

**BREAKING THE HABIT: THE ROLE OF MYCORRHIZAE IN
NITROGEN-SATURATED SOILS**

Cara Machacek

*Colorado College, 14 E. Cache La Poudre St., Colorado Springs,
CO 80946, USA*

Advisors:

John Hobbie

MBL, 7 MBL St.,

Woods Hole, MA 02543, USA

Howard Drossman

*Colorado College, 14 E. Cache La Poudre St., Colorado Springs,
CO 80946, USA*

ABSTRACT

I examined the effects of long-term fertilization on the soil fungal and, particularly, mycorrhizal communities in the Falmouth Wastewater Treatment Plant experimental forest. Mycorrhizal fungi form symbiotic relationships with plants and provide them with essential nutrients in terrestrial ecosystems. In return, fungi derive sugars from plants that can amount to 30% of a plant's primary production. I investigated whether this relationship can change in response to external nutrient loading by examining the soils and plants in a fertilization field study site that has been sprayed with treated wastewater for 18 years. I sampled organic and mineral soils near black oak (*Quercus velutina*), pitch pine (*Pinus rigida*), and black huckleberry (*Gaylussacia baccata*) plants to analyze for ergosterol content, a useful bioindicator of fungal biomass in soils. Unfertilized soils had significantly more ergosterol per gram soil than fertilized soils ($P = 0.002$), indicating higher levels of total fungal biomass in unfertilized soils. I also collected leaf samples for $\delta^{15}\text{N}$ determination. Mycorrhizal fungi preferentially use ^{14}N in producing amino acids to transfer to plants such that plants that rely on mycorrhizae for nitrogen uptake will be greatly depleted in ^{15}N and those that do not rely on mycorrhizae will have $\delta^{15}\text{N}$ values comparable to soil dissolved inorganic nitrogen. All three plant species showed dependence on mycorrhizae in unfertilized soils ($\delta^{15}\text{N}$ range in plants: -1.6 to -0.3; $\delta^{15}\text{N}$ soils: 1.9). In fertilized forests, pitch pine and black oak both showed significant departure from mycorrhizal dependence (pine $\delta^{15}\text{N}$: 5.2; oak $\delta^{15}\text{N}$: 6.8) while huckleberry was only marginally enriched in ^{15}N (huckleberry $\delta^{15}\text{N}$: 1.1). These results indicate that soil mycorrhizal communities may indeed be strongly affected by external nitrogen deposition.

Key words and phrases: Mycorrhizae; Fungi; Ergosterol; Stable isotopes; Pinus rigida; Quercus velutina; Gaylussacia baccata; Nitrogen addition

INTRODUCTION

Mycorrhizae are among the most ubiquitous symbioses in terrestrial ecosystems. They provide an effective means for nutrient uptake by plants in nitrogen and phosphorous-poor soils. The majority of terrestrial plant species rely on mycorrhizal transport as a primary source of nitrogen and mycorrhizal fungi, in turn, rely on plants for sugars (Read 1991). While these relationships are highly beneficial and oftentimes necessary to plants, they come at a high energy cost; fungi may consume up to 30% of a plant's photosynthate (Hobbie and Hobbie in press). However, plants absorb inorganic nitrogen from the soil solution and therefore may be able to conserve those sugars when soil nitrogen is available. Little is known about whether these symbiotic relationships are truly obligate or whether they become less significant when inorganic nitrogen is readily available in the soil solution. Studies indicate that the frequencies of mycorrhizal fruiting bodies (Rühling and Tyler 1991) and fungal structures involved in nitrogen transfer (Johnson et al. 2003) may decrease with fertilization. I examined the effects of fertilization on total fungal biomass and on mycorrhizal fungi specifically.

The Falmouth Wastewater Treatment Plant has sprayed purified, nutrient-rich effluent into a neighboring forest for 18 years as an experiment to evaluate both the effectiveness of the forest in removing nutrients from the wastewater and the effects of fertilization on plant growth and distribution (Jordan et al. 1997). The forest contains both treated and untreated stands of oak, pine and herbaceous plants and therefore

provides control and treatment plots in which to analyze the effects of fertilization on the plant/fungal symbiosis. Assuming identical soil nutrient concentrations and fungal abundances prior to the fertilization experiment, any current differences in fungal abundance between control and fertilized plots can be attributed to the effect of nutrient-loading through fertilization.

Mycorrhizal fungal influence on plants has been determined by numerous chemical and biological methods, including ATP assays, substrate-induced respiration assays and hyphal counting by direct microscopy (Frankland et al. 1990). Ergosterol, a structural sterol found in the cell membranes of fungi (Weete 1989), is a useful bioindicator of fungus in terrestrial ecosystems because it is produced mainly by fungi and to a small extent by microalgae (Grant and West 1986). While ergosterol is not necessarily an accurate indicator of absolute fungal biomass due to differing ergosterol to biomass ratios among fungus species (Montgomery et al. 2000) and difficulty in separating ergosterol from living and non-living sources (Mille-Lindblom et al. 2004), quantification of ergosterol does provide a reasonable estimate of the relative abundance of fungal membranes in soils (Ruzicka 2000). Stahl et al. showed that ergosterol extraction provides a more accurate and less user-biased method of quantifying fungal biomass than does hyphal counting by direct microscopy (1996). Ergosterol, when extracted from cell membranes and quantified by HPLC, appears to be one of the more effective methods in determining relative fungal abundances in soils. However, ergosterol is produced by both saprotrophic fungi, which consume non-living organic matter, and mycorrhizal fungi. Therefore it is not an indicator of mycorrhizal biomass alone but of total fungal biomass in the soil.

The degree of mycorrhizal influence on plants can be determined using stable isotope analysis. When mycorrhizal fungi produce simple amino acids to transfer to plants, they preferentially use ^{14}N such that the fungi transfer ^{15}N -poor compounds to plants and retain ^{15}N -enriched compounds (Hobbie et al. 1999). Assuming that neither plants nor mycorrhizal fungi fractionate against ^{15}N in direct nitrogen uptake (Evans 2001) and that the forest soils had similar $\delta^{15}\text{N}$ values before fertilization, different plant $\delta^{15}\text{N}$ values indicate different degrees of reliance on mycorrhizae for nitrogen uptake. Plants that rely on mycorrhizae are depleted in ^{15}N relative to the soil while plants that do not rely on mycorrhizae have $\delta^{15}\text{N}$ values comparable to those of the soil. These two methods were used to determine the effects of fertilization on total soil fungal biomass and mycorrhizal/plant interactions.

METHODS

Sample collection and preparation

Soils and plant leaves were collected from the fertilized and control oak and pine forests of the Falmouth Wastewater Treatment Plant in early November, 2005. Soil samples were collected from the base of each of three pitch pine, black oak and huckleberry plants in each plot (oak control 1 & 2 (OC1, OC2), pine control 1 & 2 (PC1, PC2), oak fertilized 1&2, (OF1, OF2), pine fertilized 1& 2 (PF1, PF2)). At each plant both an organic layer sample and a 15cm core containing organic and mineral soils were obtained. Soils and leaf samples from the three species were stored in plastic bags. Equal volumes of soils from replicate plots (e.g. OC1 and OC2; OF1 and OF2) were pooled, sieved to 1 mm and stored at 4°C in the dark.

Soil moisture, pH determination and nutrient analysis

Soils of a known weight were dried at 60°C and reweighed to determine soil moisture. The pH of the dried soils was determined using a Fisher Scientific accumet pH meter. Nitrate and ammonium were extracted from mineral and organic soils with 1M KCl, shaken 1 h, and filtered with Whatman 42 ashless filters. Extracts were stored frozen until processing. Nitrate was analyzed spectroscopically using a Lachat Quikchem 8000 Autoanalyzer. Ammonium was treated according to the procedure developed by Solarzano (1969) and analyzed using a Varian Cary 50 Scan Spectrophotometer. Phosphate was extracted with a solution of 0.0125M H₂SO₄ and 0.05M HCl and ca. 200mg activated carbon (Nelson et al. 1953). Extractions were shaken ca. 5 m and filtered with Whatman 1 ashless filters. Samples were stored frozen until treated according to Murphy and Riley (1962) and spectroscopic analysis on a Perkin Elmer LambdaBio 20 UV/Vis Spectrophotometer.

Ergosterol extraction by microwave-assisted extraction method

The microwave-assisted extraction (MAE) method procedure was based on that originally proposed by Young (1995), modified for use in soils by Grant and West (1986) and expanded upon by Montgomery et al. (2000). All reagents and solvents used in extraction and chromatography were analytical grade. Approximately 0.5 g soil was weighed into a glass vial, treated with 2 ml methanol and 0.5 ml 2M NaOH. A 100 µl spike of 100ppm ergosterol was added to one replicate prior to methanol treatment. The vials were tightly sealed, enclosed in a 1 l plastic bottle and heated in a conventional microwave at 60% maximum power for 18 seconds. The vials were cooled ca. 15 minutes and heated for an additional 17 seconds. After cooling, samples were neutralized

with 1M HCl and treated with 2 ml methanol. Samples were then extracted 3 times with pentane (2x ca. 2 ml, 1x ca. 4 ml). Organic layer samples were centrifuged for 10 min at 10,000 rpm and remaining pentane was extracted. Combined extractions were transferred to an 8 ml amber vial and stored at room temperature until processing.

Ergosterol extraction by ultrasonication

The sonication extraction procedure was based on that of Ruzicka et al. (1995). Approximately 5 g soil was treated with 10 ml hexane isopropanol (98:2, v/v) and refrigerated 72 hours. Each sample was treated with 20 ml hexane isopropanol (98:2, v/v) and immediately sonicated on ice for 200s. Each sample was allowed to settle for 30-40s and then 2 ml of the supernatant was pipetted into a 2 ml microcentrifuge tube. Samples were centrifuged at 10,000rpm for 10 minutes and the supernatant was removed and stored at 4°C until processing.

HPLC analysis

Extractions by both methods were analyzed at the Waters Lab by High Performance Liquid Chromatography (HPLC). Ergosterol was detected at 282nm. The MAE method samples were injected in 100 µl volumes onto a 25 cm Waters Symmetry C18 column with 5µm packing. Samples were eluted with 100% MeOH at 2 ml/min and detected by photodiode array with the spectra from 209-450 nm. The sonication method samples were injected in 100 µl volumes onto a Si column, eluted with 98% hexane/2% iPrOH at 1.5 ml/min, and detected by photodiode array.

Isotope analysis

Leaf samples were ground using a Thomas Scientific Wiley Mill and organic soils were ground by mortar and pestle and analyzed at the MBL Stable Isotope Lab for $\delta^{15}\text{N}$.

RESULTS

Comparison of microwave-assisted and sonication extractions

The two methods of extraction yielded dramatically different ergosterol recoveries. The sonication method yielded better recoveries for all soil types, but recoveries were frequently higher than 100% (Fig. 1). The microwave method yielded much lower recoveries than the sonication method and the recoveries were highly dependent on the soil type. The recoveries for treated and untreated soils were highly significant ($P = 0.001$), as were the recoveries for organic and mineral soils ($P < 0.001$). When the microwave method results were corrected by dividing each sample ergosterol concentration by the recovery for the spiked replicate, the two methods yielded strongly correlated results ($r^2 = 0.65$, Fig. 2).

Ergosterol across soil types

Nearly all organic soils contained significantly more ergosterol ($P < 0.05$, Fig. 3), and therefore fungal biomass, than mineral soils (Fig. 3). Treated plots contained significantly less ergosterol by soil weight ($P = 0.002$) than untreated plots in organic soils (Fig. 4). When the same comparison is made by area to evaluate the effect of soil bulk density and organic layer thickness, the significance is lost ($P = 0.154$) but the trend persists (Fig. 5). There is no significant trend in the mineral soils, which show much more variability in ergosterol content than do the organic soils (Fig. 6). Ergosterol content in organic soils was weakly negatively correlated with soil ammonium content ($r^2 = 0.21$, Fig 7) and with soil nitrate content ($r^2 = 0.43$, Fig 8).

Soil respiration

Current and past SES classes have found that control oak soils have consistently had higher microbial respiration rates than fertilized oak soils (range: 2% to 47%; Table 1). Control pine soils have generally had slightly lower respiration rates than fertilized soils (range: -26% to 26%; Table 1).

$\delta^{15}\text{N}$ of leaves and soils

See Figure 9 for all $\delta^{15}\text{N}$ results. All three plant species in the control plots were depleted in their $\delta^{15}\text{N}$ values relative to the soil. In the fertilized plots, both pine and oak were greatly enriched in their $\delta^{15}\text{N}$ values relative to the soil. Pine leaves were enriched by about 5.6‰ in the fertilized plots relative to control leaves. Oak leaves were enriched by 8.4‰ and huckleberry leaves were enriched by 1.4‰. The bulk soil was enriched by 2.2‰. The wastewater had an average $\delta^{15}\text{N}$ of 9.0‰ when determined in 2001 by SES student Jordan Kramer.

DISCUSSION

Comparison of sonication and microwave-extraction methods

The microwave-extraction method and the sonication method were both developed to improve upon the original method proposed by Sietz et al. (1977). Both methods require significantly less time, smaller solvent volumes and smaller soil samples but present new problems with extracting ergosterol. The MAE method yielded significantly lower ergosterol recoveries than the sonication method, but this may be due to modifications of the MAE method (Montgomery 2000). We used moist soils instead of air-dried soils to prevent fungal death and loss of ergosterol. The soil moisture may have contributed to the high emulsion and therefore difficult extractions. Montgomery et

al. reported $90 \pm 6\%$ ergosterol recovery from three A-horizon soils (2000), indicating that air-dried soils may have yielded better recoveries. In addition, the MAE method yielded significantly different recoveries for control and fertilized soils and for organic and mineral soils, indicating that it may not be an appropriate method for comparison across treatments and soil types. Because this study relies on the ability to compare fungal biomass across these soil types, the results from the MAE method were not suitable for comparison. The sonication method yielded significantly higher and less variable recoveries but recoveries that were frequently greater than 100%. This most likely reflects the observed solvent evaporation from loosely-sealed sample vials during transportation. This problem could be easily remedied by storing samples in tightly-sealed vials immediately following sonication and centrifugation. It should be noted, however, that there is evidence that ergosterol in solution is not extracted as efficiently as ergosterol in fungal tissue (Davis and Lamar 1992) and therefore the determined recoveries for both methods may not be reflective of the actual recovery from the soil.

Because the recoveries from the MAE method were highly consistent within a given soil type or treatment, the ergosterol results could be corrected for recovery. The sonication method recoveries, on the other hand, appeared to reflect individual sample solvent evaporation so the results could not be corrected for recovery. None the less, the MAE corrections provide significant correlation between the two methods, indicating that with better recoveries, the two methods would yield similar results. This correlation also suggests that despite the problem of solvent evaporation in the ultrasonication method, the method provides reasonably accurate estimations of ergosterol content. Because the MAE method yielded recoveries that were both low and dependent on soil type and

treatment, the results from the ultrasonication method appeared more reliable and were used in the following comparisons.

Ergosterol and fungal biomass

Ergosterol has been shown to be a reliable indicator of fungal membranes in soils, but it has been difficult to convert ergosterol concentrations to fungal biomass due to variation across species in ergosterol to fungal biomass ratios (Ruzicka et al. 2000). Also, because it is present in both saprotrophic and mycorrhizal fungi, it is an indicator of total fungal presence and not mycorrhizal presence alone. Regardless, it is a useful bioindicator of fungal presence across soil types.

Organic soils had significantly more ergosterol, and therefore fungal biomass, than did mineral soils. Organic soils are richer in nutrients and non-living organic matter than mineral soils and therefore are a more suitable environment for fungi. Because organic soils were less variable, the following correlations were made predominantly with ergosterol data from organic soils.

Studies have shown that nitrogen additions have frequently led to decreased mycorrhizal fruiting frequency, species diversity and root abundance (Wallenda and Kottke 1998). The fertilized organic soils of the Falmouth Wastewater Treatment Plant contained significantly less ergosterol than unfertilized soils, indicating that the fertilized soils have less fungal biomass. Soil ergosterol content also generally decreased with increasing nitrate and ammonium soil contents, indicating that nutrient-addition may indeed negatively affect the amount of fungal biomass in soils. The significantly

decreased microbial respiration in fertilized oak forest soils is further evidence for diminished fungal and microbial biomass with nitrogen additions.

However, fertilization may have other effects that were reflected in the soil ergosterol contents. While declines in the ergosterol of fertilized soils may suggest decreased mycorrhizal fungi, they may also reflect potential negative effects of nitrogen and water additions on saprotrophic soil fungi. The process of fertilization involves irrigation and soil saturation may inhibit fungal growth or change the structure of the soil community to one dominated by microbes. A third treatment of irrigation alone would shed light on the effects of water-logged soils on fungal growth. Fertilization may also affect fungal species distribution, changing the average ergosterol to fungal biomass ratio of the soil. An investigation into the ergosterol to fungal biomass ratios of each species and the species distribution in each treatment would indicate whether these results reflect a species effect or a plant inhibition effect.

Stable isotopes and mycorrhizal influence

All three plant species in unfertilized soils were depleted in ^{15}N relative to the soil, indicating that they likely rely on mycorrhizae for nitrogen transport in these nitrogen-limited soils. In the fertilized plots, all three plant species were enriched in ^{15}N relative to the unfertilized plants. Huckleberry followed a trend in enrichment similar to that of the soil. Enrichment of the soil probably reflects the high $\delta^{15}\text{N}$ of the sprayed effluent. Both oak and pine were dramatically enriched in ^{15}N relative to the unfertilized plots. While these plants may have been influenced to an extent by the high $\delta^{15}\text{N}$ of the effluent, studies have shown that many plants actually discriminate more against ^{15}N in

their uptake of dissolved inorganic nitrogen when soils are rich in mineral nitrogen (Evans 2001; Yoneyama et al. 1991). Therefore these results may also indicate decreased reliance on mycorrhizal fungi.

Hobbie and Hobbie (in press) designed a model that quantifies the percentage of a plant's total nutrient uptake that is derived from mycorrhizae. The model estimates the percentage of nutrients derived from fungal transfer using $\delta^{15}\text{N}$ values of soils and plant and fungal tissue according to the equations:

$$(1) \quad 100(\delta^{15}\text{N}_{\text{available}}) = (100-T)(\delta^{15}\text{N}_{\text{fungi}}) + (T)(\delta^{15}\text{N}_{\text{transfer}})$$

$$(2) \quad 100(\delta^{15}\text{N}_{\text{plant}}) = (f)(\delta^{15}\text{N}_{\text{transfer}}) + (100 - f)(\delta^{15}\text{N}_{\text{available}})$$

$$(3) \quad \Delta = \delta^{15}\text{N}_{\text{available}} - \delta^{15}\text{N}_{\text{transfer}}$$

In these equations, Δ represents the fractionation by fungi against ^{15}N during the synthesis of amino acids, f represents the percentage of plant N derived from mycorrhizae and T represents the fungal N transferred to plants. Using equations 2 and 3 and assuming a Δ of 10‰, I determined the percentage of pine, oak and huckleberry plant N that was derived from mycorrhizal fungi in the treated and untreated soils (Table 2).

Using the unfertilized soil $\delta^{15}\text{N}$ for the $\delta^{15}\text{N}_{\text{available}}$, I found that the pine and huckleberry relied on mycorrhizae for about one quarter of their total plant N while oak relied on mycorrhizae for about one third. Using the fertilized soil $\delta^{15}\text{N}$, I found that neither oak nor pine relied on mycorrhizae for any N transfer while huckleberry increased its dependence on mycorrhizae. It therefore appears that pitch pine and black oak may be capable of regulating their mycorrhizal interactions while huckleberry are not. However, using the $\delta^{15}\text{N}$ value of the spray as $\delta^{15}\text{N}_{\text{available}}$, all three plant species appeared significantly more dependent on mycorrhizae. The $\delta^{15}\text{N}$ of the soil solution might be a

more appropriate value for $\delta^{15}\text{N}_{\text{available}}$ to determine the actual dependence of plants on mycorrhizae in fertilized soils.

CONCLUSIONS

As levels of anthropogenic nitrogen deposition continue to rise, understanding the consequences of increased loading to natural ecosystems is becoming increasingly important. This study highlights one potential result of nitrogen deposition: diminishing fungal biomass in soils. The loss of mycorrhizae and other soil fungi may have significant ecological ramifications in the future. Research in this field should focus on investigation into whether the results presented in this paper are a result of plant inhibition of mycorrhizae or of nutrients and water directly changing the structure of the fungal and microbial communities. This will inevitably involve developing better methods to determine the effects of fertilization on mycorrhizal fungal biomass, separating the effects on mycorrhizal fungi from those on saprotrophic fungal biomass and evaluating the effects of irrigation on fungal biomass. With these insights we can better evaluate what future effects our current nitrogen deposition may have on these vital symbiotic relationships.

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TABLES

	Soil respiration (mg CO₂-C m⁻² hr⁻¹)						
	1997	1998	1999	1999 (2)	2002	2003	2005
Oak control	167	242	186	53	123	251	300
Oak fert	88	200	117	43	120	197	175
% increase control/fert	47%	17%	37%	19%	2%	22%	42%
Pine control	104	200	118	92	126	217	209
Pine fert	131	208	145	105	120	160	227
% increase control/fert	-26%	-4%	-23%	-14%	5%	26%	-9%

Table 1. Soil respiration by year including the percent increase from fertilized to control soils.

	$\delta^{15}\text{N}_{\text{available}}$	Δ ‰	$\delta^{15}\text{N}_{\text{transfer}}$ ‰	$\delta^{15}\text{N}_{\text{plant}}$ ‰	f ‰
Pine control	1.9 (soil)	10	-8.1	-0.4	23
Pine fert	4.1 (soil)	10	-5.9	5.2	-11
Pine fert	9 (spray)	10	-1	5.2	38
Oak control	1.9 (soil)	10	-8.1	-1.6	35
Oak fert	4.1 (soil)	10	-5.9	6.8	-27
Oak fert	9 (spray)	10	-1	6.8	22
Huck control	1.9 (soil)	10	-8.1	-0.3	22
Huck fert	4.1 (soil)	10	-5.9	1.1	30
Huck fert	9 (spray)	10	-1	1.1	79

Table 2. Estimation of f , the percentage of plant nitrogen derived from mycorrhizae, from soil and spray $\delta^{15}\text{N}$, plant $\delta^{15}\text{N}$ and an assumed fractionation (Δ) of 10‰.

FIGURES

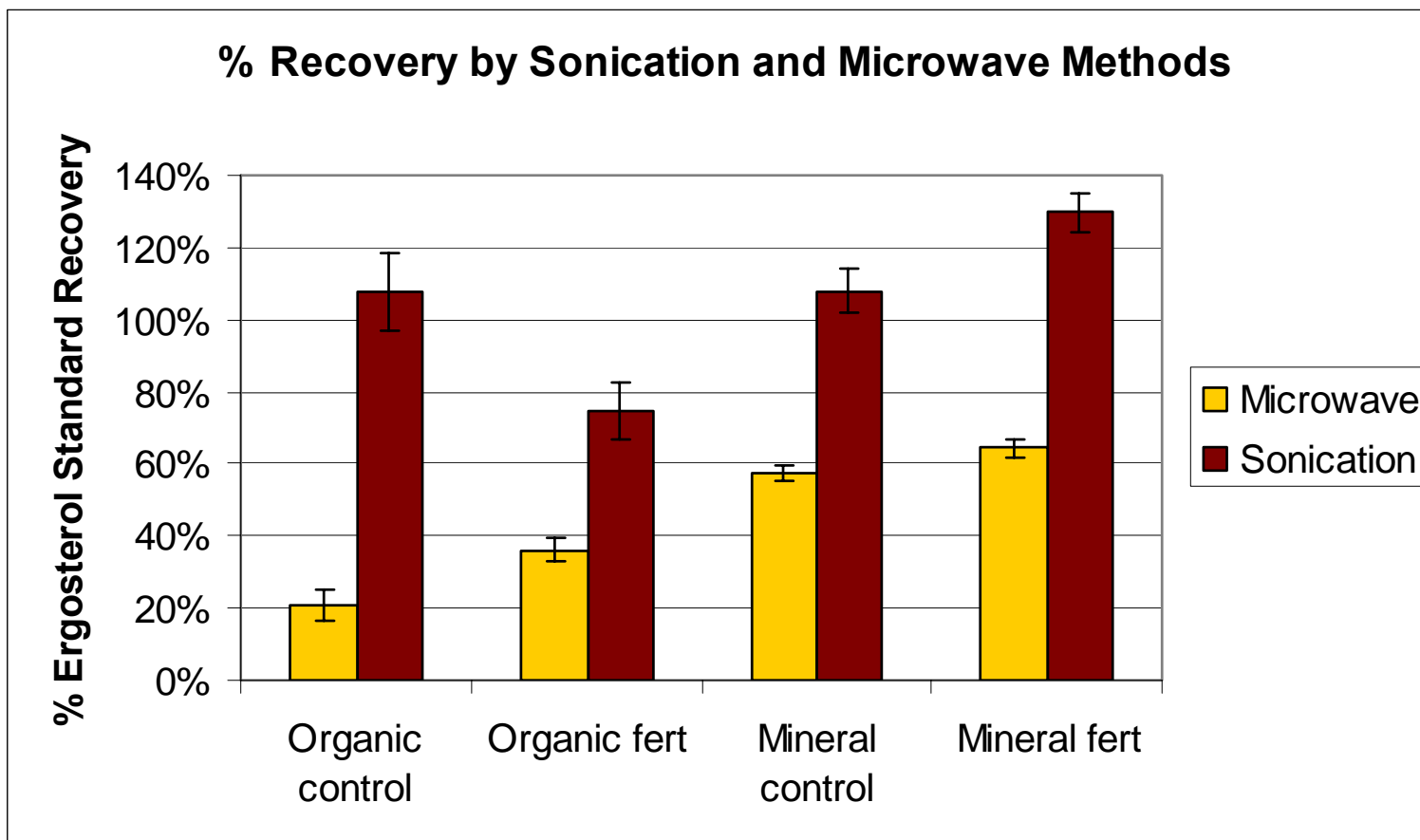


Figure 1. Comparison of the microwave and sonication methods based on % recovery of a standard ergosterol spike.

Methods Comparison After Recovery-Correction

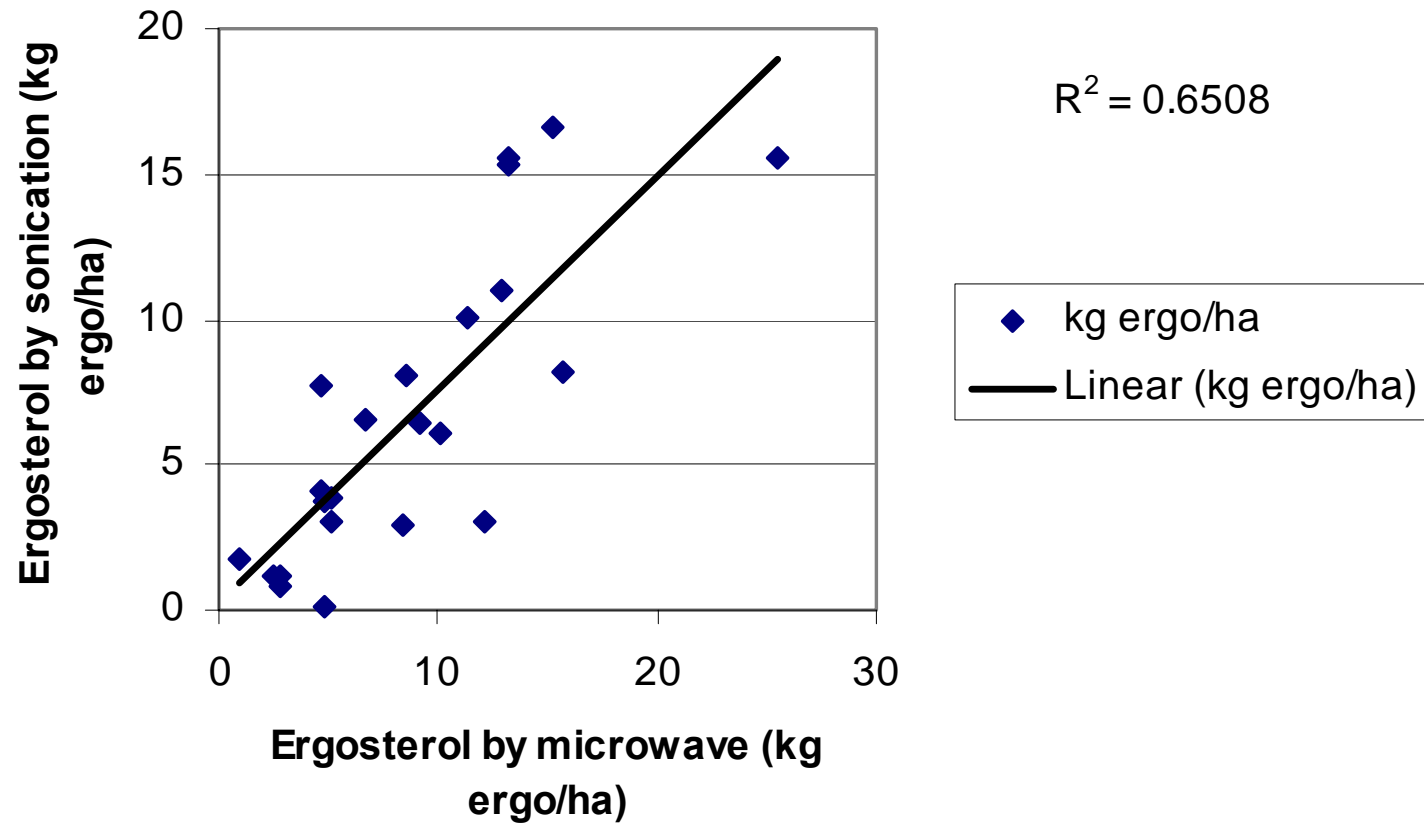


Figure 2. When the microwave method was corrected for recovery, the two methods showed a strong correlation.

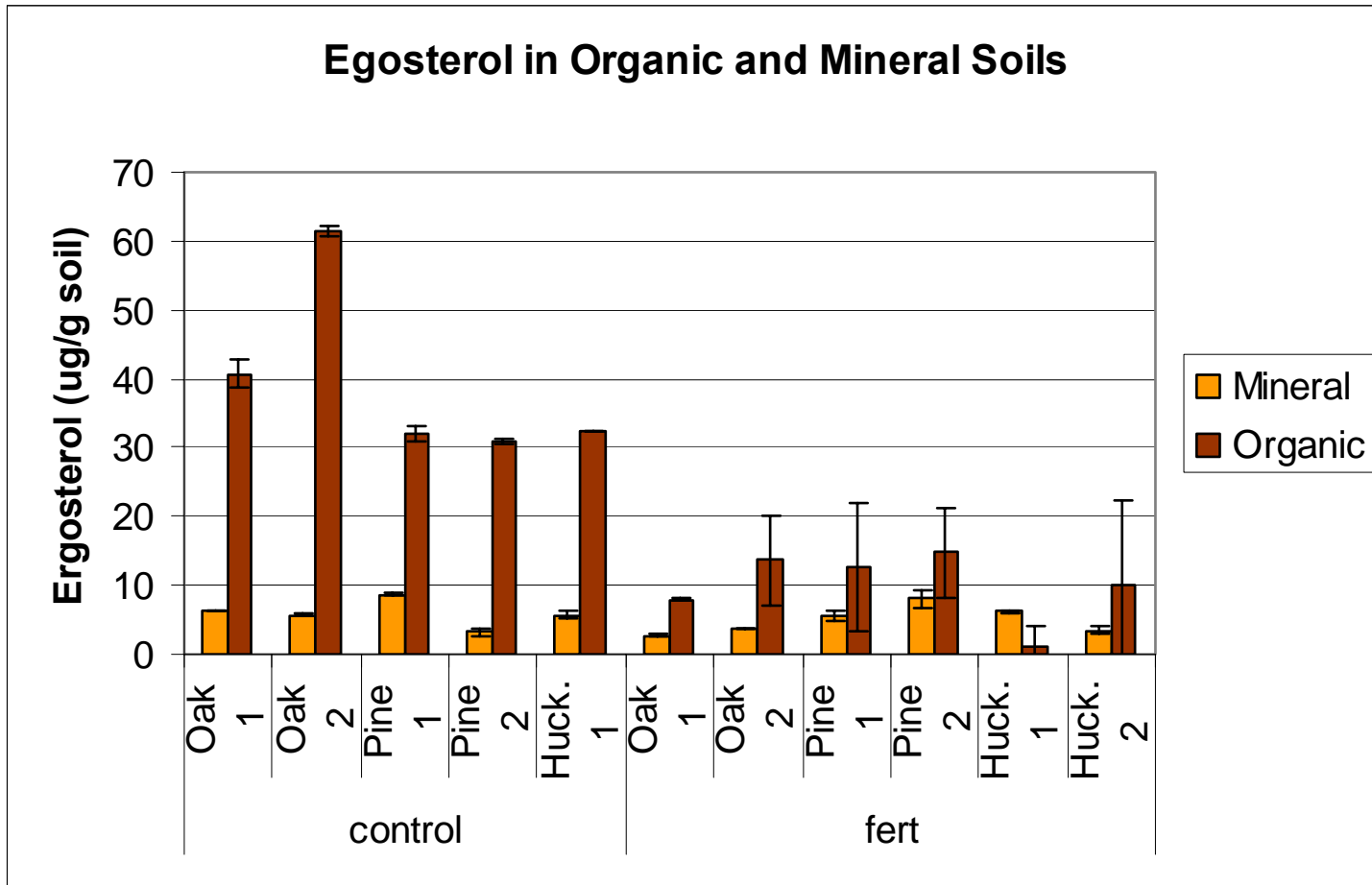


Figure 3. Comparison of ergosterol in organic and mineral soil samples.

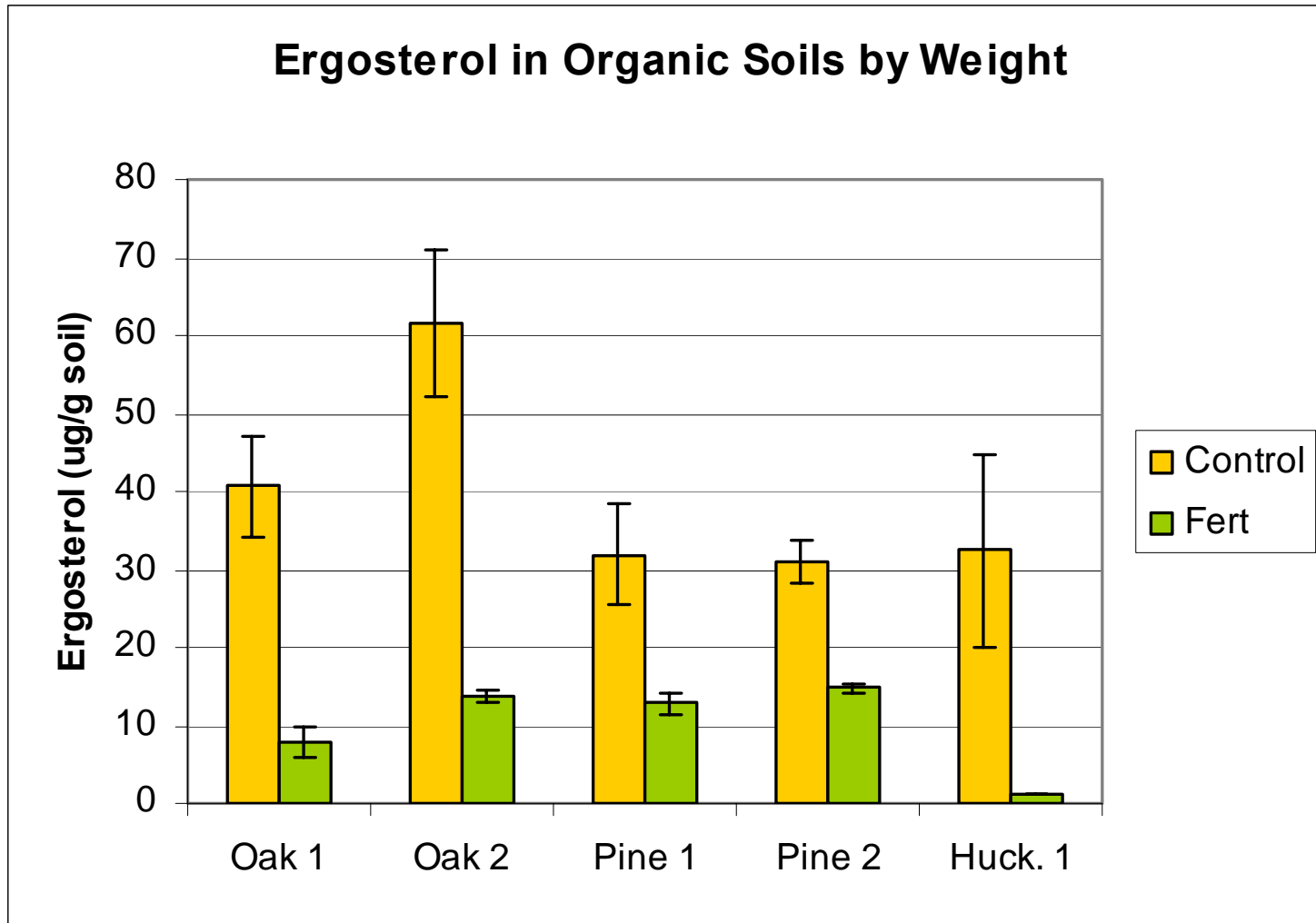


Figure 4. Comparison of ergosterol content in organic soils in treated and untreated soils (in units of μg ergosterol/g soil).

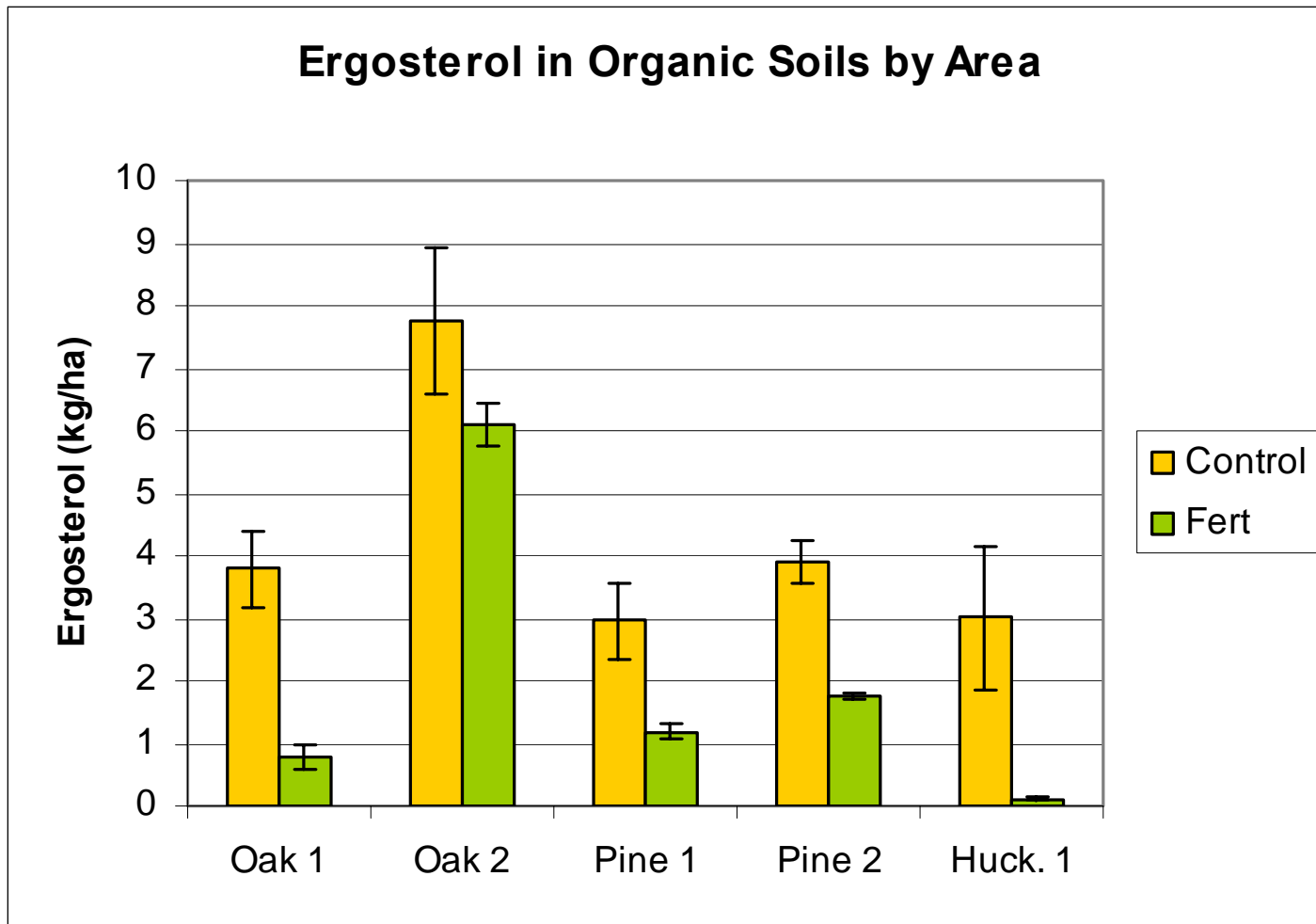


Figure 5. Comparison of ergosterol content in organic soils in treated and untreated soils (in units of kg ergosterol/ha).

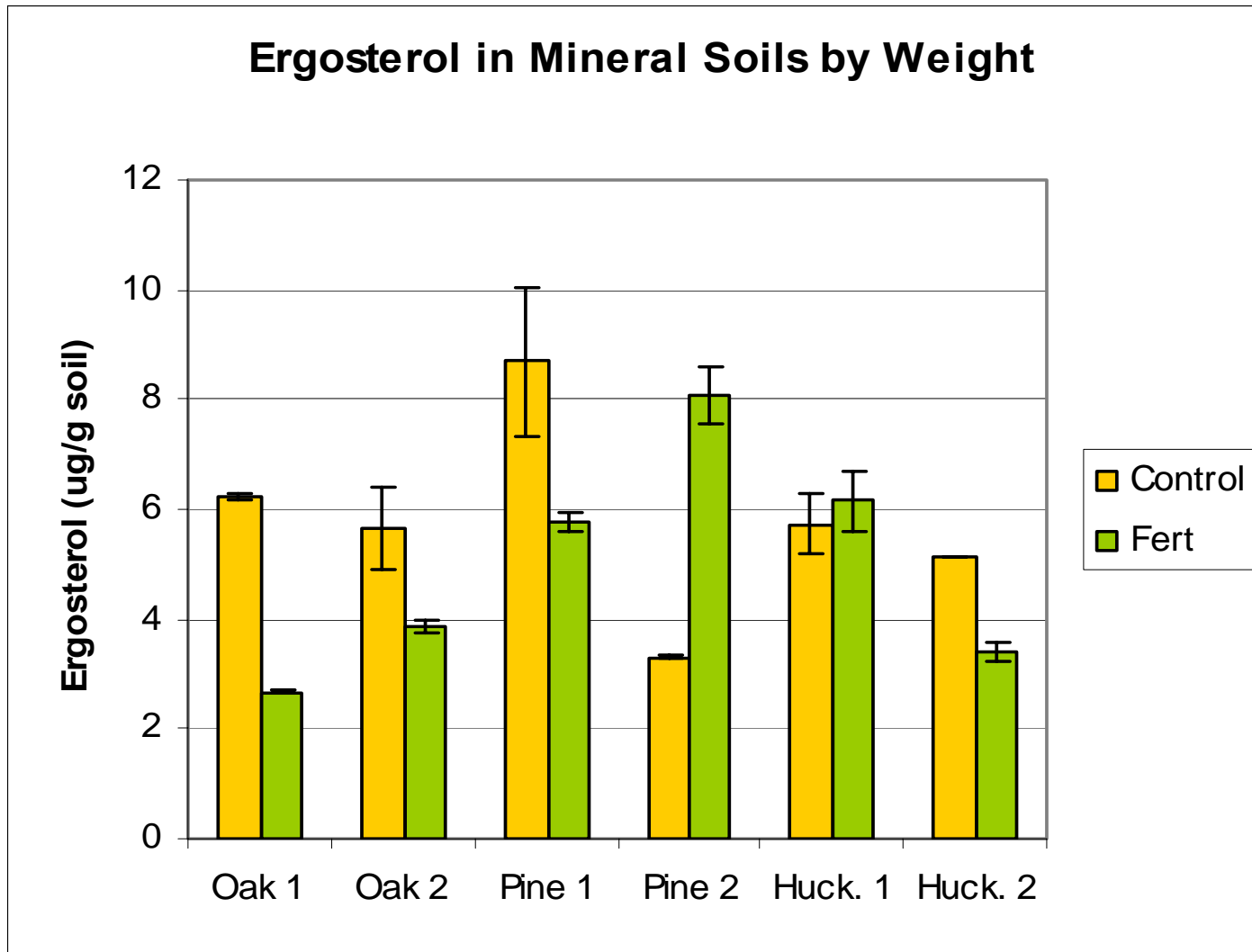


Figure 6. Comparison of ergosterol content in mineral soils in treated and untreated soils (in units of μg ergosterol/g soil).

Ergosterol vs. NH4 in Organic Soils

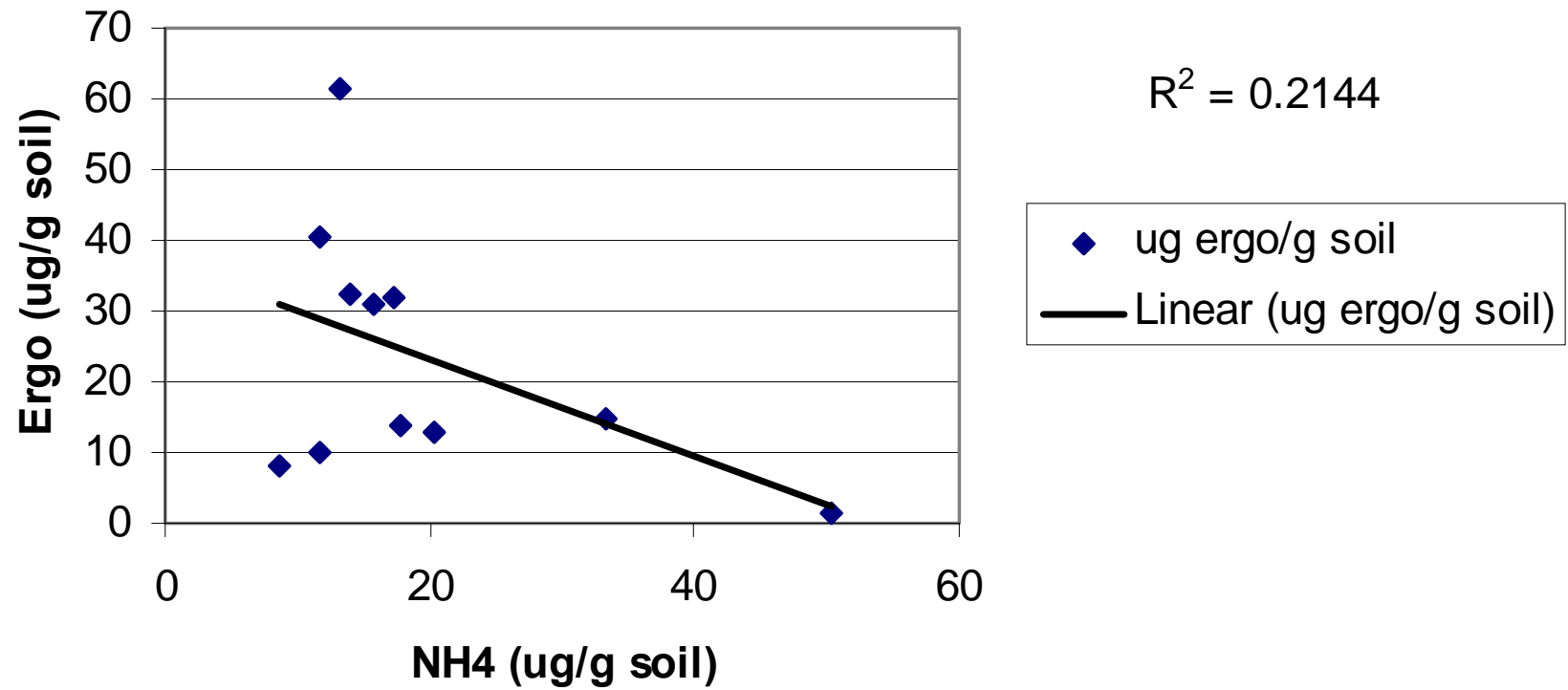


Figure 7. Correlation of ergosterol and soil ammonium content.

Ergosterol vs. NO3 in Organic Soils

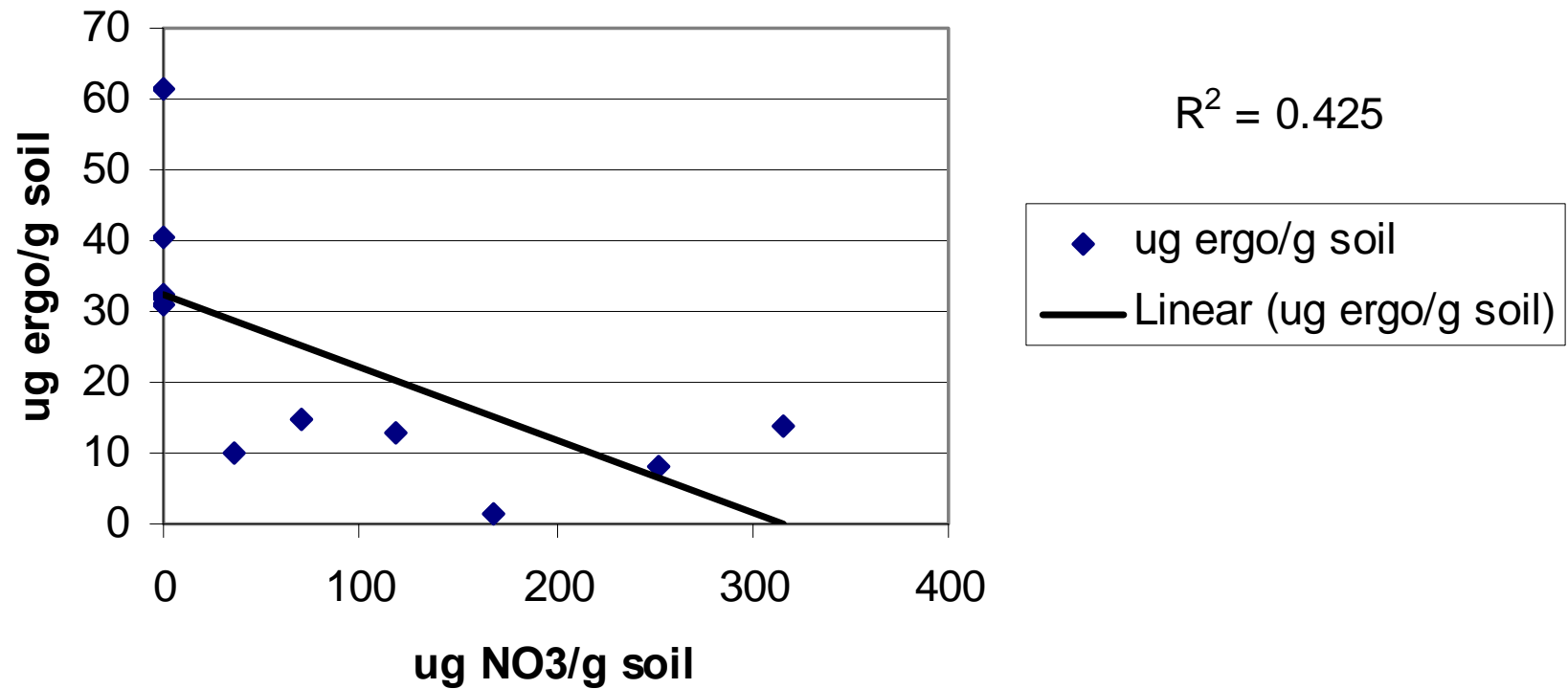


Figure 8. Correlation of ergosterol and soil nitrate content.

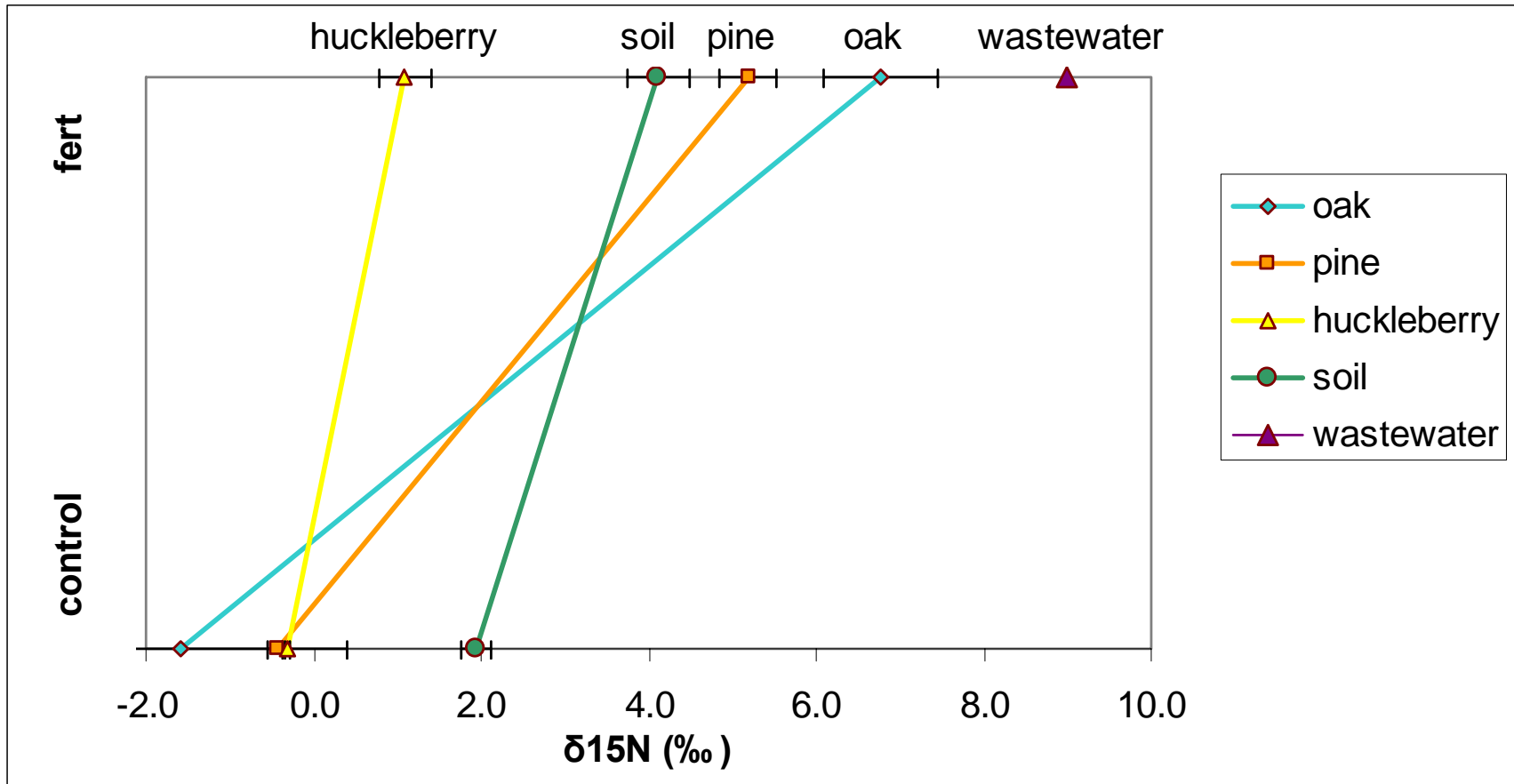


Figure 9. $\delta^{15}\text{N}$ values for plant species, soil and wastewater in control and fertilized plots.