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Effects of storage on measurements of potential microbial activities in stream fine benthic organic matter

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Abstract

Sample storage can significantly influence measured microbial activities in stream fine benthic organic matter (FBOM), possibly confounding effects of sample variability and short-term changes in activity. Denitrification potential, acetylene reduction and respiration rates, mineralizable N and extractable ammonium concentrations, and β-glucosidase and phosphatase enzyme activities of FBOM from first-order mountain streams in the western Oregon Cascade Mountains were assayed at various times after collection to determine potential storage effects. Denitrification potential, phosphatase activity, and extractable ammonium remained stable over a minimum of 11 h of storage at 5°C. Mineralizable N concentrations, respiration rates, and β-glucosidase activity all decreased within 12 h of collection. Results varied for acetylene reduction. Once assay conditions were established, denitrification potential and respiration rates were linear with incubation time. Based on paired *t*-tests, measures of acetylene reduction, denitrification potential, respiration rate, β-glucosidase activity, and phosphatase activity were generally similar at a 1-wk interval within the same stream reaches.

Keywords: Fine benthic organic matter; Microbial indicators; Storage effects; Streams

1. Introduction

As a major component of organic matter in streams, fine benthic organic matter (FBOM) is nutritionally important to stream microorganisms and invertebrates (Vannote et al., 1980; Benke et al., 1988; Cummins et al., 1989; Schlosser, 1991). Fine benthic organic matter (≤ 1 mm diameter) is derived from leaf litter and woody debris, as well as from fecal matter, riparian soil, flocculated dissolved organic matter, and autochthonous plant matter (Ward et al., 1994). Unlike leaf litter and woody debris, the decomposition dynamics of FBOM are relatively unstudied. Examination of the activity of various microbial populations (e.g., denitrifiers and nitrogen-fixers) associated with FBOM, as well as other chemical parameters (C:N ratio), provides insight into FBOM decomposition dynamics. To enhance the accuracy of data interpretation, we need to understand the effects of sample storage and perturbation on observed microbial activities.

Experimental design can quantitatively and qualitatively influence apparent measures of biogeochemical transformations. Changes occurring in microbial populations with storage time have been well documented in water samples from marine (Ferguson et al., 1984; Marrase et al., 1992) and freshwater

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environments (Reinke and de Noyelles, 1985; Amy and Hiatt, 1989), and in aquifers (Hirsch and Rades-Rohkohl, 1988). In soils, storage time has been shown to influence apparent microbial C, N, P, and ATP concentrations and enzyme activities (Tabatabai and Bremner, 1969; Pancholy and Rice, 1972; Speir and Ross, 1975; West et al., 1986). Sample perturbation also has been found to increase microbial activity rates in aquatic samples (McIntire, 1966; Logan and Kirchman, 1991) and mineralizable N in soils (Cabrera and Kissel, 1988).

Few studies have documented the effects of sample storage or perturbation on stream sediment microbial or biochemical variables. Even though sample size does not influence oxygen uptake by microorganisms in sediment and water samples, sample stirring does influence oxygen uptake (Hargrave, 1972). Other studies have found changes, often increases, in microbial potential activity after perturbation of both stream sediments (Christian and Wiebe, 1979) and marine or intertidal sediments (Oremland et al., 1984; Findlay et al., 1985; Meyer-Reil, 1987; Carman et al., 1989; Dobbs et al., 1989). When measuring lipid phosphates, an indicator of total microbial biomass, Federle and White (1982) found that sample perturbation by sieving estuarine sediments actually decreased lipid phosphates, and samples stored at 5°C showed minimal declines. Dobbs et al. (1989) report a nonsignificant increase in lipid phosphates in undisturbed marine sedimentary microorganisms after storage at 5°C for 5 h.

Researchers frequently find that neither perturbing samples (e.g., via suction, sieving, and preparing slurries), nor conducting a wide range of measurements of microbial activity at the same time is practical. Therefore, the objective of our study was to determine the stability of various microbial and chemical characteristics of FBOM during storage and analysis. An assessment of the relative stability of different microbial activities responsible for major biogeochemical transformations will enable researchers to design more satisfactory sampling schemes.

2. Materials and methods

2.1. Site description

To examine the effects of sample storage, we collected FBOM samples from two first-order streams selected to represent a range of possibly influential environmental conditions on the H.J. Andrews Experimental Forest, in the western Cascade Mountains, Oregon. One stream ran through an old-growth forest (OG) dominated by Douglas-fir (*Pseudotsuga menziesii*) and western hemlock (*Tsuga heterophylla*), and the other ran through a young Douglas-fir stand regenerating from a harvest in the late 1980s (CUT). Stream temperatures were 14 and 11°C in OG and CUT, respectively. Temporal variability was measured by sampling FBOM from the same streams twice, at a 1-wk interval. In all, we sampled 14 first-order streams running through either old growth or young Douglas-fir stands. The young stands had been harvested either 10 or 30 y before we collected samples for this study.

2.2. Experimental design

We determined the effects of short-term storage of samples by conducting potential activity measures at regular time intervals after initial collection of FBOM from two first-order streams on two separate occasions. The effects of storage time were determined by performing analyses from 3 to 15 h after collection, and comparing the rates determined after different storage times. Linearity of N_2O and CO_2 production was monitored by measuring denitrification potential and respiration on subsamples taken during the 15 h incubation period.

Temporal variability in denitrification potential, respiration, and acetylene reduction rates, and in β-glucosidase and phosphatase activities, was measured by sampling the same 14 streams twice, separated by an 8-d interval. In addition, C:N ratios were calculated for nine of these streams on each date.

2.3. Sample collection, preparation, and storage

This study was conducted under low-flow summer conditions, a time of year when terrestrial litter inputs and FBOM movement downstream are minimal, to limit the influence of environmental variability. Fine benthic organic matter was collected from crevices between rocks and the beds of first-order streams with a hand-held vacuum pump connected to a 2-L collecting jar. This sampling scheme

was designed to reduce sample spatial variability. The vacuum line was attached to a nozzle fitted with a 1-mm stainless steel screen, thus allowing benthic material ≤ 1 mm in diameter to be automatically wet sieved into the collecting jar. Samples of FBOM and stream water were transferred to polystyrene jars (500 ml). The jars were stored in the field in an insulated chest filled with stream water and ice. In the laboratory, a slurry was prepared by decanting excess stream water from the jars, and then mixing to keep the FBOM suspended during subsampling. Subsamples were dispensed by plastic syringes with enlarged openings to facilitate transfer. Samples were removed from 5°C storage to dispense subsamples immediately prior to each analysis, to limit sample exposure to ambient temperature. Storage time was determined as the time lapse between sample collection from the stream to the beginning of the assay incubation.

2.4. Laboratory analyses

Because of significant perturbation (e.g., suction, sieving, mixing, and preparing slurries) of FBOM during sampling and subsampling, we performed laboratory analyses described in the following sections to measure potential activity rates. These rates do not reflect in-stream FBOM activity rates, but rather give us a relative sense of the variability of rates among treatments and over time.

2.4.1. Denitrification potential rates

Denitrification potential was determined as N_2O emission by anaerobically incubating FBOM supplemented with glucose and $NaNO_3$ (Martin et al., 1988). Duplicate 5-ml slurry samples in 25-ml Erlenmeyer flasks were capped with rubber stoppers and purged for 3 min with argon at a rate of at least 120 cc min^{-1}. In the middle of the purge, the flasks were shaken gently to ensure removal of air bubbles. The flasks were allowed to incubate for 1 h at 24°C, and then 2 ml of sterile solution of 1 mM glucose and 1 mM $NaNO_3$ were injected through the stopper, and 2 ml of headspace gas were withdrawn with the syringe. Flasks were allowed to incubate for another hour at 24°C. At zero and 2 h time, 0.5 ml of headspace gas was removed from the flasks and assayed for N_2O in a gas chromatograph equipped with an electron capture detector. All gas chromatographs had stainless steel columns packed with Poropak-Q, either 50/80 or 80/100 mesh (Water Associates, Inc., Medford, MA).

2.4.2. Respiration rates

To determine respiration rates, duplicate 5-ml subsamples were placed in 25-ml Erlenmeyer flasks, and then capped with rubber stoppers. After incubating for 1 h at 24°C, 0.5 ml of headspace gas was analyzed for CO_2 in a gas chromatograph fitted with a thermal conductivity conductor. After incubating for an additional 2 h under the same conditions, a second headspace gas sample (0.5 ml) was collected and analyzed.

2.4.3. Acetylene reduction rates

Putative nitrogen fixation rates were determined by acetylene reduction (Weaver and Danso, 1994). Samples were prepared in the same way as for denitrification potential, except that the headspace gas contained 1.5% O_2, 12.5% acetylene, and 86% argon. After 24 h of incubation, 0.5 ml of headspace gas was removed and analyzed for ethylene in a gas chromatograph fitted with a flame ionization detector. A control was analyzed for ethylene production in the absence of acetylene.

2.4.4. Mineralizable nitrogen and extractable ammonium concentrations

For mineralizable nitrogen measurements, the microbial conversion of organic N to inorganic N in the form of NH_4–N was measured by the ''anaerobic'' incubation method (Keeney, 1982). Duplicate 10-ml sediment subsamples were added to 50-ml screw-topped test tubes, which then were completely filled with deionized water (53 ml), capped, and incubated at 40°C for 7 d. After incubation, subsamples were transferred to 25-ml Erlenmeyer flasks, and 53 ml of 4 M KCl and 0.4 ml of 10 M NaOH were added to each. The flasks were shaken for 1 h, and then analyzed with a selective ion electrode (Corning ammonium electrode, Medford, MA).

Extractable ammonium concentrations were measured by adding 50 ml of 2 M KCl to duplicate 10-ml subsamples in 250-ml Erlenmeyer flasks. The flasks were capped, shaken while incubating for 1 h in the presence of 0.4 ml 10 M NaOH, and analyzed with a selective ion electrode to determine KCl-

extractable ammonium concentration. Net mineralization was calculated as mineralizable N-extractable ammonium to account for ammonium present prior to incubation.

2.4.5. Enzyme activities

Phosphatase activity was determined according to the spectrophotometric assay of Tabatabai (1994) as modified by Zou et al. (1992). One ml of 50 mM *p*-nitrophenyl phosphate (Sigma 104) was added to duplicate 1-ml subsamples containing suspended FBOM. The tubes were shaken and then placed, along with duplicate controls with no phosphatase substrate addition, in a 30°C water bath for 1 h. After incubation, 1 ml of 50 mM *p*-nitrophenyl phosphate was added to the controls; reactions were stopped immediately with the addition of 2 ml 0.5 M NaOH and 0.5 ml 0.5 M $CaCl_2$. The assay mixtures were centrifuged for 5 min at $500 \times g$. A 0.2-ml subsample of the supernatant was diluted with 1.8 ml deionized water, and the optical density was measured at 410 nm. A standard curve was prepared from 0.02 to 1.00 μmol ml^{-1} *p*-nitrophenol. Enzyme activity was expressed as μmol *p*-nitrophenol released gdw^{-1} h^{-1}.

The β-glucosidase activity assay required the same general procedure as was used for phosphatase activity, except that the substrate was 1 ml 10 mM *p*-nitrophenyl β–D glucopyranoside, the incubation period was 2 h, and 2 ml 0.1 M tris[hydroxymethyl]aminomethane at pH 12.0, instead of 0.5 M NaOH, were added to terminate the reaction.

2.4.6. Total carbon and nitrogen

Total C and N were determined by dry combustion with a Carlo-Erba NA 1500 Series II high-temperature combustion furnace on oven-dried subsamples ground to pass through a 250-μm sieve.

2.5. Statistics

All statistical analyses were performed according to SAS (SAS Institute Inc., 1996). To examine whether or not potential activity rates changed with length of storage time, we used a one-way analysis of variance (PROC ANOVA), with time as the independent variable, to test the null hypothesis of no change. Where significant differences occurred, differences were examined by a multiple comparisons test with least significant differences (LSD).

To examine rates after the onset of incubation, linear regression (PROC REG), with time as the independent variable and activity rate as the dependent variable, was used to determine whether or not denitrification potential and respiration rates were linear over time.

For each assay, results from 14 streams, sampled twice with a 1-wk interval between samplings, were analyzed by a paired *t*-test. The difference in results from the 2 days was calculated as $d = \text{rate}_{\text{time 1}} - \text{rate}_{\text{time 2}}$, and then PROC MEANS was used to compute the probability that the difference (d) was significantly different from zero.

3. Results and discussion

The effect of FBOM storage, as determined by the comparison of rates after different storage times, varied for each analysis. Potential denitrification rates remained stable during 11–12 h of storage (Fig. 1). No significant change in rates occurred in FBOM from CUT. Although a statistically significant decrease in denitrification potential rate occurred in OG FBOM (one-way ANOVA; $F = 4.37$, $P = 0.0423$, $n = 12$), the decrease was not biologically significant. Mean rates at 8.3 and 13.3 h were 6.2 and 5.7 nmol N gdw^{-1} h^{-1}, respectively, a difference of 9%. After the onset of incubation, denitrification potential rates were linear from 2.0 to 9.5 h in FBOM collected from OG and CUT stream reaches. Linear regressions with time as the independent variable and denitrification potential rate as the dependent variable yielded $r^2 = 0.99$ and 0.97 for OG and CUT, respectively ($P \leq 0.001$, $n = 4$). Thus, gas production rates could be calculated even when the time between headspace gas analyses was delayed. Similar results have been found in sediments from a small forested stream hyporheic zone, where denitrification potential was linear for up to 3.3 h (Duff and Triska, 1990), and in pond sediment, where rates were linear for at least 40 min following nitrate amendment (Murray et al., 1989). In addition, denitrification potential was found to be linear for over 8 h in intertidal estuarine sediments (Oremland et al.,

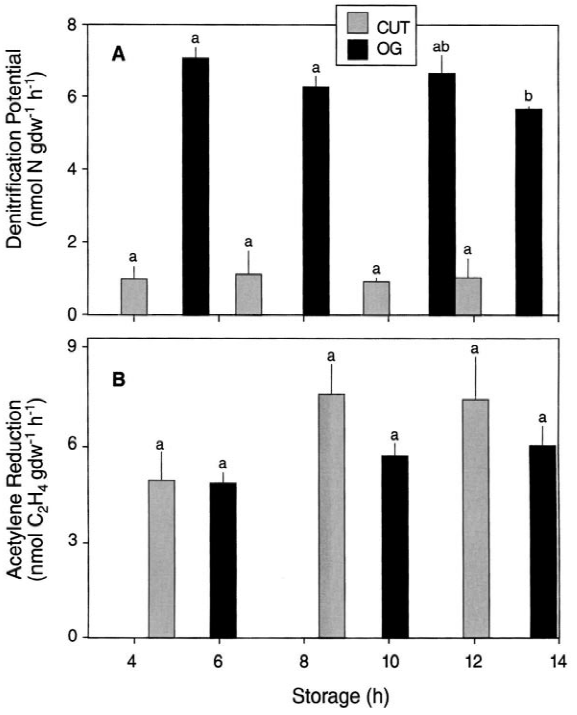

Fig. 1. Comparison of rates of (A) denitrification potential and (B) acetylene reduction measured in fine benthic organic matter collected and stored for between 3 and 14 h before initiation of rate determinations. CUT = young Douglas-fir stand, and OG = old-growth forest. Error bars = 1 S.D. Activities with different letters differ significantly ($P \leq 0.05$).

1984). Similar results have been found by Madsen (1979) and Oren and Blackburn (1979).

For acetylene reduction, conclusions about the effect of storage time are more difficult to draw. High sample variability (Fig. 1) resulted in no significant change in rate with up to 14 h of storage. However, acetylene rates in FBOM from OG and CUT followed a similar trend, and increased approximately 25 and 50%, respectively, after 8–10 h of storage, when compared to samples stored 6 h or less. Increased storage time may have allowed the development of anaerobic microsites prior to the establishment of anoxic assay conditions. Samples were stirred every 2–3 h when they were removed from storage to be subsampled for subsequent rounds of acetylene reduction assays, which likely aerated the samples. During preparation, sediments were exposed to an atmosphere with a very low O_2 concentration; however, it is possible that optimum conditions for N-fixation or the growth of N-fixers were not achieved until the resident heterotrophs had further reduced O_2 concentrations with extended incubation.

Extractable ammonium concentrations (ANOVA; $F = 308.65$ and $P = 0.0001$ for OG, and $F = 4.98$ and $P = 0.0774$ for CUT) generally remained stable during 11–12 h of storage. Although statistically significant differences occurred (ANOVA; $F = 6.03$ and $P = 0.0576$ for OG, and $F = 67.17$ and $P = 0.0007$ for CUT; $n = 8$ for both), these differences were not biologically significant. For example, concentrations at 4.3 and 6.8 h for OG FBOM were 25.9 and 23.4 μg NH_4–N gdw^{-1}, a change of less than 10%. In contrast, mineralizable N concentrations declined severely within the first few hours (Fig. 2).

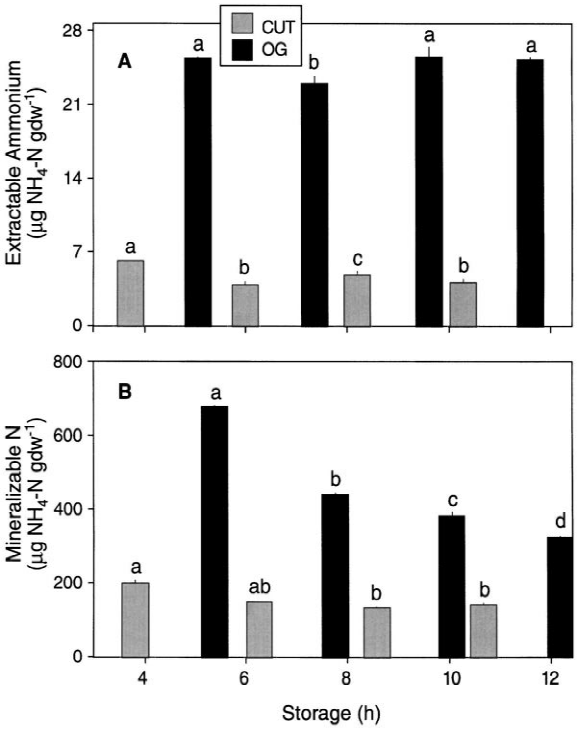

Fig. 2. Comparison of concentrations of (A) extractable ammonium and (B) mineralizable N measured in fine benthic organic matter collected and stored for between 3 and 14 h before initiation of determinations. CUT = young Douglas-fir stand, and OG = old-growth forest. Error bars = 1 S.D. Activities with different letters differ significantly ($P \leq 0.05$ for all except mineralizable N in CUT, for which $P = 0.08$).

From approximately 5 to 8 h post-collection, the apparent decrease in OG FBOM mineralizable N concentrations was 34%. Similarly, from approximately 4 to 8 h post-collection, CUT FBOM mineralizable N concentrations decreased 25%.

Given the lengthy incubation period for the mineralizable N assay (7 d), the reduction with storage time is puzzling. Particle flocculation may have occurred after the first 2 h of storage, thus resulting in a decline in available N as increased particle size effectively decreased the active surface area of particles, as well as the number of sites available for mineralization. Numerous processes can result in flocculation. The inorganic fraction of FBOM samples may have responded to increased ion concentrations in the thick slurry. In a study of microbial response to inorganic and organic particles in marine water, inorganic particles were found to flocculate within 1 h (Muschenheim et al., 1989). Use of a blender to create turbulence for 2 min was found to increase particle flocculation approximately 6-fold within 10 h (Lush and Hynes, 1973). Flocculation may also have been involved in the reductions in respiration and β-glucosidase activity rates determined after different storage times in this study (Figs. 3,4).

Another possible explanation for the decline in mineralizable N concentrations is that there was rapid decomposition of a highly labile organic N fraction within the first couple of hours after initial sampling. The rate of loss then decreased with time, a trend typical of decomposition curves.

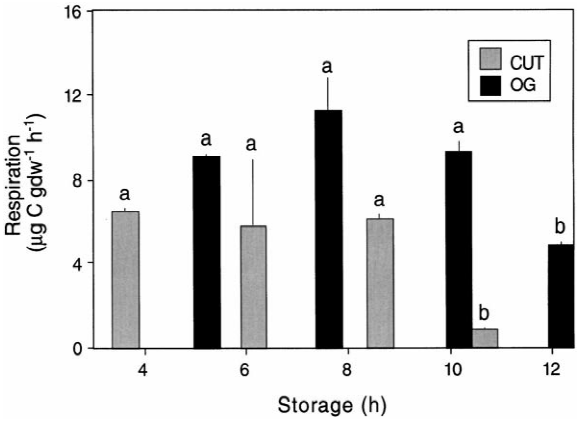

Fig. 3. Comparison of rates of respiration measured in fine benthic organic matter collected and stored for between 3 and 14 h before initiation of rate determinations. CUT = young Douglas-fir stand, and OG = old-growth forest. Error bars = 1 S.D. Activities with different letters differ significantly ($P = 0.07$ for CUT, $P \leq 0.05$ for OG).

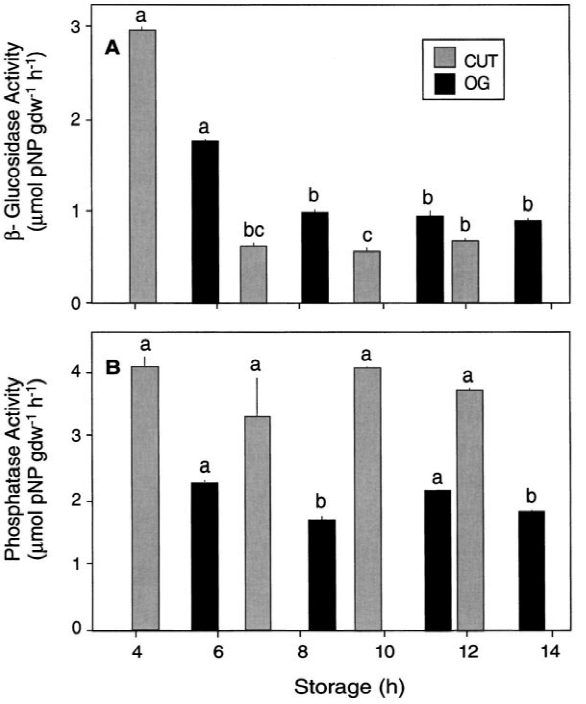

Fig. 4. Comparison of rates of (A) β-glucosidase and (B) phosphatase activity measured in fine benthic organic matter collected and stored for between 3 and 14 h before initiation of rate determinations. CUT = young Douglas-fir stand, and OG = old-growth forest. Error bars = 1 S.D. Activities with different letters differ significantly ($P \leq 0.05$).

Both respiration and β-glucosidase activities were significantly impacted by storage time (ANOVA; $F = 18.24$ and $P = 0.0085$ for OG, and $F = 5.48$ and $P = 0.0670$ for CUT) (Figs. 3,4(A)). A dramatic change occurred 8.5 and 10.0 h after sample collection, when respiration rates decreased 87 and 48% for CUT and OG, respectively. Note that in samples repeatedly tested after the onset of incubation at 24°C, rates remained steady for the same time period (not shown); respiration rates were linear from 3.0 to 9.0 h in FBOM from OG and CUT stream reaches. Linear regressions with time as the independent variable and respiration rate as the dependent variable yielded $r^2 = 0.99$ and 0.98 for OG and CUT, respectively ($P \leq 0.01$, $n = 4$ for both).

Respiration measurements were not amended, and thus represent both microbial activity and substrate availability. A reduction in substrate availability as a limiting factor to respiration is suggested by the decline in β-glucosidase activity. Beta-glucosidase activity decreased 80 and 46% between 4 and 6 h of storage for CUT and OG, respectively, approximately 4–5 h prior to the decline in respiration rates (Fig. 4). Beta-glucosidase hydrolyzes high molecular weight polysaccharides, generated by cellobiohydrolases, to low molecular weight glucose monomers, which are directly available for bacterial uptake (Münster, 1991). Evidence exists that enzymatic hydrolysis of polysaccharides and aquatic microbial uptake of sugars, such as glucose, are linked (Chróst, 1989; Chróst, 1990; Chróst and Rai, 1993). A decline in β-glucosidase activity, and subsequent decrease in available substrate for uptake, could reduce microbial growth and respiration.

Apparent decreases in activity could also be an artifact of substrate competition. Perturbance from stirring the slurry may have made naturally occurring substrate more available to microorganisms. Because the assay measures only synthetic substrate, preferential utilization by β-glucosidase of natural substrate to synthetic substrate (*p*-nitrophenyl) added during the assay would result in an apparent decrease in activity rates, because of direct competition between the natural and added artificial substrates. Prolonged exposure to low temperature (5°C storage vs. 10–14°C in situ) may also have led to decreases in β-glucosidase activity. In lake water with an in situ temperature of 7.5°C, low incubation temperatures (2–15°C) have been found to result in significantly decreased β-glucosidase activity, and in linear increases in rates up to the optimal temperature of 28°C (Chróst, 1989). Note that, in this assay, FBOM samples did incubate for 2 h at 30°C after storage.

Phosphatase activity remained relatively stable for at least 12 h of storage after collection (Fig. 4). No significant change was found in CUT FBOM (ANOVA; $F = 2.72$, $P = 0.1791$, $n = 8$). In OG, although FBOM rates vacillated significantly (ANOVA; $F = 59.18$, $P = 0.0009$, $n = 8$), differences were not biologically significant. For example, rates at 11 and 13.5 h were 8.6 and 7.3 μmol pNP gdw^{-1} h^{-1}, a decline of 8%. In streambed sediments, phosphatase activity was stable for 5 d at in situ temperatures (Marxsen and Schmidt, 1993). Similar results have been found in soils (Pettit et al., 1977; Speir and Ross, 1975; West et al., 1988). Phosphatase may be more stable when stored and more resistant to flocculation than β-glucosidase, because a greater percentage of β-glucosidase is associated with the cell surface of heterotrophic bacteria. Dissolved, free β-glucosidase and phosphatase, unassociated with microorganisms, comprised 0–30% and 18–100% of total extracellular enzyme activity, respectively (Chróst, 1989; Chróst, 1990; Wetzel, 1991).

After assessing the stability of various microbial and chemical characteristics of FBOM during storage and analysis, we used the same methods to examine within-stream variability over time. Microbial activities were measured in 14 streams on two sampling dates separated by 8 d. Despite the inherent heterogeneity of the stream environment, measures of microbial activity from a given stream were generally repeatable during a 1-wk interval, thus allowing in-stream comparisons through time and between-stream comparisons of FBOM biochemical properties. Paired *t*-tests based on the difference in rates between sampling times showed no difference for any of the activity variables (Table 1). Carbon:nitrogen ratios were significantly different between the two dates. Mean ratios for time 1 and time 2 were 23.6 and 25.9, respectively, a change of less than 10%.

One of our major concerns was that FBOM sample heterogeneity within a given stream would be so high that consistent patterns of microbial activity among streams would not be reproducible at different sampling times. We found that microbial activities and chemical characteristics of FBOM are relatively constant during the summer, when low flows and small allochthonous inputs result in relatively stable conditions. This may not, however, be the case during periods of high flow, when these variables may change rapidly over a 1-wk period (Bonin, unpublished data). Evidence exists that streams have large FBOM pools with a long residence time. These pools are supplemented by FBOM from seasonal events, such as autumn inputs of fresh litter and spring algal blooms (Cushing et al., 1993), which could greatly influence microbial activities

Table 1
Results of paired *t*-tests to test the hypothesis of no difference between assay results of FBOM from streams sampled twice at an interval of 1 wk[a]

Measure	*n*	*d*	*P*
Respiration rate (μg C gdw^{-1} h^{-1})	14	2.1 (1.2)	0.1050
Denitrification potential (nmol N gdw^{-1} h^{-1})	14	1.6 (0.9)	0.0918
Acetylene reduction (nmol C_2H_4 gdw^{-1} h^{-1})	14	8.0 (6.7)	0.2558
Beta-glucosidase activity (μmol pNP gdw^{-1} h^{-1})	14	0.2 (0.1)	0.1419
Phosphatase activity (μmol pNP gdw^{-1} h^{-1})	14	0.6 (0.4)	0.1918
Mineralizable N (μg NH_4–N gdw^{-1})	14	56.6 (32.8)	0.1082
C:N ratio	9	2.2 (0.8)	0.0226

[a] n = number of observations; d = mean of $rate_{time\ 1}$–$rate_{time\ 2}$ (standard error).

over relatively short time intervals during periods of increased flow.

4. Conclusions

The application of methods of soil and water analyses to sediments necessitates their reevaluation. We found that FBOM analyses varied in their robustness and reaction to such factors as storage time and perturbation. A comparison of rates determined after different storage times showed that extractable ammonium, denitrification potential, and phosphatase activity rates generally remained stable for 12 h of storage after collection. Respiration rates, β-glucosidase activity, and mineralizable N concentrations varied with storage time. Results varied for acetylene reduction rates. Results of biochemical analyses of FBOM from stream reaches were generally repeatable within a 1-wk period, thus supporting the validity of within-stream comparisons over time, and between-stream comparisons of FBOM characteristics.

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