RHIZOSPHERE N₂ FIXATION IN A FOREST ECOSYSTEM
IN SITU ASSAYS AND EVALUATION OF THE
ACETYLENE REDUCTION TECHNIQUE

by
Inger Börjesson

A dissertation submitted in partial fulfillment
of the requirements for the degree
of
DOCTOR OF PHILOSOPHY
in
Biology

Approved:

UTAH STATE UNIVERSITY
Logan, Utah
1983
ACKNOWLEDGEMENT

This study was supported by a grant from the US National Science Foundation and by the Ecology Center at Utah State University.

I am grateful to the members of my committe, especially to Dr. E.E. Harding for her support during field work. I also want to express my thanks to my friends that have supported me during the time that this study was in progress.

Eugor Björkström
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td><strong>Chapter</strong></td>
<td></td>
</tr>
<tr>
<td>I REVIEW OF LITERATURE</td>
<td>1</td>
</tr>
<tr>
<td>History</td>
<td>1</td>
</tr>
<tr>
<td>The N₂ Fixing System</td>
<td>3</td>
</tr>
<tr>
<td>The N₂ Fixing Organisms</td>
<td>6</td>
</tr>
<tr>
<td>Acetylene Reduction Technique</td>
<td>14</td>
</tr>
<tr>
<td>II IN SITU MEASUREMENTS OF N₂ FIXATION ACTIVITY USING THE ACETYLENE REDUCTION TECHNIQUE</td>
<td>22</td>
</tr>
<tr>
<td>Introduction</td>
<td>22</td>
</tr>
<tr>
<td>Objectives</td>
<td>23</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>23</td>
</tr>
<tr>
<td>Results</td>
<td>36</td>
</tr>
<tr>
<td>Discussion</td>
<td>45</td>
</tr>
<tr>
<td>Summary</td>
<td>50</td>
</tr>
<tr>
<td>III AN ALTERNATIVE METHOD FOR EVALUATION OF ACETYLENE REDUCTION ASSAYS</td>
<td>52</td>
</tr>
<tr>
<td>Introduction</td>
<td>52</td>
</tr>
<tr>
<td>General Aspects of Diffusion and Effusion</td>
<td>53</td>
</tr>
<tr>
<td>The Method in Current Use</td>
<td>56</td>
</tr>
<tr>
<td>The Closed System</td>
<td>62</td>
</tr>
<tr>
<td>The Open System</td>
<td>73</td>
</tr>
<tr>
<td>Summary</td>
<td>82</td>
</tr>
<tr>
<td>IV EVALUATION OF ACETYLENE REDUCTION ASSAYS CORRECTING FOR EFFUSION</td>
<td>84</td>
</tr>
<tr>
<td>Introduction</td>
<td>84</td>
</tr>
<tr>
<td>Results</td>
<td>85</td>
</tr>
<tr>
<td>Discussion</td>
<td>85</td>
</tr>
<tr>
<td>Chapter</td>
<td>Page</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>Summary</td>
<td>92</td>
</tr>
<tr>
<td>V SUMMARY</td>
<td>94</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>99</td>
</tr>
<tr>
<td>VITA</td>
<td>107</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. A survey of N₂ fixation activity on the rhizoplane of plants collected from a subalpine forest.</td>
<td>26</td>
</tr>
<tr>
<td>2. Plant species from which N₂ fixing bacteria have/have not been isolated.</td>
<td>27</td>
</tr>
<tr>
<td>3. Total N (Kjeldahl N) content, in percent of plant dry weight, of plants collected from Jebo Creek, July 1980.</td>
<td>27</td>
</tr>
<tr>
<td>4. Number of cases tested for N₂(C₂H₂) fixation activity and endogenous ethylene production in each site at different times during the 1980 and the 1981 seasons.</td>
<td>30</td>
</tr>
<tr>
<td>5. Endogenous ethylene production from the soil and from plant+soil at different times. Comparison is made to the ethylene produced in enclosures for N₂(C₂H₂) fixation assays.</td>
<td>37</td>
</tr>
<tr>
<td>6. N input by N₂ fixation in the four sites the meadow, the aspen, the fir and the spruce at different times during the vegetation period. Significance level for an increased activity in the presence of plant at each performed assay.</td>
<td>42</td>
</tr>
<tr>
<td>7. Annual N input by N₂ fixation in the four sites the meadow, the aspen, the fir and the spruce.</td>
<td>43</td>
</tr>
<tr>
<td>8. Increase in ( d(E/P)/dt ) over the time of an assay for a constant ethylene production rate and different, but constant</td>
<td>57</td>
</tr>
<tr>
<td>9. N input by N₂ fixation in the four sites the meadow, the aspen, the fir and the spruce at different times during the vegetation period. Significance level for an increased activity in the presence of plant at each performed assay. Activities are corrected for effusion.</td>
<td>87</td>
</tr>
<tr>
<td>10. Annual N input by N₂ fixation in the four sited the meadow, the aspen, the fir and the spruce. Activities corrected for effusion.</td>
<td>87</td>
</tr>
<tr>
<td>11. N inputs measured in the meadow, 8 September 1980.</td>
<td>89</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>12. Error in corrected acetylene reduction activity if the true effusion rate deviates ±lsd from the determined effusion rate.</td>
<td>91</td>
</tr>
<tr>
<td>13. Interval for the difference in acetylene reduction activity between two enclosures if their true effusion rates deviate ±lsd from the determined effusion rates.</td>
<td>91</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>1. Correlation between depth of soil in the pot and from it measured $N_2(C_2H_2)$ fixation activity.</td>
<td>39</td>
</tr>
<tr>
<td>2. Correlation between soil moisture content and measured $N_2(C_2H_2)$ fixation activity.</td>
<td>39</td>
</tr>
<tr>
<td>3. Diurnal variation in $N_2(C_2H_2)$ fixation activity.</td>
<td>40</td>
</tr>
<tr>
<td>4. $N_2(C_2H_2)$ fixation activity from plant+soil, bare soil and net activity in association with plant in each of the four sites the meadow, the aspen, the fir and the spruce at different times during the vegetation period.</td>
<td>41</td>
</tr>
<tr>
<td>5. N input by $N_2$ fixation in the soil in the four sites the meadow, the aspen, the fir and the spruce at different times during the vegetation period.</td>
<td>44</td>
</tr>
<tr>
<td>6. Hypothetical difference between diffusion from an open system and effusion from a closed system with respect to the concentration of the diffusing/effusing substance in the volume where it is initially present over time.</td>
<td>55</td>
</tr>
<tr>
<td>7. Time courses for amount propane, amount ethylene and the ratio $E/P$ for the same production rate of ethylene but different effusion rates from a closed system.</td>
<td>65</td>
</tr>
<tr>
<td>8. Relationships of interest for evaluation of acetylene reduction activity in closed system.</td>
<td>72</td>
</tr>
<tr>
<td>9. Rates of effusion and corresponding standard deviations measured from enclosures used for testing.</td>
<td>86</td>
</tr>
<tr>
<td>10. Relationship between overestimation of the $N_2(C_2H_2)$ fixation activity calculated without correction for effusion and the effusion rate.</td>
<td>88</td>
</tr>
</tbody>
</table>
ABSTRACT
Rhizosphere N₂ Fixation in a Forest Ecosystem

in situ Assays and Evaluation of the Acetylene Reduction Technique

by
Inger Börjesson, Doctor of Philosophy
Utah State University, 1983

Major Professor: Dr J.J. Skujins
Department: Biology

In situ assays of N₂ fixation activity, using the acetylene reduction technique, were performed in four successional stages of a Northern Wasatch Mountain subalpine forest ecosystem, elevation 2,800 m. Emphasis was made on rhizosphere fixation in association with Antennaria microphylla and Achillea millefolium. The vegetation period was approximately 100 days.

Assays were performed in Saran bags. A defined amount of propane was injected at initiation of the assay and acetylene was generated from CaC₂. Samples were analyzed for ethylene and propane. Data were evaluated assuming that the ethylene production was directly proportional to the increase in the ratio of ethylene to propane in the samples.

Input of N by soil free-living N₂ fixers in the meadow, the aspen, the fir and the spruce was 0.5, 0.3, 0.2 and 0.3 kg N ha⁻¹ y⁻¹, respectively. A higher activity in the presence of plant, as compared to the soil activity, was measured in 10 of 16 assays, however, the
increase was significant at three testings only. This might indicate a contribution by rhizosphere N\textsubscript{2} fixation, but to an extent lower than was expected and therefore not detectable with the method used.

Leakage of gases from the test device was not corrected for in the method used for evaluation of data. This introduces an overestimation of the obtained activities that increases exponentially with a more rapid effusion rate. Correction for effusion from a closed device can be made provided quantitative analyses of the tracer gas. To determine a small difference between enclosures with and without plants the accuracies in the effusion rates must be high. Quantitative analyses were not required for evaluation according to the method used and therefore, the obtained effusion rates have too wide standard deviations for correction of the effusion rate. It was shown that the determined effusion rates with corresponding standard deviations might obscure a low rhizosphere N\textsubscript{2} fixation activity.

Acetylene reduction assays performed in open devices can not easily be corrected for diffusion of gases. The initial very rapid diffusion from an open device leads to a vastly overestimated acetylene reduction activity, when the diffusion is not corrected for.
CHAPTER I
REVIEW OF LITERATURE

History

When life first emerged on the earth, the atmosphere provided reducing conditions and it is possible that the nitrogen transfer in the environment was non-cyclic (Postgate, 1973). During prebiotic era, abiologically fixed N accumulated in the form of NH₃. This was the N source for early life and was present in an amount sufficient to support life and its evolution for millions of years. At the time that free O₂ appeared, most of the planetary NH₃ was consumed and converted into N₂ and NO₃⁻, and the oxidizing environment enhanced the rate of decrease in the NH₃ still remaining. Nitrogen ultimately became the limiting nutrient for life, and the ability to biologically fix N₂ subsequently evolved. Exactly when this occurred is unknown, but it could have been more recent than 10⁹ years ago (Postgate, 1973).

There is evidence that demonstrates that the ability to fix N₂ emerged only once in evolutionary development. In all N₂ fixing species, where the N₂ fixing system has been investigated, the N₂ fixation is performed by an enzyme complex consisting of two different protein compounds both of which are required for maximal activity. Non-homologous combinations of the two proteins have, in 85% of the tested combinations, resulted in a catalytically active N₂ fixing system. Inactive combinations were obtained when one protein from the
strict aerobe *Azotobacter vinelandii* was combined with the complementary protein from the strict anaerobe *Clostridium pasteurianum*. In this case, the preserved homology was sufficient for the two proteins to bind together, but the binding was too tight (Emerich and Burris, 1976). A combination between *C. pasteurianum* and *Anabaena cylindrica* proteins formed an active nitrogenase (Tsai and Mortensson, 1978).

The molecular weights of the two proteins are independent of the source organisms (Eady, 1980), and sequence studies have shown a strongly conserved genetic message for the respective proteins.

During the 17th Century, including legumes in crop rotation became a practice in Europe (Aulie, 1970) and a 40-50% increase in crop yield has been estimated for the 18th Century. The reason for this impressive benefits from the introduced agricultural practice became a subject of scientific interest. When methods for chemical analysis were developed, the increase was related to the high N content of legumes. The question as to whether plants could acquire N from the atmospheric N₂ was intensively discussed among scientists during the 18th Century (Aulie, 1970). In 1883, the connection to microbiology was made when German plant chemists demonstrated a good correlation between the growth of pea plants and the presence of nodules on their roots.

*In vitro* transfers of the nitrogen fixation genes (*nif*) are commonly performed without any great difficulties (Dixon et al., 1976). Although the *nif* cluster is too large for transduction, both transformation and conjugation are likely to occur *in vivo*. Postgate (1973) postulates that microbial evolution, in regard to acquisition
of the $N_2$ fixation ability, at present is an accelerating process. New species able to fix $N_2$ are likely to appear whenever the selection pressure is strong enough.

The $N_2$ Fixing System

The nitrogenase complex

The $N_2$ fixing system consists of two components, one Mo-Fe containing protein and one protein containing Fe only. The Mo-Fe protein is commonly referred to as number 1 and the Fe protein as number 2 (Eady et al., 1972). A nomenclature descriptive for the enzymatic action of the respective components was introduced by Burris et al. (1981). The Mo-Fe protein that binds and reduces the substrate is then referred to as dinitrogenase and the Fe protein is referred to as dinitrogenase reductase as it provides the dinitrogenase with the required electrons. The two together make up the nitrogenase (Nase).

Nitrogenase. The nitrogenase is the only isolated enzyme capable of reducing a triple bond (Burns and Hardy, 1975) and it performs this reduction especially when the triple bond is bridging N to N, N to O, N to C and C to C. It makes nitrogenase a very versatile enzyme which is able to reduce substrates such as $N_2$, $C_2H_2$, $N_2O$, $CN^-$ and $N_3^-$, in addition it also reduces $H_3O^+$ to $H_2$.

In nature, the reduction of $N_2$ to $NH_3$ is the most important reaction catalyzed by nitrogenase. Industrial fixation of the chemically inert $N_2$ is a highly energy consuming process that also requires extremely strong reagents as well as anoxic and anhydrous conditions. The $N_2$ fixing organisms are, with respect to $N_2$ fixation, superior to
man as they can carry out the reduction of $N_2$ to $NH_3$ in living protoplasm surrounded by air and water. However, also for $N_2$ fixers, it takes a large amount of energy as well as a reagent providing a low potential of reducing equivalents. In vivo both energy and electrons are supplied from carbohydrates. Energy in the form of ATP, and the number of ATPs hydrolyzed, for each $N_2$ reduced, vary between investigations from as low as five to as high as 30 (Yates, 1977). The necessary electrons are transferred to the nitrogenase via a ferredoxin electron donor system (Burns and Hardy, 1975).

The dinitrogenase can bind two molecules of dinitrogenase reductase, but an almost maximal activity has been measured for a 1:1 ratio between the two components of C. pasteurianum (Burris et al., 1981). In vivo, a variation in the component ratio has been observed, 1:1 was determined for Klebsiella pneumoniae (Eady, 1973) and 2:1 for C. pasteurianum (Vandecasteele and Burris, 1970).

Dinitrogenase. The dinitrogenase is the largest of the nitrogenase components, molecular weight 220,000 daltons. Two subunits, forming a tetramer structure of $\alpha_2\beta_2$ type, have been isolated from dinitrogenase of different sources (Lowe et al., 1980). The amino acid composition of the two subunits is very similar and the molecular weights for the $\alpha$ and the $\beta$ subunits are 50,000 and 60,000 daltons, respectively.

The action center of dinitrogenase constitutes of Fe-S clusters. Molybdenum is essential for enzymatic activity. Its role is not exactly known, but it is thought to be involved in reduction of the substrate (Ljones, 1974).
Oxygen causes irreversible damage of the dinitrogenase and after 10 min exposure to air, half of the activity is lost in isolates of A. vinelandii and K. pneumoniae. The $t_{1/2}$ is considerably shorter for the strict anaerobe C. pasteurianum (Postgate, 1978).

**Dinitrogenase reductase.** The dinitrogenase reductase has the molecular weight 65,000 daltons. Biochemical data indicate that it is a dimer composed of two identical subunits (Lowe et al., 1980).

The dinitrogenase reductase is extremely sensitive to $O_2$ and is irreversibly destroyed in air, $t_{1/2}$ about 30 s (Postgate, 1978).

**Mechanism for $N_2$ fixation**

**Electron transport.** The complete mechanism for $N_2$ fixation is still not unanimously agreed upon, but present opinion favors a step-wise process of four steps for the electron transfer (Masterson and Murphy, 1980).

1. Reduction of the dinitrogenase reductase.

EPR measurements of the nitrogenase components separately and combined support the hypothesis that the electrons are transferred from ferredoxin or flavodoxin to the dinitrogenase reductase.

2. Intermolecular transfer of electrons.

The dinitrogenase reductase transfers one electron at a time to the dinitrogenase (Ljones and Burris, 1978). As mentioned earlier, the component ratio is variable and the higher ratio of dinitrogenase reductase to dinitrogenase, the faster the electron flow.

3. Intramolecular transfer of electrons.

All reductions by nitrogenase take a multiple of two electrons and
the transferred electrons, one at a time, must be stored in the dinitrogenase molecule. What feature of the dinitrogenase acts as an electron sink is so far unknown.

4. Reduction of substrate.

Reduction of substrate occurs when the dinitrogenase has reached the reducing capacity required for reduction of available substrate. The reduction of $N_2$ to $NH_3$ requires six electrons while reduction of $C_2H_2$ to $C_2H_4$ requires only two.

The role of ATP. Activated ATP, MgATP, is specifically required for nitrogenase activity. A binding of MgATP to the dinitrogenase reductase has been demonstrated for isolates of different organisms (Eady and Smith, 1979). This results in a lower redox potential and induces the electron transfer to the dinitrogenase. For a long time, the interaction with the dinitrogenase reductase was thought to be the only role of ATP, but later discoveries have revealed that MgATP also is closely related to the reduction of substrate (Rennie et al., 1978; Thorneley et al., 1979).

The $N_2$ Fixing Organisms

Distribution

$N_2$ fixing organisms are present in a wide variety of habitats, the only prerequisites are the possession of genes for nitrogenase synthesis and a source for the required energy and reducing capacity. They appear in all $O_2$ regimes, although often actively fixing $N_2$ only under low $p_{O_2}$. Some $N_2$ fixers are exclusively symbionts, others are free-living, and a number of associations with other organisms have
been observed. The carbon and energy requirements vary from heterotrophically living organisms, via chemoautotrophs to photoautotrophs. To date \( \text{N}_2 \) fixation ability has been accepted only in procaryotic organisms, but \( \text{N}_2 \) fixation has been reported in a thermophilic green algae (Yamada and Sakaguchi, 1980).

\( \text{N}_2 \) fixing bacteria are taxonomically widely distributed, but high potentials for \( \text{N}_2 \) fixation are mainly attributed to species of Azotobacteriaceae, Rhizobiaceae, Spirillaceae and, to some extent, Bacillaceae and Enterobacteriaceae. Anaerobic bacteria as Clostridium spp. and Desulfovibrio spp. are known to fix \( \text{N}_2 \) but little attention has been paid to these species, although Clostridium could have a significant influence on the \( \text{N}_2 \) fixation in waterlogged soils. Lists of \( \text{N}_2 \) fixing organisms vary slightly between authors and species are continuously added and deleted (Dalton, 1980; Postgate, 1981).

**N input by \( \text{N}_2 \) fixing organisms**

Global aspects. The turnover time for the global \( \text{N} \) is approximately \( 10^6 \) years, and 80\% of the \( \text{N}_2 \) fixed is by biological \( \text{N}_2 \) fixation. Estimations of \( \text{N} \) input to different ecosystems are presented by Delwich (1970), Burns and Hardy (1975) and Söderlund and Svensson (1976) and might be summarized as follows. 80\% of the biologically fixed \( \text{N} \) is fixed to terrestrial ecosystems and the remaining 20\% to aquatic systems, this is approximately equal to 200 and 1 kg \( \text{N} \) ha\(^{-1}\) y\(^{-1}\), respectively. The high terrestrial contribution is mainly in association with agriculture where 175 kg \( \text{N} \) ha\(^{-1}\) y\(^{-1}\) is fixed, 140 kg ha\(^{-1}\) y\(^{-1}\) by legumes. Non-agricultural land can be divided into for-
ests, grassland-meadow and others (including sediments) and the N input to each category is estimated to 10, 15 and 2 kg N ha\(^{-1}\) year\(^{-1}\), respectively. Considering the variation in area between the different ecosystems, agricultural land, grassland-meadow and forest each gets 25% of the biologically fixed N.

**N\(_2\)** fixation in soil. 

N\(_2\) fixation activity in virgin soils is attributed to cyanobacteria, free-living bacteria and nodulated wild plants. In situ measurements of N\(_2\) fixation are scarce and reported activities range from a few micrograms to hundreds of kilograms of N per hectare per year.

N input by cyanobacteria in various soils was determined to be 1-25 kg N ha\(^{-1}\) year\(^{-1}\) (Postgate, 1974; Stewart et al., 1977). A considerable higher input of N by cyanobacteria was measured in a subtropical grassland, 122 kg N ha\(^{-1}\) year\(^{-1}\) (Jones, 1977).

A maximal N\(_2\) fixation rate by free-living bacteria, Azotobacter and Clostridium, corresponding to 0.3 kg N ha\(^{-1}\) year\(^{-1}\) was calculated for various unamended soils (Postgate, 1974). Vlassak, Paul and Harris (1973) determined the contribution to N input by free-living bacteria in a grassland in the temperate zone to 2 kg N ha\(^{-1}\) year\(^{-1}\). A N input as high as 384 kg N ha\(^{-1}\) year\(^{-1}\) was measured for a tropical salt marsh (Carpenter et al., 1978). In the Arctic and sub-Arctic zones, yearly inputs in the range of micrograms N have been reported (Jurgesen and Davey, 1971; Granhall and Lid-Torsvik, 1975).

**Rhizosphere associated N\(_2\)** fixation. 

From free-living N\(_2\) fixers, a significantly higher activity has been measured in the rhizosphere of plants than in non-rhizosphere influenced soil. The higher availa-
bility of carbohydrates, originating from root exudates, lysates and litter, in the rhizosphere is considered to cause the increased activity. The rhizosphere associated $N_2$ fixation is of special importance in natural soils where the carbohydrate content is the limiting factor for $N_2$ fixation activity.

In association with *Thalassia testudinum* (marsh grass) the rhizosphere $N_2$ fixation was 10-50 kg N ha$^{-1}$ y$^{-1}$ (Capone and Taylor, 1980). The rhizosphere associated $N_2$ fixation in a tropical salt marsh, 2595 kg N ha$^{-1}$ y$^{-1}$, was six to seven times higher than was measured in the non-rhizosphere influenced soil in the same marsh land (Carpenter et al., 1978; Valiela and Teal, 1979). A potential $N_2$ fixation in association with *Paspalum notatum* was determined to 100 kg N ha$^{-1}$ y$^{-1}$ (Döbereiner, 1974). In the tropics, $N_2$ fixation associated with maize was 2 kg N ha$^{-1}$ d$^{-1}$ (vonBulow and Döbereiner, 1975).

**Environmental factors affecting the activity of free-living $N_2$ fixers**

*Light.* A stimulation of the rhizosphere $N_2$ fixation by an increased light intensity was observed both in growth chambers and in situ. The observed increase in $N_2$ fixation activity is closely related to the plant response to light. The nitrogenase activity, in itself, is independent of light.

In full sunshine, photosynthesis is active and the plant is rich in carbonaceous compounds, resulting in a large quantity of root exudates. During night, the exudation products originate from the hydrolysis of stored starch (Balandreau, 1975), which on its part is dependent on the photosynthetic activity during daytime.
**Temperature.** Assuming the $Q_{10}$ value is 2 for the nitrogenase activity, as it has been shown to be for many other biological processes, soil temperature is likely to influence the $N_2$ fixation. In the temperate zone, the soil temperature is frequently a limiting factor for the $N_2$ fixing activity (Balandreau, 1980).

The rate by which assimilates are transported through the plant is dependent on the temperature and increases with a higher temperature (West, 1971). In the climate zone, where the light intensity is high during the day and the temperature is high during night, two peaks, for daytime and nighttime, respectively, can be shown for the associative $N_2$ fixation activity (Balandreau, 1975). Under temperate conditions, where the nighttime temperature falls under 19°C, there is usually no night peak (Balandreau and Ducerf, 1979).

**Water.** An increase in the soil water content will lower the $P_{O_2}$ and provide a better oxygen regime for the $N_2$ fixing organisms.

A low water content in the air will cause closure of stomata preventing transpiration and avoiding excessive reduction in osmotic pressure. This will decrease the photosynthetic activity and a lower level of root exudates is likely to be the effect.

In the plant, the upward flow rate of water with dissolved nutrients decreases with a lower air and soil water content. In legumes, the removal of newly synthesized ammonium from the sites for $N_2$ fixation is highly dependent on the upward flow in the plant (Minchin and Pate, 1974). An accumulation of ammonium could be inhibitory for the $N_2$ fixation activity. A similar action would not be entirely unlikely for rhizosphere associated $N_2$ fixation (Balandreau, 1980).
Plant. Plants growing in waterlogged soils provide for a higher potential of the \( \text{N}_2 \) fixation activity in the rhizosphere than plants growing under more normal water regimes (Balandreau, 1980). This could possibly be due to the differences in physiology between the plants, but more probably due to the low redox potential under waterlogged conditions.

High numbers of the \( \text{N}_2 \) fixing bacterium Azotobacter paspali have been shown exclusively in the rhizosphere of Paspalum notatum (Döberleiner, 1974). Chromosome changes in wheat resulted in strains showing a higher count of \( \text{N}_2 \) fixing organisms in the rhizosphere, as compared to unmodified strains (Neal and Larson, 1976). Compounds, inhibitory to \( \text{N}_2 \) fixation, are produced from some plants, e.g. Sorghum (Balandreau, 1980) and Ambrosia (Rice, 1965). The inhibitory action for these compounds is not known.

The above factors imply that the plant genome at least could have some influence on the \( \text{N}_2 \) fixation activity in the rhizosphere. Genes coding for efficiency of photosynthesis, translocation and exudation can possibly determine the rhizosphere associated \( \text{N}_2 \) fixation activity.

Micro-organisms in the rhizosphere

Population of \( \text{N}_2 \) fixers. Aerobic, heterotrophic bacteria isolated from the roots of Oryza spp. (wetland) gave a positive \( \text{N}_2 \) fixation response in 75 to 90% of tested isolates (Watanabe, 1981). The majority of the positive species were identified as close to Pseudomonas. A higher nitrogenase activity in the rhizosphere, as compared
to the non-rhizosphere influenced soil, was found by Low and White (1976).

Some $N_2$ fixers, e.g. *Azotobacter* spp., have been isolated both from the rhizosphere of plants and from non-rhizosphere environments and a difference in abundance of the species favors the rhizosphere. Other species have only been demonstrated in more or less species specific associations with plants.

Plants with $C_4$ photosynthetic pathway are considered to selectively enhance growth of $N_2$ fixing bacteria. A stimulation of *Beijerinckia* spp. was found in the rhizosphere of certain grasses, rice and sugar cane (Döbereiner and Campelo, 1971). *Azospirillum* and *Enterobacter* have also been reported as $N_2$ fixing inhabitants of rice roots (Watanabe et al., 1979). Grasses in general stimulate growth of *Spirillum lipoferum*, as compared to legumes (Döbereiner et al., 1976) that seems to favour *Klebsiella* spp. (Evans et al., 1972).

In situ, an interaction between bacteria species in the rhizosphere has been demonstrated. On roots of rice seedlings, colonies of *Beijerinckia* were observed, except for the root tip region. An antagonistic action by actinomycetes, abundant in the soil used, and the limited exudation from the young stage of the plant contributed to the lack of *Beijerinckia* colonization at the root tip (Diem et al., 1978). A lower $N_2$ fixation activity was measured *in vitro* from the rhizosphere of rice than was obtained *in situ* (Watanabe, 1981), indicating a positive effect by interaction between the natural flora.

Site for colonization. The acidity of root exudates decreases the colonization by the acid sensitive *Azotobacter* on the root sur-
face, and they are mostly found in the rhizosphere influenced soil. Beijerinckia is acid tolerant and grows better on the root surface than in the surrounding soil, where competition from Azotobacter occurs (Döbereiner, 1974; Diem et al., 1978).

Firm evidence that \( \text{N}_2 \) fixers would invade the intercellular space between living root cells or the living root cells has not been equivocally demonstrated. Reports of inter- and intracellular colonization are derived from electron microscopic studies and the distinction between living and damaged or dead root cells is not made. In 3 to 4 weeks old roots, as much as 70% of the cortical root cells can be dead (Holden, 1975). At the end of the growing season of wheat, the root surface is separated from the living tissue by a sheath of collapsed cortical cells occupied by bacteria (Foster and Rovira, 1976). The most probable number of \( \text{N}_2 \) fixing bacteria was estimated from surface sterilized (endorhizospheric colonization) and from only washed (rhizoplane and endorhizospheric colonization) roots of maize and rice (Diem and Dommergues, 1980). Both species showed an almost exclusively rhizoplane colonization which is in agreement with results by Okon et al. (1977) and Scott et al. (1978).

The mucigel layer surrounding the roots has a high C/N ratio and speculations about this layer as a favorable site for \( \text{N}_2 \) fixers have been made. Electron microscopic studies have revealed electron transparent zones in the mucigel (Bowen and Rovira, 1976; Dayan et al., 1977) but any strong evidence for the mucigelous layer as a favorable site for \( \text{N}_2 \) fixing organisms has not been shown. In contrast, roots lacking the mucigel have an increased number of bacteria at-
tached (Old and Nicolson, 1975).

Soil micro-organisms seem unable to colonize the root apex (Diem et al., 1978; Dayan et al., 1977). This inability is attributed to the thickness of the root cap mucigel (Brown, 1975), antimicrobial substances exuded in this region of the root (Samtsevich, 1968) and the time it takes for micro-organisms to respond to and occupy the new growth (Diem et al., 1978).

Acetylene Reduction Technique

Optional methods

Prior to the development of the acetylene reduction technique, the N₂ fixation activity was determined by measuring the total N content or the isotopic enrichment in the investigated systems.

Total N content. The increase in N content in the investigated system can be determined by the Kjeldahl's method. However, the sensitivity of the method is low and applicable mainly when large variations in the N content are to be determined. The Dumas' method is more sensitive but requires more specialized equipment.

Isotopic enrichment. The isotopic method using ¹⁵N₂ is considered to give the most accurate estimate of the N₂ fixation activity. General use of the ¹⁵N method is practically restricted by its high cost, need for long incubation times and the specialized technique for analysis.

Nomenclature

The method used for determination of N₂ fixation activity is of-
ten a main concern when comparisons between investigations are made. A visually easy detectable denotation has been recommended (Masterson and Murphy, 1980), where estimations using the acetylene reduction technique is denoted $N_2(C_2H_2)$ fixation and $N_2(^{15}N)$ fixation indicates that the isotopic method has been used.

**Acetylene as substrate**

Acetylene, $C_2H_2$, as an inhibitor of $N_2$ fixation activity was observed by Schollhorn and Burris (1967) and a reduction of the $C_2H_2$ to ethylene, $C_2H_4$, by the nitrogenase was discovered by Dilworth (1966). This provided the basis for the development of an inexpensive, highly sensitive and non-destructive method for the estimation of $N_2$ fixation activity. The acetylene reduction technique is today widely applied on all types of biological $N_2$ fixing systems both in laboratory and in field studies.

The method is based on the versatility of the nitrogenase for which $C_2H_2$ is one of the substrates reduced. The product, $C_2H_4$, is quickly and easily detected using gas chromatography with a flame ionization detector. An alternative procedure for detecting ethylene is the colorimetric determination after ethylene has been oxidized to formaldehyde (LaRue and Kurz, 1973), but this method is less sensitive, slower and more complicated to perform.

Acetylene used as substrate for the nitrogenase requires a high grade of purity. Contaminants present in commercial or welding grade $C_2H_2$ have been shown to decrease the acetylene reduction activity in white clover (Tough and Crush, 1979).
Interaction between \( \text{N}_2 \) and \( \text{C}_2\text{H}_2 \)

The rate-substrate relation for nitrogenase is for practical purposes simplified to follow that for a 0 order reaction. The substrate concentration must, for the entire time of the assay, be in the enzyme controlled region. \( K_m \) values for \( \text{N}_2 \) and \( \text{C}_2\text{H}_2 \) are, in pure systems, 0.04 and 0.005, respectively (Hardy et al., 1973). When exposed to mixed substrates, 10% \( \text{C}_2\text{H}_2 \) in air, the \( \text{C}_2\text{H}_2 \) occupies 80-90% of the available sites on the nitrogenase present (Hardy and Burns, 1968). The different \( K_m \) values for \( \text{N}_2 \) and \( \text{C}_2\text{H}_2 \) are partly an effect of differences in solubility in water, \( \text{C}_2\text{H}_2 \) is roughly 65 times more soluble than \( \text{N}_2 \), in ability to permeate the lipid layer of the cell membrane and in affinity for the enzyme.

Multiple sites, possible five, or modified sites for reduction of different substrates have been suggested from results obtained by Hwang, Chen and Burris (1973). Two of those are sites for \( \text{C}_2\text{H}_2 \), one with high and one with low affinity for the substrate (Davis et al., 1979). In \( \text{C. pasteurianum} \) \( \text{C}_2\text{H}_2 \) was found to be non-competitive with \( \text{N}_2 \), whereas \( \text{N}_2 \) was a competitive inhibitor for \( \text{C}_2\text{H}_2 \) (Hwang et al., 1973).

An increase in the electron flow from dinitrogenase reductase to dinitrogenase has been observed in the presence of \( \text{C}_2\text{H}_2 \) (Thorneley and Eady, 1977). A dependency on the component ratio for the ratio \( \text{C}_2\text{H}_2 \) reduced to \( \text{N}_2 \) fixed, such as the higher ratio of dinitrogenase reductase to dinitrogenase the lower will the ratio \( \text{C}_2\text{H}_2 \) to \( \text{N}_2 \) be (Davis et al., 1975). It concludes that there is an increased reduction capacity in the presence of \( \text{C}_2\text{H}_2 \).
The nitrogenase catalyzed $H_2$ evolution is suppressed in the presence of $C_2H_2$ and the electron flow is changed to favor the reduction of acetylene over reduction of $N_2$ (Hudd et al., 1980).

Utilization of acetylene other than reduction by nitrogenase

Acetylene is metabolized by some aerobic (Kanner and Bartha, 1979) and some anaerobic (Watanabe and de Guzman, 1980) microorganisms, none included among the $N_2$ fixers. Due to the high concentration of acetylene present in assays for $N_2$ fixation activity and the relatively short incubation periods, the utilization of acetylene by non-fixers is unlikely to significantly influence the $pC_2H_2$.

Effects of the product

When exposed to $C_2H_2$, the $N_2$ fixer is deprived of the essential product from $N_2$ fixation, $NH_4^+$. The assay procedure imposes a $N$ limitation for the organism that is likely to decrease its activity (Brouzes and Knowles, 1973). This might be of special importance for the activity obtained in environments that naturally are $N$ deficient.

Ethylene is oxidized by methane oxidizing bacteria (DeBont and Mulder, 1976) and decomposition of ethylene has been demonstrated in the rhizosphere of rice roots (Döbereiner, 1974). Subsequently, some of the ethylene produced in assays for acetylene reduction could be metabolized and an activity value lower than the actual activity will be determined.

The oxidation of $C_2H_4$ is inhibited by $C_2H_2$ (DeBont, 1976) and rates of the endogenous ethylene production cannot be used as con-
trols for acetylene reduction assays (Witty, 1979a).

Many factors influence the acetylene reduction activity, some interact with the nitrogenase, others are dependent on the environment in which the assay is performed. It follows that acetylene reduction rates in different systems are not directly comparable. A standardization of the measured acetylene reduction activities to the more accurate isotopic method is required for more reliable quantifications of the N input. In cases where the acetylene reduction activity has been compared to the isotopic method, ratios for moles $\text{C}_2\text{H}_2$ reduced to moles of $\text{N}_2$ fixed from 1.5:1 to 25:1 have been reported (Hardy et al., 1973). Due to difficulties in carrying out the isotopic method, the theoretically derived ratio of 3:1 is commonly used.

In situ measurements

A method for in situ measurements of the acetylene reduction activity was designed by Balandreau and Dommergues (1973). A cylinder is arched over the system to be investigated and virtually no disturbance is imposed on the system, or the system can be enclosed in a plastic bag which necessarily causes some disturbance. An internal standard (e.g. propane) is injected into the device used for monitoring the diffusion of gases from the device. The method is applicable unless the assay is performed in environments that provide a very high rate of diffusion or those with a high water content (Knowles, 1981).
Comparisons between investigations are difficult due to differences in methodology, extrapolation from sporadically performed assays to kg N ha\(^{-1}\) y\(^{-1}\) and the common use of the ratio 3:1 for moles of C\(_2\)H\(_2\) reduced to moles of N\(_2\) fixed.

**Calculation of the acetylene reduction activity**

The formula commonly used for calculation of the acetylene reduction activity was derived by Balandreau and Dommergues (1973) in the following way:

\[
\text{ARA} = \frac{e_2v_2 - e_1v_1}{t_2 - t_1} \quad \text{eq. 1.1}
\]

where ARA is the constant rate of acetylene reduction activity from the tested system (mole acetylene reduced h\(^{-1}\))

- \(t_1\) and \(t_2\) are the 1st and the 2nd samplings times (h)
- \(e_1\) and \(e_2\) are the ethylene concentrations in the gas samples at the 1st and the 2nd time (mole ethylene ml\(^{-1}\))
- \(v_1\) and \(v_2\) are the volumes (device+soil pores) occupied by ethylene and propane at the 1st and the 2nd time (ml).

Ethylene and propane are assumed to diffuse with the same rate.

The occupied volumes \(v_1\) and \(v_2\) are determined from the relation

\[
v_n = \frac{V P}{p_n} \quad \text{eq. 1.2}
\]

where \(V\) is the volume of propane injected into the device (ml)
- \(P\) is the concentration of the injected propane (mole propane ml\(^{-1}\))
- \(p_n\) is the concentration of propane in the nth sample (mole propane ml\(^{-1}\))
- \(v_n\) is the occupied volume at the nth sampling (ml).

Hence,
where $ARA$, $e$ and $t$ are defined as for eq. 1.1 
$V$, $P$ and $p$ are defined as for eq. 1.2.

According to definitions, the $e$ and the $p$ in eq. 1.3 are given as concentrations, mole ml$^{-1}$. The ratio $e/p$ is determined for each sample and as the volume, from which the concentrations for $e$ and $p$ are measured, is the same, the ratio will have the same numerical value for $e$ and $p$ expressed in moles. With this change of view, the ratio $e/p$ is directly proportional to the gas chromatographic response for the ethylene and the propane in the sample concerned. Peak heights, mm, are often used as the output unit from the gas chromatograph and Knowles (1980) introduced the proportionality factor $e'/p'$ for the conversion from mm to mole of the ratio. It follows that

$$ARA = V P \frac{e_2}{p_2} - \frac{e_1}{p_1}$$

where $ARA$, $e$ and $t$ are defined as for eq. 1.1
$V$, $P$ and $p$ are defined as for eq. 1.2.

$$ARA = P_0 \frac{e_1'}{p_1'} \frac{e_2'}{p_2'}$$

where $ARA$ and $t$ are defined as for eq. 1.1
$P_0$ is the amount of propane injected into the device at time 0 h (mole)
$e'/p'$ is the gas chromatographic response to equal molar quantities of ethylene and propane (mole ethylene mm propane mm ethylene$^{-1}$ mole propane$^{-1}$)
$E_1$ and $E_2$ are the amounts of ethylene detected at the 1st and the 2nd sampling (mm)
$P_1$ and $P_2$ are the amounts of propane detected at the 1st and the 2nd sampling (mm).

Diffusion of gases from the device used into the soil was considered in derivation of eq. 1.3, but any correction is not indicated in the final expression. Substantial leakage occurred from the
assay devices used by Patriquin and Keddy (1978), who suggested a formula that included correction for diffusion. They assumed that in the end of an initial rapid diffusion phase, propane and ethylene occupy the same volume and that during the following slow phase of diffusion, the diffusion rate for ethylene is directly proportional to its concentration in the air phase. The observed change in the \( C_2H_4 \) concentration in the gas samples is then

\[
\frac{d(C_2H_4)}{dt} = K - r(C_2H_4)
\]

where \( \frac{d(C_2H_4)}{dt} \) is the observed increase in ethylene over time (mole h\(^{-1}\)),

\( K \) is the ethylene production rate (mole h\(^{-1}\)),

\( r(C_2H_4) \) is the diffusion rate of ethylene (mole h\(^{-1}\)).

Equal diffusion rate for propane and ethylene was assumed and \( r \) given by

\[
p_2 = p_1 e^{-rt}
\]

where \( p \) is the propane concentration (mole ml\(^{-1}\))

\( t \) is the time from initiation of the assay (h)

\( r \) is the proportionality factor for propane (h\(^{-1}\))

subscripts denote two consecutive samplings.

The acetylene reduction activity of the test material is then

\[
ARA = \frac{e_2 \ln p_1/p_2}{1 - p_2/p_1} \frac{p}{p_i}
\]

where \( ARA \) and \( e_2 \) are defined as for eq. 1.1

\( p \) is defined as for eq. 1.6

\( P \) is defined as for eq. 1.2

\( p_i \) is the propane concentration at the 1st sampling.
CHAPTER II

IN SITU MEASUREMENTS OF $N_2$ FIXATION ACTIVITY USING THE ACETYLENE REDUCTION TECHNIQUE

Introduction

According to Odum (1969) nutrient cycles are more open in an early successional stage than in a more mature one. This hypothesis was partly confirmed by Skujins and Klubek (1982), who found that mineral cycles were more closed in a mature than in a developing stage of a northern Wasatch Mountain subalpine forest ecosystem. It follows from Odum's hypothesis that $N_2$ fixation would decrease as a climax community is approached from earlier successional stages.

The high concentration of easily utilizable carbohydrates in the rhizosphere makes it a favorable site for heterotrophic microorganisms. An increase in the total number of bacteria (Diem and Dommergues, 1980) as well as a higher level of nitrogenase activity (Low and White, 1976) have been demonstrated in the rhizosphere as compared to the environment not under its influence. Most investigations of associative $N_2$ fixation activity have been performed with grasses and crops in the tropical zone (Döbereiner and Day, 1976). To date the knowledge of associative $N_2$ fixation in subalpine, subboreal and temperate coniferous forest ecosystems is very limited.

The acetylene reduction method for assaying nitrogenase activity (Burris, 1975) is sensitive and easy to carry out. An application of this method well suited for in situ studies was worked out by Baland-
reau and Dommergues (1973). In soils with moderate porosity, saturation of the N₂ fixing sites on the nitrogenase is easily achieved with 0.05 atm \( p_{C_2H_2} \) (Knowles, 1980). Empirically found values of the conversion factor from moles of \( C_2H_2 \) reduced to moles of N₂ fixed are widely varying from 1.5:1 to 25:1 (Hardy et al., 1973), this compares with the theoretically derived value 3:1. Although the ratio will affect a quantitative evaluation of measured activities, it does not influence a qualitative comparison between activities obtained under equal conditions.

**Objectives**

This investigation was designed to test two hypothesis concerning associative N₂ fixation activity:
1. N₂ fixation in association with the roots of small plants does contribute to the total input of N to a forest ecosystem;
2. Odum's hypothesis about nutrient cycles in a developing ecosystem is pertinent to associative N₂ fixation.
   Odum's hypothesis was also tested for N₂ fixation activity by free-living N₂ fixers in the soil.

**Material and Methods**

**Field area**

The field area, Jebo Creek, is of subalpine type located in the Utah State University School Forest ca. 30 km NE of Logan, elevation 2,800 m. It represents an ecological succession from the open meadow surrounded by a belt of invading aspen, *Populus tremuloides*, to a
forest dominated by fir, Abies lasiocarpa, and as a climax community, a forest of spruce, Picea engelmannii, (Schimpf et al., 1980). The vegetation period is approximately 100 days from late June to early October. During the summer, the precipitation is minimal.

In each of the successional stages, areas have been designated for research purposes. For the last six years, these areas have been fenced to exclude grazing cattle.

One site in each of the successional stages was assigned for in situ determination of associative \( N_2(C_2H_2) \) fixation activity.

Meadow. The meadow is located on a north slope. It is an open area with mainly low vegetation, grasses and small plants. A few depressions with shrubs and tall firs are present. In essence, the area is exposed to direct sunlight all day, the limited shade afforded by the groves is very local and only for short periods of the day.

The test site was 10x15 m\(^2\) and was shaded from around 3 P.M.

Aspen. The aspen belt is 10-20 m wide. Small plants here are much more protected from direct light, than in the meadow. The vegetation is much leafier and gives a greener impression.

*Lupinus argenteus* is frequent in the aspen. To avoid the influence of their nodules when testing for associative \( N_2 \) fixation, the actual test site was chosen close to the boundary meadow-aspen, but still clearly influenced by the aspen belt. The acetylene reduction assays were performed within an area 10x10 m\(^2\), exposed to direct and indirect light in equal proportions of the day.

Fir. The fir is the darkest of the sites, and most of the light reaching the forest floor is indirect. The vegetation is sparse and
restricted to areas not covered by a thick layer of needles. In early spring, there is a small stream running through the area, but at the time for the first testing for the season, it has already dried.

All tests were carried out with material outside the projection of the fir canopies and within an area 15x15 m².

**Spruce.** The spruce site is an open area in a generally dark forest. It might be questionable whether the assigned site is representative for the spruce in general. There is also some uncertainty as regards to a distinct border line between the fir and the spruce (Schimpf et al., 1980). From this point of view, the two forest sites are likely to be combined. They have, however, in the following presentation been kept separate mainly due to the differences in type of vegetation and light exposure. In contrast to the fir, the spruce is open and exposed to direct light half of the day and the vegetation is made up of grasses and small plants.

The tested plants were all within an area 8x8 m², on a slightly sloping hillside, the change in elevation not exceeding 1 m.

**Plant material**

Plant species of interest for *in situ* assays of rhizosphere associated \( N_2 \) fixation were selected from results of *in vitro* studied plant-nitrogen relations. From various plants detached roots were assayed for \( N_2(C_2H_2) \) fixation activity and the results are presented in Table 1 (D. Hansen, pers. comm.*). The presence of \( N_2 \) fixing bacteria in the rhizosphere of some plants (Table 2) was demonstrated by D.

*report for Dr. J.J. Skujins, Utah State University, Logan Ut.*
Table 1. A survey of N$_2$ fixation activity on the rhizoplane of plants collected from a subalpine forest.

<table>
<thead>
<tr>
<th>Family</th>
<th>Activity (μmole C$_2$H$_4$ g d.w.$^{-1}$ d$^{-1}$; $p_0$ = 0.02)</th>
<th>Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Sucrose</td>
</tr>
<tr>
<td>Poaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agropyron trachycaulum</td>
<td>0.003±0.007</td>
<td>0.008±0.020</td>
</tr>
<tr>
<td>Agrostis scabra</td>
<td>0.007±0.009</td>
<td>0.063±0.064</td>
</tr>
<tr>
<td>Bromus carinatus</td>
<td>0.003±0.009</td>
<td>2.554±4.510</td>
</tr>
<tr>
<td>Phleum alpinum</td>
<td>0.792±1.444</td>
<td>2.032±2.711</td>
</tr>
<tr>
<td>Poa nervosa</td>
<td>0</td>
<td>0.719±0.803</td>
</tr>
<tr>
<td>Stipa leterminii</td>
<td>0</td>
<td>1.103±0.812</td>
</tr>
<tr>
<td>S. columbiae</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trisetum spicatum</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Asteraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Achillea millefolium</td>
<td>1.923±0.548</td>
<td>0.719±0.268</td>
</tr>
<tr>
<td>* Anthemaria microphylla</td>
<td>0</td>
<td>0.088±0.126</td>
</tr>
<tr>
<td>A. engelmannii</td>
<td>0.066±0.093</td>
<td>0.126±0.178</td>
</tr>
<tr>
<td>A. integrifolius</td>
<td>0.350±0.138</td>
<td>1.748±1.136</td>
</tr>
<tr>
<td>Erigeron speciosus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>* Hieracium scouleri</td>
<td>0</td>
<td>0.016±0.023</td>
</tr>
<tr>
<td>Rudbeckia occidentalis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Senecio crassulus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. serrii</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Boraginaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Hackelia micrantha</td>
<td>1.905±2.729</td>
<td>9.908±13.135</td>
</tr>
<tr>
<td>Caryophyllaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stellaria jamesiana</td>
<td>0.057±0.116</td>
<td>1.601±0.791</td>
</tr>
<tr>
<td>Geraniaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geranium viscosissimum</td>
<td>0</td>
<td>0.356±0.346</td>
</tr>
<tr>
<td>Onagraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epilobium minutum</td>
<td>1.333±1.993</td>
<td>5.121±3.480</td>
</tr>
<tr>
<td>Polemoniaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gilia aggregata</td>
<td>0</td>
<td>0.310±0.021</td>
</tr>
<tr>
<td>* Rocaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potentilla arguta</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*indicates species tested in situ.

Mueller and D. Hansen (pers. comm.*). Determination of the total N (Kjeldahl N) content in stems and roots of plants is presented in Table 3 (J. Schijf and D. Hansen, pers. comm.*).

Final selection of plant species to be tested in situ was made upon visual observation of predominance in respective sites. Individual plants were selected for similarity in above ground biomass at

*report for Dr. J.J. Skujins, Utah State University, Logan Ut.
Table 2. Plant species from which N\textsubscript{2} fixing bacteria have/have not been isolated.

<table>
<thead>
<tr>
<th>N\textsubscript{2} fixing bacteria</th>
<th>Found</th>
<th>Not found</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Achillea millefolium</td>
<td></td>
<td>Agropyron cristatum</td>
</tr>
<tr>
<td>Agropyron spicatum</td>
<td></td>
<td>Aster foliaceus</td>
</tr>
<tr>
<td>* Antennaria microphylla</td>
<td></td>
<td>Bromus carinatus</td>
</tr>
<tr>
<td>Aster integrifolius</td>
<td></td>
<td>Epilobium minutum</td>
</tr>
<tr>
<td>* Hackelia micrantha</td>
<td></td>
<td>Gilia aggregata</td>
</tr>
<tr>
<td>* Hieracium scouleri</td>
<td></td>
<td>Phleum alpinum</td>
</tr>
<tr>
<td>Stipa lessertmannii</td>
<td></td>
<td>Poa nervosa</td>
</tr>
<tr>
<td>Trisetum spicatum</td>
<td></td>
<td>Stellaria jamesiana</td>
</tr>
</tbody>
</table>

* indicates species tested in situ.

Table 3. Total N (Kjeldahl N) content, in percent of plant dry weight, of plants collected from Jebo Creek, July 1980.

<table>
<thead>
<tr>
<th>N content</th>
<th>% of plant d.w.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem</td>
</tr>
<tr>
<td>* Achillea millefolium</td>
<td>1.69</td>
</tr>
<tr>
<td>* Antennaria microphylla</td>
<td>nd</td>
</tr>
<tr>
<td>Aster integrifolius</td>
<td>2.15</td>
</tr>
<tr>
<td>Geranium viscosissimum</td>
<td>1.60</td>
</tr>
<tr>
<td>Gilia aggregata</td>
<td>1.04</td>
</tr>
<tr>
<td>* Hackelia micrantha</td>
<td>1.04</td>
</tr>
<tr>
<td>* Hieracium scouleri</td>
<td>1.16</td>
</tr>
<tr>
<td>Penstemon cyanathus</td>
<td>1.04</td>
</tr>
<tr>
<td>Stellaria jamesiana</td>
<td>1.69</td>
</tr>
</tbody>
</table>

* indicates species tested in situ.

the time for initiation of the investigation. Differences in growth rate during the season were considered to reflect the natural variation and consequently, the tested plants were not true replications.

In situ assays were performed with Achillea millefolium, Antennaria microphylla, Hackelia micrantha and Hieracium scouleri.

Achillea millefolium. *A. millefolium* starts its growth immediately after the snow has melted. The green rosettes of leaves are frequently seen throughout most sites, except the meadow. It starts blooming in late July and is still active at the time of the first frost.
Antennaria microphylla. A. microphylla grows in dense communities. It is perennial and actively growing during the entire vegetation period. Blooming starts in the middle of July and continues until the end of the season.

Hackelia micrantha, Hieracium scouleri. In early growth stages, the two species H. micrantha and H. scouleri look very similar, and not until they bloom, at the end of August, are they easily distinguished.

Bare soil controls

In each of the four sites, vegetation-free areas were selected as controls for the $\text{N}_2(\text{C}_2\text{H}_2)$ fixation activity of the soil free-living $\text{N}_2$ fixing organisms.

Delimitation of experimental plant and bare soil units

Selected plants with attached soil, sufficient to contain virtually their entire root system, were delimited by cylinders or pots, as were the bare soil controls. To avoid the influence of fresh roots from neighbouring plants, this was done well ahead of the first assay. All vegetation, except the plant of interest, was removed from an area ca. 10 cm around each plant and completely from the bare soil controls. These areas were weeded before each assay was carried out.

In 1980, cylinders cut from PVC pipe were used for delimitation. The cylinders measured 22 cm in height and the diameter was 15 cm for A. millefolium and corresponding bare soil controls and 25 cm for the remaining plant units and their controls. Most cylinders were embed-
ded immediately after snow melt, with additional units placed at least one week before the assay was performed. They were forced as deeply as possible into the soil with a minimum of disturbance to the delimited material. It was assumed, that each cylinder was forced down deep enough to contain the entire layer in which the majority of N₂ fixing organisms are present. Since the horizontal water movement in the soil was interrupted by the cylinders, the units were watered at intervals after the first assay.

For the 1981 season, the units to be tested were transferred into flower pots of "soft" plastic, 20 cm high and slightly conic, bottom diameter 15 cm and top 18 cm. Along the sides and in the bottom of the pots 36 and 13 holes were drilled, respectively. The holes, 1.5 cm in diameter, provided some water movement through the system. Plants to be tested during the 1981 season were potted in late fall 1980 and corresponding bare soils in early spring 1981. Depth of soil in the pots was recorded in the aspen, the fir and the spruce.

Endogenous ethylene production

The endogenous ethylene production from the soil was measured at the time of each assay. These units were treated in the same manner as those for acetylene reduction assays, except for the addition of acetylene.

Experimental design

Acetylene reduction assays of the selected and potted plants and bare soils were followed over the season. Within some sites, additional units were potted for later assays during 1980 (Table 4). All
Table 4. Number of cases tested for N\textsubscript{2}(C\textsubscript{2}H\textsubscript{2}) fixation activity and endogenous ethylene production in each site at different times during the 1980 and the 1981 seasons.

<table>
<thead>
<tr>
<th>Site</th>
<th>Antennaria microphylla</th>
<th>Achillea millefolium</th>
<th>Hackelia micrantha</th>
<th>Hieracium scouleri</th>
<th>Bare soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meadow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 July 1980</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>2 (1)</td>
</tr>
<tr>
<td>27 July 1980</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>3 (1)</td>
</tr>
<tr>
<td>5 Sept 1980</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>3 (1)</td>
</tr>
<tr>
<td>21 June 1981</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Aspen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Aug 1980</td>
<td></td>
<td>2</td>
<td>2</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>5 July 1981</td>
<td>5 (1)</td>
<td></td>
<td></td>
<td></td>
<td>5 (1)</td>
</tr>
<tr>
<td>28 July 1981</td>
<td>5 (1)</td>
<td></td>
<td></td>
<td></td>
<td>5 (1)</td>
</tr>
<tr>
<td>Fir</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 July 1980</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>2 (1)</td>
</tr>
<tr>
<td>7 Aug 1980</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>4 (1)</td>
</tr>
<tr>
<td>10 Sept 1980</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>4 (1)</td>
</tr>
<tr>
<td>30 June 1981</td>
<td>5 (1)</td>
<td></td>
<td></td>
<td></td>
<td>5 (1)</td>
</tr>
<tr>
<td>16 July 1981</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Spruce</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 July 1980</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>2 (1)</td>
</tr>
<tr>
<td>7 Aug 1980</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>3 (1)</td>
</tr>
<tr>
<td>10 Sept 1981</td>
<td>5 (1)</td>
<td></td>
<td></td>
<td></td>
<td>3 (1)</td>
</tr>
<tr>
<td>10 July 1981</td>
<td>5 (1)</td>
<td></td>
<td></td>
<td></td>
<td>5 (1)</td>
</tr>
<tr>
<td>28 July 1981</td>
<td>5 (1)</td>
<td></td>
<td></td>
<td></td>
<td>5 (1)</td>
</tr>
</tbody>
</table>

Number without parenthesis: acetylene reduction assays
Number within parenthesis: endogenous ethylene production

Tests were performed during daytime, except for one sequence of test in the fir site, 9 July 1980, where the assay was extended to include one sampling after 22 h.

In 1980, the N\textsubscript{2}(C\textsubscript{2}H\textsubscript{2}) fixation rates were determined from two to four plants and two to four controls evenly distributed over the respective sites. Endogenous release of ethylene was measured from the bare soil. All sites, except the aspen, were each assayed three times over the vegetation period (Table 4).

To decrease the influence of the intrasite variation in testing hypothesis 1, plants and controls were placed in a paired arrangement for the 1981 season, and the total number of units per site was increased (Table 4). The distance between the two units in a pair did
not exceed 20 cm. Endogenous ethylene production was determined from a complete pair. With exception of the meadow, all sites were assayed twice during the summer (Table 4).

Protection of plants

The acetylene reduction test was carried out in a Saran bag, inside which limited air movement and exposure to direct sun light were likely to increase the temperature. Negative effects of these conditions were prevented, as much as possible, by shading the units when enclosed. A piece of white cotton cloth was stretched as a roof over the enclosure, high enough to allow air to circulate underneath.

A direct contact between the plant and the Saran bag was avoided with a wire arch frame, taller than the plant, inside the bag.

Soil moisture

At the time of each assay, three soil cores were collected for determination of the soil moisture content. The moisture content was calculated after having dried the soil at 85°C until constant weight.

Acetylene reduction assay

At time for the assay, the cylinders or pots with their content were lifted up, transferred into a Saran bag (CRYOVAC) and then replaced into their position in the soil. A piece of plastic tape, 3x6 cm², attached to the bag approximately 10 cm from the top of the cylinder or pot, acted as an injection port.

A vial containing calcium carbide, CaC₂, for acetylene generation was placed inside the bag, as far as possible from the plant
when present. The two metal arches were placed over the plant.

To minimize the volume, and thereby increase the concentration of produced ethylene, a portion of the air was evacuated from the bag before it was firmly twisted closed and the seal secured with a rubber band. In a few cases, the bag was first sealed and the volume afterwards adjusted with a reversed bicycle pump, having an injection needle at the air outlet.

The assay was initiated by injecting water, in excess, to the CaC$_2$ container. With a minimum time delay, a defined amount of tracer gas (propane) was injected to the Saran bag. Holes from needles were sealed with Scotch tape.

The first gas sample was taken 0.5 to 2 h after the assay was initiated. This time was considered sufficient for the gases to be evenly distributed throughout the enclosed volume. A total of four to seven samples were then collected at approximately 2 h interval in 1980 and 1 h 1981.

Within 48 h of sampling, at the latest, the collected samples were analyzed for ethylene, propane and, when present, acetylene. Gases were separated using gas chromatography.

Initially 0.2 ml of the sample was used for analysis. This amount was later increased to 0.5 ml and during 1981, 1 ml was used.

Amount of gas analyzed was recorded as peak heights that were measured manually.

**Acetylene generation**

Acetylene was generated *in situ* from CaC$_2$ (Matheson, Coleman &
Bell) according to

$$\text{CaC}_2 + \text{H}_2\text{O (excess)} \rightarrow \text{C}_2\text{H}_2 + \text{Ca(OH)}_2 + \text{H}_2\text{O}$$

From an approximate estimation of the expected volume, in which the assay was to be carried out, the amount of CaC$_2$ required to give the $p_{\text{C}_2\text{H}_2}$ 0.10 atm was calculated and used. The CaC$_2$ was weighed into a 40 ml plastic vial and transported to the site for acetylene generation.

The reaction is highly exothermic and a considerable amount of H$_2$O(v) is released. To prevent the vapor from condensing on the plant, and possibly burn it, the following precautions were taken. In 1980, the vial with CaC$_2$ was capped with a flexible rubber enclosure (Trojan no. 95) through which the water was injected, leaving a small hole through which the C$_2$H$_2$ could effuse and a closed flexible area for the vapor to condense on. In 1981, the condensation surface was made up of an approximately 4x20 cm$^2$ strip of plastic, wrinkled into the vial on top of the CaC$_2$.

**Tracer gas**

Propane, C$_3$H$_8$, was used as a tracer gas. A varying, but defined, amount was used dependent on the volume of the bag and the sensitivity of the gas chromatographic system used for analysis.

The choice of propane was based on its suitable compatibility with the available columns and its resistance to degradation in the soil (Johnson and Frederick, 1971).
Sample procedure

Gas samples were collected in serum vials, nominal volume 5 to 6 ml, sealed with serum-type rubber stoppers. From the Saran bag, 30 ml of gas was withdrawn and flushed through the sealed vial, which was provided with an outlet needle.

Prior to analysis the acetylene was precipitated with a AgNO₃ solution (David et al., 1980), a procedure that considerably decreased the time for each analysis. Commonly, a solution four times the strength given by David et al. (1980) was used.

During 1981, at least one sample per set was untreated to assure that a sufficient amount of acetylene had been generated.

Gas chromatographic system

Analysis were made using a Varian model 1700 analytic gas chromatograph. For the first series of samples, gases were separated using a 6'x1/8" (1.8 m x 3.2 mm) stainless steel column with Porapak R, mesh size 80/100 μ. This was later replaced by a 7'x1/8" (2.1 m x 3.2 mm) stainless steel column, packed with 10% Na₃PO₄ (tribasic) on a Spherosil XOB base, mesh size 100/200 μ.

The carrier gas was He with the flow rate 22 ml min⁻¹ for the Porapak R and 18 ml min⁻¹ for the column with Spherosil. Injector temperature was 60°C and detector temperature 90-100°C. For Porapak R, the column temperature was 75°C while room temperature (≈22°C) was used for the Spherosil column.

The gas chromatographic response was recorded as peak heights using a Varian aerograph model 20.
Data processing

Acetylene reduction activity. The $N_2(C_2H_2)$ fixation activity was determined according to the formula originally developed by Balandreau and Dommergues (1973) and later modified by Knowles (1980). In essence it states

$$ARA = p \frac{E_2}{P_2} \frac{E_1}{P_1}$$

where $ARA$ is the acetylene reduction activity of the enclosed material (mole ethylene produced h$^{-1}$), $P$ is the amount of tracer gas injected into the enclosure at the time for initiation of the assay (mole propane), $e'/p'$ is the gas chromatographic response to equal molar quantities of ethylene and tracer gas (mole ethylene mm propane mm ethylene$^{-1}$ mole propane$^{-1}$), $E/P$ is the ratio of amount ethylene to amount propane as recorded from one sample (mm ethylene mm propane$^{-1}$), $t$ is the accumulated time from initiation of the assay (h), and subscripts 1 and 2 denote two consecutive samplings.

In this investigation, the rate part of eq. 2.1, $\frac{d(E/P)}{dt}$, was determined from linear regression of four to seven measurements of $E/P$ vs. time. For each sampling, the median of the determined $E/P$ ratios were used for linear regression. Values with a standard residual < |2| were omitted.

Quantitative estimates. The input of N by associative $N_2$ fixation was assigned the difference between the activity obtained in the presence of plant and the activity obtained in bare soil. For the unpaired design the input of N was calculated as the difference between respective averages and for a paired design as the average of pairwise obtained differences.
Estimations of N input were made under the assumption that the conversion factor from moles C\textsubscript{2}H\textsubscript{2} reduced to moles N\textsubscript{2} fixed was 3:1. Yearly input was calculated by stepwise linear integration of the function obtained when measurements at different times over the season were connected linearly. N\textsubscript{2} fixation was assumed to proceed with the determined rate for 12 h d\textsuperscript{-1}, for 100 d y\textsuperscript{-1}. The vegetation period for the meadow was calculated from the 16th of June and for all other other sites from the 26th of June.

Statistic evaluation. The significance of determined differences was tested with the t-test. Due to the relatively low number of cases the differences were considered statistically verified at \( \alpha < 0.10 \).

For unpaired data, a version of the t-test not requiring equal variance in the two sets to be compared was used. Paired data were evaluated with a paired t-test.

Constants. Correction for the change in atmospheric pressure between USU, 0.89 atm, and the field area, 0.78 atm, was made for calculations involving gases.

The \( e'/p' \) ratio for the Porapak R column was 0.094 and for the Spherosil column 1.502.

Results

Endogenous ethylene production

The rates of endogenous ethylene production were highly variable from less than 0 mole h\textsuperscript{-1} early in the season in the fir to values above those obtained from the reduction of acetylene at the mid-sum-
mer assay in the meadow (Table 5). Rates obtained in the fir and in the spruce were considerably higher in 1980 as compared to 1981.

It was apparent that the rate of endogenous ethylene release was the highest in the meadow followed by the fir, the spruce and lowest in the aspen soil. Data from the meadow indicate that over the season

Table 5. Endogenous ethylene production from the soil and from plant +soil at different times. Comparison is made to the ethylene produced in enclosures for $N_2(C_2H_2)$ fixation assays.

<table>
<thead>
<tr>
<th></th>
<th>Endogenous ethylene production</th>
<th>% of corresponding</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n mole h$^{-1}$ encl.$^{-1}$</td>
<td>$\bar{x}_A$</td>
<td>$\bar{x}_B$</td>
</tr>
<tr>
<td><strong>Meadow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 June 1981</td>
<td>E</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>10 July 1980</td>
<td>EB</td>
<td>41</td>
<td>51</td>
</tr>
<tr>
<td>27 July 1980</td>
<td>EB</td>
<td>154</td>
<td>129</td>
</tr>
<tr>
<td>8 Sept 1980</td>
<td>EB</td>
<td>158</td>
<td>66</td>
</tr>
<tr>
<td><strong>Aspen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 July 1981</td>
<td>EA</td>
<td>-5</td>
<td>&lt;0</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>28 July 1981</td>
<td>EA</td>
<td>24</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>-19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>9 Aug 1980</td>
<td>EB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Fir</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 June 1981</td>
<td>EA</td>
<td>-4</td>
<td>&lt;0</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>-3</td>
<td></td>
</tr>
<tr>
<td>9 July 1980</td>
<td>EB</td>
<td>25</td>
<td>61</td>
</tr>
<tr>
<td>16 July 1981</td>
<td>E</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>7 Aug 1980</td>
<td>EB</td>
<td>17</td>
<td>51</td>
</tr>
<tr>
<td>10 Sept 1980</td>
<td>EB</td>
<td>16</td>
<td>42</td>
</tr>
<tr>
<td><strong>Spruce</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 July 1980</td>
<td>EB</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>10 July 1981</td>
<td>EA</td>
<td>-7</td>
<td>&lt;0</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>28 July 1981</td>
<td>EA</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>EB</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RE</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>7 Aug 1980</td>
<td>EB</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>10 Sept 1980</td>
<td>EB</td>
<td>22</td>
<td>39</td>
</tr>
</tbody>
</table>

E: endogenous ethylene production  
A: presence of Achillea millefolium  
B: bare soil  
encl.: enclosure
as much as 72% of the ethylene detected in enclosures for $\text{N}_2(\text{C}_2\text{H}_2)$ fixation assays originated from the endogenous production of ethylene. Corresponding values for the aspen, the fir and the spruce are 3, 39 and 24%, respectively.

No impact of plants on the rate of endogenous ethylene production could be found (Table 5), and their presence has been considered insignificant in this aspect. Any correction of the presented results accounting for the endogenous ethylene release is specifically indicated in the text.

$\text{N}_2$ fixation and soil depth

In the sites where the depth of soil in the pots was recorded, it varied from 10 to 15 cm. No convincing correlation between $\text{N}_2$ fixation activity from the bare soil and the depth of soil in the pot was evident (Figure 1).

Influence of environmental parameters

Soil moisture. Within the soil moisture range of 10 to 40% of g dry soil, the soil moisture content was not correlated with the $\text{N}_2(\text{C}_2\text{H}_2)$ fixation activity measured from bare soil (Figure 2).

Diurnal variation. No ethylene production was detected during the night from either bare soil or plant containing enclosures (Figure 3). During the night, a substantial decrease in ethylene was measured in one, of three, plant containing enclosure (Figure 3).
Figure 1. Correlation between depth of soil in the pot and from it measured \( N_2(C_2H_2) \) fixation activity.

Figure 2. Correlation between soil moisture content and measured \( N_2(C_2H_2) \) fixation activity.
Figure 3. Diurnal variation in $N_2(C_2H_2)$ fixation activity.

**Net input of N by associative $N_2$ fixation**

The input of N by $N_2$ fixation in association with the roots of tested plants was calculated per area ($m^2$). It was based on a 100% cover of *Achillea microphylla*, and the plant densities for *Achillea millefolium*, *H. micrantha* and *H. scouleri* being approximately 50, 20 and 20 plants $m^{-2}$, respectively.

**Meadow.** In three of four tests, the presence of *A. microphylla* correlated with an increase in the rate of ethylene production, as compared to the rate measured from the bare soil (Figure 4). At none of the tests was the higher activity in the presence of plant statistically significant (Table 6).

Annual input by associative $N_2$ fixation from *A. microphylla* was 15.7 mg N $m^{-2}$, which corrected for the endogenous ethylene production in the meadow was 4.4 mg N $m^{-2}$ (Table 7).

**Aspen.** The 1980 tested *H. scouleri* and *H. micrantha* both showed higher average activities than were measured from the bare soil, 809 and 351 $\mu$g N $m^{-2}$ d$^{-1}$, respectively (Table 6).
Figure 4. \( \text{N}_2(\text{C}_2\text{H}_2) \) fixation activity from plant+soil, bare soil and net activity in association with plant in each of the four sites the meadow, the aspen, the fir and the spruce at different times during the vegetation period.
Table 6. N input by N\textsubscript{2} fixation in the four sites the meadow, the aspen, the fir and the spruce at different times during the vegetation period. Significance level for an increased activity in the presence of plant at each performed assay.

<table>
<thead>
<tr>
<th>Plant+soil</th>
<th>Bare soil</th>
<th>Net plant (\Delta (90% CI))</th>
<th>Plant+soil and bare soil</th>
<th>Plant+bare soil significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\bar{x}\pm sd)</td>
<td>(\bar{x}\pm sd)</td>
<td>(\bar{x}\pm sd)</td>
<td>(\bar{x}\pm sd)</td>
<td></td>
</tr>
<tr>
<td><strong>Meadow</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 June 1981</td>
<td>536±65</td>
<td>491±86</td>
<td>45±123</td>
<td>514±123</td>
</tr>
<tr>
<td>10 July 1980</td>
<td>88±98</td>
<td>183±89</td>
<td>-94 (-686-498)</td>
<td>136±94</td>
</tr>
<tr>
<td>27 July 1980</td>
<td>376±126</td>
<td>273±238</td>
<td>104 (-262-469)</td>
<td>325±179</td>
</tr>
<tr>
<td>8 Sept 1980</td>
<td>919±429</td>
<td>546±149</td>
<td>373 (-282-1028)</td>
<td>759±433</td>
</tr>
<tr>
<td><strong>Aspen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 July 1981</td>
<td>392±120</td>
<td>362±45</td>
<td>31 (-120-182)</td>
<td>377±86</td>
</tr>
<tr>
<td>28 July 1981</td>
<td>252±70</td>
<td>315±81</td>
<td>-63±67</td>
<td>283±79</td>
</tr>
<tr>
<td>9 Aug 1980</td>
<td>1130±730(^a)</td>
<td>321±25</td>
<td>809</td>
<td>351</td>
</tr>
<tr>
<td></td>
<td>672±678(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fir</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 June 1981</td>
<td>244±161</td>
<td>177±135</td>
<td>67±91</td>
<td>210±144</td>
</tr>
<tr>
<td>9 July 1980</td>
<td>420±103</td>
<td>258±54</td>
<td>162 (-45-368)</td>
<td>355±118</td>
</tr>
<tr>
<td>16 July 1981</td>
<td>180±27</td>
<td>178±46</td>
<td>2±43</td>
<td>179±36</td>
</tr>
<tr>
<td>7 Aug 1980</td>
<td>270±41</td>
<td>206±50</td>
<td>64 (-2-129)</td>
<td>238±54</td>
</tr>
<tr>
<td>10 Sept 1980</td>
<td>248±90</td>
<td>245±135</td>
<td>3 (-156-161)</td>
<td>247±104</td>
</tr>
<tr>
<td><strong>Spruce</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 July 1980</td>
<td>259±126</td>
<td>441±20</td>
<td>-184 (-401-32)</td>
<td>331±135</td>
</tr>
<tr>
<td>10 July 1981</td>
<td>246±139</td>
<td>351±232</td>
<td>-105±315</td>
<td>298±189</td>
</tr>
<tr>
<td>28 July 1981</td>
<td>234±125</td>
<td>362±129</td>
<td>-128±187</td>
<td>298±137</td>
</tr>
<tr>
<td>7 Aug 1980</td>
<td>260±62</td>
<td>272±51</td>
<td>-12 (-121-94)</td>
<td>256±51</td>
</tr>
<tr>
<td>10 Sept 1980</td>
<td>407±103</td>
<td>351±70</td>
<td>67 (-469-581)</td>
<td>374±78</td>
</tr>
</tbody>
</table>

\(^a\) tested species in meadow Antennaria microphylla in other sites Achillea millefolium, unless else indicated
\(^b\) Hieracium scouleri

A. millefolium tested during the 1981 season showed a contribution to N input by associative N\textsubscript{2} fixation at the earliest assay, 5 July, however, not significant. At the second assay no positive influence of plant was detected (Figure 4).

Any seasonal input from A. millefolium associated N\textsubscript{2} fixation could not be shown from the two assays performed (Table 7).

Fir. All of the five tests showed a higher rate of ethylene
production in the presence of *A. millefolium* than was measured from the bare soil (Figure 4). For three of the tests, the increase was significant (Table 6).

Annual input of N by \(N_2\) fixation in association with *A. millefolium* was 3.8 mg N m\(^{-2}\), when the endogenous ethylene production was subtracted it was 2.4 mg N m\(^{-2}\) (Table 7).

**Spruce.** The acetylene reduction activity obtained in the presence of *H. micrantha* on 9 July, did not exceed that which was measured from bare soil at that time (Table 6).

Only the latest assay, 10 September, indicated a net positive input of N by associative \(N_2\) fixation of *A. millefolium* (Figure 4). It was, however, not statistically significant (Table 6).

Annually the acetylene reduction activity in the presence of *A. millefolium* was less than in the absence of the plant (Table 7).
**N input by soil free-living $N_2$ fixers**

An acceptable significance level for a difference between the acetylene reduction activity in the presence of plant and that measured from the bare soil was not reached in any of the assays. Based on this, the average of all measured activities was used for calculation of N input by free-living $N_2$ fixers.

A seasonal variation in the ethylene production activity was not evident (Figure 5), although the activity measured in the meadow in the fall was higher than which was measured at any of the other sites. The standard deviations for the values marked in Figure 5 are shown in Table 6.

The yearly input of N to the meadow, the aspen, the fir and the

![Figure 5](image.jpg)

**Figure 5.** N input by $N_2$ fixation in the soil in each of the four sites: the meadow, the aspen, the fir and the spruce at different times during the vegetation period.
spruce were 48±24, 33±5, 24±8 and 32±10 mg N m⁻², x±sd, respectively. Corrected for the endogenous release of ethylene, these values were 13±7, 3±5, 15±5 and 25±8 mg N m⁻², x±sd, respectively.

Discussion

Method

The described method for N₂ fixing assays is comparatively easy to carry out and presents a low cost. With hourly samplings, the optimal number of enclosures that can be handled by one person is within the range of 10 to 15, the preparation of the assay being the limiting step.

Providing an efficient condensation surface for the H₂O(v) released during C₂H₂ generation considerably lowered the humidity inside the Saran bag. At the very first assay, 9 July 1980, this was not provided for and the enclosed plants seemed to have suffered from the conditions imposed by the assay procedure, but they recovered in a few days. The plastic strip wrinkled on top of the CaC₂ was as efficient as the flexible rubber enclosure.

Time required for sampling can be shortened by using evacuated vials and a double-pointed needle. When tested in the laboratory, the variation in gas exchange was the same for both methods. Evacuation was then made by expanding the air in the sealed serum vial to approximately ten times its volume, using a large syringe.

Material

Neither A. millefolium nor A. microphylla seemed to suffer from
the assay procedure, although the parts of the leaves that were in direct contact with the plastic bag were burned. *H. micrantha* and *H. scouleri* both showed severe damage from the assay condition.

**Endogenous ethylene production**

The reported rates of the endogenous ethylene production must be carefully interpreted. The values were highly variable and at each occasion measured without replication.

The primary interest of this investigation was to determine the influence of plants on the $N_2$ fixation activity as the difference between plant+soil and bare soil systems. In this respect, the endogenous production is trivial and assumed the same for both systems.

The presence of acetylene has been shown to inhibit oxidation of ethylene (Witty, 1979a). A larger portion, than has been corrected for, of the ethylene produced in assays for $N_2(C_2H_2)$ fixation activity might originate from the endogenous release of ethylene.

**$N_2$ fixation and soil depth**

By using the described methodology and experimental design $N_2$ fixation activity was not found to be dependent on the depth of soil in the pot, as long as it exceeded 10 cm. The obtained result may be interpreted in two different ways. Either most of the $N_2$ fixation activity in the soil occurred in the upper 10 cm, or the number of replications together with the inherent variability in the method did not provide sufficient sensitivity to detect a dependency between $N_2$ fixation activity and depth of soil in the pot. Neither interpretation supports a standardization of obtained activity values to an
equal depth of soil.

Influence of environmental parameters

The variation in soil moisture content did not influence the acetylene reduction activity. This result can reflect a true independency or be an effect of the methodologies used.

The overnight rate of ethylene production was measured in the same enclosures as the daytime rate, without opening the enclosures in between. It is likely that results showing no ethylene production activity during night is due to inhibition from the previous exposure to acetylene. If this was the case, the N inputs would be twice those reported.

Associative $N_2$ fixation

Any statistically verified contribution by rhizosphere $N_2$ fixation in association with the tested plants could not be shown in more than a few cases. However, in the meadow three of four assays indicated an increased $N_2$ fixing activity in the presence of A. microphylla. A. millefolium in the aspen, the fir and the spruce gave a net increase to N input at 1 of 2, 5 of 5 and 1 of 5 assays, respectively. Taken together, a higher activity in the presence of plant was measured for 10 of 16 performed assays, although only three were within the accepted statistical limit. Both H. micrantha and H. scouleri enhanced $N_2$ fixation activity in the aspen, while in association with H. micrantha in the spruce, the activity was lower than that obtained from the bare soil. This might indicate that $N_2$ fixation acti-
vity is stimulated in the presence of plants, but to an extent lower than was expected and, therefore, was not detected with the experimental design and methodology used.

During both the 1980 and the 1981 seasons, the subjective observation that *A. millefolium* growing in the field area showed a slower growth rate and bloomed less frequently than those growing under more favorable conditions at lower elevation was made. If the physiological status of plants determines the quality and the quantity of the root exudates, plants would be less likely to exude high quality carbohydrates in large amounts when grown under physiological stress. As it is the high concentration of easily available carbohydrates in the rhizosphere that is considered the main factor for stimulated bacterial growth, stressed plants would not provide conditions for a considerable increase in the bacterial population density in their rhizosphere as compared to the non-rhizosphere environment. The slow growth of *A. millefolium* in the field area might indicate that plants here are subjected to a physiologic stress, possibly the low water availability. An increase in the bacterial population, including *N₂* fixers, in the rhizosphere of tested plants would then, as found, be less pronounced than has been reported for other investigations (Diem and Dommergues, 1980).

The obtained result could be an effect of a too low sensitivity of the assays as performed. Taking effusion of gases (discussed later) into consideration and increasing the number of replicates would increase the sensitivity of the method and provide a better basis for detecting small differences between *N₂* fixation activity in the pres-
ence and absence of plants.

**Free-living N\textsubscript{2} fixers**

The yearly input of N by non-associative N\textsubscript{2} fixation in the soil was in the meadow, the aspen, the fir and the spruce 0.48, 0.33, 0.24 and 0.32 kg N ha\textsuperscript{-1} y\textsuperscript{-1}, respectively. This is in agreement with the contribution of N by *Azotobacter* and *Clostridium* in unamended soils reported by Postgate (1974), 0.3 kg N ha\textsuperscript{-1} y\textsuperscript{-1}.

A higher N\textsubscript{2} fixation rate, 2 kg N ha\textsuperscript{-1} y\textsuperscript{-1}, was measured in a native grassland in the temperate zone (Vlassak et al., 1973), while the input of N to a sub-Arctic pine wood forest was only a few micrograms per hectare and year (Granhall and Lid-Torsvik, 1975).

**Data processing**

In eq. 2.1 it has been assumed that the ethylene production rate is constant over the time for the assay and that the rate is directly proportional to the rate by which the ratio of ethylene to propane increases. The proportionality factor is the amount of propane injected into the device used at the time of initiation of the assay. It follows that

$$\frac{de}{dt} = p \frac{d(e/p)}{dt}$$

where \(de/dt\) is the rate of ethylene production (mole ethylene h\textsuperscript{-1}), \(P\) is the amount of propane injected to the device at the time for initiation of the assay (mole propane), \(d(e/p)/dt\) is the observed increase in \(e/p\) (mole ethylene/mole propane h\textsuperscript{-1})

Eq. 2.2 is satisfied if and only if \(P=p\) for the entire time of the assay, which means that no diffusion of gases occurs from the device.
used. A decrease in detected amount of propane was observed for most enclosures used for the investigation. How the decreasing amount of propane influences the result will be discussed in Chapter IV.

In deriving eq. 2.1 Balandreau and Dommergues (1973) did consider diffusion of gases, but no correction for different rates of diffusion is indicated in the final expression. An alternative method for evaluation of \( \text{N}_2(C_2H_2) \) fixation assays, that includes a correction for diffusion, is derived in Chapter III.

**Summary**

*In situ* measurement of \( \text{N}_2 \) fixation activity, using the acetylene reduction technique, were performed in four successional stages of a northern Wasatch Mountain forest ecosystem. Assays were carried out with units of soil, some vegetation free and some containing one plant species. The difference between the activity obtained in the presence of plant and that determined from vegetation free units was considered to be due to the rhizosphere effect of the tested plant on \( \text{N}_2 \) fixation. Tested plants were *Achillea millefolium*, *Antennaria microphylla*, *Hackelia micrantha* and *Hieracium scouleri*. Data were processed according to the equation derived by Balandreau and Dommergues (1973).

The \( \text{N}_2 \) fixation in the soil of the four successional stages the meadow, the aspen, the fir and the spruce was \( 0.48 \pm 0.24 \), \( 0.33 \pm 0.05 \), \( 0.24 \pm 0.08 \) and \( 0.32 \pm 0.10 \) kg N ha\(^{-1}\) yr\(^{-1}\), respectively. In association with *A. microphylla* in the meadow, with *A. millefolium* in the aspen, the fir and the spruce the presence of plant enhanced the \( \text{N}_2 \) fixation.
in 3 of 4, 1 of 2, 5 of 5 and 1 of 5 assays, respectively, but a statistically significant contribution ($\alpha<0.10$) was obtained in three of the assays only. The found result might indicate that $N_2$ fixation in association with the tested plants does take place but to an extent lower than could be detected with the methodology used.

The low sensitivity in the methodology could be due to no correction for leakage of gases. Leakage of gases occurred from most of the enclosures used for the acetylene reduction assays and the accuracy in the measured activities could therefore be low.
CHAPTER III
AN ALTERNATIVE METHOD FOR EVALUATION OF
ACETYLENE REDUCTION ASSAYS

Introduction

The commonly used method for evaluation of \textit{in situ} determination of acetylene reduction activity was designed by Balandreau and Dommergues (1973) and suggested as the method of choice by Knowles (1980). Some of the assumptions on which their mathematical formula is based might not be acceptable in all cases. The most critical point being whether leakage of gases (diffusion and/or effusion) from the device used can be considered of trivial importance, as implied in the method by Balandreau and Dommergues (1973). This assumption has previously been considered by Patriquin and Keddy (1978), who (quoting from Knowles, 1980) "... take into account the initial rapid and subsequently slow phase of dilution of C$_2$H$_4$ and internal standard into the occupied volume ...". Their approach for omitting the critical assumption is correct, but their presentation is not in agreement with their methodology and for diffusion existing laws.

The procedure described in Chapter II differs slightly from that used by Balandreau and Dommergues (1973) and by Patriquin and Keddy (1978). They enclosed the system to be tested in a cylinder sealed at the top but open at the bottom, while the method described in Chapter II involves a complete enclosure of the tested system in a Saran bag, also suggested as an alternative by Balandreau and Dommergues (1973).
This makes a considerable difference regarding leakage of gases. In the open system, diffusion of gases from the cylinder into the surrounding soil can proceed uninterruptedly, while such an uninterrupted flow of gases is prevented from a closed system.

**General Aspects of Diffusion and Effusion**

**Differences between a closed and an open system**

The two phenomena, diffusion and effusion, are closely related and not always distinguished. For the following presentation, it is an advantage to separate the two because diffusion bears a relation to the open system while effusion is related to the closed system. Both involve an equalization of concentration between two volumes, of which one contains the diffusing substance in a higher concentration than the other one. In the case of diffusion, the two volumes are in direct contact with each other and the rate of diffusion is determined by characteristics of the medium into which diffusion occurs. Effusion implies that the two volumes are separated from each other with a membrane (a plastic bag, etc.) and the effusion occurs through orifices in the separating material. The rate of effusion is determined by the size of the passages present.

The force driving diffusion and effusion is the difference in concentration between the two volumes concerned. The concentration gradient at the boundary between the two volumes has its highest value at time 0, when all of the diffusing/effusing substance is located in one of the volumes. In an open system, there is initially
a very rapid diffusion into the volume where the concentration is 0 mole 1\(^{-1}\). The diffusing substance builds up a concentration gradient from the originally sharp boundary, with respect to the concentration of the diffusing substance, into the volume with the lower concentration. Both the concentration of the diffusing substance and its concentration gradient decrease exponentially with the distance from the originally sharp boundary. Effusion from a closed system has its highest rate at time 0 h, but the initial rate of effusion is not as rapid as that of diffusion, the membrane separating the two volumes is a hindrance. Penetration of the membrane occurs with a much slower rate than the diffusion rate of the effusing substance in the medium into which effusion occurs. The concentration of the effusing substance will remain virtually 0 mole 1\(^{-1}\) in the medium into which effusion occurs, provided that its volume is sufficiently large.

Effusion is a slower but longer lasting process as compared to diffusion. A hypothetical difference between the two systems with respect to the time course of the concentration of the diffusing/effusing substance in the volume where it was initially present is shown in Fig. 6.

Physical laws for diffusion and effusion

Frick's first and second laws of diffusion. In the middle of the 19th Century Frick studied the diffusion phenomenon and stated his first and second laws of diffusion. Both Frick's laws are derived in most textbooks in physical chemistry (Chang, 1981).
Frick's first law of diffusion is

\[ r_x = -D \left( \frac{dC}{dx} \right)_t \]  

where \( x \) is the distance from the originally sharp boundary, with respect to concentration, between the two volumes with different concentrations of the diffusing substance to the location of measurement.

\( t \) is the accumulated time from initiation of diffusion.

\( r_x \) is the diffusion rate at the distance \( x \) at time \( t \).

\( \left( \frac{dC}{dx} \right)_t \) is the concentration gradient of the diffusing substance at the distance \( x \) at time \( t \).

\( D \) is the diffusion coefficient for the diffusing substance in the medium into which diffusion occurs.

The negative sign indicates that the change in concentration is negative with respect to the direction of diffusion.

Frick's first law states that the diffusion rate at the distance \( x \) is directly proportional to the concentration gradient over \( x \) at time \( t \).

Figure 6. Hypothetical difference between diffusion from an open system and effusion from a closed system with respect to the concentration of the diffusing/effusing substance in the volume where it is initially present over time.
Frick's second law of diffusion is

\[
\left( \frac{dC}{dt} \right)_x = D \left( \frac{d^2C}{dx^2} \right)_t
\]

where \( x, t \) and \( D \) are defined as for eq. 3.1

\[
\left( \frac{dC}{dt} \right)_x \text{ is the change in concentration over time at the distance } x
\]

\[
\left( \frac{d^2C}{dx^2} \right)_t \text{ is the change in concentration gradient over time at the distance } x.
\]

Frick's second law states that at the distance \( x \) the rate of diffusion is directly proportional to the concentration gradient over \( x \), that on its part changes with time.

Graham's law of diffusion and effusion. During the same period that Frick studied diffusion, studies were also conducted by Thomas Graham, who was interested in the difference between gases with respect to diffusion. His discoveries resulted in Graham's law of diffusion and effusion, which states that the diffusion and the effusion rates of two different gases are inversely proportional to the square root of their molar mass, provided that the force driving diffusion/effusion is the same for both gases. Thus, for the two gases \( 1 \) and \( 2 \)

\[
\frac{r_1}{r_2} = \left( \frac{mw_2}{mw_1} \right)^{1/2}
\]

where \( r \) is the rate of diffusion or effusion (mole time\(^{-1}\))

\( mw \) is the molecular weight of the gas

\( 1 \) and \( 2 \) denote the two gases to be compared.

The Method in Current Use

The formula derived by Balandreau and Dommergues (1973), p. 20, is valid for enclosures from which no diffusion/effusion occurs, p.
50, or where it can be shown to be insignificant.

Considering diffusion and effusion in evaluation of the method and interpretation of results

Correction for diffusion/effusion. Diffusion and/or effusion of gases from the device used will continue during the entire time of the assay. The propane will, therefore, continuously decrease. The ethylene is produced with a constant rate and simultaneously subjected to effusion. If the diffusion rate is approximately constant during the time of sampling, the net increase of ethylene is the same for equally long periods of time at the beginning, the middle and the end of the assay. It follows that the rate by which the ratio of ethylene to propane increases is more rapid during the later stages of the assay (Table 8).

Table 8. Increase in $d(E/P)/dt$ over the time of an assay for a constant ethylene production rate and different, but constant, rates of diffusion.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Interval beginning</th>
<th>Interval middle</th>
<th>Interval end</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>No diffusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethylene produced</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>propane, average during the interval</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>$d(E/P)/dt$</td>
<td>$0.0067$</td>
<td>$0.0067$</td>
<td>$0.0067$</td>
</tr>
<tr>
<td>Slow diffusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethylene produced</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>propane, average</td>
<td>300</td>
<td>275</td>
<td>250</td>
</tr>
<tr>
<td>the interval</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$d(E/P)/dt$</td>
<td>$0.0067$</td>
<td>$0.0073$</td>
<td>$0.0080$</td>
</tr>
<tr>
<td>Rapid diffusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethylene produced</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>propane, average during the interval</td>
<td>300</td>
<td>250</td>
<td>200</td>
</tr>
<tr>
<td>$d(E/P)/dt$</td>
<td>$0.0067$</td>
<td>$0.0080$</td>
<td>$0.0000$</td>
</tr>
</tbody>
</table>
Acetylene reduction activity, calculated from the ratios E/P obtained over the time of the assay, is overestimated when diffusion/effusion occurs and is not corrected for. The degree of overestimation increases as the rate of diffusion/effusion increases and only for a tight device is the \( \frac{d(E/P)}{dt} \) constant.

An increase in the ethylene production rate was observed during the time of incubation (Boddey, 1980). A constant ethylene production rate would occur only after the presence of acetylene has created a nitrogen limitation for the microbial population in the rhizosphere. No attention was paid to diffusion as a possible cause for the observed increase in ethylene production.

The correction for diffusion introduced by Patriquin and Keddy (1978) is based on a direct proportionality between the rate of diffusion from the air phase of an open system and the concentration of the diffusing gas in the air phase. According to Frick's first law of diffusion, the rate of diffusion is proportional to the concentration gradient driving the diffusion. The correction by Patriquin and Keddy (1978) bears a relation to the closed system.

**Occupied volume at different times.** The volume, \( v_n \) in eq. 1.2 p. 25, that is occupied by ethylene and propane at different times is derived from the concentration of propane in the air phase at the respective times. Determination of the total volume from the concentration in one sample requires that the gas concerned is present in equal concentration throughout the total volume. This is not in agreement with the actual situation, where the propane diffusing from the device is present at a lower concentration as the distance from
the device increases. Frick's second law of diffusion expresses how the concentration gradient at a distance x from the opening of the device changes over time.

**Equal diffusion rate for different gases.** The difference in diffusion rate between ethylene and propane is given by Graham's law of diffusion and effusion. The rate for ethylene is 1.25 times the rate for propane.

The equal diffusion rate of ethylene and propane, that has been reported for many investigations (Balandreau and Dommergues, 1973), might be a result of the different diffusion coefficients for propane and ethylene in the soil of concern.

**One rapid and one slow phase of diffusion.** The diffusion pattern obtained from open devices shows an initial very rapid decrease in the diffusion rate and after a short time, as compared to the time of the assay, the diffusion rate appears to be approximately constant. This has been interpreted as two different processes, an initial fast drop in concentration of propane in the air phase due to a rapid diffusion into the occupied volume, followed by a slower process during which the measured decrease in concentration is due to the gas adsorbing to soil particles and dissolving into the soil water (Witty, 1979b).

The interpretation by Witty (1979b) did not consider that the diffusion itself gives the observed time course for the concentration of propane in the air phase (Figure 6). The initial rapid decrease occurs when the concentration gradient over the air-soil interface is high. The open area through which diffusion takes place in an open
system brings about a very fast increase in the propane concentration in the soil at the very vicinity of the air phase. This lowers the concentration gradient over the air-soil interface and as that is the driving force for gases diffusing from the air phase into the soil, the result is a less pronounced decrease in the propane concentration in the air phase.

Incorrect reduction of units. When examining the units for the calculation of acetylene reduction activity, it is important to distinguish the different kinds of "mole propane" that appear to be the same but actually are time dependent.

The rate, \( K' \), by which the ratio of mole ethylene to mole propane increases over time, \( \frac{d(e/p)}{dt} \) in eq. 1.3, and \( \frac{d(E/P)}{dt} \) in eq. 1.4 has the unit

\[
K': \frac{\text{mole ethylene produced}}{h \times \text{mole propane remaining at time } t \ h}
\]

According to the eqs. 1.3 and 1.4 the acetylene reduction activity is obtained by multiplying \( K' \) with "mole propane injected to the device at time 0 h". Hence,

\[
\text{ARA}: \frac{\text{mole ethylene produced}}{h \times \text{mole propane remaining at time } 0 \ h} \times \frac{\text{mole propane}}{\text{mole propane remaining at time } t \ h}
\]

The improper reduction is made when "mole propane at time 0 h" is reduced against "mole propane remaining after time t h" without introducing a proper conversion factor between the two. Only in the case when "mole propane" is constant over time, a tight device, is the performed reduction correct.

The presence of diffusion automatically gives "mole propane at
time 0 h" > "mole propane remaining at time t h" and the calculated acetylene reduction activity is overestimated.

In the eqs. 1.3 and 4 an improper reduction is also made when in the numerator the ratios of ethylene to propane at different times are subtracted in spite of different time dependent denominators. Expressed in units it is

\[ \frac{\text{mole ethylene at time } t_2}{\text{mole propane at time } t_2} - \frac{\text{mole ethylene at time } t_1}{\text{mole propane at time } t_1} \]

The approximation that "mole propane at time t_2" is equal to "mole propane at time t_1" introduces a less severe error than that introduced by assuming them equal to "mole propane at time 0 h". The approximation results in an overestimation of the calculated difference.

Presence of ethylene at time 0 h. A small amount of ethylene is generated as an impurity when acetylene is generated from calcium carbide. This ethylene is most likely negligible when high activities of acetylene reduction are measured, but must be considered when the acetylene reduction activity is low.

Introduced correction for diffusion. The differential equation by Patriquin and Keddy (1978), eq. 1.5 p. 21, do express a correction for the diffusion. However, in solving the equation for K, the ethylene production rate, a factor t, time, has wrongly been introduced in the numerator. The conversion from the ethylene production determined for the sample volume to the total ethylene produced introduces an overestimation of the activity.
The Closed System

General aspects

When assays for determination of \( N_2(C_2H_4) \) fixation activities are carried out, gases of interest are ethylene, acetylene and propane (if used as a tracer gas). None of these gases are normally present in the atmosphere and after penetration of the enclosure, the rate determining step for effusion, the gases are fast diluted in the surrounding air volume. It follows that during the entire time of the assay, the concentration of the gases of interest is virtually 0 mole ml\(^{-1}\) on the outside vicinity of the enclosure and consequently the concentration gradient driving effusion of gases is directly proportional to the inside concentration at any time. Expressed in mathematical terms the above condition gives the rate by which the inside concentration changes over time as

\[
\frac{dC}{dt} = rC \quad \text{eq. 3.4}
\]

where \( C \) is the inside concentration at time \( t \) h (mole ml\(^{-1}\))

\( t \) is the time from initiation of effusion to \( t \) h (h)

\( r \) is the proportionality factor for the gas concerned (h\(^{-1}\))

When effusion occurs, the inside concentration decreases with time and \( \frac{dC}{dt} < 0 \). The rate of effusion is a positive value and therefore defined as

\[
\text{effusion rate for the gas concerned} = -\frac{dC}{dt} = -rC \quad \text{eq. 3.5}
\]

where \( C, t \) and \( r \) are defined as for eq. 3.4

the negative sign indicates that the effusion rate decreases with a decrease in \( C \).

Solving eq. 3.4 for \( C \) during the time interval from 0 to \( t \) h
where $C_0$ is the inside gas concentration at 0 h (mole ml$^{-1}$)
$t$ is the time from initiation of effusion to t h (h)
$r$ is the proportionality factor for the gas concerned (h$^{-1}$).

Eq. 3.6 gives the time course for the inside gas concentration. Both
$C_0$ and $r$ can be determined from measurements of the gas concentration
at different times by exponential curve fit to the function described
by eq. 3.6 or by linear regression according to the rearranged form

$$\ln C = rt + \ln C_0$$

where all symbols are defined as for eq. 3.6.

A gas injected into the enclosure is quickly evenly distributed
throughout the entire air volume, and the concentration obtained from
a sample is the same as that of the total volume. The total amount of
gas in the enclosure is known at 0 h only, before some of the gas has
effused. It follows that at 0 h

$$\frac{A}{vol} = C_0$$

where $A$ is the amount of gas injected at 0 h (mole)
$vol$ is the enclosed volume (ml volume$^{-1}$)
$C_0$ is the gas concentration at 0 h (mole ml$^{-1}$).

The enclosed volume can be determined from eq. 3.8.

**Tracer gas**

By injecting a defined amount of a tracer gas into an enclosure
in which the acetylene reduction assay is to be performed, both the
enclosed volume and the effusion rate from the enclosure can be de-
terminated. For the following presentation propane has been used as the tracer gas.

Effusion rate. The effusion rate for propane is according to eq. 3.5

$$\text{effusion rate} = -r_P P$$  \hspace{1cm} \text{eq. 3.9}

where $P$ is the propane concentration at $t$ h (mole ml$^{-1}$)

$r_P$ is the proportionality factor for propane ($h^{-1}$).

The proportionality factor is determined by exponential curve fit according to eq. 3.6 that applied on propane is

$$P = P_0 e^{r_P t}$$  \hspace{1cm} \text{eq. 3.10}

where $P$ is the propane concentration at $t$ h (mole ml$^{-1}$)

$P_0$ is the propane concentration at 0 h (mole ml$^{-1}$)

$r_P$ is the proportionality factor for propane ($h^{-1}$)

$t$ is the time from initiation of the assay (h).

Eq. 3.10 is graphically presented, for the same initial propane concentration but different $r_P$ values, in Figure 7. Alternatively, the $r_P$ is determined by linear regression according to

$$\ln P = r_P t + \ln P_0$$  \hspace{1cm} \text{eq. 3.11}

where all symbols are defined as for eq. 3.10.

When the effusion rate for propane is determined the rate by which ethylene effuses is given by Graham's law of effusion. It, however, requires equal concentration for the gases to be compared, and the rates of effusion are therefore expressed per concentration present, which is equal to $-r$ for the respective gas. It follows that

$$-r_E = -1.25 r_P$$

and the effusion rate for ethylene is
Figure 7. Time courses for amount propane, amount ethylene and the ratio E/P for the same production rates of ethylene but different effusion rates from a closed system.
effusion rate = \(-r_E\) \(E = -1.25 r_P \) \(E\) 

\[\text{eq. 3.12}\]

where \(-r_E\) is the proportionality factor for ethylene \((h^{-1})\), 
\(E\) is the ethylene concentration at \(t\) \(h\) \((\text{mole ml}^{-1})\), 
\(-r_P\) is the proportionality factor for propane \((h^{-1})\).

Enclosed volume. The enclosed volume can be determined according to eq. 3.8 as

\[\text{vol} = \frac{PP}{P_0}\]

\[\text{eq. 3.13}\]

where \(\text{vol}\) is the enclosed volume \((\text{ml})\), 
\(PP\) is the amount of propane injected at \(0\) \(h\) \((\text{mole})\), 
\(P_0\) is the propane concentration at \(0\) \(h\) \((\text{mole ml}^{-1})\).

\(P_0\) is determined from the intercept of eq. 3.10 or 11 as \(P_0\) or \(\ln P_0\), respectively. To determine \(P_0\) from a sample taken at time \(0\) \(h\) is unreliable as some time must be allowed for the gases to equilibrate and the rate of effusion is most rapid at \(0\) \(h\).

Ethylene

At any time during the assay the production rate of ethylene is equal to the sum of the observed increase in ethylene over time and the rate by which ethylene effuses. It follows that

\[dE/dt = k + (-r_E) \] \(E\)

\[\text{eq. 3.14}\]

where \(k\) is the ethylene production rate \((\text{mole ml}^{-1} \text{h}^{-1})\), 
\(E\) is the ethylene concentration at \(t\) \(h\) \((\text{mole ml}^{-1})\), 
\(t\) is the time from initiation of the assay to \(t\) \(h\) \((h)\), 
\(dE/dt\) is the observed increase in ethylene \((\text{mole ml}^{-1} \text{h}^{-1})\), 
\(-r_E\) is the effusion rate for ethylene at \(t\) \(h\) \((\text{mole ml}^{-1} \text{h}^{-1})\).

Solving eq. 3.14 for \(E\) during the time for the assay, \(0\) to \(t\) \(h\), gives

\[E = \frac{r_E t \cdot e^{-r_E t}}{r_E} + E_0 \cdot e^{r_E t}\]

\[\text{if } r_E \neq 0 \text{ eq. 3.15.a}\]
\[ E = kt + E_0 \quad \text{if } r_E = 0 \quad \text{eq. 3.15.b} \]

where \( E \) is the ethylene concentration at \( t \) h (mole ml\(^{-1}\))
\( r_E \) is the proportionality factor for ethylene (h\(^{-1}\))
\( t \) is the time from initiation of the assay to \( t \) h (h)
\( E_0 \) is the ethylene concentration at 0 h (mole ml\(^{-1}\))
\( k \) is the ethylene production rate (mole ml\(^{-1}\) h\(^{-1}\)).

The first term of eq. 3.15 is the portion of the produced ethylene that remains in the enclosure at time \( t \) h. The second term is what remains at \( t \) h of the ethylene present at the initiation of the assay. The most plausible origin of ethylene present at 0 h, is that if is produced as an impurity when acetylene is generated from carbide. Ethylene is also commonly present in commercially supplied acetylene. The two kinds of ethylene, the one produced from acetylene reduction and the one originating from acetylene generation are shown in Figure 7 as \( E(p) \) and \( E(i) \), respectively.

The convenience of a linear relationship is always worth aiming for and for this purpose a rearrangement of eq. 3.15, to give a time independent intercept, is necessary. It gives

\[ E e^{-r_E t} = k e^{-r_E t} - 1 + E_0 \quad \text{if } r_E \neq 0 \quad \text{eq. 3.16.a} \]

\[ E = kt + E_0 \quad \text{if } r_E = 0 \quad \text{eq. 3.16.b} \]

where all symbols are defined as for eq. 3.15.

It follows that linear regression of

\[ E e^{-r_E t} \quad \text{vs.} \quad e^{-r_E t} - 1 \quad \text{if } r_E \neq 0 \]

\[ E \quad \text{vs.} \quad t \quad \text{if } r_E = 0 \]

in both cases gives the ethylene production rate, \( k \), as the slope of
the regression line and the initial ethylene concentration, \( E_0 \), as the intercept.

The ethylene production rate is determined from the measured ethylene concentrations over time according to eq. 3.16, and has the unit mole ml\(^{-1}\) h\(^{-1}\). The ultimate goal is to determine the acetylene reduction activity for the entire volume enclosed, and if this volume is known

\[
ARA = k \, \text{vol} \tag{eq. 3.17}
\]

where \( ARA \) is the acetylene reduction activity (mole h\(^{-1}\) vol\(^{-1}\)),

\( k \) is the ethylene production rate (mole ml\(^{-1}\) h\(^{-1}\))

\( \text{vol} \) is the enclosed volume (ml volume\(^{-1}\)).

If the enclosed volume is unknown, it can be determined according to eq. 3.13. Then

\[
ARA = PP \frac{k}{P_0} \tag{eq. 3.18}
\]

where \( ARA \) is the acetylene reduction activity (mole h\(^{-1}\) vol\(^{-1}\)),

\( PP \) is the amount of propane injected at 0 h (mole)

\( k \) is the ethylene production rate (mole ml\(^{-1}\) h\(^{-1}\))

\( P_0 \) is the propane concentration at 0 h (mole ml\(^{-1}\)).

In eq. 3.18, the \( k \) and the \( P_0 \) could be determined from the eqs. 3.17 and 3.10, respectively, but a more convenient way to obtain the \( k/P_0 \) is to use the ratio \( E/P \) in the collected samples.

The ratio \( E/P \)

The time course for \( E/P \) is illustrated in Figure 7 for different rates of effusion but for the same ethylene production rate.

The ratio \( E/P \) is given by dividing eq. 3.15 by eq. 3.10 which gives
where $E$, $E_0$, $r_E$, $k$ and $t$ are defined as for eq. 3.15. $P$, $P_0$ and $r_p$ are defined as for eq. 3.10.

A linear relationship is achieved by rearranging eq. 3.19 as

$$
\frac{E}{P} = \frac{k}{P_0} \frac{r_{pt}^t}{e^{r_{pt}}} + \frac{E_0}{P_0} e^{r_{pt}}
$$

if $r_E \neq 0$ eq. 3.19.a

$$
\frac{E}{P} = \frac{kt}{P_0} + \frac{E_0}{P_0}
$$

if $r_E = 0$ eq. 3.19.b

where all symbols are defined as for eq. 3.19.

After substituting $r_E$ for $1.25r_p$, eq. 3.12, it follows that

$$
\frac{E}{P} e^{-0.25r_{pt}} vs. e^{-1.25r_{pt}} - 1
$$

if $r_E \neq 0$ 

$$
\frac{E}{P} vs. t
$$

if $r_E = 0$

gives a straight line with the slope $k/P_0$ and the intercept $E_0/P_0$.

The $k/P_0$, determined from eq. 3.20, can be used to determine the acetylene reduction activity according to eq. 3.18.

Error when effusion is not corrected for

Evaluation of data based on the detected ethylene concentrations underestimates the acetylene reduction activity when effusion occurs and is not corrected for. This is illustrated in Figure 7, where the
slope of the line obtained from the detected ethylene concentrations vs. time decreases with a more rapid effusion rate, although the actual ethylene production rate is the same for all cases. If the acetylene reduction activity is determined as the slope of $E/P$ vs. time the activity is overestimated when effusion is not corrected for (Figure 7).

The degree of under- and overestimation is, besides the effusion rate, dependent on the initial amount of ethylene present and the time interval during the assay is performed.

**Practical aspects**

The gas concentration is from the gas chromatograph given in the unit mm sample$^{-1}$, if peak height is the output unit. If the same volume is analyzed for all samples the output unit can directly be used for evaluation of data. The eqs. 3.17 and 3.18 are then

\[ ARA = \frac{e'}{k'} \frac{\text{vol}}{\text{ss}} ; k' \text{ the slope of } E' e^{-r_E t} \text{ vs. } \frac{e^{-r_E t}}{-r_E} \]

\[ ARA = \frac{e'}{P'_0} \frac{k'}{P'_0} PP ; k' \text{ the slope of } \frac{E' p_t}{P'_0} e^{-0.25r_p t} \text{ vs. } \frac{e^{-1.25r_p t}}{-1.25 r_p} \]

where $ARA$ is the acetylene reduction activity (mole h$^{-1}$ vol$^{-1}$)

$E'$ is the ethylene concentration at t h (mm sample$^{-1}$)

$e'$ is the gas chromatographic response to ethylene (mole mm$^{-1}$)

$k'$ is the ethylene production rate (mm h$^{-1}$ sample$^{-1}$)

$P'_0$ is the propane concentration at t h (mm sample$^{-1}$)

$P'_0$ is the propane concentration at 0 h (mm sample$^{-1}$)

$PP$ is the amount of propane injected at 0 h (mole)

$p'_0$ is the gas chromatographic response to propane (mole mm$^{-1}$)

$r_E$ is the proportionality factor for ethylene (h$^{-1}$)

$r_p$ is the proportionality factor for propane (h$^{-1}$)

$ss$ is the sample volume (ml sample$^{-1}$)
t is the time from initiation of the assay to \( t \) (h).

\( \text{vol} \) is the enclosed volume (ml volume\(^{-1}\)).

\( r \) is, according to eq. 3.6, independent of the unit in which \( C \) is expressed and \( r_p \) can be determined from eq. 3.10 using the output unit, mm sample\(^{-1}\), for the propane concentrations. \( r_E \) is determined from \( r_p \) according to eq. 3.12.

Relationships of interest for acetylene reduction assays performed in closed systems, from which effusion occurs, are presented in Figure 8.

It has been assumed that the concentration on the outside of the enclosure is 0 mole l\(^{-1}\) for the gases of interest. This is true for gases effusing from the enclosure into the surrounding air space. However, holes accidentally created when the Saran bag was replaced into the soil will appear under the ground level and the effusing gases will enter the soil. If the physical properties of the soil are such that they provide a slow diffusion of the effusing gases they will be retained at the close proximity of the bag and result in a concentration greater than 0 mole l\(^{-1}\) on the outside of the enclosure.

Differences between ethylene and propane regarding their different solubilities in the soil water, adsorption to soil particles etc. and how these properties vary with the decrease in partial pressure of propane and the increasing partial pressure of ethylene are not taken into consideration.

The endogenous ethylene production is not corrected for. If the rate of the endogenous ethylene release is known, it can be treated as the ethylene produced from acetylene production.
Figure 8. Relationships of interest for evaluation of acetylene reduction activity in closed system. (See text for explanation.)
General aspects

Diffusion of a gas from the air phase of an open device out into the surrounding soil is driven by its concentration gradient over the air-soil interface. This concentration gradient decreases with time and consequently, the diffusion rate is slower the later during the assay. The rate of diffusion is also dependent on the diffusion properties of the soil for the gas concerned. Frick's second law of diffusion describes how, for a gas in the air phase, the concentration gradient over the air-soil interface changes over time, eq. 3.2 for x=0. A mathematical expression describing the relation between the gas concentration in the air phase and time can not be obtained for the entire time of the assay provided measurements of the gas concentration in the air phase only. This has the consequence that the diffusion rate can not be determined for the entire time of the assay and the initial gas concentration can not be computed.

After an initial short time period, during which the diffusion rate first is very rapid and then rapidly decreases, the change in the concentration gradient over the air-soil interface is very slow and the diffusion rate can be assumed approximately constant (Figure 6). During an interval where this approximation is acceptable

\[ C = rt + C_b \]  

where \( C \) is the gas concentration in the air phase at \( t \) h (mole \( \text{ml}^{-1} \)),  
\( r \) is the rate by which \( C \) changes during the selected interval (mole \( \text{ml}^{-1} \) h\(^{-1}\))
t is the time from the beginning of the selected interval to \( t \) h (h)

\( C_b \) is the gas concentration in the air phase at the beginning of the selected interval (mole ml\(^{-1}\)).

When diffusion occurs \( C \) is less than \( C_b \) and consequently, \( r \) is negative. The diffusion rate is positive and therefore defined as

\[
\text{rate of diffusion for the gas concerned} = -r \quad \text{eq. 3.25}
\]

For a comparison between the diffusion rates of different gases the force driving the diffusion must be the same for both gases. The driving force is the concentration gradient and

\[
\text{rate of diffusion per concentration gradient} = \frac{-r_x}{(dC/dx)_t} \quad \text{eq. 3.26}
\]

where \( -r_x \) is the diffusion rate at the distance \( x \) from the device at \( t \) h (mole ml\(^{-1}\) h\(^{-1}\))

\( (dC/dx)_t \) is the concentration gradient over \( x \) at \( t \) h (mole ml\(^{-1}\) h\(^{-1}\))

\( dC \) is the difference in gas concentration over \( x \) at \( t \) h (mole ml\(^{-1}\))

\( t \) is the time from the beginning of the selected interval to \( t \) h (h).

Eq. 3.24-26 are all valid for an interval where \( r \) is constant only.

**Tracer gas**

If propane is injected into the air phase of the assay device and its concentration in the air phase monitored over time, then the diffusion rate or propane from the air phase into the soil can be determined. During a selected interval the diffusion can be assumed constant and the time course for propane during this interval is

\[
P = r_p t + P_b \quad \text{eq. 3.27}
\]

where \( P \) is the propane concentration at \( t \) h (mole ml\(^{-1}\))
\( r_p \) is the rate by which \( P \) changes during the selected interval (mole ml\(^{-1}\) h\(^{-1}\)).

\( t \) is the time from the beginning of the selected interval to \( t \) h (h).

\( P_b \) is the propane concentration at the beginning of the selected interval (mole ml\(^{-1}\)),

and the diffusion rate for propane, from the air phase into the soil, is during the selected interval \(-r_p\).

Eq. 3.26 gives the format for the diffusion rate as it can be compared for different gases and it includes the concentration gradient. According to Graham's law of diffusion the relation between the diffusion rate for propane and the diffusion rate for ethylene is

\[
\frac{-r_E}{(dE/dx)_t} = -1.25 \frac{-r_p}{(dP/dx)_t} \quad \text{eq } 3.28
\]

where \(-r_p\) is the diffusion rate for propane over the air-soil interface during the selected interval (mole ml\(^{-1}\) h\(^{-1}\))

\((dP/dx)_t\) is the concentration gradient for propane over the air-soil interface at \( t \) h (mole ml\(^{-1}\))

\(-r_E\) is the diffusion rate for ethylene over the air-soil interface during the selected interval (mole ml\(^{-1}\) h\(^{-1}\))

\((dE/dx)_t\) is the concentration gradient for ethylene over the air-soil interface at \( t \) h (mole ml\(^{-1}\))

\( t \) is the time from the beginning of the selected interval to \( t \) h (h).

It follows from eq. 3.28 that the diffusion rate for ethylene, from the air phase into the soil, can be determined from that for propane only if their respective concentration gradients over the air-soil interface are known or equal. The concentration gradient cannot be determined from measurements of the concentration in the air phase and equal concentration gradients must be provided for in order to determine the diffusion rate for ethylene from that for propane. This can only be done by adjusting the amount of propane injected into the
air phase of the device to the ethylene production rate, so that the concentration gradient of the two gases is the same over the air-soil interface.

A complication, in determining the diffusion rate for ethylene from the diffusion rate for propane, is the different diffusion patterns for the two gases. Propane diffuses from the air phase into the soil only, while the ethylene is produced in the soil and diffuses in two directions, into the air phase and into the surrounding soil. The two gases will diffuse in similar ways, only if it can be assumed that the ethylene concentration in the air phase is the same as its concentration at the site of production at any time, and the diffusion of ethylene is considered to take place into the soil only.

The volume of concern for the assay must meet the requirement of an equal concentration of gases throughout the entire volume. If this is not fulfilled the analyzed gas concentration would be dependent on the location of sampling. For an open device, this requirement is met by the air phase only, in the soil the concentration decreases with the distance from the device. The volume of the air phase can be calculated only if the total amount of a gas and its concentration in the air phase are known at the same time. The total amount of propane in the air phase is known when a defined amount of propane is injected into the air phase at initiation of the assay, but the initial propane concentration can not be determined from measurements of the propane concentration in the air phase over time. It follows that the volume of the air phase must be predetermined. However, when the volume of the air phase and the total amount of propane injected are
known, the initial propane concentration can be calculated. The volume can then be expressed as $PP/P_0$ according to eq. 3.13.

**Defining the test material**

Acetylene, generated in the air phase of an open device, will diffuse into the soil and there build up a concentration decreases with the distance from the device. A constant ethylene production rate can be expected only when the partial pressure of acetylene is constant or high enough to saturate all of the active sites on the nitrogenase present in the volume occupied by acetylene.

The volume occupied by acetylene increases over time and at any location, the acetylene concentration decreases with time. It is therefore difficult to define the tested material and to assume a constant ethylene production rate.

**Acetylene reduction assays**

Acetylene reduction assays performed in oped devices can be evaluated from measurements of the concentrations of ethylene and propane in the air phase only if it can be assumed that the ethylene production rate from the tested material is constant, that the ethylene concentration in the air phase of the device is equal to its concentration at the site of ethylene production and that the amount of propane injected into the assay device provides equal concentration gradients for ethylene and propane over the air-soil interface. Further, that the volume of the air phase of the device used is predetermined.

Under these conditions, the ethylene production rate is equal to
the sum of the observed increase in ethylene in the air phase over time and the rate by which ethylene diffuses into the soil. It gives

\[
k = \frac{dE}{dT} + (-R)
\]

where \( k \) is the ethylene production rate (mole ml\(^{-1}\) h\(^{-1}\))
\( E \) is the ethylene concentration in the air phase at \( T \) h (mole ml\(^{-1}\))
\( T \) is the time from the initiation of the assay to \( T \) h (h)
\( \frac{dE}{dT} \) is the observed increase in ethylene over time in the air phase (mole ml\(^{-1}\) h\(^{-1}\))
\( -R \) is the diffusion rate for ethylene at \( T \) h (mole ml\(^{-1}\) h\(^{-1}\)).

The diffusion rate for ethylene can be determined only during the selected interval. Solving the differential equation 3.29 for \( E \) during the selected interval, from 0 to \( t \) h, gives

\[
E = (k - (-r_E))t + E_b
\]

where \( E \) is the ethylene concentration in the air phase at \( t \) h (mole ml\(^{-1}\))
\( k \) is the ethylene production rate (mole ml\(^{-1}\) h\(^{-1}\))
\( -r_E \) is the diffusion rate of ethylene during the selected interval (mole ml\(^{-1}\) h\(^{-1}\))
\( t \) is the time from the beginning of the selected interval to \( t \) h (h)
\( E_b \) is the ethylene concentration in the air phase at the beginning of the selected interval (mole ml\(^{-1}\)).

Eq. 3.30 shows that the slope of the regression line \( E \) vs. time is \( k - (-r_E) \) and the ethylene production rate then

\[
k = \frac{dE}{dt} + (-r_E)
\]

where \( k \) and \(-r_E\) are defined as for eq. 3.30
\( \frac{dE}{dt} \) is the observed increase in ethylene during the selected interval (mole ml\(^{-1}\) h\(^{-1}\)).

The acetylene reduction activity for the volume of the air phase is then
ARA = \frac{\text{vol} \left( \frac{dE}{dt} + (-r_E) \right) }{PP} = \frac{\text{PP}}{P_0} \left( \frac{dE}{dt} + (-r_E) \right) \quad \text{eq. 3.32}

where ARA is the acetylene reduction activity (mole h⁻¹)
vol is the volume of the air phase (ml volume⁻¹)
E is the ethylene concentration in the air phase at t h (mole ml⁻¹)
t is the time from the beginning of the selected interval to t h (h)
dE/dt is the observed increase in ethylene during the selected interval (mole ml⁻¹ h⁻¹)
-r_E is the diffusion rate of ethylene during the selected interval (mole ml⁻¹ h⁻¹)
PP is the amount of propane injected into the air phase at 0 h (mole)
P₀ is the propane concentration in the air phase at 0 h (mole ml⁻¹).

Evaluation of acetylene reduction assays is often based on the ratio of ethylene to propane in the samples collected over time. The mathematical expression for the E/P ratio is obtained by dividing eq. 3.30 with eq. 3.27. It gives

\[ \frac{E}{P} = \frac{(E - (-r_E))}{r_P t + P} \left( \frac{t}{P} + \frac{E_b}{P_b} \right) \quad \text{eq. 3.33} \]

where E, k, -r_E, t and E_b are defined as for eq. 3.30
P and P₀ are defined as for eq. 3.27
E/P is the ratio of the ethylene concentration to the propane concentration in samples from the air phase (mole ethylene ml⁻¹ mole propane ml⁻¹).

Eq. 3.33 is easily interpreted only if there is no significant diffusion during the time interval concerned, r=0. Eq. 3.33 is then

\[ \frac{E}{P} = \frac{k}{P_i} t + \frac{E_b}{P_i} \quad \text{eq. 3.34} \]

where E/P is defined as for eq. 3.33
k and E_b are defined as for eq. 3.30
P_i is the propane concentration in the air phase during the selected interval (mole ml⁻¹).
The ethylene production rate is then

$$k = P_i \frac{d(E/P)}{dt}$$  

(eq. 3.35)

and the acetylene reduction activity for the volume of the air phase

$$ARA = PP \frac{d(E/P)}{dt} \frac{P_i}{P_0}$$  

(eq. 3.36)

where $ARA$ is the acetylene reduction activity (mole h$^{-1}$)

$PP$ is the amount of propane injected into the air phase at $0$ h (mole)

$d(E/P)/dt$ is the observed increase in the ratio $E/P$ (mole ethylene mole propane$^{-1}$)

$P_i$ is the propane concentration in the air phase during the selected interval (mole ml$^{-1}$)

$P_0$ is the propane concentration in the air phase at $0$ h (mole ml$^{-1}$).

In eq. 3.36 the initial rapid diffusion is corrected for, and during the selected interval no significant diffusion was assumed.

**Error when diffusion is not corrected for**

Evaluation of the acetylene reduction assay based upon the measured ethylene concentrations underestimates the activity as the diffused ethylene is not included, eq. 3.32.

When the acetylene reduction activity is computed from the $E/P$ ratios according to Balandreau and Dommergues (1973), the diffusion is not corrected for and

$$ARA = PP \frac{d(E/P)}{dt}$$  

(eq. 1.4)

where $ARA$, $PP$ and $d(E/P)/dt$ are defined as for eq. 3.35.

Comparison between the eqs. 3.35 and 1.4 shows that the ethylene production rate is overestimated by a factor $P_0/P_i$, the propane concentration at $0$ h to the propane concentration during the selected
interval. From an open device, the initial drop in propane concentration is very rapid and $P_0$ considerably greater than $P_i$ and consequently, the acetylene reduction activity without correction for diffusion vastly overestimated.

The factor $P_0/P_i$ is determined under the condition that the propane concentration in the air phase is constant during the time interval for sampling. If it decreases during the interval concerned the overestimation is more severe.

The factor $P_0/P_i$ increases the more propane is injected into the air phase at the time for initiation of the assay, and consequently a higher acetylene reduction activity is determined the more propane is used as a tracer gas.

A speculation is that the very high, indeed, rates of nitrogen fixation activity reported from some investigations is a result of the fact, that data are evaluated without correction for diffusion. Examples of high activities are 2 kg N ha$^{-1}$ d$^{-1}$ in the rhizosphere of maize (von Bulow and Döbereiner, 1975) ans approximately 6 kg N ha$^{-1}$ d$^{-1}$ by rhizosphere associated $N_2$ fixation in a tropical salt marsh (Valiela and Teal, 1979).

Practical aspects

It is questionable, as to whether the necessary presumptions for acetylene reduction assays in open devices can be met. The most difficult requirement is to assure an equal diffusion rate for the propane injected to the device and the ethylene produced. The advantages with an open system do not balance the difficulties in assay preparation, and assays in open devices not advisable.
Two different designs have been used for assaying $N_2(C_2H_2)$ fixation activity. An open system, where a cylinder is arched over the material to be tested and a closed system, where the test material is completely enclosed in e.g. a Saran bag. Evaluation of the acetylene reduction activity is commonly based on the ratio of amount ethylene to amount propane in collected samples. A defined amount of propane is injected into the device used at the time for initiation of the assay. Diffusion/effusion of gases from the device used is not corrected for in calculation of the acetylene reduction activity (Ballandreau and Dommergues, 1973; Knowles, 1980).

Results obtained according to the above methodologies do in some cases show unexpected trends that are easily explained considering diffusion/effusion as a possible cause. However, the found results are often explained based on other reasons and it is likely that these reasons are given a too great importance by neglecting diffusion/effusion from the assay device used.

The open system requires very specific presumptions that are difficult to satisfy. It is therefore not advisable to perform acetylene reduction assays in open devices.

For the closed system, both the effusion rate and the volume of the enclosure can be derived from the time course of the initially injected propane. The acetylene reduction activity including correction for effusion is calculated according to

$$\text{ARA} = \frac{PP \cdot k}{P_0}$$
where \( k/P_0 \) is the slope of the regression line

\[
\frac{E}{P} e^{-0.25 r_p t} \quad \text{vs.} \quad \frac{1 - e^{-1.25 r_p t}}{-1.25 r_p}
\]

where \( r_p \) is the slope of

\[ \ln P \quad \text{vs.} \quad t \]

where ARA is the acetylene reduction activity for the enclosed material (mole h\(^{-1}\))

- \( P_P \) is the amount of propane injected into the device at 0 h (mole)
- \( k \) is the ethylene production rate (mole ml\(^{-1}\) h\(^{-1}\))
- \( P_0 \) is the propane concentration at 0 h (mole ml\(^{-1}\))
- \( E \) is the ethylene concentration at t h (mole ml\(^{-1}\))
- \( P \) is the propane concentration at t h (mole ml\(^{-1}\))
- \( r_p \) is the proportionality factor for effusion rate (h\(^{-1}\))
- \( t \) is the time from initiation of the assay to t h (h).

When the effusion is not corrected for the acetylene reduction activity is underestimated if evaluation is based on the measured ethylene concentrations and overestimated when evaluated based on the E/P ratio in the samples.

If the assay device is completely tight the formula by Balandreau and Dommergues (1973) is applicable.
CHAPTER IV
EVALUATION OF ACETYLENE REDUCTION ASSAYS
CORRECTING FOR EFFUSION

Introduction

The in situ investigation of N₂(C₂H₂) fixation activity reported in Chapter II was both designed and performed for evaluation according to Balandreau and Dommergues (1973) and consequently, effusion of gases from the enclosures used not corrected for in calculation of the acetylene reduction activity. The formula derived by Balandreau and Dommergues (1973), eq. 1.3 p.20, and the alternative formula eq. 3.19 in Chapter III, differ in that the former is dependent on the E/P ratio in the collected samples only, while the latter, in addition to the ratio E/P, includes the proportionality factor for the effusion rate, \( -r_p \). The ratio E/P is independent of the sample volume, while the effusion rate is determined from the actual amount of gas present in the samples and subsequently volume dependent. It follows that, unless quantitative accuracy is assured in the sampling and the analysis procedures, data collected for evaluation according to Balandreau and Dommergues (1973) are not applicable for correction of the effusion.

For the investigation presented in Chapter II, the same amount of gas was flushed through each sample vial and all samples collected from the same enclosure were analyzed using the same injection volume to the gas chromatograph. Although no emphasis was made on obtaining
quantitative estimates and the sampling technique used has been shown to give variable gas exchange in the sample vial, the use of the same routines for each sample assures some consistency regarding equal volume in the collected samples. In the following presentation data have been processed as quantitative measurements.

Results

Effusion rate

The proportionality factor for the effusion rates from the enclosures, as determined from the measured amounts of propane according to eq. 3.11, varied from slightly negative values to 0.32 h⁻¹ (Figure 9). The standard deviations of the determined effusion rates were high. Distribution of the obtained effusion rates and their relation to the corresponding effusion rates are shown in Figure 10.

N₂ fixation activity

N input by N₂ fixation in association with the roots of the tested plants and by free-living soil N₂ fixers is presented for each assay in Table 9 and annually in Table 10.

The overestimation of the determined acetylene reduction activities when effusion is not corrected for increases exponentially with a more rapid effusion rate (Figure 10).

Discussion

Effusion rate

The negative effusion rates, an increase in the amount of pro-
Figure 9. Rates of effusion and corresponding standard deviations measured from the enclosures used for testing.
Table 9. N input by N$_2$ fixation in the four sites: the meadow, the aspen, the fir and the spruce at different times during the vegetation period. Significance level for an increases activity in the presence of plant at each performed assay. Activities corrected for effusion.

<table>
<thead>
<tr>
<th>Species</th>
<th>Date</th>
<th>Plant+soil</th>
<th>Bare soil</th>
<th>Net plant</th>
<th>Plant+soil and bare soil</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meadow</td>
<td>21 June 1981</td>
<td>421±96</td>
<td>348±42</td>
<td>73±102</td>
<td>38±77</td>
<td>0.190</td>
</tr>
<tr>
<td></td>
<td>10 July 1980</td>
<td>73±54</td>
<td>95±36</td>
<td>-22 (-311 266)</td>
<td>84±39</td>
<td>0.357</td>
</tr>
<tr>
<td></td>
<td>27 July 1980</td>
<td>306±128</td>
<td>238±275</td>
<td>69 (-444 580)</td>
<td>272±196</td>
<td>0.368</td>
</tr>
<tr>
<td></td>
<td>8 Sept 1980</td>
<td>506±184</td>
<td>415±128</td>
<td>91 (-203 385)</td>
<td>467±158</td>
<td>0.307</td>
</tr>
<tr>
<td>Aspen</td>
<td>6 July 1981</td>
<td>279±88</td>
<td>330±36</td>
<td>-50 (-162 61)</td>
<td>304±68</td>
<td>-0.183</td>
</tr>
<tr>
<td></td>
<td>28 July 1981</td>
<td>216±55</td>
<td>246±36</td>
<td>-29±25</td>
<td>231±46</td>
<td>-0.029</td>
</tr>
<tr>
<td></td>
<td>9 Aug 1980</td>
<td>742±564a</td>
<td>298±22</td>
<td>444</td>
<td>151 not enough data for evaluation</td>
<td></td>
</tr>
<tr>
<td>Fir</td>
<td>30 June 1981</td>
<td>239±150</td>
<td>178±146</td>
<td>61±86</td>
<td>209±143</td>
<td>0.092</td>
</tr>
<tr>
<td></td>
<td>9 July 1980</td>
<td>322±87</td>
<td>245±131</td>
<td>78 (-83 238)</td>
<td>292±76</td>
<td>0.147</td>
</tr>
<tr>
<td></td>
<td>16 July 1981</td>
<td>172±24</td>
<td>165±36</td>
<td>7±37</td>
<td>168±29</td>
<td>0.350</td>
</tr>
<tr>
<td></td>
<td>7 Aug 1980</td>
<td>233±39</td>
<td>195±37</td>
<td>37 (-16 91)</td>
<td>214±40</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td>10 Sept 1980</td>
<td>256±79</td>
<td>321±124</td>
<td>25 (-127 177)</td>
<td>245±95</td>
<td>0.371</td>
</tr>
<tr>
<td>Spruce</td>
<td>9 July 1980</td>
<td>239±110</td>
<td>400±23</td>
<td>-166 (-353 31)</td>
<td>304±118</td>
<td>-0.067</td>
</tr>
<tr>
<td></td>
<td>10 July 1980</td>
<td>96±46</td>
<td>178±146</td>
<td>61±86</td>
<td>209±143</td>
<td>0.092</td>
</tr>
<tr>
<td></td>
<td>28 July 1981</td>
<td>215±90</td>
<td>261±155</td>
<td>-45±202</td>
<td>239±122</td>
<td>-0.322</td>
</tr>
<tr>
<td></td>
<td>7 Aug 1980</td>
<td>165±94</td>
<td>205±94</td>
<td>-100±145</td>
<td>235±103</td>
<td>0.098</td>
</tr>
<tr>
<td></td>
<td>10 Sept 1980</td>
<td>266±10</td>
<td>235±24</td>
<td>-9 (-54 34)</td>
<td>230±18</td>
<td>0.305</td>
</tr>
</tbody>
</table>

Tested species in meadow: Antennaria microphylla
in other sites: Achillea millefolium, unless else indicated

Table 10. Annual N input by N$_2$ fixation in the four sites: the meadow, the aspen, the fir and the spruce. Activities corrected for effusion.

<table>
<thead>
<tr>
<th>Species</th>
<th>mg N fixed m$^{-2}$ y$^{-1}$</th>
<th>Plant+soil</th>
<th>Bare soil</th>
<th>Net plant</th>
<th>Plant+soil and bare soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meadow</td>
<td>35.5±13.0</td>
<td>29.4±14.2</td>
<td>6.1</td>
<td>32.7±13.8</td>
<td></td>
</tr>
<tr>
<td>Aspen</td>
<td>23.0±6.2</td>
<td>29.5±2.8</td>
<td>-6.5</td>
<td>28.7±3.0</td>
<td></td>
</tr>
<tr>
<td>Fir</td>
<td>24.1±6.7</td>
<td>20.8±8.1</td>
<td>3.6</td>
<td>22.6±7.3</td>
<td></td>
</tr>
<tr>
<td>Spruce</td>
<td>29.2±5.7</td>
<td>31.5±6.4</td>
<td>-2.2</td>
<td>29.9±6.1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 10. Relationship between overestimation of the $\text{N}_2(\text{C}_2\text{H}_2)$ fixation activity calculated without correction for the effusion and the effusion rate.

pane over time, that was determined from some enclosures are most likely an effect of the inherent variability in the method used.

In 15% of the enclosures the effusion rate was greater than 0.10 h$^{-1}$ which, according to Figure 10, gives more than 25% overestimation of the uncorrected acetylene reduction activity. For 30% of the enclosures, the effusion rates were less than 0.03 h$^{-1}$, and the overestimation of the activity, when effusion was not corrected for, less than 10%. The exponential relationship between overestimation and effusion rate makes the influence from a few enclosures with a rapid
effusion rate significant, although they are few.

The high standard deviations for the determined effusion rates is due to variations in the sampling and/or the analysis procedure. Gas chromatography has a high precision in analyses and most of the variation can most likely be traced to the sampling procedure used. When tested in the laboratory the flush-through method exchanged 91±3% of the gas volume in serum vials of the type used for sampling. Under field conditions the variation could be considerably higher.

**N\textsubscript{2} fixation activity**

The most apparent difference in acetylene reduction activity when effusion from the enclosures was corrected for was found in the meadow. Before correction for effusion the yearly input of N was determined to 54.8 mg N m\textsuperscript{-2} and after correction it was 35.5 mg N m\textsuperscript{-2}. This difference was mainly influenced by the late fall assay on the 8th of September 1980, which included the enclosure with the most rapid effusion rate obtained (Table 11). For this specific enclosure the effusion rate was 0.32 h\textsuperscript{-1} and the uncorrected acetylene reduc-

| N inputs measured in the meadow, 8 September 1980. |
|---------------------------------|--:|--:|--:|
|                                | ug N fixed m\textsuperscript{-2} d\textsuperscript{-1} | Effusion rate h\textsuperscript{-1} |
|                                | Uncorrected | Corrected |                      |
| Bare soil                      | 684         | 454       | 0.081                |
|                                | 568         | 519       | 0.017                |
|                                | 388         | 272       | 0.080                |
| Plant+soil                     | 477         | 473       | 0.002                |
|                                | 1679        | 266       | 0.328                |
|                                | 853         | 696       | 0.044                |
|                                | 667         | 591       | 0.030                |
tion activity the highest of all obtained activities. Correction for effusion gave a six times lower activity. No difference in plant development that could explain the very high activity in one of the plant containing enclosures was observed.

Correcting for effusion did not significantly affect the overall conclusion presented in Chapter II, no significant increase in acetylene reduction activity in the presence of plant. However, the low precision in the determined effusion rates could obscure an influence of plant, specially if the increase is small.

When the effusion rate is correctly determined the corrected acetylene reduction activity accurately gives the true activity from the investigated system. If the true effusion rate deviates ±1 sd from the determined rate then the actual acetylene reduction activity is higher or lower than the activity corrected for the determined effusion rate (Table 12). For each individual enclosure the difference in corrected activity does not considerably differ although the error in the determined effusion rate is is ±1 sd. However, the difference in activity between two enclosures can be vastly changed if the true effusion rate differs from the determined rate with ±1 sd. Table 13 shows that corrected for the determined effusion rate the difference in acetylene reduction activity between the enclosures is 1 unit, but if the true effusion rate differs ±1 sd from the determined rate the true difference can be several times more or less.

The above example shows that in order to detect a 10% increase in the acetylene reduction activity the error introduced by a low precision in the effusion rate must be considerably decreased. As the
### Table 12. Error in corrected acetylene reduction activity if the true effusion rate deviates ±1sd from the determined effusion rate.

<table>
<thead>
<tr>
<th>Effusion rate (h⁻¹)</th>
<th>Acetylene reduction activity Uncorrected</th>
<th>Corrected for effusion rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x±sd^a</td>
<td>x±sd</td>
</tr>
<tr>
<td>0.000</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>0.020±0.013</td>
<td>10.3</td>
<td>9.6</td>
</tr>
<tr>
<td>0.040±0.017</td>
<td>10.8</td>
<td>9.4</td>
</tr>
<tr>
<td>0.060±0.019</td>
<td>11.5</td>
<td>9.2</td>
</tr>
<tr>
<td>0.080±0.021</td>
<td>12.4</td>
<td>9.0</td>
</tr>
<tr>
<td>0.100±0.023</td>
<td>13.5</td>
<td>8.8</td>
</tr>
<tr>
<td>0.120±0.024</td>
<td>14.8</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.020±0.013</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>0.040±0.017</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>0.060±0.019</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>0.080±0.021</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td>0.100±0.023</td>
<td>14.8</td>
</tr>
<tr>
<td></td>
<td>0.120±0.024</td>
<td>16.3</td>
</tr>
</tbody>
</table>

^aStandard deviations obtained from Figure 9.
^bEffusion rates as commonly obtained, Figure 9.

### Table 13. Interval for the difference in acetylene reduction activity between two enclosures if their true effusion rates deviates ±1sd from the determined effusion rates.

<table>
<thead>
<tr>
<th>Difference in activity #1-#2</th>
<th>Effusion from enclosure #1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>0.000</td>
<td>1</td>
</tr>
<tr>
<td>0.020</td>
<td>0.8-1.8</td>
</tr>
<tr>
<td>0.040</td>
<td>0.6-1.6</td>
</tr>
<tr>
<td>0.060</td>
<td>0.8-1.8</td>
</tr>
<tr>
<td>0.080</td>
<td>0.2-2.0</td>
</tr>
<tr>
<td>0.100</td>
<td>0.0-2.2</td>
</tr>
<tr>
<td>0.120</td>
<td>-0.2-2.4</td>
</tr>
</tbody>
</table>
values in the Tables 14 and 15 are representative for the effusion rates determined from the enclosures used for the in situ investigation of acetylen reduction activity, it is evident that a small increase in acetylene reduction activity could be obscured by the wide standard deviation for the determined effusion rates.

**Improvement in data**

In order to detect a small difference between enclosures with and without plants, it is necessary to increase the precision in the effusion rates determined from the enclosures. The low precision can most likely be traced to the sampling procedure rather than to the gas chromatographic analyses.

The time required for sample analysis limits the number of samples which can be collected at one testing. Replicate analyses are, of course, preferable but as the precision in the effusion rate is of great importance and most of the variation originates from the sampling procedure, it might in this case be more useful to analyze replicate samples as compared to replicate analyses of the same sample.

**Summary**

When acetylene reduction assays are performed in enclosures from which effusion occurs, the effusion of ethylene must be corrected for in order to determine the true acetylene reduction activity from the enclosed material. The required correction increases exponentially with a more rapid effusion rate, and in order to determine small activities a high precision is required for the effusion rate. For the
performed acetylene reduction assays the standard deviations for the
determined effusion rates were too wide to determine a 10% increase
in acetylene reduction activity in the presence of plant. It is pos-
sible that the obtained result, that the plants did not significantly
increase the acetylene reduction activity, is an effect of the effu-
sion rates rather than a reflexion of the actual condition.

The result of the study is that the tested hypotheses could not
be certified but neither falsified, and that further studies must be
designed to meet the requirement for correction of effusion.
CHAPTER V

SUMMARY

In situ assays of N₂ fixation activity, using the acetylene reduction technique, were performed, with emphasis on rhizosphere activity, in four successional stages, meadow, aspen, fir and spruce, of a Northern Wasatch Mountain subalpine forest ecosystem, elevation 2,800 m. The vegetation period was approximately 100 days, from late June to early October, and during the summer, the precipitation was minimal. Plant species tested for associative N₂ fixation were Antennaria microphylla and Achillea millefolium.

Assays were performed in Saran bags. Acetylene was generated from CaC₂ and a defined amount of propane was injected at the time for initiation. Samples were collected from the air phase of the enclosure and analyzed for ethylene and propane using gas chromatography. Data were evaluated according to Balandreau and Dommergues (1973) and the annual input of N determined by stepwise linear integration of activities obtained at different testings over the vegetation period.

Input of N by soil free-living N₂ fixers was in the meadow, the aspen, the fir and the spruce 0.48±0.24, 0.33±0.05, 0.24±0.08 and 0.33±0.10 kg N ha⁻¹ y⁻¹, respectively, endogenous ethylene production not being accounted for. The higher input in the meadow was mainly due to a higher acetylene reduction activity in the fall. Any seasonal fluctuation in the N₂ fixation activity was not evident in the
other sites.

A higher acetylene reduction activity in the presence of plant, as compared to the soil activity, was measured in 10 of a total of 16 performed assays; however, only at three testings was the increase in activity statistically significant, α<0.10. An input of N by N\textsubscript{2} fixation in association with the rhizosphere of \textit{A. microphylla} and \textit{A. millefolium} could not be significantly determined. Most assays showed an increase in acetylene reduction activity in the presence of plants which might indicate a contribution to N input by rhizosphere associated N\textsubscript{2} fixation, but to an extent lower than expected and not significantly detectable with the method used.

The method used for calculation of the acetylene reduction activity is valid for assay devices from which no leakage of gases occurs. However, for most of the enclosures used for testing leakage of gases did occur and therefore, the accuracy in the determined activities low.

Correction for effusion can be made for assays where the test material is completely enclosed and the size of orifices present in the enclosure determines the effusion rate. The force driving effusion is the concentration gradient over the enclosure. For a closed system, the outside concentration of gases concern for acetylene reduction assays can be assumed 0 mole l\textsuperscript{-1} at ant time and consequently, the effusion rate directly proportional to the inside concentration. The relationship between effusion rates of different gases is given by Graham's law of effusion, for which the closed system meets the necessary requirements.
A gas injected into the air phase of the enclosure is quickly evenly distributed and the concentration of gases present in the enclosure is the same throughout the entire volume. The enclosed volume can then be determined if a known amount of gas is injected and its concentration at the time for injection can be determined.

At any time during the assay the ethylene production rate is equal to the sum of the observed increase in ethylene over time and the rate by which ethylene effuses.

If a known amount of propane is injected into the enclosure for the acetylene reduction assay the acetylene reduction activity can be calculated from the ratio of ethylene to propane determined for the collected samples. Corrected for the effusion of ethylene the acetylene reduction activity is

\[ ARA = \frac{k'}{P_0} PP; \quad \frac{k'}{P_0} \text{ is the slope of } \frac{E}{P} e^{-0.25 r_P t} \text{ vs. } e^{-1.25 r_P t} - 1 \]

and \( r_P \) determined according to

\[ P = P_0 e^{r_P t} \]

where ARA is the acetylene reduction activity (mole h\(^{-1}\)),
E is the ethylene concentration at t h (mole ml\(^{-1}\)),
k is the ethylene production rate (mole ml\(^{-1}\) h\(^{-1}\)),
P is the propane concentration at t h (mole ml\(^{-1}\)),
P\(_0\) is the propane concentration at 0 h (mole ml\(^{-1}\)),
PP is the amount of propane injected at 0 h (mole),
r\(_p\) is the proportionality factor for propane (h\(^{-1}\)),
t is the time from initiation of the assay to t h (h).

The difference between the above equation and the equation by Balandreau and Dommergues (1973), that do not provide correction for effusion, is that \( k/P_0 \) according to them is the slope of \( E/P \) vs.
time. When effusion occurs, \( r_p < 0 \), the increase in E/P is more rapid with time and consequently, the acetylene reduction activity is overestimated when effusion occurs and is not corrected for. The error increases exponentially with a more rapid effusion rate.

For evaluation of data according to Balandreau and Dommergues (1973) the information obtained from the samples is the ratio of the ethylene concentration to the propane concentration which is independent of the sample volume. Determination of the effusion rate is based on the actual concentration of propane over time and the sample volume is then of importance. Data collected for evaluation according to Balandreau and Dommergues (1973) do not necessarily meet the requirement for correction of the effusion rate.

The reported study of \( \text{N}_2(\text{C}_2\text{H}_2) \) fixation activity was both designed and performed for evaluation according to Balandreau and Dommergues (1973) and no emphasis was made to assure a constant sample volume. The effusion rate could therefore not be determined with the accuracy required for a reliable correction of the acetylene reduction activities. For an error of \( \pm 1 \) standard deviation in the effusion rate, the difference in activity between a plant containing enclosure and an enclosure without plant commonly varied \( \pm 100\% \). This could possibly be one reason why no significant influence was determined in the presence of plant. The ultimate conclusion is that the tested hypotheses could not be verified but neither falsified.

Acetylene reduction assays are often performed in devices open to the soil, as the disturbance imposed on the test material then is minimal. Evaluation of such assays is possible only under very speci-
fic presumptions that are difficult to provide. Most difficult is to adjust the amount of propane injected into the device so that the diffusion rate for the propane is equal to the diffusion rate for the ethylene produced. If this can be provided for the acetylene reduction activity can be calculated for a time interval where the diffusion of propane is insignificant as compared to the propane diffused from the initiation of the assay to the beginning of the interval. It then follows that

$$ARA = PP \frac{d(E/P)}{dt} \frac{P_i}{P_0}$$

where ARA is the acetylene reduction activity (mole h\(^{-1}\)), PP is the amount of propane injected at 0 h (mole), \(d(E/P)/dt\) is the rate by which the ratio E/P increases during the interval (mole ethylene mole propane\(^{-1}\) h\(^{-1}\)), \(P_i\) is the propane concentration during the interval (mole ml\(^{-1}\)), \(P_0\) is the propane concentration at 0 h (mole ml\(^{-1}\)).

Comparison between the above equation and that by Balandreau and Dommergues it follows that without considering diffusion the acetylene reduction activity is overestimated by a factor \(P_0/P_i\). The initial very rapid diffusion from the air phase makes \(P_0\) considerably greater than \(P_i\). Thus the acetylene reduction activity is vastly overestimated when diffusion is not corrected for.
REFERENCES


calorimetric determination for ethylene. Plant Physiol. 51:1074-
1075.

The biology of nitrogen fixation. North Holland Publ. Co., Ams-
tterdam.

Ljones, T. and R.H. Burris. 1978. Evidence for one-electron transfer
by the iron protein of nitrogenase. Biochem. Biophys. Res. Com-
mun. 80:22-25.

Low. D.J. and G.J. White. 1976. Non-symbiotic N₂ fixation in the rhi-

Lowe, D.J., B.E. Smith and R.R. Eady. 1980. The structure and mecha-
nism of nitrogenase, p.34-87. In N.S. Subba Rao (Ed.) Recent ad-
vances in biological nitrogen fixation. Holmes & Meier Publ.

Masterson, C.I. and P.M. Murphy. 1980. The acetylene reduction tech-
nique, p.8-83. In N.S. Subba Rao (Ed.) Recent advances in bio-

Minchin, F.R. and J.S. Pate. 1974. Diurnal functioning of the legume

Neal, J.L. and R.I. Larson. 1976. Acetylene reduction by bacteria i-
solated from the rhizosphere of wheat. Soil Biol. Biochem. 8:
151-155.

270.

of Spirillum lipoferum at constant partial pressures of oxygen,
and the properties of its nitrogenase in cell-free extracts. J.

OId, K.M. and T.H. Nicolson. 1975. Electron microscopy of the microb-
ial colonization of roots of sand dune grasses. p.208-215. In
G. Kilbertus, O. Resinger, A. Mourey and J.A. Cancela da Fonseca
(Eds.) Biodegradation et Humification. Pierron, Sarrequeimenes,
France.

reduction) in a Nova Scotian salt marsh: its association with
4:227-244.


VITA

Inger Börjesson

Candidate for the Degree of

Doctor of Philosophy

Dissertation: Rhizosphere N₂ Fixation in a Forest Ecosystem.
   In situ Assays and Evaluation of the Acetylene Reduction Technique.

Major field: Biology (Microbiology)

Biographical Information:

   Swedish citizen.

   Education: Graduated from:
   1968 Falu Högre Almänna Läroverk, Falun. (High school)
   1971 Lärarhögskolan, Falun. (Teachers College)
   1975 Umeå University, Umeå. Fil. Kand. (BS) in mathematics and chemistry

   Continued studies:
   Umeå University, Umeå. biology and microbiology
   Swedish University of Agricultural Sciences, Uppsala. microbiology

   Professional Experience:
   Teaching:
   fall 1980 Utah State University, Logan. Teaching Assistance, soil microbiology.

   Research:
   1978-1980 Swedish University of Agricultural Sciences Uppsala. Forsknings assistant (Research assistant)