Practical article

A practical guide for restoration ecologists to manage microbial contamination risks before laboratory processes during microbiota restoration studies

Running head: Guide to minimize microbial contamination

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ABSTRACT

Environmental microbiota are becoming more conventional components of restoration ecology studies due to their functional importance in ecosystems. Studying these microbiota offers insight into how they respond to, and potentially drive, ecosystem restoration. However, *microbes are everywhere* and therefore they pose a risk to sample integrity via uncontrolled contamination, and many of these risks are introduced before entering a molecular facility. Field ecologists who have limited experience in microbial and/or molecular studies may lack the knowledge on how to mitigate microbial contamination risks and, accordingly, may find rigorous collection of microbial samples a daunting task. Here, we present a practical guide that builds on our previous paper to help manage the risks of microbial contamination when undertaking a microbiota restoration study prior to entering a molecular facility. We cover study design and planning, undertaking field sampling, and sample transport and storage. We hope to provide a useful and practical guide to restoration ecologists who wish to include a microbiota component in their studies. If done well, this inclusion offers improved research quality and ultimately enhanced restoration outcomes.

Keywords: DNA; environmental DNA; microbial contamination; microbiome; restoration genomics

Implications for practice

- Restoration ecology has seen a rapid increase in studies that incorporate microbiota, and the outcomes of these studies could be improved by better research practices that minimize the risks of microbial contamination.
- Before entering a molecular facility, restoration ecologists need to be aware of steps to minimize contamination risks during study design, planning of field sampling, field sampling, and transport and storage of samples.
- Key steps include identifying and seeking training for the required facilities, developing a simple and robust labelling system, having a rigorous approach to equipment and consumable sterilization, and ensuring storage facilities match the requirements of the samples collected.

INTRODUCTION

Environmental microbiota – the community of microbes in a defined environment – co-develop with vegetation communities and often represent a target for restoration interventions (Harris 2009), as they support many ecosystem functions and services such as nutrient cycling (Ciais et al. 2014), symbioses (Livne-Luzon et al. 2017), and human health (Liddicoat et al. 2019). Accordingly, restoration ecology has seen rapid growth of studies that incorporate them (Chua et al. 2019; Birnbaum & Egidi 2020; Farrell et al. 2020). In a restoration context, microbiota are ubiquitous and can be sampled from soils (Liddicoat et al. 2022; Birnbaum & Egidi 2020), air and dust (Mhuireach et al. 2016; Liddicoat et al. 2020; Robinson et al. 2020), human and animal subjects (Liddicoat et al. 2020; Selway et al. 2020), and plant material (Berendsen et al. 2012; Selway et al. 2020). Microbiota, however, are persistent even on cleaned surfaces or 'sterile' consumables, including sample containers, field equipment and consumables (Eisenhofer et al. 2019; Weyrich et al. 2019). These persistent microbiota present contamination risks that could affect the results of a restoration study. As such, it is important for restoration ecologists to have effective and accessible recommendations on how to minimize contamination risks from study design to entering a molecular facility, which are the steps that restoration ecologists are most likely to be responsible.

Modern DNA sequencing methods allow for microbiota to be more accurately characterized than traditional approaches, which typically use field observations or microbial culturing, as most microbiota cannot be easily observed or cultured (Berg et al. 2020). There is a vast literature exploring the contamination risks inherent in these DNA-based techniques in principle and practice (Minich et al. 2019; Weyrich et al. 2019; Cando-Dumancela et al. 2021). These studies have largely focused on molecular laboratory and downstream data

analysis methods (i.e., DNA extraction, PCR and sequencing protocols, bioinformatics), rather than steps prior to entering a molecular facility, and seldom cover issues specific to restoration ecology. Restoration ecology presents novel considerations for microbial contamination, which may not be common to other scientific disciplines – for example, restoration ecology is a practice orientated discipline with a limited history of engaging with molecular biology (Breed et al. 2019; Mohr et al. 2022). While including microbiota into restoration studies should not replace traditional ecological approaches (e.g., plant-based approaches), microbiota have a clear role for inclusion but require researchers to be enabled to incorporate them in a rigorous way.

There is growing interest in including microbiota in restoration ecology studies, but this presents the need for researchers to have the knowledge to minimize the risks of microbial contamination during their studies. We have previously provided an overview of these steps, with a focus on protocols from entering a molecular facility to downstream steps (e.g., data analysis; Cando-Dumancela et al. 2021). Here, we build on our previous work and focus on steps prior to entering a molecular facility. If these steps are not adequately managed, microbial contamination can undermine study results and result in wasted time and resources. We focus on providing simple and accessible recommendations for reducing the risks of microbial contamination, considering aspects such as study design, the planning and implementation of field sampling, and the transport and storage of samples (Figure 1).

STUDY DESIGN

Literature review

To guide study design, microbiota restoration researchers must conduct a literature review to establish aims, hypotheses/research questions, and justify methods – including a targeted

search on selection of appropriate methods that minimize contamination risks. Since our understanding of environmental microbiota is rapidly developing alongside our understanding of contamination risks, it is incumbent on the researchers to ensure they are employing up to date and appropriate methods to control, minimize and account for these risks. Ideally, microbiota restoration studies should not only examine the effects of microbial contamination, but also publish their methods and findings. This will allow future researchers to better understand and minimize contamination risks. Such transparent reporting of contamination effects should help improve research practices, help create a culture of improved transparency within the discipline, and generate greater collaboration between restoration and microbial ecologists. Below, we also go through two additional key considerations to incorporate into study designs.

Sample biomass

When planning a microbiota restoration study, researchers need to ask a fundamental question: what are the types of microbiota samples to be collected, and is the microbial biomass of the samples low (e.g., air, swabs of plant tissues) or high (e.g., soil)? This is one of the most important questions to ask early as it will have serious impacts on the protocols employed to minimize contamination risks. Low biomass samples have a very high risk of contamination both from external sources (= external contamination) and between samples (= cross contamination) and need considerable effort to be employed to control, minimize, and account for this contamination (Minich et al. 2019; Weyrich et al. 2019; Cando-Dumancela et al. 2021). While the risks are lower for high biomass samples, effort is still required to ensure sample integrity from the outset and minimize the effect of cross and external contamination in fieldwork (discussed below).

Molecular facilities

It is fundamental at this design stage to locate and gain access to the required molecular facilities, and to receive the required training for appropriate use. Increased contamination risks are introduced when microbiota restoration studies are done in inadequate facilities or by researchers who do not have adequate training. It is essential that all microbiota laboratory work is done in a dedicated facility with a unidirectional workflow, as discussed in detail elsewhere (Drengenes et al. 2019; Minich et al. 2019; Weyrich et al. 2019). Furthermore, molecular facilities often have established protocols to minimize contamination that researchers should be familiar with and follow. Collaboration with molecular labs may also be important (discussed below). Once these steps are completed, attention can now turn to planning field sampling.

PLANNING FIELD SAMPLING

Microbial contamination risks can be minimized by making a comprehensive sampling plan prior to field sampling. At this stage, the following should be considered at a minimum: experience of researchers, sample type, labelling system, consumables and equipment, and sampling kit. We explain each of these in turn.

Experience of researchers

Restoration ecologists may not be experienced in working with microbiota. As such, it may be important to work collaboratively with experienced microbial researchers and/or receive dedicated training. Such approaches should help build the capacity of restoration ecologists in microbial ecology. Training should involve: (a) understanding the biology of microbial contamination, including both sources and pathways of external and cross-contamination; (b) practicing sampling protocols (e.g., subsampling soils into collection tubes; swabbing petri

dishes for collection of aerobiome or dust samples) and use of any specialized (e.g. soil corer, sterile swabs) or custom (e.g. hand-made aerobiome sampling stations, as per Robinson et al. 2020) equipment so that proficiency is maximized and risks of mistakes are minimized.

Sample type

When planning a microbiota restoration study, a fundamental and early factor with considerable contamination implications is sample type – as described above. However, when planning the field sampling, it is essential that there is an order to sample collection to minimize contamination risks. If a study plans to collect both high and low biomass samples (e.g., a study of how restoration may lead to changes in leaf surface and soil microbial communities), the low biomass samples (i.e., the leaf surface samples) should be collected prior to the high biomass samples (i.e., soil) if possible, to minimize the risk of contaminating the low biomass samples. If it is not possible, consider having two sampling teams: one that focusses on low biomass collections and one on high biomass collections. Alternatively, if separation in sampling time or teams is not possible, then extra care (e.g., additional sterilization of consumables and equipment; duplicate task-specific sampling kits) should be applied to minimize contamination risks, but this approach is not recommended.

Even if separation of sampling time and/or teams is possible, extensive sterilization of consumables prior to sampling is required to minimize external contamination (e.g., all collection tubes), especially for low biomass samples. This sterilization process is still advised for high biomass samples to increase sample integrity. Extensive sterilization of sampling equipment between collected samples is also required to minimize cross-contamination for both sample types. Furthermore, when dealing with low biomass samples, researchers must wear contamination-minimizing protective equipment (e.g., face masks,

disposable gloves), and this is still recommended when sampling high biomass samples. It is also essential that these researchers collect field controls that characterize the background contamination of actual samples from non-target sources (e.g., consumables, field equipment). These field controls should be collected during each sampling session to enable potential exclusion of contaminating sequences during downstream analysis (discussed below).

Labelling system

A robust and efficient labelling system is essential to minimize contamination risks as it will reduce the chances that samples are mixed up and that they are easy to track and identify downstream. All the relevant information should be included in this labelling system, such as location, date, sample type, treatment, and replicate number. A good labelling system should also be simple to further help minimize any confusion and potential mistakes when sampling. Here is an example of a system that we employ:

- 1 letter for a site abbreviation (e.g., site A = A, site B = B)
- 1 number for the sampling date (e.g., sampling day 1 = 1, sampling day 2 = 2)
- 1 letter for sampling type (soil sample = S, petri dish aerobiome sample = P)
- 1 letter for treatment group (e.g., restored treatment 1 = R, degraded treatment 1 = D)
- 1 number for the replication number (e.g., replicate 1 = 1, replicate 2 = 2)

Accordingly, A1SR1 is a sample from site A, sampling day 1, soil sample, restored treatment 1, and is replicate 1. We combine this system with a meta-data spreadsheet that links this system to any field notes made on the day and extended details of the system employed (e.g., dates of sampling, GPS coordinates of sites, name(s) of samplers, weather conditions, photos of sites and surroundings).

Another consideration on labelling is that the labelling itself – both the manual handling and the equipment used – is a potential source of contamination. As such, care is needed to ensure that the labels on consumables, such as tubes and bags, do not become undecipherable after sterilizing (e.g., with bleach). If a permanent marker is used, great care needs to be employed not to smudge or remove the written label during this process. We recommend the use of high-quality adhesive labels, as these should not only be robust to impact during sterilization processes but should also lower the risk of mistakes. A note of caution when using adhesive labels is that a test should be employed to check whether they are impacted by any field conditions (e.g., rain, sun exposure), sterilization process and storage conditions (e.g., stored at -20 or -80).

Consumables and equipment

All consumables (e.g., tubes, bags) for low biomass studies must be sterilized prior to use in the field, and this is recommended also for high biomass studies – as indicated above. While proprietary bags to achieve this goal do exist (e.g., Whirl-pak® sampling bag), we recommend using lower cost and common consumables. For example, we use consumables such as high quality zip-lock bags, tubes and petri dishes and sterilize them with UV light for 30 min in a dedicated facility within a clean environment (e.g., a biosafety cabinet), which is consistent with best practice recommendations for microbiome studies (e.g., Eisenhofer et al. 2019). Field equipment (e.g., shovel, fork) should be autoclaved prior to use in the field and thoroughly sterilized with bleach and Decon 90 (a laboratory grade surface cleaning agent and decontaminant) between, collecting samples.

Sampling kit

Sterile consumables should be organized into a sampling kit, and we recommend these consumables are grouped by number of samples per site, sample type and time of sampling or for individual sites. For example, if a field trip is planned to a single site across an entire day, consumables should be grouped into the morning session, which is then stored separately from the consumables for the afternoon session. Such separation of consumables will assist in reducing both external contamination by only having subsets of consumables exposed to external environments at any point in time, as well as cross contamination by minimizing the number of consumables that are exposed to already collected samples. Below is an example of an approach to sampling kits that we employ when collecting both dust samples from petri dishes and soil samples across numerous sites:

- 1 bag per site: labelled petri dishes for dust sample collection, parafilm to seal the petri dishes, labelled tubes and zip lock bags for soil samples. Each bag is UV-sterilized prior to entering the field.
- Bag with field control materials: petri dishes and tubes for field controls for each site. This bag is also UV-sterilized prior to entering the field.
- Bag with consumables and sterile equipment: nitrile gloves, face masks, spray bottle with 5% bleach, spray bottle with 5% Decon 90, laboratory grade paper towels, scissors, permanent markers, extra parafilm. This bag is also UV-sterilized prior to entering the field.
- Bag with field equipment: hand trowel for soil sampling, plastic container for soil homogenization, UV-sterilized bags to line plastic container for soil homogenization.

FIELD SAMPLING

Appropriate and simple sampling protocols will help minimize both external and cross contamination risks. If the sampling process is overly complex (e.g., many steps), poorly

planned (e.g., no pre-labelling) or takes a long time per sample, then both *external* and *cross contamination* risks are increased. An appropriate and efficient protocol should be developed that avoids unnecessary steps in the field and minimizes the time taken to collect individual samples. As indicated above, low biomass samples should be collected prior to and/or by a separate team from high biomass samples.

Sampling station

We advise that a sampling station is established at each field site, near the sample locations. This station should allow easy access to all the necessary consumables and equipment (e.g., as described in the Sampling kit section above). At the station, equipment should be sterilized, and field control samples collected (see below).

Contamination-minimizing protective equipment

Equipment used should minimize contamination from the researcher as well as from the environment and samples, particularly for low biomass samples. Researchers should wear face masks and use disposable gloves when collecting samples, double gloves are recommended to speed up replacement of gloves, particularly when hands are perspiring and protect the sample from external contaminants. Gloves should be replaced between each sample to minimize cross-contamination risks and wearing two pairs of gloves will help minimize surface contamination when donning and doffing the outer pair. Face masks should be replaced between each sampling time period.

Sterilization

All equipment that contacts samples must be sterilized at the sampling station between collections (e.g., shovels, plastic containers to combine soil samples). This equipment should

be sterilized with bleach and/or Decon 90 by, for example, spraying these sterilizing agents onto the equipment and wiping it off with lab-grade paper towel as many times as required to clean the equipment, as residual bleach and Decon 90 may affect microbiota collected.

Here is an example of a system that we employ for sterilizing equipment when sampling soil with a hand trowel:

- After sampling with a hand trowel at one site, remove all noticeable soil and other material from the trowel
- Comprehensively spray the trowel with Decon 90, and then dry with laboratory grade paper towels
- Comprehensively spray the trowel with 5% bleach, and then dry with laboratory grade paper towels. The trowel is now ready to collect the next sample.

Field controls

Field controls should be collected to enable researchers to quantify, and potentially bioinformatically account for, background and uncontrollable microbiota (e.g., from sampling consumables and equipment). Such field controls are essential when collecting low biomass samples (e.g., air or dust samples) and are recommended for high biomass samples (e.g., soil). For example, field controls could be: (a) sample tubes and swabs that are used for dust microbiota collection, however instead of these tubes and swabs used to sample the dust from a target surface (e.g., vegetation, petri dishes), they are left open for approximately the same time taken to do the real sampling; (b) sample tubes that are used for rhizosphere microbiota collection, however instead of these tubes used to sample the rhizospheres from a target plant, they too are left open for approximately the same time taken to do the real sampling collect. These controls should be taken at each sampling site on each day since this

external contamination can be site and/or sampling time specific. It is important to account for microbial contaminants and to be able to separate the target of the sampling (e.g., soils, dust) from background and uncontrollable microbiota.

Here is an example of a system that we employ to collect field controls when collecting dust samples:

- Open the field control bag (see above) and remove the petri dish and place on the sampling station.
- Leave petri dish open for 5 minutes (= approx. time required for processing actual samples)
- Close and then seal petri dish with parafilm and store as per actual samples (e.g., freeze at -4˚C on site).

Waste management

All disposable waste material (e.g., gloves, paper towel) should be stored in a dedicated bag or container and kept separate from samples and not interacted with by researchers immediately prior to sampling, as this introduces contamination risks.

TRANSPORT AND STORAGE

Adequate transport and storage protocols will help minimize external contamination risks in addition to helping to maintain sample integrity and minimize cross contamination.

Storage in the field

After collection, samples should be stored in suitable containers that maintain the integrity of the samples. It is necessary for samples to be grouped in such a way to make downstream

processing (e.g., DNA extraction) easy and quick, as this will help minimize unnecessary handing of samples. For example, sample type (e.g., soil samples from site 1) should be carefully arranged in a storage container at the sampling station in the field and maintained as such during transport to a storage facility. Also, samples may be collected directly into tubes for freezer storage, and these tubes can be stored in dedicated boxes at this stage in the field. However, these boxes should be clearly labelled with sufficient detail to make them easy to identify (e.g., project code, sampling sites, date, and contact details). We note that groups of samples from a single study (e.g., soil samples) should be randomized within groups prior to DNA extraction to help minimize batch effects, as has been emphasized elsewhere (Eisenhofer et al. 2019; Cando-Dumancela et al. 2021).

Transport

It is essential that samples are maintained in intact containers/bags during transport. Researchers should be aware of the potential for rupturing of sample containers if transported over rough terrain or excessive numbers of samples are attempted to be placed in a container that is too small. While it is not a contamination risk *per se*, researchers should be aware that microbiota within samples can change during transport. For example, if samples are collected and then stored frozen in the field, but thaw during transport, there may be unexpected changes to the microbiota due to this change in temperature. For all samples, we recommend using a double bag system where the external bag is used only directly prior to samples being put into short-term (e.g., within the molecular lab) or long-term storage.

Long-term storage

Samples for long-term storage should be stored at -18°C or below (Delavaux et al. 2020) in a dedicated long-term storage facility (e.g., sample biobank, dedicated cold room/freezer).

Such facilities should have storage protocols that limit access and maintain a chain of custody of samples, which will not only help locate samples but also minimize sample handling, which subsequently reduces both external and cross contamination, and shifts in microbiota due to thermal fluctuations.

CONCLUSIONS

Restoration ecologists that undertake microbiota restoration studies should invest considerable effort in planning their studies to minimize the potential impact of microbial contamination. Many of these researchers may not have molecular and microbiology training and experience, but this does not absolve these researchers from the need to actively seek ways to mitigate microbial contamination risks either through collaboration or via training. While there are several studies that have focused on ways to minimize microbial contamination risks in molecular facilities and steps downstream from this, there is presently little guidance for restoration ecologists during the important steps prior to entering a molecular facility. Here we provide a simple guide that builds on our previous paper that focused on steps for restoration ecologists from a molecular facility and downstream and focus on the practical steps prior to entering a molecular facility. These steps include study design, planning of field sampling, field sampling, and transport and storage of samples stages. We outline steps at each of these stages to help minimize contamination risks. We hope that this guide will help continue to improve both the quality of microbiota restoration studies as well as subsequent restoration outcomes.

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Conflict of Interest

The authors declare no conflict of interest.

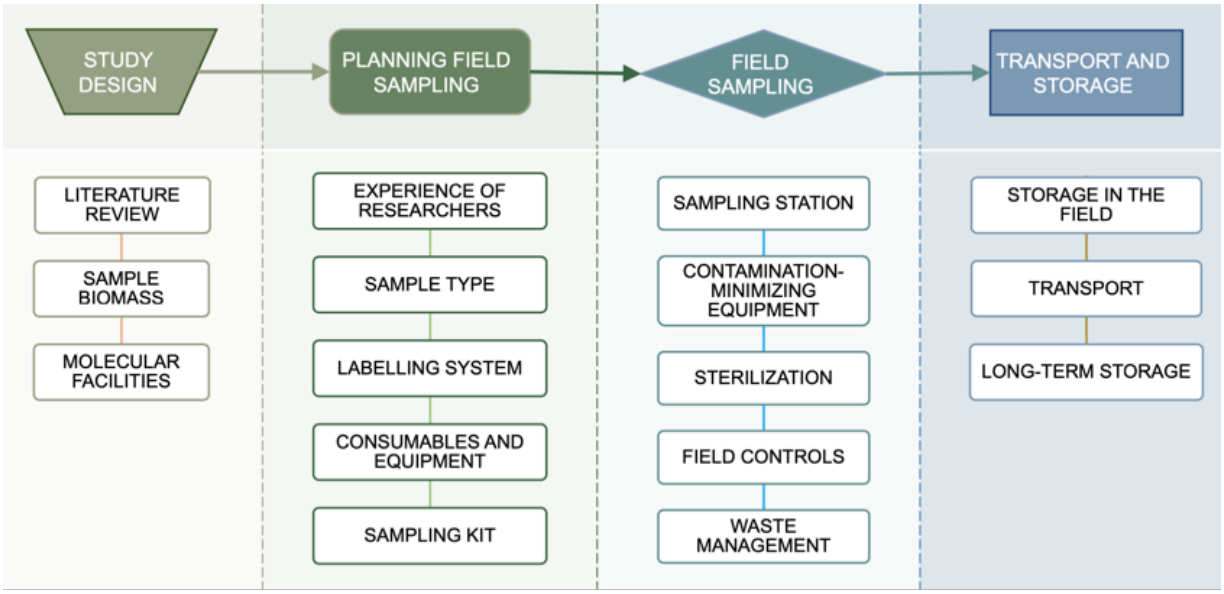

Figure 1. Flow diagram of when actions can be taken to reduce microbial contamination risks in microbiota restoration studies before entering a molecular biology facility. These actions progress from the initial study design, through the planning and implementation of field sampling, to the transport and storage of samples.

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