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Pollen chemical composition determined by infrared spectroscopy - species identification and environmental effects.

Kjemisk innhold i pollen bestemt av infrarød spektroskopi - artsbestemmelse og miljøeffekter.

Kjersti Misfjord

Preface

This thesis has been a cooperation between three institutes; The Department of Plant Sciences, The Department of Ecology and Natural Resource Management (INA) and The Department of Mathematical Sciences and Technology (IMT). Sampling of pollen was done during summer in 2013, in Jotunheimen and in Follo region. The pollen analysis took place in Nofima AS (Ås) during February. The thesis will be the last credits that will result in my degree in Biology.

I owe great thanks to my supervisors; professor Mikael Ohlson (INA) for introducing me to this thesis and being available and encouraging, and professor Achim Kohler (IMT) for giving quick lectures and being enthusiastic. I also want to thank research fellow Murat Bagcioglu (IMT) for showing me how to collect and analyze pollen, and introducing me to The Unscrambler X. At last I want to thank researcher Boris Zimmermann (IMT) for helping me to understand the results, and for thoroughly feedback. There has always been someone who had time to help me, and for that I am grateful.

In addition I would like to thank senior engineer Elin Ørmen for helping with scanning electron microscope images.

Ås, May 14, 2014

Kjersti Misfjord

Summary

In this study pollen is collected from different populations of common and widespread plants in alpine and lowland habitats. Infrared spectroscopy was used to determine chemical composition of pollen. Although this is a relatively new method used in pollen research, it has a large potential due to simple and rapid analysis that enables economical study of numerous samples. It is found that pollen chemical composition varies between species and between populations of the same species. Different environments and populations contribute to these differences. These separations are mainly due to variations in carbohydrate content, but also proteins, lipids and sporopollenin. Separation between *Ranunculus* and grass species are due to more protein and sporopollenin in *Ranunculus*. Acclimatization seems to be affected by change in carbohydrate content. *Ranunculus* and grasses appeared to respond different at the climatic conditions. The separation between species seemed to be connected to taxonomy, pollination strategy and pollen grain morphology. The variation of carbohydrates in alpine and lowland habitat can be connected to different need for energy reserves, so that pollen tube growth can still occur at low temperatures.

Sammendrag

I dette studiet er det samlet pollen fra plantens naturlige habitat. Dette er arter som har stor utbredelse og ble samlet i ulike populasjoner fra et alpint og et lavland miljø. For å finne kjemiske komponenter som kan kobles til innhold i pollen har det blitt brukt infrarød spektroskopi, som er en relativ ny metode innenfor pollenforskning. Rask og enkel utførelse gjør det mulig å få undersøkt mange pollenprøver. Det ble erfart at denne metoden egnet seg godt til å skille pollen innhold mellom ulike arter, i tillegg til å skille mellom klimatiske miljøer og ulike populasjoner. Karbohydrater var det som i hovedsak var årsaken til oppdelingen, men også lipider, proteiner og sporopollenin hadde innvirkning. *Ranunculus* skilte seg tydelig ut fra gressene ved å ha mer proteiner og sporopollenin. Det kan se ut som at tilpasning til et alpint miljø skyldes endring av karbohydrater. Det viste seg at *Ranunculus* og gressartene reagerte noe ulikt på klimatisk endring. Årsaken til variasjonene mellom arter blir koblet til pollenkornets utseende, pollineringsstrategi og slektskap. Ulike behov for karbohydrater mellom klimatiske miljø blir knyttet til tilpasning av pollenkornet energilager slik at det på en bedre måte kan utføre vekst av pollenslange.

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1 Introduction

Although pollen can be as small as 5 µm it has a major mission in plant lifespan. Pollen is essential for a plant to reproduce with a genetic variation, which in turn opens adaptations and long-term survival in an ever changing environment. During development and maturation a pollen grain store energy reserves, such as carbohydrates, proteins and lipids (Baker & Baker 1979; Pacini 1996; Pacini 2000; Stanley & Linskens 1974). Since a pollen grain does not perform photosynthesis these energy reserves are obtained from the tapetum in the mother plant (Delph et al. 1997; Pacini et al. 1985; Pacini 1996; Pacini 2000; Shivanna 2003; Stanley & Linskens 1974). A mature pollen grain contains a vegetative and a generative cell (Stanley & Linskens 1974), which are surrounded by a complex wall system (Sanger & Jackson 1971a). The inner wall, intine, is cellulose based and similar to plant cell wall (Keijzer 1987) while the outer wall, exine, is built from sporopollenin (Nepi & Franchi 2000; Stanley & Linskens 1974). Some species, often anemophilous, can be covered with an oily, sticky and often colorful material called pollenkitt (Pacini 2000). The type of energy reserves in pollen can vary with and within genus (Baker & Baker 1979). It is not clearly understood why, but research suggest that the pollen reserves are connected with selection of food for bees, the stigma-ovule distance and ecophysiological conditions (Roulston et al. 2000). Moreover it seems like starch can be connected to pollination strategy (Baker & Baker 1982). The energy reserves are metabolized during growth of pollen tube. This process requires a lot of energy in a short time, and must be able to conduct in all kind of environment.

Environmental conditions are shown to affect the chemical composition of pollen (Sears & Metcalf 1926; Van Herpen 1981; Van Herpen 1986; Vesprini et al. 2002; Vesprini & Pacini 2005) and pollen performance (Elgersma et al. 1989; Johannsson et al. 1994; Lau & Stephenson 1994; Stanley & Linskens 1974; Steinacher & Wagner 2012; Stephenson et al. 1994; Van Herpen & Linskens 1981). Pollinating plants occur in a broad range of different environments, some of which are characterized by close to extreme environmental conditions. Alpine region is characterized by a short growth season with large diurnal variation. There is also other climatic factors that are characteristic for high altitude, such as reduced atmospheric pressure, change in partial pressure and increase of areal flux of solar radiation (Körner 2003). Most of the pollen studies on environmental conditions are indoor studies, performed under laboratory conditions on plants or pollen collected in nature, such as study under different temperature (Vesprini et al. 2002). In addition the lowest temperature has been relatively high compared to what can be found in the alpine region. Some of the main effects that temperature has on pollen chemical composition is change in carbohydrate (Vesprini et al. 2002) and protein content (Van Herpen 1981). The progamic phase takes longer time with decreased temperature, but pollen tube in alpine plants still grows near to freezing temperatures. This can imply an adaption to night temperature in alpine populations (Steinacher & Wagner 2012). Since the vegetative cell in the pollen grain contain

the same organelles as a normal plant cell (Nepi & Franchi 2000; Sanger & Jackson 1971b), it is reasonable to assume that the temperature effect on plant cell affects pollen grain as well. The change in cell component is mainly due to change in the membrane lipids to maintain membrane fluidity, so the cell still can perform their physiological tasks (Quinn 1988). For example, one possible acclimatization process is increased storage of unsaturated fatty acids in chilling resistant plants that were exposed to low temperature (Lambers et al. 2008; Taiz & Zeiger 2010).

The study of pollen, the principal research area of palynology, is covering physical, chemical and physiological characterization of pollen grains. In order to achieve that, the contemporary palynological studies are mainly focused on chromatography and optical and electron microscope techniques (Weber 1998). This are also methods used for the other applications of palynology, such as paleoecology, aeropalynology, and criminology (Moore et al. 1991). Using optical microanalysis to determine plant species can be difficult since some genera, especially grasses have pollen grains with a rather similar morphology. Scanning electron microscopy (SEM) images can give a more detailed picture of the surface, but the exine (which is used for characterization in optical microanalysis) can be covered by pollenkitt (Moore et al. 1991). In addition, microscopy is a time consuming technique that usually needs sample pre-treatment. The use of vibrational spectroscopy for pollen analyses has recently started to evolve (Dell'Anna et al. 2009; Mularczyk-Oliwa et al. 2012; Pappas et al. 2003). Raman and Fourier transform infrared (FT-IR) spectroscopy present two vibrational spectroscopic techniques. Both have the ability to provide information about molecular composition and structure in a sample. FT-IR is considered to have more potential due to strong fluorescence from pollen which swamped the Raman signals (Ivleva et al. 2005; Laucks et al. 2000; Zimmermann 2010). The principle behind infrared spectroscopy is that chemical bonds absorb light in the infrared region of the electromagnetic spectrum. The frequencies of absorbed light are characteristic for molecular structure (i.e. for chemical bonds in the molecule), as well as for surrounding environments of the molecule. Thus, different molecular structures give rise to different absorption bands in the infrared spectrum allowing the investigation of chemical structure. Therefore, infrared spectroscopy can give information on molecular composition of a pollen sample such as lipids, protein and carbohydrate components. A major advantage of infrared spectroscopy compared with chemical analysis, is that samples can be investigated in the natural form without employing any pre-treatment procedures. Depending on the type of samples investigated, different sampling techniques are available in infrared spectroscopy such as diffuse reflectance infrared transform spectroscopy (DRIFTS), attenuated total reflectance (ATR), potassium bromide (KBr) pellets and microspectroscopy.

Studies which are done on pollen by using vibrational spectroscopy have mainly been focused on the discrimination between families and genera (Dell'Anna et al. 2009; Gottardini et al. 2007; Ivleva et al.

2005; Laucks et al. 2000; Mularczyk-Oliwa et al. 2012). This have been successful, and studies with a higher diversity of species have proven to be able to discriminate between congenital species, though not for all genera (Pappas et al. 2003; Schulte et al. 2008; Zimmermann 2010). Most of these studies on pollen are related to allergy research and therefore used only anemophilous species. There has been a few studies that have looked closer on specific components in pollen, such as differences of carotenoid content between species (Schulte et al. 2009). Recently it has been recognized that infrared spectroscopy has potential for plant phenology. Zimmermann and Kohler (2014) found that annual variation in populations and pollinating strategy could give variation in pollen content.

In order to further utilize infrared spectroscopy in environmental research the aim for this study was to see if this method could detect environmentally induces variations in chemical composition of pollen grains, in particular to alpine habitat conditions. Since in alpine environment pollen must be able to propagate and germinate at low temperature, it possibly requires different energy reserves than those stored in grains growing in lowland environment. Therefore, for the two habitats (alpine and lowland) it has been anticipated to detect variations in pollen chemical composition associated with pollen reserves, such as lipids and carbohydrates. The additional aim of this study was to detect and characterize variations in chemical composition between species and populations.

2 Material and methods

2.1 Study area

In this study there were two general sampling areas. One has represented an alpine environment and is located in Jotunheimen (61,6251, 8,4036), Oppland County, and the other has represented a lowland environment and is located in Follo (59,7511, 10,7671), Akershus County. Figure 1 shows pictures of the studied areas, while an overview map can be found in Figure 2. More detailed map of the locations can be found in appendix 2 and 3. The map of the locations is made in ArcGIS 10. The background was obtained from a WMS from Statens Kartverk, Topografisk norgeskart2 (2007) (Datum: WGS84, Coordinate system: UTM 32N). The data for precipitation and temperature for the studied areas has been obtained from Norwegian Meteorological Institute.

Figure 1. The two study areas: Jotunheimen to the left, Follo in the middle and to the right.

Jotunheimen is a mountain area which is known for having Norway's highest mountains, a rich plant life and a national park (see Figure 1). The area has an alpine vegetation zone, with a slightly oceanic section (Moen et al. 1999). The bedrock is consisting of charnockite to gabbro, gneiss and amphibolite (NGU 2011). The superficial deposits are dominated by moraine till and mass-movement deposits. The soil is mainly lithosols (Moen et al. 1999). Annual precipitation measured in Bøverdal, 15 km from study area, is 512mm (2004-2013). Monthly average temperature is presented in Figure 3 , measured on Juvasshøe, which is localized 8 km from study area at 1894 m.a.s.l. Since the study area is localized at a lower altitude than the weather station the temperature would be 2-3°C higher in the study area. The vegetation in the area is dominated by heather (*Ericaceae*), willow (*Salix)* and some grasses (*Poaceae*), where most of the vegetation is growing in the snow bed that provides shelter, see Figure 1. Sheep (*Ovis aries*) were grazing in the area. The woodland limit lies around 800 m.a.s.l.

Follo has a subdivided landscape with agriculture, forest and settlement. It has a boreonemoral vegetation zone, with a slightly oceanic section (Moen et al. 1999). The bedrock is consisting of gimmergneiss, gimmerschist, metasandstone and amphibolite (NGU 2011). Marine deposits is dominating the superficial deposits, and the soil changes between podsolic soil and brown soil (Moen et al. 1999). The annual precipitation is 855 mm, measured in Ås. Monthly average temperature is shown in Figure 3 . The samples were collected from four areas within Follo; Nordre Pollen, Svartskogen, Kollåsen and Gaupesteinmarka. All of them in a nature reserve or on the border to nature reserve. In this way it was possible to get samples from a close to natural site, in an area which in a large degree is affected by humans. All of the areas have that in common that they are dominated by conifers; scots pine (*Pinus sylvestris*) and norwegian spruce (*Picea abies*) with some deciduous trees in between. The forest floor is covered with mosses and heather (*Ericaceae)*, as well as with grasses and herbs in light openings. Some of the locations were more influenced by forestry than others.

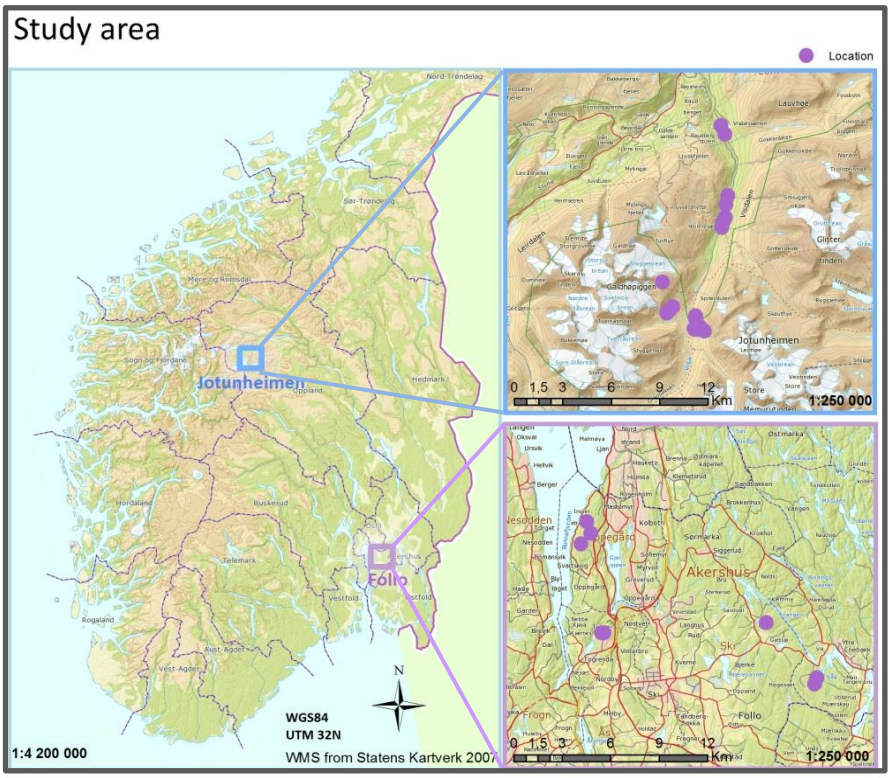


Figure 2. Overview of the study area, with a closer look at the sampling locations (dark purple). Jotunheimen has a blue frame, while Follo has a purple frame. Background map is obtained from Statens kartverk (2007).

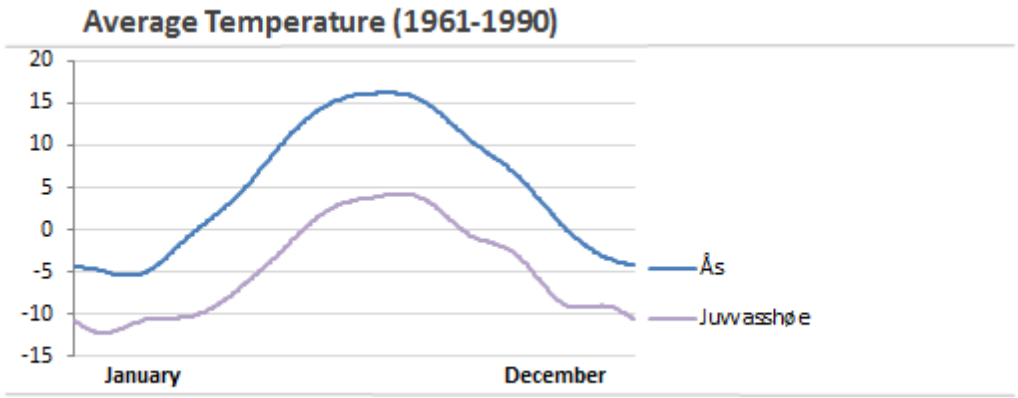


Figure 3. Annual average temperature for Ås (Follo) and Juvasshøe (Jotunheimen). Data is obtained from Norwegian Meteorological Institute.

2.2 Study species

Possible study species were selected in advance to the flowering season. The criteria for selection were that the species is abundant in lowland- and alpine habitat, that it produces enough pollen grains, that there are sufficient specimens within an area and that they are easily accessible. Six species were studied in total, and three of these were sampled in both alpine and lowland habitats (Table 1). There were also some species that were collected but not analyzed: *Juniperus communis* and *Pinus Sylvestris*. 274 samples were collected in total, and 126 of these were analyzed by infrared spectroscopy. In appendix 1, a more detailed sample list can be found.

Table 1. List of the study species, where they was collected, and number of samples used for data analysis

Species		Family	Number of samples	M.A.S.L.
Alpine - Jotunheimen				
Anthoxanthum nipponicum	Sweet vernal grass	Poaceae	12	1350-1556
Avenella flexuosa	Wavy Hair-grass	Poaceae	20	774-912
Deschampsia cespitosa	Tufted Hair-grass	Poaceae	7	774-1101
Festuca ovina	Sheep's Fescue	Poaceae	15	1209-1363
Ranunculus acris	Buttercup	Ranunculaceae	20	765-948
Ranunculus acris	Buttercup	Ranunculaceae	23	1209-1404
Lowland - Follo				
Anthoxanthum odoratum	Sweet vernal grass	Poaceae	6	81-165
Avenella flexuosa	Wavy Hair-grass	Poaceae	5	95-221
Ranunculus acris	Buttercup	Ranunculaceae	18	35-222

All of the collected species are present in large parts of Norway, although there are differences with respect to distribution of the species. *A.nipponicum* is an alpine species, while *A.odoratum* is a lowland species. Since they are extremely closely related it is of particular interest to compare these two grasses. *Deschampsia* and *Avenella* are also closely related. The flowering season of the species varies. *Festuca* and *A.odoratum* are found from May to June, while *Deschampsia*, *Avenella* and *A.nipponicum* are found from June to July. They are all anemophilous plants, and their pollen will discharge when anther mature (Pacini 1996). *Ranunculus* is found from May to September and is an entomophilous species. In Figure 4 there are pictures of some of the collected species in addition to scanning electron microscopy (SEM) images of their pollen grains. Pollen grains from grasses are typical oval with one or more pollen pores. The studied grass species only have one pore each. When pollen gets hydrated it will fold and make furrows (Heslop-Harrison 1979), which is obvious in F in Figure 4. Pollen from dicots has more complex surface structure, as can be seen on *Ranunculus* (D).

The SEM pictures were taken at the Microscopy lab, Imaging Centre, in NMBU, with help from Elin Ørmen.

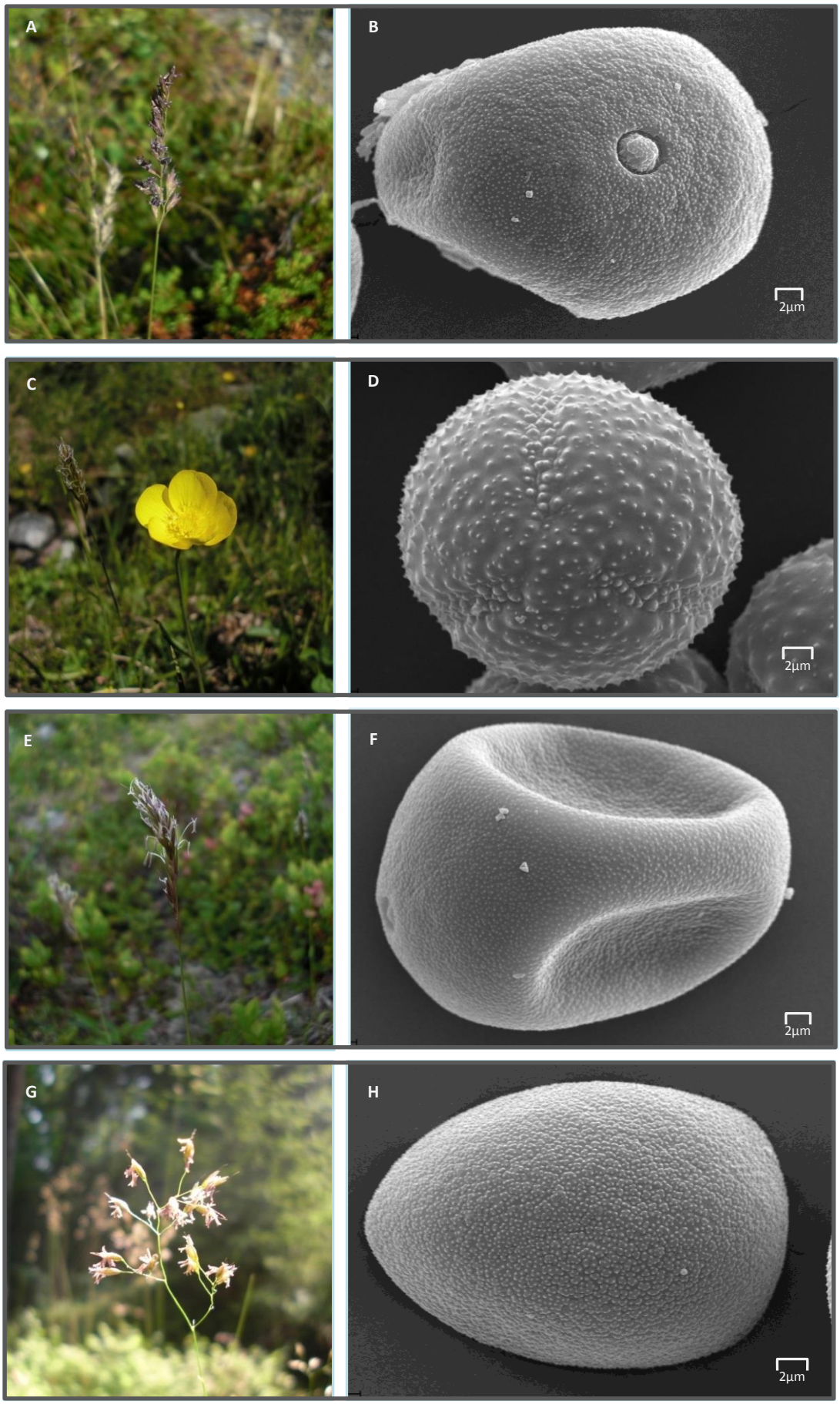


Figure 4. Some of the study species with the flowering plant is to the left, and a Scanning Electron Microscope (SEM) picture of the pollen grain to the right. **A** and **B**: *Festuca ovina* **C** and **D**: *Ranunculus acris* **E** and **F:** *Anthoxanthum nipponicum*. **G** and **H:** *Avenella flexuosa*.

2.3 Sampling of pollen

The time of the sampling in the alpine habitat was from 21.07.2013 to 26.07.2013 and in the lowland from 18.06.2013 to 01.08.2013. The samples from the Jotunheimen region were collected between 775 and 1556 m.a.s.l. In Follo samples were collected between 35 and 222 m.a.s.l. To get mature pollen grains the plant had to be collected during anthesis. The anthesis was determined by the colour of the anther, a cloud of pollen when the plant got touched, or by taking test samples. To get suitable pollen samples the weather was crucial. Preferably there should not be any rain or strong wind several days before sampling (Edwards & Jordan 1992). In addition these conditions must be present at the flowering of the plant and anthesis. The grasses were collected by cutting the flowering spike. On the buttercup the top of the flower was cut and the petals were removed. This was done to avoid that pollen grains stick to the petals. The flowers were put in a homemade paper bag, and marked with date, altitude, location and species. In each paper bag there were spikes or flowers of approximately 20 plants. A GPS (Garmin Dakota 20) was used to get coordinates for the location, and a picture was taken for each location. After collection the samples had to dry for a few days before they were cleaned for non-pollen debris, and transferred to eppendorf tubes. The eppendorf tubes were marked with a code which followed the pollen sample through further analysis. The samples were first stored at -18°C, and later at -40°C. Some of the samples were discarded because there were not enough pollen grains to make a proper sample.

Samples from different plants were collected for each species in order to represent the variation of the different populations. For each species it was tried to collect five samples at five different locations, both in alpine and the lowland habitat. This was not possible in all cases, because of the challenges described above.

2.4 Infrared analysis

For infrared spectroscopy analysis a Bruker Equinox 55 Fourier transform infrared (FTIR) spectrometer with an attenuated total reflectance (ATR) and OPUS software was used. ATR is a non-destructive sampling method that can be used on solids or liquids and requires minor or none sample preparation. The infrared beam passes a diamond where the beam is totally reflected in the interface between the diamond and the sample. Because of high refraction and the angle of the beam an evanescent wave penetrates 0, 5 - 5 µm into the sample. Because of the short penetration length the sample has to be in good contact with the crystal. If the sample absorbs energy, the evanescent wave will be attenuated or altered. This information is brought to a detector which is plotted into a spectrum on a computer (Barth 2007; Smith 2011).

Unfrozen dry pollen grains were grained in a mortal, such that both external and internal compounds were accessible for the ATR crystal. A small amount of the grained pollen sample was transferred to the diamond crystal with a spatula. A clamp ensured good contact between the sample and the crystal. Three replicates were taken of each sample. Between each replicate the pollen was brushed away and the crystal was cleaned with paper. Before each sample measurement a background spectra were taken to minimize disturbances from the atmosphere. If there was a lot of noise in a spectrum or if it stood out with a completely different shape than the other replicates, a new replicate was taken. The spectra were taken in a frequency range of 4000 cm^{-1} to 600 cm^{-1} with a 4 cm^{-1} resolution.

2.5 Data analysis

In addition to chemical information, the raw spectra of pollen contain unwanted physical variations that may affect the subsequent data analysis. To minimize these variations transformation techniques or model based pre-processing tools may be applied (Kohler et al. 2009). Transformation of spectral data may be performed by calculating the second derivative spectra, which resolves overlaying bands and which suppresses broad underlying baselines due to light scattering and variations in light source. The disadvantages of the use of derivatives are that signals of high-frequency noise may be increased, while broad peaks belonging to measured sample are suppressed (Zimmermann & Kohler 2014). Model-based techniques may be used to quantify and separate different physical l variations in the spectra and differences in optical path length and sample thickness. Extended multiplicative signal correlation (EMSC) is a model-based technique which normalizes spectra with respect to a reference spectrum and removes physical effects (Afseth & Kohler 2012; Kohler et al. 2010). For data analysis the software “The Unscrambler X” version 10.3 was used. The raw spectra was either corrected by EMSC or by applying the second derivative, by Savitzky-Golay algorithm, followed by EMSC. The second derivative was used by applying a polynomial order of two, and a window size of 15 points.

Multivariate analysis of spectral data was done by principal component analysis (PCA). Since spectral bands in infrared cover many variables and there are often several bands relating to the same biomolecules, there is a high co-linearity in FT-IR data of biological materials. PCA can handle this co-linearity and reduce a high dimensional multivariate space to a few main components, the so-called principal components. A few main principal components (PCs) are explaining the main variation in the data set. PC1 explains most of the information, while PC2 explains the second most, and so on. The new variables that refer to the principal components are called scores. When scores are plotted in a score plot, similarities and differences between groups of variables can be studied. With each principal component a so-called loading follows. The loading explains how principal component relates to the original spectral variables. When the loading is high for a given variable or region of variable, this means that this variable has high contribution to the respective principal component. Thus, a loading plot can

explain how differences in sample pattern in the score plots can be explained by spectral bands and chemical differences (Kohler et al. 2008). Samples that had noise that was not detected during ATR analysis were excluded from the analysis. The data set was grouped by wavenumber, genus, species, environment and populations.

3 Results

3.1 Pre-processing

The effects of two pre-processing techniques on the spectra are illustrated in Figure 5 and Figure 6. The strong variation patterns in Figure 5 and 6 A which are due to the effective optical path length of the infrared light are removed by EMSC (Figure 5 B and Figure 6 C). The second derivative enhances the spectral resolution. Overlaying peaks are resolved by second derivative and result in negative peaks in Figure 6 B.

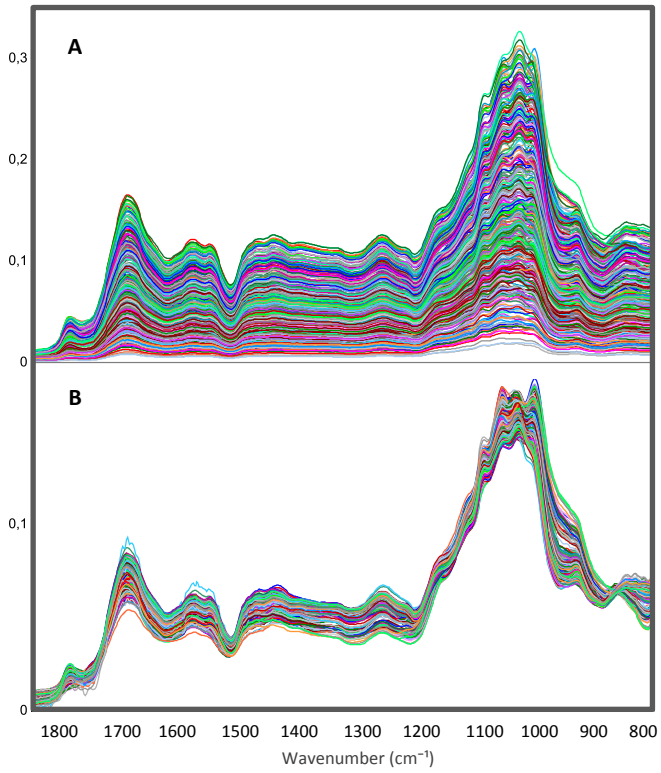


Figure 5. Vibrational spectra from all samples (126) with three replicate showing prepossessing step by step, by only doing EMSC. **A**: raw spectra. **B**: The spectra after EMSC correction

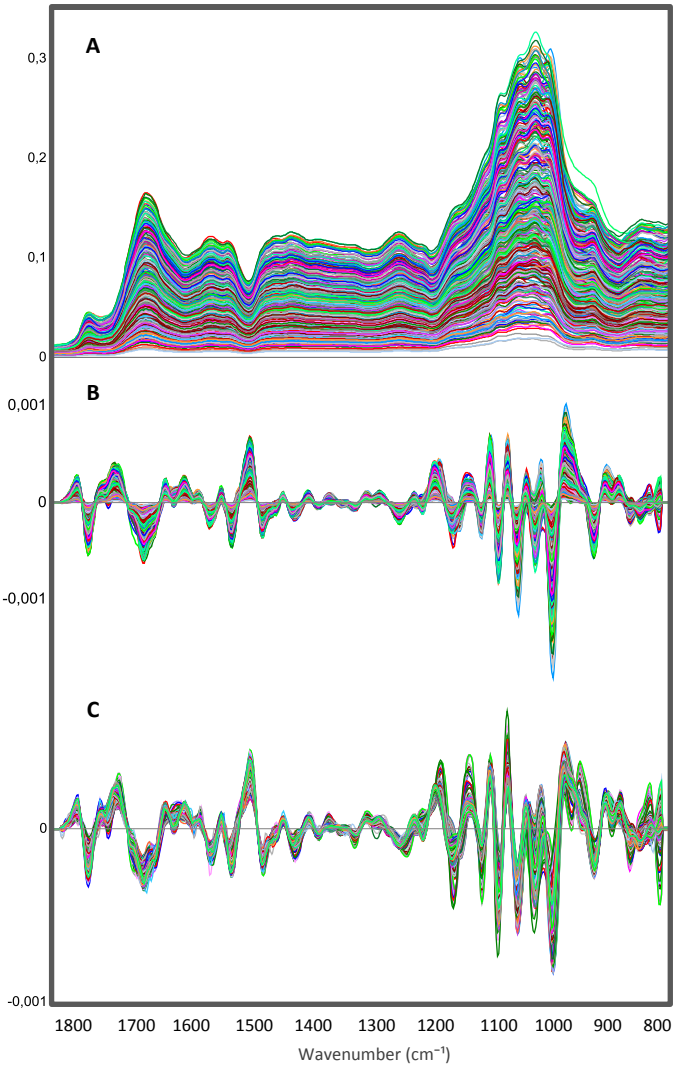


Figure 6. Vibrational spectra from all samples (126) with three replicate showing prepossessing step by step, by doing second derivative followed by EMSC. **A**: raw spectra. **B**: Second derivative spectra are resolving overlapping peaks. **C**: Spectra after EMSC correction.

In vibrational spectra of the study species, in Figure 7, the peaks are associated with different molecular groups that are specific compounds found in pollen. The signals at 3000 -2800 cm^{-1} associated with C-H stretching vibrations and at approximately 1745 cm^{-} associated with C=O stretching vibrations are characteristic for lipids. Since these two sets of signals usually correlate, it is possible to use only spectral region 1800 - 800 cm^{-1} for analysis. Signals characteristic for proteins are amide I and amide II bands associated with vibrations at 1650 cm^{-1} (C=O stretching) and 1550 cm^{-1} (NH deformation and C-N stretching). Vibrational bands characteristic for carbohydrates are associated with different vibrations (C-O-C stretch, C-OH stretch, COH deformation, COC deformation, pyranose and furanose rings vibrations) in the range 1200 - 800 cm^{-1} (Gottardini et al. 2007; Pappas et al. 2003; Shurvell 2002; Zimmermann 2010; Zimmermann & Kohler 2014). In addition specific pollen related compounds can be detected in the spectra, such as sporopollenin that is characterized by the bands at 1605, 1512, 1171 and 833 cm^{-1} which can be attributed to vibrations of aromatic rings (Zimmermann & Kohler 2014). Spectra of the studied species are very similar, and therefore are composed approximately of the same components. However, small and characteristic differences can be detected nevertheless.

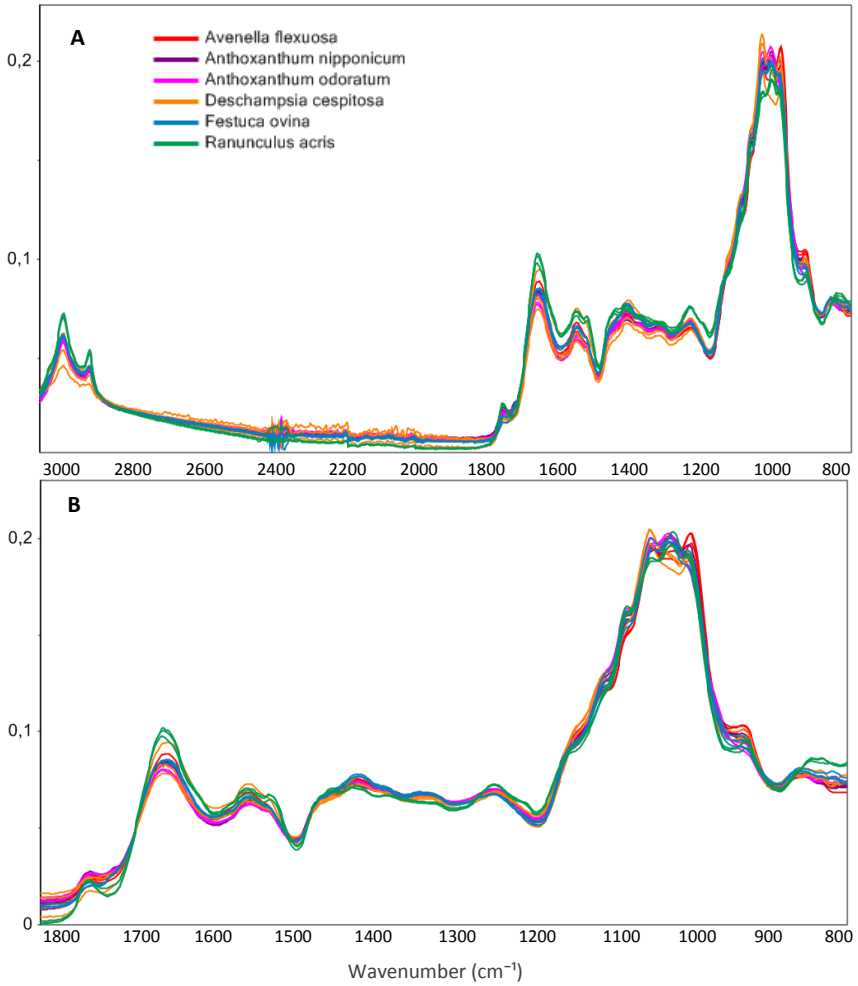


Figure 7. Vibrational spectra of the six study species, with three samples per species. **A**: showing spectra in the wavenumber range 3000 cm^{-1} - 8000 cm^{-1}. **B**: Spectra in the wavenumber range 1800 cm^{-1} - 800 cm^{-1}. Colour description; *Avenella*: red, *A.nipponicum*: purple, *A.odoratum*: pink, *Deschampsia*: orange, *Festuca*: blue, *Ranunculus*: green.

3.2 Identification of species

3.2.1. All species

A score plot of a PCA including all species is shown in Figure 8. Spectra are pre-processed by second derivative followed by EMCS. The main separation is between *Ranunculus* and grasses, while there is smaller separation between grass species as well. Some samples from *Ranunculus*, *Festuca* and *Avenella*, termed as outliers, are deviating from the rest of the samples (termed as normal samples). These outliers will be elucidated in chapter 3.2.2. The separation in the PCA plot is a combination of two principal components (PC1 and PC2), and therefore it is difficult to estimate the cause of separation. The loading plot for PC1 and PC2 can be found in Figure 9. This show that the separation is mainly due to vibrations in the band 1745 cm^{-1}, associated with lipids, 1652 cm^{-1}, associated with proteins, the band 1508 cm^{-1}, related with sporopollenin and 1080, 1043, 1016 cm^{-1} due to carbohydrates. The high loading around 987cm^{-1}, is believed to be caused by the deviated samples mention above and will be discussed in chapter 3.2.2. The grouping of the grass species will be presented in detail in chapter 3.2.3.

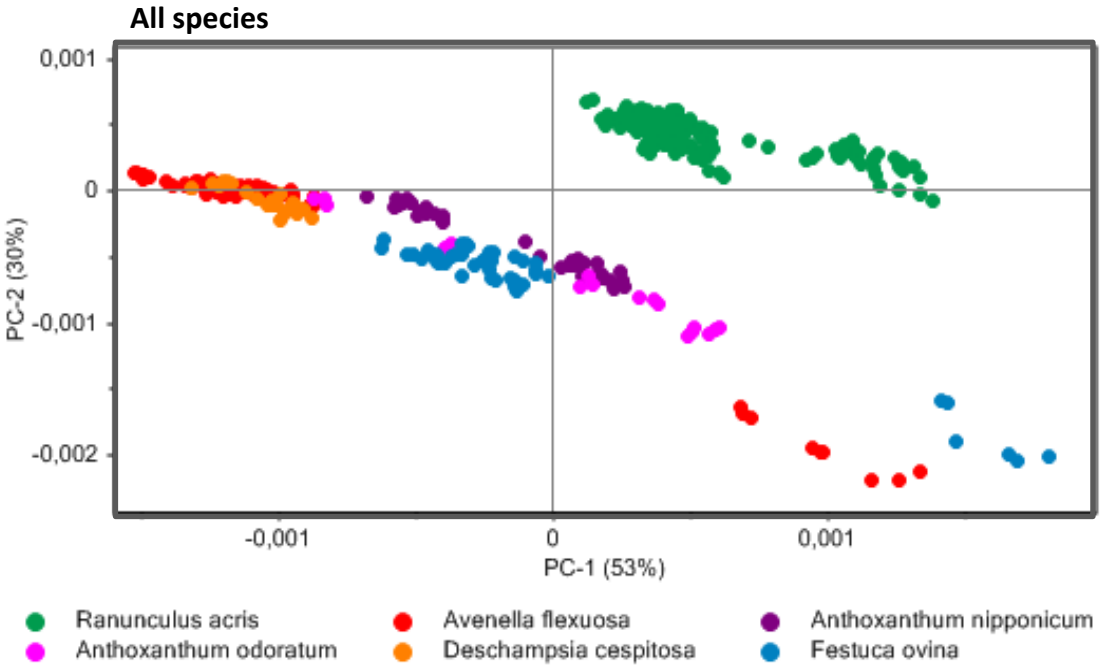


Figure 8. PCA plot of all study species (126 samples) with three spectra per samples. Spectra are pre-processed by second derivative followed by EMCS. Percentage variance of the five first PC is: 53 %, 30 %, 8 %, 2 % and 2 %. Colour description; *Avenella*: red, *A.nipponicum*: purple, *A.odoratum*: pink, *Deschampsia*: orange, *Festuca*: blue, *Ranunculus*: green.

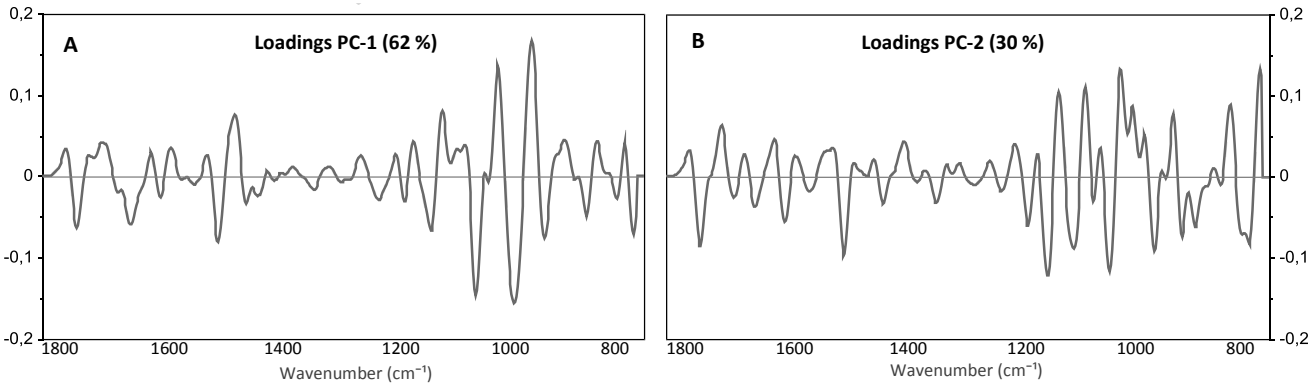


Figure 9. Loading plots for all study species. **A**: PC1 (53 %) **B**: PC2 (30 %)

When spectra are EMCS corrected without employing second derivative, the loadings explaining the cause of separation between *Ranunculus* and grasses are easier to interpret (Figure 10). In this case only one principal component is explaining the variation between *Ranunculus* and grasses. There are less samples used in this PCA because of deviating samples are more present when not employing second derivative, see chapter 3.2.2. Vibrations are especially present in amide I band at 1651 cm^{-1}, which is associated with proteins. This can also be seen in the EMSC corrected spectra (Figure 11) were *Ranunculus* has higher peak around 1650 cm^{-1} than *Avenella* (which is representing grasses). High intensity of the bands 1045, 1018 and 997 cm^{-1} can be shown as different ratio between 1045 and 997 cm^{-1} and 1018 cm^{-1}. In additions, signals at 1508 and 1606 cm^{-1} are present as well, in PC1. The presence of bands at these wavenumbers is usually associated with sporopollenin. It is noticeable that these signals are stronger in *Ranunculus* spectrum than in *Avenella* spectrum.

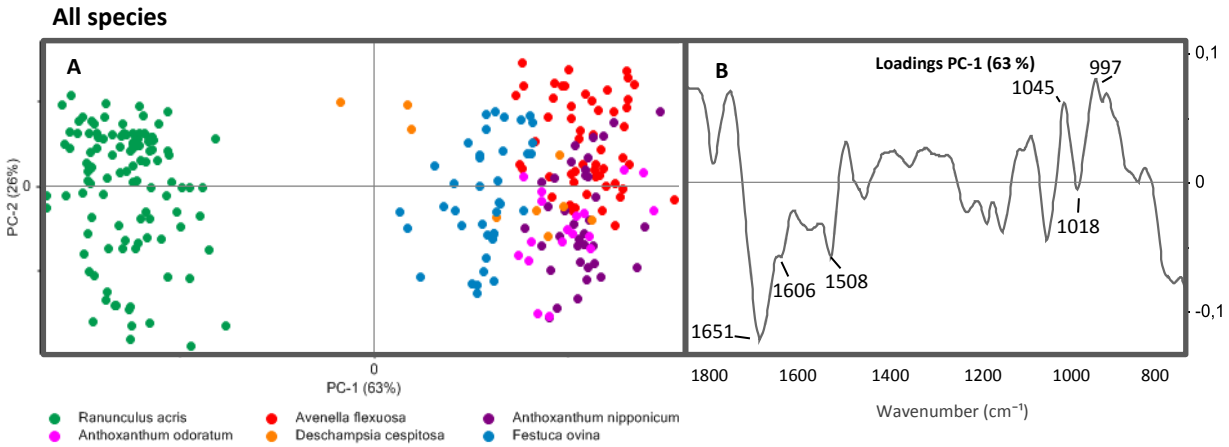


Figure 10. **A**: PCA plot of all species (99 samples) with three spectra per sample. The spectra are EMCS corrected without employing the second derivative. Percentage variance of the five first PC is: 63 %, 26 %, 5 %, 3 % and 1 %. Colour description; *Avenella*: red, *A.nipponicum*: purple, *A.odoratum*: pink, *Deschampsia*: orange, *Festuca*: blue and *Ranunculus*: green. **B**: Loading plot for PC1 (63 %).

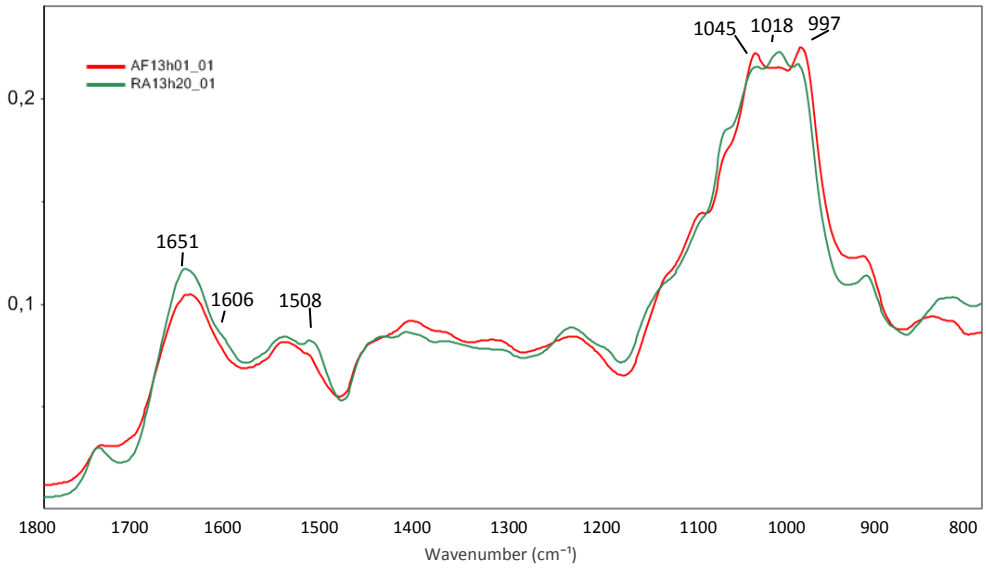


Figure 11. Vibrational spectra which show differences between *Ranunculus* (green) and grasses. *Avenella* (red). Bands which have high intensity in the PCA are highlighted. Spectra are EMSC corrected without employing second derivative.

3.2.2 Outlying samples

The outlying samples which can be seen in Figure 8 will now be investigated in more details. In Figure 12 the separate PCA plots with the corresponding loadings are presented for four species. Spectra are EMSC corrected without employing second derivative. Four different species have separation due to high intensity in the same bands, indicating the common reason for deviations. The samples are separated by high positive and negative intensity in the bands at 1136, 1080, 1045, 1020, 960, 925, 815 cm^{-1} and especially by high intensity of bands at 987 cm^{-1}. The bands between 1200 – 800 cm^{-1} are usually associated with carbohydrates. The vibrational band at 987 cm^{-1} can be associated with P-O-C asymmetric stretch which is characteristic for phospholipids (Shurvell 2002). When one spectrum from outlying group and normal group is set against each other (Figure 13) it is easier to see what is causing the separation. The outlying spectrum have lower intensities in the peaks of the bands at 987 and 925 cm^{-1}, and higher intensity in the peak at 815 cm^{-1}. The same tendency is present in all the species, but not that clear as with *Avenella*. This is expected when looking at the PCA plots since the strength of explanation of separation, shown by percentage of the principal component, varies with the species. The PC that explains separation of outlying and normal samples, has a higher explanation percent for *Avenella* (PC2: 62 %) then for *Festuca* (PC2: 25 %), *A.nipponicum* (PC2: 15 %) *and Ranunculus* (PC3: 9 %). This means that the separation between the two samples sets, outlying and normal, is largest for *Avenella*. Difference between deviated samples and normal samples are for all species except *Ranunculus* represented by PC1 and PC2. In *Ranunculus* the deviated samples are separated by normal samples by PC2 and PC3. Because this grouping was most obvious in PC3 of the *Ranunculus*, the cause of separation was studied for this case. Outlying samples from *Avenella*, *Festuca* and *Ranunculus* were excluded from the following analyses. Although the separation of outlying samples was clear for

A.nipponicum they were taken into consideration in the environmental analysis (see chapter 3.3). While lowland A.odoratum has some outlying samples, the deviation is not as strong as for the other species.

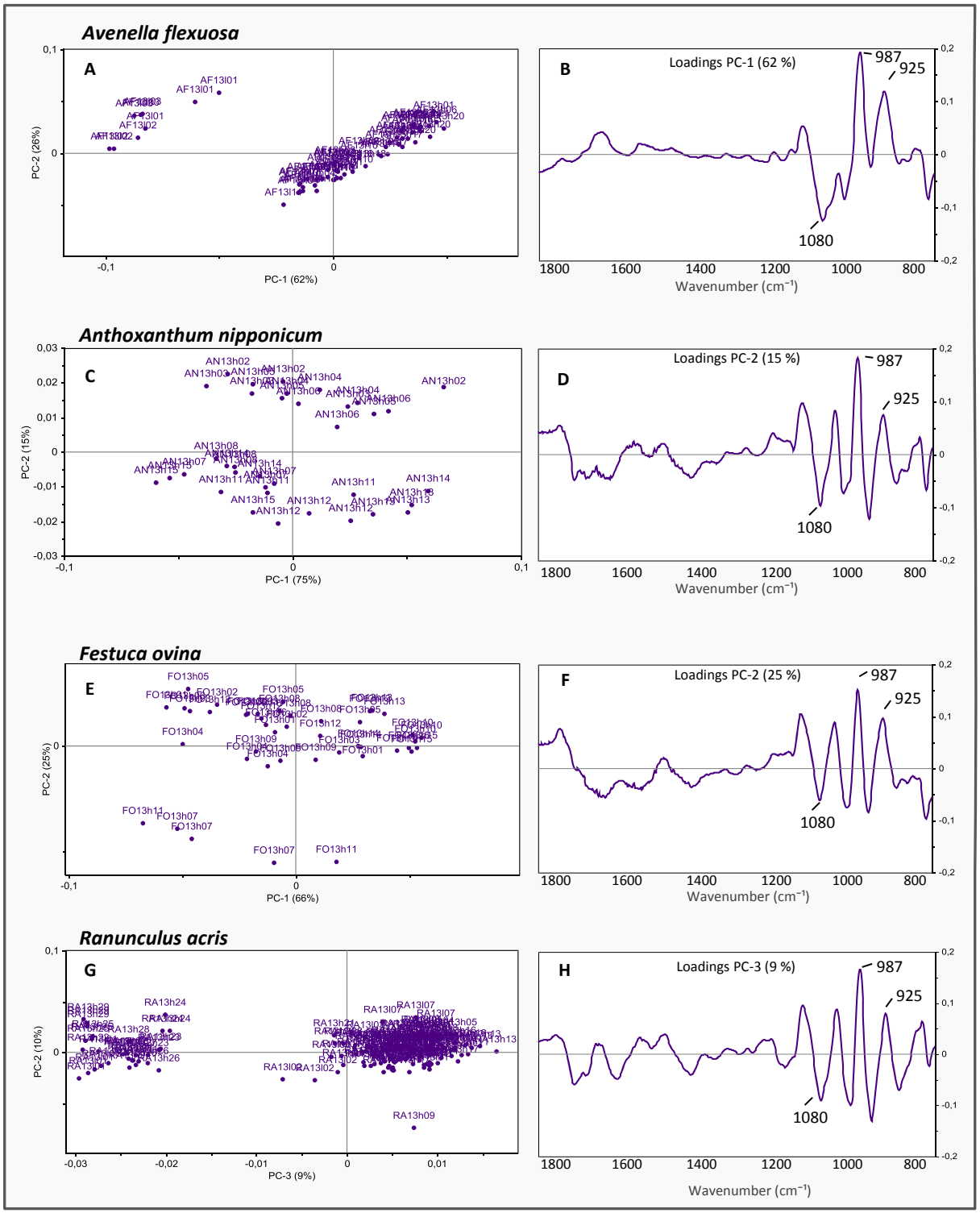


Figure 12. PCA plots and loading plot for deviations. **A**: PCA plot of *Avenella* (21 samples with three spectra per sample) Spectra are EMCS corrected without employing second derivative. Percentage variance of the five first PC is: 62 %, 26 %, 5 %, 4 % and 1 %. **B:** Loading plot from PC 1 of *Avenella*. **C**: PCA plot of *A.nipponicum* (12 samples with three spectra per sample). Spectra are EMCS corrected without employing second derivative. Percentage variance of the five first PC is 75 %, 15 %, 6 %, 2 % and 0 %. **D**: Loading plot from PC 2 of *A.nipponicum* **E:** PCA plot of *Festuca* (15 samples with three spectra per sample). Spectra are EMCS corrected without employing second derivative. Percentage variance of the five first PC is 66 %, 25 %, 5 %, 2 % and 1 %. **F**: Loading plot from PC 2 of *Festuca*. **G**: PCA plot of *Ranunculus* (52 samples with three spectra per sample). Spectra are EMCS corrected without employing second derivative. Percentage variance of the five first PC is 71 %, 10 %, 9 %, 2 % and 2 %. **H**: Loading plot from PC 3 of *Ranunculus*.

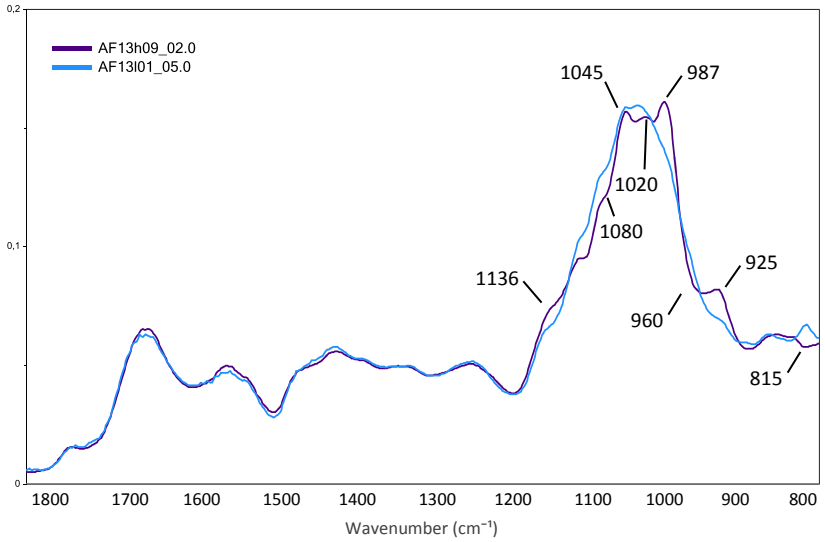


Figure 13. Vibrational spectra of *Avenella* from the two deviating groups. One spectrum from normal group (purple) and one from deviating group (indigo-blue). Wavenumbers which had high peaks in the loadings is highlighted. Spectra are EMSC corrected without employing second derivative.

Regarding the normal samples, some additional separations have been noticed for *Deschampsia, Ranunculus* and *Avenella*. Thus, the separation between normal and outlying samples was much lower for *Avenella* than *Deschampsia* and *Ranunculus*. In Figure 14 the spectrum of the two groups in *Deschampsia* are shown and the difference are clear. These separations are mainly due to high positive and negative intensity in the bands 1174, 1045, 960 and 806 cm^{-1} (Figure 15). In the further analyses, when samples were pre-processed by second derivative followed by EMSC, the deviating samples from *Ranunculus* are removed, while when pre-processed by EMSC without employing second derivative all deviating samples were removed.

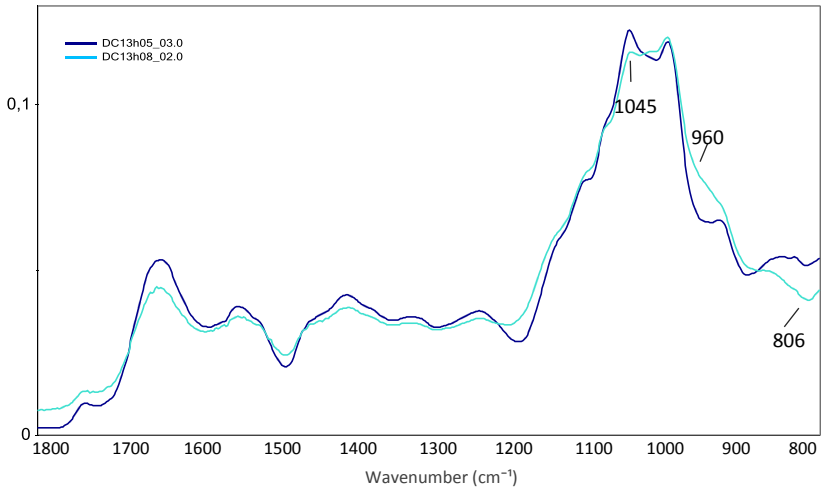


Figure 14. Vibrational spectra of *Deschampsia* from the deviating group (indigo-blue) and the normal group (dark blue). Wavenumbers which had high peaks in the loadings is highlighted. Spectra are EMSC corrected without employing second derivative.

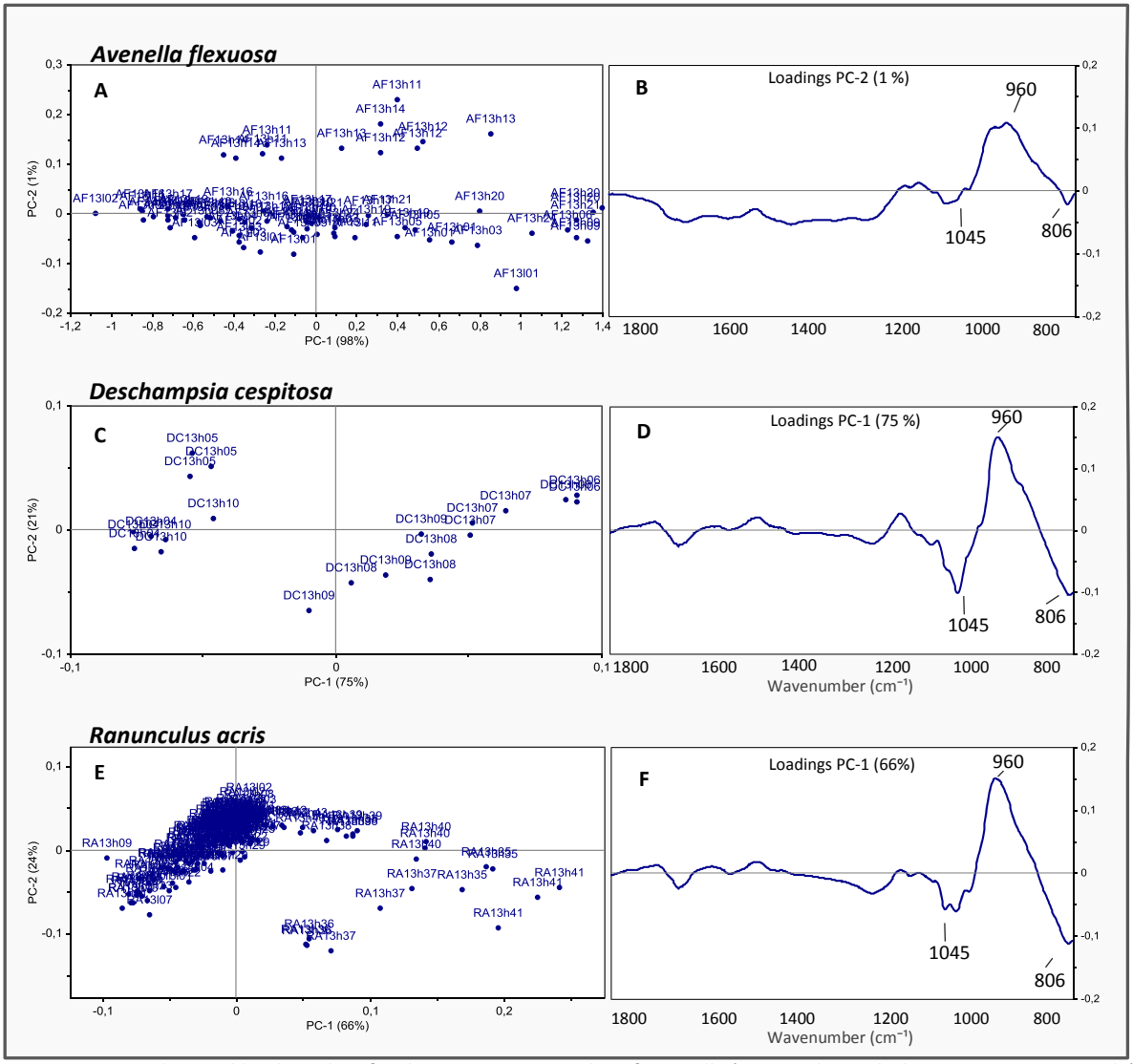


Figure 15. PCA plots and loading plots for deviations. **A**: PCA plot of *Avenella* (25 samples with three spectra per sample). Spectra are EMCS corrected without employing second derivative. Percentage variance of the five first PC is: 98 %, 1 %, 0 %, 0 % and 0 %. **B**: Loading plot from PC 1 of *Avenella*. **C**: PCA plot of *Deschampsia* (7 samples with three spectra per sample). Spectra are EMCS corrected without employing second derivative. Percentage variance of the five first PC is 75 %, 21 %, 3 %, 0 % and 0 %. **D**: Loading plot from PC 2 of *Deschampsia*. **E**: PCA plot of *Ranunculus*. Spectra are EMCS corrected without employing second derivative (61 samples with three spectra per sample). Percentage variance of the five first PC is 66 %, 24 %, 3 %, 3 % and 1 %. **F**: Loading plot from PC 2 of *Ranunculus*.

3.2.3 Grasses

In Figure 16 a PCA plot of all grasses spectra are shown. Spectra are pre-processed by second derivative followed by EMSC. It can be clearly seen that different grass species are separated from each other. Grasses which are closely related, such as *Deschampsia* and *Avenella, and A.nipponicum* and *A.odoratum,* are located close to each other in the score plot. *A.nipponicum* spectra are separated into normal and outlying samples since the outlying group was not deleted, see chapter 3.2.2. *Avenella* and *Deschampsia* separate from the other grasses by having strong vibrations in the bands 1153, 1080, 1020 and 815 cm^{-1} (Figure 16). These vibrations are probably associated with carbohydrates. PC1 and PC3 is

used in the PCA plot because PC2 is not showing any separation due to species, but has some separation due to vibrations associated with carbohydrates.

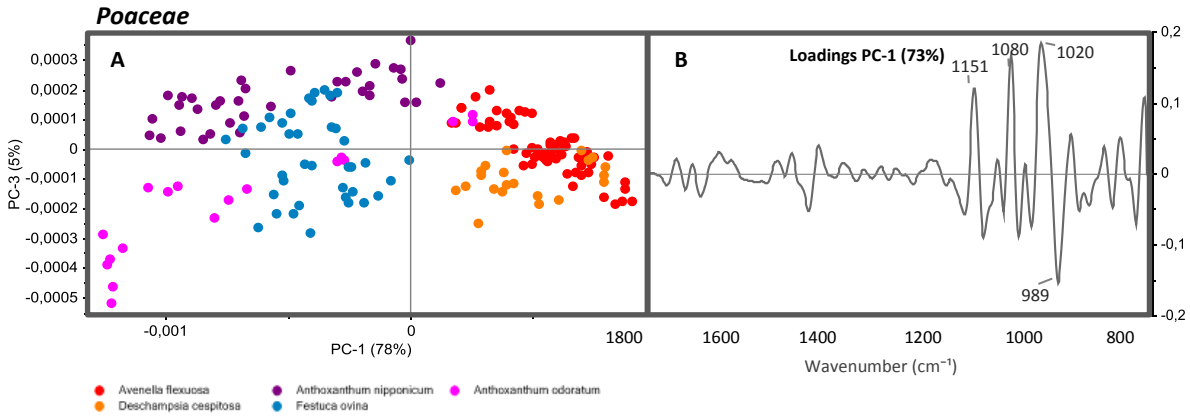


Figure 16. PCA plot of all grasses (60 samples) with three spectra per sample. Spectra are pre-processed by second derivative followed by EMCS. Percentage variance of the five first PC is: 73 %, 11 %, 5 %, 3 % and 2 %. Colour description; *Avenella*: red, *A.nipponicum*: purple, *A.odoratum*: pink, *Deschampsia*: orange, *Festuca*: blue.

By looking closer to the two closely related species *Avenella* and *Deschampsia,* it is possible to differentiate them based on their infrared spectra. The changes are mainly due to vibrational bands in the region 1160 - 810 cm^{-1} (Figure 17), which is associated with carbohydrates.

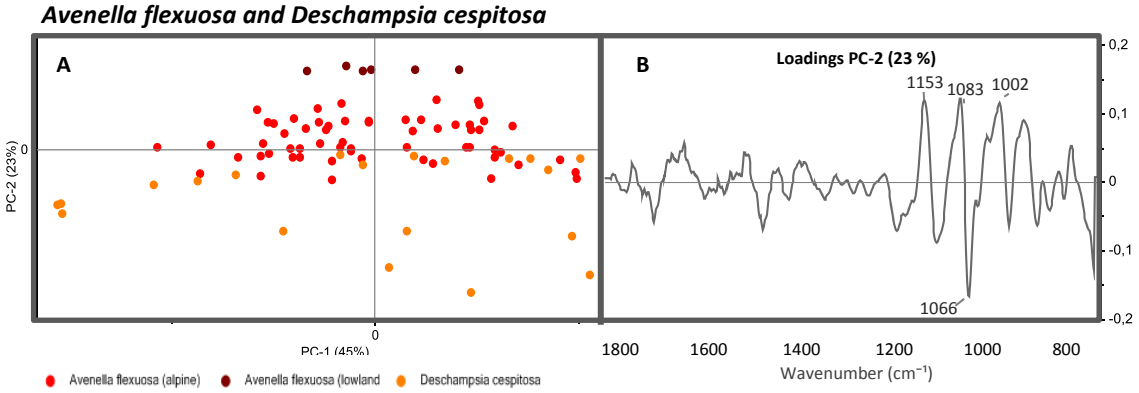


Figure 17. PCA plot of *Avenella* (23 samples) and *Deschampsia* (7 samples) with three spectra per sample. Spectra are pre-processed by second derivative followed by EMCS. Percentage variance of the five first PC is: 45 %, 23 %, 14 %, 5 % and 2 %. Colour description; *Avenella* from alpine: red, *Avenella* from lowland: dark red, *Deschampsia*: orange.

3.3 Environmental effect

In Figure 18 - 20 PCA plot of *Avenella*, *Anthoxanthum* and *Ranunculus* are shown. The samples of these species was collected both in alpine and lowland habitat, and infrared spectroscopy detects differences between these habitats. All samples are pre-processed by second derivative followed by EMCS. The

separation between alpine and lowland can be explained by some common vibrational bands in all three species. However, *Ranunculus* has a different ratio of these signals, as well as some additional signals that are explaining the difference. High intensity of the bands 1155 and 1020 cm^{-1} is found in *Ranunculus* from alpine, and in *Avenella* and *A.odoratum* from lowland. The band 1065 cm^{-1} has high intensity in *Ranunculus* from lowland and in *Avenella* and *A.nipponicum* from alpine. This indicate different ratio of these components between *Ranunculus* and the other species. These wavenumbers are probably associated with carbohydrates. In addition there is a high intensity of band at 1670 cm^{-1} in the alpine samples, both for *Ranunculus* and to some less extent for *Anthoxanthum*. Vibrational band at 1670 cm^{-1} can be associated with proteins. For the lowland samples, *Anthoxanthum* has high intensity bands that are common with *Avenella* (1080 cm^{-1}) and *Ranunculus* (1480 cm^{-1}). The environmental differences are explained by differences in carbohydrates for the two grasses, and by proteins and carbohydrates for *Ranunculus*. The separation in *Anthoxanthum* (Figure 19) is a result of a combination between two principal components PC2 and PC3. PC1 is showing the outliers mention in chapter 3.2.2. In addition to the alpine and lowland groups *Ranunculus* has a separate group (Alpine-low) which is representing a middle altitude. In the PCA plot (Figure 20) this middle altitude samples are placed approximately between lowland and alpine samples. For *Ranunculus* it is mainly PC2 which is explaining the separation. PC4 is giving some separation of the Alpine-low group, which the first three components are not showing. The loadings in PC1 have signals associated with carbohydrates, in PC3 with proteins, and in PC4 with lipids and carbohydrates.

In Figure 21 the vibrational spectra of *Ranunculus* and *Avenella* is containing one spectrum from each alpine and lowland habitat, from wavenumber 1400 cm^{-1} – 800 cm^{-1}. *Avenella* is having more separation between the two spectra. This is because the percentage, which is explaining the variance, is higher for PC2 percentage for *Avenella* (31 %) than PC2 percentage for *Ranunculus* (10 %).

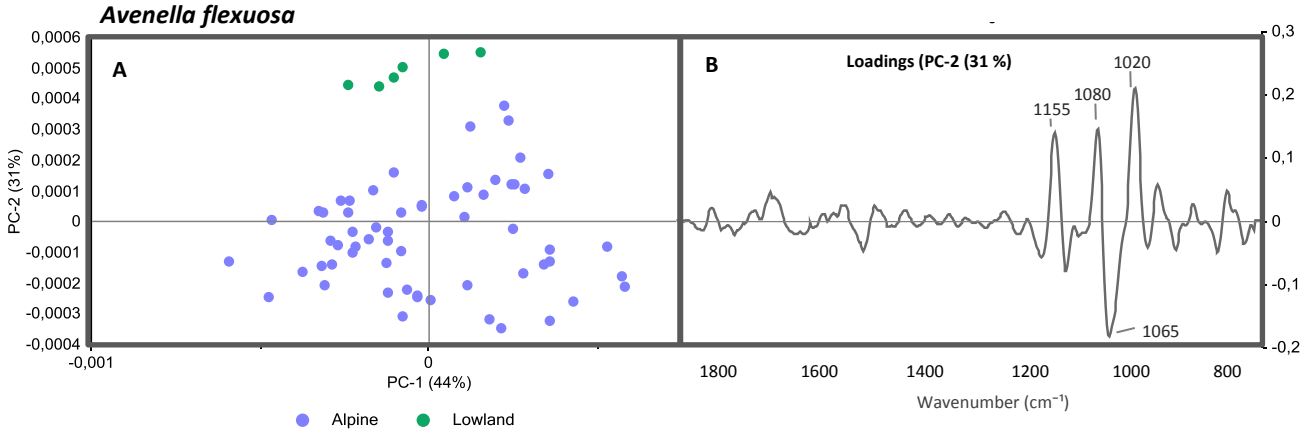


Figure 18. **A**: PCA plot of *Avenella* showing differences between alpine (blue) and lowland (green) environments (23 samples with three spectra per samples). Spectra are pre-processed by second derivative followed by EMCS. Percentage variance of the five first PC is: 44 %, 31 %, 9 %, 4 % and 3 %. **B**: Loading plot for PC2.

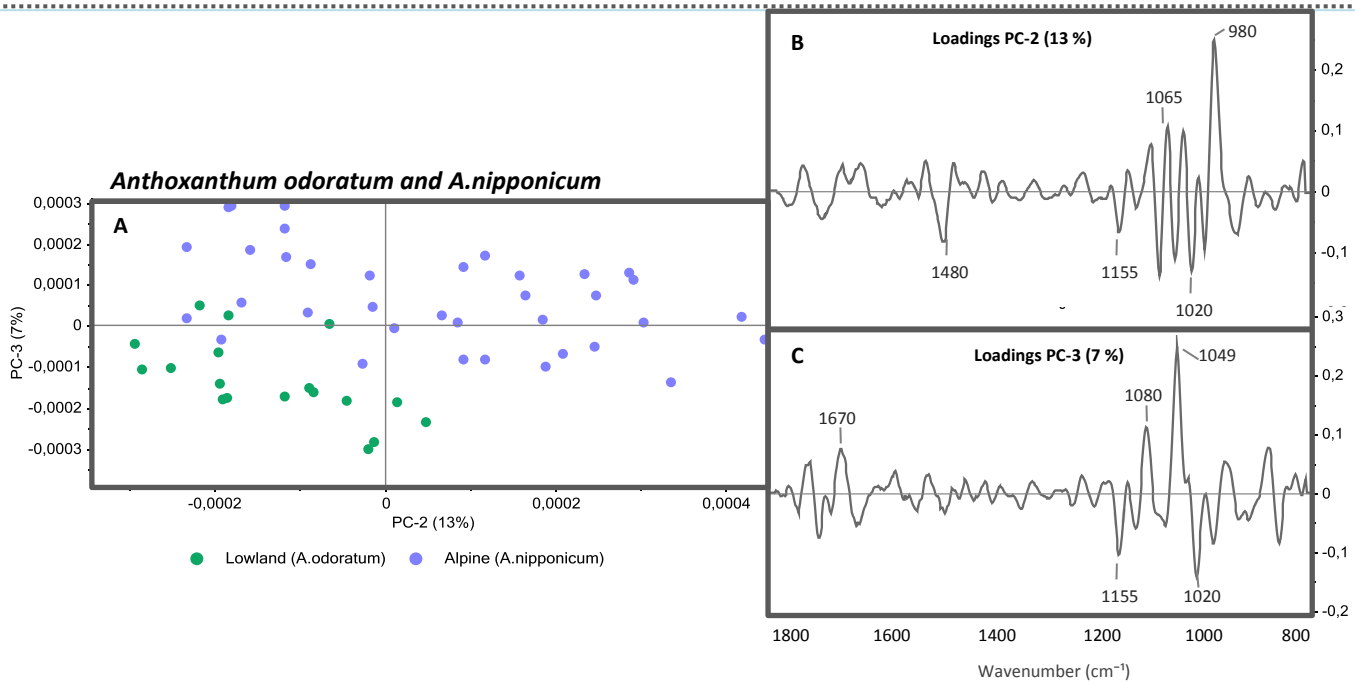


Figure 19. **A**: PCA plot of *A.nipponicum* and *A.odoratum* showing differences between alpine (blue) and lowland (green) environments. Respectively 12 and 6 samples with three spectra per sample. Spectra are pre-processed by second derivative followed by EMCS. Percentage variance of the five first PC is: 71 %, 13 %, 7 %, 2 % and 1 %. **B**: Loading plot for PC2 and PC3.

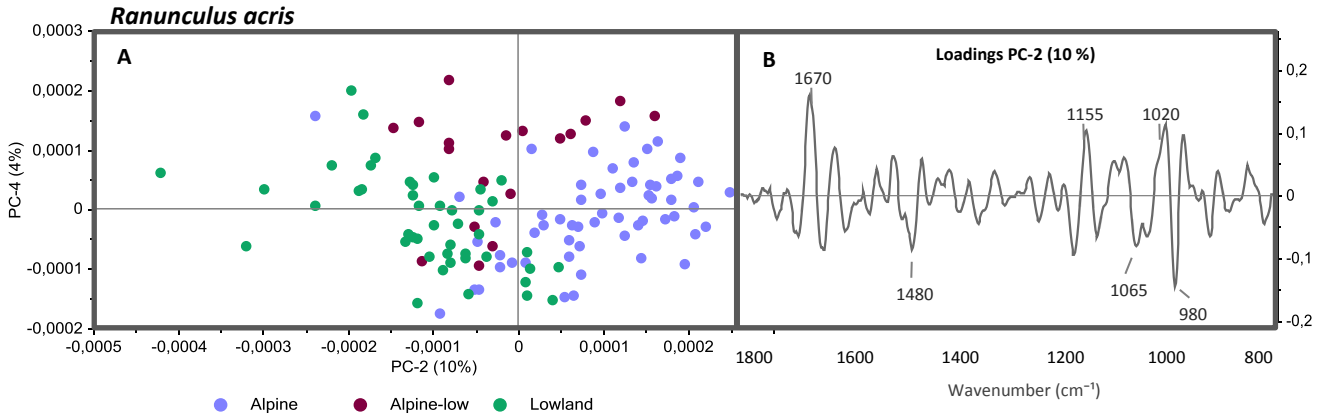


Figure 20. PCA plot of *Ranunculus* showing differences between alpine high altitude (blue), alpine low altitude (dark red) and lowland (green) environments. Respectively 20, 6 and 15 samples with three spectra per sample. Spectra are pre-processed by second derivative followed by EMCS. Percentage variance of the five first PC is: 56 %, 10 %, 9 %, 4 % and 3 %. **B**: Loading plot for PC2.

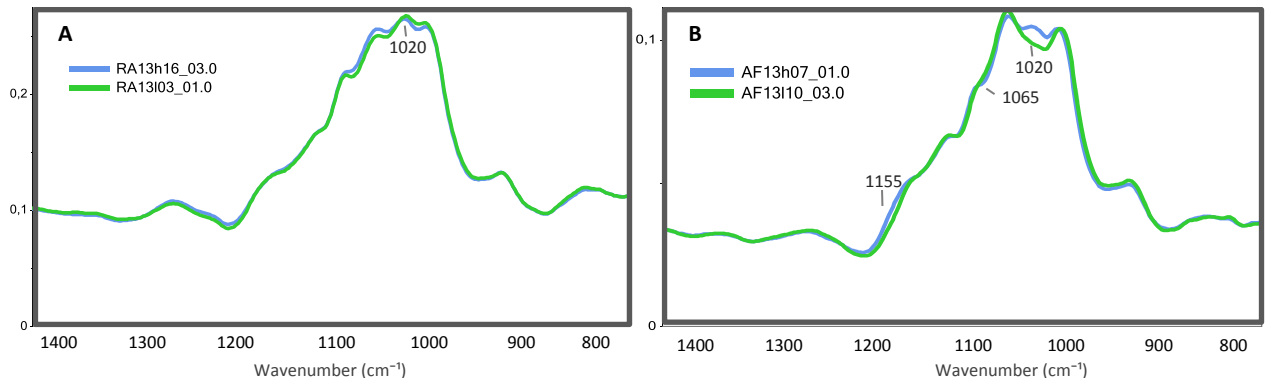


Figure 21. Vibrational spectra showing the differences between alpine and lowland environment for *Ranunculus* and *Avenella*, taken from the wavenumber range 1400 cm^{-1} - 800 cm^{-1}. The spectra are EMSC corrected without employing second derivative. The blue line is representing the alpine habitat and the green line is representing the lowland habitat. **A**: Vibrational spectra of *Ranunculus*. **B**: Vibrational spectra of *Avenella*

3.4 Variation in populations

The PCA plot in Figure 22 – 24 are showing the separation due to different populations of each species. For all the PCA plots the spectra are pre-processed by second derivative followed by EMCS. Most of the species have overlapping populations in PCA, indicating small spectral differences. These differences for *Avenella* (Figure 22) can be easily detected, and they are predominantly due to vibrations associated with carbohydrates. Although *Ranunculus* (Figure 23) has more overlapping populations, there is still some separation present and this is due to vibrations associated with carbohydrates and proteins. Figure 24 is showing the population-based PCA plots for the rest of the species. As can be seen, some separation between populations is present as well. It should be taken into account that the number of samples was smaller than in the cases of *Avenella* and *Ranunculus*. The population numbers in Figure 22 – 24 can also be found in the sample table, in appendix 1, and the maps in appendix 2 and 3.

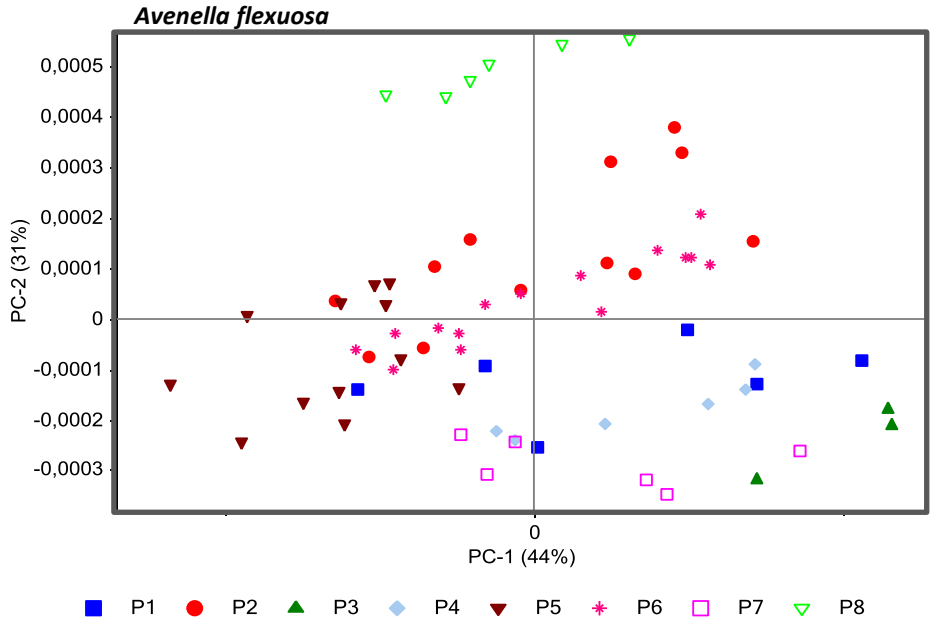


Figure 22. PCA plot of *Avenella* showing differences between 8 populations (23 samples with three spectra per sample). Spectra are pre-processed by second derivative followed by EMCS. Percentage variance of the five first PC is: 44 %, 31 %, 9 %, 4 % and 3 %. Each symbol is representing one population.

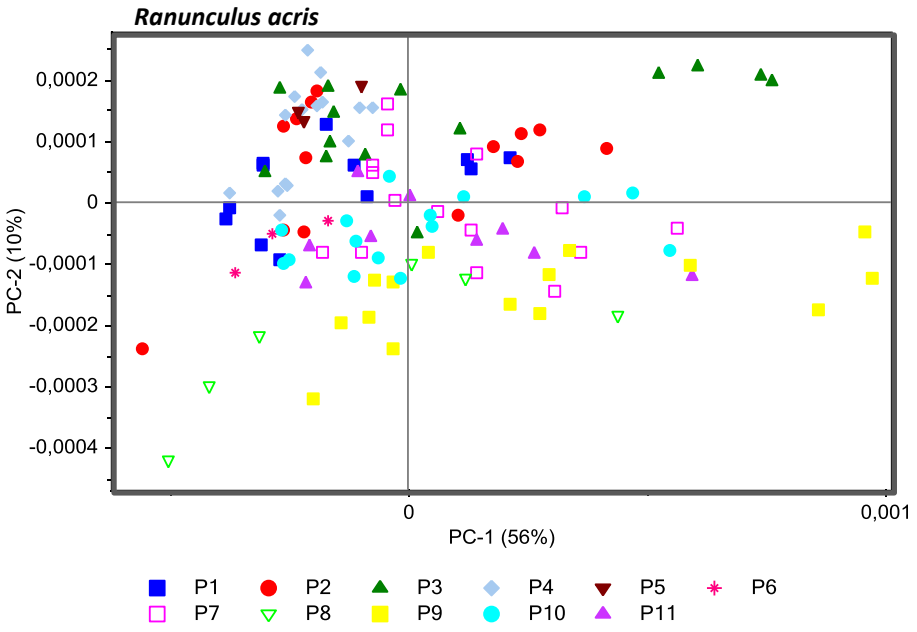


Figure 23. PCA plot of *Ranunculus* showing differences between populations (41 samples with three spectra per sample). Spectra are pre-processed by second derivative followed by EMCS. Percentage variance of the five first PC is: 56 %, 10 %, 9 %, 4 % and 3 %. Each symbol is representing one population.

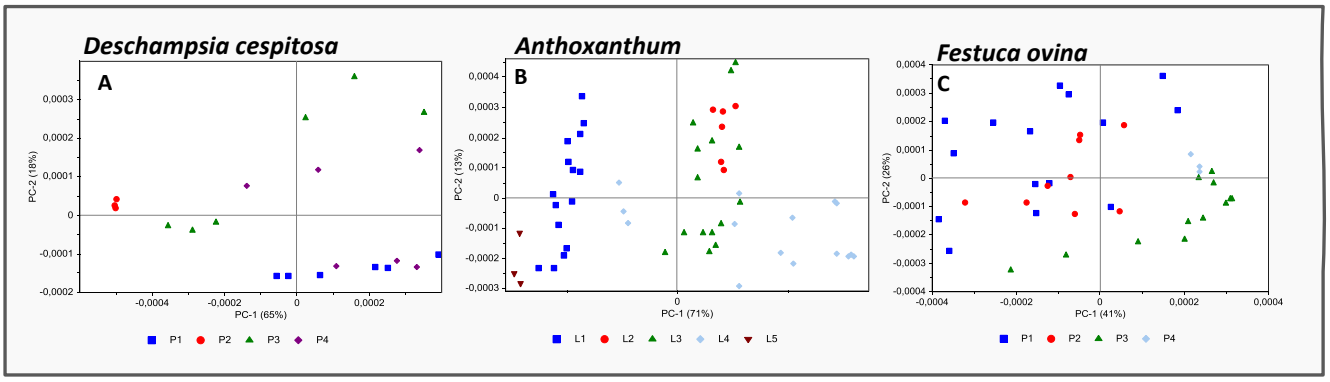


Figure 24. PCA plot showing differences by populations. All spectra are pre-processed by second derivative followed by EMCS. **A**: Deschampsia (7 samples with three spectra per sample). **B:** A.nipponicum (12 samples with three spectra per sample) and A.odoratum (6 samples with three spectra per sample). **C**: Festuca (13 samples with three spectra per sample). Each symbol is representing one population.

4 Discussion

Four main conclusions are drawn from the results: First, is possible to separate between species by differences in pollen chemical composition. Species close related have more similarities. Second, same species from different environmental habitats has different chemical composition. This chemical composition varies with *Ranunculus* and grasses. Third, different populations also have different chemical composition, but this is less clear. And last, all these differences can be detected by using infrared spectroscopy.

4.1 Separation of species

The vibrational spectra (Figure 7) of all studied species show that they have many common features in terms of chemical characteristics of their pollen. For example, the ratio among lipids, proteins and carbohydrates, which are the main components in pollen (Stanley & Linskens 1974), was rather similar among the study species. The vibrational spectra are not representing the exact amount of the different content, since the ATR technique focus more on the lower wavenumbers, thus overrepresenting the carbohydrate content. Nevertheless, the study has shown that carbohydrates are the major component of angiosperm pollen, which is in accordance to previous findings (Stanley & Linskens 1974).

Although the spectral differences are small the variation in chemical ratio and content between species has been detected. The main separation in PCA can be found between *Ranunculus* and grasses. This is expected since multiple factors separate *Ranunculus* and grasses, including different taxonomy, pollinating strategy, and pollen grain morphology. Baker and Baker (1979) have found that some species were starchy while other was starchless, and that this could be related to pollinating strategy. Similar conclusion has been reach by Zimmermann and Kohler (2014), who found a separation between entomophilous and anemophilous species based on higher carbohydrate-to-protein ratio in anemophilous pollen. This could be supported by my present study where the main separation that entomophilous *Ranunculus* and anemophilous grasses was due to vibrations associated with carbohydrates and to smaller extent to lipids and proteins. However, limitations in the method make it difficult to identify specific carbohydrates, but it is likely that they have to do with starch. Franchi et al. (1996) have found that it is possible to distinct starch in pollen grain into several types, and that these types vary between genera and within genus. Starch can also be transformed into other types of carbohydrates, such as polysaccharide, disaccharides and monosaccharide before anthesis (Pacini 2000). This can be supported by this study since it has been found that carbohydrate content varies with species, and that related species have more common chemical composition. The closely related species *Deschampsia* and *Avenella* are differing from the rest of the grasses by having the same types of

carbohydrates. Moreover, these species can be separated by having different ratio of carbohydrate components.

Vibrations associated with proteins are more visible between *Ranunculus* and grasses, where *Ranunculus* has higher ratio of proteins compared to carbohydrates. Protein content could be related to selection of food for bees (Roulston et al. 2000). So pollen that has more protein will be more likely collected by bees. This coincides good with this study were *Ranunculus* is an entomophilous species separated from grasses by vibrations associated with proteins. Entomophilous species usually have pollenkitt, which contains lipids, carbohydrates, proteins, glycoproteins, carotenoids and flavonoids (Shivanna 2003). Since *Ranunculus* probably has pollenkitt the separation could be due to this presence. Moreover, vibrational bands associated with sporopollenin seem to differ between the two taxa: *Ranunculus* and grasses. *Ranunculus* has more sporopollenin in the spectrum (Figure 11), compared to the grasses. The SEM images of the two taxa clearly show that the exine, were sporopollenin can be found, are very different. The reason for this separation is probably the difference in exine thickness, although it could be differences in sporopollenin chemical composition as well.

The outlying samples, presented in chapter 3.2.2 have lower intensity of vibrational band at 987 cm^{-1}. This decreasing absorption band is probably associated with P-O-C antisymmetric stretch of phospholipids, which has strong vibrations in 1055 - 915 cm^{-1} spectral region. The decreasing absorption of phospholipids could indicate deesterification process in pollen grains that could lead to degradation of pollen (Van Bilsen & Hoekstra 1993). It is known that phospholipids osmoregulate intracellular material by means of water-enclosing vesicles. Deesterification of phospholipids lead to increased phospholipid permeability, followed by leakage of entrapped solutes within vesicles, and finally harmful desiccation of microorganism that can be lethal. This is what most likely has happened with these samples. There is not a clear connection between these samples that can explain the degradation. The samples from *Avenella* are from one population, while all the other species have outlying samples from different populations. *Festuca* has one sample from two different populations, and the rest of the samples from these populations are not deviating. It could be old pollen, but this is not likely since pollen on grasses will dehisce right after maturation. This could indicate that the degradation occurred due to inadequate storage of samples after collecting in nature. However, some other outlying samples had more in common. All species that were deviating had been collected close to a gravel road. The sampling period was characterized by heavy road traffic and dry weather in the sampling regions. This resulted in debris that was blown to the environment in road surrounding. During pollen analysis it was noted that some samples from *Deschampsia* contained dark debris, probably of inorganic origin. It was exactly these samples that were deviating. This debris must have been so small that is was not possible

to separate it from pollen grains during cleaning of samples. The deviating samples had more of content which was causing increased absorptions at 960 and 806 cm $^{-1}$.

4.2 Environmental effect

The separation between alpine and lowland habitat for species covered in this study has mainly been based on vibrational bands associated with carbohydrates. The previous studies have found a relation between temperature and carbohydrate content in pollen. Van Herpen (1986) has found that low temperature during pollen development induce less low-molecular weight carbohydrates in pollen. In contrary to Van Herpen (1986), Vesprini et al (2002) have found that polysaccharide content decreases with lower temperature, while the content of sucrose and monosaccharaides would increase. This suggests that the cytoplasmic carbohydrates play a role in low temperature resistance. These two studies have implemented different methods. Van Herpen (1986) have measured carbohydrates for plants that have been growing in different temperature regimes where the low temperature was 19.5 °C. Vesprini et al (2002) have collected pollen from the species that are pollinating early in the spring, and stored them at different temperatures, where the low temperature was 0 °C. An alpine habitat will generally have lower air temperature than lowland so plants pollinating here must be able to tolerate temperatures down to freezing point. The plants collected from alpine region in this study have experienced temperatures close to the freezing point roughly one week prior to sampling. During a summer day the plant temperature in alpine can be 25-30°C (Steinacher & Wagner 2012). Therefore the pollen in the alpine has to tolerate both low and high temperatures. This can be similar to the environment conditions experienced by plants that flower in the early spring, which Vesprini et al. (2002) have used. However, Vesprini et al. (2002) have started temperature treatment after collecting of the plants, while in this study the temperature have affected the pollen during pollen development. Therefore, the study of Van Herpen (1986) would be more comparable with this study since the temperature treatment was started during plant growth. It should be noted that the low temperature in Van Herpen's study was 19.5°C, while the highest air temperature measured in Jotunheimen in 2013 was 15.3°C. Although, these two previous studies are not entirely comparable with the present study, the fact that the previous studies detected temperature dependent variation in carbohydrates is supported by this thesis.

Protein was also found to influence the separation between alpine and lowland habitat. In the study by Van Herpen (1981) it was found that pollen grain that had developed under low temperature had less amount of protein than grains developed at high temperature.

Plant cells are known to change composition of membrane lipids if the temperature decrease (Quinn 1988). Since pollen grains have the same organelles as plant cells, except chloroplasts, it is reasonable to

assume that with temperature decrease there will be a change in membrane lipids in pollen as well. Van Herpen (1986) have found no differences in lipid composition in pollen between plants growing in low and high temperature. It is difficult to detect difference between saturated and unsaturated lipids in the method which was used in this study, since pollen is a complex mixture of biochemicals. Though, there are no vibrations associated with lipids that are detected to separate between alpine and lowland habitat. This could suggest that, contrary to plant cells, composition of pollen lipids is not temperature dependent, and that role of carbohydrates is more important than lipids.

Temperature also has great effect on pollen performance. Although pollen performance was not directly studied in this thesis it is believed that energy reserves in pollen have an effect on pollen tube growth. Pollen tube growth is often used as a measurement of pollen performance. Reduced or more slow pollen growth when temperature is low seems to be the common result (Elgersma et al. 1989; Johannsson et al. 1994; Steinacher & Wagner 2012; Stephenson et al. 1992). Steinacher and Wagner (2012) used alpine plants in their study, and have found that pollen tube could grow close to freezing temperature. This could imply that the plant was adapted to alpine night temperatures. Although Steinacher and Wagner (2012) have used plants which can only be found in alpine environment, it is reasonable to believe that other plants which are growing in the alpine, could have some of the same effect. The pollen energy reserves could be the substances that enable pollen tube to grow in low temperature.

Ranunculus differs from *Avenella* and *Anthoxanthum* by having different ratio of main components. As discussed in chapter 4.1 there are many factors that separate *Ranunculus* from the other study species. For example, pollen of grasses will leave the anther as soon as it opens, while entomophilous pollen delay its departure (Pacini 1996). Because of this the time between maturation and germination will vary between *Ranunculus* and the grasses. These variations might give different requirements for storage compounds in pollen. Ratio could also vary with the size of the pollen grain. Pollen size has been shown to be bigger if the plant has been grown with more nutrients (Lau & Stephenson 1993; Lau & Stephenson 1994; Stephenson et al. 1994) and if it contain a lot of starch (Baker & Baker 1979). In case of significant difference in size between alpine and lowland pollen grains this would be shown in the spectra as a difference in ratio between components which can be found in external and internal parts in pollen. Sporopollenin can only be found in exine, so it would be expected to find signals associated with sporopollenin bands in the loading plot. Since this has not been detected in the study, the size of pollen grains was probably the same.

It is not only temperature that is different between alpine and lowland habitat. With increased altitude the areal flux of solar radiation will increase, which greatly affect the plants (Körner 2003). The

combination of high solar radiation and low temperature may give photoinhibition. Antioxidants are known to prevent photoinhibition, and studies have found higher amount of these compounds in alpine plant species (Sickel et al. 2012). Since cells in pollen do not have chloroplast, it will not be exposed to photoinhibition. However, pollen contains carotenoids, although their purpose in pollen is unclear. It is considered that they might protect grain against light-induces damage (Stanley & Linskens 1974). Schulte et al. (2009) have used Raman spectroscopy to find variation in carotenoids in pollen species, but this has not been possible with FTIR.

Since this study was based on collecting pollen in field there were some factors that could affect the results. The weather prior to sampling was quite different between the sites. The month until sampling in Follo was rainy while in Jotunheimen it was almost without precipitation. It has been shown that climate in advance to dehisce can affect pollen chemical composition (Zimmermann & Kohler 2014). Although the weather in Follo was more rainy and colder than usual it was not abnormal for this region, not either for Jotunheimen. *Ranunculus* in Follo was sampled both in June and in August, but there was no separation in the PCA between these samples. This could indicate that the separation due to different habitat is more important than climate in advance of sampling.

4.3 Variation in populations

This study shows that different plant populations have common variations in chemical composition. It is difficult to determine exactly what these variations are since the populations are overlapping to some extent. The main signals in PCA are associated with carbohydrates. The grouping of each population in the score plot shows that reproducibility of the measurements was good. The populations in the alpine region are geographical located closer than the populations in the lowland. The reason for this is that it was easier to find the different populations in the alpine because of the open landscape, compared with lowland. The variance between populations in alpine and lowland seems to be the same in the study species, which indicate that the samples can be separated by populations, although this difference is small.

The separation of chemical components between populations could be due to variations in microclimate and/or edaphic growth site conditions. It has been shown that nutrient could affect pollen performance and phosphorous content of pollen (Lau & Stephenson 1993; Lau & Stephenson 1994). Different plant species have basic growth requirements, i.e. given amounts of light and nutrient, in common, which allow co-existence of different species under seemingly similar habitats conditions. This may be the reason for why the separations between populations are not larger. Genetic differentiation is considered to be connected to different populations (Ohlson 1989), and this could induce the small

separations between populations in this study. The soil is not investigated in this study, and relationship between soil conditions and pollen quality is thus outside the scope of the study.

4.4 Method

Even though infrared spectroscopy has strong potential as a method to study pollen quality, there are some issues that have to be addressed. Two different pre-processing techniques have been used on the spectra, in this study. For some of the PCA there has been more convenient to use EMSC corrected spectra without employing second derivative. Although, second derivated spectra are considered to give more information since overlapping peaks are resolved. By transforming spectral set into second derivatives, some of the bands can be suppressed, especially broad bands, this can reduce the separation between samples. This could be negative because some important variations will not be shown. For example in Figure 15 this separation is not present for second derivated spectra, but only in spectral set which is EMSC correlated. Though, the separation is caused by samples that are deviating from the normal samples. PCA performed on second derivative spectra give more information. However due to overlapping peaks the interpretation of PCA results can be extremely difficult, especially if a combination of principal components is making the separation.

The ATR technique has been showed to give distinct separation between genera and species, as found by other studies (Gottardini et al. 2007; Mularczyk-Oliwa et al. 2012; Zimmermann 2010; Zimmermann & Kohler 2014). This technique has more focus on the lower wavenumbers, meaning that the region with carbohydrate will be more overestimated. In this study signals associated with carbohydrates were the most prominent in the loading plots for almost all PCAs. Still there are some PCA loading plots which have strong signals in the high wavenumber region that can be associated with proteins and lipids. By grinding pollen samples, to investigate both internal and external parts, also seem successful since there has been detected both sporopollenin and carbohydrates in the spectra. This all indicates that ATR is a suitable tool for pollen analysis.

The conventional methods for pollen analysis are more time consuming than FTIR since the samples usually need some chemical pre-treatment (Weber 1998). The FTIR method provides a lot of information on chemical composition of pollen obtained by simple and rapid measurement. Although FTIR only can provide information about the main chemical composition in pollen it is a method that offers complementary information to the quantitative methods. This gives a possibility to analyze large number of pollen samples from a variety of species and environments. Combining IR spectroscopy with other quantitative methods will give a more complete picture of pollen biology, physiology and ecology.

5 Conclusion

This study shows that alpine and lowland populations of plants differ in terms of pollen quality. In particular this is the case as regard chemical components that are related to energy reserves in pollen. Relatively large energy reserves in alpine pollen could make more suitable for this specific environment and able to grow pollen tube even at temperatures close to zero. The chemical composition of pollen was in general clearly species-specific, and there were less clear differences between populations of the same species. All this variations can be determined by the use of infrared spectroscopy, but methodological limitations make it hard to precisely determine what components that is responsible for the differentiations among species and populations.

For future research on pollen it would be interesting to study species from additional climatic environments, such as coastal areas, continental areas or areas at different latitudes, and relate this to site-specific growth conditions. Qualitative methods for careful characterization of chemical components would be an interesting and promising complement to infrared spectroscopy, for more accurate assignation of vibrational bands in infrared spectra of pollen.

6 Reference

Afseth, N. K. & Kohler, A. (2012). Extended Multiplicative Signal Correction In Vibrational Spectroscopy, A Tutorial. *Chemometrics And Intelligent Laboratory Systems*, 117: 92-99.

Baker, H. G. & Baker, I. (1979). Starch In Angiosperm Pollen Grains And Its Evolutionary Significance. *American Journal Of Botany*, 66 (5): 591-600.

Baker, H. G. & Baker, I. (1982). Starchy And Starchless Pollen In The Onagraceae. *Annals Of The Missouri Botanical Garden*, 69 (4): 748-754.

Barth, A. (2007). Infrared Spectroscopy Of Proteins. *Biochimica Et Biophysica Acta-Bioenergetics*, 1767 (9): 1073-1101.

Dell'Anna, R., Lazzeri, P., Frisanco, M., Monti, F., Campeggi, F. M., Gottardini, E. & Bersani, M. (2009). Pollen Discrimination And Classification By Fourier Transform Infrared (FT-IR) Microspectroscopy And Machine Learning. *Analytical And Bioanalytical Chemistry*, 394 (5): 1443-1452.

Delph, L. F., Johannsson, M. H. & Stephenson, A. G. (1997). How Environmental Factors Affect Pollen Performance: Ecological And Evolutionary Perspectives. *Ecology*, 78 (6): 1632-1639.

Edwards, J. & Jordan, J. R. (1992). Reversible Anther Opening In *Lilium-philadelphicum* (Lilaceae): A Possible Means Of Enhancing Male Fitness. *American Journal Of Botany*, 79 (2): 144-148.

Elgersma, A., Stephenson, A. G. & Nijs, A. P. M. (1989). Effects Of Genotype And Temperature On Pollen Tube Growth In Perennial Ryegrass *(Lolium perenne L.)*. *Sexual Plant Reproduction*, 2 (4): 225-230.

Franchi, G. G., Bellani, L., Nepi, M. & Pacini, E. (1996). Types Of Carbohydrate Reserves In Pollen: Localization, Systematic Distribution And Ecophysiological Significance. *Flora*, 191 (2): 143-159.

Gottardini, E., Rossi, S., Cristofolini, F. & Benedetti, L. (2007). Use Of Fourier Transform Infrared (FT-IR) Spectroscopy As A Tool For Pollen Identification. *Aerobiologia*, 23 (3): 211-219.

Heslop-Harrison, J. (1979). An Interpretation Of The Hydrodynamics Of Pollen. *American Journal of Botany*, 66 (6): 737-743.

Ivleva, N. P., Niessner, R. & Panne, U. (2005). Characterization And Discrimination Of Pollen By Raman Microscopy. *Analytical And Bioanalytical Chemistry*, 381 (1): 261-267.

Johannsson, M. H., Winsor, J. A. & Stephenson, A. G. (1994). *Genetic And Environmental Effects On In Vitro Pollen Tube Growth In Cucurbita*. Pollen-Pistil Interactions and Pollen Tube Growth, vol. 12. Rockville: Amer Soc Plant Physiologists. 307-309 pp.

Keijzer, C. J. (1987). The Processes Of Anther Dehiscence And Pollen Dispersal. 2. The Formation And The Transfer Mechanism Of Pollenkitt, Cell-Wall Development Of The Loculus Tissues And A Function Of Orbicules In Pollen Dispersal. *New Phytologist*, 105 (3): 499-507.

Kohler, A., Hanafi, M., Bertrand, D., Qannari, E. M., Janbu, A. O., Møretrø, T., Naterstad, K. & Martens, H. (2008). Interpreting Several Types Of Measurements In Bioscience. In Lasch, P. & Kneipp, J. (eds) *Biomedical Vibrational Spectroscopy*, pp. 333-356. USA: John Wiley & Sons.

Kohler, A., Zimonja, M., Segtnan, V. & Martens, H. (2009). Standard Normal Variate, Multiplicative Signal Correction And Extended Multiplicative Signal Correction Preprocessing In Biospectroscopy. In Brown, S. D., Tauler, R. & Walczak, B. (eds) *Comprehensive Chemometrics*, pp. 139-162. Oxford: Elsevier.

Kohler, A., Kristian Afseth, N. & Martens, H. (2010). Chemometrics In Biospectroscopy. In Chalmers, J. M. & Griffiths, P. R. (eds) vol. 1 *Handbook of Vibrational Spectroscopy*. Chichester: Wiley.

Körner, C. (2003). *Alpine Plant Life: Functional Plant Ecology Of High Mountain Ecosystems*. 2 ed. Berlin: Springer. 344 pp.

Lambers, H., Pons, T. L. & Chapin, F. S. (2008). *Plant Physiological Ecology*. 2 ed. New York: Springer. 604 pp.

Lau, T. C. & Stephenson, A. G. (1993). Effects Of Soil-Nitrogen On Pollen Production, Pollen Grain-Size, And Pollen Performance In *Cucurbita-Pepo* (Cucurbitaceae). *American Journal Of Botany*, 80 (7): 763-768.

Lau, T. C. & Stephenson, A. G. (1994). Effects Of Soil-Phosphorus On Pollen Production, Pollen Size, Pollen Phosphorus-Content, And The Ability To Sire Seeds In *Cucurbita-Pepo* (Cucurbitaceae). *Sexual Plant Reproduction*, 7 (4): 215-220.

Laucks, M. L., Roll, G., Schweiger, G. & Davis, E. J. (2000). Physical And Chemical (Raman) Characterization Of Bioaerosols - Pollen. *Journal Of Aerosol Science*, 31 (3): 307-319.

Moen, A., Lillethun, A. & Odland, A. (1999). *National Atlas Of Norway: Vegetation*. Hønefoss: Norwegian Mapping Authority. 200 pp.

Moore, P. D., Webb, J. A. & Collinson, M. E. (1991). *Pollen Analysis*. Oxford: Blackwell Scientific Publications. 216 pp.

Mossberg, B., Stenberg, L., Mossberg, B., Båtvik, S., Moen, S. & Karlsson, T. (2007). *Gyldendals Store Nordiske Flora*. Oslo: Gyldendal. 928 pp.

Mularczyk-Oliwa, M., Bombalska, A., Kaliszewski, M., Wlodarski, M., Kopczynski, K., Kwasny, M., Szpakowska, M. & Trafny, E. A. (2012). Comparison Of Fluorescence Spectroscopy And FTIR In Differentiation Of Plant Pollens. *Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy*, 97: 246-254.

Nepi, M. & Franchi, G. G. (2000). Cytochemistry Of Mature Angiosperm Pollen. *Plant Systematics And Evolution*, 222: 45-62.

NGU. (2011). *Norges Geologiske Undersøkelse: Berggrunn* (accessed: 04.02.14).

Ohlson, M. (1989). Ecotypic Differentiation And Phenotypic Plasticity In *Saxifraga hirculus* Populations In Central And Northern Sweden. *Ecography*, 12 (1): 46-53.

Pacini, E., Franchi, G. G. & Hesse, M. (1985). The Tapetum: Its Form, Function, And Possible Phylogeny In *Embryophyta*. *Plant Systematics and Evolution*, 149: 155-185.

Pacini, E. (1996). Types And Meaning Of Pollen Carbohydrate Reserves. *Sexual Plant Reproduction*, 9 (6): 362-366.

Pacini, E. (2000). From Anther And Pollen Ripening To Pollen Presentation. *Plant Systematics and Evolution*, 222: 19-43.

Pappas, C. S., Tarantilis, P. A., Harizanis, P. C. & Polissiou, M. G. (2003). New Method for Pollen Identification by FT-IR Spectroscopy. *Applied Spectroscopy*, 57 (1): 23-27.

Quinn, P. J. (1988). Effects Of Temperature On Cell-Membranes. In Long, S. P. & Woodward, F. I. (eds) vol. 42 *Plants And Temperature*, pp. 237-258. Cambridge: Company Biologists Ltd.

Roulston, T. H., Cane, J. H. & Buchmann, S. L. (2000). What Governs Protein Content Of Pollen: Pollinator Preferences, Pollen-Pistil Interactions, Or Phylogeny? *Ecological Monographs*, 70 (4): 617-643.

Sanger, J. M. & Jackson, W. T. (1971a). Fine Structure Study Of Pollen Development In *Haemanthus katherinae* Baker. 1: Formation Of Vegetative And Generative Cells. *Journal of Cell Science*, 8 (2): 289-301.

Sanger, J. M. & Jackson, W. T. (1971b). Fine Structure Study Of Pollen Development In *Haemanthus katherinae* Baker. 3: Changes In Organelles During Development Of Vegetative Cell. *Journal Of Cell Science*, 8 (2): 317-329.

Schulte, F., Lingott, J., Panne, U. & Kneipp, J. (2008). Chemical Characterization And Classification Of Pollen. *Analytical Chemistry*, 80 (24): 9551-9556.

Schulte, F., Mader, J., Kroh, L. W., Panne, U. & Kneipp, J. (2009). Characterization Of Pollen Carotenoids With In Situ And High-Performance Thin-Layer Chromatography Supported Resonant Raman Spectroscopy. *Analytical Chemistry*, 81 (20): 8426-8433.

Sears, P. & Metcalf, E. (1926). The Behaviour Of Pollen Starch In A Geranium And Its Bud Sport. *Journal OF Genetics*, 17 (1): 33-42.

Shivanna, K. R. (2003). *Pollen Biology And Biotechnology*. Enfield: Science Publishers. 301 pp.

Shurvell, H. (2002). Spectra–Structure Correlations In The Mid-And Far-Infrared. In Chalmers, J. M. & Griffiths, P. R. (eds) vol. 3 *Handbook Of Vibrational Spectroscopy*, pp. 1783-1816. Chichester: John Wiley & Sons.

Sickel, H., Bilger, W. & Ohlson, M. (2012). High Levels Of α‐Tocopherol In Norwegian Alpine Grazing Plants. *Journal Of Agricultural And Food Chemistry*, 60 (31): 7573-7580.

Smith, B. C. (2011). *Fundamentals Of Fourier Transform Infrared Spectroscopy*. 2 ed. Boca Raton: CRC press.

Stanley, R. G. & Linskens, H. F. (1974). *Pollen: Biology Biochemistry Management*. Berlin: Springer. 307 pp.

Steinacher, G. & Wagner, J. (2012). Effect Of Temperature On The Progamic Phase In High-Mountain Plants. *Plant Biology*, 14 (2): 295-305.

Stephenson, A. G., Lau, T. C., Quesada, M. & Winsor, J. A. (1992). Factors That Affect Pollen Performance. In Wyatt, R. (ed.) *Ecology and Evolution of Plant Reproduction*, pp. 119-136. New York: Routledge Chapman & Hall Inc.

Stephenson, A. G., Erickson, C. W., Lau, T. C., Quesada, M. R. & Winsor, J. A. (1994). Effects Of Growing Conditions On The Male Gametophyte. In Stephenson, A. G. & Kao, T. H. (eds) vol. 12 *Pollen-Pistil Interactions And Pollen Tube Growth*, pp. 220-229. Rockville: Amer Soc Plant Physiologists.

Taiz, L. & Zeiger, E. (2010). *Plant Physiology*. 5 ed. Sunderland, Mass.: Sinauer Associates. 782 pp.

Van Bilsen, D. & Hoekstra, F. A. (1993). Decreased Membrane Integrity In Aging Typha-Latifolia L Pollen - Accumulation Of Lysolipids And Free Fatty-Acids. *Plant Physiology*, 101 (2): 675-682.

Van Herpen, M. M. A. (1981). Effect Of Season, Age And Temperature On The Protein Pattern Of Pollen And Styles In Petunia Hybrida. *Acta Botanica Neerlandica*, 30 (4): 277-287.

Van Herpen, M. M. A. & Linskens, H. F. (1981). Effect Of Season, Plant-Age And Temperature During Plant-Growth On Compatible And Incompatible Pollen-Tube Growth In Petunia-Hybrida. *Acta Botanica Neerlandica*, 30 (3): 209-218.

Van Herpen, M. M. A. (1986). Biochemical Alterations In The Sexual Partners Resulting From Environmental Conditions Before Pollination Regulate Processes After Pollination. In Mulcahy, D., Mulcahy, G. & Ottaviano, E. (eds) *Biotechnology And Ecology Of Pollen*, pp. 131-133. New York: Springer

Vesprini, J. L., Nepi, M., Cresti, L., Guarnieri, M. & Pacini, E. (2002). Changes In Cytoplasmic Carbohydrate Content During Helleborus Pollen Presentation. *Grana*, 41 (1): 16-20.

Vesprini, J. L. & Pacini, E. (2005). Temperature-Dependent Floral Longevity In Two Helleborus Species. *Plant Systematics and Evolution*, 252: 63-70.

Weber, R. W. (1998). Pollen Identification. *Annals of Allergy Asthma & Immunology*, 80 (2): 141-145.

Zimmermann, B. (2010). Characterization Of Pollen By Vibrational Spectroscopy. *Applied Spectroscopy*, 64 (12): 1364-1373.

Zimmermann, B. & Kohler, A. (2014). *Infrared Spectroscopy Of Pollen Identifies Plant Species And Genus As Well As Environmental Conditions*. PLoS ONE, 9, 4: Public Library of Science. p. e95417. doi:10.1371/journal.pone.0095417.

Sample list

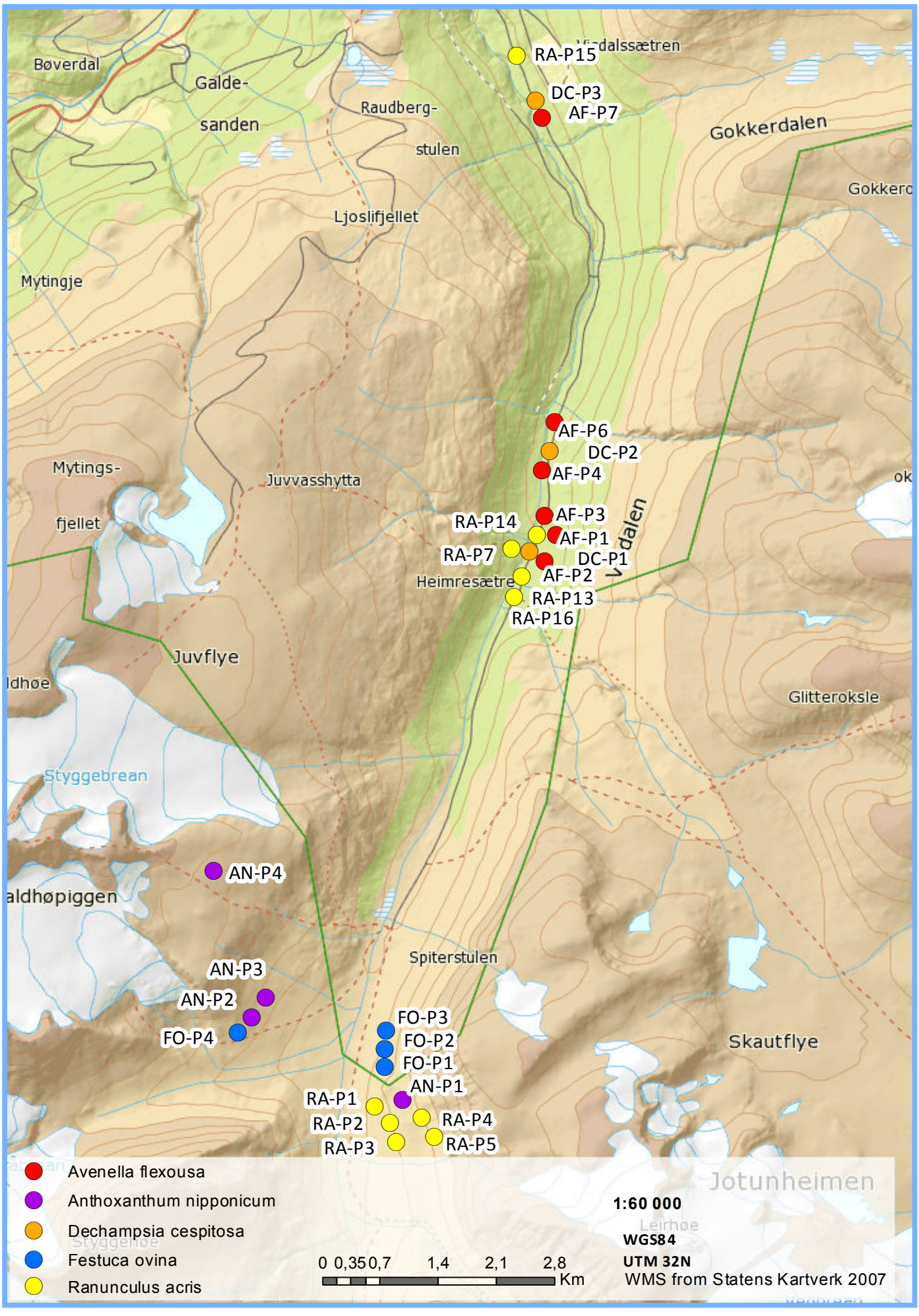
The sample list is showing the samples which is analyzed with infrared spectroscopy. The samples which have a “h” in the code number are from an alpine habitat, while those from a lowland habitat have a “l” in the code number (these are also marked with grey).

Speices	Code number	Sampling date	East coordinates	North coordinates	Altitude	Population
Anthoxanthum nipponicum	**AN13h02**	22.07.2013	468592	6830607	1350 m	AN-P1
Anthoxanthum nipponicum	**AN13h03**	22.07.2013	468592	6830607	1350 m	AN-P1
Anthoxanthum nipponicum	**AN13h04**	22.07.2013	468592	6830607	1350 m	AN-P1
Anthoxanthum nipponicum	**AN13h05**	22.07.2013	468592	6830607	1350 m	AN-P1
Anthoxanthum nipponicum	**AN13h06**	22.07.2013	468592	6830607	1350 m	AN-P1
Anthoxanthum nipponicum	**AN13h07**	23.07.2013	466792	6831596	1383 m	AN-P2
Anthoxanthum nipponicum	**AN13h08**	23.07.2013	466792	6831596	1383 m	AN-P2
Anthoxanthum nipponicum	**AN13h11**	23.07.2013	466948	6831823	1394 m	AN-P3
Anthoxanthum nipponicum	**AN13h12**	23.07.2013	466948	6831823	1394 m	AN-P3
Anthoxanthum nipponicum	**AN13h13**	23.07.2013	466948	6831823	1394 m	AN-P3
Anthoxanthum nipponicum	**AN13h14**	23.07.2013	466948	6831823	1394 m	AN-P3
Anthoxanthum nipponicum	**AN13h15**	23.07.2013	466948	6831823	1394 m	AN-P3
Anthoxanthum odoratum	**AO13l02**	18.06.2013	612447	6621505	163 m	AO-P1
Anthoxanthum odoratum	**AO13l03**	18.06.2013	612447	6621518	160 m	AO-P1
Anthoxanthum odoratum	**AO13l04**	18.06.2013	612538	6621825	149 m	AO-P1
Anthoxanthum odoratum	**AO13l05**	18.06.2013	612545	6621818	156 m	AO-P1
Anthoxanthum odoratum	**AO13l06**	18.06.2013	612543	6621826	153 m	AO-P1
Anthoxanthum odoratum	**AO13l07**	06.07.2013	597947	6630252	120 m	AO-P2
Avenella flexuosa	**AF13h01**	25.07.2013	470211	6837388	912 m	AF-P1
Avenella flexuosa	**AF13h02**	25.07.2013	470211	6837388	912 m	AF-P1
Avenella flexuosa	**AF13h03**	25.07.2013	470090	6837131	905 m	AF-P2
Avenella flexuosa	**AF13h04**	25.07.2013	470090	6837131	905 m	AF-P2
Avenella flexuosa	**AF13h05**	25.07.2013	470090	6837131	905 m	AF-P2
Avenella flexuosa	**AF13h06**	25.07.2013	470090	6837131	905 m	AF-P2
Avenella flexuosa	**AF13h07**	25.07.2013	470198	6837413	887 m	AF-P3
Avenella flexuosa	**AF13h08**	25.07.2013	470268	6838181	893 m	AF-P4
Avenella flexuosa	**AF13h10**	25.07.2013	470268	6838181	893 m	AF-P4
Avenella flexuosa	**AF13h11**	25.07.2013	470394	6838762	889 m	AF-P5
Avenella flexuosa	**AF13h12**	25.07.2013	470394	6838762	889 m	AF-P5
Avenella flexuosa	**AF13h13**	25.07.2013	470394	6838762	889 m	AF-P5
Avenella flexuosa	**AF13h14**	25.07.2013	470394	6838762	889 m	AF-P5
Avenella flexuosa	**AF13h16**	25.07.2013	470394	6838761	875 m	AF-P6
Avenella flexuosa	**AF13h17**	25.07.2013	470394	6838761	875 m	AF-P6
Avenella flexuosa	**AF13h18**	25.07.2013	470394	6838761	875 m	AF-P6
Avenella flexuosa	**AF13h19**	25.07.2013	470394	6838761	875 m	AF-P6
Avenella flexuosa	**AF13h20**	26.07.2013	470394	6838761	875 m	AF-P6
Avenella flexuosa	**AF13h21**	26.07.2013	470201	6842619	774 m	AF-P7
Avenella flexuosa	**AF13h24**	25.07.2013	470201	6842619	774 m	AF-P7

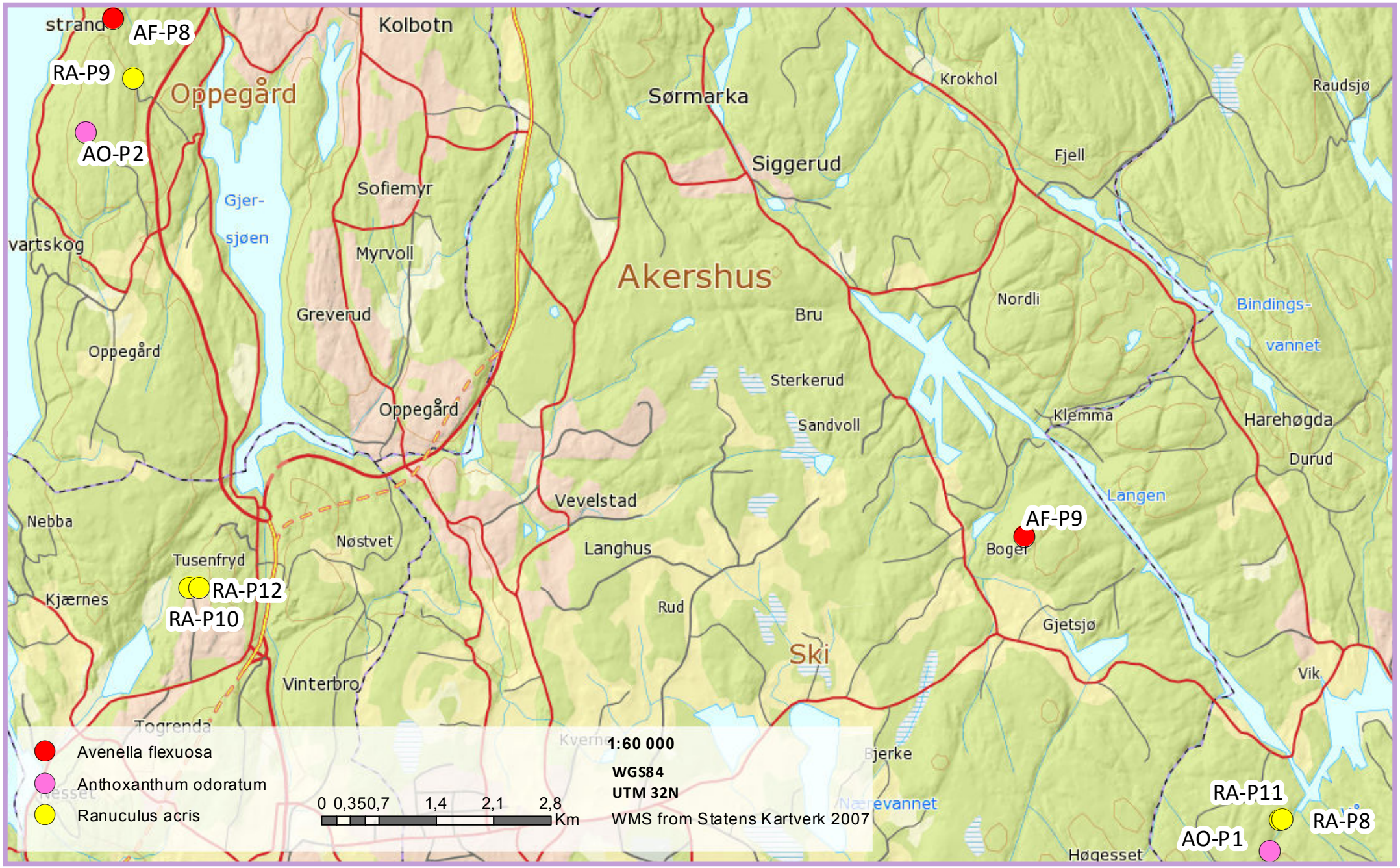
Avenella flexuosa	**AF13l01**	01.07.2013	598289	6631631	122 m	AF-P8
Avenella flexuosa	**AF13l02**	01.07.2013	598287	6631665	104 m	AF-P8
Avenella flexuosa	**AF13l03**	01.07.2013	598307	6631732	114 m	AF-P8
Avenella flexuosa	**AF13l10**	07.07.2013	609402	6625295	221 m	AF-P9
Avenella flexuosa	**AF13l11**	07.07.2013	609397	6625298	219 m	AF-P9
Deschampsia cespitosa	**DC13h04**	25.07.2013	470124	6837206	910 m	DC-P1
Deschampsia cespitosa	**DC13h05**	25.07.2013	470124	6837206	910 m	DC-P2
Deschampsia cespitosa	**DC13h06**	25.07.2013				DC-P3
Deschampsia cespitosa	**DC13h07**	25.07.2013				DC-P4
Deschampsia cespitosa	**DC13h08**	25.07.2013				DC-P4
Deschampsia cespitosa	**DC13h09**	26.07.2013	470202	6842619	774 m	DC-P5
Deschampsia cespitosa	**DC13h10**	26.07.2013	470202	6842619	774 m	DC-P5
Festuca ovina	**FO13h01**	23.07.2013	468369	6831051	1249 m	FO-P1
Festuca ovina	**FO13h02**	23.07.2013	468369	6831051	1249 m	FO-P1
Festuca ovina	**FO13h03**	23.07.2013	468369	6831051	1249 m	FO-P1
Festuca ovina	**FO13h04**	23.07.2013	468369	6831051	1249 m	FO-P1
Festuca ovina	**FO13h05**	23.07.2013	468369	6831051	1249 m	FO-P1
Festuca ovina	**FO13h06**	23.07.2013	468378	6831216	1209 m	FO-P2
Festuca ovina	**FO13h07**	23.07.2013	468378	6831216	1209 m	FO-P2
Festuca ovina	**FO13h08**	23.07.2013	468378	6831216	1209 m	FO-P2
Festuca ovina	**FO13h09**	23.07.2013	468378	6831216	1209 m	FO-P2
Festuca ovina	**FO13h10**	23.07.2013	468380	6831224	1210 m	FO-P3
Festuca ovina	**FO13h11**	23.07.2013	468380	6831224	1210 m	FO-P3
Festuca ovina	**FO13h12**	23.07.2013	468380	6831224	1210 m	FO-P3
Festuca ovina	**FO13h13**	23.07.2013	468380	6831224	1210 m	FO-P3
Festuca ovina	**FO13h14**	23.07.2013	468380	6831224	1210 m	FO-P3
Festuca ovina	**FO13h15**	23.07.2013	466611	6831407	1363 m	FO-P4
Ranunculus acris	**RA13h01**	22.07.2013	468281	6830435	1236 m	RA-P1
Ranunculus acris	**RA13h02**	22.07.2013	468281	6830435	1236 m	RA-P1
Ranunculus acris	**RA13h03**	22.07.2013	468281	6830435	1236 m	RA-P1
Ranunculus acris	**RA13h04**	22.07.2013	468281	6830435	1236 m	RA-P1
Ranunculus acris	**RA13h05**	22.07.2013	468332	6830388	1260 m	RA-P1
Ranunculus acris	**RA13h06**	22.07.2013	468332	6830388	1260 m	RA-P2
Ranunculus acris	**RA13h07**	22.07.2013	468332	6830388	1260 m	RA-P2
Ranunculus acris	**RA13h08**	22.07.2013	468332	6830388	1260 m	RA-P2
Ranunculus acris	**RA13h09**	22.07.2013	468332	6830388	1260 m	RA-P2
Ranunculus acris	**RA13h10**	22.07.2013	468468	6830375	1285 m	RA-P3
Ranunculus acris	**RA13h11**	22.07.2013	468468	6830375	1285 m	RA-P3
Ranunculus acris	**RA13h12**	22.07.2013	468468	6830375	1285 m	RA-P3
Ranunculus acris	**RA13h13**	22.07.2013	468468	6830375	1285 m	RA-P3
Ranunculus acris	**RA13h14**	22.07.2013	468468	6830375	1285 m	RA-P3
Ranunculus acris	**RA13h15**	22.07.2013	468807	6830384	1327 m	RA-P4
Ranunculus acris	**RA13h16**	22.07.2013	468807	6830384	1327 m	RA-P4
Ranunculus acris	**RA13h17**	22.07.2013	468807	6830384	1327 m	RA-P4

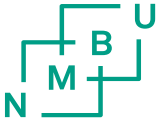
Ranunculus acris	**RA13h18**	22.07.2013	468807	6830384	1327 m	RA-P4
Ranunculus acris	**RA13h19**	22.07.2013	468807	6830384	1327 m	RA-P4
Ranunculus acris	**RA13h20**	22.07.2013	468929	6830298	1404 m	RA-P5
Ranunculus acris	**RA13h21**	24.07.2013	470015	6836772	948 m	RA-P6
Ranunculus acris	**RA13h22**	24.07.2013	470015	6836772	948 m	RA-P6
Ranunculus acris	**RA13h23**	24.07.2013	470015	6836772	948 m	RA-P6
Ranunculus acris	**RA13h24**	24.07.2013	470015	6836772	948 m	RA-P6
Ranunculus acris	**RA13h25**	24.07.2013	470015	6836772	948 m	RA-P6
Ranunculus acris	**RA13h26**	24.07.2013	470029	6836897	928 m	RA-P13
Ranunculus acris	**RA13h27**	24.07.2013	470029	6836897	928 m	RA-P13
Ranunculus acris	**RA13h28**	24.07.2013	470029	6836897	928 m	RA-P13
Ranunculus acris	**RA13h29**	24.07.2013	470029	6836897	928 m	RA-P13
Ranunculus acris	**RA13h30**	25.07.2013	470123	6837232	932 m	RA-P7
Ranunculus acris	**RA13h31**	25.07.2013	470123	6837232	932 m	RA-P7
Ranunculus acris	**RA13h32**	25.07.2013	470123	6837232	932 m	RA-P7
Ranunculus acris	**RA13h33**	25.07.2013	470123	6837232	932 m	RA-P7
Ranunculus acris	**RA13h34**	25.07.2013	470123	6837232	932 m	RA-P7
Ranunculus acris	**RA13h35**	25.07.2013	470216	6837398	925 m	RA-P14
Ranunculus acris	**RA13h36**	25.07.2013	470216	6837398	925 m	RA-P14
Ranunculus acris	**RA13h37**	25.07.2013	470216	6837398	925 m	RA-P14
Ranunculus acris	**RA13h38**	25.07.2013	470216	6837398	925 m	RA-P14
Ranunculus acris	**RA13h39**	25.07.2013	470216	6837398	925 m	RA-P14
Ranunculus acris	**RA13h40**	26.07.2013	469966	6843155	765 m	RA-P15
Ranunculus acris	**RA13h41**	26.07.2013	469966	6843155	765 m	RA-P15
Ranunculus acris	**RA13h42**	26.07.2013	469966	6843155	765 m	RA-P15
Ranunculus acris	**RA13h43**	26.07.2013	469966	6843155	765 m	RA-P15
Ranunculus acris	**RA13l01**	18.06.2013	612556	6621844	140 m	RA-P8
Ranunculus acris	**RA13l02**	18.06.2013	612554	6621834	164 m	RA-P8
Ranunculus acris	**RA13l03**	01.07.2013	598530	6630901	94 m	RA-P9
Ranunculus acris	**RA13l04**	01.07.2013	598533	6630924	98 m	RA-P9
Ranunculus acris	**RA13l05**	01.07.2013	598547	6630982	98 m	RA-P9
Ranunculus acris	**RA13l06**	01.07.2013	598540	6631002	93 m	RA-P9
Ranunculus acris	**RA13l07**	01.07.2013	598540	6631005	92 m	RA-P9
Ranunculus acris	**RA13l08**	06.07.2013	599220	6624687	67 m	RA-P10
Ranunculus acris	**RA13l09**	06.07.2013	599222	6624678	48 m	RA-P10
Ranunculus acris	**RA13l10**	06.07.2013	599217	6624669	35 m	RA-P10
Ranunculus acris	**RA13l11**	06.07.2013	599196	6624542	62 m	RA-P10
Ranunculus acris	**RA13l12**	06.07.2013	599288	6624773	90 m	RA-P10
Ranunculus acris	**RA13l13**	07.07.2013	612581	6621871	185 m	RA-P11
Ranunculus acris	**RA13l14**	07.07.2013	612575	6621881	188 m	RA-P11
Ranunculus acris	**RA13l15**	07.07.2013	612561	6621927	222 m	RA-P11
Ranunculus acris	**RA13l16**	01.08.2013	599346	6624681	80 m	RA-P12
Ranunculus acris	**RA13l17**	01.08.2013	599346	6624681	80 m	RA-P12
Ranunculus acris	**RA13l18**	01.08.2013	599346	6624681	80 m	RA-P12

Study area - Jotunheimen



Study area - Follo





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