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Exoenzyme accumulation in epilithic biofilms

Robert L. Sinsabaugh, Deborah Repert, Timothy Weiland, Stephen W. Golladay & Arthur E. Linkins
Biology Department, Clarkson University, Potsdam, New York, NY 13699, USA

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Abstract

Although exoenzyme accumulation is often proposed as an explanation for the high metabolic activity of biofilms, little is known about the abundance, distribution and turnover rates of exoenzymes within these communities. To assess accumulation, epilithic biofilm samples were collected from a fourth-order boreal river and homogenized. The resulting particles were fractionated by size and each fraction was assayed for nine exoenzyme activities, chlorophyll, and ATP.

In general, carbohydrase activities were not correlated with microbial biomass indicators; the largest pool of activity was in the aqueous phase ($<0.2\ \mu m$). Phenol oxidase, peroxidase, and phosphatase activities were largely particle-bound and often correlated with microbial biomass distribution. It was concluded that the epilithic biofilm matrix was effective at accumulating carbohydrase activity and that this accumulation may partially account for the metabolic resistance of epilithic biofilms to dissolved organic matter fluctuations.

Introduction

Although epilithon is the major nexus of autotrophic and heterotrophic activity in many streams, most of the research in this area has focused on autotrophy. However, interest in epilithic heterotrophy has been increasing. Geesey *et al.* (1977, 1978) studied the dynamics of attached and entrained bacteria in second-order mountain streams and found that attached bacteria were numerically dominant by 2–3 orders of magnitude. In addition to numeric dominance, attached bacteria have also been found to be metabolically more active (reviewed by van Loosdrecht *et al.*, 1990). For example, the specific glutamate uptake rates of biofilm bacteria have been reported as up to 26 times greater than those of planktonic bacteria (Ladd *et al.*, 1979). Despite this potential for high specific activity, several studies have shown that epilithic biofilms exhibit considerable metabolic resistance to alterations in the dissolved organic matter (DOM) composition of their perfusion medium. Often several days are required before significant metabolic acclimation is observed (Ford & Lock, 1985, 1987; Kaplan & Bott, 1983, 1985; Bott *et al.*, 1984).

Lock *et al.* (1984) incorporated these findings into a conceptual model of epilithon structure and function. Key features of their model with respect to biofilm metabolism are tight internal nutrient cycling and exoenzyme accumulation. These features, together with the typically high detritus content of epilithic biofilms (Perkins & Kaplan, 1978; Christensen & Characklis, 1990) which may pro-

vide a carbon and nutrient reservoir, could account for the slow responsiveness of a metabolically active community to alterations in the DOM milieu.

However, this model has yet to be thoroughly investigated. In particular, little is known about the types, quantities, and turnover rates of exoenzymes within epilithon. Initial descriptions of exoenzyme activities and their spatiotemporal dynamics have been presented by Sinsabaugh and Linkins (1988a) and Jones & Lock (1989). These studies have shown that activities are generally correlated with the areal concentration of organic matter. However, the nature of the association has not been investigated and may be ecologically significant.

Exoenzymes have the potential to become decoupled from the organisms that produced them. In the terminology of soil biochemistry, all extracellular enzyme activity is classified as abiontic; some abiontic activity is associated with viable microorganisms, the rest, associated with detrital particles, is termed accumulated activity (Skujins, 1978; Burns, 1983). This accumulated activity represents a temporal link between current community metabolism and past disturbance events.

To ascertain whether epilithic biofilms accumulate exoenzyme activity, we collected samples from a fourth-order boreal river, homogenized and fractioned them into five size classes, and examined the distribution of exoenzyme activity in relation to biomass indicators.

Methods

Sample collection

Epilithon samples were collected from the West Branch of the St. Regis River, a fourth-order boreal stream originating in the Adirondack Mountains of New York and flowing northward to the St. Lawrence River. The sampling site was a riffle located in the Town of Stockholm (44° 42′ 51′′ N, 74° 54′ W). Detailed descriptions of this site have been presented (Sinsabaugh & Linkins, 1988a, 1990a). On each sampling date, 6–10 large cobbles were collected from the riffle and transported to the laboratory in buckets of river water. Samples were collected on 6 July, 19 July, 21 August, 28 September, and 13 November 1989.

Sample processing

The biofilm was removed from the cobbles by thoroughly brushing each in a basin containing about one liter of river water. The surface area sampled was estimated by wrapping the rocks in aluminum foil, drying and weighing the foil, and calculating area from the mass per unit area of the foil. The total area sampled at each collection date ranged from 0.30–0.47 m^2.

To promote particle dispersion, the epilithon suspension was sonicated while being vigorously stirred. It was then immediately eluted through a nested series of 20 cm stainless steel sieves with mesh sizes of 250, 125, 63, and 38 μm, followed by rinses with distilled water. Material >250 μm, which consisted largely of invertebrates and their retreats, was discarded. Particles collected on the 125 and 63 μm sieves were rinsed, pooled, and resuspended in 50 mM, pH 5.0, acetate buffer to produce a 63–250 μm fraction.

The eluate from the sieves was further filtered through 10 μm nylon mesh. To prevent clogging of the mesh and secondary particle aggregation, the suspension was continuously sonicated during this step. Particles retained by the 10 μm mesh and the 38 μm sieve were pooled and resuspended in acetate buffer to produce a 10–63 μm fraction.

The <10 μm filtrate was centrifuged at 10000 *g* for 5 min at 4 °C. The pellets were resuspended in acetate buffer to yield a 1–10 μm fraction. The supernatant was designated as the <1 μm fraction. These designations were based on microscopic examination of the pellets and supernatants.

Finally, about 300 ml of the supernatant was filtered under low vacuum through 0.2 μm membrane filters to produce a cell-free fraction containing only dissolved matter. All suspensions

were kept on ice throughout the size sorting procedure.

This size fractionation procedure was chosen because it resulted in a roughly equitable distribution of organic matter among the fractions. Although reproducible, it was simply a device for investigating the covariance between exoenzyme activity and microbial biomass and did not reflect any natural organization of the biofilm.

Analyses

The dry mass and organic matter contents of the 63–250, 10–63, and 1–10 μm fractions were determined by filtering 25 ml aliquots through preweighed 0.45 μm glass fiber filters, then drying the filters at 105 °C, reweighing, ashing at 500 °C for 30 min, and reweighing again. For the <1 μm fraction, 100 ml aliquots were filtered through preweighed 0.2 μm membrane filters. These filters were dried at 50 °C and reweighed. The ash content could not be determined by this procedure but was assumed to be negligible.

Chlorophyll *a* concentrations were determined by filtering aliquots of each fraction as described above and placing the filters in 50 ml of 90% $MgCO_3$-saturated aqueous acetone. The filters were extracted in the dark for 24 h at 8 °C. The concentration of chlorophyll *a* was estimated by scanning extract absorbance from 500–750 nm and calculating chlorophyll concentration as 12 times the difference between peak and baseline absorbance at 664 nm. Filtrates were also scanned for chlorophyll as a check for cell fracturing during filtration; none was detected.

For ATP determinations, aliquots of each fraction were filtered and placed in 25 ml of tris-EDTA buffer. ATP was extracted by adding 25 ml of 1.2 N H_2SO_4. After an hour, the extracts were filtered and brought to pH 7.6 by addition of 5 N NaOH solution. These extracts were frozen until analyzed photometrically by the luciferin-luciferase reaction. Counting efficiency was estimated for each sample by addition of internal standards. Filtrate aliquots were also analyzed for ATP as a check for cell fracturing; none was detected.

Each fraction was assayed for acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), Beta-N-acetylglucosaminidase (EC 3.2.1.30), B-xylosidase (EC 3.2.1.37), Alpha-glucosidase (EC 3.2.1.20), Beta-glucosidase (3.2.1.21), endocellulase (EC 3.2.1.4), phenol oxidase (EC 1.10.3.1,2, EC 1.14.18.1), and peroxidase activities (EC 1.11.1.7). All assays, except alkaline phosphatase, were conducted at 21 °C in 50 mM, pH 5, acetate buffer with four analytical replicates and two controls per sample. Alkaline phosphatase activity was assayed at pH 9.0 in 200 mM Tris buffer. Activities were expressed as μmol of substrate converted h^{-1} m^{-2}, except for endocellulase activity which was given in relative units.

The substrates for the phosphatase, Beta-N-acetylglucosaminidase, B-xylosidase, Alpha-glucosidase, and Beta-glucosidase assays were p-nitrophenyl (pNP) phosphate, pNP-B-N-acetylglucosaminide, pNP-B-xylopyranoside, pNP-Alpha-glucopyranoside and pNP-Beta-glucopyranoside, respectively, in acetate buffer. Two ml of sample and 2 ml of substrate solution were placed in 5 ml polyethylene test tubes which were capped and tumbled in a platelet mixer at 3 rpm for 3 h. The final substrate concentrations were 10 mM except pNP-B-N-acetylglucosaminide which was 1.0 mM due to limited solubility. After incubation, the samples were centrifuged and 2 ml of supernatant was transferred from each into a test tube containing 0.2 ml of 1.0 N NaOH. After adding 8 ml of water to each tube, absorbance was measured at 410 nm. Duplicate controls containing 2 ml of sample suspension plus 2 ml of buffer and 2 ml buffer plus 2 ml substrate were processed in parallel.

Endocellulase activity was estimated viscometrically using the method of Almin & Eriksson (1967). One ml of sample was mixed with 2 ml of 1% carboxymethyl cellulose in a 5 ml test tube. The tubes were capped and tumbled for a variable time depending on activity. After incubation, the tubes were centrifuged and viscosity estimated by the fall velocity of the fluid in a 0.1 ml glass pipette. Activity was calculated in relative units based on the difference between initial and final viscosity.

Phenol oxidase activity was estimated by incubating 2 ml of sample with 2 ml of 10 mM L-3,4-dihydroxyphenylalanine (L-DOPA) for 1 h with continuous tumbling. Following centrifugation, the absorbance of the supernatant was measured at 460 nm. Controls were prepared by mixing 2 ml of sample with 2 ml of acetate buffer. Peroxidase activity was assayed in parallel by mixing 2 ml of sample, 2 ml of L-DOPA, and 0.2 ml of 0.3% H_2O_2. Controls contained 2 ml of sample, 2 ml of buffer, and 0.2 ml of H_2O_2. Peroxidase activity was calculated as the difference between substrate oxidation rates in the presence and absence of peroxide.

Results

The mean areal density of the five biofilm samples was 5.6 g dry mass m^{-2} (range 2.9–8.5). Most of this material was inorganic. Ash content declined with particle size, averaging 82% ± 9 (SD) in the 63–250 μm fraction, 59% ± 10 in the 10–63 μm fraction, and 47% ± 8 in the 1–10 μm fraction. The mean standing stock of AFDM was 1.7 g m^{-2}, range 1.1–2.1 (Fig. 1). Although total standing stocks varied 2–3 fold over the sampling period, the size distribution of dry mass and AFDM within the biofilm was relatively stable. For dry mass, the mean distribution among the 63–250, 10–63, 1–10, and < 1 μm fractions was 50% ± 17 (SD), 23% ± 9, 22% ± 8, and 5% ± 2, respectively. The corresponding values for AFDM were 24% ± 4, 28% ± 5, 35% ± 6, and 14% ± 5.

Unlike dry mass and organic matter, the size distributions and total standing stocks of ATP and chlorophyll *a* were quite variable (Fig. 1). ATP levels averaged 32.6 μg m^{-2} (range 11.1–56.4) and chlorophyll *a* 2.4 mg m^{-2} (range 1.2–4.5). The mean ATP/chlorophyll ratio was 14 μg/mg, within the range reported for lotic epilithon by other investigators (Rounick & Winterbourn, 1983; Lock *et al.*, 1984; Palumbo *et al.*, 1987). Using a conversion factor of 0.5 mg dry mass/μg ATP, the mean viable microbial biomass was about 16 mg m^{-2}, equivalent to 0.3% of the mean dry matter standing stock and 1% of the mean organic matter standing stock. Thus, the overall structural composition of the biofilm was about 70% mineral, 30% detritus and *ca.* 1% viable microbial biomass.

Like the biomass indicators, exoenzyme activities and distributions were quite variable (Figs. 2–4). However, two distinct groups of enzymes were identified by correlation analysis. Group 1 enzymes included only carbohydrases: Alpha-glucosidase, Beta-glucosidase, Beta-xylosidase, Beta-N-acetylglucosaminidase (Fig. 2). The common element in the distribution of these enzymes was that the largest pool of activity generally resided in the aqueous phase. These enzymes are also functionally related in that they all catalyze the hydrolysis of disaccharides into monosaccharides. The pairwise correlation coefficients within this group ranged from 0.48 to 0.60 (Pearson correlation coefficients, statistically significant at $P < 0.02$, $n = 20$). There were no statistically significant ($P < 0.05$) positive correlations between group 1 enzymes and biomass indicators.

The group 2 enzymes were acid phosphatase, alkaline phosphatase, phenol oxidase, and peroxidase (Fig. 3). The enzymes in this group were largely particle-bound. Only a small fraction of their total activities were found in the aqueous phase. This grouping was weaker than group 1; only three of the six possible pairwise correlations were statistically significant. The distribution of these enzymes tended to parallel biomass distribution; for example, alkaline phosphatase activity was correlated with ATP, AFDM, and chlorophyll *a* ($r = 0.56$–0.64, significant at $P < 0.03$), phenol oxidase with AFDM ($r = 0.64$), and peroxidase with chlorophyll ($r = 0.57$).

Endocellulase was the only enzyme that did not fall into one of these groups (Fig. 4). Although the activity distribution resembled the other group 1 carbohydrases in that there was a significant quantity of activity in the aqueous phase, the data were variable, and did not correlate with biomass indicators or other enzymes.

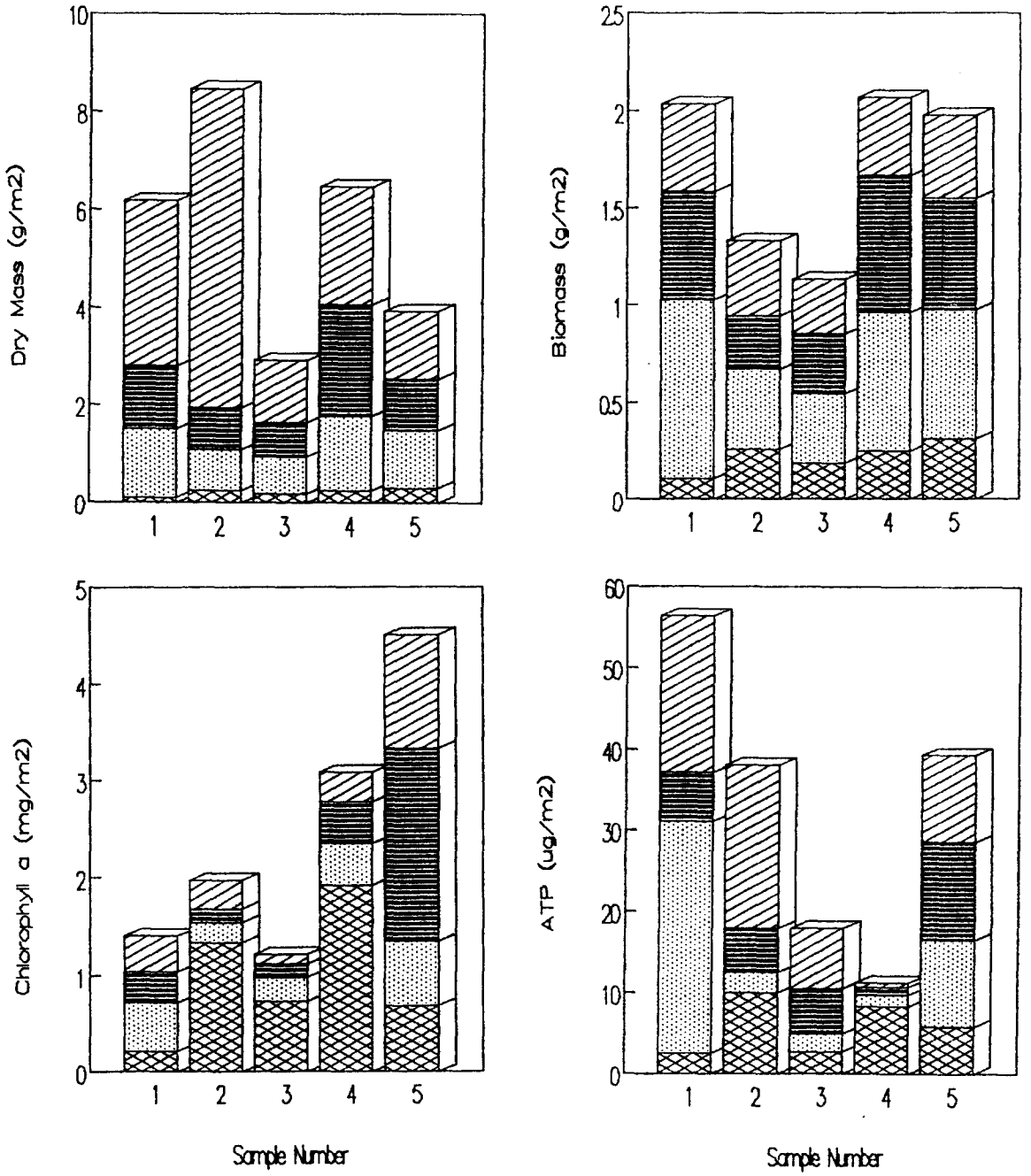

Fig. 1. The distribution of dry mass, AFDM, chlorophyll *a*, and ATP among four size fractions of lotic epilithon collected from a fourth-order riffle. The size fractions from top to bottom are 63–250, 10–63, 1–10 and $< 1\ \mu m$.

Discussion

In a prior study at the same St. Regis River site, the activities per unit area of 11 of 14 exoenzymes extracted from bulk epilithon samples were significantly correlated with AFDM (Sinsabaugh & Linkins, 1988a). When activities were expressed per g AFDM, two groups of covarying enzymes were identified. One of these clusters included

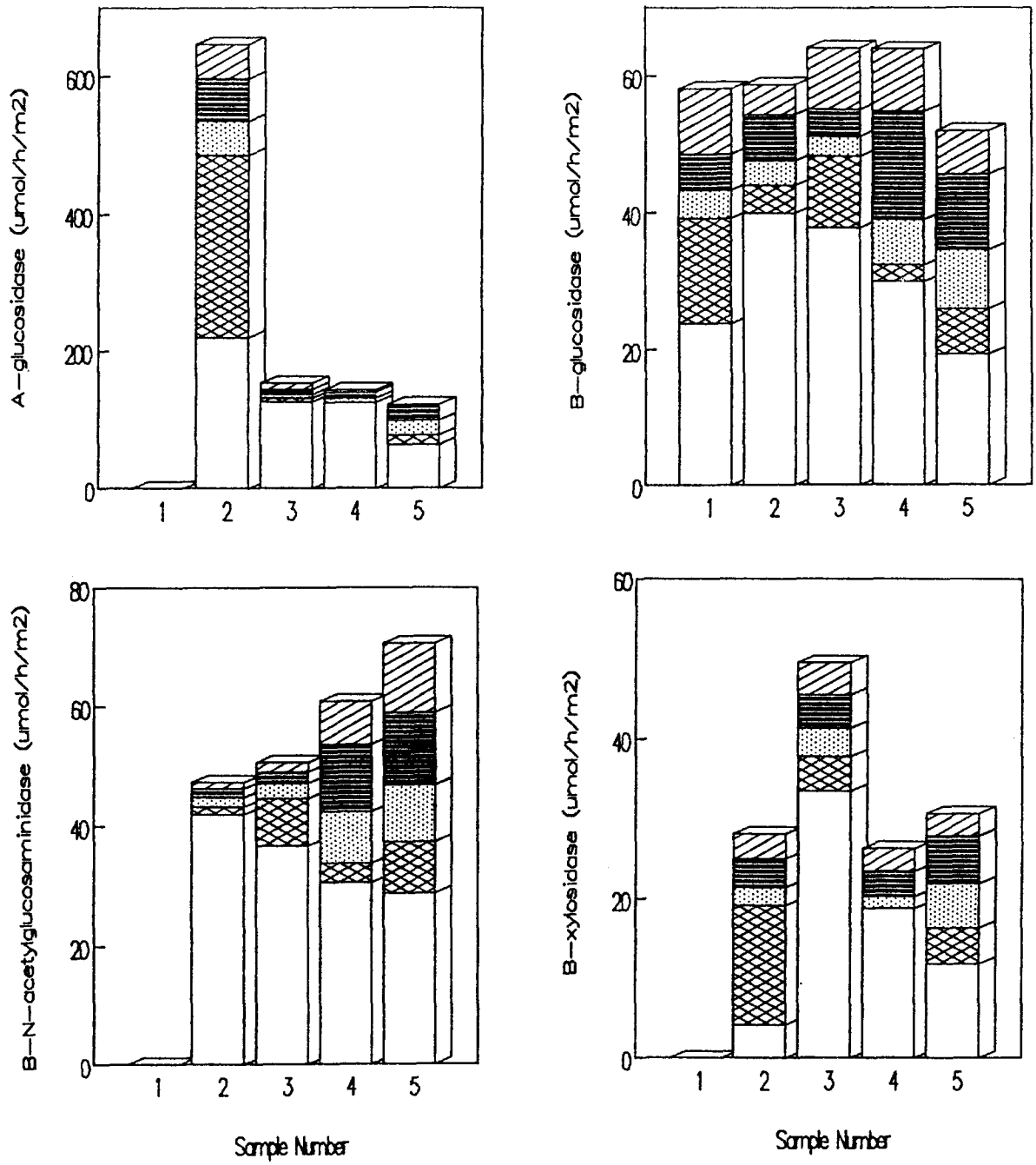

Fig. 2. The distribution of Alpha-glucosidase, Beta-glucosidase, Beta-xylosidase, and Beta-N-acetylglucosaminidase activity among five size fractions of lotic epilithon collected from a fourth-order riffle. The upper four fractions are solid phase with particle size ranges of 63–250, 10–63, 1–10 and < 1 μm. The bottom fraction is the aqueous phase (< 0.2 μm). Empty squares represent missing data.

Alpha-glucosidase, Beta-glucosidase, Beta-xylosidase, Beta-N-acetylglucosaminidase, and endocellulase, approximately corresponding to the carbohydrase group described in the Results section. The second cluster included phenol oxidase and peroxidase, sharing some similarity with our

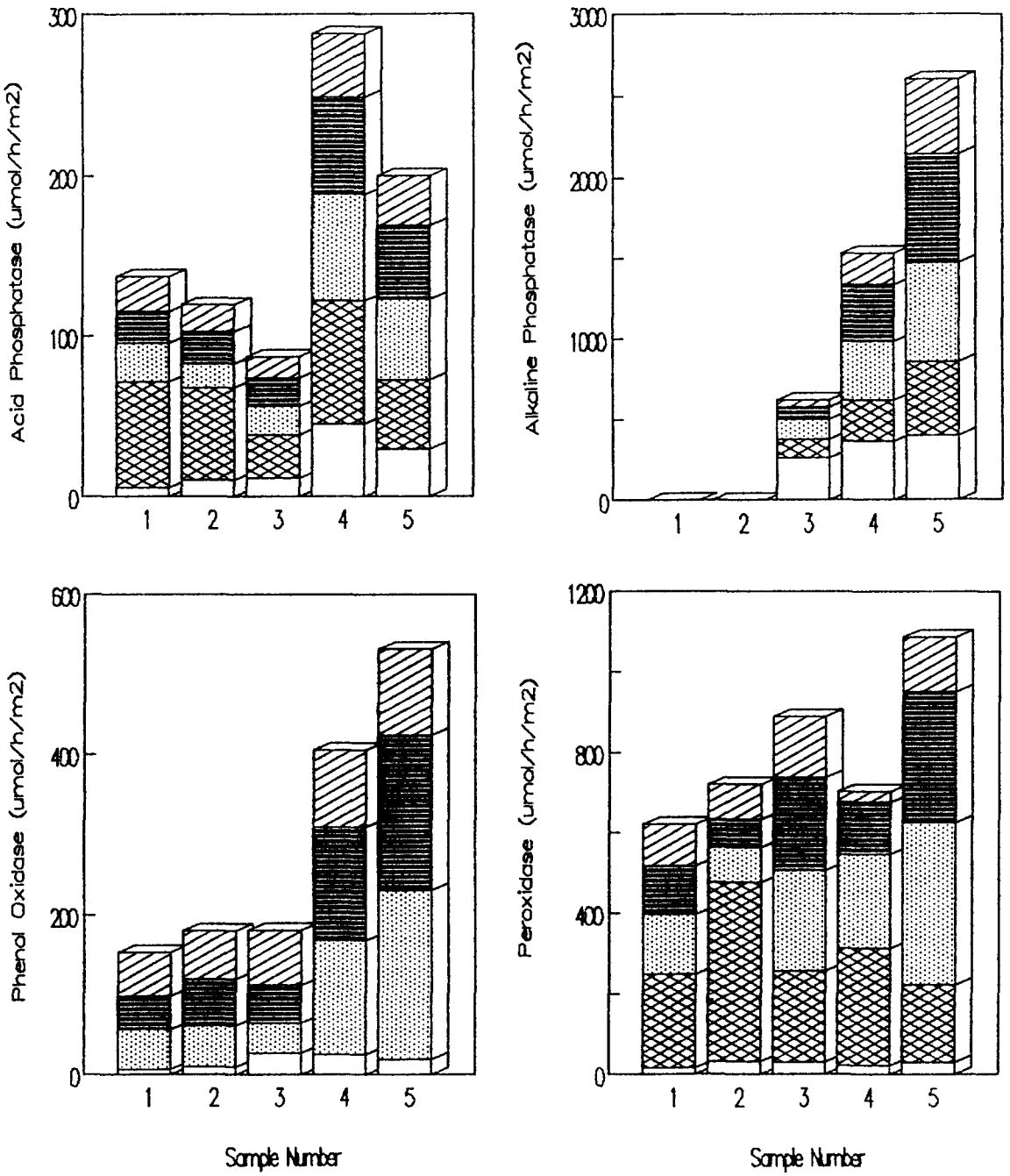

Fig. 3. The distribution of acid phosphatase, alkaline phosphatase, phenol oxidase, and peroxidase activity among five size fractions of lotic epilithon collected from a fourth-order riffle. The upper four fractions are solid phase with particle size ranges of 63–250, 10–63, 1–10, and $<1\,\mu$m. The bottom fraction is aqueous phase ($<2\,\mu$m). Empty squares represent missing data.

group 2 enzymes. These similarities suggest that the enzyme patterns observed in bulk biofilm analyses emerge, in part, from underlying distribution patterns within the biofilm. In particular, the emergence of two covarying groups of enzymes appears to be an effect of similar phase partition

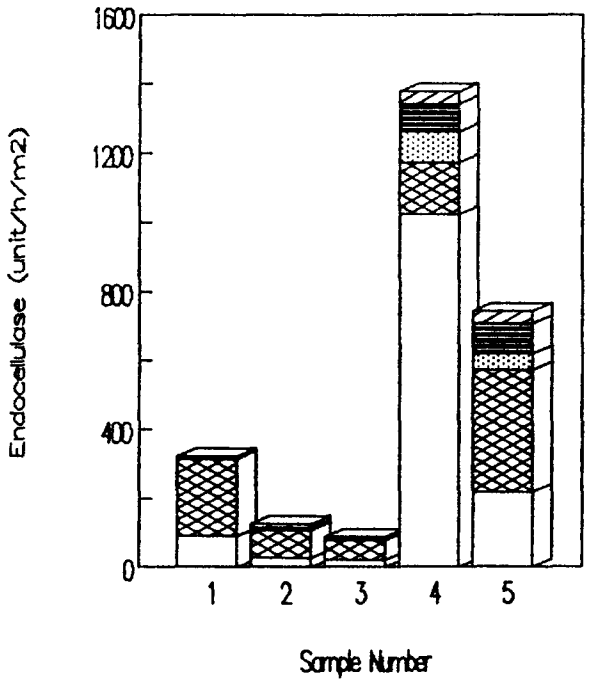

Fig. 4. The distribution of endocellulase activity among five size fractions of lotic epilithon collected from a fourth-order riffle. The upper four fractions are solid phase with particle size ranges of 63–250, 10–63, 1–10, and < 1 μm. The bottom fraction is the aqueous phase (0.2 μm).

patterns, i.e. enzymes with a high fraction of soluble activity versus more effectively immobilized enzymes. However, the pattern may be more fundamental because it recurs even in the absence of a solubility differential between the groups.

Sinsabaugh & Linkins (1990a) measured exoenzyme activities associated with coarse and fine particulate organic matter (POM) collected from the St. Regis River. The correlation pattern among enzymes was very similar to that of epilithon, even though, except for cellulase, only a small fraction ($< 10\%$) of the activity was extractable. The existence of this pattern in both POM and epilithon suggests that it results from similarities in the composition of the microflora and/or similar enzyme regulation strategies among taxa. If true, then the differences in carbohydrase extractability between POM and epilithon must result from microenvironmental interactions.

Specifically, the low solubility of the carbohydrase activity associated with POM relative to epilithic activity indicates either that exoenzyme activity is more effectively immobilized by organic substrata or that enzyme accumulation is constrained by leaching losses. Although both factors may be important for some enzymes, available evidence favors the latter. Comparison of exoenzyme activities associated with decomposing birch sticks at lotic, riparian, and upland sites shows that lotic sticks accumulate less activity and that the activity present is less extractable (unpublished data). In addition, temporal patterns of cellulase activity on decomposing leaf litter in streams appear more closely related to the absorption properties of the litter than do activity patterns on terrestrial litter which is subjected to only intermittent leaching events (Sinsabaugh & Linkins, 1988b; Sinsabaugh & Linkins, 1990a).

Thus, the relatively high extractability of epilithic carbohydrase activity suggests that the biofilm matrix (glycocalyx) promotes accumulation of the more freely deployed exoenzymes by retarding losses, as suggested by Lock *et al.* (1984). There are several possible mechanisms that could effect a net accumulation: physical enmeshment by the glycocalyx, hindered diffusion, adsorption, cation bridging (see Characklis & Marshall (1990) for a thorough discussion of transport phenomena in biofilms). Regardless of retention mechanism, the functional effect of accumulation is to spatiotemporally decouple enzyme activity from its source. This effect is evidenced by the lack of correlation between carbohydrase activities and microbial biomass within the biofilm. On a larger scale, i.e. bulk biofilm community, this decoupling apparently averages out, yielding the significant correlations between biomass and enzymic activity that have been reported (Sinsabaugh & Linkins, 1988a).

The advantage of accumulation is presumably diversion of resources from exoenzyme synthesis to microbial growth, which may contribute to the high specific activity rates observed for attached microbiota. The disadvantage is slower community response to alterations in carbon resources. To further evaluate this model, it will be necessary

to correlate the metabolic acclimation times of biofilms to various organic substrates with the characteristics, e.g. abundance, turnover, induction, of the enzymes responsible for generating assimilable products from the substrates.

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