number of branches was observed in ARC habitat. After 12 weeks, the highest number of branches was recorded in TEMP SL habitat followed by TEMP M and ALP habitats respectively (Figure 4.5). However significantly lower number of branches was observed in plants grown in ARC habitat cross all weeks (Table 4.10).

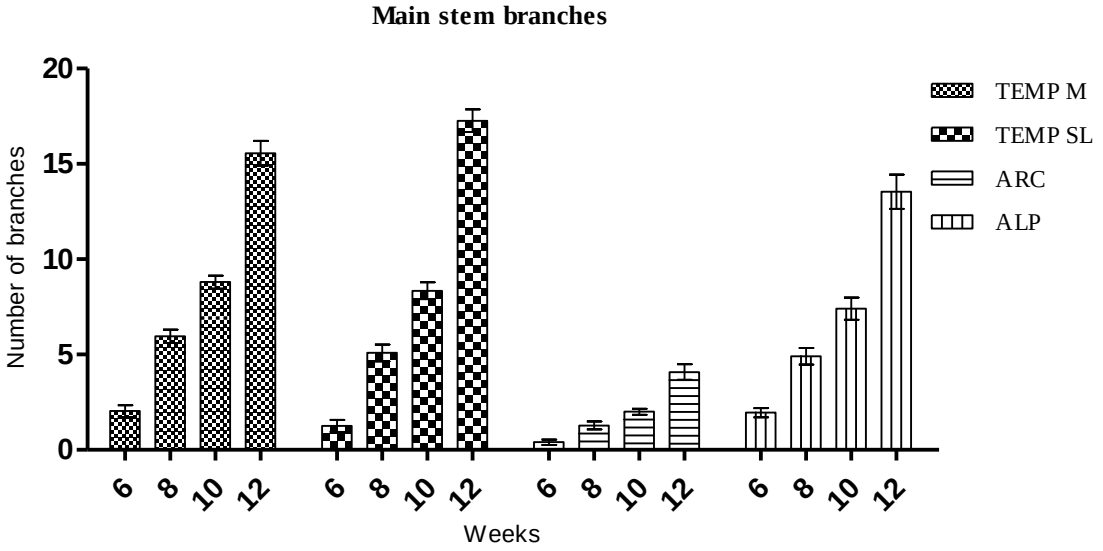

Figure 4.5: The number of branches emerged from main stem of *A. ciliata* in different growth habitats up to 12 weeks.

Table 4.10: Statistical analysis of multiple differences in main stem branches between treatment cohorts of *A. ciliata*. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Weeks	**6**			**8**			**10**			**12**		
Habitats	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP
TEMP M	ns	P ***	ns	ns	P ***	ns	ns	P ***	ns	ns	P ***	ns
TEMP SL		ns	ns		P ***	ns		P ***	ns		P ***	P **
ARC			P ***			P ***			P ***			P ***

Number of main stem branches of A. norvegica cohorts up to 12 weeks.

The number of stem branches from main stem of *A. norvegica* under different growth habitats up to 12 weeks are shown in figure 4.6. *A. norvegica* cohorts had almost similar trend as observed in *A. ciliata*, whereby a continuous increase in number of branches was observed in all habitats. The highest number of branches were obtained under TEMP SL

habitat (Figure 4.6), while lowest number of branches were obtained in ARC habitat, which differed significantly from the other growth habitats (Figure 4.6 and Table 4.11).

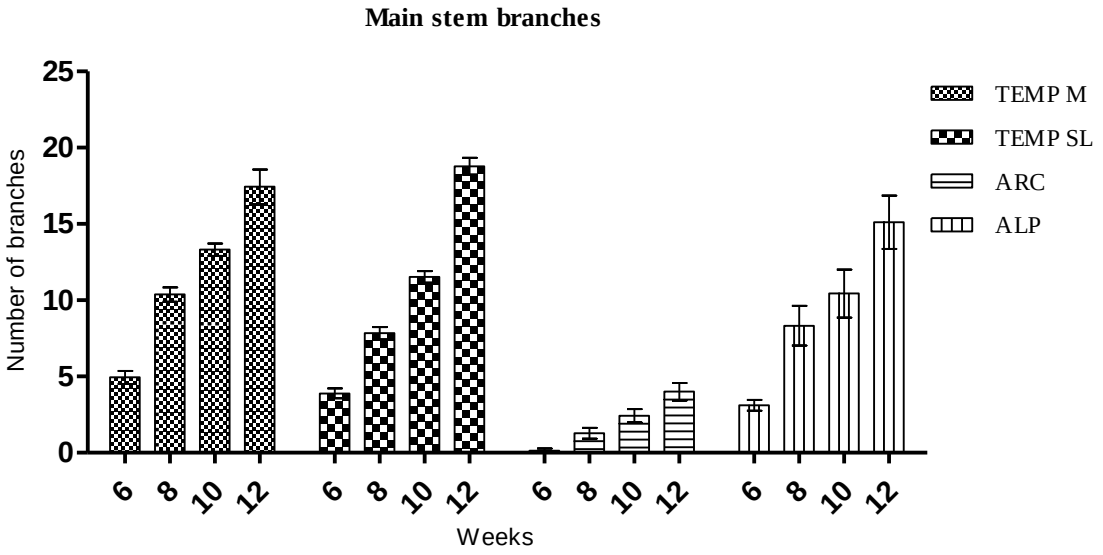

Figure 4.6: The number of branches emerged from main stem of *A. norvegica* in different treatment habitats up to 12 weeks.

Table 4.11: Statistical analysis of multiple differences in main stem branches between treatment cohorts of *A. norvegica*. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Weeks	**6**			**8**			**10**			**12**		
Habitats	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP
TEMP M	ns	P ***	P *	P *	P ***	ns	ns	P ***	P *	ns	P ***	ns
TEMP SL		P ***	ns		P ***	ns		P ***	ns		P ***	ns
ARC			P ***			P ***			P ***			P ***

Comparative number of main stem branches up to 12 weeks between Arenaria ciliata and A. norvegica in four different habitats.

Figure 4.7 further compares the number of branches from main stem between *A. ciliata* and *A. norvegica* under different habitats up to 12 weeks of growth. *A. norvegica* demonstrated the higher number of branches from the main stem as compared to *A. ciliata* in TEMP M, TEMP SL and ALP habitats.

Up to 10 weeks, number of main stem branches of *A. norvegica* cohorts was significantly higher than those recorded from *A. ciliata* main stem under all habitats (Figure 4.7A, B, D and Table 4.12), with exception of ARC habitat whereby they showed almost similar growth performance (Figure 4.7C and Table 4.12).

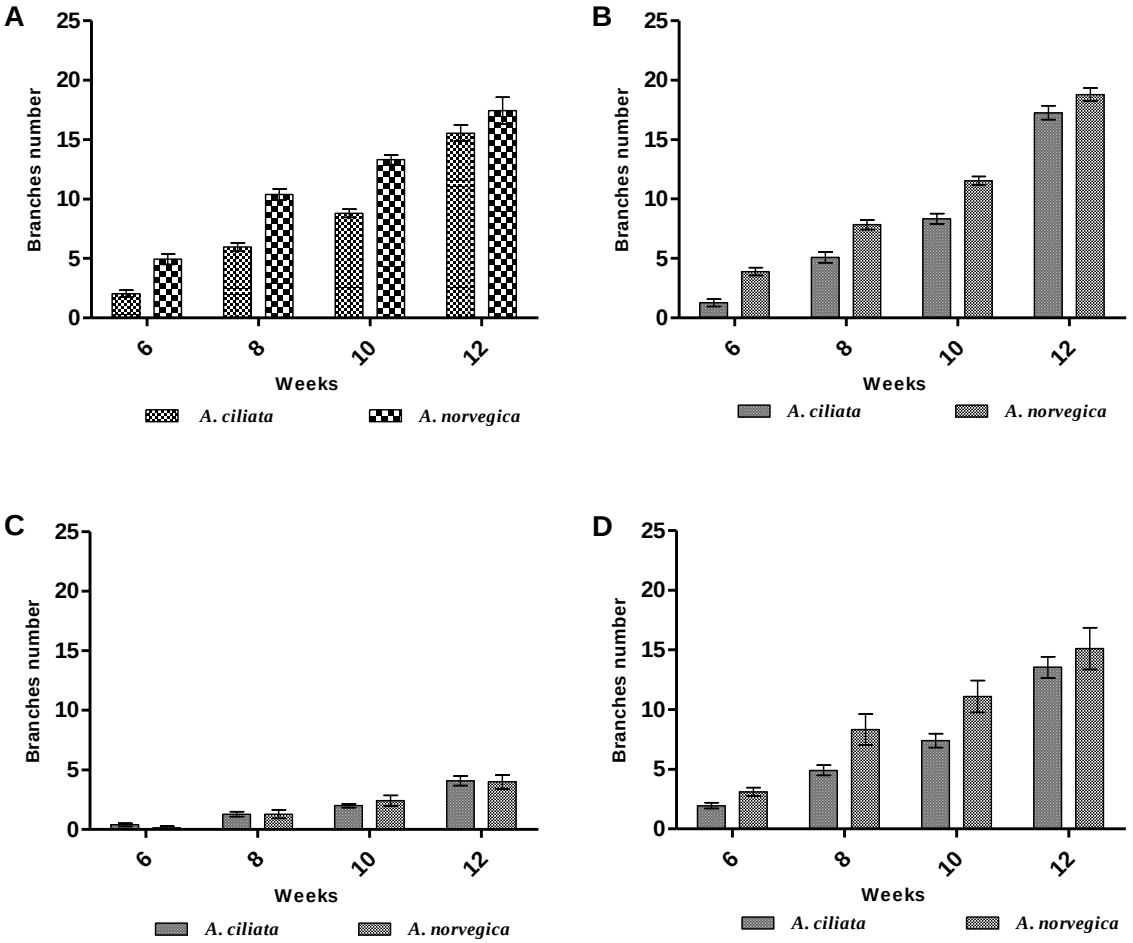

Figure 4.7: The number of branches emerged from main stem of *A. ciliata* and *A. norvegica* in different habitats up to 12 weeks. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Arctic habitat (ARC) **D.** Alpine habitat (ALP).

Table 4.12: Statistical analysis of pairwise differences in main stem branches between treatment cohorts of *A. ciliata* and *A. norvegica*. Analysis of differences were performed by T-test using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

Weeks	6	8	10	12
TEMP M	P ***	P ***	P ***	ns
TEMP SL	P **	P ***	P ***	ns
ARC	ns	ns	ns	ns
ALP	P *	P **	P **	ns

4.3.1.3.3 Number of branches overall *A. ciliata* and *A. norvegica* plants.

To proceed further on growth performance of two *Arenaria* species in different habitats, the total number of branches emerging from overall plant were also analysed under different habitats up to 12 weeks.

Total number of emerged branches of A. norvegica cohorts up to 12 weeks.

Figure 4.8 presents the total number of branches from overall plant of *A. ciliata* under different growth habitats up to 12 weeks. It was demonstrated that the number of branches were continuously increased up to 12 weeks under all habitats. The highest number of branches was observed under TEMP SL habitat, while the lowest number of branches was obtained under ARC habitat, which differed significantly form the other habitats after the sixth week (Table 4.13).

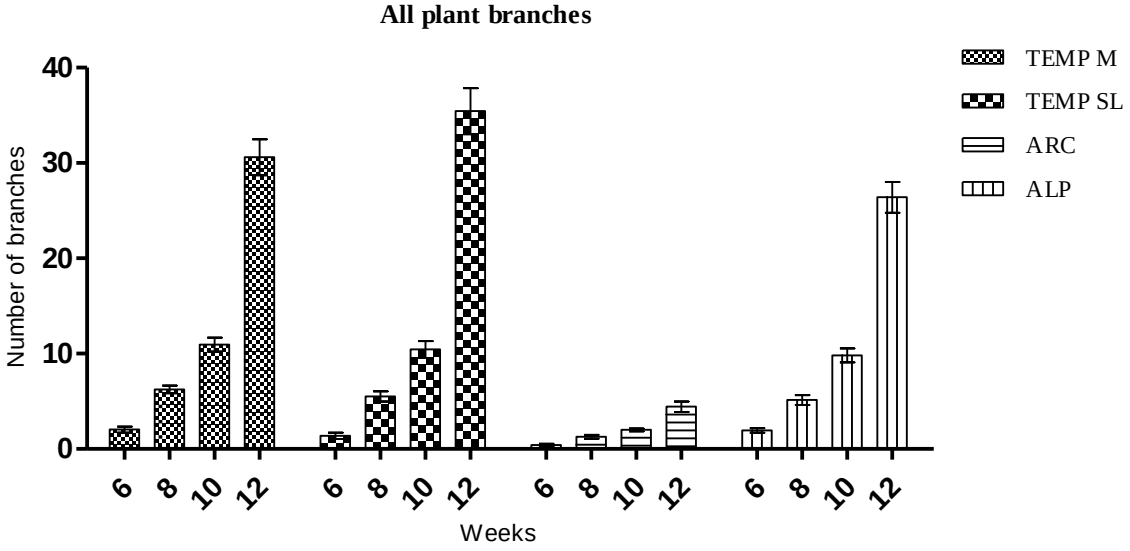

Figure 4.8: The total number of branches emerged from overall *A. ciliata* plant in different habitats up to 12 weeks.

Table 4.13: Statistical analysis of multiple differences in overall branches between treatment cohorts of *A. ciliata*. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Weeks	**6**			**8**			**10**			**12**		
Habitats	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP
TEMP M	ns	P ***	ns	ns	P ***	ns	ns	P ***	ns	ns	P ***	ns
TEMP SL		ns	ns		P ***	ns		P ***	ns		P ***	P **
ARC			***			P ***			P ***			P ***

Total number of emerged branches of A. norvegica cohorts up to 12 weeks.

The same kind of trend was found in *A. norvegica* as well (Figure 4.9), which also showed a continuous increase in total number of branches under all habitats up to 12 weeks. As

found for *A. ciliata*, the highest number of total branches were obtained under TEMP SL followed by TEMP M habitat, and they differed significantly from the ALP and ARC habitats, while the lowest number of total branches were obtained under ARC habitat (Table 4.14).

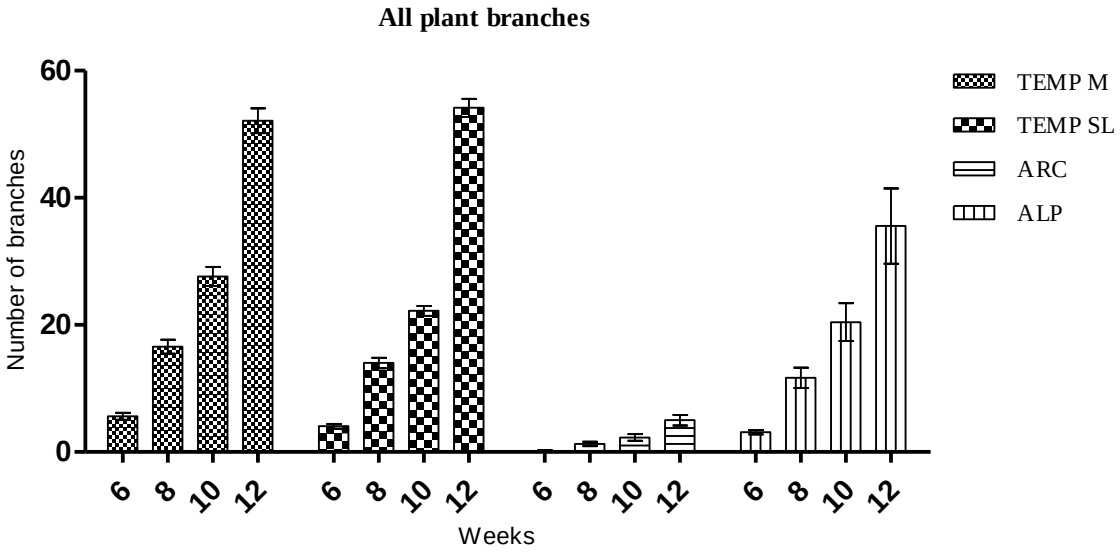

Figure 4.9: The total number of branches emerged from overall *A. norvegica* plant in different habitats up to 12 weeks.

Table 4.14: Statistical analysis of multiple differences in overall branches between treatment cohorts of *A. norvegica*. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Weeks	**6**			**8**			**10**			**12**		
Habitats	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP
TEMP M	P *	P ***	P **	ns	P ***	P *	P *	P ***	P *	ns	P ***	P **
TEMP SL		P ***	ns		P ***	ns		P ***	ns		P ***	P **
ARC			P **			P ***			P ***			P ***

Comparative total number of emerged branches up to 12 weeks between Arenaria ciliata and A. norvegica in four different habitats.

Figure 4.10 further demonstrated that *A. norvegica* got higher number of total branches as compared to *A. ciliata* under all habitats up to 12 weeks. Our results showed that growth and development of *A. norvegica* plant were much significant performance under TEMP SL and TEMP M habitats followed by ALP habitat (Figure 4.10 A, B, D and Table 4.15). However, in ARC growth conditions *A. norvegica* displayed slightly higher number of branches compared to *A. ciliata* (Figure 4.10 C)

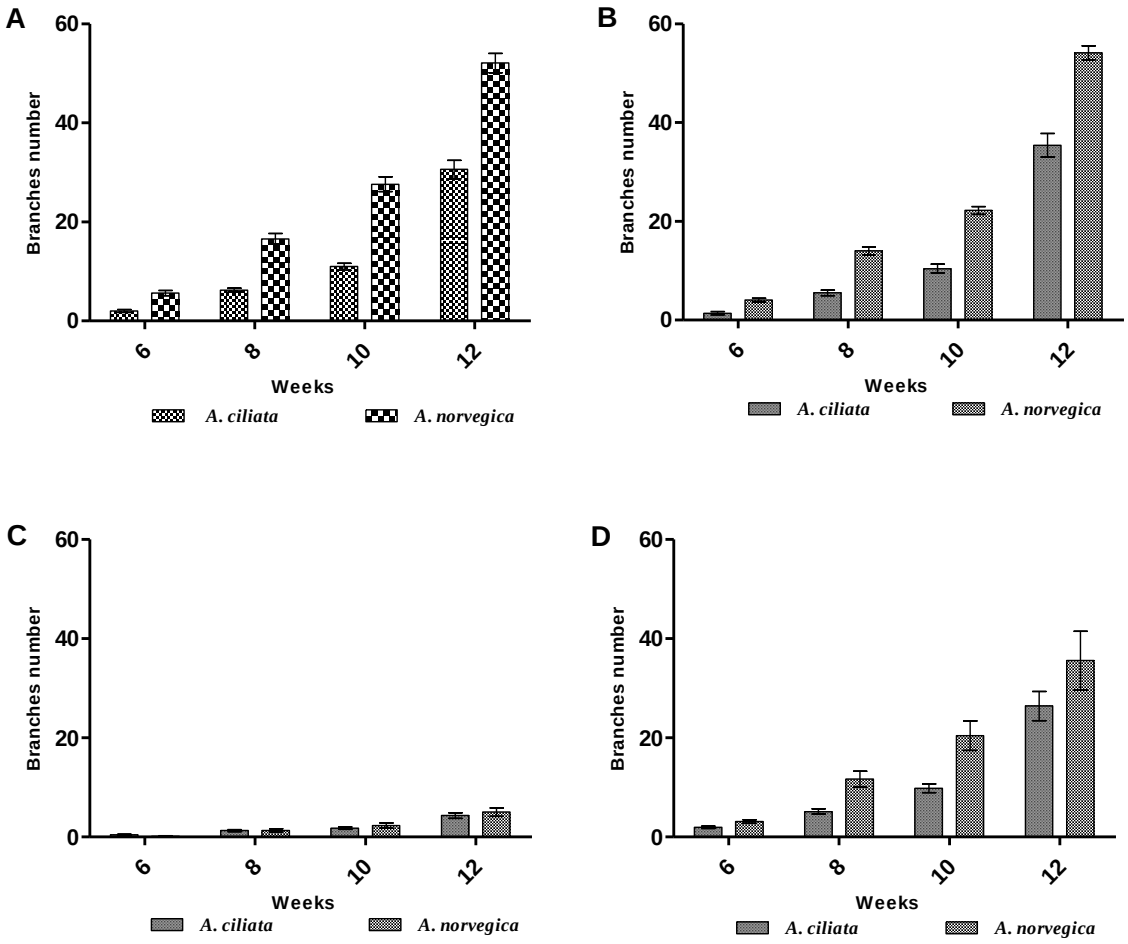

Figure 4.10: The total number of branches emerged from overall plant of *A. ciliata* and *A. norvegica* under different habitats up to 12 weeks. A. Temperate mountain habitat (TEMP M) B. Temperate sea level habitat (TEMP SL) C. Arctic habitat (ARC) D. Alpine habitat (ALP).

Table 4.15: Statistical analysis of pairwise differences in overall branches between treatment cohorts of *A. ciliata* and *A. norvegica*. Analysis of differences were performed by T-test using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****: P < 0.001).

Weeks	6	8	10	12
TEMP M	P ***	P ***	P ***	P ***
TEMP SL	P ***	P ***	P ***	P ***
ARC	ns	ns	ns	ns
ALP	P *	P ***	P ***	ns

4.3.1.3.4 Main stem growth of *A. ciliata* and *A. norvegica* plants in different habitats under summer, winter and autumn seasons.

After 12 weeks of growing season, it was difficult to count branches correctly of both two species as there was overlapping between branches. However, the main stem was still distinctable and measurable, which is one of the important factors needed to be analysed while analysing the growth performance of plants under different conditions.

Main stem growth of A. ciliata cohorts.

Main stem growth of *A. ciliata* under different habitats in summer, autumn and winter is presented in Figure 4.11. The data demonstrated that the lowest main stem length of the plant was obtained in ARC habitat. Moreover, in ARC habitat, the growth of the main stem occurred only in summer and no further growth occurred in autumn and winter (Figure 4.11). In TEMP M and TEMP SL habitat also, the main stem growth occurred in summer, however a slight growth continued even in autumn and winter as well. In ALP

habitat, the stem growth also mainly occurred in summer and it was slightly increased in autumn but in winter no further increase in stem growth occurred (Figure 4.11). In terms of main stem length, the highest main stem length of the plant occurred in TEMP SL habitat, which differed significantly from the other growth habitats (Table 4.16).

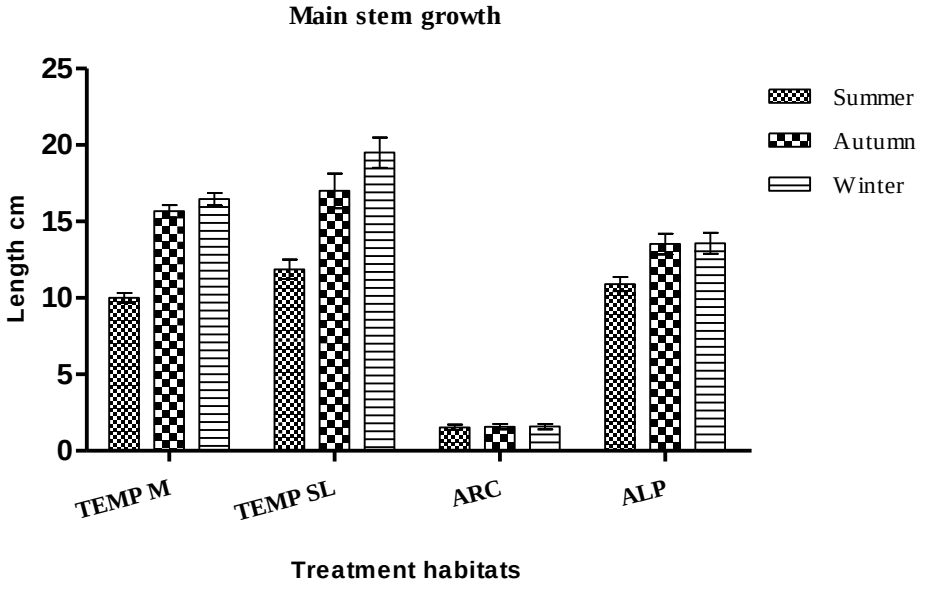

Figure 4.11: *Arenaria ciliata* main stem length measurements in different growth habitat under summer, autumn and winter seasons.

Table 4.16: Statistical analysis of multiple differences in the main stem length between treatment cohorts of *A. ciliata*. At the end of growth seasons; summer, autumn and winter. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Seasons	**Summer**			**Autumn**			**Winter**		
Habitats	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP
TEMP M	P *	P ***	P *	ns	P ***	P *	P **	P ***	P **
TEMP SL		P ***	ns		P ***	P **		P ***	P ***
ARC			P ***			P ***			P ***

Main stem growth of A. norvegica cohorts.

Figure 4.12 demonstrates the main stem growth of *A. norvegica* under different habitats in summer, winter and autumn seasons. As observed in *A. ciliata*, in case of *A. norvegica* as well, the lowest main stem growth of the plant occurred in ARC habitat. The highest main stem growth of the plant was observed in TEMP SL and TEMP M habitats, which showed similar higher growth. This was followed by ALP habitat and ARC habitat. In all the habitats tested, the main stem growth occurred mainly in summer then a significant increase in main stem was recorded in autumn. However, astonishingly no further increase in main stem length was observed in winter under the all habitat conditions.

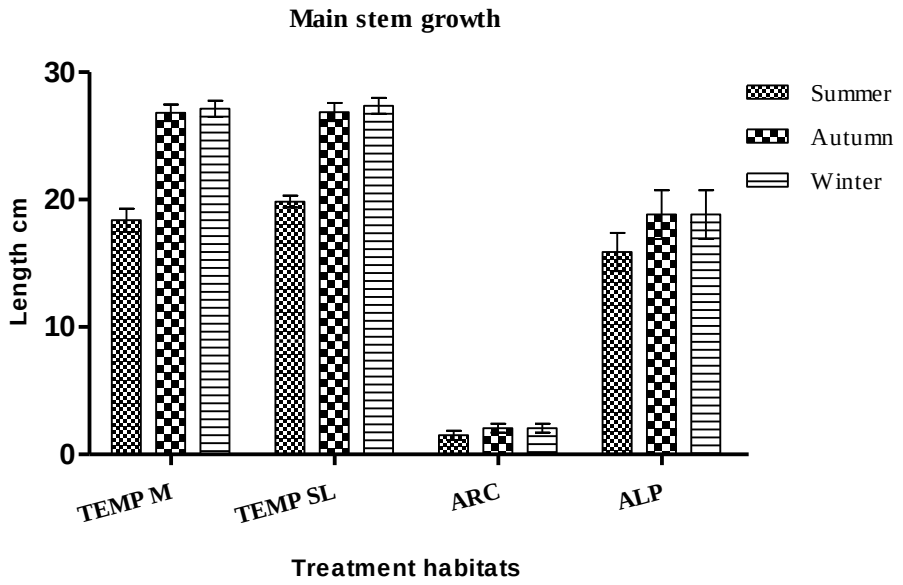

Figure 4.12: *Arenaria norvegica* main stem length measurements in different growth habitat under summer, autumn and winter seasons.

Table 4.17: Statistical analysis of multiple differences in the main stem length between treatment cohorts of *A. norvegica*. At the end of growth seasons, summer, autumn and winter. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Seasons	**Summer**			**Autumn**			**Winter**		
Habitats	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP	TEMP SL	ARC	ALP
TEMP M	ns	P ***	ns	ns	P ***	P ***	ns	P ***	P ***
TEMP SL		P ***	p *		P ***	P ***		P ***	P ***
ARC			P ***			P ***			P ***

Comparative main stem length between Arenaria ciliata and A. norvegica in different growth habitat under summer, autumn and winter.

Figure 4.13 further depicted that *A. norvegica* has significantly higher main stem length as compared to *A. ciliata* under the all growth habitats and under all seasons (Figure 4.13 A, B and D and Table 4.18), with only one exception whereby in ARC habitat under all seasons both plants showed roughly same the stem length (Figure 4.13 C and Table 4.18).

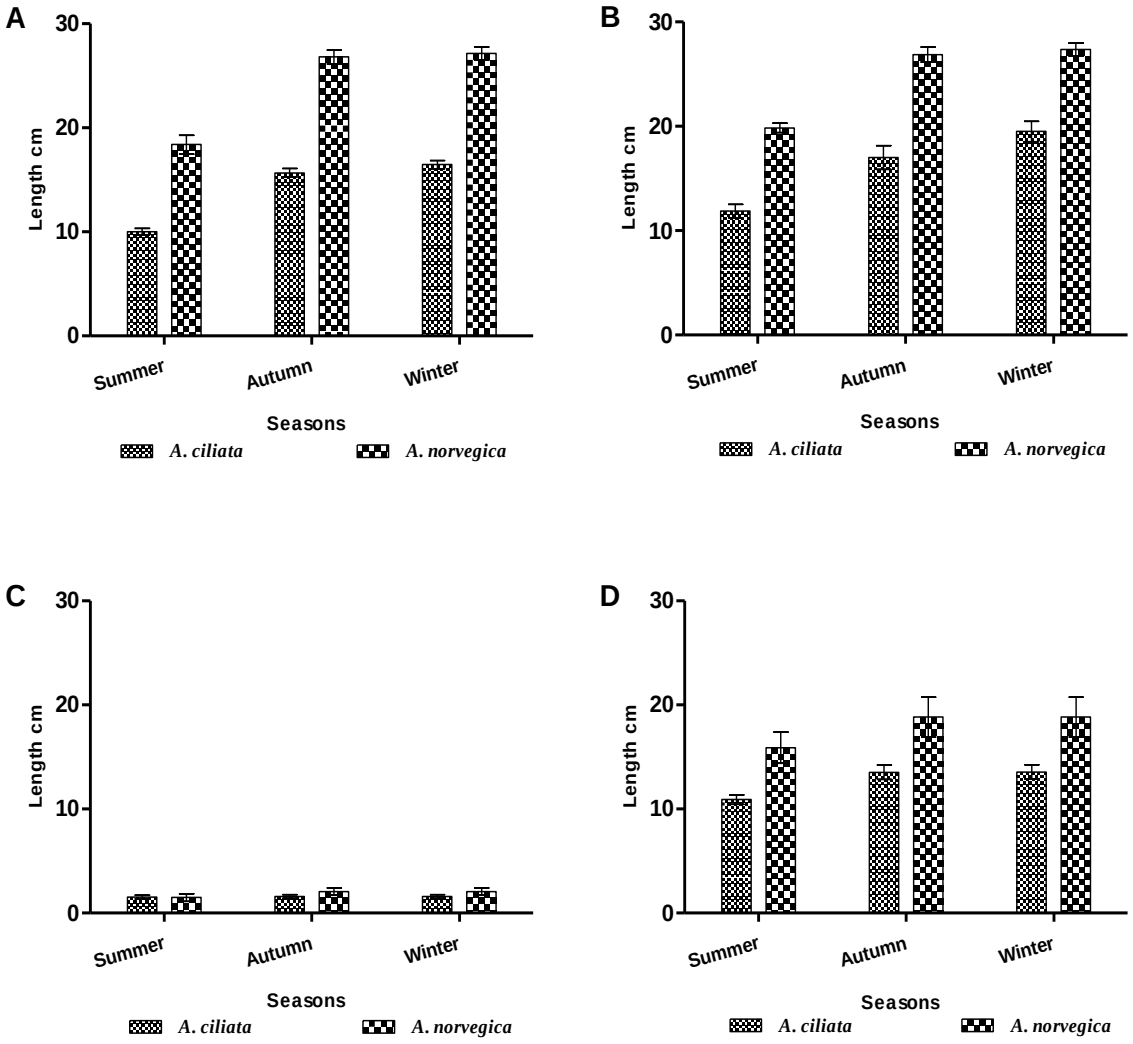

Figure 4.13: The main stem growth patterns of *A. ciliata* and *A. norvegica* plants in different habitats under summer, autumn and winter seasons. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Arctic habitat (ARC) **D.** Alpine habitat (ALP).

Table 4.18: Statistical analysis of pairwise differences in main stem length between *Arenaria ciliata* and *A. norvegica* under summer, autumn and winter seasons. (ns: P > 0.05) (**: P < 0.01) (***: P < 0.001).

Season	Summer	Autumn	Winter
TEMP M	P ***	P ***	P ***
TEMP SL	P ***	P ***	P ***
ARC	ns	ns	ns
ALP	P ***	P **	P **

4.3.1.3.5 Above ground and root biomass of *Arenaria ciliata* and *A. norvegica*

In order to proceed further, at the end of growing season above ground biomass and root biomass of both plants under different habitats was also analysed.

Above ground biomass of Arenaria ciliata and A. norvegica.

Figure 4.14 demonstrates the above ground biomass of both species under different habitats. Data demonstrated that the above ground biomass of *A. ciliata* and *A. norvegica* was highest in TEMP SL habitat, while it was lowest in ARC habitat. Under all seasons, *A. norvegica* showed the higher above ground biomass as compared to *A. ciliata.* This result illustrates that *A. norvegica* has more ability to adapt to the all treatment habitats with exception to ARC habitat they showed similar above ground biomass.

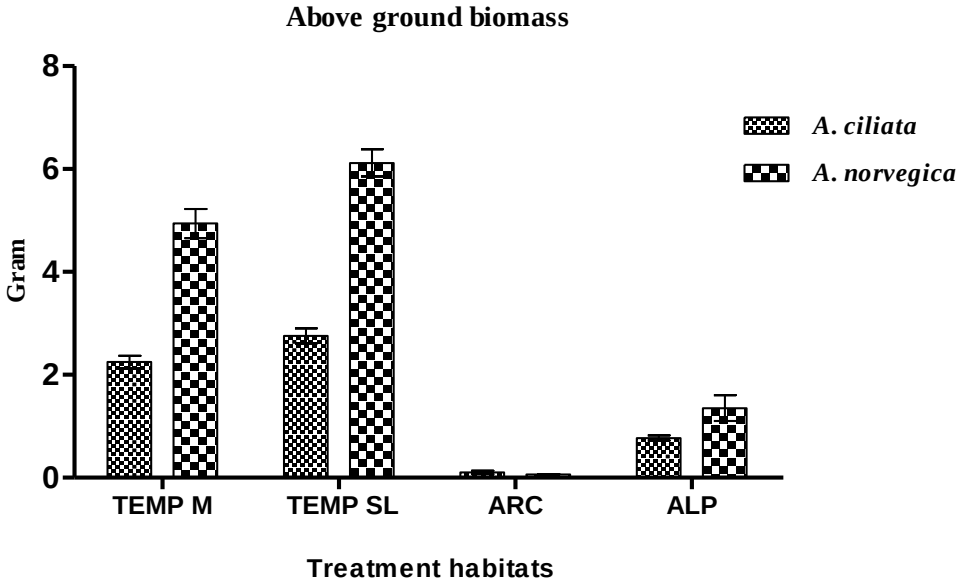

Figure 4.14: The above ground biomass of *A. ciliata* and *A. norvegica* plants grown under different habitats.

Table 4.19: Statistical analysis of multiple differences in above ground biomass between treatment cohorts. **A**. *A. ciliata* and **B**. *A. norvegica*. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

A

	TEMP SL	ARC	ALP
TEMP M	P *	P ***	P ***
TEMP SL		P ***	P ***
ARC			P **

B

	TEMP SL	ARC	ALP
TEMP M	P **	P ***	P ***
TEMP SL		P ***	P ***
ARC			P **

Table 4.20: Statistical analysis of pairwise differences in above ground biomass between treatment cohorts of *A. ciliata* and *A. norvegica*. (ns: P > 0.05) (**: P < 0.01) (***: P < 0.001).

Habitats	TEMP M	TEMP SL	ARC	ALP
P value summary	P ***	P***	ns	P**

Root biomass of Arenaria ciliata and A. norvegica.

Root biomass data of both plants grown in different habitats is shown in Figure 4.15. The trend is very similar to above ground biomass. Both plants demonstrated highest root biomass in TEMP SL habitat. In contrast, they showed the lowest root biomass in ARC habitat. Under all seasons, *A. norvegica* showed the higher root biomass as compared to *A. ciliata* (Table 4.29).

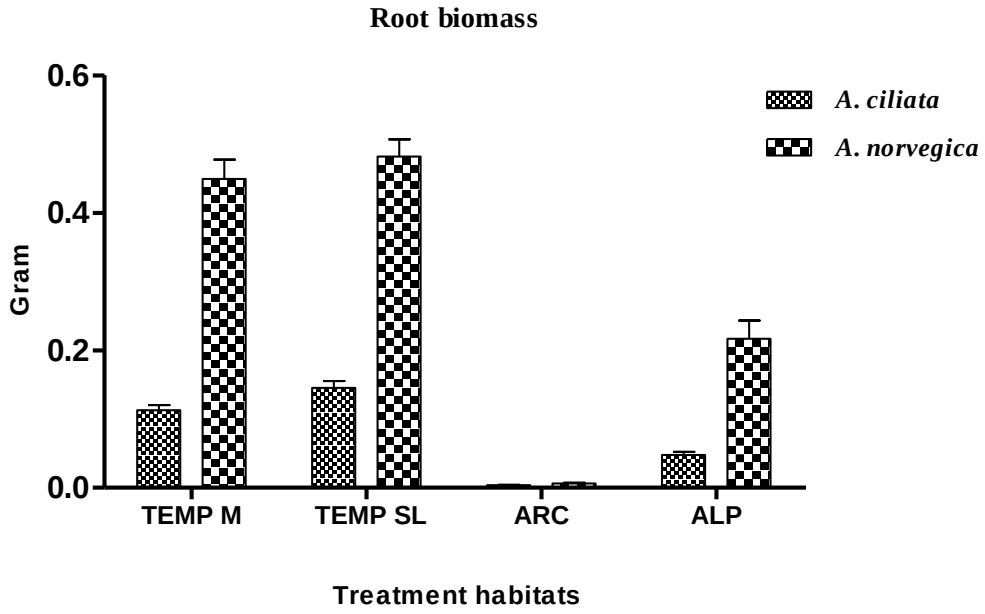

Figure 4.15: The root biomass of *A. ciliata* and *A. norvegica* plants grown under different growth habitats.

Table 4.21: Statistical analysis of multiple differences in root biomass between treatment cohorts. **A**. *A. ciliata* and **B**. *A. norvegica*. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

A

	TEMP SL	ARC	ALP
TEMP M	P *	P ***	P ***
TEMP SL		P ***	P ***
ARC			P ***

B

	TEMP SL	ARC	ALP
TEMP M	ns	P ***	P ***
TEMP SL		P ***	P ***
ARC			P ***

Table 4.22: Statistical analysis of pairwise differences in root biomass between treatment cohorts of *A. ciliata* and *A. norvegica*. (**: P < 0.01) (***: P < 0.001).

Habitats	TEMP M	TEMP SL	ARC	ALP
P value summary	p ***	p***	p**	p***

4.3.2 Leaf chlorophyll content in *A. ciliata* and *A. norvegica*.

End of summer chlorophyll content.

The chlorophyll content observed at the end of the summer season for cohorts of both species in the four treatments habitats is indicated in Figure 4.16. The chlorophyll content of *A. ciliata* was similar in TEMP M, TEMP SL and ALP habitats, however a significantly lower chlorophyll content was observed in plants grown in Arctic habitat (Figure 4.16A, Table 4.23A).

For *A. norvegica*, the ARC habitat cohort showed a significantly lower level of chlorophyll compared to the other habitat treatments (Figure 4.16B, Table 4.23B), and while there was a significant difference between the TEMP SL and TEMP M habitats, neither differed significantly from the ALP habitat, which returned a wider range of values than any other.

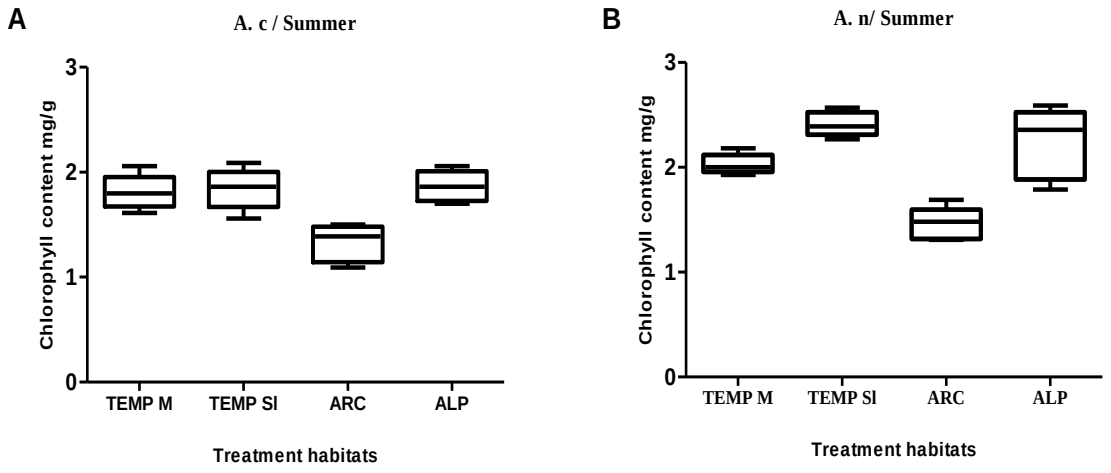

Figure 4.16: Observed end of Summer Season leaf chlorophyll content in 4 treatment habitat cohorts. **(A)** *Arenaria ciliata* and **(B)** *A. norvegica.* Figure represents mean of 5 measurements (n = 5).

Table 4.23: Statistical analysis of multiple differences in end of summer chlorophyll content between treatment cohorts of (**A**) *A. ciliata* and (**B**) *A. norvegica*. Analysis of differences were performed by Mann Whitney test using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

A

	TEMP SL	ARC	ALP
TEMP M	ns	P **	ns
TEMP SL		P **	ns
ARC			P **

B

	TEMP SL	ARC	ALP
TEMP M	P **	P **	ns
TEMP SL		P **	ns
ARC			P **

Comparative summer chlorophyll content between Arenaria ciliata and A. norvegica in four different growth habitats.

Compared to *A. ciliata*, higher content of chlorophyll was seen in case of *A. norvegica* in all habitats (Figure 4.17, Table 4.24). The lowest content was observed in Arctic habitat for both species. Similar chlorophyll content of *A. ciliata* and *A. norvegica* leaves was recorded in TEMP M, ARC and ALP habitats. However, *A. norvegica* chlorophyll content was significantly higher than *A. ciliata* chlorophyll content in TEMP SL habitat (Table 4.24).

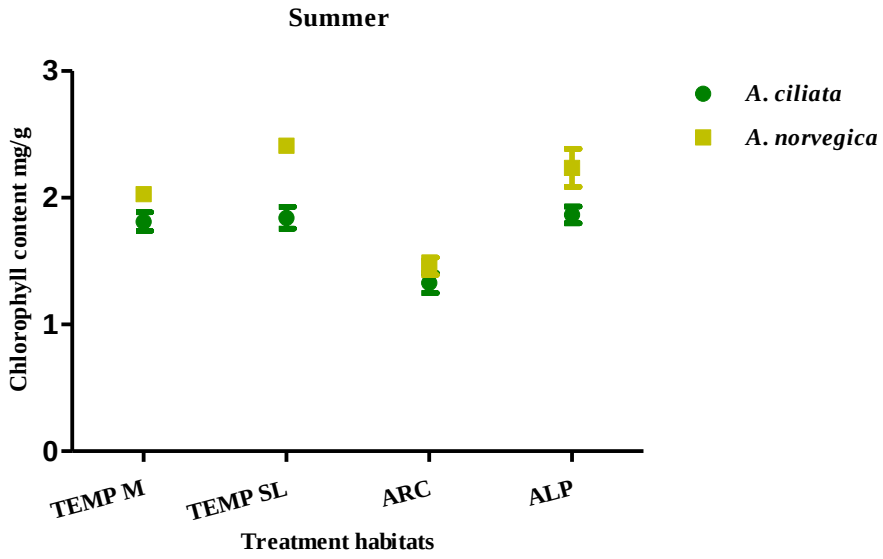

Figure 4.17: End of summer chlorophyll content of *Arenaria ciliata* and *A. norvegica* grown in different habitats.

Table 4.24: Statistical analysis of pairwise differences in end of summer chlorophyll content between treatment cohorts between *A. ciliata* and *A. norvegica*. Analysis of differences were performed by Mann Whitney test using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Growth habitats	TEMP M	TEMP SL	ARC	ALP
P value summary	P *	P ***	ns	ns

End of autumn chlorophyll content.

The chlorophyll content of both species (grown in different habitats) at the end of autumn is given in Figure 4.18. The highest chlorophyll content of *A. ciliata* leaves was observed in the TEMP M habitat and followed by TEMP SL and ALP habitats. The lowest chlorophyll content was observed in ARC habitat (Fig. 4.18 A, Table 4.25 A).

A. norvegica showed a similar trend for chlorophyll content, with the highest chlorophyll content in TEMP M habitat and followed by TEMP SL and ALP habitats (Fig 4.18B, Table 4.25 B). Similarly, the lowest chlorophyll content of the plant leaves were observed in ARC habitat.

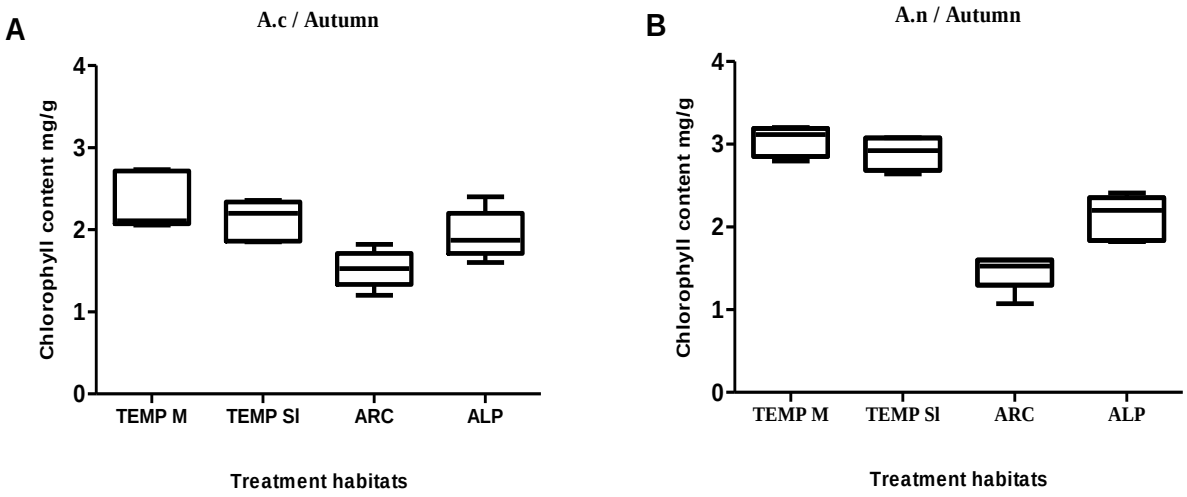

Figure 4.18: End of autumn chlorophyll content of both species grown in different habitats **A.** *Arenaria ciliata*. **B.** *A. norvegica.* Figure represents mean of 5 measurements (n = 5).

Table 4.25: Statistical analysis of multiple differences in end of autumn chlorophyll content between treatment cohorts of (**A**) *A. ciliata* and (**B**) *A. norvegica*. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A

	TEMP SL	ARC	ALP
TEMP M	ns	P **	ns
TEMP SL		P **	ns
ARC			p*

B

	TEMP SL	ARC	ALP
TEMP M	ns	P **	P **
TEMP SL		P **	P **
ARC			P *

Comparative autumn chlorophyll content between Arenaria ciliata and A. norvegica cohorts in four different growth habitats.

In relation to comparative chlorophyll content of both species, *A. norvegica* showed higher content of chlorophyll than *A. ciliata* in TEMP M and TEMP SL regimes, whereas in ARC and ALP habitats they displayed similar content of chlorophyll (Figure 4.19, Table 4.26). The lowest chlorophyll content was observed in plants of both species grown in ARC habitat (Figure 4.19).

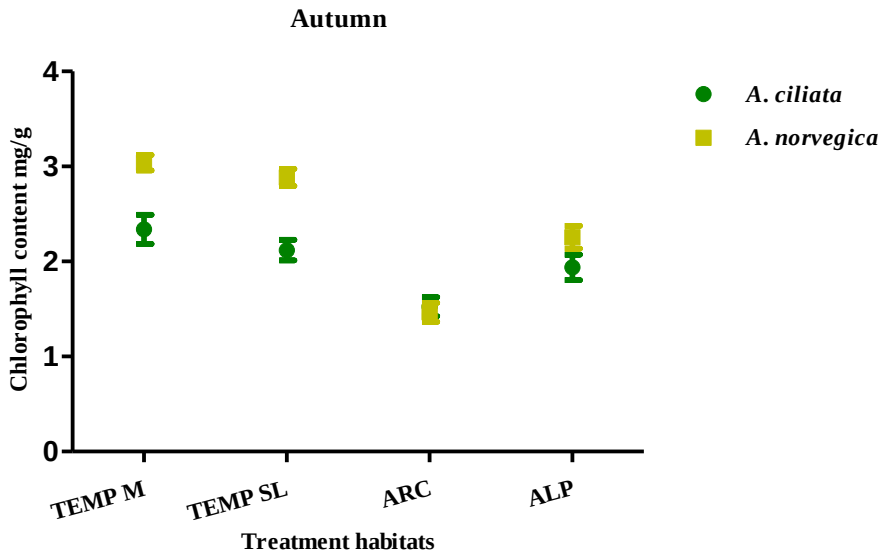

Figure 4.19: End of autumn chlorophyll content of *Arenaria ciliata* and *A. norvegica* grown in different habitats.

Table 4.26: Statistical analysis of pairwise differences in chlorophyll content between treatment cohorts of *A. ciliata* and *A. norvegica*, at the end of autumn. (ns: P > 0.05) (**: P < 0.01).

Growth habitats	TEMP M	TEMP SL	ARC	ALP
P value summary	P **	P**	ns	ns

End of winter season chlorophyll content.

The chlorophyll contents of both species (grown in different habitats) at the end of winter is given in figure 4.20. The highest chlorophyll content of *A. ciliata* at the end of winter was observed in TEMP SL habitat and followed by TEMP M habitat (Figure 4.20A). These values differed significantly, and were also significantly higher than those recorded for plants in the ARC and ALP habitats, which showed almost similar chlorophyll content (Table 4.27A).

For *A. norvegica*, the chlorophyll content were similar in TEMP M and TEMP SL habitats and these were the highest chlorophyll content of all the habitats (Figure 4.20B). As observed for *A. ciliata*, the chlorophyll content of *A. norvegica* plants was lowest in ARC and ALP habitats, both of which recorded similar chlorophyll content.

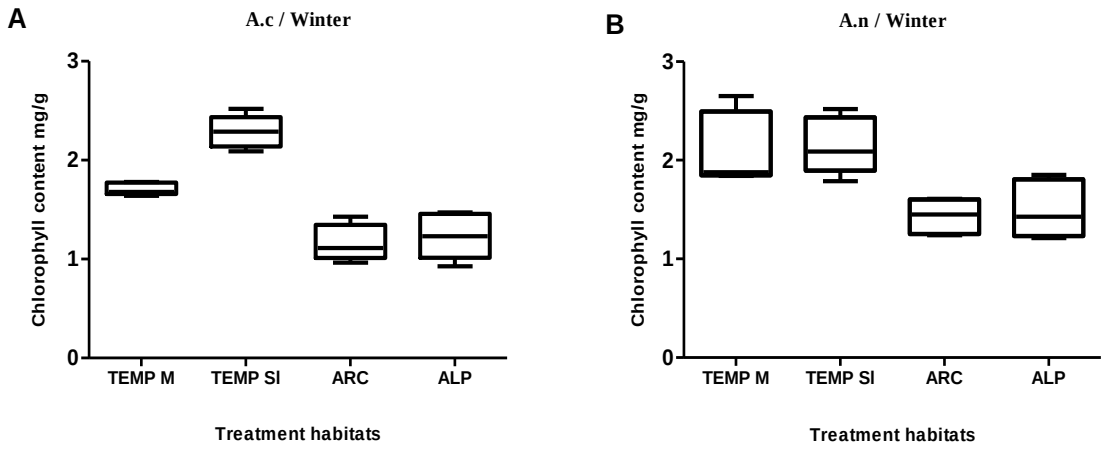

Figure 4.20: End of winter chlorophyll content *Arenaria* species grown in different habitats. **A.** *A. ciliata* **B**. *A. norvegica*. Figure represents mean of 5 measurements (n = 5).

Table 4.27: Statistical analysis of multiple differences in end of autumn chlorophyll content between treatment cohorts of (A) *A. ciliata* and (B) *A. norvegica*. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

A			
	TEMP SL	**ARC**	**ALP**
TEMP M	p *	p *	p *
TEMP SL		p **	p **
ARC			ns

B			
	TEMP SL	**ARC**	**ALP**
TEMP M	ns	p **	p *
TEMP SL		p **	p *
ARC			ns

Comparative winter chlorophyll content between Arenaria ciliata and A. norvegica cohorts in four different growth habitats.

Almost Similar chlorophyll content for *A. ciliata* and *A. norvegica* leaves was recorded in TEMP SL, ARC and ALP habitats. However, *A. norvegica* chlorophyll content was significantly higher than *A. ciliata* chlorophyll content in TEMP M habitat (Table 4.28).

The lowest chlorophyll content was observed in ARC and ALP habitats for the both two species (Figure 4.21).

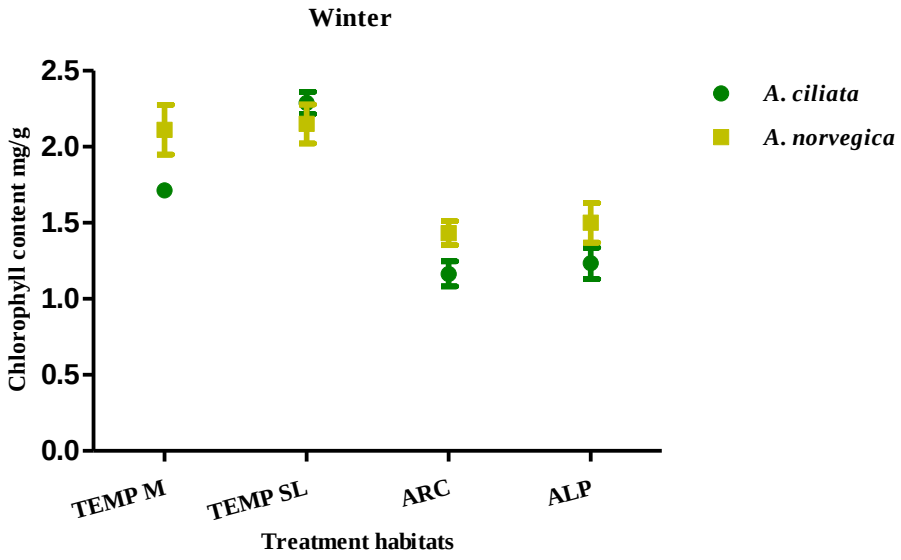

Figure 4.21: End of winter chlorophyll content of *Arenaria ciliata* and *A. norvegica* grown in different habitats.

Table 4.28: Statistical analysis of pairwise differences in end of winter chlorophyll content between treatment cohorts of *A. ciliata* and *A. norvegica*. (ns: P > 0.05) (*: P < 0.05)

Growth habitats	TEMP M	TEMP SL	ARC	ALP
P value summary	P *	ns	ns	ns

4.3.2.1 Changes in chlorophyll content with changes in growth season.

Changes in chlorophyll content of both the ecotypes under different seasons and habitats has been presented in Figure 4.22 and 4.23 to demonstrate the seasonal variation in plant chlorophyll content.

Arenaria ciliata chlorophyll content.

In case of *A. ciliata,* the highest chlorophyll content in the plant leaves was seen in TEMP M and TEMP SL habitat, while the lowest chlorophyll content was obtained in ARC habitat (Figure 4.22). All the plants were grown in summer and growth continued in autumn and winter. Under TEMP M, ARC and ALP habitat, the chlorophyll content of the plant leaves was increased from summer to autumn, but the chlorophyll content was decreased in winter. However, under TEMP SL habitat, the chlorophyll content continuously increased from summer to autumn and then in winter. Our result demonstrates that under TEMP M, ARC, and ALP habitats, the chlorophyll content dropped significantly at the end of winter compared to end of autumn (Figure 4.22 and Table 4.29).

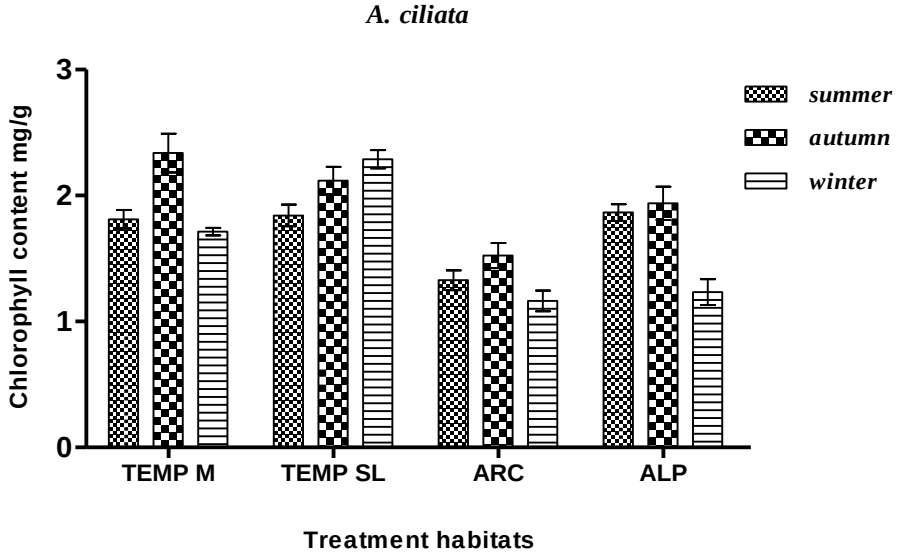

Figure 4.22: Changes in chlorophyll content of *Arenaria ciliata* with changes in growth season. Figure represents mean of 5 measurements ± SEM (n = 5).

Table 4.29: Analysis of changes in the levels of chlorophyll of *A. ciliata* leaves with seasons. Analysis of differences were performed by Mann Whitney test using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns**:** P > 0.05) (*****:** P < 0.05) (******:** P < 0.01) (*******:** P < 0.001)

Habitats	**TEMP M**		**TEMP SL**		**ARC**		**ALP**	
Seasons	AUT	WIN	AUT	WIN	AUT	WIN	AUT	WIN
SUM	p *	ns	ns	p *	ns	ns	ns	p **
AUT		p **		ns		p *		p **

Arenaria norvegica chlorophyll content.

In case of *A. norvegica*, under TEMP M and TEMP SL habitats, the chlorophyll content was very significantly increased from summer to autumn but then a sharp decrease in

chlorophyll content was observed in winter (Figure 4.23 and Table 4.30). Figure 4.23 show that, under ARC habitat no seasonal effect on chlorophyll content of the plant was determined, as the chlorophyll content in all the seasons was similar. Under ALP habitat, the chlorophyll content between summer and autumn was similar but a sharp decrease in chlorophyll content of the plant was observed in winter (Table 4.30).

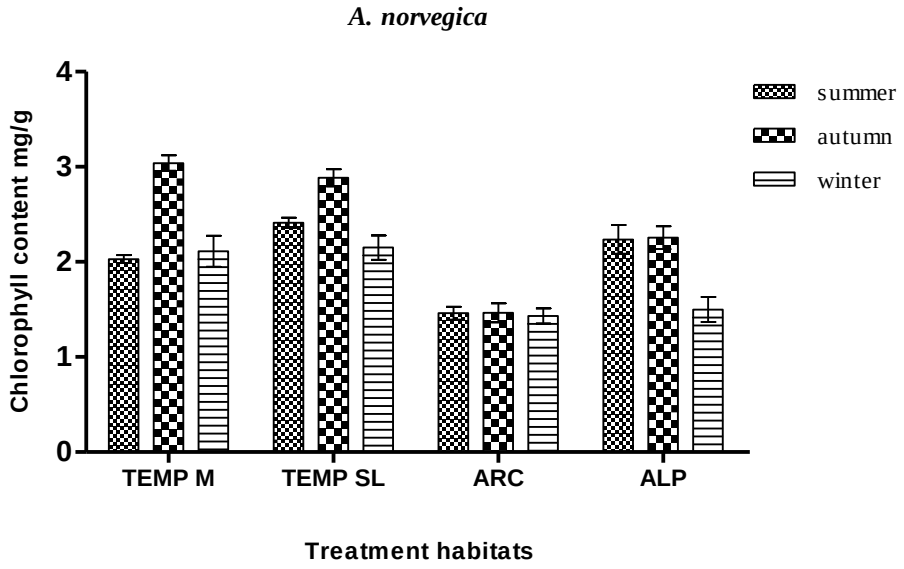

Figure 4.23: Changes in chlorophyll content of *Arenaria norvegica* with changes in growth season. Figure represents mean of 5 measurements ± SEM (n = 5)

Table 4.30: Analysis of changes in the levels of chlorophyll of *A. norvegica* leaves with seasons. Analysis of differences were performed by Mann Whitney test using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

Habitats	**TEMP M**		**TEMP SL**		**ARC**		**ALP**	
Seasons	AUT	WIN	AUT	WIN	AUT	WIN	AUT	WIN
SUM	p **	ns	p **	ns	ns	ns	ns	p *
AUT		p **		p **		ns		p *

4.4 Discussion.

Seeds of the diploid *Arenaria ciliata* germinated more slowly and with a higher percentage than the tetraploid *Arenaria norvegica* in all habitats. The lowest percentage of germination was seen in the ARC habitat for *A. norvegica* (22.2%) whereas *A. ciliata* showed a higher percentage of germinated seeds (75%) in the same ARC conditions (Table 4.2). In general, number of germinated seeds and survival of seedlings was higher in *A. ciliata* ecotypes compared to *A. norvegica.* The lowest survival of *A. norvegica* seedlings was observed in high summer temperatures of ALP habitat (Table 4.2). Plant germination from diploid seeds was generally more viable in the first stages of growth, a result consistent with germination patterns for different ploidy states in *Beta vulgaris*, as examined by Beyaz *et al* 2013 .Tetraploid plants had lower germination and seedling survival than diploid genotypes, a pattern that could be attributed to ploidy-related increased thickness of the seed coat (Sung and Chui 1995).

Seedling growth and development of tetraploid genotypes was faster and first leaf and branch emergence earlier than diploid plants in TEMP M, TEMP SL and ALP habitats. In the ARC habitat first leaf and branch of *A. ciliata* plants appeared earlier as compared to *A. norvegica* (Table 4.3). This data clearly shows that comparatively *A. ciliata* and *A. norvegica* are very well adapted to the environmental conditions of the experimental TEMP M, TEMP SL and ALP habitats. Both plant ecotypes were least well adapted to ARC habitat conditions for all parameters of their growth performance.

In terms of flowering time, *A. norvegica* flowered in three habitat treatments (TEMP M, TEMP SL and ALP) as compared to *A. ciliata* plants which showed flowering only in two habitats (TEMP M and TEMP SL) with longer number of days required for flowering. Although Stebbins Jr, (1947) reported that in tetraploid plants, flowering was

often later than diploid genotypes, in our study under greenhouse conditions of TEMP M and TEMP SL habitats, tetraploid species (*A. norvegica*) flowering time was earlier than for the diploid species (*A. ciliata*). Plants of both species growing in the Arctic habitat did not flower as their growth was too slow (Figure 4.1). *A. norvegica* flowering time was at the end of summer whereas the appearance of the first flower of *A. ciliata* was at the end of autumn. All *A. norvegica* plants grown in TEMP M and TEMP SL habitats bloomed while in ALP habitat 88.8 % of plants showed flowers with longer time required for flowering. Compared to *A. norvegica*, lower percentage of flowers was produced by *A. ciliata* plants grown in TEMP M and TEMP SL habitats; 56.5 and 69.5 respectively, (Table 4.6).

In terms of main stem growth performance, both species showed the same trend of continuous growth in the first 12 weeks (Figure 4.2 and 4.11). Much more rapid main stem growth was seen in *A. norvegica* as compared to *A. ciliata*. However, the two species showed a similar slower rate of growth in ARC habitat (Figure 4.4 and Table 4.9). This finding reveals that up to 12 weeks after seed sowing, both species performed better in the TEMP M, TEMP SL and ALP growth habitats as compared to the ARC habitat. For both species, the most rapid main stem growth occurred mainly in summer season in all treatment habitats (Figure 4.11 and 4.12). In the autumn season, both species showed continuous growth in all habitats except in ARC habitat where no further growth was recorded for *A. ciliata*.

In contrast, no further increase in main stem length was observed in winter for *A. norvegica* under all habitat conditions. Continued main stem growth of *A. ciliata* was observed under TEMP M and TEMP SL habitats during winter (Figure 4.11 and 4.12).

At the end of the entire growing season, the greatest main stem growth for both species was reported in the TEMP SL habitat while the lowest growth was observed in ARC

habitat. *A. norvegica* plants showed significantly higher main stem growth compared to *A. ciliata* plants in all habitats across all seasons, with only one exception whereby in the ARC habitat both species showed roughly same stem growth performance (Figure 4.13 and Table 4.18). Similarly, the shortest internode mean length of both species was reported in the ARC habitat, however *A. norvegica* showed significantly longer internode length in all the habitats as compared to *A. ciliata* (Table 4.3 and 4.5). This clearly highlights a different underlying genotype/ phenotype for this trait in *A. norvegica* compared to *A. ciliata*.

For main stem branches, up to 12 weeks *A. ciliata* seedlings under all treatment habitats showed continued increase in the number of branches emerging from the main stem. The highest number of branches was recorded in TEMP SL followed by TEMP M and ALP habitats. The lowest number of main stem branches was observed in ARC habitat, which is the harshest abiotic environment for *A. ciliata* compared to the other experimental treatment habitats (Figure 4.5 and Table 4.10).

In the case of *A. norvegica* similar patterns of growth performance was observed compared to *A. ciliata*, whereby the highest number of main stem branches was recorded in TEMP SL habitat followed by TEMP M and ALP habitats respectively, whereas seedlings growing under Arctic habitat treatment produced significantly lower number of branches (Figure 4.6 and Table 4.11). Compared to *A. ciliata*, a higher number of branches was observed in case of *A. norvegica* in TEMP SL, TEMP M and ALP habitats (Figure 4.7 and Table 4.12).

In terms of overall branches, both species showed the same pattern of growth performance in all habitats, with a continuous increase in total number of branches recorded up to 12 weeks. *A. norvegica* seedling growth and development were

significantly higher compared to *A. ciliata* in TEMP SL, TEMP M and ALP treatment habitats respectively, throughout the summer season. However, under ARC habitat, they showed similar total number of branches (Figure 4.10 and Table 4.15). This further demonstrates that *A. norvegica* displayed more vigorous growth in all habitats as compared to *A. ciliata,* with exception of ARC habitat whereby they showed almost similar reduced growth performance.

In terms of biomass accumulation over a growing year comprising four growing seasons (summer, autumn, winter and spring) of four different treatment habitats, for both species, the highest dry weight of above ground and root biomass was obtained in TEMP SL habitat followed by TEMP M. these values were significantly higher than recorded weights for both plant species in the ARC and ALP habitats, which also differed significantly from each other (Figures 4.14 and 4.15 Tables 4.19 and 4.21). *A. norvegica* showed significantly higher above ground and root biomass compared to *A. ciliata* in all experimental treatment habitats.

Our results clearly show that *A. ciliata* and *A. norvegica* are best adapted to the experimental growth conditions of the TEMP SL and TEMP M habitats, compared to the ALP habitat, and especially the ARC habitat, for all tested growth parameters. *A. norvegica* plants displayed greater development and vegetative growth than *A. ciliata* plants, this could be attributed to increased water and other nutrients absorption via roots in *A. norvegica* tissue, as tetraploid genotypes are known to have bigger vacuoles, and this may have a significant role in regulating cell osmotic pressure. High cell osmotic pressure in polyploid plants is associated with higher water and nutrients uptake, which in turn has the capacity to increase cell metabolic activity (Guertin and Sabatini, 2006; Beyaz *et al*., 2013). However growth performance can also be affected by genotypic

differences (in terms of entire genome variation) more than ploidy level (in terms of genome duplicate effects) as reported by Sullivan and Pfahler (1986).

Chlorophyll content is an important parameter that is regularly measured as an indicator of photosynthetic capacity of green tissues (Rao *et al*., 2001; Kamble *et al*., 2015). The chlorophyll content of both ecotypes has been measured at the end of three growing seasons; summer, autumn and winter. *A. norvegica* leaves contained higher amount of chlorophyll than *A. ciliata* leaves under TEMP M, TEMP SL and ALP habitats at the end of summer and autumn. These results agree with many previous studies that reported that the chlorophyll content in polyploid plant green tissue is higher than those with lower chromosome numbers (diploid plants), (Molin *et al*., 1982; Warner *et al*., 1987; Mathura *et al*., 2006) . However, in the ARC habitat they showed a similar content of chlorophyll whereby the lowest value was recorded in this habitat (Figures 4.17 & 4.19 Tables 4.24 & 4.26).

At the end of winter, the higher mean chlorophyll content for both species was observed in the TEMP SL habitat (2.28 mg/g for *A. ciliata* and 2.15 mg/g for *A. norvegica*, Figure 4.21) and this is associated with the higher temperature regime in this compared to other treatment habitats, as would be normal on the maritime west coast of Ireland, for which this habitat is an approximation (Table 4.1). In the other habitats at the end of winter, *A. norvegica* showed slightly but not significantly higher chlorophyll content than *A. ciliata* (Table 4.28).

Changes in chlorophyll content were also observed in relation to changes in the growing season. In case of *A. ciliata*, as the seedlings matured from summer into autumn the chlorophyll content increased; however chlorophyll content decreased sharply in winter in all the growth habitats with one exception of the cohorts grown in TEMP SL, where

chlorophyll content continuously increased over all seasons (Figure 4.22 and Table 4.29). *A. norvegica* plants grown in TEMP M and TEMP SL showed increase in chlorophyll content from summer to autumn season, however in the winter season, chlorophyll content decreased as was seen in *A. ciliata* cohorts (Figure 4.23 and Table 4.30). In the case of both species, the lowest chlorophyll content was observed in ARC and ALP habitats at the end of the winter season, as the temperature dropped to -5°C and -3°C respectively (Figures 4.22 and 4.23).

Taken together these data confirm significant differences between *A. ciliata* and *A. norvegica* in terms of growth and phenology. As determined by Dang (2012), *A. norvegica* is likely to have an evolutionary origin among the *A. ciliata* populations of northern Spain in the Late Pleistocene, whereas the Irish ecotype of *A. ciliata* shares a common origin with *A. ciliata* subsp. *pseudofrigida*. In Chapter 3, it was confirmed that the species differ in their ploidy. It is clear from these data that the separation of the two species is not just limited to geographic or ploidy aspects; the two taxa now have clearly differentiated ecophysiologies with *A. norvegica* having a more rapid growth pattern and earlier flowering phenology than *A. ciliata*, but also having lower germination rates, and poorer seedling survival, especially in more extreme summer heat, as present in Alpine habitats. Importantly, and addressing a key objective of the study, both species populations in Ireland retain the ability to tolerate freezing growth conditions, even though climatic evidence suggests that neither population has experienced pronounced winter freezing for several thousand years. Both grow well in Alpine growing conditions with freezing winters and warms summers, but also, to a much lesser extent, in Arctic conditions, with freezing winters and cool summers.

An important consideration is the extent to which this capacity to survive freezing conditions reflects overall ecophysiological ability to cope with freezing stress, especially relative to other ecotypes of the *A. ciliata* complex, and this question is addressed in the next chapter, Chapter 5.

Chapter 5

5 Investigation of the impact of seasonal light and temperature variation on photosynthesis performance in selected ecotypes of the *Arenaria ciliata* species complex.

5.1 Introduction.

5.1.1 Chlorophyll fluorescence imaging

Photosynthesis is a process by which the green tissue of plants use light energy to convert carbon dioxide and water into sugars. These processes occur in two stages within microscopic organelles called chloroplasts; termed light reactions and the Calvin cycle. The light reactions take place in the thylakoid membranes, and use light energy and water to produce chemical energy in form of ATP and NADPH, and include the components that make up Photosystem I and Photosystem II. The Calvin cycle is the process of synthesis a simple three-carbon sugar, using ATP and NADPH from the light reactions and carbon atoms from atmospheric CO_2. The Calvin cycle reactions occur in the stroma (in the inner space of chloroplasts) (Nabors, 2004).

Photosynthetic pigments, including chlorophyll and carotenoids, have important roles in light absorption for photosynthesis and they also play a crucial role in protecting the photosystem from damage by excessive radiation (Devi *et al*., 2015). Photosynthetic pigment concentrations are regulated by plants in response to environmental conditions, and this regulation is considered an adaptive response to temperature fluctuations (Close *et al*., 2006). Observation of changing pigment content is particularly relevant for plants that occupy high altitude and latitude habitats that are subject to large seasonal fluctuations in photosynthesis performance. Carotenoid pigments such as violaxanthin, antheraxanthin, and zeaxanthin are associated with these temperature extremes, and play a role in quenching excess energy that is passing through photosystem II (Gamon *et al*., 2016).

Recently, the technique of chlorophyll fluorescence imaging analysis (Chl-FI) has become one of the most useful and widely applied methods for evaluating photosynthesis performance by plant physiologists and ecophysiologists.

Based on detailed analysis of absorbed and reflected light spectra from leaf tissue, current Chl-FI imaging techniques allow for the accurate early detection of plant stress prior to stress symptoms becoming visible within plant tissues, by which time the plant can already be adversely affected (Chaerle and Van Der Straeten, 2000).

Fluorescence analysis is thus widely used as a method for describing the efficiency and functioning of photosynthesis in plants (Schreiber, 2004; Murchie and Lawson, 2013). Photosynthesis is one of the most sensitive physiological processes to temperature changes, and also one of the primary sites for stress-related disruption of cellular processes (Magaña Ugarte *et al*., 2019). Chl-FI is sensitive to relative declines in real-time energy utilisation within the photosystem that arise as a result of biotic and abiotic stress, and so provides a rapid method for identifying when plants begin to experience stress (Kalaji *et al*., 2016). These kinds of data are relevant not just for analysing performance of elite agricultural crop varieties, but also for establishing general functional limits among plant species and ecotypes.

The usefulness of Chl-FI derives from its ability to give information that is not readily available via other methods, in particular; by providing details about a plant's ability to tolerate environmental stress and give insights into the extent to which biotic and abiotic stresses have damaged the photosynthetic apparatus (Maxwell and Johnson, 2000; Hanelt, 2018).

As a non-destructive sampling method, chlorophyll fluorescence imaging provides valuable information of plant growth and vitality at specific moments in time, and also,

through repeated observations, throughout the plant life cycle, and is increasingly used to investigate the responses of plants to various changing environments (Humplík *et al*., 2015).

5.1.2 Analysis of energy flux in photosystem II using Chl-FI

Light energy that is absorbed by plant leaves can be directed into one of three pathways: (i) it can be utilized to drive the photosynthetic process, (ii) it can be dissipated in a controlled manner as heat, or (iii) it can be re-emitted as light- chlorophyll fluorescence and heat in an uncontrolled manner (Maxwell and Johnson, 2000; Bresson *et al*., 2015). Chlorophyll fluorescence analysis generates detailed information about the first pathway, by monitoring energy transfer into photosystem II (PSII) (Murchie and Lawson, 2013).

Although the amount of chlorophyll fluorescence detectable from leaf tissue is very small, typically about only 1 or 2 % of the total amount of light absorbed, informative measurements can be achieved. This is possible because any increase in the proportion of energy directed to any one of those three processes (i-iii, above) results in a decrease in the energy yield of the others. Thus quantum yields of non-photochemical and photochemical energy conversion in photosystem II can give detailed information into the extent to which PSII is using the energy absorbed by chlorophyll molecules and the extent to which excess light is damaging it (Klughammer and Schreiber, 2008; Maxwell and Johnson, 2000).

Each element of plant tissues and cells has specific wavelength absorbance, reflectance and transmittance properties. For instance, chlorophyll absorbs photons primarily in the blue spectrum (chlorophyll a, at a wavelength of 436 nm, and chlorophyll b, at 470 nm) and the red spectrum (at around 650 nm for both chllorophyll a and b) of visible light. Other light absorbance is observed for water, in the near and short wavelengths, and for

cellulose, absorbing photons in a wide range between 2200 and 2500 nm ((Fiorani *et al*., 2012; Li *et al*., 2014).

By accounting for changes in the spectrum of reflected light, in addition to the total quotient of emitted fluorescence, Chl-FI can provide detailed profiles of ongoing photosynthesis performance within leaf tissue. If a plant is placed under stress, photosynthesis reaction centres begin to close, and eventually, under extreme stress, all these centres will close. This process causes the amount of non-photochemical energy in the photosynthesising tissue to rise. If the plant has the ability to cope with and manage non-photochemical excess energy, by dissipating it as heat in a regulated manner, it has what is known as a good photoprotective capacity: this will protect photosystem II from damage, resulting in the plant being better able to tolerate stress. Chl-FI allows for the active monitoring of these changes in the proportion of absorbed energy converted in PSII to (a) photochemical energy (a measure known as Y(II), *the yield of PSII*), (b) regulated non-photochemical energy (Y(NPQ)), and finally (c) non-regulated non-photochemical energy (Y(NO)), (Figure 5.1). These three yield values equate to the three energy portions (i-iii) detailed in the introductory paragraph above.

Across the biogeographic distribution of the *Arenaria ciliata* species complex in Europe there are a wide variety of local habitat climates with specific temperature stress characteristics, from generally cold Arctic conditions in the North, to alternately freezing and warm conditions in the Alpine south, and intermediate montane and cold maritime conditions in Northwestern Europe. In all populations surveyed so far from across the range, local ecotypes have discrete genetic identities (Chapter 3) which are likely reflective of long-term isolation since the end of the Pleistocene glaciation (Dang *et al*., 2012; Howard-Williams, 2013).

In the work presented in this chapter, chlorophyll fluorescence was measured for five representative ecotypes of the *A. cilaita* complex across an array of experimental treatments with a view to establishing whether local genetic isolation has given rise to variation in baseline stress responses and critical thresholds.

Objective.

The aim of this work was to describe the impact of environmental variation, including temperature extremes, on photosynthesis performance among 5 ecotypes drawn from across the biogeographic range of the *A. ciliata* complex. The experiments focused on photosynthesis indices as a measure of stress response. The objective was to determine whether the ecotypes differed in their response to conditions in each habitat, including across seasons, as well as to extreme low and high temperature spikes, and in doing so establish whether local selection pressure has given rise to adaptation in ecophysiological performance.

5.2 Material and Methods.

5.2.1 Ecotype sample cohorts

Sampling from *A. ciliata* complex populations involved cohorts of plants that were either core to the ongoing biometric habitat study (*A. ciliata* [Ireland, 2N=40] and *A. norvegica* [Ireland, 2N=80]) or representative of three additional ecotypes (*A. ciliata* subsp. *pseudofrigida* (Svalbard, 2N=40); *A. norvegica* (Norway, 2N=80) and *A. ciliata* subsp. *bernensis* (Switzerland, 2N=200). All sampled plants were maintained under the seasonal

growing conditions of the 4-habitat biometric analysis study (Temperate Sea Level [TEMP SL], Temperate Montane [TEMP M], Alpine [ALP] and Arctic [ARC] idealised habitats, Table 4.1), or, in selected temperature extremes observations, under additional specific growing conditions. The site information of collected samples are detailed in Table 2.1 and Figure 2.1 and 2.11 (Chapter 2).

In terms of the genetic identities detailed in Figure 2. 17 and 3.10, the *A. ciliata* (Ireland) plants included in these experiments all displayed the identity RTC01, belonging to clade I (Figure 2.17). The *A. ciliata* subsp. *bernensis* plants also displayed an identity from clade I of the phylogeny, RTCAb2. The *A. norvegica* plants (Ireland and Norway) all displayed the identity RTN01 (from clade II) and *A. ciliata* subsp. *pseudofrigida* plants displayed the identity RTC14 (from clade III).

For each analysis batch, five plants from each ecotype in each habitat were analysed and chlorophyll fluorescence was measured (Figure 5.1, 5.2). All plant cohorts were cultivated and maintained at growth room facilities in Maynooth University Biology Department. All chlorophyll fluorescence measurements were conducted at Prof. Marcel Jansen's laboratory in the UCC Department of Plant Sciences. Measurements were made using Pulse-Amplitude-modulation fluorometry (PAM) and the saturation pulse (SP) method on an Imaging-PAM/ M-series machine (Walz Heinz GmbH, Effeltrich, Germany).

5.2.2 Experimental Observations

5.2.2.1. Core observations of seasonal change in Photosynthesis rates in *A. ciliata* complex ecotypes.

Core observations for each ecotype cohort were completed at the end of the summer, autumn and winter growing seasons in time with the biometric analysis experiments. These observations tracked changes in the rates of photosynthesis in response to seasonal alterations in temperature and light conditions in each treatment habitat, which represent idealised habitat settings from the biogeographic range of the species complex (Figure 5.1, 5.2). In the idealised habitats set up, there were instances of overlap between sites in terms of ambient environmental conditions. In the first case, TEMP M, TEMP SL and ALP treatments in the summer season all had the same temperature and light regime, which differed the ARC habitat (Table 4.1). In the remaining seasons of autumn and winter, each of the 4 habitats had unique temperature and light regimes. However, both the ARC and ALP habitats shared snow cover during winter (see 5.2.2.3 below), while snow was also present in the ARC habitat in the latter months of autumn.

5.2.2.2. Photosynthesis responses to heat stress in *A. ciliata* complex ecotypes.

To investigate the response of the target ecotypes to heat stress, at the end of each growing season two sets of plants from each ecotype were exposed to warmer growth conditions, one at 20 ˚C and the other at 30 ˚C, both for one week, before chlorophyll fluorescence measurements.

5.2.2.3. Impact of snow cover on Photosynthesis responses to cold (freezing) stress in the *A. ciliata* (Ireland) ecotype.

To investigate whether snow cover assists plants in tolerating cold (freezing) stress, 10 plants of *A. ciliata* ssp *ciliata* (Ireland) were placed in the Arctic habitat. Five plants were covered with snow which was gathered from freshly fallen uncompacted snow on Maynooth Campus on the starting date of the experiment, while the remaining 5 plants were left uncovered and exposed to freezing air. The two groups of plants were maintained in these conditions for three weeks and then chlorophyll fluorescence measurements were conducted.

5.2.2.4. Impact of plant age on cold stress tolerance in *A. ciliata* (Ireland) and A. *norvegica* (Ireland) ecotypes

To determine whether plant age impacts on cold tolerance in *A. ciliata* complex ecotypes, two sets of 3 and 12 month old *A. ciliata* and *A. norvegica* plants were acclimated in ARC autumn growing conditions. Photosynthesis performance was recorded for five plants from each age cohort of the two ecotypes.

5.2.3 Selection of data points for comparative analysis of light response curves.

In order to evaluate ecotype variation in photosynthesis response curves in a customised manner, multiple data points were selected for comparative analysis (Figure 5.1). These captured initial, end-point and intermediate empirical values for four key data points in the light response curves of Y(II), and Y(NO), [points 1, 3, 7 and 13] and three key data points for Y(NPQ) [points 3, 7 and 13 – *note* point 1 is always 0 for Y(NPQ)]. Each point

provides valuable information on the fate of excitation energy in photosystem II at a certain time in the light exposure window, allowing comparative insight into the health of the photosystems and the plant capacity of managing excessive excitation energy.

- Point number 1 gives the maximal quantum efficiency of PSII [Y(II)]
- Point number 3 reflects the initial phase of the plant response to the saturating pulse.
- Point number 7 reflects the health of plant photosystem II in the middle of measurements.
- Point number 13 indicates the PS II efficiency under equilibrium conditions, reflecting the capacity of the plant to cope with excess energy at maximum saturating pulse intensity. At this point successful regulation of non-photochemical energy dissipation [Y(NPQ) and Y(NO)] is indicated by a maximal ratio of Y(NPQ)/Y(NO).

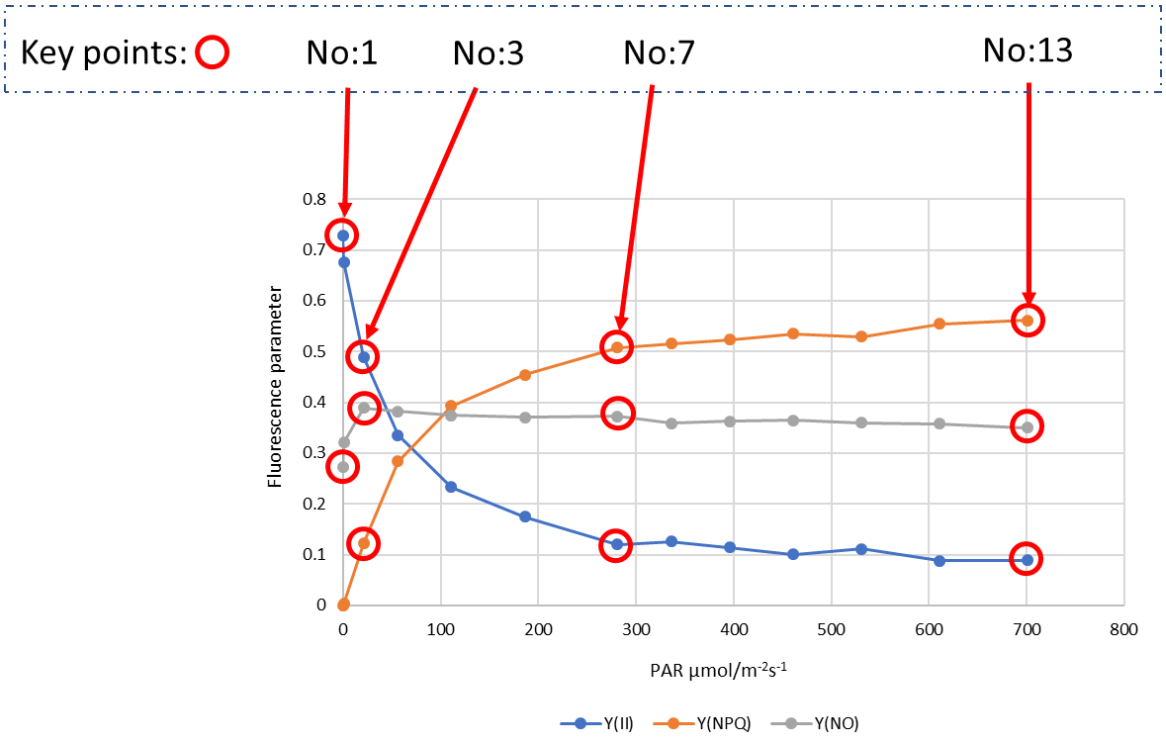

Figure 5.1: Illustrative light response curve for *A. ciliata* complex ecotypes in the current study. Y(II) a portion of absorbed energy that converted in PSII. Y(NPQ) regulated non-photochemical quenching loss in PSII (photoprotection mechanism). Y(NO) a portion of non-regulated non-photochemical loss in PSII. Data points (1-4) used for comparative analysis of ecotype responses are indicated. Point 1 captures initial reads for the plant in the acclimated state, and for Y(NPQ) is always zero.

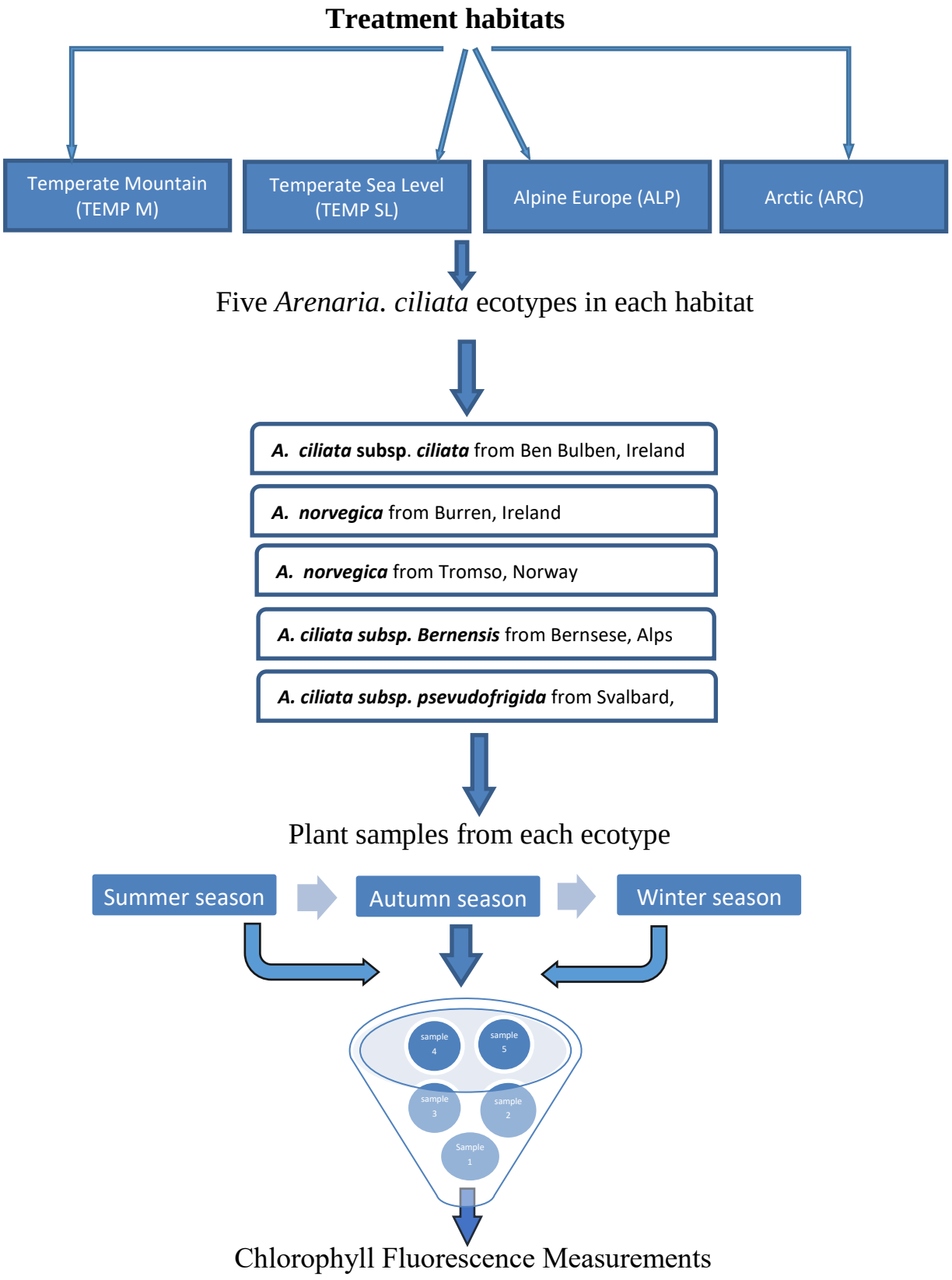

Figure 5.2: Diagram of experimental design to track seasonal changes in the rates of photosynthesis in response to changes in temperature and light in each ecotype in each treatment habitat.

5.2.4 Statistical Analysis.

A standard approach was applied for statistical analysis of changes in the levels of complementary photosystem II quantum yields Y(II), Y(NPQ) and Y(NO) of *A. ciliata* ecotypes. Following preliminary evaluation of datasets for sample size and normality, pairwise and multiple comparisons between cohort datasets were completed using T-test and ANOVA test for pairwise and multiple comparisons of parametric data, respectively, as applied in GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA.

5.3 Results

5.3.1 Core observations of seasonal change in Photosynthesis rates in *A. ciliata* complex ecotypes.

These analyses comprise 3 end of season datasets (Summer, Autumn, Winter), made up of photosynthesis observations on each of five ecotypes from *A. ciliata* complex (Figure 5.2) that were exposed to four different idealised habitat growing conditions that changed accordingly along a season timeline (Table 4.1).

Summer Season Data

Chlorophyll fluorescence measurements for A. ciliata at the end of the summer season.

Arenaria ciliata seedlings response to light intensity are given in figure 5.3. Plants grown under summer experimental conditions of TEMP, TEMP SL and ALP showed lower photosynthesis performance with initial photosystem II efficiency (Y(II) = 0.76)

compared to seedlings grown in ARC summer (Y(II) = 0.78) (Figure 5.3). At maximal PAR, Plants grown in the ARC habitat showed significantly higher ability to manage excess illumination by increasing the ratio of Y(NPQ)/Y(NO) = 2.12 compared to the other habitats ratio Y(NPQ)/(NO) = 1.39 (Table 5.1 and Appendix Table B.1).

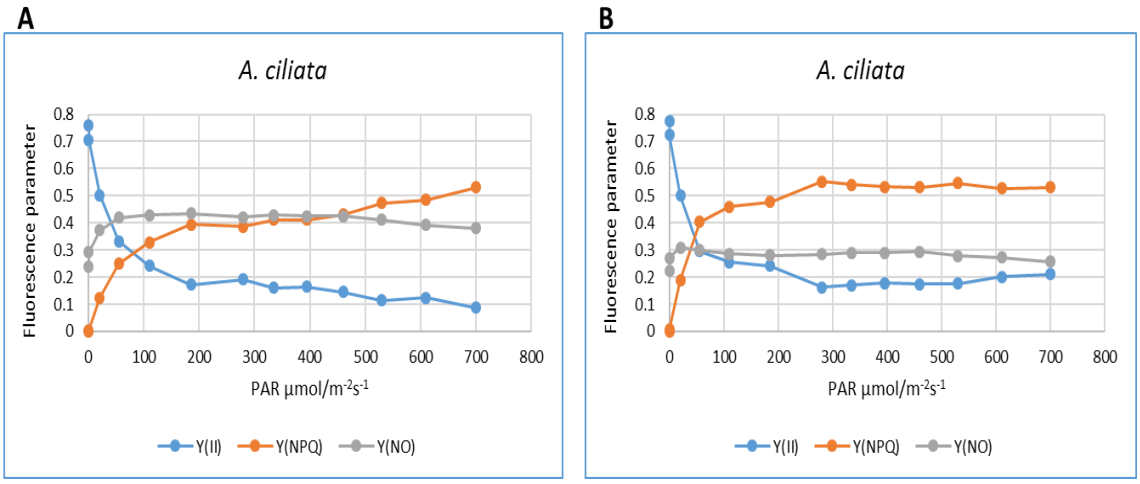

Figure 5.3: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. ciliata* (Ireland) acclimated to the summer season growth settings in 4 experimental habitat settings. **A**. Temperate Mountain (TEMP M), Temperate sea level (TEMP SL) and Alpine (ALP) habitats **B.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.1: Comparison of key data points in the end of summer light response curves of Y(II), Y(NPQ) and Y(NO) among *A. ciliata* seedlings grown in different habitat treatments. Pairwise T-tests were completed using GraphPad Prism version 5.00, GraphPad Software, San Diego, California USA. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

Habitats		ARC 9 ˚C / 14 h			
	Data points ˣ	1	3	7	13
TEMP M, TEMP SL and ALP 17 ˚C / 16 h	Y(II)	ns	ns	ns	P **
	Y(NPQ)		P **	P **	ns
	Y(NO)	ns	P *	P **	P **

Chlorophyll fluorescence measurements for A. norvegica (Ireland) at the end of the summer season.

Figure 5.4 shows the partitioning of absorbed energy in photosystem II of *Arenaria norvegica* (Ireland) green leaves at the end of the summer growth period. Higher initial photosystem II efficiency was observed in ARC habitat Y(II) = 0.77 compared to the other habitats which showed, Y(II) = 0.73, with lower values of Y(NO) and higher values of Y(NPQ) at all subsequent data points (Figure 5.4). At maximal saturating light intensity, plants acclimated in ARC habitat conditions showed a higher ratio of Y(NPQ)/Y(NO), at 1.96, compared to the other habitats, at 1.05, showing significantly greater ability to manage excess illumination (Table 5.2 and Appendix Table B.1).

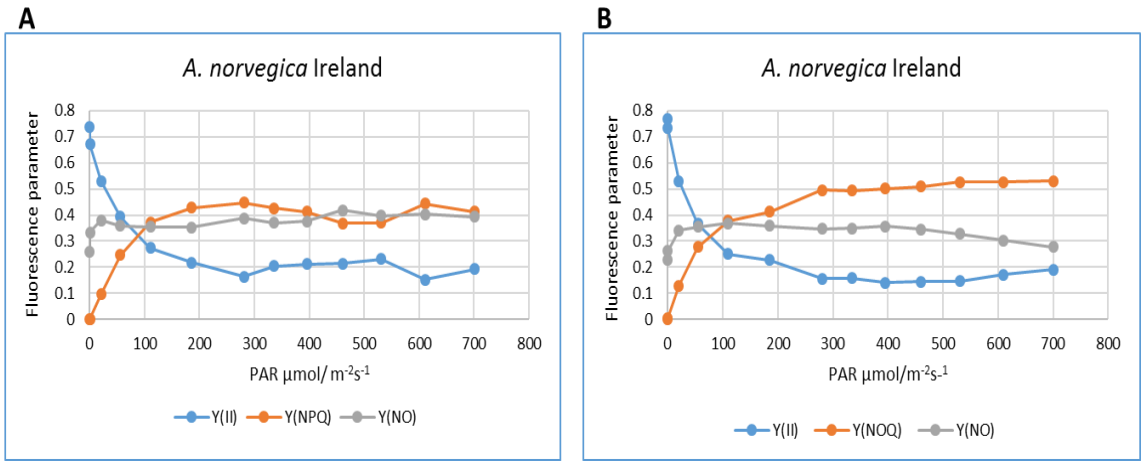

Figure 5.4: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. norvegica* (Ireland) acclimated to the summer season growth settings in 4 experimental habitat settings. **A**. Temperate Mountain (TEMP M), Temperate sea level (TEMP SL) and Alpine (ALP) habitats **B.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.2: Comparison of key data points of end of summer light response curves of Y(II), Y(NPQ) and Y(NO) among *A. norvegica* seedlings between habitat treatments. (ns**:** P > 0.05) (****:** P < 0.01).

Habitats	ARC 9 ˚C / 14 h				
	Data points ˣ	1	3	7	13
TEMP M, TEMP SL and ALP 17 ˚C / 16 h	Y(II)	ns	ns	ns	ns
	Y(NPQ)		ns	ns	P **
	Y(NO)	ns	ns	ns	P **

Chlorophyll fluorescence measurements for A. norvegica (Norway) at the end of the summer season.

Relatively Lower photosynthetic performance was observed in all *Arenaria norvegica* plants (Norway) compared to the other analysed ecotypes, with initial photosystem II efficiency around (Y(II) = 0.55) under all experimental treatment habitats (Figure 5.5). Under increasing rates of PAR, plants grown under TEMP M, TEMP SL and ALP habitats showed significantly higher values of Y(II) than those grown in ARC habitat (Table 5.3). However, at saturating light intensity, sustained photosynthesis performance was observed in plants acclimated in ARC summer conditions. In addition, they displayed an ability to dissipate excess illumination energy as heat energy in a regulated way within the photosynthetic apparatus (NPQ mechanism), showing a high photoprotective capacity (Figure 5.5 B).

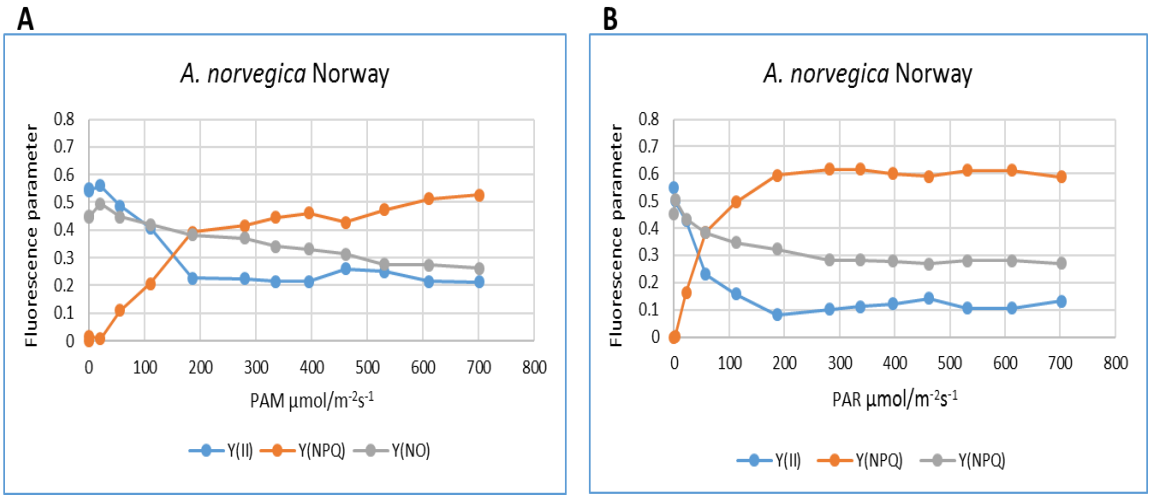

Figure 5.5: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. norvegica* (Norway) acclimated to the summer season growth settings in 4 experimental habitat settings. **A**. Temperate Mountain (TEMP M), Temperate sea level (TEMP SL) and Alpine (ALP) habitats **B.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.3: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *A. norvegica* (Norway) seedlings between treatments at the end of summer. (ns**:** P > 0.05) (*: P < 0.05) (**: P < 0.01)

Habitats	ARC 9 ˚C / 14 h				
	Data points ˣ	1	3	7	13
TEMP M, TEMP SL and ALP 17 ˚C / 16 h	Y(II)	ns	P **	P *	p*
	Y(NPQ)		P **	P *	ns
	Y(NO)	ns	ns	ns	ns

Chlorophyll fluorescence measurements for A. ciliata subsp. *bernensis (Switzerland) at the end of the summer season.*

Figure 5.6 demonstrates the photosynthesis performance of *A. ciliata* subsp. *bernensis* cohorts under different habitats in the summer season. The sample plants displayed similar photochemical efficiency of PS II between treatments, at Y(II) = 0.73 for the ARC

habitat and 0.74 for the other habitats at first point of measurements. Higher values of Y(II) and lower values of Y(NO) were recorded in ARC growth habitat as light intensity increased compared to TEMP M, TEMP SL and ALP habitats (Figure 5.6 B, A and Table 5.4). However, at high light flux density (700 µmol/$m^{-2}s^{-1}$), plants under all experimental habitats showed a similar capacity of photoprotective reactions, with a Y(NPQ)/Y(NO) ratio of 1.76 for ARC and 1.73 for the other habitats (Appendix Table B. 1).

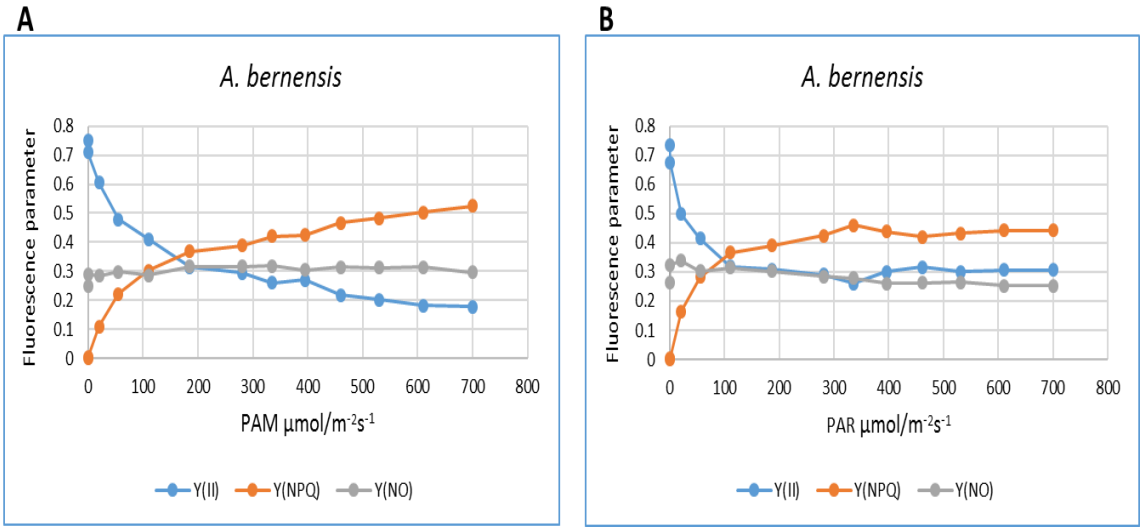

Figure 5.6: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. bernensis* (Switzerland) acclimated to the summer season growth settings in 4 experimental habitat settings. **A**. Temperate Mountain (TEMP M), Temperate sea level (TEMP SL) and Alpine (ALP) habitats **B.** Arctic (ARC) habitat. Illumination at each step is 30 seconds.

Table 5.4: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *A. bernensis* (Switzerland) seedlings between treatments at the end of summer. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01).

Habitats	ARC 9 ˚C / 14 h				
TEMP M, TEMP SL and ALP 17 ˚C / 16 h	Data points ˣ	1	3	7	13
	Y(II)	ns	P **	ns	P **
	Y(NPQ)		P *	ns	P *
	Y(NO)	ns	P **	ns	P *

Chlorophyll fluorescence measurements for A. ciliata subsp. *pseudofrigida (Svalbard) at the end of the summer season.*

A similar fraction of absorbed energy used as photochemical energy [Y(II)] was observed for all *A. ciliata* subsp. *pseudofrigida* cohorts under all experimental habitats at all time points, with initial photochemical efficiency of PS II, Y(II) = 0.77 for ARC habitat and 0.76 for the other habitats, (Figure 5.7 and Table 5.5). In terms of dissipation of excitation energy, a significant increase in the values of Y(NO) mostly in place of Y(NPQ) was seen in TEMP M, TEMP SL and ALP habitats, resulting in a in decrease of Y(NPQ)/Y(NO) to 1.05, as a compared to 2.7 in the ARC habitat. That is indicative of a high photoprotective capacity under ARC conditions (Appendix Table B. 1).

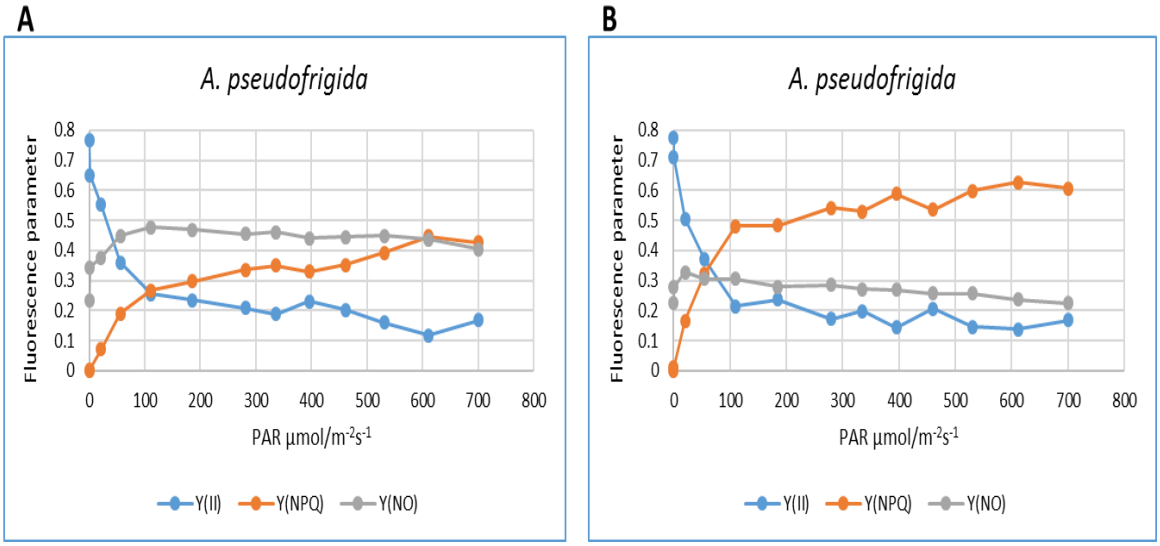

Figure 5.7: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. pseudofrigida* (Svalbard) acclimated to the summer season growth settings in 4 experimental habitat settings. **A**. Temperate Mountain (TEMP M), Temperate sea level (TEMP SL) and Alpine (ALP) habitats **B.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.5: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *A. pseudofrigida* (Svalbard) seedlings between treatments at the end of summer. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01).

Habitats	ARC 9 ˚C / 14 h			
	Data points	1	3	7	13
TEMP M, TEMP SL and ALP 17 ˚C / 16 h	Y(II)	ns	ns	ns	ns
	Y(NPQ)		P ***	p***	P***
	Y(NO)	ns	P*	p***	P***

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the TEMP SL, TEMP M and ALP habitats at the end of the summer season.

By pooling the data, it is possible to compare photosynthetic performance between five *A. ciliata* ecotypes grown in controlled environmental conditions (17^0C and 16h daylight), representing idealised summer growing conditions of Temperate mountain (TEMP M), temperate sea level (TEMP SL) and alpine (ALP) habitats (Table 5.6). In the first months of development during the summer season conditions, seedlings of *A. ciliata* (Ireland), *A. norvegica* (Ireland), *A. ciliata* subsp. *bernensis* (Switzerland) and *A. ciliata* subsp. *pseudofrigida* (Svalbard) showed good initial photochemical efficiency of photosystem II Y(II), ranging between 0.73 to 0.76 efficiency (Figure 5.3 A, 5.4 A, 5.6 A and 5.7 A).

A. norvegica seedlings (Norway) exhibited a lower initial value of Y(II) = 0.55 which differed significantly from the other analysed ecotypes. However, this ecotype showed sustained photosynthesis performance similar to the other ecotypes at the high flux quantum intensities (Figure 5.5 A and Table 5.6).

At maximal light intensity, *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* showed greater ability to cope with excess excitation energy in PS II, showing higher ratio of Y(NPQ)/Y(NO) = 2 and 1.7 respectively compared to the other ecotypes, which showed a similar ratio of Y(NPQ)/Y(NO) ranging between 1 and 1.3, (Appendix Table B. 1).

Table 5.6: Comparison of key data points of light responses curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* ecotypes acclimated to the summer season of Temperate mountain, Temperate sea level and Alpine habitats. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points [x]	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
A.c	Y(II)	ns	P***	ns	ns	P***	P***	ns	ns	ns	P***	ns	ns	ns	P***	ns	ns
	Y(NPQ)		ns	ns	ns		p***	ns	ns		ns	ns	ns		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
A. n	Y(II)					P**	ns	ns	ns	ns	P**	ns	ns	ns	ns	ns	ns
	Y(NPQ)						p***	ns	ns		ns	ns	ns		ns	ns	ns
	Y(NO)					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
A. n N	Y(II)									P***	ns	ns	ns	P***	ns	ns	ns
	Y(NPQ)										p***	ns	ns		p**	ns	ns
	Y(NO)									ns	p***	ns	ns	ns	ns	ns	ns
A. b	Y(II)													ns	ns	ns	ns
	Y(NPQ)														ns	ns	ns
	Y(NO)													ns	ns	ns	ns

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the ARC habitat at the end of the summer season.

Arenaria ciliata ecotypes grown under ARC summer conditions (9 ˚C and 14 h daylight), showed greater photosynthetic performance and higher photoprotective capacity compared to the same ecotypes grown in the other habitats.

A similar fraction of absorbed excitation energy that converted in PS II to photochemical energy [Y(II)] was seen in *A. ciliata*, *A. norvegica* and *A. ciliata* subsp. *pseudofrigida* with the same initial value of Y(II) = 0.77 (Figure 5.3 B, 5.4 B and 5.7 B). A slightly lower value of Y(II) = 0.73 was observed in *A. ciliata* subsp. *bernensis* seedlings, however this ecotype showed higher ability to photosynthesise with increasing light pulse intensities compared to the others (Figure 5.6 B).

As seen in the summer conditions of the other habitats, *A. norvegica* (Norway) showed significantly lower initial photosynthetic performance Y(II) = 0.55 compared to the other analysed ecotypes (Figure 5.5 B and Table 5.7).

All ecotypes growing in the ARC habitat during summer showed high photoprotective capacity by maintaining a high level of Y(NPQ) which kept Y(NO) at a reduced level, thus reducing damage to PS II by excess excitation energy, in contrast to relatively higher summer Y(NO) values recorded for *A. ciliata*, *A. norvegica* and *A. ciliata* subsp. *pseudofrigida* in the other three habitats (Figure 5.3 B, 5.4 B and 5.7 B).

Table 5.7: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the summer season of Arctic habitat. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points ˣ	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	P***	ns	ns	P***	P***	ns	ns	ns	P***	p*	ns	ns	P***	ns	ns
A.c	Y(NPQ)		ns	ns	ns		ns	ns	ns		ns	p***	ns		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	p***	p***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Y(II)					P***	ns	ns	ns	ns	ns	p*	ns	ns	ns	ns	ns
A. n	Y(NPQ)						ns	p*	ns		ns	ns	ns		ns	ns	ns
	Y(NO)					p***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Y(II)									p***	ns	p***	p**	P***	ns	ns	ns
A. n N	Y(NPQ)										ns	p***	p**		ns	ns	ns
	Y(NO)									p***	p***	ns	ns	p***	ns	ns	ns
	Y(II)													ns	ns	p*	p*
A. b	Y(NPQ)														ns	p*	p***
	Y(NO)													ns	ns	ns	ns

Autumn Season Data

Chlorophyll fluorescence measurements for A. ciliata at the end of the autumn season

Similar photosynthetic performance at the end of the autumn growing season was observed for *A. ciliata* (Ireland) plants growing in TEPM M, TEMP SL and ALP habitats, with initial photosystem efficiencies for photosystem II Y(II), of 0.77, 0.76 and 0.73 respectively, and sustained photosynthesis performance under increasing rates of PAR, (Figure 5.8 A, B and C). Plants grown under ARC habitat conditions showed significantly lower initial photosynthetic performance (Y(II) = 0.60) compared to the other habitats (Figure 5.8 D, Table 5.8). Plants growing in this habitat also displayed a reduced ability to manage excessive photic energy, with a marked increase of Y(NO) in place of Y(NPQ) (Figure 5.8 D and Appendix Table B. 2).

Similar, but more highly significant differences were also observed in respect of Y(NO) between the ARC habitat and the TEMP SL, TEMP M and ALP habitats (Table 5.8).

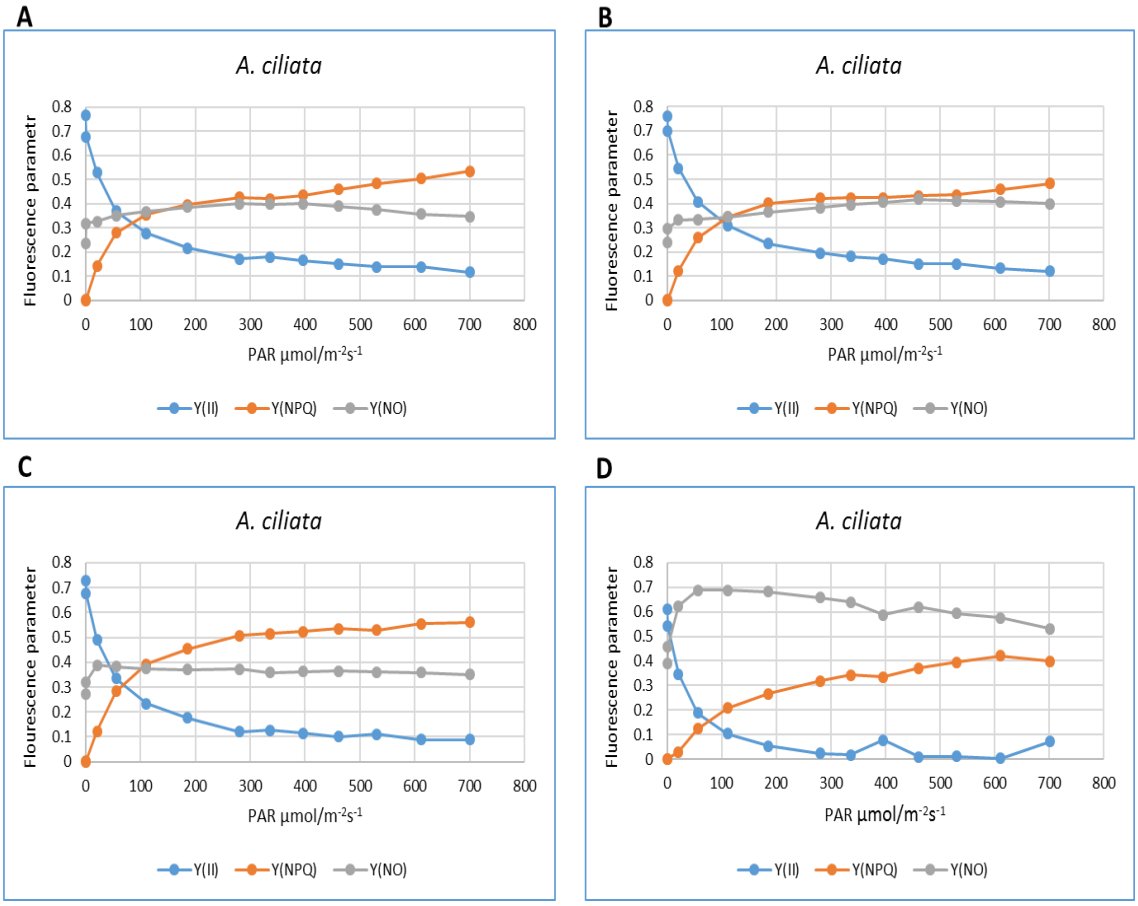

Figure 5.8: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. ciliata* (Ireland) acclimated to the autumn season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.8: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *A. ciliata* seedlings (Ireland) between treatments at the end of the autumn season. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	ns	ns	ns	P***	P***	P***	ns	ns	ns	ns	ns
	Y(NPQ)		ns	ns	ns		P***	ns	P**		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	P***	P***	P***	P***	ns	ns	ns	ns
TEMP SL	Y(II)					P***	P***	P***	ns	ns	ns	P**	ns
	Y(NPQ)						P**	ns	ns		ns	P*	P*
	Y(NO)					P***	P***	P***	P**	ns	ns	ns	ns
ARC	Y(II)									P***	P**	P*	ns
	Y(NPQ)										P***	P***	P***
	Y(NO)									P***	P***	P***	P***

Chlorophyll fluorescence measurements for A. norvegica (Ireland) at the end of the autumn season.

In the TEMP M and TEMP SL habitats, *A. norvegica* (Ireland) plants showed similar initial photosynthetic performance, with Y(II) = 0.77 and 0.78 respectively (Figure 5.9 A and B), with a slightly lower photosynthetic efficiency [Y(II) = 0.71] observed in ALP habitat (Figure 5.9 C).

The lowest performance of photosynthesis was observed in the plants acclimated in ARC habitat conditions, with initial photosystem II efficiency Y(II) = 0.65, decreasing to zero when the saturating pulse reached 200 µmol/$m^{-2}s^{-1}$ of PAR (Figure 5.9 D).

During all quantum flux densities, the plants grown under TEMP M and TEMP SL habitats displayed a greater capacity to manage excessive photic energy by dissipating it as heat Y(NPQ) and photochemical energy Y(II) compared to ALP and ARC habitats (Figure 5.9 and Table 5.9).

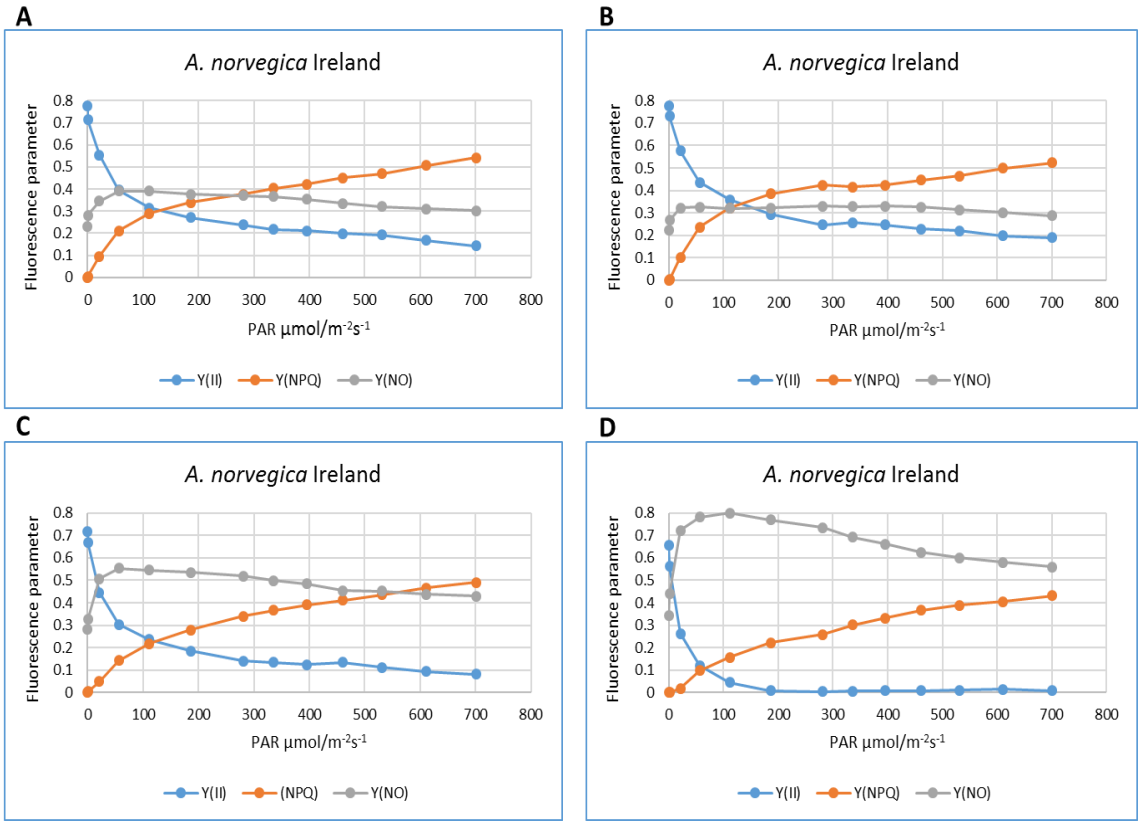

Figure 5.9: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. norvegica* (Ireland) acclimated to the autumn season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.9: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *A. norvegica* seedlings (Ireland) between treatments at the end of the autumn season. (ns**:** P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	ns	ns	ns	P***	P***	P***	P***	ns	P*	ns	ns
	Y(NPQ)		ns	ns	ns		P***	P*	P**		P*	ns	ns
	Y(NO)	ns	ns	ns	ns	P***	P***	P***	P***	ns	P**	P**	P**
TEMP SL	Y(II)					P***	P***	P***	P***	ns	P**	P*	P***
	Y(NPQ)						P***	P**	ns		P**	ns	ns
	Y(NO)					P***	P***	P***	P***	ns	P***	P**	P***
ARC	Y(II)									ns	P***	P***	P**
	Y(NPQ)										ns	ns	ns
	Y(NO)									P*	P***	P***	P**

Chlorophyll fluorescence measurements for A. norvegica (Norway) at the end of the autumn season.

Arenaria norvegica (Norway) plants grown in the TEMP M and the TEMP SL habitats showed the highest initial photosynthetic performance with photosystem II efficiency Y(II)= 0.77 and 0.76 respectively followed by 0.74 for ARC and 0.71 for ALP (Figure 5.10 A, B, C and D). At increasing photon flux density, Y(II) values decreased to almost zero in the ARC habitat, which differed significantly from the other habitats, at the same time plants grown in the ALP habitat showed greater photosynthetic performance than the other habitats (Table 5.10).

Under all growth habitats, *A. norvegica* plants showed great ability to manage absorbed energy by utilizing a high portion of it as photochemical energy via photosystem II and as heat via the regulated photoprotective NPQ mechanism, keeping Y(NO) at low values (Figure 5.10 A, B, C, D and Appendix Table B. 2). At maximal quantum flux densities, significant differences were observed in respect of Y(NO) between the ARC habitat and the other growth habitats, linked with decrease in the value of Y(II) (Figure 5.10 D and Table 5.10).

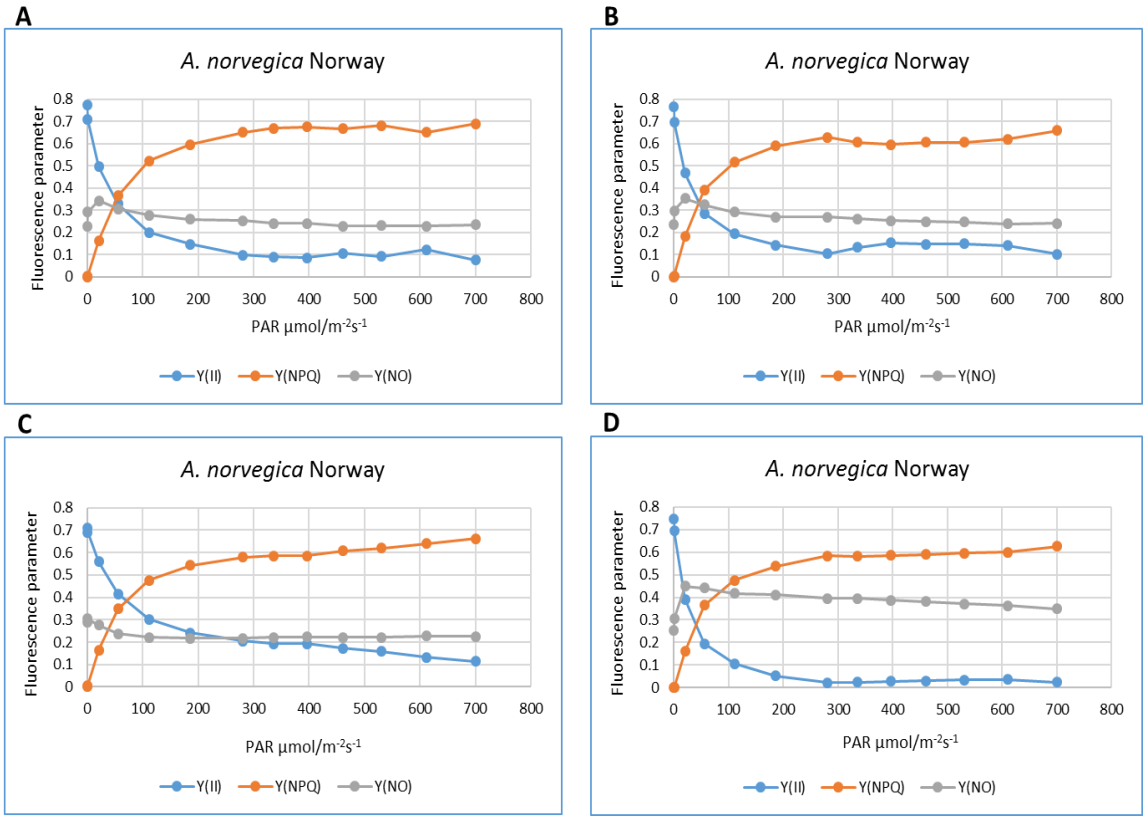

Figure 5.10: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. norvegica* (Norway) acclimated to the autumn season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.10: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *A. norvegica* seedlings (Norway) between treatments at the end of the autumn season. (ns**:** P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	ns	ns	ns	ns	P***	ns	ns	P***	P*	P**	ns
	Y(NPQ)		ns	ns	ns		ns	ns	ns		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	ns	P***	P***	P***	P***	P**	ns	ns
TEMP SL	Y(II)					ns	P*	ns	ns	P***	P***	P**	ns
	Y(NPQ)						ns	ns	ns		ns	ns	ns
	Y(NO)					ns	P***	P***	P***	P***	P**	ns	ns
ARC	Y(II)									P*	P***	P***	P**
	Y(NPQ)										ns	ns	ns
	Y(NO)									P*	P***	P***	P***

Chlorophyll fluorescence measurements for A. ciliata subsp. *bernensis (Switzerland) at the end of the autumn season.*

Figure 5.11 shows the efficiency of photosystem II of *Arenaria bernensis* (Switzerland) samples grown in four different growth conditions at the end of the autumn season. The highest fraction of absorbed energy, converted in PS II to photochemical energy Y(II), was observed in the ALP habitat followed by the TEMP SL and then the TEMP M habitats with initial photosystem II efficiency Y(II) = 0.77, 0.76 and 0.74 respectively, whereas the lowest value of Y(II) = 0.71 was recorded for the ARC habitat. (Figure 5.11 and Table 5.11).

In terms of energy loss (non-photochemical energy) in PS II, in the all habitats an increase in the value Y(NPQ) is paralleled by a decrease of Y(NO) value, showing the great ability of plants to protect PS II against damage by excess excitation energy (Figure 5.11).

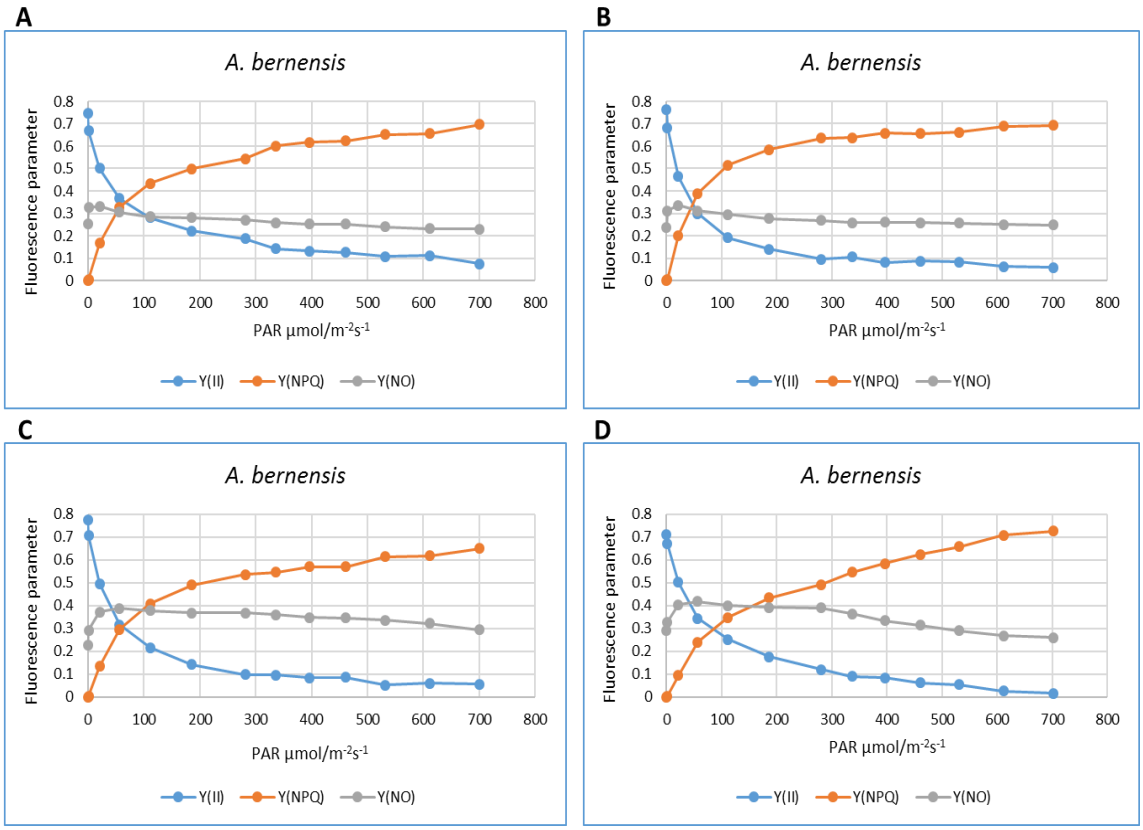

Figure 5.11: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. bernensis* (Switzerland) acclimated to the autumn season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.11: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *A. bernensis* seedlings (Switzerland) between treatments at the end of the autumn season. (ns**:** P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	ns	P*	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Y(NPQ)		ns	ns	ns		P*	ns	ns		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	ns	P*	P*	ns	ns	ns	ns	ns
TEMP SL	Y(II)					P***	ns	ns	ns	ns	ns	ns	ns
	Y(NPQ)						P***	P**	ns		P*	ns	ns
	Y(NO)					P***	P*	P*	ns	ns	ns	ns	ns
ARC	Y(II)									P***	ns	ns	ns
	Y(NPQ)										ns	ns	ns
	Y(NO)									P***	ns	ns	ns

Chlorophyll fluorescence measurements for A. ciliata subsp. *pseudofrigida (Svalbard) at the end of the autumn season.*

Figure 5.12 displays the photosynthetic performance of *Arenaria pseudofrigida* (Svalbard) plants acclimated to the autumn season of four different experimental habitats. Generally, relatively lower photosynthetic performance was recorded in all habitats compared to the other analysed ecotypes. The lowest photochemical efficiency of PS II was seen in ARC habitat with an initial value of Y(II)= 0.56 (Figure 5.12 D). This value was significantly lower than those recorded for plants in the other growth habitats, which showed almost similar initial photochemical efficiency of photosystem II, (Y(II)) ranging between 0.66 to 0.70 (Figure 5.12 and Table 5.12). With increasing photon flux intensity, a sharp decrease in values of Y(II) to zero was seen in the ARC habitat and also low values of Y(II) were observed in the ALP habitat. These values were significantly lower than those values recorded for plants grown in the TEMP M and the TEMP SL habitats, which showed similar patterns of performance (Figure 5.12 and Table 5.12).

Compared to the other habitats, higher values of Y(NO) was recorded for plants grown in the ARC habitat, which were significantly higher than that recorded for the other growth habitats where plants showed similar values. However, at high quantum intensities, the high value of Y(NPQ) was seen in ARC habitats as the other habitats showed (Figure 5.12).

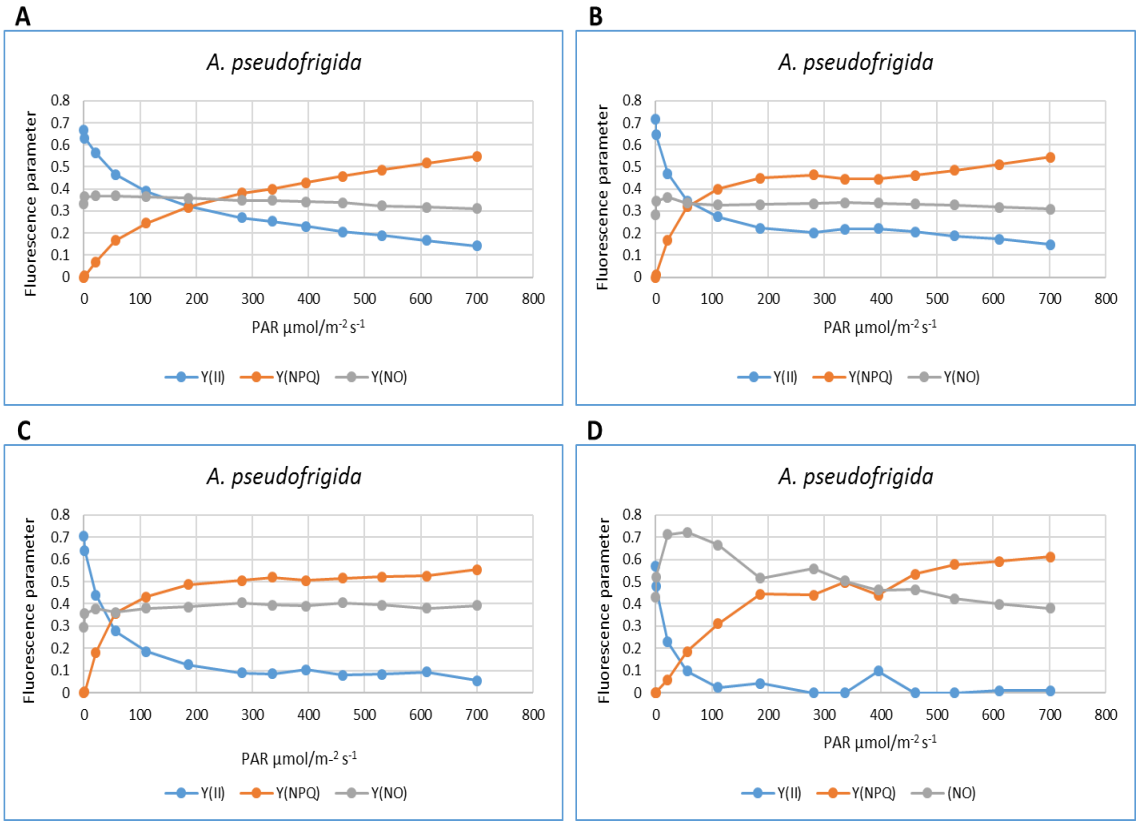

Figure 5.12: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. pseudofrigida* (Svalbard) acclimated to the autumn season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.12: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *A. pseudofrigida* seedlings (Svalbard) between treatments at the end of the autumn season. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	P*	ns	ns	P***	P***	P***	P***	ns	P**	P***	P***
	Y(NPQ)		P**	ns	ns		ns	ns	ns		P***	ns	ns
	Y(NO)	ns	ns	ns	ns	P***	P***	P***	ns	ns	ns	ns	ns
TEMP SL	Y(II)					P***	P***	P***	P***	ns	ns	P**	P***
	Y(NPQ)						P***	ns	ns		ns	ns	ns
	Y(NO)					P***	P***	P***	ns	ns	ns	ns	ns
ARC	Y(II)									P***	P***	P*	ns
	Y(NPQ)										P***	ns	ns
	Y(NO)									P***	P***	P**	ns

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the TEMP M habitat at the end of the autumn season.

At the end of autumn season in the TEMP M habitat, similar photosynthetic performance was observed in *A. ciliata*, *A. norvegica* (Ireland), *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis*, with initial efficiency of PS II, Y(II) = 0.76, 0.77, 0.77, 0.74 respectively (Figure 5.8 A, 5.9 A, 5.10 A and 5.11 A). A significant decrease in the initial value of Y(II) = 0.66 was recorded for *A. ciliata* subsp. *pseudofrigida* plants, however they showed slightly higher performance at high quantum flux intensities compared to the other ecotypes (Figure 5.12 A and Table 5.13).

Under all rates of PAR, *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* displayed greater ability to dissipate excess non-photochemical energy in form of heat via photoprotective NPQ mechanism, showing higher values of Y(NPQ) in place of Y(NO) values compared to the other ecotypes, which showed similar efficient dissipation of non-photochemical energy (Table 5.13 and Appendix Table B. 2).

Table 5.13: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the autumn season of Temperate mountain habitat. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	p***	ns	ns	ns
A.c	Y(NPQ)		ns	ns	ns		ns	p***	p**		ns	ns	p***		p*	ns	ns
	Y(NO)	ns	ns	ns	ns	ns	ns	p***	p**	ns	ns	p**	p***	p***	ns	ns	ns
	Y(II)					ns	ns	p*	ns	ns	ns	ns	ns	p***	ns	ns	ns
A. n	Y(NPQ)						ns	p***	p*		ns	p*	p**		ns	ns	ns
	Y(NO)					ns	ns	p**	ns	ns	ns	p*	ns	p***	ns	ns	ns
	Y(II)									ns	ns	ns	ns	p***	ns	p**	ns
A. n N	Y(NPQ)										ns	ns	ns		p*	p***	p*
	Y(NO)									ns	ns	ns	ns	p***	ns	ns	ns
	Y(II)													p***	ns	ns	ns
A. b	Y(NPQ)														p**	p*	p*
	Y(NO)													p***	ns	ns	ns

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the TEMP SL habitat at the end of the autumn season.

In the TEMP SL habitat autumn conditions, *A. norvegica* (Ireland) plants displayed the highest efficiency of converting a high fraction of absorbed energy to photochemical energy followed by *A. ciliata*, with an initial efficiency of Y(II) = 0.78 and 0.77 respectively (Figure 5.8 B and 5.9 B).

The lowest initial of the photochemical efficiency of PS II, Y(II) = 0.71 was observed in *A. ciliata* subsp. *pseudofrigida* plants, which was significantly lower than the other analysed ecotypes, (Figure 5.12 and Table 5.14). However, with increasing PAR intensities these plants exhibited sustained photosynthetic performance, which is similar to the performance of *A. ciliata* and *A. norvegica* (Ireland) plants.

Similar photosynthesis performance was recorded for *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* with an initial efficiency of Y(II) = 0.76 for both species (Figure 5.10 B and 5.11 B). However lower values of Y(II) were observed for the both species at high rates of PAR compared to the other ecotypes (Table 5.14).

In terms thermal energy dissipation, as is seen in the TEMP M habitat, a marked increase of Y(NPQ) in place of Y(NO) observed in cases of *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* plants, showed a greater ability to manage lost excitation energy in PS II reaction centers compared to the other ecotypes (Figure 5.8 B, 5.9 B, 5.10 B, 5.11 B, 5.12 B and Appendix Table B. 2).

Table 5.14: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the autumn season of Temperate sea level habitat. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	ns	ns	p**	ns	ns	ns	ns	ns	p*	p*	p*	p***	p*	ns	ns
A.c	Y(NPQ)		ns	ns	ns		ns	p***	p***		p**	p***	p***		ns	ns	ns
	Y(NO)	ns	ns	ns	p***	ns	ns	***	***	ns	ns	p***	p***	p***	ns	ns	p***
	Y(II)					ns	p**	p**	p**	ns	p**	p***	p***	p***	p**	ns	ns
A. n	Y(NPQ)						p*	p***	p***		p***	p***	p***		ns	ns	ns
	Y(NO)					ns	ns	ns	ns	ns	ns	ns	ns	p***	ns	ns	ns
	Y(II)									ns	ns	ns	ns	p***	ns	ns	ns
A. n N	Y(NPQ)										ns	ns	ns		ns	p**	p**
	Y(NO)									ns	ns	ns	ns	p***	ns	ns	ns
	Y(II)													p***	ns	p*	p**
A. b	Y(NPQ)														ns	p**	p***
	Y(NO)													p***	ns	ns	ns

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the ARC habitat at the end of the autumn season.

In the (-5˚C) freezing conditions at the end of autumn in the ARC habitat, *A. ciliata*, *A. norvegica* (Ireland) and *A. ciliata* subsp. *pseudofrigida* showed a similar reduction of photosynthetic performance, with initial value of Y(II) = 0.61, 0.65 and 0.56 respectively (Figure 5.8 D, 5.9 D and 5.12 D), compared to *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis*. This reduced level of photosynthesis was also remarkable when compared to their counterpart performance that growing in the other experimental habitats in the same season (Appendix Table B. 2). In these freezing conditions, *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* displayed a high efficiency of the initial value of Y(II) =0.74 and 0.71 respectively, and these ecotypes continued photosynthesising even under high rates of PAR (Figure 5.10 D, 5.11 D and Table 5.15).

In terms of thermal dissipation of excitation energy (non-photochemical) in PS II, freezing temperature induced a marked increase in level of Y(NO) mostly at cost of Y(NPQ) in *A. ciliata* and *A. norvegica* (both from Ireland) (Figure 5.8 D, 5.9 D and Table 5.15). At the same time, *A. norvegica* (Norway) and *A. ciliata* subsp.*bernensis* (Switzerland) displayed a significant increase of Y(NPQ) mainly in place of Y(NO), reflecting the greater ability of NPQ-generating reactions to protect these plants against cold damage (Table 5.15 and Appendix Table B. 2).

A. ciliata subsp. *pseudofrigida* (Svalbard) plants showed relatively lower photoprotective capacity [lower ratio of Y(NPQ)/ Y(NO)], compared to *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* (Figure 5.12 D and Table 5.15).

Table 5.15: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the autumn season of Arctic habitat. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
A.c	Y(II)	ns	ns	ns	ns	p*	ns	ns	ns	ns	p**	p***	ns	ns	p*	ns	ns
	Y(NPQ)		ns	ns	ns		p***	p***	p**		p*	p*	p***		ns	ns	p***
	Y(NO)	ns	ns	ns	ns	p*	p**	p***	p**	ns	p***	p***	p***	ns	p***	ns	p*
A. n	Y(II)					ns	ns	ns	ns	ns	p***	p***	ns	ns	ns	ns	ns
	Y(NPQ)						p***	p***	p*		p**	p**	p***		ns	p*	p**
	Y(NO)					ns	p***	p***	p**	ns	p***	p***	p***	ns	p***	p*	p**
A. n N	Y(II)									ns	ns	p***	ns	p**	p**	ns	ns
	Y(NPQ)										ns	ns	ns		p***	ns	ns
	Y(NO)									ns	ns	ns	ns	p**	ns	ns	ns
A. b	Y(II)													p*	p***	p***	ns
	Y(NPQ)														ns	ns	ns
	Y(NO)													p*	ns	ns	ns

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the ALP habitat at the end of the autumn season.

In the (4˚C) low-temperature conditions at the end of autumn in the ALP habitat, *A. ciliata* subsp. *bernensis* cohorts showed significantly higher initial photochemical efficiency of PS II, Y(II) = 0.77 compared to the other analysed ecotypes, which displayed Y(II) values ranging between 0.70 to 0.73. However, all the ecotypes showed similar photosynthesis performance at increasing PAR rates except *A. norvegica* (Norway) which exhibited higher values of Y(II) than the others (Figure 5.8 C, 5.9 C, 5.10 C, 5.11 C, 5.12 C and Table 5.16).

In terms of non-photochemical energy loss in PS II, *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* exhibited more efficient dissipation of excitation energy in PS II in form of heat by generating high levels of Y(NPQ) in place of Y(NO), compared to the other ecotypes (Figure 5.10 C, 5.11 C, Table 5.16 and B. 2).

The highest values of Y(NO) were recorded for *A. norvegica* plants (Ireland), showing reduced photoprotective capacity. These values were significantly higher than those observed for the other ecotypes (Figure 5.9 C, Table 5.16 and B. 2).

Table 5.16: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the autumn season of Alpine habitat. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	ns	ns	ns	ns	ns	p*	ns	p*	ns	ns	ns	ns	ns	ns	ns
A.c	Y(NPQ)		p***	p***	ns		ns	ns	ns		ns	ns	ns		p*	ns	ns
	Y(NO)	ns	p*	p***	p*	ns	p**	p**	p**	p*	ns	ns	ns	ns	ns	ns	ns
	Y(II)					ns	p**	ns	ns	p**	ns	ns	ns	ns	ns	ns	ns
A. n	Y(NPQ)						p***	p***	p***		p***	p***	p*		p***	p***	ns
	Y(NO)					ns	p***	p***	p***	p*	ns	p**	p*	ns	p*	p*	ns
	Y(II)									p***	ns	p*	ns	ns	p**	p**	ns
A. n N	Y(NPQ)										ns	ns	ns		ns	ns	ns
	Y(NO)									p***	ns	p**	ns	ns	p*	p***	p***
	Y(II)													p***	ns	ns	ns
A. b	Y(NPQ)														ns	ns	ns
	Y(NO)													p***	ns	ns	ns

Winter Season Data

Chlorophyll fluorescence measurements for A. ciliata at the end of the winter season.

Photosynthetic performance of *A. ciliata* (Ireland) plants grown in the winter season of four different experimental habitats shown in Figure 5.13. The photochemical efficiency of PS II was similar in TEMP M and TEMP SL habitats (Figure 5.13 A and B), however a significantly lower efficiency of photosystem II was observed in plants grown in ALP and ARC habitats which showed a similar values of Y(II) at all time points (Figure 5.13 C and D and Table 5.17).

In terms of non-photochemical energy dissipation after the initial saturating pulse, a sharp increase of Y(NO) was observed, mostly at the cost of Y(II), in both ARC and ALP habitats (Appendix Table B. 3), and these responses differed significantly compared to

TEMP M and TEMP SL habitats (Table 5.17). However at maximal quantum flux intensities, an increase of Y(NPQ)/(NO) was seen in ALP and ARC habitats, where a greater proportion of excess energy was managed via photochemical mechanisms (Figure 5.13 C and D).

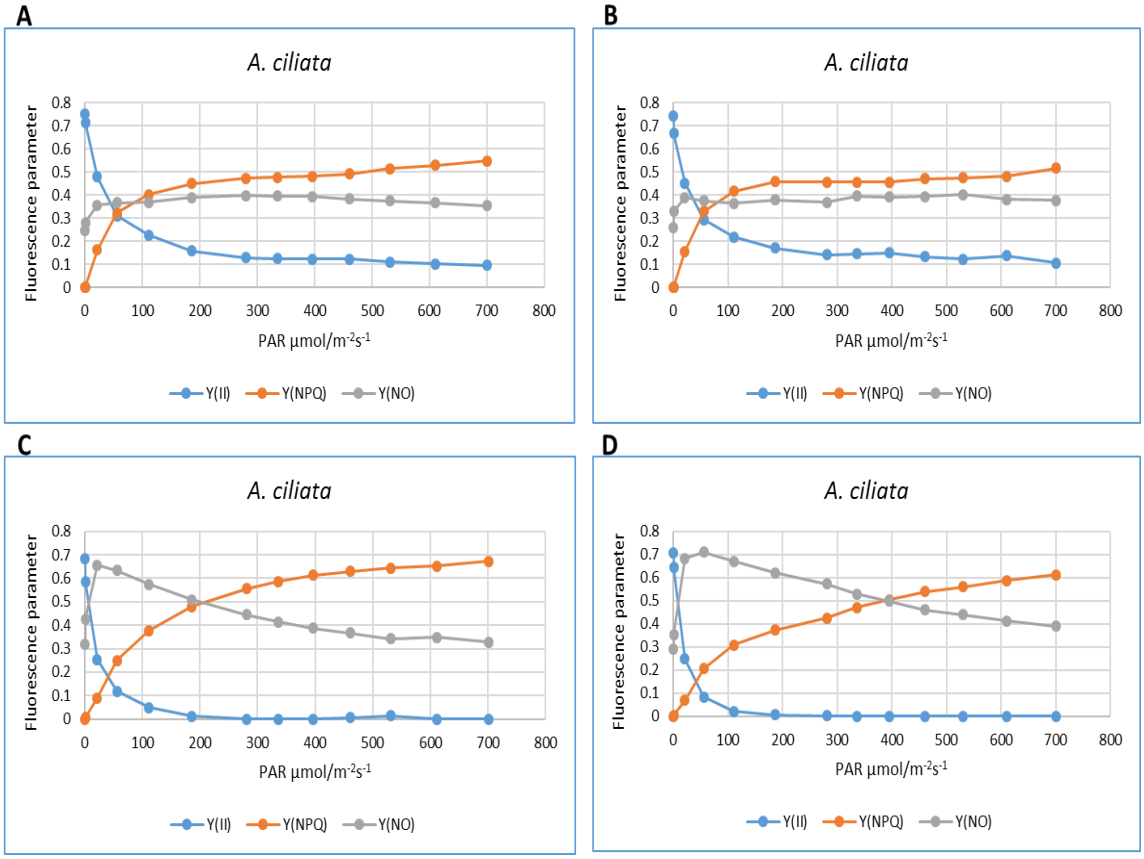

Figure 5.13: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. ciliata* (Ireland) acclimated to the winter season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.17: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) *A. ciliata* leaves (Ireland) between treatments at the end of winter season. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	ns	ns	ns	P**	P***	ns	P***	P***	P***	P*	P***
	Y(NPQ)		ns	ns	ns		P**	ns	ns		P*	ns	P***
	Y(NO)	ns	ns	ns	ns	P**	P***	P***	ns	P***	P***	ns	ns
TEMP SL	Y(II)					ns	P***	P***	P***	P***	P***	P***	P***
	Y(NPQ)						P*	ns	P*		ns	P**	P***
	Y(NO)					ns	P***	P***	ns	P***	P***	ns	ns
ARC	Y(II)									ns	ns	ns	ns
	Y(NPQ)										ns	ns	ns
	Y(NO)									ns	ns	P*	ns

Chlorophyll fluorescence measurements for A. norvegica (Ireland) at the end of the winter season.

The photosynthetic performance of *A. norvegica* plants (Ireland) grown in four different experimental habitats at the end of winter is given in Figure 5.14. Plants were grown in the TEMP M and the TEMP SL habitats exhibited similar rates of photosynthetic performance at all time points with initial photochemical efficiency of PS II, Y(II) = 0.77 and 0.76 respectively (Figure 5.14 A and B), while plants growing under the ARC and ALP habitat conditions showed significantly lower initial photosynthetic performance [Y(II) = 0.64 for ARC and 0.63 for ALP habitats] compared to plants in the TEMP M and the TEMP SL (Figure 5.14 C and D). Moreover, *A. norvegica* plants acclimated in ARC and ALP habitats displayed reduced ability to manage excess illumination dissipation. This is demonstrated in Figure 5.14 C and D, which shows an apparent increase of Y(NO) in place of Y(NPQ).

Plants grown in ARC and ALP habitats showed significantly higher values of Y(NO) than those values which recorded for the plants grown in TEMP M and TEMP SL habitats (Table 5.18).

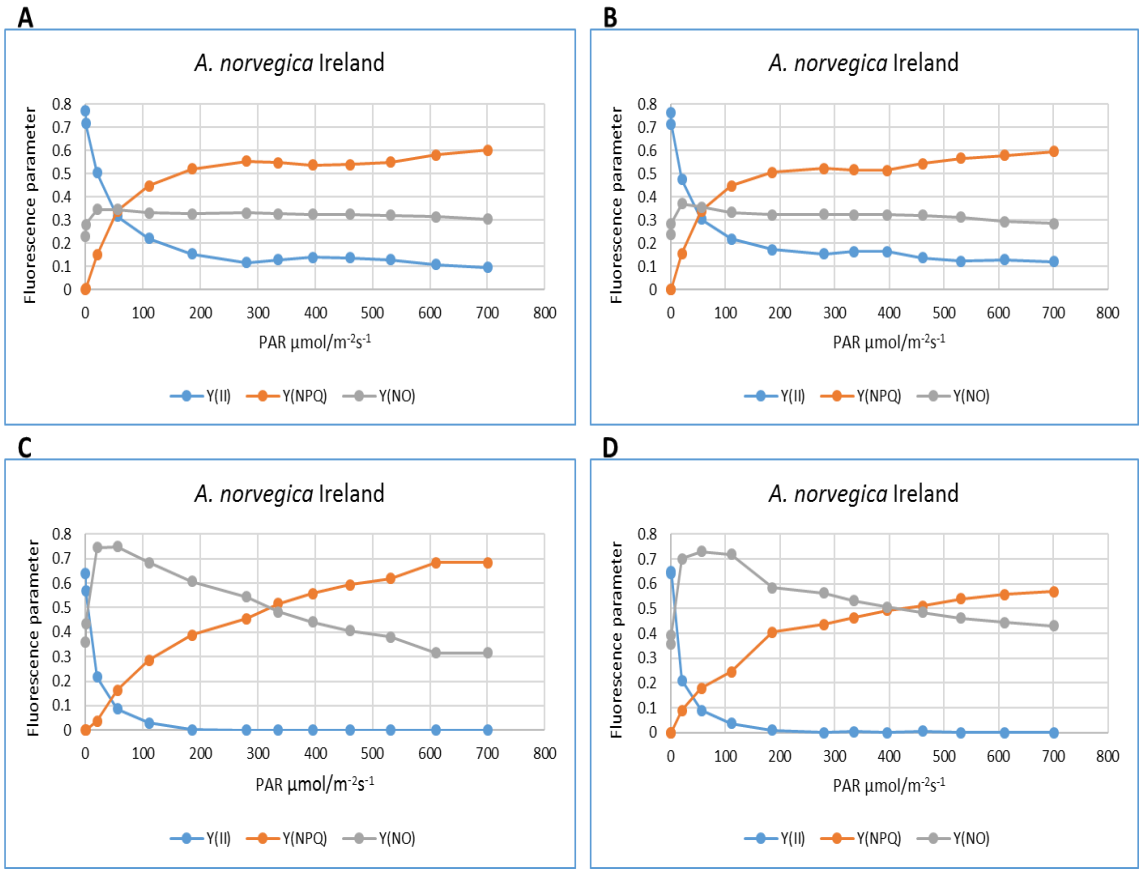

Figure 5.14: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. norvegica* (Ireland) acclimated to the winter season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.18: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) *A. norvegica* leaves (Ireland) between treatments at the end of winter season. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	ns	ns	P*	P***	P***	P***	P***	P***	P***	P***	P***
	Y(NPQ)		ns	ns	ns		P*	ns	ns		p***	ns	ns
	Y(NO)	ns	ns	ns	ns	P***	P***	P***	P**	P***	P***	P***	ns
TEMP SL	Y(II)					P***	P***	P***	P***	P***	P***	P***	P***
	Y(NPQ)						P*	ns	ns		p**	ns	ns
	Y(NO)					P***	P***	P***	P***	P***	P***	p***	ns
ARC	Y(II)									ns	ns	ns	ns
	Y(NPQ)										ns	ns	P*
	Y(NO)									ns	ns	ns	P*

Chlorophyll fluorescence measurements for A. norvegica (Norway) at the end of the winter season.

In the TEMP M and TEMP SL habitats, *A. norvegica* (Norway) plants showed similar photosynthetic performance, with initial photosystem II efficiency Y(II) = 0.74 and 0.76 respectively (Figure 5.15 A and B). Whereas significantly lower photosynthetic performance was recorded for the plants growing in the ALP and the ARC habitats which showed similar performance with initial values of Y(II) = 0.68 and 0.67 respectively (Figure 5.15 C and D and Table 5.19). During initial quantum flux intensities, significantly higher values of Y(NO) were recorded for the plants grown in the ARC winter conditions compared to the winter of other habitats (Figure 5.15 and Table 5.19). However, at high rates of PAR, an increase in the plants protective capacity was observed in all habitats through elevated energy dissipation via Y(NPQ) (Figure 5.15).

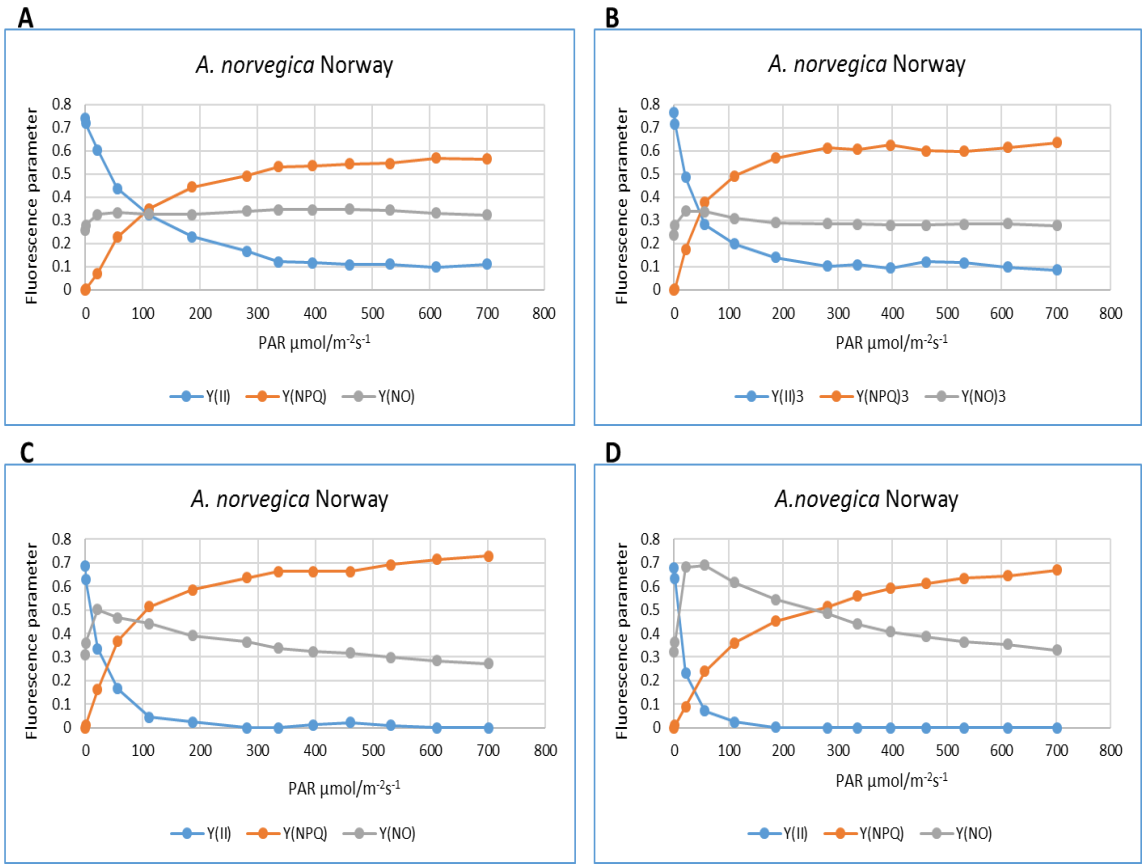

Figure 5.15: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. norvegica* (Norway) acclimated to the winter season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.19: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) A. norvegica leaves (Norway) between treatments at the end of winter season. (ns**:** P > 0.05) (***:** P < 0.05) (****:** P < 0.01) (*****:** P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	P*	ns	ns	P*	P***	P***	P***	ns	P***	P***	P***
	Y(NPQ)		P*	ns	ns		ns	ns	P**		ns	ns	P***
	Y(NO)	ns	ns	ns	ns	P*	P***	P*	ns	ns	P*	ns	ns
TEMP SL	Y(II)					P***	P***	P***	P***	P**	P***	P***	P***
	Y(NPQ)						ns	ns	ns		ns	ns	ns
	Y(NO)					P***	P***	P**	ns	P**	P*	ns	ns
ARC	Y(II)									ns	P*	ns	ns
	Y(NPQ)										ns	ns	ns
	Y(NO)									ns	P*	ns	ns

Chlorophyll fluorescence measurements for A. ciliata subsp. *bernensis (Switzerland) at the end of the winter season.*

Figure 5.16 shows detailed information of the photosynthetic performance of *Arenaria bernensis* (Switzerland) plants acclimated to the winter season of four different experimental habitats. The highest photosynthetic performance was observed in the TEMP SL habitat followed by the TEMP M and then the ALP habitats with initial photosystem II efficiency Y(II) = 0.74, 0.69 and 0.66 respectively, whereas the lowest initial value of Y(II) = 0.63 was recorded for the ARC habitats (Figure 5.16 and Table 5.20).

Under all habitats conditions, *A. ciliata* subsp. *bernensis* plants showed high photo-protective capacity against excessive photic energy, with rapid induction of Y(NPQ) in place of Y(NO) (Figure 5.16 and Appendix Table B. 3).

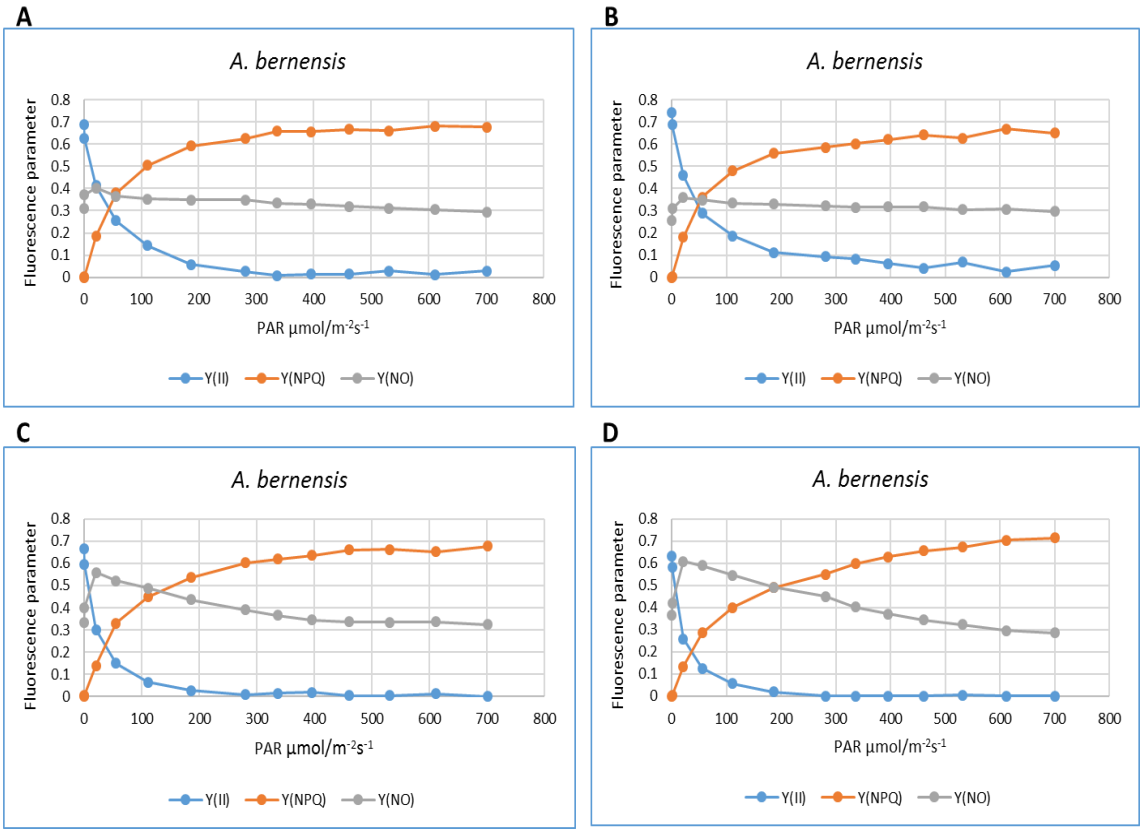

Figure 5.16: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. bernensis* (Switzerland) acclimated to the winter season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.20: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) *A. bernensis* leaves (Switzerland) between treatments at the end of winter season. (ns**:** P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	ns	P***	ns	ns	P**	ns	ns	ns	ns	ns	ns
	Y(NPQ)		ns	ns	ns		ns	ns	ns		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	ns	P**	ns	ns	ns	*	ns	ns
TEMP SL	Y(II)					P**	P***	P***	P***	P*	P**	P***	P***
	Y(NPQ)						ns	ns	ns		ns	ns	ns
	Y(NO)					P**	P***	ns	ns	P*	P**	ns	ns
ARC	Y(II)									ns	ns	ns	ns
	Y(NPQ)										ns	ns	ns
	Y(NO)									ns	ns	ns	ns

Chlorophyll fluorescence measurements for A. ciliata subsp. *pseudofrigida (Svalbard) at the end of the winter season.*

In the all experimental habitats, *A. ciliata* subsp. *pseudofrigida* (Svalbard) plants exhibited relatively lower photosynthetic performance compared to the other analysed ecotypes. Figure 5.17 B and Appendix Table B. 3 showed the highest values of Y(II) were recorded for plants grown in the TEMP SL habitat with an initial efficiency of photosystem II Y(II) = 0.62. Followed by plants grown under the TEMP M conditions with an initial value of Y(II) = 0.61 (Figure 5.17 A). The lowest initial photosynthesis was observed in the plants acclimated in ALP habitat conditions, with Y(II) = 0.46 (Figure 5.17 C) with a slightly higher photosynthetic efficiency [Y(II) = 0.49] recorded in the ARC habitat (Figure 5.17 D).

Compared to the other habitats, higher values of Y(NO) were recorded for the ARC habitat which differed significantly from the TEMP M and the TEMP SL habitats (Table 5.12), affecting photo-protective capacity [Levels of Y(NPQ)] of plants growing in the ARC habitat (Figure 5.17 D). Notably, in the ARC habitat, Y(II) photosynthesis levels remained above zero up to PAR (point 9), the highest value recorded for any ecotype.

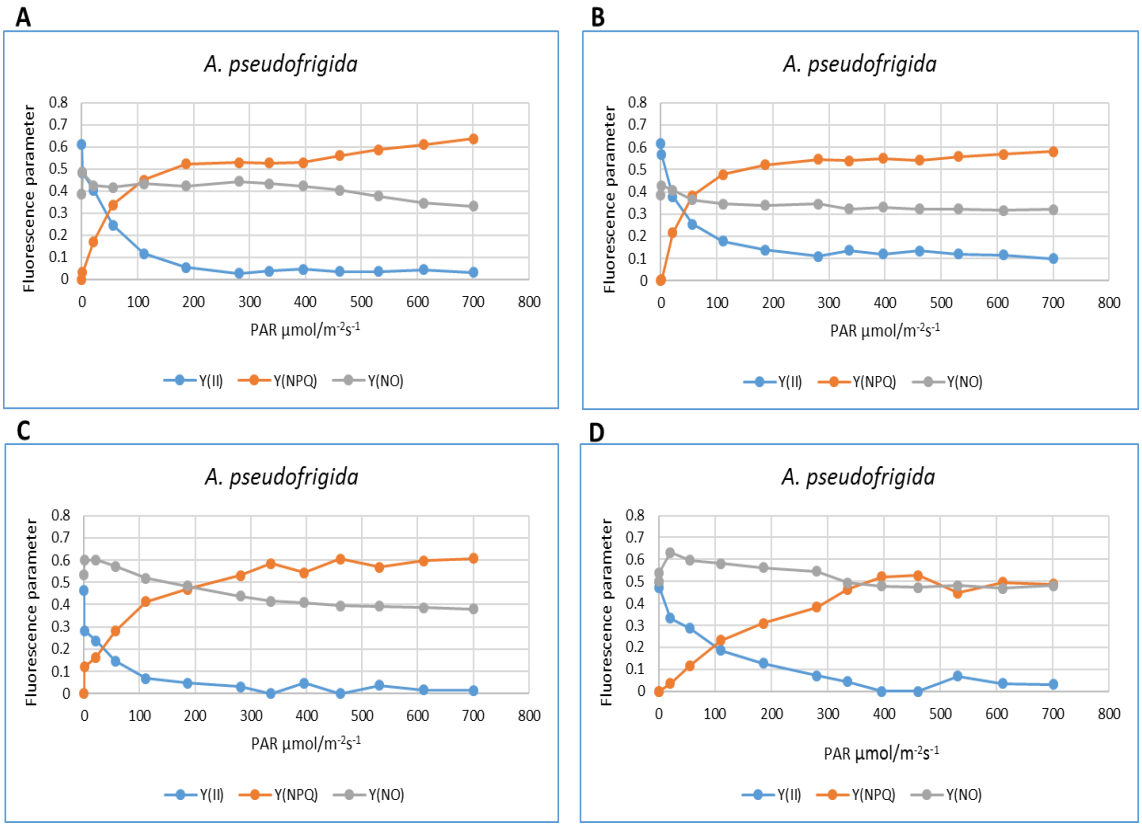

Figure 5.17: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) recorded for samples of *A. pseudofrigida* (Svalbard) acclimated to the winter season growth settings in 4 experimental habitat settings. **A.** Temperate mountain habitat (TEMP M) **B.** Temperate sea level habitat (TEMP SL) **C.** Alpine habitat (ALP) **D.** Arctic habitat (ARC). Illumination at each step is 30 seconds.

Table 5.21: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) *A. pseudofrigida* leaves (Svalbard) between treatments at the end of winter season. (ns**:** P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

Habitats		TEMP SL				ARC				ALP			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13
TEMP M	Y(II)	ns	ns	P**	P*	P***	ns	ns	ns	P***	P***	ns	ns
	Y(NPQ)		ns	ns	ns		ns	P*	p*		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	P**	P**	ns	P*	P***	P**	ns	ns
TEMP SL	Y(II)					P**	ns	ns	p*	P***	P***	P**	P**
	Y(NPQ)						P**	P**	ns		ns	ns	ns
	Y(NO)					P***	P**	P**	P*	P***	P**	ns	ns
ARC	Y(II)									ns	ns	ns	ns
	Y(NPQ)										ns	P**	ns
	Y(NO)									ns	ns	ns	ns

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the TEMP M habitat at the end of the winter season.

In relation to photosynthesis performance comparison between analysed ecotypes of *A. ciliata* complex acclimated to the winter season of TEMP M habitat. *A. norvegica* (Ireland), *A. ciliata* and *A. norvegica* (Norway) showed similar performance with initial photochemical efficiency of PS II Y(II) = 0.77, 0.75 and 0.74 respectively (Figure 5.13 A, 5.14 A and 5.15 A), whereas significantly lower photosynthesis performance was observed in *A. ciliata* subsp. *pseudofrigida* and *A. ciliata* subsp. *bernensis* which displayed these initial values of Y(II) = 0.61 and 0.69 respectively (Figure 5.16 A, 5.17 A and Table 5.22).

When Y(II) values decreased at high light intensities, all ecotypes showed good ability in protecting themselves against damage by excess excitation energy in PS II, by maximising the fraction of energy Y(NPQ) that dissipate in form of heat via photoprotective NPQ mechanism. However higher values of Y(NO) were observed in *A. ciliata* subsp. *pseudofrigida* plants compared to the other ecotypes. These values differed significantly from the other species in the initial stages of measurements (Figure 5.17 A and Table 5.22).

Table 5.22: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the winter season of Temperate mountain habitat. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	ns	ns	ns	ns	p***	ns	ns	p*	ns	p**	p*	p***	ns	p**	p*
A.c	Y(NPQ)		ns	ns	ns		p***	ns	ns		ns	p**	p***		ns	ns	p*
	Y(NO)	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	p***	p*	ns	ns
	Y(II)					ns	p**	ns	ns	p**	p*	p*	p*	p***	p**	p*	p*
A. n	Y(NPQ)						p**	ns	ns		ns	p*	ns		ns	ns	ns
	Y(NO)					ns	ns	ns	ns	p**	p*	ns	ns	p***	p***	p**	ns
	Y(II)									ns	p***	p***	p**	p***	p***	p***	p**
A. n N	Y(NPQ)										p***	p*	p***		p***	ns	p*
	Y(NO)									ns	p*	ns	ns	p***	p***	p*	ns
	Y(II)													p*	ns	ns	ns
A. b	Y(NPQ)														ns	ns	ns
	Y(NO)													p*	ns	ns	ns

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the TEMP SL habitat at the end of the winter season.

In the TEMP SL habitat winter conditions, all analysed ecotypes of *A. ciliata* complex exhibited good photosynthetic performance with initial efficiency of Y(II) ranging between 0.74 to 0.76, with exception of *A. ciliata* subsp. *pseudofrigida*, with significantly lower value of Y(II) = 0.62 (Figure 5.13 B, 5.14 B, 5.15 B, 5.16 B and 5.17 B).

In terms of excess energy dissipation, all ecotypes were successfully able to manage excess illumination dissipation by increasing values Y(NPQ) in place of values of Y(NO). Greater efficient dissipation of excess energy was seen in plants of *A. ciliata* subsp. *bernensis*, *A. norvegica* (Norway) followed by *A. norvegica* plants (Ireland), (Figure 5.14 B, 5.15 B, 5.16 B and Appendix Table B. 3), compared to *A. ciliata* and *A. ciliata* subsp.

pseudofrigida plants, which showed higher levels of Y(NO), (Figure 5.13 B, 5.17 B, Table 5.23 and Appendix Table B.3).

Table 5.23: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the winter season of Temperate sea level habitat. (ns: $p > 0.05$) (*: $p < 0.05$) (**: $p < 0.01$) (***: $p < 0.001$).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	p*	p***	p**	ns	ns
A.c	Y(NPQ)		ns	p**	p*		ns	p***	p***		ns	p***	p***		p**	p**	ns
	Y(NO)	ns	ns	ns	p***	ns	p*	p*	p**	ns	ns	ns	p*	p***	ns	ns	ns
	Y(II)					ns	ns	ns	ns	ns	ns	ns	p*	p***	p***	ns	ns
A. n	Y(NPQ)						ns	ns	ns		ns	ns	ns		p**	ns	ns
	Y(NO)					ns	ns	ns	ns	ns	ns	ns	ns	p***	ns	ns	ns
	Y(II)									ns	ns	ns	ns	p***	p***	ns	ns
A. n N	Y(NPQ)										ns	ns	ns		ns	ns	ns
	Y(NO)									ns	ns	ns	ns	p***	p**	ns	ns
	Y(II)													p***	p**	ns	ns
A. b	Y(NPQ)														ns	ns	ns
	Y(NO)													p***	ns	ns	ns

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the ARC habitat at the end of the winter season.

All analysed *A. ciliata* ecotypes grown in ARC winter (freezing conditions -5 ˚C), displayed photosynthetic performance reduction compared to their counterparts acclimated to the winter of TEMP M and TEMP SL habitats, with initial efficiency of photosystem II, Y(II) = 0.70 for *A. ciliata*, 0.64 for *A. norvegica* (Ireland), 0.67 for *A. norvegica* (Norway) and 0.63 for *A. ciliata* subsp. *bernensis*, (Figure 5.13 D, 5.14 D, 5.15 D and 5.16 D). And the lowest initial value of Y(II) = 0.49 was recorded for *A. ciliata*

subsp. *pseudofrigida* which differed significantly from the others (Figure 5.17 D and Table 5.24).

Despite the low initial value of Y(II) and high values of Y(NO), *A. ciliata* subsp. *pseudofrigida* plants showed greater ability to photosynthesising under high light flux intensities compared to other analysed ecotypes, which showed sharp decrease in the photosystem II efficiency Y(II) to zero when the saturating pulse reached 200 µmol/$m^{-2}s^{-1}$ of PAR. However, they exhibited good ability to manage excess excitation energy in PS II in a positive way by dissipating it as heat, protecting themselves against any photodamage (Figure 5.13 D, 5.14 D, 5.15 D and 5.16 D).

Both *A. ciliata* subsp. *bernensis* and *A. norvegica* (Norway) displayed significantly higher photoprotective capacity (Table 5.24), showing high ratios of Y(NPQ)/Y(NO) = 2.53 and 2.09 respectively compared to *A. ciliata* and *A. norvegica* (Ireland) which showed lower ratios of Y(NPQ)/Y(NO)= 1.6 for the former and 1.32 for the latter (Appendix Table B. 3).

Table 5.24: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the winter season of Arctic habitat. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	ns	ns	ns	ns	ns	ns	ns	p*	ns	ns	ns	p***	ns	p***	p*
A.c	Y(NPQ)		ns	ns	ns		ns	P*	P*		ns	P**	P**		ns	ns	P*
	Y(NO)	ns	ns	ns	ns	ns	ns	P**	P*	p*	ns	P**	P**	p***	ns	ns	P*
	Y(II)					ns	ns	ns	ns	ns	ns	ns	ns	p***	P*	p***	p*
A. n	Y(NPQ)						ns	P*	P*		ns	P**	P**		ns	ns	ns
	Y(NO)					ns	ns	P*	P*	ns	ns	P**	P*	p***	ns	ns	P*
	Y(II)									ns	ns	ns	ns	p***	P*	p***	p**
A. n N	Y(NPQ)										ns	ns	ns		ns	ns	p**
	Y(NO)									ns	ns	ns	ns	p***	ns	ns	p**
	Y(II)													p***	ns	p***	p*
A. b	Y(NPQ)														ns	ns	p**
	Y(NO)													p***	ns	P**	p*

Comparative chlorophyll fluorescence measurements among 5 A. ciliata complex ecotypes in the ALP habitat at the end of the winter season.

In the ALP winter (freezing conditions -3˚C), all the ecotypes showed a similar trend of photosynthesis performance to the same ecotypes which are grown in ARC habitat. *A. ciliata*, *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* exhibited almost the same photosynthesis performance with initial photochemical efficiency of photosystem II, Y(II) = 0.68, 0.69 and 0.67 respectively (Figure 5.13 C, 5.15 C and 5.16 C), with slightly lower photosynthetic efficiency (Y(II) = 0.64) observed in *A. norvegica* (Ireland), (Figure 5.14 C). The lowest initial value of Y(II) = 0.46 was recorded for *A. ciliata* subsp. *pseudofrigida* plants (Figure 5.17 C and Table 5.25).

AT the maximal flux densities (PAR), when almost all PS II reaction centres closed (Y(II)= zero), all ecotypes displayed elevated active protection against damage that can be caused by NO by maintaining a maximal ratio of Y(NPQ)/Y(NO) (Appendix Table B. 3, Figure 5.13 C, 5.14 C, 5.15 C, 5.16 C and 5.17 C).

Table 5.25: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* species acclimated to the winter season of Alpine habitat. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	p***	ns	p*	ns
A.c	Y(NPQ)		ns	ns	ns		ns	ns	ns		ns	ns	ns		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	p***	ns	ns	ns
	Y(II)					ns	ns	ns	ns	ns	ns	ns	ns	p***	ns	ns	ns
A. n	Y(NPQ)						P**	ns	ns		P*	ns	ns		ns	ns	ns
	Y(NO)					ns	P**	ns	ns	ns	P*	ns	ns	p***	ns	ns	ns
	Y(II)									ns	ns	ns	ns	p***	ns	p*	ns
A. n N	Y(NPQ)										ns	ns	ns		ns	ns	ns
	Y(NO)									ns	ns	ns	ns	p***	ns	ns	p*
	Y(II)													p***	ns	ns	ns
A. b	Y(NPQ)														ns	ns	ns
	Y(NO)													p***	ns	ns	p*

5.3.2 Photosynthesis responses to heat stress in *A. ciliata* complex ecotypes.

Photosynthesis responses at 20˚C.

Figure 5.18 demonstrates the photosynthetic performance of five mature plants of *A. ciliata* ecotypes when they exposed to warm conditions (20˚C and 16h daylight) for a week. In general, all ecotypes showed elevated photosynthesis performance. *A. ciliata* (Ireland), *A. norvegica* (Ireland), *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* (Switzerland) displayed similar initial photochemical efficiency of PS II with, Y(II) = 0.75, 0.76, 0.75 and 0.74 respectively (Figure 5.18 A, B, C and D). While lower efficiency with, Y(II) = 0.67 was reported for *A. ciliata* subsp. *pseudofrigida* (Svalbard), which differed significantly from the other ecotypes (Figure 5.18 E and Table 5.26).

With increasing rates of PAR, greater photochemical efficiency of PS II and photoprotective capacity were seen in case of *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis*, showing higher values of Y(II) and Y(NPQ) in place of Y(NO). These values in some points were significantly higher than those values recorded for the other ecotypes, which showed similar photosynthesis performance, (Figure 5.18 and Table 5.26).

In terms of thermal energy dissipation, all the ecotypes successfully regulated energy dissipation, maintaining a maximal ratio of Y(NPQ)/Y(NO), which is a sign of high photoprotective capacity (Appendix Table B. 6).

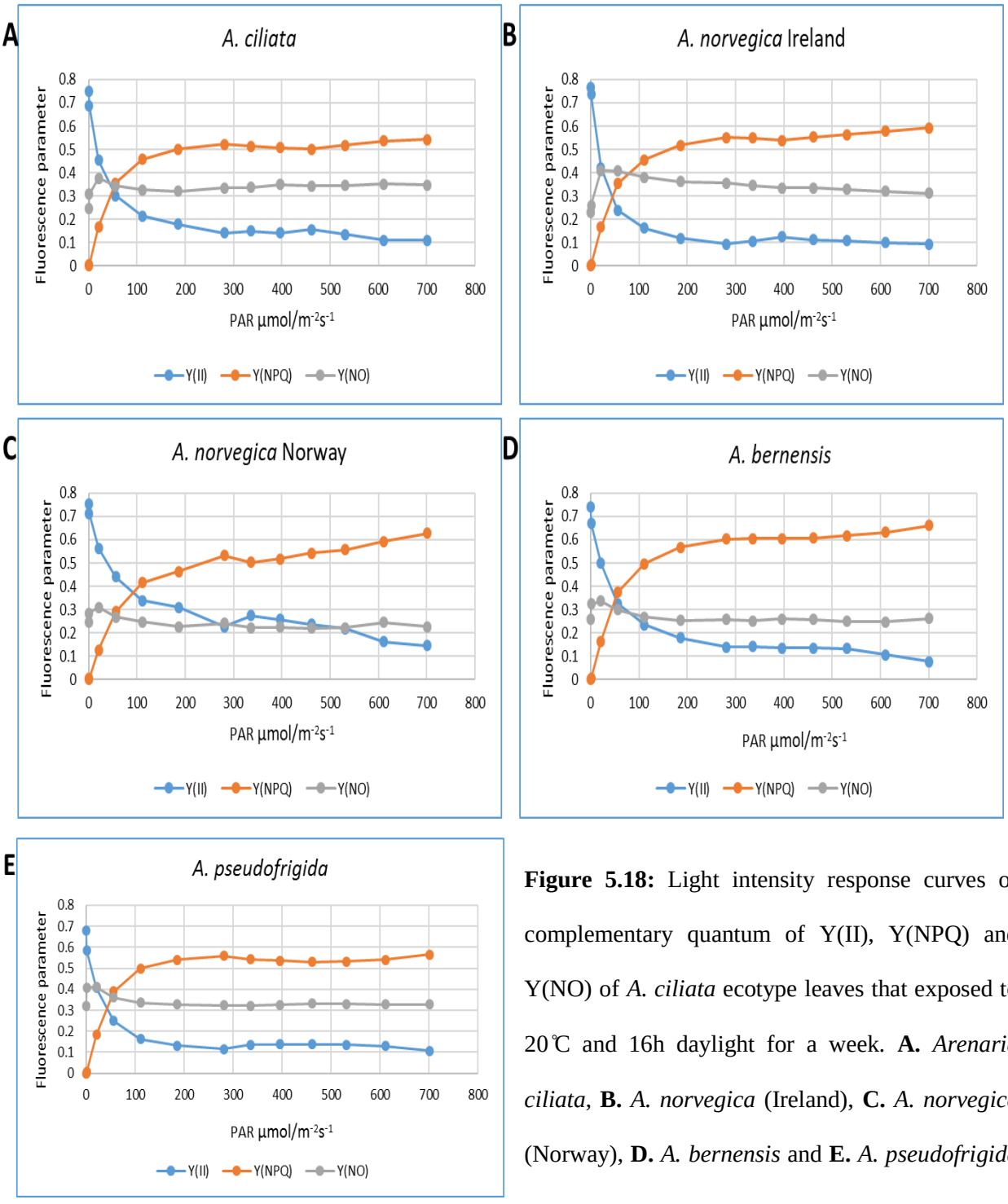

Figure 5.18: Light intensity response curves of complementary quantum of Y(II), Y(NPQ) and Y(NO) of *A. ciliata* ecotype leaves that exposed to 20℃ and 16h daylight for a week. **A.** *Arenaria ciliata*, **B.** *A. norvegica* (Ireland), **C.** *A. norvegica* (Norway), **D.** *A. bernensis* and **E.** *A. pseudofrigida*

Table 5.26: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* ecotypes exposed to warm conditions (20 °C and 16h Daylight). (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
	Y(II)	ns	ns	ns	ns	ns	p***	p*	ns	ns	ns	ns	ns	p***	ns	ns	ns
A.c	Y(NPQ)		ns	ns	ns		ns	ns	ns		ns	ns	p**		ns	ns	ns
	Y(NO)	ns	ns	ns	ns	ns	ns	p*	p***	ns	ns	ns	p*	p***	ns	ns	ns
	Y(II)					ns	p***	p***	ns	ns	p*	ns	ns	p***	ns	ns	ns
A. n	Y(NPQ)						ns	ns	ns		ns	ns	ns		ns	ns	ns
	Y(NO)					ns	p***	p**	p*	ns	p*	p*	ns	p***	ns	ns	ns
	Y(II)									ns	ns	ns	ns	p***	p***	p**	ns
A. n N	Y(NPQ)										ns	ns	ns		ns	ns	ns
	Y(NO)									ns	ns	ns	ns	p***	p**	ns	p**
	Y(II)													p***	p**	ns	ns
A. b	Y(NPQ)														ns	ns	ns
	Y(NO)													p***	ns	ns	ns

Photosynthesis responses at 30 °C.

All groups of *A. ciliata* ecotypes exposed to high temperatures (30˚C and 16 h daylight) showed a similar trend of photosynthetic performance to their counterparts, which are exposed to 20 ˚C 16h daylight, but with slightly higher photochemical efficiency of photosystem II (Figure 5.19). *A. ciliata*, *A. norvegica* (Ireland), *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* exhibited similar initial photosynthetic performance with Y(II) = 0.79, 0.78, 0.77 and 0.79 respectively, however a lower initial value of Y(II) = 0.73 was recorded for *A. ciliata* subsp. *pseudofrigida* plants. This value differed significantly from those recorded for *A. ciliata, A. norvegica* (Ireland) and *A. ciliata* subsp. *bernensis* (Figure 5.19 E and Table 5.27).

With increased light intensity, higher values of Y(II) coupled with low rates of Y(NO) were seen in case of *A. norvegica* plants (Norway) followed by *A. ciliata* subsp. *bernensis*

plants compared to the other ecotypes, which showed roughly similar behaviours (Figure 5.19 and Table 5.19).

In terms of thermal energy dissipation, all analysed ecotypes exhibited a great capacity to dissipate excess energy in the form of heat within photosynthetic apparatus, showing higher values of Y(NPQ) compared to Y(NO) values (Appendix Table B. 7). As seen for the 20˚C growing conditions, *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* displayed relatively greater photoprotective capacity compared to other analysed ecotypes (Figure 5.19).

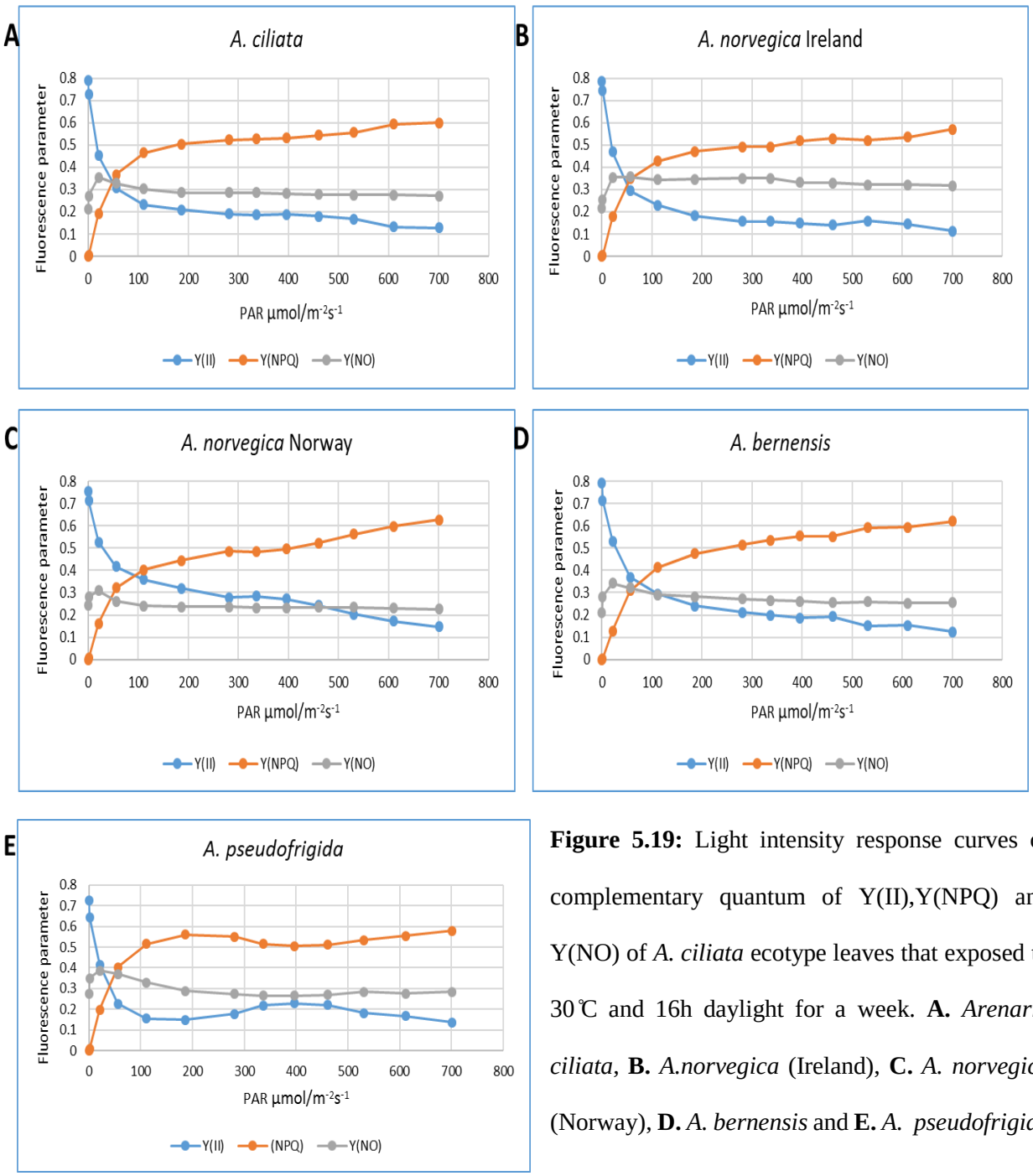

Figure 5.19: Light intensity response curves of complementary quantum of Y(II),Y(NPQ) and Y(NO) of *A. ciliata* ecotype leaves that exposed to 30℃ and 16h daylight for a week. **A.** *Arenaria ciliata*, **B.** *A.norvegica* (Ireland), **C.** *A. norvegica* (Norway), **D.** *A. bernensis* and **E.** *A. pseudofrigida*

Table 5.27: Comparison of key points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* ecotypes exposed to high temperature (30 °C and 16h Daylight). (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata species		A.n				A. n N				A. b				A. p			
	Data points	1	3	7	13	1	3	7	13	1	3	7	13	1	3	7	13
A.c	Y(II)	ns	ns	ns	ns	ns	p***	p***	ns	ns	p***	ns	ns	p***	ns	ns	ns
	Y(NPQ)		ns	ns	ns		ns	ns	ns		p**	ns	ns		ns	ns	ns
	Y(NO)	ns	ns	p*	ns	ns	ns	ns	ns	ns	ns	ns	ns	p***	ns	ns	ns
A. n	Y(II)					ns	p**	p***	ns	ns	p**	p*	ns	p***	p*	ns	ns
	Y(NPQ)						ns	ns	ns		p*	ns	ns		ns	ns	ns
	Y(NO)					ns	ns	p***	p**	ns	ns	p**	p**	p***	ns	p**	ns
A. n N	Y(II)									ns	ns	p**	ns	ns	p***	p***	ns
	Y(NPQ)										ns	ns	ns		ns	ns	ns
	Y(NO)									p*	ns	ns	ns	ns	p**	ns	ns
A. b	Y(II)													p***	p***	ns	ns
	Y(NPQ)														p**	ns	ns
	Y(NO)													p***	ns	ns	ns

5.3.3 Impact of snow cover on photosynthesis responses to cold (freezing) stress in the *A. ciliata* (Ireland) ecotype.

Figure 5.20 illustrates the difference in photosynthesis performance between two groups of mature *A. ciliata* plants in freezing conditions of Arctic habitat, one covered with snow (Figure 5.20 A) and the other group exposed to air (Figure 5.20 B). Snow-covered plants showed significantly higher initial photosynthesis performance, with Y(II) = 0.72 compared with Y(II) = 0.65 were recorded for cold air exposed plants.

At high quantum flux intensities when values of Y(II) approached zero, snow-covered plants displayed significantly higher values of Y(NPQ) and lower levels of Y(NO) than those observed in the same plants left exposed to cold air (Figure 5.20 and Table 5.28). at the maximal rate of PAR, a lower ratio of Y(NPQ)/Y(NO) = 1.67 was observed in air-

exposed plants compared with *A. ciliata* plants covered with snow, which displayed Y(NPQ)/Y(NO) = 2.66 (Appendix Table B. 4).

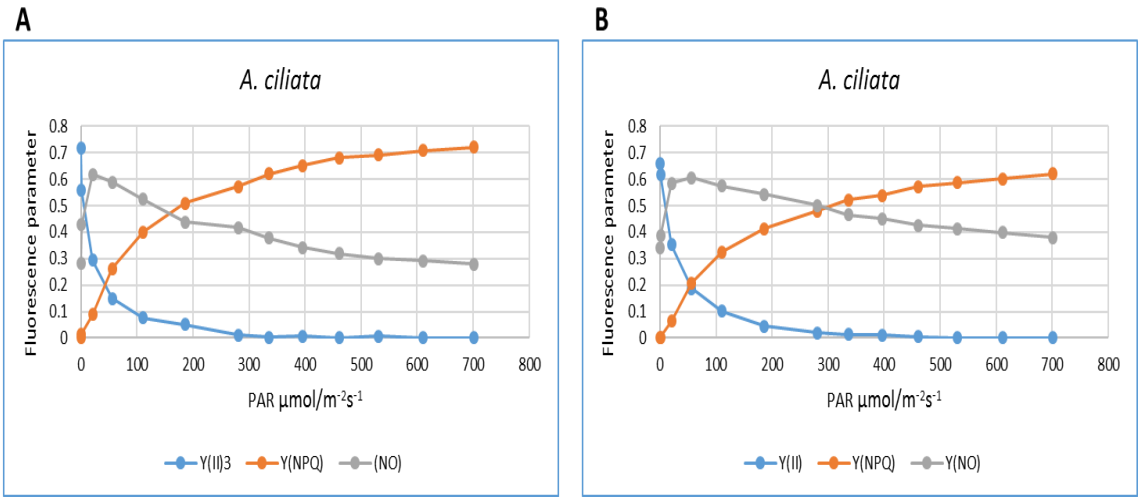

Figure 5.20: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) of *A. ciliata* spp. *ciliata* mature plants placed in winter conditions of Arctic habitat for three weeks. **A.** Plants covered with snow **B.** Plants exposed to cold air.

Table 5.28: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) between *Arenaria ciliata* plants covered with snow and plants exposed to cold air under Arctic winter conditions. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A.ciliata		Plants exposed to air			
	Data points	1	3	7	13
Plants covered with snow	Y(II)	p***	ns	ns	ns
	Y(NPQ)		ns	p**	p***
	Y(NO)	p***	ns	p*	p***

5.3.4 Impact of plant age on cold stress tolerance in *A. ciliata* (Ireland) and *A. norvegica* (Ireland) ecotypes.

Arenaria ciliata

Arenaria ciliata plants at the juvenile stage (3 months) showed less ability to photosynthesise at low temperature (-5˚C) compared to the mature plants (12 months), (Figure 5.21). The initial photochemical efficiency of PS II of juvenile plants was lower than mature plants, with Y(II) = 0.61 for juvenile plants and Y(II) = 0.65 for mature plants (Figure 5.21). Across all light intensities, mature plants displayed higher efficiency of photosystem II and improved ability to cope with excess excitation energy compared to juvenile stage plants, showing a marked increase in the values of Y(NPQ) and decrease in the values of Y(NO) (Figure 5.21 and Appendix Table B. 5). These values differed significantly from those recorded for juvenile plants (Table 5.29).

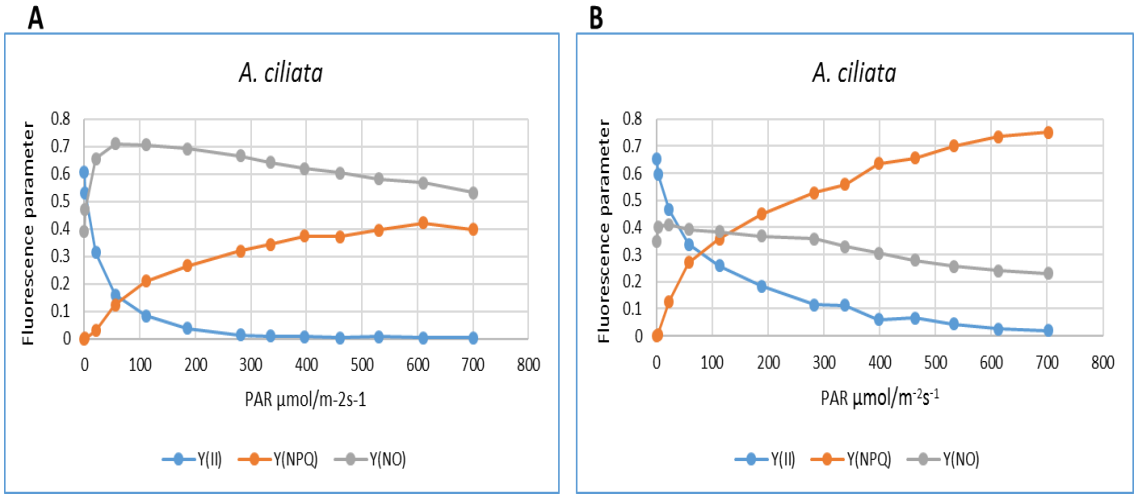

Figure 5.21: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) of *Arenaria ciliata* (Ireland), plants grown in autumn conditions of Arctic habitat. **A.** *A. ciliata* Juvenile plants **B.** *A. ciliata* mature plants.

Table 5.29: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *Arenaria ciliata* plants, juvenile stage and mature plants under Arctic autumn conditions. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. ciliata		Juvenile plants			
	Data points	1	3	7	13
mature plants	Y(II)	ns	p**	p***	ns
	Y(NPQ)		p***	p**	p***
	Y(NO)	ns	p***	p***	p***

Arenaria norvegica (Ireland)

As observed for *A. ciliata* plants, mature plants of *A. norvegica* showed greater photosynthesis performance than juvenile stage plans at low-temperature conditions, with initial photosystem II efficiency (Y(II) = 0.74 and 0.65 respectively) (Figure 5.22).

Juvenile plants showed a weak ability to dissipate excess energy (non-photochemical) in form of heat via the regulated photoprotective NPQ mechanism which results in an increase of the values of Y(NO), that can be lead to photodamage by excess illumination (Figure 5.22 A). Mature plants exhibited significantly higher values of Y(NPQ), compensating the decrease of Y(II) and lowering values of Y(NO) which are indicative of a considerable photoprotective capacity (Figure 5.22, Table 5.30 and Appendix Table B. 5).

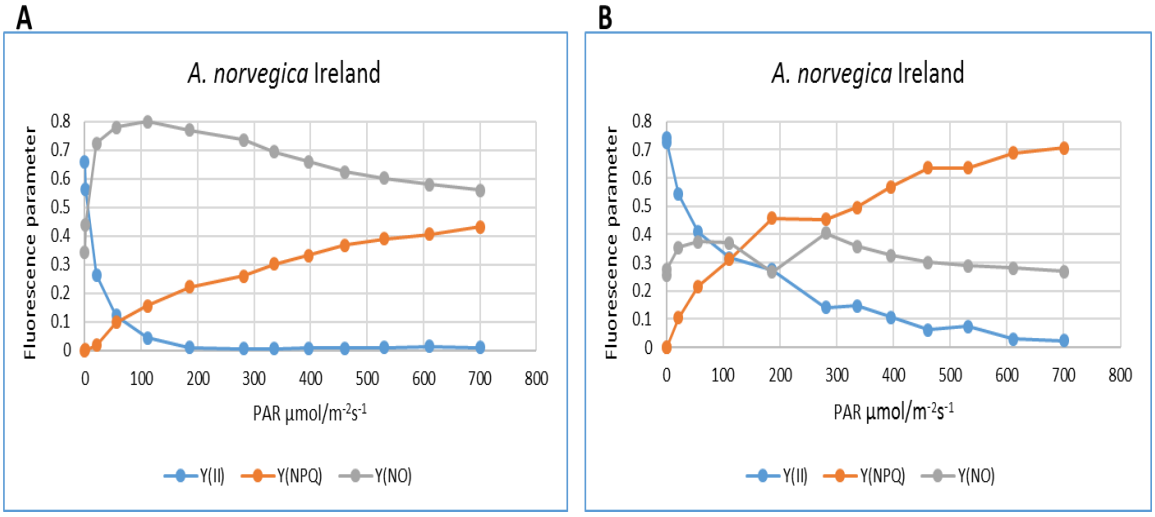

Figure 5.22: Light intensity response curves of complementary quantum yields Y(II), Y(NPQ) and Y(NO) of *A. norvegica* (Ireland), plants grown in autumn conditions of Arctic habitat. **A.** *A. norvegica* juvenile plants **B.** *A. norvegica* mature plants.

Table 5.30: Comparison of key data points of light response curves of Y(II), Y(NPQ) and Y(NO) among *Arenaria norvegica* plants, juvenile stage and mature plants under Arctic autumn conditions. (ns: P > 0.05) (*: P < 0.05) (**: P < 0.01) (***: P < 0.001).

A. norvegica (Ireland)		Juvenile plants			
	Data points	1	3	7	13
mature plants	Y(II)	p*	p***	p***	ns
	Y(NPQ)		p***	p***	p***
	Y(NO)	p*	p***	p***	p***

5.4 Discussion.

5.4.1 Seasonal variation in Photosynthesis efficiency among *A. ciliata* complex ecotypes.

Summer

In the first months of development during the summer season in the TEMP M, TEMP SL and ALP habitats (set to 17 ˚C and 16 h daylight), seedlings of *A. ciliata* (Ireland), *A. norvegica* (Ireland), *A. ciliata* subsp. *bernensis* (Switzerland) and *A. ciliata* subsp. *pseudofrigida* (Svalbard) showed good initial photochemical efficiency of photosystem II Y(II), ranging from 0.73 to 0.77 efficiency (Figure 5.3 A, 5.4 A, 5.6 A and 5.7 A). At maximal quantum flux densities, these ecotypes were able to regulate dissipation of excess energy as heat via the regulated photoprotective NPQ mechanism. However, *A. ciliata* subsp. *bernensis* seedlings presented the highest efficient dissipation of non-photochemical energy, indicated by maximizing the ratio of Y(NPQ)/Y(NO) (Appendix Table B.1).

A. norvegica (Norway) showed significantly lower initial Y(II) values of 0.54, however at the maximal flux densities (PAR) this ecotype displayed elevated photosynthesis performance and active protection against damage that can be caused by excitation energy in PS II by maintaining a high positive ratio of Y(NPQ)/Y(NO) (Appendix Table B.1).

In the cool summer conditions of the ARC habitat (9 ˚C and 14h daylight), *A. ciliata*, *A. norvegica* (Ireland) and *A. ciliata* subsp. *pseudofrigida* displayed similar photosynthetic performance (Figure 5.3 B, 5.4 B and 5.7 B) whereas, *A. ciliata* subsp. *bernensis* presented higher ability to photosynthesise at high rates of PAR (Figure 5.6 B). Notably

these performance rates were slightly higher than their counterparts' performance in the summer conditions of other habitats. On the other hand, *A. norvegica* (Norway) cohorts in ARC summer conditions exhibited a reduction of photosynthetic performance [Y(II)] compared to the same ecotype grown in the other summer experimental habitats.

Seedlings of all ecotypes growing under the summer conditions of ARC habitat showed higher photoprotective capacity compared to the response of the same ecotypes to summer conditions in other habitats. In particular *A. ciliata, A. norvegica* (Ireland) and *A. ciliata* subsp. *pseudofrigida*, which showed a marked increase of Y(NPQ)/Y(NO) from 1.39, 1.04 and 1.05 (in the standard TEMP M, TEMP SL and ALP summer habitat) to 2.12, 1.96 and 2.70 respectively (in the ARC habitat) at high photon flux density (Figure 5.3 B, 5.4 B, 5.7 B and Appendix Table B.1).

These data suggest that ecotypes grown in ARC summer conditions showed greater ability to cope with excess excitation energy in PS II, protecting the photosynthetic apparatus compared to the same ecotypes that were grown in the summer conditions of other habitats. In other words, the lower temperatures of the ARC habitat elicited less stress and/or damage to the photosystem II in this stage of growth and development (Juvenile stage).

Autumn

In the end of the autumn chlorophyll fluorescence analysis in the TEMP M and the TEMP SL habitats, *A. ciliata*, *A. norvegica* (Ireland), *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* cohorts shared a similar initial photochemical efficiency of photosystem II, Y(II) ranging between 0.76 to 0.78. (Table 5.13 and 5.14). *A. ciliata* subsp. *pseudofrigida* cohorts displayed significantly lower initial efficiency of PS II with 0.66

for the TEMP M and 0.71 for the TEMP SL habitats (Table 5.13 and 5.14). At high photon flux densities, lower values of Y(II) were observed for *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* compared to the other three ecotypes in both the two habitats. However, these two ecotypes showed greater photoprotective capacity, displaying a higher ratio of Y(NPQ)/Y(NO) compared to the other ecotypes, which showed similar ratios (Appendix Table B. 2).

In the -5˚C freezing conditions at the end of autumn in the ARC habitat, *A. ciliata* (Ireland), *A. norvegica* (Ireland) and *A. ciliata* subsp. *pseudofrigida* shared a similar reduction in photosynthesis performance [Y(II)] compared to the summer season (Figure 5.8D, 5.9D and 5.12D). This reduced performance was also noticeable when compared to the response of these ecotypes to autumn conditions in other habitats, where photosynthesis levels were elevated compared to the ARC habitat. In these freezing conditions, *A. ciliata* and *A. norvegica* (Ireland) displayed marked increase of Y(NO) mostly at cost of Y(NPQ), likely resulting in system damage as a result of excess illumination (Figure 5.8D, 5.9D and Table 5.15). At the same time, *A. ciliata* subsp. *bernensis* and *A. norvegica* (Norway) showed elevated photosynthesis performance and they displayed a greater ability to dissipate excess excitation energy in PSII, showing a marked increase of Y(NPQ) relative to Y(NO) (Figure 5.10D and 5.11D).

In the low temperature (4 ˚C) at the end of autumn conditions in the ALP habitat, all ecotypes exhibited good ability to photosynthesise with slight reduction in the initial PS II levels compared to their counterparts grown in autumn conditions of the TEMP M and the TEMP SL habitats (Figure 5.8C, 5.9C, 5.10C and 5.12 C), except for *A. ciliata* subsp. *bernensis*, which showed a similar photosynthetic performance to the same ecotype in the TEMP M and TEMP SL habitats (Figure 5.11C). As seen in the other habitats, *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* displayed higher photoprotective

capacity compared to the others followed by *A. ciliata* and *A. ciliata* subsp. *pseudofrigida*, whereas much lower efficient dissipation of non-photochemical energy was observed in *A. norvegica* (Ireland), showing high values of Y(NO), (Figure 5.9C).

Winter

In the end of winter chlorophyll fluorescence analysis in the TEMP M habitat, *A. ciliata*, *A. norvegica* (Ireland) and *A. norvegica* (Norway) presented a good and roughly the same level of photosynthesis (Figure 5.13A, 5.14A and 5.15A), whereas *A. ciliata* subsp. *bernensis* and *A. ciliata* subsp. *pseudofrigida* showed lower photosynthetic performance (Figure 5.16A, 5.17A and Table 5.22). In this habitat, all ecotypes exhibited high photoprotective capacity against any photodamage.

In the TEMP SL habitat winter conditions, all ecotypes exhibited a stable and similar trend of photosynthetic performance, with the exception of *A. ciliata* subsp. *pseudofrigida*, which showed much lower initial photochemical efficiency of PS II (Figure 5.13B, 5.14B, 5.15B, 5.16B, 5.17B and Table 5.23). This rate of performance was slightly lower when compared with the photosynthesis levels of the same plants in the autumn conditions. All analysed ecotypes showed enhanced ability to cope with excess excitation energy compared to in the TEMP M habitat, however *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* displayed greater photoprotective capacity compared to the others.

In the -5˚C freezing conditions at the end of winter in the ARC habitat, all ecotypes displayed very low photosynthetic efficiency Y(II), (Figure 5.13D, 5.14D, 5.15D, 5.16D and 5.17D). Despite this trend, both *A. ciliata* (Ireland) and *A. norvegica* (Ireland) showed a greater capacity to cope with excess excitation energy at this time than at the onset of freezing conditions at the end of the autumn growing season (Figure 5.13D and 5.14D).

At maximal PAR, *A. ciliata* (Ireland) and *A. norvegica* (Ireland) displayed an increased ratio of Y(NPQ)/(NO) from 0.74 and 0.76 at the end of autumn to 1.6 and 1.32 at the end of winter respectively (Appendix Table B. 2 and B. 3). This divergent rate of change may indicate a slower adaptation to freezing conditions compared to the ecotypes *A. norvegica* Norway and *A. ciliata* subsp. *bernensis* Switzerland, which both routinely experience freezing winter conditions in their native habitat as part of the annual seasonal cycle.

In terms of Y(II) photosynthesis rates in the ARC habitat in winter, *A. ciliata* subsp. *pseudofrigida* maintained clear photosynthetic activity up to 400 PAR, a considerably higher threshold than observed for all other ecotypes. This is notable, as the idealised ARC habitat is the home habitat of *A. ciliata* subsp. *pseudofrigida*, and it is the only ecotype to experience normally freezing conditions for extended periods of the year.

In the -3˚C freezing conditions at the end of winter in the ALP habitat, reduced photosynthetic performance was observed for all analysed ecotypes compared to the same ecotypes in the TEMP M and the TEMP SL habitats (Figure 5.13C, 5.14C, 5.15C,5.16C and 5.17C). This pattern of performance reduction was similar to the same plant responses to freezing conditions in the ARC habitat. Despite this photosynthesis reduction, all ecotypes were able to cope with excess excitation energy, showing active photoprotective capacity at a higher rate than their counterparts in the freezing conditions of ARC habitat. As seen in the all habitats in the autumn and the winter seasons, *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* plants were more efficient in dissipating excess energy in photosystem II compared to the others in this habitat. Similarly, *A. ciliata* subsp. *pseudofrigida* again showed an enhanced PAR threshold for Y(II) compared to the other ecotypes in the ALP habitat.

5.4.2 Photosynthesis responses to heat stress in *A. ciliata* complex ecotypes.

In mature plants exposed to warm growth conditions of 20˚C and 30˚C with 16 hours light, all ecotypes showed elevated photosynthesis performance and active protection against damage that can be caused by Y(NO) by maintaining this fraction of energy at low values (Figure 5.18 & 5.119). Compared to the other ecotypes, *A. ciliata* subsp. *bernensis* and *A. norvegica* (Norway) displayed higher ability to manage lost excitation energy in PS II, showing a noticeable increase in the fraction of energy that dissipated as heat via the regulated photoprotective mechanism Y(NPQ) in place of Y(NO), in both 20 and 30 degree growing conditions (Figure 5.18 and 5.19).

5.4.3 Impact of snow cover on photosynthesis responses to cold (freezing) stress in the *A. ciliata* (Ireland) ecotype.

In terms of the thermal insulation effect of covering plants with snow in freezing habitats, mature snow-covered *A. ciliata* plants displayed greater ability to cope with excess excitation light compared to the same plants left exposed to freezing air (Figure 5.20 A, B and Table 5.28). At the highest quantum flux densities, an increase of Y(NPQ)/(NO) to 2.66 was observed in snow-covered plants, compared with *A. ciliata* plants that exposed to cold air, which displayed Y(NPQ)/Y(NO) = 1.67 (Appendix Table B. 4).

5.4.4 Impact of plant age on cold stress tolerance in *A. ciliata* (Ireland) and *A. norvegica* (Ireland) ecotypes

In terms of the impact of plant age on cold tolerance in autumn freezing conditions (Arctic treatment habitat -5 ˚C, (Figure 5.21 A, B and 5.22 A, B) mature *A. ciliata* and *A.*

norvegica plants (both from Ireland) presented higher an initial efficiency of photosystem II (Y(II) = 0.65 and 0.74 respectively) and more efficient dissipation of energy as heat via the photoprotective NPQ mechanism (Y(NPQ) = 0.75 and 0.70 ,at maximal light intensity, respectively), compared to juvenile stage plants [Y(II) = 0.61 and Y(NPQ) = 0.41 for *A. ciliata* and Y(II) = 0.65 and Y(NPQ) = 0.43 for *A. norvegica*] (Appendix Table B. 5). The value of Y(NO) for juvenile plants was also higher (Y(NO) = 0.55 and 0.56 respectively) (Appendix Table B.5) reflecting passively dissipated energy that can lead to secondary damage by photoinhibition (Figure 5.21 B and 5.22 B).

5.5 Conclusions

The results presented in this chapter show that the photosynthetic performance and photoprotective capacity of *Arenaria ciliata* complex ecotypes are both different and variable, changing during different growth and developmental stages.

Over the course of the seasonal growth cycle, optimal photosynthetic performance (highest Y(II) & Y(NPQ), and lowest Y(NO)) is seen for all ecotypes in the Temperate Sea Level (TEMP SL) habitat, with weakest performance (lowest Y(II) and Y (NPQ), highest Y(NO)) recorded for the Arctic (ARC) habitat. Optimal photosynthesis rates among the seedling cohort were observed in habitats maintaining air temperatures of 10-17^{o}C. However, summer photosynthesis rates recorded for the seedling cohorts in the TEMP SL, TEMP M, and ALP habitats were lower than those independently recorded for more mature plants growing at 20˚C and 30˚C, indicating that the age of the seedlings used in these observations is a background factor in the recorded photosynthesis performance rates.

Plant age is known to be an important factor influencing the fate of excited energy within the photosynthetic apparatus (Bielczynski *et al*., 2017). All *A. ciliata* ecotypes showed that as individual plants get older their tolerance to both heat and freezing stress increases, leading to an increase of photosynthetic performance. A similar pattern of high light tolerance was observed in a study by Carvalho *et al*., 2015 conducted on *Arabidopsis* plants. The study demonstrated that high light intensity tolerance increased as plants gets older; *Arabidopsis* in the productive phase (8-week old plants) showed considerably higher photoprotective capacity [higher Y(NPQ)] than those plants in the juvenile phase (2 weeks old plants).

All analysed *A. ciliata* ecotypes showed good ability to photosynthesising at low temperature (4 ˚C) and even in the freezing conditions (-5 ˚C) they were able to photosynthesise with very low rates Y(II), however at this low rate of photosynthetic performance, plants showed a good photoprotective capacity to protect themselves against damage by excess illumination. The ability to perform photosynthesis in cold growing conditions may be crucial to the ability of cold-tolerant herbaceous species to dominate in cold habitats, as many of these species are able to continue to grow in above-freezing cold temperatures that cause tissue death and mortality in other ecophysiological categories of plants (Adams *et al*., 2001; Adams Iii *et al*., 2002; Good, 2007).

Snow cover affects photosynthetic photoprotective capacity and photosynthetic performance, and *A. ciliata* spp. *ciliata* plants from Ireland that were covered with snow in ARC habitat conditions exhibited a great ability to cope with excess light energy, dissipating it as heat via the regulated NPQ mechanism, showing significantly higher photoprotective capacity than those plants exposed to cold air in the ARC winter conditions. At the same time, snow-covered plants showed an initial increase in the Y(II) rate compared to the cold air exposed plants. Snow cover is an important component of

freezing avoidance in cold-habitat plant species, which can be seen at a large scale by the topographical distribution of certain species in cold-stress habitats in snow-cover, but not bare, areas, reflecting adaptation to ambient biotic and abiotic stresses (Charrier *et al*., 2015). Sensitive organs and meristems that covered with snow get more protection from cold temperatures, as they have less exposure to freezing temperatures compared to meristems above the snow cover (Charrier *et al*., 2015). In the freezing events (ca. -5˚C), fine root meristems are the most sensitive plant tissue that can be damaged under naked soil. Snow cover protects the roots from freezing damage by keeping the temperature above critical freezing thresholds that damage these meristems (Cleavitt *et al*., 2008; Comerford *et al*., 2013).

Under long-term heat stress (7 days at 30 ˚C), all investigated ecotypes of *A. ciliata* complex exhibited a great heat tolerance, a result that is consistent with Buchner and Neuner's (2003) findings. They demonstrated that heat tolerance of Alpine plants such as *Silene acaulis, Minuartia recurva* and *Saxifraga paniculata* increased under warmer sites conditions and in warmer years. All the analysed *Arenaria ciliata* ecotypes in the present study showed a stable photosynthetic performance and active photopeotective capacity at elevated temperatures, and all investigated plants were able to photosynthesis under high quantum flux densities, showing an initial increase in photochemical efficiency of PS II compared to the same ecotypes under 20 ˚C. Taken together, these data suggest that *Arenaria ciliata* complex ecotypes have valuable stress tolerance capacities at both ends of the temperature spectrum.

Notably, in all chlorophyll fluorescence measurements (heat, cold and freezing stress conditions), it was evident that the *A. norvegica* (Norway) and *A. ciliata* subsp. *bernensis* (Switzerland) ecotypes have higher photoprotective capacity compared with other analysed ecotypes.

Chapter 6

6.1 General discussion

This project set out to (i) characterise the ploidy diversity of the *A. ciliata* complex in Europe; (ii) describe the ecophysiological characteristics of *Arenaria ciliata* and *Arenaria norvegica*, and (iii) determine the impact of geographical climate variation on ecophysiological performance of 5 ecotypes from the *A. ciliata* complex (3 ecotypes of *A. ciliata*: *A. ciliata* (Ireland), *A. ciliata* subsp. *bernensis* (Switzerland) and *A. ciliata* subsp. *pseudofrigida* (Svalbard); and 2 ecotypes of *A. norvegica*: *A. norvegica* (Norway) and *A. norvegica* (Ireland).

Ecophysiological analyses were completed using a replicate 4-habitat treatment, comprising idealised annual climate scenarios for temperate sea level (TEMP SL, home to *A. norvegica* Ireland), temperate montane (TEMP M, home to *A. ciliata* Ireland), Alpine (ALP, home to A. *ciliata* subsp. *bernensis*) and arctic (ARC, home to *A. ciliata* subsp. *pseudofrigida* and *A. norvegica* Norway) habitats.

The utility of the project data has some limitations. In the original experimental design, it was considered to use all 5 ecotypes in the biometric analysis, however seed sterilisation using chlorine gas caused high mortality in the seed stock, and surplus seeds for full biometric analysis were only available for *A. ciliata* and *A. norvegica* – this batch of seeds was not sterilised. Among the remaining ecotypes, the number of available seeds was insufficient for full biometric analysis, and these were germinated only for the fluorescence analysis. So it was not possible to pursue a wider sampling group. In terms of habitat design, the growth chambers had limited flexibility in terms of light and temperature, and the arctic habitat presented problems. Although daylight hours extend to 24 hours through high summer in arctic habitats, in reality cloud cover is often extensive, meaning that photosynthetically usable light is limited, especially during dusk.

To facilitate this situation using the limited growth facilities available, it was decided to shorten the day length rather than attempt to build a light-filtering mechanism.

Notwithstanding these limitations, the project work presented here provides many new insights on the ecophysiology and recent evolutionary adaptation of ecotypes from the *A. ciliata* complex in Europe.

Ploidy Analysis

The DNA sequencing work presented in Chapter 2 confirmed the genetic identity and affiliation of the ecotypes included in the study, and validated that the *A. norvegica* and *A. ciliata*- *A. ciliata* subsp. *pseudofrigida*- *A. ciliata* subsp. *bernensis* ecotypes used in this study belong to known *A. norvegica* and *A. ciliata* identities, respectively.

In Chapter 3, the novel ploidy analysis protocol successfully revealed the extent of ploidy variation across the genetic lineages that comprise the *A. ciliata* complex in Europe, establishing that many independent polyploidisation events have occurred within the group, and that highest ploidy levels and ploidy diversity are associated with high elevation populations. Among *A. ciliata*- affiliated ecotypes, observed ploidy varies between 2n=40 and 2n =200, whereas for *A. norvegica* the standard observed ploidy level is 2n = 80.

Polyploidization is an important mechanism in the generation of new evolutionary lineages in areas that have been glaciated during the Pleistocene period (Stebbins, 1985; Popp *et al*., 2005). There are multiple sources of molecular evidence that reveal the rate of polyploidizations throughout the last glaciation period was high, compared to the low level of polyploids formation after the Quaternary (Brochmann *et al*., 2004). These results support the hypothesis that polyploid plants have additional stress-tolerance abilities in

strongly fluctuating environments and thus; they have been more successful than diploid plants in colonizing new areas during the postglacial period (Brochmann *et al*., 2004; te Beest *et al*., 2011; García-Fernández *et al*., 2012). The data presented here regarding ploidy variation among geographically-dispersed *A. ciliata* species and ecotypes, whose distributions are strongly influenced by ice age cycles, indicates that many separate polyploidisation events occurred through the period of rapid climate change during the late Pleistocene and Holocene. This phenomenon has played an important role in the recent evolution of *A. ciliata* complex.

Biometric Analysis

In Chapter 4, clear differences in growth patterns and phenology were identified between *A. ciliata* (2n=40, from Ireland) and *A. norvegica* (2n=80, from Ireland) when measured across the 4 idealised biogeographic climate scenarios, with the latter displaying more rapid growth, higher chlorophyll concentration, greater biomass accumulation and earlier flowering than the former. Across habitats, the temperate TEMP SL habitat (typical of *A. norvegica* in Ireland) facilitated highest levels of growth, chlorophyll accumulation and flowering, while the ARC habitat encouraged the least.

Temperature is the most important factor influencing plant growth and development stages. The rate of growth increases as temperature rise to the optimum range for the species, and slows or cess when temperature is outside the optimum range (Schlenker and Roberts, 2009). Arctic and alpine plants experience more extreme changes in temperature compared to other temperate habitats, especially in transition periods between seasons (Good and Millward; 2007). In this study, analysed species of *A. ciliata* and *A. norvegica* from Ireland showed much slower growth and development in the ARC

followed by ALP habitats compared to TEMP M and TEMP SL habitats. Our results clearly illustrate both ecotypes are very well adapted to the growth conditions of Temperate sea level habitats and Temperate mountain habitats, while they showed lower adaptation to the environmental conditions of the Arctic and Alpine habitats. Importantly, while observed growth rates were diminished in these latter two habitats, both ecotypes retained the ability to grow through harsh freezing conditions.

Plant phenology is one of the most easily observable and sensitive natural indicators of plant responses to temperature change (Barlow *et al*., 2015 Shen *et al.*, 2015). During the observation period, neither studied species flowered in the ARC habitat, and *A. ciliata* also failed to flower in the ALP habitat, while both two species were able to flower in the TEMP M and the TEMP SL habitats at different times.

This set of results showed that across habitats at all tested parameters, *A. norvegica* plants exhibited greater performance in terms of development and vegetative growth compared to *A. ciliata* plants. This could be attributed tetraploid genotypes have bigger vacuoles that have a significant role in regulating cells osmotic pressure, causing higher water and nutrients uptake that could increase cell metabolic activity (Guertin and Sabatini, 2006; Beyaz *et al*., 2013) . These data confirm a shift to a more annual flowering cycle in *A. norvegica* compared to *A. ciliata*, a change which is associated with more rapid growth, biomass accumulation, branching rate, onset of flowering, and total flower number. *A. norvegica* chloroplast identity is associated with the *A. ciliata* identities currently found in Northern Spain (Dang, 2012; Howard-Williams, 2013), however the late-Pleistocene colonisation of Northwestern Europe by *A. norvegica* is accompanied by significant deviation from *A.ciliata*, including a change in ploidy, a pronounced shift from a perennial to an annual flowering cycle, and more responsive growth in Temperate Sea Level and Mountain habitats, compared to *A. ciliata*.

Photosynthesis Analysis

In Chapter 5, ecophysiological variation among five *A. ciliata* ecotypes was assessed using fluorescence analysis of photosynthesis performance in both end-of-season observations in the 4 idealised climate scenarios and in targeted heat stress test observations. A number of adaptive trends in ecotype ecophysiology were observed.

In the abiotic stress conditions, when the photon flux density exceeds the photosynthetic capacity (Y(II)), excitation energy can cause severe damage to the photosynthetic apparatus (PSII) (Bielczynski *et al*., 2017). In order to avoid photodamage, plants activate photoprotective mechanisms within seconds or minutes, which is known as non-photochemical quenching energy (NPQ). Damaged reaction centre components (PSII) can be later replaced by PSII repair cycle (Li *et al*., 2000; Aro *et al*., 2004; Bielczynski *et al*., 2017) . Under abiotic stress conditions, high levels of Y(NPQ) indicate a high photoprotective capacity, whereas high values of Y(NO) are indicative of decreased plants ability to protect themselves against damage by excess excitation energy. In those stress conditions, photosynthetic apparatus response is aimed to generate maximal values of Y(II), with a maximal ratio of Y(NPQ)/Y(NO) to protect PS II against photodamage

(Klughammer and Schreiber, 2008; Bielczynski *et al*., 2017).

Among all *A. ciliata* ecotypes, the baseline capacity for coping with excess light energy, measured in terms of Y(NPQ), the quotient of managed non-photosynthetic energy, was very high compared with known capacities in other angiosperms (Bielczynki *et al*., 2017). Both *A. ciliata* subsp. *bernensis* (2n=200, from the Bernese Alps in Switzerland) and *A. norvegica* (2n=80, from Finnmark in Arctic Norway) showed enhanced capacity to deal with both cold and heat stress compared to the other ecotypes.

Among juvenile seedlings, the ideal growth temperature, generating the maximum Y(II) and Y(NPQ) values, was c. 9 °C, while all individual mature plants of all ecotypes showed good capacity to cope with excess light energy (Y(NPQ)) at both 20 and 30°C. In addition, when *A. ciliata* from Ireland was grown under freezing conditions, snow cover was seen to provide significant protection compared to air-exposed leaves, facilitating effective management of excess light energy (Y(NPQ)), even in temperatures as low as -4 °C.

Overall, ecotypes from habitats experiencing frequent freezing events showed elevated capacity to deal with this stress (*A. ciliata* subsp. *bernensis* from ALP, and *A. ciliata* subsp. *pseudofrigida* and *A. norvegica* from ARC). Ecotypes from more benign temperate habitats (*A. ciliata* and *A. norvegica* from Ireland) showed reduced capacity to cope with freezing stress. These patterns are more closely correlated with home habitat climate for each ecotype, than with their chloroplast haplotype lineage, pointing to the importance of local selection pressure on nuclear genomic traits in influencing ecotype physiology.

Chlorophyll and carotenoid pigments are responsible for light absorption for photosynthesis, at the same time they play an important role in protecting plants from excessive radiation (Devi *et al*., 2015). Seasonal variation (temperature and daylight) leads to changes in the concentration, composition and abundance of these photosynthetic pigments which can cause disruption in photosynthesis performance.

In Chapter 4, it was shown that *A. ciliata and A. norvegica* plants germinated in the ARC and ALP habitats displayed lower values of chlorophyll content at the end of autumn and winter seasons in these habitats compared to other habitats. In Chapter 5, the data showed that as temperatures dropped to below zero in Autumn, both of these ecotypes failed to manage excess energy successfully, showing high values of Y(NO) in place of Y(NPQ),

which can be interpreted either as a weak response to the onset of cold, or more fundamental cold damage of the NPQ generation mechanism. This kind of weak photosynthesis performance is associated with plant growth and development reduction, causing an overall reduced level of whole-plant productivity (Foyer *et al*., 2017).

All analysed *A. ciliata* complex mature plants exhibited a great photoprotective capacity, showing high values of Y(NPQ) under a variety of stress conditions; heat, cold and freezing.

In terms of the impact of plant age on cold and heat tolerance in extreme temperature events, it is known from the literature that plant age influencing the fate of absorbed energy within photosynthetic apparatus. Thus, both photoprotective capacity and photosynthetic performance will be effected by the age of a given plant (Bielczynski *et al*., 2017). A previous study conducted on *Arabidopsis* plants showed high radiation intensity tolerance increased as plant age gets older (Carvalho *et al*., 2015). All investigated *A. ciliata* complex ecotypes (12-months old plants) exhibited higher capacity to deal with excess energy when exposed to warm temperature compared to the same ecotypes in the juvenile phase (3-months old plants).

Conclusions

The experimental analysis presented in this study have improved our understanding of the basis for, and characteristics of, diversification across the *A. ciliata* species complex in Europe. A number of conclusions can be made:

(i) Polyploidisation has occurred on multiple separate occasions in isolated populations, but only in Alpine habitats.

(ii) Low ploidy number is associated with north-western colonisations near sea level by *A. ciliata* complex species.

(iii) *A. norvegica* shows clear divergence from *A. ciliata* in terms of growth and phenology, displaying an annual growth habit with accelerated growth milestones and flowering.

(iv) Despite occupying population sites with relatively moderate climate conditions, the local Irish ecotypes of both *A. ciliata* and *A. norvegica* retain the ability to grow and survive through freezing habitat conditions, with functional capacity to manage excess light in below zero temperatures via the Y(NPQ) pathway.

(v) Despite these latent abilities, these two ecotypes display a reduced capacity to cope with the first onset of freezing at the end of autumn, compared to ecotypes from habitats where seasonal freezing is consistent and/ or prolonged.

(vi) Cold stress tolerance capacity is greatest in the ecotypes of *A. ciliata* subsp. *bernensis* and *A. norvegica* (Norway), as measured in terms of PSII capacity to cope with excess energy [Y(NPQ)].

(vii) Among all European ecotypes, *A. ciliata* subsp. *pseudofrigida* from Svalbard demonstrates the strongest capacity to utilise light energy for photosynthesis in freezing conditions, as measured in terms of Y(II).

(viii) Across the entire dataset, cold stress tolerance capacity in each ecotype is more closely associated with the climate of the home population site than with evolutionary lineage.

(ix) The association between stress tolerance capacity and ploidy is unclear. It is positively associated with ploidy level in *A. ciliata*, *A. ciliata* subsp. *pseudofrigida* and *A. ciliata* subsp. *bernensis*, however in *A. norvegica* (Ireland and Norway), it is not.

(x) There is a clear improvement in cold and heat stress tolerance between juvenile and adult plants of *A. ciliata* and *A. norvegica* from Ireland

(xi) Snow cover provides protection to the photosystem in *A. ciliata* plants growing in sub-zero conditions

(xii) Adult plants of all tested ecotypes displayed great ability to deal with excess light growing at ambient temperatures of both 20℃ and 30℃.

Clinal Variation

Low and freezing temperatures are a form of abiotic stress that changes predictably with latitude and altitude and to which induce organisms to evolve multiple physiological response (Zhen and Ungerer, 2008), in a similar way to the responses observed for changing light regimes (Stinchcombe *et al*., 2004).

Latitude-based clinal variation in drought and freezing tolerance is a feature of many organism origins with widespread distributions (Zhen and Ungerer, 2008; Baruah *et al*., 2009; Zuther *et al*., 2012; Kooyers *et al*., 2015) . For example, considerable variation in freezing tolerance is observed in *A. thaliana* natural accessions with a positive correlation between latitude of plant origin and freezing tolerance (Zhen and Ungerer, 2008). Baruah *et al* (2009) report a similar pattern among wild rice accessions.

In the present study, the ecotypes with an origin from high latitude and high altitude tended to be more freezing tolerant than those from lower latitude and elevation sites.

The *A. norvegica* ecotype from Norway displayed higher ability to cope with freezing stress than *A. norvegica* plants from Ireland. Importantly, both ecotypes share the same genetic identity RTN01 in clade II of the overall phylogeny, and both also share the same ploidy level (see chapter 2 and 3). This finding suggests recent adaptive changes in the two populations related to local climate conditions.

The data also showed a positive relationship between freezing tolerance and elevation of origin of ecotypes; *A. ciliata* subsp. *bernensis* (Switzerland- 2100 m asl) exhibited greater capacity for cold stress tolerance, compared to *A. ciliata* from Ireland (475 m asl), which belongs to the same haplotype lineage (Clade I). This pattern of behaviour can be associated with ploidy level (Stebbins, 1985; Spooner *et al*., 2010; te Beest *et al*., 2011).

Across all treatments, Y(NPQ) was maximized and Y(NO) was minimized for both *A. ciliata* subsp. *bernensis* (2100m asl, 46ºN) and *A. norvegica* (Norway, 15m asl, 69ºN), compared to the other ecotypes, suggesting that in habitats with pronounced seasonal temperature extremes from freezing winters into summer heat (such as at high elevation or on the southerly margins of the arctic), stress tolerance has been positively selected for.

The overall pattern in clinal variation in *Arenaria ciliata* complex ecotypes suggests local adaptation processes have played a greater role in variance in cold stress tolerance than evolutionary divergence between lineages.

Climate Change Considerations

Field observations and models predict that cold-tolerant plants in arctic and alpine regions will face extinction risk from changes in temperature (both the seasonal cycle of temperature change and temperature extremes) and the diversity and abundance of

companion species (including mutualists, pathogens, competitors and facilitators) arising from climate change (Thuiller *et al*., 2005; Elmendorf *et al*., 2012; Pauli *et al*, 2012).

Abbott (2008) suggested that understanding the potential impact of climate change on arctic-alpine plants should focus on studying adaptive rather than neutral genetic variation, as this will help to predict how arctic and alpine plant respond to climate warming in the near future.

At the same time, Crawford (2008) argues that many plants in the arctic and alpine regions are characterized by high levels of ecotypic diversity, occupying a wide range of different environments in terms of day length, warmth and soil moisture content, and that this diversity should protect them from extinction as global temperatures get warmer. In relation to both of these perspectives, the data presented in this project identifies that the *A. ciliata* complex includes a diversity of ecotypes with different cold-stress sensitivities and growth/ flowering phenologies that are associated with prevailing conditions in their home habitat environment (most often), and their recent phylogenetic and ploidy history (less often).

A. ciliata in Ireland, and *A. norvegica* in Ireland (especially) show ecophysiological changes associated with milder climate conditions compared to ecotypes from colder habitats, including reduced capacity for coping with temperature stress through the NPQ system, in both cold and hot climate conditions. In addition, *A. norvegica* from Ireland displays a clear life history change towards growth in milder conditions, with significantly more rapid growth and biomass accumulation, and a shorter flowering cycle, compared to *A. ciliata*.

The freezing stress tolerance mechanism within *A. ciliata* species is enhanced by snow cover, whereas exposure to freezing air without snow cover causes significant tissue

damage. Because of this, warmer climate conditions that increase minimum cold temperatures in winter and/ or reduced snow precipitation have the capacity to damage plant fitness, whereas warmer summer conditions in maritime, alpine and arctic habitats will not likely reduce fitness in the same way. Taken all together, these results indicate that under warmer climate conditions there will be different likelihoods for survival for *A. ciliata* ecotypes. While ecotypes from milder habitats already show adaptive capacities for milder climate conditions, ecotypes from the Alps and Norway show enhanced capacity to cope with heat stress as well as cold stress. Recent analysis of climate change-associated vegetation change in the Italian Alps indicates that this ability to cope with higher summer temperatures is a feature of many high elevation alpine plant species (Cannone *et al*., 2007).

In this case, the most likely extinction danger will not come from changes to temperature, but from changes in competitor species, especially the migration into alpine and arctic habitats of species from more temperate habitats with more vigorous growth strategies, which will not be restricted by harsh drops in temperature.

Future conditions in Ireland

On Ben Bulben, where cool rather than freezing winters are the norm, and specific habitat conditions such as northerly aspect, high rainfall and poor soil formation are the basis for *A. ciliata* abundance, we have no local climate model to predict how climate change will impact on Alpine plant communities in this habitat. It is possible, given the long survival of this habitat in the local maritime temperate climate since the end of the Pleistocene era, that the isolated alpine plant communities on Ben Bulben will persist through climate

warming pressures. But this may depend on land management decisions at the site, especially in terms of grazing sheep.

On the Burren, microhabitat conditions at the *A. norvegica* population site are known to be changing in the present day, linked to reduced grazing pressure and increased soil formation on the karst landscape (NPWS, 2008). Despite the clear ability of *A. norvegica* to cope with warmer conditions, including several major adaptive changes compared to more cold adapted ecotypes, survival of the population may rely on the stability of the habitat, which depends on farming patterns that are often independent of climate change.

Future Research Work

The results presented here demonstrate that a wide variation in ploidy and ecophysiology exists among ecotypes of the *A. ciliata* complex. In terms of both ploidy and ecophysiology analysis, wider sampling of ecotypes would improve resolution of these results. To build on the achievements of this project, it is likely many new insights could be gained from investigation of the molecular basis of stress tolerance. In particular this could include transcriptomic and proteomic analysis of plant responses to stress. For this project, evaluation of secondary metabolite profiles was completed, however there was not enough time to analyse and interpret this data. This work will be completed in the coming year after the project is submitted. In addition, treatment of plants with multiple/ combined stress pressures, for example a combination of heat and water deficit stresses, will further improve understanding of the basis of stress responses under climate change.

Chapter 7

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Appendix A

Appendix B

Appendix A

Table A.1: Taxon identity and individual sample ploidy levels for the analysed species of the *Arenaria ciliata* group.

Taxon identity	Population code	Individual sample code	Country	Ploidy level
A. ciliata spp. *ciliata*	Ac1	Ac BB1.1	Ireland	40
		Ac BB1.4	Ireland	40
	Ac2	Ac BB2.1	Ireland	40
		Ac BB2.2	Ireland	40
		Ac BB2.4	Ireland	40
		Ac BB2.5	Ireland	40
	Ac3	Ac BB1.5	Ireland	40
		Ac BB1.8	Ireland	40
		Ac BB1.7	Ireland	40
		Ac BB2.3	Ireland	40
		Ac BB 2.2	Ireland	40
		Ac BB 2.4	Ireland	40
		Ac BB 1.9	Ireland	40
	Ac6	Ac 6.4	Italy	40
		Ac 6.5	Italy	40
		Ac 6.8	Italy	40
		Ac 6.9	Italy	80
	Ac8	Ac8.2	Italy	40
	Ac16	Ac16.27	Austria	200
		Ac16.20	Austria	200
		Ac16.17	Austria	160
	Ac17	Ac17.24	Austria	120
		Ac17.8	Austria	160
		Ac17.15	Austria	160
	Ac19	Ac19.1	Switzerland	40
		Ac19.8	Switzerland	40
	Ac20	Ac20.1	Austria	200

Table A.2: Taxon identities and individual sample ploidy levels for the analysed species of the *Arenaria ciliata* group

Taxon identity	Population code	Individual sample code	Country	Ploidy level
A. ciliata spp. *bernensis*	Ab1	Ab1	Switzerland	200
	Ab2	Ab2	Switzerland	200
		Ab2.1	Switzerland	200
A. ciliata spp. *pseudofrigida*	Ap1	Ap1	Norway	40
		Ap1.1	Norway	40
	Ap3	Ap3	Norway	40
A. gothica spp. *Meohringioides*	Ac10	Ac10.3	Spain	40
		Ac 10.14	Spain	40
		Ac10.18	Spain	80
	Ac13	Ac13.7	Spain	40
		Ac13.16	Spain	40
	Ac14	Ac14.5	Spain	40
		Ac14.15	Spain	40
	Ac15	Ac15.1	Spain	80
		Ac15.2	Spain	80
		Ac15.5	Spain	40
		Ac15.7	Spain	40
A. norvegica	An1	An1.1	Ireland	80
		An3	Ireland	80
		An3.7	Ireland	80
	An9	An9	Norway	80
		An9.2	Norway	80
		An9.4	Norway	80
		An9.5	Norway	80
	An5	An5	Scotland	80
	An8	An8	Iceland	60

Appendix B

Table B.1: Initial value of Y(II) point 1 and the partitioning of excitation energy in PS II at the point 13, highest light intensity, averaged for five juvenile plants of each *Arenaria ciliata* ecotype. At the end of summer season of 4 different habitat conditions (see Chapter 4 for habitat growth conditions).

		Y(II)		Y(NPQ)	Y(NO)	Y(NPQ)/Y(NO)
	Data point	1	13	13	13	13
TEMP M, TEMP SL & ALP						
	Ac	0.76	0.087	0.53	0.38	1.39
	An	0.73	0.19	0.41	0.39	1.05
	An N	0.55	0.21	0.52	0.26	2
	Ab	0.74	0.18	0.52	0.3	1.73
	Ap	0.76	0.16	0.42	0.4	1.05
ARC						
	Ac	0.77	0.21	0.53	0.25	2.12
	An	0.77	0.19	0.53	0.27	1.96
	An N	0.55	0.13	0.58	0.26	2.23
	Ab	0.73	0.3	0.44	0.25	1.76
	Ap	0.77	0.17	0.6	0.22	2.7

Table B.2: Initial value of Y(II) point 1 and the partitioning of excitation energy in PS II at point 13, highest light intensity, averaged for five plants of each *A. ciliata* ecotypes. At the end of autumn season of 4 different habitat conditions (see Chapter 4 for habitat growth conditions).

		Y(II)		Y(NPQ)	Y(NO)	Y(NPQ)/Y(NO)
	Data point	1	13	13	13	13
TEMP M						
	Ac	0.76	0.11	0.53	0.34	1.55
	An	0.77	0.14	0.54	0.3	1.8
	An N	0.77	0.075	0.68	0.23	2.95
	Ab	0.74	0.074	0.69	0.23	3
	Ap	0.66	0.14	0.54	0.31	1.74
TEMP SL						
	Ac	0.76	0.12	0.48	0.39	1.23
	An	0.78	0.19	0.53	0.28	1.9
	An N	0.76	0.1	0.65	0.23	2.82
	Ab	0.76	0.058	0.69	0.24	2.87
	Ap	0.71	0.14	0.54	0.3	1.8
ALP						
	Ac	0.73	0.089	0.56	0.34	1.64
	An	0.71	0.08	0.49	0.43	1.14
	An N	0.71	0.11	0.66	0.22	3
	Ab	0.77	0.056	0.64	0.29	2.2
	Ap	0.7	0.053	0.55	0.39	1.41
ARC						
	Ac	0.61	0.003	0.39	0.53	0.74
	An	0.65	0.009	0.43	0.56	0.76
	An N	0.74	0.03	0.62	0.34	1.82
	Ab	0.71	0.015	0.72	0.25	2.88
	Ap	0.56	0.008	0.61	0.37	1.64

Table B.3: Initial value of Y(II) point 1 and the partitioning of excitation energy in PS II at the point 13, highest light intensity, averaged for five plants of each *Arenaria ciliata* ecotype. At the end of winter season of 4 different habitat conditions (see Chapter 3 for habitat growth conditions).

		Y(II)		Y(NPQ)	Y(NO)	Y(NPQ)/Y(NO)
	Data point	1	13	13	13	13
TEMP M						
	Ac	0.75	0.095	0.54	0.35	1.54
	An	0.77	0.095	0.6	0.3	2
	An N	0.74	0.11	0.56	0.32	1.75
	Ab	0.69	0.02	0.67	0.29	2.31
	Ap	0.61	0.03	0.63	0.33	1.9
TEMP SL						
	Ac	0.74	0.1	0.51	0.37	1.37
	An	0.76	0.12	0.59	0.28	2.1
	An N	0.76	0.086	0.64	0.27	2.37
	Ab	0.74	0.05	0.64	0.29	2.2
	Ap	0.62	0.098	0.58	0.32	1.81
ALP						
	Ac	0.68	0	0.67	0.32	2.09
	An	0.63	0	0.68	0.31	2.19
	An N	0.68	0	0.72	0.27	2.66
	Ab	0.66	0	0.67	0.32	2.09
	Ap	0.46	0.013	0.6	0.37	1.62
ARC						
	Ac	0.7	0	0.61	0.38	1.6
	An	0.64	0	0.56	0.43	1.32
	An N	0.67	0	0.67	0.32	2.09
	Ab	0.63	0	0.71	0.28	2.53
	Ap	0.49	0.031	0.49	0.48	1.02

Table B.4: Initial value of Y(II) point 1 and the partitioning of excitation energy in PS II at the point 13, highest light intensity, averaged for five of snow-covered plants and five cold air exposed plants of *Arenaria ciliata* (Ireland) in ARC winter conditions.

A. ciliata		**Y(II)**		**Y(NPQ)**	**Y(NO)**	**Y(NPQ)/Y(NO)**
	Data point	1	13	13	13	13
Plants covered with snow		0.71	0	0.72	0.27	2.66
Plants exposed to cold air		0.65	0	0.62	0.37	1.67

Table B.5: Initial value of Y(II) point 1 and the partitioning of excitation energy in PS II at the point 13, highest light intensity, averaged for five Juvenile and five mature plants of *Arenaria ciliata* and *A. norvegica* (both from Ireland) acclimated to ARC autumn conditions.

		Y(II)		**Y(NPQ)**	**Y(NO)**	**Y(NPQ)/Y(NO)**
	Data point	1	13	13	13	13
A. ciliata						
	Juvenile plants	0.61	0.003	0.41	0.55	0.75
	Mature plants	0.65	0.02	0.75	0.23	3.26
A. norvegica						
	Juvenile plants	0.65	0.008	0.43	0.56	0.76
	Mature plants	0.74	0.02	0.7	0.27	2.59

Table B.6: Initial values of Y(II) point 1 and the partitioning of excitation energy in PS II at point 13, the highest light intensity, averaged for five mature plants of each *A. ciliata* ecotype when exposed to 20˚C and 16h daylight ('warm' conditions) for 7 days.

	Y(II)		Y(NPQ)	Y(NO)	Y(NPQ)/Y(NO)
Data point	1	13	13	13	13
Ac	0.75	0.11	0.54	0.034	1.58
An	0.76	0.09	0.59	0.31	1.9
An N	0.75	0.14	0.62	0.22	2.8
Ab	0.74	0.07	0.66	0.26	2.53
Ap	0.67	0.1	0.56	0.32	1.75

Table B.7: Initial value of Y(II) point 1 and the partitioning of excitation energy in PS II at the point 13, highest light intensity, averaged of five mature plants of each *A. ciliata* ecotype when they exposed to 30˚C and 16h daylight (high temperature) for 7 days.

	Y(II)		Y(NPQ)	Y(NO)	Y(NPQ)/Y(NO)
Data point	1	13	13	13	13
Ac	0.79	0.12	0.6	0.27	2.22
An	0.78	0.11	0.57	0.31	1.83
An N	0.77	0.15	0.62	0.22	2.8
Ab	0.79	0.12	0.62	0.25	2.48
Ap	0.73	0.13	0.57	0.28	2.03