MICROBIAL TRANSFORMATIONS OF NITROGEN IN TEXAS STRIP MINE SPOIL

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ABSTRACT

Strip mining of lignite in Texas will result in the disturbance of more than one million acres of land. The microbial component of the soil is an important consideration in attempting to return these disturbed land to pre-mining productivity, especially in relation to the cyclic transformations of nitrogen mediated by soil microbes. A study was conducted in which microorganisms in mine spoil samples from two different mining areas in Texas were enumerated, including aerobic heterotrophic bacteria, fungi, and actinomycetes, as well as nitrifiers, denitrifiers, and free-living nitrogen fixing bacteria. Numbers of these organisms were determined in undisturbed soils adjacent to the sites sampled as well as in the mine spoil. Specific studies were conducted to determine the potential for associative and symbiotic nitrogen fixation on these sites. These included an enrichment study in which glucose and lime amendments were used, and a study of unamended rates of fixation occurring in grass and legume root-soil cores. Results indicated that mine spoils are recolonized by microorganisms soon after vegetation becomes established, but populations are altered from the native condition. In nonvegetated areas numbers of microorganisms are diminished. Autotrophic nitrifiers were adversely affected by low pH, as were associative nitrogen fixing bacteria. Increased utilization of legumes in reclamation programs would be beneficial to the reclamation process.

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INTRODUCTION[1]

Currently in the United States, surface mineable lignite is becoming an increasingly attractive alternative energy resource. The constant rise in petroleum prices, along with recent advances in surface mine technology make the utilization of this resource more profitable now than before. Development of lignite is also favored by the goal of increasing the country's independence from unpredictable energy supplies abroad.

In Texas alone, 10.4 billion short tons of lignite lie near enough to the surface to be recovered by strip mining. Removal of this lignite will result in the disturbance of more than 1 million acres of land (19). The mining process produces parallel rows of spoil material, consisting of the strata removed to expose the lignite seam. These layers are inverted in this process, and are mixed further when the spoil piles are leveled. State and federal reclamation laws require strip mined land to be returned to pre-mining productivity or better. Reclaimed areas must fulfill a five-year performance bond in order to satisfy this requirement. Interest in improving the effectiveness of reclamation practices has led to many investigations into the physical, chemical, and biological properties of spoil materials. Much of the biological data deals with the suitability of various plant species for use in reclaiming mine spoil, with very little information available on the microbial communities in spoil material.

[1]Prepared according to the organization and format guidelines for Applied and Environmental Microbiology.

Several problems are encountered in attempts to revegetate mine spoils. In Texas, the materials brought to the surface often have a high percentage of clay and exhibit poor soil structure. The clay texture is not usually considered a problem in itself, and often represents an improvement over infertile sandy soils which occurred in the area prior to mining (11). However, the structural characteristics of the material do present problems for revegetation. Large columnar aggregates or massive clays tend to drain poorly and can aggravate erosion problems by increasing runoff. The poor structure also makes tillage difficult and limits the plant's ability to establish a vigorous root system. Mine spoil containing substantial amounts of shale is an even greater disadvantage, since it has in effect regressed to the first stage of soil development, the breakdown of parent material. Until a significant amount of soil has developed through weathering, very little plant life can be supported by this material. The presence of lignite in the spoil may inhibit the infiltration of water into the material (25).

Excluding residual lignite, organic matter is extremely low in spoil materials (14), as is nitrogen content (23). The nitrogen and carbon contents of lignite are relatively high (17), but the breakdown of this material is so slow that essentially none is released into the soil system. These two components (nitrogen and carbon) exert a profound effect on the biological activity in the soil.

Another problem associated with strip mine spoils is the oxidation of materials containing reduced forms of iron and sulfur, such as pyrite and marcasite (3,13). These materials are often associated with lignite

deposits since they are formed under similar conditions. Oxidation can occur both chemically and biologically, with the biological reaction occurring on the order of a million times faster than the chemical oxidation (20). The net product of the reaction is H_2SO_4 which affects not only the pH of the soil, but can also pollute ponds and streams receiving runoff from the area. In culture media, the autotrophic microorganisms which mediate the oxidation reaction can cause a drop in pH below 1.0 (2). Acid conditions in the soil increase the solubility of many elements, such as aluminum and iron, which may accumulate to phytotoxic levels (7).

Microorganisms perform many essential functions in the soil which are important considerations in strip mine reclamation. The most basic function is the decomposition of plant and animal residues, resulting in the production of humus (5). At the same time, nutrients are released from organic combination and become available for plant growth. Bacterial cells and their exudates also play an important role in aggregate stabilization (2,5).

Numerous associations occur between higher plants and microorganisms. The roots of a majority of plant species are known to interact with fungi (mostly Basidiomycetes or Phycomycetes) to form mycorrhizae, a mutually beneficial relationship in which the fungus increases the absorptive capabilities of the plant roots (22). This association enables plants to survive in areas of low fertility, since the fungus is able to extract nutrients from the soil more efficiently than can the plant roots alone. Some of the nutrients obtained by the fungus are

made available to the plant for its growth, and photosynthate from the plant is used by the fungus. Another beneficial plant-microbe interaction results in the occurrence of biological nitrogen fixation. In both symbiotic and non-symbiotic systems the microorganism is provided with photosynthate for its growth, and it in turn reduces molecular dinitrogen to plant available forms. Both of these associations have significance in reestablishing vegetation on nutrient deficient mine spoil.

Nutrient cycling in soils is accomplished through a wide variety of mechanisms, many of which depend on microorganisms. As previously mentioned, nutrients bound in organic combination are released in inorganic forms through microbial decomposition of plant residues. Microbes also mediate inorganic transformations, such as oxidation-reduction reactions of various elements including sulfur, nitrogen, and iron (2). They are capable of increasing the solubility of phosphorous and potassium through the production of organic and inorganic acids (1). Microorganisms may effect a net change in the total amount of a nutrient in the soil. This may represent an increase in nutrients, such as occurs in biological nitrogen fixation and in the transformation of carbon dioxide to organic carbon compounds by chemoautotrophs. Conversely, nutrient levels in the soil can decrease through processes which produce gaseous end products, such as denitrification and sulfate reduction. It is evident, even from this partial listing, that the complex interactions between the microbial community and higher plants are crucial in maintaining the nutrient balance within the soil system.

Processes affecting the availability of nitrogen to higher plants determine the productivity of the system, whether the area is an intensively managed cropland or a naturally occurring ecosystem. Nitrogen is the nutrient required in greatest amounts by plants, but in soils it is usually the nutrient found in shortest supply. Since the transformations which comprise the nitrogen cycle are accomplished almost exclusively by microbial processes (1), these soil inhabitants are essential in maintaining adequate vegetation in native areas, and substantially affect inputs in agricultural systems.

Mineralization of organic nitrogen-containing substrates such as proteins and nucleic acids can result in the release of ammonium in the soil if the amount of nitrogen in the substrate exceeds the microbial requirement for the nutrient (2). This process is carried out by a great diversity of microorganisms. Consequently, it is not easily disrupted by environmental stress, although the species of organisms mediating the process will change with variations in the conditions. Mineralization is essential for plant growth, since most of the nitrogen occurring in soils is in organic combination. When nitrogenous fertilizer is applied, some is immobilized by the soil microflora and later released through mineralization. In this way, the applied nitrogen becomes available to plants slowly, over a longer period of time.

When ammonium (NH_4^+) is available in soils it may undergo a two step biological oxidation to form nitrate (NO_3^-), through the process known as nitrification. This transformation is accomplished by two specific groups of autotrophic bacteria which obtain energy from the oxidation

reactions. The first group, including species of Nitrosomonas, Nitrosospira, Nitrosolobus, and related genera, oxidizes NH_4^+ to nitrite (NO_2^-). The second step, most commonly mediated by species of Nitrobacter, further oxidizes NO_2^- to NO_3^- (2). Nitrification is important because NO_3^- is more easily absorbed by most plants than is NH_4^+. Application of NH_4^+ fertilizers is often preferred, since the NH_4^+ ion is adsorbed to colloids in the soil and is less subject to leaching than is NO_3^- (5). Nitrifying bacteria gradually oxidize the applied fertilizer, making it more available for plant use. In the absence of actively growing plants the highly mobile NO_3^- produced through nitrification is easily lost from the soil by leaching or under certain conditions, through denitrification (discussed below). Excessive leaching of NO_3^- can cause eutrophication of waterways, and may pose health hazards if drinking water is contaminated. Nitrification is markedly reduced under acidic conditions, with negligible activity below pH 5.0 (8,12). For this reason, it may be adversely affected in mine spoils where problems with soil acidity develop.

A net decrease in the total amount of nitrogen in the soil may occur through denitrification. Certain heterotrophic bacteria which normally grow aerobically are capable of using NO_3^- as an alternate electron acceptor in the absence of oxygen. The NO_3^- is thereby reduced to form gaseous end products such as nitrous oxide and molecular dinitrogen which are lost from the soil (29). This loss can be substantial in heavily fertilized agricultural land (1). This process is affected by the availability of suitable organic substrates, NO_3^- levels, and in some cases by soil pH. The poor drainage characteristics of mine spoils containing a

high percentage of clay could lead to the anaerobic conditions required for denitrification during periods of high rainfall.

Biological nitrogen fixation (BNF) is the reduction of atmospheric dinitrogen (N_2) to NH_4^+ in the presence of the nitrogenase enzyme complex. N_2-fixation may occur in symbiotic associations with higher plants, such as Rhizobium with legumes and the actinomycete Frankia with certain non-legume angiosperms. Less specific relationships also exist, including the associations occuring between nitrogen fixing bacteria and the roots of grasses. These are now called associative symbioses. Both the associative and the more specific symbioses involve the utilization of plant-produced carbon compounds as substrates for microbial growth. Many free-living bacteria are able to carry out this process in soil without interactions with living plants. These diazotrophs (N_2-fixing bacteria) obtain their carbon source from the organic matter in the soil. Their input is generally quite small, but it may be an important addition to grasslands or forests where nitrogen is not lost through crop removal (1). Organisms capable of BNF have a competitive advantage over other microbes in nutrient impoverished habitats, since nitrogen availability usually limits microbial growth in soils. This advantage is lost in heavily fertilized areas, which may result in lower numbers of nitrogen-fixing bacteria. In mine spoil, factors which may limit BNF are the lack of carbon sources (either plant roots or organic matter), low pH, and the inavailability of trace elements such as molybdenum, magnesium, and iron required by the nitrogenase enzyme complex. High levels of nitrogenous fertilizers are known to inhibit nitrogen fixation (2). If properly managed, however, symbiotic nitrogen fixation

can make a significant contribution to reclamation efforts.

It is obvious from this discussion that microorganisms substantially affect the soil's ability to support plant growth. In cases where the soil has been severely disturbed, as in mine spoil, it is important to know whether the microbial community has been altered by the disturbance. If changes have occurred which adversely affect beneficial microbial activities, reestablishment of these functions becomes a consideration in effectively reclaiming the spoil. Before such detailed studies can begin, there is a need for establishing an extensive data base to indicate areas requiring further investigation. A study was conducted to determine the amount of variation in microbial numbers between various spoil materials and undisturbed soil. Specific emphasis was placed on the more easily disturbed microbial groups participating in nitrogen transformations. A more detailed study was conducted to determine the potential for BNF in the reclamation of mine spoils.

LITERATURE REVIEW

Although strip mine reclamation is an extremely active research area, very few comprehensive studies of the microflora of strip mine spoils are found in the literature. One notable exception is the study conducted by Wilson (30) in which eleven strip mine spoils in West Virginia and Pennsylvania were samples. Plate counts from four strip mined areas indicated that larger numbers of bacteria, fungi, and actinomycetes occurred in vegetated mine spoil than in nonvegetated spoil. This trend was observed even when the pH of the vegetated spoil was lower than the nonvegetated spoil. In most cases actinomycete populations were higher in undisturbed soil than in vegetated spoil, but bacteria and fungi were sometimes found in greater abundance in the vegetated spoil material. Wilson concluded that the presence of vegetation had a more significant effect on the microflora than did soil pH. A recently published study by Hersman and Temple (16) used ATP measurements to quantitate microbial activity in strip mine spoils. This study determined that ATP concentrations were higher in native range soils than in mine spoil, and that the vertical distribution of ATP in the soil profile differed between the two areas.

Carbon dioxide evolution is often used as a measure of microbial activity in soils. In the study by Reeder and Berg (26), samples of shale, fresh mine spoil, vegetated mine spoil, and undisturbed soil were monitored for CO_2 evolution. Total amounts of CO_2 evolved were essentially the same in all but the fresh mine spoil, which exhibited less CO_2 evolution. Amendment with NH_4^+ did not significantly affect the rate or amount of

CO_2 evolution. Wilson's study also included data on CO_2 evolution. Vegetated and nonvegetated mine spoils, as well as undisturbed soil were treated with combinations of $Ca(OH)_2$, straw, nitrogen, phosphorous, and potassium. In each treatment, the undisturbed soil exhibited greater amounts of CO_2 evolved than either the vegetated or nonvegetated mine spoil (30).

A discussion of microbial processes in soil as related to the reclamation of disturbed areas is found in a review by Cundell (10). Key functions mentioned include the accretion of organic matter, accumulation of plant nutrients, improved soil aggregation, and cyclic transformations of nitrogen. The study conducted by Wilson (30) included the determination of nutrient requirements of bacteria isolated from spoils, as well as their relative abilities to produce polysaccharides. Polysaccharide production is considered to be an important factor in the stabilization of soil aggregates.

The autotrophic iron and sulfur oxidizing bacteria have been more extensively studied than any other microbial group occurring in mine spoil. There has been concern that problems with acid production similar to those encountered in the eastern United States could also occur in Texas. Miller, et. al. (24) reported the difficulty of establishing vegetation on road cuts in East Texas where pyritic materials were exposed. The distribution and morphology of pyrites were studied by Arora, et. al.(3). They found that pyrite concentration was highest at the upper and lower boundaries of the lignite seam. Scanning electron microscopy was used to study the various crystalline structures formed by the mineral, many

of which were found to have very high surface areas. This large surface area may result in accellerated rates of oxidation (both chemical and biological) of the pyritic materials exposed on the surface of strip mine spoils.

An assessment of nitrogen cycling in strip mined areas was included in Wilson's study (30). Most probable number (MPN) counts of ammonifiers and denitrifiers tended to increase from the nonvegetated spoil to the undisturbed sample, with intermediate numbers occurring in the vegetated spoil. Populations of nitrifiers were very low on the mine spoils, and in some cases nitrifiers were not detected. Enrichment studies designed to determine the amount of nitrification occurring in the mine spoil materials indicated that low pH was a limiting factor for this process. Low nitrification potentials were also reported by Hons and Hossner (17) in a study of Texas mine spoils. Undisturbed soil amended with NH_4^+ converted 93% of the applied nitrogen to NO_3^- while similarly amended spoil material only converted 7% of the nitrogen applied. A study by Reeder and Berg (26) of shale and coal mine spoils in Colorado revealed a similar decrease in the rates of mineralization and nitrification occurring in the nonvegetated spoil materials compared to vegetated spoil and undisturbed areas.

In Wilson's work (30), Azotobacter was found to be absent from all of the samples tested at two mining areas. This associative nitrogen-fixing bacterium is known to be sensitive to low pH. Application of the Rhizobium-legume symbiosis to reclamation was included in a forage study conducted in Texas by Hons, et. al. (18). Legumes were found to require

high rates of phosphorous fertilizer for adequate establishment and optimum yields.

The greater part of the literature has dealt with microbial numbers in the strip mine spoils of Appalachia. To a lesser degree, information is available on the microorganisms in mine spoils in the Northern Great Plains states. Information concerning the microbial communities occurring in Texas strip mine spoil is limited. Since geological and climatic factors affecting Texas strip mining areas differ greatly from those which affect the Appalachian region and the Northern Great Plains, there is a need for research in Texas to determine the effect strip mining has on the microbial inhabitants of the soils in this region.

MATERIALS AND METHODS

Site Descriptions

Soil samples were collected at two locations for this study. Each location was sampled on two different occasions during the study period.

The first of these locations is in Cherokee County, near Troup, Texas. The site consists of test plots set up for a revegetation study conducted by Dr. L. R. Hossner of Texas A&M University, Department of Soil and Crop Sciences. These test plots were made up of overburden material transported to the study area from a test pit which had been excavated in 1977 in order to remove a bulk sample of lignite for testing.

Overburden 1 consisted of soil strata excavated from 0 - 6.1 m. This material was quite weathered and had an initial pH range of 4.0 - 5.0. Overburden 2 was made up of strata from below 14 m. It was an unweathered silty clay, with an initial pH above 7.0. The overburden materials were piled to a uniform depth of 1.2 m and leveled to conduct the revegetation field trials. Native soils in this area are of the Bowie-Ruston-Lakeland complex.

In July and November of 1980 soil samples were collected from both of the overburden plots as well as from the control plot for use in a study of microbial populations. Two separate samples were taken from each overburden plot, one from areas covered with vegetation and another from "hot spots" devoid of vegetation. All samples collected were taken from

plots under a "medium fertility" regime, consisting of 56 kg/ha N (34-0-0), 84 kg/ha P_2O_5 (0-46-0), and 56 kg/ha K_2O (0-0-60). The annual fertilizer application in April was followed by additional applications of 56 kg/ha N at monthly intervals through the summer. Lime was applied once in the spring, at rates varying from 0 - 9 metric tons/ha. Vegetation on the plots included Coastal Bermudagrass (Cynodon dactylon (L) Pers.), Kleingrass (Panicum coloratum L.), and Bahiagrass (Paspalum notatum Flugge).

The second location sampled was the Alcoa lignite mine in Milam County near Rockdale, Texas, where mining began in 1952. Soils in this area are most commonly in the Axtell series, but Padina, Rader, and Uhland soils are also found. Mining operations produce mixed overburden including strata from 0 - 30 m. Layers of shale, dolomite, and pyritic material are often found associated with the lignite seam, and subsequently become mixed with the other overburden materials.

The control sample was collected in a field adjacent to the mining area. The field had been an improved pasture prior to acquisition by the mining company in 1977, but it was not maintained after that time. Vegetation included Common and Coastal Bermudagrass, native grasses, and herbaceous species.

Another sample was obtained from a newly revegetated area which had been seeded with Gulf ryegrass (Lolium multiflorum Lam.) one week prior to sampling. Current revegetation practices include the application of 11 - 15 metric tons/ha lime and 560 kg/ha fertilizer (17-17-17). This

area was sampled again three months later when the ryegrass had become established, and after an additional 224 kg/ha fertilizer (17-17-17) had been added.

The third sample area at Rockdale had been revegetated 7 - 8 years ago in the early 1970's. Initial treatment consisted of minimal applications of lime and fertilizer, with no additional inputs since that time. The area was originally planted with Coastal and Common bermudagrass, then seeded with Yuchi-Arrowleaf clover (Trifolium vesiculosum), Crimson clover (Trifolium incarnatum), and Crown vetch (Coronilla varia) in the mid-1970's.

The fourth area sampled was mined approximately 15 years ago, before reclamation laws were in effect. The spoil material was left in heaps, with no effort to reclaim the area. The mine spoil has become recolonized by pioneer plant species through natural succession. Although disturbance species predominate, examples of most of the easily disseminated plant species in the surrounding areas can be found within the spoiled area.

During the first sample trip to Rockdale, fresh spoil material was collected, approximately 15 minutes after it was disturbed by the dragline. Its placement prior to excavation was 0 - 3 m below the surface, and it consisted primarily of oxidized materials. This sample was used for enumerating bacterial, fungal, and actinomycete populations, but was not included in further tests.

At each site composite samples were taken at a depth of 0 - 10 cm.

The samples were sealed in plastic bags and kept at ambient temperature until inoculations were made the following day. A summary of the sample descriptions is given in Table 1.

Soil Tests

Soil pH was determined with a Corning Model 125 pH meter equipped with a combination glass electrode. Electrical conductivity readings were made with a YSI Model 31 Conductivity Bridge. In both tests, a 2:1 distilled water:soil mixture was used. Percent water (Pw) in the samples was determined by drying at 110 C for 24 hr.

Inoculations

Microbial populations were enumerated by inoculating appropriate culture media with decimal dilutions of each of the soils being tested. Formulas for the media used are listed in the Appendix. Populations of bacteria, fungi, and actinomycetes were estimated by direct plate counts on tryptic soy agar (bacteria, Difco), rose bengal-streptomycin agar (fungi) and actinomycete isolation agar (Difco). On three occasions, the pour plate method was used and on one occasion (October 1980) the spread plate technique was used. Three replicate plates at each dilution were incubated at 25 C and were counted after 4 - 6 days (bacteria and fungi) or 2 weeks (actinomycetes).

Nitrogen Cycle Survey

Organisms mediating various transformations in the nitrogen cycle were enumerated using the Most Probable Number (MPN) technique (9). This method involves inoculation of three replicate tubes of the culture

TABLE 1. SOIL SAMPLE DESIGNATION

TROUP SITE: SAMPLED 7-80 AND 22-80

ABBREVIATION	DESCRIPTION
Control	Control plots
OB 1-V	Overburden 1, vegetation present
OB 1-NV	Overburden 1, no vegetation (hot spots)
OB 2-V	Overburden 2, vegetation present
OB 2-NV	Overburden 2, no vegetation (hot spots)

ROCKDALE SITE: SAMPLED 10-80 AND 1-81

ABBREVIATION	DESCRIPTION
Control	Control field
NR	Newly revegetated area
7 YR-V	7-8 yr old revegetated area, vegetation present
7 YR-NV	7-8 yr old revegetated area, no vegetation present
15 YR-V	15 yr old unreclaimed spoil, vegetation present
15 YR-NV	15 yr old unreclaimed spoil, no vegetation present
Fresh spoil	Sampled 15 min after excavation

medium with each dilution in a decimal dilution series. The number of positive growth responses (positive tubes) at each dilution is used to determine from statistical tables the approximate number of organisms present in the sample.

Numbers of nitrifying organisms were estimated using ammonium (NH_4^+) broth and nitrite (NO_2^-) broth. After 4 - 5 weeks incubation at 27 C, each tube was assayed for the presence of nitrite and nitrate using Griess' reagent (6). Denitrification potential was determined by using nitrate (NO_3^-) broth tubes containing inverted Durham tubes to collect the gaseous end products of the reaction. Tubes accumulating gas after 1 week at 27 C were scored as positive for the MPN enumeration.

Organisms capable of biological nitrogen fixation (BNF) were enumerated using a semi-solid medium lacking combined nitrogen. After two days incubation at 27 C the nitrogen-free semi-solid agar deeps were assayed for nitrogenase activity using the acetylene reduction (AR) technique (15). This involved sealing the tubes with serum stoppers, replacing 11% of the gas volume in the tube with acetylene, and after 24 hr additional incubation determining the amount of acetylene (C_2H_2) reduced by the nitrogenase enzyme to ethylene (C_2H_4). The relative amounts of these gasses were determined by gas chromatography using 0.2 ml of the gas phase injected into an Antek Model 300 Gas Chromatograph with a hydrogen flame ionization detector. Column packing was Porapak N (Waters Assoc., Framingham, Mass.) and nitrogen was used as the carrier gas. Numbers of Azotobacter, a common associative nitrogen fixing bacterium, were estimated with spread plates on Alkaline Nitrogen-Free agar. These plates

were incubated at 27 C for 3 - 4 days before counting the large mucoid colonies characteristic of Azotobacter on this medium.

Nitrogen Fixation Studies

An enrichment study was conducted using the soil samples collected during January from Rockdale sites. Half-pint jars fitted with serum stoppers were used to incubate 20 g of soil. For each soil sample three replicate jars were amended with 1% (wt/wt) glucose delivered in 10 ml distilled water. The three soil samples with the lowest pH values (7 YR-NV, 15 YR-V, and 15 YR-NV) had an additional three replicate jars amended with 1% glucose and 1 g $CaCO_3$/jar. Three unamended controls were also included for each sample. The jars were sealed and the internal atmosphere adjusted to 11% acetylene (0.11 ATM p C_2H_2). Ethylene production by the amended soils was measured after 24 and 44 hr incubation at 27 C.

The final investigation of nitrogen fixation was carried out in March 1981 at the Alcoa mine near Rockdale. The Control, NR, and 7 YR sites were as previously described. The unreclaimed 15 yr old area differed from the site originally sampled. Parts of the area sampled in March had been aerially seeded with Yuchi-Arrowleaf clover during 1971.

Soil-root core samples with intact plant tops (approximately 10 cm X 5 cm diameter) of both grasses and legumes (clovers and vetch) were collected, placed into 1 quart canning jars fitted with serum stoppers and injected with acetylene (0.11 ATM p C_2H_2) within 20 minutes of collection. After all the core samples had been collected they were

immediately transported to the laboratory and assayed for ethylene production by gas chromatography. Time elapsed between collection and first assay was 6 hr. Additional assays were run at 24 and 48 hr. Between assays the jars were kept at room temperature on a light table regulated for a 14 hr photoperiod (200 uE/m^2/sec).

At the time each core sample was taken a soil sample from the side of the resulting hole was also collected. These samples were sealed in plastic bags, kept at ambient temperature, and transported to the laboratory. The next day these samples were used to determine the Pw, pH, and electrical conductivity of the soil as described previously. Soil dilutions (10^{-2} and 10^{-3}) were prepared in order to inoculate Alkaline Nitrogen-Free agar plates to detect the presence of Azotobacter. Semi-solid nitrogen-free agar deeps were inoculated (10^{-2} only) and incubated at 27 C for a total of 72 hr, including 21 hr under acetylene, after which they were assayed for ethylene production.

RESULTS

General Survey

The values obtained in the determinations of soil pH, moisture content, and electrical conductivity are listed in Table 2. At the Troup site, sampling in July followed several weeks of drought, as is reflected by the low Pw of the samples. The moisture status of every sample was higher on the November sampling date. Soil pH increased significantly between July and November in all of the samples except OB 1-NV. In general, the areas lacking vegetation had lower pH readings than those supporting plant growth. Soluble salts (as indicated by conductivity) were highest on Overburden 2, with the greatest concentration occurring in the nonvegetated sample.

At Rockdale the pH readings from the 7 YR-NV and 15 YR-NV areas were substantially lower than those of the surrounding vegetated areas. The Pw was also much lower in the nonvegetated samples. Conductivity readings showed moderate salt accumulation in both the NR and 15 YR-NV samples, and very high salt levels in the 7 YR-NV sample (Table 2).

Plate Counts: Enumeration of bacteria, fungi, and actinomycetes revealed some differences among the samples, as seen in Table 3. In general, the vegetated samples exhibited higher numbers of organisms than non-vegetated samples. At the Troup site there was an increase in all three groups from July to November. Bacterial and actinomycete counts on OB 2-V were of the same order of magnitude as the control counts, while all other samples showed lower numbers of these organisms. Fungal counts increased

TABLE 2. SOIL pH, Pw, AND ELECTRICAL CONDUCTIVITY OF SURFACE MINE SPOIL SAMPLES FROM TROUP AND ROCKDALE COLLECTION SITES.

DATE	SAMPLE	pH	Pw	CONDUCTIVITY (mmhos/cm)
TROUP				
7-80	Control	4.8	1.2	ND*
	OB 1-V	4.4	2.0	ND
	OB 1-NV	3.4	3.5	ND
	OB 2-V	3.7	3.6	ND
	OB 2-NV	2.8	6.6	ND
11-80	Control	6.5	14.3	0.14
	OB 1-V	5.0	19.9	0.10
	OB 1-NV	3.7	19.9	0.31
	OB 2-V	5.2	32.9	0.62
	OB 2-NV	4.5	25.0	1.10
ROCKDALE				
10-80	Control	7.6	13.5	ND
	NR	7.6	19.0	ND
	7 YR-V	6.9	26.4	ND
	7 YR-NV	4.2	12.3	ND
	15 YR-V	5.8	29.8	ND
	15 YR-NV	4.3	8.5	ND
	Fresh spoil	7.3	22.7	ND
1-81	Control	7.2	10.4	0.11
	NR	7.2	11.1	1.60
	7 YR-V	6.9	34.5	0.28
	7 YR-NV	4.1	9.1	5.00
	15 YR-V	5.5	32.2	0.14
	15 YR-NV	4.8	13.2	1.40

*Not Determined.

TABLE 3. NUMBER OF BACTERIA, FUNGI, AND ACTINOMYCETES IN SURFACE MINE SPOIL SAMPLES FROM TROUP AND ROCKDALE SITES.

		NUMBER OF ORGANISMS/GRAM SOIL		
DATE	SAMPLE	BACTERIA	FUNGI	ACTINOMYCETES
TROUP				
7-80	Control	1.1×10^6	8.7×10^4	7.7×10^5
	OB 1-V	2.3×10^5	1.3×10^5	3.7×10^4
	OB 1-NV	1.7×10^4	1.7×10^4	1.7×10^4
	OB 2-V	1.1×10^6	5.8×10^4	3.1×10^5
	OB 2-NV	$<3.0 \times 10^3$	6.0×10^3	5.9×10^4
11-80	Control	1.0×10^7	6.2×10^5	1.3×10^6
	OB 1-V	1.6×10^6	3.2×10^5	1.6×10^5
	OB 1-NV	1.7×10^5	4.2×10^4	1.0×10^4
	OB 2-V	9.4×10^7	1.9×10^5	3.3×10^6
	OB 2-NV	2.7×10^6	4.9×10^4	7.2×10^5
ROCKDALE				
10-80	Control	8.7×10^6	7.7×10^4	4.7×10^5
	NR	9.5×10^6	2.2×10^4	3.7×10^4
	7 YR-V	2.6×10^7	8.6×10^4	3.8×10^5
	7 YR-NV	7.2×10^5	5.5×10^4	3.1×10^4
	15 YR-V	3.3×10^6	1.3×10^5	4.6×10^5
	15 YR-NV	2.5×10^6	9.0×10^4	2.4×10^5
	Fresh spoil	3.5×10^6	$<3.3 \times 10^2$	1.7×10^4
1-81	Control	1.5×10^7	2.8×10^5	9.3×10^5
	NR	3.4×10^7	1.1×10^4	4.2×10^5
	7 YR-V	2.1×10^7	3.1×10^5	5.6×10^5
	7 YR-NV	1.9×10^5	1.1×10^5	2.2×10^4
	15 YR-V	8.0×10^6	1.9×10^5	4.9×10^5
	15 YR-NV	1.3×10^6	1.8×10^5	2.2×10^5

in both the Control and OB 2 samples in November, while the samples from OB 1 remained essentially unchanged.

Samples collected at Rockdale also demonstrated differences in the microflora observed at the different sites. On both sampling dates there were hundred-fold differences between bacterial counts in the 7 YR-V and 7 YR-NV samples. The number of bacteria occurring in the sample from the NR site increased substantially following the establishment of vegetation in that area. Fungal counts increased by a factor of 10 between October 1980 and January 1981, with the exception of the NR area and the 15 YR-V site. Actinomycete counts were lower on non-vegetated areas, with the exception of the 15 YR-NV site, which only showed a very slight decrease in numbers. The fresh spoil sample contained populations of actinomycetes and bacteria comparable to those found in the other disturbed samples. The fungal population in this sample was much lower than in the other samples tested.

Survey of Populations Critical for Nitrogen Transformations

Nitrification and Denitrification: Populations of nitrifying bacteria were very low in the spoil samples collected at the Troup site in July 1980 (Table 4). Although the number of NO_2^- oxidizers in the Control sample was higher than in other samples, the NH_4^+ oxidizer population was low. The only spoil sample which exhibited detectable nitrification was OB 2-V. Most samples contained larger populations of nitrifiers on the November sampling date, with the exception of OB 1-NV. There was a large increase in the number of NO_2^- oxidizers in OB 2-V between July and November 1980. Denitrification potential was found to be highest in

TABLE 4. MOST PROBABLE NUMBER OF MICROORGANISMS CAPABLE OF AMMONIUM OXIDATION, NITRITE OXIDATION, AND DENITRIFICATION IN CULTURE MEDIA INOCULATED WITH MINE SPOIL SAMPLES.

DATE	SAMPLE	NH_4^+ OXIDIZERS	NO_2^- OXIDIZERS	DENITRIFIERS
TROUP				
7-80	Control	$<3.0 \times 10^3$	2.3×10^4	2.1×10^4
	OB 1-V	$<3.0 \times 10^3$	$<3.0 \times 10^3$	2.0×10^4
	OB 1-NV	$<3.0 \times 10^3$	$<3.0 \times 10^3$	3.7×10^4
	OB 2-V	7.6×10^3	9.4×10^3	9.4×10^3
	OB 2-NV	$<3.0 \times 10^3$	$<3.0 \times 10^3$	$<3.0 \times 10^3$
11-80	Control	8.8×10^4	5.4×10^4	2.8×10^4
	OB 1-V	2.6×10^4	$<3.0 \times 10^2$	1.2×10^4
	OB 1-NV	$<3.0 \times 10^2$	$<3.0 \times 10^2$	5.0×10^2
	OB 2-V	3.6×10^4	1.4×10^6	2.2×10^4
	OB 2-NV	5.7×10^3	6.1×10^4	3.9×10^3
ROCKDALE				
10-80	Control	5.3×10^4	5.0×10^3	1.7×10^5
	NR	3.0×10^4	5.3×10^3	2.8×10^4
	7 YR-V	2.0×10^4	3.3×10^5	3.1×10^4
	7 YR-NV	2.7×10^3	1.7×10^4	3.4×10^3
	15 YR-V	1.3×10^3	3.3×10^3	3.4×10^4
	15 YR-NV	5.0×10^2	1.0×10^4	8.0×10^2
1-81	Control	5.1×10^4	5.1×10^4	8.4×10^3
	NR	2.7×10^4	5.2×10^5	1.3×10^4
	7 YR-V	7.0×10^4	1.7×10^5	3.7×10^4
	7 YR-NV	2.5×10^3	2.5×10^3	1.0×10^3
	15 YR-V	5.0×10^2	6.3×10^3	3.5×10^4
	15 YR-NV	1.1×10^4	2.6×10^3	5.0×10^3

the vegetated samples on both sampling dates.

At the Rockdale site NH_4^+ oxidizers were most numerous in the Control, NR, and 7 YR-V areas. A distinct trend was not observed in the NO_2^- oxidizing populations in October, but on the January sampling date the trend paralleled that of the NH_4^+ oxidizers. As observed in the Troup samples, denitrification potential was lower on nonvegetated sites than on vegetated areas.

Nitrogen Fixation: As shown in Table 5, populations of bacteria capable of nitrogen fixation were highest on OB 1-V and OB 2-V at the Troup site. There was also an increase in the population on OB 2-NV observed in November. Nitrogen fixation potential in the Control sample was low on both occasions. Plates inoculated with the samples collected in November did not exhibit typical Azotobacter colonies at the lowest dilution used (10^{-2}). At the Rockdale site, nitrogen fixing populations were highest in the Control sample on the October sampling date. Populations increased in most of the samples from the mined sites in January 1981, with the exception of the 7 YR-NV area. Only the 7 YR-V sample exhibited populations comparable to the Control sample. The presence of Azotobacter was detected in all samples except 7 YR-NV and 15 YR-NV in October. By January, the size of these populations had decreased in all but the 7 YR-V sample, in which the Azotobacter population had increased slightly.

Nitrogen Fixation Studies

Enrichment study: The results of the enrichment study are presented in

TABLE 5. MOST PROBABLE NUMBER OF MICROORGANISMS CAPABLE OF NON-SYMBIOTIC NITROGEN FIXATION IN CULTURE AND NUMBER OF AZOTOBACTER COLONIES DEVELOPING ON ALKALINE N-FREE AGAR, FOLLOWING INOCULATION WITH MINE SPOIL SAMPLES.

		NUMBER OF ORGANISMS/GRAM SOIL	
DATE	SAMPLE	NITROGEN FIXATION	AZOTOBACTER
TROUP			
7-80	Control	$<3.0 \times 10^2$*	ND**
	OB 1-V	4.4×10^3	ND
	OB 1-NV	$<3.0 \times 10^2$	ND
	OB 2-V	3.0×10^3	ND
	OB 2-NV	$<3.0 \times 10^2$	ND
11-80	Control	5.0×10^2	<30
	OB 1-V	3.0×10^4	<30
	OB 1-NV	5.0×10^2	<30
	OB 2-V	1.1×10^3	<30
	OB 2-NV	3.2×10^3	<30
ROCKDALE			
10-80	Control	2.8×10^4	38
	NR	1.0×10^2	81
	7 YR-V	6.3×10^3	950
	7 YR-NV	2.7×10^3	<30
	15 YR-V	2.1×10^3	570
	15 YR NV	$<3.0 \times 10^1$	<30
1-81	Control	1.3×10^4	<30
	NR	4.8×10^2	<30
	7 YR-V	2.3×10^4	1,300
	7 YR-NV	1.0×10^2	<30
	15 YR-V	4.3×10^3	<30
	15 YR-NV	1.0×10^2	<30

*MPN based on semi-solid agar deeps exhibiting acetylene reduction.

**Not Determined.

Figure 1. Unamended controls did not produce detectable levels of ethylene during the study period. The glucose amended soils from all of the sample sites tested showed increased ethylene production over controls. The 7 YR-NV and 15 YR-NV samples exhibited the lowest rates of acetylene reduction. When $CaCO_3$ was added to the 7 YR-NV, 15 YR-V, and 15 YR-NV samples in addition to the glucose amendment, the AR rates increased substantially.

Core assays: As seen in Table 6, AR by grass cores exhibited very low rates and was highly variable between replicates. The highest rate of AR by grass cores occurred in cores taken from the 15 YR-V area. All of the soil samples obtained immediately adjacent to the cores had populations capable of reducing acetylene in culture. Azotobacter was absent from all of the soils adjacent to grass cores except one sample from the 15 YR-V area. Legume cores exhibited rates of AR 2 - 3 orders of magnitude greater than the grasses, and of the legumes collected, vetch demonstrated the greatest rate of AR.

Figure 1. Acetylene reduction by mine spoil samples from the Rockdale site amended with glucose alone and glucose plus lime.

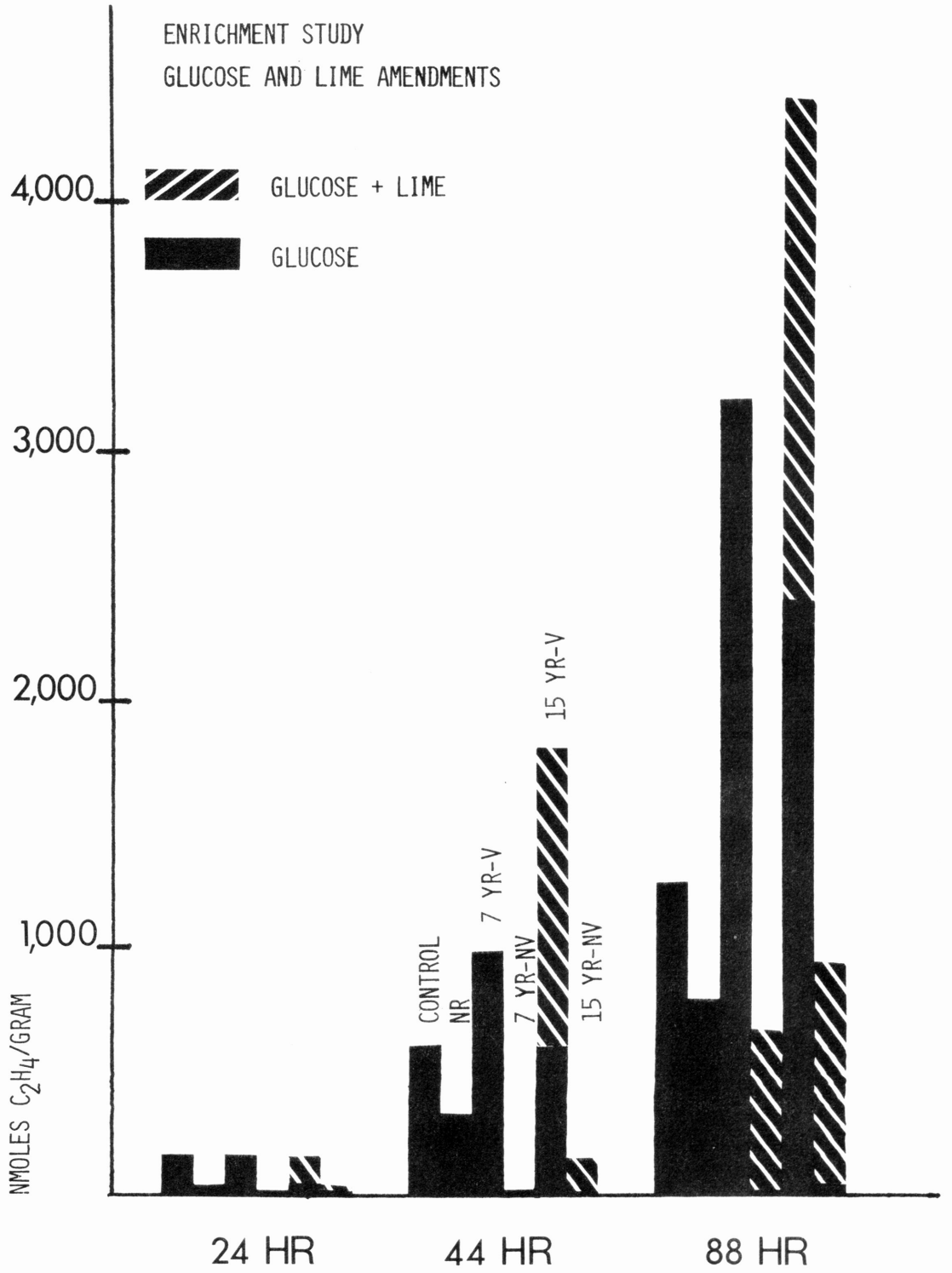

TABLE 6. ACETYLENE REDUCTION BY INTACT PLANT-SOIL CORES OF GRASSES AND NODULATED LEGUMES COLLECTED FROM ROCKDALE SITES

(nmoles C_2H_4 produced/core)

SAMPLE	DEEPS	PLATES	6 HOUR	24 HOUR	48 HOUR
CONTROL BERMUDA	6/6*	0/6**	0	0	0 - 26 ($\bar{x}$ = 9.8 ± 11.3)
NEW REVEG RYEGRASS	6/6	0/6	0	0 - 49 ($\bar{x}$ = 17 ± 20)	49 - 156 ($\bar{x}$ = 89.5 ± 39.3)
7 YR REVEG BERMUDA	6/6	0/6	0 - 43 ‡ ($\bar{x}$ = 7.2 ± 17.6) ‡‡	0 - 73 ($\bar{x}$ = 12 ± 30)	0 - 170 ($\bar{x}$ = 45 ± 66)
CLOVER	ND	ND	3359 - 5771 ($\bar{x}$ = 4564 ± 1206)	9296 - 16844 ($\bar{x}$ = 13830 ± 3997)	9185 - 16000 ($\bar{x}$ = 12279 ± 3451)
VETCH	ND	ND	11369 - 32032 ($\bar{x}$ = 20211 ± 10649)	41572 - 86635 ($\bar{x}$ = 58087 ± 24825)	39302 - 81882 ($\bar{x}$ = 56279 ± 22562)
15 YR SPOIL GRASS	3/3	1/3	0 - 560 ($\bar{x}$ = 187 ± 323)	0 - 1477 ($\bar{x}$ = 568 ± 795)	81 - 1952 ($\bar{x}$ = 859 ± 974)
CLOVER	3/3	3/3	5192 - 12613 ($\bar{x}$ = 9948 ± 4129)	8536 - 24373 ($\bar{x}$ = 17225 ± 8030)	9227 - 25152 ($\bar{x}$ = 17899 ± 8057)

* Number of N-free soft agar deeps positive for AR/total number of replicates tested.
** Number of Alkaline N-Free agar plates positive for Azotobacter/total number of replicates.
‡ Range for replicate cores. (First two columns indicate number of replicates for each sample.)
‡‡ Mean of replicates ± one standard deviation.

DISCUSSION

General Survey

Samples collected at the Troup site exhibited low pH values in July despite the application of lime earlier in the year. Lack of moisture seems to have limited the reaction of the applied lime in the soil. After the soil moisture status improved the soil pH increased substantially. This undoubtedly had an effect on the microbial inhabitants in the soil system. Soil salinity did not appear to be excessive in most of the samples collected at the Troup site. In general, conductivity readings below 1.0 mmhos/cm indicate salt levels which are not injurious to crops. The OB 2-NV sample, with a conductivity reading of 1.10 mmhos/cm, contains enough soluble salts to affect very salt sensitive plants.

Soil moisture was not extremely low on either sampling date at the Rockdale site, but notable differences occurred between the Pw in vegetated and nonvegetated samples taken from both the 7 YR and 15 YR areas. These differences may be due to higher levels of lignite in the nonvegetated samples. Lignite tends to repel water, and when mixed with soil would lead to decreased infiltration rates. Electrical conductivity readings indicated that very salt sensitive plants might be affected on the NR and 15 YR-NV areas. The 7 YR-NV area was extremely saline, falling within the range in which salt tolerant plants are affected. The salinity of this area affects the soil microflora both directly, by selecting for halophilic species, and indirectly by the decrease in the amount of vegetation present.

Plate Counts: Numbers of bacteria were most strongly related to the presence or absence of vegetation on the sample sites. At the Troup site, bacterial numbers increased by at least a factor of ten between the two sampling dates. This was most likely attributable to improved moisture status, a resulting increase in nutrient availability, higher pH, or seasonal variation. The observed increase in the bacterial numbers on OB 2-NV was much greater than the increase seen in the other samples. This may have been due to the more extreme pH limitation on OB 2-NV in July. Once the pH improved bacteria were able to proliferate, resulting in the elevated population counts observed in November. In October 1980 bacterial populations in the samples obtained at the Rockdale site were essentially the same except in the 7 YR area. At this site bacteria in the vegetated portions of the field outnumbered those in the nonvegetated areas by a factor of 1,000. This is a clear example of the effect of vegetation on microbial numbers. In January bacterial populations were higher in the Control, NR, and 7 YR-V areas than in the other samples. The greatest change in bacterial numbers between the two sampling dates occurred in the NR sample. This was probably related to the establishment of vegetation in this area. Fungal populations in the overburden materials at the Troup site were higher in the vegetated samples than in nonvegetated samples. The Control and OB 2 samples increased by a factor of 10 between July and November of 1980, while samples from OB 1 did not increase appreciably. This difference between population changes in the two overburden materials did not seem to be correlated with any of the soil properties monitored in this study. Fungal counts exhibited a high degree of uniformity on both the occasions that samples were collected from the Rockdale site. In most cases the

number of fungi increased from October to January, as might be expected because of the seasonal availability of readily decomposable plant residues. Fungal populations did not increase substantially in the NR area, where ryegrass was actively growing in January.

Actinomycetes tended to be more numerous in the vegetated samples from both Troup and Rockdale. There was a ten-fold increase in numbers in the samples collected at the Troup site in November compared to the July samples. The only exception to this trend was OB 1-NV, in which the population remained stable. This sample was the only one in which the pH did not increase markedly between the two sample dates. This could explain the lack of increase in the number of actinomycetes since acid environments do not favor their growth (2). At the Rockdale sample site actinomycete counts increased in the NR sample as vegetation became established. The 15 YR-NV sample exhibited populations of actinomycetes comparable to those in the 15 YR-V sample.

Data regarding the microbial groups indicate that mine spoils become recolonized shortly after the initial disturbance. Some changes in species composition were apparent from visual examination of the plates used to enumerate fungal populations. It was clear that fungal communities were simplified in some cases and these changes may be significant in terms of reestablishing the native microflora, along with the functions peculiar to it. The full impact of the observed changes is difficult to interpret based on this limited data.

Nitrification and Denitrification: Samples collected at the Troup site

exhibited very low populations of nitrifying bacteria. Only the OB 2-V sample contained sufficient numbers of NH_4^+ oxidizers and NO_2^- oxidizers to be detected at the dilutions used. Nitrite oxidizers were detected in the Control sample, but NH_4^+ oxidizers were not found. On the November sampling date the number of nitrifiers had increased in most of the samples, indicating that moisture stress and low pH may have been limiting factors earlier in the year. Nitrifier populations remained low in OB 1-NV, as did the soil pH. There was no discernable trend between the relative numbers of NH_4^+ oxidizers and NO_2^- oxidizers detected in the samples.

At the Rockdale site a distinct trend was observed in the populations of NH_4^+ oxidizers. Both the NR and 7 YR-V samples produced MPN counts of the same order of magnitude as the Control sample. The other three samples (7 YR-NV, 15 YR-V, and 15 YR-NV) exhibited lower populations of the autotrophs. Generally, the population tended to decrease with lower soil pH. Both groups of nitrifiers are adversely affected by acid conditions. An increase in the NH_4^+ oxidizing population was noted in the 15 YR-NV sample between October and January, while the corresponding population in the 15 YR-V sample decreases. Although a slight decrease in pH is observed in the latter sample and a slight increase occurred in the former, this explanation alone does not seem adequate to account for the differences observed. It is more probable that several factors, including the effect of pH, acted together to produce the fluctuations in population size. Trends in the numbers of NO_2^- oxidizers were not apparent on the October sample date. In January, however, the trend seemed to correlate with the pH of the sample. A marked increase was

observed in the NO_2^- oxidizing population of the NR sample, possibly due to the application of nitrogenous fertilizer, which would provide an increase in the available substrate for this organism's growth. Since these bacteria do not require organically combined carbon as an energy source, their numbers would not be expected to follow trends of vegetation. The nitrifying bacteria are critical in converting NH_4^+ to NO_3^-, the preferred ion for plant assimilation. The low numbers observed in these studies suggest that nitrification might place a limitation on revegetation. Inoculation of the soils with bacteria might prove beneficial to these systems, in conjunction with the maintenance of a suitable pH by effective liming treatments.

Denitrification is affected by both vegetation and pH trends, since it is a heterotrophic, acid sensitive process. The population size of denitrifiers in the samples collected on both sampling dates at the Troup site was directly related to the pH of the samples. This observed relationship may be equally influenced by vegetation, since the samples exhibiting higher pH values were those which supported vegetation. The samples collected at the Rockdale site demonstrate the interrelationship of these two factors in determining the population size of denitrifiers. In October, the Control and the NR sites both had soil pH values of 7.6. The numbers of denitrifiers differed by an order of magnitude between the two sites, with the newly seeded NR area exhibiting lower populations of bacteria capable of denitrification. Numbers of denitrifiers in the other samples obtained at the Rockdale site followed the previously discussed trends. In assessing these populations, it is important to bear in mind that the process only occurs under anaerobic conditions,

although even well drained soils may contain anaerobic microsites. The counts obtained in the present study indicate the potential for denitrification when soil conditions become favorable for the process. Fertilizer nitrogen could be lost due to this process, a factor which can indirectly increase the cost of effective reclamation. A full assessment of this problem would require field measurement of denitrification to confirm whether these losses actually occur.

Nitrogen Fixation: Populations of bacteria capable of nitrogen fixation were lower in samples collected at the Troup site in July than in November. The Control and nonvegetated spoil samples contained too few nitrogen fixing bacteria to be detected at the lowest dilution (10^{-2}). These organisms were detected in the vegetated overburden samples. A logical explanation for the presence of higher numbers of nitrogen fixing bacteria in spoil material than in the Control sample is the greater amount of fertilizer nitrogen which probably remained on the Control plot. The overburden materials were piled to a height of 1.2 m above the surrounding field and showed signs of erosion. In addition, grass growing in the field along the lower edges of the overburden plots was darker green than grass in other parts of the field. The observations led to the assumption that considerable fertilizer runoff was occurring from the overburden plots. If this was the case, the resultant lower fertility status on the overburden materials could favor the colonization of the material by organisms capable of nitrogen fixation. Higher fertility on the level Control plots would tend to cause a decrease in the population size of nitrogen fixing bacteria. In November a similar trend was seen, with fewer nitrogen fixing bacteria

enumerated in the Control than in the vegetated spoil samples. Azotobacter colonies did not develop on the plating medium inoculated with 10^{-2} dilutions of each of the samples collected at the Troup site.

Populations of nitrogen fixing bacteria were lower in the Rockdale site minespoil samples than in the Control. Vegetated spoil samples generally exhibited higher numbers of these organisms than did the nonvegetated samples. An exception to this was seen in the NR area, where numbers remained low after vegetation was present. This was probably due to the higher fertility in the NR area. Populations of Azotobacter were higher in the vegetated spoil samples than in the nonvegetated samples or in the Control. In the nonvegetated samples no Azotobacter was detected at the 10^{-2} dilution. In January the Azotobacter populations had declined in all of the samples except 7 YR-V. This area included patches of actively growing legumes which may have provided a suitable rhizosphere habitat in which the Azotobacter could survive. Azotobacter was not detected in the NR area which was covered in ryegrass, probably because high fertility levels would not favor its development. When nitrogen is not limiting, other bacteria compete more efficiently for available carbon sources than does Azotobacter.

Nitrogen Fixation Studies

Enrichment study: The enrichment study demonstrated the presence of a bacterial population capable of nitrogen fixation (AR) in all of the vegetated samples. The more acidic samples exhibited increased AR activity after lime was added. These results suggest that liming of acidic spoil materials will enhance their potential for nitrogen fixation.

This is a very important consideration in reclaiming land which will not be intensively managed, such as rangeland or native pasture.

Core assays: The results of the grass and legume core study have significant applications in future reclamation efforts. Although the rates of AR exhibited by the grass cores were low, they probably represent a minimum level of activity because of the early spring sampling date. It is interesting to note that the highest rate of AR by a grass core was observed in the samples taken from the 15 year old unreclaimed spoil area. This demonstration of AR associated with grass roots under low nutrient conditions indicates that associative nitrogen fixation is providing some fixed nitrogen for the soil-plant systems. Even these low values can be of significance for plant growth in habitats otherwise deprived of inputs of combined nitrogen. A full assessment of the role of associative symbioses in these systems will require more field investigation during the more active growing seasons of the grass species present. These systems could be quite significant in sites where continued fertilization practices might be abandoned.

As expected, the legume cores exhibited much higher rates of AR than did the grass cores. Extrapolation of the amount of acetylene reduced to field rates of nitrogen fixation (assuming a constant rate for 10 hr/day during a 100 day growing season, and using the theoretical C_2H_2:N_2 ratio of 3:1) results in 40 - 100 kg N/ha/100 days added to the soil by the legumes. The increased utilization of legumes in reclamation programs is to be encouraged. Not only do they reduce the amount of nitrogenous fertilizer required, but many legumes also provide good

erosion control and excellent forage.

CONCLUSIONS

1. Microbial soil inhabitants are affected by the land disturbance resulting from strip mining. The full impact of these changes on nutrient cycling should be investigated in more detail.
2. Differences in the types and numbers of microorganisms exist between spoil materials of different ages, as well as within a given disturbed site, depending on the conditions present in the spoil material.
3. Microorganisms are markedly influenced by the vegetation in an area. It appears that once vegetation becomes established the soil microorganisms will recolonize the area. It might be possible to reduce fertilizer and other inputs as an effective soil microflora becomes established.
4. Certain physiological groups of microorganisms are adversely affected by acidic conditions which are often associated with mine spoil. High acidity has a negative impact on both associative and symbiotic nitrogen fixation, as well as nitrification.
5. Additional research is needed in order to better understand and learn to maximize associative nitrogen fixation. This could prove to be an essential factor in returning mined areas to self maintaining, biologically stable ecosystems.
6. The legume - Rhizobium symbiosis can and should be included as an effective management practice in current reclamation programs. Mine spoils cropped to legumes will benefit from nitrogen and organic

matter accretion.

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APPENDIX

ALPHABETICAL LISTING OF CULTURE MEDIA

1. ACTINOMYCETE ISOLATION AGAR (Difco) (28)

Sodium caseinate	2.0 g
Asparagine	0.1 g
Sodium propionate	4.0 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$FeSO_4 \cdot 7H_2O$	1.0 mg
Agar	15.0 g
Distilled water	1,000 ml
Glycerol	5.0 g

2. ALKALINE NITROGEN-FREE AGAR (31)

Glucose	20.0 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCO_3$	2.0 g
$FeCl_3 \cdot 6H_2O$	0.1 g
$Na_2MoO_4 \cdot 2H_2O$	2.0 mg
Agar	15.0 g
Distilled water	1,000 ml

pH to 7.5

3. AMMONIUM BROTH (E. L. Schmidt, personal communication)

$(NH_4)_2SO_4$	0.5 g
$CaCl_2 \cdot 2H_2O$	536.0 mg
$MgSO_4 \cdot 7H_2O$	0.4 g
Brom Thymol Blue	2.0 mg
KH_2PO_4	204.0 mg
Chelated Iron	5.8 mg
$FeSO_4 \cdot 7H_2O$	
Na_2EDTA	
Trace Elements	0.4 mg
$Na_2MoO \cdot 2H_2O$	
$MnCl_2$	
$CoCl_2 \cdot 6H_2O$	
$ZnSO_4 \cdot 7H_2O$	
$CuSO_4 \cdot 5H_2O$	
Distilled water	1,000 ml

pH to 7.2

4. NITRATE BROTH (31)

Peptone	5.0 g
Beef Extract	3.0 g
KNO_3	5.0 g
Distilled water	1,000 ml

5. NITRITE BROTH (E. L. Schmidt, personal communication)

KNO_2	85.0 mg
$CaCl_2 \cdot 2H_2O$	13.4 mg
$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4	139.2 mg
KH_2PO_4	27.2 mg
Chelated Iron	5.8 mg
$FeSO_4 \cdot 7H_2O$	
Na_2EDTA	
Trace Elements	0.4 mg
$Na_2MoO \cdot 2H_2O$	
$MnCl_2$	
$CoCl_2 \cdot 6H_2O$	
$ZnSO_4 \cdot 7H_2O$	
$CuSO_4 \cdot 5H_2O$	
Distilled water	1,000 ml

6. ROSE BENGAL-STREPTOMYCIN AGAR (21)

Glucose	10.0 g
Peptone	5.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Streptomycin*	33.0 mg
Rose Bengal	33.0 mg
Agar	15.0 g
Distilled water	1,000 ml

*filter sterilize, add to sterile medium cooled to 50 C

7. SUCROSE NITROGEN FREE MEDIUM (27)

Sucrose	10.0 g
Yeast Extract	50.0 mg
NaCl	0.1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4	0.1 g
$CaCl_2$	2.0 mg
$FeCl_2$	1.0 mg
$NaMoO_4 \cdot 2H_2O$	2.0 mg
Brom Thymol Blue	0.6 mg
Agar	3.0 g
Distilled water pH to 7.0	1,000 ml

8. TRYPTIC SOY AGAR (Difco)

Tryptone	15.0 g
Soytone	5.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1,000 ml