James T. Anderson
Craig A. Davis *Editors*

Wetland Techniques

Volume 2
Organisms

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Preface

Wetlands are generically defined as lentic systems that take on characteristics of both terrestrial and aquatic systems where vegetation capable of growing in shallow water proliferates. However, there are many definitions of wetlands in use around the world, including a number that have ecological and legal significance. Even among these definitions, there are numerous subtle nuances that blur the lines between wetlands and either terrestrial or aquatic systems. Despite the confusion and oftentimes contradictory nature of wetland definitions, wetlands are increasingly being recognized as critical ecosystems throughout the world. In particular, we are seeing an increased awareness about the values and benefits derived from the world's wetlands. As this awareness has grown, we have also seen a greater focus on efforts to better manage, conserve, and protect wetlands. Wetland-related research has been and will continue to be critically important in providing guidance to all the efforts to better manage, conserve, and protect wetlands. In fact, there is a plethora of wetland-related literature available to wetland scientists, regulators, and managers, many of which can be found in at least two journals that are dedicated exclusively to wetlands. However, for most wetland professionals, it may be a daunting task to access much of this literature. Additionally, wetland professionals have not had a book available that covers techniques associated with wetland research, management, and regulation.

The lack of such a book has been a major void in the wetland field. In fact, wetland professionals have discussed for some time the need for a book that focused on wetland research and management techniques. We believe the development of a techniques book for a profession is a sign that the profession, in this case wetland science, is maturing. Scientific progress in a field is often advanced by the development of a techniques book because almost all studies and management actions boil down to choosing appropriate techniques, and a book focused on the topic of wetland techniques will provide fledgling scientists and managers a solid foundation for initiating research and management efforts. We have designed this three volume set for students and professionals interested in wetlands ecology,

management, and creation. We are pleased to be a part of the development and progression of our discipline through our involvement with the development of *Wetland Techniques Volume 1*: *Foundations*, *Volume 2*: *Organisms*, and *Volume 3*: *Applications and Management*.

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Acknowledgments

Wetland Techniques is our first attempt at a major book project and it was a wonderful learning opportunity as well as an eye-opening experience in regards to all the effort that goes into creating a series of books of this magnitude. We have new-found admiration for all those before us that have successfully tackled book projects for the benefit of science.

We thank the chapter authors for providing freely of their time and expertise. It has been a pleasure working with the authors and we have learned a lot more about wetlands because of them. We thank all of the chapter referees for giving their time and expertise to improve the quality of this three volume *Wetland Techniques* set through constructive reviews that greatly improved the chapters. We especially thank Rachel Hager, undergraduate student in Wildlife and Fisheries Resources at West Virginia University, for all of her help in formatting and verifying literature citations and performing numerous other tasks to improve the book. We also thank Roseanne Kuzmic, research associate in the Natural Resource Ecology and Management Department at Oklahoma State University, for assistance with verifying literature citations.

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Chapter 1
Methods for Sampling and Analyzing Wetland Algae

Steven N. Francoeur, Steven T. Rier, and Sarah B. Whorley

Abstract Algae are a biologically diverse group of aquatic photosynthetic organisms, and are often common in wetlands. Algal species vary in their optimal environmental conditions, thus the taxonomic identity of algae present in a wetland can be used to make inferences about the environmental characteristics (e.g., water quality) of the wetland in which they are found. Algae also play important roles in the ecology of wetlands. They can be highly abundant and productive, thereby supporting wetland food webs and affecting wetland biogeochemical cycles. It is hoped that this chapter will provide a useful reference for wetland scientists and managers, and also serve to introduce students to appropriate methods for the sampling and analysis of wetland algae.

1.1 Scope and Purpose

The purpose of this chapter is to introduce the reader to commonly-used techniques for the analysis of wetland algal community composition, biomass, productivity, and nutrient limitation. These techniques are suitable for use in both university-level classes and original research. Many other specialized and less-common techniques (e.g., measurement of nitrogen fixation rates or single-cell

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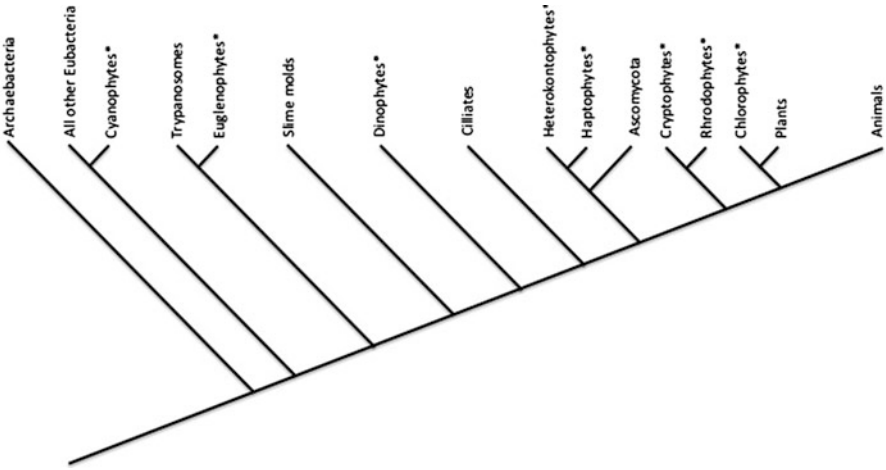

Fig. 1.1 Approximate phylogenetic tree of some algal (marked with an *) and non-algal groups. In addition to the relationships shown here, note that some algal groups (e.g., Heterokontophyta) contain both algae and non-algal organisms (Van den Hoek et al. 1995). Basic tree structure based on the 16/18s rRNA phylogenetic tree of Van den Hoek et al. (1995), with additional resolution of some eukaryotic groups from the 23s rRNA phylogenetic tree of Van den Hoek et al. (1995)

photosynthetic activity) exist, but are not considered here. This chapter also provides a basic introduction to the biology of wetland algae, and includes a set of suggested laboratory exercises and in-class activities.

1.1.1 Algal Biology and Phylogeny

Algae are generally defined as thalloid, oxygenic photosynthetic organisms which contain chlorophyll *a*, but lack multicellular gametangia (Graham and Wilcox 2000; Lowe and LaLibertie 1996). The requirement for oxygenic photosynthesis separates the algae from heterotrophs and anoxygenic microbial phototrophs, and the generally low degree of differentiation of body tissues and lack of multicellular gametangia separates the algae from the Kingdom Plantae. Thus, algae are the group of oxygenic primary producers that remain after one excludes mosses, liverworts, and vascular plants.

However, defining algae as "photosynthetic organisms that are not 'real' plants" causes the algae to be a highly polyphyletic group. In other words, there are several distinct evolutionary lineages of algae. These algal lineages are often more closely related to non-algal organisms than they are to other algae, and the genetic distance between algal groups can be very large. In evolutionary terms, humans (*Homo sapiens*) are more similar to oak trees (*Quercus* sp.) than many groups of algae are to each other (Fig. 1.1). Consequently, algae exhibit great variability in biology, biochemistry, and life history.

Algal taxonomy is currently in a state of flux. A rapidly-increasing knowledge of evolutionary histories within and among algal groups is driving revision of algal taxonomy, as taxonomic systems are revised to better reflect algal phylogeny. In addition, new algal species are still being discovered at a rapid rate. Nevertheless, reasonable modern algal taxonomic systems recognize 9–11 Divisions (= Phyla in the zoological taxonomic nomenclature) of algae (Graham and Wilcox 2000; Van den Hoek et al. 1995). Of the algal Divisions, three (Chlorophyta, Cyanobacteria, and Heterokontophyta) dominate freshwater wetlands, while the other Divisions are usually less common, but may still be abundant in certain locations. Approximately 45,000 species of algae are known to science, with the Chlorophyta (~17,000 species) and the Heterokontophyta (~15,000 species) being the most species-rich Divisions (Graham and Wilcox 2000). Many algal species remain undescribed; some experts estimate that there are millions of extant algal species (Norton et al. 1996).

Algae exhibit several general growth forms. Some algae have large complex, plant-like thalli. Very few freshwater algae have this growth form, but certain Chlorophytes with this growth form (e.g., *Chara*, *Nitella*) can be abundant in some wetlands. Much more common is the simple unicellular growth form, where algae exist as individual cells. Other wetland algae have a colonial growth form. Colonies are assemblages of cells. In some species, the arrangement and number of cells in a colony is fixed (such a colony is called a coenobium), while in other species the number and arrangement of cells within a colony can vary. The filamentous growth form is also common in wetland algae. Filaments are chains of algal cells. They may be branched or unbranched; uniseriate (made of a single chain of cells) or multiseriate. A few wetland algae (e.g., *Vaucheria*) have a siphonous or coenocytic growth form. These algae are characterized by a rarity or lack of cross walls separating cells, and thus exist as one large, multinucleate cell. Figure 1.2 shows several examples of major algal Divisions and growth forms.

The Chlorophytes, or green algae, are common in wetlands. These eukaryotic algae are usually grass-green in color, store their carbohydrate reserves as starch (and thus will stain blue-black with iodine), and have cell walls (typically made of cellulose). Chlorophytes exhibit a range of growth forms, including plant-like, unicellular, colonial, and filamentous. Some Chlorophytes are motile in the vegetative state; some species swim with flagella, while others can squirt mucilage for short-range jet propulsion.

The Heterokonts are another type of eukaryotic algae common in wetlands. These algae are usually brown, tan, golden, or yellow-green in color. Several different sub-groups of Heterokont algae occur in wetlands, but the most abundant and important wetland Heterokonts are the diatoms. Approximately 12,000 of the 15,000 known Heterokont algal species are diatoms. Diatoms store their energy reserves as lipids, and have silica-impregnated cell walls, known as frustules. They typically have a unicellular growth form, but colonies and filaments are also possible. Many diatoms are motile in the vegetative state, exhibiting crawling mobility using a structure known as a raphe. A few species can exude mucilage for short-range, jerky movement.

Fig. 1.2 The unicellular Chlorophyte Cosmarium (**a**), the unbranched filamentous Chlorophyte Spirogyra (**b**), the branched filamentous Chlorophyte Cladophora (**c**), the unicellular diatom Gyrosigma live, showing chloroplasts and oil droplets (**d**), and dead, showing siliceous frustule (**e**), the colonial diatom Tabellaria (**f**), several unbranched filaments of the Cyanophyte Oscillatoria (**g**)

The Cyanobacteria, often called the blue-green algae, are prokaryotic algae. As such, they lack the plastids found in eukaryotic algal groups, and can be easily identified by the distribution of photosynthetic pigments throughout their cells. Cyanobacteria are often blue-green in color, but may also be olive-drab or black. About 2,000 species of cyanobacteria are currently recognized (Graham and Wilcox 2000). Cyanobacteria exhibit unicellular, colonial, and filamentous growth forms. Some Cyanobacteria are motile, exhibiting a form of gliding motility across surfaces. Others can regulate their buoyancy to move vertically through the water column. Many Cyanobacteria can fix nitrogen, and thus they may dominate low nitrogen wetlands.

1.1.2 Algal Habitats

Wetland algae occupy three principal habitats. Many people are familiar with the planktonic habitat. Planktonic algae (phytoplankton) live at least a portion of their lifecycle suspended in the water column. Planktonic algae tend to have characteristics which assist them in remaining in the water column, such as: swimming motility; positive buoyancy; thin, light cell walls; and projections such as spines, which increase drag and slow sinking rates.

The second major algal habitat is benthic. In this habitat, algae live on or attached to surfaces. The benthic habitat has been divided into a number of subcategories, based upon the nature of the surface supporting algal growth (see Goldsborough et al. 2005; Wetzel 2001). These subcategories are often useful, as the nature of the substratum influences which algae are likely to grow upon it. Of the many different subcategories of benthic algal habitat, wetlands are especially rich in epiphytic (plants as substratum), epipelic (fine organic sediments/silt as substratum) and epixylic (wood as substratum) habitats. Benthic algae tend to have characteristics which assist them in remaining anchored to or moving across surfaces, such as: specialized attachment structures (mucilage stalks and pads, holdfast cells); gliding or crawling motility; and thick, heavy cell walls.

The third habitat type, metaphyton, is intermediate between planktonic and benthic habitats. Metaphytic algae are neither truly benthic or planktonic. They can arise via aggregation and clumping of planktonic algae, or (more commonly) via detachment of benthic algae from the substratum. In wetlands, metaphyton is frequently observed as algal aggregations on the water's surface (Fig. 1.3).

1.1.3 Ecological Roles

Algae can be the dominant primary producers in aquatic systems. In shallow systems such as wetlands, metaphytic and benthic algal production is especially important. Algal production in lakes and wetlands can sometimes equal or exceed

Fig. 1.3 Floating metaphyton mats in a Michigan wetland (**a**), close-up of metaphytic filaments (**b**)

the production of vascular plants (Goldsborough and Robinson 1996; Wetzel 2001). Although some algae produce toxins which discourage grazers (e.g., Vanderploeg et al. 2001), most algae are highly digestible relative to vascular plants. Algae also can be an important dietary source of essential fatty acids (Brett and Müller-Navarra 1997). Furthermore, diatoms store energy reserves in the form of lipids (Graham and Wilcox 2000), which results in these algae being an especially energy-dense food resource. In addition to providing energy and nutrient-rich particulate organic matter (algal cells) for ingestion, algae are also a source of energy-rich dissolved organic carbon molecules. Thus, wetland algae are an important food resource for many aquatic invertebrates, heterotrophic flagellates, ciliates, and bacteria (Goldsborough and Robinson 1996; Hamilton et al. 1992; Hart and Lovvorn 2003; Keough et al. 1998).

Algae affect wetland biogeochemical cycling (Vymazal 1995). Algal photosynthesis can be a key determinant of the redox state of aquatic habitats, which in turn influences the chemical form of nutrients. In particular, algal photosynthesis at the surface of illuminated sediments can keep this microzone oxygenated, thereby inhibiting phosphorus and ammonia release from sediments (Carlton and Wetzel 1988; Goldsborough and Robinson 1985). Direct nutrient uptake by algae can also greatly influence wetland biogeochemical cycles. Nutrient sequestration by wetland algae can be a major mechanism for immobilizing dissolved nutrients (Goldsborough and Robinson 1996).

Individual algal species have their own unique set of optimal conditions and tolerance limits. Thus, the taxonomic composition of algal communities reflects environmental conditions, and can be used to make inferences about characteristics such as water quality. Use of algal communities to monitor current or reconstruct past wetland environmental conditions can be complicated by factors such as hydrology and spatial heterogeneity (EPA 2002; Weilhoefer and Pan 2006), but such approaches can still provide valuable information. Several references (e.g., Lowe and Pan 1996; EPA 2002; Gaiser and Rühland 2010) provide good introductions to this topic.

1.2 Collection

Collection of algae may be done by either qualitative or quantitative methods. Qualitative collection methods do not rigorously define the water volume (phytoplankton) or surface area (benthic algae, metaphyton) from which the algal sample is collected. In contrast, quantitative collections sample known volumes or areas. Thus, qualitative collections are often faster and easier to conduct, but quantitative collection methods must be used when expression of the abundance, biomass, or metabolic activity of algae on a volume- or area-specific basis is required.

Appropriate collection methods vary by algal habitat. Recommended qualitative and quantitative collection methods for planktonic, metaphytic, and benthic algae are described below.

1.2.1 Phytoplankton

Phytoplankton in shallow surface waters can be easily sampled by filling a jar or bottle with water. A depth-integrated phytoplankton sample may be obtained from shallow waters by vertically submerging one end of a rigid acrylic tube (recommended diameter ~6 cm) while keeping the other end of the tube above the water's surface. Once the bottom of the tube has reached the desired depth, the aerial end of the tube is tightly capped with a rubber stopper. The tube is then vertically removed from the water. A tightly-sealed stopper will prevent any loss of water from the tube. The open end of the tube is placed into a clean bucket, and the stopper seal is broken, releasing the depth-integrated water sample from the sampling tube. Phytoplankton from deeper layers of water can be sampled using a Kemmerer or Van Dorn bottle. To use such a device, one opens the seals on both ends of the bottle and arms the triggering mechanism. The open bottle is lowered down a line to the desired depth; then a messenger weight is sent down the line. The messenger weight strikes the trigger mechanism and closes the bottle, thereby enclosing a water sample from the desired depth. These collection methods are suitable for qualitative sampling and can also be used for quantitative phytoplankton sampling by simply measuring the volume of water captured by the sampling device. One liter of water or more may be required to collect a measurable amount of phytoplankton biomass.

Plankton nets are frequently used for phytoplankton sampling. Plankton nets have the great advantage of pre-concentrating the plankton sample during collection, and thus are very useful in oligotrophic waters. Horizontal plankton tows are accomplished by tossing a plankton net into the water, allowing it to sink to the desired depth, and towing it back to the investigator by taking up the tow line. Smaller plankton nets can be attached to the end of a fishing line, then cast and retrieved using a rod and reel. It is imperative to employ a small mesh size (75 μm or less; finer mesh is generally better), and realize that many phytoplankton may be

smaller than the mesh openings, and thus cannot be reliably collected with a net (APHA 2005). Many plankton net design modifications and sampling procedures have been developed in attempts to use plankton nets for quantitative sampling (see APHA 2005). Simple horizontal tows of common conical plankton nets should be used only for qualitative sampling.

1.2.2 Metaphyton

Metaphyton may be qualitatively sampled by scooping material into jars or fine-meshed dip nets, suctioning material (syringes or kitchen-type turkey basters work well for this purpose), or (for cohesive, filament-based metaphyton) collecting material by hand. Quantitative collection of metaphyton requires definition of the sampling area, then carefully removing all metaphyton from the sampling area. A floating sampling frame can be used to define the sampling area; one may need to cut along the edges of the frame with sharp scissors to separate the metaphyton sample. Use of a coring tube instead of floating frame can improve quantitative metaphyton collection by physically isolating the sample area through the water column. The tube is first placed at the water's surface to define the sampled area, then gently extended downwards through the metaphyton. As the tube is extended downwards, one cuts along the edges of the tube with sharp scissors. Be sure not to disturb underlying benthic algae with the submerged end of the tube. For loosely-aggregated metaphyton, a large-diameter (10 cm) tube is recommended (EPA 2002).

1.2.3 Benthic Algae

Proper collection methods for benthic algae depend on the substratum upon which the algae are growing. Benthic algae may be collected from hard surfaces by scraping or brushing. Scalpels, razor blades, spoons, rubber spatulas, and stiff-bristled toothbrushes are commonly-used removal devices. *In-situ* scraping/brushing sample collection devices (e.g., Loeb 1981) can be employed, or substrata may be removed from the water for scraping and brushing. Move substrata slowly in the water and gently pull them through the air-water interface to avoid loss of loosely-attached benthic algae. If such losses are problematic, carefully enclose submerged substrata in resealable plastic bags (Ziplock or equivalent) with a minimum of surrounding water as early as possible in the sample collection process. Any algae dislodged during the sampling process will be contained within the plastic bag, so the contents of the entire bag should be included in the slurry of scraped benthic algal material. For some assays, it is preferable to sample algae by cutting the substratum and removing the substratum section along with its associated benthic algae, thereby ensuring the physical integrity of the benthic algal community and avoiding any concerns about imperfect algal removal during

the scraping and brushing process. If necessary, these substratum pieces can also be bagged while submerged. Benthic algae from soft sediments can be collected by coring the sediment.

Quantitative benthic algal samples are obtained by sampling a known area of substratum. Sampling devices (such as coring tubes) may be used to define the area, or algae may simply be removed from a known area (a stencil can be useful in defining the area to the scraped/brushed). The gravimetric aluminum-foil technique can be used to find the surface area of irregularly-shaped regions (e.g., rock surfaces). Once the algae have been removed from the substratum, the edges of the removal region are marked. A monolayer of aluminum foil is pressed over the removal region, taking care to avoid folding or bunching the foil. The foil is trimmed so that it matches the dimensions of the removal region, then its mass (to the nearest 0.1 mg) is compared to the mass of a known area of foil from the same roll (recommend 100 cm^2). The ratio of the masses is used to calculate the area of the removal region using the following formula.

$$\begin{aligned}\text{Size of unknown area } \left(\text{cm}^2\right) = {} & \text{foil mass from unknown area (mg)} \\ & \times \left(\text{size of known area } \left(\text{cm}^2\right)\right. \\ & \div \text{ foil mass from known area (mg)}\left.\right)\end{aligned}$$

The simple aluminum-foil technique presented here is reasonably accurate and is in common use. It can overestimate surface areas, as the wrapped foil will often be folded or bunched slightly, which artificially increases foil mass. It may also underestimate areas of objects with very rough or highly complex surface topography, as it will not be possible to press the foil completely into all topographic features. Bergey and Getty (2006) reviewed several techniques for measuring surface areas of irregularly shaped objects, developed an empirical correction factor for the foil-wrapping conversion equation, and discussed the use of wetted-layer techniques suitable for objects with very rough surfaces.

Benthic algae are often grown upon artificial substrata (see, for example, APHA 2005). Use of artificial substrata can facilitate benthic algal sampling by providing equally-sized and easily-handled algal growth surfaces, removing any among-sample substratum variability, and ensuring that sampled algal communities are of uniform age. However, benthic algal communities grown on artificial substrata will not always be representative of natural communities (Cattaneo and Amireault 1992); species composition can differ, and biomass may either be greater or less than natural communities. Consequently, their use should be confined to situations where this limitation is not a serious drawback.

The locations, number of samples obtained, and frequency of algal sampling will depend on the goals of the study. Broad recommendations have been published for algal sampling programs designed to detect water pollution (e.g., APHA 2005; Biggs and Kilroy 2000; EPA 2002). It is important to note that benthic algal communities are notoriously variable in space, and thus precise estimates may require extensive replication. For example, 8–10 replicate benthic algal samples per site are typically required to have a 95 % chance of detecting a two-fold difference in benthic algal biomass between sites (Francoeur 2001; Morin and Cattaneo 1992).

1.3 Identification, Enumeration, and Preservation

1.3.1 Taxonomic References and On-Line Resources

Although molecular tools are often used in investigations of algal phylogeny, the primary method for taxonomic identification of collected specimens remains morphological examination, generally performed using a compound microscope. Thus, illustrated taxonomic keys are of great value. Provision of a detailed algal taxonomic key is a major undertaking and outside the scope of this chapter. Wehr and Sheath (2003) and Dillard (2008) recently published comprehensive, genus-level keys of the North American freshwater algal flora. Both are useful references, although Dillard's key (Dillard 2008) does not include the most common group of heterokonts, the diatoms. Lowe and LaLibertie (1996) presented a simple illustrated key appropriate for lotic wetlands. Regional taxonomic references (e.g., Dillard 2007; Fucikova et al. 2008; Prescott 1962) can also be invaluable resources, but care must be taken regarding updated taxonomies and newly discovered and invasive taxa when using older references. The Süßwasserflora von Mitteleuropa series (e.g., Komarek and Anagnostidis 2008; Krammer and Lange-Bertalot 1986) provides a similar modern algal taxonomic reference for Europe.

In contrast to North America and Europe, many regions of the world lack modern comprehensive regional algal taxonomic references. In addition to using regional references (when available), workers in these regions should consult with taxonomic references from other regions and the primary literature, and expect to encounter many undescribed species. Even the relatively well-investigated North American and European algal floras are not completely known; new species are continually being discovered.

On-line taxonomic and image databases can also aid in proper algal identification. One must be cautious in using web-based information, as some websites contain factual errors, change content rapidly, or exhibit variable quality control. Nevertheless, useful, authoritative on-line resources exist. For example, websites such as: the Great Lakes Diatom page (http://www.umich.edu/%7Ephytolab/GreatLakesDiatomHomePage/top.html), AlgaeBASE (http://www.algaebase.org), the California Academy of Sciences diatom page (http://research.calacademy.org/izg/research/diatom), the Bowling Green State University Algal Image Laboratory (http://www.bgsu.edu/Departments/biology/algae/), and the Diatoms of the United States website (http://westerndiatoms.colorado.edu/) all provide high quality taxonomic and image databases. The Phycological Society of America website (www.psaalgae.org) maintains a list of professionally-useful on-line resources.

Formal training is highly recommended for individuals seeking to become skilled algal taxonomists. Several universities and biological field stations offer Phycology courses which include instruction in algal identification and practical taxonomy.

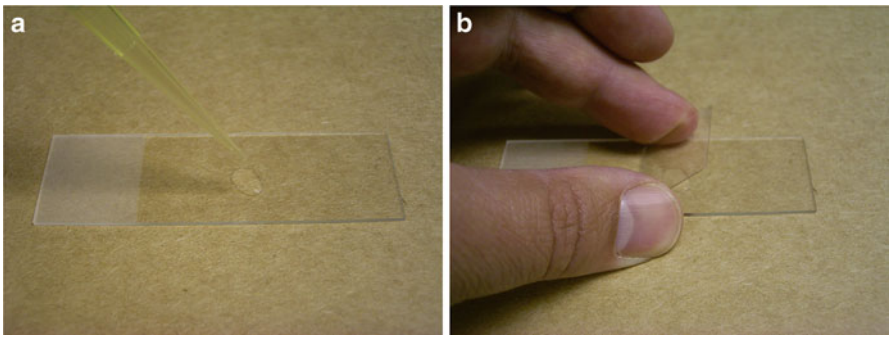

Fig. 1.4 Making a wet mount. Adding a single drop of sample to slide (**a**), placement of cover slip (**b**)

1.3.2 Slide-Making and Enumeration

Microscopy is the primary way in which algae are identified, and the principal technique used for enumeration of algal abundance. Thus, slide-making and the use of microscope counting chambers are necessary skills. The type of slide or counting chamber employed will depend on the nature of the algal sample (size and type of algae) and the type of information desired (specimen identification, relative abundance, or absolute abundance). Five different techniques (wet mount, syrup mount, diatom mount, Palmer-Maloney cell, and Sedgewick-Rafter cell) suitable for conventional upright microscopes are outlined below, along with a discussion of the appropriate use of each. If an inverted microscope is to be used, the Palmer-Maloney and Sedgewick-Rafter cells can be replaced by a settling chamber (see APHA 2005).

Wet mounts are quick and easy to make, and are suitable for use with dry objectives (total magnification usually $<500\times$). Wet mounts are made by placing one drop (recommend 20–100 µl) of algal sample onto a standard glass microscope slide, then placing one edge of a cover slip onto the slide near the drop, and finally allowing the cover slip to fall onto the drop (in a motion similar to closing the cover of a book) thereby enclosing the sample between the slide and the cover slip (Fig. 1.4). The wet mount is now ready to examine. Do not use large or multiple drops – too much liquid will cause the cover slip to float, resulting in a mobile mount and unstable microscope images. A properly made wet mount should be dry enough to be completely inverted without any movement of the cover slip. Very dry (20 µl drop size) wet mounts can be adapted for use with oil immersion lenses (total magnification ~1,000×) by ringing all four sides of the cover slip with fingernail polish to seal the cover slip to the slide. Once dry, the fingernail polish usually provides sufficient mechanical strength to allow use of oil immersion lenses.

Syrup mounts can be used with oil immersion objectives, and are thus suitable when greater magnification (up to ~1,000×) and resolution are required. Syrup mounts are also semi-permanent; when stored properly, they will last for years. The following method is adapted from Stevenson (1984). First, make a solution of

100 % Taft's syrup by combining seven volumes of 4 % formaldehyde with three volumes of light corn syrup (Karo or equivalent), and a solution of 10 % Taft's syrup by diluting a portion of the 100 % Taft's syrup 1:10 with distilled water. If the sample to be examined is not already preserved in glutaraldehyde, place an aliquot in 2–5 % (final concentration) glutaraldehyde (see Sect. 1.3.4), and allow at least 2 h of glutaraldehyde fixation before proceeding with the syrup mount. Place preserved material onto a cover slip, cover with at least an equal amount of 10 % Taft's syrup, and allow to dry; note that material will not dry completely, but will remain tacky. Repeat the addition of 10 % Taft's syrup and drying process 2–3 times. Place one drop of 100 % Taft's syrup onto a standard glass microscope slide, then place the cover slip (sample side down) onto this drop. Press gently, if necessary, and allow the 100 % Taft's syrup to harden; note that the drying process may take several days. Add additional 100 % Taft's syrup as needed if the syrup solution pulls away from the edges of the cover slip during the drying process – gently inject small amounts of 100 % Taft's syrup into gaps between the slide and the cover slip. Once dry, seal the cover slip to the slide by ringing all four sides of the cover slip with fingernail polish. After the fingernail polish is dry, the syrup mount is ready to observe.

Contact and vapor exposure to formaldehyde and glutaraldehyde are harmful. When working with solutions of these substances, you must use adequate ventilation (e.g., a fume hood) and wear eye protection and gloves. Due to harmful fumes, drying of syrup mount cover slips and slides must be conducted in a fume hood. Be careful not to close the fume hood door too far when drying cover slips – this can cause rapid air flow and upset the cover slips. For best results, store syrup mounts in a cool, dark place. Darkness prevents bleaching of color from algae. If slides become too warm, the syrup may lose viscosity and run.

Adequate observations of diatoms can sometimes be accomplished using wet mounts or syrup mounts. However, much diatom taxonomy is based upon detailed features of the diatom frustule, and observation of these features often requires the use of permanent diatom mounts. These mounts facilitate detailed observations by removing organic matter ("cleaning") and mounting frustules in a high refractive index mountant, and are compatible with oil immersion lenses.

The following cleaning method was introduced by Carr et al. (1986). It is inexpensive, safe, and generally suitable for light microscopy. Place an aliquot of sample into a large glass tube. One to 5 ml of sample is usually sufficient to make multiple slides. Add an equal amount of a 5.25 % sodium hypochlorite solution (commercially-available laundry bleach is suitable). Wait 1–3 h, depending on amount of organic material present, and periodically agitate the tube during this time. Do not exceed 3 h, as prolonged exposure to sodium hypochlorite will erode frustules. After the exposure period is finished, immediately add distilled water to fill the tube, and let stand until all diatom frustules have settled to the bottom of the tube; 8–24 h or longer, depending on the height of the tube and settling speed of the frustules. Furet and Benson-Evans (1982) measured settling speeds of 3.3 mm/h for many phytoplankton, but small diatoms sank at half that rate. Large, heavily-silicified benthic diatoms will settle more quickly. Frustules will appear as a fine white powder. Remove as much supernatant as possible without disturbing settled

frustules by carefully decanting supernatant or by using an aspirator, and refill tube with distilled water. Repeat cycles of filling, settling, and decanting until the supernatant is clear and has a pH ~7. The rinsing process may be shortened by using centrifuge tubes, and replacing the settling step with centrifugation (4,000–6,000 revolutions per minute (rpm) for 5–10 min is usually sufficient).

The one drawback of this cleaning protocol is that the sodium hypochlorite cleaning solution can erode fine structures on the frustule. Thus, samples for electron microscopy or samples containing diatoms with lightly-silicified frustules should be cleaned using alternative methods. For diatom samples with little organic matter (e.g., phytoplankton), one can often achieve reasonable cleaning by placing a few drops of material on a glass cover slip, then ashing the cover slip (either on a hotplate or in a furnace) for a few minutes. Diatoms on ashed coverslips are ready for mounting directly onto microscope slides (see below). If too much organic matter is present in the sample, residual ash will obscure frustules in diatom mounts made by this technique.

Acid-based cleaning methods are effective, even for samples with high organic matter. Many such methods exist (e.g., APHA 2005; Round et al. 1990), typically employing some combination of concentrated acid(s), heat, and/or chemical oxidizers. The following method is modified from one of several discussed by Round et al. (1990). Make a 1:1:1 mixture of algal sample, concentrated sulfuric acid and concentrated nitric acid in a glass tube or beaker, then bring this mixture to a boil. Cool, then conduct cycles of dilution with distilled water, settling, and decanting (as described above) until supernatant is clear and has a pH ~7. Handling of concentrated acid requires proper safety equipment (e.g., eye protection, gloves, lab coat). Mixing water with concentrated acid can release large amounts of heat; never hold the beaker in your hand, and whenever possible add acid to water (instead of water to acid) to reduce flash boiling and splattering. Noxious fumes can be released when boiling acids, so boiling must be done in a fume hood. For those who do not wish to employ strong acids, an alternative oxidative cleaning method using H_2O_2 and $K_2Cr_2O_7$ can also be used (see Lowe and LaLibertie 1996).

Once frustules are free of organic matter, transfer a suspension of frustules to a glass cover slip, and allow to air dry. Cover slips may also be dried using gentle heat, but this will result in uneven distribution of frustules. One must calibrate the amount of material placed onto cover slips to the density of frustules within the sample. In overloaded cover slips, frustules will lie on top of one another and present obscured views. In underloaded coverslips, few frustules will be observed. After the cover slip is dry, place one drop of Naphrax (available from Brunel Microscopes Ltd., Unit 6 Enterprise Centre, Bumpers Farm Industrial Estate, Chippenham, Wiltshire, SN14 6QA, U.K.) onto a clean microscope slide, then place the cover slip (sample side down) onto this drop. Place the slide onto a hotplate at high temperature, and heat until bubbling of the mountant greatly slows or stops. Remove slide from hotplate and gently press the cover slip with forceps tips to settle it and force out any final bubbles. The mountant will quickly cool and harden, and then the diatom mount is ready to observe. If sodium hypochlorite was used for cleaning and not completely removed by rinsing, feathery crystalline

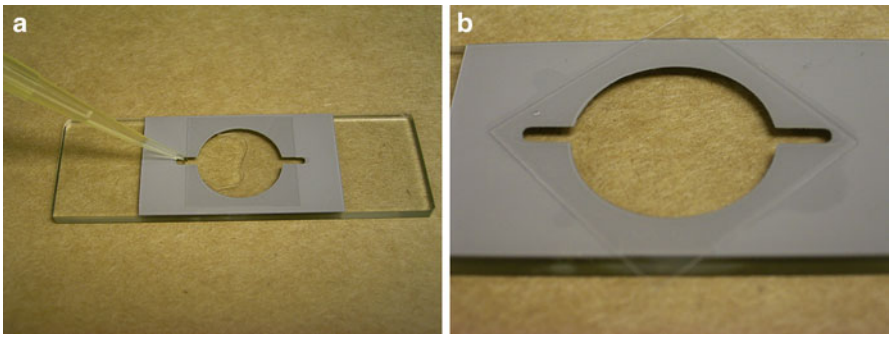

Fig. 1.5 Loading a Palmer-Maloney cell. Injecting sample under cover slip into projection on cell (**a**), rotated cover slip making a complete seal (**b**)

structures will be seen in the diatom mount. If these are objectionable, the frustule suspension remaining in the tube should be rinsed several more times before re-making the diatom mount.

Naphrax releases toluene when heated, so heating must be done in a fume hood to avoid exposure to harmful fumes. Naphrax is also expensive (>$1,000 per liter). Other, less expensive, less toxic, high refractive index mountants (i.e., Meltmount 1.704, Cargille Labs, or equivalent) may be used in its place for production of diatom mounts.

A Palmer-Maloney cell is a precision depression slide, used for examining known volumes of sample (Fig. 1.5). The sample chamber measures 17.9 mm diameter × 0.4 mm deep, for a total volume of 0.1 ml. The thickness of the Palmer-Maloney cell restricts it to use with dry objective lenses and prohibits the use of magnifications greater than ~400×. To use a Palmer-Maloney cell, one covers most of the sample chamber with a square glass cover slip (22 × 22 mm recommended), leaving open both filling projections on the sides of the chamber. A pipette tip is placed into one of the open chamber projections and gently touched to both the glass base of the Palmer-Maloney cell and the edge of the cover slip. Exactly 0.1 ml of homogenized sample is slowly injected into the counting chamber beneath the cover slip and drawn via capillary action into the chamber. Slight elevation of the side of the chamber opposite the pipette may help prevent bubbles from being trapped under the cover slip. The cover slip is then rotated 45°, so that it covers the circular chamber and both projections. The cell is now ready for use. Most algal cells will rapidly settle to the bottom of the chamber, but be sure to check the entire vertical field of view for non-settled or floating cells. Optimal algal densities within a sample result in 10–30 cells in each field of view when using a Palmer-Maloney cell. If cell densities are far outside the optimal range, adjust them via concentration or dilution.

The Sedgwick-Rafter cell is a larger precision depression slide, also used for examining known volumes of sample (Fig. 1.6). The rectangular chamber measures 50 mm × 20 mm × 1 mm deep, for a total volume of 1 ml. The greater thickness of the Sedgwick-Rafter cell prohibits the use of magnifications greater than ~200×.

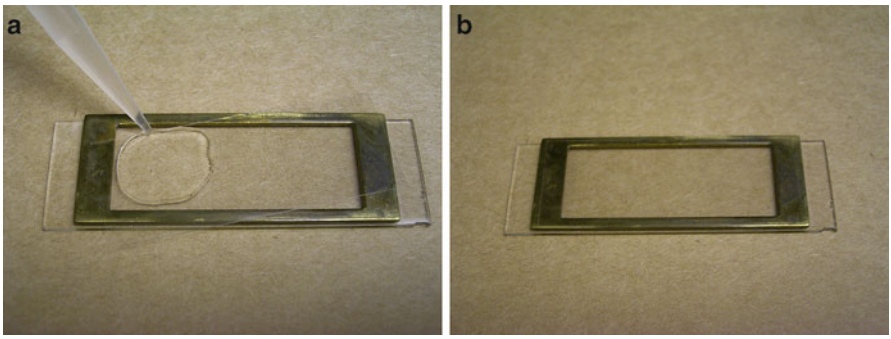

Fig. 1.6 Loading a Sedgewick-Rafter cell. Adding sample to corner of cell (**a**), cell completely sealed by cover slip (**b**)

To use a Sedgwick-Rafter cell, one covers most of the sample chamber with a rectangular cover slip placed slightly askew, so as to leave two opposite corners of the sample chamber uncovered. A 1 ml aliquot of homogenized sample material is pipetted into the chamber, and then the cover slip is squared to the cell, completely enclosing the chamber and making it ready to view under the microscope. Most algal cells will rapidly settle to the bottom of the chamber, but be sure to check the entire vertical field of view for non-settled or floating cells.

1.3.3 Relative and Absolute Abundance

Algal relative abundance (i.e., the proportional representation of different algal taxa within the community) may be assessed via semi-quantitative observation or through enumeration. Algal absolute abundance can be assessed only by enumeration of a known amount of sample material collected from a known area (benthic algae or metaphyton) or volume (phytoplankton).

Both relative abundance and absolute abundance enumerations require examination of a representative subsample. For this reason, the algal sample must be homogenized before the subsample aliquot is withdrawn. Plankton samples can simply be shaken. Metaphyton and benthic algal samples typically require the use of a hand-held or benchtop blender (~30 s) for adequate homogenization (Biggs 1987). Tough filamentous algae (e.g., *Cladophora*) may tangle on the blender blades and shaft, requiring periodic removal and manual cutting of strands with sharp scissors. After blending, rinse algae from the bender with distilled water from a squirt bottle. If absolute abundance enumerations or biomass analysis will be conducted, measure the volume of the slurry (a graduated cylinder usually works well). Remove representative subsamples by withdrawing aliquots with a pipettor. Keep slurry well-mixed by shaking, sloshing, or vigorously stirring in random directions. Do not simply swirl the slurry, as low velocities in the center of the container will allow cells to settle out of suspension. Trim the end of a disposable pipettor tip in order to create a larger opening and prevent plugging by thick slurries.

Algal densities may need adjustment to allow efficient enumeration. Excessively dilute phytoplankton samples require too much scanning to encounter cells, and excessively concentrated metaphyton or benthic algal samples will have some cells obscured by others. Phytoplankton are best concentrated by preserving a representative subsample (see Sect. 1.3.4) and allowing it to settle (8–24 h) or longer, depending on the height of the tube and settling speed of the frustules. Many phytoplankton settle at 3.3 mm h^{-1}, but small diatoms can sink at half that rate (Furet and Benson-Evans 1982). After algae have settled, remove as much supernatant as possible without disturbing settled algae by carefully decanting supernatant or by using an aspirator. For absolute abundance enumerations, the pre- and post-settling volumes must be measured, to allow calculation of the concentration factor. Other phytoplankton concentration methods are possible (see APHA 2005). To dilute metaphyton or benthic algae, simply add distilled water. For absolute abundance enumerations, the pre- and post-dilution volumes must be measured, to allow calculation of the dilution factor.

$$\text{Concentration or dilution factor} = \text{original volume (ml)} \div \text{final volume (ml)}$$

A semi-quantitative rank-based algal relative abundance system was developed by Biggs and Kilroy (2000). To conduct such an analysis, one uses an aliquot of homogenized sample material to make a slide (wet mount or Palmer-Maloney cell is suitable), and then scans the entire slide first at relatively low (100–200×) and then at medium to high (400–700×) magnification, to ensure detection of both small and large taxa. All taxa observed are listed and ranked on a 1–8 scale. The taxon with the greatest cell volume (visual integration of abundance and size) receives a ranking of 8. It is permissible to have multiple taxa ranked 8, if all are equally dominant. The remaining taxa are compared to the dominant taxon, and receive ranks from 7 (abundant) to 1 (very rare). This procedure is very rapid and can provide useful information regarding relative differences among algal communities, but it can be subject to high interobserver variability.

For an enumerative relative abundance estimate, one uses an aliquot of homogenized sample material to make a slide (wet mount or Palmer-Maloney cell is suitable), then observes randomly-selected fields of view. All algae which lay 50 % or more in the field of view are identified and enumerated. The viewing and enumeration continues until one reaches a pre-specified number of cells (usually set at 100–500 cells, with 300 being common). If the cut-off is reached partway through examining a field, all the remaining cells in the field must also be enumerated and included in the tally, to avoid any potential bias. In low-density samples, the enumeration process may be sped by examining randomly-chosen transects instead of individual fields of view. Relative abundance of each taxon in a sample is calculated by dividing the total number of cells of that taxon by the total number of cells of all taxa.

$$\text{Relative abundance of taxon} = \text{no. cells of taxon} \div \text{total no. of cells of all taxa}$$

Absolute abundance enumerations require quantitative collection of algae, i.e., sampling a known volume of water (phytoplankton) or a known surface area (benthic algae and metaphyton) (see Sect. 1.2). Load a Palmer-Maloney cell with an aliquot of homogenized sample material, then observe randomly-selected fields of view. All algae which lay 50 % or more in a field of view are identified and enumerated. The viewing and enumeration continues until one reaches a pre-specified number of cells (usually set at 300 or 500 cells). If the cut-off is reached partway through examining a field, all the remaining cells in the field must also be enumerated and included in the tally. The number of fields viewed is also counted. In low-density samples, the enumeration process may be sped by scanning and examining transects. Entire transects along the horizontal or vertical diameters (length 17.9 mm) of the circular chamber may be examined, or (in microscopes with calibrated stage mechanisms) randomly-chosen horizontal or vertical transects may be examined, with transect lengths determined from the initial and final positions of the microscope stage. The total volume of the sample observed is calculated by multiplying the area of the microscope field of view by the number of fields viewed by the depth of the Palmer-Maloney cell (400 μm), or by multiplying the diameter of the microscope field of view by the length of the transect examined by the depth of the Palmer-Maloney cell. Absolute abundance of each taxon in a sample is calculated by multiplying the total number of cells of that taxon by the total fraction of sample observed, corrected for any concentration or dilutions, then relating this figure back to original volume or area sampled.

$$\text{Total volume examined}_{\text{fields}}\ (\mu m^3) = \text{no. fields observed} \times \pi \times \text{field radius } (\mu m)^2 \times 400\ \mu m$$

$$\text{Total volume examined}_{\text{transects}}\ (\mu m^3) = \text{diameter of field } (\mu m) \times \text{length of transect } (\mu m) \times 400\ \mu m$$

$$\text{Total volume examined (ml)} = \text{Total volume examined } (\mu m^3) \div 10^{12}$$

Phytoplankton:

$$\text{Absolute abundance (cells/L)} = \text{no. cells enumerated} \times [\text{total sample volume (ml)} \div (\text{total volume examined (ml)} \times \text{dilution factor})] \div \text{total sample volume (L)}$$

Benthic Algae and Metaphyton:

$$\begin{aligned}\text{Absolute abundance } \left(\text{cells}/\text{m}^2\right) = {} & \text{no. cells enumerated} \\ & \times [\text{total slurry volume (ml)} \\ & \div (\text{total volume examined (ml)} \\ & \times \text{dilution factor})] \div \text{area sampled } \left(\text{m}^2\right)\end{aligned}$$

Both relative and absolute abundance enumerations share a number of considerations. Foremost of these considerations is taxonomic resolution. Use of species-level taxonomy provides the most information about the composition of the algal community, and is the preferred level of taxonomic resolution. However, conducting species-level enumerations requires a good deal of taxonomic expertise. In addition, some algae only display the characters required for species-level identification at certain stages in their life cycle, and these life stages may not be present in field collections. Because of these difficulties, investigators may compromise between the loss of taxonomic information and ease of algal identification, and conduct enumerations at the genus level. Enumerations are often conducted at the lowest practical taxonomic level; in such cases, some taxa are enumerated as individual species, whereas others are enumerated at the genus level. Occasionally, enumerations conducted at higher taxonomic (e.g., Division) levels are reported.

In both relative and absolute abundance enumerations, only live algae should be considered. In typical practice, cells that appear to contain cytoplasmic contents are considered "live". The presence of cytoplasmic contents is not a perfect indicator; for example, recently dead cells may still contain non-living cytoplasmic material. If the distinction between living and dead cells is critical, then one may need to employ vital staining (APHA 2005; Peperzak and Brussard 2011) or microautoradiography (Wasmund 1989). The wet mounts and counting chambers commonly used for enumerations are easily prepared and allow examination for cytoplasmic contents, but may not allow sufficient magnification or resolution to identify all algal taxa. It is often necessary to make syrup or diatom mounts to confirm the identity of some taxa.

Some taxa consist of cells that are small, numerous, or otherwise difficult to individually enumerate using wet mounts or counting chambers. In these cases, it is preferable to count algal units (i.e., individual colonies, or a specific length of filament measured using an ocular micrometer) instead of individual algal cells. Then, one carefully examines and enumerates the number of algal cells per algal unit in at least 10 units of each taxon (syrup mounts are often helpful for these detailed examinations – see Sect. 1.4.3 for ocular micrometer calibration), and uses the mean number of cells per algal unit of a particular taxon to convert the enumerated algal units to the number of algal cells of a particular taxon.

$$\text{No. of cells of taxon} = \text{no. of algal units of taxon} \times \text{mean no. of cells per unit}$$

Table 1.1 Example of a "double count" relative abundance procedure

Initial enumeration	Diatom mount	Final relative abundance
12 % *Spirogyra*	n.a.	12 % *Spirogyra*
64 % Small oval diatom	81 % *Achnanthidium*	52 % *Achnanthidium*
	19 % *Cocconeis*	12 % *Cocconeis*
24 % Large cylindrical diatom	100 % *Melosira*	24 % *Melosira*

It is often the case that some diatom taxa are indistinguishable from one another during initial enumeration, making it necessary to double count each sample (initial enumeration and a diatom mount). In such cases, the initial enumeration identifications are provisional, and may even be size/shape categories, such as "small oval diatom". The initial enumeration quantifies the relative or absolute abundances of non-diatom algae and provisional diatom categories. The subsequent enumeration of a diatom mount serves to identify the diatom taxa and to quantify the relative abundances of the taxa within the individual provisional diatom categories. These data are then used in combination to calculate the absolute or relative abundances of the diatom taxa in the sample. Consider the following example of a relative abundance enumeration (Table 1.1). The initial enumeration revealed one green alga (*Spirogyra*) and two provisional diatom categories ("small oval diatom" and "large cylindrical diatom") within the sample. Subsequent enumeration of the diatom mount revealed two taxa, *Achnanthidium* and *Cocconeis*, within the "small oval diatom" category (81 and 19 % of this category, respectively), and only one taxon, *Melosira*, within the "large cylindrical diatom" category. Thus, the provisional category "large cylindrical diatom" is replaced by the taxon name *Melosira*, the final relative abundances of *Spirogyra* and *Melosira* remain unchanged from the initial enumeration, and the final relative abundances of *Achnanthidium* and *Cocconeis* are calculated as 81 and 19 % of the relative abundance of "small oval diatom" category. Absolute abundance enumerations would be conducted in the same way; the initial enumeration absolute abundances would be partitioned using the relative abundances of diatom taxa within each provisional diatom category.

Another consideration for enumerations is the possible need for stratified counts. Algal cells can span several orders of magnitude in size, and algal abundance is often negatively correlated with cell size. Thus, if a wide range of cell sizes is present in the sample, it may not be possible to simply enumerate the sample using a single magnification. Use of a magnification great enough to identify the small abundant taxa could likely result in observing only a very small fraction of the sample, thereby missing the larger less-common taxa. Such an error can be important, as large less-common cells may actually compose the majority of the algal biomass. Stratified counts present a solution to this problem. To conduct a stratified count, use a Palmer-Maloney cell to complete an absolute abundance enumeration (i.e., enumerate cells per unit of sample volume) of smaller taxa at ~400×, then use a Sedgwick-Rafter cell to complete an absolute abundance enumeration of larger taxa at ~100–200×. Once combined into a single dataset, these enumeration results (cells per sample volume) can either be used to calculate absolute or relative algal abundances for all taxa in the sample.

Table 1.2 Effect of increasing the number of individuals enumerated on the mean number of diatom species recorded from four samples (Data from Patrick and Strawbridge 1963)

Mean number of cells enumerated	Mean number of species recorded
204	29
549	48
1,303	81
10,959	137

A final consideration for enumerations is the issue of how many cells must be counted within each sample. Three hundred cells per sample is a typical enumeration limit, and limits of 100 and 500 cells are also common. Algal communities tend to be species-rich, with a few dominant taxa and many less-abundant or rare species. As a result, the relation between the number of algal species in a community and the number of individuals within a given species appears to be approximately lognormal (Patrick et al. 1954). Given this type of abundance pattern, many of the rare taxa present within the sample will not be encountered in enumerations limited to a few hundred cells (Table 1.2). Thus, enumerations of a few hundred individuals per sample should be considered accurate only for relatively common taxa. Some workers have recommended enumerating 8,000 cells per sample when less-common taxa of interest (Patrick 1961), but even this amount of enumerated cells is unlikely to fully capture all species present. In one example, an additional 38 rare species were recorded from a single site after increasing the enumeration limit from 8,595 to 35,092 cells (Patrick et al. 1954).

1.3.4 Preservation Techniques

The goal of algal preservation is to arrest the decay process without distorting algal features, so the algal material may be examined at a later date. For most purposes, this is best done by using a chemical preservative agent. Many chemicals have been used for algal preservation (see APHA 2005); the method given below utilizes glutaraldehyde because of its excellent preservative ability. Less toxic alternatives, such as Lugol's iodine, may be used instead (see APHA 2005 for protocol), but offer markedly inferior performance. Glutaraldehyde, like most chemical preservatives, is a hazardous substance. You must use adequate ventilation (e.g., a fume hood) and wear eye protection and gloves when working with glutaraldehyde. Concentrated glutaraldehyde solutions are viscous, so be sure to allow enough time for it to be drawn into and expelled from pipette tips. For best results, use electron-microscopy grade glutaraldehyde. Aliquot stock glutaraldehyde into small containers and store in the freezer (−10 °C). Refrigerate (4 °C) working solutions to retard polymerization caused by warm temperatures and repeated freeze-thaw cycles (Rasmussen and Albrechtsen 1974).

Place a subsample of known volume (3–5 ml is usually sufficient) in a tightly-sealable vial. Phytoplankton samples may require additional volume, to allow for concentration (see Sect. 1.3.3). To each vial, add enough stock glutaraldehyde solution to make a final glutaraldehyde concentration of 1–2 % for plankton or 2–5 % for metaphyton and benthic algae. For example, a 3 ml subsample requires the addition of 0.75 ml of stock 25 % glutaraldehyde to have a final glutaraldehyde concentration of 5 %. Vials should be capped and stored in darkness. This preservation procedure conforms to Standard Methods (APHA 2005).

To prevent potentially harmful exposure, glutaraldehyde must be removed from preserved samples prior to examination. To do this, the preserved material (or a homogenous aliquot) is placed in a tube and diluted with distilled water. The algae are allowed to settle (8–24 h or longer, depending on the height of the tube and settling speed of the algae), and then the glutaraldehyde-rich supernatant is removed and discarded. This rinsing procedure should be repeated several times to fully remove glutaraldehyde and render the sample safe for use outside of a fume hood. Use of large volumes of distilled water relative to the volume of the preserved sample will reduced the number of required rinses. If the sample is to be used for absolute abundance enumerations, be sure to record the pre- and post-rinse sample volumes and account for any dilution or concentration of the sample. The rinsing process may be sped by using centrifuge tubes, and replacing the settling step with centrifugation (4,000–6,000 rpm for 5–10 min is usually sufficient, but may damage some delicate taxa).

Large, plant-like algae (e.g., *Chara*) may be adequately preserved using standard botanical techniques of pressing and drying; sometimes algae must be separated from the blotting paper using wax paper or other barriers to prevent algae from sticking (Whitton et al. 2002). If only diatoms are of interest, and if the diatom analysis is to be conducted solely via examination of siliceous frustules, then samples may simply be stored in capped vials; preservation is unnecessary as decay affects only organic material, not frustules. If samples are being stored for later extraction of DNA, then low-temperature (−80 °C) freezing without a chemical preservative is the best option.

1.3.5 Digital Image Archives

Producing and using an image archive during the enumeration process will help ensure taxonomic consistency through time and across enumerators. In addition, such an archive also facilitates harmonizing the investigator's taxonomy with newly-published taxonomic revisions. High-resolution digital image technology makes such an archive easy and inexpensive to create and curate. As each new taxon is encountered during microscopy, one or more digital images are captured. Image files receive a descriptive name (often linked to the taxon name, collection site, and magnification of image). Archived images can thus be easily retrieved, reviewed, and compared with new specimens. The best images are created using a

high-resolution digital camera mounted to the trinocular head of the microscope, but acceptable-quality images can sometimes be generated by focusing an inexpensive hand-held digital camera through one of the microscope's ocular lenses.

1.4 Biomass and Biovolume

The amount of algae present in a wetland is often of great interest. There are three common techniques for measuring algal biomass: (1) direct measures of mass, (2) quantitative analysis of pigment molecules, and (3) biovolume measurements. All of these techniques require quantitative collection of algae (see Sect. 1.2).

1.4.1 Wet Mass, Dry Mass, and Ash-Free Dry Mass

Due to the high water content of algal tissue and the difficulties of ensuring equal removal of external water from all samples, algal wet mass is an imprecise estimator of algal biomass. This technique is occasionally used for measuring the biomass of filamentous benthic algae after excess water has been removed by blotting or gentle centrifugation in a "salad spinner" (e.g., Higgins et al. 2005; Power 1990) but is not generally recommended.

Dry mass measurements remove all water from algal samples, and are thus a great improvement over wet mass. To prepare for a dry mass analysis, 47-mm glass fiber filters (0.7 μm pore size) should be placed individually into aluminum weighing pans. Identification marks can be scored on the underside of the pans using an awl or similar tool, and filters and pans should be handled with clean forceps to avoid transferring skin oils to their surfaces. The combined weight of each filter and pan combination (i.e., the pre-weight) should be measured (to 0.1 mg).

A known volume of phytoplankton sample should be filtered onto a pre-weighed filter using light vacuum ($\leq$0.3 atmospheres [atm] or 228 mmHg). For metaphyton or benthic algal samples, a homogenous slurry of known volume should be made from all algae sampled from a known area (see Sect. 1.3.3), then a measured subsample of slurry should be filtered. Load the filters with as much material as possible. If the slurry is sufficiently thick, one can dispense with the filtering procedure and simply place a known volume of slurry directly into pre-weighed pans. After returning each filter to the correct pan, wrap the weighing pans with aluminum foil to exclude dust, and dry at 105 °C to a constant weight (24 h is usually sufficient). Cool the pans in a desiccator, and reweigh. Subtract the final dry weight of each filter and pan from their pre-weight to calculate the dry mass of each sample (mg dry mass/ml filtered). For benthic algae, multiply this figure by the total volume of the slurry then divide by the area sampled to calculate the benthic algal dry mass (mg dry mass/surface area sampled).

Phytoplankton:

$$\begin{aligned}\text{Phytoplankton dry mass } (\text{mg/ml}) = &(\text{dry weight } (\text{mg}) - \text{pre-weight } (\text{mg})) \\ &\div \text{volume phytoplankton filtered } (\text{ml})\end{aligned}$$

Benthic Algae or Metaphyton:

$$\begin{aligned}\text{Benthic algal dry mass } \left(\text{mg}/\text{m}^2\right) = &(\text{dry weight } (\text{mg}) - \text{pre-weight } (\text{mg})) \\ &\div \text{volume slurry filtered } (\text{ml}) \\ &\times \text{total slurry volume } (\text{ml}) \\ &\div \text{total surface area sampled } \left(\text{m}^2\right)\end{aligned}$$

This dry mass analysis procedure conforms to Standard Methods (APHA 2005).

Dry mass can greatly overestimate algal biomass in samples containing large amounts of inorganic particles (e.g., sand grains), as these inorganic materials contribute to dry mass. Such inorganic materials are often common in benthic algal samples. Ash-free dry mass (AFDM) removes the weight of these inorganic contaminants, and thus is the best mass-based measure of algal biomass.

Preparation for AFDM measurements is similar to that for dry mass. However, the individual filters and aluminum weigh pans must be pre-ashed (1 h at 500 °C) before pre-weighing, in order to remove any contaminants. After this preliminary ashing, it is critical to handle filters and pans with clean forceps. If both dry mass and AFDM data are desired, then the protocol outlined above for determination of dry mass should be followed. If only AFDM is of interest, then the pre-weighing step in the dry mass protocol may be omitted. After measurement of dry weights, samples should be re-wrapped in aluminum foil, and ashed (1 h at 500 °C). After cooling, ashed samples must be re-wet with reagent-grade water and re-dried at 105 °C to a constant weight (24 h is usually sufficient), cooled in a desiccator, and weighed to the nearest 0.1 mg. The re-wetting step reintroduces waters of hydration to any clay in the sample – without this step water of hydration would erroneously be included with algal mass (APHA 2005). Subtract the final ashed weight of each sample (including filter and pan) from its corresponding dried weight to calculate the AFDM of each sample (mg AFDM/ml filtered). For benthic algae, multiply this figure by the total volume of the slurry then divide by the area sampled to calculate the benthic algal dry mass (mg AFDM/surface area sampled).

Phytoplankton:

$$\begin{aligned}\text{Phytoplankton AFDM } (\text{mg/ml}) = &(\text{dry weight } (\text{mg}) - \text{ash weight } (\text{mg})) \\ &\div \text{volume phytoplankton filtered } (\text{ml})\end{aligned}$$

Benthic Algae or Metaphyton:

$$\begin{aligned}\text{Benthic algal AFDM}\left(\text{mg}/\text{m}^2\right) = {} & (\text{dry weight (mg)} - \text{ash weight (mg)}) \\ & \div \text{volume slurry filtered (ml)} \\ & \times \text{total slurry volume (ml)} \\ & \div \text{total surface area sampled}\left(\text{m}^2\right)\end{aligned}$$

This AFDM analysis procedure conforms to Standard Methods (APHA 2005).

All mass-based estimates of algal biomass simply measure the mass of the sample under consideration, and assume that all the mass is algal in origin. Thus, any non-algal mass is erroneously attributed to algae. Even the best of these procedures (AFDM) includes non-algal organic matter (bacteria, protists, detritus, etc.) in its estimate of algal biomass. To avoid this problem, algal-specific biomass measures may be employed.

1.4.2 Quantitative Pigment Analysis

Quantitative analysis of pigment molecules (generally chlorophyll *a*) is an algal-specific biomass measure. Many different protocols have been developed, each using various organic solvents and instruments to extract and detect chlorophyll (e.g., APHA 2005; Sartory and Grobelaar 1984; Wetzel and Likens 2000). The protocol described below (modified from Biggs and Kilroy 2000; Sartory and Grobelaar 1984) combines the excellent extraction efficiency and low toxicity of ethanol with the ease and affordability of spectrophotometry, while avoiding the need for time-consuming maceration of samples. This technique incorporates an acidification step to correct for phaeopigments, and has been successfully applied in many aquatic systems, including wetlands (Francoeur et al. 2006; Thomas et al. 2009). More elaborate pigment analysis assays exist for use in specialized situations, such as measuring very low amounts of algal biomass and estimating coarse-scale algal community composition via discrimination amongst algal photosynthetic pigments (APHA 2005).

A known volume of phytoplankton sample should be filtered (47-mm glass fiber filter, 0.7 μm pore size) using light vacuum ($\leq$0.3 atm). For metaphyton and benthic algal samples, a homogenous slurry of known volume should be made from all algae sampled from a known area (see Sect. 1.3.3), then a measured subsample of slurry should be filtered. Use only enough material to give the filter some color; filters do not need to be heavily-loaded with algae. Visually inspect each filter and remove any green plant material present. Filters should be folded in half (algae-side in) and placed individually in 15 ml polypropylene centrifuge tubes. Do not use polystyrene tubes, as this polymer will not withstand high temperatures used in subsequent steps. Tap tubes to drive the filters to the bottom. Alternatively, the

slurry-making and filtration steps can be avoided if the substratum upon which benthic algae have grown can be cut into sections of known size and placed (with all algae still attached) directly into tubes. If samples are not to be immediately analyzed, the dry filtered samples may be stored frozen (−10 °C, darkness) in capped centrifuge tubes for 1 month. Samples from acidic waters may degrade during storage, so immediate processing is preferred in such cases (APHA 2005).

Add 5 ml of 90 % ethanol to each tube and loosely re-cap the tubes. If filters are not completely covered by ethanol, either push the filter down or add more ethanol. Place tubes in pre-heated (80 °C) water bath or dry heat block for 5 min to boil ethanol (if using a dry heat block, slightly higher temperatures and extra time may be required as a result of less efficient heat transfer). Remove tubes from heat, tighten caps, and allow tubes to steep in a refrigerator for at least 8 h (4 °C, darkness). Once ethanol has been added to samples, minimize light exposure of samples (e.g., store tubes in darkness, work in low light) to prevent degradation of extracted chlorophyll. Never expose samples to sunlight.

After steeping, any tubes that are dark green in color should be diluted prior to measurement. Assuming 5 ml of ethanol was used for extraction, a 1:2 dilution may be easily accomplished by adding an additional 5 ml of fresh 90 % ethanol to the 5 ml of ethanol already in the vial. Greater dilutions are best made by removing an aliquot of the extractant ethanol and mixing it with fresh 90 % ethanol; for example, a 1:5 dilution may be made by adding a 1 ml aliquot of extractant ethanol to 4 ml of fresh 90 % ethanol. All tubes should be vortexed or shaken to mix ethanol, then centrifuged (4,000–6,000 rpm for 2–5 min) to settle any particles.

Turn on a spectrophotometer (band pass width 1–2 nm, slit width ca. 0.2 nm), and complete warm-up and diagnostics as recommended by manufacturer. This may take over 30 min. Use 90 % ethanol to blank the spectrophotometer at the wavelength of maximal chlorophyll absorbance (usually ~665 nm; scan the absorbance of a chlorophyll extract from 660 to 670 nm and use the maximally-absorbing wavelength for all subsequent measurements; for simplicity, the text will assume that this is 665 nm), and 750 nm, to account for sample turbidity.

Pipette a known volume of sample into a 1-cm-pathlength cuvette and place into the spectrophotometer. Always: (1) hold cuvettes by the frosted edges (not the clear sides), (2) wipe the sides of the cuvette with a lintless laboratory wipe (Kim-wipe or equivalent) before placing in the spectrophotometer, and (3) place the cuvette into the spectrophotometer in the same orientation each time. Read and record the absorbance at 665 and 750 nm (a665 and a750). The a665 should be <1. If a665 is >1, then dilute the sample with additional 90 % ethanol and re-read. If a750 > 0.02, then particulate matter is likely still in suspension. Recentrifuge the sample and re-read.

After initial a665 and a750 readings are complete, add 0.3 M HCl to the cuvette, wait 30 s, and re-read and record a665 and a750. The volume of acid added should be ~1/40th the volume of ethanol in the cuvette. For example, for a standard cuvette holding 4 ml of ethanol, add 0.1 ml of HCl. Do not over-acidify the samples. If using re-usable cuvettes, be sure to rinse the cuvette with 90 % ethanol and tap it dry before re-using it for another sample. All traces of the acid and the previous sample must be removed during the rinsing process.

Continue this procedure until all samples are measured. Be sure to re-blank the spectrophotometer when necessary. The amount of chlorophyll can be calculated using the following equations. Note that all volumes are in liters (including ethanol volumes), and that ethanol volumes must be corrected for dilutions. The effective ethanol volume is simply the volume of ethanol placed in the extraction tube multiplied by the dilution of the extract (if any). For example, if a sample was extracted using 5 ml of ethanol in the tube, and the extract was diluted 1:3 prior to measuring absorbance, then the ethanol volume is 15 ml (i.e., 0.015 L).

Phytoplankton:

$$\text{Chlorophyll } a \text{ (mg/sample)} = (A_{\text{before}} - A_{\text{after}}) \times 28.66 \times \text{ethanol volume}$$

$$\text{Chlorophyll } a \text{ (mg/L)} = \text{chlorophyll } a \text{ (mg/sample)} \div \text{volume of water filtered (L)}$$

Benthic Algae or Metaphyton:

$$\text{Chlorophyll } a \text{ (mg/sample)} = [(A_{\text{before}} - A_{\text{after}}) \times 28.66 \times \text{slurry volume} \times \text{ethanol volume}] \div \text{volume slurry filtered}$$

$$\text{Chlorophyll } a \left(\text{mg/m}^2\right) = \text{chlorophyll } a \text{ (mg/sample)} \div \text{area of substratum sampled } \left(\text{m}^2\right)$$

Note:

A_{before} = a665 before acidification – a750 before acidification
A_{after} = a665 after acidification – a750 after acidification

The absorption coefficient for chlorophyll a (based on absorption coefficient of 83.4 g/L/cm and an acid ratio of 1.72, see Sartory and Grobelaar (1984) is 28.66). If substrata (with attached benthic algae) are extracted directly, then both the "slurry volume" and "volume slurry filtered" terms become 1.

Green plant tissue contains chlorophyll; if this material is not excluded from the samples, algal biomass can be greatly overestimated. In addition to assuming that all chlorophyll is of algal origin, chlorophyll-based biomass estimates assume that all algal taxa have the same invariant biomass to chlorophyll ratio. This assumption is not strictly true. For example, algal chlorophyll to biomass ratios are dependent on nutrient content and light exposure (e.g., Hessen et al. 2002; Riemann

et al. 1989). Variability in chlorophyll to biomass ratios is usually ignored, but caution is required in some situations. Typically, chlorophyll is 1–2 % of total algal dry weight (APHA 2005).

1.4.3 Biovolume

Biovolume quantification is the most time-consuming algal biomass assay. However, some consider it to be the most accurate measure (Stevenson 1996), and it is the only method that allows measurements of the biomass of individual algal species within communities.

To conduct a biovolume assay, first conduct absolute abundance enumerations (see Sect. 1.3.2) on all samples. In addition, the relevant dimensions of at least ten individuals of each algal taxon must be measured. Measurements may be accomplished by use of either a calibrated ocular micrometer or calibrated digital images. To calibrate an ocular micrometer, observe a fine-scale stage micrometer (10 μm gradations) through the microscope, and note the mathematical relation between the scale of the ocular micrometer and the known distances shown on the stage micrometer (Fig. 1.7). Repeat the calibration process for each magnification. To calibrate digital images, obtain a high-resolution image of the stage micrometer using the microscope imaging system, and determine the relation between the number of linear pixels in the image and the known distances shown on the stage micrometer. Repeat the calibration process for each magnification, and be sure to capture all calibration and data images using identical capture conditions (e.g., identical resolution and image size).

It is most efficient to conduct ocular measurements or capture digital images simultaneously with enumerations. The dimensions measured for each taxon will depend on the taxon's shape and the formula for the volume of the relevant geometric solid or combination of solids that approximate the shape of the algal cell. Wetzel and Likens (2000) present a short list of applicable geometric formulae for several appropriate shapes (e.g., sphere, cylinder, cone, elongated cube, irregular solids). Much more comprehensive lists can be found in Hillebrand et al. (1999) and Sun and Liu (2003). Alternatively, if all taxa recorded during the enumerations are included in an existing algal biovolume database (e.g., http://diatom.acnatsci.org/nawqa/2001biovol.asp), one may use these values. In either case, one simply multiplies the mean cell-specific biovolume of each taxon by the absolute abundance of that taxon in an individual sample to calculate its biovolume in that sample. This process is repeated for all taxa, and the sum over all taxa is the total algal biovolume for a particular sample. Spreadsheet programs can be used to make these calculations, and specialized biovolume calculation software is also available (e.g., BIOVOL; https://www.msu.edu/~kirschte/biovol/).

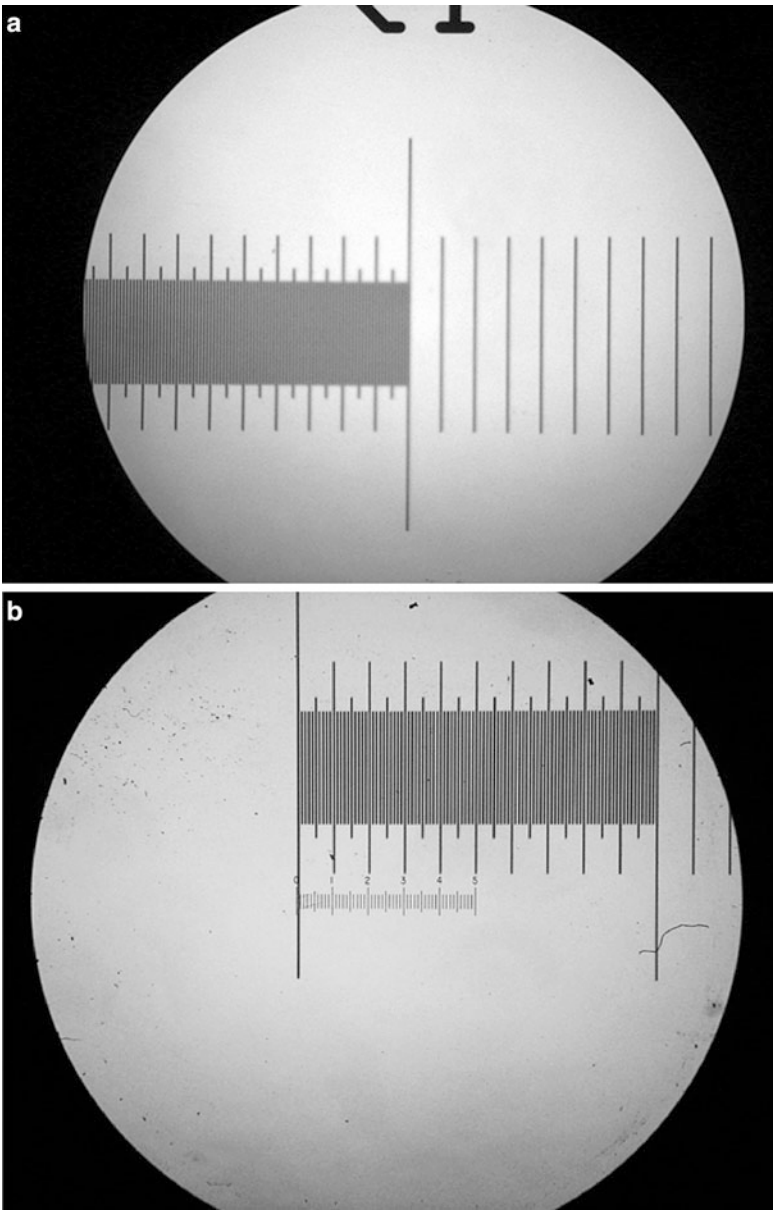

Fig. 1.7 Calibration of field diameter (**a**) and ocular micrometer scale (**b**) with a stage micrometer

Phytoplankton:

$$\text{Biovolume of taxon } \left(\mu m^3/L\right) = \text{mean cell specific-biovolume of taxon } \left(\mu m^3/\text{cell}\right) \times \text{absolute abundance of taxon } (\text{cells}/L)$$

$$\text{Total sample biovolume } \left(\mu m^3/L\right) = \Sigma\, \text{Biovolume of each taxon in sample } \left(\mu m^3/L\right)$$

Benthic Algae or Metaphyton:

$$\text{Biovolume of taxon } \left(\mu m^3/m^2\right) = \text{mean cell specific-biovolume of taxon } \left(\mu m^3/\text{cell}\right) \times \text{absolute abundance of taxon } \left(\text{cells}/m^2\right)$$

$$\text{Total sample biovolume } \left(\mu m^3/m^2\right) = \Sigma \text{ Biovolume of each taxon in sample } \left(\mu m^3/m^2\right)$$

It is possible to measure and subtract non-metabolic volumes (e.g., vacuole volume) from the cell-specific biovolume figure for each taxon. In theory, this improves the relation between biovolume and actual biomass of algae (Stevenson 1996); however, this is rarely done, due to the additional effort required to make such measurements.

1.5 Productivity and Photosynthetic Activity

Production is the flux of mass or energy in a defined location over time (Wetzel 2001). Algae can exhibit heterotrophy or mixotrophy (Tuchman et al. 2006), but algae are predominantly photoautotrophs, so algal production is often considered to be synonymous with primary production (i.e., the quantity of new organic matter or fixed energy generated via photosynthesis). Thus, algal production is generally tightly related to various measures of algal photosynthetic activity. Gross primary production (GPP) is the total amount of new organic matter produced or energy fixed over a specific interval, whereas net primary production (NPP) is net accumulation of organic matter or energy over time (i.e., GPP minus all losses, such as respiration).

1.5.1 Biomass-Based Methods

Algae exhibit high rates of growth, mortality, immigration, and emigration. Algae may also lose relatively large amounts of photosynthate (5–50 % of total incorporated carbon, see Jones and Cannon 1986) as dissolved organic carbon. Thus, unlike large, slow-growing, sessile, infrequently-grazed wetland primary producers such as emergent macrophytes, change in algal biomass through time is a poor measure of algal production, and is not generally recommended. Some errors can be reduced by measuring biomass dynamics over very short (1–3 day) intervals, but even so, production assays more directly related to algal photosynthesis are superior.

1.5.2 Oxygen-Based Methods

Oxygen dynamics present a moderately sensitive tool for measurement of algal photosynthesis and community respiration, thereby allowing calculation of both GPP and NPP. The net photosynthetic equation $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$ illustrates how oxygen production and inorganic C incorporation are linked to photosynthesis. A wide variety of whole-system and chamber-based assays have been developed, using several different oxygen measurement techniques (see APHA 2005; Bott 1996). The method related here employs chambers and instrument-based oxygen analysis. The general protocol is to isolate algae in chambers, incubate under light and dark conditions, and measure production and consumption of oxygen. Due to the inclusion of both algae and heterotrophic organisms (e.g., bacteria, zooplankton) in bottles, respiration measurements will reflect the entire community, not just algal respiration. In addition, light-dark bottle incubations cannot account for photorespiration. Methods for scaling-up short-term chamber-based assays to estimate total daily production will also be discussed.

1.5.2.1 Phytoplankton

This assay is based upon the phytoplankton protocol of Wetzel and Likens (2000). For shallow, well-mixed surface waters of most wetlands, collection of phytoplankton from a single depth (or via a depth-integrated sample) and incubation of chambers at the mean water column depth should be sufficient. If the water column is deep or stratified, then water should be collected (using a non-metallic Van Dorn bottle) and incubated at discrete depths. For the most accurate measures of whole-wetland phytoplankton production, conduct incubations at several sites within the wetland. This is especially important if some areas have different environmental or biological conditions. In such cases, chamber-based production figures should be weighted by the size of the differing areas to calculate accurate whole-system production estimates.

Prepare and calibrate an oxygen meter according to manufacturer's instructions. The oxygen meter probe must be small enough to fit inside the neck of incubation bottles. Galvanic and Clark-type probes consume oxygen and can be troublesome to calibrate and maintain. Galvanic probes consume oxygen rapidly enough to require stirring for accurate measurements and can artificially deplete oxygen levels in small incubation chambers if used continuously. Optical probes lack these shortcomings, but are more expensive.

Collect water (using non-metallic sampling equipment) and measure oxygen concentration. If the oxygen concentration is near saturation, bubble compressed nitrogen gas through the water to lower oxygen levels to ~80 % saturation. From this point on, work in subdued light, if possible, to limit photosynthesis.

Obtain glass incubation bottles of ~300 ml volume. Bottles should have ground-glass stoppers with tapered tips, to allow both a tight seal and closure without

Fig. 1.8 Light and dark bottles for photosynthesis measurements

trapping air bubbles. Commonly-available BOD (biological oxygen demand) bottles work well (Fig. 1.8). Bottles must be scrupulously clean (acid-washed and washed with phosphate-free detergent). If UV inhibition of algal photosynthesis is of concern, then bottles must be UV-transparent quartz glass. In shallow, clear waters, UV inhibition of algal photosynthesis can be significant (e.g., Belay 1981; Helbling et al. 2001). If UV effects can be ignored, then standard laboratory-quality glass is adequate, and much more economical. Dark bottles can be produced by wrapping clear bottles in two layers of black electrical tape. All bottles should have clips attached to their necks.

Gently fill three (two clear, one black) incubation bottles with water from a common vessel via a tube run to the bottom of each bottle. Flush each bottle with water for three times as long as it took to fill initially. Do not splash or agitate the water during the filling process; bottles must not contain bubbles. Immediately cap the dark bottle and one clear bottle. Avoid trapping bubbles during the capping process by overfilling the bottles, and penetrating the water's surface with the tapered tip of cap. Done correctly, excess water will flow down the outside of the bottle, and no bubbles will be trapped. Wrap aluminum foil over the cap of the dark bottle to ensure complete light exclusion. Measure the oxygen concentration in the remaining uncapped clear bottle – this will be the initial bottle (IB) measurement. Alternatively, if the oxygen probe is sufficiently small (i.e., when inserted into the bottle it displaces so little water that the bottle can later be capped without including bubbles), then dispense with the third bottle (the IB), and simply measure the initial oxygen concentrations directly in the dark and light bottles before they are capped. Using a marked, weighted, buoyed line, submerge the two capped bottles to the

desired depth. To avoid bottles shading each other, bottles should be clipped to the ends of a spreader bar (a rod with eyebolts at each end), which is attached at its center to the buoy line. If shading of bottles by a single overhead buoy is significant, the line can be attached to the center of a rod (~2–3 m long), each end of which is floated by a buoy. Incubation time begins when bottles reach the desired depth. Two to 4 h of incubation is typically sufficient and may be increased or decreased in low or high production environments. If oxygen becomes supersaturated (bubbles form) within the clear bottle, or if oxygen is totally depleted from the dark bottle, the incubation was too long. At the end of the incubation, retrieve the bottles, record the time, and immediately measure the oxygen concentrations in each bottle. These will be the dark bottle (DB) and light bottle (LB) measurements. The following formulae can be used to calculate respiratory activity, net photosynthesis, and gross photosynthetic activity. Alternatively, gross photosynthetic activity can be calculated as the sum of net photosynthesis and respiratory activity.

$$\text{Respiration } (\text{mg } O_2/\text{L/h}) = (\text{IB } (\text{mg } O_2/\text{L}) - \text{DB } (\text{mg } O_2/\text{L})) \div \text{incubation interval(h)}$$

$$\text{Net photosynthesis } (\text{mg } O_2/\text{L/h}) = (\text{LB } (\text{mg } O_2/\text{L}) - \text{IB } (\text{mg } O_2/\text{L})) \div \text{incubation interval(h)}$$

$$\text{Gross photosynthesis } (\text{mg } O_2/\text{L/h}) = (\text{LB } (\text{mg } O_2/\text{L}) - \text{DB } (\text{mg } O_2/\text{L})) \div \text{incubation interval (h)}$$

The following formulae are used to express production and respiration in carbon units, assuming a typical respiratory quotient of 1.0 and a typical photosynthetic quotient of 1.2, and employing conversion factors of 12 mg C : 32 mg O_2 (i.e., 0.375) and 1,000 L to 1 m^3.

$$\text{Respiration } \left(\text{mg C}/\text{m}^3/\text{h}\right) = [(\text{IB } (\text{mg } O_2/\text{L}) - \text{DB } (\text{mg } O_2/\text{L})) \times 1{,}000 \times 0.375] \div \text{incubation interval(h)}$$

$$\text{Net photosynthesis } \left(\text{mg C}/\text{m}^3/\text{h}\right) = [(\text{LB } (\text{mg } O_2/\text{L}) - \text{IB } (\text{mg } O_2/\text{L})) \times 1{,}000 \times 0.375] \div (1.2 \times \text{incubation interval (h)})$$

$$\text{Gross photosynthesis } \left(\text{mg C}/\text{m}^3/\text{h}\right) = [(\text{LB } (\text{mg } O_2/\text{L}) - \text{DB } (\text{mg } O_2/\text{L})) \times 1{,}000 \times 0.375] \div (1.2 \times \text{incubation interval (h)})$$

To express these parameters on an areal basis (i.e., planktonic respiration or production in a 1 m^2 vertical column of wetland water), multiply the photosynthetic or respiratory rate (mg C/m^3/h) by the water depth (m). If incubations have been conducted at multiple depths, calculate the area-specific rates individually for each depth stratum by multiplying the incubation results from a particular depth stratum by the water column height of that stratum, then sum over all strata.

For example, the following equation would be used for a 3 m-deep wetland pool, where gross photosynthesis has been measured in the 0–1 m depth stratum and the 1–3 m depth stratum.

$$\begin{aligned}\text{Gross photosynthesis}\left(\text{mg C/m}^2\text{/h}\right) = {} & \text{Gross photosynthesis}_{0-1\,\text{m}}\left(\text{mg C/m}^3\text{/h}\right) \times 1\ \text{m} \\ & + \text{Gross photosynthesis}_{1-3\,\text{m}}\left(\text{mg C/m}^3\text{/h}\right) \times 2\ \text{m}\end{aligned}$$

Photosynthetic and respiratory parameters may also be expressed on a biomass-specific basis. To do this, collect phytoplankton samples for biomass analysis (see Sect. 1.4) concurrently with sample collection for the production assay, then multiply volume-based photosynthesis or respiration results by the volume-specific phytoplankton biomass for that assay. If expressing photosynthesis in mg C/m^3/h, recall that there are 1,000 L in 1 m^3.

If daily production (i.e., production over a 24 h cycle) is desired, it is best to conduct sequential incubations, beginning at sunrise and continuing until sunset, and sum the results. If this labor-intensive effort is not possible, then irradiance measurements (μmol photons/m^2/s) should be made at frequent intervals from sunrise to sunset (an automated sensor/datalogger is of great value here), and a single incubation can be conducted near mid-day (~10:00–14:00). The relation between the measured production and the irradiance during the incubation can be used to estimate production for other periods of the day, in which production is unknown, but irradiance was measured. For example, gross photosynthesis during the period 06:00 until 10:00 h can be estimated from measured values of gross photosynthesis during the period 10:00 until 14:00, mean irradiance during the period 10:00 until 14:00 and mean irradiance during the period 06:00 until 10:00.

$$\begin{aligned}&\text{Gross photosynthesis}\left(\text{mg C/m}^3\text{/h}\right)_{06:00-10:00} \\ &= \left(\text{Gross photosynthesis}\left(\text{mg C/m}^3\text{/h}\right)_{10:00-14:00} \div \text{mean irradiance}_{10:00-14:00}\right) \\ &\quad \times \text{mean irradiance}_{06:00-10:00}\end{aligned}$$

This time-integration method assumes that phytoplankton photosynthesis is perfectly proportional to irradiance. This assumption can be violated in shallow waters due to photoinhibition, and in nutrient-poor waters due to nutrient limitation.

1.5.2.2 Metaphyton

Metaphytic photosynthetic activity can be measured using the same equipment and techniques as phytoplankton, with the exception that small subsamples of metaphyton should be added to each bottle (e.g., Scott et al. 2007). A separate set of phytoplankton bottles should also be incubated, and their values subtracted from the metaphyton bottles to correct for respiration or production of phytoplankton

included in the metaphyton bottles. Alternatively, the water can be filtered (0.7 μm pore size glass fiber filter) to remove phytoplankton before filling bottles. Because metaphyton subsamples can vary substantially in biomass, the biomass of metaphyton subsamples in the incubation bottles should be measured after the production assay (see Sect. 1.4), and all terms expressed on a biomass-specific basis. Because incubation bottles vary in volume, the volume of each bottle should be individually measured and the exact bottle volume used in conversions of volume-specific to biomass-specific units. Separate quantitative metaphyton biomass sampling (see Sects. 1.2 and 1.4) can establish metaphyton biomass in the wetland, and these values can be used to convert biomass-specific production measurements from bottle incubations to area-specific (m^2 of wetland) values. The following equations present example calculations for correcting for gross photosynthesis of phytoplankton (if water was not filtered prior to filling bottles), and for converting biomass-specific metaphytic gross photosynthesis to area-specific metaphytic gross photosynthesis.

$$\begin{aligned}&\text{Gross photosynthesis}_{\text{metaphyton}}\ (\text{mg } O_2/\text{L/hr}) = \\ &\quad [(\text{LB}_{\text{metaphyton}}\ (\text{mg } O_2/\text{L}) - \text{DB}_{\text{metaphyton}}\ (\text{mg } O_2/\text{L})) \\ &\quad - (\text{LB}_{\text{phytoplankton}}(\text{mg } O_2/\text{L}) - \text{DB}_{\text{phytoplankton}}\ (\text{mg } O_2/\text{L}))] \div \text{incubation interval (h)}\end{aligned}$$

$$\begin{aligned}&\text{Gross photosynthesis}\ \left(\text{mg C}/\text{m}^2/\text{h}\right) = \\ &\quad \text{gross photosynthesis (mg C/mg metaphyton/chl a/h)} \\ &\quad \times \text{metaphyton biomass}\ \left(\text{mg chl a}/\text{m}^2\right)\end{aligned}$$

Full daily estimates of metaphytic photosynthesis and production can be produced in the same manner as for phytoplankton.

1.5.2.3 Benthic Algae

Benthic algal production can be measured in a similar fashion, using light and dark chambers instead of bottles (APHA 2005). Chambers should be sized appropriately for the substratum, incubation time, and level of oxygen production or consumption. Chambers approximately 20 cm in diameter and 30 cm high are often recommended (APHA 2005), but smaller chambers have been used successfully. Chambers should have a lateral port, sealed with a resealable stopper for insertion of an oxygen probe, or a septum suitable for piercing with a needle-type probe. Recently developed optical sensing technology (non-invasive oxygen sensors; Presens, or equivalent) could perhaps be used measure oxygen directly through the clear chamber wall, thereby eliminating the need for a potentially-leaky sensor port. The chamber should also include some means of mixing the water prior to measurement. A small, manually operated, propeller-shaped stirring paddle is traditional, but simply including a magnetic stir bar inside the chamber allows

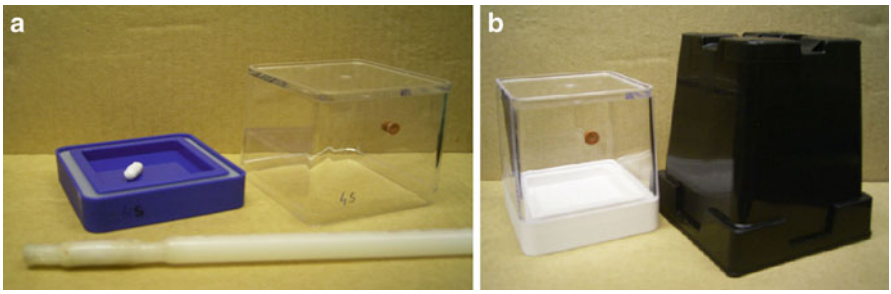

Fig. 1.9 Benthic incubation chambers with magnetic stirring system (**a**) and removable dark cover (**b**)

for mixing with a magnetic wand held outside the chamber, and eliminates the need for an additional hole (and potential leak point) in the chamber wall. Figure 1.9 shows a smaller, fully-enclosable chamber with septum port, magnetic stirring system, and removable dark cover. Since most wetlands do not have appreciable water movement, this static water chamber design is suitable. In lotic wetlands, a pump-driven recirculating chamber design (e.g., Biggs and Kilroy 2000) should be used to replicate the *in-situ* current. If phytoplankton activity is likely to introduce substantial error in estimates of benthic algal activity, then a separate set of phytoplankton bottles should also be incubated, and their values subtracted from the benthic algal results to correct for phytoplankton included in the benthic algal chambers.

Benthic algae on hard substrata can be gently placed into a fully-enclosable chamber (i.e., a chamber in which the top and bottom can be sealed together to completely enclose the algae) and chambers incubated submerged on the bottom of the wetland. Benthic algae on soft substrata may require use of open-bottom chambers, which are pressed into the sediments. Make sure no bubbles are captured in the chambers. Measure oxygen concentrations in the chambers at the beginning and end of the incubation period, stirring to ensure that the water within the chamber is fully mixed immediately prior to measurement. Typical incubation times range from 2 to 6 h. Respiration and photosynthesis are calculated as for phytoplankton, with the exceptions that the initial oxygen measurement of the individual chamber replaces the initial bottle oxygen measurement. One may either employ separate light and dark chambers for simultaneous respiration and production measurements (analogous to the phytoplankton light and dark bottles), or employ a single clear chamber for sequential respiration and production measurements. When using a single chamber for sequential measurements, cover the clear chamber with a removable dark cover for the respiration incubation, then remove the cover and conduct the light incubation. A single oxygen measurement between the dark and light incubation is sufficient as both a final value for the respiration incubation and an initial value for the production incubation. Although sequential incubations increase the total time needed to make a complete production and respiration measurement, they avoid complications caused by benthic algal biomass

differing between light and dark chambers, and can eliminate the need to bubble chamber water with nitrogen gas to reduce oxygen concentrations. Volume-specific measurements of respiration and production should be converted to area-specific units by measuring the volume of water enclosed in each chamber after the incubation (in fully-enclosed chambers be sure to account for the volume of occupied by benthic algae and substrata; volume measurements of open bottom chambers are facilitated by choosing areas of level sediments and pressing the chamber a known distance into the sediments) and measuring the area of benthic algae contained in the chamber, then using this factor to convert volume-specific units to area-specific units. For example, consider the conversion of volume-specific net photosynthesis to area-specific net photosynthesis, for a chamber with a water volume of 6.28 L, covering a benthic algal area of 0.0314 m^2 (a cylindrical open-bottom chamber 20 cm diameter and 20 cm tall after being seated in the sediments). Recall that there are 1,000 L in 1 m^3.

$$\text{Net photosynthesis } \left(\text{mg C}/\text{m}^2/\text{h}\right) = \text{Net photosynthesis } \left(\text{mg C}/\text{m}^3/\text{h}\right) \times 6.28 \text{ (L)} \div 1,000 \div 0.0314 \left(\text{m}^2\right)$$

Measurement of benthic algal biomass within the chamber (see Sect. 1.4) can provide the information needed for converting area-specific units to biomass-specific units. The following equation presents an example calculation for converting area-specific benthic algae gross photosynthesis to biomass-specific benthic algal gross photosynthesis.

$$\text{Gross photosynthesis (mg C/mg benthic algal chl a/h)} = \text{Gross photosynthesis } \left(\text{mg C}/\text{m}^2/\text{h}\right) \div \text{benthic algal biomass } \left(\text{mg chl a}/\text{m}^2\right)$$

Full daily estimates of benthic algal photosynthesis and production can be produced in the same manner as for phytoplankton.

1.5.3 ^{14}C-Based Methods

Radiocarbon uptake can be used in place of oxygen production for measurement of algal photosynthesis. ^{14}C-based photosynthesis measurements are considerably more sensitive than oxygen-based methods, and are thus are superior to oxygen-based techniques when algal biomass is low, incubation times must be kept short, or slight changes to photosynthetic activity are of interest. *In-situ* ^{14}C incubations in sealed containers can be conducted for measuring *in-situ* algal production in a manner similar to that previously described for oxygen-based methods in section 1.5.2 (see APHA 2005; Wetzel and Likens 2000). However, concerns about potential release of radioactive material into the environment have caused some

institutions to discourage use of *in-situ* ^{14}C incubations. The procedure outlined here describes laboratory-based ^{14}C incubations, suitable for investigating photosynthetic responses of algae from circumneutral or alkaline wetlands to altered environmental conditions (e.g., nutrients, pollution).

1.5.3.1 Phytoplankton

Sample phytoplankton as described for the oxygen-based method. Collect an additional water sample for measurement of the pH and alkalinity of wetland water by completely filling a screw-cap opaque polyethylene bottle. Place samples in a cooler, and transport to laboratory.

Place 130 ml of phytoplankton sample into a sealable clear bottle and a sealable dark bottle. Add 1.0 ml of $NaH^{14}CO_3$ solution (total activity added 74–185 kilobecquerels [kBq]) and record time of addition to the nearest minute. Cap bottles, swirl to mix radiolabel, and place in plant growth chamber set to wetland surface water temperature, with saturating light intensity (recommend 400 μmol/m^2/s). Incubate up to 4 h, record the incubation end time to the nearest minute, and place all samples in complete darkness to stop any photosynthesis. Immediately filter the contents of each bottle onto a 25 mm diameter, 0.45 μm pore-size mixed cellulose ester membrane filter (Millipore HA or equivalent). Do not exceed a vacuum pressure of 0.3 atm (228 mmHg). Dry filters in a desiccator. Place a sealable container (Tupperware or equivalent) into a functioning radioisotope-approved fume hood. Add a small dish of concentrated HCl and (if desired) a small dish of CO_2 sorbent to the container and close the lid. Expose filters to HCl fumes for 10 min to convert residual inorganic ^{14}C on the filters to $^{14}CO_2$ gas, then open the container while still in the fume hood. Wait 5 min, then remove filters.

Place individual filters into 20 ml scintillation vials. The filter should be positioned flat on the bottom of the vial, algae-side up. Add 10 ml of a water-, salt-, base-tolerant low-toxicity scintillation fluor (EcoLume, MP Biomedical, or equivalent) to each vial, let sit at least 8 h (or longer, until vial contents are clear), then count on scintillation counter, using internal standards to automatically correct for counting efficiency and report results as disintegrations per minute (DPM).

Measure temperature, pH, and alkalinity of water sample as soon as possible, ideally during phytoplankton incubation. Measure alkalinity by placing a known amount of water (recommend 50 ml) in a beaker and titrating with acid of precisely-known strength (usually 0.1 or 0.2 N) until a pre-selected pH endpoint is reached (APHA 2005).

If the initial pH of the water is greater than 8.3, first titrate to a pH of 8.3, and calculate the "phenolphthalein alkalinity" using the following formula:

$$\text{Alkalinity}\,(\text{mg}\,CaCO_3/\text{L}) = (\text{volume acid used(ml)} \times \text{normality of acid} \times 50{,}000) \div \text{volume of water titrated(ml)}$$

Measure the total alkalinity of the sample by titrating to a lower pH endpoint, then applying the same formula. The total alkalinity acid volume is total amount of acid required to reach the low pH endpoint, and thus includes the acid used in the phenolphthalein alkalinity titration (if conducted). For routine analysis, use a lower pH endpoint of 4.5. For more accurate alkalinity measurements, select the pH endpoint based on the alkalinity of the water; in waters of ~30 mg $CaCO_3$/L use an endpoint of 4.9, in waters of ~150 mg $CaCO_3$/L use an endpoint of 4.6, and in waters of ~500 mg $CaCO_3$/L use an endpoint of 4.3. For very soft waters (>20 mg $CaCO_3$/L), see APHA (2005) for an appropriate alkalinity assay and calculations.

Apply the following formula to calculate the amount of ^{12}C available in the water:

$$\begin{aligned} {}^{12}\text{C content of water (mg } {}^{12}\text{C/L)} = {} & (\text{total alkalinity (mg } CaCO_3\text{/L)} \\ & - \text{ phenolphthalein alkalinity (mg } CaCO_3\text{/L))} \\ & \times 0.240 \end{aligned}$$

Calculate the amount of ^{14}C available within each phytoplankton bottle by applying the following formula (note 1 kBq = 60,000 DPM):

$$^{14}\text{C available (DPM/bottle)} = {}^{14}\text{C activity added to each bottle (kBq)} \times 60,000$$

Calculate the amount of ^{14}C assimilated by each light-exposed phytoplankton sample by subtracting background and correcting for isotopic fractionation by applying the following formula:

$$^{14}\text{C assimilated (DPM/bottle)} = \left(\text{DPM}_{\text{light bottle}} - \text{DPM}_{\text{dark bottle}}\right) \times 1.06$$

If only a portion of each bottle was filtered, then apply the following formula instead:

$$\begin{aligned} {}^{14}\text{C assimilated (DPM/bottle)} = {} & \{[\text{DPM}_{\text{light bottle}} \times (\text{total volume}_{\text{light bottle}} \\ & \div \text{ volume filtered}_{\text{light bottle}})] \\ & - [\text{DPM}_{\text{dark bottle}} \times (\text{total volume}_{\text{dark bottle}} \\ & \div \text{ volume filtered}_{\text{dark bottle}})]\} \times 1.06 \end{aligned}$$

The rate of ^{12}C assimilation by algae is then calculated as follows. Recall that there are 1,000 L in 1 m^3.

$$\begin{aligned} {}^{12}\text{C assimilated (mg } {}^{12}\text{C/m}^3\text{/h)} = {} & ({}^{12}\text{C content of water (mg } {}^{12}\text{C/L)} \\ & \times {}^{14}\text{C assimilated (DPM/bottle.)} \times 1,000) \\ & \div {}^{14}\text{C available (DPM/bottle.)} \\ & \div \text{incubation interval (h)} \end{aligned}$$

This figure can also be expressed on an areal or biomass-specific basis, in the same way as was discussed for oxygen-based photosynthesis measurements.

If the total rate of photosynthetic C-fixation is of interest, then one must measure both the amount of C assimilated by algae and retained in biomass, and the amount of C fixed by algae and lost into the water as soluble photosynthate. To quantify the amount of soluble photosynthate lost during the incubation, collect the individual filtrates from the post-incubation filtration of each sample. For each filtrate, measure the volume, and place a 6 ml aliquot of the filtrate into a scintillation vial. Place vials into a functioning radioisotope-approved fume hood, then acidify to pH 3 with 3 % H_3PO_4 and gently sparge for 4 min with CO_2. Add 13 ml of a water-, salt-, base-tolerant low-toxicity scintillation fluor (EcoLume, MP Biomedical, or equivalent) to each vial, let sit at least 8 h (or longer, until vial contents are clear), and count on a scintillation counter as described above. Apply the same equations used for calculating C assimilation to calculate the rate of photosynthetically-fixed C lost into the water as soluble photosynthate. Be sure to account for only counting a fraction of the filtrate when calculating ^{14}C assimilation. The total rate of photosynthetic C fixation (mg $^{12}C/m^3/h$) is the sum of the ^{12}C fixed and assimilated into cells and the ^{12}C fixed and lost into solution.

1.5.3.2 Metaphyton

Metaphytic photosynthesis can be measured in a similar manner. Collect metaphyton and wetland water and return to the laboratory. Place small (recommend ~1 cm^3) subsamples of metaphyton into a clear 20 ml glass scintillation vial and a dark scintillation vial. Add 4.9–14.7 ml of filtered (0.7 μm pore size glass fiber filter) wetland water to each vial. For each 4.9 ml of wetland water in the vial, add 0.1 ml of $NaH^{14}CO_3$ solution (total activity added 7.4–55.5 kBq). Cap vials, record time of addition and swirl to mix. Place vials on their side, and incubate 2 h in a plant growth chamber, as above. At the end of the incubation, record the time, and place all samples in complete darkness to stop any photosynthesis. Filter onto pre-weighed, 25 mm diameter, 0.45 μm pore size mixed cellulose ester filters (rinse vials once with distilled water to remove any adherent algae), and dry in a desiccator. Because metaphyton subsamples can vary substantially in biomass, weigh the dried samples to determine the dry mass of the metaphyton subsample in each incubation vial. If filters contain only small, evenly distributed amounts of metaphyton, fume and radioassay samples as described for phytoplankton. If filters contain large clumps of metaphyton, then self-quenching and color quenching may cause inaccuracies in the radioassay. Fume, digest, decolorize, and radioassay these filters using the procedure described below for benthic algae. Measure wetland water alkalinity as previously described, and apply the formulae listed above for phytoplankton. Modify the ^{14}C assimilation calculation as follows, to correct for differences in metaphyton biomass between light and dark bottles:

$$\begin{aligned} {}^{14}\text{C assimilated}(\text{DPM/bottle}) = &\left[\left(\text{DPM}_{\text{light bottle}} \div \text{dry mass}_{\text{light bottle}}\right)\right. \\ &\left. - \left(\text{DPM}_{\text{dark bottle}} \div \text{dry mass}_{\text{dark bottle}}\right)\right] \\ &\times \text{dry mass}_{\text{light bottle}} \times 1.06 \end{aligned}$$

The total amount of ^{12}C assimilated by metaphyton is then calculated as follows:

$$\begin{aligned} {}^{12}\text{C assimilated } \left(\text{mg } {}^{12}\text{C/mg dry mass/h}\right) = {}& {}^{12}\text{C content of water } \left(\text{mg } {}^{12}\text{C/L}\right) \\ & \times \text{incubation volume (L)} \\ & \times {}^{14}\text{C assimilated (DPM/bottle)} \\ & \div {}^{14}\text{C available (DPM/bottle)} \\ & \div \text{dry mass}_{\text{light bottle}} \text{ (mg)} \\ & \div \text{incubation interval (h)} \end{aligned}$$

If expression in biomass-specific units other than dry mass (e.g., per unit chlorophyll, or per unit AFDM) are desired, create a conversion factor by measuring dry mass and other biomass parameters on separate samples of metaphyton collected at the same time and location as those used in the production assay, then multiply dry mass-specific C assimilation values by this conversion factor. For example, to convert to chlorophyll-specific C assimilation, use the following formula.

$$\begin{aligned} {}^{12}\text{C assimilated } \left(\text{mg}{}^{12}\text{C/mg chl } a\text{/h}\right) = {}& {}^{12}\text{C assimilated } \left(\text{mg } {}^{12}\text{C/mg dry mass/h}\right) \\ & \times \text{metaphyton dry mass to chl } a \text{ ratio} \\ & \text{(mg dry mass/mg chl } a\text{)} \end{aligned}$$

If the total rate of photosynthetic C fixation C is of interest, then one can determine the amount of C fixed by algae and lost into the water as soluble photosynthate as described for phytoplankton.

1.5.3.3 Benthic Algae

Benthic algal photosynthesis is measured in a similar manner. Collect benthic algae and wetland water and return to the laboratory. Do not disrupt the benthic algal community. If algae are growing on small substrata or substrata that can be cut into small sections (e.g., plant litter), place small (recommend ~2 cm^2) subsamples of known area into a clear 20 ml glass scintillation vial and a dark scintillation vial. Add 4.9–14.7 ml of filtered (0.7 μm pore size glass fiber filter) wetland water to each vial. For each 4.9 ml of wetland water placed in the vial, add 0.1 ml of $NaH^{14}CO_3$ solution (total activity added 7.4–55.5 kBq). Cap vials, record time of addition and swirl to mix. Place vials on their side, and incubate 2 h in a plant growth chamber, as above. At the end of the incubation, record the time. Place all samples in complete darkness to stop any photosynthesis. Filter algae and substratum onto 25 mm diameter, 0.45 μm pore size mixed cellulose ester filters (rinse vials once with distilled water to remove any adherent algae) and dry in a desiccator. Fume the entire sample mass (i.e., algae, filter, and substratum) as described above for phytoplankton. Place the entire sample mass into a 15 ml polyethylene centrifuge tube, add 5 ml of 0.5 M NaOH, and heat at 80 °C for 1 h

in a dry heat block to dissolve algae and filter. Do not use polystyrene centrifuge tubes, as they will melt. Let tubes sit overnight after heating. Place 100 µl of the resulting alkaline digest into glass scintillation vial, add an equal volume of fresh 30 % H_2O_2 to remove any color, and let sit for 24 h. Add 10 ml of a water-, salt-, base-tolerant low-toxicity scintillation fluor (EcoLume, MP Biomedical, or equivalent) to each vial, let sit at least 8 h (or longer, until vial contents are clear), then count on scintillation counter, using internal standards to automatically correct for counting efficiency and report results as disintegrations per minute (DPM). If scintillation counts are too low, scintillation vials may be re-made using greater volumes of alkaline digest. Measure wetland water alkalinity and calculate ^{12}C content of water (mg ^{12}C L^{-1}), ^{14}C availability (DPM $bottle^{-1}$), and ^{14}C assimilation (DPM $bottle^{-1}$) as previously described for phytoplankton.

The total amount of ^{12}C assimilated by benthic algae is then calculated as follows:

$$\begin{aligned} {}^{12}\text{C assimilated } \left(\text{mg } {}^{12}\text{C/cm/h}\right) = ({}^{12}\text{C content of water } \left(\text{mg } {}^{12}\text{C/L}\right) \\ \times \text{ incubation volume (L)} \\ \times {}^{14}\text{C assimilated (DPM/bottle)} \\ \div {}^{14}\text{C available (DPM/bottle)} \\ \div \text{ area of substratum } \left(\text{cm}^2\right) \\ \div \text{ incubation interval (h)} \end{aligned}$$

If benthic algae are growing on large substrata that cannot be sectioned, then one must increase the size of incubation containers. It is not practical to filter large amounts of benthic algae, so after the incubation, one must scrape or brush algae from the substratum to make a slurry, quantitatively subsample that slurry for radioisotopic analysis, and correct final production values to account for using only a subsample of material. Be careful not to splatter radioactive material when making these slurries.

For benthic algae growing on soft sediments, collect samples by coring with clear and opaque plastic tubes. Keep cores in their natural orientation in the coring tubes during transport to the laboratory and during incubations. Use coring tubes as incubation containers (cover open tops of coring tubes with clear or opaque caps or plastic wrap to avoid evaporation), and incubate as described above. Following incubation, the top 1 cm of sediments should be collected and filtered, dried, fumed, digested, cleared, and radioassayed as described above. Benthic algae growing on soft sediments obtain some of their inorganic C from the sediments. Typical C assimilation formulae assume 100 % of inorganic C is obtained from the water, and thus underestimate epipelic photosynthesis. For best accuracy, one should conduct a separate experiment to quantify the fraction of algal inorganic C arising from sediments (see Vadeboncoeur and Lodge 1998), or one may assume that inorganic C from water and sediments are used approximately equally, and thus multiply the C assimilation values provided by traditional formulae by 2.

To express the results of benthic algal ^{14}C incubations in biomass-specific units, create a conversion factor by measuring area-specific benthic algal biomass on separate samples of benthic algae collected at the same time and location as those used in the production assay. If the total rate of photosynthetic C fixation is of interest, then one can determine the amount of C fixed by algae and lost into the water as soluble photosynthate as described for phytoplankton (for epipelic communities, be sure to include both the filtrate from the sediment and the overlying water from the incubation).

The ^{14}C production assays outlined here employ strong bases and oxidizers, as well as low levels of radioactive material. All personnel must receive proper training and use appropriate protective equipment (e.g., gloves, eye protection, lab coats). Proper laboratory procedures must be in place for the use, monitoring, storage, and disposal of radioactive material. Always keep in mind that dissolved inorganic ^{14}C will become $^{14}CO_2$ gas under acidic conditions – never acidify samples unless they are in a fume hood. Consult with your institution's Radiation Safety Officer for specific policies and procedures.

1.6 Extracellular Enzyme Activity

Algae can produce and secrete appreciable quantities of extracellular phosphatase (e.g., Cembella et al. 1984; Chrost 1996; Chrost and Overbeck 1987). Phosphatase cleaves phosphate groups from large phosphate-containing molecules, increasing the supply of phosphate available for algal uptake. Some phosphatase activity is constitutive, but additional extracellular phosphatase activity can be induced when phosphate supply is insufficient to meet algal metabolic demands (e.g., Bruckmeier et al. 2005; Fitzgerald and Nelson 1996). Thus, production and secretion of extracellular phosphatase is often indicative of phosphorus stress (Hameed et al. 1999; Rose and Axler 1998), and the relative amount of phosphorus stress among sites or over time can generally be inferred via comparison of phosphatase activity. In some instances, large changes in taxonomic composition and resulting inherent differences in phosphatase production abilities of algal communities have made cross-system comparisons difficult (Cao et al. 2010).

Whole-community phosphatase activity can be measured using a fluorometric assay, where fluorogenic artificial substrate is converted to a fluorescent product when hydrolysed by phosphatase. The results obtained will be an integrated measure of the extracellular phosphatase activity of all algae and other phosphatase-producing organisms (e.g., bacteria, fungi) in the sample. Other assays have been developed for examining phosphatase activity of individual cells, allowing for discrimination between enzyme activities of different microbial groups, and even individual algal species (e.g., Espeland et al. 2002; Novotna et al. 2010; Sharma et al. 2005). The method described below is designed to measure whole-community phosphatase activity of living organisms in their natural state, and is thus suitable for physiological experiments as well as intersite

and temporal comparisons. It has been successfully employed in wetlands (e.g., Francoeur et al. 2006).

Collect, filter (0.7 μm pore size), autoclave, and cool water from wetland field site. Use this water to fill incubation vials, make methylumbelliferone sodium salt (Sigma Chemical, or equivalent vendor) standards (0–5,000 nanomolar [nM] is a reasonable range for benthic samples) and make a concentrated substrate stock solution (1,000 nM) of 4-methylumbelliferyl phosphate disodium salt (Sigma Chemical, or equivalent vendor). If the low solubility of methylumbelliferyl phosphate in pure water makes the generation of a concentrated stock solution difficult, the methylumbelliferyl phosphate can be first dissolved in a small volume of organic solvent, then dissolved in water (Hoppe 1983). Use only autoclaved labware for making and storing enzyme substrate solutions. If enzyme substrate solution is to be kept any length of time, store frozen (−10 °C).

Carefully collect phytoplankton or benthic algal samples. Do not disrupt the physical structure of benthic algal community; instead section the substratum (with attached algae) into pieces of known size; pieces of ~3 cm^2 are recommended. If standardization of enzyme activity to biomass is desired, collect extra replicate samples for biomass analysis (see Sect. 1.4).

Place benthic algae into autoclaved glass scintillation vials, and add 1 ml of autoclaved wetland water. Also prepare algae-free controls by adding 1 ml of autoclaved wetland water to empty vials. Once all vials are loaded, add 2 ml of concentrated stock solution to achieve a final methylumbelliferyl phosphate disodium salt concentration of 660 nM. Record the time of addition for each vial to the nearest minute. Incubate vials for 30 min. Note that incubation conditions should be chosen with care and carefully controlled, as many factors, including temperature and photosynthetic activity, can affect enzyme activity.

Turn on a plate-reading fluorometer and complete warm-up and diagnostics as recommended by manufacturer. Ensure the appropriate wavelengths will be used for excitation and emission of methylumbelliferone (~365 nm excitation, ~445 emission). Prepare a 96-well plate by adding 100 μl of pH 10 sodium carbonate-bicarbonate buffer (pHydrion, or equivalent) to each well. Note that the alkaline buffer equalizes pH among all samples and standards, and maximizes the pH-dependent fluorescence of methylumbelliferone; it will not halt phosphatase activity in benthic algal communities (Espeland and Wetzel 2001). Immediately before the end of the incubation, add 100 μl of methylumbelliferone standards to the appropriate wells of the plate. Run standards in duplicate. At the conclusion of the incubation, remove a 100 μl aliquot from each sample or control vial and add to the appropriate well of the 96-well plate, and record the time to the nearest minute. Immediately measure the fluorescence of each well of the plate. Alternatively, if a plate reading fluorometer is not available, reactions can be run in individual autoclaved glass tubes, and fluorescence can be measured using a single-well fluorometer. This alternative is much slower than a plate-reading fluorometer; pay special attention to recording incubation times and ensuring they do not become excessively long. Use the standard curve of methylumbelliferone fluorescence and the incubation time to calculate the methylumbelliferyl phosphate hydrolysis

rate of each sample (nmol/h). These rates can be expressed on an areal basis (nmol/h/cm^2) by dividing by the size (area) of the benthic algal pieces used in the assay, or on a biomass basis (nmol/h/biomass) by dividing by the mean biomass of replicate biomass samples.

For phytoplankton samples, add 1 ml of phytoplankton sample to each vial, in place of the benthic algal material and 1 ml of autoclaved wetland water. Construct algae-free controls by adding 1 ml of filtered (0.7 μm pore size glass fiber filter) wetland water in place of the 1 ml of phytoplankton. Once all vials are loaded, add 2 ml of concentrated stock solution to achieve a final methylumbelliferyl phosphate disodium salt concentration of 660 nM. Incubation times may need to be increased, to allow for adequate hydrolysis, and methylumbelliferyl phosphate concentrations could likely be reduced while still maintaining saturating conditions. Prepare a 96-well plate by adding 100 μl of pH 10 sodium carbonate-bicarbonate buffer (pHydrion, or equivalent) to each well. To correct for possible fluorescence quenching by suspended phytoplankton cells, prepare fluorescence standard stock solutions at 1.33 times the desired final concentrations (i.e., for a final concentration of 25 nM, prepare a stock solution of 33.33 nM). Immediately before the end of the incubation, add 75 μl of methylumbelliferone standard stock solutions and 25 μl of fresh phytoplankton suspension to the appropriate wells of the plate. Run standards in duplicate. Load sample wells by adding a 100 μl aliquot from each sample or control vial and add to the appropriate well of the 96-well plate, and recording the time to the nearest minute. Conduct the fluorescence assay and calculate hydrolysis rates and enzyme activities as above, but express on a volumetric basis (nmol/h/ml) by dividing by the volume of phytoplankton added to an individual vial, or on a biomass basis (nmol/h/biomass) by dividing by the mean biomass of replicate biomass samples.

If assay conditions are approximately saturating, the hydrolysis rates calculated from the assay will estimate the V_{max} of phosphatase. By definition, hydrolysis rates at saturation (V_{max}) depend only on the activity of the enzyme, and thus are independent of substrate concentration. Under subsaturating conditions, measured hydrolysis rates are less than V_{max}, and depend upon both the intrinsic activity of the enzyme and the concentration of the substrate. Thus, enzyme assays should be conducted under saturating conditions (or as near to saturating as logistically possible) in order to ensure that the measured hydrolysis rates are not affected by substrate concentration. Preliminary experiments should be conducted to ensure that the assay conditions (amount of algae, substrate concentration, incubation time, temperature, etc.) are saturating.

To conduct such a preliminary experiment, simply prepare algae-containing and control vials as described above, but use a range of final methylumbelliferyl phosphate concentrations (0–1,000 nM is recommended) in the vials. Incubate vials and measure hydrolysis rates as described above, then use these results to calculate V_{max} and K_m. These two parameters are estimated from the data, using iterative least-squares nonlinear regression (Systat, or equivalent statistical software) and the standard Michaelis-Menten equation.

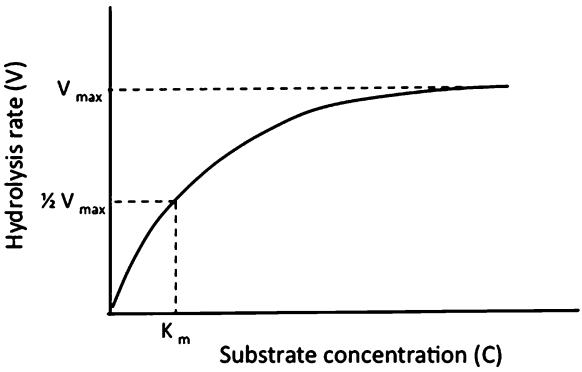

Fig. 1.10 Michaelis-Menten plot

$$V = (V_{max} \times C) \div (K_m + C)$$

where V is the measured hydrolysis rate at substrate concentration C, V_{max} is the maximal reaction velocity, and K_m is the half-saturation constant (Fig. 1.10).

To ensure approximate saturation for the particular assay conditions in question, one should employ a methylumbelliferyl phosphate substrate concentration that is well above K_m and results in a measured hydrolysis rate approximating the calculated value of V_{max} (see Francoeur et al. 2006). Alternatively, one can dispense with conducting preliminary saturation experiments, and employ a range of methylumbelliferyl phosphate substrate concentrations in every assay (e.g., Sinsabaugh et al. 1997). V_{max} and K_m can then be calculated from assay data as described above. Such an approach increases the workload required to complete an individual assay, but provides additional information about phosphatase kinetics.

1.7 Nutrient Content, Uptake and Limitation

1.7.1 Algal Nutrient Content

There are a number of procedures for determining the nutrient content of phytoplankton, periphyton and metaphyton. The two procedures described below are for determining the nitrogen and phosphorus content of living material. These methods can be easily carried out on algal slurries or on material that has been drawn down onto pre-combusted, pre-weighed, glass fiber filters (0.7 μm pore size).

1.7.1.1 Phosphorus Content

The following procedure is a slight modification of a method originally described by Stockner and Armstrong (1971). Dry samples (either as slurries or on acid-washed glass fiber filters) at 105 °C in acid-washed borosilicate glass vials

or beakers. Weigh dried material and container to the nearest 0.1 mg, place in a 500 °C muffle furnace for 1 h and reweigh. Extract phosphorus from ash in 5 mL of 1 M HCl at 80 °C for 1 h. Dilute samples to 50 mL with ultra-pure water and analyze using the ascorbic acid method described in Standard Methods (APHA 2005). A long path-length cuvette (recommend 10 cm) should be used in the spectrophotometer in order to maximize the sensitivity of this method. Standard reference material (e.g., tomato leaves from the National Institute of Standards and Technology) should be analyzed along with the samples to establish phosphorus recovery efficiency (see Hill and Fanta 2008; Hill et al. 2009; Mulholland and Rosemond 1992 for examples).

1.7.1.2 Nitrogen Content

Nitrogen is best analyzed in conjunction with carbon using an elemental analyzer (CHN). Dry samples (either as slurries or on glass fiber filters) at 105 °C and weigh on microbalance. Follow the manufacturer's directions for analysis of samples on the elemental analyzer. Standard reference material (e.g., tomato leaves from the National Institute of Standards and Technology) should be analyzed along with the samples.

1.7.2 Nutrient Limitation

Algal growth and production can be constrained by shortage of many different nutrients (Borchardt 1996; Carrick and Lowe 2008; Wetzel 2001), but nitrogen (N) and phosphorus (P) are the principal limiting nutrients for freshwater algae. Assessing the presence and strength of nutrient limitation and determining the identity of the limiting nutrient has value for both basic scientific investigations and applied management actions. Algal nutrient limitation may be inferred via analysis of algal nutrient content and examination of tissue stoichiometry. Empirical determination of nutrient limitation can be achieved via nutrient enrichment experiments.

1.7.2.1 Stoichiometric Inferences

Relative intracellular carbon (C), nitrogen (N), and phosphorus (P) content are expected to fall within a relatively narrow range. Redfield (1958) proposed an optimal molar C:N:P ratio of 106:16:1 for marine phytoplankton. Hillebrand and Sommer (1999) demonstrated that this ratio was 119:17:1 for marine benthic algae. Utilizing an extensive literature review, Kahlert (1998) found that the optimal molar ratio was 158:18:1 for freshwater periphyton. Substantial deviations from these optimal N:P ratios may indicate which nutrient (N or P) is likely to be limiting. Healy and Hendzel (1980) recommend that N:P $>$ 22 be considered

evidence of phytoplankton P-limitation. Hillebrand and Sommer (1999) reported that N:P < 13 and N:P > 22 are indicative of N- and P-limitation in benthic algae, whereas the literature review of Kahlert (1998) suggested slightly more extreme ratios (N:P < 12, N:P > 32).

It must be remembered these algal nutrient ratios are determined from bulk measurements of particulate nutrients. Thus, these ratios are affected by nutrients contained in detritus and non-algal organisms, and cannot account for the diverse nutrient requirements of individual algal species. In addition, N:P ratios can only indicate which nutrient is in relatively shorter supply. Regardless of the numerical value of the N:P ratio, other factors (e.g., light, Si, Fe) might actually be limiting algal growth and production. Examination of multiple nutrient ratios (e.g., C:P, N:C) can partially alleviate this problem. Despite these constraints, examination of algal nutrient ratios can provide a rapid, useful indication regarding the identity of potential limiting nutrients. Values are often most useful when there are extreme departures from the optimal ratio or when drawing comparisons between experimental treatments or sampling sites that are subjected to similar environmental conditions other than N and P loading.

1.7.2.2 Empirical Determination

Nutrient uptake rates can also be used to elucidate relative nutrient limitation and have been shown to closely track other measures of limitation such as algal responses to nutrient-diffusing substrata and the C:N:P ratios of the algal community (e.g., Flecker et al. 2002; Webster et al. 2003). Empirical measures of nutrient uptake are carried out by placing the algal assemblage of interest into an enclosed container, where ambient light and temperature are maintained. These containers may range from BOD (biological oxygen demand) bottles (if the researcher is only interested in phytoplankton) to larger open-bottom mesocosms for simultaneously measuring nutrient uptake by assemblages associated with the water column and the benthos. It is also possible to place natural (e.g., macrophyte stems) or artificial substrata into open-topped containers. In all cases, these containers will have an isolated volume of water that is then spiked with the nutrient or nutrients of interest. Depending on the question, salts of nitrate (e.g., $NaNO_3$) or ammonium (e.g., NH_4Cl) are usually used as a nitrogen source and phosphate (e.g., Na_2HPO_4) is used as a phosphorus source. Samples are collected at the beginning and end of a set incubation time and analyzed for the nutrient(s) of interest using standard methods (APHA 2005). Water motion can have a strong effect on algal nutrient uptake rates (e.g., Borchardt 1996). Thus, use static incubations for samples from habitats with little or no water movement. If water in chambers is stirred or circulated to mimic lotic conditions, ensure equal water movement in all chambers. Nutrient uptake rate in the chambers can be calculated using the following formula:

$$V = (C_i - C_f) \times L \div t \times A$$

where V is uptake rate in μg nutrient/area/h, C_i and C_f are the initial and final nutrient concentrations in the chamber respectively, L is chamber volume in liters, t is time in hours, and A is the area of the substratum. Note: Depending on the question, biomass (e.g., dry mass, AFDM, chlorophyll *a*) of the assemblage (see Sect. 1.4) may be substituted for area (A) in the above equation.

Nutrient limitation can be assessed by incubating assemblages at multiple nutrient concentrations. The relation between concentration and uptake rate should follow a pattern that is mathematically equivalent to Michaelis-Menten model for enzyme kinetics (Borchardt 1996; see Fig. 1.10).

$$V = (V_{max} \times S) \div (K_s + S)$$

where V is the uptake rate of the nutrient, V_{max} is the maximum uptake rate, K_s is the half saturation constant, and S is the nutrient concentration. The parameters V_{max} and K_s can be obtained by first measuring nutrient uptake in at least 10 experimentally manipulated nutrient concentrations and then fitting the nutrient concentrations and uptake rates to the Michaelis-Menten equation (see above) using a nonlinear regression procedure found in most statistical packages. This approach can also be used to estimate uptake rates at ambient concentrations (see Payn et al. 2005). Estimates of the uptake parameter V_{max} can be particularly useful for evaluating nutrient limitation in algal assemblages since algae have been shown to increase short term uptake rates in response to nutrient limitation, whereas K_s tends to be a more static species-specific characteristic (e.g., Rivkin and Swift 1982). Therefore, a higher V_{max} for a particular nutrient likely indicates a greater degree of limitation by that nutrient.

There are several important considerations when conducting nutrient uptake studies. The first is that spiking with unlabeled forms of nutrients measures net uptake rates, not total uptake rates, since a certain indistinguishable fraction of each nutrient is continuously being turned over. Next, nutrient concentrations need to be set high enough so that differences can be detected by the analytical methodology being used, but not so high that all concentrations fall above saturating levels. Working with labeled nutrients such as ^{15}N, which is a stable isotope of nitrogen, or ^{32}P or ^{33}P, which are radioactive isotopes of phosphorus, can help to circumvent all of these problems because they are detectable at extremely low concentrations. In addition to measuring total uptake rates, labeled nutrients can be used to detect turnover rates and, in the case of nitrogen, rates of nitrification and denitrification. However, specific use of stable or radioactive isotopes in nutrient uptake studies is beyond the scope of this chapter. A final issue concerns non-biological uptake of phosphorus. In addition to biological uptake, phosphate can be sorbed to inorganic particles such as clay and calcium carbonate and be effectively taken out of solution (Withers and Jarvie 2008). When possible, algal-free control containers should be used to measure and correct for non-biological uptake.

Nutrient-diffusing substrata (NDS) provide an effective, economical way to empirically assess benthic algal nutrient limitation. NDS experiments are common in streams and lake littoral zones, and they are also useful in wetland systems

(Goldsborough et al. 2005). Many NDS designs have been introduced since the classic work of Fairchild et al. (1985). Three of the more common designs are based upon clay flowerpots, centrifuge tubes with porous disks, and jars capped with a porous membrane. The method given below is of the latter type. It blends several features from Biggs and Kilroy (2000) and Winterbourne (1990), is convenient to construct and deploy, and has been used successfully in wetlands (Whorley 2008).

Obtain 75 mL plastic jars (ht = 4.5 cm, diameter = 5 cm) with screw-top lids (Fig. 1.11); make sure there are two lids for each jar. Bore 38 mm diameter holes through the center of one set of lids. Clean all jars and lids with phosphate-free detergent (Liquinox or equivalent) and acid wash (soak in 10 % HCl for 2 h, then rinse copiously in distilled water). After drying, label jars "C", "N", "P", or "N&P" with a permanent marker, and place a dab of hot glue adhesive approximately one-third of the distance from the bottom of the jar (Fig. 1.11). The number of jars to prepare depends of the desired statistical power of the experiment. Five or six replicates per treatment are frequently employed, but 8–10 replicates of each nutrient treatment are required for the typical NDS experiment to have a 95 % chance of detecting a doubling of benthic algal biomass by nutrient enrichment (Francoeur 2001).

Make four solutions (Control, Nitrogen, Phosphorus, and Nitrogen & Phosphorus treatments) of 2 % agar. Agar solutions are made by dissolving powdered microbiological-grade agar in boiling distilled water. Sodium nitrate ($NaNO_3$) is added to the liquid Nitrogen agar, to a final concentration of 0.5 M (20 g $NaNO_3$ per liter of agar solution), sodium phosphate ($Na_3PO_4 \cdot 12H_2O$) is added to the Phosphorus agar, for a final concentration of 0.05 M (19 g $Na_3PO_4 \cdot 12H_2O$ per liter of agar solution), and both are added to the Nitrogen & Phosphorus for final concentrations of 0.5 M $NaNO_3$ and 0.05 M $Na_3PO_4 \cdot 12H_2O$. Agar containing sodium phosphate will turn brown. If desired, nutrient supply ratios can be adjusted by altering nutrient concentrations in the agar, or other nutrients or inhibitors can be included in the agar. If any modifications are made, then release rates should be re-quantified (see below).

Pour hot liquid agar into the appropriate jars and allow to cool and solidify. Agar shrinks slightly as it cools, so jars should be topped off with additional hot liquid agar to ensure that the jars are completely filled. The surface of the solidified agar should be level or convex, not concave. Placing jars on trays or aluminum foil for filling will make clean up of spilled agar much easier. Once agar is solidified, cap jars with intact lids, place in resealable containers (Tupperware or equivalent) with damp paper towels, and store refrigerated (4 °C). Agar-filled jars may be stored in this way for several months.

To complete NDS construction, remove intact lids from jars. Cover the agar with the desired growth surface, and attach to the jar by capturing the growth surface between the jar and the bored-out screw cap. Hardened ashless filter paper (Whatman 542, or equivalent), nitrocellulose filters (0.8 mm pore size), and circles of ultrafine nylon mesh (5 mm pore size, Nitex or equivalent) are all appropriate growth surfaces. Nitex mesh is the most resistant to abrasion, while the others can be subsectioned after collection for multiple analyses.

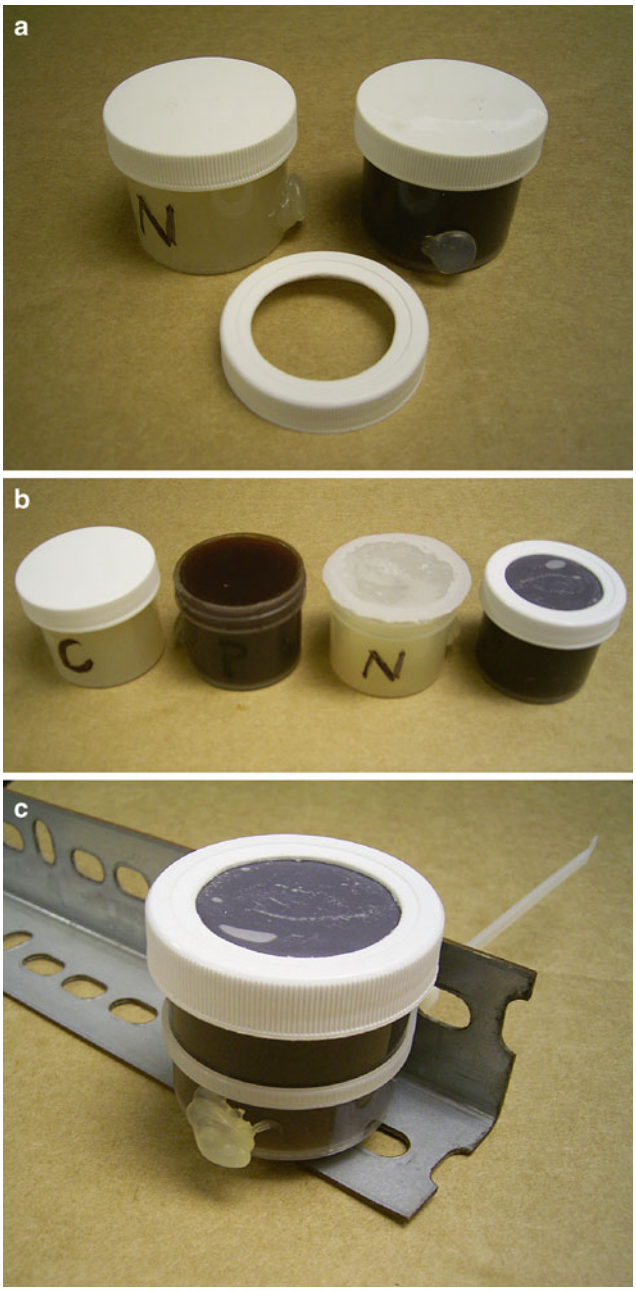

Fig. 1.11 Nutrient-diffusing substrata design and construction. A bored-out lid with sealed jars of N (white) and P (brown) enriched agar (**a**), from left: a sealed jar of agar, an open jar, jar covered with growth surface, jar with growth surface anchored by bored-out lid (**b**), jar attached to angle iron, ready for deployment (**c**)

Attach individual NDS jars to perforated angle iron by placing a ratcheting cable tie around the jar (between the dab of solidified glue and the lip of the jar) and through the perforations in the angle iron. In lotic systems, short (~40 cm) sections of perforated angle iron can be used, with jars placed relatively close together (8–10 cm between jars), so long as angle iron sections are oriented perpendicular to the current. In lentic systems, use longer (~1 m) sections of perforated angle iron and space jars further apart (20–25 cm between jars) to prevent nutrients diffusing from one jar from influencing algae growing on other jars.

In wetlands with relatively hard bottoms, perforated angle iron sections (with attached NDS jars) can be bolted to cement patio blocks, then placed into the wetland. Alternatively, the perforated angle iron sections (with attached NDS jars) can be cable-tied to PVC stakes which have been driven into wetland sediments. The latter method is superior in soft-bottomed wetlands.

After a sufficient incubation period, NDS should be retrieved. Incubation times must allow for colonization and adequate algal growth, yet not be so long that senescence and sloughing cause loss of algal biomass. Fourteen to 28 days is usually sufficient, but this depends on environmental conditions (light, temperature, etc.). To retrieve NDS, jar lids are unscrewed, and growth surfaces are placed directly into centrifuge tubes for chlorophyll analysis (see Sect. 1.4.2). After chlorophyll extraction, nylon mesh may be cleaned and re-used. If filter paper has been used as the growth surface, sections of known area (recommend 10–20 mm diameter) may be cut with a cork borer for alternative uses (e.g., preserved for later microscopy and determination of responses of individual species) before placing the remainder of the filters into centrifuge tubes. Should algal communities be loosely-attached, growth surfaces may be bagged underwater. In such cases, the water in the collection bags should be filtered and extracted for chlorophyll along with the NDS growth surface. Significantly greater benthic algal biomass on nutrient-enriched relative to control substrata is evidence of nutrient limitation.

Incubation durations should not exceed the nutrient delivery period of the NDS. Nutrient release rates from the jar-based NDS design presented here are shown in Fig. 1.12. To generate these data, NDS jars (5 mm Nitex mesh as growth surface) were placed in beakers of distilled water, which were periodically sampled for dissolved nutrients (automated analysis using EPA-approved methods, Seal Analytical 2005) and refilled with fresh distilled water. Measurements were corrected for background nutrient levels in the distilled water. Table 1.3 displays nutrient release data from the jar-based design and literature values given for two other common NDS designs (clay flowerpots, Fairchild et al. 1985; vials with ceramic disks, Rugenski et al. 2008) for comparative purposes. To ensure consistency among the studies, only data from room-temperature experiments utilizing 0.05 M P or 0.5 M N agar were considered. Nutrient release rates generally decline over time for all designs, and clay flowerpots release nutrients at lower concentrations and more slowly than the other two designs. The NDS jar design presented here release relatively little NO_3 after 24 days, and thus should not be used for long (30+ days) incubations.

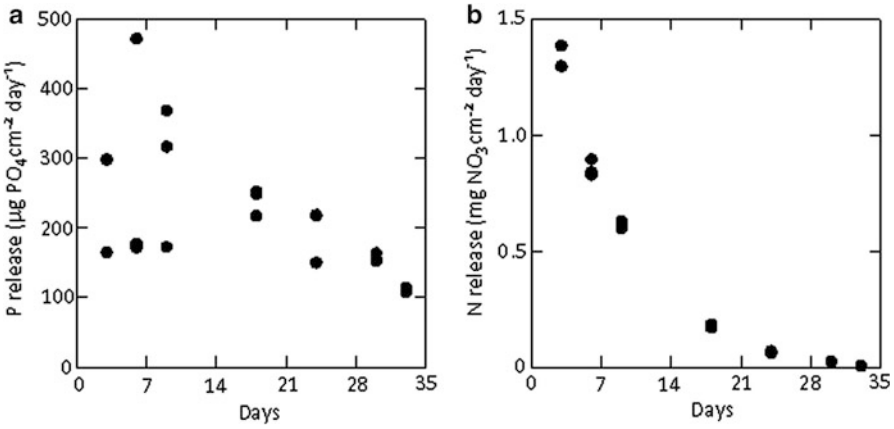

Fig. 1.12 Area-specific nutrient release rates of P (µg P cm^{-2} day^{-1}) (**a**) and N (mg N cm^{-2} day^{-1}) (**b**) from NDS jars over 33 days

Table 1.3 Initial (flowerpots, Day 6; vials Day 0; jars, Day 3) and final (flowerpots, Day 23; vials, Day 21; jars, Day 24) area-specific nutrient release rates for N (mg NO_3 cm^{-2} day^{-1}) and P (µg PO_4 cm^{-2} day^{-1}) from three NDS designs

	Initial release rate		Final release rate	
Design	N	P	N	P
Jars (This study)	1.3	231.5	0.06	195.5
Clay flowerpots (Fairchild et al. 1985)	0.71	44.8	0.30	33.4
Vials with ceramic disks (Rugenski et al. 2008)	4.5	678.1	0.50	113.0

NDS experiments are not applicable to all wetland algae. They are obviously ineffective for phytoplankton, nor can they capture the responses of benthic algae that slowly or sporadically colonize new substrata (e.g., *Chara*, *Cladophora*). Open or closed mesocosm designs have been employed for conducting nutrient enrichment experiments for such communities. Finally, nutrient enrichment experiments provide the most accurate results for algal communities which naturally obtain nutrients from the water column (e.g., phytoplankton, metaphyton, benthic algae growing on rocks or plant detritus). Epipelic algae can gain substantial nutrients from the sediments, so results of NDS experiments may not reflect in-situ epipelic algal nutrient status (see Vadeboncoeur and Lodge 2000).

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Student Exercises

The following laboratory exercises and in-class activities are designed to build skills in several common techniques used for analysis of wetland algae, introduce students to natural patterns and variability exhibited by wetland algae, and provide experience in conducting, analyzing and interpreting data.

Classroom Exercises

The following activities are designed as short in-class activities.

1. Make a high-quality wet mount. Recall that a satisfactory wet mount can be inverted without the cover slip moving along the glass microscope slide.
2. Properly load a Palmer-Maloney cell and a Sedgewick-Rafter cell.
3. Use a stage micrometer to measure the area of your microscope's field of view at each magnification. Calculate the volume of sample that you can observe in a single field of a Palmer-Maloney cell and a single field of a Sedgewick-Rafter cell at each magnification.
4. Use a stage micrometer to calibrate your microscope's ocular micrometer at each magnification.
5. Distribute an algal sample to everyone in the class, and agree upon the use of one algal taxon. Measure the biovolume of one individual from that taxon and share the data. How different are the values? How many individuals need to be measured before the mean biovolume stabilizes?

Laboratory Exercises

The following activities are designed as laboratory exercises.

1. Qualitatively collect algae from a nearby wetland. Examine samples under the microscope using wet mounts, and refer to appropriate taxonomic references. How many different genera are you able to discern? Are there any patterns in the presence of taxa across the three major habitats (planktonic, metaphytic and benthic) or across different substrata (e.g., epipelic vs. epiphytic)?
2. Using the qualitative algal samples from Laboratory exercise #1, chemically clean the material and make diatom mounts. Examine samples under the microscope, and refer to appropriate taxonomic references. How many different diatom genera are you able to discern? Is this more or fewer than recorded using wet mounts? Are there any patterns in the presence of diatom taxa across the three major habitats (planktonic, metaphytic and benthic) or across different substrata (e.g., epipelic vs. epiphytic)?

3. Quantitatively collect and measure the biomass of algae in the three major habitats (planktonic, metaphytic and benthic) in a nearby wetland. On a per m^2 basis, which habitat supports the most algal biomass? Do you think the differences in algal biomass between the habitat types is substantial? Why or why not?
4. Using the O_2 technique, quantify algal production in the three major habitats (planktonic, metaphytic and benthic) in a nearby wetland. On a per m^2 basis, which habitat supports the most algal production? Do you think the differences in algal production between the habitat types is substantial? Why or why not?
5. Construct NDS and deploy them in a nearby wetland. After 21 days, retrieve the NDS and assay algal biomass. Did nutrient availability constrain benthic algal biomass? If so, which nutrient(s) was/were limiting?
6. Collect a quantitative algal sample. Use a Palmer-Maloney cell or a Sedgewick-Rafter cell to measure algal cell densities. How similar are each person's or group's cell density values?

Chapter 2
Methods for Sampling and Analyzing Wetland Soil Bacterial Community

Aixin Hou and Henry N. Williams

Abstract Wetland soil, a heterogeneous environment highly modified by its hydrologic condition and vegetation, provides habitats for a variety of aerobic and anaerobic bacteria. Sampling the wetland soil bacterial community involves collection of bulk or rhizosphere soil or both, depending on the purpose of the study. In any case, it is crucial to assure that random, but representative samples are collected to provide meaningful data and to meet the purposes of the study. Approaches to the analyses of the bacterial communities in wetland soils may be divided into two general categories: cultivation-based and cultivation-independent techniques. The first category relies on laboratory cultivation and the second is based on the analyses of indicator molecules such as DNA extracted directly from soil samples. The primary cultivation-independent methods include 16S rRNA gene-based cloning library, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and fluorescence in situ hybridization (FISH). More recently, high-throughput technologies, such as next-generation DNA sequencing (e.g., 454 pyrosequencing and Illumina sequencing) and GeoChip, were developed to generate large amounts of genetic information allowing more in-depth and comprehensive assessment of bacterial communities. This chapter focuses on some practical approaches commonly used for sampling and analyzing bacterial communities in wetland soils.

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2.1 Introduction

Highly diverse bacterial populations are present in wetland soils and mediate many vital biogeochemical processes, including those involved in carbon, nitrogen, phosphorus, sulfur, and iron cycles. The bacteria that inhabit the anoxic hydric soils of wetlands are often responsible for the formation of an oxic surface layer and a redox stratification of the oxygen-depleted zone, which is a typical characteristic of wetland soils (Zehnder and Stumm 1988). The permanent or periodic flooding of wetland soils and the presence of wetland plant roots create dynamic oxic-anoxic interfaces that provide habitats for a wide variety of aerobic and anaerobic microbes. The ability to characterize the bacterial community structure in wetland soils is fundamental to the understanding of wetland functions such as regulating the cycling, retention, and release of nutrients and soil carbon. These wetland functions have demonstrated significant effects on water quality (Richardson and Marshall 1986) and global carbon cycling (Roulet 2000). Nonetheless, the study of bacterial communities in wetlands is still fairly new relative to other ecosystems (Weber and Legge 2010).

Wetland plants modify the surrounding soil environment in various ways. In addition to contributing substantial amounts of oxygen to the surrounding anoxic soil, plant roots also excrete organic carbon compounds which provide a readily available energy source for bacteria in the rhizosphere. Therefore, bacterial communities in the rhizosphere differ quantitatively and qualitatively from those in the bulk soil, generally presenting higher population densities. Depending on the purpose of the study, either bulk or rhizosphere soil or both should be sampled for bacterial community analyses. A variety of approaches have been developed for analyses of various aspects of the bacterial community in environmental samples and many of these may be applied with minor modifications to investigations of wetland soil bacterial communities. Approaches to the analyses of the bacterial community in wetland soils may be divided into two general categories: (i) cultivation-based methods which rely on laboratory cultivation such as dilution plating and (ii) cultivation-independent techniques which are based on the analyses of indicator molecules such as DNA extracted directly from soil samples. Each of these particular methods has its advantages and limitations as discussed below. This chapter focuses on some practical approaches commonly used for sampling and analyzing bacterial communities in wetland soils.

2.2 Wetland Soil Sampling

2.2.1 Sampling Considerations

Soil, including wetland soil, is a complex environment in which soil aggregates of various sizes and plant roots may provide distinct microhabitats for the soil

bacteria. Wetland soil is therefore heterogeneous with regard to its composition and the distribution of bacterial communities, resulting in variability at both the microscale (micrometer to millimeter) and macroscale (meter) levels (Van Elsas and Smalla 1997). Given the heterogeneity and variability in wetland soils, representative sampling is important for any type of soil bacterial community analysis. A sampling strategy should be developed prior to sample collection to ensure representative samples of the study area are collected in order to provide meaningful data and meet the goals of the study (see Chap. 1 of Vol. 1 for a more detailed description of sampling strategies). General principles of and considerations on how to develop an appropriate sampling strategy for collection of soil samples have been discussed by a number of authors (e.g., Petersen and Calvin 1996; Isaacs and Srivastava 1989; Van Elsas and Smalla 1997), and can be applied to wetland soil sampling for bacteria. In any case, it is crucial to assure the representativeness and/or randomness of sampling.

Several sampling strategies typically considered by investigators include judgment, simple random, stratified random and systematic sampling. In judgment sampling, the investigator judges which are the most "typical" sites from which to draw the sample. One should bear in mind that samples selected in this manner are inherently biased and usually do not allow for a statistical treatment of the data generated. They should, therefore, be used merely as a source for such purposes as the isolation of bacteria. In simple random sampling, each possible sample site must have an equal chance of being selected for sampling. Thus, they meet the prerequisite of being random for statistical analyses of the data produced. When multiple samples are collected over a study field or site, the random samples provide an estimate of the mean and variance of the mean which determines the usual confidence limits around the mean. Simple random samples are appropriate for purposes such as the characterization of fields by mean parameter values, variation, and spatial distribution (Van Elsas and Smalla 1997). Stratified random sampling separates the study area into a number of subareas on the basis of some selected properties (e.g., hydrologic condition or plant species of the area) and simple random samples are taken from each subarea. This sampling strategy allows for separate characterization of each subarea and to a certain degree, increases the precision of estimates over the entire area. One disadvantage, however, is that it may require a large number of samples to be collected.

Systematic sampling attempts to ensure that the whole study area is well covered by the sample sites selected. Samples are collected from predetermined points at equal distances from each other, either in one (e.g., along a line) or two (e.g., plots in a field) dimensions. Both theoretical and empirical studies have shown in general that systematic sampling has greater precision than simple random or stratified random samples. It is useful for more precise and systematic documentation of a parameter across the entire study area. Detailed discussions of these distinguishable sampling methods are presented by Petersen and Calvin (1996) for the purpose of general soil analysis.

In practice, the choice of the sampling method and the number of samples usually depend on both statistical and practical considerations, in addition to the objective of the study. The statistical considerations involve the degree of

variability of the data expected and the level of precision of the data needed. The practical considerations include the number of samples that can be handled in the laboratory in a reasonable time and with reasonable investment of resources. Virtually, all practical studies are limited in resources and, therefore, the sampling strategy chosen usually is a compromise between statistical and practical considerations. This is especially true when it comes to a global analysis of microbial community, which tends to be laborious and have high costs per sample. One effective approach to reduce variations and increase precision yet limit the number of samples analyzed is to compose (combine and mix) multiple individual samples. For DNA-based analyses, genomic DNA's extracted from multiple individual samples often are pooled prior to community analyses and variances between the composites are compared.

2.2.2 Sample Collection

Depending on the purpose of the study, bulk soil samples, rhizosphere soil samples, or both may be required. For instance, bulk soil samples can be used if a study is to determine the impacts of wetland restoration methods such as sediment slurry addition on soil bacterial communities. Rhizosphere soil should be sampled if an experiment is to address how different wetland plant species may affect the microbial communities inhabiting the rhizosphere. When a study aims at determining the abundance of bacteria in wetland soils, both bulk and rhizosphere soil samples may be collected. The size of samples to be collected usually range from less than 100 g to several kilograms, which primarily depends on the experimental requirements. In most cases, several hundred grams of soil is sufficient for microbiological and soil chemical assays.

Various types of tools, ranging from spoons and spatulas to hand augers and sample corers, can be used to collect bulk soil samples. Often, a hand auger is used for sampling smaller soil volumes from different depths. Typically, the top 10–15 cm of the sediment is sampled for studies of the microbial community in wetlands. In wetland soils with dense plant roots or high contents of peat, a side-filling auger with a cutting frame (e.g., Russian peat corer; Fig. 2.1) is especially useful to collect uncompressed samples. The sampling tool needs to be pre-sterilized before use in the field and thoroughly cleaned, preferably disinfected, between sample collections in order to prevent sample cross-contamination. Handling of the soil sample should be done in an aseptic manner, which may include wearing pre-sterilized, disposable gloves and using pre-sterilized liners for transferring the soil sample from core samplers to sample containers. Sterile plastic bags (e.g., Whirl-Pak bag) or plastic bottles can be used as sample containers. If samples are collected for analyses of anaerobic bacterial communities, they should be filled into sterile serum bottles sealed with butyl rubber stoppers. In oil-contaminated wetlands, EPA certified glass jars should be

Fig. 2.1 A Russian peat corer is used to recover a core sample at a salt marsh, Barataria Bay, Louisiana

used to store oiled soil samples when petroleum carbons in the samples will be analyzed along with microbial communities.

Rhizosphere soil samples can be obtained by digging out lumps of soil containing plants with a stainless steel spade or by carefully excavating plants from soil with a clean, disinfected shovel, making sure a substantial amount of soil is intact around the roots to facilitate recovery of the entire rhizosphere. Bulk and rhizosphere soil can be dissected later in the laboratory. Soil that can be easily removed from the lumps by shaking and that contains no roots is regarded as bulk soil. Soil that remains on the roots after removal of loose soil by shaking is defined as rhizosphere soil. However, depending on soil texture and moisture content, the amount of adhering soil may vary considerably (Dandurand and Knudsen 1997). In some cases, researchers have been forced to remove most of the soil by hand leaving a thin layer of soil (no more than a few millimeters thickness) on the root system which is regarded as rhizosphere soil (Otte et al. 1991, 1995).

Ambient environmental data should be collected at the time of soil sample collection for later reference. Documenting microbial data along with environmental and geochemical data (e.g., soil pH, redox potential, nutrient concentrations, etc.) is extremely valuable but currently difficult to find in many existing databases (Stahl and Tiedje 2002). In addition, GPS coordinates should be recorded to ensure that the same sites are sampled on each occasion in studies that involve temporal variations of the microbial community.

2.2.3 Sample Transport and Storage

Field soil samples should be transported to the laboratory as soon as possible after they are collected. To ensure they do not change substantially before analysis, samples are commonly transported on ice (4 °C) in a cooler. When ice is used, one precaution during transport is to not allow water from the melting ice to contact the samples. In practice, individual sample bags can be placed into a larger plastic zip bag, and then put on ice. It is worth noting that, in tropical climates, where topsoil temperatures may reach as high as 50–60 °C, transport at ambient temperature may be more appropriate if analysis takes place shortly after transport to the laboratory (Van Elsas and Smalla 1997).

For cultivation-based analyses such as Colony Forming Units (CFU) counts, samples should in principle be processed as soon as they arrive in the laboratory, since storage of soil samples invariably changes the soil microbiology (Anderson 1987). Cultivation-independent methods do not require immediate analysis of soil samples, since nucleic acids can be preserved in frozen soil for a long period. Hence, if both types of methods are used in a study, an aliquot of the soil sample can be stored frozen either at or below −20 °C (ideally −80 °C) until DNA extraction and the remaining soil processed as soon as possible for cultivation-based analyses.

2.2.4 Sample Processing

The purpose of soil sample processing in the laboratory is to make the samples ready for analyses. Roots, stones, shells (in case of coastal wetland soils), and other debris in soil should be removed before the assays are performed. This process usually is facilitated with sterile forceps and can be done in the plastic bags (i.e., without a need of taking the soil out of the sample bags) when the size of samples is small. After coarse materials are removed, soil samples should be thoroughly homogenized, and again, this step can be completed in the sample bags by hand when working with smaller size samples. If greater homogeneity is required, soil can be passed through a 2- or 4-mm metal sieve. Peat samples which contain high concentrations of organic matter can be homogenized by cutting the peat material into small fragments (about 0.5 cm) with sterile scissors (Dedysh et al. 2006). Once samples have been processed, immediate analysis is required. For general characterization and study of culturable bacterial communities, cells must be separated from soil particulates before they are inoculated onto laboratory medium. Many bacteria can produce extracellular polymeric substances (mainly polysaccharides) which promote the irreversible adhesion of cells to soil particulates. Inefficient detachment of bacteria from the soil matrix may result in loss of a significant fraction of the community and is one of the major sources of bias in community analysis. Separation is often accomplished by mixing 1 part of soil in 9 parts of an aqueous solution (commonly phosphate buffered saline or 0.85 % NaCl solution)

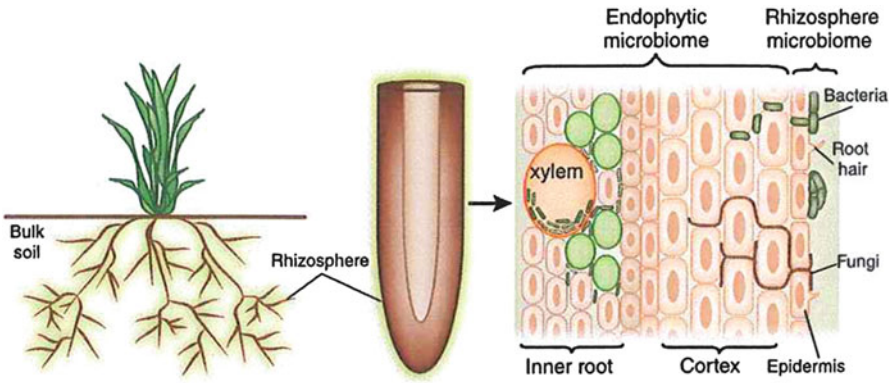

Fig. 2.2 The rhizosphere microbiome includes bacteria and fungi that are recruited from bulk soil and colonize the root surface and inhabit the soil up to 1 to a few millimeters from the surface. The endophytic microbiome includes species that infiltrate the root cortex and live as endophytes until their release back into the soil upon root senescence (Reprinted by permission from © Macmillan Publishers Ltd: Nature Biotechnology (Hirsch and Mauchline 2012). All Rights Reserved. Nature Publishing Group permission (http://www.nature.com/nbt/index.html) is acknowledged)

and shaken vigorously on a vortex mixer for 1 min at room temperature (Calheiros et al. 2010). Separation can also be accomplished by suspending 1 part of soil in 50 parts of sterile water. The suspension is then treated in a laboratory stomacher at 240 rpm for 5 min (Dedysh et al. 2006). The treated suspension is subjected to serial dilution and plating that will be discussed below.

Rhizosphere soil can be processed by cutting root segments with attached soil into pieces with sterile scissors, followed by separation of rhizosphere bacteria (i.e., those adhering to the root surface and inhabiting the surrounding soil; Fig. 2.2). A variety of methods (e.g., washing, vortexing, and Stomacher homogenizer) have been developed to separate bacterial cells. Washing followed by centrifugation (Nikolausz et al. 2004) is frequently used for separation of the rhizosphere fraction of wetland soils.

2.3 Analysis of Bacterial Community

It has long been recognized that the majority of microorganisms present in the environment may not be readily cultivated in the laboratory using traditional techniques. Biodiversity estimates show that the number of bacterial species in a single gram of soil can be enormous, ranging from 10^4 (Roesch et al. 2007; Torsvik et al. 1990, 2002) to 10^7 species (Gans et al. 2005) per gram of soil. Currently, less than 1 % of this diversity is considered to be cultivable by traditional techniques, and therefore, cultivation-based estimates of bacterial diversity may be largely biased (Schloss and Handelsman 2003). To bypass limitations and biases associated

with laboratory cultivation, a number of bacterial community profiling techniques have been developed based on the analysis of indicator molecules such as DNA or lipid extracted directly from environmental samples. Nevertheless, methods based on laboratory cultivation are still important and widely used for various bacterial community analyses, in particular enumeration and characterization of specific groups of bacteria (e.g., methanogens, nitrifying bacteria, oil-degrading bacteria). If a suitable selective medium is available, the cultivation-based strategy may still be the most sensitive approach for identification and enumeration of small, well-defined bacterial groups. Moreover, there is still a need for environmental isolates to conduct more comprehensive studies on the physiology and functionality of bacteria. Dedysh (2011: 12) stated that 'The newly isolated microorganisms, however, are a unique source of novel, unexpected findings, which may revise many old paradigms in our knowledge. They also provide the means to study cell biology, to verify hypotheses emerging from genome sequence data, and to adjust the currently used molecular detection techniques as well as our ideas about the functional role of these microbes in the environment'. The following sections will cover the most commonly used cultivation-dependent and -independent approaches for analyses of wetland soil bacterial communities.

2.3.1 Cultivation-Based Methods

Soil bacterial communities have traditionally been analyzed based on the isolation of individual strains on solid medium, followed by characterization and clustering of the isolates into operational taxonomic units (OTUs) according to phenotypic or genotypic characteristics. This type of analysis allows comparisons of the types of bacteria present (composition), the number of types (richness), and the relative abundance of different types (structure), which are the three major elements of bacterial community diversity in a sample. In addition, it provides total CFU counts (population of culturable bacteria) of a sample on the selected medium. Due to the limitations and biases inherent in laboratory cultivation, this approach only provides relative measures of the partial bacterial community, but not global pictures of community diversity in situ. Nevertheless, if samples are treated in a uniform manner, measures of community diversity derived from a culture collection can identify meaningful differences between soil bacterial communities (Dunbar et al. 1999). In particular, this strategy is appropriate for enumeration and characterization of specific groups of interest when a suitable selective medium is available.

2.3.1.1 Preparation of Media

The first step in cultivation methods is the preparation of media. A variety of culturing media have been developed to culture soil bacteria, ranging from simple,

commercially available media to media requiring a punctilious and skillful assembly to prepare in the laboratory. When designing or selecting a medium to use, it is important to understand the basic concepts of medium composition that may support or limit bacterial growth. The major nutritional requirements for bacterial growth that must be considered include carbon sources, energy sources (i.e., electron or hydrogen sources), nitrogen sources, electron acceptors, sources of other major mineral nutrients (e.g., sulfur, phosphate, potassium, magnesium, and calcium) vitamin requirements, and trace metal requirements (Gottschal et al. 1992; Tanner 1997). A good review of nutritional considerations for bacterial cultivation is provided by Tanner (1997).

A recipe for a basic basal medium for cultivation of bacteria adapted from Tanner (1997) is described in Table 2.1 (see Tanner (1997) for discussion of the selection of the various components included in the table and their roles). This basal medium consists of a mineral solution, vitamin solution, and trace metal solution. It can be used as a general-purpose medium to grow many types of bacteria of interest from wetland soils with the appropriate selection of carbon sources, energy sources, and electron acceptors. However, one point that should be kept in mind for use of this basal medium is it may require some modification for certain groups or species of bacteria. For instance, ammonium in the mineral solution presented in Table 2.1 would be removed from a medium designed to culture potential nitrogen-fixing bacteria. For recovery of sulfate reducing bacteria from wetland soil samples, ammonium chloride should be replaced with ammonium sulfate to increase the amount of sulfate available as an electron acceptor.

Carbon and energy sources for bacterial growth are usually considered together while working with chemoorganotrophs since they often use a single organic compound to satisfy both requirements. Virtually any organic substrate may be considered as a carbon and energy source, but some substrates, such as glucose, may be used by many species of bacteria, while others may be used by only a few species. Many chemolithotrophs can use carbon dioxide as a sole carbon source; others may require organic carbon such as acetate as a carbon source or for specific biosynthetic reactions. Major energy sources for chemolithotrophs are hydrogen, sulfide, and other reduced sulfur compounds (Tanner 1997). Phototrophic bacteria (both photoautotrophs and photoheterotrophs) use light as the energy source for their growth. Photoautotrophs can use carbon dioxide as the sole source of carbon and photoheterotrophs use simple organic compounds such as acetate, pyruvate, malate, and ethanol as carbon sources. The nitrogen requirements of bacteria can range from inorganic nitrogen (ammonium and nitrate) to organic nitrogen compounds such as urea and amino acids. Nitrogen gas can be used as a sole nitrogen source for nitrogen-fixing bacteria. Oxygen, nitrate, sulfate and carbon dioxide are common electron acceptors that can support bacterial respiration.

Although there are very useful generalities in regard to various requirements of bacteria for carbon and energy sources, nitrogen sources, and electron acceptors (as discussed above), the exact nutritional requirements of many strains of bacteria are still unknown. For this reason and for convenience, soil bacteria are often cultured on media containing undefined components (e.g., yeast extract or soil

Table 2.1 A general-purpose basal medium used for culturing bacteria

Component	Amount	Description
Mineral solution	**g/l**	A solution containing the major inorganic components required for microbial growth. Add and dissolve each component in order. The mineral solution can be stored at room temperature
NaCl	80	
NH_4Cl	100	
KCl	10	10 ml of the mineral solution is used for 1 liter of the basal medium
KH_2PO_4	10	
$MgSO_4 \cdot 7H_2O$	20	
$CaCl_2 \cdot 2H_2O$	4	
Vitamin solution	**mg/l**	A solution designed to meet the water-soluble vitamin requirements of many microorganisms. Store at 4 °C in the dark
Thiamine-HCl	10	
Thiamine-HCl	5	10 ml of the vitamin solution is used for 1 liter of the basal medium
Riboflavin	5	
Calcium pantothenate	5	
Thioctic acid	5	
p-Aminobenzoic acid	5	
Nicotinic acid	5	
Vitamin B_{12}	5	
Mercaptoethanesulfonic acid (MESA)	5	
Biotin	2	
Folic acid	2	
Trace metal solution	**g/l**	A solution designed to meet the trace metal requirements of many microorganisms. Store at 4 °C
Nitrilotriacetic acid Adjust pH to 6 with KOH	2.0	0.5–5 ml of the trace meal solution is used for 1 liter of the basal medium
$MnSO_4 \cdot H_2O$	1.0	
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.8	
$CoCl_2 \cdot 6H_2O$	0.2	
$ZnSO_4 \cdot 7H_2O$	0.2	
$CuCl_2 \cdot 2H_2O$	0.02	
$NiCl_2 \cdot 6H_2O$	0.02	
$Na_2MoO_4 \cdot 2H_2O$	0.02	
Na_2SeO_4	0.02	
Na_2WO_4	0.02	
Buffer		The buffer used is selected for each medium
TES (pK_a 7.4) for neutral pHs		1–20 g of the selected buffer is used for 1 liter of the basal medium
$NaHCO_3$ for CO_2-containing gas phases		
MES (pK_a 6.1) for acidic pHs		
phosphate for lactic acid bacteria or thiobacilli		
TAPS (pK_a 8.4) or glycine (pK_a 9.8) for alkaline pHs		
Final pH adjustment with NaOH	7.0–7.5	This should be adjusted as required for individual cultures or experiments

This medium is adaptable to pure culture or ecological studies with the appropriate selection of carbon sources, energy sources, and electron acceptors. A general source of growth factors, such as yeast extract (0.1–2.0 g/l), must be included for many studies

extract) or on undefined media. Yeast extract is one of the most efficient and commonly used undefined medium components (Atlas 2004). This complex mixture of acetate, amino acids, peptides, nitrogen bases, fermentable carbohydrates, vitamins, trace metals, and phosphate can support the growth of most known bacterial strains. A concentration of yeast extract as low as 50 mg/L can enhance the recovery of microorganisms from environmental samples in an otherwise defined medium (Tanner 1997). Soil extract from the source sediment contains a complex mixture of nutrients which promote the growth of indigenous bacteria and, therefore, increase the recovery of culturable bacteria. Soil extract has been routinely used in culture media for examining bacteria of wetland soils (e.g., Dedysh et al. 2006).

Commonly used undefined media for the isolation, cultivation, and maintenance of a wide variety of heterotrophic bacteria from freshwater wetland samples include nutrient broth, Luria-Bertani (LB) broth, and tryptic broth (Difco Laboratories, Inc., Detroit, Mich.). These commercially available media are pre-mixed powders and can easily be prepared. Many microbiologists use the agar plates made of one or two of them for total heterotrophic bacteria counts in an environmental sample. Bacteria from saline environments generally require salt or salts for their cultivation, and in most cases, this requirement can be met by adding 10–20 g of sodium chloride per liter of medium. Marine agar is often used for cultivating heterotrophic bacterial community from salt marsh samples. In addition, there are literally thousands of microbiological medium formulations available from a few handbooks or manuals (Atlas 2004; Balows et al. 1992; DIFCO Laboratories 1985; Gherna et al. 1992) that can be consulted for specific groups of bacteria, such as oil-degrading bacteria, sulfate-reducing bacteria, methanogens, or methanotrophs. It should be pointed out that many soil bacteria may be divided into two operationally defined classes: copiotrophs that have higher growth rates on rich complex media and oligotrophs that grow better on similar media used by copiotrophs but diluted by a factor of 10–100. Therefore, it may be best to use two sets of agar plates containing both rich and dilute media in order to ensure that the widest diversity of aerobic heterotrophs are recovered (Ogram and Feng 1997). Acidic (typically pH values below 4.0) northern peatlands are nutrient-poor by nature; the total concentration of mineral nutrients in these wetlands is typically in the range of 5–50 mg L^{-1}, which dramatically contrasts to most commonly used nutrient media with a salt content of 1–3 g L^{-1}, explaining why most peat-inhabiting bacteria do not grow on conventional media (Dedysh 2011). Strongly dilute, acidic media have been demonstrated to be one major prerequisite for successful cultivation of bacteria from the peat bog environment (e.g., Cadillo-Quiroz et al. 2009; Dedysh et al. 1998; Kip et al. 2011).

Once a medium formulation is determined based on the objective of the study, one can prepare the medium basically by constituting all of the medium components at the proper concentration in solution, and then adjusting the pH. However, two precautions during medium preparation should be followed. First, a gelling agent (commonly agar) should be added after the pH is adjusted. Second, components that break down under the conditions of autoclaving

(generally heating the medium to 121 °C at 100-kPa gauge pressure for 10–20 min) should be filter sterilized using a sterile filter with a pore size of 0.2 μm, and then added to the autoclaved medium solution after it has cooled.

2.3.1.2 Isolation of Strains

Cultivation of bacteria from environmental samples on synthetic media is perhaps the single largest source of bias in cultivation-based community analysis. Thus, the method of cultivation and incubation conditions is of primary importance. Strain isolation is typically accomplished by dilution plating. The suspension of bulk soil or rhizosphere soil sample (see above) is serially diluted in tenfold steps in sterile buffer (commonly phosphate buffered saline), and the aliquots (a volume of 100 or 200 μl per plate) from appropriate dilutions are spread onto solid growth medium to produce between 30 and 300 colonies per plate. The plates are then incubated at an appropriate temperature for a suitable time period in the dark. The control of the incubation temperature is important as bacterial populations within communities can have different temperature optima. The recovery of microorganisms from many common environmental samples can be inhibited by incubation at 37 °C, one of the common temperatures of laboratory incubators, while room temperature of 20–23 °C incubation is better for many environmental samples (Tanner 1997). Temperatures around 25 °C have been frequently used for cultivating bacterial community from various wetland samples (e.g., Calheiros et al. 2010; Williams and Crawford 1983; Zenoff et al. 2006). Incubation time is also of great importance because growth rates among soil bacteria can be highly variable. For instance, some bacteria are capable of forming visible colonies on solid growth medium after less than 1 day of incubation; others may require a few weeks of incubation to appear on plates. We found CFU counts of coastal marsh sediments on marine agar incubated at 25 °C increased approximately by 20–80 % when incubation time was extended from 1 to 2 days. Thus, plates should be examined daily for the appearance of new colonies to increase the recovery of the culturable bacterial community.

Oxygen is one of the primary physiochemical factors that affect bacterial growth and should be considered and adjusted when cultivating bacteria from environmental samples. Regulating the availability of oxygen is in particular critical to studies of bacterial communities in wetland sediment. Wetland ecosystems are characterized by hydric soils and hydrophilic plant communities (Mausbach and Parker 2001). Their fluctuating hydrology gives rise to interplay between aerobic and anaerobic processes in sediments (Davidsson et al. 1997). It is well established that wetlands support anaerobic bacteria such as methanogens, sulfate reducers, fermenters, acetogens, and denitrifiers (Conrad 1996), which are major functional groups involved in the cycling of soil carbon and nutrients in wetlands and are of particular interest to many microbiologists. Standard plating medium and incubation conditions (exposure to air) are appropriate only for isolation of aerobes. As a rule of thumb, all steps in preparation of medium and inocula for the "anaerobic community" should be performed under an oxygen-free gas stream (commonly

nitrogen) and the inoculated medium incubated in anaerobic conditions. Typically, anaerobic medium is prepared by boiling the medium under nitrogen gas (N_2) to reduce dissolved oxygen below a detection limit of 20 ppb, sealing the medium and transferring it into an anaerobic chamber where it is dispensed for further manipulation or sterilization. The Coy anaerobic chamber, one of the commonly used in many laboratories, can easily maintain an oxygen level of 0 ppm and is easy to use and maintain. Anaerobes can be handled in such a chamber much as the way aerobes are handled in an open biosafety hood. The Coy chamber also can be used as anaerobic incubator, and plates and cultures can be incubated inside of the chamber at a desired controlled temperature. Often, anaerobic bacteria are cultivated in specialized glassware such as Hungate anaerobic culture tubes, and the Most Probable Number (MPN) method is used for enumeration of their populations in environmental samples. The butyl rubber stopper used with the Hungate tubes allows for the syringe transfer technique which largely alleviates the difficulties of the general culture of strictly anaerobic bacteria. In addition, the culture of many extremely strict anaerobes such as methanogens requires a low redox potential of the medium which elimination of oxygen alone will not achieve. Thus, reducing agents must be incorporated into the medium after oxygen has been removed. Cysteine, sulfide, thioglycolate, dithionite, glutathione, yeast extract, ascorbic acid, and dithiothreitol are primary reducing agents used for the culture of anaerobes (Tanner 1997). A general cysteine-sulfide reducing agent has been widely used by many researchers. It can be stored in an anaerobic environment for at least a year and can be prepared as described by Tanner (1997). The mixture of cysteine-sulfide can be added at 1–10 ml per liter of anaerobic medium. However, it needs to be pointed out that many conventional reducing agents such as cysteine-sulfide and ascorbic acid may not support growth of methanogenic consortia from acidic peatlands (Sizova et al. 2003), whereas titanium (III) citrate has been successfully used to isolate several peat-inhabiting methanogens (Bräuer et al. 2006; Cadillo-Quiroz et al. 2009) and facultatively anaerobic bacteria (Pankratov et al. 2012; Sizova et al. 2007).

After incubation, the number of colonies appearing on the plates is counted. Plates containing between 30 and 300 colonies are examined and the colonies randomly selected for further characterization. The number of colonies that should be chosen for further analysis for each sample is usually determined by practical considerations of plans for further analysis and the investigator's limitations and resources. Selected colonies should be purified by streaking (Fig. 2.3) them onto fresh medium at least two successive times. Purified isolates are stored in 15 % glycerol at −80 °C for future analyses.

2.3.1.3 Strain Identification

Once culture collections of a sample have been established, a number of different phenotypic and genotypic methods can be employed for strain identification and classification (Louws et al. 1996). Primary methods include characterization based

Fig. 2.3 Streaking for purification of bacterial colonies on an agar medium

on colony morphology, growth substrates, lipid analysis, and nucleic acid analysis such as whole-cell hybridization, DNA fingerprinting, and 16S rRNA gene sequencing. Each of these methods permits a certain level of phylogenetic classification. Characterization of environmental isolates by colony morphology is the simplest, oldest means of differentiating between species for the purposes of diversity analysis. The method groups similar colony types into morphotypes based on the color, size, and shape of colony and level of mucoidy at various stages of colony age. However, this crude grouping system does not allow identification of species but may be useful to reduce the number of isolates for further analyses by more sophisticated means. Traditionally, environmental isolates have been most commonly identified by growth on specific substrates (Kennedy 1994), and various systems are commercially available for this type of analysis. The BIOLOG system manufactured by Biolog, Inc. (Hayward, CA) is among the most commonly used. Its latest generation redox chemistry enables testing and microbial identification of aerobic Gram-negative and Gram-positive bacteria in the same test panel. Thus, Gram stain and other pre-tests are no longer needed. Strain identification also may be based on the presence of specific cellular lipids. There are a variety of fatty acids in the lipids of bacterial cells. Different bacterial taxa have different patterns of combinations of these fatty acids (Dembitsky et al. 1992) and the distinct characteristic patterns can be analyzed quantitatively to provide taxonomic information at the species level (White et al. 1979). Thus, one can identify the unknown strain by comparison of its fatty acid pattern with those of reference strains in an existing database. Phospholipid fatty acids (PLFA) are a group of lipid components of the

microbial cell membranes and are commonly used in this type of analysis. One limitation associated with this method is the limited database of environmental strains, which may not allow many unknown environmental strains to be identified.

The most powerful, precise method of determining the phylogenetic position of an isolate is by analysis of nucleic acid. This type of analysis has become the most popular and commonly used tool for accurately deducing phylogenetic and evolutionary relationships among environmental isolates. The technique is based on the concept that rRNAs, in particular 16S and 23S rRNA, are highly conserved between different species of bacteria and archaea throughout evolution (Weisburg et al. 1991) and thus very useful as a measure of phylogenetic relationships of environmental isolates (Lane 1991; Olsen and Woese 1993). The frequencies of compositional changes (substitution rates) at different positions in the 16S and 23S rRNA molecules (a good length of about 1,500 and 3,000 nucleotides for 16S and 23S, respectively) vary greatly by at least two orders of magnitude. The diversity of rates of change within the rRNAs makes it possible that these molecules can be utilized over a wide spectrum of phylogenetic distances, from the full span of the universal tree at one extreme to distinctions among species within the same genus (Olsen and Woese 1993). The regions of the molecules that change at a very slow rate over evolutionary time are characteristic of broad phylogenetic groupings such as domains, while the ones that change at a faster rate are characteristic of narrower phylogenetic groupings such as the *Proteobacteria*, and the ones that change fastest are characteristic of individual species (Lane 1991).

Among commonly used methods for rRNA characterization are whole-cell hybridization with labeled synthetic oligonucleotide probes, sequencing of the 16S rRNA genes, and DNA fingerprinting such as rep-PCR genomic fingerprinting and restriction fragment length polymorphism (RFLP). Whole-cell hybridization with rRNA-targeted fluorescent probes (usually Cy3-labeled) can be used to quickly screen large numbers of isolates toward specific phylogenetic groups at levels from domain (*Bacteria, Archaea*, and *Eukarya*) to narrower phylogenetic groups such as *Betaproteobacteria, Alphaproteobacteria, Actinobacteria, Gammaproteobacteria, Firmicutes, and Bacteroidetes* and to numerous individual species. Standard procedures basically involve transferring of the cultures to a DNA binding membrane, lysing with a detergent (sodium dodecyl sulfate or sodium hydroxide), fixing of the released DNA to the membrane, and hybridizing with the labeled probe. The presence of the genes is typically detected by autoradiography. A great number of probes for identification of a range of phylogenetic groups have been developed. However, probe design relies on the quality of the rRNA database. The rRNA diversity of environmental microorganisms only has been partially described despite great progress made in maintaining and enlarging the database over the last two decades. Our limited knowledge of the rRNA sequences of many environmental microorganisms is still one major limitation associated with this approach.

Direct sequencing of individual 16S rRNA genes from genomic DNA isolated from individual colonies allows precise phylogenetic placement of the isolates. This procedure provides much more information on molecular evolutionary

genetics of the isolates relative to colony hybridization with oligonucleotide probes. The procedure involves several steps as follows: isolation of genomic DNA from selected colonies, PCR-amplification of the 16S rRNA genes using primers specific to these genes, separation of the amplification products by agarose gel electrophoresis, extraction of the amplification products from the gel, and cloning and sequencing of these DNA fragments for phylogenetic characterization. These general steps are similar to those used in 16S rRNA gene-based cloning libraries for bacterial community analysis that will be discussed below.

A rapid way to distinguish closely related environmental isolates is by means of DNA fingerprinting (e.g., Rep-PCR). DNA fingerprints are generated when DNA fragments of the genome are separated on agarose or polyacrylamide gels. This approach, regardless of the specific technique, generates a banding pattern, or fingerprint, unique to the genome under investigation. Rep-PCR is based on the use of DNA primers specific to naturally occurring, highly conserved, repetitive DNA sequences within bacterial genomes and the PCR reaction (Versalovic et al. 1991; de Bruijn 1992). Three families of repetitive sequences have been identified; they are the 35–40 base pair (bp) repetitive extragenic palindromic (REP) sequence, the 124–127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX sequence. These appear to be located in distinct, intergenic positions around the genome (Versalovic et al. 1994). The corresponding protocols to selectively amplify distinct regions located between these repetitive DNA elements in the genomes are referred to as REP-PCR, ERIC-PCR, and BOX-PCR genomic fingerprinting, respectively. Collectively the three methods are termed rep-PCR genomic fingerprinting. The PCR-amplified fragments can be separated in a gel, yielding a profile referred to as a rep-PCR genomic fingerprint (Versalovic et al. 1994). Computer assisted pattern analysis programs (e.g., GelCompar) can be used for the identification and classification of microbes using cluster analysis algorithms. Overall, rep-PCR is a highly reproducible and simple method to identify bacterial species and strains and to deduce phylogenetic relationships between strains. A detailed description of rep-PCR genomic fingerprinting protocols (i.e., generation of raw data, comparison of fingerprints, and different algorithms used to find groupings in the data and to identify specific strains in a database using their genomic fingerprints) can be found in Rademaker and de Bruijn (1997).

2.3.2 Cultivation-Independent Methods

Conventional cultivation-based methods usually are laborious and time-consuming, and due to their inevitable selectivity, only recover a fraction of a complex microbial community. To overcome difficulties and limitations associated with laboratory culture, various cultivation-independent methods have been developed for studying microbial communities in environmental samples including wetland soil samples. Among commonly used ones are the16S rRNA gene-based cloning

library, denaturing gradient gel electrophoresis (DGGE), T-RFLP, and fluorescence in situ hybridization (FISH). Since most of these methods involve an initial step of PCR amplification of DNA directly extracted from environmental samples, we will first discuss the basic procedures for genomic DNA extraction from wetland soils and for PCR amplification of the DNA fragment of interest, followed by discussion of specific techniques for bacterial community analysis.

2.3.2.1 DNA Extraction

Protocols for the isolation and purification of bacterial community DNA from soil may generally be grouped into two categories. The first approach lyses bacterial cells in the presence of soil. This direct lysis approach employs high temperature (typically 70 °C), lysozyme or harsh detergent (e.g., sodium dodecyl sulfate), and mechanical disruption (typically by glass beads or sands) for cell lysis. To increase the lysis efficiency, soil samples can be subjected to three repeated cycles of grinding with liquid nitrogen using a sterile pestle and a mortar (Dedysh et al. 2006) or three cycles of freezing and thawing, followed by further extraction steps. The freed DNA can be recovered and purified by procedures such as cesium chloride (CsCl) equilibrium density centrifugation or phenol-chloroform-isoamyl alcohol extraction. The DNA can be precipitated with ethanol (70 %) and the purified DNA re-suspended in TE buffer or sterile distilled water. Once the DNA has been purified, it can then be subjected to further manipulation and analysis. The second approach, bacterial fractionation, first extracts intact bacterial cells from soil by bringing the entire soil sample into suspension via homogenization in buffer (e.g., phosphate buffer, soluble sodium solution, or calcium solution). The suspension is then centrifuged at low speed to remove larger particulates, and the unattached bacterial cells are subsequently recovered by high speed centrifugation. Subsequent bacterial lysis and DNA recovery can be conducted by using procedures that combine cell wall digestion by lysozyme and proteinase, incubation at high temperature, and the removal of contaminants with polyvinylpolypyrrolidone and through equilibrium density centrifugation similar to those of direct lysis (Holben 1997). Or, lysis can be done directly in the thermocycler at a temperature of 98 °C after the cells are purified by removing humic materials (Pepper 1997). Both approaches have their advantages and disadvantages. However, in general, the direct lysis method recovers larger amounts of DNA and is more suitable for bacterial community-level analyses, relative to the bacterial fractionation method. A report by Holben (1997) describes the two approaches in detail and makes useful recommendations on the selection of either approach as appropriate for the characteristics of a given environmental sample.

A number of parameters that may affect the recovery of bacterial community DNA from soil and sediment samples should be considered when selecting a method. Two of the most important are bacterial biomass and contaminants in the sample. The bacterial biomass of a sample can determine how much soil is needed to produce usable amounts of bacterial community DNA. An average bacterium contains 9×10^{-9} μg

of DNA so that theoretically about 1.1×10^8 bacteria are needed to produce 1 μg of DNA. For samples of most surface soils, typically with total bacterial counts in the range of 10^8–10^{10} cells per g soil, 1 g samples may be sufficient to obtain $\geq$1 μg of DNA, while low-biomass soils may appear to be very difficult to produce sufficient DNA. In such cases, the bacterial fractionation approach should be considered since it allows recovery and concentration of bacteria prior to lysis (Holben 1997). Studies of wetland soil bacterial communities usually include the top 5–20 cm of bulk soil and often 0.2–1.0 g of soil are used for DNA extraction, although some investigators have used 5 or 10 g of soil to extract bacterial community DNA.

Contaminants are of great concern in the recovery and purification of bacterial DNA from wetland soils. Compounds such as polyphenolic substances (e.g., humic and fulvic materials) are frequently encountered in wetland soils and can co-purify with DNA. Such contaminants in a DNA extract can result in two major problems. First, they confound DNA quantitation because of their significant light absorbance at 260 nm, the wavelength used for DNA quantitation. Second, they interfere with subsequent analyses of the DNA, in particular those involving enzymatic reactions such as PCR. Other major contaminant types include protein and RNA. Various DNA purification approaches as well as commercial kits are available, and many of them involve equilibrium density centrifugation, large-scale precipitation, preparative electrophoresis, exclusion chromatography, or organic extractions. Holben (1997) observed that a high degree of purity may be achieved through removal of polyphenolic compounds from solution by using polyvinylpolypyrrolidone and through two rounds of equilibrium density centrifugation. The bacterial community DNA localizes in a very discrete band in the central region of the gradient while the contaminating proteins float, the RNA is pelleted, and the remaining contaminants are dispersed throughout the gradient.

One should keep in mind that modification and optimization of existing protocols may be required to make the protocol especially suitable for particular sample types (e.g., peat bog samples containing high contents of organic matter and oil-contaminated sediment samples with large amounts of petroleum compounds). In addition, for convenience, researchers have increasingly turned to commercial kits such as UltraClean soil DNA kits or PowerSoil DNA extraction kits from MO BIO Laboratories, Inc. (Solana Beach, Calif.). Lauber et al. (2009) used a MoBio PowerSoil DNA extraction kit following the manufacturer's instructions, with an additional incubation step at 65 °C for 10 min followed by 2 min of bead beating to limit DNA shearing to obtain DNAs from soil samples collected from 88 sites representing a wide range of ecosystem types across North and South America in a pyrosequencing-based assessment of soil bacterial community structure.

The quantity and quality of DNA can be checked using a spectrophotometer (e.g., a Nanodrop instrument). The quantity is determined by measuring optical density at A260 nm wavelength. The A260/A280 nm wavelength ratio should be between 1.8 and 2.0 for high quality DNA preparations. Should the ratio be lower than 1.8, it may indicate that the DNA preparation is not sufficiently clean for enzymatic application, and the DNA would require re-purification. Following isolation, the DNA is stored at −20 °C or −80 °C.

2.3.2.2 PCR Amplification

The purpose of PCR amplification is to increase sensitivity of detection of a DNA sequence present in trace amounts in total community DNA extracted from a sample. PCR is an enzymatic reaction allowing amplification of target DNA via a repetitive process in vitro. Theoretically, during each cycle of PCR, any target DNA present in the reaction is copied and the amount of the DNA therefore doubles, as expressed below:

$$P = (2)''T$$

where P is the product of PCR, n is the number of PCR cycles, and T is the initial number of template DNA copies with which the reaction was started. In practice, 25 cycles of PCR result in approximately a one million-fold increase in the amount of initial DNA (Pepper 1997). This amplification produces target DNA in sufficient concentrations to be purified through gel electrophoresis and visualized under UV light after staining with ethidium bromide.

The choice of the primer sequences largely determines whether or not a target DNA sequence can be successfully PCR-amplified. Generally, most primers are two short sequences (a primer pair, 16–30-bp) that can anneal at specific, different sites on the chromosome and thus initiate the amplification of target DNA. Primer sequences can be designed manually based on the known sequences of the target DNA, which are available in the NCBI database (http://www.ncbi.nlm.nih.gov). The retrieved sequences from the database can be aligned using the ClustalX program (Thompson et al. 1997) and the sequence alignment opened with the GenDoc program (http://www.nrbsc.org/gfx/genedoc/index.html). Alternatively, primer sequences can be deduced by the use of primer design software. The primers can be specific to a given species, genus, or broader groups of bacteria depending on the aim of the investigator. Once potential primers have been identified, the degree of primer specificity and the sensitivity of amplification need to be evaluated. If a primer pair is required to amplify specific DNA at a species or genus level, the pair must only allow amplification of the target species- or genus-specific DNA and not any other, even closely related, species- or genus-specific DNA. Total bacterial community analyses of a wetland soil sample often are conducted on 16S rDNA. PCR amplifications can be performed by using universal primers from conserved regions of DNA so as to allow amplification of 16S rDNA fragments present in all bacteria. Among others, the bacteria-specific primers Eub9f and Eub1492r (Lane 1991), 27f (5′-AGAGTTTGATC CTGGCTCAG-3′) and 1392R (5′-GACGGGCGGTGTGTAC-3′) (Lane 1991), and BSF 343/15 (TACGGRAGGCAG) and BSR 926/20 (CCGTCAATTYTTTRAGTT) (Wilmotte et al. 1993) have been used in 16S rDNA-based bacterial community analyses of wetland soils (Brofft et al. 2002; Dedysh et al. 2006; Hartman et al. 2008). The degree of specificity of primers can intentionally be varied by primer design, and also by changing the annealing temperature in PCR protocols. Generally, the specificity of amplification increases with increasing annealing temperature due to a decrease in the number of base pair mismatches

allowable for hybridization. However, there are trade-offs between specificity and sensitivity. First, there is an associated decrease in sensitivity along with increased specificity (Pepper 1997); second, the extent of preferential amplification of multitemplate PCR is in relation to increasing annealing temperature with certain primers such as the 63F primer hybridizing to phylogenetically highly conserved regions of 16S rRNA genes (Sipos et al. 2007). In general, the maximum allowable annealing temperature is 10 °C less than the melting temperature which depends on percent G + C (Pepper 1997). Sensitivity of amplification is important when a target DNA is in low concentration in a soil sample. Sensitivity can be evaluated by using whole cells or pure genomic DNA (see Pepper (1997) for details). One way to increase sensitivity is increasing the number of cycles. However, increasing the number of cycles may result in PCR bias, especially when subsequent analyses such as T-RFLP or sequencing are employed. Normally, the number of cycles used in PCR is around 25 and should not exceed 35.

A PCR protocol involves a PCR reaction mixture and a thermal cycle profile. The reaction mixture must contain all the components needed for PCR amplification, including DNA polymerase, dNTPs, buffer, primers, and DNA template. The various components are added at the proper concentration to a polypropylene tube usually yielding a total volume of 20–50 μl. The thermal cycle profile comprises repeated cycling of temperatures to (i) denature template double stranded DNA resulting in a single strand, (ii) anneal primers to the denatured (single-stranded) template DNA, and (iii) extend from the 3′ ends of primers to complete the synthesis of a copy of each single strand. A standard thermal cycle profile for 16S rDNA amplification typically consists of an initial denaturation at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 or 55 °C for 30 s or 1 min, and extension at 72 °C for 2 min, and a final extension step at 72 °C for 7 min. The temperature and length of time for each step and the number of cycles can be modified and optimized for specific PCR amplifications, depending on the purpose of the experiment. For every PCR assay, the use of negative controls that contain no added DNA as template is required to check laboratory contamination.

PCR products can be separated by electrophoresis in a 1 % agarose gel and visualized after staining with ethidium bromide (0.5 mg per liter). The band with expected size or others of interest can be excised and the DNA extracted with commercial kits such as QIAquick gel extraction kit (Qiagen, Valencia, CA). This purification procedure is necessary because sometimes PCR products generate multiple bands. At this stage, purified PCR products are ready for further analysis of the bacterial community.

The technique involved in PCR amplifications has revolutionized molecular biology methodologies with the advantages of increased speed and sensitivity, and relatively low costs. However, there are also some well-known disadvantages associated with PCR amplification, leading to pitfalls in PCR-based community analysis. For instance, a bias in the amplification of particular sequences as a result of preferential annealing to particular primer pairs (i.e., primer bias; Suzuki and Giovannoni 1996), or an increase in the incidence of chimeric PCR products with

increasing numbers of PCR cycles (Wang and Wang 1996) reduces the quality of quantitative characterization of bacterial communities. Possible biases in the relative rates of PCR amplification of rRNA genes due to differences in copy number and proximity of the operons (Farrelly et al. 1995) may also have a bearing on the quantitative interpretation of the microbial community data gained by PCR-based methods. In addition, there may be potential bias of separate PCRs, and in order to reduce such bias, often the amplicons of two independent PCRs are combined before further analysis such as cloning.

2.3.2.3 16S rRNA Cloning Library, RFLP, and Sequence Analysis

One standard approach to analysis of bacterial communities within a soil sample is to extract soil community DNA, PCR-amplify 16S rDNA fragments using universal primers, and construct a 16S rRNA gene clone library, followed by RFLP screening and subsequent sequence analysis of the clones or directly by sequence analysis. This approach identifies or characterizes individual members of a community and generates the most detailed information concerning their phylogenetic affiliations by sequencing each of the individual clones. Since the PCR products of community 16S rDNA are heterogeneous molecules of similar sizes originating from the various organisms present in the sample, populations of molecules need to be separated by cloning for further analysis. The purified PCR products can be cloned in vectors specifically designed for this purpose using commercial kits such as TOPO TA cloning kits (Invitrogen Corp., Grand Island, NY). Clones with inserts of target DNA are screened on LB plates containing 50 μg/mL of kanamycin (kanamycin as the selective agent).

Once the clone library of a sample has been created, the number of clones subject to further sequence analysis may be reduced by RFLP grouping. RFLP analysis is a technique exploiting variations in homologous DNA sequences, in which the purified DNA from individual clones is digested into pieces by a variety of restriction enzymes and the resulting restriction fragments are separated by gel electrophoresis according to their lengths. Theoretically, differences in sequence of a specific gene between different clones may result in a different number of sites recognized by the restriction enzymes, and thus generate different restriction patterns. The gels can be stained with ethidium bromide and photographed under UV light. The DNA fragment size of each band that appears in the gel is determined by comparison to the marker DNA bands using software such as Quantity-One (BioRad Laboratories, Hercules, CA). Restriction patterns of individual clones within the clone library can be sorted and grouped into different phylotypes. A phylotype is usually defined as a group of 16S rDNA sequences that have indistinguishable restriction patterns. The clones with identical patterns for restriction profiles are grouped into the same phylotype (i.e., OTU). The number of phylotypes and the number of clones in each of them often are used for analysis of community diversity (composition, richness, and structure). It should be noted that the restriction endonucleases (such as *Taq*I, *Hae*III, and *Sau*3A) that recognize 4 bp, rather

than 6 bp, are usually used in RFLP analysis of 16S rDNA since this rRNA gene is only approximately 1.5 kb in length and highly conserved. Also, in practice, at least two restriction enzymes should be used in independent digestions to improve resolution of restriction patterns.

Following RFLP grouping, usually one representative from each phylotype is sequenced using ABI BigDye (Applied Biosystems, Inc., Grand Island, NY) on a sequencer such as ABI 3700 capillary DNA sequencer or ABI Prism 377 DNA sequencer as specified by the manufacturer. Alternatively, and more often now due to the rise of inexpensive DNA sequencing technologies, investigators have chosen to sequence all clones included in the library. Sequencing all clones will avoid RFLP-associated biases which can cause an underestimation of community diversity. It has been observed that some clones with identical RFLP patterns may have sequences with <97 % sequence similarity among them (Bae and Hou 2007, unpublished data).

DNA sequences should be deposited in GenBank where each unique sequence will be assigned an accession number. The sequences can be compared to those in the NCBI Blast (Benson et al. 2000) and RDP sequence classifier databases (Maidak et al. 2001) for identification. One should keep in mind that amplification of mixtures of DNA molecules may result in the formation of so-called chimeras (i.e., amplification products composed of the 3′ end of one molecule and the 5′ end of another, usually present in less than 10 % of the clones obtained). Therefore, sequences, in particular poorly matching sequences (<65 % identity), should be screened for chimeric recombination using a program such as RDP Chimera Checker available through the RDP homepage (http://www.rdp.life.uiuc.edu). Phylogenetic analyses can be carried out using computer programs such as the PHYLIP program package (Felsenstein 1989) or the Mothur pipeline. Phylogenetic trees of sequences in the clone library are constructed using methods such as the neighbor-joining (Saitou and Nei 1987) in the MEGA program (Tamura et al. 2011). Robustness of phylogenetic inference is evaluated by bootstrap analysis based on 1,000 replications.

The separation of a mixture of PCR amplicons from community DNA samples can be carried out by cloning as discussed above or by fingerprinting techniques such as DGGE and T-RFLP (referred to as whole library or total library in contrast to the clone library) that will be discussed below.

2.3.2.4 DGGE

The DGGE analysis of 16S rRNA gene fragments amplified by PCR from DNA directly extracted from soil samples is a rapid profiling procedure for analysis of soil bacterial communities. It has been successfully used for comparison of the microbial community structures in wetlands (e.g., Hadwin et al. 2006). This technique separates DNA fragments based on their mobilities on an acrylamide gel containing a linearly increasing gradient of DNA denaturants (commonly formamide and urea). In general, DNA fragments richer in G-C pairing (3 hydrogen

bonds per pairing) are more stable and remain double-stranded until reaching higher denaturant concentrations, relative to those richer in A-T pairing (2 hydrogen bonds). Double-stranded fragments migrate faster in an acrylamide gel, while denatured fragments migrate slower or stop in the gel. Thus, DNA fragments that differ in sequence can be separated by electrophoresis on the gel under increasingly denaturing conditions. A typical standard DGGE analysis consists of loading of PCR products onto the denaturing gradient gel, separation of the products by electrophoresis in buffer (usually 1 × TAE buffer: 40 mM Tris–acetate, 1 mM EDTA, pH 8.0), staining with DNA binding fluorescent dyes (e.g., SYBR Green or silver), and visualization of the gel under UV light. Known DNA standard fragments of various sizes may be used for comparison to the DNA bands formed on the gels. A detailed description of DGGE analysis is presented by Heuer et al. (2001). It is worth mentioning that an additional acrylamide gradient (e.g., 6–9 %) may be used to enhance the bands' resolution and sharpness (Gomes et al. 2005).

Ideally one 16S rDNA band in the gel corresponds to one bacterial species so that the number of bands reflects the bacterial diversity of the sample. The intensity of each band relative to the intensity of all bands in the sample can be used to estimate the relative abundance of various corresponding species. Bacterial community profiles can be analyzed with cluster analysis. The cluster analysis is based on Pearson's correlation index and can be done by UPGMA (unweighted pair-group method with arithmetic averages, also known as average linkage) (Smalla et al. 2007). The DNA fragments of each band can be excised, cloned and sequenced for phylogenetic characterization as discussed in the section of "the16S rRNA cloning library, RFLP, and sequence analysis". The easier approach to characterization of differentiating bands by cloning and sequencing is perhaps the great advantage of DGGE when compared to other fingerprinting methods such as T-RFLP. The major drawback of DGGE is the potential for gel-to-gel variation (Nunan et al. 2005) which can complicate comparisons between gels and laboratories. Using known standards may help solve this problem. Moreover, gels of complex communities may look smeared due to the large number of bands. This makes DGGE more suitable for samples with fewer species. In addition, it is possible for some phylogenetically distantly related taxa to generate the same DGGE band, thus resulting in an underestimation of the true diversity in the sample.

2.3.2.5 T-RFLP

Another rapid bacterial community profiling procedure is T-RFLP, which was originally developed to identify mycobacteria (Avaniss-Aghajani et al. 1996) and has now been automated to process multiple samples in a short time-span to obtain information on microbial community structure of environmental samples. In this approach, 16S rRNA genes in a soil sample are PCR-amplified using a fluorescently-labeled primer (or both primers, each labeled with a different fluorescent dye) to yield a mixture of end-labeled 16S rRNA genes. These PCR products

are then digested with restriction endonuclease to produce labeled terminal restriction fragments (T-RFs). *Hha*I (recognizes 4 bp fragments) is the most commonly used restriction enzyme in such a digestion due to its higher heterogeneity relative to several others including *Alu*I, *Hae*III, *Msp*I, and *Scr*FI (Ulrich and Becker 2006). The digested product is mixed with an internal size standard (e.g., GeneScan 1000 Rox, Applied Biosystems) that is labeled with a different fluorescent dye. The fragments are then denatured by heating (usually at 95 °C) and separated by electrophoresis using either gel- or capillary-based systems, with laser detection of the terminal, end-labeled fragments using an automated analyzer (e.g., ABI 310 DNA sequencer, Applied Biosystems). T-RFs are evaluated by software such as GeneScan Analytical Software. Comparison of the migration time of the T-RFs with internal standards permits accurate sizing of the fragments to within ± 1 nucleotide. Two forms (electropherogram and numerical table) of output can be generated from such a T-RFLP analysis. The electropherogram presents the profile of a bacterial community as a series of colored peaks of varying heights. The T-RFLP profile can provide information on the number and size, in bp, of T-RFs present in the profile as well as the height of each T-RF peak. The numerical output consists of a table that includes the size (in bp, calculated by comparison with the internal standard) of each of the T-RF peaks and the height (relative to the amount of fluorescence detected) of each peak. Ideally, each T-RF represents a single species, although in practice bacteria of different species frequently share one T-RF (therefore, it is recommended that two or three different restriction enzymes be used for accurate identification of a microorganism on the basis of T-RF size). The height of each peak can be used to determine the relative abundance of corresponding microorganisms of a community (Osborn et al. 2000). The Jaccard distance (1 − Jaccard coefficient) is commonly used to analyze T-RFLP profiles of the bacterial communities based on presence/absence of T-RFs and their relative abundance. The Jaccard coefficient is equal to the ratio of the number of T-RFs in common between two profiles to the total number of T-RFs present in both profiles. The Ward algorithm (Ward 1963) is applied to create a dendrogram when clustering the distance metrics.

Studies have proven that T-RFLP is a robust and reproducible methodology for soil microbial community analysis, yielding community fingerprint profiles with high resolution (Tiedje et al. 1999; Osborn et al. 2000; Smalla et al. 2007). The technique has been used to examine total sediment bacterial communities and specific functional groups in wetland soils (e.g., Angeloni et al. 2006). The major merit of T-RFLP is its suitability for routine analysis of large numbers of samples due to its reproducibility and capability of being automated. Moreover, when comparing the potential of DGGE and T-RFLP to unravel the bacterial community diversity of complex environmental samples, T-RFLP appears to have greater resolution power (Horz et al. 2001; Nunan et al. 2005), although both techniques lead to similar general findings (Moeseneder et al. 1999; Nunan et al. 2005; Smalla et al. 2007). T-RFLP also suffers from a number of limitations. As in the case of DGGE, phylogenetically different species of bacteria may share the same T-RF, which will lead to an underestimation of the overall diversity within a bacterial

community. On the other hand, the generation of partially digested fragments due to incomplete digestion by the restriction enzyme may lead to an overestimation of the diversity (Osborn et al. 2000).

2.3.2.6 FISH

The composition of bacterial communities in soil samples can also be directly analyzed by rRNA-targeted nucleic acid probes. FISH is an excellent example of such techniques (Amann and Ludwig 2000). Unlike PCR-based community analysis methods (i.e., 16S rRNA gene-based cloning library, DGGE, and T-RFLP), FISH does not rely on PCR amplification but rather it uses fluorescent oligonucleotide probes specific for different bacteria to detect the presence or absence of specific DNA sequences in bacterial cells. The two antiparallel strands in the DNA molecule are held together by hydrogen bonds. If the hydrogen bonds are broken with heat or chemicals, the two complementary strands are able to re-bind to each other when conditions become favorable, which provides the basis for DNA molecular hybridization. The basic elements of FISH include an oligonucleotide probe and a target sequence. Bacterial FISH probes are often primers for the 16s rRNA region, and are labeled with the cyanine dyes Cy3 or Cy5, or Fluorescein-11-dUTP. A set of labeled probes specific for species (e.g., L. ferrooxidans and A. ferrooxidans), groups (e.g., α-, β-, and γ-subclass of Proteobacteria and Gram-positive bacteria with high or low GC content), and domains (Bacteria and Archaea) are commercially available, and have been used in FISH analyses of bacterial communities in wetlands (Dedysh et al. 2006; Kobabe et al. 2004; Nicomrat et al. 2006; Zhang et al. 2008). The FISH protocol primarily involves (i) soil sample fixation, either directly in ethanol (96 %) or in a freshly prepared 4 % (wt/vol) paraformaldehyde/phosphate-buffered saline (PBS) solution (pH 7.2), (ii) hybridization of the fixed samples to the respective fluorescent probes, and (iii) staining of the hybridized cells with the universal DNA stain 4, 6-diamidino-2-phenylindole (DAPI). The hybridized and DAPI-stained cell counts are determined under epifluorescence microscopy with a high-pressure mercury bulb, for instance, with a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with the Zeiss filters.

The FISH protocol has become a powerful tool for investigation of the composition of complex bacterial communities over the past two decades. The technique is not subject to the biases inherent in the PCR reaction. Moreover, it is able to reveal both qualitative and quantitative knowledge on the composition of bacterial communities. One of the limitations and biases of FISH is the high variability in the effectiveness of the detection of target cells. A quantitative review (Bouvier and del Giorgio 2003) of published papers on the percentage of cells detected using the common EUB338 probe (% Eub) in aquatic ecosystems reports that the % Eub ranges from 1 to 100 % in the different published papers, with an average of 56 %. Factors that influence the detection of bacterial cells using FISH include a methodological component such as the fluorochrome type

and the stringency conditions of the reaction as well as an environmental component such as the type of ecosystem and dominant phylogenetic group. Another bias of FISH may be the limited availability of relevant probes that may discriminate bacteria at the appropriate taxonomic level. It also needs to be pointed out that background signals of non-bacterial soil particles may complicate results of FISH in soil samples when using a single fluorescent probe. In such cases, a threefold staining procedure of the target-cells may warrant a more accurate assessment (Kobabe et al. 2004).

2.3.2.7 High-Throughput Technologies

Recently, a number of high-throughput technologies (e.g., next-generation DNA sequencing and GeoChip) have been developed to permit a more comprehensive assessment of bacterial communities at finer levels of taxonomic resolution. The 454 platform, the first of such next-generation platforms, is a highly parallel noncloning pyrosequencing system capable of sequencing up to 100 million bases in one 4-h run (Margulies et al. 2005). Pyrosequencing has the potential to revolutionize sequencing studies, including characterizing microbial community diversity (Hamady et al. 2008). This sequencing method has been successfully used to study the microbial community in soils (Roesch et al. 2007) and oceans (Sogin et al. 2006). In addition, multiplexed high throughput pyrosequencing of individual genes (e.g., 16S gene) by tagging or barcoding with short nucleotides (Huse et al. 2007; Roesch et al. 2007; Hamady et al. 2008; Meyer et al. 2008) has been developed to process many samples simultaneously. Studies demonstrate that the 454 pyrosequencing platform may generate tremendous sequencing information and is a powerful tool for studying the bacterial community. However, pyrosequencing is virtually constrained by cost limitations and a relatively high per-read error rate. More recently, the introduction of the Illumina sequencing has vastly decreased cost per sequence, resulting in a growing interest in amplicon sequencing on Illumina. Although Illumina sequences were once thought to be unsuitable for microbiome profiling due to their short sequencing reads, new improvements on sequencing platforms and software tools have circumvented the problem. Caporaso and colleagues (2011) demonstrated that short sequences generated by Illumina could be used to successfully reconstruct the bacterial communities in environmental samples. They also demonstrated that this technology could possibly allow large-scale studies that analyze thousands of samples simultaneously to profile microbial communities at an unprecedented spatial and temporal resolution (Caporaso et al. 2011).

GeoChip is another robust technology to study the structure and function of microbial communities in environmental samples. Although conventional molecular methods, such as PCR-based cloning and FISH are very useful in providing snap shots for bacterial diversity, structure and function, they failed to provide a full picture of microbial activities and dynamics, especially on a large scale and in a parallel and high throughput fashion. To overcome such obstacles for studying

microbial communities in natural settings, a microarray-based, high-throughput technology, functional gene arrays has been recently developed. This type of arrays contains probes from the genes involved in key microbially-mediated biogeochemical processes, such as C, N, and S cycling, and phosphorus utilization, so such arrays are also called GeoChip. So far, the GeoChip has been used in a variety of studies such as assessing the effects of contaminants on microbial communities (Wu et al. 2006), characterizing metabolic diversity of microbial communities at deep-sea hydrothermal vents (Wang et al. 2009) and in the deep-sea Horizon oil plume (Hazen 2010), characterizing grassland microbial community structure under elevated CO_2, and determining spatial scaling of microbial community diversity in a forest soil (Zhou et al. 2008). The results from various studies demonstrate that GeoChip works very well with the samples from natural environments such as soils, marine sediments and ground waters, and is a powerful tool for profiling microbial community differences in general, and for linking community structure to functions. The latest version of GeoChip contains more than 50,000 probes from genes involved in C, N, S cycling, organic contaminant degradation, and metal resistance.

2.4 Summary

The study of soil bacterial communities in wetlands is relatively new in comparison to many other ecosystems, despite their important roles in controlling biogeochemical cycling at landscape scales. This is partly due to the difficulties and inconvenience of gaining access to the sites and collecting wetland soil (bulk or rhizosphere) samples. Developing a sampling strategy prior to sampling is crucial in order to assure that random, but representative samples, of the study area are collected to provide meaningful data and to meet the purposes of the study. In practice, the choice of the sample type and the number of samples to be collected depends on the objective of the study, statistical requirements, and practical considerations. Virtually the sampling strategies of all practical studies are a compromise between statistical and practical considerations due to limited resources.

A number of cultivation dependent and independent methods have been used to analyze the bacterial communities of various wetland soil samples. Generally, the commonly used cultivation-based methods start with the isolation of individual strains on solid medium (e.g., dilution plating method) or, less frequently, in liquid medium (e.g., MPN method). Selection and preparation of media is therefore crucial to such approaches. Despite very useful generalities regarding various requirements of bacteria for carbon and energy sources, nitrogen sources, electron acceptors, the exact nutritional requirements of many strains from natural ecosystems are still unknown. For this reason (and also for convenience), strains of soil bacteria are often isolated using undefined media or media containing undefined components. Soil extract, containing a variety of undefined compounds

that may support the growth of many indigenous bacteria, has been frequently used for cultivation of bacteria from wetland soils. Also, it is recommended that two sets of agar plates containing higher and lower nutrient concentrations, respectively, be used to ensure recovery of the widest diversity of aerobic heterotrophs, in particular for studies of many oligotrophic wetlands. In addition, several types of wetlands are quite unique in regard to their environmental conditions, requiring special considerations on medium selection. For instance, salt marshes are in close proximity to coastal water and high salinity is characteristic of these wetlands. Accordingly, media containing high contents of salts should be utilized to isolate bacterial strains from such environments. *Sphagnum*-dominated peatlands, one of the most extensive types of northern wetlands, typically have pH values below 4.0 and total concentrations of mineral nutrients between 5 and 50 mg L^{-1}. Such wetlands require strongly dilute, acidic media for successful cultivation of the indigenous peat-inhabiting bacteria. Although now technologically out of date due to the rise of modern molecular biological techniques, cultivation-based methods were the first strategy for analyzing the composition and function of bacterial communities, and are still appropriate for studies that require characterization of individual isolates or for identification and enumeration of small, well-defined bacterial groups. Moreover, the newly isolated microorganisms provide an invaluable source and means to study many aspects of microbiology. It is worth noting that during the last decade cultivation of uncultured bacteria from acidic northern wetlands has received much attention due to their important role in the global carbon budget. Significant breakthroughs have been made in uncovering the microbial diversity in these systems by modifying media and designing novel isolation strategies. Yet, cultivation of slow-growing, oligotrophic bacteria from the acidic, cold, nutrient-poor and water-saturated environments still remains challenging (Dedysh 2011).

There is no doubt that cultivation-based methods suffer from the problem of limited culturability, retrieving only a small portion of the complex microbial communities from environmental samples including wetland soil samples. In the last several decades, modern molecular biological techniques have been increasingly applied to gain a global view of soil bacterial communities. Some of the most commonly used molecular methods for wetland soil bacterial community analysis include 16S rRNA gene-based cloning library, DGGE, T-RFLP, and FISH. Unlike the cultivation-dependent methods that rely on the isolation of individual strains, these cultivation-independent methods are based on analyses of bacterial community DNA directly extracted from soil samples and subsequent PCR amplification of target genes. The cloning library approach can identify the individual members of the community at the genus or species level through the sequencing of clone libraries and provide DNA sequence data suitable for the supplementation of ribosomal databases. Due to the rise of less expensive DNA sequencing technologies, this method has experienced widespread application in the study of wetland soil bacterial communities. DGGE and T-RFLP offer an alternative to the tedious and relatively expensive cloning-based approach, and have been frequently applied for assessing the diversity, composition, and dynamics of bacterial communities from wetland soils. Instead of being used to identify single bacteria

in the community to the level of species, these rapid community profiling procedures typically are employed to demonstrate genetic differences or similarities among larger numbers of samples. Once standardized, the techniques (in particular T-RFLP analysis) are reproducible and robust for monitoring shifts in community structure, perhaps more appropriate for specific groups in the community structure, over time or among different treatments. Regardless of the specific method, cloning library, DGGE and T-RFLP, are all subject to the PCR-inherent biases. Different from the PCR-based techniques, FISH bypasses the step of PCR amplification. Although coming with limitations, the method is suited to investigate the composition of complex bacterial communities.

Analysis of bacterial communities in environmental samples is driven by technology. Lately developed high-throughput technologies, such as next-generation DNA sequencing and GeoChip, may generate huge amounts of genetic information making in-depth and comprehensive assessment of bacterial communities possible. Although these technologies have not been specifically used to analyze bacterial communities in wetland soils, they have proven to be a powerful tool for profiling microbial communities in a wide range of environmental samples. It is highly likely that such robust techniques will see a wide application in future studies of wetland bacterial communities.

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Chapter 3
Methods for Sampling and Analyzing Wetland Fungi

Steven L. Stephenson, Clement Tsui, and Adam W. Rollins

Abstract Most fungi are terrestrial, but representatives of all major groups of fungi along with three groups of fungus-like organisms (water molds, slime molds and lichens), usually studied by mycologists, can be found in wetlands. The primary ecological role of the fungi and water molds in wetland habitats is to decompose dead plant material—both woody and herbaceous debris as well as dead bryophytes. Although sometimes present in wetlands, slime molds and lichens occur almost exclusively on emergent (dry) substrates. Because the vast majority of fungi and fungus-like organisms associated with wetlands are microscopic, efforts to document their distribution and patterns of occurrence often pose a real challenge to ecologists. This chapter reviews some of the more useful and effective methods that can be used to study these organisms in wetland habitats. These include collecting specimens directly in the field, isolating specimens from substrate samples placed in moist chamber cultures and obtaining specimens on various types of organic baits.

3.1 Introduction

Fungi (singular: fungus) are a large, diverse and ecologically important group of eukaryotic organisms found in every ecosystem on earth. These organisms constitute a separate kingdom, distinct from both plants and animals, from which they appear to have diverged more than one billion years ago (Bruns 2006). With a few

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exceptions, the vegetative body of a fungus is made up of microscopic filaments called hyphae. The latter are usually extensively branched, have a cell wall consisting largely of chitin, and are either septate or aseptate (coenocytic), depending upon the group of fungi involved. Collectively, the system of hyphae making up a single fungus is referred to as a mycelium. Because they lack the photosynthetic pigments found in plants and algae, fungi have a heterotrophic mode of nutrition. In contrast to animals, which feed by ingestion of organic material, fungi obtain their nutrition by extracellular digestion that is facilitated by enzymes secreted from the hyphae; they then absorb the solubilized breakdown products (Webster and Weber 2007). Both sexual reproduction and asexual reproduction occur in fungi, although some species seem to have either lost the capability for sexual reproduction or do so only infrequently. Both types of reproduction generally involve the production of microscopic spores on or within some type of fruiting structure. The spores represent the primary means of dispersal, but once these reach a suitable substrate and germinate, the fungus can proliferate rapidly by means of hyphal growth and potentially colonize the entire substrate (Stephenson 2010).

In most traditional taxonomic treatments of the kingdom Fungi, five phyla have been accepted as being "true" fungi: Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota, and Basidiomycota (Alexopoulos et al. 1996; Stephenson and Stempen 1994). In addition to these "true" fungi, there are three other groups of "fungus-like" organisms that have long been studied by mycologists. The first example is the group known as water molds, which have often been treated in the context of the true fungi as the phylum Oomycota although they actually belong to an entirely different kingdom (the Chromista). Water molds have a vegetative body consisting of hyphal-like filaments that superficially resemble the hyphae of fungi and they obtain their food in the same manner. However, water molds also possess a number of other features that indicate they are not closely related to fungi. The most important of these is that the cell wall contains cellulose-like compounds and not chitin. The slime molds, members of yet another kingdom (the Amoebozoa), are a second group of fungus-like organisms. Some slime molds produce fruiting structures similar to, albeit usually much smaller than, those of certain macrofungi. Other than this, slime molds share few other features in common with the true fungi. However, they have been traditionally studied along with fungi and are typically included in most mycology textbooks (Stephenson and Stempen 1994). The members of yet another group, the lichens, are more than just fungi. These organisms are fungi that have established a mutualistic symbiotic relationship with another organism (either an alga or cyanobacterium, or a combination of the two) that enables them to survive under conditions that could not be tolerated by the fungus alone. This "composite" organism is usually very different in appearance from what it would be with only the fungus present, and the fungal component might not be recognized as such (Stephenson 2010). Because some representatives of each of these three groups can be found in wetlands and the fact that mycologists have traditionally considered them along with the true fungi, they are considered herein.

The ability to recognize a particular fungus and then to assign it to the proper taxonomic group is, with rare exceptions, dependent upon features of the spores and fruiting structures produced by the fungus in question. Although the fruiting structure is macroscopic in some members of the Ascomycota and many members of the Basidiomycota, the vast majority of fungi produce spores on or within fruiting structures that are too small to be observed in any kind of detail without the use of a microscope. The Chytridiomycota and water molds produce motile, flagellated spores (called zoospores), but this type of spore is not found in any of the other groups. Even for those forms that produce macroscopic fruiting structures (usually referred to as fruiting bodies), the occurrence of the fungus itself in a wetland is often not immediately apparent when these structures are not present. This is because the vegetative body of most fungi is limited in extent, highly dispersed, or more or less completely immersed within a particular substrate. As such, surveys for these organisms are more likely to involve isolation from samples collected in nature and brought into the laboratory for analysis than by direct detection in the field (Stephenson 2010).

The total number of species of fungi and fungus-like organisms found on the Earth is not known, but it almost certainly exceeds one million and some estimates are appreciably higher (Hawksworth 2001). Since no more than about 100,000 species of fungi have been described to date, it follows that there are tremendous numbers of fungi yet to be discovered. It seems likely that many of these are associated with ecosystems (e.g., tropical forests) that only recently have been the focus of relatively intensive mycological studies. However, even in temperate regions of the world there are certain types of habitats that remain understudied, and among these are wetlands.

3.2 Fungi and Fungus-Like Organisms Found in Wetlands

All of the major groups of fungi and fungus-like organisms occur in wetlands, but some groups are much better represented than others, and particular examples are associated with only certain kinds of wetland situations. The primary role of the true fungi in all types of ecosystems in which they occur is to decompose dead plant material, but some fungi attack and live on or within other living plants, animals, or even other fungi. Fungi that decompose dead plant material are called saprotrophs, whereas those that feed on living hosts are called parasites if the host is harmed, but not killed and pathogens if their presence produces a condition (disease) that has the potential of resulting in the death of the host. The distinction between parasite and pathogen is not necessarily absolute, and a parasite may become a pathogen over time or under a different set of circumstances (Stephenson 2010). In addition, as discussed in more detail elsewhere in this chapter, fungi also form beneficial mutualistic associations with many vascular plants (e.g., mycorrhizae in roots and endophytes in other tissues) and certain types of algae and cyanobacteria.

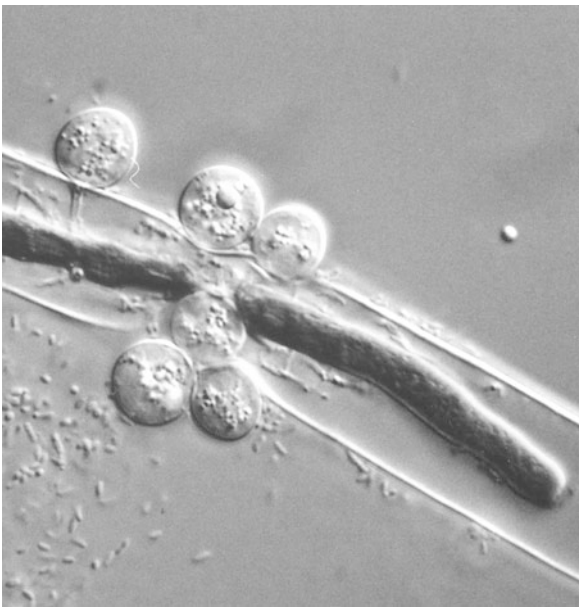

Fig. 3.1 Chytrids on the filament of a green alga (Published with kind permission of © Peter Letcher 2014. All Rights Reserved)

3.2.1 Chytridiomycota

The members of the Chytridiomycota, known as chytrids or chytrid fungi, are considered to be the most primitive of the true fungi, and they are the only group of fungi that have flagellated cells for sexual reproduction and dispersal. There are approximately 1,000 described species of chytrids, and members of the group are essentially ubiquitous, being found in most types of habitats and occurring from the polar regions to the tropics. The majority of species are thought to occur in terrestrial habitats such as forest, agricultural, and deserts soils, but the group is also well represented in freshwater habitats, including streams, ponds, lakes, marshes, and bogs. A few species tolerate saltwater and can be found in estuaries (Freeman et al. 2009). Most chytrids are saprotrophs, feeding upon plant and animal debris introduced into the habitats in which they occur. However, some species are parasites of algae or small aquatic animals (Fig. 3.1). One species, *Batrachochytrium dendrobatidis*, has been associated with population declines of some species of amphibians (Longcore et al. 1999). The flagellated cells (zoospores) that chytrids produce give them the potential of dispersing readily from one host or microsite to another. The vegetative body of a chytrid is essentially unicellular and thus extremely small; most species are recognizable only under the high power objective of a microscope. The usual way to obtain chytrids for study is to "bait" a sample of water or a soil suspension for those species that occur in aquatic and terrestrial habitats (Stevens 1974; Shearer et al. 2004). Chytrids often appear on such baits within a few days, and their frequency of occurrence and sheer numbers give some indication of just how common and widespread these organisms are in nature. The

Fig. 3.2 Zoosporangium (containing numerous zoospores) of a water mold

techniques used to sample for chytrids in wetlands are described in more detail later in this chapter.

3.2.2 Water Molds

As noted in the introductory section, the water molds are not true fungi, but they are morphologically similar to fungi and occur in some of the same habitats. Members of the group are common freshwater organisms and also occur in moist soil. Most of the approximately 600 species are saprotrophs, but a few are parasites of algae and other forms of aquatic life. Specimens of water molds are usually obtained in the same manner as described earlier for chytrids, although the actual baits used are somewhat different (Stevens 1974), as will be discussed in more detail below in the section on culturing zoosporic fungi. Like the chytrids, water molds produce motile zoospores (Fig. 3.2). However, water molds differ from chytrids in that they produce a mycelium-like structure. Often, an example of the latter can be observed directly in nature as a white, cottony halo that forms around the body of a dead insect or fish floating on the surface of a quiet pool. In spite of the fact that they are not fungi (and actually belong to an entirely different taxonomic group), water molds are often considered together with the chytrids as "zoosporic fungi" in ecological studies or biodiversity inventories of the type described in this chapter.

Fig. 3.3 Dung colonized by a zygomycete

3.2.3 Zygomycota

As a group, fungi assigned to the Zygomycota are terrestrial, although many of them tend to be confined to moist places. Most members of the Zygomycota (or "zygomycetes" as they are known in a more informal sense) are saprotrophs, but some species are parasites or pathogens of plants, animals and even other fungi. Although usually rather inconspicuous, they are often exceedingly common in soil and on the dung of animals as well as also occurring on many other types of substrates, including many of those found in wetland habitats. It is sometimes possible to notice the presence of their mycelia on fresh organic debris as a fuzzy grey growth that is similar in appearance to what one observes on moldy bread or a piece of fruit that has gone bad (Fig. 3.3). Both of these more familiar situations usually involve a member of the Zygomycota. The latter are often the first group of fungi to colonize such substrates (Stephenson 2010).

3.2.4 Glomeromycota

The Glomeromycota, also known as the arbuscular mycorrhizal fungi, were once considered to be part of the Zygomycota. Members of this group form intimate relationships with vascular plants in which some of the fungal hyphae making up the organism live inside cells of the root, forming what is referred to as an endomycorrhizal association. The species of fungi involved in endomycorrhizal associations cannot survive without their plant host. Both the plant and the fungus benefit from the association. These fungi do not produce fruiting bodies and occur

Fig. 3.4 Fruiting bodies of *Mitrula paludosa* on partially submerged dead leaves (Published with kind permission of © Martin Schnittler 2014. All Rights Reserved)

as non-septate hyphae growing inside root cells. Members of this group are not known to reproduce sexually, but they produce exceedingly large, multinucleate spores that often exceed 80 μm in diameter. Identification is based largely upon diagnostic features of these spores.

3.2.5 Ascomycota

The Ascomycota is a large and heterogeneous assemblage of fungi, and members of the group occur in every type of habitat examined to date and exhibit an amazing diversity of form and function. Many are saprotrophs and play an important role in the decomposition of dead plant material, whereas others are parasites or pathogens of plants and animals. Unlike the other groups of fungi covered thus far, some of the Ascomycota (or "ascomycetes" as they are known in a more informal sense) produce fruiting structures (fruiting bodies) of sufficient size to be conspicuous in nature (Stephenson 2010). Many of the more common examples are characterized by a fruiting body that is shaped like a cup or bowl, with the spore-producing hyphae forming a layer over the upper surface. Such fungi are often referred to as cup fungi. However, others produce fruiting bodies with shapes more difficult to characterize (Fig. 3.4). The spores produced in a fruiting body are sexual spores, but reproduction by means of asexual spores (conidia) is more characteristic for the group as a whole. The term "mitosporic ascomycetes" is given to members of the phylum Ascomycota in which only asexual spores are produced. These fungi are truly ubiquitous in nature, where they are of considerable (albeit, often little appreciated) ecological importance in all types of habitats.

3.2.6 Basidiomycota

Most of the large, conspicuous fruiting bodies encountered in nature are produced by members of the Basidiomycota (or "basidiomycetes" as they are known in a more informal sense). The various different kinds of fungi that make up this phylum

Fig. 3.5 Fruiting bodies of a species of *Galerina* on mosses in a wetland

are distinguished from one another on the basis of where the spore-producing hyphae are located and the overall shape of the fruiting body (Alexopoulos et al. 1996; Stephenson 2010). Among the more familiar members of the basidiomycetes are the mushrooms, polypores and puffballs. As is also the case in the ascomycetes, the spores produced in fruiting bodies are sexual spores. However, basidiomycetes are unlike ascomycetes in that most species typically do not produce any type of asexual spore. In wetland habitats, the fruiting bodies of basidiomycetes can be found on woody substrates above water level. In mountain bogs with an extensive cover of *Sphagnum* and other mosses, fruiting bodies also can be found on the raised areas (hummocks) that usually exist in bogs or directly associated with mats of mosses elsewhere throughout the bog (Fig. 3.5).

3.2.7 Slime Molds and Lichens

As already noted, the slime molds (phylum Myxomycota) are not fungi, and what we recognize as a lichen consists of more than just a fungus. However, most of the vegetative body of a lichen (or thallus) is made up of fungal tissue, and the fungus involved is almost always an ascomycete. Both slime molds and lichens are almost exclusively terrestrial, although there are a few records (Lindley et al. 2007) of members of the former group occurring in aquatic habitats. The fruiting bodies of slime molds usually occur on substrates above the water, including woody debris, bryophytes and leaf litter (Fig. 3.6). The occurrence of lichens in wetlands is generally restricted to elevated substrates (e.g., either living or dead but still

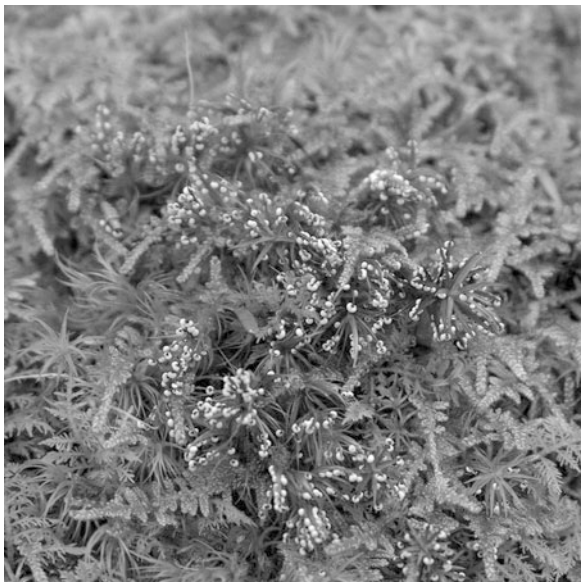

Fig. 3.6 Myxomycete fruiting bodies on the leaves of a moss (Published with kind permission of © Randy Darrah 2014. All Rights Reserved)

Fig. 3.7 Lichen on an elevated area (hummock) in a high-elevation bog (Published with kind permission of © Jason Hollinger 2014. All Rights Reserved)

standing trees), but they also commonly occur in the raised areas (e.g., the *Polytrichum-Sphagnum* hummocks) that exist in mountain bogs (Gibson 1982). Various species of *Cladonia* are often found in such situations (Fig. 3.7). Interestingly, although lichens are predominantly terrestrial, the oldest known fossil lichen is from the Rhynie chert in Scotland, which consists of very fine-grained sediments

deposited in a tropical or subtropical marsh-like setting that was subject to periodic inundation by water more than 400 million years ago (Taylor et al. 1997).

3.3 Ecological Roles of Fungi in Wetland Habitats

The primary ecological role of fungi in wetland habitats is to decompose dead plant material—both woody and herbaceous debris as well as bryophytes in those wetlands in which these organisms are present. As a group, fungi have the capability to decompose an incredible diversity of organic substrates, although a particular species may be limited to one or a few types of substrates. For example, fungi that decompose woody debris are not the same species that decompose either herbaceous debris or bryophytes. In some instances, a single species of fungus is confined to an even more restricted range of substrates. For example, some fungi decompose only the woody debris of conifers, while others are restricted to woody debris from angiosperms.

Decomposition of all types of woody debris is primarily accomplished by various ascomycetes and basidiomycetes. All of these fungi possess the enzymes required to degrade cellulose, but there are far fewer species (mostly basidiomycetes along with only a few ascomycetes) that have the capability of decomposing lignin (Hudson 1991).

Taxa of wood-decomposing fungi are often assigned to two categories on the basis of whether or not they can degrade both cellulose and lignin or just cellulose alone. The members of the first category (the so-called "white-rot" fungi) have the enzymes necessary to degrade both cellulose and lignin more or less simultaneously. The residual material that is left behind has a somewhat fibrous appearance and is very pale in color, looking as if it had been bleached. In contrast, wood degraded by members of the second group (the so-called "brown-rot" fungi) is brown in color and tends to be broken up into somewhat cuboidal fragments that quickly disintegrate into a powdery brown residue. In the both instances, the structural integrity of the wood is lost. Common and widespread examples of white-rot fungi are *Trametes versicolor* and *Daldinia concentrica*, whereas *Fomitopsis pinicola* and *Laetiporus sulphureus* are among the better known brown-rot fungi. The term "soft-rot" is sometimes applied to situations in which only the outermost layers of wood are subject to decay. Soft rots occur only in wood that has an unusually high level of moisture, which is often the case for woody substrates in wetlands. Most of the fungi involved are ascomycetes, with species of *Chaetomium* among the most common and best known. Only the cellulose of the wood is degraded by soft rot fungi (Stephenson 2010).

Leaves and other non-woody plant parts (e.g., fruits and seeds) represent an entirely different type of substrate than wood or bark. Considerable diversity exists for the leaves of plants, and what might be termed a "typical leaf" from a common and widespread angiosperm is markedly different from the needle-like leaf of many conifers. Moreover, angiosperm leaves vary from relatively "soft" and readily decomposed examples to those that are rather "tough" and fairly resistant to

decomposition. The former would include the leaves of virtually all herbaceous plants, whereas the leaves of such plants as *Rhododendron maximum* and *Kalmia latifolia* would represent the latter category. Spores of fungi can reach a leaf while it is still attached, and the extent to which the leaf serves as a "spore trap" is related to such factors as its size, position on the tree, surface (smooth or hairy) and whether or not the spore lands when the leaf is moist. Studies have shown that numerous spores are already present on older leaves prior to leaf-fall. Once a leaf falls from a plant, it can be invaded by soil-inhabiting fungi or, if the leaf becomes submerged, various aquatic fungi. The fruiting bodies of certain basidiomycetes (e.g., species of *Marasimus* and *Collybia*) often occur in abundance on dead leaves, which simply reflects their biological role as litter-inhabiting saprotrophs. The fungi associated with fruits and seeds are not necessarily the same ones found on leaves. In some cases, a particular species may be restricted to the substrate represented by a certain type of fruit or seed (Stephenson 2010). Two examples are *Mycena luteopallens*, which occurs only on the husks of hickory (*Carya*) nuts and walnuts (*Juglans*), and *Strobilurus conigenoides*, a species found only on old fruits of magnolia (*Magnolia*).

An individual living plant is a complex and spatially diverse structure that represents a habitat that supports a diverse assemblage of fungi. Some of these fungi (termed epiphytes) colonize the surfaces of living leaves and stems, but others (termed endophytes) occupy internal tissues. Many of these (called foliar endophytes) occur inside leaves, whereas others are associated with stem tissues. It has been increasingly apparent that even a healthy plant has an assemblage of endophytes present, and there is considerable evidence that this situation is beneficial to the plant itself. For example, certain endophytes may increase the tolerance of the plant to the effects of temperature extremes and drought situations. Endophytes have been described as having biologically active secondary metabolites that serve to protect the plant against herbivory (Clay 1990) or have antibacterial or antifungal activity (Fisher et al. 1984). In return, the endophyte receives photosynthates from the plant. Many endophytic fungi are transmitted from one generation to the next by inoculating the seeds produced by the host plant. Interestingly, when the host plant dies, the endophytic fungi already present apparently play a role in the early stages of decomposition prior to the appearance of other species of fungi more typically associated with this process in the particular habitat where the plant occurs (Gessner et al. 2007; Van Ryckegem et al. 2007). Studies specifically directed towards the endophytes of wetland plants appear to be lacking, but these fungi, most of which are ascomycetes, have received considerable study in some groups of plants (e.g., grasses) that are not uncommon in some types of wetlands.

Some fungi form a symbiotic relationship with the roots of trees and other plants. This relationship, which is called a mycorrhizal association, is mutually beneficial to both the plant and the fungus. The fungus enables the plant to take up nutrients that would otherwise be unavailable, and the plant provides nutrition for the fungus. The majority of plants on Earth are involved in these associations. In some instances, the mycorrhizal association is so essential to the plant that the latter would not survive without its fungal partner. There are two fundamentally different types of

mycorrhizal associations—ectomycorrhizal (usually involving a basidiomycete) and endomycorrhizal (most often involving a member of the Glomeromycota). In the former, the fungus produces a covering of hyphae (called a sheath or mantle) around the outside of smaller rootlets of the host plant. Other hyphae invade the cortex of the rootlet but do not disrupt the individual cells. In endomycorrhizal associations, no sheath is formed and hyphae of the fungus actually invade cells of the cortex of the rootlet. Perhaps 80 % of all vascular plants form mycorrhizal associations with fungi. Endomycorrhizal associations are predominant, but many of the trees (e.g., oak [*Quercus*], beech [*Fagus*], willow [*Salix*], spruce [*Picea*] and fir [*Abies*]) that are important in temperate and boreal regions of the Northern Hemisphere are ectomycorrhizal. However, the fungi that form ectomycorrhizal associations do not normally survive in areas that are permanently flooded (Bauer et al. 2003; Jurgensen et al. 1997), which limits their occurrence in wetlands. Moreover, saturated soil conditions, such as those characteristic of wetlands, also have been reported to restrict the growth of endomycorrhizal fungi, most likely as a result of low oxygen levels (Slankis 1973). Nevertheless, endomycorrhizal fungi are known to be present in many types of wetlands (Hoewyk et al. 2001; Rickerl et al. 1994) and certainly play important roles in the growth of numerous species of wetland plants (Dunham et al. 2003).

The various types of ecological associations that exist for fungi and plants in wetland habitats are most apparent for vascular plants, although bryophytes also must be considered in those instances in which at least some representatives of the group are present. Although both chytrids and water molds commonly colonize dead parts of vascular plants that are introduced into the aquatic habitats where they occur and these organisms are easily isolated in laboratory culture from this type of material (e.g., plant pollen is a standard "bait" for chytrids), they are ecologically more important for their roles as parasites of phytoplankton (chytrids) and primary colonizers of the bodies of aquatic invertebrate animals (water molds). Chytrids are often surprisingly abundant on filamentous algae and diatoms, and some species are known to severely deplete local populations of their algal hosts (Webster and Weber 2007). Water molds, including species belonging to the common and widespread genera *Saprolegnia* and *Achlya*, quickly colonize the bodies of aquatic insects (Dick 1970) and other invertebrates. In addition, some species of both chytrids and water molds are parasites of larger animals found in aquatic habitats, including crayfish and fish. In some instances, their ecological impact can be considerable.

3.4 Wetlands as a General Habitat for Fungi

Fungi are primarily terrestrial organisms, but numerous species can be found in wetlands. Some of these are aquatic forms while others are restricted to substrates that are above water level. For the most part, fungi are aerobic organisms, and they do not thrive in situations where an oxygen deficit develops. Interestingly, some

otherwise terrestrial fungi are able to survive in aquatic habitats, but they do not complete their life cycle, at least what is known of it, in water. By definition, truly aquatic forms normally do complete their entire life cycle in the aquatic habitats in which they are found. For the majority of these, their whole evolutionary history seems to have been in water (Hudson 1991). They possess motile zoospores as the primary unit of dispersal and the latter function only in an environment with water present. As already noted, both chytrids and the fungus-like water molds share this feature.

More fungi are associated with forested wetlands (pocosins, swamps, bottomland hardwood communities, glades and bogs) than non-forested freshwater and saltwater marshes. This is a direct result of the greater substrate heterogeneity in wetland situations with trees and other woody plants present. In other words, the wider the range of available substrates, the larger the number of fungi likely to be present. Few fungi can tolerate salt water, which limits their occurrence in saltwater marshes. In acidic mountain bogs, the low pH represents another limiting factor for fungi, either directly on the fungi themselves or indirectly as a result of the constraints it places on the other organisms that cannot survive under conditions of low pH. Such wetlands often contain rare species of plants and animals (Grafton and Eye 1982). The same situation is likely to be true for fungi, but the fungi of acidic mountain bogs are understudied.

Regardless of the type of wetland being considered, the numbers and types of fungi present are determined largely by the type and nature of the substrates available, the relative abundance of those substrates and their condition (i.e., whether submerged or not, living or dead, and the stage of decomposition when the latter is the case). For example, the fungi involved in the decomposition of coarse woody debris are generally not the same as those associated with twigs derived from the same plant, and the fungi found on submerged woody debris are different from those that occur on the same type of woody debris located above the water level. Moreover, as already noted, the assemblages of fungi that decompose woody debris from conifers—which are important in some mountain bogs—and angiosperm trees are often quite different.

3.5 Fungi and Fungus-Like Organisms Associated with the Different Microhabitats Found in Wetlands

The composition and structure of the vegetation and thus the potential range of plant hosts available and the ultimate sources of the input of dead plant material vary considerably for different types of wetlands (Ellis and Chester 1989; Richardson and Gibbons 1993; Sharitz and Mitsch 1993). For example, Rentch and Anderson (2006) listed more than 1,700 species of vascular plants for wetland and riparian habitats in West Virginia. Approximately 38 % of these were plants that usually to almost always occur in such habitats. Obviously, only a smaller

subset of this total would be found in a particular habitat, but even then the number of species present can be impressive. For example, Brown (1982) recorded 281 species for a swamp in western Maryland.

Many of the fungal parasites or pathogens associated with living plants are rather host specific, sometimes to the point of being restricted to a single species or genus. For example, this is usually the case for two groups of basidiomycetes, the rust fungi and the smut fungi. Members of the latter group are particularly common on grasses and sedges, both of which are common wetland plants. Saprotrophic microfungi associated with living plants tend to be much less host specific, with the same species potentially occurring on a wide range of different hosts. Conceivably, the greater the biodiversity of potential host species in a particular wetland, the greater the biodiversity of fungi associated with these hosts. The majority of saprotrophic microfungi found on living plants are ascomycetes.

Once the plant is no longer alive, it represents a source of material that is subject to decomposition, and the nature of the material itself is the main factor determining just what fungi are involved. Ascomycetes are the most important group for both submerged and emergent nonwoody substrates as well as submerged woody substrates, whereas basidiomycetes also play an important role for emergent woody substrates. Zygomycetes commonly occur on nonwoody substrates in moist microhabitats such as at the margin of a shallow pool, chytrids and water molds can colonize submerged nonwoody substrates, and both slime molds and lichens are sometimes found on emergent woody substrates. Slime molds occasionally occur on emergent nonwoody substrates but are more likely to be found on both nonwoody and woody substrates in moist microhabitats. As mentioned earlier, neither slime molds nor lichens are decomposers. However, the vegetative stage in the slime mold life cycle feeds primarily upon bacteria, which are abundant in all types of dead plant material except for acidic bogs, where the low pH is a limiting factor.

Although they receive much less attention than the fungi that decompose wood or litter, there is a group of fungi which decompose bryophytes. In those wetlands where bryophytes are abundant, such as in many mountain bogs dominated by *Sphagnum* mosses, it would be a mistake to dismiss the ecological importance of these bryophilous ("moss-loving") fungi. Thormann and Rice (2007) summarized the literature on the fungi associated with peatlands and reported that 601 species of fungi have been identified from such habitats. It has been proposed that fungi are the dominant microbial decomposers (even more so than bacteria) in relatively acidic situations such as *Sphagnum*-dominated bogs (Andersen et al. 2006). For example, 55 species of fungi were reported to be associated with *Sphagnum fuscum* from one bog sampled in Alberta, Canada (Thormann et al. 2001). The group of bryophilous fungi includes a number of species of basidiomycetes, ascomycetes, and zygomycetes, but most examples produce fruiting bodies that are relatively small and thus not easy to spot in the field. Among the more common bryophilous fungi are species in the genus *Galerina*, whose fruiting bodies often occur in small clusters on mats of bryophytes (Fig. 3.5). The bryophilous fungi are another group that has received relatively little study.

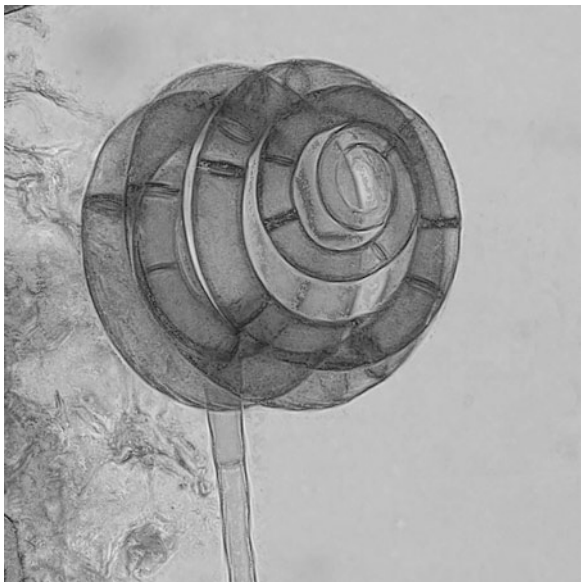

Fig. 3.8 Reproductive propagule of an aero-aquatic fungus (Published with kind permission of © Jerry Cooper 2014. All Rights Reserved)

The majority of mitosporic ascomycetes are terrestrial, but those found in aquatic habitats include some distinctive examples. One such ecological group is made up of the aero-aquatic fungi, which occur on dead leaves, twigs, and other types of dead plant material submerged in water. Interestingly, as long as the mycelium of an aero-aquatic fungus is below the surface of the water, formation of asexual reproductive structures does not take place. However, when the water level drops and the mycelium of an aero-aquatic fungus is exposed to air under moist conditions, formation of asexual reproductive structures takes place. The asexual propagules produced are too complex to be considered spores but have the same function. Each propagule consists of either a spherical network enclosing an open space or a tightly coiled and hollow helical structure (Fig. 3.8). Both types trap air inside and can float on the surface of the water once liberated. The unique structural configuration of these structures appears to be an adaptation for dispersal by water.

The yeasts are among the most common of all fungi in nature. Both the ascomycetes and basidiomycetes include taxa that are considered as yeasts, but the best known yeasts are ascomycetes. By definition, yeasts are fungi that are unicellular and reproduce by budding. Budding is a method of asexual reproduction, and some yeasts appear to have lost the capability for sexual reproduction. However, many other yeasts do reproduce sexually, although sometimes only rarely. Yeasts are exceedingly common on the surfaces of living plants, and they also can be found in soil and water. However, relatively few studies have been directed towards the occurrence of yeasts in wetland habitats, although there is little doubt that these organisms are associated with every available type of substrate present. Thormann et al. (2007) summarized the information available on yeasts in

peatlands and indicated that 75 species had been reported as associated with this habitat. It appears that these yeasts play an important role in the initial decomposition of plant debris by feeding upon the simple polymers that leach out of dead and dying plants.

3.6 General Methods/Techniques Used to Sample the Various Groups of Fungi

The vast majority of fungi are microscopic. Although some basidiomycetes and a few ascomycetes produce macroscopic fruiting structures, these structures tend to be rather ephemeral in nature. Only the fungus-like lichens are characterized by vegetative structures that persist for a considerable period of time. As such, sampling for fungi is a real challenge, and the methods and/or techniques used for one group are rarely appropriate for other groups. Prior to initiating any field survey for the fruiting structures of ascomycetes, basidiomycetes, slime molds or lichens, an investigator should conduct some preliminary background work on the system to be studied. This would involve such things as assessing the types and relative abundance of the each of the various substrates present along with determining the most appropriate conditions (e.g., immediately after a period of rainy weather) for carrying out field surveys. Having some knowledge of what to look for, where to look, and how to actually collect any specimens that turn up as a result of the collecting effort are absolutely essential.

Temperature and rainfall are the major factors that determine when macrofungi produce fruiting bodies in nature. The latter is relatively less important in wetlands, where moist conditions are not necessarily dependent solely upon periods of rainfall. Nevertheless, the occurrence of fruiting bodies is affected by the amount of moisture in the soil or the substrate within which the mycelium of the fungus occurs, which can range from saturated to dry, depending upon the type of wetland and the time of year. If the only objective is to determine what species occur in a particular area of wetland, then obtaining good baseline data may involve intensive collecting every 1–2 weeks throughout the fruiting season in order to maximize the number of species collected. It is widely known that a particular species of fungus may not produce fruiting bodies every year. This underscores the need for long-term (at least several years) studies, which may not be practical in every situation, to document most of the species for the area being investigated. In many instances, especially when surveying larger areas, the opportunistic sampling protocol described by Cannon and Sutton (2004) is appropriate. This method simply involves walking through the entire area in a random fashion to maximize the probability of finding fruiting bodies of most of the species present at the time sampling is being carried. Otherwise, a plot can be delimited and the sampling effort confined to just the area within the plot. This allows the calculation of abundance measures for the fruiting bodies of the various macrofungi present.

Moreover, the use of a plot is especially appropriate when the fruiting bodies are so small as to be easily overlooked and thus require a more intensive examination (often on the hands and knees) to detect. This is often the case for some of the fruiting bodies of the species of *Galerina* that are associated with mats of bryophytes or the small cup fungi that are found on the surface of moist soil. Since the advent of digital photography, it is often standard practice to document field-collected fruiting bodies of macrofungi with images, which can be invaluable in making identifications (e.g., when comparing a particular fruiting body with the illustrations available in most field guides and taxonomic monographs).

Except for those few basidiomycetes and ascomycetes that produce fruiting bodies that are tough, leathery or woody, which is case for certain species (e.g., most polypores, some corticoid fungi and many members of the Xylariaceae) that occur on decaying wood, field-collected fruiting bodies should be dried to preserve many of the features considered when making an identification. Prior to drying, it is exceedingly useful to prepare a spore print (which reveals the color of the spores in mass) for those fungi (mostly agarics and boletes) for which this is possible. This simply involves placing the cap (or a section of the cap) on a piece of paper, covering this under a cup, bowl or other container in order to maintain moist conditions, and allowing it to remain in place for several hours (usually overnight). Spores that fall from the cap produce the spore print. Afterwards, the fruiting bodies should be dried by placing them on some sort of drying apparatus. Commercial food dehydrators are usually the best option. Larger fruiting bodies should be split in half from top to bottom before being placed on the dryer. This practice speeds the drying process, prevents the interior of the fruiting body from decaying and stops the feeding activities of any insect larvae that might be present (Lodge et al. 2004). Once dry, fruiting bodies can be placed in small paper boxes, paper bags or plastic bags for permanent storage.

As mentioned in the introductory information provided for chytrids and water molds, the most common method of sampling for this group of organisms involves the use of baits, either directly in the field or under laboratory conditions. The former involves placing suitable baits in the water, allowing them to remain in place for several days, and returning the baits to the laboratory. In order to confine the items used as baits, they are usually placed in some sort of trap (e.g., a small-mesh wire cage or perforated plastic basket), which is suspended in the water by a piece of nylon cord. Some of the more commonly used baits include boiled hemp (*Cannabis*) seeds, dead insects, pieces of fruits (e.g., pear or apple), hair, and small pieces of cellophane (Bruns 2006; Stevens 1974). The length of time the baits are left in place will depend upon conditions, especially the temperature of the water. However, a period ranging from several days to a week is usually sufficient during most of the year. In winter, it may be best to recollect the baits after a somewhat longer period of 10 days to 2 weeks. Once the baits have been collected from the water, they are transported to the laboratory and kept under cool conditions (no more than 20–25 °C) until examined for the presence of both chytrids and water molds. Conversely, it is possible to collect water molds directly in the field by examining substrates upon which they are likely to be present. Examples include

floating or submerged small animals (e.g., insects) surrounded by an obvious halo of white threads. Water molds are usually easy to recognize from their coarse, stiff and radiating hyphal-like structures. Chytrids are too small to be spotted in the field but can be found when filaments of algae are collected from the water, brought back to the laboratory and examined under a compound microscope.

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Student Exercises

Laboratory Exercises

The exercises outlined below provide opportunities to examine the diversity of wetland fungi. Because it has been estimated that 95 % of the world's fungi have yet to be discovered (Hawksworth 2001) and many of those species that are known can be identified only by experts for the particular group involved, one can anticipate that it will be possible to assign many of the fungi likely to be encountered only to a

major taxonomic or ecological group. Detailed field observations provide a basis for developing a better understanding of the effects of various environmental factors on the distribution and abundance of wetland fungi. As such, it is worthwhile to record the temperature of the water when collecting samples. This is particularly important when an effort will be made to isolate and culture the fungi likely to be present. Some fungi tend to grow better at the temperature of the environment from which they were isolated than at room temperature. An effort should be made to identify at least the more abundant plants both surrounding and within the wetland, since they represent the primary sources of plant-derived debris introduced to the wetland itself. If possible, data should be obtained on the physical and chemical characteristics of the water present at a collection site. If the prerequisite equipment is available, such things as levels of dissolved oxygen, pH, and concentrations of nitrate and phosphate should be determined. Having such data allows the conditions present in different wetlands or different portions of the same wetland to be compared.

Hawksworth DL (2001) The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol Res 105:1422–1432

Laboratory Exercise #1: Biodiversity of Macrofungi, Slime Molds and Lichens

As already mentioned, the appearance of the fruiting bodies of macrofungi and slime molds in nature is both sporadic and variable through time, whereas lichen thalli are persistent and thus can be collected at any time of the year. As such, any effort to assess the diversity of the first two groups for a particular wetland may not be possible on a given visit. However, when favorable conditions do exist, the "opportunistic sampling method" described above can be used to carry out a survey of fruiting bodies that have developed under natural conditions in the field. In order to quantify the results obtained in such a survey, it is important to utilize a predefined plot size (e.g., 10 by 10 m or 20 by 50 m) and a predefined search time (e.g., 15 min or 1 h) for the area being examined. The type of substrate (e.g., bark, wood, dead leaf or bryophyte) from which each fruiting body was collected and whether the substrate was (a) emergent, (b) on the ground, or (c) submerged should be noted and recorded. If carried out as an educational activity, student participants can be divided into two or more equal numbered groups, with each group establishing at least one replicate plot. For example, two groups of five students could each sample a different wetland plot of the same dimensions in a particular wetland, using the same search time. The results obtained by the two groups could be compared, with the objective of providing answers to a number of questions. For example, how similar were the two plots? What were the differences? Where there any differences in the numbers and types of fungi recovered from different substrates (e.g., wood vs. litter) and the same substrate occurring under different conditions (e.g., aerial woody debris versus submerged woody debris)?

Specimens of macrofungi that have a fleshy texture should be wrapped in sheets of wax paper or aluminum foil, placed in wax paper "sandwich" bags or put in small plastic boxes for temporary storage. Plastic boxes of the type used for fishing tackle are especially appropriate. It is useful to add some type of organic padding (e.g., mosses or pieces of leaves) to the compartment of the box in which a small specimen is to be placed. This prevents the specimen from rolling around and potentially becoming damaged. Wrapped specimens of larger fleshy fungi are best placed in some type of rigid-sided container (bucket, pack basket or pasteboard box) large enough to hold multiple specimens. This protects individual specimens from being crushed while being transported from the field to the laboratory. Specimens of macrofungi with a woody or leathery texture can be placed in small paper bags and, because of their relative toughness, do not have to be handled with as much care.

Paper bags are also appropriate for storing specimens of lichens, while fishing tackle boxes are invariably used for slime molds. Since many of the latter are relatively fragile, providing at least some organic padding for each specimen is strongly recommended. Boxes containing slime molds should be opened immediately upon returning from the field to enable the specimens to air-dry. Otherwise, specimens will be quickly colonized by various filamentous fungi. Dried specimens can be placed in small pasteboard boxes for permanent storage (Stephenson and Stempen 1994). Wet or damp specimens of lichens should be air-dried as soon as possible for the same reason. Simply placing a specimen on a piece of newspaper in the laboratory is usually enough, although drying specimens in a food dehydrator in the same manner as already described for fleshy fungi is sometimes appropriate.

Stephenson SL, Stempen H (1994) Myxomycetes: a handbook of slime molds. Timber Press, Portland

Laboratory Exercise #2: Biodiversity of Non-zoosporic Microfungi

The occurrence of various non-zoosporic microfungi associated with wetlands is often difficult to assess, but at least some data on their ecological distribution and relative abundance can be obtained with the use of direct observation with a microscope (either a field microscope or a standard microscope in the laboratory) and various culturing techniques. Two of the most widely used culturing techniques are "baiting" and the use of what are referred to as moist chamber cultures (Fuller and Jaworski 1987; Shearer et al. 2004). Both direct observation and culturing involve collecting samples of substrates that fungi typically colonize in nature. Examples include pieces of semi-submerged or fully submerged woody debris and submerged or emergent portions (dead leaves and stems) of wetland vascular plants such as sedges. Pieces of decorticated woody debris tend to be particularly productive (Fig. 3.9). It is helpful to have either a small knife or a pair of plant cutters to remove small pieces of woody debris from large logs or trees or shrubs still rooted in the water. Samples should be placed in a plastic bag, and a small amount of water added to maintain moist conditions. However, the samples should not be flooded. All samples should be kept cool while being brought to the laboratory. In the

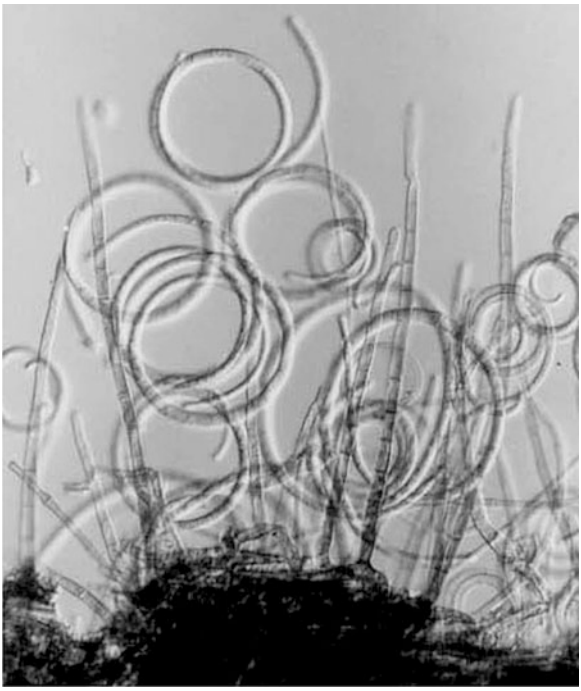

Fig. 3.9 Hyphae and asexual spores of a mitosporic ascomycete (*Helicosporium* sp.) on a piece of submerged wood

laboratory, samples can be examined under a stereomicroscope to check for the presence of fungi, and small amounts of material can be removed with fine-pointed forceps or a dissecting needle and transferred to a glass slide. Once a coverslip has been added, the fungi present in the material on the slide can be checked under a compound microscope. Adding a very small amount of a stain such as methylene blue often causes the vegetative and reproductive structures of fungi to be more easily discernable on the slide. Identification of these fungi is difficult, but notes can be made on any fruiting bodies or other distinctive features observed (e.g., shapes and sizes of conidia). In this way, it is possible to derive at least some estimate as to the diversity of taxa associated with a particular substrate or different types of substrates.

Culturing these same samples (or other samples collected in the same manner) usually yields additional taxa that are not evident through direct observation. The use of moist chamber cultures is a particularly effective way of isolating fungi from all types of substrates. A moist chamber is prepared by first lining the bottom of a Petri dish, glass finger bowl, culture dish or other suitable container with filter paper or a piece of paper towel cut to the appropriate size. Sample material is then placed on the filter paper, a small amount of distilled water is added, and the container set aside for one to several days. If the intent is to maintain the culture for more than 2–3 days, it will be necessary to either place a cover over it (the lid for a Petri dish) or add additional water. The cultures should be examined periodically to note the appearance of various fungi. The same method can be used for slime molds,

although cultures usually have to be maintained for a least several weeks to obtain fruitings (Stephenson and Stempen 1994).

A second method of culturing involves attaching a series of "organic baits" (either pieces of woody debris or small-mesh nylon bags containing samples of litter) to a cord anchored to the bottom of a wetland with a brick or a metal stake (Shearer et al. 2004). The organic baits should not be too close to one another, since this may affect the flow of water. One or more of the baits should be retrieved periodically (e.g., once a month or every 2 months for examination). Once they are collected, samples should be placed in plastic bags containing several pieces of paper towels to absorb excess water and stored temporarily in a small cooler away from temperature extremes until taken back to the laboratory.

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Shearer CA, Langsam DM, Longcore JE (2004) Fungi in freshwater habitats. In: Mueller GM, Bills GF, Foster MS (eds) Biodiversity of fungi: inventory and monitoring methods. Elsevier Academic Press, Amsterdam, pp 513–531

Stephenson SL, Stempen H (1994) Myxomycetes: a handbook of slime molds. Timber Press, Portland

Laboratory Exercise #3: Biodiversity of Zoosporic Fungi

Chytrids and water molds are relatively easy to isolate from samples of water and organic debris that are collected in the field and brought back to the laboratory (Stevens 1974; Shearer et al. 2004). The first step involves collecting a small sample of water with at least some obvious organic debris present. Samples are best collected in a small (25 or 100 ml) screw cap tubes, but most types of containers can be used. Various types of baits are then added to the water in the tubes. Among the most appropriate baits to use are pollen grains, hemp seeds, pieces of snake skin or insect exoskeletons. Once again, samples should be kept away from temperature extremes while being brought back to the laboratory. In the laboratory, the baits in the containers can be examined for the presence of the target organisms in the manner described in the next section.

Shearer CA, Langsam DM, Longcore JE (2004) Fungi in freshwater habitats. In: Mueller GM, Bills GF, Foster MS (eds) Biodiversity of fungi: inventory and monitoring methods. Elsevier Academic Press, Amsterdam, pp 513–531

Stevens RB (ed) (1974) Mycology guidebook. University of Washington Press, Seattle

Classroom Exercises

All of the techniques described thus far can be adapted for classroom activities, since most of these are relatively simple and do not require any specialized

equipment. For example, it is possible to have students prepare moist chamber cultures. Each student can prepare one or several moist chamber cultures with a particular type of substrate material or several different types of material, using the same method described above. Students would be expected to check their cultures periodically (at least once a week) and record any fungi that have appeared. Checking the culture under a dissecting microscope is best, but a hand lens can be used if dissecting microscopes are not available. If the students do have access to compound microscopes, they should prepare wet mounts of any filamentous fungus observed. This will allow them to make note of features that are not necessary apparent under the lower magnification available with a dissecting microscope. Various types of information can be complied in this type of activity, including the relative abundance of fungi on different types of substrates (e.g., dead leaves versus woody twigs) and estimates of the numbers of different taxa associated with a particular substrate.

Another possible classroom activity involves "baiting" for chytrids and water molds. Once again, the methods used are the same as already described. A wide range of wetland substrates (surface soil, various types of sediments, woody twigs, and different types of plant debris) in addition to samples of water from the wetland could be investigated, and students can experiment with different types of baits. These baits would be examined periodically for the appearance of chytrids (a compound microscope would be required) or water molds (often readily apparent with the naked eye, but better observed with a hand lens or under a dissecting microscope). The same type of information outlined above should be recorded.

As a variation on this basic activity, different types of substrates (e.g., small fruits and seeds, flower parts and small pieces of leaves from glasses and broad leaf plants) could be collected from the wetland being studied, brought back to a laboratory, autoclaved (to kill any fungi already present) and then used as baits for a series of cultures established from the same sample material. This would allow the students to determine which baits were most effective at attracting zoosporic fungi and whether there were different assemblages of fungi associated with the different types of baits.

Students can gain an appreciation of the morphological diversity of the fungi they have isolated in the laboratory or collected in the field by preparing drawings and detailed descriptions of the fungi in question. Notes should be made of such features as color, shape, and size of any obvious fruiting or vegetative structures as well as the shape and size of any spores that happen to be present.

One important aspect of any classroom activity is having the students become familiar with what is involved in keeping a complete and accurate record of biological research. For example, each student could be expected to prepare a small report on one or more of the fungi observed in a particular activity. This would include (a) where the fungus or sample yielding the fungus was found, including a general description of the wetland involved; (b) when the sample was collected or the fungus actually observed; and (c) how it was collected or isolated (i.e., a description of the techniques used). In addition, the report could include a labelled sketch of the fungus, with diagnostic features pointed out.

Classroom Exercise #1: Isolation of Zoosporic Fungi

As noted earlier in this chapter, obtaining chytrids and water molds on baits under laboratory conditions is relatively easy to do and simply involves collecting samples of water, soil, mud or plant debris in the field, bringing these back to the laboratory, placing each sample in a culture dish, glass beaker or other suitable container, adding water (either distilled water or water from the study site) if the sample being examined is soil, mud or plant debris, and then adding baits to the surface of the water. For chytrids, pine (*Pinus*) pollen grains work exceedingly well, whereas boiled hemp seeds or dead insects are among the most effective baits for water molds.

Another method used to isolate zoosporic fungi directly from field-collected substrates involves placing a small portion of substrate material along with a small amount of water in a Petri dish prepared with water agar or corn meal agar supplemented with streptomycin and penicillin. Dead, but still intact dead leaves, should be rinsed gently with sterile distilled water, cut into small pieces with scissors, and several of these small pieces should be placed on the surface of the agar in a Petri dish. Approximately 0.5 ml of sterile distilled water is then added to each Petri dish, and the dish should then be incubated at 25 C for 24 h. After this period of time, the baits and leaf pieces should be observed under a dissecting microscope with magnification ($\geq$30×). The presence of chytrids and water molds is best determined by carefully examining the margins of the leaf pieces or baits.

Classroom Exercise #2: Isolation of Non-Zoosporic Fungi

The methods used to isolate non-zoosporic fungi in the laboratory are essentially extensions of field-based efforts to survey these fungi. Samples of woody debris or dead portions of herbaceous plants, two of the more productive substrates, should be rinsed with sterile tap water or distilled water to remove mud and debris and then used to prepare a series of moist chamber cultures of the type already described. The cultures should be kept at room temperature and under ambient levels of light. It is important to maintain moist conditions in each culture by using a lid to cover Petri dishes, stacking Petri dishes on top of each other, or enclosing the culture in a plastic bag. Samples should be examined for the presence of fruiting bodies under a dissecting microscope (at least 50×) after 1 week and then on a daily basis for a period of up to a month or more. Fruiting bodies of wood-inhabiting ascomycetes may require weeks to develop, whereas reproductive structures and conidia of mitosporic fungi often appear in just a day or two.

Some of the microfungi associated with woody twigs and dead leaves can be induced to form spores under laboratory conditions if the sample material is placed in a conical flask with sterile water or water collected from the study site and then subjected to forced aeration for several days (Tsui et al. 2003). The type of small air pump commonly used with a household aquarium works well for setting up an aeration flask. If some of the bubbles or foam that forms on top of the water in the flask is transferred to a glass slide, a cover slide added, and the slide viewed under a

compound light microscope, it is usually possible to observe the spores of various mitosporic ascomycetes and the spore-like propagules produced by aero-aquatic fungi. The latter are especially common in water that is somewhat stagnant. It is important to note that identification in many of these fungi is based largely upon features of their spores and spore-like structures.

Another isolation method that can be used for soil and samples of water involves placing soil particles or a small amount of water directly on the agar surface of a plate prepared with some type of media suitable for the growth of microfungi. The media used vary considerable, but in general, low-nutrient media work best. Among these are cornmeal agar, potato glucose agar and peptone-yeast agars (Stevens 1974). The temperature and light conditions that are most effect will need to be determined through experimentation. Some fungi grow well and produce conidia (usually necessary for identification) under low levels of light, while others appear to require at least some exposure to high light levels. Many of the more common microfungi appear on such media, sometimes in great profusion. To reduce the abundance of fungi appearing in a plate, the sample of water or soil can be diluted. This simply involves thoroughly mixing a small amount of soil in a measured amount (the larger the amount, the greater the dilution) of distilled water in a small tube, adding approximately 0.5 ml of the resulting suspension to a plate and spreading this over the surface of the agar. Colonies of various yeasts appear quickly in these plates, and their sheer abundance is clear evidence of how common these fungi are in wetland and other habitats.

Stevens RB (ed) (1974) Mycology guidebook. University of Washington Press, Seattle

Tsui CKM, Hyde KD, Hodgkiss IJ (2003) Methods for investigating the biodiversity and distribution of freshwater ascomycetes and anamorphic fungi on submerged wood. In: Tsui CKM, Hyde KD (eds) Freshwater mycology. Fungal Diversity Press, Hong Kong, pp 195–209

Literature for Identifying Fungi

As noted earlier in this chapter, identification of most fungi beyond the group to which they belong is difficult, sometimes even for mycologists. Although sources of information do exist, many of these are highly technical. However, there are a number of field guides and similar publications that are relatively non-technical and thus suitable for use by someone without a high level of expertise relating to fungi. These include both general treatments of all fungi (Alexopoulos et al. 1996; Stephenson 2010) as well as publications dealing with specific groups (Lincoff 1981; Fuller and Jaworski 1987; Stephenson and Stempen 1994).

Alexopoulos CJ, Mims CW, Blackwell M (1996) Introductory mycology, 4th edn. Wiley, New York

Fuller MS, Jaworski A (1987) Zoosporic fungi in teaching and research. Southeastern Publishing Corporation, Athens

Lincoff GH (1981) The Audubon Society field guide to North American mushrooms. Alfred A. Knopf, Inc., New York

Stephenson SL (2010) The Kingdom fungi: the biology of mushrooms, molds, and lichens. Timber Press, Portland

Stephenson SL, Stempen H (1994) Myxomycetes: a handbook of slime molds. Timber Press, Portland

Glossary – Wetland Fungi

Agaric	a type of fleshy fruiting body produced by some macrofungi; it is characterized by a cap with gills (upon which the spores are produced) present on the underside
Bait	a small piece of organic material, such as a hemp seed, that is placed out in nature or added to a sample of water collected from nature, where it serves as a substrate to isolate an organism of interest (e.g., a chytrid)
Bolete	a type of fleshy fruiting body produced by some macrofungi that is characterized by a cap that contains many small tubes on the underside within which the spores are formed
Bryophilous	either living on or producing fruiting bodies in association with bryophytes
Cap	the portion of a fleshy fruiting body produced by some macrofungi that sits on top of the stalk and contains the spore producing region; a more technical term is "pileus"
Chitin	a nitrogen-containing polymer that serves as the primary structural component of the cell wall of fungi; also found in the exoskeletons of arthropods
Coenocytic	a cell that contains multiple nuclei
Conidium (plural: conidia)	asexual spores produced by non-zoosporic microfungi
Ectomycorrhizal	a mutually beneficial relationship that develops between a fungus (typically a basidiomycete) and the root cells of a living plant; the hyphae of the fungus do not penetrate the root cells
Endomycorrhizal	a mutually beneficial relationship that develops between a fungus (typically a glomeromycete) and the root cells of a living plant; the hyphae of the fungus do penetrate some of the root cells
Endophyte	a fungus that lives inside the tissues of a living plant
Epiphyte	a fungus that lives on or produces fruiting bodies on the surface of a living plant

Eukaryotic	an organism made up of cells with a nucleus
Fruiting body	a reproductive structure produced by fungi; the spores are produced within or on the surface of this structure
Germination	the process by which the initial hypha of a fungus emerges from a spore
Heterotroph	an organism that cannot manufacture its own food and instead derives its food from some outside source, either dead organic matter or a living organism; all fungi are heterotrophs
Hypha (plural: hyphae)	one of the microscopic, thread-like filaments making up the body of a fungus
Life cycle	the series of developmental changes through which a fungus passes from its inception (usually as a germinated spore) to the mature state in which a fruiting structure is produced
Lignin	a structural compound associated with plant cell walls in woody tissues
Macrofungus (plural: macrofungi)	a fungus that produce fruiting bodies (mostly above ground) that are fleshy and large enough to be noticed by a casual observer in the field
Microfungus (plural: microfungi)	a fungus that produce fruiting bodies that are small and inconspicuous and therefore not easily detected in the field
Moist chamber culture	a simple isolation technique in which a substrate sample is brought into the lab, placed in a container and kept moist in order to observe the organisms that develop
Mushroom	a common name often used to describe the type of fruiting body produced by some macrofungi
Mutualistic	an association between two organisms in which both members benefit
Mycelium (plural: mycelia)	a collection of interwoven hyphae making up the body of the fungus
Mycology	the scientific study of fungi
Mycologist	a person who studies fungi
Parasite	an association in which an organism derives its food from a second organism (the host), usually without killing the latter
Pathogen	an association in which an organism derives its food from a second organism (the host), ultimately killing the latter
Polypore	a type of tough and often resistant fruiting body produced by some macrofungi in which the spores develop within tiny tubes, thus giving the spore-bearing surface a porous appearance

Puffball	a type of more-or-less globose fruiting body formed by some macrofungi in which the spores are produced internally in a large mass; the spores are released when the outer wall ruptures in some fashion
Saprotroph	an organism that obtains its food from dead organic matter
Septum (plural: septa)	a cross-wall (often with a tiny opening or pore) that forms inside the hyphae of many fungi, dividing a hypha into cell-like units
Spore	the reproductive propagule of a fungus
Spore print	a technique used to help in the identification of some macrofungi by providing samples of spores for microscopic examination and allowing the investigator to determine the color of the spores in mass; a spore print is obtained by placing a portion of a relatively fresh fruiting body on a piece of paper and allowing the spores to be deposited
Substrate	the substance or material upon which a fungus lives, feeds or produces its fruiting bodies
Zoospore	a motile spore that is capable of moving through its environment by means a flagellum; produced by chytrids and the fungus-like water molds

Chapter 4
Methods for Sampling and Analyzing Wetland Protozoa (Protists)

Marianne Borneff-Lipp and Matthias Duerr

Abstract Wetlands have been promoted for use in rural communities in developed as well as in developing countries as an appropriate technology to be handled with low operational maintenance costs. The removal of protozoan pathogens is an important, yet often neglected issue, particularly in countries, where water obtained from sewage plants is used for irrigation. This chapter is focussed on the detection and the removal of protozoa in natural and constructed wetlands, since protozoan parasites are most frequently identified as pathogens with increasing medical and economical consequences. Different methodologies for collection, storage, record keeping, transportation, analyses of samples and for identification of protozoa are discussed, for instance immunofluorescence microscopy, fluorescence in-situ hybridization (FISH) and polymerase chain reaction. Advantages and disadvantages of different technologies of wetlands and a field trial application are presented.

4.1 Introduction

Wetlands are promoted in recent literature as attractive and cost-effective technology for waste water treatment. However, health concerns are legitimate whenever water, that has passed a treatment plant, is used for agricultural application (e.g., as irrigation water). The World Health Organization has compiled standards governing the hygienic quality of irrigation water (WHO 1989). These standards can only be achieved if raw water entering the treatment wetland is treated adequately. Untreated water may contain a high amount of microorganisms, so that crops might become contaminated by bacteria and protozoa, as well, after irrigation with waste water. Moreover, the discharge of insufficiently treated sewage water into rivers, lakes and ponds may contaminate drinking water resources, as well.

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Whereas removal of bacteria in wetlands is well documented (Baeder-Bederski et al. 2004), there is only limited information about the fate of protozoa in wetland plants. However, the removal of protozoan pathogens is an important yet often neglected issue, since infections with protozoan parasites, such as *Cryptosporidium* and *Giardia* spp. are most frequently associated with severe diarrhea, occurring after ingestion of infective oocysts, which are voided in the feces of an infected person or animal. Cysts from human or animal feces can enter surface water directly or through runoff from the fields, where manure or sewage water is used as fertilizer.

4.2 Protozoa Biology

Protozoa are defined as a large collection of organisms, which are eukaryotic and unicellular. Most of them employ chemoheterotrophic nutrition, but certain species can practice photoautotrophy (Horan 2003). The current trend is to classify them as a Subkingdom of the Kingdom Protista. With a wide range of species – around 50,000 – they play an important role in raw and wastewater and in potable water as well (Ross 1999). Among the most frequent microbial causes of death worldwide, six are eukaryotic including the agents of malaria, African sleep sickness and amoebic dysentery.

In order to understand the contribution of protozoa to aquatic ecosystems, it is essential to identify and classify them. This is done on the basis of their morphology, in particular regarding their ability to move. The classification published by Ross (1999), formally adopted from Jahn (1979) recognizes four major Phyla of protozoa:

1. *Mastigophora* or flagellated protozoa (e.g., Euglena),
2. *Sarcodina* or amoeba like protozoa (e.g., Amoeba),
3. *Sporozoa* or parasitic protozoa (e.g., Plasmodium), and
4. *Ciliophora* or ciliated protozoa (e.g., Paramecium).

4.2.1 Nutrition

Protozoa possess a wide range of feeding strategies. Certain members are primary producers and capable of photoautotrophic nutrition, in addition to the more usual chemoheterotrophic nutrition (Horan 2003). All protozoa are using phagocytosis for building cellular material. A solid food particle is enclosed in a vacuole, covered with a membrane. Dissolved nutrients are removed from the cell by fusion of the vacuole with the cell surface membrane. A food vacuole is “living” for around 20 min; this time is reduced if the cell is not feeding.

In addition to phagocytosis, there are other mechanisms by which a protozoan can obtain energy. Some of them are participating in symbiotic relations with photosynthetic organisms; others are considered to take up dissolved nutrients. However, this mechanism may not play any role for the free-living protozoa outside of a laboratory culture (Horan 2003). He describes a number of different feeding patterns: filter feeders, raptorial feeders and diffusion feeders.

Filter feeding involves the creation of a feeding current, which is then passed through an organelle to filter out the solid particles in the water. In the flagellates, this is a collar of straight, rigid tentacles. For the ciliates, the water is passed through an arrangement of parallel cilia. The clearance between the tentacles in the collar and the parallel ciliates dictates the size of particle that is retained (Horan 2003).

Raptorial feeding is practiced in small flagellates and amoebae. In this mode, water currents are driven against the cell using a hairy anterior flagellum. Particles which make contact with a lip-like structure on the protozoa are phagocytized. Diffusion feeding is practiced by the sarcodines. The suctorians are common protozoa in activated sludge and they feed by diffusion. They are attached to a floc particle by a stalk including tentacles. They have bundles of tentacles supported with an internal cylinder of microtubules. Ciliates touching these tentacles become attached and immobilized and are drawn through the tentacle into the suctorian.

Within the latter two modes of nutrition, the protozoa display a certain degree of selective feeding. Larger forms of amoebae are carnivorous, eating mainly ciliates and flagellates, whereas the smaller amoebae feed primarily on bacteria. The suctorians feed almost exclusively on holotrichous and spiriotrichous ciliates, with hypotrichs, flagellates and amoebae rarely being captured. For more detailed information, see Horan (2003).

4.2.2 Reproduction

For the free-living protozoa, an increase in cell size is followed by asexual reproduction such as binary fission. Horan (2003) describes this procedure as follows: A single cell divides into two and generates two daughter cells. As the protozoan is not symmetrical, the two daughter cells are not identical initially; however, the differences soon disappear. Sexual reproduction only occurs in times of stress or adversity, for instance, if the food supply diminishes (Horan 2003).

The flagellated protozoa undergo division along a longitudinal plane and the ciliated protozoan divide along a transverse plane. The suctorial lead a sedentary lifestyle spent attached to particulate material. Thus, a method of asexual reproduction involving fission would lead to a rapid increase in their population with increased competition for food and space. Consequently, they divide by budding in which the new cell appears as a slight protuberance on the parent cell surface. This is evaginated and liberated, but unlike its parent, it is motile in its immature stage, for around 30 min, which allows it to migrate and reduce crowding.

The most common form of protozoan reproduction is known as binary fission in which the organism divides into two equalized daughter cells. In the ciliated

protozoa, binary fission is usually transverse with the posterior end of the upper organism forming next to the anterior end of the lower one. However, when environmental conditions begin to change, sexual reproduction generally becomes more prevalent. Sexual reproduction allows for the mixing of DNA among the various strains (asexual daughters) of a local protozoan population. A genetically diverse population is more able to adapt to changing conditions. In the competitive world of a wastewater treatment wetland, the more diverse the gene pool for a species, the greater the likelihood that it will persist over a wide range of changing environmental and operating regimes (Horan 2003).

4.3 Risks of Acquiring Waterborne Infections

Protozoa in water are primarily focused on those species that are used as host organisms to complete their life cycle. Sporozoa, for instance, are able to use different sites in the human body, such as the skin, eyes, gut, blood, muscle and thus cause diseases (Table 4.1).

One species of special concern to humans is *Cryptosporidium parvum* (Phylum Sporozoa Subphylum Apicomplexa), which detected in 152 species of mammals so far. Infections in humans are well documented, occurring after ingestion of infective oocysts (in most cases use of oocysts refers to both oocysts and cysts), which are voided in the feces of an infected person or animal (Slifko et al. 2001). Diarrheal symptoms usually last for 7–10 days (Thielman and Guerrant 2004). Cysts from human or animal feces can enter surface water directly or through runoff from fields where manure or sewage sludge is used as fertilizer (Bukhari et al. 1997). The most important source of surface water contamination in densely populated areas is sewage effluent from wastewater treatment plants (Hänninen et al. 2005).

Giardia is a flagellated protozoan (Phylum Metamonada) that has been found in more than 40 animal species (Meyer 1994). Fecal-oral transfer of *Giardia* cysts is the major route of transmission of giardiasis, as indicated by the high prevalence in developing countries with poor standards of hygiene and sanitation, in day-care centers and nurseries (Pickering and Engelkirk 1990). Waterborne outbreaks of giardiasis have been reported for some 30 years (Moore 1969; Brodsky et al. 1974;

Table 4.1 Diseases associated with protozoan infections of humans (Horan 2003)

Niche	Protozoan	Condition
Skin	*Leishmania*	Cutaneous leishmaniasis
Eye	*Acanthamoeba*	Corneal ulcers
Gut	*Giardia, Entamoeba, Cryptosporidium*	Giardiasis, Cryptosporidiosis
Bloodstream	*Plasmodium, Trypanosoma*	Malaria, African sleeping sickness
Spleen	*Leishmania*	Visceral leishmaniasis
Liver	*Entamoeba, Leishmania*	Visceral leishmaniasis
Muscle	*Trypanosoma*	Chaga's disease

Craun 1990). In the USA, *Giardia* is the most commonly identified pathogen in outbreak investigation, with more than 100 waterborne outbreaks, based on epidemiological evidence. Waterborne outbreaks have also been reported in Australia, Canada, New Zealand, Sweden, and the United Kingdom. These outbreaks have been linked to consumption of untreated surface water contaminated by human sewage (Craun 1990).

In addition to being parasites, protozoan populations are also subject to parasitism by a large number of fungi, bacteria and other protozoa. The opportunistic human pathogen, *Legionella pneumophila*, is able to parasitize the ciliated protozoan, *Tetrahymena pyriformis* (Phylum Ciliophora, Subphylum Oligohymenophora). This mechanism may help its distribution and survival in aquatic environments (Fields et al. 1984).

4.4 Wetland Principles and Protozoan Survival

Today, most wastewater treatment plants make use of the natural self purification capacity of wetlands and other aquatic ecosystems, which results from the presence and action of microbial communities. Thus, biological sewage treatment plants might be regarded as "constructed" ecosystems.

Artificially constructed wetlands may be installed either as vertical or as horizontal flow filter constructions. Horizontal systems may be further classified, depending on the pathway of water flow, as surface and subsurface flow systems. Constructed wetlands have demonstrated effective removal of protozoan parasites mainly with horizontal subsurface flow gravel-based systems (Rivera et al. 1995). Within these configurations, the wastewater is flowing through the media and also below the surface level. The influence of vegetation in constructed wetlands on parasite removal is still unclear. Utilization of reeds (*Phragmites* spp.) for instance, is common in most types of constructed wetlands; however, the presence of marsh plants in subsurface flow gravel beds may not be a significant factor for the removal of protozoan parasites (Quiñónez-Díaz et al. 2001).

Recent documentation on wetland systems concerning wastewater quality improvement has focused on the efficiency of removing waterborne pollutants. Several studies have shown the effectiveness of wetlands in treating domestic wastewater for reduction of biochemical oxygen demand, suspended solids and nitrogen (Karpiscak et al. 1996). In the meantime, constructed wetlands are known as an attractive and cost-effective wastewater treatment alternative compared to conventional processes. However, information on removal of pathogenic microorganisms is limited; only a few studies have been performed to determine the fate of pathogenic microorganisms and protozoan parasites at the same time (Stott 2003). A variety of processes have been identified concerning the removal of bacterial and viral pathogens, but there is a lack of detailed studies of parasite removal and inactivation mechanisms.

The survival of protozoans in wetlands depends on a large collection of parameters, such as biological, chemical and physical environmental conditions. Although survival can vary widely, typical survival times for protozoa in sewage are <30 days (Stott 2003). Conventional secondary biological treatment processes do not inactivate parasites respectively protozoa. Robertson et al. (2000) reported no reduction in *Cryptosporidium* oocysts viability during aerobic biological treatment. Robertson et al. (1999) also reported higher amounts of viable *Giardia* cysts in treated effluents (>20 %) than in influents (<10 %). Even tertiary treatment, using filtration and chlorination may not remove protozoa completely (Stott 2003).

Natural wastewater treatment systems such as ponds and wetlands with longer retention times appear to be more effective in removal than conventional systems. Most studies on parasites in wetlands have concentrated on the efficiency of removal of nematode ova, and not protozoan oocysts. The difficulty in determining accurate removal efficiencies was demonstrated by a study from Canada (Roach et al. 1993); the final effluent contained significantly higher cyst concentration (17 cysts/L) than in raw wastewater (1 cyst/L).

Using constructed wetlands with and without plants (*Phragmites* spp.), a recent study evaluated protozoan survival (Redder et al. 2010). However, there is still a lack of studies that may define the parameters involved. In conclusion, the study demonstrated that removal of protozoans via constructed wetlands turns out to be a combined effect of physical, chemical and biological effects.

Although information on protozoan removal is available, further studies are needed to understand fully the processes for inactivation. Only at that stage of knowledge, an improvement of strategies and techniques can be achieved. Hereby, the occurrence of parasitic diseases can be minimized and public health will be safeguarded (Stott 2003).

4.5 Detection of Protozoa in Wetlands

For isolation and enumeration of oocysts from wetlands, specific methods are required to improve removal efficiency due to the low densities of protozoans. There are several reasons for low detection rates and the need to determine presence (Horan 2003; Smith and Grimason 2003; Redder et al. 2010):

1. Oocysts can pass through physical and chemical barriers,
2. They are chlorine insensitive,
3. They have to be detected in water in spite of the absence of other indicator organisms,
4. They can cause epidemic disease in consumers of contaminated potable water,
5. As *Giardia* and *Cryptosporidium* are obligate parasites, their numbers cannot be augmented by conventional in vitro culture methods, and
6. The minimum infectious dose for humans is generally low.

Sampling and detection necessitates multiple stages such as sample collection and concentration, separation of oocysts from contaminating debris, and detection identification of oocysts. Due to the oocysts occurring at low densities in wetlands, a system that enables their efficient recovery from large volumes of water is required. Currently, filtration followed by immunofluorescence detection are recommended by official authorities for monitoring treated water (United States Environmental Protection Agency [USEPA 1998]; United Kingdom Government 1999a), but it is also the most effective methods for isolating and enumerating waterborne oocysts.

All sampling procedures should follow the general principles mentioned below (Kator and Rhodes 2003):

Personal safety equipment must be worn. A mask covering the mouth and nose, laboratory glasses, and a pair of gloves (non-sterile) are mandatory attire.

Good laboratory practices are to be followed. Collect water in clean containers or bottles. Identify and record prior to the sampling process, the following information (date, time, place of collection, the name of the person collecting the sample, and any unusual condition).

Sampling locations should reflect the aim of the detection process and consider pollution sources and hydrographic parameters, including water flow, direction, circulation, wind speed, etc.

Containers, respectively bottles. The bottles should be protected against any contamination before, during, and after collection and during transportation.

Keep the container unopened until filling starts and during sampling, keep the closure free from contamination. To collect a sample, hold the bottle near to the water source and plunge the neck into the water by using a clean, handling instrument like a laboratory clamp or a commercial holding device, or plunge a clean vessel into the water and then fill the water into the bottle neck (peristaltic pumps and sterile tubings or syringes can be used for sampling in shallow water). Storage in ice is recommended to reduce likelihood of highly enriched samples changing in composition, if temperature is not reduced. Samples should be processed as soon as possible and the use of additional spiked samples for all detection methods that are non-regulatory methods is recommended.

Standard methods are preferred and should be used whenever possible. Specification of the sample materials (e.g., water quality), target organisms (e.g., bacteria, protozoa, viruses), and other factors at the site that may affect sampling (e.g., pH and temperature, physiological obstacles) should be recorded.

4.5.1 Sample Collection and Concentration Methods

Protozoan sample collection, storage, and transport are important initial elements within the whole process of detection within wetlands. The most careful enumeration might be useless if poor sampling and storage is practiced. Also, adequate sample volumes for detecting the target organisms must be considered.

There are two different situations to follow in sampling water from wetlands. Large volume sampling includes the collection of a sample over a period of hours at a defined flow rate. Small volume sampling, typically a volume of 10–20 L, is taken as a so-called grab sample. In case there is no prior knowledge concerning the occurrence or temporal distribution of oocysts in the sample tested, large volume sampling is useful as the sample is taken over a long-time period. In contrast, grab samples can provide higher recovery efficiencies than large volume sampling and are collected readily. A compromise between both regimes is the collection of numerous grab samples over the large volume sampling period in order to generate one composite sample (Smith and Grimason 2003).

4.5.1.1 Large Volume Sampling

Large volumes (~100–1,000 L) of water are filtered through a special filter [in literature the following products are suggested: (e.g., yarn wound cartridges) (CUNO Europe SA, flow rate 1.5 L/min), polypropylene cartridges (Filterite, flow rate 4 L/min), compressed foam (Genera Technologies Filtra-MaxTM Crypto Dtec, flow rate 1–2 L/min), HV pleated membrane cartridge filter, Pall Envirochek® flow rate 4 L/min)]. These entrap oocysts and other particulates of similar and larger size. Oocysts and other particulars attached to the yarn wound and polypropylene cartridges are eluted by immersing the cut, teased filter in large volumes of a mild detergent (0.01 % Tween 80 in deionized water containing an antifoaming agent).

The resulting washings from these cartridges sometimes amounts to 5 L and require further concentration by centrifugation. The compressed foam cartridge requires expensive and dedicated manufacturer's equipment to elute oocysts which are concentrated onto flatbed membranes and eluted by massaging the membrane in a dilute detergent solution.

4.5.1.2 Small Volume Sampling

Oocysts present in grab samples can be concentrated either by membrane filtration or flocculation. In literature, the following procedure is recommended. The sample is filtered through either a flatbed 42 mm, 1.2–2 μm cellulose acetate or polycarbonate membrane (flow rate ~150 ml/min) or a pleated membrane capsule (e.g., Gelman Envirochek, flow rate 2 L/min) using a peristaltic pump.

The concentrated materials are recovered by "scraping" the surface of the membrane together by washing with dilute detergent, followed by further concentration using centrifugation. However, while it is relatively easy to filter 10–40 L of low-turbidity water, with some high-turbidity waters it is possible to filter only 1–2 L. As with cartridge filtration, a range of recovery efficiencies has been reported for flatbed membranes. An average recovery of 9 % for *Cryptosporidium* and 49 % for *Giardia* has been reported in literature (Nieminski et al. 1995). Other authors suggested in a study about the recovery efficiency of several different

membranes that 1.2-mm cellulose-acetate membranes will result in higher recovery (30–40 % and 50–67 %, for *Cryptosporidium* and *Giardia*, respectively (Shepherd and Wyn-Jones 1996)) than the 2-mm polycarbonate membranes (22–36 % and 41–49 % (Ongerth and Stibbs 1987)).

Another established method for concentrating oocysts is the calcium carbonate flocculation procedure developed by Vesey et al. (1994). By adding calcium chloride and sodium bicarbonate and adjusting the pH to 10.0 with sodium hydroxide, a precipitate of calcium carbonate is formed. After the precipitate has settled, the supernatant fluid is removed by aspiration, the calcium carbonate is dissolved with sulfamic acid, and the sedimented material is resuspended. Recovery efficiencies using this method have been reported to be as high as 70 % for both *Cryptosporidium* and *Giardia*. More recent work has demonstrated that this is the upper limit of the detection efficiency and that recoveries are usually lower.

The search continues for new methods of concentrating water samples to detect the presence of protozoans and many techniques have been evaluated, including cross-flow filtration, continuous-flow centrifugation, and vortex-flow filtration (Whitmore 1994) as well as a number of proprietary systems. There continues to be much debate over which method is most appropriate. Realistically no single method is suitable for all situations. The choice of method should be made with due regard to a number of factors, including the purpose of sampling, the water quality, and the facilities in the laboratory that will perform the analysis. Ideally, the method chosen should efficiently concentrate as large a sample as possible and yield a concentrate that can be examined easily. Some workers prefer a small volume of water to examine the entire concentrate. Others take large samples and examine only a fraction of the final concentrate. Both approaches are valid, but the methods used to concentrate small volumes tend to be easier to perform and generally have higher recovery efficiency.

4.5.2 Separation Methods

Concentration of *Cryptosporidium* oocysts and *Giardia* cysts is based almost exclusively on particle size. Extraneous debris may interfere with the successful detection of oocysts. Some form of separation technology is therefore required.

4.5.2.1 Density Centrifugation

Density centrifugation is often used to separate oocysts from background debris and thus reduce the amount of material to be examined. Of particular interest was the finding of Bukhari and Smith (1996) that sucrose density centrifugation selectively concentrated viable, intact *Cryptosporidium* oocysts.

4.5.2.2 Immunomagnetic Separation

The principle of this technique is that specific antibodies are attached to magnetizable particles. The oocysts attach to the magnetizable particles and are isolated from this debris with a strong magnet. There are potential sources of failure. For example, many of the commercially available monoclonal antibodies to *Cryptosporidium* or *Giardia* are of the IgM type, and are therefore of low affinity since they have not undergone affinity maturation or isotype switching.

However, the real benefit of a good separation technique is seen with samples that have yielded a highly turbid concentrate. In these samples, immunomagnetic separation seems to perform less efficiently. The use of antibodies of higher affinity may improve the recovery efficiency of oocysts from high turbidity samples.

4.5.2.3 Flow Cytometry

Flow cytometry has been attempted with environmental samples to detect *Cryptosporidium* oocysts, but it was found that the sensitivity was insufficient to distinguish oocysts. Incorporation of a cell-sorting facility enabled oocysts to be sorted efficiently from background material. Vesey et al. (1994) describe this method to work well for *Giardia* cysts, too.

Water concentrates are stained in suspension with fluorescein isothiocyanate-labelled (FITC-labelled) antibodies and passed through the fluorescence-activated cell sorter (FACS). Particles with the fluorescence and light-scattering characteristics of oocysts are sorted from the sample stream and collected on a microscope slide or membrane filter, which is then examined by epifluorescence microscopy to confirm the presence of oocysts.

The procedure is not sufficiently specific or sensitive for the count of sorted particles to give a definitive indication of the number of oocysts present since others may cross-react with the monoclonal antibody and have similar fluorescence characteristics. However, the confirmation by epifluorescence microscopy can be performed much more easily and reliably than direct microscopy of non-sorted samples (WHO 2011).

4.5.3 Identification Methods

The enumeration and detection of oocysts from water requires specific methods because they can pass through physical and chemical barriers. Another problem is that for instance *Giardia* and *Cryptosporidium* spp., as obligate parasites, cannot be cultured by conventional in vitro-methods, and also, they usually will occur in relatively low concentrations in the aquatic environment. However, there are several methodologies available for detection of these protozoans, which are described below (WHO 2011).

4.5.3.1 Immunofluorescence Microscopy

Detection of *Cryptosporidium* oocysts and *Giardia* cysts relies on epifluorescence microscopy, which may be used to examine material deposited on multi-well slides or membrane filters. The oocysts are specifically stained with monoclonal antibodies which have been either labelled directly with FITC or labelled during staining with an FITC-labelled anti-mouse antibody, whereas staining with a directly labelled antibody seems to produce less nonspecific binding and can make preparations easier to examine.

Several anti-*Cryptosporidium* and anti-*Giardia* antibodies are commercially available, but apparently there is no single antibody that is preferred for all purposes. Some antibodies cross-react with other members of the genera and therefore cannot be used to specifically identify *C. parvum* or *G. intestinalis*.

4.5.3.2 Fluorescence In-situ Hybridization (FISH)

Fluorescence in-situ hybridization (FISH) has been suggested as a tool for the specific detection of *Cryptosporidium parvum* (Lindquist 1997). The FISH method may be combined with the immunofluorescent assay (IFA) method. However, the FISH-fluorescence signal is relatively weak, which makes microscopic interpretation difficult.

4.5.3.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is one of the most extensively tested procedures for detection of specific sequences of nucleic acids down to species or genus level. However, difficulties have been reported with application of the PCR technique to water concentrates. As a result of PCR's sensitivity to the concentration of many compounds within the reaction mixture, inhibition of the DNA amplification process may happen. Nevertheless, many authors plead for the detection of *Cryptosporidium* oocysts by PCR.

As pointed out within the WHO (2011) Guidelines for Drinking Water Quality, the sensitivity of the standard PCR was reported to be one cyst in water samples and moreover it has been reported that amplification of heat shock-induced mRNA using the same heat shock protein (HSP) primers was indicative of viable *Giardia* cysts.

The use of PCR for the detection of oocysts in water concentrates might be preferred rather than direct microscopic examination, since the procedure can be automated, allowing several samples to be handled simultaneously. Also, it may be possible to distinguish viable from non-viable oocysts.

Some claim to be able to detect a single oocyst in a water concentrate by using a procedure involving reverse transcription (RT-PCR) where the target sequence

codes for the *Cryptosporidium* HSP 70 (Stinear et al. 1996). However, the method is not quantitative and thus may be of limited value in some circumstances. The use of RT-PCR against induced mRNA, a nucleic acid with a short half life, overcomes the concern that false-positive results could be obtained either from non-viable oocysts or from free DNA.

Many scientists prefer to view the object directly. For the future, a combined approach might be the solution, with molecular techniques being used as a screening tool, followed by microscopic examination, when positive results are obtained (WHO 2011).

Currently, filtration is considered as the most effective method in combination with immunofluorescence detection. This method can also be used for direct counting when conjugated with fluorochromes, such as fluorescein isothiocyanate (FICT). These methods are recognized officially by the USEPA (1995) and the United Kingdom (United Kingdom Government 1999b) for monitoring treated water.

4.6 Langenreichenbach, Germany Case Study

4.6.1 Field Trial Application

The following description of a field trial is provided to demonstrate what additional conditions are to be considered, in order to meet the practical conditions of constructed wetlands. In one of our studies, which was supported by German Federal Governmental Funding (BMBF-No.: 02WA0108), the microbiological water quality was evaluated by determining not only fecal indicators, such as *Escherichia* (*E.*) *coli*, Enterococci, coliform bacteria, but also other microbiological parameters like colony forming units (cfu) and protozoan parasites as well.

A pilot plant system was set up in 2000 by the UFZ Centre for Environmental Research Leipzig-Halle (Germany) in the village of Langenreichenbach near Leipzig, Germany (Fig. 4.1). The water resource was a main sewer carrying municipal raw sewage of about 10,000 population equivalent to the plant. The raw water was mechanically pre-treated in a straw filter. The plant itself consisted of 14 coated steel container elements each measuring 6.7 m^2, which were filled with identical wastewater from a ring piping. Different scenarios for evaluating different constructive elements and following microbiological and chemical parameters were tested from 2002 to 2005.

To study the influence of two filter materials, seven basins were filled with washed sand (grain size, 0–2 mm Heinrich Niemeier GmbH & Co KG, Sprotta, Germany), while the other seven filters were filled with a mixture of expanded clay (Fibo Exclay Deutschland GmbH, Lahmstedt, Germany) and sand, grain size 2–4 mm and 0–2 mm, respectively. This quality has been developed especially for comparative tests to examine the influence of different types of filter materials.

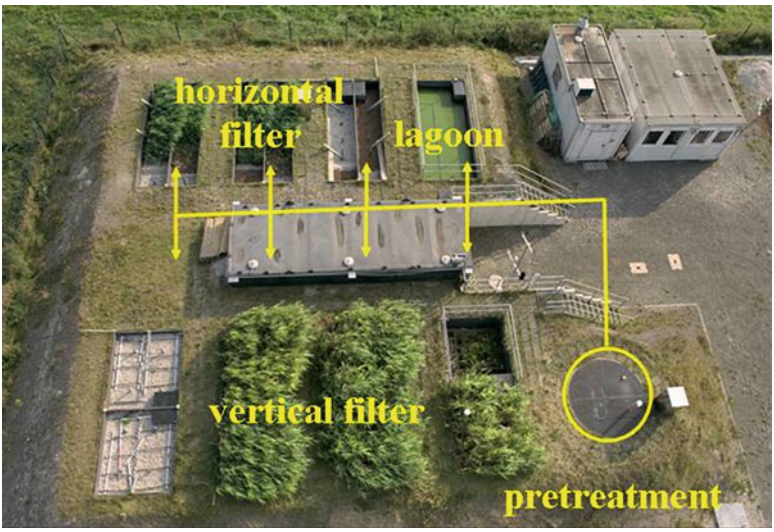

Fig. 4.1 Pilot wetland at Langenreichenbach, Germany (Redder et al. 2010) (Published with kind permission of © [Elsevier] [2010])

The filters were planted with reed (*Phragmites australis*) with a density of 6 plants/m^2. To determine the influence of the reed on the reduction performance, 4 of 14 filter beds remained unplanted.

After running through the pilot plant, the water was then returned to the municipal sewage plant. Combinations of six different soil filters were tested within the pilot project (Table 4.2). In this two-stage operation, the water – discharged from the first vertical or horizontal flow filter – was transferred at intervals to the second horizontal flow filters using peristaltic pumps. This specific setup was operated for 12 months.

4.6.2 Parasitological Analysis

Samples were collected every two weeks for a period of nine months. Sampling spots were selected within the system as follows: (1) influent, raw wastewater; (2) effluent of the first filter flow; (3) effluent from the second filter flow; (4) effluent from a facultative pond (without filter material and without reeds).

Sample collection and processing was performed according to the USEPA filtration method "ICR Protozoan Method for Detecting *Giardia* cysts and *Cryptosporidium* oocysts in Water by a Fluorescent Antibody Procedure" (EPA/814-B-95-003; US EPA 1995). The test method includes detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in water by a fluorescent antibody procedure (Fig. 4.2).

Table 4.2 Sample sites of the pilot plant at Langenreichenbach, Germany (Redder et al. 2010)

Filter bed combinations	Flow	Filter material	Plants	
Combination I	1st stage	Vertical	Clay/sand	*Phragmites australis*
	2nd stage	Horizontal	Sand	
Combination II	1st stage	Vertical	Sand	*Phragmites australis*
	2nd stage	Horizontal	Clay/sand	
Combination III	1st stage	Horizontal	Clay/sand	None
	2nd stage	Horizontal	Sand	
Lagoon	–	–	None	None

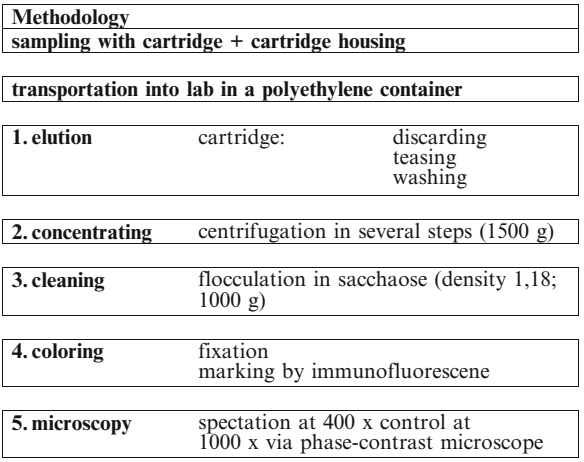

Fig. 4.2 Methodology of protozoan sampling and identification in German study (Redder et al. 2010)

Protozoa were concentrated from a large volume (100 L) by retention on a yarn-wound filter, 1 µm nominal porosity (Micro-Wynd® MW D-PPPY, Cuno Europe SA, Mainz, Germany). Retained particulates were eluted from the filter using an eluting solution (Tween 80) and were concentrated by centrifugation (1,500 × g for 10 min). *Cryptosporidium* oocysts and *Giardia* cysts were separated from other particulate debris by flotation on a Percoll-sucrose solution with a specific gravity of 1.18. A monolayer of the water layer and Percoll-sucrose interface is placed on a membrane filter, indirectly stained with fluorescent antibody Hydrofluor™-Combo (Indirect Immunofluorescent Detection Procedure for *Giardia* cysts and *Cryptosporidium* oocysts in Environmental Samples, Strategic Diagnostics Inc., Newark, DE, USA), and examined under the fluorescence microscope.

Using epifluorescence (Axiolab®, Zeiss, Jena, Germany), slides were scanned at 400× magnification for apple-green fluorescence of *Cryptosporidium* oocysts and *Giardia* cysts shapes. If apple-green fluorescing cyst and oocyst structures were observed, *Cryptosporidium* and *Giardia* were identified at a 1,000× magnification. Cysts and oocysts were classified according to specific criteria (immunofluorescence, size and shape). Each well was scanned systematically and the number of oocysts were counted and documented as counts per actual volume per sample. Results were reported in terms of the categories per 100 L.

According to WHO guidelines (1989), enumeration of cysts and oocysts was determined as total number of protozoa per liter and the performance of removal was expressed as a $\log_{10}$ reduction factor ($\log_{10}$ (concentration of protozoa) influent – $\log_{10}$ (concentration of protozoa) effluent of filterbed).

4.6.3 Results Obtained at the Pilot Plant

A total of 93 samples were gathered at the pilot plant at Langenreichenbach. The influent contained a primary mean concentration of 150 *Giardia* cysts/100 L and 14 *Cryptosporidium* oocysts/100 L. *Cryptosporidium* oocysts only appeared sporadically in the influent of the pilot plant. In the effluent of the 2-stage systems, less than 1 oocyst/100 L was identified. The lagoon is had negligent efficacy. The mean concentration of *Giardia* in the influent was 150 cysts/100 L (±100) and for *Cryptosporidium* 13.6 oocysts/100 L (±14.7) respectively. The passage of the 1st stage of the combination of filter beds resulted in a comparably high removal rate of both *Cryptosporidium* and *Giardia* spp.

Nevertheless, low concentrations of protozoa were still found in samples taken at the effluent of the first filter. In the effluent of the second stages, less than 1 oocyst/100 L was found in most of the samples, with average recovery level of oocysts and cysts being 6.5 and 14.7 %, respectively. Because of the low amount of protozoa in the effluent of the second stages, no significant difference between the primary effluent of the effluent of the first filter and each of the effluents of the second filter was determined.

The average removal of *Giardia* ranged between 0.08 and 1.58 $\log_{10}$ with the highest reduction rates in the first stages of the treatment process in all combinations. In the effluent of the treatment process, less than 1 oocyst/100 L was detected in all combinations. The $\log_{10}$ reduction factor of *Cryptosporidium* oocysts varied between 0.25 and 1.58 with highest in the first step of combination II. The average $\log_{10}$ reduction factor (RF) for the two-stage system (combination I) was 2.2. Similar reductions were observed for combination II ($(\log_{10}) = 1.9$) and for the unplanted combination system III ($\mathrm{RF}(\log_{10}) = 2.0$). However, there was no difference between the pretreatment and the effluent of the lagoon for *Cryptosporidium* or *Giardia* oocysts.

Therefore, it can be assumed that filtering of the wastewater is the main effective mechanism reducing the protozoan parasites. The removal of *Cryptosporidium* oocysts and *Giardia* cysts, however, seemed to be independent of the filter types used. *Phragmites australis* generally did not have a noticeable influence on the removal of the protozoan parasites tested.

4.6.4 Conclusion Field Trial

Constructed wetlands appear to be an alternative to municipal plants. Our study demonstrated constructed wetlands in a pilot and a field scale do achieve reduction rates of ≈2 log for the protozoan pathogens *Cryptosporidium* oocysts and *Giardia*

cysts. These results are in agreement with an Italian study that investigated municipal wastewater plants and observed a *Giardia* cyst reduction rate of 1–2 logs (Caccio et al. 2003).

In our study, the most important results were that several consecutive stages appeared to be the only alternative to guarantee the removal of protozoan pathogens. This two stage system consisting of a subsurface horizontal flow filter led to an almost complete removal of parasite pathogens in the pilot plant at Langenreichenbach.

Small particle sizes (0–2 mm sand particles) seemed to favor parasite reduction by direct mechanical removal. Particulate matter accumulating especially within the first 10–20 m in the reed bed, suggests that parasite eggs might be removed by mechanical filtration in subsurface flow systems (Williams et al. 1995; Stott 2003).

Within the pilot plant at Langenreichenbach, limiting the hydraulic load to a range of 40–60 mm/day turned out to be essential. Higher loads might have led to a breakdown of the system by overloading (Baeder-Bederski et al. 2005).

In our study, the microbiological water quality was evaluated and fecal indicators were determined, especially *E. coli* (DIN 19650 1999; WHO 1989). Generally, at the input of the pilot plant at Langenreichenbach, *E. coli* was detected up to an average of 10^7 cfu/100 ml. All filterbeds exhibited reduction factors up to 5 with a range of 0.5–5.9. Concerning the lagoon, the reduction factor was 1.4 with a range of 0.4–2.5. *E. coli* concentrations were relatively constant. The highest reduction rates were achieved with horizontal flow filter beds. Although natural wastewater treatment systems in general are known to be more effective at removal of protozoan parasites than conventional (mechanical) systems (Stott 2003), the rate of reduction usually turned out to be relatively low in comparison to bacterial removal. No direct correlation between protozoan parasites reduction and those of other indicator organisms could be shown. These results are found in other studies.

The lack of correlation between bacterial indicator organisms and parasites underlines the necessity of adequate diagnostics for parasitic load in hygienized water. Monitoring a range of indicator organisms in reclaimed effluent is more likely to be predictive concerning presence of parasitic pathogens, and a need for additional pathogen monitoring in reclaimed water in order to protect public health is suggested (Harwood et al. 2005).

4.7 Outlook

In summarizing the current techniques for sampling and detection of protozoans, it is obvious that additional research in improving water and sewage treatment practices in treatment wetlands is needed. Timely and efficient detection of infectious *Cryptosporidium parvum* and *Giardia lamblia* in environmental samples requires the development of rapid and sensitive techniques. A major factor complicating proper detection is the problem of efficiently concentrating cysts from environmental samples, while limiting the presence of extraneous materials.

Molecular-based techniques are the most promising methods for sensitive and accurate detection in the near future (e.g., a multiplex PCR for the simultaneous detection of *Cryptosporidium parvum*, *Giardia lamblia* and other waterborne protozoan pathogens; Carey et al. 2004).

In conclusion, the tested filter systems within the treatment wetland field trial are able to reduce the number of protozoa to some extent and thus to reduce the potential risk of infections associated with wastewater reuse. *Phragmites australis* seems to have little influence on protozoan (as well as bacterial) reduction performance.

Wastewater effluents from the tested types of plants may be used as irrigation water (e.g., for open areas and parks according to the WHO guidelines); however, in the event of strong evidence for high parasitic load, these requirements might not be met. Therefore, point intensive control of protozoan parasites is necessary, with this being the most important issue since the literature shows no direct correlation to other indicator organisms. Thus, any use of the obtained water must be accompanied by close quality controls.

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Student Exercises

The following will introduce students to wetland protozoan life history, sampling, identification, and ecology.

Classroom Exercises

1. Make specific plans for collections and storage record.
2. Note chains of custody for the use of signatures to record individual responsibility for the sample.
3. Describe the mostly recognized methodologies for sampling of protozoa.
4. Note the minimum particle size for filter beds in constructed wetlands.
5. Note the actual main technologies for constructed wetlands.
6. Describe methods for detection of protozoans.
7. List advantages and disadvantages in waste water planted and unplanted containers.
8. Summarize risks of protozoan contamination in constructed wetlands.
9. Describe new technologies for analyzing protozoans in water of wetlands.
10. Describe your own perspective of wastewater management in the future.

Chapter 5
Sampling and Processing Aquatic and Terrestrial Invertebrates in Wetlands

James T. Anderson, Florencia L. Zilli, Luciana Montalto, Mercedes R. Marchese, Matthew McKinney, and Yong-Lak Park

Abstract Obtaining unbiased samples of aquatic and terrestrial invertebrates from wetlands provides unique challenges due to the varied life history strategies of invertebrates as well as the heterogeneity present within a wetland. Many sampling devices are useful in more than one sampling environment within a wetland but the effectiveness of most methods varies among and within wetlands as well as between users. In this chapter, we emphasize field collecting techniques and address laboratory sorting methods. When possible, the advantages and disadvantages of each method are listed and suggestions are provided to reduce bias and unwanted variability in sample collection. Sampling devices for benthic (grabs, single and multiple cores, nets, and artificial substrate), water-column (open cylinder, emergence trap, activity trap, sweep net), epiphytic (box samplers, quadrat samplers), flying terrestrial (aerial net, flight intercept trap, light trap, malaise trap), and non-flying terrestrial (sweep net, aspirator, vacuum sampler, Berlese-Tullgren funnel, mist net) invertebrates are presented and discussed.

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5.1 Introduction

Wetland invertebrates are comprised of a diverse assemblage of aquatic and terrestrial species. The various wetland physiognomic characteristics (e.g., open water, emergent vegetation, forested vegetation), invertebrate life stages (i.e., larvae, pupae, adult), modes of existence (i.e., skaters, planktonic, divers, swimmers, clingers, sprawlers, climbers, and burrowers), functional feeding groups (i.e., collectors, engulfers, parasites, piercers, scrapers, shredders), and habitat specialization (e.g., aerial, benthic, endogeic, nektonic) make for unique challenges in sampling and quantifying invertebrates. Moreover, the sheer number of species makes identification problematic, often requiring specialists for identification to species, and local or regional taxonomic guides. For this reason, many studies and identification manuals are at the family or genus level.

Invertebrate studies in wetlands are conducted for a variety of reasons. Invertebrates are integral to the functioning of wetlands, and are often viewed as surrogates for wetland health. Indeed, a number of indices of biotic integrity and rapid assessment protocols focus on wetland (Gernes and Helgen 2002; Veselka 2008) or stream and river invertebrates (Angradi et al. 2009; Whittier and Van Sickle 2010). Invertebrates are abundant and taxonomically rich, which contributes to their value as study specimens (Dodson 2001). Long- and short-term hydrologic cycles, water quality, and vegetative communities associated with wetlands influence many adaptive strategies of invertebrates (Wiggins et al. 1980; Doupe and Horwitz 1995; Anderson and Smith 2000; Brooks 2000). Thus, resource managers have used invertebrates to quantify and qualify water quality in wetland ecosystems (Wallace et al. 1996). Invertebrates contribute to other wetland functions by assisting in litter decomposition, nutrient cycling (Cummins 1973; Merritt et al. 1984) and plant community regulation (Weller 1994). Hence, invertebrates indirectly aid in the transfer of nutrients from the sediments, detritus, and water column to higher-level organisms. They also have direct impacts on wildlife species by providing a prey base for other wildlife species. Because numerous avian species, particularly waterfowl and other waterbirds, depend on invertebrates for food (De Szalay and Resh 1996; Gonzalez et al. 1996; Anderson and Smith 1998, 1999; Anderson et al. 2000), researchers can assess avian productivity by sampling invertebrates. As well, they are important in the diets of anurans (Anderson et al. 1999; Lima and Magnusson 2000; Peltzer and Lajmanovich 2007), fish (Toft et al. 2003; Shervette et al. 2007; Parker et al. 2009) and some mammals (Conaway 1952; Errington 1963). Invertebrate communities have even been used as indicators for delineating wetland boundaries (Euliss et al. 2002). It is clear that invertebrates play a vital role in wetland function, and thus are integral in analyzing the health of these ecosystems (Zilli and Marchese 2011).

The objectives of this chapter are to outline the various benefits and pitfalls of a variety of wetland invertebrate sampling techniques. As with any technique, the choice of an appropriate sampling device depends on the study objectives. Wetland type, location, and depth; season; logistical and ecological considerations; local variation; and even personal preference and experience can influence the choice of

sampling device. Hence, this chapter should serve as a guide for choice of technique rather than as the definitive statement on which technique or protocol to use.

5.2 Selecting a Sampling Device and Developing a Sampling Design

Before a sampling device can be chosen, study objectives must be clearly articulated to ensure that the technique used is appropriate for the question posed. For example, if densities are required, that will entail a different sampling intensity and methodology than if the goal is to obtain an unbiased representative sample of organisms, to calculate an index of biotic integrity (IBI). Too often, we place a large emphasis on technique rather than focusing on the overall objectives of the survey. In the end, methods cannot compensate for a poorly designed study (see Chap. 1 of Vol. 1). Details of subsequent statistical analyses to be applied and needs for comparison of results with other studies should be linked with the overall survey objectives and must also be assessed in the planning phase (Keith 1991).

Sampling techniques and study design will also depend on the invertebrate community being studied as well as the conditions of their associated environments. For example, a clumped distribution of invertebrate populations could be related to heterogeneity in environmental factors and microhabitat preference, or could depend on the natural tendency of some species to aggregate and thus produce a contagious distribution, without the influence of environmental factors. For reasons such as these, the distribution and the number, spacing and timing of samples should be carefully determined in relation to the overall aim and duration of the study, seasonal and daily fluctuations in invertebrate activity, and the levels of taxonomic and ecological analysis necessary to answer research questions posed. Logistical and financial considerations will also play a major role in the sampling design and techniques used (Fig. 5.1).

Careful thought and documentation are essential during sampling plan preparation and will help to eliminate misunderstanding later in the sampling effort. Such planning should include: (1) identification of specific study objectives; (2) sample processing, transportation, and preservation techniques; and (3) level of taxonomic expertise needed. Another important component of sample planning is a review of existing information for the study area (e.g., consultation of local experts, governmental and non-governmental reports, bathymetric maps, hydrological surveys, geomorphological analyses, identification of potential contamination sources, existence of current or previous disturbance activities, previous research on the topic of interest and the particular wetlands of interest, climatic information from past decades). The inspection of the actual study area also is important before a sampling program is developed in order to assess the validity of historical data and identify any possible changes (Mudroch and MacKnight 1991). In addition, exploratory sampling is also often desired in order to provide preliminary

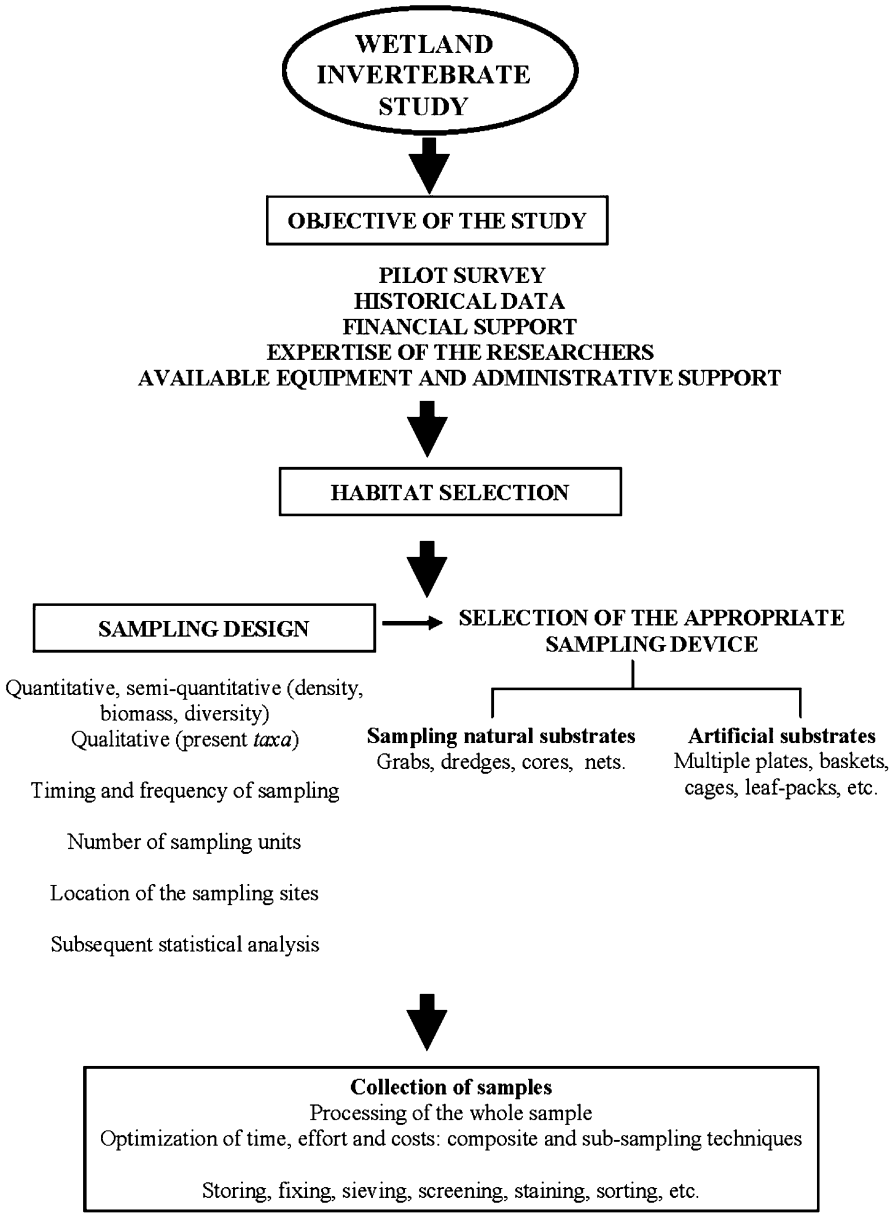

Fig. 5.1 Flow chart summarizing the process implemented in designing and performing a wetland invertebrate study

information about the sites or material being analyzed, and to determine accessibility of sampling sites and the time needed to perform the sampling. Another important issue for consideration is to determine the available resources for the project (e.g., personnel, time, and instruments).

Prior to collecting the data, it is important to determine the type of data needed in order to adjust the sampling design. Choice of sampling device should be thought of in terms of qualitative, semi-quantitative, and quantitative sampling. In a qualitative sampling design, the aim of the research is to determine which species, genera, or families are present, in order to obtain general information with which to estimate the relative abundance or percentage of taxa (e.g., number of Oligochaeta/total number of benthic macroinvertebrates) or functional feeding groups present (e.g., percentage of collectors/total invertebrates collected or, the percentage of burrowers/total number of invertebrates; Merritt and Cummins 1996), for both taxonomic and biogeographic studies. In this type of sampling, large samples should be collected to account for any rare species present in relatively low numbers. Qualitative sampling provides comparable values, when samples are collected within the same habitat and when the sampling device is the same for all samples. A qualitative sampling regime might be turned into a semi-quantitative sampling method if sampling effort (distance or time) is taken into account during the sampling procedure. Uniform sampling efforts should be considered when planning this type of sampling (taking into account the same distance or time for collecting each sample).

In quantitative sampling, the size of the sampling unit is known. Because of this, in studies involving a quantitative sampling design, it is possible to determine the density (number of individuals/area or volume), biomass (usually a dry weight/area or volume), diversity, richness or other metrics, within the same habitat or among different habitats allowing for comparative studies.

Quantitative sampling requires strict considerations on sampling site location, sampling frequency, and number of sample units (sample size) to be collected during each time interval (Merritt and Cummins 1996). The number and size of samples depend on the variability and distribution of organisms in the area and the level of accuracy and precision needed for the research. However, in most cases replicated quantitative sampling provides the strongest, most interpretable data (Kerans et al. 1992). For all of these reasons, an exploratory survey of a selected wetland, using a qualitative sampling approach, may precede an investigation that requires a more quantitative sampling design.

The timing and frequency of sampling should be related to the goal of the study. For example, if the goal of the study is to analyze the population development of some species, or their secondary production, then the sampling frequency must be adapted to take into account the duration of their life cycle and phenology (Benke 1984, 1993). In quantitative studies of macroinvertebrate communities, the sampling frequency may range from 3 to 4 days to a month or more (Anderson and Smith 2000). In geographically large studies (e.g., biogeographic distribution) or for calculating IBIs, seasonal (Selego et al. 2012) or yearly sampling (Mereta et al. 2012) might be enough. In large rivers, with large and complex floodplains (e.g., secondary channels, shallow lakes, aquatic-terrestrial transitional zones), such as the Paraná, Paraguay, Amazonas, and Orinoco Rivers, the sampling frequency may depend on the hydrologic regime and connectivity. However, in nearly all

cases a temporal sampling scheme is desired (Cummins 1962). Surveys may be intensive, with many replicate samples in each of a limited number of habitats or sites or the investigation may require extensive surveys with few sampling dates but including a large number of sites or environments.

Sampling is often conducted during diurnal time periods, but in some instances crepuscular or night sampling is more conducive to obtaining certain segments of the community or population (e.g., collection of insect pupal exuviae [sloughed skin] after the emergence, drift at different hours, different behavioral activities). Many taxa are seasonal in appearance and also may show well-marked patterns of activity in relation to weather or time of day (New 1998). Invertebrate activity is influenced by temperature, precipitation, wind, sunlight, and other meteorological factors. For these reasons, the best comparisons are drawn from samples collected under similar conditions. Understanding insect drift, which mainly occurs at night, may require a sampling design with a 24-h basis (Waters 1965; Brittain and Eikeland 1988).

In quantitative sampling, the sampling device usually collects a small area of the substrate or volume of water and consequently a major decision involves how many sampling units (sample size) are to be collected to ensure adequate sampling. As many benthic invertebrates show clumped or aggregated distribution, a large variation is encountered in sampling populations and small samples are statistically inaccurate (Elliott 1977). To avoid this problem a large number of sampling units should be collected, but this may be laborious, mainly in relation to sorting procedures. Sampling design must consider a balance between adequate sample sizes and also take into account the resources needed to process and handle the samples.

Selection of a sampling device for wetland invertebrates is contingent upon myriad factors as outlined above. Additionally, wetlands provide unique challenges for sampling due to complex hydrologic, physiognomic, and edaphic properties. Sampling devices and processes are often designed to sample one or more of the invertebrate communities inhabiting a particular niche or area of a wetland (Table 5.1). To decide whether the sampling device is adequate for a certain objective and substrate type, a pilot study should be carried out to correctly identify the devices that might be employed according to habitat characteristics. Sometimes multiple sampling devices or a device different than the one originally identified will be necessary to best meet the objectives of the study. Investigators should always conduct a preliminary study to evaluate the best methods for their particular situation (Anderson and Smith 1996).

In the sections below, we detail many of the methods that have been used in the past to sample invertebrates. It is important to realize that most methods can be used to sample multiple communities thus techniques may be described in more than one section. However, because wetland scientists often are sampling a particular component of the macroinvertebrate community or in some instances the entire community, it is important to describe the various uses for each device even at the expense of some repetition.

Table 5.1 Definitions of various invertebrate components inhabiting wetlands

Invertebrate component	Definition
Aerial	Free-flying invertebrates that live in, near, or around wetlands or that predominantly live in terrestrial environments and fly over wetlands
Benthic (Benthos)	Invertebrates that live in the bottom substrate or on submerged surfaces of a wetland
Endogeic	Invertebrates that live in non-flooded (dry or saturated) soil within wetlands on a seasonal or semi-permanent basis
Epibenthic	Invertebrates that live on the surface of bottom substrates
Epigeic	Ground-dwelling and subsurface invertebrates that live in plant litter or debris on the soil surface
Epiphytic	Invertebrates that inhabit above- or below-water portions of wetland and aquatic plants
Herbaceous vegetation	Invertebrates that live primarily within the stems of or on the leaves or stalks of terrestrial herbaceous vegetation, grasses, and shrubs
Nektonic	Free-swimming invertebrates that live in the water-column
Neustonic	Invertebrates that inhabit the surface water of a wetland
Soil surface	Terrestrial invertebrates that primarily live a terrestrial existence on the soil surface but often occur in dry wetlands or recently flooded wetlands
Trees	Invertebrates that live primarily within the canopy of trees
Zooplankton	Free-floating invertebrates that live in the water-column

5.3 Sampling Benthic Invertebrates

Benthic invertebrates or benthos are invertebrates that inhabit the upper layers of sediment and detritus in wetland and aquatic systems. The composition of the benthic fauna is usually diverse and the sediment or substratum they inhabit is highly variable. For this reason, many different sampling devices and techniques have been developed. During the selection of sampling devices and techniques, it is important to understand that not every species or taxonomic group will have the same probability of being caught by any given sampling device or technique. Such complexity is reflected in a general lack of methods that enable simultaneous assessment of all of the various taxonomic groups present and generally substantial effort is required to assess the entire community. The characteristics of an ideal sampling device for quantitative benthic sampling are shown in the following box.

The basic characteristics of an ideal quantitative benthic sampling device (Edmondson and Winberg 1971; Brinkhurst 1974):

1. The sampler must penetrate sufficiently deep into the sediment to trap all of the organisms inhabiting the sediment column beneath the surface area sampled.
2. The sampler should enclose the same surface area each time.

(continued)

(continued)

3. The depth sampled should be uniform across the area sampled.
4. The area of the sample must be quantifiable.
5. There must not be pressure waves to displace the finest surface sediments or organisms during the approach to the bottom. During descent the sampler should not disturb the sediment or the enclosed fauna so as to reduce the number of organisms present before the sampler is closed.
6. The closure of the sampler should be such as to preclude any loss of sediment or sample during retrieval. The closing mechanisms, if using jaws, should be strong enough to shear through twigs or other obstructions.

Sampling devices used in qualitative sampling studies need not necessarily conform to these requirements.

Many samplers are capable of collecting a sample in fine-grain sediments; however, fewer are suitable for sampling harder sediments containing major quantities of sand, gravel, firm clay or till (Mudroch and Azcue 1995). Selecting an effective sampling device may depend on considerations of the type of habitat present (e.g., current velocity, macrophyte cover, detritus in bottom sediments) as well as the overall sampling design and the objectives of the study. Some basic requirements should be met in order to capture a quantitative sample of populations living over and within the sediments. Samplers must penetrate into the sediments to a sufficient depth to allow collecting all the organisms inhabiting a defined area (Wetzel and Likens 1979). The sampling device should close completely to avoid the loss of sediments and organisms during the retrieval and should not disturb the sediments or allow the organisms to escape prior to collection. The efficacy of benthic sampling devices varies greatly. The advantages and disadvantages of some sampling devices are described in Table 5.2 and the adequacy of different sampling devices for different type of habitats is detailed in Fig. 5.2. Benthic invertebrates of streams can be collected from a unit area of the substratum or they can be caught as they drift downstream. Sampling devices for benthic invertebrates can be grouped in the following categories: grabs, cores, dredges, nets, and artificial substrates.

5.3.1 Grabs

Grabs consist of a pair of jaws in the shape of a quarter-cylinder. Closure of these devices around a sample may depend upon the weight of the device to close the jaws around a sample as in the Petersen and Ponar grabs (Fig. 5.3a), or closure may depend upon a modification consisting of the addition of two long arms as in the Van Veen grab (Fig. 5.3b). In large rivers and flooded bottomlands such as the Paraná, Paraguay, or Uruguay which typically have sandy sediments and high

Table 5.2 Advantages and disadvantages of sampling devices employed in benthic studies (Summarized from Edmondson and Winberg 1971; Brinkhurst 1974; Elliott and Drake 1981; Rosenberg and Resh 1982; Downing 1984; Klemm et al. 1990; Mudroch and MacKnight 1991; Resh and Jackson 1993; Merritt and Cummins 1996; Brandimarte et al. 2004)

Sampling device	Design	Advantages	Disadvantages
Grabs			
Ponar	Consisting of two jaws with lateral screens added to the jaws	The protection of lateral screens, allows a higher speed of descent and the avoidance of the speed up of water pressure	The standard model is heavy and difficult to handle, usually requiring a boat equipped with a windlass
	Closes automatically by an arrangement of the rope to raise and lower the device	Higher penetration than other devices	May have problems in substrates with stones, impeding the proper closing of jaws and losing material during the retrieval
		The lower sized ones can be manually handled without boats or windlass use	Smaller models are inappropriate for sampling on flowing waters
		It can be constructed in different sizes	
Petersen	Consisting of two jaws of about a quarter of cylinder in shape	It can be constructed in different sizes	Low penetration efficiency. Limited to sampling of epibenthic organisms but not useful for sampling of burrowing organisms
	Closes automatically by an arrangement of the rope to raise and lower the device		Causes a disturbance in soft sediments when descent is performed at higher speed
			May have problems in substrates with debris, stones, gravels, etc. that may impede the proper closing of jaws, losing material during the retrieval
			The standard model is heavy and difficult to handle, usually requiring a boat equipped with a windlass
VanVeen	Modification of the Petersen grab, with a closing mechanism consisting of two long arms	More efficient closing mechanism than the Petersen grab	Low penetration efficiency. Not useful for sampling of burrowing organisms
		Useful in the majority of substrate types and habitats	May have problems in substrates with stones impeding the proper closing of jaws and losing material during the retrieval
		It can be constructed in different sizes	The larger models are heavy and difficult to handle, usually requiring a boat equipped with a windlass

(continued)

Table 5.2 (continued)

Sampling device	Design	Advantages	Disadvantages
Tamura	Consisting of two jaws with upper window on the top	Very efficient in deep rivers with sandy sediments	It is not effective in muddy sediments
	Closes automatically by metal device (like a piston) that tightly close the jaws	The design reduce the pressure waves in the sediments when is lowered and the jaws close completely resulting in reduced losses during the retrieval	
		Low disturbance of water/substrate surface	
		High penetration efficiency	
		It can be constructed in different sizes	
Ekman-Birge	Consisting of a box with a pair of spring operated jaws that retain the sample within the box. Two doors on the upper top of the box	The upper doors of the box help to avoid the shock wave, with a relatively nondisruptive sampling	Often manufactured from much lighter material than the before mentioned grabs, which reduces their penetration in hard substrates and make it less useful for high speed flowing waters
	Commonly operated by a messenger, but also by a self-closing device (e.g. Rawson 1947)	Useful in shallow habitats (poles instead of cables or ropes for improved penetration in substrates)	It may be difficult to operate the closing mechanism when the jaws are blocked with debris, etc., and, when the grab is turned around in the bottom substrate
	Many modifications of the original design have been made	Useful for sampling vertical profiles (modified for the insertion of horizontal dividers after retrieval)	
		In very soft mud material may be lost through the top because of the grab sinking too far in the sediment	
		It can be constructed in different sizes	
Cores	Usually cylindrical in shape and often with a closing device at the upper end that prevents the loss of the sample	The multiple models may be useful to simultaneously obtain many sampling units and to avoid the problem of using simple cores when benthic invertebrates are scarce	The relative small sample obtained (mainly for those of small extraction area) make then inappropriate when benthic organisms are scarce
	They may be single or multiple corer devices	The collection of low volume allows the processing of a high number of sample units in a short time	In some cases, epibenthic organisms may be lost by the shock wave generated by the core

	Can be manually handled or may have a closing mechanism that functions by gravity or by messengers	Easily handled in shallow and vegetated habitats (manual models) Higher penetration in soft sediments. High performance in soft sediments Low disturbance of water/substrate surface Useful in vertical distribution sampling	Low penetration in deep habitats
Dredges	Consisting of cylinders of known surface, and a suction mechanism (air or water pumps or compressors)	The same advantages of cores and useful in different type of habitats Allow for more precision of benthic invertebrate population estimates	The same disadvantages of cores and also expensive devices needed (vacuum pumps, compressors, etc.)
Nets	Consist on devices of different size (surface of the net mouth, filtering area) and sieves of different mesh size	Cheap, of low weight and easily transported. Useful in flowing and still water	Refluxes of water by pore may generate lateral loss of invertebrates Restricted to shallow habitats (most of them)
Surber net	Devices with nets that delimit a known surface of substrate that is generally perturbed to remove the invertebrate that are captured in the nets	Known sampled area Easy to manufacture and transport	Difficulties to set in some types of substrates Limited efficacy in low flowing waters Only for habitats of <50 cm depth
Hess net	Devices with nets that delimit a known surface of substrate that is generally perturbed to remove the invertebrate that are captured in the nets	Known sampled area Easy to manufacture and transport Useful in vegetated habitats	Difficulties to set in some types of substrates Limited efficacy in low flowing waters Limitations around the depth of sampling
Artificial substrates	Consisting on substrates that imitate habitat characteristics of interest, providing a substrate for benthic invertebrate colonization	Allow to obtain information that could not be collected in any other way Reduce variability among sample units High control over variables Not destructive sampling of the environment Cheap and easy manufacturing of devices	May have selective properties over organisms Inadequate for short-term studies in most cases Loss of devices by vandalism, animals, extreme drying or flooding phases

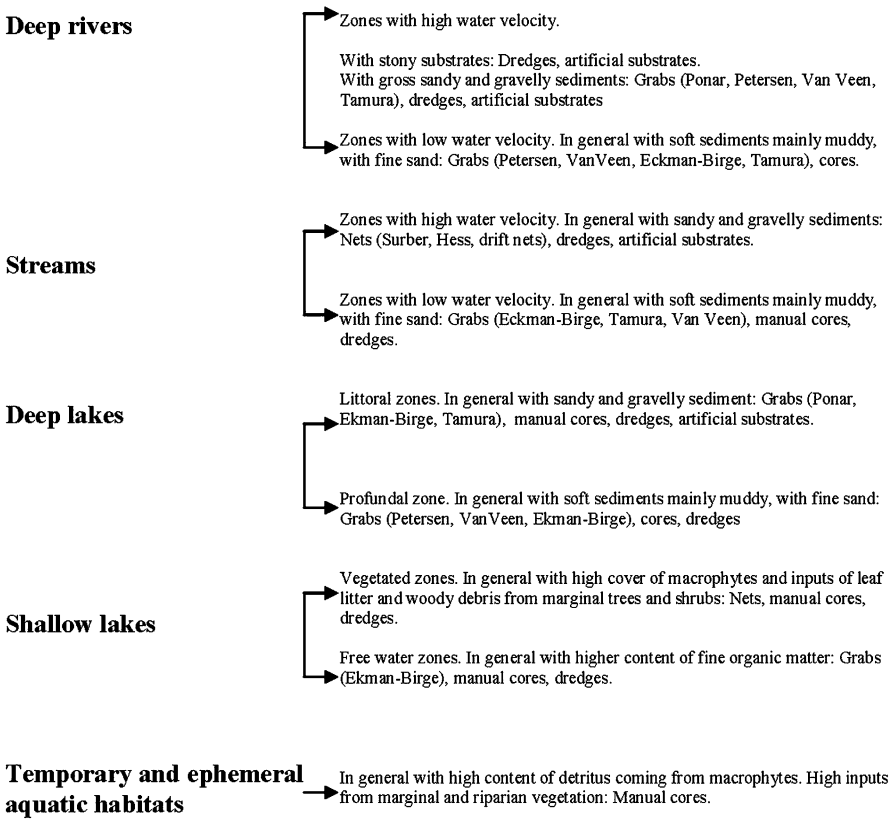

Fig. 5.2 Adequacy of different sampling devices for different types of habitats (Brinkhurst 1974; Downing 1984; Merritt and Cummins 1996; Brandimarte et al. 2004)

current velocity, the Tamura grab (Fig. 5.3c) is extremely effective. This sampler is designed to reduce the pressure waves in the sediments when it is lowered and heavy springs ensure that the jaws close completely, typically resulting in only minor losses during the retrieval. Another type of grab sampler consists of a box with a pair of spring operated jaws that retain the sample within the box and doors on the upper top of the box (Ekman-Birge, Fig. 5.3d). These devices are commonly operated by a messenger but also by a self-closing device (Rawson 1947) and are most often used in lakes and large rivers. Usually the weight of the grab sampler is adequate to allow penetration of soft sediments; however, in some cases alternative designs must be considered for harder sediments. For example, Ekman samplers mounted on a pole are more effective for collecting samples from hard sediments such as clay, sand and compacted materials and for this reason are more frequently used to sample in wetlands (Brinkhurst 1974). Many modifications to the original sampler designs now exist with which to sample a variety of taxa and habitats. It is necessary to handle grab samplers carefully because accidental closure of jaws can cause injuries.

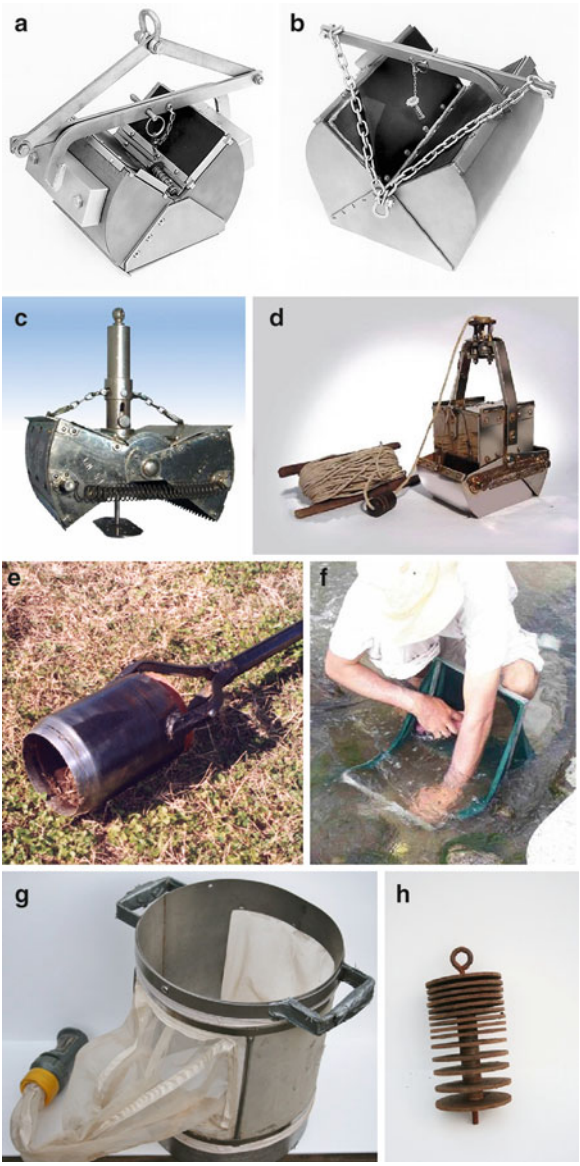

Fig. 5.3 Collection devices used for sampling benthic invertebrates. Grabs: (**a**) Ponar, (**b**) Van Veen, (**c**) Tamura, (**d**) Ekman-Birge; Cores: (**e**) Single core; Nets: (**f**) Surber; (**g**) Hess cylinder; and Artificial Substrate: (**h**) Hester-Dendy (**a**, **b**: Published with kind permission of © Rickly Hydrological Company, Inc., Columbus, Ohio, USA 2012. All Rights Reserved; **c**, **d**, **e**, **f**: Published with kind permission of © Laboratorio de Bentos, Instituto Nacional de Limnologia (CONICET-UNL), Argentina 2012. All Rights Reserved. **g**, **h**: Published with kind permission of © Ann Anderson, West Virginia University, Morgantown, West Virginia, USA 2014. All Rights Reserved)

5.3.2 Cores

A variety of core sampler designs have been created for sampling and collecting benthic invertebrates. Core samplers are usually cylindrical tubes that are pushed into the sediment by hand, usually sealed in some manner to form a vacuum, and all benthos is then entrapped within the corer. There are single or multiple core devices that extract substrate columns and can be manually handled (Fig. 5.3e). The core

sample is then sieved in the field or bagged and brought to the laboratory for processing. Corers are designed to be inserted into the substrate to a set depth (usually 8–12 cm) for complete sample removal or can be inserted to a deeper depth and then the bottom of the core sample is cut off and removed. A multiple core unit permits several samples to be taken simultaneously (Euliss et al. 1992). In shallow environments, plastic or metal tubes may be simply pushed into the sediment directly or with different adaptations. Such cores also have been used at greater depths with SCUBA (Wetzel and Likens 1979).

Core samplers built longer than the depth of sediment to be sampled are often advantageous because the bottom of the longer core will penetrate into more consolidated material forming a tighter seal and in this way prevent the sample from falling out of the corer. Core samplers are most commonly built from polyvinyl chloride (PVC) or steel pipe, although plexiglass designs have been described. Hyvönen and Nummi (2000) describe an 86 mm diameter steel tube, 45 cm in height, with handles with which to sample benthic invertebrates in 25–75 cm water depths. Plexiglass corers 65 cm in diameter have been used; however, these devices captured few invertebrates and displayed large coefficients of variation compared to other sampling techniques such as a stovepipe sampler (Turner and Trexler 1997). Stovepipe samplers are generally created from metal stovepipes which result in relatively large cylindrical enclosures. Stovepipe sampler sizes vary but samplers 34 cm in diameter and 60 cm tall have been described (Turner and Trexler 1997). Stovepipe samplers are able to sample both benthic and water-column invertebrates simultaneously (Merritt et al. 1984).

5.3.3 Dredges

Dredges consist of cylinders of known surface, and a suction mechanism (vacuum pumps, compressors) that are employed for quantitative sampling. The suction device collects benthic invertebrates, sediments, and water. The invertebrates can be separated from the sediments and water in the field, or instead all the collected material may be retained and the benthos may be separated later in the laboratory. Dredges are useful in some instances for scraping natural stony or rocky substrates.

5.3.4 Nets

Nets consist of mesh of various sizes (e.g., variation in the surface of the net mouth or filtering area) and sieves of different screen sizes. The collection of benthic organisms are obtained by disturbing the bottom (e.g., hand turning of stones, stones lifted and cleaned in the mouth of the net, kicking with feet, D nets) and allowing the local current to carry the removed material into the net device. The device can be slowly moved along the study area over a fixed distance (e.g., 10 m each) or over a fixed time (e.g., 3 min). Drift nets are useful in streams (Waters 1965).

Some nets are attached to a device that delimits a known surface of substrate that is generally perturbed to remove the invertebrates that then drift with the current and are collected in the nets (e.g., Surber [Fig. 5.3f], Hess cylinder [Fig. 5.3g]). When used in streams with a stony bottom, the surfaces of the stones are scraped with a brush and everything on their surface is removed and collected in the nets. The stones' surface should be measured, generally distinguishing the part inserted in the bottom and the part above it (colonization area) estimating simply by assuming the area of the stone as a geometrical figure (Dusoge 1966).

5.3.5 *Artificial Substrates*

Artificial substrates consist of structures that imitate habitat characteristics of interest, providing a substrate for benthic invertebrate colonization. The sampling substrates' dry weight (e.g., leaf-packs) or area (e.g., plates) is measured before placing it in the field. When stones are the substrate used, colonization area should be measured as the part above the bottom, assuming the area of stones as geometrical figures (Dusoge 1966). In some cases, as when the study site has been widely disturbed by anthropogenic activities, it may be desirable to provide a substrate composed by materials from outside the study site (e.g., cages or baskets with rock or other substrates, multiple plates, synthetic materials such as polyethylene fibers). One of the most widely used synthetic samplers is a multiplate sampler, comprised of a metal bolt and nut that holds a series of round wooded plates with spacers in between, to provide colonization access by invertebrates (Hester and Dendy 1962) (Fig. 5.3h). This sampler can be easily disassembled to remove invertebrates. According to the objectives, the period of time for colonization is important when using these types of sampling devices. Artificial substrates may be collected from the study site and returned to the laboratory for analysis.

5.3.6 *Comparative Methodologies for Benthic Sampling*

Environmental conditions and data requirements often determine the methods and devices selected for sampling. Grab samplers, dredges, and net samplers are useful devices for quantitative sampling as they sample a known surface of substrate which allows for the calculation of the number of the individuals per unit of area (usually as the number of individuals per square meter), allowing for the comparison of densities among different devices and investigations. When the substrate is covered by aquatic plants or mollusk shells, then core samplers can be more secure than jaw samplers. Core samplers and some grabs (e.g., Ekman-Birge) are useful for determining benthic invertebrate vertical distribution such as in the analysis of the importance of benthos for the circulation of matter in the habitat (Kajak 1971). In such a study, the core sample is fractioned with dividers in horizontal fractions in

order to understand distributions at different substrate depths. Nets are mainly useful when qualitative or semi-quantitative sampling is carried out and the results may be expressed as number of individuals collected for sampling effort unit (i.e., time or distance). All the sampling devices have some common disadvantages (Merritt and Cummins 1996). If devices collect from large areas, then there is often a tendency for a reduction in the total number of samples collected due to the increased time involved with sample processing. In contrast, if the area sampled is small then the variability among replicates increases. Sampling errors may occur if multiple investigators collect samples or if there are sample irregularities such as variations in substrate particle size. In addition, abundance of invertebrates in samples can be underestimated when highly mobile organisms escape during sample collection.

Some invertebrates can be scarce in benthic samples, but quite numerous in traps because of their high level of activity (Kajak 1971). Benthos that inhabits the substrate–water interface for all or part of the life cycles often can avoid collection using traditional sampling devices. In such cases, special methods of study are required, such as epibenthic trawls composed of glass funnels and jars. When sampling is required in deeper wetlands, large grab samplers such as the Ekman dredge, which can be used at greater depths, are often the most effective sampling device (Kajak 1971).

Sampling may be done by walking in most wetlands, but wetlands on the margins of lakes, streams, and rivers may necessitate the use of boats. In some of these circumstances, sampling by divers is probably the most reliable procedure. Using divers also often allows the use of cameras and underwater photography which can be particularly useful when large rocks are studied. For example, in the Salado River (Santa Fe Province, Argentina) dive sampling was successfully used to collect the invasive bivalve *Limnoperna fortunei* which had attached to bridges and blocks (Ezcurra de Drago 2013, personal communication). Electric shock is often used for sampling in flowing water environments in order to dislodge invertebrates from substrates after which they are collected from the drift (Fahy 1972).

5.4 Sampling Water-Column Invertebrates

Invertebrates inhabiting the open water areas of a wetland are generally referred to as nektonic (i.e., free-swimming within water column), neustonic (i.e., inhabiting surface water), or zooplankton (i.e., free-floating invertebrates). Some invertebrates may live within the wetlands' water-column for substantial periods of time, whereas others are transitory as benthic larvae transform into adults, moving through the water-column into the air or vegetation. A number of techniques have been used to sample wetland invertebrates in the water-column including activity traps, emergence traps, nets, and enclosure type samplers.

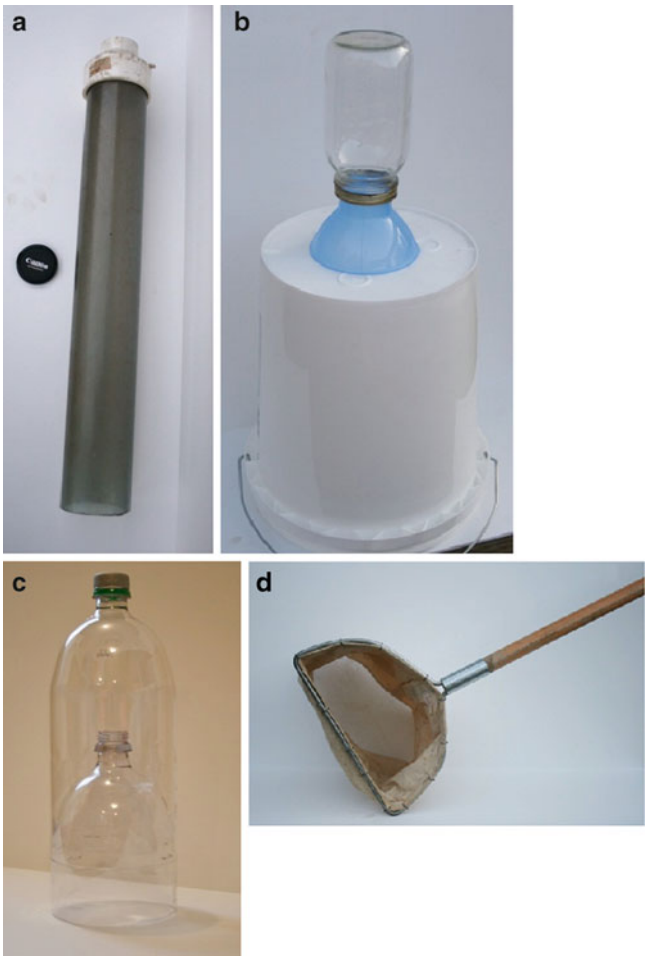

Fig. 5.4 Collection devices used for sampling water column invertebrates. (**a**) Open Cylinder, (**b**) Emergence Trap, (**c**) Activity Trap, and (**d**) Sweep Net (Published with kind permission of © Ann Anderson, West Virginia University, Morgantown, West Virginia, USA 2014. All Rights Reserved)

5.4.1 Open Cylinders

A variety of open cylinder traps have been used to sample invertebrates (Fig. 5.4a). Clear PVC pipe is commonly used now (Anderson and Smith 1996), but this concept was derived from the use of a graduated cylinder that had the bottom cut off (Swanson 1978). Wider cylinders reduce the chance of escape but smaller diameters are more effective in dense vegetation. Rubber stoppers or corks can be used to facilitate removing the enclosed sample of water with associated invertebrates. One design has a small hole in the top stopper that can be easily plugged with one finger

after the cylinder is placed in the water to prevent the loss of the sample as the bottom of the sampler is moved towards the surface. Before the bottom seal is broken by removing the sampler from the water, the bottom cork should be added to prevent loss of water. For smaller diameter samplers, the operators hand can easily serve as the bottom seal. These devices are easy to use and result in quantitative samples.

5.4.2 Emergence Traps

Emergence traps have been used to study populations of insects with aerial stages as part of their life history (Jónasson 1954; Sublette and Dendy 1959; Sandberg 1969). Emergence traps have been designed to capture invertebrates before they reach the surface of the water, at the surface of the water, or above the surface of the water (Jónasson 1954). The basic design consists of a glass, plastic, or metal capture vessel (usually a jar) attached to the top of an inverted funnel or bucket (Fig. 5.4b). Funnels are usually mesh, netting or screening with a wire frame to provide a consistent size and support. Wire framed and staked traps can be set directly on the bottom in shallow water wetlands or suspended above the bottom substrate by attaching to poles. Alternatively, plastic or wooden floats can be used to allow some surface movement of the traps. It is important that the capture vessel contain air so the invertebrates will continue to congregate in the trap. Emergence traps in the water have been paired with similarly designed emergence traps in riparian zones or along wetlands to capture those invertebrates that crawl out of the water on to land for emergence (Paetzold and Tockner 2005). Emergence traps are sometimes referred to as activity traps (Brinkman and Duffy 1996); however, we will refer to funnel-type traps with funnels facing downward as emergence traps and traps with funnels oriented horizontally as activity traps. Emergence traps can provide a quantitative sample if the bottom area covered is consistent.

5.4.3 Activity Traps

Activity traps or funnel traps have been used in a variety of settings including both lakes (Pehrsson 1984; Elmberg et al. 1992) and wetlands (Armstrong and Nudds 1985; Neckles et al. 1990; Hyvönen and Nummi 2000). Activity traps are generally comprised of glass, metal or plastic jars with funnels on one or both ends; traps are generally set to capture horizontal movements of invertebrate at specific depths (Fig. 5.4c). Jars often range from 1 to 4 L (Hyvönen and Nummi 2000). Funnel sizes also vary based on application and size of the jar. Advantages of activity traps include ease of use, ability to use in vegetated littoral zones (Hyvönen and Nummi 2000), and the ability to capture mobile predators such as dytiscids (Murkin et al. 1983; Mackey et al. 1984). A disadvantage of activity traps is their inability to obtain quantitative estimates of density (Murkin et al. 1983) unless placed

vertically in a water body, like an emergence trap (Pieczynski 1961; Brinkman and Duffy 1996). Minnow traps also can be used as activity traps but are generally placed on the bottom substrate. The mesh size of minnow traps limits the size of invertebrates retained (Turner and Trexler 1997); as a result, they are often used to catch large numbers of crayfish and similar-sized invertebrates. Traps placed on the bottom of the substrate are especially effective for capturing mobile benthic invertebrates.

Trap contents from both emergence and activity traps are collected by gently lifting the traps from the water, unhooking the funnels, which can be held on by bungee cords or other material, and draining the water through a sieve. Both devices generally provide a relatively clean sample. Density or relative abundance estimates can be calculated and temporal sampling regimes can be incorporated.

5.4.4 Sweep Nets

Sweep nets, most commonly D-frame sweep nets (Turner and Trexler 1997) and modified sweep nets (i.e., the handle is bent up 45° so the handle projects straight above the water when the net lies flat on the bottom surface; Murkin et al. 1983) are also effective at capturing invertebrates inhabiting the water column. D-frame sweep nets are among the most widely used devices for sampling a variety of invertebrates. They have a simplistic design and are simple to use. A conventional D-frame net has a mesh size of 1.2 mm and a mouth area of 690 cm^2 (Turner and Trexler 1997) (Fig. 5.4d). One potential concern with the use of a D-frame net is the lack of a consistent sampling area. To take this into account, most studies try to standardize the length of their "sweeps" to either 0.5 or 1.0 m in length (Turner and Trexler 1997). In this manner, by knowing the size of the net opening and the length of the sweep, scientists can estimate the volume of water sampled and calculate densities. The modified sweep net is moved from the bottom to the surface and by obtaining water depth the volume sample can be calculated. However, sweep nets are best used as qualitative or semi-quantitative sampling devices and are most effective at capturing a diversity of specimens rather than obtaining quantitative samples. For these reasons, sweep nets are probably best used for IBI or other indices where a diversity of specimens is more important than density estimates. Sampling with sweep nets in vegetation is particularly problematic and researchers should understand the limitations of the devices in these situations.

5.4.5 Other Techniques for Water-Column Sampling

The above techniques are those most commonly used in wetlands. However, a variety of other methods have been used sporadically in wetlands. Light traps or interception nets such as Shannon or Malaise have been used above the surface of

the water or wetland vegetation (Edmondson and Winberg 1971). Traps may be baited to increase attractiveness for specific taxa. The adult stage of different insects can be effectively collected with light traps, including imagoes and subimagoes of mayflies (Ephemeroptera) and stoneflies (Plecoptera) (Domínguez et al. 2006). Hand-picking or netting with common entomological nets and drift nets is an effective means of collecting specific invertebrates, particularly those that occur on the surface of water (neustonic) such as water striders (Gerridae), as well as floating pupae and pupal and larval casts.

5.4.6 Comparative Methodologies for Water-Column Sampling

Several studies have compared multiple techniques within water columns. Like most decisions regarding sampling technique, the final decision comes down to study objectives and logistics and deciding on which trade-offs you can accept, because no technique is perfect. Activity traps on the bottom and at mid-water level performed similarly (Hyvönen and Nummi 2000). In Delta Marsh Manitoba, presence of fish and predatory invertebrates in activity traps appeared to cause a slight decline in total invertebrate abundance based on comparative samples with sweep nets and presence or absence of fish and predatory invertebrates within activity traps (Murkin et al. 1983). Moreover, density estimation is problematic in activity traps but it can also be problematic in sweep samples. However, activity traps provide a cleaner sample resulting in less processing time, increase standardization of sampling, work well in both open water and vegetated wetlands and can be incorporated into temporal sampling designs (Murkin et al. 1983). Cylinders are effective at collecting invertebrates between vegetation and can provide quantitative estimates (Anderson and Smith 2000). The diameter of the opening influences capture probability; too small a diameter allows mobile invertebrates to escape and too large a diameter causes excess disturbance of vegetation and increases the chance of loss during removal of the sample from the water.

5.5 Sampling Epiphytic Invertebrates

Epiphytic invertebrates inhabit the above- and below-water portions of wetland and aquatic plants but are most commonly associated with the below-water component. Other names that have been used in the past include phytomacrofauna, phytophilous fauna, macroperiphytonic fauna, and phytomacrobenthos (Murkin et al. 1994). Epiphytic macroinvertebrates are one of the more difficult components to measure due to the complexity of the substrate. In addition, some epiphytic invertebrates

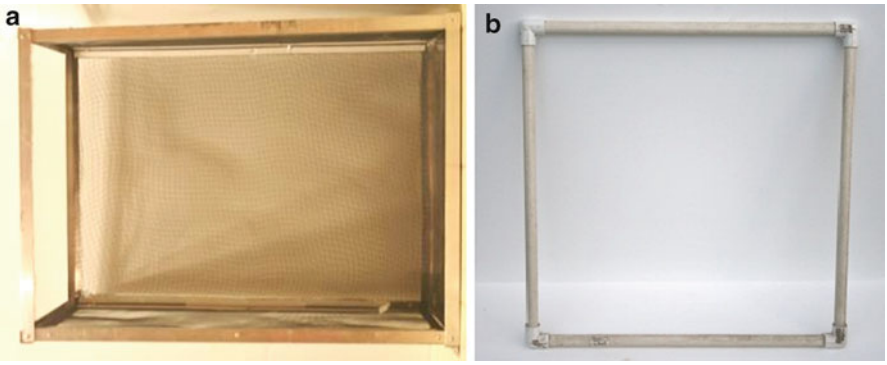

Fig. 5.5 Collection devices for sampling epiphytic invertebrates. (**a**) Box sampler, and (**b**) Quadrat sampler (Published with kind permission of © Ann Anderson, West Virginia University, Morgantown, West Virginia, USA 2014. All Rights Reserved)

inhabit the stems of plants and thus are not easily sampled using most standard techniques.

Many invertebrate studies have attempted to quantify invertebrate abundance and biomass on particular species of plants (Colon-Gaud and Kelso 2003). Other studies assess invertebrates on a per unit area of the bottom of a wetland rather than on surface area of plants (Anderson and Smith 2000). Determining the surface area of plants and or quantifying invertebrates in particular plants is generally a time-consuming process, so unless the study question is specifically concerning density or abundance on specific plants an approach that samples a unit area may be easier to achieve.

5.5.1 Box Samplers

Box samplers enclose a volume of water and the associated vegetation. One common form of a box sampler is the model A sampler (Gerking 1957) now commonly referred to as the Gerking sampler. A Gerking sampler, constructed from a 14 cm diameter acrylic tube with two sliding plexiglass doors attached to the bottom, was used to sample a single stem of softstem bulrush (*Scirpus validus*) (Brinkman and Duffy 1996). This sampling technique generally consists of cutting a plant with scissors and then closing the door of the sampler. This technique has also been tried with larger boxes (50 × 50 cm) and a single sliding door designed to sample multiple plant stems within the water column (Anderson and Smith 1996; Fig. 5.5a). The larger size reduces the overall escape of mobile invertebrates while placing the sampler; however, larger boxes are also more difficult to seal resulting in increased loss of specimens. Samplers also can have the sample plate sharpened to facilitate

closing the door in dense vegetation (Murkin et al. 1994). Using scissors to cut the stems before closing the door reduces the incidence of pulling plant roots, and associated soil, into the sampler. A modification of the box type sampler is the suitcase sampler (Colon-Gaud and Kelso 2003). This device was designed to sample submerged vegetation and water in deeper areas and can be lowered to a specific depth. These various versions of the box samplers are effective for isolating invertebrates associated with the vegetation along with a volume of water. Some researchers have suggested that techniques for using the box sampler is most effective on water-column invertebrates, and in some ways is just a modified open cylinder or column sampler. However, these techniques can also be used to sample epibenthic and potentially even some benthic invertebrates depending on the amount of substrate disturbance which takes place.

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5.5.2 Quadrat Sampling

Quadrats, along with a gentle clipping of the vegetation, have been used to sample invertebrates from submerged vegetation (Downing and Cyr 1985) and emergent vegetation (Anderson and Smith 1996; Fig. 5.5b). However, mobile invertebrates including amphipods, odonates, coleopterans, and hemipterans may avoid being sampled with this method (Downing and Cyr 1985). This bias can be at least partially overcome by the use of open cylinder samplers placed gently between stems of vegetation (Anderson and Smith 1996). Column sampler widths should be maximized to reduce escaping of mobile invertebrates while simultaneously being size appropriate for vegetation density to avoid double-sampling of vegetation with both the quadrat and the water-column sampler. As vegetation begins to die and decompose, the speed of collection increases, which likely reduces escapes.

5.5.3 Other Techniques

There are a number of other techniques that have been used to sample epiphytic invertebrates. Throw traps are square or circular cages (1 m^2) that are open on both the top and the bottom, with mesh enclosing the sides. Mesh size (2 mm is common) and height of the trap varies based on the needs of the study design. Invertebrates within the frame can be removed with a D-frame net, smaller hand-held nets, or bar seines (Turner and Trexler 1997). Bar seines are rectangular frames, generally the same width as the throw trap but only half the length, and covered with netting. Bar seines are used in a similar fashion to a fish seine. Activity traps (described in the water-column sampling section) may be used to capture some invertebrates associated with vegetation (Hyvönen and Nummi 2000).

A variety of artificial substrates have been used to sample invertebrates. One design was comprised of four 2.5 cm wide × 35 cm long ribbons of green electrical tape attached to a plastic pot filled with gravel (Flanagan and Rosenberg 1982; Brinkman and Duffy 1996). Small pieces of Styrofoam™ were attached to the ends of the ribbon to provide flotation. Artificial substrates were placed in the water and retrieved 7 days later using the Gerking sampler. The standardized Hester-Dendy samplers also can be deployed at specific depths above the substrate by using floats or stakes (Hester and Dendy 1962). Some research has suggested that due to low capture rates compared to other techniques that artificial substrates have limitations for use in wetlands (Brinkman and Duffy 1996). Visual inspection surveys, which may include dissecting decaying logs and other vegetation to remove embedded invertebrates, has also been proposed (Lenat 1988).

Sweep nets are used in vegetated wetlands but are more frequently considered more appropriate for open-water sampling. The density and type of vegetation, water depth, and other factors influence the sampling efficiency of sweep nets and because of this preclude the ability to compare samples taken from differing vegetative structures. D-frame nets are not recommended for quantitative sampling in vegetated wetlands (Downing 1984), but can be effective for capturing invertebrates quickly for use in education programs or in other situations where only qualitative information is needed.

5.5.4 Comparative Studies

The quadrat sampler in conjunction with a cylinder sampler has been shown to be more effective in thick vegetation than a box sampler (Anderson and Smith 1996). This is likely as a result of invertebrate escape and loss due to the cumbersome nature of sampling with this device in vegetated wetlands. Another concern with box and stovepipe samplers is the contamination with benthos and benthic invertebrates during sampling (Downing and Cyr 1985). However, if benthic sampling is specifically incorporated into the study design, then this technique

may prove useful. The Gerking device was found to collect more invertebrates than the artificial substrate design comprised of green electrical tape described above. As with techniques for sampling other components of the invertebrate community, the choice of technique comes down to study objective as each technique has its pros and cons. However, under most circumstances when quantitative estimates are desired a box sampler or a quadrat sampler would seem most appropriate for sampling epiphytic invertebrates.

5.6 Complementary Techniques

It is important to remember that each device is only effective for sampling a portion of the invertebrate community. For this reason, many studies use multiple techniques in combination to get a more comprehensive picture of the invertebrates present throughout a wetland. Various combinations such activity traps and benthic corers (Hyvönen and Nummi 2000), emergence traps and corers (Whiteside and Lindegaard 1980), and benthic cores, quadrats of clipped vegetation, and open cylinders (Anderson and Smith 2000) have been used in studies to provide a more complete picture of the invertebrate community than any one technique alone. Turner and Trexler (1997) evaluated eight samplers in vegetated wetlands of the Florida Everglades and recommended that the funnel trap, D-frame sweep net, and 1-m^2 throw trap be used over a stove pipe sampler, Hester-Dendy, minnow trap, benthic corer, and a plankton net. This recommendation was based on the maximum number of individuals per sample, maximum number of taxa sampled, precision, and labor involved. The D-frame sweep net, funnel trap, and stovepipe sampler collected the most individuals and also had the greatest evenness of taxa. However, as previously mentioned others have indicated that the D-frame sweep net should never be used for quantitative estimation within vegetated wetlands.

5.7 Sampling Terrestrial Invertebrates

Terrestrial invertebrates include round worms (Phylum Nematoda), earthworms (Annelida), slugs and snails (Phylum Mollusca), and arthropods such as insects (Class Insecta), hexapods (Class Entognatha), millipedes (Class Diplopoda), centipedes (Class Chilopoda), spiders and mites (Class Arachnida), and isopods (Class Malacostraca). These terrestrial invertebrates inhabit a variety of habitats including wetlands, and play key roles as herbivores, carnivores, omnivores, and detritivores. While some insects can fly, other groups of invertebrates, including many immature stages of insects cannot fly. Therefore, terrestrial invertebrates occurring in wetlands can be sampled from the air as well as on plants, inside plants, on the ground, or under the soil.

In general, living plants provide food or shelter for a great many species of terrestrial invertebrates. They may live on or within flowers, fruits or seeds, leaves,

stems, barks, branches, and roots (herbivores). Pollinator invertebrates (e.g., bees and butterflies) visit flowers and predatory invertebrates also use living plants to obtain their prey and to hide themselves (carnivores). When plants or trees die and decay, some terrestrial invertebrates recycle plant materials (detritivores). Felled trees, dead stumps, or leaf litters also serve as food sources for many terrestrial invertebrates.

The surface of soil or topsoil is one of the most common places to find terrestrial invertebrates. They can be found under stones, inside dead animals, or inside dung which provides shelter as well as food. Many insects, round worms, and earthworms live under the soil surface. Some of these invertebrates move between the soil surface and deeper in the soil, depending on soil temperature and humidity of the soil. For example, grubs (Insecta: Coleoptera: Scarabaiedae) move up to the soil surface during summer and move down in the soil during winter.

Adult insects generally have two pairs of wings so that they can fly. Insect flight activity is highly correlated with ambient temperature, precipitation, and wind speed. Sunny, warm, and calm days are usually the best times to see many flying insects. Flies, ants, and termites often swarm during a sunny, warm days, especially after a light rain. Flying insects are generally easy to spot, but sometimes hard to catch, especially nocturnal insects flying at night such as moths. For these reasons, many techniques have been developed for sampling flying invertebrates.

5.8 Sampling Techniques for Flying Invertebrates

Sampling flying insects poises unique problems. Sampling techniques are classified as active or passive methods. Active methods include the use of aerial nets. Passive sampling methods for flying invertebrates include flight intercept trap, malaise trap, sticky trap, pheromone trap, and pan trap.

5.8.1 Aerial Nets

Portable aerial nets are composed of a mesh bag attached to a 30.5, 38.1, or 45.7 cm wire frame that is connected to a metal or wooden handle (Fig. 5.6a); the length of the handle varies according to sampling application (commonly 91.4 cm). Net handles may also be modified into a telescoping style that permits the user to sample insects at otherwise unreachable heights, such as the Monarch Net (Bioquip Products, Rancho Dominguez, CA, USA).

When using an aerial net one must learn to swing the net at the targeted insect or area and then quickly twist the handle. If done correctly, this will cause the deepest part of the mesh net to swing around the wire frame, closing off the net opening to anything captured during the swinging motion. It is important when sampling

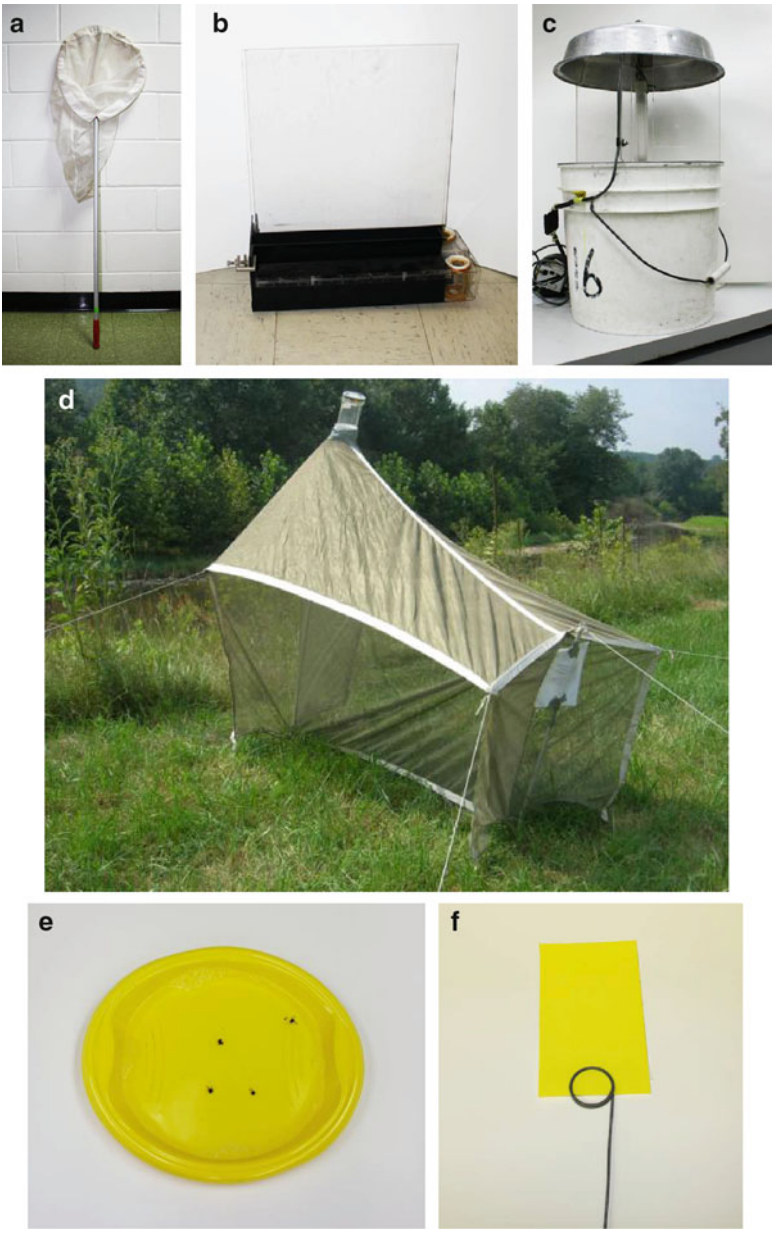

Fig. 5.6 Collection devices for sampling flying terrestrial invertebrates in wetlands. (**a**) Aerial net, (**b**) Flight intercept trap, (**c**) Light trap, (**d**) Malaise trap, (**e**) Pan trap, and (**f**) Sticky trap (Published with kind permission of © West Virginia University Entomology Lab, West Virginia University, Morgantown, West Virginia, USA 2014. All Rights Reserved)

multiple areas for a study to always use the same diameter net at each site. This insures that the same volume is collected in the bag during each collecting effort. Aerial nets with the proper modifications are very easy to transport. However, it is difficult to perfectly standardize collecting attempts with aerial nets. This is especially true when multiple individuals are collecting for the same project, as each individual's collecting effort will be slightly different (even when great care is taken). Aerial netting is useful for sampling all flying insects, especially Lepidoptera and Odonata.

5.8.2 Flight Intercept Traps

Flight intercept traps are constructed from a rectangular piece of fabric (usually a fine mesh) which is tied between two trees or poles in a natural flyway. A trough is dug below the fabric and the trough is lined with plastic or alternatively, collection containers are placed in the trough. A dilute propylene glycol solution is then poured into the trough or containers. When a flying invertebrate hit the fabric it will drop and land in the propylene glycol solution, which acts as a preservative until the insect in the trough can be collected. A variation of the flight intercept trap is the "window trap." Window traps utilize a clear piece of glass or plastic in place of a piece of mesh fabric (Fig. 5.6b).

By using the same sized flight intercept trap at different collecting sites, results for diversity or relative abundance of captured invertebrates can be safely compared. Also, the passive nature of the trap allows for long-term collecting at a site (such as bi-weekly samples). However, pre-made flight intercept traps may be expensive. Additionally, digging a trough in some areas may be difficult and time consuming. Flight intercept traps are useful for collecting most flying insects, but are especially useful for collecting Coleoptera, which often drop when they make contact with a barrier.

5.8.3 Light Traps

Light traps (Fig. 5.6c) take advantage of a behavior possessed by some insects known as "positive phototaxis" (an attraction toward light). Whenever you see an abundance of moths at a gas station at night, you are witnessing positive phototaxis in action, and the gas station is acting as a light trap. Light traps used for sampling insects are usually built with two intersecting sheets of metal placed over a bucket fitted with a funnel. A light (mercury vapor or black light) is placed at the point of intersection. Insects fly toward the light and bump into the metal plates, causing them to fall. When they fall they drop down the funnel and into the bucket, which contains an appropriate killing agent. Light traps are most effective on cloudy nights during a new moon. Lights may also be placed near white "light sheets"

(often just a bed sheet). Insects will fly and land on the white sheet, making them easy to hand collect.

A special technique called "light cycling" may be used to collect some taxa at light sheets. This technique is carried out by running the light for 30 min and then shutting it off for 10 min. Some insects will sit at the edge of the light and only move into the area after the light has been shut off. After the light is turned back on, the insects will often then fly to the sheet where they can be collected. Mantidflies (Neuroptera: Mantispidae) are readily captured by using this technique. If conditions are favorable, a large volume of specimens can be caught using light traps. Because mercury vapor bulbs get very hot, there are some safety concerns when using light traps. In addition, because the light needs power to run, it is necessary to purchase and constantly refill generators or use rechargeable batteries to run the traps. This makes the traps both expensive and time consuming. Finally, because all the insects fall into the same collecting chamber, Lepidoptera collected in the trap may cover other specimens in scales, which is undesirable. Lepidoptera, Coleoptera, Neuroptera, and Megaloptera may all be collected in significant number by using light traps.

5.8.4 Malaise Traps

Malaise traps (Fig. 5.6d) are similar to flight intercept traps, but are used to collect flying invertebrates which move up, rather than drop down, when they hit a barrier (negative geotaxis). To capture these insects the rectangular mesh is given a roof in the shape of a scalene triangle. Insects that come in contact with the mesh wall will crawl up and follow the roof into a collecting jar placed at the apex of the triangle. The collecting jar is similar to a minnow trap in that it uses an inverted cone to prevent the escape of collected specimens.

By digging a trough below the malaise trap (see Flight Intercept Trap), it may simultaneously act as a malaise trap and a flight intercept trap. However, Malaise traps are relatively expensive which may limit the number that can be put in the field. Malaise traps are especially good for collecting Diptera and Hymenoptera, though they can effectively be used to collect other flying insects.

5.8.5 Pan Traps

Pan traps (Fig. 5.6e) may be made from a number of containers such as: solo cups, gallon jugs, and pie pans (from where they get their name). These containers are partially filled (2/3 of the volume) with soapy water. Because soap breaks the surface tension of the water, flying insects which land in the pan traps have a high chance of drowning. Pan traps are often painted with fluorescent blue and yellow paints. The fluorescent paints cause the traps to mimic flowers, which will

then be very attractive for investigation by Diptera and Hymenoptera. For best results, pan traps should be placed 3–4 m away from each other in open areas to reduce competition between traps. When using pan traps in wetlands it may be necessary to create a raised platform to keep the traps out of the water during the wet season.

Collecting samples from a pan trap is most efficient when using a fine mesh aquarium net. The contents of the pan trap can be dumped into the net and then transferred straight into a preserving agent (usually ethanol). This method is much faster than plucking individual specimens from a pan trap. Pan traps are inexpensive to produce and easy to deploy in the field. During the dry season, pan traps may need to be visited frequently as they will be prone to dry out quickly. Also, after a season in the field some containers will become brittle and need to be replaced. Pan traps are very good at catching Diptera and Hymenoptera, but they are also effective for catching a number of other invertebrate orders.

5.8.6 Sticky Traps

Sticky traps (Fig. 5.6f) are any of a variety of traps that employ the use of sticky substances such as Tanglefoot ® (Tanglefoot Co., Grand Rapid, MI) or Stickem-Special® (Helburg 1979) to trap insects on their surface. The most common sticky trap is a 7.6 cm × 12.7 cm yellow card. By coating a cylinder with a sticky substance a sticky trap can be made to collect insects flying in all directions. Sticky traps are often deployed in fields and greenhouses for pest monitoring purposes. To deploy sticky traps in wetlands, you must clip the trap onto a stake or similar object on or near the ground or above the water.

Sticky traps are relatively inexpensive and easy to use. However, due to the nature of the trap, it is difficult to collect specimens without harming them, often making identification difficult. For this reason, we do not suggest using sticky traps for wetland sampling. Sticky traps tend to collect Diptera, Thysanoptera, and some small Hemiptera.

5.9 Sampling Techniques for Non-flying Invertebrates

5.9.1 Sweep Netting

Sweep nets (Fig. 5.7a) are similar in structure to aerial nets (see Aerial Nets above), the primary difference being that sweep nets use a thick canvas bag rather than the fine mesh bag of an aerial net. Sweep nets are used to collect insects in areas of highly dense brush where aerial nets may become torn. As with the use of aerial nets, learning the proper technique when using a sweep net, will help prevent the

Fig. 5.7 Collection devices for sampling non-flying terrestrial invertebrates in wetlands. (**a**) Sweep net, (**b**) Aspirator, (**c**) Vacuum sampler, (**d**) Berlese-Tullgren funnel, (**e**) Beating sheet, (**f**) Emergence, (**g**) Mist, and (**h**) Pitfall trap (Published with kind permission of © West Virginia University Entomology Lab, Morgantown, West Virginia, USA 2014. All Rights Reserved)

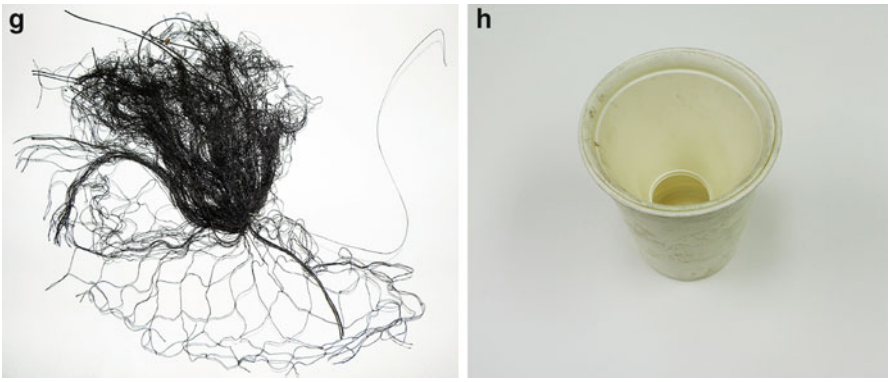

Fig. 5.7 (continued)

escape of insects collected. Because sweep nets use a thick canvas net bag, it is difficult to see what has been collected while specimens are inside the net, which can make transferring the net's contents to a kill jar difficult. Sweep nets are useful in collecting a wide variety of insect orders, with Hemiptera and Orthoptera being some of the more frequently encountered orders.

5.9.2 Aspirators

Aspirators (Fig. 5.7b), sometimes called "pooters," are used to collect a variety of tiny invertebrates. Aspirators consist of a plastic cylinder which has a long plastic tube connected to one end and a short plastic tube connected at the other end. The short plastic tube contains a mesh screen or, in more expensive models, an air filter. By aiming the long plastic tube at a small invertebrate and sucking through the short tube, the insect is captured in the plastic cylinder. There are a few slightly different configurations for aspirators; however, the basic function is similar. Aspirators are good for collecting diminutive invertebrates which are otherwise extremely fragile. In addition, aspirators are small and highly portable. Learning effective techniques for using aspirators can take some practice, but when mastered can be very useful for collection of tiny invertebrates.

5.9.3 Vacuum Sampling

A vacuum sampler such as the D-Vac vacuum insect collector (Rincon-Vitova Insectaries Inc., Ventura, C.A., USA) (Fig. 5.7c) uses the power of suction to collect insects (somewhat like an enormous aspirator). Vacuum samplers may be powered

by a generator or by a gas powered motor worn on the back. A length of hose is connected to the engine running the vacuum, and this hose can be used in a standard vacuum fashion, or (if it has a handle) can be swept like a net. It is also possible to create a vacuum sampler rather than purchase one. Because vacuum samplers pull in a set volume of air and the hose has a defined circumference, vacuum samplers are useful for standardizing collecting at different locations. However, the vacuum requires fuel to run and is not very portable. Additionally, vacuum samplers using the gas powered backpack mower may be heavy. Vacuum samplers can be used to collect most insect taxa, and may be useful for collecting non-insect invertebrates as well.

5.9.4 Berlese-Tullgren Funnels

Berlese-Tullgren funnels (Fig. 5.7d) are used to extract invertebrates from soil samples. The soil is placed onto a piece of hardware cloth set inside a funnel. A light bulb is turned on above the funnel. The light bulb slowly dries out the soil, and any invertebrates within the soil continually migrate into moist soil. Eventually (after several days) they fall through the hardware cloth and down the funnel. A vial with 70 % ethanol collects the specimens as they emerge from the funnel. Berlese-Tullgren funnels are often simply called Berlese funnels or Tullgren funnels. Berlese-Tullgren funnels are one of the most effective methods for removing small invertebrates from soil samples. Also, they can also be made entirely from materials found in hardware stores if pre-made Berlese-Tullgren funnels are too expensive for a particular project. Berlese-Tullgren funnels may take 6 or more days to fully dry out the soil sample. This means processing a significant number of samples either requires a great deal of time or several Berlese funnels. Additionally, there is the possibility that some invertebrates will be captured in the dry soil clumps, unable to migrate down to the collecting vial (Bioquip Products, Rancho Dominguez, CA). The Berlese-Tullgren funnels are adept at capturing Protura, Diplura, Collembola, Pseudoscorpionida, and any other soil dwelling invertebrate.

5.9.5 Beating Sheet

Beating sheets (Fig. 5.7e) are used to collect insects from vegetation where sweep netting is not effective, such as bushes. A piece of canvas has a small pocket sewn into each corner, and a wooden dowel is placed in opposite corners, so that two wooden dowels cross each other to form an ‘X’ pattern. The beating sheet is placed beneath the target plant and then the plant it struck several times with a heavy object (a big stick works well for this). Any insects on the plant should fall off onto the beating sheet, which can then be collected with forceps. Alternatively, a white bed sheet can be laid beneath the plant to be sampled, which will act in a similar manner

to a beating sheet. Using a bed sheet has the advantage of being cheaper and more portable, but it is not as easy to deploy in the field. Beating sheets are one of the best ways to collect insects found in dense bushes which are otherwise difficult to sample from. However, beating sheets are not good for collecting insects which fly or move very fast, as the technique relies on the insects staying on the sheet to be collected. Beating sheets are effective for collecting larval Lepidoptera and other slow moving insects.

5.9.6 Emergence Traps

Emergence traps (Fig. 5.7f) are a broad category of traps designed to collect invertebrates as they emerge from a substrate. The design of an emergence trap is dependent on the target invertebrates and the location the invertebrates have been found. A simple emergence trap design is a simple dome that is placed on the ground. Insects emerging from beneath the dome are then trapped inside and can be collected. More advanced designs add collection heads, killing agents, and substrate specific modifications to the emergence trap. Emergence traps allow monitoring of exact date of species emergence, which is useful in natural history studies and for pest management applications. Also, freshly emerged specimens should be in the best possible condition, which could be a consideration for taxonomists and collectors. To use emergence traps, some idea as to where the targeted insect will emerge from is important. Locating these areas may be difficult or impossible in some cases.

5.9.7 Mist Nets

Mist nets (Fig. 5.7g) are primarily used to collect burrowing crayfish (Decapoda). Mist nets are extremely simple devices, consisting of a string tied off to a bundle of fine mesh material. The string is then attached to a stick or secured in some other fashion near the entrance of a crayfish burrow, and the mesh is shoved into the burrow entrance. Crayfish have a tendency to clean out their burrow entrances, so when they encounter the blocked entrance they use their chelae (claws) to remove the mist net. Often when removing the mist net, their chelae become entangled in the net, and they retreat back into their burrows. It is then only a matter of tugging on the secured string to remove the crayfish from the burrow. Mist nets are exceptionally portable, inexpensive to make, and reasonably effective at catching crayfish. However, caught crayfish have a tendency to fight to resist being pulled from their burrows, and on occasion will drop off their entangled chelae (which will later regenerate) to get free of the mist net.

5.9.8 Pitfall Traps

Many invertebrates spend the vast amount of their foraging and nesting within detritus on the forest floor. Pitfall traps (Fig. 5.7h) are used to collect these invertebrates. The simplest pitfall trap is a cup placed in a hole in the ground, so that the lip of the cup is flush with the ground. It will collect any non-flying invertebrate that falls into it, most of which will not be able to climb back out of the cup. Adding dilute propylene glycol to the trap greatly increases the catch, as invertebrates caught in the mixture will drown and not have an opportunity to escape. Additionally, propylene glycol acts as a preservative, reducing the frequency with which the traps must be checked.

Pitfall traps may be modified in several ways. First, catch can be increased by placing a wall (such as a 6–12 cm tall board) between two pitfalls. When most invertebrates encounter a wall they will follow it until it terminates. With a pitfall trap on each end, the wall acts to extend the length of the trap and can catch invertebrates bi-directionally. Another useful modification of the pitfall trap is to place several small holes in the collecting cup, and then place the collecting cup inside another cup before putting the trap in the ground. This serves two purposes; first, by placing the pitfall trap in the secondary cup you can easily remove the trap from the ground without disturbing the soil. Second, if the pitfall trap gets filled with water, the water will drain into the secondary cup, potentially causing the collecting cup to float. This may prevent specimens in the trap from being washed out. Another useful modification to pitfall traps are rain covers. These can be made from chicken wire covered in tin foil, and are useful for keeping rainwater out of traps. Finally, there is a variation of the pitfall trap called a drift fence. Drift fences are long walls made from aluminum flashing or silt-fence which contain many pitfall traps along the length of the wall. Depending on the size of the pitfall traps used, a variety of taxa, including vertebrates, may be collected in the trap. Pitfall traps collect many nocturnal invertebrates that would otherwise be difficult to collect unless hand collecting at night with a headlamp.

There are several disadvantages to using pitfall traps when sampling for invertebrates. First, pitfall traps will frequently collect non-target taxa, including small vertebrates such as salamanders (Caudata). Second, during the wet season, it is difficult to use pitfall traps in wetlands, as the traps will be submerged in many types of wetlands. Third, anything that makes digging difficult makes using pitfall traps difficult (such as digging in rocky terrain). Finally, mammals sometimes take an interest in pitfall traps, especially if ethylene glycol is used as a preservative instead of propylene glycol. This is due to ethylene glycol's sweetness. A bittering agent (such as quinine) may be added to the chosen preservative to reduce mammalian interest, saving both your trap and the mammal. Pitfall traps are good for collecting some Coleoptera (such as Carabidae), Diplopoda, Chilopoda, some Araneae, and other invertebrates that spend their time on the forest floor.

5.10 Processing and Identification of Invertebrates

One of the most time consuming components of wetland invertebrate sampling is sorting and processing invertebrate samples. As one would expect, larger samples take more time to process than smaller samples but the number of invertebrates collected in smaller samples is also lower. The more extraneous material collected in a sampler such as vegetation and sediment also adds to the overall time for sample processing. Therefore, consideration of sampling devices should also take into account the amount of time necessary to sort and process invertebrates.

5.10.1 Sieving, Sub-sampling and Compositing

Once obtained, the sample must be properly processed in the field and stored. All the samples should be immediately labeled with the sampling site, date of sampling, site location (e.g., coordinates from a global positioning system), identification of the subsample or composite sample, and other pertinent data. The identification of the contents may be done in or on the container of the sample and it is also desirable to make further annotations on a notebook. Permanent marker works well on the outside of the container. Thick paper or card stock, often placed within a plastic envelope inside of the container makes a good long-term tag for labeling the contents.

To reduce sample volume in the field, the materials can be passed through a sieve, being careful to avoid the destruction or washing away of the smallest organisms by rough sieving (Fig. 5.8a). The sieves can be brass or stainless steel commercial models, but commercial or homemade sieves composed of nylon cloth will reduce fragmentation of individuals. Floating sieves with wooden frames reduce accidental loss when working in deep muddy water or over the side of a boat (Fig. 5.8b). Many wetland studies use a 500 μm mesh size (No. 35 U.S. sieve) to retain macroinvertebrates (Huener and Kadlec 1992). The 200–250 μm mesh size is recommended to avoid the loss of the small size specimens, but in first-order streams the commonly used sieve sizes are $>$300 μm. Benthic core samples and epiphytic samples are best initially sieved in the laboratory. Although it is possible to identify and count all the invertebrates in a sampling unit, sorting can be time consuming, mainly in relation to the large quantities of material (e.g., sand, gravel, detritus, clay, silt) that occur in bottom sediment or vegetated samples. This is a serious problem, when large areas are sampled, when large sample sizes are obtained, or when a rapid assessment is required (such as in biomonitoring and bioassessment). The use of different techniques and instruments may positively improve this task.

Techniques that subsample or composite samples are useful in solving many of the problems described and also to obtain the information necessary for short-term biomonitoring. These techniques are often used to reduce the cost and labor involved

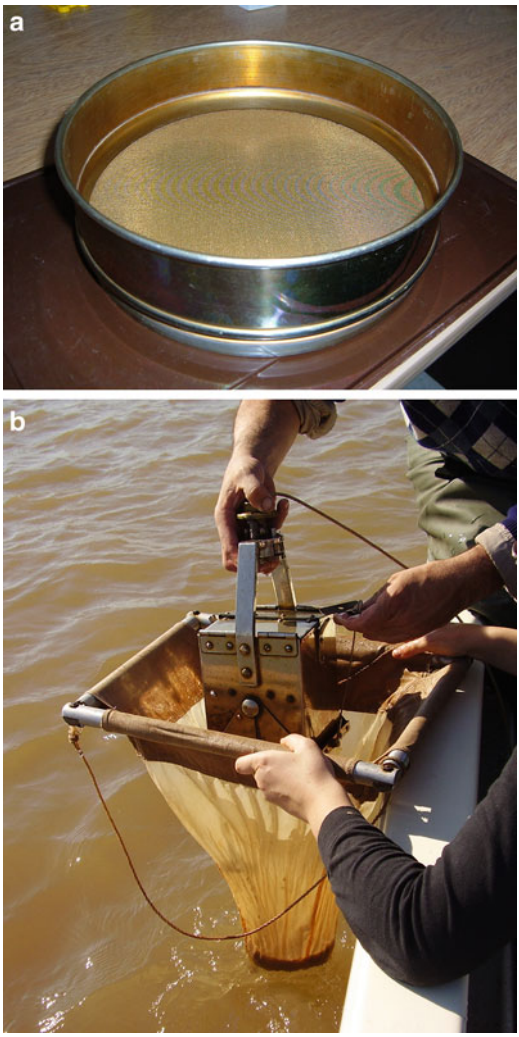

Fig. 5.8 Sieves used for sorting and processing wetland invertebrate samples. (**a**) Standard sieve and (**b**) Floating sieve with wooden frame (Published with kind permission of © Laboratorio de Bentos, Instituto Nacional de Limnologia (CONICET-UNL), Argentina 2014. All Rights Reserved)

with analyzing a large number of samples. Composite sampling may also reduce intersample variance due to the heterogeneity of the sampled material and also increase the amount of material available for analysis. In general, unrepresentative and heterogeneous material (e.g., shells, leaves, stones, debris) are removed and documented in a field log (notebook), prior to homogenizing (mixing). The removal may be done by hand or with screen or sieves of appropriate mesh size. Sometimes larger organisms such as mussels or crayfish must be removed. Then, the mixing of sediments is done prior to subsampling or compositing.

Subsampling is useful when large numbers of individuals of the same taxa are collected (Merritt and Cummins 1996) in large samples or when a great number of sample units need to be sorted. Two basic types of sub-sampling are commonly used:

1. *Analysis of a fixed fraction of sample*: for each sample, a fixed volume is obtained (subsample). The whole subsample or a selected quadrant (selected manually or with a grid) is then spread in a tray or Petri dish that is settled down on a stereoscopic microscope.
2. *Analysis of a fixed number of organisms*: A fixed number of organisms (generally 100–500) are randomly picked from the original sample (Brandimarte et al. 2004). This is an appropriate technique for some rapid assessment techniques, but results may be inadequate for presence or absence analyses and for the applications of indexes based on richness (Somers et al. 1998).

King and Richardson (2002) indicated that fixed counts (minimum of 200 individuals) should be considered as minimum subsample sizes for wetlands and that the fixed number was superior to the fixed area approach.

Compositing refers to combining two or more samples and analyzing the resulting pooled sample (Keith 1991). The composite sample from multiple sites can be used when the objective is to determine if the sites are different in terms of richness or abundance and the knowledge of the variance of each site is not critical for the study (Merritt and Cummins 1996). This technique is a practical, cost-effective way to obtain average characteristics for a particular site; often a large number of samples are collected, composited and then subsampled. The subsampling and compositing might be accomplished in the field or in the laboratory and sometimes a combination of both techniques is used.

5.10.2 Sample Sorting and Preservation

To further improve the separation of organisms from the substrate, the samples may be sieved (with the appropriate mesh size) as described above either in the field or the laboratory, and then stained with Erythrosin, Congo red dye, Rhodamine B dye, rose Bengal, pholxine B, or other dyes prior to sorting, to increase the visual contrast with the sediments (Mason and Yevich 1967; Brinkman and Duffy 1996). Separation of the material by screens or sieves of different mesh size, reduces the time of sample processing. Larger material (e.g., greater than 1,000 μm) may be directly separated, while the smaller-sized materials would benefit from sorting with a stereoscopic microscope or low power scanning lens (2×–4×). Some samples, particularly those collected from the water column, have little debris and invertebrates are easily found, collected, and preserved in the field.

Generally, for sample preservation, 70–95 % ethanol is commonly used, but propanol also has been used (Brinkman and Duffy 1996; Merritt and Cummins 1996). Ideally the soft bodied organisms (e.g., oligochaetes, leeches) should be fixed immediately after collection in 5–10 % buffered formalin to fix the tissue. Specimens may then be preserved and stored in ethanol. However, formalin has also been used to store complete samples prior to processing. Others have suggested that certain taxa (e.g., Hirudinea, Hydracarina and Trematoda) should not be fixed

when taxonomic identification at species level is necessary and they should be separated alive and stored in special solutions (Thorp and Covich 2001).

When the substrate contains mainly sand or gravel, flotation and elutriation may be useful techniques using sugar or salt solutions (Kajak et al. 1968; Brandimarte et al. 2004). In the absence of invertebrates with high specific weight (such as mollusks), the use of a saturated sodium chloride or magnesium sulfate solution, results in a low loss of organisms (Turner and Trexler 1997; Brandimarte and Anaya 1998). An elutriator, which is a mechanical sorting device that quickly helps separate various weights of material by moving water and air through a cylinder containing the sample, allows removal of invertebrates from the sample material (Lauff et al. 1961; Worswick and Barbour 1974; Whitman et al. 1983). The efficiency of any of these techniques should be estimated by analyzing 10 % of the samples held in reserve.

Invertebrates may be sorted alive and picked in the field by using a tray, a good lighting device, and a magnifying glass. Laboratory behavioral sorting methods have also been described (Fairchild et al. 1987; Brinkman and Duffy 1996). With this technique core samples are placed on a mesh hardware cloth basket that is suspended into an 18.9 L bucket. The bucket is filled with tap water and placed in a trough containing 30 cm of cool water. The top of the buckets are covered with black plastic and a 100 W light bulb is suspended 10 cm above the bucket to provide heat and create a thermal gradient. The invertebrates leave the core and move to the bottom of the bucket where they are easily removed via sieving. Recovery efficiency of this technique is estimated at 42 % (Brinkman and Duffy 1996) so additional processing of the core may be necessary if quantitative densities are desired.

Sometimes, such as when isotopic analyses or nutritive content are the aim of the study, the samples must be stored frozen without adding any fixing substance. In genetic studies, the cleaned organisms must be conditioned in ethanol. When gut content is the focus of the study, then special techniques (e.g., heat exposure, freezing) are used, as formalin or alcohol may produce regurgitation and loss. Placing invertebrates in hot water or warming water up to a lethal temperature will kill larvae but leave them in a more relaxed state for preserving, in this way facilitating later identification (Winterbourn and Gregson 1981). Introducing soda water or carbon dioxide into the collecting jar to anesthetize the invertebrates also should result in a more relaxed state. However, when this technique is used, invertebrates will still need to be killed with alcohol or formalin (Winterbourn and Gregson 1981).

5.10.3 Handling Terrestrial Invertebrate Samples

After sampling terrestrial invertebrates, they may need to be killed and transported. Because some invertebrates are very brittle, some equipment and supplies are needed to handle samples properly.

Fig. 5.9 Kill jars are useful for killing terrestrial invertebrates collected in wetlands (Published with kind permission of © West Virginia University Entomology Lab, Morgantown, West Virginia, USA 2014. All Rights Reserved)

5.10.4 How to Kill Sampled Terrestrial Invertebrates

5.10.4.1 Kill Jar

A kill jar is generally made by making a mixture of plaster of Paris and water that is poured into a clean jar with air-tight lid (Fig. 5.9). Then a killing agent such as ethyl acetate is added to saturate the plaster of Paris. Adding a few strips of soft paper or similar material to the jar can help to keep the bottle dry and prevent captured specimens from mutilating each other.

There are many killing agents available. Ethyl acetate and carbon tetrachloride are inexpensive and generally harmless to human unless inhaled in excess or swallowed. Chloroform and cigarette lighter fluid will kill most insects quickly; these are cheap, non-toxic, and readily available. Cyanide also can be used instead of ethyl acetate, but cyanide is more hazardous although it last much longer than ethyl acetate. Live invertebrates can be killed by placing them in the kill jar. The invertebrates need to be removed from the kill jar as soon as they are killed and placed in a container to carry. Kill jars need to be recharged with a killing agent when they do not kill invertebrates effectively any more. Never place small delicate insects in the same bottle that contains large insects.

5.10.4.2 Vials with Alcohol

Vials filled with ethanol are the most commonly used method in the field to kill soft-bodied and small terrestrial and aquatic invertebrates (Fig. 5.10). In general, 80 % ethanol or isopropanol are used for terrestrial invertebrates with a firm exoskeleton such as beetles, true bugs and many bees. For killing caterpillars or soft-bodied invertebrates, Peterson's K.A.A. mixture and Huffacker's X.A. mixture are commonly used solution.

Fig. 5.10 Vials of ethanol are commonly used to kill aquatic and terrestrial wetland invertebrates (Published with kind permission of © West Virginia University Entomology Lab, Morgantown, West Virginia, USA 2014. All Rights Reserved)

A K.A.A. solution consists of commercial kerosene (1 part), 95 % ethanol (10 parts), and acetic acid (2 parts). This mixture produces quick kills, rapid penetration and some inflation of tissues. Most larvae killed in a K.A.A. mixture lose their pale colors, especially the greens. X.A. mixture is made of a mixture of xylene (1 part) and 95 % ethanol (1 part) and is frequently used for killing caterpillars. However, this solution does not work well for many aquatic larvae and soft bodied flies and bees. Specimens killed in this solution should be transferred to and preserved in 95 % alcohol as soon as they are fully distended. Do not place hard-bodied invertebrates into the solutions because specimens can be inflated and turned to abnormal shapes.

5.10.5 How to Handle Dead Terrestrial Invertebrate Samples

In the field, it is handy to use temporary containers for transporting captured and killed invertebrate specimens (Fig. 5.11). Moths, butterflies and dragonflies with their wings folded together above their backs can be placed in individual envelopes made from rectangular sheets of paper or cellophane. After an insect is in the envelope, the edges are folded to secure the envelope.

Paper boxes and small tins make good transporting and storage containers. To effectively use these containers, place layers of cellucotton or facial tissue on the bottom, then place a layer of invertebrates on top of it. Additional layers of cellucotton or facial tissue should then be placed on the top of a layer of invertebrates.

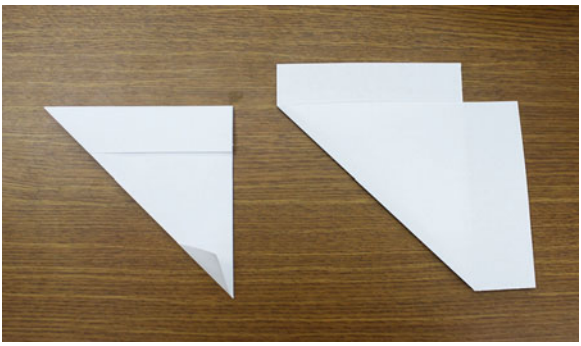

Fig. 5.11 Temporary containers for transporting captured and killed invertebrate specimens such as dragonflies and moths (Published with kind permission of © West Virginia University Entomology Lab, Morgantown, West Virginia, USA 2014. All Rights Reserved)

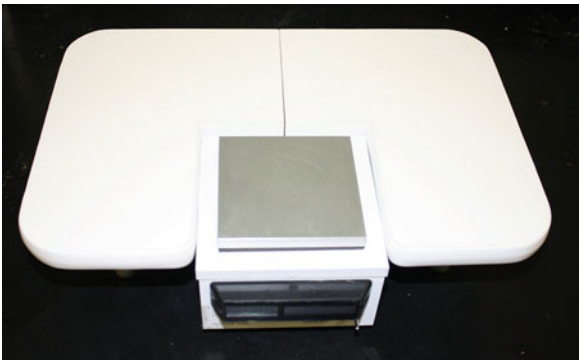

Fig. 5.12 Chilling table used to immobilize terrestrial invertebrates (Published with kind permission of © West Virginia University Entomology Lab, Morgantown, West Virginia, USA 2014. All Rights Reserved)

5.10.6 How to Handle Live Terrestrial Invertebrate Samples

Sometimes, sampled terrestrial invertebrates need to be transported live. Different life stages of the same species will often vary in their ease of handling. To effectively handle tiny or soft-bodied terrestrial invertebrates such as insect eggs and mites, fine paintbrushes or camel hairs are used. Various types of forceps are commercially available including entomological forceps for handling large terrestrial invertebrates and soft forceps for handling soft-bodied invertebrates such as caterpillars, earthworms, and snails.

In some circumstances, it is necessary to immobilize living insects for closer examination. Carbon dioxide can be used to immobilize terrestrial invertebrates especially insects for easier handling. However, carbon dioxide can kill invertebrates when they are exposed for an extensive period. Some insects can also be immobilized by placing them for a few minutes in a refrigerator at 4 °C (Fig. 5.12). An electric-powered chilling table has also been used to immobilize insects (Berry et al. 1978). However, note that carbon dioxide and cold immobilization can cause significantly higher mortality.

5.10.7 Identification of Invertebrates

The immature stages of insects are common in wetlands. However, for the identification of many insects to species, the adult stage is required. In some studies of diversity and in taxonomic analyses, a high level of taxonomic resolution is necessary and genus or species level taxonomy is required (Resh and McElravy 1993; King and Richardson 2002); family or even lower level of identification is often appropriate depending on project goals and knowledge of taxonomy within a region. The need for adult specimens can be solved either by rearing the larvae to the adult stages or by collecting associated adult and immature stages (i.e., larvae, pupae, cast larval or pupal exuviae, pharate adult, adults). For example, adult odonates can be readily captured using light-weight entomological nets. Rearing or culture techniques in the field or laboratory differ for various taxa but some standard techniques have been developed for insects (Corkum and Hanes 1989; Hargeby 1986) and other invertebrate taxa (El-Emam and Madsen 1982). The rearing techniques may consist of maintaining larvae and pupae until emergence occurs or obtaining eggs from adult females and rearing to newly hatched larvae (Gerberg 1970). Moreover, pupae rising to the water surface and adults that have just emerged indicate the kinds, number and biomass of insects leaving a unit area or a volume of habitat per unit time. The sex ratio, phenological pattern of emergence, voltinism, effect of predation on insects, and secondary production estimations can all be determined using invertebrates maintained in captivity.

Automated computer identification, which relies on machine-learning algorithms, has been used on stoneflies (Lytle et al. 2010), spiders (Do et al. 1999), and other groups (Watson et al. 2004; MacLeod 2007). Most of these techniques are still in the developmental stages and have limited taxonomic capabilities. However, correct classification rates can exceed 90–95 % (Lytle et al. 2010) which likely meets or exceeds novice classification rates. Likewise, DNA techniques are being increasingly used to help identify and classify invertebrates (Geraci et al. 2010). Many of these techniques are most useful for appropriate taxonomic classification and identification should be based primarily on morphology to keep costs reasonable.

Taxonomic keys and identification guides are essential for investigations of wetland invertebrates. The following citations are just a few of the many available guides that are critical for proper identification of aquatic invertebrates inhabiting wetlands: Fernández and Domínguez (2001); Lopretto and Tell (1995); Merritt et al. (2008); Smith (2001) and Thorp and Covich (2010).

Fernández HR, Domínguez E (editors) (2001) Guía para la determinación de los Artrópodos Bentónicos Sudamericanos. Editorial Universitaria de Tucumán, Serie: Investigaciones de la UNT, Tucumán, Argentina. 282 pp.

Lopretto EC, Tell G (editors) (1995) Ecosistemas de aguas continentales. Metodologías para su estudio. First Edition. Ediciones Sur, La Plata, Argentina. 1400 pp.

Merritt RW, Cummins KW, Berg MB (editors) (2008) An introduction to the aquatic insects of North America. Fourth edition. Kendall/Hunt Publishing Company, Dubuque, Iowa. 1158 pp.

Smith DG (2001) Pennak's freshwater invertebrates of the United States: Porifera to Crustacea. Fourth Edition. John Wiley and Sons, New York, New York. 664 pp.

Thorp JH, Covich, AP (editors) (2010) Ecology and classification of North American freshwater invertebrates. Third edition. Academic Press, San Diego, California. 1021 pp.

Although there are many resources for identifying terrestrial invertebrates, we suggest a series of books published as Pictured Key Nature Series by Wm. C. Brown Company Publishers (Dubuque, Iowa). This series includes very useful taxonomic keys and identification guides for major arthropods: Bland (1978); Chu and Cutkomp (1992); Kaston (1978) and McDaniel (1979).

Bland RG (1978) How to know the insects. Third edition. Wm. C. Brown Company Publishers, Dubuque, Iowa. 409 pp. 1157

Chu HF, Cutkomp LK (1992) How to know the immature insects. Second edition. Wm. C. Brown Company Publishers, Dubuque, Iowa. 346 pp.

Kaston BJ (1978) How to know the spiders. Wm. C. Brown Company Publishers, Dubuque, Iowa. 272 pp.

McDaniel B (1979) How to know the mites and ticks. Wm. C. Brown Company Publishers, Dubuque, Iowa. 335 pp.

In addition, a couple of good resources with photographs or pictorial keys are available: Marshall (2006); Iowa State University (2012).

Marshall SA (2006) Insects: their natural history and diversity with a photographic guide to insects of eastern North America. Firefly Books, Buffalo, New York. 736 pp.

Iowa State University (2012) Bugguide: identification, images, and information for insects, spiders and their kin. http://bugguide.net

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Student Exercises

The following are some brief ideas for introducing students to wetland invertebrate sampling, identification, and ecology. If you are a wetland invertebrate specialist, you probably already have your own favorite classroom, laboratory, and field exercises. However, if invertebrates are not your main forte, the following ideas may provide you with a starting point for incorporating invertebrates into your wetlands course. These can be used as presented or modified to suit your individual needs.

Classroom Exercises

Classroom Exercise 1: Understanding Taxonomy

The following exercise known as the "Nuts and Bolts" lab was adapted from Dr. Robert Whitmore's Vertebrate Natural History course at West Virginia University. The exercise is designed to acquaint your students with development and use of a dichotomous key. This assignment is most appropriate for introductory courses or for interdisciplinary courses where students may not have a solid background in biology. Assign students to work in a group and provide each group with a jar containing various small articles. You can populate each jar with the same set of items or make unique sets for each group. Examples of items might include rubber bands, dice, pebbles, small pieces of cloth, etc. Assume that each article is a wetland invertebrate. Students should examine these animals very carefully, and compare each piece with every other piece. As they examine the differences and similarities among the "organisms," they should decide on their degree of relationship.

Part 1: Instruct students to place these "species" in some type of "classification" where they would show a degree of relationship based on their shape or "morphology" (the function of each article may also be used as a classification character). Classifications should reflect "true relationships". Have the students address the following questions.

1. List the characteristics used to group and split the taxa, and explain why you placed these species together in a given taxon at a given rank.
2. What makes some characters more important than others in your classification?
3. What characters do you consider trivial/awkward if any? Why?
4. Are functional or nonfunctional characters useful in determining relationships?
5. To what extent did arbitrary decisions and criteria influence your results?

Part 2: After the students have decided on a classification scheme, they should construct a dichotomous identification key. This key should be a ready identification of your "species". The dichotomous key, meaning forking regularly into two nearly equal branches or segments, is based on an orderly elimination of the

characters that do not fit the case in hand. This particular key considers only two possibilities at one time.

The key should be arranged to give the user the choice of two alternatives. In arriving at a choice, one should carefully read all of the characteristics that apply to both choices and then decide which of the alternatives best fits the animal. When they decide which choice to follow, they should proceed with it to the next alternative which is indicated by the number at the right of the page. Below is a very simple example.

A Simple Key for Identification of a Few Biological Objects

1A	Vascular plant	2
1B	Vertebrate animal	3
2A	Leaf margins without obvious indentations; total length greatly exceeds maximum width	Fescue (Grass)
2B	Leaf margins indented; total length equal or less than maximum width	Red Maple
3A	Feathers present	4
3B	Feathers absent	7
4A	Beak length greater than 4 times the width	5
4B	Beak length equal to or less than 4 times beak width	6
5A	Toes connected to each other by skin (webbed)	Gull
5B	Toes not webbed	Heron
6A	Dominant color red	Cardinal
6B	Dominant color blue	Blue Jay
7A	Body mostly covered with hair	Squirrel
7B	Body not covered with hair	Turtle

Classes that already have the fundamentals down regarding use of dichotomous keys (but are not adept at identifying invertebrates) can skip the jars containing artificial contents and go straight to vials containing real specimens. Specimens can come from previous collections or research or from the field laboratory exercises below.

Classroom Exercise 2: Selection of Sampling Devices and Creation of Study Design

As we have learned in this chapter, there are numerous methods and techniques for collecting invertebrates from wetlands. Each method has its own biases and the advantages and disadvantages of each technique will vary greatly under the particular circumstances. For each of the following scenarios, identify an appropriate sampling device and a study design. For the study design, think about number of

samples, sample location, timing, and frequency of sample collection to meet objectives. Justify your choice of technique and design. Are there any potential biases or issues with your technique and design?

(A) You work for a state wildlife management agency within their wetland mitigation program. Your boss wants you to evaluate function of created wetlands in comparison to natural wetlands. In particular, your objective is to obtain an unbiased estimate of food availability (invertebrates) based on biomass and density for red-spotted newts (*Notopthalamus viridescens*) inhabiting these natural and created wetlands. Red-spotted newts generally select prey items within the water-column, on vegetation, and in the substrate. Each of the wetlands you choose to study (5 of each) has an average water depth of 50–60 cm and varies from about 0–125 cm deep. Wetlands are primarily covered in broad-leaved cattail (*Typha latifolia*) with about 10 % open water.

(B) You are in charge of developing the first dragonfly and damselfly atlas for you state or province. The atlas should provide the distribution and relative abundance of each species on a county-by-county basis. In essence, for each county you are recording and documenting a species' presence and their relative abundance (rare, uncommon, common, abundant) based on criteria that you will set.

(C) Little whirlpool ram's-horn snail (*Anisus vorticulus*) is a rare gastropod species that occurs in wetlands throughout a number of European countries including England, Germany, and Poland among others. In England, they have been identified as important umbrella species for their relation to high quality wetlands and their association with other invertebrates (Osmerod et al. 2009). Even though they can be associated with high quality wetlands, they are known to occur in highly altered systems including drainage ditches designed to drain wetlands to facilitate grazing by livestock. However, much of their basic life history remains unstudied. Develop a sampling methodology to better understand their population dynamics within these altered wetland systems.

Osmerod SJ, Durance I, Terrier A, Swanson AM (2009) Priority wetland invertebrates as conservation surrogates. Conserv Biol 24:573–582

(D) You work as a biologist on a national wildlife refuge located along a major migratory pathway for spring migrating shorebirds. The refuge has water control capabilities on eight impoundments totaling about 1,200 ha of wetlands. These wetlands are managed as moist-soil management units. Thus, they are primarily vegetated with annual plants and by the time you start your spring drawdowns there is less than 5 % vegetative cover. You want to experiment with drawdown timing and water management to maximize shorebird use and invertebrate foods (benthic invertebrates). You have already designed a strategy to sample shorebird use every other day for an 8-week period. How will you sample their food sources?

Classroom Exercise #3: Understanding Species Diversity Through Sampling

Species diversity is a common measure of species composition and numbers in a community. Higher species diversity exists when many equally or nearly equally abundant species are present. A higher diversity also indicates a more complex community with more species interactions. This classroom exercise is designed for student to calculate and understand species richness and diversity with data sampled in different ways.

In this exercise, Margalef's species richness index (D_a) and Simpson's species diversity index (D_s) will be used. Students are advised to use hypothetical data sets provided below.

	Number of individuals collected with different sampling methods (Hypothetical data set)			
Species	Method 1	Method 2	Method 3	Method 4
n1	100	100	10	10
n2	100	90	10	10
n3	100	80	10	10
n4	100	70	10	10
n5	100	60	10	10
n6	100	50	10	10
n7	100	40	10	10
n8	100	30	10	10
n9	100	20	10	10
n10	100	10	100	10

Species richness: Margalef's species richness index is calculated as,

$$D_a = \frac{(s-1)}{\log N}$$

where s is number of species and N is number of individuals.

Species diversity: Simpson's species diversity index is calculated as,

$$D_s = 1 - \frac{\sum n_i(n_i - 1)}{N(N-1)}$$

where n_i is number of species i and N is number of individuals.

Based on calculations of indices, examine the effect of sampling methods on species richness and diversity indices. Also, discuss about advantage and disadvantage of using Margalef's species richness index (D_a) and Simpson's species diversity index (D_s) to compare structures of different communities or compare different sampling methods.

Further study: students are encouraged to examine the data sets further with other indices. Diversity indices are well presented in many ecology books, and a list of references below may be helpful for students to understand the indices.

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Peet RK (1974) The measurement of species diversity. Annu Rev Ecol Syst 5:285–307

Maurer BA, McGill BJ (2011) Measurement of species diversity. In: Magurran A, McGill B (eds) Biological diversity: frontiers in measurement and assessment. Oxford University Press, Oxford

Laboratory Exercises

Field Laboratory Exercise #1: Using a Diversity of Sampling Devices

The purpose of this exercise is to allow students the opportunity to use and understand how to operate a variety of different wetland invertebrate sampling devices. Obtain examples of all the aquatic and terrestrial samplers that we described in the chapter. Go to a wetland that has varied topography and structure so a variety of sampling sites are available such as open water, emergent vegetation, trees, shrubs, and mudflats are available. Have each student deploy and operate each sampling device within the appropriate circumstances. Each student should collect multiple samples to ensure that they have the procedure down. Samples can be deposited back in the wetland if not needed or a few samples can be kept and preserved in the field or brought back to the laboratory for processing and use in other lab exercises. Setting up stations with one or two devices and having groups of students rotate among stations often works best with larger class sizes. After using a device students should write a few notes on their perceived advantages and disadvantages of using each device. Students should type a report comparing and contrasting each device's effectiveness.

Field Laboratory Exercise #2: Epiphytic Invertebrate Sampling

Epiphytic invertebrates present particularly unique challenges in sampling invertebrates due to the complexity that the plant shoots, water, and substrate provide. Assign students to work in pairs or small groups and select two or three different sampling methods for epiphytic invertebrates such as a box sampler, stovepipe sampler, quadrat, throw trap, or D-frame net for them to use. Have them collect an equal number of samples using each assigned method from a homogenous stand of vegetation within a wetland that has standing water. Remove the invertebrates from the sampling device and preserve in ethanol. Use one vial for each sample. Be sure to label all containers with all relevant information. Take the samples back to the laboratory or do an analysis in the field depending on logistics. Depending on the

identification proficiency available within the class you can conduct: (1) total counts of invertebrates captured, (2) separate into morphological types or major categories (worms, snails, bugs), or (3) identify to family or genus. After counting and recording data, place the samples back in the vials, and add new ethanol. These vials of specimens can be reused later in more formal identification labs if desired.

Field Laboratory Exercise #3: Terrestrial Invertebrate Sampling in Wetlands

This exercise is performed over a several day period, and is especially appropriate for long field trips. The purpose of the exercise is to highlight how different collecting techniques are appropriate for collecting different target taxa. As mentioned in this chapter, there are a multitude of methods for collecting terrestrial invertebrates within dry wetlands, and each method is particularly useful for collecting certain terrestrial invertebrate groups. Divide students into small groups and assign each group a different collecting technique. We suggest pit fall traps, beat sheets, malaise traps, pan traps, and berlese funnels (soil sampling). Each group should then deploy their traps in the field. It is best if all methods are deployed in the same type of habitat, though this is not absolutely necessary. Other sampling methods, such as light trapping, may be very good for highlighting the lessons of this exercise, but be sure to consider the logistics of using such methods when teaching a class.

After a predetermined amount of time (3 days–1 week will be adequate), have the students collect the specimens from the traps and bring them into the classroom. Now the groups can count the number of specimens collected in their traps (abundance) and identify them to order (diversity). Gather the data from each group and share it with the rest of the class. The students should notice which traps collect the most terrestrial invertebrates and which traps collect a large number of specimens from a certain order (such as Diptera in pan traps). As a follow up to this exercise, you may have the students write a short report on why they think certain collecting techniques are more effective for collecting certain taxa.

Chapter 6
Wetland Fish Monitoring and Assessment

Michael D. Kaller, William E. Kelso, and Joel C. Trexler

Abstract Fish sampling is an important component of wetland research, management, conservation, monitoring, and assessment programs, and studies of fish abundance, productivity, and community structure can provide important information about wetland condition and health. In this chapter, we discuss considerations specific to wetland sampling, including issues such as the phenology of wetland use by transient fishes and sampling constraints in hydrologically-dynamic habitats. We review both active techniques, which involve moving the gear to the fish, and passive techniques, which involve the fish moving to the gear, and differentiate gears based on their ability to provide qualitative or quantitative data. Because wetlands vary considerably in their hydrology, physicochemistry, habitat structure and biotic community composition, we review and recommend a wide variety of collection techniques, including seines, minnow traps, gill and entrapment nets, electrofishing, throw and drop traps, weirs, and trawls. Problems and solutions related to gear calibration and gear bias also are addressed, and we provide examples and exercises that demonstrate common approaches to sampling wetland fishes.

6.1 Wetland Fish Sampling

Although defined by periodically or permanently saturated (hydric) soils that support characteristic water-tolerant (hydrophytic) vegetation, the term wetlands can be applied to a tremendous diversity of systems from depressional habitats that

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are dry for periods of time ranging from days to years, to frequently or permanently inundated swamps and marshes. Variable water levels, shallow depths, and periodic drying result in physical and chemical conditions that are unique to wetlands and differentiate these transitional habitats from more permanent lentic and lotic systems. Wetlands can support diverse resident and transient fish assemblages that are economically and ecologically important. In these systems, research and management activities often focus on aspects of recreational and commercial production, as well as the effects of natural and anthropogenic factors on water quality, habitat structure, fish assemblage composition, and trophic web dynamics. Wetlands that are periodically dry must be connected to permanently flooded habitats to sustain fish populations. Episodic movements of fishes into and out of these wetlands can make fish sampling a difficult task. The dynamic nature of wetland hydrology poses unique challenges for resident fish species, as well as for researchers and managers wishing to assess wetland health and productivity and evaluate wetland restoration efforts based on fish community structure.

In this chapter, we present guidelines for selection of passive and active gears that have been used in wetland fish research. Passive gears require the fish to enter them and become trapped, whereas active gears capture fish either by moving faster than fish do or by enclosing fish quickly. This chapter complements published summaries of techniques for sampling fishes in open water (Lester et al. 2009; Pope et al. 2009; Hayes et al. 2012; Hubert et al. 2012; Reynolds and Kolz 2012) and specialized wetland situations (e.g., McIvor and Odum 1986; Rozas and Minello 1997; Connolly 1999; Steele et al. 2006), and includes lesser-studied systems such as prairie potholes and freshwater coastal wetlands. We begin the chapter by briefly discussing development of a wetland fish sampling project, followed by a discussion of wetland characteristics and their potential effects on sampling design. We then move on to descriptions of fish sampling gears and their relative effectiveness under different sampling conditions.

6.1.1 Wetland Fish Abundance and Productivity

Hydrology varies substantially among wetland types, and the effects of hydrological drivers on fish population and community dynamics and function is a common focus for wetlands research. Such research requires assessing characteristics such as density, taxonomic diversity, biomass, and productivity (including individual growth rates, food habits, measures of condition, recruitment into harvestable or catchable size, etc.) over a range of taxonomic levels, from single-species (autecological) descriptions (e.g., Kneib 1984) to characterizations of assemblages or guilds (i.e., species groups; Elliot et al. 2007) or communities (i.e., all fishes present in the system; Able et al. 2001). Further, wetland fishes are often sampled with other aquatic biota, such as crustaceans and insects (e.g., Peterson and Turner 1994; Hanson and Riggs 1995). Because of gear selectivity, no single gear or sampling technique adequately samples all fishes throughout their life history across all

habitats (Sect. 6.3.1.4). Thus, determination of the specific research objectives(s) in terms of fish abundance, biomass, productivity, and taxonomic scope is very important, as these decisions will guide gear and technique selection (Sect. 6.3).

6.1.2 Wetland Fish Communities

Wetlands are integral features of freshwater and coastal marine systems around the world that support highly diverse and productive fish communities (e.g., Elliot et al. 2007; Abell et al. 2008). Most wetland ecosystems support resident fishes that not only play key roles in the aquatic-terrestrial trophic web, but also provide recreational and commercial fisheries harvests worth billions of dollars annually (Woodward and Wui 2001). In addition, the seasonal availability of suitable wetland areas as spawning, nursery, and foraging habitat is a critical life history requirement for numerous transient fish species (Jude and Pappas 1992; Saint-Paul et al. 2000; Nagelkerken and van der Velde 2002; Manson et al. 2005). The importance of wetlands as fish habitat has been tied not only to high rates of productivity that result in abundant food resources, but also to the structural complexity that reduces predatory mortality of early life stages (Beck et al. 2001; Rehage and Loftus 2007). However, the dynamic hydrology, landscape complexity, and dense vegetation structure that make wetlands such productive fish habitats sometimes limit the options available to effectively sample wetland-dwelling larval, juvenile, and adult fish. We will cover several aspects of wetland fish species and community assessment, as well as wetland characteristics that must be considered in the design of a sampling project. We will then discuss the advantages and disadvantages of gears that can be used to collect qualitative and quantitative fish samples in these shallow-water systems.

6.1.2.1 Wetlands Fish Community Assessment

A tremendous amount of research has been directed at understanding the environmental relationships, ecological roles, and economic importance of wetland-dwelling fishes. Within the last decade, numerous studies have addressed a diversity of topics dealing with wetland fish communities, including species coexistence and dispersal (DeAngelis et al. 2005), responses to human perturbation (Trebitz et al. 2009a), relationships with physicochemical characteristics such as salinity (Lorenz and Serafy 2006), flooding (da Silva et al. 2010), and hypoxia (McNeil and Closs 2007), seasonal use of wetlands as dry-season refugia (Rehage and Loftus 2007), effects of wetland connectivity on assemblage composition, nursery value, and fisheries harvests (Meynecke et al. 2008a; Sheaves and Johnston 2008; Bouvier et al. 2009; Roegner et al. 2010), and the value of fishes as prey for wetland predators (Lorenz and Serafy 2006; Collazo et al. 2010). Results of these studies demonstrate the unique character of wetland fish communities and the nature and

variability of environmental factors that result in significant spatial and temporal changes in fish species distributions and community composition. Moreover, they highlight the integral role that wetlands play in the life histories of resident and transient fish species, the similarly important roles that fishes play in wetland trophic webs, and ultimately the importance of wetlands and the organisms they support to humans.

There have also been widespread efforts to develop and apply assessment indices of habitat quality based on fish community structure in estuarine (e.g., Breine et al. 2010) and freshwater wetlands (e.g., Galatowitsch et al. 1999; Teels et al. 2004), particularly in the Great Lakes (Uzarski et al. 2005; Seilheimer et al. 2009). Results indicate varying success in terms of detecting anthropogenic effects on habitat quality with fish community metrics (Meng et al. 2002; Bhagat et al. 2007; Bilkovic and Roggero 2008; Franco et al. 2009), although some studies have reported successful discrimination of low and high quality habitat (Seilheimer and Chow-Fraser 2006, 2007), with consistency among sequential samples (Harrison and Whitfield 2006). As with other fish-based research efforts, correct assessment of habitat quality depends on accurate description of the fish community, which can be strongly influenced by the choice of sampling gears (Connolly 1999; Rozas and Minello 1997; Ruetz et al. 2007). Ineffective gears, inadequate or non-standard effort, or poor design can strongly bias fish community-based assessments (Nijboer and Schmidt-Kloiber 2004; Bakus et al. 2007; Hewitt and Thrush 2007; Hirst and Jackson 2007; Bonar et al. 2009). We will discuss gear size and species selectivity, standardization approaches, and some design considerations in Sects. 6.3.1.3 and 6.3.1.4.

6.2 Wetland Sampling Considerations

All aquatic habitats have unique characteristics that affect sampling design and accuracy, but wetland fish community assessments are complicated by seasonal and ontogenetic changes in wetland use by transient and resident fish species (e.g., Ley et al. 1999; Franco et al. 2006), as well as the complex and sometimes ephemeral nature of wetland water levels, physicochemistry, and habitat structure. In the following section we briefly identify some biological and physicochemical factors that can affect sampling design and efficiency in shallow water systems.

6.2.1 Phenology of Wetland Use

The use of wetlands as spawning and nursery habitat by transient fishes has been widely reported in both freshwater and marine systems (e.g., Jude and Pappas 1992; Able et al. 2001; Lewis and Gilmore 2007). This seasonal presence and absence complicates characterization of some wetland fish species and fish communities, often requiring the use of multiple gears in different locations at

different times of the year to accurately describe the abundance and species composition of resident and transient assemblages. Furthermore, wetland drying can lead to dramatic increases in fish density for short periods, followed by substantial and persistent drops in density if regional wetland habitats and associated deepwater refuges dry (Lowe-McConnell 1987; Loftus and Eklund 1994; Mosepele et al. 2009). An effective sampling program would necessarily require a thorough understanding of transient fish ecology, including spawning times, movement patterns, ingress/egress routes, and wetland habitat preferences, which could then be integrated into a seasonal/spatial sampling design for fish during their wetland residency, and/or during their wetland migration period (e.g., Kwak 1988; Rozas and Minello 1997).

6.2.2 Shallow Depths

Wetland hydrology is highly dependent on position in the landscape, geology, precipitation, and other factors (Acreman et al. 2007). However, many wetlands experience substantial changes in water elevation, flow, and physicochemistry associated with variable groundwater inputs and periodic rainfall events, such as large river and coastal marsh systems subject to river flood pulses and seasonally high rainfall (e.g., Sabo et al. 1999; Rutherford et al. 2001; Thomaz et al. 2007; Dussaillant et al. 2009). During low water periods, or in permanently shallow wetlands, the use of larger boats and towed gears is typically not feasible. Depending on bottom substrate composition, these systems may be wadeable for sampling or may be sampled from small boats, canoes, or kayaks. Extremely soft-bottomed wetlands should probably not be waded because of safety concerns for sampling crews, and sampling in these systems is probably most effective from a small watercraft. Passive sampling devices such as gill nets (Sect. 6.3.2.4), and minnow traps (Sect. 6.3.2.2) may be most effective because of they can be deployed with relatively little intrusion into unstable sites. Enclosure traps (drop, throw, and pull-up Sects. 6.3.2.7 and 6.3.2.9) may be preferred if substrates support ready access because they can be set to sample the entire water column and provide density information. Push-nets mounted on small boats and light traps may be effective for collecting larval fish (Sect. 6.3.2.7), and backpack electrofishing (Sect. 6.3.2.3), and other manually-operated gears, such as seines (Sect. 6.3.2.1) may also be effective in areas where maneuverability is an overriding sampling requirement. Although the use of sonar technology to discern fish density and has increased substantially in recent years (Mueller et al. 2006), discrimination typically declines in shallow water (<1.0 m), limiting the usefulness of these gears in many situations. Ultimately, the choice of sampling gear (s) in shallow wetlands will be determined by accessibility, maneuverability, safety, and research needs.

6.2.3 Physical Habitat Structure

In addition to shallow depths, most wetland systems are characterized by significant habitat complexity from submerged, emergent, and floating aquatic plants and woody debris (e.g., Lougheed et al. 2001; Rolon and Maltchik 2006). Wetland macrophyte communities can exhibit complex changes in species composition by season, depth, and flooding regime (Prado et al. 1994; Van Geest et al. 2005), all of which will likely affect fish abundance and distribution (Brazner and Beals 1997) and gear effectiveness, necessitating consideration of habitat structure as the sampling design is developed (Chick et al. 1992; Rozas and Minello 1997; Connolly 1999). For example, electrofishing efficiency in wetland habitats is often reduced because fish are lost or unseen in dense vegetation (e.g., Klein Breteler et al. 1990). In estuarine marshes, fish abundance and distribution is often tied to vegetative structure, as well as physical discontinuities such as marsh edges and oyster reefs (La Peyre and Birdsong 2008; Schneider and Winemiller 2008; Stunz et al. 2010). The structural complexity characteristic of most wetlands relates directly to the nursery function associated with many wetland types, but from a sampling standpoint, it can strongly influence choice of fish sampling gear. In freshwater systems, electrofishing may still be an option, although minnow traps and even short seine hauls may also be feasible. Other gears such as throw/drop traps allow sampling near underwater discontinuities (Rozas and Minello 1997), and application of the toxicant rotenone (Sect. 6.3.5.2) within a confined area (Lorenz et al. 1997) may also be effective, although only appropriate when complete confinement is feasible and regulations permit use.

6.2.4 Water Chemistry

Many wetlands undergo seasonal changes in physicochemistry that can influence not only fish behavior and movement, but also sampling gear efficiency. For example, specific conductance can be extremely low in freshwater wetlands (e.g., <30 µS/cm in Canadian lakes; Jeffries et al. 2010), which can significantly reduce the effective field of capture of electrofishing gear (e.g., Monzyk et al. 1997; Rabeni et al. 2009). High specific conductance can also affect electrofishing catch rates and operational characteristics (e.g., the use of alternating versus direct current; Hill and Willis 1994; Rabeni et al. 2009). High abiogenic turbidity in shallow wetlands caused by wind-driven sediment suspension, or elevated biogenic turbidities caused by eutrophication and high algal densities can also reduce the effectiveness of fish detection during electrofishing.

Extremely low dissolved oxygen levels are common in both freshwater and coastal marine wetland systems (e.g., Killgore and Hoover 2001; Hagy et al. 2004). Rising and falling water levels coupled with seasonal hypoxia can affect fish

behavior, distribution, and survival in floodplain wetlands (e.g., McNeil and Closs 2007), which complicates effective assessment of fish community composition and abundance. The effects of hypoxic conditions on fish behavior are variable and species specific (Pollock et al. 2007). Some fishes respond to hypoxia by moving to more physiologically tolerable habitats, which could increase their susceptibility to passively deployed gears as dissolved oxygen levels decline. Conversely, fish unable to escape hypoxic conditions would likely decrease energy expenditures for feeding and movement (Chapman and McKenzie 2009; Chu et al. 2010), which would reduce the effectiveness of most passive gears. In estuarine marshes, salinity can vary substantially at timescales ranging from hours to months (Fernandes and Achuthankutty 2010), and these salinity changes are often reflected in the species composition of the fish community sampled at various times and locations in the marsh (Lorenz and Serafy 2006). Elevated conductivity can eliminate the use of electrofishing in these systems, necessitating the use of other gears such as seines, passive nets and traps.

6.2.5 *Water Movement*

Although wetlands are often lacustrine in nature, characterized by rainfall-driven water levels and minimal directional water movement, tidal and riparian floodplain wetlands often experience significant directional water flow that can cue the ingress or egress of transient fishes (e.g., Kwak 1988; Fernandes 1997; Dresser and Kneib 2007; Hering et al. 2010). These movements can facilitate fish collections by passive gears, such as fish traps or flume nets that can intercept individuals as they move with or against the current (e.g., McIvor and Odum 1986; Laffaille et al. 2000), but they can also complicate sampling efforts (Connolly 1999). In estuarine marshes, some fishes move with the ebbing tide while others remain in the upper marsh (e.g., mummichog *Fundulus heteroclitus*; MacKenzie and Dionne 2008). As with rapidly changing salinity, collection of sedentary and mobile species will complicate the sampling design if the study objectives involve determination of resident fish community structure. Characterizing the relative abundance (the number of species *i* / sum of all fish collected) and density (number of fish per unit area) of tidal marsh fishes is particularly challenging and may require integration of several sampling methods.

6.2.6 *Substrate Composition*

Freshwater and marine wetlands often have soft, unconsolidated substrates with high organic content (Mitsch and Gosselink 2007), which can influence fish

sampling in two ways. First, soft sediments may preclude the use of seines, backpack electrofishing, or other gears that require researchers to walk on the substrate during sampling (Rabeni et al. 2009). Combined with shallow depths, this may create a particularly problematic situation for sampling fishes, perhaps requiring the use of shallow-draft boats and paddles or push poles to set and retrieve passive gears. Second, mud and silt substrates can reduce the horizontal intensity of electrical fields (Scholten 2003), reducing electrofishing efficiency and likely requiring increased power to maintain fishing effectiveness.

6.2.7 Ice Cover

In northern latitudes, wetlands are often subject to ice cover for periods ranging from days to months. Ice and snow cover can have significant effects on fish community structure, particularly where hypoxia-related winterkill is common (Magnuson et al. 1985). Ice cover obviously eliminates the use of boats to sample fish, but it does provide access to all parts of a waterbody (given sufficient ice thickness, see Berry et al. 2012 for guidelines) for the use of stationary gears such as nets and traps (Tonn and Magnuson 1982; Hubert et al. 2012), underwater visible light cameras and video recorders (Lagler 1956; Mueller et al. 2006), echo sounders (Crawford and Jorgenson 1990), and acoustic imaging devices, such as dual-frequency identification sonar systems (DIDSON™; Mueller et al. 2006). However, it should be remembered that net and trap effectiveness is dependent on the magnitude of fish feeding (baited trap) and movement (unbaited trap and net) activities, both of which tend to decline with decreasing water temperatures (e.g., Suski and Ridgway 2009). Light cameras and video recorders are limited by turbidity, which can reduce their usefulness in many wetlands. Echo sounders can discriminate among fish and fish sizes when species are known or suspected (Foote 1987; Crawford and Jorgenson 1996; Boswell et al. 2008a), which may be useful in water bodies with limited fish faunal diversity, such as a trout and sculpin dominated bog or a estuarine wetland with a simple fish community structure, such as Gulf of Mexico coastal flats dominated by Bay anchovy and Gulf menhaden (Boswell et al. 2007). In some situations, dual-frequency identification sonar systems can identify fish species and number up to 16 m under ice cover (Mueller et al. 2006). However, when species composition is unknown, echo sounders are not useful for discriminating among species. Lastly, spears, lures, and telemetry have been employed to sample through the ice (Hayes et al. 2012). Active methods are subject to the same constraints of limited fish activity in cold water (e.g., Suski and Ridgway 2009). Sampling fishes through the ice remains a challenge, but promising advances in acoustical technology may offer possibilities for fish community assessment during a time period that has historically received little sampling effort.

6.2.8 Crew Safety

Crew safety is a critical issue in the design and implementation of a fish sampling program, because no data are worth exposing field crews to dangerous field conditions. Some gear and gear deployment protocols have in-built risks to crews and fish (see Dotson 1982; Jennings et al. 2012; Reynolds and Kolz 2012), and the responsibility for reducing risk lies with the project planner and manager. Drowning is a constant risk during aquatic sampling, particularly when sampling from boats, canoes, or kayaks, and collisions and other boating accidents can also be fatal (Berry et al. 2012). Consequently, boat sampling crews should never be comprised of less than two individuals, crews should have completed a National Association of Safe Boating Law Administrators approved training course, life jackets should be worn, and flotation devices should be readily available. Training and preparation can prevent and minimize risks to field crews.

Electrofishing is inherently dangerous and exposure to electrical currents can cause death or other serious injury (Professional Safety Committee 2008; Reynolds and Kolz 2012). Prior to sampling, all electrofishing equipment, including all wires, connections, and ground, should be inspected (Reynolds and Kolz 2012). At all times during electrofishing, insulated gloves and insulated, closed-toe shoes should be worn. During electrofishing, one person not involved in manipulating nets or probes should have the sole responsibility for overseeing the operation and quickly disabling current if necessary. Electrofishing should not be conducted during rain, wet, or slippery conditions. The minimum amperage and voltage that stuns fish should be used to protect crews and fish. Those unfamiliar with electrofishing should seriously consider an instructional course, such as that offered by the United States Fish and Wildlife Service at the National Conservation Training Center, Shepherdstown, West Virginia. Other gears, such as gill nets, can also be hazardous to field personnel through entanglement during deployment and retrieval. At a minimum, exposed buttons or zippers should be covered by tape or cloth flaps to minimize these risks.

Fish are often preserved in the field for later identification with known or suspected toxic preservatives. Aldehyde-based sample preservatives, such as formalin, can be harmful to field crews and sample handlers (Songur et al. 2010), and non-toxic alternatives, such as alcohol, freezing, and propylene-glycol based formaldehyde alternatives (e.g., Carosafe, Carolina Biological Supply Company, and Formalternate, Flinn Scientific) should be explored. Developing and adhering to standard operating procedures (SOP) can substantially reduce safety risks (Professional Safety Committee 2008).

6.2.9 Responsible Fish Handling

Precautions also need to be taken so that fish are not harmed unnecessarily during collection and processing (American Society of Ichthyologists and Herpetologists,

American Fisheries Society, American Institute of Fisheries Research Biologists 1988; Fisheries Society of the British Isles 2006, 2011). Crew leaders and members should be trained in proper fish collection and handling protocols to reduce risks to the fish (Jennings et al. 2012). In addition to ethical concerns, poor fish handling can directly influence density and relative abundance estimation, specifically in mark-recapture and depletion-style sampling (Jennings et al. 2012). Projects should be in compliance with university or agency policies regarding animal care and welfare (e.g., institutional animal care and use procedures; Fisheries Society of the British Isles 2006, 2011).

6.3 Gear and Technique Selection

Fish sampling decisions often balance the reality of time and available resources with the desire to increase precision and accuracy of estimates (Brown et al. 2012). Generally, researchers seek to allocate effort to best estimate parameters of the fish sample (e.g., mean CPUE or fish length) while minimizing variance of the estimates (Underwood 1981; Underwood and Chapman 2003). Selection of particular gears and methods can significantly influence the quality of estimated fish population, assemblage, and community characteristics. No fish collecting gear is free from issues of bias and non-random variance in estimates. Since bias is unavoidable, sampling programs should ideally characterize bias for their application and account for it when interpreting the results (see Sect. 6.3.3). In this section, we outline recommended gear and sampling techniques for specific wetland habitats, research objectives, and taxonomic scopes.

6.3.1 Introduction to Gear and Technique Types

In the following sub-sections, we have chosen terminology that is consistent with open water fish sampling literature to provide the reader opportunities for comparison and reference to the broader fish and fisheries literature. For several gears, we provide diagrams of gear and technique deployment, although we have not included detailed gear descriptions such as schematics, photos, and construction details, which are readily available elsewhere (see Hayes et al. 2012; Hubert et al. 2012; Reynolds and Kolz 2012). The emphasis of this section is to match gear and technique to specific wetland sampling scenarios with sampling guidance.

6.3.1.1 Quantitative vs. Qualitative

Quantitative fish sampling methods produce measures of fish abundance (e.g., relative abundance for communities or mark-recapture estimates for specific taxa), biomass,

or diversity that are integer values (e.g., counts, ages). Data can include direct measurements of fish or habitat characteristics (e.g., fish length, water temperature), assemblage or community metrics (e.g., indices of diversity, similarity, or dissimilarity), or ratios (e.g., catch-per-unit-effort or CPUE). Note that when effort is standardized CPUE is no longer treated as a ratio. In these cases, CPUE becomes an index of fish density. However, care should be taken to document effort and, if effort varies among trips or among habitats because of field conditions or crew experience, CPUE should be treated as a ratio or rate.

In contrast to quantitative sampling, qualitative fish sampling methods produce data that are nominal, or without an ordered relationship among delineated categories (e.g., presence/absence, fish sex, species lists), or ordinal, with some ordered relationship among delineated categories (e.g., descriptions of frequency of occurrence in terms of rare, uncommon, common, and ubiquitous). These data are not interchangeable, and each has a place in fish research projects (see Legendre and Legendre 1998; McKenzie 2005; Newcomb et al. 2007; Brown et al. 2012). Although quantitative methods can yield qualitative data following categorization (e.g., CPUE could be converted to low, medium, and high catch), conversion of qualitative data to quantitative data is impossible after sampling (see Milliken and Johnson 2002, 2009). Thus, the quantitative or qualitative nature of the data that are needed to address the objectives of the study must be decided prior to selection of a sampling method. In the following sections, we emphasize quantitative sampling techniques, but also include a few qualitative alternatives that may be appropriate for specific situations.

6.3.1.2 Active vs. Passive Techniques

Fish sampling techniques traditionally have been separated into active and passive methods based on whether fish move to the gear, termed passive, or whether the gear is moved to the fish, termed active (Hubert et al. 2012; Hayes et al. 2012). Active techniques share the advantages of spatial and temporal specificity (i.e., area sampled and exact time of collection are known, which may be important to diet, feeding, or movement studies) and shorter duration of sampling, and they may have less inherent selectivity and yield larger sample sizes (Hayes et al. 2012). Importantly, active methods typically rely much more on operator skill (Reynolds and Kolz 2012), frequently require specialized training, usually involve more sophisticated and expensive equipment (e.g., electrofishing boats or hydroacoustic arrays), and often require more labor than passive gears (Hayes et al. 2012).

Conversely, passive gears commonly involve limited time and labor to deploy and retrieve, they are usually cheaper and simpler to construct, and require less specialized training (Hubert et al. 2012). However, effectiveness of most passive gears is limited by inherent species, size, and behavioral selectivity (e.g., these gears are often poor at collecting sedentary, territorial, or structure-oriented fish; Hubert

et al. 2012). Further, in order for a fish to be captured by a passive gear, it must encounter the gear, become entangled, trapped, or hooked, and be retained by the gear until retrieval. Fishes often differ regarding trap behavior (e.g., "trap-happy" and "trap-shy" fish), probability of capture (e.g., mesh size too large or small or encountered at the wrong angle), probability of retention (e.g., more mobile fishes may escape entrapment gears more frequently than more sedentary fishes), and probability of encountering a trap (e.g., more mobile fish have a greater probability of encountering a trap). Corrections for one or more of these rates and probabilities or efficiencies have been developed for some gear types (e.g., Rudstam et al. 1984; Millar and Fryer 1999; Obaza et al. 2011; Section 6.3.3), which may increase the accuracy of CPUE estimates. Although some debate exists about the usefulness of abundance indices estimated from passive gear CPUE (Hayes et al. 2012; Hubert et al. 2012), it is generally assumed that CPUE is reflective of fish density. The accuracy of this assumption depends on whether the activity of all fish varies little enough to be ignored throughout a study (across space, or time, or both) and density variation is the primary driver of variation in CPUE (Prchalova et al. 2008; Obaza et al. 2011). In wetlands, where water levels fluctuate seasonally, this is a particularly dubious assumption. Some researchers call CPUE from passive devices 'activity density' to acknowledge these dual sources of variation. An additional disadvantage of passive gear is that lost or unrecovered gear can continue to capture and kill animals, termed ghost fishing (Guillory 1993; Matsuoka et al. 2005). Despite the potential disadvantages of passive techniques, these methods remain popular because of lower costs, labor, and effort, and may be useful in circumstances where access or available resources are limited.

Logistics may be the most important determining factor in the choice of active versus passive sampling methods. Boat-mounted gears such as shallow water trawls, electrofishing units, and hydroacoustic arrays are effective active methods of fish assessment, but all require boat launches suitable for fairly large watercraft [but see Chick et al. 1999, 2004 and Jackson and Noble 1995 for alternative electrofishing gears]. Because wetlands are often shallow and may be spatially isolated from a suitable boat launch, these active methods may not be feasible. Conversely, passive gears, such as entrapment nets and gill nets, can be deployed from the shore or from small vessels, including inflatable rafts.

Additionally, the type and level of human activity in a wetland may influence the choice of active or passive gears. Because passive gears are often left unattended, theft or vandalism of deployed gear may be problematic, and replacement may be expensive (Hubert et al. 2012). Active methods are not always preferable, however. For example, both diurnal and nocturnal electrofishing (the latter of which is typically more effective) are often unpopular with local residents, particularly those who engage in commercial or recreational fishing or reside on the shoreline. Avoiding conflicts may be impossible, but educational and outreach efforts may be able to minimize negative reactions. We recommend that experienced local fisheries specialists be consulted concerning recommended active or passive approaches for sampling fishes.

6.3.1.3 Standardized Methods

In addition to considering qualitative versus quantitative and active versus passive techniques, the use of standardized methods should be considered. Over the last two decades, consistency among studies for larger scale comparisons and meta-analyses has become increasingly important to fisheries researchers (Bonar et al. 2009). Whereas not all studies would benefit from standardized methods (e.g., studies with unique habitats, rare species, logistic difficulties, and other special considerations), standardized methods offer many benefits, including increased ease of comparisons over time and across geographic space, greater generalization of results, and improved communication among fisheries scientists and managers (Bonar et al. 2009). Consequently, management efforts can be better monitored and assessed over time, and localized changes in fish populations, assemblages, or communities can be better understood from a regional perspective. In 2009, the American Fisheries Society published *Standard Methods for the Sampling of North American Freshwater Fishes*, which offers a handy reference for methods that are comparable across studies and regions. In the following sections, we have indicated which techniques are recommended standardized methods.

Additionally, standardized methods offer benefits in data analysis. As mentioned in Sect. 6.3.1.1, CPUE may be analyzed as a ratio or rate, often with a generalization of the linear model based on the Poisson distribution (Tschernij et al. 2004; Shono 2008), or with more conventional parametric techniques if effort was standardized (i.e., effort becomes a constant that may be ignored). It should be noted that standardized data may still be zero-rich and may still require generalized linear model methods (Wharton 2005). Because many of the methods in the next sections generate CPUE, the benefit of standardized effort to analysis may be great, and we suggest that researchers seriously consider adopting standardized methods.

6.3.1.4 Gear and Technique Selectivity

Generally, all gears and techniques are biased, i.e., there are fish species or sizes that are sampled effectively (in proportion to their abundance), and those that are either over-represented or under-represented in the collections. For example, electrofishing is well documented to be size selective, under representing both small fishes because of limited surface area for development of electrical potential between the fish and water, and very large fishes that can often detect and avoid the electrical current (Reynolds and Kolz 2012). Efficacy and selectivity of fish sampling gears and techniques have been extensively evaluated in open water habitats (see references in Millar and Fryer 1999; Pope et al. 2009; Lester et al. 2009; Hayes et al. 2012; Hubert et al. 2012; Reynolds and Kolz 2012). Similarly, Rozas and Minello (1997) and Connolly (1999) have specifically addressed gear selectivity in wetland fish sampling, although both reviews directed recommendations at specific assemblages and situations. Limited direct comparisons among gear types and techniques have

Table 6.1 Selected studies on fish sampling gear comparisons and recommended gears by wetland type

Habitat	Gears compared	Recommendation	Reference
Freshwater marshes	Fyke net and boat DC electrofishing	Multiple gears	Ruetz et al. (2007)
Freshwater marshes	Drop trap and throw trap	Throw trap	Kushlan (1981)
Freshwater ponds	Beach seine, drop net, gill net, rotenone	Drop nets, beach seines	Beesley and Gilmour (2008)
Freshwater embayment	Bag seine, barge-style DC electrofishing, boat DC electrofishing	Barge-style DC for smaller fish, boat DC for larger fish	Jackson and Noble (1995)
Freshwater pond/lake	Beach seine, fyke net, gill net, purse seine, trap net	Trap net and gill net	Hayes (1989)
Freshwater floodplain wetlands	Dip nets, electrofishing, gill nets, light traps	Electrofishing, gill nets, light traps	Knight and Bain (1996)
Estuarine pond, pools	Minnow trap and seine	Seine	Layman and Smith (2001)
Estuarine submersed aquatic vegetation	Bottomless pop net, boat AC electrofishing, and seine	Bottomless pop net	Serafy et al. (1988)
Intertidal salt marsh	Fyke net and trap net	Fyke net	Varnell and Havens (1995)
Shallow salt grasses	Drop trap and visual assessment	Drop trap for richness	Bobsien and Brendelberger (2006)
Shallow saltwater bay	Drop net and push net	Drop net	Evans and Tallmark (1979)

been performed in wetlands (Table 6.1), but a review of published studies suggests that wetland type is an important factor in understanding gear selectivity because of differences among flooding regimes (Sect. 6.2.5), physical structure (Sect. 6.2.3), or water chemistry (Sect. 6.2.4). In addition to these reports, some gear and validation studies have been performed with marked and recaptured populations, and others have compared gear variations (e.g., different mesh sizes for traps; Table 6.2). Consequently, our discussions and recommendations of gear types in Sect. 6.3.2 have been divided among different types of wetland habitats.

We recommend the following approach when considering the selectivity of various gears and sampling techniques. First, the research objective(s) and taxonomic scope of the study should be the clearly stated. Certain gears and techniques are superior at collecting specific fish species or assemblages, but no single gear or technique completely samples a fish community. If community-wide sampling is desired, multiple gears should probably be used (Ruetz et al. 2007). Second, if regional recommendations, unpublished data (e.g., regional theses, dissertations, or

Table 6.2 Gear variations and validation studies in wetlands or other shallow water habitats

Comparison	Conclusion	Reference
Beach seine	More efficient for mid-water compared to benthic fishes	Lyons (1986)
Beach seine	First haul reasonably describes assemblages	Allen et al. (1992)
Gill net	Selectivity is bell-curve shaped around mesh size	Pope et al. (1975)
Gill net	Fish girth should be 1.25 less than mesh size	Hamley (1980)
Throw trap	1 m^2 trap had limited bias against large fish	Kushlan (1981)
Throw trap	Estimates ranged from 59 to 110 % actual density	Rozas and Reed (1994)
Throw trap	Estimates were within 90 % of actual density	Steele et al. (2006)
Throw trap	Effective sampling area of 1 m^2 trap was 0.81 m^2	Jacobsen and Kushlan (1987)
Trap net	2.5 cm nets biased toward larger sizes	Laarman and Ryckman (1982)
Trap net	Sensitive to soak time, location, season	Hamley and Howley (1985)

agency reports), or regional assessment protocols (e.g., U.S. Environmental Protection Agency's Great Lakes Coastal Wetlands REMAP) exist, we strongly suggest that these methods be used to increase consistency among studies. For example, drop and throw enclosure traps were recommended by Rozas and Minello (1997) for shallow estuaries in the Gulf of Mexico, and gear choices for large- and small-bodied fishes in Great Lakes freshwater coastal wetlands were outlined by Ruetz et al. (2007). Similarly, employing commonly-used regional sampling methods, such as multi-gear sampling protocols in Great Lakes (e.g., Jude and Pappas 1992; Brazner 1997; Brazner and Beals 1997) and isolated/semi-isolated freshwater wetlands (e.g., Rahel 1984; Main et al. 2007; Clark-Kolaks et al. 2009) will increase data consistency and comparability (Sect. 6.3.1.3). Third, if regional recommendations or conventions are not available, guidelines included in Sect. 6.3.2 should prove useful in the assessment of gear characteristics and selectivity. Lastly, we encourage researchers to consider adding direct gear and technique comparisons to their studies. Ample opportunity exists to expand the number of direct comparisons in the literature, and these studies are invaluable for improving wetland fish collection methods.

6.3.1.5 Special Considerations for Specific Objectives

Within the scope of fish assessment in wetlands, a number of objectives exist beyond the assessment of abundance, density, biomass, or productivity. Food habits and food resources are often important goals. Food resources are discussed elsewhere in this text; however, herein we include some information about food habit assessment. Food habits can offer insights into potential for individual and population growth and individual fish health. Additionally, classifying fishes by their food habits is 1 of several useful species traits, including reproductive modes and habitat associations, that may be used in assessment [see Poff 1997 and Goldenstein and

Meador 2004, 2005 for more information on the use of species traits in assessment]. Food habits may be determined by the sacrifice of fish to remove stomachs, non-lethal gastric lavage techniques that flush gut contents from live fishes, and often non-lethal tissue based methods, including amino acid (you-are-what-you-eat) analyses and measures of stable isotope ratios. Additionally, tissue samples may be obtained for genetic analyses. Active capture methods are preferable for food habit assessment or tissue sampling, which is most effective when tissues are removed from live or recently dead fish. In passive methods, fish may vomit or continue to digest gut contents when caught and may begin to decay, if sampling occurs over a period of time, rendering these methods less preferable for food habit research or tissue sampling. Other goals that need special consideration include meristic and morphometric studies, including assessment of condition (see Pope and Kruse 2007). When condition, such as Fulton's condition factor or relative weight, is part of the planned assessment, similar to tissue sampling, methods should be avoided that may have decay or mass loss following prolonged delays between capture and retrieval. Gill or trammel nets (Sect. 6.3.2.4) are not recommended for any of these goals.

6.3.2 Recommended Techniques

In this section, wadeable habitats are defined as shallow areas where walking can occur unimpeded at depths that do not limit the use of sampling gear. Non-wadeable habitats can be sampled from watercraft, but are either too deep for commonly used methods such as backpack electrofishing, or consist of unconsolidated substrate that may be dangerous or be subject to wading-related problems, such as high turbidity levels.

Intermittent habitats such as seeps, springs, playas, and vernal pools offer special sampling challenges because of their transient nature and shallow depths. These habitats are typically seasonally flooded (i.e., a few days to a few months), although some playas and seeps may be flooded for longer periods of time between drying. Resident fish and other aquatic organisms may have specialized life history traits to wait out dry periods, such as the lungfish *Proptopterus* spp. (Fishman et al. 1986), or may only access these habitats during wet periods (e.g., fishes moving to floodplain wetlands for reproduction). Consequently, timing is very important in sampling intermittent wetlands, which are often very shallow and not amenable to many sampling gears. However, water levels on some floodplains may vary by 10 m or more during the year from wet to dry periods (e.g., Ouachita River floodplain as described in Sheftall 2011), and these systems will require a flexible sampling approach, perhaps relying on techniques in this section.

Recommended gears and techniques for freshwater wadeable and intermittent habitats are bag or beach seines (active, quantitative, standardized method; Sect. 6.3.2.1), gill nets (passive, quantitative, standardized method; Sect. 6.3.2.4), entrapment nets (passive, quantitative, standardized method; Sect. 6.3.2.5), backpack, bank, or barge electrofishing (active, quantitative, standardized method;

Sect. 6.3.2.3), and minnow traps (passive, quantitative; Sect. 6.3.2.2). Given the small and shallow nature of these habitats, bag seines and minnow traps (e.g., Hanson and Riggs 1995; Batzer 1998) may be the best choices and should be used in combination if assemblage or community-wide data are desired. If habitats are too obstructed for seining or targeted fishes are not vulnerable to minnow traps, shallow-water electrofishing techniques (e.g., King and Crook 2002) are a viable active option, and entrapments nets may also be effective (Poizat and Crivelli 1997). Other gears that may be more preferable in these habitats include drop nets (active, quantitative; Sect. 6.3.2.7; see Beesley and Gilmour 2008), throw traps (active, quantitative; Sect. 6.3.2.8; see Baber et al. 2002), and dip nets (active, qualitative; see Main et al. 2007).

Some wadeable freshwater wetlands, such as bayous, shallow prairie potholes, and floodplain ponds, are characterized by periodic drying and variable periods of inundation (e.g., some floodplain ponds may be inundated 6 or more months of the year, and some potholes may be inundated for several years between drying). These wetlands can have significant habitat complexity in the form of woody debris, live trees and shrubs, and submerged, emergent, and floating vegetation, all of which add complexity to sampling. Further, these habitats are more prone to soft substrates than intermittent wetlands, which typically have more compacted substrate from frequent drying. However, permanent wetlands can also have deeper, open areas free of obstacles, so preliminary trips to the study sites will be valuable in the selection of sampling technique.

Recommended gears for freshwater wadeable habitats are bag or beach seines (active, quantitative, standardized method; Sect. 6.3.2.1), gill nets (passive, quantitative, standardized method; Sect. 6.3.2.4), entrapment nets (passive, quantitative, standardized method; Sect. 6.3.2.5), backpack, bank, or barge electrofishing (active, quantitative, standardized method; Sect. 6.3.2.3), and minnow traps (passive, qualitative; Sect. 6.3.2.2). The likelihood of increased densities of bottom debris and natural structure in these habitats can limit the effectiveness of seining (but see Walker and Applegate 1976; Scheerer 2002), and minnow traps (but see Rahel 1984; He and Lodge 1990; Snodgrass et al. 1996). Consequently, backpack, bank, barge, or shallow-draft boat electrofishing (Dunson and Martin 1973; McIvor and Odum 1988; Chick et al. 1999, 2004), entrapment nets (e.g., Cross et al. 1995; Paukert and Willis 2002), and gill nets (Hayes 1989; Knight and Bain 1996; Schrage and Downing 2004) are probably the best options, and should be used in combination if assemblage or community data are desired (e.g. Rahel 1984; Knight and Bain 1996).

Spatially isolated habitats such as deep prairie potholes and isolated swamps often support a diversity of emergent, submerged, and floating vegetation types [e.g., a pond ringed with cattail (*Typha* spp.) may also have lily pads (*Nelumbo* spp.) and coontail (*Cabomba* spp.)]. This structural complexity can significantly increase fish diversity, but can also reduce sampling efficiency, often requiring the use of multiple gears to effectively characterize the resident fish community. The level of complexity is closely related to the duration and frequency of inundation. Therefore, these habitats may vary widely in sampling difficulty.

In these types of habitats, gill nets (passive, quantitative, standardized, Sect. 6.3.2.4), entrapment nets (passive, quantitative, standardized, Sect. 6.3.2.5), boat electrofishing (active, quantitative, standardized, Sect. 6.3.2.6), and minnow traps (passive, qualitative, Sect. 6.3.2.2) are probably the most effective fish sampling gears. If launch facilities exist, boat electrofishing in combination with gill nets and/or entrapment nets is likely the best combination. Otherwise, inflatable craft, canoes, or kayaks can be used to may deploy gill nets and/or entrapment nets. In shallow (<1 m) dense submersed or emergent vegetation, pop nets (active, quantitative, Sect. 6.3.2.7; Higer and Kolipinski 1967; Serafy et al. 1988) and throw traps (active, quantitative, Sect. 6.3.2.8; Rozas and Odum 1987a; Streever and Crisman 1993; Jordan et al. 1997, 1998; Castellanos and Rozas 2001) may be viable options, but the type and density of vegetation will largely determine which gears are effective.

In deeper wetlands connected to larger aquatic systems, such as river embayments, bottomland hardwood floodplain ponds, drowned river mouths, and Great Lake marshes, seasonal changes in water depth, flow velocities, and water chemistry and the ingress and egress of transient fishes can all affect the sampling design. Riverine floodplain wetlands are typically influenced by the annual food pulse, and many fishes exploit these wetlands for reproduction and feeding. These transients may remain in the wetland for long periods of time, and may become trapped if floodwaters recede quickly. Accordingly, choice of sampling gear will depend on timing of the sampling in relation to the flood pulse.

Recommended gears for connected wetlands are gill nets (passive, quantitative, standardized, Sect. 6.3.2.4), entrapment nets (passive, quantitative, standardized, Sect. 6.3.2.5), boat electrofishing (active, quantitative, standardized, Sect. 6.3.2.6), pop nets (active quantitative, Sect. 6.3.2.7), and minnow traps (passive, qualitative, Sect. 6.3.2.2). Boat electrofishing (Jude and Pappas 1992; Batzer et al. 2000; Clark-Kolaks et al. 2009; Trebitz et al. 2009a) is robust to the variability added by transient fishes, and can be combined with gill nets and entrapment nets to broaden the diversity of fishes collected (e.g., Brazner 1997; Brazner and Beals 1997; Uzarski et al. 2005; Ruetz et al. 2007; Clark-Kolaks et al. 2009). Pop nets are effective in high vegetation density wetlands (Petering and Johnson 1991; Jude and Pappas 1992; Gilchrest and Schmidt 1997), and seines may be the best option in structurally simple littoral zones (Sect. 6.3.2.1; Leslie and Timmins 1992; Brazner 1997; Brazner and Beals 1997).

By their nature, intertidal flats share many characteristics with intermittently flooded freshwater wetlands (Sect. 6.3.2.1), with similar recommended gears and techniques, including bag or beach seines (active, quantitative, standardized, Sect. 6.3.2.1), gill nets (passive, quantitative, standardized, Sect. 6.3.2.4), entrapment nets (passive, quantitative, standardized, Sect. 6.3.2.5), and minnow traps (passive, qualitative, Sect. 6.3.2.2), all of which have proven effective (e.g., Varnell and Havens 1995). For small fishes, shallow pans (e.g., Kneib 1984) or plastic containers (e.g., Yozzo and Smith 1998) buried at marsh level can be used to trap fish as tides recede. Bottomless lift nets and drop nets (Sect. 6.3.2.7; Rozas 1992; Lorenz et al. 1997) may be particularly well suited in dense vegetation, although

retrieval of fish after the net is raised can be problematic, perhaps requiring the use of the toxicant rotenone (Sect. 6.3.5.2) to aid in fish recovery.

Wetlands with longer term inundation in estuarine habitats include shallow sand or silt salt marshes, mangroves, and shallow, rocky fjords that are inundated by river flood pulses or tides. Consequently, a wide variety of gear types and techniques have been employed in these habitats, depending on the targeted species of fish, accessibility, substrate composition, water clarity, and habitat complexity, including emergent and submerged vegetation. Large transient fishes can cause sampling problems in these habitats because of their ability to destroy deployed gears (e.g., adult red drum *Sciaenops ocellatus* moving through shallow marsh creeks), hence gear choice may vary seasonally depending on fish movements.

Although seines are not recommended in rocky estuarine areas because of the difficulty in maintaining contact between the substrate and the bottom of the net, gill nets (active, quantitative, standardized, Sect. 6.3.2.4), entrapment nets (passive, quantitative, standardized, Sect. 6.3.2.5), and minnow traps (passive, quantitative, standardized, Sect. 6.3.2.2) are likely to be effective fish sampling gears. Drop traps (active, quantitative, Sect. 6.3.2.9) or throw traps (active, quantitative, Sect. 6.3.2.8) may also be useful if substrates are fine enough to allow effective contact with the bottom of the trap (Evans and Tallmark 1979). In structurally complex mangrove wetlands with firm substrates, effective gears include seines (Sect. 6.3.2.1; Shervette et al. 2007), throw traps, and drop traps (Kushlan 1974; Lorenz et al. 1997). Gill and entrapment nets may prove difficult to deploy, but bottomless lift nets (Sect. 6.3.2.7; McIvor and Silverman 2010) and cast nets (Lin et al. 2003) may be effective alternatives.

Several active and passive gears, such as seines (Williams and Zedler 1999; Layman and Smith 2001; Gelwick et al. 2001; Akin et al. 2003) throw traps (Rozas and Minello 1997; Bush Thom et al. 2004; Kanouse et al. 2006), drop traps, gill nets, entrapment nets, and minnow traps (West and Zedler 2000) will likely be effective in salt marshes with sandy or silty substrates and vegetative cover. In wetlands with firm substrates and large open areas, all of these gears can provide representative fish samples, although each gear will exhibit species-specific sampling bias. Specific habitat conditions may preclude the use of certain gears, e.g., seines and entrapment would probably be difficult to use in wetlands with very soft substrates, and deployment of gill or entrapment nets may not be possible in dense cover.

Non-wadeable fjords, estuaries, salt marsh creeks and ponds often support a diverse aquatic plant community that will be an important factor governing the choice of fish sampling gear(s). Tides may also be an important consideration, and fish weirs (Sect. 6.3.2.11; Kneib 1991), flume nets (Sect. 6.3.2.11; McIvor and Odum 1986), or trawls (Sect. 6.3.2.11) may be highly effective sampling techniques for fishes that move with tidal flows. Sampling programs in these deeper, open systems will likely encounter seasonal influxes of transient fishes (Sect. 6.2.1), which may also affect sampling design.

Effective gears and techniques for low tidal energy wetlands are drop and throw traps (active, quantitative, standardized, Sect. 6.3.2.8; Kjelson et al. 1975; Rozas and Minello 1997), gill nets (passive, quantitative, standardized, Sect. 6.3.2.4),

entrapment nets (passive, quantitative, standardized, Sect. 6.3.2.5), and minnow traps (passive, qualitative, Sect. 6.3.2.2). In many cases, combinations of drop or throw traps with gill or entrapment nets (e.g. Hettler 1989; Connolly et al. 1997; Plunket and La Peyre 2005) will improve the diversity of fishes in the collections. In open water habitats with few benthic obstructions, small otter or beam trawls may be an effective sampling gear (Macauley et al. 1999; Martinho et al. 2008; Selleslagh and Amara 2008; Trebitz et al. 2009b), particularly when combined with other gear types (Mendoza-Carranza et al. 2010). In more vegetated habitats, drop (e.g., Rakocinski et al. 1992; Roth and Baltz 2009; Piazza and La Peyre 2009) or throw traps are likely the best options (Rozas and Minello 1997), although bottomless lift nets (Sect. 6.3.2.7; McIvor and Silverman 2010) and pop nets (Connolly et al. 1997; Connolly 1999) may also be good alternatives if sample sites are sufficiently shallow. In high tidal energy wetlands, trawls, flume nets or weirs (passive, quantitative, Sect. 6.3.2.10; Rozas and Odum 1987a, b; Peterson and Turner 1994; Kimball et al. 2010) may be the best choice. Entrapment nets may be effective if the wetland has suitable open areas and firm substrates, but gill nets are not recommended because of potentially strong currents.

6.3.2.1 Use of Seining in Freshwater Wadeable Habitats

A seine consists of a synthetic material mesh net strung between poles, termed brailes, with a weighted lead line on the bottom and a floating line on the top (additional floats are optional). Seines are typically constructed as beach seines (simple net panel) and bag seines, with the latter having a box-like section of extra netting (usually in the middle) that improves capture success and acts as a holding area for collected fishes. Mesh can vary by size of opening, measured as bar (distance from one knot to its adjacent knot) or stretch (distance from one knot to its opposite knot when the net is stretched) size, and the orientation of the opening, which may be square, diamond, or oval shaped. Mesh size sets the maximum size that a fish, or other aquatic organism and debris, can pass through the seine, and also affects net drag and speed of sampling. Although smaller meshes collect smaller individuals, they may be prone to increased drag and clogging. Larger meshes often capture smaller fishes with reduced clogging, but the unknown number of smaller fish that may have passed through the net reduces the accuracy and precision of CPUE estimates. Seines can be virtually any length that is practical for the sampling situation. Smaller (3–6 m) seines can be better suited to shallow littoral habitats because of their maneuverability, whereas larger seines may be more appropriate in open estuarine habitat because of increased spatial coverage (e.g., 46 m; Kelso 1979). In order to maximize the comparability of seine data, net length and sampling techniques should be standardized among hauls.

Deploying seines quantitatively in wetland habitats can be more difficult than other settings, if substrates are soft or underwater obstructions are abundant; however, quantitative seining is a low-cost, versatile, and rapid active gear choice. In suitable habitats, seines are well suited to capture near-shore and mid-water

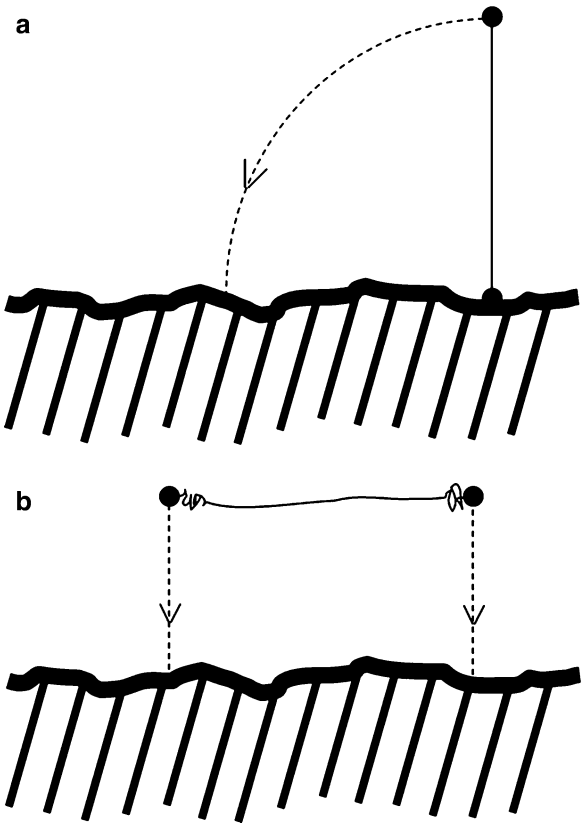

Fig. 6.1 Two approaches are recommended for quantitative seining. (**a**) The seine is stretched perpendicular to shore with the distal end, relative to shore, moved as quickly as possible in an arc to the shoreline. (**b**) Approximately one-third of the seine is bunched at each end with one-third of the seine stretched between the brailes, which may be several meters apart. The brailes are then moved quickly toward the shoreline enclosing the sampling area. *Dark line* indicate seine, *dark circles* indicate crew, *dashed lines* indicate direction of movement. Shoreline is indicated by *thick black lines* with angled hashing

species, and are often more effective at capturing smaller bodied fishes than electrofishing (Ruetz et al. 2007). In addition, seines can be used by one person, are depth limited only by the height of the net and crew members, and are among the least harmful methods for capturing fishes, which makes them an ideal choice when live capture is a necessity (e.g., sampling rare or uncommon fishes). However, seine sampling also has several disadvantages. Quantitative seining is not as effective as some other methods for capturing larger and more mobile fishes, nets typically contain substantial bycatch (e.g., invertebrates, other aquatic and semi-aquatic organisms, and anything else that is in the water column), and may be prone to clogging, which could reduce sampling effectiveness. Although a useful gear for sampling wetland fishes, it may be necessary to supplement seining with another technique, depending on research objectives.

Seines can be quantitatively deployed in two ways (Fig. 6.1). One method is to collapse about one-third of the net at each end, move offshore that distance, move parallel to the shoreline for a few meters so that the sample will be from an undisturbed volume of water, move both ends to shore as quickly as possible, and carefully retrieve the seine, making sure the lead line stays in contact with the

substrate as much as possible. This method will enclose an area or volume of water of known dimension, allowing calculation of fish density (usually fish/m^2 or fish/m^3) and CPUE. A second method is known as a "quarter-haul", in which one braile is held on the shoreline while the other end of the net is extended perpendicular to the shoreline and is then swept to shore as quickly as possible, again making sure the lead line remains in contact with the bottom. Area sampled can be calculated from the length of the net, and fish density and CPUE can be calculated. One advantage of the quarter-haul method is that deeper water can be sampled with the assistance of watercraft, although maintaining good contact between the lead line and bottom may be problematic.

Prior to sampling, decisions also need to be made regarding the distance of individual hauls and number of hauls to be performed. Net length and distance sampled dictate area sampled, and consistent sample distances simplify CPUE estimates and offer the benefits of data standardization. Distance sampled is relative to the size of the water body and littoral habitat structure, and research suggests that multiple hauls are necessary for the estimation of relative abundance or density, particularly when working with rare or uncommon fishes (Allen et al. 1992). Section 6.3.5 illustrates the advantages of additional hauls for population estimation. Fortunately, seining is not a time consuming activity, therefore, using multiple hauls and correcting for capture efficiency (Hayes et al. 2012) is recommended.

6.3.2.2 Use of Minnow Traps in Freshwater Wadeable Habitats

Minnow traps typically are typically constructed of a fine metal mesh cylinder with inwardly-pointing conical openings at each end, which allows fish to easily enter, but not easily exit, the trap. Although named for the most common use, minnow traps may be used to collect many small bodied, non-minnow fishes and are often used to catch young-of-the-year specimens of sunfish, including green (*Lepomis cyanellus*) and longear sunfish (*Lepomis megalotis*), northern pike (*Esox lucius*), and even grayling (*Thymallus thymallus*) in high altitude wetlands. Minnow traps are usually small (<0.5 m in length), with two sections opening in the middle allowing access to collected fishes. Because of their popularity among recreational anglers for the collection of bait fish, minnow traps are widely available at a low cost in a variety of sizes. They are usually set in groups at different distances from shore at variable or consistent depths depending on targeted fish taxa (Fig. 6.2). Attaching a float or line from the trap to a pole anchored in the bottom will reduce lost gear and ghost fishing (Sect. 6.3.1.2).

Minnow traps can be set anywhere in the water column on stakes, and can be highly effective for sampling small benthic and structure-oriented fishes. Although traps are usually fished with baits, baiting can contribute to species selectivity (i.e., some baits may be unattractive to some fishes). In structurally complex habitats, minnow traps can be disguised with vegetation or woody debris to reduce "trap happy" and "trap shy" effects. Minnow traps and other pot gears do not have a long history of use in fishery-independent sampling (i.e., data used in fishery

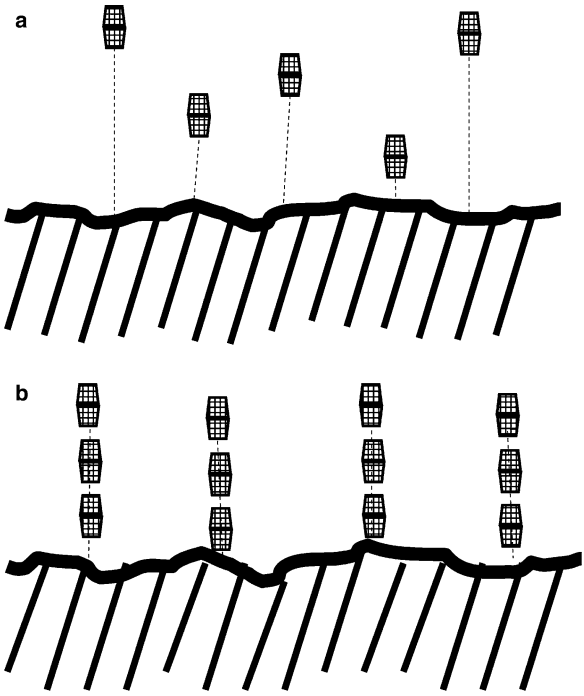

Fig. 6.2 Minnow traps may be set a varying distances from shore to sample different depths (**a**) or along depth gradients (**b**). Minnow traps are tethered to shore by rope (*dashed lines*). Shoreline is indicated by *thick black lines* with angled hashing

management not derived from commercial or recreational harvest; Hubert et al. 2012). Although they are not useful for calculating relative abundance or density because of species differences in trap susceptibility and unknown sampling range, they can be useful for describing the age structure and condition of susceptible taxa (Hubert et al. 2012). Although they can be highly biased for or against various fish species, minnow traps they can be quite effective under certain situations and can be an excellent complement to other gears, specifically when benthic, cryptic, and structure-oriented fishes are part of the targeted community.

6.3.2.3 Use of Backpack, Bank, and Barge Electrofishing Freshwater Seasonally or Longer Flooded Wadeable Habitats

In freshwater habitats, electrofishing is probably the most common form of fish sampling in North America. Electrofishing is popular because it has generally low associated mortality (although this is dependent on the electrofishing parameters and the species of interest), has low bias for diet studies, offers quantitative measures of density and relative abundance, and can yield high quality data quickly (Reynolds and Kolz 2012). Many freshwater studies have investigated the properties of electrical transmission, effects of voltage, amperage, and pulse choices on sampling efficiency, and the effects of electrofishing characteristics on fish mortality (e.g., Martinez and Kolz 2009; Miranda and Kidwell 2010; Reynolds

and Kolz 2012). Although electrofishing gear has been developed for low to intermediate salinities (up to a specific conductance of 25,000 micro-Siemens, approximately 16 parts/thousand), relatively few studies have used these units relative to more established and more frequently used saltwater gears. Less electrical power is needed to stun fish for collection in freshwater because power transfer is more readily accomplished in a more favorable water-to-fish conductivity gradient (Reynolds and Kolz 2012), and electrofishing is probably best used in freshwater and very low salinity wetlands.

The popularity of electrofishing as a sampling technique arises in part from the ability to sample large spatial areas in a short period of time. Electrofishing is versatile, less habitat selective (i.e., it samples both bottom-oriented and pelagic fishes simultaneously), and can result in low mortality (Bardygula-Nonn et al. 1995; Habera et al. 1996; Dolan and Miranda 2004; but see Snyder 2003). However, electrofishing gear is quite costly compared to nets or traps, and is more dependent on user skill and experience than many gears (Hayes et al. 2012; Reynolds and Kolz 2012). Further, very high or very low water specific conductance limits efficiency (Rabeni et al. 2009; Reynolds and Kolz 2012), as do soft substrates (Scholten 2003), dense aquatic vegetation that reduces electrical field size and traps stunned fish (Killgore et al. 1989; Klein Breteler et al. 1990; Miranda and Pugh 1997; but see Perrow et al. 1996; Chick et al. 1999), water depths beyond the electrical field range, and highly turbid or deeply stained waters that reduce netting efficiency. Fishes also vary in susceptibility to electrofishing, with larger and coarse scaled fishes more susceptible than smaller, fine-scaled, and cryptic taxa (Grabowski et al. 2009). Section 6.3.3 offers methods that may mitigate some of these concerns.

Electrofishing works by establishing an electric circuit in the water, usually by direct current (DC) and occasionally by alternating current (AC). Electrofishing with DC is far more common because of reduced injury to fish and increased safety for field crews (Snyder 2003; Reynolds and Kolz 2012). However, some situations may call for the use of AC, including the use of parallel wire systems which can be effective in complex structure where seining is ineffective and high levels of fish mortality are not problematic (Basler and Schramm 2006; Burns 2007; Hitt and Angermeier 2011). Ideally, electrofishing should induce electrotaxis, or involuntary movement along the direction of electrical current towards the anode, which brings the fish into the range of dip nets. If current exceeds the levels that induce electrotaxis, fish may undergo narcosis (unconsciousness) or tetany (involuntary muscle contractions). Fish experiencing narcosis or tetany can surface after the sampling team has passed, or can remain on the bottom in the case of benthic fishes without swim bladders and may not be detected by field crews. In addition, excessive power transfer may result in trauma, including stress, hemorrhaging, fracturing, and mortality, which may be immediate or delayed (Snyder 2003). Consequently, settings that may efficiently induce electrotaxis in one fish species or size range may induce tetany in other members of the fish assemblage or community. Before sampling, careful review of the literature and consultation with experienced professionals regarding the most effective electrofishing settings is recommended.

Commonly, electrofishing gear consists of a power source (e.g., a deep-cycle DC battery or AC gasoline generator with an AC-DC converter), a transformer, a control unit or pulsator, one or more safety switches (e.g., foot pedals, thumb switches), a cathode, and an anode. Older units may not have all of these components (e.g., may be wired directly to the generator) and may be effective. However, we recommend sampling with modern units equipped with redundant safety features and control units with selectable current type, wave form, pulse rate, and voltage.

Electrofishing units vary in their intended depth of operation, spatial coverage (i.e., effective electrical field), and amount of voltage generation. Backpack electrofishing units are the least powerful with the smallest effective electrical field and shallowest operational depths (usually <1.0 m). Backpack electrofishing units mount the power source, usually a deep-cycle DC battery, and pulsator unit on a backpack frame for ease of mobility and comfort by the operator. The cathode may be a hand-held non-conductive pole with an exposed metal square or ring, a square or trapezoidal float, or a heavy gage wire with an un-insolated tip. The anode is usually a hand-held non-conductive pole with an exposed metal diamond or ring and thumb-depressed safety switch. Backpack units also commonly feature a tilt switch that cuts power when the backpack is tilted beyond a certain angle to reduce the possibility of shocking after a fall, and a water sensor that cuts power when water contacts the battery or pulsator housing.

Bank electrofishing units, including parallel wire designs (Burns 2007) that create a broad field of current between two wires usually arranged from bank to bank with current passing from one wire to the next, throwing electrode designs that project and retrieve the electrode from cover or deep water, and handheld anode designs similar to backpack units, are used in deeper water (limited by wader height) and when larger, more powerful electrical fields are needed. A bank electrofishing unit has a gasoline generator and pulsator that are placed on the shore close to the water, with a submerged heavy gage wire cathode and hand held anode(s) attached to 50–100 m of cable. The anode may be exposed heavy gage wire in a parallel wire design, modified for throwing and retrieval, or designed similarly to a backpack unit. Cable length is limited by diminishing electrical current with increasing cable length, as well as reduced sampling efficiency with increasing distance between the cathode and anode(s). Barge-mounted electrofishing units mounting the generator and pulsator in a small barge or johnboat, which solves bank electrofishing issues of decreased efficiency as cable and anode-cathode distances increase, as well as constant snagging of long anode cables during sampling. The boat is wired to be the cathode, and the anode pole is usually attached to 2–3 m of cable. Generally, the anode is operated similar to a backpack unit but also may be modified into a throwing electrode. Efficiency is high because the anode and cathode are in close proximity, and the generator allows a larger effective electrical field than can be generated with backpack units. However, barge units are expensive and can be cumbersome if water depth or obstructions inhibit barge movement.

The optimal electrofishing unit for a sampling situation will ultimately be dictated by water depth, size of the sample area, specific conductance of the water, substrate, and the amount of voltage needed to effectively sample the taxa of interest. Soft, silty substrates are known to diminish the effectiveness of electrofishing (Scholten 2003; Reynolds and Kolz 2012) and will require greater power input than sandy and rocky substrates. Wave form, pulse rate, and voltage can be varied by the operator (see Reynolds and Kolz 2012 and guidelines provided by unit manufacturers), but as a general guideline, we recommend selecting the continuous (preferable) or pulsed DC, the lowest practical voltage to generate a safe and effective amperage (2–3 amps is usually sufficient in shallow waters), and a high pulse rate for soft rayed (but not salmonids, Reynolds and Kolz 2012) or strong-swimming fishes, a low pulse rate for spiny-rayed fishes, and a very low pulse rates for catfishes (Corcoran 1979).

Prior to electrofishing, one should consider whether to deploy block nets to spatially isolate the sampling areas. Deploying block nets allows estimation of fish per unit area, whereas sampling without block nets results in abundance estimations based of fish collected per time shocked over a set distance (CPUE), such as fish per minute (Bohlin et al. 1990; Reynolds and Kolz 2012). Block nets may not be feasible because of site-specific characteristics (e.g., weedy, shallow shorelines that impede net deployment) and use of block nets in these conditions may actually reduce efficiency (Perrow et al. 1996). However, when feasible, block nets probably enhance sampling efforts and estimation of density and relative abundance.

Electrofishing is usually best performed in the spring or fall of the year to reduce fish stress and maximize efficient collection of fishes of many sizes. Although electrofishing is often more effective at night (e.g., Dumont and Dennis 1997), night sampling has additional logistic (e.g., lights) and safety considerations that, in combination with increasing restrictions on night sampling from permitting agencies and unpopularity with shoreline landowners, have made it less common than in the past. Typically, sampling involves either continuously electrofishing a given area/distance, or sampling discrete habitat patches such as woody debris piles, known as the fractional or point abundance method. In the area/distance electrofishing method, an electrofishing sample consists of low-speed (e.g., 1,000 s/100 m) movement along the entire shoreline, or randomly-selected portion(s) of the shoreline in larger wetlands (Fig. 6.3). If current is present, movement should proceed from down-current to up-current, because fish often orient to face up-current. If fishes are removed during multiple samples taken over the same area, particularly if block nets are used, fish abundance, density, and capture probability (Dauwalter and Fisher 2007) can be estimated by depletion (Sect. 6.3.5). In the fractional or point abundance sample method, habitats are selected *a priori* and are often surrounded by block nets (Perrow et al. 1996; Scholten 2003; Lapointe et al. 2006; Fig. 6.3), at which point the electrical current is applied and fishes are collected. The continuous electrofishing method is advantageous because multiple habitat types are sampled, which can increase sample species diversity. However, this method can underestimate density and relative abundance and does not permit association of specific fishes with specific habitats.

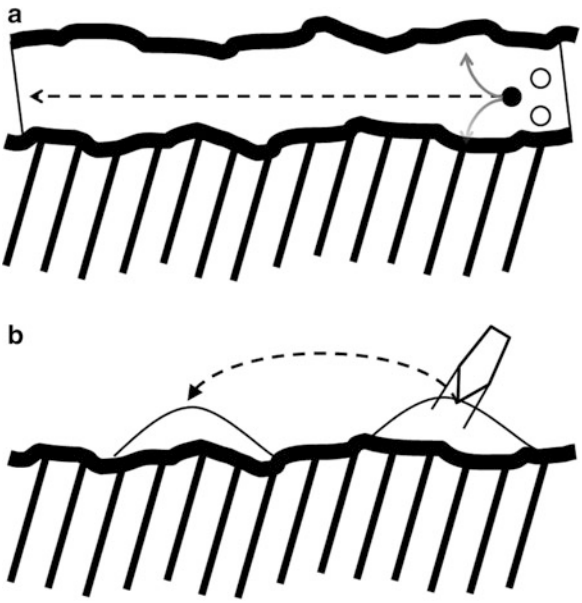

Fig. 6.3 Continuous electrofishing usually occurs over a preset distance along a shoreline conducted by a three person crew (**a**). *Dark circle* indicates crew member with a backpack electrofishing unit or the person who would carry the electrode in a bank electrofishing deployment. *Gray lines* suggest movement of electrode. *Open circles* depict crew members netting fish. Within an enclosed, habitat-specific area, point abundance sampling is an effective alternate depicted here by a boat (**b**). During both methods, time sampling is recorded, usually in seconds, to quantify effort. *Black lines* depict block nets in both methods

The point-sample method can provide better density and species-specific habitat use data (Lapointe et al. 2006; Janáč and Juradja 2007; Sect. 6.3.2.6), but usually covers less area and is more time-consuming.

6.3.2.4 Use of Gill Nets in Freshwater Non-wadeable and Spatially Isolated Habitats

Gill nets are one of the oldest fish sampling gears, and are able to provide quantitative estimates of fish density or activity density, and community composition, usually measured in CPUE. Gill nets typically consist of vertically-oriented monofilament net panels suspended between a foam core float line and a weighted lead-core line. Fish encountering the net become tangled in the mesh by their body, opercula, teeth, spines, or other projections. Gill nets can have mesh of uniformly-sized mesh openings, or may be constructed of successive panels of different mesh size, usually called experimental gill nets, to sample a range of fish species and sizes. A degree of gill net species and/or size selectivity can be accomplished by adjusting the mesh size, although individuals of some species that are smaller than

the mesh opening may still be susceptible to capture because of morphology (e.g., catfish spines). Gill nets are often fished overnight, which can result in substantial fish mortality. However, if fish survival is important, they can be set as "strike nets", which involves staying with the net after it is set, removing fish as they are captured and minimizing duration of the set ("soak time"), which substantially increases fish survival (e.g., Nieland et al. 2002). Another option that may improve survival of captured fish is the trammel net, which consists of a loose, small-mesh panel hung behind a coarse-mesh panel; fishes encountering the smaller-mesh panel swim through the coarse mesh and are held in the resulting pocket. Trammel nets are less size selective than gill nets and may be highly effective in shallow water (Hubert et al. 2012). However, trammel nets are less popular than gill nets because of higher price, lower availability, and increased handling time during retrieval.

Gill nets have several advantages as a sampling gear, including low cost, wide availability, long service life, ease of use, and versatility. Because they are widely used in commercial fisheries, many suppliers exist for gill nets, and custom nets of particular lengths and heights can be constructed for most sampling situations. Although gill nets slowly degrade from normal wear and tear and exposure to UV light, they are readily repaired (see Gebhards 1996). Gill nets typically require little training for deployment and retrieval, although fish removal takes practice. Gill nets also are quite versatile, and can be set horizontally anywhere in the water column to collect fishes that inhabit surface, pelagic, or benthic habitats, or vertically to investigate depth-specific fish distributions (Kohler et al. 1979). Lastly, gill nets fish bi-directionally, which can provide information about prevailing directions of fish movement, e.g., onshore versus offshore, or ingress versus egress of transient wetland species.

Regardless of their advantages and adaptability to various sampling situations, gill nets also have several disadvantages that may preclude their use. They are selective regarding fish size and activity and are more effective for larger taxa (Hubert et al. 2012), which is why they are often used in combination with other gears. Hamley (1980) suggested that the optimum fish girth for capture was 1.25 times the perimeter of the mesh opening. Consequently, capture efficiency is curvilinear, often bell-shaped (Pope et al. 1975), with reduced sampling efficiency of larger and smaller individuals. Unless they are set as strike nets, gill nets are not appropriate for diet or physiology studies because captured fish exhibit high stress levels, often vomit, and continue to digest food after capture. Monofilament gill nets have relatively low gear bias associated with net visibility (Jester 1977), but net avoidance may occur in clear waters. Gill net efficiency is also related to variations in soak time, hence standardization is important. Generally, as the net captures more fish, available space for additional captures decreases, and gear visibility increases. If gill nets are deployed in currents or in windy locations, large anchors are necessary to eliminate unwanted movement, or the loss of nets that can result in ghost fishing. Gill nets do not sample well in dense vegetation or in areas with large amounts of woody debris, and entrapment nets (Sect. 6.3.2.5) may be a better option. Lastly, because gill net effectiveness is dependent on fish activity, collections are typically biased against sedentary, habitat-oriented, or highly

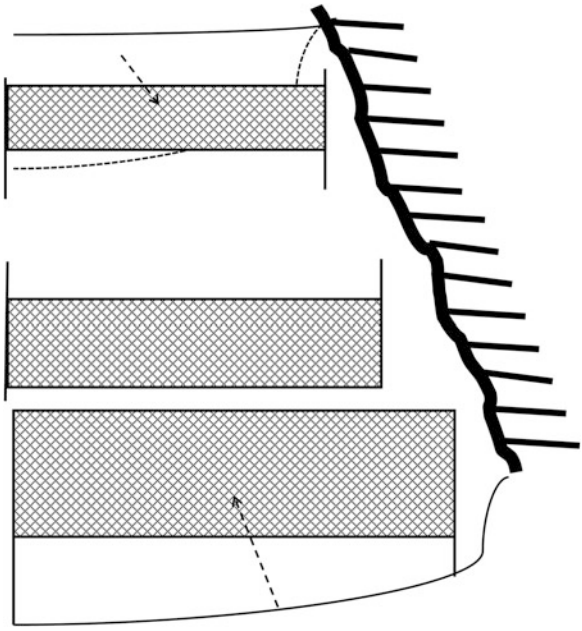

Fig. 6.4 Gill net sets parallel or angled to the shoreline are effective at sampling active fishes. Gill nets may be set at varying depths, as shown. *Dashed lines* indicate anticipated directions of fish movement

territorial fishes. In addition, variables that affect fish movements (e.g., season, water temperature, water level fluctuations, weather) will affect sampling efficiency, and these conditions should be noted and incorporated into the sampling design, if possible.

Gill net deployment is highly variable because of the numerous possible combinations of sampling depth, panel length, and mesh configurations. We recommend experimental gill nets because of their ability to capture a wider range of fish sizes, unless specific species/size classes are being targeted. In shallow waters, nets are usually anchored at the shoreline and set perpendicularly out into deeper water, and if multiple experimental gill nets are used, it would be best to alternate the mesh size that is close to shore. Sets that are parallel or angled to the shoreline may be useful if onshore/offshore fish movements increase susceptibility to the net or are of interest in the study (Fig. 6.4).

Gill nets should be transported in a container designed to reduce net tangling (Fig. 6.5), which allows simultaneous deployment of both float and lead lines and helps keep the net straight. Deployed nets are usually anchored at both ends with marker buoys at one or both ends, which is particularly important if the net would present a boating hazard. Care should be taken so that gill nets do not snag crew members or any other objects on the boat that might cause the nets to tear. Net retrieval generally begins at the deeper end, and if an apparatus similar to Fig. 6.5 is used, net retrieval simply involves pulling the marker float or buoy to the boat, unhooking the anchor, feeding the floating and lead lines onto their respective poles, and catching the net material in the basket. Although removal of fish

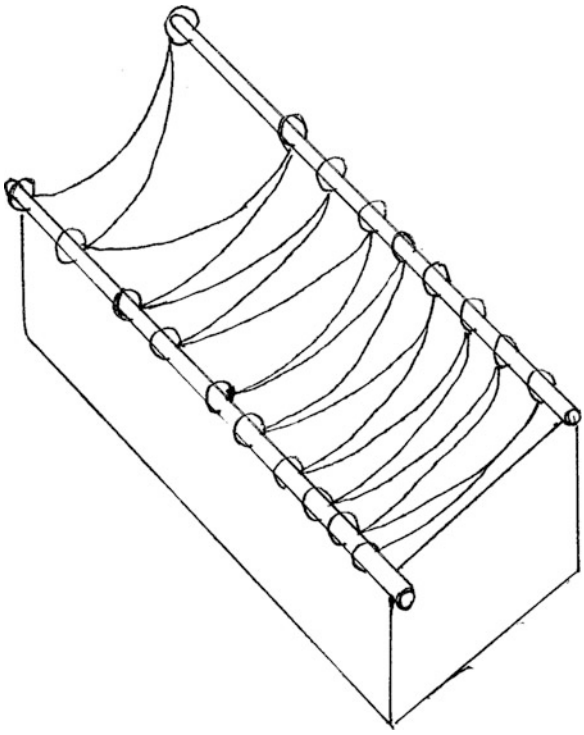

Fig. 6.5 Gill net tangling may be avoided by the use of poles and removable rings. Rings are attached to the gill net at 1 m increments. Rings then slide the gill net onto and off of poles that keep the gill net hanging vertically and reduce tangling. During storage, the vertical position assists in drying the net

encountered in the net can be difficult and time-consuming, particularly in strike nets where minimal fish mortality is desired, a number of tools have been suggested to assist removal (see Lagler 1978; Hubert et al. 2012).

Special circumstances may require alteration of standard gill netting methods. Deployment of several shorter nets may be more desirable in vegetated or debris filled habitats than setting a single longer net. Gill nets may be set under ice (Hubert et al. 2012), although reduced fish movements in cold water may limit the success of this technique. Commercial fishers sometimes herd fishes into gill nets set in a circle or semi-circle, which might be effective in shallow wetland habitats and might address some of the biases against sedentary, territorial, or otherwise non-active fishes. However, because this technique would add another unknown variable to the gear bias (species differences in susceptibility to herding techniques), results should probably be treated as qualitative data. Soak time can also be varied depending on targeted fishes and objectives of the study. Strike nets are typically set during the day, when evidence of capture (e.g., buoy or net movement) is more readily observed. Depending on the species of interest, strike nets may need to be checked at frequent intervals (e.g., 15 min), as smaller fishes are less likely to provide visual evidence. Gill nets can also be set overnight to sample both dawn and dusk, which are frequently times of peak fish movement, or for short time intervals throughout the day to target specific movement periods.

6.3.2.5 Use of Entrapment Gear in Freshwater Non-wadeable and Spatially Isolated Habitats

Hoop, fyke, and trap nets are passive, quantitative gears with many of the same passive gear advantages (e.g., low cost because of extensive use in commercial fishing) and disadvantages (e.g., potential size selectivity; Laarman and Ryckman 1982; but see Kraft and Johnson 1992; Hubert et al. 2012) as gill nets (Sect. 6.3.1.2). However, these nets work by entrapping, rather than entangling fish, which generally leads to much less mortality of captured fish (Hopkins and Cech 1992; Krueger et al. 1998; Booth and Potts 2006). Entrapment gears can be fished in stronger currents than gill nets, and generally work best when set on firmer substrates. They appear to be particularly successful at sampling cover-oriented fishes, which are less readily sampled by gill nets, and are well suited for sampling tidal creeks or riparian wetlands with seasonal ingress and egress of transient fishes. However, entrapment gears fish uni-directionally, and placement of the net opening (e.g., facing inshore, offshore, up-current, down-current) is an important variable that can affect sampling efficiency. Fish can also escape from these traps, particularly small fishes, and we suggest putting marked individuals of the focus species into the last compartment of a net, submerging it for the projected soak time, and assessing escapement upon retrieval. Even with these potential concerns, entrapment nets are a popular and useful sampling gear because of their low mortality, low cost, and reduced size selectivity.

Hoop nets, which are constructed of a set of concentric metal or wooden frames (usually round or rectangular) connected by mesh (usually nylon), are an effective sampling gear in lotic freshwater systems (e.g., Pugh and Schramm 1998). Typically, inward-facing mesh funnels are attached to the second and fourth hoops to hinder fish escapement, and the distal end of the net (cod end) is usually cinched by a drawstring for easy fish removal (see Hubert et al. 2012). Hoop nets are typically anchored from the cod end, with the open end facing down current. Marker buoys attached to the first frame aid in net recovery and ensure the net will be retrieved from the open end, concentrating fish in cod end.

Although similar to hoop nets, fyke and trap nets have leads (long vertical mesh panels, often reaching from bottom to surface) that guide fishes toward the net opening (Fig. 6.6). One to three leads may be attached to a fyke net, with two leads set in a "V" shape and three leads set in a fan shape from the front of the net. Fyke nets are often constructed with a rectangular frame at the net opening for stability and strength, with round frames for the body of the net ("modified" fyke nets; Hubert et al. 2012). Although functionally identical, trap nets typically have rectangular rather than circular frames. Because of the leads, fyke and trap nets theoretically sample a larger area than hoop nets, assuming fishes are moving along the path intercepted by the leads, and can be particularly effective for benthic species. However, although potentially very useful in lentic wetland habitats, these nets are less useful in moving water without considerable efforts to make sure the leads are well-anchored.

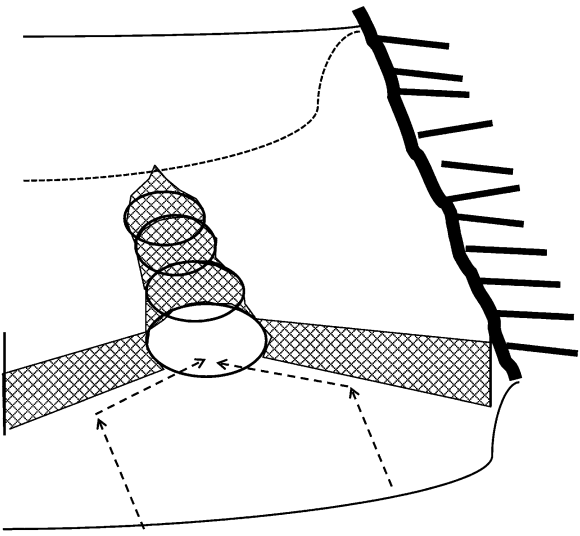

Fig. 6.6 Fyke nets are often set in anticipated fish movement paths along shorelines (**a**). *Dashed lines* indicate fish movement along shoreline into fyke net. Hoop nets, which lack the characteristic wings of fyke nets, should be set with then open end facing down current because most fish orient and move up current

Fyke and trap nets are more tedious and time consuming to deploy than hoop or gill nets, as the leads must be anchored firmly. Leads may require float and lead lines, and marker buoys are recommended to prevent loss of the net and reduce boating hazards. Retrieval is similar to a hoop net, and the leads should be constructed so that they can be easily detached from the net to facilitate retrieval. It may be possible to remove fish from fyke and trap nets without bringing the entire net onboard, which allows a single net to be fished for consecutive sampling periods.

Similar to gill nets, entrapment net efficiency is related to soak time, habitat characteristics, and time of year. Research suggests that single night sets are as effective as two night sets (Brady et al. 2007), and net saturation and increased fish stress can occur during longer soak times (see Sect. 6.3.2.4). Lastly, because entrapment nets are dependent on fish activity, standardization of sampling protocols regarding time of year (Cross et al. 1995) and other variables that affect fish activity is recommended (see Hamley and Howley 1985).

6.3.2.6 Use of Boat Electrofishing in Freshwater Non-wadeable and Spatially Isolated Habitats

Boat electrofishing typically allows greater sampling speed, areal coverage, and electrical power generation than backpack, bank, or barge electrofishing methods (Sect. 6.3.2.3), but with greater initial expense and upkeep. Boat electrofishing units can be as simple as placing barge or bank units on small flat-bottom craft (e.g., Jackson and Noble 1995). Although the use of hand-held anodes in these types of boat units may limit sampling effectiveness (Jackson and Noble 1995), smaller

boats and airboats can sample much shallower habitats and may be better suited for wetlands that lack large boat launch facilities. Typical electrofishing boat units consist of large generators and boom-mounted anodes mounted on custom built hulls (Reynolds and Kolz 2012) or airboats (Chick et al. 1999, 2004).

Boat electrofishing units often use the hull as the cathode, although chains or other submerged wires can also be used. Anodes are attached to the end of one or two booms mounted on the front of the boat, which creates a large effective area for collecting fish. Two-boom electrofishing units usually have two netters, one of which should operate a foot pedal safety switch. The boat driver, often the most experienced crew member, should also have access to a safety switch either on the pulsator or mounted on the boat. The boat driver and the netters should be in constant communication regarding potential hazards (e.g., low hanging branches, underwater obstructions), and slow electrofishing speeds will minimize risks to crew and equipment.

Boat electrofishing has all of the advantages and disadvantages listed in Sect. 6.3.2.3 with several additional considerations. It is important not to try and quickly cover a large area, as fish vary in their response time and susceptibility to netting, particularly under turbid conditions. However, slower boat speeds may increase the risk of harm to shocked fish from continued exposure to electrical current (Snyder 2003). Specific guidelines regarding electrofishing protocols are problematic given the range of habitats, water chemistry conditions, and fish species encountered in freshwater wetlands. However, familiarity with the potential electrical outputs of the electrofishing unit combined with preliminary assessments of unit performance will allow researchers to balance the pace of sampling with appropriate power settings to minimize fish injury. In addition, despite additional power generation in boat electrofishing units, electrical fields may still be small in soft substrate, low specific conductance wetlands. In these habitats, we have found that electrical fields generated at the highest practical settings are effective to a maximum depth of 2.5 m. Importantly, the large generators and strong electrical currents used with boat electrofishing make it among the most hazardous sampling gears (see Sect. 6.2.8).

Boat electrofishing can be conducted with continuous or point abundance techniques (Lapointe et al. 2006) described in Sect. 6.3.2.3. Generally, continuous sampling occurs along shorelines in the littoral zone, although in shallow wetlands, open water sampling may also be effective, particularly for pelagic taxa such as shad *Dorosoma* spp. Shocking effort is typically standardized by time (e.g., 900 seconds (s) on-time for continuous runs, repeated 60 s on-times for point sampling). Recent integration of point abundance sampling with historical continuous sampling methods in the Atchafalaya River Basin indicate that the combined methods increase species richness in the samples and better represent fish community composition in complex habitats that include open water, floating and submerged macrophytes, and woody debris (MDK and WEK, unpublished data). We recommend trying both methods during preliminary studies to determine the best method(s) for collecting fish to address the study objectives.

6.3.2.7 Use of Pop and Lift Nets in Freshwater Non-wadeable, Habitats Connected to Permanent Water Bodies

Although not commonly used to sample wetland fishes, pop nets and lift nets have an advantage of reduced fish avoidance and have been found to provide quantitative density estimates for a number of taxa (Bagenal 1974; Hewitt 1979; Larson et al. 1986). Pop nets (and electrified pop nets; Petering and Johnson 1991) sample water-column dwelling fishes in a volume of water defined by the dimensions of the net. The net, which often resembles a conical plankton net, is attached to a floating frame and is submerged and held on the bottom by a weighted release mechanism prior to sampling (Bagenal 1974; Larson et al. 1986; Dewey et al. 1989). The release mechanism allows the net to be set, left undisturbed for a period of time, and then remotely triggered so that it collects fish as it rises to the surface. Lift nets (Higer and Kolipinski 1967; Rozas 1992; McIvor and Silverman 2010) are attached to poles or other structures that allow the net to be lifted from the bottom to enclose shallow-water organisms. Nets can be constructed with bottoms, so that the net is simply placed on the substrate, or can be open on the bottom, in which case the side netting is buried in trenches dug into the substrate. Targeted fishes are isolated in a known sampling area when the sides are raised (Rozas 1992; McIvor and Silverman 2010) and can be collected by lifting the net (with bottom), or with dip nets or small seines in bottomless nets. Lift and pop nets provide quantitative estimates of fish density and relative abundance (Hayes et al. 2012), and probably deserve wider use in situations with wary fishes or underwater obstructions (e.g., cypress knees, mangrove roots) that would preclude the use of other gears. Although these nets sample small areas, necessitating high levels of replication for sparsely distributed or uncommon fishes, they share the advantages of high portability (Connolly 1999), low cost, and effectiveness in densely vegetated habitats (Dewey et al. 1989). They may create bias by creating structure on the substrate that could attract some species causing them to be over-represented in the final collections.

6.3.2.8 Use of Throw Traps in Brackish or Estuarine Wadeable and Seasonally or Daily Inundated Habitats

A throw trap is an active and quantitative gear that functions by quickly enclosing a discrete volume of water, which is then sampled with dip nets or small seines to collect fishes within the enclosure (Fig. 6.7). Because enclosure sampling has been used extensively to collect amphibians (e.g., Mullins et al. 2004), invertebrates (e.g., Waters and Knapp 1961; Turner and Trexler 1997; Dorn et al. 2005), and fishes (e.g., Kushlan 1981; Jacobsen and Kushlan 1987; Rozas and Minello 1997; Steele et al. 2006), the sampling characteristics of these gears are well understood. Throw traps can be round or square enclosures open at the top and bottom that are usually less than 100 cm in height and enclose areas of 0.25–4 m^2. They can be solid structures made of light weight material, such as aluminum, or can be a mesh

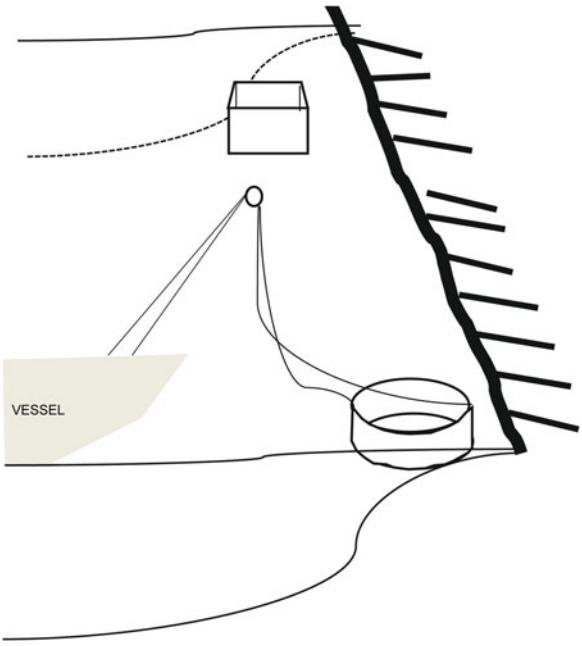

Fig. 6.7 Throw (*top*, *square*) and drop (*bottom*, *round*) traps enclose areas for quantitative sampling

cylinder fitted with a weighted bottom frame and a floating top frame (Wegener et al. 1973; Miller and Guillory 1980). Smaller throw traps often need more replication than larger throw traps to describe diversity or abundance (Krebs 1999; Steele et al. 2006), and balancing portability and the potential for increased replication is an important consideration in the selection of throw traps size.

In addition to known area and volume, throw traps have additional advantages of relatively low cost, high portability, rapid deployment, low personnel requirements, very low fish mortality, and high efficiency in open or densely vegetated habitats (Jacobsen and Kushlan 1987; Rozas and Minello 1997; Steele et al. 2006; Troutman et al. 2007). Conversely, throw trap effectiveness can be limited by depth, inadequate sealing on uneven or vegetated substrates (allowing fish escapement), and fish size (throw traps are most effective at capturing smaller individuals; Jordan et al. 1997). Many fishes are wary of avian and other over-head predators (e.g., Giles 1984; Angradi 1992; Malavasi et al. 2008), and caution should be taken to minimize shadows that are cast when the trap is thrown (Miller and Guillory 1980). Jacobsen and Kushlan (1987) noted the effective sampling area of a throw traps was smaller than its area, which may be an important consideration during interpretation of results.

Prior to sampling, the number of deployments in targeted habitat(s) should be determined based on desired levels of precision (more precision usually requires more throws), expectations of fish density (greater density usually requires fewer throws), and trap size (smaller traps would typically require more throws). Throwing techniques require practice to ensure that a high proportion of deployments sink

and seal properly on the bottom. The number of dip net/seine sweeps required within the deployed trap to adequately estimate abundance and diversity appears to be related to fish density (Steele et al. 2006), and complete removal of all fishes may not be necessary (i.e., depletion sampling may be adequate, Sect. 6.4.2).

6.3.2.9 Use of Drop Traps in Brackish or Estuarine Non-wadeable Habitats

Drop traps and drop nets (e.g., Kjelson et al. 1975; Beesley and Gilmour 2008) are another form of active, quantitative enclosure samplers (Sect. 6.3.2.8) that have been used with insects (e.g., Mason and Blocker 1973), crocodiles (e.g., Webb and Messel 1977), and other wildlife (e.g., Ramsey 1968). Drop traps are usually larger versions of throw traps, consisting of bottomless cylindrical or square enclosures of 1–2 m area and 1–1.5 m height constructed of fiberglass or aluminum (Kahl 1963; Kushlan 1974; Zimmerman et al. 1984; Fig. 6.7). Drop traps and nets can be suspended above the water surface by ropes attached to pulleys mounted on a fixed structure, on floating structures, or from an arm projecting from the front of a boat. All variations have trigger releases that allow the drop trap or net to fall into the water. Drop traps can be effective in sand-bottom habitats (Evans and Tallmark 1979), and have been widely used in the Florida Everglades (e.g. Kushlan 1974, 1981; Lorenz et al. 1997) and saline and freshwater marshes of the northern Gulf of Mexico (Rakocinski et al. 1992; Roth and Baltz 2009; Piazza and La Peyre 2009), where they are considered particularly useful for sampling submerged vegetation (Rozas and Minello 1997).

Similar to throw traps, drop traps have the important advantage of known sampling areas and volumes, and their greater weight and vertical deployment often improves proper contact with the substrate. They have compared favorably with other sampling gears in direct comparisons (Table 6.1) and in meta-analyses of fish community composition (Rozas and Minello 1997). However, drops traps can be expensive, drop sampling is a relatively slow process, and deployment and fish collection tend to disturb the surrounding habitat, requiring movement to new locations or a substantial time period prior to re-deployment. However, the design of the drop trap, boat, and crew experience may mitigate these disadvantages (see Lorenz et al. 1997).

A standard acclimation period should be used after maneuvering the trap net into position; (Kjelson et al. 1975) recommend waiting 10 min between erection of the drop trap structure and sampling. Similar to throw traps, target fishes may be wary of over-head movement (e.g., Giles 1984; Angradi 1992; Malavasi et al. 2008), and caution should be taken to minimize shadows cast during movement of the trap and the acclimation period. When the drop trap has been deployed, fishes can be collected with dip nets, or the enclosed volume of water can be pumped out, with fish collected in dip nets or plankton nets attached to the pump (Rakocinski et al. 1992; Roth and Baltz 2009). If the trap is made of impermeable substance, such as parachute cloth, it may be possible to use rotenone to facilitate fish removal; the rotenone can be neutralized with potassium permanganate before removing the enclosure (Lorenz et al. 1997).

6.3.2.10 Use of Weirs and Flumes in Brackish or Estuarine Non-wadeable Habitats

Weirs are among the oldest techniques for collecting fish in moving water, and have historically been employed to capture fishes during spawning migrations (Hubert et al. 2012). McIvor and Odum (1986) and Kneib (1991) described applications of weirs in tidally-influenced coastal marsh habitats, with fish captured as they moved through marsh channels. Weirs are usually constructed of stone, wood, or netting (typically called flume nets) and, much like fyke nets, are designed to guide fish into a collecting area for removal. The 20-m long by 1.5-m wide flume nets described by (McIvor and Odum 1986) have been used in several studies (Rozas and Odum 1987b, c; Kimball et al. 2010), but net size depends on the fishes of interest and the morphology of the channels being sampled, and wider and shorter nets have been effective (Rountree and Able 1992; Peterson and Turner 1994). These nets can be designed to fish uni-directionally or with two open ends to sample fishes moving in both directions, e.g., movement onto and off of the marsh during a tidal cycle (Peterson and Turner 1994). Although often constructed to intercept all fishes moving along a channel, weirs can effectively describe the movements of marsh fishes even when only part of the channel is sampled (Kneib 1991).

Weirs share many of the same sampling features as entrapment nets, including very low mortality and reduced size selectivity. Although somewhat complicated to deploy, they can be designed to be portable for short-term studies (Kimball et al. 2010), but can also be constructed for long-term sampling. Weirs are effective in dense vegetation (McIvor and Odum 1986; Kneib 1991) and are well suited for sampling large areas (Peterson and Turner 1994; Connolly 1999), largely because of the mobility of many marsh fishes (Kneib 1997). Although generally considered resistant to fouling (McIvor and Odum 1986), weirs that are deployed for extended periods of time are vulnerable to weather hazards and damage from debris (Hubert et al. 2012). McIvor and Odum (1986) suggested that weir sampling was not practical in highly fragmented marshes. In addition, weirs suffer the same biases against sedentary and for cover-oriented fish as entrapment nets (Sect. 6.3.2.5).

6.3.2.11 Use of Trawls in Brackish or Estuarine Non-wadeable Habitats

Although shallow and structurally complex wetland habitats often require passive and surface-oriented active gears to sample fish, marsh channels, sea grass beds, open wetland lakes, and deeper estuarine areas may be amenable to trawling (Guest et al. 2003; Martinho et al. 2008; Selleslagh and Amara 2008). Trawls are framed (beam trawls) or unframed (otter trawls) conical nets that are usually towed along the bottom at speeds up to 2 m/s. This makes trawls particularly effective for benthic species, but in shallow habitats, trawls can be designed to fish nearly the entire water column. Depending on boat design and the size of the net, trawls can be fished behind the boat, or to the side of the boat on outriggers. Trawl efficiency can vary among species and size classes depending on trawl design (width, height), mesh size, boat

speed, and sampling location (see Rozas and Minello 1997; Hayes et al. 2012), but they can sample a large area in a short period of time, and can sample a diversity of taxa (Allen and Herbinson 1991; Able et al. 2001; Rotherham et al. 2008).

As the name implies, beam trawls rely on a fixed frame to hold the mouth of the net open. The net is typically attached to top and side frames, with the side frames also functioning as runners to facilitate movement along the substrate (e.g., Reiss et al. 2006). Chains can also be attached between the runners to flush benthic taxa off the bottom and into the net. Beam trawls are quite adaptable as a sampling gear in terms of size, shape, and mesh configuration, and may be particularly effective for certain taxa, such as juvenile flatfishes (Carlson et al. 2000; Le Pape et al. 2003). Otter trawls are frameless nets, the sides of which are attached at the top and bottom to lines that extend to the otter boards, which are connected to the towing cables at such an angle that they exert downward and outward force as the trawl is pulled through the water. Although otter trawls can be easier to use than beam trawls because the boards and net collapse upon retrieval, the net opening can change dimension during sampling depending on trawl speed and the amount of material in the net (Koenig and Coleman 1998), and they may be strongly biased against larger fish (Rulifson 1991). Even with these sampling deficiencies, both beam and otter trawls have been used extensively to sample juvenile and adult fish in shallow water habitats, and may yield data on specific taxa that are not sampled effectively with other gears.

6.3.3 Calibration, Bias, and Efficiency of Wetlands Fish Sampling

Under typical conditions, all sampling methods are subject to some form of inefficiency and bias; estimating, controlling, and adjusting for these issues in the final data, if possible, is desirable of all fisheries studies (Hamley 1975). Efficiency is a measure of the presence of a species, a size class, or an age class in samples relative to its actual abundance in the wetland. Two sources of inefficiency are typical in fisheries data: efficiency of capture or entrainment by sampling devices and efficiency of clearing or counting fish once captured or entrained. For example, in an enclosure sampler, some fraction of fish in the sampled area escape the trap before it seals, and only a fraction of those captured are removed and counted (Rozas and Minello 1997; Jordan et al. 1997). The final number collected is a fraction of those fish from total number in the area sampled and is equal to the product of the proportion captured (capture efficiency) and the proportion of those captured that are removed and counted (clearing efficiency). Bias arises from systematic under or over representation of species, size classes, or age classes relative to their presence in the environment (Brown et al. 2012). For example, capture efficiency is commonly different between day and night sampling (e.g., Pierce et al. 2001), therefore combining such data without adjustment will yield bias. Similarly, some species are more efficiently sampled than others, yielding biased estimates of community composition (e.g., Lyons 1986; Parsley et al. 1989). In practice, such bias is unavoidable, but can be

controlled if standardized throughout a study by use of consistent, standardized methods (Bonar et al. 2009). This is particularly important when calibration studies are impractical and bias is unknown; it is prudent to assume bias and inefficiency is present unless demonstrated otherwise. Since a 'true' density of fish is seldom known in field conditions, some approximate measure of density is required for calibration. Calibration is accomplished by identifying a correction factor for data gathered relative to a standard (e.g., Bayley et al. 1989; Chick et al. 1999). Treatment with rotenone within an area surrounded by block nets is oft used standard (see Bayley et al. 1989; Chick et al. 1999), and where permissible, offers a very close to 'true' density. Regardless of the calibration method, calibration can offer insights into capture and clearing efficiencies.

Electrofishing has several important biases that should be addressed including fish size and species (see Sect. 6.3.2.3). Electrofishing has an additional source of bias that is unlike many of the sampling methods in the previous sections, which is dependence on the visual detection of fishes during sampling. Conceptually, visual detection is much like the clearing efficiency of a fish trap or enclosure in that during electrofishing, some fraction of fish are never detected in the sampled area resulting in sample bias (i.e., when block nets are deployed and multiple electrofishing passes occur, electrofishing capture efficiency is assumed to be close to 100 % because fish escape from sampling area is impeded and avoidance of electrical current is very difficult, thus, errors in visual detection are considered analogous to clearing efficiency). Water clarity, crew experience, fish coloration (e.g., cryptic species), and rapid sampling speed all may reduce visual acuity and the probability of observing the fish (i.e., detection probability), which decreases confidence in estimates of relative abundance, density, or CPUE. Further, because size and species biases may synergistically confound visual detection (e.g., small, cryptic fishes are difficult to observe and difficult to shock), error introduced by these biases has been traditionally unmeasured and undocumented (i.e., error was unknown because the sampler did not know that the fish was missed). As such, this error could not be accounted for in subsequent studies because it is specific to fish species, fish sizes, field conditions, and crew experience. Standardized methods, as well as, mark-recapture and removal methods (Sect. 6.4.2; Hayes et al. 2007) reduce these biases by accounting for fish capture histories and catchability; however, the biases are never completely eliminated.

Three other options exist to describe errors in detection or clearing efficiency during electrofishing. One could conduct a calibration study comparing electrofishing against a standard, such as rotenone treatment of an enclosed area (see Chick et al. 1999), or in a comparable location where a complete census may be conducted (e.g., a pond that may be drained by a water control structure). Alternatively, electrofishing shares many sampling characteristics with other visually-based sampling methods, such as point counts and distance-based transect counting methods, and solutions developed for these terrestrial sampling methods, such as sightability functions (Steinhorst and Samuel 1989) and detection probability estimation (McKenzie and Kendall 2002) can be applied to electrofishing data (e.g., Hayer and Irwin 2008; Peoples and Frimpong 2011). One method is to estimate detection probabilities from multiple sampling events in a given habitat (see Box 6.1), which

Box 6.1: Detection Probability Example

Estimation of detection probabilities can help understand the confidence one may place on estimates of relative abundance or density. Estimation of detection probability generally assumes that detection follows a logistic function bounded by 0 (no detection) and one (detection). Therefore, for a two pass removal, adapted from (McKenzie et al. 2006), the detection probability of a given species depends on its capture history by iterative solving of

$$\begin{aligned}\mathrm{Ln(L)} = {} & C_1 * [(e^{(b00*psiint)}/(1+e^{b00*psiint})) * (e^{(b0*pint)}/(1+e^{(b0*pint)})) * (e^{(b0*pint)}/(1+e^{(b0*pint)}))] \\ & + C_2 * [(e^{(b00*psiint)}/(1+e^{b00*psiint})) * (e^{(b0*pint)}/(1+e^{(b0*pint)})) * (1-(e^{(b0*pint)}/(1+e^{(b0*pint)})))] \\ & + C_3 * [(e^{(b00*psiint)}/(1+e^{b00*psiint})) * (e^{(b0*pint)}/(1+e^{(b0*pint)})) * (1-(e^{(b0*pint)}/(1+e^{(b0*pint)})))] \\ & + C_4 * [(e^{(b00*psiint)}/(1+e^{b00*psiint})) * (1-((e^{(b0*pint)}/(1+e^{(b0*pint)}))) * (1-((e^{(b0*pint)}/(1+e^{(b0*pint)})))))]\end{aligned}$$

where C_1 has a value of 1 if a species was detected in both removals and a 0 otherwise, C_2 has a value of 1 if a species was detected in only the first removal and a 0 otherwise, C_3 has a value of 1 if a species was detected in only the second removal and a 0 otherwise, C_4 has a value of 1 if a species was not detected in both removals and a 0 otherwise, b0 is the detection probability (i.e., the probability of visually detecting an animal that was actually present), b00 is the site occupancy probability (i.e., the probability that an animal was present, whether detected or undetected), pint is the intercept value for the detection probability, psiint is the intercept value for the site occupancy probability, and ln (L) is the log-likelihood value for determining the best value for b0 with higher values indicating the best b0.

Begin with 1 for b0, b00, pint, and psiint and solve for L. The process changes the value of b0 iteratively until L is maximized. A spreadsheet or computer program will greatly speed the process.

For example, if a largemouth bass was collected only on the second removal, we would start by setting b0 to one and decrease the value to 0 in 0.01 increments:

$$\begin{aligned}-1.966 = {} & 0*[(e^{(1*1)}/(1+e^{(1*t)})) * (e^{(1*1)}/(1+e^{(1*1)})) * (e^{(1*1)}/(1+e^{(1*1)})) \\ & + 0 * [(e^{(1*1)}/(1+e^{(1*t)})) * (e^{(1*1)}/(1+e^{(1*1)}))*(1-(e^{(1*1)}/(1+e^{(1*1)})))] \\ & + 1 * [(e^{(1*1)}/(1+e^{(1*t)}) * (e^{(1*1)}/(1+e^{(1*1)} * (1-(e^{(1*1)})/(1+e^{(1*1)}))))] \\ & + 0 * [(e^{(1*1)}/(1+e^{(1*t)}) * (1-(e^{(1*1)}/(1+e^{(1*1)})) * (1-(e^{(1*1)}/(1+e^{(1*1)})))))]\end{aligned}$$

We would find that L is maximized at −1.69 when b0 is less than 0.01.

$$\begin{aligned}-1.69 = {} & 0 * [(e^{(1*1)}/(1+e^{(1*t)})) * (e^{(1*0.01)}/(1+e^{(1*0.01)})) * (e^{(1*0.01)}/(1+e^{(1*0.01)})) \\ & + 0 * [(e^{(1*1)}/(1+e^{(1*t)})) * (e^{(1*1)}/(1+e^{(1*0.01)})) * (1-(e^{(1*0.01)}/(1+e^{(1*0.01)})))] \\ & + 1 * [(e^{(1*1)}/(1+e^{(1*t)})) * (e^{(1*1)}/(1+e^{(1*0.01)})) * (1-(e^{(1*0.01)}/(1+e^{(1*0.01)})))] \\ & + 0 * [(e^{(1*1)}/(1+e^{(1*t)})) * (1-(e^{(1*0.01)}/(1+e^{(1*0.01)})) * (1-(e^{(1*0.01)}/(1+e^{(1*0.01)}))))]\end{aligned}$$

(continued)

Box 6.1 (continued)

This means that based on our two removals, the detection probability of largemouth bass in this wetland is very low (0.01). This makes sense when one considers that we did not collect a largemouth bass on the first pass. Usually, the first pass is the most efficient and collects the most fish. Therefore, in this wetland, we have low detection for bass. Of course, this example is over simplified. Typically, detection probabilities would be estimated following multiple sampling efforts (e.g., Peoples and Frimpong 2011) to provide more realistic estimates.

can provide insight into the relative confidence of CPUE, relative abundance, or density estimates. For example, if CPUE for largemouth bass was reported as ten fish per hour with a detection probability of 0.90, one would have higher confidence in the CPUE estimate than if the detection probability was estimated at 0.50. Another method is to adjust relative abundance, density, or CPUE estimates based on detection probability estimates (McKenzie et al. 2006). Detection probabilities can be calculated by hand or in a spreadsheet, with PROGRAM PRESENCE (http://www.mbr-pwrc.usgs.gov/software/presence.html), PROGRAM MARK (http://warnercnr.colostate.edu/~gwhite/mark/mark.htm), publically-available MS EXCEL spreadsheets (http://www.uvm.edu/rsenr/vtcfwru/spreadsheets/occupancy/occupancy.htm), or with SAS/STAT or R. However, both detection probability methods rely on either at least one detection of a fish species during a sampling period or knowledge that the fish species could occur in the given habitat, based on prior sampling or other gear. Another method would be to employ a state space modeling approach (e.g., Dennis et al. 2006). State space models use repeated sampling to estimate observational error separately from what is termed process error, which is the error related to the parameters of interest (e.g., time or environmental factors). State space models are much more demanding of data and require a substantial time series of sampling. A full discussion of calibration, detection probability estimation, and state space approaches is beyond the scope of this chapter, and we suggest references herein for further reading. It should be noted that extremes in conductivity, high turbidities, or fishes that are not susceptible to electrofishing may result in biases that none of these methods may rectify. In these situations, sampling biases may preclude the use of electrofishing, and other gears should be employed.

Capture efficiency and bias are important concerns in interpreting data obtained from traps. Encounter traps such as minnow traps, gill nets, and fyke nets are subject to bias by temporal, spatial, or species-specific variation in movement rates because these methods depend of fish to enter them to be collected (Fago 1998; Prchalova et al. 2008; Obaza et al. 2011). Fish movements often change with time of day, season, weather, prey movements, and the presence of predators (i.e., decreasing movements of bluegill in the presence of largemouth bass; Savino and Stein 1989), all of which could increase or decrease susceptibility to a passive gear. In enclosure traps, fish capture efficiency may be a function of the water depth, water clarity, and habitat

complexity sampled (Chick et al. 1992; Loftus and Eklund 1994; Jordan et al. 1997; Rozas and Minello 1997). Chick et al. (1992) found little difference in capture efficiency with different size and shape throw traps ranging from 2.27 to 1.0 m^2, but recommended a 1-m^2 trap because of efficiency of sample processing with the smaller trap. Jordan et al. (1997) found little bias in capture efficiency across a range of emergent plant stem density in the Everglades (18–677 stems/m^2). They estimated capture efficiency at 80 % and clearing efficiency as 83 %, for a total under-estimate of 66 % of fish actually present (20 % of fish were not caught in the trap and 17 % of those caught were not removed). Use of throw traps in emergent plants densities exceeding those examined is subject to increasing inefficiency, while very low plant cover and clear water are also problematic because fish see the sampler and leave before a sample can be collected.

Many studies require repeated sampling in a study area to track community changes through time. This creates the potential for sampling bias through changes in the habitat complexity by repeated visits or alteration of the community itself by removal without replacement. Wolski et al. (2004) estimated the impact of repeated sampling in marshes by comparing long-term sampling plots to nearby areas where no sampling had been conducted. These authors discussed a trade-off in study design between distributing samples in space, potentially combining data from more than one target population, and repeating samples in space, potentially altering the target population through habitat destruction. Preliminary studies documenting scaling effects on sampling variability should be used to arrive at a sampling design seeking to minimize such bias. However, long-term studies are challenging because of uncertainty in how future conditions and environmental perturbations will affect spatial scaling patterns.

6.3.4 Sampling Wetland Fish Larvae

The relative effectiveness of active and passive gears that can be used in wetland habitats to collect larval fish depends on larval behavior as well as the physical structure and physicochemical characteristics of the habitat, particularly depth, water clarity, and specific conductance (Kelso et al. 2012). As it affects sampling design and gear choice, larval behavior mostly involves interspecific and ontogenetic differences in movement, habitat specificity, and phototaxis. Some littoral species spawn in shallow water but move to pelagic habitats for a period of time (e.g., bluegill *Lepomis macrochirus*; Werner 1969), making them vulnerable to plankton nets that are pushed in front of, or towed beside or behind a boat. Ichthyoplankton nets typically range up to 1 m in diameter, but smaller-diameter nets from 0.3 to 0.5 m, although sampling less volume per unit time, may be more maneuverable and effective in shallow water. Net mesh depends on the size of target larvae, but most nets employ mesh sizes of 200–500 μm. In order to minimize the effects of net clogging and reduced net efficiency in turbid water, it is important to select the largest mesh size that will collect the taxa of interest. Mounting flow

meters inside and outside the net will allow estimation of filtered volumes as well as filtering efficiency (e.g., Smith et al. 1968).

In contrast to fishes that occupy open water habitats as larvae, some species remain closely associated with cavities (e.g., catfishes *Ictalurus* spp.) or other submerged structure (e.g., vegetation, bowfin *Amia calva*) during early development (Ross 2001). Although these species may not be captured by towed nets and traps until they leave the nest, they can often be collected with artificial nesting structures and may be vulnerable to dip nets, suction devices, or electrofishing gear depending on depth, substrate, and water clarity (Kelso et al. 2012). An additional behavioral consideration in sampling fish larvae is phototaxis, or movement towards or away from a light source. Many taxa are positively phototactic, and light traps can be effective in shallow habitats (Niles and Hartman 2007). However, light responses vary among species, as well as larval developmental periods (Bulkowski and Meade 1983; Gregory and Powles 1985). These differences in light responses suggest that light traps may be most appropriate for determining species presence/absence.

Several other types of gears have been used to collect larval fishes depending on specific habitat characteristics. Fine-mesh seines (Scheidegger and Bain 1995), pop nets (Paradis et al. 2008), drop nets (fishing down through the water column; (Rowe and Taumpoepeau 2004), dip nets (Talbot and Able 1984), and electrified dip nets (King and Crook 2002) have all proven to be effective larval fish gears, and might be particularly useful under turbid conditions when light trap effectiveness would be limited. Electrofishing units modified for fractional or point abundance sampling have also been employed to collect larvae and juveniles in shallow floodplain habitats (Copp and Penaz 1988). It is important to remember that although larval density is relatively easy to calculate with towed nets, it is more problematic (and likely less accurate) to determine with most other gears, i.e., it is difficult to sample a pre-determined area or volume of water and to maintain constant effort in structurally complex habitats. Under such circumstances, larval presence/absence and CPUE can be recorded, with statistical comparisons of intraspecific relative abundance or density patterns between habitats and time periods. Interspecific comparisons may also be possible, but will depend on the relative susceptibilities of different species to the gear(s) being used.

6.3.5 Additional Sampling Techniques

Most of the sampling methods outlined so far have been used extensively to collect fishes in a diversity of wetland habitat types under variable physicochemical conditions. These additional techniques have proven to be successful under specific circumstances related to habitat, water quality, and study objectives. Although not as commonly used as other methods for studying wetland fishes, these techniques may provide data on abundance, behavior, and population characteristics that cannot be otherwise obtained.

6.3.5.1 Visual Sampling

Fishes have been counted and observed visually with video and acoustic cameras (Mueller et al. 2006; Meynecke et al. 2008b) as well as snorkeling and SCUBA gear, in both freshwater and saltwater habitats (e.g., Bobsien and Brendelberger 2006). Visual methods can be as effective as electrofishing for assessing fish abundance (Mueller 2003), and these techniques are highly effective for quantifying fish-habitat associations of both adult (e.g., Brosse et al. 2001) and juvenile (Searcy and Sponaugle 2001) fishes, as well as documenting fish behaviors under natural conditions (e.g., Stoner et al. 2008). Moreover, visual sampling is a good alternative when sampling must be minimally invasive to the habitat and have no fish mortality. Obviously, visual methods are highly dependent on water clarity, and highly turbid or stained water in many wetlands may preclude the use of a visual census. Conversely, if targeted wetlands are low in turbidity with substrates that are not easily disturbed by movement, visual sampling could be an effective sampling alternative (Thurow et al. 2012).

6.3.5.2 Explosives and Toxicants

Detonating cords (e.g., Metzger and Shafland 1986), and fish toxicants such as rotenone (Swingle 1954) have been used to sample fish in shallow waters, and are currently used for nuisance species control (e.g., Gresswell 1991; Dinger and Marks 2007; Hamilton et al. 2009; Finlayson et al. 2010). Although rotenone can provide a relatively unbiased sample of the taxonomic composition and size distribution of fishes in a given area, neither explosives nor toxicants have any real advantages over other fish collection gears, and both methods are highly regulated. Explosives can damage habitat, and toxicants, which often result in nearly 100 % mortality, can spread outside the intended sampling area, causing unintended mortality of other aquatic organisms (e.g., Dinger and Marks 2007; Hamilton et al. 2009; Finlayson et al. 2010). Although rotenone has been used in wetland restoration efforts, particularly for control of common carp *Cyprinus carpio* (Wilcox and Whillans 1999), we generally do not recommend the use of either method as a wetland fish collecting technique.

6.3.5.3 Fish Marking and Telemetry

Determination of fish population sizes and movement patterns with mark-recapture and telemetry methods are extensively covered elsewhere (e.g., Hayes et al. 2007; Rogers and White 2007; Pine et al. 2012), but several points may be important in the design of wetland fish sampling programs. Numerous minimally-invasive and low cost options exist for marking fishes. Fin clipping or fish branding, while relatively quick and easy, are less preferred techniques because of their associated risks of infection and mortality. Colored elastomer, alpha-numerically coded visible implant

elastomer (VIE) tags, coded wire tags (CWT), coded passive integrated transponder (PIT) tags, t-bar tags, bachelor button, Peterson discs, Carlin tags, dart tags, and spaghetti tags all offer low impact and low cost means of identifying individuals (Pine et al. 2012). We recommend the use of sub-cutaneous or internal tags, such as elastomer, VIE tags, PIT tags, and CWT because of their greater retention (up to 99 %; Hale and Gray 1998), low potential for infection or irritation (Buckley and Blankenship 1990), and reduced interference with fish activity (Scheirer and Coble 1991; Mourning et al. 1994; but see Catalano et al. 2001). If individual identification is not necessary, fish can be marked in large groups by feeding fish radioisotope-laced foods, or with oxytetracycline (OTC, immersion or in food), which leaves a permanent fluorescent mark on bony structures (Unkenholz et al. 1997).

Telemetry is a relatively expensive fish assessment method, but it is the most accurate method for determining spatial and temporal patterns of fish movement and habitat use, which is virtually impossible with other data collection methods. The decision of whether to use radio or ultrasonic transmitters will generally be dictated by the salinity or specific conductance of the water. Although ultrasonic transmitters are superior in brackish or saltwater habitats where attenuation from suspended solids reduces the effectiveness of radio signals (Cooke et al. 2012), complex habitats inhibit reception due to scattering of the signal. However, ultrasonic signals can be difficult to pick up in structurally-complex habitats. Radio transmitters are more easily detected and triangulated with hand-held antennae, but the effectiveness of these tags typically decreases with depth, even in fresh water.

The choice between internally (usually abdominal cavity) and externally attached transmitters is equally important. External mounts generally require less training and oversight than surgical implantation. Both attachment methods can negatively impact tagged fish through infection, reduced swimming ability, and increased predation (Ross and McCormick 1981; Adams et al. 1998; Sutton and Benson 2003). If internal implantation is desired, (Brown et al. 2011) and references therein outline best practices for the internal implantation of transmitters. Ideally, transmitters should not affect fish behavior or swimming capabilities, and general guidelines suggest that transmitters be between 2 and 5 % body weight in air (Jepsen et al. 2001, 2002) and should not exceed 12 % of body weight in air (Brown et al. 1999). Because transmitter life is directly related to battery size and weight, a balance must be struck between research needs and acceptable impact on the fish. Study objectives, transmitter size and battery life, size of the fish, and habitat characteristics should all be considered carefully in the design of a telemetry project.

6.3.5.4 Hydroacoustics

Several hydroacoustic methods have been employed to obtain fish abundance, movement, and habitat use data. One approach that has been used to assess movements of fishes in tidal creeks is a passive integrated transponder (PIT) system. With this technique, fish are implanted with PIT tags, and their movements are recorded as they move past stationary antenna detection arrays (Adams et al. 2006; Meynecke

et al. 2008b). Unfortunately, the detection range of PIT tags is small, which probably limits the usefulness of this technique to situations where fish move repeatedly through a confined channel. Recently, shallow-water, side-scan hydroacoustics has generated interest as a fish sampling method in wetlands and marshes sufficiently deep for wading and too saline for electrofishing (e.g., Gerlotto et al. 2000; Boswell et al. 2007, 2010; Tátrai et al. 2008). The use of echo-sounding, which is a bottom-oriented form of hydroacoustics, is a well-developed technology with roots in open water fisheries that stretch back nearly 80 years (Rudstam et al. 2012). In the last two decades, technological advances have allowed side-facing hydroacoustics to be employed in shallow water habitats. Hydroacoustics involves the interpretation of returned acoustical energy, the echo, from fishes (primarily the gas bladder) and other objects. Because fish return energy at certain ranges that are different than the surrounding water and substrate, debris and other objects can be filtered out, with the resulting data containing measurements of returned acoustical energy from fish encountering the beam. These energy measurements are then converted to fish lengths by target strength-fish length equations (Hartman and Nagy 2005), and can be converted to biomass as well (Boswell et al. 2008b). Hydroacoustic devices come in single, dual, or multi-beam models that offer increasing powers of discrimination and functionality in a variety of water depths and clarities. Dual-frequency units, such as the DIDSON™, offer imaging that can be used to identify fish types and even species in some cases, particularly for individuals over 10 cm total length. However, submerged vegetation, air bubbles in algal mats and bottom sediments, and other reflective objects can obstruct sonar penetration and limit the range of wetland habitats where these methods are applicable. Although initial equipment costs are high and intensive training is necessary, these methods offer promise as a non-invasive, non-lethal alternative to other fish collecting gears. Because of the expense and technical expertise required, hydroacoustic methods have received limited application in wetland fish studies. It is likely that hydroacoustic studies will become more common as the technology evolves and more researchers become familiar with its applications.

6.3.5.5 Hook and Line

At times, the more traditional gears recommended in this chapter may not be permitted, logistically feasible, available, or effective in capturing targeted fishes, and hook and line sampling may be the only reliable alternative. For example, in low-salinity Louisiana coastal wetlands that are not amenable to electrofishing, large alligator gar (*Atractosteus spatula*) are vulnerable to jug lines (passive, semi-quantitative; DiBenedetto 2009), which consist of baited hook(s) attached to a float with high pound test line and a steel leader. Hook and line sampling also includes trotlines (passive, semi-quantitative) and angling (active, semi-quantitative). Trotlines typically consist of a series of baited hooks on leaders (1–2 m in length) attached at intervals along a ground line, and can be particularly effective for benthic fishes catfishes, carps, and sturgeons. Angling with live or artificial baits is frequently used to capture fish for marking, attaching or implanting radio or

ultrasonic transmitters, or obtaining tissue samples. Angling data is not usually expressed as CPUE, although fish per day, or fish per 20 casts with a specific bait, etc., might be useful as a qualitative measure of fish abundance among habitats within or between wetlands. For jugline and trotline sets, CPUE is usually expressed in units of deployment time (e.g., fish per day) or gear (e.g., fish per 100 hooks). Depending on hook size and bait choice, hook and line methods can be highly size and species selective (e.g., Grixti et al. 2007), but may be the only alternative for the targeted species if other methods are unsuitable. Community composition and CPUE data generated by hook and line data should be considered qualitative. However, within species, hook and line sampling may yield quality data on length structure, age and growth, genetic composition, toxicology, and physiology (e.g., Shilling et al. 2010; Bacon et al. 2011).

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Laboratory Activities and Problem Sets

In the following sections, we offer field and laboratory activities and additional exercises to illustrate topics discussed in this chapter. Note that field fish sampling may require authorization or notification of local regulatory agencies. Please adhere to your institution's policies regarding animal care and use during these exercises.

Baited Versus Unbaited Traps

Goal: To determine the influence of bait type on potential sampling bias in minnow traps.

Overview: Passive sampling is quite popular for fisheries studies. However, biases may be introduced by gear type and method of deployment. This field or laboratory exercise examines biases that may occur as the result of sampling choices.

Supplies for field trip version: Minnow traps ($\geq$6), bait (commercial fish food and commercial fish attractant such as Berkley trout bait), rope, stakes (equal to minnow traps), standard aquarium dip nets (1–2) buckets, sorting pans (2–3), fish identification guides such as Freshwater Fishes of Virginia (Jenkins and Burkhead 1994).

Supplies for laboratory version of experiment: Aquaria, no larger than 10 gal or 38 L (6), hardware cloth (fine mesh) fashioned into minnow traps (cylinder 7–10 cm in diameter, 15–20 cm long, with 5 cm, or other dimension less than the diameter, openings), bait (as above), standard aquarium dip nets.

Methods for Field Trip Version

The evening before the planned trip, minnow traps should be baited and deployed in a nearby wetland. At least two minnow traps should be used for each treatment: unbaited; baited with commercial fish food; and baited with commercial fish attractant. All six minnow traps should be secured by rope to stakes driven into the banks and deployed along the shoreline a sufficient depth to cover the trap. Deployment and retrieval times should be noted. Students should empty the contents of each trap into individual holding buckets or directly into sorting pans if few fish are caught. Students should then identify and enumerate fish and estimate catch per unit effort (CPUE) as the number of fish of each species in a trap divided by the number of hours deployed. Next, students should calculate the arithmetic mean CPUE for each of the three trap treatments and answer the questions below.

Methods for Laboratory Version

Instructor(s) will stock three aquaria with similar densities of one or more species of fish obtained from a local wetland, bait shop or pet store. Instructors or students should build small minnow traps prior to the experiment with narrow openings sufficient for fish entry. Two minnow traps will be deployed during the laboratory period in three aquarium treatments: (1) one trap with fish food and one with fish bait; (2) one trap with fish food and one with no bait; and (3) one with fish bait and one with no bait. Time of trap deployment into the aquaria should be noted. Students will observe fish movement into minnow traps and record the species

and number of fish in each trap at the end of the laboratory period. CPUE will be estimated as the number of fish of each species in a trap divided by the number of minutes passed since the traps were placed into the aquaria (Hubert et al. 2012)

$$\text{CPUE} = \sum (n_1 + n_2 + \ldots n_i)/t_i$$

where n_1 is the number of fish of the first species, n_2 is the number of fish of the second species, n_i is the number of fish in the last species, and t_i in the number of minutes a trap was deployed. Students can calculate the arithmetic mean CPUE for each trap type (two traps each for commercial food, commercial bait, and unbaited). Students should then answer the questions below.

Questions for Reflection and Study

1. Which trap type exhibited the highest mean CPUE, combining all fish species that were collected? Did any single fish species differ from this pattern (i.e., did any species become trapped at a higher CPUE in another type of trap than the one that collected the most overall fish)? Did anything that you observed about the trap or bait suggest why CPUE was highest in this type of trap?
2. Do you believe that your experiment has evidence of trap bias? Did bait type matter? Explain and defend with your data.
3. Could your data be comparable with a minnow trap study that used another kind of bait? Why or why not?
4. Consult a regional fish guide, such as Freshwater Fishes of Virginia (Jenkins and Burkhead 1994), about life history and habitat requirements of the fish with the highest CPUE in each trap type. Does something about their life history or habitat requirements suggest why the fish was attracted to that trap type?

Jenkins RE, Burkhead NM (1994) Freshwater fishes of Virginia. American Fisheries Society, Bethesda, 1079 pp

Removal Sampling and the Influence of Increasing Sampling Effort

Goal: Introduce removal sampling and common estimators of population size associated with removal sampling.

Overview: In small, enclosed wetlands or wetlands where habitats may be segregated from the surrounding areas, researchers can use closed population methods to estimate fish population size. One historically popular method is population estimation by removal. In removal sampling, fish are collected and removed or held while additional fish are collected. A minimum of two collections are needed, and often additional collections are recommended because the

additional collections enhance the population estimate. This experiment may be conducted in the field by seining or electrofishing, whichever is available, or by dip net in the laboratory.

Supplies needed for a field version: Seine or backpack electrofishing unit (depending on availability and suitability given local conductivity), gloves and waders, long-handled nets (electrofishing only), buckets or large cooler or fish basket to hold fish, block nets (if sampling a small area of a larger wetland).

Supplies needed for laboratory version: table top, simulated fish (e.g., small plastic vials or packing peanuts) and a sampling "net" (e.g., an inverted shoe box).

Methods for Field Version

A small, enclosed wetland should be selected or a small area within a larger wetland should be enclosed by block net. The ideal area would be about one-eight acre, if block nets are deployed. Students should conduct 10-m quantitative seine hauls (Fig. 6.1) or 100-m electrofishing passes (Fig. 6.3). Ideally, no fewer than four hauls or passes should be conducted; however, if very few fish are caught on the second and third haul or pass, the fourth haul or pass may be omitted. At the end of each haul or pass, fish should be identified and enumerated and then transferred to a holding bucket, cooler, or basket, until the final haul or pass when all fish can be returned to the wetland. Students should record the number and type of fish in each haul or pass to answer the questions in this section.

Methods for Laboratory Version

A small table top should be covered with random patches of "fish", and the "sampler" should be given to a blindfolded student to randomly place on the table. After each sample is taken, the remaining "fish" should be collected and re-distributed on the table top. For best results, the sampler should probably be able to cover about 20 % of the table top. For each sample, students should record the number of "fish" and set them aside. After at least three samples, students should be able to answer the questions in this section.

Questions for Reflection and Study

The Zippin method is commonly used to estimate fish populations with two removals (Hayes et al. 2007). The Zippin method is a maximum likelihood method and differs from the regression based DeLurly method more frequently used in the past. The Zippin method assumes all fishes had equal vulnerability to being sampled by the selected gear, equal effort was expended for each sample, and the probability of capture (catchability) was equal for each sample. The Zippin method

also requires that the first sample yield more fish than the second sample. The Zippin method is

$$\text{N-hat} = n_1^2/(n_1 - n_2)$$

where N-hat is the estimate of the fish population, n_1 is the number of fish removed in the first sample, and n_2 is the number of fish removed in the second sample. When we estimate, we also desire to know the precision of the estimate. For the Zippin method, we can estimate the precision of the estimate by its variance

$$V(N) = n_1^2 n_2^2 (n_1 + n_2) \Big/ (n_1 - n_2)^4$$

where V(N) is the variance of the Zippin estimate, n_1 is the number of fish removed in the first sample, and n_2 is the number of fish removed in the second sample.

The Zippin method is not possible for more than two removals. For three removals, the following formula is used following Hayes et al. (2007) citing Junge and Libosvarksy (1965) as cited in Seber (1982)

$$\text{N-hat} = 6X^2 - 3XY - Y^2 + Y\sqrt{\left(Y^2 + 6XY - 3X^2\right)}/18(X - Y)$$

where N-hat is the population estimate, X is $2n_1 + n_2$ and Y is $n_1 + n_2 + n_3$. Again, it is always of interest to estimate variance, which is estimated by finding q or catchability first as

$$\text{q-hat} = 3X - Y - \sqrt{\left(Y^2 + 6XY - 3X^2\right)}/2X$$

where q-hat is the catchability estimate, X is $2n_1 + n_2$ and Y is $n_1 + n_2 + n_3$. Then, we estimate variance as

$$V(N) = \text{N-hat}(1 - \text{q-hat})\text{q-hat} \Big/ (1 - \text{q-hat})^2 - \left[t(1 - \text{q-hat})^2 \text{q-hat}^{(t-1)}\right]$$

where V(N) is the estimate of the variance of the fish population, q-hat is the estimate of catchability, and t is the number of removals.

1. Estimate the fish population size and variance by the Zippin method with the first two removals. Then estimate the fish population by the three removal method with the first, second, and third removals. Estimate catchability and the variance of the three removal population estimate.

   1a. Do the fish population estimates differ?
   1b. If smaller variance may be assumed to suggest greater precision, did adding another removal increase precision?
   1c. Given your experience sampling, do you think that adding another removal is worth the difference in precision?

For the field version only:

1d. Given that you do not know the actual number of fish present, do you believe that removal sampling provides a reasonable estimate of the fish present?
1e. Can you get insights from the variance? Defend your answer with your data.

For the laboratory version only:

1d. Show your estimates to the instructor, who will reveal the actual number. Which estimate was closer to the real number?
1e. Given your experiences, how confident are you that removal sampling may offer reasonable estimates of fish populations?

2. Another common assessment is to describe the taxonomic diversity of fishes in a wetland. One common and readily estimated measure of diversity is the Shannon index, also termed the Shannon-Wiener or Shannon-Weaver Index. The index ranges from a low end near one, which indicates low species richness (low number of species) and evenness (few species numerically dominate the community), to 3.5, which indicates high species richness (higher number of species) and evenness (numbers spread among the species). The Shannon Index is found by

$$H' = -\Sigma_i^s [p_i \ln(p_i)]$$

where p_i is the proportion of an individual species. For example, if 3 species are present in a sample at 35, 25, and 15 individuals, $H' = -\{[(35/75)\ln(35/75)]+[(25/75)\ln(25/75)]+[(15/75)\ln(15/75)]\} = -[(-0.36)+(-0.37)+(-0.32)] = 1.05$ suggesting low diversity of species and dominance by 1 species.

2a. Estimate H' for the first removal, then for the first 2 removals combined, and then for all removals combined.
2b. Does adding removals increase H'? What does this mean?
2c. Is H' sensitive to the number of removals?

Hayes DB, Bence JR, Kwak TJ, Thompson BE (2007) Abundance, biomass, and production. In: Guy CS, Brown ML (eds) Analysis and interpretation of freshwater fisheries data. American Fisheries Society, Bethesda, pp 327–374

Junge CO, Libosvarsky J (1965) Effects of size selectivity on population estimates based on successive removals with electrofishing gears. Zoologicke Listy 14:171–178

Seber GAF (1982) The estimation of animal abundance and related parameters. Blackburn Press, Caldwell, , pp 672

Additional Exercises

1. Suppose a fish manager is tasked to sample a wetland in order to determine its ecological health. She assumes that a wetland with high fish diversity (a Shannon

index above 2.5 for this small wetland) and high fish density (more than100 fish per acre) would indicate a healthy ecosystem. She conducts removal sampling by seine hauls in a 1-acre enclosed portion of the wetland. The hauls produced 56 fish, 32 fish, and 13 fish. The hauls produced 9 emerald shiners, 7 fathead minnows, 5 common carp, 9 largemouth bass, 4 bluegill, 7 pumpkinseed sunfish, 9 green sunfish, 3 sand shiners, 6 warmouth bass, 5 chain pickerels, 3 golden shiners, 9 Johnny darters, 9 white crappies, 5 brown bullheads, and 11 yellow bullheads. Use the estimators given in Sect. 6.4.3 to determine if the wetland would be considered healthy.

2. Suppose the same fish manager in question 1 visited a second wetland. This time, the hauls produced 39 fish, 41 fish, and 18 fish. Use the estimators given in Sect. 6.4.3 to determine if the wetland would be considered healthy. The hauls produced 19 emerald shiners, 7 fathead minnows, 5 common carp, 1 largemouth bass, 1 bluegill, 8 pumpkinseed sunfish, 19 green sunfish, 3 sand shiners, 3 warmouth, 2 chain pickerels, 3 golden shiners, 2 Johnny darters, 2 white crappies, 12 brown bullheads, and 11 yellow bullheads. Use the estimators given in Sect. 6.4.2 to determine if the wetland would be considered healthy.
3. A wetland manager decided to sample a large wetland with boat electrofishing and gill nets. The electrofishing sampled for 45 min and collected 100 fish of 7 species. The gill nets soaked for 12 h and collected 292 fish in 11 species. Which gear exhibited the higher CPUE? Why might the electrofishing have collected fewer species than the gill net?
4. A wetland manager sampled a boat-accessible wetland with point abundance electrofishing and a drop sampler. The point abundance electrofishing sampled 900 s in five 100 m^2 areas surrounded by block nets, yielding 14, 25, 9, and five fish. The drop trap of 1 m^2 was deployed 10 times yielding 1 fish, 0 fish, 3 fish, 0 fish, 0 fish, 1 fish, 0 fish, 0 fish, 0 fish, and 2 fish. Estimate fish density per 1 m^2 for both methods. Which method estimated a higher mean fish density?

Chapter 7
Wetland Wildlife Monitoring and Assessment

Matthew J. Gray, Michael J. Chamberlain, David A. Buehler, and William B. Sutton

Abstract Monitoring wetland wildlife is complex and requires use of various techniques to obtain robust population estimates. Herpetofauna, birds and mammals frequently inhabit wetlands and adjacent uplands. Sampling herpetofauna can include passive techniques such as visual encounter and breeding call surveys, and capture techniques that use nets and traps. Common bird monitoring techniques include scan surveys, point counts, nest searches, and aerial surveys. Some mammals, such as bats, can be sampled with audio devices, whereas mark-recapture techniques are most effective for other taxa. For all groups, the techniques used depend on the monitoring objective and target species. This chapter describes various techniques for monitoring populations of wetland wildlife. If these techniques are incorporated into a robust sampling design, they can be used to document changes in species occurrence, relative abundance, and survival.

7.1 Introduction

Wetland wildlife (e.g., freshwater turtles, amphibians) are some of the most imperiled taxa in the world. Many species that use wetlands (e.g., waterbirds) have great economic and recreational importance. Thus, monitoring wildlife populations in wetlands is a fundamental component of management and conservation. Monitoring data can be used to document species distribution, estimate relative abundance, and track population change over time. Monitoring data also are useful in evaluating wildlife responses to management and conservation strategies. If monitoring data are collected using an

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unbiased sampling design, they can be used for making science-based, adaptive management decisions.

Monitoring wetland wildlife requires a combination of techniques to sample effectively the species that are present. Four wildlife groups that commonly use wetlands for portions of their life cycle include amphibians, reptiles, birds, and mammals. This chapter outlines standard procedures for sampling these animals, which includes passive methods, such as visual or auditory surveys, and techniques where animals are captured. Below, we describe some considerations for monitoring wildlife in wetlands.

7.2 Monitoring Considerations

The first step in selecting a monitoring technique is to identify the management, conservation, or research question (Witmer 2005). If determining species presence is the goal, techniques that target animal detection (e.g., call surveys, animal tracks) can be used. Techniques that produce count data (e.g., visual encounter surveys, scan sampling) can be used if estimating relative abundance is an objective. Lastly, if the goal is to develop a model that predicts population trends, a combination of techniques will be needed that estimate survival, reproduction, and relative abundance (e.g., mark-recapture). Sampling duration and costs typically increase from determining species presence to developing a predictive population model. Therefore, matching sampling techniques with the monitoring objective is key to ensuring appropriate data are collected considering the available resources (Witmer 2005).

Regardless of the technique used, rarely can 100 % detection of individuals present be ensured when sampling wetlands. Wetlands contain dense vegetation that aids in concealment, and many wetland wildlife are secretive or cryptically colored which reduces detectability. Detection also can be affected by observer experience, weather conditions, and time of day or year. Given that species detection is imperfect within a wetland, occupancy or relative abundance estimates can be biased depending on the sampling conditions or target species. Analytical techniques are available to correct for imperfect detection (see MacKenzie et al. 2006), and typically involve repeated sampling in a designated area. For very rare species, detection can be near zero, thus alternative sampling approaches (e.g., adaptive cluster sampling, Thompson and Seber 1996) may be needed. Sampling intensity and duration also should be considered, and correspond with monitoring objectives and animal life history. For example, breeding bird surveys should occur during late spring and early summer, whereas sampling for resident wildlife (e.g., rodents) could occur throughout the year. The goal of this chapter is not to discuss possible study designs (e.g., random, stratified, systematic, cluster) or analytical methods (e.g., occupancy modeling, calculating detection probabilities, Jolly-Seber population estimation) associated with monitoring wildlife populations, but to present techniques used to sample wetland wildlife. We recommend readers consult a statistician or biometrician to assist with designing an unbiased sampling

approach and analyzing monitoring data. Chapter 1 (Vol. 1) covered several of these basic sampling principles, and various classic texts exist for guidance (Williams et al. 2002; Montgomery 2005; McComb et al. 2010; Zar 2010). Additionally, we define statistical terms and concepts that are used in this chapter (Box 7.1).

Box 7.1

The accuracy of a given population estimator can be defined as how close the actual estimate is to the true population value. The precision of the estimator is defined as how much variability there is in the estimate, based on repeated sampling. Ideally, a monitoring method should produce accurate and precise estimates. The accuracy of a given method can vary based on the species being monitored and the local setting under which monitoring is occurring. The precision of the method is a function of the inherent variance associated with the technique used and sample size. Consideration also must be given to the area and time period being sampled to ensure that sampling is representative of the population parameter being estimated. A census technique is defined as any method in which the goal is to count all individuals in the population. An index of relative abundance is any method in which a parameter estimate is counted that is related to total population size. All population estimation techniques can be affected by bias, a measure of the difference between the expected value of a given population parameter and the true value. Bias can result from many potential sources, including effects related to the behavior of the species being targeted and the ability and experience of the observer. For most methods, there are three sources of variability for population estimates: spatial variability, temporal variability, and detectability. Spatial variability results because not all sites occupied by a given species have the same size, habitat configuration, and density. A sampling framework is needed (e.g., stratified approach), as a result, to ensure that the samples are representative of the areas being occupied by the target species. Temporal variability results because populations are dynamic and change by time of day, stage of the life cycle, and year. Sampling needs to account for the sources of temporal variability. Detectability can be defined as the probability a given individual will be detected by the observer given that it is present to be detected. Detectability can vary based on species, observer, weather, time of day, season, and habitat conditions. Wherever possible, estimates of detectability should be used to adjust indices of relative abundance to reduce bias.

Additional considerations during sampling include animal welfare and personal safety. In most cases, wildlife collection permits need to be acquired prior to sampling if animal capture is part of the study design. Further, capture, handling, and marking techniques may require approval by an institutional animal care and use committee. Some techniques described below (e.g., capturing snakes and bats)

can be dangerous and require training by an expert or immunizations. We recommend that novice biologists consult experts for initial hands-on training if dangerous wildlife will be handled. Many wetland wildlife species harbor zoonotic pathogens so standard biosafety precautions, such as wearing disposable gloves and disinfecting equipment, should be practiced. Biologists and researchers can contribute to pathogen pollution (i.e., introduction of novel pathogens into a population, Cunningham et al. 2003) while sampling wetlands by unintentionally translocating pathogens among populations on fomites. Thus, all sampling gear and footwear should be disinfected before moving among wetlands. A solution of 10 % bleach or 2 % Nolvasan® (chlorhexidine diacetate) with a contact time of 10 min will inactivate most pathogens.

7.3 Monitoring Herpetofaunal Populations

Herpetofauna include animals in the Classes *Amphibia* and *Reptilia*. Various species of amphibians and reptiles use wetlands and their adjacent terrestrial habitats. To obtain the most representative estimates of species occurrence, relative abundance, or other demographic indices, a combination of aquatic and terrestrial sampling techniques are typically required. Below, we summarize aquatic and terrestrial sampling techniques for amphibians and reptiles; some methods (e.g., funnel and pitfall traps) can be used for both groups. We also summarize techniques for marking herpetofauna for mark-recapture studies.

7.3.1 Amphibians

Most amphibian species in the temperate regions of the world have a complex life cycle that involves development in aquatic systems as larvae and in terrestrial systems as juveniles and adults (Wells 2007). Thus, sampling amphibian populations associated with wetlands typically involves a combination of techniques that target both ecosystems (Dodd 2009). Information collected in the wetland zone of aquatic systems typically addresses questions related to reproductive effort, larval production, and possible recruitment; whereas, data collected in the terrestrial environment provide information on survival and recruitment of juveniles, adult survival and population size, and dispersal. Techniques involve passive counts, capture methods, and marking individuals for survival and dispersal estimates. Below are some approaches for sampling amphibians in aquatic and terrestrial systems; we encourage readers to consult Dodd (2009) for additional details.

Fig. 7.1 Amphibian sampling techniques in the aquatic environment. Egg mass surveys for anurans (**a**) and ambystomatid salamanders (**b**), dip-net sampling (**c**), frog-call recorders (**d**), seine sampling (**e**), larval enclosure sampling (**f**), and aquatic minnow traps (**g**) (Published with kind permission of © Matthew Gray, William Sutton, and David Steen 2014. All Rights Reserved)

7.3.1.1 Aquatic Sampling

Many amphibian species oviposit floating egg masses or attach eggs to emergent or submersed vegetation in wetlands (Fig. 7.1; Wells 2007). Egg mass counts can be used as an index of adult population size and reproductive effort (Paton and Harris 2010). For some amphibian species (e.g., *Ambystoma maculatum*, Petranka 1998:80) females can lay multiple clutches, thus egg mass counts need to be adjusted if inferences are made on per capita reproduction or adult population size. Wells (2007:501) summarized information on average number of egg masses and clutch size for several anuran species, while Petranka (1998) provides information on oviposition strategies of salamanders. Egg mass identification can be done reliably to genus; species-level identification requires more experience and typically knowledge of breeding species and their phenology at a site. Most amphibian identification guides (e.g., Dorcas and Gibbons 2008; Niemiller and Reynolds 2011) and websites include photos of egg masses. Most egg masses are deposited in the littoral zone of wetlands in water that is <60 cm (Wells 2007),

thus sampling should focus in areas close to the water's edge. Some species prefer to lay egg masses amongst vegetation (e.g., *Pseudacris* spp.), whereas other species prefer more open water (e.g., *Lithobates catesbeianus*). Counts should be performed at least twice per year (i.e., spring and summer) to incorporate breeding phenology (Paton and Harris 2010). Amphibian movements and breeding tend to be associated with precipitation (Wells 2007), hence targeting sampling within 48 h of a rain event likely increases egg mass detection. If a site is sampled multiple times, egg mass counts can be adjusted for changes in detection associated with rainfall. Counts are typically performed along a transect or within a designated area, and search time and number of observers is recorded to standardize relative abundance estimates (Paton and Harris 2010). To estimate relative abundance per species, divide number of egg masses counted per species by the collective minutes searched for all observers then divide this quotient by the number of observers. This estimate can be compared among years and sites if egg mass detectability is similar.

Amphibian larvae in the temperate regions include frog tadpoles and salamander larvae. Tadpoles of various species can be found in wetlands; the most commonly encountered salamanders in North American wetlands belong to the Ambystomatidae and Salamandridae families (Wells 2007). The most common capture techniques include nets, traps and enclosure sampling (Skelly and Richardson 2009). Schmutzer et al. (2008) used a combination of seine nets in deeper water and dip nets in shallow water to sample larval amphibians. Typically, seine nets (0.48-cm mesh) are pulled over a specified distance for relative density estimates, and are most effective if emergent vegetation is absent (Fig. 7.1). Dip netting can be done at sampling points along transects that traverse the elevational gradient of the wetland (Schmutzer et al. 2008) or in random locations within the emergent vegetation zone (Fig. 7.1). For truly random sampling, a 1-m^2 grid should be overlaid on a geo-referenced image of the wetland in a GIS and cells randomly generated for sampling locations. Similar to egg masses, sampling can be standardized by recording the number of dips taken over a specified duration and the number of individuals that participate (Skelly and Richardson 2009). There are a variety of dip nets that can be used; however, we recommend one with a large opening (e.g., 40 $\times$ 40 cm) and deep net (>50 cm) with fine mesh (<0.25 cm). Dip nets should be plunged down into the water including the leaf litter and quickly scooped upward. Alternatively, nets can be dragged through the water for a specified distance. Net contents, which may include litter and substrate, should be carefully sorted to detect larvae. Dip nets can be destructive to habitat and cause injury to larvae if the net frame strikes them; thus, dipnetting may not be ideal for threatened species or frequent, long-term monitoring.

The most common type of trap used to capture amphibian larvae is a minnow trap, which contains two opposing funnels that taper inward (Fig. 7.1; Skelly and Richardson 2009). Larvae are naturally directed into the tunnel, and after passing through a small opening are unable to find the opening again. Minnow traps should be placed in shallow water with at least 10 % of it exposed to provide air if adult salamanders are captured and should be checked every 12–24 h (Skelly and

Richardson 2009). If minnow traps are placed in deepwater zones of a wetland, they should be tethered to a permanent structure (e.g., tree or stake at the edge of the wetland) to prevent the traps from sinking or floating away, and to facilitate relocation. Drechsler et al. (2010) describe the design for a modified funnel trap that has greater capture efficiency than traditional traps.

Enclosure samplers are either rectangular (box-type) or circular, and are designed to enclose a designated area for sampling (Mullins et al. 2004; Skelly and Richardson 2009). A very simple, circular enclosure can be created by cutting the bottom off of a 120-L garbage can (Fig. 7.1). Enclosures are placed into water with about 5 cm of the bottom sunk into the substrate and the contents netted. Nets should be small (20 × 13 cm) with fine-mesh and a sturdy handle; most aquarium nets are too flimsy. Nets are repeatedly dipped through the entire water column and surface area for a minimum of ten times (Werner et al. 2007). Dipping should cease after ten consecutive dips result in no captures (Werner et al. 2007). Similar to the other methods, enclosures can be randomly or systematically placed in the wetland. For all procedures, captured larvae can be placed in a holding container until they can be identified and enumerated. Dr. Ronn Altig has written several keys for the tadpoles of North America (Altig 1970, 1987), and collaborated in developing a U.S. Geological Survey website (http://www.pwrc.usgs.gov/tadpole/). Tadpole identification can be difficult and requires knowledge of unique combinations of the vent, spiracle, and eye positions on the body, oral disc morphology, and dentition. Petranka (1998) provides descriptions of most salamander larvae in North America.

7.3.1.2 Terrestrial Sampling

Postmetamorphic amphibians can use terrestrial habitat within a considerable distance from a breeding wetland. In a review by Semlitsch and Bodie (2003) of the core terrestrial habitat for 32 North American amphibians, they reported that amphibians used habitat within 159–290 m of their breeding site. Smith and Green (2005) also reported that 40 of 90 (44 %) amphibian species reviewed moved <400 m, with salamanders being less mobile in general compared to anurans. Most amphibians acquire food resources necessary for growth and survival, estivate and hibernate, and disperse between wetlands in the uplands (Wells 2007). Thus, sampling terrestrial systems around wetlands for amphibians is a fundamental component of population monitoring. As with larvae, it is recommended that multiple sampling methods are used to increase the likelihood of detecting all amphibian species (Ribeiro-Junior et al. 2008; Farmer et al. 2009).

One of the most common techniques used to document anuran species occurrence is advertisement call surveys. Advertisement calls are produced by adult males of most frog and toad species during breeding to attract females (Wells 2007). Anuran calls are unique among species, and most species can be reliably identified with practice. Several CDs are available with anuran calls from North America (e.g., Elliot et al. 2009). Calls can be recorded by observers or automated

recording devices that are deployed overnight (Fig. 7.1; Dorcas et al. 2010). Procedures for performing call surveys vary, but the most widely used approach in North America follows protocols outlined by the North American Amphibian Monitoring Program (NAAMP, http://www.pwrc.usgs.gov/naamp/). The NAAMP is composed of routes randomly located throughout North America where volunteers listen for breeding frogs at ten stations per route. The NAAMP protocol specifies that call surveys are performed between 30 min following official sunset and 0100 h. Surveys are performed for 5 min only, and during that time, all frog species heard are recorded along with an index of relative abundance (Burton et al. 2007). Several studies suggest that 5 min is adequate to detect most breeding anurans (Shirose et al. 1997; Gooch et al. 2006; Burton et al. 2007). A call index = 1 when calls from different males do not overlap, = 2 when calls overlap but individual males can be distinguished, and = 3 when calls overlap and individual males are indistinguishable (Burton et al. 2007).

Call surveys are inherently biased for most anuran communities if detection is not corrected, because acoustical properties, including sound power and call frequency, differ among species (Dorcas et al. 2010). Additionally, ambient conditions can impact detection positively or negatively (e.g., during rain events or windy nights, respectively; Dorcas et al. 2010). Observers also differ in their ability to detect species and record similar abundance (Burton et al. 2007). Thus, most experts recommend that call surveys should be used to document species occurrence only (Dorcas et al. 2010). Surveys should be performed at least once monthly from early spring through summer to encompass most of the anuran breeding season (Wells 2007). Performing surveys within 48 h following a rain event may increase the likelihood of species detection, because call frequency is correlated with precipitation in many species (Wells 2007).

More detailed information on adult population size and processes (e.g., survival, dispersal) can be measured using capture-recapture techniques. The most common capture techniques used in the terrestrial environment include: drift fences with pitfalls, artificial cover objects, funnel traps, and visual encounter surveys (Willson and Gibbons 2010). Drift fences can completely or partially enclose a wetland, or be constructed as single segments or an array (Fig. 7.2; Willson and Gibbons 2010). Drift fences can be made of various materials, but plastic erosion fencing with 60-cm wooden stakes tends to be least expensive and is easy to erect. Although more expensive than erosion fencing, metal flashing or hardware cloth is more durable – usually, erosion fencing is usable for 1–2 years only. The bottom of the fence should be buried to prevent amphibians from crawling underneath; soil from holes dug for pitfalls (discussed below) can be used to bury the fence. If the goal is to estimate adult breeding population size or number of metamorphosed juveniles produced, drift fences should be placed near the wetland. Gray et al. (2004) standardized drift fence placement at 10 m above the expected high waterline and parallel to the wetland. Single drift fence segments or arrays can be erected between wetlands or along terrestrial contours to identify movement corridors and estimate dispersal rates.

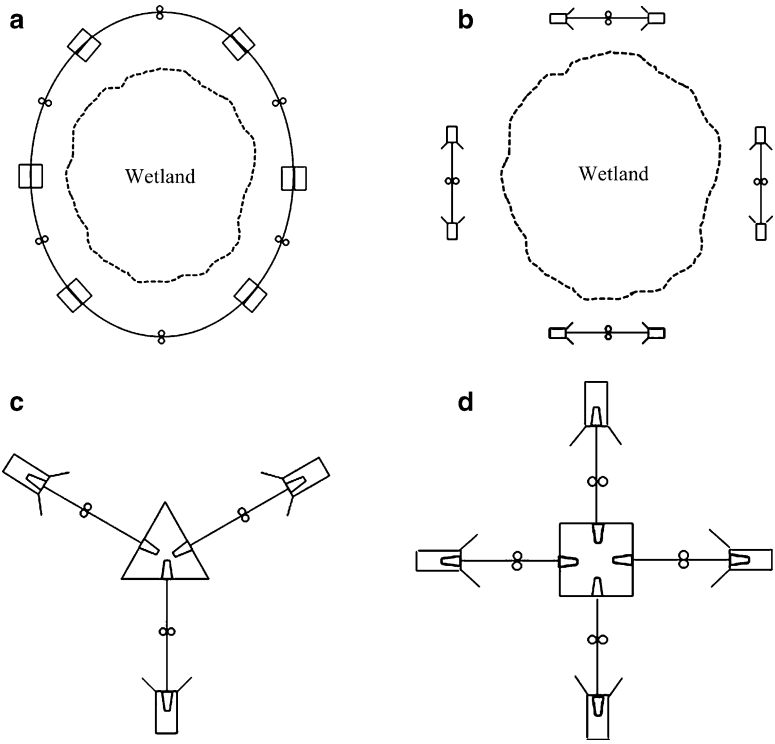

Fig. 7.2 Drift-fence arrays for sampling herpetofauna in the terrestrial environment. Wetlands fully (**a**) and partially (**b**) encircled with drift fence and pitfalls. Three- (**c**) and four-fence (**d**) arrays, which include a large box trap at the center (Figure 7.2c published from Sutton et al. (2010) with kind permission of Current Zoology 2010. Figure 7.2d modified from Burgdorf et al. (2005) and published with kind permission of the Society for the Study of Amphibians and Reptiles 2005

Pitfalls are placed adjacent to the drift fence typically every 10 m and at the ends of fence sections to capture amphibians that intercept the fence (Fig. 7.3; Gray et al. 2004). Standard placement is two opposing pitfalls; one on each side of the fence (Willson and Gibbons 2010). However, digging holes near each other for pitfalls can result in the dirt collapsing between them. Thus, an alternative design is to place one pitfall on alternating sides of the fence every 5 m (Burton et al. 2009), which results in the same pitfall density. Pitfalls can be made of various materials but large (19-L) plastic buckets tend to capture the greatest number of species (Willson and Gibbons 2010). Pitfall captures for most amphibian communities will be biased, as many tree frog (Hylidae) species can climb out and large ranid frogs (e.g., *Lithobates catesbeianus*) can jump out (Willson and Gibbons 2010). Usually, 1-cm holes are drilled in the bottom of pitfalls to allow water to drain during rain events, and a moist sponge is placed in the pitfall to prevent desiccation of captured amphibians. However, in arid or hot regions, a sponge may be insufficient to keep amphibians moist, thus some water (e.g., 5 cm) can be put in pitfalls to reduce desiccation. If water is

Fig. 7.3 Pitfall traps (19-L plastic buckets) used to catch amphibians and reptiles. Pitfall traps must be installed flush with the ground and as close to the drift-fence to be as effective as possible (Published with kind permission of © Matthew Gray and William Sutton 2014. All Rights Reserved)

added, the sponge should remain in the pitfall to allow small mammals captured incidentally to climb out of the water. When pitfalls are not covered, they should be checked at least every 24 h, and we recommend they are opened at least 2 days per week. Typically, captured amphibians are measured, marked uniquely, and released on the opposite side of the fence that they were captured. Covering pitfalls with bucket lids for 24 h between capture events can prevent immediate recapture and biases in population estimates. Drift fences that completely encircle wetlands should be checked daily or partitions removed to allow unrestricted movement when sampling is not occurring. Similar to other techniques, opening pitfalls during rain events can facilitate amphibian captures due to greater movement.

Artificial cover objects can be used as a technique to supplement species detection; however in general, this method does not provide good estimates of population size because recapture rates tend to be low (Fig. 7.4; Bailey et al. 2004a, b). Given that amphibians desiccate easily (Wells 2007), cover objects can provide moist microhabitat during the day. Cover objects (e.g., 120 × 120 cm or smaller) are usually made of untreated plywood or corrugated tin (Willson and Gibbons 2010). The odds of catching amphibians are often greater under wood objects, whereas reptile captures tend to be greater under tin (Grant et al. 1992; Hampton 2007). Objects should be deployed for at least 1 month prior to sampling so that suitable microclimate conditions develop under the object. Funnel traps (discussed in the following section) are often used in combination with drift fences and cover objects to capture amphibians and reptiles (Willson and Gibbons 2010).

Amphibians are often found amongst leaf litter and under natural cover objects (e.g., logs, stones) in the terrestrial environment (Wells 2007). Thus, searching for amphibians under natural cover objects has become a standardized sampling method. Visual encounter surveys can be time- or area-based. For both survey types, natural cover objects are searched for amphibians. Searching during nights with rain can increase the likelihood of detecting individuals. Crump and Scott (1994) describe three levels of search intensity: Level 1 = counts of amphibians on the surface only, level 2 = level 1 and amphibians detected under natural cover objects, and level 3 = previous levels and intense searches through leaf litter and the interior of decaying logs. Level 2 is most commonly used because level 3 destroys

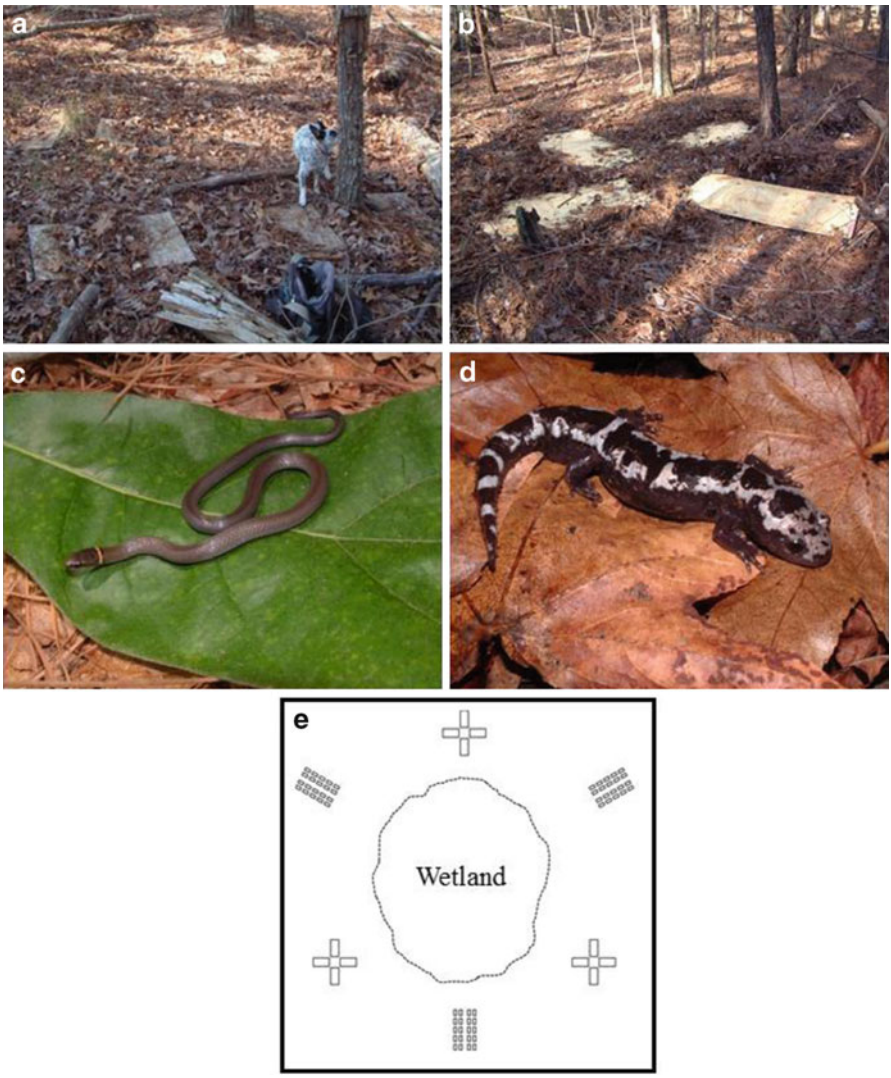

Fig. 7.4 Cover objects such a small (**a**) and large (**b**) plywood boards can placed directly on the ground to survey amphibians and reptiles. Fossorial species such as ring-necked snakes (*Diadophis punctatus*; **c**) and marbled salamanders (*Ambystoma opacum*; **d**) can be sampled using this technique. A variety of coverboard sizes can be used to obtain a more complete sample of the herpetofaunal community and can be distributed at increasing distances from the wetland area (**e**) (Published with kind permission of © William Sutton 2014. All Rights Reserved)

amphibian habitat, and detection is low for level 1 except during rain events. Similar to egg and larval sampling, time-based surveys involve searching for a designated duration (e.g., 30–60 min) and adjusting the number of detected amphibians for the amount of time searched and number of searchers used.

Area-based searches can be in plots or belt transects. Plots are typically 10 × 10 m or 25 × 25 m, with larger plot sizes used when amphibian densities are low (Crump and Scott 1994). The most common transect dimensions are 50 or 100 m in length and 1 or 2 m in width (Marsh and Haywood 2009). Plots or transects can be randomly or systematically placed in a sampling area; adaptive cluster sampling is recommended for species that are uncommon or have a clustered distribution (Marsh and Haywood 2009).

7.3.2 Reptiles

Worldwide, reptiles are found in a wide variety of habitats. Specifically, wetlands provide habitat for many reptile species, with some species endemic to these ecosystems. Reptiles are an important component of wetland ecosystems, as their species diversity is equal to or higher than amphibians in some regions of the United States (Russell et al. 2002). Most reptiles associated with wetlands use both terrestrial and aquatic habitats. Therefore, it is necessary to use multiple sampling techniques to monitor reptile communities effectively. In this section, we discuss standardized reptile sampling techniques including drift-fences, artificial cover objects, aquatic trapping techniques, and visual encounter surveys.

7.3.2.1 Aquatic Sampling

Similar to amphibians, visual encounter surveys can be used to sample aquatic reptiles in wetland ecosystems. Visual encounter surveys are commonly used to detect basking turtles (e.g., map turtles [*Graptemys geographica*]), semi-aquatic snakes (e.g., northern water snakes [*Nerodia sipedon*]), and crocodilians (Fig. 7.5). Sampling should be performed at a distance (20 m or greater) to prevent disturbance. We recommend establishing viewing stations systematically around a wetland. Blinds can be erected to aid with entry and departure without disturbing basking individuals. Kayaks or similar watercrafts can be used to quietly traverse transects on larger bodies of water or rivers. Binoculars or a spotting scope should be used to aid in detection and identification of species and individuals. Basking reptiles are frequently detected on emergent structures (e.g., logs and stumps) and along the banks of wetlands or rivers that are devoid of vegetation. We recommend performing basking surveys for turtles and snakes late morning through mid-day (10:00–15:00 h when water temperatures are between 15 and 25 °C (Coleman and Gutberlet 2008). Nocturnal surveys from watercrafts with a bright spotlight (≥200,000 candlepower) are often performed for detecting crocodilians (Fujisaki et al. 2011). Visual encounter surveys can be used to estimate species occurrence or relative abundance. Mark-resight techniques with highly visible marks (discussed later) are generally necessary to estimate relative abundance. However, certain occupancy-based analyses permit simultaneous estimation of

Fig. 7.5 Common aquatic reptile sampling techniques. Visual encounter surveys can be used to spot basking turtles (**a**) and semi-aquatic snakes (**b**). Baited hoop traps are commonly used to sample aquatic turtles (**c**), whereas crayfish traps (**d**) permit sampling in deeper water and may increase detectability of secretive semi-aquatic snakes such as *Farancia* spp. (Published with kind permission of © Sean Sterrett, William Sutton, and David Steen 2014. All Rights Reserved)

occupancy, abundance, and detection probability based on presence-absence or count data (Fujisaki et al. 2011; Royle and Nichols 2003; Royle et al. 2005).

Baited hoop traps and basking traps are the primary method used to sample aquatic turtles. Hoop traps are a series of large hoops wrapped with netting material with an elliptical funnel-like entrance on one side of the trap (Fig. 7.5). Hoop traps are typically baited with canned fish to attract turtles. To prevent captured turtles from drowning, traps should be placed in shallow water with a portion of the top exposed. If sampling in deeper water is necessary, one or more 2-L plastic bottles should be placed inside the trap as buoys to create a breathing space, or traps can be tethered to a tree on the edge of the river or wetland. Turtles are known to escape from hoop traps, thus they should be checked a minimum of two times per day to reduce trap escapes (Frazer et al. 1990; Brown et al. 2011). Hoop traps can be modified to include fyke nets or leads that function as an underwater drift-fence to

direct turtles into a central trap (Vogt 1980; Glorioso et al. 2010). We recommend using fyke traps in isolated portions of a wetland, such as a narrow cove to intercept swimming turtles (Vogt 1980). Lastly, a basking trap is a square floating structure with an open center and a net or wire basket attached underneath (Brown and Hecnar 2005; Gamble 2006). Turtles bask along the frame of the trap and are captured when they fall into the center, which typically occurs as the trap is approached by the researcher. Basking traps should be placed in areas where turtles are likely to bask, such as shallow wetlands with abundant woody structure.

To increase the likelihood of species detection, we recommend using at least two trap types and placing them in a variety of depths and wetland types (Glorioso et al. 2010). Regardless of trapping method, all traps must be checked daily to minimize capture mortality. Trapping effort can be calculated by multiplying the number of traps set by the total number of sampling events. To standardize capture success at a particular site, divide the total captures by the overall sampling effort.

Aquatic funnel traps (i.e., minnow traps), as used for sampling larval amphibians, are an effective method to sample aquatic and semi-aquatic snakes (Willson et al. 2008). We recommend using different aquatic minnow traps with varying funnel sizes and mesh openings to increase the number of snake species and size classes captured (Willson et al. 2008). Using commercial crayfish traps or modified trash can funnel traps will permit sampling at greater water depths and may target larger species missed with traditional funnel traps (Fig. 7.5; Johnson and Barichivich 2004; Luhring and Jennison 2008). Funnel traps can be placed singly throughout a wetland or as part of an aquatic drift-fence array. If drift-fences are used in tandem with aquatic funnel traps, we recommend the rectangular style minnow traps because they fit flush against the side of the fence, which will increase capture probability (Willson and Dorcas 2004). To reduce the number of escapes and trap-induced mortality of turtles, all traps should be checked daily (Willson et al. 2005, 2008).

7.3.2.2 Terrestrial Sampling

Terrestrial visual encounter surveys for reptiles are commonly used to sample reptile populations. Techniques similar to those discussed in the amphibian section (e.g., searching forest litter, turning cover objects) are most effective for detecting reptiles. To make data comparable among wetlands, it is essential to implement a unbiased sample design and standardize effort among individuals involved in the survey.

Reptiles commonly seek shelter under cover objects, such as large logs, for protection from predators, thermoregulation sites, and nesting habitat. Researchers can exploit these behaviors by using artificial cover objects to sample reptiles (Fig. 7.4; Russell and Hanlin 1999). Many species of reptiles use corrugated tin at a greater frequency compared to wooden cover objects (Lamb et al. 1998), but certain species (e.g., litter dwelling snakes) will frequently use wooden cover objects (Felix et al. 2010). Black plastic sheeting also has been used to sample snakes and lizards (Adams et al. 1999; Kjoss and Litvaitis 2001). Because cover

objects can be used to sample amphibians and reptiles, we recommend using a combination of wood and tin cover objects of various sizes (small [48 cm × 30.5 cm], medium [121.9 × 60.0 cm], and large [243.8 cm × 121.9 cm]) if sampling reptiles and amphibians is an objective. Placing cover objects at increasing distances from the edge of the wetland into the terrestrial environment will also permit sampling of both semi-aquatic and terrestrial reptile species. As with amphibians, cover objects should be deployed at least 1 month before sampling begins.

To avoid disturbing the microhabitat and negatively affecting occupancy rates, cover objects should be sampled only 1–2 times per week (Dodd 2003). Each cover object can be numbered to assess use patterns and trends (Fellers and Drost 1994). We recommend that researchers record environmental covariates (e.g., air temperature, relative humidity, and % cloud cover) to account for possible abiotic factors influencing detections (Joppa et al. 2009). Once individuals are captured, they should be processed according to study objectives (e.g., mass and body length measurements, genetic samples), and assigned an individual- or plot-specific mark to account for recaptures.

Certain reptile species may be difficult to detect due to large yearly dispersal patterns, cryptic coloration, or secretive life history patterns. Drift-fences with pitfall and funnel traps are commonly used to capture reptiles (Fig. 7.6). We recommend using aluminum flashing (60–90 cm in height) instead of silt fence to sample reptiles, because the metal surface deters climbing and trespass of individuals. Drift fences set for reptiles are often erected as X- or Y-shaped arrays with pitfalls and rectangular double-entrance funnel traps placed at various locations along the fence. Single-entrance funnel traps also can be placed at the terminus of each fence, with a 1-m section of fence angled at 45° from each trap corner to direct reptiles into the traps (Sutton et al. 2010). Arrays can include a large, central box trap for capturing larger snake species; schematics for these traps have been detailed elsewhere (Fig. 7.6; Burgdorf et al. 2005; Sutton et al. 2010). Small doors can be installed on the sides of box traps to assist with safe removal of venomous snakes (Steen et al. 2010). A water source should be added to traps to prevent dehydration of captured individuals. To increase capture efficiency, the drift-fence should be shaped to extend 15–20 cm into the funnel entrance and fit flush with the dimensions of the trap funnel. It is important that funnel and pitfall traps are installed flush along the vertical surface of the fence to prevent reptiles from circumventing the trap (Jenkins et al. 2003). Design and placement of drift fence arrays should be planned prior to study implementation and should correspond with study objectives (Fig. 7.2; Todd et al. 2007), as discussed in the amphibian section.

7.3.3 Methods for Marking Herpetofauna

Studies that seek estimates of population size, survival, or dispersal require the recognition of previously captured individuals (Williams et al. 2002). A myriad of

Fig. 7.6 Drift-fence arrays with large box traps used to sample the reptile community, especially large snakes such as timber rattlesnakes (*Crotalus horridus*) and black racers (*Coluber constrictor*) (Published with kind permission of © William Sutton and David Steen 2014. All Rights Reserved)

marking techniques have been developed for a variety of wildlife species (Silvy et al. 2005), with some more successful than others. For a marking technique to be effective and result in unbiased parameter estimates, it cannot affect survivorship or behavior of the individual and must provide a permanent and easily detectable mark (Ferner 2010). Additionally, application of the mark should not cause undue stress or pain. Typically, anesthesia is unnecessary but topical analgesics (e.g., Orajel®) can be applied to reduce pain. We recommend that researchers consult a wildlife veterinarian for correct dosage if an analgesic is used, because some analgesics contain chemicals (e.g., benzocaine), which can function as a euthanizing agent.

The methods below have been approved previously by U.S. Institutional Animal Use and Care Committees (Ferner 2010). The most common method for marking lizards, anurans, and salamanders is removing a toe(s) from the hind or front foot (i.e., toe-clipping) that corresponds to a pre-determined numerical scheme (Woodbury 1956). Sharp scissors that are disinfected in 2 % chlorhexidine diacetate or 95 % EtOH can be used to remove digits. Excisions should be made at the lowest

joint to reduce bleeding and regeneration. For frogs with webbed feet, the webbing should be cut prior to excising the toe. Silver nitrate sticks can be used to stop bleeding and a topical antibiotic applied to the excision site to reduce risk of infection. Multiple pairs of scissors should be used because each pair should soak in disinfectant for at least 1 min between animals.

Marking schemes have been developed for amphibians (Donnelly et al. 1994; Ferner 2010) and lizards (Enge 1997), and most schemes can account for many unique individuals. For lizards, the longest (fourth) toe of the hind foot should not be clipped, and removal of >1 toe per foot should be avoided. In general, toe clipping arboreal frogs or lizards is not recommended (Ferner 2010). Thumbs on the front feet of male anurans should never be clipped due to their importance for amplexus (Ferner 2010). Toe-clipping has been shown to have both negligible (Paulissen and Meyer 2000; Dodd 1993) and negative impacts (Bloch and Irschick 2005; Schmidt and Schwarzkopf 2010) on climbing and running behaviors in lizards. Amphibian responses to toe-clipping are similarly disparate. For example, McCarthy et al. (2009) found that in salamanders, the likelihood of recapture decreased with the number of removed toes, whereas other research has found limited or no impacts of toe-clipping on normal amphibian behaviors (Ott and Scott 1999; Liner et al. 2007; Phillott et al. 2007). If toe-clipping is not an option, additional marking options include branding (Clark 1971; Ferner 2010), paint marking (Jones and Ferguson 1980; Simon and Bissinger 1983), and injectable colored elastomers (Schmidt and Schwarzkopf 2010).

Similar marking schemes exist for turtles and snakes; however, marks must be applied using different methods. Turtles can be individually marked by notching within the first and last four marginal scales on either side of the carapace using a sharp-edged metal file or rotary tool (Cagle 1939; Honegger 1979; Enge 1997). Tools should be disinfected between individuals. Other inexpensive marking options include branding the plastron (Clark 1971) or painting identifying features on the carapace using permanent paints. Snakes can be marked by using sterilized scissors or fingernail clippers to remove ventral (Brown and Parker 1976; Spellerberg 1977) or subcaudal (Blanchard and Finster 1933) scales, according to a pre-determined numbering pattern (Enge 1997). Care must be taken not to cut the scales too deeply, as infection may result (Honegger 1979). Other related marking methods include using medical cautery units (Winne et al. 2006) or colored injectable elastomers (Hutchens et al. 2008) to apply a semi-permanent mark.

A more expensive but highly effective method to individually mark herpetofauna is using passive integrative transponder (PIT) tags. A PIT tag is a small microchip encased in a glass container that transmits a signal, which is interpreted with an electronic reader as a unique serial number (Fig. 7.7; Gibbons and Andrews 2004). PIT tags can be implanted either subcutaneously or intraperitoneally in snakes (Keck 1994), in the abdominal skin midway between the limb and the plastron in turtles (Buhlmann and Tuberville 1998; Rowe and Kelly 2005), and in the abdominal cavity of anurans and salamanders (Ferner 2010). PIT tags are implanted using a large syringe, so care must be taken not to damage internal organs. To reduce the chance of infection, the PIT tag along with the syringe needle must be disinfected before injection into the organism. PIT tags appear to have few

Fig. 7.7 Subcutaneous injection of a Passive Integrative Transponder (PIT) tag into *Anaxyrus cognatus* (**a**), PIT tag scanner (**b**), and PIT tag under skin of *Spea multiplicata* (**c**) (Published with kind permission of © Matthew Gray and Sumio Okada 2014. All Rights Reserved)

negative impacts on survival and growth rates of turtles (Rowe and Kelly 2005) and snakes (Keck 1994; Jemison et al. 1995). PIT tags have been used to mark anurans and salamanders (Hamed et al. 2008; Ferner 2010); however, use of this technique should be limited to larger species. This technique results in a permanent mark that is easy to differentiate; however, newly marked individuals are at risk of losing tags through the PIT tag injection site. Vetbond® and other veterinary grade skin adhesives can be used to close the injection site (Ferner 2010). We recommend a secondary mark (e.g., scute/scale mark or toe clip) in addition to the PIT tag to ensure that recaptured individuals are not overlooked.

7.4 Monitoring Bird Populations

Monitoring bird populations in wetlands is challenging because many species are migratory, and use of a given wetland may vary during different seasons. For the purposes of this chapter, we divide the seasons into functional life-cycle stages (breeding, migration, and wintering), and discuss different methods for the following species groups: waterfowl, wading birds, shorebirds, secretive marsh birds, songbirds and raptors (Table 7.1). Species within these groups comprise most avifauna associated with wetlands in North America. For more detail on monitoring bird populations, please see Bibby et al. (2000) or Ralph et al. (1993).

7.4.1 Waterfowl

There are 70 species of waterfowl (Order Anseriformes: Ducks, Geese and Swans) in North America. Most of these species nest in Canada and the northern latitudes of the United States and migrate south during winter. Waterfowl are monitored and

Table 7.1 Avian monitoring methods by waterbird group

	Waterfowl			Shorebirds			Wading birds			Secretive marshbirds			Songbirds			Raptors		
Monitoring method	B[a]	M	W	B	M	W	B	M	W	B	M	W	B	M	W	B	M	W
Population status and trends																		
Aerial counts	x	x	x				x	x	x							x	x	x
Point counts													x		x	x		x
Mist-netting													x	x	x		x	
Transect counts	x			x			x			x			x		x	x		
Territory mapping													x					
Call-back surveys										x		x	x			x		
Migration counts		x			x			x									x	
Reproduction																		
Nest monitoring	x			x			x			x			x			x		
Brood counts	x																	
Survival																		
Band recoveries		x	x														x	
Radio telemetry	x	x	x	x	x	x	x	x	x	x			x			x	x	x
Activity																		
Radio telemetry	x	x	x	x	x	x	x	x	x	x			x		x	x	x	x
Other methods																		
Stable isotopes	x		x	x		x	x		x	x		x	x		x	x		x
Genetic markers	x		x	x		x	x		x	x		x	x		x	x		x

[a]Breeding, Migration, Winter

managed in North America under the guidance of the North American Waterfowl Management Plan (NAWMP). The NAWMP is a formal agreement among the United States, Canada and Mexico to set population and habitat goals for continental waterfowl populations (NAWMP Planning Committee 2004). A primary objective of NAWMP is to restore and maintain continental waterfowl populations at approximately 62 million breeding ducks (NAWMP Planning Committee 2004). Several monitoring programs exist to ensure accurate and precise estimation of waterfowl population sizes each year. By using estimates of breeding pairs, brood production, and overwinter survival, harvest regulations can be set to ensure that populations are maintained at desired levels. Below are some monitoring programs and techniques used to estimate waterfowl populations.

7.4.1.1 National Programs

Waterfowl populations are monitored during the breeding season and winter. The U.S. Fish and Wildlife Service and the Canadian Wildlife Service, along with state and provincial agencies, collaborate to implement the North American Waterfowl Breeding Population and Habitat Survey, which has been estimating duck and goose populations annually on the major breeding grounds in North America since 1955. This program relies on aerial surveys of over five million

square kilometers of wetlands from fixed-wing aircraft and helicopters. Surveys are conducted in May and early June in the principal waterfowl breeding grounds of North America, including the north-central United States and Canada (i.e., the Prairie Pothole Region), Alaska, and the eastern United States and Canada. Ground surveys are used in combination with aerial surveys to adjust estimates for visibility bias. Surveys are flown along fixed transects at low altitude (ca. 50 m above ground level), and waterfowl pairs are counted on individual wetlands. The sampling design allows the data from these transect surveys to be extrapolated to the entire population based on the area covered. A detailed description of this methodology and results from the annual survey are available (e.g., U.S. Fish and Wildlife Service 2011). To estimate the number of waterfowl that are expected during the fall migration (i.e., fall flight index), breeding population estimates are combined with estimates of habitat conditions, adult summer survival, and projected fall age ratio (young/adult, U.S. Fish and Wildlife Service 2011). To estimate wintering populations, the Mid-winter Waterfowl Population Survey has been conducted annually by state and federal wildlife agencies since 1935. This aerial survey covers the four migratory flyways in the United States and parts of Mexico. Results of the survey are reported annually for each of the four migratory flyways in North America, but are not comparable because of differences in survey methodology among flyways.

7.4.1.2 Monitoring Recruitment

Waterfowl recruitment was monitored annually by estimating number of broods on wetlands in the breeding grounds; however, this survey was discontinued in 2004 due to budget constraints. Individual studies, however, continue to report results on various species of interest. Recruitment can be monitored by searching for nests and counting broods. Nest searches can include systematically searching wetlands for diving duck nests or in adjacent uplands for many dabbling duck species. Once nests are located, they are monitored for activity every 3–5 days until the eggs hatch. Estimates of nest success are calculated based on the number of successful nests divided by the total number of nests monitored $\times$ 100 % (i.e., raw nest success), or by less biased methods involving calculation of the number of nest survival days using the Mayfield method (Mayfield 1975). Contemporary nest survival analyses, such as the nest analysis module in Program MARK and the logistic exposure model, also exist and are similar to predictions using the Mayfield method (Rotella et al. 2004). Brood counts can be conducted by aerial surveys, or if species-specific data are required, from the ground. These surveys are typically performed in July.

7.4.1.3 Band Recovery Programs

Analysis of band return data is the cornerstone of monitoring mortality rates for waterfowl and estimating population size. All banding data are administered

Fig. 7.8 U.S. Fish and Wildlife Service biologist affixing a leg band to a wood duck (*Aix sponsa*, *left*), and a hunter with a banded mallard (*Anas platyrhynchos*, *right*) (Published with kind permission of © Clayton Ferrell and Barry Pratt 2014. All Rights Reserved)

through the U.S. Geological Survey's Bird Banding Lab and Canada's Bird Banding Office of the Canadian Wildlife Service. The U.S. Fish and Wildlife Service, Canadian Wildlife Service, state and provincial agencies, and other non-governmental conservation organizations (e.g., Ducks Unlimited) cooperate in banding waterfowl during the summer (Fig. 7.8). Because waterfowl are hunted, bands are often reported by waterfowl hunters when a bird is harvested (Fig. 7.8). Band return data can be used to estimate the proportion of the population that is being harvested, track movements of birds, and estimate population size and over-winter survival. Because the banding data are age- and sex-specific, estimation of mortality rates can be for each sex and age class. In addition, hunter surveys are conducted annually by the U.S. Fish and Wildlife Service to estimate waterfowl harvest by species. Band return data and hunter harvest surveys are important in determining mortality rates and are used, along with breeding population estimates, for setting waterfowl hunting regulations annually.

7.4.1.4 Other Monitoring Methods

Several other methods are important for monitoring waterfowl populations. Radio telemetry has been used extensively to monitor survival, habitat use, activity, and movements during all stages of the annual cycle (i.e., breeding, migration, and wintering). The presence of stable isotopes of carbon, nitrogen, oxygen, and sulfur has been used as markers to determine the natal origin of individuals that were harvested on the wintering grounds (Hebert and Wassenaar 2005). Stable isotope analyses also are used to conduct trophic studies in wetlands and assess diets of waterfowl. Genetic markers have been used to identify waterfowl species and sub-species, where physical characteristics prevent differentiation. This approach

has been used to discriminate among sub-species of Canada goose (*Branta canadensis*, Mylecraine et al. 2008).

7.4.2 Shorebirds

There are 49 species of shorebirds (Order *Charadriiformes*) that regularly breed in North America and warrant monitoring at local, regional or continental scales (Brown et al. 2001). Similar to waterfowl, shorebirds breed at northern latitudes in North America, but typically migrate farther distances. Many species of shorebirds that breed in northern Canada migrate to Central and South America, resulting in round-trip distances exceeding 20,000 km (Helmers 1992). Considering that flight is energetically demanding (Loesch et al. 2000), migrating shorebirds must land frequently to acquire high-energy food resources (Skagen and Knopf 1993). Mudflats and shallowly flooded wetlands are the primary foraging habitats used by migrating shorebirds (Helmers 1992). Widespread wetland loss in the continental United States has presumably led to less foraging and resting habitat for shorebirds than what was historically available (Brown et al. 2001). Over half of North American shorebird species are in decline, with most species representing long-distance migrants (Brown et al. 2001). Bart et al. (2007) estimated that 23 of 30 shorebird species in northeastern North America were experiencing declines. Obtaining accurate and precise estimates of shorebird population sizes is fundamental to ensuring conservation of this imperiled group of wetland fauna. A monitoring strategy for these species has been developed under the North American Shorebird Conservation Plan (Howe et al. 2000).

7.4.2.1 National Programs

Skagen et al. (2003) outlines the major components of the continental monitoring plan for shorebirds, which is the Program for Regional and International Shorebird Monitoring (PRISM). Shorebird populations are monitored by natural resource organizations and private partners in key breeding areas, during migration at key stopover areas, and on the wintering grounds. The continental survey is based on surveying 10–16 ha wetland plots, selected as a sample from a geographic information system of wetland areas. A rapid assessment approach is used to count shorebirds in these plots, based on point counts, area searches, or transect counts. More intensive methods are used on a subsample of plots to develop a correction factor for the rapid assessment density estimates. In temperate areas where roads are available, the North American Breeding Bird Survey can produce reliable results for common species. However, rare or imperiled species require a focused sampling approach. At stopover sites in the continental United States, ground counts can be conducted during the 6–8 week period when most shorebirds migrate (i.e., April–May for spring migration and August–September for fall migration). Monitored sites are visited every

Fig. 7.9 Scan sampling with a spotting scope (**a**), lesser yellowlegs (*Tringa flavipes*) foraging on a mudflat (**b**, *left*), king rail (*Rallus elegans*) responding to a callback survey (**b**, *right*), and a great blue heron (*Ardea herodias*) rookery (**c**) (Published with kind permission of © Clayton Ferrell and Matt Gray 2014. All Rights Reserved)

7–10 days during this period. Shorebirds are counted using a spotting scope or binoculars via scan sampling (Fig. 7.9). Scan sampling involves viewing a defined area over for a specified short duration (i.e., 3–5 min) with binoculars or spotting scope and counting the number of individuals present in the area by species. Wintering grounds surveys are being conducted in similar fashion in the United States, although a standardized effort needs to be developed for Central and South America. Similar to waterfowl, stable isotope analyses have been conducted for some priority species, such as the red knot (*Calidris canutus*), to link breeding areas to specific wintering sites (Atkinson et al. 2005).

7.4.2.2 Monitoring Survival and Recruitment

Survival for shorebird species can be monitored by a variety of methods. Banding return analyses have been useful for documenting range-wide movements of shorebirds; however, banding data generally have not been useful for estimating survival because of low band return rates, unlike waterfowl which are harvested. As such, most banding studies for shorebirds typically are used in a local area to answer questions about short-term survival and population turnover at a particular site (Gratto-Trevor 2004). Radio telemetry has been used to track short-distance movements, survival and habitat use. Satellite transmitters are being used to track long-distance migration by researchers at the Alaska Science Center of the U. S. Geological Survey for species of conservation concern (e.g., curlews [*Numenius*] and godwits [*Limosa*], http://alaska.usgs.gov/science/biology/shorebirds/index.php).

Monitoring recruitment can be done using standard nest monitoring protocols. Once nests are located, they can be checked every 3–5 days until hatching similar to waterfowl. Broods can be monitored post-hatching from blinds or survey stations to estimate brood survival until fledging. Obtaining accurate chick counts in shorebird broods from visual surveys can be challenging due to their small size and cryptic coloration. To increase detection, small radio transmitters can be attached to a subsample of chicks in each brood.

7.4.3 Wading Birds

Species of herons, egrets, storks, ibises, flamingos, and spoonbills are classified as long-legged wading birds. There are 38 species of wading birds in North America. Wading birds exhibit a wide range of life history strategies. Some species are largely non-migratory, others are exclusively migratory, and some species are migratory only in the northern portion of their range. Wading birds are predatory in their foraging approach, feeding in aquatic habitats on fish, amphibians, reptiles, crustaceans, and other invertebrates. Many of these species are colonial nesters in rookeries, which provide opportunities for population monitoring unique to this group.

7.4.3.1 National Programs

An international conservation plan has been drafted for waterbirds, including waders (Kushlan et al. 2002). The U.S. Geological Survey Patuxent Wildlife Research Center hosts a Waterbird Monitoring Partnership, with the goal of coordinating and standardizing efforts to monitor waterbird populations in North America. Because many species of waders are colonial nesters, the best time to monitor populations is during the breeding season in nesting colonies (Steinkamp et al. 2003). Populations can be monitored through a two-stage approach. First, nesting colonies are located (Fig. 7.9). Depending on the area being covered, this can be done by ground-based, boat, or fixed-wing aircraft surveys. Once a colony is located, then colony visits can determine the species composition and the number of active breeding pairs. Breeding pairs can be counted directly or the total number of nests can be counted. The number of nests generally exceeds the number of breeding pairs because not all nests are actively used in a given breeding season. If the colony is relatively small (e.g., $<$100 breeding pairs), then a complete count may be possible. As colonies become larger, a standardized sampling approach is needed. Typically, fixed-width belt transects are walked through the colony. Breeding pair estimates per unit area from the sample can be extrapolated to the entire colony to estimate population size. Repeat visits may be necessary to account for imperfect detection and temporal variability during the breeding season. Colonies with dense vegetation and poor visibility from below require aerial surveys for monitoring. Aerial surveys can either directly count individual nests or adults, or aerial photos can be taken and inspected in the lab.

7.4.3.2 Monitoring Survival and Recruitment

There are a limited number of studies documenting survival and recruitment in wading birds (Cezilly 1997). Survival rates have traditionally been monitored via banding studies; however, large numbers of individuals need to be banded to yield sufficient returns to estimate survival. This problem can be mitigated by using auxiliary markers, such as color leg bands or patagial tags, so that banded individuals do not require recapture to be identified. Radio telemetry studies can also yield reliable survival estimates, although many species of wading birds may disperse outside the study area, and aerial tracking is required to discriminate between dispersal and mortality.

Monitoring of recruitment can often be accomplished via direct observation at nesting colonies. Nest success can be determined from repeated observation of active pairs. Counts of young produced in active nests can be made from the ground but are typically biased low because of poor visibility into nests. Climbing to a subsample of nests or combining ground-based observations with aerial observation or photography can account for visibility biases.

7.4.4 Secretive Marsh Birds

Species of rails, bitterns, coots, moorhens, gallinules, and grebes are classified as secretive marsh birds, because their skulking behavior makes them difficult to detect by conventional means. These species use freshwater and brackish marshes throughout North America. Most of these species breed across the continent and are migratory, wintering in the southern United States, Mexico, and the Caribbean. They are generally cryptically colored, nest on the ground, and spend most of their time on the ground in dense vegetation. Detection of these species in wetlands for population monitoring is problematic. As a result, specialized protocols have been developed.

7.4.4.1 National Programs

The North American Breeding Bird Survey is inadequate for monitoring secretive marsh birds because road access to wetlands is limited and passive point-count methods are inefficient at detecting these species. As a result, an independent national monitoring program for marsh birds, involving standardized count protocols and a sampling framework, has been developed (Conway 2011; Johnson et al. 2009). The count protocol is based on point-count monitoring stations that are located $\geq$400 m apart in wetlands that are representative of an area. The number of point counts conducted on each site is based on the level of precision desired by the researcher or natural resource manager (i.e., more points typically yield better precision) and available resources. The protocol involves a passive 5-min point count in which all species of interest that are heard or seen are recorded. Focal individuals are recorded during the first 1-min interval, and the distance to the individual at first detection is visually estimated. Playback recordings are then broadcast on a 30-s playback, 30-s silence interval for each focal species. Broadcast of playback recordings of many marsh birds has been shown to increase detection (Conway and Gibbs 2005). These data yield an index of relative abundance (individuals per species per point) that can be adjusted to estimate density using distance sampling and time-to-detection functions, which is discussed in greater detail in Sect. 7.4.5.2.

7.4.4.2 Measuring Survival and Recruitment

Few telemetry studies have been conducted during the non-breeding season, which would allow for seasonal estimates of survival. Although some of the secretive marsh birds are hunted (e.g., rails and gallinules), banding data are sparse and have been ineffective for monitoring survival (Eddleman et al. 1988). Capture and banding efforts by natural resource agencies and researchers are limited, and hunting pressure is too low to provide sufficient recaptures to produce reliable

survival estimates. Monitoring productivity can be done by nest searching, monitoring nest success, and counting number of young fledged. Finding nests of these species can be challenging, because nests are cryptic and often located in areas that are difficult to access. Radio telemetry has aided nest finding for some species if adults can be captured prior to the nesting season.

7.4.5 Songbirds

A great diversity of songbird species use wetlands during part of their life cycle. Wetlands serve as productive breeding, stopover, and wintering sites for songbirds, because of the abundance of invertebrates and seed sources for food. Some songbirds are wetland specialists, such as the marsh wren (*Cistothorus palustris*), whereas other species use wetlands as one option in an array of potential habitats. Riparian zones along waterways provide important habitat for songbirds, even if they are not classified as a wetland.

7.4.5.1 National Programs

Several national programs monitor songbird population status and trends, and may be useful for monitoring populations of some wetland species. The North American Breeding Bird Survey (BBS) was established in 1966, and is the primary continental monitoring program for songbirds. The BBS is based on a stratified random sample of 40 km (25 mile) roadside routes conducted once during the early breeding season (late May and early June) each year. Breeding Bird Atlases (BBA) map the breeding distribution of all birds, including wetland species, across a given state or province. The Christmas Bird Count (CBC) is another long-term national monitoring program conducted by volunteer bird watchers that contains data on distribution and relative abundance of songbirds, including wetland-dependent species, during early winter. The CBC is based on volunteer bird watchers visiting a given area (e.g., portion of a county) for a prescribed period of time (observers $\times$ hours $=$ party hours), and recording the species and number of individuals encountered within that prescribed area. A training program is required to participate in the BBS, and CBC counts are performed by local volunteer groups with an experienced coordinator that is responsible for ensuring data quality. The Monitoring Avian Productivity and Survivorship (MAPS) program sponsored by Point Reyes Bird Observatory is another national program that produces estimates of survival and productivity. The MAPS program is based on constant-effort mist netting during the breeding season. The value of MAPS for monitoring wetland birds, however, is limited because very few MAPS stations are located in wetlands.

7.4.5.2 Count Methods

Songbird populations in wetlands can be monitored using various count methods including point counts, transect counts, passive constant-effort mist-netting, and territory mapping. Point and transect counts are the most easily accomplished, whereas mist-netting and territory mapping are more time intensive. Songbird vocal behavior, visibility within the wetland, and accessibility of the wetland determine which method might be most appropriate. Point counts are used extensively for monitoring during the breeding season (May–June in the contiguous United States) when songbirds are most vocal. Raw counts need to be adjusted for the likelihood of detection to yield unbiased results. Point counts are typically conducted for 5 or 10 min with bird detections recorded in 1-min intervals to allow for time-to-detection (Alldredge et al. 2007) or time-removal (Farnsworth et al. 2002) analyses. Distance to individual birds are usually estimated within 0–25, 26–50, 51–75, 76–100 and >100 m distance bands to allow for distance-detection analyses (Buckland et al. 2001). By taking time to detection and distance into consideration, relative abundance estimates can be converted to unbiased estimates of bird density for each species. Repeated visits to point count stations can be used to estimate bird occupancy (Mackenzie et al. 2006). Territory mapping is an alternative method, which involves typically eight repeated visits to a survey plot (10–20 ha), and mapping the location of all songbirds detected within the plot (Bibby et al. 2000). Thereafter, maps from all visits are overlaid and territories of individual males are mapped based on consistent detections over time. Territory mapping generally yields the most reliable density estimates (breeding pairs per ha) of any of the methods discussed.

Transect counts or constant effort mist-netting typically are more appropriate during migration or winter because detections are lower when songbirds generally are not vocalizing. Transect methods involve walking a fixed distance across a target area, recording all birds seen or heard and the distance from the observer to the bird. Program Distance (http://www.ruwpa.st-and.ac.uk/distance/) can fit probability density functions to distance-based counts and estimate bird density. Constant effort mist-netting involves setting an array of mist nets in a target area and banding all songbird species captured. Capture effort (net-hours) is recorded based on the number of nets used times the number of hours the nets are set. An index of relative abundance can be generated from these data based on captures per species per net-hour.

7.4.5.3 Measuring Survival and Recruitment

Survival estimates for most songbirds are difficult to obtain. Radio transmitters for most songbirds typically have less than 30 days of battery life, thus telemetry is of limited value for estimation of seasonal or annual survival rates. As a result, banding studies with recapture or resighting are about the only method available

for estimating seasonal or annual survival. Often individual birds are banded with a unique combination of color bands to enable individual identification upon resighting (Bibby et al. 2000).

Recruitment can be measured by standard nest monitoring methods outlined above or by using videography. Nest success, the number of young fledged from successful nests, the number of renesting attempts, and the number of broods produced define fecundity for a given species. Nest success is typically monitored by determining daily nest survival and analyzing the data with the nest module in Program MARK or the logistic exposure model (Rotella et al. 2004). Patterns of juvenile dispersal and site fidelity vary widely for songbirds (Greenwood and Harvey 1982). Stable isotopes, in conjunction with genetic markers, are being used to link songbird breeding and wintering grounds (Hobson and Wassenaar 1996).

7.4.6 Raptors

Many raptors (hawks, falcons, eagles, kites, and owls) regularly use wetlands for nesting, during migration, or winter. Raptor habitat is defined by the availability of suitable prey and suitable nest and roost sites. Different habitats can often meet these requirements, thus few raptors would be considered obligate wetland species. Monitoring raptor population status and trends in wetlands is challenging, because their low relative abundances and large home ranges make detection problematic.

7.4.6.1 National Programs

Raptor monitoring is included in the previously discussed national monitoring programs (e.g., BBS, BBA and CBC), and these data are useful for estimating range wide or regional population status and trends. Raptors are also monitored across a network of continental “hawk watch” monitoring stations during migration.

7.4.6.2 Count Methods

Raptors can be monitored in individual wetland sites by conducting nest searches and monitoring the number of active pairs. Nests can be located either by aerial, boat, or ground-based surveys. Some species (eagles and osprey) have nests that can be easily detected during surveys. Point counts have been used during breeding and non-breeding seasons to monitor populations of more common species. Roadside point-count routes, similar to BBS, have been used, because long distances (e.g., 40 km) can be surveyed within a morning, increasing the probability of detection. Surveys involving broadcast of species vocalizations have also been effective for monitoring raptors to enhance detections (Mosher et al. 1990).

7.4.6.3 Measuring Survival and Recruitment

Survival rates can be estimated by banding and recapture or resighting of raptors. Color leg bands and patagial tags have been used to aid in individual identification. Survival rates have also been estimated through radio and satellite telemetry studies for many species. Satellite telemetry has also aided in identification of migratory pathways and developing linkages between breeding and wintering grounds. Stable isotopes have been used for this purpose as well.

Recruitment can be monitored through traditional nest monitoring methods. The nests of many raptors are visible to ground-based observers for monitoring nest success and number of young produced. Aerial surveys have also been used effectively for monitoring reproduction in eagles and ospreys (Fraser et al. 1984).

7.5 Monitoring Mammal Populations

A diversity of mammals occupy wetland environments, and sampling methodologies for monitoring mammals are numerous and variable, depending on the species of focus. We have separated mammals into groups including bats, small mammals, large rodents, and carnivores. Monitoring techniques for each group are well established, yet emerging techniques offer ways to improve our abilities to make inferences about mammal behavior, activity, and abundance.

7.5.1 Bats

Bats occupy a variety of habitats, including a diversity of wetland types. Bat activity is primarily nocturnal and crepuscular, and because of their size and mobility, is somewhat difficult to monitor. Bats emit high-frequency sounds, and use the echoes (echolocation) of these sounds to locate food and navigate at night. Echolocation allows bats to judge the size and location of objects in their flight path, and the speed of flight. Bats spend diurnal periods at roost sites, which may include cavities, caves, tree foliage, and various man-made structures such as bridges and buildings. Most bats are insectivores, but some bats eat fruits, vertebrates, and blood. Not surprisingly, efforts to monitor bats typically focus on roosting or foraging locations, and foraging location is ultimately determined by the species of bat encountered. We encourage readers to consult Kunz et al. (2009a, b) for further details on monitoring bats.

7.5.1.1 Acoustical Surveys

Because bats are elusive and sometimes difficult to study, much attention has focused on detecting and analyzing vocalizations to make inferences about the ecology and behavior of bats. Acoustic monitoring often focuses on estimating bat activity across habitat types (Gannon and Sherwin 2004), evaluating resource use, or describing behavior (Barlow and Jones 1997). Acoustic surveys are a noninvasive method to survey bats across broad geographic extents at relatively low cost. Acoustic surveys rely on being able to compare calls heard in the field to reference collections, but they have inherent biases because the likelihood of detection probably differs among bat species (Brigham et al. 2004). Nonetheless, acoustic surveys can provide qualitative and quantitative assessments of bat activity and habitat associations. The quality of data gained from any acoustic survey will ultimately depend on the quality of the library of calls from known species available to the observer. Observers should attempt to standardize equipment used during acoustic surveys if comparisons among sites or habitats are desirable. Acoustic detectors can be used in practically any habitat, and multiple units can be deployed by one person, making them efficient for collecting large amounts of data across broad spatial scales (Rodhouse et al. 2011). Various song meters and recognition software, such as SonoBat, are available, which detect high-resolution full spectrum sonograms. Song Scope® by Wildlife Acoustics (http://www.wildlifeacoustics.com/) is a versatile software that can analyze calls from bats, birds and anurans, thus permitting simultaneous monitoring of these groups. For bats, many researchers use Anabat or Anabat II detectors in combination with recognition software to survey bats along specific habitat features such as streams, as well as in habitats unable to be sampled effectively with other techniques (e.g., open expanses of marsh; Hayes et al. 2009).

7.5.1.2 Live Capture

There are various methods used to capture bats, but mist nets, handheld nets and harp traps are the most common (Kunz et al. 2009a, b, Fig. 7.10). In wetlands, capturing most bat species represented in the community will require multiple capture techniques given the diversity of habitat types available to bats. The type of capture method used ultimately depends on the abundance of bats in the sampling area and the expected number of flying bats that will be encountered. Areas where bats are traveling routinely, feeding or drinking are ideal capture sites regardless of the method used. Once a capture technique is selected, there are many ways to implement the technique, as mist nets, harp traps, and even handheld nets can be used in a myriad of ways (see Kunz et al. 2009a, b). Mist nets are most commonly used to capture bats, and typically set up on the ground (as with songbirds), within the tree canopy, or suspended across narrow waterbodies. Mist nets should not be used if the possibility exists of capturing large numbers of bats in

Fig. 7.10 Mist nets (**a**) are effective at capturing bats in a variety of trapping situations. Harp nets (**b**) are often used at the entrances and exits to roosting structures, and should be used if capturing large numbers of bats is possible (Published with kind permission of © Steven Castleberry 2014. All Rights Reserved)

short time periods. Mist nets should be checked several times per hour, and bats removed as quickly as possible. Harp nets can be used to capture large numbers of bats near the openings of caves and other roosting structures. Handheld nets also can be used to capture bats exiting roosting structures.

Special care should be taken when removing bats from traps to prevent injury to the bat and observer. Similar to birds, determine the direction from which the bat entered the trap, and attempt to remove the bat from that direction. Researchers attempting to capture bats should wear leather gloves. The thickness of the glove should be chosen considering the size and aggressiveness of the bat species handled. Rabies virus is the most significant public health concern related to bats, so individuals that frequently handle bats should be vaccinated.

7.5.1.3 Radio Telemetry

Because telemetry involves capture of individual bats, it is a relatively invasive and costly form of monitoring. However, properly designed telemetry studies (e.g., Ratti and Garton 1994; Millspaugh and Marzluff 2001) can provide observers with movement and behavioral data that are impossible to obtain with other techniques. Transmitters can be attached to bats in a variety of ways depending on the size of the bat. Telemetry studies on small bats typically use surgical glue to attach transmitters on the back between the scapulars, whereas larger bats may be able to wear collar or necklace transmitters. Regardless of the attachment, transmitters should weigh less than 5 % of the body weight of the bat, including

the adhesive or attachment used. Monitoring of bats with telemetry typically uses either homing (e.g., to locate a roost site) or triangulation, with one or multiple observers. If movement ecology is a primary objective, the use of multiple receiving stations and simultaneous azimuths should be considered (see review by Amelon et al. 2009).

Researchers using radio telemetry to monitor bat populations need to consider the target number of animals to be monitored and the desirable number of relocations per animal. In general, if monitoring fine-scale movements is an objective, more relocations are necessary. Conversely, study objectives may make it necessary to monitor greater numbers of individuals, potentially resulting in fewer relocations per individual. Monitoring bats with radio telemetry requires careful consideration of research designs prior to capture and marking.

7.5.1.4 Roost Surveys

Bats use roosts for extended periods of time and for various reasons (e.g., hibernation), so surveying roosts to monitor bat populations is commonly used. Roost surveys may be used to determine colony size, or to monitor temporal trends in abundance (Warren and Witter 2002). Typically, repeated visits to roost sites through time are necessary to provide inferences on changes in colony size. Because populations of bats may use several roosts through time, it is critical to locate roosts being used in the area, and determine the number of roosts in the area being studied (Hayes et al. 2009). Field staff responsible for monitoring roosts should strive to minimize disturbance to roosts and roosting bats, as increased disturbance can result in roost abandonment and population declines (Mann et al. 2002).

Bats roost individually or in large aggregations in a variety of sites, including caves, under bridges and in buildings, as well as in trees. Monitoring roosts in wetlands could involve any of these sites, and each introduces unique challenges to those conducting the monitoring efforts. Caves and other subterranean roosts, as well as buildings and bridges, can either be sampled internally to directly count numbers of bats, or externally to count bats as they exit. Internal surveys can result in disturbance, but can provide fairly precise estimates of bat abundance. With the recent emergence of white-nose syndrome, field gear and footwear should be disinfected with 10 % bleach for a minimum of 10 min to inactivate the fungus (*Geomyces destructans*) associated with this disease. White-nose syndrome is a disease responsible for significant deaths in bat populations throughout the eastern United States. Infrared cameras and night vision goggles allow precise estimates of the numbers of bats emerging from roost sites (Kunz et al. 2009a, b), as long as all roost openings are monitored simultaneously. Monitoring bats that roost in trees can be difficult, because exit points are difficult to determine and bats roosting in trees tend to show low fidelity to roost sites (Barclay and Kurta 2007). Radio telemetry is an effective way to determine selection of tree roosts and fidelity to roost sites so that monitoring programs can be established in areas.

7.5.2 Small Mammals

A rich diversity of terrestrial small mammals use wetlands. Small mammals are typically secretive and nocturnal, and serve numerous ecological roles important in wetland ecosystems. Specifically, small mammals serve as seed dispersers, prey for many vertebrate species, and are important in nutrient cycling (Dickson 2001). Because of their variable sizes and niche differences, adequate monitoring of small mammal communities requires multiple techniques, with the understanding that small mammal populations fluctuate considerably throughout the year. Typically, population size of many small mammal species peaks during fall and is lowest during early spring prior to the first reproductive pulse. Monitoring strategies should be designed to encompass the entire small mammal community, ranging from the smallest (e.g., shrews) to the largest (e.g., woodrats) species.

7.5.2.1 Live and Removal Trapping

Snap-traps can be used to provide unbiased estimates of species composition, relative abundance, occurrence, and distribution of small mammals. However, because using snap-traps involves removing individuals from the population, this technique should be used only once annually on the sites being studied. Snap-traps should be placed along transects rather than grids to maximize capture rates and increase the probability of sampling all species present (Pearson and Ruggiero 2003). We recommend placing traps along microhabitat attractive to small mammals, such as woody debris and trails. Traps can be placed systematically along transects at regular intervals (e.g., 10–15 m) or stratified in different wetland types. Sampling duration will depend on specific objectives of the monitoring program, but generally traps should be operated for several days to a week to adequately estimate species composition. There are two general sizes of snap traps: those designed to sample smaller mice and shrews, and those capable of capturing larger mice and rats. To improve accuracy of population estimates, both trap sizes should be used. Pre-baiting snap traps can improve capture rates once sampling begins, but if pre-baiting is not possible, field staff should consider lengthening the trapping period by up to 4–6 nights (Ritchie and Sullivan 1989).

Live trapping small mammals is used widely, and various trap designs (e.g., Longworth, Sherman) are available to capture species ranging from shrews to larger rodents. Typically, using multiple live-trap types simultaneously more adequately samples the entire small mammal community (Anthony et al. 2005). Trap arrangement will depend on whether estimating species occurrence or density is an objective. Live traps can be placed along transects similar to snap-traps or randomly within wetland habitats to estimate species occurrence or composition. However, if density estimates are needed, traps should be placed using a grid or web design (Greenwood 1996). Spacing among traps usually ranges from 10 to 25 m, and traps are usually baited with peanut butter or grains (e.g., oats), often in combination.

Pre-baiting live traps can improve capture success, although results among studies are highly variable and often conflicting (Edalgo and Anderson 2007).

7.5.2.2 Pitfall Trapping

As discussed, pitfall trapping can be used to study herpetofauna, but this technique can be equally effective at evaluating occurrence and spatial distribution of some small mammals. For example, fossorial small mammals that have locally confined movements, such as shrews, are readily captured in pitfall traps (Laakkonen et al. 2003). Pitfall traps can be more effective and efficient than other methods at sampling small mammals, particularly with rare species (Umetsu et al. 2006). However, small mammals captured in pitfall traps can suffer mortality in the traps, so traps should be covered while sampling is not occurring. Pitfall traps vary in size and shape, but all consist of a collection device (e.g., buckets, drums, or tins) buried in the ground with the top level with the ground. Drift fences that funnel animal movements into the pitfalls can improve capture rates. Pitfall traps should be placed in areas most likely to be encountered by the species of interest. For additional details on pitfalls and drift fence designs, please see the previous sections on amphibian and reptile monitoring.

7.5.3 Wetland-Dependent Rodents

Nutria (*Myocastor coypus*) are indigenous to South America, but were introduced in North America primarily for production and sale of their pelts (Kinler et al. 1987). They are most abundant in freshwater and brackish wetlands, and construct burrow systems along banks that range from simple tunnels to complex systems with multiple entrances and exits (Bounds et al. 2003). Nutria forage on various species of wetland plants, and their foraging and burrowing activity can significantly compromise the function and quality of wetlands (Shaffer et al. 1992). Monitoring for nutria sign and evidence of foraging can provide estimates of relative abundance and be used to make decisions on when population control is needed. Signs of nutria include burrows, resting structures, paths or runs where animals exit the water, evidence of rooting or excavating plants, plant herbivory, and feces (Gosling and Baker 1991). Generally, these signs increase as density increases, and the amount of destruction often is greater during winter when food resources are limited.

Beavers (*Castor canadensis*) are the largest rodent in North America, and have well developed family units called colonies. Beavers consume primarily herbaceous and woody plants, and the leaves, buds, bark, and twigs of woody plants are typically the most important component of the diet throughout their range. Beavers are a true keystone species, as their dam-building and foraging activities have profound effects on wetlands. Surveying beaver populations often

involves estimating numbers of colonies and mean colony size (Novak 1987). Colony size can be difficult to determine, and varies spatially and temporally. Aerial surveys have been widely used to estimate size of beaver populations, and are typically conducted during fall. If precise estimates of beaver abundance are desired, beavers can be trapped in box traps, snares, or specially designed traps (e.g., Bailey or Hancock traps; Baker and Hill 2003). If trapping is used, field staff should use enough traps to capture all colony members as quickly as possible, as beavers become trap-shy quickly.

Muskrats (*Ondatra zibethicus*) are widely distributed in wetlands throughout North America. Muskrats require emergent, submersed, or shoreline vegetation, and often use this vegetation for forage and to construct houses. Houses provide protection from predators and safe areas for raising young, and coupled with burrows and tunnels along banks of wetlands, are signs of muskrat presence. Counting houses is used widely to monitor muskrat populations, and can be conducted by air or boat (Erb and Perry 2003). Abundance of houses can vary seasonally, as water levels and spring breeding activity can result in either decreases or increases in the number of houses constructed (Palmisano 1972). If an estimate of muskrat density is desirable, mark-recapture techniques using cage traps are effective (Clark and Kroeker 1993). Traps should be baited with fruits or vegetables, and covered with natural vegetation to provide concealment and increase capture success. Captured muskrats should be carefully removed from the trap by grabbing their tail, and placed head first into a wire cone for marking with standard ear tags (Erickson 1963).

7.5.4 Carnivores

Terrestrial carnivores are prominent species in wetland systems, and include truly carnivorous species such as river otter (*Lontra canadensis*), as well as the omnivorous raccoon (*Procyon lotor*). Carnivores range in size from diminutive weasels, to larger canids such as the coyote (*Canis latrans*), and even large felids such as bobcats (*Lynx rufus*) and cougars (*Puma concolor*). Carnivores are secretive species, being most active during crepuscular and nocturnal periods. As such, monitoring techniques are highly variable, with most relying on attracting animals to a site where they are captured or sign is collected (e.g., tracks, hair). Additionally, passive techniques (e.g., remote cameras) can be used to monitor abundance or distribution. Similar to many other mammals, monitoring carnivore populations requires a working knowledge of potential species at a site and a willingness to use multiple techniques.

7.5.4.1 Live Trapping

Cage traps are widely used to capture mammals in wetlands, including raccoons, river otters, mink (*Neovision vision*), and beaver. Animals captured in cage traps

Fig. 7.11 Various mammals (e.g., bobcat [*top*], mink [*middle left*], river otter [*middle right*]) can be effectively and humanely captured in foot-hold traps; wire cage traps also are effective at capturing small mammals (e.g., raccoon) (Published with kind permission of © Ryan Williamson and Mike Byrne 2014. All Rights Reserved)

typically must be sedated or restrained if handling is necessary. Field staff responsible for chemical immobilizing mammals should refer to Kreeger and Arnemo (2007) for information on appropriate methods and drugs for safely removing individuals from traps. If marking animals is necessary to meet management objectives, cage traps are effective and efficient. Foot-hold traps and other restraining traps (e.g., snares) can be used to capture larger carnivores, and capture rates for many species (e.g., coyotes, bobcats) increase dramatically when using these trap types (Fig. 7.11). Trapping with foot-hold traps requires skill and training, as well as the ability to remove captured animals safely from the trap. As such, monitoring carnivores through trapping with restraining traps is time and

labor intensive. However, if research objectives require radio marking of species such as coyotes and large felids, using foot-holds to capture individuals may prove most efficient (Shivik et al. 2005). Alternative designs to foot-restraint traps, such as the EGG™ trap, have been shown to be more effective and efficient than cage traps in capturing raccoons (Austin et al. 2004).

7.5.4.2 Camera Surveys

Many carnivores are rare and elusive, so remote cameras offer opportunities to observe behavior, estimate abundance, and evaluate occurrence of various species across broad spatial scales. Remote cameras have numerous advantages over other survey techniques, producing relatively large datasets with minimal effort and labor. Likewise, cameras can be set to detect carnivores of varying sizes and because animals do not have to exhibit any particular behavior (e.g., stepping into a trap) to be photographed, they have little bias (Kays and Slauson 2008). However, the cost of initiating a camera survey can be substantial relative to other passive monitoring techniques (e.g., track surveys, scent stations).

Most surveys designed to monitor carnivores should use either active infrared or passive infrared sensors. Active sensors require multiple units, whereas passive sensors only require one sensor component; passive sensors are easier to set up and are manufactured widely. Regardless of the sensor used, most camera sets are aimed at game trails or baited sites. Field staff using remote cameras should become familiar with features of the cameras used, and develop standardized protocols in regards to the height of deployment and the distance between the camera and the intended target.

Beyond deciding which types of cameras to use and how to deploy them, those using remote cameras to survey carnivores also need to consider whether or not to use bait, and whether camera avoidance (through flashes) is a concern (Wegge et al. 2004). Likewise, the cost of purchasing individual cameras, monitoring units in the field, and reviewing photographs should be considered before initiating a camera survey. Designing a camera survey will ultimately be an exercise in balancing the best data with the most efficient use of available cameras. Typically, cameras should be spaced relative to the home range characteristics of the species' studied (Kays and Slauson 2008), although the arrangement of cameras within the surveyed area can be highly variable (Gompper et al. 2006).

7.5.4.3 Genetic Sampling

The use of noninvasive hair sampling methods has increased rapidly during the past decade, and is now being used widely to sample carnivores in a variety of habitat types, including wetlands (Kendall and McKelvey 2008). Hair collection methods vary, but generally use baited sets to encourage carnivores to deposit hair (e.g., hair corrals, Fig. 7.12), or unbaited sets where natural behaviors facilitate the animal

Fig. 7.12 Non-invasive hair collection site for black bears (*Ursus americanus*) with bait (*top inset*) and barbed wire (Published with kind permission of © Michael Hooker, Jared Laufenberg, and Carrie Lowe 2014. All Rights Reserved)

leaving hair samples (e.g., rub trees used by bears). Regardless, hair collection provides genetic samples that can be used to estimate density using mark-recapture methods, and can allow researchers to track individuals on the study site (e.g., estimate survival through time). Hair samples also can be used for stable isotope analyses to learn about carnivore diets or migratory movements (Fox-Dobbs

et al. 2007). However, analyzing large numbers of hair samples can be costly, and genetic material recovered in the field can degrade rapidly in wetland environments. Monitoring of collection stations should be frequent enough to recover hair in a timely manner so as to minimize degradation of DNA. This monitoring protocol will differ by species and site (Goosens et al. 1998). Degradation of hair samples is influenced by environmental factors, but a good rule of thumb is to collect samples at least every 7 days.

Amplifying DNA from fecal samples has gained attention recently as a way to monitor carnivore abundance and individual ecology (e.g., prey selection by known individuals). Feces contain sloughed epithelial cells, and a single sample typically provides enough material to recover DNA multiple times. Species depositing feces can often be identified with considerable success, although identification of individuals is highly variable (McKelvey et al. 2006). Fecal samples rapidly deteriorate, so field staff should be trained on ways to dry samples in a manner that allows them to be stored prior to analysis. Drying and storage protocols differ greatly among species, hence someone interested in initiating a study using fecal genotyping should either conduct pilot studies to determine which technique(s) are most effective in their particular situation, or use techniques proven successful on similar species (Schwartz and Monfort 2008).

7.5.4.4 Track and Sign Surveys

During the course of normal daily activities, carnivores leave signs that can be used to determine species distribution, relative abundance, and occurrence across the landscape. Sign surveys are inexpensive, use the tracking medium on site (e.g., snow, sand), and can be conducted by practically anyone trained to identify the track of interest. Likewise, scat surveys are commonly conducted to determine carnivore diets, but the ability to identify species and individuals, as well as the recent use of dogs to locate scat (see MacKay et al. 2008 for a thorough review) have increased the usefulness of scat surveys. Additionally, some species deposit scat in a way that is easily detected or can be used to survey population trends (e.g., river otters; Kruuk et al. 1986; Heinemeyer et al. 2008). Regardless, the probability of detecting a species and monitoring abundance ultimately depends on survey effort and access to the study site and survey area (Harrison et al. 2004).

Beyond simply noting tracks left through normal activities, monitoring carnivore populations using tracks typically involves the use of track plots, track plates, or scent stations. Track plates require the use of manufactured materials, whereas track plots and scent stations typically rely on prepared substrates (e.g., sand, sifted dirt). Scent stations are sites where tracking substrate (soil, sand) is prepared or placed, then baited with something that serves as an olfactory attractant to stimulate visitation by carnivores. Scent stations have been widely used to estimate occurrence, distribution and relative abundance of carnivore species (Sargeant et al. 2003; Zielinski et al. 2005). Track plates are metal plates coated with smoke residue that provides a high-quality tracking medium, and have been used

to survey numerous species worldwide (Ray and Zielinski 2008). Track plates are either designed to be closed (contained within a structure that the animal enters) or open (no structure), and may be used to target specific species or groups of species. Scent stations and track plots will readily detect most felids and canids, hence they are widely used in a variety of habitats. However, a number of factors, such as weather, season, space use patterns, and species density, are known to affect how individuals respond to track stations (Harris and Knowlton 2001); these should be considered when designing and implementing track surveys. Likewise, scent stations provide more reliable data when used across large spatial scales and with large samples of stations. Ultimately, numbers of stations deployed and the choice of survey design will depend on logistical constraints. We encourage researchers using scent stations to carefully consider recent assessments of sampling designs (Sargeant et al. 2003) when implementing scent station surveys.

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Student Exercises

Laboratory Exercise #1: Herpetofaunal Sampling Laboratory

Location and Time: Herbaceous or forested wetland with standing water during the growing season (ideally spring or summer).

Description: The goal of this field lab will be to build competency in common techniques used to sample amphibian and reptile communities. Activities described herein could be assigned in their entirety or only portions of the exercise used. If completed in its entirety, students will have an understanding how to use pitfalls, funnel traps, and nets to capture amphibians, use basking traps to capture turtles, and collect biological information on captured amphibians and turtles.

Supplies and Equipment: Silt fencing with attached wooden stakes, 3-lb sledge hammer, rake, tape measure, shovel, six 19-L (5-gal) buckets, six sponges, six rectangular funnel traps, dip nets, seine net, basking turtle trap, snake tongs, calipers, organism bags, spring scales, and disposable gloves (worn while handling animals). Given that live animals will be captured and handled, scientific collection permits must be secured, and an Institutional Animal Care and Use Committee (IACUC) protocol may be required by your institution.

Set-up Instructions: Identify a wetland for sampling and remove leaves, debris, and herbaceous vegetation in a 0.5-m wide band 10 m above the high waterline and parallel to the wetland for 40 m. Dig holes for 5-gal pitfalls every 10 m, with two pitfalls paired at each end. Pitfall tops should be flush with the top of the ground. Erect fencing such that it passes next to each pitfall and 0.5 m past the end pitfalls. Cover the base of the fence with soil to prevent trespass of animals. Fill pitfalls with 2.5 cm of water from the wetland and place one sponge in each pitfall. Place one funnel trap on each side of the fence, and one trap in shallow water ($<$10 cm) in each cardinal quadrant of the wetland. Construct a basking trap following Brown and Hecnar (2005), and place in water $>$1 m depth.

Sampling Instructions: After deployment of traps, check in $<$24 h. Identify all captured juvenile and adult amphibians and reptiles in pitfall and funnel traps, and measure the snout-to-vent length and mass. Amphibians can be placed in plastic bags when processing but should be rehydrated with water from the wetland before release. Lizards can be placed in cloth bags or plastic containers. When handling lizards, avoid grabbing by the tail because most species will autotomize it as an anti-predator response. Captured snakes should only be handled after verifying they are not venomous; non-venomous snakes can be temporarily held in a well-secured pillowcase. Venomous snakes should not be handled and be removed from traps using snake tongs or snake tubes. Aquatic and terrestrial turtles can be placed in 5-gal buckets or large plastic containers. Care should be taken when handling snapping turtles because their bite can cause injury. Larval amphibians can be sampled using dip and seine nets following Schmutzer et al. (2008). Identify and

enumerate all larval amphibians by species. As a second exercise, determine developmental stage according to Gosner (1960).

Brown CL, Hecnar SJ (2005) Capture success of northern map turtles (*Graptemys geographica*) and other turtle species in basking vs. baited hoop traps. Herpetol Rev 36:145–147

Gosner, KL (1960) A simplified table for staging Anuran embryos and larvae with notes on identification. Herpetologica 16:183–190

Schmutzer AC, Gray MJ, Burton EC, Miller DL (2008) Impacts of cattle on amphibian larvae and the aquatic environment. Freshw Biol 53:2613–2625

Laboratory Exercise #2: Amphibian Marking Laboratory

Location and Time: This laboratory can be completed in combination with the Herpetofaunal Sampling Laboratory or in the classroom with preserved laboratory specimens.
Description: The purpose of this laboratory is to practice common herpetofaunal marking methods. Activities in this laboratory can include marking techniques for adult or larval anurans. If this laboratory is completed in its entirety, students will have the experience necessary to mark amphibians using scissors, Visible Implant Elastomers (VIE), and Passive Integrated Transponder (PIT) tags.

Supplies: Wild captured or preserved amphibians (3 per student or team), surgical grade scissors, VIE mix and injection syringes (Northwest Marine Technologies, Inc.), PIT tag supplies (PIT tags, tag reader, and injection syringe [Biomark, Inc.]), 95 % EtOH or 2 % chlorhexidine diacetate solution, disposable gloves, and appropriate marking schemes. If live amphibians are captured, scientific collection permits must be secured, and an IACUC protocol may be required by your institution.

Set-up and Instructions: Wild amphibians can be captured during the Herpetofaunal Sampling Laboratory, and preserved specimens (e.g., *Necturus*, *Lithobates*) can be acquired from most biological supply companies. If wild animals are used, it is important to sanitize all marking instruments using 95 % ETOH or 2 % chlorhexidine diacetate solution. Prior to the lab, review and select one of the published marking schemes for amphibians (see Donnelly et al. 1994 or Ferner 2010). Students should work on a stable, clean surface. Distribute at least three amphibians to each student or group (i.e., one amphibian per marking technique). Students should wear disposable gloves and change them between animals. Practice toe clipping as described in the chapter. The excision should be made at the most proximal joint; bones and thumbs should not be cut. Excise the appropriate toes to number the individual as #234. Once completed, use a different animal for VIE marking. The elastomer should be injected under the skin where very little pigment occurs; the ventral side of most amphibian legs is a good location. Care should be taken to slide the needle under the skin (forceps can help) so as to not pierce muscle or organs. When working with live amphibians, students should work in pairs, with one student holding the animal securely. Students should conceive schemes that

allow for individual or batch marking using different VIE colors and marking locations on the amphibian. Finally, practice injecting a PIT tag under the skin mid-body on ventral and dorsal sides. Scan the tag prior to and after injecting. At the conclusion of the laboratory, students should discuss advantages and disadvantages of each technique. Processing time for each technique should be recorded and considered.

Donnelly MA, Guyer C, Juterbock JE, Alford RA (1994) Techniques for marking amphibians. In: Heyer WR, Donnelly MA, McDiarmid RW, Hayek LAC, Foster MS (eds) Measuring and monitoring biological diversity: standard methods for amphibians. Smithsonian Institution Press. Washington, DC, pp 277–284

Ferner JW (2010) Measuring and marking post-metamorphic amphibians. In: Dodd CK (ed) Amphibian ecology and conservation: a handbook of techniques. Oxford University Press, Oxford, pp 123–142

Laboratory Exercise #3: Small Mammal Trapping Laboratory

Location and Time: Perimeter of a forested or emergent wetland in areas not subject to permanent flooding, or the adjacent upland can be used; performed preferably during fall.

Description: The goal of this lab will be to expose students to basic live-trapping techniques used to estimate abundance, density, or distribution of small mammals. This lab will require students to establish trapping grids prior to setting traps, set traps in the afternoon, and check all traps the following morning. Students will gain an understanding of how to establish trapping grids, set traps, capture and mark small mammals, and collect basic morphological and demographic data.

Supplies: Sherman or Longworth traps, flagging tape, peanut butter and oats, wax paper, clear plastic bags, spring scale, and disposable gloves. Given that live animals will be captured and handled, scientific collection permits must be secured, and an IACUC protocol may be required by your institution.

Set-up Instructions: Identify an area that is not permanently flooded with suitable herbaceous or woody vegetation to harbor small mammals. Establish a trapping grid with a minimum of 25 traps (e.g., 5 × 5 matrix) with each trap placed 10 m apart. Place flagging at each trap site so that traps can be quickly relocated. Prior to setup, mix oats and peanut butter together. Cut 3 × 3 in. squares of wax paper, and place ½ teaspoon of peanut butter mixture in center of wax paper, fold the ends together, and twist so that the peanut butter mixture is inside a pouch of wax paper.

Bait each trap by placing the bait balls (inside of wax paper) at the back of each trap. Traps should be placed in areas likely to be used by small mammals, such downed woody debris, stumps, etc. Place one trap at each trapping site during the afternoon or evening prior to the day that traps will be checked. Trap sites should be spatially referenced using a GPS unit to ensure re-location of the sites during monitoring.

Sampling Instructions: All traps should be checked <16 h after being set. Mammals captured in traps can be removed by opening the door of the trap, placing a clear plastic bag over the trap door, and turning the trap over to drop the captured individual into the bag. Each person handling mammals should wear disposable gloves. Mammals can be carefully removed from the bag by pinching the fur around the back of the neck. Larger mammals, such as cotton rats (*Sigmodon hispidus*), can be further restrained by holding their tails with the other hand. Identify mammals to species and determine sex. Small mammals can be identified using Peterson (2006). Body weight can be determined by closing the plastic bag briefly, and hanging the bag from a spring scale. Capture data should be summarized by species, and inferences made about their association with different cover types. For instance, students could simultaneously measure habitat characteristics (e.g., canopy cover, vegetation type, vegetation density) at sites where mammals are successfully captured and compare them to sites where mammals are not captured.

Peterson RT (2006) Peterson field guide to mammals of North America, 4th edn. Houghton-Mifflin, Boston

Laboratory Exercise #4: Evaluating Wildlife Sign for Surveys

Location and Time: In-class laboratory with slide presentation.

Description: The goal of this lab is to train students to identify tracks and sign of common mammals likely to be encountered in various wetland habitats. The slide presentation is designed to provide students with information on how to identify tracks based on numbers of toes, distance between front and rear feet, morphological characteristics of feet among species, as well as gait patterns. The lab is most effective if students have plaster casts of the species covered in the presentation so that they can view tracks and study them.

Supplies: Slide presentation (PDF format) by Mike Chamberlain is available for use at: http://fwf.ag.utk.edu/mgray/WetlandBook/WildlifeSignsLab.pdf. If track casts are unavailable, a reference collection can be created using Plaster of Paris available at craft stores.

Set-up Instructions: The presentation describes how to identify tracks of mammals based on numbers of toes. Specifically, students should be instructed on ways to recognize 2-toed hooved species, 4-toed species with heal pads, 5-fingered species, and species with 4 front toes and 5 hind toes. Mammals occupying wetlands vary by locale, but larger, more common species, such as white-tailed deer, feral hog, coyote, red fox, gray fox, bobcat, cottontail and swamp rabbits, raccoon, opossum, muskrat, beaver, mink, river otter, and black bear, are covered in this lab.

Laboratory Exercise #5: Waterbird Identification, Sexing and Aging Laboratory

Location and Time: Indoor laboratory during fall or spring. This lab should be conducted prior to the Waterbird Population Monitoring field lab.

Description: The goal of this lab will be to expose students to waterbirds that are of management and conservation interest in the state and region. In addition, sexing and aging techniques by plumage will be demonstrated for species where this information is of management interest, such as waterfowl. The students will be responsible for identification of the species that are presented in the lab and sexing and aging for a subset of those species.

Supplies: Photographs of species of interest and study skins where possible, waterfowl wings for males and females of species of interest, bird field guide, and waterfowl wing sexing and aging guide (Carney 1992). Also, a slide presentation by Matthew Gray on the identification of North American waterfowl is available: http://fwf.ag.utk.edu/mgray/wfs560/WaterfowlID.pdf.

Classroom Instruction: Develop a list of waterbirds that students will be responsible for learning including waterfowl, wading birds, shorebirds, and secretive marsh birds. Include species that are generally of management interest for the state or region, including species that will likely be encountered during the field lab. Develop a slide presentation in which the instructor reviews the identification characteristics of the species on the list. The instructor should also review the sexing and aging techniques for species of interest. After the presentation is complete, the students will break into 2-person teams to review the specimens that are available using their field guides to make a positive identification. In addition, they can use the U.S. Fish and Wildlife Service sexing and aging guide for waterfowl wings as additional practice (see below).

Lab Proficiency Quiz: When each student (or team) believes they have mastered identification, they can attempt a proficiency quiz. The quiz should include images of birds at varying distances. They will be declared proficient if they correctly identify >70 % of the birds. Students should demonstrate proficiency prior to the field lab.

Carney SM (1992) Species, age and sex identification of ducks using wing plumage. U. S. Department of the Interior, U.S. Fish and Wildlife Service, Washington, DC. Northern Prairie Wildlife Research Center, Jamestown. http://www.npwrc.usgs.gov/resource/tools/duckplum/index.htm

Laboratory Exercise #6: Waterbird Population Survey Laboratory

Location and Time: Wetlands with open water and mudflats during fall or spring.

Description: The goal of this field lab will be to demonstrate population survey methods for waterfowl, shorebirds and wading birds, and allow students to practice the methods, analyze the data, and interpret the results.

Supplies: Binoculars and spotting scopes, laser rangefinders, study area maps, bird field guides, clipboards, data sheets, and 1-m stakes for each student team.

Set-up Instructions: Identify a wetland with open water and mudflats for survey. Divide the wetland area into sampling units based on geographic area (e.g., cardinal quadrants) or habitat. Use the maps, laser rangefinders, and stakes to delineate the spatial extent of each survey location. Each location should survey approximately the same viewable area. Divide the class into 2–4 person teams and assign each team to a location.

Survey Instructions: At the beginning of the laboratory, explain the goals of the exercise and review the count protocols and identification for species likely to be encountered. If there are species that are difficult for novices to identify (e.g., various sandpiper species), group these as morpho-species (e.g., western, least and semi-palmated sandpipers might be counted simply as "sandpipers"). Deploy the teams to conduct the counts, ideally within 3 h of sunrise or sunset. Each team should spend the first 30 min scanning the area and identifying the waterfowl, wading birds and shorebirds to species. The last 15 min of the count period will be spent estimating a count for each species. As an additional exercise that students can practice focal surveys, where bird activities are recorded (e.g., feeding, walking, swimming, inactive, sleeping, antagonistic, alert) for 1 min. Students are encouraged to read Davis and Smith (1998) for an example of collecting and analyzing activity budget data.

Data Analyses: Data from each team should be entered into a database, including date, time, study area(s), environmental conditions (temperature, wind speed and direction, precent cloud cover, precipitation), observers, species and counts. Each team will summarize the data collected by the entire class to make inferences about waterbird use of the area. After conducting this exercise over several years, students can begin to look for seasonal or yearly changes in species composition and abundance, and develop hypotheses for why these demographics may be fluctuating.

Written Assignment: Each team will be responsible for writing up a lab report, summarizing the objectives, methods, and results from the surveys and discussing the implications of the results and answering critical questions about changes in waterbird populations. Additionally, each team may present their results orally, and a class discussion can explore the lessons learned from this experience.

Davis CA, Smith LM (1998) Behavior of migrant shorebirds in playas of the Southern High Plains. Condor 100:266–276

Index

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