Chapter 22

Preservation of freshwater fishes in the field

by

Dirk Neumann
Zoologische Staatssammlung München
Section Ichthyology / DNA-Laboratory
Münchhausenstr. 21, 81247 Munich, Germany
Email: Dirk.Neumann@zsm.mwn.de

Abstract

Collecting in rivers, streams or lakes is challenging, and the fishing efficiency is highly depending on habitat conditions and the selection of fishing gear. While some fishing gear can be very targeted, most gear types result in the capture of non-specific by-catch. Therefore, careful and thorough planning of any field project is essential in ensuring the collection of undamaged, well preserved samples, including the targeted species. Planning not only includes preparatory work before heading into the field such as applying for permits and the selection of suitable fishing gear for the specific habitat and species to be sampled, but also putting together comprehensive sampling equipment for the activities to be undertaken and assembling adequate personnel to handle all sampling events during the specified time period.

Key words: Fishing gear, fishing methods, fishes, preservation, tissue sampling

1. Introduction

“If field workers understand how specimens are processed and used in museums, they will prepare better specimens. If collection users understand how animals are collected and preserved in the field, they will make better use of the specimens. If all of us understand how collections are managed, specimens will be better utilised and preserved for the future.” (Simmons, 2002). Besides your own research interests, specimens in natural history collections serve as valuable representatives of natural populations for other scientists for decades or even centuries. Therefore, all collections from natural (fish) populations should be made careful and with the best preservation procedures to ensure the highest quality of the preserved specimens and tissues for future research. Collection of ancillary material (additional specimens, species or amount of tissue) should be evaluated against the time, effort and money invested in the sampling. With collecting becoming increasingly difficult both due to budget and permitting issues, collaborative collecting is becoming more prevalent and cost effective.

2. Permits, regulations and responsibilities

Ecologists and Biologists working with freshwater fishes have to cope with many regulations and obligations. Besides national and international regulations and provisions on species covered under the *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES), additional permits and licences are necessary or required for endangered, threatened or protected species and have to be considered in the planning of a proposed field work project. These should not only include the required permitting legislation, but also consider the ethical treatment of the collected specimens. Minimizing actions and conditions that might induce physiological stress, physical damage and injury are not only a matter of animal welfare, but may have a direct impact on the quality of the preserved fishes in the field.

2.1. Collecting permits

Prior to the start of any field programme and depending on which country the field work is to be conducted in, all necessary permits for the proposed fishing and sampling activities in the study area must be obtained. This includes official research permits on multiple national/federal levels, fishing permissions, a valid fishing licence at least for the fishing person (mandatory in Europe and North America) and especially local permits (*i.e.* allowance/authorisation of local fishing right holders, communities, village chiefs, etc.). For entry and collecting in several National Parks additional permits might be necessary, negotiated and permitted by the administration of the National Park itself. Official and local permits should include the name of each field crew member on the permit, and the explicit permissions for entry of special geographic locations such as National Parks, restricted/prohibited areas, private land etc., and allowance of

collection of specific species (especially CITES, threatened, endangered or protected species) including any potential by-catch or the collection of other disciplines if applicable. Applications for these official and local permits should be made well in advance of the planned field work with adequate time for processing (and return) of the permits. Illegal fishing without permission may result in fines and other penalties (including prison time in some countries). Any permit conditions should be strictly adhered to (limitation of number of specimens of individual species, return of unwanted material to the environment, return of collected material to the country of origin after the collecting trip, restrictions on methods of collection, etc.).

2.2. Export / Import Permits

An export permit is required if the sampling/field work is carried out in a foreign country and the samples have to be transported from this country or state for further study and analysis (and/or final deposition). If the material is deemed for final deposition at the destination institution, the export permit should specifically allow the permanent export from the host country and transfer of ownership. The transfer of ownership may be negated by conditions such as export of CITES, endangered or protected species, or by other stipulations regarding the return of identified material to the host country. Again, as with collecting permits, any conditions or stipulations of the export permit must be adhered to. Import permits are required for the import of CITES species, but may also be required for the destination country and should also stipulate permanent deposition at the destination institution.

If the collected material is to be transported across federal/state, provincial or county boundaries, or exported and imported to different countries (*e.g.* for entry into the EU), national or international animal health and veterinary regulations may also apply requiring certificates for transportation, *i.e.* health or veterinary certificates, as detailed in Section 5 (Trade measures, importation/exportation procedures and health certification) of the *Aquatic Animal Health Code* as provided by the *Office International des Epizooties* (OIE). For further reading follow the OIE-link given below to Internet based information.

2.3. Additional permits and licences

Besides the above, further permits and licences may be required for any proposed fishing activities, including but not limited to:

- Valid electro-fishing licence at least for the collector who operates the dip net/the anode pole.
- Valid skipper's licence for the boat driver and/or coxswain (either a person of the fishing crew or a hired skipper) who knows the particular river very well (invaluable in navigating unknown, unchartered or dangerous waters).
- Diving certification for all persons who will be engaged in SCUBA sampling.
- Additional permission may also be needed for certain collecting methods, *e.g.* the use of rotenone (especially in freshwater environments), spear

guns, gill nets or any other method not routinely allowed by the general public.

2.4. Conservation and species protection regulations

Besides the relevant permits outlined above, the planned fieldwork has to comply with international wildlife regulations and provisions. The conservation status of the target species, and any potential by-catch in the specific collection area, needs to be evaluated before undertaking the collecting trip, to ensure that the necessary permits are in place prior to collection. Some species are protected or regulated by special legislation, such as CITES, the *International Union for Conservation of Nature* (IUCN), the *European Nature Conservation Legislation* or the *Endangered Species Act* (ESA), etc. Additionally, national or federal regulations on endangered species and species protection may apply or demand the obtaining of exemptions for catching certain species during closed seasons as well as undercutting minimum size limits.

2.5. Fish handling procedures and ethical concerns

Fishes are extremely sensitive animals that require fundamentally different handling requirements compared to other vertebrates due to their physiochemical make-up. Unnecessary by-catch together with careless handling and injury to specimens can result in increased mortality rates and must be avoided. Unnecessary (physiological) stress, inadequate handling or manipulation of specimens in the field will result in discoloured, damaged specimens with limited or no scientific value. Unless the fishes are not already dead (*e.g.* gill net fishing), fishes have to be euthanased prior to tissue sampling. An overdose of approved anaesthetic immobilizes the fish and allows more efficient processing and sampling of the catch and reduces potential pain at contact with the fixative.

Appropriate ichthyological anaesthetics include:

- Chlorobutanol (trichloro-2-methyl-2-propanol, CAS-No. 57-15-8); a saturated solution of approximately 1-2 tablespoons per litre will narcotise within seconds and has no known negative degrading effects on the DNA extracted from tissues. Possible issues:

  a) For euthanasia, dip specimens 3-4 times for few seconds into the anaesthetic, but do not leave them for longer periods in the fluid; the adhering narcotic on the gill surface is sufficient for sedation.

  b) Species with thick mucilaginous layers (*e.g.* eels, sturgeons) show increased mucus secretions after Chlorobutanol treatment caused by the high salt concentrations; especially those species should be narcotised by repeatedly short dipping for only few seconds.

  c) Low ambient temperatures and metabolism rates of fishes during autumn/winter demand higher Chlorobutanol concentrations which may lead to vascular gill swellings and subsequent gill haemorrhage due to the high salt concentrations of the anaesthetic.

d) Air breathing fishes are hardly affected and cannot be efficiently narcotised with this method.

e) Chlorobutanol is extremely dangerous if ingested and may cause irritation of skin and eyes (see Appendix for further information and follow link for MSDS provided in section 7, internet based information).

- MS-222 (9-Tricaine Methane Sulfonate, CAS-No. 886-86-2) is the only approved substance (Europe, North America) for anesthesia of fishes; the fine powder can be dissolved in much lower concentrations (10 mg/l, thus avoiding possible negative effects of high salt concentrations of the narcotic; irritant but less irritating as Chlorobutanol (see Appendix for further information and follow link for MSDS provided in section 7, internet based information).
- Clove oil (CAS-No. 8015-98-2) is a natural analgesic, the main ingredient Eugenol is used as narcotic mainly for marine organisms; Eugenol is water insoluble, for usage emulsify 1-5 ml clove oil in alcohol; irritant and hazardous in case of skin contact (see Appendix for further information and follow link for MSDS provided in section 7, internet based information).
- Carbon dioxide can be an effective narcotic and is easily available *e.g.* carbonated bottled water or soda; narcosis can take longer with this method and may cause body contortions and muscle spasms that may affect the quality of the preserved specimens.

After short sedation in above detailed anaesthetics, or after electrofishing, fishes recover in well-oxygenated and ambient temperate water usually within minutes. For recovery, they should be placed in a separate tank or bucket. Only fully recovered specimens should be carefully released back into their environment to prevent injuries, damage or predation while still tranquilised.

2.6. Fishing relevant safety issues

Dangerous situations during fishing can arise within seconds leading to serious injury or fatalities.

Always ensure a firm, stable footing when collecting near or in water – especially when wading in fast flowing streams, in deep water or in case of low visibility of the water. Be especially cautious around slippery surfaces such as exposed, wet rocks, submerged substrates and vegetation or on moveable and uneven surfaces (sand, boulders etc.), and wear appropriate footwear such as rafting or canoeing shoes. Remember that waders can exacerbate such circumstances by causing extra drag and weight, especially when suddenly infiltrated by water. Attempt to strip off waders if in trouble.

When sampling with nets, beware of entanglement and drag caused by the net which can pull you off balance or into deeper water. If in trouble, release one or both ends of the net to maintain your footing and allow the net to drift with the current to minimize the contact surface and to reduce the velocity pressure and drag force of the net. In case of net fishing from a boat, avoid that the propeller

snags on static or grounded nets or catches the net during retrieving it into the boat. Human life is at all times more valuable than any sampling equipment!

When electrofishing, special precaution should be taken against electrocution by using rubber boots and gloves. Make sure that only certified/trained persons are allowed to operate the electrofishing device (mandatory in several countries). In the event of a member of the team falling into the water or otherwise coming into contact with electric current, cut immediately the electric circuit by removing either the anode pole or cathode rod from the water. Stop the engine before trying to help imperilled persons! Negligence of these simple principles will endanger further lives.

When diving or snorkelling, ensure that all safety precautions are taken and that one member of the team is always on shore to assist in an emergency.

A mobile or satellite phone and GPS device should always be part of sampling safety/emergency equipment to allow for rapid contact of and position location by emergency crews in the event of an accident.

2.7. Fluid fixation/preservation hazards

Exposure to aqueous solutions or fumes of formaldehyde should be avoided by always working in well-ventilated areas or fresh air and through the use of approved protective equipment. Formaldehyde is not only noxious but is also a known carcinogen. Minimise direct exposure as best as possible (*e.g.* only open the fixation container to add further specimens). Latex gloves provide no protection against formaldehyde, use Nitrile or Neoprene gloves for protection. Do not wear soft contact lenses which absorb formaldehyde vapours and will trap them against the eyes (Cohen *et al.*, 1979, cited in Simmons, 2002).

3. Fishing strategies (fishing gear, fishing methods)

Most fishing methods applied for ichthyological sampling catch unspecific, thus it is difficult (or impossible) to target individual species without some element of by-catch. This by-catch can however be reduced through the employment of appropriate, habitat specific sampling techniques, while the collection of multiple species may necessitate the use of multiple techniques at a given site. Fishing efficiency is affected by multiple factors (specifications and selectivity of the gear, seasonal variation, habitat conditions, fish size, etc.) which, in return, directly influence the collection efficiency of the field project. The fishing gear has to be selected in terms of operational efficiency and the availability of target species with regards to the sampling location. “Target species” is used here as a technical term and may include single species confined to specific habitats (*e.g.* specific pelagic fish species confined to open-water habitats which must be targeted with specific net gear), but can also include the complete fish fauna from a specific collection site (*e.g.* for taxonomical collections of fish faunas from previously unexplored rivers or lakes). Depending on what is defined as “target species” for the specific field trip or collection event, the fishing gear has to be selected. During the survey, it might be necessary to adjust the fishing methods to provide the required quality and quantity of the catch. A variety of

gear types or repeated fishing at the same spot might be necessary to ensure the widest possible range of fish species and life stages. Spawning and migratory behaviour of the target species, habitat preferences of different life stages of the same species and a basic knowledge of physical stream parameters, such as velocity, conductivity, stream size, water depth, water temperature, underground conditions (muddy/sandy/rock) are crucial for successfully fishing and sampling. Aspects that influence fishing efficiency are:

Water depth

- Deep water bodies such as lakes or large rivers favours long-line fishing, ground nets, fish weirs (if velocity allows).
- The length of the anode rod limits electrofishing (which normally ends 1 m below the water surface).

Water conductivity

- The salt concentrations in rivers and streams (depending on the geological conditions of the drainage area), at estuaries or entering freshwater streams in euryhaline lakes, in tidal pools and estuaries may vary extremely (*e.g.* favours or limits electrofishing).
- Different salt tolerances/preferences of euryhaline/stenohaline target species.

Water clarity

- Influences snorkelling/SCUBA diving.
- Electrofishing efficiency is strongly influenced by the clarity of the water.

Velocity

- Most static nets can only be deployed in shallow/stagnant water or in the direction of the current at moderate velocity.
- Higher water velocity drastically decreases electrofishing efficiency because stunned fishes will drift faster while increased drag on the anode pole will reduce its manoeuvrability.

Different behaviour/activity of target species

- Pelagic/demersal/benthic species.
- Diurnal/nocturnal species.
- Aestivation/hibernation.
- Spawning migrations.

Remark: Small species sheltering themselves in the shallow water from large nocturnal predators are easily caught at night in the shallow water using headlamps, handnets or a beach seine.

Habitat conditions

- Cobbles, boulders and rocks shelter lithophile or rheophile species.

- Benthic species may hide in muddy or sandy ground.
- Large stones, branches and trunks of trees minimize application of net gear.

Be aware that all of these parameters and conditions can change and normally do change within minutes during heavy rain events, *e.g.* in the rainy season. This not only strongly influences the fishing events following after the rain event because of increased turbidity or cloudiness of the water. Heavy rains (especially overnight) might also necessitate immediate removal of static fishing gear to avoid damage or loss.

Generally speaking, there are two different fishing methods – active and passive. For passive methods (such as gill nets or fish traps), personnel are only required for deployment and retrieving of the gear. Active methods require (with few exceptions) at least two people to actively operate the fishing gear during the collecting period. While active gear can be adapted during fishing *e.g.* for difficult habitat conditions, passive gear cannot. A combination of active and passive fishing methods will raise the sampling efficiency at a collecting site through setting of passive gear before the start of any active method and retrieving thereafter.

3.1. Selective (active) fishing methods

3.1.1. **Cast (throw) net** (Fig. 1A)

Operation

- Small net thrown onto the surface in a circular formation.
- After sinking to the ground the net is closed and retrieved.
- Requires trained skilled person to successfully use a cast net (for further information follow link provided at Internet based information below).

Specifications

- Small mesh sizes (usually 0.8, 1.0, 2.0 cm).
- Net should have bottom pockets to hold the catch.

Application

- Used in streams, rivers, lakes.
- Can be operated either wading in shallow water or from the boat.
- Allows for fairly directed and selective fishing.
- Can be used for retrieving live fish for bait.
- Only applicable at low or moderate water velocities (net will collapse in higher currents before reaching the ground).

Remarks

- The sample site should be free from obstacles like fallen trees, branches, roots, cobbles or boulders to allow closure of the net.
- Repeated casting at the same spot can scare off nearby fishes through the splashing of the net.

3.1.2. Seine (beach/pole seine) (Fig. 1A)

Operation

- Easily deployed from shore, wading in shallow water or from a boat.
- Requires minimal instruction and training.

Specifications

- Large variety of mesh sizes, lead lines and floats are available.
- Net specifications dependant on habitat and size of the target species.
- Should have a bunt (cod end) to effectively trap the catch.

Application

- Effective in most habitats but especially in larger streams, rivers and lakes.
- Shorelines should be free of obstacles to allow net to be pulled onto shore for effective specimen collection.
- Low to moderate water current.
- Beach seine with a fine mesh (up to 1.0 cm) should be shorter (approx. 10 m) to ensure that the seine can be pulled quickly enough against the current.
- Net filament can be either a strong visible (cotton) yarn or a less perceptible nylon or polyester, which provides lower visibility and detection by the catch.
- The lead line must be kept at ground level and ahead of the float line to prevent fish from escaping under the net.
- The float line must be raised well above the water surface when pulling the seine towards the shore to prevent fish from jumping over the net.
- Operating larger beach seines (20 m or more) requires a larger mesh size (to lower the velocity pressure) and more people to pull the net (because of increased drag force).
- Pole seines are usually operated from boats in deep water, or by wading in calm water (*e.g.* lakes).

Remarks

- Water depth and current can affect the efficiency of a seine net.

- If the water conditions allow, nets should be set or operated from row boats (to prevent any propellers from damaging or curling up the net) or manually by multiple people.
- This method has the advantage of being operated quietly, reducing the possibility of scaring fish from the sampling area.

3.1.3. **Frame net** (Fig. 1B)

Operation

- Individual fishing in shallow water.
- Along beaches and under overhanging shoreline vegetation (except for stands of dense weeds).
- For kick-net sampling in shallow riffles.

Specifications

- Either as solid aluminium or metal frame (60 x 40 cm), collapsible (two solid connectable parts) or foldable frame with a solid bar and two movable arms.
- Fine mesh (2.0-4.0 mm) to collect small species and fish fry.
- Approx. 30-40 cm deep net sack.

Application

- In shallow riffles, in creeks, smaller streams, slip-off slopes of larger rivers.
- Frame net is rammed into the soft bottom of the river vegetation and quickly lifted up.
- During sorting of the catch the frame must be kept well above the water surface while the net sack should remain dipped into the water.
- As a kick-net it can be either dragged by the current over larger boulders and rocks or scraped against the current, lifting smaller cobbles and stones and trapping smaller lithophilic fishes.

Remarks

- Favour aluminium frames in areas in which strong-electric fish occur (*e.g.* electric eels in South America or electric catfishes in Africa) due to the amplifying effects of the metal frame.
- Keep the net sack closed when crossing/wading through moderate to strong flowing water to reduce the drag force.

3.1.4. Angling

Operation

- Moderately selective method especially for clear water habitats.
- Species can be targeted through specific baiting.

- Fish size and species depends on hooks (size and form) and bait used.

Specifications

- Different types of fish hooks and monofilament lines, weights, baits, fishing rods and reels.

Application

- Can be employed from shore or boat.
- Highly biased method for fish size and species.
- Most effective if the angler has a specific knowledge of the habitat and habitat preferences of the target species.

3.1.5. **Hand nets** (Fig. 1B)

Operation

- Very selective method which allows also observation of fish activity, behaviour and habitat occupation (while snorkelling or SCUBA sampling).
- For collection of relatively small species in shallow water or from the surface of deeper water.
- Allows specific and selective sampling of individuals (*e.g.* breeding pairs, gobies and their symbiotic shrimps, etc.).
- Can also be used underwater for snorkelling or SCUBA collection.

Specifications

- Commercially available (aquarium) hand nets.
- Fine mesh size on varying size circular or square frame with wooden or metal handle.

Application

- Additional method for collecting specimens that may escape from seine nets.
- For underwater rotenone collections.
- For chasing specimens in very shallow pools and streams where larger seines are rendered ineffective.

3.1.6. Spear (Spear gun, Hawaiian sling or pole spear)

Operation

- Most selective collection method to target individual specimens.

Specifications

- Varying types, sizes, power and spear type (single or multi-barbed).
- Rubber band or air powered spear guns with barbed spear.
- Pole spear – long spear powered by rubber band (Fig. 1D).
- Hawaiian sling – smaller rubber band powered spear.
- Hand spear – thrown into shallow water or at species close to the surface of deeper water.

Application

- In any water body.
- Usually used underwater while snorkelling or SCUBA diving (although restricted or illegal in some countries).
- Requires sufficient underwater visibility.
- Can also be used from the surface on shore or from a boat.
- Hawaiian sling: operated and fired much like a slingshot; rubber bands attached to tube or block through which spear is drawn back and aimed.
- Pole spear: rubber band is held in the hand while pole is drawn back through the hand to produce tension in the rubber band; aimed through extending arm in front of face (Fig. 1D).
- Hand spear: varying length and barbs; effective in collecting flatfish in shallow water.

Remarks

- Spear collection is usually prohibited on SCUBA for the general public and specific permission may be necessary.
- Care should also be taken not to lose the spear and not to fire towards hard substrates or surfaces.
- Caution should be exhibited when using spears to ensure that they are not aimed towards other people or misfired during handling.

3.1.7. Electrofishing

Operation

- Common survey method to collect specimens as well as estimate abundances, density and species composition of fish populations.
- Uses electricity both to stun and attract the fish with the positive pole before capture (Galvanotaxis).
- Needs two persons for operation, one operating the dead man's switch and the electrofishing device, the other for catching and collecting the stunned fish.

Specifications

- Effectiveness depends heavily on water conductivity.
- Influenced by the size of the water body and riverbed conditions (soft or hard ground).
- Various models are available that generate varying strength of electric field.
- Pulsed or non-pulsed direct current (DC).
- Portable battery (Fig. 1B) or gas powered backpack models for small rivers and streams or large stationary models are available which are operated either from shore or a boat.
- The positive pole (anode) usually forms a ring at the end of a 2 m fibreglass or wooden pole and holds fine mesh net.
- The negative pole (cathode) comprises a braided copper cable which trails several meters behind the operator in the water.
- Pulse speed, voltage gradient and current influence and trigger Galvanotaxis and cause fish to turn into the electric field and to be attracted towards anode pole.
- Rubber gloves and rubber boots must be worn to isolate the operator and collector and to prevent electrocution.

Application

- Most useful in small creeks, streams, rivers and shallow (littoral) zones e.g. in lakes, in smaller rapids.
- Additional barrier nets can be placed downstream to collect the catch.
- Operator must be trained and in several countries a valid electrofishing licence is required.

Remarks

- Anode rods with dead man's switch included in the rod should be preferred instead of electrofishing devices with separate dead man switches operated by additional crew members (*e.g.* boat driver) for safety reasons, as the electrofisher operating the anode rod has the best overview in case of emergency.
- Stunned fish must be continuously removed from the electric field and be collected in plastic buckets or containers by additional fishing crew members to avoid injuring the fish through long term muscle contraction or tetanus.
- A continuous tetanus may easily break the backbone of smaller specimens.
- Fishing should be conducted in upstream direction so that disturbed debris and sediment are washed downstream and the visibility of the water remains good.

- Also minimises the escape effect of the cathode and allows for easy collection of the stunned fish that are swept downstream towards the collecting crew.
- The electric field depends not only on the conductivity of the water, but also on the ring size of the anode rod (see Table 1).
- Bedrock and sand bottom are insulating and support a stronger electric field in the free water column while soft or muddy bottoms are weakening and may even cause a collapse of the electric field (this might necessitate shortening the cathode length and thus the surface area with a cable strap).
- Wearing Polaroid glasses increases the visibility of both targeted fish and obstacles under water.
- Using a metal boat as cathode is strictly prohibited in several European countries for safety reasons.
- For further practical information please refer to Section 5.4.1 of the Fish Collection Methods and Standards Manual (website address see below at Internet based Information).

3.1.8. Fish market

To obtain a fast overview for the fish biodiversity especially in large rivers or lakes, it is always valuable to explore and obtain specimens from local fish markets. Local fishermen know the specific fishing grounds in their area well and normally apply a variety of different fishing methods which can provide a surprisingly high species richness. Depending on the climate and geographical region, these fish markets are either stocked early in the morning (before sunrise) or late in the afternoon. It is best to visit fish markets during these times to ensure freshness. Specimens should be sampled immediately on the market or might be carried cooled (*e.g.* placed on ice if available, but might be problematic in remote areas) possible for later sampling to ensure specimen and tissue viability. Fresh fishes are easily recognised by their transparent fins and vividly red coloured gills when lifting the gill cover. In most cases these specimens will need to be purchased and should be purchased to be fixed as voucher specimens, but in some cases either fishermen are not willing to sell their catch (*e.g.* fishermen depending on subsistence fishing in remote villages) or a specimen of a rare species might be too large to fit in any preservation container. In cases like this, ask for allowance of tissue sub-sampling of gill filaments or fins and photograph the specimen, so that the images can be taken to serve as vouchers. Fresh and freshly smoked and dried (not fried) specimens obtained from a fish market are even suitable for DNA sampling. As much authentic collecting information as possible should be obtained from the fishermen (fishing location, habitat conditions, fishing gear, fishing time, etc.), keeping in mind that might be imprecise or wrong, as some fishermen are not willing to reveal exact locations or fishing methods. Be aware that on large fish markets in major cities or capitals valuable species or complete catches might be carried by truck over hundreds of kilometres, stacked in layers of ice and sawdust to be sold for higher revenues. Sawdust in the fish baskets or on the

market place and bent or deformed fish bodies because of being stacked for hours or days are good indicators for this practise.

Commercial fishing methods are omitted here, since they are not applicable for small scale scientific surveys in freshwater environments. To expedite large lakes with trawling equipment, a commercial fishing crew and fish trawler should be hired.

3.2. Unselective (passive) methods

3.2.1. **Gill net** (Fig. 1C)

Operation

- Set across rivers and streams or on lakes (usually at night).
- Left in place for longer periods to allow specimens to become entrapped.
- Sampling areas should be free of underwater obstacles and have a moderate to low current.

Specifications

- Different types of net gear highly specific for species and fish size (standard gill nets, for targeting multiple fish sizes multi-mesh gill nets or enmeshing/trammel nets are used).
- Usually constructed of monofilament so as to be strong and invisible, but also as (visible) multifilament nets.
- Mesh sizes, floaters and weights might be optimised depending on the target species.
- Lead and float line should be sufficient to ensure net remains perpendicular to any current.
- Single nets can be combined to larger panels.

Application

- Highly effective when set in moderate to slow current in rivers, streams and lakes as a single net or as multiple sets in a staggered arrangement parallel to the shore, blocking preferred habitats or shelters (*e.g.* weeds or littoral zones).
- Can be effective for collection of species other nets will not catch (nocturnal, deeper water species).
- Net should be tied off to shore or a heavy weight to ensure net remains in place while floats should be large enough to ensure net does not get dragged under.
- For level inventories nets and panels employed at different depths (*e.g.* for target species with vertical or diurnal/nocturnal migrations).

Remarks

- Monofilament nylon nets should be cleaned after usage from any biofilm or algae coatings to maintain the invisibility.
- Areas with high fish abundances might require repeated control of the nets to minimize the number of killed fishes and possible negative effects on the fish population.
- Reducing the net size (single nets vs. nets combined to large panels) also helps to reduce the catch and avoids over-sampling.
- Might also be used as stop net for electrofishing or invisible seine in small streams with low risk of damages of floating debris (branches, leaves, etc.).
- For further practical information on gill nets and their application please refer to Section 5.3.2 of the Fish Collection Methods and Standards Manual (website address see below at Internet based Information).

Fig. 1 (next page). Active and passive fishing gear. A. Small beach seine (8 m) with lead line and floaters and approx. 1.20 m deep bunt; cast net with bottom pockets, diameter 8.0 m; B. Left, front: battery powered DEKA 3000 electrofishing device (out of production) with anode rod, collapsible frame net (net fixed with Velcro strip to aluminium frame); Middle: collapsible frame net, aluminium frame 60 x 40 cm, mesh size 2.0 mm, net sack 40 cm deep, frame included in special pocket in the net and secured with Velcro strip; Far right: Large dip net (approx. 1.0 x 1.2 m) with lead line and lateral short poles for manoeuvring the net; Front: staple dip nets (10 mm mesh, aluminium frame / 2 mm mesh, stainless steel frames, which might also be used as anode dip net for electrofishing), and hand nets; C. Monofilament gill net with lead line and floats (20 m, 10 mm mesh), fish trap with two traps and connecting wing (approx. 5 m long); Front: foldable fish traps without and with wings; D. Rubber band powered pole spear. (Photos D by M. Geiger, all other photos by D. Neumann).


A
B
C
D

3.2.2. Long-line fishing

Operation

- Short lines (up to 20 m) for sampling along shores or obstructive habitats like rapids or block stone embankments.
- Longer anchored lines (50 m and more) for sampling the river bed of large and deep rivers.
- Multiple hooks on branching lines and baiting may affect sampling efficiency.

Specifications

- Strong braided hook links with high strength fibres for maximum strength and abrasion resistance should be used.
- The length of the hook links should not exceed half the distance of the single hook links to each other to prevent the entangling of the hook lines.
- Small barbed hooks in combination with strong hook links allow catches of small to large species and minimize injuries and improves unhooking.
- Long-lines are usually anchored to the shore or substrate, additional weighting with lead is not necessary.

Application

- Long-line fishing is the only known method to sample the riverbed of large/deep rivers.
- Long lines for river bed sampling must be employed and anchored from a boat and marked with buoys.
- This method is even applicable in strong currents.
- Application of 20 m long-lines from the shore/river bank requires two persons (one holding the baited line which is anchored to the substrate, the other is throwing the anchored free end perpendicular to the beach into the river/stream).
- In strong currents or rapids, the free end fixed to a buoy may be released with the current, the depth of the long-line can be adjusted with length of the buoy line holding the free end.

Remark

- Secure the hooks immediately after unbaiting/unhooking within polystyrene foam to avoid entangling of hooks and lines.

3.2.3. **Fish traps (Minnow traps/Fyke nets)** (Figs 1C, 2)

Operation

- Small portable wire or net baskets.
- Traps with wings designed to guide especially small demersal and/or benthic species into the trap.
- Fyke nets are large hoop nets that act as funnels to trap swimming fish.

Specifications

- Either small foldable net or wire traps with internal funnels leading to a collection chamber.
- For small species, up to 60 cm length and 30 cm opening diameter with small mesh size.
- For large (demersal/nocturnal predatory) species, larger traps with 5-20 mm mesh size, 2 m or longer with opening diameters up to 75 cm.
- Larger traps are available commercially or from local fishermen but more difficult to handle and normally inappropriate for scientific sampling (these can be set by the collector or can be purchased from fishermen with existing nets – the benefit here being talking advantage of local knowledge and skill).
- Either with two wings (up to 3 m length) which are attached to one trap, or two traps which are attached to one wing.

Application

- Usually placed with or without bait in the shallow, calm or low current water near the shoreline.
- Assembled and placed before dusk and removed in the early morning.
- Traps and attached wings can be placed under overhanging vegetation, littoral zones and in front of weeds.
- Fyke nets may set at the intersection of smaller creeks to block the free passage.
- Traps should be monitored regularly for (nocturnal) predators.

Fig. 2. Wagenia fishing with large bamboo fish traps in the Congo rapids at Kisangani (Democratic Republic of Congo). (Photo by U. Schliewen).

3.2.4. Fishing using ichthyocide (Rotenone)

Operation

- Traditional fishing method employed from indigenous tribes *e.g.* in Africa and South America.
- Utilising the extract from roots of certain plant species (especially those belonging to the genus *Lonchocarpus* and *Derris* or *Tephrosia*, the latter used locally in many villages in the Democratic Republic of Congo).
- Pulverised roots are placed in the water to release the active ingredient.
- Depending on the affected area, needs 5-10 persons with dip nets to collect the fish.
- Rotenone fishing can be a useful alternative in inaccessible habitats, SCUBA collections in deeper water and for the collection of cryptic and hole dwelling, otherwise inaccessible species.

Specification

- Commercially used broad-spectrum insecticide, pesticide and piscicide.
- Available either as fine brown powder or emulsified liquid.

- Classified as IATA air dangerous good (toxic, Class 6.1).

Application

- Powdered Rotenone must be solved in water using an emulsifying agent (usually detergent).
- Liquid Rotenone can be applied directly but should be diluted 1:10 first.
- After mixing, Rotenone is easily spread into the environment using smaller containers like plastic bags, bottles or buckets.
- Affected fishes show suffocating symptoms and either turn gaspingly to the water surface or fall to the bottom.
- Fishes should be euthanased in an anaesthetic immediately after capture (prior to death) to avoid further pain and irreversible abduction of the lower jaws and opercles (results in poor quality of preserved specimens).

Toxicity

- Extremely toxic to insects and aquatic life including fish – easily absorbed through the gills or trachea.
- Interrupts the electron transport chain in the NADH complex in mitochondria of aquatic animals and insects.
- Has only minor and transient environmental side-effects.
- Low toxicity to humans or higher vertebrates.
- Poorly absorbed by the skin and gastrointestinal tract of mammals (see Appendix for further information and follow link for MSDS provided in section 7, internet based information).

Remarks

- Rotenone is the most effective tool available because only small quantities are necessary and well suited for small scale sampling of cryptic, hidden fishes or shoreline fish communities.
- Application area should be free of thicker mud deposits with sufficient water visibility to allow easy detection and effective collection of those specimens falling to the bottom (specimens are easily covered by disturbed mud and become soon invisible for collecting).
- Sampling locations with strong currents should be avoided or the habitat should have some mechanism of containing the spread of the rotenone.
- Spread of rotenone to adjacent areas should be prevented (areas of current flow affecting easily larger areas than required).
- In small streams or creeks, block affected area up- and downstream with gill nets prior to rotenone application to catch those specimens which flee from the rotenone or affected specimens that drift with the current.
- Great care should be taken in frequented areas of nearby villages.

- Rotenoning from shore, boat or SCUBA should be evaluated regarding efficient collecting of affected fishes from the environment.
- The environment should be evaluated with this in mind together with an estimation of the number of specimens that will be affected in order to minimize large scale effects on populations and the environment.

4. Sampling and fixation

This section covers only the collection and sampling techniques for freshwater fishes for scientific (zoological) purpose, *i.e.* tissue samples and fixing voucher specimens in the field for final deposition in Natural History Collections.

The handling time needed to set and retrieve the fishing gear, and subsequent time to sort, tissue sample and fix the specimens, is often underestimated. As a general rule, 1 to 2 minutes per specimen should be calculated for retrieval, tissue sampling and fixation. This adds up to about 6.5 hours for 200 samples processed. Experienced crews with highly efficient sampling workflows will need less time, untrained ones may need even more. Also, this time frame can be greatly increased by any ancillary sampling requirements such as measurement and photographing. Tissue viability and natural coloration will quickly fade. Both can be extended by keeping the fishes alive as long as possible or through cooling the freshly dead specimens with ice.

All of these factors should be taken into consideration when planning a sampling event as these may determine: a) the number of specimens that can realistically be handled (how many of them can be photographed and/or individualised and tissue sampled); b) the number of sites that can be sampled in a day. This is especially critical when collecting previously unexplored regions and faunas to ensure well-preserved, well-documented, straight specimens and individualised tissue samples. Poorly documented and/or preserved specimens resulting from overambitious sampling events are not only of limited scientific value but are a waste of time and money (Figs 4E,G and 5F).

4.1. Necessary equipment and chemicals

Table 1 gives a list of minimally required field equipment for the adequate sampling and fixation of fishes in the field. This list omits any recommendations for personal equipment (such as multi-tools, headlamps for night-fishing, tripod chairs, rain covers, etc.). Appropriate footgear for fishing is discussed above. This suggested list may need to be adapted relative to the planned sampling and depends on the duration of the trip, destination and mode of field transportation which may allow only a minimal subset of this gear.

As a general rule, all preservation and fixation chemicals (together with any other hazardous substances like Rotenone) should be kept strictly separated from each other as well as from other field gear (especially personal items) to prevent any risk of personal injury and also contamination of tissues samples by residual formalin or formalin vapours. Under humid climates, formalin vapours may condense during cooler nights inside the box containing the sampling gear and may adversely affect other gear and degrade tissue sample quality.

Dissection tools, documentation materials, DNA-vials, towels and additional plastic ware for DNA-vouchers should be packed in a durable (aluminium) container that will withstand the rigours of rough field conditions.

Additional gear may be necessary for specialised sampling routines such as electrofishing (protective boots and gloves) or for live fish maintenance during collection (tubs, oxygen supply) etc.

	Items/Gear type	**Specifications**	**Remarks**
Documentation	pre-numbered field lists or field book	water resistant laser or ink jet print	numbering coherent with fish-ID tags and vial numbers
	pre-numbered field tags	water and formalin-resistant	*e.g.* paper printed or numbered plastic tags
	paper or transparent paper (2-3 sheets)	should be acid free	for additional location data to be included to the preserved specimens
	2 graphite lead pencil or graphite monolithe pencil	with graphite lead	industrial polymere lead pencils are made of coloured polymers not waterproof and rub off
	2 pigment ink pen	water & alcohol resistant pen	*e.g.* EDDING 1880 profipen, Securline MarkerII
	1 GPS device	positioning	should allow positioning to nearest 5 m and should receive satellite signals in forested areas
	1 camera	analog or digital	documentation of life colouration
	photo cuvette/photo aquarium	30 x 5 x 10 cm	documentation of life colouration
	(negative/slide) films or memory cards	storage medium	sufficient amount
Fishing gear (minimum) – non electrofishing	1 pole/beach seine	mesh size max. 10 mm	
	2 gill nets	monofilament nylon net, mesh size 10 mm	for small species
	2 gill/enmeshing nets	monofilament nylon net, mesh size 20-60 mm	for large species, mesh size depending on target species

	2 aquarium nets	20 x 10 cm	for juveniles and to sort the catch; 2 nets per person for snorkeling
	1-2 frame nets	60 x 40 cm with a 30-40 cm deep bunt	preferably collapsible
Fishing gear (optional) – non electrofishing	1 cast net	mesh size 6-8 mm, with bottom pockets, diameter 4-8 m	net diameter depending on the cast netting skills
	2-3 minnow traps or fyke nets	fine mesh, with funnels and collection chamber	trap should not exceed 30 cm diameter, wing not 3 m
	1-2 longlines	20 hooks/20 m line with small barbed hooks and strong hooklings	application either from shore or boat (requires additional anchors & buoys)
	angling/hook & line	1 spool and several small barbed hooks	especially in third world countries for fishing kids
Electrofishing gear	electrofishing device	see 3.1	suited model size and generated power dependent on habitat conditions and water conductivity
	anode rod with electro-shock dip net	anode ring with dip net (mesh size 6-8 mm)	electric field depending on the ring size; large ring (30- 40 cm) = larger but more diffuse field; small ring (20 cm and less) = more concise field
	cathode cable	braided copper cable	standard length 2-3 m; length might be adjusted (see under 3.1)
	1-2 dip nets	firm net ring, diameter ca. 30 cm	1 dip net per person (except for the electrofisher and operator of the device)
	2-4 buckets	10-20 l	for collecting the catch during electrofishing
	and/or 1-2 tubs	80-90 l	for collecting and holding the catch during electrofishing

	Electro-shock low voltage gloves	size depending on the persons	natural rubber gloves for use on circuits of up to 1000 volts
	Wellington boots/hip waders	size depending on the persons	
Preservation gear	1 set of dissecting tools	1 fine scissors 1 re-usable scalpel 2-3 sharp blades 1 medium forceps (serrated tips, manipulation of formalin vouchers) 1 probe 1 box of needles	minimum equipment list; two separate sets of dissecting tools for manipulation of tissue and formalin samples are mandatory to prevent formalin contaminations of DNA tissues; larger fishing crews should operate with at least two dissecting kits
	4% aqueous buffered formalin solution	10-20 l; ca. 1:9 dilution of concentrated formalin (37%)	be sure the formalin is buffered, or add additional buffer to keep the pH stable (especially in hot climates); 1 l 37% formaldehyde in solution as back-up and for injection of large specimens
	1 syringe (with strong needles and spare needles)	50 ml	for formalin injection of large specimens
	anaesthetic	minimum quantity: 2 l in solution	carry sufficient additional crystalline anaesthetic as back-up
	2-3 towels		to remove adhesive fish slime from the hands after sorting the catch (contamination risk) and to padding the photo aquarium for transportation
	multi fold sanitary paper towels (1-2 pack)	recycling quality, as supplied in towel dispensers	paper towels in recycling quality remain stable if soaked with fixative solution (compared to toilet paper or kitchen towels)

	2 square plastic food containers	30 x 20 cm (as large as available)	minimum number; should be as leak proof as possible (rely on quality products) to prevent formalin leakage
	1 aquarium net	20 x 10 cm	to dip the fish in the anaesthetic
	5-10 PE plastic bottles	1-2 l	for direct fixation without prior pre-fixation of small specimens
	1 measuring jug	500-1000 ml	to pour the fixative into the fixation containers
	2 plastic kegs (optional)	20 l	round plastic kegs with red screw-on lids with air and watertight O-Ring rubber gaskets, UN-X approved for storage and further fixation of pre-fixed specimens
	cheese cloth/cotton cloth (optional)	sufficient	for enwrapping preserved specimens for transportation
	2 plastic kegs	60 l	as specified above, one for formalin, one for anaesthetic storage,
	2 plastic buckets (optional)	10-20 l	for transportation/sorting of the catch
	2 large plastic tubs (optional)	ca. 100 l	for holding of captured fish; required for storage during electrofishing
Tissue sampling gear	separate set of dissecting tools	separate blades for re-usable scalpel 1 fine scissors 1 fine forceps (with smooth tips)	manipulation of DNA-tissues
	2.0 ml self-standing microtubes	200-400	pre-numbered, prefer renowned high-quality brands
	1-2 storage boxes	100-200 place boxes	large boxes are easier to handle compared to small 81 place boxes
	toilet paper	1 coil	required for cleaning of scissors/forceps after each sampling

	centrifuge tubes	50-100, self-standing form	for preservation of small fishes as DNA-voucher
	96% distilled ethanol	1 l	as back-up and to fill the centrifuge tubes

Table 1. Field and preservation equipment.

4.2. Documentation – general aspects

The precise, accurate, detailed documentation of all associated data is of vital importance for any biological sampling. The more information is collected on the geographic, taxonomic and habitat characteristics of specimens, the more valuable these specimens become to the scientific community. Ideally, exact location should be pinpointed with the aid of a GPS unit. This may entail single point data, start and end points of a transect or corners of a rectangular area. If a GPS unit is not available or practical, precise written, descriptive locality information becomes that much more important.

At all times it is crucial that all associated data (geographic, taxonomic and habitat) be stored together with the samples to ensure correct interpretation and matching of this data to specimens. All field notes, labels and ancillary documentation should be taken on weather proof, alcohol and formaldehyde resistant paper and with good quality ink or pencil (see Table 1). It is helpful to draw a picture of the sampling location highlighting features of interest (vegetation, current direction, physical features, etc.), habitat specifications and exact sampling location(s), gear used and species collected. As collecting gear or sampling site might be very specific for species or sizes, the precise documentation of the gear types used at the respective habitats adds valuable biological and ecological information to the collected specimens. This allows conclusions on daytime and habitat preferences of species or different live stages of the species. A comprehensive list (but not be limited to) of information to be collected is included in the Appendix (Documentation of Collecting Event). Any semi-accurate information must be omitted, *e.g.* habitat or locality information for specimens purchased from the fish market (see comments in section 3.1, fish market), unless the exact location is known.

4.3. Landing, sorting and euthanizing the catch

Any catch must be landed carefully. This applies especially to landing net collected species where entangling may cause damage to the specimens, *e.g.* curling beach seines with the catch and debris and stones up the beach. Instead leave the net gear (and the fishes) in the shallow water (being sure to elevate the edges of the net to prevent escape) and retrieve the specimens as quick as possible. This may be unavoidable in the case of gill nets that are left in the water for extended periods. Fishes should be removed from entanglements as carefully as possible to ensure no further damage is done to the specimen. Specimens should always be removed head first to avoid fin damage or de-scaling. Care should be taken with spines as they can damage the net, the fish and the collector. Bony spines (and their serration) are valuable

diagnostic characters for many species. Be aware that some species have poison glands associated with bony spines which can cause painful injuries or numbing and the affected area may cause dizziness or even severe allergic shock.

Living specimens recovered from electrofishing or retrieved alive from fish traps or hooks might require temporary holding in large buckets or tubs for later euthanasing or photo documentation of live colouration. The water temperature should be ambient and the containers placed in the shade protected from direct sun. Covering the container reduces stress and prevents escaping. Overcrowding should be prevented and the water should be exchanged at regular intervals to reduce physiological stress. Additional (pure) oxygen-supply from oxygen-bottles may be mandatory (*e.g.* for holding during electrofishing in several European Countries).

Rapid sorting of the catch is crucial:

- to obtain an overview of the number of included species;
- to separate those specimens required for DNA sampling;
- to release or fix the remaining specimens.

Especially in hot climates, specimens die rapidly and this immediately starts autolysis of the guts which may compromise tissue removal, particularly in predatory and herbivorous species. Sort the catch according to species or genera required for sampling. Dead specimens are either set aside for later tissue-sampling or immediately fixed in formalin. Specimens should be placed in appropriately sized containers where the total ratio of animal tissue to fixative does not exceed 1:3, enabling the specimens inside to float. Small dead specimens (up to 10 cm total length) for formalin fixation are immediately collected head first either into 50 ml centrifuge tubes (Figs 3D & 4H) or PE-plastic bottles (1-2 l). Plastic containers (buckets, bottles, tubes, jars, etc.) should be constructed of UV resistant material to ensure they maintain their integrity. Insert a folded (to prevent rubbing and removal of lettering), water resistant label with sampling data written either with pigmented ink or pencil and fill the bottle to the top with the fixative solution (to prevent desiccation of specimens extremities). Leave the container horizontal for at least 1 hour to ensure that the specimens remain straight and do not bend during pre-fixation. After this pre-fixation, the container can be turned in upright position for thorough fixation of specimens (1-3 days, depending on specimen size, Figs 5D & 5F). Remaining specimens should be kept alive and fresh as long as possible to keep (and document) their living colouration. Dead specimens can also be placed on ice to maintain tissue integrity and colouration, especially if photographing or additional treatments are needed. Specimens may be euthanized immediately before tissue sampling and fixation by (repeated – if necessary) exposure to a suitable anaesthetic for few (5-10) seconds (see 2.4 for anaesthetics and doses). Sort the fish to be tissue sampled according to species as best as possible, since carrying forward the same species information into the tissue sampling lists is much more convenient and less time consuming. Euthanize only enough specimens as can be processed by the sampling crew in a single session. Depending on the species and fish size, if

opercular movements have ceased for ca. 5 minutes, the fish can be considered to be dead. Be aware that air breathing species, such as some Cyprinids, many Silurids, Gouramis and Lungfishes, or fishes with low respiration rates (*e.g.* during winter), are less perceptible for water soluble anaesthetics and may require repeated exposure to be euthanased. The live colouration should be documented (written description or photographed) prior to or shortly after euthanasing, but before fixation, since the pigment cells will relax and expand turning specimens dusky after treatment.

Fig. 3. Preservation and dissecting gear. A. Mobile tissue sampling station and documentation in a van: right, keg drumstores anaesthetic; left, 4% formaldehyde solution; euthanased specimens are sorted and tagged on fresh paper towels to minimise mucus contaminations, dissecting tools cleaned with absolute ethanol and toilet paper; B. Dissecting tools including two smooth forceps, scalpel blades and a small scissor for tissue sampling (bottom), different sized probes and needles for raising and fixing fins, larger serrated forceps for manipulating formalin specimens, large scissor and Luer Lock syringe for penetration of the abdominal cavity of larger specimens; C. Pre-numbered 2 ml storage vials (NUNC), 200 vials per rack, with corresponding gill tags (smooth tracing paper 110 g/m² printed with HP DeskJet 600 with original HP Cartridge no. 29); different sizes of the same numbers for tagging of smaller (first two numbers only) or larger (complete tag) specimens; D. Self-standing 15 ml and 50 ml centrifuge tubes (TPP) for fixation of small specimens and fixation container (commercially available plastic food container). (Photos A, B, D, F by S. Beyer, all other photos by D. Neumann).

4.4. Tagging of specimens and preservation of DNA-tissues

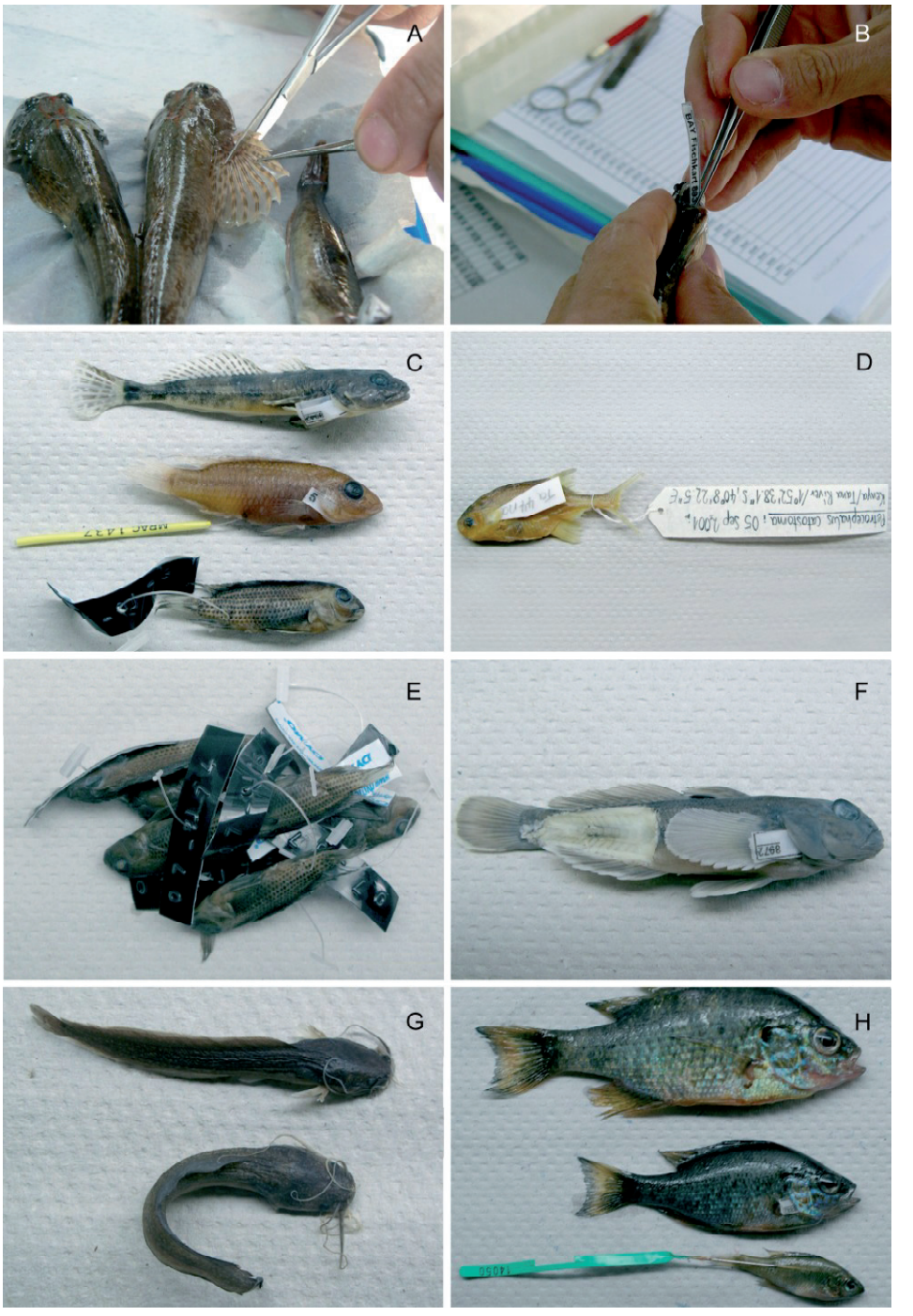
While formalin fixation varies only in relation to specimen size, tissue sampling necessitates exact and clean working to avoid cross-contamination of tissue samples, and should be done by a well-trained crew to speed up tissue sampling. Efficiently tagging and sampling of specimens requires two people, one for preparing, sorting and tagging, and the other for tissue sampling. At all times the link between specimen and tissue should be maintained both in field notes and through tagging of specimen and labelling of tissue tube. Tissue sampling requires a separate set of dissecting tools to avoid formalin contamination of tissues (for basic tissue sampling set up and requirements see Figs 3A-D). A set of freshly dead specimens (4-6, depending on size) are placed (sorted to species) with the head to the right on multi-fold sanitary paper towels to absorb residual mucus (Fig. 4A). Tissue tags can either be applied directly into the muscular tissue (Fig. 4C bottom, 4H bottom) or into the anus (Fig. 4C middle) using commercially available ribbon tags, t-end pins or similar (Fig. 4C), poly streamer tags (Fig. 4H, bottom) or tied through the gills (Figs 3C, 4B). Specimens are always tagged on the right side of the body, regardless which tag type is applied. In some species they may also be tied around the tail (Fig. 4D) ensuring that the tag will not work itself loose or become disassociated from the specimen. Tubes should be labelled with pencil or indelible ink, or both with information linking the specimen and tissue (Fig. 3C). Insertion of labels into tubes should be avoided to prevent contamination of tissue. The sampling crew should verify species and tissue tag numbers and tissue vial numbers during sampling to avoid mistakes during handling.

All tissues are sampled always from the right hand side of the specimen as the left hand side is traditionally used for measuring and photography. Muscle tissue is preferred to prevent having to gut the specimen for heart or liver tissue. Muscle tissue is usually removed from above the pectoral fin or on the caudal peduncle and should not alter the contour of the specimen (Fig. 4F). Muscular samples from the right abdominal region (behind the anus) are preferred if specimens have been dead for any length of time or if they have started decaying.

Fin clips are commonly sampled but may yield less DNA (quality and quantity). Usually only the lower portion of the pectoral fin is sampled, so that the total fin length remains unchanged and fin rays are still countable (both are diagnostic characters) (Fig. 4A, 4H top specimen). Depending on the specimens size, it may be necessary to cut the pectoral and pelvic fin, including the muscular fin bases (Fig. 4H, specimen in the middle), or to abduct the complete caudal peduncle (if more than one specimen is available) to receive enough tissue. The latter method should not be the first choice, because identification especially of small species under field conditions is extremely difficult, and recognising that the only minute specimen of a potentially new species lost its diagnostic character together with the caudal peduncle thereafter will neither improve the specimen, nor its condition. Cutting off bony spines has to be avoided for the same reasons, since most spines bear diagnostic characters and are essential for species identification as the left spine might be broken or missing (Fig. 4G).

It is best to use a new disposable scalpel blade for each tissue extraction, or to clean the scalpel blades or scissors after abducting the fins to prevent cross-contamination from one specimen to another. For this purpose, wipe and clean all tools (scissors, forceps, and scalpel) after each processed specimen best with 96% ethanol. If 96% ethanol is not easily accessible in the field, clean thoroughly with dry towel or toilet paper. While contamination between of allopatric species separated for a long period on a geologic time scale might be detected, it is impossible to detect cross-contaminations of just recently split or hybridising species. Same applies for any population genetics. The forceps for tissue manipulation should have smooth tips rather then serrated ones, to allow better cleaning of the tips and to avoid contaminations from residual mucus adhering to the serration. Excess mucus or debris should be removed from the tissue extraction site using paper towels or similar after which scales should be removed and the area cleansed (using 96% ethanol).

Fig. 4 (next page). Tissue sampling and tagging. A. Fin sampling of the lower portion of the right pectoral fin of euthanased sculpins; paper towels remove residual mucus; B. Gill tags should be folded with a small hook at the proximal end of the tag which is applied between 1st and 2nd gill arch (at least 2/3 of a gill tag should be covered – and secured – from the opercle); C. Application of different tags (from above): gill tag printed on smooth tracing paper with HP 600 DeskJet printer, T-anchor tags (Hall Print) applied into anus are firmly attached by piercing the gut canal (mind not to damage genital papillae during tagging to allow sexing of specimens thereafter), DYMO-tags applied with t-end pins and commercial tag guns (badly damaging especially small specimens); D. Small Mormyrid with handwritten tag (pigmented ink on durable paper) tied to the tail (museum gill tag easily lost and inappropriate for field conditions); E. Coiled up specimens removed from a museum jar: large t-end pins and even larger rigid plastic tags fixed to small specimens impede straight fixation and further damage them during museum storage; note that the imprint of DYMO-tags gets illegible in alcohol after few years; F. Muscular tissue samples should be removed from the tail (behind the anus) without altering the contour of the specimen. G. Diagnostic pectoral spine removed in the lower specimen for tissue sampling. H. Abduction of the fin base in smaller specimens might raise the DNA-content of the tissue sample; alternative PE-plastic streamer tag attached to a needle, applied in rostral direction into the muscular tissue causing minimal damage to the specimen (Caution: for museum storage plastic tags should be replaced because of potential corrosion from denaturing agents and/or high alcohol concentrations). (All photos by D. Neumann).


A
B
C
D
E
F
G
H

Tissues should be placed immediately into labelled tubes (preferably good quality, self standing, gasketed tubes), filled with 96% ethanol and tightly sealed (Fig. 3C). Cryo-tubes, which are especially designed for cryo-storage at deep temperatures, might cause problems under hot climates because of high evaporation losses. Repeated cases of evaporation losses of 50% or more have been reported. Be aware that in such cases the concentration of the residual ethanol may be far below 96%, since ethanol below 80% evaporates as pure ethanol. Tubes with 50% evaporation losses are inappropriate for tissue preservation and should be discarded since the ethanol concentration might have dropped below 40%. Prefer 96% distilled instead of chemically dried ethanol (concentrations of 99% or higher) as residual low boiling benzines used for drying the ethanol might degrade the DNA. The amount of tissue in any tube should not exceed one third of the total volume to allow for efficient and rapid preservation of tissues. If not avoidable, replenish with new ethanol after 2 hours. Tissue may also be cut into smaller pieces or macerated to facilitate this process.

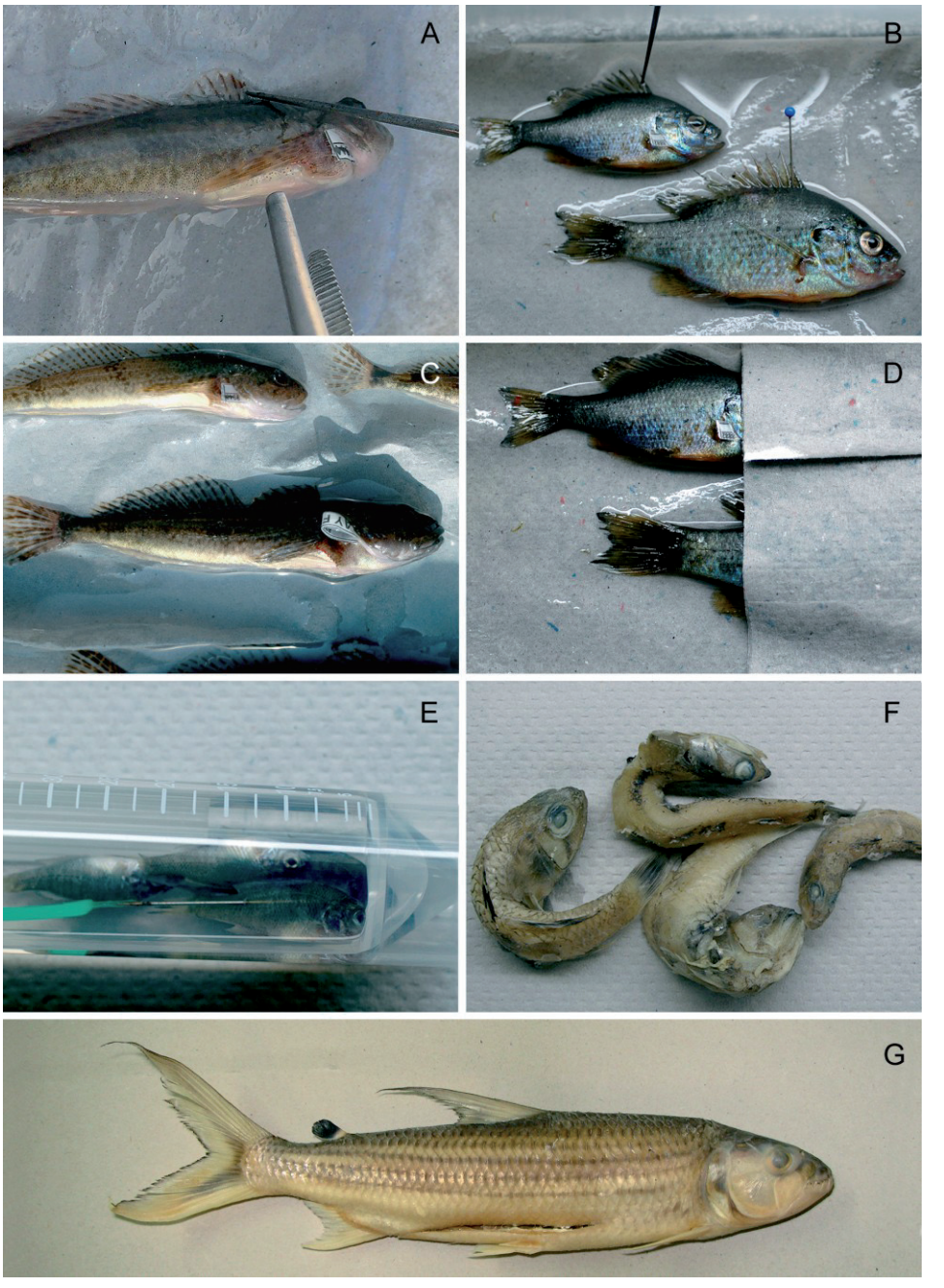
If no field tags are available for some reason, species should be sampled in order and by size to provide a mechanism of verification. For smaller specimens, whole specimens may be placed in tubes as tissue voucher but either photographs should be taken as vouchers or other specimens undoubtedly of the same species can serve as surrogate vouchers (not preferable).

4.5. Formalin fixation of specimens

Fixation and preservation is not the same. Preservation should only follow adequate fixation. Fixation stops autolysis by cross-linking and degrading proteins into amino acids by the formation of covalent bonds and coagulates cell contents to insoluble substances, whereas preservation alters the hydrogen bonding pattern and preserves the specimen by de-hydrating it (Simmons, 2002). Therefore, DNA-extraction of ethanol preserved tissues is possible (with DNA quality strongly depending on quick and efficient tissue dehydration), while extraction from formalin fixed tissues yields only short degraded gene fragments, depending on the number of cross-links and bonds which prevent the uncoiling of the DNA-Helix and thereby disabling the bonding of DNA polymerase.

The fixative should penetrate the specimens as rapidly as possible to prevent decomposition, especially of the guts and their contents. This initial or pre-fixation should be done within 10 minutes of the specimen dying.

Fig. 5 (next page). Fixation of fish. A. Prevent fusiform specimens from turning during prefixation; B. Prefixation of unpaired fins requires roughly 5 minutes; C. Orientate specimens in the one direction with sufficient spacing; D. Specimens stacked in different layers head on tails separated with formalin soaked paper towels; E. Small specimens (tissue sampled or ethanol vouchers) fixed head first horizontally in 50 ml centrifuge tubes; F. Nearly impossible to measure these bent specimens and useless for other morphological approaches; G. Opening abdominal cavity of large specimens allows quick fixation of the guts. (All photos by D. Neumann).


A
B
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D
E
F
G

This 10-min time frame is crucial and requires a disciplined and experienced DNA-sampling crew and fixation routine.

The most common fixative is a 4% (or 3.7%) aqueous formaldehyde solution; in the tropics and for larger specimens, a higher concentration of 10% should be considered for quick pre-fixation (= 1:4 dilution of 37%, concentrated formaldehyde solution with tap or river water). Use only buffered, methanol stabilized formaldehyde solution (Simmons, 2002) for fixation to maintain a stable pH range as formaldehyde is unstable and oxidises in water into formic acid and to prevent decalcification especially of the often minute and fragile bones of fish skulls. While decalcification begins at a pH of 6.4 and below, clearing of soft tissues may already start at a pH of 7.0! Additionally, unbuffered formalin causes subsequent problems during later museum storage. Omitting the buffer from the formalin will shift the pH inside collection jars to acidic ranges. If you are dependent on locally available, unbuffered formalin because of transportation limitation (*e.g.* IATA aviation restrictions), this can be buffered through the addition of Sodium Phosphate Dibasic Anhydrous (CAS 7558-79-4) and Sodium Phosphate Monobasic Monohydrate (CAS 10049-21-5) in a ratio of 6 grams and 4 grams per litre respectively, or by adding a carbonate buffer; even chips of marble or limestone will help. Be aware that it might be necessary to add more then 6 (4) grams per litre of buffer, if you are depending on river water with low (acidic) natural pH values (*e.g.* streams draining rainforest or granite soils) for dilution of higher concentrated formalin to receive a 4% aqueous formaldehyde solution.

The diffusion rate of formaldehyde through the animal tissue is crucial and this may be slowed by thick or swollen mucus layers (*e.g.* in eels or sculpins) or size of the specimens. Simple formalin permeation (placing specimens directly into the fixative) is sufficient for fixation of small specimens up to 10 cm. Cover the bottom of the fixation container with 1-2 paper towels and a small amount of formalin, preventing the specimens from floating. This should allow the specimens to be fixed in a natural position. Better quality specimens may be obtained by raising the dorsal and pectoral fin with a probe thereby fixing the spines against the formalin soaked paper towel together with spreading the caudal fin. This improves counting of spines and soft rays of preserved specimens. If necessary, hold the raised spines in place with a probe or fix one of the first spines with a small needle. The minute muscles responsible for the fin movements are fixed within 5-10 minutes (Figs 5A-B). This may not be possible for all specimens due to time constraints.

Gill covers and branchiostegal apparatus should be in a normal position, the mouth should be slightly open, and the jaws should not be abducted. If a specimen has suffocated prior to relaxation in the anaesthetic, hold the fish and simultaneously keep the gill covers closed with one hand, while carefully closing the mouth and setting the branchiostegal apparatus back into a normal position with the other. Cut a finger off a disposable glove, push the specimen into the closed end and place it carefully into the fixative. Locked pectoral spines of catfishes should be released by pulling the spine carefully backwards (caudal direction) and turning it (beware of serrated or poisonous spines). All specimens which are placed in one layer into the fixative should be orientated in the same

direction. Take care that the new layer is sufficiently moist with formalin and place the next batch of specimens in the opposite direction (heads to tails) and raise the new fins by placing them on the bodies of the previous layer. The vertical distance between single specimens should be wide enough to unfold the caudal fins (Figs 5C-D).

This method of stacking the specimens in different layers of paper towels has several advantages:

- Pre-fixation and fixation can be done in the same container while limiting the necessary amount of formalin and minimizing the amount of formalin vapour.
- Specimens inside the layers will support each other resulting in straighter preserved specimens for natural history collections (compare Fig. 5F).
- Different sampling locations can be separated inside one fixation box (in this case pay attention to fold the outer ends of the towels upward to prevent that single specimens slip from their layer and mix with other locations).
- During transportation the arrangement in layers stabilises the specimens during fixation.
- The layers will also reduce movement of free formalin solution inside the box which stabilises the box during transportation and lowers the risk of potential spillage.

For specimens ranging from 10-20 cm, the permeation method is sufficient for pre-fixation only (10-20 minutes). After this time, the specimens need to be immersed in 4% formaldehyde solution to ensure high penetration rates (*e.g.* by stepwise filling the fixation box to the lowest layer with formalin).

For good fixation results and efficient penetration of specimens larger then 20 cm, the fixative may need to be injected into the body cavity. Assure that formalin/fixative is injected only into the cavity and not into the muscular tissue, which leads to intramuscular tissue rupture and formalin swelling of the tissues. Penetrate the abdomen laterally through of the anus or the belly near the (scaleless) base of the pelvic or pectoral fins with a syringe and needle (preferably a Luer Lock syringe and needle to prevent the needle from being propelled off the end of the syringe due to pressure build up inside the specimen's belly). Caution: To prevent eye damage through exposure to formaldehyde always wear protective eyewear (safety glasses or goggles) and turn the specimen away from your face when injecting and removing the needle.

Specimens larger than 30 cm should either be injected with 10-37% formaldehyde solution (37% for specimens larger than 50 cm) or the abdominal cavity should be opened with a sharp scalpel. Insert the scalpel into the anus (blade in parallel direction with the inner body cavity), cut 2-3 scale rows upwards in a dorsal direction and then turn in a rostral direction and parallel to the ventral border of the belly so that the natural contour of the specimen remains unchanged (Fig. 5G). Care should be taken to avoid damages of the genital papilla to ensure sexing of the specimen and to cut only the lateral right side of the body to keep the pre-anal measurements of this specimen.

Large specimens need to be placed in larger UN-approved, tight closing plastic kegs. For fixation it is important to leave the drums in a horizontal position for at least a day for good fixation results and to avoid deformation of specimens. For transportation, place the keg horizontally in a plastic box to collect any potential leakage and secure against rolling.

4.6. From fixation to preservation

Small to medium sized specimens should be left in sufficient formaldehyde solution in the fixation box for at least one week. Larger specimens should be checked periodically to ensure adequate fixation of the entire specimen – the belly should be firm, muscular tissue should be moderately hard, leaving no thumb imprint behind after manual inspection. If the gut contents of specimens (mainly herbivorous) start to decay, fermentation may cause gas to build in the gut cavity distending the specimen. In this case, the abdominal cavity needs to be opened and washed out thoroughly. Care should be taken when opening the abdominal cavity of such a specimen to prevent chemical eye burns and this can be done underwater to alleviate any pungent smell emanating from the specimen.

After fixation of specimens, the transfer to the preservation fluid has to be done in several steps to remove residual formalin from the specimens, and to avoid dehydration or cell rupture. First, wash the specimens by rinsing in water or by immersing in water and exchanging the water several times on a daily basis. This procedure should be repeated, until no or only a moderate formalin smell is perceptible. Then transfer the specimens into a 20% ethanol solution for 1-2 days (for specimens up to 5 cm), one week (specimens up to 20 cm) or 1-2 weeks (larger specimens), and repeat this procedure with 40% and 60% solutions before finally transferring the specimens into 70% ethanol. From personal observations, specimens originating from high water conductivity environments (especially European Cyprinids) seem to be highly susceptible to ethanol dehydration and thereby should not be transferred directly from formalin into 70% ethanol to avoid shrinkage.

5. Packing for transport

Returning samples from the field, including DNA-tissues as well as formalin preserved specimens might give rise to some unforeseen difficulties. Specimens should therefore always be packed in such a way that: a) they are not damaged during the transit; b) the specimen can be stored under stable conditions inside the containers for several weeks. This might be necessary if an airline refuses to carry formalin samples and you are forced to return the samples by surface mail from third world countries.

5.1. Transport on the road from the field site

Packing requirements of the specimens strongly depends on the road conditions.

5.1.1. On paved roads

Preservation and fishing gear has to be packed in such a way to prevent leakage from formalin containers and personal injury in case of emergency braking. All field gear should be secured in the trunk with straps, as for oxygen bottles and especially larger drums containing formalin or anaesthetics. Formalin containers should be placed in larger plastic or aluminium boxes to avoid any formalin leakage into the trunk particularly for bendy roads. Formalin and ethanol fixation gear always has to be stored in separate boxes to avoid any formalin contamination of the ethanol gear. Square plastic food containers are never 100% leak-proof and should be filled only to 5 cm below the top to minimise potential leakage. Formalin vapours emanating from the trunk into the cabin might cause dizziness or sickness to the driver. Plastic keg drums with formalin specimens should be carried horizontally or nearly horizontally (*e.g.* if a drum is placed and strapped into a tub) to avoid bending of only weakly fixed (larger) specimens inside the drum.

5.1.2. On gravel roads or rough tracks

For the transport of specimens for long distances and days over rough roads or dirt tracks, the packing of specimens requires additional precaution to avoid physical damage to them. Smaller PE-plastic bottles and kegs should be filled to the top with fixative leaving no air inside, to keep specimens in stable condition inside. Any free air space inside will shake both fluid and specimens vigorously after every road bump likely damaging the fins and abrading specimen-ID labels or DNA-tags. To protect fixed specimens in a keg or drum, wrap medium to large specimens in formalin soaked cloth. Each wrap should include only (consecutive series of DNA sampled) specimens from the same location to allow identification of specimens in case single field tags become illegible. Small fixed specimens can be easily separated and secured in disposable tea bags and several of those bags should be wrapped in cloth. For packing and returning material from foreign countries, decant the formalin and separate and pack the specimens in the same way. Additionally, single wraps inside the kegs may be packed inside aquarium bags and sealed tightly with rubber bands to minimise the risk of formalin leakage from the drums during transport. This method is not suited for pre-fixed or only weakly fixed specimens because the weaving pattern of the cloth (or specimens itself!) will imprint into the epidermis of the specimens!

5.2. Transport as carry-on luggage on board on aircraft

Carrying pure alcohol in hand or checked luggage onboard an aircraft is strictly prohibited (IATA, 2009). This applies for all DNA-samples *e.g*. placed in 2.0 ml tubes. Salt-based DNA-buffers (*e.g*. DMSO) or buffers with a volume less then 24% ethanol are not regulated. Alcohol based buffers should be referred to as “DNA-buffer” only when entering or leaving Islamic countries to avoid problems at customs. Aqueous formaldehyde solutions with less than 25% formaldehyde are not regulated under dangerous goods whereas solutions with more than 10% are classified as aviation regulated liquid (UN 3334). Concentrations less

than 10% are not regulated at all. To comply with the current IATA regulations, specimens should be wrapped in formalin (4%) soaked moist cheese-cloth, sealed in PE-plastic bags inside the drum as described above, and must be packed leak-proof in and best in UN-approved plastic kegs or drums. Note: There will be new regulations and amendments for shipping and transport of natural history specimens on board of aircraft in the 52st Ed. of the IATA Dangerous Goods Regulations affective on 1 January 2011 and perhaps in future; please keep yourself updated to comply with the regulations!

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7. References

IATA, 2009. Dangerous Goods Regulations, 50th Edition. International Air Transport Association, Montreal - Genf: 880 PP.

SIMMONS, J.E. 2002. Herpetological Collecting and Collections Management. *Society for the study of amphibians and reptiles, Herpetological Circular* 31: 1-153.

8. Further reading

For further information especially on net gear, electrofishing and fishing methods in general, the “Fish Collection Methods and Standards (Version 4.0)” is very valuable. This free Guideline issued by the Government of British Columbia (http://ilmbwww.gov.bc.ca/risc/pubs/aquatic/fishcol/assets/fishml04.pdf) can also be viewed (http://ilmbwww.gov.bc.ca/risc/pubs/aquatic/fishcol/index.htm) online.

9. Additional Internet based Information

CITES: http://www.cites.org

IUCN: http://www.iucnredlist.org

OIE: http://www.oie.int/eng/en_index.htm

How to throw a cast net: http://www.ausfish.com.au/castnet/

Aquatic Animal Health Code, Cpt. 5:
http://www.oie.int/eng/normes/fcode/en_sommaire.htm

Name	CAS-Number	Properties	Availability	Safety	IATA
Chlorobutanol	57-15-8	white, coarse crystalline powder	www.sciencelab.com VWR International	Irritant (eyes, skin) and extremely dangerous if ingested	not regulated
Ethanol (96%)	64-17-5	colourless liquid	www.sciencelab.com Sigma-Aldrich VWR International	Irritant (eyes, skin)	class 3
Eugenol	97-53-0	liquid oil		Irritant (skin), inhalation hazard; seek immediate medical attention in case of serious contact	may be regulated as UN 3334
Formaldehyde solution (10%)	5-00-0	colourless liquid	www.sciencelab.com Sigma-Aldrich VWR International	Irritant (skin), inhalation and ingestion hazard; carcinogenic, mutagenic and teratogenic effects (suspected or possible); classified developmental toxin to male/female reproductive system	concentrations of 10% and below may be regulated as UN 3334; concentrations of 10-24% regulated as UN 3334; less then 25% regulated as class 8 (UN 2209)
MS-222	886-86-2	white, fine crystalline powder	Sigma-Aldrich VWR International	Irritant (skin, eyes and respiratory system)	not regulated
Rotenone		brownish fine powder or dark	Sigma-Aldrich	Irritant (eyes and inhalation); after eye contact and ingestion seek	class 6.1

		brown fluid		immediate medical attention; Lethal oral dose (Human): 143 mg/kg	
Disodium Hydrogen Phosphate	7558-79-4	clear or white crystalline hygroscopic powder	www.sciencelab.com VWR International	Dust may cause irritation (eyes and respiratory system)	not regulated
Sodium Dihydrogen Phosphate	10049-21-5	clear or white crystalline hygroscopic powder	www.sciencelab.com VWR International	Dust may cause irritation (eyes and respiratory system)	not regulated

11. Appendix 2 - Material Safety Data Sheets (MSDS)

Chlorobutanol: http://www.sciencelab.com/xMSDS-Chlorobutanol-9923417

Disodium hydrogenorthophosphate:
http://www.chemicalbook.com/ProductMSDSDetailCB1242667_EN.htm

Ethanol: http://www.sciencelab.com/msds.php?msdsId=9923956

Eugenol: http://www.sciencelab.com/xMSDS-Eugenol-9924007

Formaldehyde solution (10%):
http://www.sciencelab.com/msds.php?msdsId=9924096

MS-222:
http://caligula.bcs.deakin.edu.au/bcs_admin/msds/msds_docs/Ethyl%203-aminobenzoate%20methanesulfonate%20salt%20(Sigma%20A5040).pdf

Rotenone:
http://caligula.bcs.deakin.edu.au/bcs_admin/msds/msds_docs/Rotenone.pdf

Sodium Dihydrogen Phosphate:
http://www.sciencelab.com/msds.php?msdsId=9925021

12. Documentation of Collecting Event

Collecting Event

- Field number (usually provided by the collector) *e.g.* XYZ-2009/01 (three-letter country code-year/sample number).
- Date of collection – start and end date if necessary.
- Gear used – type or combination of different gear types.
- Gear specifications – mesh size, depth, exposure time, voltage/current (as precise and detailed as possible).
- Habitat specifications referred to employed gear type – (gill net in front of weeds, kick-net sampling in riffles, etc.).
- Habitat conditions referred to collecting site (hard/soft/sand bottom).
- Full names of all collectors (first, middle and last names).

Locality

- Drainage – might be hard to discern *e.g.* for small streams or swamps.
- Geopolitical designation (country, state/province, county, etc.).
- Detailed locality string – usually in ‘named place’ and ‘location to named place’ format.
- Highlight any distinctive features like road and river crossings (indicate any distances by road or linear – *e.g.* 5 miles West of…).

- Any other geographic subdivision (State, Province, Department, Village or Township, County (?) etc.).
- Latitude and longitude.
- Geodetic datum.

Specimen

- Identification to family, genus or species level (if known, elsewise unspecific as *e.g.* as “Cyprinidae indet.”, “Barbus sp.” to allow re-identification of tissues in cases of erroneous sampling lists).
- Any distinctive characteristics – colouration, morphological characters as these may be lost during fixation and preservation.
- Size – in millimetres as standard or total length (SL or TL) or as disc width for rays etc. (can slow down specimen processing and can be done at a later time).
- Weight – in grams (often times not practical in the field and may be done later).

Habitat

- Habitat specifications – road side ditch, floodplain, swamp, forest stream, rapids, etc.
- Peculiar habitat conditions – leaf litter, deadwood, dense weed standings, boulders, etc.
- Climate – cloud cover, precipitation etc.
- Season – rainy/dry season, summer/winter.
- Stream type – white water, clear water, black water.
- Water and ambient temperature.
- Water clarity.
- Water pH.
- Water conductivity.
- Current strength and direction.
- Associated vegetation.
- Associated species not collected.

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