APPENDIX B. METHODS OF ANALYSIS FOR WATER QUALITY VARIABLES

Several methods used to analyze certain water quality variables are discussed briefly in this section. Additionally, relevant publications are referenced as appropriate. Some tips on analysis will also be presented to help users to be efficient in determinations. As with any environmental analysis, the most efficient strategy when learning a new technique is to visit a laboratory where it is routinely performed. The methods that will be discussed include those for light transmission; total suspended solids; total nitrogen, total phosphorus, and dissolved inorganic nitrogen and phosphorus; conductivity; chlorophyll *a* and ash free dry mass (AFDM) for algal biomass; and microscopic identification to determine the algal taxa present. Brief discussion of secondary indicators of eutrophication (algal production, dissolved oxygen concentrations, limiting nutrients and macroinvertebrates) will also be presented. As discussed above, determination of other factors such as hydrology, geology, soil characteristics may also be necessary.

PHYSICAL WATERBODY CHARACTERISTICS

LIGHT TRANSMISSION

Total suspended solids and dissolved humic compounds can absorb light and limit algal biomass. As periphyton biomass increases, particulate matter sloughed and eroded from the periphyton also increases, reducing transparency. Light transmission measurements may be required. Light transmission can be measured using turbidity meters (transmissometers or turbidometers). Use of these meters is described in Standard Methods (APHA 2000). A quick method for determining light transmission is use of a black disk and an underwater periscope (Davies-Colley 1988). The path length for transparency is measured horizontally in shallow streams, as opposed to vertically in lakes, reservoirs and deep rivers or estuaries. The vertical water column in relatively clear-water, gravel/cobble bed streams/rivers is usually insufficient to determine Secchi disk depth.

LIGHT AVAILABILITY

Light availability can be measured directly with a light meter as photon flux density (μmole quanta m^{-2} s^{-1}), but such measurement vary temporally. Measures of % canopy cover, TSS and average water depth, light transmission with a black disk or periscope, and stream direction provide measures of relative availability of light which can be related to a regional average. Light intensity varies so much during a day or with weather from day to day that indicators of relative light intensity may be a more precise indicators of light availability than one-time measurements of light intensity.

Light availability for photosynthesis can be reduced by the amount of total suspended sediment (TSS) in the water column, light attenuation caused by dissolved compounds, river depth, and channel shading. In addition to scouring algae, TSS also attenuates light to benthic algae. Dissolved organic humic compounds can absorb light, and if they are present in high enough concentrations, they can prohibit algal growth. Similarly, forest canopies can shade stream channels (Dodds et al. 1996). This shading can lead to rivers with relatively high nutrient concentrations, but with negligible sestonic or benthic algal biomass. In such cases, nutrient control may have few immediate benefits.

If there is seasonally high TSS or shading (e.g., deciduous forests), the high nutrients may cause excessive periphyton algal biomass only during certain times of the year. An example of this would be

streams where snow melt is common in the spring; this could lead to high levels of TSS and low algal biomass, but during stable flows in summer, low TSS and high algal biomass. Finally, very deep channels will not usually have excessive algal biomass except at the margins, since limited amounts of light reach most of the bottom (Allan 1995), and sestonic algae are mixed frequently throughout the water column, which reduces available light while increasing respiration (Welch 1992). Therefore, net productivity (gross production minus respiration) decreases with depth of mixing.

FLOW AND VELOCITY

Flow and velocity measurements are important for determining nutrient loadings, concentrations, and distributions. Flow volume or discharge is easily calculated based on stream channel area and velocity. Velocity is typically measured with a stream gauge or current meter. See http://water.usgs.gov/pubs/circ1123/collection.html for more details.

TOTAL SUSPENDED SOLIDS AND VOLATILE SOLIDS

It can be useful to quantify total suspended solids because of their effect on light attenuation, and the determination of volatile solids may be of interest to determine if the total suspended solids are from organic sources. The methods for total suspended solids and volatile solids are presented in Standard Methods (APHA 2000).

TEMPERATURE

Temperature can be an important variable in determining alkalinity, saturation, and rates of chemical and biological reactions. It is a simple but useful measurement to include in a sampling regime. Methods for temperature measurement in the field and laboratory are described in Standard Methods (APHA 2000).

CHEMICAL WATERBODY CHARACTERISTICS

NUTRIENT ANALYSES

Nutrient analyses are the most important indicators for determining sources of nutrients and for monitoring the effectiveness of control programs. The analyses for soluble reactive phosphorus and dissolved inorganic nitrogen are mentioned first because they are the forms available for algal uptake and because they are the forms determined (after digestion) for total nitrogen and total phosphorus. In general, determinations of nutrient concentrations by field kits are only adequate to identify potential problems. If many nutrient assays are required to define the problem accurately, laboratory procedures are more cost effective and have greater sensitivity.

In nutrient-poor systems, levels of dissolved inorganic nutrients are generally near the limits of detection of the assays used. For example, phosphate levels in excess of 30 µg/L saturate uptake by algae, but this is the lower limit of detection in many laboratories. Care must be taken that the assay procedure used matches the question being asked.

The assay for dissolved or soluble reactive phosphorus from Standard Methods (APHA 2000) should be followed. A common source of contamination that causes problems with soluble reactive phosphorus analysis is the use of phosphate containing detergents to wash laboratory equipment. It is good practice to use phosphate-free detergents in the laboratory for this reason. Another important problem is the source of low-phosphorus water for dilution and blanks. Absorbance should be very low (0.001-0.003 absorbance units per cm) for such purposes.

The soluble reactive phosphorus assay does not determine only phosphate, because the chemicals in the assay react with some dissolved organic compounds that contain phosphorus other than ortho-phosphorus. It has been demonstrated that increased phosphorus deficiency in algae in natural systems leads to a lower percentage of biologically available phosphate in the chemically determined soluble reactive phosphorus (e.g., Dodds 1995). Unfortunately, the identity of the remaining fraction of soluble reactive phosphorus is unclear, so soluble reactive phosphorus values from natural waters are difficult to interpret, unless the values are fairly high (e.g., above 10 mg/L). In some such cases (e.g., groundwater or wastewater input), a large portion of the soluble reactive phosphorus may actually be in the form of phosphate, and the assay will provide a fairly accurate measure of the phosphate immediately available for algal consumption. The soluble reactive phosphorus assay is particularly useful to determine phosphate in sewage (where most soluble reactive phosphorus is phosphate) and to analyze digested samples for total phosphorus. A method for the analysis of PO_4^{3-} (orthophosphate) is also available in Standard Methods (APHA 2000).

Analysis of ammonium is straightforward with the phenate method (APHA 2000). Note that ammonium (NH_4^+) is the ion that identifies the available nutrient, and ammonia (NH_3) is the gas, known as unionized ammonia, which is the fraction that can cause toxicity. Contamination of ammonium assays can occur from scratched glassware and airborne ammonia gas, which can come from smoke (tobacco and otherwise), cleaning products with ammonia, and newly cut grass. Care should be taken to avoid these potential sources of contamination.

Nitrate is commonly measured by reduction to nitrite in a copper-cadmium reduction column (APHA 2000). Nitrite can be analyzed alone to correct estimates of nitrate, but in most studies of streams, nitrite is assumed to be a relatively small fraction of nitrate and as such is not accounted for. Cadmium is toxic and difficult to handle and dispose. Some packaged nitrate kits use cadmium pillows that are added to the sample. Appropriate precautions for handling and disposing of samples are recommended if these kits are used. Other (e.g., hydrazine) nitrate techniques may be more prone to interference or reduced efficiency. Automated analysis methods with segmented flow autoanalyzers are commonly used to speed processing and maintain sensitivity. Ion chromatography can be used successfully for nitrate determinations, but it should be kept in mind that this method is not sensitive enough for nitrate values typical of many moderately productive systems.

Total nitrogen and total phosphorus require digestion to dissolved inorganic forms before analysis. There are a number of available techniques. An important point is that the efficiency of digestion of organic materials varies with procedures and waters being analyzed. Regardless of the procedure chosen, solutions with known concentrations of organic compounds (e.g., urea for nitrogen, ATP for phosphorus) should be added to natural water samples in known concentrations and analyzed to check for complete digestion.

Persulfate digestion is commonly used for total phosphorus. This procedure can be modified to oxidize organic phosphorus to phosphate, as well as organic nitrogen to nitrate (Ameel et al. 1993). Careful attention to pH of the samples is necessary in these digestions (the digest must remain alkaline for nitrogen digestion, but if too much persulfate is used, it may not become acidic later in the digestion and incompletely decompose the phosphorus) and appropriate concentrations of fresh reagents should be used to allow for complete digestion of both organic nitrogen and phosphorus.

Persulfate digestion converts all forms of nitrogen except N_2 gas to nitrate. If nitrate analysis is not easily accomplished in the laboratory, it may be desirable to use a Kjeldahl digestion procedure (APHA 2000) for total nitrogen analysis. In this procedure, all nitrogen forms but nitrate, nitrite and nitrogen gas are digested to ammonium. If this procedure is employed, it is still necessary to analyze for nitrate and nitrite to determine total nitrogen. Because the sensitivity and accuracy of the cadmium reduction method for nitrate are greater than analyses for ammonium, and the toxicity and corrosiveness of the digestion procedure are less, persulfate digestion and nitrate analysis is usually preferred to Kjeldahl.

Analyses for TN, TP, phosphate, and nitrate can also be used to calculate water column N:P ratios.

CONDUCTIVITY AND PH

Conductivity may serve as a first indicator of total nutrients (although it indicates total ions which are much more abundant than, and not always closely correlated to, nutrients), and pH may be of interest as a variable indicating impairment. Both of these analyses are most easily accomplished with electronic probes. Refer to Standard Methods (APHA 2000) for the particulars of the analysis.

DISSOLVED OXYGEN

Analyses of dissolved oxygen, for measurements of primary production and determination of low oxygen demand should be done with a titrametric method or polarographic sensor. The titrametric is more accurate, but more time consuming. Standard Methods should be consulted for these analyses (APHA 2000). If diurnal measurements of oxygen are performed, procedures outlined by Marzolf et al. (1994) should be followed for small streams.

ORGANIC CARBON

Analysis of organic carbon (dissolved) may be problematic because incomplete digestion of dissolved organic carbon is common. This has been most thoroughly investigated for marine samples (Perdue et al. 1993). However, similar problems have been documented for freshwater samples (Kaplan 1992). High temperature catalyzed analyzers provide more complete digestion and generally yield reliable results.

ALGAL AND PLANT ATTRIBUTES

COLLECTION OF ALGAL SAMPLES

The choice of methods for sample collection is dependent upon the intent of algal sample analysis. These methods are reviewed in the Revised Rapid Bioassessment Protocols for Streams and Rivers (Stevenson and Bahls 1999), so only a brief overview will be presented in this document. Sampling for

assessments of the biotic integrity of algal assemblages should be more thorough and extensive spatially than sampling for algal assessments of water quality. Thorough assessments of biotic integrity would call for multihabitat sampling over large reaches of the stream to find as many species and habitats within the stream or river as possible. Targeted habitat sampling (most commonly samples of algae from rocks in riffles) can provide collections that provide indications of biotic integrity or water quality. A third major alternative is to the use of artificial substrata that have the advantage of controlling variability among streams due to substratum type, but the disadvantage of having to visit the field two times (to place and retrieve substrata) and the concern that non-natural assemblages are being sampled. Targeted habitat sampling is usually recommended, is employed by most State programs, and is known to be successful. Efforts should be made to sample more than one riffle, particularly if an important goal of sampling is to assess benthic algal biomass in a stream.

The collection of algal samples can be a complex exercise due to the variability of stream features such as depth, substrata, flow velocity, and bottom characteristics. Holding some of these variables relatively constant by selecting a habitat zone with a narrow range for these variables was suggested earlier. Another approach is using artificial substrata which are easier to sample than natural substrata but which have several drawbacks. Artificial substrata are more likely to be vandalized, and they often tend to alter the flow regime around them resulting in silt deposition. The use of artificial substrata limits the ability to move to a different area where conditions are more acceptable, as can be done when using natural substrata. Perhaps the biggest drawback of artificial substrata is their inability to promote colonization by certain forms of algae, especially the massive filamentous forms. This issue is discussed further below in connection with the best methods of sampling various algal growth forms.

While there is a great variety of algal taxa, there are two main growth forms of algal communities: thin biofilms and long filaments. Many single-celled and colonial forms of attached algae appear to the naked eye as a biofilm of slippery, gelatinous material (often referred to technically as slime) on river rocks. This material can be easily sampled by using a template method.

Template Method

A template is a piece of flat, flexible, waterproof material in which a window of about 2.5 cm to 5 cm per side is cut. This template is placed in the center of the upper surface of a rock collected from the sample site, and a razor blade is used to scrape together all the material in the window. The material is then placed in a small water tight container (snap-shut plastic petri dishes, vials, or a piece of aluminum foil), and stored on ice in the dark until frozen.

This procedure is greatly facilitated by selecting smooth rocks. To avoid bias, sample points should be selected randomly. Then, rocks are selected blindly until one is chosen that is between 10 and 20 cm (in some regions of the country one is allowed to take a quick look for snakes first). If the rock's surface is too rough to sample, it should be replaced and the process continued until a rock of the right size and smoothness is selected.

The biofilms sampled by the above method form fairly quickly on artificial substrata and often the thickness and composition of this film is quite similar to that on nearby natural substrata in a matter of weeks (Watson unpublished). However, some of the more complex attached algae, most noticeably *Cladophora glomerata*, attach to rocks using a basal holdfast cell which supports a long filament. *Cladophora* holdfasts often survive short exposure and drying out and the scour that removes the

filament. The holdfasts spread over the rock and support more massive growths in subsequent years. After several years of flows that are too low to dislodge and roll the river rocks over, *Cladophora* may take the form of massive tangled branched filaments streaming several meters long. Since the massive growths take several years to develop, they can not be produced on artificial substrata which are likely to wash away during spring floods. Hence, such growth forms must be sampled from natural substrata. An example of a method for sampling from natural substrates is the hoop method.

Hoop Method

The template method does not work well for sampling the massive growth form (long string filaments) mentioned above. It is possible to be standing in a sea of waving *Cladophora* and pick up a random rock that has no *Cladophora* on it, or that has a tangled mass hanging by a few threads a few inches from the rock. The preferred way to sample such a growth form is to place a heavy metal hoop about 0.3 to 0.5 meter in diameter on the bottom of the stream (at the randomly selected point) and collect all the filamentous material inside the hoop. This often involves cutting the filaments around the hoop and picking up the filaments and rocks inside the hoop. The collection should be brought to shore in a tub where the filaments should be removed from the rocks. Razor blades and paint scrapers work well; hack saws are generally unnecessary. Wrapping these large samples in aluminum foil will facilitate the drying, weighing and ashing process. The collection of 10 to 20 replicates of such samples at a series of high biomass sites will represent a large volume.

Freezing samples not only helps preservation, but cells are ruptured, facilitating chlorophyll extraction. Samples collected by templates should be frozen at -10°C on return to lab, and analyzed for chlorophyll *a* and AFDM within 2 weeks to a month. Laboratory methods for analyzing algal biomass for chlorophyll and ash free dry weight (AFDM) are discussed below. The same sample can be analyzed for both chlorophyll *a* and AFDM. After chlorophyll analysis, the extracted sample is poured into an aluminum weigh boat, the solvent is evaporated, and AFDM analysis is performed on the boat. This facilitates determining the chlorophyll to AFDM ratio on these samples.

Due to the abundance of material collected using the hoop method, it is not possible to extract all the chlorophyll from these samples. Hence, only small subsamples of each large sample are analyzed for chlorophyll and AFDM, and the large samples are analyzed for AFDM only. Their chlorophyll content is estimated using the chlorophyll to AFDMratio determined from the subsamples. These samples are handled as follows: 1) before freezing the samples collected by the hoop method, take each sample and spread it out; 2) collect many tiny subsamples from all over this bulk sample; 3) chop and mix the subsamples; 4) make at least 4 replicate composite samples from this well mixed pile; 5) place these in small containers and process as you do the template samples; 6) analyze the remaining bulk sample for AFDM; and 7) use the chlorophyll to AFDM ratio of the small composite samples to estimate the chlorophyll in the bulk sample (consider the variability in the chlorophyll/AFDM ratio of the composite subsamples as well as the variability in biomass of the large samples).

COLLECTION OF MACROPHYTE SAMPLES

Macrophyte sampling is commonly performed 1) to qualitatively assess the distribution of vegetation in an area or 2) to quantitatively measure primary productivity (gauged by changes in biomass). Caution must be taken when sampling macrophytes for biomass determination to ensure that the appropriate portions of macrophytes (above and below ground) are collected. (Macrophytes may have up to 90%

underground biomass.) After collection, macrophyte samples may be dried and combusted to determine AFDM in a manner comparable to that for algal samples. Macrophyte sampling and biomass determinations are discussed in Wetzel and Likens (1991).

ALGAL BIOMASS - % COVER OF BOTTOM BY NUISANCE ALGAE

Methods have been described in the literature (e.g., Sheath and Burkholder 1985) to estimate algal biomass in the stream by visual observation. In the Revised Rapid Bioassessment Protocols for Rivers and Streams (Stevenson and Bahls 1999), a rapid periphyton survey is described that provides an in-stream assessment of algal biomass. The technique is simple and can be used by professionals or volunteers with little training. Two steps are involved as the stream bottom is observed at multiple sites (usually >9) through a viewing bucket (clear-bottom bucket submerged in stream for clear observation of the stream bottom). First, percent cover of filamentous algae over the stream bottom is assessed. Then thickness and percent cover of microalgae is assessed. A ranking system is used to quantify thickness of microalgal mats. The advantages of this rapid periphyton survey are that it allows for rapid assessment of algal biomass, particularly filamentous algal green biomass, and it covers large regions of the stream (thus accounting for the great spatial variability in algal biomass).

CHLOROPHYLL *a*

The most commonly used determinant of benthic algal biomass is chlorophyll *a*. Chlorophyll *a* is often a superior indicator of biomass compared to determination of AFDM because non-algal material can contribute to biomass. Chlorophyll *a* is used because it occurs in all common photosynthetic organisms. Other forms of chlorophyll can inflate estimates of algal biomass, because the amount per cell can be more variable. In addition, counts of algal cells and biovolume are often used as a determinant of biomass. These counts are time consuming and require taxonomic expertise, and thus are rarely done and will not be considered here. The general methods for biomass determination are well described by Steinman and Lamberti (1996) and Stevenson (1996); the interested reader should consult these references and others cited herein.

Chlorophyll is determined in seston on filtered material and from benthic material either from cores, artificial substrata, or scraped and extracted substrata. In general, artificial substrata yield higher chlorophyll *a*/AFDM values than natural substrata (Dodds et al., unpublished), and this should be kept in mind when selecting the method to be used. However, measurement of area and extraction of pigment is easier with artificial substrata.

Chlorophyll analyses without an acidification step to correct for chlorophyll degradation products (phaeophytin correction) are occasionally encountered. This acidification is essential for periphyton because dead cells that contain phaeophytin can remain in the assemblage, and lead to biomass overestimates. A fluorometric method with narrow band filters that correct for phaeophytin but omit the acidification step was recently introduced (Welschmeyer 1994).

Determination of phaeophytin concentrations may be useful not only for correcting chlorophyll *a* concentrations, but also as an indicator of periphyton degradation. Wetzel and Likens (1991) give a method for determining both chlorophyll *a* and phaeophytin concentrations. The ratio of chlorophyll *a* to phaeophytin gives an indication of periphyton growth and activity.

Generally, a ratio of 9:1, acetone:water, is used as an extractant. We have found that hot 90% ethanol extraction (Sartory and Grobbelaar 1984) offers some advantages. Primarily, material need not be scraped from the substratum, and grinding of the sample is not required. Rather, the entire sample of substratum and periphyton is placed in a heat resistant (autoclavable) plastic bag with extractant and heated to 80 ºC for 5 min. Ethanol fumes are also less noxious than acetone fumes.

The preferred procedure is to use a spectrophotometer to read absorbance, because the relatively dense solutions of extracted chorophyll are common for periphyton samples. Very dense solutions of chl must be avoided for spectrophotometry and fluorometry to prevent analytical errors; the problem is of greater concern in fluorometry. In spectrophotometry, solutions of greater than 1.5 absorbance units per cm at 665 nm should not be analyzed. Fluorometric analysis should not be attempted with samples having more than 0.5 absorbance units per cm at 665 nm. Dilution with extractant can bring samples to within the appropriate absorbance range.

AFDM AND ALGAL CELL BIOVOLUME

Methods for AFDM and algal cell biovolume are covered by Steinman and Lamberti (1996) and Wetzel and Likens (1991), respectively. Ash-free dry-weight values have been used in conjunction with chlorophyll a as a means of determining the trophic status (autotrophic vs. heterotrophic) of streams (Weber 1973). The Autotrophic Index (AI) is calculated as:

AI = AFDM (mg/m2) / chlorophyll *a* (mg/m2).

As suggested before, these should be relied upon as supplementary methods, and the large degree of time required for biomass determinations by cell counts and biovolume estimates should be considered.

ALKALINE PHOSPHATASE ACTIVITY

Analysis of alkaline phosphatase activity (APA) is used to determine phosphorus limitation in algae. Alkaline phosphatases are enzymes produced by algae to break down organic phosphorus compounds and release bioavailable (PO_4) P (Steinman and Mullholland 1996). Studies have shown that lower levels of P result in higher levels of APA and vice versa (Klotz 1992). The most common method for APA analysis is a fluorometric method described by Hill et. al. (1968).

ALGAL SPECIES COMPOSITION

Different methods can be used to assess algal species composition depending upon the objective of the assessment (Whitton et al. 1991; Whitton and Rott 1996; Lowe and Pan 1996; Stevenson 1998; Stevenson and Pan 1999; Stevenson and Bahls 1999). For example, if the objective of the assessment is to determine if nutrient conditions meet a drinking water use, then analysis of all algae in samples may be desirable to determine if taste and odor algae are present. If the objective is to get an indication of nutrient conditions, trophic status, or biotic integrity, then analysis of species composition of diatoms only may be sufficient. The latter is less time consuming that an analysis of all algae in samples. The methods for analysis of algal species composition in samples can be found in Standard Methods (APHA 2000), the Revised Rapid Bioassessment Protocols (Stevenson and Bahls 1999) or in Lowe and LaLiberte (1996).

Although time consuming, it may be desireable to determine the types of algae present that are thought to be creating problems. The methods necessary for such determinations are described in Lowe and LaLiberte (1996) and in Standard Methods (APHA 2000). In general, taxa determination, especially to species, requires expertise, similar to that required for precise water chemistry and macroinvertebrate assays. Such fine level determinations may be useless if not conducted by experienced taxonomists. Some companies provide algal identification and analysis services that may be useful for those lacking such expertise. The reputation of prospective companies should be verified. In general, total algal biomass is of greater concern than taxonomic composition to those wishing to control eutrophication and its effects.

MACROINVERTEBRATE ANALYSIS

Macroinvertebrates may indicate water quality problems and some monitoring programs may want to evaluate biomass and diversity of macroinvertebrates. There is little precedence for this in stream eutrophication studies, and the analysis of macroinvertebrates to species is time consuming. Methods to assess stream macroinvertebrates have recently been reviewed (Hauer and Resh 1996). Generally, identification of most animals to species is required for accurate indices to be constructed, so it is important that such analyses be carried out by individuals with taxonomic expertise.

COMMUNITY METABOLISM ANALYSES

Productivity/respiration (P/R) ratios can be determined by the upstream-downstream method with dissolved oxygen data and estimates of atmospheric reaeration (Odum 1956; Marzolf et al. 1994) or light/dark, flow-through chambers (Hickey 1987; Dodds and Brock 1998). P/R ratios measured using chambers are generally higher than those measurements obtained from upstream-downstream methods. Even in streams with heavy algal growths, it is rare to find P/R ratios in excess of one (1) using upstream-downstream methodology. Both methods convert the diel changes in dissolved oxygen into actual rates of productivity. The diel range in dissolved oxygen indicates the magnitude of gross productivity and can be used to monitor ecological integrity in streams and rivers of similar velocity, depth, and turbulence.

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