Chapter 10

Sampling continental freshwaters

by

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Abstract

This chapter provides a summary of methods used for collecting freshwater organisms, covering algae, aquatic macrophytes, and invertebrates. It does not deal with aquatic fungi or freshwater vertebrates, which are dealt with in other chapters. After a preliminary introduction, subsequent sections deal with major subdivisions of biota based on taxon and/or body size. We also discuss sampling special habitats, with the subterranean environment (*sensu lato*) and anquialine waters covered in particular detail. We do not pretend to be exhaustive in the presentation of well-known techniques frequently included in freshwater techniques texts, but rather we emphasize ‘tricks of the trade’ employed by the authors that are rarely described in print. Sampling, sorting and fixing methods are suggested for each major group. The references included, some of them websites, will complement the methods described here.

Key words: algae, aquatic vascular plants, invertebrates, subterranean habitats, anchialine waters

1. Introduction

Life in fresh waters is extremely varied from any point of view, be that size, morphology or behaviour. Texts devoted to promoting 'the importance of life in soil or life in water' tend to emphasize extremes, as if extremes provide the most compelling justification for studying anything. Fresh waters do not lack extremes, but they are a poor argument for defending one's cause. For this chapter, the 'cause' is how to sample freshwater habitats for eukaryotic organisms (excluding vertebrates, which are dealt with in different chapters). At first glance this may seem a straightforward proposition, but many questions immediately arise, among the most important of which is: 'sampling for what purpose?' We distinguish between Taxon-specific, Biodiversity and Ecological sampling, but our main emphasis will rest on Taxonomic sampling.

On the other hand, we do not pretend that we have discovered the many techniques and methods that already populate books, review articles and web pages. We describe techniques that we use currently in our taxonomic practice and provide rarely published hints and tricks that give them a personal flavor. But we do not pretend to be exhaustive. Additional information is available in documents that can be found on the internet, review chapters, monographic books, etc.

We particularly emphasize sources of information easily found on the internet for free; including construction of inexpensive sampling devices and ways to use them. We feel this is important given that the areas of greatest interest for taxonomic research are often in developing countries, where it may be difficult to find prefabricated samplers or where they are so costly as to be prohibitive for the local taxonomist (traditionally a not very well funded professional).

However, the improvement of our knowledge of freshwater biota does not only depend on adequate sampling methods but also on advances in molecular techniques and improvement of image-processing hardware and software.

1.1. Ranges in body size and species-richness of taxa in freshwater habitats

Truly knowing the number of species now living on Earth is a Herculean and likely impossible task. Specialist taxonomists who consult the meritorious work of Chapman (2009) realize that some of his counts of species richness are significant underestimations of the number presently known. For instance, Plecoptera total 2,274 in Chapman (2009) but are raised to 3,497 in the stonefly chapter of the Freshwater Animal Diversity Assessment (see Balian *et al.*, 2008 for a summary of the project). However, the total count by Chapman seems to be fairly accurate.

In the following Table 1 we present a list of the main freshwater taxa with an indication of their approximate range in body size and number of species. It would be useful to include some idea of abundance, but this is too variable. Both variables could give an idea of what amount of diversity may be found or lost

depending on the mesh size and sampling method used. Suggestions for sampling particular groups are provided in additional tables below.

Taxon	Size Range	Number of Species
Microalgae		> 50,000
Macroalgae		>19,000
Aquatic vascular plants		2614
Microinvertebrates		> 15,000
Nematoda	0.2-2 mm	>2000
Gastrotricha	100-300 μm	320
Rotifera	100-500 μm	1498
Tardigrada	50-500 μm	62
Cladocera	0.2-18. 0 mm	620
Copepoda	0.3-3.2 mm	2814
Ostracoda	0.4-30 mm	1936
Syncarida	0.5-2.0 mm	240
Halacaridae	140-2000 μm	56
Oribatida	0.3-0.8 mm	86
Hydrachnidia	0.3-3.0 mm	> 6000
Macroinvertebrates		>87,000
Porifera	2-3 cm up to 40 m^2	219
Coelenterata	2-25 mm	<20
Turbellaria	5-30 mm	1303
Nemertea	> 30 mm	22
Nematomorpha	1-100 cm	326
Oligochaeta	0.1-4 cm	>1200
Polichaeta		168
Hirudinea	0.5-45 cm	482
Bryozoa		88
Anostraca	7-100 mm	307
Notostraca	10-58 mm	15
Conchostraca	2-16 mm	<200
Branchiura Argulidae	5-25 mm	113
Cumacea		21
Tanaidacea		4
Mysida	10-30 mm	72
Isopoda	5-20 mm	>994
Amphipoda	5-25 mm	1870
Decapoda	15-130 mm	>2662
Collembola		>103
Ephemeroptera	3-28 mm	3046

Plecoptera	6-50 mm	3497
Odonata	10-45 mm	5680
Hemiptera		4810
Hymenoptera		150
Megaloptera	25-90 mm	328
Neuroptera	6-8 mm	118
Trichoptera		12,627
Coleoptera		18,000
Diptera		27,141
Gastropoda	2-70 mm	>3800
Bivalvia	2-250 mm	1026

Table 1. Size range and global number of species of freshwater taxa (From FADA Project [see Balian *et al*., 2008]; Pennack, 1978; McLaughlin, 1980; Bartsch, 2004; Thorp & Covich 2001 and other sources).

1.2. Categories of sampling strategies: Taxon-specific, Biodiversity Survey and Ecological

Although this may go against common usage, we would like to keep the distinction between *taxonomy* and *biodiversity*. It is not merely rhetorical as it affects contents, procedures and aims.

In taxonomy, which following Darwin could be loosely defined as '*the empirical evidence for speciation*', the objective is to have the full representation of a certain clade or taxon, in its worldwide distribution. It leads to narrow taxon-focused sampling schemes, usually of a qualitative nature, elevated status of rare specimens (even a single specimen may be important if it is the sole representative of a new species) and very selective in sorting and fixing procedures. In biodiversity surveys the objective is to garner an overview of a variety of taxa in a geographical area during a certain time period. It usually has a wide taxon focus, mainly uses semi-quantitative sampling schemes and is compatible with use of a modest number of general fixatives.

Both contrast clearly with sampling for ecological goals, as the latter usually involves testing hypotheses with either observational or experimental designs, and, in consequence, is problem focused, quantitative or at least replicable.

1.3. A 'pattern' cycle of Taxonomic sampling

Under a taxonomically oriented project, the sampling cycle can be subdivided into a presampling, sampling, field sample manipulation, transportation, laboratory manipulation and sample maintenance. The emphasis in this chapter is mainly on actual sampling although occasional information is provided on the other steps of the sampling cycle. Below we briefly describe the other parts of the cycle before moving to taxon- and habitat-specific chapters.

Presampling involves defining the objective of the sampling, target organisms and sites, and compiling a list of material needed. A comprehensive list of sampling

material that can be adapted and enhanced for particular objectives may be found on http://pubs.usgs.gov/of/2002/ofr-02-150/. In addition some safety measures should be taken (more on this below).

Field manipulation may involve on-the-spot sorting for organisms that should be brought alive to the laboratory (*e.g.* Tricladida) or are so delicate so that they need *in situ* fixing (*e.g.* Ephemeroptera). One should decide in advance what the target groups demand so as to have enough time and material to process samples as required. This is also the time to decide whether duplicate samples or subsamples should be fixed in different fixatives, *e.g.* absolute ethanol for molecular analysis and the right fixative for taxonomic analysis. Additionally, one may need to quickly record morphological information that may disappear or be difficult to obtain in fixed material (*e.g.*, eye pattern in leeches). Digital imaging of live specimens will likely become common in the near future.

Transportation may be done with the sample already fixed or kept at low temperature with a field refrigerator or inside a container with ice.

Finally, laboratory manipulation may involve additional sieving and sorting, subsampling and transferring to the final fixative. Regular revision of fixative and sample conditions on a yearly basis may be desirable. In some special cases, fixed samples can be stored at low temperature to allow for future molecular studies. For samples coming from fragile habitats the process of sample maintenance is of utmost importance given the value of the material.

1.4. General remarks on classifying water bodies

From a practical point of view, the most important criteria to classify inland water bodies is ease of access, in particular, wadeable versus non-wadeable waters. Everything becomes more problematic when waters cannot be easily accessed on foot, especially if one is sampling in remote areas. This pragmatic subdivision of freshwater habitats is not the most common classification. We mention two other categories of classification. One is the IUCN Habitats Authority File (http://www.iucn.org/themes/ssc/sis/authority.htm) where freshwater habitats are classified in the context of all other Earth habitats, the other, our favorite, is that of Elton & Miller (1954) in which a wide variety of different aquatic habitats are summarized along two axes: current speed and size (Table 2). Additional axes could be added to increase the number of habitats covered.

That said it is very common that in taxon-specific sampling the researcher goes alone or in small groups to the field, carrying relatively little equipment. Impermeable rubber boots are an essential element for sampling freshwaters. Waders can be more troublesome, especially in deep places with a swift current. However, boots limit the depth were the researcher can get into: mainly shallow streams and ponds. For deeper ponds and lagoons, besides boats (which are frequently not available), there is the individual solution used by fisherman known as ‘float tubes’. Basically they are a floating device (round or in u) where the sampler gets into with fins and diving boots (if the water is cold) and may advance moving the fins. The floating device includes different kinds of pockets for

storage. Prices are below 100 €. For deep water sampling, however, a boat is essential.

	Very small	Small	Medium	Big	Very big
Quiet	Treehole	Small pond (< 17 m^2)	Pond (< 0,4 ha)	Small lake (< 40 ha)	Large lake or sea
Slow	Trickle	Ditch	Channel		
Medium	Small stream	Lowland stream	Lowland river	Big river	Estuary
Swift	Spring	Torrent	Swift torrent		
Vertical or	Drip	Small waterfall	Medium waterfall	Big waterfall	Very steep

Table 2. Different aquatic habitats summarized along two axes: current speed and size.

Subaquatic viewers (Fig. 2), which may be as simple as a bucket with the bottom replaced by clear glass, can be extremely useful in shallow or deeper water, specially when the flow is high or the water is not very transparent.

Fig. 1. Observing in wadeable waters. (Photo by Maria Eugenia Cañadas)

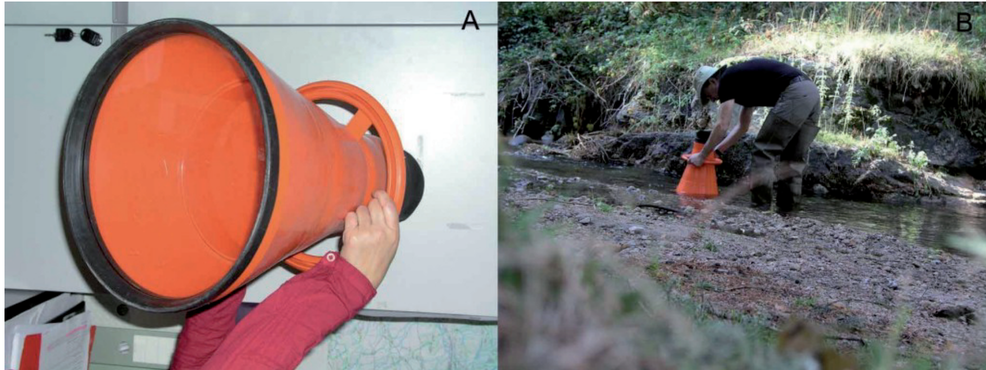

Fig. 2. The view bucket. A. Bottom; B. Correct usage (Photos by Antonio G Valdecasas)

1.5. Safety notes

To our knowledge there has not been an exhaustive study of risks for freshwater field work similar to the report of Nancy Howell (1990). However, many of the risks evaluated in that report are similar to those which natural history researchers confront.

When planning a sampling trip to a remote or poorly known area, information and recommendations such as those included in Johnson *et al.* (2008) "Handbook of Expedition and Wilderness Medicine" can be extremely useful. Information and common sense are key words for a successful sampling trip.

And finally, care should be taken when dealing with fixatives, as many of them are toxic and should be manipulated under safe conditions. The product information labels to proceed as required.

1.6. Additional information and some general web pages

The U.S. Geological Survey has published a set of books under its National Water-Quality Assessment (NAWQA) program that are available at http://water.usgs.gov/nawqa/. Some of them are mentioned in the sections below.

Especially useful are the IBP Handbooks (International Biological Program) published by Blackwell in the 1960's and 70's. Relevant for the organisms dealt in this chapter are Vollenweider's (1969, 1974) manual on primary production, where a very detailed review of techniques to sample phytoplankton, periphyton and macrophytes may be found. The IBP handbook nº 17 edited by Edmondson & Winberg (1971) includes chapters on zooplankton, benthos of standing and flowing waters, periphyton interstitial fauna and a review of emergence traps plus a chapter on sorting and counting organisms. Hauer & Lamberti (2007) cover a great diversity of stream-specific methods.

Another series of books that may help when planning or revising the information available on faunistically or floristically still poorly known countries is the set of Limnology in Developing Countries books, published by the International Association of Limnology (SIL). Four volumes have been published up to date.

The best way to get advice on particular items concerning freshwater sampling and organisms is to address the specialists themselves. Many of them may be found through the help of learned society and international organizations devoted to the scientific study of water habitats. We include below three of them:

- The International Society of Limnology (http://www.limnology.org/index.shtml)
- The Freshwater Biological Association (http://www.fba.org.uk/index.html)
- The North American Benthological **Association(http://www.benthos.org/index.aspx)**

1.7. References

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VOLLENWEIDER, R.A. (Ed.) 1969, 1974. *A manual on methods for measuring primary production in aquatic environments*. IBP handbook nº 12. Blackwell Scientific Publications, Oxford: 213 pp (225 pp.).

2. Sampling continental algae

Algae are the dominant autotrophic organisms in many aquatic systems, including fresh and brackish waters. Some of them are the sole photosynthetic organisms in extreme habitats (Ciniglia *et al*., 2004). Presently four Kingdoms are included in the polyphyletic group called 'algae' (Cavalier-Smith, 2004).

2.1. Safety notes

Where toxic algae are expected, it is recommended to use gloves. Some toxic species can produce aerosols that may affect the respiratory system (Cheng *et al*., 2007). Also, care should be taken when sampling water bodies inhabited by invasive species to avoid accidentally dispersing them to other water bodies.

Almost all fixatives are toxic if inhaled and it is recommended to carry out all the fixation procedures in well-ventilated areas or under a fume hood in the case of formaldehyde. Ideally the material should be taken alive to the lab in a portable fridge (dark and cold conditions). Fixation should be done then in the lab with gloves and safety glasses. If it is compulsory to fix the material in the field it is advisable to transport all the reactive agents inside air-tight boxes to avoid accidental inhalation. All the materials used for fixation and their remains should be disposed of appropriately.

2.2. Sampling the plankton

To collect the diversity of phytoplankton typically present in standing water bodies, there are different kinds of plankton nets and sampling bottles. Both allow vertical and horizontal sampling, but bottles allow calculations of the density of cells when counted under sediment chambers with a known volume and with inverted microscope (Utermohl method).

It is advisable to carry out a preliminary observation of the material to see the movements of the organisms, their true colors, and some structures that are not detected in fixed material, such as a contractile vacuole (Chlorococcales). The use of Lugol (IIK) allows gentle fixation that keeps the flagellum but is of short duration. Formaldehyde (2-4%) allows a long-term fixation. Alternatively glutaraldehyde (2-3%) may be used as its vapors narcotizes motile species and facilitates their microscopic study (see Table 3).

If the study is focused on a single taxonomic group then the routine can be simplified. If the groups have a tough envelope alternative reagents may be employed (see Table 3).

When doing molecular studies one must have a duplicate in absolute ethyl alcohol or at –20ºC.

For ecological studies it is often enough just to know which general categories of algae are present. Flow cytometry allows discrimination of cells by size, shape and pigments. This type of evaluation can be done either in the field with portable equipment or in the laboratory.

2.3. Sampling the benthos

Before beginning sampling it is useful to do a visual inspection of the area under study to establish its heterogeneity and take samples from all the microenvironments available.

Microphytobenthos are algae whose presence can only be detected by the color of the substrate. To sample these communities it is useful to use a brush, scalpel or jacknife on hard substrates, and PVC cylinders or Petri dishes on soft substrates. If the hard substrates cannot be taken out of the water it is necessary to use tubular samplers that can be held securely inside the water (Steinman *et al*., 2007). Methods of fixation are similar to that used with phytoplankton.

In some cases when substrates are scarce, or if one desires to compare algal assemblages between two locations while holding the substrate type constant, then artificial substrate can be used: microscope slides, stones, plastic materials, bricks, tiles, etc. However, artificial substrates are selective and different substrates will not necessarily be colonized by the same arrays of algae (Cairns, 1982).

Macrophytobenthos are macroalgae that can easily be recognized in the field with the naked eye and can be separated from the substrate with scalpels or razors if they develop on hard substrate, or with hooks or a potera (squid jig) when forming meadows on soft substrate. It is important to get the basal portions of macroalgae, as they may be essential for the taxonomic identification. Specimens can be fixed with formaldehyde or processed as is done for vascular plants (see below). Dry material can be used for molecular studies without further treatment (see also chapter 7).

2.4. Sampling shallow and deep waters

See the introduction.

2.5. Sampling special habitats

2.5.1. Caves and hypogean environment

To preserve these fragile ecosystems, especially when sampling stalactites, stalagmites or close to old remains of primitive human artifacts, sampling methods that are not very aggressive such as adhesive paper or moistened filter paper are to be used (more information in § 6 to 8)

2.5.2. Endophytic algae on aquatic plants

The host plant is collected (*Lemna* sp., *Sphagnum* sp.) and preserved in formaldehyde.

Table 3 summarizes in more detail the algae groups, their habitats and growth forms, number of species and appropriate fixatives.

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Taxon	Gross habitat	Specific habitat	Habit	Number of species	Recommended fixative	Extra treatment	DNA preservation
Microalgae							
Cyanophyceae	Standing/flowing waters	Epipelon, epiliton, epifiton, plancton	Unicellular, colonies, filaments	2664 (1)	Formaline or drying	EDTA or light acid treatment	Absolute methanol, frozen (-20ºC), dried
Rhodophyceae	Standing/flowing waters	Epiliton, epifiton	Unicellular, colonies, filaments	5000-6000 (3)	Formaline or drying		Absolute methanol, frozen (-20ºC)
Prasinophyceae	Standing water	Plancton	Unicellular	300 (2)	Lugol or formaline, better if study alive	Narcotizing previous fixing	Absolute methanol, frozen (-20ºC)
Chlorophyceae	Standing/flowing waters	Plancton, epilithon, metaphyton	Unicellular, colonies, filaments	3500 (2)	Lugol or formaline	EDTA or light acid treatment	Absolute methanol, frozen (-20ºC)
Trebouxiophyceae	Standing/flowing waters	Plancton, metaphyton	Unicellular, colonies		Lugol or formaline		Absolute methanol, frozen (-20ºC)
Charophyceae	Standing/flowing waters	Epiliton, epipelon	Unicellular, colonies, filaments	11700 (2)	Lugol or formaline		Absolute methanol, frozen (-20ºC)
Bacillariophyceae	Standing/flowing waters	Epipelon, epiliton, epifiton, plancton	Unicellular, colonies	20000 (2)	Formaline	Organic matter oxidation	Absolute methanol, frozen (-20ºC)
Chrysophyceae	Standing water	Plancton	Unicellular, colonies	1250 (2)	Lugol, formaline, glutaraldehyde		Absolute methanol, frozen (-20ºC)
Glaucophyceae	Standing water	Plancton	Unicellular, colonies	15 (2)	Lugol, formaline, glutaraldehyde		Absolute methanol, frozen (-20ºC)
Synurophyceae	Standing water	Plancton	Unicellular, colonies		Lugol, formaline, glutaraldehyde		Absolute methanol, frozen (-20ºC)
Xantophyceae	Standing water	Plancton	Unicellular, colonies, filaments, siphonous	600 (3)	Lugol, formaline, glutaraldehyde		Absolute methanol, frozen (-20ºC)
Cryptophyceae	Standing water	Plancton	Unicellular	200 (2)	Lugol, formaline, glutaraldehyde, better if study alive		Absolute methanol, frozen (-20ºC)
Prymnesiophyceae	Standing water	Plancton	Unicellular	500 (2)	Lugol formaline, glutaraldehyde		Absolute methanol, frozen (-20ºC)

Dinophyceae	Standing water	Plancton	Unicellular, colonies	2000 [2]	Lugol, formaline, glutaraldehyde	Hypochlorite treatment	Absolute methanol, frozen (-20ºC)
Euglenophyceae	Standing water	Plancton/benthos	Unicellular, colonies	1600 [2]	Lugol, formaline, glutaraldehyde		Absolute methanol, frozen (-20ºC)
Raphidophyceae	Standing water	Plancton	Unicellular	36 [2]	Lugol, formaline, glutaraldehyde, better if study alive		Absolute methanol, frozen (-20ºC)
Eustigmatophyceae	Standing water	Plancton	Unicellulat		Lugol, formaline, glutaraldehyde, better if study alive		Absolute methanol, frozen (-20ºC)
Macroalgae							Absolute methanol, frozen (-20ºC)
Rhodophyceae	Flowing water	Epiliton	Filaments, laminar	5000-6000 [3]	Formaline or drying		Absolute methanol, frozen (-20ºC), dried
Ulvophyceae	Standing/flowing waters	Epipelon, pleuston	Filaments, laminar	300 [2]	Formaline or drying		Absolute methanol, frozen (-20ºC), dried
Charophyceae	Standing/flowing waters	Rhizobenthos	Filaments	11700 [2]	Formaline or drying	EDTA or light acid treatment	Absolute methanol, frozen (-20ºC), dried
Phaeophyceae	Flowing water	Epipelon	Filaments, laminar	1600 [2]	Formaline or drying		Absolute methanol, frozen (-20ºC), dried

[1] Guiry & Guiry, 2009; [2] Corliss, 2000; [3] Graham & Wilcox, 2000

Table 3. Gross and specific habitats, number of species and fixatives for freshwater algae

3. Sampling aquatic plants

3.1. Introduction

This section is dedicated to the sampling of vascular aquatic macrophytes *sensu stricto*, those plants that complete their life cycle when all their parts are submerged or floating in the water (Den Hartog & Segal, 1964). This group of plants is called ‘hydrophytes’ (hydro = water, phyte = plant).

There are other plants that are usually included in the generic term ‘aquatic plants’ but they are properly amphibian as only their basal part is submerged when they reach their maximum development. These plants are generally known as ‘helophytes’ (helo= swamp), and typical genera are *Phragmites*, *Sparganium*, *Typha* and *Eleocharis*. They are collected and preserved as any other terrestrial plant.

Within vascular aquatic plants several biological types based on their morphological characters or the relation with the substratum can be distinguished, including rooted (rhizophytes) or floating in the water and completely submerged (pleustophyte) (Den Hartog & Segal, 1964; Cirujano *et al*., 2002). There are aquatic vascular plants that are very noticeable with big leaves and flowers that float on the water surface (*Nymphaea, Nuphar*) and other inconspicuous, that live completely submerged with fine leaves and minute flowers (*Zannichellia, Althenia,* Callitriche). Within both extremes we find a varied range of plants.

The smaller aquatic plants are always more delicate and care should be taken when they are picked up and prepared, as they should retain their flowers and fruit that are often necessary for proper identification.

3.2. Preparing for the sampling trip

Before going to the field it is necessary to prepare the material that should include the following: high rubber boots, swimsuit if sampling clean temperate waters; a medium sized hoe (with a 80 cm wooden handle and a 15 x 8 cm flat end) that will help to extract the plants and increase sampling reach; a note book and a pencil tied to it; card labels (Haynes, 1984)

Obviously, not all aquatic ecosystems are the same. In shallow waters (up to 1 m) sampling is easy as we can get to the bottom easily. For deeper waters it is necessary to use a boat and an aquatic viewer or scuba glasses to see the distribution of aquatic vegetation if the transparency of the water allows it. Fine and rigid hooks with weights attached to a rope can help to sample rooted plants in deep waters. A little practice is necessary to operate the hooks.

3.3. Preparing sampled material

Fine and delicate plants should be deposited in a tray with a small amount of water and a card sheet position below it, arranging the specimens so that they

can be clearly seen with flowers and fruits clearly exposed. It is better to have few well-arranged plants than many crowded ones. With practice it will be possible to dispense with the tray and arrange the plants directly on the card submerging the card at the place being sampled. Strong plants can be arranged directly on the cards. If they are very big, like water lily one should select a small leaf that fits onto the card and cut the flower in half or keep only a representative part of it. The card with the plant should be placed between two sheets of newspaper and with some more sheets between the next cards to act as blotting paper.

Each card requires a label with an identification number. The best practice is to use always the same numbering system and to use correlative numbering with the initials of the name and surname of the collector.

In the notebook write this identification number and any relevant observations regarding the sampled site: locality, area names, date, depth of water, if it was clean or contaminated, etc. and if possible measure the dissolved O_2, pH and salinity. It may be interesting to make a sketch of the spatial arrangement of the vegetation.

Once finished, the cards will go to the field press to tighten them a bit. It is not necessary to tighten too strong as aquatic plants are not woody and if we do it, they will stick strongly to the card and it will not be easy to split them without fracturing.

3.4. Arriving home or the lab

Once at working place the newspaper sheet should be changed and the material pressed again. This process is repeated until the specimens dry completely.

If the material is going to be deposited in a public collection it is necessary to fill a complete card with the name of the plant, if known, locality, geographical coordinates if known, date, collector’s name and person who has identified the material.

The material that arrives at a public collection usually undergoes another preparation process, transferring the plants to standard sheets, being numbered and registered and finally frozen at -20ºC to eliminate insects and other small creatures that live in stored plants (Forman & Bridson, 1989).

Fig. 3. Sampling hooks. (Photo by Santos Cirujano).

Fig. 4. Preparing a herbarium specimen. A. Arranging the specimen on a Bristol card; B. Example of a herbarium specimen. (Photos by Santos Cirujano).

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4. Sampling microinvertebrates

4.1. Defining microinvertebrates

It is not easy to define the precise size range ascribed to freshwater microinvertebrates. As a reference, the microplankton made up by adult and juvenile crustaceans, rotifers and protozoa have body lengths between 50 and 1000 µm, and sometimes exceed 1500 µm. To get a representative sample of these organisms, plankton nets with net mesh size between 25 to 50 µm diameter will be enough, although mesh size up to 100 µm or more could be used taking in account that filtering efficiency is typically diminished due to clogging.

The main fractions in freshwater zooplankton are protozoans (not properly invertebrates), Rotifera, Cladocera and Copepoda (Cyclopoida and Calanoida). Other microinvertebrate groups like Nematoda, Gastrotricha, Tardigrada, etc., are represented in different habitats of standing and flowing waters (see Table 4 for a summary of the habitats of the different groups). This can also be found in the heleoplankton (= swamp) of shallow waters and in the littoral area of deep waters, running waters, interstitial, ponds and in moss, lichens and phytotelmata (see section on special habitats).

To take microinvertebrate samples one should consider the kind of habitat: pelagic and littoral zone of deep waters, shallow standing waters, running and subterranean waters, interstitial and aquatic vegetation.

4.2. Sampling methods

The study of plankton is very well treated in many limnological treatises (see also the section on algae). Prior to sampling, the researcher should be prepared to deal with variation in vertical and horizontal distribution of the organisms in response to physicochemical gradients like light, dissolved oxygen, temperature, pH, and salinity.

To capture the microinvertebrate fraction of the plankton, one can sample horizontally by trawling from a boat at reduced speed or from a fixed location vertically to get a complete profile from bottom to surface. In this latter case we will have an integrated sample of the entire water column that will include those organisms that only live at certain depth where environmental circumstances are adverse for all the other species (*e.g.*, deep anoxic waters of the hypolimnion of lakes in temperate climates).

In eutrophic lakes with abundant seston it is advisable to use nets with mesh size of 100 µm or more. If the conventional plankton nets of 25-50 µm mesh size are used these will rapidly be clogged up, making only short trawling distances possible.

If we would like to ascertain which species live at different depths, it is necessary to use sampling bottles to take localized samples at designed depths or to use a water vacuum pump run by electric batteries. In both cases the sample should be filtered though a mesh size similar to that used for plankton nets and the concentrated sample stored in a small volume. Samples taken with bottles or vacuum pumps are useful for quantitative studies as sampling volume is accurately known. In the case of an oligotrophic lake the amount of water filtered to get an adequate sample of zooplankton could be 50 l or more.

In shallow standing waters it is necessary to take samples of the heleoplankton. In this case, the samples can be taken by hand using a triangular net with a smaller mesh size, slightly above 100 µm, as in this habitat it is usual to find an abundant aquatic vegetation that will reduce the net filtering capacity very quickly. The net should have at its posterior end a plastic container of 50 to 250 ml where the filtered organisms remain. If we are looking for epiphytic microinvertebrates associated with plant surfaces small fragments of that vegetation must be taken and placed in a bottle with a wide mouth previously submerged close to the plant.

In shallow water, it is common that depth increases rapidly as we depart from the shore and it is not possible to sample using only rubber boots. An alternative is to employ a small inflatable boat as the sampling gear is usually less and lighter than that used in the case of deep waters. Another possibility is to use float tubes (see 1.4)

Microinvertebrates can also be found among sand grains in running and still waters. To sample this interstitial milieu one can make a hole in the sand and collect the water that flows to it (see also section on subterranean aquatic habitats).

To sample the benthos of deep waters it is recommendable to use dredges or grabs of a certain weight, thrown from a boat. It is possibly the most complicated

sampling technique due to the size and weight of the gear used. In shallow waters it is enough to drag the net over the bottom surface avoiding disturbing excessively the water to avoid the clogging of the net and to make it easier later to look at the samples under the microscope. In deep littoral areas it is useful to use small dredges.

In shallow running waters and in the higher reaches of streams and rivers there will rarely be much zooplankton, and it is enough to use the same sampling methods employed for the shallow littoral water of lagoons and ponds. The middle and lower reaches of large rivers may contain potamoplankton which will require plankton nets and a small boat if the water current is sufficiently slow.

Fig. 5. Sampling in shallow waters. (Photo by Jose Luis Velasco).

4.3. Fixing protocols

Sample-fixation protocols vary depending on the circumstances and objectives of the sampling trip. Duplicate samples that are examined without fixation are especially helpful to identify soft body forms that distort when in contact with a fixative. It is advisable to keep the unfixed sampled refrigerated and in some cases to add a narcotic agent to slow the fast movements that make identification of some species difficult. Narcotic derivates of cocaine have been used since long ago. The most common ones now are bupivacaine, tricaine and procaine. This last one is used as a 0,04 % solution for 16 h, although the duration will depend on the concentration of the narcotic agent and the response of the different species to it. Other methods to slow moving animals with less legal

problems are to add a volume of boiling water equal to the volume of the sample or carbonate water up to 20% of the total water sample. A viscous material like methyl-cellulose that slows down the animals' movements while keeping them alive may help.

The fixative most commonly employed is neutral formaldehyde at 3-5% final solution, although higher concentrations may be advisable when there is abundant organic matter. When using formaldehyde it is very important to avoid contacting it with bare skin or inhaling its toxic vapors. Other fixatives commonly used are ethyl alcohol at 30-50% and Lugol's solution at 4-5%. Table 4 provides a list with recommended fixatives.

Some rotifer species require observation of the structure of the trophi for proper identification, so it will be necessary to eliminate the soft parts that surround them. To do this it will be necessary to get a great amount of individuals with a micropipette and leave them in a 1 ml chamber with a few drops of sodium hypochlorite at 10%. When there are only a few specimens it is recommendable to observe the dissolution process of the organisms to track the location of the trophi as these parts are usually smaller than 45 µm and can easily be lost.

There are several options to prepare samples for microscopic identification. For quantitative works it is necessary to do precise counts of population density and use Utermöhl sedimentation chambers that allow microscopic observation of the concentrated sample in the bottom. This is equivalent to a flat chamber of 1 ml capacity and it helps the illumination system of the inverted microscope. The height of the tube of the chamber determines the amount of sample volume to observe – from 1 to 100 ml – using the larger chambers for samples with fewer specimens and vice versa.

In qualitative work with taxonomic purpose the objective will be to have the best illuminating condition for the sample. It is convenient to do preparations that allow the best optical condition using the classical crystal slide and a normal microscope in the case of samples with big concentrations of organisms. In the case of scarce samples use flat chambers like Sedgewick-Rafter or composed chambers that allow one to sort through bigger sample volumes. In this case it will be useful to use an inverted microscope to get the magnification equivalent to those attained in a normal microscope, excepting the immersion objectives.

Taxon	Major habitat	Subhabitat	Recommended sieve or net mesh-size (for adults or fully developed aquatic stages)	Taxonomic fixative
Microinvertebrates				
Nematoda	Any flowing or standing water	Sand, mud, debris, vegetation	35 µm	85% alcohol or 5% formalin
Gastrotricha (a)	Mainly standing waters	Debris and on aquatic vegetation (periphyton). Interstitial water in sandy beaches.	Use a 250 um mesh to remove larger particles and organisms, and examine the material that goes through the mesh	2% Osmic acid/Bouin's fixative
Rotifera (a)	Mainly (but not restricted to) standing waters	Plankton, interstitial and periphyton	45 µm	Hot water treatment first to prevent them from contracting and then place in 30-50% EtOH
Tardigrada	Flowing and some standing waters	Moss and aquatic vegetation	45 µm	85% alcohol or 5% formalin
Cladocera	Generally standing but some in flowing waters	Plankton, benthos, macrophytes, interstitial habitats	90-150 µm	95% EtOH or 5% sugared formalin solution for killing and storing in 70% EtOH
Copepoda	Any flowing or standing water	Plankton, benthos, interstitial habitats	60-200 µm Finer mesh for cave copepods	70% alcohol
Ostracoda	Any flowing or standing water	Vegetation, benthos, interstitial habitats	180 µm	4% formalin 2 days and store in 70% alcohol

Syncarida	Generally flowing waters but some in standing waters	Interstitial waters and caves	100 µm	4% formalin 2 days and store in 70% alcohol
Halacaridae	Standing and flowing water	Interstitial waters, mosses and caves	100 µm	70% alcohol
Oribatida	Standing and slow flowing water	Debris and vegetation	250 µm	70% alcohol
Hydrachnidia	Clean flowing or standing water	Standing, flowing and interstitial waters	250 µm	Koenike's fluid
Macroinvertebrates				
Porifera	Clean flowing or standing water	Growing on any stable submerged substrate	Hand picking	Drying/70% alcohol
Coelenterata	Clean flowing or standing water	Attached to substrate	Hand picking	Bouin's fluid
Turbellaria (a)	Any flowing or standing water	Benthos, on and under rocks, among vegetation and subterranean	Bait, hand picking, 100 µm	Hot Bouin's to fix followed by storage in 70% EtOH
Nemertea	Mainly standing waters	Among vegetation	Hand picking	Anesthetized followed by 70% alcohol
Nematomorpha	Standing and flowing water	Necton, benthos and among vegetation	250 µm	70% alcohol
Oligochaeta	Any flowing or standing water	Benthos, among vegetation and subterranean	180 µm	70% alcohol
Polychaeta	Flowing and interstitial waters	Benthos	180 µm	Bouin's fluid
Hirudinea (a; for eye number and arrangement)	Standing and flowing waters	Benthos and among vegetation; on fish	Hand picking and 180 µm	Anesthetized followed by Schaudinn´s fluid

Bryozoa	Clean flowing or standing water	Attached to stable submerged substrates	Hand picking	Anesthetized followed by Bouin's fluid
Anostraca	Standing waters	Necton/benthos	250 µm	85% alcohol or 5% formalin
Notostraca	Standing waters	Benthos	250 µm	85% alcohol or 5% formalin
Conchostraca	Standing waters	Benthos	250 µm	85% alcohol or 5% formalin
Branchiura Argulidae	Standing and flowing waters	On fish hosts and free-swimming	Hand-picking and 250 µum	70% EtOHY
Cumacea	Saline/Brackish coastal lagoons	Necton	250 µm	70% alcohol
Tanaidacea	Saline/Brackish coastal lagoons	Necton	250 µm	70% alcohol
Mysida	Flowing and standing waters	Necton	500 µm	4% formalin 2 days and store 70% alcohol
Isopoda	Flowing waters mainly but some in standing water	Benthos and subterranean	180 µm	70% alcohol
Amphipoda	Flowing waters mainly but some in standing water	Benthos and subterranean	180 µm	70% alcohol
Decapoda	Flowing waters mainly but some in standing water	Benthos	Baited traps and 1 mm Y	4% formalin 2 days and store 70% alcohol

Collembola	Standing water	Surface film	250 µm	70% EtOHY with drop of detergent to break surface tension
Ephemeroptera	Any flowing or standing water	Benthos and among vegetation	250 µm	70% alcohol
Plecoptera	Flowing waters mainly	Benthos	250 µm	70% alcohol
Odonata	Standing water mainly but also many in flowing	Benthos and among vegetation	250 µm	70% alcohol
Hemiptera	Any flowing or standing water	Benthos, nekton, among vegetation, on surface	250 µm	70% alcohol
Hymenoptera	Flowing and standing waters	Benthos, among vegetation and parasitoid in aquatic insects	250 µm	70% alcohol
Megaloptera	Flowing waters	Benthos and among vegetation	250 µm	70% alcohol
Neuroptera	Flowing waters	Benthos and among vegetation	250 µm	70% alcohol
Trichoptera	Any flowing or standing water	Benthos and among vegetation	250 µm	70% alcohol
Coleoptera	Any flowing or standing water	Benthos, nekton and among vegetation	250 µm	70% alcohol
Diptera	Any flowing or standing water	Benthos, plankton (for Chaoboridae) and among vegetation	250 µm	70% alcohol
Gastropoda	Any flowing or standing water	Benthos and among vegetation	250 µm or hand picking	Anaesthetize and then 75% EtOH

Bivalvia	Any flowing or standing water	Benthos and among vegetation	350 µm or hand picking	Anaesthetize and then 75% alcohol

Table 4. Major invertebrate taxa, their habitats, and recommended mesh sizes and fixatives. (a) = best examined alive (from Balian *et al*., 2008; Pennack, 1978; McLaughlin, 1980; Bartsch, 2004; Thorp & Covich, 2001; and other sources)

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5. Macroinvertebrate collection and extraction

'Macroinvertebrates' represent neither a taxonomic nor an ecological category, but rather are defined operationally, on the basis of the size of mesh on which organisms are retained (see Table 1). In marine ecology, the cut-off between macrofaunal and meiofaunal invertebrates is 1 mm (Herman & Dahms, 1992), whereas in freshwater the usual cut-off is 500 µm (Carter & Resh, 2001); however, depending on the study, the lower size boundary of 'macroinvertebrate'

can range from 250 µm to 1 mm. A species can technically be both micro- and macro- at various stages in its life cycle. Most macroinvertebrates are associated to a greater or lesser degree with some sort of substrate, such as macrophytes or gravel, and are rarely completely planktonic. They are thus often referred to as the "benthos" in contrast with zooplankton. The vast majority of freshwater macroinvertebrate species are insects. Representatives of other groups of arthropods (crustaceans, arachnids) and of numerous other phyla (Porifera, Bryozoa, Plathelminthes, Cnidaria, Mollusca, Annelida) also frequently fall into the macroinvertebrate size category.

Benthic macroinvertebrates are probably the best-surveyed of the freshwater invertebrate fauna, in part because many countries have biomonitoring programs specifically aimed at assessing the 'health' of fresh waters via the diversity and abundance of macroinvertebrates (*e.g.*, RIVPACS in the United Kingdom, STAR-AQEM in the European Union, AusRivAS in Australia, CABIN in Canada, as well as various state-specific protocols of the U.S. Environmental Protection Agency (Carter & Resh, 2001)) (see References for websites). These programs are focused mostly on running waters, but protocols for assessment of lakes and wetlands also exist, and are being developed at a rapid rate (*e.g.*, Boix *et al.,* 2005; Mack, 2006). Rosenberg *et al*. (2001) provide an on-line bibliography of methods and protocols for assessing benthic macroinvertebrate diversity (http://www.emanrese.ca/eman/ecotools/protocols/freshwater/benthics/references.html). Although much effort is expended on such assessment, the 'biodiversity' measured is seldom at the species level (Carter & Resh, 2001). For example, the CABIN protocol requires identification only to family level (http://cabin.cciw.ca/Application/Downloads/cabin_protocol.doc). STAR-AQEM procedures differ depending on the country. For instance, samples from Germany are usually identified up to species level whereas samples from Greece are identified only up to family level (Clarke *et al*., 2006). Instead, emphasis is typically on rapid sampling and processing in order to assess large numbers of sites. Despite the poor taxonomic resolution associated with many biomonitoring programs, because the intent of their collecting methods is to maximize higher-taxon richness as rapidly as possible at a given site, combining the methods of these programs with more careful taxonomy will result in a good overview of biodiversity for most of the typically sampled types of water bodies (streams, lakes, wetlands).

The following sections review these general methods as well as more taxon-specific or time consuming means of collecting and extracting macroinvertebrates. A very comprehensive survey, with illustrations of devices, is provided by Merritt *et al*. (2008).

5.1. Habitat-based sampling

The main considerations when sampling macroinvertebrates are: (i) is the water flowing or standing?; (ii) is the substrate hard or soft?; (iii) can the substrate be reached by a wading human? (iv) is the substrate bare or covered with macrophytes? (v) is an areal estimate of abundance needed or is the intent to maximize diversity?

5.1.1. Shallow running water

Wadeable streams are among the most tractable and most frequently studied habitats for macroinvertebrates. The literature on sampling methods is enormous, and has been summarized by Resh (1979), Peckarsky (1984), and others. For biodiversity estimation, the most common method is kick-sampling, in which a D-shaped net (shaped like a semicircle, flat on the bottom) is held downstream in the wake of the collector, who shuffles through the substrate backwards for a predetermined distance or time. Both riffles (water moving rapidly over stones) and pools should be sampled in order to collect invertebrates with different sensitivities to oxygen levels and rate of flow. If the stream has undercut banks, one can collect by pushing the net underneath the stream bank overhang. For pools, the collector can bring up silt and debris in the D-net and rinse excess silt from the net by dipping the net bag repeatedly into the water before examining sample in a tray. A general rule for maximizing diversity that holds for both running and standing water habitats is to sample substrates that differ in morphology, be it grain size (*e.g.* cobble vs gravel) or leaf shape or density (*e.g*. mosses vs reeds). In water bodies with mostly uniform and monotonous substrates such as mud, sand or cement, it is the small areas with diverse structure where most macroinvertebrate diversity will accumulate. Take out pieces of submerged wood and let them dry to encourage insects to emerge from the crevices (Thorp & Covich, 2001). Even human-made objects such as discarded bottles or shopping carts will create diversity in substrate and flow regimes. The STAR-AQEM biomonitoring protocol emphasizes the importance of sampling all microhabitats that have a minimum 5% coverage of the total substrate (Hering *et al*., 2004).

With regard to areal sampling, perhaps the most common method for streams is the Surber-type sampler in its various incarnations, which share the features of having a defined (usually square) demarcated area, ideally with basal foam to accommodate irregularities in the substrate, with a downstream capture net. Substrate within the demarcated area is disturbed to a particular depth, with cobble being lifted and rubbed, so that dislodged animals are carried by the current into the net. For Surber and kick-sampling, variation among individual human samplers with regard to vigour of moving the substrate can affect number and diversity of animals collected. An electric pump sampler such as that described by Brooks (1994) can increase efficiency of extraction of animals from stream substrate, and possibly also reduce inter-individual variation in sampling effort.

5.1.2. Deep/rapid running water

For streams that are too deep or rapidly-flowing to allow safe wading, placement and subsequent collection of artificial substrates of known area will allow for estimation of richness and densities of macroinvertebrates. Tiles, bricks or wire baskets of stones can be fixed to the stream bottom and left for weeks or months to be colonized. Larval black flies (Simuliidae) can be collected using plastic tape hung in the current (Hamada *et al*., 1997). These methods can of course also be used in shallow waters. Use of artificial substrate comes with many caveats,

however, including variation in the attractiveness of textures of substrates to invertebrates, and need for conditioning of certain substrates (*e.g.*, bricks) to leach out chemicals and/or allow an algal biofilm to build up. Air-lift samplers can be used to access stony substrates of moderately deep flowing water (see illustration at http://www.uwitec.at/html/river_benthos.html), but often require two or more operators as well as complicated equipment. Grab or drag-type samplers will work better than air-lifts for soft sediments of large rivers (Drake & Elliott, 1983).

5.1.3. Shallow standing water

Many of the collecting methods useful in running water are also applicable to standing water habitats. The wadeable margins of lakes and wetlands are typically sampled qualitatively using a modified form of kick-sampling, in which the 'downstream' flow is created by the movement of the sampler rather than the water. In a macrophyte rich zone one must move the net up and down in a sine wave through the water column as one walks in order to sample the entire range of vertical habitat. In contrast, in macrophyte free zones, almost all macroinvertebrates will be confined to the bottom substrate, which should be gently disturbed by the feet of the sampler or the edge of the net. For rapid evaluation of the benthic invertebrate diversity of a large area, care should be taken not to collect too much organic substrate or macrophytes, as sorting through this material can be very time consuming. If material is to be picked in the field rather than preserved and examined in the lab, collected macrophytes can be put in a bucket with water from the site, and the water poured into white trays for examination. Because some organisms will cling to the macrophytes, the plants can be set aside in dry trays and periodically examined for invertebrates that attempt to escape from the drying macrophytes. This works particularly well for adult beetles (Clifford, 1991). Masses of vegetation and other debris can also be taken back to the lab and left overnight in water-filled containers. In response to declining oxygen levels in the middle of the debris, many otherwise cryptic organisms (especially hydras and flatworms) will move to the sides of the containers or accumulate on the surface film (Clifford, 1991; Slobodkin, 2001). Snails and leeches can also be collected from the sides of the container once the debris has been removed.

Estimating areal-based abundance of invertebrates from macrophyte-rich sites is much more difficult than from uniform and relatively flat substrates such as mud, sand, or gravel. Several hand-operated or automated cutting devices have been created for harvesting known basal areas of rooted macrophytes (Downing, 1984), but estimation of surface area of the plants is an additional problem. Possibly determining surface area of known dry weights of macrophytes will allow this.

5.1.4. Deep lakes

The benthos of deep standing water is usually sampled with a grab-type sampler, dropped from a boat (Downing, 1984). Coring tubes can also be used; when the end of the tube is capped, the vacuum in the tube prevents sediments from falling

out (under ideal circumstances). The speed at which the grab or coring tube hits the substrate can affect what it captures, as in water bodies with light flocculent layers (*e.g*., gyttja) a fast moving sampler can blow away this animal rich layer without collecting it, whereas in a heavier sandy substrate a slow-moving or lightweight sampler may not penetrate deeply enough. A dredge can also be used for areal sampling if pulled for a known distance, but this is difficult to control. Probably SCUBA-diving is the best way to ensure consistent areal sampling of deep lake benthos, though even in this case divers must take care to avoid kicking up the flocculent layer. SCUBA-diving or snorkeling is also an efficient way to collect large-bodied but sparsely distributed or attached organisms (*e.g.* mussels, sponges, bryozoans).

SCUBA-diving based methods are treated in more detail in chapter 11. Trapping

Diversity and abundance of certain groups of freshwater insects can be estimated using emergence traps (Davies, 1984). These traps collect aerial adults of most insect orders, perhaps with the exception of adult Hemiptera (which do not necessarily become airborne upon adulthood) and Coleoptera and Megaloptera (most of which pupate on land). One advantage of emergence traps for biodiversity estimation is that sexually mature individuals (or subimagos, in the case of Ephemeroptera) are collected, and this is the stage on which species-level keys are usually based. Emergence traps are probably not ideal for areal estimation of densities, as many aquatic insects move from their region of larval development to a more confined area (*e.g*. near shore for Odonata) prior to emergence. For those Diptera that emerge vertically (*e.g*. Chironomidae, Chaoboridae), emergence traps may provide a good estimate of areal productivity. Malaise traps set up over streams will provide a good biodiversity estimate for adult aquatic insects, although it will be difficult to localize the place of origin. Adults of some aquatic insects can also be collected by pheromone traps (*e.g.* Trichoptera), but these are very taxon-specific and hence would not be useful for broadly aimed surveys.

An ecologically specialized mode of sampling involves activity traps. For running water, these are drift nets, which collect the invertebrates that have voluntarily or catastrophically entered the water column. This is particularly valuable for larval mayflies (Ephemeroptera) (Clifford, 1991). In standing water, floating bottle traps, with or without luminescent lures such as plastic glow-sticks (*e.g*. Barr, 1979) can be used in shallow or deep waters. Baiting is another type of activity-related collecting methods. The bait itself may be colonized by macroinvertebrates (*e.g*. a small piece of liver left for a few hours in the water will attract flatworms; Clifford, 1991) or the bait may be inside a trap (*e.g*. minnow traps for collecting crayfish; Hobbs, 2001). Other methods aimed at collecting large crustaceans such as crayfish include visiting burrows at night with a flashlight and net to collect the animals when they emerge to forage. Palaemonid shrimp can apparently also be collected at night with the aid of a headlight, as they can be targeted by their red eye-shine (Hobbs, 2001).

Fig. 6. Sampling macroinvertebrates. A. Kick-sampling with D-net in Alberta, Canada; B. Sampling a stream in Saskatchewan, Canada; C. Setting up drift net in stream in Alberta, Canada; D. Drift net in stream Alberta, Canada. (Photos by Heather Proctor)

5.2. Processing samples

5.2.1. Preservation

Collected samples may be mass-preserved in the field and later sorted at the laboratory. They may also be picked at the field site and the organisms individually dropped into preservative, or they may be returned alive to the laboratory for extraction. The first method has the advantage of including all organisms in the sample, but on the negative side, much organic and inorganic substrate is likely to also be included. Picking in the field minimizes extraneous materials but is very likely to be biased towards large and active macroinvertebrates (especially if the person doing the picking is inexperienced), and will underestimate the true diversity of the sample. If the full sample is to be preserved, 10% formalin at a 1:3 ratio of formalin: sample is a good initial preservative. Samples should be transferred into 70% EtOH in the lab after

approximately 3 days in the formalin (CABIN protocol, http://cabin.cciw.ca/Main/cabin_about.asp?Lang=en-ca). The initial process kills specimens quickly with a minimum of fluid preservative and fixes tissues without dissolving exoskeletal calcium (*e.g*. in ostracods). Replacement of formalin with EtOH makes sorting less hazardous. This procedure is good for many taxa but not for all, *e.g.* not for water mites (Hydrachnidia), which ideally should be killed and preserved in a mixture of ~45% glycerol: 10% glacial acetic acid: 45% water (Koenike's Fluid or GAW). One of the benefits to sorting in the field is that taxon-specific methods of preservation may be used (for an overview of such methods, see Clifford, 1991).

5.2.2. Extraction of invertebrates from samples

Sorting live samples in the laboratory will provide the greatest opportunity for maximizing observed diversity from a sample. It also allows use of behavioural methods of extracting invertebrates from the 'background noise' of sediments or macrophytes. For extraction of oligochaetes from substrate, Brinkhurst & Gelder (2001) suggest spreading clean sand over the sample or putting the sample on a screen set over clean water. The worms will then actively migrate into the sand or water and be more easily picked out against this background. Some seldom used but potentially valuable methods of extracting invertebrates from macrophyte samples involve use of light and/or heat. Organisms may be encouraged to move out of masses of vegetation or other substrates by creating a thermal gradient, with the coolest zone being periodically examined for invertebrates (*e.g*. Kolasa, 2001). If this is combined with a light gradient, negatively phototactic organisms may be encouraged to move to the dark, cool end of the gradient. Berlese-Tullgren funnels, although usually used for extraction of soil invertebrates (http://www.eman-rese.ca/eman/ecotools/protocols/terrestrial/arthropods/soil-litt.html), can be used to extract a wide range of apparently 'rare' invertebrates (*e.g.* aquatic Lepidoptera) from macrophytes that have been drained of most of their water (Proctor, pers. obs.). Desiccation caused by the light bulb's heat induces the normally clinging animals to move away from the drying vegetation, deeper into the funnel, and thence into the collection vial.

If a sample is mass-preserved, interference by substrate is a major problem. If the invertebrates clearly differ in size from the mean particle size of the substrate, then sieves can be used to separate the two, though damage to delicate body parts (particularly devastating to Ephemeroptera) may occur with over-vigorous sieving. If they differ in density, then elutriation via bubbling air may separate the usually less dense invertebrates from particles of substrate. Hydrocarbon flotation with kerosene differentially floats objects whose outside structure has affinities to the hydrocarbon (*e.g.* cuticle of arthropods) (Proctor, 2001), but it is not known whether this method is suitable only for relatively small animals (< 5 mm) or whether it will float larger-bodied animals as well. Some biomonitoring programs employ subsampling trays in which the preserved sample is spread out and a certain number of randomly selected squares within a grid are completely sorted. Clarke *et al*. (2006) emphasize how important it is to distribute the sample evenly across the tray to avoid subsampling errors.

Fig. 7. Berlese-Tullgren funnels. (Photo by Heather Proctor).

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6. Subterranean aquatic habitats

Hypogean life exists in a continuum through different types of karstic, porous and fissured aquifers. Subterranean aquatic habitats vary in void size (*e.g.* tiny pores in sandy aquifer, caves), degree of interconnectedness between voids, and

strength of hydrological connection with the surface environment (*e.g*. sinkholes, deep aquifers). The subterranean biodiversity is still underestimated, however in several places might surpass the epigean diversity of a certain area (Danielopol, 1989). Organisms range in body size from less than 1 μm up to 10 mm in some crustaceans. There are two significant differences between surface freshwater and groundwater, first related to composition of the fauna and second to the high endemism within groups (Sket, 1999; Gibert & Deharveng, 2002).

Freshwater subterranean fauna range widely in taxonomic diversity but are dominated by crustaceans, while in surface freshwater habitats insects prevail. This makes groundwater more similar with marine waters than to freshwater (Deharveng *et al*., 2009). Second, endemism is a rule in groundwater of which fauna is reduced in distribution and frequently limited to few aquifers, and only few species are recorded across large areas. Moreover, the subterranean populations are generally smaller in size in comparison with the epigean ones, and subsequently the species and mainly the endemics are more vulnerable to extinction. When sampling subterranean fauna care should be taken not to over-collect or damage these small and isolated populations. Hence, only collect the minimum number of specimens required for taxonomic purposes. We distinguish the following groundwater habitats: the hyporheic (or interstitial); the hypothelminorheic; springs; wells; deep water table aquifers and caves.

6.1. The hyporheic (interstitial) habitat

The hyporheic or interstitial habitat were first time observed and investigated in the surface rivers (Chappuis, 1942, 1946; Leruth, 1938; Orghidan, 1955; Motaş, 1958). The interstitial is a surface - subsurface hydrological exchange zone (*i.e*. an ecotone) of which extent vertical or horizontal is difficult to be defined without detailed hydrodynamic and/or hydrochemical measurements. The hyporheic zone is temporally dynamic and determined by porosity and relative volume of water recharging the groundwater zone from the channel, or the channel from the aquifer.

6.1.1. Methods of sampling the hyporheic habitat

The hyporheic habitat is sampled by the Karaman-Chappuis technique (only within the shore stream sediments), Bou-Rouch pump, standpipe cores and freezing cores. Artificial substrates and baited/un-baited traps (in both shore and riverbed sediments) might be used (detailed in the section of methods for sampling invertebrates of interstitial lotic waters).

Sampling by the Karaman-Chappuis method

This method involves digging a shallow pit in the shore sector bordering to a stream, allowing it to fill with water, and then filtering the accumulated water (ideally 5-10 l). The method was developed by both Karaman (1934) and Chappuis (1942) to sample the fauna in the water beneath gravel banks at the margins of rivers and streams in both surface and underground. The method is rapid, not time consuming and does not require a specific device, except the

plankton net. The method allows collecting of a large array of interstitial organisms while causing little damage to them. When using this method, the distance from the hole to the river and the depth of the hole should be recorded.

Sampling by Bou-Rouch pumping

The method was developed by Bou & Rouch (1967) and involves the pumping of interstitial water into a stand pipe with a peristaltic pump, driven at various depths into the sediments of a stream. At one end the core has rows of holes, allowing the water and sediments to be extracted. By pumping, a disturbance is created that maintains an interstitial flow around the pipe sufficient to dislodge the hyporheic organisms. The optimal sample volume of water pumped has been estimated to be 1-10 (Boulton *et al*., 2004). For an accurate estimation in numbers of taxa and individuals, the number of replicates could vary between 3-5 times of 5 l (Malard *et al*., 2003). It is assumed that in the first 5 l, 76-100 % of the taxa found in 10 l is collected, providing the best density estimate for organisms living in close proximity to sediments. Some authors recommend that the first 0.2-0.5 l of water to be discarded, to avoid the risk of contamination with surface water and its biota (Danielopol, 1976; Boulton *et al*., 1992). A strong pumping rate is recommended to avoid bias in estimating of hyporheic density. Hence, organisms adhering less tightly to the substrate, (i.e. cyclopoids, ostracods, isopods, and amphipods) may be more easily captured; while others have some abilities to resist mild vacuum pressure. Currently, the Bou-Rouch method is extensively used in hyporheic investigations, although some studies indicate that insect larvae and especially later instars of chironomids are underrepresented in samples (Fraser & Williams, 1997). The Bou-Rouch method has a few disadvantages: i) it is not strictly quantitative because faunal density and diversity cannot be expressed per volume of hyporheic sediments, but comparisons between samples of equal volume are still possible with caution; ii) it is limited in collection at different depths and to streams with sandy and fine gravl sediments; and iii) certain invertebrates may be damaged during the pumping.

Fig. 8. Sampling percolation water. (Photo by Ioana Meleg).

Sampling by standpipe cores

An alternative method perhaps most often used to collect both chemical and invertebrate samples with less impact on organisms, consists of pumping hyporheic water from specific depths in the streambed using permanently installed standpipe wells (Taglianti *et al*., 1969; Palmer & Strayer, 1996). The advantages of the method are related to low habitat disturbances and the option to use the standpipes for long term monitoring. Additional investigations can be performed with the help of a transparent standpipe 5 cm in diameter installed into the sediments. A video-camera equipped with a light can be introduced into the pipe, and hence in situ observations of sediments and its fauna can be performed. Disadvantages of the method are that the organisms colonizing a permanent core differ significantly, in terms of both composition and abundance, from those animals collected from a newly installed well (Hakenkamp & Palmer, 1992). Further, the samples taken sequentially from a well cannot be used as replicates, because a 48 h period between sequential samples from the same well does not allow adequate time for recovery by the fauna in the immediate vicinity of the pipe. Other sources of bias in samples from colonization of permanent wells include the trapping action in the non-perforated segments of the pipes and the possible attraction of predators/scavengers (Bretschko & Klemens, 1986).

Sampling by freeze coring

In this method, the fauna is paralyzed by an electric field and then the core is frozen with liquid nitrogen (Stocker & Williams, 1972; Hynes, 1974; Bretschko, 1985). This is a more quantitative method than those described above; however, it has the disadvantage that removing a series of frozen cores from a stream bed destroys the habitat for an undetermined, but extended, period. Also, there are several logistic constraints related to weight of the equipment and the core removal from a relatively high depth that is relatively difficult.

6.2. The hypothelminoisrheic habitat

The hypothelminorheic habitat is a submerged interstitial between soil and rocky beds. Meštrov (1962) defined this habitat as: "*Il est constitué par les sols humides des montagnes, riches en matières organiques et traversés par des filets d'eau courante*". It has often been included among subterranean habitats because it harbors a fauna dominated by species with typical morphological traits associated with subterranean life (Fiers & Gheene, 2002; Culver *et al.,* 2006). This habitat is hypothesized to play a significant role in active colonization's by the surface dwelling organisms of the subterranean realm. The hypothelminorheic habitat may be sampled by using a hand held manually peristaltic pump and filtering the water through a plankton net. Additionally, a cut off water bottle with bait could be used.

6.3. Springs

Springs can be viewed as access points to collect the fauna from epikarst, vadose zone and phreatic zone of an aquifer. They are natural resurgences of groundwater that surfaces through rock faults or fractures that may form a marsh (helocrene), pond (limnocrene), or a brook (rheocrene). Springs may be supplied by water from un-consolidated or consolidated sediments (*i.e.* karst). In a helocrene spring, water seeps out off the ground slowly and is usually temporarily confined to small holes or ditches; while in the limnocrene springs, water comes out of the ground and creates a pond at the source, before flowing out slowly. The pond is usually deeper than in helocrene springs, so that water is permanent. Springs are very heterogeneous and may differ significantly in features (*e.g*. substrate, amount of aquatic vegetation, and degree of shading by spring side vegetation), water chemistry (*e.g*. pH and ionic content), and biotic composition (*e.g.* presence or absence of specific competitors, predators and/or parasites). Being a transition area between groundwater and surface water (ecotone), springs host a mixed assemblage of epibenthic organisms, stygobites (species living exclusively in groundwater) and crenobiont taxa (*i.e.* characteristically occurring in springs). Their investigations are useful for monitoring the quality of groundwater, and for comparing the adaptations of surface and subterranean life.

6.3.1. Methods of sampling springs

There are no specific requirements to sample springs and various methods could be combined taking into account their heterogeneity. The most used method is direct sampling with a drift net in rheocrene springs. These simply consist of a net fixed in place and left to capture organisms as they are washed out of the ground. Noll (1939) describes a spring sampler consisting of a double funnel of bronze wire netting, fixed to a glass flask and sealed with a rubber ring. The device is dug into the mouth of the spring's issue point and removed after several hours. The bottom layers and bed sediments of both helo- and limnocrene springs can be sampled with a pond net, Hess sampler, Surber sampler, Bou-Rouch pump, a freeze core, artificial substrates and traps with baits. The Bou-Rouch pump may clog if silt or fine sand is present. Springs large enough to be accessed by divers can be sampled by installing a large net at the exit and the bottom sediments are shaken dislodging fauna that are after that washed out and into the net.

6.4. Wells

Wells are "open points" within the phreatic zone of porous or karst aquifers.

There are three methods of faunal sampling in wells related to their depth: (i) filtering the water to a Cvetkov net (Cvetkov, 1968), (ii) bait traps, and (iii) pumping the water with a surface-mounted pump.

Sampling with the Cvetkov net is well suited for large wells and requires a dynamic movement of the mesh that allows the sediments and associated animals living at the bottom to be captured through the water column.

Baited containers or nets should be left for at least 12 h to attract the organisms within. Baits can be installed also in a stand pipe for few hours and then water is pumped by using different devices (Husmann, 1964; Danielopol & Niederreiter, 1987; Boulton *et al*., 1992; Hakenkamp *et al.,* 1994).

Water pumping is suitable for wells less than 8 m deep, and a volume of at least 50 l is required (Malard *et al.*, 1997). This method is often considered quantitative, with the number of organisms collected related to the volume of water pumped. For a well deeper than 8 m, pressure pumps are required (see below).

Fig. 9. A modified version of the Cvetkov net, adapted to sample in shallow wells. (Photo by Damia Jaume).

6.5. Deep water table aquifers

Investigation of aquifers with the water table located deeper than 8 m below the surface requires a pressure pump. Access to the deeper phreatic groundwater can be reached by piezometers (of varying diameters from 2.5 – 20 cm) installed at many places into the water table. A tube can be inserted into a piezometer or borehole and connected to a pump. The flow generated by this is then passed through a sieve or net, or into a tank for holding sediments and fauna.

Several pumps have been tested for their ability to extract water and fauna: centrifugal (Danielopol, 1983; Notemboom & Boessenkool, 1992; Rouch *et al*., 1993), pneumatic and air-lift pumps (Malard *et al*., 1994). The main difficulty in using the pumps is to remove the organisms with little damage and the lifting of water and suspended particles efficiently. However, all have a limitation: they could not be used to provide samples at a certain depth within an aquifer. When choosing a suitable device for pumping, it should take into account also the possibility of measuring simultaneously biological and chemical parameters. The centrifugal pump seems to be efficiently used for both. Some studies show that the turbine of the centrifugal pump damages large animals like isopods and amphipods, but it extracts micro-invertebrates in good condition. For instance, Notemboom & Bosessenkool (1992) successfully extracted the groundwater copepod *Parastenocaris germanica*. The advantages are that it provides macro-invertebrates in good conditions and is also less expensive. Its limitation is related to the depth from where the water is extracted, which should be at least 50% of the total depth of the well (Roscoe Moss Company, 1990; Malard *et al*.,

1994). The pneumatic pump does not have this problem as long as sufficient pressure is provided, but the cost is 15 times higher.

6.6. Caves

Caves can be viewed as access points to an aquifer and often contain a large variety of aquatic habitats. From the entire array of the subterranean realm, caves are the best sampled. Cave aquatic fauna include a variety of organisms, but is dominated by invertebrates actively or accidentally arriving in underground (Gibert *et al*., 2005). Such invertebrates potentially inhabit a diversity of subsurface waters (Rouch, 1986; Danielopol, 1989) and are not necessarily restricted to caves. Relating to the rocks in which caves are formed, limestones, gypsum and lava caves can be recognized. The most investigated caves are those from karst aquifers formed in limestones and dolomites where the dissolution of calcium and magnesium carbonate creates a three-dimensional network of interconnected openings (i.e. a drainage network). Two aquatic zones can be distinguished within a cave: unsaturated zone (or vadose) and saturated zone (or phreatic). Each zone contains a large array of aquatic habitats that can be sampled by a combination of methods described above.

An unsaturated zone is partially filled with water that flows by gravitation through deep underground. At the top of the vadose zone is a perched aquifer called epikarst (Mangin, 1974, 1975; Klimchouk, 2004). It is an area of higher porosity and permeability that extends a few meters below the karst surface (Malard *et al*., 2003). The epikarst permeability decreases with depth and temporary or permanent springs may appear at the contact between epikarst and the less fractured rock. Cave biologists have found a considerable number of both terrestrial and aquatic organisms in drips and seeps percolating from the cave ceilings that are washed out of the epikarst and found later on in pools and even streams (Bobič, 1993; Brancelj, 2004; Sket *et al*., 2004; Brancelj & Culver, 2004; Pipan, 2005; Pipan & Brancelj, 2004; Pipan & Culver, 2005a; Camacho *et al.,* 2006; Moldovan *et al.,* 2007). The percolating water seems to be rich in organisms where numerous specimens of Copepoda, Nematoda, Oligochaeta and Ostracoda, as well as Turbellaria, Rotifera, Archiannelida [‘archiannelids’ are no longer considered to be a monophyletic taxon, so perhaps ‘polychaetes’ would be better], Gastropoda, Araneae, Acarina, Bathynellacea, Isopoda, Amphipoda, Diplopoda, Collembola, Coleoptera, and Diptera larvae are found.

Methods of sampling the epikarst

Water from drips and trickles can be sampled by directing the water through a funnel into a plastic container (Fig. 10). To avoid the loss of the animals, the container is perforated and covered with a plankton net (60-100 μm). The containers can be kept in the cave for a period of 1-4 weeks, but a longer time is required for ecological investigations (1-2 years). Collections can be made at a certain interval of time in relation with the purpose of study, but should cover a rainy period. In order to minimize changes due to births and deaths of various organisms, collection intervals of 10 days are advised. For long term monitoring, the collection could be done monthly. The devices must be located in an area

where water infiltration is more frequent (Rouch, 1968). The number of the trickles selected to be sampled, vary in relation to cave development, water infiltration availability, thickness of the ceiling and not at least the purpose of study. A priori investigations are necessary to detect the location of potential trickles that could be inactive for a limited period of time during a year. The distance between the trickle samples could vary from 1 m up to 1 km corresponding to the same cave or to large cave systems.

Epikarst can be accessed also by sampling the drip pools (gours), puddles, small rivulets, and small pools in the top of stalagmites (Pipan, 2005). They may receive water and organisms from the surrounding fractures (Rouch, 1968). Most hypogean crustaceans appear to prefer pools with fine silt at the bottom, although they are occasionally seen in crystal-lined gours. Water bodies supplied by surface water (epiphreatic waters) appear after periods of floods and form pools, puddles and lakes of different sizes. They should be differentiated by the previous pools feed by subsurface water of the vadose zone.

Fig. 10. Sampling percolation water. (Photo by Ioana Meleg).

6.7. Pools, puddles and epiphreatic waters

6.7.1. Methods of sampling pools, puddles and epiphreatic waters

These habitats can be sampled by filtering the water through a mesh net of 60-150 µm. Small hand pumps and even pipettes can be used to collect the water

and then filtered through the net. Because most species live in sediments (if they are present in the pools) they must be shaken before. Some large crustaceans like amphipods, leave small trails at the surface silt of a pool bottom after feeding, which hence could be an indicator of their presence. The large animals easily seen in pools (more common in large ones), can be collected by hand using forceps or pipette. If the pools are dry, clean water could be added and filtered after few hours (Pleşa, 1972). It is assumed that the organisms that live within the small fissures around the pool might be found in the pool water. In large pools a large quantity of water can be filtered, although the sediments accumulated in the net may make sorting the material difficult.

The vadose zone of a cave may also include large lakes, exogenous rivers that sink into a cave from the surface, and endogenous rivers (autogenic streams) originated from the drainage of rainfall infiltrating through the soil and vadose zone. The subterranean rivers could flow on a bed-rock with or without sediments and hence, interstitial habitat could be available along the entire stream or parts of it within a cave. The interstitial sediments of the rivers sinking from the subsurface are inhabited by a large array of organisms drifting from outside (especially at the entrance of the river underground), and hence, if the sampling aim is to get only stygobites, sampling these rivers should be avoided. Endogenous streams are more likely to contain solely hypogean fauna.

6.8. Subterranean lakes

6.8.1. Methods of sampling subterranean lakes

In large lakes a zooplankton net attached to a length of rope can be used. The net will need to be weighted in order to be thrown from the edge of the water body effectively and sink to the bottom where the invertebrates could be found. Small traps with baits can be used (Chappuis, 1950) for a short period of time (about 1 hour), however, they are not recommended since they can attract large predators like amphipods that may devour the fauna that has gathered. However, sampling by baits in cave environment should be used with caution, since the food is scarce, and the bait will then become a long-lasting focus of attraction which could destroy small and localized populations of hypogean fauna.

6.8.2. Methods of sampling the sediments of subterranean rivers and lakes

Methodologies to collect epibenthic macro- and micro-invertebrates in subterranean environments are similar to those for epigean streams.

The phreatic zone of a cave includes voids which are completely filled with water at equal pressure (water table) or higher than atmosphere, and hence the water flows through a hydraulic gradient. Fauna inhabiting this zone is similar to that found in the water bodies of the vadose zone.

a) Sampling by artificial substrates consists of using a plastic or PVC tube of about 25-30 cm long filled with a synthetic rope (Vervier, 1990). The device is covered by a net in order to prevent the loss of the animals when the device is

pulled out from the sediments. The tubes placed into sediments should be colonized by the organisms living between the interstitial spaces. They are best suited for upstream/downstream studies or studies designed to test for changes in communities over time (Coleman & Hynes, 1970; Hynes, 1974; Mathieu *et al*., 1984, 1991; Tabacchi, 1990). Artificial substrates provide a relative representative sample of the actual community which is living on a certain surface area of a stream. The method offers the advantages of samples collection from locations that cannot be sampled because of substrate or depth and is non-destructive for the site. There are few disadvantages related to this method: (i) the colonization rates differ from site to site; (ii) the species in sampler may be different than stream bottom; (iii) the long exposure times (6-10 weeks) and, not at least (iv) the vulnerability of samplers to vandalism.

b) Sampling by traps. A container with holes at the bottom and covered by a mesh net allowing the water and the organisms to flow through it can be used as trap. The neck of the container forms a narrow funnel, allowing easy access by invertebrates to the trap, but impeding their exit. It is recommended that the traps are buried in the sediments and kept from 2 hours up to 1-2 days. Baits can be used, and it is expected that wandering invertebrates will move upstream and enter the trap following the smell of the bait in the water. For baits, salami and meat is more attractive than cheese or fish. Trapping is a semi-quantitative method useful to capture large carnivorous like amphipods, isopods and decapods. They are more efficient in interstitial sediments with the water flows rather low. The number of species found by trapping is higher than for pumping, which means that a more complete range of the faunal community is present in the trapped samples.

c) Sampling from deep underground by using devices for pumping. Air lift samples could be also used in siphons if the gallery allows the transportation and usage, although the technique is expensive and not usually used in routine sampling. In large conducts and siphons (submerged tunnels) within the phreatic zone, sampling can be performed by scuba diving (Fig. 11). Divers may carry a funnel with a net used to filter the water while moving upstream, or by scraping the walls and than collect the material deposit that potentially could contain animals.

Fig. 11. Cave diver sweeping the water column with a simple, hand-held plankton net. (Photo by Damia Jaume).

Many of these sampling methods are discussed and illustrated in the PASCALIS project Sampling Manual available at: http://pascalis.univ-lyon1.fr/index.html

Concerning fixation of organisms in this particular habitats we refer to the sections on micro- and macroinvertebrates.

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7. Sampling anchialine habitats

The term anchialine (Greek anchialos = near the sea) is used to designate salinity-stratified coastal aquifers affected by marine tides but with no surface connection with the sea (Holthuis, 1973; Stock *et al*., 1986). Anchialine environments include pools excavated in calcareous or volcanic debris or hard substratum, coastal tectonic faults extended below sea level, drowned limestone caves and lava tubes, and the network of flooded narrow fissures and cracks developed in coastal aquifers and accessible only via bore-holes or hand-dug wells. Most of the inhabitants of these environments are of direct marine derivation and display troglomorphic traits, such as regressed eyes and body pigmentation, and elongation of appendages. Crustaceans are the predominant faunistic group, including a representation of primitive, high rank taxa not found anywhere else aside these habitats (*i.e.* Remipedia, Thermosbaenacea, Mictacea, Platycopioida), or of genera displaying extremely disjunct distribution patterns (Iliffe, 2000).

Whereas several of the most remarkable dwellers of anchialine environments live beneath the halocline in locations only reachable by SCUBA diving, others can be easily captured from the surface using very basic equipment. Here we describe some techniques and devices to sample in a broad array of anchialine habitats.

7.1. Completely submerged chambers and passages in drowned caves

Sampling in these habitats requires advanced cave-diving skills. Usually, modified hand-held plankton nets are used to sweep the water column in search of swimming animals (copepods, thermosbaenaceans) that concentrate around

haloclines, or near the floor, walls and ceiling of the cave passages. A useful tool is a plankton net 30 cm in diameter and 1 m long provided with a short, curved handle to be easily operated and transported by a diver (Fig. 11), and that can be easily closed by constriction with one hand or with the add of an elastic strap once the capture has been produced. The net can be used repeatedly during the same dive.

Ordinary small glass vials or jars are used to pick up individual specimens from the water column. Even tiny animals, such as cyclopinid copepods, are revealed as bright spots by the beam of diving torches, and can be captured by hand with a vial. This technique is especially adequate when dealing with fragile animals that loose limbs with ease, such as thermosbaenaceans.

More sophisticated devices, enabling sampling in cracks and fissures, include a vast array of aspirators and suction bottles, the so-called "Sket bottle" represents the high of technology as well as of simplicity on that respect (Chevaldonné *et al*., 2008).

7.2. Cave lakes and anchialine pools

Sampling can be done directly with a small hand-held plankton net (21 cm in diameter, 35 cm long) screwed to a telescoped, extensible (up to 3 m) handle (Fig. 12). The folded handle and unscrewed net can be carried out with ease along the narrow cave passages, and assembled to reach the deeper parts of the pools and cracks from the shore. Several groups of animals that are never attracted by bait, such as metacrangonyctid amphipods, or that live mainly on rotting, submerged wood (atlantasellid isopods, many bogidiellid amphipods) are caught with nets of this sort.

An indirect way of sampling involves the settlement of baited traps on the bottom of the lakes and pools, which are left for a few hours or several days depending on the target group. Cirolanid isopods seem to be attracted by bait during the first few hours only and then disappear; in contrast, some amphipods (such as niphargids, pseudoniphargids and salentinellids) concentrate and persist in the traps by days. The animals are attracted irrespective of the type of bait (whether fish, meat or cheese); nevertheless, due to its compactness, using a piece of sausage has demonstrated to be unbeatable on that respect and is here highly recommended. A simple trap can be constructed using a broad mouth, stout plastic flask with the bottom cut and removed, and with the central portion of the screwing cap cut and adapted to retain a piece of Nytal mesh (Fig. 13). The trap is ballasted with several pieces of lead and is hung by means of a string. A hook of thick metal wire is used to retain the bait in place. The trap lacks of any device to avoid the animals to escape. In order to impede eels, crayfishes or brachyuran crabs to get in and damage the trap or predate on the eventual animals concentrated inside, two pieces of stout plastic grid united with elastic string can be used to block the entrance and to protect the piece of Nytal mesh.

A very simple trap (Fig. 14) consisting of a plastic bottle with the central portion of the stopper drilled to set a narrow pipe has demonstrated to work very well for cirolanid isopods (they concentrate in the bottle and cannot escape), or to sample

on muddy or salty bottoms (where the other type of traps easily collapse with sediment).

Fig. 12. Small plankton net screwed to an extensible handle. The folded handle and unscrewed net can be carried out with ease along the narrow cave passages, and assembled to reach even the deeper parts of the pools and cracks from the shore (Photo by Damia Jaume).

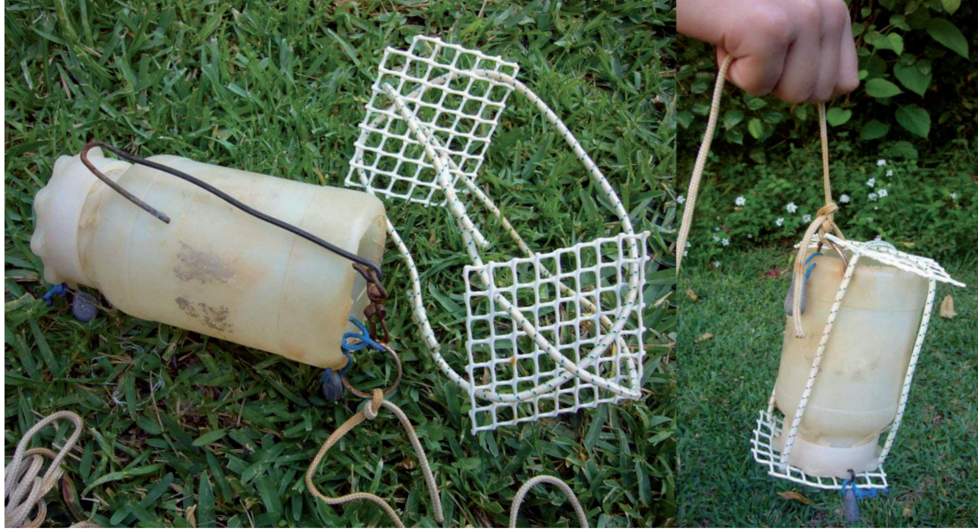

Fig. 13. A simple trap to sample stygofauna in cave lakes and pools. The plastic grid frames can be added to protect the animals eventually attracted by bait from crabs, eels or other potential predators. (Photo by Damia Jaume).

Fig. 14. A simple trap for cirolanid isopods, designed by French biospeleologist Dr. Claude Boutin. (Photo by Damia Jaume).

Sampling inaccessible aquifers: wells and pumps

Shallow brackish-water wells are commonplace in coastal, upraised coral reef terraces and volcanic outcrops, and frequently represent the only pathway to sample aquifers otherwise inaccessible. In addition, these wells (due to accumulation of bird and bat droppings, vegetation remains, animal carcasses, etc.) support high populations of stygobitic crustaceans. Sampling can be undertaken by means of a modified, broader-than-long version of the so-called Cvetkov net (see Cvetkov, 1968), 30 cm in diameter and with the portion corresponding to the funnel reduced to a length of ca. 23 cm; this has proved to work particularly well in these habitats, where the depth of the water column is usually less than 1 m (Fig. 9). Wells provided with pumps and where nets cannot be deployed can be sampled by directly filtering the extruded water, although the specimens eventually caught are frequently damaged (Fig. 15). Finally, it is recommended to ask the landowner for permission if baited traps are to be set in wells that provide water for people or livestock.

Fig. 15. Filtering water directly from a fixed pump at a coastal well. (Photo by Damia Jaume).

7.3. Additional information and web references

Iliffe's web page on anchialine waters warrants a visit (http://www.tamug.edu/cavebiology/index2.html)

7.4. References

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8. Sampling in other special habitats

8.1. Phytotelmata

Phytotelmata (Greek phyton + telm = plant + pond) are small bodies of water held by plant leaves, inflorescences (especially bromeliads) or in tree holes (Fig. 16). They are considered temporary water bodies, even if the habitats themselves are permanently available. Due to their bounded nature and relatively low species richness, phytotelms have been used as models for various ecological processes including dispersal, colonization, species interactions and founder effect.

Fig. 16. Bromeliad *Neoregelia* cf. *princes.* (Photo by Sanda Iepure).

The organisms found in phytotelms include temporary and permanent inhabitants. However, few studies have been devoted to estimate total species richness and species composition (Frank & Fish, 2008). By far the most complex communities developed in phytotelms are in the wet tropics (Menzel, 1926; Tressler, 1941, 1956; Nodt, 1956; Torales *et al*., 1972; Reid, 1993). They include algae and representatives of many taxa of freshwater invertebrates: ostracods *Metacypris maracaoensis* Tressler, 1941 found in epiphytic bromeliads in Puerto Rico and Collier County, Florida (Tressler, 1956); harpacticoid crustaceans *Attheyela* and *Elaphoidella* in neotropical bromeliads; or diptera the mosquito *Wyeomyia mitchellii* (Theobald), 1905 originally described from Jamaica, and known also from other islands of the Greater Antilles, eastern Mexico, and Florida. Almost any plant is a potential host for invertebrates in the small amount of water accumulated by the receptacle, but some groups appear to favor phytotelmata and may be considered specialists, *i.e.* the cyclopids crustaceans *Tropocyclops jamaicensis* Reid and Janetzky, 1996 present in bromeliads from Jamaica (Reid & Janetzky, 1996; Reid, 2001) and *Paracyclops bromeliacola* Karaytug & Boxshall, 1998 originally described from bromeliads in Brazil by Karaytug & Boxshall (1998). In temperate areas tree holes developed at the junction between the trunk and limbs are probably the best habit for small invertebrates. They are less studied in comparison with other phytotelms, and hence a low number of species are known from this habitat.

To sample phytotelms water is extracted by using a pipette and then filtered through a mesh net. A manufactured tool could be successfully used especially to collect insect's larvae. It consists of a 50 cm long endoscopic tube with a diameter of 5 mm and an opening of approximately 4 mm attached to the long snout of a 50 ml syringe. The diameter of the tube should be not less than 5 mm, otherwise it could be blocked by debris present in the leaf axils, such as seeds,

leaves and dirt. Conversely, wider tubes can push apart bromeliad leaf axils, not only damaging them but causing increased leakage of water and animals.

8.2. Mosses and leaf litter

Aquatic (*Sphagnum*, *Hypnum*) and terrestrial mosses in humid conditions host a wide variety of invertebrates (Uniyal, 2000). Although water in *Sphagnum* tends to be quite acidic, this type of moss seems to harbor the richest fauna (Gerson, 1982). The hyaline dead cells in leaves retain rotifer, nematodes, various algae or cyanobacteria and numerous taxa of insects. Scourfield (1953) found many species of copepods (*Bryocyclops*, *Muscocyclops*) living in mosses or seeps in rock outcrop where moss and algae are present. Common species are to be found (*Bryocyclops pygmaeus* Sars, 1863) or rare species like *Stolonicyclops heggiensis* (Reid & Spooner, 1998).

Normally aquatic taxa of invertebrates have been reported from sodden leaf litter from scattered areas around the world, including New Zealand beach forest litter, (Harding, 1958), Australian forests (Dendy, 1895; Plowman, 1979), a sedge meadow in the Canadian tundra (Bliss *et al*., 1973), the Paramo region in the Colombian Andes (Sturm, 1978), and a wet campo marsh in sub-tropical Brazil (Reid, 1984). In Europe, where extensive forests of *Fagus silvatica* exist, leaves form a dense layer that retains water and animals live mainly in the deeper more humid layers (Nielsen, 1966; Schaeffer, 1991; Dumont & Maas, 1998). Fiers and Gheene (2000) surveyed soil nematodes in Belgium, and found a large number of copepods in the litter sample in spite of using an inadequate method for collecting this normally aquatic group. Some of the species found display particular traits of a subterranean inhabitant like *Graeteriella unisetigera* (Graeter, 1908), and hence, the authors suggested that leaf litter was important in the dispersal and population maintenance of stygofauna.

Mosses and leaf litter may be sampled by washing the substrate through a mesh. For the leaf litter a corer for soil samples with a diameter of 2-5 cm could be used, and remove 2-5 kg of soil. The depth of the sample depends on the vegetation type and could range from the surface to a depth of 40 cm. In the laboratory a small amount of soil (representing about 5% of the entire sample) is suspended in distilled water. The mixture is sieved with a mesh net and the fractions smaller than 2 mm are suspended in distilled water. This solution could be sieved again through a smaller mesh than the previous one, and the retained residue is fixed in 4% formaldehyde. The residue is afterwards centrifuged once in distilled water, once in a 50% solution of Ludox® and water. The organisms are further sorted under the stereo-microscope. See also the section on macroinvertebrate extraction from macrophytes using Berlese-Tullgren funnels. This method will extract many of the arthropods from wet moss and litter, but is not appropriate for most soft-bodied invertebrates.

8.3. References

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