Handbook of field protocols for using REFA methods to approximate ecosystem functions

Version 1.0

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Introduction

Rapid ecosystem function assessment (REFA) methods have been proposed as a fast, easy, and cost-efficient solution to approximate ecosystem functions in a standardized way (Meyer et al. 2015). While REFA has already been applied (Hertzog et al. in press, Leidinger et al. in press), no systematic and detailed description of the field protocols and implementation of the method is currently available. This handbook summarizes the details of applying REFA methods for measurements of ecosystem functions in the field. Many REFA methods can be carried out simultaneously at shared sampling points (primary and secondary productivity both above and below ground, decomposition, and herbivory). However, some of the assays depend on animals to behave naturally while interacting with the sampling equipment (predation, seed dispersal, pollination). To ensure that animals are disturbed as little as possible, these proxies should be measured at a separate sampling location within a site (see Leidinger et al. in press for an example of a sampling design).

Primary productivity

Aboveground primary productivity: Standing biomass

Aboveground primary productivity is estimated by measuring standing biomass. For herbaceous vegetation, productivity can be approximated as the standing biomass at peak biomass (Woodwell and Bourdeau 1965, Ni 2004), since it has predominantly been produced in the year leading up to the sampling date. Plant biomass is sampled in 20 cm x 50 cm subplots. To delineate the area, a frame is placed on the ground and biomass is harvested within the frame by cutting at ground level. All biomass reaching into the space above the frame from outside the sampling area is also cut. All standing biomass, including woody components and senescent leaves, is harvested. These biomass samples can also serve as the basis for herbivory assessments. After sampling, the biomass is dried for 48 hours or until dry at 70°C and weighed. Samples can be separated into different groups (grasses, herbs, shrubs) to gain additional information. Also, it may be necessary to separate dead plant material from living material in systems in which large quantities of dead biomass accumulate due to low decomposition rates, low grazing pressure and lack of fire. In systems with high herbivory rates and especially in systems grazed by livestock, temporary fences can help to approximate true primary productivity.

Needed materials and equipment: scissors, frame of 20 cm x 50 cm or square of string (20 cm x 50 cm) with pegs fixed at the corners (Figure 1), paper bags for storing biomass, drying oven, fine scale balance

Figure 1 Harvesting of a biomass sample on an area of 20 cm x 50 cm marked with a string and pegs fixed to the corners of the rectangle. Photo by Isabelle Studer.

Estimates of productivity in forests and other vegetation types that contain woody plants require special methodologies due to the increased structural complexity of both the whole plant community and individual plants which combine a large standing (dead) biomass with additional annual growth. Separating these two components is a major challenge, yet not impossible. It is feasible to separate current year growth from accumulated biomass by visual aids like wood growth rings (in temperate climates) and bud-scale scars (Whittaker 1961). Completely harvesting large enough forest plots is often impossible. To overcome this limitation, the harvesting of representative individuals, or of individuals of varying sizes, and the subsequent calculation of regressions between easily measured variables, e.g. height, and biomass has been used to extrapolate stand biomass (Ovington 1957, Woodwell and Bourdeau 1965).

Belowground primary productivity: Root standing biomass

It is important to note that net primary productivity has an above- and a below-ground part. Below-ground production of plants comprises the accumulated root biomass plus additional exudates and root litter and can be a substantial fraction of above-ground productivity (e.g., Ravenek et al. 2014). Equivalent to the measurements above ground, the standing biomass of roots can be used as a relatively easy-to-measure approximation (Ni 2004). To sample root biomass, a volumetric soil sample of 10 cm depth and a diameter of 20 cm is taken using a split corer (Kempson Bohrer; Electro-Technik-Schuller, Darmstadt, Germany). The corer is driven into the soil with the help of a large 8 kg hammer and a protective plate made of hard plastic. Before taking the soil core, all aboveground biomass is removed from the sampling area using scissors and knives. Soil cores are soaked in water for several hours to facilitate separation of roots from the soil and are stirred manually while in suspension. Roots are separated by washing the soil-root mixture through a large sieve (mesh size 2 mm) by continuous stirring and adding of water. Stones in the sieve are removed manually. The liquid filtrate is then filtered through a smaller sieve (mesh size 1 mm) to retrieve small particles of root litter. Roots and root litter are dried for 48 hours at 70 °C before weighing.

Needed materials and equipment: soil corer (diameter 20 cm; Figure 2a-d), protective plate (Figure 2b-d), large hammer (Figure 2d), sturdy plastic bags for transport of soil cores from the field, scissors, large sieve (2 mm mesh size), fine sieve (1 mm mesh size), buckets, hose, paper bags for storing roots, drying oven, fine scale balance

Figure 2 A, B: 20 cm diameter split corer with the protective plate; C: The corer is driven into the soil to a depth of 10 cm using a large hammer. Photos by Jan Leidinger

Saprophagous food-web

Decomposition: Weight loss of standardized materials

The quantity of accumulated dead organic matter depends on the rate with which new dead material is accumulated, the rate with which it is decomposed and also on human use (e.g. removal of wood from forests or hay from grasslands). Consequently, standing litter stocks are ambiguous to interpret, making rates of decomposition a better proxy for this ecosystem function. Decomposition of natural litter is highly dependent on the occurring community of decomposers, environmental conditions and the properties of the leaf litter. To standardize the assay and make results comparable, decomposition of a neutral substrate (wooden sticks of standardized dimensions) is used (Reed et al. 2005). Tongue depressors bought from medical supply have proven best for this purpose. Before burying, the tongue depressors are dried and weighed. Next, they are buried in a horizontal position at a depth of approximately 10 cm. To place the sticks into the soil, a spate incision is made, and individual sticks are placed in the gap with a colored nylon cord flung around it to mark their position on the surface and thus facilitate

recovery of the sticks. Additional marking might be necessary to relocate samples when retrieving the sticks after exposure in the field. Sticks should remain buried in the ground during 4–6 weeks. Depending on the conditions at the site, this period might need to be shortened (high decomposition rates) or extended (low decomposition rates). After exposure, the sticks are carefully retrieved by digging up the site where the marking cord of a sample is located using a shovel or spade. Make sure to insert the spade about 15 cm deep and a few centimeters away from the marking and then pull the handle of the spade towards you to push up the soil containing the wooden stick. Any wooden sticks damaged or destroyed during extraction cannot be used to determine weight loss. All wooden sticks that are retrieved intact are carefully washed with water to remove dirt and rinsed with 70% ethanol to eliminate all microbial activity. The cleaned sticks are dried at 70°C for 48 hours and then weighed to establish the weight loss. Three to five sticks should be buried per site to yield reliable results.

Needed materials and equipment: wooden tongue depressors, spate, colored plastic cord, additional marking for the field sites, ethanol, drying oven, fine scale balance

Decomposer abundance: Belowground invertebrate biomass

The decomposition of dead organic material is often accelerated by the physical breakdown of materials by saprophagous soil fauna. The total biomass of soil fauna can be used as an approximation for the potential of these organisms to break down litter material. Invertebrates are sampled quantitatively by taking a large (20 cm diameter, 10 cm depth, Figure 2) soil core (Edwards 1991, Southwood and Henderson 2009). Before extracting the soil core, all aboveground biomass is removed using scissors. For stabilization, the cylindrical soil cores are placed inside a plastic tube matching the dimensions of the soil core. Samples should be immediately placed in closed plastic bags and stored in a shaded location until transportation. Within less than 8 hours after sampling, soil cores have to be placed upside-down in a Tullgren-Funnel (soil surface facing downwards and resting on the mesh over the funnel). To extract the soil fauna, a common light bulb (30 Watts) is placed over each funnel. The light and the heat drive the soil fauna away from the bulb and eventually out of the soil core, so that individuals fall through the funnel and can be collected in a plastic bag containing ethylene glycol. Samples are incubated over a period of 10 days to allow for the soil cores to completely dry out. After the incubation period, soil cores are discarded, and the contents of the ethylene glycol bag transferred to jars with 70% ethanol. Due to the presence of debris in most samples, they need to be cleaned following the procedure described below for the processing of the above-ground suction samples. From the cleaned samples, the abundance and fresh weight of below-ground arthropods can be determined as described below.

Needed materials and equipment: soil corer (diameter 20 cm, depth 10 cm), protective plate, large hammer, sturdy plastic bags for transport of soil cores from the field, plastic rings of 20 cm diameter to protect soil cores during transport, Tullgren-Funnels (can be built from large funnels, coarse mesh, and light bulbs, Figure 3), ethylene glycol, plastic bags, rubber bands, ethanol, micro strainer (<< 1 mm mesh size), hard tweezers, soft tweezers, Petri dishes, stereo microscope, tally counter, tissue paper; fine scale balance

Figure 3 A: Stabilizing the soil core with a plastic ring; B: Wrapping the stabilized cores in a plastic bag for transport; C: Extractor for soil fauna built from large water bottles cut in half to create funnels. A plastic bag filled with some ethylene glycol is fixed to the end of the funnels; D: Soil cores are placed on a mesh on top of the funnel and heated from above using a light bulb.

Consumer food-web

Aboveground secondary productivity: Aboveground invertebrate biomass

In most ecosystems, plants are linked to a large number of consumers both via trophic and non-trophic interactions. The total biomass of consumers can be used to approximate the magnitude of the phytophagous-food-web in an ecosystem. REFA focuses on invertebrate consumers because they are relatively easy to sample and show a close link to site conditions at the scale of sampling plots. The abundance and fresh weight of invertebrates are established through suction-sampling (Brook *et al.* 2008). An area of 0.25 m² of vegetation is covered with a cylindrical, fine-meshed gauze-cage of 0.4 m diameter to prevent individuals from escaping. The lower rim of the cage should connect to the soil surface very tightly to effectively trap insects inside the cage. The cage also should be put in place in one quick motion to minimize disturbance and to prevent the escape of individuals before the cage is installed. Once the cage is set up, it is sampled with a leaf blower set to suction mode, e.g. D-Vac (Stihl SH 86), equipped with a fine mesh textile bag that is inserted into the suction tube (Figure 4C). Sampling takes place twice for 1.5 minutes with a 30-second break in between. In each 90-second interval, all vegetation inside the cage and the inside of the cage itself is vacuumed at least once. To standardize sampling between sites, sampling is always conducted between 11 am and 1 pm under dry conditions. Samples, including debris and plant material, are then immediately transferred to jars containing 70% ethanol using a plastic funnel.

Samples are filtered through a micro strainer (mesh size << 1 mm) in the laboratory to separate them from ethanol, and then transferred to a large strainer (mesh size 5 mm) inside a plastic tray and covered with water. Large floating debris is removed from the sample using forceps after being rinsed with water to avoid accidentally removing invertebrates. The tray containing the large strainer with the sample in it is placed on an orbital shaker at 38 rpm for 60–90 minutes to separate a coarse fraction in the strainer from a fine fraction in the tray below the strainer. Large invertebrates remaining in the coarse fraction are removed by hand, counted, and placed in ethanol, after which the coarse fraction is discarded. The remaining fine fraction of the sample is filtered through a micro strainer (mesh size << 1 mm) to remove water and transferred to a Petri dish. After adding a small amount of water to facilitate handling, the sample is examined in a line-by-line pattern using a stereo microscope, and the number of individuals is recorded using a tally counter. Individuals are removed from the sample and added to those extracted from the coarse fraction. For samples without large pieces of debris, the creation of a coarse fraction is skipped by omitting the large strainer. The fresh weight of each sample is established after spreading individuals on tissue paper, then transferring them back to a petri dish in the same order while allowing for a minimum drying time of 1 min (Gehaka AG200, max=201 g, d=0.1 mg, e=1 mg). Determining the fresh weight after storage in 70% ethanol standardizes water content and avoids the destruction of the sample otherwise necessary to determine dry weight. If necessary, dry weight biomass can be derived from the slope between fresh and dry weight determined by previous calibrations.

Needed materials and equipment: fine mesh cage (Figure 4A), leaf blower (adapted for suction rather than blowing), fine mesh filters (Figure 4B), funnel, sampling jars, ethanol, large strainer

(5 mm mesh size), micro strainer (<< 1 mm mesh size), plastic tray, hard tweezers, soft tweezers, Petri dishes, tally counter, stereo microscope, tissue paper, fine scale balance

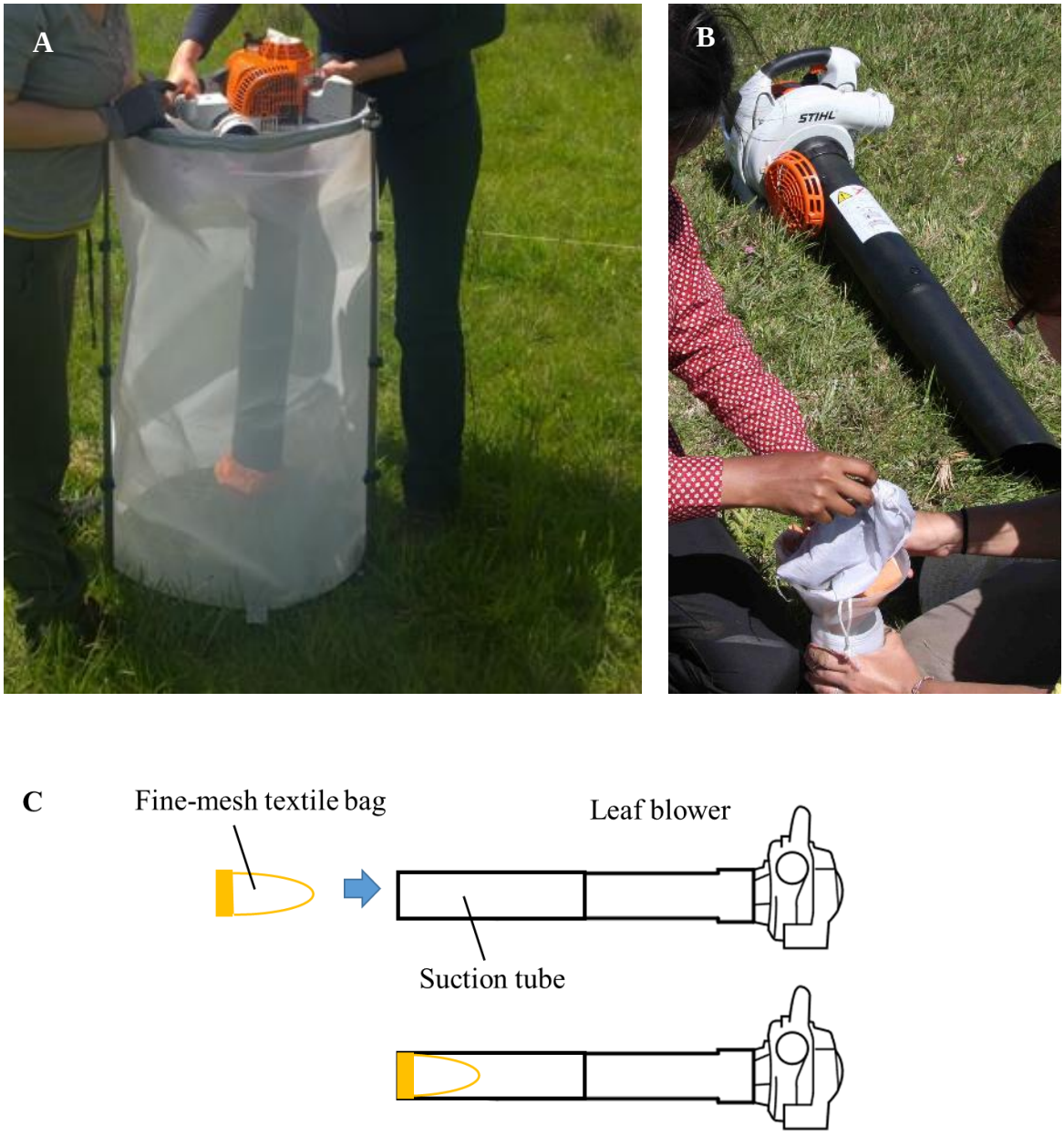

Figure 4 A: Using a fine-mesh cage to prevent arthropods from escaping and a leaf blower set to suction mode with a fine-mesh textile bag inserted into the suction tube; B: Emptying of the bag into a sampling jar using a funnel; C: Setup of leaf blower with fine-mesh textile bag. The bag is folded over the rim of the suction tube and fixed with strong elastic bands.

Herbivory: Proportion of damaged leaves

Herbivory, i.e. the consumption of living plant material by animals, is the link between the primary producers and higher trophic levels. Herbivory sustains the consumer community and can profoundly impact vegetation structure, community composition, and plant physiology (Hulme 1996, Weisser and Siemann 2004 and references therein). A difference has to be made between herbivory by (large) vertebrates and herbivory by invertebrates. REFA focusses on herbivory by invertebrates which is omnipresent, albeit on lower levels than vertebrate herbivory (Weisser and Siemann 2004). To quantify invertebrate herbivory, leaves are screened for evidence of damage by herbivores. Fresh leaves from the above-ground plant biomass sample are used for this measurement. To keep the measurements fast and standardized, leaves are only scored as showing damage (1) or being undamaged (0), as methods estimating the removed leaf area (Loranger et al. 2014, Meyer et al. 2017) are too labor intensive for large scale monitoring. The proportion of leaves that show any damage (Souza et al. 2013), or fall within categories of no, low, medium, or high damage (Christie and Hochuli 2005), has been used as an approximation of herbivore pressure. For this measure to be informative, it is necessary to evaluate a large number of leaves per site (at least 200) and to ensure that leaves are a random subset of the leaves in the biomass sample both regarding their herbivore damage and plant species composition. If plant biomass is separated into different categories or species groups, leaves for the herbivory estimate should be drawn proportionally to the estimated share of biomass in the sample. Each leaf is then screened individually for evidence of herbivore damage using a magnifying glass and scored as showing damage (1) or being undamaged (0).

Needed materials and equipment: plastic box with moist sponge (to store leaves before measurements), magnifying glass, tally counter

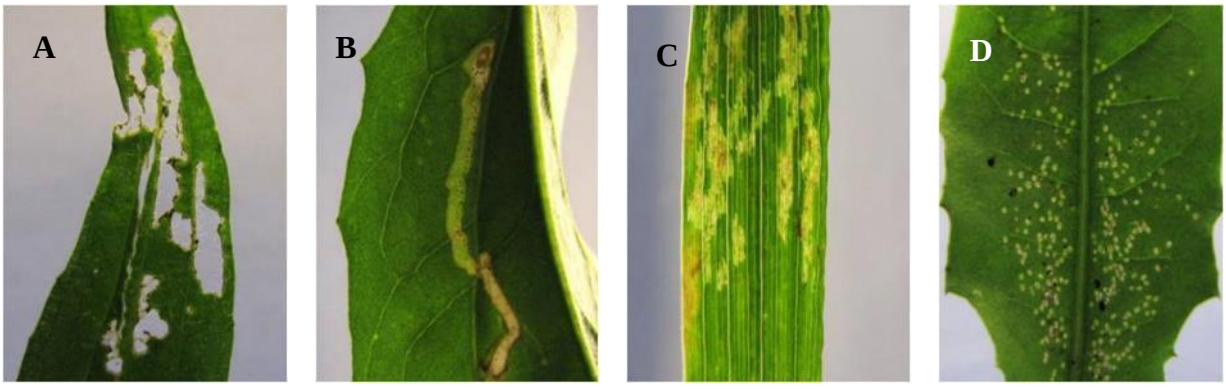

Figure 5 Different types of herbivore damage to leaves. A: chewing damage; B: mining damage; C: rasping damage; D: sucking damage. Photos by Hannah Loranger.

Predation: Standard prey removal or caterpillar dummies

Consumers are not a homogenous group as not all individuals are phytophagous feeders on plant material. Consumers of higher orders feed on other animals, transferring energy within the group of secondary consumers. Direct assessment of predation on insects in a standardized procedure is made difficult by several factors, including the size of the animals and the infrequency of attacks (Low et al. 2014). To estimate predation pressure, predation marks in caterpillar dummies made from plasticine are evaluated. Exposing caterpillar dummies has been used for surveys of both invertebrate and vertebrate predators (Loiselle and Farji-Brener 2002, Koh and Menge 2006, Posa et al. 2007, Ruiz-Guerra et al. 2012). This method has the additional advantage that the predation marks on the caterpillars can be counted and attributed to different feeding types or even species, as is done in most of the cited studies.

Dummies are produced from green plasticine (e.g. noris club plasticine, Staedtler, Nürnberg, Germany). The plasticine is pushed through a modified meat mincer from which the cross knife has been removed and the grinding plate been replaced by a solid plate with one or two round holes of 6 mm diameter. Pushing the plasticine through this modified mincer produces long strands of plasticine. These are rolled between two glass plates, without applying pressure, to smooth out the surface. Next, the now smooth strands are cut into 2 cm long pieces using a razor blade. Finally, the dummies are individually pinned into storage boxes with a styrofoam inlay using insect needles. Dummies are to be handled carefully to avoid leaving fingerprints or finger nail marks on the surface of dummies. Per sampling point, ten dummies are set up along a transect by pinning them to the ground with their insect needle on a 3 cm x 2 cm earth-colored paper (to keep the surface of the dummies clean) at a distance of 1 m from each other. For specific research questions, dummies can also be exposed in other strata by pinning them to the vegetation (leaves, twigs, stems). Experience has shown that attack rates are higher on the ground and exposure on the ground has the additional advantage of being feasible at all sites independent of the local vegetation (Geisthardt 2014, Hertzog et al. in press). Exposure time affects the number of marks found on the dummies (Solovyeva 2015). Since different groups of arthropods are active during day and during night (Solovyeva 2015), dummies should be exposed for at least 24 hours and up to a maximum of 72 hours. Exposure durations should be recorded and used as a covariate when analyzing the data. Upon retrieval, dummies are transferred to a storage box where they are again pinned to styrofoam with a label attached to the needle beneath the dummy. Finally, dummies are examined under a stereo microscope and marks are quantified and attributed to either arthropod, bird, mammal predators or slugs (Figure 6). Individual predators often leave multiple marks in a dummy (unpublished data). Therefore, the number of marks per dummy is not a suitable response variable to approximate predation pressure. Instead, the proportion of dummies that show at least one mark is used to approximate predation. For this reason, it is important to expose at least ten dummies per site. The proportion of dummies with marks should be analyzed in a generalized linear model with binomial error distribution for the effects of any explanatory variables.

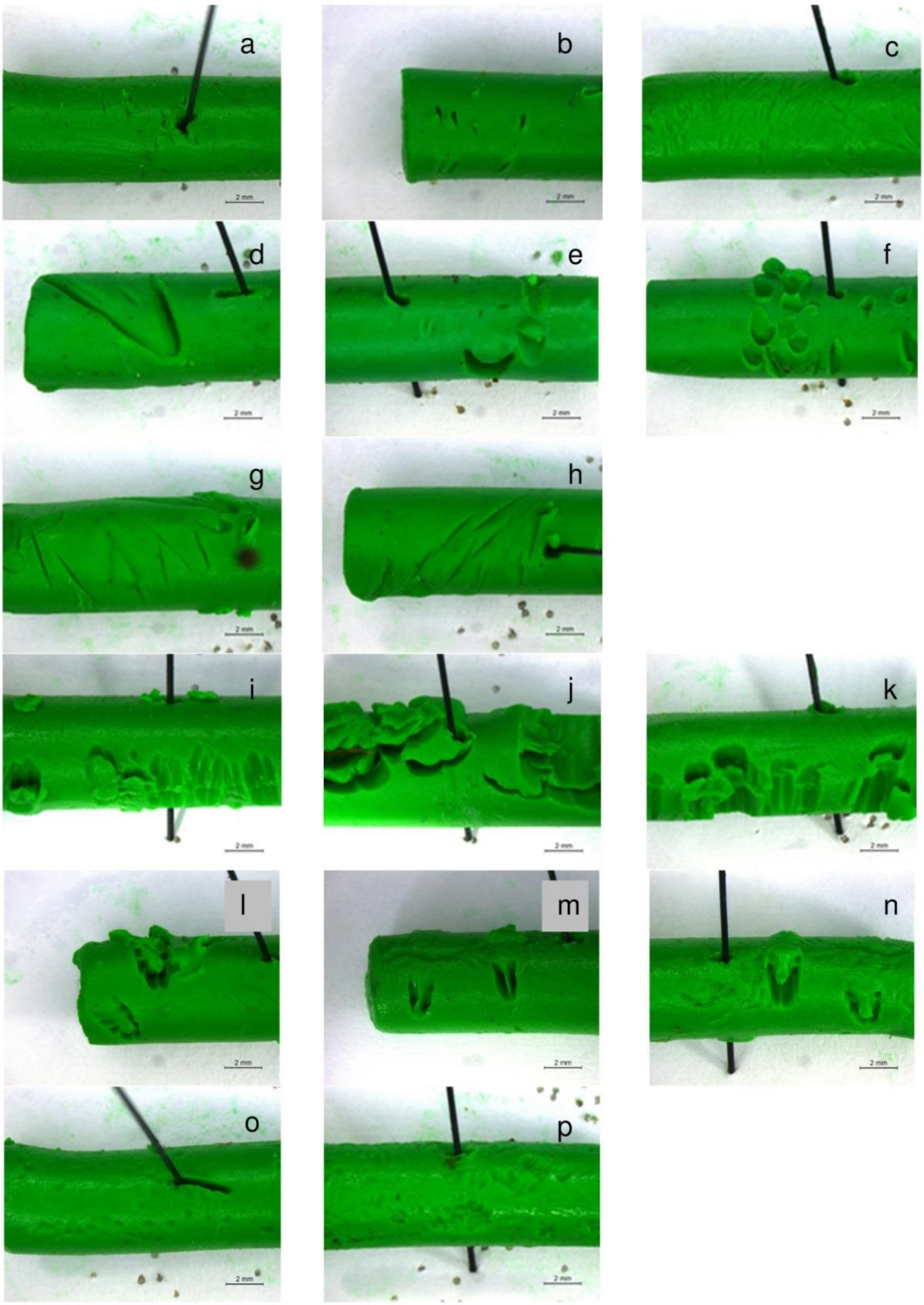

Figure 6 Examples of bite marks by arthropods (a-c), birds (d-f), claw marks by birds (g, h), typical bite marks by small mammals (i-k), bite marks by shrew (l-n), rasping marks by slugs (o, p). From Solovyeva (2015).

Needed materials and equipment: plasticine, modified meat mincer, glass plates, razor blades, insect needles, storage boxes, styrofoam, brown wrapping paper, stereo microscope (alternatively magnifying lens), small labels (ca. 5 mm x 20 mm).

Pollination: Pan traps

One major mutualistic interaction between plants and animals is the transfer of pollen between flowers that is carried out in large parts by flying insects. As such, pollination is a prerequisite for seed setting, and the loss of pollinators can cascade to the loss of plant species (Bond 1994). The potential for pollination is approximated based on the occurrence of pollinating insects. Active sampling (netting of flower visitors) is highly dependent on the skill of the sampler (e.g., Leong and Thorp 1999, Nielsen et al. 2011) and the availability of flowers. According to Westphal et al. (2008) and also Nielsen et al. (2011), pan traps are the most effective method to assess overall pollinator richness while minimizing collector biases, costs, and time effort even if not all trapped individuals are pollinators.

Pan traps are used in three different colors (yellow, blue, UV-white) as previous research has shown that there are differences in color preferences of different pollinators. By using the three different colors, the pollinator community is sampled more thoroughly. Pans mounted on wire loops that are attached to wooden sticks with cable fixers and tape allow to adapt the set-up to varying sampling heights (Figure 7). Traps should be mounted at a standardized height relative to the height of the vegetation (Vega et al. 1990, Tuell et al. 2009, Nuttman et al. 2011). If a sampling site is to be replicated, traps should be spaced by at least 3–5 meters(Droege et al. 2010). Pan traps are filled with water containing a small amount of neutral detergent. Ideally, one sampling period lasts from sunrise to sunset to cover all groups of pollinators with different diurnal activity patterns. After sampling ends, the individuals in each pan are counted. If sampled insects should be processed further (determination of pollinator groups, biomass) the contents of the pans are poured through a tea filter and stored in a jar with 70% ethanol. Insects are later removed from the filter bags under a stereo microscope using tweezers. The fresh weight of each sample is assessed following the procedure specified for the weighing of suction samples.

Needed materials and equipment: pans (of colors white, blue, and yellow), wooden sticks, wire loops, tape, cable fixers, neutral detergent, tea filters, sampling jars, ethanol, stereo microscope, hard tweezers, soft tweezers, petri dish, tissue paper, fine scale balance

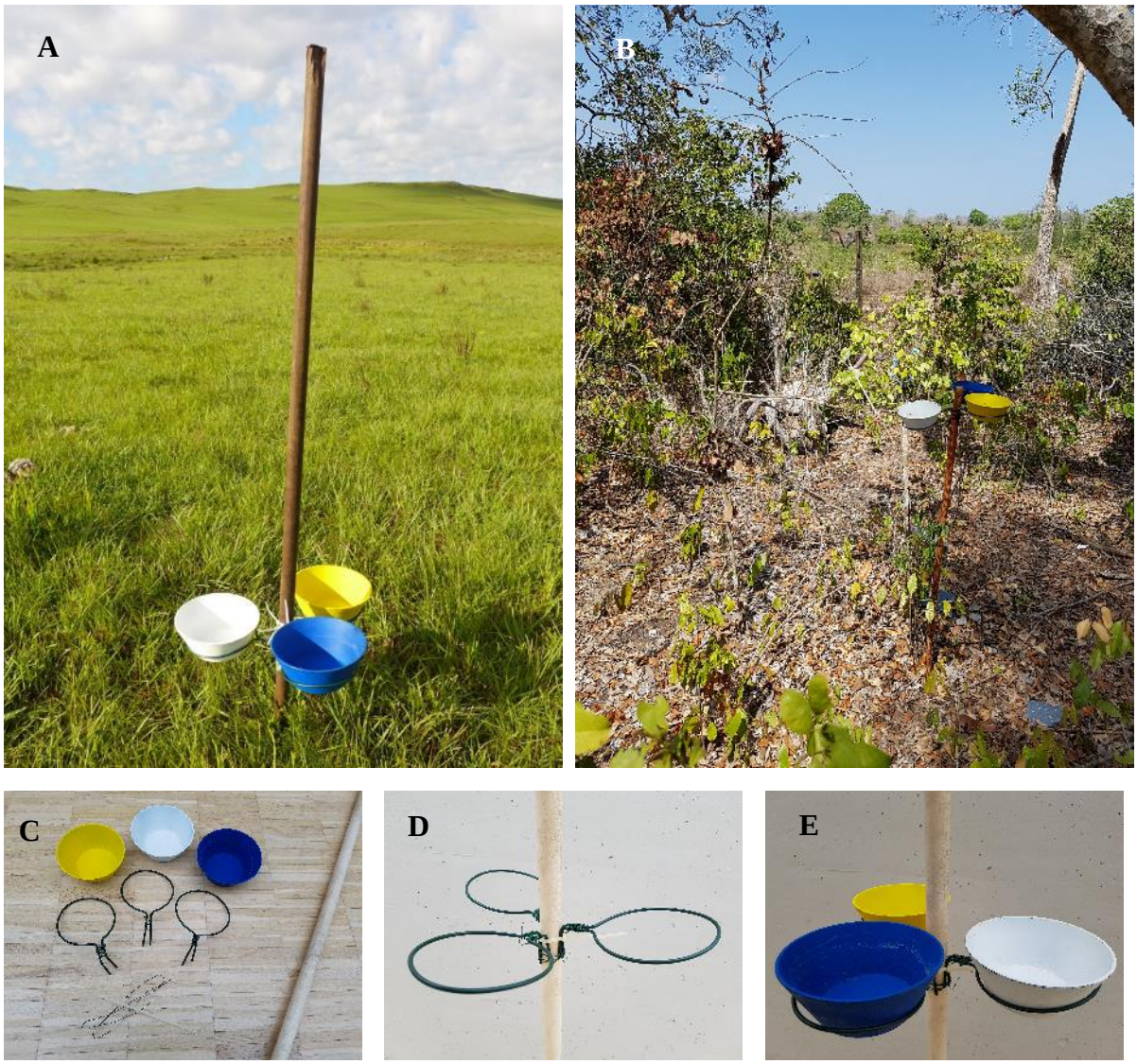

Figure 7 Setup of the different colored pan traps sitting in loops made from sturdy wire fixed to a pole using cable fixers. A: Pan traps in an open grassland vegetation in South Brazil; B: Pan traps at the forest edge in East Kenia; C: Material for one set-up of pan traps; D: Wire loops fixed to the pole with a cable fixer and stabilized by wire; E: Set-up including the three pans. Photos by Isabelle Studer (A) and Sebastian Meyer (B-E)

Seed dispersal: Grain removal

The second mutualistic interaction between plants and animals that contributes to the successful regeneration of terrestrial plants is seed dispersal (Bond 1994). Seed dispersal benefits plants because of reduced density-dependent seed and seedling mortality (Harms et al. 2000). Also, competition with adult plants may be lower away from parent plants, and dispersal allows plants to reach suitable new habitats. The disappearance of experimentally exposed standardized seeds is monitored and their removal probability calculated. Removal experiments are routinely used in

studies on seed fate (Forget and Wenny 2004). While they have often been interpreted as seed predation, newer studies stress the importance of secondary seed dispersal showing that often up to half of the removed seeds are dispersed and germinate (Van der Wall et al. 2005). The removal of seeds quantifies the potential of seed dispersal to occur in a habitat, as seeds that are not removed are evidently not dispersed. To make results comparable, independently of habitat or continent, removal assays are conducted with a standardized seed. Easily obtainable hulled sunflower seeds are used in the assays. 25 seeds are exposed on the ground on plastic plates with a regular grid of 5x5 wells spaced by 2 cm (Figure 8A). Plates are checked at regular intervals of 60–90 min for the remaining number of grains. It is recommended to check the plates 5–10 min after installation and keep monitoring them at 10 min intervals for the first hour in case of high removal activity. During each check, the number of remaining seeds per plate is recorded. A seed is counted as removed when it has left the seed plate. Thus, a seed removed from its well but still lying on the plate is recorded as present, while a seed lying next to the plate (and all seeds that are not visible anymore) is recorded as removed. A datasheet with a table as shown in Table 1 can be used to record the data during the seed dispersal assays in the field. To decrease small scale variability, we recommend to expose three seed plates spaced by at least 1 m at each sampling point (Figure 8B), to make the discovery of the plates by potential dispersers more independent from each other.

Figure 8 Exposition of sunflower seeds in grassland. A: Exposition of 25 hulled sunflower seeds in a 5x5 grid spaced by 2 cm; B: Arrangement of three seed plates spaced by 1 m. Photos by Andrea Sepperl (A) and Isabelle Studer (B)

The resulting data on the number of seeds remaining after different times elapsed since the start of the experiment allows establishing the removal hazard at each plate by performing a survival analysis, e.g. using the survival package in R (Therneau 2015). Removal hazards are frequencies quantifying removal events per individual per minute. They can be derived from a table in which the data is structured as depicted in Table 1 by using the R-code provided below. Only the number of minutes passed since the beginning of the experiment needs to be calculated for each

check conducted in the field. Finally, removal hazards can be translated into removal probabilities as is done at the end of the script provided. Resulting removal probabilities, in this case, quantify the probability of a given seed to be removed within 1 hour. Removal probabilities are calculated for each of the three plates separately, so that removal probability at each sampling point is estimated by three values as indicators of overall seed dispersal potential. Using removal probabilities as an indicator for dispersal is superior to using the number of seeds remaining after a fixed period or the time needed for a given number of seeds to be removed because both these alternative measures are unable to estimate reliable numbers for cases with high and low removal activity. That is because at a single time point either no seeds are left anymore in one case while in the other case no seeds have been removed yet. This problem is resolved by repeatedly checking the seed plates. Consequently, extending exposition of the plates overnight does not add much useful information because of the high probability of all seeds to be removed in the morning. To estimate reliable removal probabilities at night would require repeated checking of the number of remaining seeds during the night. Using a time lapse camera taking photographs of the seed plates at given time intervals would collect the same data without the need of continuous manual monitoring during the measurements. The removal probabilities can be used as the response variable in models testing for the effect of site properties on dispersal.

Needed materials and equipment: seed plates, hulled sunflower seeds

Table 1 Structure of a data sheet to collect data from the seed removal assays with 3 plates per site exposing 25 seeds each

Site		**Time**	**Number of seeds remaining**		
			Plate 1	**Plate 2**	**Plate 3**
1	Start	8:00	25	25	25
1	1st check	8:09	25	22	23
1	2nd check	8:20	21	15	16
1	3rd check	9:53	21	14	14
1	4th check	etc.			

R-code to calculate removal probabilities for the exposed seeds

```
#load package
library(survival)
#read in data
seeds<-read.table("Example_seed_removal.txt",header=TRUE,sep="\t",stringsAsFactors=TRUE)

nsites <- 4 #define the number of sites, 4 in the example file
hazard <- data.frame(matrix(NA, nrow=nsites*3, ncol=4))
names(hazard) <- c("site", "plate", "hazard", "status")

#the following loop recasts the data from a format easy to collect in the field (number of seeds
#that remain at the time of a check) into the format needed for the survival analysis (time when
#each individual seed was last seen on a plate). Next, the loop performs the survival analyses,
#and extracts the removal hazard for each seed plate.
counter=1
for (a in 1:nsites) {
  temp <- seeds[seeds$site==a,]
  new <- data.frame(matrix(NA, nrow=75, ncol=4))
  names(new) <- c("site", "plate", "gone", "status")
  new$plate <- rep(c(1,2,3), each=25)
  new$site<-temp$site[1]
  t1 <- t2 <- t3 <- 0; for (i in 2:length(temp[,1])) {
    t1 <- c(t1, temp[i-1,3]-temp[i,3])
    t2 <- c(t2, temp[i-1,4]-temp[i,4])
    t3 <- c(t3, temp[i-1,5]-temp[i,5])
  }
  if (any(c(t1, t2, t3)<0)) {
    hazard[I(counter*3-2):I(counter*3),1:4] <- c(rep(a, 3), 1,2,3, NA, NA, NA, rep("Error:
Increasing number remaining seeds", 3))
  } else {
    new$gone[1:25] <- c(rep(temp$time_since_start, times=t1),
rep(temp$time_since_start[length(temp[,1])], times=temp[length(temp[,1]),3]))
    new$gone[26:50] <- c(rep(temp$time_since_start, times=t2),
rep(temp$time_since_start[length(temp[,1])], times=temp[length(temp[,1]),4]))
    new$gone[51:75] <- c(rep(temp$time_since_start, times=t3),
rep(temp$time_since_start[length(temp[,1])], times=temp[length(temp[,1]),5]))

    new$status[1:25] <- c(rep(1, times=25-temp[length(temp[,1]),3]), rep(0,
times=temp[length(temp[,1]),3]))
    new$status[26:50] <- c(rep(1, times=25-temp[length(temp[,1]),4]), rep(0,
times=temp[length(temp[,1]),4]))
    new$status[51:75] <- c(rep(1, times=25-temp[length(temp[,1]),5]), rep(0,
times=temp[length(temp[,1]),5]))

    model1 <- with(new[1:25,], survreg(Surv(gone, status) ~ 1, dist="exponential"))
    temp2_1 <- exp(-(summary(model1)$table[,1]))
    model2 <- with(new[26:50,], survreg(Surv(gone, status) ~ 1, dist="exponential"))
    temp2_2 <- exp(-(summary(model2)$table[,1]))
    model3 <- with(new[51:75,], survreg(Surv(gone, status) ~ 1, dist="exponential"))
    temp2_3 <- exp(-(summary(model3)$table[,1]))

    hazard[I(counter*3-2):I(counter*3),1:4] <- c(rep(a, 3), 1,2,3, c(temp2_1, temp2_2, temp2_3),
rep("OK",3))
  }
counter <- counter+1
}
hazard$hazard <- as.numeric(hazard$hazard)
#calculate removal probabilities for time periods of 60 minutes
hazard$remprob <- 1 - exp(hazard$hazard * -60)
#wirte the results in a file
write.table(hazard, file="ResultsCalculationSeedRemovalProbability.csv", sep=";",
row.names=FALSE)
```

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