Seventh Quarter Progress Summary for the IPEC project titled,

"Using Plants to Remediate Petroleum-Contaminated Soil - Project Renewal"
EPA Grant Number: R827015-01-0

Principal Investigators:

Greg Thoma, Dept. of Chemical Engineering, 3202 Bell Engineering, University of Arkansas, Fayetteville, AR 72701, (479) 575-7374, gthoma@uark.edu

Susan Ziegler, Dept. of Biological Sciences, 416 Science Building, University of Arkansas, Fayetteville, AR 72701, (479) 575-6342, susanz@uark.edu

Duane Wolf, Dept. of Crop, Soil, and Environmental Sci., 115 Plant Sciences, University of Arkansas, Fayetteville, AR 72701, (479) 575-5739, dwolf@uark.edu

Industrial Collaborator:

Robert Reynolds, Shuler Drilling, 3514 West Hillsboro, El Dorado, AR, (870) 863-7234

Institution: University of Arkansas

EPA Project Officers: Bala Krishnan
Project Period: September 1, 2003 to August 31, 2004 *extended to December 31, 2005*
Project Amount: $157,212
Research Category:

This report covers the March 1, 2005 to May 31, 2005 period and summarizes our current IPEC phytoremediation studies that consist of an on-site field project in southern Arkansas and a mathematical modeling project.

Progress Summary/Accomplishments:

Field Study

Materials and Methods

The field site in El Dorado, AR is located in a bermed crude oil storage/separation facility that was the site of an intentional spill in 1997 by vandals. The experimental plots consist of four replicates of the following treatments: (1) nonvegetated-nonfertilized control, (2) ryegrass (*Lolium multiflorum L.*) - fescue (*Festuca arundinacea Schreb.*) + fertilizer, and (3) bermudagrass (*Cynodon dactylon (L.) Pers.*) - fescue + fertilizer. Each field plot has 12 microplots (>soil socks=) that contain homogenized soil that allow monitoring of the field treatments, on a smaller scale, with less effect of field variability of the contaminant levels. The spring sampling is scheduled for June 2005 (t=66 mo).

Results and Discussion

As part of the Quality Assurance Project Plan, we have summarized data for the oil degraders, root parameters, and soil nutrient levels for samples collected during the Spring and Fall 2004. A previous report summarized data prior to 2004 sampling. Precision was calculated as the Relative Percent Difference (RPD)=[(n1-n2)/mean]100. Range was calculated as (n1-n2) for a duplicate analysis of a given sample. Completeness was determined as the percent of the required samples actually collected and analyzed.

OIL DEGRADER NUMBERS

A. Precision: A total of four samples were analyzed in duplicate for Alkane, Petroleum, and PAH degrader numbers and the precision estimates for one Alkane and two PAH degrader numbers exceeded the corrective action limit of 0.3 log (Fig. 1). The 0.3 log unit limit appeared to be too restrictive for the parameters analyzed. When the data were calculated as RPD, only one PAH degrader exceeded 20% (Fig 2). This was due to the extremely low values of PAH degraders. We have modified the Quality Assurance Project Plan to reflect this change in our method of estimating precision for this parameter.
B. Bias: Not applicable for the analyses.
C. Completeness: Experimental protocol required collection of 12 samples for each of the two sample times and a total of 24 samples were collected and analyzed (Table 1).

ROOT PARAMETERS

A. Precision: Plant root length, surface area, diameter, and volume were measured in duplicate for 6 samples over the two sample times and all values were below the corrective action limit of 20% (Fig. 3).
B. Bias: Not applicable for the analyses.
D. Completeness: Experimental protocol required collection of 12 samples for each of the two sample times and a total of 24 samples were collected and analyzed (Table 1).

SOIL NUTRIENT LEVELS

A. Precision: Soil chemical parameters were measured in duplicate for four samples over the two sample times. The calculated RPD for pH, electrical conductivity (EC), P, and K has a QC corrective action limit of 10% while Ca, Mg, Na, Zn, and Cu is 20%. For pH and K, all RPD values were below the 10% limit (Figs. 4 and 5). Due to the low extractable P concentrations in the soil, two of the four values exceeded the 10% level. Two of the four EC values were above the 10% limit due to low EC values found in the control plots which resulted in high RPD values. All 4 of the Zn RPD values fell below the 20% correction action limit. Three of the four Ca, Mg, Na, and Cu values were within the QC limit. For the Cu, the very low levels resulted in the marginal RPD value for sample A1.
B. Bias: Not applicable for the analyses.
C. Completeness: Experimental protocol required collection of 12 samples for each of the two sample times and a total of 24 samples were collected and analyzed (Table 1).

MICROBIAL

PLFA Method Development/Improvement

It was necessary to improve the detection limit for the PLFA analysis of our soils collected as part of the experiments outlined. In order to adequately assess the role of bacterial versus fungal contributions in the degradation of contaminants proposed we need to resolve unique biomarkers. We have been successful in isolating and analyzing bacterial biomarkers for stable isotopic composition. Those PLFA unique to fungi, however, are present in far less abundance even when fungi are abundant. It is the low abundance of those PLFA unique to fungi that make it problematic to detect and analyze them for stable isotopic composition. We sought to reduce our blank background levels in order to decrease our detection limit. Dr. Robert Findlay, University of Alabama, provided some assistance in our pursuit of lower detection limits. He has had over 20 years experience with various sample types and is able to detect pg quantities of PLFA, however, he does not currently analyze PLFA for stable isotopic composition. Graduate assistant Jill Baird spent 4 days in Findlay's laboratory during October 2004 in order to learn how to make clean solid phase extraction columns and use a different saponification and methylation technique. The manufactured silicic acid columns available have a number of trace contaminants that contribute to the PLFA blank background and thereby increase detection limits for the method.

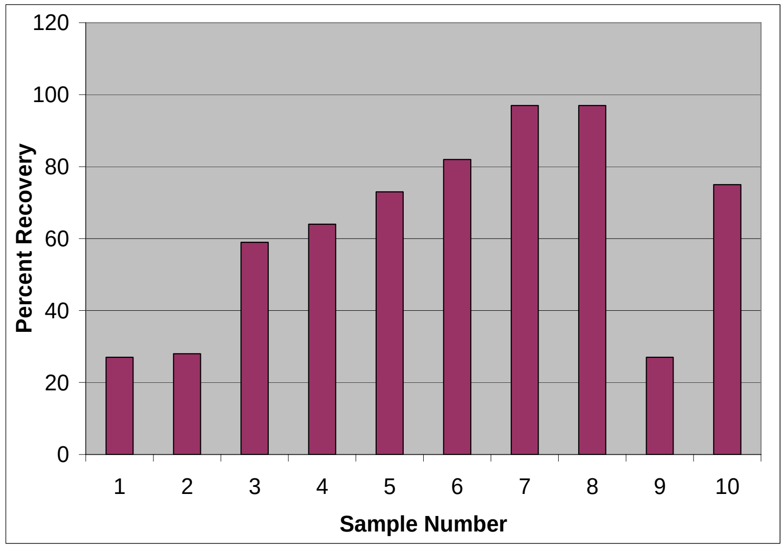

Figure 1. based upon recovery of phospholipid standard added at beginning of entire process or prior to lipid class separation.

Initially we found very poor recoveries from our new lipid class separation columns using very small (<30 μg) quantities of pure phospholipid standards (<30%;Figure 1). This was somewhat of a mystery and appears to be the result of excessive adherence of the phospholipids to the silicic acid requiring more methanol rinsing then practical. When we tested recovery of the same standards spiked into replicate subsamples of soil we achieved a much higher recovery (59-97%) which improved as we improved our technique. It appears that with the Captina Silt Loam soil used in the ^{13}C -hexadecane experiment we can achieve at least a 70% recovery of phospholipid fatty acids.

As mentioned above, we have now instituted this new approach outlined in Findlay (2004). Changes in the approach used to saponify and methylate the phospholipid fractions from soil extracts has necessitated the development of a new stable isotope standard. Previously we spiked samples with 19:0, with known stable isotopic composition, prior to methylation. This standard was used to correct the stable isotopic composition of sample PLFA for the added methyl group. The new approach does not methylate free fatty acids so we extensively analyzed a series of phospholipids for the stable isotopic composition of the individual fatty acids associated with each using the old approach. Now with each set of samples extracted and analyzed we run a phospholipid standard mixture containing fatty acids of known isotopic composition. This is used to correct each sample but also to assess the performance of the GCIRMS system and methylation process itself.

Precision for the GCIRMS analysis using this new approach appears to remain below 4% as indicated by replicates of the phospholipids standard mixture run between June and August 2005 when we had the method completely tested (Figure 2). These samples were run after extensive analysis of the 4 PL standards for the stable isotopic composition of the individual fatty acids in each PL. The standard deviation of any standard PLFA derived from a PL was always <1.3 per mil. Multiple analyses of individual samples suggests precision for actual samples is slightly higher: less than or equal to 10%.

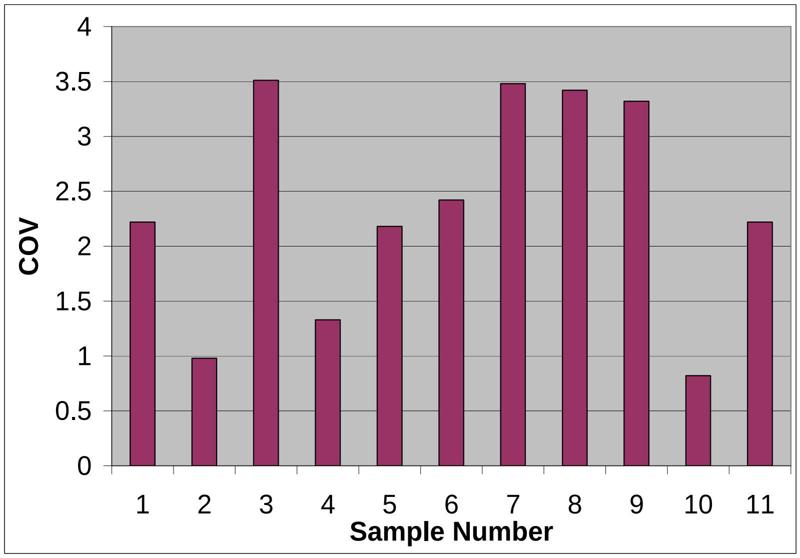

Figure 2. Values represent the average relative standard deviation (coefficient of variance) of the 4 phospholipid standards in the standard test mixture run with each GC-IRMS analysis made for the hexadecane experimental samples.

PUBLICATIONS/PRESENTATIONS: March 1, 2005 to May 31, 2005

Abstracts and titles of poster or oral presentations given during this quarter include:

Savin, M.C., P.J. Tomlinson, K.J. Davis, S.E. Ziegler, G.J. Thoma, and D.C. Wolf. 2005. Using nematode diversity and maturity indicies to assess ecosystem recovery during phytoremediation of a crude oil-contaminated soil. *In* Soil Ecology Society 10th Biennial International Conference. 22-25 May 2005. Argonne National Laboratory, Argonne, IL.

Thoma, G., T.B. Lam, S.E. Ziegler, and D.C. Wolf. 2005. Novel approaches to measurement of rhizosphere effects in phytoremediation of oil contaminated soils. *In* 3rd International Phytotechnologies Conference. 20-22 April 2005. Atlanta, GA.

Abstracts and titles that have been submitted for presentation as posters or presentations in the future include:

Alba, O., D.C. Wolf, E.E. Gbur, Jr., J.D. Mattice, K.J. Davis, and G.J. Thoma. 2005. Phytoremediation of pyrene: Analysis of nitrogen amendments and root parameters. *In* Annual Meetings Abstracts [CD-ROM]. ASA, CSSA, SSSA, Madison, WI.

Alba, O., D.C. Wolf, J.D. Mattice, K.J. Davis, and G.J. Thoma. 2005. Phytoremediation of pyrene-contaminated soil: Nitrogen additions and plant root parameters. *In* 12th Annual International Petroleum Environmental Conference. 8-11 November 2005. Houston, TX. Integrated Petroleum Environmental Consortium, Tulsa, OK.

Alba, O., D.C. Wolf, J.D. Mattice, K.J. Davis, and G.J. Thoma. 2005. Influence of urea-N and plants on the remediation of pyrene in Captina silt loam. *In* 26th Annual Soc. Environ. Toxicol. Chem. Conference. 13-17 November 2005. Baltimore, MD. Society of Environmental Toxicology and Chemistry, Pensacola, FL.

Karim, K., G.J. Thoma, D.C. Wolf, P.M. White, Jr., O. Alba, and K.J. Davis. 2005. A five-year field

study to evaluate phytoremediation of a crude oil-contaminated soil. *In* 12th Annual International Petroleum Environmental Conference. 8-11 November 2005. Houston, TX. Integrated Petroleum Environmental Consortium, Tulsa, OK.

Savin, M.C., P.J. Tomlinson, K. Karim, G.J. Thoma, K.J. Davis, and D.C. Wolf. 2005. Assessing phytoremediation of a crude oil-contaminated soil using nematode diversity and maturity indices. *In* 12th Annual International Petroleum Environmental Conference. 8-11 November 2005. Houston, TX. Integrated Petroleum Environmental Consortium, Tulsa, OK.

Thoma, G.J., T.B. Lam, K. Karim, D.C. Wolf, and S. Ziegler. 2005 Assessment of the rhizosphere effect in phytoremediation of PAHs. *In* American Institute of Chemical Engineers Annual Meeting 30 Oct. – 4 Nov. 2005, Cincinnati, OH. American Institute of Chemical Engineers, New York.

Manuscripts submitted:

None

Manuscripts published:

Krutz, L.J., C.A. Beyrouty, T.J. Gentry, D.C. Wolf, and C.M. Reynolds. 2005. Selective enrichment of a pyrene degrader population and enhanced pyrene degradation in bermudagrass rhizosphere. Biol. Fert. Soils. 41:359-364.

Ziegler, S.E., P.M. White, Jr., D.C. Wolf, and G.J. Thoma. 2005. Tracking the fate and recycling of ^{13}C-labeled glucose in soil: Lessons for stable isotope-labeling and biomarker studies. Soil Sci. (in press).

FUTURE ACTIVITIES:

We plan to conduct the Spring 2005 sampling at the El Dorado field site in June 2005 (t=66 mo) and complete the required microbial, plant, and soil analyses. The TPH analysis and PLFA analyses on previously collected samples are currently nearing completion. A laboratory ^{13}C-hexadecane study has been completed and data are being analyzed. Manuscripts on rhizosphere microbial degrader numbers and a novel approach to measure rhizosphere effects are being prepared.

Supplemental Keywords:

Rhizosphere; rhizodegradation; species selection; Arkansas; South Central United States

Relevant Web Sites:

Remediation Technologies Development Forum: **www.rtdf.org**; IPEC: **ipec.utulsa.edu**

Table 1. Number of samples collected and completeness of sample analyses for critical measurements.

	Sample Time (months)		
	53	57	Total
---------- Field Study ----------			
Samples Collected			
Oil Degrader Numbers	12	12	24
Root Parameters	12	12	24
Soil Nutrient Levels	12	12	24
Samples Analyzed			
Oil Degrader Numbers	12	12	24
Root Parameters	12	12	24
Soil Nutrient Levels	12	12	24
Completeness (%)			
Oil Degrader Numbers	100	100	100
Root Parameters	100	100	100
Soil Nutrient Levels	100	100	100

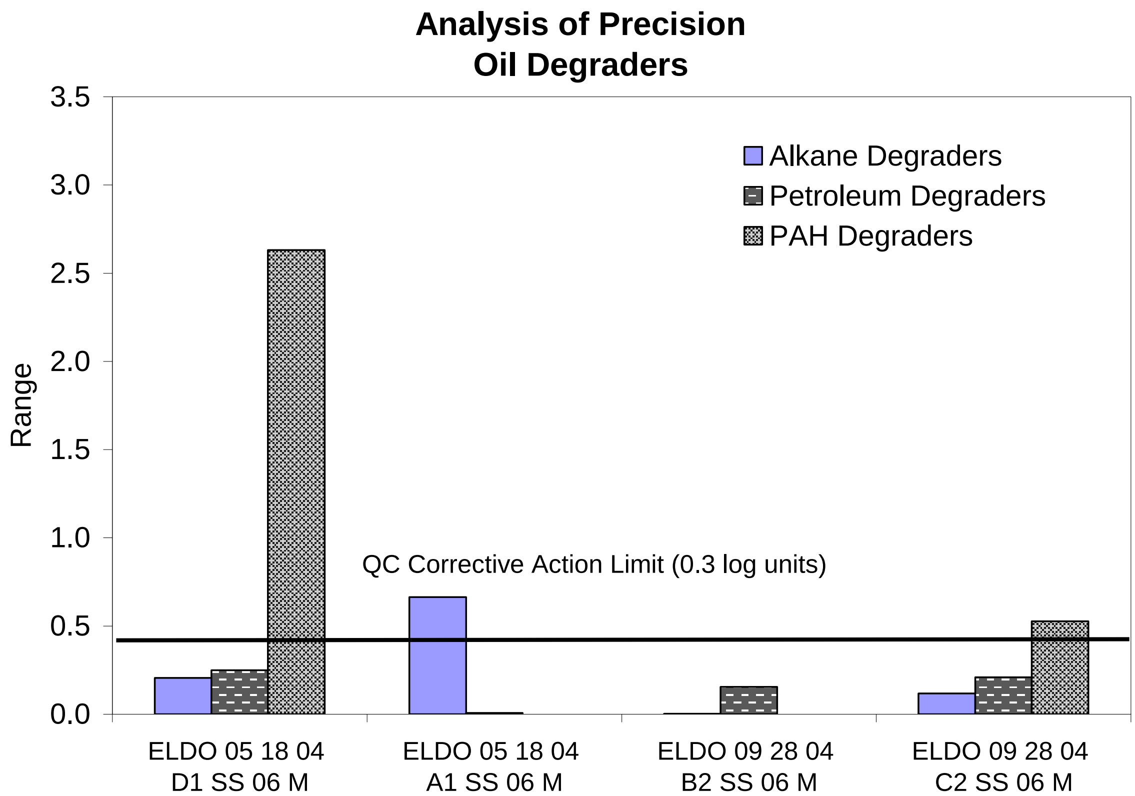

Fig. 1. Oil degrader precision results for four soil samples from the IPEC study at El Dorado, AR, during 2004.

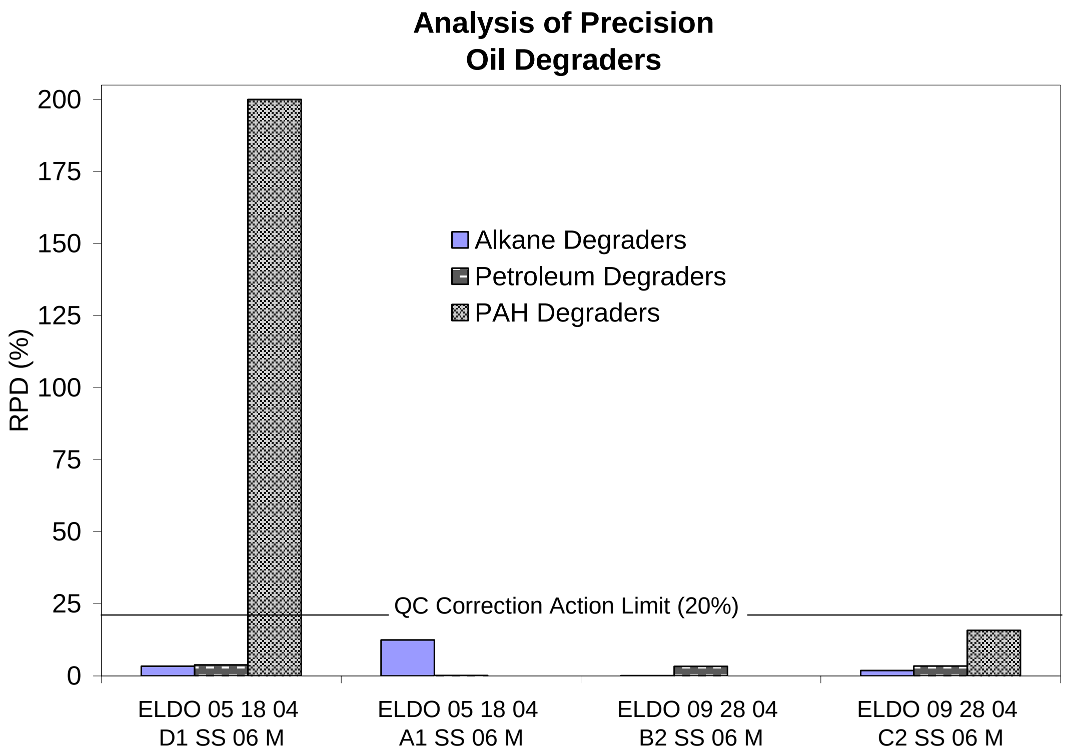

Fig. 2. Oil degrader precision results for four soil samples from the IPEC study at El Dorado, AR, during 2004.

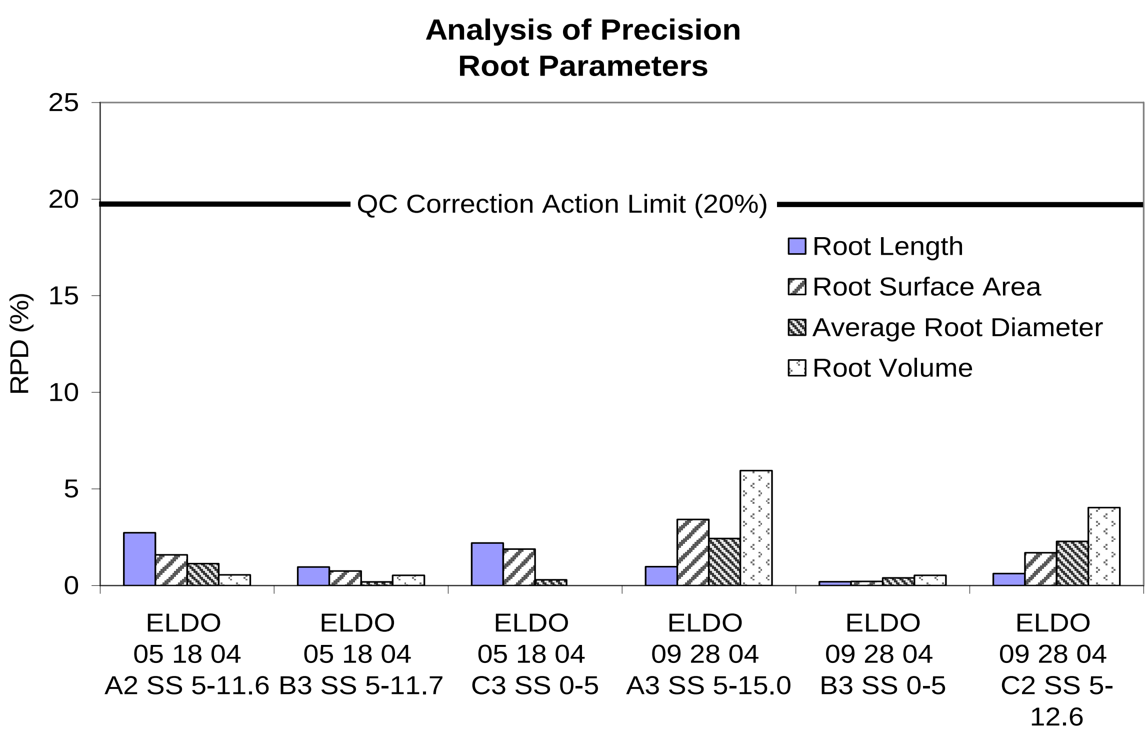

Fig. 3 The root parameter precision results for six soil samples from the IPEC study at El Dorado, AR, during 2004.

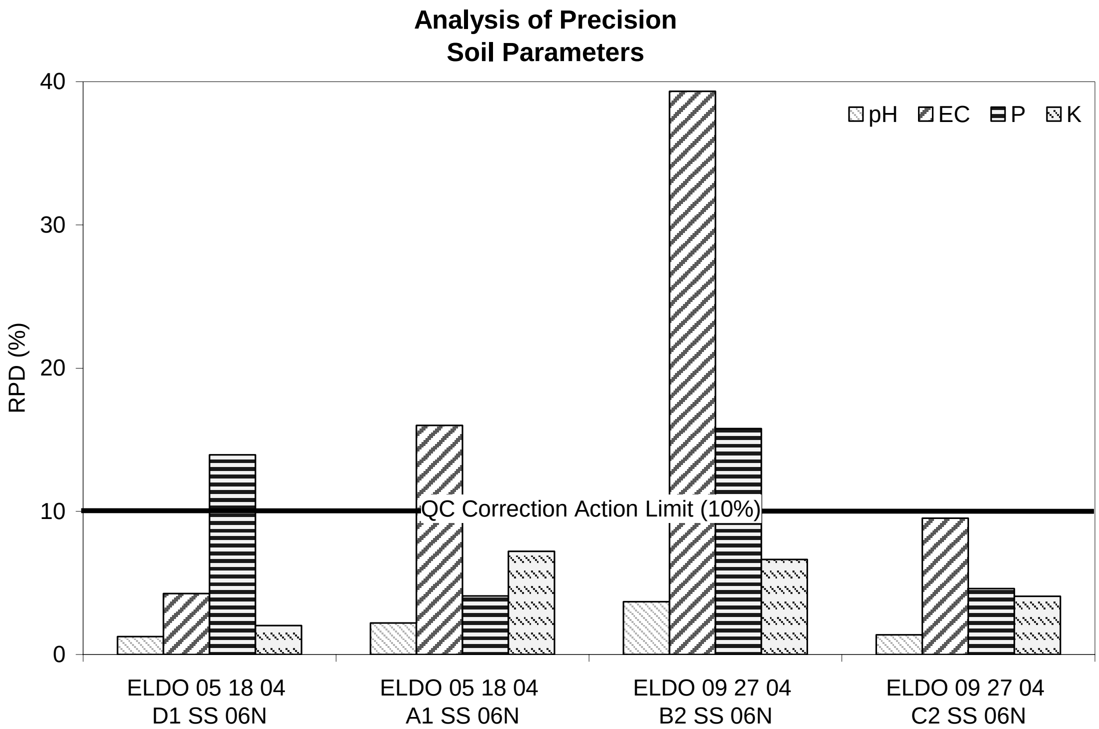

Fig. 4. Soil pH, electrical conductivity (EC), and Mehlich 3 extractable P and K precision results for four soil samples from the IPEC study at El Dorado, AR, during 2004.

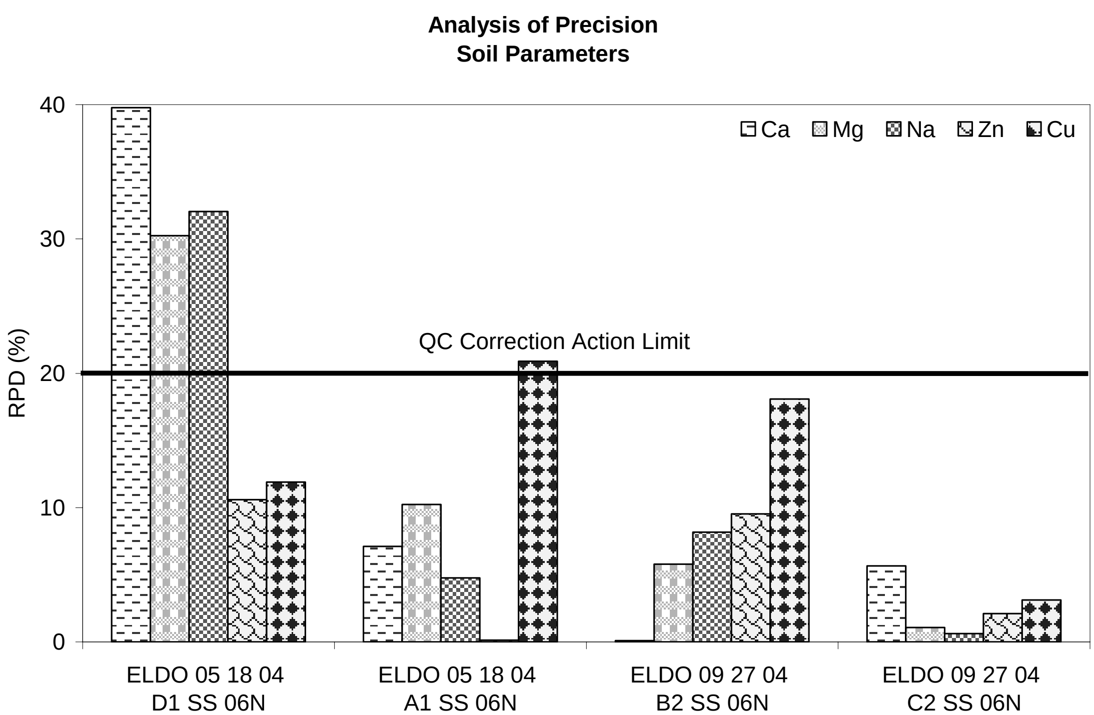

Fig. 5. Mehlich 3 extractable Ca, Mg, Na, Zn, and Cu levels in soil for four soil samples from the IPEC study at El Dorado, AR, during 2004