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CANADIAN THESES ON MICROFICHE / THÈSES CANADIENNES SUR MICROFICHE

NAME OF AUTHOR/*NOM DE L'AUTEUR* James Douglas Salahub

TITLE OF THESIS/*TITRE DE LA THÈSE* The Effect of Crude Oil On Anaerobic Nitrogen Fixation In a Muskeg Ecosystem

UNIVERSITY/*UNIVERSITÉ* University of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED/*GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* Master of Science

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1977

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* J. N. Campbell

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THE EFFECT OF CRUDE OIL ON ANAEROBIC NITROGEN FIXATION IN A MUSKEG ECOSYSTEM

by

J.D. SALAHUB

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

FALL, 1977

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

THE EFFECT OF CRUDE OIL ON ANAEROBIC NITROGEN FIXATION IN A MUSKEG ECOSYSTEM

submitted by ..... J. D. SALAHUB

in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

.....
Supervisor

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Date Oct 5, 1977

DEDICATION

To my many friends who made it possible by giving their encouragement.

To my parents who saw me through all the discouraging times, and especially to my wife, Kathleen A.J. Nelson, for simply being Katy.

ABSTRACT

An investigation was conducted into the effects of an accidental oil spill on the anaerobic microflora and their associated N_2-fixation in a northern Alberta muskeg. The investigation was divided into three sections.

1. Taxonomic Studies. An increase in the number of anaerobes classified as clostridial type in the oil impregnated soil was observed, as previously reported. Of these isolates, about 17% (13/77) were tentatively identified as Clostridium perfringens and an additional 10% (8/77) were shown to produce at least one clostridial exotoxin.

2. N_2-fixation by pure cultures of Clostridium pasteurianum W5. Optimal conditions for C_2H_2 reduction by whole cell cultures included incubation at 37°C, pH 7.2, C_2H_2 concentration 0.1 Atm. and a gas:liquid ratio of 10:1. Under these conditions, C_2H_2 reduction was linear with time for at least four hours. Only actively growing cultures reduced C_2H_2. The amount of nitrogenase (C_2H_2) activity present was proportional to cell concentration. It was also demonstrated that crude oil in concentrations up to 16% (v/v) had very little effect on the C_2H_2-reducing ability of actively growing cultures.

3. N_2-fixation by mixed cultures in oil spill and muskeg soils. Temperatures in the muskeg ranged from 7°C to 16°C and the pH of muskeg samples was as low as 3.7. The oil

spill tended to raise the pH of samples slightly. Although these conditions differ greatly from the optimum conditions for C. pasteurianum W5 nitrogenase, they were duplicated as closely as possible in the laboratory. In situ C_2H_2-reduction assays indicated that the oil spill was more active (4.9 n mole/h/g) than control sites (0.6 n mole/h/g) on the average. Laboratory experiments however showed the reverse trend, with average values of 0.5 n mole/h/g in spill samples and 1.0 n mole/h/g for control samples. The net long term effect of the oil spill on anaerobic N_2-fixation appears to be negligible.

Under the conditions of this study, it was found that the use of activated steel wool to obtain anaerobic conditions was incompatible with the C_2H_2-reduction assay, as the steel wool alone reduced C_2H_2 abiologically in significant quantities.

ACKNOWLEDGEMENTS

I am grateful to Dr. J.N. Campbell for guiding and stimulating this research and for his friendship and moral support during many years of graduate and undergraduate study.

I would also like to thank Drs. F.D.Cook and D.W.S.Westlake for their valuable advice and encouragement, Roger Phillippe and Bob Salahub for technical assistance and Phil Fedorak for his advice and assistance with gas chromatography and computer programing.

I gratefully acknowledge the financial support of the people of Canada (Arctic Land Uses Research Program, Department of Indian Affairs and Northern Development and National Research Council of Canada, Operating grants A-1743 (Campbell) and A-3687 (Westlake)) and of the University of Alberta, without which this work would not have been possible.

To all the members of the Department of Microbiology I extend my thanks for their many suggestions and encouragements throughout my graduate program.

In addition, special thanks are extended to Ms. Rita Radvony for an excellent job of drafting the figures for this thesis.

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INTRODUCTION

In this era of fuel shortages and energy crises, the transportation of northern crude oils by pipeline through regions of tundra and muskeg has become a virtual certainty. Along with this is the certainty that some of this oil will be spilled onto the tundra and muskeg, resulting in clean-up problems of immense proportions. Once physical means of picking up the oil have been exhausted, it becomes the job of the hydrocarbonoclastic bacteria and fungi to convert the oil into harmless substances and to allow the regrowth of plants. Fortunately, the ability to degrade at least some fraction of oil is found within several genera and these genera are widely dispersed in nature[80,99].

The microbiological study of the effect of oil spills falls into two major divisions. The first is the microbial biodegradation of the crude or refined hydrocarbons. Equally as important is the study of the effects of the hydrocarbons on the normal activities of the microbial flora of the ecosystem[2], such as the decomposition of protein, cellulose, lipids and chitin[117,124] and other organic carbon compounds, the transformation of nitrate, nitrite and ammonia, and the fixation of atmospheric [illegible] and nitrogen. The current work will concentrate on N_2-fixation in a situation where hydrocarbon biodegradation is known to be occurring[129].

I. Biodegradation of Hydrocarbons

The microbial biodegradation of crude and refined hydrocarbons has been under study for several decades both from the point of view of producing protein or other economically important metabolic products[1] and of trying to speed up the clean-up of accidentally spilled petroleum. Dissolved aromatic hydrocarbons were found to be toxic to non-hydrocarbonoclastic bacteria of the genera Serratia and Vibrio [16] and crude and fuel oils were toxic to many types of bacteria[124,5]. Griffin and Calder[45] have hypothesized that this toxic effect is bacteriostatic, caused by a membrane effect, rather than bacteriocidal in nature, and have demonstrated its reversability by addition of organic nutrients. Some aromatics, such as naphthalene[73], benzoate and methylbenzoates[82], catechol, phenol, and ortho, para and meta cresol[95] were however readily broken down and used as a carbon and energy source by some species of Pseudomonas, Achromobacter and Nocardia [6,67,73,81]. The pathways, intermediates[41,107] and regulatory mechanisms [49,82,95,112] have been investigated for aerobic biodegradation of aromatic compounds, and straight and branched chain alkanes. This breakdown is primarily via an oxygenase-dependent attack. Anaerobic breakdown of aromatic hydrocarbons [109,112] occurs by an unknown mechanism possibly involving the anaerobic insertion of oxygen. Attack on aliphatic hydrocarbons [19,112] may occur by way of a dehydrogenase with nitrate as the terminal electron acceptor in species of

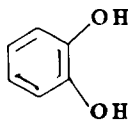
Pseudomonas, but evidence for a similar dehydrogenation of hydrocarbons by sulfate reducing anaerobes has so far been inconclusive and contradictory [55,112] except for the very short chain n-alkanes, CH_4, C_2H_6, and possibly n-octadecane [26]. The reactions by which hydrocarbons are degraded biologically are summarized as follows;

A. Aerobic.

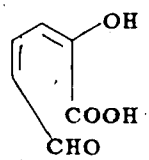
Aliphatics.

$CH_3(CH_2)_{14}CH_3$ + O_2 ----------> $CH_3(CH_2)_{14}COOH$
n-hexadecane Oxygenase n-hexadecanoic acid

Aromatics.



+ O_2 ----------> Oxygenase



Catechol

2-hydroxy muconic semialdehyde

B. Anaerobic.

$CH_3(CH_2)_5CH_3$ + X ----------> $CH_3(CH_2)_4CH=CH_2$ + XH_2
n-heptane dehydrogenase n-hept-1-ene

When crude oils are attacked by bacteria, the medium chain n-alkanes, nC_{16}-nC_{37}, are the first to be degraded followed by the aromatic fraction [56]. Generally the susceptibility of an oil to bacterial attack was related to the quality of the oil [20,21,55,117,123,130] A high quality oil contains a low weight percentage of sulphur, has a low density and a high saturates content [55]. Initially at least, there is an increase in the quantity of long chain n-alkanes and asphaltenes during bacterial attack [119,120,121,122], at least part of which appears to be freshly synthesised

material.

In as much as oil is very high in carbon but low in nitrogen, it is easy to appreciate that biodegradation of oil and recovery of soil after oil spills is considerably advanced by the application of fertilizers containing available nitrogen and phosphorus, such as urea phosphate [57, 25, 129]. Any N_2 fixed by microorganisms in the vicinity of the spill will decrease the amount of fertilizer that would otherwise have to be added. It is therefor of interest to discover the extent of N_2-fixation in the ecosystem and the effect of the oil spill upon it.

II. Nitrogen Fixation

Since the earliest recorded speculations by Davey in 1813 that legumes obtained "Azote" from the air, to the first experimental demonstration of bacterial N_2-fixation by Jodin in 1862, and the proof of symbiotic fixation in legumes by Hellriegel and Wilfarth in 1888 as cited in Burns and Hardy [15], and Quispel [91], the emphasis in the study has been on the legume-*Rhizobium* symbiosis. However, since the subject has been extensivly reviewed elsewhere [2, 15, 36, 74, 86, 92, 114, 132] and since legumes are not a significant part of the muskeg flora, they will not be discussed further in this thesis.

Non-symbiotic N_2-fixing organisms can be divided into four groups, the aerobic photosynthetic blue-green bacteria, the anaerobic photosynthetic bacteria, and aerobic- and anaerobic- non-photosynthetic bacteria. The photosynthetic

N_2-fixing bacteria [104] include species of the Thiorhodaceae and Athiorhodaceae and possibly the Chlorobacteriaceae. These bacteria fix N_2 only under reducing conditions and are probably of significance only in deep meromictic lakes. The Cyanophyceae or "blue-green algae" fix significant amounts of N_2 under aerobic or microaerobic conditions [104]. Heterocystous filamentous genera such as Anabaena, Nostoc and Tolypothrix make major contributions to the amount of N_2-fixation in muskeg [10,43], and tundra soils [3,4,44] as well as many other ecosystems [58,59,64]. Certain strains of the Cyanophyceae are able to form symbiotic associations with higher plants [11]. In contrast to the legume-Rhizobium symbiosis, the bacteria are not taken inside the plant cells in nodules, although special receptacles may develop to hold the bacteria and allow greater surface contact [94,106]. This association may also be very simple, involving merely an adherence of the bacteria to the plant roots, as has been shown to occur in muskeg, between blue green bacteria and the moss Sphagnum [10], or slightly more intimate with the bacteria being epiphytically or intracellularly associated, as is the case between Nostoc and Sphagnum lindbergii [43]. In any event, the bacteria do not change form, and are capable of N_2-fixation in the non-associative state under environmental conditions.

Aerobic, non-symbiotic and non-photosynthetic N_2-fixation is the province of the Azotobacteraceae [2,13,79] with some contribution from Mycobacterium [24,133], some of

the Methylomonadaceae [103,133], and at least one Spirillum [84,85,133]. Nitrogen fixation by Pseudomonas sp. has occasionally been reported, but these reports are discounted by Mulder and Brotonegoro [79] as being due to incorrect classification of bacteria belonging to other genera.

There are also some bacteria that grow aerobically but fix N_2 only under anaerobic conditions. Foremost amongst these are the N_2-fixing strains of Klebsiella [133], but also included are some strains of Bacillus [15,50] and the N_2-fixing Escherichia coli hybrid developed by Dixon and Postgate [32], which contains the nif gene from Klebsiella pneumoniae.

Anaerobic fixation is carried out by Desulfovibrio, Desulfotomaculum [15,133] and by four species of Clostridium; C. butyricum, C. biejerinckii, C. acetobutylicum and C. pasteurianum [13].

Since muskegs are primarily anaerobic environments, the anaerobic and facultatively anaerobic bacteria can be expected to predominate except at the surface. Aerobes, and in particular the Cyanophyceae account for a major portion of the N_2 fixed by the surface layers of a muskeg [10]. Although Christensen [18] found no organisms in Alberta muskegs capable of fixing N_2 under aerobic conditions using nitrogen free mannitol medium, several types of mire from Scotland were shown to be able to reduce C_2H_2 in the presence of oxygen [128]. In an oil spill situation, however, the blue-green bacteria and other strict aerobes would be

largely killed off by the oil [63] and would reappear only slowly.

III. Nitrogenase

The structure and function of the enzyme system referred to as nitrogenase, has been the subject of several recent reviews [11,15,68,104,108,133]. It will therefor be dealt with only briefly.

Nitrogenases from the various diazotrophs each exhibit unique properties, but most of them are surprisingly similar. The differences tend to be related to how the enzyme is adapted to the unique physiology and metabolism of the producing organism, while the similarities are related to the enzyme function [15]. The enzyme system known as nitrogenase mediates the overall reaction;

$$N_2 + 3H_2 \longrightarrow 2NH_3 .$$

It consists of two non-haem, iron-sulphur proteins [11,133]. Component I has a molecular weight of 220,000 and contains four subunits of two types, two of 50,000 M.W. and two of 60,000 M.W., along with twenty to thirty Fe^{++} ions and acid labile sulphur groups and two Mo^{++} ions. The Mo^{++} and some of the Fe^{++} are contained on a small co-factor [11] and the Mo[illegible] to be involved at the active site of the enzyme. [illegible]ent II weighs 55,000 to 60,000 daltons and is compo[illegible] subunits of equal weight, with four Fe^{++} and acid lab[illegible]ar groups per molecule [133]. Both components as well as [illegible] enzyme are extremely sensitive to oxygen [63] [illegible] wh[illegible]er the source bacteria are

aerobes or anaerobes. This caused considerable difficulty during early attempts to isolate the enzyme [17]. For example, Component I from K. pneumoniae loses 60% of its activity in ten minutes at 0.2 atmospheres of O_2 and Component II loses 70% of its activity after only one minute at the same pO_2 [34]. Component II from most systems is cold labile and must be stored at room temperature. This is especially important with cell free extracts and purified enzyme preparations [68,76,77].

Both components are required for the reduction of N_2 to NH_3. Generally speaking what happens is illustrated in Figure 1. Component II is reduced by passage of electrons from ferredoxin or another suitable reducing agent [1]. The reduced Component II then binds ATP and undergoes a conformational change [2] that results in a lowering of the Eh from -280 mV without ATP to -400 mV with ATP [133]. At the same time, Component I binds molecular nitrogen [3] at a Mo^{++} ion in a configuration that weakens the interatomic bonds. The two components then come together [4] in a complex and in an energy requiring process, electrons are passed from Component II to Component I [5]. In a second energy requiring step [6] the electrons are passed from Component I to N_2. Ammonia and orthophosphate are then released and the two components separate [7]. Six electrons are required for the reduction of one molecule of N_2 to NH_3. Current theory proposes a step-wise reduction with a diimide, HN=NH, as an intermediate, but if any intermediate

FIGURE 1

THE SEQUENCE OF EVENTS IN NITROGENASE ACTIVITY

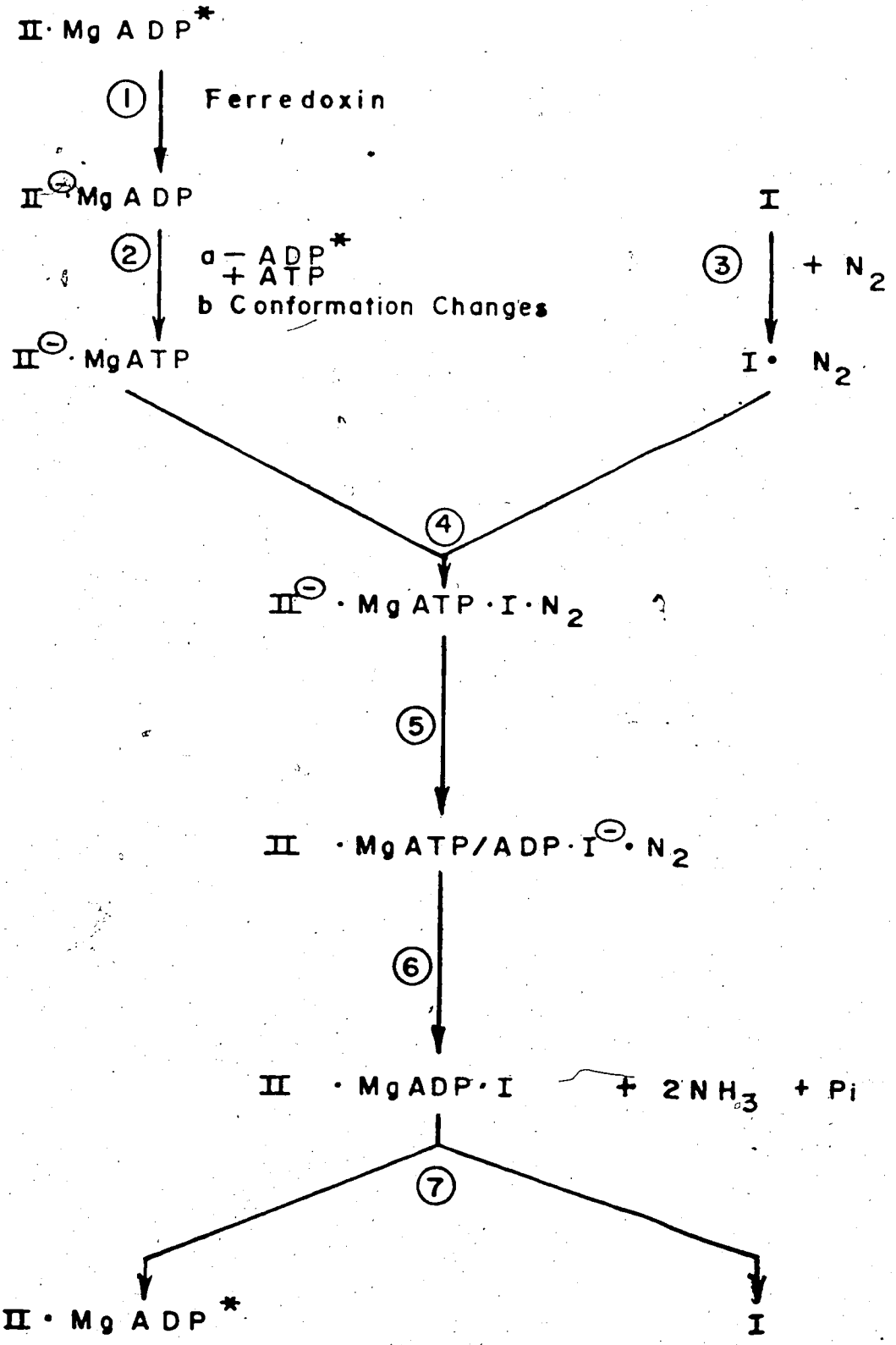
I = Component I, the complete Mo-Fe-protein.

II = Component II, the Fe-protein.

1. Reduction by ferredoxin, flavodoxin, SO_2^-, hydroquinone or viologen dyes.
2. a. ADP replaced by ATP.
   b. Conformational change.
3. Binding of molecular nitrogen.
4. Components I and II combine.
5. Protein-Protein electron transfer.
6. Protein-substrate electron transfer and release of product.
7. Separation of components.

* The point of ADP release is uncertain.

Adapted from Yates, 1976.


II · Mg A D P*
1
Ferredoxin
II⊖ Mg A D P
2
a — ADP*
+ ATP
b Conformation Changes
II⊖ · MgATP
I
3
+ N2
I · N2
4
II⊖ · MgATP · I · N2
5
II · MgATP/ADP · I⊖ · N2
6
II · MgADP · I
+ 2NH3 + Pi
7
II · Mg A D P*
I

is formed it is not released from the active site.

One of the points on which the various systems differ is in the source of reducing power. In saccharolytic clostridia and probably other anaerobes, reducing power comes from the pyruvate phosphoroclastic system [15], with ferredoxin as the carrier [68], passing electrons to Component II two at a time. In cases of Fe limitation, flavodoxin can substitute for ferredoxin in vitro at least. In Azotobacter and other aerobes the electron source is probably $NADPH_2$ [8,40] and the electron carrier can be either an iron-sulphur protein (Azotobacter ferredoxin) or a flavoprotein (azotoflavin) [68].

The reduction of atmospheric nitrogen is an energy-consuming reaction. Energy is required to generate reducing power and further energy is required for the enzymatic reduction of N_2. This energy is supplied in the form of ATP [34]. The additional requirement for Mg^{++} suggests that the active form of ATP is the magnesium:ATP chelate. The source of ATP in saccharolytic clostridia is the pyruvate phosphoroclastic system and in Azotobacter it is oxidative phosphorylation. Both components of nitrogenase are required for ATP hydrolysis but the hydrolytic site appears to be on Component II [111]. The enzyme complex will hydrolize ATP in the absence of both a substrate and an electron source at a slow rate [54]. In the presence of a reductant the hydrolysis of ATP results in the passage of electrons to protons at normal rates and the evolution of

molecular hydrogen at a ratio of 2ATP per H_2 [54]. The reductant is not consumed and H_2 is not evolved in the absence of ATP. Even in the presence of N_2, some H_2 is still evolved. For every two electrons passed through the nitrogenase system *in vitro*, anywhere from two to twenty molecules of ATP are hydrolized to ADP and orthophosphate, however the ratio is usually between 4 and 5 ATP/$2e^-$ [68]. This is affected by temperature, pH, and the ratio of Component I to Component II. Yates [133] notes that 15 ATP are required to completely reduce one N_2 molecule *in vitro*, but speculates that more might be required *in vivo*.

Nitrogen reduction by nitrogenase is competitively inhibited by H_2 and CO [69]. All functions, ATP hydrolysis, electron transfer and substrate reduction are inhibited by ADP which may indicate that ADP has a regulatory role [78]. In addition, nitrogenase is competitively inhibited by a whole series of alternate substrates. The enzyme, it seems, is promiscuous and will recognize and reduce almost any triple bond, and specifically those shown in Table 1. Of these, the reduction of C_2H_2 to C_2H_4 has proven to be the most usefull.

IV. THE ACETYLENE REDUCTION ASSAY

Dilworth [31] and Schöllhorn and Burris [97], working independently, discovered that C_2H_2 was a competitive inhibitor of N_2-fixation. They reasoned that since C_2H_2 is the closest analogous compound in the carbon series to N_2, being both isoelectronic and isosteric to N_2, it might function as an inhibitor and aid in the study of the mode of

TABLE 1

REDUCTIONS CATALYZED BY NITROGENASE

Reactants	Products
1. $N_2 + 6H^+ + 6e$	$2NH_3$
2. $CH_3N{\equiv}C + 6H^+ + 6e^-$	$CH_3NH_2 + CH_4$ ($C_2H_4 + C_2H_6$)
3. $HCN + 6H^+ + 6e^-$	$CH_4 + NH_3$
4. $HCN + 4H^+ + 4e^-$	CH_3NH_2
5. $HN_3 + 2H^+ + 2e^-$	$N_2 + NH_3$
6. $N_2O + 2H^+ + 2e^-$	$N_2 + H_2O$
7. $C_2H_2 + 2H^+ + 2e^-$	C_2H_4
8. $2H^+ + 2e^-$	H_2

Adapted From: Yates, 1976.

action of the assay. Dilworth used manometry to measure the inhibition and infra-red spectroscopy to show the conversion of C_2H_2 to C_2H_4 by the cis addition of two protons. Schöllhorn and Burris [98] used mass spectrometry and gas chromatography to detect and quantitate C_2H_4 production, but it was left to Stewart, Fitzgerald and Burris [105] to point out the usefulness of this reaction as an indirect assay for nitrogenase activity. Within two years of its discovery, the use of C_2H_2 reduction as an assay had been proven and extended to almost every conceivable situation [48,105].

In brief, a sample to be assayed is placed in an airtight container and the atmosphere is replaced by one containing C_2H_2. The sample is incubated under the desired conditions for a period of time then the reaction is stopped and a sample of the gas phase is analysed by gas liquid chromatography where the C_2H_2 and C_2H_4 are separated and

measured.

The source of nitrogenase may be anything from a purified enzyme [14,25,132], to a whole cell culture [23], detatched legume nodules [71,102], entire plants [65], grass roots [27], rice roots [134,135], Mangrove leaves [42], wood [100], soil [46,110,115], rheotrophic peat [127], and rumen contents [62].

Nitrogenase tolerates from 1% [65] to 20% [9] C_2H_2 during the assay, with 10% [132] being most frequently used. Oxygen is either ridgidly excluded or added in amounts up to 40% [35], and CO_2 is sometimes added in amounts up to 5% [7,100,102]. The balance of the support gas may be air, N_2, He or Ar. Incubation time varies from minutes [65] to days [127] and the role of other parameters such as water content, temperature and illumination [60,61,113] depend upon the system being assayed.

The most frequent means of terminating the assay is removal of a sample of the gas phase [48] which is stable and may be stored for analysis in gas tight containers [96,126] for several weeks. However this is not always convenient and in such cases the reaction has been stopped by injection of 50% trichloroacetic acid (TCA) [105] or 6N H_2SO_4 [48] and storage of the gas in the incubation chamber. For work with pure cultures in liquid this method is adequate, but in samples containing large amounts of solid matter such as nodules or soil, slow penetration of the acid into the sample can result in error [47].

Gas chromatographic analysis has been done with several types of columns [47]. One of the most popular is Porapak R [105], which is typically used as the packing material in a 6mm by 2m column and run at an oven temperature of 55°C with N_2 carrier gas at a flow rate of 60 cc/min. The injector port and detector temperatures are 80°C and 65°C respectively [93]. Phenyl isocyanate/Porasil C is preferred for studies of aquatic systems because it resolves CH_4 which is produced in these systems and can interfere with measurement of C_2H_4 [37]. Detection is by hydrogen-flame ionization, and quantitation can be by measuring peak height [9] or area by manual methods or electronic integration. Acetylene can be used as an internal standard [48], but the use of high purity C_2H_4 as an external standard is much more usual [14]. Hanson [46] used C_2H_6 as an internal standard which can be adjusted to the range of the expected C_2H_4 concentration.

Since six electrons are required to reduce one molecule of N_2 to $2NH_3$ and only two electrons are required to reduce one molecule of C_2H_2 to C_2H_4, a molar ratio for C_2H_4:N_2 of 3:1 could in theory be used to convert C_2H_2 reduction data into N_2-fixation data. In practice, a ratio of from 3:1 to 4.5:1 was found for Azotobacter [48].

The C_2H_2 reduction assay has several advantages over the earlier methods of measuring nitrogenase activity [48], but recently certain limitations have been revealed.

A particular case that bears on this research is that

of the interaction of alkane-producing and-metabolizing bacteria with C_2H_4 and C_2H_2. Both C_2H_2 and C_2H_4 inhibit CH_4 production in marine sediments [88] and these same sediments are believed to reduce C_2H_2 to C_2H_4 by a mechanism other than the nitrogenase system. If the methanogenic bacteria involved are capable of N_2-fixation, a point that has yet to be decided, the inhibition of CH_4 production might also indicate an inhibition of nitrogenase. Systems containing methanogenic bacteria must therefor be handled with discretion as they may cause either an over or an under-estimation of nitrogenase activity.

Nitrogen-fixing bacteria which grow with CH_4 as the sole carbon source fail to produce C_2H_4 when exposed to C_2H_2 [29]. They would not grow on either C_2H_2 or C_2H_4 alone. When growing with CH_4 as a carbon source, they co-oxidize $^{14}C_2H_4$ to water soluble non-volatile products, possibly including ethylene glycol [38]. It was later shown that although these bacteria can co-oxidize many of the lower alkanes [30] including C_2H_6, C_3H_8, C_4H_{10}, and C_6H_{14}, as well as C_2H_4 and CO, they are totally inhibited by the presence of C_2H_2 and their only likely effect on the C_2H_2 reduction assay is that the N_2-fixation for which they are responsible would not be measured.

Bacteria have been isolated which will grow on C_2H_4 as a sole source of carbon, but C_2H_2 completely inhibits these organisms. Bacteria were also found which would grow at the expense of C_2H_2 [28] but these are not N_2-fixers and the

amount of C_2H_2 used by them is not likely to prejudice the results of the assay for nitrogenase. They do not co-oxidize C_2H_4.

Soil bacteria and fungi produce C_2H_4 in the absence of C_2H_2 [90] and Cook and Smith [22] showed that the amount produced was greatest in water saturated soils. They attributed the production to anaerobic spore forming bacteria. Ethylene is produced by green plants as a hormone [90].

Commercial supplies of C_2H_2 may contain detectable amounts of C_2H_4 [47].

Van Straten and Schmidt [113] demonstrated that excess water inhibits C_2H_2 reduction by detached soybean nodules and suggested that it might be due to restriction of gas diffusion into and out of the nodule. Rice and Paul [93] also pointed out the difference in diffusion rates through water of C_2H_2 and N_2 and suggested that in waterlogged soils nitrogenase activity was limited by the amount of N_2 penetrating into the soil column whereas C_2H_2 which is much more soluble in water would not be limiting. They note that because of this, the C_2H_4:N_2 molar ratio used for converting C_2H_2 reduction assay results to N_2-fixation values will probably be much greater than the theoretical 3:1 when waterlogged soils are involved. They caution that C_2H_2 reduction should be backed up by direct measurement;

> "$^{15}N_2$-incorporation measurements determined the actual nitrogen-fixation under the given conditions, whereas the C_2H_2 reduction assay

measured the nitrogen-fixing potential of the organisms."

Flett et al [37] have indicated that C_2H_4 is 8.1 times more soluble in water than N_2 is and cautioned that agitation of all samples should be uniform as it affects the rate of gas exchange. The ratio of sample volume to gas phase volume can affect the sensitivity of the assay [37] with a larger sample giving greater sensitivity with the same size gas volume. A sample size of no greater than one tenth of the gas volume is recommended by Waughman [126] in order to minimize variation between samples.

Finally, it has been shown that C_2H_2 inhibits both cell proliferation and nitrogen accumulation in C. pasteurianum [12] regardless of whether cells are growing on N_2 or combined nitrogen. In N_2-fixing cultures the inhibition is relieved only when the amount of C_2H_2 present has been reduced to 0.025 Atm, an amount much lower than the usual substrate levels.

V. OCCURRENCE OF CLOSTRIDIUM SPECIES IN MUSKEG

A significantly greater number of clostridia were found to occur in the soil or water below oil spills than in similar samples without oil [129]. Since the anaerobic breakdown of oil by Clostridium is unlikely [112] it was felt that they must be present to take advantage of some other feature of the oil spill. This could be something as simple as a change in the pH, or as complex as utilization of secondary metabolites produced by bacteria that did utilize

the oil.

The following study represents an investigation into the effect of crude oil on anaerobic N_2-fixation and is treated in three sections; 1. an evaluation of conditions and bacteria found in the muskeg, including a survey of pathogens, 2. field studies of the effect of crude oil on nitrogenase activity in muskeg under anaerobic conditions, including *in situ* studies and laboratory assays of field samples, 3. *in vitro* studies of the effect of crude oil on the nitrogenase activity of pure cultures of *C. pasteurianum* W5 under controlled conditions.

MATERIALS AND METHODS

I. Materials

All reagents used were reagent grade and were obtained from commercial sources.

Biotin was purchased from Calbiochem, p-aminobenzoic acid from J.T.Baker Chemical Co., 3,3-dimethylglutaric acid from Aldrich Chemical Company, Inc., and L-cysteine from Sigma.

All gases used were Linde tank gases supplied by a local supplier. The acetylene was atomic absorbtion grade and the ethylene was 98% C_2H_4. The ethylene was gas chromatographically pure, but the acetylene showed trace amounts of methane and ethylene as contaminants.

II. Organisms

1. Stock Cultures: Cultures of Clostridium botulinum, Clostridium novyi, Clostridium perfringens, Clostridium tetani and Clostridium pasteurianum W5 were obtained from the University of Alberta, Department of Microbiology culture collection.

2. Field Isolates: All isolates were anaerobic rods, and were obtained from various muskeg and oil spill sites in the following manner;

i. Isolates 1 - 24 were obtained by enrichment and selection as Gram positive, sporogenous, SO_3^{2-} reducers and were provided by Dr. F.D. Cook, Department of Soil Science, University of Alberta. A listing of the source of these isolates may be found in Appendix II.

ii. Isolates 25 - 29 were selected by survival of pasteurization followed by repeated streaking on reduced blood agar (RBA).

iii. Isolates 30 - 45 were obtained by enrichment as sporogenous organisms able to grow in the absence of combined nitrogen, on Nitrogen Free Medium B (NFB), and purified by streaking on Nitrogen Free Agar (NFA) and on RBA.

iv. Isolates 46 - 72 were selected as Gram-positive sulfite reducers.

v. Isolates 73 - 77 were enriched and selected for growth in the absence of combined nitrogen, on Skinners Medium (SM).

III. Growth Media

The formulae of all media used along with their respective abbreviations are given in Appendix I.

IV. Incubation Conditions

1. **Time and Temperature**: Stock cultures and isolates were maintained as streak plates on RBA and/or NFA in anaerobic jars held at room temperature (23°C). Cultures were transfered to new plates every 60 to 90 days. Liquid cultures of C. **pasteurianum** W5 were incubated at 37°C for from 12 to 133 hours as required for the various experiments. TSN agar plates were incubated for 48 hours at 46°C, and EYA plates were incubated at 35°C. Cultures on all other media were incubated at 30°C for 24 to 48 hours.

2. **Monitoring of pH**: The pH of the various media,

cultures and assay materials was measured with a Radiometer pH Meter 51.

3. Conditions of Anaerobiosis: Anaerobic conditions were obtained in the following ways.

i. Gaspak System: Anaerobic jars (BBL) were used with the gaspak system (BBL). The methylene blue impregnated strip indicator insured that a redox potential of at least -49 mV [53] was obtained with each incubation. These indicator strips were also used in the large desiccators and assay vessels.

ii Gas Replacement: Large desiccators were evacuated to a vacuum of 20 mm Hg and filled with O_2 free N_2. This was repeated three times and a small amount of CO_2 (approximately 3%) was then introduced and the desiccator sealed. Liquid cultures of N_2-fixing organisms were continuously purged with this same gas mixture. The gases were rendered free of oxygen by passage through a column of hot (350°C) copper turnings [52,66].

iii. Steel Wool: Residual oxygen was removed from the large desiccators and assay vessels by placing pads of steel wool which had been activated with copper sulfate [89,101] into the containers before sealing.

iv. Nitrogen, Argon Flush: Vessels used for the acetylene reduction assay were flushed with either scrubbed N_2 or scrubbed Ar for five minutes.

v. Purging: Liquid cultures of C. pasteurianum W5 were grown in Erlenmeyer flasks (250 ml or 1000 ml) with a

continuous flow of O_2 free gas being bubbled through the culture at a rate of approximately 250 ml/min. and exiting via a Bunsen valve. The purging gas consisted of N_2 with from 3% to 5% CO_2. Each gas was separately passed through a Hungate column, then through a flask of sterile distilled water to allow re-humidification before being mixed just prior to entering the growth flask.

The use of pre-reduced media [33,51] was found to be of no benefit with the isolates obtained in this project.

vi. Indicators: In all cases where an indicator of anaerobiosis was used, it was methylene blue, either as a Gaspak indicator strip for anaerobic jars and large CO_2 reduction assay vessels, or as a boiled solution of methylene blue (0.065 mg/ml). This solution was not added to media, but whenever the state of reduction of a culture was required, an aliquot of the culture was placed into a Hungate tube and a drop of the methylene blue solution injected.

V. Estimation of Growth

1. Most Probable Number (MPN): Five tube MPN counts were conducted using either B 10 + SO_3^{2-} or SM. Blackened tubes were counted as positives with the B 10 + SO_3^{2-} medium and tubes showing growth plus gas in the inverted Durham vial were positive in SM. Numbers of positives were converted to bacterial counts according to the tables found in Standard Methods For the Examination of Water and Wastewater [39].

2. Optical Density (OD): Estimations of growth of liquid cultures of C. pasteurianum W5 were made by measuring the absorbance at 600 nm or 660 nm in a Bosch and Lomb, Spectronic 20 equipped with a special cuvette holder that allowed insertion of Hungate tubes.

VI. Sampling of Muskeg

1. For removal for analysis in the laboratory: Samples ranging in size from a few grams to several kilograms comprising the upper 50 cm of the soil and liquid were removed by shovel and stored at 4^0C in plastic bags from which most of the air had been squeezed. Samples were normally analyzed within 14 days of removal.

2. For assay in situ: Samples were removed by shovel and immediatly placed into large polyethylene containers (9.5cm diameter x 21 cm high). Immediately thereafter the samples were assayed for nitrogenase activity by the C_2H_2 reduction assay. Gas samples were returned to the laboratory for analysis. Large samples (700 g) were weighed upon return to the laboratory after assay, while small (150 g) samples were weighed into the containers in the field using a three beam balance.

VII. Muskeg Analysis

1. pH: Approximately 10 g of each sample were weighed into a 50 ml beaker, combined with an equal amount (w/v) of water and stirred thoroughly then allowed to stand at room temperature for one hour. The pH of the aqueous portion was then measured with a Radiometer PHM51 pH meter.

2. Temperature: A recording thermometer (Tempscribe; Bacharach, Pittsburgh) was placed in the muskeg for 24 hrs. or 13 days with the probe buried in the 10-20 cm layer.

3. Moisture (Dry Weight): Duplicate aliquots (approximately 5 g) from each sample were weighed into pre-weighed aluminum foil pans and placed in an oven at 80°C. The pans were removed from the oven after 48 hours and at irregular intervals thereafter, allowed to stand on the bench top for one hour to return to room temperature and rehydrate, then weighed. After weighing the samples were returned to the oven for further heating. This process continued until a constant weight was achieved, which in some cases was 45 days. The dry weight and % moisture of the samples was calculated.

4. Oil Content: Aliquots of soil from oil spill and control samples were weighed (approximately 5 g) into 63 ml serum bottles. Each bottle received 30 ml of chloroform, was stoppered, thoroughly shaken then allowed to stand at room temperature for 48 hrs. The sample was then emptied onto Whatman #2 filter in a Buchner funnel (9 cm) and the liquid phase drawn off by vacuum. The bottle was washed with at least two washes of chloroform which were poured through the funnel, and additional chloroform was used to wash the sample until the filtrate ran clear. The filtrate was then transferred quantitatively to a 500 ml separatory funnel. The vacuum flask was rinsed twice with chloroform then once with

water. The washings were added to the separatory funnel which was then shaken thoroughly and allowed to stand until separation had occurred. The chloroform layer was drawn off into a pre-weighed 250 ml beaker and the aqueous layer was washed at least twice with 25 ml aliquots of chloroform, or until the interface was clear of visable oil. These washings were added to the beaker which was then covered loosely with aluminum foil and set in a fume hood where the chloroform was allowed to evaporate to dryness. The beakers were reweighed in 14 days and again in 16 days to ensure constant weight. The percent oil in the samples was calculated.

5. Soil Nutrients: Two samples, one control and one from the oil spill, were submitted to the Soil and Feed Testing Laboratory of the Alberta Department of Agriculture for routine soil analysis. These samples were analysed for content of nitrogen (as NO_3^- and as NH_4^+), phosphorus, potassium, sulphur, aluminum and manganese, as well as for salinity, organic matter content and texture.

VIII. Nitrogenase Assay

The N_2 Fixing capacity of the various samples was estimated by measuring the nitrogenase activity, using modifications of the C_2H_2 reduction assay technique of Stewart and Hardy [48,105] and quantitating the amount of C_2H_4 produced by gas liquid phase chromatography (GC).

1. Sample Preparation:

i. In the Laboratory: Aliquots were aseptically taken from within the bulk of the stored samples and placed in

either large (1500 ml) polyethylene containers (Prig-O-Seal), (150 g sample) or in 63 ml serum bottles, (5 g sample). When liquid cultures of C. pasteurianum W5 were used, 5 ml aliquots were assayed in Hungate tubes (18 ml) or serum bottles. The serum bottles were sealed with rubber serum stoppers and the large containers with lids fitted with serum stoppers. Samples which had been stored at 4^0C were routinely preincubated with an atmosphere of O_2 free N_2 for two hours to allow for temperature equilibration. The head gas was then removed and replaced with a mixture containing 90:10 air:C_2H_2, 90:10 N_2:C_2H_2, or 90:10 argon:C_2H_2. Removal of head gas was either by evacuation with a vacuum pump to a pressure of 30 mm Hg followed by injection of the required amounts of the other gases, or by constant flushing with the major gas for five minutes then replacement of the required amount with C_2H_2 by syringe. The containers were then incubated at either 23^0C or 30^0C for from one to 24 hours.

ii. In Situ In the Field: Aliquots of either 700 g or 150 g were placed in large polyethylene containers and sealed as described previously. The containers were then placed into the hole from which the sample had been removed and the head gas was removed using a hand operated Nalgene vacuum pump and repl ed with either a 90:10 air:C_2H_2 mixture or a 90:10 argon:C_2H_2 mixture. The samples were incubated in situ for periods ranging from 24 hours to two weeks.

2. Handling of Gas Samples: At various times during and at the end of the incubation periods, aliquots of the head gases were removed for analysis. These gas phase samples were handled in one of three ways;

i. Immediate Analysis: When practical, a 0.5 cc aliquot of the head gas was removed from the assay with a 1 cc plastic disposable syringe and injected directly into the gas chromatograph.

ii. Displacement and Capture: When GC analysis lagged behind sample assays, an aliquot of the head gas was captured by injecting 20 ml of distilled water into the serum bottle and forcing the gas into a second syringe. The gas was then injected into a Hungate tube which had been previously filled with distilled water, and allowing the water to be forced out through a 22 gauge needle. When samples were to be taken before the end of the assay, 5cc head gas samples were removed by syringe and placed in Hungate tubes over water. Head gas samples (50 cc) from large containers, both in the field and the lab, were removed by syringe and injected into pre-evacuated serum bottles. In all cases where gases later to be assayed were held in serum bottles or Hungate tubes. Needle punctures in the septa were sealed with silicone high vacuum grease.

iii. Delayed Sampling: End time samples of assays of C. pasteurianum W5 were held in the reaction vessel, after the reaction had been stopped by injecting 1 ml of 50% (w/v) trichloroacetic acid (TCA).

3. Gas Liquid Phase Chromatography: Gas chromatographic analysis was carried out on 0.5 cc samples on a Hewlett-Packard 5700A Gas Chromatograph with a H_2 flame ionization detector, combined with a 3370B Integrator and a 7127A Strip Cha Recorder. A 6' by 1/8" glass column packed with Phenyl Isocyanate on Porasil C (80-100 mesh) was used with N_2 carrier gas at a flow rate of 10 cc/min. The injector, oven and detector temperatures were 150°C, 27°C and 300°C respectively. Retention times were 50, 80 and 145 seconds for methane, ethylene and acetylene respectively.

4. Assay in the Presence of Oil: Nipisi crude oil was dissolved in benzene to yield a 10% (v/v) solution, and dispensed into serum bottles, so as to deliver from 0.001 to 1 ml of oil per bottle. The benzene was then moved by continual flushing with air until no visual or olfactory traces remained. Cultures to be assayed were injected into the serum bottles and preincubated for two hours with the oil to allow dispersion of the oil in the culture medium and uptake by the cells of any soluble oil components.

5. Standard Curve: Volumetric flasks (200 ml) were filled to the mark with water, glass beads were then poured in until the water rose to the top of the neck, allowing for the volume occupied by the internal part of a serum stopper. The water was then poured out and a serum stopper was fitted, resulting in a flask with a gas volume of exactly 200 ml. A volume of ethylene, calculated to contain 4×10^{-4} moles of ethylene allowing for variations in barometric

pressure and the purity of the cylinder gas (98%) (J.L.Neal, Agriculture Canada, Research Station, Lethbridge: personal communication), was injected into the first of a series of flasks. Similarly, $4x10^{-5}$ moles was injected into a second flask. From these two flasks 100 fold serial dilutions were made so that a standard series was achieved from 10^1 pico mole/0.5 cc to $1x10^6$ pico mole/0.5 cc. From these flasks 0.5 cc aliquots were removed and analysed by gas chromatography and from the integrator output a standard curve was drawn relating log mV to log p moles C_2H_4. Each experiment was converted using the standard curve which had been run at the same time in order to eliminate any variation in the detector response with time.

6. Calculations: The millivolt integrator output from acetylene reduction assay samples was converted to p moles of C_2H_4 by a computer program using the slope and intercept of the standard curve in the formula $y=ax+b$ where a=slope, b=intercept, x=log p moles C_2H_4, and y=log mV. The program also allowed for subtracting blank values from experimental values to compensate for the C_2H_4 contaminant in the C_2H_2 supply, and for calculating the amount of C_2H_4 produced per g or ml of sample by using the ratio between the volume injected and the head space volume of the container, which it also calculated, based upon experimentally determined weight to volume ratios for the samples and the measured volume of the assay container. When also given the percent moisture and incubation time, the results were expressed as

p moles/h/g dry weight, as well as p moles/h/g wet weight.

IX. Acetylene Reduction by Activated Steel Wool

Samples of commercial grade 00 steel wool were activated as previously described then placed into 1.5 litre assay vessels which contained no other sample. They were then assayed for acetylene reduction for a period of two hours.

RESULTS AND DISCUSSION

I. Evaluation of Microflora and the Environmental Parameters of the Muskeg

1. Sample Sites: A sketch of the main features of the Nipisi No.1 oil spill is given in Figure 2 which also shows the location of the various sample sites within the spill and most of the control sites. Samples were taken from several of the sites at more than one time. Details of each sample, including site, date, depth and a description of the material taken can be found in Appendix III. The Nipisi No.2 spill is located approximately two kilometers west of the Nipisi No.1 spill along a feeder pipeline that runs parallel to the road past the two spills. The Rainbow spill is approximately one kilometer north of the two Nipisi spills on another road. The Norman Wells spill is located at Norman Wells in the Northwest Territories, along the MacKenzie River valley.

2. Profile of Physical Parameters of Sample Sites:

i. Temperature: Temperature was monitored at site B with a recording thermometer, at a depth of 10 - 20 cm, over a seven day period in June and again for one day in September. Figure 3 gives the results of the warmest and coldest days from June and the day in September.

ii. pH: The pH was determined for many of the samples obtained from spill and control sites. These values appear in Appendix IV along with a classification of the samples as to their physical nature, ie; organic, inorganic, or

FIGURE 2

OUTLINE, MAIN PHYSICAL CHARICTERISTICS, AND SAMPLE SITES OF NIPISI NO. 1 SPILL

S = source of the spill.

A - W = sample sites.

——— perimeter of spill and retaining ditch.

═══ road.

—·—·— cutline.

Approximate scale is 1:5000.

Distances outside the spill are not to scale:

a. from site A to the road is about 100 m.

b. from the road to site U is about 100 m.

The perimeter of the spill is approximately 240 m and it has an area of about 14 ha. It is located at Pt.LSD 6-24-79-8-W5.

Adapted from Westlake and Cook, 1975.

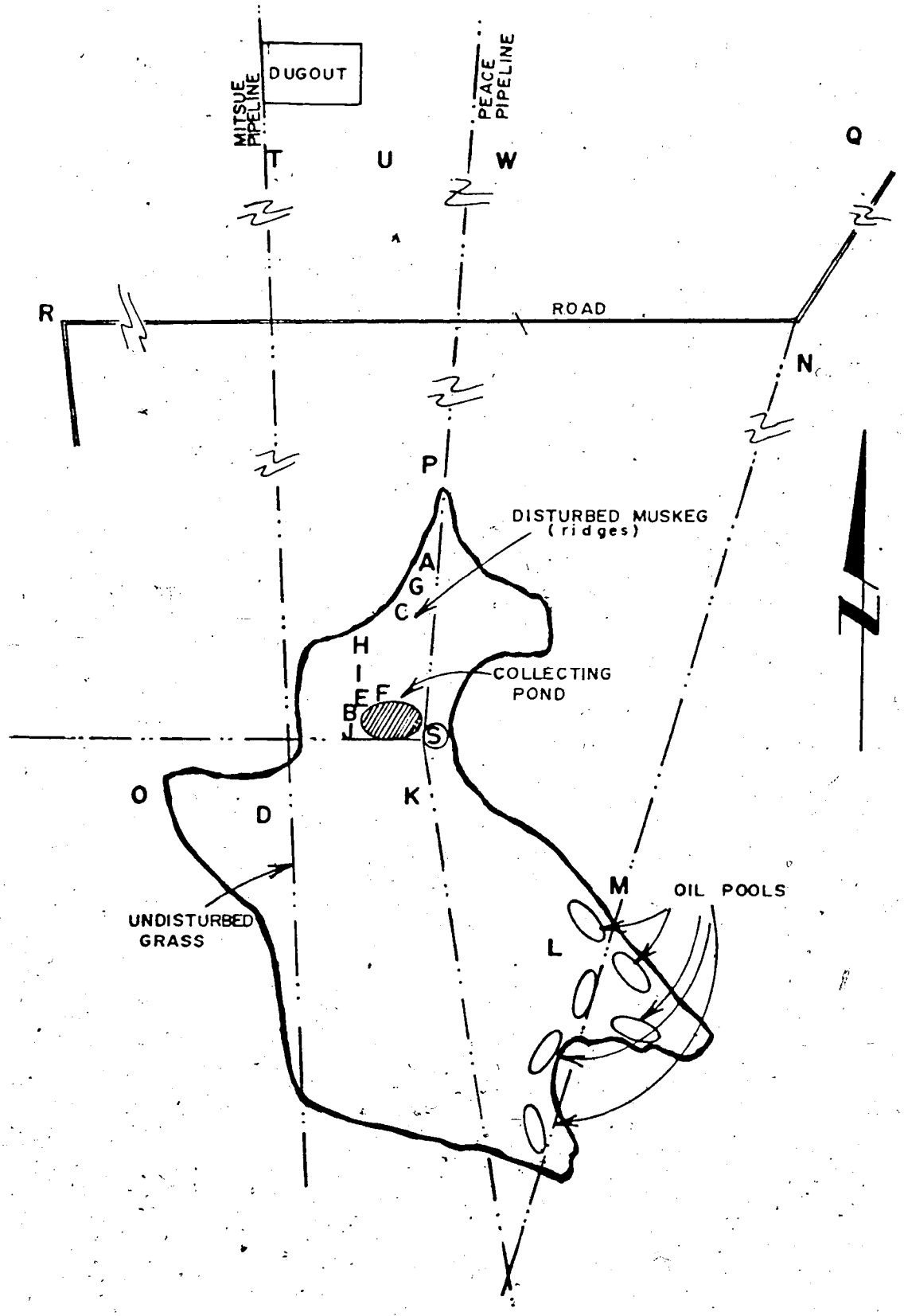
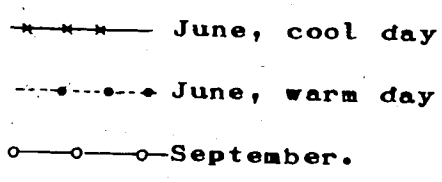

MITSUE PIPELINE
DUGOUT
PEACE PIPELINE
T
U
W
Q
R
ROAD
N
P
DISTURBED MUSKEG (ridges)
A
G
C
H
COLLECTING POND
E
F
B
I
S
O
D
K
M
OIL POOLS
L
UNDISTURBED GRASS

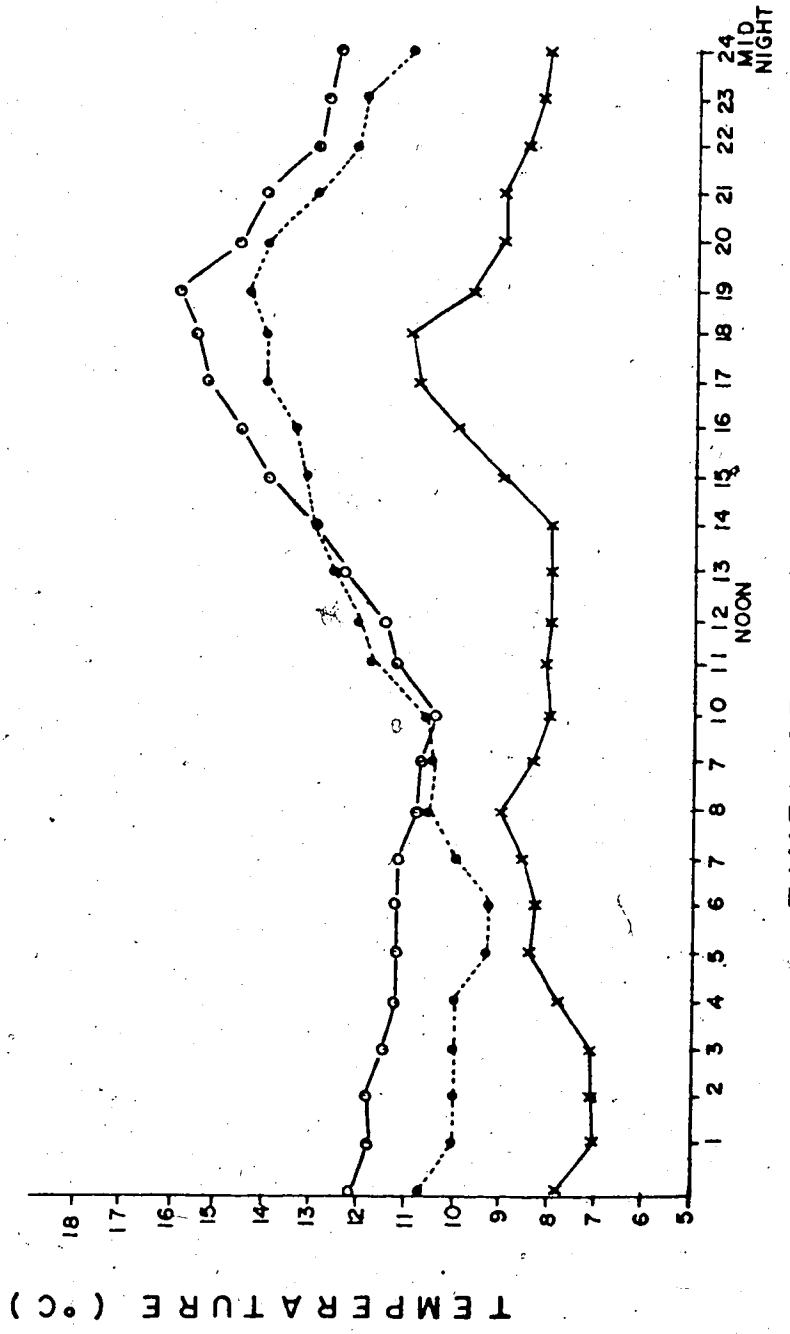
FIGURE 3

DIURNAL TEMPERATURE FLUCTUATIONS IN

THE NIPISI NO. 1 OIL SPILL

The probe of a recording thermometer was placed 10 cm below the surface at site B in the spill and the temperature was recorded for seven days in June and one day in September of 1975.




TEMPERATURE (°C)
18
17
16
15
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9
8
7
6
5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
NOON
MID NIGHT
TIME OF DAY

aqueous, and are summarized in Table 2. Muskeg and the soil and water associated with them are acidic. The occurence of an oil spill has caused the average pH of each sample type to increase toward neutrality. Although the pH alone is probably not the only cause, it could well be one of the more important factors involved in the "Clostridial Bloom", since most bacteria, including the clostridia, prefer a pH near neutrality [2].

iii. Water Content: Dry weight determinations on a group of samples revealed that the moisture content ranged from 10% for a coarse sand through 95% for moss samples. Most samples contained more than 50% water (Appendix IV). The control samples were significantly wetter thar se from the spill, with an average of 82% moisture a ared to 53%. This reflects the disturbed nature of the spill, where the vegetation has all been knocked down into the muskeg and the bottom detritis and clay have been brought to the surface. The control sites on the other hand tended to be open with lots of moss hummocks and standing water. In some cases the measured moisture content may be higher than the true value for the spill samples, since the method of determining dry weight (80^0C) tends to drive off not only water but also some of the volatile components of the oil. This is exemplified by the values for sample S/58b which are 70% moisture and 38% oil, which is already over 100% and does not include the solid matter.

iv. Oil Content: The amount of oil, as chloroform

TABLE 2

SUMMARY OF PHYSICAL PARAMETERS OF SAMPLES

Soil Type*1	pH	Percent Moisture*2	Percent Oil*2*3
A. Control Samples			
O	4.51 ± 0.46 (13)*4	89.00 ± 14.09 (11)	0.240 (1)
I	5.70 (1)	22.38 (1)	--- (0)
I/O	5.28 ± 0.04 (2)	77.10 ± 24.10 (3)	0.475 (1)
A	5.62 (1)	--- (0)	--- (0)
ALL	4.73 ± 0.59 (17)	82.18 ± 22.85 (15)	0.358 ± 0.166 (2)
B. Oil Spill Samples			
O	4.89 ± 0.62 (23)	72.05 ± 23.55 (17)	14.012 ± 15.620 (6)
I	7.32 ± 0.63 (15)	18.12 ± 8.74 (11)	4.576 ± 6.872 (5)
I/O	5.87 ± 0.29 (6)	65.01 ± 18.63 (5)	8.323 (1)
A	6.35 ± 0.27 (6)	--- (0)	--- (0)
ALL	5.91 ± 1.19 (50)	53.00 ± 31.28 (33)	9.606 ± 12.260 (12)

*1 A=Aqueous, I=Inorganic, O=Organic. The soil types are more fully described in the footnote to Appendix IV.
ALL=a mathematical average of all of the samples.

*2 Each value used for % moisture or oil is itself the mean of duplicates, the standard deviation of each is shown in Appendix IV.

*3 Percent Oil is the amount of chloroform extractable material in the sample divided by the total weight of the sample, which includes the weight of soil + oil + water, times 100.

*4 Numbers in parenthesis are the number of values included in each average.

extractable material, in selected samples is shown in Appendix IV. Since chloroform was a to extract some

material from the organic fraction of the control samples, it can be assumed that similar quantities were extracted from the organic matter in the spill samples. Those spill samples which contained very low amounts of $CHCl_3$-extractable material contained, in all likelihood, no oil.

v. Soil Nutrients: Analysis for plant nutrients of one sample from a control site and one from the spill was conducted by the provincial soil testing laboratory. The results appear in Table 3. There is considerably more

TABLE 3

SOIL TEST REPORT

	Control Sample S/27	Spill Sample S/26
Nitrogen		
NH_4^+-N (ppm)	59.5	15.6
NO_3^--N (ppm)	0	1
Phosphorous (ppm)	1	2
Potassium (ppm)	7.5	20
Aluminum (ppm)	3.8	1.8
Manganese (ppm)	4.0	10.8
Sulphur	Low	Low
Sodium	Very low	Very low
Organic matter	Very high	Very high
Conductivity (mmhos)	0.1	0.1
pH	4.5	5.0

ammonia-nitrogen in the control sample than in the spill sample. The control values of 59.5 ppm for an air dried

sample converts into 22 kg/ha, which would be considered a high level in a mineral soil (Bulk density of about 1). The bulk density of the muskeg samples however is only about 0.1 (90% moisture) which would mean an actual concentration of about 2.2 kg/ha, or 6 ppm in the muskeg. In peat soils, only about 10% of the ammonia present is in solution, with the rest being held on the exchange complex of the soil (W.B.McGill, University of Alberta, Department of Soil Science: personal communication). This would mean that there would be approximately 0.6 ppm ammonia-nitrogen in solution in the control areas and only 0.16 ppm in the spill areas of the muskeg. Both of these values are low when compared to mineral soils, but are estimated to be at or above the minimum levels required for bacterial uptake (W.B.McGill, personal communication)

The level of nitrate-nitrogen is negligible in both samples. The other mineral nutrients (P,K,S) are all low by agricultural standards. Aluminum and Mn are present in amounts that would be toxic to cereal crops according to the information received with the soil test report.

3. Isolation, Identification and Enumeration:

i. Clostridia: In order to confirm the existence of a "Clostridial Bloom", counts were carried out on a series of spill and control samples. Five tube MPN counts, the results of which appear in Table 4, were done using B10 + SO_3 medium. Growth with blackening was interpreted as the ability to reduce sulfite and the ability to withstand

pasteurization at 80°C for 20 minutes was taken as evidence that spores were present. Anaerobic, sulfite reducing, spore formers were assumed to be members of the genus Clostridium. As can be seen from Table 4, there is an increase of approximately twenty fold in the number of clostridia in samples taken from the spill over those taken from control sites. This is not nearly as pronounced as the "Bloom" reported by Westlake and Cook [129] which showed increases of as much as three thousand fold. The increase is not as great for aqueous samples as for soil samples. The total population of sulfite reducers is the same inside the spill as outside.

As pointed out by Westlake and Cook [129], this technique gives a count of spores present rather than a number of spore forming organisms present. There does not appear to be any consistent relationship between the total population of sulfite reducers in a sample and the number of spores.

To confirm the presence of clostridia, isolates (46 - 72) were obtained by enriching for sulfite reduction. Standard taxonomic tests, listed in Appendix V, were used to characterize the isolates.

ii. Nitrogen-Fixing Anaerobes: It was hypothesized that since several species of Clostridium are able to fix atmospheric N_2, and since large numbers of clostridia have been found in association with oil spills, that clostridia of the N_2-fixing varieties might also be enriched, and that they might make significant contributions to N_2-fixation in

TABLE 4

NUMBERS OF SULFITE REDUCING ANAEROBES IN SAMPLES

Sample Number	Sample Type*1	Counts*2/Gram dry weight or /ml Before Pasteurization	After Pasteurization*3
A. Control Samples.			
S/16	A	3.3×10^2	1.7×10^1
S/19	I	<1	<1
S/27	O	1.8×10^4	6.6×10^3
S/28	O	2.1×10^3	1.1×10^2
S/30	O	4.0×10^4	2.8×10^2
B. Spill Samples.			
S/10	A	7.9×10^1	<1
S/13	A	7.0×10^1	2
S/14	A	2.2×10^1	9
S/15	A	4.0×10^1	<1
S/17	A	7.9×10^1	3.3×10^1
S/18	A	3.3×10^2	4.6×10^1
S/7	I	2.4×10^6	4.2×10^5
S/8	I/O	2.5×10^4	1.2×10^4
S/11	O	1.4×10^3	1.6×10^1
S/12	O	1.6×10^4	1.3×10^4
S/20	O	1.9×10^4	3.4×10^3
S/21	O	1.5×10^4	4.4×10^3
S/22	I/O	2.4×10^4	2.4×10^4
S/23	I	1.3×10^2	4.0×10^2
S/24	I	4.1×10^3	4.1×10^2
S/25	I	9.0×10^3	2.9×10^4
S/26	O	1.2×10^5	1.9×10^4

*1 Symbols are explained in Appendix IV.
*2 Most Probable Number Counts expressed as counts per gram dry weight of soil (O and I samples), or as counts per ml (A samples).
*3 20 minutes at 80°C.

the spill. Five tube MPN counts were therefore conducted in Skinner's medium, as described in Materials and Methods. Parallel counts were conducted on pasteurized samples to detect spores. Table 5 reveals that relatively few of the

anaerobic diazotrophs were present as spores and that their

TABLE 5

VIABLE COUNTS OF NITROGEN-FIXING ANAEROBES IN SAMPLES OF MUSKEG SOIL

Sample Number*1	Sample Type*2	Counts/Gram wet weight Before Pasteurization	After Pasteurization*3
A. Control Samples.			
S/31	I	5.4×10^4	2.0×10^2
S/32	I	2.1×10^6	2.0×10^2
S/33	O	3.5×10^6	<1
B. Spill Samples.			
S/34	O	7.9×10^3	<1
S/35	O	1.7×10^5	5.0×10^2
S/26	O	2.4×10^4	<1

*1 See Appendix III for descriptions of samples.
*2 Symbols are explained in Appendix IV.
*3 20 minutes at 80°C.

numbers were approximately the same in the spill samples as in control samples.

To confirm the presence of clostridial diazotrophs cultures were enriched and isolates obtained and characterized as previously described.

Three N_2-fixing isolates (73,76,77) were obtained from control samples which were tentatively identified as Clostridium sp. on the basis of their being obligately anaerobic, Gram-positive, spore formers. Another two (74,75)

were obligately anaerobic N_2-fixing bacteria, but were not spore formers and were of uncertain Gram reaction. One isolate (72) was a possible Klebsiella sp., a Gram negative asporogenous rod which fixes N_2 anaerobically but grows as a facultative anaerobe in complex media.

Isolates from the spill samples fall into three classes. Five isolates (31,33,34,45,54) are either aerotolerant clostridia or facultative Bacillus species. They are Gram-positive, spore forming rods that fix N_2 under anaerobic conditions, but are able to grow in the presence of air on a complex medium. Four others (41,44,48,63) were possible Klebsiella types and the remaining two were Gram-positive rods which do not form spores. One (46) is obligately anaerobic and the other (35) is facultatively so.

iii. Pathogens: If clostridia are present in large numbers, it is also probable that some of them would be pathogenic, since many species of Clostridium show varying degrees of pathogenicity for man [13]. All isolates on hand were screened for possible pathogenicity by growing them on RBA to test for haemolysis, and on EYA to test for lipase and lecithinase production [33,51]. Some additional isolates (25-29) were obtained by enrichment on RBA, and many but not all of the isolates were tested for proteolytic activity and for the ability to grow and reduce sulfite in the presence of neomycin at 46°C [72] which is indicative of C. perfringens. Appendix V contains the detailed results and a summary is presented in Table 6. All isolates that were

positive on TSN agar were also negative for lipase, positive for lecithinase and β haemolytic with the exception of isolate No. 10 which differed only in being α haemolytic. This pattern also fits C. perfringens. The total number of

TABLE 6

SUMMARY OF ISOLATES FROM MUSKEG SOIL SHOWING PATHOGENIC CHARACTERISTICS

	Number of isolates giving positive results			
Sites	Haemolysis*1	Lecithinase*2	Lipase*3	TSN*4
Control	1 α 8 β	1	1	1
Spill	11 α 32 β	16	3	12

*1 Growth with haemolysis on RBA.
*2 Growth with opacity on EYA.
*3 Growth with a pearly layer on EYA.
*4 Growth with blackening on TSN agar. See Appendix V for details.

isolates is too low to allow any statements to be made about relative numbers of pathogens in the spill as compared with outside the spill, however, the occurrence of C. perfringens appears to be quite consistent. A further study would be required to determine if enrichment of pathogens in general or specifically of C. perfringens were occurring.

II. NITROGEN FIXATION

A. PURE CULTURE ANALYSIS

1. Evaluation of Parameters:

i. General Principle: Analysis for N_2-fixation was in all cases done using the C_2H_2 reduction assay, which depends upon the non-specificity of the enzyme nitrogenase. This allows the substitution of C_2H_2 for N_2 and subsequent measurement of the C_2H_4 produced. Because it is an indirect assay and subject to the variations mentioned in the introduction, caution must be exercised in the interpretation of the results. This is especially true for the choice of a conversion factor for translating moles C_2H_4 produced into moles N_2 reduced.

ii. Anaerobiosis: Conditions of anaerobiosis were obtained as described in Materials and Methods.

a. Degree of Anaerobiosis: Since the Eh of methylene blue at pH 7 is -49 mV at the point at which all color is lost [53], at which point 1% or less of the indicator is oxidized, all cultures and assays were assured of being reduced to this point or lower. Jacob [53] has shown that purging with O_2-free N_2 lowers the redox potential of a nutrient solution to -250 mV and that the oxygen concentration simultaneously drops to 5% or less within minutes. Although a low redox potential is routinely used as an indicator of anaerobic conditions, this is not necessarily so [87,116]. It is possible to experimentally maintain a low Eh and still have high levels of O_2 present

in solution, or to have low O_2 levels and a high Eh. Under normal culture conditions however the two factors usually parallel each other, and except for work with fastidious anaerobes it should not be necessary to resort to potentiometric monitoring of dissolved oxygen.

Many clostridia [70,75] are able to tolerate fairly high levels of oxygen (ca. 3-5% O_2) and during growth will drive the Eh downwards to -250 mV or lower, thus helping to maintain their preferred environment.

b. Steel Wool: Some early C_2H_2 reduction assays were attempted using the activated steel wool technique to obtain anaerobiosis. It was later shown however that the activated steel wool alone resulted in the reduction of C_2H_2 to C_2H_4 (Table 7) in sufficient quantities to introduce major errors. Iron has been shown to form linear complexes with N_2 [15] which alter the N-N bond properties. These two techniques were therefore not used together.

iii. The Effect of Physical Conditions: In order to determine the optimal conditions for the C_2H_2 reduction assay on whole cell cultures of C. pasteurianum W5, several experiments were conducted using cells grown on either NFB or WW medium.

a. Time: Three experiments were carried out to determine the relationship between C_2H_2 reduction and the length of the assay. Cells were grown overnight in NFB, experiment C, or WW medium, experiment A and B, then dispensed into sterile pre-evacuated Hungate tubes (5

TABLE 7

NON-BIOLOGICAL ACETYLENE REDUCTION BY ACTIVATED STEEL WOOL

Weight of Steel Wool	Acetylene Added	Ethylene Produced n moles/h/g
0.0	0.1 Atm.	0
0.0	0.1	0
21.64	0.0	-*
21.86	0.0	0
5.53	0.1	157
5.23	0.1	68
10.52	0.1	66
10.45	0.1	164
14.56	0.1	0
14.92	0.1	0
21.92	0.1	51
22.02	0.1	93

* Gas phase lost.

ml/tube). Experiment B used cells from the same culture as experiment A which were diluted 1:2 with fresh medium immediately prior to commencement of the assay. An atmosphere of 10% C_2H_2 and 90% Ar was introduced and incubation was started at 37°C (A&B) or room temperature (C). At the designated times, replicate tubes (3 in A and B, 4 in C) were removed from incubation and the reaction was stopped by injection of TCA. Analysis for C_2H_4 revealed (Figure 4) that there was a linear relationship between time and C_2H_4 production between 60 and 90 minutes (experiments A&B) and that dilution of the culture caused a decrease in C_2H_4 production which was proportional to the dilution factor. The slope of line A is 4.7 whereas that for line B is 2.5. Experiment C however shows that the relationship

FIGURE 4

KINETICS OF ACETYLENE REDUCTION BY

CLOSTRIDIUM PASTEURIANUM W5

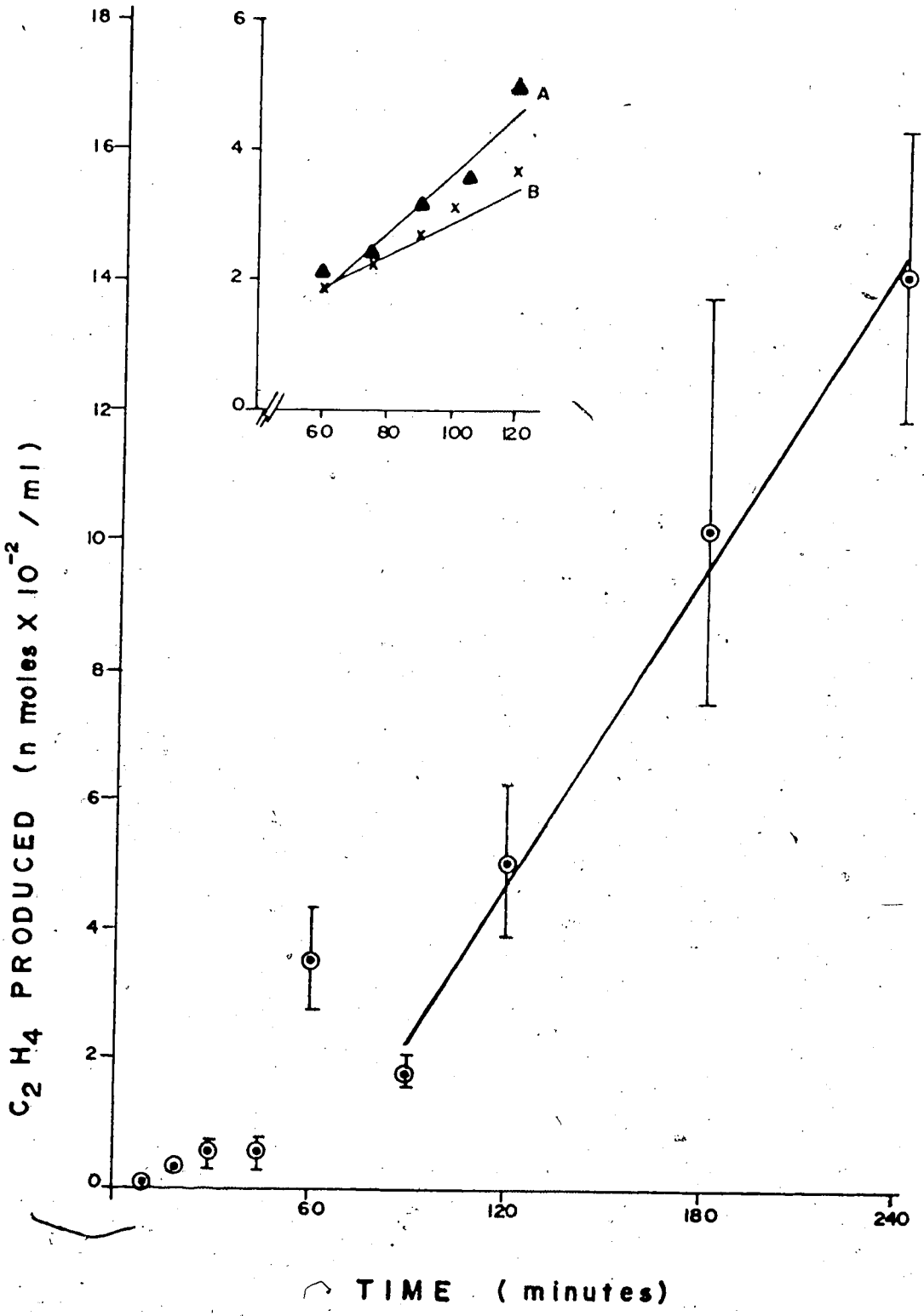
Actively growing cultures of C. pasteurianum W5 were incubated with 0.1 Atm. C_2H_2 as described in the text.

▲ Experiment A.

× Experiment B.

⊙ Experiment C.

Error bars represent ± one standard deviation. Lines were plotted by the least squares linear regression method.


18
16
14
12
10
8
6
4
2
0
60
120
180
240
$C_2 H_4$ PRODUCED (n moles X 10^{-2} / ml)
TIME (minutes)
6
4
2
0
60
80
100
120
A
B
x
x
x
x

between time and reduction is linear between 90 and 240 minutes but erratic below 90 minutes. It was suspected that this irregular behavior in experiment C was possibly due to the reaction not being stopped instantaneously, since in this experiment only 1 ml of 10% (w/v) TCA was used whereas in the other experiments, 1 ml of 50% (w/v) TCA was injected. Regardless of the possible explanation, 120 minutes was chosen as the length preferred for assays, since all three experiments show a linear relationship in this region.

b. Temperature: Cells were grown in NFB at room temperature to an OD_{600} of 0.3, then dispensed into Hungate tubes as before. Acetylene and Ar were added and replicate tubes were incubated in water baths at various temperatures. After one hour, reactions were stopped with TCA and analysis for C_2H_4 was carried out. C. pasteurianum W5 shows (Figure 5) a distinct and fairly sharp temperature optimum for C_2H_2 reduction in the region of 37°C. This is also its optimum for growth [13].

c. Concentration of Cells: Cells were grown in WW medium at 37°C to an OD_{660} of 0.6, then dispensed into Hungate tubes in aliquots ranging from 0.5 ml to 5.0 ml, then brought to 5 ml by addition of fresh media. The actual relative concentration was determined for each tube by measuring its OD_{660}. Incubation was for one hour at 37°C under 10% C_2H_2, 90% Ar, after which the reaction was stopped with TCA. Figure 6 shows a linear relationship between cell

FIGURE 5

THE EFFECT OF TEMPERATURE ON THE REDUCTION OF ACETYLENE BY *CLOSTRIDIUM PASTEURIANUM* W5

Actively growing cultures of *C. pasteurianum* W5 were incubated with 0.1 Atm. C_2H_2 as described in the text.

Each point represents the mean of five replicate samples and the error bars are ± one standard deviation.

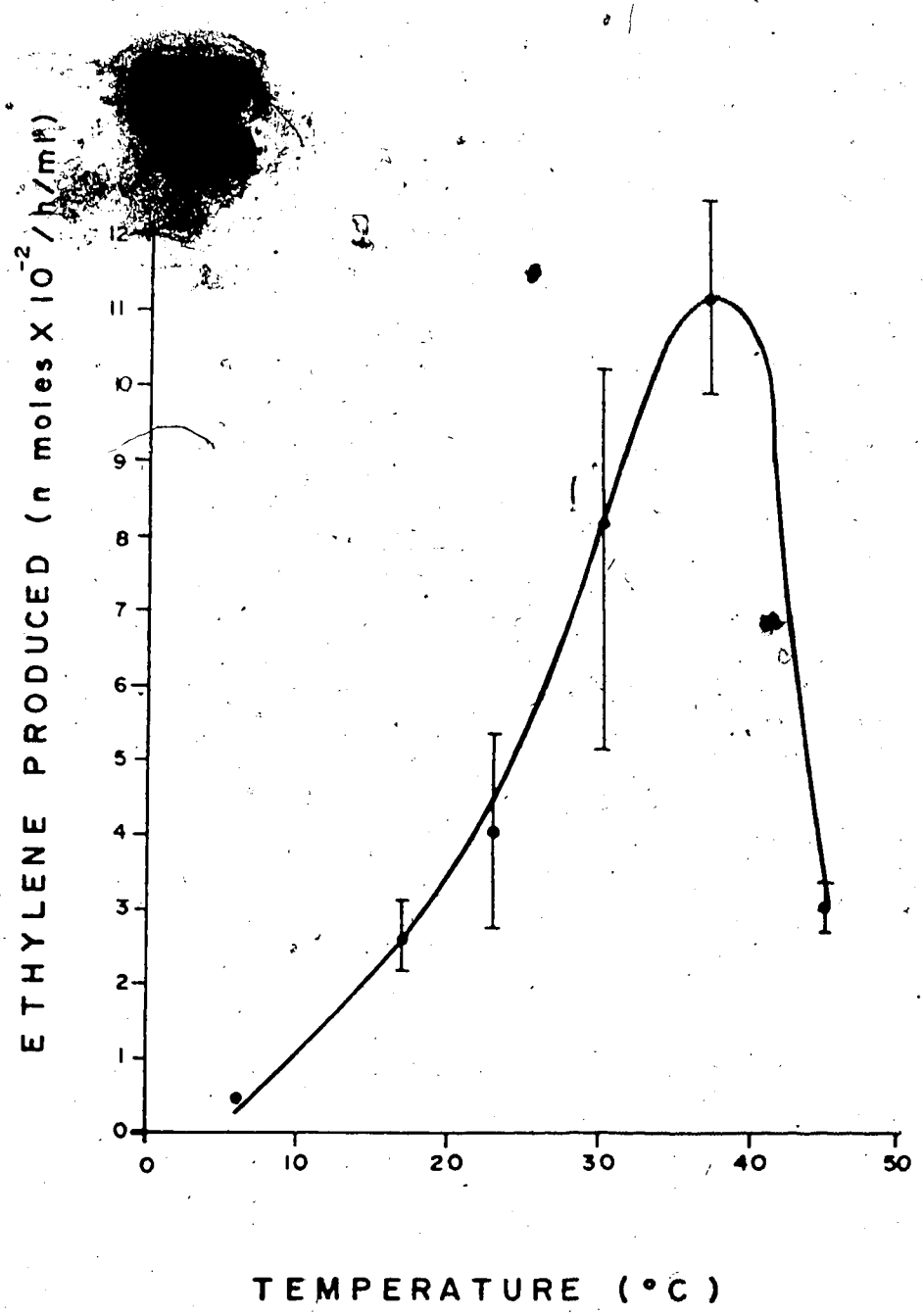
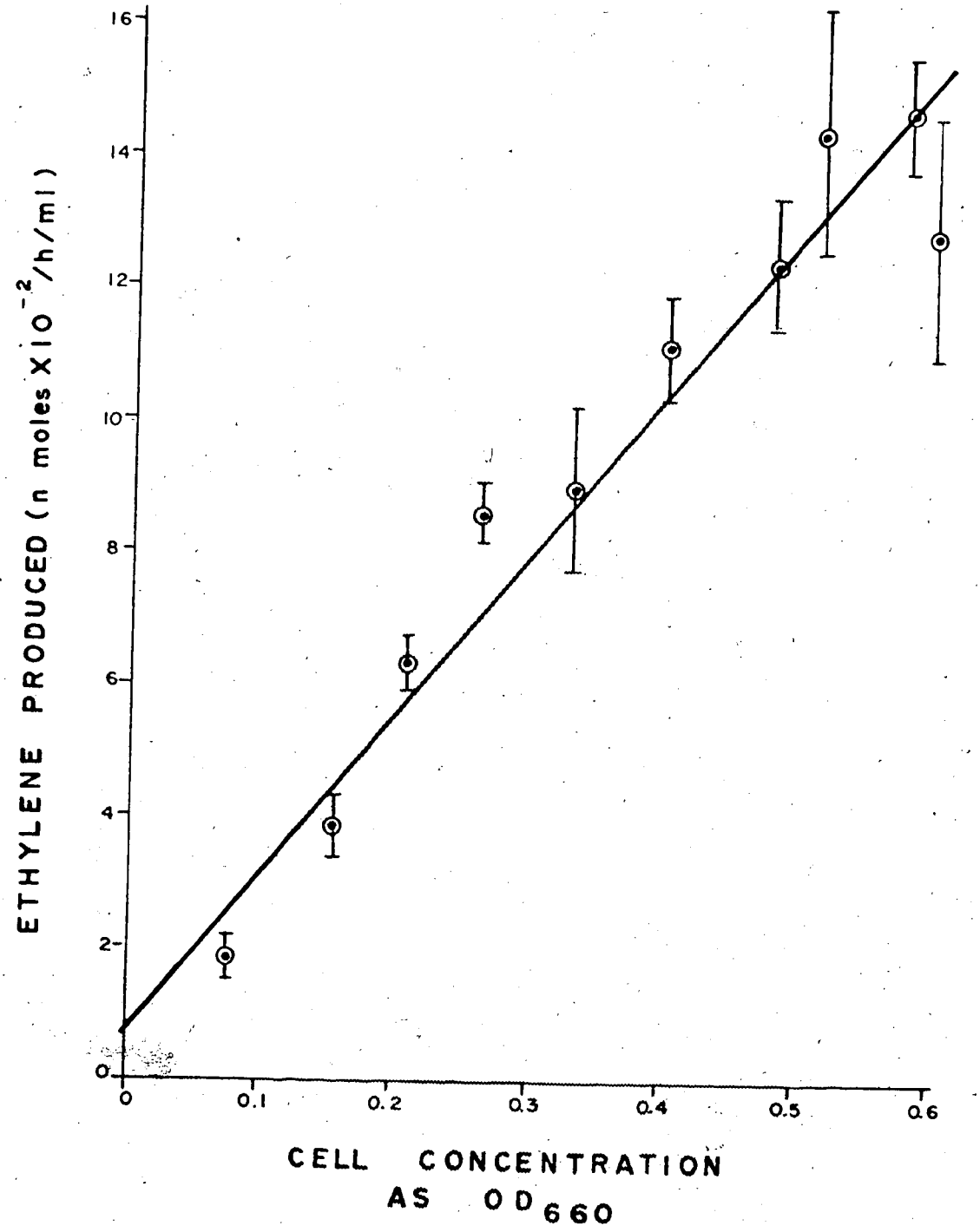

ETHYLENE PRODUCED (n moles X 10^{-2} / h / ml)
0
1
2
3
4
5
6
7
8
9
10
11
12
0
10
20
30
40
50
TEMPERATURE (°C)

FIGURE 6

THE EFFECT OF CELL NUMBER ON REDUCTION OF ACETYLENE BY *CLOSTRIDIUM PASTEURIANUM* W5

Actively growing cultures of *C. pasteurianum* W5 were incubated with 0.1 Atm. C_2H_2 as described in the text.

Each point represents the mean of four replicates and the error bars are ± one standard deviation. The line was plotted by the least squares linear regression method.


ETHYLENE PRODUCED (n moles X10^{-2}/h/ml)
16
14
12
10
8
6
4
2
0
0
0.1
0.2
0.3
0.4
0.5
0.6
CELL CONCENTRATION AS OD_{660}

number (OD_{660}) and C_2H_2 reduction.

d. Age of Cells: C. pasteurianum W5 was grown for one growth cycle in NFC mediun (250 ml) in a 1 l flask in which 1 g of $CaCO_3$ had been placed before autoclaving. The $CaCO_3$ acted as a buffer and as a source of CO_2. Inoculum (10% v/v) was from a stationary phase culture in the same medium. Incubation was at room temperature. Growth and nitrogenase activity were monitored for 133 hours by periodic removal by syringe of 5 ml aliquots which were placed in Hungate tubes and assayed for C_2H_2 reduction over a 1 hour incubation. The tubes were shaken at the start and again 5 minutes before the end of the assay. At one hour, growth was estimated by measuring OD_{600}, blanked against uninoculated media which had received exactly the same treatment. Acetylene reduction was then measured by removal of an aliquot of head gas followed by immediate analysis. The results are shown in Figure 7.

The rather lengthy lag period was probably caused by the sub-optimal temperature and by the use of an old inoculum which likely consisted largely of spores. Heat shocking was not employed. The significant observation from this experiment was that nitrogenase activity occurred only during active growth. When the rate of increase in OD_{600} dropped between 30 and 40 hours, the C_2H_2 reducing activity also dropped to just detectable levels. Although active growth and C_2H_2 reduction were both finished by 40 hours, the OD_{600} continued to rise in a more gradual manner until

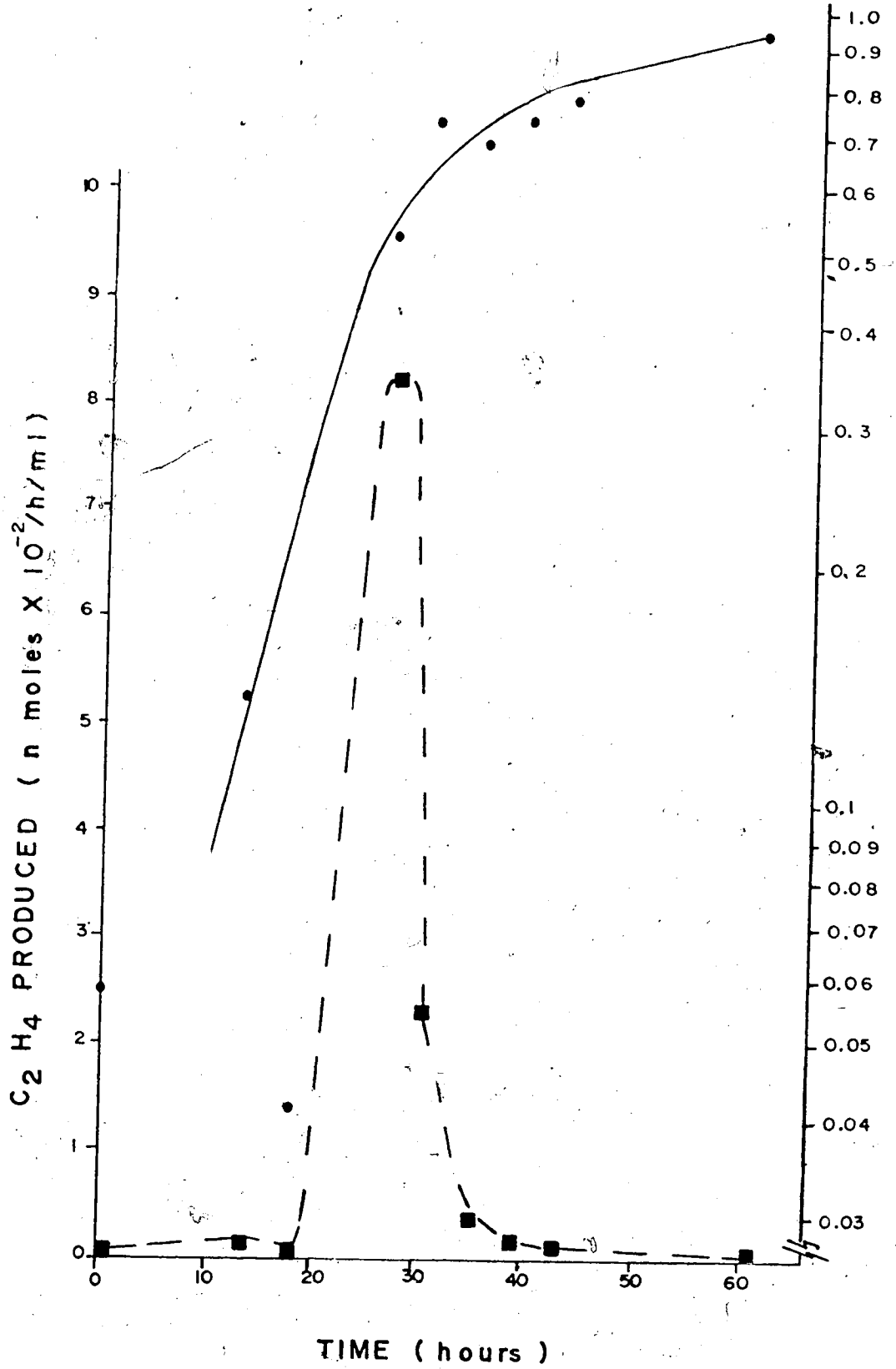
FIGURE 7

THE EFFECT OF THE AGE OF CELLS ON THE REDUCTION OF ACETYLENE BY CLOSTRIDIUM PASTEURIANUM W5

C. pasteurianum W5 was grown and assayed as described in the text.

Cell growth as OD_{660}.

Nitrogenase activity.


C_2H_4 PRODUCED (n moles X 10^{-2}/h/ml)
OD600
TIME (hours)
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

it had reached a value of 1.5 by 133 hours. It is, difficult to explain this increase in OD_{600} since growth without additional input of combined nitrogen seems unlikely. Some additional growth may occur on the products of autolysis. however this is probably insignificant since it reflects only a turn over [illegible]s. It is more likely due to reabsorption of NH_4^+ excreted during the period of N_2-fixation. Alternately, the increase in OD_{600} may simply reflect a change in the light scattering properties of the cells as they mature and begin to form spores.

e. Acetylene Concentration: Five ml aliquots of an actively growing culture in WW medium at 37°C were placed in pre-evacuated Hungate tubes and an atmosphere consisting of from zero to 96% (v/v) C_2H_2 with the balance being Ar was introduced. Incubation was for one hour at 37°C and activity was stopped with TCA. In addition to the standard analysis for C_2H_4 produced, the quantity of C_2H_2 in the head gas was determined by gas chromatography. Figure 8 shows that an optimum concentration of C_2H_2 exists in the region of 10% with a very rapid rise in activity as C_2H_2 concentration increases to optimum, and a slower drop as the level increases further. It is obvious that in order to minimize variation in the assay caused by minor errors in measuring the C_2H_2 into the assay vessels, concentrations of C_2H_2 must be kept above 5%. Similarly, in order to avoid inhibitory effects, concentrations above 20% should be avoided. The second line on Figure 8 shows how much C_2H_2 was actually

FIGURE 8

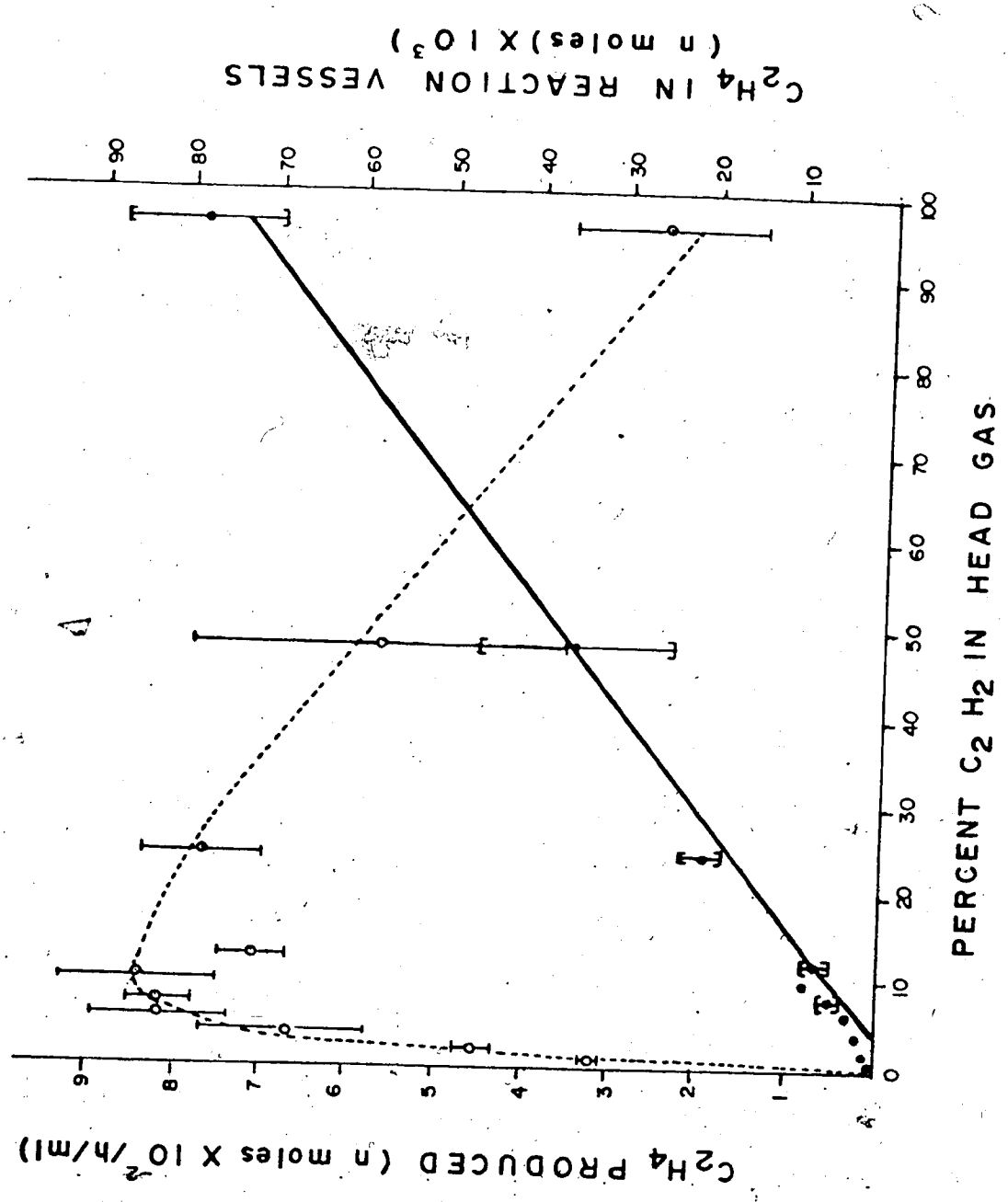
THE EFFECT OF THE CONCENTRATION OF ACETYLENE ON THE REDUCTION OF ACETYLENE BY *CLOSTRIDIUM PASTEURIANUM* W5

Actively growing cultures of *C. pasteurianum* W5 were incubated as described in the text.

-o-----o----- Nitrogenase activity.

—●———● The amount of C_2H_2 actually found in the assay vessel after the assay.

Each point represents the mean of triplicates and the error bars are ± one standard deviation.


C_2H_4 IN REACTION VESSELS
(n moles) X 10^3
PERCENT C_2H_2 IN HEAD GAS
C_2H_4 PRODUCED (n moles X 10^{-2}/h/ml)

delivered into each tube and illustrates, especially at the higher concentrations, how the variations in concentration of C_2H_2 between replicates influences the degree of variation in the replicates when C_2H_4 is measured.

f. pH: Aliquots (15 ml) of culture actively growing in WW medium at 37°C were dispensed into Ar filled Hungate tubes. This and all following manipulations were carried out at 4°C so as to minimize growth during handling. The tubes were then centrifuged for 15 minutes at top speed in a clinical centrifuge. The supernatant was drawn off by syringe and replaced by 10 ml of WW medium in which the phosphate buffer had been replaced by a β,β' dimethylglutamic acid:NaOH buffer at various pH values between 3.2 and 7.6. The pellet was resuspended and a 5 ml aliquot from each tube was transferred to an Ar filled 63 ml serum bottle. The head gas was adjusted to 10% C_2H_2 : 90% Ar and the cultures assayed for two hours at 37°C, then stopped with TCA and analysed.

A fairly broad pH optimum occurs near pH 7 (Figure 9) and no nitrogenase activity was detectable below pH 5.

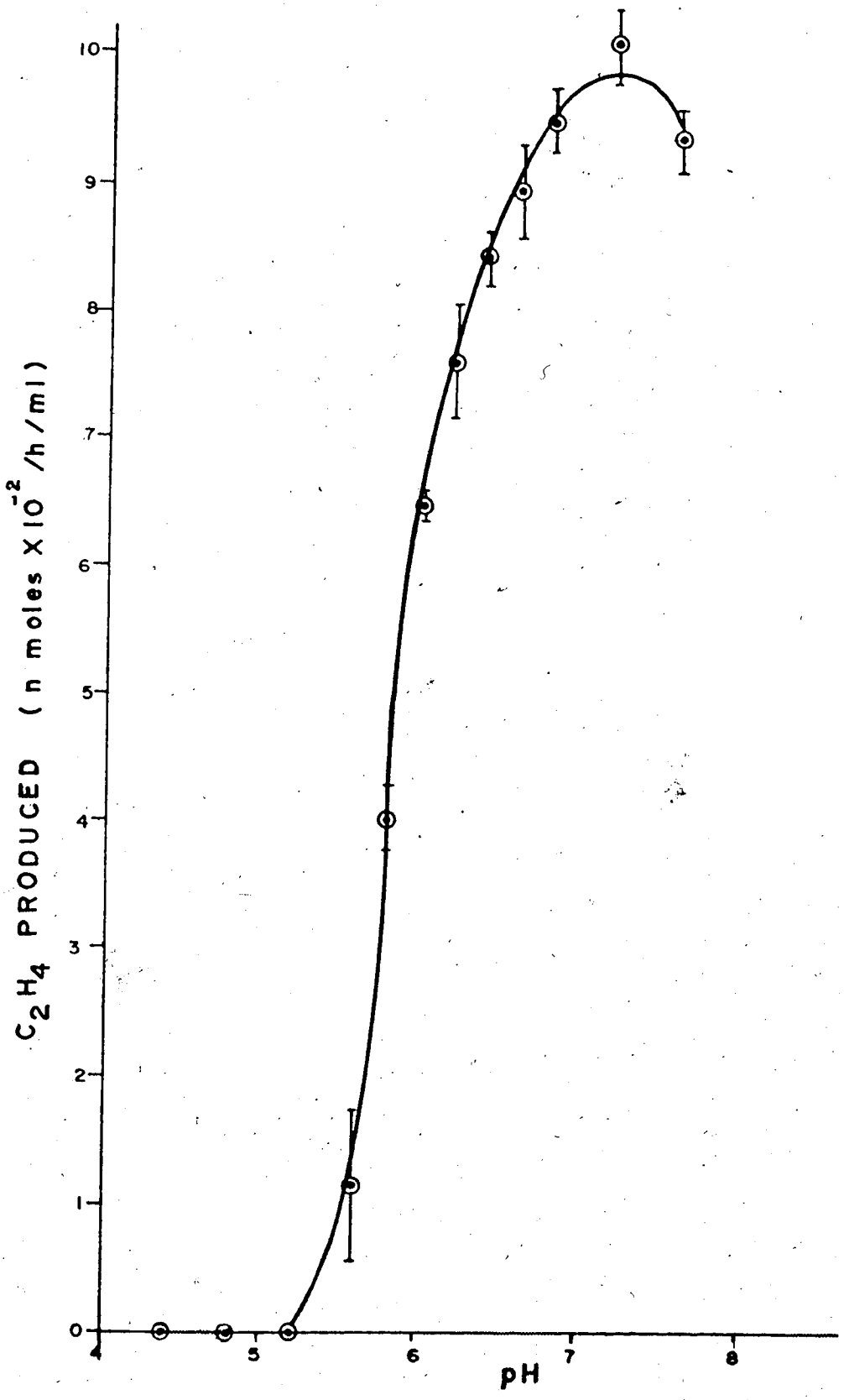
g. Gas:Liquid Ratio: Later assays were done in serum bottles rather than Hungate tubes to allow a greater volume of C_2H_2 to be used and thus reduce the effect of measuring error. Flett et al [37] have shown that the ratio of head gas to aqueous phase affects the proportion of C_2H_4 in the gas phase, and indicate that when the gas phase is small, very little of the C_2H_4 is in the gas phase and that at this

FIGURE 9

THE EFFECT OF pH ON THE REDUCTION OF ACETYLENE BY *CLOSTRIDIUM PASTEURIANUM* W5

Actively growing cultures of *C. pasteurianum* W5 were incubated in the presence of 0.1 Atm. C_2H_2 as described in the text.

Each point represents the mean of triplicates and the error bars are ± one standard deviation.


C_2H_4 PRODUCED (n moles X 10^{-2} /h/ml)
0
1
2
3
4
5
6
7
8
9
10
4
5
6
7
8
pH

point small variations in the ratio of gas to liquid can strongly affect the balance. However when the liquid phase is kept below 20% of the total volume, almost all of the C_2H_4 is in the gas phase and minor variations of the gas:liquid ratio will have little effect.

h. Summary: The conditions chosen for the assay of nitrogenase activity in C. pasteurianum W5 by C_2H_2 reduction include; 1. a two hour assay period, 2. growth and assay both at 37°C since this is the optimum temperature for both, 3. an atmosphere containing 10% C_2H_2 and 90% Ar, 4. a pH of 7.2 and 5. actively growing cells.

Under these conditions, the amount of C_2H_4 reduced is proportional to the population of N_2-fixing bacteria and estimates of the active population can be made from C_2H_4 production measurements.

iv. Interfering Reactions: To investigate the possibility of the production or consumption or either C_2H_2 or C_2H_4 by some competing non-biological reaction(s), various combinations of the assay reagents as specified in Table 8 were tested for the ability to change C_2H_4 concentrations in the headspace gas. The support gas was Ar. Reaction was for two hours at 37°C and was stopped with TCA.

Neither C_2H_2 nor C_2H_4 are evolved by any combination of non-biological components of the assay system (Table 8). It is possible that a very small amount of C_2H_4 was absorbed by the oil or by the cells, but if so, the amount was within experimental error and therefore is not of significance. The

TABLE 8

INDIVIDUAL COMPONENTS AND COMBINATIONS THEREOF ASSAYED FOR INTERFERENCE WITH THE ACETYLENE REDUCTION ASSAY

Composition*1	Ethylene Produced n moles/ml/h*2
Oil	0 ± 0
Medium	0 ± 0
Medium & Oil	0 ± 0
Culture	0 ± 0*3
Culture & Oil	0 ± 0*3
Ethylene	17,307 ± 1,346*3
Ethylene & Oil	16,471 ± 946
Ethylene & Medium	18,779 ± 322
Ethylene & Medium & Oil	18,323 ± 952
Ethylene & Culture	14,238 ± 2,039*3
Ethylene & Culture & Oil	13,215 ± 719*3
Acetylene	1 ± 0.1*3
Acetylene & Oil	0.8 ± 0.4
Acetylene & Medium	1.1 ± 0.3
Acetylene & Medium & Oil	1.1 ± 0.5
Acetylene & Culture	967.1 ± 117.8*3
Acetylene & Culture & Oil	808.1 ± 13.9*3

*1 Oil = 1 ml of Nipisi crude oil, untopped.
Medium = WW medium, 5 ml aliquots.
Culture = Actively growing C. pasteurianum W5 in WW medium, 5 ml aliquots.
Ethylene = 5 cc of 98% pure C_2H_4.
Acetylen = 5 cc of C_2H_2.

*2 Ethylene was measured and calculated as if all assays were of biological nature and producing C_2H_4 by reduction of C_2H_2.

*3 Mean of duplicates, plus or minus one standard deviation, and corrected to an assumed 5 ml sample volume. Assays not marked by *3 were quadruplicates.

supply of C_2H_2 contained a measurable amount of C_2H_4, for which allowance must be made.

2. The Effect of Crude Oil on N_2-Fixation in Pure Culture: In order to test the hypothesis that oil inhibits

nitrogenase activity, various amounts of Nipisi crude oil were dispensed as a 10% (v/v) solution in benzene into serum bottles. The bottles were flushed with air until all of the benzene had been removed, then flushed with Ar. Aliquots (5 ml) of an actively growing culture of C. pasteurianum W5 in WW medium were injected and allowed to equilibrate for two hours at 37°C. An aliquot of head gas was then replaced with C_2H_2 to create an atmosphere of 10% C_2H_2, 90% Ar and incubation was commenced at 37°C. After two hours the assay was stopped with TCA.

Three controls were included one received 16.67% oil and 5 ml of sterile medium but no bacteria. This ensured that no C_2H_4 was present in the oil or produced during incubation. The second contained no oil, but received 10 ml of benzene which was then evaporated off. This served as a check that no inhibitory or stimulatory trace substances were provided by the solvent. The final control received no oil or solvent and was assayed for nitrogenase activity immediately, without the equilibration period. This showed that no serious loss of activity was caused by pre-incubation under Ar for two hours.

The results in Table 9 indicate that there may be a slight inhibitory effect at higher concentrations of oil. This effect may however simply reflect the fact that both C_2H_2 and C_2H_4 are soluble in petroleum.

TABLE 9

THE EFFECT OF CRUDE OIL ON ACETYLENE REDUCTION BY PURE CULTURES OF CLOSTRIDIUM PASTEURIANUM W5

Amount of Oil (%)*1	Ethylene Produced n moles/ml/h*2
0.00	149 ± 13
0.02	165 ± 22
0.05	180 ± 18
0.10	181 ± 15
0.50	149 ± 31
0.99	190 ± 11
1.96	16 ± 13
2.91	15 ± 12
3.85	15 ± 10
4.76	167 ± 4
9.09	141 ± 14
16.67	91 ± 27
Solvent Control*3	164 ± 12
Preincubation Control	199 ± 11
No Culture Control	0 ± 0

*1 Amount of oil as % of total liquid volume, culture plus oil.
*2 Mean of triplicates, plus or minus one standard deviation.
*3 See text for explanation of controls.

B. MIXED FLORA ANALYSIS

1. Evaluation of Parameters:

i. General Principle: In working with natural samples such as muskeg it is not possible to establish optimum conditions as was done for the pure culture experiments. The best that can be done is to measure these parameters in the field or in the samples taken and where possible to reproduce them in the laboratory.

ii. Time: Since the population of N_2-fixing anaerobes

is considerably less in the muskeg than in pure culture, the two hour assay time established in sect[illegible] II,A,iii,a is insufficient. Twenty four hour assays [illegible] convenient and have been shown to be successful [illegible] muskeg systems [10].

iii. Temperature: Although the optimum temperature for C. pasteurianum W5 is 37°C, the temperature of the muskeg goes through a diurnal cycle and also varies from day to day as shown in Figure 3. The summer temperature ranges from about seven to sixteen degrees with a normal day time temperature of 12 to 13 degrees. The laboratory assays of muskeg samples were therefore conducted at 13°C.

iv. Age of Cells: Since the muskeg ecosystem is approximately a steady state system, there will be cells present at all stages of growth. As shown in Figure 7, only cells that are actively growing will have nitrogenase present, spores or resting cells will not contribute to the value of C_2H_2 reduction obtained.

v. Concentration of Acetylene: Since the atmospheric concentration of 10% C_2H_2 (Section II,A,1,iii,e) was optimal for cells in pure culture, it was used as a first approximation for the same cells in mixed cultures in natural materials.

vi. pH: In pure culture, C. pasteurianum W5 has a pH optimum of 7.2 and fixation is totally inhibited below pH 5.2 (Figure 9). Since the muskeg and oil spill samples have pH values for the most part below 7 (Appendix IV) and many samples are below 5.2, it is obvious that either other

organisms are contributing to N_2-fixation in th[illegible]eg or that other unknown factors are involved which allow the clostridial nitrogenase to function at lower pH levels than normal.

vii. Samples Removed to the Laboratory: In theory, samples which are assayed *in situ* in pots placed in the hole from which the sample was taken have the advantage of maintaining environmental conditions. In practice however, samples removed to the laboratory for assay are equally as good, since it is relatively easy to maintain a temperature in the range shown (Figure 3) to be maintained *in situ*. In fact there is an advantage to laboratory temperature control in that pots placed in the field are subject to a greenhouse effect which tends to raise the temperature. There are also the additional advantages of being able to handle large numbers of samples more convenien[illegible]in the laboratory than in the field where such things as accurate balances and cylinders of gas are difficult to transport and operate.

2. The Effect of Crude Oil on Nitrogen Fixation by Muskeg Samples:

i. *In Situ* Experiments: Two experiments were conducted to measure the *in situ* N_2-fixation in the oil spill as compared to control areas. In the first, samples of approximately 700 g (see Appendix VI for location and description of individual samples), were placed in large (1500 ml) polyethylene containers, and the containers replaced in the hole from which the samples were removed.

Ten percent of the head gas was replaced by C_2H_2. At zero time and again in 13 days, duplicate 50 cc samples of the head gas were removed and returned to the laboratory in pre-evacuated serum bottles for analysis. After sampling, the head gas was restored to its former composition of 10% C_2H_2 : 90% Air. The results of this experiment appear in Table 10.

TABLE 10

IN SITU AEROBIC ACETYLENE REDUCTION BY MUSKEG AND OIL SPILL SAMPLES

Sample Number	C_2H_2 Received	Elapsed Time (h)	Ethylene Produced moles/g Wet Wt.*1
Control Sites			
F4	0.1 Atm.	0	
		312	48 ± 81
F6	0.1 Atm.	0	0
		312	118 ± 0
Spill Sites			
F1	0.1 Atm.	0	0
		312	-*2
F2	0.1 Atm.	0	0
		312	23 ± 5
F3	0.1 Atm.	0	0
		312	0*3
F5	0.1 Atm.	0	0
		312	-*4

*1 Duplicate gas phase samples from the same assay vessel, plus or minus one standard deviation.
*2 Lost
*3 Control Container, received no sample.
*4 A small C_2H_4 peak may have been present, but if so it was masked by a peak from the volatile portion of the oil.

The second experiment was essentially the same as the first except that the samples were approximately 150 g of sub-surface material (Appendix VI). The containers were evacuated five times with a hand operated vacuum pump and refilled with Ar from a beach ball. The final atmosphere was 10% C_2H_2 : 90% Ar, and a control was included at each site to check for C_2H_4 being produced by plants or soil bacteria. The experiment was planned as a 48 hour assay but persistent heavy rains made access impossible. The results, shown in Table 11, indicate that the rate of C_2H_2 reduction decreases with time and shorter assay periods would be desirable. Very short assays during the heat of the day would tend to give high estimates of fixation, but a 24 hour period would probably give reasonable results.

Three of the four pots which had not received C_2H_2 showed measurable amounts of C_2H_4, indicating that the indigenous soil flora is producing C_2H_4. The amounts however are not large and can be compensated for with suitable controls. The appearance of C_2H_4 in pot 98 which had received C_2H_2 but not a sample indicates that there may be diffusion of externally produced C_2H_4 into the containers or that the polyethylene containers themselves are releasing C_2H_4, probably when warmed by the sun.

The two experiments show contradictory trends with respect to the oil spill, with the aerobic assay showing less fixation in the spill than in control sites, and the

TABLE 11

ANAEROBIC IN SITU ACETYLENE REDUCTION BY MUSKEG AND OIL SPILL SAMPLES

Sample Number	C_2H_2 Received	Elapsed Time (h)	Ethylene Produced n moles/g Wet Wt.
Control Sites			
F7	0	0	0
		3.85	0
		22.08	0
		400.83	0
F8	0.1 Atm	0	0
		3.83	11.1
		22.07	26.6
		400.82	74.4
F13	0	0	0
		376.75	2.0
F14	0.1 Atm	0	0
		376.52	414.4
Spill Sites			
F9	0	0	0
		19.75	2.9
F10	0.1 Atm	0	0
		19.37	163.4
		398.62	1,621.5
F11	0		0
		379.05	1.8
F12	0.1 Atm	0	0
		378.83	2,172.5
Control, No Sample*[1]			
F8	0.1 Atm	0	0
		378.25	2.8

*[1] The amount of C_2H_4 produced/g is calculated on the basis of an assumed 150 g sample.

anaerobic assay showing a four fold increase within the spill. The small number of samples in either experiment makes it difficult to form any conclusions and points out one additional advantage to laboratory assays over field assays in locations where motility of the researcher is

inhibited.

ii. Laboratory Experiments: In order to conduct a more extensive measurement of the C_2H_2 reducing ability of the mussel and oil spill, samples S/38 through S/59d were [illegible]ned and removed to the laboratory. Six (5 g) subsamples were weighed into serum bottles and sealed. In each set of 6 subsamples, three were designated as controls. One, a sterile control was autoclaved for 60 minutes at 121°C, incubated at room temperature for twelve hours then re-autoclaved. The second control was a blank and received no C_2H_2. The third control received 1 ml of $(NH_4)_2SO_4$ at a concentration of 11.792 mg/ml which corresponds to approximately 0.5 mg NH_4-N/g of sample. This is well above the level shown to inhibit nitrogenase synthesis [10,103]. All controls and samples were then flushed for five minutes with O_2 free N_2 containing approximately 3% CO_2 and allowed to equilibrate for 24 hours at 13°C. All were then flushed for five minutes with O_2 free Ar and with the exception of the blanks 10% of the head gas was replaced with C_2H_2. After an incubation of 24 h at 13°C, the assay was stopped by displacing and capturing [126] the head gas with water.

The results as shown in Appendix VII, and summarized in Table 12, indicate that in most cases the ammonia control totally inhibited N_2-fixation. In a few cases (eg. S/39b, Appendix VII) the lower value after subtracting the ammonia control as compared to the value with the sterile control subtracted suggests that a low level of nitrogenase was

TABLE 12

SUMMARY OF ACETYLENE REDUCTION BY MUSKEG AND OIL SPILL SAMPLES

Site	Ethylene Produced (Minus sterile Control) p moles/h/g Wet Wt.	p moles/h/g Dry Wt.
Control *1	995 ± 1,385	10,653 ± 18,058
Spill *2	533 ± 972	3,580 ± 7,133
Ratio Spill:Control	0.54	0.34

Site	Ethylene Produced (Minus Ammonia Control) p moles/h/g Wet Wt.	p moles/h/g Dry Wt.
Control*1	950 ± 1,401	9,654 ± 18,361
Spill*2	523 ± 975	3,563 ± 7,193
Ratio Spill:Control	0.55	0.37

*1 Mean of 15 samples, plus or minus one standard deviation.

*2 Mean of 33 samples, plus or minus one standard deviation.

present in the samples which was not inhibited by NH_4. This would indicate that it was pre-existing enzyme and that a slow rate of turnover was occurring.

The results with the sterile control values subtracted are the values which are used in comparisons with other data since they represent total levels of nitrogenase activity, not just that synthesised during the experiment. Only one sample, S/52b, produced any C_2H_4 in the absence of C_2H_2.

The levels of nitrogenase activity expressed as p moles/h/g dry weight (Appendix VII,A) are included primarily for comparison with the work of other researchers. However, the wet weight values (Appendix VII,B) are more meaningful for comparisons within the experiments of this study, since

there are vast differences in the water content of the samples. As can be seen from Table 12, when the results are expressed in terms of dry weight there is an apparent large inhibition of nitrogenase in the spill, however when these same results are put in terms of wet weight, the inhibition is not nearly so pronounced. Table 13 shows the breakdown of nitrogenase activity with depth in the muskeg. Only the

TABLE 13

ACETYLENE REDUCTION BY MUSKEG AND OIL SPILL SAMPLES ACCORDING TO SAMPLE DEPTH

	Ethylene Produced	
	p moles/h/g Wet Wt.	p moles/h/g Dry Wt.
Control Sites		
0 - 10 cm	568 ± 254	10,369 ± 3,268
10 - 20 cm	657 ± 766	6,838 ± 7,746
20 - 50 cm	1,323 ± 1,819	13,171 ± 24,632
over 50 cm	57 -	6,343 ± -
Spill Sites		
0 - 5 cm	620 ± 1,349	3,569 ± 7,568
5 - 10 cm	444 ± 738	1,966 ± 3,812
10 - 20 cm	507 ± 763	4,570 ± 9,291
20 - 50 cm	55 ± -	97 ± -

20-50 cm level in the control sites show a marked difference. This level includes the interface between the water column, which contains only grass and clumps of moss, and the clay bottom, which has a mixture of inorganic soil with roots and detritus in varying stages of decay. If, as

proposed by J.W.Costerton (Department of Microbiology, University of Calgary, personal communication), most bacteria in natural environments are attached to surfaces, then this layer could be expected to have the richest population since the decaying plant material would provide both substrate and surface for adhesion. In general however there is little variation of nitrogenase activity within the upper layers in the control sites. In the spill sites, the activity was lower overall and did not increase in the 20-50 cm layer. This probably reflects the fact that the spill area is much disturbed and the stratification does not exist as it does in the control sites.

An attempt to correlate the nitrogenase activity with other parameters observed revealed that there were no close correlations, but that some trends did occur. Samples which contained over 1% chloroform extractable oil did not demonstrate an appreciable amount of nitrogenase activity, the highest being sample S/53b which contained 8.3% oil and produced 564 p moles C_2H_4/h/g wet weight. Individual samples which did not contain oil were not necessarily more active, but the upper limit for these was 5,267 p moles C_2H_4/h/g wet weight, an order of magnitude higher. The lower limit in both cases was zero. There was no correlation between moisture content and nitrogenase activity but most of the more active samples were of high moisture content. This appears to be only a reflection of the higher water content in the control samples. Contrary to what would be expected

from the study of C. pasteurianum W5, samples in the neutral to slightly alkaline pH region showed very little acetylene reduction activity. All of the samples containing significant nitrogenase activity fell in the range from pH 4.1 to pH 6.1 with the most active samples being between pH 4.5 and 5.5. This trend towards more activity might suggest that organisms more acid tolerant than C. pasteurianum W5 are responsible for N_2-fixation in the muskeg soil. It is also possible that the samples of the type of soil which for other reasons best supported N_2-fixing anaerobes all had, fortuitiously, a low pH. The few samples which had pH values of 7 or higher were all inorganic soils with low moisture content, 21% or less (Appendix IV). Of this group, only sample S/59d had any nitrogenase activity.

When the results are compared with the type of soil involved (Table 14) it becomes apparent that the majority of the fixation is occurring at the clay-detritus interface layer in the control sites. The pure inorganic soil samples show very little activity regardless of source and amongst samples of organic soil, there is actually a slight increase in activity in the spill as compared to the control sites.

This very dramatically points out that in studies of this nature it is not sufficient to simply compare a control area with the spill area, but rather a complete survey of the area should be conducted and only those samples which are similar in character should be compared.

TABLE 14

SUMMARY OF ACETYLENE REDUCTION ACTIVITY ACCORDING TO SOIL TYPE

Site	Ethylene Produced (p moles/h/g Wet Wt.)		
	I*1	Q	I/Q
Control	268 (1)*2	491 (11)	3,083 (3)
Spill	247 (11)	790 (17)	287 (5)
All	249 (12)	673 (28)	1,336 (8)

*1 Symbols are explained in Appendix IV.

*2 Numbers in brackets are the number of samples used to arrive at each value. Each sample is itself the mean of triplicate assays, see Appendix VII,B.

III. General Discussion

The existence of a "Clostridial Bloom" in oil spills as proposed by Westlake and Cook [129] has been supported by the present study, however it was not nearly so pronounced in this study as indicated in their report. It is probable that the very existence of a bloom as well as its magnitude varies with the season and with local conditions. It is not surprising therefore to find variations in the numbers of clostridia found in different years.

It should be pointed out that in all of these tests, only those anaerobes capable of reducing sulfite were counted. This technique would therefore not distinguish *Clostridium* from *Desulfotomaculum*, nor would it include those clostridia unable to reduce sulfite.

Although no special effort was made to enumerate the pathogens in various areas of the muskeg, approximately 17% of the isolates obtained by all methods, have tentatively been identified as *C. perfringens* and an additional 10% shown to produce at least one recognizable toxin. With one quarter of the anaerobic isolates showing signs of pathogenicity, it is not unreasonable to conclude that pathogens are common in the muskeg and oil spill.

The muskeg was found to be a relatively hostile environment, with low pH and low levels of inorganic nutrients. The spillage of oil resulted in a slight increase in pH, and concentration of P, K, and Mn and a slight reduction in Al and NH_4-N. The water content of the oil

covered muskeg was also reduced.

Muskeg is an aquatic ecosystem. In as much as the average sample of muskeg has a moisture content in excess of 50% and the samples vary extensively, the use of wet weight is preferable to dry weight for comparison of samples from this kind of soil. If C_2H_2 reduction values are expressed relative to dry weight, the difference in moisture content between test and control samples will introduce a false variation. This will also occur if dry weight measurements are used in converting C_2H_2 reduction data to kg N_2/ha of land.

The standard deviation for most assays was large, (Figures 5 through 10) even where the analysed gas samples were from the same assay vessel (Table 10). The problem was partly alleviated (Figure 9) by the use of a container which allowed a high gas:liquid volume ratio [37], but it appears that substantial variation is introduced by the method used to handle the gaseous samples. It was found that the plastic disposable syringes used have a pronounced "memory" for both C_2H_2 and C_2H_4, therefore a new syringe was used for each sample. Although these syringes are calibrated to 0.01 cc, it is possible that the accuracy is noticably less than this, resulting in large errors when small quantities are measured. As the standard injection was 0.5 cc, a variation of only 0.01 cc would result in an error of 2%. It is therefore suggested that gas tight glass syringes be used for C_2H_2 reduction assays, or that quantitation be against

an internal standard such as ethane.

It was generally found that the variation between samples from within the control or spill areas was as great as the variation between the two areas. There were even large variations between subsamples from the same sample. It is believed that future studies on muskeg ecosystems must employ different criteria for selection of controls. Only those samples which are similar with respect to basic parameters such as temperature, pH, moisture content and soil type can be compared when trying to determine the effect of the an experimental parameter such as oil on the muskeg.

Blasco and Jordan [10] reported values of 6.4 ± 5.3, 76.4 ± 14.2, 27.8 ± 25.9 and 56.2 ± 3.8 n moles/h/g dry weight for anaerobic C_2H_2 reduction in muskeg in Ontario. The Nipisi control samples by comparison were not as active, with 10.3 ±3.3, 6.8 ± 7.7 and 13.2 ± 24.6 n moles/h/g dry weight for samples from similar depths, however the variability between the samples is about the same.

The effect of the oil spill on the level of nitrogenase activity in the soil is difficult to assess. The pure culture work with C. pasteurianum W5 shows that there is little effect on the already formed enzyme in growing cells. J. Danforth (Department of Microbiology, University of Alberta, unpublished data) demonstrated using Azotobacter vinlandii in the presence of oil in concentrations up to 1% (v/v) that the oil had, if anything, a slightly stimulatory

effect on acetylene reduction by cells grown in the presence of oil.

When natural materials were studied, due to the great variability of the samples, some experiments show an increase whereas others show a decrease in nitrogenase activity. For *in situ* assays, aerobic assays show a repression by oil whereas anaerobic assays show an enhancement. When samples were moved to the laboratory, the overall indication was that a small decrease in enzyme activity occurred in the spill. When these data were broken down according to the type of material in each sample, it was found that inorganic soils contained the same level of nitrogenase activity in the spill as outside. The organic samples from within the spill exhibited slightly more activity than those from control sites, and the mixed inorganic/organic samples were quite a bit more active in control sites.

D. C. Jordan (personal communication) found that application of 90 weight crankcase oil to Devon Island tundra resulted in an 87.3% inhibition of nitrogenase activity within 24 hours. Since N_2-fixation at this location was due almost entirely to blue-green bacteria, the inhibition was likely due to initial killing of the Cyanophytes. Studies on the long term effects of this artificial spill are continuing.

It appears that, although there is an initial decrease in N_2-fixation when an oil spill occurs, over a period of a

few months or years, the overall level returns to very nearly the normal value in the strata below the oil.

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APPENDIX I

Formula of Media.

BLOOD AGAR (BA)

Trypticase Soy Broth (BBL)	30 g
NaCl	5 g
Agar (Difco)	15 g
Distilled water	950 ml

Autoclave for 15 min at 121°C then cool to 47°C, add 50 ml of sterile sheeps blood and dispense into slants or plates.

B 10 ± SO_3^{2-}

K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4$	0.2 g
NaCl	0.2 g
$MnSO_4$	0.05 g
$NaMoO_4$	0.05 g
$CaSO_4$ (Saturated solution)	10 ml
Yeast Extract (Difco)	5 g
Peptone (Difco)	5 g
Ferric Phosphate	4.7 g
Distilled water to make	1000 ml

Adjust pH to 7.3 with NaOH, then autoclave for 15 min. After cooling, dispense into sterile tubes (10 ml/tube) and add 0.1 ml of 10% (w/v) Na_2SO_3 (filter sterile) to each tube.

BUFFERED THIOGLYCOLATE SOLUTION

(Marshall et al, 1965)

Solution A

K_2HPO_4	5.7 g
$NaHCO_3$	2.8 g
Distilled water to make	100 ml

Solution B

$HSCH_2COONa$	13.3 g
Distilled water to make	100 ml

Autoclave solutions A and B separately then mix 35 ml of solution A with 15 ml of solution B, and add to TSN agar at the rate of 25ml per litre after the agar has been autoclaved and allowed to cool to below 47°C.

DILUTION MEDIUM (DM)

NaCl	10 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
Na-Thioglycolate	2 g
Distilled water to make	1000 ml

Adjust pH to 7.0 with NaOH, dispense into tubes or bottles then autoclave. These dilution blanks were used immediately, or heated in a boiling water bath for 10

minutes and cool just prior to using.

EGG YOLK AGAR (EYA)
(Dowell and Hawkins, 1974)

Trypticase (BBL)	40 g
Na_2HPO_4	5 g
NaCl	2 g
$MgSO_4$ (5% aqueous solution)	0.2 ml
Glucose	2 g
Agar (Difco)	25 g
Distilled water to make	1000 ml

Adjust the pH to 7.3 then autoclave for 15 min at 121°C, cool to 60°C, then add the yolk of two eggs, mix and pour the plates. The eggs must be from chickens on an antibiotic free ration. The egg shells were decontaminated before the yolks were separated by immersing the eggs in a beaker of 95% ethanol for one hour.

NITROGEN FREE AGAR (NFA)

Glucose	10 g
Sucrose	10 g
$CaCl_2$	10 mg
$MgSO_4$	10 mg
$FeSO_4$	3 mg
$NaMoO_4 \cdot 2H_2O$	1.5 mg
K_2HPO_4	15.66 g
KH_2PO_4	1.36 g
Biotin	2 ug*
p-Aminobenzoic Acid	0.8 mg
Na-Thioglycolate	2 g
Agar (Difco)	15 g
Phenol Red	10 mg
Distilled water to make	1000 ml

*The vitamins were filter sterilized and added after the autoclaved medium (15 min at 121°C) had cooled to approximatly 47°C. After gentle but thorough mixing, plates were poured.

NITROGEN FREE MEDIUM B (NFB)

NFB is identical to NFA except that the agar and phenol red are omitted.

NITROGEN FREE MEDIUM C (NFC)

NFC is identical to NFB except that the K_2HPO_4 and KH_2PO_4 are omitted. When used, an excess of $CaCO_3$ is added to the flasks prior to autoclaving to provide a buffer system and a source of CO_2.

NUTRIENT GELATIN (NG)

Bacto Beef Extract (Difco)	3 g
Bacto Peptone (Difco)	5 g
Bacto Gelatin (Difco)	120 g
Distilled water to make	1000 ml

The solid ingredients were disolved in the distilled water then warmed to 50°C to melt the gelatin and poured into tubes (10 ml/18x150 mm tube) then autoclaved for 15 min at 121°C.

REDUCED BLOOD AGAR (RBA)

Reduced blood agar is the same as blood agar (BA) except for the addition of 0.5 g/liter of L-cysteine HCl to the broth before autoclaving.

SKINNERS MEDIUM (SM)

(Skinner, 1971)

K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$MnSO_4 \cdot 7H_2O$	0.01 g
$CaCl_2$	0.01 g
Sucrose	10 g
Yeast Extract (Difco)	1 mg
$Na_2MoO_4 \cdot 2H_2O$	0.025 mg
Trace Elements Mixture	1 ml
Soil Extract	10 ml
Na-Thioglycolate	1 g
Distilled water to make	1000 ml

The medium was dispensed into tubes (10 ml/tube), Durham tubes were added and the medium was autoclaved for 15 min at 121°C. If not used immediately it was heated for ten minutes in a boiling water bath then cooled just before using. Incubation was in an atmosphere of N_2 which was free of O_2 or H_2 but with a trace of CO_2 added.

SOIL EXTRACT (SE)

(Skinner, 1971)

Equal weights of garden soil and water were heated at 55°C for minutes, filtered through Whatman No.1 paper then autoclaved and stored until required.

TRACE ELEMENTS MIXTURE (TEM)
(Skinner, 1971)

$Na_2MoO_4 \cdot 2H_2O$	0.05 g
$Na_2B_4O_7 \cdot 10H_2O$	0.05 g
$CoCl_2 \cdot 6H_2O$	0.05 g
$CdCl_2 \cdot 2.5H_2O$	0.05 g
$CuSO_4 \cdot 5H_2O$	0.05 g
$ZnSO_4 \cdot 7H_2O$	0.05 g
$MnSO_4 \cdot H_2O$	0.05 g
$FeCl_3 \cdot 6H_2O$	0.05 g
Distilled water to make	1000 ml

The trace elements mixture was stored saturated with CO_2 and used at a rate of 1 ml per liter of media to give final concentrations of 5×10^{-8} g/ml of each compound.

TSN AGAR (TSN)
(Marshall et al, 1965)

TSN agar (BBL) was prepared and used in accordance with the instructions on the bottle. Buffered thioglycolate solution was added after autoclaving.

WESTLAKE AND WILSON MEDIUM (WW)
(Westlake and Wilson, 1959)

Sucrose	20 g
K_2HPO_4	15.66 g
KH_2PO_4	1.36 g
$MgSO_4$	0.25 g
$CaCl_2$	0.05 g
Fe (as $FeSO_4 \cdot 5H_2O$)	3 ppm
Mo (as $NaMoO_3$)	1.5 ppm
Biotin	0.002 mg *
p-Aminobenzoic Acid	0.8 mg *
Distilled water to make	1000 ml

* The vitamins were filter sterilized separately and added to the bulk of the medium after autoclaving.

APPENDIX II

Source of Isolates

Isolates provided by Dr.F.D.Cook were obtained from the following sources (F.D.Cook; Personal Communication).

Isolate	Source
1	Nipisi bog water, oil free.
2	Surface of 48" pipe section, No.13 Interprovincial Pipeline.
3	As for 2.
4	As for 2.
5	Swan Hills plot 18, 10-20 cm.
6	A colonial variant of 5.
7	Nipisi spill No.2, 8-20 cm oily.
8	As for 7.
9	Nipisi spill No.1, water under oil.
10	As for 9.
11	Muskeg 1/2 mile north of Nipisi No.1 spill.
12	Nipisi bog water, oil free.
13	Tar sand, Fort McMurray, Alberta.
14	Imperial Oil; Formation water, North Cantel field.
15	Liard River Hot Springs, Mile 495 Alaska Highway, B.C.
16	Scraping from Rainbow pipeline No.3.
17	As for 16
18	Scraping from Rainbow pipeline No.1.
19	As for 18.
20	As for 18.
21	As for 18.
22	As for 18.
23	Scraping from Interprovincial Pipeline, 48" pipe, site No.1.
24	A colonial variant of 23.

APPENDIX III

Sites and Descriptions of Muskeg Samples

Sample	Obtained	Location and Description
S/1	18/10/74	Nipisi spill No.1. North end of the spill in an area disturbed by a tracked vehicle. The sample was taken from 10 cm below the surface of a ridge between two tracks. (SITE A, FIGURE 1) Sample consists of oily moss but is fairly well drained.
S/2	18/10/74	From the same site as S/1, but the sample was taken from 10 cm below surface of the hollow of the track. (SITE A, FIGURE 1) Sample is moss with much flowing oil.
S/3	18/10/74	Outside the dike at the east side spill in the seismic line, 0-10 cm (SITE M, FIGURE 1)
S/4	18/10/74	From the same site as S/3, but sample is decaying moss and mud from the 10-20 cm level. (SITE M, FIGURE 1)
S/5	18/10/74	Nipisi spill No.1. From the east side of the spill, just to the north west of a pile of straw bales. Sample is water saturated moss from the 0-10 cm level. (SITE M, FIGURE 1)
S/6	18/10/74	In the bog just south of the road at the Texaco lease, about 1 km north east of the spill, along the seismic line. Sample consists of decaying moss from the 10-20 cm level. (SITE N, FIGURE 1)
S/7	9/6/75	Nipisi spill No.1, oil-soaked surface soil from near the collecting pond, 0-10 cm level. (SITE B, FIGURE 1)
S/8	9/6/75	Same site as S/7, sample is subsurface (10-20 cm) moss, clay, grass. (SITE B, FIGURE 1)
S/9	9/6/75	Nipisi spill No.1. Oil from the collecting pond.

Sample	Obtained	Location and Description
S/10	9/6/75	Water from the trench by the "bridge" at the north of the spill, contains some oil. (SITE C, FIGURE 1)
S/11	9/6/75	Nipisi spill No.1, material from the 10-20 cm level under standing oil in the region of dead trees in the western bulge of the spill. Sample is moss, spruce needles and twigs, all very oily. (SITE D, FIGURE 1)
S/12	9/6/75	Material from the 10-20 cm level just outside the dike at the west of the spill. (SITE O, FIGURE 1)
S/13	9/6/75	Nipisi spill No.2. Pond water from east of the plots in a burned area.
S/14	9/6/75	Nipisi spill No.2. Pond water from north of the plots in the burned area.
S/15	9/6/75	Nipisi spill No.2. Pond water from north east of the plots in the unburned area.
S/16	9/6/75	Rainbow spill. Water from a pond in the spill area (north of the shed), but showing no oil on the surface.
S/17	9/6/75	Rainbow spill. Water on oil soaked soil, southwest from shed.
S/18	9/6/75	Rainbow spill. Water from below oil in a pond at the end of the causeway, south-southwest of the shed.
S/19	9/6/75	Nipisi spill No.2. Control soil (clay-sand) from near the woods on the path to the spill.
S/20	9/6/75	Nipisi spill No.2. Oil soaked material from the burned area.
S/21	9/6/75	Nipisi spill No.2. Oil soaked material from the unburned area.
S/22	9/6/75	Rainbow spill. Bog material from below oil (moss and clay/mud).
S/23	9/6/75	Rainbow spill. Soil (gleyed clay) from below oil, southwest from the shed.

Sample	Obtained	Location and Description
S/24	9/6/75	Rainbow spill. Soil (clay) from below oil, southwest from the shed.
S/25	9/6/75	Rainbow spill. Oil soaked surface soil from northwest of shed.
S/26	9/6/75	Nipisi spill No.1. Oil soaked muskeg material from just west of the plots, north of the collecting pond. (SITE E, FIGURE 1)
S/27	9/6/75	Nipisi spill No.1. Oil free muskeg material from the edge of the cut line 25 meters north of the dike at the bridge. (SITE P, FIGURE 1)
S/28	22/6/75	Control. Subsurface muskeg material (moss) from 20 meters west of the road at a point approximately 3 km east and north of the point where the Peace River pipeline crosses the road north of the Nipisi No.1 spill. (SITE Q, FIGURE 1)
S/29	22/6/75	Nipisi spill No.1. Oil soaked muskeg from just north of the collecting pond. (SITE F, FIGURE 1)
S/30	22/6/75	Control. Subsurface dead and decaying moss from 30 meters west of the road approximately 2.4 km west of Nipisi No.1 spill, just after the road turns south. (SITE R, FIGURE 1)
S/31	14/9/75	Control site. Approximately 100 meters north of the road in the middle of the Mitsue pipeline cut line, and directly north of Nipisi spill No.1. Sample is clay from below approximately 30 cm of water. (SITE T, FIGURE 1)
S/32	14/9/75	Control site. Approximately 1 meter east of S/31. Sample is grey (gley) mud from below 30 cm of water. (SITE T, FIGURE 1)
S/33	14/9/75	Control site. Two meters east of S/31. Sample is sphagnum from 10-30 cm below the surface of the water. (SITE T, FIGURE 1)

Sample	Obtained	Location and Description
S/34	14/9/75	Nipisi spill No.1. Oil soaked sphagnum from 10-20 cm below the surface of a ridge between two tracks. (SITE A, FIGURE 1)
S/35	14/9/75	Nipisi spill No.1. Sample is oil and oil soaked sphagnum from the depression of a track (10-20 cm level). (SITE A, FIGURE 1)
S/36	17/10/75	Norman Wells Spill No.2. Sample is control tundra (2-2).
S/37	17/10/75	Norman Wells Spill No.2. Sample is tundra from within the spill (2-8).
S38	4/10/76	Control Site. Approximately 100 meters north of the road at Nipisi spill No.1., between the Mitsue and Peace River pipelines. The site is just into a clump of dead bush at the east of a grassy clearing. Five samples were taken at this site; a; surface moss (0-10 cm), living sphagnum. b; subsurface moss (10-20 cm), dead but not decaying moss. c; bottom muck. At a depth in water of approximately 50 cm a solid clay bottom appears. The interface from which the sample was taken is made up of a mixture of mud and decaying grass and sphagnum. (SITE U, FIGURE 1) d; bottom clay (below 50 cm of water). e; bottom muck, similar to c.
S/39	4/10/76	One meter west of S/38, in an area of tall grass standing in 50 cm of water. (SITE U, FIGURE 1) a; grass and water from upper 40 cm. b; grass and moss from the 40-50 cm level.
	0/76	Two meters west of S/38, sample is roots and decaying grass from the 40-50 cm level, just above the clay. (SITE U, FIGURE 1)
S/	76	Three meters west of S/38, in the centre of a grassy clearing. Sample is bottom

Sample	Obtained	Location and Description
		grass. (SITE U, FIGURE 1)
S/42	4/10/76	Four meters west of S/38. Sample is bottom grass from the 40-50 cm level. (SITE U, FIGURE 1)
S/43	4/10/76	Five meters west of S/38, at the edge of the grassy area. Sample is grass and clay from the 40-50 cm level. (SITE U, FIGURE 1)
S/44	4/10/76	Six meters west of S/38, in a hummock of sphagnum. Sample is moss from 10-20 cm below surface (just above the water level). (SITE U, FIGURE 1)
S/45	4/10/76	Control site. In a clump of live spruce to the east of the Peace River Pipeline cut. Approximately 100 meters east of S/38. Sample is decomposing moss from 100-150 cm below surface. (SITE W, FIGURE 1)
S/46	4/10/76	Same location as S/45. Sample is moss and grass from the 0-20 cm level. (SITE W, FIGURE 1)
S/47	4/10/76	Approximately three meters south of S/45. Sample is dry moss taken from the centre of a hummock, (10-20 cm level). (SITE W, FIGURE 1)
S/48	4/10/76	Nipisi spill No.1. From the disturbed area at the north end of the spill, about four meters east of the bridge. Sample is a composite core of the top 50 cm and is composed of oil soaked moss and grass. (SITE G, FIGURE 1)
S/49	4/10/76	Nipisi spill No.1. Same location as S/48. Samples are; a; surface crust of grass, moss and oil, less than 1 cm thick. b; 1-5 cm level, oily fibrisol. c; 5-10 cm level, oily fibrisol. d; 10-20 cm level, oily fibrisol. (SITE G, FIGURE 1)
S/50	4/10/76	Nipisi spill No.1. Mud from the ditch around the spill, taken at the northwest

Sample	Obtained	Location and Description
		bight. (SITE H, FIGURE 1) a; surface, 0-10 cm, oily mud. b; 10-20 cm layer, no visible oil.
S/51	4/10/76	Nipisi spill No.1. Site is approximately half way between the ditch and the collecting pond, (SITE I, FIGURE 1), in an area of oil soaked dry grass. There is quite a bit of regrowth of grass in this area. a; surface crust, oily fibrisol. b; 5-10 cm, oily fibrisol. c; 10-20 cm, oily fibrisol.
S/52	4/10/76	Nipisi spill No.1. Site is four to six meters north of the collecting pond in an area of tracks and ridges made by a tracked vehicle. Samples were taken from the top of a ridge. (SITE F, FIGURE 1) a; 0-5 cm, dry oily moss. b; 5-10 cm, packed oily moss and clay. c; 10-20 cm, packed oily moss and clay.
S/53	4/10/76	Nipisi spill No.1. In the bottom of the rut adjacent to the ridge where sample S/52 was taken. (SITE F, FIGURE 1) It was very wet here and the standing water ran into the hole as the samples were being taken so some mixing will have occurred. a; 0-5 cm, very wet very oily moss mixed with clay. b; 5-10 cm, same as a. c; 10-20 cm, same as a.
S/54	4/10/76	Nipisi spill No.1. Site is at the northwest edge of the collecting pond, about 50 cm from the oil. (SITE B, FIGURE 1) a; 0-5 cm, oil soaked sandy clay. b; 5-10 cm, sandy clay. c; 10-20 cm, clay.
S/55	4/10/76	Nipisi spill No.1. Bottom mud from the collecting pond. The pond has 5-7 cm of thick oil on the surface then water for a depth of about 15 cm at the point sampled and a clay mud bottom.
S/56	4/10/76	Nipisi spill No.1. Soil from a mound

Sample	Obtained	Location and Description
		about three meters west of the collecting pond and at an elevation of about one meter above the oil/water level. (SITE J, FIGURE 1) a; 0-5 cm, clay, some plant material, no visible oil. b; 5-10 cm, clay, no oil. c; 10-20 cm, clay, no oil.
S/57	4/10/76	Nipisi spill No.1. Site is about 30 meters southeast of the collecting pond, on the top of a mound of dry oily peat moss. (SITE K, FIGURE 1) a; 0-5 cm, very dry moss, some oil. b; 5-10 cm, as for a. c; 10-20 cm, as for a.
S/58	4/10/76	Nipisi spill No.1. Approximately 200 meters southeast of the collecting pond, in the middle of the spill, where all of the trees had been knocked down and the surface is covered with an interconnecting mat of criss crossed branches interspersed with oil soaked clumps of dead grass and moss. Standing pools of oil abound. (SITE L, FIGURE 1) a; 0-5 cm, very oily moss and assorted small twigs. b; 5-10 cm, same as a. c; 10-20 cm, same as a.
S/59	4/10/76	Nipisi spill No.1. Approximately ten meters east of the collecting pond on a mound of clay by the pipeline valve. (SITE "S", FIGURE 1) a; 0-1 cm, a surface crust of course sand and some oil. b; 1-5 cm, course damp sand. c; 5-10 cm, clay with some sand. d; 10-20 cm, clay.

APPENDIX IV

PHYSICAL PARAMETERS OF MUSKEG AND WATER SAMPLES FROM CONTROL AND OIL SPILL SITES

Sample Number*[1]	Sample Type*[2]	pH	Percent Moisture*[3]	Percent Oil*[4]
A. Control Samples				
S/16	A	5.62	---	---
S/19	I	5.70	---	---
S/27	O	4.05	---	---
S/28	O	3.90	---	---
S/30	O	4.10	---	---
S/38a	O	4.50	95.18 ± 0.17	---
b	O	4.62	96.16 ± 0.26	---
c	I/O	5.25	49.36 ± 30.41	---
d	I	---	22.38 ± 0.01	---
e	I/O	---	92.84 ± 0.50	0.475 ± 0.232
S/39a	O	5.40	47.67 ± 4.30	---
b	O	4.55	94.72 ± 1.46	---
S/40	O	4.80	95.08 ± 0.55	---
S/41	O	4.91	91.02 ± 0.93	---
S/42	O	4.70	92.62 ± 0.37	---
S/43	I/O	5.30	89.11 ± 1.12	---
S/44	O	4.95	96.41 ± 0.30	---
S/45	O	4.35	90.88 ± 0.18	---
S/46	O	4.55	94.11 ± 0.10	0.240 ± 0.009
S/47	O	3.78	85.14 ± 0.76	---
B. Oil Spill Samples				
S/7	I	6.45	---	---
S/8	I/O	5.45	---	---
S/10	A	6.59	---	---
S/11	O	4.70	---	---
S/12	O	4.20	---	---
S/13	A	6.65	---	---
S/14	A	5.95	---	---
S/15	A	6.40	---	---
S/17	A	6.10	---	---
S/18	A	6.40	---	---
S/20	O	6.00	---	---
S/21	O	5.85	---	---
S/22	I/O	5.34	---	---
S/23	I	6.55	---	---
S/24	I	6.73	---	---
S/25	I	6.22	---	---
S/26	O	5.15	---	---
S/48	O	4.30	91.12 ± 0.36	---
S/49a	O	3.70*[5]	33.01 ± 2.60	---

Sample Number*1	Sample Type*2	pH	Percent Moisture*3	Percent Oil*4
b	O	4.15	82.10 ± 1.24	12.065 ± 1.688
c	O	4.35	88.12 ± 0.63	---
d	O	4.29	89.28 ± 0.35	---
S/50a	O	5.30	92.85 ± 0.03	---
b	O	5.08	95.33 ± 0.01	0.135 ± 0.056
S/51a	O	5.98	76.37 ± 4.01	---
b	O	5.22	79.94 ± 0.93	---
c	O	4.95*5	87.59 ± 0.24	0.849 ± 0.028
S/52a	O	5.20	17.83 ± 0.87	28.630 ± 0.979
b	I/O	6.20	46.86 ± 7.77	---
c	I/O	5.58	63.29 ± 0.52	---
S/53a	I/O	5.95	46.91 ± 0.96	---
b	I/O	6.08	82.00 ± 0.40	8.323 ± 1.199
c	I/O	5.98	85.99 ± 1.27	---
S/54a	I	7.44	14.14 ± 2.35	---
b	I	7.64	20.76 ± 1.23	16.070 ± 5.544
c	I	8.10	14.11 ± 0.36	---
S/55	I	6.92	42.58 ± 1.13	5.924 ± 0.267
S/56a	I	7.54	20.33 ± 1.34	---
b	I	7.42	16.94 ± 0.18	0.186 ± 0.112
c	I	7.70	18.18 ± 0.91	---
S/57a	O	4.80*6	36.34 ± 16.14	---
b	O	5.10*5	60.36 ± 1.61	---
c	O	5.14	82.29 ± 2.12	4.965 ± 0.155
S/58a	O	4.42	54.20 ± 4.50	
b	O	4.11	70.03 ± 0.30	37.526 ± 2.425
c	O	5.15	88.03 ± 0.54	---
S/59a	I	7.43	10.67 ± 0.12	0.326 ± 0.047
b	I	7.32	11.20 ± 0.47	---
c	I	8.12	15.22 ± 0.49	---
d	I	8.23	15.19 ± 0.34	0.374 ± 0.026

*1 Refer to Appendix III for detailed descriptions the various samples.

*2 A = aqueous, samples containing no solid matter.
I = inorganic, samples were composed of clay or sand or a combination of the two, but contained no visable organic matter.
O = organic, samples were composed of moss, grass and decaying vegetable matter but contained no visable clay or sand.
I/O = inorganic/organic, samples were a mixture of the two types.

*3 Mean of Duplicates, plus or minus one standard deviation.

*4 Mean of Duplicates, plus or minus one standard deviation. % Oil as chloroform extractable material.

*5 In order to recover sufficient aqueous phase on which to perform the pH measurement, it was necessary to add twice the usual amount of water.

*6 This sample was so dry that six times the normal amount of water were required.

APPENDIX V.

CHARICTERISTICS OF CLOSTRIDIAL SPECIES AND ISOLATES FROM CONTROL AND SPILL SITES

Species / Isolate	Source	Lecithinase	Lipase	Haemolysis	TSN	$SO_3^{=}$	Gelatin	N_2	O_2	Spores	Gram	Morphology
C. botulinum		+	-	Y	-	+	-	--	-	+ (aδb)	+	S,P,STS,SV
C. novyi		-	-	β	-	-	-	--	-	+ (aδb)	+	S-M,O
C. perfringens		+	-	α	+	±	-	--	-	+ (aδb)	+	M-L,SL
C. tetani		+	-	Y	-	+	-	--	-	+ (aδb)	+	M-L,SL
C. pasteurianum WS		-	-	Y	-	-	-	++	-	+ (aδb)	+	M-L,SL,STS,SV
Isolates from control samples.												
1		+	-	β	-	+	+	-	-	+ (a)	+	M-L,SL
11			-	β	+	+	+	-	-	+ (aδb)	+	S,STS,SV
12			+	β	-	+	+	-	-	+ (aδb)	+	S,P,CS
36	S/6		-	β		±		-	-	- (b)	-	S
37	S/6			β				-	-			
38	S/6		-	β								
39	S/6	-	-	Y				-	-	- (b)	-	S-M,SL
40	S/6	-	-	Y		±		-	-	+ (b)	±	M,SL,TS,SV,FS
42	S/6	-	-	Y		±		-	-	-	-	M
43	S/6	-	-	Y		±		-	-	+ (b)	±	S,FS,O
69	S/27	-	-	α				-	-	+ (b)	±	M,FS
70	S/27	+	-	Y		±		-	-	+ (b)	+	S,P,CS,SV
71	S/27	-		β		±		-	-		+	M
72	S/27	-	-	Y		-		-	-	+ (b)	+	S,P,CS,SV
73	S/32			Y		-		+	+	- (b)	-	M-L
74	S/32	-	-	Y		-		++	-	+ (aδb)	+	S,P,CS,SV
75	S/32			Y		-		++	-	-	+	M
76	S/32			Y		-		++	-	-	+	M
77	S/32			Y		-		++	-	+ (b)	+	S,P,CS,SV
								++	-	+ (a)	±	S-M,
Isolates from spill sites.												
2		-	-	Y	-	+	-	-	-	+ (aδb)	+	M-L,SL,TS,SV
3		-	-	Y	-	+	-	-	-	+ (aδb)	+	M-L,SL,TS,SV
4		-	-	Y	-	+	-	-	-	+ (aδb)	+	M-L,SL,TS,SV
5		+	-	β	+	+	+	-	-	+ (aδb)	+	L,SL,CO,TS,SV
6		+	-	β	+	+	+	-	-	+ (aδb)	+	L,SL,CO,TS,SV
7		+	-	β	+	+	+	-	-	+ (aδb)	+	L,SL,STS,SV
8		+	-	β	+	+	+	-	-	+ (aδb)	+	L,SL,STS,SV
9		+	-	β	+	+	+	-	-	+ (aδb)	+	L,SL,STS,SV
10		+	-	α	+	+	+	-	-	+ (a)	+	L,SL,CO,
13		+	-	β	+	+	+	-	-	+ (aδb)	+	S-M,CS,SV
14		+	-	β	+	+	+	-	-	+ (a)	+	M-L,SL,
15		+	-	β	+	+	+	-	-	+ (aδb)	+	M-L,SL,STS,SV
16		-	-	Y	-	+	-	-	-	+ (aδb)	+	M-L,SL,STS,SV
17		-	-	Y	-	+	-	-	-		-	M
18		-	-	β	-	+	-	-	-	+ (a)	+	M
19		-	-	β	-	+	-	-	-		-	S
											-	S

Species / Isolate	Source	Lecithinase	Lipase	Haemolysis	ISN	$SO_3^=$	Gelatin	N_2	O_2	Spores	Gram	Morphology
20		-	-	β	-	+	-	-	-		-	S
21		-	-	β	-	+	-	-	-		-	M
22		-	-	β	-	+	-	-	-	+ (a)	+	M
23		+	-	β	+	+	+	-	-	+ (a)	+	M-L,SL
24		+	-	β	+	+	+	-	-	+ (a)	+	M
25	S/1	-	-	β	-	+	-	-	-	+ (a&b)	+	M,TS,SV
26	S/1	-	-	γ	-	+	-	-	+	+ (a)	±	M
27	S/3	+	-	β	-	+	-	-	-	+ (a&b)	+	M,STS,SV
28	S/3	-	-	β	-	+	-	-	-	+ (a&b)	+	S-M,chains
29	S/3	-	-	β	-	+	-	-	-	+ (a&b)	+	S,P,CS,SV,PS
30	S/3	-	-	γ		-		-	-	+ (b)	+	S,CS,SV
31	S/3	NG	NG	β		-	-	+	+	+ (b)	±	S,CS,SV
32	S/3	-	-	β		-		-	-	- (b)	+	S
33	S/3	NG	NG	β		-	-	+	+	+ (b)	+	S
34	S/3	NG	NG	γ		-	-	+	+	+ (b)	+	S
35	S/4	-	-	γ		-	-	+	+	- (b)	+	S
41	S/4	NG	NG	γ		-	-	+	+		±	
44	S/4	NG	NG	β		-	-	+	+		±	
45	S/3	NG	NG	β		-	-	+	+	+ (b)	+	S-M
46	S/7	-	-	γ		-		+	-	- (b)	+	S,P
47	S/7	-	-	γ		-		-	-	+ (b)	±	S,PS
48	S/7	-	-	γ		-		+	+	- (b)	-	S
49	S/7	-	-	γ		-		-	+	- (b)	-	S-M
50	S/7	-	-	γ		+		-	-	+ (b)	±	S,P,TS,SV,PS
51	S/7	-	-	γ		+		-	-	+ (b)	±	M,SL,STS,SV,PS
52	S/8	-	-	β		-		-	-	- (b)	+	S-M,SL
53	S/8	-	-	β		-		-	-	- (b)	±	M
54	S/8	-	-	γ		-		+	+	+ (b)	+	S-M,PS
55	S/17	-	-	β		-		-	-	+ (b)	+	S-M,STS,SV
56	S/17	-	-	α		-		-	-	- (b)	+	S-M,chains
57	S/17	-	-	α β		-		-	-	- (b)	+	M
58	S/20	+	-	β		-		-	-	+ (b)	+	S-M,STS,SV
59	S/20	+	+	α		+		-	-	+ (b)	+	S,CS
60	S/20	NG	NG	α β		-		-	-	- (b)	+	M,SL,
61	S/20	-	-	γ		+		-	-	- (b)	+	S-M,SL
62	S/20	+	+	α		-		-	-	- (b)	+	S-M,SL
63	S/20	-	-	γ		-		+	+	- (b)	±	M-L,
64	S/21	-	-	α		-		-	-	+ (b)	+	S,P,CS,SV,PS
65	S/21	-	-	α		-		-	-	+ (b)	+	S,P,CS,SV,PS
66	S/21	-	-	α		-		-	-	+ (b)	+	S,P,CS,SV,PS
67	S/26	-	-	α		-		-	-	+ (b)	+	S,P,CS,SV,PS
68	S/26	-	-	γ		+		-	-	- (b)	+	L,SL

1. Source. The number of the muskeg sample from which the isolate was obtained, see Appendix III for details. Isolates for which no source number is given will be found in Appendix II.
2. Lecithinase. Growth on EYA and the ability to turn the medium opaque for a distance beyond the edge of the colony indicates that the isolate produces the toxic Lecithinase C. NG = No Growth.

3. Lipase. The production of a pearly layer on the surface of colonies growing on EYA indicates that the organism produces the toxin Lipase. NG = No Growth.
4. Haemolysis. Growth on RBA with lysis of red blood cells, α = partial lysis, β = complete lysis, and γ = no lysis.
5. TSN. The medium TSN agar contains Neomycin, Polymyxin and sodium sulfite. Any bacterium which can grow and reduce sulfite at 46°C in the presence of these antibiotics is presumed to be C. perfringens. Positive colonies have a dark grey to black appearance due to FeS.
6. $SO_3^=$. B10 + $SO_3^=$ medium was used to screen for sulfite reducing anaerobic bacteria. A black FeS precipitate indicated a positive result.
7. Gelatin. Liquifaction of Nutrient Gelatin indicates that the bacterium being tested is proteolytic in nature.
8. N_2. Bacteria that are able to fix atmospheric nitrogen were able to grow through successive transfers on NFA. Two symbols (++ or --) indicate that the isolate was also tested on Skinners medium. Growth plus the production of gas was considered a positive result.
9. O_2. Positive indicates that the isolate was able to grow on Blood agar at atmospheric oxygen levels.
10. Spores. a = Survival in pure culture of pasteurization at 80°C for 20 minutes, b = Visual observation of spores in either a Gram stain preparation or a spore stain done with malachite green.
11. Gram. The results of the Gram Stain.
12. Morphology. Cellular morphology according to the following key. All isolates are rods. S = short, M = medium, L = long, P = plump, SL = slender, CU = curving, TS = terminal spores, CS = central spores, STS = subterminal spores, SW = the cell is swollen by the spore, FS = free spores, G = ghosts.

APPENDIX VI.

DESCRIPTIONS OF IN SITU ASSAY SAMPLES

Sample	Obtained	Weight, Location and Description
F1	9/6/75	681.45 g from the north west corner of the collecting pond. The site is oil soaked soil (clay-sand), but the sample is from the 5-10 cm level which shows no visible oil.
F2	9/6/75	659.89 g from two meters west of the plots, sample is oil soaked very wet bog material, mainly grass and moss, from the 5-10 cm level.
F3	9/6/75	Zero control, received no sample.
F4	9/6/75	890.68 g from outside the spill area. The site is twenty meters north of the bridge over the dike. Sample is moss and grass from the 5-10 cm level.
F5	9/6/75	618.78 g from 50 meters southwest of the collecting pond in an area of dead spruce. Sample is very wet and very oily moss with many twigs.
F6	9/6/75	833.45 g from outside the dike ten meters west of site F5. The sample is water soaked peat moss which had been disturbed when the dike was made. 5-10 cm level.
F7	27/8/75	141.0 g. Approximately 1 km north east of Nipisi No. 1 spill, and 100 meters east of a Texaco well head by the bend in the road. The sample is red mud and detritus from below 20 cm of water in a trench in the centre of a cut line.
F8	27/8/75	145.5 g. Identical to F7.
F9	27/8/75	134.0 g. Twenty five meters north of the collecting pond and 4 meters west of the plots in Nipisi No. 1 spill. Sample is oil soaked very wet grass and moss from the 2-10 cm level.
F10	27/8/75	175.0 g. Identical to F9.

Sample	Obtained	Weight, Location and Description
F11	27/8/75	154.2 g. Ten meters west of the collecting pond, where a plank is laid over a trench. Sample is oil soaked clay which is fairly dry from the 2-10 cm level.
F12	27/8/75	150.2 g. Identical to F11.
F13	27/8/75	150.3 g. 100 meters north of the road, due north from Nipisi No. 1 spill. Sample is detritus consisting of moss and grass from below 50 cm of water.
F14	27/8/75	150.9 g. Identical to F13.
F15	27/8/75	0.0 g. Site is identical to F13.

APPENDIX VII

ACETYLENE REDUCTION BY MUSKEG AND OIL SPILL SAMPLES

A.-Sample	Ethylene Produced (p moles/h/g Dry Weight) Minus Sterile Control	Minus Ammonia Control
S/38a	8,058 ± 1,976	2,242 ± 1,976
b	17,786 ± 15,777	16,833 ± 15,777
c	5,339 ± 2,918	5,242 ± 2,918
d	345 ± 160*[1]	345 ± 160*[1]
e	73,514 ± 31,150	73,514 ± 31,150
S/39a	3,289 ± 767	3,289 ± 767
b	1,838 ± 804	105 ± 804
S/40	1,895 ± 545	878 ± 545
S/41	5,468 ± 7,411	4,472 ± 7,411
S/42	5,239 ± 4,552	4,598 ± 4,552
S/43	11,730 ± 8,391	11,282 ± 8,391
S/44	6,313 ± 3,389	4,829 ± 3,389
S/45	6,343 ± 9,761	7,187 ± 9,761
S/46	12,679 ± 11,554	12,138 ± 11,554
S/47	-38 ± 67	-142 ± 67
S/48	11,450 ± 2,209	11,339 ± 2,209
S/49a	193 ± 277	208 ± 277
b	1,717 ± 1,884	1,605 ± 1,884
c	1,725 ± 2,351	1,372 ± 2,351
d	-96 ± 101	-5 ± 101
S/50a	19,522 ± 2,580	19,453 ± 2,580
b	27,552 ± 14,480	28,106 ± 14,480
S/51a	19,937 ± 861	19,893 ± 861
b	11,744 ± 485	11,825 ± 485
c	14,883 ± 5,594	14,896 ± 5,594
S/52a	-15 ± 20	-9 ± 20
b	211 ± 66	189 ± 66
c	48 ± 59	29 ± 59
S/53a	1,087 ± 762	1,000 ± 762
b	3,133 ± 1,128	3,082 ± 1,128
c	1,186 ± 1,342	1,144 ± 1,342
S/54a	80 ± 59*[2]	79 ± 59*[2]
b	68 ± 39	30 ± 39
c	9 ± 6	-11 ± 6
S/55	97 ± 18	46 ± 18
S/56a	109 ± 156	135 ± 156
b	484 ± 125	466 ± 125
c	124 ± 37	113 ± 37
S/57a	41 ± 19	1 ± 19
b	44 ± 6*[2]	-1 ± 6*[2]
c	68 ± 31	172 ± 31
S/58a	-9 ± 20	44 ± 20
b	--*[3]	109 ± 29
c	-12 ± 12	53 ± 12
S/59a	44 ± 20	45 ± 20

A.- Sample	Ethylene Produced (p moles/h/g Dry Weight) Minus Sterile Control		Minus Ammonia Control	
b	122 ±	21	115 ±	21
c	170 ±	97	150 ±	97
d	1,942 ±	588	1,923 ±	588 p57 58

B.- Sample	Ethylene Produced (p moles/h/g Wet Wt.) Minus Sterile Control		Minus Ammonia Control	
S/38a	388 ±	95	153 ±	95
b	684 ±	606	648 ±	607
c	2,704 ±	1,477	2,654 ±	1,477
d	268 ±	125*1	268 ±	125*1
e	5,267 ±	2,232	5,267 ±	2,232
S/39a	1,722 ±	401	1,722 ±	401
b	97 ±	42	5 ±	42
S/40	94 ±	27	44 ±	27
S/41	490 ±	666	401 ±	666
S/42	387 ±	336	339 ±	336
S/43	1,278 ±	914	1,229 ±	914
S/44	226 ±	121	173 ±	121
S/45	579 ±	891	656 ±	891
S/46	747 ±	681	715 ±	681
S/47	-5 ±	10	-21 ±	10
S/48	1,017 ±	196	1,007 ±	196
S/49a	130 ±	186	139 ±	186
b	307 ±	337	287 ±	337
c	205 ±	279	163 ±	279
d	-11 ±	11	-1 ±	11
S/50a	1,396 ±	195	1,391 ±	185
b	1,287 ±	677	1,313 ±	677
S/51a	4,713 ±	204	4,702 ±	204
b	2,357 ±	97	2,373 ±	97
c	1,847 ±	694	1,849 ±	694
S/52a	-13 ±	17	-7 ±	17
b	112 ±	36	100 ±	36
c	18 ±	22	11 ±	22
S/53a	577 ±	405	531 ±	405
b	564 ±	203	554 ±	203
c	166 ±	188	160 ±	188
S/54a	69 ±	50*2	69 ±	50*2
b	54 ±	31	23 ±	31
c	8 ±	5	-9 ±	5
S/55	55 ±	10	26 ±	10
S/56a	87 ±	125	107 ±	125
b	402 ±	104	387 ±	104
c	102 ±	30	93 ±	30
S/57a	27 ±	12	1 ±	12
b	18 ±	2*2	-1 ±	2*2
c	12 ±	5	30 ±	5
S/58a	-4 ±	9	21 ±	9
b	--*3		33 ±	9

B. Ethylene Produced (n moles/h/g Wet Wt.)

Sample	Minus Sterile Control	Minus Ammonia Control
c	-1 ± 2	6 ± 2
S/59a	40 ± 18	40 ± 18
b	108 ± 19	102 ± 19
c	144 ± 82	127 ± 82
d	1,646 ± 499	1,630 ± 499

All values are the mean of triplicate samples, plus or minus one standard deviation.

*1 Blanks were not done for this sample as not enough material was available.

*2 Duplicates only, one replicate lost.

*3 Sterile control lost.