

## Partitioning stoichiometric components of epilithic biofilm using mixing models

Gaston E. Small<sup>1</sup>\*, Rebecca J. Bixby<sup>2</sup>, Caner Kazanci<sup>3,4</sup>, and Catherine M. Pringle<sup>5</sup>

<sup>1</sup>Department of Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, MN 55108

<sup>2</sup>Department of Biology, University of New Mexico, Albuquerque, NM 87131

<sup>3</sup>Department of Mathematics, University of Georgia, Athens, GA 30602

<sup>4</sup>Faculty of Engineering, University of Georgia, Athens, GA 30602

<sup>5</sup>Odum School of Ecology, University of Georgia, Athens, GA 30602

### Abstract

Epilithic biofilms are an important and complex food resource in shallow aquatic ecosystems. Standard methods of sampling these biofilms typically yield a combination of algal cells, other organic material, and inorganic sediment. Physical methods of isolating algal fractions rely on differences in density among biofilm components, and clean separation is not always possible. Here, we explore the application of linear mixing models to the problem of estimating nutrient content of biofilm components. This method is based on the assumption that variation in bulk sample elemental composition within a sampling site is due to differences among samples in relative composition. By using standard methods to quantify organic content and algal biomass within biofilm samples, the mixing model produces estimates of nutrient content of each end member. To test this method, we analyzed the phosphorus (P) content of algal cells, non-algal organic material, and inorganic sediment associated with biofilm samples that increase in P-content 4-fold across a natural P gradient in lowland streams in Costa Rica. Our analyses show that nearly all of the observed increase in biofilm P from high-P streams was due to elevated P-content of algal cells, rather than P sorbed to inorganic sediments or in P-rich heterotrophic bacteria. Results from the mixing model were supported by results from density fractionation. This linear mixing model approach complements empirical methods of separating complex food resources and may lead to a better understanding of trophic interactions in aquatic food webs.

A central objective of food-web ecology is characterizing food resources that are important to given consumers (e.g., Lindeman 1942; Sterner and Elser 2002). In cases where a “food resource” is a mixture of living microscopic organisms

and detritus, sampling and analyzing this material as a homogeneous resource may misrepresent important characteristics of a complex food resource and could lead to errors in quantifying trophic relationships.

Benthic algae are an important basal food resource in many aquatic food webs (Lamberti 1996). Even in forested streams, algae can contribute disproportionately to energy flow relative to their biomass (e.g., Mulholland et al. 2000; March and Pringle 2003; Lau et al. 2009) due to their rapid turnover rate and relatively high nutritional value. Benthic algae are found in complex biofilms, which, in addition to algal cells, can include bacteria, fungi, microzoans, algal detritus, and detritus of other aquatic or terrestrial origin, all contained within a polysaccharide matrix secreted by these organisms (Lock et al. 1984). Algal cells typically constitute < 15% of the carbon in these biofilms (Frost et al. 2005). Standard methods for collecting benthic algae (Steinman et al. 2006) result in samples that include a mixture of different biofilm components, and can also include substantial amounts of inorganic sediment.

Although aquatic ecologists have traditionally considered

\*Corresponding author: E-mail: gesmall@umn.edu

### Acknowledgments

We are grateful to M. Hidalgo for field assistance in Costa Rica. The Organization for Tropical Studies provided logistical support. T. Maddox and L. Dean at the University of Georgia Analytical Chemistry Laboratory assisted with laboratory analyses. J. C. Finlay, M. R. Whiles, G. Taylor, and two anonymous reviewers provided comments that greatly improved the manuscript. Research funding was provided by the National Science Foundation Long-Term Research in Environmental Biology program (DEB 0545463). During the writing of this manuscript, G.E.S. was supported by an Environmental Protection Agency (EPA) Science to Achieve Results (STAR) award (FP-91687101-0). EPA has not officially endorsed this publication and the views expressed herein may not reflect the views of the EPA.

epilithic biofilm as a homogeneous food resource, benthic consumers may feed selectively (Arsuffi and Suberkropp 1985; Lodge 2006) or differentially assimilate various biofilm components (Wotton 1994). Evidence from whole-stream  $^{15}\text{N}$  additions has shown that some grazers become more isotopically enriched than any of their food resources (e.g., Mulholland et al. 2000; Rezanka and Hershey 2003), indicating that these taxa are primarily feeding on a more-enriched component of this bulk food resource. Accurately measuring food resource quality is essential in understanding consumers' rates of growth and nutrient recycling (Sterner and Elser 2002). However, despite the importance of epilithic biofilm in many aquatic food webs, quantifying the elemental composition of different components of this complex food resource remains a challenge.

As an alternative to reporting elemental composition of bulk biofilm samples, physical separation is a major step toward distinguishing between the characteristics of different biofilm components. Hamilton et al. (2005) developed a promising method for density fractionation in colloidal silica, which separates samples into lighter (predominantly algal) and heavier (predominantly detrital) components. However, samples with higher-density algae (e.g., diatoms) may not separate cleanly from detritus that is similar in density (e.g., McNeely et al. 2007), effectively limiting the utility of this method.

Here, as a complementary approach to physical methods of biofilm separation, we explore the potential of using linear mixing models to estimate nutrient composition of biofilm components. Mixing models have been used extensively in the geosciences and more recently in stable isotope ecology (e.g., Fry 2006). Frost et al. (2005) used a simple mixing model to show that carbon:phosphorus (C:P) ratios in biofilm can be determined largely by the C:P ratio of algal cells, even when algae constitute a small fraction of total biofilm mass. Here, we apply an inverse mixing model approach to address the problem of estimating nutrient content of algal and nonalgal biofilm fractions. Instead of using known characteristics of end-members to estimate the relative composition (as in isotope mixing models), this application uses known values of the relative contributions of end members (based on measurements of ash-free dry mass and algal biomass) to estimate the elemental composition of each end member.

This approach is based on two central assumptions:

- (1) A given component of a biofilm sample (e.g., algae, nonalgal organic matter, or sediment) has uniform stoichiometric properties among multiple samples within a given stream reach.
- (2) Therefore, variation in stoichiometric properties among multiple samples within a stream is due to differences in algal content and total organic matter content.

Whereas these assumptions are unlikely to hold strictly true (e.g., different algal species may vary in elemental composition due to differences in optimal nutrient ratios and polysaccharide production, and variation within individual cells within a species can result from differences in cell age,

metabolism, and physiology), this assumption of elemental uniformity is applied only among replicate samples collected within a given reach, where such deviation may be minimized. The resulting mixing model produces estimates of the mean elemental composition of each biofilm component within that reach, so violations of these assumptions result in larger variance associated with these means. Our purpose here is to evaluate whether a simple model that utilizes measurements that are commonly made by investigators can be used to extract additional information quantifying nutrient composition of algal and non-algal components of biofilm. Because this method relies on extrapolation, a large range in the relative composition among replicate samples is required to produce well-constrained estimates of nutrient content for each end-member.

We present a two-compartment mixing model, which estimates biofilm P content from organic and inorganic components, as well as a three-compartment mixing model, which provides separate estimates of the P content of algal and non-algal organic material in addition to inorganic material. We implement this approach using a dataset of biofilm stoichiometry from a natural P-gradient in streams in lowland Costa Rica. We evaluate the model results by comparing them with results from the density fractionation method.

#### Two-compartment mixing model

The P-content (%P by dry mass) of organic and inorganic components of biofilm can be estimated using the equation:

$$\%P_{\text{composite}} = \frac{(\%sed)}{100} \times (\%P_{\text{sed}}) + \frac{(\%org)}{100} \times (\%P_{\text{org}}) \quad (1)$$

where  $\%P_{\text{composite}}$  is the measured P-content of a composite biofilm sample,  $\%sed$  and  $\%org$  are the fractions of inorganic sediment and organic material, respectively, in the biofilm sample (as determined by  $\%ash\text{-free dry mass [AFDM]}$ ),  $\%P_{\text{sed}}$  is the P-content of inorganic sediment (relative to dry mass), and  $\%P_{\text{org}}$  is the P-content of organic material (relative to dry mass) within biofilm. For this analysis, each sample from a given site requires measurements of  $\%sed (= 100 - \%AFDM)$ ,  $\%org (= \%AFDM)$ , and  $\%P$  for the composite sample. As described above, we assume that  $\%P_{\text{sed}}$  and  $\%P_{\text{org}}$  do not vary among samples from a given stream reach. The effect of inorganic material associated with biota (e.g., silica in diatom frustules) on calculated values is addressed below.

To solve for the unknown variables  $\%P_{\text{sed}}$  and  $\%P_{\text{org}}$ , regression analysis can be used to examine a possible relationship between  $\%org$  and composite sample  $\%P$ . Extrapolating to 0% organic matter (i.e., pure inorganic sediment) and 100% organic matter (i.e., sediment-free biofilm) provides predictions of the P-content (and 95% confidence intervals) for these end-members. While regression analysis is typically used to examine relationships between an independent and dependent variable, in this application, regression is used to generate estimates and confidence intervals of the two end-members. A nonsignificant  $P$  value indicates that the two end-

members are similar in magnitude (see example models in Web Appendix 1).

One potential drawback of this mixing-model approach is that it can produce negative estimates of %P for one of the end members, which, of course, is a physical impossibility. For such estimates, when 95% confidence intervals include zero, we recommend setting that end member to zero to avoid inflating the predicted value for the other end member. For cases in which the model generates a negative estimate for which the 95% confidence interval does not include zero, a linear model would not be appropriate for that dataset.

### Three-compartment mixing model

The organic component of biofilm samples can be further divided into algal and non-algal material if algal biomass is quantified. Although chlorophyll is often used as an indicator of algal biomass (Steinman et al. 2006), chlorophyll:C ratios of algae are highly variable (Frost et al. 2005), limiting the value of chlorophyll in this approach. Where diatoms compose the bulk of algal biomass in biofilm samples, total algal C can be estimated from algal biovolume measurements, using a conversion factor of 0.145 mg C mm<sup>-3</sup> (Hessen et al. 2003; note that we assume this value for pelagic diatoms also applies to benthic diatoms). To estimate the contribution of algal cells to the total biomass of the organic component of biofilm, the algal C value is then divided by the measured total C for the bulk biofilm sample (based on the assumption that the %C of algal cells is equal to the %C of the overall organic component of biofilm). The contribution of algae to the total biofilm dry mass (including associated inorganic matter) is then calculated by multiplying the above term by the %org for the sample.

A three-compartment mixing model can then be used to estimate biofilm P-content of inorganic, algal, and non-algal organic fractions using the following equation:

$$\%P_{\text{composite}} = \left(\frac{\%sed}{100}\right) \times (\%P_{\text{sed}}) + \left(\frac{\%alg}{100}\right) \times (\%P_{\text{alg}}) + \left(\frac{\%org}{100}\right) \times (\%P_{\text{org}}) \quad (2)$$

where %P<sub>composite</sub> is the measured P-content of the composite biofilm sample, and %sed, %alg, and %org are the fractions of biofilm mass composed by inorganic sediment, algal cells, and non-algal organic matter, respectively. Non-algal organic matter is assumed to be the difference between calculated algal biomass and total organic matter in each sample: %org = 100 - (%sed + %alg).

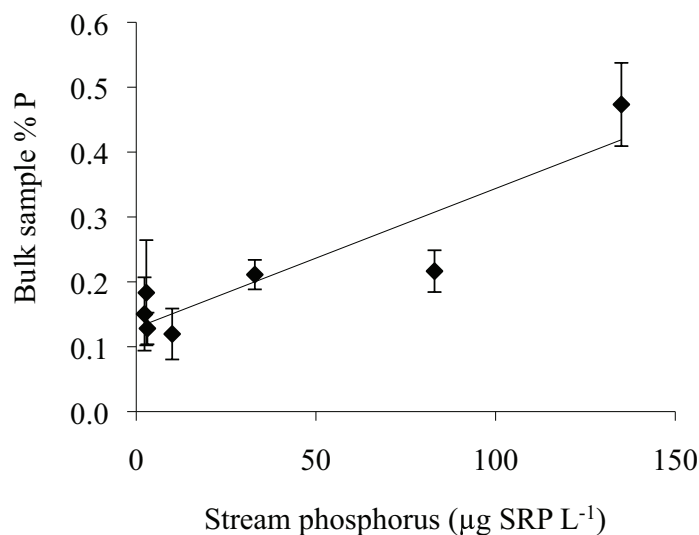
The three unknown variables, %P<sub>sed</sub>, %P<sub>alg</sub>, and %P<sub>org</sub> can be estimated using multiple regression, with measured variables %alg and %sed used as independent variables, and %P<sub>composite</sub> as the dependant variable. %P, along with associated confidence intervals, can then be estimated for each end member (pure inorganic sediment = 100% sed, 0% alg; pure algae = 0% sed, 100% alg; pure nonalgal organic material = 0% sed, 0% alg; see example models in Web Appendix 1).

## Applying the biofilm mixing-model

### Background

La Selva Biological Station, on the Caribbean slope of lowland Costa Rica, has been the focus of numerous studies in tropical stream ecology and biogeochemistry because of its unique geomorphological landscape features (e.g., Pringle and Triska 1991; Rosemond et al. 2002; Ramírez and Pringle 2006; Triska et al. 2006). Some streams at La Selva receive groundwater inputs containing elevated levels of P and other solutes (Pringle et al. 1993; Genereux et al. 2009). These streams range in soluble reactive phosphorus (SRP) from 2-150 µg L<sup>-1</sup> and low-solute streams are P-limited, for both heterotrophic processes (e.g., microbial respiration, Ramírez et al. 2003; organic matter decomposition, Rosemond et al. 2002) and primary production in light-gaps (Pringle and Triska 1991). Due to low light levels as a result of the forest canopy, diatoms compose > 90% of algal biomass in these study sites.

In these study streams, the P-content of epilithic biofilm increases 5-fold with increasing stream P levels across the natural P-gradient, leading to a 2-fold enrichment in P-content of invertebrate consumers (Small and Pringle 2010). Biofilm P-content increased from 0.1 %P (by dry weight) to 0.5 %P (Fig. 1), exceeding values reported from other nutrient-rich streams (e.g., Stelzer and Lamberti 2001; Cross et al. 2003; Bowman et al. 2005). However, how P is partitioned among different components of the biofilm could have important implications for its bioavailability. For example, biofilm samples contain large amounts of inorganic sediment (mean across all sites: 77 ± 1% by dry weight). Abiotic P-sorption is an important process in these streams, which are characterized by fine, clay-rich sediment (e.g., Triska et al. 2006), and P-sorption to sediment was shown to be a significant long-term



**Fig. 1.** Phosphorus content (relative to dry mass) of bulk biofilm samples (mean ± SE) collected from unglazed ceramic tiles in seven streams across natural P-gradient ( $R^2 = 0.83$ ,  $P = 0.004$ ).

P sink during an 8-y experimental P-addition to a naturally low-P stream (Small et al. 2008). Therefore, abiotic P-sorption could account for much of the elevated P-content in biofilm samples, potentially overestimating the amount of P available to benthic grazers. Within the biotic component of biofilm, P-enrichment of the composite sample could be caused by higher P-content of algal cells (due to luxury P-consumption: Rhee 1972; Klausmeier et al. 2008), P-enriched polysaccharide excretions outside algal cells (Noe et al. 2003), or by higher concentrations of P-rich bacteria within the biofilm (Sterner and Elser 2002). Using natural variability among replicate samples within streams to partition the P-content of these various components of biofilm could lead to a more ecologically relevant understanding of how this important food resource responds to high levels of dissolved P.

### Materials and procedures

In March and June 2006, biofilm samples were collected in seven streams that range naturally in soluble reactive phosphorus (SRP) from 2–151  $\mu\text{g L}^{-1}$ . At each sampling date, four replicate samples were collected from ceramic tiles and from natural substrata (described in Small and Pringle 2010). Each biofilm sample was transferred to a clean beaker and diluted to a known volume. While stirring, three subsamples (40–100 mL) were filtered onto pre-combusted and pre-weighed Whatman glass fiber filters (0.7  $\mu\text{m}$  pore size). One filter was dried at 60°C for 24 h, weighed to nearest 0.1 mg, combusted at 500°C for 2 h, and reweighed to determine ash-free dry mass. The second filter was placed in an aluminum foil pouch and frozen for later analysis of chlorophyll *a*. Chl *a* was measured on a Turner Designs model 10-AU fluorometer (Turner Designs) using standard methods (APHA 1998). A third filter was dried for later carbon and phosphorus analysis. This filter was subsampled using a hole-punch, and corrected for filter weight using holes punched from blank filters. Carbon content of biofilm was measured using a Carlo Erba NA 1500 CHN analyzer (Carlo Erba). For P-analysis, samples were acid-digested (Aqua Regia double acid; Jones et al. 1991) and analyzed spectrophotometrically (ascorbic acid method). Ground pine needles (US National Institute of Standards and Technology, 1575a) were used as external standards for P-analyses.

Because previous work had shown highly elevated biofilm P-content in the high-P Arboleda-30 site (Small and Pringle 2010), we collected additional data from this site to better understand how P was partitioned in these samples. We collected a 5 mL subsample for diatom identification and cell biovolume calculation from each biofilm sample collected. A minimum of 300 valves were enumerated and identified along transects on microscope slide coverslips using a Zeiss Universal research microscope (Carl Zeiss) with brightfield immersion optics at 1250 $\times$ . Identifiable valve fragments along each transect were categorized by size relative to whole valves and mathematically reconstituted to whole-valve units and fractions. We calculated the biovolume for each taxon by measur-

ing dimensions of 10 cells and using published geometric equations (Hillebrand et al. 1999).

As an independent check on algal P-content, in June 2006, we used the density-fractionation method described by Hamilton et al. (2005) to compare P-content of heavy and light fractions (after centrifuging in colloidal silica) from the seven study streams across the natural P-gradient. Due to the small amount of material recovered from light fractions, replicate samples were pooled for analysis ( $n = 1$  for each of the seven study sites). Each heavy and light fraction was subsampled and analyzed for AFDM, chl *a*, and nutrient content as described above.

For each of the seven study streams for which biofilm P-content is available, we estimated the %P of organic and inorganic fractions using the two-compartment mixing model described above. Regressions were performed using SAS (SAS Institute 2001). We then used the results of the 2-compartment mixing model to test for a relationship between stream SRP and %P of each of the biofilm components, also using regression analysis.

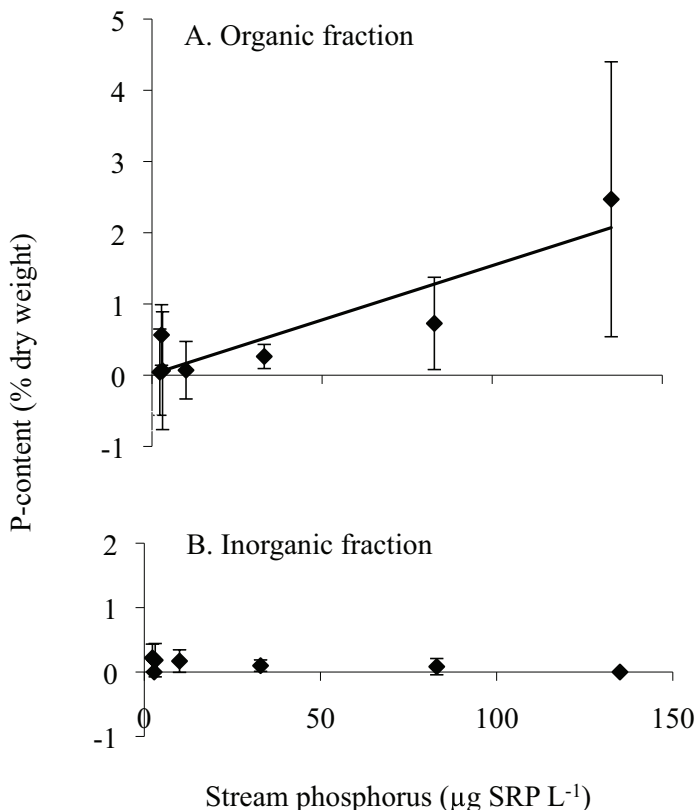
### Assessment

Across our seven study streams, estimated %P of the organic fraction ranged from 0.04% to 3.68%, and increased with stream SRP ( $P = 0.001$ ,  $R^2 = 0.82$ , Fig. 2A). In contrast, estimated %P of the inorganic fraction remained negligible even at high SRP concentrations (Fig. 2B). Estimates of %P<sub>sed</sub> ranged from -0.24% to 0.22%, and confidence intervals for %P<sub>sed</sub> for five of the seven streams included zero. Although two of the seven estimates for inorganic fraction %P<sub>sed</sub> were negative, 95% confidence intervals included 0 for both of these streams, and the model was recalculated setting these two values to zero to avoid inflating estimates of %P<sub>org</sub> (Table 1).

The mixing model estimates of organic and inorganic P-content were generally supported by results of density fractionation by centrifuge. Light fractions had higher mean organic matter content ( $34 \pm 9\%$ ) relative to the heavy fractions ( $15 \pm 1\%$ ). Both heavy and light fractions had similar P-content (all below 0.2 %P, relative to dry mass), except for in the high-P Arboleda-30 site, where the light fraction was 1.04 %P compared to 0.28 %P for the heavy fraction (Fig. 3).

From the two-compartment mixing model, we can conclude that P-enrichment measured in composite biofilm samples does not appear to be due to P sorbed to associated inorganic sediment. Furthermore, results suggest that the actual P-content of sediment-free biofilm in the high-P streams may be 10-fold higher than values measured based on composite samples (i.e., with 75% inorganic sediment by dry weight). In the Arboleda (SRP = 151  $\mu\text{g L}^{-1}$ ), composite biofilm samples had a mean %P of 0.32, compared with the estimated P-content of 3.68% for the organic fraction. We note that a small fraction of the “inorganic sediment” in our analysis is composed of diatomaceous silica (see “Discussion” below). Nevertheless, these results indicate that, in high-P streams, failing to

correct for inorganic sediment in biofilm samples could lead to a substantial underestimate of the actual P-content of this food resource, when measured as % by mass.



**Fig. 2.** Estimated P-content (relative to dry mass) of (A) organic and (B) inorganic end-members of biofilm ( $\pm 95\%$  CI) versus stream soluble reactive phosphorus (SRP) in the seven study streams. The estimated P-content of the organic component of biofilm increases with increasing dissolved P-levels ( $R^2 = 0.82$ ,  $P = 0.001$ ), while the P-content of inorganic sediment associated with these samples is negligible across all study streams.

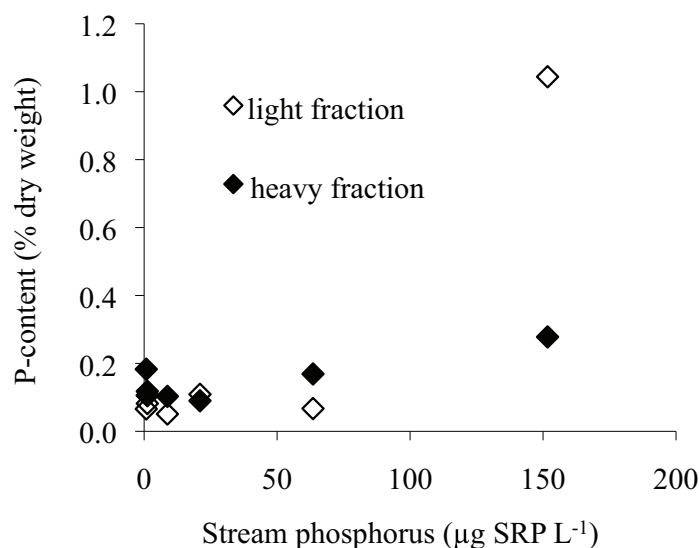
**Table 1.** Estimates of P-content (as %P by dry weight) of organic and inorganic content in biofilm samples from seven stream sites that vary widely in soluble reactive phosphorus (SRP) due to differential inputs of high-solute groundwater. Each site name includes a number indicating approximate elevation (m above sea level). The model produced negative estimates of %P<sub>sed</sub> for two sites (Arboleda-30 and Sura-60). Because 95% confidence intervals for these estimates included zero, %P<sub>sed</sub> was set at zero for these two sites (shown in **bold**) to produce new estimates of %P<sub>org</sub> for these sites.

Site	SRP ( $\mu\text{g L}^{-1}$ )	SE	<i>n</i>	<i>R</i> <sup>2</sup>	<i>P</i> value	%P <sub>org</sub>	Upper 95%	Lower 95%	%P <sub>sed</sub>	Upper 95%	Lower 95%
Arboleda-30	151.7	10.9	21	0.57	<0.001	3.68	5.06	2.29	-0.24	0.02	-0.50
				0.49	<0.001	2.47	3.00	1.93	<b>0.00</b>		
Sura-30	63.5	9.8	20	0.15	0.10	0.73	1.37	0.08	0.08	0.21	-0.04
Saltito-60	21.0	2.9	21	0.09	0.18	0.26	0.43	0.09	0.10	0.19	0.01
Salto-60	8.8	1.6	17	0.03	0.53	0.07	0.47	-0.33	0.17	0.34	0.00
Sura-60	1.2	1.2	14	0.61	<0.001	0.71	1.06	0.36	-0.05	0.08	-0.18
				0.54	< 0.001	0.57	0.71	0.43	<b>0.00</b>		
Piper-30	1.2	1.3	13	0.02	0.69	0.04	0.65	-0.56	0.22	0.43	0.01
Saltito-100	0.8	1.0	17	0.10	0.22	0.06	0.89	-0.76	0.18	0.44	-0.07

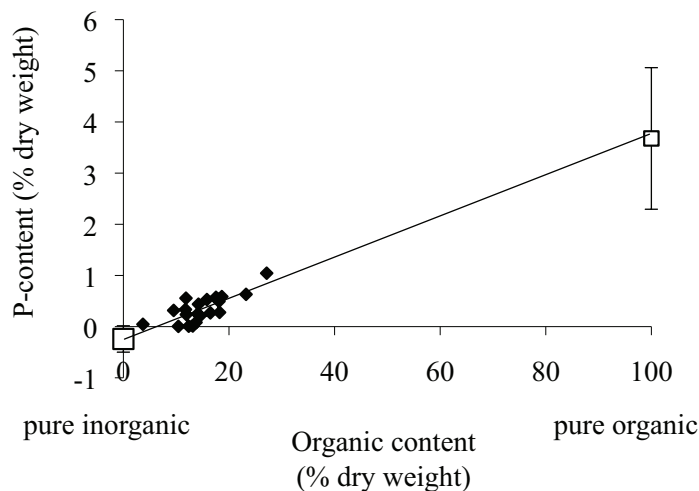
### Three-compartment mixing model

The two-compartment model above showed that biofilm in the high-P Arboleda stream has a highly P-enriched organic fraction (Fig. 4). We applied a three-compartment mixing model to determine whether this additional P occurs in the algal or non-algal components of the organic fraction. Because diatoms comprise the bulk of the overall algal cells in these samples, we estimated algal biomass using algal biovolume measurements for each replicate sample for this stream. We used data from eight biofilm samples from natural substrate and ceramic tiles collected in this stream in March 2006 (bulk sample %P, AFDM, and algal biomass) to solve for the three unknown parameters (%P<sub>sed</sub>, %P<sub>alg</sub>, and %P<sub>org</sub>) using multiple regression analysis.

The resulting model has an *r*<sup>2</sup> value of 0.80 (Fig. 5A). Estimated %P<sub>alg</sub> is  $22.88 \pm 17.11$  (estimate  $\pm$  95% C.I.), compared



**Fig. 3.** Phosphorus content (relative to dry mass) of light (open symbol) and heavy (shaded symbol) fractions of biofilm, following density fractionation, versus stream soluble reactive phosphorus (SRP).

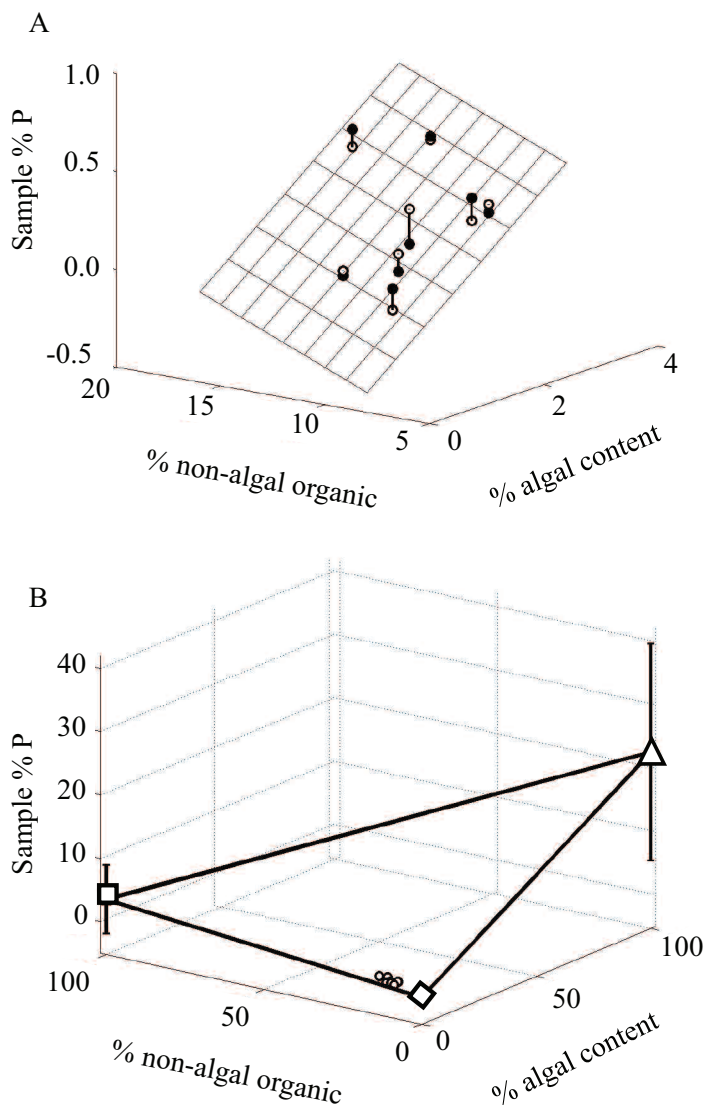


**Fig. 4.** Relationship between % organic matter and bulk sample P-content (relative to dry mass) for 21 biofilm samples in the high-P Arboleda-30 site ( $R^2 = 0.57$ ,  $P < 0.001$ ). Black diamonds represent data points (combined samples from natural and artificial substrate), and open squares represent estimated P-content ( $\pm 95\%$  CI) for end members representing pure inorganic and organic fractions of biofilm samples.

with much lower estimates for  $\%P_{\text{sed}}$  of  $-0.76 \pm 0.69$  and for  $\%P_{\text{org}}$  of  $3.74 \pm 5.41$  (Table 2). This analysis indicates that algal cells have higher P-content than other components of this biofilm, although we note that 95% confidence intervals are overlapping for algae and nonalgal organic matter. The large uncertainty associated with estimated  $\%P_{\text{alg}}$  is due to the fact that extrapolation is most severe in this dimension (Fig. 5B), due to the relatively low algal biomass in all samples.

Because the inorganic fraction is the largest component of biofilm mass, the negative estimate for  $\%P_{\text{sed}}$  inflates the estimated P-content of algae. Forcing the P-content of inorganic sediment to be zero (model  $r^2 = 0.75$ ) yields estimates of  $18.65 \pm 23.28$  for  $\%P_{\text{alg}}$  and  $-1.15 \pm 4.27$  for  $\%P_{\text{org}}$  (Table 2). Finally, forcing the P-content of both inorganic sediment and non-algal organic matter equal to zero (i.e., assuming all biofilm P is contained in algal biomass) yields an estimated  $\%P_{\text{alg}}$  of  $12.68 \pm 6.88$  (model  $r^2 = 0.73$ ; Table 2).

Our estimates of algal P-content are likely further inflated due to the fact that much of diatom dry mass is composed of silica (Lund 1965), which, in our analysis, is attributed to inorganic sediment (i.e., estimates of algal %P reflect metabolic biomass of algal cells). If we add the silica mass (assumed to be 60%; Lund 1965) back to the algal compartment, the estimated algal P-content becomes 5.07 %P, similar to reported values of algal cells grown in high-nutrient conditions ( $\sim 5\%$  %P; Nielson et al. 1996). Regardless of whether diatomaceous silica is considered to be algal biomass or inorganic sediment, results from this mixing model strongly suggest that the high biofilm P-content from the high-P Arboleda stream is almost entirely due to P-enriched algae,



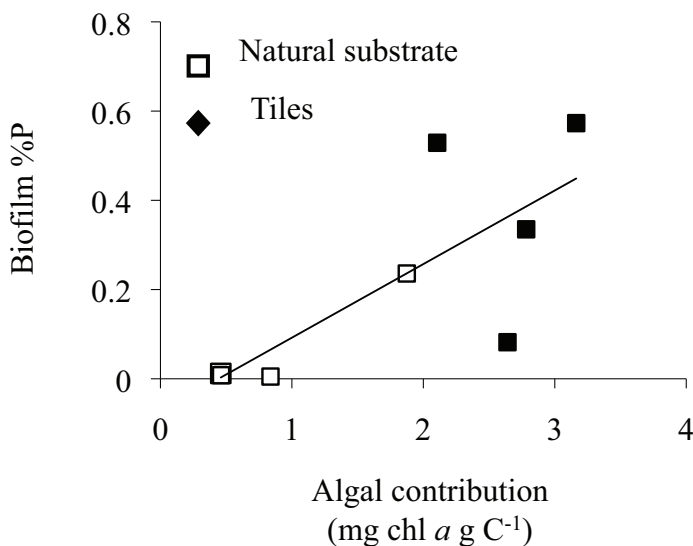
**Fig. 5.** Application of three-compartment mixing model to biofilm samples from high-SRP Arboleda-30 site ( $R^2 = 0.80$ ,  $P = 0.02$ ). (A) Projection showing fit of data points onto plane determined from least-squares regression. Shaded circles show actual data points, and open circles represent the projection of those points onto the plane to illustrate model fit. (B) Extrapolation of this plane produces estimates (and 95% confidence intervals) of P-content (relative to dry mass) of the three end-members: inorganic sediment (diamond), non-algal organic matter (square), and algae (triangle). Error bars for inorganic sediment are obscured by symbol. Measured data points are shown as small circles.

rather than to P sorbed to inorganic sediment or to high concentrations of P-rich bacteria. Even though algal cells compose only 1–3% of the mass of biofilm samples, our results suggest that algal cells may account for nearly 100% of the measured P. Results from the three-compartment mixing model are independently supported by the positive relationship between biofilm %P and chl *a*:C ratio for these eight replicate samples ( $R^2 = 0.57$ ,  $P = 0.03$ , Fig. 6), which indicates that increasing biofilm P-content is associated with increases



**Table 2.** Estimates of P-content (%P by dry weight) for algae, non-algal organic matter, and inorganic sediment, from the high-P Arboleda-30 site. In the first model, solutions to the 3-compartment mixing model are shown. In the second model, %P of inorganic sediment is fixed at zero (shown in **bold**), since the model produced a negative estimate of %P<sub>sed</sub>. In the third model, P-content of both non-algal organic matter and inorganic sediment are fixed at 0 (i.e., all P is assumed to be in algal cells).

Model $r^2$	P value	Algae			Non-algal organic matter			Inorganic sediment		
		Estimate	Lower 95%	Upper 95%	Estimate	Lower 95%	Upper 95%	Estimate	Lower 95%	Upper 95%
0.80	0.02	22.88	5.77	39.99	3.74	-1.67	9.15	-0.76	-1.45	-0.07
0.75	0.02	18.65	-4.63	41.93	-1.15	-5.42	3.12	<b>0.00</b>		
0.73	0.01	12.68	5.80	19.56	<b>0.00</b>			<b>0.00</b>		



**Fig. 6.** Relationship between epilithic biofilm P-content (relative to dry mass) and relative algal contribution to biofilm biomass, measured as chl  $a:C$  ( $R^2 = 0.57$ ,  $P = 0.003$ ). Samples from natural substrate are shown as open squares (note overlapping datapoints), and samples collected from tiles are shown as shaded diamonds.

in chl  $a$  content. This analysis also shows that biofilm collected from tiles had a higher P-content than biofilm collected from natural substrate, and that biofilm from tiles alone show no relationship between algal contribution and bulk sample %P.

## Discussion

Through the use of algebraic mixing models to analyze an epilithic biofilm dataset, we determined that the high P-content of epilithic biofilm in a highly P-enriched stream in Costa Rica was due to P-enriched algal cells rather than other organic material or inorganic sediment. Our results indicate that it is possible to use inherent variability among replicate samples to tease apart the qualitative characteristics of various components of biofilm.

Results derived from this approach are a coarse approximation, as they depend on extrapolation and multiple assumptions. Precision in these estimates is highly dependent on the

degree of heterogeneity among replicate samples. Errors in measuring independent (AFDM, chl  $a$ , algal biovolume) and dependent (%P) variables influence the resulting estimates. Also important is our assumption that a given component of biofilm at a given sampling site and date has similar elemental composition among replicate samples. While this assumption could be violated due to local heterogeneity in flow or substrata (indicated by lack of fit in the regressions), our estimates should not be biased by random variation in these values (i.e., this method estimates site-specific mean values for the elemental composition of each component, and a violation of the underlying assumptions would result in higher variation around this mean). It is necessary to know what mechanisms drive variation in P-content in all biofilm components, as well as in sediment, to spatially and temporally delimit the sampling regime, so future development of this method should focus on examining biofilm heterogeneity at different spatial and temporal scales. Finally, our linear mixing model approach assumes no interactions in nutrient content between different components of a biofilm, but the importance of nutrient exchange between bacteria and algae within a biofilm may be dependent on stream nutrient levels (Scott et al. 2008), potentially adding complexity to these relationships. Despite these limitations, the mixing model approach presented here allows additional information to be extracted from readily available data to address ecologically relevant questions. We see this algebraic method as complementary to methods of physical separation such as centrifugation or other methods that have been used to estimate qualitative characteristics of pure algae within bulk samples (Marty and Planas 2008).

Measuring the quality of food resources is an important task for ecologists, and the question of how empirical analyses of food resources correspond to the actual food resources ingested and assimilated by consumers merits further exploration. Although the case study presented here focused on partitioning biofilm P content, the same approach could be used to partition isotopic signatures of biofilm components. Also, given the importance of allochthonous organic matter in food webs in many forested streams (e.g., Vannote et al. 1980; Wallace et al. 1997), a similar mixing model approach may be useful in separating the isotopic and nutrient content of various detrital components (e.g., terrestrial plant material, fungal

biomass, bacterial biomass) in leaf packs and sedimentary environments. Application of this inverse mixing model approach should be empirically tested when applied to any system, but the general concept is applicable to the problem of partitioning qualitative components of any heterogeneous mixture, as long as the underlying assumptions are met. Dealing with heterogeneous food resources remains a challenge in food web studies, but our hope is that the approach presented here may help elucidate some of the qualities of the various components of these resources and allow for an improved understanding of trophic interactions.

## References

- American Public Health Association [APHA]. 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association.
- Arsuffi, T. L., and K. Suberkropp. 1985. Selective feeding by stream caddisfly (Trichoptera) detritivores on leaves with fungal-colonized patches. *Oikos* 45:50-58 [doi:10.2307/3565221].
- Bowman, M. F., P. A. Chambers, and D. W. Schindler. 2005. Changes in stoichiometric constraints on epilithon and benthic macroinvertebrates in response to slight nutrient enrichment of mountain rivers. *Freshw. Biol.* 50:1836-1852 [doi:10.1111/j.1365-2427.2005.01454.x].
- Cross, W. F., J. P. Benstead, A. D. Rosemond, and J. B. Wallace. 2003. Consumer-resource stoichiometry in detritus-based streams. *Ecol. Lett.* 6:721-732 [doi:10.1046/j.1461-0248.2003.00481.x].
- Frost, P. C., H. Hillebrand, and M. Kahlert. 2005. Low algal carbon content and its effect on the C:P stoichiometry of periphyton. *Freshw. Biol.* 50:1800-1807 [doi:10.1111/j.1365-2427.2005.01449.x].
- Fry, B. 2006. Stable isotope ecology. Springer. [doi:10.1007/0-387-33745-8].
- Genereux, D. P., M. Webb, and D. K. Soloman. 2009. Chemical and isotopic signature of old groundwater and magmatic solutes in a Costa Rican rain forest: Evidence from carbon, helium, and chlorine. *Water Resour. Res.* 45:W08413 [doi:10.1029/2008WR007630].
- Hamilton, S. K., S. J. Sippel, and S. E. Bunn. 2005. Separation of algae from detritus for stable isotope or ecological stoichiometry studies using density fractionation in colloidal silica. *Limnol. Oceanogr. Methods* 3:149-157.
- Hessen, D. O., T. Andersen, P. Brettum, and B. A. Faafeng. 2003. Phytoplankton contribution to sestonic mass and elemental ratios in lakes: implications for zooplankton nutrition. *Limnol. Oceanogr.* 48:1289-1296 [doi:10.4319/lo.2003.48.3.1289].
- Hillebrand, H., C. D. Durselen, D. Kirschtel, U. Pollingher, and T. Zohany. 1999. Biovolume calculation for pelagic and benthic microalgae. *J. Phycol.* 35:403-424 [doi:10.1046/j.1529-8817.1999.3520403.x].
- Jones, J. B., B. Wolf, and H. A. Mills. 1991. Plant analysis handbook 1: methods of plant analysis and interpretation. Micro-Macro Publishing.
- Klausmeier, C. A., E. Litchman, T. Daufresne, and S. A. Levin. 2008. Phytoplankton stoichiometry. *Ecol. Res.* 23:479-485 [doi:10.1007/s11284-008-0470-8].
- Lamberti, G. A. 1996. The role of periphyton in benthic food webs, pp. 533-572. *In* R. J. Stevenson, M. Bothwell, and R. L. Lowe (eds.), *Algal ecology*. Academic Press [doi:10.1016/B978-012668450-6/50046-1].
- Lau, D. C. P., K. M. Y. Leung, and D. Dudgeon. 2009. Are autochthonous foods more important than allochthonous resources to benthic consumers in tropical headwater streams? *J. North Am. Benthol. Soc.* 28:426-439 [doi:10.1899/07-079.1].
- Lindeman, R. L. 1942. The trophic-dynamic aspect of ecology. *Ecology* 23:399-418 [doi:10.2307/1930126].
- Lock, M. A., R. R. Wallace, J. W. Costerton, R. M. Ventullo, and S. E. Charlton. 1984. River epilithon: Toward a structural-functional model. *Oikos* 42:10-22 [doi:10.2307/3544604].
- Lodge, D. M. 2006. Selective grazing on periphyton: a determinant of freshwater gastropod microdistributions. *Freshw. Biol.* 16:831-841 [doi:10.1111/j.1365-2427.1986.tb01020.x].
- Lund, J. W. G. 1965. The ecology of freshwater phytoplankton. *Biol. Rev.* 40:231-293 [doi:10.1111/j.1469-185X.1965.tb00803.x].
- March, J. G., and C. M. Pringle. 2003. Food web structure and basal resource utilization along a tropical island stream continuum, Puerto Rico. *Biotropica* 35:84-93.
- Marty, J., and D. Planas. 2008. Comparison of methods to determine algal  $\delta^{13}\text{C}$  in freshwater. *Limnol. Oceanogr. Methods* 6:51-63.
- McNeely, C., J. C. Finlay, and M. E. Power. 2007. Grazer traits, competition, and carbon sources to a headwater-stream food web. *Ecology* 88:391-401 [doi:10.1890/0012-9658(2007)88[391:GTCACS]2.0.CO;2].
- Mulholland, P. J., J. L. Tank, D. M. Sanzone, W. Wollheim, B. J. Peterson, J. R. Webster, and J. L. Meyer. 2000. Nitrogen cycling in a forest stream determined by a  $^{15}\text{N}$  tracer addition. *Ecol. Monogr.* 70:471-493.
- Nielson, S. L., S. Enríquez, C. M. Duarte, and K. Sand-Jensen. 1996. Scaling maximum growth rates across photosynthetic organisms. *Funct. Ecol.* 10:167-175 [doi:10.2307/2389840].
- Noe, G. B., L. J. Scinto, J. Taylor, D. L. Childers, and R. D. Jones. 2003. Phosphorus cycling and partitioning in an oligotrophic Everglades wetland ecosystem: a radioisotope tracing study. *Freshw. Biol.* 48:1993-2008 [doi:10.1046/j.1365-2427.2003.01143.x].
- Pringle, C. M., and F. J. Triska. 1991. Effects of geothermal waters on nutrient dynamics of a lowland Costa Rican stream. *Ecology* 72:951-965 [doi:10.2307/1940596].
- , G. L. Rowe, F. J. Triska, J. F. Fernandez, and J. West. 1993. Landscape linkages between geothermal activity and solute composition and ecological response in surface



- waters draining the Atlantic slope of Costa Rica. *Limnol. Oceanogr.* 38:753-774 [doi:10.4319/lo.1993.38.4.0753].
- Ramírez, A., C. M. Pringle, and L. Molina. 2003. Effects of stream phosphorus levels on microbial respiration. *Freshw. Biol.* 48:88-97 [doi:10.1046/j.1365-2427.2003.00973.x].
- , and C. M. Pringle. 2006. Fast growth and turnover of chironomid assemblages in response to stream phosphorus levels in a tropical lowland landscape. *Limnol. Oceanogr.* 51:189-196 [doi:10.4319/lo.2006.51.1.0189].
- Rezanka, K. M., and A. E. Hershey. 2003. Examining primary producer-consumer interaction in a Lake Superior tributary using <sup>15</sup>N-tracer, grazer-reduction, and nutrient-bioassay experiments. *J. North Am. Benthol. Soc.* 22:371-387 [doi:10.2307/1468268].
- Rhee, G. 1972. Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. *Limnol. Oceanogr.* 23:10-25 [doi:10.4319/lo.1978.23.1.0010].
- Rosemond, A. D., C. M. Pringle, A. Ramírez, M. J. Paul, and J. L. Meyer. 2002. Landscape variation in phosphorus concentration and effects on detritus-based tropical streams. *Limnol. Oceanogr.* 47:278-289 [doi:10.4319/lo.2002.47.1.0278].
- SAS Institute. 2001. SAS Version 8.2 for Windows. SAS Institute Inc.
- Scott, J. T., J. A. Back, J. M. Taylor, and R. S. King. 2008. Does nutrient enrichment decouple algal-bacterial production in periphyton? *J. North Am. Benthol. Soc.* 27:332-344 [doi:10.1899/07-108.1].
- Small, G. E., and others. 2008. The dynamics of phosphorus retention during an eight-year P-addition in a Neotropical headwater stream. *Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie* 30:551-554.
- , and C. M. Pringle. 2010. Deviation from strict homeostasis across multiple trophic levels in an invertebrate consumer assemblage exposed to high chronic phosphorus enrichment in a Neotropical stream. *Oecologia* 162:581-590 [doi:10.1007/s00442-009-1489-4].
- Steinman, A. D., G. A. Lamberti, and P. R. Leavitt. 2006. Biomass and pigments of benthic algae, pp. 357-380. *In* F. R. Hauer and G. A. Lamberti, *Methods in stream ecology*, 2nd ed. Academic Press.
- Stelzer, R. S., and G. A. Lamberti. 2001. Effects of N:P ratio and total nutrient concentration on stream periphyton community structure, biomass, and elemental composition. *Limnol. Oceanogr.* 46:356-367 [doi:10.4319/lo.2001.46.2.0356].
- Sterner, R. W., and J. J. Elser. 2002. *Ecological stoichiometry: the biology of elements from molecules to the biosphere*. Princeton Univ. Press.
- Triska, F. J., C. M. Pringle, J. H. Duff, J. Avanzino, and G. Zellweger. 2006. Soluble reactive phosphorus transport and retention in tropical rainforest streams draining a volcanic landscape in Costa Rica: In situ SRP amendment to streams and laboratory studies. *Biogeochemistry* 81:145-157 [doi:10.1007/s10533-006-9048-0].
- Vannote, R. L., G. W. Minshall, K. W. Cummins, J. R. Sedell, and C. E. Cushing. 1980. The river continuum concept. *Can. J. Fish. Aquat. Sci.* 37:130-137 [doi:10.1139/f80-017].
- Wallace, J. B., S. L. Eggert, J. L. Meyer, and J. R. Webster. 1997. Multiple trophic levels of a forest stream linked to terrestrial litter inputs. *Science* 277:102-104 [doi:10.1126/science.277.5322.102].
- Wotton, R. S. 1994. Particulate and dissolved organic matter as food, p. 235-288. *In* R. S. Wotton [eds.], *Biology of particles in aquatic systems*. CRC Press.

Submitted 18 June 2010

Revised 4 March 2011

Accepted 15 April 2011