4. Species and interactions

AE Eycott & A Jentsch (editors)

Impacts of climate change on ecosystems and their functioning are, to a large extent, mediated through the impacts on and responses of the **living organisms** in the ecosystem. Climate change can be expected to have direct effects at all levels of biological organisation, with interactions (competition, mutualism, predation, decomposition, etc.) leading to often complex indirect impacts (e.g. Lurgi et al., 2012; Millon et al., 2014). Understanding how organisms, individually and in interaction with each other, are affected by and feed back to changes in the Earth system, is therefore an important research challenge.

Monitoring species and interactions is the key to understanding the processes that drive changes in taxonomic and functional biodiversity, which underpins ecosystem functioning and biodiversity conservation value. In this chapter we provide guidance on quantifying the consequences of climate change for a broad range of organisms and ecological processes, across levels of organisation, through experimental or observational approaches. Many of the methods discussed are suitable for experimental or observational approaches to other global changes, for example to nutrient manipulation experiments or CO_2 enrichment studies but we focussed on methods appropriate at scales of tens to hundreds of centimetres, with a preference for repeatable types of measurement. The majority of the protocols are about plants as they comprise the majority of biomass in most terrestrial ecosystems and their sessile nature means that exposure to treatments is consistent (Moise & Henry, 2010). The remaining protocols are on first-order plant-animal interactions that mediate changes in vegetation dynamics following climate – and other global-change drivers.

At the **level of populations**, we cover vital rates such as reproduction, recruitment, growth, mortality, and phenology, as well as the overall impacts on the dynamics and growth rates of populations. At the **community level**, we provide guidance on the assessment of impacts on plant, invertebrate, and microbial species composition, abundance, and biodiversity, as well as on seed banks and propagule rain. With regards to **species interactions**, we cover pollination, vertebrate and invertebrate herbivory, and pathogens. We also provide a short motivation for and link to the plant traits protocol handbook (Pérez-Harguindeguy et al., 2013). This chapter does not cover organism responses at the individual level, which are, to some extent, dealt with in **chapter 5 on Stress physiology**.

References

Lurgi, M., López, B. C., & Montoya, J. M. (2012). Novel communities from climate change. *Philosophical Transactions of the Royal Society B: Biological Sciences, 367*(1605), 2913-2922.

Millon, A., Petty, S. J., Little, B., Gimenez, O., Cornulier, T., & Lambin, X. (2014). Dampening prey cycle overrides the impact of climate change on predator population dynamics: a long-term demographic study on tawny owls. *Global Change Biology, 20*(6), 1770-1781.

Moise, E. R. D., & Henry, H. A. L. (2010). Like moths to a street lamp: exaggerated animal densities in plot-level global change field experiments. *Oikos, 119*(5), 791-795.

Pérez-Harguindeguy, N., Diaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., … Cornelissen, J. H. C. (2013). New handbook for standardised measurement of plant functional traits worldwide. *Australian Journal of Botany*, 61(3), pp.167-234.

How to cite a protocol:

E.g. Data on phenology was recorded using the method described in protocol 4.5 Aboveground plant phenology in the Supporting Information S4 Species and interactions in Halbritter et al. (2020).

Halbritter et al. (2020) The handbook for standardised field and laboratory measurements in terrestrial climate-change experiments and observational studies (ClimEx). *Methods in Ecology and Evolution*, *11*(1) 22-37.

How to cite an updated protocol version:

E.g. Data on phenology was recorded using the method described in protocol 4.5 Aboveground plant phenology in the Supporting Information S4 Species and interactions in Halbritter et al. (2020), using the updated protocol version, Date, available in the online version: www.climexhandbook.uib.no.

Halbritter et al. (2020) The handbook for standardised field and laboratory measurements in terrestrial climate-change experiments and observational studies (ClimEx). *Methods in Ecology and Evolution*, *11*(1) 22-37.

Table of contents

4.1 Sexual plant reproduction

Authors: Gillespie MAK[1], Hegland SJ[1]

Reviewers: Töpper JP[2,3], Muffler L[4,5]

Measurable unit: various (see below); Measurement scale: plot; Equipment costs: €; Running costs: €; Installation effort: low; Maintenance effort: low; Knowledge need: low; Measurement mode: manual

Plant reproduction refers to an individual plant's ability to produce offspring and can be vegetative or sexual. Vegetative reproduction is considered elsewhere (see protocol 4.3 Plant demography), and this section considers only reproduction via flowers and seeds. Reproduction via flowers and seeds is important for many plant species, even those capable of vegetative or clonal regeneration, because it allows the introduction of genetic variation and thus adaptation to and colonisation of new areas (Baskin & Baskin, 2014). A major issue for sexual plant reproduction today is the so-called pollinator crisis, with suggestions that the abundance and diversity of (mainly insect) pollinators have been strongly reduced in many areas of the world due to various types of global change (e.g. Biesmeijer et al., 2006; Potts et al., 2010). Plant sexual reproduction is also directly affected by climate change through different pathways. For example, various aspects of plant reproduction are likely to be sensitive to changing temperatures including flower size, flower number, seed set, seed size, seed number, and fruit to flower ratio (Arft et al., 1999; Liu et al., 2012; Meineri et al., 2014), as well as nectar and pollen production (Scaven & Rafferty, 2013). There are also documented impacts of elevated carbon dioxide and altered precipitation patterns on plant physiology, which can further affect reproductive fitness positively or negatively (Erhardt & Rusterholz, 1997; Jablonski et al., 2002; Reyer et al., 2013). These aspects of reproduction can be incorporated into protocols for observational or experimental climate-change studies, but could also be investigated as part of other global-change drivers. For example, plant sexual reproduction and plant–pollinator interactions can be affected directly and indirectly by nitrogen deposition (Hoover et al., 2012). Changes in these reproduction variables can have important impacts on community dynamics, as declining reproductive success of some species, and improved success in others, is likely to affect community composition. In particular, changes may have a cascading impact on animal populations that rely on flowers, seeds, or fruits. Pollinators may be affected by changes in flower number, flower size, nectar, and pollen amount and quality (Scaven & Rafferty, 2013). Seed and fruit consumers, including humans, will be impacted by the size, quantity, and quality of their food.

4.1.1 What and how to measure?

It is initially important to define what constitutes an "open" flower or inflorescence before beginning measurements, because plants open and wither their flowers gradually. There is little available information to construct a consensus on this, but we suggest that a flower is considered open if an insect pollinator can access the nectaries or stigma/anther (insect-pollinated plants) or that stigma and anthers are exposed (wind-pollinated plants) so that reproduction can take place. For some types of flowers it will be difficult to study a single flower and the study unit will then be the inflorescence. When counting or measuring seeds and fruits, it is important that they are ripe or mature, or that mature and immature individuals are differentiated and counted separately. For

example, Pato & Ramón Obeso (2012) counted *Vaccinium myrtillus* seeds that were mature ("filled and large") and aborted ("unfilled and small") separately. The bewildering array of seed and fruit forms in nature precludes a comprehensive review of benchmarks here, but we suggest that efforts are made to establish these standards from literature covering the study species before undertaking measurements (e.g. Molau & Mølgaard, 1996). More information can also be found in protocol 4.2 Seed germinability, viability, and dormancy. These definitions are subsequently important to the timing of these measurements. Preferably, measurements should be made at frequent intervals (e.g. once every 3 to 7 days) during anthesis, but if this is not practical, single measurements should be made at peak flowering. If the plant species exhibit more than one flowering periods or peaks, the same measurements should be made for each period. If this is not possible, it is important to reference this in any subsequent reporting.

A large number of variables can be measured easily, but these are likely to depend on the target species and research question. For example, the ITEX manual (Molau & Mølgaard, 1996) suggests different reproductive response variables for different species, ranging from inflorescence length, size of individual flowers, and bulbils per shoot for *Bistorta vivipara* to number of flowers per plot, seeds per flower, and mean seed weight for *Dryas octopetala*. Despite this variation in measurement approach, the common aspects sampled by many studies often include the following (per plot or individual):

- Flower number
- Mean flower or inflorescence size
- Nectar produced
- Pollen produced
- Mature fruit and/or seed number
- Mature fruit and/or seed weight

These variables can allow the calculation of other variables such as seed number per flower, seeds to ovule ratio (i.e. seed set), fruit to flower ratio (i.e. fruit set), and cumulative fitness (accumulation of traits expressed during a plant's life; Hargreaves et al., 2014, see below). At the plot level, reproductive structures such as flowers, inflorescences, and fruits can easily be counted, and it is recommended that these are combined with percent cover estimates of flowers. However, this method may result in the underrepresentation of less common species. It may therefore be preferable to mark up to 20 plants, genets, or ramets per plot for each of the most common species as recommended by ITEX (Molau & Mølgaard, 1996) and count the number of reproductive structures per individual. Plot size will therefore be relative to the size and density of the target species. Alternatively, some counts can be conducted at the plot level, in which case we recommend plot sizes of 0.5 m^2 for dominant forbs and low-growing shrubs (e.g. Baude et al., 2016), 1–4 m^2 for sparser or larger vegetation (e.g. Gillespie et al., 2017), and up to 0.25 ha for trees (Carrer et al., 2018). In addition, fruits, seeds, seed heads, catkins, capsules, or other seed structures can be collected for weighing, dissecting, and counting at the laboratory. Fruits can be collected in small plastic zip-lock bags if freezing or refrigerating (Pérez-Harguindeguy et al. 2013), otherwise these and seeds/seed structures can be collected in paper bags. Between collection and further processing, fruits can be kept frozen in plastic bags (for longer periods) or at room temperature in paper bags (e.g., if germination experiments are to be conducted; see Molau & Mølgaard ,1996), and seeds can be kept in a dry place at room temperature for two months (Molau & Mølgaard, 1996). Nectar and pollen production requires slightly more time and effort. To sample nectar, flowers should be bagged

with fine mesh for 24 hours to allow the nectaries to fill without being depleted by insects (Figure 4.1.1 left). Nectar should then be extracted using microcapillaries (Figure 4.1.1 right) or rinsing techniques before being analysed for sugar content with a refractometer modified for small volumes (Baude et al., 2016). Pollen grains can be counted under a microscope and are sampled by collecting the anthers of freshly opened flowers (Baude et al., 2016).

Figure 4.1.1 Nectar sampling. An *Erica cinerea* ramet enclosed in a net bag to exclude insect pollinators (left). Extracting floral nectar using a microcapillary pipette (right). Photos: Mathilde Baude.

When plants are exposed to treatments or repeated observations the individual plants or flowers, or plots, need to be mapped and tagged for resampling or for later collection of reproductive units. Individual plants can be marked with metal tags as suggested in Molau & Mølgaard (1996), although the authors of this protocol have previously used various other methods such as cut lengths of drinking straws or cable ties and colour beads. The method of tagging is not important and is not usually reported in studies, but plants should be given individual codes written onto the tag with permanent marker or be assigned a colour-coding scheme. Note that it can be difficult to find markings later in the season in locations where the vegetation is likely to grow significantly. Large herbivores may also remove markings accidentally, so researchers should consider whether it is appropriate to erect plot, block, or site fencing. At the plot level, metal tubes can make useful markers as they can be inserted into the soil for later recovery with a metal detector if aboveground markings may be disturbed by herbivory, trampling, or other factors. They can also serve as corner holders for frame quadrats. Alternatively, plastic tubing can make suitable plot markers (e.g. Cooper et al., 2011), although they tend to be attractive to grazing animals.

Interpretation of the variables covered here is straightforward. The variables can be interpreted as the reproductive effort of a plant (e.g. Arft et al., 1999; Lambrecht et al., 2007; Barrett & Hollister, 2016) or its reproductive fitness (e.g. Marchin et al., 2014; Xiao et al., 2016; Lemoine et al., 2017). The figures derived alone will not necessarily be meaningful, but the comparison of these variables between treatments or against baseline data can provide a picture of a species' reproductive fitness and differences or changes in reproductive effort. For example, individual plants with relatively more and larger flowers or inflorescences, and a higher production of nectar and/or pollen can be considered to make a greater reproductive effort because they are more likely to attract pollinators and have higher chances of seed production and dispersal. Similarly, those individuals with relatively more or heavier seeds can be said to have greater reproductive fitness, because the chances of successful reproduction are higher. In some studies spanning numerous sites or landscapes, care

should be taken over these interpretations because reproductive variables can vary widely between populations and locations (Breza et al., 2012) and with local environmental conditions (Fernández et al., 2015). Reproductive variables can also be size dependent within species and populations (Meineri et al., 2014). For these reasons, it may be advisable to include covariates such as temperature, soil moisture, relative humidity, precipitation, and plant size in any statistical analysis (Meineri et al., 2014).

Fitness is a relative measure of reproductive success and can be estimated from reproduction. It is used to assess local adaptation among populations from different habitats (Kawecki & Ebert, 2004) and a standard response variable in reciprocal transplant experiments and common gardens. It can be advisable to assess cumulative fitness (e.g. *mean survival probability per population* X *mean number of inflorescences per population*; Joshi et al., 2001; Halbritter et al., 2015), which often cannot be estimated from the performance of a subset of life stages (e.g. number of flowers or seed set; Hargreaves et al., 2014). A plant can, for example, produce flowers and seeds, but the seeds are not fertile.

Where to start

Arft et al. (1999), Molau & Mølgaard (1996), Reyer et al. (2013)

4.1.2 Special cases, emerging issues, and challenges

If the aim of the study is to assess the importance of animal pollination to sexual reproduction it may be important to combine data collection on plant reproduction with assessments of pollinator visitation (see protocol 4.12 Pollinator visitation). In addition, for a more detailed insight on the importance of pollinators to plant reproduction one should include pollen limitation experiments (Burd, 1994; Ashman et al., 2004). By comparing reproductive output from supplemental hand-pollinated flowers with flowers that receive *in situ* flower visitation (open-pollinated), one may estimate whether female reproduction in a given environment is pollen limited. Comparing bagged flowers with open pollinated flowers can further allow an assessment of the importance of pollinators in plant sexual reproduction in a given environment. To estimate population and fitness consequences of global changes researchers may also need to germinate seeds, for example in a seed-sowing experiment (Hegland & Totland, 2007; see protocol 4.2 Seed viability, germinability and dormancy), or by performing population modelling (Ashman et al., 2004; protocol 4.3 Plant demography). Reproductive data are essential parts of demographic studies and data on seed and fruit yields, germination rates, and offspring survival are essential for the construction of demographic models.

Recent work on plant nectar production has investigated the “scaling-up” of plant-level measurements to habitat- and countrywide-scale estimates of nectar production. Baude et al. (2016) measured the nectar production for 175 common British plant species and modelled the nectar of a number of others and combined these values with survey data of plant species cover in a range of British habitat types and remotely sensed habitat cover for the entire country. Although the nectar measurements were from only two populations at most, the estimates demonstrated the most productive habitats and regions in the country. Such upscaling of plant traits could be further explored in other habitats and regions and in combination with other variables. Furthermore, a

database of such traits could be maintained and added to when additional measurements are available for future modelling efforts (Baude et al., 2016).

4.1.1 References

Theory, significance, and large datasets

Arft et al. (1999), Baude et al. (2016), Scaven & Rafferty (2013)

More on methods and existing protocols

Baude et al. (2016), Molau & Mølgaard (1996)

All references

Arft, A. M., Walker, M. D., Gurevitch, J., Alatalo, J. M., Bret-Harte, M. S., Dale, M., … Wookey, P. A. (1999). Responses of tundra plants to experimental warming: Meta-analysis of the international tundra experiment. *Ecological Monographs, 69*, 491-511.

Ashman, T. L., Knight, T. M., Steets, J. A., Amarasekare, P., Burd, M., Campbell, D. R., … Wilson, W. G. (2004). Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology, 85*, 2408-2421.

Baskin, C. C., & Baskin, J. M. (2014). *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination* (2nd ed.). Elsevier.

Barrett, R. T., & Hollister, R. D. (2016). Arctic plants are capable of sustained responses to long-term warming. *Polar Research, 35*, 9.

Baude, M., Kunin, W. E., Boatman, N. D., Conyers, S., Davies, N., Gillespie, M. A. K., … Memmott, J. (2016). Historical nectar assessment reveals the fall and rise of floral resources in Britain. *Nature, 530*, 85-88.

Biesmeijer, J. C., Roberts, S. P. M., Reemer, M., Ohlemuller, R., Edwards, M., Peeters, T., … Kunin, W. E. (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science, 313*, 351-354.

Breza, L. C., Souza, L., Sanders, N. J., & Classen, A. T. (2012). Within and between population variation in plant traits predicts ecosystem functions associated with a dominant plant species. *Ecology and Evolution, 2*, 1151-1161.

Burd, M. (1994). Bateman principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Botanical Review, 60*, 83-139.

Carrer, M., Castagneri, D., Popa, I., Pividori, M., & Lingua, E. (2018). Tree spatial patterns and stand attributes in temperate forests: The importance of plot size, sampling design, and null model. *Forest Ecology and Management, 407*, 125-134.

Cooper, E. J., Dullinger, S., & Semenchuk, P. (2011). Late snowmelt delays plant development and results in lower reproductive success in the High Arctic. *Plant Science, 180*, 157-167.

Erhardt, A., & Rusterholz, H. P. (1997). Effects of elevated CO_2 on flowering phenology and nectar production. *Acta Oecologica, 18*, 249-253.

Fernández, J. D., Lorite, J., Bosch, J., & Gómez, J. M. (2015). Variation in the reproductive success of a narrow endemic plant: Effects of geographical distribution, abiotic conditions and pollinator community composition. *Basic and Applied Ecology, 16*, 375-385.

Gillespie, M., Baude, M., Biesmeijer, J. C., Boatman, N. D., Budge, G. E., Crowe, A., … Kunin, W. E. (2017). A method for the objective selection of landscape-scale study regions and sites at the national level. *Methods in Ecology and Evolution, 8*(11), 1468-1476.

Halbritter, A. H., Billeter, R., Edwards, P. J., & Alexander, J. M. (2015). Local adaptation at range edges: comparing elevation and latitudinal gradients. *Journal of Evolutionary Biology, 28*(10), 1849-1860.

Hargreaves, A. L., Samis, K. E., & Eckert, C. G. (2014). Are species' range limits simply niche limits writ large? A review of transplant experiments beyond the range. *The American Naturalist, 183*(2), 157-173.

Hegland, S. J., & Totland, Ø. (2007). Pollen limitation affects progeny vigour and subsequent recruitment in the insect-pollinated herb *Ranunculus acris*. *Oikos, 116*, 1204-1210.

Hoover, S. E. R., Ladley, J. J., Shchepetkina, A. A., Tisch, M., Gieseg, S. P., & Tylianakis, J. M. (2012). Warming, CO_2, and nitrogen deposition interactively affect a plant-pollinator mutualism. *Ecology Letters. 15*(3), 227-234.

Jablonski, L. M., Wang, X. Z., & Curtis, P. S. (2002). Plant reproduction under elevated CO_2 conditions: a meta-analysis of reports on 79 crop and wild species. *New Phytologist, 156*, 9-26.

Joshi, J., Schmid, B., Caldeira, M. C., Dimitrakopoulos, P. G., Good, J., Harris, R., ... Mulder, C. P. H. (2001). Local adaptation enhances performance of common plant species. *Ecology Letters, 4*(6), 536-544.

Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters, 7*(12), 1225-1241.

Lambrecht, S. C., Loik, M. E., Inouye, D. W., & Harte, J. (2007). Reproductive and physiological responses to simulated climate warming for four subalpine species. *New Phytologist, 173*, 121-134.

Lemoine, N. P., Doublet, D., Salminen, J. P., Burkepile, D. E., & Parker, J. D. (2017). Responses of plant phenology, growth, defense, and reproduction to interactive effects of warming and insect herbivory. *Ecology, 98*, 1817-1828.

Liu, Y., Mu, J., Niklas, K. J., Li, G., & Sun, S. (2012). Global warming reduces plant reproductive output for temperate multi-inflorescence species on the Tibetan plateau. *New Phytologist, 195*, 427-436.

Marchin, R. M., Dunn, R. R., & Hoffmann, W. A. (2014). Are winter-active species vulnerable to climate warming? A case study with the wintergreen terrestrial orchid, *Tipularia discolor*. *Oecologia, 176*, 1161-1172.

Meineri, E., Skarpaas, O., Spindelböck, J., Bargmann, T., & Vandvik, V. (2014). Direct and size-dependent effects of climate on flowering performance in alpine and lowland herbaceous species. *Journal of Vegetation Science, 25*, 275-286.

Molau, U., & Mølgaard, P. (1996). *ITEX Manual - International Tundra Experiment* (2nd ed.) Copenhagen: Danish Polar Centre. http://ibis.geog.ubc.ca/itex/PDFs/ITEXmanual.pdf

Pato, J. & Ramón Obeso, J. (2012). Growth and reproductive performance in bilberry (Vaccinium myrtillus) along an elevation gradient. *Ecoscience, 19*(1), 59-68.

Pérez-Harguindeguy, N., Diaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., … Cornelisson, J. H. C. (2013). New handbook for standardised measurement of plant functional traits worldwide. *Australian Journal of Botany*, 61(3), 167-234.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution, 25*, 345-353.

Reyer, C. P. O., Leuzinger, S., Rammig, A., Wolf, A., Bartholomeus, R. P., Bonfante, A., … Pereira, M. (2013). A plant's perspective of extremes: terrestrial plant responses to changing climatic variability. *Global Change Biology, 19*, 75-89.

Scaven, V. L., & Rafferty, N. E. (2013). Physiological effects of climate warming on flowering plants and insect pollinators and potential consequences for their interactions. *Current Zoology, 59*, 418-426.

Xiao, Y., Li, X. H., Cao, Y. S. & Dong, M. (2016). The diverse effects of habitat fragmentation on plant-pollinator interactions. *Plant Ecology, 217*, 857-868.

Authors: Gillespie MAK[1], Hegland SJ[1]

Reviewers: Töpper JP[2,3], Muffler L[4,5]

Affiliations

[1] Department of Environmental Sciences, Western Norway University of Applied Sciences, Sogndal, Norway

[2] Norwegian Institute for Nature Research, Bergen, Norway

[3] Department of Biological Sciences, University of Bergen, Bergen, Norway

[4] Experimental Plant Ecology, Institute of Botany and Landscape Ecology, University of Greifswald, Greifswald, Germany

[5] Plant Ecology, Albrecht-von-Haller Institute for Plant Sciences, University of Goettingen, Goettingen, Germany

4.2 Seed germinability, viability, and dormancy

Authors: Eycott AE[1,2], Wilfahrt PA[3]

Reviewers: Vandvik V[2, 4], Tielbörger K[5]

Measurement unit: proportion; Measurement scale: plot; Equipment costs: €; Running costs: €; Installation effort: low; Maintenance effort: low; Knowledge need: low (viability and germinability) to high (dormancy); Measurement mode: manual

Seeds are the sexual regeneration stage of plants. They are a means of plant dispersal in space and in time, allowing plants to exploit suitable habitat when and where it is available (Venable & Lawlor, 1980; Fenner & Thompson, 2005). Seed dispersal in space and in time can also reduce sibling and parent–offspring competition (Nathan & Muller-Landau, 2000). We include here all seed-like structures, for example, achenes from apomictic plants.

Seed germination can occur whenever the germination requirements are met, such as suitable environmental conditions (i.e. temperature and moisture: some species require rather specific light or temperature regimes). However, a seed or dispersal unit lying on the soil may not germinate for one of four reasons: i) the seed is inviable because the seed never formed an embryo and is in effect an empty case; ii) the seed is inviable because it died at some point; iii) the seed is viable but conditions are not suitable for germination; or iv) the seed is viable but dormant, that is, the seed has some innate mechanism that inhibits germination under conditions that are otherwise suitable. Dormancy is geographically and phylogenetically widespread (Baskin & Baskin, 2014). Dormancy can be imposed by external conditions or be an innate property of the seeds themselves, for example via an undeveloped embryo or an impermeable seed coat (hard-seededness). It can be broken by, for example, light (the red:far-red ratio is particularly important), scarification of the seed coat, temperature cycles, or extrinsic chemical signals such as smoke (Keeley & Fotheringham, 2000; Pons, 2000; Baskin & Baskin, 2014). Some species produce a mixture of dormant and non-dormant seeds, the ratios of which may be affected by climate (Wagmann et al., 2012). Other forms of global change such as nitrogen deposition can also affect the proportion of dormant to non-dormant seeds (Chen et al. 2019).

Understanding seed viability, dormancy, and germination is important for understanding plant community responses to disturbance, population dynamics, and competitive interactions. If the dormancy mechanism is known, then the proportion of seeds germinating before and after a dormancy-breaking mechanism has been applied can be compared. In the context of climate-manipulation experiments, changes in the proportion of seeds which are viable can tell us about reproductive fitness and changes in the proportion which are dormant tell us about adaptation to changing environmental conditions (Ooi et al., 2009; Shetsova et al., 2009; Walck et al., 2011). Treatment conditions mimicking a range of climate projections may be replicated either in the laboratory (e.g. Ooi et al., 2009) or in the field (e.g. Meineri et al., 2013). The effect of increased nitrogen can interact with climate effects: for example, Longas et al. (2016) show that increased nitrogen widens the thermal range at which *Buglossoides arvensis* will germinate. For precise climate manipulations, laboratory experiments may be preferred over the imprecise or noisy conditions of field sites. In this section we outline methods to investigate in the laboratory whether a seed is alive, alive but dormant, or dead. These methods link to protocols on reproductive success (see protocol

4.1 Sexual plant reproduction), demography (protocol 4.3 Plant demography), propagule rain (protocol 4.7 Propagule rain), and the seed bank (protocol 4.8 The soil seed bank (buried seed pool)).

4.2.1 What and how to measure?

Seed germinability

Seed germinability is usually measured using emergence tests. Seeds are sown onto growth medium (e.g. 1% agar or moist filter paper) or damp compost, kept in appropriate conditions for germination, and the proportion of seeds which produce a radicle is recorded. The number of seeds used for germination experiments depends on availability and varies between studies (Hobbie & Chapin, 1998; Shetsova et al., 2009; Ooi et al., 2014), but numbers around 100 per treatment and species are advisable. Experiments of this nature require a good understanding of the behaviour of the seeds of your study species and a good deal of forward planning (Thompson & Booth, 1993). Baskin & Baskin (2014) provide a list of guidelines for laboratory studies of germination.

Collect seeds at maturity. How to collect seeds depends on the architecture of the seed-bearing part of the target species and descriptions of the plants can be found in floras. The timing of seed collection is important and should be done at the time when the seeds have ripened, but ideally before they disperse. The outer part of the dispersal unit will often dry out or change colour when the seeds start to ripen, particularly for animal-dispersed fleshy fruits. For annuals, biennials, and perennials which make seasonal stems, the whole flowering structure or even plant is likely to be dying back. For seeds dispersed from capsules, the capsules split open or the seeds start to rattle. For seeds that are wind dispersed and have a pappus (e.g. Asteraceae), the inflorescence can be covered with netting to prevent the seeds dispersing before collection because dispersal can be very sudden and happen immediately after maturity is reached (Figure 4.2.1). Add the bag only after the flower has been pollinated and begins to senesce. In wet areas the seeds can start to become infected by fungus if they are covered so the bag technique may not be appropriate. In species where dormancy is broken by a dry or cold season, or by fire, it is possible to collect seeds after the dormancy-breaking event has passed provided that they are large enough to find (or remain on the parent plant, e.g. *Pinus sylvestris* or other serotinous cones; Goubitz et al., 2002), but note that some mortality may have occurred during that time.

Figure 4.2.1 A net bag placed over a flower of *Leontodon autumnalis* to collect seeds. Photo: Deborah Davy.

Check seeds for the presence of an embryo. This is tricky to do non-invasively but seed cases which are completely empty shells, or are relatively easily squashed between the fingers in comparison to healthy seeds, should be discarded.

Test and use seeds immediately after harvesting. This is not always possible, for example in the middle of a field campaign, in which case the seeds should to be stored under optimal conditions. It is important to know the suitable storage conditions for different species as some seeds do not

tolerate drying: these are called recalcitrant seeds (this can be checked in Baskin & Baskin, 2014 or on the Kew Seed Information Database at http://data.kew.org/sid/). Keep non-recalcitrant seeds cool (ideally below 5 °C but at least below 25 °C), air dry, and in the dark. Recalcitrant seeds should be kept cool and dark and slightly damp (but not soaking wet). Longer term storage can be attained for non-recalcitrant seeds by drying to 5–7% moisture and freezing (Band & Hendry, 1993).

Check that seeds can imbibe water. If your seeds sit in water without any hint of softening or swelling, they may either be dead or be hard-seeded (physical dormancy). If they are physically dormant, the coating may need to be broken gently, for example with a needle or some sandpaper (Baskin & Baskin, 2014, p. 150).

Use intact natural dispersal units. This is because the pericarp (parts of the dispersal unit formed by the parent plant) first protects the seed, for example from drying out, and second may be rather securely attached such that detachment damages the seed. Removing the pericarp can also change the germination rate (see Baskin & Baskin, 2014, pp. 12–13 for a list of examples) – which is acceptable for viability testing but not for testing germination of the seeds under "natural" conditions (Baskin & Baskin, 2014, p. 13). It is of course difficult to access some seeds, for example, the fruit of an almond, without removing the tough endocarp but in this case collection and storage should be in the natural dispersal units, only removing them when the seed is ready to be tested.

It is vital that the conditions the seeds are placed under are suitable for their germination and that they are given adequate time. Suitable germination conditions can be implied from conditions at a site where new individuals of the species are frequent or determined experimentally. These may include fluctuations in photoperiod or temperature or both (Thompson, 1993). Regarding duration, Baskin & Baskin (2014) caution against running experiments for longer than four weeks, but this recommendation relates to finding the dormant proportions and where dormancy could be broken by germination conditions. Milbau et al. (2009) monitored their Arctic seeds for 13 weeks and Mondoni et al. (2012) incubated alpine seeds for 48 weeks, albeit with a temperature-induced period of germination suppression during the experiment. Establishment of maximum germinability (for use in population models) takes considerably longer than recording relative germinability (for example, for comparing between manipulation treatments). For maximum germinability experiments, decide beforehand a length of time during which no new germinations will trigger the end of the experiment. Ungerminated seeds can be tested for viability (see below).

If the mechanism for breaking dormancy is known, then that should be applied before starting the germination test. For example, for temperate, high-elevation and high-latitude areas, vernalisation is the most common dormancy-breaking mechanism applied – seeds should be kept slightly damp and refrigerated for four months (this can be shorter for climates with warm winters; alternatively seeds can be gathered after winter). Seeds with a hard coat can be scarified by gently rubbing the seeds with medium sandpaper on a flat surface until chips of testa (seed coat) can be seen (Thompson & Booth, 1993). Some seeds need to be kept warm and dry (Thompson & Booth, 1993). A fresh seed control can be used to determine dormancy fractions (proportion of the seeds that are dormant and non-dormant). If the mechanism for breaking dormancy is not known, some caution should be applied to subsequent germination data. Determining what kind of dormancy a species has is challenging and most probably outside the scope of climate-manipulation experiments, but a protocol is given in Baskin & Baskin (2014), along with a table of dormancy type for many species and advice on methods for breaking dormancy.

Seed viability

Seed viability is a more specific test of whether the seed embryo is still alive. There are three main groups of methods: chemical, physical, and non-invasive. The most common **chemical method** is to expose the embryo to tetrazolium solution. The method works best on large grass and tree seeds, but is challenging with very small seeds due to difficulties in handling and visual assessment (although not impossible, see van Waes & Debergh, 1986). The seed coat is cut open very carefully with a scalpel, without damaging the embryo (practice on spare material). Then 2,3,5-triphenyl tetrazolium chloride (usually 0.1–1% w/v) is dropped onto the embryo. If the embryo goes pink or red, there is living tissue in the embryo which has reduced the tetrazolium chloride (colourless) to formazan (pinkish red) via dehydrogenase enzymes. The International Seed Testing Association protocol (ISTA, 2018) is the ***gold standard*** for viability tests, but that protocol requires 2500 seeds per test. **Physical methods** involve poking the seed to see if the embryo is fleshy rather than hollow inside, although note that some plants produce hard and hollow seeds (Baskin & Baskin, 2014 p. 10). It is therefore critical to cut the seed open and examine the embryo under a stereo microscope (see Pake & Venable, 1996) and so poking and cutting could be considered the ***bronze standard*** viability test, although note that the method is destructive and that the structure of the seeds of the study species should be studied and understood beforehand. **Non-invasive methods** such as the use of X-rays (Foucat et al., 1993; Dell'Aquilla, 2007) are either under development or very expensive and outside the scope of this protocol.

Where to start

Baskin & Baskin (2014)

4.2.2 Special cases, emerging issues, and challenges

In all cases where material is handled in the laboratory, care must be taken to minimise artefacts of the laboratory conditions, such as by accounting for soil temperature differences relative to air temperature, or properly replicating the diurnal cycle of light and temperature (Ooi et al., 2009, 2014). Likely, warming is the easiest global change variable to manipulate, which is appropriate given the propensity toward temperature as the dominant control of dormancy and germination (Baskin & Baskin, 2014). Germination tests from different treatment populations in identical laboratory conditions may fail to account for altered dormancy effects from treatments, but can still capture population-level changes in other seed traits that influence viability (Walck et al., 2011).

4.2.3 References

Theory, significance, and large datasets

Baskin & Baskin (2014) contains a list of known dormancy types. Seed Information Database at Kew Gardens (data.kew.org/sid/) is an extensive source of information on dormancy, dispersal, germination requirements, and more.

More on methods and existing protocols

Baskin & Baskin (2014), ISTA (2018)

All references

Band, S. R., & Hendry, G. A. F. (1993). Seed collecting, cleaning and long-term storage. In G. A. Hendry, & J. P. Grime (Eds.), *Methods in Comparative Plant Ecology: A Laboratory Manual*. Springer Science & Business Media.

Baskin, C. C., & Baskin, J. M. (2014). *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination* (2nd ed.). Elsevier.

Chen, Y., Zhang, L., Shi, X., Ban, Y., Liu, H., & Zhang, D. (2019). Life history responses of spring-and autumn-germinated ephemeral plants to increased nitrogen and precipitation in the Gurbantunggut Desert. *Science of the Total Environment, 659*, 756-763.

Dell'Aquila, A. (2007). Pepper seed germination assessed by combined X-radiography and computer-aided imaging analysis. *Biologia Plantarum, 51*(4), 777-781.

Fenner, M., & Thompson, K. (2005). *The Ecology of Seeds*. Cambridge: Cambridge University Press.

Foucat, L., Chavagnat, A., & Rennou, J.-P. (1993). Nuclear magnetic resonance micro-imaging and X-radiography as possible techniques to study seed germination. *Scientia Horticulturae, 55*(3-4), 323-331.

Goubitz, S., Werger, M. J. A., & Ne'eman, G. (2002). Germination response to fire-related factors of seeds from non-serotinous and serotinous cones. *Plant Ecology*, 169(2), 195-204.

Hobbie, S. E., & Chapin, F. S. (1998). An experimental test of limits to tree establishment in Arctic tundra. *Journal of Ecology, 86*(3): 449-461.

ISTA (2018). International Rules for Seed Testing. [https://www.seedtest.org/en/home.html].

Keeley, J. E., & Fotheringham, C. J. (2000). The role of fire in regeneration from seed. In M. Fenner (Ed.), *Seeds: The Ecology of Regeneration in Plant Communities* (2nd ed., pp. 311-330). Wallingford: CABI.

Longas, M. M., Chantre, G. R., & Sabbatini, M. R. (2016). Soil nitrogen fertilisation as a maternal effect on *Buglossoides arvensis* seed germinability. *Weed Research, 56*(6), 462-469.

Meineri, E., Spindelböck, J. P., & Vandvik, V. (2013). Seedling emergence responds to both seed source and recruitment site climates: a climate change experiment combining transplant and gradient approaches. *Plant Ecology*, 214(4), 607-619.

Milbau, A., Graae, B. J., Shevtsova, A., & Nijs, I. (2009). Effects of a warmer climate on seed germination in the subarctic. *Annals of Botany*, *104*(2), 287-296.

Mondoni, A., Rossi, G., Orsenigo, S., & Probert, R. J. (2012). Climate warming could shift the timing of seed germination in alpine plants. *Annals of Botany*, *110*(1), 155-164.

Nathan, R., & Muller-Landau, H. C. (2000). Spatial patterns of seed dispersal, their determinants and consequences for recruitment. *Trends in Ecology & Evolution, 15*(7), 278-285.

Ooi, M. K., Auld, T. D., & Denham, A. J. (2009). Climate change and bet-hedging: interactions between increased soil temperatures and seed bank persistence. *Global Change Biology*, *15*(10), 2375-2386.

Ooi, M. K., Denham, A. J., Santana, V. M., & Auld, T. D. (2014). Temperature thresholds of physically dormant seeds and plant functional response to fire: variation among species and relative impact of climate change. *Ecology and Evolution*, *4*(5), 656-671.

Pake, C.E., & Venable, D. L. (1996). Seed banks in desert annuals: implications for persistence and coexistence in variable environments. *Ecology, 77*(5), 1427-1435.

Pons, T. L. (2000). Seed responses to light. In M. Fenner (Ed.), *Seeds: The Ecology of Regeneration in Plant Communities* (2nd ed., pp. 237-260). Wallingford: CABI.

Shetsova, A., Graae, B. J., Jochum, T., Milbau, A., Kockelbergh, F., Beyens, L., & Nijs, I. (2009). Critical periods for impact of climate warming on early seedling establishment in subarctic tundra. *Global Change Biology*, *15*(11), 2662-2680.

Thompson, K. (1993). Germination at alternating temperatures. In G. A. Hendry, & J. P. Grime (Eds.), *Methods in Comparative Plant Ecology: A Laboratory Manual*. Springer Science & Business Media.

Thompson, K., & Booth, R. E. (1993). Dormancy breaking. In G. A. Hendry, & J. P. Grime (Eds.), *Methods in Comparative Plant Ecology: A Laboratory Manual*. Springer Science & Business Media.

van Waes, J. M., & Debergh, P. C. (1986). In vitro germination of some Western European orchids. *Physiologia Plantarum*, 67(2), 253-261.

Venable, D. L., & Lawlor, L. (1980). Delayed germination and dispersal in desert annuals: escape in space and time. *Oecologia, 46*(2), 272-282.

Wagmann, K., Hautekeete, N. C., Piquot, Y., Meunier, C., Schmitt, S. E., & van Dijk, H. (2012). Seed dormancy distribution: explanatory ecological factors. *Annals of Botany*, *110*(6), 1205-1219.

Walck, J. L., Hidayati, S. N., Dixon, K. W., Thompson, K., & Poschlod, P. (2011). Climate change and plant regeneration from seed. *Global Change Biology*, *17*(6), 2145-2161.

Authors: Eycott AE[1,2], Wilfahrt PA[3]

Reviewers: Vandvik V[4], Tielbörger K[5]

Affiliations

[1] Faculty of Biosciences and Aquaculture, Nord University, Steinkjer, Norway

[2] Department of Biological Sciences, University of Bergen, Bergen, Norway

[3] Department of Disturbance Ecology, BayCEER, University of Bayreuth, Bayreuth, Germany

[4] Department of Biological Sciences and Bjerknes Centre for Climate Research, University of Bergen, Bergen, Norway

[5] Institute of Evolution and Ecology, Plant Ecology Group, University of Tübingen, Tübingen, Germany

4.3 Plant demography

Authors: Linstädter A[1,2], Töpper JP[3,4]

Reviewer: Tielbörger K[5]

Measurement unit: counts and mm; Measurement scale: plot; Equipment costs: €€; Running costs: €; Installation effort: low; Maintenance effort: low; Knowledge need: medium; Measurement mode: manual

Plant demography is the study of population size (abundance) and its underlying parameters. It describes the state of a particular population of a species and how this state changes through time (Gibson, 2015). Following a population and recording demographic variables for consecutive time steps allows us to build population models (Caswell, 2001; Merow et al., 2014a). With these models we can study the mechanisms of abundance changes. Key questions for climate-change studies would be, for example, "Does the population size decline because of an increased mortality or due to a reduced reproduction?" or "Does the population structure change during population decline, i.e. are certain plant sizes, ages, or stages declining more than others?" The processes underlying changes in population size are called "vital rates" (Franco & Silvertown, 2004). These are survival, individual growth, and fecundity (sexual and clonal reproduction). In addition, the classical demographic equation also includes immigration and emigration. Such mechanistic insights improve our understanding of the ecological processes linking changing environmental conditions to abundance responses (Fridley, 2017; Töpper et al., 2018). Climate-change studies providing such links through demographic approaches thus contribute to generating more accurate projections of future vegetation responses (Pagel & Schurr, 2012; Merow et al., 2014b) and lead to more effective measures in mitigation, conservation, and restoration contexts (Elzinga et al., 1998). Studies on plant demography and population modelling are particularly well suited to investigate mechanistic responses of plant populations to a range of environmental and other global-change drivers and changes in these drivers. These can be either observational studies of, for example, climate (Adler & HilleRisLambers, 2008; Dalgleish et al., 2011; Nicole et al., 2011; Sletvold et al., 2013; Shryock et al., 2014; Treurnicht et al., 2016), competition/facilitation (Buckley et al., 2003; Adler & HilleRisLambers, 2008), fire frequency (Evans et al., 2010), land use (van der Meer et al., 2014; Johansen et al., 2016), herbivory (Miller et al., 2009), and nitrogen deposition (Nicole et al. 2011), or experimental studies of climate (Pfeifer-Meister et al., 2013; Salguero-Gomez et al., 2013; Farrer et al., 2014; Gornish, 2014; Töpper et al., 2018; Cui et al. 2018), competition/facilitation (Olsen et al., 2016), land use (Ehrlén et al., 2005; Sletvold et al., 2013), and nitrogen deposition (Farrer et al. 2014, Gornish 2014).

4.3.1 What and how to measure?

Sampling strategy

The two cardinal aspects of plant demography (abundance and vital rates) require specific consideration when designing the sampling strategy for a climate-change study. As most studies are set up in small-stature vegetation such as grasslands, the following sections mainly refer to herbaceous and small woody plant species; see section 4.3.2 for specific recommendations for trees and large shrubs. A complete census of a plant population is rarely feasible (Gibson, 2015); hence

population size and its temporal variation is usually estimated in study plots (Gross, 2002, but see Münzbergová & Ehrlén, 2005 for alternatives). The minimum number of individuals required to build population models ranges from 300–400 for matrix projection models and down to c. 100 for regression-based integral projection models (cf. Ramula et al., 2009). Note that lower numbers may also work for rare and/or endangered species for example, under the regression approach, as long as the individual regressions can be parameterised appropriately. Usually these numbers cannot be accommodated in a single study plot, and hence, the adequate number, size, and minimum distance of study plots needs to be determined depending on the size and average density of the study species. Large and rare plants require larger plots (or more plots if the study approach restricts plot size to a certain limit); the guidelines for the minimum size of plots for aboveground biomass estimation (see protocol 2.1.1 Aboveground plant biomass) may be used as a rough guideline for the maximum size of demographic study plots within the framework of climate-change studies. As with aboveground biomass plots, we recommend a nested plot design (Figure 2.1.1.1a in protocol 2.1.1 Aboveground plant biomass) or a multi-plot approach (Figure 2.1.1.1b in the same protocol).

For herbaceous vegetation, plot size ranges from 4 m² for the savanna bunchgrass S*tipagrostis uniplumis* (Zimmermann et al., 2008), over 1 m^2 for the perennial forb *Plantago lanceolata* (Wardle et al., 2014), 0.0625 m^2 for the small perennial forb *Viola biflora* (Olsen et al., 2016; Töpper et al., 2018), and down to 0.04 m^2 and 0.01 m² for the small annual forbs *Carrichtera annua* (Salguero-Gomez et al., 2012) and *Arabidopsis thaliana*, respectively (see Figure 4.3.1C). Plots are usually randomly chosen, but with the obvious restriction that they need to contain the study species (unless "empty" plots are of particular purpose, e.g. when studying invasiveness). Unless one is already very familiar with the plant species' morphology and phenology, as well as with other organisms in the habitat (such as herbivores), it is highly recommended to carry out a pilot study for testing the efficiency of field methods and for an assessment of the study design (Gibson, 2015).

When installing the study plots it is important to mark them properly so that plot position is stable and easy to retrieve. A good approach is to mark plot corners (see protocol 2.1.1 Aboveground plant biomass for details). Aboveground plot tags and individual tags should be replaced immediately when discovered to be damaged or removed (e.g. through animal trampling, snow movement, or human interference). When studying species that can "move" by means of vegetative growth, it is important to deal with emigrants and immigrants properly so as to ensure no bias is introduced. Migration can be studied as yet another "vital rate", but it can also be excluded if not relevant to the research question.

Estimating abundance

Gold standard

For plant abundance, individuals of the study population are counted in each study plot.

Bronze standard

Counting can be challenging for clonal plants; here usually proxies such as cover or presence in sub-plots are used and they serve as relative measures of abundance (e.g. for comparing climate-manipulation treatments, see also protocol 2.1.1 Aboveground plant biomass).

Measuring vital rates

The vital rates of survival, growth, and fecundity are highly variable in time and require repeated observations of plant populations in the field. They can be assessed from the same study plots used for abundance estimation by linking the state of the population in sampling event *t*+1 to that of the previous sampling event *t*. Please note that other variables such as seed viability and germinability also contribute to plant demography. However, these variables do not require a repeated observation of plant populations in the field; see protocol 4.2 Seed viability, germinability and dormancy for their respective sampling protocols.

Measuring survival

Gold standard

The gold standard for measuring survival requires marking individuals and monitoring their fate over time. Survival is then measured by re-finding a living individual recorded at the previous sampling event. For very dense populations, monitoring all individuals within the study plots might be too time-consuming, and a subsample of individuals may be monitored per plot. Note that the total population size in all replicate plots of a population (i.e. experimental treatment, gradient level) should still reach the recommended minimum number of individuals (see above).

Keeping track of each individual plant's identity is critical (Gibson, 2015). This can either be ensured by tags or marks placed next to the individual plant (options 1–5 in Table 4.3.1), or by tagging or marking the individuals themselves (options 6–12 in Table 4.3.1, where we also give the main advantages and disadvantages of the alternative methods). Practical details can be derived from an illustration of recommended options (Figure 4.3.1). Another option that could also be combined with physical marks is to record individual plants using repeat photography. This approach is particularly valuable for plants with a well-defined canopy, such as perennial bunchgrasses (Zimmermann et al., 2015). It could also be used for tree populations, where aerial photography is preferable. In general, it is advisable to note each individual's position by measuring co-ordinates within the study plots (Gibson, 2015). This information can be used to create population maps for the study plots: another useful way for monitoring individuals.

Bronze standard

The bronze standard for survival is performing a census of the established study plots during repeated sampling events: a survival rate is then calculated per plot. This census approach is easier and less time-consuming than monitoring individuals' fates: however it does not allow the monitoring of individual performance.

Measuring growth

Gold standard

Growth is estimated as the change in "developmental state" of surviving individuals from one sampling event to the next. The developmental state is described by a state variable, such as measures of stage, age, or size, which depends on the species' life-form and cycle. As plant species often lack the well-defined stages found in animals (but see Jongejans & de Kroon, 2005) and age is

hard to assess, tracking an individual plant's size is the gold standard for estimating growth. For herbaceous species and small woody species (e.g. bilberry – *Vaccinium myrtillus*), plant size can be estimated via a direct measure of a single trait such as plant height, or the tuft area of bunchgrasses (Zimmermann et al., 2015), or rosette area (Jongejans et al., 2011). However, plant size can also be estimated as aboveground biomass (dry mass), based on several measured plant traits (Wardle et al., 2014; Seldal et al., 2017).

Bronze standard

Similar to plant abundance, growth can also be assessed more rapidly as average growth per plot. For this purpose, changes in a population's average plant size or biomass are tracked on observation plots. For monospecific stands, changes in plot-level biomass can be used to estimate a population's growth rate (see protocol 2.1.1 Aboveground plant biomass for details on measuring aboveground biomass). If plant abundance is known for an observation plot, average individual growth rate per plot and individual can be calculated. Again, the bronze standard does not typically allow tracking of individual performance. In the case of clonal plants though, a plot approach may be the only feasible option.

Measuring fecundity

Gold standard

Fecundity comprises both sexual and clonal reproduction (but note that clonality can also be classified as a separate vital rate; e.g. Töpper et al., 2018). Assessing fecundity thus implies a series of measures that links the outcome of sexual and/or clonal reproduction in year t+1, i.e. number and size of established recruits (seedlings or clones), to the state variable of individuals present in year t. In practice, this usually includes measures of whether or not an individual produces seeds or asexual propagules, the number of propagules produced, and the number and size of recruits. This would require, ideally, monitoring the fate of seeds or asexual propagules through the consecutive stages of the recruitment process, which are emergence, survival, and growth through the growing season and survival through the unfavourable season (Zimmermann et al., 2008). Monitoring the individual fate of sexual and asexual recruits is very time-consuming, hence we recommend more rapid methods as a gold standard. Seedlings should only be counted in study plots. In cases of clonal growth, it might additionally be required to note their position on a plot map to be able to separate them from eventual clonal recruits at the following census. A seedling's individual fate commences being tracked if they have survived the first unfavourable season (see Wardle et al., 2014; PlantPopNet protocol, nd). Similarly, clonal reproduction is best recorded when finding clonal recruits the following year. Seed production is often measured indirectly via the number of flowers or fruits and a constant describing the number of seeds per fruit. Measurement of plant sexual reproduction is more closely described in protocol 4.1 Sexual plant reproduction.

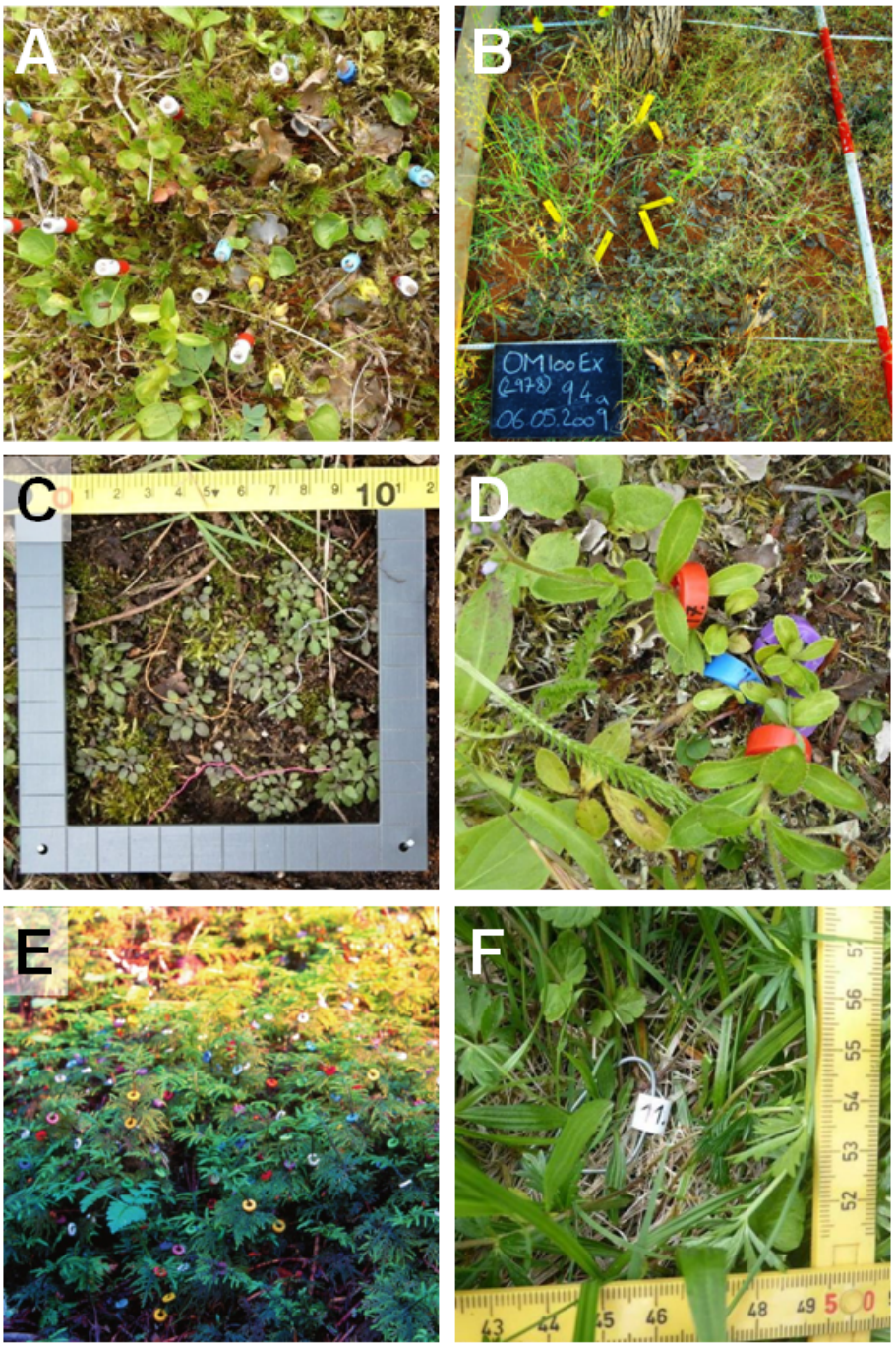


Figure 4.3.1 Recommended tags and marks in plant population biology. **A:** Wooden toothpicks labelled with numbered and colour-coded beads on top, placed to the left of individuals of *Viola biflora* (yellow), *V. palustris* (blue), and *Veronica alpina* (white and red). **B:** Labelled plastic stakes placed close to individuals of the perennial bunchgrass *Stipagrostis uniplumis* monitored in a 1 m² study plot. **C:** Colour-coded thread loops at the rosette base of *Arabidopsis thaliana* individuals monitored in a 1 dm² study plot. **D:** Numbered, colour-coded plastic rings around the basal stems of *Veronica officinalis* individuals. **E:** Numbered, colour-coded plastic rings marking clonal segments of the moss *Hylocomium splendens*. **F:** Metal wire loops labelled with a numbered plastic bead at the rosette base of *Plantago lanceolata* individuals; one end of the loop in place. Photo credits: A, D, F: Siri Lie Olsen; B, C: Anja Linstädter; E: Knut Rydgren.

Table 4.3.1 Tags and marks used in plant population ecology with their main advantages and disadvantages, and with recommendations for their use. Options 1–5 are placed next to the plant while options 6–12 are placed on the plant. Table modified and extended from Gibson (2015).

Tag or mark	Costs	Effort	Durability	Coding	Disadvantages	Our recommendation
1. Toothpicks	Low	Low	Very low	Numbered beads	Coding difficult; easily lost	For small plants; good for geophytes (Figure 4.3.1A)
2. Wooden stakes	Low	Low	Low	Flags	Will rot, markings will fade	Not recommended
3. Plastic stakes	Medium	Low	Low	Numbers on stakes or flags	Easily lost (lightweight)	For isolated herbaceous plants (Figure 4.3.1B)
4. Metal stake flags	High	Low	Medium	Flags	Plastic flags fade and rip quickly	For isolated herbaceous plants
5. Metal stakes	High	Low to high	Very high	Flags; numbers welded onto stakes	Expensive; may be stolen due to material value	For > 5 year studies on herbaceous plants
6. Thread	Low	Medium	Low	Colour coding	Difficult to create a loop	For small rosette plants (Figure 4.3.1C)
7. Paint or ink marks on leaves	Low	Low	Very low	Colour coding	Can fade or wash off; potentially toxic	Not recommended
8. Plastic straw collars	Low	Medium	Low	Flags	Easily lost	Not recommended
9. Plastic rings	Medium	Low	Medium	Numbers; colour coding possible	Not suitable for very small plants	Flexible, durable system for herbaceous plants (Figure 4.3.1D); also for clonal plants (Figure 4.3.1E)
10. Wire loops	Medium	Medium	High	Flags	Time-consuming to install	Durable system; for woody plants (stem), rosette plants (base, with rod, Figure 4.3.1F)
11. Steel or aluminium tags	High	High	Very high	Colour coding; number stamped on tag	Expensive and time-consuming to install	Very durable; for woody plants
12. Paint marks on stem	Low	Low	Medium	Colour or number coding	Can fade or wash off	Easily discernible; for woody plants (in combination with metal tags)

The link between seeds and seedlings has to be established through germination and seed-bank experiments (if the species maintains a seed bank), yielding the probability of a seed becoming an established seedling at the following census and the probability of ungerminated seeds surviving in the soil (e.g. Quintana-Ascencio et al., 2003; Zimmermann et al., 2008; see also protocols 4.2 Seed viability, germinability, and dormancy and 4.6 The soil seed bank (buried seed pool)). The specific measurements for vital rate estimation vary between species based on their life cycle, the vital rates involved, and the chosen state variable. It is thus crucial to understand the life cycle of the study species prior to doing a demographic study. Another important consideration to be addressed is whether the population is to be measured before or after reproduction (pre-reproduction census v. post-reproduction census), as this has crucial implications for the life-cycle graph that underpins further analysis of the demographic data (Caswell, 2001). Furthermore, the appropriate number of censuses may vary between certain groups of species. While there is usually one census per year for perennial plant species, annuals require several censuses per year (e.g. Salguero-Gomez et al., 2012). For very short-lived annual plants, such as *Arabidopsis thaliana*, biweekly visits are appropriate (Arany et al., 2005).

Where to start

Gibson (2015), Gross (2002)

4.3.2 Special cases, emerging issues, and challenges

Measuring species that exhibit vegetative dormancy. These species constitute a special case in terms of life cycle and modelling of demographic data (Lesica & Steele, 1994). Individuals might fail to sprout for one or more years before showing up again. In such species, it is impossible to determine in the field whether a missing plant is dormant or dead. Monitoring a population for several years can yield that information, the necessary time-span being dependent on the maximum number of years an individual of the study species can remain dormant. However, at both start and end of the demographic study, there will be some years where it will be impossible to distinguish transitions out of dormancy from clonal recruitment (start) and transitions into dormancy from mortality (end). In long-term studies, the start- and end-years are hence often excluded from analysis (Gremer et al., 2012), or unobservable life-states can be statistically modelled (Shefferson et al., 2001). In short-term studies, usually neither of these approaches are possible and researchers are hence forced to make assumptions about whether an unobserved plant is dead or dormant (Olsen et al., 2016; Töpper et al., 2018).

Measuring trees and larges shrubs. Given the temporal and spatial scale of climate-change studies, full demographic studies are often not feasible for these long-lived woody species. In the add-on protocol to the International Drought Experiment for tall stature ecosystems (IDE, 2016), it is recommended to only monitor the dieback and survival of adult trees. However, if a climate-change study is specifically set up in vegetation dominated by trees and large shrubs, the gold standard would be to additionally measure i) growth and ii) fecundity, i.e. the germination and fate of tree seedlings and saplings. Specific options for tagging large woody plants are given in Table 4.3.1. Numerous allometric models have been established to estimate the size of trees and shrubs (and, hence, growth) based on one or few state variables (see compilation of models by Henry et al.,

2013). Among these variables, "stem diameter at breast height" (dbh; measured at 1.3 m above the ground) is the most prominent one. Although growth is most often measured via annual censuses or over longer periods (Malhi et al., 2004), intra-annual measurements are also possible, for example with the aid of dendrometer bands (McMahon & Parker, 2015). For tropical trees, a single allometric model has recently been established that provides reliable estimates of tree size (biomass) based on only three state variables – stem diameter, tree height, and wood specific gravity (Chave et al., 2014). This model allows the estimation of individual-level and stand-level growth (see protocol 2.1.1 Aboveground plant biomass).

Building population models. In addition to simple statistical analysis of demographic variables of interest, a demographic dataset addressing the entire lifecycle of a species can be used to build population models. The major modelling techniques available for this today comprise "matrix models" (Caswell, 2001) for annual and perennial plants and "integral projection models" (Easterling et al., 2000) for perennials. Another upcoming method is "integrated population models", a Bayesian modelling approach capable of integrating demographic data from different sources such as field trials and museum materials (Kéry & Schaub, 2012).

4.3.3 References

Theory, significance, and large datasets

Caswell (2001), Easterling et al. (2000), Henry et al. (2013), Merow et al. (2014a), Rydgren & Økland (2002), Salguero-Gomez et al. (2015)

More on methods and existing protocols

Gibson (2015), PlantPopNet protocol (nd), Wardle et al. (2014)

All references

Adler, P. B. & HilleRisLambers, J. (2008). The influence of climate and species composition on the population dynamics of ten prairie forbs. *Ecology, 89*(11), 3049-3060.

Arany, A. M., de Jong, T., & van der Meijden, E. (2005). Herbivory and abiotic factors affect population dynamics of *Arabidopsis thaliana* in a sand dune area. *Plant Biology, 7*, 549-556.

Buckley, Y. M., Briese, D. T., & Rees, M. (2003). Demography and management of the invasive plant species *Hypericum perforatum*. II. Construction and use of an individual-based model to predict population dynamics and the effects of management strategies. *Journal of Applied Ecology, 40*(3), 494-507.

Caswell, H. (2001). *Matrix Population Models: Construction, Analysis, and Interpretation* (2nd ed). Sunderland, Massachusetts: Sinauer Associates, Inc. Publishers.

Chave, J., Réjou-Méchain, M., Búrquez, A., Chidumayo, E., Colgan, M. S., Delitti, W. B. C., … Vieilledent, G. (2014). Improved allometric models to estimate the aboveground biomass of tropical trees. *Global Change Biology, 20*, 3177-3190.

Cui, H., Töpper, J. P., Yang, Y., Vandvik, V., & Wang, G. (2018). Plastic population effects and conservative leaf traits in a reciprocal transplant experiment simulating climate warming in the Himalayas. *Frontiers in Plant Science, 9, 1069*.

Dalgleish, H. J., Koons, D. N., Hooten, M. B., Moffet, C. A., & Adler, P. B. (2011). Climate influences the demography of three dominant sagebrush steppe plants. *Ecology, 92*(1), 75-85.

Easterling, M. R., Ellner, S. P., & Dixon, P. M. (2000). Size-specific sensitivity: Applying a new structured population model. *Ecology, 81*, 694-708.

Ehrlén, J., Syrjänen, K., Leimu, R., Begona Garcia, M., & Lehtilä, K. (2005). Land use and population growth of *Primula veris*: an experimental demographic approach. *Journal of Applied Ecology, 42*(2), 317-326.

Elzinga, C. L., Salzer, D. W., & Willoughby, J. W. (1998). *Measuring and Monitoring Plant Populations*. Denver, USA: Bureau of Land Management, U.S. Department of the Interior.

Evans, M. E. K., Holsinger, K. E., & Menges, E. S. (2010). Fire, vital rates, and population viability: a hierarchical Bayesian analysis of the endangered Florida scrub mint. *Ecological Monographs, 80(*4), 627-649.

Farrer, E. C., Ashton, I. W., Knape, J., & Suding, K. N. (2014). Separating direct and indirect effects of global change: a population dynamic modeling approach using readily available field data. *Global Change Biology, 20*(4), 1238-1250.

Franco, M., & Silvertown, J. (2004). A comparative demography of plants based upon elasticities of vital rates. *Ecology, 85*, 531-538.

Fridley, J. D. (2017). Plant energetics and the synthesis of population and ecosystem ecology. *Journal of Ecology, 105*, 95-110.

Gibson, D. J. (2015). *Methods in Comparative Plant Population Ecology*. Oxford: Oxford University Press.

Gornish, E. S. (2014). Demographic effects of warming, elevated soil nitrogen and thinning on the colonization of a perennial plant. *Population Ecology, 56*(4), 645-656.

Gremer, J. R., Crone, E. E., & Lesica, P. (2012). Are dormant plants hedging their bets? Demographic consequences of prolonged dormancy in variable environments. *The American Naturalist, 179*, 315-327.

Gross, K. (2002). Efficient data collection for estimating growth rates of structured populations. *Ecology, 83*, 1762-1767.

Henry, M., Bombelli, A., Trotta, C., Alessandrini, A., Birigazzi, L., Sola, G., … Valentini, R. (2013). GlobAllomeTree: international platform for tree allometric equations to support volume, biomass and carbon assessment. *Iforest - Biogeosciences and Forestry, 6*, 326.

IDE (2016). Protocol: Drought Experiments in tall stature (forest and shrubland) ecosystems. Add-On protocol to the International Drought Experiment. http://wp.natsci.colostate.edu/droughtnet/files/2016/12/IDE_Forest-Protocol_final_3.2.pdf (accessed Oct 6 2017)

Johansen, L., Wehn, S., & Hovstad, K. A. (2016). Clonal growth buffers the effect of grazing management on the population growth rate of a perennial grassland herb. *Flora, 223*, 11-18.

Jongejans, E., & de Kroon, H. (2005). Space versus time variation in the population dynamics of three co-occurring perennial herbs. *Journal of Ecology, 93*, 681-692.

Jongejans, E., Shea, K., Skarpaas, O., Kelly, D., & Ellner, S. P. (2011). Importance of individual and environmental variation for invasive species spread: a spatial integral projection model. *Ecology, 92*(1), 86-97.

Kéry, M., & Schaub, M. (2012). *Bayesian Population Analysis using WinBUGS – A Hierarchical Perspective*. Academic Press.

Lesica, P., & Steele, B. M. (1994). Prolonged dormancy in vascular plants and implications for monitoring studies. *Natural Areas Journal, 14*(3), 209-212.

Malhi, Y., Baker, T. R., Phillips, O. L., Almeida, S., Alvarez, E., Arroyo, L., … Higuchi, N. (2004). The above-ground coarse wood productivity of 104 Neotropical forest plots. *Global Change Biology, 10*, 563-591.

McMahon, S. M., & Parker, G. G. (2015). A general model of intra-annual tree growth using dendrometer bands. *Ecology and Evolution, 5*, 243-254.

Merow, C., Dahlgren, J. P., Metcalf, C. J. E., Childs, D. Z., Evans, M. E. K., Jongejans, E., … McMahon, S. M. (2014a). Advancing population ecology with integral projection models: a practical guide. *Methods in Ecology and Evolution, 5*(2), 99-110.

Merow, C., Latimer, A. M., Wilson, A. M., McMahon, S. M., Rebelo, A. G., Silander, J. A. (2014b). On using integral projection models to generate demographically driven predictions of species' distributions: development and validation using sparse data. *Ecography, 37*, 1167-1183.

Miller, T. E. X., Louda, S. M., Rose, K. A., & Eckberg, J. O. (2009). Impacts of insect herbivory on cactus population dynamics: experimental demography across an environmental gradient. *Ecological Monographs, 79*(1), 155-172.

Münzbergová, Z., & Ehrlén, J. (2005). How best to collect demographic data for population viability analysis models. *Journal of Applied Ecology, 42*, 1115-1120.

Nicolè, F., Dahlgren, J. P., Vivat, A., Till-Bottraud, I., & Ehrlén, J. (2011). Interdependent effects of habitat quality and climate on population growth of an endangered plant. *Journal of Ecology*, *99*(5), 1211-1218.

Olsen, S. L., Töpper, J. P., Skarpaas, O., Vandvik, V., & Klanderud, K. (2016). From facilitation to competition: temperature-driven shift in dominant plant interactions affects population dynamics in semi-natural grasslands. *Global Change Biology, 22*, 1915-1926.

Pagel, J., & Schurr, F. M. (2012). Forecasting species ranges by statistical estimation of ecological niches and spatial population dynamics. *Global Ecology and Biogeography, 21*, 293-304.

Pfeifer-Meister, L., Bridgham, S. D., Little, C. J., Reynolds, L. L., Goklany, M. E., & Johnson, B. R. (2013). Pushing the limit: experimental evidence of climate effects on plant range distributions. *Ecology, 94*(10), 2131-2137.

PlantPopNet protocol (nd). http://plantago.plantpopnet.com/protocol (accessed January 15th 2018)

Quintana-Ascencio, P. F., Menges, E. S., & Weekley, C. W. (2003). A fire-explicit population viability analysis of *Hypericum cumulicola* in Florida rosemary scrub. *Conservation Biology, 17*(2), 433-449.

Ramula, S., Rees, M., & Buckley, Y. M. (2009). Integral projection models perform better for small demographic data sets than matrix population models: a case study of two perennial herbs. *Journal Applied Ecology, 46*, 1048-1053.

Rydgren, K., & Økland, R. (2002). Life-cycle graphs and matrix modelling of bryophyte populations. *Lindbergia, 27*, 81-89.

Salguero-Gomez, R., Siewert, W., Casper, B. B., & Tielbörger, K. (2012). A demographic approach to study effects of climate change in desert plants. *Philosophical Transactions of the Royal Society B: Biological Sciences, 367*, 3100-3114.

Salguero-Gómez, R., Jones, O. R., Archer, C. R., Buckley, Y. M., Che-Castaldo, J., Caswell, H., … Vaupel, J. W. (2015). The Compadre Plant Matrix Database: an open online repository for plant demography. *Journal of Ecology, 103*, 202-218.

Seldal, T., Hegland, S. J., Rydgren, K., Rodriguez-Saona, C., & Töpper, J. P. (2017). How to induce defense responses in wild plant populations? Using bilberry (*Vaccinium myrtillus*) as example. *Ecology and Evolution, 7*, 1762-1769.

Shefferson, R. P., Sandercock, B. K., Proper, J., & Beissinger, S. R. (2001). Estimating dormancy and survival of a rare herbaceous perennial using mark-recapture models. *Ecology, 82*, 145-156.

Shryock, D. F., Esque, T. C., & Hughes, L. (2014). Population viability of *Pediocactus bradyi* (Cactaceae) in a changing climate. *American Journal of Botany, 101*(11), 1944-1953.

Sletvold, N., Dahlgren, J. P., Øien, D. I., Moen, A., & Ehrlén, J. (2013). Climate warming alters effects of management on population viability of threatened species: results from a 30-year experimental study on a rare orchid. *Global Change Biology, 19*(9), 2729-2738.

Töpper, J. P., Meineri, E., Olsen, S. L., Rydgren, K., Skarpaas, O., & Vandvik, V. (2018). The devil is in the detail: non-additive and context-dependent plant population responses to increasing temperature and precipitation. *Global Change Biology*, doi:10.1111/gcb.14336

Treurnicht, M., Pagel, J., Esler, K. J., Schutte-Vlok, A., Nottebrock, H., Kraaij, T., ... Schurr, F. M. (2016). Environmental drivers of demographic variation across the global geographical range of 26 plant species. *Journal of Ecology, 104*(2), 331-342.

van der Meer, S., Dahlgren, J. P., Mildén, M., & Ehrlén, J. (2014). Differential effects of abandonment on the demography of the grassland perennial *Succisa pratensis*. *Population Ecology*, *56*(1), 151-160.

Wardle, G., Buckley, Y., & PlantPopNet steering group. (2014). PlantPopNet: A spatially distributed model system for population ecology. Ecology Society of Australia 2014 Annual Conference, Alice Springs NT, Australia

Zimmermann, J., Higgins, S. I., Grimm, V., Hoffmann, J., Münkemüller, T., & Linstädter, A. (2008). Recruitment filters in a perennial grassland: The interactive roles of fire, competitors, moisture and seed availability. *Journal of Ecology, 96*, 1033-1044.

Zimmermann, J., Higgins, S. I., Grimm, V., Hoffmann, J., & Linstädter, A. (2015). Local density effects on individual production are dynamic: Insights from natural stands of a perennial savanna grass. *Oecologia, 178*, 1125-1135.

Authors: Linstädter A[1,2], Töpper JP[3,4]

Reviewer: Tielbörger K[5]

Affiliations

[1] Institute of Crop Science and Resource Conservation (INRES), University of Bonn, Bonn, Germany

[2] Range Ecology and Management Group, Botanical Institute, University of Cologne, Cologne, Germany

[3] Norwegian Institute for Nature Research, Bergen, Norway

[4] Department of Biological Sciences, University of Bergen, Bergen, Norway

[5] Institute of Evolution and Ecology, Plant Ecology Group, University of Tübingen, Tübingen, Germany

4.4 Bud dormancy depth

Authors: Malyshev AV[1]

Reviewers: Muffler L[1,2], Wilfahrt P[3]

Measurement unit: days to budburst, proportion of budburst; Measurement scale: plot; Equipment costs: €; Running costs: €; Installation effort: low; Maintenance effort: medium to high; Knowledge need: low; Measurement mode: manual

Vegetative bud dormancy is a characteristic of many northern plant species describing a requisite cold period for budburst to occur in spring (Harrington et al., 2010). Tracking changes in bud dormancy depth allows the determination of the required temperature sums needed for budburst, thereby increasing the mechanistic understanding of subsequent phenology changes (Laube et al., 2014; Olsen, 2014; Pagter et al., 2015). Artificial warming is necessary to quantify bud dormancy depth and, when combined with photoperiod manipulation, can also be used to separate effects of photoperiod-sensitivity in certain species (Basler & Körner, 2012; Way & Montgomery, 2014; Malyshev et al., 2018). The effect of increased temperatures on bud dormancy depth depends on seasonality of warming and may increase or decrease bud dormancy depth, which may delay or advance budburst, respectively (Kalcsits et al., 2009; Pagter et al., 2015). Temperature-mediated bud dormancy changes have been incorporated into bud phenology models (Kobayashi & Fuchigami, 1983; Schaber & Badeck, 2003; Harrington et al., 2010; Schmitz et al., 2014; Harrington & Gould, 2015) and can lead to better accuracy in predicting spring budburst. Growing season changes can, in turn, be better projected, leading to better estimates of changes in primary productivity. The influence of any environmental factor on bud dormancy state can be measured with this method, taking samples from a group of studied plants growing under different conditions.

4.4.1 What and how to measure?

Bud dormancy state and depth can be quantified via destructive measurements across time. Buds gradually enter the endo-dormant stage from late summer to autumn (timing is species-specific) where dormancy "depth" increases and this inhibition is later broken by species-specific periods of cold temperature (Sogaard et al., 2008). Only after this internal inhibition is broken will buds enter eco-dormancy where budburst is advanced by warming temperatures, although warm temperatures occuring during endo-dormancy may affect budburst, at least in some species (Harrington & Gould, 2015). Therefore, environmental factors may increase or decrease bud dormancy depth and in turn spring phenology depending on the timing of events or manipulations and studied species. In addition to the destructive bud dormancy measurements, spring plant phenology can also be recorded on individual plants (see protocol 4.5 Aboveground plant phenology) to predict species- or community level spring bud burst.

Dormancy state and depth is estimated from the percentage of buds that burst and the mean time required for each bud to burst under optimal growing conditions. Such conditions are termed forcing requirements, where a temperature of approximately 20 °C (Dennis, 2003; Cooke et al., 2012) and a photoperiod of 16 hours (Balandier et al., 1993; Harrington et al., 2010; Junttila & Hanninen, 2012) are considered optimal. Dormancy depth is quantified by the amount of time required for budburst

to occur under forcing conditions: the shorter the forcing period, the lower the bud dormancy depth. Although breaking of endo-dormancy is a gradual and continuous process, more than 50% budburst is generally considered as the point at which bud dormancy is broken (Welling et al., 2004; Anzanello et al., 2014). Forcing requirements should be expressed in a standardised way as temperatures vary constantly; growing degree days (GDD) can standardise this variation and is calculated as:

$$GDD = \sum_{t_0}^{t_1} (\frac{T_{max} - T_{min}}{2} - T_{base})$$

where t_0 is the starting day of the warming period, t_1 is the day at which budburst is observed, T_{max} and T_{min} are daily maximum and minimum air temperature, respectively, and T_{base} is a constant representing a minimum temperature threshold required for budburst activity (often set at 5 ˚C; Polgar & Primack, 2011). Fu et al. (2016) additionally provide functions to calculate growing degree forcing units that capture non-linear accumulation of forcing units.

It is also useful to quantify bud dormancy at two different day lengths (8 h and 16 h are suggested) for each plant group at each sampling date to establish the sensitivity of bud dormancy to photoperiod, which can reveal non-linear responses to warming (Malyshev et al., 2018).

Where to start

Balandier et al. (1993), Champagnat (1989), Dennis (2003), Primack et al. (2015)

Method selection and sample size

Bud dormancy depth can be tested anytime after the terminal buds for next years' growth, have formed, which usually happens between August and September. Leaves, if present, are removed from plants, or twig cuttings as described below, prior to measuring dormancy depth. For small tree seedlings the best method to test bud dormancy depth involves using whole plants, while for adult trees, twig cuttings provide a reliable substitute (Primack et al., 2015). A minimum of five potted tree seedlings per treatment per species/ecotype are suggested, although ten replicates are preferable. Tree seedlings are transferred to a greenhouse or climate chamber set at the above-mentioned forcing conditions. The percentage of budburst is recorded on each tree seedling every two days until 100 % budburst occurs or no further buds burst for four weeks. Total percentage of budburst, rate of budburst (time to reach 50 % budburst), and mean forcing requirement are recorded as parameters used to gauge dormancy depth. Budburst and leaf out are detected with the appearance of the first distinguishable leaf tip and when a leaf has unfolded, respectively (Li et al., 2003; Basler & Körner, 2014; Fu et al., 2014; Vitasse et al., 2014).

Two main methods exist for making twig cuttings of the plants: 1) whole twigs are cut 20–30 cm in length (Primack et al., 2015 and references therein) and 2) single node cuttings are cut, having only one lateral bud, with cuts approximately 3–5 cm below and 1 cm above the bud (Champagnat, 1989; Sønsteby & Heide, 2014). Ten replicates of twig cuttings from at least three different individuals per species are suggested. Method 1 is preferable to mimic field conditions as closely as possible if time is not an issue (up to 3 months for species developing deep dormancy), and gives a more realistic estimation of dormancy depth. Method 1 is also advised when leaf development is to be observed to

completion (compared to timing of budburst alone) as the smaller twig segments in Method 2 may contain insufficient resources (e.g. sugars) for prolonged leaf growth. Method 2 is used when only relative (across treatments) warming-induced changes in bud dormancy over time is of primary interest (Boyer & South, 1989; Champagnat, 1989; Sønsteby & Heide, 2014).

Twig-cutting preparation

The length and diameter of twig cuttings should be kept similar across species to ensure similar resources available for budburst and leaf unfolding. The use of previous years' shoots is suggested to standardise the ages of buds tested. All twig cuttings are placed in deionised water either in large containers containing several twigs (Method 1) or inserted into foam/plastic pads floating on water (Sønsteby & Heide, 2014; Method 2). Deionised water should be used to standardise trials and twigs should be treated with a disinfectant to prevent mould growth during the incubation period (Basler & Körner, 2012; Laube et al., 2014). The water must be changed twice a week and the twigs need to be recut at least weekly to ensure sufficient water uptake by the cuttings, each time cutting off 0.5–1 cm from the stem base. Measured parameters mirror those used for whole tree seedlings.

4.4.2 Emerging issues, methods, and challenges

Changes in the timing of transition between bud dormancy states and their depths as a result of climate change will need to be studied to explain the underlying mechanism driving changes in leaf phenology. The main challenge in bud dormancy quantification is in reducing the time required to determine the bud dormancy depth at a particular point in time. Bud dormancy depth can potentially be estimated faster at the expense of being less comparable among plant groups. Here, cell division activity of leaf primordia (Cooke et al., 2012) and concentrations of specific hormones and sugars (Li et al., 2003; Chao et al., 2007; Basler & Körner, 2014) can be analysed from collected bud/twig samples. Furthermore, genetic, epigenetic, and physiological changes have been documented during changes in bud dormancy (Rios et al., 2014). Extensive preliminary analyses are required to establish precise grading of cell division in different species, with potentially species-specific genetic, hormonal, and physiological changes driving bud dormancy. Whether key compounds such as abscisic acid (Giraudat et al., 1994) are directly related to the dormancy depth across species is unknown but the search for them could be useful to enable faster quantification of bud dormancy depth.

4.4.3 References

Theory, significance, and large datasets

Champagnat (1989), Chao et al. (2007), Cooke et al. (2012), Harrington & Gould (2015)

A database on bud dormancy depth of a large selection of species is available for download from: https://hdl.handle.net/10355/53250

More on methods and existing protocols

Dennis (2003), Junttila & Hanninen (2012), Malyshev et al. (2018), Sønsteby & Heide (2014), Vitasse et al. (2014)

All references

Anzanello, R., Fialho, F. B., dos Santos, H. P., Bergamaschi, H., & Marodin, G. A. B. (2014). Bud dormancy in apple trees after thermal fluctuations. *Pesquisa Agropecuária Brasileira, 49*, 457-464.

Balandier, P., Gendraud, M., Rageau, R., Bonhomme, M., Richard, J. P., & Parisot, E. (1993). Bud break delay on single node cuttings and bud capacity for nucleotide accumulation as parameters for endo- and paradormancy in peach trees in a tropical climate. *Scientia Horticulturae, 55*, 249-261.

Basler, D., & Körner, C. (2012). Photoperiod sensitivity of bud burst in 14 temperate forest tree species. *Agriculture and Forest Meteorology, 165*, 73-81.

Basler, D., & Körner, C. (2014). Photoperiod and temperature responses of bud swelling and bud burst in four temperate forest tree species. *Tree Physiology, 34*, 377-388.

Boyer, J. N., & South, D. B. (1989). Seasonal changes in intensity of bud dormancy in loblolly pine seedlings. *Tree Physiology, 5*, 379-385.

Champagnat, P. (1989). Rest and activity in vegetative buds of trees. *Annals of Forest Science, 46*, 9-26.

Chao, W. S., Foley, M. E., Horvath, D. P., & Anderson, J. V. (2007). Signals regulating dormancy in vegetative buds. *International Journal of Plant Developmental Biology, 1*, 49-56.

Cooke, J. E. K., Eriksson, M. E., & Junttila, O. (2012). The dynamic nature of bud dormancy in trees: Environmental control and molecular mechanisms. *Plant, Cell & Environment, 35*, 1707-1728.

Dennis, F. G. (2003). Problems in standardizing methods for evaluating the chilling requirements for the breaking of dormancy in buds of woody plants. *Hort Science, 38*(3), 347-350.

Fu, Y. S. H., Campioli, M., Vitasse, Y., De Boeck, H.J., Van den Berge, J., AbdElgawad, H., … Janssens, I. A. (2014). Variation in leaf flushing date influences autumnal senescence and next year's flushing date in two temperate tree species. *Proceedings of the National Academy of Sciences USA, 111*, 7355-7360.

Fu, Y. S. H., Liu, Y., De Boeck, H. J., Menzel, A., Nijs, I., Peaucelle, M., … Janssens, I. A. (2016). Three times greater weight of daytime than of night-time temperature on leaf unfolding phenology in temperate trees. *New Phytologist, 212*, 590-597.

Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P. C., … Vartanian, N. (1994). Current advances in abscisic acid action and signalling. *Plant Molecular Biology, 26*, 1557-1577.

Harrington, C. A., & Gould, P. J. (2015). Tradeoffs between chilling and forcing in satisfying dormancy requirements for Pacific Northwest tree species. *Frontiers in Plant Science, 6*, 1-12.

Harrington, C. A., Gould, P. J., & St. Clair, J. B. (2010). Modeling the effects of winter environment on dormancy release of Douglas fir. *Forest Ecology and Management, 259*, 798-808.

Junttila, O., & Hanninen, H. (2012). The minimum temperature for budburst in *Betula* depends on the state of dormancy. *Tree Physiology, 32*, 337–345.

Kalcsits, L. A., Silim, S., & Tanino, K. (2009). Warm temperature accelerates short photoperiod-induced growth cessation and dormancy induction in hybrid poplar. *Trees – Structure and Function, 23*(5), 971-979.

Kobayashi, K. D., & Fuchigami, L. H. (1983). Modeling bud development during the quiescent phase in red-osier dogwood (*Cornus sericea* L.). *Agricultural Meteorology, 28*(1), 75-84.

Laube, J., Sparks, T. H., Estrella, N., Höfler, J., Ankerst, D. P., & Menzel, A. (2014). Chilling outweighs photoperiod in preventing precocious spring development. *Global Change Biology, 20*, 170-182.

Li, C. Y., Junttila, O., Ernstsen, A., Heino, P., & Palva, E. T. (2003). Photoperiodic control of growth, cold acclimation and dormancy development in silver birch (*Betula pendula*) ecotypes. *Physiologia Plantarum, 117*, 206-212.

Malyshev, A. V., Henry, H. A. L., Bolte, A., Arfin Khan, M. A. S., & Kreyling, J. (2018). Temporal photoperiod sensitivity and forcing requirements for budburst in temperate tree seedlings. *Agricultural and Forest Meteorology, 248*, 82-90.

Olsen, J. E. (2014). Effect of alternating day and night temperature on short day-induced bud set and subsequent bud burst in long days in Norway spruce. *Frontiers in Plant Science, 5*, art691.

Pagter, M., Andersen, U. B., & Andersen, L. (2015). Winter warming delays dormancy release, advances budburst, alters carbohydrate metabolism and reduces yield in a temperate shrub. *AoB Plants, 7*, plv024.

Polgar, C. A., & Primack, R. B. (2011). Leaf-out phenology of temperate woody plants: from trees to ecosystems. *New Phytologist, 191*, 926-941.

Primack, R. B., Laube, J., Gallinat, A. S., & Menzel, A. (2015). From observations to experiments in phenology research: investigating climate change impacts on trees and shrubs using dormant twigs. *Annals of Botany, 116*(6), 889-897.

Rios, G., Leida, C., Conejero, A., & Badenes, M. L. (2014). Epigenetic regulation of bud dormancy events in perennial plants. *Frontiers in Plant Science, 5*, art247.

Schaber, J., & Badeck, F.-W. W. (2003). Physiology-based phenology models for forest tree species in Germany. *International Journal of Biometeorology, 47*, 193-201.

Schmitz, J. D., Guédon, Y., Herter, F. G., Leite, G. B., & Lauri, P. É. (2014). Exploring bud dormancy completion with a combined architectural and phenological analysis: The case of apple trees in contrasting winter temperature conditions. *American Journal of Botany, 101*, 398-407.

Sogaard, G., Johnsen, O., Nilsen, J., & Junttila, O. (2008). Climatic control of bud burst in young seedlings of nine provenances of Norway spruce. *Tree Physiology, 28,* 311-320.

Sønsteby, A., & Heide, O. M. (2014). Chilling requirements of contrasting black currant (*Ribes nigrum* L.) cultivars and the induction of secondary bud dormancy. *Scientia Horticulturae, 179*, 256-265.

Vitasse, Y., Basler, D., & Way, D. (2014). Is the use of cuttings a good proxy to explore phenological responses of temperate forests in warming and photoperiod experiments? *Tree Physiology, 34*, 174-183.

Way, D. A., & Montgomery, R. A. (2014). Photoperiod constraints on tree phenology, performance and migration in a warming world. *Plant, Cell & Environment, 38*(9), 1725-1736.

Welling, A., Rinne, P., Viherä-Aarnio, A., Kontunen-Soppela, S., Heino, P., & Palva, E. T. (2004). Photoperiod and temperature differentially regulate the expression of two dehydrin genes during overwintering of birch (*Betula pubescens* Ehrh.). *Journal of Experimental Botany, 55*, 507-516.

Authors: Malyshev AV[1]

Reviewers: Muffler L[1,2], Wilfahrt P[3]

Affiliations

[1] Experimental Plant Ecology, Institute of Botany and Landscape Ecology, University of Greifswald, Greifswald, Germany

[2] Plant Ecology, Albrecht-von-Haller Institute for Plant Sciences, University of Goettingen, Goettingen, Germany

[3] Department of Disturbance Ecology, BayCEER, University of Bayreuth, Bayreuth, Germany

4.5 Aboveground plant phenology

Authors: Halbritter AH[1], Gillespie MAK[2], Estiarte M[3,4]

Reviewers: Wohlgemuth T[5], Peñuelas J[3,4]

Measurement unit: days, growing degree days, counts; Measurement scale: plot; Equipment costs: €-€€; Running costs: €; Installation effort: low to medium; Maintenance effort: high (frequent recording); Knowledge need: medium (species knowledge); Measurement mode: manual or data logger

Phenology refers to the timing of a species' seasonal recurrent life-cycle events – the phenophases (see Table 4.5.1). Plants living in seasonal environments adjust the timing of their vegetative and reproductive phenophases in line with conditions favourable for each activity, be it growth, flowering, seed set, or other activity. Co-ordination to promote the optimal timing of each phenophase is important for survival, plant growth, and reproduction. Minimising the risk of freezing of newly formed leaves and flowers, adjusting leaf senescence to lengthen the active season but optimising nutrient resorption before frosts, or adjusting the timing of flowering in animal-pollinated plants with the peak occurrence of their pollinators to optimise fertilisation are examples of how this timing can be important. Thus phenology can affect demography (Inouye, 2008; Miller-Rushing et al., 2010; Scranton & Amarasekare, 2017), species distribution (Chuine, 2010), biodiversity and community composition (van Vliet et al., 2003; CaraDonna et al., 2014), trophic interactions such as plant–pollinator interactions (Thackeray et al., 2016) or outbreak of disease (van Vliet, 2010), and alter biochemical cycling (Keeling et al., 1996; Cleland et al., 2007; Peñuelas et al., 2009; Heberling et al., 2019). Understanding shifts in the phenology of different and interacting species provides researchers with more information to forecast the impacts of climate change on plant communities and ecosystem functioning. In Northern Hemisphere temperate and boreal zones, northern range limits are caused by the inability to finish fruit maturation and southern ranges are defined by lacking cold temperatures (chilling) in wintertime that are necessary to break bud dormancy (Chuine, 2010).

Plants use environmental cues as seasonal triggers and to control their development. The main cues are photoperiod, snow cover, timing of snowmelt, air and soil temperature, soil moisture, precipitation, and exposure to cold, which, excluding daylength, are likely to be modified under climate change. Consequently, phenology can be used as a proxy to document the effects of changes in climate. Over the last few decades, the phenology of many species across taxonomic groups and regions has shifted due to climate change (Peñuelas & Filella, 2001; Parmesan & Yohe, 2003; Menzel et al., 2006; Cleland et al., 2007; Wolkovich et al., 2012), with many species advancing their phenology due to warmer spring temperatures. However, species have reacted in varying ways, partly because different species respond to different environmental cues (e.g. Vitasse et al., 2009). Plant phenology can also be impacted by other global-change drivers, such as nitrogen deposition and elevated CO_2 (Cleland et al., 2006; Stevens et al., 2018).

4.5.1 What and how to measure?

Visual phenological monitoring

Aboveground plant phenology is recorded by dating as accurately as possible the occurrence of the phenophases of interest related to the vegetative and/or reproductive events. Here, only aboveground phenological events visible to the naked eye are considered; not events that need instrumentation to be detected, such as stem and belowground growth or physiological events (see protocols 2.1.1 Aboveground plant biomass, 2.1.2 Belowground plant biomass and chapter 5 Stress physiology).

Phenological events are spread across the entire growing season and the data collection can be very time consuming. The most commonly recorded phenophases in climate-change studies are the first occurrence and maturation of the vegetative and reproductive phenophases listed in Table 4.5.1 (for a more detailed description of phenophases in trees see Table 1 in Finn et al., 2007). Phenophases can overlap (e.g. buds and flowers); for detailed studies, it is important to record all concurrent phases.

Table 4.5.1 The most commonly recorded vegetative and reproductive phenophases in climate-change studies.

Vegetative phases	Dormant winterbuds
	Breaking leaf buds
	Green leaves, needles, or rosette visible
	Stem or shoot elongation
	Lammas growth (second shoots grown in summer; e.g. Battey, 2003)
	Senescence / leaf or needle colouring
	Leaf or needle fall
Reproductive phases	Bud development ceased in autumn
	Budburst
	Emergence of petals
	Open flowers / anthesis
	Flower withering
	Fruit / seed maturation
	Seed dispersal

Defining beforehand when a certain phenophase is reached is important for consistency, especially when different species are compared, and different people are involved in the data collection. For example, flowering is generally defined when the petals are open, revealing the reproductive structure and the flower is ready to be pollination (if insect pollinated). Some species produce a single flower, while others produce several flowers together in a spike or umbel. For plants with a more complicated flowering structure, flowering is defined when the first flower on any spike or umble is has opened (see Haggerty & Mazer, 2008 for species with different flowering architectures and definitions of different phenophases). For large species, phenophases are better defined as a

fraction of plant organs reaching a stage, for instance 50% yellow leaves is taken as date for leaf senescence, or alternatively, the proportion of branches at a certain stage, i.e. flowers open (Morellato et al., 2010).

Typically, data are recorded for plants within experimental plots and further expressed as plot aggregated values, although sometimes records are done at plot or ramet level. Phenological monitoring requires repeated assessment of the presence and absence of phenophases (Denny et al., 2014). Repeatedly recording the presence and absence of different phenophases provides more information than recording a single "event" (Diez et al., 2012). From repeated recordings, multiple events of each phenophase (e.g. onset, duration, end) can be assessed and extracted. This is also useful in habitats with no defined start of the growing season (e.g. tropics), where plants produce leaves, flowers, and fruits all year round.

The duration of the recording period will depend on the level of detail required by the phenophases under study and on the specific growth cycle and life form of the investigated species. For example, some species flower only for a couple of days, while other species flower for several months (Gentry, 1974; Opler et al., 1980) and require a different sampling duration and frequency. Ideally, the time intervals for phenological monitoring range from every day to once a week, depending on how fast species change their phenophases. For example, to accurately detect the onset and end of anthesis, recording must be more frequent in species flowering just for a few days than in species with longer flowering periods (see above).

Abundance of phenophases

In addition to the presence/absence of phenophases, it is recommended to record the abundance of specific life stages (e.g. number of open flowers for a species), especially for continuous records. This is a simple additional effort that provides information on the date of peak events, for example number of open flowers, which can be important for pollinators.

Field operation

For continuous observations, it is important to mark the plots and plants or ramets to make sure the same area and individuals are monitored each time. In some systems (e.g. heavily grazed areas), fencing might be preferable to protect the plants and monitoring plots from large herbivores.

Interpretation

Phenophases can be defined temporally as a point in time (i.e. onset or end) or a duration. The onset or end of a phenophase can be defined by its mean start date or end date. The duration of a phenophase can be defined as the period from the mean start date to the mean end date. The onset of a phenophase is one of the most commonly used variables in phenological studies (e.g. Oberbauer et al., 2013). However, the onset and end date or duration of a phenophase do not necessarily respond in the same way to climate change, suggesting that only focusing on the onset might be misleading (CaraDonna et al., 2014).

The onset or end date of a phenophase is calculated as the first or last day a phenophase is observed. Often, the mean date when the first 10–25% of plants start to flower or budburst is used (Jentsch et al., 2009). If the abundance of different phenophases is recorded, the peak of a phenophase can be calculated. For this, the date when 50% of the individuals are in a certain phenophase is commonly used (CaraDonna et al., 2014; Gugger et al., 2015).

Phenological dates on their own are often meaningless and it is the comparison between different species, treatments, time periods, or across time periods that make them useful. Since changes in phenophases are often triggered by environmental cues, it is highly recommended to complement the phenological monitoring with continuous meteorological records. The variability in the meteorology can be correlated to within- and among- year variations in the phenophases at site level and to compare with the sensitivity to experimental treatments (see protocol 1.5 Meteorological measurements). The most useful variables to record for phenological monitoring are: air temperature, soil temperature, and soil moisture (Carbognani et al., 2016; Theobald et al., 2017). In cold environments (i.e. alpine and arctic habitats), the timing of snowmelt should always be recorded because it is an important driver that defines the start of the growing season (Körner, 2003). Climate data can also be used to calculate the cumulative temperature to reach a phenophase (often as growing degree days above a temperature threshold), which is a measure of the temperature requirement (i.e. energy) of a species to reach a phenophase.

To study the phenological response to changes in temperature, often the temperature sensitivity of a species is calculated, which is the change of a phenological event (in days) per change in temperature, ΔT (Wolkovich et al., 2012). In a climate warming experiment, the temperature sensitivity can be calculated as:

$$(\text{phenological event date}_{i,\text{warm}} - \text{phenological event date}_{i,\text{control}}) / \Delta T$$

But see Kenan et al. (2019) for challenges using this simplistic metric for temperature sensitivity and a robust alternative.

Where to start

Denny et al. (2014), Elmendorf et al. (2016), Finn et al. (2007), Haggerty & Mazer (2008)

4.5.2 Special cases, emerging issues, and challenges

Automated phenological monitoring

Ground-based, observational phenological monitoring is labour-intensive and expensive and usually only applicable at a local scale. It is thus difficult to upscale to the community or ecosystem level. More recently, “near-surface” phenological monitoring has been undertaken with automated digital cameras (Sonnentag et al., 2012; Brown et al., 2016), which can provide a measure of greenness at a broad spatial and temporal scale containing valuable information on leaf phenology (Richardson et al., 2007). Digital cameras capture colour changes in the vegetation between green-up and senescence colours (red, green, blue, RGB) in the visible spectrum or infrared spectrum (Ide & Oguma, 2010; Sonnentag et al., 2012; Nijland et al., 2014).

Satellite image-aided analysis and satellite remote sensing measure the reflectance of the vegetation from which the normalised difference vegetation index (NDVI) can be calculated. NDVI is an index for the green biomass of the vegetation (Tucker, 1979; Gamon et al., 1995) and can be used to measure the green-up and senescence at a landscape scale. Ground-based observations and hemispherical photography can be used to verify the reliability of satellite data (Schwartz et al., 2002; Karlsen et al., 2009; Rautiainen et al., 2012; Zhang et al., 2015), and also highlight methodological limitations for NDVI data (e.g. noise in the satellite data because of clouds).

Different approaches, challenges, and emerging methods

A common source of long-term datasets to analyse past phenology are museum specimens and historical recordings (MacGillivray et al., 2010; Bartomeus et al., 2011). More recently, citizen-science has contributed to the collection of large phenology datasets (e.g. Miller-Rushing & Primack, 2008; Crimmins et al., 2009).

Comparative studies manipulate plants or plots over a short time period (1–4 years) and the species' response in phenology is measured. Such experiments allow direct manipulation of environmental variables to help disentangle potentially interacting factors (Rafferty et al., 2013). The limitations of such experiments are that they are often accompanied by unwanted or unrecorded side effects (e.g. warming can be correlated with a drying effect).

Alternative approaches are to combine long-term datasets with experimental approaches and/or models to provide a more complete picture of species responses to future climate change (Rafferty et al., 2013). A useful approach is to systematically replicate experiments along environmental gradients to understand the underlying variation of species- and site-specific patterns that many studies show (Dunne et al., 2003; Delnevo et al., 2017). More recently, many national and international phenology networks (van Vliet et al., 2003; Denny et al., 2014; Elmendorf et al., 2016), have developed standardised protocols for phenological observations that allow comparisons across species, environments, phenophases, and time.

Phenology is highly linked with physiology, and interdisciplinary studies combining these two fields can improve the mechanistic and evolutionary understanding of phenology (Forrest & Miller-Rushing, 2010). To study the effect of climate change on plant–pollinator interactions (e.g. Bartomeus et al., 2011; Rafferty & Ives, 2011; Kudo & Ida, 2013; Gillespie et al., 2016), data from broad species networks are required, i.e. counting the number of insect visits to flowers (also see protocol 4.13 Pollinator visitation). Setting up such networks is time consuming and species identification skills for both plants and insects are needed. It is also challenging to combine species networks with climate manipulations, because animals are mobile and use a larger spatial area compared to plants. A useful supplement to phenological studies, is to investigate the consequences of changing phenologies on plant fitness (i.e. quantify survival and/or reproductive output; see protocol 4.1 Sexual reproduction) at the population level (Miller-Rushing et al., 2010; Kudo & Ida, 2013; Forrest, 2015), which could improve our understanding of potential future species distributions. Finally, phenological data are often sparsely and unevenly sampled, there are uncertainties around observations (e.g. the exact date a flower opens is not captured), and forecasting phenology is affected by multiple factors. Novel approaches and statistical methods from other fields can provide more robust tools to analyse phenological data (Diez et al., 2014; Pearse et al., 2017).

4.5.3 References

Theory, significance, and large datasets

More on theory: Hudson & Keatley (2010); Large data sets such as the European Phenology Network: van Vliet et al. (2003) and the USA National Phenology Network: Schwartz et al. (2012)

More on methods and existing protocols

Beuker et al. (2016), Denny et al. (2014), Elmendorf et al. (2016), Haggerty & Mazer (2008), Law et al. (2008)

All references

Bartomeus, I., Ascher, J. S., Wagner, D., Danforth, B. N., Colla, S., Kornbluth, S., & Winfree, R. (2011). Climate-associated phenological advances in bee pollinators and bee-pollinated plants. *Proceedings of the National Academy of Sciences USA*, *108*(51), 20645-20649.

Battey, N. H. (2003). Plant culture: thirteen seasonal pieces: August—learning about summer. *Journal of Experimental Botany*, *54*(389), 1797-1799.

Beuker E., Raspe S., Bastrup-Birk A., Preuhsler T. & Fleck S. (2016). Part VI: Phenological observations. In *Manual on Methods and Criteria for Harmonized Sampling, Assessment, Monitoring and Analysis of the Effects of Air Pollution on Forests*. ICP Forests.

Brown, T. B., Hultine, K. R., Steltzer, H., Denny, E. G., Denslow, M. W., Granados, J., ... Sánchez-Azofeifa, A. (2016). Using phenocams to monitor our changing Earth: toward a global phenocam network. *Frontiers in Ecology and the Environment*, *14*(2), 84-93.

CaraDonna, P. J., Iler, A. M., & Inouye, D. W. (2014). Shifts in flowering phenology reshape a subalpine plant community. *Proceedings of the National Academy of Sciences USA*, *111*(13), 4916-4921.

Carbognani, M., Bernareggi, G., Perucco, F., Tomaselli, M., & Petraglia, A. (2016). Micro-climatic controls and warming effects on flowering time in alpine snowbeds. *Oecologia*, *182*(2), 573-585.

Chuine, I. (2010). Why does phenology drive species distribution? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1555), 3149-3160.

Cleland, E. E., Chiariello, N. R., Loarie, S. R., Mooney, H. A., & Field, C. B. (2006). Diverse responses of phenology to global changes in a grassland ecosystem. *Proceedings of the National Academy of Sciences USA*, *103*(37), 13740-13744.

Cleland, E. E., Chuine, I., Menzel, A., Mooney, H. A., & Schwartz, M. D. (2007). Shifting plant phenology in response to global change. *Trends in Ecology & Evolution*, *22*(7), 357-365.

Crimmins, T. M., Crimmins, M. A., & Bertelsen, C. D. (2009). Flowering range changes across an elevation gradient in response to warming summer temperatures. *Global Change Biology*, *15*(5), 1141-1152.

Delnevo, N., Petraglia, A., Carbognani, M., Vandvik, V., & Halbritter, A. H. (2017). Plastic and genetic responses to shifts in snowmelt time affect the reproductive phenology and growth of *Ranunculus acris*. *Perspectives in Plant Ecology, Evolution and Systematics*, *30*, 62-70.

Denny, E. G., Gerst, K. L., Miller-Rushing, A. J., Tierney, G. L., Crimmins, T. M., Enquist, C. A., ... Weltzin, J. F. (2014). Standardized phenology monitoring methods to track plant and animal activity for science and resource management applications. *International Journal of Biometeorology*, *58*(4), 591-601.

Diez, J. M., Ibáñez, I., Miller-Rushing, A. J., Mazer, S. J., Crimmins, T. M., Crimmins, M. A., ... Inouye, D. W. (2012). Forecasting phenology: from species variability to community patterns. *Ecology Letters*, *15*(6), 545-553.

Diez, J. M., Ibáñez, I., Silander, J. A., Primack, R., Higuchi, H., Kobori, H., ... James, T. Y. (2014). Beyond seasonal climate: statistical estimation of phenological responses to weather. *Ecological Applications*, *24*(7), 1793-1802.

Dunne, J. A., Harte, J., & Taylor, K. J. (2003). Subalpine meadow flowering phenology responses to climate change: integrating experimental and gradient methods. *Ecological Monographs*, *73*(1), 69-86.

Elmendorf, S. C., Jones, K. D., Cook, B. I., Diez, J. M., Enquist, C. A., Hufft, R. A., ... Schwartz, M. D. (2016). The plant phenology monitoring design for the national ecological observatory network. *Ecosphere*, *7*(4), e01303.

Finn, G. A., Straszewski, A. E., & Peterson, V. (2007). A general growth stage key for describing trees and woody plants. *Annals of Applied Biology*, *151*(1), 127-131.

Forrest, J. R. (2015). Plant–pollinator interactions and phenological change: what can we learn about climate impacts from experiments and observations? *Oikos*, *124*(1), 4-13.

Forrest, J. R., & Miller-Rushing, A. J. (2010). Toward a synthetic understanding of the role of phenology in ecology and evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences, 365,* 3101–3112.

Gamon, J. A., Field, C. B., Goulden, M. L., Griffin, K. L., Hartley, A. E., Joel, G., ... Valentini, R. (1995). Relationships between NDVI, canopy structure, and photosynthesis in three Californian vegetation types. *Ecological Applications*, *5*(1), 28-41.

Gentry, A. H. (1974). Flowering phenology and diversity in tropical Bignoniaceae. *Biotropica*, *6*, 64-68.

Gillespie, M. A., Baggesen, N., & Cooper, E. J. (2016). High Arctic flowering phenology and plant–pollinator interactions in response to delayed snow melt and simulated warming. *Environmental Research Letters*, *11*(11), 115006.

Gugger, S., Kesselring, H., Stöcklin, J., & Hamann, E. (2015). Lower plasticity exhibited by high- versus mid-elevation species in their phenological responses to manipulated temperature and drought. *Annals of Botany*, *116*(6), 953-962.

Haggerty, B. P., & Mazer, S. J. (2008). *The Phenology Handbook: a guide to phenological monitoring for students, teachers, families, and nature enthusiasts.* Santa Barbara: University of California.

Heberling, J. M., McDonough MacKenzie, C., Fridley, J. D., Kalisz, S., & Primack, R. B. (2019). Phenological mismatch with trees reduces wildflower carbon budgets. *Ecology letters*, *22*(4), 616-623.

Hudson, I. L., & Keatley, M. R. (Eds.) (2010). *Phenological Research*. Netherlands: Springer.

Ide, R., & Oguma, H. (2010). Use of digital cameras for phenological observations. *Ecological Informatics*, *5*(5), 339-347.

Inouye, D. W. (2008). Effects of climate change on phenology, frost damage, and floral abundance of montane wildflowers. *Ecology*, *89*(2), 353-362.

Karlsen, S. R., Høgda, K. A., Wielgolaski, F. E., Tolvanen, A., Tømmervik, H., Poikolainen, J., & Kubin, E. (2009). Growing-season trends in Fennoscandia 1982–2006, determined from satellite and phenology data. *Climate Research*, *39*(3), 275-286.

Keeling, C. D., Chin, J. F. S., & Whorf, T. P. (1996). Increased activity of northern vegetation inferred from atmospheric CO_2 measurements. *Nature*, *382*(6587), 146-149.

Keenan, T. F., Richardson, A. D., & Hufkens, K. (2019). On quantifying the apparent temperature sensitivity of plant phenology. *New Phytologist*.

Körner, C. (2003). *Alpine Plant Life: functional plant ecology of high mountain ecosystems*. Netherlands: Springer.

Kudo, G., & Ida, T. Y. (2013). Early onset of spring increases the phenological mismatch between plants and pollinators. *Ecology*, *94*(10), 2311-2320.

Law, B. E., Arkebauer, T., Campbell, J. L., Chen, J., Sun, O., Schwartz, M., ... Verma, S. (2008). *Terrestrial Carbon Observations: Protocols for vegetation sampling and data submission*. Rome: FAO.

MacGillivray, F., Hudson, I. L., & Lowe, A. J. (2010). Herbarium collections and photographic images: Alternative data sources for phenological research. In I. L. Hudson, & M. R. Keatley (Eds.), *Phenological Research* (pp. 425-461). Netherlands: Springer.

Menzel, A., Sparks, T. H., Estrella, N., Koch, E., Aasa, A., Ahas, R., ... Chmielewski, F. M. (2006). European phenological response to climate change matches the warming pattern. *Global Change Biology*, *12*(10), 1969-1976.

Miller-Rushing, A. J., & Primack, R. B. (2008). Global warming and flowering times in Thoreau's Concord: a community perspective. *Ecology*, *89*(2), 332-341.

Miller-Rushing, A. J., Høye, T. T., Inouye, D. W., & Post, E. (2010). The effects of phenological mismatches on demography. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1555), 3177-3186.

Morellato, L. P. C., Alberti, L. F., & Hudson, I. L. (2010). Applications of circular statistics in plant phenology: a case studies approach. In I. L. Hudson, & M. R. Keatley (Eds.), *Phenological Research* (pp. 339-359). Netherlands: Springer.

Nijland, W., De Jong, R., De Jong, S. M., Wulder, M. A., Bater, C. W., & Coops, N. C. (2014). Monitoring plant condition and phenology using infrared sensitive consumer grade digital cameras. *Agricultural and Forest Meteorology*, *184*, 98-106.

Oberbauer, S. F., Elmendorf, S. C., Troxler, T. G., Hollister, R. D., Rocha, A. V., Bret-Harte, M. S., ... Jarrad, F. C. (2013). Phenological response of tundra plants to background climate variation tested using the International Tundra Experiment. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1624), 20120481.

Opler, P. A., Frankie, G. W., & Baker, H. G. (1980). Comparative phenological studies of treelet and shrub species in tropical wet and dry forests in the lowlands of Costa Rica. *Journal of Ecology*, *68*(1), 167-188.

Parmesan, C., & Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature*, *421*(6918), 37-42.

Pearse, W. D., Davis, C. C., Inouye, D. W., Primack, R. B., & Davies, T. J. (2017). A statistical estimator for determining the limits of contemporary and historic phenology. *Nature Ecology & Evolution*, *1*(12), 1876-1882.

Peñuelas, J., & Filella, I. (2001). Responses to a warming world. *Science*, *294*(5543), 793-795.

Peñuelas, J., Rutishauser, T., & Filella, I. (2009). Phenology feedbacks on climate change. *Science*, *324*(5929), 887-888.

Rafferty, N. E., & Ives, A. R. (2011). Effects of experimental shifts in flowering phenology on plant–pollinator interactions. *Ecology Letters*, *14*(1), 69-74.

Rafferty, N. E., CaraDonna, P. J., Burkle, L. A., Iler, A. M., & Bronstein, J. L. (2013). Phenological overlap of interacting species in a changing climate: an assessment of available approaches. *Ecology and Evolution*, *3*(9), 3183-3193.

Rautiainen, M., Heiskanen, J., & Korhonen, L. (2012). Seasonal changes in canopy leaf area index and MODIS vegetation products for a boreal forest site in central Finland. *Boreal Environment Research, 17,* 72-84.

Richardson, A. D., Jenkins, J. P., Braswell, B. H., Hollinger, D. Y., Ollinger, S. V., & Smith, M. L. (2007). Use of digital webcam images to track spring green-up in a deciduous broadleaf forest. *Oecologia*, *152*(2), 323-334.

Schwartz, M. D., Reed, B. C., & White, M. A. (2002). Assessing satellite-derived start-of-season measures in the conterminous USA. *International Journal of Climatology*, *22*(14), 1793-1805.

Schwartz, M. D., Betancourt, J. L., & Weltzin, J. F. (2012). From Caprio's lilacs to the USA National Phenology Network. *Frontiers in Ecology and the Environment*, *10*(6), 324-327.

Scranton, K., & Amarasekare, P. (2017). Predicting phenological shifts in a changing climate. *Proceedings of the National Academy of Sciences*, *114*(50), 13212-13217.

Sonnentag, O., Hufkens, K., Teshera-Sterne, C., Young, A. M., Friedl, M., Braswell, B. H., ... Richardson, A. D. (2012). Digital repeat photography for phenological research in forest ecosystems. *Agricultural and Forest Meteorology*, *152*, 159-177.

Stevens, C. J., David, T. I., & Storkey, J. (2018). Atmospheric nitrogen deposition in terrestrial ecosystems: its impact on plant communities and consequences across trophic levels. *Functional Ecology*, *32*(7), 1757-1769.

Thackeray, S. J., Henrys, P. A., Hemming, D., Bell, J. R., Botham, M. S., Burthe, S., ... Mackay, E. B. (2016). Phenological sensitivity to climate across taxa and trophic levels. *Nature*, *535*(7611), 241-245.

Theobald, E. J., Breckheimer, I., & HilleRisLambers, J. (2017). Climate drives phenological reassembly of a mountain wildflower meadow community. *Ecology*, *98*(11), 2799-2812.

Tucker, C. J. (1979). Red and photographic infrared linear combinations for monitoring vegetation. *Remote Sensing of Environment*, *8*(2), 127-150.

van Vliet, A. J. (2010). Societal adaptation options to changes in phenology. In I. L. Hudson, & M. R. Keatley (Eds.), *Phenological Research* (pp. 75-98). Netherlands: Springer.

van Vliet, A. J., de Groot, R. S., Bellens, Y., Braun, P., Bruegger, R., Bruns, E., ... Maggi, M. (2003). The European phenology network. *International Journal of Biometeorology*, *47*(4), 202-212.

Vitasse, Y., Delzon, S., Dufrêne, E., Pontailler, J. Y., Louvet, J. M., Kremer, A., & Michalet, R. (2009). Leaf phenology sensitivity to temperature in European trees: Do within-species populations exhibit similar responses? *Agricultural and Forest Meteorology*, *149*(5), 735-744.

Wolkovich, E. M., Cook, B. I., Allen, J. M., Crimmins, T. M., Betancourt, J. L., Travers, S. E., ... Ault, T. R. (2012). Warming experiments underpredict plant phenological responses to climate change. *Nature*, *485*(7399), 494-497.

Zhang, Z., Song, X., Chen, Y., Wang, P., Wei, X., & Tao, F. (2015). Dynamic variability of the heading–flowering stages of single rice in China based on field observations and NDVI estimations. *International Journal of Biometeorology*, *59*(5), 643-655.

Authors: Halbritter AH[1], Gillespie MAK[2], Estiarte M[3,4]

Reviewers: Wohlgemuth T[5], Peñuelas J[3,4]

Affiliations

[1] Department of Biological Sciences and Bjerknes Centre for Climate Research, University of Bergen, Bergen, Norway

[2] Department of Environmental Sciences, Western Norway University of Applied Sciences, Sogndal, Norway

[3] CSIC, Global Ecology Unit CREAF-CSIC-UAB, Bellaterra, Spain

[4] CREAF, Cerdanyola del Vallès, Spain

[5] Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland

4.6 The soil seed bank (buried seed pool)

Authors: Vandvik V[1], Eycott AE[2,3]

Reviewers: Tielbörger K[4], Wilfahrt P[5]

Measurement unit: number of seeds m^{-2} or m^{-3}; Measurement scale: plot; Equipment costs: €; Running costs: €; Installation effort: medium; Maintenance effort: high; Knowledge need: high (seedling identification skills); Measurement mode: manual

When mature and viable seeds reach the soil surface, germination may either take place immediately or be delayed for a shorter or longer period of time. If germination is delayed for an extended time, the seeds on or in the soils are said to form the soil seed bank (e.g. Fenner and Thompson 2005; Baskin & Baskin, 2014). Seeds can enter and persist in the soil seed bank for a variety of reasons, chiefly because i) the environmental conditions are not suitable for germination (such as in winter or under a drought or dry season when the temperature or water required for metabolic activity are not available to the seed), ii) the germination requirements of the species are not met (specific light or temperature regimes, etc.), or iii) the seeds are dormant (i.e. they have evolved intrinsic physical or physiological mechanisms for delaying germination until the dormancy is broken or lost, which can happen either in response to specific environmental cues or as a random process). Depending on characteristics of both the species and the environment, seeds can thus remain in the soil seed bank for a range of timespans, from very short-lived seeds surviving only a few days via seeds surviving a few months allowing them to germinate in a particular season, to seeds surviving in the seed bank for years to decades and even centuries. The world record to date of documented seed survival in the soil was set when a team of Russian researchers were able to successfully germinate embryos of *Silene stenophylla* excavated from the Siberian permafrost, placed inside a modern seed coat and endosperm. The subsequent plants survived to fertile adulthood − the seeds were 30,000 years old (Yashina et al., 2012).

Soil seed banks thus offer plants the opportunity to disperse through time. This has fundamental implications for population and community dynamics, as has been recognised by ecological and evolutionary theory, and documented in numerous empirical studies. On ecological timescales, seed banks represent local “biodiversity reservoirs” that can contribute to local population persistence and biodiversity maintenance through temporal storage effects (Chesson & Huntly, 1997; Faist et al., 2013; Plue & Cousins, 2013), remnant populations (Eriksson, 1996; Plue et al., 2008; Auffret & Cousins, 2011), and the maintenance of a functionally diverse belowground species pool available for germination in response to environmental variability or change (Kalamees & Zobel, 2002; Dostal, 2005; Clark et al., 2007; Enright et al., 2007; Måren & Vandvik, 2009; Anderson et al., 2012; del Cacho & Lloret, 2012). On evolutionary timescales, seed banks increase the mean generation times of populations, thereby affecting the potential rate and even direction of evolutionary change (Brown & Venable, 1986; Evans & Cabin, 1995; Evans & Dennehy, 2005). Seed banks allow evolution of risk-spreading mechanisms such as bet-hedging germination strategies (Cohen, 1968; Evans & Dennehy, 2005; Ayre et al., 2009; Gremer & Venable, 2014) and contribute to the maintenance of genetic and trait diversity within local populations (Cabin et al., 2000; Ayre et al., 2009; Lundemo et al., 2009; Mandak et al., 2012), thus providing a potential source of resilience in the face of disturbance or

environmental change. As pointed out by Alexander et al. (2012), disentangling the role of seed banks in community and population dynamics is essential for our general understanding of basic ecological patterns and processes in plant communities. For example, understanding the relative importance of "dispersal through time" via seed banks v. long-distance dispersal through space is essential for understanding metapopulation dynamics and metacommunity assembly (Vandvik & Goldberg, 2006). In this way seed banks can be considered biodiversity reservoirs, containing components of the plant community which are not necessarily present aboveground (Vandvik et al., 2016).

Persistent seed banks are particularly important in environments that are characterised by unpredictable environmental fluctuations, such as, for example, subtropical deserts. In such environments, seed dormancy can provide a hedge against extinction during unfavourable years and helps to exploit, on average, more favourable conditions (Cohen, 1968). As climate plays such an important role in seed production (see protocol 4.1 Sexual plant reproduction), dormancy-breaking, and germination (see protocol 4.2 Seed viability, germinability and dormancy), seed banks can be expected to respond to climate change. The methods discussed here are also relevant to other kinds of global-change experiments or observations (e.g. nitrogen desposition; Plassman et al., 2008), including interactions between climate and other drivers such as nitrogen deposition (e.g. Ochoa-Hueso & Manrique, 2010).

A number of classification schemes has been developed for seed banks (Csontos & Tamas, 2003) and Thompson et al. (1997) pragmatically recognise three main types: *transient species* where seeds typically remain in the soil less than a year before germinating or dying, *short-term persistent species* that remain in the soil for 1–5 years, and *long-term persistent species* that remain in the soil more than 5 years. While these types do not closely match the different ecological and evolutionary roles of seed banks described above, there is some relationship (e.g. species with evolutionary adaptations involving soil seed banks tend to be long-term persistent). It is worth noting that transient species are generally not considered to be part of the soil seed bank *sensu stricto* and are hence often avoided in sampling soil seed banks (see paragraph on timing of sampling). In addition to seed banks in the soil, some authors consider ripe seeds still on the parent plant but no longer dependent on the parent (serotinous seeds) to also be part of the seed "pool" (Lamont & Enright, 2000). In this protocol we only describe methods for sampling soil seed banks.

4.6.1 What and how to measure?

There are four principal ways of measuring the density and longevity of soil seed banks, each with its inherent strengths and weaknesses. Each of them involves some disturbance of the soil in treatment plots and so experimental designs must allow adequate space regardless of which method is eventually used. In the **greenhouse germination method***,* used in the great majority of soil seed-bank studies, seed banks are sampled by taking soil cores of varying depths, treating as necessary to break dormancy, and then spreading these soils in trays in a greenhouse under conditions suitable for germination. The emerging seedlings are then counted and identified.

In the major alternative approach, the **seed extraction method**, the seeds are extracted from the soil and then identified to species and counted under a stereomicroscope. In comparative studies, the

seed extraction method often reveals more species than the greenhouse germination method (e.g. Brown, 1992), which may be due to the failure to provide suitable conditions for germination and/or break dormancy in germination studies, but also because the seed extraction method may overestimate the seed bank by including dead seeds in the total count (Fenner & Thompson, 2005). While the viability problem can be circumvented, for example using a tetrazolium viability test (see protocol 4.2 Seed viability, germinability and dormancy), the seed extraction method is relatively labour intensive, especially in systems with many small-seeded species, and the method is not widely applied.

Another method is to monitor **seedling emergence *in situ*** (e.g. Kadmon & Shmida, 1990; Castillo & Stevenson, 2010; Siewert & Tielbörger, 2010; Plue et al., 2017), which may be better than the greenhouse method at promoting the germination of specialist species with particular environmental requirements and thus gives a more realistic picture of the recruitment potential from the soil seed bank (e.g. Plue et al., 2017). The method also allows for larger individual samples. Also, because of the high level of realism, the *in situ* method arguably investigates another life-history transition, seedling recruitment, rather than quantifying the size and species composition of the soil seed bank *per se.* These methods require the exclusion of the natural seed rain, however, which may be difficult in climate-manipulation studies and may also affect the microenvironment (e.g. netting might incur shading and increased moisture), which again might affect germination and survival probabilities. See Kadmon & Shmida, 1990 and Siewert & Tiellbörger, 2010 for how this can be achieved in desert annual communities.

A final method is to **bury bags of seeds** in the soil, and then exhume these after a given time (211 such studies with a duration ranging from 1−120 years are reported in Fenner & Thompson, 2005; see Telewski & Zeevaart, 2002 for the 120- year experiment). While this yields very direct estimates of seed longevity in soil, which may be very useful in demographic studies and other species-level approaches, it is less suited for assessment of the entire seed-bank community as such, and will therefore not be considered further here (see below for alternative approaches to assessing seed longevity).

Recognising the different strengths and weaknesses of these different methods, we follow the major practice in the literature (see e.g., Fenner & Thompson, 2005) and recommend germination from soil cores as the ***gold standard*** protocol for assessing seed-bank density and species composition. We also suggest a number of measures such as bulk reduction and careful selection of germination (pre-)treatments to reduce dormancy and other issues with this method (ter Heerdt et al., 1996; Måren & Vandvik, 2009). The aim of these suggestions is to maximise the representation of the true viable persistent seed bank by the sampling.

Timing of sampling: this depends on the research question, but if you are interested in the persistent seed pool (*sensu* Thompson & Grime, 1979), then sampling should be done after the main seasonal germination peak and before the main seed dispersal period. For example, in temperate and boreal systems, this generally means that sampling should be done in the early to mid summer, before peak biomass (and, crucially, after spring germination peak and before the following autumnal seed dispersal peak). If the focus is instead on the range of seeds available at the start of the growing season, select the time between the peak of unseasonable conditions (usually dry season at low latitudes and winter at high latitudes) and the onset of growing season germination. In that way,

species needing a seasonal dormant period, such as vernalisation, have received those conditions, reducing the need for dormancy-breaking treatments during sample processing (Thompson et al., 1997). If this is not practical, it may be necessary to subject the samples to artificially-induced conditions reflecting the dormant period (also known as stratification). Samples from damp environments should not be completely dry during stratification (Baskin & Baskin, 2014).

Field sampling of seed banks from the soil − spatial sampling strategy: soil seed banks are notoriously patchy (Thompson et al., 1997) and sampling methods should be carefully chosen and the resulting data interpreted with this in mind. For studies aiming to achieve a representative sample of the seed-bank community, the classical recommendation has been to take many small samples rather than a few larger ones (Molau & Mølgaard, 1996; Thompson et al., 1997). The number of samples (and total volume) needed varies with the research question and the study system, where fewer and smaller samples are needed in systems with higher seed densities (see discussion in Thompson et al., 1997).

Samples intended for **direct comparisons with aboveground vegetation data** should be taken at a grain size and sampling effort comparable to that vegetation, yet published studies typically report seed bank sampling area of 1−5% of the corresponding vegetation sampling area (this concerns both individual samples and total datasets; Thompson et al., 1997; Vandvik et al., 2016). The species–area relationships of the soil v. vegetation are surprisingly similar across scales, regions, and habitats (see field data and literature review reported in Vandvik et al., 2016), suggesting that direct comparisons of the biodiversity of vegetation and seed banks with unmatched sampling grain sizes are not valid, at least not for highly spatially structured data such as species richness. Comparable data for species richness can be achieved by increasing sampling effort and grain size for the seed bank to be comparable to the aboveground vegetation, but also by sampling the vegetation at a finer gain (i.e. nested or sub-plot designs, see protocol 4.8 Plant community composition). These results also imply that studies aiming to detect with some degree of certainty all species present in the seed bank should use similar grain sizes and sampling efforts (per unit area) as the vegetation. This may prove untenable, in many cases.

For **less spatially structured responses** such as community composition and some similarity and diversity indices (see below), comparing vegetation and seed bank data collected using different sampling effort and grain sizes may still be appropriate. It may be necessary to take several small subsamples and bulk them together at the plot scale (Figure 4.6.1). For studies aiming to compare community composition of the vegetation and seed bank, Plue & Hermy (2012) recommend a minimum sample of 3% of a plots' surface area with subsamples taken along a systematic grid covering the whole plot. Samples taken from closer than 30 cm are not independent in composition across a range of habitats, and so for sufficiently large plots it will be beneficial to space the subsamples at a greater distance than this (Plue & Hermy, 2012). Whatever the sampling strategy, the sample should be of a fixed area and depth per site or treatment.

Sampling depth: the majority of seeds are found in the upper layers, i.e. the upper 2 cm. If the aim is to recover as much as possible of the seed bank composition and diversity, it may be more cost-effective to sample a larger area than greater depths, unless the seed bank of the deeper layers is of interest (e.g. for time-series or age estimations; see below). However, for a full description of the seed bank, 10 cm is the minimum recommended depth in deep soils: more than 40 cm is most likely

excessive (Thompson et al., 1997; Eycott et al., 2006). The depth of a seed indicates how long it has been in the soil (although not exactly) and so many seed bank studies keep soil layers separate in order to make inferences about the age of the seeds of different species (Figure 4.6.1). Divisions could then be: i) 0–1 cm, ii) 1–3 cm, iii) 3–5 cm, and iv) 5–10 cm, as was used in ITEX (Molau & Mølgaard, 1996).

Figure 4.6.1 Soil seed bank sampling. Cylindrical metal cores of known dimensions are useful for sampling known depths and volumes of soil. a) A stout stick of slightly smaller diameter than the core is being used to gently push the sample out of the core, with a handle fitted to the core to ease extraction from the ground. Both these and a rubber mallet can be useful for sampling hard or dense soils. b) Cores can be divided into different layers and c) replicate cores can be sampled from across vegetation plots and either be combined to a composite sample or kept separate for more detailed spatial analyses. Photos: Vigdis Vandvik.

Lab preparation of seed bank samples: the soil should be passed through a large-grain sieve to remove stones, roots, and large leaves and to homogenise the sample (e.g. 2 mm sieve; Molau & Mølgaard, 1996). Use a bigger grid size than the largest reasonably expected seed. Take care to not damage the seeds but ensure that all the soil sticking to the roots or stones gets broken up. If suitable (e.g. for loamy soils) the sample can then be washed through a small-grid sieve using liberal amounts of water (a hand-held shower can be useful) to reduce the bulk sample and concentrate the seeds in the sieve (ter Heerdt et al., 1996). Note that, for example, *Juncus* seeds can be 0.3 mm and orchid seeds even smaller, so this sieve should be smaller than the smallest seed reasonably expected. In testing this approach, ter Heerdt (1996) found that it greatly reduces soil bulk and thereby decreases greenhouse space need and/or increases the original sample volume that can be processed. The volume-reduced samples germinated to higher percentages than unconcentrated samples, and 81–100% of the seeds remained viable, suggesting the method causes relatively little harm to seeds. The samples should then be laid out in a thin layer (< 0.5 cm, because even this little soil cover creates uneven environmental conditions and can inhibit germination in light-demanding species) over a thick layer of sterile substrate, for example 5 cm of a 1:1:1 mix of sterile peat, potting soil, and perlite, as this allows moisture conditions to be kept as constant as possible, thereby ensuring seedlings are kept alive until they can be identified (Måren & Vandvik, 2009). Note that germination requirements, and therefore optimal conditions for seedling emergence, may differ between study systems and this should be reflected in the greenhouse protocols (e.g. full light or shade; seeds on top of soil or covered by a thin layer of substrate; high, low, constant, or variable temperatures or light; high or low moisture). In any case, the conditions should be optimised so as to maximise germination in the system at hand (see 4.6.2. *Special cases* below).

Greenhouse methods: trays should be placed in random order in a greenhouse and should be rotated to new positions at regular intervals (Figure 4.6.2). Control trays of the same sterile substrate used for the samples should be randomly interspersed among the samples to control for

contamination from in and around the greenhouses, for example airborne seeds arriving via ventilation systems (Eycott et al., 2006). The greenhouse light and heat regime should reflect optimal conditions for the seeds or for the study system, for example samples from the high arctic should have 24-hour light, while for alpine and low arctic sites 16–18 h has been used (Molau & Mølgaard, 1996). The Handbook of Field Sampling Protocols for Biodiversity Indicator Monitoring (James Hutton Institute, 2011) suggests between 15 and 18 °C for 12 h for samples from temperate regions.

Visit the samples regularly and uproot seedlings as soon as they can be identified (manuals of seedling identification are available for some ecosystems, e.g. Muller, 1978; Garwood & Tebbs, 2009). Species which need to flower in order to be identified should be moved into new pots so as to not inhibit further germination (Figure 4.6.2). The amount of time for which the samples should be kept depends on the germination ecology of the component species and how important it is to capture infrequent species. Ter Heedt et al. (1996) found that with their sieving method, 95% of seeds from temperate marshland had germinated within six weeks, whereas a study in temperate mixed old-growth forest observed new species emerging after three years (Jaroszewicz, pers. comm.). In a greenhouse without artificial lighting, a year will permit all species with day length-related germination requirements to have the opportunity to germinate. In reality, whilst longer is probably better, many studies will be constrained instead by logistical limitations.

Data reporting and calculations: the data should be reported in seeds per species per m^2 or m^3, and information about both the total m^2 and total m^3 sampled (i.e. sampling area and sampling depth) and the grain and extent (subsample spatial sampling design; sample mixing) should be reported. Data on species composition and various similarity and diversity indices can be calculated from these data.

Figure 4.6.2 Soil seed bank sample processing. a) Sieving to remove large stones and roots. b) Laying out samples as evenly as possible. It is usually necessary to move samples around regularly, even in well-lit greenhouses. c) Seedlings are removed and the roots brushed gently so that other seeds are not carried on the roots. d) Individuals which cannot be identified to species from the seedlings must be carefully transferred to new pots to avoid shading out other germinating individuals. Photos: Vigdis Vandvik.

Where to start

Ter Heerdt et al., (1996), Måren & Vandvik (2009), Molau & Mølgaard (1996), Plue et al. (2012), Thompson et al. (1997).

4.6.2 Special cases, emerging issues, and challenges

Special cases for particular environments *– this is not an exhaustive list*

- Wetland samples may need to be placed in troughs to support inundation-demanding species, but that may also inhibit others (Poiani & Johnson, 1988).
- Soil seed bank samples from in the polar desert need only be very shallow – even only 1 cm (Molau & Mølgaard, 1996).
- Annual plant communities, such as from agricultural fields or deserts, have particular challenges and opportunities and may need to be sampled and analysed in different ways (see Kadmon & Schmida, 1990; Siewert & Tielbörger, 2010).
- Some seeds, particularly tropical forest trees, are recalcitrant – they do not tolerate drying, so the samples should not be allowed to get dry (moisture content of seed below 30%). Tropical tree seeds can also be very large and so may need hand-selecting at the sieving stage.

- There are relatively few tropical seed-bank studies in the same way as there are relatively few tropical climate-manipulation studies. See Garwood (1989) for a review, updated for forests by Esaete et al. (2014, Appendix 1).
- If the mechanisms by which seed dormancy is broken are unknown or difficult to reproduce experimentally, one option is to store the freshly produced seeds under field conditions during the unfavourable season (e.g. winter or dry season). This can promote dormancy breaking by the natural cues.

4.6.3 References

Theory, significance, and large datasets

Thompson et al. (1997), Vandvik et al. (2016) for a review of seed bank species-area relationships.

More on methods and existing protocols

Plue & Hermy (2012), Plue et al. (2017) for a review of sample sizes and spacings

All references

Alexander, H. M., Foster, B. L., Ballantyne, F., Collins, C. D., Antonovics, J., & Holt, R. D. (2012). Metapopulations and metacommunities: combining spatial and temporal perspectives in plant ecology. *Journal of Ecology, 100*(1), 88-103.

Anderson, T. M., Schütz, M., & Risch, A. C. (2012). Seed germination cues and the importance of the soil seed bank across an environmental gradient in the Serengeti. *Oikos, 121*(2), 306-312.

Auffret, A. G., & Cousins, S. A. O. (2011). Past and present management influences the seed bank and seed rain in a rural landscape mosaic. *Journal of Applied Ecology, 48*, 1278-1285.

Ayre, D. J., Ottewell, K. M., Krauss, S. L., & Whelan, R. J. (2009). Genetic structure of seedling cohorts following repeated wildfires in the fire-sensitive shrub *Persoonia mollis* ssp. *nectens*. *Journal of Ecology, 97*(4), 752-760. 227

Baskin, C. C., & Baskin, J. M. (2014). *Seeds. Ecology, Biogeography and Evolution of Dormancy and Germination* (2nd ed.). Academic Press.

Brown, D. (1992). Estimating the composition of a forest seed bank: a comparison of the seed extraction and seedling emergencemethods. *Canadian Journal of Botany, 70*, 1063-1612.

Brown, J. S., & Venable, D. L. (1986). Evolutionary ecology of seed-bank annuals in temporally varying environments. *The American Naturalist*, 127, 31-47.

Cabin, R. J., Marshall, D. L., & Mitchell, R. J. (2000). The demographic role of soil seed banks. II. Investigations of the fate of experimental seeds of the desert mustard *Lesquerella fendleri*. *Journal of Ecology, 88*(2), 293-302.

Castillo, L. S., & Stevenson, P. R. (2010). Relative importance of seed-bank and post-disturbance seed dispersal on early gap regeneration in a Colombian Amazon forest. *Biotropica, 42*(4), 488-492.

Chesson, P., & Huntly, N. (1997). The roles of harsh and fluctuating conditions in the dynamics of ecological communities. *The American Naturalist*, 150, 519-553.

Clark, C. J., Poulsen, J. R., Levey, D. J., & Osenberg, C. W. (2007). Are plant populations seed limited? A critique and meta-analysis of seed addition experiments. *The American Naturalist*, *170*(1), 128-142.

Cohen, D. (1968). A general model of optimal reproduction in a randomly varying environment. *Journal of Ecology,* 56, 219-228.

Csontos, P., & Tamás, J. (2003). Comparisons of soil seed bank classification systems. *Seed Science Research*, 13(2), 101-111.

del Cacho, M., & Lloret, F. (2012). Resilience of Mediterranean shrubland to a severe drought episode: the role of seed bank and seedling emergence. *Plant Biology, 14*, 458-466.

Dostal, P. (2005). Is the population turnover of patchy-distributed annuals determined by dormancy dynamics or dispersal processes? *Ecography, 28*, 745-756.

Enright, N. J., Mosner, E., Miller, B. P., Johnson, N., & Lamont, B. B. (2007). Soil vs. canopy seed storage and plant species coexistence in species-rich Australian shrublands. *Ecology, 88*, 2292-2304.

Eriksson, O. (1996). Regional dynamics of plants: a review of evidence for remnant, source–sink and metapopulations. *Oikos, 77*, 248-258.

Esaete, J., Eycott, A. E., Reiniö, J., Telford, R. J., & Vandvik, V. (2014). The seed and fern spore bank of a recovering African tropical forest. *Biotropica*, 46(6), 677-686.

Evans, A. S., & Cabin, R. J. (1995). Can dormancy affect the evolution of post-germination traits – the case of *Lesquerella fendleri*. *Ecology, 76*, 344-356.

Evans, M. E. K., & Dennehy, J. J. (2005). Germ banking: bet-hedging and variable release from egg and seed dormancy. *Quarterly Review of Biology, 80*, 431-451.

Eycott, A. E., Watkinson, A. R., & Dolman, P. M. (2006). The soil seedbank of a lowland conifer forest: the impacts of clear-fell management and implications for heathland restoration. *Forest Ecology and Management, 237*(1-3), 280-289.

Faist, A. M., Ferrenberg, S., & Collinge, S. K. (2013). Banking on the past: seed banks as a reservoir for rare and native species in restored vernal pools. *AoB Plants, 5*, plt043.

Fenner, M., & Thompson, K. (2005). *The Ecology of Seeds*. Cambridge: Cambridge University Press.

Garwood, N. C. (1989). Tropical soil seed banks: a review. In M. A. Leck, V. T. Parker, & R. L. Simpson (Eds.), *Ecology of Soil Seed Banks* (pp. 149-209). San Diego, CA: Academic Press Inc.

Garwood, N. C., & Tebbs, M. C. (2009). *Seedlings of Barro Colorado Island and the Neotropics*. Comstock Publishing Associates/Cornell University Press.

Gremer, J. R., & Venable, D. L. (2014). Bet hedging in desert winter annual plants: optimal germination strategies in a variable environment. *Ecology Letters, 17*, 380-387.

James Hutton Institute (2011). *Handbook of Field Sampling Protocols for Biodiversity Indicator Monitoring*. http://www.hutton.ac.uk/sites/default/files/files/A%20Handbook%20of%20biodiversity%20monitoring%20protocols(1).pdf

Kadmon, R., & Shmida, A. (1990). Spatiotemporal demographic processes in plant populations: an approach and a case study. *The American Naturalist*, *135*(3), 382-397.

Kalamees, R., & Zobel, M. (2002). The role of the seed bank in gap regeneration in a calcareous grassland community. *Ecology, 83*, 1017-1025.

Lamont, B. B., & Enright, N. J. (2000). Adaptive advantages of aerial seed banks. *Plant Species Biology*, *15*(2), 157-166.

Lundemo, S., Falahati-Anbaran, M., & Stenøien, H. K. (2009). Seed banks cause elevated generation times and effective population sizes of *Arabidopsis thaliana* in northern Europe. *Molecular Ecology, 18*, 2798–2811.

Mandak, B., Zákravský, P., Mahelka, V., & Plačková, I. (2012). Can soil seed banks serve as genetic memory? A study of three species with contrasting life history strategies. *PLoS ONE, 7*, e49471.

Måren, I. E., & Vandvik, V. (2009). Fire and regeneration: the role of seed banks in the dynamics of northern heathlands. *Journal of Vegetation Science, 20*, 871-888.

Molau, U., & Mølgaard, P. (1996). *ITEX Manual - International Tundra Experiment* (2nd ed.) Copenhagen: Danish Polar Centre. http://ibis.geog.ubc.ca/itex/PDFs/ITEXmanual.pdf

Muller, F. M. (1978). *Seedlings of the North-Western European Lowland.* Dordrecht: Springer.

Ochoa-Hueso, R., & Manrique, E. (2010). Nitrogen fertilization and water supply affect germination and plant establishment of the soil seed bank present in a semi-arid Mediterranean scrubland. *Plant Ecology, 210*(2), 263-273.

Plassmann, K., Brown, N., Jones, M. L. M., & Edwards-Jones, G. (2008). Can atmospheric input of nitrogen affect seed bank dynamics in habitats of conservation interest? The case of dune slacks. *Applied Vegetation Science, 11*(3), 413-420.

Plue, J., & Cousins, S. A. O. (2013). Temporal dispersal in fragmented landscapes. *Biological Conservation*, *160*, 250-262.

Plue, J., & Hermy, M. (2012). Consistent seed bank spatial structure across semi-natural habitats determines plot sampling. *Journal of Vegetation Science,* 23, 505-516.

Plue, J., Hermy, M., Verheyen, K., Thuillier, P., Saguez, R., & Decocq, G. (2008). Persistent changes in forest vegetation and seed bank 1600 years after human occupation. *Landscape Ecology, 23*(6), 673-688.

Plue, J., Thompson, K., Verheyen, K., & Hermy, M. (2012). Seed banking in ancient forest species: why total sampled area really matters. *Seed Science Research, 22*(2), 123-133.

Plue, J., Colas, F., Auffret, A. G., & Cousins, S. A. O. (2017). Methodological bias in the seed bank flora holds significant implications for understanding seed bank community functions. *Plant Biology, 19*(2), 201-210.

Poiani, K. A., & Johnson, W. C. (1988). Evaluation of the emergence method in estimating seed bank composition of prairie wetlands. *Aquatic Botany*, *32*(1-2), 91-97.

Siewert, W., & Tielbörger, K. (2010). Dispersal-dormancy relationships in annual plants: putting model predictions to the test. *The American Naturalist*, *176*(4), 490-500.

Telewski, F. W., & Zeevaart, J. A. (2002). The 120-yr period for Dr. Beal's seed viability experiment. *American Journal of Botany, 89*(8), 1285-1288.

Ter Heerdt, G. N. J., Verweij, G. L., Bekker, R. M., & Bakker, J. P. (1996). An improved method for seed-bank analysis: seedling emergence after removing the soil by sieving. *Functional Ecology*, *10*, 144-151.

Thompson, K., & Grime, J. P. (1979). Seasonal variation in the seed banks of herbaceous species in ten contrasting habitats. *Journal of Ecology*, *67*(3), 893-921.

Thompson, K., Bakker, J. P., & Bekker, R. M. (1997). *The Soil Seed Banks of North West Europe: Methodology, Density and Longevity* (Vol. 1). Cambridge: Cambridge University Press.

Vandvik, V., & Goldberg, D. E. (2006). Sources of diversity in a grassland metacommunity: quantifying the contribution of dispersal to species richness. *The American Naturalist*, *168*, 157-167.

Vandvik, V., Klanderud, K., Meineri, E., Måren, I. E., & Töpper, J. P. (2016). Seed banks are biodiversity reservoirs: species–area relationships above versus below ground. *Oikos*, *125*, 218-228.

Yashina, S., Gubin, S., Maksimovich, S., Yashina, A., Gakhova, E., & Gilichinsky, D. (2012). Regeneration of whole fertile plants from 30,000-y-old fruit tissue buried in Siberian permafrost. *Proceedings of the National Academy of Sciences USA*, *109*(10), 4008-4013.

Authors: Vandvik V[1], Eycott AE[2,3]

Reviewers: Tielbörger K[4], Wilfahrt P[5]

Affiliations

[1] Department of Biological Sciences and Bjerknes Centre for Climate Research, University of Bergen, Bergen, Norway

[2] Department of Biological Sciences, University of Bergen, Bergen, Norway

[3] Faculty of Biosciences and Aquaculture, Nord University, Steinkjer, Norway

[4] Institute of Evolution and Ecology, Plant Ecology Group, University of Tübingen, Tübingen, Germany

[5] Department of Disturbance Ecology, BayCEER, University of Bayreuth, Bayreuth, Germany

4.7 Propagule rain

Authors: Eycott AE[1], Wilfahrt PA[2]

Reviewers: Vandvik V[1,3], Tielbörger K[4]

Measurement unit: number of seeds $m^{-2}yr^{-1}$; Measurement scale: plot; Equipment costs: €; Running costs: €; Installation effort: low to medium; Maintenance effort: low to medium; Knowledge need: high (seed identification); Measurement mode: manual

Propagule rain (also called seed rain) is the supply of plant propagules to a local area over a given time. Propagule rain is a critical component of plant demography, community, and metacommunity dynamics. It is linked to changes in diversity (Vandvik & Goldberg, 2005), plant eco-evolutionary trade-offs (Mouquet et al., 2004), rates of migration and range expansion (Lockwood et al., 2009), and the colonisation and invasion of habitats (Levine & D'Antonio, 1999).

In this protocol we describe methods to quantify the propagule rain. A combination of input of local propagule production and dispersal from off-site locations is considered, because separating these is notoriously difficult. We distinguish between propagule rain and soil seed bank measurement as their methodology for detection is distinct (see protocol 4.6 The soil seed bank (buried seed pool)). Quantifying propagule rain may help explain shifts in population demographics or community composition by defining the potential of species to recruit new genetic individuals. This has clear application within the context of climate-change studies and may be relevant to other global-change studies but is rarely included in study designs. Propagule rain is a process likely to be affected by experimental infrastructure such as open-topped chambers or CO_2 blowers as they affect wind speed (e.g. Fuhrer 1994) but we are unaware of any measurements of this effect.

4.7.1 What and how to measure?

There are two broad types of methodology for detecting propagule rain. First, there are seed trap-based options where seeds are intercepted at or just above ground level, removed, and identified. Trap-based methods can be used in vegetation types (e.g. forests) where destruction of the vegetation is not feasible or the source of the germinating propagules is not of interest. Sticky traps favour wind-dispersed seeds and expose seeds to predators and rainwash: they are occasionally used but not recommended (Kollman & Goetze, 1998; Chabrerie & Alard, 2005). Second, there are various ways to expose a seed-free substrate to the seed rain upon which you monitor emergence of seedlings *in situ*. This has the advantage of monitoring germination as it would occur under local conditions, but the disadvantage of causing greater disturbance to experimental plots. A "special case" method developed by Kadmon & Shmida (1990) and Siewert & Tielbörger (2010) can distinguish between propagule sources (local v. non-local propagule origins) and it can identify the actual consequences of seed rain for population dynamics, but is destructive and only suitable under particular circumstances (strictly annual species).

Seed traps

Excluding the non-recommended sticky traps (see above), there are two common designs for seed traps: mats and funnels. Whichever you use, the traps should be exposed throughout the whole season of seed dehiscence, with regular checking and cleaning.

The use of mats is more common in grassland and dwarf shrubland. Plastic doormats with ~1 cm tines are placed under the vegetation and the seeds which are caught in the tines are removed and recorded (Figure 4.7.1, Figure 4.7.2). Mat size varies from 25 x 25 cm to match vegetation recording plot size (Boixaderas, 2012) up to 50 x 80 cm to maximise trapped material (Birks & Bjune, 2010). To dislodge the seeds, the mats may be tapped with a hammer in the field (Molau & Mølgaard, 1996) or flushed with a shower attachment if the mats are removed to a laboratory. Particular care must be taken to dislodge seeds bearing hooks or awns.

Figure 4.7.1 A 25cm x 25 cm doormat seed trap being set up in the field. This one has been covered by a cage to stop sheep from chewing or trampling the mat. The vegetation underneath was cut to ground level to keep the mat flat. Photo: Inger Elisabeth Måren.

In forests, seed traps made of funnels with mesh bags at the base are more common, and can be very efficient (Chabrerie & Alard, 2005). Litterfall traps can be used for this (see the ICP Forests litterfall protocol; Ukonmaanaho et al., 2016, although this may collect a very large sample for sorting, p. 8). Funnels have to be set below the vegetation layer of interest: for low-growing vegetation which potentially involves destructive digging in order to place the trap below ground but for trees it is effective. Funnels catch more seeds than horizontal rough surfaces such as mats (Johnson & West, 1988).

Funnels catch more seeds than horizontal rough surfaces such as mats (Johnson & West, 1988).

Unless a reference collection is already available, it is recommended to make one from local specimens of confirmed identity (Molau & Mølgaard, 1996). Lay the samples out to find the seeds: it is recommended to lay them out on dark paper or cloth (Molau & Mølgaard, 1996). A stereomicroscope is necessary to identify seeds. Many of the seeds may be dead or empty and should be omitted from the count; see protocol 4.2 Seed viability, germinability and dormancy for methods to test viability.

Monitoring seedling emergence on a seed-free substrate in situ

In the simplest form of this type of method, a deep layer of turf is extracted and inverted so seed-free soil becomes the top layer (e.g. 35 cm depth, Pakeman et al., 1998). When compared to a turf that has been disturbed but not inverted (i.e. extant vegetation removed), this allows for distinction between germination from the seed bank v. dispersed propagules while maintaining “field” conditions. The subsoil must be checked for deeply-buried seeds when using this approach. Less

destructive is to use pots of sterile soil, but this exposes seeds to predators (Kollman & Goetze, 1998; Chabrerie & Alard, 2005). Such "field conditions" approaches measure germination under realistic conditions which could have a positive or negative effect depending on your research question. The substrate should be exposed before the season of seed dispersal until after the season of seed germination.

4.7.2 Special cases, emerging issues, and challenges

Identifying the source of newly germinating propagules allows for an enhanced understanding of local and metapopulation dynamics, species' range expansions and contractions, and gene flow between populations (Kadmon & Shmida, 1990). Under particular circumstances the spatial source of propagules germinating in a particular patch can be further refined into those originating from local dispersal and longer distance dispersal. For very patchy populations, this may even be generalisable to dispersal from adults inside and outside the patch. One method for distinguishing these sources has been successfully applied in an annual plant system (Siewert & Tielbörger, 2010). In this method, extant vegetation was removed with a glyphosphate herbicide prior to seed production and germination rates compared with those in control plots (unherbicided) over the following year. Dispersal distances in the system can be measured simultaneously by removing plants within a certain radius and counting emerging seedlings along the radii. Additional census plots are necessary in the denuded areas to differentiate germinants that arrived from recent dispersal to those that were dormant in the pre-existing seed bank. This may be accomplished by covering additional subplots with netting that is permeable but fine enough to prevent dispersed seeds from reaching the subplot. By monitoring germination in the following year, this yields three subplot types with nested information on germinant sources: i) denuded and netted subplot germinants are recruited from the soil seed bank, ii) denuded subplot germinants are recruited from dispersal and from the soil seed bank, and iii) control subplot germinants are recruited locally from dispersal, and from the soil seed bank (Siewert & Tielbörger, 2010). While these techniques are powerful in detailing spatial and temporal dynamics of propagule rain, they are limited to situations where i) the destruction of extant vegetation is feasible within the study design and ii) the system is composed of strictly annual species, as perennial vegetation may

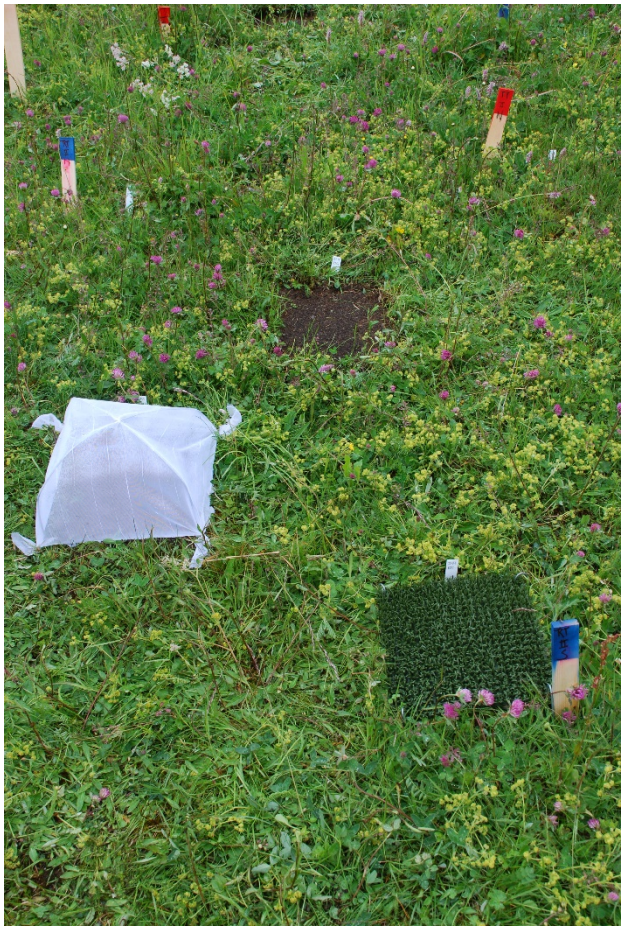


Figure 4.7.2 A combined method. A seed trap (bottom right) made of 25 x 25 cm plastic doormat measures the seed rain, bare soil (top) measures combined effects of seed bank and seed rain, and bare soil covered in mesh (middle left) measures emergence from the seed bank only. Photo: Vigdis Vandvik.

resprout either from nearby or belowground, even following glyphosate application (Wilfahrt pers. obs.).

4.7.3 References

Theory, significance, and large datasets

Nathan & Muller-Landau (2000)

More on methods and existing protocols

Birks & Bjune (2010) or Molau & Mølgaard (1996) for doormats; Siewert & Tielbörger (2010) for the patch clearance based method.

All references

Birks, H. H., & Bjune, A. E. (2010). Can we detect a west Norwegian tree line from modern samples of plant remains and pollen? Results from the DOORMAT project. *Vegetation History and Archaeobotany, 19*(4), 325-340.

Boixaderas, M. R. (2012). *Plant dispersal in a changing climate. A seed-rain study along climate gradients in Southern Norway*. MSc thesis, University of Bergen, Bergen. Available at bora.uib.no/handle/1956/6575

Chabrerie, O., & Alard, D. (2005). Comparison of three seed trap types in a chalk grassland: toward a standardised protocol. *Plant Ecology, 176*(1), 101-112.

Fuhrer, J. (1994). Effects of ozone on managed pasture: I. Effects of open-top chambers on microclimate, ozone flux, and plant growth. *Environmental Pollution, 86*(3), 297-305.

Johnson, C. K., & West, N. E. (1988). Laboratory comparisons of five seed-trap designs for dry, windy environments. *Canadian Journal of Botany*, 66(2), 346-348.

Kadmon, R., & Shmida, A. (1990). Spatiotemporal demographic processes in plant populations: an approach and a case study. *The American Naturalist*, *135*(3), 382-397.

Kollmann, J., & Goetze, D. (1998). Notes on seed traps in terrestrial plant communities. *Flora, 193*(1), 31-40.

Levine, J. M., & D'Antonio, C. M. (1999). Elton revisited: a review of evidence linking diversity and invasibility. *Oikos*, *87*(1), 15-26.

Lockwood, J. L., Cassey, P., & Blackburn, T. M. (2009). The more you introduce the more you get: the role of colonization pressure and propagule pressure in invasion ecology. *Diversity and Distributions*, *15*(5), 904-910.

Molau, U., & Mølgaard, P. (1996). *ITEX Manual - International Tundra Experiment* (2nd ed.) Copenhagen: Danish Polar Centre. http://ibis.geog.ubc.ca/itex/PDFs/ITEXmanual.pdf

Mouquet, N., Leadley, P., Mériguet, J., & Loreau, M. (2004). Immigration and local competition in herbaceous plant communities: a three-year seed-sowing experiment. *Oikos*, *104*(1), 77-90.

Nathan, R., & Muller-Landau, H. C. (2000). Spatial patterns of seed dispersal, their determinants and consequences for recruitment. *Trends in Ecology & Evolution, 15*, 278-285.

Pakeman, R. J., Attwood, J. P., & Engelen, J. (1998). Sources of plants colonising experimentally disturbed patches in an acidic grassland, in eastern England. *Journal of Ecology*, *86*(6), 1032-1041.

Siewert, W., & Tielbörger, K. (2010). Dispersal-dormancy relationships in annual plants: putting model predictions to the test. *The American Naturalist*, *176*(4), 490-500.

Ukonmaanaho, L., Pitman, R., Bastrup-Birk, A., Breda, N., & Rautio, P. (2016). Part XIII: Sampling and Analysis of Litterfall. In: UNECE ICP Forests Programme Co-ordinating Centre (Ed.), *Manual on Methods and Criteria for Harmonized Sampling, Assessment, Monitoring and Analysis of the Effects of Air Pollution on Forests*. Eberswalde, Germany: Thünen Institute for Forests Ecosystems. [http://www.icp-forests.org/manual.htm].

Vandvik, V., & Goldberg, D. E. (2005). Distinguishing the roles of dispersal in diversity maintenance and in diversity limitation. *Folia Geobotanica*, *40*(1), 45-52.

Authors: Eycott AE[1,2], Wilfahrt P[3]

Reviewers: Vandvik V[2,4], Tielbörger K[5]

Affiliations

[1] Faculty of Biosciences and Aquaculture, Nord University, Steinkjer, Norway

[2] Department of Biological Sciences, University of Bergen, Bergen, Norway

[3] Department of Disturbance Ecology, BayCEER, University of Bayreuth, Bayreuth, Germany

[4] Department of Biological Sciences and Bjerknes Centre for Climate Research, University of Bergen, Bergen, Norway

[5] Institute of Evolution and Ecology, Plant Ecology Group, University of Tübingen, Tübingen, Germany

4.8 Plant community composition

Authors: Jaroszynska F[1], Eycott AE[2,3], Vandvik V[1], Halbritter AH[1]

Reviewers: Peñuelas J[4,5], Reinsch S[6], Wilfahrt PA[7], Wohlgemuth T[8]

Measurement unit: percentage cover, counts, presence/absence; Measurement scale: plot; Equipment costs: €; Running costs: -; Installation effort: low to medium; Maintenance effort: -; Knowledge need: high (species identification); Measurement mode: manual

Plant community composition is the description of the vegetation in a fixed area by the species present and, often, their abundance. Plant community composition is commonly recorded in vegetation science and is frequently assessed in climate-manipulation studies (Elmendorf et al., 2015; Kröel-Dulay et al., 2015) as well as in other global-change studies (Borer et al., 2014) and along climate and other environmental gradients (e.g. Stevens et al., 2004). Measurements of vegetation composition indicate the plant species' responses to experimental treatments and environmental gradients in space and time, for example by changes in extinction, colonisation, and/or abundance. Plant communities can respond relatively rapidly (over a timeframe of a few years) and reasonably consistently to a range of different types of climate-manipulation experiments (Elmendorf et al., 2015). Differences between plots, treatments, and sites can be quantified by standardising compositional measurements. Plant community composition provides information on plant−plant interactions (competition, facilitation, etc.), trophic interactions such as herbivory, and more general aspects of ecosystem function (e.g. seasonality, carbon dynamics, or disturbance intensity). Combining compositional data with species functional traits is a powerful tool for inferring more mechanistic changes to communities and their function (see protocol 4.16 Functional traits; Díaz et al., 2004; Garnier et al., 2007; Enquist et al., 2015).

4.8.1 What and how to measure?

There are three main methods of estimating species composition in climate-change studies: i) visual estimation of the proportion of the sample area covered by each species, either as percent or cover classes, ii) the point-intercept method, and iii) direct estimation of the number of individuals of each species. The choice of method depends on the ecosystem and the scientific question. For example, to estimate species' percentage cover or to apply the point-intercept method, a clear view from directly above the vegetation is needed. In all cases, plots should be permanently marked at the corners or given precise GPS coordinates for revisitation purposes (for information on plot selection and marking, see protocol 2.1.1 Aboveground plant biomass). The plot should be located in representative vegetation, by which we mean that areas with unusually tall, short, species-rich, or species-poor vegetation in relation to the surroundings should be avoided.

We suggest the following for selecting which method is best.

i) Visual estimation of species' percentage cover is preferred if:

- Rare species are part of the ecosystem or of interest to the research
- The vegetation is in the form of a dense mat, such as is common among bryophytes or lichens or cushion plants, which are better estimated by their cover and height

- Taxonomic expertise is high.

ii) The point-intercept method is best if

- Both species composition and a proxy for biomass are needed (see also protocol 2.1.1 Aboveground plant biomass, non-forest system)
- Vegetation has a highly layered canopy, where the leaf area index (LAI) is greater than one
- The 3D space occupancy of plant species is of interest
- Dominant plant species are the main interest, with low importance of rare species
- The surveying team is large and/or inexperienced, as this method is more objective than visual estimation
- Time is not limiting.

iii) Direct estimation of number of individuals per species is best if:

- You work in forests or shrubland where the distinction of individuals is clear. However, understorey vegetation is still better recorded as percent cover or with the point-intercept method. Moreover, woody species cover should also be estimated at multiple strata (e.g. herb, shrub, canopy layer; see Peet et al., 1998)
- Vegetation is sparse enough such that individual ramets or genets are distinguishable
- Depending on the research questions, woody individuals are sometimes counted only above a threshold diameter at breast height (DBH, e.g. 10 cm or 12 cm) or counted in size classes.

Plot size. As a minimum, this should be the smallest area which makes it possible to recognise the ecosystem or habitat type. Even though rules have been developed over several decades for minimal areas of vegetation types in the context of phytosociology (e.g. Mueller-Dombois & Ellenberg, 1974), no set rules have emerged for wider use. A reasonable plot size depends on the organism size, the research question, and the focal response variables, and can range from a few square centimetres to hectares. Here is a list of things to consider:

- Larger individual constituent plants (e.g. trees) require larger plots, whereas small or fine-scaled organisms (e.g. bryophytes or grasses) require smaller plots. At the very least, a plot should contain several individuals.
- Plots should neither be so small as to miss frequent species altogether, nor so large that it becomes easy to overlook occasional or rare species. This implies differently-sized plots for different organisms and habitats, ranging from tens-of-centimetre scales for small-statured organisms and fine-scaled habitats (bryophytes, small vascular plants, low-productive grasslands, tundra, mire structures) via approximately metre-scaled plots (more productive grasslands, heathlands, forest understorey) to tens of metres (trees, forest).
- Plot size should not exceed treatment area and there should ideally be a buffer zone (an area around the edge which is treated but not recorded).
- If the plot size is large compared to the constituent individuals, it may be helpful to record community data in subplots (Figure 4.8.1) by dividing the whole plot up and either recording

all subplots or by taking a randomly assigned subset. Subplot analyses can also be useful if there are likely (and potentially interesting) edge effects in the experiment, or if particular species groups or life-history stages differ considerably in size and spatial structure – in this case not all life-stages are recorded at all sampling scales (see Vandvik, 2004). Patterns can be captured more consistently by averaging over several subplots, i.e. using smaller subplots spread over the area, than by using a single large subplot of equivalent total area (Green, 1979).

Co-variables. The research question and habitat type will determine the taxonomic sub-group(s) of interest (e.g. vascular plants, cryptograms) and these should be separated to the species level. However, at a minimum, all other sub-groups should be measured at an aggregated level along with additional variables explaining the plot structure. Such variables may include the cover (or pin-point hits) of various forms of substrate (e.g. rocks, bare soil), litter, or animal faeces. Grazing or other disturbance of the plot (e.g. trampling, rodent nesting) should also be noted.

The height of the vegetation should be measured, becaused it can be used to estimate biomass (see protocol 2.1.1 Aboveground plant biomass). It can be difficult to measure the 'average' vegetation height, so there are some options depending on your vegetation type (see Stewart et al., 2001). In direct measurement, there needs to be a predetermined method for deciding what height you are trying to measure – the very tallest, the highest leaf, or the height of a fixed percentage of the biomass. We reccomend ignoring extruding flowering stems, instead taking the highest leaf, and measure several subpoints per plot. Otherwise employ a 'floating disc' – a circular plastic disc with a small circular hole in the centre. A cylindrical stick marked up with centimets (or millimetres depending on the vegetation height) is placed in the middle of the plot and the disc slipped over the top and dropped down. The disc should be heavy enough to press down extruding strands of grass but not enough to leave a permanent mark in the vegetation. The floating disc method works best in taller vegetation (Stewart et al., 2001).

For studies where finer-scale patterns or species dynamics are of interest, life stages of plant species can be recorded. For example, seedling count or reproductive status give information about recruitment and reproduction, although these are more thoroughly covered in the plant population dynamics section (see protocol 4.3 Plant demography).

Timing. All methods should be carried out during the peak growing season to derive a complete understanding about which species dominate the growth and which species play a role in peak growing season interactions. The plant community assessment can be aligned with estimation of aboveground biomass (see protocol 2.1.1 Aboveground plant biomass). Depending on both the study question and the vegetation type, two assessments per year may be needed to cover the whole species diversity, for example one in spring and one in early summer in deciduous woodland with many spring geophytes, or in surveys where demography such as life-history stages are recorded (e.g. seedlings, sterile, fertile individuals, see protocol 4.3 Plant demography). Additional assessments can be conducted earlier and later than peak growing season if within-year dynamics are of interest. For instance, in mixed prairies of the US, peak growing season differs for the co-dominant C_3 and C_4 plant residents, meaning single sampling dates may bias compositional estimates (Ode et al., 1980).

Visual estimation of species cover

Visual estimation of cover, for example percent cover, is most commonly used in mesic and productive grassland systems, for small plots at ground layer in forests, and in dwarf-shrub systems. Percent cover captures whole-plot species composition, thus making this technique suitable for studies where the presence of rare species is of interest.

The percent cover of each vascular plant species is estimated visually, usually at peak growing season. All species whose canopy intersects with permanently marked plots should be captured both as species presence and species' occupied space. Percent cover is the vertical projection of each species onto an imagined two-dimensional plane that is parallel to the ground, taking into account all aboveground plant parts. Thus, the total plot cover can reach more than 100%, allowing for overlapping plants within the canopy, and the cover can include both species rooted inside and outside the plot boundary. In addition, we advise that you estimate the percent cover of surface substrate types (bare soil and/or bare rock) and litter: record only the substrate which is visible when looking directly through the vegetation from above.

Estimation in categories may save time relative to pseudo-exact cover-estimates, and for decades, categorical scales such as decimal (Londo, 1975) and coarser estimates (e.g. DAFOR or DOMIN scales, Braun-Blanquet, 1964) have been used. Presence measures over several subplots is another useful compromise if it is not possible to perform percent cover calibration between several observers but rare species are of interest (and therefore point-quadrat is not appropriate). However, we recommend that percent cover is estimated directly, at as fine a resolution as possible, because continuous data have better analytical properties and it increases the potential to be used in cross-study analyses − it is easier to convert from a more detailed scale to a less detailed one than the other way round. Direct estimation of percent cover has the advantage that it allows translation to continuous numeric values for further analysis, even though the error around estimates can be high (particularly in mid-range values, 25−75%). A conversion table for Londo and other common abundance-class measurements to fixed percent cover points is found in Annex 1 of the ICP Forests vegetation sampling protocol (Canullo et al., 2016): note that scale data should still be analysed as non-continuous data even after conversion to percentages. While it is difficult to estimate cover precisely, we present techniques in the next paragraph to guide estimations and increase data quality.

Several techniques are available for reducing error rates in direct percent cover estimation. Practice and cross-referencing with an external reference and across field workers is key. Printed shapes of known area can be used as guides to visual area estimation (Figure 4.8.1b). Some studies maintain a full integer percentage scale but use 0.1 or "present" for items below 1% cover (Vandvik et al., submitted). Subplots can be used to guide the estimation, for example, in a quadrat with a 5 x 5 grid of subplots, each subplot will represent 4% area (Figure 4.8.1a). Subplots can also be useful for collecting additional data such as fine-scale spatial distribution, species co-occurrence at the fine scale, and various kinds of frequency data, and they may be useful for downstream data checking and corrections. For example, a species occurring in only one subplot cannot have greater cover than the percent of the plot occupied by one subplot. There are examples of what different amounts of cover look like at https://cnps.org/wp-content/uploads/2018/03/percent_cover_diag-cnps.pdf and a worked example of percent cover calculated from a photograph in Chen et al. (2010).

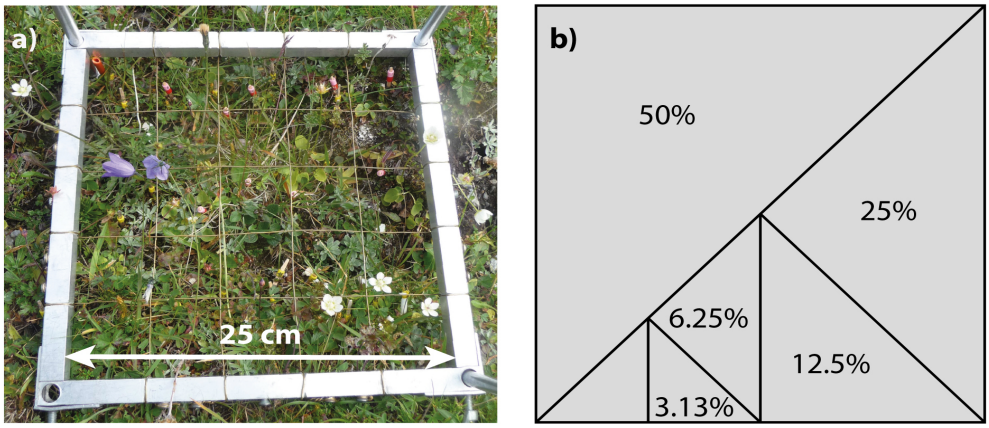


Figure 4.8.1: Species presence, abundance, fertility etc. can be measured both at the whole-plot and sub-plot scales, and printed cards with shapes of different area can be used to aid visual estimation at the plot scale. a) Quadrat (25 x 25 cm) used for visual cover estimation with 5 x 5 subplots, each subplot will represent 4% of the total plot area. b) Printed field card with different areas representing 50, 25, 12.5, 6.25, and 3.13% of the total area. Photo: Siri Lie Olsen.

Control plots are very important for data quality control in plant compositional measurements. The species turnover might vary between treatments but is not expected to be large between years for control plots and is therefore a source of information about overlooked or misidentified species. Finally, if species are hard to identify when only vegetative organs are present, a record of their reproductive status gives an indication of the reliability of the identification, which can then be cross-referenced to a year when that species was observed with reproductive organs.

Point-intercept method

The point-intercept method is primarily used to measure plant cover, but it can indirectly quantify plant frequency and total aboveground biomass (Jonasson, 1988; see protocol 2.1.1 Aboveground plant biomass). It is suited for studies focused on the more common species within a community, where the leaf area index (LAI) is greater than one, or where 3D space occupancy of plant species is of interest. One benefit of the point-intercept method is that it can be used to aggregate the cover of different species – in visual estimation of percent cover, vertical overlap of individuals within a species cannot be accounted for when aggregating data into groups at the analysis stage.

Where accurate estimates of the dominant species within a given community are more relevant to the research question, the point-intercept method can be a less subjective approach than percent cover (Ferris-Kaan & Patterson, 1992; Traxler, 1997; Bonham, 2013). This is because potential observer bias is reduced as quantification is more objective (a plant is touched by the pin or not). Furthermore, all plants touched by the pin must be identified so it may be less likely that inconspicuous individuals are overlooked. As a result, however, the technique often misses rare species, particularly where point frequency is low. The point-intercept method is preferred when the recording team is inexperienced or is large, but experienced botanists may find that this method uses more time compared to the percent cover method, particularly if there are few hard-to-recognise species in their system.

The technique works by lowering a pin vertically into the vegetation at a predetermined point, either on a grid (Pauli et al., 2015) or along a transect (INCREASE, 2014), and the identification of each plant

touched by the pin on its route to the ground is noted. The GLORIA project (Pauli et al., 2015) uses a knitting needle (approx. 30 cm) of 2 mm diameter, while longer pins are needed in taller vegetation (see Figure 4.8.2). Multiple hits of the same species are recorded, in addition to its life stage (alive/dead). The point at which the pin is lowered is predetermined to reduce bias and allow for accurate resurveying. Cover is expressed as the total number of hits per species as a proportion of the total number of pin measurements. The number of pins needed for adequate cover estimations is large (Ferris-Kaan & Patterson, 1992; Traxler, 1997; Bonham, 2013): the GLORIA project uses 100 points per 1m^2 plot while the INCREASE project uses 300 points distributed along transects up to 4 m (INCREASE, 2014).

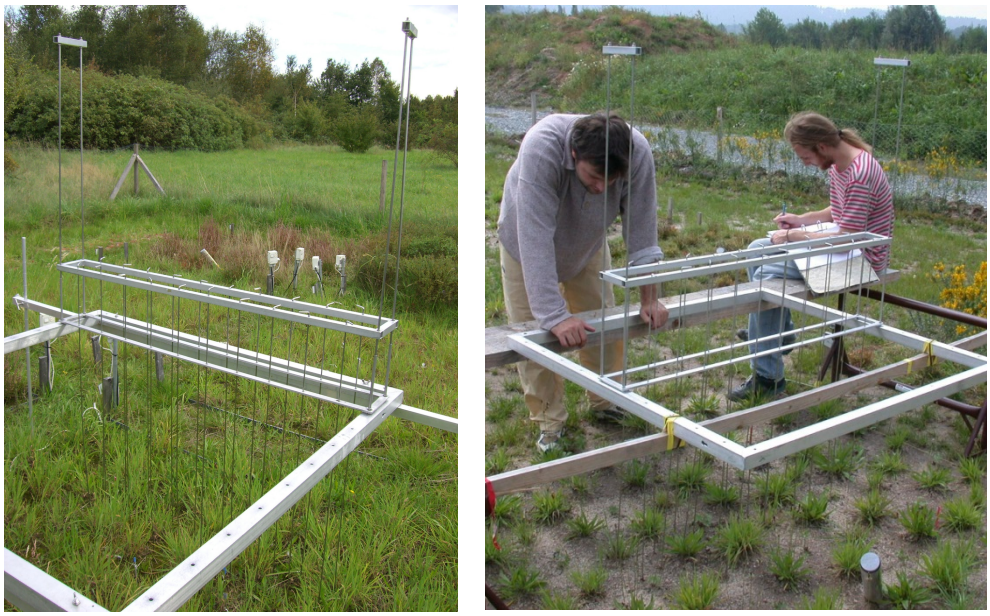


Figure 4.8.2: Point intercept method. Photo: Juergen Kreyling, EVENT experiment.

Direct estimation of number of individuals per species

Recording abundance by number of individuals (i.e. stem density) is most often used in forests where individuals are distinct (although this can be effectively combined with cover estimates of all species at multiple strata, e.g. herb layer, shrub layer, canopy layer; see Peet et al., 1998) or in systems where vegetation is sparse (e.g. deserts, annual plant communities). A nominal scale of species abundance may be more suitable when the number of individuals varies by several orders of magnitude. Occurrence is then classed into 1−10, 10−100, 100−1000, or >1000 individuals present. It is important to understand the life cycle of all of the species in order to decide whether to count ramets (distinct stems) or genets (distinct genetic individuals; see protocol 4.2 Plant demography). Individual count measurements are usually combined with some kind of individual size data.

Indices calculated from raw compositional data

Species compositional data can be used to extract species presence for presence/absence analyses such as species richness (count data), while abundance allows for calculation of a wider variety of indices, such as community weighted trait means (Funk et al., 2017; see next paragraph), beta-diversity (Chao et al., 2005), species diversity (e.g. Shannon index), and evenness (Hill, 1973; Smith & Wilson, 1996). The choice of the most appropriate diversity index is dependent on the question and

often a matter of quite some discussion in the ecological community: Magurran (2003) provides detailed guidance on this. Additionally, calculating relative abundances (as opposed to absolute abundance) allows for comparison of dominance patterns in plots that differ in productivity (Heckman et al., 2017).

Plant community compositional data can be combined with continuous numerical trait values to give mean trait values for plots, which are currently a popular way to compare ecological function between treatments and between sites or even different ecosystems. In Europe, species indicator values (a "soft" trait, i.e. not one which is physiologically established and non-plastic) are often applied to compare the environment of plots or subplots (examples of the lists of indicator values include Ellenberg et al., 1991; Landolt et al., 2010; for reviews of the utility of indicator values see Zeleny & Schaffers, 2012; Diekmann, 2003). The trait value for each species is multiplied by the percent cover for that species (or added if presence/absence data are used), then all the totals are added together and divided by the sum of all the percent cover values for the plot to obtain a mean plot value called a community-weighted mean (see protocol 4.16 Functional traits; Funk et al., 2017). Villéger et al. (2008) provide alternative weighted approaches for situations requiring categorical traits.

Where to start

Bonham (2013), Ferris-Kaan & Patterson (1992), Peet et al. (1998).

4.8.2 Special cases, emerging issues, and challenges

Accuracy of taxonomic identification

Plants should be identified to species where possible and taxonomy should follow standard nomenclature. Ensuring that everyone working in the field uses a consistent set of plant names reduces the potential for error during data analysis. The Plant List (TPL; http://www.theplantlist.org/) and the Taxonomic Name Resolution Service (TNRS; Boyle et al. 2013) are working lists of primarily vascular plants, and both are available as a R packages for data analysis: *Taxostand* (Cayuela et al., 2012) and *taxize* (Chamberlain & Szöcs, 2013; Chamberlain et al., 2016).

Where time or taxonomic knowledge is limited, or to answer different ecological questions, an estimation of plant functional group cover is taken. In this case, graminoids, non-leguminous forbs, leguminous forbs, bryophytes, shrubs, and trees are common groupings, and group cover can be estimated as described above for herbaceous systems. This information can also be inferred from full species composition.

Observer bias and survey repeatability

It is important to calibrate cover estimates of all recorders to reduce observer bias. It is ideal to have two or more recorders working together per plot, but as a minimum all recorders should calibrate their estimates at the start of the project or field season by separately analysing a set of the same plots and comparing their findings. This should be repeated, using new plots each time, until the estimates of all recorders are within predefined acceptable bounds. Recalibration throughout the

field season is advised. Observer calibration is not as critical for the point-intercept method, but all recorders should thoroughly familiarise themselves with the protocol to avoid errors in sampling procedure (e.g. missing points within the point grid). As well as keeping a record of the observer for each plot, external data such as weather should be recorded as it has been shown to affect the quality of surveys (Burg et al., 2015).

Estimating the species pool of an experimental site

The species pool around a long-term climate change experiment can influence plot level change in species composition over time. It can be useful to know how large the local species pool is to understand the heterogeneity in species diversity across an experimental site and to get estimates of so-called "dark diversity" (absent species; Pärtel et al. 2011). The ITEX species pool protocol outlines methods for quantifying the size and diversity of the species pool around long-term monitoring ecological plots.

All species in an area of 100 – 250 m radius around an experimental site are recorded (Figure 4.8.3). The sampling is started in the centre of the site recording the species all species in a 0.0625, 1 and 5m^2 radius. From there, all new species are recorded walking in larger and larger circles around the mid point (roughly 10 m appart). The location for the starting point and all new species are marked with a GPS, to get their distance from the centre. For more details on the method see Species pool protocol for the International Tundra Experiment Network (ITEX) (Rixen et al., 2019).

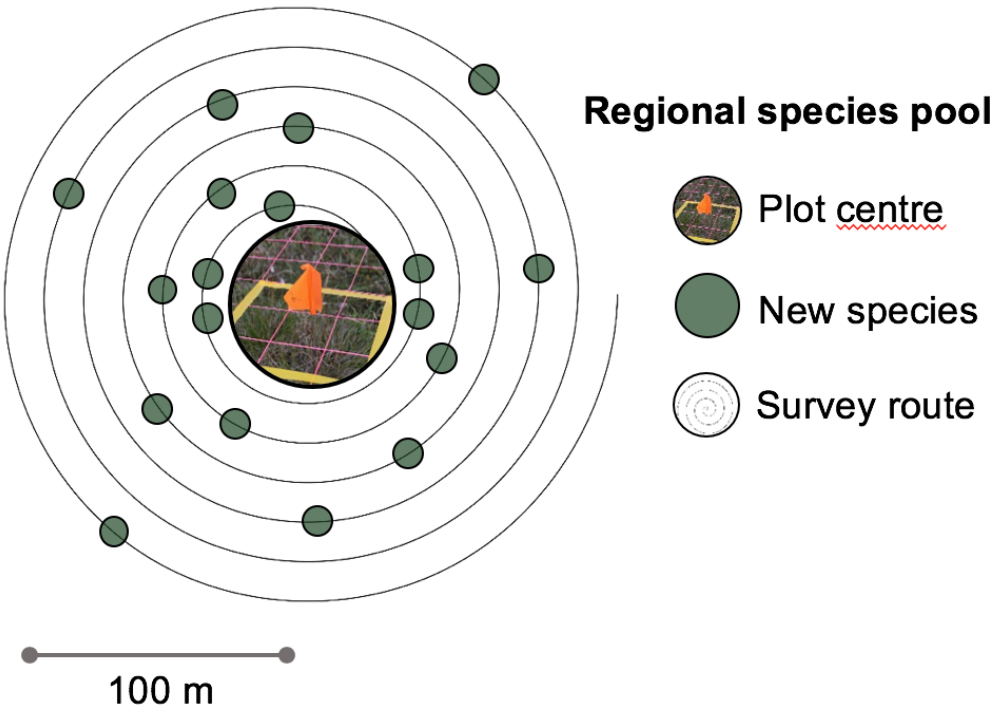


Figure 4.8.3: Sampling scheme for collecting data on the regional species pool for a climate change experiment. Picture from Rixen et al. 2019.

Species community data analysis

Methods for analysis of vegetation compositional data vary: one of the most popular is ordination. Ordination reduces the complexity of the data to positions on one to several axes. It is a useful way to visualise the data and see which samples are similar in composition, and there are methods to relate this to environmental conditions (Borcard et al., 2018). Choice of ordination method depends on the underlying data structure and analytical aim, although there remain "strongly divergent" opinions on the usefulness of particular methods (Minchin & Oksanen, 2015; see other papers within the same issue for defences of the various methods). Guidance for choosing amongst the parametric forms of ordination can be found in Chapter 5 of Jongman et al. (1995). For analytical packages offering ordination techniques, the vegan package in R (Oksanen et al., 2018) is the most widely used at this time and Canoco is also popular (Šmilauer & Lepš, 2014). In both the visual estimation of percent cover and in the point-intercept method, the distribution of values for individual plants follows a non-normal distribution and should be analysed accordingly (Damgaard, 2009).

4.8.3 References

Theory, significance, and large datasets

Elmendorf et al. (2015) summarise and compare the outcomes of various warming experiments on plant community composition in tundra environments and consider the pros and cons of different methods. Peñuelas et al. (2013) compare and integrate climate-change effects at different levels of biological organisation from the genome to the region, including the community. Franklin et al. (2016) conceptually embed vegetation analysis and small-scale climate-manipulation experimentation within the different common types of environmental-change monitoring and modelling which together form our knowledge base for forecasting future landscapes.

More on methods and existing protocols

Plant cover is described in depth in many of the climate-manipulation experiment protocols. We draw particular attention to the protocols from GLORIA (Pauli et al., 2015, pp. 38-43), SeedClim (Vandvik et al., submitted), and INCREASE (INCREASE, 2014, pp. 6-7) projects. Standardised illustrations of percent cover values are presented in Law et al. (2008, p22). Well-used protocols from distributed experiments other than climate manipulations include NutNet (at https://nutnet.umn.edu/exp_protocol, section a) and the Herbivory Network. There are also a few protocols from large-scale monitoring networks which integrate field and canopy layer monitoring for forests within the same sampling system, of which ICP forests and the UK Environmental Change Network are two examples (Rodwell et al., 1996; UNECE ICP, 2016).

All references

Bonham, C. D. (2013). *Measurements for Terrestrial Vegetation*. Chichester: John Wiley & Sons.

Borcard, D., Gillet, F., & Legendre, P. (2018). *Numerical Ecology with R*. Springer International Publishing.

Borer, E. T., Harpole, W. S., Adler, P. B., Lind, E. M., Orrock, J. L., Seabloom, E. W., & Smith, M. D. (2014). Finding generality in ecology: a model for globally distributed experiments. *Methods in Ecology and Evolution*, *5*(1), 65-73.

Boyle, B., Hopkins, N., Lu, Z., Garay, J. A. R., Mozzherin, D., Rees, T., ... Lowry, S. (2013). The taxonomic name resolution service: an online tool for automated standardization of plant names. *BMC Bioinformatics*, *14*(1), 16.

Braun-Blanquet, J. (1964). *Pflanzensoziologie. Grundzüge der Vegetationskunde*. Vienna: Springer.

Burg, S., Rixen, C., Stöckli, V., & Wipf, S. (2015). Observation bias and its causes in botanical surveys on high-alpine summits. *Journal of Vegetation Science, 26*(1), 191-200.

Canullo, R., Starlinger, F., Granke, O., Fischer, R., & Aamlid, D, (2016). Part VI.1: Assessment of ground vegetation. In: UNECE ICP Forests Programme Co-ordinating Centre (Ed.), *Manual on Methods and Criteria for Harmonized Sampling, Assessment, Monitoring and Analysis of the Effects of Air Pollution on Forests* (pp. 1-18). Eberswalde: Thünen Institute of Forest Ecosystems [http://www.icpforests.org/Manual.htm].

Cayuela, L., Granzow-de la Cerda, Í., Albuquerque, F. S., & Golicher, D. J. (2012). Taxonstand: An R package for species names standardisation in vegetation databases. *Methods in Ecology and Evolution, 3*(6), 1078-1083.

Chamberlain, S., & Szöcs, E. (2013). taxize - taxonomic search and retrieval in R. *F1000Research, 2*, 191.

Chamberlain, S., Szöcs, E., Boettiger, C., Ram, K., Bartomeus, I., Baumgartner, J., … O'Donnell, J. (2016). taxize: Taxonomic information from around the web. R package version 0.7.8. https://github.com/ropensci/taxize.

Chao, A., Chazdon, R. L., Colwell, R. K., & Shen, T. J. (2005). A new statistical approach for assessing similarity of species composition with incidence and abundance data. *Ecology Letters, 8*(2), 148-159.

Chen, Z., Chen, W. E., Leblanc, S. G., Henry, G. H., & Chen, W. (2010). Digital photograph analysis for measuring percent plant cover in the Arctic. *Arctic*, *63* (3), 315-326.

Damgaard, C. (2009). On the distribution of plant abundance data. *Ecological Informatics, 4*(2), 76-82.

Díaz, S., Hodgson, J. G., Thompson, K., Cabido, M., Cornelissen, J. H. C., Jalili, A., ... Band, S. R. (2004). The plant traits that drive ecosystems: evidence from three continents. *Journal of Vegetation Science, 15*(3), 295-304.

Diekmann, M. (2003). Species indicator values as an important tool in applied plant ecology – a review. *Basic and Applied Ecology, 4*(6), 493-506.

Ellenberg, H., Weber, H. E., Düll, R., Wirth, V., Werner, W., & Paulissen, D. (1991). Zeigerwerte von Pflanzen in Mitteleuropa. *Scripta Geobotanica, 18*, 1-248.

Elmendorf, S. C., Henry, G. H., Hollister, R. D., Fosaa, A. M., Gould, W. A., Hermanutz, L., ... Magnusson, B. (2015). Experiment, monitoring, and gradient methods used to infer climate change effects on plant communities yield consistent patterns. *Proceedings of the National Academy of Sciences USA, 112*(2), 448-452.

Enquist, B. J., Norberg, J., Bonser, S. P., Violle, C., Webb, C. T., Henderson, A., ... Savage, V. M. (2015). Scaling from traits to ecosystems: developing a general trait driver theory via integrating trait-based and metabolic scaling theories. *Advances in Ecological Research, 52*, 249-318.

Ferris-Kaan, R., & Patterson, G. S. (1992). *Monitoring Vegetation Changes in Conservation Management of Forests* (Forestry Commission Bulletin 108). London: HMSO.

Franklin, J., Serra-Díaz, J. M., Syphard, A. D., & Regan, H. M. (2016). Global change and terrestrial plant community dynamics. *Proceedings of the National Academy of Sciences USA, 113*(14), 3725-3734.

Funk, J. L., Larson, J. E., Ames, G. M., Butterfield, B. J., Cavender-Bares, J., Firn, J., ... Wright, J. (2017). Revisiting the Holy Grail: using plant functional traits to understand ecological processes. *Biological Reviews, 92*(2), 1156-1173.

Garnier, E., Lavorel, S., Ansquer, P., Castro, H., Cruz, P., Dolezal, J., ... Grigulis, K. (2007). Assessing the effects of land-use change on plant traits, communities and ecosystem functioning in grasslands: a standardized methodology and lessons from an application to 11 European sites. *Annals of Botany*, *99*(5), 967-985.

Green, R. H. (1979). *Sampling Design and Statistical Methods for Environmental Biologists*. New York: John Wiley & Sons.

Heckman, R. W., Halliday, F. W., Wilfahrt, P. A., & Mitchell, C. E. (2017). Effects of native diversity, soil nutrients, and natural enemies on exotic invasion in experimental plant communities. *Ecology, 98*(5), 1409-1418.

Hill, M. O. (1973). Diversity and evenness: a unifying notation and its consequences. *Ecology*, *54*(2), 427-432.

INCREASE (2014). An integrated network on climate change activities on shrubland ecosystems: final report. Department of Geosciences and Natural Resource Management, University of Copenhagen. https://cordis.europa.eu/result/rcn/164418_en.html

Jonasson, S. (1988). Evaluation of the point intercept method for the estimation of plant biomass. *Oikos 52*(1), 101-106.

Jongman, R. H. G., ter Braak, C. J. F., & van Tongeren, O. F. R. (1995). *Data Analysis in Community and Landscape Ecology*. Cambridge: Cambridge University Press.

Kröel-Dulay, G., Ransijn, J., Schmidt, I. K., Beier, C., De Angelis, P., De Dato, G., ... Peñuelas, J. (2015). Increased sensitivity to climate change in disturbed ecosystems. *Nature Communications, 6*, 6682.

Landolt, E., Bäumler, B., Erhardt, A., Hegg, O., Klötzli, F., Lämmler, W., ... Urmi, E. (2010). *Flora Indicativa: Ökologische Zeigerwerte und Biologische Kennzeichen zur Flora der Schweiz und der Alpen*. Haupt, Bern.

Law, B. E., Akebauer, T., Campbell, J. L., Chen, J., Sun, O., Schwartz, M., … Verma, S. (2008). *Terrestrial Carbon Observations: Protocols for vegetation sampling and data submission*. Rome: FAO.

Londo, G. (1975). Dezimalskala für die vegetationskundliche Aufnahme von Dauerquadraten. In W. Schmidt (Ed.), *Sukzessionsforschung*. *Bericht über das Internationale Symposia der Internationalen Vereinigung für Vegetationskunde, 1973* (pp. 316-317). Netherlands: Springer.

Magurran, A. E. (2003). *Measuring Biological Diversity*. Oxford: Wiley-Blackwell.

Minchin, P. R., & Oksanen, J. (2015). Statistical analysis of ecological communities: progress, status, and future directions. *Plant Ecology, 216*(5), 641-644.

Mueller-Dombois, D., & Ellenberg, H. (1974). *Aims and Methods of Vegetation Ecology*. New York: Wiley.

Ode, D. J., Tieszen, L. L., & Lerman, J. C. (1980). The seasonal contribution of C_3 and C_4 plant species to primary production in a mixed prairie. *Ecology, 61*(6), 1304-1311.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., ... Wagner, H. (2018). Vegan: Community ecology package. R package version 2.5-2. https://CRAN.R-project.org/package=vegan.

Pauli, H., Gottfried, M., Lamprecht, A., Niessner, S., Rumpf, S., Winkler, M., ... Grabherr, G. (2015). *The GLORIA Field Manual – Standard multi-summit approach, supplementary methods and extra approaches* (5th ed.). Vienna: Austrian Academy of Sciences & University of Natural Resources and Life Sciences.

Pärtel, M., Szava-Kovats, R., & Zobel, M. (2011). Dark diversity: shedding light on absent species. *Trends in Ecology & Evolution, 26*(3), 124-128.

Peet, R. K., Wentworth, T. R., & White, P. S. (1998). A flexible, multipurpose method for recording vegetation composition and structure. *Castanea*, *63*(3), 262-274.

Peñuelas, J., Sardans, J., Estiarte, M., Ogaya, R., Carnicer, J., Coll, M., ... Filella, I. (2013). Evidence of current impact of climate change on life: a walk from genes to the biosphere. *Global Change Biology*, *19*(8), 2303-2338.

Rixen, C., Daskalova, G., Bjorkman, A., and Normand, S. (2019). Species Pool Protocol for the International Tundra Experiment Network (ITEX). OSF. June 16. https://osf.io/agdfq/ DOI: 10.17605/OSF.IO/AGDFQ

Rodwell, J. S., Sykes, J. M., & Helps, M. B. (1996). *The UK Environmental Change Network Protocols for Standard Measurements at Terrestrial Sites Protocol V: Vegetation*. Swindon: UK Natural Environment Research Council.

Šmilauer, P., & Lepš, J. (2014). *Multivariate Analysis of Ecological Data Using Canoco 5*. Cambridge: Cambridge University Press.

Smith B., & Wilson J. B. (1996). A consumer's guide to evenness indices. *Oikos, 76*, 70-82.

Stevens, C. J., Dise, N. B., Mountford, J. O., & Gowing, D. J. (2004). Impact of nitrogen deposition on the species richness of grasslands. *Science, 303*(5665), 1876-1879.

Stewart, K. E. J., Bourn, N. A. D., & Thomas, J. A. (2001), An evaluation of three quick methods commonly used to assess sward height in ecology. *Journal of Applied Ecology, 38*(5), 1148-1154.

Traxler, A. (1997). *Handbuch des Vegetationsökologischen Monitorings: Methoden, Praxis, angewandte Projekte. Teil A: Methoden*. Vienna: Umweltbundesamt.

UNECE ICP Forests Programme Co-ordinating Centre (Ed.) (2016). Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on

forests. Eberswalde: Thünen Institute of Forest Ecosystems. http://icp-forests.net/page/icp-forests-manual

Vandvik, V. (2004). Gap dynamics in perennial subalpine grasslands: trends and processes change during secondary succession. *Journal of Ecology, 92*, 86-96.

Vandvik, V., Klanderud K., Skarpaas O., Telford R. J., Halbritter A. H., & Goldberg, D. E. (submitted to *Ecology Letters*). Context-dependencies in biodiversity response to climate change are reduced by expressing abiotic environments in biological terms.

Villéger, S., Mason, N. W., & Mouillot, D. (2008). New multidimensional functional diversity indices for a multifaceted framework in functional ecology. *Ecology, 89*(8), 2290-2301.

Zelený, D., & Schaffers, A. P. (2012). Too good to be true: pitfalls of using mean Ellenberg indicator values in vegetation analyses. *Journal of Vegetation Science*, *23*(3), 419-431.

Authors: Jaroszynska F[1], Eycott AE[2,3], Vandvik V[1], Halbritter AH[1]

Reviewers: Peñuelas J[4,5], Reinsch S[6], Wilfahrt PA[7], Wohlgemuth T[8]

Affiliations:

[1] Department of Biological Sciences and Bjerknes Centre for Climate Research, University of Bergen, Bergen, Norway

[2] Department of Biological Sciences, University of Bergen, Bergen, Norway

[3] Faculty of Biosciences and Aquaculture, Nord University, Steinkjer, Norway

[4] CSIC, Global Ecology Unit CREAF-CSIC-UAB, Bellaterra, Spain

[5] CREAF, Cerdanyola del Vallès, Spain

[6] Centre for Ecology and Hydrology, Environment Centre Wales, Bangor, UK

[7] Department of Disturbance Ecology, BayCEER, University of Bayreuth, Bayreuth, Germany

[8] Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland

4.9 Soil microbial community composition

Authors: Radujković D[1], Verbruggen E[1]

Reviewer: Vik U[2]

Measurable unit: number and relative abundance of microbial taxa; Measureable scale: plot; Equipment costs: €€; Running costs: €€; Installation effort: medium; Maintenance effort: -; Knowledge need: medium to high; Measurement mode: manual

The microbial community composition represents the number and relative abundance of microbial taxa in a given system. This measure provides insight into the diversity and variability of the relative abundances of microbial taxa and thus aspects of their community dynamics. Moreover, changes in overall soil microbial community composition may point to corresponding changes in the various processes in which these communities are involved (Zogg et al., 1997; Balser & Firestone, 2005; Strickland et al., 2009). Microbes in soil are essential for decomposition of organic matter (Allison & Martiny, 2008), they can play a key role in long-term carbon storage (Clemmensen et al., 2013), and they are important drivers of biogeochemical cycling processes, including carbon and nitrogen cycling (Prosser et al., 2007; Falkowski et al., 2008). Specific microbial functional groups (such as nitrifying bacteria, mycorrhizal fungi, plant parasites) may affect ecosystem functioning by altering nutrient availability or plant productivity (van der Heijden et al., 2008). Mycorrhizal fungi, for example, are associated with up to 90% of terrestrial plants (Smith & Read, 2008) and they could have important effects on plant productivity (Wilson et al., 2016; Yang et al., 2016) and carbon dynamics in soil. It has been demonstrated that changes in different environmental factors (e.g. precipitation, CO_2, temperature, nutrient concertation) can cause shifts in microbial community composition (Zogg et al., 1997; Castro et al., 2010; Nemergut et al., 2014). These changes may, directly or indirectly, affect important ecosystem process (e.g. carbon cycling), thereby mediating the feedback responses to global change (Davidson & Janssens, 2006; Pold & DeAngelis, 2013).

4.9.1 What and how to measure?

Gold standard

With the development of high-throughput DNA sequencing techniques, the composition of soil microbial communities can be studied in more detail at a lower cost than using traditional culture-dependent approaches (Shokralla et al., 2012). High-throughput molecular identification of microbial communities requires the isolation of nucleic acids from environmental samples, followed by DNA amplification using primers (small manufactured sections of DNA) that bind specifically to phylogenetically conserved regions of genes, which flank so called barcode markers (Winsley et al., 2012). The accuracy of these analyses are strongly dependent on the choice of primers (Klindworth et al., 2013). Genes encoding components of the nuclear ribosomal units (small subunit, SSU; large subunit, LSU; internal transcribed spacer, ITS) are by far the most commonly used genetic markers for taxonomic identification of microorganisms (Lindahl et al., 2013). Current high-throughput sequencing techniques allow the simultaneous sequencing of millions of reads (Bartram et al., 2011), which are then clustered into operational taxonomic units (OTUs; typically at 97% sequence similarity) and assigned to taxonomic/functional groups using various bioinformatical tools and

reference databases. The changes in microbial community composition exposed to certain climate treatments (e.g. warming, drought) compared to control communities, can be statistically assessed based on the differences in the number and relative abundance of OTUs between these communities and/or changes in relative abundance of taxonomic/functional groups.

Bronze standard

Phospholipid fatty acid analysis (PLFA) is another culture-independent method that is commonly used to assess the changes in microbial community composition. It has been demonstrated that PLFA analyses and genetic sequencing can detect similar overall patterns in bacterial community composition (Orwin et al., 2018). Compared to genetic sequencing, PLFA has a very limited taxonomic resolution, especially for groups other than bacteria, but unlike sequencing it can provide quantitative information about microbial biomass (Brewer et al., 2015). It can thus be preferred in cases when quantitative shifts in both biomass and broad functional groups (fungi, gram-positive v. gram-negative bacteria) are to be delineated. For a detailed protocol and possible applications of PLFA see Frostegård et al. (1993) and Frostegård et al. (2011), respectively.

Soil sampling and storage

Soil samples are collected using soil corers, usually at depths of 0–5 cm and/or 5–10 cm (e.g. Rinnan et al., 2007; Kuffner et al., 2012; Hayden et al., 2012). The corers must be cleaned between the samples in order to avoid cross-contamination. When collecting samples for fungal analysis, it should be borne in mind that fungi can have very long mycelia and thus it is recommended to keep a minimal distance of 3 m between different samples when independence is required for statistical analysis (Lindahl et al., 2013). Typically, a few soil samples (e.g. for a good representation of a plot, samples can be taken in four corners and the centre). Depending on the study question, samples can be taken one time only (e.g. in the peak of the growing season, if the aim is to examine the effect of treatments at the peak of vegetation growth) or multiple times in the same plot (e.g. if the aim is to examine inter- or intra-annual changes in community composition). The samples can be stored in sterile plastic ziplock bags. After sampling, the soil is sieved (2 mm mesh size is a standard in soil science), taking care to prevent contamination. The samples should be kept in a cold place and processed as soon as possible to avoid the degradation of DNA and microbial growth (Rochelle et al., 1994). Over longer time periods, samples can be optimally stored by freezing at -20 °C or -80 °C (Song et al., 2016). Alternatively, they can be freeze-dried (Lindahl et al., 2013) or stored in pure ethanol (Hale et al., 2015) or commercially available preservation solutions.

DNA extraction

Most extraction methods are based on direct cell lysis which generally provides high yields of DNA with relatively short processing times (Robe et al., 2003). Commercially available soil DNA extraction kits provide detailed protocols for extraction procedures. Because of the typical low sample size for extraction (0.25–0.5 g dry weight) care should be taken to thoroughly homogenise material for subsampling, or isolate DNA from multiple technical replicates. Ideally, extraction should yield high and uniform amounts of DNA and minimal concentrations of amplification inhibitors (Lindahl et al.,

2013). DNA yield can be assessed and concentrations can be adjusted through dilution. The same DNA extraction protocol should be used for all samples (Tedersoo et al., 2010) ensuring that potential extraction-related biases are equally distributed across all samples.

The procedures described next are sometimes outsourced to a commercial laboratory (even including taxonomic annotation of obtained sequences), or can be performed in-house when facilities are available.

DNA amplification – PCR

Following extraction, DNA is amplified using primers that target a barcode marker region which is conserved within a particular microbial group (prokaryotes, eukaryotes, fungi, arbuscular mycorrhizal fungi), but includes variable regions that allow the distinction at the phylogenetic level of interest (Lindahl et al., 2013). The primers also include artificial barcode sequences that allow identification of different samples after sequencing, or these barcodes are added in a second step. Amplification of the marker is accomplished by successful binding of the two primers to the flanking sections, and generating copies of it through a "polymerase chain reaction" (PCR). In order to assess the variation resulting from stochastic processes during laboratory work, replicate PCR reactions can be performed using independently obtained DNA extractions from the same sample (Kauserud, et al., 2012). PCR conditions (see e.g. Bartram et al., 2011; Klindworth et al., 2013; Zhang et al., 2016) need to be optimised to the marker region and lab conditions (e.g. enzymes and thermal cycler), where the annealing temperature in particular deserves attention. Optimal annealing temperatures between 45 and 68 °C differ depending on primer sequence and length and are, as a rule of thumb, set at 5 °C below the calculated temperature of the lowest primer melting point (T_m) (Roux, 2009).

To assess the success of a PCR, the products are visualised on an agarose gel where presence and length of a product can be determined. If the annealing temperature is too low (primers do not anneal specifically to the target region) there will be more bands visible on the gel (more than expected based on natural length variation of the marker); if it is too high (primers do not anneal to target region at all) there will be no bands on the gel. The optimal annealing temperature for a particular primer pair can be determined by gradually increasing the annealing temperature (gradient PCR). PCRs can also fail due to different inhibitors present in the starting template. A 5–100-fold dilution of the template may dilute out the inhibitor (Roux, 2009). Other possible solutions in case of PCR failure include re-extraction, re-amplification, ethanol precipitation, changing the number of PCR cycles, or adding stabilising proteins such as bovine serum albumin (BSA) (see Roux, 2009 for more details on optimisation of PCR process).

Primer choice

There are multiple valid reasons to choose one primer-pair over another for a particular group of microbes. Main reasons are i) the sequencing technology used: some instruments (e.g. Illumina Miseq) work optimally with DNA sequences between 250–500 base pairs (bp) in length, while others (e.g. PacBio) can sequence whole DNA strands with thousands of bp; ii) sequence variability: ideally there should be a so called "barcode-gap" (Schoch et al., 2012) making it easy to delineate within-species v. between-species variability, however this varies between taxonomic groups and markers and so choice will often be a trade-off where higher quality data for one group will come at a cost of

another; and iii) historical reasons will cause a marker for a group of interest to have a much better representation in databases (e.g. 16S/18S rRNA for many microbial groups), which means that even when in principle other regions would be more suitable, having a well-filled database to compare against will improve the quality of the eventual data.

Bacteria. The 16S rRNA gene (encodes SSU in prokaryotes) has been by far the most commonly used genetic marker for analyses of bacterial communities (Klindworth et al., 2013) for a number of reasons: it is present in all bacteria; it contains both highly conserved regions and hypervariable regions; and it is sufficiently long (1,500 bp) for bioinformatic purposes (Janda & Abbott, 2007). The combination of Bakt_341F and Bakt_805R primers (Herlemann et al., 2011) can be used to amplify variable regions V3 and V4 of 16s rRNA gene. This primer set was evaluated by Klindworth et al. (2013) as one of the most efficient in amplifying a wide range of bacterial phyla.

Fungi. Molecular analyses of fungal communities mainly rely on amplification of the ITS region (spanning the ITS1, 5.8S, and ITS2 regions), which was selected as the universal genetic barcode for fungi (Schoch et al., 2012). However, whether the ITS1, ITS2, or a combination of these two regions is better suited for characterisation of fungal communities is still under debate (Blaalid et al., 2013). The ITS1 region is frequently amplified using the combination of IT1f and ITS2 primers (Op De Beeck et al., 2014; Smith & Peay, 2014). fITS7, gITS7, and fITS9 primers target binding sites in the 5.8S region and together with the ITS4 primer, they can be used to amplify the ITS2 region (Ihrmark et al., 2012). The combination of ITS1f and ITS4 primers span both ITS regions together with 5.8S region (Smith & Peay, 2014).

Arbuscular mycorrhizal fungi (AMF) – Glomeromycota. For AMF analysis, the most commonly used marker region is SSU (18S rDNA in eukaryotes), followed by LSU(28S rDNA) and ITS rDNA region (Öpik et al., 2014). The SSU rDNA region alone is not suitable for identification of species (Öpik et al., 2014), but in the cases when species resolution is not the primary goal, primers that target SSU region, for example AML1 and ALM2, designed by Lee et al. (2008), can provide useful information regarding the overall AMF community composition. Primer set SSUmAf–LSUmAr (1800 bp) and SSUmCf–LSUmBr (1500 bp) developed by Krüger et al. (2009) spans a fragment covering the partial SSU, the entire ITS, and the partial LSU rDNA region. This combination of primers enables detection of additional AMF, but the sequences are too long for some high-throughput sequencing and alternative sequencing methods must be used (Schlaeppi et al., 2016).

Protists. A comprehensive overview of different SSU primers designed to target protists is provided by Adl et al. (2014). However, Adl et al. (2014) conclude that none of the examined primers had a high specificity at taxonomic levels higher than genus. The combination of primers TAReuk454FWD1 and TAReukREV3 (Stoeck et al., 2010) that targets the V4 region of SSU, can be used for detection of a wide range of eukaryotic lineages (Mahé et al., 2017). A recently developed combination of primers (ITS3 primer mixes, ITS4ngs) described in Tedersoo et al. (2015) that target ITS2 region can be used to characterise certain protist groups: Cercozoa, Ciliophora, and Chlorophyta, as well as soil animals (Acari, Nematoda, Collembola, Rotifera, Annelida) which are thought to be the most abundant and species-rich eukaryotic taxa in soil (Tedersoo et al., 2015). Given the paraphyletic nature of protists (spanning the entire eukaryotic phylogenetic tree), no primers specifically targeting this group as a whole can be designed. For this reason, samples containing a high concentration of plant, fungal, or

animal DNA, such as when one aims to elucidate the protists that are part of their "microbiomes", are at risk of primarily generating non-target sequences.

Library preparation and sequencing

Following purification from PCR artefacts (primers and primer-dimers), different samples with specific barcodes are equimolarly pooled into a single library ready for sequencing. The Illumina MiSeq platform (Illumina Inc; San Diego, CA, USA) is currently the most commonly used platform for high-throughput sequencing of environmental microbial samples. This platform enables sequencing of 200–550 bp-long paired-end reads (forward and reverse) which is, in most cases, enough to cover the entire marker region for different microbial groups. Longer reads can be sequenced using single molecule real-time (SMRT) methodology (PacBio; Manlo Park, CA, USA).

Quality control and bioinformatics analyses

UPARSE (Edgar, 2013), QIIME (Caporaso et al., 2010), and mothur (Schloss et al., 2009) are some of the most commonly used bioinformatics pipelines that allow quality filtering and construction of OTUs from next-generation sequencing reads. The main result of these analyses is an OTU table (Figure 4.9.1). The downstream analyses (e.g. standardisation of read number through downsampling (Weiss et al., 2017), calculation of alpha and beta diversity) can be performed using QIIME and mothur, but also in statistical programs such as R (e.g. using the 'vegan' or 'phyloseq' packages). Typically, OTU tables are used to create distance matrices, which include pairwise distances between the microbial communities of different samples (Figure 4.9.1). It should be noted that for bacterial sequences, it is common to create phylogenetic trees and use phylogenetically informed distance metrics (i.e. UniFrac). Statistical analyses on distance matrices or OTU tables can be performed using various multivariate types of analyses such as perMANOVA, ANOSIM, and ordination methods (e.g. PCoA, (G)NMDS, CCA, an example is depicted in Figure 4.9.1).

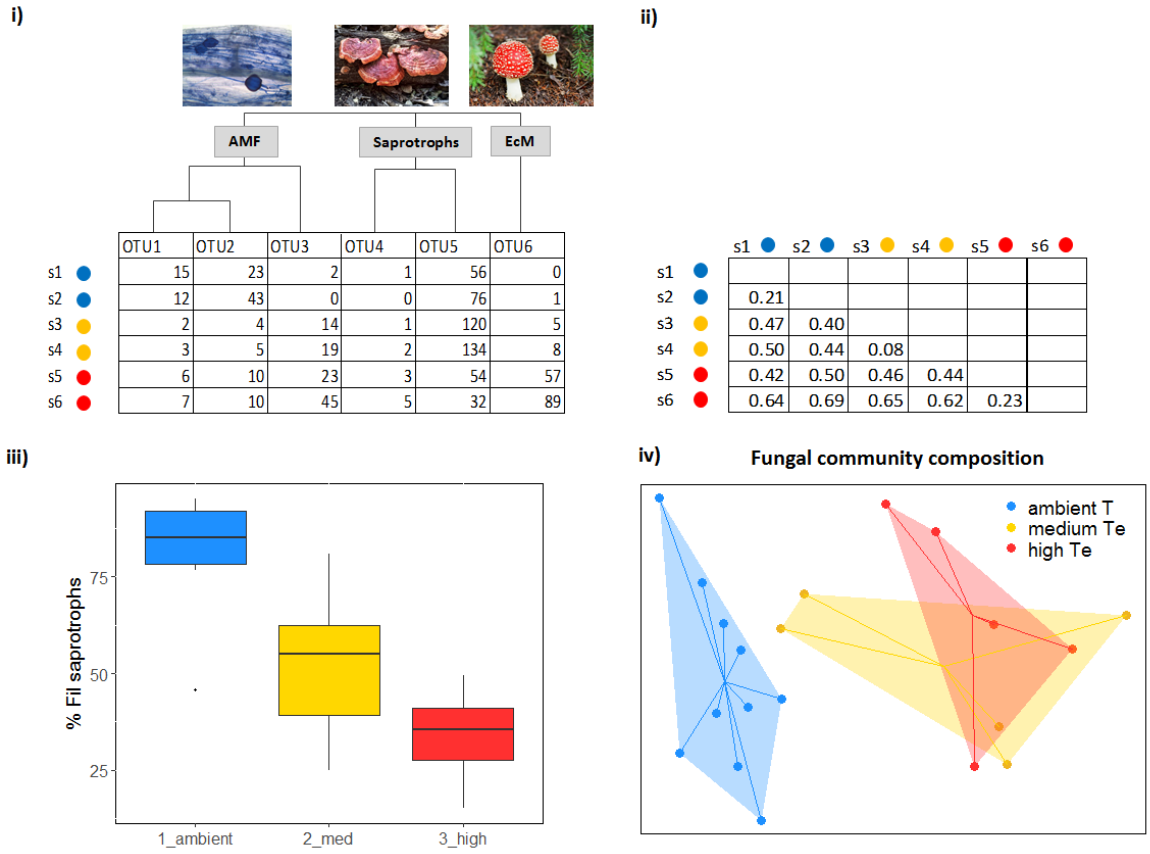


Figure 4.9.1 Main output of microbial genetic sequencing. Simplified representation of an OTU table containing the number of fungal OTUs in soil samples (s1–6) exposed to 3 different treatments (depicted in different colours). Different OTUs are assigned to taxonomic and/or functional groups by comparing them against a database. ii) Based on the OTU table it is possible to quantify the dissimilarities between the samples and summarise them in a distance matrix. Lower panels show the differences between soil fungal communities exposed to different intensities of natural warming, based on a subset of actual data from the ForHot natural experimental site (microbial data: Radujković et al. 2018; ForHot experiment: Sigurdsson et al. 2016). iii) The relative abundance (% of the total amount of sequences in a sample) of filamentous saprotrophic fungi exposed to different intensities of warming and iv) the multidimensional ordination of samples based on Bray-Curtis distances. Points and the corresponding polygons are coloured according to temperature elevations (Te): blue – ambient temperatures; orange – medium temperature elevation; (+3 °C to +5 °C); red – high temperature elevation (+7 °C to +11 °C).

Taxonomic identification is performed by aligning sequences to the reference sequences (using BLAST or other methods implemented in UPARSE/QIIME/mothur) deposited in publicly available databases. An overview of different databases is given by Santamaria et al. (2012). For instance, the Greengenes database contains a collection of bacterial 16s rDNA sequences (DeSantis et al., 2006), UNITE is a comprehensive reference database for fungal ITS sequences (Abarenkov et al., 2010), and PR^2 (Protist Ribosomal Reference) database is suitable for annotation of protist SSU sequences (Guillou et al., 2013). Other databases, such as Silva (Quast et al., 2013) and Ribosomal Database Project (Cole et al., 2014) contain collections of SSU and LSU sequences for various groups of prokaryotic and eukaryotic microorganisms. Following the taxonomic assignment, fungal OTUs can also be assigned to different functional categories (i.e. saprotrophic fungi, white rot decomposers, yeasts, plant pathogens, mycoparasites, animal parasites, arbuscular mycorrhizal fungi – AMF, ectomycorrhizal fungi – EcM) by matching their genus/family level with the known lifestyles (e.g. as in Tedersoo et al., 2014) using specialised tools such as FUNGuild (Nguyen et al., 2016).

Where to start

Bartram et al. (2011), Lindahl et al. (2013), Roux (2009), Shokralla et al. (2012), Tedersoo et al. (2010)

4.9.2 Special cases, emerging issues, and challenges

The methods of molecular analysis of microbial communities are evolving very rapidly with the development of new technologies. Previously commonly used 454 pyrosequencing is now almost entirely replaced by Illumina sequencing by synthesis. SMRT technology, such as PacBio, is now being increasingly used since it can provide longer reads (albeit with high error rates). The choice of sequencing platform is therefore currently a trade-off between the quality of the produced reads and the maximum length of the reads (Kennedy et al., 2018), but these or other platforms will likely become cost-efficient at low error rates in the near future.

Recently, there has been a lot of discussion regarding the common practices for bioinformatics analysis of sequencing data. The conventional approach is to perform clustering of OTUs, usually based on 97% similarity. However, this approach has been challenged and it has been proposed that instead of OTU clustering, amplicon sequence variants (ASVs) should be used. It is argued that, compared to OTUs, ASVs represent a biological reality independent of the data analysis, they have a better taxonomic resolution, they can be validly compared across different studies, and they are not limited by incomplete reference databases (Callahan et al., 2017). However, ASVs are highly sensitive to the quality of the data and this approach could be problematic for downstream analysis due to significantly increased diversity. While OTU clustering still remains the most common approach, it would be useful to also report the sequence variants in order to enable the effective comparison between different studies.

4.9.3 References

Theory, significance, and large datasets

Allison & Martiny (2008), Falkowski et al. (2008), Smith & Read (2008), van der Heijden et al. (2008), Zogg et al. (1997)

More on methods and existing protocols

Adl et al. (2014), Ihrmark et al. (2012), Klindworth et al. (2013), Op De Beeck et al. (2014), Smith & Peay (2014)

All references

Abarenkov, K., Henrik Nilsson, R., Larsson, K.-H., Alexander, I. J., Eberhardt, U., Erland, S., … Kõljalg, U. (2010). The UNITE database for molecular identification of fungi - recent updates and future perspectives. *New Phytologist*, *186*(2), 281-285.

Adl, S. M., Habura, A., & Eglit, Y. (2014). Amplification primers of SSU rDNA for soil protists. *Soil*

Biology and Biochemistry, *69*, 328-342.

Allison, S. D., & Martiny, J. B. (2008). Resistance, resilience, and redundancy in microbial communities. P*roceedings of the National Academy of Sciences USA, 105*(Supplement 1), 11512-11519.

Bartram, A. K., Lynch, M. D. J., Stearns, J. C., Moreno-Hagelsieb, G., & Neufeld, J. D. (2011). Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Applied and Environmental Microbiology*, *77*(11), 3846-3852.

Balser, T. C., & Firestone, M. K. (2005). Linking microbial community composition and soil processes in a California annual grassland and mixed-conifer forest. *Biogeochemistry, 73*(2), 395-415.

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M., & Kauserud, H. (2013). ITS1 versus ITS2 as DNA metabarcodes for fungi. *Molecular Ecology Resources*, *13*(2), 218-224.

Brewer, S., Techtmann, S. M., Mahmoudi, N., Niang, D., Pfiffner, S., & Hazen, T. C. (2015). Co-extraction of DNA and PLFA from soil samples. *Journal of Microbiological Methods, 115*, 64-66.

Callahan, B. J., McMurdie, P. J., & Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME journal, 11*(12), 2639.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335-336.

Castro, H. F., Classen, A. T., Austin, E. E., Norby, R. J., & Schadt, C. W. (2010). Soil microbial community responses to multiple experimental climate change drivers. *Applied and Environmental Microbiology, 76*(4), 999-1007.

Clemmensen, K. E., Bahr, A., Ovaskainen, O., Dahlberg, A., Ekblad, A., Wallander, H., … Lindahl, B. D. (2013). Roots and associated fungi drive long-term carbon sequestration in boreal forest. *Science*, *339*(6127), 1615-1618.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., … Tiedje, J. M. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, *42*(D1), D633-D642.

Davidson, E. A., & Janssens, I. A. (2006). Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. *Nature*, *440*(7081), 165-173.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069-5072.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, *10*(10), a2604.

Falkowski, P. G., Fenchel, T., & Delong, E. F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science, 320*(5879), 1034-1039.

Frostegård, Å., Tunlid, A., & Bååth, E. (1993). Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different

heavy metals. *Applied and Environmental Microbiology, 59*(11), 3605-3617.

Frostegård, Å., Tunlid, A., & Bååth, E. (2011). Use and misuse of PLFA measurements in soils. *Soil Biology and Biochemistry, 43*(8), 1621-1625.

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., … Christen, R. (2013). The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. *Nucleic Acids Research*, *41*(Database issue), D597-604.

Hale, V. L., Tan, C. L., Knight, R., & Amato, K. R. (2015). Effect of preservation method on spider monkey (*Ateles geoffroyi*) fecal microbiota over 8 weeks. *Journal of Microbiological Methods*, *113*, 16-26.

Hayden, H. L., Mele, P. M., Bougoure, D. S., Allan, C. Y., Norng, S., Piceno, Y. M., … Hovenden, M. J. (2012). Changes in the microbial community structure of bacteria, archaea and fungi in response to elevated CO_2 and warming in an Australian native grassland soil. *Environmental Microbiology*, *14*(12), 3081-3096.

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., & Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME Journal*, *5*(10), 1571-1579.

Ihrmark, K., Bödeker, I. T. M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., … Lindahl, B. D. (2012). New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology*, *82*(3), 666-677.

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology, 45*(9), 2761-2764.

Kauserud, H., Kumar, S., Brysting, A. K., Nordén, J., & Carlsen, T. (2012). High consistency between replicate 454 pyrosequencing analyses of ectomycorrhizal plant root samples. *Mycorrhiza*, *22*(4), 309-315.

Kennedy, P. G., Cline, L. C., & Song, Z. (2018). Probing promise versus performance in longer read fungal metabarcoding. *New Phytologist, 217*(3), 973-976.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glockner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), e1.

Krüger, M., Stockinger, H., Krüger, C., & Schüßler, A. (2009). DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. *New Phytologist, 183*(1), 212-223.

Kuffner, M., Hai, B., Rattei, T., Melodelima, C., Schloter, M., Zechmeister-Boltenstern, S., … Sessitsch, A. (2012). Effects of season and experimental warming on the bacterial community in a temperate mountain forest soil assessed by 16S rRNA gene pyrosequencing. *FEMS Microbiology Ecology*, *82*(3), 551-562.

Lee, J., Lee, S., & Young, J. P. W. (2008). Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. *FEMS Microbiology Ecology*, *65*(2), 339-349.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., … Kauserud, H.

(2013). Fungal community analysis by high-throughput sequencing of amplified markers - a user's guide. *New Phytologist*, *199*(1), 288-299.

Mahé, F., de Vargas, C., Bass, D., Czech, L., Stamatakis, A., Lara, E., … Dunthorn, M. (2017). Parasites dominate hyperdiverse soil protist communities in Neotropical rainforests. *Nature Ecology & Evolution*, *1*, 91.

Nemergut, D. R., Shade, A., & Violle, C. (2014). When, where and how does microbial community composition matter? *Frontiers in Microbiology, 5*, 497.

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., … Kennedy, P. G. (2016). FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology*, *20*, 241-248.

Op De Beeck, M., Lievens, B., Busschaert, P., Declerck, S., Vangronsveld, J., & Colpaert, J. V. (2014). Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. *PLoS One*, *9*(6), e97629.

Öpik, M., Davison, J., Moora, M., & Zobel, M. (2014). DNA-based detection and identification of Glomeromycota : the virtual taxonomy of environmental sequences. *Botany*, *92*(2), 135-147.

Orwin, K. H., Dickie, I. A., Holdaway, R., & Wood, J. R. (2018). A comparison of the ability of PLFA and 16S rRNA gene metabarcoding to resolve soil community change and predict ecosystem functions. *Soil Biology and Biochemistry, 117*, 27-35.

Pold, G., & DeAngelis, K. M. (2013). Up against the wall: the effects of climate warming on soil microbial diversity and the potential for feedbacks to the carbon cycle. *Diversity, 5*(2), 409-425.

Prosser, J. I., Bohannan, B. J., Curtis, T. P., Ellis, R. J., Firestone, M. K., Freckleton, R. P., ... Osborn, A. M. (2007). The role of ecological theory in microbial ecology. *Nature Reviews Microbiology, 5*(5), 384.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590-D596.

Radujković, D., Verbruggen, E., Sigurdsson, B. D., Leblans, N. I., Janssens, I. A., Vicca, S., & Weedon, J. T. (2018). Prolonged exposure does not increase soil microbial community compositional response to warming along geothermal gradients. *FEMS Microbiology Ecology*, *94*(2), fix124.

Rinnan, R., Michelsen, A., Bååth, E., & Jonasson, S. (2007). Fifteen years of climate change manipulations alter soil microbial communities in a subarctic heath ecosystem. *Global Change Biology*, *13*(1), 28-39.

Robe, P., Nalin, R., Capellano, C., Vogel, T. M., & Simonet, P. (2003). Extraction of DNA from soil. *European Journal of Soil Biology*, *39*(4), 183-190.

Rochelle, P. A., Cragg, B. A., Fry, J. C., John Parkes, R., & Weightman, A. J. (1994). Effect of sample handling on estimation of bacterial diversity in marine sediments by 16S rRNA gene sequence analysis. *FEMS Microbiology Ecology*, *15*(1-2), 215-225.

Roux, K. H. (2009). Optimization and troubleshooting in PCR. *Cold Spring Harbor Protocols*, *2009*(4), pdb.ip66.

Santamaria, M., Fosso, B., Consiglio, A., De Caro, G., Grillo, G., Licciulli, F., … Pesole, G. (2012). Reference databases for taxonomic assignment in metagenomics. *Briefings in Bioinformatics*, *13*(6), 682-695.

Schlaeppi, K., Bender, S. F., Mascher, F., Russo, G., Patrignani, A., Camenzind, T., … van der Heijden, M. G. A. (2016). High-resolution community profiling of arbuscular mycorrhizal fungi. *New Phytologist*, *212*(3), 780-791.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., … Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537-7541.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., … Schindel, D. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences USA*, *109*(16), 6241-6246.

Shokralla, S., Spall, J. L., Gibson, J. F., & Hajibabaei, M. (2012). Next-generation sequencing technologies for environmental DNA research. *Molecular Ecology*, *21*(8), 1794-1805.

Sigurdsson, B. D., Leblans, N. I., Dauwe, S., Gudmundsdottir, E., Gundersen, P., Gunnarsdottir, G. E., ... Maljanen, M. (2016). Geothermal ecosystems as natural climate change experiments: The ForHot research site in Iceland as a case study. *Icelandic Agricultural Sciences, 29*, 53-71.

Smith, D. P., & Peay, K. G. (2014). Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. *PLoS ONE*, *9*(2), 090234.

Smith, S. E., & Read, D. J. (2008). *Mycorrhizal Symbiosis*. Academic Press.

Song, S. J., Amir, A., Metcalf, J. L., Amato, K. R., Xu, Z. Z., Humphrey, G., & Knight, R. (2016). Preservation methods differ in fecal microbiome stability, affecting suitability for field studies. *mSystems*, *1*(3) e00021-16.

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M. D. M., Breiner, H. W., & Richards, T. A. (2010). Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Molecular Ecology*, *19*(suppl. 1), 21-31.

Strickland, M. S., Lauber, C., Fierer, N., & Bradford, M. A. (2009). Testing the functional significance of microbial community composition. *Ecology*, *90*(2), 441-451.

Tedersoo, L., Nilsson, R. H., Abarenkov, K., Jairus, T., Sadam, A., Saar, I., … Kõljalg, U. (2010). 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. *New Phytologist*, *188*(1), 291-301.

Tedersoo, L., Bahram, M., Polme, S., Koljalg, U., Yorou, N. S., Wijesundera, R., … Abarenkov, K. (2014). Global diversity and geography of soil fungi. *Science*, *346*(6213), 1256688.

Tedersoo, L., Bahram, M., Cajthaml, T., Põlme, S., Hiiesalu, I., Anslan, S., … Abarenkov, K. (2015). Tree diversity and species identity effects on soil fungi, protists and animals are context dependent. *The ISME Journal*, 10, 346-362.

van der Heijden, M. G., Bardgett, R. D., & van Straalen, N. M. (2008). The unseen majority: Soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters*, *11*(3), 269-310.

Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., ... Hyde, E. R. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome, 5*(1), 27.

Wilson, H., Johnson, B. R., Bohannan, B., Pfeifer-Meister, L., Mueller, R., & Bridgham, S. D. (2016). Experimental warming decreases arbuscular mycorrhizal fungal colonization in prairie plants along a Mediterranean climate gradient. *PeerJ*, *4*, e2083.

Winsley, T., van Dorst, J. M., Brown, M. V., & Ferrari, B. C. (2012). Capturing greater 16S rRNA gene sequence diversity within the domain Bacteria. *Applied and Environmental Microbiology, 78*(16), 5938-5941.

Yang, G., Yang, X., Zhang, W., Wei, Y., Ge, G., Lu, W., … Zhang, Y. (2016). Arbuscular mycorrhizal fungi affect plant community structure under various nutrient conditions and stabilize the community productivity. *Oikos*, *125*(4), 576-585.

Zhang, Y., Dong, S., Gao, Q., Liu, S., Zhou, H., Ganjurjav, H., & Wang, X. (2016). Climate change and human activities altered the diversity and composition of soil microbial community in alpine grasslands of the Qinghai-Tibetan Plateau. *Science of the Total Environment*, *562*, 353-363.

Zogg, G. P., Zak, D. R., Ringelberg, D. B., White, D. C., MacDonald, N. W., & Pregitzer, K. S. (1997). Compositional and functional shifts in microbial communities due to soil warming. *Soil Science Society of America Journal*, *61*(2), 475-481.

Authors: Radujković D[1], Verbruggen E[1]

Reviewer: Vik U[2]

Affiliations

[1] Centre of Excellence PLECO (Plants and Ecosystems), Biology Department, University of Antwerp, Wilrijk, Belgium

[2] Section for Genetics and Evolutionary Biology (EVOGENE), Department of Biosciences, University of Oslo, Oslo, Norway

4.10 Soil micro- and mesofauna community composition

Authors: Konestabo HS[1] & Kardol P[2]

Reviewer: Birkemoe T[3]

Measurement unit: abundance per area or gram soil; Measurement scale: plot; Equipment costs: €; Running costs: €; Installation effort: low to high; Maintenance effort: low to medium; Knowledge need: medium to high (taxonomic knowledge); Measurement mode: manual

Soil micro- and mesofauna are small invertebrates of ~0.1–2 mm in length, living in soil or the litter layer. For microfauna, we focus on soil nematodes (also called roundworms), which are among the most numerous soil organisms in most terrestrial ecosystems with densities up to 3–4 million m^{-2} (Bardgett et al., 1999; Van den Hoogen et al., 2019). Soil nematodes also display high taxonomic and functional diversity. Based on the morphology of their mouth parts, soil nematodes can be attributed to one of the following feeding groups (Yeates et al., 1993; Fig. 4.10.1): 1) endo-parasitic plant-feeders (feeding from within the plant), 2) ecto-parasitic plant feeders (feeding on plant tissue from outside the plant), 3) bacterial feeders, 4) fungal feeders, 5) omnivores, and 6) carnivores. Endo-parasitic plant feeders enter the plant root and feed on deep cell layers, while ecto-parasitic nematodes feed from outside the plant on outer cortical cells and root hairs. Omnivorous nematodes feed on bacteria, amoebae, flagellates, and bacterial-feeding, fungal-feeding, and plant-feeding nematodes (Yeates et al., 1993), while carnivorous nematodes feed on other groups of nematodes and on other organisms such as enchytraeids (Yeates et al., 1993). Entomopathogenic nematodes are not considered in this protocol. Springtails (Collembola), mites (Acari), and enchytraeids or potworms (Enchytraeidae) are generally considered as mesofauna, while diplurans (Diplura), proturans (Protura), and jumping bristletails (Archaeognatha) are sometimes also included in this group. The mesofauna are a functionally diverse group, dominated by decomposers or detritivores, but also include microbivores, herbivores, fungivores, and predators. Mesofauna decomposers may feed directly on decaying organic matter, or on bacteria and fungi associated with detritus (Hopkin, 1997). Microbivorous mesofauna can be generalists, feeding on different kinds of bacteria and fungi, or have strong feeding preferences (Verhoef et al., 1988; Chen et al., 1995). Predatory mesofauna can be found within the Diplura, mites of the order Mesostigmata and the sub-order Prostigmata, and there are also some predatory springtails (e.g. *Friesea* sp.), feeding on other micro- and mesofauna. Mesofauna feeding activities may exert strong top-down control on decomposition processes in the soil through cascade effects on the activity of microorganisms, and may influence primary production and plant N accumulation (Santos et al., 1981; Setälä et al., 1998; Hedlund & Sjögren-Öhrn, 2000; Cortet et al., 2003). Species richness and functional diversity as well as abundance and biomass of mesofauna greatly influence soil decomposition processes (Setälä et al., 1991; Mebes & Filser, 1998; Cortet et al., 2003).

Together with macroinvertebrates and earthworms, micro- and mesofauna are important in driving litter decomposition, nutrient turnover, and plant productivity, and hence, how ecosystems respond to natural and anthropogenic environmental changes. Micro- and mesofauna are also important bio-indicators for effects of climate change on the functioning of terrestrial ecosystems as these organisms are sensitive to shifts in the soil environment (e.g. Kardol et al., 2010). Although the direct effect of small temperature changes (1–3 °C) on soil fauna may be small (Sjursen et al., 2005; Alatalo et al., 2015, 2017; De Long et al., 2016), changes in water availability, increased number of freeze-thaw cycles, changing plant cover and species composition, and changes in nutrient availability are

expected to have larger impacts (Konestabo et al., 2007; Kardol et al., 2010; Elmendorf et al., 2012; Krab et al., 2015). Land-use change, nitrogen deposition, habitat disturbances, and plant invasions have all been shown to affect micro- and mesofauna abundance either directly or indirectly through effects on plant cover and soil biochemistry (Bardgett & Cook, 1998; Bedano et al., 2006; Hågvar & Klanderud, 2009; Leinaas et al., 2015). Because of the close relationships between micro- and mesofauna and other below- and aboveground communities, it is recommended to include these functionally diverse groups in studies of climate- and global-change effects on ecosystem functioning. Classifying micro- and mesofauna into functional groups is of particular interest as it informs on how climate change affects belowground trophic relationships and the flow of energy in soil food webs.

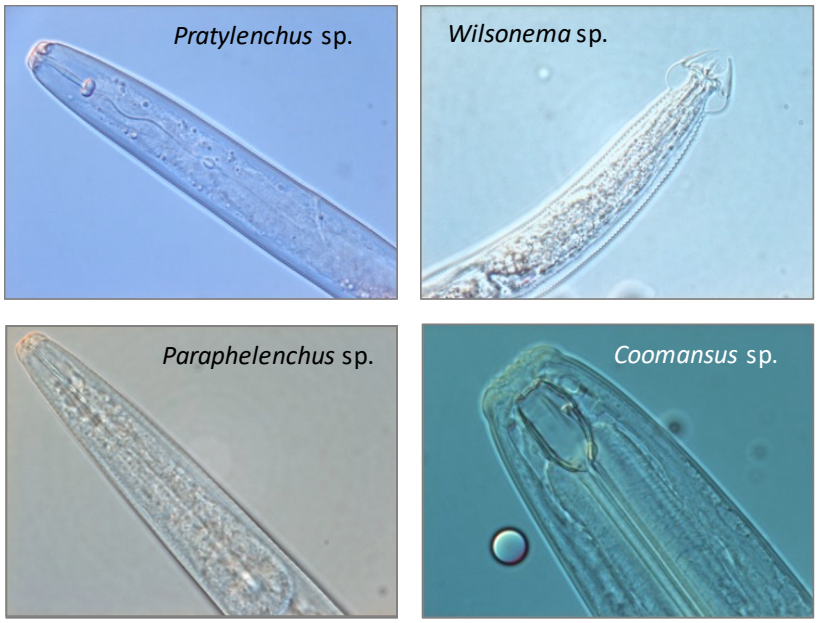


Figure 4.10.1 Examples of plant-feeding (upper left), bacterial-feeding (upper right), fungal-feeding (bottom left) and carnivorous (bottom right) nematodes.

Here, we describe the recommended sampling methods for 1) extraction of nematodes from soils and from plant roots, and 2) extraction of soil and litter mesofauna.

4.10.1 What and how to measure?

Micro- and mesofauna are quantified by counting the number of individuals per area or per gram of soil and/or litter. Endoparasitic nematodes can also be quantified per gram of root tissue. Standardised sampling and extraction methods for soil invertebrates, including more details on some of the most common methods described here, can be found in ISO Standards 23611: 1-4 (ISO, 2006; 2007a, 2007b; 2018). Micro- and mesofauna are relatively stationary organisms compared to macroinvertebrates and insects (Ojala & Huhta, 2001 and references therein), and usually complete their life-cycle within the same habitat. However, the different taxonomic groups of micro- and mesofauna vary in size, activity level, and how they burrow into the soil matrix. Thus, there is not one single extraction method that will cover all organisms. Each method's efficiency depends on a number of factors including soil properties, vegetation cover, the animal's behavioural responses, their association with water or air pockets, and their tolerance to drought and heat.

Some soil organisms have inactive stages that will not be captured by active extraction methods (see below). Seasonal variation in abundance and vertical distribution are common and should be taken into account when comparing between years. The vertical distribution might also differ between life stages. Generally, large variation in abundances over a small spatial scale should be expected when comparing sites within or between growing seasons. To fully capture soil micro- and mesofauna responses to climatic changes, samples should be taken at different times across the years. Seasonal dynamics, and hence, the required number of samplings, depend on the climatic zone and on

vegetation type (for example, evergreen vs. deciduous forests). But, generally 3–4 samplings would capture most of the seasonal variation. When resources only allow one sampling per year, soils are best collected at peak standing biomass, or towards the end of the growing season.

Soil core sampling

The most common sample unit for measuring micro- and mesofauna are soil cores.

For nematodes, small-diameter soil cores (1–5 cm diameter) are collected from the plot, typically using a tubular soil probe (Fig. 4.10.2). In order to get a representative sample of the plot, it is recommended to collect 5–20 small cores (subsamples) and bulk them into one composite sample per plot. Collecting several small samples is also less destructive to the plot than one large sample. The total size of the composite soil sample should be about 100–200 ml. The subsamples can be collected haphazardly, or according to a fixed sampling grid. Here, it is important to keep in mind the spatial configuration and heterogeneity of the plant community. Notably, soil samples collected from beneath individual plant species give different results compared to soil samples randomly collected across the plot (Kardol et al., 2010). Samples are often collected from the upper 10 cm of the soil profile (the litter layer is generally not included). However, depending on the research question and the type of ecosystem, samples can also be collected from greater soil depth (e.g. 0–10 cm, 10–20 cm, 20–30 cm). Alternatively, samples can be collected according to soil horizons or soil layers (e.g. O-horizon/humus layer, A- horizon/top soil, B-horizon/sub soil).

Figure 4.10.2 Metal soil corer used for mesofauna samples (left; photo: Heidi S. Konestabo) and a tubular soil probe commonly used to collect soil samples for nematode extraction (right; photo: Paul Kardol).

For mesofauna, *intact* soil cores including the litter layer are extracted from the habitat, using a metal soil corer (Fig. 4.10.2). Where the plant cover is low (typically mosses, lichens), the plant cover can be included in the sampled soil core. In habitats with a plant cover reaching above 2–3 cm in height, the plant cover can be removed by cutting it just above the litter layer, or gently bending it to the sides, exposing the soil and litter layer below. The soil cores should be large enough to include a representative estimate of the mesofauna without destroying the plots. Usually, a sample diameter of 4–10 cm is chosen depending on the heterogeneity of the habitat, plot size, and the extraction facilities available. Cores should be taken from a representative section of the habitat. A large core size demand less replicates; 3–12 replicates are commonly used. The depth of the cores depends on the habitat, but as mesofauna are most often found in the uppermost layers of the soil and in the litter layer, a depth of ~5 cm is often sufficient. It can, however, be useful to perform a pilot

investigation of the patchiness and the soil profile before deciding on the sample size, depth, and replication.

Nematode extraction methods

Upon collection, soil and/or root samples for nematode extraction should be stored in plastic bags or containers, not be exposed to high temperatures during the sampling campaing (a simple cooler does the job), and be refrigerated (± 4°C) at the earliest opportunity. Samples should be extracted as soon as possible after collection although storage for 1–2 weeks is generally not a problem. Prior to extraction, bulk soil samples can be carefully passed through a 1-cm sieve and mixed well for homogeneity. Mixing should be done gently to avoid damage to the nematodes.

A variety of different methods have been developed for the extraction of free-living and plant-parasitic nematodes. The most commonly used methods can be classified based on the motility, the specific density, and the size of nematodes (EPPO, 2013). None of the methods allows extraction of all stages of all nematode taxa at 100% efficiency, but for analysis of nematode abundance and community composition in climate-change studies this is not a major problem. It is important to realise though that the extraction efficiency depends on the soil type; extracting nematodes from sandy soils is easier than from clay or organic soils (or humus). The final choice of the extraction method depends on the availability of facilities, costs of equipment, water use, and labour. Van Bezooijen (2006) and EPPO (2013) provide detailed comparisons of the costs and benefits of different nematode extraction methods.

Baermann funnels: One of the most commonly used methods for extraction of nematodes from soils is the use of Baermann funnels. This simple method makes use of the active movement of nematodes. Soil samples are wrapped in filter or tissue paper, or cheese cloth, and then placed into funnels filled with water and equipped with a piece of tubing at the stem, closed with a clamp. Instead of wrapping the sample in paper or cloth, the sample can also be spread out on a small sieve inserted into the funnel; the larger extraction surface increases the extraction efficiency. To further increase the extraction surface, the Baermann funnel method can be modified by using larger dishes (see, 'Oostenbrink dish' below) or trays where the soil sample can be spread in a thin layer. The nematodes will crawl out of the wet soils and then passively sink to the bottom of the funnel stem where they can be collected. The duration of the extraction depends on the soil type, the sample size, and the extraction surface, but typically 24–72 hours is sufficient. It is critical that the soil samples never dry out. Advantages of Baermann funnels (and derived tray methods) are their simplicity and low costs. Disadvantages are their low extraction efficiency for large samples and for immotile nematodes.

Oostenbrink elutriators: Another commonly used method for the extraction of free-living soil nematodes from soil, especially in larger nematode labs, is the use of Oostenbrink elutriators (Oostenbrink, 1960), particularly for larger soil samples. The method typically consists of two phases. The first phase makes use of differences in size, shape, and sedimentation rate between nematodes and soil particles, while the second phase makes use of nematode mobility (just like Baermann funnels). In the Oostenbrink elutriator (Fig. 4.10.3), an upward water stream makes the nematodes and fine soil particles float in the upper extraction column, whereas heavier soil particles settle in the lower part. The suspension in the extraction column is then let out and poured onto a set of four 45 µm sieves. In the second phase, the debris washed from the sieves can be further cleaned by the use

of 'Oostenbrink dishes'. These are plastic or stainless steel dishes with a cross piece and an extraction sieve equipped with milk filters (see EPPO, 2013 for an example). The dishes are generally incubated for ~48 hours at room temperature during which the nematodes move through the filters into the water in the dish. During incubation the filters must be kept moist at all times. Verschoor & De Goede (2000) give more information on the overall extraction efficacy and parameters influencing the extraction efficacy. Advantages of Oostenbrink elutriators are the generally high efficiency and standardisation of extractions. Major disadvantages are the high costs of the custom-made equipment, and the physical space needed for the permanent installation of the elutriators. This method is therefore only recommended for labs where nematodes are extracted on a more or less routine basis.

To note, methods based on nematode motility do not capture slow-moving and inactive nematodes, or eggs. The number of nematodes moving out of a soil sample further depends on extraction duration and sample type. The efficiency usually increases when the debris layer is thin. The water temperature also influences nematode motility in the soil sample and therefore the numbers of nematodes in the final suspension.

Figure 4.10.3 Oostenbrink elutriators at the Netherlands Institute of Ecology (NIOO-KNAW in Wageningen, the Netherlands. Photo: Freddy ten Hooven.

Centrifugal flotation: Free-living soil nematodes can be separated from soils by centrifugation because of the difference in specific gravity between nematodes and soil particles. Soil samples are suspended in a highly-concentrated sugar solution: based on specific gravity the nematodes float, while the soil particles sink. This so-called centrifugal flotation method can be used for all types of soil, but is more efficient for loose, sandy soils than for aggregated clay or loam soils. To handle larger samples and obtain higher extraction efficiency, pre-extraction of the sample is recommended to get a more concentrated soil suspension prior to centrifugation. Pre-extraction can be done with Oostenbrink elutriators (see above), for example, or with a simple sieving procedure (top: 180 µm; bottom: 38 µm or 32 µm). The actual separation method then consists of two centrifugation cycles. In the first cycle, the concentrated soil suspension is centrifuged so all particles with a specific gravity >1 precipitate; this includes the nematodes. The supernatant is then discarded. In the second cycle, concentrated sugar solution is added to the sediment from the first phase, and the sediment is

brought into suspension. After centrifugation, the nematodes float in the supernatant, whereas the soil particles are precipitated to the bottom of the centrifugation tube. The supernatant is passed through a 25 μm sieve, after which the nematodes can be rinsed into a vial or tube. An advantage of the centrifugation method is that not only active nematodes but also slow-moving and inactive nematodes are extracted. More detailed directions for the centrifugal flotation method can be found in Jenkins (1964) and Van Bezooijen (2006). Another advantage of the centrifugal flotation method is that no special equipment is needed. The main piece of equipment is a standard table top centrifuge which can be found in most ecology and microbiology labs. Other than that, one needs a set of (portable) sieves and some standard labware and disposables. This means that this method can easily be applied at most universities and research institutes.

Extraction of nematodes from plant roots: Several methods have been developed for extraction of nematodes from roots (and other plant material), mostly in agricultural sciences. For analysis of nematodes from plant roots in terrestrial climate-change studies, Baermann funnels (see above) or a funnel spray apparatus (also known as 'mist chamber' or 'mistifier') are generally most suitable. Making use of nematode mobility and sedimentation rate, both these methods extract most active nematodes from roots. When root samples are moistened in water, nematodes crawl out and sink. The funnel spray apparatus consists of Baermann funnels equipped with small sieves, under a spray nozzle (Fig. 4.10.4). The washed roots are then placed in sieves with little legs which prevent the sieves from touching the water surface in the funnels. The nematodes will fall into the funnel where they settle. Nematodes should be collected from the funnels every day or two. The total duration of extraction depends on the type of root material and the nematode taxa, but typically 2–7 days is sufficient. Here, it is important to mention that part of the nematode population within plant roots is in the form of eggs which may hatch over time. If those numbers are of interest to the research question, samples need to be incubated for much longer, up to several weeks, allowing enough time for the eggs to hatch. Compared to normal Baermann funnels (as described above for soil samples), the funnel spray method yields nematodes in better condition, and the extraction efficiency is also higher. Cutting the roots into small pieces further enhances the extraction efficiency (Van Bezooijen, 2006). A disadvantage is that the funnel spray matter uses high amounts of water.

Figure 4.10.4 Mist chambers at the University of Tennessee, Knoxville, USA. Photos: Paul Kardol.

Fixation, preservation, and identification: Upon extraction from soils and/or plant roots, nematodes are collected as a clear suspension in water. For concentrating nematode suspensions for counting or fixation, the natural nematode sedimentation rate is used. The amount of fluid can be reduced by

sieving the suspension, or by pipetting the liquid after the nematodes have settled. Nematodes can be counted and identified alive, or nematodes can be fixed and preserved and counted and identified at a later stage. Live nematodes can be kept alive and in good condition for several days when refrigerated at ± 4°C. Nematode identification, however, is easier done when the nematodes are dead and fixed. Usually the best results are obtained by killing the nematodes rapidly through heating (up to 65–90°C) followed by immediate fixing. With a hot fixative, these two steps can be combined. Cooling must happen fast, because an extended heating period causes deformations (Van Bezooijen, 2006). Formalin (4%) is most commonly used as a fixative. Note, that formalin is harmful to human health and therefore fixation work must be carried out in a fume hood. After fixation, nematodes can be transferred to other preservatives. Nematodes can be counted and/or identified to broad taxonomic groups (i.e. family level) in suspension using an inverted microscope. Alternatively, for higher taxonomic resolution (i.e. genus- or species-level), nematodes should be mounted on slides (see Van Bezooijen, 2006 for detailed instructions), and identified using a compound microscope. For ecological studies, typically all nematodes in the sample are counted after which a random subset (± 150–200 individuals) of the nematodes is identified. For inference on "function", nematodes are then often allocated to feeding groups (see above).

Where to start

EPPO (2013), Van Bezooijen (2006)

Mesofauna extraction methods

The mesofauna should be extracted from the soil and litter immediately after sampling, but storage for a short time (1–2 weeks) at 4–5 °C is common when there are practical limitations to extraction capacity. This is unlikely to affect the overall extraction results. Extraction of mesofauna from soil cores can be done either by active or passive extraction. Active extraction relies on mesofauna migration towards a collection device in response to a stimulus, for example temperature or water availability, and is by far the most common extraction method used (Andre et al., 2002). Passive or mechanical extraction methods are more commonly used when sampling larger soil organisms, and rely on the physical separation of the fauna from the soil by hand-sorting, flotation, or chemical extraction.

For a critical review of different soil core extraction methods, including comparisons of efficiency and consequences for biodiversity estimates, see Andre et al. (2002).

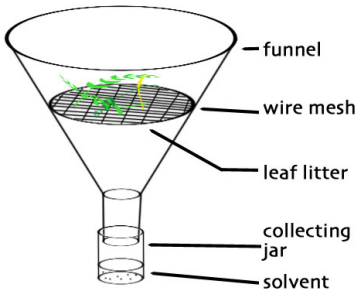


Figure 4.10.5 Schematic drawing of a Tullgren funnel. Re-used with permission from Dr. Grant Brown, University of St. Andrews. http://biology.st-andrews.ac.uk/gardenlife/sampling.html

Active mesofauna extraction methods

Heat and light extraction: Extracting mesofauna from soil cores by heat and light is the most common method for assessing springtails, mites, diplurans, and proturans. Heat extraction will also frequently extract earthworms, enchytraeids, bristletails, insect larva, and small adult insects and spiders, although other sampling methods are more suitable for these organisms (see protocol 4.11. Macroinvertebrate community composition). Heat and light extraction is often referred to as Berlese or Tullgren extraction, as the method was first developed and described by Berlese (1905) and Tullgren (1917). Later modifications to the extraction method have been described by MacFadyen (1961), Petersen (1978), and Andrén (1985). The principle of the method is to slowly heat and dry the soil sample from the top, while placing a collection beaker below, creating a temperature and moisture gradient which will induce movement of the mesofauna towards the collection beaker (Fig. 4.10.5). The set-up most commonly used today consists of a temperature-controlled top chamber, where heating elements (e.g. light bulbs) are used to create a set heating temperature, and a cooling chamber at the bottom (Fig. 4.10.6). Extraction temperature usually starts at room temperature and should be increased up to 60 °C over the course of about a week. Samples should be completely dry when extraction ends. Soil cores are placed inside the extraction chamber upside down, with the litter layer facing downwards, on top of a mesh to prevent soil particles from falling into the collection beaker below.

Figure 4.10.6 Automated heat and light extractor, with a temperature controlled top (heating) and bottom (cooling) chamber. Soil cores are placed on top of a mesh, and a collection beaker with preservative is placed below, cooled by the bottom chamber. Photo: Heidi S. Konestabo.

The collection beaker should contain a liquid for killing and preserving the animals, for example 70% ethanol, benzoic acid, propylene glycol, or ethylene glycol. The extraction method can also be used for collecting live specimens: the collection beaker should then be layered with a substrate to keep the extracted animals moist, such as moist plaster of Paris.

Moulting microarthropods as well as larval or nymphal stages of many prostigmatid mites are inactive and will not be extracted by heat. The mesh, on which the soil samples rest during extraction, might also discriminate against larger sized animals if the mesh size is small. The extraction apparatus also needs electricity for heating, which can be limiting in some places. An alternative using only drought has been described by Owen (1987). Mesh bags are filled with a soil/litter sample and placed inside an outer cloth bag. When the sample is left to dry out, the mesofauna will actively move out of the mesh bag and fall into a collection beaker in the bottom of the outer bag.

Wet extraction: O'Connor wet funnel extraction (O'Connor, 1955) is primarily used to extract enchytraeids from the soil. Here, the same principle of using a heating source to create a temperature gradient in the sample as for the Berlese-Tullgren method is used. A mesh or sieve is placed inside a water-filled funnel or a bowl, with room for the collection of animals below. The funnel or bowl is filled with tap water covering the mesh, and the soil sample is placed on the mesh

so that it is completely soaked. Increasing the temperature gradually for 8–24 hours, the enchytraeids move away from the heat and towards the bottom of the funnel. By opening the funnel from below, or removing the mesh with the soil sample, the water with the live enchytraeids can be collected. The extraction method can also be used without heating if the extraction time is extended to several days. The enchytraeids should be collected daily if this method is used. Enchytraeids are best identified alive, preferably within 2 days after extraction. The water samples should be kept at 5–10 °C, and the water should be changed daily to avoid deoxygenation.

Passive mesofauna extraction methods

Hand sorting: Hand sorting of field soil samples may potentially recover all mesofauna, including non-mobile forms such as eggs and dormant or immobile stages. However, the method is laborious and time-consuming, and it is difficult to detect and collect the smaller microarthropods efficiently. It works best with loosely structured soil types without roots or clay aggregates. For a comparison of hand-sorting techniques and time restraints, see Schmidt (2001).

Flotation: Extraction by flotation relies on the hydrophobic properties of the cuticle of the organisms, or their specific gravity. The soil is suspended in water, and animals are collected from the water surface. This method can be useful when sampling loosely structured soil with few roots and clay particles, and with low organic content. Stirring or centrifuging the soil can increase the method's efficiency. The method extracts inactive as well as active stages; however, only species with a hydrophobic cuticle will float on the water's surface. Using a more dense solution such as brine or magnesium sulfate can extract all soil organisms with a specific gravity lower than the solution. The animals are then collected at the bottom of the fluid, and the samples will often have to be sieved or hand sorted after extraction by flotation. A detailed description of flotation devices and solutions is found in Edwards (1991).

Pitfall traps, sticky traps, and field sampling by hand

Larger, surface-dwelling mesofauna, especially large springtail and mite species, as well as bristletails, can be collected using similar methods as described for macroinvertebrate sampling (see protocol 4.11. Macroinvertebrate community composition). Pitfall traps or sticky traps will catch organisms moving actively around on the soil or litter surface. Collection of animals directly from the soil or litter surface can be efficient, but this method is less suitable for assessment of soil communities as there may be an overrepresentation of migrating or aggregating individuals. A brush, soft tweezers, or a pipette tip aspirator can be used to collect individuals by hand (Davidson & Broady, 1996; Sinclair & Sjursen, 2001).

Morphological identification

Identification of mesofauna is the most time-consuming part of the procedure and good taxonomic knowledge is needed. Species or family identification is usually done under a compound microscope, while coarse sorting and identification to functional groups can be carried out using a stereo microscope. Several identification keys to orders, families, and species exist: for springtails (Gisin,

1960; Fjellberg, 1998, 2007), mites (Balogh & Balogh, 1992; Krantz & Walter, 2009), and enchytraeids (Nielsen & Christensen, 1959; Schmelz & Collado, 2010).

Where to start

Andre et al. (2002), Edwards (1991), O'Connor (1955), Petersen (1978)

4.10.2 Special cases, emerging issues, and challenges

Genetic sequencing

Genetic sequencing or molecular barcoding techniques to identify soil microbial communities have been widely applied, but such techniques for characterising nematodes and soil mesofauna are still relatively new, and only recently have seen an increase in application. For nematodes, taxa abundances can be determined using quantitative polymerase chain reaction (qPCR) techniques. For analyses of the taxonomic and functional composition of soil nematode communities, molecular diversity analyses can be used, such as DGGE, T-RFLP, and high-throughput sequencing (HTS). qPCR may reveal lower abundances than traditional morphological approaches. On the other hand, HTS produces higher taxonomic resolution, while relative HTS and relative morphological nematode data show very strong correlations (Geisen et al., 2018). Therefore, a combination of molecular and morphological approaches would provide the most detailed characterisation of soil nematode community responses to climate change (Geisen et al., 2018). Interestingly, Geisen et al. (2018) also indicate that while material costs are higher for molecular methods compared to morphological methods, the reduction in labour costs easily offsets the extra costs when sample sizes increase. This would particularly be the case in high-income countries. For more details on the progress and application of molecular nematode analyses, and comparisons of traditional morphological approaches and new molecular approaches, we refer to Sapkota & Nicolaisen (2015), Geisen et al. (2018), and Wilschut et al. (2019). For mesofauna, Oliverio et al. (2018) compare traditional morphological identifications of heat-extracted soil arthropod families with DNA meta-barcoded heat-extracted arthropods and DNA meta-barcoded bulk soil. The results are strongly correlated. However, discrepancies due to the lack of arthropod species in the reference databases and due to the presence of DNA from fragments of dead specimens in the bulk soil were found, as well as specific primer biases.

4.10.3 References

Theory, significance, and large datasets

Andre et al. (2002), Bardgett (2002), Coleman & Whitman (2005), Coleman et al. (2018), Edwards (1991), Ferris et al. (2001)

More on methods and existing protocols

Andre et al. (2002), Edwards (1991), Geisen et al. (2018), ISO (2006, 2007a, 2007b, 2018), O'Connor (1955), Oliverio et al. (2018), Petersen (1978), Sapkota & Nicolaisen (2015), Yeates et al. (1993)

All references

Alatalo, J. M., Jagerbrand, A. K., & Cuchta, P. (2015). Collembola at three alpine subarctic sites resistant to twenty years of experimental warming. *Scientific Reports, 5*, 10.1038/srep18161.

Alatalo, J. M., Jaegerbrand, A. K., Juhanson, J., Michelsen, A., & Luptacik, P. (2017). Impacts of twenty years of experimental warming on soil carbon, nitrogen, moisture and soil mites across alpine/subarctic tundra communities. *Scientific Reports, 7*, 10.1038/srep44489.

Andre, H. M., Ducarme, X., & Lebrun, P. (2002). Soil biodiversity: myth, reality or conning? *Oikos, 96*(1), 3-24.

Andrén, O. (1985). Microcomputer-controlled extractor for soil microarthropods. *Soil Biology & Biochemistry, 17*(1), 27-30.

Balogh, J., & Balogh, P. (1992). *The Oribatid Mites Genera of the World*. Budapest: Hungarian Natural History Museum.

Bardgett, R. D. (2002). Causes and consequences of biological diversity in soil. *Zoology, 105*(4), 367-374.

Bardgett, R. D., & Cook, R. (1998). Functional aspects of soil animal diversity in agricultural grasslands. *Applied Soil Ecology, 10*(3), 263-276.

Bardgett, R. D., Cook, R., Yeates, G. W., & Denton, C. S. (1999). The influence of nematodes on belowground processes in grassland ecosystems. *Plant and Soil, 212,* 23-33.

Bedano, J. C., Cantú, M. P., & Doucet, M. E. (2006). Influence of three different land management practices on soil mite (Arachnida: Acari) densities in relation to a natural soil. *Applied Soil Ecology, 32*(3), 293-304.

Berlese, A. (1905). Apparecchio per raccogliere presto e in gran numero piccoli arthropodi. *Redia, 2*, 85-90.

Chen, B., Snider, R. J., & Snider, R. M. (1995). Food preference and effects of food type on the life history of some soil Collembola. *Pedobiologia, 39*, 496–505.

Coleman, D. C., & Whitman, W. B. (2005). Linking species richness, biodiversity and ecosystem function in soil systems. *Pedobiologia, 49*(6), 479-497.

Coleman, D. C., Callaham, M. A., & Crossley, D. A. (2018). *Fundamentals of Soil Ecology* (3rd edition). Academic Press.

Cortet, J., Joffre, R., Elmholt, S., & Krogh, P. H. (2003). Increasing species and trophic diversity of mesofauna affects fungal biomass, mesofauna community structure and organic matter decomposition processes. *Biology and Fertility of Soils, 37*(5), 302-312.

Davidson, M. M., & Broady, P. A. (1996). Analysis of gut contents of *Gomphiocephalus hodgsoni* Carpenter (Collembola: Hypogastruridae) at Cape Geology, Antarctica. *Polar Biology, 16*(7), 463-467.

De Long, J. R., Dorrepaal, E., Kardol, P., Nilsson, M.-C., Teuber, L. M., & Wardle, D. A. (2016). Contrasting responses of soil microbial and nematode communities to warming and plant

functional group removal across a post-fire boreal forest successional gradient. *Ecosystems, 19,* 339-355.

Edwards, C. A. (1991). The assessment of populations of soil-inhabiting invertebrates. *Agriculture Ecosystems & Environment, 34*(1-4), 145-176.

Elmendorf, S. C., Henry, G. H. R., Hollister, R. D., Bjork, R. G., Bjorkman, A. D., Callaghan, T. V., ... Wookey, P. A. (2012). Global assessment of experimental climate warming on tundra vegetation: heterogeneity over space and time. *Ecology Letters, 15*(2), 164-175.

EPPO European and Mediterranean Plant Protection Organization (2013). PM 7/119 (1) Nematode extraction*. EPPO Bulletin,* 43, 471-495.

Ferris, H., Bongers, T. & De Goede, R. G. M. (2001). A framework for soil food web diagnostics: extension of the nematode faunal analysis concept. *Applied Soil Ecology, 18,* 13-29.

Fjellberg, A. (1998). The Collembola of Fennoscandia and Denmark: Part I: Poduromorpha. *Fauna Entomologica Scandinavica, 35*, 1-184.

Fjellberg, A. (2007). The Collembola of Fennoscandia and Denmark: Part II: Entomobryomorpha and Symphypleona. *Fauna Entomologica Scandinavica, 42*, 1-266.

Geisen, S., Snoek, L. B., ten Hooven, F. C., Duyts, H., Kostenko, O., Bloem, J., … van der Putten, W. H. (2018). Integrating quantitative morphological and qualitative molecular methods to analyse soil nematode community responses to plant range expansion. *Methods in Ecology and Evolution, 9,* 1366-1378.

Gisin, H. (1960). *Collembolenfauna Europas*. Genève: Museum d'Histoire Naturelle.

Hedlund, K., & Sjögren-Öhrn, M. (2000). Tritrophic interactions in a soil community enhance decomposition rates. *Oikos 88*(3), 585-591.

Hopkin, S. P. (1997) *Biology of the Springtails (Insecta: Collembola).* Oxford: Oxford University Press.

Hågvar, S., & Klanderud, K. (2009). Effect of simulated environmental change on alpine soil arthropods. *Global Change Biology 15*(12), 2972-2980.

ISO (2006). Soil quality - Sampling of soil invertebrates - Part 2. In *Sampling and extraction of micro-arthropods (Collembola and Acarina)* (Vol. ISO 23611-2:2006).

ISO (2007a). Soil quality - Sampling of soil invertebrates - Part 3. In *Sampling and soil extraction of enchytraeids* (Vol. ISO 23611-3:2007).

ISO (2007b). Soil quality - Sampling of soil invertebrates - Part 4. In *Sampling, extraction and identification of soil-inhabiting nematodes* (Vol. ISO 23611-4:2007).

ISO (2018). Soil quality - Sampling of soil invertebrates - Part 1. In *Hand-sorting and extraction of earthworms* (Vol. ISO 23611-1:2018).

Jenkins, W. R. (1964). A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Disease Report 48*(9)*,* 692.

Kardol, P., Cregger, M. A., Campany, C. E., & Classen A. T. (2010). Soil ecosystem functioning under climate change: plant species and community effects. *Ecology, 91*(3), 767-781.

Konestabo, H. S., Michelsen, A., & Holmstrup, M. (2007). Responses of springtail and mite populations to prolonged periods of soil freeze-thaw cycles in a sub-arctic ecosystem. *Applied Soil Ecology, 36*(2-3), 136-146.

Krab, E. J., Cornelissen, J. H. C., & Berg, M. P. (2015). A simple experimental set-up to disentangle the effects of altered temperature and moisture regimes on soil organisms. *Methods in Ecology and Evolution, 6*(10), 1159-1168.

Krantz, G. W., & Walter, D. E. (Eds.) (2009). *A Manual of Acarology* (3rd edition). Texas: Texas Tech University Press.

Leinaas, H. P., Bengtsson, J., Janion-Scheepers, C., Chown, S. L. (2015). Indirect effects of habitat disturbance on invasion: nutritious litter from a grazing resistant plant favors alien over native Collembola. *Ecology and Evolution, 5*(16), 3462-3471.

Macfadyen, A. (1961). Improved funnel-type extractors for soil arthropods. *Journal of Animal Ecology, 30*(1), 171-184.

Mebes, K., & Filser, J. (1998). Does the species composition of Collembola affect nitrogen turnover? *Applied Soil Ecology, 9*(1-3), 241–247.

Nielsen, C. O., & Christensen, B. (1959). The Enchytraeidae. Critical revision and taxonomy of European species. (Studies on Enchytraeidae VII). *Natura Jutlandica, 8-9*, 1-160.

O'Connor, F. B. (1955). Extraction of enchytraeid worms from a coniferous forest soil. *Nature, 175*, 815-816.

Ojala, R., & Huhta, V. (2001). Dispersal of microarthropods in forest soil. *Pedobiologia, 45*(5), 443-450.

Oliverio, A. M., Gan, H. J., Wickings, K., & Fierer, N. (2018). A DNA metabarcoding approach to characterize soil arthropod communities. *Soil Biology & Biochemistry, 125*, 37-43.

Oostenbrink, M. (1960). Estimating nematode populations by some selected methods. In J. N. Sasser, & W. R. Jekins (eds.), *Nematology* (pp. 85-102). Chapel Hill, NC: The University of North Carolina Press.

Owen, J. A. (1987). The 'Winkler extractor'. *Proceedings and Transactions of the British Entomological and Natural History Society, 20*(4), 129-132.

Petersen, H. (1978). Some properties of two high-gradient extractors for soil microarthropods, and an attempt to evaluate their extraction efficiency. *Natura Jutlandica, 20*, 55-122.

Santos, P. F., Phillips, J., & Whitford, W. G. (1981). The role of mites and nematodes in early stages of buried litter decomposition in a desert. *Ecology 62*(3)*,* 664-669.

Sapkota, R., & Nicolaisen, M. (2015). High-throughput sequencing of nematode communities from total soil DNA extractions. *BMC Ecology, 15,* 1-8.

Schmelz, R. M., & Collado, R. (2010). A guide to European terrestrial and freshwater species of Enchytraeidae (Oligochaeta). *Soil Organisms, 82*(1), 1-176.

Schmidt, O. (2001). Time-limited soil sorting for long-term monitoring of earthworm populations. *Pedobiologia, 45*(1), 69-83.

Setälä, H., Tyynismaa, M., Martikainen, E., & Huhta, V. (1991). Mineralization of C, N and P in relation to decomposer community structure in coniferous forest soil. *Pedobiologia, 35*, 285-316.

Setälä, H., Laakso, J., Mikola, J., & Huhta, V. (1998). Functional diversity of decomposer organisms in relation to primary production. *Applied Soil Ecology, 9*(1-3), 25-31

Sinclair, B. J., & Sjursen, H. (2001). Terrestrial invertebrate abundance across a habitat transect in Keble Valley, Ross Island, Antarctica. *Pedobiologia, 45*(2), 134-145.

Sjursen, H., Michelsen, A., & Jonasson, S. (2005). Effects of long-term soil warming and fertilisation on microarthropod abundances in three sub-arctic ecosystems. *Applied Soil Ecology, 30*(3), 148-161.

Tullgren, A. (1917). En enkel apparat för automatiskt vittjande av sållgods. *Entomologisk Tidskrift, 38*, 97-100.

Van Bezooijen, J. (2006). Methods and techniques for Nematology. Script, 112 p. https://www.wur.nl/upload_mm/f/9/3/10aac0cb-8289-400a-a6e5-c4687461d138_MethodsandTechniquesforNematology.pdf [accessed on 15 May 2019].

Van den Hoogen, J., Geisen, S., Routh, D., Ferris, H., Traunspurger, W., Wardle, … & Bardgett, R.D. (2019). Soil nematode abundance and functional group composition at a global scale. Nature, 1.

Verhoef, H. A., Prast, J. E., & Verweij, R. A. (1988). Relative importance of fungi and algae in the diet and nitrogen nutrition of *Orchesella cincta* (L.) and *Tomocerus minor* (Lubbock) (Collembola). *Functional Ecology, 2*(2), 195-201.

Verschoor, B. C., & De Goede, R. G. M. (2000). The nematode extraction efficiency of the Oostenbrink elutriator-cottonwool filter method with special reference to nematode body size and life strategy. Nematology, 2, 325–342.

Wilschut, R. A., Geisen, S., Martens, H., Kostenko, O., de Hollander, M., ten Hooven, F. C., ... van der Putten, W. H. (2019). Latitudinal variation in soil nematode communities under climate warming-related range-expanding and native plants. *Global Change Biology,* DOI: 10.1111/gcb.14657

Yeates, G. W., Bongers, T., De Goede, R. G. M., Freckman, D. W., & Georgieva, S. S. (1993). Feeding-habits in soil nematode families and genera – an outline for soil ecologists. *Journal of Nematology 25*(3), 315-331.

Authors: Konestabo HS[1] & Kardol P[2]

Reviewer: Birkemoe T[3]

Affiliations

[1] The Science Library, University of Oslo, Norway

[2] Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, 901-83 Umeå, Sweden

[3] Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, Ås, Norway

4.11 Macroinvertebrate community composition

Author: Birkemoe T[1]

Reviewers: Sverdrup-Thygeson A[2], Gillespie MAK[3], Eycott AE[4,5]

Measurement unit: number or relative abundance per area; Measurement scale: plot; Equipment costs: €; Running costs: €; Installation effort: low to medium; Maintenance effort: medium to high; Knowledge need: high (species knowledge); Measurement mode: manual

Terrestrial macroinvertebrates, that is invertebrates generally above 2 mm in size, mainly include insects, spiders, millipedes, centipedes, molluscs, and crustaceans (isopods), and most assemblages are functionally diverse, with high numbers of detritivores, fungivores, herbivores, and predators. Invertebrates, therefore, can significantly affect primary production, nutrient cycling, and decomposition. From a global perspective, macroinvertebrates are likely to modify responses of ecosystem processes to climate and global change. The high functional diversity and large number of interactions between macroinvertebrates and vegetation, fungi, and dead organic matter strongly suggest incorporating these into studies of terrestrial ecosystems and certainly into ongoing climate-change studies.

Macroinvertebrates vary from millimetres to centimetres in size and range from sedate species living their entire life on a few square metres to highly active species covering many kilometres during their lifetime. Many species also have life stages with highly different habitat requirements, such as some Lepidoptera that have herbivorous larvae but adults that feed on floral nectar. The effect of macroinvertebrates on vegetation or ecosystem processes is therefore highly dependent on the geographical and temporal scale of measurement. Pollinators, herbivores, detritivores, and predators forage on available flowers, plants, dead organic matter, or prey, respectively. Suitable food items may change during the arthropod life stages and species-specific phenology results in large variation in species composition throughout the year. Pollinators are discussed in protocol 4.12 Pollinator composition.

4.11.1 What and how to measure?

There are two main strategies for quantifying macroinvertebrates: i) estimate densities per area or ii) use relative measurements based on arthropod activity (Southwood & Henderson, 2000). The distinction between these two strategies is not always clear, however, as absolute estimates are rarely obtained. Use of traps can also be further divided into passive and active trapping – passive traps catch insects by chance and active traps attract species by bait, colour, chemical lures, or light (Leather, 2004). As this protocol strives to describe methods that quantify invertebrates in climate-manipulated plots, attraction of species from larger areas is likely to include higher densities or species that are not strictly relevant to the plot. This limitation also applies to other studies focussing on local species composition. For a thorough discussion of all sampling techniques, see Southwood & Henderson (2000) and Leather (2004).

When estimating macroinvertebrates it is essential to recognise that their behaviour changes with time of day, season, weather conditions, and life stage, influencing activity and habitat use. Care should therefore be taken to minimise this variation (i.e. sample in equal conditions or preferably at

the same time) when communities are compared between different sites. As there is no method that catches all macroinvertebrate groups, we will not define a gold standard, but rather describe the options related to the scale of plots and microhabitat of sampling.

Table 4.11.1. Overview of methods of macroinvertebrate sampling. Suggested plot size is noted with an x. Subplots indicate that the sampling may need to be repeated in order to represent the plot scale.

		Plot scale			
	Methods	**≤ 0.25 m²**	**0.25 m² – 1 m²**	**1 m² – 10 m²**	**≥ 10 m²**
Vegetation	Suction	x	x	subplots	subplots
	Visual search	x	x	subplots	subplots
	Removal of vegetation for extraction	-	x	subplots	subplots
	Vegetation beating	-	-	x	x
	Sweep-netting	-	-	x	x
	Emergence traps	x	x	subplots	subplots
	Malaise, window, and sticky traps	-	-	-	x
	Fogging	-	-	-	x
Soil surface	Suction	x	x	subplots	subplots
and litter	Visual search	x	x	subplots	subplots
	Pitfall traps	-	x	x	subplots
	Removal of litter for sieving/extraction	x	x	subplots	subplots
	Emergence traps	x	x	subplots	subplots

Densities per area

In the methods described here, the sampling area is generally known and the invertebrates are estimated per unit area.

Suction or vacuum sampling has proven highly useful in grasslands and agricultural systems and is particularly efficient in sampling insects from grasses and herbs. A suction sampler (Figure 4.11.1) catches macroinvertebrates such as spiders, beetles, planthoppers, and true bugs (Brook et al., 2008; Zentane et al., 2016). It is driven by a petrol engine that sucks arthropods out of an area defined by the aperture of the sampling tube. Both wide-hosed (>20 cm in diameter) and narrow-hosed (<15 cm in diameter) types are used, but narrow-hosed types are usually most efficient and therefore recommended (Ozanne, 2004). Quadrats can be laid out and the nozzle run through the vegetation for a standard time

Figure 4.11.1 Vacuum or suction sampling in a closed area. Photo: Anders Endrestøl, NINA.

period (e.g. 1 minute per 25 cm^2; denser vegetation may require more time per unit area). A cylinder or tent with a known cross-sectional area can also be placed on the ground, the hose inserted through the top and the contents vacuumed up. This will ensure that fewer insects are being lost or being drawn into the area from the surroundings (Cherill, 2015). In one study using a 15.7 cm diameter nozzle, 16 seconds of sampling was enough to sample 90% of all beetles, spiders, planthoppers, and true bugs in grass turves covered by the nozzle compared to turf extraction and Berlese funnel techniques (Brook et al., 2008). The sampling efficiency depends on vegetation height and invertebrate groups present, as well as on the proficiency of the operator in emptying the collection bag (active insects can be lost). On the downside, a suction sampler will also suck up debris, removing more than just the macroinvertebrates from the site. Also, the method is sensitive to rain and dew, so collection should only be carried out in dry conditions. The method can be used for small to medium sized plots (Zentane et al., 2016; Facey et al., 2017; Table 4.11.1).

Sweep-net capture is an easily-available method shown to efficiently sample invertebrates in shrubs and low vegetation (Figure 4.11.2). The method can be standardised by a given number of sweeps per area or by length of sweeping time (e.g. 5 or 10 minutes; Ozanne, 2004). Higher vegetation complexity requires longer sweeping times or sweep numbers. The net should be moved in a figure-of-eight motion while moving forward to avoid overlap between sweeps. It has been found to be more efficient at catching larger arthropod species and less efficient with smaller species than suction sampling in coastal sage scrub, for example (Buffington & Redak, 1998). It was also demonstrated as an efficient way to catch Hymenoptera, Diptera, small Coleoptera, and arachnids in forests (Canaday, 1987). The efficiency of sweep-netting depends on the operator, with factors such as speed of net movement impacting catches, but it is also affected by vegetation structure, species composition, vertical distribution, weather conditions, and daily activity cycles of the insects sampled (pp. 267-8, Southwood & Henderson, 2000). Sweep-netting should not be carried out during wet weather. The method can be used in medium to large plots (Table 4.11.1).

Figure 4.11.2 Sweep-netting in a grassland. Photo by John Rostron, CC BY-SA 2.0.

Vegetation beating is an efficient method of invertebrate sampling of shrub land or forest vegetation and might be regarded as a method of estimating densities per area or recording a relative measure (see below) depending on the species in question (Southwood & Henderson, 2000; Ozanne, 2004). It can be used on flowers, branches, twigs, or leaves and be standardised by using a defined number of similar force beats with a

Figure 4.11.3 Beating for insects in dead branches. Photo: Fritz Geller-Grimm, CC BY-SA 3.0

stout stick on a similar substrate and area. The invertebrates falling down are collected in horizontal canvas trays or an "upside down umbrella" of a standardised size and removed individually with an aspirator or soft forceps (Figure 4.11.3; Samways et al., 2009). As flying insects might escape quickly, it can be advantageous to have two people collecting from the tray. Beating was found to be the best method for collecting Hymenoptera and Coleoptera in understorey vegetation in a forest in Virginia using 10 beats and a 1 m^2 sheet below the vegetation (Rohr et al., 2007). The method is dependent on dry weather and can be used in medium to large plots (Table 4.11.1). **Visual search** or direct counting can be used to track relatively immobile or conspicuous invertebrates on plants, such as certain butterfly larvae and adults, crickets, and snails (pp. 150-1, Southwood & Henderson, 2000). Counts of spider webs may also represent a proximate measurement of population size. Care must be taken not to disturb the animals when counting. For example, many caterpillars and aphids purposefully fall from vegetation when sufficiently disturbed as a defence reflex. The search should be carried out during a fixed time interval at a consistent time of the day, and preferably within a few days to minimise differences in population development, temperature, and moisture conditions. Depending on the animals in question, the method can be used in small to large plots. The size of animals and plot size will determine the time needed for observations (Table 4.11.1).

Removing vegetation and litter within an area with a corer and transferring it to a plastic bag followed by extraction in Berlese funnels can give a higher number of macroinvertebrates than suction sampling and could provide absolute estimates for grass turves (Brook et al., 2008; Zentane et al., 2016). Removed litter may also be hand sorted or sieved before extraction. When electricity is unavailable, a Winkler bag can be used, which works on the same principle as a Berlese funnel, but without heat (Samways et al., 2009). Removal of vegetation and litter are highly destructive to the plots, but may potentially work at all scales by sampling several subsets of the plots (Table 4.11.1). Dead wood structures may also be broken open and inspected and the remaining invertebrates extracted or placed in emergence traps (see below).

Emergence traps can be used to estimate insects in pieces of dead wood, fungi, litter, soil, and vegetation (Samways et al., 2009). The material is enclosed in a dark net or box with a mounted collection tube to which the insects are attracted by light. Ground emergence traps, i.e. a floorless black tent mounted on the ground with a collection tube in an upper corner, can be efficient for estimating densities of ground-living flies and nesting solitary bees as well as for dating their emergence. Emergence traps can catch insects present as larvae or pupae at the time of mounting if left for a longer time period. Parasitoids can be efficiently captured when emerging from particular substrates. The traps can be deployed in the field or transferred to the lab. When mounted on the ground, the vegetation can be affected by the trap, and removal of material will obviously disturb plots. Depending on material enclosed in the trap, this trap type can be applied to plots of all sizes (Table 4.11.1). However, the size and number of traps needed is highly dependent on the density of insects in the study area. When numbers are particularly low, such as in the High Arctic, they are likely to be inefficient.

Relative measures

In the methods described above, the relative abundance of invertebrates is estimated per unit area. With the trapping devices described below, the sampling area is generally unknown as the species

composition and abundances depend on invertebrates actively entering the trap. Thus, when interpreting the data, differences in numbers of the same species should be considered.

Pitfall traps catch active, mainly polyphagous predators, foraging on the soil surface. The major taxonomic groups caught are ants, wandering spiders (Aranae: Lycosidae, Clubionidae), rove beetles (Coleoptera: Staphylinidae), and ground beetles (Coleoptera: Carabidae) (Southwood & Henderson, 2000; Woodcock, 2004). In polar areas where several insect groups tend to stay close to the soil surface, pitfall trapping has also been used to compare catches of butterflies, midges, and flies (Høye et al., 2013). Pitfall trapping is one of the oldest and most-used techniques and a large number of studies have focused on trapping efficiency relative to trap construction and placement (Woodcock, 2004). Generally, a pitfall trap (Figure 4.11.4) consists of a jar dug into the ground so that the rim is level with the soil surface and half filled with some sort of liquid preservative (saline solution or propylene glycol are the least attractive to animals and have low toxicity, to which a few drops of detergent is added to break the surface tension). An upper jar diameter of 6–9 cm and a 6–7 cm depth works well. A roof, 3–4 cm above the trap entrance, prevents overflowing during rain and dilution of preservative as well as blocking debris which could create insect escape routes from falling in.

Use of a transparent roof minimises the influence of roofs on trap catches (Woodcock, 2004). Trapping periods depend on the rate of preservative evaporation (heat) and dilution (rain), number of animals caught (traps can fill up), and the research question, but generally range from 2 days in the tropics to a maximum of 4 weeks in colder areas. Use of two layers of traps, with only the inner trap removed during each emptying session, ensures minimal soil disturbance. The trap catches depend on the vegetation structure immediately surrounding the traps and, if comparing sites with highly different structure, removing vegetation immediately around the trap should be considered (Woodcock, 2004). Digging pitfall traps is destructive to plots and affects trap catches for a few days after installation. Pitfall traps can work on small to large plot sizes (Table 4.11.1).

Figure 4.11.4 Pitfall trap. Photo: Anne Sverdrup-Thygeson, NMBU, Norway.

Malaise traps, **sticky traps**, **pan traps**, and **interception traps** are all ways to target flying insects and their effective use depends on flight activity. As most climate-manipulation plots are relatively small (<10 m^2), flight-based insects traps are likely to catch insects from a much larger area or those merely passing by, and thus introduce too much noise for plot-related effects. However, they might be good for baseline data backing up plot-level studies, gradient studies, or studies of changes through time, and a short description is therefore given. **Malaise traps** are tent-like structures attached to the ground and measuring up to 2 m high. A traditional malaise trap consists of a fine mesh net intercepting flight in two directions and a roof leading the insects into a sampling jar with preservative. They come in several sizes and

colours, each affecting trap catches (bicoloured is the most used). A standard Townes-type trap usually needs about 2 m^2 for proper mounting. Small and mostly flying invertebrates such as diptera (herbivores, detritivores, predators) and hymenoptera (herbivores, parasitoids) are particularly well sampled. Malaise traps work in damp conditions and may therefore be used as an alternative to suction sampling or sweep-netting if the understorey is constantly wet (Ozanne, 2004). **Sticky traps** catch flying insects such as pollinators, herbivores, or their parasitoids and predators, and have been used in Free Air CO_2 Enrichment (FACE) studies (Facey et al., 2017) and studies of parasitoid communities on herbivores (http://www.helsinki.fi/foodwebs/parasitoids/). The catch depends on trap colour, height, and position (vertical, angled, horizontal) (Young, 2005). **Interception traps (window traps)** are traps undetected by flying insects and are most commonly used to catch beetles, but bees and other flying insects are also captured. Fully transparent material, often Perspex or acrylic, are used either in a cross shape or single panel and when a flying insect hits the trap, it falls down into a collecting bottle or tray with preservative and detergent (see pitfall traps above). Malaise traps, sticky traps, and window traps can be placed close to objects (trees, shrubs, dead wood) to increase the focus of the trap catches. For traps targeting pollinating insects such as pan traps, see protocol 4.12 Pollinator composition.

Where to start

Leather (2004), Samways et al. (2009), Southwood & Henderson (2000)

4.11.2 Special cases, emerging issues, and challenges

The use of image-based observations for estimating animals and plants is an emerging and highly interesting method (Burton et al., 2015; Steenweg et al., 2017). Combined with effective image-analysis, this has a large potential for future species monitoring at least of the larger and easily identified macroinvertebrates. The use of environmental DNA (eDNA), that is sampling of DNA from the environment, also has a high potential for identification and monitoring of elusive and small species such as invertebrates (Bohmann et al., 2014). Invertebrates might also be pulverised and identified by DNA-barcoding. This can be time-efficient as compared with sorting and visual identification. To ensure the best material for barcoding, the macroinvertebrates should preferably be stored in 96% ethanol.

We have not included canopy invertebrates in this protocol as they are outside the usual plot size for climate manipulation experiments. To sample these structures, traps (for example interception traps or sticky traps) could be mounted hanging from branches. If the canopy is very high, walkways, cranes, drones or other devices might be needed to access the area. Chemical knockdown can be used from the ground. Fogging or misting include insecticides (natural pyrethrum or synthetic pyrethroids, the first is most quickly broken down by UV-radiation) and can only be carried out under calm and dry conditions. The macroinvertebrates are collected at the ground, or higher up, by collection mats or trays. For more information about canopy sampling, see Ozanne (2014) and Samways et al. (2009).

Including flying macroinvertebrates such as insects in plot-level climate studies is challenging. Their occurrence might not relate to the effect of the treatment as they visit the plots only within snapshots of time. Thus care should be taken when interpreting the results and high sample sizes are preferable. Another important challenge is the removal of invertebrates from the plots. With the exception of visual search, which can only be used for some few species, the methods described in this chapter will potentially disturb ongoing interactions within the plots. Whether long time activated traps or repeated collections (vacuum sampling, sweep net, beating or fogging/misting) are the least disturbing method is unknown and likely to depend on species and plot size. Use of subplots for macroinvertebrate collection only are recommended if ecosystem processes, species interactions or other organism groups are to be measured simultaneously.

4.11.3 References

Theory, significance, and large datasets

Leather (2004), Samways et al. (2009), Southwood & Henderson (2000)

More on methods and existing protocols

Facey et al. (2017), Leather (2004), Samways et al. (2009), Southwood and Henderson (2000)

All references

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., … de Bruyn, M. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution, 29*(6), 358-367.

Brook, A. J., Woodcock, B. A., Sinka, M., & Vanbergen, A. J. (2008). Experimental verification of suction sampler capture efficiency in grasslands of differing vegetation height and structure. *Journal of Applied Ecology, 45*(5), 1357-1363.

Buffington, M. L., & Redak, R. A. (1998). A comparison of vacuum sampling versus sweep-netting for arthropod biodiversity measurements in California coastal sage scrub. *Journal of Insect Conservation, 2*(2), 99-106.

Burton, A. C., Neilson, E., Moreira, D., Ladle, A., Steenweg, R., Fisher, J. T., … Boutin, S. (2015). Wildlife camera trapping: a review and recommendations for linking surveys to ecological processes. *Journal of Applied Ecology, 52*(3), 675-685.

Canaday, C. L. (1987). Comparison of insect fauna captured in six different trap types in a Douglas-fir forest. *The Canadian Entomologist, 119*(12), 1101-1108.

Cherill, A. (2015). Suction sampling of grassland invertebrates using the G-vac: Quantifying and avoiding peripheral suction effects. *European Journal of Entomology, 112*(3), 520-524.

Facey, S. L., Fidler, D. B., Rowe, R. C., Bromfield, L. M., Nooten, S. S., Staley, J. T., … Johnson, S. N. (2017). Atmospheric change causes declines in woodland arthropods and impacts specific trophic groups. *Agricultural and Forest Entomology, 19*(1), 101-112.

Høye, T. T., Post, E., Schmidt, N. M., Trøjelsgaard, K., & Forchhammer, M. C. (2013). Shorter flowering seasons and declining abundance of flower visitors in a warmer Arctic. *Nature Climate Change, 3*, 759.

Leather, S. R. (Ed.) (2004). *Insect Sampling in Forest Ecosystems*. Oxford: Wiley-Blackwell.

Ozanne, C. M. P. (2004). Sampling methods for forest understory vegetation. In S. R. Leather (Ed.), *Insect Sampling in Forest Ecosystems* (pp. 58-76). Oxford: Wiley-Blackwell.

Rohr, J. R., Mahan, C. G., & Kim, K. C. (2007). Developing a monitoring program for invertebrates: guidelines and a case study. *Conservation Biology, 21*(2), 422-433.

Samways, M. J., McGeoch, M. A., & New, T. R. (2009). *Insect Conservation. A Handbook of Approaches and Methods*. Oxford: Oxford University Press.

Southwood, T. R. E., & Henderson, P. A. (2000). *Ecological Methods* (3rd ed.). Oxford: Blackwell.

Steenweg, R., Hebblewhite, M., Kays, R., Ahumada, J., Fisher, J. T., Burton, C., … Rich, L. N. (2017). Scaling-up camera traps: monitoring the planet's biodiversity with networks of remote sensors. *Frontiers in Ecology and the Environment, 15*(1), 26-34.

Woodcock, B. A. (2004). Pitfall trapping in ecological studies. In S. R. Leather (Ed.), *Insect Sampling in Forest Ecosystems* (pp. 37-57). Oxford: Wiley-Blackwell.

Young, M. (2005). Insects in flight. In S. R. Leather (Ed.), *Insect Sampling in Forest Ecosystems* (pp. 116-145). Oxford: Wiley-Blackwell.

Zentane, E., Quenu, H., Graham, R. I., & Cherrill, A. (2016). Suction samplers for grassland invertebrates: comparison of numbers caught using Vortis™ and G-vac devices. *Insect Conservation and Diversity, 9*(5), 470-474.

Author: Birkemoe T[1]

Reviewers: Sverdrup-Thygeson A[1], Gillespie MAK[2, 3], Eycott AE[4,5]

Affiliations

[1] Faculty of Environmental Sciences and Natural Resource Management, University of Life Sciences, Ås, Norway

[2] Department of Arctic and Marine Biology, Faculty of Biosciences, Fisheries and Economics, UiT—The Arctic University of Norway, Tromsø, Norway

[3] Department of Engineering & Natural Sciences, Sogn & Fjordane University College, Sogndal, Norway

[4] Faculty of Biosciences and Aquaculture, Nord University, Steinkjer, Norway

[5] Department of Biological Sciences, University of Bergen, Bergen, Norway

Halbritter et al. (2020) The handbook for standardised field and laboratory measurements in terrestrial climate-change experiments and observational studies (ClimEx). *Methods in Ecology and Evolution*, *11*(1) 22-37.

4.12 Pollinator composition

Authors: Gillespie MAK[1], Hegland SJ[1]

Reviewer: Sydenham MAK[2]

Measurable unit: number of individuals/species, various indices; Measurement scale: site; Equipment costs: €; Running costs: €; Installation effort: low; Maintenance effort: low; Knowledge need: high; Measurement mode: manual

Pollinators are usually insects that assist a wide range of flowering plants with pollination, i.e. they visit flowers to feed on nectar or pollen and inadvertently transport pollen to conspecific flowers. Sampling pollinators in experimental or observational studies can provide variables of community composition, species richness, and pollinator abundance. Pollination is important for a range of flowering species that rely on animals for cross-pollination (Ollerton et al., 2011), and both the composition and diversity of the pollinating community of a particular habitat can be important drivers of plant fitness, productivity, and biodiversity (Hoehn et al., 2008; Bartomeus et al., 2013). Pollinator communities are thought to be particularly sensitive to climate change through spatial and temporal mismatches between pollinators and their food plants (Hegland et al., 2009; Gonzalez-Varo et al., 2013). These changes may occur due to different changes in distribution and/or phenology of interacting species (Hegland et al., 2009) and are likely to impact vegetation community composition, agriculture, and the tolerance of habitats and ecosystems to changes (Gonzalez-Varo et al., 2013). Such effects are best studied over long time periods, but the potential for mismatch can be investigated using plot-level manipulations (e.g. Rafferty & Ives, 2012). At much broader scales, changes in pollinator community composition, diversity, and abundance can drive local or regional extinctions of key species and specialist plants and animals (Allen-Wardell et al., 1998) with habitat fragmentation and loss as the most important global driver (Potts et al., 2010). The protocols detailed here can therefore be used at a range of scales to study a number of global-change drivers. In general, serious impacts on food production have also been forecast in the event of severe declines in pollinator communities and diversity (Gallai et al., 2009), although it is often the more generalist and common species that contribute the most per capita to crop-pollination (Kleijn et al., 2015; Senapathi et al., 2015). Nevertheless, in acknowledgement of the role pollinators play in both agricultural and wild ecosystems, many countries have, or are developing pollinator monitoring protocols (Westphal et al., 2008; Gezon et al., 2015).

4.12.1 What and how to measure?

There are a number of ways to assess the pollinator community of a site, with varying degrees of accuracy and time and skill required. The most common method, which is used by some established national monitoring schemes, involves collecting pollinators by pan-trapping, sweep netting, or a combination of the two (Westphal et al., 2008; Gezon et al., 2015). A plot-level approach can be taken, particularly when combined with plant studies (see also protocol 4.13 Pollinator visitation), but it should be noted that pan traps are active traps in that they attract flying insects from an unknown area. They are, therefore, only likely to be suitable for landscape scale studies that use

natural gradients as a proxy for manipulations, (e.g. space for time substitutions, Blois et al. 2013). Pan traps usually consist of a cluster of circular bowls measuring approximately 15 cm in diameter, each sprayed with UV reflecting paint. A combination of coloured bowls to reflect the prevailing flower colours in the region is usually selected to account for the range of colour preferences of local flower visitors. For example, one white, one yellow, and one blue bowl for each trap is commonly employed in northern Europe (Westphal et al., 2008). The bowls should be installed at the height of the surrounding vegetation and placed no closer than 5 m apart to avoid inter-trap competition (Droege et al., 2010). In short vegetation (e.g. grasslands, tundra), the bowls can simply be placed on the ground or held in place using a stake. Mounting the bowls on a large stake is recommended for taller vegetation types. Bowls should be half filled with water and a drop of liquid detergent added to break the surface tension. Traps are often emptied before 48 h (Westphal et al., 2008) after setup to prevent decomposition of the insect material. The trapping time may be shorter due to practical considerations or high insect abundance, but normally covers at least one flying day, i.e. 12 daytime hours. For longer trapping periods (e.g. a week), a mixture of water and clear propylene glycol (50:50) can be used (Rubene et al., 2015), although this approach incurs the risk of rainfall diluting the mixture and causing it to overflow as well as masking of the reflecting surface of the bowls due to the greater volume of insects. Propylene glycol acts as a preserving agent in this mixture, but other work has suggested that antifreeze for mobile home drinking water systems could provide a less toxic (although less effective) alternative (Thomas 2008). Traps should ideally be placed out in standardised weather (e.g. relatively high temperature, low wind, no rain) to ensure as many species are flying as possible or record the weather variables to control for their influence. To remove captured material, the liquid should be preserved (particularly if it contains glycol) by sieving over another trap or other vessel, and the insects emptied into a plastic ziplock bag. The specimens can then be frozen before drying and mounting or identifying. If specimens cannot be frozen on the day of removal, a quick spray of hand sanitizer or ethanol will help with preservation. Storing in ethanol should be avoided where possible, as it can fade the colouration of species like bumblebees.

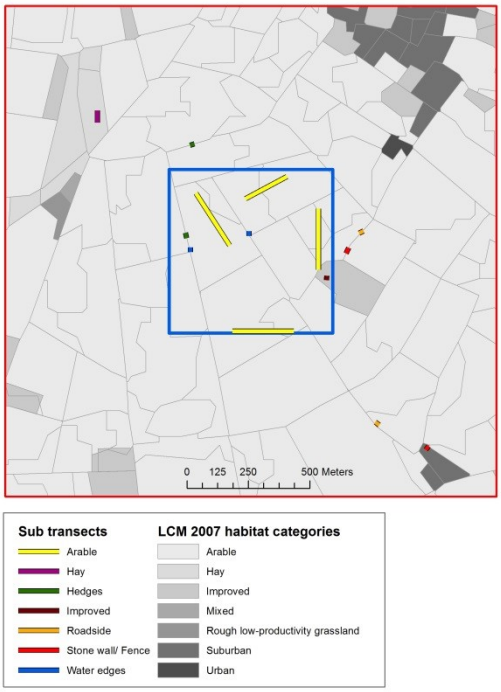


Figure 4.12.1 Representative transect allocation example from Gillespie et al. (2017). The majority of this 2 x 2 km landscape is arable agriculture (93.6%), so 940 m of the 1 km linear transect has been allocated to this habitat type. The transects are split into 4 sub-sections for practicality. The remaining 60 m of transect is allocated to hay meadow (4.7% land cover; 1 x 50 m sub-transect) and improved grassland (1.3%, 1 x 10 m sub-transect). The habitat types rough-low productivity grassland (0.3%) and mixed woodland (0.1%) have no transect segments as the cover is considered to be too small. There are also 2 x 20 sub-transects for linear features (hedges, roadsides, walls/fences and water edges).

For sweep-netting approaches (also see protocol 4.11 Macroinvertebrates community compisition), the only installation required is to determine the location of transects and the time when surveys are conducted. If the aim of the study is to survey as much of the community of a site as possible, it may be desirable to place temporary transects deliberately through a range of habitats at peak flowering,

and complete those transects at different times of the day to capture early and later flying insects. Alternatively, variable transects can be chosen whereby observers walk steadily in a set direction passing from one cluster of floral resources to the next, rather than along a fixed straight line (Westphal et al., 2008). However, if the study question requires a more representative and/or random sample of a set area, it is recommended that transects are placed remotely before fieldwork to avoid bias, and that the order of transects are randomised during each sampling session. For example, in a pollinator study of British landscapes, Gillespie et al. (2017) allocated sub-sections of a 1-km transect proportionately depending on the areal cover of different habitat types (Figure 4.12.1).

More recently, studies have shown that vane-traps (hanging bowls or bottles with cross-vane panels mounted at the opening) may provide a complementary method for sampling wild bees as they capture species not caught by pan traps and sweep-netting (Rhoades et al., 2017; Figure 4.12.2). The sampling protocol for vane traps largely follows that of pan traps and is not discussed in detail here. In general, the choice of method depends on resources available and the research question. While the trapping methods are less labour intensive than sweep-netting, they only provide information on the presence and relative abundance of pollinator species, whereas sweep-netting allows the recording of plant species visited and the construction of plant–pollinator networks (see protocol 4.13 Pollinator visitation). Furthermore, while pan traps attract a wide range of pollinators, the method can under-sample larger and stronger flying insects (Cane et al., 2000) and the choice of trap colour and trapping date can bias the sample (Wilson et al., 2008). Moreover, the efficiency of different colours of pan traps depends on the background vegetation which can act as a confounding factor that is difficult to measure (Saunders & Luck, 2013; see below). Overall, pan traps and sweep-netting may reveal similar species compositions, but pan traps have been found to catch more specimens and to be the superior method of the two in terms of detecting species richness (Westphal et al. 2008). Whichever method is chosen, it is recommended that sites are sampled several times during the flying season of target species as many pollinators have seasonal flight activity patterns (Oertli et al., 2005).

Figure 4.12.2 Different pollinator traps. A cluster of pan traps set up to collect pollinators (left). Photo: Catherine Jones. Sweep-netting for pollinators along a predetermined transect (right). Photo: Stein Joar Hegland.

A further consideration for method choice and sampling schedule is the expense of post-capture identification. While some netted insects can be identified to species in the field depending on collector skill, all methods often require a large post-trapping effort to identify all specimens to species level. Depending on the trapping effort and availability of skilled taxonomists, this can be time-consuming and expensive. For this reason, all sampled insects must be placed in a preservation medium (e.g. >80% ethanol) or frozen for storage before pinning, labelling, and identification.

Following the identification of insect material, several variables can be derived: total number of insects and of each species group, species richness and diversity indices of the total catch and for each group, and species composition are the most common. Species composition data are the most detailed as they do not require the data to be summarised into a single metric, but these matrices are also sometimes the most challenging to interpret and analyse. A number of multivariate statistical approaches exist to analyse compositional data, from exploratory approaches such as principal component analysis to more comparative approaches such as canonical analysis (Legendre & Legendre, 2012). Conversely, species abundance, richness, and diversity data can be used as dependent variables in most univariate statistical approaches. However, while diversity indices (see also protocol 4.8. Plant community composition) are designed to try and capture aspects of abundance and species richness in a single figure, each index is thought to be biased in some way and is the subject of debate (Loreau, 2010). The researcher should therefore consider carefully which index is most relevant to the research question. Furthermore, care should be taken when interpreting data from pan traps as it is difficult to ascertain the boundaries of the trapping area.

Bronze standard

Pollinators of some species can be sampled by visual observation or via netting using the transect method outlined above but without the use of taxonomists to identify specimens to species level. This method requires either good identification skills of the observer, or the use of a more coarse identification system whereby species are classified according to their size, to a broader taxonomic classification scheme (e.g. bumblebees, honeybees, solitary bees, hoverflies, other flies, wasps) or by their functional traits (e.g. long-tongued v. short-tongued bees). The specific grouping will depend on the system studied and the research questions. Group-level approaches are often precise enough to answer broad ecological questions (e.g. Hegland & Totland, 2012).

Where to start

Gezon et al. (2015), Gillespie et al. (2017), Hegland & Totland (2012), Westphal et al. (2008)

4.12.2 Special cases, emerging issues, and challenges

Perhaps one of the main issues with this protocol is that it is often not possible to define the scale of the study. As flying insects are mobile organisms with ranges that vary from species to species (10 km for honey bees, 100 m for some parasitoid wasp species), it can be impossible to determine the boundary of the study area. This may be less of a problem for central-place foragers (e.g. bees) that show steep decay curves in abundance as the distance to source habitats increase than for non-central-place foragers (e.g. hoverflies) which are more ephemerally distributed throughout the landscape (Jauker et al., 2009). For single site, plot-level studies this renders the protocol to be more of a background data-gathering procedure. For wider, landscape scale studies the protocol can be a useful sampling procedure, but it is important that the study landscapes are sufficiently separated in space to avoid overlapping populations. Gillespie et al. (2017), for example, ensured that study landscapes were at least 6 km apart.

A further challenge to researchers is the placement of traps on site, which is also dependent on the research question. For example, to attempt to complete a census of a single site, Westphal et al. (2008) set out 5 traps spaced 15 m apart. In contrast, to compare pollinator composition between field sites, Gillespie et al. (2017) placed 5 traps within a 660 x 660 m study square, with traps no closer than 50 m apart. Furthermore, the capture of pollinating insects in pan traps depends in part on the amount of floral resources in the local area (Dafni et al., 2005). Pan traps are supposed to simulate a food source to adult insects, so if there are a lot of floral resources available in the landscape, the appearance and attraction of the traps may be diminished compared to landscapes with fewer floral resources. In any case, it is recommended to record the number and types of flowers in the immediate (1 m radius) vicinity of the trap to use as a covariate in analysis. Vegetation information on wider scales may also be needed, although this has not been explored adequately to date.

Despite the above uncertainties, these methods often result in a large number of specimens and any collection will contain an enormous range of information. For example, many non-target species will be caught in pan traps and these specimens can themselves hold important information about the study areas. It is recommended that they are preserved and expertise sought to identify these insects

further. For example, following a pollination study in the UK comparing organic and conventional agricultural landscapes (Gabriel et al., 2013), the "by-catch" was examined for tachinid fly specimens and a further paper published addressing a similar research question (Inclan et al., 2014).

4.12.3 References

Theory, significance, and large datasets

Dafni et al. (2005), Droege et al. (2010), Gezon et al. (2015), Legendre & Legendre (2012), Westphal et al. (2008)

More on methods and existing protocols

Bartomeus et al. (2013), Cane et al. (2000), Gillespie et al. (2017), Rhoades et al. (2017), Rubene et al. (2015), Saunders & Luck (2013)

All references

Allen-Wardell, G., Bernhardt, P., Bitner, R., Burquez, A., Buchmann, S., Cane, J., … Walker, S. (1998). The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. *Conservation Biology, 12*, 8-17.

Bartomeus, I., Park, M. G., Gibbs, J., Danforth, B. N., Lakso, A. N., & Winfree, R. (2013). Biodiversity ensures plant–pollinator phenological synchrony against climate change. *Ecology Letters*, *16*, 1331-1338.

Blois, J. L., J. W. Williams, M. C. Fitzpatrick, S. T. Jackson, & S. Ferrier. 2013. Space can substitute for time in predicting climate-change effects on biodiversity. Proceedings of the National Academy of Sciences *110*(23), 9374-9379

Cane, J. H., Minckley R. L., & Kervin, L. J. (2000). Sampling bees (Hymenoptera: Apiformes) for pollinator community studies: Pitfalls of pan-trapping. *Journal of the Kansas Entomological Society, 73*, 225-231.

Dafni, A., Kevan, P. G., & Husband, B. C. (Eds.) (2005). *Practical Pollination Ecology*. Cambridge, Ontario: Enviroquest.

Droege, S., Tepedino, V. J., Lebuhn, G., Link, W., Minckley, R. L., Chen, Q., & Conrad, C. (2010). Spatial patterns of bee captures in North American bowl trapping surveys. *Insect Conservation and Diversity, 3*, 15-23.

Gabriel, D., Sait, S. M., Kunin, W. E., & Benton, T. G. (2013). Food production vs. biodiversity: comparing organic and conventional agriculture. *Journal of Applied Ecology, 50*, 355-364.

Gallai, N., Salles, J.-M., Settele, J. & Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics, 68*(3), 810-821.

Gezon, Z. J., Wyman, E. S., Ascher, J. S., Inouye, D. W., & Irwin, R. E. (2015). The effect of repeated, lethal sampling on wild bee abundance and diversity. *Methods in Ecology and Evolution, 6*, 1044-1054.

Gillespie, M. A. K., Baude, M., Biesmeijer, J., Boatman, N., Budge, G. E., Crowe, A., … Kunin, W. E. (2017). A method for the objective selection of landscape-scale study regions and sites at the national level. *Methods in Ecology and Evolution, 8*(11), 1468-1476.

Gonzalez-Varo, J. P., Biesmeijer, J. C., Bommarco, R., Potts, S. G., Schweiger, O., Smith, H. G., … Vila, M. (2013). Combined effects of global change pressures on animal-mediated pollination. *Trends in Ecology & Evolution, 28*, 524-530.

Hegland, S. J., & Totland, Ø. (2012). Interactions for pollinator visitation and their consequences for reproduction in a plant community. *Acta Oecologica, 43*, 95-103.

Hegland, S. J., Nielsen, A., Lázaro, A., Bjerknes, A. L., & Totland, Ø. (2009). How does climate warming affect plant-pollinator interactions? *Ecology Letters, 12*, 184-195.

Hoehn, P., Tscharntke, T., Tylianakis, J. M., & Steffan-Dewenter, I. (2008). Functional group diversity of bee pollinators increases crop yield. *Proceedings of the Royal Society B: Biological Sciences, 275*, 2283-2291.

Inclan, D. J., Cerretti, P., & Marini, L. (2014). Interactive effects of area and connectivity on the diversity of tachinid parasitoids in highly fragmented landscapes. *Landscape Ecology, 29*, 879-889.

Jauker, F., Diekötter, T., Schwarzbach, F., & Wolters, V. (2009). Pollinator dispersal in an agricultural matrix: opposing responses of wild bees and hoverflies to landscape structure and distance from main habitat. *Landscape Ecology, 24*, 547-555.

Kleijn, D., Winfree, R., Bartomeus, I., Carvalheiro, L. G., Henry, M., Isaacs, R., … Potts, S. G. (2015). Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. *Nature Communications, 6*, art7414.

Legendre, P., & Legendre, L. (2012). *Numerical Ecology* (3rd English ed.). Amsterdam: Elsevier.

Loreau, M. (2010). T*he Challenges of Biodiversity Science*. Oldendorf/Luhe: International Ecology Institute.

Oertli, S., Muller, A., & Dorn, S. (2005). Ecological and seasonal patterns in the diversity of a species-rich bee assemblage (Hymenoptera: Apoidea: Apiformes). *European Journal of Entomology, 102*(1), 53-63.

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos, 120*, 321-326.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution, 25*, 345-353.

Rafferty, N.E. & Ives, A.R. (2012) Pollinator effectiveness varies with experimental shifts in flowering time. *Ecology, 93*(4), 803-814.

Rhoades, P., Griswold, T., Waits, L., Bosque-Perez, N. A., Kennedy, C. M., & Eigenbrode, S. D. (2017). Sampling technique affects detection of habitat factors influencing wild bee communities. *Journal of Insect Conservation, 21*, 703-714.

Rubene, D., Schroeder, M., & Ranius, T. (2015). Estimating bee and wasp (Hymenoptera: Aculeata) diversity on clear-cuts in forest landscapes - an evaluation of sampling methods. *Insect Conservation and Diversity, 8*, 261-271.

Saunders, M. E., & Luck, G. W. (2013). Pan trap catches of pollinator insects vary with habitat. *Australian Journal of Entomology, 52*, 106-113.

Senapathi, D., Carvalheiro, L. G., Biesmeijer, J. C., Dodson, C. A., Evans, R. L., McKerchar, M., … Potts, S. G. (2015). The impact of over 80 years of land cover changes on bee and wasp pollinator communities in England. *Proceedings of the Royal Society B: Biological Sciences, 282*, 8.

Thomas, D B, 2008. Nontoxic antimfreeze for insect traps. *Entomological News*. *119*(4), 361-365

Westphal, C., Bommarco, R., Carre, G., Lamborn, E., Morison, N., Petanidou, T., … Steffan-Dewenter, I. (2008). Measuring bee diversity in different European habitats and biogeographical regions. *Ecological Monographs, 78*, 653-671.

Wilson, J. S., Griswold T., & Messinger, O. J. (2008). Sampling bee communities (Hymenoptera: Apiformes) in a desert landscape: Are pan traps sufficient? *Journal of the Kansas Entomological Society, 81*, 288-300.

Authors: Gillespie MAK[1], Hegland SJ[1]

Reviewer: Sydenham MAK[2]

Affiliations

[1] Department of Environmental Sciences, Western Norway University of Applied Sciences, Sogndal, Norway

[2] Norwegian Institute for Nature Research, Oslo, Norway

4.13 Pollinator visitation

Authors: Hegland SJ[1], Gillespie MAK[1]

Reviewer: Sydenham MAK[2]

Measurement unit: number of pollinators per flower per time unit/various network descriptors; Measurement scale: site, plot, plant or flower; Equipment costs: €€€; Running costs: € Installation effort: low; Maintenance effort: low; Knowledge need: medium; Measurement mode: manual

Data on pollinator visitations are usually collected via observations of interactions between animal pollinators and flowers and can be recorded at one or several sites. Such pollinator visitation observations can be used across a wide range of climate- and global-change studies. The data are relevant both when performing experiments on plant or insects and when observing fine- to coarse-scale impacts of various factors. The data are specifically used to i) calculate the pollinator visitation rate, ii) describe the structure of individual plant–pollinator interaction networks (see Protocol 4.12 Pollinator composition), and iii) describe the structural differences between networks if several study sites are surveyed.

The pollinator visitation rate (also called "flower visitation rate", e.g. the number of pollinators visiting a flower per unit time) is the most commonly used variable to assess animal pollination and its contribution to plant reproduction (Garibaldi et al., 2013). Animal pollination is the most common pathway of sexual reproduction in plants and is considered a key regulating ecosystem service due to the mutual benefits for plants and pollinators. Nearly 90% of wild flowering plants depend on animal pollination to varying degrees (Ollerton et al., 2011) and pollination contributes up to 35% of global crop production volume (Klein et al., 2007; IPBES, 2016). As plant–pollinator interactions are thought to be sensitive to global climate changes through spatial or temporal mismatches or by interacting with other global drivers such as habitat loss and invasive species (Hegland et al., 2009; Schweiger et al., 2010), consequent changes in visitation rate may impact seed and fruit production in plants and crops. For example, reduced visitation rates may increase pollen limitation and decrease crop production (Allen-Wardell et al., 1998; Potts et al., 2010). Other studies have demonstrated potential climate-induced mismatches by manipulating flowering phenology and observing pollinator visitation (Rafferty & Ives, 2011; Gillespie et al., 2016). The protocols described here are relevant for studying a wide range of non-climatic anthropogenic influences on pollinator visitation such as habitat loss and fragmentation, invasive species, vegetation composition, and land-use change. Independent of any study aim, the variables can be collected as part of site census work, gradient studies, site comparisons, or plot-level experiments. However, it should be noted that it is often difficult to manipulate the pollinator side of the interaction as they operate on coarser scales than plants.

Plant–pollinator network descriptors have become common variables in the ecological literature. Network structure descriptors provide the researcher with the tools to compare communities in time and space by statistical network patterns (Bascompte et al., 2003; Olesen et al., 2008; Jordano, 2016). In particular, the structural descriptors of mutualistic networks may indicate their robustness to perturbations (Rezende et al., 2007) such as climate change (Hegland et al., 2010). While many diverse networks are considered to be robust against the perturbations associated with climate warming (Hegland et al., 2009), there is much still to learn about these food webs and global change (Schmidt et al., 2017).

4.13.1 What and how to measure?

Measuring the pollinator visitation rate necessarily includes the counting of both pollinators and flowers and is largely done non-destructively to avoid influencing the number of possible visits. However, the aim of the study influences the choice of scale (flower, plant, plot, site) and precision in the recordings. Plot-level studies often provide the best quality pollinator data, but plant-level recordings represent the highest quality data for both pollination and plant reproductive studies. Plot-level studies can be combined with plant/flower-level studies by tagging plants/flowers.

Plot-level recordings are often used for community studies where both plant and pollinator related questions are in focus. Such studies enable the observation of pollinator visitations to several plant species simultaneously and may involve the determination of pollinators to taxonomic group or species level (Hegland & Totland, 2005). Plot-level recordings are usually conducted within a set area, for example 1–5 × 1–5 m plots (or the circular equivalent), large enough to obtain the variation of plants and pollinators that are in focus and easily perceived by the observer(s) available. The plots are often permanent within or across seasons, but may also be single-census plots depending on the aim of study. First, the number of pollinator visitations to each plant is recorded within a set time-period, and second, the number of flowers or inflorescences for each flowering species is counted. The time-period of observation ranges in many studies from a few minutes to a few hours, depending on the study system and insect density. For example, in the High Arctic a low density of flying insects requires longer (e.g. 2 x 20 min per plot per day; Olesen et al., 2008) than, for example, in a temperate grassland (e.g. 1 x 10 min per plot per day; Hegland & Totland, 2005). Researchers must adapt to the system-specific properties and be guided by previous studies in similar environments (see also below).

Plant-level recordings are often used when research questions mainly relate to plant reproduction of one species (see also protocol 4.1 Plant sexual reproduction) or to pollination by specialist pollinator species. In these cases, the observations are the same as plot-level studies, but a number of individual plants or flowers of the focal species are marked and observed repeatedly. Recordings at this level are also used when observing pollinator visitation and plant reproduction at the plant level is difficult, for example in trees. In all cases, it is recommended that observations are carried out on multiple days and across multiple sites (Fijen & Kleijn, 2017).

To measure network structure descriptors and interaction turnover it is necessary to construct plant–pollinator networks, which also requires the sampling of species interactions (Figure 4.13.1). This can involve either standardised observation periods (often 2 x 20-min; e.g. Olesen et al., 2008) as described above, or preferably a transect method standardised by distance, time, or both (e.g. 25 m and/or 5 min subtransect; e.g. Westphal et al., 2008; Hegland et al., 2010; see also protocol 4.12. Pollinator composition). However, at higher latitudes and elevations sufficient data may only be attainable via the observation or plot method due to low species diversity and a reduced occurrence of interactions (Olesen et al., 2008). In the transect method, the observer walks along transects recording pollinators observed feeding from flowers or capturing them by netting for later identification (Hegland et al., 2010). In both cases, the flower species is also recorded. Plant–pollinator networks are then constructed, from which descriptors can be calculated (Nielsen & Bascompte 2007; Hegland et al., 2010; Jordano, 2016).

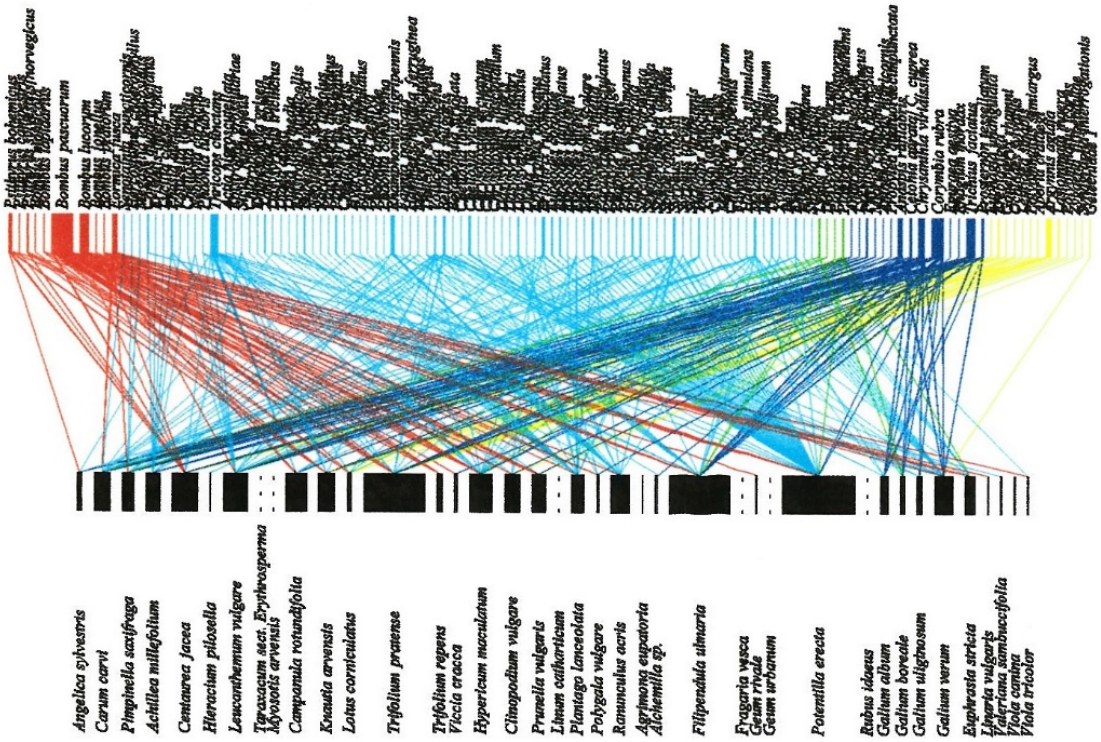


Figure 4.13.1 An example of a plant—pollinator network.

When using permanent plots or transects it is essential to mark and map their location to enable accurate resampling. Metal tubes may be inserted into the soil for relocation with a metal detector if other aboveground markings (e.g. plastic tubes, pegs, flags) may be disturbed by grazing, trampling, or other factors. When catching pollinators for later identification, much more equipment is required (sweep-net, storage vials, preservation materials; see also protocol on 4.12. Pollinator composition) and expert taxonomists may be required to determine specimens to species level (Prys-Jones & Corbet, 2011). If transects or observation periods are to be timed, a timer is required and it is recommended that *in situ* weather data are collected (temperature, rainfall, cloud cover, wind speed and direction) as these may be important covariates. Where possible, observations of insect visitation should be constrained to standardised weather conditions such as calm days with no rain and relatively high temperatures above to ensure the majority of the pollinator community are in flight (Totland, 1993, 1994; Kleijn et al., 2015).

Interpretation

The most common way to use and interpret pollinator visitation data is to use the number of flower visits by a pollinator species (i.e. *Bombus terrestris*), a pollinator group (*Bombus* sp.), or all pollinators to a single or multiple flowers per unit of time (Ricketts et al., 2008; King et al., 2013; Fijen & Kleijn, 2017). Alternatively, at the plot level the number of visits to flowers in a plot divided by the number of flowers for a set time period can be calculated (Lazaro et al., 2013). However, a recent study has warned against analysing proportions as both the numerator and denominator can have distinct and unknown error distributions. It may be preferable, therefore, to analyse the number of visitors as

count data and use the number of flowers as an "offset" variable (a component of the linear predictor of a Poisson model held constant, while other covariates are evaluated; Crawley, 2013) to account for sampling effect (Reitan & Nielsen, 2016). The rate of pollinator visitors to a plant or plot can be interpreted in a number of ways, depending on the research question. For example, a decline in visitation rate over time can be an indication of the effectiveness or state of the pollinator community (Ricketts et al., 2008), or, in comparative studies, as the degree of attraction of the plant/plot to insect visitors (Lazaro et al., 2013). More attractive or rewarding plant species and plots are likely to attract a greater number of total visits and, depending on the research design, visits from a wider range of species or taxonomic groups. These variables can also provide an indication of the relative size and composition of the local pollinator community, with more visits by a diverse range of pollinators expected in an area with a rich and healthy community.

When constructing plant–pollinator networks there are many potential statistics to calculate based on the two-part matrices (reviewed in Bascompte & Jordano, 2007), although care must be taken with their use as they can be sensitive to sample size (Nielsen & Bascompte, 2007). Basic metrics such as number and diversity of plant and pollinator species and the number of interactions can easily be calculated. In addition, nestedness, connectance, link density, and the degree-strength relationship are descriptors of mutualistic networks that provide an estimation of robustness against perturbations and losses in interactions (Bascompte et al., 2003; Rezende et al., 2007; Hegland et al., 2010). For example, networks with greater levels of connectance and nestedness have a stronger core of generalist species which subsequently aid the persistence of specialised interactions (Bascompte & Jordano, 2007). In general, the analysis of mutualist networks can be a complex undertaking and readers should become familiar with some key studies (see *Where to start* below) and theory before planning such a study.

Where to start

Bascompte & Jordano (2007), Hegland & Totland (2005), Hegland et al. (2010), Olesen et al. (2008), Rezende et al. (2007)

4.13.2 Special cases, emerging issues, and challenges

A key issue to determine is the length of time of the observation period, which depends on a number of factors. For example, the researcher should consider the time available for observations, the number of focal species, methods of studies with similar research questions, and the required sample size when choosing the time period. Furthermore, a recent study found that the minimum observation time required to accurately represent visitation rate varies between days and field locations with observations peaking in the middle of the day and at 29 ˚C (Fijen & Kleijn, 2017). Fijen & Kleijn (2017) conclude that the time taken for a set number of pollinators to visit the plot or plants may provide a more accurate estimate of visitation rate.

An additional emerging issue is the distinction between flower visitors and effective pollinators. In the absence of observations to the contrary, a flower-visiting insect touching the sexual organs of a flower may be assumed to be a pollinator and included in plant–pollinator networks (Hegland et al., 2010). However, in some cases the insect may not be effective as a pollinator, taking the floral reward without providing the pollination service, or may be an inefficient pollinator (King et al.,

2013). King et al. (2013) therefore propose the use of the variable "single-visit deposition" of pollen on virgin stigmas as a more practical measure of pollinator effectiveness. They further highlight the need to consider the effectiveness of insect-visitors in plant–pollinator network studies. In the strictest sense, if data on pollen deposition or pollinator effectiveness is lacking, flower-visitors should only be considered to be pollinating if they i) come into contact with the stigma and ii) have morphological features (e.g. hairs) that would allow them to carry and deposit pollen, otherwise they should be referred to as flower-visitors.

Methods described above for constructing plant–pollinator networks are "phyto-centric" in that the focus is on the visitors to the plant species. Alternative "zoo-centric" methods, such as sampling the pollen attached to the bodies of pollinators are less common (Jordano, 2016), perhaps because they require identification of both insect and pollen species. In some cases, such as the identification of pollen attached to museum specimens (Bartomeus et al., 2011), the extra identification work is not required, however. Nevertheless, combining data from both phyto- and zoo-centric studies could provide a more complete analysis of interactions and ecosystem services provision (Jordano, 2016). Networks can be constructed as qualitative or quantitative food webs containing information on interaction strength or species abundance with the quantitative approach containing most information (Memmott, 1999).

With some extra effort, planning, and species identification, plots and data used for calculating visitation rates may also be used to derive estimates of flowering phenology, pollinator composition and diversity, plant–pollinator networks, etc. (Westphal et al., 2008; Fijen & Kleijn, 2017); see also protocol 4.5 Aboveground plant phenology and 4.12 Pollinator composition.

The field of pollinator networks is constantly developing and understanding spatial turnover, and subsequently the heterogeneity among sites or communities, is central to many biodiversity issues. For example, new metrics such as the H2' index, which measures the degree of specialisation in networks, are thought to be more sensitive to perturbations (Blüthgen et al., 2006; Hoiss et al., 2015). Likewise, the identity of interacting species within plant–pollinator networks varies along environmental and spatial gradients (Trøjelsgaard et al., 2015), which potentially aids the long-term persistence and capacity for evolutionary adaptation under climate change (Burkle & Alarcon, 2011). The focus on interaction-turnover is relatively recent with many questions remaining unanswered (Burkle et al., 2016).

4.13.3 References

Theory, significance, and large datasets

Fijen & Kleijn (2017), Jordano (2016), King et al. (2013), Ricketts et al. (2008)

More on methods and existing protocols

Fijen & Kleijn (2017), Hegland et al. (2010), King et al. (2013)

All references

Allen-Wardell, G., Bernhardt, P., Bitner, R., Burquez, A., Buchmann, S., Cane, J., … Walker, S. (1998). The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. *Conservation Biology, 12*, 8-17.

Bartomeus, I., Ascher, J. S., Wagner, D., Danforth, B. N., Colla, S., Kornbluth, S., & Winfree, R. (2011). Climate-associated phenological advances in bee pollinators and bee-pollinated plants. *Proceedings of the National Academy of Sciences USA, 108*, 20645-20649.

Bascompte, J., & Jordano, P. (2007). Plant-animal mutualistic networks: the architecture of biodiversity. *Annual Review of Ecology Evolution and Systematics, 38,* 567-593.

Bascompte, J., Jordano, P., Melian, C. J., & Olesen, J. M. (2003). The nested assembly of plant-animal mutualistic networks. *Proceedings of the National Academy of Sciences USA, 100*, 9383-9387.

Blüthgen, N., Menzel, F., & Blüthgen, N. (2006). Measuring specialization in species interaction networks. *BMC Ecology, 6*, art9.

Burkle, L. A., & Alarcon, R. (2011). The future of plant-pollinator diversity: understanding interaction networks across time, space, and global change. *American Journal of Botany, 98*, 528-538.

Burkle, L. A., Myers, J. A., & Belote, R. T. (2016). The beta-diversity of species interactions: untangling the drivers of geographic variation in plant–pollinator diversity and function across scales. *American Journal of Botany, 103*, 118-128.

Crawley, M. J. (2013). *The R Book*. Chichester: John Wiley & Sons.

Fijen, T. P. M., & Kleijn, D. (2017). How to efficiently obtain accurate estimates of flower visitation rates by pollinators. *Basic and Applied Ecology, 19*, 11-18.

Garibaldi, L. A., Steffan-Dewenter, I., Winfree, R., Aizen, M. A., Bommarco, R., Cunningham, S. A., … Klein, A. M. (2013). Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science, 339*, 1608-1611.

Gillespie, M. A. K., Baggeson N., Cooper E. J. (2016). High Arctic flowering phenology and plant-pollinator interactions in response to delayed snow melt and simulated warming. *Environmental Research Letters, 11*(11): 115006.

Hegland, S. J., & Totland, Ø. (2005). Relationships between species' floral traits and pollinator visitation in a temperate grassland. *Oecologia, 145*, 586-594.

Hegland, S. J., Nielsen, A., Lázaro, A., Bjerknes, A. L., & Totland, Ø. (2009). How does climate warming affect plant-pollinator interactions? *Ecology Letters, 12*, 184-195.

Hegland, S. J., Dunne, J., Nielsen, A., & Memmott, J. (2010). How to monitor ecological communities cost-efficiently: the example of plant-pollinator networks. *Biological Conservation, 143*, 2092-2101.

Hoiss, B., Krauss, J., & Steffan-Dewenter, I. (2015). Interactive effects of elevation, species richness and extreme climatic events on plant-pollinator networks. *Global Change Biology, 21*, 4086-4097.

IPBES. (2016). *Summary for policymakers of the assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food*

production. Secretariat of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, Bonn, Germany.

Jordano, P. (2016). Sampling networks of ecological interactions. *Functional Ecology, 30*, 1883-1893.

King, C., Ballantyne, G., & Willmer, P. G. (2013). Why flower visitation is a poor proxy for pollination: measuring single-visit pollen deposition, with implications for pollination networks and conservation. *Methods in Ecology and Evolution, 4*, 811-818.

Kleijn, D., Winfree, R., Bartomeus, I., Carvalheiro, L. G., Henry, M., Isaacs, R., … Potts, S. G. (2015). Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. *Nature Communications, 6*, art7414.

Klein, A. M., Vaissiere, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences, 274*, 303-313.

Lazaro, A., Jakobsson, A., & Totland, Ø. (2013). How do pollinator visitation rate and seed set relate to species' floral traits and community context? *Oecologia, 173*, 881-893.

Memmott, J. (1999). The structure of a plant-pollinator food web. *Ecology Letters, 2*, 276-280.

Nielsen, A., & Bascompte, J. (2007). Ecological networks, nestedness and sampling effort. *Journal of Ecology, 95*, 1134-1141.

Olesen, J. M., Bascompte, J., Elberling, H., & Jordano, P. (2008). Temporal dynamics in a pollination network. *Ecology, 89*, 1573-1582.

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos, 120*, 321-326.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution, 25*, 345-353.

Prys-Jones, O. E., & Corbet, S. A. (2011). *Bumblebees* (3rd ed.). Exeter: Pelagic Publishing.

Rafferty, N. E., & Ives, A. R. (2011). Effects of experimental shifts in flowering phenology on plant-pollinator interactions. *Ecology Letters, 14*(1), 69-74.

Reitan, T., & Nielsen, A. (2016). Do not divide count data with count data; a story from pollination ecology with implications beyond. *PLoS ONE, 1*1, :e0149129.

Rezende, E. L., Lavabre, J. E., Guimaraes, P. R., Jordano, P., & Bascompte, J. (2007). Non-random coextinctions in phylogenetically structured mutualistic networks. *Nature, 448*, 925-928.

Ricketts, T. H., Regetz, J., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., Bogdanski, A., ... Viana, B. F. (2008). Landscape effects on crop pollination services: are there general patterns? *Ecology Letters, 1*1, 499-515.

Schmidt, N. M., Hardwick, B., Gilg, O., Hoye, T. T., Krogh, P. H., Meltofte, H., … Roslin, T. (2017). Interaction webs in arctic ecosystems: determinants of arctic change? *Ambio, 46*, S12-S25.

Schweiger, O., Biesmeijer, J. C., Bommarco, R., Hickler, T., Hulme, P. E., Klotz, S., … Settele, J. (2010). Multiple stressors on biotic interactions: how climate change and alien species interact to affect pollination. *Biological Reviews, 85*, 777-795.

Totland, Ø. (1993). Pollination in alpine Norway: flowering phenology, insect visitors, and visitation rates in two plant communities. *Canadian Journal of Botany, 71*,1072-1079.

Totland, Ø. (1994). Influence of climate, time of day and season, and flower density on insect flower visitation in alpine Norway. *Arctic and Alpine Research, 26*, 66-71.

Trøjelsgaard, K., Jordano, P., Carstensen, D. W., & Olesen, J. M. (2015). Geographical variation in mutualistic networks: similarity, turnover and partner fidelity. *Proceedings of the Royal Society B: Biological Sciences, 282*(1802), 2014.2925.

Westphal, C., Bommarco, R., Carre, G., Lamborn, E., Morison, N., Petanidou, T., … Steffan-Dewenter, I. (2008). Measuring bee diversity in different European habitats and biogeographical regions. *Ecological Monographs, 78*, 653-671.

Authors: Hegland SJ[1], Gillespie MAK[1]

Reviewer: Sydenham MAK[2]

Affiliations

[1] Department of Environmental Sciences, Western Norway University of Applied Sciences, Sogndal, Norway

[2] Norwegian Institute for Nature Research, Oslo, Norway

4.14 Plant pathogen and invertebrate herbivory

Authors: Wilfahrt P[1], Halliday FW[2]

Reviewers: Barrio IC[3], Te Beest M[4,5]

Measurable unit: damage severity/community load; Measurement scale: plot; Equipment costs: €; Running costs: none; Installation effort: low; Maintenance effort: low; Knowledge need: medium; Measurement mode: manual

Quantifying damage severity on plant organs from invertebrate herbivores and viral, bacterial, and fungal pathogens (herein collectively called plant enemies) helps connect often difficult-to-measure abundances of these enemies to plant community and species dynamics. Changes in enemy damage can reflect bottom-up ecosystem responses to changes in plant diversity resulting from changes in the local environment, such as increased CO_2 or temperature (Mitchell et al., 2003; Rúa et al., 2014). Plant enemies can also be directly affected by environmental changes and drive top-down changes to plant hosts due to changes in enemy abundance (Mordecai, 2011; Gillespie et al., 2013) or activity (Barrio et al., 2016). Understanding links between changes to the physical environment and plant-enemy interactions is critical as climate change can cause range expansion or increased abundance of plant enemies (Garrett et al., 2006). This protocol is applicable to other global-change drivers, such as soil eutrophication, as they can also affect the abundance and impacts of natural enemies at the species and community level (Halliday et al., 2019).

4.14.1 What and how to measure?

Damage by pathogens and invertebrate herbivores is typically more localised than that of vertebrate herbivores (Kotanen & Rosenthal, 2000). Vertebrates typically remove entire organs and damage is often quantified at the whole plant scale, as opposed to damage within plant organs that often characterise invertebrate consumers and plant disease. For vertebrate herbivory we refer to protocol 4.15 Vertebrate herbivory. This section focuses on aboveground invertebrate plant damage. Belowground plant enemies are more challenging to assess and are addressed in the subsection 4.14.2 *Special cases, emerging issues, and challenges*.

Leaf damage

Gold standard. The most commonly assessed plant organs for enemy damage are leaves as they receive the majority of aboveground damage and this type of damage is highly apparent (Barrio et al., 2017). Foliar damage is optimally assessed by collecting fully expanded, moderate-aged leaves from target species, scanning them, and quantifying the percentage of the leaf that has enemy damage using image analysis software (Parker et al., 2015). Leaves should be kept cool and moist during this period to avoid desiccation and shrinkage that will bias results. The percent leaf area with necrosis (cell death), chlorosis (chlorophyll insufficiency), or the area removed or affected by other feeding activities is symptomatic of enemy damage. As a minimum, leaf damage should be partitioned into pathogen and invertebrate herbivory damage, but it is recommended to further partition by damage type listed in Table 4.14.1. Examining a subset of leaves under magnification and

with a source of light through and against the leaf surface is recommended to ensure that categorisation into damage type is accurate (Barrio et al., 2017; see Figure 4.14.1). Here, researchers should look for evidence of mechanical damage (e.g. shredding, tearing of plant fibre) that is symptomatic of invertebrate damage, different shaped lesions with differently coloured moulds or powders that are symptomatic of fungal damage, or lesions without coloured moulds or powder that are symptomatic of bacterial or viral lesions (Liu et al., 2016). When none of these signs are evident, chlorosis is likely due to other environmental stresses (e.g. potassium deficiency) and should not be attributed to enemies. The effects of phloem-feeding insects are difficult to quantify, as sucking damage can be nearly impossible to detect. Nonetheless, the effects of these herbivores might be as relevant as other damage types (Kozlov et al., 2015b).

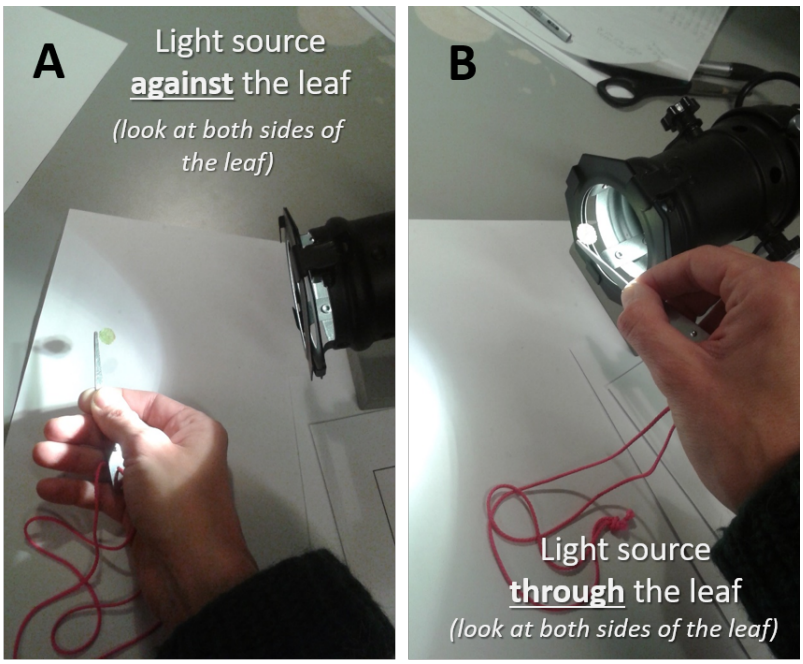


Figure 4.14.1 Using a light source can increase the apparency of damage. Be sure to check both sides, and also hold the leaf to the light to detect damage that may be less conspicuous.

The selection of focal plant species on which to measure damage will depend on the study system. For studies on single plant species or experimental communities that are maintained at specific compositions, a minimum of 20 leaves per species per plot or site should be sampled from different individuals (selected haphazardly) and at different plant heights, or split evenly among individuals when less than 20 individuals are present (Mitchell et al., 2002). Because damage can be strongly determined by leaf age (e.g. Halliday et al., 2017a), leaves should be selected by either stratifying across age classes (e.g. Halliday et al., 2017b) or through a randomisation procedure (e.g. Zvereva & Kozlov, 2019). In certain regions, such as tundra ecosystems, the frequency of damaged leaves is often low, thus larger sample sizes of at least 100 leaves per sampling unit are recommended (Barrio et al., 2017). For studies on natural vegetation, species selection should be based on vegetation cover surveys (see protocol 4.8 Plant community composition). Ideally all species will be sampled, but when species diversity is too great for this to be feasible, rare species can be removed (for example, Heckman et al. (2016) only used species with at least 5% cover in any plot). It should be noted that sampling fewer plant species will likely bias estimations of community damage severity (Zvereva & Kozlov, 2019), as rare species are more likely to avoid enemy damage (Mordecai, 2011) and rarity can arise as a consequence *of* enemy avoidance (Klironomos, 2002). Additionally, as the number of species sampled increases, it may be sensible to reduce replicate leaves per species, although we recommend never going below 5 leaves per species per plot.

Multiple surveys of permanently marked plants may reveal important components of enemy damage such as rates of herbivory (Anstett et al., 2016) or phenological characteristics and species

interactions among plant enemies (Halliday et al., 2017a). Depending on the research questions, the advantages of repeated measurements should be considered, although the time investment can be considerable. As such, we believe single surveys are, in most situations, adequate (Kozlov & Zvereva, 2017) and typically the only option given the destructive method we recommend as the gold standard. When only one damage survey is feasible, it is ideally matched to the peak growing season of the vegetation, unless there is a specific phenological focus of the study (e.g. spring budburst). An additional temporal consideration is that chronic, small-scale enemy damage can have stronger effects in the long-term than a short-term, large-scale enemy damage event (Mitchell, 2003; Zvereva et al., 2012).

Quantifying foliar damage in forest systems presents additional challenges due to the size of individual plants. In cases where significant portions of a tree are visibly damaged, it may be more practical to record foliar damage as a percentage of the individual affected, as opposed to surveying individual leaves (Eichhorn et al., 2016). In this scenario, damage to the crown should be recorded by estimating percent damage in 10% increments (i.e. 0%; 1–10%; 11–20% etc.). In temperate and boreal forest systems, enemy damage for woody species is often recorded at a higher level of specificity for damaging agents than herbaceous species, and you should refer to Tables IV-8 to IV-11 in the ICP Forests Protocol for possible causal agents (Eichhorn et al., 2016).

***Bronze standard*.** When scanning leaves is not an option, visual assessments of the percent of leaf tissue damaged by plant enemies can be estimated in the field. Quantifying percent damage for leaves is frequently aided by digitised images of known severity such as those provided for agricultural crops (James, 1971). When this technique is used, it is critical to have one party sampling all plants to avoid observer bias between samplers (e.g. Johnson et al., 2016). When multiple parties are required, they should "calibrate" their damage estimates on sample plants to avoid this bias as much as possible. Note that because of this bias, this method makes cross-study comparisons such as relative rates of damage inadvisable. Kozlov & Zvereva (2017) found that leaf scanning, measurements with a grid or dot grid method and visual assessment using cover classes yielded statistically indistinguishable estimates when specific plant–insect species herbivory was studied. Nevertheless, we recommend leaf scanning when possible as plant pathogen damage can be difficult to estimate accurately (Cooke, 2006) and scanning is the least subjective method, making between-research group comparisons possible. When visual assessment of percent leaf damage is the chosen method, the value of repeated sampling of leaves should be assessed as discussed above. A last option is to simply record presence or absence of damage on plant leaves, as has been recommended as a rapid assessment tool in other protocols (Meyer et al., 2015). However, we recommend against this when possible, as the loss of information is significant.

Damage to other plant organs

Stem damage is more difficult to assess as a percentage because these organs are generally not functionally two-dimensional like leaves. Thus, for herbaceous systems where stem damage may be important, it is recommended that 20 individuals per species of interest per plot are haphazardly selected and stem damage is recorded as presence/absence and partitioned by damage type when applicable (Roy et al., 2004). Damage prevalence can be calculated as the percentage of individuals showing damage symptoms across the sampled population. Stem damage to trees, however, can be

recorded using the same 10% increment classes as crown damage to estimate the average percentage of the stem circumference that is damaged along the entire bole (Eichhorn et al., 2016).

Plant reproductive organs are also susceptible to enemy damage, which can severely reduce plant fitness. Given the high degree of specialisation of inflorescences and seeds, a standardised method for assessing damage severity within such a structure is impractical (Stowe et al., 2000). Instead, damage prevalence can be measured by the percentage of damaged individuals (Abbate & Antonovics, 2014). Within individuals, the percentage of damaged organs can be recorded as a metric for damage severity (e.g. percent flowering tillers with damaged inflorescences, Groppe et al., 2001; percent damaged seeds, Asikainen & Mutikainen, 2005). Accounting for post-dispersal seed loss from invertebrates and pathogens requires experimental approaches that exclude enemies, generally by applying pesticide (Hulme, 1994) or fungicide (Clark & Wilson, 2003) to a known quantity of seeds and comparing post-exposure germination rates to a control. Studies of damage prevalence also pose difficulties. First, reproductive organs are often shorter lived and more variable in seasonal production than stems and leaves (McCall & Irwin, 2006). Thus, frequent surveys are required throughout the reproductive period, especially since certain periods (e.g. flowering) where damage is conspicuous may only last a few days. Second, differentiation between damage to specific reproductive structures is challenging, particularly as consumers may damage multiple structures simultaneously. Furthermore, damage to different reproductive structures, such as anthers v. calyxes, do not equally affect a plant's reproductive abilities. Collectively, this makes damage surveys of reproductive organs inadvisable at a community level without a high degree of expertise. It is thus advised that damage prevalence of reproductive structures be quantified at a population level only for species with known and observable damage symptoms.

Interpretation

When assessing foliar damage on a single host species, foliar enemy damage can be quantified by calculating damage severity at the plant species level as the average percentage area damaged per leaf. However, because total leaf area varies among plant species, it is inadvisable to compare damage severity among multiple plant species within a community. Instead, severity can be scaled up to the community level for among-host and cross-community comparisons by calculating the community load, which weights damage severity by each plant species' abundance (Mitchell et al., 2002):

$$l = \frac{\sum_{i=1}^{n} s_i a_i}{\sum_{i=1}^{n} a_i}$$

where n is the number of species surveyed, s_i is the damage severity in the i^{th} species, and a_i is the abundance (e.g. cover, biomass) of the i^{th} species. Observed increases in damage severity along a gradient or in response to experimental treatments indicate increased transmission, which could be attributed to factors such as increased host susceptibility or increased abundance or propagule supply of the damaging agent (Keesing et al., 2010). Increases in community load indicate a shift in plant host composition towards more competent host species. However, changes in community load have complicated relationships with plant host richness, where competing mechanisms may result in increased host richness either diluting damage severity (Liu et al., 2016) or amplifying it (Halliday et al., 2017b).

Where to start

Halliday et al. (2019), Kozlov & Zvereva (2017), Mitchell et al. (2002), Mordecai (2011), Parker et al. (2015)

4.14.2 Special cases, emerging issues, and challenges

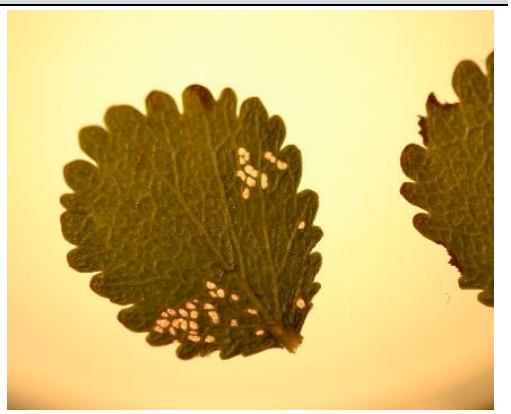
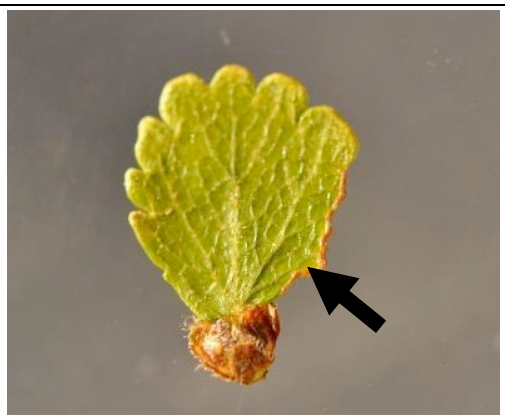
Species identification of fungal pathogens may be possible in some cases with laboratory microscopy and taxonomic expertise (Liu et al., 2016); otherwise it requires more arduous and costly culturing and sequencing techniques. The identification of microbial species from disease symptoms is challenging. High-throughput sequencing can be used to identify operational taxonomic units (OTUs) in various plant organs, but distinguishing pathogenic from non-pathogenic OTUs is difficult (Turner et al., 2013). For fungal species, fungal colonies can be cultured from diseased plant-organ fragments and fungal mycelia can be subsequently subjected to DNA sequencing and identified through comparison to sequences obtained from known pathogens (Hersh et al., 2012; Nguyen et al., 2016). However, care must still be taken in assigning pathogenic status to these fungi, as non-pathogenic organisms (e.g. saprotrophs) may be associated with observed diseases. Assigning pathogenic status to a single organism with confidence requires fulfilling several conditions derived from Koch's postulates (Plowright et al., 2008). Even then, some pathogens may remain latent within host tissue long before exhibiting disease symptoms (Photita et al., 2004).

Likewise, invertebrate communities require extensive effort for sampling using vacuum suction, sweep-netting, and pitfall traps (see protocol 4.11 Macroinvertebrate community composition), along with high taxonomic expertise (Siemann, 1998). Functional group identification based on damage type may be more practical when either high effort or taxonomic expertise is not an option (Table 4.14.1). However, quantifying enemy species richness from observed damage is inadvisable because, first, different enemies may cause highly similar damage symptoms (Kozlov et al., 2016) and second, differences in sampling efforts between plots due to differences in plant species richness can strongly misrepresent estimates of enemy richness and require site-based rarefaction (Halliday et al., 2017b).

Assessing the severity and prevalence of belowground plant enemies is highly challenging despite the importance of plant–soil feedbacks in structuring plant communities (Mordecai, 2011). Current alternative approaches include growing plant species in soil that was previously cultivated by conspecifics and comparing performance to soils cultivated by other species (Kulmatiski et al., 2008). This approach can only measure aggregate effects, meaning that potential effects of soil enemies can be masked by soil mutualists. A second approach is to leverage recent advances in sampling the soil microbial community (see protocol 4.9 Soil microbial community composition) and use developing techniques to separate organisms into functional groups that are pathogenic (Aguilar-Trigueros et al., 2014). Even then, this cannot be fully confirmed without rigorous fulfilment of a set of criteria such as Koch's postulates or some variation thereof (Plowright et al., 2008). In the case of root herbivores, the effects of herbivory may be experimentally manipulated by simulating damage (Zvereva & Kozlov, 2012) or by manipulating herbivore densities (Kozel et al., 2017).

Table 4.14.1 List of plant enemy types and descriptions of most apparent types of damage. Table adapted from ICP-forests protocol.

Class	Description	Picture
CAUSAL AGENT - MICROBES		
Leaf spot	Small round spots appearing on leaves. Fungal or bacterial.	 Photo: Erin Mordecai
Powdery mildew	White, powdery spots that appear on leaves and stems. Fungal.	 Photo: Anna-Liisa Laine
Downy mildew	Yellow patches on the leaf surface that expand and turn brown. Spores are visible under magnification. Fungal.	 Photo: Yawen Lu
Rust	Yellow to red hair-like structures covering leaves and stems. Often easily rubbed off with fingers. Fungal.	 Photo: Kayleigh O'Keefe

Blight	A general term for lesions that cause rapid chlorosis which expands and may eventually cause necrosis. Fungal, bacterial, or viral.	 Photo: Xiang Liu
	CAUSAL AGENT - INVERTEBRATES	
Skeletonised/ scraping	Removal of leaf epidermal tissue, causing sections of the leaf to appear translucent. (Image is of leaf with backlight to help identify damaged areas)	 Photo: Isabel Barrio
Chewing	Missing segments of leaf either as sporadic spots across the leaf or large sections.	 Photo: Isabel Barrio
Galling	Abnormal growths on plant tissue.	 Photo: Isabel Barrio

Mining	Visible, unbroken trail on the leaf surface, often moving erratically.	

Photo: Isabel Barrio

4.14.3 References

Theory, significance, and large datasets

Kozlov et al. (2015a), Mitchell et al. (2003), Mordecai (2011), Nguyen et al. (2016)

More on methods and existing protocols

ICP Forests protocol (Eichhorn et al., 2016)

All references

Abbate, J. L., & Antonovics, J. (2014). Elevational disease distribution in a natural plant–pathogen system: insights from changes across host populations and climate. *Oikos*, *123*(9), 1126-1136.

Aguilar-Trigueros, C. A., Powell, J. R., Anderson, I. C., Antonovics, J., & Rillig, M. C. (2014). Ecological understanding of root-infecting fungi using trait-based approaches. *Trends in Plant Science*, *19*(7), 432-438.

Anstett, D. N., Nunes, K. A., Baskett, C., & Kotanen, P. M. (2016). Sources of controversy surrounding latitudinal patterns in herbivory and defense. *Trends in Ecology & Evolution*, *31*(10), 789-802.

Asikainen, E., & Mutikainen, P. (2005). Preferences of pollinators and herbivores in gynodioecious *Geranium sylvaticum*. *Annals of Botany*, *95*(5), 879-886.

Barrio, I. C., Bueno, C. G., & Hik, D. S. (2016). Warming the tundra: reciprocal responses of invertebrate herbivores and plants. *Oikos*, *125*(1), 20-28.

Barrio, I. C., Lindén, E., Te Beest, M., Olofsson, J., Rocha, A., Soininen, E. M., ... Kozlov, M. V. (2017). Background invertebrate herbivory on dwarf birch (*Betula glandulosa-nana* complex) increases with temperature and precipitation across the tundra biome. *Polar Biology*, 40(11), 2265-2278.

Clark, D. L., & Wilson, M. V. (2003). Post-dispersal seed fates of four prairie species. *American Journal of Botany*, *90*(5), 730-735.

Cooke, B. (2006). Disease assessment and yield loss. In B. Cooke, D. Jones, & B. Kaye (Eds.), *The Epidemiology of Plant Diseases* (pp. 43-80). Dordrecht: Springer.

Eichhorn, J., Roskams, P., Potočić, N., Timmermann, V., Ferretti, M., Mues, V., … Wulff, S. (2016). Part IV: Visual Assessment of Crown Condition and Damaging Agents. In UNECE ICP Forests Programme Coordinating Centre (Ed.), *Manual on Methods and Criteria for Harmonized Sampling, Assessment, Monitoring and Analysis of the Effects of Air Pollution on Forests*. Eberswalde: Thünen Institute of Forest Ecosystems.

Garrett, K. A., Dendy, S. P., Frank, E. E., Rouse, M. N., & Travers, S. E. (2006). Climate change effects on plant disease: genomes to ecosystems. *Annual Review of Phytopathology*, *44*, 489-509.

Gillespie, M. A. K., Jónsdóttir, I. S., Hodkinson, I. D., & Cooper, E. J. (2013). Aphid–willow interactions in a High Arctic ecosystem: responses to raised temperature and goose disturbance. *Global Change Biology*, *19*(12), 3698-3708.

Groppe, K., Steinger, T., Schmid, B., Baur, B., & Boller, T. (2001). Effects of habitat fragmentation on choke disease (*Epichloë bromicola*) in the grass *Bromus erectus*. *Journal of Ecology*, *89*(2), 247-255.

Halliday, F. W., Umbanhowar, J., & Mitchell, C. E. (2017a). Interactions among symbionts operate across scales to influence parasite epidemics. *Ecology Letters*, *20*(10), 1285-1294.

Halliday, F. W., Heckman, R. W., Wilfahrt, P. A., & Mitchell, C. E. (2017b). A multivariate test of disease risk reveals conditions leading to disease amplification. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1865), 20171340.

Halliday, F. W., Heckman, R. W., Wilfahrt, P. A., & Mitchell, C. E. (2019). Past is prologue: host community assembly and the risk of infectious disease over time. *Ecology Letters*, *22*(1), 138-148.

Heckman, R. W., Wright, J. P., & Mitchell, C. E. (2016). Joint effects of nutrient addition and enemy exclusion on exotic plant success. *Ecology*, *97*(12), 3337-3345.

Hersh, M. H., Vilgalys, R., & Clark, J. S. (2012). Evaluating the impacts of multiple generalist fungal pathogens on temperate tree seedling survival. *Ecology*, *93*(3), 511-520.

Hulme, P. E. (1994). Post-dispersal seed predation in grassland: its magnitude and sources of variation. *Journal of Ecology*, *82*(3), 645-652.

James, W. C. (1971). An illustrated series of assessment keys for plant diseases, their preparation and usage. *Canadian Plant Disease Survey,* 51, 39-65

Johnson, M. T. J., Bertrand, J. A. & Turcotte, M. M. (2016). Precision and accuracy in quantifying herbivory. *Ecological Entomology*, *41*(1), 112-121.

Keesing, F., Belden, L. K., Daszak, P., Dobson, A., Harvell, C. D., Holt, R. D., ... Myers, S. S. (2010). Impacts of biodiversity on the emergence and transmission of infectious diseases. *Nature*, *468*(7324), 647-652.

Klironomos, J. N. (2002). Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature, 417*(6884), 67.

Kotanen, P. M., & Rosenthal, J. P. (2000). Tolerating herbivory: does the plant care if the herbivore has a backbone? *Evolutionary Ecology, 14*, 537–549

Kozel, A. V., Zvereva, E. L., & Kozlov, M. V. (2017). Impacts of root herbivory on seedlings of three species of boreal forest trees. *Applied Soil Ecology, 117-118*, 203-207

Kozlov, M. V., & Zvereva, E. L. (2017). Background insect herbivory: impacts, patterns and methodology. *Progress in Botany, 79*, 313-355.

Kozlov, M. V., Lanta, V., Zverev, V., & Zvereva, E. L. (2015a). Global patterns in background losses of woody plant foliage to insects. *Global Ecology and Biogeography*, *24*(10), 1126-1135.

Kozlov, M. V., Stekolshchikov, A. V., Söderman, G., Labina, E. S., Zverev, V., & Zvereva, E. L. (2015b). Sap-feeding insects on forest trees along latitudinal gradients in northern Europe: a climate-driven patterns. *Global Change Biology*, *21*(1), 106-116.

Kozlov, M. V., Skoracka, A., Zverev, V., Lewandowski, M. & Zvereva, E. L. (2016). Two birch species demonstrate opposite latitudinal patterns in infestation by gall-making mites in Northern Europe. *PLoS ONE*, *11*(11), e0166641.

Kulmatiski, A., Beard, K. H., Stevens, J. R., & Cobbold, S. M. (2008). Plant–soil feedbacks: a meta-analytical review. *Ecology Letters*, *11*(9), 980-992.

Liu, X., Lyu, S., Zhou, S., & Bradshaw, C. J. (2016). Warming and fertilization alter the dilution effect of host diversity on disease severity. *Ecology*, *97*(7), 1680-1689.

McCall, A. C., & Irwin, R. E. (2006). Florivory: the intersection of pollination and herbivory. *Ecology Letters*, *9*(12), 1351-1365.

Meyer, S. T., Koch, C., & Weisser, W. W. (2015). Towards a standardized rapid ecosystem function assessment (REFA). *Trends in Ecology & Evolution, 30*(7), 390-397.

Mitchell, C. E. (2003). Trophic control of grassland production and biomass by pathogens. *Ecology Letters*, *6*(2), 147-155.

Mitchell, C. E., Tilman, D., & Groth, J. V. (2002). Effects of grassland plant species diversity, abundance, and composition on foliar fungal disease. *Ecology*, *83*(6), 1713-1726.

Mitchell, C. E., Reich, P. B., Tilman, D., & Groth, J. V. (2003). Effects of elevated CO_2, nitrogen deposition, and decreased species diversity on foliar fungal plant disease. *Global Change Biology*, *9*(3), 438-451.

Mordecai, E. A. (2011). Pathogen impacts on plant communities: unifying theory, concepts, and empirical work. *Ecological Monographs*, *81*(3), 429-441.

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., ... Kennedy, P. G. (2016). FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology, 20*, 241-248.

Parker, I. M., Saunders, M., Bontrager, M., Weitz, A. P., Hendricks, R., Magarey, R., ... Gilbert, G. S. (2015). Phylogenetic structure and host abundance drive disease pressure in communities. *Nature*, *520*(7548), 542-544.

Photita, W., Lumyong, S., Lumyong, P., McKenzie, E. H. C., & Hyde, K. D. (2004). Are some endophytes of *Musa acuminata* latent pathogens? *Fungal Diversity, 16*, 131-140.

Plowright, R. K., Sokolow, S. H., Gorman, M. E., Daszak, P., & Foley, J. E. (2008). Causal inference in disease ecology: investigating ecological drivers of disease emergence. *Frontiers in Ecology and the Environment, 6*(8), 420-429.

Roy, B. A., Güsewell, S., & Harte, J. (2004). Response of plant pathogens and herbivores to a warming experiment. *Ecology*, *85*(9), 2570-2581.

Rúa, M. A., McCulley, R. L., & Mitchell, C. E. (2014). Climate drivers, host identity and fungal endophyte infection determine virus prevalence in a grassland ecosystem. *Journal of Ecology*, *102*(3), 690-699.

Siemann, E. (1998). Experimental tests of effects of plant productivity and diversity on grassland arthropod diversity. *Ecology*, *79*(6), 2057-2070.

Stowe, K. A., Marquis, R. J., Hochwender, C. G., & Simms, E. L. (2000). The evolutionary ecology of tolerance to consumer damage. *Annual Review of Ecology and Systematics*, *31*(1), 565-595.

Turner, T. R., James, E. K., & Poole, P. S. (2013). The plant microbiome. *Genome Biology*, *14*(6), 209.

Zvereva, E. L., & Kozlov, M. V. (2012). Sources of variation in plant responses to belowground insect herbivory: a meta-analysis. *Oecologia, 169*, 441-452.

Zvereva, E. L., & Kozlov, M. V. (2019). Biases in studies of spatial patterns in insect herbivory. *Ecological Monographs*, e01361.

Zvereva, E. L., Zverev, V., & Kozlov, M. V. (2012). Little strokes fell great oaks: minor but chronic herbivory substantially reduces birch growth. *Oikos*, *121*(12), 2036-2043.

Authors: Wilfahrt P[1], Halliday FW[2]

Reviewers: Barrio IC[3], Te Beest M[4,5]

Affiliations

[1] Department of Disturbance Ecology, BayCEER, University of Bayreuth, Bayreuth, Germany

[2] Department of Biology, University of North Carolina, Chapel Hill, USA

[3] Department of Natural Resources and Environmental Sciences, Agricultural University of Iceland, Reykjavík, Iceland

[4] Environmental Sciences, Copernicus Institute of Sustainable Development, Utrecht University, Utrecht, the Netherlands

[5] Centre for African Conservation Ecology, Nelson Mandela University, Port Elizabeth, South Africa

4.15 Vertebrate herbivory

Authors: Speed JDM[1], Barrio IC[2], Ravolainen VT[3]

Reviewers: Te Beest M[4,5] & Wilfahrt P[6]

Measurement unit: various (see below); Measurement scale: site to plot (herbivore presence), plot to individual (herbivore damage); Equipment costs: €–€€; Running costs: €; Installation effort: low; Maintenance effort: low; Knowledge need: medium; Measurement mode: manual

Vertebrate herbivory can have dramatic impacts on ecological dynamics at the scale of individual plants, populations, communities, and ecosystem functioning. Herbivore impact on vegetation can be either direct through biomass removal (including grazing, browsing, uprooting, bark stripping) and trampling or indirect through, for example, altered nutrient cycling, vector (e.g. seed) dispersal, disturbance, and changed competitive environments. Herbivory is nearly omnipresent across the world's ecosystems (Burkepile & Parker, 2017) and its impact on vegetation and ecosystems can outweigh that of climatic change (Post & Pedersen, 2008). However, climate-change studies often fail to quantify herbivory, even though this can be a confounding effect of climatic manipulations.

Assessments of vertebrate herbivory must account for spatial and temporal variation (Austrheim et al., 2011) and may be scale dependent (Senft et al., 1987). Vertebrate herbivores themselves respond directly to environmental change, for example by changing their behaviour, movement patterns, or population dynamics. However, when manipulating environmental change in experiments, treatments may inadvertently affect the activity of vertebrate herbivores, for example, open topped chambers can operate as partial exclosures of vertebrate herbivores (Post & Pedersen, 2008). Assessing vertebrate herbivory is therefore important to avoid confounding effects in climate-change and other environmental manipulation experiments and should be carried out at both site and plot scales. Assessing vertebrate herbivory is also valuable within observational studies. Different species of herbivore can have distinct impacts on ecosystems (e.g. van der Plas et al., 2016); therefore it can be useful to identify vertebrate herbivores to species, or group, where possible.

4.15.1 What and how to measure?

Vertebrate herbivory typically manifests as the removal of plant tissues or organs and can be measured through signs of tissue or organ removal (incidence of shoots or leaves with signs of grazing, browsing, or other types of herbivory) or estimates of biomass removal. However, because vertebrate herbivory is often difficult to detect due to complete removal of individuals, it is often recorded using signs of herbivore presence or activity (e.g. faecal densities, trampling, or disturbance signs). The presence or activity of herbivores should be assessed at a site level, while plant tissue removal is most commonly measured at a plant level, or in small sampling plots.

Herbivore presence or activity

The presence, density, and species composition of vertebrate herbivores is most commonly estimated through indirect assessments of faecal (dung) densities (Figure 4.15.1). These often correlate well with herbivore densities and herbivore species can be identified from the dung

(Putman, 1984): many region-specific field guides to identifying animals from tracks and signs exist. Comparisons across sites with different herbivore assemblages are challenging because the identity and relative abundance of different herbivore species can lead to distinct impacts on vegetation. However, indices of "herbivore pressure" can be approximated based on metabolic requirements of each species, as a way to make comparisons at broader spatial scales (Austrheim et al., 2011). For large herbivore species, regional density data may be available (e.g. livestock or hunting statistics) for the study sites, but given the spatial and temporal scale hierarchies of herbivory (Senft et al., 1987) these are often of incorrect spatial or temporal resolution (Bernes et al., 2015).

Figure 4.15.1 Faeces of herbivores, like those of A) hoary marmots (*Marmota caligata*), B) pink-footed goose (*Anser brachyrhynchus*), or C) reindeer (*Rangifer tarandus*) can be used to infer the presence or activity of herbivores. Photos: Isabel C Barrio

Faecal densities, typically estimated as densities of pellet groups, can be estimated along line transects (e.g. Cromsigt et al., 2009) or by area (e.g. quadrat) approaches (e.g. Ims et al., 2007). Observer bias (Jenkins & Manly, 2008) and detection probabilities (Marques et al., 2001) should ideally be accounted for. Care should be taken that the spatial scale is appropriate given the ecology of the plant–herbivore interactions in consideration, relative to the size of the environmental-manipulation study plots. For example, some herbivores are likely to have an impact at a scale larger than the experimental plots, such as wide-ranging animals. In these cases, recording herbivore presence at the site level is desirable, because herbivory might be spatially variable and thus more difficult to detect in smaller plots. If the interest of the study is in assessing temporal (e.g. inter-annual) variation in herbivore densities in a certain area, permanent plots or transects can be marked, where faecal pellets are removed during each visit.

Care should be taken in the interpretation of faecal densities at smaller spatial scales since dung and feeding localities become decoupled within daily ranges (with higher faecal densities at, for example, rest sites). Dung densities estimate defecation rates, not necessarily herbivory rates since defecation may vary with season, diet, etc. Furthermore, there is a need to account for dung decay that varies with dietary and environmental factors (Marques et al., 2001): this may be achieved using measurements of dung decomposition under field conditions (e.g. Sitters et al., 2014) or repeated visitation of the same pellet groups (Tsaparis et al., 2009). Dung detection can also vary across different sized species, with a lower chance of detecting pellets of smaller species, or across different habitat types, with a lower chance of detecting dung in denser (ground) vegetation.

A number of other approaches can be used to estimate herbivore presence and densities. These include direct detection of herbivores using camera traps (Brodie et al., 2012) including adaptations for rodent herbivores and subnivean activity (Soininen et al., 2015). Camera traps are more effective

to monitor visitation than pellet counts, particularly for species with smaller pellet sizes and closely related species, such as co-occurring deer species (Pfeffer et al., 2018). In addition to these, indirect signs of herbivore presence or activity can be used to record herbivore activity. These include track plots (Lyra-Jorge et al., 2008) or trampling indicators that detect passage of animals, or the presence of rodent runways, winter nests, or latrines (Sutherland, 2006). Indirect indicators can, in the same way as dung surveys, be quantified along transects or using area approaches. More labour-intensive and direct methods such as live-trapping or direct observations can also be applied where conditions allow, taking care to standardise effort between treatments. For live-trapping, this involves standardising the number of traps, bait amount (if used), and duration of trapping period. For direct observation this involves standardising duration of observation, with ideally the same observer between sites. In all cases, these parameters should be reported in the methods.

In some cases, signs of herbivore presence are not easily assigned to a certain herbivore species, or they may only give an indication of relative abundance, for example when quantifying indirect signs of herbivore presence: nevertheless, this information is valuable to approximate "herbivore pressure" at each site and may provide at least some qualitative information for comparisons across sites.

Plant damage

While herbivore presence indicates the potential for impacts of vertebrate herbivores on vegetation and ecosystems, direct measurements of herbivory are more valuable in assessing how herbivores have affected individual plants. Since herbivory involves the removal of plant tissues, this requires assessment of plant parts that are no longer present. This tends to be simpler in practice for woody plant species than for herbaceous plant species. Exclusion of herbivores is one of the most direct ways of assessing the amount of biomass removed by the animals. Often, using size-selective exclosures is the only possible option to separate between different size classes of herbivores (Kartzinel et al., 2014).

For woody plant species, browsed shoots can be observed in the field (Figure 4.15.2). Browsing pressure is generally assessed as the proportion of shoots that have been browsed. Depending on the shoot structure of the plant species all shoots can be assessed, or in the case of

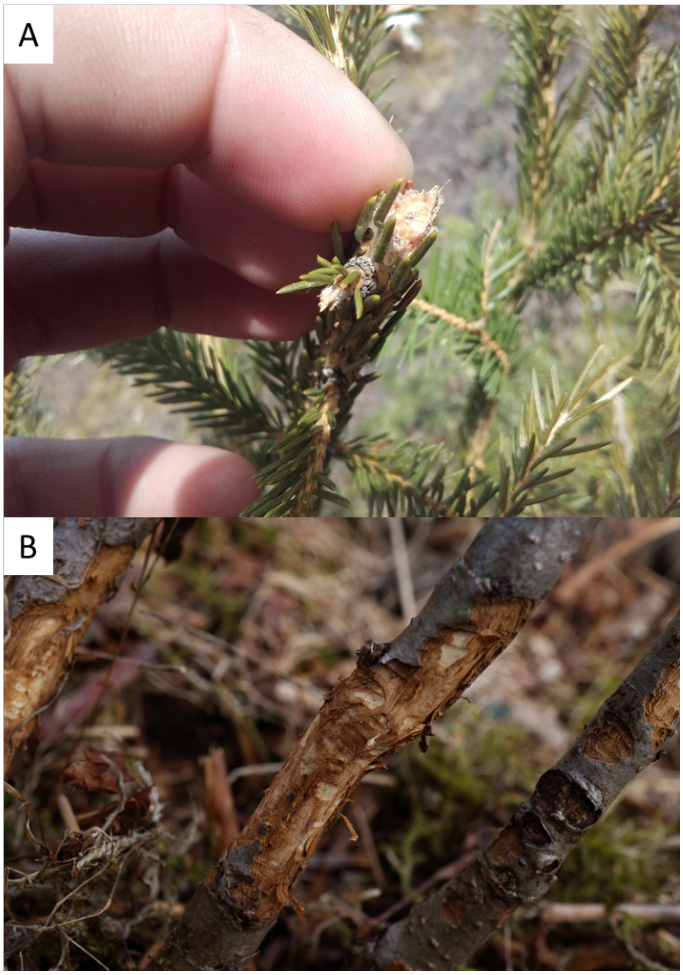


Figure 4.15.2 Direct browsing signs in boreal forest ecosystems. A) *Alces alces* browsing on *Picea abies* and B) rodent gnawing on small stems of *Sorbus aucuparia*. Photos: Anders L. Kolstad

many shoots (e.g. over 100), a subsample, for example every third shoot (Bilyeu et al., 2007). It is possible, in some cases, to distinguish herbivore species (or functional groups) on the basis of the browsing marks since these relate to herbivore dental morphology. The location of browsing signs relative to height and shoot diameter can also be used to partition browsing between herbivore species, although factors such as snow depth also need to be taken into account. Damage to plant parts also depends on plant defensive strategies (see protocol 4.16 Functional traits). Two main factors determining plant structural defence include leaf dimensions and spinescence (see Pérez-Harguindeguy et al., 2013). A recently developed bite size index measures both dimensions simultaneously by using human bites as a proxy to simulate the bite size of a medium-sized mammalian herbivore (Charles-Dominique et al., 2015).

Grazing on herbaceous vegetation can also be assessed as the proportion of shoots grazed or by quantifying grassland heterogeneity as a proportion of short grass (e.g. Cromsigt & Te Beest, 2014). Here, however, it is rarely possible to distinguish herbivore species from grazing marks. These methods are less robust due to the potential for whole organs to be removed and the difficulties in observing grazing signs. As such, herbivore exclusion is the recommended approach for assessing herbivore impacts on herbaceous vegetation, although this would require additional experimental plots.

Timing is crucial for assessing tissue removal by herbivores since browsing and grazing marks become less visible as time since the event increases. Grazing signs are particularly transient. Repeated assessments are therefore recommended, with a minimum frequency of annually for browsing and monthly for grazing.

Camera trap installation

Most approaches for quantifying herbivore densities or herbivory require no prior installation of equipment other than permanent marking of plots or transects if repeated visits are planned, or installation of fences, exclusion cages, or track plots. An exception is the use of automatic trail cameras, also known as wildlife cameras or camera traps, to assess the presence and species of herbivores (Figure 4.15.3). The use of camera traps in ecological monitoring has greatly increased in recent decades (Ahumada et al., 2011; Meek et al., 2014) and cameras are now widely available from a number of manufacturers and for a range of budgets. Placement and installation will depend on the specific research questions. Most studies stress the importance of adequate sampling effort for adequate community-level inferences (Hamel et al., 2013; Cusack et al., 2015). Cameras are usually installed by attaching them to nearby trees or posts, but the height and orientation of the camera (horizontal or perpendicular to the ground) should be considered as they will maximise detection of species of different body size (Tobler et al., 2008; Glen et al., 2013). Most models allow the user to set detection levels, number of photos in a burst, or video recording, and some units can use mobile telephone networks to send images. Maintenance will depend on the battery life and image storage capacity of the unit deployed, but this is becoming less of an issue nowadays. The occurrence of false positive (camera triggered but not by target) and false negative images (camera not triggered by herbivore) needs to be considered (see e.g. Newey et al., 2015 for further details). Motion-triggered settings are more affected by weather, as extremes in temperature or motion caused by wind will increase false positives. The use of time-lapse rather than motion-triggered cameras can overcome the problem of false negatives (Hamel et al., 2013), and the use of time-lapse at short intervals has

been recommended to optimise detection rates. Images require careful visual assessment to identify herbivores within images (to species level and to separate from other animals in the image). Detection rates can be interpreted as a proxy of the intensity of herbivore pressure over a given area or time.

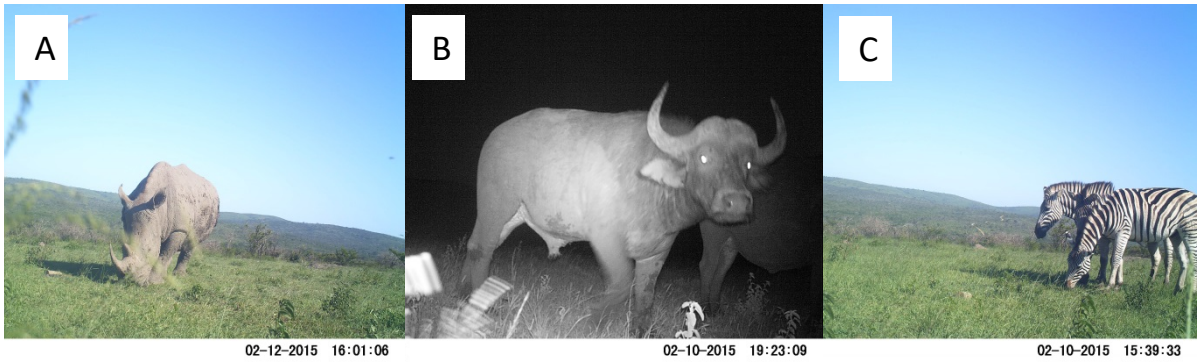


Figure 4.15.3 Images from camera traps: A) White rhino (*Ceratotherium simum*), B) African buffalo (*Syncerus caffer*), and C) zebra (*Equus quagga*), which can be used to monitor herbivore presence, densities, and/or activity. Photos: Joris PGM Cromsigt and Elizabeth le Roux.

Where to start

Faecal counts: Marques et al. (2001), Putman (1984); camera trapping: Meek et al. (2014); plant damage: Bilyeu et al. (2007)

4.15.2 Special cases, emerging issues, and challenges

Some species of herbivore show specific activities or behaviours that will have unique impacts on vegetation, for example digging by burrowing species (Huntly & Reichman, 1994), grubbing and uprooting by wildfowl (Speed et al., 2009) or suidae (Bueno et al., 2009), and bark stripping by some ungulates (Gill, 1992). Quantifying these types of activities, most commonly through transect or area approaches as outlined for other herbivory signs above, can give additional information on herbivore use of an area. Metabarcoding and other environmental DNA techniques are becoming more common approaches for the identification of plant species eaten by herbivores based on faecal samples or the identification of herbivore species themselves through DNA retained on bite marks (Nichols et al., 2015).

4.15.3 References

Theory, significance, and large datasets

Burkepile & Parker (2017), Post & Pedersen (2008), Senft et al. (1987)

More on methods and existing protocols

Bilyeu et al. (2007), Glen et al. (2013), Pfeffer et al. (2018)

ITEX herbivory protocol (tundra habitat specific):
http://herbivory.biology.ualberta.ca/files/2016/11/itex-herbivory-protocol_2016.pdf

All references

Ahumada, J. A., Silva, C. E., Gajapersad, K., Hallam, C., Hurtado, J., Martin, E., ... Rovero, F. (2011). Community structure and diversity of tropical forest mammals: data from a global camera trap network. *Philosophical Transactions of the Royal Society B: Biological Sciences, 366*(1578), 2703-2711.

Austrheim, G., Solberg, E. J., & Mysterud, A. (2011). Spatio-temporal distribution of large herbivores in Norway from 1949 to 1999: has decreased grazing by domestic herbivores been countered by increased browsing by cervids? *Wildlife Biology, 17*, 1-13.

Bernes, C., Bråthen, K. A., Forbes, B. C., Speed, J. D. M., & Moen, J. (2015). What are the impacts of reindeer/caribou (*Rangifer tarandus* L.) on arctic and alpine vegetation? *Environmental Evidence, 4*(1), 4.

Bilyeu, D. M., Cooper, D. J., & Hobbs, N. T. (2007). Assessing impacts of large herbivores on shrubs: tests of scaling factors for utilization rates from shoot-level measurements. *Journal of Applied Ecology, 44*(1), 168-175.

Brodie, J., Post, E., Watson, F., & Berger, J. (2012). Climate change intensification of herbivore impacts on tree recruitment. *Proceedings of the Royal Society B: Biological Sciences, 279*(1732), 1366-1370.

Bueno, C., Alados, C., Gómez-García, D., Barrio, I. C., & García-González, R. (2009). Understanding the main factors in the extent and distribution of wild boar rooting on alpine grasslands. *Journal of Zoology, 279*(2), 195-202.

Burkepile, D. E., & Parker, J. D. (2017). Recent advances in plant-herbivore interactions. *F1000Research, 6*, 119.

Charles-Dominique, T., Midgley, G., & Bond, W. (2015). An index for assessing effectiveness of plant structural defences against mammal browsing. *Plant Ecology*, *216*(10), 1433-1440.

Cromsigt, J. P., & Te Beest, M. (2014). Restoration of a megaherbivore: landscape-level impacts of white rhinoceros in Kruger National Park, South Africa. *Journal of Ecology, 102*(3), 566-575.

Cromsigt, J. P., van Rensburg, S. J., Etienne, R. S. & Olff, H. (2009). Monitoring large herbivore diversity at different scales: comparing direct and indirect methods. *Biodiversity and Conservation, 18*(5), 1219-1231.

Cusack, J. J., Dickman, A. J., Rowcliffe, J. M., Carbone, C., Macdonald, D. W., & Coulson, T. (2015). Random versus game trail-based camera trap placement strategy for monitoring terrestrial mammal communities. *PLoS One, 10*(5), e0126373.

Gill, R. (1992). A review of damage by mammals in north temperate forests: 1. Deer. *Forestry, 65*(2), 145-169.

Glen, A. S., Cockburn, S., Nichols, M., Ekanayake, J., & Warburton, B. (2013). Optimising camera traps for monitoring small mammals. *PLoS One, 8*(6), e67940.

Hamel, S., Killengreen, S. T., Henden, J. A., Eide, N. E., Roed-Eriksen, L., Ims, R. A., & Yoccoz, N. G. (2013). Towards good practice guidance in using camera-traps in ecology: influence of

sampling design on validity of ecological inferences. *Methods in Ecology and Evolution, 4*(2), 105-113.

Huntly, N., & Reichman, O. (1994). Effects of subterranean mammalian herbivores on vegetation. *Journal of Mammalogy, 75*(4), 852-859.

Ims, R. A., Yoccoz, N. G., Brathen, K. A., Fauchald, P., Tveraa, T., & Hausner, V. (2007). Can reindeer overabundance cause a trophic cascade? *Ecosystems, 10*, 607-622.

Jenkins, K. J., & Manly, B. F. J. (2008). A double-observer method for reducing bias in faecal pellet surveys of forest ungulates. *Journal of Applied Ecology, 45*(5), 1339-1348.

Kartzinel, T. R., Goheen, J. R., Charles, G. K., DeFranco, E., Maclean, J. E., Otieno, T. O., ... Pringle, R. M. (2014). Plant and small-mammal responses to large-herbivore exclusion in an African savanna: five years of the UHURU experiment. *Ecology, 95*(3), 787-787.

Lyra-Jorge, M. C., Ciocheti, G., Pivello, V. R., & Meirelles, S. T. (2008). Comparing methods for sampling large- and medium-sized mammals: camera traps and track plots. *European Journal of Wildlife Research, 54*(4), 739.

Marques, F. F., Buckland, S. T., Goffin, D., Dixon, C. E., Borchers, D. L., Mayle, B. A., & Peace, A. J. (2001). Estimating deer abundance from line transect surveys of dung: sika deer in southern Scotland. *Journal of Applied Ecology, 38*(2), 349-363.

Meek, P., Ballard, G., Claridge, A., Kays, R., Moseby, K., O'Brien, T., ... Tobler, M. (2014). Recommended guiding principles for reporting on camera trapping research. *Biodiversity and Conservation, 23*(9), 2321-2343.

Newey, S., Davidson, P., Nazir, S., Fairhurst, G., Verdicchio, F., Irvine, R. J., & van der Wal, R. (2015). Limitations of recreational camera traps for wildlife management and conservation research: A practitioner's perspective. *Ambio, 44*(4), 624-635.

Nichols, R. V., Cromsigt, J. P., & Spong, G. (2015). Using eDNA to experimentally test ungulate browsing preferences. *SpringerPlus, 4*(1), 489.

Pérez-Harguindeguy, N., Diaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., … Cornelisson, J. H. C. (2013). New handbook for standardised measurement of plant functional traits worldwide. *Australian Journal of Botany*, 61(3), 167-234.

Pfeffer, S. E., Spitzer, R., Allen, A. M., Hofmeester, T. R., Ericsson, G., Widemo, F., ... Cromsigt, J. P. (2018). Pictures or pellets? Comparing camera trapping and dung counts as methods for estimating population densities of ungulates. *Remote Sensing in Ecology and Conservation, 4*(2), 173-183.

Post, E., & Pedersen, C. (2008). Opposing plant community responses to warming with and without herbivores. *Proceedings of the National Academy of Sciences USA, 105*(34), 12353-12358.

Putman, R. J. (1984). Facts from faeces. *Mammal Review, 14*(2), 79-97.

Senft, R. L., Coughenour, M. B., Bailey, D. W., Rittenhouse, L. R., Sala, O. E., & Swift, D. M. (1987). Large herbivore foraging and ecological hierarchies. *Bioscience, 37*(11), 789-799.

Sitters, J., Maechler, M. J., Edwards, P. J., Suter, W., & Olde Venterink, H. (2014). Interactions between C:N:P stoichiometry and soil macrofauna control dung decomposition of savanna herbivores. *Functional Ecology, 28*(3), 776-786.

Soininen, E. M., Jensvoll, I., Killengreen, S. T., & Ims, R. A. (2015). Under the snow: a new camera trap opens the white box of subnivean ecology. *Remote Sensing in Ecology and Conservation, 1*(1), 29-38.

Speed, J. D. M., Woodin, S. J., Tømmervik, H., Tamstorf, M. P., & van der Wal, R. (2009). Predicting habitat utilization and extent of ecosystem disturbance by an increasing herbivore population. *Ecosystems, 12*(3), 349-359.

Sutherland, W. J. (2006). *Ecological Census Techniques: A Handbook*. Cambridge: Cambridge University Press.

Tobler, M., Carrillo-Percastegui, S., Leite Pitman, R., Mares, R., & Powell, G. (2008). An evaluation of camera traps for inventorying large-and medium-sized terrestrial rainforest mammals. *Animal Conservation, 11*(3), 169-178.

Tsaparis, D., Katsanevakis, S., Ntolka, E., & Legakis, A. (2009). Estimating dung decay rates of roe deer (*Capreolus capreolus*) in different habitat types of a Mediterranean ecosystem: an information theory approach. *European Journal of Wildlife Research, 55*(2), 167-172.

Van der Plas, F., Howison, R. A., Mpanza, N., Cromsigt, J. P., & Olff, H. (2016). Different-sized grazers have distinctive effects on plant functional composition of an African savannah. *Journal of Ecology, 104*(3), 864-875.

Authors: Speed JDM[1], Barrio IC[2], Ravolainen VT[3]

Reviewers: Te Beest M[4,5] & Wilfahrt P[6]

Affiliations

[1] Department of Natural History, NTNU University Museum, Norwegian University of Science and Technology, Trondheim, Norway

[2] Department of Natural Resources and Environmental Sciences, Agricultural University of Iceland, Reykjavík, Iceland

[3] Norwegian Polar Institute, Fram Centre, Tromsø, Norway

[4] Environmental Sciences, Copernicus Institute of Sustainable Development, Utrecht University, Utrecht, the Netherlands

[5] Centre for African Conservation Ecology, Nelson Mandela University, Port Elizabeth, South Africa

[6] Department of Disturbance Ecology, BayCEER, University of Bayreuth, Bayreuth, Germany

4.16 Functional traits

Author: Gya R[1], Wilfahrt P[2]

Reviewer: Halbritter AH[1], Vandvik V[1]

Trait-based ecology has seen a steady rise in recent decades by helping explain patterns of how organisms affect and mediate ecosystem processes and functioning (Violle et al., 2007; de Bello et al., 2010). Functional traits are morphological, physiological, or phenological features measurable at the individual level that link an individual's performance to its biotic and abiotic environment (Webb et al., 2010). Plant functional traits are particularly well-studied and traits such as relative growth rate, leaf stoichiometry, and photosynthetic rate can yield mechanistic insights into demographics, species interactions, and ecosystem processes (Wright et al., 2004; Pérez-Harguindeguy et al., 2013; Díaz et al., 2016). Incorporating traits into climate-change studies can greatly increase our ability to understand how plants and other organisms mediate changes to both the physical and biotic environment.

Pérez-Harguindeguy et al. (2013) compiled an extensive overview with protocols for measuring plant traits and we do not replicate that effort here. Rather, we discuss how traits should be incorporated into climate-change studies, highlight traits that are most likely to be useful (Table 4.16.1), and direct readers to the handbook by Pérez-Harguindeguy et al. (2013) for instructions on how to measure them. Although we focus on climate-change studies in this protocol, many of the traits mentioned in this section are also relevant for other global-change studies on topics such as land-use change (Garnier et al., 2006), invasive plants (Drenovsky et al., 2012), and disturbance (Mouillot et al., 2013). While the trait handbook gives a broad coverage, there are potentially important suites of traits that are not covered here, for example floral traits important for pollination (see Hegland & Totland, 2005; Pellissier et al., 2010). and plant modularity (Klimešová et al., 2019). We also encourage readers to consult the stress physiology section, which provides protocols for additional growth- and stress response-related plant traits that are highly relevant to global-change studies (see Chapter S5). We also provide a short overview of trait ecology and databases for other organisms than plants (see below).

Traits can be broadly partitioned into two groups: *response traits* that relate to how community structure and diversity are affected by environmental factors and *effect traits* that drive changes in ecosystem functioning (Lavorel & Garnier, 2002; Suding et al., 2008; Funk et al., 2017). Often traits can fall into either category, with the focus of the study defining how they are used. Response traits facilitate predictions of how communities will change with climate change and can be studied both through extant communities along climatic gradients (Guittar et al., 2016) and as species turnover from climate-change experiments (Hudson et al., 2011). Effect traits facilitate an improved understanding of the underlying processes of ecosystems properties such as carbon and water dynamics (Pappas et al., 2016). Combined, trait-based approaches can lead to important insights of the causes and consequences of changing plant communities in response to climate-change and other global-change factors.

A second important partition to consider for trait-based studies is the relative importance of *inter-* and *intra-specific* trait variability. A global meta-analysis of plant communities revealed that, on average, intraspecific variation accounted for 25% of the variation within communities, but this

ranged from 2% to 67% for different traits (Siefert et al., 2015). Generally, the relative importance of intraspecific variability decreases as the geographic scale of study increases (Albert et al., 2011): the magnitude of contribution is also habitat specific, with intraspecific trait variability being relatively greater in species-poor and colder habitats (Siefert et al., 2015). Thus, we recommend strongly that researchers collect trait data from within their own study area, although this relaxes for studies focused on broad-scale geographic variation or traits that are known to be relatively non-plastic (e.g. wood density). For robust inference, it is recommended to sample as many species as possible, although the most abundant species should be prioritised and, as a rule of thumb, an acceptable coverage is achieved by sampling the species representing 80% or more of the relative abundance at the plot scale (Pakeman & Quested, 2007). Typically, at least five individuals per species per sampling unit (experimental plot, site, or species, depending on the research question and trait data resolution of the particular study) should be sampled (see Appendix 1 of Pérez-Harguindeguy et al., 2013).

4.16.1 What and how to measure?

Gold standard

We recommend sampling traits *in situ*, and, in the case of experiments, within each of the experimental treatments and controls (i.e. at the plot scale), with at least five measurements per species per site/plot. For community-focused research, measure the most abundant species that together represent 80% or more of the total community abundance per plot (Pakeman & Quested, 2007). Abundance may be determined by cover, biomass, or other metrics appropriate for the study (see protocol 4.8 Plant community composition and 2.1.1 Aboveground plant biomass). If you are specifically interested in certain species, in the rare species, and/or in biodiversity issues, you may need higher numbers of species and/or all plots or treatments where a particular species is present.

For protocols on how to measure these traits, see Pérez-Harguindeguy et al. (2013). Traits that are especially relevant for climate-change research are presented in Table 4.16.1.

Bronze standard

When traits cannot be collected *in situ* for all treatments, species trait data can be collected from site-level sampling, or they can be complied from other sources. Several open-source trait databases exist that provide functional traits for a large number of plant species (TRY – Kattge et al., 2011; BIEN – Enquist et al., 2016; TTT – Bjorkman et al., 2018). When using traits from such databases, it is highly recommended to select trait values from individuals sampled under as similar as possible conditions, habitat(s), and climate(s) to the current study site (Cordlandwehr et al., 2013). When traits are collected *in situ*, it is highly recommended to add the trait data to these global repositories to aid further empiricial studies, meta-analyses, and ecological modelling (Gallagher et al., 2019).

Interpretation

The most common metric used in trait-based studies is the community weighted mean. This combines the relative abundance of species with their trait value and provides a central tendency of a community-trait score (Funk et al., 2017). De Bello et al. (2011) provide methods for decomposing the variance contributions of inter- v. intra-specific variation to community weighted means.

However, focusing solely on single-trait means may miss important trends in the data and additional moments such as variance, skewness, or kurtosis may be used to infer processes such as stability or the relative strengths of environmental filters v. biotic interactions (Enquist et al., 2015). Additionally, a variety of tools have emerged to calculate multivariate trait indices such as functional richness and evenness, which can lend greater support to inferences on the processes structuring communities (Villéger et al., 2008).

Where to start

Enquist et al. (2015), Funk et al. (2017), Pérez-Harguindeguy et al. (2013), Violle et al. (2012)

4.16.2 Special cases, emerging issues, and challenges

Currently, existing trait protocols and databases for plants do not include cryptogams. Nonetheless, there have been advances in developing trait protocols for cryptogams such as bryophytes and lichens (Cornelissen et al., 2007; St. Martin & Mallik, 2017) and soil crusts (Mallen-Cooper & Eldridge, 2016). While no large database has been developed, there have been renewed calls for the integration of cryptogram trait ecology into plant-based trait studies (Deane-Coe & Stanton, 2017; St. Martin & Mallik, 2017).

Trait-based ecology has proliferated in many non-plant taxa as well, offering substantial value to climate-change studies. However, given the increased trait specialisation across and within the other organismal domains, we do not cover any other taxa in detail here. Considerations of response and effect traits and inter- and intra-specific variability also apply to non-plant taxa. The availability of trait databases is highly variable across guilds of species and include the PanTHERIA database for mammals (Jones et al., 2009), an amniote database for birds, reptiles, and mammals (Myhrvold et al., 2015), the GlobalAnts database that includes both abundance and trait data linked to local assemblages (Parr et al., 2017), and the FUNGuild database which has begun classifying fungal operational taxonomic units identified by high throughput sequencing into functional guilds (Nguyen et al., 2016). This is not a comprehensive list of trait databases (many databases exist for aquatic organisms), and regional scientific societies often curate their own such databases for a variety of organisms.

In line with the goal to make climate change experiments more compatible, data more available, and science more transparent, we encourage the same mentality with newly collected trait data. One opportunity is through the Open Traits Network (Gallagher et al., 2019), which fosters an international alliance of researchers and institutions working towards open data and workflows to improve the way we work with functional traits.

Halbritter et al. (2020) The handbook for standardised field and laboratory measurements in terrestrial climate-change experiments and observational studies (ClimEx). *Methods in Ecology and Evolution*, *11*(1) 22-37.

1

Table 4.16.1 Selected traits from Pérez-Harguindeguy et al. (2013) that may be of particular value for climate-change research with their relevance as response or effect traits, and links to relevant internal protocols. Other traits not listed here may still be situationally informative. Note that Pérez-Harguindeguy et al. (2013) does not cover the full range of traits.

Trait name	Description	Relevance to climate change studies	Relevant protocols
Whole plant			
Life history and maximum plant lifespan	Classification (annual, biennial, perennial) or quantification of plant life span	**Effect:** Distinguishing between dominant life-history categories, e.g. perennial or annual, informs carbon and nutrient cycling *and expected rate of species turnover*. Decreases or increases in life span affect these rates	4.3 Plant demography
Plant height	Maximum vegetative height of free-standing, mature individual	**Response:** Indicates position in vertical light gradient, competitiveness for light capture, growth potential **Effect:** Used in allometric equations for estimating biomass	2.1.1 Aboveground plant biomass
Spinescence	Quantifies type, size, and density of spines, prickles, and thorns	**Response:** Indicator of vertebrate pressure on plants	4.15 Vertebrate herbivory
Leaf area:sapwood area ratio	Capacity for water transport and mechanical strength	**Response:** Balance between transpiration and stem water supply **Effect:** Indicates potential for transpiration	3.7 Sap flux
Root-mass fraction	Proportion of plant dry mass found in roots	**Response:** Indicates plant strategy for belowground foraging. Increase may indicate nutrient-poor soils, BUT can also occur in nutrient-rich sites where competition is high	2.1.2 Belowground plant biomass
Relative growth rate and components	Increase in relative size of plant across a defined time interval. Can be separated into leaf, stem, and root mass components	**Response:** Increases or decreases to vital rates may indicate shifts in competitive dominance. Separation into components indicates trade-offs, e.g. between aboveground and belowground allocation strategy **Effect:** Growth rate determines rate of carbon sequestration and nutrient cycling	2.1.1 Aboveground plant biomass 4.3 Plant demography
Water-flux traits	Plant stature on hydrological fluxes *external* to plant (e.g. free throughfall, retention followed by evaporation, stemflow)	**Effect:** Impacts hydrologic cycle of system	3.8 Ecosystem water stress
Leaf			
Specific leaf area (SLA)	Leaf area (fresh) divided by dry mass	**Response:** Higher values indicate resource-acquisitive strategies; lower	2.1.1 Aboveground plant

	(LMA, leaf mass per area, is simply inverse of SLA). Part of leaf economic spectrum	values indicate resource-conservatism **Effect:** When leaves are collected in a known area, the dry mass multiplied by total SLA gives the leaf area index (LAI), a useful parameter in modelling productivity and water stress	biomass 3.8 Ecosystem water stress See Breda (2003) for more information on measuring LAI
Leaf dry-matter content	Leaf dry weight divided by water saturated fresh weight	**Response:** Negatively correlated to relative growth rates and resource capture and usage. Similar to SLA, but independent of leaf size **Effect:** Negatively correlated to litter decomposition rates	
pH of green leaves or leaf litter	pH of green or senesced leaf tissue (generally yield the same values)	**Response:** Positively related to palatability and digestibility to herbivores **Effect:** Persists in leaf litter, affecting decomposition rates	
Leaf N and P concentration	Total amount of N or P per unit of leaf dry mass	**Response:** Positively correlated with growth rates and nutritional quality for consumers **Effect:** Single or co-limitation may limit primary production	2.1.6 Foliar stoichiometry and resorption protocol
Light-saturated photosynthesis	Carbon dioxide assimilation with full light	**Response:** Positively correlated to resource acquisition capacity **Effect:** Relates to biomass accumulation and carbon sequestration	2.1.3 Leaf-scale photosynthesis
Leaf dark respiration	Measure of basal metabolism and rough correlate to night-time respiratory carbon flux	**Response:** Sensitive to rises in temperature, related to resource acquisition-conservatism **Effect:** Determines net primary production, which is the difference between photosynthesis and respiration	2.1.4 Plant respiration
C-isotope composition (water-use efficiency)	Analysis of ^{13}C:^{12}C indicates ratio of photosynthesis to transpiration	**Response:** May indicate inter- and intra-specific shifts in water-use efficiency in plants in response to environmental change **Effect:** Traces where carbon is allocated during CO_2 uptake	2.2.3 Soil CO_2 (and other trace gas) fluxes
Electrolyte leakage (frost sensitivity)	Cell membranes ruptured following frost damage reduces retention of solutes	**Response:** Sensitivity to frost damage may explain how species sort along thermal gradients or probability of a species to withstand frost events	
Leaf water potential	Indicates status of water in leaf by measuring pressure required to induce leaf water loss	**Response:** Can quantify drought tolerance across individuals, populations, or species **Effect:** Can indicate soil water potential when measured pre-dawn. Night-time transpiration or xylem cavitation may disrupt this equilibrium	3.4 Soil water potential
Litter decomposability	Mass loss of leaves or litter	**Response:** Influenced by temperature and microbial activity	2.2.6 Litter decomposition

	contained in litter bags	**Effect:** Differing rates of decomposition results in different rates of CO_2 and nutrient release	
Stem			
Stem-specific density	Volume of fresh stem biomass divided by dry mass	**Response:** May indicate changes in water availability, pressure from consumers, resistance to disturbance events **Effect:** Critical for carbon storage. Often used in allometric equations to estimate biomass	2.1.1 Aboveground plant biomass
Xylem conductivity	Ability to move water from soil to leaves; measured by rate of water flow per xylem area and per unit gradient of pressure	**Response:** Low conductivity leads more rapidly to drought-induced leaf damage **Effect:** High conductivity increases transpiration rates	
Vulnerability to embolism	When air gets into xylem tissue, it rapidly expands and blocks water flow. Measured by building a xylem conductivity curve	**Response:** Positively related to mortality risk during drought (i.e. inversely correlated with drought tolerance)	
Belowground			
Specific root length	Ratio of root length to root dry mass	**Response:** Higher values are associated with more rapid nutrient uptake ability but decreased root longevity	2.1.2 Belowground plant biomass
Root-system morphology	Primarily defined by three components: depth, lateral extent, and exploration intensity (fine-root biomass per unit soil volume). These can be further refined into parameters at different soil depths	**Response:** Indicative of the resource space where plants forage for soil nutrients, and the competitiveness of areas where they do (intensity) **Effect:** The distribution of root biomass at different depths is a useful trait for modelling productivity and water stress	2.1.2 Belowground plant biomass 3.8 Ecosystem water stress
Regenerative			
Dispersal syndrome	Categorical trait detailing main vector of dispersal	**Response:** Turnover at the community level may indicate possible dispersal limitations or explain recent invasions	4.7 Propagule rain
Dispersule size and shape	Dry mass and variance of length, width, and thickness of dispersule	**Response:** Dispersule size is related to seed-bank persistence and therefore long-term community recruitment dynamics	4.6 The soil seed bank (buried seed pool)

	(i.e. seed and associated structures)		
Seed mass	Dry mass of seed without associated structures (e.g. fruit)	**Response:** Indicates parental investment per unit offspring. Higher seed mass may confer initial stress tolerance to seedlings, lower seed mass allows more offspring per unit energy investment and often longer individual seed persistence	4.6 The soil seed bank (buried seed pool)

4.16.3 References

Theory, significance, and large datasets

Bjorkman et al. (2018), Enquist et al. (2015), Jones et al. (2009), Kattge et al. (2011), Kleyer et al. (2008), Myhrvold et al. (2015), Nguyen et al. (2016), Parr et al. (2017)

More on methods and existing protocols

Klimešová et al (2019), Pérez-Harguindeguy et al. (2013)

All references

Albert, C. H., Grassein, F., Schurr, F. M., Vieilledent, G., & Violle, C. (2011). When and how should intraspecific variability be considered in trait-based plant ecology? *Perspectives in Plant Ecology, Evolution, and Systematics*, *13*(3), 217-225.

Bjorkman, A. D., Myers-Smith, I. H., Elmendorf, S. C., Normand, S., Thomas, H. J., Alatalo, J. M., ... Baruah, G. (2018). Tundra Trait Team: A database of plant traits spanning the tundra biome. *Global Ecology and Biogeography*, *27*(12), 1402-1411.

Breda, N. J. (2003). Ground-based measurements of leaf area index: a review of methods, instruments and current controversies. *Journal of Experimental Botany, 54*(392), 2403-2417.

Cordlandwehr, V., Meredith, R. L., Ozinga, W. A., Bekker, R. M., Groenendael, J. M., & Bakker, J. P. (2013). Do plant traits retrieved from a database accurately predict on-site measurements? *Journal of Ecology*, *101*(3), 662-670.

Cornelissen, J. H. C., Lang, S. I., Soudzilovskaia, N. A., & During, H. J. (2007). Comparative cryptogam ecology: a review of bryophyte and lichen traits that drive biogeochemistry. *Annals of Botany*, *99*(5), 987-1001.

de Bello, F., Lavorel, S., Díaz, S., Harrington, R., Cornelissen, J. H. C., Bardgett, R. D., ... da Silva, P. M. (2010). Towards an assessment of multiple ecosystem processes and services via functional traits. *Biodiversity and Conservation*, *19*(10), 2873-2893.

de Bello, F., Lavorel, S., Albert, C. H., Thuiller, W., Grigulis, K., Dolezal, J., ... Lepš, J. (2011). Quantifying the relevance of intraspecific trait variability for functional diversity. *Methods in Ecology and Evolution*, *2*(2), 163-174.

Deane-Coe, K. K., & Stanton, D. (2017). Functional ecology of cryptogams: scaling from bryophyte, lichen, and soil crust traits to ecosystem processes. *New Phytologist*, *213*(3), 993-995.

Díaz, S., Kattge, J., Cornelissen, J. H. C., Wright, I. J., Lavorel, S., Dray, S., ... Garnier, E. (2016). The global spectrum of plant form and function. *Nature*, *529*(7585), 167.

Drenovsky, R. E., Grewell, B. J., D'antonio, C. M., Funk, J. L., James, J. J., Molinari, N., ... Richards, C. L. (2012). A functional trait perspective on plant invasion. *Annals of Botany*, *110*(1), 141-153.

Enquist, B. J., Norberg, J., Bonser, S. P., Violle, C., Webb, C. T., Henderson, A., ... Savage, V. M. (2015). Scaling from traits to ecosystems: developing a general trait driver theory via integrating trait-based and metabolic scaling theories. *Advances in Ecological Research, 52*, 249-318.

Enquist, B. J., Condit, R., Peet, R. K., Schildhauer, M., & Thiers, B. M. (2016). Cyberinfrastructure for an integrated botanical information network to investigate the ecological impacts of global climate change on plant biodiversity. *PeerJ Preprints, 4*, e2615v2.

Funk, J. L., Larson, J. E., Ames, G. M., Butterfield, B. J., Cavender-Bares, J., Firn, J., ... Wright, J. (2017). Revisiting the Holy Grail: using plant functional traits to understand ecological processes. *Biological Reviews*, *92*(2), 1156-1173.

Gallagher, R. V., Falster, D. S., Maitner, B. S., Salguero-Gomez, R., Vandvik, V., Pearse, W. D., ... & Andrew, S. C. (2019). The Open Traits Network: Using Open Science principles to accelerate trait-based science across the Tree of Life. *EcoEvoRxiv*.

Garnier, E., Lavorel, S., Ansquer, P., Castro, H., Cruz, P., Dolezal, J., ... Grigulis, K. (2006). Assessing the effects of land-use change on plant traits, communities and ecosystem functioning in grasslands: a standardized methodology and lessons from an application to 11 European sites. *Annals of Botany*, *99*(5), 967-985.

Guittar, J., Goldberg, D., Klanderud, K., Telford, R. J., & Vandvik, V. (2016). Can trait patterns along gradients predict plant community responses to climate change? *Ecology*, *97*(10), 2791-2801.

Hegland, S. J., & Totland, Ø. (2005). Relationships between species' floral traits and pollinator visitation in a temperate grassland. *Oecologia*, *145*(4), 586-594.

Hudson, J. M. G., Henry, G. H. R., & Cornwell, W. K. (2011). Taller and larger: shifts in Arctic tundra leaf traits after 16 years of experimental warming. *Global Change Biology*, *17*(2), 1013-1021.

Jones, K. E., Bielby, J., Cardillo, M., Fritz, S. A., O'Dell, J., Orme, C. D. L., ... Connolly, C. (2009). PanTHERIA: a species-level database of life history, ecology, and geography of extant and recently extinct mammals. *Ecology*, *90*(9), 2648-2648.

Kattge, J., Díaz, S., Lavorel, S., Prentice, I. C., Leadley, P., Bönisch, G., ... Cornelissen, J. H. C. (2011). TRY–a global database of plant traits. *Global Change Biology*, *17*(9), 2905 2935.

Kleyer, M., Bekker, R. M., Knevel, I. C., Bakker, J. P., Thompson, K., Sonnenschein, M., ... Klotz, S. R. G. M. (2008). The LEDA Traitbase: a database of life-history traits of the Northwest European flora. *Journal of Ecology*, *96*(6), 1266-1274.

Klimešová, J., Martínková, J., Pausas, J. G., de Moraes, M. G., Herben, T., Yu, F. H., ... & Altman, J. (2019). Handbook of standardizied protocols for collecting plant modularity traits. *Perspectives in Plant Ecology, Evolution and Systematics*, 125485.

Lavorel, S., & Garnier, É. (2002). Predicting changes in community composition and ecosystem functioning from plant traits: revisiting the Holy Grail. *Functional Ecology*, *16*(5), 545-556.

Mallen-Cooper, M., & Eldridge, D. J. (2016). Laboratory-based techniques for assessing the functional traits of biocrusts. *Plant and Soil*, *406*(1-2), 131-143.

Mouillot, D., Graham, N. A., Villéger, S., Mason, N. W., & Bellwood, D. R. (2013). A functional approach reveals community responses to disturbances. *Trends in Ecology & Evolution*, *28*(3), 167-177.

Myhrvold, N. P., Baldridge, E., Chan, B., Sivam, D., Freeman, D. L., & Ernest, S. K. (2015). An amniote life-history database to perform comparative analyses with birds, mammals, and reptiles. *Ecology*, *96*(11), 3109-3109.

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., ... Kennedy, P. G. (2016). FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology*, *20*, 241-248.

Pakeman, R. J., & Quested, H. M. (2007). Sampling plant functional traits: what proportion of the species need to be measured? *Applied Vegetation Science*, *10*(1), 91-96.

Pappas, C., Fatichi, S., & Burlando, P. (2016). Modeling terrestrial carbon and water dynamics across climatic gradients: does plant trait diversity matter? *Phytologist*, *209*(1), 137-151.

Parr, C. L., Dunn, R. R., Sanders, N. J., Weiser, M. D., Photakis, M., Bishop, T. R., ... Chick, L. (2017). GlobalAnts: a new database on the geography of ant traits (Hymenoptera: Formicidae). *Insect Conservation and Diversity*, *10*(1), 5-20.

Pellissier, L., Pottier, J., Vittoz, P., Dubuis, A., & Guisan, A. (2010). Spatial pattern of floral morphology: possible insight into the effects of pollinators on plant distributions. *Oikos*, *119*(11), 1805-1813.

Pérez-Harguindeguy, N., Díaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., ... Cornelisson, J. H. C. (2013). New handbook for standardised measurement of plant functional traits worldwide. *Australian Journal of Botany*, *61*(3), 167-234.

Siefert, A., Violle, C., Chalmandrier, L., Albert, C. H., Taudiere, A., Fajardo, A., ... Dantas, V. (2015). A global meta-analysis of the relative extent of intraspecific trait variation in plant communities. *Ecology Letters*, *18*(12), 1406-1419.

St. Martin, P., & Mallik, A. U. (2017). The status of non-vascular plants in trait-based ecosystem function studies. *Perspectives in Plant Ecology, Evolution and Systematics*, *27*, 1-8.

Suding, K. N., Lavorel, S., Chapin, F. S., Cornelissen, J. H. C., Díaz, S., Garnier, E., ... Navas, M.-L. (2008). Scaling environmental change through the community-level: a trait-based response and-effect framework for plants. *Global Change Biology*, *14*(5), 1125-1140.

Villéger, S., Mason, N. W., & Mouillot, D. (2008). New multidimensional functional diversity indices for a multifaceted framework in functional ecology. *Ecology*, *89*(8), 2290 -2301.

Violle, C., Navas, M.-L., Vile, D., Kazakou, E., Fortunel, C., Hummel, I., & Garnier, E. (2007). Let the concept of trait be functional! *Oikos*, *116*(5), 882-892.

Violle, C., Enquist, B. J., McGill, B. J., Jiang, L., Albert, C. H., Hulshof, C., ... Messier, J. (2012). The return of the variance: intraspecific variability in community ecology. *Trends in Ecology & Evolution*, *27*(4), 244-252.

Webb, C. T., Hoeting, J. A., Ames, G. M., Pyne, M. I., & LeRoy Poff, N. (2010). A structured and dynamic framework to advance traits-based theory and prediction in ecology. *Ecology Letters*, *13*(3), 267-283.

Wright, I. J., Reich, P. B., Westoby, M., Ackerly, D. D., Baruch, Z., Bongers, F., ... Flexas, J. (2004). The worldwide leaf economics spectrum. *Nature*, *428*(6985), 821.

Author: Gya R[1], Wilfahrt PA[2]

Reviewer: Halbritter AH[1], Vandvik V[1]

Affiliations

[1] Department of Biological Sciences and Bjerknes Centre for Climate Research, University of Bergen, Bergen, Norway

[2] Department of Disturbance Ecology, BayCEER, University of Bayreuth, Bayreuth, Germany