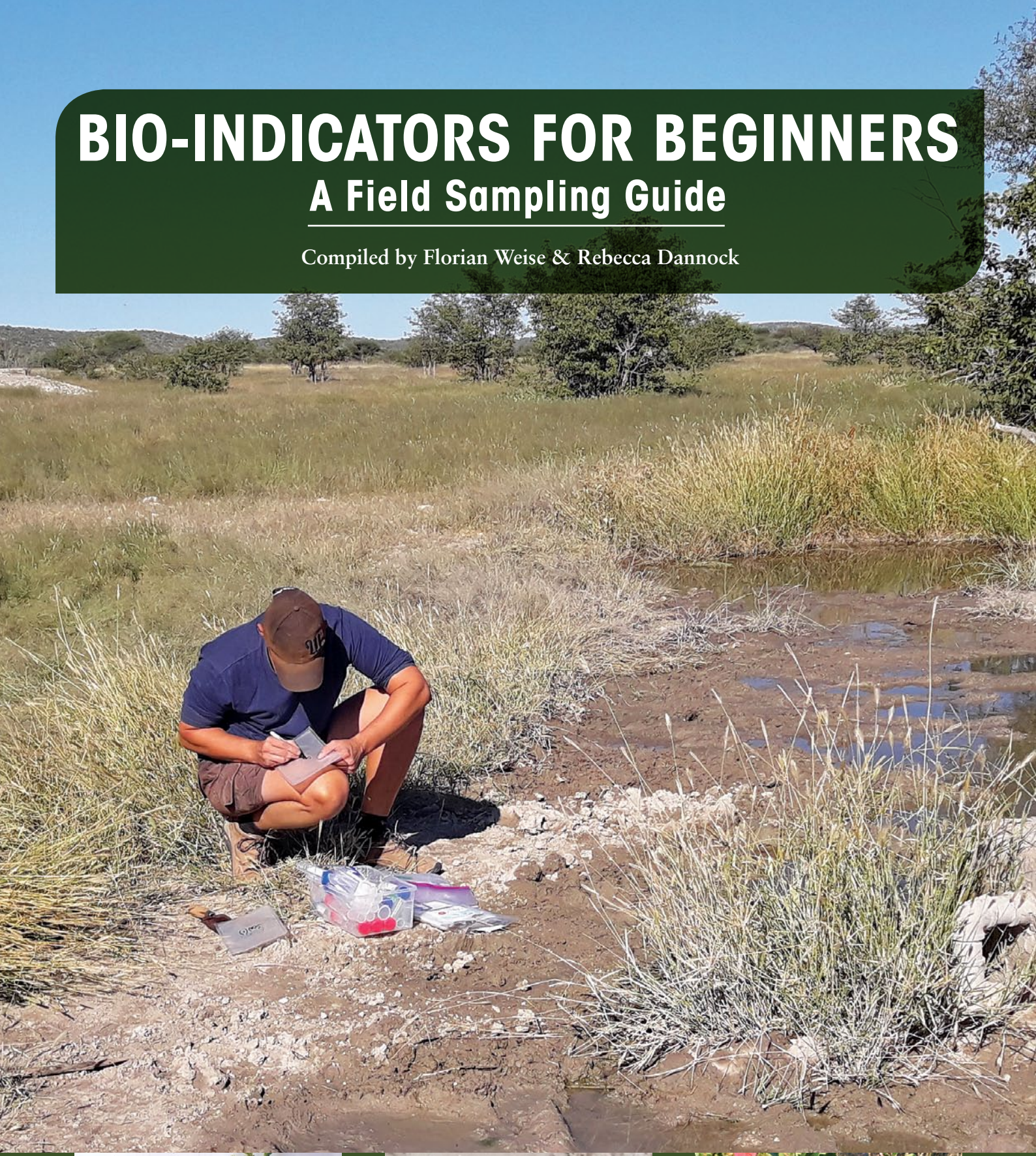
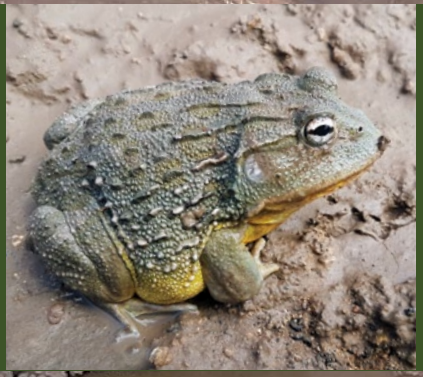
BIO-INDICATORS FOR BEGINNERS

A Field Sampling Guide

Compiled by Florian Weise & Rebecca Dannock





BIO-INDICATORS FOR BEGINNERS

A Field Sampling Guide

Compiled by Florian Weise & Rebecca Dannock

With content contributions by William Versfeld, Stéphanie Périquet, Theresa Zett, and John Mendelsohn

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ONGAVA RESEARCH CENTRE
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Contents

Foreword

I had the pleasure of visiting the Ongava Research Centre and meeting Dr Florian Weise in July 2019 when I was invited by Namibia's then Ministry of Environment and Tourism (MET) to assess biomaterial collections in Namibia. Looking back on my report, I noted then "Dr Florian Weise has shown a keen interest in the running of the Biobank component, and his key purpose is to preserve genetic compositions of local populations, but also with a view of using materials that best conserve environmental conditions". Having read the content of this field guide, I cannot help but smile that his vision has given rise to this valuable resource.

With the ongoing annihilation and worldwide loss of species in recent decades, the need for well-managed biodiversity collections has become even more obvious. These collections, when well-curated, are like museums – they create records of different times and places, which in turn become a wealth of data for those periods. A species or environment which is considered widespread today, may be threatened tomorrow. And with developing technology, a sample that does not yield many results right now could provide an abundance of knowledge in a few years' time.

This field guide does not concentrate on specific biomaterials. Instead it is all-encompassing: it provides a how-to for students, researchers, or just for interest's sake for sampling across all trophic levels – plants, soils, bones, vertebrates, invertebrates and DNA;, how to sample, what to sample, and the data to collect to add value to the samples; describing detailed workflows and processes.

These collections of environmental samples, or bioindicators, can offer insights on the genetics of species, environmental contaminants and toxins, pollution, diseases, nutrition, and natural minerals in plants and soils, amongst many others.

Provision has also been made in great detail for the data associated with these samples in the form of a field app – EpiCollect – and later to be able to download the data into a well-managed database. A sample is only as valuable as the data collected and stored with the sample.

To increase the range and quality of samples and data stored, the need for a well-coordinated network of collections, and collaborations between government, NGOs, and private research institutions is important for the longevity of these repositories. This could prevent smaller collections disappearing, and secure valuable Namibian resources for future conservation and research studies.

Ensuring transmission of knowledge from collector to collection, from field sampling to research, it is critical for these repositories to have a strong backbone from the foundation.

Bioindicators for beginners – A Field Sampling Guide is an excellent reference and first step towards streamlining the collecting methodology for Namibian repositories. It is my wish that this field guide for bio-indicator sample collection, processing and storage inspires young Namibian students to not only involve themselves in preserving these natural resources, but also to become more involved in the research and conservation opportunities that evolve from them.

Kim Labuschagne
Biobank Curator
South African National Biodiversity Institute (SANBI)

Authors' Note

Environmental changes are apparent the world over. Much speculation exists as to what drives these changes. Some may simply be due to natural fluctuations that occur on different time and geographic scales, many of which we do not yet comprehend, whereas other fluctuations may be man-made. These anthropogenic influences may be reversible through human intervention, if deemed unsustainable. Thus, disentangling cause and effect remains a fundamental challenge for science.

While remote sensing methods have advanced tremendously in recent years and offer additional insight into the ecological functioning of our environment, physical samples are still needed to ground-truth results, put them into perspective and measure specific physical and bio-chemical characteristics. Organisms respond to and preserve environmental conditions, often storing changes in their tissues or expressing them in their appearance and occurrence, thus indicating when and how changes occur. With rapidly evolving analytical methods, we can measure these attributes, sometimes only requiring minute chemical traces, but to do so we need physical samples.

We have compiled this field guide to give a brief overview of the basic principles and key concepts of bio-indicator research, alongside an outline of common sampling methods and useful sample types. We describe techniques and procedures from a two-year pilot study in northern Namibia, a country without a concerted bio-indicator programme to date. We give practical examples and present sampling considerations in the context of a challenging research environment, recognising its remoteness, its seasonal and annual environmental fluctuations, and the difficult local logistics.

Our work is mainly intended for lay people and aspiring students, not expert scientists. We hope to excite others to get involved in bio-indicator research, to go out and try to understand how and why their environment is changing, by designing projects that align with existing efforts in a collaborative fashion. We aim to give simple practical guidance for novices and highlight the fact that bio-indicator research need not be complicated. Indeed, sampling methods can often be improvised, yielding valuable samples for long-term collections. None of our thoughts and examples are chiselled in stone, they are a compilation of current applications.

Methods will continue to evolve and, undoubtedly, we will need to revise and adapt approaches. We invite others to suggest additions and updates, this is intended to be a living, dynamic document. Since we cannot anticipate the analytical prowess available to science in decades to come, our intention is to demonstrate how bio-indicator samples can be stored in their original state for prolonged time periods so that future scientists can extract the maximum amount of knowledge and insight from these samples using ever-evolving technology.

Florian Weise and Rebecca Dannock

Acknowledgements

Many of the thoughts and materials presented here have their origin in the great work of others. A profound body of bio-indicator knowledge already exists, and we encourage novices to explore it. We have drawn from it profusely. Discussions with many Namibian and international colleagues improved our approach and concept, for instance initial thoughts by Kim Labuschagne of South African National Biodiversity Institute (SANBI). For this field guide, substantial content contributions also came from other Ongava Research Centre staff, especially Stéphanie Périquet and William Versfeld who assisted with the entire design and implementation of our pilot study, as well as senior guidance and perspective by John Mendelsohn. Elizabeth Shangano, Fillemon Shatipamba, Simeon Naholo and Theresa Zett deserve special credit for their tireless help with sample collection, processing, and storage. We are deeply grateful to Namibia Nature Foundation and Nedbank Go Green grant for their financial support that enabled us to pursue this work over several years and to set up sample storage facilities in a remote area of Namibia, both key prerequisites for measuring environmental change.

We would also like to thank Ray Weil, of the University of Maryland, who kindly reviewed the *Soil Samples* chapter and allowed us to reprint the *Importance of Soils in the Ecosystem* figure in that chapter. Further thanks go to those who have kindly allowed us to reprint their materials throughout this guide: Professor Ian Hodkinson for the tables in the Introduction and *Whole Animal* chapter; and Professor Miller for the table in the *Introduction* and the figure in the *Whole Animal* chapter, the International Atomic Energy Agency (IAEA) for their graph in the *General Methods and Tools* chapter and Professor Brian Spratt for the EpiCollect workflow figure in the *Epicollect 5* chapter.

We thank the management and Directors of Ongava Game Reserve for their support. John Mendelsohn and Ken Stratford deserve special mention for their guidance of this project.

Introduction

Why Bio-Indicator Sampling?

The world's natural environment is changing rapidly and in many ways. The changes we observe may be caused by human activities, for example through increasing greenhouse gas emissions stemming from accelerating industrialisation. However, they can also be part of the inherent variation of natural systems and ecological cycles, such as long-term fluctuations in local rainfall patterns. Although changes are apparent, their causes, drivers and effects are often poorly understood. Many of our knowledge gaps stem from a lack of standardised data, an absence of regular monitoring and sampling programmes, weak research facilities, and funding insufficiencies.[1]

Measuring and understanding environmental change requires samples, such as materials collected from biological indicators (bio-indicators). These are organic materials or organisms that are sensitive to, and suitably reflect, changes in environmental conditions. Bio-indicators should not be confused with biobanks or biodiversity monitoring programmes. The concept of bio-indicators and bio-monitors took flight during the 1980s when environmental impacts caused by 150 years of industrialisation became more apparent and scientific measuring methods advanced to new heights.[2] When collected regularly, bio-indicators enable measurement of specific changes, driven by natural variation or human activities.[1, 3, 4, 5, 6] Scientists the world over, from various scientific disciplines, are trying to understand which changes are largely man-made, and can thus be mitigated or reversed. This reflects a global effort to maintain ecological functioning for future generations.

> *Regular bio-indicator collection aims to provide a long-term repository of biotic and abiotic materials that preserves environmental conditions and reflects their changes for future analysis.*

The question of what and how to collect and record remains a global challenge.[7] Yet, with analytical methods rapidly improving, historical samples from different taxa and various trophic levels can be used to measure changes and their drivers over time. Analyses spanning multiple sites can reveal ecological processes and trends across regions with bio-indicators enabling investigations into species biogeography, disease ecology, and habitat quality,[1, 3, 6] among many others. Trend analyses can tell us how the environment is changing and how these changes might impact ecological processes and organisms. The choice of specific bio-indicators and how these are sampled are determined by the purpose of each project, the type of change being assessed, and the type of analysis envisaged. The keys to a successful programme are to define its purpose before sampling commences and be as consistent as possible so that samples collected over long periods can be compared with each other, or with those collected in other areas. An excellent summary of the general concept of bio-indicators, and indeed compulsory reading for novices, is Bioindicators: Using Organisms to Measure Environmental Impacts.[4]

Thousands of organisms have already been used to document a variety of changes[3, 4] and worldwide, more and more environmental programmes rely on specimens collected by citizen scientists to increase sample sizes. The uses for these samples vary widely, from fish skeletons helping governments monitor fish populations[8] to soil samples helping scientists find the next biomedical discovery.[9] While applications may vary, so do the types of samples that can benefit science. For example, scats from echidnas are being collected to understand their health,[10] feathers are used to assist in water resource management decisions[11] and water samples shed light on water quality.[12] As citizen science becomes more prevalent and the samples collected increase in variety, these efforts increasingly find their way into bio-indicators studies. For instance, in national parks across the United States, citizen scientists already collect dragonfly larvae to help monitor the bioaccumulation of mercury.[13] Engaging citizen scientists in the collection of bio-indicator samples has opened new possibilities that may lead to more robust studies with broader geographic representation, more consistent sampling efforts and, not least, covering a larger number of organisms and materials. Environmental health is a public concern and many wish to get involved.

How Many and How Much to Sample?

The simple answer is: *Don't just collect anything*. Haphazard, unstructured indicator sampling fills up storage space very quickly. It also unnecessarily costs a lot of money and time. While sample sizes should be big enough to account for individual variation and population variance, one can determine minimum required thresholds to avoid over-sampling. Statistical power tests are great tools in determining these minimum sample size thresholds, ensuring that researchers can detect meaningful trends in the attributes that are being measured, while considering the degrees of change that are considered important or significant. The choice of power testing depends on the attributes measured and the strength of changes that people want to detect. Power testing is commonly used in medical sciences, but is used much less by biologists. Novice investigators can seek help from biostatisticians when determining minimum sample sizes for indicator collection.

An excellent open-source online resource is Power and Sample Size Determination by Sullivan[14]

Many environmental changes can now be detected and assessed from small, sometimes minute, indicator species and sample volumes. For instance, blowflies and ticks make excellent indicators for a vast number of environmental changes and disturbances and can be collected in large quantities while requiring very little storage space.[15, 16, 17, 18, 19] Therefore, indicators and indicator repositories need not be voluminous to be meaningful. Prior to starting a collection, researchers should familiarise themselves with the specific analytical requirements and, if only part of an organism is required to answer the question, only that part should be stored as opposed to entire organisms.

When to Sample?

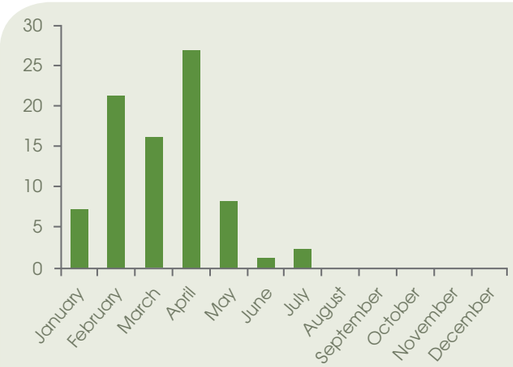
Sampling times can be very different indeed, depending on when suitable bio-indicators are available and accessible. For example, woody plant seeds that form part of ORC's collection are not available throughout the year as they start developing during the long rains, ripen in the months after, and eventually drop off the trees. Therefore, their occurrence is highly seasonal and collectors need to consider the timing of sample availability when planning projects. Similarly, many invertebrate species have highly episodic occurrence cycles (e.g., crickets), thus preventing annual collection.

If multiple indicators are collected as part of the same programme, it is advisable to select species and materials that can be collected simultaneously, to reduce the amount of time and resources required for collection. A concentrated, short sampling season may then be sufficient to complete annual collection.

Where to Sample?

Collectors must also consider where they can obtain samples easily, reliably and safely. Many species of plants and animals, have patchy geographic distributions, or occur clumped in certain habitat pockets. This influences the time and effort spent in collecting specimens.

For long-term trend analysis it is crucially important to account for individual variance. Thus, indicator specimens should be collected repeatedly from the same site or organism, such as water samples from the same waterhole across different seasons, or seeds from the same tree over many years. Having fixed sampling stations is one way to



The number of ripe woody plant seeds collected on Ongava Game Reserve (2019-2021). The distribution shows a highly seasonal pattern with most samples (88%) collected between February and May.

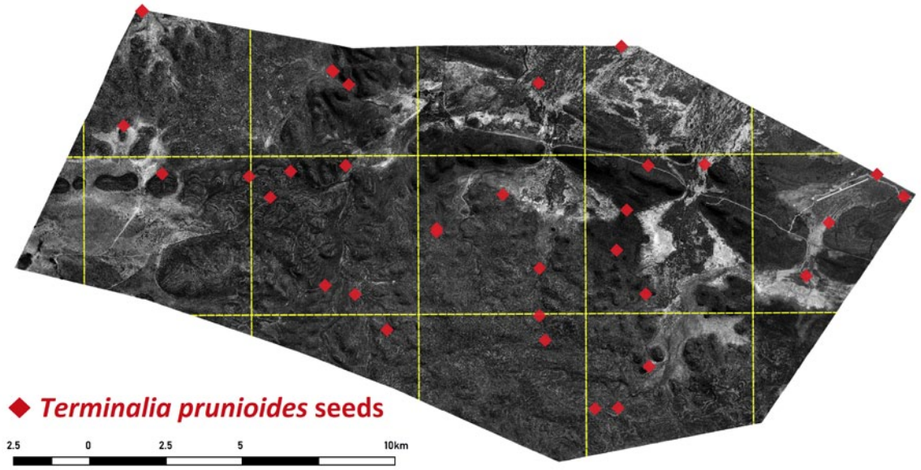
ensure that individual variance can later be measured and accounted for. This is not possible if an entire organism is collected as the sample source has now been removed from the environment. In these cases, it is advisable to collect the same type of organism from the same site at regular intervals, to be able to measure and account for other, external influences on variance.

As bio-indicator research is concerned with measuring changes in ecosystem condition of particular sites, collectors should avoid sampling highly mobile species such as migrants as their attributes may reflect the environmental conditions from other areas.

If multiple sample types are collected for inference, researchers often use nested sampling. For example, the point of interest may be a waterhole, and samples from other trophic levels are collected immediately around this focal point, e.g., the nearest tree, grass patch, soil etc. Nested analysis examines multiple important measurements including minerals, trace elements, forage quality and nutrients, disease presence, pollution etc. Since each sample responds differently to environmental change and stress, multiple measurements can provide a more comprehensive picture about whether change is significant and worrying or simply due to environment's dynamism stemming from fluctuation conditions. Thus contributing to a more detailed understanding of a location's changes.

Useful guidance on selecting appropriate sampling approaches can be found in works by Johnson Jr. et al.[20], Wheater et al.[21] and Williams and Brown.[22]

Researchers should not be afraid to adjust and adapt protocol in light of new information or circumstances, such as new analytical techniques. Change should be implemented as needed, based on a firm justification. It is advisable to conduct pilot studies before entire collection protocols are amended.



Map of the Ongava Game Reserve in northern Namibia showing the locations of Terminalia prunioides *trees from which seed samples are collected year after year. The sampling trees are approximately evenly scattered across the reserve, ensuring environmental change is recorded across the entire area of interest. While it is useful to achieve a broad sampling spread across each study area, the collection of indicator samples will always be influenced by the local distribution of different species across environments and logistical constraints. A grid cell approach can be used to maximise and assess the geographic spread of collected samples – here, grid cells have a size of 30 km², roughly 10% of the reserve's area.*

What to Sample

Choosing the right samples

"Bio-indicators may be micro- and macro-organisms, and include their activities or functions" Vimal Chandra Pandey[23]

The most useful and most frequently collected bio-indicators are plants and invertebrates,[3, 5, 24] or their products. Terrestrial invertebrates can yield dozens of change indicators of habitat management, degradation, restoration and improvement.[1] Animal and plant products such as teeth, and seeds provide simple and inexpensive long-term records of environmental conditions, often without requiring specialised storage facilities.

"Bioindicators include biological processes, species, or communities and are used to assess the quality of the environment and how it changes over time" Emily Holt and Scott Miller[4]

Useful bio-indicators combine a set of key characteristics. For example, they allow retrospective studies of environmental change by being present at the same site over many years. Suitable indicator species are generally widely distributed, common and cover a range of environments, thus enabling multi-site comparison. Good bio-indicators can be easily and safely sampled over prolonged periods. Their collection should have no or negligible consequences for the source population's sustainability.

Table 1: Criteria for useful bio-indicators (table adapted from Holt and Miller[4]). Regardless of the geographic region, type of disturbance, environment, or organism, good bioindicators often share several characteristics.

Criterion	Characteristics
Good indicator quality	• Provides measurable response (sensitive to disturbance or stress but does not experience immediate mortality) • Response reflects the whole population / community / ecosystem • Response reflects the degree of disturbance or contamination • Adequate local population density
Abundant and common	• Common occurrence • Relatively stable despite moderate climatic and environmental variability
Well-studied	• Ecology and life history well understood • Taxonomically well documented and stable
Economical/ commercial importance	• Easy and cheap to survey • Species already being harvested for other purposes • Public interest in or awareness of the species

Suitable bio-indicators combine multiple characteristics

In addition to these key characteristics, the impact of sampling can be reduced, and the value of a bio-indicator repository can be increased by the following considerations:

- Least invasive collection methods with a focus on animal/plant products rather than organisms;
- Low concern conservation status of the focal populations (i.e., sampling non-threatened species);
- Rapid population turnover or short generations (where sampling of live specimens is necessary);
- Reliably identifiable to avoid the pitfalls of comparing apples with pears;
- Conveniently collectable in numbers that satisfy minimum statistical assumptions;
- Appropriate inclusion of different taxa and strata, including abiotic materials, plants as well as animals to enable comprehensive investigations at various trophic levels;
- Simple, long-term storage without loss of important attributes;
- Utility for a variety of analytical methods; and
- Use in other bio-indicator studies.

Consideration of these criteria will greatly minimise adverse effects on local populations while allowing a large variety of useful indicators to be sampled non-invasively, including animal and plant products such as seeds and faeces. Selecting immobile indicators, plants for instance, considerably increases the ease and feasibility of sampling. If researchers are primarily interested in determining trends over time, it is crucially important that the selected indicator occurs reliably in good numbers to enable regular sampling. Regardless of the type of indicator being collected, it is paramount that collectors can identify materials and species unambiguously during field work.

Useful indicators can be found across all ecosystems and ecosystem levels.[3, 4, 25, 26] Selecting specific indicators for long-term sampling depends on the question that researchers wish to understand or address. Carignan and Villard[25] provide very useful guidance on strategically choosing appropriate indicators. Similarly, Hodkinson and Jackson[3] outline how different invertebrates can be used to assess changes in terrestrial and aquatic ecosystems.

Table 2: Selected recent examples of the suggested use of invertebrates as indicators of habitat management, degradation, restoration, and improvement (reprinted with permission from Hodkinson, I.D. & Jackson, J.K.[3])

Change indicated	Invertebrate group	Reference
Grassland topsoil removal	Carabid beetles	Sieren and Fischer 2002
Land management practice	Ants	Andersen et al. 2002
	Dispersing insects	Mora et al. 2004
Extent of logging	Spiders	Willett 2001
	Dung beetles	Davis et al. 2001
	Stream macroinvertebrates	Bojsen and Jacobsen, 2003
Mining disturbance in savanna	Grasshoppers	Andersen et al. 2001
General ecosystem health	Many invertebrates	Hilty and Merenlender 2000
Landscape/ecosystem sustainability	Many invertebrates	Paoletti 1999b
	Soil invertebrates	Duelli et al. 1999
	Earthworms	Paoletti 1999a
Impact of genetically modified crops	Invertebrates	Haughton et al. 2003
Soil management	Soil invertebrates	Enami et al. 1999
Change in general habitat quality	Bees and wasps	Tscharntke and 1998
Forest restoration	General invertebrate community	Jansen 1997
Farming impacts	Protozoa	Foissner 1997
Forest degradation	Tiger beetles	Rodriguez et al. 1998
	Various insects and nematodes	Lawton et al. 1998
Sheep grazing	Several insect groups	Gibson et al. 1992
Grassland management	Coleoptera and Orthoptera	Jonas et al. 2002
Pollutant effects on forest	Scolytid beetles	Grodzki 1997
Forest disturbance	Butterflies	Hamer et al. 1997
	Moths (Arctiidae and Notodontidae)	Summerville et al. 2004
Forest management	Mycetophilid flies	Okland 1994
	Forest floor invertebrates	Schowalter et al. 2003
	Longicorn beetles	Maeto et al. 2002
Grassland habitat disturbance	Hemiptera Auchenorrhyncha	Nickel and Hildebrandt 2003
Urbanization	Carabid beetles	Sustek 1992
Habitat fragmentation	Ants, Coleoptera, Arenaea, Diptera, other Hymenoptera	Gibb and Hochuli 2002
Water quality/habitat integrity	Benthic invertebrates	Kashian and Burton 2000
Stream restoration	Benthic invertebrates	Muotka et al. 2002

A simplified representation of a energy chain.

In addition to the large body of bio-indicator literature, guidance on how to structure a sampling regime can also be found in the general ecology texts. Some very useful examples on ecological sampling include Chapter 3 of Johnson Jr. et al.,[20] Wheater et al.[21] and Williams and Brown[22]

Sampling Across Trophic Levels

Environmental changes, especially those caused by toxins and pollutants, usually ripple through ecosystem layers, or trophic levels. A trophic level is the group of organisms that occupy the same level of a food chain. All organisms in an ecosystem belong to a trophic level. The lowest level consists of primary producers, which synthesise food from solar or chemical energy, by utilising nutrients and minerals from soil and water. Each subsequent level obtains its energy from the levels below.

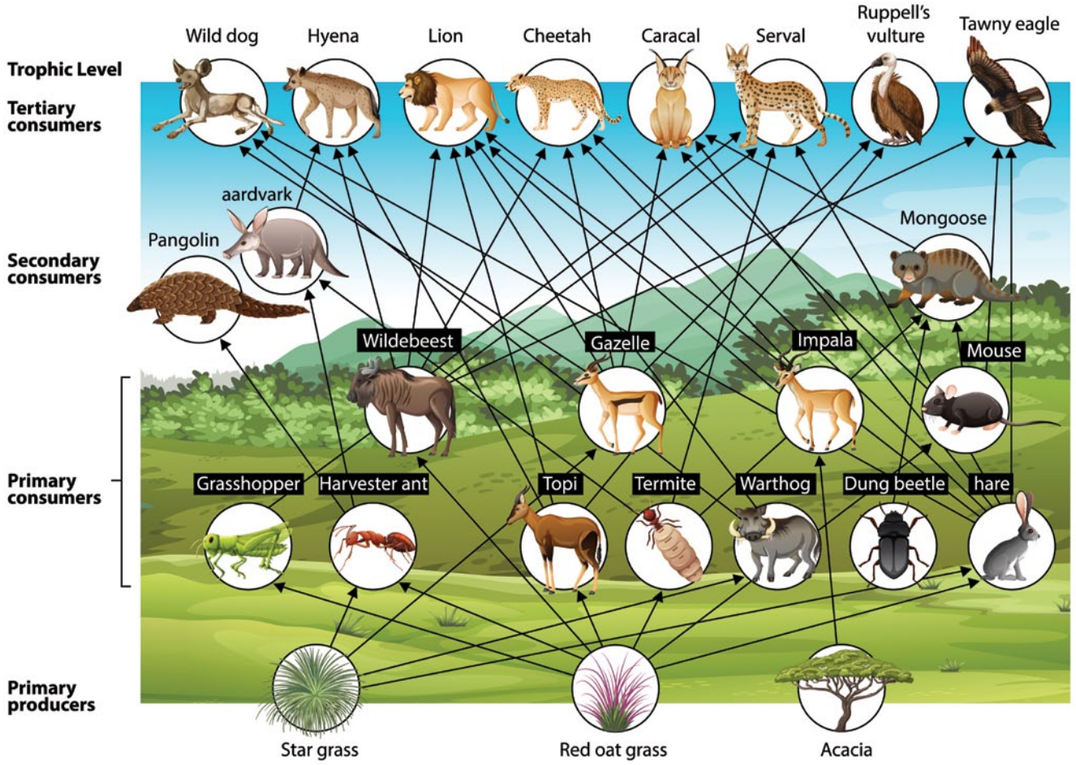
As with energy, heavy metals such as lead, cadmium, and mercury are also passed from one trophic level to another, as organisms accumulate or metabolise chemicals and are then consumed by other organisms. The aim of ORC's bio-indicator programme is to collect samples from different trophic levels to represent and consider all ecosystem layers and understand inter-connections. All organisms metabolise and accumulate chemicals in different ways, with some being more susceptible to certain substances than others. What is toxic to one species may not be toxic to another. Stable compounds such as mercury, however, accumulate up the food chain, as very few organisms manage to break down these robust molecules and or convert them into less hazardous substances (see figure below). The accumulating effect of highly stable, dangerous molecules typically leads to higher concentrations at higher trophic layers, reaching toxic levels.

By sampling indicators at various trophic levels, we can examine at which stage pollutants or toxins (for example) enter a food chain, allowing us to take countermeasures if these happen to be introduced by human activities.

Ecosystems and natural cycles, however, are more complex than simple linear connections like a food chain. Indeed, energy and nutrients as well as hazardous substances are passed along entire trophic webs with multiple organisms interacting with each other both vertically (along a particular food chain) and horizontally (within a specific trophic layer). While this makes indicator research more complicated, as well as disentangling cause and effect, researchers should focus on manageable and achievable projects, by starting to investigate well-known connections and inter-relations within the trophic web.

We will address the many questions surrounding how to sample bio-indicators in the following chapters.

FOOD WEBS



Example of an African savannah trophic web showing the various inter-linkages at vertical and horizontal levels (reprinted from Vecteezy.com under the Vecteezy License Agreement).

Further Reading

At the time of writing this guide, there were more than 100,000 peer-reviewed bio-indicator studies in the scientific literature. Here, we outline just a few that provide ample guidance, background, and inspiration. One need not reinvent the proverbial wheel, as the major principles, concepts and applications of bio-indicators are well established.

We have access to a profound body of literature that helps us to design and implement meaningful indicator studies in a robust fashion. For instance, a very useful overview of the general topic and its relevance is given by Holt and Miller: *Bioindicators: Using Organisms to Measure Environmental Impacts*.[4] This excellent summary addresses the questions of "*How do we assess the impacts of human activities on natural ecosystems?*" and "*What can the biota tell us about the environment and its response to natural stress?*". Holt and Miller describe what bio-indicators are, what their purposes are, and a set of key characteristics of good bio-indicators.

There are several insightful reviews of bio-indicator applications and of materials and biological taxa used in indicator studies. Carignan and Villard[25] examine the selection of indicator species at various trophic levels, and provide guidance of how to approach the selection process. Hodkinson and Jackson[3] critically evaluate the use of terrestrial and aquatic invertebrates as indicators to monitor change in ecosystems. They comprehensively review their suitability for assessing a range of environmental problems, including pollution, long-term habitat degradation and the possible recovery of ecosystems. In addition to topic- or taxa-specific reviews, several scientific journals are now dedicated to bio-indicator sampling and associated research methods, including:

- Ecological Indicators
- Journal of Environmental Bioindicators (four volumes between 2006-2009)
- Environmental and Sustainability Indicators

These provide a wealth of information on why, where, how and when to study particular bio-indicators, with articles covering applied studies from nearly all trophic levels of the world's different ecosystems.

Several books comprehensively deal with different aspects of bio-indicator sampling and the inferences and knowledge that can be drawn from them. They emphasise the importance of integrative studies and how this applies to ecosystem management practices. Not least, the featured studies reveal astonishing ecological insights and cover a large variety of suitable indicator materials and biological taxa, ranging from minute trace elements to complex organisms, and ultimately ecosystem functions. To ensure sampling and inference frameworks are robust, researchers can also consult a range of ecological sampling literature when designing bio-indicator programmes (for examples, Wheater et al.[21] and Williams and Brown.[22] Further literature specific to each sample type is within the following chapters.

A selection of useful books for further reading on field sampling techniques and bio-indicators.[21,27,28]

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General Methods & Tools

Safety Considerations

Safety is an important consideration when collecting samples. The collection of any sample may come with risks to be considered, including sun exposure, dealing with living animals, plants with thorns etc. However, sample collectors must pay particular attention to the risk of disease transmission.

When collecting and processing samples from animals (whole animal, bones and teeth, tissue, hair, faeces), consider the risk of disease transmission, such as anthrax, when determining sampling protocols. If the sample source may cause harm to humans, collectors must wear a mask and gloves. Single-use safety equipment such as gloves cannot be re-used and must be disposed of safely, to avoid further contact.

Properly clean your hands and anything else that comes in contact with specimens with disinfectant after collection; this will ensure you are clear of ticks, fleas, mites, bacteria, viruses, other disease agents and/or dangerous contaminants that the specimen may have had. If you are in doubt about the safety of collecting a specimen, it is best to not collect it.

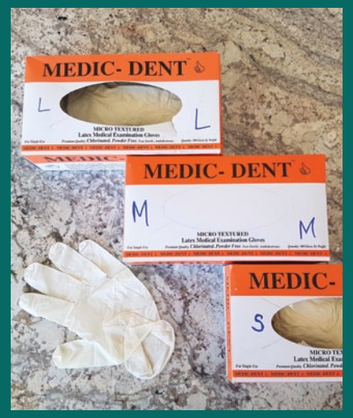
After handling samples, ensure that detergent or disinfectants are used for all person and equipment cleaning. Do not just use water. Non-disposable equipment such as bandanas used for face protection must be thoroughly cleaned with disinfectant or detergents before re-use. Ensure you always carry detergent and water, or a sanitiser that does not require water, during sampling.

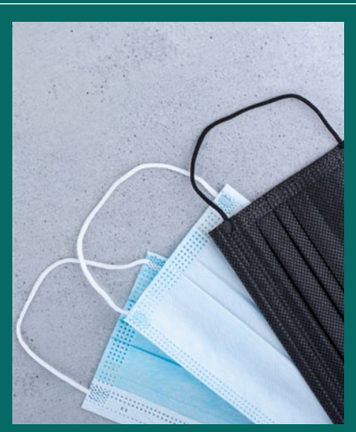
Collectors should also carry first aid kits in the field and keep kits in the lab to ensure they are prepared for any minor cuts, scrapes, and burns that may occur. Betadine, as shown in *Processing Tips* on page 16 is a useful antiseptic.

Research and Collection Permits

The legal requirements for bio-indicator collection differ from country to country. Prior to collecting samples, collectors must ensure they have the necessary permission(s) to extract samples from the natural environment and that they comply with the relevant legal frameworks. Collectors should be aware that in some cases multiple permits may be necessary for legal collection, transport, and analysis of specific samples. For instance, if highly endangered species form part of the collection or sampling is undertaken in specially protected areas. Ensure that collection permit details are recorded during the collection stage, but also by researchers analysing samples and then disseminating results. Noting the permit number(s) on sample labels is essential.

Personal Protective Equipment





Left - Gloves: A selection of gloves (sized small to large) should always be available for collecting and processing samples; gloves prevent sample contamination and reduce the risk of disease transmission.
Centre - Masks: To avoid disease transmission, use a mask, or simply a bandana or other piece of fabric doubled or tripled over and tied around your face, when collecting in areas with serious air-borne diseases (see also sample-specific chapters).
Right - Gloves: Gloves are useful for collecting samples and are important to avoid cross-contamination.

Collection Protocols

Before collecting any animal or plant materials, it is pertinent to identify the species correctly, so that the sample adds value to the collection. If unsure of the species, collectors should consult a field identification book, or app, to determine the species.

If ad-hoc sampling is conducted, where samples are collected opportunistically, it is important to always have suitable sampling kits available. At a minimum, a sampling kit should contain clean, unused containers and bags to hold samples, an array of sampling tools, personal protective, and any items required for transport (see section on *Sample Transport*). If only specific sample types are collected, a sampling kit suited to the particular requirements can be carried.

When collecting samples, ensure that each is given its own bag or container and that samples are not mixed, to avoid cross-contamination. Further, ensure that tools are properly cleaned between collections. Samples must be labelled in the field with enough information to differentiate them from one another. Long-term labelling will be done in the office/lab and so field labels only need to contain enough information for accurate distinction.

For any samples collected, accurate data recording is imperative. These data will enable others to process and analyse the sample later. Nowadays, various digital data recording apps can be downloaded onto smart phones, enabling quick and accurate data recording in the field, even in off-line mode without network connectivity. These apps can be programmed to contain data fields for any sample characteristics that need to be recorded, such as the number of specimens collected, sample condition, location detail, collector information etc.

Dates should be recorded considering international differences, to avoid confusion. Internationally, different formats have been adopted. However, these can be misleading, for instance between American and British notation. The 6th of August 2021 in American notation would be 08/06/2021 whereas the British notation would be 06/08/2021. An external collaborator receiving samples for analysis might be confused. The easiest, and internationally, accepted option is to numerically record dates with the YYYY MM DD notation, starting with the year, then month, and ending with the specific day, in our case 20210806. Collectors should be consistent recording dates using only this notation.

Since these apps are linked to the smart phone being used for recording, they can utilise the phone's in-built functionalities, for instance, to attach photos to the record and automatically record important information like date, time, and GPS location. Digital data recording in the field reduces the risk that paper records may be lost or damaged during field work. Digital recording also prevents data transcription errors as well as uncertainties arising from illegible handwriting, while improving data integration with electronic databases and reducing storage needs. Once collectors are back in Wi-Fi range, the digital records are simply uploaded to a server platform and can easily be accessed for integration into any digital database format, such as an Excel file.

Containers

Containers can be different shapes, sizes, and materials, depending on the specimens. Often it is not necessary to purchase special collection containers as there are many household items that can be used as substitutes for collecting and storing samples.

Field Identification Books

These are useful to confirm correct sample identification in the field or lab. They are particularly valuable to inexperienced collectors.

Useful Tools

Top to bottom: tweezers (collection of teeth, hair, termites etc.), hand shovel (collection of soils and grasses), markers (in field and in lab labelling), soft brush (removes soil from plant samples), and scissors (packaging samples) for collecting and preparing samples for storage. Tools should always be cleaned before each use to prevent cross-contamination.

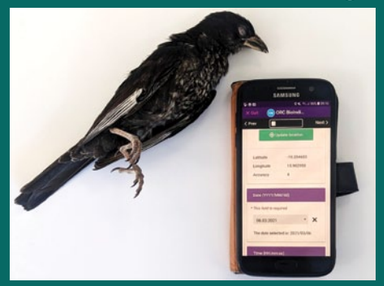
Multi-Tool

A multi-tool is useful as a back-up tool for when other tools have been forgotten or lost. As with all other tools, it must be kept clean with disinfectant before each sample collection.

Collection Bags

From left to right: vacuum seal bags, large paper bags, zip lock bags and small paper bags. Special plastic polymer bags should be used for long-term freezing to prevent sample contamination and disintegration; standard plastic bags often degrade when stored in a freezer. Cheap paper bags are often sufficient for field collection of samples, for instance, for seeds, grass, and soil samples.

Smart Phone with Recording App



A smart phone enabled with a digital recording app (EpiCollect), pictured here being used to record the collection of a sub-adult female buffalo weaver. Digital data recording in the field reduces time spent transcribing data from paper records and reduces errors that stem from transcribing these data. Different open-access field data recording apps are now available for collectors.

Sample Transport

As with sample collection, it is important that there is no cross-contamination of samples during transport; ensure that sample bags and containers are kept sealed and separated from one another. Often, multiple samples of the same type or species are collected on the same day, for instance plant seeds or grasses. Where these samples are not transferred straight into a sealable container for transport, particular consideration must be given to avoiding cross-contamination. For example, grass samples may be transported to the research facility or field station in paper bags, with a risk of cross-contamination between bags placed next to each other, or loss of materials from non-sealed bags. A small stapler can be used to temporarily seal the bag, reducing the risk of cross-contamination. In case of doubt over cross-contamination between samples, the affected samples should be discarded.

Several sample types (see sample-specific chapters) also require cooling during transit. For these samples, ensure you have a cooler or portable refrigerator for transport between the collection location and where processing for long-term storage will occur. If using a cooler, freezer bricks, ice packs or well-sealed containers or bottles filled with frozen water will help keep the samples chilled during transport until they can be stored in a freezer. When using a portable refrigerator, ensure that it is turned on and chilled to the required temperature prior to sample collection.

Mini Stapler for Transporting

To mitigate the risk of cross-contamination, sample bags can be closed with a stapler to contain each sample safely. The bags can easily be opened to process and prepare samples for permanent storage.

Styrofoam Cooler

A styrofoam box with ice packs for transporting samples. Alternatively, a plastic cooler box or small fridge can be used for short distances. Ice packs can be improvised by freezing water in plastic bottles.

Processing Tips

All sample types require specific processing steps (see sample-specific chapters). However, it is important to note that while some processing requires specific equipment, many procedures can be done using homemade or readily available equipment. Careful consideration must be given to what processing is required for a specific sample type prior to collecting it. Part of this consideration should be what processing is required immediately upon return to the lab/office, and which steps can be undertaken later. For example, many samples require intermediate processing steps such as drying after field collection, whereas additional procedures such as the application of specific preservatives may only occur later during final preparation for long-term storage.

Storage Protocols

It is critically important that specimens are stored in such a manner that they can later be found easily, be easily identified, and be stored long-term without, or with minimal, deterioration of the sample.

While digital data records may already be recorded during field collection, a clearly written label with a complete set of key information is also required for each sample. An example label is presented here, however, it is important to consider sample specific information labelling needs (see sample-specific chapters). Affixing labels, as well as sealing packages, is best done with a clear tape to enable easy sample identification, without needing to open the packaging. Alternatives can be lamination or inserting the sample label into the transparent vacuum sealing bag.

While an updated electronic meta-database with all sample information should be kept in a digital format, it is also imperative that stored samples are labelled clearly. For analysis, samples may need to be transported to processing labs or other external sites where personnel may not have access to the original information. Therefore, labels must be written legibly so that the information contained remains clear and unambiguous for sample processors and analysists. For permanent freezer storage, pencil-written labels are usually preferable as the graphite does not smudge or wipe off easily. Labels written with permanent marker may wipe off easily from plastic and glass containers under moist conditions, while the writing may smudge on paper bags, for instance.

Long-term storage must reflect the needs of specific sample types and analyses planned. For more detail on specific storage requirements, see the sample-specific chapters. Storage solutions may include simple everyday items (e.g., paper bags), cheap items (e.g., silica bead bags, etc.) or more specialised solutions (e.g., freezers and vacuum sealers).

For long-term freezer storage, the freezer must: freeze at or below -14°C; not have automatic defrost cycles or if it does, the automatic defrost cycles must be able to be turned off (there must be consistent freezing to avoid sample thawing and decomposition); and be able to retain sub-zero temperatures for several days in the event of a power outage (particularly important in remote locations and locations with a power supply that is frequently interrupted). For instance, ORC uses deep freezers with the automatic defrost cycle turned off and the units can hold deep-freeze temperatures for a minimum of 64 hours.

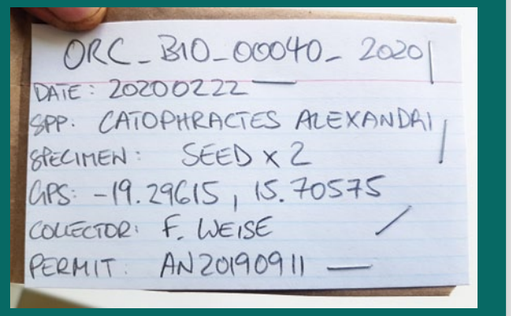
Plant Press

A tool for dry pressing plant samples such as grass. Heavy weights like books and sheets of cardboard can be used for an improvised press.

Labelling Tools

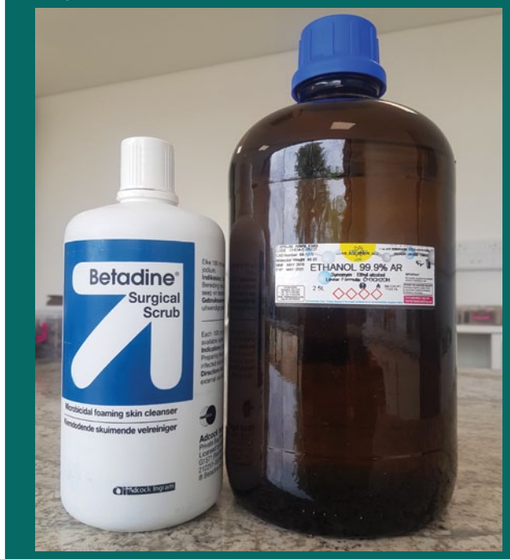
For clear and efficient storage, all samples must be marked uniquely and appropriately, using suitable materials such as pencils and permanent markers on thick and large enough paper stock. Each sample should have its own unique label.

Labels



Correct, durable labels are as important as the sample itself. Labels must (at least) note collection date and location, species, collector, permit number

Disinfectants and Preserving Agents



Betadine is a useful antiseptic for protection against a variety of germs. A clean workspace is critical for sample processing.
Ethanol is used as a preserving agent for different sample types, but Ethanol based storage may result in sample discolouration.

Freezer

Freezers are crucial for reliable long-term storage of many sample types (see sample-specific chapters) and should not auto-defrost.

Silica Bead Bags

Different silica bead bags for absorbing moisture from indicator samples during storage. For example, plant seeds. Reducing moisture is a critical consideration for long-term storage.

Vacuum Sealer

Vacuum sealers extract air and moisture from the bag prior to sealing, thus reducing the likelihood the sample may deteriorate. Vacuum sealing bags should be made from special plastic polymers that do not degrade when frozen.

Packaging Tape

Clear tape is useful for packing samples in a sealed environment without obstructing what is inside the bag or written on the label.

Plastic Storage Bags

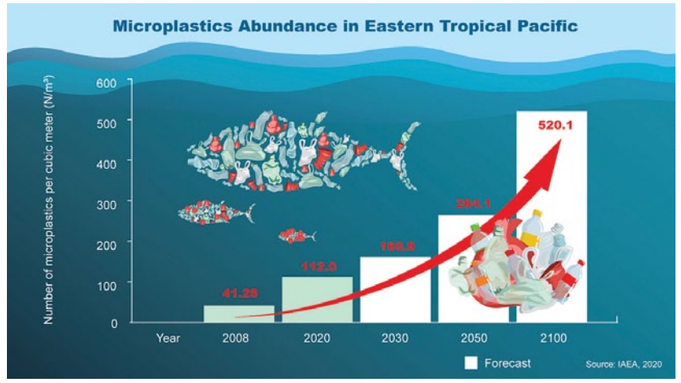
Ensure samples are stored in special polymer bags designed and tested for long-term storage (right), rather than standard plastic bags such as shopping bags (top left) or zip seal bags (bottom left).

Case Study: Why Appropriate Long-Term Storage of Bio-Indicator Samples is Important – Microplastic Pollution

One major objective of bio-indicator sampling is to establish long-term trends in environmental pollution, and its effect on living organisms. An increasingly prominent case is microplastic pollution, especially in regards marine and soil ecosystems. Microplastics are synthetic polymer compounds less than 5mm in length. These particles mostly originate from the mechanical erosion of discarded plastic packaging. Microplastics also come in the form of specially designed microbeads that have long been used in beauty products and toothpaste, for instance. Minute plastic particles (of any origin) generally pass-through filtration systems and end up in our oceans and soils. Here, they might get swallowed or absorbed by a wide range of animals and plants or be deposited in sediments where they further degrade. Field studies and experiments have shown that growing accumulation in the environment has serious consequences. For example, microplastics alter the morphology and productivity of plants. They also block the digestive tracts of animals, eventually resulting in starvation and death. Well-studied examples include fish, sea turtles, and birds, though many other taxa are affected.

Similarly, soil density, soil aeration and porosity change as microplastics accumulate. Microplastics also chemically impact organisms. Oysters, for instance, which were exposed to minute pieces of polystyrene produced fewer eggs and less mobile sperm, thus reducing their ability to reproduce. Fish suffered considerable liver damage when consuming microplastic-enriched food, thus limiting their ability to metabolise other pollutants such as pesticides. Human consumption of microplastics via food such as fish is speculated to have a whole range of deleterious effects, such as causing cancer.

For these reasons, measuring microplastic accumulation in the environment is a useful and important application of the bio-indicator concept. Their example further emphasises that the appropriate long-term storage of bio-indicator samples is not a trivial issue. Microplastics come from macroplastics which disintegrate over time under the influence of temperature and mechanical grinding, step by step turning into smaller and smaller pieces. The same happens when storing standard plastic bags and containers in a freezer for long periods of time. Hence, using the incorrect packaging materials can easily lead to contamination and, therefore, influence results measured later. If samples that have been stored in freezers for many years were used for microplastic studies in the future, the contamination measured may be skewed by how the sample was stored, no longer reflecting the sample's original characteristics at the time of collection. Special polymer bags are thus recommended for long-term freezer storage. Vacuum sealing bags, for example, have been tested by the food industry to remain stable during freezer storage, as otherwise they would contaminate the food that is stored within them. Since we cannot anticipate the sensitivity of analytical methods in the years to come, it is critical that we use the most stable storage conditions available to us to prevent contaminated or biased samples in the future.



Microplastics abundance diagram (reprinted with permission from International Atomic Energy Agency (IAEA))

Useful Links

What are microplastics?, article, online resource.

Microplastics, encyclopaedic entry, online resource.

You're literally eating microplastics. How you can cut down exposure to them, article, online resource.

From Fish to Humans, A Microplastic Invasion May Be Taking a Toll, article, online resource.

Microplastics Are a Big—and Growing—Part of Global Pollution, article, online resource.

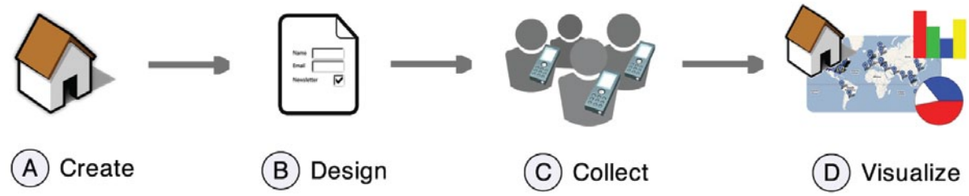
Interesting Studies

Microplastic pollution in seawater and marine organisms across the Tropical Eastern Pacific and Galápagos, by Alfaro-Núñez, A., Astorga, D., Cáceres-Farías, L., Bastidas, L., Soto Villegas, C., Macay, M. and Christensen, J.H.

Microplastic pollution in a rapidly changing world: Implications for remote and vulnerable marine ecosystems, by Horton, A.A. and Barnes, D.K.A.

Microplastic Shape, Polymer Type, and Concentration Affect Soil Properties and Plant Biomass, by Lozano, Y.M., Lehnert, T., Linck, L.T., Lehmann, A. and Rillig, M.C.

Methods – Epicollect 5: Field Data Recording App



The Epicollect workflow. Website creation is undertaken at www.epicollect.net (A), followed by online form design (B). A project is loaded onto one or more phones and, following data gathering and synchronisation (C), data are visualised using Google Maps and charts at the project website (D). Data can also be entered or downloaded directly via the project website. (Reprinted with permission: Aanensen et al. (see Useful Links*))*

Field Data Recording Apps

A variety of digital data recording apps are now available, see *Useful Links* (page 16). One of these is Epicollect, which enables flexible and efficient field data recording using a modern smart phone. A detailed Epicollect workflow description can be found in Aanensen et al., see *Useful Links*.

Epicollect

Basic Requirements

- Smart phone with standard functions such as camera, GPS chip for location detection, etc.
- Occasional internet access to upload field data and photos.

Key Steps

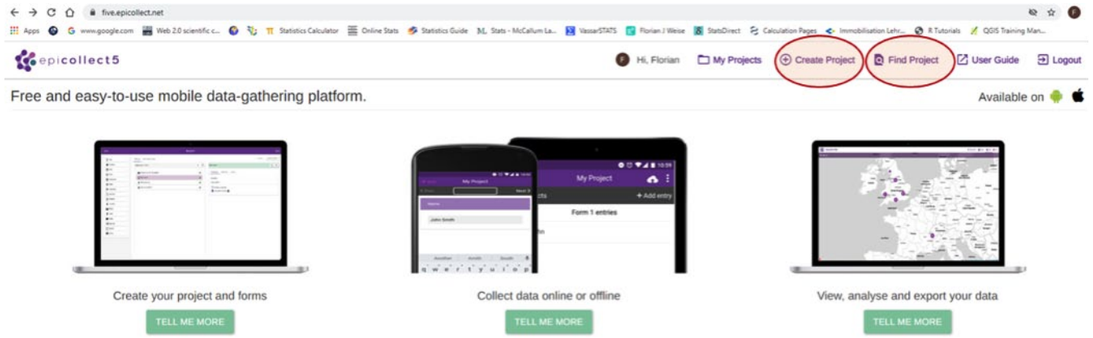
- Register an account online; create your own project at **https://five.epicollect.net/** or join an existing one.
- Download the app to your phone.
- Set up your form online and synchronise it with your phone using the app (see Useful Links for a tutorial)
- Add relevant users to the project.

Advantages

- Avoids the risk of paper records being damaged or lost during field work.
- Minimises the time required to transcribe field data records into the database.
- Avoids data transcription errors resulting from illegible handwriting.
- Field data can be recorded in offline mode (no network required) and are uploaded later.
- Easy and fast integration of records with other electronic data filing systems, e.g., the sample database.
- Data can easily be shared with other bio-indicator programmes.
- Users can easily be added or removed from the project at any time.
- Epicollect utilises standard smart phone functionalities like the in-built camera, GPS chip for location acquisition, etc, allowing collectors to carry less equipment.
- Several data such as time and date information can be automatically recorded.
- Specific data fields can be made compulsory (e.g., species and number collected), assuring that all important sample characteristics are indeed recorded during field work.
- Data fields can also be pre-set as multiple-choice answers, so there is no ambiguity in information collectors record, e.g., who is collecting specimens and the types of samples collected.
- Data collection forms can easily be changed (if needed); the updated forms can be synchronised when internet access is available.
- There is an open user community where online discussion fora exist for users to seek advice and help: **https://community.epicollect.net/** or **https://github.com/epicollect**.
- Online tutorials are available for the use of Epicollect for data recording.
- The Epicollect data recording app operates on a variety of smart phone types.

Disadvantages and Cautions

- Data are stored on an external server which may have security implications for sensitive data.
- If photos accompany field data, there are two options: record the photo through the app interface or attach a pre-recorded photo from the phone's image folders. The photos uploaded to the online data server are heavily compressed and this may obscure important detail. See



Screenshot of the Epicollect 5 online platform. Red circles indicate where new users can create projects or join an existing one.

Appendix for information on taking and using photos in EpiCollect.

- The original high-resolution photos recorded with the app using the phone's camera are stored in a difficult-to-find folder on the phone, making subsequent extraction tedious. Also, if photos are recorded through the app interface, they are stored with long, cryptic file names on the phone. Pre-capturing photos simply using the phone's camera and subsequently attaching them to the record via the app interface might be the more feasible approach to link images to data records.
- The phone may break or be lost before field data records are uploaded to the server.
- The phone needs to be sufficiently charged for use in the field, especially during long sample collecting sessions; might require carrying a power bank or other charging device.
- Anyone collecting samples on a regular basis needs to be added as a user and use the app consistently.
- Data recorded with one recording device are not instantly synchronised with other devices. This bears the risk of assigning the same sample ID to different samples if multiple collectors are working in the filed simultaneously. Synchronisation of records is only achieved via the online platform, after all collectors have uploaded their field data.

Useful Links

8 Apps for Data Collection in Research, Web resource by teamscope.

The 5 best data collection tools in 2020: The best apps for gathering data in the field Web resource by Eposito, E.

EpiCollect+: linking smartphones to web applications for complex data collection projects [v1], PDF Resource by Aanensen, D.M., Huntley, D.M., Menegazzo, M., Powell, C., and Spratt, B.G.

Creating a Form in Epicollect 5 to Use for Field Survey, YouTube resource by Joyce, K.

Whole Animals

Background

Whole animals are useful bio-indicators for assessing contaminant and disease presence, and changing habitat conditions. Depending on the planned analyses, or compound being tested for, certain tissues are more useful than others; different parts of the body are responsible for different functions and absorb and store materials differently. Collecting whole animals is valuable as it enables their use for a variety of indicator purposes. There are numerous ways whole animals can be useful bio-indicators:

- Birds accumulate mercury, lead, and other heavy metals in their **feathers**.
- Most animals accumulate heavy metals in **internal organs (e.g., kidneys and liver), bones, teeth** as well as other organs like the brain and muscle tissue.
- Increased levels of **mutations** in frogs can indicate the presence of toxins.

A range of invertebrate and vertebrate species across trophic levels and biomes (aquatic, terrestrial and aerial) can be used as indicators, see table below. Invertebrates are well-suited to determine a breadth of anthropogenic impacts on the environment, including habitat management consequences, degradation, restoration and improvement. While invertebrates are the most useful and most frequently collected bio-indicators, there are suitable indicator organisms in all animal phyla. Due to their semi-permeable skin and high sensitivity to environmental change, amphibians make excellent indicators. In contrast, mammals are collected less often due to their size, resulting in storage limits.

Collection can occur without negative effects on local populations, for example, by non-invasive sampling of window strike mortalities or roadkill. During a 10-month trial period, ORC collected 31 window strike specimens (16 species) and four roadkill specimens (three species).

Table 3: Selected examples of the suggested use of indicator invertebrates for evaluating habitats for biodiversity, condition, and structure (reprinted with permission from Hodkinson, I.D. & Jackson, J.K., 2005)

Change indicated	Invertebrate group	Reference
Terrestrial		
General (habitat continuity)	Fungivorous beetles	Sverdrup-Thygeson 2001
General (quality)	Spiders	Riecken 1999, Paoletti and Hassall 1999
	Diptera	Frouz 1999
	Coccinellid beetles	Iperti 1999
	Syrphid flies	Haslett 1997b, Sommaggio 1999
	Staphylinid beetles	Bohac 1999
	Cryptostigmatic mites	Behan-Pelletier 1999
	Rare beetles	Franc 1994
	Tiger beetles	Pearson and Cassola 1992
	Butterflies	Brown and Freitas 2000
Landscape and habitat features	Lepidoptera, spiders, carabid beetles	Jeanneret et al. 2003
Agroecosystems	Heteropterous bugs	Fauvel 1999
	Ants	Peck et al. 1998
	General invertebrates	Buchs et al. 2003
Savanna grassland	Dung beetles	McGeoch et al. 2002
Grassland	Collembola	Greenslade 1997
Forest	Fungivorous insects	Jonsell and Nordlander 2002
Boreal forest	Coleoptera	Jonsson and Jonsell 1999
Rangeland	Ants	Andersen et al. 2004
Aquatic		
Aquatic ecosystems (general)	Interstitial invertebrates	Claret et al. 1999
	General invertebrates	Charvet et al. 1998
River (typology)	Lotic invertebrates	Cayrou et al. 2000
Stream (habitat integrity)	Benthic invertebrates	Buffagni and Comin 2000
Stream (morphological integrity)	Benthic invertebrates	Jansen et al. 2000

Table 3 (continued): Selected examples of the suggested use of indicator invertebrates for evaluating habitats for biodiversity, condition, and structure (reprinted with permission from Hodkinson, I.D. & Jackson, J.K., 2005)

Change indicated	Invertebrate group	Reference
Aquatic		
Lakes	Chironomid midges	Brodersen and Lindegaard 1999
Ponds	Odonata and Trichoptera	Briers and Biggs 2003
Streams	Plecoptera	Helesic 2001
Headwater streams	Macron vertebrates	Heino et al. 2003a
Rivers	Benthic invertebrates	Lang 2000
Seasonal and temporary wetlands	Aquatic invertebrates	Euliss et al. 2002
Freshwater littoral	Macroinvertebrates	White and Irvine 2003

Sample collection and transport

Whole animal-specific equipment

- Gloves (face masks and body suits where needed)
- Nets or traps for live capture
- Containers and bags
- Styrofoam box with ice packs
- Standard vacuum sealer (i.e., for food)
- Vacuum sealing bags made from special plastic polymers that do not degrade and contaminate specimens under freezing conditions
- Freezer (no auto-defrost function); or
- Ethanol (99%)

Sample selection

Before starting: When planning a collection, researchers should consult with local experts and other regional or global collections to ensure their work ties in with existing efforts, thus ensuring maximum scientific value and effect (see *Useful Links* on page 24). Coordination with museums, biobank projects, and universities is strongly recommended. Collectors should refrain from picking up any dead animal they find; only collect those species and/or tissues with indicator value as otherwise collections might quickly expand beyond a manageable size.

*Photos showing the difference in body size, plumage, and beak colouration of two Southern grey-headed sparrows (*Passer diffusus*). Collectors should be familiar with indicator phenotypes to ascertain accurate sample ID and to be able to distinguish between adult and non-residents. Specimen ID photos should always show characteristic features that enable independent verification of species ID.*

Species selection: It is important to collect common and abundant species, rather than rare or cryptic ones, to ensure collection remains reliable, and probable, over time. Selected species should be easily and unequivocally identifiable with confidence by a range of collectors; avoid selecting species that are similar looking as this may lead to misidentification.

Indicator organisms should have a well-understood ecology to ensure that interpretation of changes and effects is accurate. Species should be naturally re-occurring annually and be safe and easy to collect. The species should have good indicator qualities: measurable response of change effects, for example toxin or heavy metal accumulation in bird feathers or livers and kidneys.

Migratory species: Globally distributed species are useful bio-indicators as they allow analyses across different habitats and continents. However, specimens must reflect local conditions. Avoid collecting adult migrants; instead, collect young migrants before their first journey/dispersal to ensure the local conditions are being reflected – young migrants from different areas can then be compared.

Collecting whole animal or animal parts: While whole animal collection should be the norm when considering a wide range of indicator purposes, specific indicator analyses can be catered for by collecting only the relevant tissue or body part, thus reducing storage requirements.

Temporal Trend Analysis: Annual hatchlings/broods should be collected so that the collector is certain of the sample's environmental time stamp. For instance, ORC collects Marsh terrapin hatchlings, which can be aged accurately. Older terrapins, however, are difficult to age as they may lay dormant underground for several years during drought conditions. Invertebrates can also be challenging to age. Mopane worm (emperor moth) larvae may lay dormant in substrate under mopane trees for years and may thus may not represent the environmental conditions from the year of hatching and/or collection. It is important that indicator collectors are familiar with such traits.

Collection

The process of collection depends on the species being sampled and the location(s) being accessed. However, there are some key considerations no matter what is being collected, or where.

Safety: A core consideration of collecting whole animals is to minimise safety risks. Part of this is understanding local diseases and their reservoirs and vectors as well as their transmission routes to ensure safety during collection. Further, where poisoning of specimens is suspected, collectors should exercise caution and wear gloves and other protective gear to avoid exposure. These samples must be labelled clearly so that laboratory personnel are also aware of the risk of secondary exposure. See the *General Methods and Tools* section for further information.

Collection of dead animals is safer than collecting live animals. Other safe collection methods include sweep netting and pitfalls traps. Immature specimens such as the larvae of many moths or beetle hatchlings are very easily collected, highly useful and representative, and many are widely distributed, in large numbers.

Recording: Record as much information as possible for each specimen collected. For example, cause of mortality (if known), age, sex, etc.

Bagging: Ensure the whole animal is bagged appropriately before transport to ensure there is no cross-contamination with other samples.

Transporting: Cool dead specimens as soon as possible after collection, ideally during transport, to prevent further degradation and decomposition. One can use a portable freezer or cool box with ice packs.

Sample processing and storage

The over-riding aim of collecting whole animals is to maintain these specimens in their original condition as much as possible and for as long as possible. Our diagnostic capabilities are evolving rapidly and whole samples may enable a new range of indicator analyses in the future, while already supporting a large spectrum of valuable investigations.

By preserving the whole animal with its various organ systems, we may enable analyses that are not available or understood at the time of collection, allowing deeper insights into environmental change and its causes.

Live collected animals: Humane euthanasia is necessary for specimens that are collected alive. For cold blooded animals this can be achieved by chilling the organism to sub-zero temperatures in a freezer, gas fumigation, or insertion into a preservative (for example, 99% ethanol).

Storage options: For indicator purposes, the easiest option for preservation is deep freezing the entire sample so that tissues are preserved in their original states, enabling a range of different analyses later. Where freezing can be reliably done, the use of additional preservatives is not necessary.

Where long-term freezing isn't guaranteed, collectors may store materials in preservatives such as ethanol (99%). Note that ethanol discolours specimens, thus making subsequent identification based on pelage or skin colouration difficult. The storage medium may also have to be replaced every few years. Museums retain specimens for decades in preservatives, even though genetic material may slowly degrade. A useful way of preserving DNA in tissue samples is to freeze samples submerged in ethanol; the ethanol does not freeze, preventing tissue crystallisation, but it maintains the sample in a stable storage medium in case the freeze cycle is interrupted.

Whole animals can also be preserved with a 10% formalin solution that is injected into the heads, limbs, body cavity and holes poked into body tissues. These samples are moved into permanent ethanol storage after about 24 hours. This procedure, however, can become cumbersome where large numbers of specimens are collected in a short amount of time.

If collectors are interested in preserving genetic material specifically, rather than the whole organism, tissue extracts may be preserved using RNA Later (a specialised RNA storage medium), substantially reducing storage space requirements while ensuring adequate preservation of genetic material.

Generally, all whole organism and tissue material samples should be kept out of direct sunlight and at the lowest temperatures possible to avoid unnecessary tissue degradation.

*Vacuum sealing a male Violet-eared waxbill (*Uraeginthus granatinus*) for permanent deep freeze storage in a special polymer bag. Labels should show the species' Latin name. The Violet-eared waxbill is also known as the Common grenadier, potentially confusing species ID at a later stage.*

*Vacuum sealed Spotted thick knee (*Burhinus capensis*) road mortality showing the storage space implications of whole animal collections.*

Preparing for freezer storage: Specimens can be vacuum sealed to remove water and oxygen from the storage environment. This process also reduces storage space requirements when compared with storage in containers and it allows easy shipment when samples need to be sent for analysis.

Freezing considerations: To preserve samples as best as possible, immediately store them in a freezer. When specimens have been labelled and packaged for long-term storage, they should be packed into a permanent storage freeze, with the location noted in the database. For long-term storage, consider:

- Freeze at or below -24°C
- There must be consistent freezing. If your freezer has a defrost mode, it must be turned off.
- Most freezers will hold sub-zero temperatures for several days if they are not opened regularly and seals are intact. To ensure optimum preservation conditions, ORC uses special freezers that reliably hold deep-freeze temperatures for three to four days in case of power failure.

Common analyses and purposes

- Toxicity and pollution (e.g., herbicides, air contamination, micro- and nano-plastics, soil, and water toxicants);
- Overall habitat quality and condition;
- Disease incidence and ecology (e.g., from specimen's blood, saliva, organs, or faeces);
- Minerals and trace element composition;
- Growth adaptations to changing habitat and climate characteristics;
- Microplastic accumulation and effects, (e.g., fish);
- Voucher specimens; and
- Genetic variation and genome changes over time.

Useful Links

The South African National Biodiversity Institute (SANBI), contact, an institute which oversees and coordinates various biodiversity and indicator monitoring programmes in South Africa.

National Zoological Gardens, contact, organisation with a BioBank that oversees collection and repository of important biological materials such as sperm, tissue, blood products, DNA etc.

A toolkit to determine policy-relevant biodiversity data, PDF resource.

Terrestrial and Aquatic Invertebrates as Bioindicators for Environmental Monitoring, with Particular Reference to Mountain Ecosystems by Hodkinson, I.D. & Jackson, J.K. (2005), PDF resource, use of invertebrates as bio-indicators and the range of chemical factors that can be bio-monitored using invertebrates.

How do ecologists select and use indicator species to monitor ecological change? Insights from 14 years of publication in *Ecological Indicators*, by Siddig, A.A.H., Ellison, A.M., Ochs, A., Villar-Leeman, C., and Lau, M.K., PDF resource, information on aligning collections with sampling at other sites.

Bioindicators: Using Organisms to Measure Environmental Impacts, by Holt, E.A. and Miller, S.W., web resource, using whole animals to describe the environment and its response to stress.

Selecting indicator species to monitor ecological integrity: A review, by Carignan, V. and Villard, M-A., PDF resource.

Interesting Studies

Bioindicators: the natural indicator of environmental pollution, by Parmar, T.K., Rawtani, D. and Agrawal, Y.K.

Ground beetles (Coleoptera: Carabidae) as bioindicators, by Rainio, J. and Niemelä, J.

Global biogeography and ecology of body size in birds, by Olson, V.A., Davies, R.G., Orme, C.D.L, Thomas, G.H., Meiri, S., Blackburn, T.M., Gaston, K.J., Owens, I.P.F. and Bennett, P.M.

Avian Feathers as Bioindicators of the Exposure to Heavy Metal Contamination of Food, by Markowski, M., Kaliński, A., Skwarska, J., Wawrzyniak, J., Bańbura, M., Markowski, J., Zieliński, P. and Bańbura, J.

Fish as bioindicators for trace element pollution from two contrasting lakes in the Eastern Rift Valley, Kenya: spatial and temporal aspects, by Plessl, C., Otachi, E.O., Korner, W., Avenant-Oldewage, A. and Jirsa, F.

Usefulness of Bioindicators and Biomarkers in Pollution Biomonitoring, by Hamza-Chaffai, A.

Biomonitoring of Bees as Bioindicators, by Ruiz, J.A., Gutierrez, M. and Porrini, C.

Frogs and toads as biological indicators in environmental assessment, by Simon, E., Puky, M., Braun, M. and Tothmeresz, B.

Migratory birds shrinking as climate warms, new analysis of four-decade record shows, news article.

Dirty birds show just how catastrophic air pollution used to be, news article.

Bone and Tooth Samples

Background

Bones and teeth combine a wide range of useful characteristics making them excellent bio-indicators. Along with antlers, horns, and hairs, they are important elemental storage tissues and, thus, are among the most informative indicator materials available. Other valuable characteristics, making them ideal indicators, include them being:

- **Abundantly available** in the environment from carcasses (often very conspicuous);
- **Safely collected using non-invasive sampling** (natural deaths, road kills, by-catch or window strikes);
- **Suitable for long-term storage not requiring specialised facilities;**
- **Of low risk of accidental contamination;**
- **Great absorbers and stores of elements,** and so they contain deposits of many chemical elements, enabling retrospect trend analyses including contamination, presence, and concentrations of specific elements; and
- **Non-volatile tissues,** having very low degradation/decomposition rates and are thus available in the environment and retain their indicator quality over long periods, reliably preserving conditions for centuries, if not longer.

Bone is composed of protein collagen and mineral crystals (bone salts). Bone salts largely consist of hydroxyapatite, carbonate, calcium, and citrate and also have smaller amounts of other elements such as sodium and potassium, as well as a number of trace elements which can include rare earths and metals from land contamination. The relatively slow turnover rate of ions in bone salts means that bones may even store trace ions such as uranium and lead.

Teeth consist of a crown (area above gums), and either a singular root (incisors), dual roots (lower molars) or trial roots (upper molars). They are composed of dentine (major component, surrounding the pulp), cementum and enamel (outer layer). Both enamel and dentine's inorganic components are largely composed of calcium, phosphorous, magnesium and carbon trioxide. The pulp (soft tissue) has the highest water content.

Sample collection and transport

Bone and tooth-specific equipment

- Paper bags or other containers
- Gloves
- Disinfectant/Sanitiser
- Respiratory mask or face covering
- Pliers (tooth extraction) and diamond saw, drill or chisel for tooth or bone separation (as required)

Selection

Limit collection to what can be stored and used in analyses; storing large vertebrae or leg bones can take up a lot of space. Further, beware of sampling materials from migrants or introduced animals as these samples may represent the conditions of other areas, rather than the local ecosystem.

Collection

Safety: There is considerable risk of disease/infection transmission when handling carcasses, especially where diseases are endemic. Take safety precautions (see *General Methods and Tools* chapter).

Collect: Bones from dried carcasses can be collected in paper bags or other suitable containers. Teeth are easily extracted from old carcasses with pliers and then bagged or put into containers. Often a sub-sample – a shard of a larger bone or only a few molar teeth – is sufficient to conduct the analyses required. It is usually unnecessary to collect all teeth, or entire bones, if they are large.

Transport: Transport in container/bag. No cooling or special treatments are required.

Sample processing and storage

Bone Processing

Processing bones requires removal of connective tissues, cartilage, soft tissues, fat, fibres, and marrow. This process will result in the strong smell of decomposing flesh; the location of bone processing should be chosen bearing this

Carcasses provide ample and safe opportunity for collection of bones and teeth.

Giraffe skull with easily extractable teeth (extraction using pliers).

in mind. Processing bones is a three-step process, but each step can be altered depending on preference, condition of the bone prior to processing, and available resources. Be sure work safely; use gloves, face coverings, and thoroughly disinfect/sanitise your hands, tools, and work areas.

1. Decomposition (where samples are not already decomposed/flesh-free): Bones can be left out in the open, with sun (UV radiation) exposure. Micro-organisms and invertebrates will remove decomposable tissue from the sample. Another simple process is to submerge the bone in water and expose it to UV radiation, which will eventually dissolve all the connective tissue. These processes are effective; however, both methods may require weeks or months before the sample is entirely clean. One may choose to skin or de-flesh the bone manually first to speed up the decomposition process. Regardless of the method chosen, be sure to protect the sample from scavengers; a simple wire mesh cage is usually sufficient.

Chemical agents can be used to speed up this process (for example, ammonium hydroxide or acetone); however,, ensure they will not have a deleterious effect on the planned analyses. For instance, it is not advisable to submerge bones in a hydrogen peroxide bath (as is often done for animal taxidermy); this process induces oxidative stress, which affects bone mineral density and stiffness, thus destroying sample properties and diminishing the sample's scientific value. Also avoid boiling or simmering the bone in water. While this process is cheap and simple, it can also damage the bones.

2. Removing remaining tissue: If any tissue remains, pick it off with tweezers or wash the bone while gently removing the tissue.

3. Degreasing: Once all flesh/tissue has been removed from the bone, place the sample in a container filled with water and dish soap and seal the container. Soaking should occur for a week or longer. If, after week one, the bone is still greasy, replace the soapy water and leave it to soak for another week. Repeat this procedure until the bone no longer is greasy.

Storage

Storage of dry bones and teeth in a cool, dark place, in paper bags, is usually sufficient. In humid climates, silica beads can be added to minimise moisture. While more costly, freezer storage is also an option.

Common analyses and purposes

Bones and teeth are such versatile bio-indicators that almost all analytical techniques can be, and have been, used for trace element analysis. These tissues are generally analysed to answer four main questions:

- Which species have been/are present in the area;
- How have environmental changes affected local species – comparing tooth/bone growth changes with concurrent environmental conditions allows a detailed understanding of responses to environmental change;
- What changes have occurred in elemental (chemical) composition and their specific concentrations over time; and
- Is there presence and/or accumulation of deleterious chemical compounds (e.g., heavy metals) in the environment?

When analysing teeth, either the whole tooth can be analysed, or particular sections may be analysed separately. Specific analyses will depend on the quantity of the sample and the elements being examined. However, non-destructive techniques are generally preferred to retain the sample for additional purposes. Common analyses for assessing element composition include:

- X-ray fluorescence (XRF) or particle-induced X-ray emission (PIXE);
- Neutron activation analysis (NAA);
- Atomic absorption spectroscopy (AAS) or atomic emission spectroscopy (AES);
- Anodic stripping voltammetry (ASV); and
- Mass spectrometry (MS).

Useful Links

The multi-elemental analysis of bone. A review, PDF resource, summarises methods for bone analysis.

The analysis and levels of lead in human teeth: a review, PDF resource, summarises methods for tooth analysis.

Minor and Trace Elements in Human Bones and Teeth, PDF resource, International Atomic Energy Agency.

Interesting Studies

Elemental Analysis of Bone, Teeth, Horn and Antler in Different Animal Species Using Non-Invasive Handheld X-Ray Fluorescence, by Buddhachat, K., Klinhom, S., Siengdee, P, Brown, J.L., Nomsiri, R., Kaewmong, P., Thitaram, C., Mahakkanukrauh, P. and Nganvongpanit, K.

Teeth as Indicators of Environmental Pollution with Lead, by Kamberi, B., Koçani, F. and Dragusha, E.

Hair and bone as predictors of tissular mercury concentration in the western Alaska red fox, *Vulpes vulpes* by Dainowski, B.H., Duffy, L.K., McIntyre. J. and Jones P.

Impact of anthropogenic activities on the concentration of trace elements in toe bones of the common toad (*Bufo bufo*). Simon, E., Kundarat, J.T., Braun, M., Kovacs, B., Andrasi, D and Tothmeresz, B.

Hair Samples

Background

The stability of hair tissues means hair samples can be very useful indicators not requiring special preservation or refrigeration for most analytical purposes. Hair samples also enable a wide range of analyses and large numbers of specimens can often be **collected cheaply and easily**, without trauma/pain, for example from carcasses. Hair is also an ideal indicator that can be **easily stored and transported**.

The hair shaft emerges from the root; the shaft below the skin is part of the living follicle, and above the skin is a tube of dead cells. Certain analyses require hair from below the skin, while others do not. Hair can be analysed to determine:

- Trace element presence/concentration over time;
- Individual identification (via DNA) and species distribution;
- Relatedness of populations;
- Hormone and stress levels of individuals;
- Point of origin of trafficked animals; and
- Disease/disease loads within populations.

Potential sources of hair samples, from top to bottom: week-old elephant carcass being sampled for mitochondrial DNA, day-old zebra carcass for disease/trace element analysis.

Sample collection and transport

Hair-specific equipment

- Paper sample envelopes
- Pliers/strong tweezers/scissors
- Rubber bands
- Gloves, sanitiser, and wipes

Selection

Age: Depending on the analyses required, most hair samples can be collected from old carcasses, and can be stored for many years, without refrigeration or preservatives. Indeed, scientists have sequenced the woolly mammoth's genome from 20,000-year-old hairs while another study using 30-year-old, dry-stored hair successfully extracted mitochondrial DNA (mtdNA). Exposure to sunlight degrades samples more quickly, so sampling from sheltered areas will improve sample quality. Some hair components are less stable than others (e.g., nuclear DNA (nDNA)) and thus degrade more quickly; when selecting hair sources, be sure to consider the type of analysis planned.

Body part(s): There are variations in hair characteristics depending on the body part the hair is from; protein differs across the body, as does the amount of contact with the environment (e.g., UV radiation). As such, it is best to always collect hair from the same body part or collect samples from multiple body parts, in cases where the analytical procedures are yet unknown or where samples may be used as part of a larger study from multiple sources. When collecting samples from multiple body parts, it is best to collect them from the head, back, a leg and the tail.

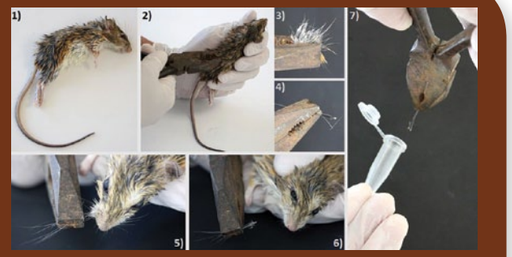
Quantity: Aim for approximately a pencil's thickness for each sample, to ensure there is enough hair.

Collection

Protocol: Ensure that collection tools are clean and void of any materials that may contaminate the hair prior to collecting each sample. Make sure not to touch hair (genomic DNA from fingerprints can contaminate the hair), particularly the follicles, other than with the sanitized equipment (such as tweezers or gloves) and unused sampling envelopes to minimise contamination. Multiple hairs comprising one sample should be banded together, noting if the band is at the root end, or the opposing end.

mtDNA collection: Cut the hair from as close to the skin as possible, recording the body part the sample came from. Bind the hairs and place into an envelope and seal. For hair collected for DNA analysis, surface DNA decreases in quantity the further it is collected from the root due to longer exposure outside of the epidermis. Hence, it is always best to collect from as close to the root as possible, keeping the full length of the hair to increase the sample's size.

nDNA and other collections: Use tweezers or pliers to pull the hair away from the skin; beware that pulling at an angle may leave the follicle behind. Confirm that the follicle is attached by checking there is a bulb/thickening at the base



Hair pulling with roots and follicles attached. When the animal is dry and hair is difficult to extract, submerge the animal in warm water for several minutes to soften the skin tissue for easier extraction (1). Use pliers (or other tools) that have a good grip. To extract hair with roots and follicles still attached, hold the animal firmly and extract the hair with a quick, forceful pull (2, 5). Assess the hair against a dark backdrop (3), the roots and follicles should be clearly visible (4). Whiskers are often easier to extract than body hair, as they are embedded in soft muzzle tissue (5). Once enough hairs with roots and follicles attached are extracted (6), place them into the storage container (7) and add Ethanol as a preserving agent before freezing – if freezing is not possible, dry storage (at room temperature) in a paper bag is sufficient for subsequent DNA analysis.

of the shafts. Place hairs in a sampling envelope, seal it and note which body part the hair was collected from.

Transport: Transport in sample envelopes. Cooling or other treatments are not required for transport.

Sample processing and storage

Processing

To reduce surface contamination, wash the sample using water and detergents or solvents. Chemicals used in washing should be chosen considering the planned analysis. If the chemical's influence on test diagnostics is unknown, do not wash the hair.

Storage

First, ensure that hair is completely dry. For most analyses (mtDNA extraction, trace element detection, etc.) hair can be stored in the collection envelope, in an airtight container with other envelopes. Silica bags should be added to the container to minimise moisture, particularly in humid environments.

For nDNA or hormone analysis or when diagnostic techniques are unknown at the point of storage, the samples should be frozen at -20˚C in the collection envelope, in an airtight container. As nDNA is prone to degradation, it is preferable to extract the DNA from the hair sample upon collection and store as DNA, rather than storing the hair. However, this will not always be feasible given the facilities available.

Common analyses and purposes

- Mitochondrial DNA testing for lineage, species distribution, genetic relatedness of populations/ individuals;
- Nuclear DNA analysis for mapping genomes;
- Mineral analysis (e.g., neutron activation analysis) to determine trace elements (led, etc.);
- Stable isotope ratio analysis (i.e., mass spectrometry) to assess disease loads, nutrition, hormone levels; and
- Steroid extraction methods to assess hormone and stress levels.

Useful Links

Hair Collection, PDF resource by Kendall, K.C. and McKelve, K.S.

Interesting Studies

A quantitative evaluation of two methods for preserving hair samples by Roon, D.A., Waits, L.P. and Kendall, K.C.

Noninvasive genetic sampling tools for wildlife biologists: A review of applications and recommendations for accurate data collection by Waits, L.P. and Paetkau, D.

Efficient DNA extraction from hair shafts by Almeida, M., Betancor, E., Fregel, R., Suarez, N.M. and Pestano, J.

Hair as a bio-indicator: Limitations and complications in the interpretation of results by Evans, G.J. and Jervis, R.E.

Hair After Death by Wilson, A.S. and Tobin, D.J.

Mammalian hair as an accumulative bioindicator of metal bioavailability in Australian terrestrial environments by McLean, C.M., Koller, C.E., Rodger, J.C., and MacFarlane, G.R.

Scientists Sequence Woolly-Mammoth Genome by Penn State

Trace Elements in Hair: Relevance to Air Pollution by Chikawa, J., Salter, J., Takaaki Tsuchida, H.S, Ueda, T., Yamada, K. and Yamamoto, S.

A novel method using hair for determining hormonal levels in wildlife by Koren, L., Mokady, O., Karaskov, T., Klein, J., Koren, G. and Geffen, E.

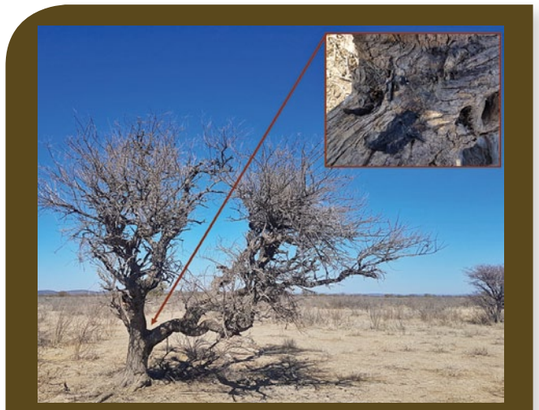
Density and population size estimates for North Cascade grizzly bears using DNA hair-sampling techniques by Romain-Bondia, K.A., Wielgusa, R.B., Waits, L., Kaswormc, W.F., Austin, M. and Wakkinene, W.

Faecal Samples

Background

Faecal samples are commonly used bio-indicators; they are abundantly available, easy, and cheap to collect and store and non-invasive to collect. Fresh faeces (scat) and fossilised faeces (coprolites) can be useful bio-indicators, however, each enables a different set of analyses.

Faeces enable a range of investigations; assessing animal health, nutrition and stress, the presence of disease, parasites, and contaminants as well as genetic analyses with an accurate reference time stamp. New technology even allows ancient **coprolites** to be examined for diet composition and health of individuals living in the distant past. Further, ancient DNA analyses can be performed with coprolites.



Cheetah marking tree in northern Namibia. Inset shows fresh cheetah scat deposited by a resident male. Known scat deposition sites such as marking trees and latrines enable regular sample collection and improve confidence in correct species identification.

Sample collection and transport

Faecal-specific equipment

- Wildlife scat field identification guidebook
- Gloves
- Containers or paper bags
- Styrofoam/cool box with ice packs
- Disinfectant/Sanitiser

Selection

Prior to collecting faeces, it is important to be able to reliably identify the species that deposited the faeces - retrospectively identifying species from faeces is very costly (for example, using genetics).

Scat age: Ideally, scat samples should be fresh (usually soft to the touch in arid/semi-arid environments). Long exposure to UV radiation and high temperatures reduces sample value, for example because UV radiation destroys genetic material and pathogens. To determine scat age, softness and colour can be used as proxies; UV radiation bleaches scat, so that older scats often appear lighter in colour. However, scat colour can also be misleading; colouration is also strongly influenced by what an animal consumed (above). Hard, solid scats are usually old and therefore, less useful.

Species identification: Some species' scats are distinguishable from similar species by their size or by location. For example, cheetahs often defecate in trees, while hyenas use latrines. This can simplify locating samples and species identification. Scat shape, segmentation and content analysis are other ways of identifying species correctly, while genetic analysis remains the most reliable approach.

Left: Size and colour differences of fresh lion scats. The black scat (bottom left) was approximately 24 hours old and its surface dry, whereas the lighter sample (centre) was less than 4 hours old and its surface moist. The colouration of scat can indicate scat age, but in this case was influenced by the diet of the animals. Both specimens were soft and moist on the inside.
Right: Cheetah scats at different stages of decomposition, with various degrees of discolouration due to aging (i.e., bleaching from UV radiation). Scat ages (top to bottom): 1 week; 7 weeks; 4 months; 11 months.

Transport: safe transportation of three scat specimens wrapped in the collection glove

Processing: three lion scat portions (~40g each) from the same scat sample. Packaged portions facilitate easier storage and transport.

Storage: size comparison of storage space requirements of differently packaged lion scat samples: portioned samples in tubes (bottom left) and containers (top left); 15 RNA Later vials (centre); two full scat samples (top-right and right).

Collection

Safety: See General Methods and Tools chapter – without gloves and correct sanitation there is a very real risk of disease transmission.

Collect: For simple and safe collection, samples can be picked up by gloved hand, placed in their container and then be wrapped in the glove by turning the glove inside out from the wrist down over the container, thus preventing hand contact with the specimen and contamination. This way the glove will cover the sample.

Prepare for transport: Samples can be transported wrapped in the collection glove to prevent spillage and contamination. This will also reduce the smell during transport. Ideally, samples should be cooled during transport using a Styrofoam box and ice packs, or a portable cooler.

Sample processing and storage

For easier storage and transport, it is advisable to sub-divide large faeces into smaller portions (aliquots) before freezing as sub-division post-freezing is more difficult. When there are multiple portions from the same scat specimen, label each container uniquely; for example, ORC_BIO_00216a, ORC_BIO_00216b etc.

When samples will be used for **disease, hormonal, and similar purposes**, freeze faecal samples immediately.

For **RNA preservation and bio-banking**, preserve faecal extracts using *RNA Later* (a specialised RNA preservation medium), which substantially reduces storage space while preserving genetic materials appropriately.

For **diet composition studies**, dry out scats in paper bags in a shaded environment for several weeks, selecting an area where the smell will not become problematic. Store dried scat in paper bags or vacuum sealed bags.

To extract and analyse information from **coprolites**, freeze the coprolites and grind them into powder prior to analysis.

The **sample size required** depends on the analyses; for disease, contaminant, and hormonal analyses 40-50g is sufficient, for RNA Later or bio-banking the samples can be very small, whereas samples for determining diet composition should be as complete as possible.

Common analyses and purposes

- Identification of environmental pollution/contaminants; for example, presence of macro- or micro-plastics;
- Genetic studies; using dead epithelial cells from the lining of the gut, e.g., for occurrence/distribution studies or assessing population numbers;
- Hormonal assays; hormones and their metabolites are excreted with scat (as well as in saliva, urine, and hair) and can be used, for example, to investigate animal stress levels by examining glucocorticoids;
- Population structure and health as well as heredity; including estimates of population size, sex ratios and the interrelatedness of all the animals in an area; and
- eDNA studies, such as retrieving genetic fragments of carnivore prey or assessing local plant composition (usually at genus level) from DNA fragments found in herbivore scat.

Useful Links

Exposure to Animal Faeces and Human Health: A Systematic Review and Proposed Research Priorities, research article, online/PDF resource, detailing health effects of faecal exposure

Common methods for fecal sample storage in field studies yield consistent signatures of individual identity in microbiome sequencing data, research article, online, detailing effectiveness of different storage techniques for microbiome sequencing

Interesting Studies

Measuring stress in mammals using fecal glucocorticoids: Opportunities and Challenges, by von der Ohe, C.G. and Servheen, C.

Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: A literature review, by Keay, J.M, Singh, J. Gaunt, M.C. and Kaur, T.

Garbage in guano? Microplastic debris found in faecal precursors of seabirds known to ingest plastics by Provencher J.F., Vermaire , J.C., Avery-Gomm, S., Braune, B.M. and Mallory, M.L

Plastic Microfibers Found in the Stool Samples of Wild Animals by Donlon, M.

Evaluating blood and excrement as bioindicators for metal accumulation in birds, by Berglund, A.M.M.

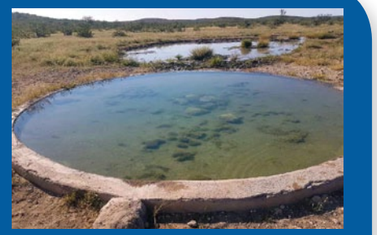
Heavy Metal Contamination in Ranthambore National Park: Feces as Bioindicators by Gupta, V. and Bakre, P.

Feces of captive wild mammal use as bio-indicator of heavy metal pollution in urban air, by Gupta, V.

Arthropods as bioindicators of the red fox foraging activity in a Mediterranean beach-dune system, by Ricci, S., Colombini, I. Fallaci, M., Scoccianti, C and Chelazzi, L.

Water Samples





Example source, from left to right: permanent waterhole with overflow, seasonal pond, and permanent waterhole

Background

Potential water sources for indicator collection are rivers, streams, dams, permanent waterholes, seasonal puddles/dams, and groundwater sources. Depending on the area, and its rainfall patterns, pure rainwater may also be collected.

The importance of water lies in it being vital for metabolic processes of all living organisms. While some animals are more water-dependent than others, most will drink when practicable. Different parts of the water source (edge, overflow etc.) may be used by different species, sexes and at different times of the year. Water is used in all plant and animal cells, organs, and tissues. It is crucial for:

- carrying nutrients to all body cells and transporting oxygen to the brain;
- allowing the absorption and assimilation of minerals, vitamins, amino acids, glucose, and other substances;
- keeping body tissue hydrated, including acting as a lubricant and cushion for joints;
- flushing out toxins and waste through urination, defecation, and perspiration; and
- body temperature regulation.


Equipment from left to right: 50 mL plastic containers, Parafilm, and Styrofoam box with ice packs (if a portable freezer isn't available)

Sample collection and transport

Water-specific equipment (*see image*)

Collection location

The collector should bear in mind that water quality will vary depending on the collecting location. For instance, samples will differ between the surface vs. below surface level and from the edge of a waterhole vs. the inlet, etc.

Care should be taken to standardise the collection point of water samples. Ideally, samples reflect the water that animals have access to for drinking.

Pumped waterholes (with inlet): We recommend that at least two samples are taken:

1) a surface sample, collected at the waterhole's edge furthest away from the inlet; and
2) a sub-surface sample collected from the inlet.

Pumped waterholes (with inlet and overflow): Three samples should be taken from these water sources:

1) and 2) as above; and
3) an additional surface sample from the overflow which will account for greater variability.



Sampling locations from left to right: At the inlet, at the edge of a pumped waterhole and at the edge of a natural waterhole.

Natural waterholes: We recommend collecting three samples, including:

1) a surface sample, collected from the edge of the waterhole;
2) a sub-surface sample; and
3) a second surface sample, collected from approximately 1 m distance from the edge, noting the distance from the water body's edge and whether the water was disturbed when collecting this sample (i.e., through wading in).

Sampling methods

Plan: Decide which water bodies are relevant (or of interest) and may become fixed sampling points, i.e., water collection is repeated regularly for subsequent comparisons. Regardless of the type of water body, a minimum of two samples should be collected per locality.

Collect: Using the plastic container (ideally 50 mL), collect water up to 90% of the container's volume (45 mL for a 50 mL container). Filling the container only to about 90% of its total volume allows for expansion of the sample when it is frozen (as per storage requirements) without cracking the container and destroying the sample.

Record: In addition to standard data (e.g., Sample Database appendix), you should also make a note of the water source type (i.e., dam, borehole-fed waterhole, etc.) and if the water is flowing or stagnant. If there are obvious sources of contamination (e.g., animal carcasses in the water, or faecal matter) make note of these too.

Transport: Cool the samples as soon as possible after collection, ideally already during transport, to minimise chemical processes and associated degradation. Do this by using a portable freezer or cool box with ice packs.

Freeze: Ensure the samples are frozen as soon as possible to stop chemical processes entirely and to preserve the original composition as much as possible; either by using portable freezer or by bringing samples to the storage location as soon as possible. Ideally, water samples are transported frozen to the laboratory and only thawed prior to analyses.

Sample storage

Samples must be stored frozen, at a temperature of between -10°C and -24°C. Accidental thawing should be avoided to ensure the original composition of the sample.

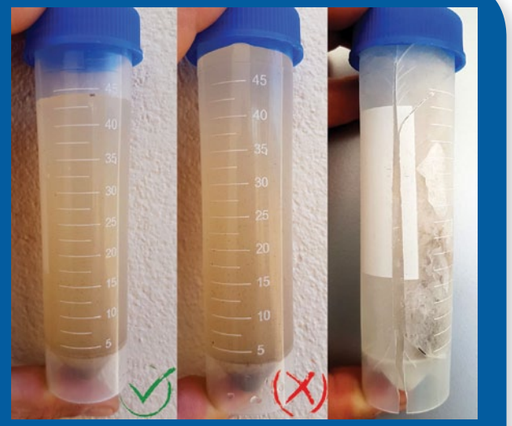
Parafilm wrap should be used to seal off the gap between container and cap to prevent leakage.

Common analyses

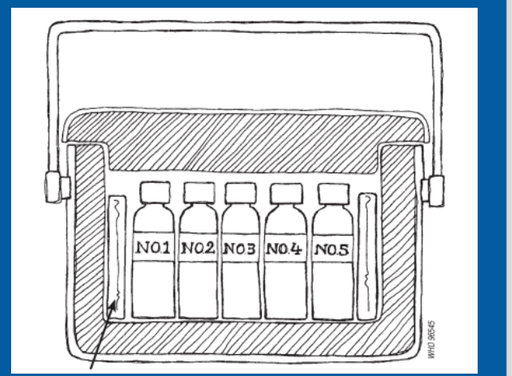
- Chemical and mineral composition, e.g., sodium, calcium, magnesium, chloride, sulphate, carbonate, bicarbonate, organic carbon
- Acidity (pH-value), salinity, alkalinity (or hardness) and dissolved oxygen
- Pathogen/Microbial load
- Toxicity, heavy metals (like lead, mercury, copper), agrochemicals (specific pesticides or fertilisers) and contaminants (e.g., pharmaceuticals and personal care products)
- Groundwater stable isotope analysis
- Aquatic micro-diversity from DNA as well as eDNA (environmental DNA) for confirmation of a variety of taxa



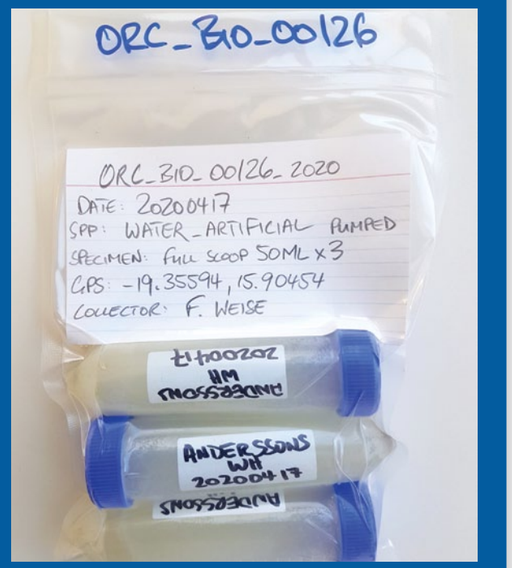

Directions, from left to right, of the simple application of Parafilm to a water sample to seal the container for long-term freezing.



Don't completely fill the container



Transport upright, using ice packs where a portable freezer is not available World Health Organization (2017): Figure re-printed with permission under license: CC BY-NC-SA 3.0 IGO*)*



Clearly mark all samples before storage

Useful Links

Guidelines for drinking-water quality, 4th edition, incorporating the 1st addendum. Online: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO.

National Field Manual for the Collection of Water-Quality Data, U.S. Geological Survey, PDF resource.

Water sampling and analysis, World Health Organization, PDF resource, source of modified cool box and ice pack drawing (page 57).

Environmental DNA Sampling Protocol—Filtering Water to Capture DNA from Aquatic Organisms, U.S. Geological Survey, PDF resource.

Interesting Studies

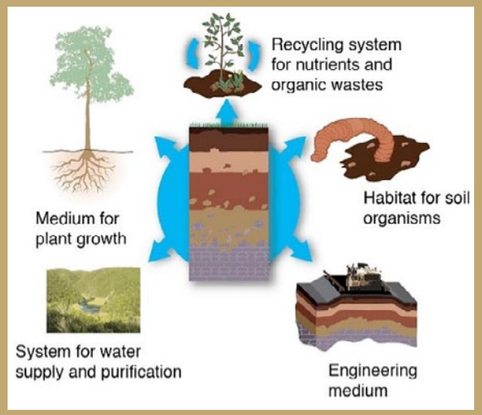
Groundwater stable isotope profile of the Etosha National Park, Namibia, by Riddell, E.S., Kilian, W., Versfeld, W. and Kosoana, M.

Soil Samples

Background

Sources include, but are not limited to, soil from freshly built termite mounds (not old soils) and the edge of permanent waterholes. Termite mounds are a useful source as termites process organic plant matter at all stages of decomposition, influencing the chemical structure of soil by returning nutrients such as nitrogen into the soil. They also blend different soils together, from deep below the soil surface, often with organic materials and in doing so improve soil fertility. Termites also loosen and help form soil, altering its physical structure, leading to better drainage and plant growth. Waterhole edge soil can reveal information on nutrient presence, toxicity, soil protozoans, disease agents, soil quality and soil composition. It is important to note the source of the soil due to these differences. **Soil is vital in:**

- Providing minerals and nutrients for plant growth;
- Holding and purifying water;
- Natural filtration;
- Carbon sequestration;
- Storing and recycling nutrients; and
- Containing soil protozoa, e.g., for soil oxygen.



Importance of soil in the ecosystem (Reprinted from What are Soils? by Needelman (2013), redrawn from The Soils Around Us by Weil and Brady (2017))

Sample collection and transport

Soil-specific equipment

Suitable non-degradable plastic bag (should be large enough to contain about 1kg of substrate)

- Hand shovel and/or spoon
- Small pickaxe for loosening substrate, where necessary

Collection

Collection points: Ongava Research Centre collect two types:

1) at the edge of permanent waterholes; and
2) at *Macrothermes* spp. termite mounds, collecting only fresh soils from new builds, not old soils.

Collection quantity: Minimum amount should be 750 grams per soil specimen to ensure an adequate amount for analysis.

Equipment handling: It is important to clean the collecting tools thoroughly after collecting each sample to prevent cross-contamination between subsequent samples. Clay soils, for instance, stick to the shovel and may accidentally be added to the next sample.

Sample processing and storage

Processing

Soils should be dried at low temperatures (19o -23o C). High-temperature oven-drying will degrade proteins and prevent some future analyses. For example, environmental DNA (eDNA, DNA that is released from organisms to the environment) will not survive high temperatures and so soils dried at high temperatures will not be usable to analyse eDNA.

Bear in mind that the more clumped soils will take longer to dry and require more storage volume than fine-grain soils.

Soil sampling sources. From left to right: two termite mounds with fresh builds; the edge of a permanent waterhole.

Collection of soil samples, from left to right: Collecting from beside a permanent waterhole, collecting from a termite mound, and collection tools including labelled, filled sampling bags.

Soil processing and storage, top left to bottom right: samples at collection point, samples after drying (note the reduction in volume), preparation for storage (note labelling and silica bags) and soils ready for permanent storage.

Storage

Storage conditions (freezing or dry storage) should be chosen based on the planned analyses.

Soil chemistry changes over time, as biological and chemical processes break down or combine compounds over time. These processes change once the soil is removed from its natural ecosystem (flora and fauna that penetrate the sampled area) and environment (temperature, moisture, and solar light/radiation cycles). As a result, analysis is improved if the soil is analysed soon after collection, usually within 24 hours. Chemical changes can be slowed during storage and transportation by freezing it. Air drying can also preserve the soil sample for many months.

Soil samples, whether dry-stored or frozen, should be stored away from UV radiation and moisture.

Common analyses

Several analyses can be employed when determining soil characteristics:

- Toxicity (e.g., via measuring heavy metal deposition such as lead concentration);
- Common mineral soil contaminants (e.g., arsenic, barium, cadmium, copper, mercury, lead, and zinc);
- Contamination (e.g., pesticides);
- Micro-plastics and nano-plastics (e.g., testing for the presence and effect of these plastics in the soil);
- Disease (e.g., pathogen presence, pathogen diversity, pathogen load);
- Compounds (e.g., sulphur, nitrogenous and cyanide compounds);
- Nutrients including ***major nutrients (nitrogen, phosphorus, potassium), secondary nutrients (sulphur, calcium, magnesium) and micro-nutrients (iron, manganese, copper, zinc, boron, molybdenum, chlorine)***;
- Metals (e.g., lead, copper), which are one of the most tested for groups in environmental analysis;
- e-DNA (e.g., micro and macro biodiversity, see Leempoel et al. 2020 in Useful Links, below);
- Soil protozoans, among other characteristics, can be detected using soil protozoan bioassays (e.g., abundance, individual dominance, biomass dominance and frequency of important protozoans; and
- Inorganic compounds (e.g., chloride, nitrate, nitrite, sulphate, phosphate, sulphide, ammoniacal nitrogen).

There are also a variety of chromatography-based soil analyses available that determine several soil characteristics including the presence of speciated phenols, petroleum hydrocarbons, such as range organics (GRO) and extractable petroleum hydrocarbons (EPH), and other compounds (e.g., volatile organic compounds (VOCs), semi-volatile organic compounds (SVOCs) and pesticides).

Useful Links

A Guide to Collecting Soil Samples for Farms and Gardens, Oregon State University, PDF resource

Soil Survey Field and Laboratory Methods Manual, U.S. Department of Agriculture, PDF resource

A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity, Proceedings of the Royal Society B: Biological Sciences, Journal Article resource, Kevin Leempoel, Trevor Hebert, Elizabeth A. Hadly, 2020, 287 (1918): 20192353

Soil protozoa as bioindicators in ecosystems under human influence, Oxford, Book Chapter PDF resource, Foissner, W., 1994, In: Darbyshire, J.F. (Ed.), Soil Protozoa. CAB, Oxford, pp. 147–193.

Grass Samples

Background

Plants, as primary producers, accumulate materials directly from their environment (from soil and water) and so can be used to understand the health of the environment. Plants are widely used as biological indicators; they can reveal nutrient levels and toxicity of an environment, including soil and air pollution (including the frequency, distribution, and degree of pollution events) and determine herbicide toxicity levels. Valuable plant indicators include fungi, mosses and lichen, grasses, and tree bark, rings, and leaves. **Grasses are useful plant indicators;** while any plant is a useful indicator, not all are easy to collect across a vast area. Grasses, on the other hand, not only exist across different environments, and indeed the world, but are also easy and safe to collect.

Examples of the uses of plants/plant parts as suitable indicators include:

- Tree bark and leaves used to determine roadside pollution from transport gases (air pollution);
- Seagrasses as indicators of trace metal pollution;
- Leaves from urban trees revealing lead concentrations and isotope ratios; and
- Grasses that have been genetically engineered to respond to the toxicity of the environment; the colour of these grasses is dependent on the presence, and concentration, of toxins in the surrounding soil.

Using indigenous grass species will increase the relevance and reliability of bio-indicator testing. It is important to select the best species for the attribute being tested and to know and recognise the species that are being collected.

Clockwise from top-left: Various field collection and storage bags; hand shovel; and plant press for specimen drying.

Sample collection and transport

Grass-specific equipment

- Plant guide, hand lens and ruler for field identification
- Secateurs and shovel for collection and excavation
- Paper bags; large and small
- Rubber bands for sealing bags
- Labels
- Water to moisten specimens once placed in bags (needed for long transport only);
- Plant press and newspaper (only required in the lab/ office - can be improvised using cardboard sheets and heavy objects)

From left to right, different grass identification photos, all Tragus berteronianus*: whole grass in situ; whole grass removed from substrate in one piece; and whole specimen ID photo*

Selection

Species: Grasses are a particularly challenging family of plants to identify due to their often-ambiguous characteristics and similarity between species and/or locally overlapping sub-species. As such, it is advisable to select indicator species with clear, unmistakeable characteristics that are easy to recognise by a range of collectors unfamiliar with the whole range of grass species. Ideally, projects should collect a combination of annual and perennial grasses.

Plant parts: As different parts of the plant are responsible for different functions, they also store different materials, so, in order to undertake comprehensive analyses using grasses, it is important to collect the whole plant including roots, rhizomes (underground stems), stolons (stems/runners growing along the soil surface) and mature seeds (important to test germination responses to environmental change).

Ensure you are collecting samples that are **fruit/flower bearing** as sterile material will hold less scientific value and make species identification much more challenging.

Collection

Record: Take a photo of the grass before collecting it.

Collect: Collect the whole plant. Be as careful as possible to keep the plant intact, including underground organs as well as flowers and seeds. Collect at least two specimens of the same species per site. ORC collects three specimens of the same grass species at each site. This allows us to distribute two specimens for analysis while retaining one specimen in our long-term repository.

Verification: Take another photo of the whole plant, at the collection site that shows all details clearly. This will enable you to gain independent species identification.

Prepare for transport: Place your plants into paper bags, ensuring that there is no cross-contamination between seeds of different plants. If transport to the storage site is far, lightly spray the grass with water to keep the plant from wilting during transport. Secure the bags with rubber bands to prevent any loss of materials. Label each bag.

Sample processing and storage

Processing

Make sure you process your specimens as soon as possible after they are collected to prevent wilting of leaves. Remove soil by carefully brushing it away or washing it off if needed. Then, where possible, you should attempt to separate grass clumps without damaging the plant so that there are smaller items to press.

Dry press your specimens between cardboard or paper sheets using a plant press or heavy items such as books. If the specimen does not fit into the press, without hanging over the sides, you can carefully fold into a V or N shape to ensure it fits.

Seeds and fruits should not be pressed but should be dried at room temperature (see chapter Seeds) and stored with the specimen to prevent loss of these materials.

Storage

Samples can either be freezer stored, without prior drying, or dry stored after pressing. Freezer storage, while providing a less variable environment, will require considerable freezer space over longer periods, leading to difficult logistics and increased costs.

When dry storing grasses it is more important to consider the moisture and humidity of the storage environment than room temperature. Also see Storage Considerations in the Seed Samples chapter for more information regarding moisture, light, and temperature considerations for storage. After drying and pressing, whole plants can either be stored in a pressed booklet or in brown paper bags.

Whole grass specimen from left to right: at collection site; prior to drying and pressing; after drying and pressing for dry storage; and vacuum sealed for freezer storage.

Be vigilant for insects and pests that may infiltrate long term storage, resulting in damage to samples. In large collections, there may be a need to fumigate storage cabinets periodically, e.g., Namibia's National Botanical Research Institute does this twice annually.

Common analyses

- Nutrient composition and absorption;
- Toxicity and pollution (e.g., herbicides, air contamination, road-side emissions from vehicles, micro- and nano-plastics, soil toxicants);
- Overall habitat quality;
- Growth adaptations to changing soil and climate characteristics;
- Germination success and growth characteristics in relation to environmental variables; and
- Genetic diversity and changes.

Useful Links

Guide for Collecting Plants, National Botanical Research Institute (NBRI) National Herbarium of Namibia (WIND), PDF resource. **This is a simple but excellent open-access plant collection guide.** Note that the NBRI, upon request, also assists others with plant identification where necessary.

How to Collect Plants, Royal Botanic Gardens Sydney, PDF resource

Laboratory manual for collecting and processing of plant samples, Papua New Guinea Forest Authority, PDF resource

Interesting Studies

Grasses as Bio-indicators of Air Pollution in Coal Mines of Yellandu, Khammam District, Andhra Pradesh by K. Prameela, M.A. Singara Charya

Characterization of Poaceae (grass) species as indicators of the level of degradation in a stretch of riparian forest in Matutina, Brazil, by V. Londe, J.C. da Silva

The application of tree bark as bio-indicator for the assessment of Cr(VI) in air pollution, by K.L. Mandiwana, T. Resane, N. Panichev, P. Ngobeni

Evaluation of prairie grass species as bioindicators of halogenated aromatics in soil, by S.D. Siciliano, J.J. Germida, J.V. Headley, doi.org/10.1002/etc.5620160318

Microplastic effects on plants, by M.C. Rillig, A. Lehmann, A. Abel de Souza Machado, G. Yang

Growth responses of African savanna trees implicate atmospheric [CO2] as a driver of past and current changes in savanna tree cover, by B.S. Kgope, W.J. Bond, G.F. Midgley

Comparison of grass and soil conditions around water points in different land use systems in semi-arid South African rangelands and implications for management and current rangeland paradigms, by S. Siyabulela, S. Tefera, I. Wakindiki, M. Keletso

Seed Samples

Background

Plant seeds provide excellent and easily collected bio-indicators. They require very little effort in terms of collection, processing or long-term storage. Seeds can be accurately assigned to a specific time period, a year's growth season. Seeds contain the plant's genetic make-up and preserve each growth season's environmental profile because they store available nutrients for the seedling to grow. Seeds store three major classes of chemical compounds, including sugars (carbohydrates), fats and oils (lipids) and proteins and the quantities of these compounds may vary with the type of seed. Further, seed banks are important to preserve indigenous plant diversity and can be used to restore the indigenous vegetation composition of denuded ecosystems.

Sample collection and transport

Seed-specific equipment

- Brown paper bag
- General sampling equipment, see *General Methods and Tools* section

Collection

Seeds should be collected directly from the tree. Per plant, a minimum of three seeds should be collected, ideally five to seven. Seeds should be collected from approximately 12-15 plants per species every year. The distribution of sampled plants should represent each species' occurrence throughout different habitats in the study area.

Plants produce seeds at different times of the year, but generally during the rainy season or immediately after. The timing of seed collection should coincide with seed maturity, which also varies by plant species. Seeds should not be collected too early during their development, but ideally before they drop off the plant. Collectors should familiarise themselves with each species' seed production period, and general timing of seed maturity, in their area.

Depending on the purpose of analysis, seeds should be collected from the same plants year after year, or from different plants across subsequent years.

Sample processing and storage

Processing

Seeds can be room-dried at between 18°-23°C for about 2-3 weeks. During drying, seeds should not be exposed to direct sunlight as this may damage their genetic material. Once the seeds have dried, a fine spray coating of sanitising agent (e.g., 99% Ethanol) can be applied to prevent fungal growth/moulding (ideal, but not necessary). Seeds should generally not be dried above >25°C; drying at higher temperatures may damage seeds.

Seed field collection from top-left to bottom-right: tree with mature seeds, mature seeds close-up, field sample bag with collection details, picking seeds, adding seeds to the bag, entering collection record in EpiCollect App.

Storage

Seeds have a remarkable ability of dormancy. Woody plant and grass seeds are generally long-lived. Seeds naturally retain their characteristics for dozens of years or centuries in low-moisture environments. For long-term storage, controlling and minimising moisture and humidity in the storage environment is far more important than controlling ambient temperature. Most seeds can be stored at room temperature, or slightly colder. Improved seed longevity is achieved at a storage temperature of 5°C. As a general rule, each one percent decrease in moisture content of a seed nearly doubles the safe storage period. As such, it is important to dry seeds prior to storage and to keep them dry. Reducing moisture will prevent fungal growth and moulding as well as unwanted seed germination.

Storage considerations

Moisture: Ensure to remove all moisture. Use a paper bag as they continue to absorb moisture and enable air flow, thus preventing moulding. For further drying, silica beads or salt bags can be added to the storage bag as a moisture-absorbing agent.

Light: Seeds should be stored out of direct sunlight and in a dark environment, e.g., a brown paper bag.

Temperature: Low temperatures are desirable for maintaining seed viability. Each 12°C decrease in temperature will roughly double the safe storage period. Remember that if both temperature and moisture cannot be controlled, then moisture is the most critical factor to be controlled.

Optional storage processes: Some projects find it useful to vacuum pack seeds, especially vegetable seeds, as they remain viable for several years. However, seeds need to be dryer when vacuum packed; generally, the moisture content of vacuum-sealed seeds should be two to four percent lower than that required for conventional storage.

*Room-dried seeds from top to bottom, Velvet corkwood (*Commiphora mollis*) seeds and Mopane (*Colophospermum mopane*) seeds*

For simple storage, paper bags with dry seeds inside can be stored in a normal plastic container in a dry, dark corner of a room. The bags should be placed on top of a bed of silica beads to keep the seeds dry.

Common analyses and purposes

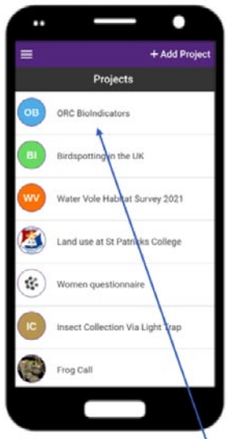
- Toxicity and disease screening
- Genetic variation and plant variety protection
- Germination success and viability (seed vigour)
- Chlorophyll content as a seed quality indicator
- Nutrient availability

Useful Links

Seed Pre-Treatment Guide, Food and Agriculture Organizations of the United Nations, web resource

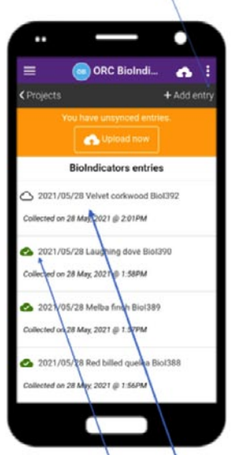
Left to right: Process of labelling seeds, processed seed sample ready for storage and an example of an annual collection volume.

APPENDIX I – Using Epicollect for Bio-indicator Field Data Recording

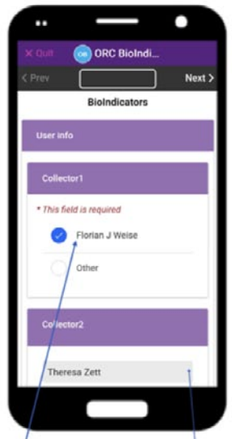


Select the Bio-Indicators project from your EpiCollect list.

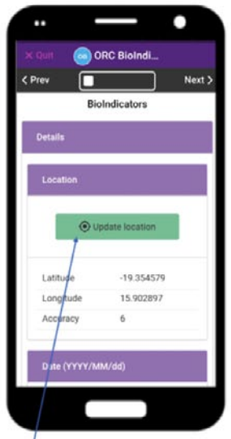
To record another sample, simply press **+ Add entry** and your data collection form opens.



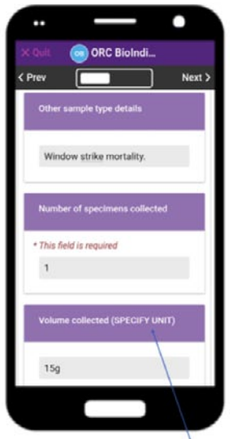
Your data entries are listed chronologically. Green clouds show data entries already uploaded to the server. White clouds indicate entries that still need to be uploaded.



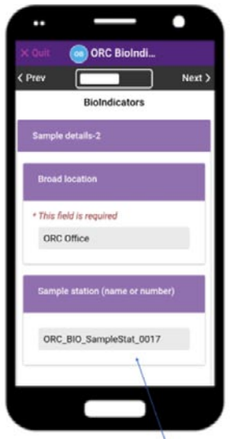
Collectors can be listed in a multiple-choice selection, or names be entered manually.



EpiCollect can record the sample location by using the phone's in-built GPS chip. Note that this also works in off-line mode.



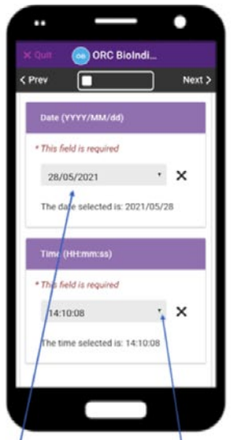
Sample information can be text or numerical, depending on which data are required. Specific instructions are useful for clarity.



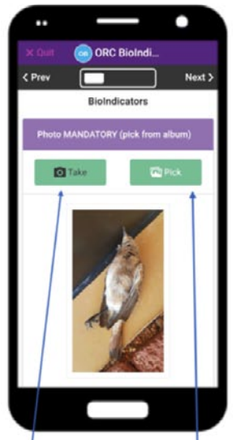
It is helpful to record whether samples are collected opportunistically or at fixed sampling stations.

ORC uses a double-verified field sample ID system. The assigned field ID must be unique and entered twice correctly to avoid confusion with identifying samples later on.

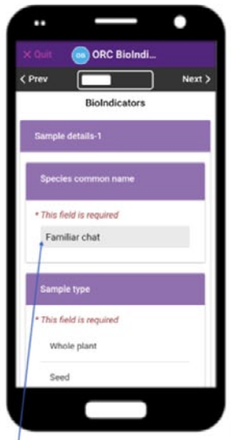
Additional, supporting information can always be added at the end of the data collection form. The data can be very useful for analysts and results interpretation later on.



You can set EpiCollect to record date and time information automatically, like here. You can still manually change this information if necessary.

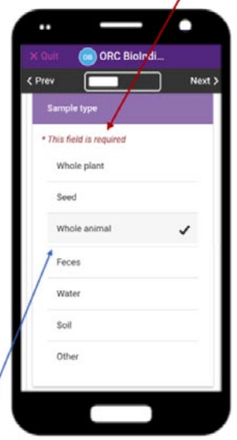


You can either record your sample photo during data recording, or select a pre-recorded photo from the phone's image folders. Photos are important to verify detail or species identification.

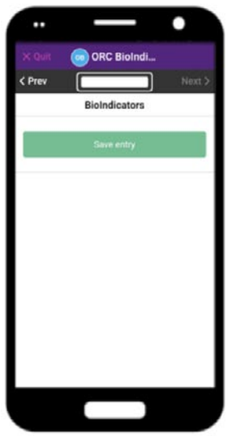


Important sample information are entered either manually, or using pre-defined multiple choice lists.

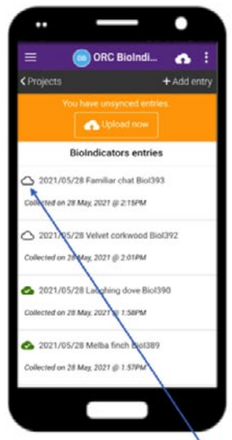
Required data fields have to be filled with information – otherwise you cannot complete and save the data record.



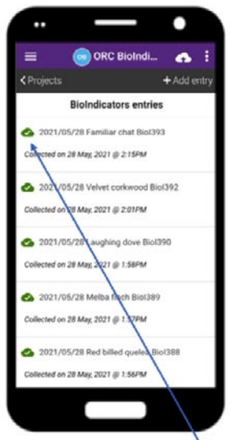
The project administrator decides which data fields are compulsory. This assures all important data are recorded in the field.



Finally, save your data entry. You can always change the recorded information at a later stage.



Your sample entry will appear at the top of the list and still needs to be uploaded to the online server and database. You can add more sample entries in the meantime.



When in internet network range, simply upload data and photos and the entry's cloud icon will change to added sample.

APPENDIX II – Batch export and renaming of images from Epicollect projects with R

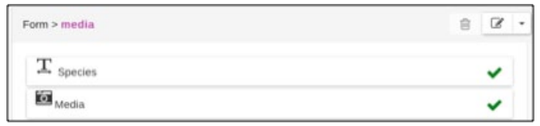
Background

One potential challenge with using data recording apps like Epicollect can be that the associated photo records are sometimes stored in difficult-to-find folders on the phone and Epicollect attaches long, cryptic file names (see *Epicollect 5 – Field Data Recording App* chapter). This makes image extraction from the recording device and archiving sample photos rather tedious, especially if this is done manually. However, R users can automate this process. The following steps explain how to batch-export images with R and rename them, if necessary. Additional information can be found at https://developers.epicollect.net.

Process

The project used for this working example is called *"Export media"* and contains only a single, simple data form named *"media"* with no sub-branches. The purpose of this process is to export photos from Export Media. The R code that enables users to do this will need to be modified for the specific project that photos need to be extracted from, or if users wish to export other type of media.

The form *media* has only two questions: Species (text entry for species name) and Media (the associated picture).



A note of caution

When taking photos using the Epicollect app, they are automatically resized to a resolution of 1024 x 768 pixels (landscape or portrait, with an aspect ratio of 4:3). If data collectors wish to save high resolution images, they need to take sample photos with the phone's camera first and then import the image into the Epicollect record form using the "Pick" function. Epicollect also resizes imported photos but maintains the original aspect ratio.

Prepare Your Epicollect Data Form

The following assumes that your project is Private. If it is Public, extraction is easier since you do not need to specify *Client ID* and *Client Secret* as explained below.

Create an App

In Epicollect's project details page, go to the *Apps* page (located in the bottom left ribbon) and select *CREATE NEW APP*. Choose a name and click create. In our example the app is called *Media*.

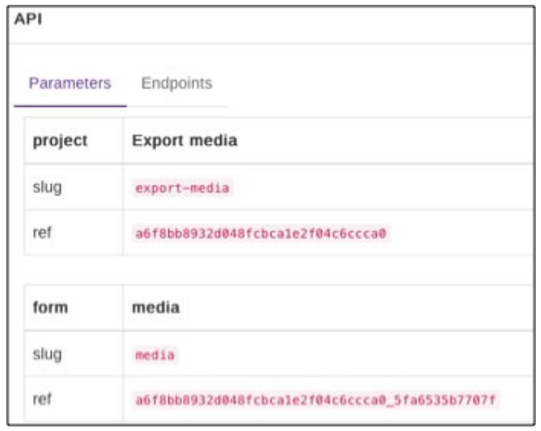
Make a record of the *Client ID* and *Client Secret*. You will need these for the R script.

Obtain API Information

In Epicollect's project details page, go to the API page (located in the bottom left ribbon).

Make a record of the project's *slug ref* and form *slug ref* details. You will need these later for the R script.

These two steps can be undertaken after the project is created and data have already been collected.



	A	B	C	D	E	F	G
1	ec5_uuid	created_at	uploaded_at	created_by	title	1_Species	2_Media
2	435a7aa2-dca2-4f76-a91e-4dc8cabe5d19	2020-11-07T08:18:05.185Z	2020-11-07T08:18:12.000Z	User1	435a7aa2-dca2-4f76-a91e-4dc8cabe5d19	Leopard	435a7aa2-dca2-4f76-a91e-4dc8cabe5d19_1604737082.jpg
3	4844990a-4473-4504-825d-9026375e1a74	2020-11-07T08:17:49.719Z	2020-11-07T08:18:09.000Z	User1	4844990a-4473-4504-825d-9026375e1a74	Lion	4844990a-4473-4504-825d-9026375e1a74_1604737067.jpg
4							

Export Your Data in .csv (Excel) Format

Access the project data online (Epicollect website) and exporting them as a csv file to your computer. Above is what the csv file looks like for the example project.

Retrieve Your Associated Media Using R

The below R script allows users to access the images from the Epicollect website, rename them based on their field ID (from the exported csv) and save the files onto their machine (e.g., storing them into a bio-indicator sample database).

In the below code script, the following variables need to be modified to fit your data:

- *cID:* found on the Epicollect project page
- *secret:* found on the Epicollect project page
- *proj.slug:* found on the Epicollect project page
- *form.ref:* found on the Epicollect project page
- *setwd("/Users/ORC/Desktop/"):* where your csv data file is located on your computer.
- *form-1__media.csv:* the name of the csv file containing the data.
- *data$imagename <-…:* how you choose to name your images.
- *setwd("/Users/ORC/Desktop/test"):* where to save the images on your machine.

```
install.packages("httr") # install the httr package
library(httr) #load the package
cID <-"  2062"      # client ID
secret <- "DrWm8WCGjNpiXZ13YnPYbqlWUwydCGnJ52WOfCSq" # client secret
proj.slug <- "export-media" # project slug
form.ref<- "a6f8bb8932d048fcbca1e2f04c6ccca0_5fa6535b7707f" # form reference
res <- POST("https://five.epicollect.net/api/oauth/token",
            body = list(grant_type = "client_credentials",
                        client_id = cID,
                        client_secret = secret))
http_status(res) # testing access to the site, the last line should be "Success: (200) OK"
token <- content(res)$access_token

setwd("/Users/ORC/Desktop/") # location of the csv file containing the data
data <- read.csv("form-1__media.csv",sep=",") # load the csv file
data$imagename <- paste(data$X1_Species,"-",seq(1:nrow(data)),".jpg",sep="") # create names for the
image files using data contained in the csv file, here species and number sequence

for (i in 1:nrow(data)) { # loop on the number of rows in the dataset
  Photo <- data$X2_Media(i) # Actual file name from row i in the csv
  writeFile <- data$imagename(i) # name of the file to be saved
  imageURL <- paste0("https://five.epicollect.net/api/export/media/", proj.slug, "?type=photo&format=entry_
original&name=", Photo)
  setwd("/Users/ORC/Desktop/test") # location of the csv file containing the data
  httr::GET(imageURL, add_headers("Authorization" = paste("Bearer", token)), write_disk(path = writeFile,
overwrite = TRUE)) # write the image as a file, overwrite = TRUE means that if the file already exists, it will be
overwritten without warning
  }
```

Useful Links

Batch export and rename media from Epicollect forms, R script, online open-access resource.

APPENDIX III - Sample Database

Similar to museum collections or any other structured sampling effort, bio-indicators should be accompanied by a proper information database. While professional information archiving systems are now universally available, for instance the *Specify 7* platform, a simple and easy-to-handle electronic database (for example in Excel format) that contains all sample details is sufficient for most bio-indicator projects. It is important that the database is updated regularly to keep track of valuable information associated with each sample. It is also crucial that the database is regularly backed up to prevent the loss of information, e.g., as may arise from computer failure or file corruption.

The database should contain all sample details. Databases may have different formats and styles between projects, but they should be de developed so that external collaborators (e.g., analysists) can easily understand the information contained.

*Table 4: The 32 sample characteristics and attributes that are recorded in Ongava's bio-indicator collection database. The Example column shows the complete entry for seven seeds that were collected from a Velvet corkwood tree (*Commiphora mollis*) on the 25th of February 2021.*

Database Info Field	Explanation	Example
Field Sample ID	The temporary ID recorded during field collection and entered into Epicollect – this should be different from any other samples collected but may still differ in style or format from the final bio-repository ID.	Biol279
Bio-Repository ID	The final, unique ID code assigned to each sample before storage. The code should contain some reference to the collecting organisation and unique sample number. This ID code is listed on the sample label and the storage container. It is used for any formal correspondence about samples. Ongava's bio-indicator coding contains four pieces of information, as below: ORC = collecting organisation: Ongava Research Centre BIO = indicates the sample repository: Bio-indicators 00279 = unique sample number 2021 = year when the sample was collected	ORC_BIO_00279_2021
Electronic Record	The name of the digital data recording app if field data were recorded using such an app: e.g., Epicollect.	Epicollect
Photo Reference	Photo file name or digital app name depending on how the sample photo was recorded.	Epicollect
Research/Collecting Permit	Research or sample collecting permit number(s), if necessary. Any legal authorisation information relevant to the sample.	Permit: AN20190911 Certificate: RCIV00012019
Collector	Name of the person(s) who collected the sample in the field.	Florian J Weise
Species_Code	A random 4-digit code assigned to each indicator species/material collected. The random code does not change even if Latin or vernacular species names might change due to taxonomic re-classification. The list of all permanent unique species/material codes is stored in a separate database sheet.	D5F6
Species_Latin	Linnaean name – can be multiple if species was re-classified taxonomically.	*Commiphora mollis*
Species_Common	Vernacular/common species name – can be multiple names.	Velvet corkwood
Number of Specimens	The number of individual specimens comprising this sample. Here 7 seeds were collected from the same tree.	7
Volume	An indication of the weight/volume of the entire sample. This might be important later on if samples need to be shipped to external laboratories etc. This also helps with estimating storage requirements (e.g., freezer space) over multiple years of collection.	5 grams
Year_Collected	Numeric.	2021
Month_Collected	Numeric.	02
Day_Collected	Numeric.	25
Study Area	The broader study area name. For example, a protected area or specific property.	Ongava Game Reserve
Location	A more specific description or name of the locality where the sample was collected, e.g., waterhole name or similar.	Onduri
Latitude	GPS coordinate North/South reading in decimal degrees format.	-19.39470

*Table 4 (continued): The 32 sample characteristics and attributes that are recorded in Ongava's bio-indicator collection database. The Example column shows the complete entry for seven seeds that were collected from a Velvet corkwood tree (*Commiphora mollis*) on the 25th of February 2021.*

Database Info Field	Explanation	Example
Longitude	GPS coordinate East/West reading in decimal degrees format.	15.84668
Fixed sample station ID	For some trend analyses it is imperative to collect samples from the same location or organism (e.g., tree) across multiple years. These sites should have a fixed permanent sample station ID so that other collectors can identify and find them easily. A list of all permanent sampling stations with their corresponding GPS coordinates is maintained in a separate database sheet.	ORC_BIO_SampleStat_0084
Type Code	What type of sample was collected: e.g., seed, water, soil, faeces?	Seed
Processing_Notes	Any particular notes on processing steps taken after field collection of the sample. How was the sample treated before final storage? And for how long? This is important information for analysts as the bio-chemical composition of the sample may be altered and influence the results.	Room drying for 41 days at temperature of 22°C
Preservative(s)	Any preservative/reagent/chemicals applied to the sample for cleaning or preservation. These should be listed in detail.	Ethanol 99% spray coating; Silica beads added to bag for long-term drying
Processor	Name of the person(s) who processed the sample before final storage.	Florian J Weise
Date_Processed	The date that pre-storage processing or sample preparation started: (YYYYMMDD format).	20210225
Date_Stored	The date that the sample was stored: (YYYYMMDD format).	20210516
Box_ID	The ID of the container/box where the sample is permanently stored. This aids others in finding samples later on. All permanent storage boxes must be labelled clearly.	ORC_BIO_Box_009
Location in Box	ID of the bag or container that contains the sample.	Bag: ORC_BIO_00279_2021
Storage Location	A description of the final storage location, including relevant building, room, and equipment information. This may be a freezer, shelf, cupboard, or similar location. Storage freezers, cupboards etc must be labelled clearly. This aids others in finding samples later.	ORC general laboratory: Cupboard: Bio-Indicators 002
Storage Temperature	The temperate of the final storage location given in degrees Celsius.	19°C
Storage Container	The type of container the sample is permanently stored in. This aids others in finding samples within a specific storage box later. It also aids analysts in assessing whether the sample may have been contaminated by the container during long-term storage. For example, plastic bags disintegrate over time and may thus contaminate the sample with polymers.	Paper bag (brown)
Collection_Notes	Any other important sample detail that may be relevant for future analyses. For example, the sex or age of a specimen. Cause of mortality, if known. Information about identification uncertainties. Freshness of scat samples, colour detail etc.	Ripe seeds collected from the tree. Dark red colouration. Seed pods closed.
Storage_Notes	Once per year, the collection manager should assess whether samples are still stored at their indicated locations and whether samples are in good order (no moulding or other contamination). Dates are given in YYYYMMDD format.	Location verified during sample inventory on 20210602.

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