The Determination of Leaf Processing Rates and Fungal Biomass via a Chemical Index

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Abstract

The goal of this study was to determine leaf processing rates and to determine fungal biomass accumulations on different leaf species in different seasonal environments. Sugar maple (*Acer saccharum*) and river birch (Betula nigra) leaves were incubated in two Northcentral Pennsylvania streams; Mill Creek, which is a second order stream, and Big Bear Creek, which is a third order stream. Leaves were incubated for 7 to 35 days during the summer and for 14 days during both the early and late fall. Weekly water chemistry and aquatic hyphomycetes spore counts were done during the incubation periods. Incubated samples were analyzed in the lab using HPLC to determine the presence of ergosterol, which is a membrane lipid of aquatic fungi. The surface areas of the incubated leaves were measeure pre- and post- incubation to determine processing rates. Additionally, incubated samples were heated in a muffle oven to determine percent organic content. Sugar maple leaves had higher fungal biomass accumulations during the summer and early fall, but declined slightly during the late fall. River birch had smaller fungal biomass accumulations during the summer, but peaked during the late fall. Leaf processing rates for both leaf species were significantly lower during the fall studies. Invertebrate colonization on incubated leaf samples was also significantly lower during the fall studies. Spore counts were significantly higher during the late fall than both the summer and early fall.

Introduction:

Stream order as defined by Cummins (1975) is a classification system used to describe the physical and biological properties of a stream, stream order ranges from type 1 (headwaters) to type 12 (mouth). Low order streams are classified from type 1 to type 3. These low order streams are typically small headwater springs and streams that are light and nutrient poor. The biota of headwater to order type 3 streams greatly depends on allocthonous organic materials as their primary energy sources. This allocthonous material usually takes the form of fallen leaves that enter the stream (Vannote et. al 1980). Many factors play a role in leaf breakdown in a stream. Once leaf litter enters a stream, it is subject to physical abrasion, microbial degradation, and invertebrate fragmentation (Graca 2001). Temperature, water chemistry, leaf species, and the colonization of macroinvertebrates and microorganisms affect leaf breakdown rates (Suberkropp and Chauvet 1995). Studies have shown leaf breakdown rates were higher at times of high nutrient levels. Additional studies have shown that pollutants actually slow down the leaf breakdown process (Pascoal et. al 2001).

Fresh leaf litter contains plant polymers which cannot be digested by animal consumers. In order for leaves to be consumed, microbial colonization and degradation is necessary for the litter to become a suitable food source for aquatic organisms (Suberkropp 2001). Macroinvertebrate shredders are the primary consumers of leaf litter in a stream. They are responsible for consuming coarse particulate organic matter (>1mm) and for the fragmentation of leaf litter into fine particulate organic matter (<1mm-0.005mm) that can be consumed by other macroinvertebrates (Cummins 1975). Shredders are known to have different preferences for different leaves within the same

stream. Differences in leaf toughness and nutrient content may play a role in shredder preference. Invertebrates feeding on leaf litter play an important role in the incorporation of plant material into secondary production (Graca 2001). A food web of a freshwater stream can be seen in Figure 1.

The presence of a microbial community appears to be attractive to shredders. Fungal hyphae that colonize leaves give the leaves a higher nutritional value and in turn make them more appealing to macroinvertebrate consumers (Graca 2001). The presence of a fungal biomass on leaf litter in streams can be related to the presence of aquatic hyphomycetes spores, or conidia. In the Northern Hemisphere, peak spore concentrations have been found in autumn and winter. Minimum spore concentrations are expected to occur in the summer months (Thomas et al 1989). Spore concentrations have been found to be positively correlated with the amount of leaf litter present in a stream (Suberkropp 1997). In addition to conditioning the leaves for invertebrate consumption, fungi in culture has been shown to cause changes in leaf litter that are similar to changes observed in the stream, suggesting that fungi function as a decomposer of leaf litter (Suberkropp 2001).

Ergosterol is a membrane component found predominantly in aquatic hyphomycetes, which are freshwater fungi (Newell 1992). Past studies have shown that ergosterol is not present in vascular plants, making it useful in the determination of a fungal index (Gessner and Chauvet 1994). Pure ergosterol can be quantified using HPLC to generate a calibration curve which can be used to determine the unknown concentration of ergosterol within a processed leaf litter sample. An ergosterol to fungal

biomass conversion factor was developed by Gessner and Chauvet (1992) to quantify the fungal biomass in a leaf litter sample.

This study examined the presence of fungal biomass in Sugar Maple (*Acer saccharum*) and River Birch (*Betula nigra*) leaves that were incubated at two separate sites (Mill Creek and Big Bear Creek in Lycoming County, PA). The study was conducted during the summer and fall to determine seasonal differences in the colonization of fungal biomass on incubated leaf samples. Relationships between fungal biomass and environmental factors were examined. These factors include water chemistry, the presence of aquatic hyphomycetes, leaf processing rates, and invertebrate populations. This study is the third and final project in a series of studies that have been conducted during the past three years under a research grant from Merck/AAAS. Emily Stricker completed an honors project summarizing the first year of the grant, while Christina Panko used the second year study to finish her honors project. In addition to continuing a third year of data collection, my study further divided the fall data into two separate study periods to determine any differences in fungal biomass and leaf processing rates occurring between the early fall and late fall time periods.

Methods:

Study Sites

The study was performed in the summer from June 2002 to July 2002 and in the fall during September 2002 and October 2002 at two study sites within the Loyalsock Creek watershed in Lycoming County, PA. The first study site was on Mill Creek, which is a second-order stream that flows between Rose Valley Lake and Loyalsock Creek in Montoursville, PA. The Mill Creek site is located downstream from agricultural and

residential property. The second study site was on Big Bear Creek, which is a third-order stream found near Barbours, PA that functions as a tributary to Loyalsock Creek. Second and third order streams are considered to be small headwater streams that are heavily shaded. Allochthonous material is heavily relied upon for the overall productivity of both streams. An EPA habitat assessment score indicated that Big Bear Creek had a habitat assessment score of 187, while Mill Creek had a lower score of 141 (Plafkin et al. 1989).

Physical and Chemical Water Analysis

Physical and chemical water analyses were conducted once a week during the summer leaf incubation periods. These analyses were conducted approximately once every two weeks during the fall study. A hand-held YSI model 55 DO meter was used to measure dissolved oxygen (ppm) and temperature (°C). A Swoffer Model 2100 flow meter was used to measure velocity (m/s). A meter stick and tape measure were used to measure depth and width of the creeks. Velocity, width, and depth were used to calculate the total volume of water. Alkalinity (ppm $CaCO_3$), nitrate (ppm NO_3^-), nitrite (ppm NO_2^-), orthophosphorus (ppm PO_4^{3-}), total phosphorus (ppm PO_4^{3-}), and pH were measured within 24 hours after collection of samples in the laboratory following Standard Methods procedures (American Public Health Association 2000). A titration method (using 0.2 N H_2SO_4) was used to measure alkalinity and pH was determined with a Corning pH Meter Type 440. Nitrate, nitrite, orthophosphorus, and total phosphorus were measured on a HACH DR/4000 Spectrophotometer. Standards were performed on all instruments.

Leaf Processing Rates

Sugar Maple (*Acer saccharum*) leaves were collected from a tree on the Lycoming College campus and River Birch (*Betula nigra*) leaves were collected at a site along Mill Creek on Dr. Zimmerman's property. Leaves from both sites were collected in early September and then again in early October. The October leaves were collected post abscission. The dried leaves were stored in a cold room at approximately nine degrees Celsius. Surface areas (cm^2) of each leaf were measured using a LI-COR Model LI-3000A area meter. Leaf packs were created from the measured leaves. Five sugar maple leaves were attached to a numbered brick with a rubber band to create one leaf pack. Five river birch leaves were attached to a numbered brick with two rubber bands to create one leaf pack. Each brick was placed in a mesh laundry bag.

River Birch and Sugar Maple leaf packs were placed in Mill Creek at Zimm's site on June 4th. Leaf packs of both species were placed in Big Bear Creek (BBC) Site #11 on June 5th. One week later and weekly thereafter for five weeks, one leaf pack of each species was removed from Mill Creek and Big Bear Creek and brought to the lab. In the lab, invertebrates were collected from each leaf and preserved in 70% ethanol. The leaves were rinsed with deionized water and post-incubation surface areas were measured. Processing rates, or k-values were calculated from the surface area measurements. From their k-values, leaves were identified as fast, medium, or slow decomposers (Petersen and Cummings 1974). Leaves with a k-value greater than 0.010 were identified as fast decomposers. Medium decomposers have k-values between 0.005 and 0.010. Slow decomposers have k-values less than 0.005. The same procedure was

followed for both collection periods in the fall, except that the fall leaf packs were all removed after a two week incubation period.

After surface areas were measured, the leaves were placed into a drying oven at 80°C for 24 hours. The dried leaves were ground with a mortar and pestle and placed into pre-weighed crucibles. The crucibles were then weighed again to determine the weight of the leaf material. Next, the crucibles were heated in a muffle oven at 550°C for 3 hours. After heating, the leaves were weighed again and percent organic content was calculated from the weight loss due to muffling.

Fungal Biomass:

Fungal biomass was determined through the use of High Pressure Liquid Chromatography following the procedure of Newell et al. (1988). In the lab, leaves were removed from the bricks and invertebrates were collected and preserved in 70% ethanol. Ten leaf discs were cut from each pack with a 16-mm cork borer. The leaf discs were weighed on an analytical balance and then placed in a round bottom flask with 25 mL HPLC grade methanol. The flasks were refluxed in an 80 °C water bath for 30 minutes. After 30 minutes, 5 mL of 10% KOH was added and refluxed for an additional 30 minutes. The process of saponification released ergosterol through the hydrolysis of sterol esters (Volker et al. 2000). After cooling to room temperature, the solution was filtered by water aspiration into a filter flask through a glass frit (coarse, 40-60 um) to remove any debris. The filtrate was transferred to a 65-mL screw cap vial where ergosterol was extracted with pentane. First, 5 mL of 20% aqueous NaCl was added to induce separation. Three separate portions of pentane were added to the vial in the amounts of 10 mL, 5 mL, and 5 mL. After each portion of pentane, the vial contents

were mixed and the pentane layer containing ergosterol was extracted. The extracted portions were allowed to dry overnight under a hood. The next day, 1.0 mL HPLC grade methanol was pipeted into the vial and sonicated to dissolve the ergosterol residue. The solution was filtered through a 13mm 0.45um nylon membrane Whatman filter.

The filtered solution was injected into a High Pressure Liquid Chromatography (HPLC) instrument. Ergosterol peaks were monitored at 282 nm, where there is maximum absorption. The HPLC instrument consisted of a Waters 510 pump, a Whatman Partisil 5 OD5-3 25-cm x 4.6-mm column, a 100uL sample loop, and a Waters 991 photodiode array detector with Millennium software. Ergosterol eluted at a flow rate of 1.5 mL/min in methanol between 5.2 and 5.7 minutes.

Standard ergosterol solutions were prepared from 0.1068 g ergosterol (Aldrich 95%) diluted to 200 mL with HPLC grade methanol for a concentration of 507.3 ug/mL. Next, 5 mL of the 507.3 ug/mL solution were diluted to 100 mL with HPLC grade methanol for a concentration of 25.365 ug/mL. Also, 1 mL of the 507.3 ug/mL solution was diluted to 100 mL with HPLC grade methanol for a concentration of 5.073 ug/mL. These two standard solutions were injected at different volumes to give amounts of ergosterol ranging from 0.1015 ug to 0.5073 ug. A standard curve was created by plotting the known ergosterol amounts against their peak areas on the Kaleidagraph program.

Before samples were injected onto the column, a baseline was monitored for approximately 20 minutes to ensure no impurities were in the column. The peak areas from injected samples were entered into the standard curve's line equation to determine ergosterol amounts. The dissolution (1 mL) and injection volumes (60 uL) were taken

into account to determine the ug ergosterol/10disc sample. Weight percent ergosterol was then calculated and converted to ug fungal biomass/mg leaf detritus with a 182.6 g fungal biomass/g ergosterol conversion factor (Gessner and Chauvet 1992).

Additional studies were undertaken to determine the accuracy and efficiency of the extraction and determination procedure. Weekly standard solutions were injected to ensure the accuracy of the standard curve. Recovery studies were performed to determine the efficiency of the procedure. This was achieved by processing fresh leaves with a known amount of ergosterol standard and analyzing using the HPLC to determine the ergosterol's percent recovery. Approximately a 98% recovery occurred. Non-incubated leaves were processed and analyzed to determine any naturally occurring ergosterol peaks. Tests were also performed to determine the effect of freezing and refrigerating sample leaf discs before being processed.

Aquatic Hyphomycetes Spore Analysis:

Each week during the summer, 300 mL water samples from both sites were filtered through a Millipore filter with 8um pore size. The samples were filtered into a Whatman membrane filter flask with a Model DOA-P104-AA Gast pump. The filters were dried and then placed into Petri dishes where they were flooded with 0.01% Trypan blue in lactic acid to kill and stain the spores. The dishes were heated at 55 °C for 55 minutes. The filters were then cooled and mounted on microscope slides. The spores were counted and recorded as the number of spores per 300 mL water sample. This procedure was repeated in the fall study, with water samples being analyzed once every two weeks.

Each week during the summer, 10 mL of creek water from both sites were placed into test tubes. Leaf discs were cut from fresh leaves of both species taken from the cold room. One leaf species was placed into a test tube containing water from one creek site. This was done with both Sugar Maple and River Birch for both sites. The test tubes were then put into the cold room for three days. On the third day, the leaf discs were removed and mounted on slides. The discs were fixed on each slide with 0.1% cotton blue in lactic acid and then flooded with 0.01% Trypan blue in lactic acid. Spores were then counted on each slide.

Invertebrate Data:

Macroinvertebrates were collected from analyzed leaf discs, as well as by the Surbur and kick sample methods. Macroinvertebrates from the leaves were collected each week, preserved in 70% ethanol, and later identified. Surbur and kick samples were collected monthly in the summer and once during the fall. Surbur samples were counted to determine the density of macroinvertebrates per square meter. The kick samples were used to identify 100 macroinvertebrates picked at random to determine the food web composition, species diversity, and to calculate an EPA Rapid Bioassessment (Plafkin et al 1989) of the study sites.

Results:

Fungal Biomass

Sugar maple leaves placed in Mill Creek during the summer study displayed the greatest fungal biomass after 14 days of incubation. They reached a peak of 7.10 ug fungal biomass/mg detritus. A decline in fungal biomass occurred through the 21 to 35 day incubation periods. Sugar maple leaves that were incubated in Mill Creek for 14

days during the early fall study displayed an average of 5.79 +/-1.46 ug fungal biomass/mg detritus. Statistical analysis performed at the 0.05 alpha level showed no significant difference (P=0.518) between the fungal biomass results for the summer and early fall study of sugar maple leaves incubated in Mill Creek for 14 days. A significant difference (P=0.0003) was found to exist between the summer data for 14 days and the late fall 14 day incubation data for sugar maple leaves in Mill Creek. The leaves incubated during the late fall achieved an average of 1.20+/-0.0910 ug fungal biomass/mg detritus. Fungal biomass accumulation during the late fall was significantly lower than the data from the summer study. The fungal biomass accumulation during the early fall was significantly higher than the fungal biomass accumulation during the late fall (P=0.006) for sugar maple leaves incubated in Mill Creek for 14 days..

River birch leaves placed in Mill Creek during the summer study displayed the greatest fungal biomass after 35 days of incubation. They reached a peak of 6.70 ug fungal biomass/mg detritus. River birch leaves that were incubated for 14 days in the summer displayed 3.17 ug fungal biomass/mg detritus. No significant decline in fungal biomass occured on the river birch leaves even after 35 days of incubation, suggesting that a longer incubation period would display greater amounts of fungal biomass. River birch leaves placed in Mill Creek during the early fall achieved an average of 9.58+/-0.590 ug fungal biomass/mg detritus after 14 days of incubation. The late fall study of river birch leaves incubated in Mill Creek for 14 days revealed an average of 33.1+/-1.43 ug fungal biomass/mg detritus. Statistical analysis determined that the early fall fungal biomass accumulations were significantly higher than the summer fungal biomass accumulations (P=0.0110). The late fall fungal biomass accumulations were significantly

higher than both the summer fungal biomass accumulations (P=0.00302) and the early fall fungal biomass accumulations (P<0.0001) for river birch leaves incubated in Mill Creek for 14 days.

Sugar Maple leaves placed in Big Bear Creek during the summer study displayed the greatest fungal biomass after 35 days of incubation. They reached a peak of 3.91 ug fungal biomass/mg detritus. No decline in fungal biomass occurred, also suggesting that a longer incubation period may lead to greater amounts of fungal biomass. During the summer, fungal biomass reached 1.86 ug fungal biomass/mg detritus after 14 days of incubation. During the early fall study, fungal biomass accumulations averaged 4.67+/-0.0545 ug fungal biomass/mg detritus after 14 days of incubation, which was significantly higher than the summer study (P=0.0005). The late fall study achieved an average of 2.30+/-0.131 ug fungal biomass/mg detritus after 14 days of incubation, which was not significantly higher than the summer study (P=0.0988). The early fall study achieved significantly greater amounts of fungal biomass than the late fall study for sugar maples leaves incubated in Big Bear Creek for 14 days (P<0.0001).

River birch leaves placed in Big Bear Creek during the summer study displayed the greatest fungal biomass after 7 days of incubation. It is hypothesized that ergosterol contaminated glassware or instrumentation lead to the amount of fungal biomass analyzed after the 7 day incubation period. After discounting the 7 day incubation period data, the greatest fungal biomass was displayed after 28 days of incubation with a peak of 3.17 ug fungal biomass/mg detritus. The 35 day incubation period data showed a decline of 1.31 ug fungal biomass/mg detritus. The 14 day incubation period achieved 2.05 ug fungal biomass/mg detritus. The early fall study achieved an average of 1.74 ug fungal

biomass/mg detritus with a standard deviation of 0.0667 after 14 days of incubation, which was not significantly different than the summer data (P=0.0557). The late fall study achieved an average of 19.4 ug fungal biomass/mg detritus with a standard deviation of 0.491 after 14 days of incubation, which was significantly higher than the summer data (P=0.00107). The late fall fungal biomass accumulations were significantly higher than the early fall fungal biomass accumulations for river birch leaves incubated in Big Bear Creek for 14 days (P<0.0001).

The summarized fungal biomass data for the summer study can be found in Figures 2A, B, C, and D. Comparisons of fungal biomass between the seasons can be found in Figure 3.

During the late fall study, duplicate samples were run to determine the repeatability of the results from the HPLC. The average standard deviation from three sample preparations was +/-1.46.

Another supplementary study was performed to determine the effect of refrigeration and freezing on incubated leaf samples. Incubated sugar maple leaves were taken from Mill Creek. Three leaf discs were cut from each leaf with a 16-mm cork borer. Ten discs were immediately analyzed for ergosterol, ten discs were placed in the cold room at approximately 9°C, and ten discs were placed in the freezer. This process was done three times throughout the summer study. One set of frozen and one set of refrigerated leaves were analyzed after 9 days, a pair of sets was analyzed after 10 days, and a pair of sets was analyzed after 16 days. The frozen leaf discs all experienced slight losses in fungal biomass ranging from an 8% to a 25% loss in fungal biomass. The leaf discs refrigerated for 10 days displayed a 36% increase in fungal biomass. The leaf discs

refrigerated for 9 days displayed a 20% loss in fungal biomass. The leaf discs refrigerated for 16 days displayed a 46% loss in fungal biomass. These results weakly suggest that long term refrigeration may lead to losses in the determination of fungal biomass.

***Hyphomycetes spore analysis*:**

During the summer study, Mill Creek averaged 700 spores/L. The early fall study averaged 689 spores/L, while the late fall study averaged 4533 spores/L in Mill Creek. There was not a significant difference between the summer and early fall spore counts in Mill Creek ($P=0.948$). The spore counts for the late fall were significantly higher than both the summer study ($P=0.0374$) and the early fall study ($P=0.0359$).

During the summer study, Big Bear Creek averaged 185 spores/L. The early fall study averaged 80 spores/L, while the late fall study averaged 716 spores/L. There was no significant difference between the summer and early fall spore counts in Big Bear Creek ($P=0.264$). The late fall study spore counts were significantly greater than both the summer ($P=0.00369$) and early fall studies ($P<0.0001$).

The summer spore counts for Mill Creek were significantly higher than the summer spore counts for Big Bear Creek ($P=0.00122$). The early fall study spore counts were also significantly greater in Mill Creek than Big Bear Creek ($P=0.0126$). There was no significant difference between the late fall studies at both sites ($P=0.274$).

Identification of some of the most common spores was attempted. *Flagellospora curvula* was predominant early in the summer study. As the summer progressed, *Lunulospora curvula* gradually took over predominance. *Tetrachaetum elegans*, *Clavariopsis aquatica*, and *Clavatospora longibrachiata* appeared frequently throughout

the summer study. During the fall studies, *Flagellospora curvula* once again was dominant. Identification was made using the book Guide to Aquatic Hyphomycetes by C.T. Ingold. The spore data can be viewed in Figures 4A and 4B.

The leaf disc method was attempted during the summer study. It was unsuccessful and discontinued during the fall studies. Because there was no movement of water within the test tube, the spores had no way to reach the leaf discs and colonize.

Leaf Processing Rates

The summer k-values for sugar maple differed greatly between the Mill Creek and Big Bear sites. Sugar maple leaves from Mill Creek had an average k-value of 0.0422, which designated them as fast decomposers. Sugar maple leaves from Big Bear Creek had an average k-value of 0.00195, which designated them as slow decomposers. The greater spore population in Mill Creek may have lead to the leaves becoming better conditioned for colonization and consumption by invertebrates leading to greater decomposition.

The summer k-values for river birch designated the leaves to be slow decomposers at both sites. Mill Creek leaves had a k-value of 0.00309, while Big Bear Creek leaves had a k-value of 0.00154. Once again, Mill Creek had slightly higher numbers, possibly caused by the greater spore population.

The fall k-values for sugar maple at Mill Creek were 0 for both fall studies, designating sugar maple as a slow decomposer. The early fall k-value for sugar maple at Big Bear Creek was 0.000356, designating sugar maple as a slow decomposer. The late fall k-value for Big Bear Creek was 0.00012, designating sugar maple as a slow

decomposer. The fall k-values for river birch were 0 at both sites during the early and late fall studies, which designated river birch as a slow decomposer.

The percent organic content steadily decreased for both species of leaf at Mill Creek during the summer study. Sugar maple percent organic content values ranged from approximately 91% to 45%. Sugar maple percent organic content decreased sharply until the incubation period between 28 and 35 days, where the percent organic content leveled off. The leveling of the organic content occurred at approximately the same time as a decrease in fungal biomass was occurring, suggesting a relationship between the two parameters. The organic content of river birch ranged from approximately 89% to 65%. River birch percent organic content in Mill Creek remained relatively constant until the incubation period between 28 and 35 days. At that time, percent organic content began to decrease more sharply. The time period of this decrease in organic content corresponds to the time period when fungal biomass was at its highest peak. The percent organic content results from both sugar maple and river birch in Mill Creek suggest that as fungal biomass levels increase, percent organic content levels will decrease. One explanation for this relationship is that as fungal biomass increases, the organic content of the leaves is being conditioned for consumption by invertebrates. The percent organic content data for Mill Creek can be found in Figure 5A.

The percent organic content data for both species of leaf in Big Bear Creek followed a pattern similar to that of Mill Creek. The major difference between the two sites is that sugar maple organic content ranged from 93% to 75% and river birch organic content ranged from 93% to 80%. The percent organic content data for Big Bear Creek can be found in Figure 5B.

The percent organic content for sugar maple in Mill Creek at 14 days during the summer was 77%. The early fall percent organic content for sugar maple at 14 days dropped to 72% and the late fall percent organic content for sugar maple in Mill Creek dropped only slightly to 76%. Sugar maple's percent organic content for Big Bear Creek after 14 days of incubation in the summer was 92%. The early fall percent organic content for sugar maple in Big Bear Creek was 87%, and the late fall organic content was 86%.

The percent organic content for river birch in Mill Creek at 14 days during the summer was 90%. The early fall percent organic content was 88%, and the late fall percent organic content was 89%. River birch's percent organic content for Big Bear Creek after 14 days of incubation in the summer was 92%. The early fall percent organic content for river birch leaves in Big Bear Creek was 78%, while the late fall percent organic content was 88%.

Invertebrate Data

During the summer study, maple leaves after seven days incubation at Mill Creek did not contain any invertebrates. However, after fourteen days incubation on maple leaves there was a large increase in the number of invertebrates (132 invertebrates). The number of invertebrates decreased significantly at day twenty-one and continued to decrease at days twenty-eight and thirty-five. At day thirty-five the number of invertebrates had decreased to 24 invertebrates on the sugar maple leaves. River birch leaves also did not contain any invertebrates after seven days incubation. The number of invertebrates increased at day fourteen and again at day twenty-one but then decreased at days twenty-eight and thirty-five. The total number of invertebrates on the river birch

leaves at Mill Creek peaked at day twenty-one with 56 invertebrates. At day thirty-five the number of invertebrates had decreased to 25 invertebrates. Overall, sugar maple appeared to have the greatest density of invertebrates at Mill Creek. The invertebrate percentages for Mill Creek can be seen in Figure 6

Also during the summer study, maple leaves incubated at Big Bear Creek for seven and fourteen days did not contain any invertebrates. At twenty-one days the maple leaves contained 32 invertebrates. The number of invertebrates decreased at twenty-eight days incubation but then increased sharply to 59 invertebrates after thirty-five days incubation. River birch trees incubated at Big Bear Creek for seven days did not contain any invertebrates. After fourteen days incubation the leaves contained two invertebrates. The number of invertebrates increased slightly after twenty-one days but then decreased slightly after twenty-eight days. A peak was hit at 35 days with a total number of invertebrates at 53. Overall, sugar maple appeared to have the greatest density of invertebrates at Big Bear Creek. The invertebrate percentages for Big Bear Creek can be seen in Figure 7.

The densities of invertebrates on the leaves from the fall studies were much smaller than the summer densities. During the early fall study, maple leaves from Big Bear Creek only contained 7 total invertebrates. No other incubated leaf samples from the early fall contained any invertebrates at all. During the late fall study, maple leaves from Big Bear Creek contained 6 invertebrates, while Birch leaves incubated in Mill Creek achieved 41 total invertebrates.

During the summer study the density of invertebrates on the incubating leaves was directly proportional to the level of ergosterol on the leaves. During the fall study,

the greatest density of invertebrates positively correlated with the highest levels of ergosterol for the late fall on river birch leaves incubated in Mill Creek. The invertebrate population composition obtained from the kick samples can be seen in Figure 8.

The density of invertebrates was consistently higher at Mill Creek than Big Bear Creek. Surbur densities at Mill Creek were 567 invertebrates/square meter in late May, 1444 invertebrates/square meter in late June, and 678 invertebrates/square meter in late September . Surbur densities at Big Bear Creek were 322 invertebrates/square meter in late May, 611 invertebrates/square meter in early July, and 88 invertebrates/square meter in late September. The surbur density data can be seen in Figure 9.

Water Chemistry

The summer water chemistry data showed Mill Creek to have significantly higher pH values than Big Bear Creek ($P=0.0003$). Mill Creek also had significantly higher alkalinity ($P<0.0001$), temperature ($P=0.0309$), and total dissolved solids ($P<0.0001$). Big Bear Creek had significantly higher dissolved oxygen ($P=0.0143$) than Mill Creek.

For Big Bear Creek, the early fall had a significantly higher pH than the summer ($P=0.00426$). The late fall had significantly higher nitrite concentrations than the early fall ($P=0.0304$). The early fall had significantly lower dissolved oxygen values than both the summer ($P=0.0177$) and the late fall ($P=0.0270$). The early fall also had significantly higher temperature readings than the summer ($P=0.0151$) and the late fall ($P=0.00485$).

For Mill Creek, the early fall had a significantly higher pH than the summer ($P=0.0185$). The early fall also had significantly higher alkalinity values than both the summer ($P=0.00384$) and the late fall ($P=0.000934$). Nitrate values were significantly

higher in the late fall than the summer (P=0.0343) and the early fall (P=0.00892). The early fall had a significantly higher temperature than the late fall (P=0.0005).

Discussion:

Allochthonous organic matter has long been considered the main energy source for the stream biota of low order streams. In order for the energy from this allochthonous material to enter the stream system, the material must be fed upon by decomposers. Macroinvertebrates, particularly shredders, are the primary organisms responsible for feeding upon allochthonous material. Before allochthonous material, such as leaf litter, is consumed by the macroinvertebrates, it must be conditioned by aquatic fungi. Aquatic fungi spores known as aquatic hyphomycetes must colonize the leaf litter in a process called conditioning. Studies have shown that the levels of fungal biomass on leaf litter is strongly correlated with shredder abundance. The most agreed upon explanation for this data is that leaves conditioned with fungal biomass are more attractive and nutritious to macroinvertebrates (Graca 2001). Other factors shown to be responsible for leaf breakdown in streams are specific leaf species, temperature, water chemistry, and habitat (Ostrofsky 1997).

The fungal biomass levels for the summer study were higher in sugar maple than river birch at both study sites. In addition, both species achieved greater amounts of fungal biomass in faster times in Mill Creek as opposed to Big Bear Creek. The most likely explanation for this difference would be the significantly higher spore densities found in Mill Creek. A greater density of spores in the water would lead to greater fungal

biomass accumulations (Iqbal and Webster 1973). Mill Creek also had significantly higher pH and temperature values than Big Bear Creek. Studies have shown that lower pH values may lead to a decrease in microorganism colonization, which in this case would be the fungal biomass (Solada et al 2000).

The fungal biomass levels for sugar maple in Mill Creek during the early fall study showed no significant difference from the summer values. During the late fall study, fungal biomass levels for sugar maple in Mill Creek were significantly lower. These results are unexpected because during the late fall spore densities were at their highest. Sugar maple leaves in Big Bear Creek displayed their highest amounts of fungal biomass during the early fall. The temperature in Big Bear Creek was the highest during the early fall, which may have encouraged greater fungal biomass accumulation.

The fungal biomass levels for river birch during the late fall were significantly higher than both the summer and early fall levels at both sites. These high levels correspond to the time period when the spore densities at both sites were at their highest.

The data from the two previous studies is consistent with current data in the fact that sugar maple displayed the highest fungal biomass levels in the summer and river birch displayed the highest levels in the fall. Data from the second year study shows sugar maple to have gained a significantly greater amount of fungal biomass in the fall when compared to the summer. The data from the first study year and the current data showed sugar maple values that did not increase significantly during the fall incubation period.

The summer study's leaf processing rates showed sugar maple to be a fast decomposer in Mill Creek, but a slow decomposer in Big Bear Creek during the summer

and at each site during the fall. River birch was a slow decomposer at each site during each season. Sugar maple and river birch have both been shown to be fast decomposers (Peterson and Cummins 1974). The only results that are consistent with previous research are the sugar maple k-values during the summer study in Mill Creek. The percent organic content for both leaf species decreased by greater percentages during the summer than during either fall study. The greater competition for invertebrate colonization during the fall may have lead to lower k-values and a lower decrease in percent organic content. During the summer study, very little allochthonous material had entered the stream, therefore the invertebrate population had fewer options for consumption. During the fall study, a large amount of seasonal leaf fall had entered the stream which created more competition for invertebrate colonization. Additionally, the surbur invertebrate densities were lower during the fall, which meant even fewer invertebrates were available to colonize a larger amount of leaf detritus.

The invertebrate densities on the incubated leaf samples during the summer study were positively correlated with the ergosterol peaks of the leaf samples. This would be expected because as the ergosterol levels increase on the leaf samples, the leaves are becoming better conditioned and more attractive and nutritious to the invertebrates (Suberkropp 1997). After reaching their peaks, both the invertebrates and the ergosterol levels would steadily decline as the invertebrates consumed the fungal biomass and moved on to other allochthonous material. Leaves in Mill Creek tended to have greater invertebrate densities. This may be related to the fact that Mill Creek is located downstream from a residential and agricultural area. The organic pollution in Mill Creek would be higher than that of Big Bear Creek, which would lead to greater numbers of

invertebrates. During the fall studies, the only significant amount of invertebrate colonization occurred on river birch leaves in Mill Creek during the late fall study. These river birch leaves had accumulated the greatest amount of fungal biomass of any incubated leaf sample during the fall. It would be expected that river birch would achieve the greatest invertebrate colonization.

In order to come to a definite conclusion about what factors or combination of factors determines fungal biomass accumulation and leaf processing rates, many more studies would have to be performed in an attempt to isolate certain factors. Additionally, a possible future project would be to inoculate agar plates with aquatic hyphomycetes under different conditions, such as varying pH, temperature, or nutrient levels, and observe what conditions promote the greatest fungal biomass growth. From this study and the previous two, it can be determined that leaf processing is not controlled by one or two factors, but rather by many variables that are all connected.

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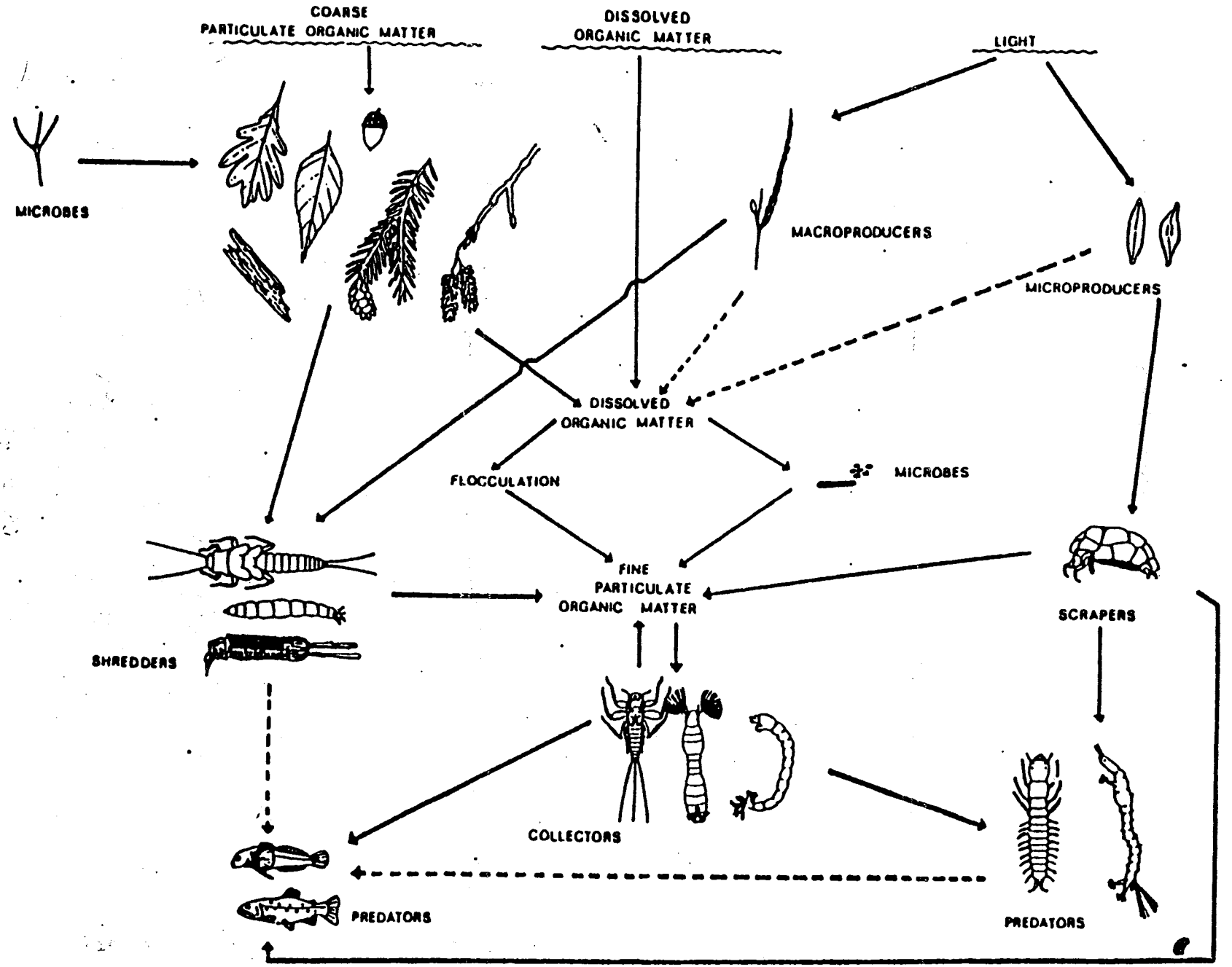
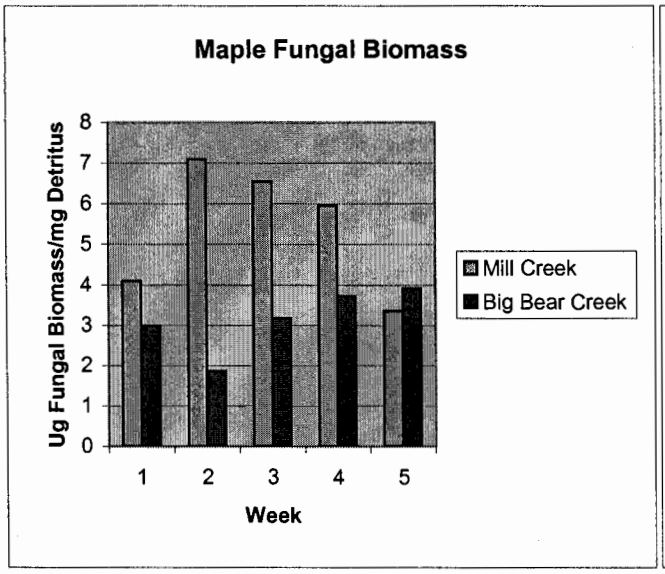


Figure 1 Food Web of a Freshwater Stream

Figure 2 Comparisons of Fungal Biomass From Summer Study

A



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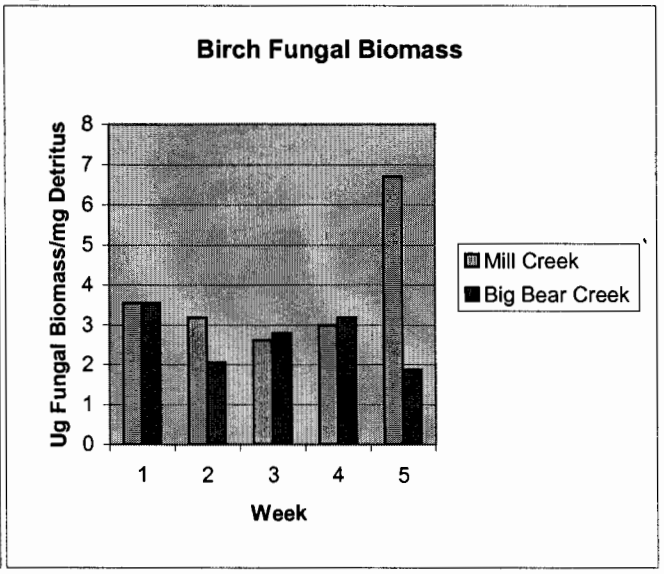

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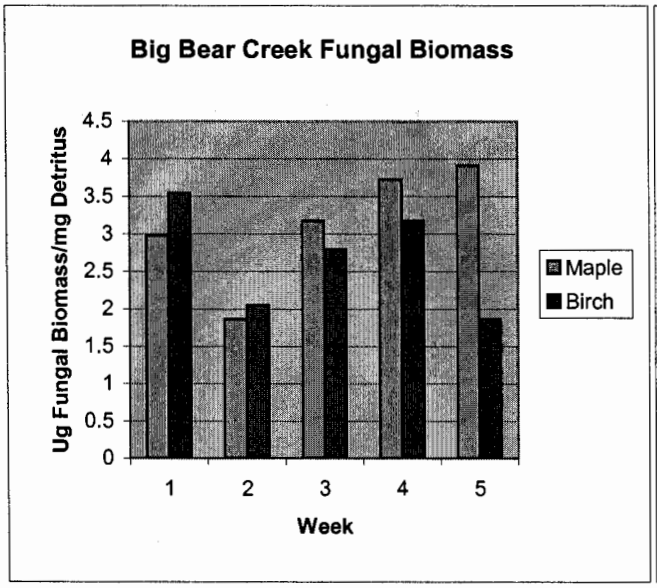

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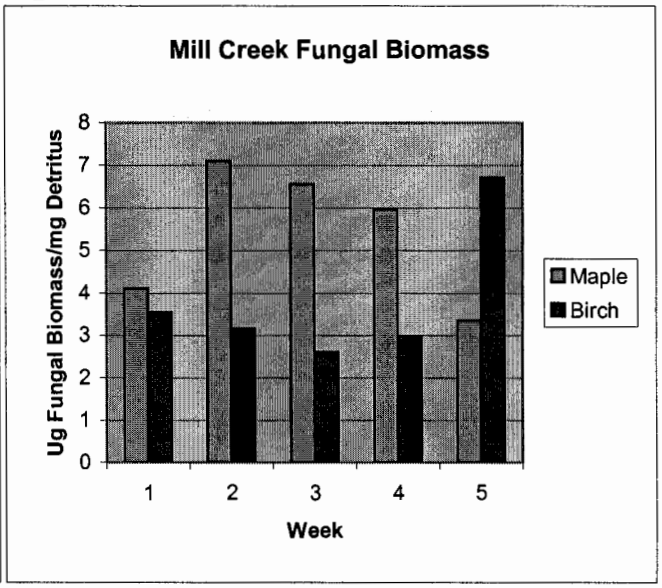
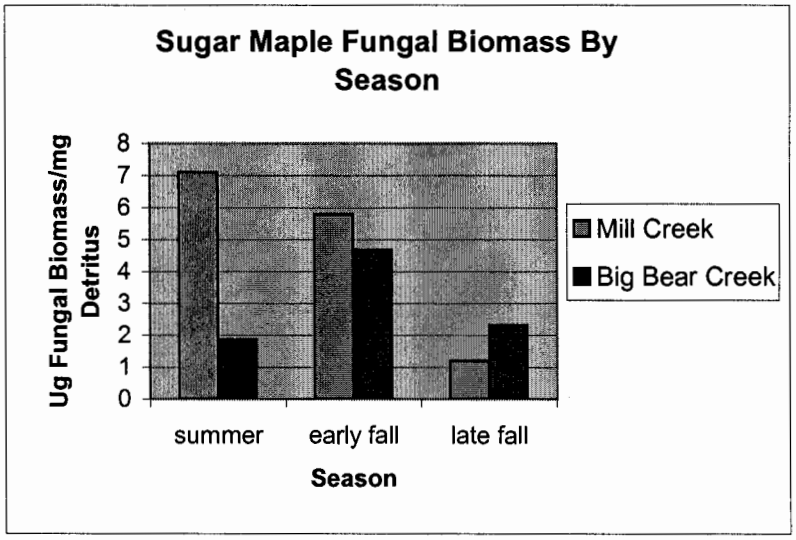


Figure 3 Comparison of Fungal Biomass by Season

A



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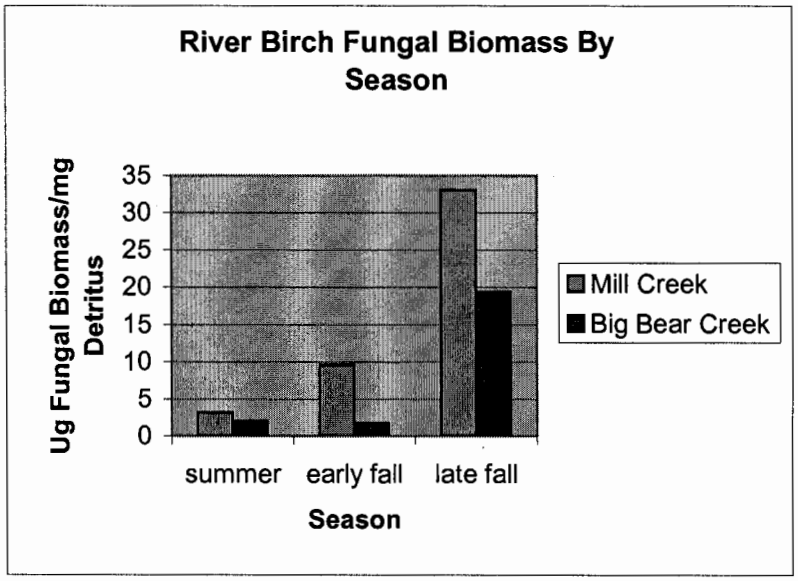
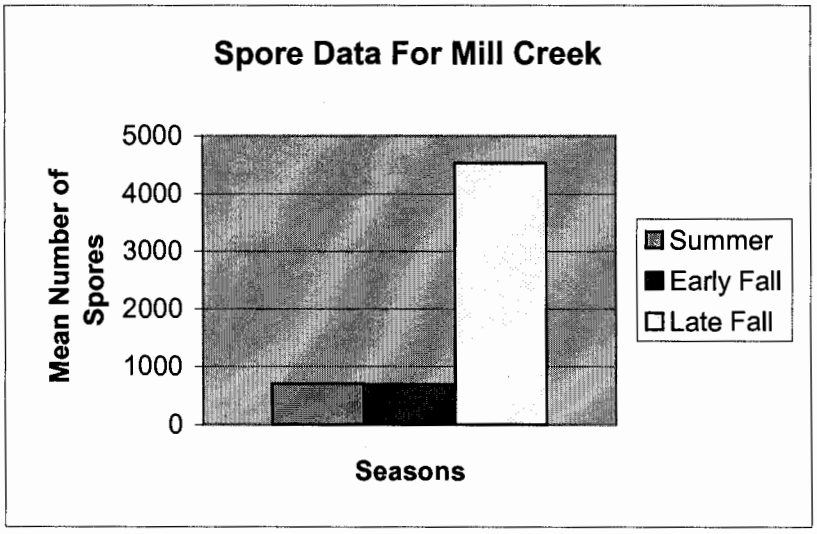


Figure4 Spore Data for Mill Creek and Big Bear Creek

A



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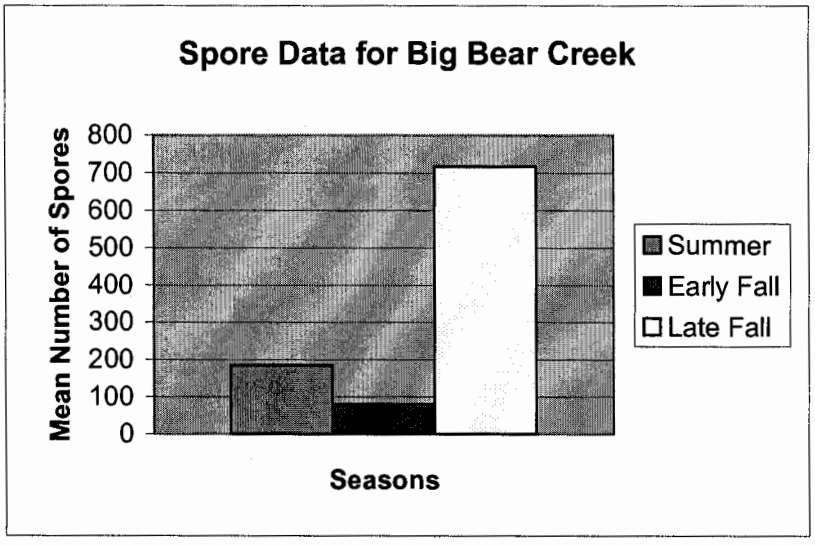


Figure5A

Percent Organic Content of Mill Creek Leaves

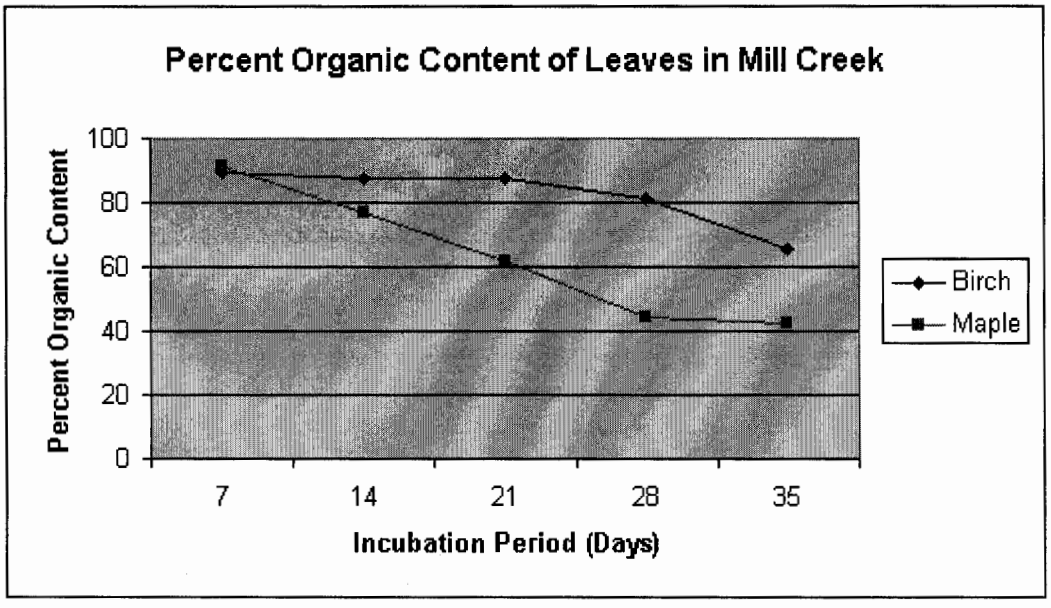
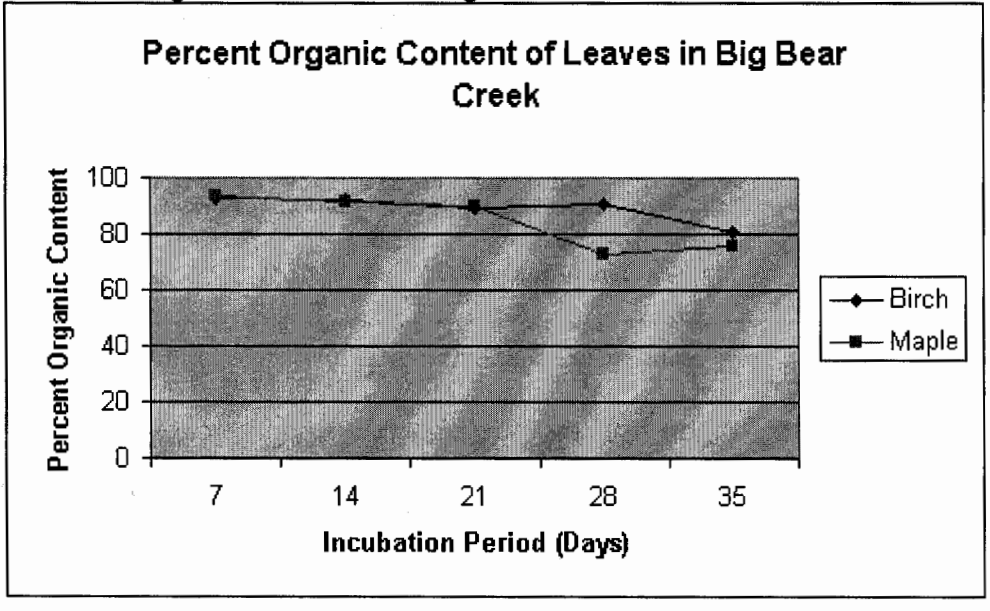


Figure 5B

Percent Organic Content of Big Bear Leaves



Figures 5A,B- Percent Organic Content of Each Leaf Species at Mill Creek and Big Bear Creek

Figure 6 **Mill Creek Inverterbrate Percentages for the Summer Study**

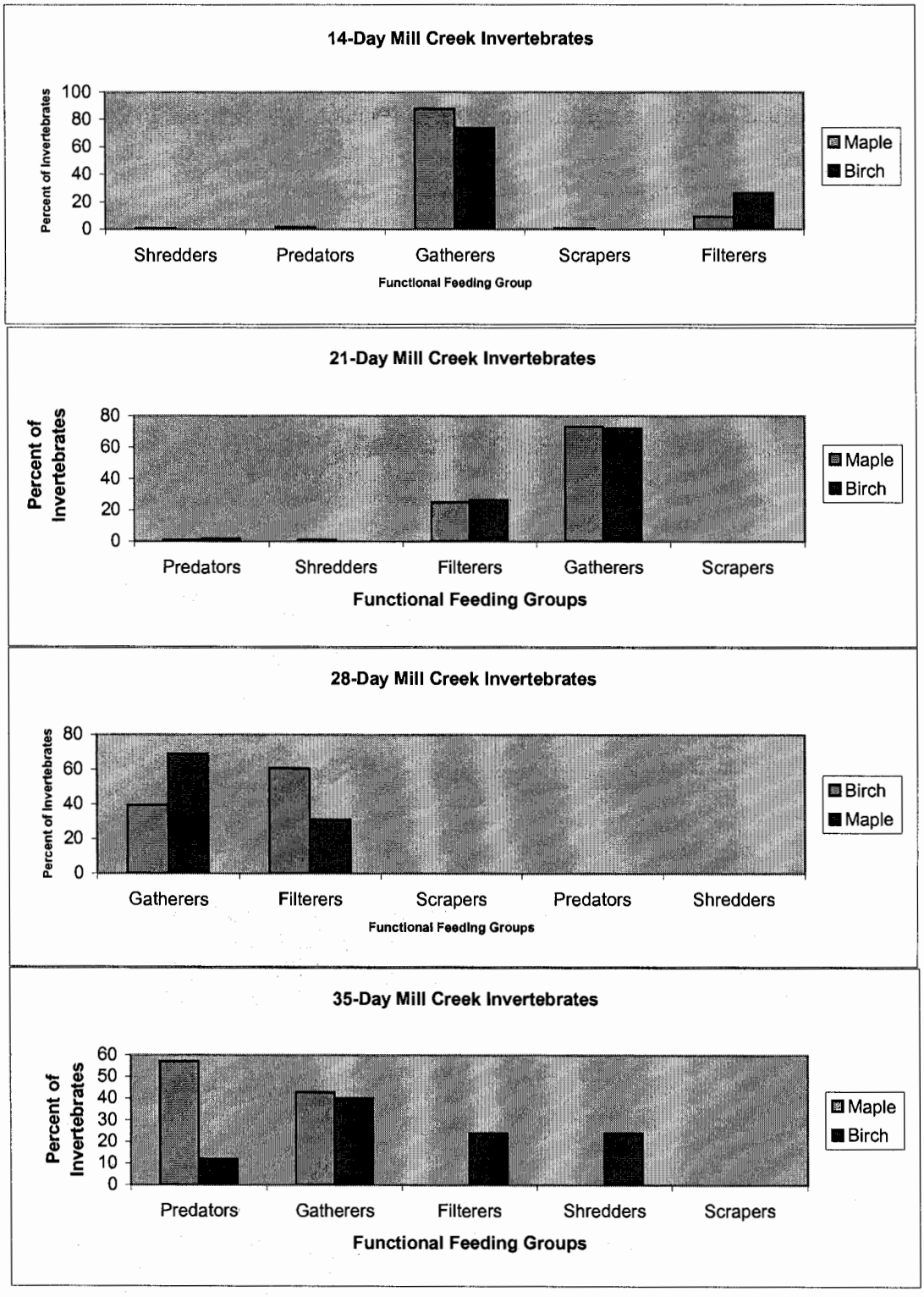
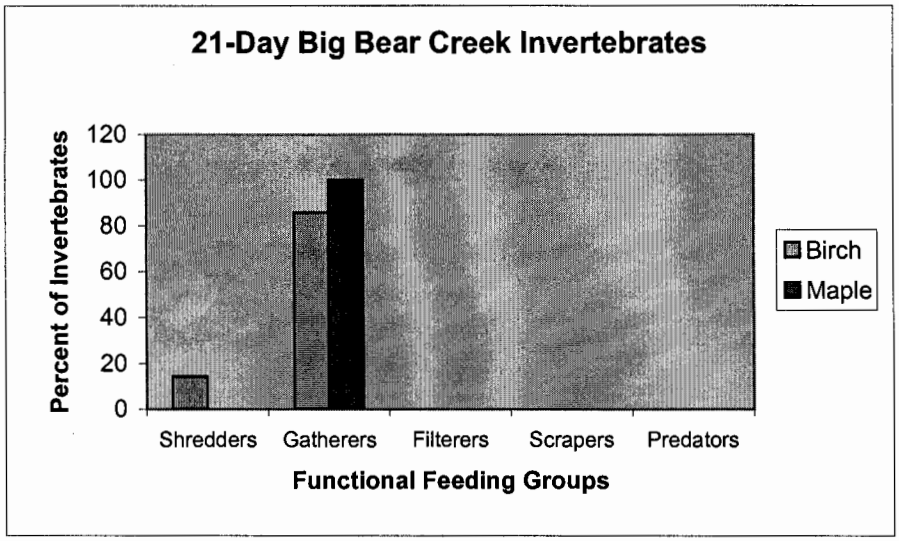


Figure 7 Big Bear Creek Invertebrate Percentages for the Summer Study



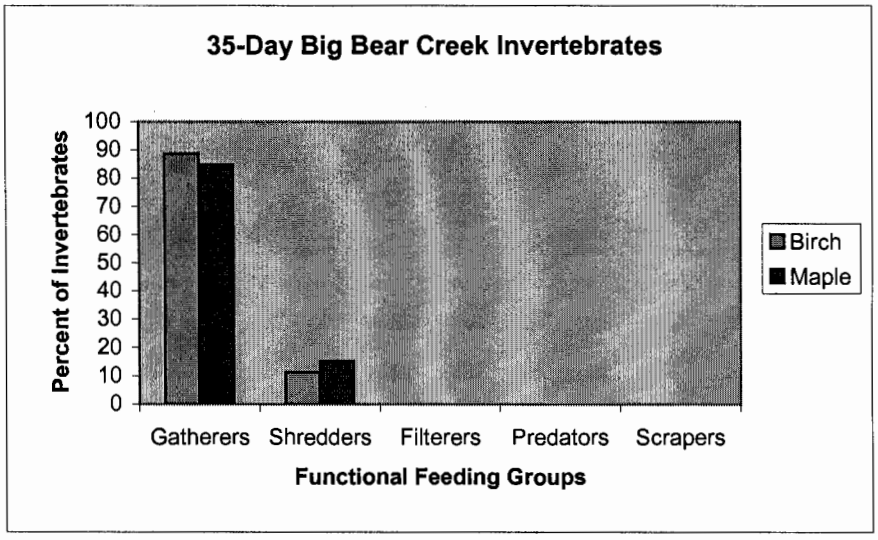
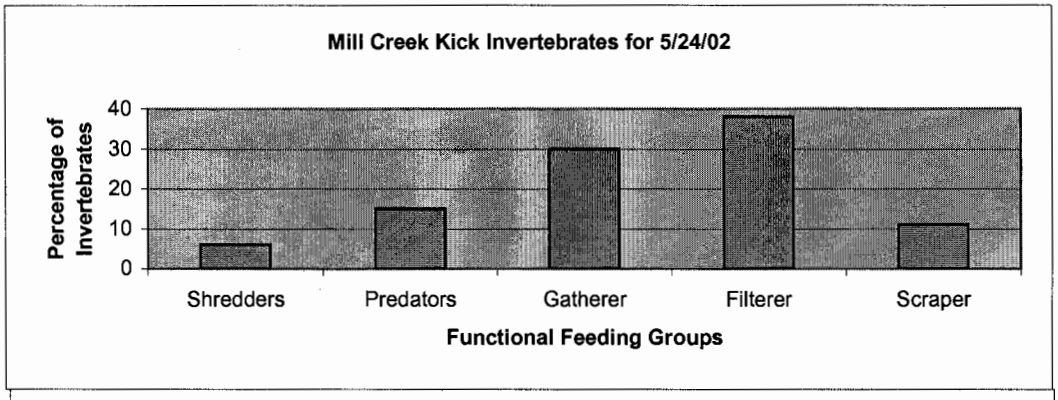
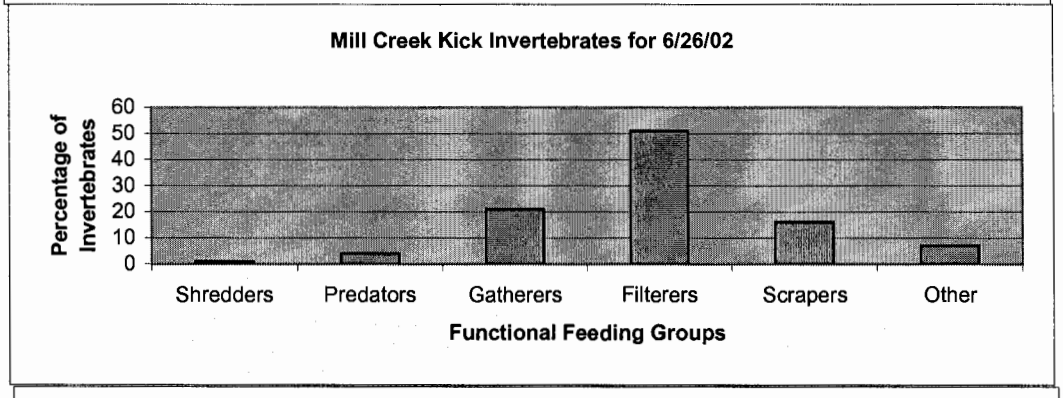
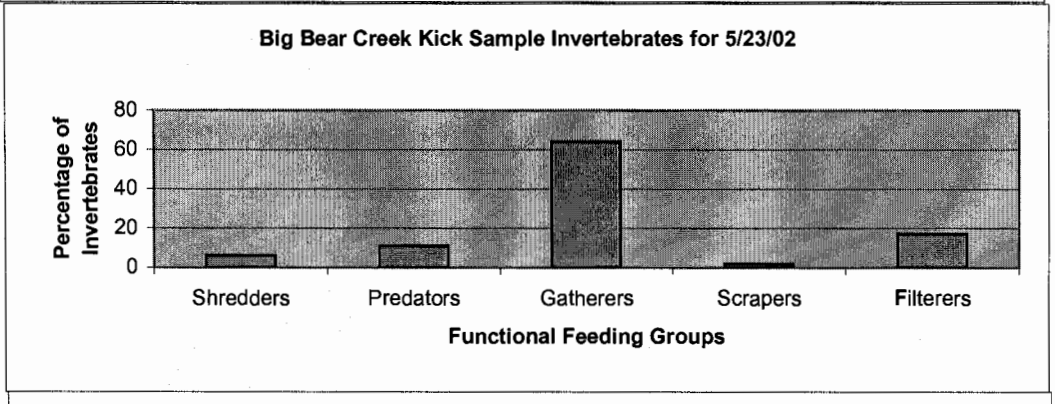


Figure 8 Inverterbrate Population Compositions from Kick Samples







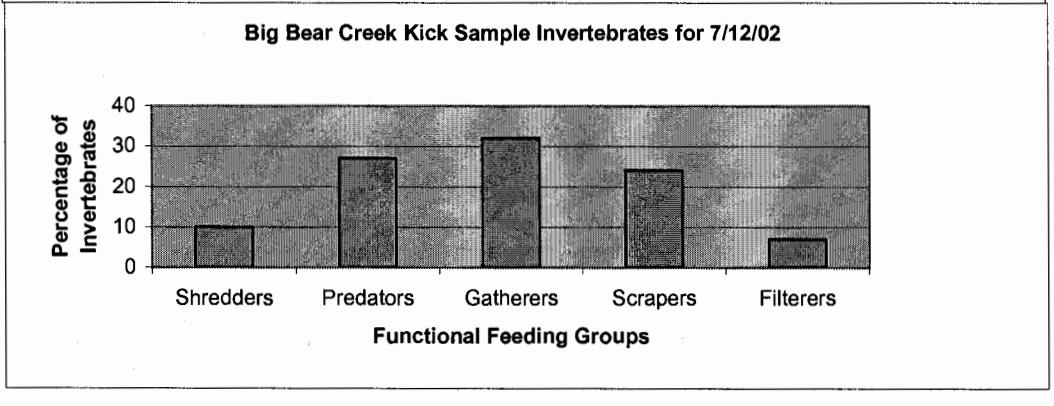
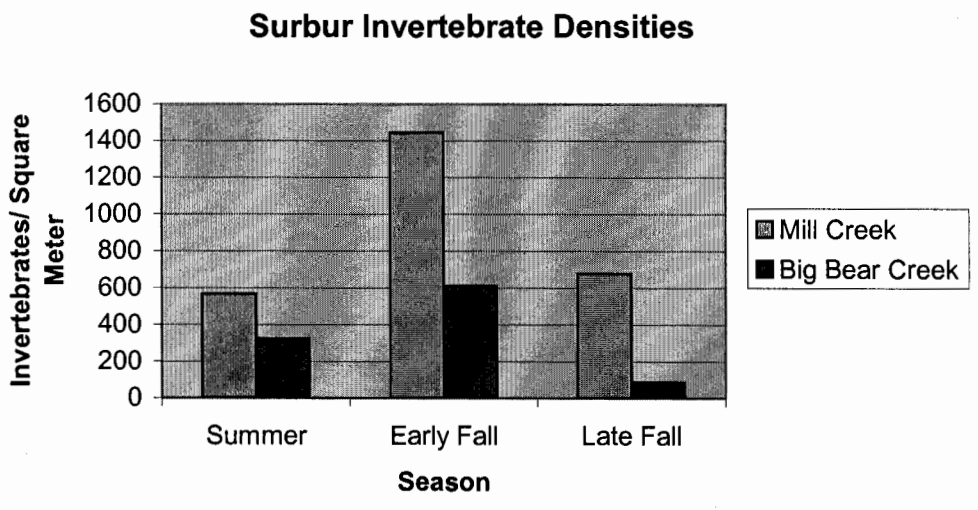


Figure 9	Surbur Data for Each Season in Mill Creek and Big Bear Creek



Appendix I
Sample Calculations

Sample Calculations:

1.) Percent Organic Content:
(Crucible weight + pre-burn weight)-post-burn weight = ash-free dry weight
(ash-free dry weight/pre-burn weight)*100 = percent organic content

2.) Leaf Processing Rates:
-[{ln(post-incubation surface area/pre-incubation surface area}/incubation length]

3.) Ergosterol standard:
0.1068 g of 95.0% ergosterol in 200mL methanol

$$\frac{0.1068\text{g erg} * 0.95 * (u\text{g } 10^6)}{200 \text{ mL MeOH}} = 507.3\ u\text{l erg/mL MeOH}$$

4.) Working Standard:
1.0 mL of ergosterol standard diluted to 100.0mL with MeOH
use equation $C_1V_1 = C_2V_2$

5.) Working Standard Curve:
Inject the amounts of ergosterol standard to establish a working curve

Injection volume (*u*l)	Inj vol. x standard vol	Final concentration
5	(0.005mL)(5.073 *u*g/mL)	0.0254 *u*g
10	(0.010mL)(5.073 *u*g/mL)	0.051 *u*g
20	(0.020mL)(5.073 *u*g/mL)	0.101 *u*g
40	(0.040mL)(5.073 *u*g/mL)	0.203 *u*g
60	(0.060mL)(5.073 *u*g/mL)	0.304 *u*g
80	(0.080mL)(5.073 *u*g/mL)	0.406 *u*g

6.) Equation of a Line
Once the working curve is plotted on the Kaleidagraph, the equation of a line was found by linear fit to be:
*U*g erg. = 1.2393 * 10^{-6} * peak area - .009477

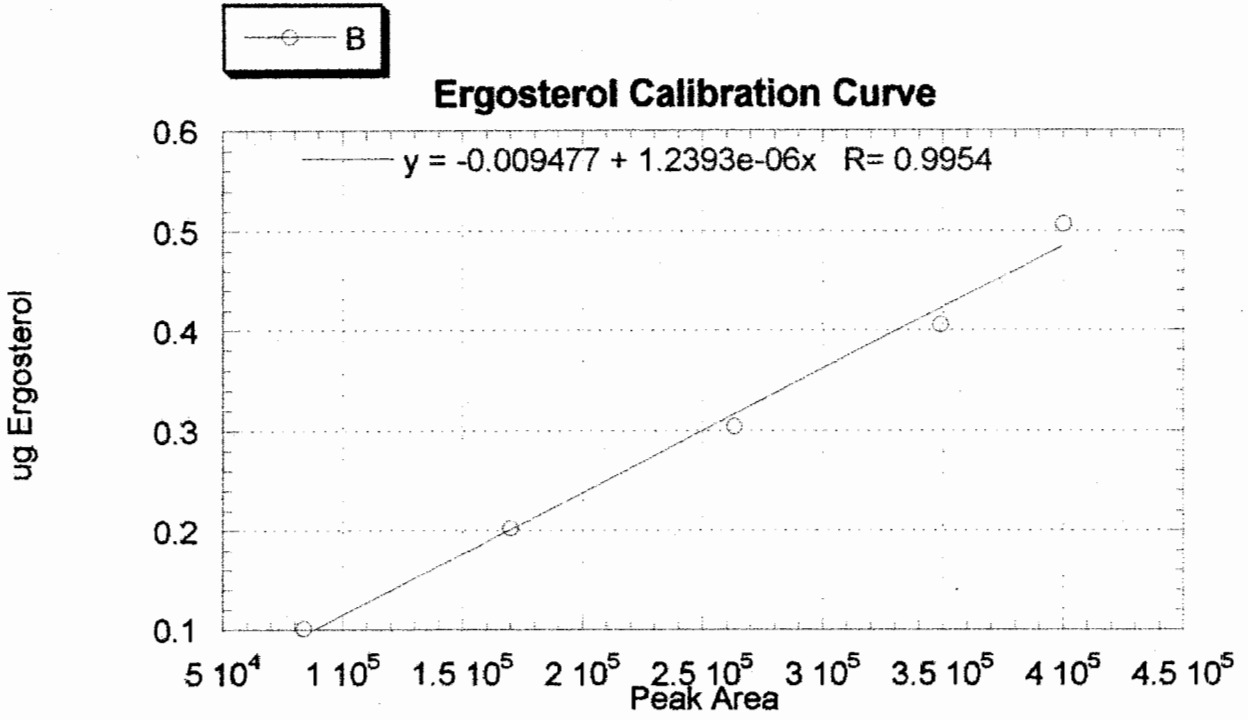
7.) Accounting for dissolution volumes:
$$\frac{u\text{g ergosterol}}{\text{mL Injected}} * 1.00 \text{ mL (dissolution volume)} = \text{ug ergosterol/ refluxed sample}$$

8.) Weight % Ergosterol:
$$\frac{\text{Total } u\text{g ergosterol/10 discs}}{\text{Leaf weight/10discs}}$$

9.) Fungal Biomass Determination:

$$\text{conversion factor} = \frac{182.6\text{g fungal biomass}}{\text{g ergosterol}}$$

Appendix II
Ergosterol Standard Curve

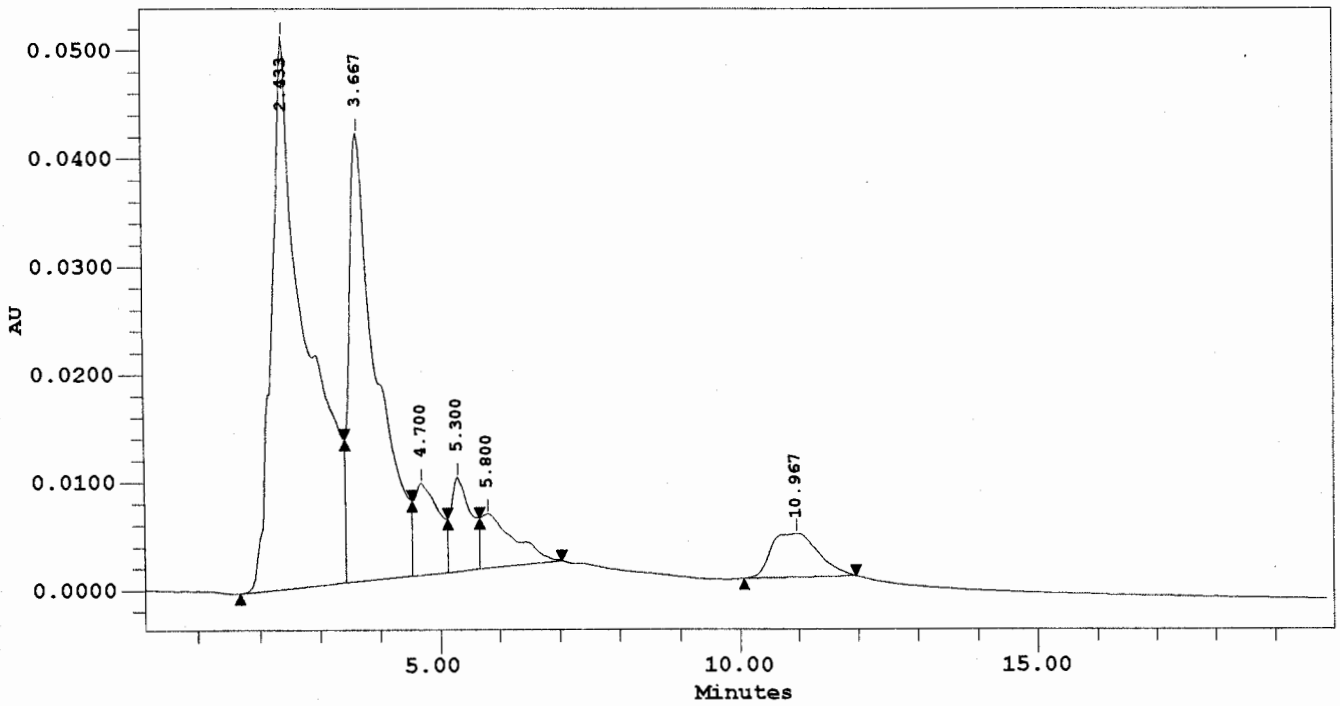

B
Ergosterol Calibration Curve
y = -0.009477 + 1.2393e-06x R= 0.9954
ug Ergosterol
0.6
0.5
0.4
0.3
0.2
0.1
5 10^4
1 10^5
1.5 10^5
2 10^5
2.5 10^5
3 10^5
3.5 10^5
4 10^5
4.5 10^5
Peak Area

Appendix III
Sample HPLC Chromatograms

Report Method: MetCarb_RM Version: 2.15
For Sample: Birch/BBC(4) Vial: 6 Injection: 1 Channel: 991M
Proc Chan: PDA_282.0nm Processed: 07/08/86 09:47:13 AM
Channel Descr: PDA 286.4 nm

Lycoming College, Department of Chemistry

Project Name:	Ergosterol	Sample Name:	Birch/BBC(4)
Vial:	6	SampleOrigin:	
Sample Type:	Unknown	Solvent:	MeOH
Injection:	1	FlowRate:	1.500
Channel:	991M	Level:	
Date Acquired:	07/08/86 09:26:55 AM	Volume:	60.00 ul inj
SampleWeight:	1.00000	Run Time:	20.0 min
Acq Meth Set:	Ergosterol_MS		
Processing Method:	Ergosterol_PM		



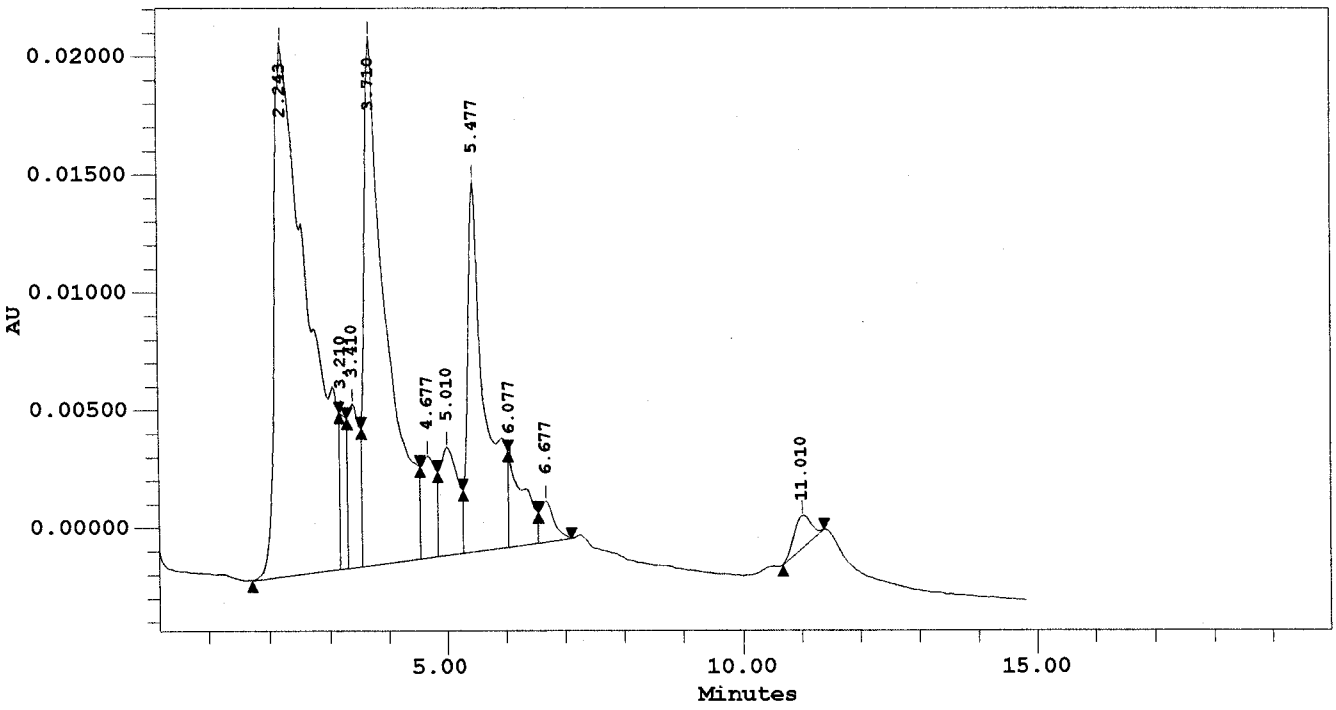
Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Int Type	Start Time (min)	End Time (min)	% Area
1		2.433	2053532	51180	BV	1.667	3.433	48.41
2		3.667	1320458	41536	VV	3.433	4.533	31.13
3		4.700	247492	8488	VV	4.533	5.133	5.83
4		5.300	201327	8691	VV	5.133	5.667	4.75
5		5.800	195130	5025	VB	5.667	7.033	4.60
6		10.967	224106	4041	BB	10.067	11.967	5.28

Millennium Results Report		July 11, 1986			Page: 1 of 1
Report Method:	MetCarb_RM		Version:	2.15	
For Sample:	Maple/BBC (5)	Vial: 2	Injection:	1	Channel: 991M
Proc Chan:	PDA_282.0nm		Processed:	07/11/86 09:41:19 AM	
Channel Descr:	PDA 286.4 nm				

Lycoming College, Department of Chemistry

Project Name:	Ergosterol	Sample Name:	Maple/BBC (5)
Vial:	2	SampleOrigin:	
Sample Type:	Unknown	Solvent:	MeOH
Injection:	1	FlowRate:	1.500
Channel:	991M	Level:	
Date Acquired:	07/11/86 09:26:08 AM	Volume:	60.00 ul inj
SampleWeight:	1.00000	Run Time:	20.0 min
Acq Meth Set:	Ergosterol_MS		
Processing Method:	Ergosterol_PM		



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Int Type	Start Time (min)	End Time (min)	% Area
1		2.243	884326	22665	BV	1.710	3.177	39.50
2		3.210	52088	6562	VV	3.177	3.310	2.33
3		3.410	90941	6956	VV	3.310	3.543	4.06
4		3.710	589468	22455	VV	3.543	4.543	26.33
5		4.677	72771	4322	VV	4.543	4.843	3.25
6		5.010	98121	4596	VV	4.843	5.277	4.38
7		5.477	322381	15787	VV	5.277	6.043	14.40
8		6.077	72841	3672	VV	6.043	6.543	3.25
9		6.677	29033	1747	VB	6.543	7.110	1.30
10		11.010	26756	1363	BB	10.677	11.377	1.20

Millennium Results Report July 8, 1986 Page: 1 of 1

Report Method: MetCarb_RM Version: 2.15

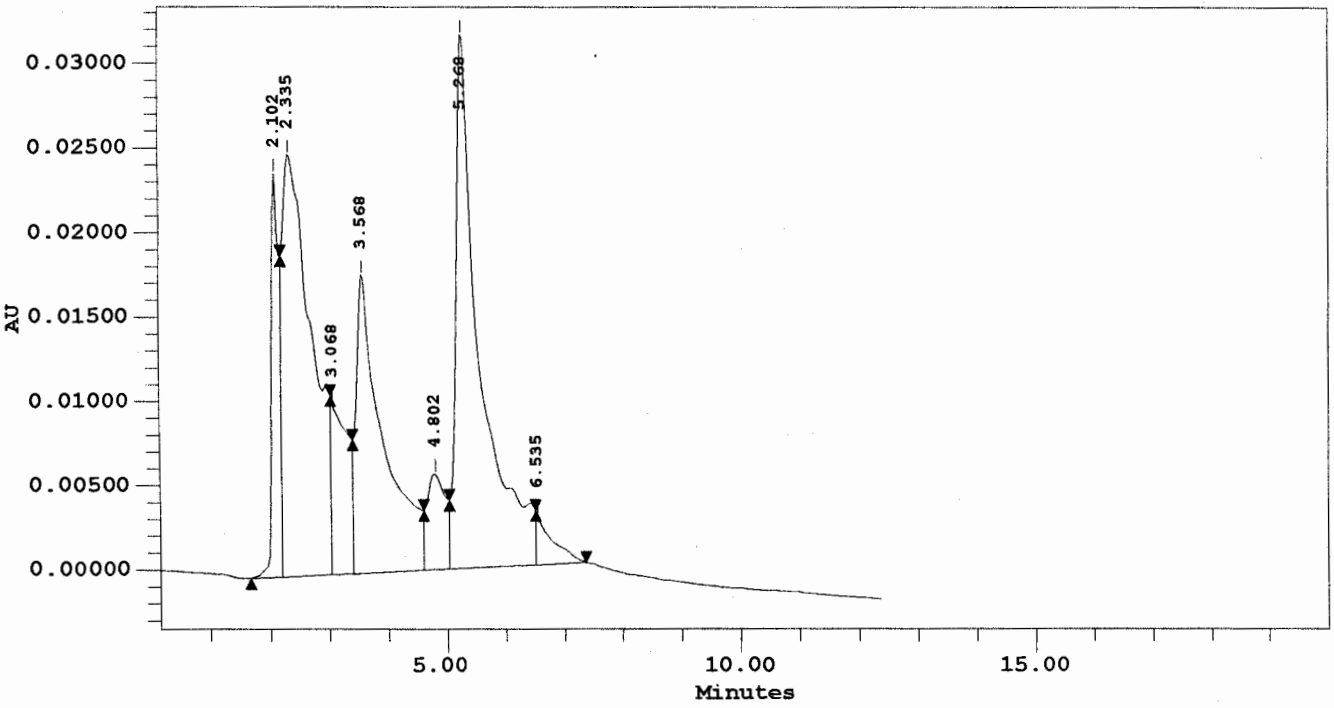
For Sample: Maple/Zimm's(4) Vial: 4 Injection: 1 Channel: 991M

Proc Chan: PDA_282.0nm Processed: 07/08/86 09:01:26 AM

Channel Descr: PDA 286.4 nm

Lycoming College, Department of Chemistry

Project Name:	Ergosterol	Sample Name:	Maple/Zimm's(4)
Vial:	4	SampleOrigin:	
Sample Type:	Unknown	Solvent:	MeOH
Injection:	1	FlowRate:	1.500
Channel:	991M	Level:	
Date Acquired:	07/08/86 08:48:35 AM	Volume:	60.00 ml inj.
SampleWeight:	1.00000	Run Time:	20.0 min
Acq Meth Set:	Ergosterol_MS		
Processing Method:	Ergosterol_PM		



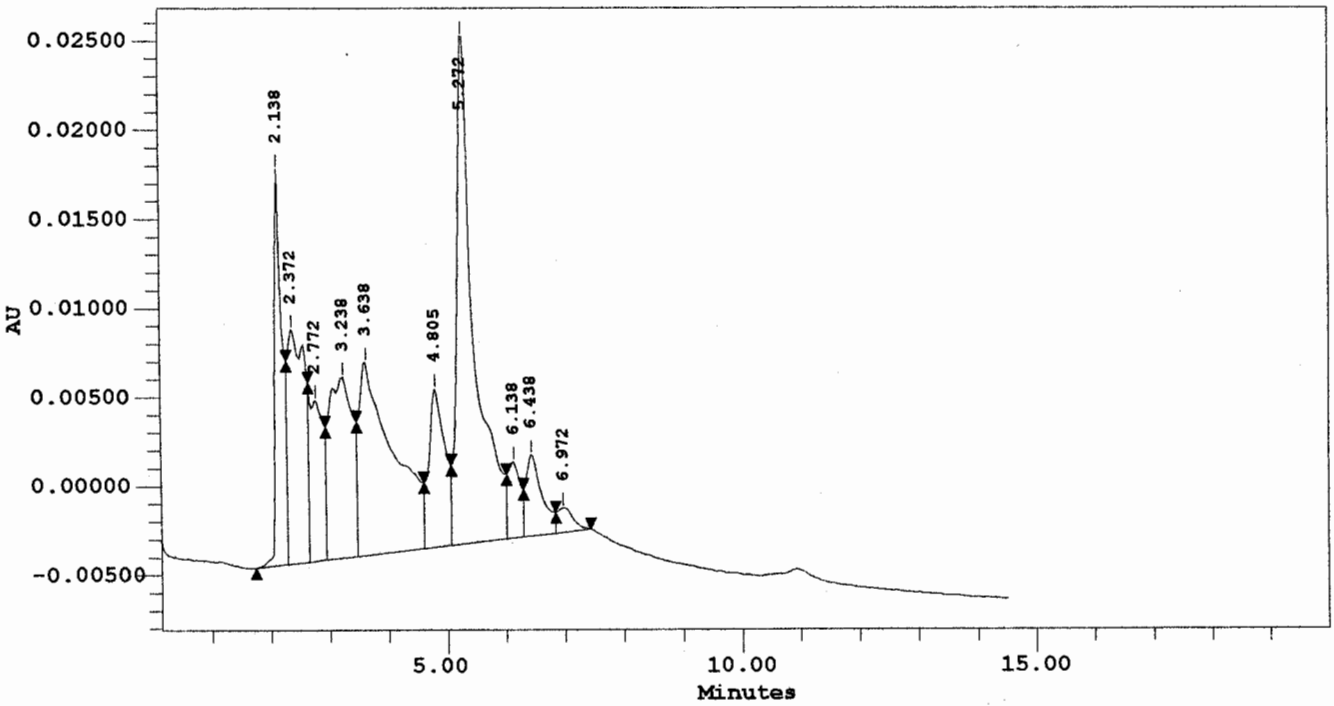
Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Int Type	Start Time (min)	End Time (min)	% Area
1		2.102	212377	23896	BV	1.668	2.202	7.23
2		2.335	868746	24976	VV	2.202	3.035	29.59
3		3.068	197885	10098	VV	3.035	3.402	6.74
4		3.568	574528	17655	VV	3.402	4.602	19.57
5		4.802	123507	5677	VV	4.602	5.035	4.21
6		5.268	900052	31570	VV	5.035	6.502	30.66
7		6.535	58888	2946	VB	6.502	7.368	2.01

Report Method: MetCarb_RM Version: 2.15
For Sample: Maple/Freezer(2 Vial: 3 Injection: 1 Channel: 991M
Proc Chan: PDA_282.0nm Processed: 07/18/86 08:59:55 AM
Channel Descr: PDA 286.4 nm

Lycoming College, Department of Chemistry

Project Name:	Ergosterol	Sample Name:	Maple/Freezer(2)
Vial:	3	SampleOrigin:	
Sample Type:	Unknown	Solvent:	MeOH
Injection:	1	FlowRate:	1.500
Channel:	991M	Level:	
Date Acquired:	07/18/86 08:45:01 AM	Volume:	60.00
SampleWeight:	1.00000	Run Time:	20.0 min
Acq Meth Set:	Ergosterol_MS		
Processing Method:	Ergosterol_PM		



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Int Type	Start Time (min)	End Time (min)	% Area
1		2.138	189530	22272	BV	1.738	2.272	8.31
2		2.372	264798	13157	VV	2.272	2.638	11.61
3		2.772	154169	9056	VV	2.638	2.938	6.76
4		3.238	284866	10157	VV	2.938	3.472	12.49
5		3.638	442816	10830	VV	3.472	4.605	19.41
6		4.805	171787	8906	VV	4.605	5.072	7.53
7		5.272	596380	28522	VV	5.072	6.005	26.14
8		6.138	65730	4282	VV	6.005	6.305	2.88
9		6.438	85876	4540	VV	6.305	6.838	3.76
10		6.972	25655	1365	VB	6.838	7.438	1.12

Report Method: Default Version: 2.15

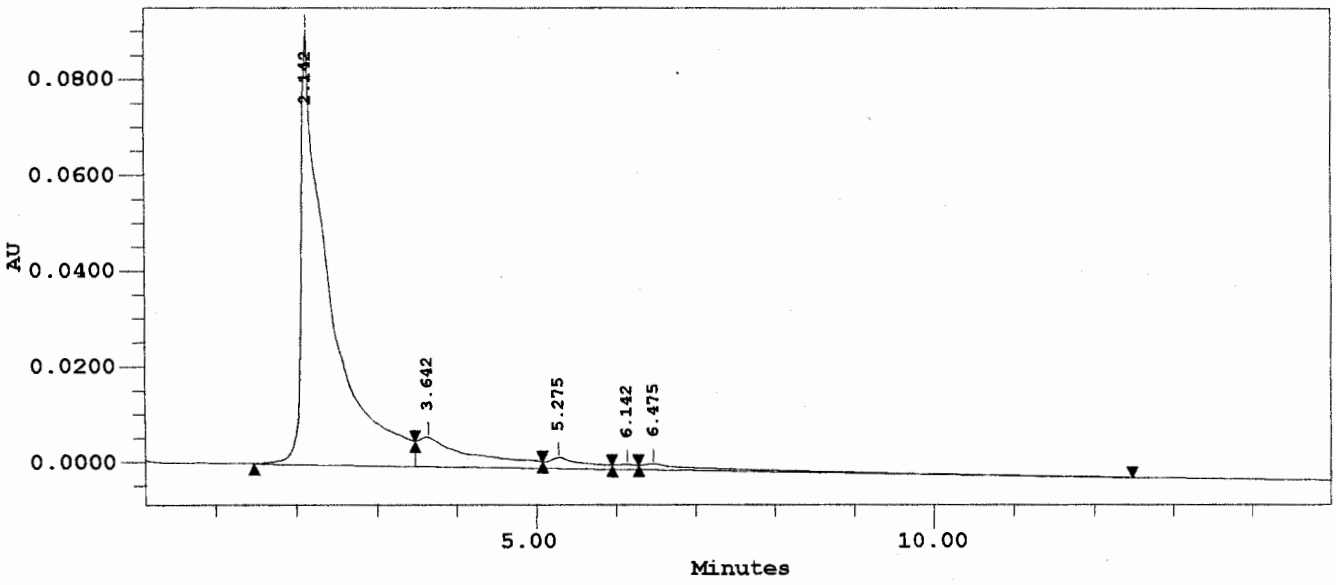
For Sample: BBCBrch-9/16 Vial: 2 Injection: 1 Channel: 991M

Proc Chan: Ergo_282 Processed: 12/10/86 03:11:53 PM

Channel Descr: PDA 282.0 nm

Millennium Sample Information

Project Name:	Ergosterol		
Sample Name:	BBCBrch-9/16		
Vial:	2	Sample Type:	Unknown
Injection:	1	Volume:	30.00
Channel:	991M	Run Time:	15.0 min
Date Acquired:	12/10/86 02:56:28 PM	Date Processed:	12/10/86 03:11:53 PM
SampleWeight:	1.00000	Dilution:	1.00000
Acq Meth Set:	Ergosterol_MS		
Processing Method:	Ergosterol_PM		



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		2.142	2141507	90784		BV
2		3.642	290608	6134		VV
3		5.275	71353	2331		VV
4		6.142	18368	1004		VV
5		6.475	84499	1316		VB

Report Method: Default Version: 2.15

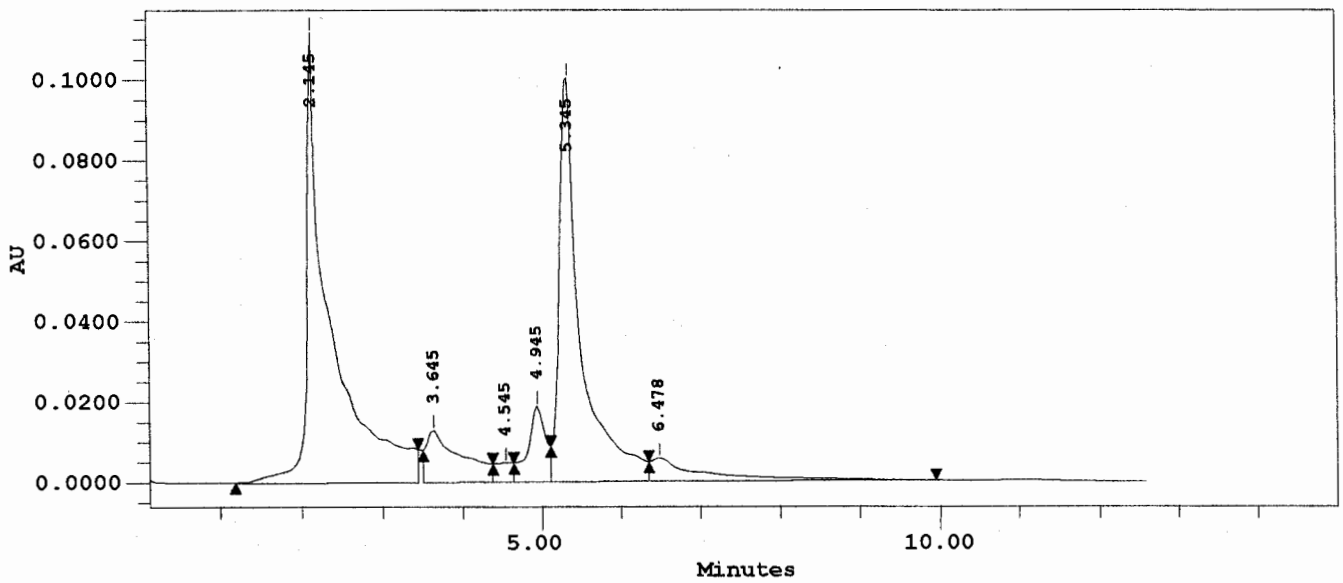
For Sample: ZimmBrch-11/11 Vial: 4 Injection: 3 Channel: 991M

Proc Chan: Ergo_282 Processed: 12/10/86 05:41:33 PM

Channel Descr: PDA 282.0 nm

Millennium Sample Information

Project Name:	Ergosterol		
Sample Name:	ZimmBrch-11/11		
Vial:	4	Sample Type:	Unknown
Injection:	3	Volume:	30.00
Channel:	991M	Run Time:	15.0 min
Date Acquired:	12/10/86 05:28:37 PM	Date Processed:	12/10/86 05:41:33 PM
SampleWeight:	1.00000	Dilution:	1.00000
Acq Meth Set:	Ergosterol_MS		
Processing Method:	Ergosterol_PM		



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		2.145	2249702	111820		VV
2		3.645	389747	12968		VV
3		4.545	74563	4784		VV
4		4.945	292059	18827		VV
5		5.345	1888883	100222		VV
6		6.478	263295	5724		VB