Chytrid protocol – Instructions and aims

Background

This is a brief instruction for sampling and for completing the form "Protocol-chytrid sampling". The sampling protocol is designed to include relevant information about sampling site (e.g breeding pond) and/or amphibians sampled in the terrestrial environment. It should also be stated in the protocol if water samples are taken for eDNA-analysis and/or swabbing of animals. Moreover, it is appreciated if sites and sampling is documented by photographs and short films (a smart phone is good enough).

Swabbing

The main goal is to find out if *Batrachochytrium dendrobatidis* (Bd) is present or not in a given population of amphibians, within a limited budget. Based on previous studies it is recommended to sample at least 20-30 individuals from a metapopulation (swabbing) in order to be able to detect Bd in a population if it is present. There are differences in prevalence between years and between season within the same population. Furthermore, swabbing may underestimate prevalence. From a scientific point of view, it is best to analyse each sample separately, but the costs will be higher so therefore it may be better to pool samples from e.g. three individuals of the same species from a site for analyses. But this pooling should be done in the lab, not in the field. All samples taken should be individually and stored in separate tubes!

It is advisable to sample species during breeding period (or froglets/toadlets when leaving the ponds in late summer). When selecting species for sampling it is also apt to sample species that are relatively easy to capture/handle and species previously known to be infected at a comparatively high rate.

Suitable species to swab is green frogs, fire-bellied toads and other toads. Newts may also be suitable and should preferably be sampled late during their breeding season (May/June).

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Informative instructions (and film clip) for swabbing can be found at AmphibiaWeb (https://amphibiaweb.org/chytrid/swab_protocol.html) and can be summarized as follows:

- Preferably, capture amphibians by hand. Wear gloves when swabbing animals and change gloves between animals. If you are using a dip net, be aware that Bd zoospores could be caught on the net and transferred between individuals, therefore, use different nets whenever possible, or disinfect (Virkon® or ethanol) the net as often as you can.
- To avoid problems during analyses, the amphibian should be comparatively clean before swabbing. Use tap-water if necessary.
- Swab the underside or ventrum of adult/metamorphs up to 30 times, depending on size of the animal. Remember you are actually scraping small amounts of tissue from the skin. Some pressure must be applied, but this does not mean that you must squash the animal. to swab from are the drink patch, thighs and webbing between the toes.
- Air dry the swab for approximately 5 minutes, avoid direct sunlight if possible (if conditions are too humid to air dry then store in 95% EtOH).
- Break swab ~3cm from tip and drop into empty screw cap tube. The swab stick should not touch or bump against the top of the vial. Screw the cap on the vial and store in the shade.
- Samples can be kept at room temperature for a week or maybe longer, but it is best to keep the samples cool and placed as soon as possible, in a - 4° C freezer (the kind you have at home is fine). Avoid extreme high temperature and direct sunlight. Samples may be stored in a freezer for many months without problems.
- Depending on laboratory routines, storage liquid of swabs (and filters) may be recommended due to the DNA extraction method used. This must be clarified before sampling occurs and added to the protocol.

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Detection of Bd by analysis of eDNA from water samples

Another goal is to use different analytical methods for cost-efficient sampling of Bd. By taking water samples these can be analysed for Bd (and amphibian species if required).

Example of methods can be found in: Kamoroff, C. and Goldberg, C.S. 2017. Using environmental DNA för early detection of amphibian chytrid fungus Batrachochytrium dendrobatidis prior to a ranid die -off. Diseases of Aquatic Organisms, 127: 75-79.

In the above given reference, it is recommended to collect a 50 ml water sample every 40 m along the shoreline. Every 5 water samples should then be combined into at least 250 ml composite sample. Several scientists also filter water on site, which requires equipment and filters. Recommended filter sizes must be decided in collaboration with the laboratory performing the analyses.

Information in Swedish can also be found at: http://www.nrm.se/forskningochsamlingar/centrumforgenetiskidentifiering/miljoovervakarensdnaskola.9004349.html

In summary, the recommendation for taking water samples for analyses of eDNA is:

- to only sample smaller ponds (maximum 2 500 m2)
- take water samples during breeding season
- based on experiences in Sweden and Norway we would recommend taking 300-500 ml water samples for waterbodies 100-2 500 m2, but 100 ml should be enough if the water body is smaller (for example rock pools)
- pool five water samples taken along the shoreline to get the wanted water volume. Avoid sediment and organic matter
- do not fill the bottles completely since water expand in the freezer
- keep water samples cool and dark until frozen
- avoid repeated freezing and thawing of water samples because DNA quality may be affected
- if filtering water in the field contact lab for instructions, and add information to the protocol on sampled volume, type of filter being used, and if relevant also solution being used for preservation of filters

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Other information

In order to get an indication of how useful analysis of eDNA from water samples is in comparison to traditional swabbing of animals, it is suggested to take water samples from at least two sites (in each country), where also amphibians (at least 20-30 individuals) have been swabbed at the same period of time. The budget limits the number of sites.

In the protocol for sampling, specific information about the aquatic habitat and other information is needed. This information will be used to assess parameters that could potentially interfere with analysis of eDNA and the infection rates of amphibians. The combination of measurements of pH, water colour and conductivity give a good picture of the type of water that has been sampled.

For example, conductivity (using a meter, make sure to specify the unit, mS/m is recommended) gives good information about type of water that is being sampled. Conductivity is high (> 500 mS/m) when the water is influenced by sea water. In freshwater, it gives a good relationship with the calcium concentration ($y=1.3117x + 4.0659$, $r2 = 0.80$, $n>100$) and thus potentially the trophic status of the pond. Conductivity of < 5 mS/m corresponds to low concentration of Ca (< 5 mg/l), and values above 50 mS/m to more calcium rich habitats (> 50 mg/l).

This material has been developed within the framework of the project *the Nordic Network for Actions Against Chytridiomycosis* with grants from *the Nordic Council of Ministers*.

Protocol - chytrid sampling

General information

Investigator:

Sampling date:

Coordinate system:

X/Y:

County/Municipality:

Site name:

Habitat type (water/terrestrial):

Air temperature:

Sample type (swab/water sample*):

*Water volume sampled (ml):

Sample id:

Aquatic habitat

Water temperature:

Conductivity (mS/m):

pH:

Wetland area (m2):

Water colour (clear, slightly brown, brown):

General information

Affected by salinity (Y/N):

Temporary/permanent:

Percentage of shaded water surface (%):

Percentage of surface covered by plants (%):

Connected to stream/ditch:

Surrounding land-use:

Amphibian species observed:

Previous records of amphibian species:

Other information:

Clear Slightly coloured Brown

Sample-ID	Species	Life stage	Dead/Alive	Other comments