

Different methods and storage duration affect measurements of epilithic extracellular enzyme activities in lotic biofilms

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Abstract Extracellular enzyme activity (EEA) is becoming increasingly common for measuring biofilm function in streams. Different methods for enzyme assays may yield results that cannot be compared among studies, and duration of sample storage may also affect EEAs, leading to erroneous conclusions. We compared two frequently used methods for measuring phosphatase (PHOS), leucine aminopeptidase (LAMP), β -glucosidase (GLU), and β -xylosidase (XYLO) by conducting assays with intact and disrupted epilithic biofilms grown on tiles in three streams. Storage duration effects on EEA were documented with intact and disrupted biofilms kept in the dark at 4°C for 3 and 5 days. Intact biofilms had significantly less EEA than disrupted biofilms for all enzymes ($P < 0.01$). The two methods gave conflicting EEA results among streams, and ratios of disrupted to intact EEAs for each enzyme were not consistent among the three streams. PHOS was significantly greater than day 1 measurements when stored as

disrupted (Day 3 = 210%, Day 5 = 199% increases) and intact biofilms (Day 3 = 375%, Day 5 = 240% increases). LAMP activities were significantly less when stored as disrupted biofilms (Day 3 = -49% decrease) and greater when stored as intact biofilms (Day 5 = 72% increase). GLU (Day 3 = 313% increase) and XYLO (Day 3 = 121%, Day 5 = 188% increases) were significantly greater when stored as intact biofilms. The magnitude of change for all EEAs was inconsistent among streams, indicating that a consistent correction factor cannot be used to account for variation associated with storage duration. Consistent methods must be used and storage time should be minimized, preferably to the day of sampling, for valid inter-study comparisons. Conclusions can significantly differ between the two methods, therefore having implications for inter-study comparisons, understanding of biofilm function and dynamics, and environmental management decisions.

Keywords Enzyme methods · Phosphatase · Environmental assessment · Periphyton · Nutrients · Carbon

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Introduction

Biofilms grow on all substrata in lotic ecosystems and are comprised of algae, bacteria, fungi, and other microorganisms in a matrix of extracellular polymeric substances (EPS; Christensen & Characklis,

1991). EPS is the main component of the biofilms, but various nutrients, proteins, and dissolved, fine, and coarse particulate organic matter also contribute to its structural composition. Epilithic habitats often dominate the benthic surfaces in many small to mid-sized streams (<500 km² drainage areas), and epilithic biofilms are therefore important components of lotic processes, such as energy transfer, nutrient spiraling, and carbon assimilation (Lock et al., 1984; Chappell & Goulder, 1994; Romani & Sabater, 1999). The tight proximity of these organisms and EPS can contribute to intense resource cycling within biofilms and important interactions among biofilm components (Lock et al., 1984; Rier et al., 2007; Scott et al., 2008). Most organic matter, polymers, and proteinaceous compounds require extracellular enzyme hydrolysis as they are too large for transport across microbial cell membranes (Chróst, 1991). Extracellular enzyme activities (EEAs) are important for characterizing resource acquisition and function of epilithic biofilms when rocks dominate the substrata in lotic systems (Sinsabaugh & Linkins, 1988; Ainsworth & Goulder, 2000), and cycling of organic matter is more important in epilithic biofilms than in the water column of shallow streams (Romani & Sabater, 1999). Quantifying this extracellular enzyme activity (EEA) provides valuable insight into biological processes and ecosystem function of benthic biofilms, along with assessments of human impacts (Brown & Goulder, 1999; Sabater et al., 2007). Phosphatase (PHOS) activity has been especially useful for assessing impacts of nutrients on biofilm communities (Stevenson et al., 2008; Scott et al., 2009). Leucine aminopeptidase (LAMP), β -glucosidase (GLU), and β -xylosidase (XYLO) activities are important for understanding nitrogen and carbon dynamics in biofilms (Ainsworth & Goulder, 2000; Romani & Sabater, 2000; Espeland et al., 2001).

Two methods of EEA measurement are most common in the literature: (1) intact biofilms incubated with a fluorogenic substrate, in the dark on rotary shakers, with quantification of fluorescent product in the supernatant as a measure of EEA (Chappell & Goulder, 1992, 1994; Romani & Sabater, 1999, 2001), and (2) disrupted or suspensions of biofilms with fluorogenic substrates (Sinsabaugh & Linkins, 1988; Findlay et al., 2001; Findlay &

Sinsabaugh, 2003). Regardless of assay method, results may or may not be affected by the amount of time between sampling and quantification of EEAs. No literature has explicitly assessed potential problems, if any exist, associated with storage duration in aquatic systems, and most publications using EEAs do not report the duration of sample storage if assays were not conducted immediately upon return to a lab. If EEAs do not change throughout storage duration, then documentation of this consistency may be beneficial for studies covering vast geographical areas that require longer storage times. An empirical study of storage duration and EEAs would benefit future research because reliability of results may depend on the time between sampling and assays.

Novel techniques may be required for certain research questions (Francoeur et al., 2001), but different methods used to examine a similar question (e.g., environmental assessments of nutrients or carbon dynamics) should be tested as they might yield non-comparable results or conflicting conclusions for similar ecosystems. This research addressed three questions important to the reliability and comparability of EEA measurements using four commonly assayed enzymes (PHOS, LAMP, GLU, and XYLO): (1) are EEAs different between intact and disrupted biofilm assays, (2) if so, do they have conflicting results, and (3) do EEAs change when biofilms are stored as either disrupted or intact samples? The first and second questions are important for establishing whether results are comparable between the two methods, and the third question could be very important because many studies do not report the amount of time between sampling and quantification of EEA. It is probably widely accepted that immediate analysis is preferred, but some studies cover vast geographical areas, and immediate EEA analysis might not be physically possible. Therefore, documentation of storage time effects, if any exist, on the quality of results is imperative. This study does not explore biotic or abiotic causes of EEA variation; the results are only intended to address sampling and analytical concerns for results and conclusions. We emphasize that our main objective is not to determine which method is better, but to compare results for the two methods to evaluate if inter-study comparisons could be made using either technique.

Materials and methods

Study sites

During June 2008, 72 non-glazed ceramic tiles (each 25.8 cm²) were staked flush to the substratum at a location with homogeneous depth, current, and light, within riffle habitats of three streams (Shade River, Pratts Fork, and Big Bailey) in southeastern Ohio, USA. Stream chemistry of sites was representative of typical streams in the Western Allegheny Plateau of southeastern Ohio with pH of 8.1, 7.8, and 8.0, conductivity of 460, 570, and 620 $\mu\text{S cm}^{-1}$, and temperature of 19.1, 19.5, and 19.2°C for the Shade River, Pratts Fork, and Big Bailey, respectively. Riffles had rocky substrata, were 2–4 m wide, and had open canopies. Tiles were colonized for 6 weeks, which is adequate time for mature biofilms to develop (Gale et al., 1979). Water tight containers were used to transport tiles from field sites to the laboratory to minimize physical disturbance to biofilms.

Enzyme analysis

Fluorescent methylumbelliferone (MUB) linked substrates from Sigma–Aldrich were used for measuring phosphatase (M8883), β -glucosidase (M3633), and β -xylosidase (M7008) activities of biofilms, and the fluorogenic substrate leucine 7-amido-4-methylcoumarin (L2145) was used for measuring leucine-aminopeptidase activity (Sigma–Aldrich, St. Louis, MO). Fluorescence was measured using a microplate reader with excitation wavelength of 365 nm and emission wavelength of 455 nm (Synergy HT, Bio-Tek, Winooski, VT, USA). Preliminary experiments of EEA using extra tiles from the three streams and different substrate concentrations (10, 50, 100, 300, 600 μM) over time indicated that 300 μM (final concentration) with 20–40 min incubations were appropriate for assays of these four enzymes. Fresh substrate and standard solutions were made prior to each analysis day because some substrates and fluorescent standards deteriorate within 3 days (DeForest, 2009). Enzyme analysis for comparisons between intact and disrupted biofilms began immediately upon return to the lab on day 1.

For EEA measurements of disrupted biofilms, the samples were prepared by removing the biofilm of nine randomly selected tiles in each stream using a razor

blade and clean toothbrush ($n = 27$). Each sample was homogenized for 30 s with a tissue tearer at high speed (Biospec products, Inc, Bartlesville, OK, USA), which dispersed cells without damaging membranes. For quantifying EEA, 96-well black polystyrene microplates with 300 μl wells (Whatman Inc., Florham Park, NJ, USA) were used [12 columns \times 8 rows (analytical replicates for determination of mean fluorescence)]. For dispensing samples, substrates, and standards efficiently, 8-channel multi-step pipettes were used. Column 1 was a blank and contained 200 μl of filtered stream water (buffer) to account for background fluorescence of stream water. Column 2 was a reference standard containing 100 μl of either 10 μM MUB or 10 μM 7-amino-4-methylcoumarin (COUM) and 100 μl of filtered stream water as a known emission coefficient to determine standard curves for calculating EEA as nmol h^{-1} . The reference standard also accounts for any possible differences in the fluorescence of assays caused by different pH, which is important because MUB fluorescence increases with pH (Chróst & Krambeck, 1986). Column 3 was a negative control containing 100 μl of substrate and 100 μl of filtered stream water to account for any fluorescence by the substrate itself. Columns 4–6 were used to determine the EEA for each sample. Column 4 was a quench containing 100 μl of homogenized sample and 100 μl of MUB or COUM to determine the fluorescence masked by the sample. Column 5 was a sample control containing 100 μl of sample and 100 μl of filtered stream water to account for natural fluorescence of the sample (biofilm). Column 6 was the assay and contained 100 μl of sample and 100 μl of the appropriate 600 μM substrate. Columns 7–9 and 10–12 accommodated two more samples (three samples per plate). The microplate loading order was buffer (filtered stream water), sample, MUB or COUM standard, and finally substrate. Immediately after adding substrate, plates were incubated in the dark at 20°C (all three streams were within 1°C of the incubation temperature) and read after 20 and 40 min. The pH values of the three streams were above 7.8 and did not require any buffer solution to increase fluorescence and therefore two measurements could be made. EEAs were very similar between the two times; therefore, the mean was used to provide a more robust quantification. EEAs were calculated as in DeForest (2009). The pH of samples after analysis was identical to the initial pH, which indicated no problems of

fluorescence intensity changing as a result of pH variation. Activities were reported as $\text{nmol h}^{-1} \text{cm}^{-2}$ for simplicity, as we were only interested in describing the differences of EEA, and biomass was homogeneous among tiles as indicated by low standard errors.

Extracellular enzyme activity (EEA) measurements of intact biofilms were conducted by placing each tile in a 58 ml sterile Whirl-Pak (Nasco, Fort Atkinson, WI, USA) with 10 ml of 300 μM substrate, which completely submerged the tile and biofilm. Nine randomly selected tiles from each stream were used for each enzyme analysis [$n = 27$ for each enzyme, (36 tiles from each stream to provide 9 tiles for each of the 4 enzymes)]. As in previous studies of intact biofilm EEA, the tiles were incubated in the dark on a rotary shaker (80 oscillations min^{-1}) for 40 min in the laboratory. The supernatant was dispensed into the microplates and read immediately. All columns were the same as in disrupted biofilm analysis, but a quench could not be calculated, or a sample control because only the 300 μM substrate supernatant was used in the analysis. Assays of intact biofilms were corrected for natural fluorescence of substrate and filtered stream water as in the disrupted biofilm assays.

For documenting the effect of storing samples as disrupted biofilms, three tiles from each stream ($n = 9$) were scraped on day 1 and assayed for EEA. Disrupted samples were then stored in the dark at 4°C and EEA quantified again on days 3 and 5. Three tiles from each stream were stored in filtered stream water as intact biofilms in the dark at 4°C and then disrupted for immediate EEA assays on days 3 and 5. Day 1 measurements were considered the most accurate quantification of potential enzyme activity, and were used as reference values for measuring how EEA changed with duration and type of storage.

Statistical analysis

Data were log transformed to meet normality and homogeneity of variance assumptions. For testing differences between intact and disrupted EEAs, a 2×2 factorial ANOVA was conducted. Intact versus disrupted biofilm was a fixed factor, with stream as a block to account for inter-stream differences of environmental variables. A one-way ANOVA was conducted on intact biofilms to test for significant

differences of each EEA among streams, and another was conducted using disrupted biofilms. A 2×2 mixed model ANOVA (stream \times time) tested for significant differences of each EEA on days 1, 3, and 5. Accounting for inter-stream differences was important because different environments can affect EEA regardless of biomass (Chappell & Goulder, 1992; Romani & Sabater, 2000). For ANOVAs, a Tukey–Kramer multiple comparisons test was used to detect significant differences among groups and treatments. All analyses were conducted using Number Cruncher Statistical Systems 2004 (NCSS, Kaysville, UT, USA).

Results

Tiles from each stream had homogenous algal biomass (mean and standard error in mg m^{-2} ; Shade = 38.9 ± 1.9 , Pratts = 114.0 ± 4.9 , Big Bailey = 47.6 ± 2.4). Enzyme activities were significantly different ($P < 0.05$) among the three streams (Table 1), showing the necessity of the two-factor ANOVAs and mixed model ANOVAs in subsequent analyses to account for among stream variations. Using pooled results from the three streams ($n = 27$), all EEAs (PHOS, LAMP, GLU, and XYLO) were significantly greater ($P < 0.05$) in disrupted biofilms than in intact ones (Fig. 1). The ratio of GLU:XYLO was greater in disrupted biofilms than intact biofilms ($P < 0.01$), and the ratio of PHOS:LAMP was greater in intact biofilms than disrupted biofilms ($P < 0.01$). PHOS activity of intact biofilms was 49%, LAMP 8%, GLU 48%, and XYLO 65% of that for disrupted biofilms.

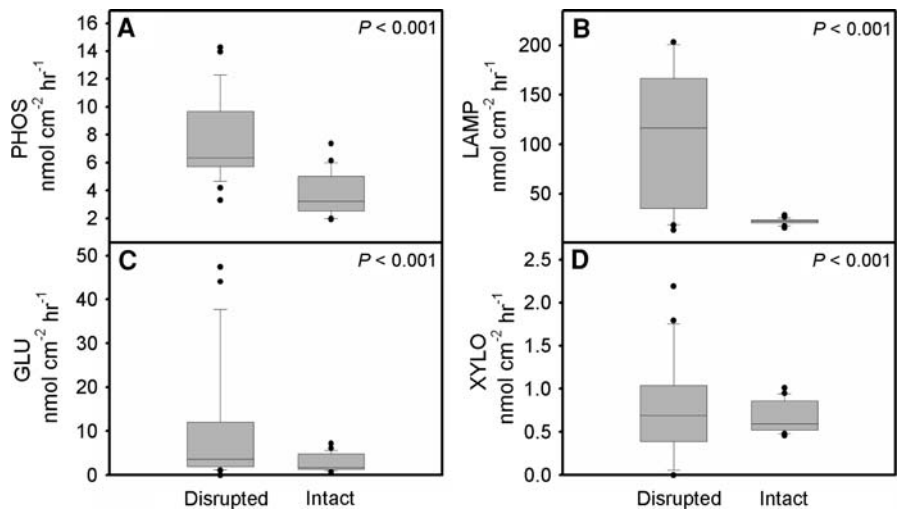
When analyzing each stream independently ($n = 9$ for each stream), patterns of EEAs were different depending on whether intact or disrupted biofilms were used (Table 1). Both methods indicated similar differences of statistical significance for PHOS and LAMP activities among the three streams. PHOS was similar in Big Bailey and Shade River, but both were significantly less than Pratts Fork ($P < 0.01$) as indicated by both intact and disrupted assays. LAMP was similar in Pratts Fork and Shade River, but both were significantly greater than Big Bailey ($P < 0.01$). The two methods had different results for GLU and XYLO (Table 1). Intact assays showed that GLU activity was significantly different for each stream,

Table 1 Means of extracellular enzyme activities assayed on day 1 for disrupted and intact biofilms in the three streams

Enzyme	Big Bailey (nmol h ⁻¹ cm ²)			Pratts Fork (nmol h ⁻¹ cm ²)			Shade River (nmol h ⁻¹ cm ²)		
	Intact	Disrupted	Ratio	Intact	Disrupted	Ratio	Intact	Disrupted	Ratio
Phosphatase	2.98 (0.18) ^a	6.12 (0.81) ^a	2.1	5.58 (0.29) ^b	9.86 (0.93) ^b	1.8	2.66 (0.31) ^a	6.45 (0.64) ^a	2.4
Leucine aminopeptidase	7.84 (0.31) ^a	27.0 (3.15) ^a	3.4	9.46 (0.37) ^b	174.1 (16.6) ^b	18.4	9.51 (0.31) ^b	138.7 (11.2) ^b	14.6
β -glucosidase	1.70 (0.27) ^a	2.88 (0.51) ^a	1.7	2.67 (0.20) ^b	28.29 (5.03) ^b	10.6	1.05 (0.07) ^c	2.94 (0.30) ^a	2.8
β -xylosidase	0.78 (0.08) ^a	0.36 (0.09) ^a	0.5	0.41 (0.03) ^b	1.34 (0.17) ^b	3.3	0.38 (0.01) ^b	0.70 (0.08) ^c	1.8

Values in *parentheses* are one standard error of the mean ($n = 9$ for each stream). Ratios are disrupted divided by intact values. Different *letters* indicate significant differences among treatments using Tukey–Kramer multiple comparison tests. *Note: Superscripts* compare intact and disrupted biofilm values separately

Fig. 1 Extracellular enzyme activities from disrupted and intact biofilm assays ($n = 27$ for each method). PHOS = Phosphatase, LAMP = Leucine aminopeptidase, GLU = β -glucosidase, XYLO = β -xylosidase. Boxes are inter-quartile ranges, lines are medians, whiskers are 90th % values, and circles are outliers

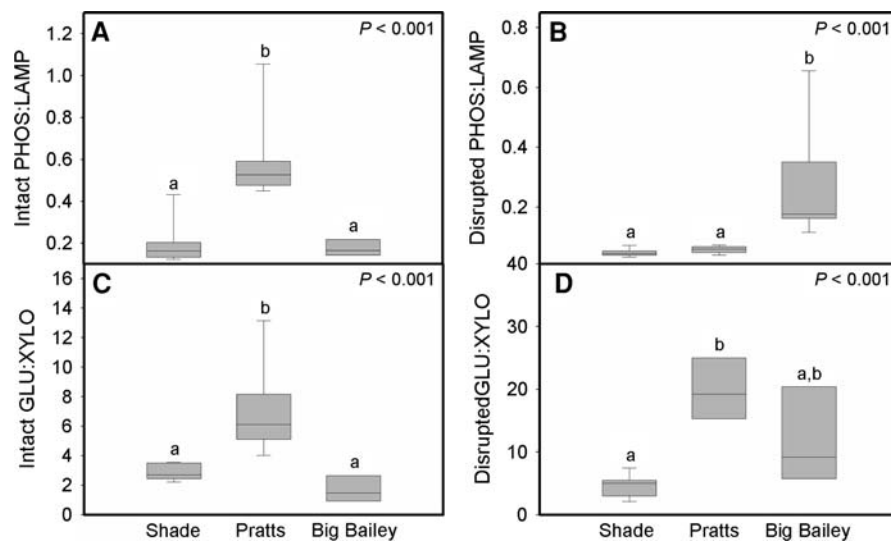


with activity being greatest in Pratts Fork, intermediate in Big Bailey, and least in the Shade River ($P < 0.01$), but disrupted assays showed that GLU was similar in Big Bailey and Shade River, and significantly greatest in Pratts Fork ($P < 0.01$). Intact assays showed that XYLO activity was similar in Pratts Fork and Shade River, and significantly greatest in Big Bailey ($P < 0.01$), but XYLO was significantly greatest in Pratts Fork, intermediate in Shade River, and least in Big Bailey ($P < 0.01$). In each stream, EEA of each enzyme was always significantly greater ($P < 0.05$) in disrupted biofilms than intact ones, but the magnitude of difference varied among streams. Ratios of disrupted to intact assays for each enzyme were not consistent among the three streams, which indicated that intact EEAs were not always a set proportion of disrupted EEAs (Table 1). For example, the ratio of disrupted to intact LAMP activity was 3.4, 18.4, and 14.6 for Big Bailey, Pratts Fork, and Shade River, respectively (Table 1).

PHOS:LAMP ratios and GLU:XYLO ratios among streams were not concordant between the two methods (Fig. 2). For intact assays, PHOS:LAMP was similar in the Shade River and Big Bailey, and was significantly greatest in Pratts Fork (Fig. 2A), but PHOS:LAMP was similar in the Shade River and Pratts Fork, and was significantly greatest in Big Bailey (Fig. 2B). GLU:XYLO was similar in the Shade River and Big Bailey, and significantly greatest in Pratts Fork for intact assays (Fig. 2C), but GLU:XYLO for disrupted assays, Shade River was significantly less than Pratts Fork, and Big Bailey was intermediate and not significantly different from the other two streams (Fig. 2D).

Storage time affected EEAs when samples were scraped on day 1 and then stored as disrupted biofilms until days 3 and 5 (Fig. 3A). PHOS activity was significantly greater ($P < 0.05$) on days 3 and 5 than on day 1. LAMP activity was significantly less ($P < 0.05$) on day 3 than day 1 ($P < 0.05$). GLU and

Fig. 2 Phosphatase:leucine aminopeptidase ratios ($n = 27$ for each method) for intact (A) and disrupted (B) biofilms among the three streams. β -glucosidase: β -xylosidase ratios for intact (C) and disrupted (D) biofilms among the three streams. Different letters indicate significant differences among streams using Tukey–Kramer multiple comparison tests following analysis of variance



XYLO activities were not significantly different among days ($P > 0.05$). General trends of EEA were mostly consistent among the three streams, but their magnitudes varied (Table 2). For example, PHOS was 351% greater on day 3 in Pratts Fork, but only 143% greater in Shade River, and GLU was 188% greater on day 5 in Shade River, but was 4% less in Big Bailey (Table 2).

Extracellular enzyme activity (EEA) varied greatly when tiles were stored as intact biofilms and then scraped on days 3 and 5 (Fig. 3B). PHOS and XYLO activities were significantly greater ($P < 0.05$) on days 3 and 5 than on day 1. LAMP exhibited a similar response to samples stored as disrupted biofilms, but had significantly greater activity on day 5 ($P < 0.05$). GLU activity was significantly greater on day 3 than on day 1 ($P < 0.05$). Storage time effects varied among streams (Table 2). For example, PHOS was 437% greater on day 5 in Big Bailey, but only 257% greater in Shade River, and XYLO was 487% greater on day 3 in Big Bailey, but only 246% greater in the Pratts Fork (Table 2).

Discussion

Intact assays represent the natural structure of and conditions in stream biofilms. However, even with oscillations mimicking stream flow for intact assays, some of the methylumbelliferone (MUB) linked

substrate may not reach the inner portion of the biofilm due to slow diffusion and boundary layer effects. Chappell & Goulder (1992) warned that when intact biofilms are assayed by adding substrates to overlying water, only enzymes near the biofilm–water interface will have access to the substrates, therefore missing relatively inaccessible enzymes deeper within the biofilm. Intact assays may be of interest for relationships between EEAs and water column chemistry, but this ignores channels and voids within biofilms that are important for nutrient and ion transport to deeper portions (Battin et al., 2003b). However, the extent to which nutrients and compounds are transported throughout biofilms also depends on current velocities and turbulence at the biofilm–water column interface (Battin et al., 2003b). If biofilms were left intact for EEA assays and incubation times were consistent, what effect would thicker biofilms have on the reliability of results? As biofilms become thicker, diffusion through the biofilm is slowed (Stevenson & Glover, 1993), and the ability of substrate to reach all depths of the biofilm and the subsequent release of fluorescent product to the supernatant for analysis within the experimental time is likely low. Therefore, much of the EEAs and interactions within biofilms could be under estimated or misinterpreted when using intact assays.

Disrupted biofilms may provide a more comprehensive representation of potential EEAs in biofilms. The entire biofilm matrix is analyzed, indicating both

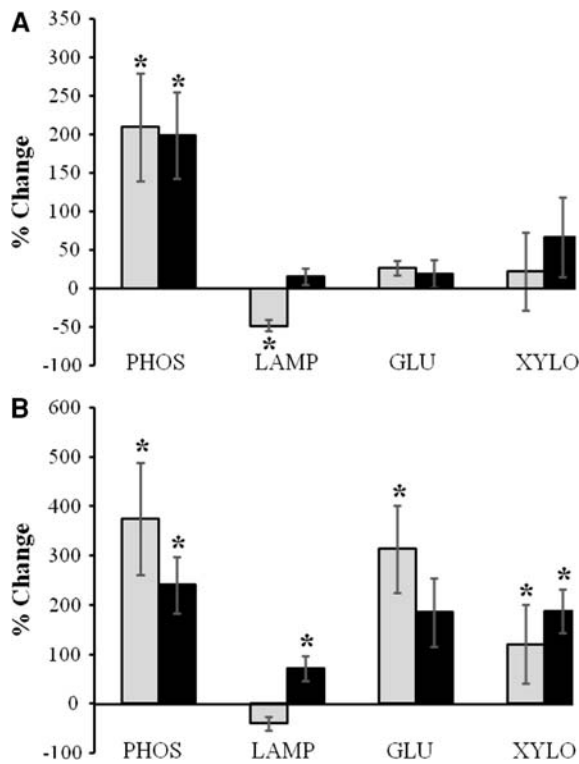


Fig. 3 Storage time effects on extracellular enzyme activities (EEA) as measured by percent change. PHOS = Phosphatase, LAMP = Leucine aminopeptidase, GLU = β -glucosidase, XYLO = β -xylosidase. *Gray bars* are day 3 measurements, *black bars* are day 5 measurements (*error bars* are ± 1 standard error). *Asterisks* denote significant difference from day 1 values (ANOVA, $P < 0.05$). **A** Samples disrupted on day 1 and then stored as disrupted biofilms until days 3 and 5 ($n = 9$). **B** Samples stored as intact biofilms and then disrupted for EEA analysis on days 3 and 5 ($n = 9$)

resource acquisitions within biofilms and at the biofilm–water column interface. Micro-scale architecture and channels or voids enhance the transport of nutrients and other compounds throughout biofilms (Battin et al., 2003a, b). Thompson & Sinsabaugh (2000) found matrix enzymes to be an extremely important resource contribution to biofilm community dynamics as they represent a substantial portion of total biofilm EEA. Disrupted biofilm assays would eliminate potential problems with biofilm thickness and could lead to more consistent, comparable, and nearly instantaneous results of potential enzyme activity among streams. Without assaying disrupted biofilms, much of the EEA could be missed from deeper portions of the biofilm matrix. If relationships between EEAs and biomass are of interest, disrupted biofilms would provide a better link because enzymes and biomass from the entire biofilm are analyzed. Many studies that used intact assays subsequently related EEAs to bacterial and algal biomass of the entire biofilm, although EEAs deep within the biofilm may not have been quantified. This method would lead to reporting of lower than actual EEAs standardized to biomass (i.e., EEAs from intact assays, which are significantly less than from disrupted assays, are then standardized to biomass from the entire biofilm, leading to less activity per biomass unit than what are likely true values). Potential problems with using disrupted biofilms may be that relatively inactive enzymes could be stimulated, or that intracellular enzymes might be released if cells are lysed during the disruption process. With regards

Table 2 Effect of storage time on extracellular enzyme activity for each stream

Streams	Phosphatase		Leucine aminopeptidase		β -glucosidase		β -xylosidase	
	Day 3 (%)	Day 5 (%)	Day 3 (%)	Day 5 (%)	Day 3 (%)	Day 5 (%)	Day 3 (%)	Day 5 (%)
Scraped Day 1, then stored as disrupted biofilm								
Bailey	435*	366	80	140	157	96	165	213
Pratts	351*	301*	40*	95	121	73	139	179
Shade	143	230*	35*	111	101	188*	34*	107
Stored as intact biofilm, then scraped on Days 3 and 5								
Bailey	659*	437*	89	199*	645*	392	487*	382*
Pratts	525*	327*	54*	154*	349*	139	246	266*
Shade	241	257*	38	164	245	326	100	215

Values are reported as the mean percent deviation from the mean of day 1 measurements (e.g., a value of 100% would be interpreted as equal activity to day 1). For each stream, $n = 3$. *Asterisks* denote significant differences from day 1 values ($P < 0.05$)

to the first concern, if biofilms are disrupted and immediately assayed, latent enzyme stimulation would likely be minimized, but further research is needed to examine the rate at which bacteria and algae are capable of producing EEAs. The second concern can be addressed by using minimally damaging techniques.

PHOS, LAMP, GLU, and XYLO activities of intact biofilms were all significantly less than disrupted biofilms. This result has implications for inter-study comparisons using different methods as the magnitude of activity measured between intact and disrupted biofilms was substantial and inconsistent among streams (Table 1). Depending on the enzyme, EEAs were on average 187–1,213% greater in disrupted assays than in intact assays. XYLO had the least difference between intact and disrupted biofilms, which may result from particulate organic matter accumulating in higher layers of mature biofilms (nearer the water interface) and primarily being of allochthonous origin (Romani, 2000). Algal exudates are most likely the dominant source of polysaccharides broken down by bacterial GLU activity in biofilms (Somville, 1984; Romani & Sabater, 2000), but algae can also stimulate XYLO activity as a result of photosynthesis (Espeland et al., 2001). Diatoms, a prominent component of lotic biofilms, secrete large amounts of extracellular polymeric substances for cell attachment to substrata and as a result of excess photosynthate, which then become substantial and important components of biofilm structure (Hoagland et al., 1993; Staats et al., 1999). The differences of GLU and XYLO between intact and disrupted biofilm assays have implications for understanding carbon dynamics within biofilms and carbon cycling in streams.

Differences between the two methods could also cause potential problems with interpreting significant or non-significant results. Although this study did not focus on how environmental variables affect EEAs, intact and disrupted biofilms showed similar significant differences for PHOS and LAMP, but conflicting significant differences among streams for GLU and XYLO activities (Table 1) and different patterns of GLU:XYLO and PHOS:LAMP ratios among streams (Fig. 2). PHOS:LAMP ratios are useful for documenting nutrient dynamics and limitation in streams (Sala et al., 2001). The current study showed different patterns of PHOS:LAMP ratios between the

two methods, which would lead to different interpretations of nutrient dynamics. The statistically significant differences of EEAs between the two methods gave conflicting results with regards to environmental impacts on biofilm function. This difference further confounds inter-study comparisons, the understanding of biofilm function and dynamics, and ultimately management efforts, because one method may indicate a certain pattern that could be contradicted by results from the other method.

Duration of sample storage had significant effects on extracellular enzyme activities, regardless of being stored as intact or disrupted biofilms. PHOS changed most drastically with storage duration, which creates problems for understanding nutrient dynamics in streams if samples are not assayed immediately. Variability of PHOS activity associated with storage duration has also been observed for soil samples (DeForest, 2009). The magnitude of change for enzyme activities differed among streams, and EEAs on days 3 and 5 were rarely similar to day 1 measurements (Table 2). As a result, researchers cannot rely on adding or subtracting a certain percentage depending on the duration of storage as the difference has no consistent pattern. Each stream sample can potentially respond differently to storage duration. Freezing is another potential method for storing samples, but this was not tested in the current study, and its practicality is unknown for lotic systems. Freezing samples before conducting assays gives unreliable EEA measurements, but assaying samples and then freezing the fluorescent product can be useful if immediate determination of fluorescence is not possible (Chróst & Velimirov, 1991). Boiling or chemical alteration (e.g., HCl, NaOH, or HgCl₂ preservation) can be useful for preserving samples, but only after assays with appropriate substrates are performed (Marxsen & Fiebig, 1993; Christian & Karl, 1995; Bélanger et al., 1997). These various preservation methods can have potential problems associated with increased fluorescence and loss of sensitivity, but HCl addition could be most effective (Bélanger et al., 1997). The pH needs to be raised before reading in a fluorometer, which adds more steps, complexity, and time for conducting assays. As a note of caution, the aforementioned methods of preservation were not conducted on freshwater epilithic biofilms, which are very different, both structurally and functionally, from aquatic sediments and

the water column. With broad scale stream sampling, storage of samples before conducting assays poses a greater problem than storage afterward. Minimal storage duration before conducting assays is crucial for attaining reliable EEA results, especially because EEAs in samples from different streams do not change in a consistent way.

Conclusions

Extracellular enzyme activities are important for understanding processes within aquatic biofilms and provide valuable data to assess natural and anthropogenic impacts on ecosystem function. How researchers measure EEAs can significantly affect results and their interpretations, which has implications for restoration, management, and conservation strategies of lotic systems, along with general understanding of biofilm function, ecology, and biotic or abiotic interactions. In the worst case scenario, results from one method might lead to one plan of action, while results from another might lead to a conflicting management plan. Further research is needed to empirically identify evidence for which method may be more advantageous for which types of research questions. Arguments in favor of one method or the other have been made, but no research has empirically tested or validated hypothetical opinions and concerns. Research needs to document the potential limitations and causes of problems associated with each method. The results of our research were simply intended to raise concern that differences exist between two commonly used methods for assaying extracellular enzyme activities from epilithic habitats in streams. These differences could potentially perpetuate conflicting findings and conclusions in peer-reviewed literature, which would hinder the advancement of aquatic science. Before conducting studies, preliminary assays using different substrate concentrations and incubation times must be conducted to identify optimal methods, which will generate reliable and valid results that could otherwise be affected by environmental differences among regions and stream types. Samples must be assayed as soon as possible to improve consistency, preferably the day of sampling, especially because the direction and magnitude of change can vary among streams, which could lead to conflicting or unexpected results (e.g., relationships with nutrients or carbon sources might be over or under

estimated and significant results may be detected when no actual significance exists, or vice versa). Storage time and method should be reported in the literature when presenting results due to the EEA variability.

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