Developing Methodologies to Assess the Influence of Nutritional and Physical Characteristics of *Hydrilla verticillata* on Its Biological Control Agents

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PURPOSE: This technical note describes experimentation used to develop methods of culturing *Hydrilla verticillata* (L. f.) Royle (hydrilla) to obtain plants with consistent but wide-ranging nutritional and physical characteristics. Results of this research will allow laboratory testing of the impacts of the structural and nutritional quality of hydrilla on insect and fungal pathogen biological control agents.

BACKGROUND: Four insect biological control agents have been released in the United States as management tools for the control of hydrilla. They include two weevil species, *Bagous affinis* Hustache and *B. hydrillae* O'Brien, and two leaf-mining fly species, *Hydrellia pakistanae* Deonier and *H. balciunasi* Bock. While considerable effort has gone into the release of these agents, only the two fly species have become established, with *H. pakistanae* being more successful based on establishment success and range expansions (Buckingham, Okrah, and Thomas 1989; Buckingham, Okrah, and Christian-Meier 1991; Bennett and Buckingham 1991; Center et al. 1997; Grodowitz et al. 1997, 2000). In addition, research is also progressing on the application of endemic and exotic fungal pathogens for hydrilla control, especially the use of *Mycoleptodiscus terrestris* (Gerd.) Ostazeski, a native pathogen shown to be effective on both hydrilla and Eurasian watermilfoil (*Myriophyllum spicatum* L; Shearer 2001).

Though the two leaf-mining flies have exhibited extensive range expansions throughout the Southeast (especially *H. pakistanae*), field populations have typically remained below levels found damaging to hydrilla under controlled laboratory and greenhouse conditions (Wheeler and Center 2001). Reasons for low population increases in the field are unknown but may be caused by the impact of a complex of abiotic and biotic factors, including temperature, parasitism, predation, and plant physical and nutritional properties. For example, Wheeler and Center (1996) demonstrated significant increases in developmental times and in mortality of *H. pakistanae* larvae reared on field-collected hydrilla with greater leaf toughness; their findings further indicated leaf toughness to be negatively correlated with plant tissue nitrogen concentration.

Similarly, little information is available on fungal pathogen impacts in relationship to the physical and nutritional characteristics of hydrilla. However, there are frequently major differences in *M. terrestris* pathogenicity that are unaccounted for, and may be related to a variety of factors including plant nutritional composition.[1]

To further understand how the nutritional status of hydrilla influences agent establishment, survival, and subsequent plant impact, it is necessary to develop methods of culturing hydrilla of

[1] Personal Communication, 2002, Dr. J. Shearer, Research Plant Pathologist, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

consistent but wide-ranging nutritional and physical characteristics. Past efforts toward this goal have been only partially successful. Wheeler and Center (2001) obtained only marginal differences in nitrogen concentration using an N:P:K enriched sand mixture to obtain varying levels of hydrilla tissue nitrogen concentration. Nitrogen differences obtained in these experiments were at the low end of field-grown hydrilla with the high fertilizer treatments reaching nitrogen levels of only approximately 2 percent. It has been shown that whole plant nitrogen concentrations in field hydrilla can range from 1 percent to almost 4 percent (Zimba, Hopson, and Colle 1993).[1] Even less information is available that examines other potentially important nutritional components including phosphorus, calcium, lipids, fiber, carbohydrates, etc.

This report provides detailed information on procedures used to grow hydrilla with wide-ranging nutritional compositions with relatively low variability among individual replications. This is an important first step in understanding nutritional influences on the effectiveness of hydrilla insect and fungal pathogen agents. For this report, information will be provided concerning plant tissue nitrogen and phosphorous concentrations, and leaf hardness.

MATERIALS AND METHODS, EXPERIMENT 1: The first experiment was conducted May through June 1998 in a controlled growth-chamber facility at the U.S. Army Engineer Research and Development Center (ERDC), Vicksburg, Mississippi. Temperature was maintained at 25 °C with simulated sunlight at 350 $\mu E\ m^{-2}\ s^{-1}$ for 14 hr d^{-1}. Hydrilla was maintained in aquaria consisting of clear, Lucite columns (150 cm in height, 15 cm diameter with 20 L volume) with 3.5-L removable bases (Figure 1). For more detailed descriptions of the columns and environmental chamber see Barko and Smart (1980).

Fertilization/Sediment Treatments. Sediment used in the study was collected from Brown's Lake, ERDC, and was processed in the laboratory to obtain three fertilization/sediment treatments. One treatment was a low-fertility, "Used" sediment, rendered nitrogen-poor due to previous growth of submersed macrophytes. The second was "Unused," fresh sediment, with no artificial nutrient amendment. The third was "Fertilized," fresh sediment, prepared by adding 0.7g $NH_4Cl\ L^{-1}$ wet sediment.

The sediment treatments were mixed separately and poured into plastic containers (volume = 1,050 ml) to a depth of 12 cm and provided a surface area of 90 cm^2. The containers were transferred individually to each column base and positioned as indicated in Figure 1. Sediment physical and chemical characteristics are summarized in Table 1, as determined by analytical procedures described in Barko et al. (1988). Based on these data and results of previous studies (Barko et al. 1988; McFarland, Barko, and McCreary 1992), the nitrogen level in the "Used" sediment treatment was acutely growth limiting.

CO_2 Treatments. For each sediment type, two CO_2 treatments were utilized, including a "High" level and a "Low" level of CO_2 aeration. A single air line to each column delivered filtered, humidified air, either with or without a CO_2 addition (Smart and Barko 1985). The

[1] Unpublished Data and Personal Communication, 2002, Dr. R. M. Smart, U.S. Army Engineer Research and Development Center, Lewisville Aquatic Ecosystem Research Facility (LAERF), #1 Fish Hatchery Road, Lewisville, TX 75056.

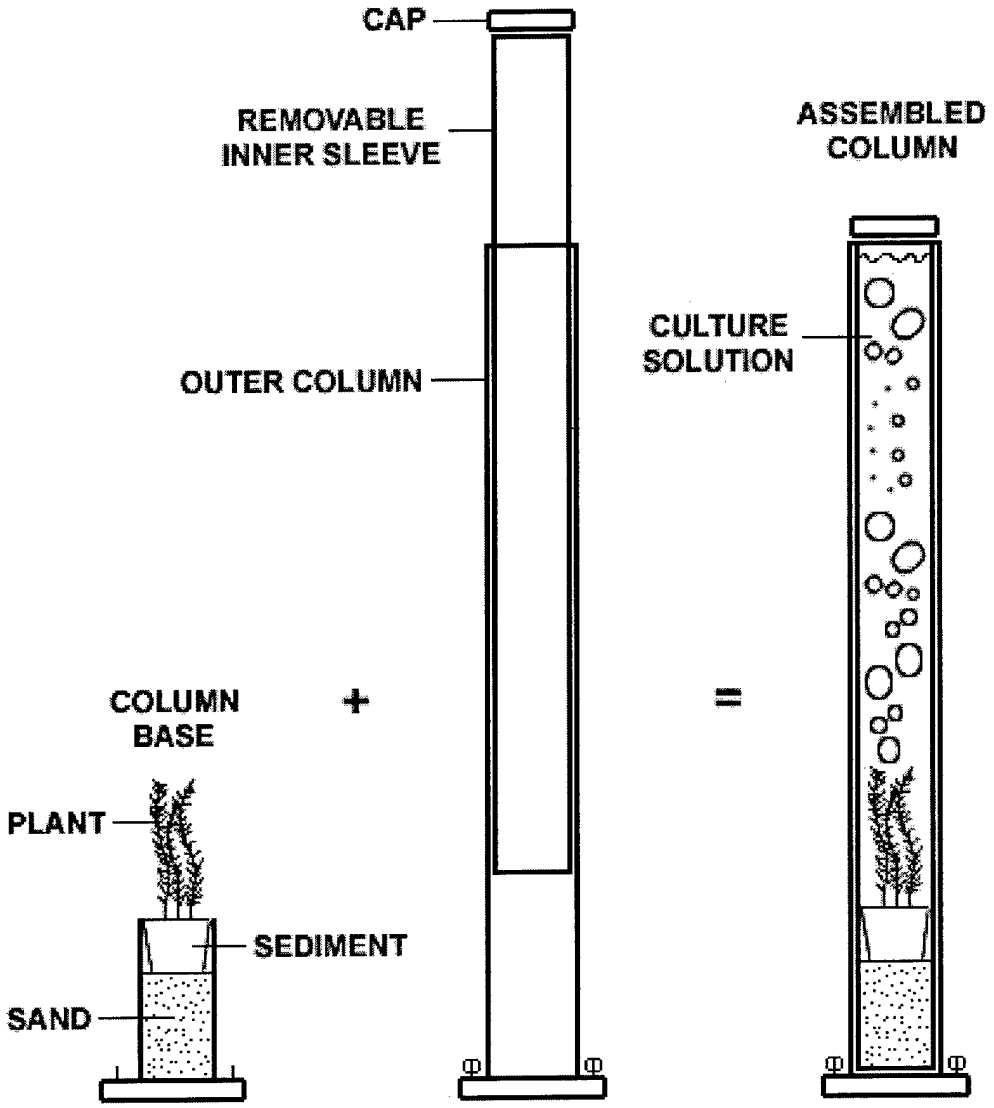

Figure 1. Assembly of Lucite columns used in Experiment 1

"Low" CO_2 treated columns received only ambient air (350 µL CO_2 L^{-1}) while the "High" CO_2 treated columns received air enriched tenfold in CO_2 concentration. A diffuser stone at the tip of each airline was lowered near the base of the plants, to allow gentle mixing of the solution with a gaseous stream of either CO_2 treatment.

Planting/Experiment Setup. Apices of hydrilla were clipped to 20 cm from six-week-old plants cultured under temperature and light conditions similar to those used in the experiment and described previously. The plant stock was originally established from a field collection made several months earlier from the San Marcos River, Texas. Three hydrilla sprigs were planted per sediment container, with basal ends of the sprigs buried 5 cm deep in the sediment. After planting, the sediment was covered with a thin layer of clean sand (1 cm deep) to minimize sediment disturbance and algal growth inside the columns. Once assembled, each column was filled with 15 L of the general-purpose culture solution described in Smart and Barko (1985).

Table 1
Physical and Chemical Characteristics of Brown's Lake Sediment [1]

Variable [2]	Used	Unused	Fertilized
Physical			
Moisture content, %	27.83 ± 0.11	27.60 ± 0.31	30.25 ± 0.14
Dry wt. density, g ml^{-1}	1.36 ± 0.01	1.38 ± 0.02	1.29 ± 0.01
Ash content, %	2.83 ± 0.01	2.72 ± 0.00	2.81 ± 0.23
Coarse particles, % [3]	10.73 ± 0.84	10.09 ± 1.40	9.57 ± 0.23
Fine particles, % [4]	89.27 ± 0.84	89.93 ± 1.42	90.43 ± 0.22
Silt content, %	76.77 ± 0.84	79.93 ± 1.42	77.87 ± 0.19
Clay content, %	12.50 ± 0.00	10.00 ± 0.00	12.57 ± 0.03
Chemical			
Exch. NH_4-N, mg g^{-1}	0.01 ± 0.00	0.06 ± 0.00	0.21 ± 0.00
Avail. PO_4-P, mg g^{-1}	0.11 ± 0.00	0.13 ± 0.00	0.13 ± 0.00

[1] Sediment was processed in the laboratory to obtain three sediment treatments (i.e., used, unused (fresh), and fertilized). Means and standard errors are presented, based on triplicate determinations for each variable.
[2] Except for moisture content, all listed variables were determined on a per gram dry sediment basis.
[3] Coarse particles = sand (or particles > 50 μ in diameter).
[4] Fine particles = silt + clay (or particles < 50 μ in diameter).

Upon preparation (at 25 °C), the solution had a pH of 7.9 and an elemental composition (mg L^{-1}) of the following: $Na^+ = 16.0$, $K^+ = 6.0$, $Ca^{+2} = 25.0$, $Mg^{+2} = 6.8$, $Cl^- = 44.2$, $HCO_3^- = 51.8$ and $SO_4^{-2} = 26.9$).

Study Design. Study 1 consisted of six treatment combinations (i.e., three fertility treatments by two CO_2 treatments) and each combination was assigned to four replicate columns. The hydrilla in each column was allowed to grow for five weeks and was subsequently harvested to assess structural and nutritional differences.

Plant Structural Quality Evaluations: Structural quality was evaluated based on laboratory assessments of plant morphology, leaf hardness, and plant biomass distribution. Plant biomass was harvested and separated into above- and belowground portions by clipping aboveground biomass at the sand surface; belowground biomass was retrieved by rinsing over a 1-mm mesh sieve to remove attached sand, sediment, and other debris. Gross morphologies were assessed by measuring maximum and average shoot lengths, and by directly counting the number of stem apices. Other determinations included measures of nodal frequency and inter-nodal diameter in the upper 20 cm of the stem, number and length of lateral and midrib spines, and leaf length. Leaf hardness, as a physical measure of leaf toughness and a possible indicator of *Hydrellia* larva's ability to penetrate a leaf, was determined using a self-constructed electronic penetrometer. The penetrometer consisted of a 250-g load cell attached to a data logger that evaluated resistance levels coming from the load cell as it deformed while penetrating the leaf surface. The load cell resistance levels were calibrated for weight using a 200-g certified standard weight. A 0.2206-mm^2 diameter probe was attached to the cell and used to pierce the leaf surface. All leaf hardness measurements were expressed in g mm^{-2}. This procedure was developed from that described in Wheeler and Center (1996).

Nutritional Determinations. All plant material was oven-dried to constant weight at 80 °C and ground to a fine powder for tissue analyses. Dry weights obtained for shoot (aboveground) and root (belowground) biomass were used to calculate total biomass production and root-to-shoot ratios.

The nutritional status of aboveground plant tissues was determined following tissue digestion in a mixture of sulfuric acid and hydrogen peroxide (Allen et al. 1974). N and P in the digestates were measured colorimetrically using a Lachat Instruments (Milwaukee, WI) QuikChem Autoanalyzer, employing a molybdate method for P and a salicylate method for N. Percent N in the tissues was multiplied by a factor of 6.25 to calculate percent crude protein in accordance with Allen et al. (1974). Other elemental components, (i.e., K, Mg, and Ca) were measured directly using a Perkin-Elmer atomic absorption (AA) spectrophotometer (Wellesley, MA). The accuracy of analytical procedures was verified by including National Bureau of Standards reference materials as part of the experimental sample sets. Unless otherwise noted, all tissue nutrient concentrations are reported here on a dry weight basis.

MATERIALS AND METHODS, EXPERIMENT 2: This experiment was conducted June through August 1999, in a greenhouse facility, at the ERDC. Hydrilla was grown in large (~1,200-L), white, fiberglass tanks, 150 cm long by 90 cm wide by 90 cm deep. Each tank was filled to a depth of 83 cm, with culture solution prepared as described in the previous experiment. The solution was continuously circulated and thermally controlled (±1 °C) using one Remcor® Products Company (Glendale Heights, IL) circulator per tank with capacities for both heating and cooling. Maximum midday PAR (photosynthetically active radiation) levels inside the tanks averaged approximately 400 $\mu E\ m^{-2}\ s^{-1}$ during a photoperiod of approximately 14 hr.

Fertilization/Sediment Treatments: Surficial sediment dredged from Brown's Lake was processed in the laboratory to obtain "Used" and "Fertilized" sediment as described for Experiment 1. The prepared sediment was poured 8 cm deep in plastic containers (24.3 cm long by 24.3 cm wide by 10 cm deep) and was allowed to settle for several days just prior to planting. Compositional characteristics at the initiation of the study were similar to those of "Used" and "Fertilized" sediment presented in Table 1.

CO_2 Treatments. The two CO_2 treatments, as in the first experiment, consisted of ambient air (350 $\mu L\ CO_2\ L^{-1}$) and air increased tenfold in CO_2 concentration. Each tank was aerated continuously using twin airlifts, providing filtered, humidified, compressed air (or amended air) at a rate of about 2.5 $L\ min^{-1}$. The procedure used to aerate the experimental tanks was identical to earlier work performed by Barko, Smart, and McFarland (1991).

Planting/Experiment Setup. Apical cuttings of hydrilla, 20 cm in length, were planted to a sediment depth of approximately 5 cm. Eight cuttings were planted per sediment container, and were obtained from 5-week-old greenhouse cultures established from collections from the San Marcos River, Texas. Sediment surfaces were covered with about 2 cm of washed silica sand to minimize physical and chemical exchanges between sediment and the culture solution. Half the tanks (i.e., four) were established for a growth period of ten weeks, while the other half were established for four weeks. Plants growing for ten weeks were kept at 18 °C, while those growing for four weeks were kept at 28 °C. The shorter growth period was assigned to plants at

28 °C to minimize potential nitrogen shortages due to increased growth rate at high temperature. Planting dates were staggered so that the harvest date was the same for both ten-week-old and four-week-old treated plants.

Study Design. Hydrilla was cultured under different treatment conditions (eight total) by varying levels of sediment fertility, CO_2, and temperature/growth period. The resulting eight treatment combinations were assigned to separate tanks, and five containers of hydrilla were planted in each.

Plant Structural Quality Evaluations. Structural quality of the plants was assessed based on the measurements discussed for Experiment 1. However, since no differences were noted in spine number or leaf length, these were not quantified for Experiment 2.

Nutritional Determinations. Analytical Research Services of the University of Georgia, Athens, GA, performed the nutritional analysis of aboveground plant tissues. They used a basic feed analysis known as a Proximate Analysis to provide crude estimates of the major nutritional groups including ether extractable compounds (EE; as a measure of fat content), crude protein (CP), ash (or mineral content), crude fiber (CF; cell-wall constituents), and nitrogen-free extract (NFE, representing soluble carbohydrates). Procedures for these analyses were provided at http://bluehen.ags.udel.edu/homepage/anfs/ansc251/lectures/Feeds/Feed_Analysis.html but are no longer available. However, they are provided at the end of this manuscript as an appendix. In addition, several additional mineral analyses were also accomplished using atomic absorption spectroscopy including total phosphorous (P), calcium (Ca), and magnesium (Mg) concentrations.

STATISTICAL ANALYSIS: All experimental data considered in this article were analyzed statistically using Statistica (StatSoft 1999) including ANOVA and linear regression. Unless otherwise noted, statements of significance made throughout the text refer to a 5 percent level or less of statistical confidence.

RESULTS AND DISCUSSION: The major objective of these experiments was to examine the feasibility of producing plants of widely varying nutritional composition (e.g., nitrogen and phosphorous concentrations) and physical characteristics (e.g., leaf toughness) but maintain low variability between replications of a specific treatment combination. In this regard we were highly successful in producing plants with wide nutritional compositions. For example, percent crude protein concentrations for plant tissues grown in "Fertilized" sediments supplied with "Low" CO_2 were almost threefold higher than tissues obtained from plants grown in "Used" sediments supplied with "High" CO_2 (Figure 2). Similarly, the same treatment combinations produced wide ranges (> 3.5-fold) in phosphorous concentrations (mg g^{-1}).

Similar ranges were observed for protein and phosphorous for tissues collected from plants grown under conditions described for Experiment 2. In this case, highest percent crude protein (22.5 percent) occurred for the Short/Warm, Fertilized sediment, High CO_2 treatment combination (Figure 3). This was close to 4.5 times higher than that observed for the Long/Cool, Used sediment, Low CO_2 and the Long/Cool, Used sediment, High CO_2 treatment combinations.

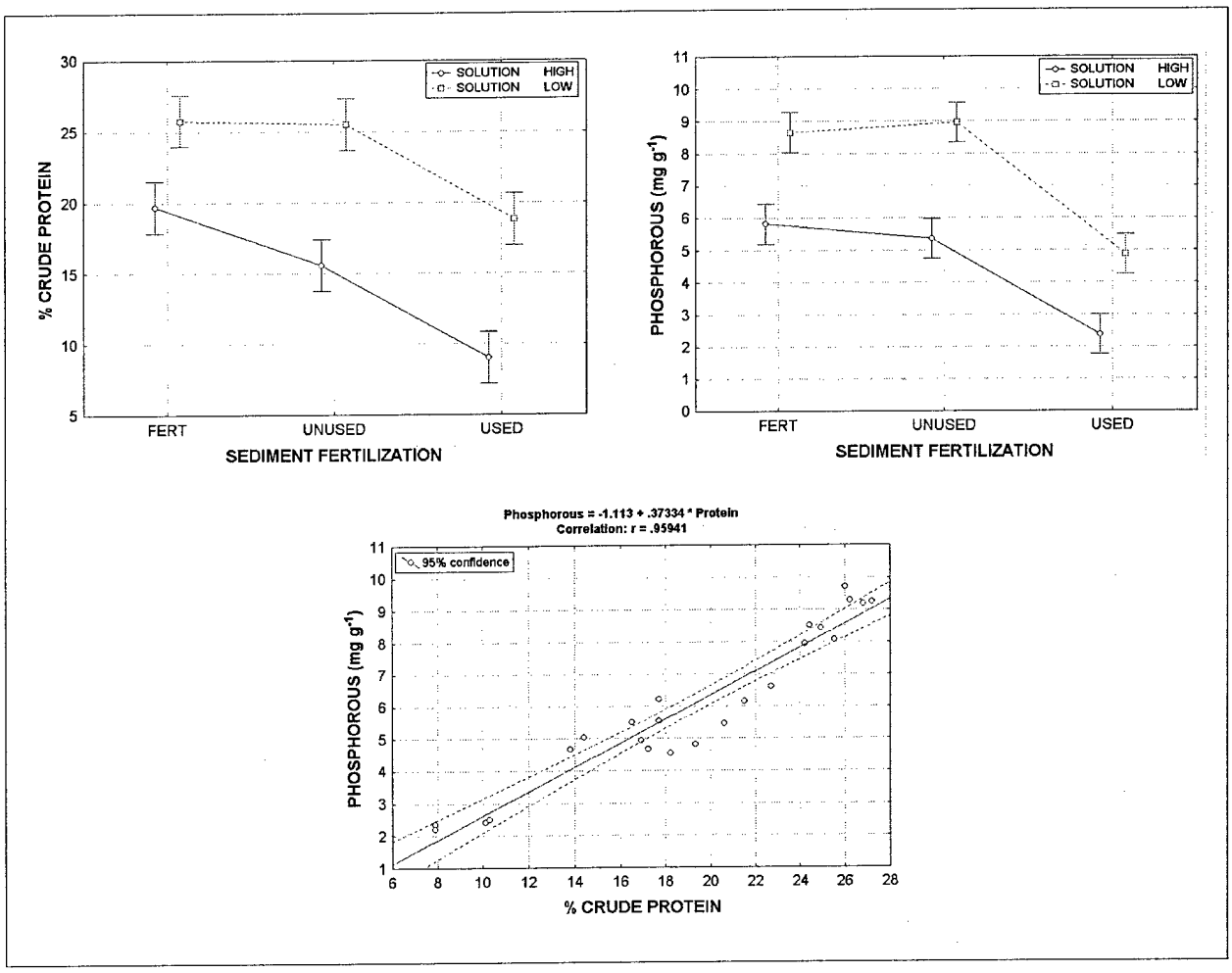

Figure 2: Percent crude protein and total phosphorous concentrations (mg g^{-1}) for plants grown under treatment combinations described for Experiment 1. Both the main treatment effects were statistically significant at: CO_2 Solution – F = 147.7, df = 1, 18, p < 0.0000; Fertilization – F = 56.1, df = 2, 18, p , 0.000; while the interaction term was only borderline significant (F = 3.2, df = 2, 18, p = 0.0660)

Similar ranges were observed for phosphorous concentrations where the Short/Warm, Fertilized sediment, High CO_2 treatment combination was over two times higher than both the Long/Cool, Used sediment, Low CO_2 and the Long/Cool, Used sediment, High CO_2 treatment combinations.

In addition, highly significant linear relationships between percent crude protein and phosphorous (mg g^{-1}) were noted for both experiments (Experiment 1: $p < 0.0001$, $r = 0.95941$; Experiment 2: $p < 0.0001$, $r = 0.89397$; Figures 2, 3, and 4) again indicating consistency in the plant culturing procedures for the different experiments. These graphs also illustrate the wide range of both protein and phosphorous concentrations formed in plant tissues under these experimental situations. For example, in Experiment 1, percent crude protein ranged from about 8 percent to a high of over 27 percent with phosphorous concentrations increasing linearly from 2 mg/g to over 9 mg/g (Figure 4). It is interesting that while positive linear relationships were noted for both experiments, the slope of these lines differed significantly ($F = 43.0$, $p = 0.034$,

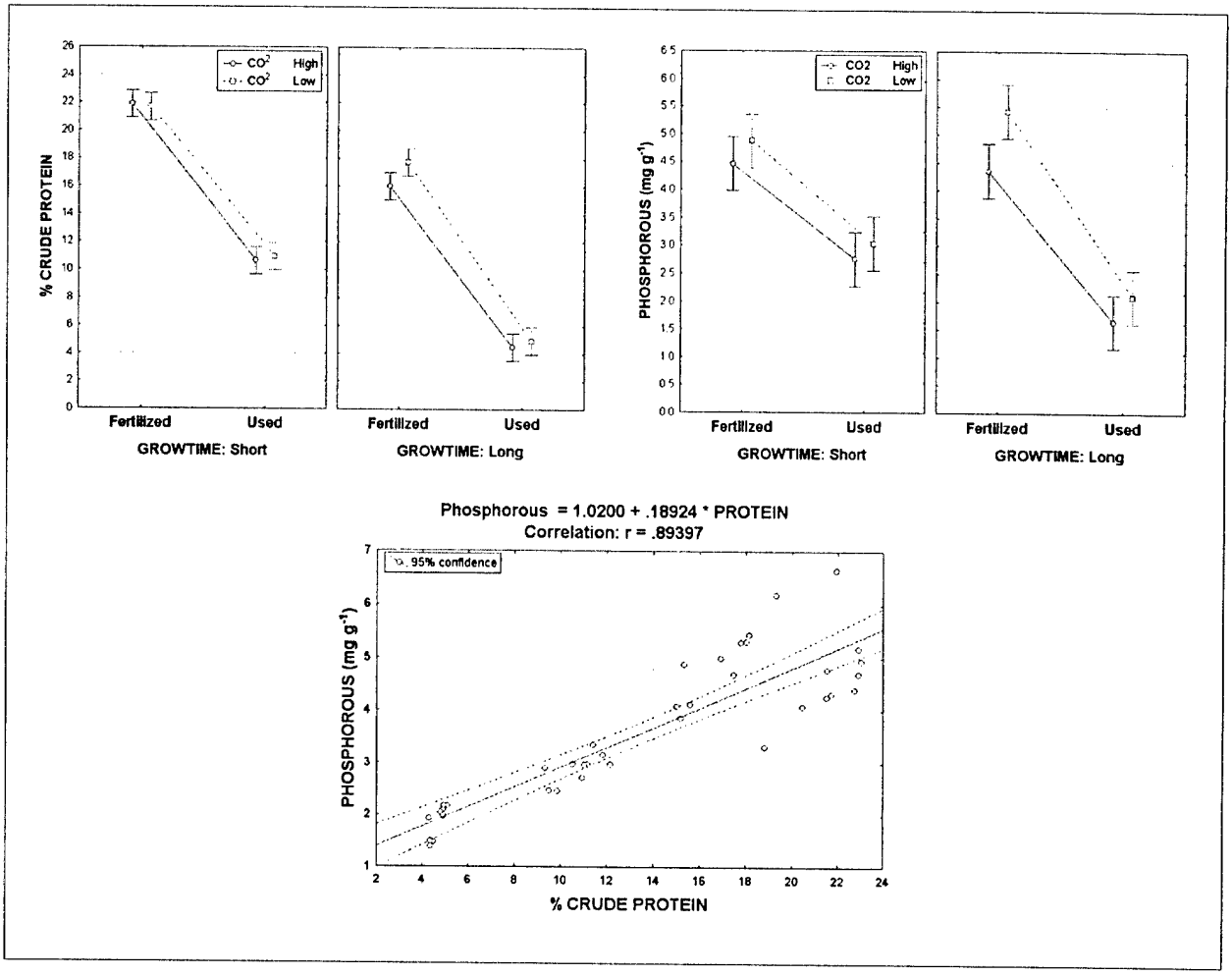

Figure 3: Percent crude protein and total phosphorous concentrations (mg g^{-1}) for plants grown under treatment combinations described for Experiment 2. Only the Fertilization and Growtime treatments were significant for crude protein (Fertilization - F = 1143.6, df = 1, 32, p < 0.0000; Growtime = F = 257.3, df = 1, 32, p < 0.0000). This differed for phosphorous where all main effects were significant as well as the Fertilization and Growtime interactive term (CO_2 – F = 10.7, df = 1, 32, p = 0.0025; Fertilization – F = 202.9, df = 1, 32, p < 0.0000; Growtime – F = 5.7, df = 1, 32, p = 0.0228, Fertilization X Growtime interactive term – F = 13.6, df = 1, 32, p = 0.0008)

df = 1) indicating that relationships between nitrogen and phosphorous are dependent on experimental and culturing conditions (Figure 4).

In addition to producing plants with wide ranges in nutritional parameters, minimizing variability among replicates within each treatment combination was also important. Generally, these experimental setups produced plants that had relatively consistent nutritional composition within a specific treatment and experiment type (i.e., variation among replicates was low for a given experimental setup). For example, coefficients of variation for the different treatment combinations for crude protein ranged from less than 2.5 percent to only about 15.0 percent of the mean (Table 2). By definition, "coefficient of variation" is a statistic used to describe the amount of variation in a characteristic or response of a population (Snedecor and Cochran 1980). The overall mean coefficient of variation for each experiment was not significantly different

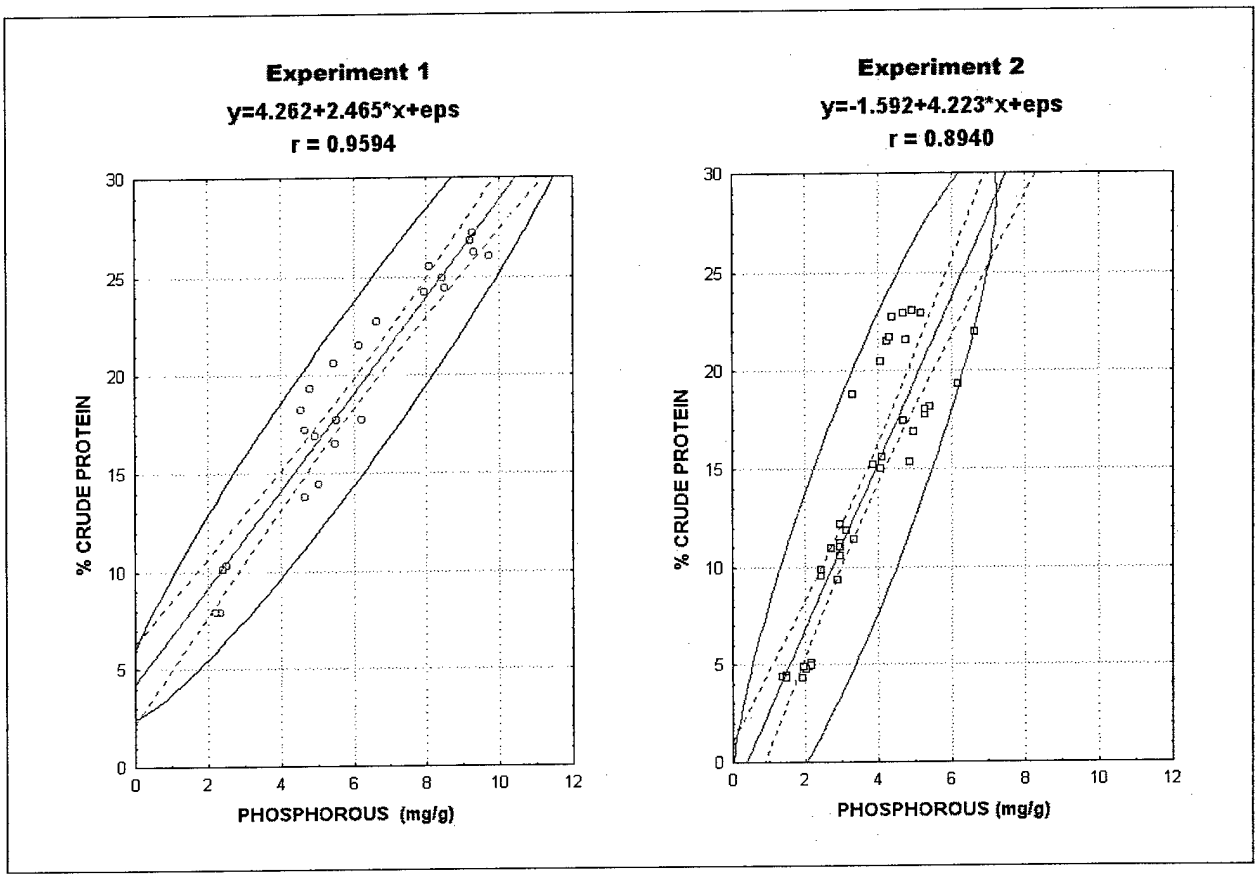

Figure 4: Relationships between percent crude protein and phosphorous (mg g^{-1}) for both experiments

(df = 1, 12; F = 2.21; p = 0.163). However, the overall mean coefficient of variation for percent crude protein for Experiment 1 was somewhat higher; i.e., Experiment 1 was equal to 9.6 percent while the mean percent crude protein coefficient of variation for Experiment 2 was lower, being only 6.8 percent. These variance components are relatively low considering that the number of replicates in each experiment was low and that biological measurements inherently have high variability. Similarly, low coefficients of variation were observed for phosphorous measurements (Table 2).

While nitrogen and phosphorous concentrations varied significantly in both experiments, differences in leaf hardness were less prevalent. Leaf hardness, an important physical characteristic shown to influence larval growth and survival (Wheeler and Center 1996), was unresponsive to treatment combinations provided in the first experiment (Solution – df = 1, 18, F = 2.76, p = 0.1137; Sediment – df = 2, 18, F = 1.17, p = 0.3331, interaction – df = 2, 18, F = 2.21, p = 0.1385; Figure 5). However, statistical differences were imposed by the Growtime variable in Experiment 2 (Growtime – df = 1, 32, F = 47.23, p < 0.0000; Figure 6), where hardness was almost three times higher for plants grown for a longer period under cooler temperatures.

Table 2
Coefficient of Variation for Means Generated for Each Treatment Combination and Experiment for the Nutritional Parameters Crude Protein (percent dry weight) and Phosphorous (mg g^{-1})

Treatment			Coefficient of Variation (% of Mean) % Crude Protein	Coefficient of Variation (% of Mean) Phosphorous (mg/g)
Experiment 1				
CO_2	**Sediment**			
High	Fertilized		14.38	12.62
High	Unused		11.64	12.61
High	Used		14.70	5.17
Low	Fertilized		4.89	8.45
Low	Unused		4.23	6.75
Low	Used		7.76	8.31
Experiment 2				
CO_2	**Sediment**	**Grow/Temp**		
High CO_2	Fertilized	Short/Warm	4.58	6.37
High CO_2	Fertilized	Long/Cool	6.86	10.88
High CO_2	Used	Short/Warm	8.82	11.17
High CO_2	Used	Long/Cool	5.66	16.96
Low CO_2	Fertilized	Short/Warm	7.90	25.26
Low CO_2	Fertilized	Long/Cool	8.28	8.768
Low CO_2	Used	Short/Warm	9.72	5.92
Low CO_2	Used	Long/Cool	2.26	4.16

It is interesting that no correlations were detected between leaf hardness and plant tissue nitrogen concentration. Wheeler and Center (1996) observed strong relationships between these variables with harder leaves associated with lower concentrations of nitrogen. Because of this correlative effect, it was impossible to separate the influence that leaf toughness had on larval survival and development from that caused by tissue nitrogen levels. However, the plant culturing methods described here allowed the development of plants with varying nitrogen percentages with no significant changes in leaf hardness. Hence, the impact of nitrogen on larval survival, fecundity, and development can be ascertained without influence of leaf hardness. However, more research is needed to determine what factors influence the formation of tougher or harder leaves in hydrilla since this has been implicated as an important factor. One reason for using different concentrations of CO_2 in these experiments was to vary alkalinity since changes in alkalinity were thought to be responsible for producing variation in leaf hardness. However, changes in alkalinity due to increased CO_2 concentration apparently had little or no effect on leaf hardness.

Overall, these procedures for plant culturing provide researchers with excellent tools for assessing the impact of nutrition on insect herbivores and plant pathogens of hydrilla. Because plants can be grown with wide-ranging nutritional compositions but with minimal variation between replications and batches, test plant material can be readily available for experimental purposes. This was an important first step in evaluating the influence of nutritional composition on biological control agents of hydrilla.

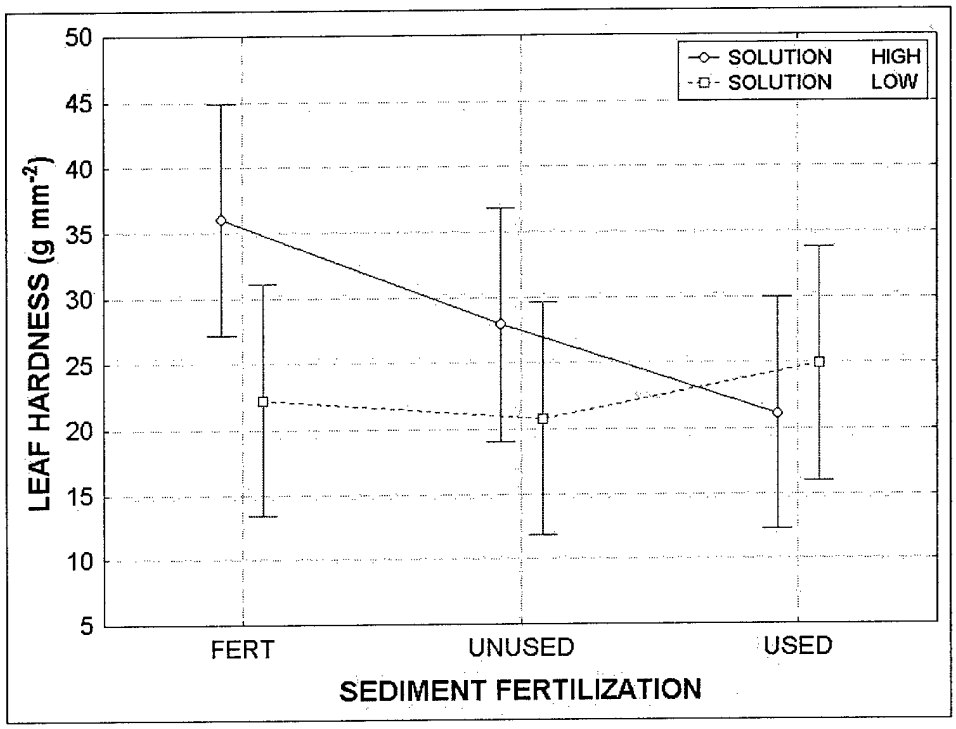

Figure 5: Leaf hardness (g mm^{-2}) for treatment combinations used for Experiment 1. Note that bars surrounding means are 95-percent confidence intervals and no significant differences were noted for any variable

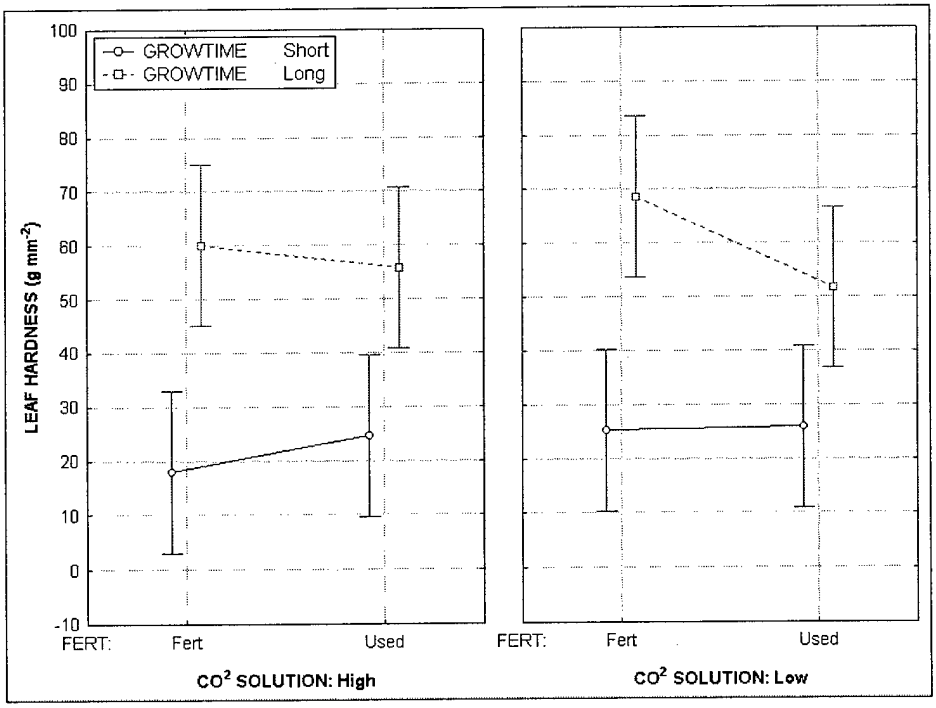

Figure 6: Leaf hardness (g mm^{-2}) for treatment combinations used for Experiment 2. Note bars surrounding means are 95-percent confidence intervals and significant differences were noted for the variable Growtime only (df = 1, 32, F = 47.23, p < 0.0000) where higher leaf hardness occurred for plants grown for longer periods and under cooler temperatures

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Appendix A: Proximate Analysis Procedures as Taken from the Web Address -

http://bluehen.ags.udel.edu/homepage/anfs/ansc251/lectures/Feeds/Feed_Analysis.html

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Evaluating the Nutritional Quality of Feedstuffs
Proximate Analysis

MARGINAL NUTRITION

Steps in Feed Ingredient Analysis

Obtain a Representative Sample

The sample must be REPRESENTATIVE of the entire lot of an ingredient

Coring Hay

- Lots vary by size
  - ° Feed bag, bin, truck, or rail car
- Lots vary by storage technique
  - ° Baled hay, silage
- Sampling techniques and equipment also vary
  - ° Grain core sampler
  - ° Hay core sampler
  - ° Silage sampling

Prepare Sample

Wiley Mill

- Reduce particle size to ensure representative "sample of the sample"
- Grind sample to pass through a 1-mm sieve

Proximate Analysis

- Developed in Germany in 1860's
- Relatively rapid, inexpensive
- Requires small sample size
- Involves six analytical procedures

Feed Label

  - ° Dry Matter Determination
  - ° Ether Extract Determination
  - ° Crude Fiber Determination

° Crude Protein Determination
° Ash Determination
° Calculation of Nitrogen-Free Extract

Proximate Analysis

Dry Matter Determination

- Removal of water from sample
- One of three methods is used:
  1. Dry at 100-105 °C for 8 hr or overnight.
  2. Dry under vacuum at 60 °C.
  3. Dry in forced-air oven at 150 °C for 2 – 4 hr.

Dry Matter (DM) Calculation					
Status	Dish & Sample		Dish Wt.		Sample Wt.
Before Drying	3.19 gm	-	1.14 gm	=	2.05 gm
After Drying	3.04 gm	-	1.14 gm	=	1.90 gm
Percent DM = Sample Wt. After Drying / Wt. Before Drying * 100% = 92.7% DM					

Ether Extract Determination

- Designed to determine the LIPID or FAT content of feed ingredients
- Involves extraction of lipid and lipid-soluble components with an organic solvent
- Method:

  1. Weigh 2-g sample into sample thimble.
  2. Weigh extraction beaker.
  3. Extract with anhydrous ether on Goldfisch apparatus at a condensation rate of 4-6 drops/sec for 4 hr.
  4. Weigh extract and calculate percent ether extract.

Ether Extract (EE) Calculation					
Before Extraction	Thimble & Sample		Thimble Wt.		Sample Wt.
	6.65 gm	-	4.39 gm	=	2.26 gm
After Extraction	Beaker & Extract		Beaker Wt.	=	Ether Extract Wt.
	42.25 gm	-	41.98 gm	=	0.27 gm
Percent DM = Ether Extract Wt. / Sample Wt. * 100% = 11.9% EE					

- Extract contains lipids, plant pigments (e.g., chlorophyll, carotene)
- Non-lipid portion may represent as much as 25-40% of the total extract for some plant materials

Crude Fiber Determination

- Designed to determine the STRUCTURAL CARBOHYDRATE component of feeds
- Based on removal (by acid and alkali digestion) of all other components of feed
- Method:
    1. Weigh 2-g sample into Berzelius beaker.
    2. Add 200 ml 1.25 N H_2SO_4. Place on fiber rack and reflux for 30 min. Filter, rinse, return to beaker.
    3. Add 200 ml 1.25 N NaOH. Reflux for 30 minutes.
    4. Filter, rinse and dry in pre-weighed crucible.
    5. Weigh crucible containing the residue (crude fiber plus ash).
    6. Ash at 500 °C for 2 hr, cool, weigh crucible containing the ash.
    7. Calculate percent crude fiber.

Rinse and Filter

Crude Fiber (CF) Calculation	
Sample Weight	2.58 gm
Crucible Weight	31.14 gm
Crucible & Dried Residue	31.27 gm
Crucible After Ashing	31.24 gm
Dried Residue Weight	0.13 gm
Residue Ash Weight	0.10 gm
Crude Fiber Weight	0.03 gm
Percent DM = Crude Fiber Wt. / Sample Wt. * 100% = 1.2% CF	

Crude Protein Determination

- Designed to determine the PROTEIN content of feed ingredients
- Based on assumption that proteins contain 16% nitrogen

NITROGEN BY KJELDAHL METHOD (as in John Kjeldahl)

- Determination is based on assumptions:
  - ° All nitrogen in the ingredient is in the form of protein
  - ° All protein in the ingredient contains 16% nitrogen (100/16 = 6.25)
- Exceptions:
  - ° Milk protein contains 15.7% N (6.38)
  - ° Wheat protein contains 17.5% N (5.71)
  - ° Crude protein contains true protein and non-protein nitrogen (amino acids, amides, urea, etc.)
- Method:
  1. Weigh 0.5- to 2-g sample, place in Kjeldahl flask.
  2. Add concentrated sulfuric acid and heat for 2 hr.

Johan Kjeldahl

N in Sample + H_2SO_4 → $(NH_4)_2SO_4$

Distilling Off NH_3

  3. Add concentrated sodium hydroxide to flask and distill off ammonia.

$(NH_4)_2SO_4$ + NaOH → NH_3 + Na_2SO_4 + H_2O

  4. Trap NH_3 in boric acid solution containing an indicator.
  5. Titrate with standardized H_2SO_4 to determine N content.
  6. Calculate percent crude protein (N x 6.25).

Crude Protein (CP) Calculation	
Sample Weight	2.0196 gm (2019.6 mg)
Amount of standardized acid required to titrate N in sample (each ml = 2 mg N)	16.54 ml
Mg N in sample	33.08 mg
Percent N in sample = mg N in sample / mg sample * 100 % = 1.64% N	
Percent Crude Protein = % N * 6.25 = 10.25 % CP	

Ash Determination

- Designed to determine the MINERAL (inorganic) component of feed ingredients
- Based on ignition of all organic matter
- Method:
  1. Weigh 2-g sample into pre-weighed crucible.
  2. Ash at 600 °C for 2 hr in pre-heated "muffle furnace."
  3. Cool in desiccator, weigh.
  4. Calculate percent ash.

Muffle Furnace

Ash Calculation					
Status	Crucible & Sample		Crucible Wt.		Sample Wt.
Before Ashing	11.97 gm	-	9.39 gm	=	2.58 gm
After Ashing	9.48 gm	-	9.39 gm	=	0.09 gm
Percent Ash = Ash Wt. / Sample Wt. * 100% = 3.49% Ash					
Percent Organic Matter = 100 – Percent Ash = 96.51% Organic Matter					

Calculation of Nitrogen-Free Extract

- Designed to provide an estimate of the SOLUBLE CARBOHYDRATE component (sugars and starches) of feed ingredients

% NFE = 100 – (% H_20 + % Ether Extract + % Crude Fiber + % Crude Protein + % Ash)

Nitrogen Free Extract (EE) Calculation		
Dry Matter 92.7%		
	% As Fed Basis	% Dry Matter Basis
1. Ether extract (crude fat)	11.03	11.9
2. Ash (minerals)	3.24	3.49
3. Crude protein (N * 6.25)	9.50	10.25
4. Crude fiber	1.11	1.2
5. Moisture (100 - %DM)	7.3	0
Sub-total	32.18	26.84
6. Nitrogen-free extract	67.82	73.16
Total	100%	100%

Limitations of Proximate Analysis "Quantitative not Qualitative"

- Crude Protein
  - ° Nitrogen can come from NPN, urea, or amino acids
- Ash
  - ° Total mineral content is not separated into individual components
- Ether Extract
  - ° No information on individual fatty acids
  - ° Not just lipids in extract – waves and pigments have no nutritional value

- Nitrogen-Free Extract
  - ° Determined by difference errors in other analysis are compounded
- Crude Fiber
  - ° Does not differentiate structural components
- No vitamin determination

VAN SOEST ANALYSIS

Van Soest Method of Fiber Analysis (Detergent Method)

ADF Apparatus
Reflux of Acid

- Neutral Detergent Analysis (NDF):
  - ° Hemicellulose
  - ° Cellulose
  - ° Lignin
- Acid Detergent Analysis (ADF)
  - ° Cellulose
  - ° Lignin
- Acid Detergent Lignin Analysis (ADL)
  - ° Lignin
- NDF – ADF = Hemicellulose
- ADF – ADL = Cellulose
