Sampling protocol for the freshwater mussel *Simpsonaias ambigua* (Salamander Mussel) in Canada

Isabel Porto-Hannes, Kelly McNichols-O'Rourke, Margaret Goguen, Maggie Fang and Todd J. Morris

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Simpsonaias ambigua, Salamander Mussel (previously known as Mudpuppy Mussel) is a freshwater mussel of the Family Unionidae. This species is unique from other unionids as it uses *Necturus maculosus* (Mudpuppy), as a host instead of a fish. This mussel is found within medium to large rivers or lakes, in areas of swift current almost exclusively beneath large, flat stones or under ledges of rock walls. Salamander Mussel was assessed as Endangered in Canada in 2001 and this status was re-affirmed in 2011. This document describes three different field methods that can be used to find live *S. ambigua* in Ontario with step-by-step instructions: traditional surveys in wadable water (Rock Flipping), environmental DNA (eDNA), and capturing the host to inspect for signs of encysted glochidia (Mudpuppy Capture). Rock Flipping in wadable rivers was shown to be the best method for detecting extant populations of *S. ambigua* and is the only method that allows for an estimation of population health (direct handling permitting counting and measuring of individuals). The other two methods are suitable when exploring large areas for the first time, as they help narrow the search area using traditional surveys or when Rock Flipping is not possible.

RÉSUMÉ

Porto-Hannes, I., McNichols-O'Rourke, K., Goguen, M., Fang, M., and Morris, T. J. 2021. Sampling protocol for the freshwater mussel *Simpsonaias ambigua* (Salamander Mussel) in Canada. Can. Tech. Rep. Fish. Aquat. Sci. 3411: vii + 60 p.

Simpsonaias ambigua, ou mulette du Necture (auparavant appelée mulette du Necturus) est une moule d'eau douce de la famille des unionidés. Cette espèce se distingue des autres unionidés, car elle utilise comme hôte *Necturus maculosus* (necture tacheté) plutôt qu'un poisson. On trouve cette moule dans les eaux agitées de lacs et rivières de moyenne ou grande taille, presque exclusivement sous de grosses pierres plates ou sous des bordures rocheuses. La mulette du Necture a été désignée comme espèce en voie de disparition au Canada en 2001, et ce statut a été renouvelé en 2011. Le présent document décrit trois méthodes– accompagnées d'instructions détaillées – qui peuvent être utilisées sur le terrain pour trouver des spécimens vivants de *S. ambigua* en Ontario : faire des relevés classiques en eaux peu profondes (retourner des pierres), effectuer une analyse de l'ADN environnemental (ADNe) ou capturer l'hôte de la mulette du Necture pour chercher des signes de glochidies enkystées (capturer un necture tacheté). Le retournement de pierres en eaux peu profondes s'est révélé être la méthode la plus efficace pour détecter les populations existantes de *S. ambigua.* De plus, c'est la seule méthode qui permet d'estimer l'état de santé de la population (manipuler les mulettes permet de les compter et de les mesurer). Les deux autres méthodes conviennent au moment de l'exploration initiale de grandes zones, car elles permettent de restreindre la zone de recherche pour ensuite effectuer des relevés classiques, et dans les situations où il est impossible de retourner les pierres.

PREFACE

Simpsonaias ambigua (Say, 1825), Salamander Mussel (previously known as Mudpuppy Mussel) is a freshwater mussel of the Family Unionidae. It is small (approximately 42 mm in length and 20 mm in height), with a thin, elongated and elliptical shell. This species is unique in that it uses an aquatic salamander with external gills (*Necturus maculosus* (Rafinesque, 1818), Mudpuppy), as a host instead of a fish like all other North American freshwater mussels of the order Unionida (Howard 1915). *Simpsonaias ambigua* is found within medium to large rivers or lakes, in areas of swift current burrowed in mud, silt, sand or gravel (Watson et al. 2001). This mussel is almost exclusively found beneath large, flat stones or under ledges of rock walls and this habitat most likely facilitates contact with its amphibian host (Howard 1915).

Simpsonaias ambigua was assessed as Endangered by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) in 2001 (Watson et al. 2001) and federally listed under the *Species at Risk Act* (SARA) in Canada in 2003 (Morris and Burridge 2006). The status was then reassessed and confirmed as Endangered in 2011 (COSEWIC 2011). *Necturus maculosus* is considered not at risk (COSEWIC 2000, Gendron 2000); however, its status is being re-evaluated in 2021 (COSEWIC 2021).

In Canada, only 59 live individuals were recorded between 1965 and 2017 (LGLUD 2018). Historical records (i.e., before the year 2000) of *S. ambigua* shells or live individuals were from Bear Creek-North Sydenham River (Clarke 1981, Metcalfe-Smith et al. 1998), the Detroit River (Clarke 1985), the St. Clair River delta (Metcalfe-Smith et al. 1998) and the Thames River (Metcalfe-Smith et al. 1998). Currently, the Canadian distribution of the species is believed to be restricted to the middle reach of the East Sydenham River in southwestern Ontario (COSEWIC 2011). A total of 37 live specimens at five sites in the East Sydenham River have been observed through informal and formal surveys conducted by Government of Canada agencies and Academic researchers since the original COSEWIC assessment in 2001; however, no additional specimens have been observed in the Thames River (Metcalfe-Smith et al. 2007).

Despite the large effort that has taken place to survey freshwater mussels in Ontario, detailed knowledge of *S. ambigua* population densities, structure and current distribution are lacking (Metcalfe-Smith et al. 1998, Metcalfe-Smith et al. 1999, COSEWIC 2011). The specialized habitat characteristics of this species makes it unlikely to be encountered during most mussel community assessments in southern Ontario, resulting in a paucity of data on its distribution and abundance. These data are critical for future recovery efforts to restore or augment populations of *S. ambigua.* Between 2018 and 2020 three survey methods were developed and implemented to find extant populations of *S. ambigua* in Canada. This technical report describes these three methods in detail and provides step-by-step guidance for their implementation.

SAMPLING PROTOCOLS

1.0 CONVENTIONAL SURVEY METHODS “ROCK FLIPPING”

Simpsonaias ambigua is found within medium to large rivers or lakes, in areas of swift current burrowed in mud, silt, sand or gravel (Watson et al. 2001). This mussel is almost exclusively found beneath large, flat stones or under ledges of rock walls and this habitat probably facilitates contact with its host, *N. maculosus* (Howard 1915). Other potentially suitable habitats are among roots of emergent vegetation, large woody debris and undercuts of banks (Howard 1915, Bogan and Locy 2009). In Ontario, qualitative timed-searched surveys to find freshwater mussels (Family Unionidae) often focus on areas such as riffles which are considered the preferred habitat of most mussel Species at Risk (SAR) (Metcalfe-Smith et al. 2007). Furthermore, in order to assess the distribution and demographics of freshwater mussel SAR, a systematic sampling design is often employed. These methods have proven insufficient to survey *S. ambigua* because of its clustered distribution, low population abundance and unique habitat preferences; therefore, a method specifically designed to target *S. ambigua* would be a better approach. Here, we describe a survey method called “Rock Flipping” that targets *S. ambigua*’s habitat.

The search area and the method; qualitative or semi-quantitative, depends on the survey objectives. Qualitative searches are appropriate when investigating an area for the first time for the presence of *S. ambigua*. Search time should be adjusted based on the research goals. The Rock Flipping method described here is qualitative; however, a semi-quantitative approach could be adopted by spatially bounding the area in which the Rock Flipping is conducted.The use of quadrats is highly discouraged given the clustered and low density distribution of this species (Reid and Morris 2017). Rock flipping should be implemented with a minimum of three people.

Live *S. ambigua* in the Sydenham River, Ontario, have been found almost exclusively in pockets of fine gravel underneath large flat rocks. No live mussels have been found where the sediment underneath the rock was too coarse (e.g., very coarse gravel or cobble, >32 mm) or too fine (e.g., silt). Furthermore, live mussels were found under flat rocks that were somewhat stable on the substrate (e.g., no wobble or not easily moved) and that had no large openings nor were completely filled with sand or silt. Therefore, large, stable, flat rocks with pockets of fine gravel should be targeted when using this method. Rock Flipping is most effective when the river is wadable but can be employed using SCUBA in deeper areas. The current report will only address wadable applications.

An important consideration is that this method involves lifting rocks and removing sediment, which could potentially disturb Salamander Mussel and Mudpuppy habitat; therefore, it is extremely important to recreate the habitat after the search is concluded. Details will be explained in the step-by-step instructions below.

1.1 Salamander Mussel Survey

1. See Appendix A for a complete packing list of equipment required for this survey and an example of the field data sheet.
2. Upon arrival at the site, prepare for the day (e.g., put on waders, pack equipment for transport, etc.).
3. At the river, start the survey by recording GPS coordinates at the starting point and set timer to zero. Record other relevant information related to the project on the data sheet (Appendix A).
4. At a site, an initial 30 minute search (90 person-minutes or 1.5 person-hours for a crew size of three three surveyors), is conducted by wading or snorkeling while carefully lifting large flat rocks or looking under submerged debris. Search time should be adjusted based on the research goals.
5. Before a rock is lifted pay attention to the position of the rock and the space between the rock and the bottom of the river because if live mussels are found, the habitat must be recreated as much as possible when putting the mussels back underneath the rock. Rocks should be lifted from the upstream edge whenever possible.
6. Before lifting any flat stones or debris, two nets with a wide (~30 cm) D-rim should be placed at the downstream end of the rock to catch any mussels that could be dislodged while searching (Figure 1.1 A).
7. To search for Salamander Mussel, scoop out the sediment under the rock by hand. Dip nets can be used to scoop fine sediments but the best method is to use both hands. Pay attention to the type of sediment that is found under the rock because the same type of sediment must be replaced if live individuals are found (Figure 1.2).

Figure 1.1. Rock Flipping sampling steps.
Sampling steps to find Salamander Mussel (A-C), buoy marking a rock (D) and sample processing (E).

Figure 1.2. Reconstruction of Salamander Mussel habitat after collection. Pay attention to the type of sediment that is found under the rock because the same type of sediment must be returned if live individuals are found. In this image, sediment similar to that originally found underneath the rock was collected at the bank and placed in a plastic container to then put underneath the rock.

8. Place the sediment in the wooden sieve and collect any live or spent shells of Salamander Mussel (Figure 1.1. B, C).
9. If live mussels are found stop the timer and continue with the next step. If no live mussels are found continue to step 11.
10. Place the live individuals in a mesh bag or in a receptacle with river water (see below Section 1.2 Processing Live Salamander Mussel, Step 1). Individuals from the same rock should be placed together in the same mesh bag/receptacle, do not mix mussels from different rocks/structures.
11. Take latitude and longitude GPS coordinates of the location that was searched. Place a small numbered buoy to mark the structure (Figure 1.1 D). Structures (e.g., rock, sunken debris, etc.) that have been searched are marked with numbered buoys to avoid re-sampling and/or allow the return of individuals to the same rock from where they were removed after processing (see below Section 1.3 Mussel Return).
12. If a rock needs to be found in subsequent sampling events, place a stake with a PIT (Passive Integrated Transponder) tag in the sediment underneath the rock. Record the PIT tag number.
13. After the search under the rock is complete, ensure that the area under the rock is clear (e.g., no hands, feet, etc.; Figure 1.3), by checking on every crew member before laying the rock back down. Only when everyone has stepped away and cleared the area, proceed to lower the rock slowly and carefully. Always try to lay down and position the rock as it was originally found. If live mussels were found, remember to re-start the timer after putting the rock down.
14. Continue searching until the 30 minutes are reached. If live individuals are encountered during the first 30 minutes increase the total search time to 60 minutes (180 person-minutes or 3 person-hours) for a crew of three, or target time based on project objectives.

15. All mussels should be processed at the end of the search time. If live mussels need to be processed before the completion of the timed-search survey, the search time should be stopped and resumed after processing is complete.

Figure 1.3. Caution about lifting rocks.
Some rocks are very large and heavy. Do not attempt to lift a rock if it is too heavy. If a team member is getting tired of holding the rock and it must be put down before the search is complete, notify all surrounding crew members that a break is required or if there is a need to change positions. Never let go of the rock without warning.

16. At the end of the survey, a GPS coordinate is recorded as the “end of the search point”.
17. The field sheet must be completed for each site regardless of whether or not live animals were found. Other live unionids found during the search should be recorded on the data sheet.

1.2 Processing Live Salamander Mussel

1. If live *S. ambigua* individuals are found, place them in small mesh bags completely submerged in water to allow water flow or place a 20 L bucket with holes in the stream near the shore using rocks to keep it on the bottom and from floating downstream. Place a pond basket inside the bucket to hold Salamander Mussel (Mackie et al. 2008). Mussels must be kept cool in shaded areas. Label each mesh bag or pond basket with the number of the rock where the mussels were found, do not mix mussels from different rocks/structures (see Section 1.1 Salamander Mussel Survey Step 11 above).

2. Measure the length and height of each live mussel to the nearest millimeter using vernier calipers and take photographs.
3. When photographing a mussel, place it in a container with sand so the mussel lays flat (Figure 1.1 E). Take a picture of the side (lateral view) and the top (beak sculpture) (Figure 1.4). Always make sure to include a ruler for scale and avoid shadows which may cause image distortion.
4. After processing is complete, the live mussels are returned to the river following the steps outlined in Section 1.3 Mussel Return.

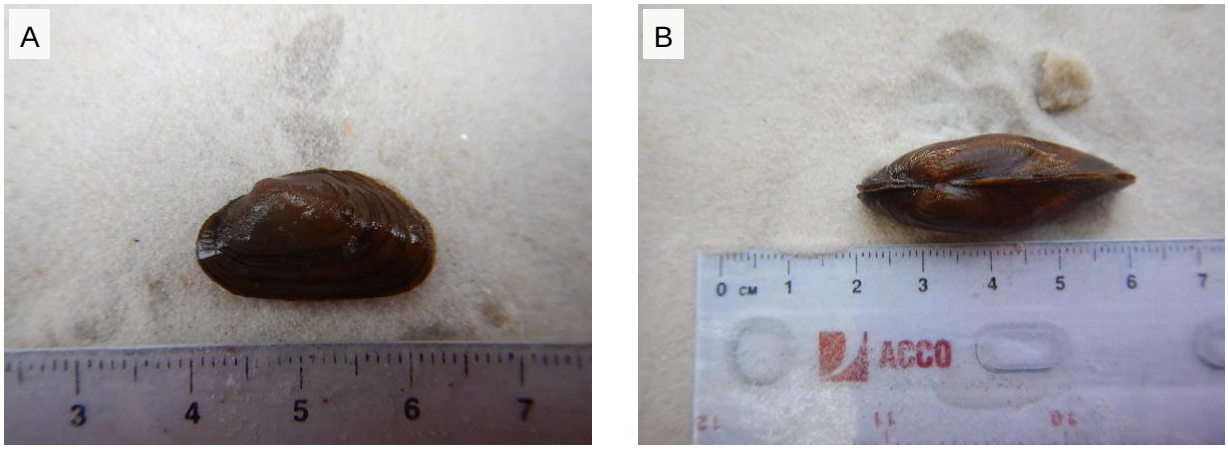

Figure 1.4. Salamander Mussel shell images. Lateral view (A) and beak sculpture (B) shell images.

1.3 Mussel Return

1. Be sure to place the mussels under the same rock/debris from where they were originally collected.
2. The sediment that was removed from underneath the rock must be replaced before putting the mussels back.
3. Locate sediment with the same or similar characteristics as the sediment that was originally removed (Figure 1.2). This can be located around the rock, but you may have to search for it.
4. Fill ¾ of the interstitial areas with the sediment.
5. Place the mussels on the sediment, then cover them with a small amount of sediment. Make sure the sediment that covers the mussels is leveled with the surrounding areas and that no mussels are above the sediment, otherwise they may be crushed when the is replaced.
6. The rock should be carefully placed to make sure that there are no large openings (e.g., space between the bottom of the river and the rock), that the rock is lying flat

and that it does not wobble. If necessary, place other smaller rocks on top and around the main rock. Be careful not to block all of the entrances underneath the rock, as this would prevent the entry of the *N. maculosus*.

2.0 eDNA SAMPLING

Environmental DNA (eDNA), the DNA shed by organisms into their environments, can be extracted from water samples and used to detect specific organisms, including endangered or rare organisms (Goldberg et al. 2011, Thomsen et al. 2012), or communities of organisms in aquatic ecosystems. The use of eDNA can supplement traditional biological monitoring efforts and inform conservation efforts (Taberlet et al. 2012, Kelly et al. 2014, Thomsen and Willerslev 2015). Furthermore, the use of eDNA has been proven to be a successful tool for freshwater mussel detection (Currier et al. 2017, Sansom and Sassoubre 2017).

The protocol described here is specifically for the collection of in-situ water samples to detect eDNA using an OSMOS eDNA backpack sampler (hereafter referred to as OSMOS) manufactured by Halltech Environmental Inc. (hereafter referred to as Halltech). There are other available eDNA in-situ samplers such as the *eDNA sampler* from Smith-Root (https://www.smith-root.com/edna/edna-sampler). Furthermore, there is an extensive body of literature describing other methods to capture eDNA from water samples. We encourage readers to review available literature during the project planning process to determine which method is appropriate. In addition, the protocol described here assumes that the reader is familiar with basic eDNA methods and terminology.

To increase *S. ambigua* eDNA detection from river samples, a larger volume of river water can be filtered in-situ using the OSMOS (in contrast to filtering at the laboratory). In-situ filtering using the OSMOS can be applied to any eDNA study with the corresponding modifications according to the target species and the research objectives. Laboratory procedures will not be described in this document; however, a publication that details *S. ambigua* species-specific primer-probes and quantitative polymerase chain reaction (qPCR) conditions is in preparation and this information is currently available from the authors upon request.

The decontamination procedures listed in this protocol are for sampling Salamander Mussel eDNA. For other eDNA projects these methods must be revised to meet the decontamination standards required for that particular project. For example, some protocols suggest using a 50% bleach solution and/or require bleaching waders, boots, and all equipment. Using a higher concentration of bleach solution is recommended when the risk of cross-contamination is high; however, residual bleach will degrade the eDNA from samples resulting in false negatives. This must be weighted during the project design stage. Cross-contamination in eDNA sampling is a great problem, if the decontamination steps explained in this protocol are followed, there should not be any contamination issues when collecting water samples to detect Salamander Mussel eDNA. If carrying out live animal surveys (e.g., Rock Flipping), the eDNA sampling should be completed first to avoid contamination.

There are many types of filters to capture eDNA made of different materials and of different pore sizes, and the proper type must be determined based on specific project goals. We used 0.8 µm pore size cellulose nitrate filters (GE Healthcare Whatman™ Type WCN Cellulose Nitrate Membranes). These were effective at capturing *S. ambigua* eDNA in the field but are very brittle; the tweezers may poke holes in the filter and they may break easily when folding them for storage in transport tubes. Other filters such as polycarbonate filters (EMD, Millipore, Germany) may be more flexible. Regardless of the filter used, holes or small rips are not serious issues; however, the more intact the filter is the better.

The time a sample takes to filter depends on the target volume and the sediment load of the waterbody. Clearer water will filter faster, for example 500 mL of distilled water is filtered in one minute or less; river samples do not filter that fast especially if turbidity ishigh. While filtering, pay attention to the flow rate displayed on the OSMOS settings display screen (Figure 2.4). If the flow is reduced to almost 0 L/min this means that the filter is clogged. Also pay attention to the pressure. The OSMOS is set up to have a threshold of -60 kPa, when the machine is close to that threshold, it automatically reduces the flow to prevent pump damage. If the pressure exceeds the pressure threshold and the flow is close to zero, stop filtering (even though the target volume has not beenreached). Further instructions are included below in Section 2.3.2 eDNA SampleCollection. When the waterbody to be sampled is highly turbid an additional filter with larger pore size may be needed to remove larger particles. The OSMOS has the option of using a pre-filter stage (Figure 2.1 A), where this filter can be placed. The use of the prefilter stage is not described in this protocol and if it is required, optimization of the filter material and pore size is highly recommended.

Determining how and where the filters will be stored and how to best preserve the eDNA are important considerations as well. Storing the filters in transport tubes that are kept cold (<5ºC) in a cooler with ice is sufficient until the filters can be stored in a freezer (no more than 24 hours, Pilliod et al. 2013). However, if samples need to be shipped/transported for processing and refrigeration is not possible or reliable during transport, the use of DNA preservatives such as ethanol or buffers may be used as an alternative.

The OSMOS requires cleaning and special precautions that are summarized in Section 2.3.3. Contact information for Halltech is listed in Section 2.5 in case maintenance or part replacement for the OSMOS is required.

2.1 Equipment Preparation at the Laboratory

1. See Appendix B for a complete packing list of equipment required for this survey and an example of the field data sheet.
   a. A small control cooler or container with a lid (~5 L capacity) must be decontaminated with bleach in the laboratory (repeat decontamination in the field if you suspect some contamination has occurred inside of the cooler). Be sure to use nitrile gloves and change them if any laboratory surfaces or other laboratory objects are touched. This control cooler/container will be used to take the field control blank at each site, see Section 2.3.1 Collection of Control Field Blank.
   b. Mix one part bleach, nine parts distilled water for a 10% bleach solution to clean the inside of the cooler or container. For convenience this mix can be put in a squirt bottle to bring in the field for gear decontamination when necessary.
   c. Leave the bleach solution in the control cooler/container for 10 minutes.
   d. Properly dispose of the bleach solution.
   e. Rinse the cooler or container thoroughly at least 3 times with distilled water until bleach residue has been cleared.
   f. Let the cooler air dry or dry with clean paper towels. Use a new pair of gloves to do this.
   g. After decontamination, seal the lid of the cooler/container with tape and do not open it again until arrival to the first sampling site.
2. Decontaminate a ~2-3 L plastic bottle with 10% bleach solution as described above and fill with distilled water (the volume of water needed varies according to the volume and number of field control blanks, so adjust this volume according to the sampling needs, but always bring more than is required as a precaution). The plastic bottle can be Nalgene or similar. This water will be used to collect the control blank at the field site. Do not take the control blank in the laboratory.
3. All OSMOS filter housing parts (Figure 2.1) must be decontaminated following the same bleaching method as the control cooler (Step 1b).

NOTE: The filter housing has two rubber rings, these must not be confused. There is a flat rubber ring that goes inside the final stage (Figure 2.1 B) and a rubber O-ring that goes on the inlet stage Figure 2.1 B, C).

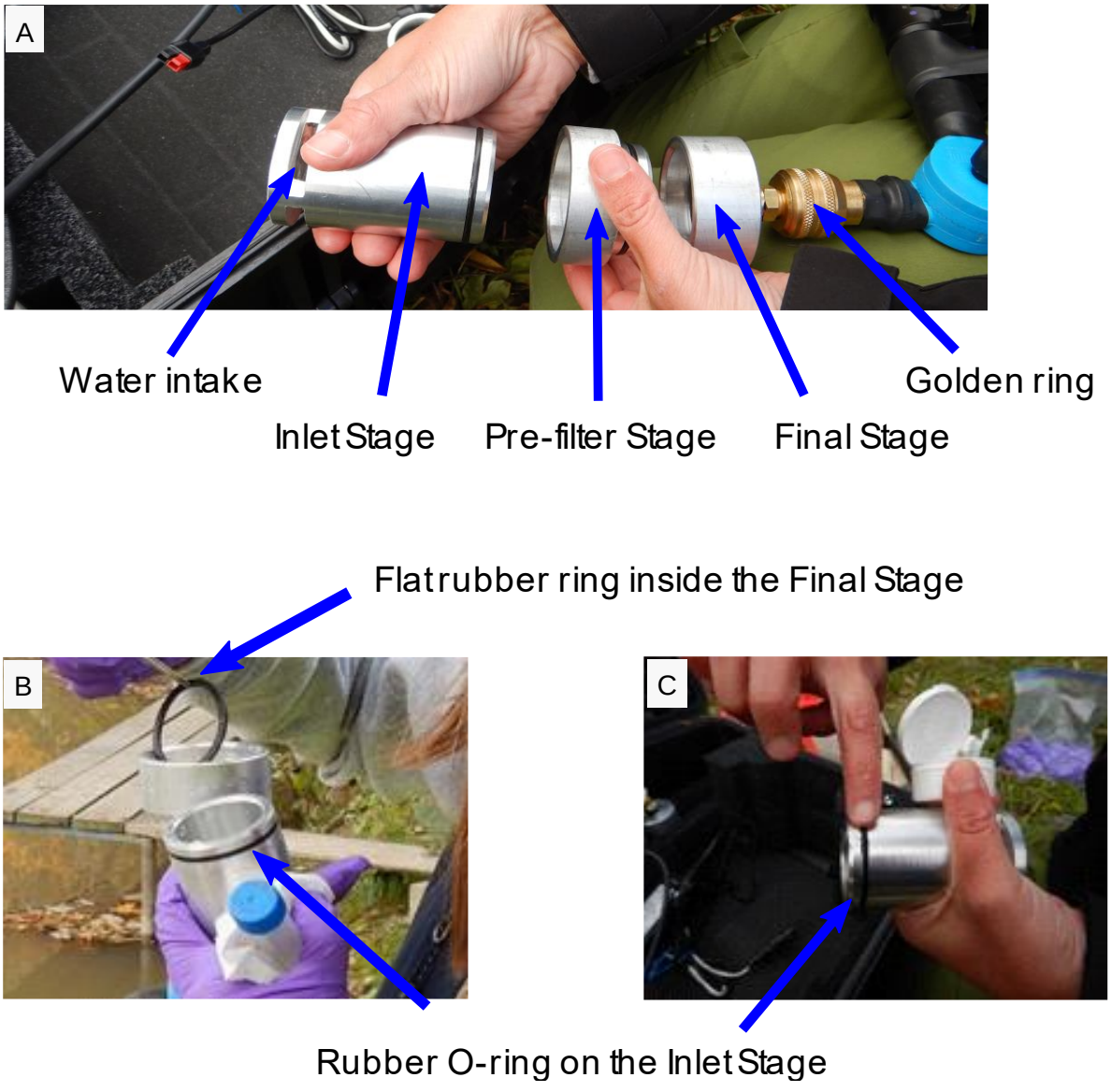

Figure 2.1. OSMOS eDNA backpack sampler filter housing parts.
OSMOS backpack filter housing main components (A). The filter housing has two rubber rings: a flat ring inside the final stage and an O-ring on the inlet stage (B). Silicone application on the rubber O-ring on the inlet stage (C).

a. In the laboratory, wipe off any silicone lubricant from the housings before placing them in the bleach solution. Then submerge the filter housing parts in the 10% bleach solution for 10 minutes.
b. Remove from bleach and rinse with distilled water at least 3 times until all bleach is removed.
c. Let the filter housing parts air dry on clean paper towels.

d. When fully dry, the outer rubber O-ring on the inlet stage (Figure 2.1 C) must be lubricated. The recommended silicone is *Silicone Lubricant, Master plumber* (SKU1036C). Put on gloves and apply a SMALL (e.g., 0.5 cm in diameter) amount of silicone to the rubber O-ring using your finger while it is attached to the inlet stage as in Figure 2.1 C. The rubber O-ring should look smooth and slightly shiny after the silicone is applied, there should be no lumps of silicone around/on the rubber O-ring nor on the inlet stage. The silicone ensures a tight seal; however, if too much silicone is used the filter housing inlet stage (Figure 2.1 A) will slide off and it may get lost in the field. To test if the amount of silicone is appropriate, hold the filter housing from the final stage with the inlet stage facing down. If the inlet stage slides down, it means there is too much silicone. Store the silicone in the OSMOS pelican case in case more silicone is required while in the field. Silicone only needs to be applied after the filter housings are cleaned (for further information see 2.3.3 Cleaning and Caring for the OSMOS After Field Sample Collection).
e. Using clean gloves, place the <u>assembled and lubricated</u> filter housings (inlet stage + final stage, Figure 2.1 A) in a new resealable bag or large Whirl-pack® and label as "clean". Before putting the inlet stage on the final stage (Figure 2.1 A), make sure that the flat rubber ring (Figure 2.1 B) is inside the final stage. Silicone should <u>not</u> be put on the flat rubber ring. If the flat rubber ring is missing there will not be a proper seal and the OSMOS will not work properly. Furthermore, the flat rubber ring holds the filter in place.

NOTE: Filter housings must be decontaminated before using them again. In the field, filter housing parts can be decontaminated by spraying 10% bleach and leaving it for 10 minutes, rinsing 3 times with distilled water and drying with clean paper towels. This distilled water is in addition to the distilled water that will be used for the control blanks (See Appendix B for an approximate total volume of distilled water needed to rinse off equipment in the field per day- filter housings and tweezers-, but always do the calculations based on the project needs). Ensure to have enough distilled water in case decontaminating is required in the field. Check the amount of silicone lubricant on the O-ring on the inlet stage to see if it shouldbe reapplied after cleaning in the field. Alternatively, at the end of the day, the filter housingscan be decontaminated in bulk in a clean plastic container following Steps 3 a-e above.

4. Hydrogen peroxide will be used to decontaminate tweezers in the field. If no hydrogen peroxide is available tweezers can be decontaminated using 10% bleach solution. Adjust the volume of the 10% bleach solution and distilled water needed for equipment decontamination in the field (See Appendix B for an approximate total volume of distilled water needed to rinse off equipment in the field per day). If using hydrogen peroxide, the rinsing water is not needed.

5. Charge the OSMOS batteries (29V 10Ahr Lithium Ion Batteries). One battery can last for 1 or 2 days of sampling (if taking 4 samples per site at 6-7 sites). It is always important to charge the battery prior to going to the field.
    a. Take the battery out of the OSMOS back pack battery holder (Figure 2.2 A).
    b. Plug the cable with the red and black ends into the corresponding red and black plugs on the battery.
    c. For a fully charged battery, charge for 5 hours. Batteries can be charged overnight safely.
    d. Place the charged battery in the OSMOS battery holder (Figure 2.2 A).
6. To check the battery levels, while operating the OSMOS, press the UP/DOWN control buttons (Figure 2.2 A) to view the remaining battery charge. There is also a low battery warning alarm when the battery is below the minimum charge required.

2.2 Assembling the OSMOS eDNA Backpack Sampler

Assembly of the OSMOS constitutes the assemblage of the backpack unit (Figure 2.2 A, B) and the tripod (Figure 2.2 C). Tripod use is not necessary at all sites, but it does facilitate the use of the backpack sampler and the telescopic pole from the shore (Figure 2.2 C, D). An initial evaluation of the sampling site (e.g., water depth, flow, access to the water) must be completed before deciding if the tripod is required. Assemble the OSMOS following the directions below (Setting up the backpack unit). The backpack assemblage should occur at the truck to avoid carrying unnecessary equipment to the sampling site. Furthermore, the control blank should be taken by the truck (see Section 2.3.1 Collection of Control Field Blank) and for this task the backpack must be assembled. The backpack can be partially disassembled (e.g., removal of the inflow hose) when driving between sampling locations.

<u>Setting up the backpack unit</u>

1. After arriving at the site, assemble the backpack by the truck.
2. Securely attach the inflow and outflow tubes to the backpack unit (Figure 2.2 B).

NOTE: The attachments may not fit securely if there is debris in the locking spring mechanism.

3. Attach the remote control to the unit (Figure 2.2 B).
4. Turn on the unit by opening the waterproof casing and flicking the ON-OFF switch (Figure 2.2 A).

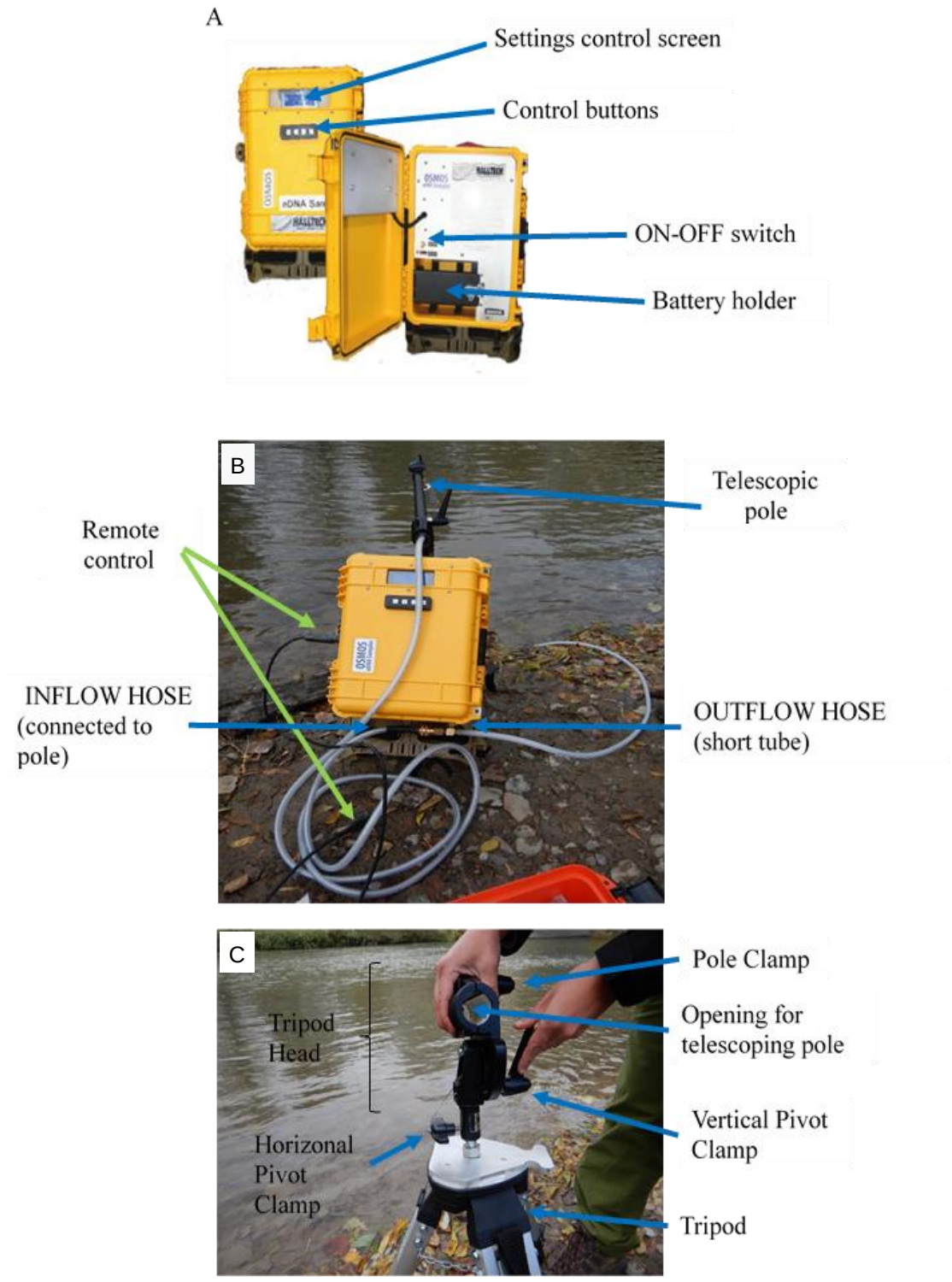

Figure 2.2. OSMOS eDNA backpack sampler parts and assemblage.
Assembly of the OSMOS constitutes the assemblage of the backpack unit (A, B) and the tripod (C).

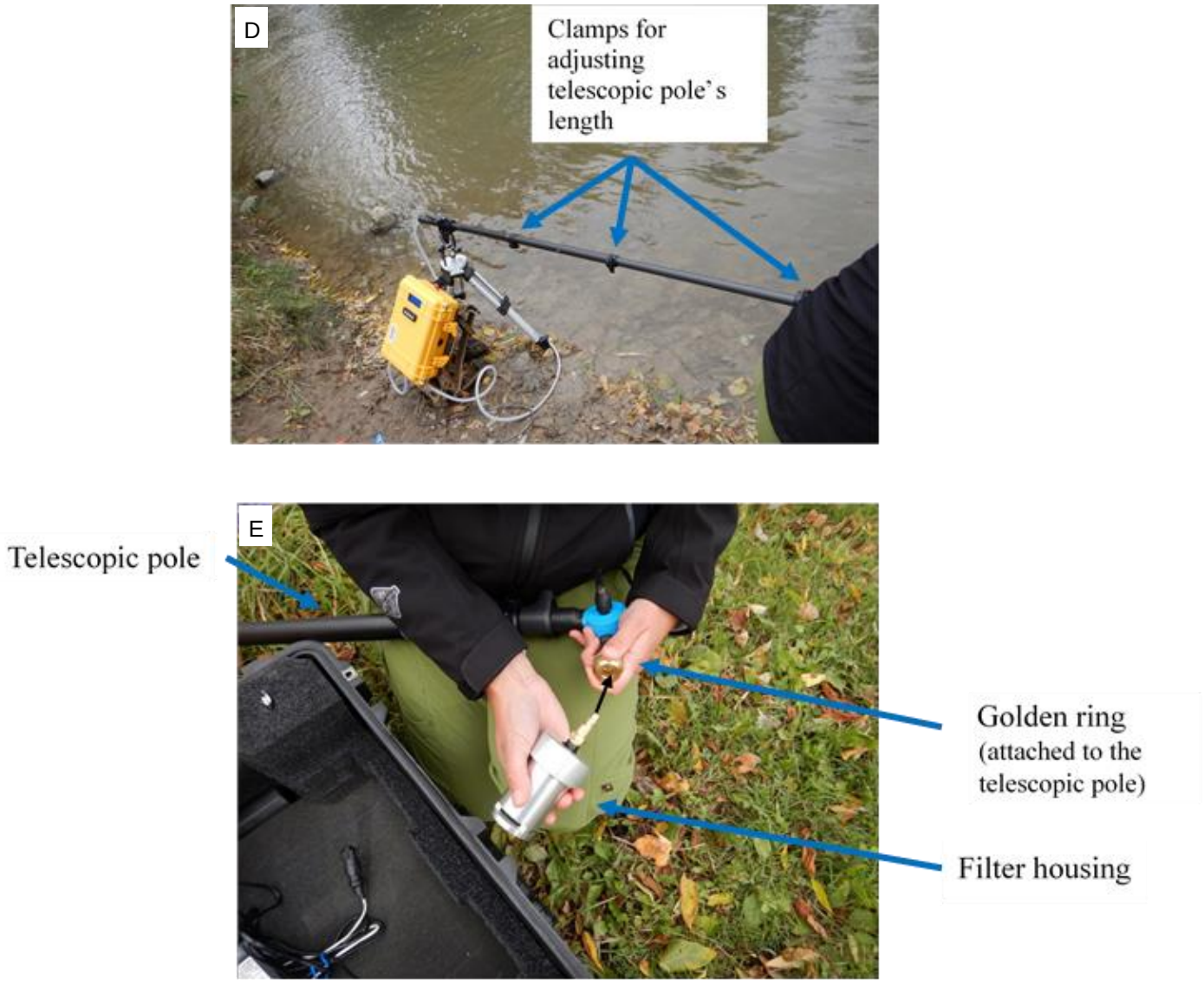

Figure 2.2. (continued). OSMOS eDNA backpack sampler parts and assemblage.
The length of the telescopic pole can be adjusted (D). The filter housing is attached to the end of the telescopic pole (E).

2.3 Field eDNA Sampling

2.3.1 Collection of Control Field Blank

1. A field blank must be taken at each site before sampling begins. For convenience this is done at the truck, not at the river.
2. Two people are needed, one to control the OSMOS and one “clean” person to handle the filters.
3. To take the field control blank, pour ~700 mL – 1 L of distilled water into the decontaminated control cooler/container (or additional volume if the control blank volume is > 500 mL).
4. The backpack should be assembled at this point, if not, follow Section 2.2 (Setting up the Backpack Unit, Steps 1-4) but do not assemble the tripod. The tripod is not needed for this step.
5. Before attaching the filter housing (Figure 2.2 E) to the telescopic pole, the filter needs to be inserted into the final stage of the housing.
    a. Put gloves on.
    b. Remove a decontaminated filter housing (final stage + inlet stage) from the bag labeled “clean”.
    c. Remove the inlet stage (Figure 2.1).
    d. Hold the inlet stage in one hand while you put the filter in the final stage. Otherwise, place the inlet stage on a paper towel in a secure location (e.g., out of foot traffic, prevent rolling, etc.).
    e. Decontaminate tweezers (if they are not decontaminated) by pouring ~30-50 mL of hydrogen peroxide OR 10% bleach solution in a small plastic container, immerse and stir the tweezers in the solution for approximately 15-20 seconds. If using hydrogen peroxide simply wipe down the tweezers with a paper towel. If cleaned with the 10% bleach solution, rinse 3 times with distilled water and then dry them with a paper towel. Wrap the tweezers in paper towel while filtering the blank to avoid contamination of the tweezers.

NOTE: All needed tweezers can be decontaminated in the laboratory and used in the field as needed. Do not use the same tweezer more than once without decontaminating. *Decontaminate the tweezers in the field as needed, also change the gloves as often as you consider necessary based on your exposure to possible contamination sources. If you are unsure of whether or not you should change your gloves, change them to be safe.*

    f. Dispose of the hydrogen peroxide or bleach solution according to the appropriate environmental, health and safety protocols (a new solution is needed to decontaminate the tweezers when taking a control blank).

g. Take a filter using tweezers. The filters can be very thin, check that only one filter was selected and that there is no blue paper (or divider, this may vary according to filter type/manufacturer). Properly discard the dividers.
h. Remove the flat rubber ring that is on top of the metal mesh (Figure 2.1 B and Figure 2.3 A) (this is not the rubber O-ring that is on the outside of the inlet stage). The rubber flat ring can be held on your pinky finger or wrapped in a clean paper towel.
i. Put the filter in the final stage on top of the mesh (Figure 2.3 B). The filter should lay flat on top of the mesh with either side facing upwards (Figure 2.3 B). Once the flat rubber ring is placed on top of the filter (see next step) the filter will be secure. If the filter is pushed down with the tweezers, the filter may break.

NOTE: Do not push the filter with your fingers, never touch the filter with fingers as this could contaminate the filter.

j. Place the flat rubber ring on top of the filter (Figure 2.3 C).
k. Gently push down the sides of the flat rubber ring with the tweezers. This way the filter will be secured in place.

NOTE: The filter should lay flat, if for some reasons there are folds (extremely rare), discard the filter and start again.

l. Attach the inlet stage to the final stage. Press tightly to make sure the inlet stage is secured correctly in the final stage.
m. Connect the filter housing to the end of the telescopic pole (Figure 2.2 E). Pull the exterior of the golden ring connector upwards and hold it in this position (Figure 2.2 E). Insert the final stage into the hole and release the golden ring to close the connector. The golden ring should return to the initial position. There is no need to screw the golden ring. Check that the final stage is secured by gently pulling onthe filter housing as if trying to remove it.

6. Ensure that the outflow hose (Figure 2.2 B) is pointing away from gear and/or the crew as water will come out of the hose once the OSMOS begins filtering.

Figure 2.3. Filter placement in the housing.
Remove of the flat rubber ring that is on top of the metal mesh (A), put the filter in the final stage on top of the mesh (B) then place the flat rubber ring on top of the filter (C).

7. Filter 500 mL (or target volume) of distilled water following these instructions:
   - If the OSMOS is not on, turn it on by flicking the power switch to the ON position (Figure 2.2 A).
   - When the OSMOS is turned on, the setting screen will light up (Figure 2.4).
   - Using the 'BACK', 'UP', and 'DOWN arrows in conjunction with the ENT (next) control button (Figure 2.4) change the settings:
     - **Volume**: set to 500 mL (or target volume) pressing UP/DOWN. Then press ENT.
     - **Pressure**: leave as the default (-60 kPa), press ENT.
     - **Pre-filter:** unless you are using a pre-filter stage (Figure 2.1 A) leave as the default (no pre-filter). Press ENT.
     - **Hose length:** is the length of the inflow hose (Figure 2.2 B). Leave as the default, Press ENT. (*NOTE: If sampling in deep water and the hose length changes, this needs to be adjusted).*
     - **Remote Switch**: this is for the remote control, press the UP/DOWN arrow to change to YES. Press ENT.
     - **Heater:** If the OSMOS has a heater choose the appropriate settings. Press ENT.

Figure 2.4. Settings control screen.

   - On the final screen after all the settings have been programed, press RUN to start filtering (or press the black button of the remote control). Use BACK if a setting needs to be changed.
   - If *set volume* option in the OSMOS was set to a specified volume (best option), the machine will beep when it is close to reaching the desired volume. At this point invert the filter housing (i.e., inlet stage is facing up) and press the black button of

the remote control which will stop the filtering and wait for the machine to beep again.

- Keep the filter housing inverted.

8. Remove the inlet stage of the housing filter. Gloves are not needed to remove the inlet stage because this part will not be re-used.
9. Now, put on new gloves.
10. Decontaminate the tweezers as explained above in this section (Step 5. e → f).
11. Remove the black, flat rubber ring using tweezers. You can place the rubber ring on your pinky finger or wrap it in a clean paper towel.
12. Remove the filter from the final stage using the tweezers and fold the filter. There are two ways to fold the filter: the quesadilla fold or the burrito fold (see below for instructions). For the 0.8 µm pore size cellulose nitrate filters (GE Healthcare Whatman™ Type WCN Cellulose Nitrate Membranes), the burrito fold works the best.

Burrito fold: with the tweezers grab one end of the filter and fold inwards 2/3 of the way. Hold the filter with the tweezers on an angle (if placed on a 90º angle the tweezers may poke the filter) gently walk the tweezers up and down to flatten the filter. Then grab the folded part fold again. Walk the tweezers to flatten the filter. Grab the filter and put it in the 5 mL transport tube.

Quesadilla fold: with the tweezers grab one end of the filter and fold it in half. Hold the filter with the tweezers on an angle (if placed on a 90º angle the tweezers may poke the filter) gently walk the tweezers up and down to flatten the filter. Then fold it in half again so it looks like a quesadilla or a piece of pizza. Grab the filter and put it in the 5 mL transport tube.

13. Label the tube on the lid and sides with the site abbreviation, date and the word “Blank”.
14. Place the tube in the cooler with ice.
15. Discard gloves after collecting the blank.
16. Remove the final stage from the telescoping pole, make sure to put the flat rubber ring back in the final stage. Place the inlet stage and final stage in a bag labeled “used”. This filter housing should not be used again until decontaminated.
17. Write additional notes on the field sheet addressing any issues with the control blank collection (e.g., filter broke or was folded during filtration, etc.).
18. Discard the used gloves.

2.3.2 eDNA Sample Collection

1. See Appendix B for a complete packing list of equipment required for this survey and an example of the field data sheet.

2. After collecting the field control blank at the truck, proceed to pack a dry bag with the items needed to collect water samples from the river.
3. At the river, if sampling in the water, follow Option 1. If sampling from the shore, follow Option 2. A minimum crew of two people is required to operate the OSMOS and collect water samples. Always designate one person as the “clean” person, who will be the only one handling the filters.

Option 1: Sampling in the water and wearing the backpack (no tripod)

A. Ensure the following is done prior to wearing the backpack in the water as it may require opening the waterproof casing again.
   - Unit is powered on.
   - Unit is responsive and functioning properly (e.g., setting control screen is not frozen, Figure 2.4).
   - Remote control is attached and working properly (e.g., advances setting selections when pressed).
   - Settings have been input prior to entering the water in case the system freezes and must be restarted (see below for detailed instructions).

B. While standing on the shore, put on new gloves, grab a new/decontaminated filter housing and connect it to the telescopic pole (Figure 2.2 E). This housing can be used to take all samples at a given site. It will be removed and placed in the “used” bag after sampling is complete.

C. The filter housing should not have a filter in the final stage (placing the filter in the final stage of the housing just before sampling occurs decreases the chances of contamination). After the filter housing is secured, proceed to place the filter following Section 2.3.1 Collection of Control Field Blank Steps 5. a → m. Keep in mind that in this case the filter housing is connected to the telescopic pole and it will remain connected throughout the duration of the sampling (unless issues arise, see Section 2.4 eDNA Backpack Troubleshooting).

NOTE: Placing the filter in this sequence is in contrast to the method for collection of a control blank, where the filter is placed in the final stage before the housing is connected to the telescopic pole.

D. Proceed to the river.

E. The best section of the river to sample is at a thalwag (e.g., fastest part of the river) where the river water is well mixed. Choose a sampling location that is safe for the crew. Depending on the project objectives other sampling designs may be employed, for example sampling at several points across the river channel (transects).

F. Walk into the river below your chosen location and walk upstream to your sample location. Orient the the pole upstream of the person with the backpack.

G. Lower the filter housing into the water. Keep the water intake openings of the inlet stage (Figure 2.1) submerged in the water at all times.
H. Press the remote control (black button) to begin taking the sample.
I. Filter 3 L (or target volume) per sample following these steps:
    - When the OSMOS is turned on, the setting screen will light up.
    - Using the 'BACK', 'UP', and 'DOWN' arrows in conjunction with the ENT (next) control button (Figure 2.4) change the settings as required by the protocol.
    - After all the settings have been programmed and ready to filter, press RUN to start filtering (or press the black button on the remote control). Use the BACK button if a setting needs to be changed.

NOTE: The outflow hose makes strange noises during submersion when it lifts out of the water; however, this is no cause for concern.

J. If the *set volume* option in the OSMOS was selected (best practice), the machine will beep when it is close to reaching the desired volume. At this point, invert the filter housing (i.e., inlet stage is facing up) and press the black button of the remote control to stop pumping and wait for the machine to beep again.

NOTE: Inverting the housing is very important because the water that has gone through the filter but that remains in the inflow hose has to go through the OSMOS in order to obtain an accurate volume of filtered water. Also see Section 2.4 eDNA Backpack Troubleshooting.

K. While keeping the filter housing inverted:
    - Put on new pair of nitrile gloves and remove the filter following Section 2.3.1 Collection of Control Field Blank Steps 8 → 18. Plus the following steps:
    - For eDNA water samples write with a permanent marker on the top of the lid: site abbreviation, replicate # and date. On the side of tube write: site abbreviation, replicate #, date and the volume of water filtered (displayed in the OSMOS settings display screen as "quantity").
    - Fill out the field sheet with all information related to each sample. Include water temperature taken by the OSMOS as well as taken with the thermometer.
    - Put on a new pair of gloves.
    - Use a new pair of tweezers or decontaminate the tweezers that were used.
    - Take another filter and place it in the final stage of filter housing as explained above.

NOTE: After filtering the first sample, the final stage may be a bit wet, so the filter will get wet, which is okay. It will also appear that there is a bubble as seen in Figure 2.3 B, but that is just a part of the filter that is still dry. If, after filtering the desired volume, there is evidence that there was a bubble (clean circle with no debris/dirt) or that the filter was

folded, place the filter in the 5 mL transport tube and make notes; however, another sample must be taken to replace this one.

- Repeat sampling until at least 3 replicates (or target number of replicates) are collected per site.

Option 2: Sampling from land using the tripod

A. Find a suitable location to set up the tripod. At this point the backpack should be assembled as the Blank has been taken.

NOTE: There is no rule to find a particular spot for tripod set up. Close to the water, on level ground is better but not always possible. The telescopic pole has to pivot from the water where the sample is taken to the bank where the filter will be placed and removed, so consider a clear space (e.g., no trees or tall vegetation, or the river bank) so the pole can move freely. However, keep in mind that the pole can move up/down, right/left to bypass obstacles.

B. Set the tripod onto the ground securely. The tripod legs have sharp ends that can be pushed into the ground, but they do not always secure the tripod to the ground, someone must stay with the tripod at all times.
C. Attach the tripod head onto the tripod (Figure 2.2 C).
D. Thread the telescoping pole through the opening on the tripod head and tighten the pole clamp so it does not slide out (Figure 2.2 C).
E. Extend the telescoping pole to the required length (Figure 2.2 D).

NOTE: <u>Keep a hand on the set-up at all times</u> as it is prone to tipping, especially when the telescopic pole is extended.

F. Pivot the pole (horizontal pivot clamp, Figure 2.2 C) towards the person handling the filter housing.
G. Connect a new/decontaminated filter housing without a filter to the telescopic pole (Figure 2.2 E), no gloves are needed to connect the filter housing. This housing can be used to take all samples at a given site. It will be removed and placed in the “used” bag after sampling is complete.
H. After the filter housing is secured, place a new filter following Section 2.3.1 Collection of Control Field Blank Steps 5. a → m. Keep in mind that in this case the filter housing is connected to the telescopic pole and it will remain connected through the duration of the sampling (unless it needs to be replaced because of any issues that may rise, see troubleshooting section).
I. At this point the inlet stage is facing upwards, turn the telescoping pole so the inlet stage faces down.

J. Pivot the telescopic pole with the filter housing towards the water where the sample will be taken.
K. Extend the horizontal pivot clamp and move the pole until it is positioned at the desired location.
L. Lock the pivot clamp in position.
M. Adjust the vertical positioning (vertical pivot clamp) of the filter housing into the water. Secure the vertical pivot clamp at a point where the entire filter housing is out of the water (~5 cm). When the handle is released, the housing will be lowered by its own weight. Make necessary adjustments. Keep the openings of the inlet stage (where water enters the stage) submerged in the water all the time. Even though the whole filter housing can be submerged under water, it is better if only the inlet stage openings are submerged.

NOTE: Careful readjustments may be needed during sampling if the pole starts moving down.

N. Ensure all clamps are secured and the filter housing is appropriately submerged prior to starting the sample collection.
O. If the OSMOS is not on, turn it ON by flipping the switch to the ON position (Figure 2.2 A).
P. Ensure that the outflow hose (Figure 2.2 B) is pointing away from gear and/or the crew as water will come out of the hose once filtering begins.
Q. Filter 3 L (or target volume) per sample following these steps:
    - When the OSMOS is turned on the setting screen will light up.
    - Using the 'BACK', 'UP', and 'DOWN arrows in conjunction with the ENT (next) control button (Figure 2.4) change the settings as required by the protocol.
    - After all the settings have been programmed and you are ready to filter, press RUN to start filtering (or press the black button of the remote control). Use the BACK button if a setting needs to be changed.
R. If *set volume* option in the OSMOS was set (best option), the machine will beep when it is close to reaching the desired volume. At this point invert the filter housing (e.g., inlet stage is facing up), press the black button of the remote control and wait for the machine to beep again.
S. Keeping the filter housing inverted (Figure 2.5), pivot the pole towards the shore.
T. Put on new pair of nitrile gloves and remove the filter following Section 2.3.1 Collection of Control Field Blank Steps 8 → 18. Plus the following steps:
    - With a permanent marker write on the top of the lid: site abbreviation, replicate # and date. On the side of tube: site abbreviation, replicate #, date and the volume of water filtered (displayed in the OSMOS settings display screen as "quantity").
    - Record all the information related to each sample on the field sheet. Include water temperature taken by the OSMOS as well as taken with the thermometer.

Figure 2.5. Collecting filtered samples and replacing filtered samples
Keeping the filter housing inverted pivot the pole towards the shore to change the filter.

U. Change gloves to a new pair.
V. Take a new pair of tweezers or decontaminate the tweezers you were using (see Section 2.3.1 Collection of Control Field Blank, Step 5. e → f).
W. Take another filter and place it in the final stage of the filter housing as explained above (Section 2.3.1 Collection of Control Field Blank, Steps 5. a → m). Repeat sampling until at least 3 replicates (or target number of replicates) are collected per site.

NOTE: If the filter looks folded or ripped after filtering, place it in the 5 mL transport tube and make notes; however, another sample must be taken to replace this one.

2.3.3 Cleaning and Caring for the OSMOS After Field Sample Collection

- At the end of a sampling day:

1. Decontaminate all of the used filter housings with 10% bleach solution as described in Section 2.1 Equipment Preparation at the Laboratory Steps 3. a → e (including adding the silicone lubricant).
2. Decontaminate tweezers and other tools needed for the next day of sampling.
3. Decontaminate the cooler/plastic container for collecting the field control blank.
4. Fill the water bottle with distilled water to collect the field control blanks the next day, if required.
5. If the OSMOS backpack, hoses and/or tripod have an excess of mud wipe down with a wet paper towel.

- At the end of the sampling session/week:

If the OSMOS is not going to be used for a couple of days or will be stored indeterminately the unit must be flushed with clean water and dried out:

1. At the laboratory assemble the OSMOS (just the backpack) as if you will be taking a field control blank.
2. In a utility sink fill a clean bucket with distilled water.
3. Connect the filter housing (without a filter) to the telescoping pole. The filter housing does not have to be decontaminated but it should be clean.
4. Submerge the housing in the bucket.
5. Before powering the machine ON to start pumping water, make sure the outflow hose (Figure 2.2 B) is inside the sink.
6. Power the machine ON and filter/pump (e.g., let water run through the machine) for 10 minutes to clean and flush the components.
7. As the machine operates ensure that the bucket does not run out of water.
8. After 10 minutes while the OSMOS machine is still pumping water, remove the housing from the water so all the water drains out of the hoses. Do not let the machine run dry for too long. As soon as water is no longer running through the outflow hose, stop the machine.
9. Power the machine OFF.
10. Disconnect the hoses.
11. Let the hoses and the machine air dry in the laboratory. The machine must be completely dry before storing it.

12. Take apart the filter housings and decontaminate with 10% bleach and let each part air dry separately.
13. Store when completely dry. The flat rubber ring in the final stage (Figure 2.1 C) can be stored separately (not inserted in the final stage) but make sure all small parts are stored together (same compartment in the pelican case).
14. Wipe down the OSMOS backpack, hoses and the tripod with a wet paper towel. All mud and dirt should be removed from the surface.

NOTE: Halltech recommended not to run bleach through the machine. If over time there is bacteria/fungus growing in the hoses contact Halltech for maintenance.

2.4 eDNA Backpack Troubleshooting

- Pump making irregular noises

1. Check that the battery is not running low.
   a. Press the UP/DOWN buttons (Figure 2.4) to view the amount of battery left. There is also a low battery warning alarm when the battery is below the minimum charge required.
2. Irregular noises such as gurgling, or a higher pitch humming sound may be an indication of air leaking into the system when sampling.

Potential sources of leakage could be:

1. Lack of silicone on the rubber O-ring on the final stage of the filter housing.
   a. Add more silicone to the outer black rubber O-ring of the inlet stage.
   b. OR replace the inlet stage and/or final stage as some may not fit together as securely as they should.
2. A bad connection between inflow tube to the back pack unit – inspect the locking spring mechanism and ensure it is secure. Debris could affect this mechanism.
3. Filter not laying flat in the final stage of the housing.
   a. Missing the black, flat rubber ring.
   b. Improper placement of the filter or the black, flat rubber ring on top could result in the filter bunching up during sampling.
   c. Uneven wiring in the mesh of the final stage of the filter housing may affect how the filter fits. Request for Halltech to replace the mesh.

- Control screen on the backpack is unresponsive when pressing the buttons

1. Check if it is also unresponsive when pressing the remote control – sometimes the settings can be set to respond to the remote control only.
2. Restart the machine – the ON/OFF switch can be accessed inside the waterproof casing.

- Clogged filters and water remaining in the hoses

If the OSMOS must be manually stopped before the set volume is reached because of a clogged filter, the unit will not have cleared the water out of the hoses. This creates two problems: first, the displayed volume is incorrect and second, the water may travel back down the hose and out the housing, which will push the filter out from under the black, rubber flat ring. To deal with this, stop the OSMOS with the remote control, invert the housing, note the volume of water, press the remote control again and let the water run until the hoses are clear. Record the final volume after the water has been cleared from the hoses.

2.5 Halltech Contact Information

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3.0 MUDPUPPY CAPTURE

An alternative method to identify sites (e.g., reach) where live *S. ambigua* may be present consists of finding the host *Necturus maculosus*, Mudpuppy, and inspecting for signs of glochidial (larvae) infestation (encysted glochidia). Mudpuppy gills have not been examined for glochidial infestation in Canada; therefore, this needs further investigation. To achieve this, Mudpuppy are captured, then visually inspected for the presence of suspected glochidia. Mudpuppy are easy to capture (when abundant) and it is cost-effective (Murphy et al. 2016).

Mudpuppy can be captured by two methods: manual survey and trapping. There are two manual surveying techniques: “noodling under rocks” and “nocturnal survey”. These methods are especially effective in shallow, clear water and it is successful year round (Matson 1990, Nickerson et al. 2002, Trauth et al. 2007). “Noodling under rocks” works best in late spring and early summer when Mudpuppy are guarding their offspring and staying under flat rocks or debris (Petranka 1998).

“Nocturnal surveys” were designed to catch Hellbenders (*Cryptobranchus alleganiensis*) from June-September (Foster 2006), but it has been proven to be effective for locating Mudpuppy especially during the winter given that this is the time of the year when Mudpuppy is more active (A. Haines, Buffalo State College, personal communication, 2018; F. Schueler, Fragile Inheritance Natural History, personal communication, 2011). In brief, this method consists of placing bait in the stream while surveyors watch with spotlights for animals from an elevated position (A. Haines, personal communication, 2018). Because the nocturnal survey method was not employed during our surveys, there is no detailed protocol for this technique described in this document; consult the above mentioned references for more information about this method.

“Noodling under rocks” is carried out during the day and consists of wading or snorkeling while flipping large flat rocks (Murphy et al. 2016) and looking under submerged debris. The duration of time spent surveying each site is relative to the total area of available habitat. Detailed methods are similar to what is described in Section 1.1 Salamander Mussel Survey, but instead of looking for the Salamander Mussel, you are looking for Mudpuppy. Some additional steps and considerations are described below in Section 3.1 Capturing Mudpuppy: Manual survey.

Mudpuppy can also be trapped using modified funnel-type minnow traps (Gendron et al. 1997, Gendron 1999, McDaniel et al. 2009, Chellman et al. 2017). The detailed protocol of this method is described below in Section 3.2 Trapping. For the modified funnel-type minnow traps the two openings of the trap were widened to allow the entry of adult Mudpuppy (Gendron et al. 1997, McDaniel et al. 2009). This type of trap has been used to trap Mudpuppy from the Lake Champlain basin, the St. Lawrence River basin, and the lower Great Lakes without any mortality or injury of the animal (Gendron et al. 1997, McDaniel et al. 2009, Chellman et al. 2017).

Although trapping has shown year round success (reviewed by Murphy et al. 2016), it is most effective from late fall through early spring as this is the primary foraging period for Mudpuppy (Shoop and Gunning 1967, Matson 1990). This is also the time of the year when encysted Salamander Mussel glochidia were observed on Mudpuppy gills (DFO unpublished data). McDaniel et al. (2009) had high Mudpuppy trapping success in the East Sydenham River, Ontario, during the winter and early spring. We had the most success trapping when water temperature ranged between 0ºC - 6ºC. In 2018-2020 these temperatures occurred in the months of November and March.

When water temperature is below freezing, the traps can become filled with ice (Figure 3.1). This is a problem in shallow rivers with flowing water, but is not a problem if trapping in deep pools or in lakes. Deep pools are great places to set the traps because they do not fill with ice; however, the surface of the river can freeze, making it very difficult to put the traps in the river as well as to retrieve the traps. A mallet or other tool will be needed to break the ice and can be labour intensive.

Be sure to check the weather before the field trip. If there is a chance of heavy rain or snow melt that could result in a significant increase in the water level, this may be problematic for flashy rivers such as the Sydenham River. Assess how water changes may affect placement and retrieval of the traps and plan accordingly.

The traps should be installed at sunset if possible and checked in the morning the following day to minimize the time individuals spend in the traps. This reduces stress, injury and potential cannibalism. The maximum time the traps should remain in the water may vary depending on the time of the year. A Mudpuppy could stay in the trap between 12 to 16 hours, but no more than 24 hours. Based on personal observations as well as previous studies (e.g., Gendron et al. 1999) Mudpuppy trapped for 16-24 hours have shown no signs of stress, injuries or death. No more than 24 hours should pass between setting and checking the traps.

From our experience, traps placed close to overhanging vegetation worked the best. However, there is a chance that the traps may get tangled in the vegetation. The team must weigh the risk of entering the water to retrieve traps if they become tangled. If entering the water to retrieve the traps is not possible, then traps should be placed where retrieval can be done safely from shore. When trapping occurs in late fall-early spring, the water can be cold posing an increased risk to safety. Do not enter the water if it is not safe. For our purposes, the best way to secure the traps was to wrap the rope around trees on the banks and secure them with carabiners attached to the ends of the ropes (see protocol below for details).

Traps can also be attached to a cinder block that is secured with a metal cable to a tree; however, retrieval of the cinder blocks is not very efficient and it does not allow the location of the deployed traps to change, which is sometimes necessary given the field conditions (e.g., ice accumulation, water levels, etc.). On the other hand, the use of cinder blocks can be used if traps are to be set in the middle of the river or in parallel line traps

(see McDaniel et al. 2009). The best way to set up and secure the traps depends on the study objectives and field conditions.

The number of traps per site and the number of sites must be determined based on the project goals. To bait the traps, freshwater wild caught fish, frozen marine fish, store-bought canned sardines, smelt or cat food can be used. If using freshwater wild caught fish it must be from the same river that the traps are being set in. Never move wild caught fish or bait across rivers. If using store-bought frozen fish, use marine fish instead of freshwater fish to decrease the introduction of potential invasive species.

When this protocol was designed, suspected encysted glochidia were clipped from the gill filaments to verify that they were glochidia. The protocol to anesthetize and clip the gill filaments is included in Section 3.2.4 Clipping Glochidia, but this is not necessary every time a Mudpuppy is trapped; visual inspection of encysted glochidia (Section 3.2.3 Handling of Mudpuppy for Visual Inspection) may be sufficient. An approved animal care protocol may be required to either visually inspect Mudpuppy for signs of glochidia and/or to clip gill filaments. Check with the appropriate Animal Care Committee or designated authority before conducting any work involving Mudpuppy. Also consider that obtaining approval may take some time (possibly months).

Figure 3.1. Ice accumulation in the traps.
Ice accumulated in the traps when water temperature is below freezing and traps were placed in shallow/fast water.

3.1 Capturing Mudpuppy: Manual Survey

The manual survey described here is “noodling under rocks”, detailed methods are described above in Section 1.1 Salamander Mussel Survey, but instead of looking for Salamander Mussel, you are looking for Mudpuppy. Handling of Mudpuppy should

follow an approved handling protocol recommended by the appropriate animal care committee (e.g., CCAC 2004)

These are some additional steps and considerations:

1. Lift the rock slowly, Mudpuppy tends to hunker down as the rock is lifted.
2. Wait until suspended sediment settles to look for Mudpuppy.
3. If you see a Mudpuppy gently direct it towards the net.
4. Place Mudpuppy in a bucket with fresh river water during inspection (Figure 3.2 A-C). Mudpuppy must be kept in plastic containers with river water at all times except when taking measurements (Figure 3.2 D; see Section 3.2.3 Handling of Mudpuppy for Visual Inspection, Step 5). During the summer, keep the bucket in a shaded area if possible and change the water regularly to keep Mudpuppy cool. Also, during handling Mudpuppy may release mucus.
5. All lifted rocks will be placed in their original location and orientation in order to maintain the integrity of the habitat.
6. When handling Mudpuppy, wear clean gloves. Substances on the hands (e.g., anti-bacterial hand sanitizers, insect repellants and sunscreen) can be absorbed directly through an amphibian's skin potentially causing health problems.

Figure 3.2. Mudpuppy handling and data acquisition.
Mudpuppy processing can occur on the bank (A) or in the back of the truck depending on the environmental conditions. Mudpuppy must be kept in plastic containers with river water at all times (B, C). Measuring vessel (D). Images taken in the winter/early spring**.**

3.2 Trapping

3.2.1 Equipment Preparation at the Laboratory

1. See Appendix C for a complete packing list of equipment required for this survey and an example of the field data sheet.
2. Prepare the minnow traps by widening the two side openings. The number of traps depends on the study objectives.
   a. Prior to going to the field, modify the minnow traps by cutting the two ends of the trap so that the diameter of the opening is maximum 6.0 cm. Enlarging the opening allows the entry of adult Mudpuppy.
   b. Cover the edge of the opening with electrical or duct tape to prevent injury to Mudpuppy or other non-target species by sharp exposed wires while entering/leaving the trap.
3. Depending on how the traps will be laid out in the field, ropes with the trap hooks can be cut and pre-assembled in the laboratory (Figure 3.3).

4. Cut the target length of a polyester rope. The length of the rope and number of traps per rope depend on the project's objectives. For example, we placed five traps per rope spaced every 5 m. We left 10-15 m of extra rope at each end; therefore, we cut the rope 40-50 m long. We also had a second trap set up with two traps per line where each rope was 15 m; the first trap was placed on one end, and the second trap 5 m from the end leaving ~10 m of loose rope.
5. At this point only the trap hooks are being attached to the rope (each trap will be assembled and hooked in the field). Secure a trap hook to the rope where each trap will be placed (Figure 3.3). We used "U" bolts and nuts, but heavy duty cable ties can be used as well.
6. Tie flagging tape (of different colour than the rope) to the rope at the location of each trap. This makes it easy to find the hooks in the field to secure the traps
7. Place one large carabiner at each end of the rope. Using carabiners is the best and most versatile way to secure the traps to trees or logs on the banks, see below.
8. Wrap the rope on a reel if available. It is important to store and transport the ropes in a manner that prevents or reduces tangling.

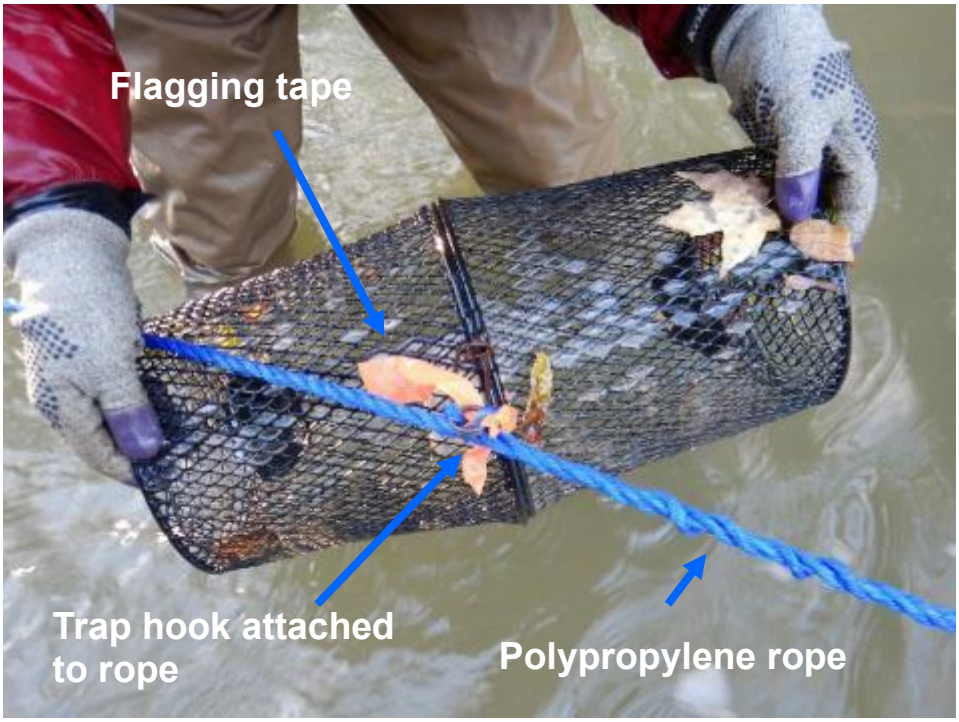

Figure 3.3. Traps set up.
Traps are attached to pre-setup ropes (rope with trap hooks and flagging tape) in the field.

3.2.2 Field Sampling

1. See Appendix C for a complete packing list of equipment required for this survey and an example of the field data sheet.
2. Check the weather and ensure clothing/gear is appropriate for conditions (e.g., dry suits in cold weather).

NOTE: Dress according to the weather and water temperature. Dress in layers as it is better to be overly warm than cold. During very cold days or if going in the water it is very important to use toe warmers. ALWAYS wear a life jacket!

3. The traps should be installed at sunset if possible and checked in the morning the following day, minimizing the time Mudpuppy spends in the traps. A Mudpuppy could stay in the trap between 12 to 16 hours, but not more than 24 hours.

NOTE: Check sunset times and plan the trap set up accordingly. It may take a long time to suit up, get to the river and set up the traps. Leave extra time in case there are some setbacks, you do not want to be in the river when it is dark, if possible. SAFETY FIRST.

Also, if setting traps at multiple sites and it is not possible to install all of them at sunset, it is okay if some are set earlier, but be sure to check these traps <u>first</u> the next day.

4. Find a location to place the traps. If conditions allow (e.g., appropriate water level), each trap will be placed on the bottom of the stream close to Mudpuppy preferred habitat using cinder blocks (McDaniel et al. 2009), otherwise traps will be placed from the shoreline (see below).
5. Lay out the ropes on a clear space if possible near the banks (Figure 3.4 A).
6. Place the bait in each trap (Figure 3.4B).

NOTE: When baiting the traps, it is better to leave the canned bait in the can without the lid. Some canned sardines or small filets will disintegrate in the water as soon as the trap is submerged. Leaving the bait in the can protects the bait (Figure 3.4 B).

7. Hook the traps to the rope (Figure 3.4 B).
8. Before tossing the line, secure the rope to a structure by wrapping the end of the line around the structure and securing it with the carabiner (Figure 3.5 A, B). Keep in mind the water level may change overnight. If there is a chance of rain and an increase in the water level, secure the trap lines to a structure that will not be underwater the next day so that you can still access it safely.
9. One person can cast a rope with five traps, but it is best if two or three people cast the trap line. Toss the traps as far out from the bank as you can, that way the current (if any) will take the rope downstream (Figure 3.5 C, D).
10. Traps must be completely submerged under water and secured to the shoreline.

Figure 3.4. Traps set up in the field.
Lay out the rope (A), put the bait in the traps and then hook the traps to the rope (B).

11. Traps are placed for one night per site, unless there are no captured Mudpuppy, then the traps will be placed for additional nights until a Mudpuppy is trapped (number of nights may vary according to the project's objectives).
12. The next day plan to arrive at the site before sunrise to get in the water to check or retrieve the traps with the first light when safe to do so.
13. Slowly pull in the rope checking each trap as you reach it (Figure 3.6). If a live Mudpuppy is found, slowly open the trap while keeping it underwater (especially if the air temperature is below freezing). Remove Mudpuppy and place it in a bucket or open container with fresh river water. Change the water as needed; during handling as Mudpuppy will release mucus.

NOTE: Do not warm up the water (e.g., water in the heated truck), or use distilled water.

Figure 3.5. Traps set up in the river.
Rope with traps can be secured by attaching it to logs or living trees along the bank (A, B). Once the rope is secured, the traps can be tossed into the river (C, D).

14. If a Mudpuppy is trapped, then all traps will be removed (depending on the study objectives; for example mark-recapture, you may want to leave the traps).
15. All non-target species present in the traps must be immediately released back into the water. Take notes of the trapped non-target species.
16. If air temperature is below freezing, all inspections should be carried out in the back of the truck or other temperature controlled area. Otherwise, inspection can be conducted on a flat area of the river bank or by the truck (Figure 3.2).

Figure 3.6. Checking the traps.

3.2.3 Handling of Mudpuppy for Visual Inspection

1. See packing list (Appendix C) for required equipment when visually inspecting Mudpuppy and an example of the field data sheet. If the weather is suitable consider bringing the “Mudpuppy processing kit” and other necessary equipment to the site.
2. Inspection of Mudpuppy can be carried out by a two-person team to minimize the handling time as it should not take more than 5 minutes per animal.
3. The animal should be restrained with an open flat hand applying even pressure over the animal's entire body. Avoid holding or applying any pressure to the tail (CCAC 2004).
4. Visually inspect for signs of glochidial infestation in the gills, the ventral edge of the tail and in-between the toes (Megan Bradley, US Fish and Wildlife Service, personal communication, 2018; Figure 3.7). If necessary a hand lens can be used to look for encysted glochidia.

5. Record sex, total length (snout–vent length), and if possible, total weight of all animals.

NOTE: These are non-invasive procedures; therefore, Mudpuppy will remain awake (i.e., no use of anesthetics). Mudpuppy must remain submerged in water during the inspection (except during a brief period of time while weighing). This method does not inflict any pain on Mudpuppy and handling time will be minimized to avoid stress.

6. After inspection, Mudpuppy should be returned to the river.

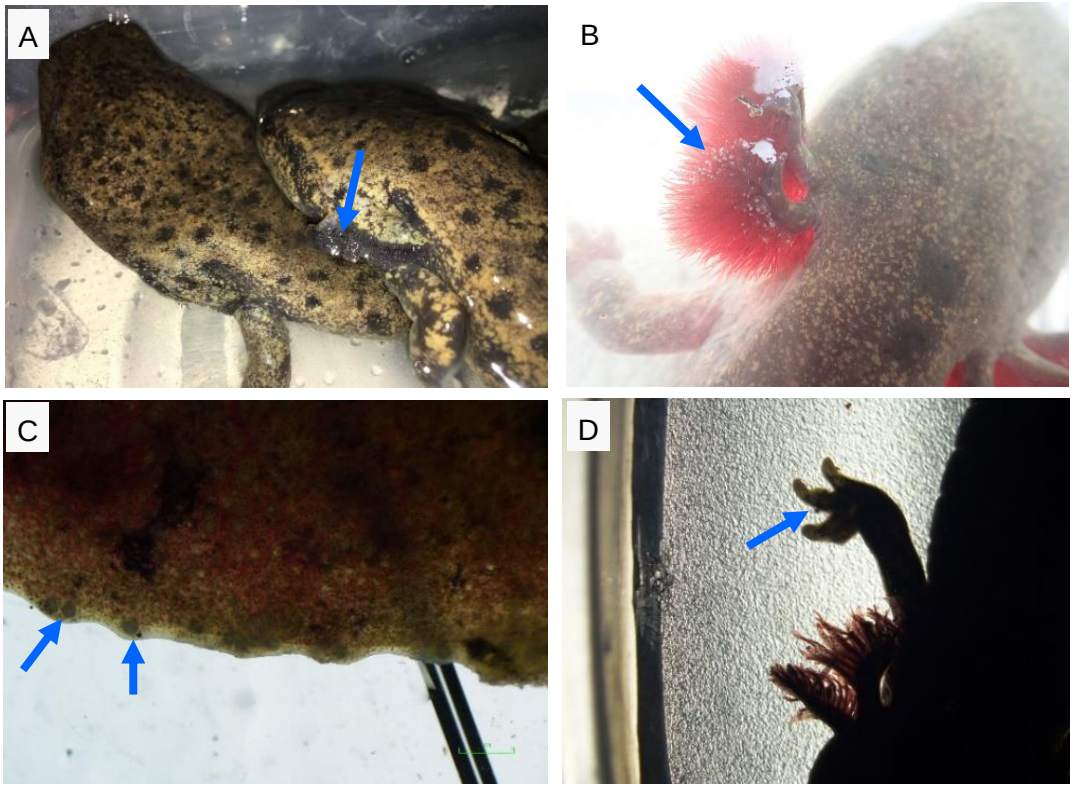

Figure 3.7. Signs of glochidia infestation in a Mudpuppy.
Glochidia are mostly found on the gills (A, B), but can also be found on the ventral edge of the tail (C) and/or in between the toes (D). Blue arrows indicated encysted glochidia. Images (A, C and D) courtesy of Megan Bradley, Genoa National Fish Hatchery, US Fish and Wildlife Service.

3.2.4 Clipping Glochidia

If necessary, to verify that the suspected glochidia are actually glochidia or to collect glochidia for species confirmation, a gill filament clip containing the structure can be carried out.

Procedure:

1. To clip gill filaments, animals are anaesthetized by submersion in a mild solution (0.25 g/L, 0.025%) of MS-222 (Tricaine methane sulfonate) following the Animal Care Committee (ACC) Standard Operating Procedure (SOP)- 10 Mudpuppy Blood sampling protocol (2002) recommendations (Appendix D). Even though blood samples will not be collected, the ACC SOP- 10 Mudpuppy Blood sampling protocol is specific to Mudpuppy and it describes the anesthesia methods for handling animals in the field, during the winter. The proposed dose of 0.025% MS-222 is recommended when water temperature ranges between 0°C - 6°C (Gendron et al. 1997), which is the water temperature the trapping and handling of Mudpuppy occurs. This dose was sufficient to anaesthetize Mudpuppy with no subsequent mortality in this study as well as studies by Gendron et al. (1997) and McDaniel et al. (2009).
2. Gill filaments are cut using sharp scissors or nail clippers. The scissors must be cleaned with soap (e.g., mild dish detergent) and disinfected with 95% ethanol prior to the procedure. Instruments (e.g., tweezers and scissors) should be disinfected using 95% ethanol between use for each animal to prevent any possible spread of disease or parasites from one individual to the next.
3. Gill filaments are very slippery, so the cutting instrument must be very sharp. Sometimes the gill must be held with tweezers while cutting; however, it is very challenging to cut the filaments.
4. Gill filament samples are placed in a 1 mL tube with 95% ethanol. Some water carried on the tweezers may end up in the ethanol tube. Change the ethanol within 24 hours by gently removing the majority of the ethanol-water solution with a plastic pipette and replacing it with 95% ethanol.

NOTE: The ethanol dehydrates and changes the colour of the tissue which is not ideal for taking good quality pictures. If you need to take nice pictures, take them as soon as possible and/or consider fixing the gills in a different solution (e.g., formalin). In our case they were fixed in ethanol so the DNA was preserved.

5. After gill samples are taken, animals are placed in a fresh river water bath until they have fully recovered. Any animals that do not recover will be euthanized using a 10 g/L (0.1%) solution of MS-222 following the GWACC-107 – Fish Euthanasia protocol (2018). (GWACC stands for GLLFAS/ Water Science and Technology Directorate (WSTD) Animal Care Committee (ACC).

6. Gill filament clips are stored in >95% ethanol and transported to the laboratory for further analyses. Place all gill filaments from one individual in the same tube. Label the tube with Mudpuppy ID, site ID and date. If necessary, gill filaments can be further separated into individual tubes at the laboratory.
7. In the laboratory, the glochidia-like structure will be analyzed under a microscope to verify that the structure is a glochidium.
8. Subsequently, DNA can be extracted for genetic barcoding using mitochondrial genes.

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APPENDICES

Appendix A. Salamander Mussel Rock Flipping sampling packing list and suggested field data sheet

NOTE: This is a suggested list of items needed, use of additional items and exact quantities must be determined based on each project's needs.

Task	Item
Personal gear	Waders
	PFDs/life jackets
	Sunblock
	Bug repellant
	Surveying gloves
Survey equipment	Mesh bags
	D-frame aquatic dip net
	Wood sieve
	Small orange buoys/floaters
	Small plastic boxes with lids
	Field sheets
	Vernier calipers
	Clip board (with pencils, eraser pencil sharpener)
	Camera + charger + memory card
	Sand box to take pictures + ruler
Other	GPS location tracker-trace device
	GPS + charger
	Water quality sampling and monitoring meters
	First aid kit
	Drybag backpacks (various sizes)

Simpsonaias ambigua collection data sheets

Date: _____ **Coordinates:** _____

Site name: _____ _____

Site ID: _____

Waterbody: _____

Time arrival _____ **Time departure** _____

Site description _____

(Where did you park? Access to water, etc.) _____

Habitat description _____

_____

Collectors: _____

Time search:

people _____ Time searched (total) _____

Start coordintaes _____ End coordinates _____

Sample number	Rock#/Coordinates	Length/height (mm)	Sex (F/M)	Gravid	Swab	Gonad/ Water tubes	Picture #	Notes

Other unionid species?

_____

_____

_____

Appendix B. eDNA Sampling using OSMOS backpack sampler packing list and suggested field data sheet

NOTE: This is a suggested list of items needed, use of additional items and exact quantities must be determined based on each project's needs.

Task	Item
Personal gear	Waders
	PFDs/life jackets
	Sunblock
	Gloves
	Bug repellant
Other	GPS location tracker-trace device
	GPS + charger
	Camera + charger + memory card
	Water quality sampling and monitoring meters
	First aid kit
	Drybag backpacks (various sizes)

(List continuation on next page)

Task		Item
eDNA	OSMOS	OSMOS- Backpack sampler in pelican case
		Tripod and telescopic pole with hoses
		OSMOS battery- charged
		Pivot clamp (in pelican case)
		Silicone gel
		Filter housing (Inlet and Final stage)
	Sample collection	eDNA filters
		Tweezers-preferably blunt end
		50 mL transport tubes
		Storage cooler (to store eDNA samples)
		Ice pack/ice cubes (ice is preferred)
		Decontaminated control cooler/plastic container with lid for blanks
		~ 3-4 L of distilled water in decontaminated plastic bottle to rinse bleach off items (e.g., filter housing and tweezers) in the field per day.
		~500 mL per site of distilled water for Control Blanks
		Nitrile gloves (various sizes) in resealable bag
		Bag to dispose of used gloves
		Thin permanent marker
		Paper towels
		Bottle hydrogen peroxide (HP)* or 10% bleach solution**
		* If using HP, ~50 mL container to submerge tweezers
		**If 10% is brought, also bring 1 L of distilled water
		Spray bottle (for 10% bleach)
		1 L Bottle bleach
		Denatured ethanol (200 proof)
		Plastic pipettes 1mL (to add ethanol to filters) with 200 µL mark
		Large resealable bags or Whirl-packs®
		Field sheets (eDNA)
		Clip board (with pencils, eraser, pencil sharpener)

eDNA sampling using OSMOS

Water body: _____ Date: _____
Site Name _____
GPS coordinates _____
Crew: _____
Notes: _____
Location where the sample was taken (bank, mid-channel), other remarks. _____

Sample #	Filter pore size	Filtered volume	Notes
BLANK			

Water temp _____
Discharge/water levels _____

eDNA sampling using OSMOS

Water body: _____ Date: _____
Site Name _____
GPS coordinates _____
Crew: _____
Notes: _____
Location where the sample was taken (bank, mid-channel), other remarks. _____

Sample #	Filter pore size	Filtered volume	Notes
BLANK			

Water temp _____
Discharge/water levels _____

Appendix C. Mudpuppy Capture, gill clips and glochidia visual inspection packing list and suggested field data sheet

NOTE: This is a suggested list of items needed, use of additional items and exact quantities must be determined based on each project's needs.

Task		Item
Personal gear		Drysuits + boots
		PFDs/ life jackets
		Neoprene gloves
		Hand and feet warmers
Mudpuppy Trapping	**General kit**	Duct tape
		Electrical tape
		Flagging Tape
		Cable ties- 48 inch black extra heavy duty
		Bag for garbage
	Setup	Metal clothesline
		Cutting board and knife
		Bait
		Traps
		Polypropylene rope with trap pins and carabiner (wrapped on reels)
	Setup kit	EXTRA trap pins
		Pliers
		U-bolts
		Wire cutter
		Flashlights
		AA batteries for flashlights (extra)
		Headlamps
		EXTRA carabiner
		Large scissors

Task		Item
Processing Mudpuppy		Clip board
		Field Sheets
		Pencils
		Vernier calipers
		Wooden measuring trough/board
		Stereoscope
		Magnifying lamp
		Scale (300 grams)
		Tupperware to weigh Mudpuppy
	Mudpuppy processing kit	Hand lens
		Nitrile gloves (various sizes)
		Long transparent plastic containers to hold Mudpuppy while taking biopsy
		Labeled 1 mL tubes with Ethanol 95% in box
		Metal tweezers
		Metal small scissors (mustache/nail)
		Whirl-packs
		Small resealable bags
	Anesthesia and biopsy	MS-222 Stock solution (2.5 g/L) (amber jar)
		Plastic containers with lids for MS-222 bath + recovery
		Plastic beaker (500 mL)
		Mild dish detergent
		Ethanol (95%) (sterilize scissors)
		Small plastic container to rinse scissors
		Paper towels
		Plastic funnel 6 inch wide
		Plastic waste containers for MS-222 (1 Gal)
Other		GPS location tracker-trace device
		GPS + charger
		Camera + charger and memory card
		Water quality sampling and monitoring meters
		First Aid Kit
		Drybag backpacks (various sizes)

FRONT SIDE

Mudpuppy trapping and *Simpsonaias ambigua* encystment

Date: _____ Site: _____ Coordinates: _____

_____

Diagram of traps palcement

Trapping effort

Time arrival site: _____ Time crew got out water: _____

Time traps went in: _____

out/checked: _____ Total time: _____ Notes: _____

_____

Number of traps:	Bait used	Amount of bait	Notes

Trapped Mudppupy data

No.	Weight (g)	Sex	Length (cm)	Attached glochidia (Y/N)	Time in MS-222	Gill filament biopsis (Y/N)	Time in recovery bath	Mudpuppy Recovered (Y/N)	Picture #
1									
2									
3									
4									
5									
6									
7									

Additional notes: _____

_____

_____

_____

BACK SIDE

Mudpuppy trapping and *Simpsonaias ambigua* encystment

Date: _____ Coordinates: _____
Site: _____ _____

Site conditions (when checking traps AM):

Air temp: _____ Water temp: _____
Other: _____

Water quality (measured upstream end of trap line):

Trap location (from map)							
Water depth							
ODO (mg/L)							
ODO %							
Cond (uS/cm)							
pH							
TDS (mg/L)							
Salinity							
Turbidity							
Flow 1							
Flow 2							
Flow 3							
Flow 4							

Appendix D. ACC SOP-10. Mudpuppy Blood Sampling

ACC SOP-10. Mudpuppy Blood sampling. Pamela Martin and Tana McDaniel, Environment Canda (Reprinted).

ACC SOP-10 Mudpuppy Blood Sampling | **Revised: Nov 2002/Dec 2006**
Ontario Region - Wildlife Toxicology | Canadian Wildlife Service
National Wildlife Research Centre | Environment Canada
Author: Pamela Martin / Tana McDaniel | ACC Reviewed: date

Blood sampling of mudpuppies in winter

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1. Scope and Field of Application

Blood sampling is a viable alternative to lethal sampling methods for measuring biomarkers and contaminant levels in either wild caught or captive animals (Bishop and Martinovic 2000). Blood plasma can be used to measure circulating levels of hormones, DNA, albumin, lipophilic contaminants, heavy metals, and standard blood chemistry variables. This document describes the method used by Gendron et al. (1997) to sample wild caught adults.

2. Purposes

To ensure the safe and humane conduct of this sampling technique in mudpuppies.

3. Considerations

Since the blood samples are often used to measure compounds that are sensitive to capture methods, particularly in terms of the timing of sampling, care must be taken to ensure that blood sampling is appropriate. Measurement of corticosterone, for example, usually requires relatively little stress to the study animal prior to sampling, so some capture methods may reduce the validity of the measurement. Typically, the blood plasma but not the cellular fraction is used to measure biomarkers and contaminants, and access to a centrifuge, generator and cold storage below -20 °C is required. Also, bleeding mudpuppies entails some failure in obtaining a blood sample, so more individuals are required than the target number of blood samples. To ensure an adequate final sample size, assuming that 30% of the attempts will fail should give a good safety margin. Mudpuppies also vary in size; trapped individuals are expected to range in weight

between 50 to 400 g. In order to increase blood sampling success, and reduce physiological stress that may be induced by sampling from small individuals, only those animals greater that 200 g will be sampled for blood. Amphibians are ectothermic, and their physiological rates track the ambient temperature. Sudden extreme temperature changes can be stressful, and in some cases lethal to amphibians. Care must be taken to maintain animals at stream temperature during all stages of blood sampling including anesthesia and recovery. Ideally such sampling will be conducted during times of year when ambient air temperature is similar to water temperature. Animals will be handled at the collections site with no transportation required. They will be held in buckets of water taken from the stream immediately prior to collection, thus keeping them at the ambient temperature to which they are accustomed. These animals are completely aquatic and respire through the use of external gills. Care must be taken to minimize handling time in the air. Mudpuppies will be handled using gloves and placing in a plastic bag as the plastic reduces rubbing of the mucous skin coating. As the animals are frequently captured in very shallow water (< 0.5 m) it is not suspected that ambient daylight will present a problem.

The dose of anaesthetic that we are suggesting (0.025% MS-222) is higher than the recommended dose of 0.01% MS-222. The lower dose has been found to be effective at room temperature (20C). Sampling by CWS will take place in winter months when the water temperature is between 0 C and 10 C. Since mudpuppies are ectothermic their metabolic rate is dependent upon temperature and slows considerably at these low temperatures. Gendron (pers. comm.) has found that the uptake of MS-222 was reduced considerably at 0C to 5 C, and was also inhibited by water hardness levels typical of southern Ontario (60 to 120 mg/L). The anaesthetic dose used by Gendron et al 1997 for winter mudpuppy sampling was 0.25 g/L (0.025% MS-222). This was used on animals that ranged in weight from 50 grams to 400 grams, and was conducted at a site with water hardness that ranged from 60 to 120 mg/L and at water temperatures that were below 5C. If field conditions vary considerably from the conditions of Gendron et al 1997, then it is understood that we would revert to the recommended dose of 0.01% or 0.1 g/L of MS-222 until further testing has been completed. If either the times for anesthesia or recovery vary from the approximate time of 4 minutes recommended by Gendron (pers com) then rates will be adjusted, observations made, and an updated SOP will be submitted. MS-222 stock solutions must be kept refrigerated and maintained at a pH 7. The shelf life for a solution is approximately 2 months and is stored in an amber glass bottle to protect it from light. Unused solution and diluted bath is returned to the lab and disposed of according to protocols for the disposal of hazardous wastes.

4. Materials and Equipment

Adults:

cryomarkers to label cryovials
1 mL cryovials to store plasma
27 gauge needles
1 cc syringes
heparin to prevent clotting of blood
centrifuge tubes to hold blood while spinning
micro-centrifuge to spin blood
generator to power centrifuge
shipping dewars (2) for storing plasma at -80 C
Glass Pipets and bulbs
Sharps disposal container
2 plastic containers to hold anesthesia bath and euthanasia bath
1 bucket for holding and recovery bath
MS-222 to make euthanasia and anesthesia bath
pH meter
Foam holding tray

5. Procedure

(1) Anesthesia

A) Preparation of baths:

Pre-prepare an MS-222 stock solution in the lab by dissolving 2.5 grams of MS-222 in 1 litre of de-chlorinated tap water. **Do not** use distilled water as this will place the animal under osmotic stress. Adjust the pH to 7.1 using NaHCO3.

Prepare an anesthesia bath of 0.025% (or 0.25 g/L) MS-222, by adding one part stock solution to 9 parts fresh river water. This bath should be made daily to ensure that it is always fresh. If the bath becomes foamy during use, it should be discarded and a new bath prepared with fresh river water. The bath should be prepared just prior to use so it is a fresh as possible. If the air temperature is below 0 °C then care must be taken to prevent the bath from freezing - this can be accomplished by keeping the bucket sitting in a shallow portion of the stream.

Prepare a euthanasia bath of 0.1% (1 g/L) MS-222 by adding 4 parts stock solution to 6 parts river water.

A recovery bath can be made simply by filling a large plastic bucket with river water. This can be changed between animals to insure that it is fresh.

B) Anesthesia: Place the mudpuppy in the anesthesia bath and monitor. The animal should be anesthetized within 4 minutes. Rinse the animal quickly in the recovery bath to halt absorption of anesthetic. If the animal is not anesthetized in a reasonable period of time, i.e. 6 minutes, then the concentration of the bath should be adjusted.

(2) Blood sampling

A foam holder is used to secure the mudpuppy during blood sampling. This holder consists of a 1 x 2 x 3 feet piece of upholstery foam with two v-shaped slits cut into it, that are 30 and 20 cm in length and 5 cm wide. The mudpuppy is placed in a slit in the foam with the ventral surface exposed. This will hold the mudpuppy firmly, but without damaging its delicate skin. The foam will be soaked in water prior to each use.

Blood will be obtained from the caudal vein by inserting a 27 gauge needle into the underside of the animal near the base of the tail fin (behind the cloaca). The needle is inserted vertically (with a slight angle) through the musculature of the tail toward the vertebral column. The objective is to tap into the caudal vein just before the backbone. Before inserting the needle, the plunger of the syringe will be pulled partially out to create a small vacuum so that when the vein is encountered blood will flow into the syringe (we will use a 1 ml syringe). The syringes will be coated with heparin before use to prevent the clotting of blood. Once the vein has been located the plunger is pulled out further slowly until sufficient blood has been collected (0.5 to 1 ml of blood). Care will be taken not to pull too fast: the heart beat of the mudpuppy can be very low especially if captured during the cold season at water temperature of 4C.

(3) Recovery

The animals will be immediately placed in a recovery bath consisting of fresh river water at ambient water temperature. The mudpuppy will be monitored and should recover within 4 to 6 minutes. The animal will be released when it has regained the ability to swim. Any animals that do not recover from the anesthetic will be euthanized by immersion in the euthanasia bath and their bodies kept for later dissection and tissue collection.

(4) Preparation of sample

Blood samples will be placed in heparinized eppendorf tubes and centrifuged in the field. Plasma will be separated and transferred to labeled cryovials and placed in liquid nitrogen in the field. Storage subsequent to analysis will be at -80C.

6. References

Gendron AD, CA Bishop, R Fortin and A Hontela. 1997. In vivo testing of the functional integrity of the corticosterone-producing axis in mudpuppy (Amphibia) exposed to chlorinated hydrocarbons in the wild. Environ. Toxicol. Chem. 16: 1694-1706.

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