Chapter 33

Phosphorus Limitation, Uptake, and Turnover in Benthic Stream Algae

Alan D. Steinman[1] and Solange Duhamel[2]
[1]Annis Water Resources Institute, Grand Valley State University; [2]Lamont–Doherty Earth Observatory, Columbia University

33.1 INTRODUCTION

Increased loading of nutrients into streams and lakes has become one of the major environmental problems facing society today. Non–point source pollution, associated with changing land use patterns and practices, has resulted in increased impairments to water bodies (e.g., Jordan et al., 1997; Carpenter et al., 1998; Allan, 2004; Dodds, 2006; Kaushal et al., 2011; Michalak et al., 2013). These impairments include cultural eutrophication, harmful algal blooms, thermal pollution, increased sedimentation, and increased loadings of contaminants, such as metals, pesticides, oil, and grease. The ability of an aquatic ecosystem to assimilate these stressors without exhibiting impairment depends largely on its biology (i.e., species composition and abundance, elemental stoichiometry), chemistry (i.e., nutrient quantity and quality), geology (i.e., underlying lithology), and geomorphology (i.e., constrained or unconstrained valley floor).

In stream ecosystems, benthic algae and bacteria represent a potentially important biotic sink for pollutants, such as excess nutrients (Mulholland and Rosemond, 1992; Bernhardt et al., 2003; Cardinale, 2011), although adsorption to stream sediments also can be a major sink, especially for phosphorus (Mulholland, 1996; Stutter et al., 2010). Determining the rates at which nutrients are taken up and released can provide important information in assessing how large a nutrient load a stream, lake, or estuary can process before its integrity is negatively impacted (cf. Dodds, 2003; Withers and Jarvie, 2008; Steinman and Ogdahl, 2015).

The nutrient that we focus on in this chapter is phosphorus (P). Inorganic P is commonly considered the element most likely to limit primary production in freshwater ecosystems (Schindler, 1977; Hecky and Kilham, 1988; Hudson et al., 2000; but see below). Although P concentrations in healthy plants are relatively low, usually ranging from 0.1% to 0.8% of dry mass (Raven et al., 1981), P is an essential element. Some of the more important functions played by P in plants include being a structural component of "high-energy" phosphate compounds [e.g., ADP and adenosine triphosphate (ATP)], nucleic acids, several essential coenzymes, and cell membrane constituents (phospholipids), as well as being involved in the phosphorylation of sugars.

Although a significant number of stream studies have indicated that P limits the growth of benthic algae (e.g., Stockner and Shortreed, 1978; Elwood et al., 1981; Peterson et al., 1983; Bothwell, 1989; Dodds et al., 1997), the phenomenon is not universal (McCall et al., 2014), and P is by no means the only limiting nutrient in lotic ecosystems. Nitrogen (N) has been found to be the limiting nutrient in some streams (Grimm and Fisher, 1986; Hill and Knight, 1988; Lohman et al., 1991; Tank and Dodds, 2003), whereas other lotic systems can be colimited by N and P (Rosemond et al., 1993; Perrin and Richardson, 1997; Francoeur, 2001; Tank and Dodds, 2003), micronutrients (Pringle et al., 1986), or light (Hill et al., 1995; Carey et al., 2007; Johnson et al., 2009).

In this chapter, three different aspects of P utilization by benthic algae will be covered: (1) assessment of P limitation, (2) measurement of P uptake rates, and (3) determination of the release rate of P (expressed as the turnover rate). We note two caveats regarding this chapter. First, we focus exclusively on inorganic P; it is likely that dissolved organic phosphorus plays an important, albeit relatively undefined role, in the nutrient dynamics of freshwater algae (cf. Hwang et al., 1998; Pant et al., 2002), especially in P-limited environments (Karl and Björkman, 2015). However, treatment of

Methods in Stream Ecology. http://dx.doi.org/10.1016/B978-0-12-813047-6.00011-5

Methods in Stream Ecology, Third Edition, 2017, 197–218

this topic is beyond the scope of this chapter (but see Chapter 24). Second, although we use the term benthic algae throughout the chapter, it should be noted that the benthic algae attached to submerged substrata in streams usually exist as part of a complex assemblage variously referred to as periphyton, *aufwuchs*, or biofilm. This assemblage usually consists of algae, bacteria, fungi, and meiofauna (see Chapters 9–11, 14) that exist within a mucilaginous, polysaccharide matrix (Lock et al., 1984), and each biotic group has different affinities for P. Indeed, even within a group, P uptake and cycling may be influenced by the abundance of different species and growth forms (cf. Steinman et al., 1992; Davies and Bothwell, 2012).

33.1.1 Assessment of P Limitation

Nutrient limitation in algae can be assessed in several different ways, including elemental composition of biomass, nutrient enrichment bioassays, enzymatic activities, and physiological responses. Elemental composition can suggest nutrient limitation because the proportions of carbon (C), N, and P, while confined to a relatively narrow range in algae (Hall et al., 2005), nonetheless vary in response to both ambient nutrient concentrations in the water and ambient light conditions (Finlay et al., 2011; Drake et al., 2012). The ratios of C:N:P have profound ecological implications, as nutrient stoichiometry at the base of trophic food webs can influence or be influenced by trophic level interactions, population dynamics, taxonomic structure at the community level, and ecosystem level processes such as nutrient limitation and cycling (Hillebrand and Kahlert, 2001; Frost et al., 2002; Stelzer and Lamberti, 2002; Hillebrand et al., 2008). Kahlert (1998), in a review of the literature, found that the optimal (i.e., conditions without nutrient limitation or surplus) C:N:P ratio of freshwater benthic algae was 158:18:1 (molar), which deviates from both the Redfield ratio of 106:16:1 (Redfield, 1958; but see Geider and La Roche, 2002) derived from mixed phytoplankton populations and the ratio of 119:17:1 obtained for marine benthic microalgae (Hillebrand and Sommer, 1999). It is likely that carbon-rich detritus (cf. Cross et al., 2003), the inclusion of macroalgae (cf. Hillebrand and Sommer, 1999), and the carbon content of the mucilaginous biofilm matrix account, at least in part, for the higher C:P ratio of Kahlert's benthic algae compared to Redfield's planktonic algae. When C, N, or P become limiting in the environment, this condition can be reflected in a lower level of nutrient present in the algal cell. For example, if P concentration becomes growth limiting in a stream, tissue C:P and N:P ratios would be expected to increase because the algae make more efficient use of the P incorporated into cells. Moreover, certain microorganisms have evolved mechanisms to reduce their P requirements. For example, some phytoplankton species can partially substitute sulfur for P in membrane lipids (Van Mooy et al., 2009); more controversial has been the suggestion that an aquatic bacterium may be able to partially substitute arsenic for P in nucleic acids (Wolfe-Simon et al., 2011), although this work has been challenged (e.g., Reaves et al., 2012). For freshwater planktonic algae, C:P values >129 and N:P values >22 (as opposed to Redfield ratios of 106 and 16, respectively) suggest at least moderate P deficiency in algae (Hecky et al., 1993). However, these ratios increase considerably for freshwater benthic algae, where P deficiency is suggested if C:P values exceed 369 and N:P values exceed 32 (Kahlert, 1998). See Chapter 36 for more information and exercises on elemental composition.

Nutrient enrichment bioassays involve the addition of nutrients to a stream, either in the form of diffusing substrata (see Chapter 31), fertilizer pellets (Steinman et al., 2011), or solute injections (see Chapters 30 and 31). The enrichment lasts for some designated period of time, and its effect is evaluated by change in biomass (see Chapter 12) or primary productivity (see Chapter 34) in enriched compared to unenriched systems.

An enzymatic assay that has proven to be a reliable indicator of P limitation is whole-community *phosphatase* activity (PA). The phosphatase enzyme hydrolyzes phosphate ester bonds, thereby releasing orthophosphate (PO_4) from organic P compounds. PA is quantified by measuring the amount of hydrolysis produced after the phosphatase enzyme comes in contact with an added organic P substrate, thereby releasing PO_4. The most common type of phosphatase assayed in freshwater systems is alkaline phosphatase, which hydrolyzes phosphomonoesters. In contrast, phosphodiesterase hydrolyzes phosphodiesters, while adenosine triphosphatase hydrolyzes ATP. PA is regulated by P concentration present in the water (Dyhrman and Ruttenberg, 2006; Duhamel et al., 2010); PA is generally repressed as inorganic P concentrations increase. Conversely, PA generally increases as inorganic P concentrations decline in aquatic ecosystems (Healey, 1973; Wetzel, 1981; Currie et al., 1986; Espeland et al., 2002; Scott et al., 2009; Ellwood et al., 2012). Thus, PA has been used to infer P limitation for aquatic microflora (Healey and Hendzel, 1979; Burkholder and Wetzel, 1990; Hernandez et al., 2002; Newman et al., 2003). Based upon their results from phytoplankton culture studies, Healey and Hendzel (1979) suggested that phosphatase levels above 0.003 mmol P mg chlorophyll a^{-1} h^{-1} indicate moderate P deficiency and levels above 0.005 mmol P mg chlorophyll a^{-1} h^{-1} indicate severe P deficiency. It should be noted that these values are specific to the environmental conditions and substrate that they used: pH 8.5, 35°C, and 10 μM *o*-methylfluorescein phosphate (which they found to support saturated PA). In systems with complex organic P substrates (e.g., dystrophic

systems), PA can lead to biased conclusions if the assays do not include the appropriate phosphatases (cf. Pant et al., 2002); in addition, normalizing to chlorophyll may inflate algal-derived phosphatase rates as it does not account for bacterial biomass, which can be substantial in some periphyton mats.

The application of enzyme-labeled fluorescence (ELF) has opened up research avenues in the use of PA to detect P limitation. Although the details of this method are beyond the scope of this chapter, a brief review helps illustrate possible future directions of this field. Rengefors et al. (2001) used ELF to differentiate PA at the species-specific scale for phytoplankton. ELF results in a fluorescent product when it reacts with alkaline phosphatase, which precipitates at the site of enzymatic activity (i.e., cell surface). This method makes it possible to determine not only which species contains PA but also the relative quantity of PA (Nedoma et al., 2003; Duhamel et al., 2009). ELF also has been used to (1) differentiate whole-community PA from that of individual bacterial cell PA in wetland periphyton biofilms (Espeland et al., 2002), (2) determine the contribution of microbes to overall phosphatase production in periphyton mats inhabiting *Utricularia* traps (Sirová et al., 2009), and (3) localize AP within freshwater macroalgal epiphyte assemblages (Young et al., 2010). ELF labeling of both intact and homogenized periphyton mats may result in interesting insights regarding where P limitation is occurring within the biofilm matrix (Sharma et al., 2005).

33.1.2 P Uptake Rates

The relationship between the nutrient concentration in the water and the rate at which nutrients are taken up by algae can be described by a hyperbolic function (Fig. 33.1). The Michaelis–Menten equation for enzyme kinetics is often used to describe this function:

$$V = V_m(S/(K_s + S)) \tag{33.1}$$

where V = nutrient uptake rate, V_m = maximum nutrient uptake rate, S = concentration of the nutrient, and K_s = half-saturation constant (or nutrient concentration at which nutrient uptake is one-half the maximal uptake rate). From a biological perspective, there are two critical considerations in Fig. 33.1. First, nutrient uptake rates become saturated as nutrient concentration increases. Empirical studies have shown that saturation of P uptake can occur at very low concentrations in both individual benthic diatom cells (<1 µg/L; Bothwell, 1989) and whole streams (<10 µg/L; Mulholland et al., 1990). Thus, investigations examining P uptake in benthic algae must consider the possibility that saturation will influence uptake kinetics even at relatively low concentrations. Second, the constant K_s provides a useful index of a cell's affinity for a nutrient—a lower K_s suggests a greater affinity for the nutrient, which can confer a competitive advantage when the nutrient is present at low concentrations. Generally, taxa that have low K_s values have a competitive advantage at low nutrient concentrations. However, K_s values appear to be fixed and do not appear to vary much under different environmental conditions. Rather, the physiological reason why nutrient-limited algae often increase their short-term nutrient uptake rates when exposed to elevated nutrient concentrations is because of an increase in V_m and not a change in K_s

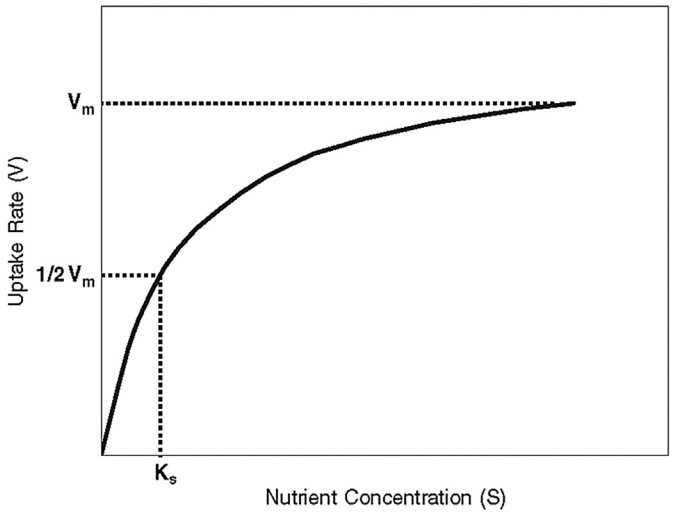

FIGURE 33.1 Relationship between nutrient concentration (S) and nutrient uptake rate (V). V_{max}, the maximum nutrient uptake rate; K_s, the half-saturation constant or the nutrient concentration at which the uptake rate is one-half of the maximum uptake rate.

(Darley, 1982; Lohman and Priscu, 1992). However, multiphasic P uptake systems have been documented in unicellular algae (Rivkin and Swift, 1982; Jansson, 1993), suggesting that K_s values are not static. From an energetic perspective, increasing V_m makes more sense, as a change in K_s requires the alteration of existing enzyme structures or the induction of an alternative enzyme, whereas increasing V_m requires only the activation or additional synthesis of an existing enzyme (Rivkin and Swift, 1982). Of course, over the long term, elevated nutrient levels may lead to an altered community structure, resulting in dominance by new algal or bacterial species, which may have greater V_m or different K_s values, thereby changing nutrient kinetics in the benthic community.

It is also important to distinguish between nutrient-limited *uptake* rates (above) and nutrient-limited *growth* rates. The relationship between nutrient concentration and algal growth can be modeled using either the Monod model or the Droop model. The Monod model relates algal growth to the external concentration of nutrients in the water, whereas the Droop model relates algal growth to internal (cellular) concentration of nutrients. For additional details on these models, see Droop (1974), Rhee (1978), Kilham and Hecky (1988), and Borchardt (1996).

33.1.3 P Turnover Rates

This portion of the chapter is designed to examine P turnover rates in benthic stream algae. P turnover may provide an index of internal cycling in the algal community. Once an algal cell takes up P from the external medium, the P can be incorporated into structural elements, maintained in a labile pool, or excreted from the cell. Cells that are phosphorus limited may be less likely to release the P they have taken up (back to the external medium) than cells that are phosphorus replete (but see Cembella et al., 1984; Borchardt et al., 1994). Thus, the P turnover rate in algae (i.e., loss of P from algal cell relative to total algal P) may be lower in P-limited cells than P-saturated cells, assuming both the P-limited and P-replete cells have similar metabolic activities and are exposed to similar grazing pressures (Steinman et al., 1995). One way to measure P turnover in algae is to label the cells with a P *radioisotope* (e.g., ^{32}P or ^{33}P) in the laboratory, place the algae back into the natural or a controlled environment, and then measure the amount of radioactive P present in the cells over time. This gives an apparent P turnover rate, as turnover is being estimated from the entire periphyton matrix and not from individual cells.

33.1.4 Overview of Chapter

This chapter examines P limitation and uptake in benthic algae collected from a relatively low- and a relatively high-P stream. In theory, the benthic algae growing in the low- and high-P streams should have adapted to the different ambient conditions and exhibit different ecological attributes (Fig. 33.2). Specifically, if the algae in the low-P stream are P-limited, they should have lower biomass and greater C:P ratios than algae collected from the high-P stream, all else being equal (although intense internal nutrient cycling within the periphyton matrix can compensate for low external nutrient concentrations; Dodds, 2003; Mulholland et al., 1994). In addition, algae in the low-P stream should have greater phosphatase activities, lower K_s values, lower total P uptake rates (i.e., computed as mass per unit time), and lower apparent P turnover rates (greater retention) than algae from the high-P stream (Fig. 33.2).

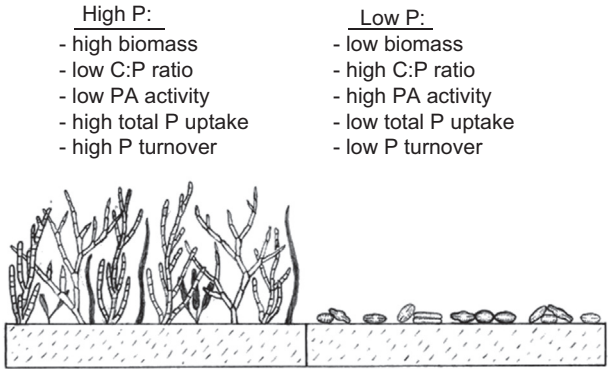

FIGURE 33.2 Phosphorus-related attributes for hypothetical attached algal communities exposed to high-P (left side) and low-P (right side) conditions.

33.2 GENERAL DESIGN

This chapter describes the methodology to measure P limitation, uptake, and turnover in benthic algae. Although valuable information will be gleaned from any of these methods in isolation, we recommend combining them when possible to gain a broader understanding of P-related processes in streams.

Site selection should include two streams, one with relatively high P (e.g., >20 μg PO_4-P/L, if available) and one with relatively low P (e.g., <5−10 μg PO_4-P/L, if available). If differences in algal response are to be detected, it is critical that the algae be exposed to ecologically meaningful differences in nutrient concentration. We recommend using an undisturbed stream (if available) for the "low-P" system, where there are few obvious impacts (e.g., point source inputs, unnatural absence of riparian vegetation, livestock in streams). For the "high-P" system, use streams receiving either agricultural runoff or point sources containing high P (e.g., sewage effluent) or clarifying tanks at sewage treatment facilities (Davis et al., 1990). If all streams in the region have low levels of P, then it may be possible to enrich a stream with P for a sustained period of time (e.g., >4 weeks) to create high-P conditions (e.g., Steinman, 1994; Cross et al., 2003) or, alternatively, enrich a stream with N to force P limitation. These changes can be done through the use of nutrient-diffusing substrata (see Chapter 31) or solute additions (see Chapter 30) if permitted by local regulatory agencies. Regardless of which streams are selected, collect algae from sites in the two streams that are comparable in terms of other environmental conditions (e.g., irradiance level, current velocity, discharge, temperature, grazer density) to the greatest extent practicable.

33.2.1 Basic Method 1: Phosphatase Activity

This method consists of two parts: an assay of PA, followed by normalizing activity to an estimate of biomass (see Chapter 12). Normalizing PA to Chlorophyll fails to account for bacteria in the production of PA, likely resulting in an overestimation of PA. Alternatively, PA can be normalized to ash-free dry mass (AFDM) (see Chapter 12), which does account for bacteria (and other organic matter). However, this approach may underestimate PA, as AFDM includes materials such as fungi and detritus, which are not directly involved in PA. PA also can be normalized to surface area, but in that case, rates may be more reflective of how much (or how little) active biomass is present than the P concentrations in the water column.

Phosphatase acts on a variety of organic P compounds. Synthetic substrate analogs are used to measure PA in natural samples. After hydrolysis of the PO_4 moiety, these substrates release a product that can be assayed colorimetrically (e.g., 5-bromo-4-chloro-3-indolyl phosphate) or fluorometrically [e.g., methylumbelliferyl phosphate (MUF-P), 3-*o*-methylfluorescein (MFP)]. While fluorogenic substrates are considered to be the most sensitive, colorimetric assays are preferred in environments with high biomass as they are easier to perform (Hernandez et al., 2002; Hoppe, 2003; Jansson et al., 1988; Manafi et al., 1991). In this method, we add a commercially available compound, *para*-nitrophenyl phosphate (*p*-NPP), to determine the PA present. When PO_4 is hydrolyzed from *p*-NPP, *p*-nitrophenol (*p*-NP) is formed, which can be measured spectrophotometrically.

33.2.2 Basic Method 2: Chemical Composition (C:P Ratio in Algal Tissue)

This method consists of three components: measurement of algal AFDM (and conversion to C), acid digestion of combusted matter to obtain dissolved P leached from ashed algal tissue (Solórzano and Sharp, 1980), and then measurement of inorganic P in oxidized algal material according to standard methods. Ideally, the C concentration in algae would be measured with an elemental analyzer (see Chapter 36). However, this instrument is not always available. Consequently, in this method we present an alternative approach to estimate C, based on measurement of AFDM (see Chapter 12), which is easy to perform but less accurate than elemental analysis. We assume C is 53% of AFDM, a reasonably accurate assumption for most algal communities (Wetzel, 1983).

33.2.3 Basic Method 3: Net Nutrient Uptake—Stable Phosphorus

This procedure involves the measurement of net loss of dissolved inorganic phosphorus from the water in which the algae are growing. Because certain classes of organic P compounds are partially hydrolyzed by the standard molybdenum blue assay used to measure dissolved inorganic P concentration (see below), results are typically reported as soluble reactive phosphorus (SRP). The method consists of three components: sample water during the incubation, measure SRP in water samples, and measure algal biomass. Water is sampled at the start of the incubation, and thereafter at 30 and 60 min, and

analyzed for SRP according to standard methods (APHA et al., 1995). Large changes in the biomass:water volume ratio resulting from water sampling during the incubation period should be avoided by minimizing sample volumes or number of samples. If the water volume in the incubation chambers is low, the 30 min samples can be omitted. We recommend that volumetric change be limited to <10% of initial volume during sampling. Ideally, chambers attached to pumps that could recirculate water during the incubation would be used (Fig. 33.3), as water velocity will influence the uptake rate of P in benthic stream algae (Whitford and Schumacher, 1964). However, if chambers and pumps are not available, the method can still be completed by using large (2 L) glass chambers and stir plates. An open petri dish is glued to the bottom of the chamber, into which is placed a stir bar. Then coarse-meshed screening (e.g., chicken wire) is placed over the petri dish, thereby creating a shelf onto which are placed the substrata with attached algae. The rotation speed of the stir bar is varied until it matches approximately the current velocity in the sampled streams.

The use of stable (i.e., nonradioactive) elements to measure *net nutrient uptake* rate is dependent on the initial nutrient concentration in the ambient water being high enough to measure the remaining nutrient at the end of the incubation period. For example, if P concentrations are low at the beginning of the incubation, they may be below the detection limit at the end. Another potential problem with this measurement is that if nutrient regeneration rates from algae are similar to nutrient uptake rates (i.e., the community is at steady state with respect to nutrient dynamics), then no net uptake will be measured, even if total uptake rates are appreciable. An alternative approach is to add nutrients to stream water. This elevates nutrient concentrations above ambient levels, ensuring that concentrations at the end of the incubation will still be high enough to be measured, and temporarily increases nutrient uptake rates above rates of nutrient regeneration. However, this approach measures only nutrient uptake potential at the higher concentration and may be an overestimate of ambient uptake rate depending on the degree of enrichment (see Mulholland et al., 1990, 2002). Alternatively, a nutrient addition

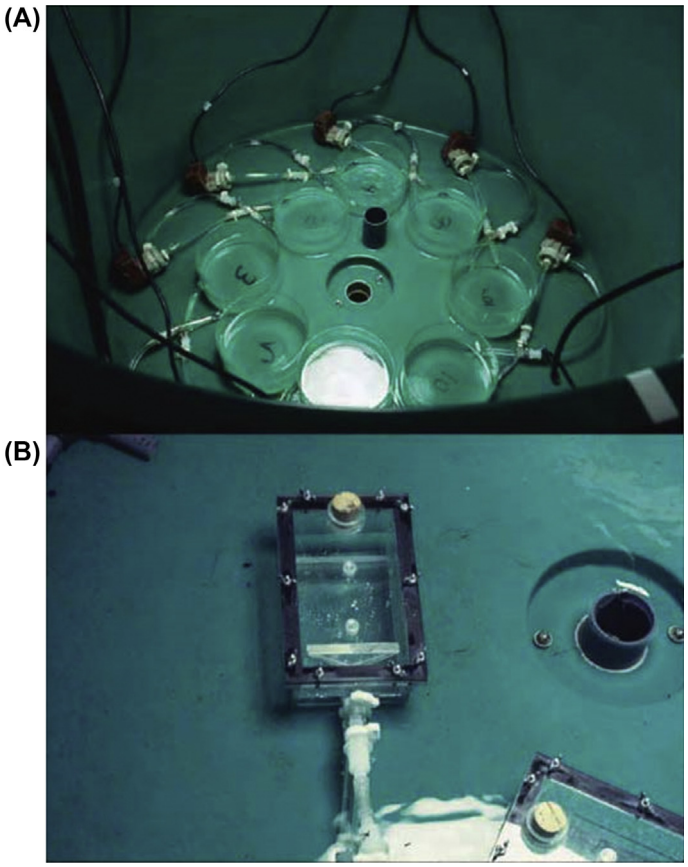

FIGURE 33.3 Examples of incubation chambers that have been used for P uptake studies. (A) 2-L glass chambers fitted with adapters to accept tubing attached to submersible pumps. Pumps circulate water within the chambers. Placing adjustable clamps on tubing line can reduce flow rate, if so desired. (B) 1-L plexiglass chamber with detachable lid. Lid attaches to main body of chamber with wing nuts; gaskets provide a leak-proof seal. Chambers are attached to submersible pumps. Note the large port (far end with cork) in lid, which allows an oxygen meter to be placed directly in the chamber to measure metabolism. The two small ports allow for injection of radioisotope into the chamber.

approach that involves multiple levels of nutrient enrichment can be used to approximate total uptake rate at ambient nutrient concentration (Payn et al., 2005; see also Chapter 30). In this chapter, we provide instructions for measuring net uptake rates at ambient nutrient concentrations (i.e., without enrichment).

Although we recommend the measurement of SRP in this analysis, we note that SRP may overestimate the true concentration of phosphate in the water sample. That is because the soluble portion of SRP may include colloidal P in the filtrate, while the reactive portion of SRP may include organic forms of P that react with the reagents (Rigler, 1966; Hudson et al., 2000). The difference between SRP and phosphate concentration is most distinct in waters with very low-P concentrations but becomes less noticeable as ambient P concentration increases.

33.2.4 Advanced Method 1: Gross Nutrient Uptake (Phosphorus Radiotracer)

This procedure involves measuring the loss of $^{33}PO_4$ added to the water in which the algae are growing.[1] Stable and radioisotopic tracers are important tools to monitor and quantify microbial growth and metabolism, as well as the cycling of elements. Because P has only one stable isotope (^{31}P), the use of radioactive isotopic tracers of phosphorus (i.e., ^{32}P or ^{33}P) can be critical in quantifying P fluxes in aquatic environments. P radioisotopes are particularly useful as they can be added to the sample at trace levels, and thus not perturb the steady-state phosphate concentration. This is particularly relevant in P-limited environments with very low PO_4 concentrations, where P radioisotopes allow rigorous estimation of the uptake kinetic parameters and the turnover time of phosphate and organic P substrates (e.g., ^{33}P-glucose-6-phosphate, ^{33}P-ATP).

Recent studies also have used variations in the natural abundance of the stable ^{18}O isotope bound to P ($\delta^{18}O_P$) to study P transformations and determine P sources in aquatic systems (Colman et al., 2005; McLaughlin et al., 2006; Elsbury et al., 2009; Young et al., 2009). P is strongly bound to oxygen (e.g., orthophosphate, polyphosphate), and in many aquatic ecosystems the oxygen isotopic composition of $\delta^{18}O_P$ is not in isotopic equilibrium with ambient water, and may thus reflect the phosphate sources to water bodies. These analyses require sophisticated instrumentation and are not included in this chapter.

Uptake rates will be calculated in this method from algae growing in high-P and low-P streams. This method should use algae growing on small artificial substrata [e.g., unglazed ceramic cylinders (Steinman et al., 1991b) or unglazed tiles placed in the streams for a period long enough to acquire an algal community similar to natural substrata]. The use of small artificial substrata allows AFDM to be measured directly on the substratum without it being physically removed, thereby minimizing contact with radioactive P in the algae. In addition, if the turnover option is to be completed (see below), the P in the algae on these substrata can be extracted with relative ease.

33.2.5 Advanced Method 2: Phosphorus Turnover

This method involves measuring the rate at which radiolabeled P, incorporated into algal biomass, is lost from the algal assemblage over time.[2] Ideally, this procedure will be piggybacked on the prior method of measuring P uptake rates using ^{33}P. The method consists of four parts: radiolabeling of algae, oxidation of labeled algae, extraction of P from ash, and measuring radioactivity in subsamples of extracted material.

After the ^{33}P uptake method is completed, substrata are either returned to the high- and low-P streams if allowed, or placed into lab-based recirculating chambers or aquaria containing either high or low concentrations of P. Four substrata are sampled from each stream on four occasions over a 10-day period. The algae on the substrata are oxidized, and ^{33}P is extracted from the ash. A subsample of this extract is diluted, placed into scintillation cocktail, and assayed for radioactivity using liquid scintillation spectroscopy.

P turnover for each stream is calculated as the first-order rate constant of the decline in ^{33}P activity over time (slope of relationship between ln [^{33}P] in algae vs. time). A mean activity is calculated on each date from the four substrata collected and used in the regression with time. For the purposes of this method, we recommend normalizing ^{33}P content to unit area

1. Extreme caution must be exercised when using radioisotopes. Users must consult with the radiation safety officer at their institution. We recommend the use of ^{33}P, instead of ^{32}P, because of its lower energy, although it is more expensive. Even with the relatively low maximum energy of ^{33}P (0.248 MeV), the small amount of radioactivity used (0.5 mCi/L), and the short half-life of the isotope (25.4 days), all handling of the isotope must be done with extreme care.

2. Extreme caution needs to be exercised when using radioisotopes. See the prior cautionary note. In addition, because this exercise involves potentially placing radiolabeled algae back into the natural environment, users must consult with the radiation safety officer at their institution regarding restrictions or other potential concerns about this protocol. If this option is not viable, labeled algae can be placed into chambers or aquaria filled with water of high and low-phosphorus concentration, to evaluate the influence of P concentration on turnover.

of substratum, as opposed to biomass, although this approach assumes relatively similar biomass levels among substrata or that sufficient samples are collected on each date to take into account the natural variability of biomass in the system. If ^{33}P content is expressed per unit biomass, it becomes necessary to introduce a growth correction factor to account for any net growth during the period of the experiment (because the amount of radioactivity per unit biomass in the sample will decline due to dilution by the accrual of new, nonlabeled biomass). Also, if the extracted ^{33}P samples are counted on different days over the period of the turnover experiment, they must be corrected for radioactive decay from the start of the experiment; because of the short half-life of ^{33}P (25 days), some of the decline in ^{33}P content in algae will be the result of radioactive decay. As a solution to this problem, all of the ^{33}P extract samples for the entire turnover study can be assayed on the scintillation counter at the same time at the end of the study, thereby minimizing the need to correct for decay.

33.3 SPECIFIC METHODS

33.3.1 Basic Method 1: Phosphatase Activity

33.3.1.1 Preparation Protocol

1. At least 1 month, and preferably 3–6 months, prior to the experiment, place approximately 50 of the 3 cm × 3 cm unglazed ceramic tiles or ceramic cylinders (Steinman et al., 1991b; Du-Co Ceramics Co., Saxonburg, PA: http://www.ceramics.com/duco/) in riffle habitats of the two streams to be sampled (50 tiles allows several teams of students to replicate this analysis); riffles should have similar physical characteristics (i.e., current velocity, flow, irradiance, substrate). We recommend substrates of small size because this allows them to be placed directly into an extraction jar at the time of sampling, without having to remove algae from the surface. If unglazed tiles are used, and are purchased attached to each other in sheets (as opposed to individual tiles), place the entire sheet in the stream, which minimizes the likelihood of tiles being lost if high discharge occurs.
2. At least five tiles per stream should be analyzed (four for PA and one control per stream). Alternatively, small rocks can be used but they must be small enough to fit in the incubation jars and be submersed in a small volume of water.
3. Label two acid-washed plastic containers (c. 30 cm × 30 cm = 900 cm^2) by stream name or type (high-P; low-P).
4. Label 10 wide-mouth glass incubation jars (30 mL or large enough to contain the substratum) by stream type (high-P or low-P stream): one tile per jar for each of the 4 PA tiles and one control per stream type (10 total).
5. Prepare 150 mM *p*-NPP solution (add 2.78 g of *para*-nitrophenyl phosphate to 50 mL of ultrapurified deionized water).

33.3.1.2 Field Collection Protocol

1. Collect tiles and filter water [filter about 500 mL of stream water into an acid-washed 1-L plastic bottle using a hand pump and a Whatman GFF or Gelman Type A/E glass fiber filter (GFF)] from each stream.
2. Fill the two acid-washed plastic containers (one labeled high P and the other labeled low P) with the appropriate stream water, and place five tiles (one extra per stream for PA in case of loss; control treatment has no tile) from each stream inside the container. Attach the lids to completely water-filled containers (which minimizes tile movement), and place the containers in a cooler to be transported back to the laboratory.

33.3.1.3 Laboratory Protocol

1. Using an automatic pipette, transfer 20 mL of filtered stream water (use more water if needed to completely submerse substratum) to each of the 10 incubation jars labeled for PA (5 jars for each stream type). In the laboratory, separate the sheet of tiles into individual tiles if necessary (ignore any glue that may remain attached to individual tiles following separation), and place one tile into each incubation jar. Leave one jar per stream without a tile (control).
2. Using an adjustable volume 1-mL pipette (set to 0.4 mL), transfer 0.4 mL of the *p*-NPP solution into the water (units of mmol/L) in each of the incubation jars (or proportionately more if water volume is >20 mL), cap the jar, and gently mix. Incubate the jars at room temperature for 30 min, gently hand swirling the jars every 3–5 min.
3. After 30 min, filter the water in each jar by removing the water using a 25-mL plastic syringe and filtering it through a 0.45 μm pore size syringe-mounted filter (e.g., Syrfil-MF, Costar Corp., Cambridge, MA) and collecting 10 mL of filtrate in a labeled glass scintillation vial.
4. Add 0.05 mL of 1N NaOH to each vial containing the 10 mL of filtrate from each incubation jar to bring the pH up to ~10 (for maximum color development of nitrophenol). Measure the absorbance of each filtrate at 410 nm against deionized water using a dual-beam spectrophotometer and a 1-cm pathlength cuvette.

5a. Biomass as chlorophyll *a*: Remove the tile from each jar, rinse it by immersing it into filtered, unamended stream water, and place it in a small plastic jar or centrifuge tube containing a known volume of 90% acetone that is sufficient to cover the substratum if measuring chlorophyll; follow the procedures in Chapter 12 for chlorophyll analysis.

5b. Biomass as AFDM: Remove the tile from each jar, rinse it by immersing it into filtered, unamended stream water, and scrape the material off the tile following the procedures for measuring AFDM described in Chapter 12.

33.3.1.4 Data Analysis

1. PA is calculated from the absorbance of the *p*-NPP solution as follows:

$$\mathrm{PA} = (\mathrm{Abs}_{\mathrm{sample}} - \mathrm{Abs}_{\mathrm{blank}}) \times 58 \times \mathrm{Volume}_{(\mathrm{inc})} \tag{33.2}$$

where $\mathrm{Abs}_{\mathrm{sample}}$ = absorbance reading of sample at 410 nm, $\mathrm{Abs}_{\mathrm{blank}}$ = absorbance reading of control at 410 nm (filtered stream water only, to correct for natural phosphatase in water), $\mathrm{Volume}_{(\mathrm{inc})}$ = volume of stream water in which each algal sample is incubated (in L); [if 20 mL is used (as described in this method), this value will be 0.02]. Use Table 33.1 for data entry and calculations. The value 58 in Eq. (33.2) is the specific absorbance (at pH > 10) of nitrophenol, which is the hydrolysis product of *p*-NPP.

2. PA of the periphyton community on each cylinder is computed as the amount of NPP hydrolyzed [mmol of nitrophenyl (NP) produced] per unit biomass for each sample, to obtain chlorophyll-normalized or AFDM-normalized PA (units of mmol mg chlorophyll a^{-1} 0.5 h^{-1} or mmol mg $AFDM^{-1}$ 0.5h^{-1}, respectively). If phosphatase levels are very low, the incubation period can be extended to 1 h, and the values are reported per hour. Alternatively, PA could be normalized by tile surface area to obtain area-specific PA (units of mmol cm^{-2} 0.5 h^{-1}).

33.3.2 Basic Method 2: Chemical Composition

33.3.2.1 Preparation Protocol

1. If tiles are to be used for this experiment (in lieu of rocks), place approximately 50 of the 3 cm × 3 cm unglazed ceramic tiles or ceramic cylinders (Steinman et al., 1991b) in selected high-P and low-P streams to be sampled (50 tiles allows several teams of students to replicate this analysis). Tiles should be placed in streams at least 1 month, and preferably 3–6 months, prior to the experiment.

2. Label two acid-washed plastic containers (30 cm × 30 cm) by stream name or type (high-P; low-P).

3. Combust six acid-washed 10-mL glass beakers at 500°C for 1 h. Cut out 6 pieces of aluminum foil that are large enough to cover the top of each beaker. Lightly etch the sample number onto the foil with a pointed object (do not write it on the beaker because it will burn off during combustion, potentially contributing to dry mass and leaving one unable to track individual samples).

TABLE 33.1 Sample data sheet for determination of phosphatase activity (PA). See also Online Worksheet 33.1.

Date:				Stream:				
Sample	Absorbance (410 nm)	Net Abs. (Absorbance Minus Sample Blank)	Volume (L)	PA (mmol ½h^{-1})	Chlorophyll *a* (mg)	Chl-Specific PA (mmol mg^{-1} ½h^{-1})	Ash-Free Dry Mass (AFDM) (mg)	AFDM-Specific PA (mmol mg^{-1} ½h^{-1})
Blank								
1								
2								
.								
.								
n								

33.3.2.2 Field Collection Protocol

1. Collect rocks or ceramic tiles from each stream.
2. Fill the two plastic containers (one labeled high P and the other labeled low P) with the appropriate stream water, and place three small rocks/tiles from each stream inside the container. Attach the lids to completely water-filled containers (which minimizes rock/tile movement) and place the containers in a cooler to be transported back to the laboratory.

33.3.2.3 Laboratory Protocol

1. Follow the general procedures outlined in Chapter 12 for determination of AFDM, including the following modifications. After the algae are removed from each rock/tile, add the slurry (make sure the volume is less than 10 mL) to the bottom of the precombusted, acid-washed 10-mL tared, glass beaker. Cover the top of the beaker with numbered aluminum foil. Dry the beaker to constant weight at 105°C (c. 24–48 h). Remove the beakers from the drying oven and transfer them to desiccators until weighing.
2. After the beakers have been weighed, place them in a muffle furnace at 500°C for at least 4 h (make certain the oven is at 500°C before timing), remove, and allow beakers to cool to room temperature in a desiccator and reweigh.
3. Using a 5-mL pipette, add 5 mL of 2N HCl to the beaker, label the beaker with the sample number, and replace the aluminum foil with parafilm over the beaker to prevent evaporation. Acid extraction of ashed material should last at least 24 h. Place beakers in the laboratory hood during the extraction period.
4. After extraction, transfer contents of each beaker to a 500-mL volumetric flask. Rinse the beaker with deionized water and pour rinse water into the volumetric flask as well. Bring the total volume in the volumetric flask to 500 mL by adding ultrapurified deionized water (this will result in a leachate of 0.02N HCl).
5. Pour each sample into separate acid-washed plastic bottles, label accordingly, and analyze using standard methods for analysis of P in water (APHA et al., 1995; see below).[3]

33.3.2.4 Data Analysis

1. Calculate the amount of carbon in the sample by multiplying the AFDM by 0.53. Carbon content is estimated by assuming that 53% of AFDM is composed of carbon (Wetzel, 1983). Although this value may vary slightly among algal groups and environmental conditions, the variance is low (±5%) compared to other cellular constituents. Alternatively, you may follow the guidance in Chapter 36, which calls for estimating carbon content by assuming that 30% of DM is composed of carbon. Use Table 33.2 for data entry and calculations.
2. Calculate the concentration of P in each sample by comparing its absorbance against a standard curve developed from the standards analyzed. The total amount of P (in mg) is then calculated by multiplying the P concentration by 0.5 (because the total volume of diluted leachate is 0.5 L).
3. The C:P ratio is calculated by dividing the total C by the total P in each sample (converted to the same mass units) and then multiplying by 2.58 (to convert to a molar basis). Compare the ratio to the Redfield ratio (106:1) and analyze the differences between the high-P and low-P streams.

33.3.3 Basic Method 3: Net Nutrient Uptake (Stable Phosphorus)

33.3.3.1 Preparation Protocol

1. At least 1 month, and preferably 3–6 months, prior to the experiment, place approximately 50 of the 3 cm × 3 cm unglazed ceramic tiles or ceramic cylinders (Steinman et al., 1991b) in selected high-P and low-P streams to be sampled. If tiles were purchased in sheets, and uptake is to be normalized by AFDM, then tiles should be preashed to remove attached glue, which otherwise would be included in the AFDM measurement. If tiles are purchased individually or uptake is to be normalized by chlorophyll *a*, then no preashing is necessary.
2. Label two acid-washed plastic containers (30 cm × 30 cm) by stream name or type (high P; low P).
3. Label six acid-washed 50-mL collection bottles according to treatment (high P vs. low P) and time ("initial," "30 min," and "60 min").

3. Standards for P analysis must be made in 0.02 N HCl to be comparable to that of samples. Make sure to use personal protective equipment when handling reagents.

TABLE 33.2 Sample data sheet for determination of chemical composition (italicized letters in formulae refer to column letter). See also Online Worksheet 33.1.

Date:			Stream:			
	A	*B*	*C*	*D*	*E*	*F*
Sample	Beaker + Dried Material	Beaker + Ashed Material	Ash-Free Dry Mass (AFDM) = *A* − *B*	Carbon (mg) (AFDM × 0.53)	Phosphorus (mg) From Digestion and SRP Analysis	Molar C:P [(*D*/*E*) × 2.58]
1						
2						
3						
.						
.						
.						
n						

4a. Label 20 aluminum weigh boats by etching the bottom of the boat with a sharp edge to designate sample number, if measuring biomass by AFDM or

4b. Label 20 small plastic jar or centrifuge tube to designate sample number, if measuring biomass by chlorophyll *a*.

33.3.3.2 Field Collection Protocol

1. Collect tiles and filter water (filter 1 L of stream water into an acid-washed 1-L plastic bottle using a hand pump and a Whatman GFF or Gelman Type A/E GFF) from each stream.

2. Place 10 tiles into a labeled plastic container (high P or low P) per team, which is filled with appropriate stream water. Attach the lids to completely water-filled containers (which minimizes tile movement), and place the containers in a cooler to be transported back to the laboratory.

33.3.3.3 Laboratory Protocol

1. Transfer the 1 L of filtered stream water and tiles into one incubation chamber per stream (the number of tiles placed in each chamber will be dependent on the amount of biomass attached to the substratum; a general rule of thumb would be at least 10 tiles if biomass is low and 5 to 10 tiles if it is high). Ideally there would be multiple chambers with 5 to 10 tiles per stream, not just one chamber per stream type, to have true replication but that can become prohibitively expensive. As a consequence, we default here to just one chamber per treatment stream.

2. Using a 30-mL automatic pipette, remove 30 mL of stream water from each chamber and transfer to the sample bottle labeled "initial." Filter the 30-mL water samples through a 0.45 μm pore size syringe filter (e.g., Syrfil-MF, Costar Corp., Cambridge, MA). Start either the pumps or the stir bar in the chamber.

3. Remove 30 mL of stream water at 30 and 60 min after the start of the incubation, and transfer the water to the appropriately labeled bottle. Filter the samples as in step 2. If the water samples are not going to be analyzed for SRP within a few hours, place the bottles in the refrigerator (for storage up to 1 week) or freezer (for storage >1 week) until they can be analyzed for SRP levels (APHA et al., 1995; see below).

4. After 60 min, remove the tiles from the chamber. If normalizing uptake by AFDM, see step 5; if normalizing uptake by chlorophyll *a*, see step 6.

5a. Place the tiles in an appropriately labeled aluminum weigh boat and dry the tiles to constant weight at 105°C (c. 24−48 h). Remove the weigh boats from the drying oven and transfer them to desiccators until weighing.

5b. After the weigh boats have been weighed, place them in muffle furnace at 500°C for at least 4 h (make certain the ovens are at 500°C before timing), remove, and allow them to cool to room temperature in a desiccator and reweigh.

Calculate AFDM as the difference between the dry mass and the combusted mass, and sum the AFDM of all substrates in each chamber.

6. Place each tile in a small plastic jar or centrifuge tube containing a known volume of 90% acetone that is sufficient to cover the substratum if measuring chlorophyll; follow the procedures in Chapter 12 for chlorophyll analysis.
7. Analyze water samples for SRP (see below).

33.3.3.4 Soluble Reactive Phosphorus Analysis (Adapted From APHA et al., 1995)

1. Make up appropriate reagents:
   a. H_2SO_4 solution: Add 140 mL concentrated sulfuric acid to 900 mL of ultrapurified deionized water.
   b. Ammonium molybdate solution: Dissolve 15 g of ammonium molybdate in 500 mL of ultrapurified deionized water (store in polyethylene bottle in the dark).
   c. Ascorbic acid solution: Dissolve 2.7 g of ascorbic acid in 50 mL of ultrapurified deionized water. Make immediately before using or keep frozen.
   d. Antimony potassium tartrate solution: Dissolve 0.34 g of antimony potassium tartrate in 250 mL ultrapurified deionized water.
   e. Mixed reagent: Combine 25 mL of sulfuric acid solution, 10 mL of ammonium molybdate solution, 5 mL of antimony potassium tartrate solution, and 5 mL of ascorbic acid solution. Use within 6 h of preparation.
   f. Phosphorus standards: (1) Stock solution—Dissolve 0.2197 g of anhydrous K_2HPO_4 in 1 L of ultrapurified deionized water (1.00 mL = 50 μg P/L); (2) Prepare four standard curve solutions by diluting from stock solution—a typical range is 5−1000 μg/L but this may be extended in either direction depending on the concentrations in the selected streams and sensitivity of instrumentation; (3) Develop a standard curve of absorbance versus SRP concentration.
2. Add 3.0 mL of mixed reagent to 30 mL of standard and all samples and mix thoroughly.
3. Wait for at least 20 min, but not longer than 1 h, and measure absorbance of solution at 885 nm against distilled water on a spectrophotometer using 10-cm pathlength cuvettes (shorter pathlengths can be used, but this is not recommended as analytical sensitivity is reduced).
4. Calculate SRP concentration (μg/L) by comparing absorbance of sample against the standard curve.

33.3.3.5 Data Analysis

1. Plot the SRP concentration versus time to determine whether or not the relationship appears to be linear. Calculate the net P uptake rate using the following formula:

$$V = ([C_o - C_f] \times L)/t \tag{33.3}$$

where V = net uptake rate (μg P/h), C_o = initial SRP concentration, C_f = final SRP concentration, L = incubation volume (in L), and t = time period of incubation (h). The net P uptake rate should then be normalized to either total biomass in the incubation (e.g., AFDM or chlorophyll *a*) or total substratum surface area. Use Table 33.3 for data entry and calculations.

TABLE 33.3 Sample data sheet for determination of net P uptake. See also Online Worksheet 33.1.

Date:	Stream:
Time (min)	**Soluble Reactive Phosphorus Concentration (μg/L)**
0	
30	
60	
Calculated uptake rate: Total ash-free dry mass (AFDM) or chlorophyll *a* in sample: Uptake per unit AFDM (μg P mg AFDM^{-1} min^{-1}): Uptake per unit chlorophyll *a* (μg P mg chlorophyll a^{-1} min^{-1}):	

33.3.4 Advanced Method 1: Gross Nutrient Uptake (Phosphorus Radiotracer)

(Review cautionary notes on use of radioisotopes described previously.)

33.3.4.1 Preparation Protocol

1. At least 1 month and preferably 3–6 months, prior to the experiment, place approximately 100 small unglazed ceramic tiles or ceramic cylinders in selected high-P and low-P streams to be sampled. If tiles are used, they should be preashed to remove attached glue, which otherwise would be included in the AFDM measurement.
2. Label acid-washed plastic containers (30 cm × 30 cm) by stream name or type (high-P; low-P).
3. Each team assigned to a stream should have six 25-mL scintillation vials, each containing 15 mL of scintillation cocktail, labeled according to treatment (high P or low P) and time (background, 10, 20, 30, 45, and 60 min).

33.3.4.2 Field Collection Protocol

1. Collect 5 (uptake only) or 16 (uptake and turnover) tiles and water (filter 1 L of stream water into an acid-washed 1-L plastic bottle using a hand pump and a Whatman GFF or Gelman Type A/E GFF) from each stream.
2. Place the 5 (uptake only) or 16 (uptake and turnover) tiles per stream into labeled acid-washed plastic containers (high P or low P), which are filled with stream water. Attach the lids to completely water-filled containers (which minimizes tile movement) and place containers in a cooler to be transported back to the laboratory.

33.3.4.3 Laboratory Protocol

1. Transfer the 1 L of filtered stream water and 5 (uptake only) or 16 (uptake and turnover) tiles into each incubation chamber. An extra tile will be collected from each stream but remain unlabeled by isotope; this tile will serve as a background control (see Section 33.3.5.2).
2. Transfer approximately 50 mL of the filtered stream water to a 60-mL acid-washed plastic bottle, which will be analyzed for SRP concentration (see Section 33.3.3.4).
3. Remove 1 mL of water from each chamber just prior to the ^{33}P injection.[4] Transfer this water to the appropriately labeled scintillation vial (background) and mix thoroughly.
4. Inject 0.5 mCi of carrier-free $^{33}PO_4$ (as either orthophosphoric acid or phosphate salt dissolved in water) with a micropipette into each chamber. The micropipette tip will be extremely radioactive, so it should be removed immediately from the pipette after use and discarded in a radioactive waste bin.
5. Remove 1 mL of water from each chamber at 10, 20, 30, 45, and 60 min after the start of the incubation. Transfer the water to the appropriately labeled scintillation vial and mix thoroughly.[5]
6. After the 60 min sample is collected, carefully remove the tiles from the chamber using forceps or tongs **(remember, the material attached to the tiles will be radioactive and should be handled with great care)**. These tiles will then be processed for AFDM measurement, if turnover is not to be measured. For AFDM measurement, follow the procedures outlined below (step 7), making sure to avoid touching the radioactive material (keeping the tiles inside the beaker at all times minimizes this risk). If P turnover is to be measured, it is recommended that the tiles remain in the radiolabeled chamber water for an additional 5 h (6 h total) to allow for greater incorporation of ^{33}P by the algae. Radiolabeled tiles are then transported back out to the streams (if permitted) or returned to recirculating chambers/aquaria if turnover is to be measured. **Cautionary notes**: Wear gloves, safety glasses, and lab coats at all times when handling radioactive samples (consult the local radiation safety officer at your institution for guidance and specific regulations associated with your site). Store the radioactive water from the incubation in sealed and labeled carboys until the radioactivity decays to background levels. It is recommended to store the water for at least 10 half-lives before disposal (the half-life of ^{33}P is 25.4 days).
7. Finish measuring AFDM according to the methods outlined above, with the important modification of *not* removing the algae from the substratum. Instead, weigh the substratum with attached algae, and calculate AFDM as the difference in mass before and after combustion. Double-bag, seal, label, and store the radioactive waste until the radioactivity decays

4. If there are obvious signs of seston in the chamber water, it will be necessary to filter the subsamples before they are added to the scintillation vials (to remove radioactively labeled particulate material). This can be done by removing approximately 3 mL of water from each chamber with a 15-mL syringe, filtering the water through a 0.45-μm pore size syringe filter (e.g., Syrfil-MF, Costar Corp., Cambridge, MA) into a small beaker and then pipetting 1 mL of this filtrate into the scintillation vial.
5. The first sample is not taken until 10 min to allow complete mixing of radioisotope within the chamber.

to background levels (10 half-lives or ~250 days). Place the radioactive chambers in an appropriately labeled containment bin until reuse.

8. Count each scintillation vial for 10 min on a liquid scintillation counter (the counting efficiency for ^{33}P is generally >90%, and because the sample matrix is the same for all samples, no correction for counting efficiency is needed). No decay correction is needed if all samples are counted within a few hours of each other.
9. SRP concentration of the initial stream water will be measured according to the methods outlined above (Section 33.3.3.4).

33.3.4.4 Data Analysis

1. Gross P uptake rate is measured using the first-order rate coefficient of radiotracer depletion in the water (k), the concentration of SRP in the stream water, and the water volume during the incubation (Steinman et al., 1991a). This procedure consists of three steps:
   a. Calculate k by regressing the ln-normalized scintillation count data (minus the background value determined from the sample collected just prior to ^{33}P injection) against time. Use Table 33.4 for data entry and calculations (also see Fig. 33.4).
   b. Gross P uptake rate is then estimated by multiplying k by the SRP concentration and by the water volume in the incubation chamber. Based on the data from the sample data sheet and depicted in Fig. 33.4, k (−0.0038/min = −0.0228/h) is multiplied by 6.2 (SRP concentration) and 1.0 (L of water in chamber). This rate is in units of μg P/h.
   c. Gross P uptake rate should then be normalized to the biomass in the chamber (μg P mg AFDM^{-1} h^{-1}) or by surface area of substrata in the chamber (μg P cm^{-2} h^{-1}). The gross uptake rate is divided either by the total AFDM in the chamber (e.g., 70.4 mg based on the sample data sheet) or total substrata surface area in chamber (e.g., 160 cm^2 based on the sample data sheet) to obtain a normalized uptake rate.

33.3.5 Advanced Method 2: Phosphorus Turnover

33.3.5.1 Preparation Protocol

1. Combust 40 acid-washed 10-mL glass beakers at 500°C for 1 h. Cut out 40 pieces of aluminum foil that are large enough to cover the top of each beaker. Gently etch the following information onto the foil with a pointed object (do not write it on the beaker because it will burn off during combustion, potentially contributing to dry mass and leaving one unable to track individual samples): stream type (high-P or low-P), sampling date (Day 0, 2, 5, or 10), and replicate (a–d) (i.e., 16 beakers/stream type).

TABLE 33.4 Sample data sheet for determination of gross phosphorus uptake (radiotracer). See also Online Worksheet 33.1.

Date:		Stream:	
Time (min)	**Counts per Minute (CPM ^{33}P)**	**CPM Minus Background**	**ln (CPM Minus Background)**
0 (Background)	49.4		
10	2009.8	1960.4	7.581
20	1939.1	1889.7	7.544
30	1841.7	1792.3	7.491
45	1761.8	1712.4	7.446
60	1669.4	1620	7.390

Calculated uptake rate constant (k): −0.0038/min
Stream water soluble reactive phosphorus concentration (μg/L): 6.2
Calculated uptake rate (μg P min^{-1}): 0.024
Ash-free dry mass (AFDM) (mg) or surface area (cm^2) in sample:
AFDM = 70.4 mg; surface area = 160 cm^2
Uptake per unit AFDM (μg P mg AFDM^{-1} min^{-1}): 0.000341
Uptake per unit surface area (μg P cm^{-2} min^{-1}): 0.00015

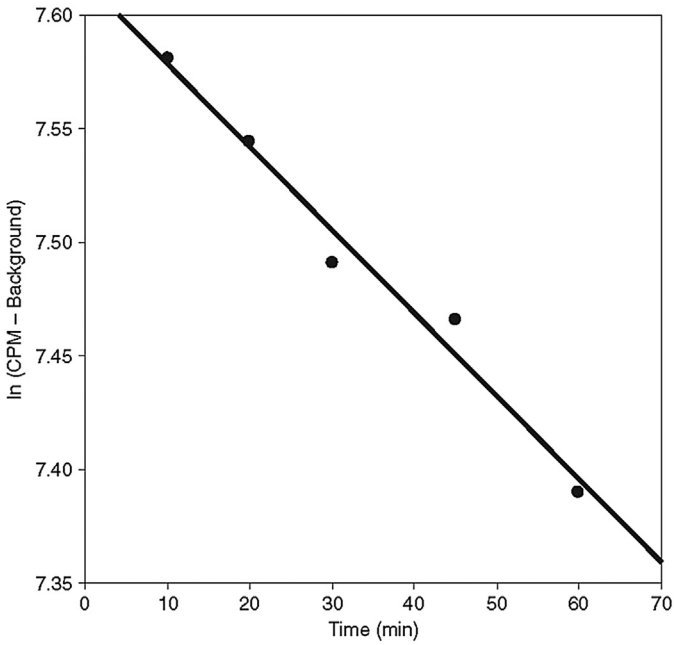

FIGURE 33.4 Radioactivity of ^{33}P in water incubated with periphyton exposed to grazing snails. Background activity of ^{33}P has been subtracted from measured activity and the data ln-normalized. Data used to generate the figure are based on real experiments and are included in the total P uptake sample data sheet (Table 33.3). *CPM*, counts per minute.

33.3.5.2 Field Placement and Collection (Option 1)

This option is to be followed if permission is obtained by the appropriate authorities to place radiolabeled material in the selected streams. If permission cannot be obtained, follow the steps in Section 33.3.5.3 (Option 2, below).

1. After completion of the radioisotopic uptake study (Section 33.3.4.3), carefully remove the tiles with forceps or tongs from each chamber, place them into plastic containers, and transport them to the high-P and low-P streams.
2. Place tiles in locations that have similar current velocity, irradiance level, temperature, and grazer density in both streams, if possible. Remove four radioactive tiles from each stream at 1 h [the 1 h incubation allows "day 0" samples to be rinsed by "cold" (i.e., nonradioactive) stream water prior to sampling to wash off adsorbed residual ^{33}P] and again at 2, 5, and 10 days, and place them in appropriately labeled and tared 50-mL glass beakers. One additional unlabeled tile should be processed from each stream; any radioactivity associated with the algae on this tile will be subtracted off all other counts, as it represents the naturally occurring background radioactivity (or the background activity associated with the scintillation counting of samples). The laboratory procedures associated with this option are included in Section 33.3.5.4 (below).

33.3.5.3 Chamber/Aquarium Placement and Collection (Option 2)

For this option, radiolabeled tiles are placed in either a high-P or low-P recirculating chamber or aquarium with unlabeled and filtered (0.45 μm) water from each stream. Ideally, the water in each chamber should be changed daily to minimize released ^{33}P from being taken up again. However, this option is time-consuming and can generate a considerable volume of contaminated waste. If water is not changed, it should be recognized that the calculated turnover rates will represent underestimates of true turnover.

1. After completion of the radioisotopic uptake study (Section 33.3.4.3), carefully remove the 17 tiles (16 labeled; 1 unlabeled) with forceps or tongs from each chamber, place them into plastic containers, and transport them to the unlabeled high-P or low-P chambers. If possible, chambers should be exposed to light and temperature regimes that are similar to ambient conditions and be fitted with a means to circulate or move the water (e.g., aeration or stirring).
2. Remove four tiles from each chamber at 1 h (the 1-h incubation allows "day 0" samples to be rinsed by stream water prior to sampling to wash off adsorbed residual ^{33}P) and again at 2, 5, and 10 days, and place them in appropriately labeled, tared 50-mL glass beakers. An additional unlabeled tile also should be processed from each stream; any radioactivity associated with the algae on this tile will be subtracted off all other counts, as it represents the naturally occurring background radioactivity.

TABLE 33.5 Sample data sheet for determination of P turnover. See also Online Worksheet 33.1.

Date:		Stream:			
	ln (Counts per Minute)				
Replicate	Background	Day 0 Minus Background	Day 2 Minus Background	Day 5 Minus Background	Day 10 Minus Background
A					
B					
C					
D					

33.3.5.4 Laboratory Protocol (Applicable for Both Options 1 and 2)

1. On the day of collection (days 0, 2, 5, and 10), place the beakers in a drying oven and dry the tiles to constant weight at 105°C (usually 24–48 h).
2. Once the tiles reach a constant dry mass, weigh the dried tiles, and combust them for a minimum of 4 h at a full 500°C. Remove tiles, allow them to cool to room temperature in a desiccator, and reweigh.
3. Using a 10-mL pipette, add 10 mL of 2N HCl to the beaker (make sure this is enough to cover all the periphyton), label the beaker with the appropriate sample designation, and place Parafilm over the beaker. Leaching of ashed material should last at least 24 h. Place beakers in the laboratory hood.
4. After a minimum exposure of 24 h to the acid, add 10 mL of deionized water to each beaker (to reduce acidity to 1N), swirl the beaker gently to mix thoroughly, and pipette 1 mL of the diluted leachate to a scintillation vial containing 15 mL of scintillation cocktail.
5. Count each sample on a liquid scintillation counter for 10 min. It is recommended that all samples from the turnover experiment be counted during the same run (within several hours of each other) at experiment's end to preclude the need to apply a radioactive decay correction factor. The counts are used to determine turnover rate.

33.3.5.5 Data Analysis

1. P turnover rate is computed by linear regression of ln [^{33}P] counts in algae versus time in stream (in days). P turnover rate is therefore expressed as a first-order turnover rate constant (days^{-1}). The background radioactivity associated with unlabeled tiles should be subtracted from each sample count prior to performing the ln-transformation and the regression, and the regression should be based on a mean value derived from the four substrata sampled on each day. Use Table 33.5 for data entry and calculations.
2. It should be emphasized that this determination of P turnover rate may not be an accurate physiological index of total algal P turnover rate. This is because not all P pools within the algal cells will have reached isotopic equilibrium during the 6 h of ^{33}P exposure during the uptake part of the experiment (cf. Scinto and Reddy, 2003). However, the approach described should provide a reasonable basis for comparing turnover rates in the more rapidly cycling P pools between different streams.

33.4 QUESTIONS

33.4.1 Limitation: Phosphatase Activity

1. Was phosphatase activity greater in the low-P stream, as hypothesized? If not, what might explain this result?
2. What other factors besides P concentration and biomass might influence the PA in the two streams?
3. Why is it important to normalize the PA data by an index of biomass?
4. Phosphatase is an inducible enzyme. That is, it is synthesized upon metabolic demand, as opposed to a constitutive enzyme, which is always present. What advantage is there to an organism in maintaining phosphatase as an inducible enzyme?

33.4.2 Limitation: Chemical Composition

5. C:P ratios substantially greater than 106:1 (on a molar basis) suggest P deficiency in planktonic algae (the Redfield ratio). Why is the C:P ratio suggesting P deficiency different in plankton compared to benthic algae (>360:1)?
6. Some algal species have greater carbon demands than others because of more carbon-based compounds in their cell walls. How would this type of demand influence the interpretation of the C:P ratio?
7. Many algal species exhibit "luxury uptake" of phosphorus (Rier et al., 2016), whereby they take up excessive amounts of P when it is available (e.g., during high-P conditions) and then store the P intracellularly (in polyphosphate bodies). How would luxury uptake of P influence the chemical composition ratio of benthic algae?

33.4.3 Net Uptake: Stable Phosphorus

8. Were the net uptake rates similar or different between the two streams? If they were different, what might account for this difference?
9. Sometimes, no net uptake is measured during an incubation (i.e., the amount of P measured at the start of the experiment is the same as that at the end of the experiment). Assuming that the algae are biologically active and actively taking up phosphorus, what might explain this result?
10. Would you expect the same P uptake rates by algae irrespective of which method of measuring uptake was used (i.e., stable vs. radioisotopic P)? If so, why? If not, how might the rates differ and why?

33.4.4 Total Uptake: Radiolabeled Phosphorus

11. Were the gross uptake rates similar or different between the two streams? If they were different, what might account for this difference? In which stream do you expect to measure the greater uptake of ^{33}P? Why?
12. By keeping the incubation time short in this exercise, you minimize the possibility that any radioactive P that was taken up could be rereleased within the incubation period (i.e., minimize the possibility of recycling). Thus, the radioactive P removed from the water is assumed to represent the total or *gross nutrient uptake* rate. How does this differ from net uptake rate (i.e., which measure should be greater)? Why?

33.4.5 Turnover

13. Were the P turnover rates similar or different between the two streams? If they were different, what might account for this difference?
14. If the ^{33}P was allowed to come to complete isotopic equilibrium within the algae during the uptake part of the experiment, would you expect measured P turnover rates to be greater or lower than those measured? Why?
15. How might the thickness of the periphyton matrix influence turnover rates? What about grazing activity?

33.5 MATERIALS AND SUPPLIES

Letters in parentheses indicate in which method (A = Basic Method 1, B = Basic Method 2, C = Basic Method 3, D = Advanced Method 1, or E = Advanced Method 2) the item is used.[6]

Field Materials

1-L polyethylene bottles (A, C, D)
Cooler (A, B, C, D)
Hand pumps with GFF (or equivalent) filters (A, C, D)
Holder to transport beakers to and from field (E)
Tupperware containers to accommodate tiles or rocks (A, B, C, D, E)
Unglazed ceramic tiles (e.g., tiles measuring 3 cm × 3 cm or ceramic cylinders) (A, C, D, E)

Laboratory Materials (excludes SRP analysis; see Section 33.3.3)

1N NaOH (A)

6. Any use of isotope requires specific laboratory protocols. These protocols are available from the Safety and/or Risk Management Department at the institution or laboratory licensed for isotope use. These protocols must be followed carefully. It is essential that gloves (we recommend double gloving, using vinyl gloves directly over the hands and disposable gloves over the vinyl ones), lab coats, and safety glasses be worn at all times.

2N HCl (B, E)
10-mL glass beakers (B, D)
25-mL plastic syringes with syringe holders; 0.45 μm pore size (A, C, D)
25-mL scintillation vials (A, D, E)
50-mL glass beakers (E)
50-mL polyethylene bottles (C)
60-mL polyethylene bottles (D)
100 mL polyethylene bottles (B)
100-mL volumetric cylinders (B)
150 mM *para*-nitrophenyl phosphate solution (add 2.78 g of *para*-nitrophenyl phosphate to 50 mL of ultrapurified deionized water) (A)
90% acetone (90 parts acetone with 10 parts saturate magnesium carbonate solution)
Aluminum foil (B, E)
Aluminum weigh boats (C, D)
Carrier-free ^{33}P isotope (0.5 mCi/chamber; order from PerkinElmer: http://www.perkinelmer.com/Catalog/Family/ID/Phosphorus33%20Radionuclide%20Orthophosphoric%20Acid%20in%201mL%20HClfree%20Water) (D)
Course-bristled toothbrushes (B)
EcoLume Scintillation Cocktail (ICN Scientific, Costa Mesa, CA) (D, E)
Large pans or trays (B)
Parafilm (B, E)
Plastic jars or centrifuge tubes (A)
Reagents for SRP analysis (B, D)
Wide-mouth glass incubation jars (30 mL or larger for larger substrata) (A)

Laboratory Equipment

Analytical balance (A, B, C, D, E)
Automatic pipettes (1 mL; 10 mL; 30 mL) (A, B, C, D, E)
Desiccators (B, C)
Drying oven (B, C, D, E)
Liquid Scintillation Counter (D, E)
Muffle furnace (B, C, D, E)
Recirculating chambers (either with pumps or stirrers to circulate water) (C, D)
Spectrophotometer (narrow band width: 0.5−2.0 nm) and cuvettes (1 and 10 cm pathlength) (A, B, C, D)

ACKNOWLEDGMENTS

This chapter is devoted to the memory of Pat Mulholland, who was the coauthor on previous editions and whose influence spirals throughout this chapter. May his memory serve as a blessing. The authors thank Mary Ogdahl and Gary Lamberti for their constructive comments on the chapter and Eric Nemeth for his artistic assistance. These experiments, and several of the methods, were developed during research supported by the National Science Foundation's Ecosystem Program, the US Department of Energy's Office of Science, Office of Biological and Environmental Research, and the Michigan Department of Environmental Quality.

REFERENCES

Allan, J.D., 2004. Landscapes and riverscapes: the influence of land use on stream ecosystems. Annual Review of Ecology, Evolution, and Systematics 35, 257−284.

APHA, AWWA, WEF, 1995. Standard Methods for the Examination of Water and Wastewater, nineteenth ed. American Public Health Association, Washington, DC, USA.

Bernhardt, E.S., Likens, G.E., Buso, D.C., Driscoll, C.T., 2003. In-stream uptake dampens effects of major forest disturbance on watershed nitrogen export. Proceedings of the National Academy of Sciences of the United States of America 100, 10304−10308.

Borchardt, M.A., 1996. Nutrients. In: Stevenson, R.J., Bothwell, M.L., Lowe, R.L. (Eds.), Algal Ecology. Academic Press, San Diego, CA, USA, pp. 183−227.

Borchardt, M.A., Hoffmann, J.P., Cook, P.W., 1994. Phosphorus uptake kinetics of *Spirogyra fluviatilis* (Charophyceae) in flowing water. Journal of Phycology 30, 403−412.

Bothwell, M.L., 1989. Phosphorus-limited growth dynamics of lotic periphytic diatom communities: areal biomass and cellular growth rate responses. Canadian Journal of Fisheries and Aquatic Sciences 46, 1293−1301.

Burkholder, J.M., Wetzel, R.G., 1990. Epiphytic alkaline phosphatase on natural and artificial plants in an oligotrophic lake: re-evaluation of the role of macrophytes as a phosphorus source for epiphytes. Limnology and Oceanography 35, 736–747.

Cardinale, B.J., 2011. Biodiversity improves water quality through niche partitioning. Nature 472, 86–89.

Carey, R.O., Vellidis, G., Lowrance, R., Pringle, C.M., 2007. Do nutrients limit algal periphyton in small blackwater coastal plain streams? Journal of the American Water Resources Association 43, 1183–1193.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., Smith, V.H., 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecological Applications 8, 559–568.

Cembella, A.D., Antia, N.J., Harrison, P.J., 1984. The utilization of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective: Part 1. Critical Reviews in Microbiology 10, 317–391.

Colman, A.S., Blake, R.E., Karl, D.M., Fogel, M.L., Turekian, K.K., 2005. Marine phosphate oxygen isotopes and organic matter remineralization in the oceans. Proceedings of the National Academy of Sciences of the United States of America 102, 13023–13028.

Cross, W.F., Benstead, J.P., Rosemond, A.D., Wallace, J.B., 2003. Consumer-resource stoichiometry in detritus-based streams. Ecology Letters 6, 721–732.

Currie, D.J., Bentzen, E., Kalff, J., 1986. Does algal-bacterial phosphorus partitioning vary among lakes? A comparative study of orthophosphate uptake and alkaline phosphatase activity in fresh water. Canadian Journal of Fisheries and Aquatic Sciences 43, 311–318.

Darley, W.M., 1982. Algal Biology: A Physiological Approach. Blackwell Scientific, Oxford, UK.

Davies, J.-M., Bothwell, M.L., 2012. Responses of lotic periphyton to pulses of phosphorus: P-flux controlled growth rate. Freshwater Biology 57, 2602–2612.

Davis, L.S., Hoffmann, J.P., Cook, P.W., 1990. Seasonal succession of algal periphyton from a wastewater treatment facility. Journal of Phycology 26, 611–617.

Dodds, W.K., 2003. The role of periphyton in phosphorus retention in shallow freshwater aquatic systems. Journal of Phycology 39, 840–849.

Dodds, W.K., 2006. Eutrophication and trophic state in rivers and streams. Limnology and Oceanography 51, 671–680.

Dodds, W.K., Smith, V.H., Zander, B., 1997. Developing nutrient targets to control benthic chlorophyll levels in streams: a case study of the Clarke Fork River. Water Research 31, 1738–1750.

Drake, W.M., Scott, J.T., Evans-White, M., Haggard, B., Sharpley, A., Rogers, C.W., Grantz, M.E., 2012. The effect of periphyton stoichiometry and light on biological phosphorus immobilization and release in streams. Limnology 13, 97–106.

Droop, M.R., 1974. The nutrient status of algal cells in continuous culture. Journal of the Marine Biological Association of the United Kingdom 54, 825–855.

Duhamel, S., Gregori, G., Van Wambeke, F., Nedoma, J., 2009. Detection of extracellular phosphatase activity at the single-cell level by enzyme-labeled fluorescence and flow cytometry: the importance of time kinetics in ELFA labeling. Cytometry. Part A: The Journal of the International Society for Analytical Cytology 75A, 163–168.

Duhamel, S., Dyhrman, S.T., Karl, D.M., 2010. Alkaline phosphatase activity and regulation in the North Pacific Subtropical Gyre. Limnology and Oceanography 55, 1414–1425.

Dyhrman, S., Ruttenberg, K.C., 2006. Presence and regulation of alkaline phosphatase activity in eukaryotic phytoplankton from the coastal ocean: implications for dissolved organic phosphorus remineralization. Limnology and Oceanography 51, 1381–1390.

Ellwood, N.T.W., Di Pippo, F., Albertano, P., 2012. Phosphatase activities of cultured phototrophic biofilms. Water Research 46, 378–386.

Elsbury, K.E., Paytan, A., Ostrom, N.E., Kendall, C., Young, M.B., McLaughlin, K., Rollog, M.E., Watson, S., 2009. Using oxygen isotopes of phosphate to trace phosphorus sources and cycling in Lake Erie. Environmental Science and Technology 43, 3108–3114.

Elwood, J.W., Newbold, J.D., Trimble, A.F., Stark, R.W., 1981. The limiting role of phosphorus in a woodland stream ecosystem: effects of P enrichment on leaf decomposition and primary producers. Ecology 62, 146–158.

Espeland, E.M., Francoeur, S.N., Wetzel, R.G., 2002. Microbial phosphatase in biofilms: a comparison of whole community enzyme activity and individual bacterial cell-surface phosphatase expression. Archiv für Hydrobiologie 153, 581–593.

Finlay, J.C., Hood, J.M., Limm, M.P., Power, M.E., Schade, J.D., Welter, J.R., 2011. Light-mediated thresholds in stream-water nutrient composition in a river network. Ecology 92, 140–150.

Francoeur, S.N., 2001. Meta-analysis of lotic nutrient amendment experiments: detecting and quantifying subtle responses. Journal of the North American Benthological Society 20, 358–368.

Frost, P.C., Stelzer, R.S., Lamberti, G.A., Elser, J.J., 2002. Ecological stoichiometry of trophic interactions in the benthos: understanding the role of C:N:P ratios in lentic and lotic habitats. Journal of the North American Benthological Society 21, 515–528.

Geider, R., La Roche, J., 2002. Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. European Journal of Phycology 37, 1–17.

Grimm, N.B., Fisher, S.G., 1986. Nitrogen limitation in a Sonoran Desert stream. Journal of the North American Benthological Society 5, 2–15.

Hall, S.R., Smith, V.H., Lytle, D.A., Leibold, M.A., 2005. Constraints on primary producer N:P stoichiometry along N:P supply ratio gradients. Ecology 86, 1894–1904.

Healey, F.P., 1973. Inorganic nutrient uptake and deficiency in algae. Critical Reviews in Microbiology 3, 69–113.

Healey, F.P., Hendzel, L.L., 1979. Fluorometric measurement of alkaline phosphatase activity in algae. Freshwater Biology 9, 429–439.

Hecky, R.E., Kilham, P., 1988. Nutrient limitation of phytoplankton in freshwater and marine environments: a review of recent evidence on the effects of enrichment. Limnology and Oceanography 33, 796–822.

Hecky, R.E., Campbell, P., Hendzel, L.L., 1993. The stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. Limnology and Oceanography 38, 709–724.

Hernandez, I., Niell, F.X., Whitton, B.A., 2002. Phosphatase activity of benthic marine algae. An overview. Journal of Applied Phycology 14, 475–487.
Hill, W.R., Knight, A.W., 1988. Nutrient and light limitation of algae in two northern California streams. Journal of Phycology 24, 125–132.
Hill, W.R., Ryon, M.G., Schilling, E.M., 1995. Light limitation in a stream ecosystem: responses by primary producers and consumers. Ecology 76, 1297–1309.
Hillebrand, H., Kahlert, M., 2001. Effect of grazing and nutrient supply on periphyton biomass and nutrient stoichiometry in habitats of different productivity. Limnology and Oceanography 46, 1881–1898.
Hillebrand, H., Sommer, U., 1999. The nutrient stoichiometry of benthic microalgal growth: redfield proportions are optimal. Limnology and Oceanography 44, 440–446.
Hillebrand, H., Frost, P., Liess, A., 2008. Ecological stoichiometry of indirect grazer effects on periphyton nutrient content. Oecologia 155, 619–630.
Hoppe, H.G., 2003. Phosphatase activity in the sea. Hydrobiologia 493, 187–200.
Hudson, J.J., Taylor, W.D., Schindler, D.W., 2000. Phosphate concentrations in lakes. Nature 406, 54–56.
Hwang, S.-J., Havens, K.E., Steinman, A.D., 1998. Phosphorus kinetics of planktonic and benthic assemblages in a shallow subtropical lake. Freshwater Biology 40, 729–745.
Jansson, M., 1993. Uptake, exchange, and excretion of orthophosphate in phosphate-starved *Scenedesmus quadricauda* and *Pseudomona* K7. Limnology and Oceanography 38, 1162–1178.
Jansson, M., Olsson, H., Pettersson, K., 1988. Phosphatases; origin, characteristics and function in lakes. Hydrobiologia 170, 157–175.
Johnson, L.T., Tank, J.L., Dodds, W.K., 2009. The influence of land use on stream biofilm nutrient limitation across eight North American ecoregions. Canadian Journal of Fisheries and Aquatic Sciences 66, 1081–1094.
Jordan, T.E., Correll, D.L., Weller, D.E., 1997. Effects of agriculture on discharges of nutrients from coastal plain watersheds of Chesapeake Bay. Journal of Environmental Quality 26, 836–848.
Kahlert, M., 1998. C:N:P ratios of freshwater benthic algae. Archiv für Hydrobiologie, Special Issues: Advances in Limnology 51, 105–114.
Karl, D.M., Björkman, K., 2015. Dynamics of dissolved organic phosphorus. In: Hansell, D.A., Carlson, C.A. (Eds.), Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, Burlington, Ontario, Canada, pp. 233–334.
Kaushal, S.S., Groffman, P.M., Band, L.E., Elliott, E.M., Shields, C.A., Kendall, C., 2011. Tracking nonpoint source nitrogen pollution in human-impacted watersheds. Environmental Science and Technology 45, 8225–8232.
Kilham, P., Hecky, R.E., 1988. Comparative ecology of marine and freshwater phytoplankton. Limnology and Oceanography 33, 776–795.
Lock, M.A., Wallace, R.R., Costerton, J.W., Ventullo, R.M., Charlton, S.E., 1984. River epilithon: toward a structural-functional model. Oikos 42, 10–22.
Lohman, K., Priscu, J.C., 1992. Physiological indicators or nutrient deficiency in *Cladophora* (Chlorophyta) in the Clark Fork of the Columbia River, Montana. Journal of Phycology 28, 443–448.
Lohman, K., Jones, J.R., Baysinger-Daniel, C., 1991. Experimental evidence for nitrogen limitation in a northern Ozark stream. Journal of the North American Benthological Society 10, 14–23.
Manafi, M., Kneifel, W., Bascomb, S., 1991. Fluorogenic and chromogenic substrates used in bacterial diagnostics. Microbiological Reviews 55, 335–348.
McCall, S.J., Bowes, M.J., Warnaars, T.A., Hale, M.S., Smith, J.T., Warwick, A., Barrett, C., 2014. Phosphorus enrichment of the oligotrophic River Rede (Northumberland, UK) has no effect on periphyton growth rate. Inland Waters 4, 121–132.
McLaughlin, K., Kendall, C., Silva, S.R., Young, M., Paytan, A., 2006. Phosphate oxygen isotope ratios as a tracer for sources and cycling of phosphate in North San Francisco Bay, California. Journal of Geophysical Research: Biogeosciences 111, G03003.
Michalak, A.M., Anderson, E.J., Beletsky, D., Boland, S., Bosch, N.S., Bridgeman, T.B., Chaffin, J.D., Chog, K., Confesor, R., Daloğlu, I., DePinto, J.V., Evans, M.A., Fahnenstiel, G.L., He, L., Ho, J.C., Jenkins, L., Johengen, T.H., Kuo, K.C., LaPorte, E., Liu, X., McWilliams, M.R., Moore, M.R., Posselt, D.J., Richards, R.P., Scavia, D., Steiner, A.L., Verhamme, E., Wright, D.M., Zagorski, M.A., 2013. Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions. Proceedings of the National Academy of Sciences of the United States of America 110, 6448–6452.
Van Mooy, B.A., Fredricks, H.F., Pedler, B.E., Dyhrman, S.T., Karl, D.M., Koblížek, M., Lomas, M.W., Mincer, T.J., Moore, L.R., Moutin, T., Rappé, M.S., Webb, E.A., 2009. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature 458, 69–72.
Mulholland, P.J., 1996. Role of nutrient cycling in streams. In: Stevenson, R.J., Bothwell, M.L., Lowe, R.L. (Eds.), Algal Ecology. Academic Press, San Diego, CA, USA, pp. 609–633.
Mulholland, P.J., Rosemond, A.D., 1992. Periphyton response to longitudinal nutrient depletion in a woodland stream: evidence of upstream-downstream linkage. Journal of the North American Benthological Society 11, 405–419.
Mulholland, P.J., Steinman, A.D., Elwood, J.W., 1990. Measurement of phosphorus uptake length in streams: comparison of radiotracer and stable PO_4 releases. Canadian Journal of Fisheries and Aquatic Sciences 47, 2351–2357.
Mulholland, P.J., Steinman, A.D., Marzolf, E.R., Hart, D.R., DeAngelis, D.L., 1994. Effect of periphyton biomass on hydraulic characteristics and nutrient cycling in streams. Oecologia 98, 40–47.
Mulholland, P.J., Tank, J.L., Webster, J.R., Bowden, W.B., Dodds, W.K., Gregory, S.V., Grimm, N.B., Hamilton, S.K., Johnson, S.L., Martí, E., McDowell, W.H., Merriam, J.L., Meyer, J.L., Peterson, B.J., Valett, H.M., Wollheim, W.M., 2002. Can uptake length in streams be determined by nutrient addition experiments? Results from an interbiome comparison study. Journal of the North American Benthological Society 21, 544–560.
Nedoma, J., Štrojsová, A., Vrba, J., Komárková, J., Šimek, K., 2003. Extracellular phosphatase activity of natural plankton studied with ELF97 phosphate: fluorescence quantification and labelling kinetics. Environmental Microbiology 5, 462–472.

Newman, S., McCormick, P.V., Backus, J.G., 2003. Phosphatase activity as an early warning indicator of wetland eutrophication: problems and prospects. Journal of Applied Phycology 15, 45–59.

Pant, H.K., Reddy, K.R., Dierberg, F.E., 2002. Bioavailability of organic phosphorus in a submerged aquatic vegetation-dominated treatment wetland. Journal of Environmental Quality 31, 1748–1756.

Payn, R.A., Webster, J.R., Mulholland, P.J., Valett, H.M., Dodds, W.K., 2005. Estimation of stream nutrient uptake from nutrient addition experiments. Limnology and Oceanography: Methods 3, 174–182.

Perrin, C.J., Richardson, J.S., 1997. N and P limitation of benthos abundance in the Nechako River, British Columbia. Canadian Journal of Fisheries and Aquatic Sciences 54, 2574–2583.

Peterson, B.J., Hobbie, J.E., Corliss, T.L., Kriet, D., 1983. A continuous flow periphyton bioassay: tests of nutrient limitation in a tundra stream. Limnology and Oceanography 28, 582–595.

Pringle, C.M., Paaby-Hansen, P., Vaux, P.D., Goldman, C.R., 1986. In situ assays of periphyton growth in a lowland Costa Rica stream. Hydrobiologia 134, 207–213.

Raven, P.H., Evert, R.F., Curtis, H., 1981. Biology of Plants, third ed. Worth Publishers, New York, USA.

Reaves, M.L., Sinha, S., Rabinowitz, J.D., Kruglyak, L., Redfield, R.J., 2012. Absence of detectable arsenate in DNA from arsenate-grown GFAJ-1 cells. Science 337, 470–473.

Redfield, A.C., 1958. The biological control of chemical factors in the environment. American Scientist 46, 205–221.

Rengefors, K., Pettersson, K., Blenckner, T., Anderson, D.M., 2001. Species-specific alkaline phosphatase activity in freshwater spring phytoplankton: application of a novel method. Journal of Plankton Research 23, 435–443.

Rhee, G.Y., 1978. Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. Limnology and Oceanography 23, 10–24.

Rier,, S.T., Kinek,, K.C., Hay, S.E., Franceoeur, S.N., 2016. Polyphosphate plays a vital role in the phosphorus dynamics of stream periphyton. Freshwater Science 35, 490–502.

Rigler, F.H., 1966. Radiobiological analysis of inorganic phosphorus in lakewater. Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie 16, 465–470.

Rivkin, R.B., Swift, E., 1982. Phosphate uptake by the oceanic dinoflagellate *Pyrocystis noctiluca*. Journal of Phycology 18, 113–120.

Rosemond, A.D., Mulholland, P.J., Elwood, J.W., 1993. Top-down and bottom-up control of stream periphyton: effects of nutrients and herbivores. Ecology 74, 1264–1280.

Schindler, D.W., 1977. The evolution of phosphorus limitation in lakes. Science 195, 260–262.

Scinto, L.J., Reddy, K.R., 2003. Biotic and abiotic uptake of phosphorus by periphyton in a subtropical freshwater wetland. Aquatic Botany 77, 203–222.

Scott, J.T., Lang, D.A., King, R.S., Doyle, R.D., 2009. Nitrogen fixation and phosphatase activity in periphyton growing on nutrient diffusing substrata: evidence for differential nutrient limitation in stream periphyton. Journal of the North American Benthological Society 28, 57–68.

Sharma, K., Inglett, P.W., Reddy, K.R., Ogram, A.V., 2005. Microscopic examination of photoautotrophic and phosphatase-producing organisms in phosphorus-limited Everglades periphyton mats. Limnology and Oceanography 50, 2057–2062.

Sirová, D., Borovec, J., Černá, B., Rejmánková, E., Adamec, L., Vrba, J., 2009. Microbial community development in the traps of aquatic *Utricularia* species. Aquatic Botany 90, 129–136.

Solórzano, L., Sharp, J.H., 1980. Determination of total and dissolved phosphorus and particulate phosphorus in natural waters. Limnology and Oceanography 25, 574–578.

Steinman, A.D., 1994. The influence of phosphorus enrichment on lotic bryophytes. Freshwater Biology 31, 53–63.

Steinman, A.D., Ogdahl, M.E., 2015. TMDL reevaluation: reconciling phosphorus load reductions in a eutrophic lake. Lake and Reservoir Management 31, 115–126.

Steinman, A.D., Mulholland, P.J., Kirschtel, D.B., 1991a. Interactive effects of nutrient reduction and herbivory on biomass, taxonomic structure, and P uptake in lotic periphyton communities. Canadian Journal of Fisheries and Aquatic Sciences 48, 1951–1959.

Steinman, A.D., Mulholland, P.J., Palumbo, A.V., Flum, T.F., DeAngelis, D.L., 1991b. Resilience of lotic ecosystems to a light-elimination disturbance. Ecology 72, 1299–1313.

Steinman, A.D., Mulholland, P.J., Hill, W.R., 1992. Functional responses associated with growth form in stream algae. Journal of the North American Benthological Society 11, 29–243.

Steinman, A.D., Mulholland, P.J., Beauchamp, J.J., 1995. Effects of biomass, light, and grazing on phosphorus cycling in stream periphyton communities. Journal of the North American Benthological Society 14, 371–381.

Steinman, A.D., Ogdahl, M.E., Wessell, K., Biddanda, B., Kendall, S., Nold, S., 2011. Periphyton response to simulation nonpoint source pollution in the lower Muskegon River watershed. Aquatic Ecology 45, 439–454.

Stelzer, R.S., Lamberti, G.A., 2002. Ecological stoichiometry in running waters: periphyton chemical composition and snail growth. Ecology 83, 1039–1051.

Stockner, J.G., Shortreed, K.R., 1978. Enhancement of autotrophic production by nutrient addition in a coastal rainforest stream on Vancouver Island. Journal of the Fisheries Research Board of Canada 35, 28–34.

Stutter, M.I., Demars, B.O.L., Langan, S.J., 2010. River phosphorus cycling: separating biotic and abiotic uptake during short-term changes in sewage effluent loading. Water Research 44, 4425–4436.

Tank, J.L., Dodds, W.K., 2003. Nutrient limitation of epilithic and epixylic biofilms in ten North American streams. Freshwater Biology 48, 1031–1049.

Wetzel, R.G., 1981. Long-term dissolved and particulate alkaline phosphatase activity in a hardwater lake in relation to lake stability and phosphorus enrichments. Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie 21, 369–381.

Wetzel, R.G., 1983. Limnology, second ed. Saunders College Publishing, Philadelphia, Pennsylvania, USA.

Whitford, L.A., Schumacher, G.J., 1964. Effect of a current on respiration and mineral uptake in *Spirogyra* and *Oedogonium*. Ecology 45, 168–170.

Withers, P.J.A., Jarvie, H.P., 2008. Delivery and cycling of phosphorus in rivers: a review. Science of the Total Environment 400, 379–395.

Wolfe-Simon, F., Blum, J.S., Kulp, T.R., Gordon, G.W., Hoeft, S.E., Pett-Ridge, J., Stolz, J.F., Webb, S.M., Weber, P.K., Davies, P.C.W., Anbar, A.D., Oremland, R.S., 2011. A bacterium that can grow by using arsenic instead of phosphorus. Science 332, 1163–1166.

Young, M.B., McLaughlin, K., Kendall, C., Stringfellow, W., Rollog, M., Elsbury, K., Donald, E., Paytan, A., 2009. Characterizing the oxygen isotopic composition of phosphate sources to aquatic ecosystems. Environmental Science and Technology 43, 5190–5196.

Young, E.B., Tucker, R.C., Pansch, L.A., 2010. Alkaline phosphatase in freshwater *Cladophora*-epiphyte assemblages: regulation in response to phosphorus supply and localization. Journal of Phycology 46, 93–101.