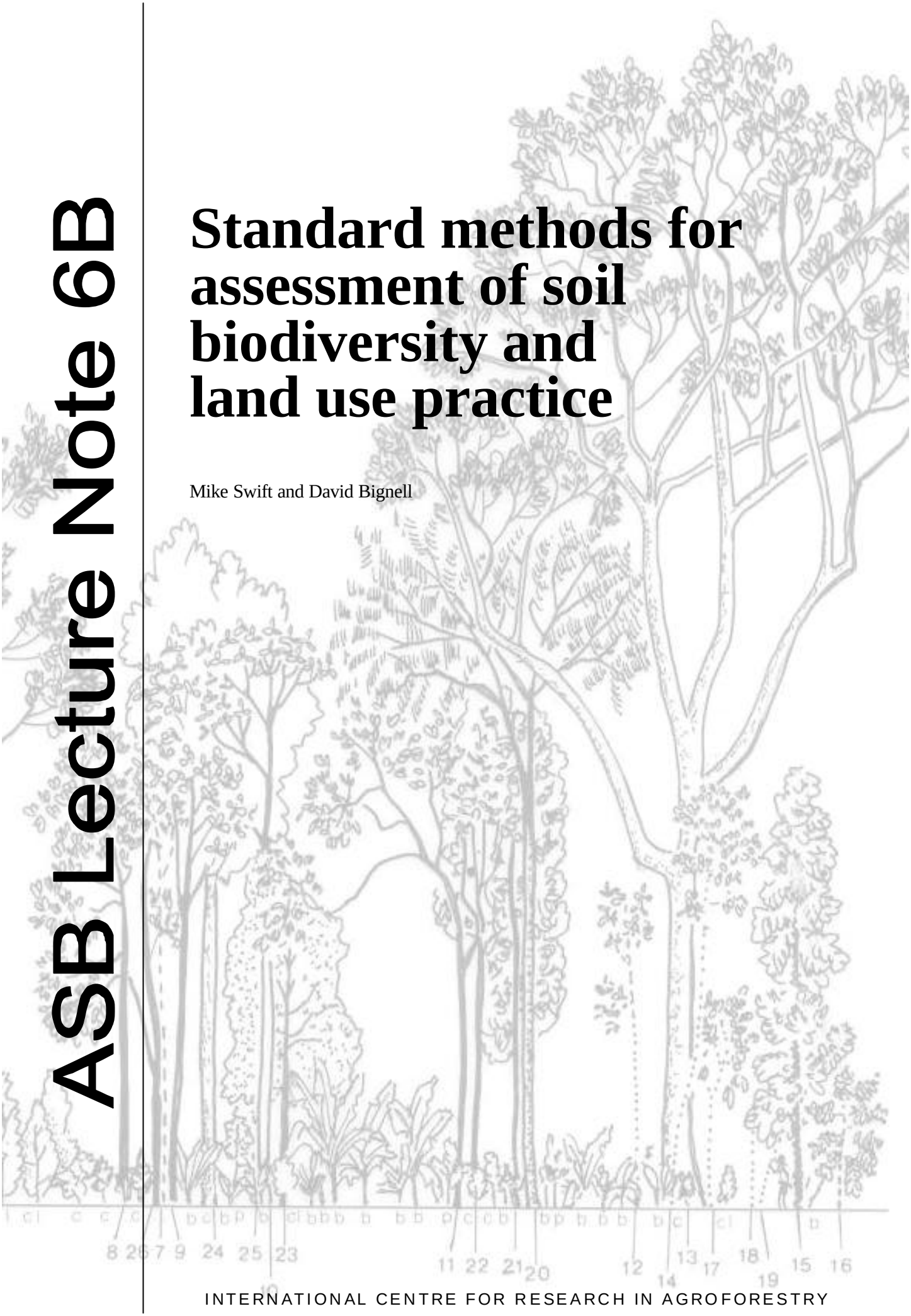
ASB Lecture Note 6B

Standard methods for assessment of soil biodiversity and land use practice

Mike Swift and David Bignell



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Lecture Note 6B

ALTERNATIVES TO SLASH-AND-BURN PROJECT

STANDARD METHODS FOR ASSESSMENT OF SOIL BIODIOVERSITY AND LAND USE PRACTICE

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Preface

Agenda 21 calls for the conservation of biodiversity as well as actions to ensure sustainable development in the agricultural and other sectors. A major feature of global change in the tropics is that of landuse associated with agricultural intensification (Lavelle *et al.* 1997). In addition to plants, soil is the habitat of a diverse array of organisms: archaea, bacteria, fungi, protozoans, algae and invertebrate animals, the activities of which contribute to the maintenance and productivity of agroecosystems by their influence on soil fertility (Hole 1981, Lavelle 1996, Brussaard *et al.* 1997). This is mediated through four basic activities:

1. **Decomposition of organic matter**, which is largely carried out by bacteria and fungi, but greatly facilitated by soil animals such as mites, millipedes, earthworms and termites which shred the residues and disperse microbial propagules. Together, the microorganisms and the animals are called decomposers, but the term litter transformers has now come to be used to describe these animals, where they are not also ecosystem engineers (see below). As a result of decomposition, organic C can be released as CO_2 or CH_4, but also incorporated into a number of pools within the soil (soil organic matter or SOM) which vary in their stability and longevity, but are generally in equilibrium with each other and the inflows and outflows of C from the system.

2. **Nutrient cycling**, which is closely associated with organic decomposition. Here again the microorganisms mediate most of the transformations, but the rate at which the process operates is determined by small grazers (micropredators) such as protozoa and nematodes. Larger animals may enhance some processes by providing niches for microbial growth within their guts or excrement. Specific soil microorganisms also enhance the amount and efficiency of nutrient acquisition by the vegetation through the formation of symbiotic associations such as those of mycorrhiza and N_2-fixing root nodules. Nutrient cycling by the soil biota is essential for all forms of agriculture and forestry. Some groups of soil bacteria are involved in autotrophic elemental transformations, i.e. they do not depend on organic matter directly as a food source, but may nonetheless be affected indirectly by such factors as water content, soil stability, porosity and C content, which the other biota control.

3. **Bioturbation**. Plant roots, earthworms, termites, ants and some other soil macrofauna are physically active in the soil, forming channels, pores, aggregates and mounds, or by moving particles from one horizon to another, in such ways as affect and determine physical structure and the distribution of organic material. In doing so, they create or modify microhabitats for other, smaller, soil organisms and determine soil properties such as aeration, drainage, aggregate stability and water holding capacity.

Therefore they are also called soil ecosystem engineers (Stork and Eggleton, 1992; Jones *et al*. 1994, Lawton 1996, Lavelle *et al.* 1997). For animal soil ecosystem engineers it is usual to add to the definition that they also form faeces which are organo-mineral complexes, stable over periods of months or more (Lavelle *et al.* 1997).

4. **Suppression of soil-borne diseases and pests**. In natural ecosystems, outbreaks of soil-borne diseases and pests are relatively rare, whereas they are common in agriculture. It is widely assumed that low plant species diversity renders agroecosystems vulnerable to harmful soil organisms by reducing overall antagonisms.

A large number of soil animals, in all size categories, are predators which feed on other animals within the same general size category or are consumers of fungal tissue. Protozoans, nematodes and some mites are micropredators ingesting individual

microorganisms or microbial metabolites. All these predatory activities will have a regulatory role contributing to population stability.

The soil biota (and hence soils as a whole) are responsive to human-induced disturbance like agricultural practices, deforestation, pollution and global environmental change, with many negative consequences including loss of primary productivity, loss of cleansing potential for wastes and pollutants, disruption of global elemental cycles, and feedbacks on greenhouse gas fluxes and erosion. At the same time, global food supply depends on intensive agriculture. As intensification proceeds, above-ground biodiversity is reduced, one consequence of which is that the biological regulation of soil processes is altered and often substituted by the use of mechanical tillage, chemical fertilizers and pesticides. This is assumed to reduce below-ground diversity as well, which if accompanied by the extinction of species may cause losses of function and reduce the ability of agricultural systems to withstand unexpected periods of stress, bringing about undesirable effects. Scientists have begun to quantify the causal relationship between i) the composition, diversity and abundance of soil organisms ii) sustained soil fertility and iii) environmental effects such as greenhouse gas emission and soil carbon sequestration.

Large numbers of farmers in the tropics have limited access to inputs but are nonetheless forced by circumstances to drastically reduce the complexity of their agroecosystems in an attempt to intensify production. An alternative solution is to intensify while at the same time retaining a greater degree of above-ground diversity. The maintenance of diversity of crops and other plants in cropping systems is widely accepted as a management practice which buffers farmers against short-term risk. Enhanced biodiversity and complexity above-ground contributes to the re-establishment or protection of the multiplicity of organisms below-ground able to carry out essential biological functions. This can be considered at both the field and the landscape level to enhance structural complexity and functional diversity, especially in degraded lands.

It is as yet an unresolved question what relationship exists between species diversity, functional diversity (the number of functional groups), functional composition (the nature of the functional groups) and the occurrence and intensity of ecological processes. More precisely, what is the minimum number of functional groups, and species within functional groups, to ensure soil resilience against natural and anthropogenic stress? Circumstantial evidence and intuition suggest that stress and disturbance affecting functional groups that are composed of relatively few species are the most likely to cause loss or reduction of ecosystem services. To the best of our knowledge this holds for shredders of organic matter, nitrifying and denitrifying bacteria, bacteria involved with C1 compound and hydrogen transformations, iron and sulphur chemolithotrophs, mycorrhizal fungi and bioturbators.

Beyond the challenge of assessing the soil fertility benefits of retaining or enhancing the biodiversity of the soil community lies the question of giving this economic value at the level of the farm, the nation and the globe. We can distinguish between the short term benefits of soil fertility after slash-and-burn conversion of forests in a long-fallow rotation and the value of the deforested land for permanent agriculture or plantation use. Apart from these values of the forest for local users and newcomers, there are additonal "environmental service" functions, such as biodiversity conservation, watershed protection and mitigation of greenhouse gas emissions, which are values for the outside world, though difficult to quantify. At the soil functional level, the short-term benefits of some types of soil biodiversity may be relatively easily evaluated, for example the gain in nitrogen from introducing N-fixing bacteria, but others are less easily specified, for example the improvements to structure resulting from the introduction of earthworms. While many farmers maintain above-ground biodiversity for a variety of reasons, the linkage between this and soil fertility may or may not be part of the

perceived value. The enhancement of soil biodiversity by the retention of crop residues and other organic matter and by limitations in the use of pesticides will also have associated labour costs which are part of the assessment.

Origins of this Manual

The manual describes sampling and laboratory assessment methods for the biodiversity of a number of key functional groups of soil biota. The methods were assembled and the protocols drafted by a number of scientists affiliated with the Tropical Soil Biology and Fertility Programme (TSBF), the EU-funded Macrofauna Network, the NERC (UK)-funded Terrestrial Initiative in Global Environmental Research (TIGER), and in particular, the UNDP-GEF funded Alternatives to Slash and Burn Project (ASB).

The objectives and working hypotheses of ASB are as follows; the methods were assembled to provide a standardized basis for achieving the first objective.

Objectives and Working Hypotheses

1. To characterize soil biodiversity occurring in natural forest, current land uses resulting from slash and burn agriculture and the "best-bet" alternatives to them.
2. To establish the relationship between the above-ground and the below-ground biodiversity across current and alternative land use systems.
3. To identify "entry points" for improved land management through introduction and/or management of soil biota. The "entry points" might include better understanding of indigenous knowledge and more effective utilization of available technologies.

The objectives were developed to test the following hypotheses:

- Agricultural intensification results in a reduction of soil biodiversity leading to a loss of ecosystem services detrimental to sustained productivity.
- Above-ground and below-ground biodiversity are interdependent across scales of resolution from individual plant communities to the landscape.
- Agricultural diversification (at several scales) promotes soil biodiversity and enhances sustained productivity.
- Sustainable agricultural production in tropical forest margins is significantly improved by enhancement of soil biodiversity.

The data sought

After a number of actual field campaigns, it is possible to give a more specific idea of the information required from sampling each land use:

- What are the following biodiversity parameters:
  taxonomic richness at species and strain (rhizobia) level
  abundance and biomass of taxa
  abundance and biomass of functional groups (FGs)
  relative proportions of FGs
  Shannon-Wiener Index, Simpsons and associated evenness

- How is the land-use defined in terms of
  apparent cropping or fallow usage
  basic physical and chemical soil properties; slope and aspect
  above-ground vegetation character
  climatic averages and actual rainfall to sampling date
  precise history of use and management since undisturbed forest

- Compared with other landuse systems in the regional/local chronosequence, and measured against the best available natural forest:
  what taxa and/or functional groups are particularly affected?
  what trends do they show in relation to landuse type?
  are there trends in the data related to factors other than landuse?
  what is the significance of the changes for soil fertility and other ecosystem services?
  how will crop productivity be affected in this or future landuses?

I. Introduction: characterization of the soil biota and methodological approaches

Key Functional Groups

The taxonomic diversity of the soil biota is so high that inevitably some selection must be made. The taxonomic groups described below were selected on the basis of their diverse functional significance to soil fertility (hence the term *"target taxa"*); and their relative ease of sampling (Figure 1).

1) Earthworms, which influence both soil porosity and nutrient relations through channeling, and ingestion of mineral and/or organic matter.

2) Termites and ants, which influence a) soil porosity and texture through tunnelling, soil ingestion and transport, and gallery construction; b) nutrient cycles through transport, shredding and digestion or organic matter.

3) Other macrofauna such as woodlice, millipedes and some types of insect larvae which act as litter transformers, with an important shredding action on dead plant tissue, and their predators (centipedes, larger arachnids, some other types of insect)

4) Nematodes, which a) influence turnover in their roles as root grazers, fungivores, bacterivores, omnivores and predators b) occupy existing small pore spaces in which they are dependent on water films and c) usually have very high generic and species richness.

5) Mycorrhizas, which associate with plant roots, improving nutrient availability and reducing attacks by plant pathogens.

6) Rhizobia and, when relevant, other N-fixing microsymbionts which transform N_2 into forms available for plant growth.

7) Microbial biomass, which is an indirect measure of the total decomposition and nutrient recycling community of a soil. Microbial biomass is contributed by three very diverse taxa: fungi, protists and bacteria (including archaea and actinomycetes), but it is not usually practical to separate these during measurements. Microbial biomass estimation usually depends on relatively crude chemical methods (lysis of cells, followed by determinations of total N (and P), conversion of these values to a C equivalent, and comparisons with unlysed control samples). It may thus have relatively low resolution, but assesses the decomposer community as a whole.

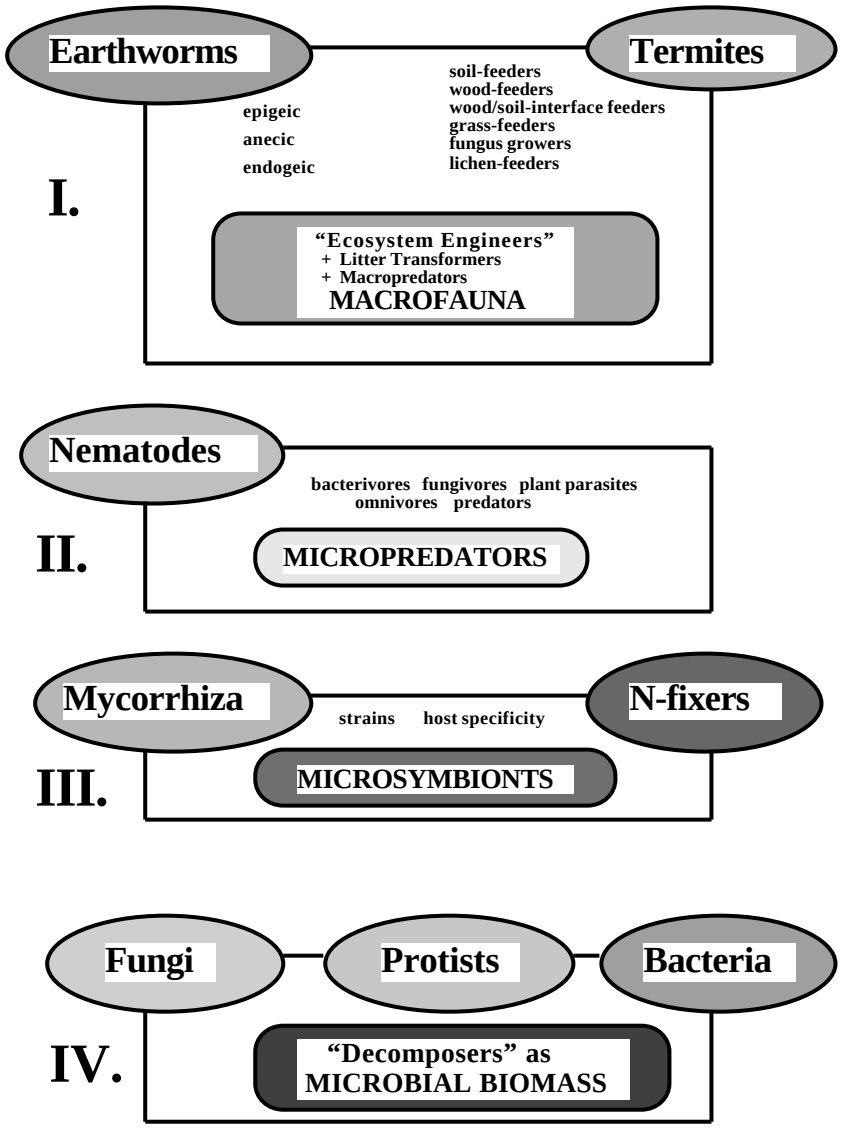


Figure 1. Main "function groups" (capitals), subsidiary functional groups and target taxa (ovals) sampled in the ASB project.

Sampling design: overall strategy

Macrofauna, microbiota and soil (for physical and chemical analyses) are sampled in transects, for which the optimum size is 40 x 4m. However, for the quantitative sampling of termites and for a number of above-ground studies (particularly plant functional attributes and C sequestration) quadrats of 40 x 5m have been deployed, and it seems advisable to standardize both above-ground and below-ground work at 40 x 5m (Figure 2). In further amendments to the procedures, pitfall trapping of surface-active invertebrates and a 100m qualitative transect for termites have been added to the sampling. These can take place along one flank of the transect (pitfalls) or in parallel at about 5-10m distance (termite transect). These modifications are intended, in part, to contribute elements of true biodiversity to the dataset by achieving resolution at the species level, but also to mitigate the variability of data from short transects on groups with typically patchy distributions. Replication of transects in each site is also desirable, as it facilitates statistical analysis of the data obtained, though this may not always be practical where time and funding are limited.

NB In small plots, highly dissected cropping systems or on difficult terrain, it is not necessary for the transect to be both linear and contiguous. For example, where the greatest linear dimension of a particular land-use is <40m, two parallel transects of 20m sample with the same theoretical efficiency as one of 40m. Similarly, a transect can be bent through angles up to 90^{o} to sample plots of irregular shape or to avoid significant natural features such as streams, steep slopes or rock outcrops. Tree falls should, however, be included in the transect if this is appropriate to its existing line and length, and not bypassed.

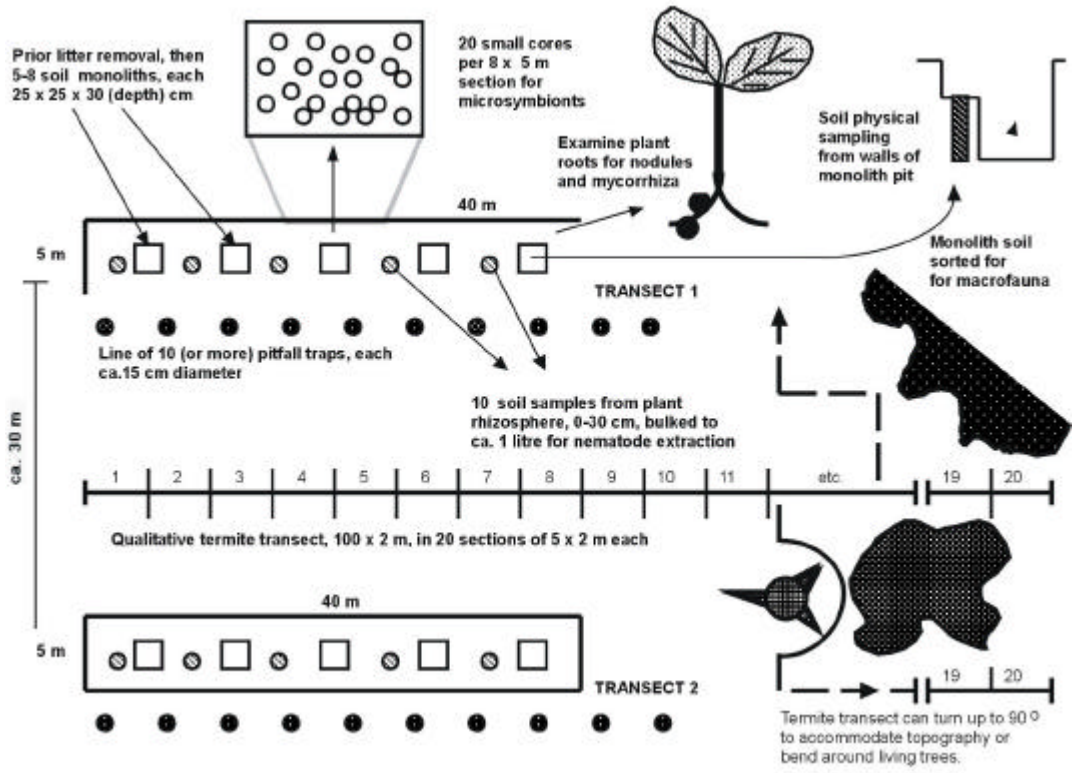


Figure 2. Transect layout and sampling scheme for below-ground biodiversity

Land Use Selection and Characterization

Soil biota are expected to vary with land-use and their diversity to broadly diminish along the chronosequence represented by undisturbed forest, logged-over forest, recently cleared and burned forest, cropping systems, derived pastures and recently established fallow. In any locality, therefore, baseline sampling must be carried out in whichever land use can be identified as the most natural (undisturbed) control site available, preferably closed-canopy forest. However, full site characteristics and classification (and therefore accurate site description) cannot be obtained from apparent land use alone. Concurrent or prior sampling must therefore be carried out for a suite of basic physical and chemical soil properties, including bulk density, texture (S/S/C ratios), pF, pH, total C, total N, exchangeable cations, available P, CEC, Al^{3+} and H^{+}. It is suggested that soil cores taken for these analyses should be from completely undisturbed ground but immediately adjacent to each monolith trench (the outer trench wall is probably the best place), thus providing the opportunity for correlating soil properties with the presence/absence of particular taxa and functional groups. A precise site history is also desirable (though not always obtainable), together with GPS coordinates, altitude, slope, aspect, annual rainfall, mean temperature and humidity, rainy days, length of dry season, and cumulative seasonal rainfall up to the sampling

date. Description of sites can be completed by the above-ground vegetation character. Features such as mean canopy height, crown cover percent, basal area, domin cover/abundance scores for ground flora, litter accumulation and abundance, plant species and generic richness may assist in arranging sites along botanical diversity gradients which have some relationship to their actual positions in the chronosequences and disturbance intensifications.

II. Macrofauna

Procedures follow Anderson and Ingram (1993), making use of pitfall traps together with the digging of soil monoliths of dimensions 25x25x30 (depth) cm. An additional 100x2m sampling transect is used for termites.

2.1 Sampling from the 40 x 5 m transect

a. 5-10 sampling points (for monoliths) are located and marked. These should be equally spaced along the transect. The larger the number of monoliths, the more comfortable the subsequent statistical analysis of the data obtained (see below). It is suggested that 8 should be the target, although 5 will suffice as a minimum number.

b. 10 pitfall traps are installed at roughly 4m intervals along one flank of the transect. The traps are put in during the afternoon or early evening and emptied 24 hours later. Each trap contains a little water, with a few drops of detergent added to immobilize specimens by drowning. Glass jars of about 10-15cm mouth diameter make suitable traps. Depth of the traps is not critical, but the mouth must be exactly flush with the surface of the ground. A sloped cover (for example an inverted petri dish, or a piece of plywood or plastic), supported on twigs over the jar, is useful to keep rain out.

c. At each sampling point, litter is removed from within a 25cm quadrat and hand-sorted at the site. Following this the exact position of the monolith of is marked with a wooden or metal quadrat of 25x25cm outside dimensions.

d. Isolate the monolith by cutting down with a spade a few centimetres outside the quadrat and then digging a 20cm wide and 30cm deep trench around it. NB. In a variant of the method, all invertebrates longer than 10 cm excavated from the trench are collected; these will be mainly large millipedes and earthworms with very low population densities but representing an important biomass. Their abundance and biomass can be calulated on the basis of 0.42m^2 samples, i.e. the width of the block plus two trench widths, squared.

e. Divide the delimited monolith block into three layers, 0-10cm, 10-20cm and 20-30cm. This can be done conveniently using a machette or parang held horizontally and grasped at both ends. Hand-sort each layer separately. If time is short or the light poor (sorting in closed canopy forest is usually difficult after about 3.30 pm), bag the soil and remove to a laboratory. Ants can be extracted by gently brushing small (handful) quantities of soil through a course (5mm) sieve into a tray: the sieve retains the ants. Bagged soil should be kept out of direct sunlight and sorted within 24 hours (but preferably sooner).

f. Record the number and fresh weight of all animals and identify to at least the taxonomic and functional levels indicated in Table 2 (but preferably further). The presence and weight of termite fungus combs (if any) should also be noted. If a balance is not available in the field, fresh weight can be approximated for preserved specimens by weighing them after light blotting.

g. Make a list of species, if possible grouped into subfamilies or families. Within each of these, use generic names to generate alphabetical orders. Combine the results from pitfall traps and monoliths to compile this list.

2.2 Recording and expressing the data

i) Fully identified species should be listed with the full binomial and descriptive authority:
e.g. *Dorylus laevigatus* Smith

Morphospecies should be listed by letter:
e.g. *Crematogaster* sp. A
Crematogaster sp. B
……..etc.

Species identified only to genus should be listed without numbers:
e.g. *Colobobsis* sp.

Incorporate the species list into a table showing the sites where each occurred.

ii) Estimate abundance as nos m^{-2}, from each monolith (multiply the raw number per monolith by 16 (except earthworms and millipedes, see above), combining data for all species. Calculate an arithmetical mean. To estimate the 95% confidence limits the primary data should be transformed as $\log_{10}$ (x+1). If there are not too many zeros, this should roughly normalize the data and produce homogeneous variances from group to group. In difficult cases a loglog transformation can be tried. Apply descriptive statistics to the transformed dataset, including 95% confidence limits, and back transform to obtain a geometric mean. Quote means for untransformed data, together with the (back-transformed) geometric mean and confidence limits for log (x+1) transformed data. The transformed data can be used for histograms and site-to-site comparisons (Eggleton *et al*., 1996).

Prepare a summary table, for example:

Table 1. Termite numerical density in 7 sites across a forest disturbance gradient in Jambi province, central Sumatra: (specimen data)

Site	Arithmetical mean, nos m^{-2} (n=5)	Geometric mean, nos m^{-2} (n=5)*	95% confidence limits*
BS1, Primary forest	2892	971	347-12827
BS3, Logged over	163	65	22-977
BS6, *Paraserianthes*	512	47	5-16445
BS8, Rubber	128	11	2-1046
BS10, Jungle rubber	211	25	2-9772
BS12, Alang-alang	3	2	0-20
BS14, Cassava	26	10	2-534
Litter	46	15	3-64
0-10 cm	106	80	43-148
10-20 cm	55	44	24-78
20-30 cm	49	4	1-50

* back-transformed.
Parametric ANOVAs can be performed on the log (x+1) transformed data. For example:

Between treatments (sites): $F_{(6,28)} = 4.064$; p= 0.005
Betweeen strata: $F_{(3,16)} = 2.299$; not significant.

iii) Estimate biomass as g m^{-2} in a similar way. Use fresh weight or the mass of blotted preserved specimen, if possible. Avoid the use of dry weight because of the different oven temperatures used by different scientists and the variable water content of different types of organism. Where insect specimens in a range of sizes are available, an alternative method is to calibrate live biomass against head width in representative specimens covering the whole size range. The weight of unknowns can then be estimated from the curve. For log transformations of data, it is most convenient to work in (mg + 1), then back-transform and express as g.

Prepare a summary table, as above.

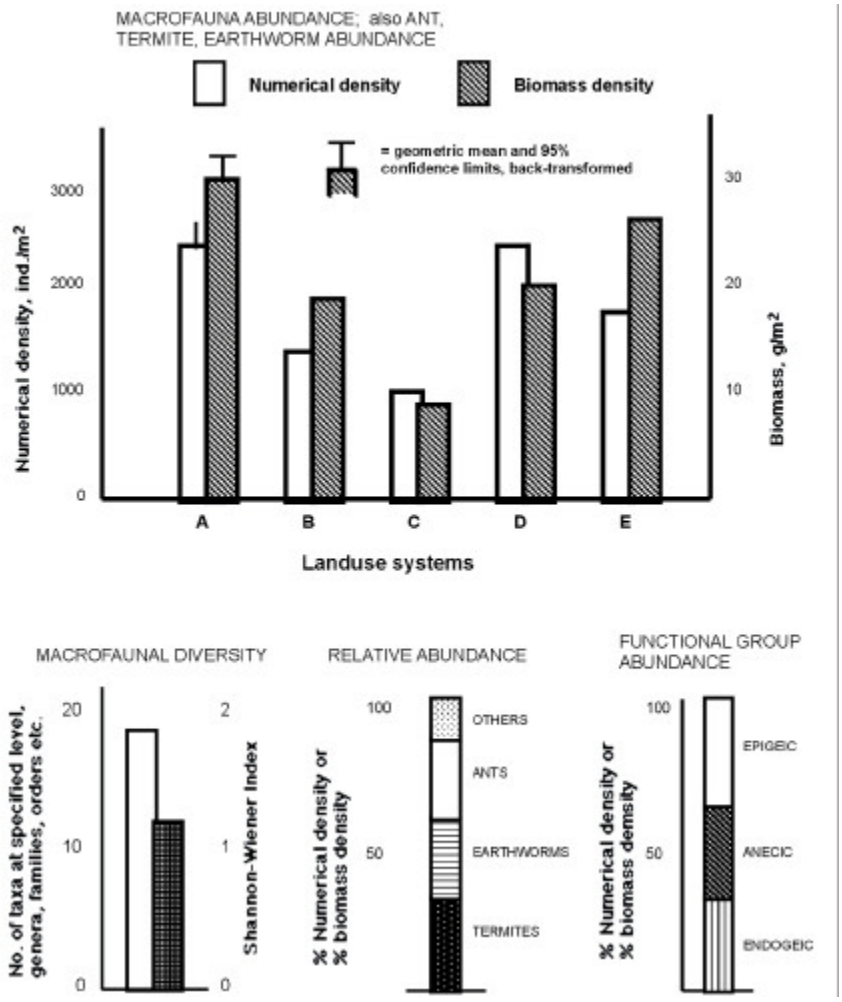


Figure 3. Presentation of abundance and biomass data for macrofauna

Figure 4. Presentation of diversity and abundance data in histogram form

Show species/morphospecies richness, abundance and biomass graphically, as illustrated in Figure 3.

2.3 Analysis

The following steps should be followed:

i) Carry out a non-parametric ANOVA (Kruskal-Wallis) on each datatset to see if there is a significant difference across the sites (or treatments). This can be followed by pairwise comparisons between sites using the Mann-Whitney U test. Matrices can be prepared for the following data:

- total numerical density
- total biomass density
- number of taxonomic orders

- earthworm numerical density
- earthworm biomass density
- earthworm species richness
- termite numerical density
- termite biomass density
- ant numerical density
- ant biomass density
- ant species richness
- coleopteran numerical density
- coleopteran biomass density
- millipede numerical density
- millipede biomass density

Alternatively, if time is short, groups can be pooled together, e.g. all macrofauna, ants and termites combined, macrofauna other than ants and termites combined, etc.

As an illustration:

Table 2. Specimen comparison of termite abundance an biomass in 7 sites across a forest disturbance gradient in Jambi province, central Sumatra.

a. <u>Termite abundance</u>

H = 14.64; p<0.025>0.01.

BS1							
BS3	**(1>3)						
BS6	ns	ns					
BS8	**(1>8)	ns	ns				
BS10	**(1>10)	ns	ns	ns			
BS12	***(1>12)	**(3>12)	*(6>12)	ns	ns		
BS14	***(1>14)	ns	ns	ns	ns	ns	
	BS1	BS3	BS6	BS8	BS10	BS12	BS14

{For each parameter, overall ANOVA is carried out by the non-parametric Kruskal-Wallis method and pairwise site comparisons by one-tailed Mann-Whitney. * p <0.05; ** p<0.025; *** p <0.005. Numbers in brackets refer to the sites. ns, not significant (p>0.05).}

b. <u>Termite biomass</u>

H = 16.49; p<0.025>0.01.

BS1							
BS3	*(1>3)						
BS6	ns	ns					
BS8	***(1>8)	ns	ns				
BS10	*(1>10)	ns	ns	ns			
BS12	***(1>12)	***(3>12)	*(6>12)	ns	ns		
BS14	***(1>14)	ns	ns	ns	ns	ns	
	BS1	BS3	BS6	BS8	BS10	BS12	BS14

{For each parameter, overall ANOVA is carried out by the non-parametric Kruskal-Wallis method and pairwise site comparisons by one-tailed Mann-Whitney. * p <0.05; ** p<0.025; *** p <0.005. Numbers in brackets refer to the sites. ns, not significant (p>0.05)}.

ii) An overall quantitative synthesis of data for macrofauna can be attempted using a matrix similar to the following:

Region	Landuse System				
	A = natural control site	B	C	D	E
e.g. Pasir Mayang	x = 80	x = 67	x = 50	x = 95	x = 57
		p = 0.1	p = 0.04	p = 0.11	p = 0.05
		% = -16	% = -38	% = +19	% = -29

where, x = average of monoliths
p = level of significance for a comparison with the control site by an appropriate statistical test.
% = percentage difference between the mean of each landuse and the control site, with an indication (+/-) of the direction of change (increase or decrease).

The control site is selected as the least disturbed local landuse; in most cases this would be closed-canopy forest, preferably primary, or else old growth secondary or disturbed primary forest.

iii) Functional group analysis.

Soil invertebrates can be classified according to their feeding habits and distribution in the soil profile as follows:

Epigeic species, which live and feed on the soil surface. These invertebrates effect litter comminution and nutrient release, but do not actively redistribute plant materials (though the comminuted material may be more easily transported by wind or water than the material from which it was derived). Mainly a variety of arthropods, for example ants, beetles, cockroaches, centipedes, millipedes, woodlice, orthopterans, together with gastropods and small, entirely pigmented earthworms. Surface-active macrofauna will include those organisms sampled by pitfall traps.

Anecic species, which remove litter from the soil surface through their feeding activities. Considerable amounts of soil, mineral elements and organic matter may be redistributed through these activities, accompanied by physical effects on soil structure and hydraulic properties. Earthworms and non soil-feeding termites are the main groups in this category, but also some arachnids.

Endogeic species, which live in the soil and feed on organic matter and dead roots, also ingesting large quantities of mineral material. The two main groups are earthworms and soil-feeding termites.

Assemblages can be compared by the relative proportions of species or recognizable taxonomic units which can be allocated to one (or more) of the functional categories (e.g. Figure 4).

iv) Summary figures can be prepared as follows:

v) It is recommended that an additional functional group classification be attempted for macrofauna, using the following categories:

Ecosystem engineers (usually ants, termites, anecic and endogeic earthworms). Large invertebrates (> 1 cm, but sometimes smaller) ingesting a mixture of organic material

and mineral debris, forming stable, long-lived faeces which are organo-mineral complexes.

Litter transformers (usually non-social arthropods, epigeic earthworms and molluscs) ingesting a mixture of organic matter and microbial biomass and forming short-lived holorganic faeces. This group includes large sized (> 1 cm) and medium-sized (0.2 - 1 cm) invertebrates, but some smaller animals (e.g. many mites) may have analogous functions.

Macropredators.

Keystone species (e.g. termites and arguably some litter transformers) providing physical niche opportunities for lower-level organisms and determining the community structure of those organisms.

c. Sampling for termite species richness and functional group composition

This method was developed for forests or sites recently derived from forests (as in slash-and-burn agriculture) and follows the formal description by Jones and Eggleton (2000), using a transect of 100 x 2m. The data obtained are qualitative, but are considered to have relatively high resolution, and are therefore treated separately from the monoliths and pitfall traps. The method is low-impact, is suitable for non-termite specialists and can be completed by two people in two or three days.

i) Equipment required: compass, 30 m tape measure, or (preferably) 100m nylon rope or string marked into 20 x 5m sections using yellow or orange fluorescent ribbon or waterproof tape, 2m pole. For each person, a sharpened machette or parang, trowel, high-sided plastic or metal tray, two pairs of forceps, ca. 40 stoppered vials (approx. 1 x 5 cm) containing 80% alcohol (more vials in termite-rich habitats). A short fixed-bladed camping knife (ca. 8-10 cm) may also be useful for probing wood and soil.

ii) Lay out a transect line of 100 m adjacent to the 40 x 5 m transect and approximately parallel with it, though at sufficient distance to avoid any mutual disturbance. The transect should be placed to run through visually homogeneous surroundings, avoiding such features as large streams, cliff edges or fresh skid trails which are not suitable habitats for termites. However, the transect can incorporate other natural features of the biotic environment which contribute to its physical heterogeneity, such as hill slopes, narrow stream gullies or small canopy gaps. Some subjective judgement is often necessary to decide on the most suitable line, especially where the treatment plots concerned are small. Transect lines do not need to be absolutely linear, and can be turned through angles of up to 90^{o} to avoid natural obstacles, as long as they do not re-intersect with themselves. In small plots a transect can be turned, successively, through two 90^{o} angles to run back towards the starting point, but the two main "arms" of the transect should be at least 15m distant. Alternatively, two 50m transects can be run in parallel. If the line runs through the middle of a tree with a very large basal area, the transect can be bent to one side at that point, as long as at least a part of the rooting system falls within the 2m wide sampling belt. Make a note of the starting point, initial compass bearing of the transect line and any major directional changes. If two 40 x 5m transects are employed for replicated monolith and other sampling, the termite transect should run between them (as in Figure 2), but allowing sufficient space to avoid mutual interference.

iii) The 5 x 2m sections are sampled sequentially, each for a total of one person hour (30 minutes per section for a team of two experienced collectors) and the following microniches are investigated in detail: surface soil and litter down to a depth of about 5cm; deep accumulations of litter and soil between large buttress roots; dead wood in all stages of decay; termite carton or soil runways on tree trunks and other vegetation;

subterranean, epigeal and arboreal termite nests and mounds to a height of 2m above ground level (including purse nests suspended on vegetation). Soil, litter and woody items can be rapidly dissected in trays; this is often helpful in revealing termites in cryptic niches, or when light is poor. Sticks should be broken into pieces and tapped onto the trays with moderate force to displace any termites they contain. Larger items of dead wood should be chopped up, bearing in mind that they may be infested in one part and not another. Rotting wood partly incorporated into the topsoil or covered in soil sheeting will frequently contain termites. Mounds and nests may be inhabited by species other than, or in addition to those building them; it is therefore advisable to check the periphery and the base of the structure, as well as its central chambers.

iv) Representatives of the termites discovered should be preserved in alcohol, wherever possible including soldiers as well as the worker caste. One specimen tube should be used for each population (or apparent colony) encountered. A label should be added into the alcohol on which is noted (NB in pencil or waterproof ink, not ball-point!):

Date and transect identification number.

The section number along the transect where the specimen occurred (1,2,3, etc. ..... 20)

The microhabitat concerned (new dead wood, rotten wood, mound, soil, litter, soil at tree root etc.)

The information is important in establishing the nature of the termite community (especially functional group diversity) and for constructing a species-accumulation curve. To avoid confusion in termite-rich sites, the labels should be written as soon as the termites have been placed in the specimen tubes. Elsewhere, labels can be written when the searching of each section of the transect is completed. However, the 30 minutes allocated should include labelling time.

v) A short training or orientation period is usually necessary before inexperienced collectors can sample with the same efficiency as experts. Ideally, a training transect of 50 or 100 m should be sampled first, with the guidance of an experienced collector. Collectors should work steadily (rather than frantically) through each 30 minute sampling period and aim to maintain the same level of sampling efficiency in all sections of the transect. For this reason, and to minimize the necessity of having to work in poor light, it is recommended that no more than 12 sections be completed in any one day. It is also helpful to rest for a few minutes between sections. Two experienced collectors can therefore complete a transect comfortably in two days. In most sites there is no need to collect every termite found, and very common species might be passed over after being initially sampled in order to search for rarer or more cryptic forms, or to find soldiers in species that have relatively low soldier/worker ratios (but bearing in mind that some species are soldierless). It is impossible to sample efficiently in heavy rain, so it is permissible to interrupt collection until better conditions return. Work can be divided between the collectors in any mutually agreed way, for example for each to work in the 1 m belt on opposite sides of the line, to divide each section into two subsections each of 2.5 x 2m, for one to sample wood and mounds, while the other examines soil and tree roots, etc, depending on the nature of the site and the section-to section topography. It is recommended that soil should be dug up in at least a dozen well separated places per section of transect. In transects where few termites are encountered, it is important to observe the sampling protocol exactly, in spite of the small returns, and not to curtail the work.

vi) It is helpful if specimen tubes are "cleaned" (i.e. the termites separated from soil and other debris, fresh alcohol added and new neat labels written) on the same day as the collection takes place, or as soon as possible afterwards. This speeds subsequent

processing of the material by expert taxonomists, and is another reason why a minimum of two days should be allowed for each transect attempted. The termites should be separated into recognisable taxonomic units, either morphospecies or named species. By treating each 5m section as an independent sample, a species accumulation curve can be constructed. Ten random sequences of sections are generated by drawing 20 sections at random without replacement. The number of species found in each section is then used to calculate the cumulative number of species for each selection of the ten sequences. Finally, the mean cumulative number of species of the ten sets of 20 sections is calculated for each section and a species accumulation curve drawn. The curve should be asymptotic (i.e. should rise, then flatten out) indicating that few new species would be found by additional sampling in the area. The actual species richness of the site can be obtained by using the first-order jack-knife estimator (Palmer, 1991). The numbers of species and morphospecies encountered per transect can range from just a few to more than 50, depending on the site and the biogeographical region.

vii) Although the basic information generated is **species richness**, other information can be obtained from the transect. Most important is **functional group diversity**; this can be obtained by noting the numbers of species and morphospecies in the following trophic categories:

Soil-feeders. Termites distributed in the soil profile, the organic litter layer and/or epigeal mounds, feeding deliberately on mineral soil, apparently with some degree of selection of silt and clay fractions. Although the ingested material is highly heterogeneous, there are higher proportions of soil organic matter and silica, and lower proportions of recognisable plant tissue than in other groups (Sleaford *et al.*, 1996).

Soil/wood interface-feeders. Termites feeding in highly decayed wood which has become friable and soil-like, or predominantly within soil under logs or soil plastered on the surface of rotting logs or mixed with rotting leaves in stilt-root complexes. This group is synonymous with "intermediate feeders", *sensu* deSouza and Brown (1994), but not the same as the category "rotten wood-feeders" recognized by Collins (1989).

Wood-feeders. Termites feeding on wood and excavating galleries in larger items of woody litter, which may become colony centres. This group also includes termites having arboreal, subterranean or epigeal nests but feeding elsewhere, and many Macrotermitinae cultivating fungus gardens. "Wood" includes dead branches still attached to living trees and dead standing trees as well as fallen larger items which are fresh, or in all except the terminal stages of decay.

Litter-foragers. Termites that forage for leaf litter, live or dry standing grass stems and small woody items, usually cutting the material before consumption or portage to the nest system. This includes some subterranean and mound-building Macrotermitinae, as well as certain Nasutitermitinae that forage on the surface of the ground, and at least one lower termite, *Hodotermes mossambicus*, with a similar habit. Foraging termites are usually more conspicuous than other feeding types, by reason of the numerous galleries or soil sheets constructed over wood, litter and the surface of the ground, foraging holes opening to the surface from subterranean passages or the formation of uncovered columns of individuals (usually mixtures of foraging workers and soldiers).

Specialized- and incidental-feeders. This category follows the listing of termite foods given by Wood (1978), and includes species feeding on fungi, algae and lichens on tree bark (for example *Hospitalitermes hospitalis* in SE Asia; species of *Constrictotermes* and *Ruptitermes* in S. America), dung-feeders and scavengers of vertebrates corpses (probably consumed opportunistically, although dung is arguably a form of decayed litter), and also certain secondary inhabitants of termite mounds which feed on the organic-rich linings of internal chambers as obligate inquilines (for example

Ahamitermes and *Incolitermes* in Australia: Gay and Calaby, 1970; *Ophiotermes* and *Tuberculitermes* in West/Central Africa: Eggleton and Bignell, 1997).

The categories are not mutually exclusive and many species will take food from at least two sources, especially under unfavourable conditions. Identification of the functional group can be made by reference to abdominal colour in live specimens (soil-feeders and soil/wood interface-feeders are darker), site of discovery (in wood, in soil, etc) and other aspects of biology such as nesting site (arboreal nesters are usually non soil-feeders), absence of soldiers (generally indicates soil-feeders) and taxonomic affiliation (e.g. all Macrotermitinae are non soil-feeders; most Apicotermitinae are soil-feeders or soil/wood interface-feeders). If allocation to functional group is difficult, a useful approximation is to divide the species into "soil-feeders" (soil-feeders and soil/wood interface-feeders as defined above) and "non-soil-feeders" (all the rest). The distribution of species between these categories indicates termite community structure; forest communities are often dominated by soil-feeders, but disturbance or drying-out generally increases the proportions of other functional groups.

Similarly, it is possible to compare termite assemblages on the basis of nesting types by allocating species to the following categories:

Wood nesting. Termites whose colony centres are normally within dead logs or standing trees. Sometimes the dead wood is gradually replaced with carton material or fungus comb.

Hypogeal nesting. Termites whose colony centres are below the ground. Centres are often poorly defined and amorphous (especially in the soldierless Apicotermitinae), with little obvious internal structure, although some have complex underground nests (e.g. many Macrotermitinae). This group includes many species which are facultative secondary inhabitants of epigeal mounds.

Epigeal mounds. Termites whose colony centres are above ground (but excluding arboreal mounds), free-standing or associated with tree buttresses. These mounds are usually well defined and highly complex structures, built to species-typical specifications but with a tendency to become more irregular as they age through erosion, additions and occupation by secondary inhabitants.

Arboreal mounds. Nests attached to trees at various heights, usually made of carton. In most cases the nests are connected to the ground by covered runways, which may assist in distinguishing some arboreal termite nests from those of ants.

Some indication of **relative abundance** may be obtained from the frequency of encounter of individual species (number of sections where they were found, out of 20). Also in the sense that the whole transect represents a single large sample, it is arguably possible to assign dominance and therefore to derive indices of species diversity and equitability. It is also possible that the overall number of encounters (i.e. the number of times separate colonies are discovered in the transect as a whole) may be a useful population parameter. For discussion of full range of information obtainable from transects, see the discussions in Eggleton and Bignell (1995), Eggleton *et al.*. (1997) and Jones and Eggleton (2000).

III. Nematodes

i) From each 40 x 5 m transect, collect 10 samples at approx. 4m intervals. To make the samples, two parallel zig-zag lines are marked along the transect, with 10 evenly spaced sampling points on each line. For each sample, two cores are taken to 30cm depth at opposite points on the zig-zag line, using a carbon steel tube, then combined to

make a pool of at least 500g of soil. Samples must be bagged and sealed, to avoid desiccation, but kept out of direct sunlight. Samples can be temporarily stored in an insulated box for transportation to the laboratory, and then at 4°C until extraction, which should be performed as soon as possible.

ii) Nematodes are extracted quantitatively by combining the flotation and sieving methods of Flegg and Hooper (1970). 300g of soil from each sample is added to 2 litres of water and shaken for 30 seconds. Soil particles are allowed to settle for 2 min., then the suspension is passed through 50-mesh (297mm) and 400-mesh (37mm) screens. After extraction on the second screen, the specimens are killed by gentle heating in water at 60°C and preserved with Golden fixative (8:2:90 mixture of formalin, glycerin and water) at room temperature. Nematode suspensions are then further clarified by a modification of the centrifugation-sugar flotation method of Jenkins (1964). The suspension is centrifuged at 3500 rpm for 5 minutes and the supernatant discarded. The residue is then re-suspended in sucrose solution (45.6%) and centrifuged at 1000 rpm for 1 min. Nematodes are collected by passing the supernatant through a 37-mm screen and washed into additional Golden fixative, to make a final volume of 15ml in which the formalin concentration is 3%.

iii) The nematode population is counted by randomly removing 1ml of solution and determining the mean of 15 fields for each of three aliquots in a counting chamber. The remainder of the specimens are processed to pure glycerine by Seinhorst quick rehydration with ethanol (Hooper, 1970) and permanently mounted on glass slides for identification and photography. 100 nematodes from each transect replication are randomly selected and identified, wherever possible to genus level, and then allocated to trophic (= functional) group. Data should be expressed as the frequency and abundance of each trophic group per unit volume of soil and compared using standard soil nematode diversity indices.

The following functional groups are recognized:

bacterivores
fungivores
plant parasites
omnivores
predators

iv) Nematode populations can be described by the Maturity Index (MI), a measure based on the ecological characteristics of nematode taxa (Bongers, 1990; Freckman and Ettema, 1993). In the scheme, nematode taxa, except for plant-feeders, are classified on a scale of 1-5, with colonizers (short life cycles, high reproductive rates, tolerant to disturbance) = 1, and persisters (long life cycles, low colonization ability, few offspring, sensitive to disturbance) = 5. The Index is calculated as the weighted mean of the constituent nematode taxa values:

$MI = \sum (v_i \times f_i)$,

Where v_i is the colony-persister (c-p) value assigned to taxon i, and f is the frequency (dominance) of taxon i in the sample. MI is a measure of disturbance, with lower values indicating a more disturbed environment and higher values characteristic of less disturbed sites.

IV. Microsymbionts: Rhizobia

4.1 Introduction

The nitrogen-fixing bacteria, known collectively as rhizobia, form nodules on roots (and exceptionally on stems) of some species of Leguminosae and on roots of *Parasponia* spp. Leguminosae comprise about 18000 species distributed in the sub-families Caesalpinoideae (1929 spp., mainly woody tropical plants), Mimosoideae (2727 spp., mainly woody tropical, sub-tropical and temperate) and Papilionoideae (13000 spp., largely herbaceous, see Sutherland and Sprent, 1993). The extent of the symbiosis with rhizobia among Leguminosae is variable and still the subject of active research. Until 1989, only 57% of genera and 20% of species had been examined for nodulation, and the proportions of species able to nodulate were 23%, 90% and 97% among Caesalpinoideae, Mimosoideae and Papilionoideae, respectively (Faria *et al.*, 1989). Although extensive searches for new nodulating genera and species have been made, especially in Brazil (Faria *et al.*, 1989; Moreira *et al.*, 1992), it is thus still possible that many new symbioses will be identified in natural ecosystems (e.g. tropical forests).

Traditionally, the taxonomy of rhizobia was based on strains isolated from temperate crop plants. However, now that isolates from other species and regions are available, and new techniques of molecular genetics have been developed, the taxonomy has been revolutionized, with 12 species added to the original five described (Table 3) and further revisions are expected (Graham *et al.*, 1991).

To isolate and enumerate rhizobia from a diverse microbial population, such as occurs in the soil, a method is required which clearly separates rhizobia from other species. The plant infection technique makes use of the nodulation process itself to estimate rhizobial populations in soil. Further culture and characterization of the rhizobia from the nodules thus formed can provide information on the taxonomic composition of rhizobial populations and the degrees of specificity between particular strains and candidate hosts. Although some host plants are considered highly promiscuous (i.e. show low rhizobial specificity) no one promiscuous host can be inoculated by all existing rhizobia, and conversely, there is no existing rhizobia strain sufficiently promiscuous to nodulate all legumes. A similar argument applies to rhizobia with low host specificity. Thus, to evaluate rhizobial diversity in soil it is desirable to make use of a variety of candidate host plant species, and the more that are employed, the greater will be the variety of rhizobial strains recognized.

The bioassay for rhizobia can make use of promiscuous hosts (e.g. Odee *et al.*, 1977), grown on field soil samples or inoculated with soil suspensions in the lab, and then compared with species nodulating naturally in the site. Although some of the latter associations may be relatively specific, the comparison of rhizobia isolated from naturally formed nodules with those sampled via the bioassay provides a useful check on the accuracy of the laboratory procedure. For instance, *Macroptilium atropurpureum* is one of the widely accepted promiscuous hosts (Vincent, 1970), but in most cases it is predominantly nodulated by *Bradyrhizobium* species (Woomer *et al.*, 1988), although it can also be nodulated by *Rhizobium* spp. Similarly, Lewin *et al.* (1987) demonstrated that *Vigna unguicalata*, usually considered a *Bradyrhizobium* host, in fact has very low specificity and can be nodulated by fast-growing *Rhizobium* spp. Preliminary experiments are therefore recommended for each soil environment before the final choice of host plants is made for the bioassay.

Nitrogenase is the enzyme responsible for the reduction of nitrogen gas to ammonia. It alternatively reduces, among other substrates, acetylene to ethylene (Dilworth, 1966; Schollhorn & Burris, 1966). This reaction is used as a technique for the measurement of

nitrogenase activity. The great advantage of acetylene reduction assay (ARA) is its great sensitivity and speed. It is also not expensive and relatively simple to carry out, even under field conditions. Although the use of ARA for quantitative estimates of N_2 fixation contribution to plant nutrition has been widely criticized (Boddey, 1987; Giller, 1987), it is very useful for the simple detection of N_2-fixers. For instance, as nodule anatomy varies widely in shape and size, the less experienced may confuse them with structures not induced by rhizobia. It can be also used for confirmation of new rhizobia symbiosis or other symbiosis forming structures like nodules (Moreira et al., 1992). Nitrogenase activity constitutes valuable information because in many cases it is impossible to verify if nodules are still viable and effective (red colour inside) as for isolation they must be intact. However, it must be considered that nodules without nitrogenase activity can be senescent or ineffective.

4.2 Requirements

Fieldwork: for *soil sampling*: alcohol, insulated cold-box, sterilized plastic bags (300 ml), spatula, large plastic bags (5 l) and small soil corer; for *nodule sampling*: small scissors, spade, hoe, mattock, forceps, shovel, screw cap tubes with silica gel or anhydrous $CaCl_2$; for *plant vouchers*: alcohol, press, old newspapers.

Laboratory work: for *rhizobia enumeration*: 1 ml and 5 ml pipettes, diluent solution, 1 l and 125 ml Ehrlenmeyer flasks, orbital shaker, sterile plastic bags (125 ml, as growth pouches) or glass tubes (150 x 20 ml or 200 x 30 mm), racks for growth pouches or tubes, nutrient solution, seeds of promiscuous host plants, controlled environment room (temperature, light, humidity); for *rhizobia isolation and culture characterization*: petri dishes, 95% alcohol, 0.1% $HgCl_2$ (acidified with conc. HCl at 5 ml/l), sterilized water, forceps, yeast-mannitol-mineral salts agar medium, pH 6.8; for *nitrogenase activity* (by acetylene reduction) Kitasato erlenmeyers, rubber ball (of the type used inside footballs), 1 ml gas-tight syringes, 5 ml vacutainers, 10 ml (or larger) vials with rubber stoppers, calcium carbide (CaC_2), Gas chromatograph equipped with flame ionization detector (FID) and Poropak RN column for acetylene/ethylene determinations. NB Nitrogenase assays can be performed in the field.

4.3 Methods

Soil sampling: small amounts of soil are cored to a depth of 20 cm from 20 points distributed within each 8 m section of the 40 x 5m transect. Each set of 20 samples is bulked to form a composite sample of about 300g and placed inside a sterile plastic bag.

Alternatively, if resources permit, three or more composite samples can be collected per transect. All sampling materials (corers, spatulae, hoe etc.) must be flamed before and after sampling at each site to avoid the introduction of exotic rhizobia. Steps inside the transect should be limited and litter must be removed just before sampling takes place (rhizobial sampling should therefore be the first priority before other groups are addressed). Soil samples should then be transferred to the laboratory in an insulated container (preferably at 4^oC) as soon as possible. If time and resources are short, a single bulked sample can be collected from 20 cores distributed across the whole transect. A second bulk sample of about 200g should be collected in a non-sterile plastic bag for soil physical and chemical analysis.

Nodule sampling: Leguminous species inside the transect should be identified and collected. It is helpful if those which are able to nodulate are known in advance, in which case the collection can be confined to these species. For herbaceous plants, the whole root system can be removed from the soil (using hoe, spade or mattock as required), with care not to accidentally sever existing nodules. Nodules of woody plants

must be discovered by excavation of the roots, taking care to explore the finer ramifications where nodulation is more commonly found. The nodules are then excized (leaving a piece of root to facilitate manipulation) and stored individually in screw-cap tubes containing desiccant. At least 50 nodules should be collected per site, and be representative of all nodulating species in the transect. Occasional nodules may be too large for the ordinary screw-cap tubes and should be stored in a larger container.

Nitrogenase activity can be measured in the field on individual nodules, just after sampling, or in the laboratory (Figure 5). The nodule is put in a 10ml (or larger if needed) vial with a rubber stopper. Acetylene is produced in a Kitasato ehrlenmeyer by the reaction of CaC_2 with water (Figure 6), and 1ml of this gas injected into the nodule-containing vial. After 1 hour (or less) 1ml of headspace gas is removed and transferred to a vacutainer for the analysis of ethylene in the laboratory by gas chromatography.

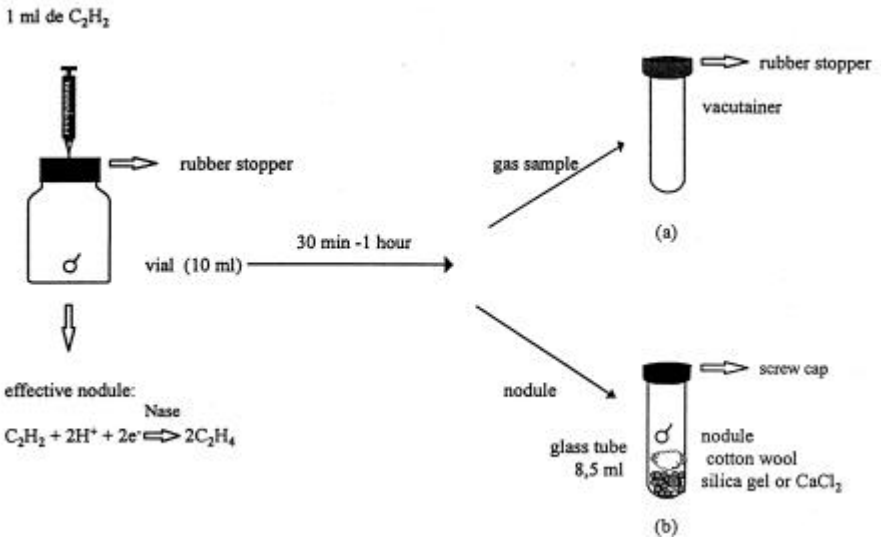


Figure 5. Field work: a) taking gas samples from nitrogenase-mediated acetylene reduction and b) storage of nodules until isolation in the lab.

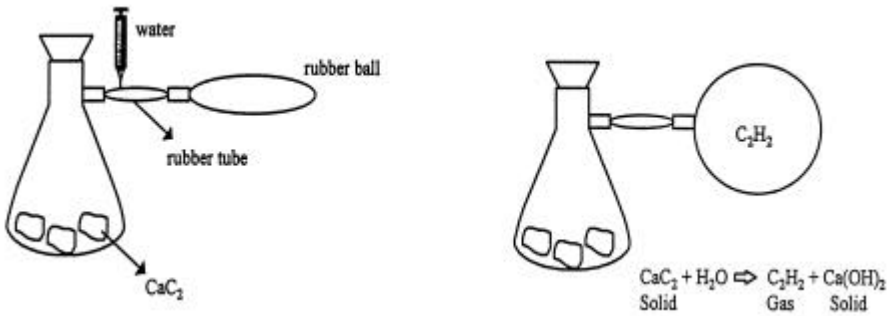


Figure 6. Acetylene production in the field or laboratory.

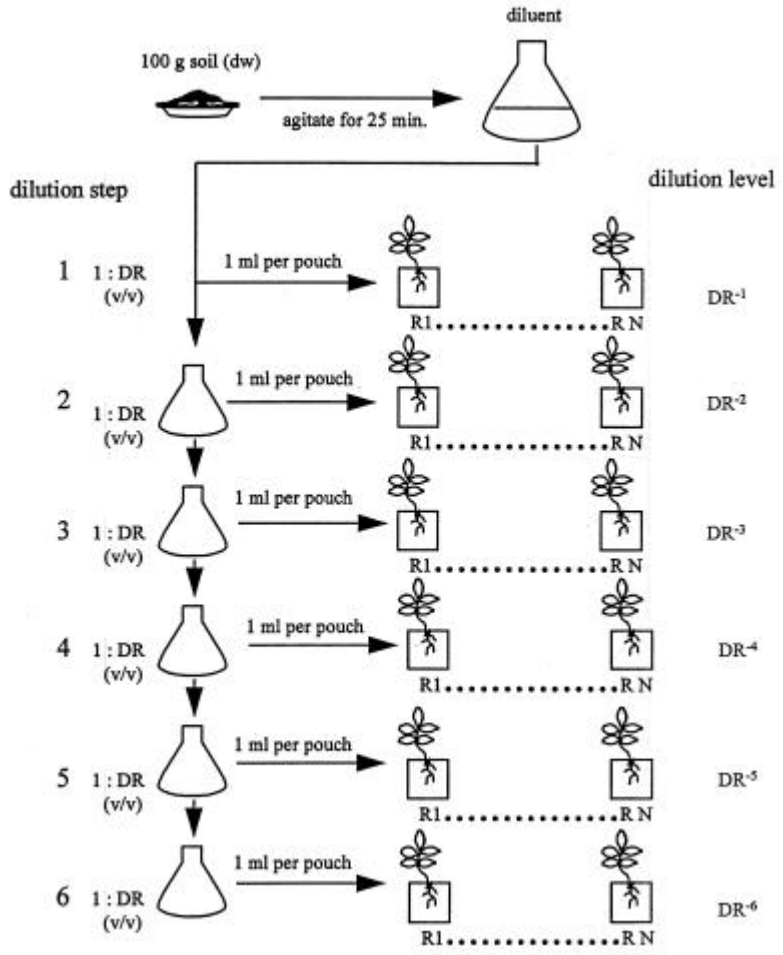


Figure 7. Method for calculation of most-probable-number of rhizobia cells in soil by the plant infection technique. Base dilution rates (DR) can vary from 2 to 14.5 and replicate numbers per dilution (N) from 2 to 5. (Adapted from Woomer, 1993 and Vincent, 1970).

Plant voucher specimens: Voucher specimens of nodulating plant species must be collected with careful attention to labelling (see below) and, if possible, the inclusion of flowers and fruits. The specimens should then be sent to a Herbarium for identification, accompanied by an identification card as in the following example:

Project: ASB Soil biodiversity and land-use practice.
Collector: Fatima Moreira.
Date: 2 May 1997.
Locality: Pedro Peixoto, State of Acre, Brazil.
Altitude: 500 m.
Species vulgar name: faveira.
Species scientific name:
Voucher number: 05.
Nodule characteristics: indeterminate growth, size 0.5 to 1.5 cm.
Site description: pasture, cattle grazing, open with few stumps.
Soil type: sandy loam.
Plant characteristics: herbaceous with mature fruits.
Other comments: seeds collected, yellow flowers.

Rhizobia counting: Soil samples are submitted to serial dilutions before candidate host plants are inoculated (Figure 7). The dilution ratios vary between 2.0 and 14.5, depending on the expected concentration of cells in the soil sample, i.e. greater dilution for soils with more rhizobia. However it is still necessary to inoculate host plants at all dilutions (see below). Also, at each dilution used, replication of the bioassay (2-5 times)

should be employed. Plants are grown under controlled environment conditions (Vincent, 1970), and examined for nodule formation after 15 days. Populations of rhizobia are estimated by the Most Probable Number method (Woomer *et al.*, 1990).

Rhizobia isolation and characterization: Rhizobia are isolated from nodules collected in the field and from those obtained under laboratory bioassay. In the latter case, it is necessary to use nodules obtained at each inoculum dilution level, in order to address those strains which are rare in the soil sample, as well as common ones.

The first step is to surface sterilize the nodules by a brief immersion in 95% alcohol, followed a longer immersion up to 3-4 mins. in $HgCl_2$ (Na or Ca hypochlorite, or H_2O_2 can be substituted) and washing in several rinses of sterile water (Vincent, 1970). The nodule is then crushed in a few drops of sterile water, using forceps, and a loopful of this suspension is streaked onto an agar medium. In the case of desiccated nodules, they should first be soaked in sterile water to improve their wettability by the sterilants. Immersion times in $HgCl_2$ should be adjusted to nodule size (shorter for smaller nodules). Composition of the yeast-mannitol-mineral salts agar medium (especially pH and carbohydrate source) can be varied to take account of particular soil conditions (Date and Halliday, 1979; 1989; Souza *et al.*, 1984; Elkan, 1991). Bromothymol blue can be included as an indicator, as pH changes caused by rhizobial growth may be useful in genus identification. Other characters include growth rate (TAIC-time of appearance of isolated colonies), the extent of extracellular polysaccharide deposition, colony shape and colony colour. The main generic descriptors are:

Allorhizobium, *Rhizobium* and *Sinorhizobium*: colonies circular, 2-4mm in diameter, but usually coalesce due to copious extracellular polysaccharide production, convex, semi-translucent, raised and mucilaginous, most with a yellowish center (due to pH indicator), fast growers (TAIC -3 days).

Mesorhizobium: same as *Rhizobium*, but intermediate growers (TAIC 4-5 days).

Bradyrhizobium: colonies circular, do not exceed 1 mm in diameter, extracellular polysaccharide production from abundant to little (the latter generally in those strains taking >10 days to grow), opaque, rarely translucent, white and convex, granular in texture, produce an alkaline pH shift, slow or very slow growers (TAIC 6 or more days).

Azorhizobium: colonies circular, 0.5mm in diameter with a creamy colour, very little extracellular polysaccharide production (much less than in *Bradyrhizobium*), produce an alkaline pH shift, fast to intermediate growers (TAIC 3-4 days).

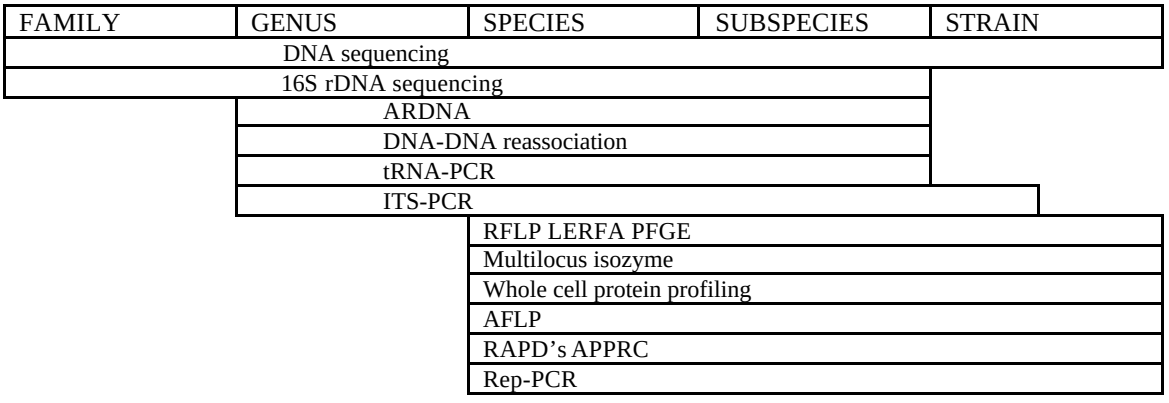
Rhizobia generally are non-spore forming G^- rods, usually containing poly-β-hydroybutyrate granules refractile under phase contrast microscopy. Isolates with these characteristics must be reconfirmed as rhizobia by demonstration that they will again nodulate a test host plant under bacteriologically controlled conditions (Vincent, 1970). Table 3 gives references in which full details of species characteristics are given, in addition to Jordan (1984) and Elkan (1991). Strain diversity within species may be high, both genetic and phenotypic (for example symbiotic, cultural morphological and physiological traits) and it is thus necessary to define the level of diversity which is appropriate to characterize particular genera and strains. Additional characters can include serology (Dudman and Belbin, 1988), cell lipopolysaccharide (de Maagd *et al.*, 1988), total protein patterns by SDS-PAGE (Hames and Rickwood,, 1985; Nwaga *et al.*, 1990; Moreira *et al.*, 1993), small subunit RNA sequence (Young and Haukka, 1996), plasmid profiles (Giller *et al.*, 1989), intrinsic antibiotic resistance (IAR, Kingsley and Bohool, 1983), multilocus enzyme electrophoresis (Selander *et al.*, 1986) and growth on different C sources (Dreyfus *et al.*, 1983). Numerical cluster analysis of an adequate number of strains and comparison with rhizobial type strains can permit the

characterization of large populations. Genetic characterization (DNA, 16S rDNA or 23S rDNA sequencing; DNA:DNA homology based on 23S rDNA) is time-consuming and requires specialized equipment, and is usually confined to representatives of clusters only. Minimum standards for the description of new genera and species are given by Graham *et al.* (1991). Bacterial classification methods and their resolution levels are summarized in Figure 8.

Table 3. Current classification of legume nodule nitrogen fixing bacteria species

Genera	Species
Rhizobium (Frank, 1889)*	*R. leguminosarum* (Frank, 1889)*, biovars *phaseoli, trifolii, viceae* (Jordan, 1984) *R. tropici* (Martinez-Romero *et al.*, 1991)* *R. etli* (Segovia *et al*., 1993)* *R. giardinii* (Amarger *et al*., 1997) *R. gallicum* (Amarger *et al*., 1997) *R. hainanense* (Chen *et al*., 1997) R. huantlense *(Wang* et al., *1998)* *R. mongolense* (Van Berkum *et al.*, 1998)
Sinorhizobium (de Lajudie *et al.*, 1994) *	*S. meliloti* (Dangeard, 1926; de Lajudie *et al.*, 1994)* *S. fredii* (Scholla & Elkan, 1984; de Lajudie *et al.*, 1994)* *S. saheli* (de Lajudie *et al.*, 1994)* *S. teranga* (de Lajudie *et al.*, 1994)* *S. medicae* (Rome *et al*., 1996[a])
Mesorhizobium (Lindström *et al.*, 1995; Jarvis *et al.*, 1997)	*M. loti* (Jarvis *et al*., 1982)* *M. huakuii* (Chen *et al.*, 1991)* *M. ciceri* (Nour *et al.*, 1994a)* *M. tianshanense* (Chen *et al*., 1995)* *M. mediterraneum* (Nour *et al.*, 1995)* *M. Plurifarium* (de Lajudie *et al.*, 1995)* *M. amorphae* (Wang *et al*., 1998)
Undefined	*Rhizobium galegae* (Lindström, 1989)*
Bradyrhizobium (Jordan, 1982)*	*B. japonicum* (Jordan, 1982)* *B. elkanii* (Kuykendall *et al.*, 1992)* *B. liaoningense* (Xu *et al.*,1995)*
Azorhizobium (Dreyfus *et al.*, 1988)	*A. caulinodans* (Dreyfus *et al.*,1988)
Allorhizobium (de Lajudie *et al.*, 1998)	*A. undicola* (de Lajudie *et al*., 1998)

* these references in Young and Haukka (1996)



Key to Figure 8: ARDRA, restriction analysis; tRNA-PCR or ITS, amplification and analysis of inter tRNA spacer regions; RFLP PFGE, restriction fragment length polymorphism-pulse field gel electrophoresis; AFLP, amplified length polymorphism; RAPD-APPCR, random amplified polymorphic DNA-arbitrary primed PCR; rep-PCR, genomic fingerprinting.

Figure 8. Bacterial classification methods, adapted from de Bruijn *et al*., 1997.

V. Microsymbionts: Mycorrhizas

The methods are developed from those described by Anderson and Ingram (1993). 20 random small auger cores of about 50g each are taken to a depth of 20cm in each 5m section of the transect. The samples are bulked together and mixed thoroughly after collection, and aggregates hand-broken. The transect as a whole therefore yields 8 bulked samples of about 1kg per sample. Each sample is then placed in a plastic bag and air-dried (with the tops of the bags rolled down) for 24 hours under cover, then brushed through a 2mm mesh and stored at 4°C.

Mycorrhizal spore extraction is carried out according to Sieverding (1991). Spores are separated from soil by a wet sieving method using a graded series of sieving baskets (45 through 710 mesh). Three replicates of ca. 100g each should be processed from each sample. Collected spores are identified under a stereomicroscope, by attention to colour, size and shape, following the key of Schenck and Perez (1990). Fine roots on the sieves are also collected and stained with acid fuchsin. Characterization of mycorrhizal infection follows evidence of vesicles, arbuscules and surface hyphae (Merryweather and Fitter, 1991).

Further evaluation of the variety of mycorrhizal infection potential in soil samples can be obtained by bioassay. 300 g of soil sample are used as growth medium for the host plants *Vigna unguiculata* and *Pennisetum americanum.* Mycorrhizal infection and sporuation are evaluated (as above) after 3 months growth in a greenhouse.

At present, the best available measure of mycorrhizal diversity is the number of types present per soil, i.e the sum of spore types from soil extraction and bioassay infections. Typical figures for tropical forests and forest-derived soils by the above methods range 4-20+ types.

VI. Microbial biomass carbon

The method is based on Amato and Ladd (1988). It is time-consuming, both during the preparation for fumigation and the subsequent extraction and assay stages. Therefore laboratory logistics and the allocation of technician time should be planned accordingly.

In the field, soil should be sampled with a spade from the top 10-10cm, after the clearance of dead wood and leaf litter. The number of samples taken should be sufficient to calculate confidence limits for the data produced, i.e. at least 5 samples per transect, but two per 5m section is a better number, making 16 in total, or two bulked samples per section, making a total of 8. At each sampling point about 500g of soil should be placed in a clean plastic bag and taken immediately to the laboratory and brushed firmly through a coarse sieve to remove stones and pieces of wood. The soil should then be air-dried for about 24 hours, subsampled for the determination of moisture content (by evaporation to dryness at 105°C) and rebagged for further processing, which should begin as soon as possible, and not more than 48 hours later. Soil waiting for processing can be stored at 4°C, but must not be frozen or oven dried.

The first step is to determine water-holding capacity (WHC). This need not be done on more than one or two samples per transect, as long as the site is pedologically homogeneous.

i) Place about 50 g of soil in a large filter funnel plugged loosely with glass wool and clamp to stand in a beaker. Pour about 100 ml of water onto the soil and leave overnight.

ii) Weigh the moist soil and funnel, then dry in an oven at 105°C for 24 hours and weigh again. Remove the dry soil from the funnel and weigh this alone.

Calculate WHC as $\frac{\text{(weight wet soil) – (weight dry soil)}}{\text{(weight dry soil)}}$ = g H_2O/g dry soil

WHC is then used to adjust all soil samples subsequently weighed out to 40% WHC (=8.0 g H_2O per 20 g soil), taking account of the moisture already present (from the earlier determination of moisture content). 20 g subsamples adjusted in this way are then ready for fumigation and/or extraction.

iii) Weigh two subsamples of 20g each of air-dried soil into small glass beakers and adjust to 40% WHC by the addition of water. One subsample will be fumigated with chloroform and then extracted with KCl solution and the other, which is a control, will be extracted with KCl solution without exposure to chloroform. Mark each beaker accordingly, using pencil and tape (not felt-tip pens).

iv) Place the samples for fumigation in a large vacuum desiccator containing a beaker with about 50ml of chloroform. Add a few glass beads to the chloroform, close the desiccator and evacuate (using either an electrical or water-jet vacuum pump) until the chloroform starts boiling. Seal the desiccator and leave in the dark for exactly 10 days at 25°C. Leave the control soil samples, loosely covered with foil, under the same conditions, but without exposure to chloroform.

v) After 10 days, extract each soil sample with 63ml of 2M KCl for one hour, using an end-over-end (or rotary) shaker. Centrifuge for 2-5 mins at 2500g to separate soil and supernatant. Carefully remove 10 ml of supernatant with a graduated plastic syringe, fit a Millipore prefilter (AP20 013 00) to the syringe and expel the supernatant through the prefilter into a clean 10ml screw-cap plastic or glass vial.

This filtrate can then be frozen pending further processing and analysis, but note that until this point is reached the timetable of the procedure must be adhered to strictly.

vi) Prepare the following reagents freshly (quantities suitable for about 60 samples):

2M KCl solution, 1 litre.

50% Analar-grade ethanol in distilled water, 1 litre.

4N Acetate buffer: add 544g sodium acetate to 400ml water and stir while heating until dissolved. Cool to room temperature and add 100ml of glacial acetic acid and make up to 1 litre; check the pH is 5.51 ± 0.03.

Ninhydrin reagent: dissolve 2g ninhydrin and 0.2g hydrindantin in 50ml 2-methoxy ethanol, add 50ml acetate buffer and mix; store in a dark glass reagent bottle.

2.5×10^{-4} M leucine solution: prepare a stock solution of 2.5×10^{-2} M by dissolving 0.3279g of L-leucine in 100ml 2M KCl.. For the assay, dilute 1ml the stock solution with 99 ml of 2M KCl.; this standard contains 7 µg N per 2ml.

2.5×10^{-4} M ammonium sulphate solution: prepare a stock solution of 2.5 x 10-2 M by dissolving 0.1652 g Analar-grade ammonium sulphate in 100ml 2M KCl. For the assay, dilute 1 ml of stock solution with 99ml of 2M KCl; this standard contains 7µg N per 2ml solution.

vii) Use the standard leucine and ammonium sulphate solutions to check the linearity of the colour development with ninhydrin and calibrate the reagent as follows:

Make up four tubes containing 1ml of solution with 0, 0.7, 1.4, 2.1 and 2.8µg N, respectively (i.e. use 0, 0.2, 0.4, 0.6 and 0.8ml of diluted stock and make up to 1ml with 2M KCl). Put 1ml of each of these standards in each of three tubes. Add 1ml of freshly prepared ninhydrin to each, mix with a vortex and boil in a water bath for 15 mins. Cool the tubes in cold water and add 5 ml of 2M KCl to each. Seal with parafilm, shake vigorously for 5 mins and immediately read optical density at 570nm against a blank tube of 2M KCl. Average OD for the three replicates.

viii) For each subsample filtrate, prepare three tubes:

A blank containing 1 ml 2M KCl.

Sample 1, containing 0.5ml of filtrate from the fumigated samples + 0.5ml 2M KCl.

Sample 2, containing 0.5ml of filtrate from the unfumigated samples + 0.5ml 2M KCl.

Add 1 ml of freshly prepared ninhydrin to each, mix with a vortex and boil in a water bath for 15 mins. Cool the tubes in cold water and add 5ml of 2M KCl to each. Seal with parafilm, shake vigorously for 5 mins and immediately read optical density at 570 nm against a blank tube of 2M KCl.

xi) Microbial biomass carbon (MBC) is defined as 21 x the release of ninhydrin-reactive N from soils fumigated for 10 days at 25°C. The calculation of results is therefore:

MBC = ([t_{10}abs – $t_0$abs – y intercept]/slope) x (sample dilution) x (extraction volume/dry soil mass) x (21).

And should be expressed as µg C per g dry weight of soil.

VII. Soil physical and chemical properties

In preparing this section we have assumed that a professional soils laboratory will be engaged to process samples for soil chemistry. We therefore provide a protocol for soil sampling and for the determination of bulk density, the principal physical attribute of relevance for faunal biodiversity and plant growth, together with summary of approaches to chemical analysis. This is because chemists around the world have not agreed on one particular way of extracting, digesting and characterising the major nutrients, so suites of properties determined in standard analyses vary from one laboratory to another.

Soil physical properties are affected by soil macrofaunal activity and in turn affect soil macrofaunal activity. While land-use systems have an effect on soil macrofauna through the different operations executed by farmers and the type of vegetational cover, the soil properties in a site may affect the magnitude of the response to any disturbance in the system. Therefore soil properties need to be determined to evaluate to what extent changes in macrofaunal density, diversity and activity as a consequence of changes in land-use are directly due to soil physical properties.

i) Site preparation and sampling regime.

When sampling the soil in a transect, care has to be taken that the soil is not trampled before sampling, especially in land-use systems not regularly subject to human traffic, as this will compact the soil considerably. In transects without monoliths, 3-5 samples should be taken from each layer in each of 5 to 10 positions. If the transect is laid out for monolith digging, soil samples should be taken close to the monoliths but before work on the monoliths is started to avoid compaction. Alternatively, if time and

resources are limited, samples can be taken from the walls of the monolith pit. This has the advantage that monolith faunal contents and soil properties are then directly comparable. A sample taken to determine bulk density can be used to determine a series of other physical and chemical properties.

ii) Bulk density

Soil bulk density is a measure of the amount of solid phase soil particles pr unit volume. Bulk density data are required to convert soil chemical properties such as nutrient concentrations to nutrient quantities in a soil horizon. Bulk density data also provide a measure of the level of compaction and indicate if processes such as water infiltration and gas exchange are likely to be impeded.

To take a core sample for bulk density determination, steel corers, a small trowel and a knife with a blade of at least 5 cm length are required. Where samples are to be kept in the core (e.g. as for bulk density) two lids for each core are required. Cores should only be transported in heavily padded containers, e.g. suitcases or cooler boxes. If samples are not to be kept in the core (e.g. for soil chemistry), plastic or paper bags are required to transfer the soil to the laboratory.

As the properties of most tropical soils change considerably through the first 10 to 20 cm of depth, it is advisable to take samples in small increments. Standard steel corers are 5 cm long, with an inner diameter of 5.04 cm to give a volume of exactly 100 cm^3. Cores of other dimensions are available, but we suggest that 5 cm length is suitable to resolve incremental changes in soil properties with depth in tropical forest, or forest-derived soils.

For a sample of the first 5 cm soil depth, the ground is first cleared of litter and a location selected with an even surface. The core is placed on the surface and driven vertically into the soil until the upper edge is level with the ground. It is essential to ensure that the core is driven vertically, to avoid unintended compaction or the formation of voids in the core. This can be assured with the use of a core-holder with a long handle (ca. 70 to 100 cm), which is first driven into the soil with a mallet.

Once the upper edge of the core is flush with the soil surface, the surrounding soil is carefully excavated and removed with the trowel until the lower edge is reached. The trowel is then inserted under the core, and the core lifted out. The top end of the core is then covered to retain the soil, and the lower end carefully cut flush with the edge of the core with the knife and covered. This sample is then suitable for the determination of bulk density, and in addition other physical properties such as saturated or unsaturated conductivity, or the low range of pF-characteristics. For soil chemistry alone, the sample can be displaced from the core into a suitable bag.

For samples at depths greater than 5 cm , the soil should first be planed to 5 cm depth, and the sampling process repeated as at the soil surface. This process can be used to sample to about 20 cm. For deeper samples, it is advisable to dig a small pit and sample the sides from inside the pit (this reduces the compaction effects associated with exposing the deeper layers).

To determine bulk density, the core is dried to 0% moisture and weighed. Weight (g) is divided by the volume of the core (cm^3), to give bulk density in g cm^{-3} (= mg mm^{-3}). The method is suitable for all soils that do not contain large stones or gravel.

iii) Soil chemistry

Soil samples are ground to 0.5 mm. pH is determined in a water suspension at a soil/water ratio of 2.5. Ca^{2+}, Mg^{2+}, K^+ and P are extracted by the melich-3 procedure (Melich, 1984). Cations are determined by atomic absorption spectrophotometry and P

by the malachite green colorimetric procedure (Motomizu *et al.*, 1983). Organic C is determined by chromic acid digestion and spectrophotometric procedure (Hearns 1984). Total N is determined using the Kjeldahl method for digestion and an ammonium electrode determination (Bremner, 1982; Bremner and Tabatabai, 1972).

VIII. Site sampling: Timing and sequencing

As almost all sites have distinguishable wet and dry seasons, different land-uses provide different microclimatic conditions throughout the year. The magnitude of fluxes also varies, with relatively stable environments in forests and plantations, but dramatic changes may take place in other sites associated with clearance, burning, tillage, harvesting etc. Sampling is therefore recommended to take place under the most stable conditions available, towards the end of the rainy season and at the maximum biomass of crops (before senescence).

To minimize unintended disturbance, each LUS should be sampled in the following sequence: earthworms (qualitative survey) ---> pitfalls ---> soil microbiota (including nematodes and root nodulation/mycorrhizal infection) ---> monoliths ---> cores for soil physics and chemistry. A team of 10-12 people (perhaps with additional assistance for soil-sorting) can complete this work in one day if the number of monoliths dug is 5. Experience has shown that 8 or more monoliths require two days to complete, including soil-sorting, not allowing for interruptions due to rain. It is impractical to have more than about 12 people working on the transects, due to excessive trampling.

The qualitative termite sampling should not affect other collections, if conducted at sufficient distance (10-20m) from the 40 x 5 m transect. It can therefore be carried out concurrently with the sampling of other groups, but allowing for the fact it will take at least two days to complete. Termites are not particularly sensitive to disturbance from unrelated sampling, but the line of the termite transect should be established at the outset and collectors should avoid unnecessary disturbance or the clearance of vegetation from this line before termite sampling begins.

VIII. Synthesis

i) A qualitative synthesis can be given by answering the following questions:

- what is the effect of each land-use system on biodiversity?
- which groups change the most with disturbance and along the land use gradient?
- what is the relationship between the functional group changes and the degree of sustainability of each land-use?

ii) The data presented and the arguments developed should make use of alpha biodiversity (richness of strains, species and higher taxonomic groupings per site), abundance (numerical density) and biomass (biomass density). Although rapid assessment methods cannot provide equal information on all groups, the individual target taxa have been selected to yield at least one of these parameters. For example termites and rhizobia will give good data on species or strain diversity; earthworms will indicate macrofaunal biomass and ants and other macrofauna will indicate overall abundance and higher taxonomic diversity. Mycorrhizas and nematodes are unlikely to be identified beyond genus level (functional group level, perhaps, for nematodes), but are important indicators of soil functions.

iii) The overall dataset required is as follows:

Diversity at the strain, species, genus and higher taxonomic level for target taxa, as appropriate.

Abundance as individuals or CFUs m^{-2}.

Biomass as g m^{-2} (normally on a wet-weight basis).

Taxonomic community composition as % per taxon (based on relative abundance and relative biomass)

Functional community composition as % per functional group (based on relative abundance and relative biomass)

Diversity indices, as appropriate.

iv) Further analysis should proceed in the sequence:

Means per land-use.

ANOVAs to distinguish real variability between land-uses from sampling error.

Correlation matrices for response parameters against soil properties and land-uses.

Cluster and Principal Component Analyses to position sites and land-uses on disturbance gradients and identify the outcomes of particular disturbance events.

Correlation of AG and BG responses.

Spatial extrapolation.

Reinterpretation with weighting factors for endemics, invasive species, keystone species and pests.

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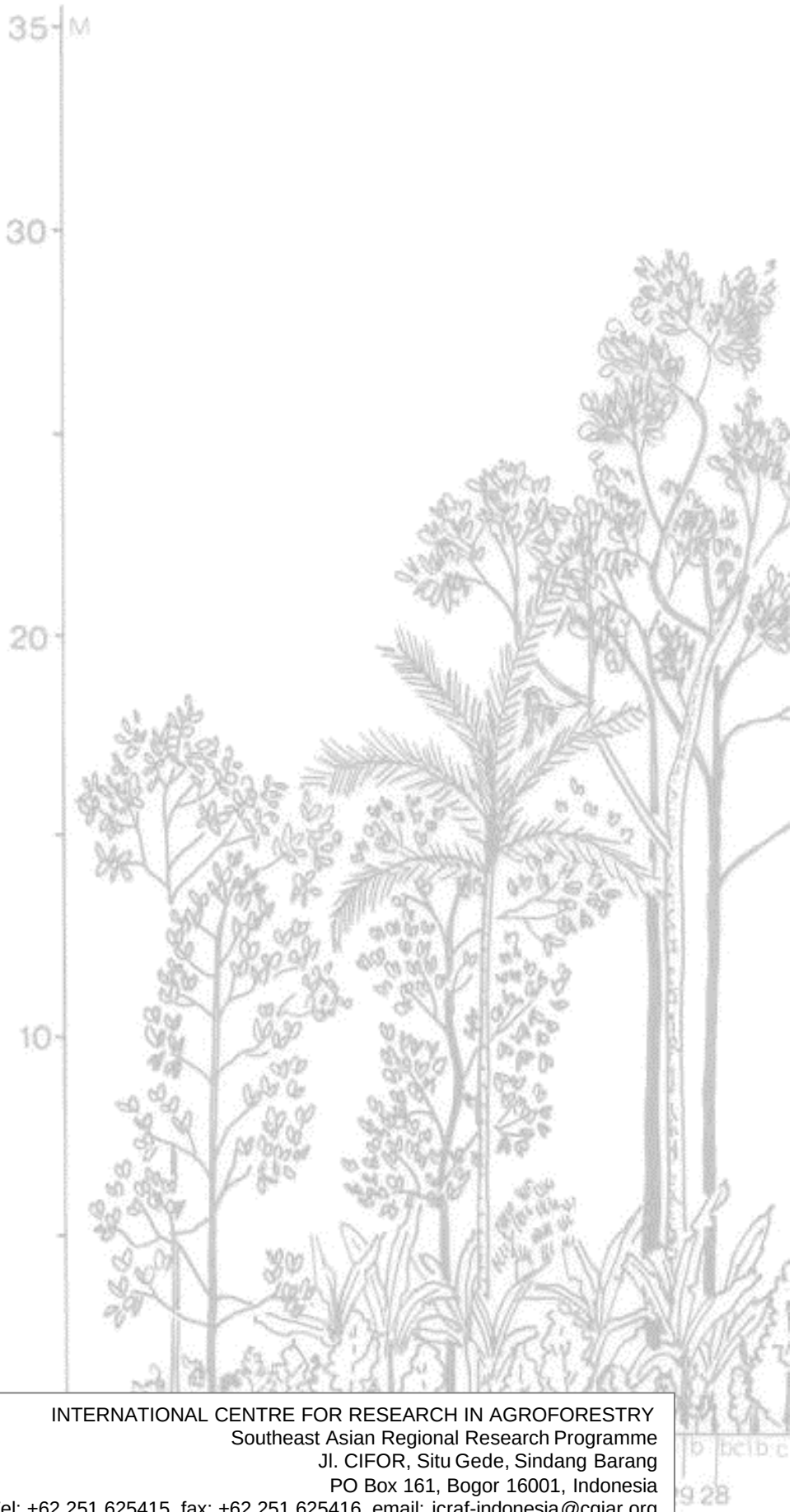

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