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MORPHOLOGICAL ASPECTS OF CATTAIL TRANSPLANTS and MICROBIOLOGICAL ASPECTS OF ORGANIC MATTER IN EXTREME ACIDIC CONDITIONS ON BASE METAL TAILINGS (UP-B7-020)

Morphological Assessment of Cattail Transplants in Acidic Tailings

FINAL REPORT

by

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ABSTRACT

Two sets of experiments were brought together under this study - the establishment of cattail populations on acidic tailings and an investigation of the ameliorating effects of organic amendments on acid mine drainage water. Both these elements are essential components in the development of a self-sustaining biological treatment process for acid mine drainage.

Cattail transplant experiments yielded varying degrees of success. The root/rhizome system was investigated from a morphological point of view to obtain evidence on the processes which lead to either death or survival of the plants. It was found that root damage during transplanting is likely to be the main factor influencing survival and, accordingly, transplanting should take place prior to root development.

Several organic amendments were added to acidic water on a pyrrhotite-covered tailings area. By 1987, and more frequently in 1988, pH increases from 2.5 to 5 were noted in isolated pockets. A diverse microbiological fauna was identified in samples of the organic material. Based on a set of laboratory tests, the microbiological processes which are most likely to be responsible for the noted changes in pH, were defined in this study.

Samples obtained in the test cells in January 1988 contained significant numbers of sulfate reducing bacteria and ammonifying bacteria. The parameters for the microbiological neutralization process have been identified. The organic matter is decomposing to provide cellulose which, in turn, will be further broken down by fungi and bacteria to protein and sugars. These are the essential components required for the ammonifiers and iron and sulfate reducers.

Abstract

SOMMAIRE

On a rapproché deux séries d'expériences dans le cadre de cette étude : l'implantation de populations de quenouilles sur des étendues de résidus acides et une enquête ayant trait aux effets bénéfiques des modifications organiques sur les eaux d'écoulement acides des mines. Ces deux éléments sont essentiels pour mettre au point un procédé autonome de traitement biologique des écoulements de nature acide dans les mines.

Les expériences portant sur la transplantation de quenouilles ont apporté des résultats mitigés. Le systbme racines/rhizomes a été étudié d'un point de vue morphologique pour determiner quels sont les processus qui mènent soit à la mort, soit à la croissance des plantes. On s'est rendu compte que les chances de survie des plantes étaient vraisemblablement accrues si les racines n'étaient pas endommagées au moment de la transplantation et, par conséquent, celle-ci devrait être effectuée avant que les racines ne se développent.

On a ajouté des modificateurs organiques à l'eau acide d'un bassin de résidus couvert de pyrrhotine. *Dbs* 1987, et plus souvent encore en 1988, on remarquait que le pH passait de 2,5 à 5 dans des poches isolées. On a trouvé dans des échantillons de matière organique une faune microbiologique variée. D'après une série de tests en laboratoire, les processus microbiologiques qui sont le plus vraisemblablement responsables des changements de pH remarqués ont été définis dans cette Qtude.

Abstract

Les échantillons obtenus en janvier 1988 dans les cellules témoins contenaient une quantité importante de bactéries ammonifiantes et de bactéries faisant baisser le taux de sulfate. On a établi les paramètres du processus de neutralisation microbiologique. La matière organique se décompose pour donner de la cellulose, laquelle à son tour sera séparée en protéines et en glucides par des champignons et des bactéries. I1 s'agit là des éléments essentiels requis pour les agents ammonifiants et les réducteurs de fer et de sulfate.

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1.0 INTRODUCTION

In many cases, acid generating waste sites produce seepages which require treatment. At present, in order to improve the seepage water, water is collected and subjected to neutralization. For the past years, Boojum Research has addressed various aspects of seepages on an experimental basis in an effort to develop a conceptual framework for a self-sustaining treatment system. This is part of the ongoing research of Ecological Engineering which aims to produce close-out conditions which are both environmentally acceptable and self-sustaining, no longer requiring the expensive treatment currently necessary.

The results from two series of experiments led to an impasse, as transplanted cattails gave inconsistent growth results following overwintering. In a second series of experiments, organic amendments which were intended to ameliorate the extreme acidic conditions produced only sporadic pH increases. This work, therefore, addressed the results of these experiments on a different level. Cattail root/rhizome morphology was used to determine the state of transplanted individuals and the organic amendments were tested for microbial activity in the laboratory.

The backgrounds and objectives of both experiments are given below by way of introduction to Sections 1 and 2 of this report.

It had been noted at various acidic tailings areas where cattail transplant experiments were underway, that growth was impaired or death occurred at various times after transplanting. Several variables could be contributing to the unsuccessful establishment of cattails in acidic tailings, some of which were indicated by the ongoing experiments, i.e. time of transplant and/or type of root/rhizome amendment.

The obvious reason for a continued search for those factors responsible for this failure is the existence of small but persistent stands of naturally colonized cattails on many highly acidic tailings sites (Kalin, 1984). It was clear therefore, that the pragmatic approach we were taking of continuing our transplant experiments would prove futile if we could not specifically delineate those factors responsible for the success or failure of the individual establishments.

Section 1 of this report has as its objective the investigation of cattail root/rhizome systems from previous transplant experiments at the morphological level to determine (a) the causes of death; and (b) the reason for successful growth.

The background for section two consists of an experimental test section of a pyrrhotite-covered waste management area which was

established in 1986, where various organic amendments were added to acidic water contained in series behind the tailings dam. In isolated pockets, pH increases from 2.5 to 5 were noted by 1987 and more frequently in 1988, A diverse microbiological fauna was identified in association with samples taken from the pools with amendments. Given the presence of this fauna, a more detailed assessment of the microbiological processes was carried out.

The objective of Section 2 has been to address the microbiological aspects of the test cells with organic amendment, specifically, to identify the sulphate reducing processes which are suspected to occur in test cells with AMD surface water.

By combining the results of section 1 and 2 of the investigation, it will be possible to define the parameters, which will hopefully lead to a self-sustaining microbiological treatment process of seepage water from acid generating waste material,

SECTION 1: Cattail Root/Rhizome Morphology

1.2 METHODS AND MATERIALS:

1.2.1 Cattail Collection Technique

Cattail rhizomes were collected from a number of locations along a spill area of an abandoned tailings site. The majority of these sites represented points of cattail transplants performed in August of 1987.

Several possible treatments had been done at each site at time of transplanting. These included: no amendment (N); straw (S); and, straw with lime (SL).

Transplant sites were designated as seepage, second opening, first opening and beach, corresponding to increasing distances from the tailings mass. Besides collection of previously transplanted cattails, cattails were also collected from a number of additional sites representing controls for comparisons with developmental status of transplanted individuals. These controls included cattails from the site which was used as a source for cattails for transplant (source); a natural stand of cattails present at the border of the second opening (Nat. Stand); individuals from Nat

Stand colonizing onto the tailings (Colonization),; an isolated clump of plants representing a seed germination and establishment event into the second opening from Nat. Stand (Seed Stand), from area Y1 which is a natural stand site existing for at least 15 years (Y1); and plants transplanted two years previously by Paul Davies onto tailings at an additional site (Paul).

Cattail plants were carefully excavated from the sites using a shovel, and were gently washed to remove excess soil. Plants were then stored in plastic bags in a cooler until the following day when they were examined and preserved for further study.

Observations on cattail growth were done at a number of levels which can be divided into stages. Stages correspond to those originally detailed in the unsolicited proposal preceding this report. Each stage is described below with particular reference to any techniques which were utilized during that stage.

1.2.2 Observation Methods

Stage 1: This involved an overall assessment of the developmental status of the plants, The following parameters were measured: number of new shoots/plants; length of rhizome between new shoots and the parent plant; and height of all new shoots. The plants were also photographed at this stage.

Stage 2: After examination of the plants from Stage 1, selected rhizomes, roots and shoots of plants from the different sites were fixed in 70% formalin; acetic acid; 95% ethyl alcohol (FAA) in a ratio of 1:1:18. Fixed plant material was hand sectioned for anatomical evidence of deleterious affects of tailings metals on cattail growth. Thick hand sections were photographed using a Zeiss stereomicroscope SV-8 at variable magnifications. To demonstrate the localization of metals in the rhizome tissue, we utilized a modification of the method proposed by McNary (1960). Unmordanted haematoxylin in pH 7.0 phosphate buffer binds to metals, producing a dark red coloration after several minutes of staining. This stain was particularly useful in enhancing observations on the localization of metals in thick sections of rhizomes.

rhizomes.

Stage 3: Plants fixed from stage 3 were sectioned for use in scanning electron microscopy (SEM). To prepare tissue for SEM, plant material preserved in FAA was first washed in 70% ethanol, before being transferred through a graded ethanol series to absolute 100% ethanol. Tissue was then critical point dried in an Omar SPC-1500 critical point dryer, mounted on metal stubs and coated for five minutes with gold palladium in a Techron Hummer V sputter coater. Material was observed at 10 kv using a Hitachi 570 SEM.

1.3 RESULTS AND DISCUSSION:

Before proceeding with a discussion of results from this study, some introductory remarks are necessary to describe basic features of the anatomy, morphology and development of cattail plants. Figure 1.1 diagrammatically illustrates many of these features.

Individual cattail plants consist of a rosette of opposite leaves (about 6 per side) which reproduce vegetatively through the production of underground stems (rhizomes). These rhizomes initially develop as small vegetative buds in the axil (at the base) of each leaf. Thus each plant has the capacity to produce

vertically (S_L, Figure 1.2A), and becomes an upright shoot (new plant) (S_V, Figure 1.2A: arrows, Figure 1.2B). Rhizomes characteristically produce roots at regular intervals along their length (arrow, Figure 1.2A), although the majority of roots tend to be produced at the bases of upright shoots (Figures 1.2B, 1.2D).

Anatomically (in section), rhizomes consist of two distinct zones, an outer cortex (C, Figure 1.2G, Figures 1.3A, 1.3B), and an inner pith (Pi, Figure 1.2G, Figure 1.3B). The pith is predominantly composed of tightly packed storage cells containing large quantities of starch granules (large arrow indicating starch granules in pith cells, Figure 1.3A). The cortex is largely composed of stellate (star-shaped cells) with large amounts of air space between cells (Figure 1.3A). The pith is bordered on its periphery by an endodermis (EN, Figure 1.3A), which acts as a barrier to lateral movement of substances from the cortex into the pith. The cortex is bordered on the outside by the exodermis which forms the outer 'skin' of the rhizome and acts as a barrier to transport of materials from the external environment into the rhizome (EX, Figure 1.2G, Figure 1.3A). Vascular bundles are found scattered throughout the cortex and pith in particular (small arrows, Figure 1.2G; VB, Figure 1.3A; small arrows, Figure 1.3B). The primary function of vascular bundles is to transport water from the roots to the shoots and to transport sugars produced during

photosynthesis from the leaves to the rhizome for storage (as starch in the pith).

The anatomy of roots is very similar to that of rhizomes, except that there is no pith region and this is replaced by a solid cylinder of vascular tissue (rather than many individual vascular bundles) termed a stele (large arrow, Figure 1.3C; Figure 1.3D). Rhizomes produce roots laterally from the pericycle, which is a thin layer of tissue found just beneath the endodermis. Root initiation is shown clearly in Figure 1.2I. Roots may also produce lateral roots from their pericycle (large arrow, Figure 1.3D).

Stage 1: Examinations of gross morphology of cattails indicated substantial information on the affects of tailing metals and the amendment treatments on growth. Quantitative data comparing parameters of growth indicate that transplanted cattails performed better the greater the distance from the tailing source (Figure 1.4). Furthermore, and of interest to the transplanting program, the amendments had significant positive affects on cattail development. This is particularly true in the case of the straw and lime amendments which had the greatest number of new shoots per plant at both the first and second openings (Figure 1.4). For length of rhizome and new shoot height, straw and straw and lime amendments both showed similar development, both performing better

than no amendment cattails (Figures 1.5B, 1.5C).

At the second opening site, transplanted cattails with straw and straw and lime amendments grew better in all respects (Figures 1.5A-C), than cattails in the natural stand and natural colonization. They also performed as well as or better than control plants from the Source population and from Y1, both of the latter representing natural stands.

Of particular interest is the fact that plants from the Seed Stand far outperformed any other plants in this investigation. Implications of this are referred to more fully in the discussion,

Root development was assessed by examining the level of new root initiation from the bases of new cattail shoots. These roots could be identified by their whitish colour and intact root tips (Figures 1.2A, 1.2B)). In contrast, older roots were invariably brown in colour and heavily mineralized. This latter point will be discussed in more detail in the results presentation for Stage 2 work.

Amendment type had a dramatic affect on root development. The straw and lime amendment in particular, greatly enhanced both the extent of new root initiation as well as the growth of these new

roots. In unamended sediments, few new roots were initiated and those present were only a maximum of several cms. in length, The straw treatment showed similar, but slightly better development. With the straw and lime amendment, 20 - 30 new roots tended to be present on larger new shoots and these often had grown as much as 10 cms. in length (Figure 1.2B). At the first opening, development on the latter plants was comparable to or better than that of plants from the natural stand or that of plants from Y1 (Compare Figures 1.2A and 1.2B). The dramatic effect of straw and lime amendments on the enhancement of root and rhizome growth is shown when one compares Figure 1.2C (seepage, no amendment) with 2D (seepage, straw/lime). Note the almost complete lack of roots and rhizome growth in Figure 1.2C, in contrast with substantial root growth in Figure 1.2D.

Overall growth, besides varying significantly between amendments versus controls, also showed a large decrease as one progressed from the beach towards the tailings seepage. This decline was evidenced by almost total loss of new shoot initiation and new root development, Deleterious affects of metals also became much more obvious towards the tailing source. This is discussed in detail under results for Stage 2 analyses of development.

Stages 2 and 3: Results from Stage 2 and Stage 3 studies of cattail development confirmed findings from Stage 1 concerning the value of the amendment treatments for promoting growth. In addition though, these results also provided considerable information about progressive stages in cattail death which are useful in indicating the best possible transplant method for ensuring future success.

Microscopic studies of roots, rhizomes and shoot, using both sectioned and whole material, indicated the following findings:

- Damage to roots and rhizomes from transplanting (i.e. loss of the growing tip) caused rapid uptake of metals through cut surfaces leading to the rapid mineralization of roots
- In the case of new roots, the following steps leading to their death were observed: Root tips would begin to accumulate metal oxides on their tips and external surfaces (arrow, Figure 1.3F versus Control, Figure 1.3E). These metals would then begin to encroach through the exodermis of the root causing death of the exodermal layers and underlying cortex (Figures 1.3G and 1.3H). As this accumulation increased and root tips began to blacken, a profusion of secondary roots would often be initiated and grow out just back from the tip (Figure 1.2K).

These roots often exhibited abnormal growth with multiple branching occurring on the same root. Shortly after this time, the primary roots would die, becoming heavily mineralized. Generally, roots showed earlier onset of this dying syndrome at sites closer or on the tailings mass. On the tailings mass itself, roots died rapidly almost at initiation, leaving black scars on the rhizome or shoot base where they had previously been initiated. Within the different amendment types, this process always occurred slowest with the straw and lime amendment.

Root death was closely tied in with death of rhizomes. Sectioned rhizomes showed first signs of mineral damage at points of entry of lateral roots into the cortex. This can be seen clearly in Figure 1.2G, where darkening of the cortex tissue indicates localized death of cells. After staining of this section (Figure 1.2H), the zone of damage can be seen to be much more widespread. An interesting fact to note is that this damage does not extend into the pith area, which remains white. Close examination of rhizomes with the naked eye indicated that the location of these internal zones of death could be seen externally as darkened spots appearing beneath the rhizome surface.

Anatomically areas of metal uptake in the cortex, as further elucidated by haematoxylin staining, would grow in size until most

of the cortex was blackened. Only in the final stages of rhizome death was this zone seen to encroach into the pith area (Figure 1.2I). Therefore, rhizomes were often seen which had a completely black cortex but a pith which remained white. The method of slow rhizome death just described was more typically seen in cattails with less extreme conditions, such as those sites further away from the tailings mass, or in amended conditions. Staining of young shoots with haematoxylin identified young leaves as containing large amounts of metals, although the young developing leaves appeared to be healthy.

In sites closer to the seepage or in places where direct contact occurred between rhizomes and tailing sediments, death of rhizomes was more dramatic. At these locations external damage to the rhizome surface was often visible. Metals, because of their corrosive nature, appeared to eat their way right through the rhizome surface leading to rapid spread of metals through the cortex, with subsequent death of the rhizome following shortly thereafter (arrow, Figure 1.2J).

1.4 *SUMMARY* AND CONCLUSIONS:

Results from the morphological/anatomical study have yielded valuable information which can be used towards planning a sound cattail transplant program for the year of 1988.

This study has clearly shown that amendments at time of transplanting can be very beneficial towards ensuring cattail growth and reproduction. This is particularly the case with the straw and lime amendment, indicating that it is the best treatment to continue investigation with.

Nevertheless, overall findings from the study suggest that direct contact between roots and rhizomes with tailing sediments result in rapid death of the organs involved. This suggests that difficulties in the promotion of further growth will be reached when lateral spread of cattail organs extends beyond the immediate area of the initial amendment.

Evidence from studies of iron plaque formation on cattail roots indicate that it is highly pH specific. At low pH, iron is predominantly in ferrous soluble form and as pH increases, iron is precipitated out into the substrate (MacFie and Crowder, 1987). This in part explains the tendency for much greater root mineral

damage (from plaque) to occur as one gets closer to the seepage. Furthermore, at a given site, the addition of lime to the tailings causes a rapid rise in pH, probably causing precipitation of harmful metals. This in turn would allow root growth to proliferate without metal damage.

Another crucial factor which appears to hamper cattail transplant success, is the damage done to roots at the time of excavation prior to subsequent transplant. Evidence presented here indicates that cut faces of roots may lead to rapid mineralization and death of these roots. The process of this mineralization also leads to metal accumulation in the rhizome cortex and in the shoot system. In both cases, vigour of the plants is undoubtedly reduced, making them more susceptible to death, Nevertheless, evidence indicates that although localized death may occur in the cortex of the rhizomes via this method, death of rhizomes would only occur after a protracted period of exposure. A crucial factor here is whether metals actually can penetrate the pith area, causing loss of storage materials essential for renewed growth, particularly in the Spring months. This does not appear to be an immediate result of metal uptake by roots, since observations here indicate pith damage only occurs under extreme conditions, as, for example, at the seepage.

1.5 RECOMMENDATIONS:

The observations discussed with respect to cattail establishment strongly suggest that it is imperative to transplant cattails with minimal damage. The best possible method to ensure this would be to transplant cattails in the Spring prior to initiation of new roots.

An alternative approach to overcome the damage problem would be to use plants grown from seed. Such a strategy might allow the additional problem of contact death described above to be overcome in the following way. If seedlings are hardened during development by growth in diluted tailings sediments, it may be possible to get seedlings to acclimatise to sediments with high metal concentrations. Seedlings treated in this way may suffer less of a shock, and show more resistance upon exposure to tailings conditions.

In the process of producing plants from seed, it is possible that certain plants may turn out to be more resistant to metals than others. Ideally, if plants showing that characteristic are found, they could be cloned for future considerations for mass transplanting. Certainly evidence from the natural stands at the 2nd opening, Y1 and Source, indicate variability for resistance to

metal damage. This point is best exemplified by evidence of the successful establishment and prolific growth of the clone Seed Stand from the 2nd opening.

SECTION 2: Microbiology of Organic Amendments

2.2 METHODS AND MATERIALS

2.2.1 Sample Collection and Handling

Samples of water and amendment material were collected in the different test cells through holes drilled with an ice auger on January 27, 1988. The samples were transported in a cooler and stored in a refrigerator at Dearborn. After 24 h, all samples were subjected to an ATP test, microscopic observations and tested for SRB (sulphate reducing bacteria). Five days later, pH was determined with a probe specifically designed to obtain the measurement in the samples with minimal introduction of oxygen. After 19 to 27 days, the microcosms were set up, addressing several conditions. At the same time, a second set of observations and determinations was obtained, consisting of pH, ATP, microscopic observations, SRB and ammonifiers determinations.

The microcosms were set up inside an anaerobic hood in 40 ml Wheaton vials representing layers in the pre-bog acid creek (water, amendment and amendments and tailings). These microcosms were incubated at an ambient temperature of 22°C. The conditions tested were anaerobic plus nutrient (0.1% peptone / 0.25% lactate),

anaerobic no nutrients added (sealed vials), aerobic/anaerobic (cotton plug), and Pentachlorophenol (3,000 ppm) treated microcosms, representing an abiotic control. Pentachlorophenol (PCP) is a strong toxic substance, killing most living organisms. After 12 to 19 days, the tests previously described were repeated for all treatments.

Figures 2.1 to 2.3 are photographs of the appearance of the field samples. The arrows indicate the regions of the sample bottles from which aliquots were removed for analysis. It was decided to analyze these sub-samples rather than homogenize the samples, in order to minimize possible trauma to the microorganisms caused by changes in pH and redox conditions during mixing.

It was noted during testing on January 28, that both the amendment 3 test cell samples had a sulphide odour. Odour was not detected in the other samples.

Figures 2.4 to 2.8 are representative photographs of the sample microcosm vials.

2.2.2 ATP (Adenosine Triphosphate) Assays

ATP was measured by the firefly luciferase method using a Turner Designs Model 20 e Photometer,

ATP was extracted from the microorganisms in the samples by a technique developed by Dearborn and found to be successful for AMD samples. Cell walls were lysed with ethanol; the ethanol was removed by filtration through a glass fibre filter; the cells were further lysed by passing acetone through the filter; finally, ATP from the organisms in the filter were leached with trisodium phosphate buffer. The extracts were further diluted in trisodium phosphate buffer containing Mg-EDTA in order to prevent inhibition of firefly luciferase due to metal ions,

2.2.3 Test for SRB's (Sulphate reducing bacteria)

In order to estimate the order of magnitude number of viable SRB's, decimal serial dilutions of sample were performed in media capable of supporting the growth of these organisms, All media contained lactate as a carbon source and iron to indicate growth which resulted in blackening of the media when the organisms produced H_2S.

Initially, API medium RP38 was used for this purpose. Each vial contained a nail to poise the redox potential.

In samples where sulphide odour was detected, subcultures of positive cultures were performed in order to ensure that blackening of the medium was not due to sulphide already formed in the sample.

In subsequent analyses, tests were performed in deep agar tubes of Postgate E and F media in an attempt to obtain more quantitative precise results. Postgate E medium was selected to provide a count of sulphate reducing bacteria while Postgate F medium was used to detect additional sources of microbially produced sulphide such as sulphide from sulphite reduction and decomposition of organo-sulphur proteins. Since counts obtained using Postgate F medium never exceeded those with Postgate E, only Postgate E medium was used in the microcosm analyses.

Although it was possible to obtain actual black colony counts for many samples, counts for many other samples were obscured by extensive blackening. Therefore, to simplify reporting, results are only shown as order of magnitude levels.

2.2.4 Estimation of Ammonifying Microorganisms

Order of Magnitude estimates of ammonifying microorganisms were obtained by preparing decimal serial dilutions of sample in Caslin medium (Methods for Microbiological Analysis of Waters, Wastewaters, and Sediments, Canada Centre for Inland Waters, 1978).*

2.3 RESULTS AND DISCUSSION

In Table 2.1, the pH measurements for the samples and their microcosms are presented. The three amendment types are coded as previously described in Figures 2.1 to 2.3. The values taken on January 27 were determined on-site, where sample temperatures were -1° C. The samples were placed in an insulated container and transported to Dearborn where they were immediately stored at 4° C. All pH readings taken during January and February therefore occur when the microbial populations in the sample are in a cold environment. Consequently, it is important to observe that in two of the samples (1.3 and 3.7), an increase in pH of more than one pH unit occurred during this cold storage period.

The "Microcosm" experiment, initiated between February 16 and 24, demonstrates the pH changes in the samples which are subjected to various conditions at room temperature (approximately 22° C)

The "anaerobic" condition essentially represents shifting a portion of the sample from refrigerated to room temperatures. It can be seen that this results in a further pH increase in the 1.3 and 3.8, 3.10 and 1.1 samples. The highest pH observed for the anaerobic test condition was a value of 5.7. Both the 3.7 sample and the 1.3 sample attained this pH.

The trends for pH increases in the microcosms supplemented with nutrients (peptone and lactate) are slightly more difficult to interpret for many of the samples because nutrient addition itself immediately caused a pH rise in the samples to pH 4.2. However, it is evident that the nutrients stimulated pH increases in the 1.1, 1.3 and 3.7 and 3.8 samples.

No pH rise was observed in any of the aerobic microcosms. On the contrary, aerobic conditions resulted in a pH depression. It is speculated that the drop in pH was caused by the growth of Thiobacillus ferroxidans.

With the exception of the 1.1 sample, no pH rise was observed in any of the microcosms poisoned with PCP (abiotic control). This indicates that acid neutralization process is biological. By examining Figure 2.7, it can be seen that sample 1.1 had a very high proportion of sediment. The presence of this sediment probably restricted access of the PCP to the microbial populations in the vial. Therefore, some limited biological activity may still have been occurring.

Tables 2.2 to 2.10 summarize the results of the microbiological tests conducted on the samples and their microcosms. In addition, some of this data has been extracted from these tables to prepare Tables 2.11 and 2.12.

By overviewing Tables 2.2 to 2.10, it can be seen that a diverse group of microorganisms were observed in the site samples. Representatives of bacterial, fungal and algal species were observed. Only bacteria were detected in the control sample.

Samples from the test cells all had significantly more viable biomass levels than the test cell samples as indicated by their ATP (Adenosine Triphosphate) content. Although ATP levels were indicative of the biovolumes observed during the microscopic examinations, no correlation with the neutralization process was

evident.

In contrast, as summarized in Table 2.11, there appears to be a direct correlation with the neutralization process and SRB12 (sulphate reducing bacteria) SRB'S and ammonifiers.

Four of the five samples in the anaerobic microcosms displayed pH increases of at least one pH unit after February 2. However none of these organisms grew in the samples which displayed no pH increase or an increase less than one pH unit after February 2.

The only exception in the above relationship appears to be sample 3.10. Nevertheless, SRB's and ammonifiers did grow in this sample when the nutrients peptone and lactate had been added. Furthermore, when peptone and lactate were added to the other microcosms, SRB's and ammonifiers grew only those which displayed a pH increase of at least one unit.

Table 2.12 summarizes the effects that these nutrients had on the samples which displayed one unit pH increase. In every case, a pH increase was observed and in the majority of cases, increases in the SRB counts and ammonifiers counts occurred.

Thus, the results in Table 2.11 and Table 2.12 demonstrate the probable involvement of SRB and ammonifiers with the neutralization process. The possibility of the involvement of iron reducers cannot be excluded based on the present data and those could also contribute to the neutralization process.

It is surprising that in most cases, the SRB and ammonifier counts are relatively low. Perhaps, the counts are underestimates. This could occur if the cultural conditions of the microbial tests were insufficient to support the growth of the major strains of these organisms. In addition, it is probable that many of the cells of these types of organisms are associated in clumps and could attach to particulates. In microbial enumeration techniques based on cultural methods, a clump of microorganisms or group of microorganisms attached to a particle is counted as a single cell. It is also possible that additional types of microorganisms could be responsible for neutralization of seepage acidity. Iron reducing bacteria are such a group.

Further examination of Table 2.12 also suggests that there is a relationship in occurrence of SRB's and ammonifiers. In each sample where SRB's were detected, ammonifiers were also found. Two ecological relationships of these organisms are probable. First, ammonia, which is produced by the ammonifiers is a nitrogen source

for SRB's. Second, the production of ammonia may raise the pH of the samples to pH 4. Growth of SRB's below pH 4 has not been documented.

It is of interest to note that sometime after February 2, 1988, during storage in the refrigerator, the 1.1, 1.3 and 2.4 samples became frozen. In the microcosm experiment, growth of both SRB's and ammonifiers occurred in all of these samples. Therefore, the members of the ecosystem leading to the process may be considered safe from irreversible damage caused by winter temperatures.

The interaction of these organisms is one of many interactions which probably occur in this ecosystem. Figure 2.9 summarizes the activities which are likely to be involved.

2.4 SUMMARY AND CONCLUSIONS

A. The experiments have demonstrated that microbial neutralizationof acid seepage is possible in samples obtained under winter conditions and AMD originating from pyrrhotite.

B. The microbial process was active at 4°C and recovered following one freeze/thaw cycle.

C. Ammonifiers and sulphate reducing bacteria appear to play key roles in the process. Compared to the control, the samples from the experimental sites had greater microbial population diversity (according to microscopic analyses) and higher biomass levels. The correlation of ATP levels with the bio-volume quantities (estimated microscopically) indicates that the majority of the organisms were viable.

D. Some of the amendments provided sufficient nutrients for the neutralization process. However, the effect of the nutrient additions in the microcosm studies indicate that there is a potential to improve the process.

E. It was demonstrated that the microbial acid neutralization process requires anaerobic conditions. Aerobic incubation of the samples resulted in generation of acidity rather than alkalinity.

2.5 RECOMMENDATIONS

A literature review was carried out to define the approach which could be taken to proceed with refinement of the process and determine parameters which could be utilized to develop an effective seepage treatment system. In Table 2.13, sulphate reduction rates are summarized fromthe literature. It is evident, that reduction rates can be increased in a bio-film lab reactor by several orders of magnitude. The approach to be taken for the development of a self-sustaining neutralization process however, is not immediately apparent. Clearly the microbiology itself and the interactions of decomposition and aerobic and anaerobic degradation are complex. One must assess the microbial interactions as outlined in Figure 2.9 in detail.

The identification of all aspects which are likely to contribute to the neutralization of the acidic water, particularly taking account of the rate limiting aspects of the process would require an extensive financial commitment without guarantee of success. It is, therefore, recommended that a more pragmatic approach be taken, utilizing the existing literature as a guide (list of references given in the Appendix).

APPENDIX

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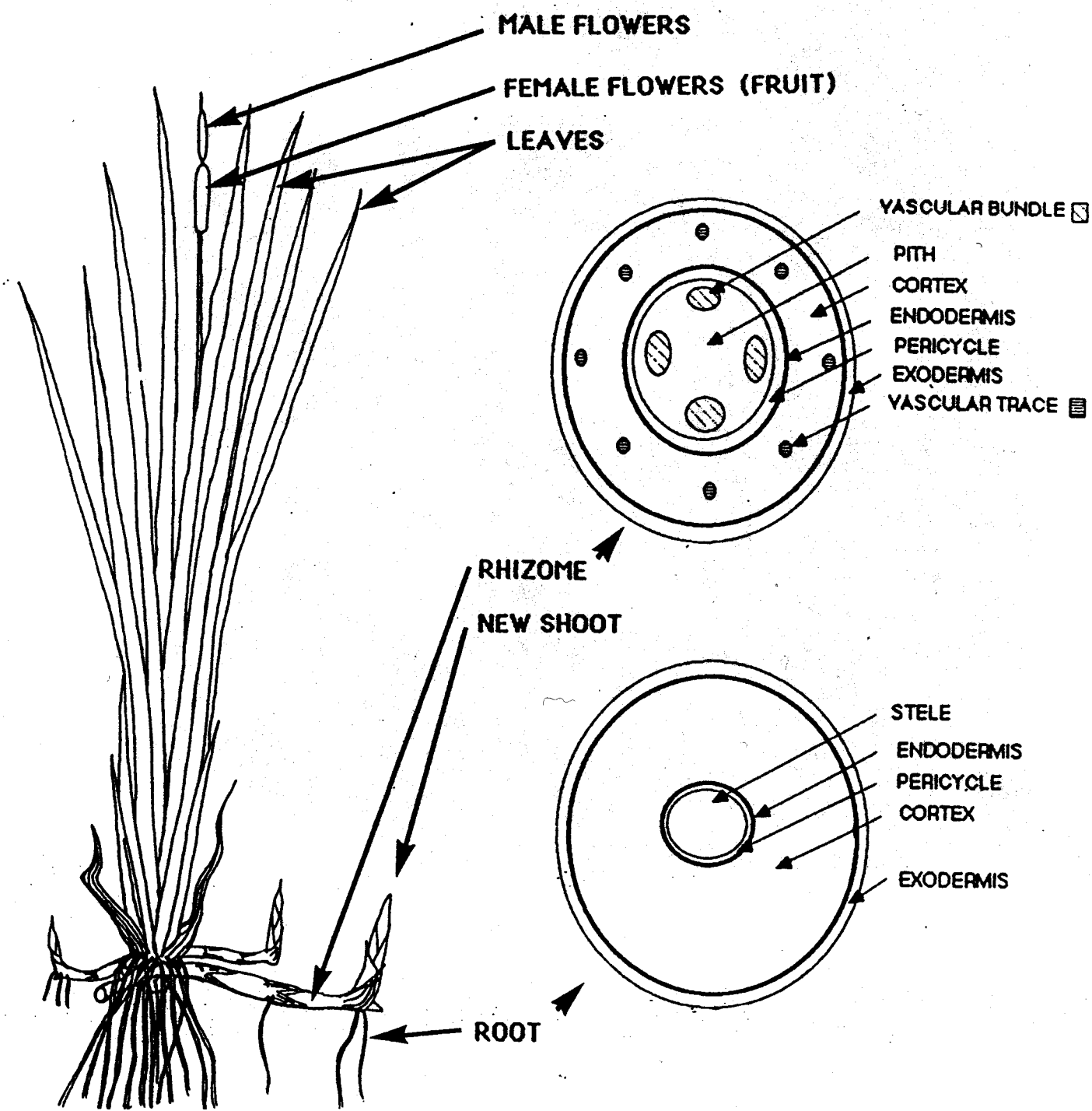
Figure 1.1: Cattail Morphology and Anatomy



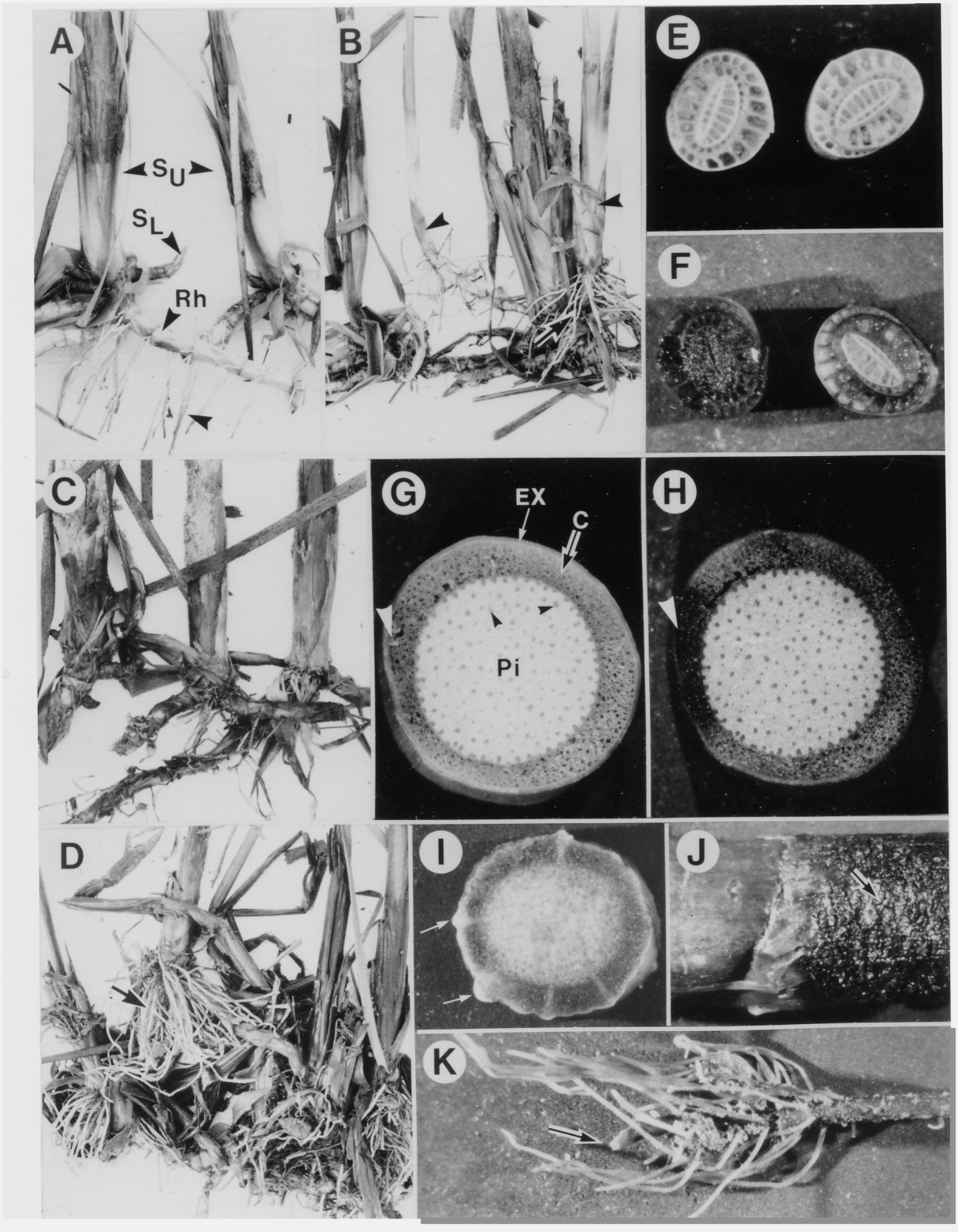


Fig. 1. 2

FIGURE 1.2

GROSS MORPHOLOGICAL FEATURES OF CATTAIL GROWTH

A. Cattail from site Y1, upright shoots (S_u), lateral shoot (S_L), rhizome (Rh), roots (arrow).

B. Cattail from the 2nd opening treated with straw and lime. Arrows indicate upright shoots.

* Note the root proliferation on the upright shoot to the right.

C. Cattail from the seepage with no amendment.

* Note the lack of new growth.

D. Cattail from the seepage amended with straw and lime. Extensive new root growth has occurred (arrow).

E & F. Sectioned shoot showing young leaves. F has been stained with haematoxylin to show the tremendous accumulation of metals in the shoot system. X 4

G & H. Section of a rhizome unstained (g) and stained (H). Metal damage from uptake is indicated by the large white arrow. Note that this damage has not spread to the pith (Pi). Exodermis (EX), cortex (C), small arrows indicate vascular bundles. X 4

I. Damage from metal uptake which has spread into the pith. Arrows indicate lateral roots.

J. External metal damage on a rhizome (arrow) due to direct contact with tailing sediments. X 4

K. Lateral root proliferation on a root after damage to the root tip. X 8

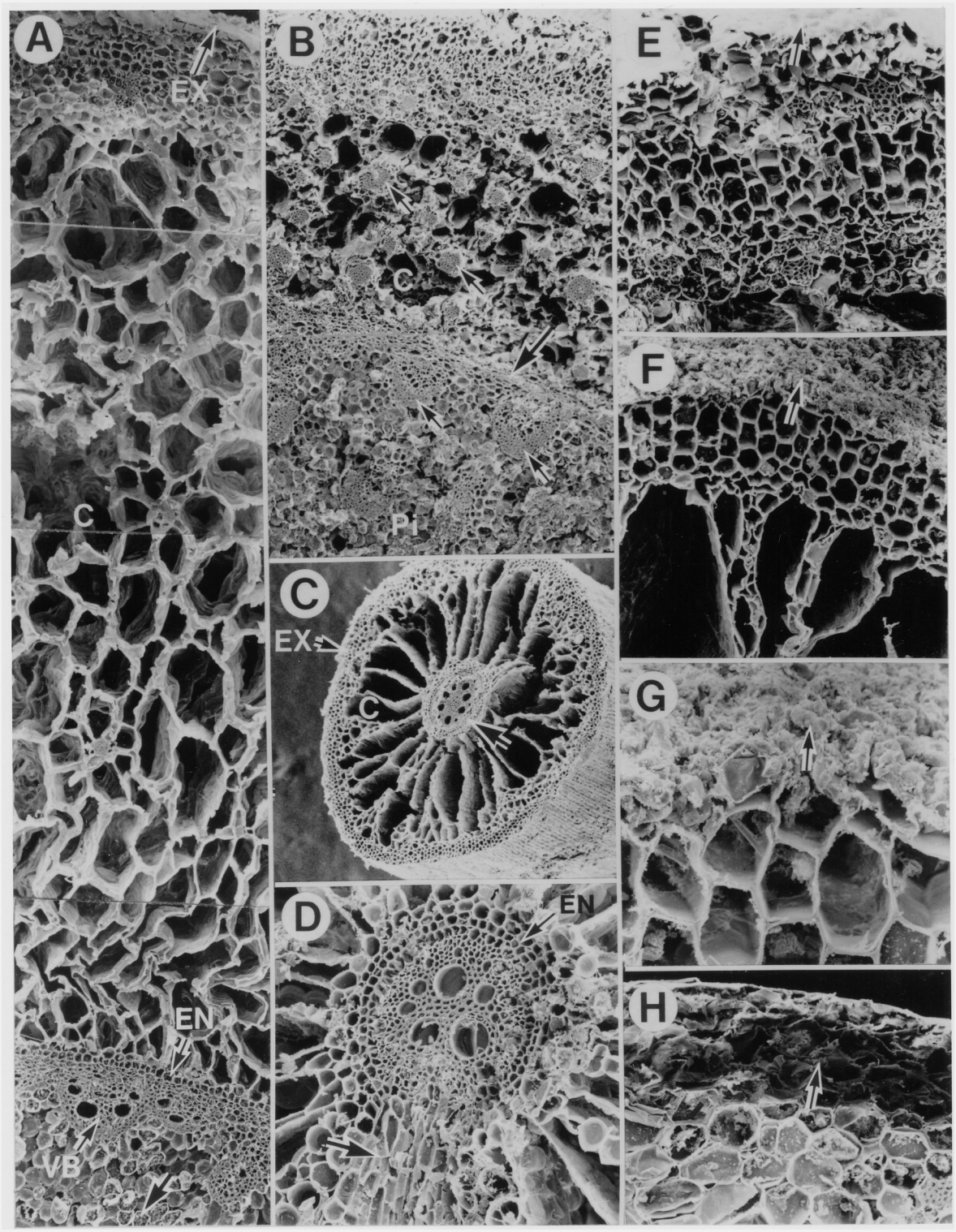


Fig. 1. 3

FIGURE 1.3

SEM SECTIONS OF CATTAIL RHIZOMES

A & B. Sections of rhizomes

A. Note the overall anatomy of the rhizome. Cortex (C), Endodermis (EN), Vascular bundle (VB), large arrow indicating pith cells containing starch granules, exodermis (small arrow). X 100

B. A younger, more compact rhizome than that shown in Figure A, Endodermis (large arrow), vascular bundles (small arrows), X 100

C. Section of a root. Stele (large arrow). X 50

D. Close-up of a stele from a root similar to that shown in Figure C. large arrow indicates a site of lateral root initiation, X 250

E. Close-up of the exodermis of a healthy root. Note that there is no metal accumulation on the outer surface of the exodermis. X 250

F. Root showing signs of metal accumulation. The exodermis surface is coated with metal particles and these metals are beginning to encroach into the cortex layers. X 250

G. Close-up view of metals in the outer layers of the cortex and beginning to accumulate in cortical cells. X 1000

H. Later stage of root death due to metal accumulation. A band of dead cells is present within the cortex. X 250

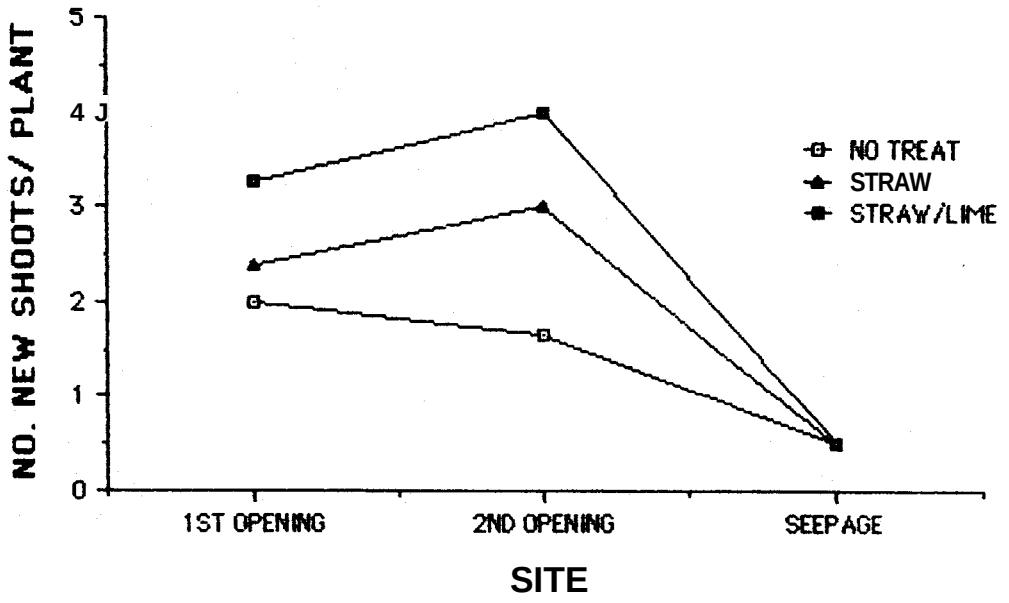
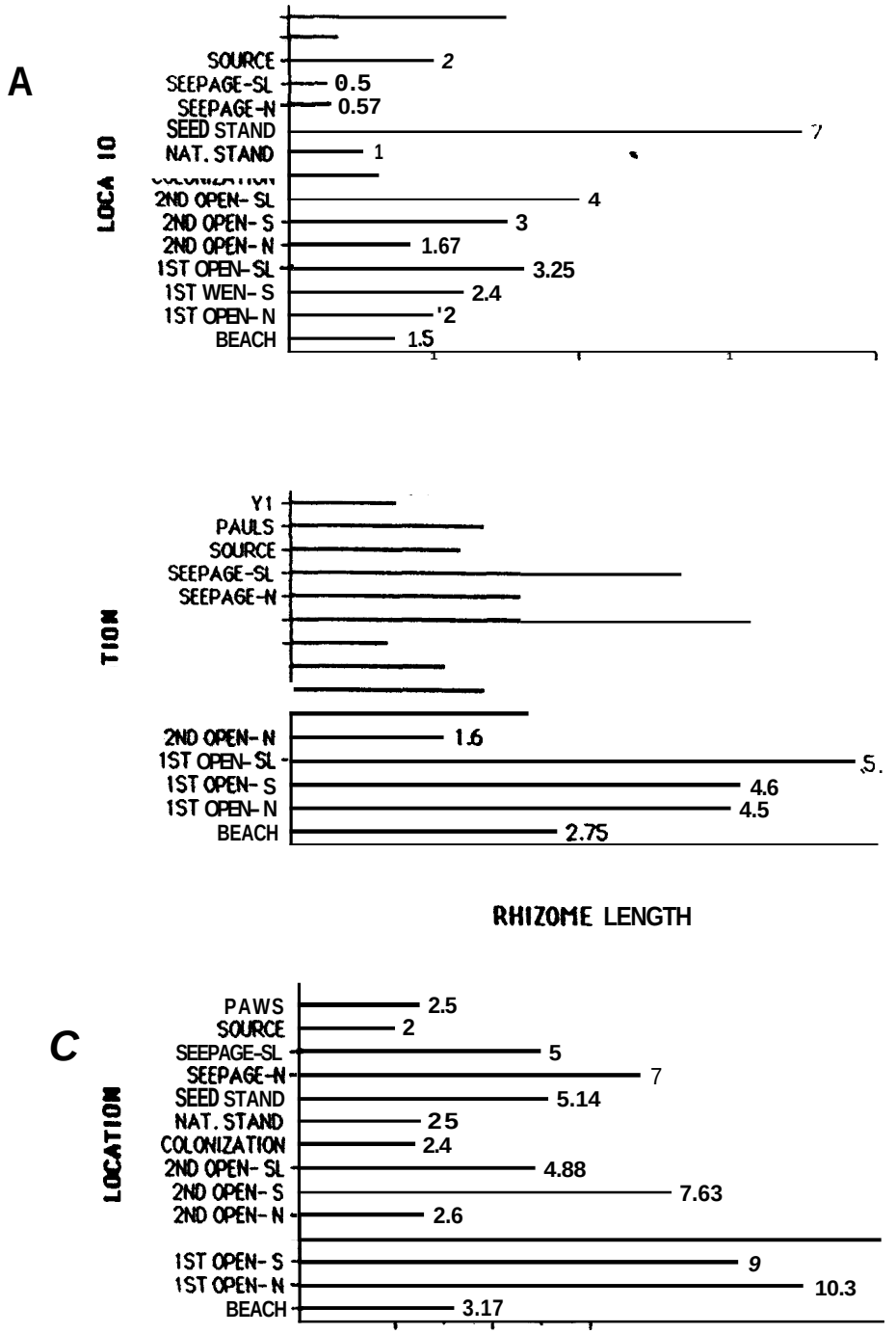


FIGURE 1.4: Affect of amendment type on number of new shoots produced per plant, expressed as a function of transplant location.

Note: Most adverse conditions (pH 1.5) encountered at seepage, and best conditions (pH 3.5) at first opening.



FIGURES 1.5A,B and C: Growth characteristics expressed as new shoots per plant, length of rhizome and height *of* new shoots

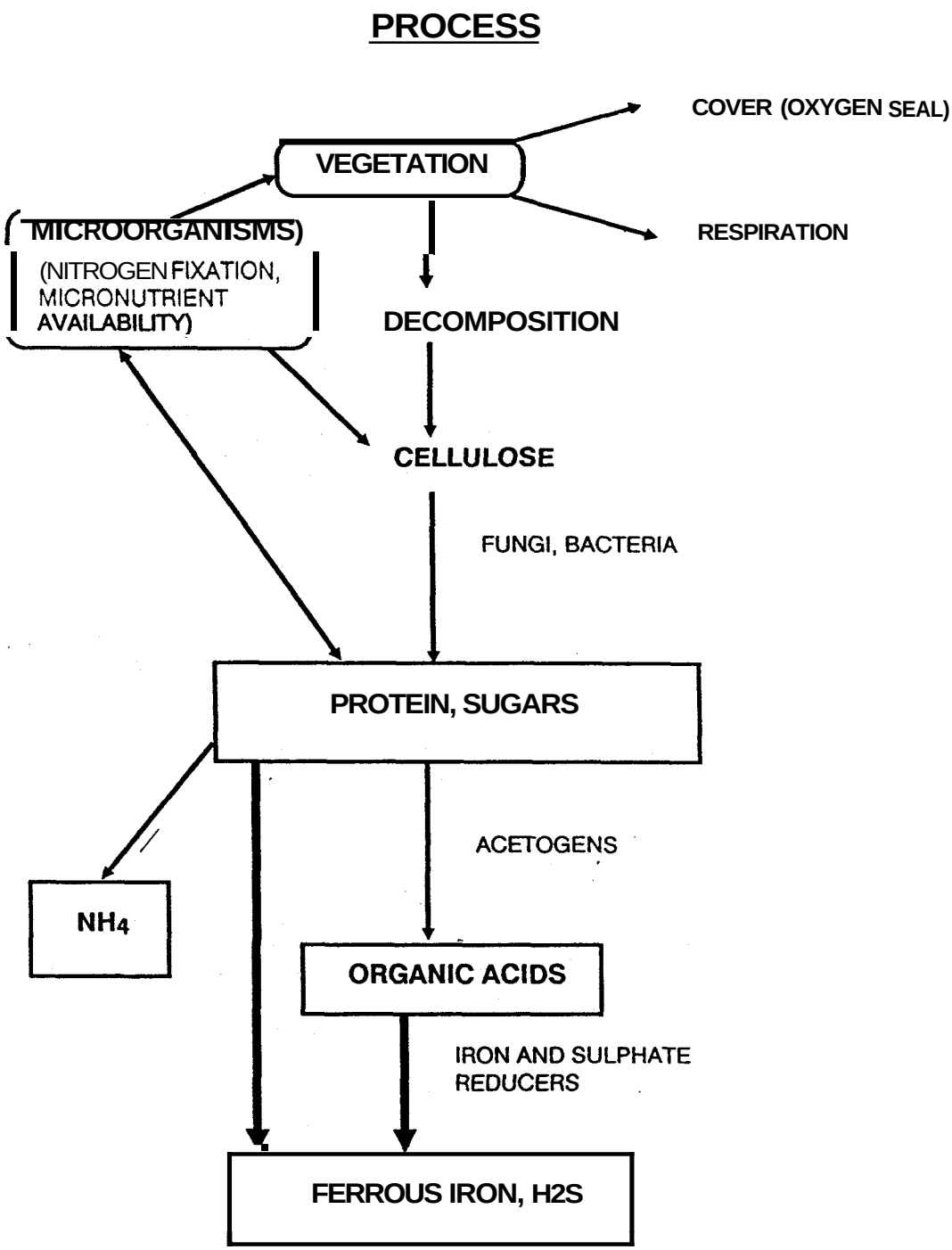


Figure 2.9: Interactions Proposed in Neutralization Process

Table 2.1: pH Values of Samples and Their Microcosms
April 5, 1988

1988 Date									
Jan. 27	3.6	--	--	--	3.5	--	--	3.9	6.2
Feb. 2	3.5	3.1	3.3	3.3	3.5	3.4	4.1	4.4	3.6
Feb.16-24	3.2	2.6	2.9	2.9	3.0	2.6	5.3	4.7	3.9
Microcosm Experiment: March 7, 1988									
Anaerobic & Nutrient	6.5	5.7	4.2	4.2	4.8	5.2	6.3	5.9	6.0
Anaerobic	2.5	2.8	2.4	2.3	6.6	4.4	5.7	5.7	5.1
Aerobic	2.2	2.5	2.0	2.3	2.5	2.5	3.0	2.4	2.4
Abiotic Control PCP	2.7	3.0	2.5	2.5	2.7	4.1	6.2	3.7	3.5

Note: pH Values could not be obtained due to frozen pH probe

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Summary of Microbiological Tests
January to March 1988

TABLE 2.2: Sample 0.0 - Control. No Amendment

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water Bottom	3.6	0.083	NG (API)	--	10 E5	<10 E4	<10 E4
Feb.5/88	Water-Top	3.5	--	--	--	--	--	--
Feb.16/88	Shaken Sample	3.2	0.17	NG (E) NG (F)	NG	10 E5	<10 E4	<10 E4
Mar.7/88	Microcosms:							
	PCP	2.7	0.18	NG (E)	NG	--	--	--
	Aerobic	2.2	0.41	NG (E)	NG	--	--	--
	Anaerobic	2.5	0.44	NG (E)	NG	--	--	--
	Lactate + Peptone	4.5	0.55	NG (E)	NG	--	--	--

NOTES: A blank (-) indicates that a test was not done
NG = no growth from an innoculum of 1.0 mL
API = American Petroleum Institute Medium RP 38
(E) = Postgate Medium E
(F) = Postgate Medium F
* = Sample had frozen in refrigerator and was allowed to stand for 4 to 7 days before initiation of microcosm experiment

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Summary of Microbiological Tests
January to March 1988

TABLE 2.3: Amendment type 2.4

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water (law)	--	1.10	10 (API)	--	10 E6	<10 El	<10 E4
	Upper Sediment	--	0.69	1 (MI)	--	10 E5	<10 E4	<10 E4
	Lower Sediment	--	2.00	lo (MI)	--	10 E6	10 E6	10 E4
Feb.5/88	Water	3.1	--	lo (API)	--	--	--	--
Feb.24/88*	Water (law)	2.6	1.10	NG (F)	NG	10 E7	<10 E4	<10 E4
	Upper Sediment	2.4	0.96	NG (F)	NG	10 E7	<10 E4	<10 E4
	Lower Sediment	--	2.60	NG (E)	10	10 E7	10 E6	10 E4
Mar.8/88	Microscoms:							
	PCP	3.0	1.70	NG (E)	NG	--	--	--
	Aerobic	2.5	4.40	NG (E)	NG	--	--	--
	Anaerobic	2.8	10.00	1 (E)	NG	--	--	--
	Lactate + Peptone	5.7	78.00	10 E3 (E)	10	--	--	--

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Summary of Microbiological Tests
January to March 1988

TABLE 2.4: No Treatment, between amendment type 2 and 1

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water	--	0.42	NG (AA)	--	10 E5	<10 E4	<10 E4
	Sediment	--	27.00	10 (API)	--	10 E5	<10 E4	10 E4
Feb.5/88	Water	3.3	--	--	--	--	--	--
Feb.17/88	Shaken Sample	2.9	0.92	1 (E)	NG	10 E6	<10 E4	<10 E4
Mar.7/88	Microcosms:							
	PCP	2.5	0.22	NG (E)	NG	--	--	--
	Aerobic	2.0	0.36	NG (E)	NG	--	--	--
	Anaerobic	2.4	0.34	NG (E)	NG	--	--	--
	Lactate + Peptone	4.2	0.62	NG (E)	NG	--	--	--

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Summary of Microbiological Tests
January to March 1988

TABLE 2.5: Amendment type 1.1

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water	--	0.85	1 (API)	--	10 E6	10 E4	<10 E4
	Sediment	--	470.00	NG	--	10 E5	10 E6	10 E5
Feb.5/88	Water	3.3	--	--	--	--	--	--
Feb.19/88	Shaken Sample	2.9	4.30	NG (E)	1	10 E7	10 E4	10 E4
Mar.7/88	Microcosms:							
	PCP	2.5	0.31	NG (E)	NG	--	--	--
	Aerobic	2.0	2.40	NG (E)	NG	--	--	--
	Anaerobic	2.3	2.90	NG (E)	NG	--	--	--
	Lactate + Peptone	4.2	0.72	NG (E)	NG	--	--	--

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Summary of Microbiological Tests
January to March 1988

TABLE 2.6: Amendment Type 1.2

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water	--	4.60	10 (API)	--	10 E6	<10 E4	<10 E4
	Upper Sediment	--	21.00	10 (API)	--	10 E6	<10 E4	<10 E4
	Lower Sediment	--	41.00	10 (API)	--	10 E5	<10 E4	<10 E4
Feb.5/88	Water	3.4	--	--	--	--	--	--
Feb.24/88*	Water	2.6	2.80	NG (E) 1 (F)	1	10 E8	<10 E4	10 E6
	Upper Sediment	2.6	23.00	10 (E) 1 (F)	10	10 E6	<10 E4	<10 E4
	Lower Sediment	--	42.00	10 (E) 1 (F)	10 E2	10 E5	<10 E4	<10 E4
Mar.7/88	Microcosms:							
	PCP	4.7	0.31	NG (E)	NG	--	--	--
	Aerobic	2.5	41.00	10 (E)	10	--	--	--
	Anaerobic	4.6	12.00	10 (E)	10 E3	--	--	--
	Lactate + Peptone	5.2	11.00	10 E5 (E)	10 E3	--	--	--

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Summary of Microbiological Tests
January to March 1988

TABLE 2.7: After amendment type 1

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water (top)		3.00	1 (MI)	--	10 E6	10 E4	<10 E4
	Water (bottom)		4.40	10 (AA)	--	10 E6	10 E5	10 E5
	Sediment		100.00	lo (MI)	--	10 E6	10 E6	10 E6
Feb.5/88	Water	4.1	--	--	--	--	--	--
Feb.18/88*	Water (top)	5.3	1.80	10 E2 (E)	10	10 E8	10 E6	<10 E4
	Water (bottom)			1 (F)				
	Sediment	--	6.70	10 (E)	10 E3	10 E9	<10 E4	10 E4
				10 (F)				
Mar.7/88	Microcosms:							
	PCP	4.2	0.30	NG (E)	NG	--	--	--
	Aerobic	3.0	4.10	10 (E)	10	--	--	--
	Anaerobic	5.7	8.10	10 (E)	10	--	--	--
	Lactate + Peptone	6.3	0.23	10 (E)	10	--	--	--

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Summary of Microbiological Tests
January to March 1988

TABLE 2.8: Amendment type 3. first test call

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water (top)	3.8	7.90	1 (API)	--	10 E6	10 E5	10 E4
	Water (bottom)	3.8	4.10	1 (API)	--	10 E5	10 E5	<10 E4
	Sediment	--	16.00	10 (API)	--	10 E6	10 E5	<10 E4
Feb.5/88	Water	4.4	--	--	--	--	--	--
Feb.19/88	Water (top)	4.7	3.50	10 E2 (E) 10 (E)	10 E2 10 (F)	10 E7	<10 E4	<10 E4
	Water (bottom)	4.7	4.50	10 E2 (E) 10 (F)	10 E2 (E) 10 (F)	10 E8	10 E6	<10 E4
	Sediment	--	5.60	10 (E) 10 (F)	10 (E) 10 (F)	10 E2	10 E7	10 E4
Mar.7/88	Microcosms:							
	PCP	3.7	0.17	NG	NG (E)	--	--	--
	Aerobic	2.6	6.10	10 (E)	10	--	--	--
	Anaerobic	5.7	5.20	10 (E)	10 E3	--	--	--
	Lactate + Peptone	5.9	0.75	10 E3 (E)	10 E3	--	--	--

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Summary of Microbiological Tests
January to March 1988

TABLE 2.9: Amendment type 3. second test cell

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water (top)	4.2	2.90	10 (API)	--	--	--	--
	Water (bottom)	4.2	3.80	1 (API)	--	10 E6	10 E4	<10 E4
	Sediment	--	14.00	--	10 E6	10 E5	<10 E4	
Feb.5/88	Water	3.6	--	--	--	--	--	--
Feb.19/88	Water (top)	3.9	2.80	10 E2 (E) 10 (F)	10 E3	10 E7	10 E7	<10 E4
	Water (bottom)	3.9	2.20	10 E2 (E) 10 (F)	10 E3	10 E7	10 E6	<10 E4
	Sediment	--	16.00	10 E4 (E)	10 E3	10 E8	10 E7	<10 E4
Mar.7/88	Microcosms:							
	PCP	3.5	0.72	NG (E)	NG	--	--	--
	Aerobic	2.4	21.00	10 (E)	10 E3	--	--	--
	Anaerobic	5.1	7.60	10 (E)	10 E3	--	--	--
	Lactate + Peptone	6.0	3.20	10 E5 (E)	10 E5	--	--	--

LEVACK
PRE-BOG ACID CREEK

Summary of Microbiological Tests
January to March 1988

TABLE 2.10: After Amendment type 3, No Treataent

Date	Test Zone	pH	ATP (ng/mL)	Sulfate Reducing Bacteria (number/mL)	Ammonifiers (number/mL)	Microscopic Direct Count (counts/mL) Bacteria	Fungi	Algae
Jan.28/88	Water (bottom)	3.5	0.29	NG (API)	--	10 E5	<10 E4	<10 E4
Feb.5/88	Water	3.5	--	--	--	--	--	--
Feb.17/88	Shaken Sample	3.0	0.80	1 (E) 1 (F)	1	10 E6	<10 E4	>10 E4
Mar.7/88	Microcosms:							
	PCP	2.7	2.30	NG (E)	NG	--	--	--
	Aerobic	2.5	1.80	NG (E)	NG	--	--	--
	Anaerobic	4.6	1.60	NG (E)	10	--	--	--
	Lactate + Peptone	4.8	1.30	1 (E)	10	--	--	--

Levack - Pre-Bog Acid Creek

Table 2.11: Relation of SRB and Ammonifier Counts
To Acid Neutralization in Anaerobic Microcosms

Samples which displayed pH increases of at least one pH unit after Feb.2	SRB's (Numbers per mL)	Ammonifiers (Numbers per mL)
No Treatment after type 3	NG	NG
Amendment type 1	10	10 E3
After Amendment type 1	10	10
Type 3 cell - #1	10	10 E3
Type 3 cell - #2	10	10 E3
Samples which displayed no pH increase or an increase less than one pH unit after Peb. 2		
Control, no amendment	NG	NG
type 2	NG	NG
No treatment, after type 3	NG	NG
Water layer. type 1	NG	NG

NG = no growth from an inoculum of 1 mL

Note: SRB's and ammonifiers grew in the Sample 3.10 microcosm in which the nutrients peptone and lactate had been added. When peptone and lactate were added to the other microcosms, SRB's and ammonifiers grew only those which displayed a pH increase of at least one unit.

Effect of Lactate/Peptone Supplement

TABLE 2.12

	final pH without addition	final pH with addition	increase in SRB count	increase in ammonifiers count
No Treatment After Type 3	4.6	4.8	+	+
Amendment Type 1	4.6	5.2	+	+
Type 3 cell #1	5.7	6.3	0	0
Type 3 cell #2	5.7	5.9	+	0
	5.1	5.9	+	+

\+ = increase was observed.

0 = no change was observed.

Examples of Sulphate Reduction Rates

TABLE 2.13

ENVIRONMENT	SULPHATE REDUCTION RATE mg SO_4 L^{-1} day^{-1}	COMMENTS	REFERENCE
Acidic peat	**17**	Highest seasonal rate observed.	Spratt et al **(1987)**
Fresh water sediments receiving acid mine drainage.	**864**	These rates are higher than any other values reported previously for coastal marine or lake sediments. Results are average of triplicate samples (CV<50%)	Herliky & Mills **(1985)**
Lab reactor	**1920**	Lactic acid as carbon source (Starkey's medium).	Cork & Cusanovich **(1978)**
Lab reactor	**2300**	Molasses as carbon source (assume lactic acid produced).	Maree et al **(1987)**
High rate sulfate reduction annular biofilm lab reaction.	**214,000**	Lactic acid as carbon source. Temperature = 20°C.	Neilsen **(1987)**