Chemometric Analysis of Compositional Variation in Bison and Cow Patties: A Biogeochemistry– Environmental Chemistry Experiment

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Abstract: Manure has been used as a fertilizer throughout recorded history; however, the high levels of nutrients in farm and feedlot runoff present many environmental problems. Analyses of nutrient cycling in manure and soil litter are important to issues such as global warming and land use. In this laboratory, chemical differences in aged manure patties from buffalo (*Bison bison*) and cow (*Bos taurus*) raised on ranches versus nature preserves are examined. These measurements offer a chance to introduce the basics of chemometrics such as factorial experimental design and multivariate analysis in an environmental context that captures student interest. These important methods are appropriate to instrumental analysis or environmental chemistry, but are rarely integrated into these courses.

Introduction

Nitrogen is usually the limiting nutrient in the formation of enzymes imperative to photosynthesis and respiration. Over the last several decades, human activity has approximately doubled the amount of nitrogen that is fixed naturally each year. Cattle feedlots alone contribute about 4×10^{11} g N yr^{-1} [1]. Although this is a relatively small fraction of the total, the high concentration of nitrogen in farm and feedlot runoff creates significant local problems in ground water and streams [2].

As a method for restoring a natural balance, there is interest in trying to re-establish ecological communities that have evolved in a given locale. In North America, bison rather than cattle were the dominant herbivore on the plains and in the mountain valleys of the American West. After being hunted nearly to extinction, small herds are being re-introduced in national parks and preserves as well as being raised commercially for food.

Buffalo (*Bison bison*) meat has a lower percentage of fat than that from cows (*Bos taurus*) and correspondingly higher protein percentages. Bison are also reported to have higher digestion coefficients for their feed [3]. These observations led a junior-level undergraduate class in environmental biogeochemistry to conjecture that bison will be more efficient with their nitrogen than cows grazing the same range. If true, their manure might then be less damaging to the environment as fewer nitrates might be released.

To partially test this hypothesis, two pairs of bison and cattle raised on adjacent ranches that had equivalent soil, climate, solar aspect, and vegetation were selected for sampling after a discussion about two-level sampling designs. Variables such as the age, growth rate, fodder selection, and gender of the animal were not studied in the time frame of the course. The class hypothesized that when controlled for variations in weathering of the patties, bison manure would have a higher ratio of carbon to nitrogen than cow manure. For the sake of simplicity, it was assumed that bison will conserve other mineral nutrients similarly. These hypotheses led to a variety of chemical analyses that are of relevance to biogeochemistry, such as the measurement of nitrogen, carbon, phosphorus, trace minerals, and the kinetics of decomposition.

In this experiment, students are expected to use the scientific literature and standard handbooks to obtain background information and procedures. The students are also required to use the instructions to authors from a relevant scientific journal to prepare a paper that is the expected outcome of this five-day laboratory. An introduction to Global Position and Geographic Information Systems (GPS and GIS), and soil and plant survey maps may also be relevant, but is not necessary. As weathering of samples was a factor, some creativity in method development for dating the samples was necessary, adding an important exploratory component.

Experimental

Experimental Design. In order to monitor the many variables inherent in environmental analyses, an appropriate sampling design must first be constructed [4]. The use of balanced two-level factorial designs followed by ANOVA is sometimes difficult to achieve in real field studies, as not all possibilities may be available. The underlying assumption of normality in each variable may also be inaccurate. For example, in this experiment, the three variables tested are bison versus cow, fresh versus old patties, and nature preserves versus commercial ranches. The first variable is categorical and it comfortably conforms to the two-level model with about fifty bison or cows available to be sampled at each location. The second variable, age, is a continuous distribution and a regression model is a more logical choice. The last variable, location, is a two-level categorical variable that fits the model, but differences in the forage selected by bison compared to cows may confound the design. Students in a variety of disciplines should be introduced to the common difficulties encountered in experimental designs. They should also learn how to work with the limitations of incomplete statistical designs.

Sample Collection. The class collected a total of twenty samples of fresh and aged cow and bison manure from the Nature Conservancy's Zapata–Medano Ranch in the San Luis Valley of Colorado and from commercial bison ranches and cow ranches that were near each other in South Park, Colorado. A 50-to-100-cm^3 portion of the interior of each patty was sealed in a polyethylene bag and refrigerated in a portable cooler. After being mixed in the bag,

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Table 1. Summary of the Data for the Full-Factorial Experimental Design Analysis

Animal:	Bison	Bison	Cow	Cow
Source:	Nature Preserve.	Ranch	Nature Preserve.	Ranch
Fresh (3-day average age)/weathered (18-day average age)				
Major components (%)				
Moisture	82.6/10.3	80.8/17.0	83.0/30.9	79.5/9.1
Nitrogen	1.48/1.20	1.41/1.39	1.28/1.26	1.44/1.26
Carbon	37.6/32.7	38.1/44.2	42.6/41.0	38.6/39.8
Minor components (ppm)				
Total Chlorophylls	1037/27	862/14	332/4	384/13

portions were dried overnight at 100 °C to obtain the percent moisture so that the other analyses could be based on the dry weight.

Soil samples were obtained by the class at each of the four locations with a 10-cm soil corer. A combination of pH, sieving, and flotation was used to classify the alluvial soils as fine, loamy calcareous entisols (pH of slurry = 9.8 ± 0.4) at the preserve and fine, loamy noncalcareous entisols (pH = 5.8 ± 0.3) at the ranches. Some mollisols were also present in the wetter areas of the preserve. These values were in agreement with published values looked up by the class [5].

Representative forage plants were collected from each site by cutting samples at least 2 cm above the ground. Dominant plants identified in 10-m^2 plots at the nature preserve were various rushes (*Juncus sp.*) and sedges (*Carex sp.*) with salt grass (*Distichlis spicata*), blue grama grass (*Bouteloua gracilis*), and *plantago sp*. also present. Larger plants not sampled but found in drier areas included greasewood, rabbitbrush, and four-wind saltbush. The major forage plant at the ranches, which were grazed much more heavily, was blue grama with small amounts of thistle (*Cirsium sp*.), sage (*Artemisia frigida*), and a shrub (*Gutierrezia Sarothrae*) that is an indicator of over-grazing. There was also lichen (*Rhizoplaca haydenii*) covering portions of bare patches on the ground that has not been previously reported in Colorado.

Sample Dating. The class estimated the age of the manure samples in the field by a visual inspection. This was later quantified in the laboratory by counting the relative numbers of different insect larvae and recording their stages of maturity. The average sample temperature at the site was used in a formula for horn-fly larvae to obtain the mean time spent in each larval stage [6]. Alternative dating methods (not employed here) that have been related to the age of manure samples, and which may be implemented in some undergraduate programs, include measuring bacterial and fungal survival rates as well as monitoring the surface oxidation–reduction potential and pH over time [7–9]

Instrumental. The class ground and dried samples that were analyzed the next day for carbon and nitrogen using a combustion analyzer (Carlo Erba NC2100). In order to measure the rate of decomposition of the original organic nitrogen, the sum of the chlorophyll and pheophytin-a were measured on samples that had not been dried. The absorbance at 664 nm (HP 8452A) of a 90% acetone 10% water extract (corrected for sample water content) was used for this analysis [10]. Extracts of 0.500 g were diluted to 40.0 mL and the concentration was measured using an absorption coefficient of 11.59 ppm (0.863×10^4 m^2 mol^{-1}) [11, 12]. The extracts had negligible remaining particulate absorbance at 750 nm after centrifugation at 3400g for 20 min. (Beckman J2-HS). Fresh aqueous extracts were prepared in the same way with subsequent filtering for anion analysis by ion chromatography (Dionex Model 40 with an AS4A-SC column). Elemental analysis of 1.00-g samples was conducted by ashing at 580 ºC and then diluting with 20.0 mL of 5% HNO_3 (trace metal grade) for analysis by ICP–OES (Thermo Jarrell Ash Atomscan-16).

Statistical Analysis. Results of the various determinations were entered into the statistical package Minitab version 12 (State College, PA) for analysis using both linear regression and a custom two-level factorial design. The data were further processed using multivariate regression and cluster analysis as an introduction to multivariate analysis methods [13].

The ability to develop and analyze customized experimental designs and to carry out cluster analyses is not available in all desktop computer statistics packages [14]; however, educational institutions commonly support Minitab, Systat, or SPSS (SPSS, Chicago, IL) for introductory statistics courses and these packages are also available in desktop versions.

Results and Discussion

Experimental Designs. A summary of the mean results for the eight possible combinations of the three variables appears in Table 1. (Replicate sample data used elsewhere is not shown. An important point to make with students is that full-factorial designs do not require replicates in order to make error estimates if interaction terms are assumed to be negligible.)

As expected, weathering decreased the mean values and increased the variability over the age of these samples. The most significant effects are seen in those components that are not recycled within the patty (moisture and total chlorophylls). Over the estimated lifetime of these samples (three to four weeks), percent carbon does not change significantly, as cellulose and lignin in the plant fibers are relatively robust [15]; however, nitrogen loss through gaseous emissions is expected and is variable depending upon moisture levels and whether aerobic or anaerobic conditions are present.

A full factorial design such as that presented in Table 1 can be readily calculated by hand if appropriate computer packages are unavailable or if more in-depth training is desired [16]; however, Minitab allows customized two-level experimental designs (even incomplete designs) if the user assigns factors (variables) in a column with a –1 or 1 for each variable to indicate a high or low level, respectively. For example, cow, commercial ranch, and “fresh” samples were assigned a –1 in Table 2, and their counterparts were labeled with a +1. The third order interaction was used to obtain the replication error in this example because interactions between the variables might be of interest, but a three-way interaction is unlikely to be significant. (In the field, replicates were also collected in the original design so that greater degrees of freedom existed in the results reported elsewhere in this paper.)

The results of the statistical analysis suggest that the age and the original chlorophyll concentration are the most significant effects for the chlorophyll and that the next most significant is the animal variable. If replicates are included in the design, greater than 95% significance was obtained for these determinations.

Most upper-level college students are familiar with linear regression and it makes sense to apply this approach to age (or time)-dependent data. As manure dries out and is weathered, the nitrogen that is bound up in organic molecules such as chlorophyll is converted by bacterial degradation to NH_4^+, and then potentially to NO_3^-, if it is not otherwise re-utilized during the process [17]. A plot of the natural logarithm of the chlorophyll plus pheophytin (chlorophyll that has lost its Mg^{2+}) concentration versus time yields a straight line with high confidence (p = 0.000) for this first-order decay process (Figure 1). The slope can be used to obtain the half-life ($t_{1/2}$ =

Table 2. Statistical Analysis of the Experimental Design For the Total of the Chlorophyll Analogs

Estimated effects and coefficients for "total" (coded units)					
Term	Effect	Coef.	St.Dev. Coef	T	P
Constant	334.12	25.62	13.04	0.049	
Animal	301.75	150.87	25.62	5.89	0.107
Source	31.75	15.88	25.62	0.62	0.647
Age	639.25	319.63	25.62	12.47	0.051
Animal source	62.25	31.13	25.62	1.21	0.438
Animal age	289.75	144.87	25.62	5.65	0.111
Source age	29.75	14.88	25.62	0.58	0.665

Analysis of variance for "total" (coded units)						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main effects	3	1001403	1001403	333801	63.54	0.092
2-way interactions	3	177430	177430	59143	11.26	0.215
Residual error	1	5253	5253	5253		
Total	7	1184087				

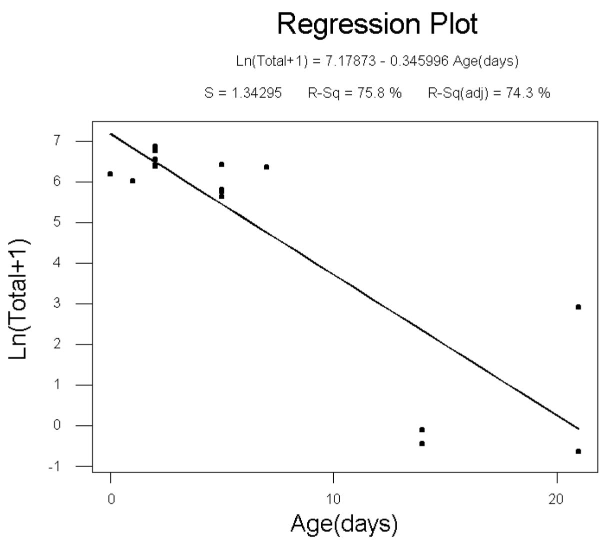

Figure 1. Loss of chlorophylls over manure with time. Total chlorophyll and pheophytin used to determine the age of manure patties. A half- life of 2 days is obtained from this mixed cow and bison data.

2 days) for the loss of the original organic nitrogen. The intercept (the constant in the experimental design results) can also be used to estimate the initial concentration of total chlorophylls (1311 ppm). This value is a little higher than the measured concentration in fresh grass clippings (895 ppm) although the difference is not significant. As expected for samples that have passed through the digestive tract, the chlorophyll analog in these samples is primarily pheophytin rather than chlorophyll.

Although a multiple-regression model poorly fits the two-level design used for the location and animal variables, it still achieves significance (p = 0.034) for a multiple regression of the C-to-N ratio versus the chlorophyll concentration. (The animal and location variables were entered as ±1.) When the variables' effects are examined in this manner, a greater significance for the animal is obtained than for the location, and the bison data are (on average) about twice as high as the cow data in the initial chlorophyll concentration. A two-sample t test (pooled s_x) of the data for the total chlorophylls in the fresh manure (Table 1) gives a significant difference (p = 0.014) between bison and cow.

As the organic nitrogen is transformed, NO_3^- levels might be expected to increase temporarily and then decrease as the overall nitrogen levels are lowered. Although it was difficult to measure NO_3^- by ion chromatography in these concentrated samples due to the presence of large amounts of Cl^-, the general trend (data not given) supports this expectation (p = 0.022) as well as suggesting that NO_3^- is rapidly reabsorbed by biota living in the manure. The remaining anion data (not presented) did not exhibit any significant differences for these variables. This suggests that phosphorus is less mobile than nitrogen (as expected) and that washout was not significant in these arid regions or Cl^- should also vary along with the NO_3^-.

In Figure 1 it is apparent that the samples chosen do represent two different populations in the time domain (new and old samples); therefore, our earlier assumption of a two-level factorial design with bison versus cow, old versus fresh sample, and commercial versus nature preserve, was not unreasonable.

Multivariate Analyses. A number of different multivariate methods are commonly employed in various branches of science that are relevant to environmental chemistry. One of the easiest of these for students to grasp is cluster analysis. In this method, a "profile" of measurements made on a sample is compared to the profile on the other samples, and those samples with similar profiles are clustered together. Sample clusters with increasingly divergent composition are connected in a tree (dendrogram) with branches that are further and further apart. There are a number of nuances that affect the best choice of clustering method, but good results are generally obtained on metric data (e.g., composition data) with Ward's method on squared Euclidean distances, once the data is standardized to remove the weighting of one component over another. Some care should be taken to look for outliers, and in this case it is more appropriate to log-transform the data instead of the more common "normalization" [18]. These steps are readily achieved in Minitab simply by clicking the appropriate boxes. In Table 3, the elemental data that were obtained using ICP are presented.

In Figure 2, a dendrogram based on this elemental data for three manure samples for each of the four experimental design combinations (excluding age), is presented. Samples 1 through 3 correspond to bison on the preserve, 4 through 6 to cows on the preserve, 7 through 9 to bison on the ranches, and 10 through 12 to cows on the ranches. The dominant grass from each location is presented in the same order for samples 13 to 16.

Clearly, the replicate samples from the same location cluster together, as would be expected. At the next level of connection, it becomes apparent that the location is also significant, as those samples from the Nature Conservancy Preserve (cow or bison) cluster together, and those from the two different South Park commercial ranches also cluster together, but with greater variability. It is also interesting to note that the cattle's variance is greater than the bison's. This may reflect a wider range of fodder utilization by cattle.

Table 3. Element Data for Manure and Plant Samples

Sample	Ca	Cu	Fe	K	Mg	Mn	P	Pb	Sr	Zn
Buf-Nat-1	11,924	9	181	2,787	3,569	110	3,163	9	101	17
Buf-Nat-2	11,129	13	193	2,939	3,592	79	3,126	10	97	18
Buf-Nat-3	12,072	8	222	3,217	3,662	97	4,040	10	97	25
Cow-Nat-1	11,772	4	147	4,258	981	95	2,221	6	65	9
Cow-Nat-2	8,988	5	280	1,415	1,616	84	1,440	6	55	6
Cow-Nat-3	11,017	9	223	1,104	3,120	118	2,198	10	63	22
Cow-Ran-1	12,769	7	666	8,200	2,836	73	3,791	12	106	19
Cow-Ran-2	31,412	21	1,116	5,484	6,931	156	2,581	24	289	42
Cow-Ran-3	8,823	7	873	5,451	2,255	102	2,408	15	67	21
Buf-Ran-1	12,581	14	764	4,895	3,399	171	4,790	15	109	53
Buf-Ran-2	12,544	15	727	3,136	4,666	175	3,455	19	117	44
Buf-Ran-3	15,615	17	978	5,199	3,337	171	3,605	17	116	73
Buf-Plnt-Nat	4,169	4	61	17,102	1,920	11	1,487	6	12	10
Cow-Plnt-Nat	3,583	3	43	6,913	2,598	31	908	7	23	4
Cow-Plnt-Ran	4,262	6	121	9,545	1,575	34	1,580	10	24	14
Buf-Plnt-Ran	3,499	4	323	6,156	1,507	20	1,625	12	17	14

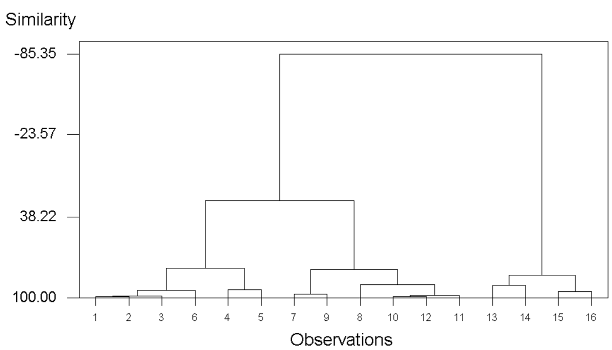

Figure 2. Cluster analysis of plant and manure samples. Manure samples 1–3 were obtained from bison on a nature preserve; 4–6 are from cows on the same preserve, 7–9 from bison on a commercial ranch, and 10–12 from cows on a nearby ranch. Dominant grass samples from the preserve (13 and 14) and the ranches (15 and 16) are also shown. The variables are the elemental profiles of these samples.

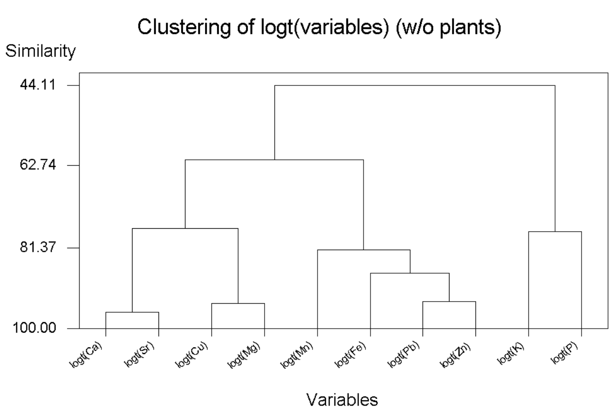

Figure 3. Cluster analysis of element samples. An R-mode cluster analysis of the manure samples illustrating the relationship between the elements used to cluster the samples in Figure 2. Three major clusters are present, which represent the major nutrient elements (K and P), the differences in the alkaline earths present in the underlying soil (Ca, Mg, and Sr), and the transition metals.

Finally, the completely dissimilar plant samples are connected in Figure 2 at a “negative” similarity.

The variables that were used to construct the first dendrogram can also be examined with a cluster analysis by choosing “cluster variables” (Figure 3). This dendrogram shows how close the variables or “factors” used to differentiate the manure samples are to each other. (The plant data was excluded because variations between manure and plants were not factors being studied in this experiment.)

This plot is sometimes referred to as a “poor-man’s factor analysis” as it gives us a handle on the number and nature of the factors that separate our data into clusters. Students find this much easier to interpret than jumping directly to a principle component analysis. From Figure 3, we can see that there are three major clusters of elements, and if we treat *K* and *P* as two different clusters, there may even be four factors. This is precisely what is found if a scree plot is constructed using factor analysis. From a chemical perspective, these clusters make sense as they reflect the major groupings found in the periodic table; thus, alkaline earth elements lump together as do transition metals. These clusters reflect, not just basic chemistry, but the impact of the location variable (with the more calcareous soil in the San Luis Valley), the animal variable (the bison had higher levels of transition metals), and the ranch versus the preserve variable (the ranches had higher metals). The higher level of transition metals in the bison manure may be due to a species variation in what fodder is selected or to differences in how the animal processes its food. Higher levels of iron and selenium and lower levels of calcium have been observed in bison meat compared to beef [19, 20]. These variables may be further elucidated with a discriminant function or factor analysis depending on the time available.

Conclusions

Careful experimental designs greatly increase the significance of results from small sample sizes as well as offering the possibility of employing more powerful methods of analysis. In this study the class found that there were significant differences in many of the components analyzed, especially the total chlorophyll analogs and transition metal levels. These differences may be a result of the fodder choices made by each animal, or they may reflect how the animals process the plant foods or both. Although the initial hypothesis that bison would have a higher C to N ratio than cows was not shown to be significant, the bison do seem to have higher ratios of organic nitrogen compared to inorganic nitrogen than the cattle, assuming the total chlorophylls and pheophytins represent the majority of the organic nitrogen. If this ratio is real, it represents a difference that has significant environmental consequences, as the organic nitrogen may be more easily assimilated by other biota without leading to emission of N_2O, an important greenhouse gas. Further study of these differences and of ammonia nitrogen and gaseous emissions should be enlightening. A student-initiated follow-up research project to more fully quantify the nitrogen cycling in bison manure in various biomes is already under way.

This study also provides an exercise for introducing chemometric principles to environmental science or instrumental analysis students, and a class can readily devise many other similar studies. For example, elk and antelope browse on the same land as bison and cow. In what ways does their dung reflect this similarity, and in what ways does it differ? Schools that cannot afford extended field trips might visit the local zoo, farm, or animal shelter. Do animals with different alimentary canals process their diets more effectively, and just how efficient are each of them? What kind of energy advantages does this represent in the wild?

Finally, this study illustrates a new use of chlorophyll and pheophytin measurements that are readily adapted to a host of other nitrogen cycling experiments. The measurements are easily learned, the reagents are relatively safe, and the equipment required is widely available. When coupled with measurements of the ash and estimations of the carbon content from muffle furnace combustion, fairly sophisticated analyses can be obtained from simple methods.

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