DANUBEPARKS

Field Protocol for eDNA sampling of Danube Crested Newt (*Triturus dobrogicus*)

Danube Crested Newt (*Triturus dobrogicus*)

Geographic range

Danube Crested Newt is found in lowland regions of the Danube River and its tributaries up to about 300 m upper elevation. First part of the range is in the Pannonian Plain, from eastern Czechia and Austria to western Ukraine and Romania, reaching the Đerdap in Serbia. The second lies downstream of the Đerdap, covering northern Serbia, Bulgaria, Romania, and parts of Ukraine and Moldova near the Danube Delta. The species' range is bordered by hilly areas with other crested newt species, with hybridization zones in areas where they overlap.

Habitats

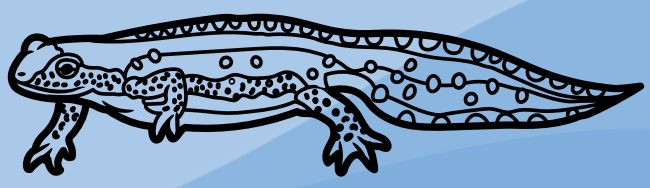
Danube Crested Newt inhabits lowland areas open habitats with mixed deciduous forests and groves, bushlands, flooded meadows and swamps. Also it can be found in agricultural landscapes (irrigation channels) villages and agricultural areas. For reproduction, the Danube Crestet Newt use small, still-water bodies such as ponds, oxbow lakes, channels, ditches, and flooded quarries that can be inhabitant with fishes.

Population Phenology

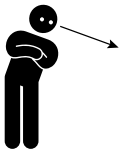
In different parts of the range, migrations from terrestrial habitats to breeding sites start from mid-February through April. In the water habitats they are usually hidden in the vegetation at the bottom, and they are more active during the night. Females lay about 100 - 200 eggs during 2 - 3 weeks. Embryonic and larval development take about 2-2.5 months. Metamorphosis has been recorded in July - early September and later. Adult and subadult individuals are known to stay in water for a long time, up to and beyond 6 months.

Population trends

Current population trend is decreasing and is listed as Least Concern species by the The IUCN Red List of Threatened Species. Subpopulations are rapidly declining as a result of habitat loss from associated wetlands in the floodplains of the Danube River and its main tributaries. Over the last 10 years, a rough estimate of population decline is 10–20%



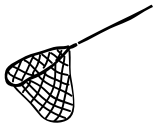
Survey methods for Danube crested newts



Daytime Visual Searching involves walking along the water's edge, stopping periodically to observe for newts at different life stages.

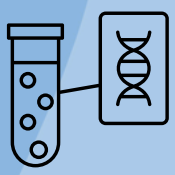
Egg Searching involves looking for newt eggs on leaves in the water. The surveyor carefully unfolds leaves to find eggs, which remain exposed once unfolded. This method is effective from April to June, the newt egg-laying period.

Torching involves slowly scanning the water's edge with a flashlight at dusk, avoiding rainy or cold conditions. This method is best from mid-March to mid-June.



Netting can be conducted from March to June, involving moving a net in a figure-eight motion for 15 minutes per 50 meters. Netting can disturb the habitat and spread invasive species.

Bottle Trapping is a highly invasive method, which involves using a plastic bottle partly submerged and set in the evening. This technique requires specific training due to risks to newts and small aquatic animals.



eDNA Survey Method: Environmental DNA (eDNA) refers to the genetic material that organisms leave behind in their surroundings. Newts constantly release DNA into their surroundings through urine, feces, cells, gametes, shed skin etc. In aquatic environments, this DNA can remain detectable for up to three weeks. By extracting DNA from water samples, researchers can analyze it to confirm the presence or likely absence of Danube Crested Newts in the habitats. The ability to detect species-specific DNA sequences in environmental samples through methods like PCR and DNA sequencing technologies has transformed species monitoring. eDNA analysis of water samples offers a faster, more affordable, and more reliable way to detect the presence or absence of smooth newts compared to traditional survey methods. This non-invasive approach eliminates risks of harm, stress, predation, and accidental death often associated with invasive techniques.

Field Protocol for Danube crested newts eDNA sampling

Field sampling should be conducted by a trained volunteer or professional. A single visit to the target site for sampling Danube crested newts eDNA should take place between mid-April and June, aligning with the newts breeding season. Samples of water with eDNA can be collected at any time of day and under most weather conditions, including light rain. However, it's advisable to avoid heavy rain, as it can complicate sampling and increase the risk of cross-contamination, such as splashes of mud potentially carrying newt DNA from wet ground. Characteristic for chosing good qualitytarget site: slow moving or still water, rich submerged vegetation, availability of deeper parts for hiding, small abundance or no fish present.

IMORTANT NOTES

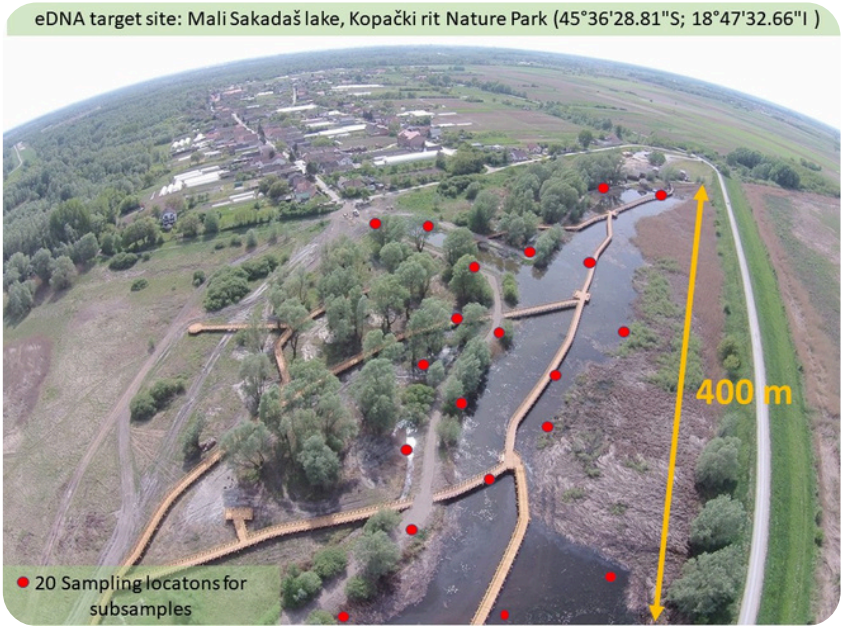
1. Collect your water subsamples before you do any other surveys.

2. Take the subsamples whilst standing on the bank of the water body, a garden cane can be used for difficult to reach water bodies (attach sampling ladle with tape or string).

3. Don't tread in the water itself either before or during collection of the DNA water sample as there is a considerable risk of contaminating your sample by bringing in DNA in mud and water from other areas on your boots and equipment.

Water sample collection

To maximizes the likelihood of detecting eDNA in the water sample from one target site collect water subsamples from different locations within target site. The aim is to spread the locations for collecting water subsamples out evenly along max 400 m stretch for larger water bodies.



Subsampling Process

At each target site, take 20 water subsamples from 20 different locations within the site. Distribute subsampling locations across both open water and vegetated areas, if both are present. Avoid water less than 10 cm deep to maximize eDNA yield.

Site Accessibility

If some areas of the water body are not accessible, position subsampling locations as evenly as possible across the accessible area without physically entering the water.

Consideration for eDNA Distribution

Due to eDNA's patchy distribution (which depends on animal movement within the water), collecting from multiple locations enhances the chance of eDNA capture.

Pooling Subsamples

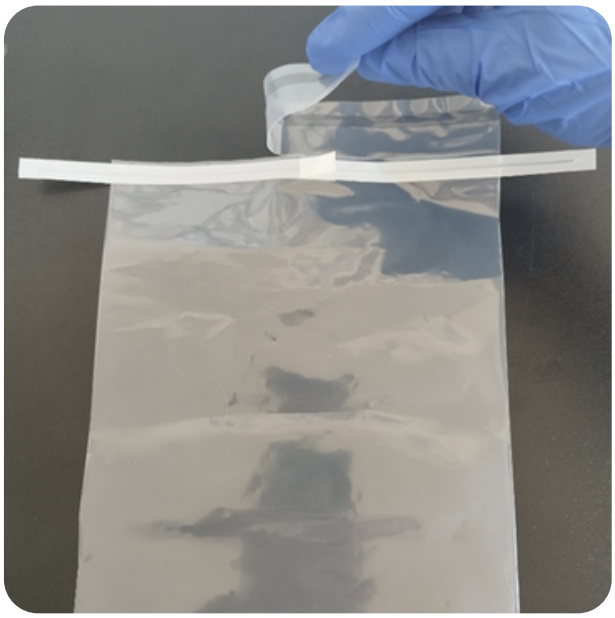
After collecting the 20 subsamples, combine them into one composite sample to represent the entire target site.

ADAS Filter Kit contents

1. sterile 30 mL sampling ladle
2. 0.45 µM filter
3. Filter sealing cap
4. sterile Whirl-Pak bag
5. 10ml syringe containing preservative
6. 50ml syringe
7. pair of sterile gloves

Sample Collection

1.Open filter kit and put on a pair of gloves



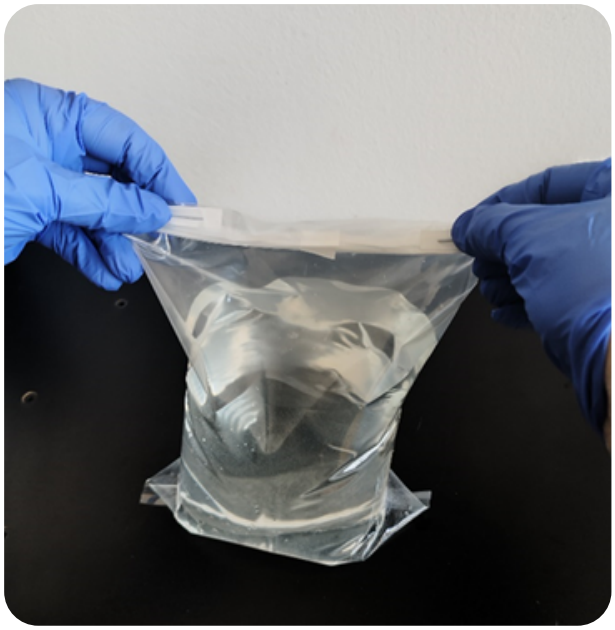
2.Open the sterile Whirl-Pak bag by tearing off the clear plastic strip along the perforated line, then pull the tabs

3.Collect 20 subsamples of 30 mL of water from using the sampling ladle and empty each subsample into the Whirl-Pak bag

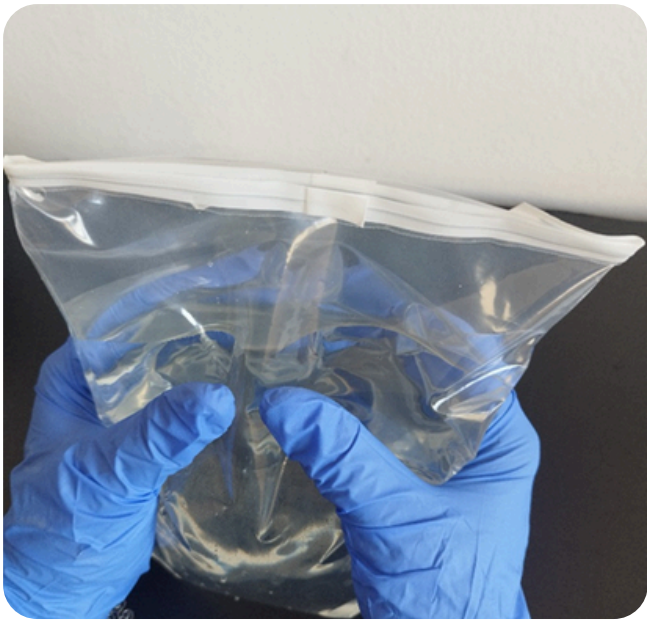
IMORTANT NOTE

Before you take each ladle subsample, be sure to mix the water column by gently using the ladle to stir the water from the surface to close to the bottom WITHOUT disturbing the mud in the bottom. DNA 'sinks' and so will often be present in larger amounts close to the bottom of water column. It is important not to collect sediment as this may cause inhibition of the PCR analysis which could lead to an inconclusive result.

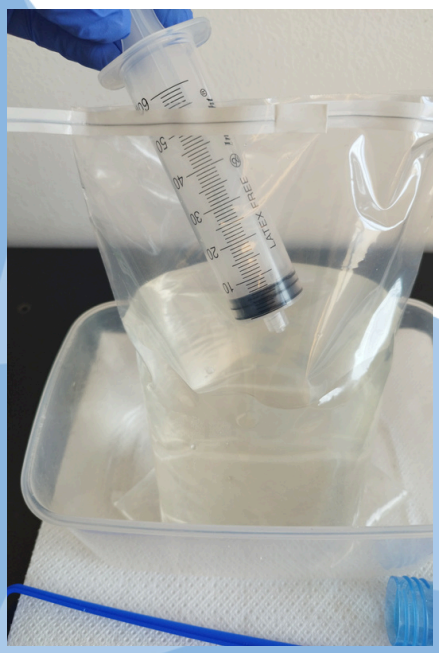
Sample preservation

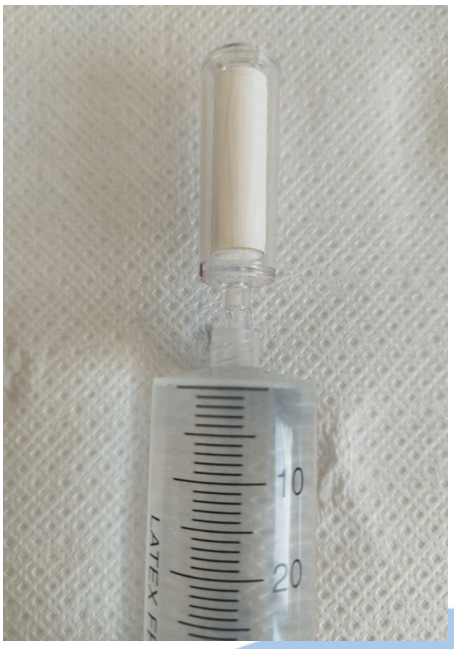


1.When you have collected 20 subsamples, close the bag securely using the top tabs (fold over several times and bend tabs over)

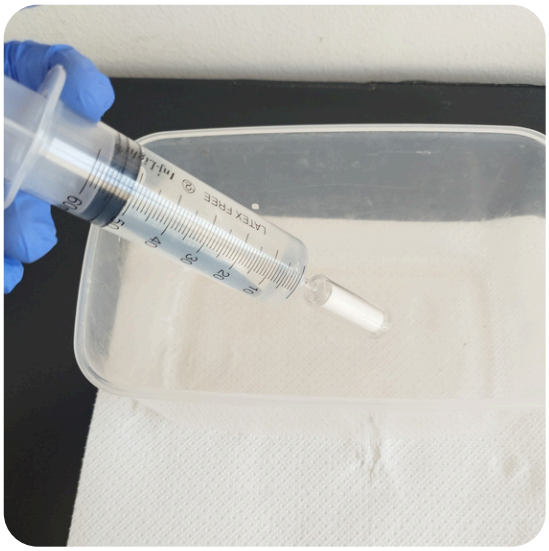


2.Shake the Whirl-Pak bag for 10 seconds. This mixes any DNA across the whole water sample.





3.Draw up water into the 50 mL syringe and then attach the syringe to the filter unit using the luer lock. Filter the water through the column. Keep repeating this process. Differences in turbidity could clog the filter up at a lower volume but please try to filter at least 200 mL water and up to 500 mL water.



4. Once water has been filtered (volume to be recorded on sample record sheet), flush any excess water out of the filter unit using the empty 50 mL syringe as follows: Draw up air into the syringe and attach to the filter unit and push the air through, this will force residual water out of the filter. This will not remove all the water, so tap the residual water out onto some tissue.

5. Using the 10mL syringe filled with preservation solution attach the syringe to the filter unit and very gently push into the filter until it starts to come out of the top.

Sample labelling, storage and mailing

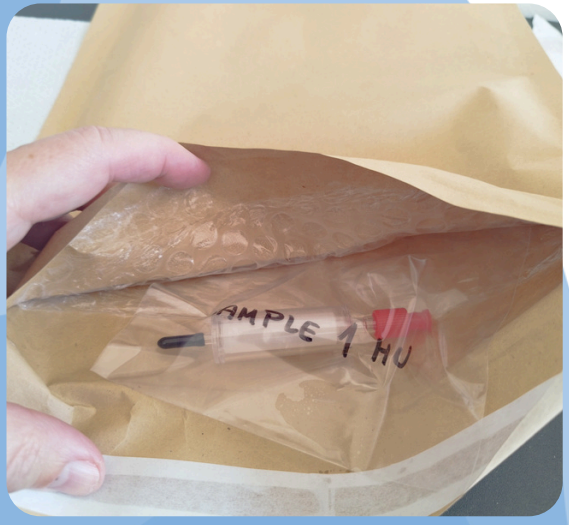
6. Screw/push caps (use the luer lock cap from the 10ml syringe and the black push on cap in the bag) onto each end of the filter unit and label the filter unit accordingly with a permanent marker i.e. “Mali Sakadaš CRO”, or “Sample 1.HU”. etc.

7. Label after applying the preservation solution as the preservation solution could wash off the marker pen. Place the filter into the grip seal bag and label the bag with the sample name.

8. Empty the remaining water from the whirl-Pack bag

9. Place all used gloves, pipettes, rubbish into the sampling bag and dispose.

10. Prior to mailing please store filter refrigerated (2-4°C) or in a cool place.

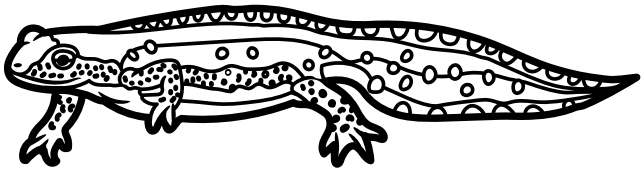


11. Mail filter with post office mail, in secured envelope to following address:

Assoc. Prof. Dr. Lidija Kalinić,
Sub-Department of Biochemistry and Molecular Biology
Department of Biology
Josip Juraj Strossmayer University of Osijek
Adress: Ulica cara Hadrijana 8/A, 31000 Osijek, CROATIA

Sample data form

12.Fill out the sample data form (scan the QR code to do this). Data requested: sample name; volume of water filtered; sample target site; sampler; date and time sample taken, sample condition; site condition; and storage prior to mailing.



Field Protocol for eDNA sampling of Danube Crested Newt (*Triturus dobrogicus)* is developed in the frame of Danubeparks project:

This protocol sholud be cited as: Mikuska A (2024) Field Protocol for eDNA sampling of Danube Crested Newt (*Triturus dobrogicus*). DANUBEPARKS

LITERATURE