TECHNIQUE FOR MEASUREMENT OF NON-SYMBIOTIC NITROGEN FIXATION IN SOME MANITOBA SOILS USING ^{15}N

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ABSTRACT

An apparatus was designed in which a soil sample can be aerated by a continuous air flow and modified to allow aeration by diffusion as well. Studies were conducted with this apparatus comparing non-symbiotic nitrogen fixation under continuous air flow with aeration by diffusion under aerobic and anaerobic conditions. Fixation of nitrogen was determined by using a gas mixture enriched with ^{15}N.

The preliminary studies indicated very small amounts of nitrogen fixation under aerobic conditions with aeration by continuous flow for an Altona soil. Significant ^{15}N enrichment of the soil was measured under aerobic conditions with soil aeration by continuous flow and diffusion on a non-enriched Portage I soil while no significant enrichment was measured under anaerobic conditions with either method of soil aeration.

Nitrogen fixation studies with a modified apparatus using 53.2 atom % ^{15}N resulted in appreciably higher ^{15}N enrichment of the soil but only when energy was supplied. Under anaerobic conditions (80% A and 20% N_2), with 0.5% glucose plus 0.5% cellulose as an energy source, the diffusion method of aerating the soil sample resulted in 0.54 and 0.48 ppm of nitrogen being fixed per day in two separate studies with a Portage III soil, whereas the continuous air flow method resulted in 0.20 and 0.06 ppm of nitrogen being fixed per day under the same conditions. Under "semi aerobic" (60% A, 20% N_2 and 20% O_2 with no supplemental oxygen added during 14 day incubation period) and aerobic (60% A, 20% N_2 and 20% O_2) conditions, with added energy, 0.09 and 0.03 ppm nitrogen were fixed per day where the sample was aerated by diffusion.

Under these conditions, no fixation took place where the sample was aerated by continuous air flow.

When no energy source was added, no significant nitrogen fixation occurred in the Portage III soil under either "semi aerobic", aerobic or anaerobic conditions regardless of method of aeration.

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I INTRODUCTION

The ability of certain free living organisms to fix molecular nitrogen has been well established in pure culture studies. These studies have shown that many of these organisms exist in soils and therefore are capable of adding nitrogen to soils. Since nitrogen is one of the major nutrients and also the most costly nutrient required for good crop growth, the significance of this process to agriculture is apparent. In the past, difficulties in measuring nitrogen gains due to non-symbiotic fixation have hindered the evaluation of the quantities of nitrogen added to soils in field and laboratory studies. Despite the availability of the sensitive tracer ^{15}N technique in recent years to measure the nitrogen gains in soils attributable to non-symbiotic nitrogen fixation, the agricultural significance still has not been clearly established. A search of the literature revealed a very limited application of this technique in laboratory studies involving nitrogen fixation in soils. Presumably the cost of the tracer ^{15}N and associated equipment required, such as a mass spectrometer and high vacuum apparatus have been a deterent to the use of this technique. A review of the literature also revealed a lack of standard techniques for using tracer ^{15}N for laboratory nitrogen fixation studies in soils.

To date, no studies have been conducted in Manitoba to ascertain the significance of non-symbiotic nitrogen fixation in soils. In view of the large amount of nitrogen required for annual crop production and the need for a better understanding of nitrogen transformation in soils, it seemed worthwhile to study non-symbiotic nitrogen fixation in Manitoba soils. The objectives of the present study were:

(1) To design an apparatus allowing soil aeration by continuous flow and by diffusion.

(2) To evolve a simple method of introducing an artificial atmosphere, including tracer N-15, into a confined apparatus.

(3) To study non-symbiotic nitrogen fixation in some Manitoba soils as affected by method of aeration and readily available energy supply.

II LITERATURE REVIEW

A. Occurrence and Abundance of Asymbiotic Nitrogen Fixers:

The ability of certain free living organisms to convert molecular nitrogen into combined nitrogen has been well established since Winogradsky first demonstrated nitrogen fixation in a strain of butyric acid bacilli and since Beijerinck discovered the aerobic nitrogen fixing bacteria of the *Azotobacter* genus. Since that time, many additional organisms have been isolated that are capable of fixing molecular nitrogen. Lindstrom, Burris and Wilson (39) demonstrated nitrogen fixation by the photosynthetic non-sulfur purple bacterium *Rhodospirillum rubrum*. With the application of N-15 tracer techniques, Lindstrom *et al.*(41) showed fixation by photosynthetic bacteria of the genera *Chromatium* and *Chlorobacterium*.

Lindstrom *et al.*(40), also showed fixation by non-sulfur purple bacteria of the genera *Rhodopseudomones* and *Rhodospirillum*. Sisler and ZoBell (64) observed nitrogen fixation by a species of autotrophic, anaerobic sulfate reducing bacteria of the genus *Desulfovibrio*. Hamilton and Wilson (25), using the ^{15}N tracer technique confirmed *Aerobacter aerogenes* as a nitrogen fixer under anaerobic conditions. Hino and Wilson (28) reported nitrogen fixation by a facultative aerobe which they reported as being similar in general morphological and biochemical properties to *Bacillus polymyxa*. Nitrogen fixation in the aerobic *Pseudomonas* spp., was reported by Anderson (2), and Paul and Newton (53). Proctor and Wilson, using ^{15}N uptake detected fixation in six strains of *Pseudomonas* and eight of *Achrombacter* (58). While results of nitrogen fixation by actinomycetes have been mostly negative, Metcalfe and Brown (4) studied two species of *Nocardia* which possessed the ability to fix

nitrogen. Nitrogen fixation has now been shown to take place in at least twenty species belonging to seven different genera of blue green algae or Cyanophyceae (34).

Although numerous organisms have been isolated and demonstrated the ability to fix molecular nitrogen in vitro, the distribution and density of many of these organisms in soil has not been clearly established. The classical Azotobacter have global distribution although they are by no means ubiquitous (34). Becking (5) in a global survey reported Azotobacter in 33 percent of 392 samples. The density of Azotobacter in soil under normal conditions is exceedingly low in comparison to the high counts of bacteria usually obtained in soils by cultural methods (34). Numbers of Azotobacter range from 10 to 1.6×10^6 per gram of soil with most figures in the range of 10^3 per gram of soil (10, 21, 32, 42, 44, 66). The clostridia, of which the Clostrium butyricum species is the largest group, is practically ubiquitous (34). Bredermann (8) examined 134 soil samples of global distribution and found butyric acid clostridia in all except five samples.

Reliable densities of clostridia are exceedingly difficult to obtain (62). Dilution counts or anaerobic plate counts have usually shown figures from sporadic to 10^5 per gram with some reports exceeding 10^8 per gram under favorable conditions (34).

While species of the other genera that are known to fix nitrogen are present in soils, little is known of their distribution and numerical significance in soils.

B. Requirements for Nitrogen Fixation.

1) Energy - A readily available source of energy or carbon is

essential for non-symbiotic nitrogen fixation. In *in vitro* studies, glucose or mannitol are generally considered favorable nutrients for *Azotobacter*. However, it is unlikely that sufficient quantities of these compounds are present in soils. Winogradsky (70) pointed out that the natural carbon sources for *Azotobacter* in soils probably are simple compounds such as ethanol, butanol, acetate, propionate, butyrate, etc. that arise as fermentation products of various high-molecular substances (*i.e.* cellulose and hemi-cellulose). The range of carbon sources utilized by the clostridia are simple sugars, including pentoses, di- and poly-sacharides, pectic substances, but no organic acids except possibly lactate.

The bulk of the organic matter added to the soil is dead plant residue that consists chiefly of celluloses, hemi-celluloses and lignin (34). Although cellulose may undergo decomposition by a variety of micro-organisms, in soil the main organisms responsible appear to be the aerobic cytophages, cellvibrios, fungi and actinomycetes and some facultative aerobic bacteria (34). *Azotobacter* apparently is unable to use the metabolic by-products, or the quantities of by-products produced are too small, of such aerobic cellulose decomposers as *Sporocytophaga*, *Cytophaga* (34), *Cellvibrio*, *Cellulobacillus*, *Streptomyces* sp., *Micromonospora chalcea* and *Trichoderma viride* (31). Significant nitrogen fixation under aerobic conditions may be expected by *Azotobacter* with cellulose when in association with facultative aerobes such as *Corynebacterium* (31, 36).

Aerobic decomposition of straw in soil apparently does not yield sufficient simple compounds for appreciable aerobic nitrogen fixation to take place (17, 35). Contrary to these reports however, Rice *et al.*(60),

using a soil-straw mixture with one and five percent straw added, found significant amounts of nitrogen fixed under aerobic conditions with the moisture status of the soil-straw mixture at field capacity, although greater amounts were fixed under water-logged conditions with added straw.

The clostridia appear quite adept at entering into association with some aerobic cellulose decomposers to fix significant quantities of nitrogen, especially when the incubation atmosphere is alternated between atmospheric air and nitrogen gas (67). Rice et al.(60) found maximum fixation using a thin soil-straw layer of 3 to 4 mm saturated with water but incubated in air. They suggested that aerobic cellulolytic organisms converted the straw to simpler intermediates which were then utilized by the nitrogen fixing clostridia.

2) Oxygen - The partial pressure of oxygen has a dual effect on the obligatory aerobes. Growth rates are increased markedly by improved aeration (1, 68) although growth may take place at partial pressures of oxygen as low as 0.008 atm (63, 65). Tschapek and Giambiagi (63) and Garbosky (20) reported that the oxygen demand of Azotobacter chroococcum decreased with decreasing organic nutrient concentration of the medium, thus behaving like a micro-aerophile. This phenomenon appears to be true of aerobic bacteria generally (72).

While improved aeration increases the growth rate of Azotobacter and other aerobes, high partial pressures of oxygen (pO_2 = 0.2 atm) tend to decrease the efficiency of nitrogen fixation (amount fixed per gram energy consumed) because oxygen then competes with gaseous nitrogen for the available hydrogen needed for fixation (51).

More recently Dalton and Postgate (16) have confirmed this phenomenon. They postulated two mechanisms whereby cell free extracts of

Azotobacter vinelandii protected the components of the nitrogenase. Firstly, some steric arrangement of the components results in oxygen tolerance by excluding oxygen from the oxygen-sensitive sites or by stabilizing the complex so that oxygen could not damage it, and secondly, respiration is used to scavenge oxygen from the vicinity of the nitrogen fixing sites. The second postulation, that respiration protects the oxygen-sensitive sites, was first suggested by Philips and Johnson (56), because under excess oxygen, Azotobacter vinelandii consumed sugar at rates greater than those necessary for energy requirements.

Maximum nitrogen fixation, where nitrogen gas was kept at a constant partial pressure of 0.16 atm, was found by Parker and Scutt (51) at a partial pressure of oxygen of 0.1 atm. Myerhof and Burk (46) found a decreasing growth rate but an increasing economy of fixation with decreasing partial pressures of oxygen down to about 0.001 atm. Other workers (49, 63) also found increased nitrogen fixing efficiency at low partial pressures of (pO_2 = 0.02 - 0.04 atm) but decreased efficiencies at higher partial pressures (pO_2 = 0.2 - 0.4 atm) with fixation ceasing completely at an oxygen partial pressure of 0.6 atm (49).

The effect of oxygen on the facultative aerobes is even more severe than for the aerobic organisms. In Bacillus polymyxa, an oxygen content of 1 percent (0.01 atm) completely stops nitrogen fixation (24), while Pengra and Wilson (54) report that nitrogen fixation stopped at pO_2 = 0.05 atm in Aerobacter aerogenes. Hamilton and Wilson (25) also found inhibition of nitrogen fixation by Aerobacter aerogenes grown aerobically. On the other hand, Jensen (33) found this species to fix nitrogen equally well under aerobic or anaerobic conditions.

The effect of oxygen gas on the clostridia and other anaerobic

nitrogen fixers would appear to be a lessening of growth at low pressures but nothing definite is known about the specific effect on nitrogen fixation (34) although recently workers have shown that nitrogen fixation by cell free extracts of C.pasteurianum and B.polymyxa is strongly inhibited by oxygen (12, 13, 23).

3) Reaction - Growth of Azotobacter in soils is usually favoured by a pH in the range of 7.2 to 7.6 with either nitrogen gas or combined nitrogen, although they have been found in soils with a reaction as low as 6.0 (34). The clostridia, on the other hand, can tolerate a wider pH range, but optimum growth is usually also found in near neutral soils (34). Optimum nitrogen fixation activity in enzyme extracts of C.pasteurianum, however, is favored by a pH in the range of 6.3 to 6.5 (41).

4) Mineral Requirements - The mineral requirements of the various aerobic and anaerobic nitrogen fixers has not been studied in great detail although some elements have received considerable attention. Bartels (7), first discovered the requirement of Azotobacter chroococcum for molybdenum, which has since led to the conclusion that it is essential for the nitrogen fixation process for many other aerobic and anaerobic organisms (6, 19, 24, 37, 55, 59, 68). Some of these organisms can replace molybdenum with vanadium in the nitrogen fixation process (6, 37, 68).

Nicholas et al.(47) suggests that small amounts (0.001 ppm) of cobalt may be necessary for the growth of A. vinelandii although it may not be specific for the nitrogen fixation process.

The requirement for iron in the nitrogen fixation process was shown by Grau and Wilson (24) for B.polymyxa while additions of calcium did not influence fixation by this organism, although other workers (11)

have shown a requirement for calcium for most organisms. Greaves and Anderson (22) showed a requirement for sulfate-sulfur by Azotobacter chroococcum. Phosphate requirement by Azotobacter has long been known and confirmed by many workers (32, 43, 71). However, critical values do not appear to have been established.

C. Quantities of Nitrogen Fixed in Soils.

Increases in nitrogen in field soils have often been reported but seldom has the evidence been such that gains in nitrogen can be unquestionably attributed to non-symbiotic nitrogen fixation (50). The reliability of field studies is debatable due to the fact that fluctuations in nitrogen content of the soil occur as a result of leaching, upward movement of soluble inorganic nitrogen, atmospheric sources of fixed nitrogen and denitrification (17). Thus in order to get an accurate estimate of nitrogen fixation, these processes must be quantified, a task not easily accomplished accurately in field studies. As a result, most nitrogen fixation studies are conducted under laboratory conditions in small closed systems where conditions can be controlled.

Laboratory studies with soils enriched with energy materials indicate that 10 to 20 mg of nitrogen are fixed per gram of energy equivalent to glucose (34). Thus when enriched, efficiency of nitrogen fixation in soils appears to be the same in terra as in vitro (34). Jensen (30), in experiments with straw, in "moderate moist nitrogen-poor soil" or pure sand kaolin mixture found no appreciable nitrogen fixation although Azotobacter multiplied vigorously. He concluded that the lack of nitrogen fixation was due to insufficient soluble organic material from the straw as addition of Ca-lactate caused significant nitrogen fixation. Water saturated sand-soil plus oat straw (1 - 5% straw) mixture showed

significant ($P < 0.01$) nitrogen gains of 16, 73 and 93 ppm when incubated 28, 150 and 250 days, respectively (30). Barrow and Jenkinson (4) reported similar results in that no significant nitrogen fixation was encountered in mixtures of soil and straw unless the mixture was water saturated.

Delwiche and Wijler (17), using the ^{15}N tracer technique, failed to detect nitrogen fixation in various non-enriched soils or soils amended with straw, grass or grass roots. However, they did detect significant nitrogen fixation when the soil was enriched with glucose or sucrose or when an inverted sod disc without added energy was incubated under aerobic conditions. Gains of nitrogen reached or exceeded 40 lbs. per acre six inches only when glucose or sucrose was added as a substrate. Less than 4 lbs. of nitrogen per acre six inches was fixed in the other 46 of 50 samples studied.

Rice and Paul (60) found fixation equivalent to 42 to 52 Kg/ha in soils at field capacity and 13 to 150 Kg/ha in water-logged soils when the soil was amended with one percent straw or less. Fixation was measured using the ^{15}N tracer technique. When soils amended with 5 to 20 percent straw were incubated under water-logged conditions, both the ^{15}N and Kjeldahl techniques showed fixation rates of 500 to 1000 Kg/ha. They concluded that atmospheric aerobic conditions provided favorable environment for aerobic organisms which stimulated decomposition of the straw to provide energy material for anaerobic nitrogen fixing organisms flourishing in the water-logged soil.

Chang and Knowles (14), using the ^{15}N tracer technique, agreed with the results of Delwiche and Wijler (17) that significant fixation under aerobic conditions was only measured when glucose was added.

However, they measured significant fixation under anaerobic conditions with a variety of organic and inorganic soils with and without glucose added. Fixation rates ranged from 1.63×10^{-4} to 29.2×10^{-4} mgms per day per gram dry soil.

Knowles (38) using the data of Chang and Knowles (14), corrected for temperature, estimated annual fixation rates for soils incubated under aerobic conditions with glucose added of 5.0 to 34.6 kg/ha/year. Under anaerobic conditions, without glucose, fixation rates of 0.1 to 44.0 Kg/ha/year were measured. With glucose added, fixation rates were estimated at 39 to 73 Kg/ha/year.

Laboratory studies by Porter and Grable (57) showed nitrogen fixation by both photosynthetic and non-photosynthetic organisms by sod mats incubated in an aerobic atmosphere containing ^{15}N. In a 10 day incubation period, mats in the dark fixed from 0.76 to 1.90 Kg/ha and illuminated mats fixed from 3.72 to 6.96 Kg/ha.

D. Techniques for Measuring Non-symbiotic Nitrogen Fixation.

1) Chemical Measurement of Nitrogen Gains - The classical method of measuring nitrogen fixation in soils has been the measurement of total nitrogen before and after incubation, usually by the Kjeldahl method. There are several factors which make this method of measuring nitrogen fixation not too satisfactory: (a) Only a net difference is measured, so that processes such as denitrification may obscure the total amount of nitrogen actually fixed; (b) The quantities of nitrogen fixed non-symbiotically in soils usually are quite small and therefore not easily detected by the Kjeldahl method unless extensive replication is employed. Bremner (9) pointed out that if a soil containing 0.1 percent nitrogen is incubated in normal air, an increase of one percent in the total nitrogen

content of the soil due to fixation cannot be reliably demonstrated by the Kjeldahl method without extensive replication and statistical treatment of the data. If the same sample is incubated in an atmosphere of N_2 containing 60 atom percent excess ^{15}N, an increase of only 0.02 percent in the total N content of the sample due to fixation can be detected by mass spectrometer analysis, as the atom percent ^{15}N of the sample will increase from 0.366 percent to 0.378 percent. Thus the isotopic method is at least 50 times as sensitive as the Kjeldahl method for establishing nitrogen fixation under these conditions.

Despite the advantages of sensitivity that this method has over the Kjeldahl method, Hüser (29) listed several disadvantages: (a) the incubation experiment with N-15 must be conducted in an air tight system; (b) expensive equipment such as a mass spectrometer, ^{15}N gas and high vacuum apparatus must be used; (c) the evacuation of the closed incubation chamber to introduce ^{15}N gas may affect the micro-organisms or plants in the system.

Bremner (9) pointed out that the validity of two basic assumptions in tracer ^{15}N investigations has not been clearly established. These are that the behavior of ^{15}N in physical, chemical and biological processes is identical to that of ^{14}N, and that these processes do not, therefore, lead to variations in the relative abundance of the nitrogen isotopes in soils and other natural materials.

2) Incubation Vessels - A variety of containers have been used for incubating soils in the laboratory for the purpose of measuring respiration and nitrogen transformations. Warburg vessels, Erlenmeyer flasks, desiccators and various jars are commonly used. In nitrogen fixation studies using ^{15}N gas, Chang and Knowles (14) and Delwiche and

Wijler (17) employed vacuum desiccators as their incubation chambers. Rice and Paul (60) utilized a stainless steel chamber whereas Porter and Grable (57) used glass chambers of about 800 ml volume constructed from standard 71/60 tapered joints. In all cases, aeration of the soil sample presumably was by diffusion.

Microbial activity is influenced by the size of the soil sample and surface/volume ratio of the incubation sample. Harmsen and van Schreven (26), in their review on soil nitrogen transformations, observed that in nitrification studies, nitrate accumulation is favored by an increase in the amount of soil used during incubation. They also observed when the soil volume is kept constant, larger amounts of nitrate nitrogen are produced when the soil is incubated in a deep layer as compared to a shallow layer and that when both soil volume and soil surface/volume ratio are kept constant, vigorous aeration results in less nitrification.

This volume and surface/volume ratio effect was also shown by Clark (15) in separate studies on organic matter decomposition and nitrification. Consistently more carbon dioxide was produced per gram of soil when 25 grams of soil + 0.5% alfalfa meal was incubated as compared to 100 grams + 0.5% alfalfa meal. Thus the volume effect was negative. However, in nitrification studies, the volume effect was positive; that is, the more soil used the greater the amount of nitrate produced per gram of soil used. Clark (15) also showed that when the soil volume was kept constant, decreasing the surface/volume ratio increased nitrification. Subsequent studies showed that the favorable influence of a decreasing surface/volume ratio is partly or wholly a function of a more favorable (higher than normal) carbon dioxide content in the soil atmosphere.

Gas chromatographic analysis indicated that the CO_2 content was higher in the depths of soil samples with a narrow surface/volume ratio as compared to a wide surface/volume ratio.

Parr and Reuszer (66) observed that, during studies on the effect of oxygen concentration and flow rate of the aerating gas on the decomposition of straw in soil, decomposition was favored by increasing flow rate at each of several oxygen levels used. They also observed that decomposition was favored by an increase in the oxygen concentration at a given flow rate, although the amount of oxygen supplied did not explain satisfactorily all aspects of their data.

No work was found in the literature on the volume effect, surface/volume ratio effect or effect of method of aeration when volume and surface/volume ratio are constant with respect to non-symbiotic nitrogen fixation in soils.

II MATERIALS AND METHODS

The investigations reported in this study include studies of experimental techniques. These methods are not described in a general methods section, but for purposes of clarity, are discussed along with the results in the appropriate subsection. Analytical procedures employed during the course of the investigation and soil characteristics are outlined in this section.

(1) Soils.

Three soils were used during the course of the study. Some of the characteristics are listed in Table I.

TABLE I

Some Characteristics of Soils Studied

Soil characteristics	Soil type		
	Altona	Portage I	Portage III
Texture	VFSCL	Si CL	CL
pH	7.3	7.5	7.3
Conductivity - mmhos/cm	0.5	0.8	0.5
$CaCO_3$	Absent	V.L.	V.L.
NO_3-N - ppm	12.2	6.3	1.3
Exchangeable - K ppm	1000	410	319
$NaHCO_3$ - Extr. - P ppm	72	11	22
Kjeldahl - Nitrogen %	0.412	0.421	0.287
Atom % ^{15}N	0.3650	0.3672	0.3672

The Portage I and III and Altona soils are described by Ehrlich et al. (18) as moderately well and well drained, black members of the Portage and Altona Associations respectively. The Portage soils were obtained from two separate cultivated sites (Portage I from SE 24-12-6 W;

Portage III from NE 13-12-7 W) on which a cereal crop had been grown. The Altona soil was obtained from a cultivated summerfallow field. The soils were air dried immediately after sampling, ground to pass a 2 mm sieve and stored at room temperature in polyethylene bags for future use.

(2) Soil pH.

The pH of the soil was determined electrometrically by the use of a Fisher Combination Electrode on a Coleman Metrion III pH meter. A soil : distilled water ratio of 1 : 1 ($^{v}/v$) was used. The pH of the supernatant above the soil was measured.

(3) CO_2 Adsorbed in KOH.

Carbon dioxide produced during incubation of soil samples was adsorbed in KOH during incubation. The amount of CO_2 adsorbed was determined by the procedure outlined by Bremner (9).

(4) Conductivity.

The conductivity of the soil was determined electrometrically by the use of a conductivity cell, type CDC104 on a direct reading Radiometer Conductivity meter. A soil : distilled water ratio of 1 : 1 ($^{v}/v$) was used. The conductivity of the supernatant above the soil was measured.

(5) Carbonate Content.

The method outlined by Ridley (61) was used. A one gram soil sample was digested in 10% HCl for 10 minutes. The CO_2 evolved was sucked through a drying and adsorption train and then adsorbed by Ascarite. The weight of CO_2 adsorbed was determined and expressed as percent $CaCO_3$.

(6) NO_3-N.

Nitrate nitrogen determinations were made on the soil samples using the colorimetric nitrophenol disulfonic acid method as modified

by Harper (27).

(7) Phosphorus.

Sodium bicarbonate extractable phosphorus was measured on the soil using the procedure outlined by Olsen et al.(48).

(8) Potassium.

Exchangeable potassium was extracted from the soil with neutral 1 N ammonium acetate. Potassium was determined using a Baird-Atomic KY-2 flame photometer. No correction was made for water soluble potassium.

(9) Total Nitrogen.

Total nitrogen on the soil for characterization purposes was determined by the Kjeldahl procedure as outlined by the AOAC (3).

(10) Total Nitrogen for ^{15}N Determinations.

Total nitrogen for ^{15}N analysis was determined using a procedure outlined by Bremner (61) with the following modications:

(a) During the predigestion treatment, 1.0 ± 0.1 gram of reduced iron was added instead of 5.0 ± 0.1 grams.

(b) Since the digestion heaters employed did not have temperature controls, digestion time during pretreatment and final digestion were reduced to 30 minutes and $1\frac{1}{2}$ hours from 45 minutes and 5 hours, respectively. It was found that the digestion times given by Bremner resulted in the mixture going dry in the digestion flask.

(11) Conversion of Ammonium-N to N_2

The solution from the Kjeldahl determination was acidified and placed on a hot plate and evaporation continued until the solution contained 3 to 5 mgms N per ml.These concentrates were stored at about 4°C until required for further analysis.

The concentrated ammonium nitrogen solutions were used for N_2 production on a vacuum system according to the following procedure[1]:

(a) Close all stopcocks except #5 on vacuum line as illustrated in Figure 1.

(b) Turn on rotary pump and the water on the Diffusion pump.

(c) Plug in Diffusion pump after pumping noise ceases on rotary pump (2 to 3 minutes).

(d) Add 2 to 3 ml of ammonium-N solution (containing 6 to 10 mg NH_4-N) into one arm of a Rittenberg vessel illustrated in Figure 2.

(e) To the other arm add 2 to 3 ml of KI- NaOBr solution, being careful not to let any drop in the NH_4-N solution.

(f) Attach Rittenberg vessel to the vacuum line at the 10/30 female joint. Also attach gas sample tube illustrated in Figure 3 to the vacuum line at the 10/18 male joint.

(g) With valves open on Rittenberg vessel and gas sample tube, open stopcocks #4, #6, #8 and #11 to degass the solutions (1 to 3 minutes).

(h) Immerse Rittenberg vessel in liquid nitrogen and then open stopcock #1. Reduce the pressure to <1 micron (measure pressure on Micro McLeod Vacuum gauge).

(i) Close valve on reaction vessel by rotating the Y-tube while still immersed in liquid nitrogen.

(j) Remove liquid nitrogen and thaw contents of Rittenberg vessel by immersing in water.

(k) Warm gas sample tube with heat gun for a few minutes.

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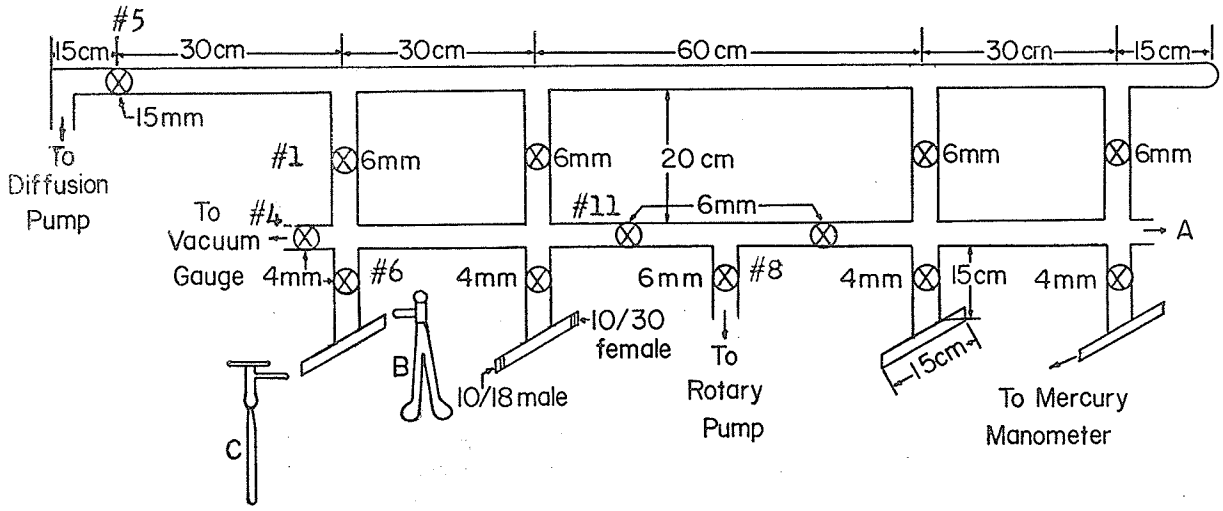
(1) Standard procedure used at U. of M. - C. M. Cho, unpublished data.



⊗ - High-vacuum Stopcock

A - To O_2, A, N_2 Gas Cylinders

B - Rittenberg Reaction Vessel

C - Gas Sample Tube

FIGURE 1. Vacuum line for oxidation of ammonium nitrogen and introduction of gases into incubation vessels.

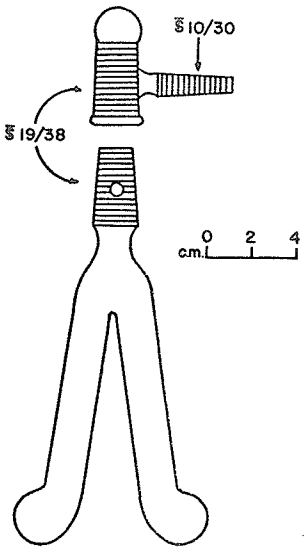


FIGURE 2. Rittenberg reaction vessel for oxidation of ammonium nitrogen.

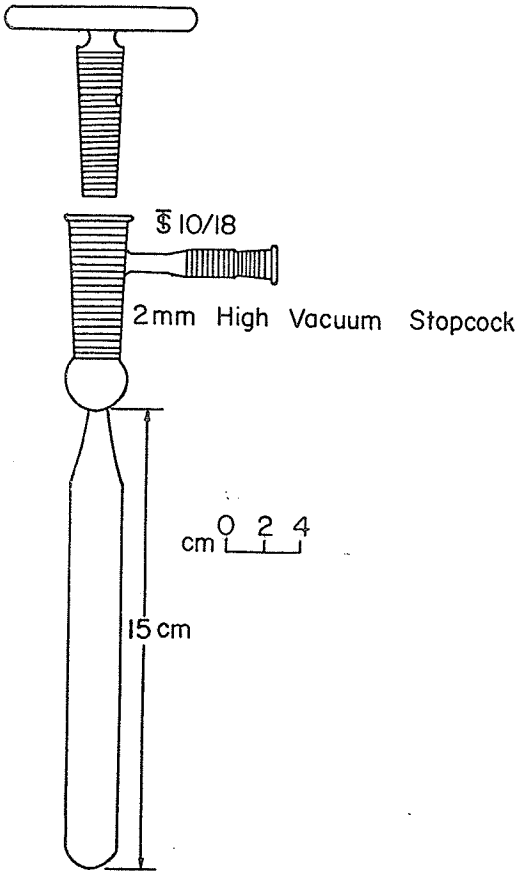


FIGURE 3. Gas sample tube for collecting gas sample for mass spectrometer analysis.

(l) After sample is thawed, mix the NH_4-N solution with the KI- NaOBr solution by tilting the Y-tube so that the NH_4-N solution runs into the KI- NaOBr solution.

(m) After the reaction ceases, immerse Rittenberg vessel in liquid nitrogen for 10 minutes.

(n) Close stopcock #6 and immerse gas sample tube in liquid nitrogen. Open valve on Rittenberg vessel by turning the Y-tube section 180° and allow N_2 gas to diffuse into gas sample tube for 10 minutes.

(o) Close valve on gas sample tube and remove from vacuum rack and determine Atom % ^{15}N on gas sample by connecting gas sample tube to inlet system of a mass spectrometer.

(12) Atom % ^{15}N.

Atom % ^{15}N on the soil sample was determined on a gaseous sample of N_2, derived from oxidation of ammonium-N with hypobromite, as described in the previous subsection, on a MAT GD 1500 mass spectrometer. Atom % ^{15}N was calculated from measured ion current intensities of mass 28 and 29 using the single collector scanning method, according to the formula: atom % $^{15}N = \frac{100}{2R + 1}$ where R is the ratio of ion current intensity of mass 28/29 (61).

Atom % ^{15}N on incubation gas samples was determined by collecting a gas sample from the incubation vessel. The gas sample was introduced into the inlet system of the mass spectrometer and ion current intensities for mass 28, 29, 30 and 32 recorded. Atom % ^{15}N was calculated using the formula: atom % $^{15}N = \frac{N^{29} + 2N^{30}}{2(N^{28} + N^{29} + N^{30})} \times 100$ (61),

where N^{28} = ion current intensity due to mass 28

N^{29} = ion current intensity due to mass 29

N^{30} = ion current intensity due to mass 30.

All ^{15}N determinations were made using the single collector scanning method. This method was chosen instead of the double collector ratio determination method because calibration data indicated that the single collector scanning method gave more accurate determinations as shown by Figure 4. Nitrogen standards of known isotopic abundance were prepared and atom % ^{15}N determined on the mass spectrometer by the two methods. Plotting the observed atom % ^{15}N against the real values showed that the single collector scanning method gave a straight line with a slope of 1, whereas the double collector ratio (non scanning) determination gave a slope of less than one. It can be seen from the plotted lines that even at low atom % ^{15}N values, the double collector ratio determination becomes greater as the atom % ^{15}N increases. This difference is partly due to the contribution of mass 30 ($^{15}N^{15}N$), which increases as the percent ^{15}N increases, to the 28 peak and partly due to the contribution of mass 30 from NO produced but not completely removed during oxidation of $NH_4^+ \rightarrow N_2\uparrow$. These interferences are not a source of error when the single collector scanning method is used.

Since most of the atom % ^{15}N calculations were made based on mass 28 and 29 only, the assumption is made that the equilibrium constant for the reaction $^{14}N^{14}N + {}^{15}N^{15}N = {}^{14}N^{15}N + {}^{14}N^{15}N$ is 4.0 which is the theoretical value. This was experimentally verified on the mass spectrometer used. Data in Table II indicates the K values calculated, according to the following formula: $K = \frac{(^{14}N^{15}N)^2}{(^{14}N^{14}N)(^{15}N^{15}N)}$, from the observed peak heights obtained on four separate ^{15}N determinations using the single

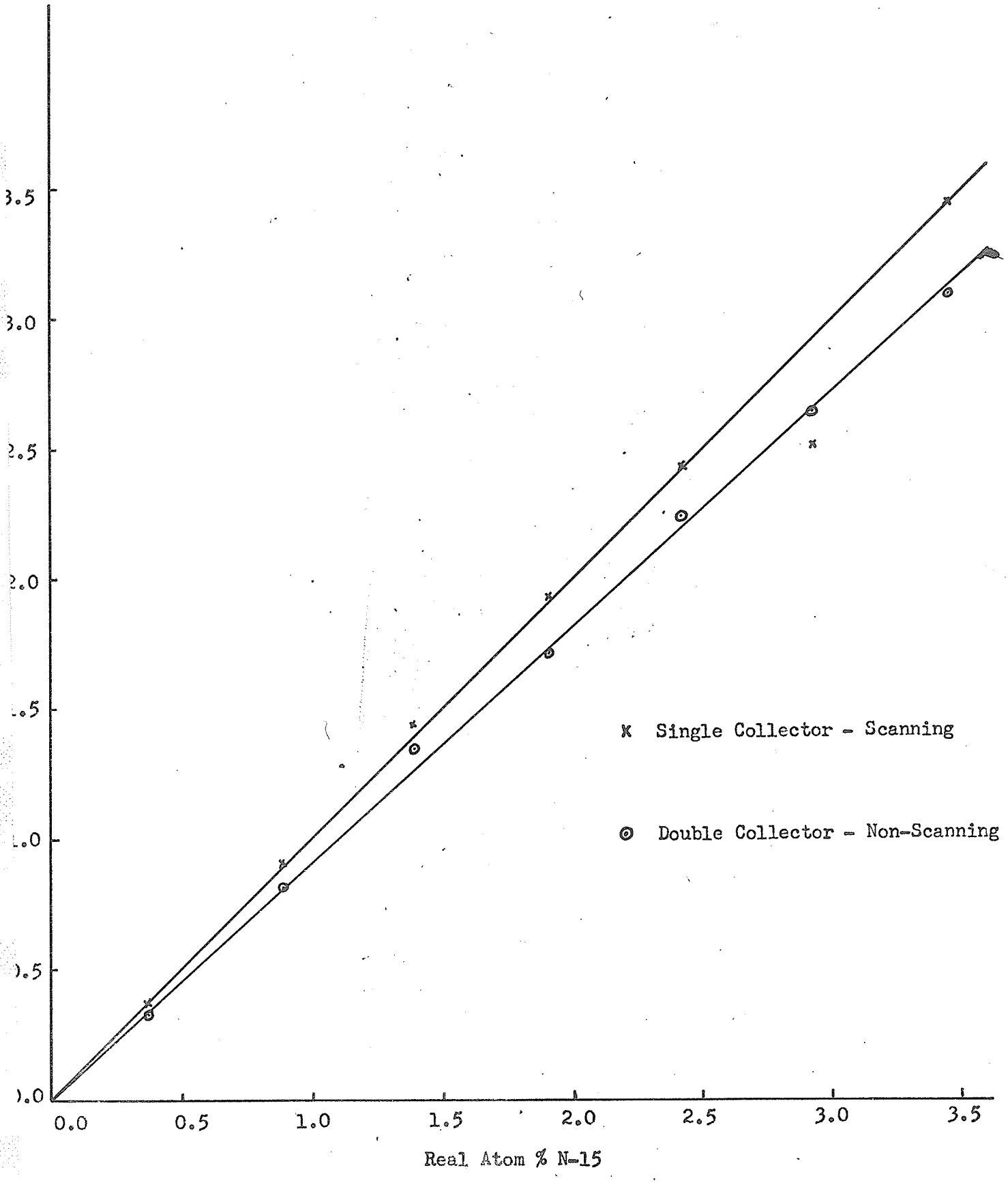


Figure 4. Calibration Curves for scanning and non-scanning methods of determining N-15

collector scanning method.

TABLE II

Observed Equilibrium Constants of N_2^{28}, N_2^{29} N_2^{30}

Derived from $(NH_4)_2SO_4$ + $(^{15}NH_4)_2SO_4$ Mixtures.

Atom % ^{15}N	Peak Heights (mm)			
	$^{14}N^{14}N$	$^{14}N^{15}N$	$^{15}N^{15}N$	K
1) 5.54	520	61.0	1.78	4.02
2) 5.53	423	49.5	1.45	3.99
3) 28.6	900	710	143	3.93
4) 53.7	385	875	515	3.86

(13) Nitrogen Fixation Rates.

Nitrogen fixation rates based on biological N-15 incorporation into the soil sample were calculated using the formula:

$$\text{ppm N Fixed} = \frac{(A) \times (B) \times 10^4}{C}$$

where A = Percent total nitrogen in soil sample.

B = Atom percent ^{15}N excess in soil.

C = Atom percent ^{15}N excess in incubation gas.

III RESULTS AND DISCUSSION

A. Design of Experimental Apparatus and Preliminary Incubation Experiments.

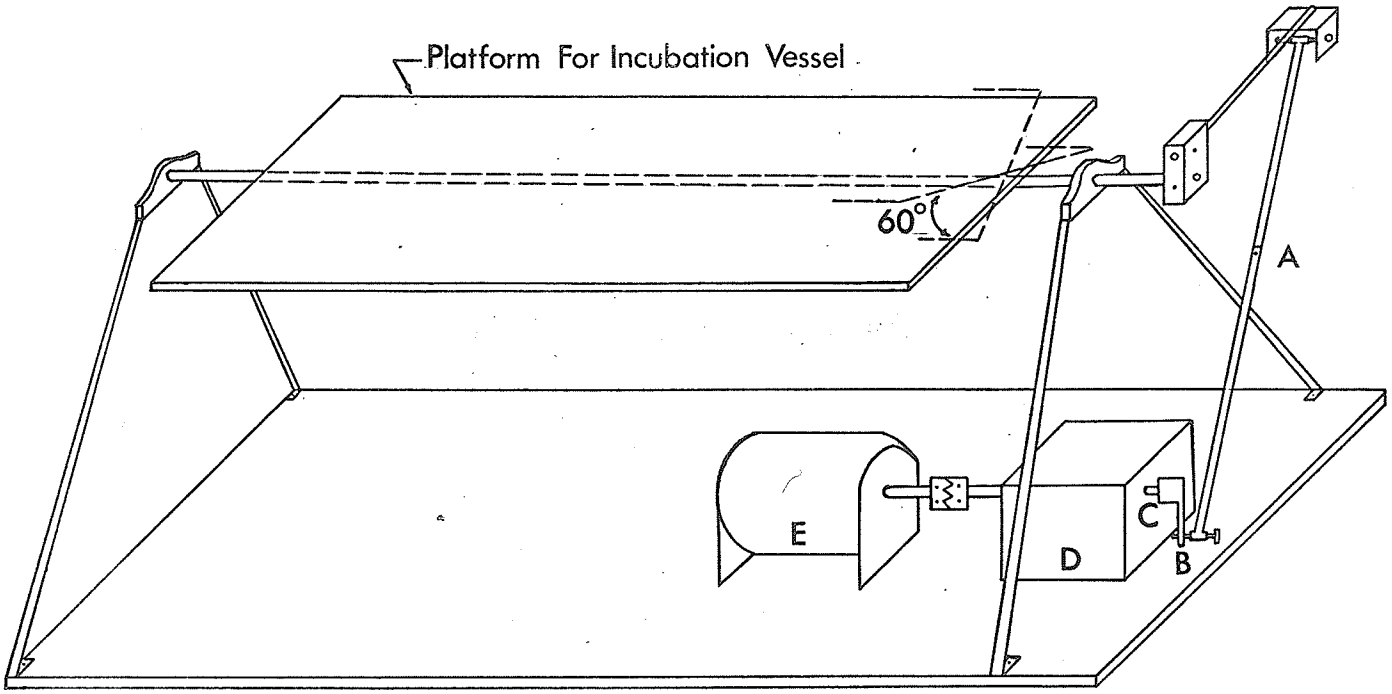
1) Design of Reciprocating Apparatus.

In order to achieve continuous air flow in the incubation vessel, it was necessary to design an apparatus that would mechanically reciprocate through a 60° arc at a constant rate. The apparatus is illustrated Figure 5. The unit is powered by a 1/4 h.p. 1800 RPM electric motor, connected to a speed reducer with a gear ratio of 900 : 1, thus reducing the output shaft of the reducer to 2 rpm. The platform holding the incubation vessels is connected to the output shaft of the speed reducer through an eccentric arm and connecting arm, thus providing the reciprocating action. The magnitude of the arc through which the platform reciprocates is determined by the adjustable connecting arm and the fixed length of the eccentric arm. For the experiments conducted in this study, the arc was adjusted to 60° which resulted in sufficient reciprocating action to achieve continuous air flow in the incubation vessel.

2) Design of Preliminary Incubation Vessel.

An incubation vessel was designed in which a soil sample can be aerated by continuous air flow in a closed system. The apparatus basically consists of two - one litre Erlenmeyer flasks connected at the base by Jaygon tubing and at the mouth of the flasks by a system of glass T-joints, rubber stoppers, Jaygon tubing and a plexiglass cylinder to hold a soil sample. A mercury manometer is attached to the system to monitor gaseous pressure in the closed system. The incubation vessel is illustrated in Figure 6.

The liquid phase volume of the incubation vessel in the



A - Adjustable Connecting Arm
B - Eccentric Arm
C - Output Shaft - 2 rpm.
D - Speed Reducer, Gear Ratio 900 : 1
E - ¼hp., 1800 rpm. Motor

FIGURE 5 Reciprocating apparatus

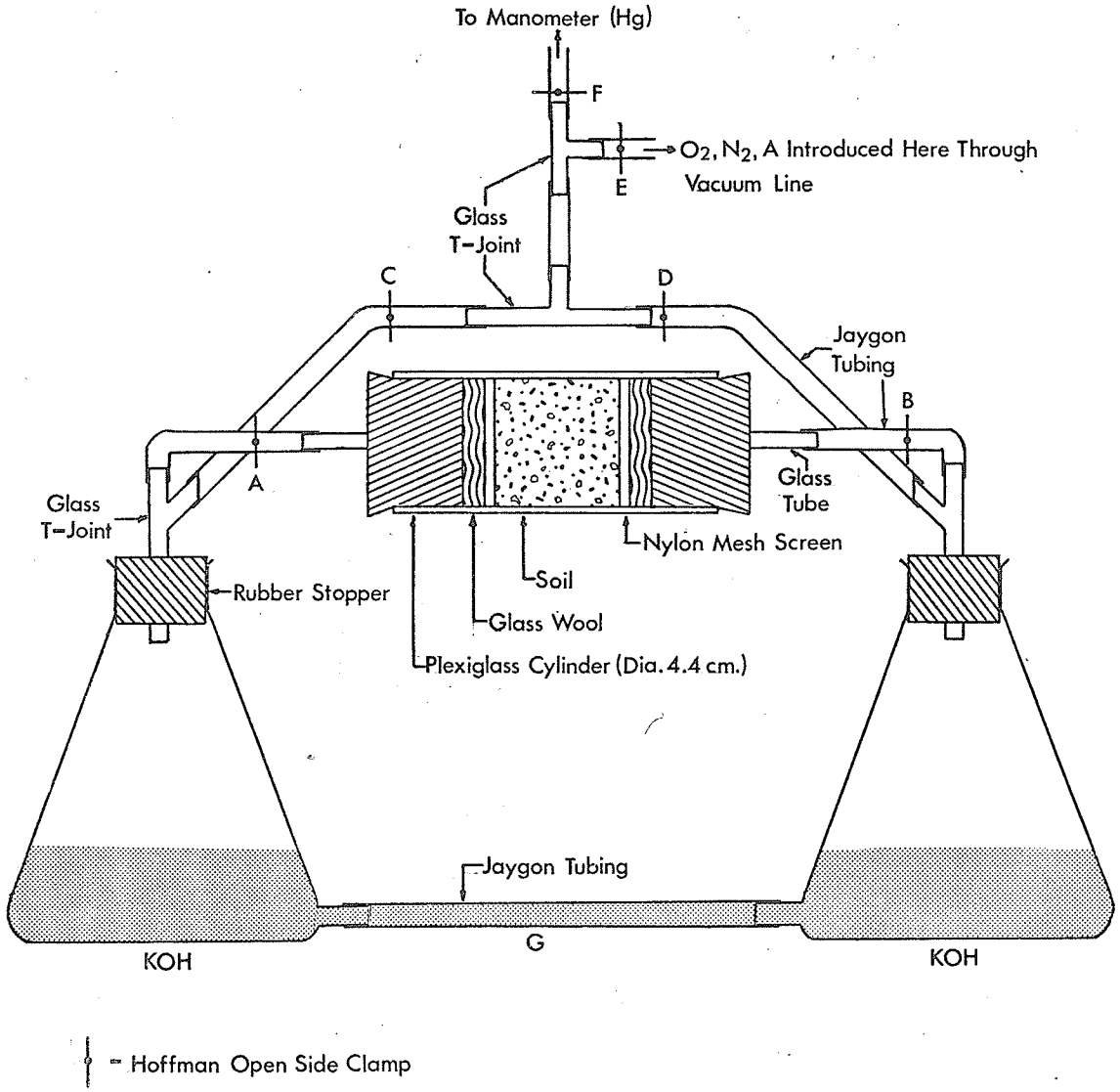


FIGURE 6. Incubation vessel for continuous air flow.

experiments conducted was 1.2 litres while the gaseous phase volume, including pore spaces in the soil sample at the moisture content used, was about one litre.

Continuous air flow is achieved by placing the incubation vessel on the reciprocating apparatus described in the previous subsection. The reciprocating action causes the potassium hydroxide in the flasks to flow back and forth from one flask to the other through the connection at the base of the flask. This in turn forces the gas mixture to flow back and forth through the soil sample, provided clamps A and B are open and clamp C or D of the bypass is closed.

The purpose of the continuous air flow is to ensure that the sample is well aerated with the gas mixture employed in the system and to remove excessive build-up of gaseous products in the soil. The potassium hydroxide, in addition to helping achieve a continuous air flow, adsorbs CO_2 flushed out of the soil.

The attached mercury manometer is used to monitor any pressure changes in the system and to maintain aerobic conditions by indicating the need for oxygen as indicated by a decrease in pressure in the system.

The introduction of the artificial gas mixture into the system is accomplished by connecting the incubation vessel to the vacuum line described in the Materials and Methods section. The incubation vessel is evacuated and flushed with argon three times with fifteen to twenty minute flushing periods. After the fourth evacuation, the system is brought to one atmosphere with the desired gas mixture by introducing each gas separately through the vacuum line and measuring the amount of gas introduced with a mercury manometer. The ^{15}N is also intnoduced into the system through the vacuum line and the transfer of the gas is

achieved by diffusion. This necessitates that the ^{15}N gas be introduced first so that a large pressure differential exists between the incubation vessel and ^{15}N source container.

3) Preliminary Studies with Incubation Vessel.

1) Nitrogen Fixation Experiment with Incubation Vessel Using ^{15}N to Measure Fixation.

A preliminary nitrogen fixation study was conducted with the experimental incubation vessel and reciprocating apparatus. The experiment consisted of two replicated treatments: a control treatment in which the gas mixture in the incubation vessel consisted of 20% oxygen, 20% nitrogen and 60% argon; and an ^{15}N enriched treatment in which the gas mixture was the same except the nitrogen gas was enriched with 19 atom percent ^{15}N.

Four - fifty gram lots of 2 mm size air dry soil were thoroughly mixed with 2 grams of vermiculite and 10 cc of water and placed in plexiglass cylinders. Each cylinder was then connected to the incubation vessel with a layer of glass wool at each end and a nylon mesh screen as shown in Figure 6. Potassium hydroxide (0.1N) was placed in the incubation flasks to absorb CO_2 and to facilitate continuous air flow in the closed system. The system was assembled as shown in Figure 6 and evacuated to 2 cm mercury and allowed to stand for 15 - 20 minutes to check the system for air leaks. If no leaks were found, the gas mixtures were introduced as described in the previous subsection 1B. The samples were incubated for 30 days at room temperature which ranged from 20 to 30°C. Aerobic conditions were maintained by periodic additions of O_2 as indicated by a decrease in pressure as measured by the attached manometer.

After incubation, the apparatus was dismantled and a portion of

each soil sample transferred to a weighed beaker for moisture determination at 100°C for 24 hours. The remainder of the sample was used for total nitrogen analyes and ^{15}N enrichment on the Kjeldahl nitrogen determined. The results of the experiment are shown in Table III.

TABLE III

^{15}N Enrichment and Amount of Nitrogen Fixed by Soil in a Test of Experimental Apparatus(1).

Treatment Gas Mixture (Volume Percent)			Atom Percent ^{15}N	Atom % ^{15}N Excess over Control(2)	Nitrogen Fixed	
					ppm/day	Mgms/50 gs/30 days
O_2	N_2	A				
20	20	60	0	Control	-	-
20	20	60	19	0.0104*	0.07	0.106

(1) All values listed are means of two replicates.

(2) Atom % ^{15}N in control sample was 0.3688.

NOTE:* Indicates a gain of at least 0.01 atom % ^{15}N over control and denotes significant nitrogen fixation (P. W. Wilson (69)).

The results indicated that under the conditions of this experiment, a significant (P= 0.01) biological enrichment of ^{15}N in the soil incubated under an atmosphere which contained ^{15}N gas was measured as compared to the soil incubated under an identical gas mixture but without ^{15}N. This provided positive and quantitative evidence that non-symbiotic nitrogen fixation had occurred during the incubation period.

The sensitivity of the ^{15}N technique is apparent from the following calculations. Since the total nitrogen in the soil samples incubated amounted to 196.5 mgms, and the amount of nitrogen fixed as calculated from the ^{15}N enrichment amounted to 0.106 mgms, the percent

fixation detected is $\frac{0.106}{196.5} \times 100 = 0.054\%$. Bremner (9) pointed out that when a soil is incubated in normal air, extensive replication of the Kjeldahl analysis is required to show an increase of one percent in the total nitrogen due to fixation. Thus the ^{15}N technique as employed is almost 20 times as sensitive as the Kjeldahl method. An additional advantage is that extensive replication is not required with the ^{15}N technique.

The sensitivity of the ^{15}N technique depends in part on the atom % ^{15}N employed in the incubation gas mixture, i.e. the higher the enrichment of the gas mixture, the more sensitive the technique. However, since the cost of ^{15}N gas is about $250 per litre (at S.T.P.) of ^{15}N gas enriched at 54 atom % ^{15}N, the cost of ^{15}N gas has a bearing on the atom percent ^{15}N enrichment used. This is especially so when the incubation vessel has a gas volume of 1.2 litres as was the case in this experiment. The cost of ^{15}N per sample then amounts to about $25 when 20 atom % ^{15}N is the final concentration of ^{15}N used and the total volume percent nitrogen in the incubation vessel is 20%.

ii) A Comparison of Methods of Aeration on Nitrogen Fixation in Soil.

The incubation vessel designed in which a soil sample can be aerated by a continuous air flow in a closed system is a departure from the normal diffusion method of aeration in closed systems when soils are incubated to study microbial activity. An experiment was designed to compare the two methods of soil aeration on non-symbiotic nitrogen fixation in an unamended soil.

Fixation of nitrogen was determined using a gas mixture enriched with ^{15}N. The incubation vessel illustrated in Figure 6 was used for

aeration by continuous flow in a closed system and is subsequently referred to as the "continuous flow" treatment. A one litre Erlenmeyer flask with the soil sample spread in a thin layer on the base of the flask was used for aeration by diffusion and is subsequently referred to as the "diffusion" treatment.

Four-eighty-gram samples of Portage I soil were placed in plexiglass cylinders as shown in Figure 6, and water added to bring to a moisture content of 30 percent by weight. One litre of 0.1N KOH was added to the incubation vessel to absorb CO_2 as illustrated in Figure 6. Another four-eighty-gram samples were spread evenly on the base of one-litre Erlenmeyer flasks and water added to bring to a moisture content of 30 percent by weight. Small vials placed on the base of the flask containing 1.0N KOH were used to absorb CO_2.

The incubation vessels were evacuated and checked for air leaks and then flushed three times with argon gas. After the fourth evacuation, the atmosphere in the vessels was brought to one atmosphere pressure with gas mixtures shown in Table IV, using the procedure previously outlined.

After the gas mixtures were introduced into the incubation vessels, they were placed in a constant temperature room and incubated at 32±0.5°C for 30 days. Aerobic conditions were maintained by periodic additions of oxygen as indicated by a decrease in the pressure in the closed system measured by the attached mercury manometers.

After incubation, a portion of each sample was taken for moisture determination and dried at 100°C for 24 hours and the remainder of the soil was used for nitrogen determinations. Biological ^{15}N enrichment of the soil was measured on the Kjeldahl nitrogen obtained. The observed ^{15}N enrichment and amounts of nitrogen fixed are shown in Table V.

TABLE IV

Composition of gas mixtures used in nitrogen fixation study

Gas Mixture	Aeration Method	Replicate	Composition of Gas Mixtures (Volume Percent)			Atom % ^{15}N
			N_2	O_2	A	
Aerobic	Cont.flow	I	21	21	58	11
		II	21	21	58	9
Aerobic	Diffusion	I	21	21	58	6
		II	21	21	58	19
Anaerobic	Cont.flow	I	20	0	80	11
		II	20	0	80	14
Anaerobic	Diffusion	I	20	0	80	19
		II	20	0	80	10

TABLE V

Observed ^{15}N enrichment and amount of nitrogen fixed in a Portage I soil

Gas Mixture(1)	Aeration Method	Replicate	Atom % Excess ^{15}N in Soil (2)	Nitrogen Fixed ppm/day	Mgms N Fixed/ 80 gms/30 d.
Aerobic	Cont.flow	I	0.012*	0.129	0.310
		II	0.010*	0.132	0.316
Aerobic	Diffusion	I	0.006	N.S.	-
		II	0.011*	0.070	0.168
Anaerobic	Cont.flow	I	0.005	N.S.	-
		II	0.008	N.S.	-
Anaerobic	Diffusion	I	0.008	N.S.	-
		II	0.005	N.S.	-

(1) See Table IV for percentage composition of gas mixtures.

(2) Atom % Excess ^{15}N in soil = Normal abundance of ^{15}N (0.366) subtracted from Atom % ^{15}N observed in soil.

NOTE: * Indicates a gain of at least 0.010 atom % ^{15}N over control and denotes significant nitrogen fixation (Wilson (69)).

Significant ($P = 0.01$) ^{15}N enrichment of the soil was observed only under aerobic conditions where the soil was aerated by continuous flow and in one replicate where the soil was aerated by diffusion. It should be noted that the other replicate in the aerobic, diffusion treatment, where the observed ^{15}N enrichment of the soil was not significant, also corresponded to the lowest level of ^{15}N enrichment in the gas mixture (6 atm % ^{15}N). The low enrichment in the atmosphere was due to insufficient ^{15}N gas on hand. Assuming the same fixation rate in replicate I as in replicate II, it can be calculated that an enrichment of 0.004 atom % excess ^{15}N in the soil would be significant fixation with 6 atom % excess ^{15}N in the atmosphere. However, since the statistically significant ^{15}N enrichment value as calculated from the standard deviation is 0.010, Wilson (69), the measured value of 0.006 atom % ^{15}N excess is not considered significant.

No significant ^{15}N enrichment of the soils incubated under anaerobic conditions was observed, regardless of aeration method.

The results of aerobic vs. anaerobic nitrogen fixation obtained are in disagreement with other workers (14, 17, 60) who have shown that anaerobic conditions usually result in more nitrogen fixation than aerobic conditions. The reason for the abnormal results is not apparent from this study.

The results also indicated that under aerobic conditions, more nitrogen was fixed when the soil was aerated by continuous flow as compared to aeration by diffusion. These differences may not be surprising when the soil surface/volume ratios of the incubation vessels are examined. The surface/volume ratio of the continuous flow treatment is 0.3 as compared to 1.4 for the diffusion treatment. Clark (15) and

Harmsen and Von Schreven (26) have shown that decreasing the surface/volume ratio while keeping the weight of soil constant resulted in an increasing amount of NO_3-N being produced per gram of soil incubated. Further studies showed that this could be attributed to the higher soil CO_2 content in the soil with a low surface/volume ratio as compared to one with a higher suface/volume ratio. Since soil CO_2 content was not measured in this fixation study, it was not possible to relate this phenomena to higher CO_2 content in the soil air. Presumably, the continuous air flow method of aerating the soil should result in a continuous flushing of CO_2 out of the soil. Unfortunately, the amount of CO_2 produced and absorbed in the KOH was not measured in this study. It would appear that the shallow layer of soil in the diffusion treatment provided a less favorable soil air atmosphere for nitrogen fixation than the narrow column of soil in the continuous flow treatment. It is possible that the big difference in the surface/volume ratio in this study was more important than the method of aeration and thus masked any differences due to methods of aeration.

iii) Studies to Determine Effect of Gas Mixtures and Method of Aeration on the Final Moisture Content of the Soil.

The maintenance of a constant soil moisture content during studies of microbial activity in incubation studies is important. Results of the preliminary nitrogen fixation study with the continuous flow incubation vessel indicated the soil moisture level did not change appreciably in a four week incubation period as it only dropped from 22.5 to 19.1%. Subsequent studies with the same incubation vessel indicated that the composition of the gas mixture apparently has a marked effect on the final moisture content of the soil sample. Data shown in Table VI

are the results of separate incubation studies in which various gas mixtures and soil samples were employed.

TABLE VI

The effect of the composition of the gas mixture on the moisture content of several soils when aerated by continuous flow

Composition of Gas Mixtures (Volume Percent) N_2	O_2	A	Soil Used	Percent Moisture of Soil (Dry weight basis) Initial	Final	Incubation Period Days
0	20	80	(50 grams Kaolinite	15.0	8.0	30
80	20	0	+ 50 grams Sand)	15.0	2.0	30
0	20	80	(")	14.4	12.5	21
80	20	0		14.5	2.6	21
21	21	58	(80 grams	30.0	23.5	30
20	0	80	Portage I)	30.0	25.4	30
20	20	60	50 grams Altona	22.5	19.1	30

While decreases in moisture content were observed in all cases, the largest decreases occurred when the percent nitrogen in the gas mixture was highest. Since a large volume of potassium hydroxide (0.1N to 1.0N) is used to absorb CO_2 and facilitate continuous air flow, it is likely that some decrease in soil moisture content is probably due to the adsorption of moisture by the potassium hydroxide. However, this does not explain the differences in final moisture content between the high and low gaseous nitrogen content treatments.

B. Design of Modified Incubation Vessel and Handling of ^{15}N Incubation Gases.

Experience during the preliminary nitrogen fixation studies with

the preliminary incubation vessel (Figure 6) indicated that the vessel required modification to reduce the amount of ^{15}N gas required and to simplify the procedure of introducing ^{15}N gas into the incubation vessel. By reducing the amount of ^{15}N gas required, it is possible to increase the ^{15}N enrichment of the nitrogen gas and still reduce the cost of ^{15}N gas per sample.

In comparing the two methods of soil aeration in preliminary studies, the results indicated higher aerobic nitrogen fixation when the soil was aerated by a continuous air flow in a closed system as compared aeration by diffusion. However, the comparison was confounded since the surface/volume ratios of the soil samples differed by a factor of five. The modified incubation vessel was designed to reduce this confounding factor so that a more valid comparison of the effect ofmethods of aeration could be made on nitrogen fixation.

1) Design of Modified Incubation Vessel.

An incubation vessel was designed which retained the original principles of the preliminary incubation vessel described. The major changes involved decreasing the total size of the apparatus, resulting in a decrease in the gas volume, liquid volume and soil sample container. The vessel consists of a narrow glass cylinder with a constriction in the centre, thus forming two liquid-gas sections, joined by a small passage through the constriction. To each section a glass tube is attached near the centre at the top of the section and these tubes are connected with Jaygon and glass tubing to a plexiglass soil sample container fitted with rubber stoppers at each end. The vessel is illustrated in Figures 7 and 8.

In the incubation vessel where the soil is to be aerated by a

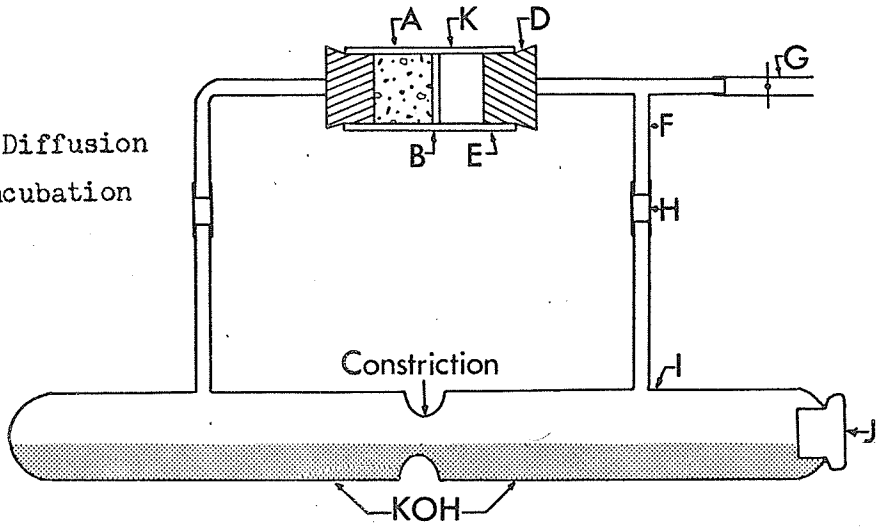


FIGURE 8 - Diffusion type incubation vessel.

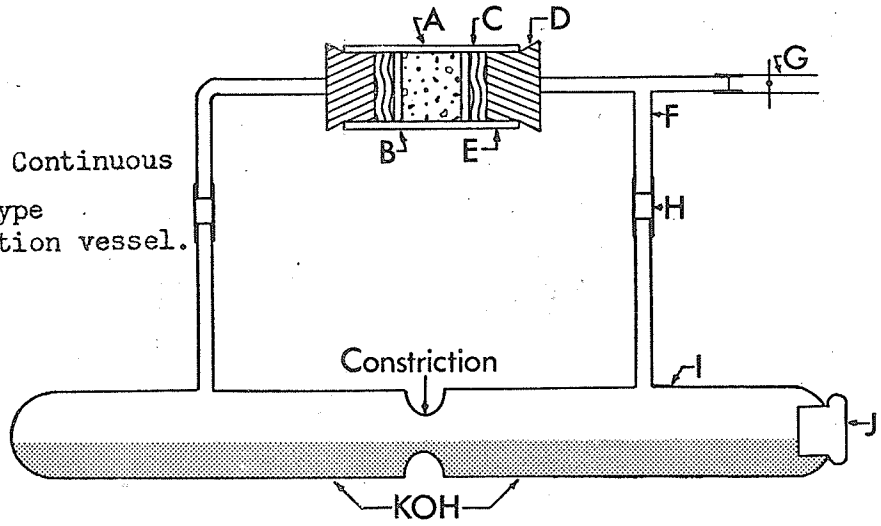


FIGURE 7 - Continuous flow type incubation vessel.

A - Soil
B - Screen
C - Glass Wool
D - Rubber Stopper
E - Plexiglass Cylinder 2.5 cm. dia.
F - Glass T-Joint
G - Jaygon Tubing (Vacuum Line Connection)
H - Jaygon Tubing
I - Glass Liquid-gas Container
J - Serum Stopper
K - Air Space
| - Hoffman Open Side Clamp

continuous air flow, a small glass wool filled air space is left between the rubber stopper and the nylon screen adjacent to the soil sample. This allows the forced air to distribute over the entire soil surface area and be forced through the soil more easily. This is illustrated in the soil sample container arrangement in Figure 7. The continuous air flow is achieved by placing 30 cc. potassium hydroxide in the incubation vessel and placing the vessel on a reciprocator, illustrated in Figure 5.

The incubation vessel in which the soil is to be aerated by diffusion is similar to the continuous flow except that a larger air space is left at one end of the soil sample container while the other end is closed to the incubation gas mixture. This arrangement is illustrated in Figure 8. Aeration of the soil is by diffusion of the gas mixture into the soil from the air space which is connected to the remainder of the gas and liquid sections. During incubation, the vessel is placed on a platform remaining in a stationary state to insure that all gaseous exchanges are by diffusion only.

The total internal volume of the "continuous air flow" and "diffusion" type of incubation vessel are 100 and 105 cc. respectively. Of this volume, 30 cc. is liquid, 20 cc. is soil plus screens and glass wool and 50 and 55 cc. are gas volume. The additional 5 cc. in the gas volume of the "diffusion" type vessel is accounted for by the air space in the soil sample container.

The soil surface/volume ratios of the "continuous air flow" and "diffusion" type of vessel are 0.54 and 0.27 respectively. Although the weight of soil used, the diameter and length of the soil core are identical, the surface/volume ratios are different by a factor of two because

the soil core in the "diffusion" type vessel is in contact with the gas mixture at one end of the core only, as compared to both ends of the soil core in the "continuous air flow" type vessel.

Evacuation and introduction of diluent gas and initial oxygen are accomplished through connection "G" in Figure 7 and 8 by attaching the vessel to the vacuum line illustrated in Figure 1 opposite the manometer connection.

Supplemental oxygen and ^{15}N gas are introduced into the vessels through serum stopper "J" in Figure 7 and 8. These procedures are discussed in the next subsection.

2) Injection of ^{15}N Gas and Supplemental Oxygen into Modified Incubation Vessel.

i) Assembly of ^{15}N gas container and Toepler pump.

Nitrogen-15 gas usually is obtained in break-seal glass clyinders. Since the contents are usually at less than atmospheric pressure, problems are encountered in getting specific quantities transferred to each incubation vessel unless a complicated transfer system is used. A simplified procedure was developed to transfer the ^{15}N gas from the source container to the incubation vessel. A high vacuum stopcock and a glass "break-seal" breaking device are attached to the ^{15}N source container as illustrated in Figure 9. The ^{15}N container and the "break-seal" breaking device are attached to a modified Toepler pump as illustrated in Figure 10. After evacuating the area between stopcock "A" and the "break-seal", stopcock "A" is closed and the "break-seal" is broken by propelling the metal bar against it. The metal bar is accelerated with a magnet. Stopcock "A" is then used to release ^{15}N gas from the container into the Toepler pump as required.

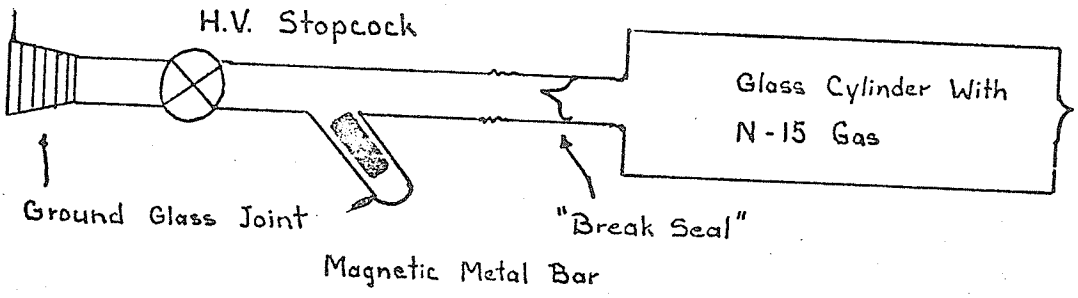


FIGURE 9 - ^{15}N source container on "break seal" breaking device.

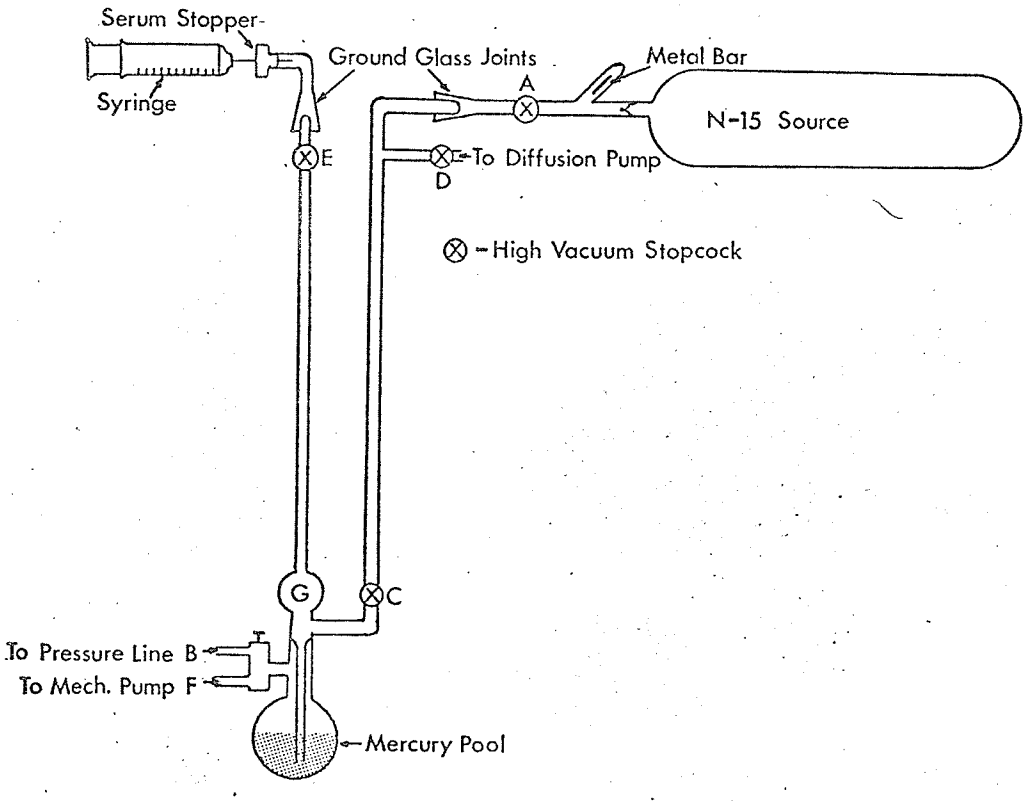


FIGURE 10 - Apparatus for extracting ^{15}N with syringe.

ii) Injection of ^{15}N and other gases.

With the incubation vessel attached to the vacuum line, the vessel is evacuated and filled with argon gas to flush out atmospheric air. This procedure is repeated twice and after the third evacuation, it is filled with oxygen and argon for an aerobic mixture or with argon only for an anaerobic gas mixture. For the aerobic gas mixture, oxygen and argon are added in the proportion of 1 : 3 by volume. Just prior to the introduction of the ^{15}N gas, 10 - 12 cc. of gas mixture are removed from the incubation vessel with a hypodermic syringe with the needle inserted through the serum stopper. ^{15}N gas is then added according to the following procedure using the apparatus illustrated in Figure 10:

(a) With stopcock A, C and D closed, open stopcock E and F and evacuate gas phase above mercury pool.

(b) Open stopcock D and evacuate. After pumping noise stops, slowly open stopcock C and evacuate.

(c) Close stopcock C and D after complete evacuation

(d) Open stopcock B very slowly until mercury rises to the half way mark in bulb G. Close stopcock B and attach hand pressure pump or pressure line to B.

(e) Open stopcock A and allow ^{15}N gas into Toepler pump. Close A after a few seconds.

(f) Slowly open stopcock C and allow ^{15}N gas to bubble through mercury in G until the mercury level has dropped to the level in the pool or no more ^{15}N will bubble through the mercury. Close stopcock C.

(g) Insert syringe needle into serum stopper with the syringe plunger completely in the syringe.

(h) Open stopcock B very slowly until mercury stops rising in Toepler pump. With B open, apply pressure with the hand pump or pressure line. This brings the pressure of the ^{15}N gas in Toepler pump to greater than one atmosphere and causes the ^{15}N to flow into the syringe, slowly pushing the syringe plunger out. Close stopcock E when the plunger has moved out to the 10 cc. mark as indicated by the gradations on the syringe.

(i) Remove syringe and immediately insert needle into serum stopper in incubation vessel and inject ^{15}N into vessel by slowly pushing plunger in.

Supplemental oxygen is added to the incubation vessel using a similar procedure except the oxygen supply is obtained in steel cylinders under pressure.

In order to determine whether the method of adding the ^{15}N gas to the incubation vessel led to contamination from the air during the time the syringe with ^{15}N is withdrawn from the Toepler pump and inserted into the incubation vessel, ^{15}N purity checks were performed after the gas was injected into the incubation vessel. Three experiments were conducted. In each study, the incubation vessel was evacuated twice and flushed with argon to remove atmospheric nitrogen. After the third evacuation, the vessel was filled with argon to one atmosphere. Ten cc. of argon was removed and 10 cc. of ^{15}N injected as described earlier. Immediately after injection of ^{15}N (within 10 minutes) a gas sample was extracted for ^{15}N analysis. In the first study, a second sample

was extracted after 24 hours, to check on leaks into the apparatus. In the second and third studies, the second sample was extracted after 48 and 96 hours respectively. The results of the ^{15}N analysis on the gas samples extracted are shown in Table VII.

TABLE VII

Atom percent ^{15}N in gas samples extracted from incubation vessels after injection of ^{15}N with hypodermic syringe.

Run No.	(^{15}N Source = 54.0 Atom % ^{15}N) Sampling Time (Hours after Injection)			
	0 Hours	24 Hours	48 Hours	96 Hours
1	52.0	51.9	-	-
2	52.2	-	53.3	-
3	53.1	-	-	51.3
Average	52.6			

The results of the 0 hour sampling indicate that the contamination of the ^{15}N gas with atmospheric nitrogen was minor as the atom % ^{15}N decreased from 54 to 52.6, a reduction of only 2.6 percent (1.4 percentage points). This reduction could be due to leakage of atmospheric nitrogen into the syringe during transfer of gas to the incubation vessel, and incomplete evacuation of gas sample tube used for collecting ^{15}N gas sample.

The results of the second sampling at 24 and 96 hours respectively, indicated a further small decrease in the atom % ^{15}N while a small increase was recorded at the 48 hour sampling. The decreases could be attributed to incomplete flushing of atmospheric nitrogen gas from the soil sample and leakage of atmospheric nitrogen into the system. The latter is not likely significant as checks at mass 32 revealed no significant amounts of oxygen present in the gas sample extracted. No

reason is apparent for the small increase in the atom % ^{15}N at the 48 hour second sampling as compared to the 0 hour sampling in the second run.

C. Nitrogen Fixation Studies with Modified Incubation Vessels.

The preliminary studies of nitrogen fixation with the original incubation vessel indicated that more fixation was observed under aerobic than anaerobic conditions. More aerobic nitrogen fixation was recorded when the soil was aerated by continuous flow than when aerated by diffusion. Since the spatial arrangement of the soil samples in the two methods of aeration were quite different, resulting in widely different surface/volume ratios, the comparison on method of aeration was confounded.

The modified incubation vessels were designed to allow a truer comparison between the two methods of aeration as the spatial arrangement for each type of aeration is identical, although the surface/volume ratios are not equal.

Two studies were conducted to compare the effect of (a) an aerobic and anaerobic gaseous atmosphere; (b) two methods of soil aeration, by diffusion and by continuous air flow; (c) the addition of energy in the form of glucose plus cellulose and without additional energy on non-symbiotic nitrogen fixation. Each treatment was replicated twice, resulting in sixteen samples for each study. In the first study, the aerobic gas mixture was termed "semi aerobic" since the oxygen level was not maintained at its original level, resulting in the atmosphere becoming increasingly less aerobic.

In each study, eight eighteen-gram samples of Portage III soil containing 0.5% glucose + 0.5% cellulose by weight were placed in plexiglass cylinders as shown in Figures 7 and 8 with four in each type of vessel. Sufficient water was added to bring the moisture content up to the levels indicated in Table XII. Another eight eighteen-gram samples of non-enriched

Portage III soil were placed in plexiglass cylinders as shown in Figures 7 and 8, with four in each type of vessel and the moisture content brought up the levels shown in Table XII.

Thirty ml. of 4% KOH (40 grams/l) solution was added to each incubation vessel through opening "J" with the serum stopper removed.

The incubation vessels were evacuated and checked for air leaks and then flushed twice with argon. After the third evacuation, the vessels were brought to one atmosphere pressure with an aerobic or anaerobic gas mixture by procedures described previously. The percentage gaseous compositions are listed in Table VIII.

TABLE VIII

Gaseous Composition for Nitrogen Fixation

Gas Mixture	Volume Percent Composition of Gases			Atom Percent ^{15}N
	Oxygen	Argon	Nitrogen	
Aerobic	20	60	20	53.2
Anaerobic	0	80	20	53.2

The eight incubation vessels in each study assembled as shown in Figure 8 referred to as soil aeration by continuous flow treatments, were placed on a reciprocator. The other eight vessels in each study, assembled as illustrated in Figure 7 and referred to as soil aeration by diffusion treatments, were placed on a stationary platform. All samples were incubated at $25 \pm 0.5°C$ for 14 days in the first study and 21 days in the second study.

In the first study, no supplemental oxygen was added during the 14-day incubation period. In the second study, supplemental oxygen was

added as required. The oxygen requirement was determined by measuring the pressure deficit in the incubation vessel by inserting a hypodermic needle connected to a small 15 cm manometer, into the serum stopper. The supplemental oxygen was injected with a hypodermic shringe to bring the pressure deficit to zero.

After incubation each sample was weighed and dried at 50°C for 24 hours to determine its moisture content. Total nitrogen and atom % ^{15}N were determined on each sample. The results are reported in Tables IX and X. In the second study, CO_2 adsorbed in the KOH was determined.

In both studies, significant ^{15}N enrichment of the soil was obtained under an anaerobic atmosphere for both methods of soil aeration only when energy in the form of glucose and cellulose was supplied. Under a "semi aerobic" and an aerobic atmosphere, significant ^{15}N enrichment of the soil occurred only when the soil was aerated by the diffusion method and energy was added.

No significant ^{15}N enrichment was observed when the energy amended soil was aerated by continuous flow under "semi aerobic" or aerobic conditions. In both studies, no significant ^{15}N enrichment of the soil occurred without added energy for either method of aeration, regardless whether the incubation gas was aerobic or anaerobic.

In both studies, more nitrogen was fixed per day under an anaerobic than under a "semi aerobic" or aerobic atmosphere as data in Tables IX and X indicate. These results are contradictory to the results obtained in the preliminary study reported on (Table V), but are in agreement with results reported in the literature by other workers (14, 17, 60). It should be noted that the soil used in the preliminary study

TABLE IX

Observed ^{15}N enrichment and rate of nitrogen fixation in a Portage soil incubated for 14 days
(All values means of two replicates)

Incubation Atmosphere	Aeration Method	Energy Added	Atom Percent ^{15}N Excess(1)	Nitrogen fixed ppm/day	Nitrogen fixed Mgms/18 gms /14 days
"Semi Aerobic"	Continuous flow	+	0.0086	N.S.	-
		-	0.0066	N.S.	-
	Diffusion	+	0.0252**	0.087	0.022
	Diffusion	-	0.0021	N.S.	-
Anaerobic	Continuous flow	+	0.0522**	0.201	0.051
		-	0.0053	N.S.	-
	Diffusion	+	0.1398**	0.539	0.136
	Diffusion	-	0.0052	N.S.	-

(1) Observed atom percent ^{15}N - 0.3672 (natural abundance of ^{15}N in Portage III soil - 16 determinations $\bar{x} = 0.3672 \pm 0.0056$) = atom percent ^{15}N excess.

P <.01 = 0.0144 atom % ^{15}N excess

P <.05 = 0.0110 atom % ^{15}N excess.

TABLE X

Observed ^{15}N enrichment and rate of fixation in a Portage soil incubated for 21 days

Incubation Atmosphere	Aeration Method	Energy Added	Atom Percent ^{15}N Excess(1)	Nitrogen fixed ppm/day	Nitrogen fixed Mgms/18 gms /21 days
Aerobic	Continuous flow	+	0.0009	N.S.	-
		-	0.0036	N.S.	-
	Diffusion	+	0.0151*	0.030	0.011
	Diffusion	-	0.0	N.S.	-
Anaerobic	Continuous flow	+	0.0224**	0.058	0.022
		-	0.0005	N.S.	-
	Diffusion	+	0.1843**	0.473	0.179
	Diffusion	-	0.0036	N.S.	-

(1) Observed atom percent ^{15}N - 0.3672 = atom percent ^{15}N excess.

was not obtained from the same location as the soil in the present study although both soils belong to the Portage Association.

The results also indicate that under both the "semi aerobic" and aerobic and anaerobic gas mixture, more nitrogen was fixed when the energy amended soil was aerated by diffusion than by continuous air flow. Under the anaerobic gas mixture, the differences ranged from 2.7 to 8.0 fold. Under the "semi aerobic" or aerobic gas mixture, no fixation was measured when the soil was aerated by continuous flow while 0.087 and 0.030 ppm/day nitrogen was fixed in the 14- and 21-day incubation periods, respectively. These results appear to be in agreement with the general view that anaerobic nitrogen fixation in soils is more significant than aerobic fixation. The results reported in this study could be explained on the basis of the degree of anaerobiosis existing in the soil sample. Ranking of the soil samples on the basis of anaerobiosis would probably place them in the following order of decreasing anaerobiosis: Anaerobic gas mixture, aeration by diffusion > anaerobic gas mixture, aeration by continuous flow > "semi aerobic and aerobic gas mixture, aeration by diffusion > "semi aerobic" and aerobic gas mixture, aeration by continuous flow. It is significant that in both studies, the quantity of nitrogen fixed decreased in the same order, that is the largest amount was fixed under the anaerobic gas mixture with aeration by diffusion and none was fixed under the "semi aerobic" or aerobic gas mixture with aeration by continuous flow.

Data presented in Table XI from the 21-day incubation study indicate that less CO_2 was produced when the energy amended soil was aerated by diffusion under aerobic and anaerobic conditions than when aerated by diffusion under the same conditions. This could be due either

to less CO_2 being produced or less being removed from the soil in the aeration by diffusion treatment. It is probable that the continuous air flow type of aeration would flush more CO_2 from the soil than aeration by diffusion. Harmsen and von Schreven (26) showed that when both soil volume and the surface/volume ratio are kept constant "vigorous aeration" resulted in less nitrification than "less vigorous aeration". They attributed this to higher soil CO_2 content in the less vigorously aerated soil.

TABLE XI

Carbon dioxide adsorbed during 21-day incubation period in nitrogen fixation study in energy enriched soil.
(All values averages of two replicates.)

Incubation atmosphere	Aeration method	Mgms CO_2 adsorbed
Aerobic	Continuous flow	120
	Diffusion	100
Anaerobic	Continuous flow	47
	Diffusion	38

TABLE XII

Moisture contents of soil before and after incubation as affected by method of aeration during nitrogen fixation studies.
(All values averages of eight replicates.)

Aeration method	Percent Moisture(1)			
	14-day incubation		21-day incubation	
	Initial	Final	Initial	Final
Continuous flow	36.5	35.2	27.8	25.1
Diffusion	36.5	35.0	27.8	26.2

(1) Initial percent moisture determined by amount of water added to soil. Final moisture determined by drying soil at 50°C for 24 hours.

Since the method of aeration used could affect the soil moisture

content and thus affect microbial activity, initial and final soil moisture contents were recorded and are reported in Table XII.

No appreciable changes occurred in the soil moisture contents during incubation. It is not likely that the small changes contributed to the differences in the amount of nitrogen fixation between the two types of aeration.

V SUMMARY AND CONCLUSIONS

An incubation vessel was designed which would allow aeration of a soil sample by a continuous air flow in a closed system when placed on a reciprocating apparatus. Preliminary studies of non-symbiotic nitrogen fixation using an ^{15}N enriched atmosphere indicated that the incubation vessel could be used for nitrogen fixation studies. The results of the initial studies showed that:

(1) The gas volume of the vessel was so large that sufficiently ^{15}N enriched nitrogen gas resulted in costly experiments.

(2) The preliminary comparison of the effect of two methods of aeration on nitrogen fixation resulted in more nitrogen fixed when the soil was aerated by continuous flow as compared to aeration by diffusion. These differences were not strictly attributable to the methods of aeration as the incubation vessels used resulted in the soil samples having widely different surface/volume ratios.

(3) Aerobic nitrogen fixation with unamended soil was greater than anaerobic fixation.

As a result of these studies, the incubation vessel was modified, retaining the original principal of aeration by continuous flow in a closed system, but was reduced in size resulting in a gas volume of 50 - 55 cc. as compared to 1.2 l in the original vessel. This allowed for the use of highly enriched nitrogen gas (50 atom % ^{15}N) as compared to 10 - 20 atom % ^{15}N used originally, while still reducing the cost of ^{15}N from $25 to about $3 per sample. Experiences during the investigation indicated that a high level of ^{15}N enrichment in the gas was desirable in order to achieve significant ^{15}N enrichment in the soils, especially

when no energy is added to the soil.

A simplified procedure of introducing ^{15}N into the incubation vessel was developed and tests showed that the procedure did not result in appreciable contamination of ^{15}N gas with atmospheric air. This procedure can also be used to introduce supplementary oxygen during the incubation period to maintain aerobic conditions.

Aerobic and anaerobic soil nitrogen fixation were studied using two methods of aeration and soil with and without energy in the form of glucose and cellulose added. The results of two studies indicated that:

(a) No significant ^{15}N enrichment of the soil was observed regardless of method of aeration for both aerobic and anaerobic conditions without energy added.

(b) Anaerobic nitrogen fixation for both methods of aeration was greater than or equal to "semi aerobic" or aerobic nitrogen fixation under the same conditions with added energy.

(c) Anaerobic nitrogen fixation in soil with added energy with aeration by diffusion was 0.54 and 0.48 ppm/day as compared to 0.20 and 0.06 ppm/day with aeration by continuous flow under the same conditions in the 14- and 21-day incubation periods respectively.

(d) Semi aerobic and aerobic nitrogen fixation in energy amended soil with aeration by diffusion amounted to 0.09 and 0.03 ppm/day as compared to none with aeration by continuous flow under the same conditions in the 14- and 21-day incubation periods respectively.

(e) During continuous flow aeration using 4% KOH to force air through the soil sample, the moisture content of the soil

did not change appreciably.

(f) The continuous air flow method of aeration resulted in more CO_2 being produced under aerobic and anaerobic conditions than aeration by diffusion during 21 days of incubation.

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