

**EFFECTS OF NITROGEN ON TANNIN CONCENTRATIONS IN  
OAKS AND ON PALATABILITY OF OAK FORESTS**

**Michael A. Fichman**

Haverford College, 370 Lancaster Avenue, Haverford, PA 19041

Semester in Environmental Science, The Ecosystems Center, Marine Biological  
Laboratory, Woods Hole, MA 02543

## ABSTRACT

This investigation clarifies the response dynamics of temperate forest systems to nitrogen loading as mediated by the biochemical defenses of black oak (*Quercus velutina*) and the feeding preference of the isopod *Armadillidium vulgare*. Tannins are secondary metabolites which defend plants from herbivory by protein precipitation and increased acidity.

In a nitrogen fertilization experiment at Falmouth, MA Wastewater Treatment Facility, *Q. velutina* responded to increased nitrogen loading by decreasing leaf tannin concentrations. When offered leaf suspensions of varying tannin concentrations, *A. vulgare* preferentially consumed low tannin foodstuffs.

Plant defensive strategy is dictated by both resource availability and herbivory. A plant must invest in a strategy dominated by either growth or defense based on the availability of nutrients, water and light and the danger of herbivory. Nutrient stressed plants invested more in defense than did fertilized plants. Animals can preferentially consume particular food types in order to minimize the negative effects secondary metabolites may have on digestion or to avoid consuming highly acidic foodstuffs. *A. vulgare* preferred to consume low tannin, low acidity foodstuffs, like those produced by fertilized plants.

Nitrogen fertilization of temperate soils leads to increased food palatability by negatively affecting leaf tannin concentrations.

*Keywords:* Tannin, Herbivory, Secondary Metabolites, Nitrogen Addition, *Quercus velutina*, *Armadillidium vulgare*, Phenolics, Quantitative Defense, Palatability

## INTRODUCTION

The degree to which a plant invests in defense by producing palatability-reducing secondary metabolites like tannins is a function of both nutrient availability and top-down pressures exerted by herbivores. Two traits in woody plants which have proved evolutionarily sustainable are 1) the ability to function in a nutrient limited environment and 2) chemical resistance to browsing (Aber and Melillo, 2001, Bryant, *et al.*, 1983, Bryant and Kuropat, 1980).

In black oak (*Quercus velutina*) and many other species, the principal form of defense from herbivory is tannins. Tannins are a group of polyphenolic compounds which have a high molecular weight (between 500 and 3000 grams per mole) and have the ability to precipitate proteins, which made them valuable to the leather tanning

industry and gave them their name (Figure 1). In fact, the word “tannin” comes from an ancient Celtic word for “oak.” Tannins are secondary metabolites, meaning that they do not play a role in primary biosynthetic or metabolic pathways (Lambers *et al.*, 1998, Hagerman, 1988, Aber and Melillo, 2001).

The manufacture of tannins as a deterrent to herbivory is considered a “quantitative defense.” Quantitative defenses such as thorns or tannins are a safer investment than so-called qualitative defenses such as toxins because of the propensity for herbivore species to coevolve in order to adapt to specific toxic compounds (Lambers *et al.* 1998).

Woody plant responses to bottom-up pressures, such as nitrogen limitation or water stress are well-known. Plants which have a tendency towards quantitative defense will increase leaf phenolic concentrations in times of water stress and nutrient limitation and decrease concentrations in times of nutrient excess (Kouki and Manetas, 2002). Explanations of both the strategy behind quantitative defense and the dynamics of nutrient allocation are less established. Despite disagreement on the nature of biochemical regulatory mechanisms which may or may not regulate production of some tannin types (Kouki and Manetas, 2002) explanations of defense strategy can be concisely framed in economic terms.

Herms and Mattson (1992) suggest that any shortage which limits plant growth more than photosynthesis will spur an increase in the production of secondary metabolites relative to a zero-resource-stress level. The investment of resources in defense can be viewed in terms of an opportunity cost to the plant. Would resources be better spent on growth (Bloom *et al.*, 1985)?

Animals have developed physiological and behavioral strategies to deal with so-called “constitutive defenses,” they can either attempt to detoxify the defensive compounds or avoid certain plant types or particular food types of a certain species (Rhoades, 1985). Animals also can preferentially consume particular food types in order to minimize the negative effects secondary metabolites may have on digestion or to avoid consuming highly acidic foodstuffs (Valiela *et al.*, 1979).

This preference by herbivores can control rates of decay and rates of system nutrient cycling by selectively degrading litter or by exerting grazing or browsing pressures which spur changes in plant chemistry (Valiela *et al.*, 1979, Bryant *et al.* 1983).

My investigation clarifies the reactions of *Q. velutina*, the isopod *Armadillidium vulgare*, and temperate forest plants and systems to nitrogen loading by showing that nitrogen fertilization of temperate soils positively controls food palatability by negatively affecting leaf tannin concentrations. At present, literature covers the effect of fertilization on secondary metabolites (Herms and Mattson, 1992). The effect of secondary metabolites on herbivore food preference is also documented (Valiela *et al.*, 1979, Robbins *et al.*, 1987, Lill and Marquis, 2000, Rietsma *et al.*, 1988, Valiela and Rietsma, 1984, Buchsbaum *et al.*, 1984). However, no studies explicitly link fertilization to herbivore and detritovore food preference in eastern North American temperate forests. My study also adds to the current understanding of the response of temperate forests as it pertains to the global nitrogen cycle by illustrating some of the dynamics of nitrogen sequestration and cycling within a particular ecosystem type.

## **STUDY AREA**

The Falmouth, Wastewater Treatment Facility's Experimental Forest (henceforth WTF) contains several forested plots in an oak climax stage (Figure 2). Some of these plots are subjected to regular spraying of treated wastewater effluent from the treatment facility. At the time of this study's completion, the WTF had yet to bring denitrifying systems online.

Since June 1988, the WTF has disposed of wastewater into the same forested areas each June through December. (Jordan, 1997) Two of these fertilized plots were used as experimental plots (called OF1 and OF2). Other areas of the WTF forest have not been sprayed and are also in climax stage. Two of these plots were used as control plots (called OC1 and OC2).

The wastewater effluent has a nitrogen concentration of between 1048 and 1481  $\mu\text{mol/L}$  with an average organic nitrogen concentration of 193  $\mu\text{mol/L}$  (Jordan, 1997). Both control and fertilized plots had soil profiles which contained O, A, E, B<sub>1</sub> and B<sub>2</sub> profiles above the C layer sandy loam soils. The C layer lay below a depth of 30 cm (Personal observations).

The understory layers of control plots were made up principally of blueberry (*Vaccinium sp.*), sweetfern (*Comptonia peregrina*) and huckleberry (*Gatkyssacua sp.*). Fertilized plots contained much pokeweed (*Phytolacca Americana*), oriental bittersweet (*Celastrus orbiculatus*), poison ivy (*Toxicodendron radicans*) and Virginia creeper (*Parthenocissus quinquefolia*).

Rainfall and groundwater levels for the water year preceding sample collection in October, 2003 were above average (USGS , public communications).

## METHODS

I collected soil samples from the organic layer (10x10 cm transect squares) and from 5 cm depth intervals to 30 cm (cores) in pre-excavated soil pits. Leaf samples were collected by cutting regular amounts of leaves from the canopy layer and from the lower leaf layers in October, 2003, prior to leaf fall. All leaves were collected from mature trees. The leaves were then dried at 50°C. Specimens of the isopod *Armadillidium vulgare* were collected from fertilized plots, kept in terrariums at room temperature and fed dead *Q. velutina* leaf matter.

### **Soil Dissolved Inorganic Nitrogen (DIN) analysis**

Soils were extracted by vacuum filtration using Whatman 42 Ashless 55 mm filters with 50 ml of 1 M KCl per 5.0 g organic soil or 10.0 g mineral soil. Each extractant was then divided into two replicate samples. The extractant was tested for  $\text{NH}_4^+$  using the Solarzano (1969) colorimetric analysis for  $\text{NH}_4^+$  by phenol hypochlorite at 640 nm on a Shimadzu UV-1601 Spectrophotometer with a KCl matrix for blanks and standards. The  $\text{NO}_3^-$  concentration of the extractant was determined by colorimetric analysis with the Lachat<sup>TM</sup> analyzer again using a KCl matrix for blanks and standards.

### **Tannin Analysis and C:N Determination**

Leaf samples were ground in a Wiley Mill and each plot's samples were homogenized. Using extraction procedures adapted from Hagerman (1988), 2.0 g of leaf matter was washed with 20.0 ml of 70% acetone and placed in a 50 ml centrifuge tube. The tube was then submerged in a sonicating bath (American Scientific, 4.6 Quart) for 30 minutes to lyse cells of their constituent parts and extract unwettable matter from the leaf tissue. The samples were then centrifuged for 10 minutes at 2500 x g. The supernatant

was removed and measured and the process of washing, sonicating, centrifuging and decanting repeated three times. The first two extractants were combined as were the second two extractants. The first extractant combination was assumed to contain 75% and the second 20% of total phenolics (Hagerman, 1998). Each extractant combination was then split into two replicate samples.

Tannin content was determined by a Prussian Blue colorimetric assay for total phenolics (Price and Butler, 1977) using a standard curve of Tannic Acid (Sigma Aldrich, ACS reagent, 1701.23 g/ mole). Standards and blanks were dissolved in a 70% acetone matrix. Samples were diluted 1:10 with 70% acetone. After timed additions of 0.10 M ammonium iron (III) sulfate dodecahydrate (Arcos Organics, C.A.S. 7783-83-4, in 0.10 M HCl) and 0.008 M potassium ferricyanide (Sigma Aldrich, ACS reagent), absorbance was read at 720 nm on a the same Shimadzu spectrophotometer used above.

Leaf carbon and nitrogen content were measured using a CHN analyzer (Perkins Elmer Series II 2400 CHNS/O Analyzer).

### **Preferential Feeding Assay**

I used *A. vulgare* to test for influence of tannins upon herbivore food preference using an experimental approach adapted from Valiela and Rietsma (1984) and Valiela *et al.* (1979). Four wells of a six-welled culture plate (Corning Costar<sup>TM</sup> No.: 3506) were filled with ground *Q. velutina* leaves from one of the WTF plots suspended in Bacto-agar (Becton Dickinson<sup>TM</sup>). Three of the leaf suspensions wells were spiked with tannic acid: one with 0.25 mM, one with 0.5 mM and one with 1.0 mM. These concentrations were chosen so as to match the order of magnitude of the tannin concentrations of leaf matter (Figure 3). The pH of each tannin spike was measured.

I used two control plate types to assess the effect of leaf suspension upon feeding choice and the effect of the tannin spikes. One control plate contained spiked and unspiked wells with no suspended leaves to control for the effect of suspending leaves. The other control plate contained two unspiked wells of pure agar and one unspiked suspension for each of the four plots to control for the effect of tannin spiking. Each plate type was replicated once.

In each experiment, four *A. vulgare* specimens were allowed three hours in which to feed upon the material in the plate. *A. vulgare* would shred plates with its legs if left in too long, thus three hours was the maximum time-scale for the assay. The assay was carried out at room temperature in complete darkness. I then examined each plate for marks made by the mouthparts of *A. vulgare* using a dissecting microscope. These marks were counted for each well. A characteristic bite mark can be seen in Figure 4. It is not known if *A. vulgare* can digest the polysaccharides which make up Bacto-agar.

## **RESULTS**

### **Soil DIN Analysis**

Fertilized plot soils displayed a DIN content roughly two to three times that of the control plot (Figure 5). Nitrogen in the form of  $\text{NH}_4^+$  exceeded  $\text{NO}_3^-$  by two orders of magnitude in  $\text{g N} / \text{m}^2$  in all plots except Oak Fert. 2, where this difference was only one order of magnitude (Table 1). This calculation of DIN represents an aggregate measurement of bio-available nitrogen to a depth of 30 cm.

### **Tannin Analysis and C:N Determination**

Leaf matter from fertilized plots was found to contain less tannin than leaf matter from control plots (Figure 6). Leaf matter from fertilized plots also had a lower ratio of C:N than leaf matter from control plots (Figure 7).

Both tannin concentration and C:N ratio were found to correlate negatively with soil DIN concentrations, with  $R^2$  values of 0.32 and 0.89, respectively. (Figures 6 and 7).

### **Preferential Feeding Assay**

Overall, *A. vulgare* preferred to consume plant material and/or leaf suspensions which had a low tannin content and a more neutral pH (Figures 8 and 9).

Control plates which contained no leaf suspensions showed that the subjects preferentially bite plates with low or no tannin concentration (Figure 10).

Control plates which had leaf suspensions showed dissimilar trends. In the non-spiked plates with leaf suspension, increased tannin concentration correlated weakly with increased consumption of the medium. In these plates, the C:N ratio of the suspended leaves had no effect on preference.

## **DISCUSSION**

Negative correlations between soil DIN and leaf tannin concentrations and between tannin concentrations and food palatability suggest that nitrogen availability is a controlling factor in food palatability.

The soils in fertilized plots clearly show retention of at least some of the nitrogen added through spray irrigation. There was a negative correlation between concentrations of soil DIN concentration and leaf tannin so as to suggest that control plots were operating under a greater degree of nitrogen limitation than fertilized plots. The decrease

in secondary metabolite concentrations in fertilized leaves implies that *Q. velutina* has reached a maximum rate of net assimilation of nutrients such that secondary metabolism decreases in accordance with the Herms and Mattson (1992) model (Figure 11).

Trees in fertilized plots seem to be investing in a strategy of growth with less regard to defense than trees in control plots. Fertilized oak plots have higher above-ground Net Primary Production (NPP) than control plots by almost 100% in long-term studies done at the WTF site (2003 Semester in Environmental Science students, unpublished data, Figure 12).

The more dynamic growth (and subsequent high volume of turnover) in fertilized plots also lessens the likelihood of a particular leaf being exposed to herbivory. In a nutrient limited plot, deterring rapid turnover is an advantageous strategy, as leaves tend to lose half of their nitrogen during leaf fall (Bryant *et al.*, 1983). So in an effort to protect nitrogen resources, leaves in control plots have allocated nutrients and biomass in a higher C:N ratio and more tannins per gram of leaf matter. One would expect a similar trend to prevail across all treatments immature trees, where below ground storage is not as great, mortality is high, photosynthesis is limited and the threat of mammalian herbivory is magnified (Bryant *et al.*, 1983).

Despite low  $R^2$  values for correlations between percentage of bite marks and tannin concentration, the prevailing trend is that tannins have an influence in deterring herbivory both through their protein-precipitating ability (Figure 8) and through acid inhibition (Figure 9). These results are consistent with both terrestrial and aquatic studies which suggest that increased concentrations of secondary metabolites negatively affect food preference in most species (Robbins *et al.*, 1987, Lill and Marquis, 2000, Rietsma *et*

*al.*, 1988, Valiela *et al.*, 1979, Valiela and Rietsma, 1984, Buchsbaum *et al.*, 1984).

Although the evidence is not statistically significant, the tannin concentrations in leaf suspensions appeared to have a greater effect on food preference than the nitrogen content of the leaf matter. Variability in animal behavior on short time-scales made the feeding assay data less robust than the other data sets, but the unavailability of *A. vulgare* specimens late in the fall season prevented the collection of a more expansive data set.

A reason for the success of *Q. velutina* is its ability to deter browsing while thriving in a nutrient-limited environment and to balance the costs of defensive adaptation with growth well enough to dominate a particular niche (Figure 13) (Rhoades, 1985). Animals have the ability to adapt and modify behavior through individual experience or social models in order to determine which foods to eat. Browsing favors an increase in numbers of unpalatable species. However, if the biomass of a generally unpalatable plant is not high enough to saturate the toxin tolerances of a browser, the species may be eliminated by browsing (Bryant *et. al*, 1991). Although this study did not specifically test the effects of top-down controls, it is worth noting the influence that such factors may have had upon the results of this investigation.

Aber *et al.* (1998) show that in New England hardwood forests nitrogen retention efficiency decreases over time with high nitrogen input. Perhaps the veritable explosion of high quality biomass in a fertilized system could lead to defoliation and possible tree death by overbrowsing or a shift to a strategy of more quantitative defense (Figure 14). Most likely, the WTF forest leaches some of the nitrogen deposited by the wastewater irrigation into groundwater and then into the fragile estuarine system of West Falmouth Harbor, generating a risk of eutrophication (Schlesinger, 1997).

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John E. Hobbie, whose advisorship and sage advice were greatly appreciated and enhanced this project significantly, Gus Shaver for his expertise on herbivory, Ken Foreman for his knowledge of the literature, Ian Washbourne for his immense aid in conducting chemical analyses, Chris Neill for his help in John's absence, Ivan Valiela for his expansive exploration of feeding inhibition and his advice, Pat Micks, Leslie Graham and Jen Bowen for lab help, Jordan Kramer for moral support, Marselle Alexander for attempting to consume an acorn despite attempts to deter her and to the Marine Biological Laboratory for putting all these people at my fingertips.

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## FIGURES AND TABLES

Table 1. Soil DIN, NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> by plot

Plot	total NH4 (g/m <sup>2</sup> )	total NO3 (g/m <sup>2</sup> )	Total inorganic N (g/m <sup>2</sup> )
OC1 Total	1.452	0.028	1.481
OC2 Total	1.034	0.049	1.083
OF1 Total	4.198	0.052	4.250
OF2 Total	3.097	0.557	3.654
OC Avg	1.243	0.039	1.282
OF Avg	3.648	0.305	3.952

Figure 1. Some examples of Tannins.

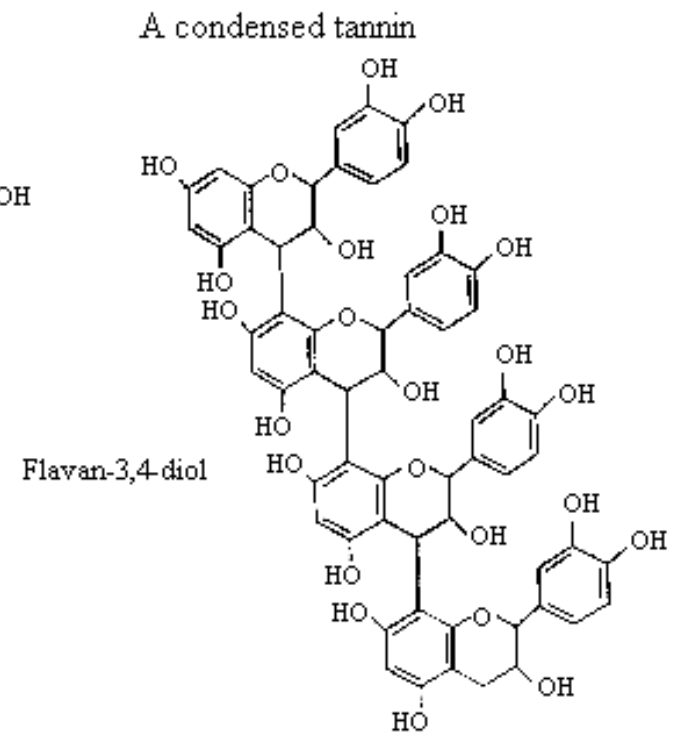
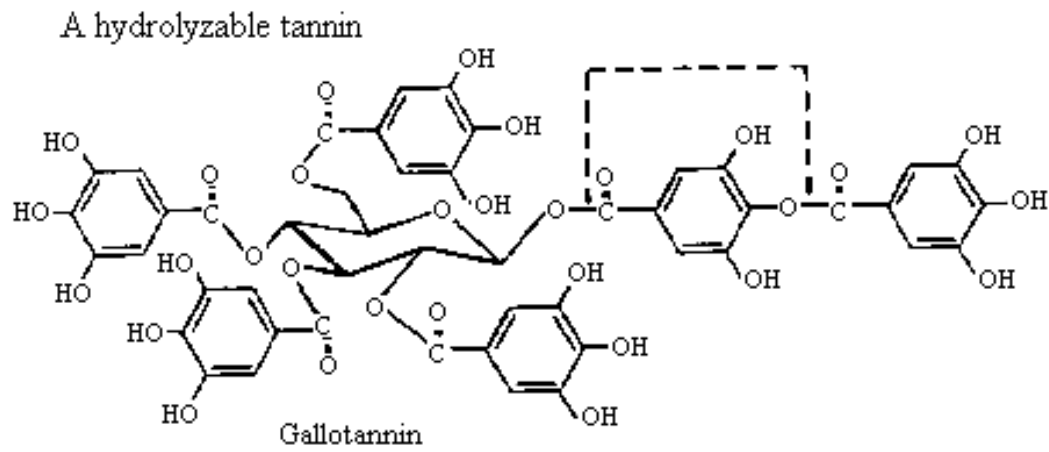


Figure 2. Falmouth, Massachusetts WTF, from Jordan, Nadelhoffer and Fry (1997)

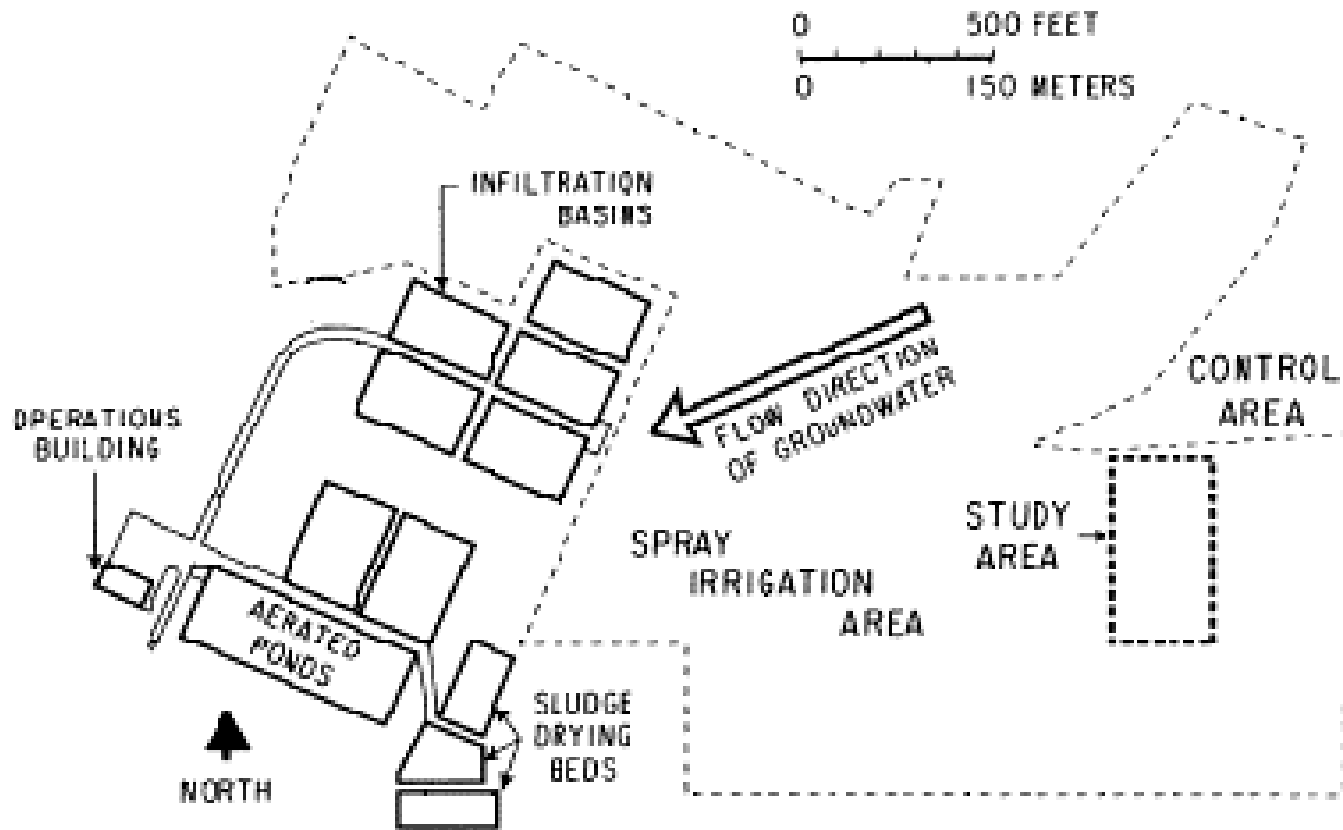
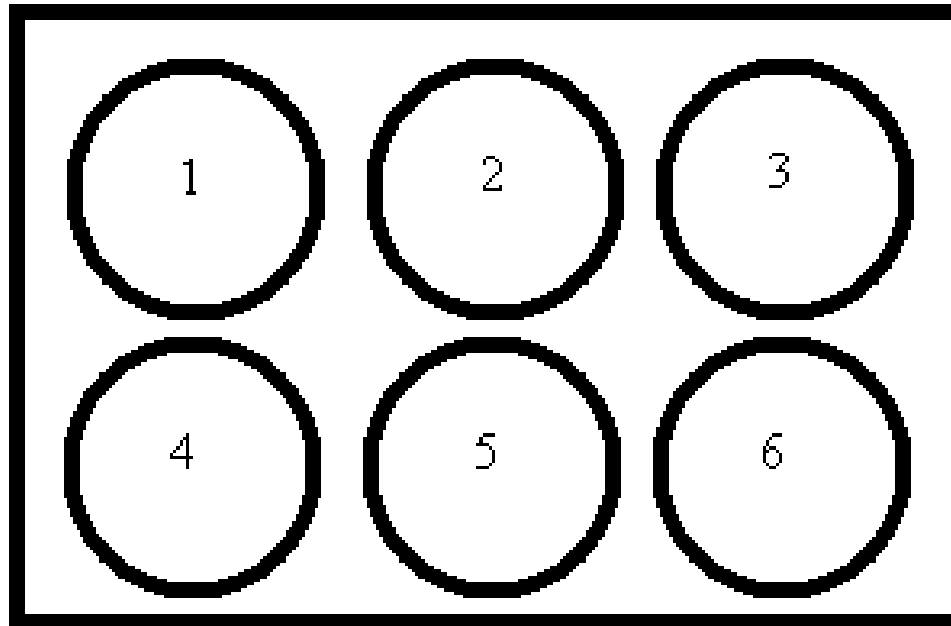


Figure 3. Setup of typical experimental bite-mark assay plate.



1. Leaf suspension only 2. Pure agar 3. Leaf suspension + 0.25 mM tannic acid 4. Leaf suspension + 0.5 mM tannic acid 5. Pure agar 6. Leaf suspension + 1 mM tannic acid

Figure 4. A characteristic *A. vulgare* bite-mark in agar. Note the suspended leaf matter. Photo by Michael A. Fichman, 2003

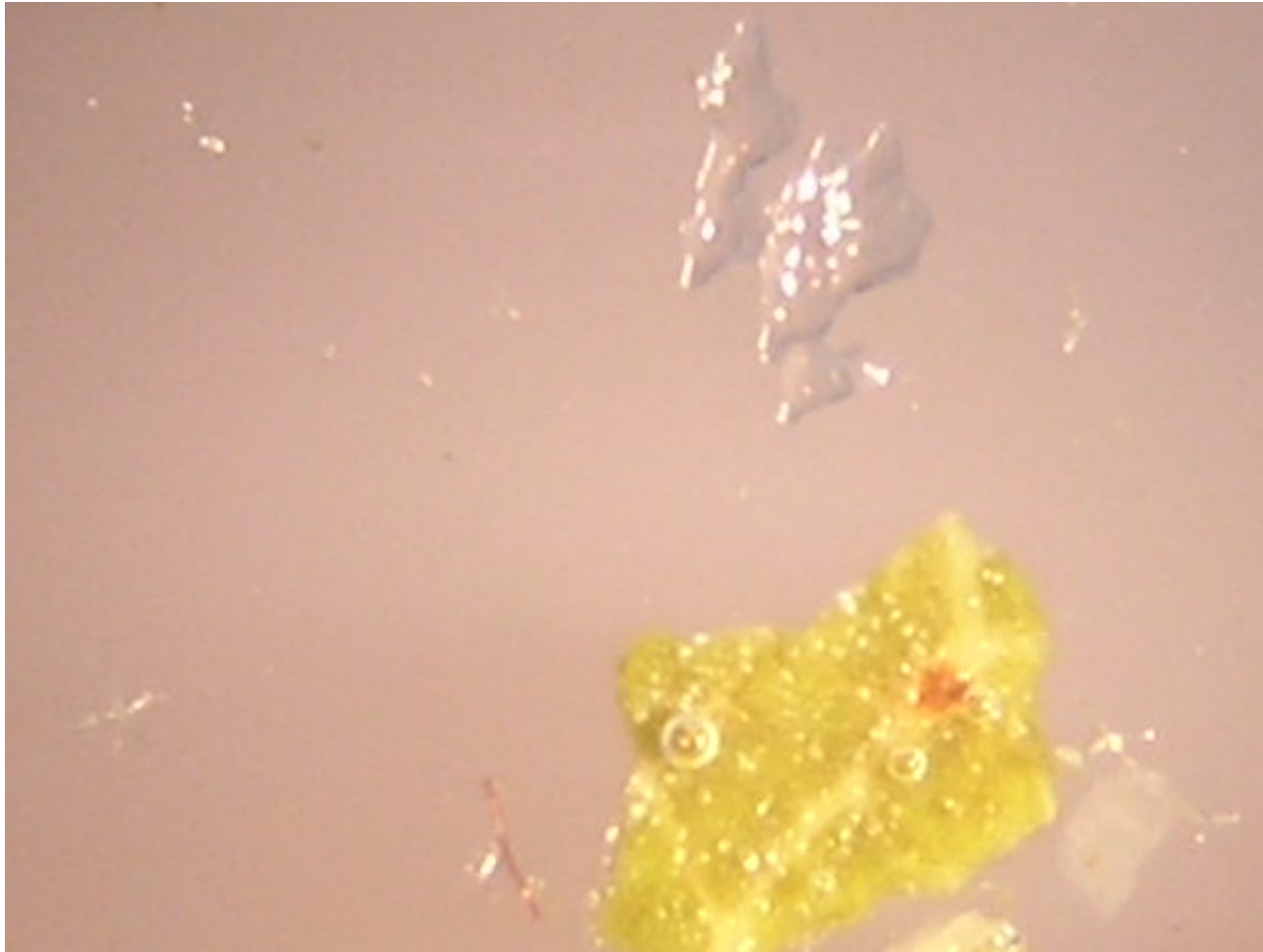


Figure 5. Soil DIN, as measured from the top 30 cm of the soil profile.

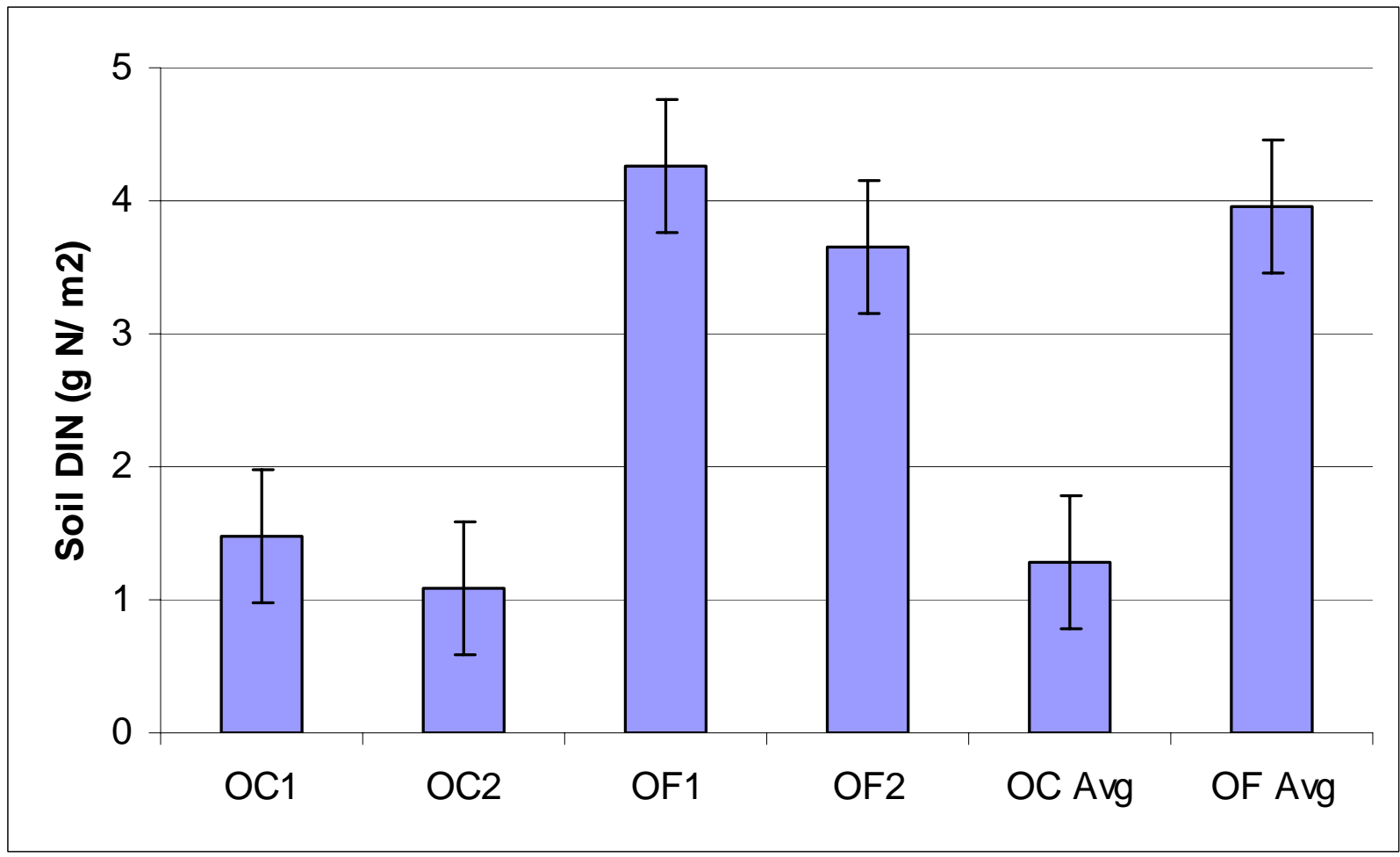


Figure 6. Leaf tannin concentration versus soil DIN

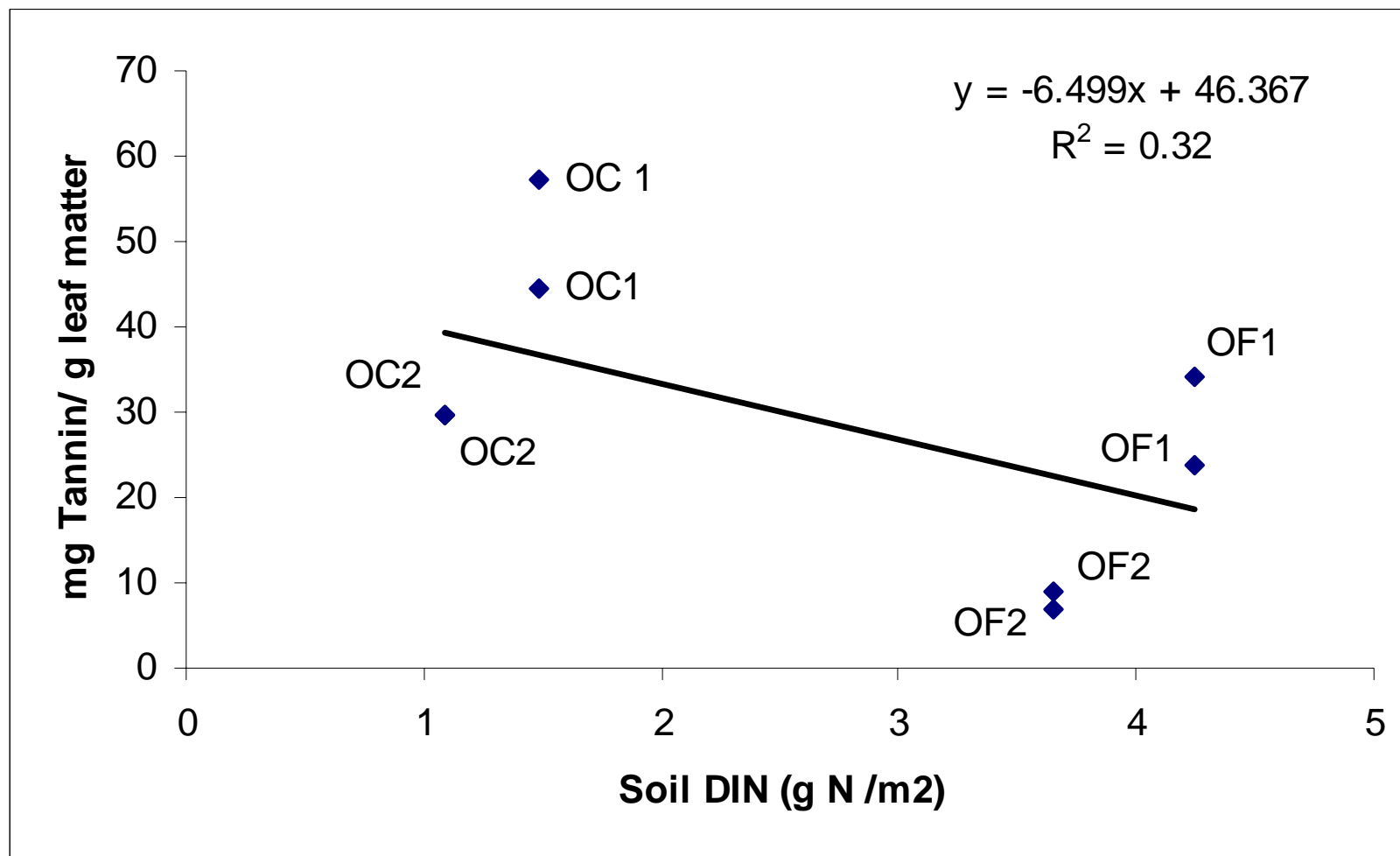


Figure 7. Leaf C:N ratios versus soil DIN

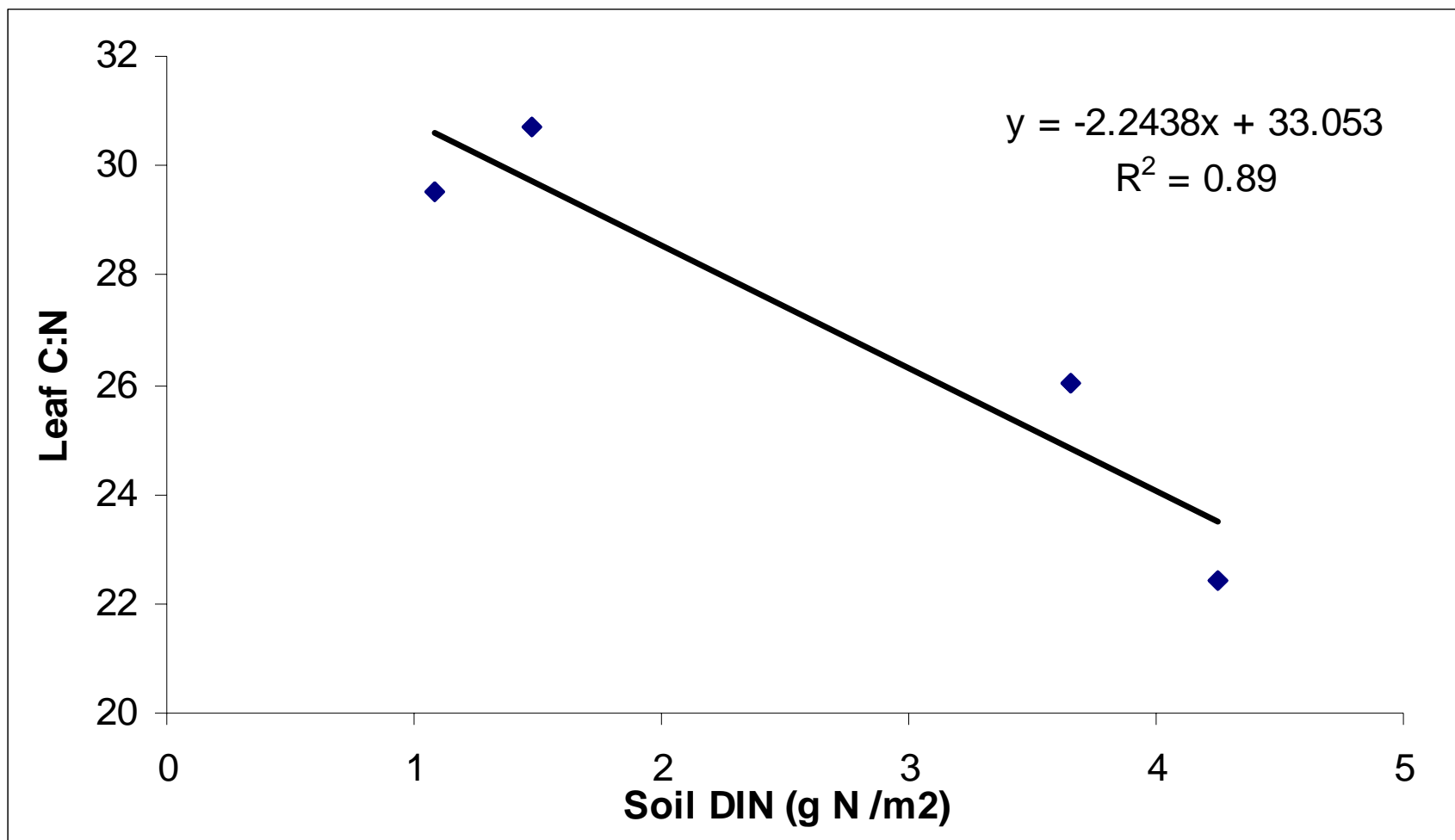


Figure 8. Bite-mark percentages versus tannin concentrations in wells containing suspended leaves

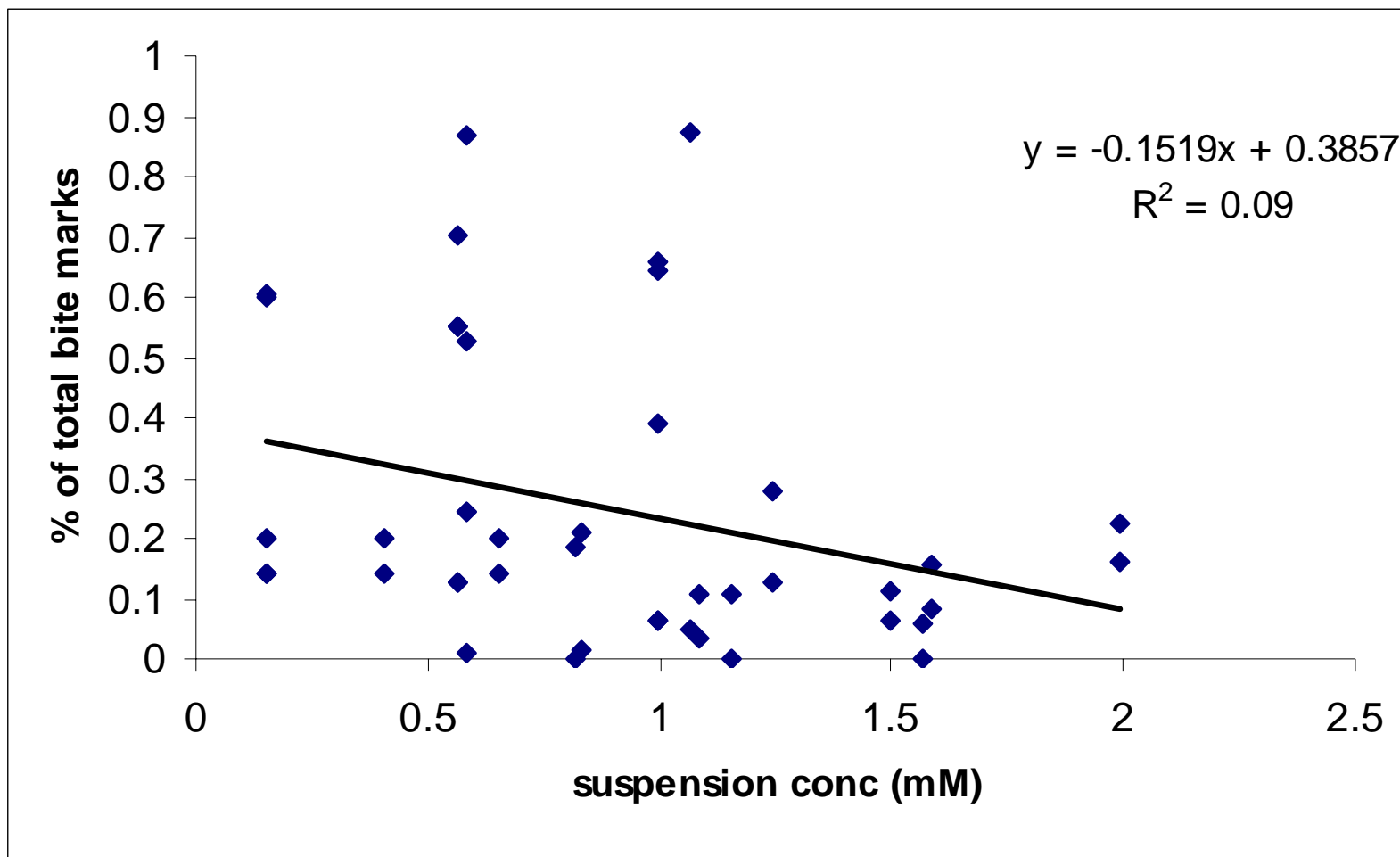


Figure 9. Bite mark percentages versus suspension pH in wells containing suspended leaves

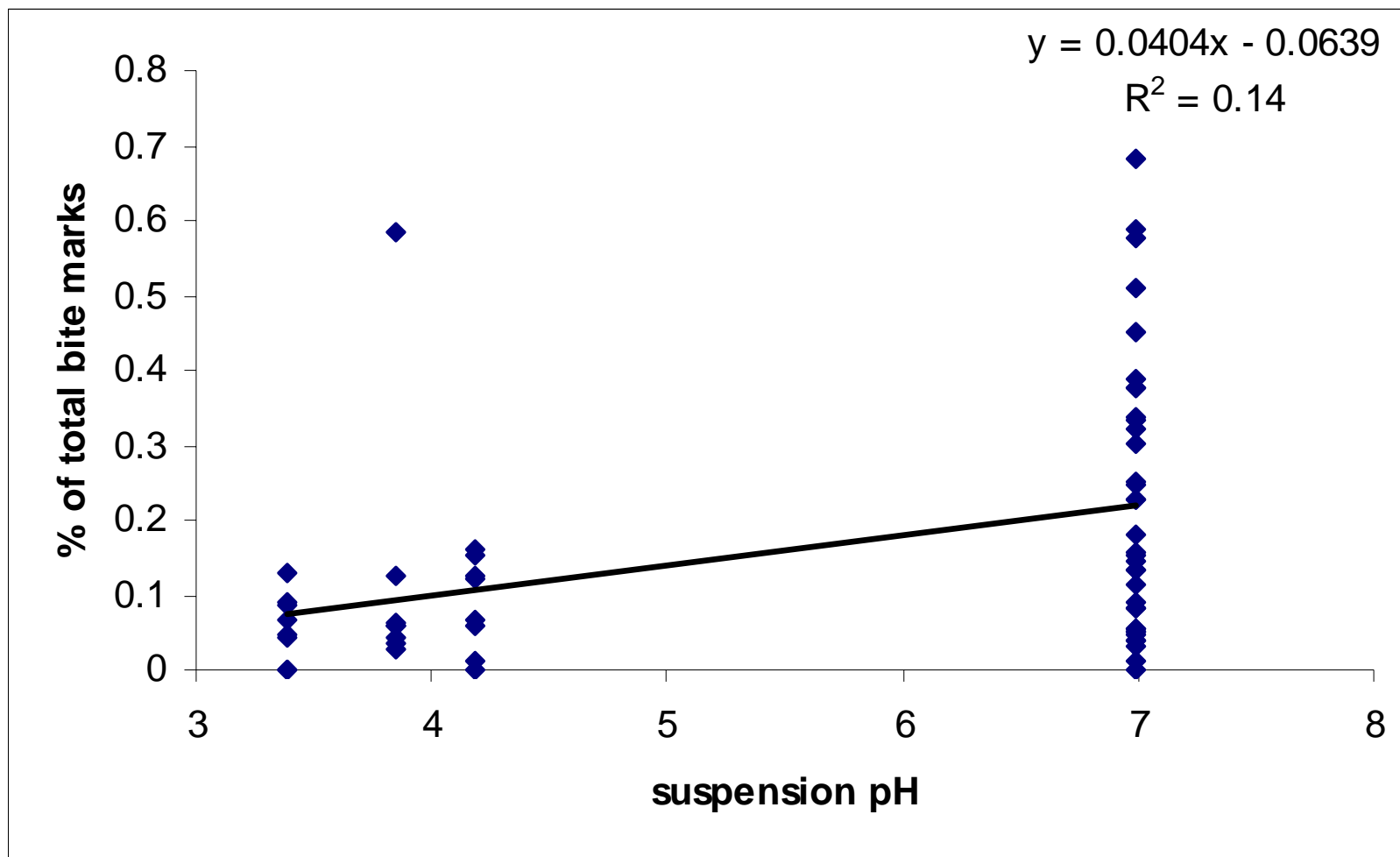


Figure 10. Bite mark percentages in versus tannin concentration in control plates containing spiked wells without suspended leaves

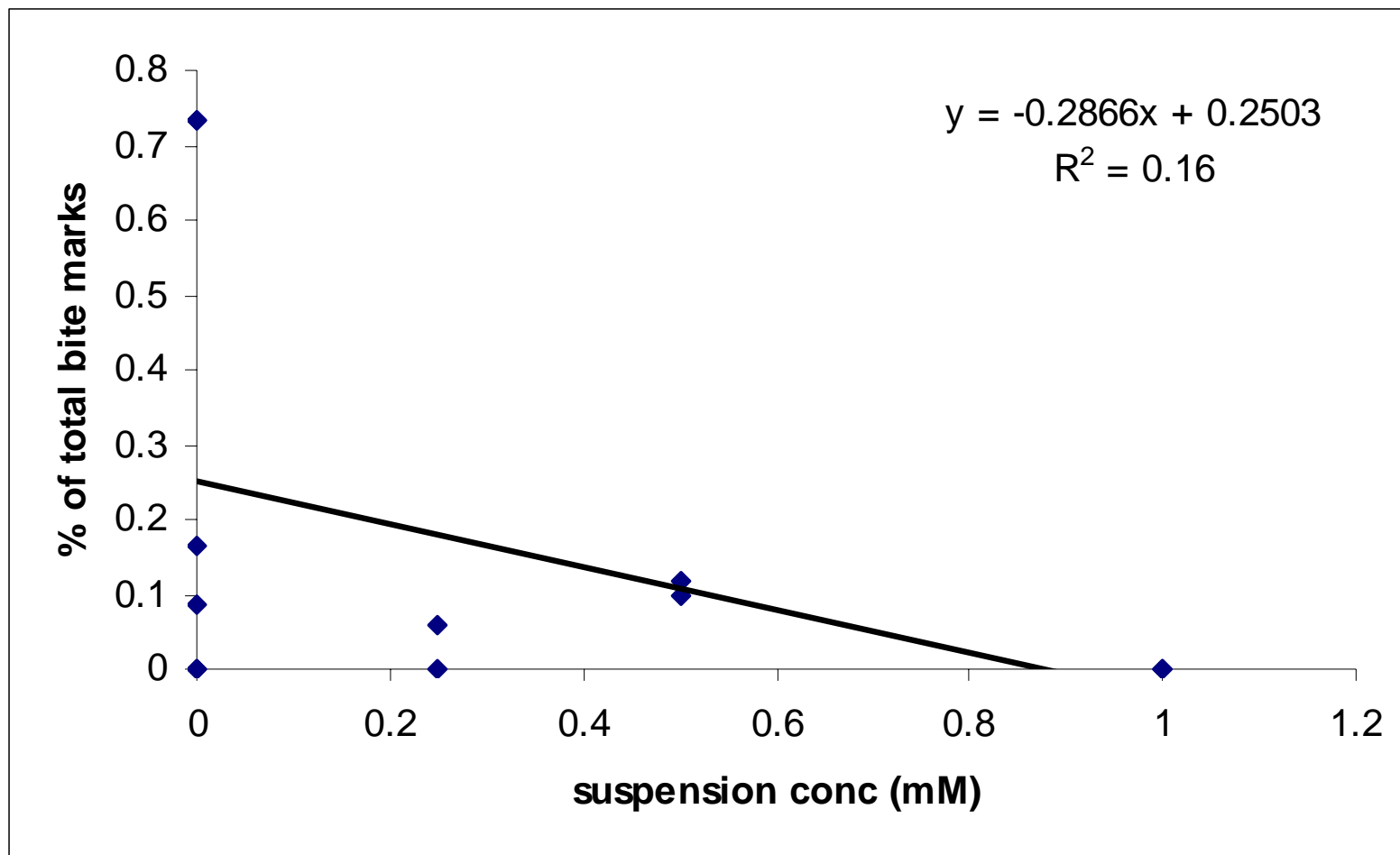


Figure 11. A hypothetical model showing the reactions of net nutrient assimilation rate, relative growth rate and secondary metabolism (i.e. production of tannins) in response to changing nutrient availability. Fertilized plots must lie to the right of the maximum rate of secondary metabolism because their tannin concentration has decreased due to high nutrient availability. The control plots may fall anywhere to the left of the fertilized plots. Adapted from Herms and Mattson (1992).

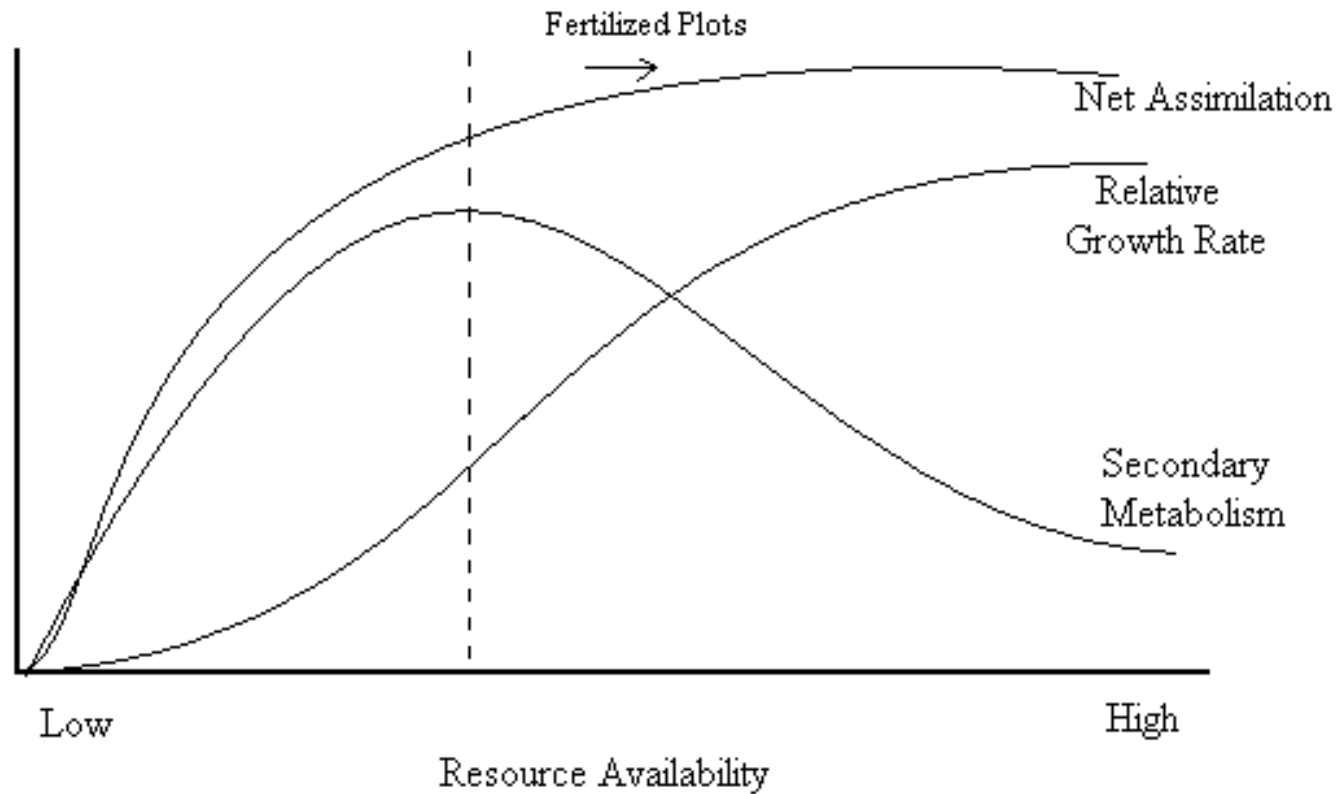


Figure 12. Above Ground Net Primary Production estimate in Falmouth WTF Experimental Forest. Data collected by Semester in Environmental Science students from the Marine Biological Laboratory, 2003.

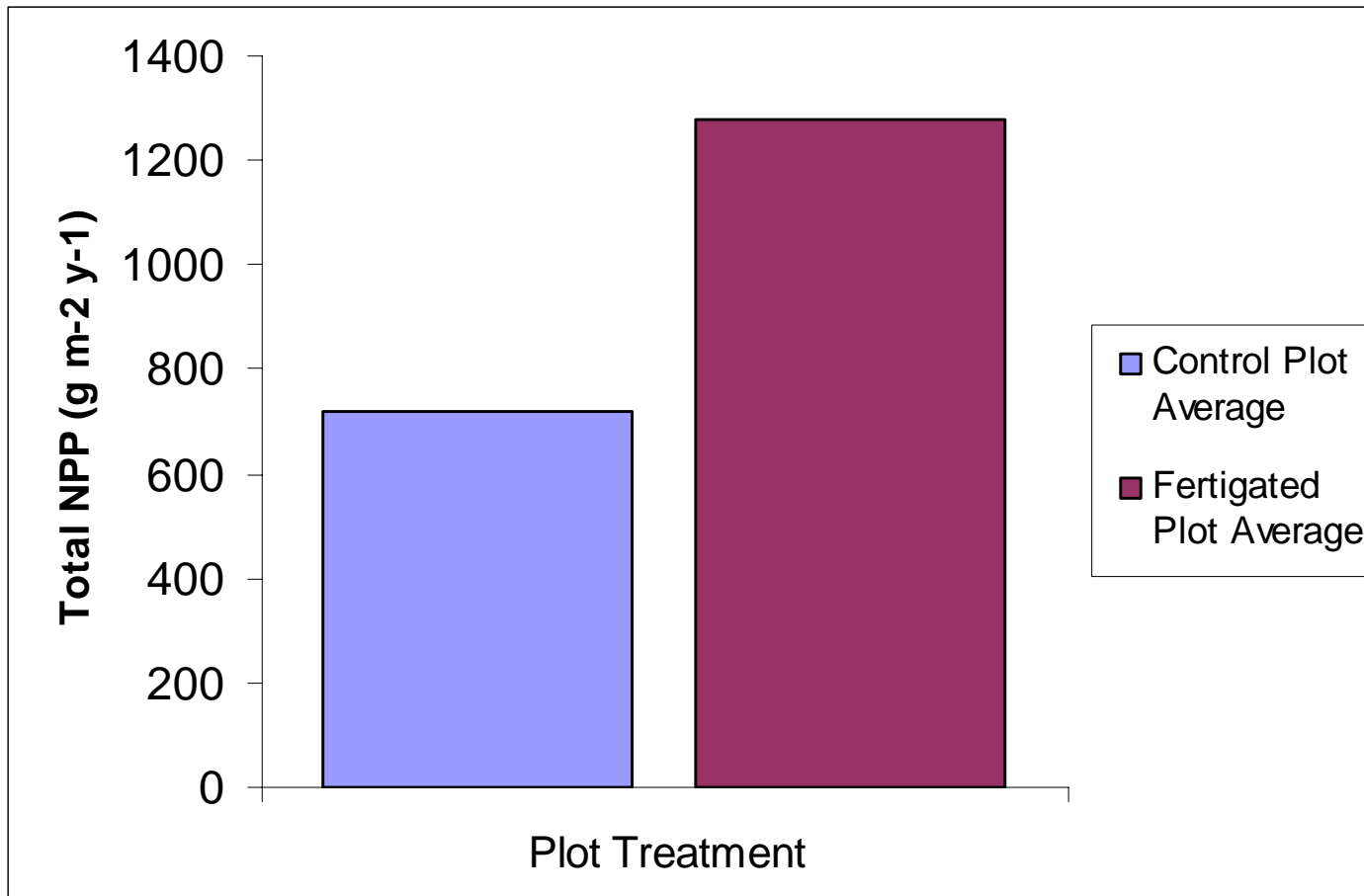


Figure 13. “Interactions between higher plants and animals involving secondary plant compounds. Attack by herbivores leads to the evolution of protection with defense compounds (defensive adaptations in producers). At the same time, there is a selection against production of defense compounds, since it incurs a cost. Defensive adaptations in plants lead to the evolution of offensive adaptations in consumers. These offensive adaptations are selected against because they incur some costs (Rhoades, 1985).” Illustration adapted from Rhoades (1985) copyright by the University of Chicago, caption text by Lambers, Chapin and Pons, 1998.

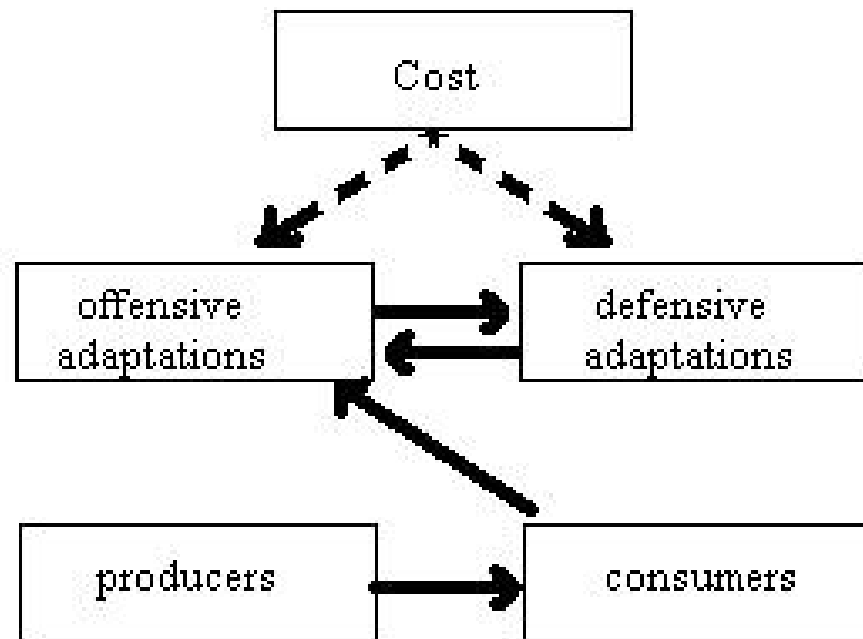


Figure 14. A theoretical progression of plant-herbivore interactions in response to fertilization: In Stage 4 trees which have not been defoliated by intense herbivory will respond to increased grazing/browsing by elevating C:N levels and tannin concentrations and preferentially increasing below ground biomass (Bryant *et al.*, 1983). In both of these scenarios, the herbaceous elements of a temperate forest system would not be able to optimally remove nitrogen from soils such that under continual fertilization, some of the added nitrogen would leach to groundwater.

