FRESHWATER MOLLUSCS

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RAPID BIOASSESSMENT METHODS FOR FRESHWATER MOLLUSCS

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Introduction

Freshwater molluscs are found worldwide, occurring on all continents except Antarctica. There are approximately 1,200 species of freshwater bivalves, 97% of which belong to eight primary freshwater families: Unionidae, Margaritiferidae, Hyriidae, Mycetopodidae, Iridinidae, and Etheriidae (all Unionoida or freshwater mussels), Sphaeriidae, and Cyrenidae (both Veneroida) (Graf 2013). The world's freshwater gastropod fauna comprises approximately 4,000 described species (Strong *et al*. 2008). Many species are globally imperiled and freshwater molluscs are considered to be the most threatened group of animals in the world (Williams *et al*. 1993; Lydeard *et al*. 2004; Johnson *et al*. 2013).

Freshwater mussels (unionoids) are an integral component of aquatic ecosystems. Freshwater mussels can comprise >90% of the benthic biomass of rivers and an individual mussel can filter 40 L of water each day (Tankersley & Dimock 1993; Pusch *et al*. 2001; Strayer 2008). In addition, their shells function as substrate for many organisms including caddisflies, mayflies and other aquatic insects. Unionoids are often described as ecosystem engineers due to the direct and indirect physical effects that they have on freshwater ecosystems (Gutiérrez *et al*. 2003). Freshwater mussels also provide important direct services to humans, such as water purification, serving as an important prey for several mammals and commercial fishes, and providing a direct source of protein. Given their importance within aquatic ecosystems, the cascading consequences of unionoid declines can be considerable (Haag 2012; Vaughn *et al*. 2015).

Freshwater snails graze on biofilms on rocks and vegetation, and some are suspension or deposit feeders. Gastropods can numerically dominate benthic stream communities and may exceed 50% of the invertebrate biomass. Gastropods are the principal grazers in many aquatic habitats and significantly influence algal primary productivity, playing a pivotal role in aquatic food webs and nutrient cycling (Johnson *et al*. 2013; Pyron & Brown 2015).

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Freshwater molluscs are ideal organisms for rapid biological surveys. Many are conspicuous and for the most part are easily and inexpensively sampled. They are sensitive to anthropogenic disturbance and are considered excellent indicator species. Freshwater mussels are sometimes colloquially referred to as “aquatic canaries in the coalmine” or “livers of the rivers” due to their sensitivity to changes in the environment and water quality and their water filtering capacity.

Freshwater mussels have been harvested for a variety of purposes, including for food, buttons, natural pearls, and as seed material for the commercial production of marine pearls. They were collected and utilized by indigenous people, particularly the mound-building tribes of North America, at least as early as 5400 years ago (Saunders *et al*. 1997). Mussels were not only eaten, but also used for tempering pottery and for making utensils, tools, and jewelry (Lucey 2000; Serrand & Cummings 2014). Freshwater snails also serve as a food source for humans in many parts of the world.

Some species of freshwater molluscs are highly invasive and can change the functioning of ecosystems, cause considerable damage to crops (e.g., some *Pomacea* spp.), spread diseases like schistosomiasis or liver flukes, are biofoulers that impact industry (e.g., zebra, quagga and golden mussels in the genera *Dreissena* and *Limnoperna*), or are detrimental to other wildlife (e.g., New Zealand mudsnails, *Potamopyrgus antipodarum* impacting trout) (Bequaert 1928; Strayer 2010; Sousa *et al*. 2014; Van Bocxlaer *et al*. 2014; Cummings & Graf 2015; Pyron & Brown 2015).

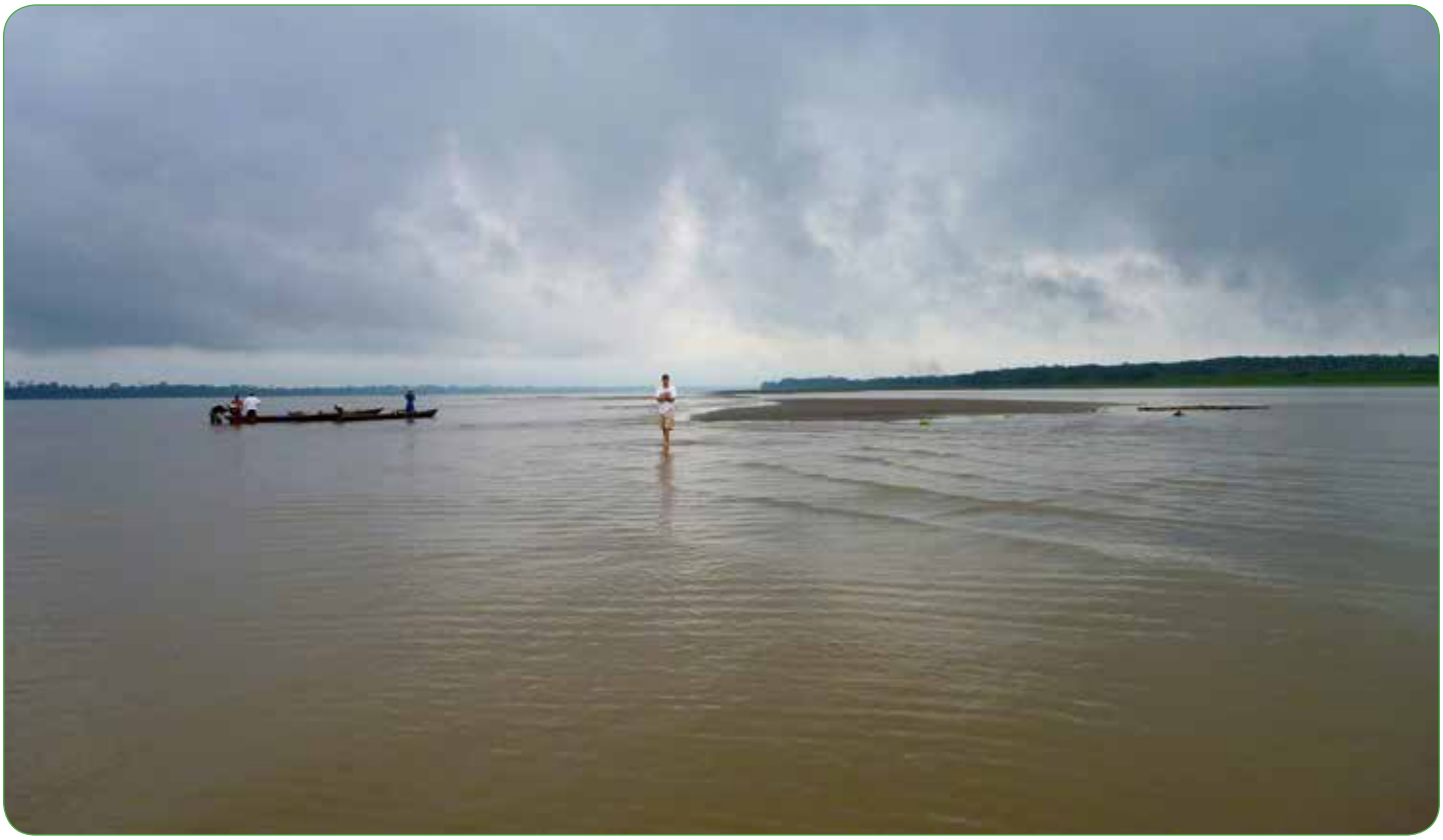

Figure 1
An example of a large river. The Rio Amazonas just outside of Iquitos, Peru.

Core Standardized Methods

Because of the wide variety of habitats occupied by freshwater snails and bivalves, no single sampling method is applicable across all species. The methods presented here will work on all continents and in both temperate and tropical ecosystems. Before fieldwork commences it is essential to do a thorough review of museum collections, the literature and to contact experts to compile data on what species are known from, or could potentially occur, in the study area. A comprehensive risk assessment should be carried out to obviate health and safety issues.

The definition of what constitutes a site varies, but in general, a site is typically an area that can be reasonably searched without traveling a great distance in a relatively short period of time. An area of about 100-300 meters of stream encompassing most of the habitats (i.e., pools, riffles, runs) is a good rule of thumb.

We highlight some basic safety rules for any mollusk survey:

- Avoid working alone, particularly in remote regions.
- Avoid sampling rivers that are in spate.
- Avoid sampling from steep or unstable banks unless equipped with appropriate safety gear, and always test the depth and stability of waterbodies before entering the water.
- Be careful when transporting and handling flammable or toxic liquids (e.g. formaldehyde and ethanol).
- Beware of potential hazards including broken glass, needles, discarded medical equipment, etc., especially when sampling urban rivers.
- Wash hands carefully with soap or a sanitizing spray after the work and before drinking or eating.
- Wear protective equipment (e.g. wet-suits, waders, rock-fishing boots, and gloves to prevent cuts and abrasions).
- Carry at least one first-aid kit.
- Let someone else know where you are going, and carry an Emergency position indicating Radio
- Beacon (EPIRB), mobile phone or satellite phone. Establish a reporting protocol for checking in at the end of each day.
- Be mindful of potential infectious diseases; in case of any eventual symptoms the surveyor should seek medical attention.

In some countries (e.g., Australia) protocols have been developed for sampling in waterbodies containing crocodiles (e.g., DERM 2011) and training is available via crocodile awareness programs to prepare people for fieldwork in waterbodies where these reptiles may be present. Precautions for working in crocodile-infested waterbodies include:

- Using local knowledge whenever possible – local inhabitants will often know if there are large crocodiles in the area.
- Always work in teams of two or more people: one person samples while another keeps watch, holding a whistle to use as an alarm if a crocodile is sighted.
- Avoid sampling sites with treacherous terrain such as steep, muddy banks and turbid waters
- Set up defensive barriers such as sturdy nets around the sampling area.

For rapid surveys, the methods used are habitat dependent. We have identified 5 major habitat types where freshwater molluscs are most frequently found: large rivers, medium-sized rivers and creeks (wadeable streams), lakes (natural and artificial impoundments), wetlands, springs and caves (Fig. 1-4). The following protocols are applicable to all of the habitat types with some modification. However, as most surveys will be conducted in wadeable streams and to a lesser extent on large rivers, the following methods were developed with those habitats in mind.

A wide variety of techniques are used to survey freshwater bivalves and large gastropods, and the method used will depend on the goals of the study and the resources available (Strayer & Smith 2003). The method chosen will influence and limit the way data can be interpreted so it is important to be clear on the objectives of the biodiversity assessment. Sampling methods for molluscs can be categorized as either qualitative or quantitative.

Qualitative sampling includes visual and tactile searches of the streambed, dip net sweeps, use of brail hooks for mussels, searches of the stream bank for shells, and under rocks for gastropods or inside dead bivalves for some fingernail clams (Fig. 5-8). Quantitative methods may include dredging, use of grab samplers or, more usually, quadrats or linear transects distributed over a defined area according to a defined sampling strategy. Quadrats require excavation of the substrate combined with sieving so that buried mussels, and small or cryptic molluscs are not overlooked.

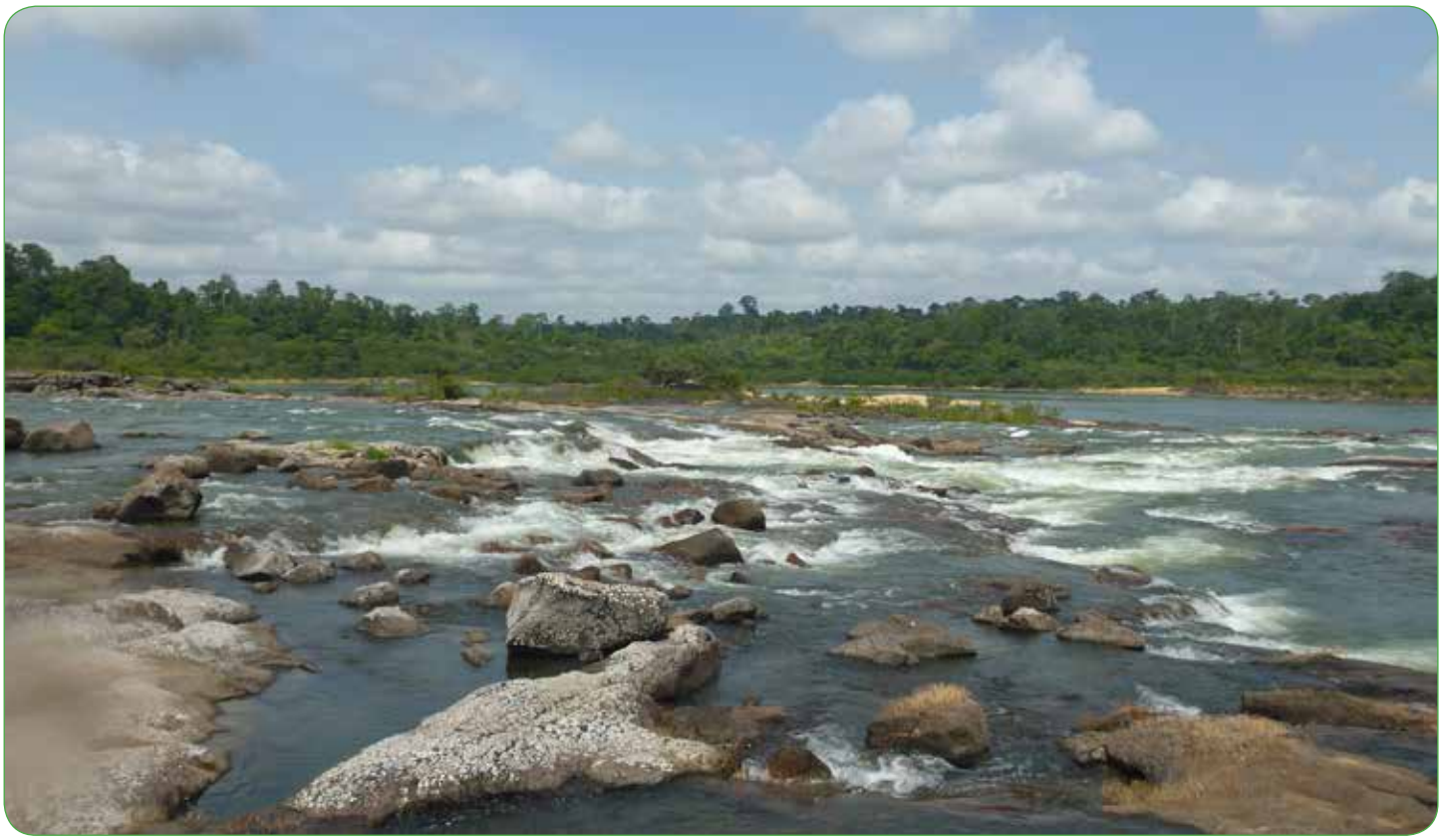

Figure 2
An example of a medium to large river. The Rio Xingu, Para, Brazil.

Surveys for molluscs based on visual or tactile searches of quadrats or a fixed area of stream bed, usually for a set time, are best considered to be semi-quantitative since detectability will vary with water clarity and substrate type, and they will be biased against small individuals and species (Hornbach & Deneka 1996).

If the main goals of a survey are to develop a species inventory or to detect rare or threatened taxa, then a qualitative survey should be adequate. However, where estimates of population density or age structure are required, quantitative methods will need to be used. Quantitative sampling should be used in baseline or monitoring studies where the objective is to assess changes in populations over time. Probability-based designs and quadrat sampling are recommended to provide estimates of uncertainty for abundance estimates and greater power for detecting change (Lindenmayer & Likens 2010; Downes *et al*. 2002).
The most common method for collecting freshwater mussels and conspicuous gastropods is simply tactile sampling in the substrate or picking up shells along the shoreline. Viewing buckets and snorkeling can supplement hand sampling, but they are only effective in clear water. Rakes or dredges can be used in shallow water with sandy or fine substrates to bring bivalves to the surface. Snails on vegetation and floating debris can easily be sampled, shaking them in to a white bucket or pan. Small snails and bivalves can be detected using stacked sieves or a kitchen strainer to process small amounts of detritus or sediment throughout the sampling site.

As stated above, no single sampling method is applicable for all species or groups due to the wide variety of habitats occupied by freshwater molluscs. Therefore, for rapid assessments we recommend the following four-step approach.

1. Reconnaissance surveys

A good indicator of current or past presence of molluscs in a waterbody is to search for stranded shells and shell middens along the stream bank and on logs and boulders in the waterbody. Often gastropod and bivalve shells accumulate in debris piles left by floods. The composition of shell middens can provide an indication of changes in the composition of the molluscan community over hundreds or thousands of years (Walker *et al*. 2001, Haag 2012). Consultation with local people can also be helpful in locating where to conduct surveys (Fig. 9-10).

Reconnaissance surveys are essentially exploratory with no set time limit. The intention is to determine presence and spatial distribution of molluscs at a site. A reconnaissance survey of the site will provide data on what molluscs occupy the area and the locations of mussel concentrations within a site, information that can be used to decide upon a more robust sampling method.
If the reconnaissance survey indicates that molluscs are clustered into particular habitats or areas, a stratified search should be done, with greater emphasis given to those areas where they appear to be most abundant. If the site is large then stratify the site by habitat or set out equally spaced transects or cells to ensure coverage of the entire site.

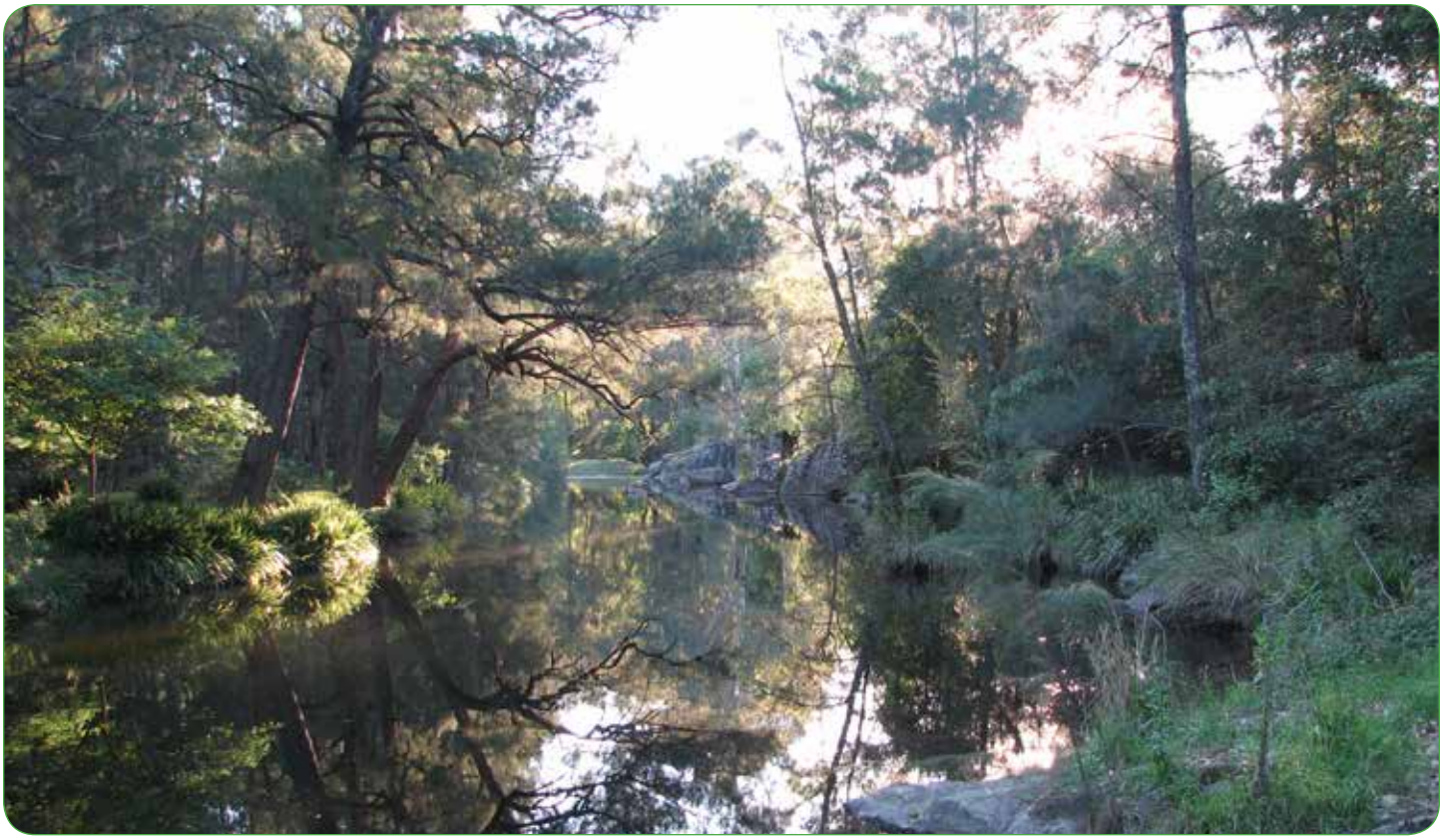

Figure 3
An example of a medium to small river. The Paterson River, a tributary of the Hunter River, Australia.

The available range of substrates should be explored, (e.g. mud, sand, rock, submerged logs, vegetation, and floating debris). In both large rivers and small streams efforts should be made to cover all available habitat types present at the site including riffles, pools, slack water, including searching along the banks versus center of the channel; lakes should be surveyed in quiet, protected bays as well as on exposed shores. Some ampulariid, lymnaeid and pomatiopsid snails can be often found at a considerable distance from the water, so the floodplain area should also be checked.

2. Timed searches for conspicuous bivalves and gastropods

A timed search across the range of habitat types is a rapid and effective technique for determining the species present at a site. The area to be searched depends on the habitats present but a length of about 100-300 m is a good rule of thumb. A variety of search methods may be used, depending on conditions. Viewing boxes are useful in clear, wadeable streams whereas tactile searches to a depth of 40 cm are appropriate in turbid water. Snorkeling, SCUBA or a surface supplied airline (hookah) are necessary for sampling in deeper water, especially in large rivers or lakes (Fig. 11-12).

Figure 4
An example of a small river. An unnamed tributary of the Luangua River, Zambia, Africa.

Rainfall generally suspends sediments in the water increasing turbidity, so surveys should be avoided in the period during or immediately following the rainy season. Additionally, water level is generally higher and the flow stronger during these periods, hampering the actions of the surveyors and decreasing efficiency. Surveys should preferably be undertaken in the dry season when water levels and water clarity are optimal for detecting molluscs. Whilst the time of day is generally not critical for mollusc activity, surveys should be conducted when light availability is good. Surveying during periods with low water clarity, high flow or high water levels will result in detecting fewer species, lower abundances and bias in species composition for the most conspicuous taxa. The suggested conditions for conducting the assessment applies for all steps (sections 2, 3, and 4).

We recommend undertaking timed searches at a site for a minimum of 4 person-hours. However, it has been demonstrated that, in North America, 4 person-hours detects about 60% of expected species. Ten person-hour searches captured more than 70% of all species at over 70% of the sites tested (Huang *et al*. 2011). No studies have been conducted in tropical streams to assess the time required to collect percentages approaching 70%.

Specimens should be placed in separate mesh bags (colors work well) at 1 person-hour intervals as they are collected so that sampling adequacy can be estimated from species accumulation curves. For a 4-person survey team this would require changing storage bags at 15 minute intervals.

The search times suggested here should be reviewed following analysis of species accumulation curves and adjusted accordingly to ensure that the majority of species are collected. The point of diminishing returns, where the curve flattens out, is a sensible time to cease sampling. An estimate of the total number of species present should be made using an estimator of species richness such as the Chao-1 estimator (Gotelli & Chao 2013).

3. Timed searches for small bivalves and gastropods (<2 cm length)

For this method we recommend undertaking timed searches in each habitat type for a minimum of 2 person-hours. A complete survey for freshwater snails and small bivalves will include sampling both benthic surveys and a variety of other substrates including macrophytes, crevices of rocks and wood, other types of floating debris and leaf litter. For sediment sampling, a “kick net” Surber bottom sampler with a rectangular or triangular opening should be used in flowing water. Save the sediments from each sample into lidded buckets for lab analysis or dump the sediments into white trays for sorting and identification in the field, collecting all specimens with forceps or plastic Pasteur pipettes.

For aquatic vegetation and other loose debris, flush the sample into a bucket or run a dip-net several times through it, examining the net contents carefully for small snails such as the hydrobiids, limpets, and small planorbids. Small kitchen strainers and white trays or buckets can be used as cheap and effective alternatives. For strainers, the mesh should have a maximum diameter of 1 mm to capture small or newly-hatched gastropods.

Figure 5
A brail (also called a crowfoot bar) with mussels attached to the hooks. Mississipp River, Illinois, USA.

4. Quantitative Sampling

The methods described above work well for answering basic questions about species presence or absence and richness, but timed searches tend to miss small individuals buried in the sediment and may give biased estimates of absolute abundance, proportional composition of species, and size structure owing to differences in detectability among species and individuals of different sizes. While not completely eliminating bias, implementation of strict search protocols will greatly reduce sampling bias and improve the precision of counts, allowing comparisons among sites.

Quantitative sampling overcomes these shortcomings, providing unbiased estimates of population parameters but it is time-consuming and increases survey costs considerably (Miller & Payne, 1988). Quantitative methods are usually conducted in the form of line transects or quadrat samples, although mark and recapture methods are occasionally used to estimate population abundance and other demographic parameters (Villella *et al*. 2004).

Typically, quadrats are placed on the bottom, and all substrate is removed to a depth of about 10 centimeters and passed through a series of sieves. This method is especially effective in recovering juvenile mussels and small species that are easily missed by hand grabbing (Fig. 13).

Molluscs, especially freshwater mussels, are often spatially aggregated in waterbodies. In this situation, stratified random sampling is a good choice for estimating abundance, especially when combined with an initial reconnaissance survey to delineate areas where molluscs are clustered at a site (Christman 2000). Systematic sampling is also a good choice as it is easy to implement and ensures that quadrats are spatially distributed throughout the site. The number of quadrats required to achieve a desired precision (d) is often expressed as the percentage deviation (p) from the mean ($\bar{x}$) and it depends on the variability of the count data among quadrats (s^2). The required sample size is expressed as:

$$n = \frac{(4s^2)}{(p\bar{x})^2} = \frac{(4s^2)}{d^2}$$

(Thompson 2012). At low densities (e.g. < 1 m^{-2}) the number of samples required achieve a precision of 25% of the mean may exceed 100 x 0.25 m^2 quadrats (Dunn 2000).

A large survey effort is required to establish the presence of rare taxa at a site. This is exacerbated for small or cryptic species that have low detectability (λ). Assuming that rare species are randomly dispersed, the following relationship can be used to estimate the power of a sampling program for the species for a given number of quadrats (n).

$$\text{Sampling power} = 1\text{-}\exp(-\bar{x}\lambda n)$$

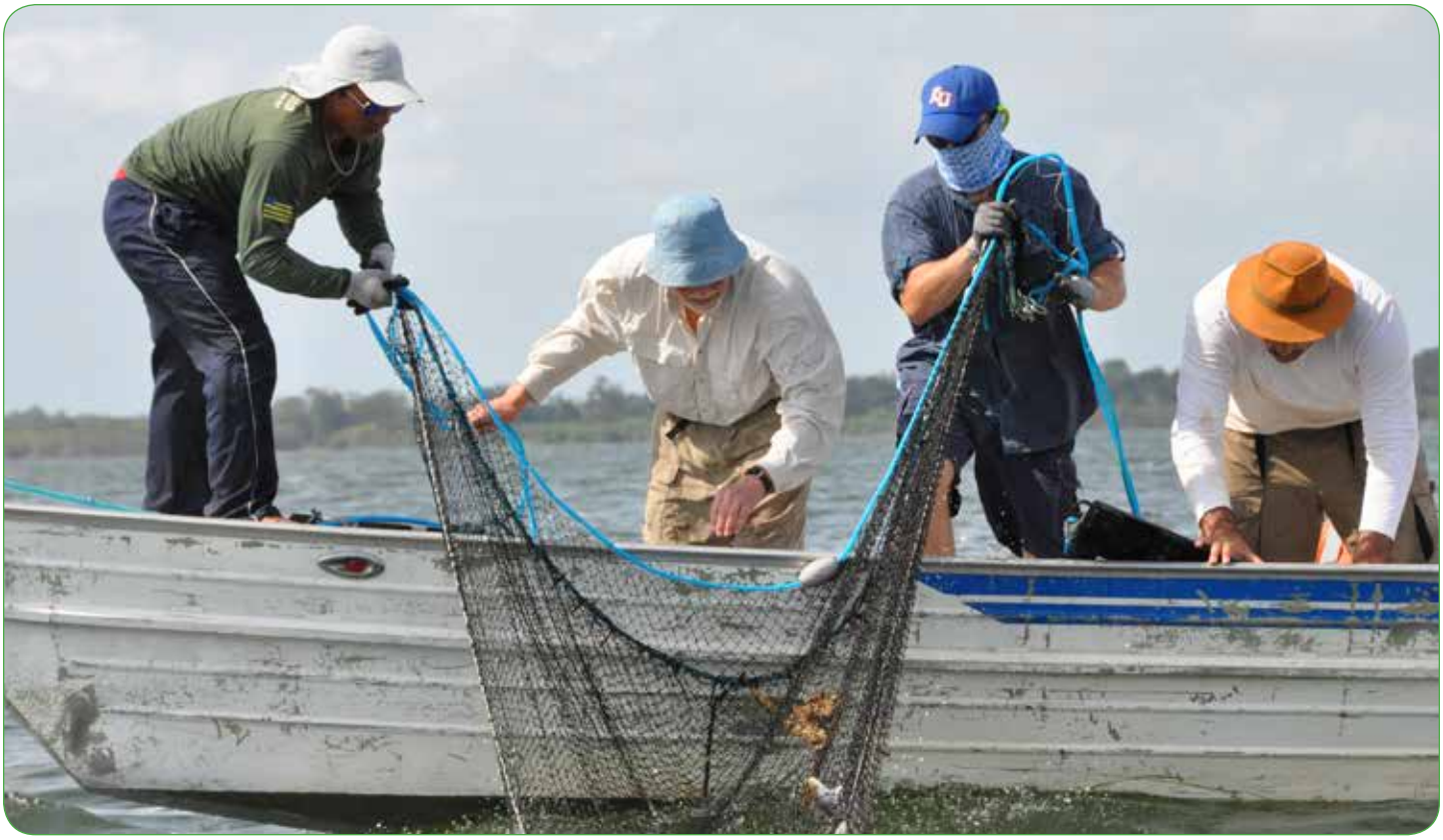

Figure 6
A dredge (modified Missouri trawl) used to sample for large molluscs in the deep water (~25 m) of the Rio Xingu, Para, Brazil.

For example, to have a 90% chance of detecting an uncommon species occurring at a mean density of $\bar{x} = 0.1$ individuals/m^2 with a detectability of $\lambda = 0.9$, a sample size of 100 quadrats (size 0.25 m^2) would be required. However, if the aim is to assess regional molluscan diversity, it should be remembered that for rare species it is often more effective to survey more sampling sites less intensively than to spend a lot of time searching a limited number of sites (Mackenzie & Royle 2005). For further details on quantitative sampling methods and different probability sampling designs, see Strayer and Smith's book 'A Guide to Sampling Freshwater Mussel Populations'.

Innovative Methods

A number of innovative methods are now available which can be used to maximize the number of species collected. The use of remote operated vehicles (ROV) equipped with a camera might be useful for visual searches of lakes and big rivers in deep water. The use of side-scan sonar can also be a valuable tool to detect the location of mussel aggregations (Powers *et al*. 2015).

The use of metabarcoding techniques with Environmental DNA (eDNA) water samples can be an alternative tool for the detection and quantification of molluscs in distinct freshwater habitats (Bronnenhuber & Wilson 2013; Goldberg *et al*. 2013; Deiner & Altermatt 2014; Mächler *et al*. 2014). Although these techniques have not yet been mastered, further technological development should increase their accuracy and importance for aquatic surveys in the very near future.

Supplemental and Habitat Dependent Methods

Large Rivers & Lakes

Large rivers present a challenge to sampling benthic organisms like molluscs. Murky water, strong currents and water depths limit tactile sampling to river margins, point bars and shallow side channels, in the absence of SCUBA or surface supplied air. Many sampling regimes involving diving and transect sampling have been developed in North America for use in large rivers (Smith *et al*. 2001; Villella & Smith 2005). Dredges are also an important tool for sampling benthic animals in deepwater habitats (Miller *et al*. 1989; Herzog *et al*. 2009). Additionally, dredges may allow for spatial and temporal comparisons and in some cases assessment of secondary production of molluscan species (Sousa *et al*. 2005, 2007, 2008). The Mini-Missouri Trawl has been used in collecting mussels in the Rio Xingu, Brazil with great success. The efficacy has not been tested and further studies are needed to assess their overall ability to capture and detect a representative sample of those habitats.

Crocodilians are a serious hazard in many tropical rivers throughout Africa, southeast Asia, South America, and northern Australia and entering the water is not always possible. In these circumstances, dredges dragged behind boats or thrown from the shore can provide qualitative samples of the benthos, although these are only effective on sandy or soft mud substrates. Samples from replicate runs or throws should be kept separate so that sampling efficiency can be estimated, and allow statistical comparisons. Trawls should be standardized by trawling set distances or times so that replicate trawls can be compared.

Figure 7
Conspicuous gastropods (Family Viviparidae) living on the underside of a large flat rock in the Wabash River, Illinois, USA.

TABLE 1: Equipment

	Small River		Large River		Lakes	Wetlands	Springs
	Timed	Quant.	Timed	Quant.			
Mesh bags (xx mm mesh)	x	x	x	x	x	x	x
Photo camera	x	x	x	x	x	x	x
GPS	x	x	x	x	x	x	x
Large-mouth double lid plastic Jars (var. sizes, 15-1000 ml)	x	x	x	x	x	x	x
5L buckets with lids	x	x	x	x	x	x	x
Reversing pliers	x	x	x	x	x		
1 mm sieve	x		x		x	x	x
Kitchen strainer	x		x		x	x	x
Waders	x	x			x	x	
Viewing scopes	x	x			x		
Snorkeling equipment	x	x			x		x
Surber benthic sampler	x	x	x	x	x	x	
Quadrats (50 x 50 cm)		x		x	x	x	
Boat			x	x	x		
Dredge			x	x	x		
Scuba or Hookah Diving Gear			x	x	x		

For genetic studies the following supplies are needed:

Ethanol/RNA later
1. 5 ml Micro-centrifuge tubes with O-ring seal screw cap
Dissection kits (including scissors, pincers and scalpels)
Vernier calipers
Paper towels
Lighters or matches
Plastic Disposable Pipettes
Disposable swabs

Figure 8
Fingernail clams (Family Sphaeriidae) living inside a large dead shell of a freshwater mussel (Family Unionidae).

Wetlands, Springs, and Caves

These are specialized habitats and methods for standard sampling in these environments are nonexistent. Protocols 1 & 3 for wadeable streams should be used and modified as needed.

Seasonal and Biogeographical Considerations

Other factors that affect sampling besides stream size are flooding and droughts. We recommend sampling at or near the dry season. High water levels increases turbidity preventing effective visual sampling, and areas that are usually dry may be inundated. High flows also make it difficult to operate dredges or safely employ SCUBA in larger rivers or snorkeling in smaller streams.

Biogeographical or regional considerations to consider include the numbers of species found in temperate as opposed to tropical systems. The temperate systems of North America and tropical Asia are hotspots of freshwater molluscan diversity and the number of person-hours spent searching sites in these regions should be increased to improve sampling adequacy.

Spatial scale of surveys

The geographical scope of the biodiversity survey will be set by the program goals. Biodiversity surveys may be focused on a single reach of a small stream or lake, river basin or the geographic range of a species spanning multiple drainage basins. The spatial design is critical and site selection needs to be spatially distributed, covering all likely habitats and watersheds. Use of Geographic Information Systems (GIS) and advanced eco-informatics models such as Ecological Niche Modelling (ENM), which combines statistical or machine-learning algorithms with spatially geo-referenced environmental data and information contained in historical records, may aid in site selection (Daniel & Brown 2013; Prié *et al*. 2014). A limitation of ENMs is that they identify regions of potential habitat suitability for a species based on its realized ecological niche in relation to environmental (usually climatic) predictors. However, some freshwater mussels and gastropods have restricted geographic ranges that are a consequence of past climatic or geological events (e.g. Ponder 1991, Strong *et al*. 2008) and their distributions may not be accurately modelled by ENMs. In addition, and for unionoids, the life cycle depends on fish hosts. Therefore, ENM models should also include data on host distribution and density. When assessing regional molluscan diversity, specialized habitats characterized by long-term hydrological stability, including the headwaters of streams and spring-fed waterbodies, should be targeted as these are often favored by gastropod groups such as hydrobiids (e.g. Ponder 1991).

Vouchers, Identification, and Data Management

It is extremely important to document species occurrences with vouchers, if at all possible, and to deposit then in an established museum to allow verification of the identification of specimens found in the study. Coordination with the host country museum should be made for depositing vouchers. Data without vouchers have far less value and are more often ignored by researchers than those documented by specimens. At a bare minimum, photographs of all target taxa collected should be taken. Small specimens that cannot be determined in the field should be returned to the lab for identification.

Accurate locality data are essential. A global positioning system (GPS) unit should be employed to record the geospatial coordinates of the samples. Field notes, including detailed ecological observations or demographic data, (sizes, ages, sex ratios, etc.), are also desirable to fully document the survey. Specimens should always be labeled in the field with complete and clear locality data using a pencil or indelible ink and waterproof paper. At a minimum, labels should include the following data: Body of Water (e.g., Stream or Lake); Country; Latitude/Longitude; Date of Collection; and Collector(s). Other data that are helpful include Drainage; State (or Province / Department, etc.), Secondary political divisions; and Common Location (Distance, Direction, and Location – i.e. 5 km SSE Manaus).

The objectives of the study will dictate the number of specimens to be collected, and in some cases a single voucher will suffice. A study on variation in shell shape, size, etc. may require retention of more specimens. If the specimens are to be used for anatomical studies, they should be narcotized and relaxed, if possible, before being placed in fixative. Commonly used relaxing agents include MS-222, chloroform, menthol crystals, and phenobarbital. Placing live molluscs directly into a fixative solution causes the animals to tightly close their shell and prevents the fluid from fully penetrating the tissues. Small wedges or pegs should be inserted between the shells of bivalves or opercula of snails to allow the fixative to enter. With the increased interest in molecular genetics, specimens should be fixed in 95% ethanol in the field, if possible. Distilled spirits may also work if ethanol is unavailable. Denatured ethanol should be avoided. It may be sufficient to voucher shells instead of live animals to document occurrences. Additional references and curatorial methods for bivalves and gastropods can be found in Cummings and Bogan (2006) and Dillon (2006) respectively.

Non-lethal sampling, in lieu of whole animal preservation, for genetic research can be done if large numbers of individuals are required or the target species is rare. This is done by swabbing the mantle cavity of unionoids or taking tissue clips from either the mantle or the foot for genetic analysis. A protocol developed for collecting genetic samples of bivalves and gastropods is given below. The equipment needed is listed in Table 1. The examples shown are for freshwater mussels.

Figure 9
A large shell deposit along the banks of the Illinois River, Illinois, USA.

Protocol for taking Tissue Samples for Genetic Analysis

If the specimen is not vouchered, take a photo of the specimen with a locality label beside it and a reference for size (e.g., a coin or ruler). For whole animals collect 3-6 animals from each population (river or lake) to use as vouchers and place each specimen in a separate vial of an appropriate volume (>5x the total volume of the specimen/clip) with ethanol (>95%). For bivalves and operculate gastropods the following surgical procedures should be applied to allow the ethanol to enter and preserve the tissue. Anesthetize the animals (e.g., in a 2-phenoxyethanol solution 0.4%): for bivalves, while holding the specimen, insert a knife or scalpel between the shell valves and sever the muscles (one on each side), thus opening the mussel. Using the point of the knife, separate the mantle a little on each shell valve and place the shells on a cloth or paper for about 15 minutes to dry them a little. For operculate gastropods, it is necessary to puncture the operculum.

For small tissues take 20-25 clips (one per specimen) per species at each site. Hold the shell in your hand and, using reverse pliers, open the valves wide enough to insert small scissors and pincers (Fig. 14). Cut a small (0.5 cm) piece off of the tip of the foot or remove a small piece of the mantle. Place the tissue clip in a labeled tube filled with ethanol. Replace the ethanol after a couple of days. Return the animals to the exact same places where they were caught. After processing each specimen, clean the scalpel blade with paper, rinse it with ethanol, and carefully cauterize the tips of scissors and pincers with a lighter.

Figure 10
Local men showing large colonies of the cementing bivalve *Etheria elliptica* Lamarck, 1807 (Family Etheriidae) in Zambia, Africa.

Tissue snips and swab tips are then placed in 1.5 ml plastic tubes with RNAlater or high concentration ethanol to be returned to the lab. Population size can be estimated using genetic analysis if a sample of at least 25 individuals is taken.

Conservation Significance of Molluscs

Freshwater molluscs can be used as sentinel species since they are among the most sensitive species in fresh waters, especially the younger life stages such as the larvae and juveniles. Larvae of most species spend a variable amount of time in the water column and the duration of the larval stage is quite sensitive to the chemistry and physical characteristics of the water. For instance, an increase in water temperature is often correlated with a dramatic decrease of the larval lifespan in many species of bivalves (Taeubert *et al*. 2014). Increased levels of pollutants, metals, nutrients, such as ammonia and other nitrogen compounds, may accumulate in the sediments inhabited by benthic molluscs. These are generally deleterious to juveniles and may cause recruitment failure in the population. Consequently, the U.S. Environmental Protection Agency bases the acceptable threshold value of ammonia for good water quality on the tolerance level of freshwater mussels (EPA 2013). In this context, freshwater molluscs are generally threatened by any major change in hydrology and channel geomorphology, water quality or other kind of disturbance. It has been observed that in many streams where habitat is apparently intact, (i.e. there are no obvious impacts to the water body, and the streams continue to support relatively healthy fish, and insect faunas), the mollusc fauna is declining, especially the bivalves (Haag & Williams, 2014).

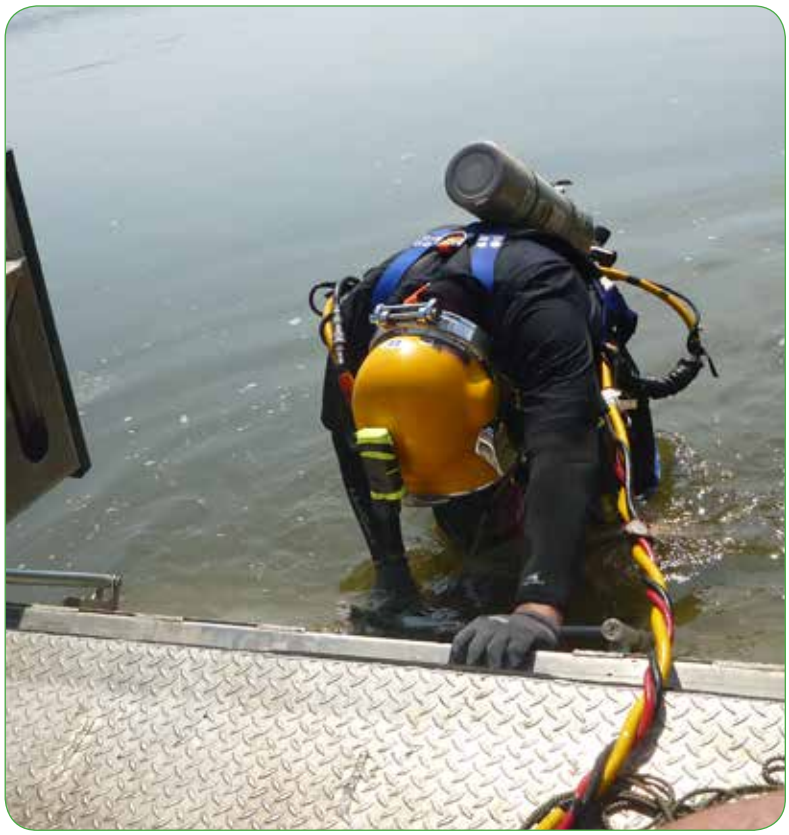

Figure 11
A diver, using surface supplied air commonly referred to as a "Hookah rig", to sample in large, deep rivers. Mississippi River, Illinois, USA.

Given the high conservation importance of freshwater molluscs and their global decline, it is imperative to implement a standardized sampling protocol. This chapter is an attempt to cover this gap and will permit comparisons at different spatial and temporal scales, providing the basic information needed to assess the conservation status of molluscs.

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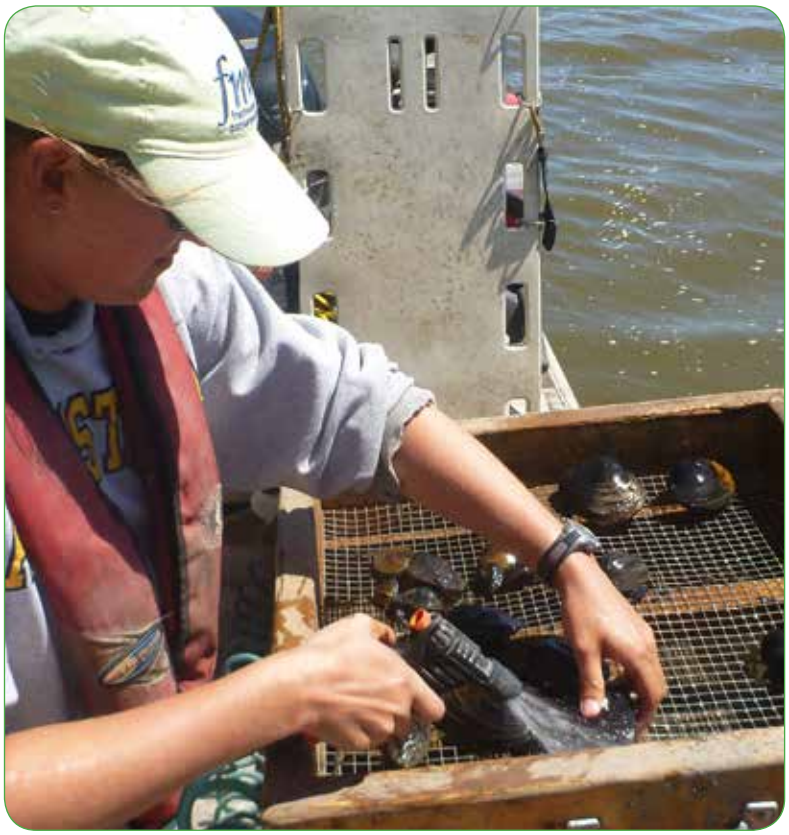

Figure 12
A cleaning station set up to wash and sort molluscs collected by divers.

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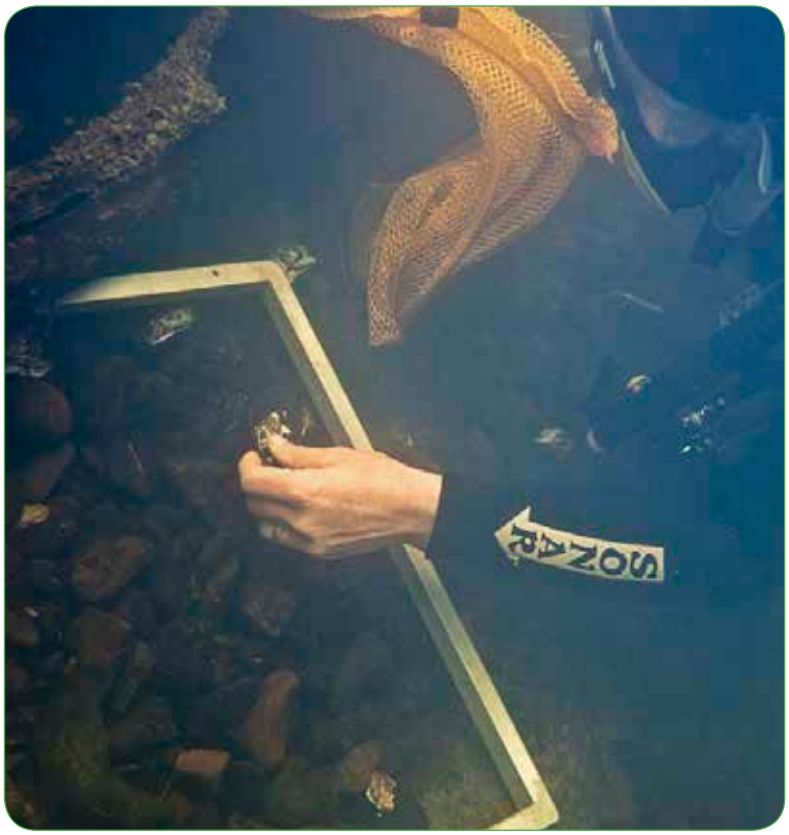

Figure 13
A quadrat frame used to conduct quantitative surveys for freshwater mussels and large gastropods.

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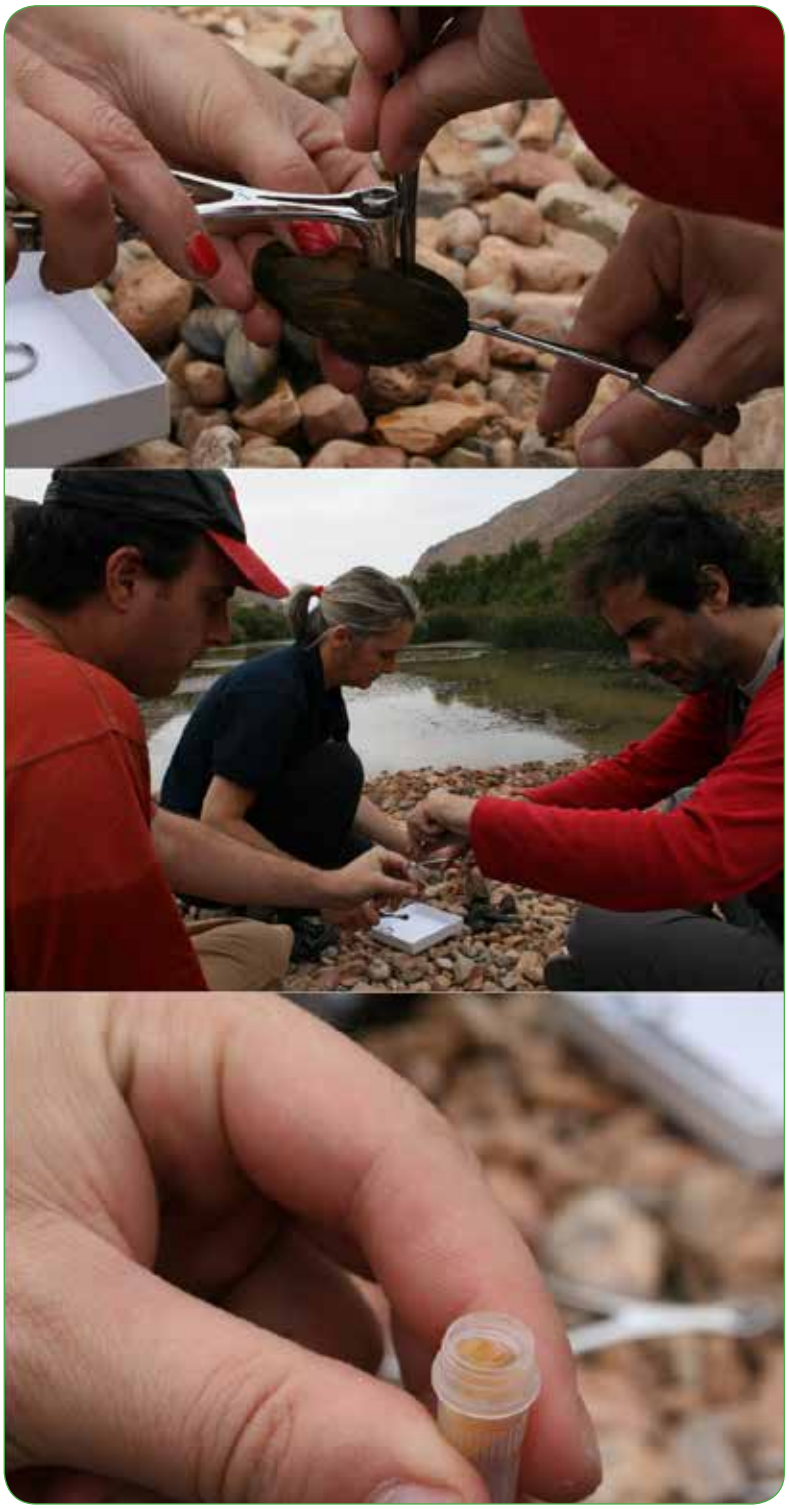

Figure 14
Collection of tissue clips of freshwater mussels for genetic analysis in the Oued Noun, Morocco.