The Effects of Saturating Levels of Nitrate and Ammonia on a Microbial Mat from the Sippewissett Marsh, MA after a Common Physical Disturbance

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Introduction

Microbial mat communities are found in a variety of environments ranging from hot spring outflows to marine intertidal zones to Antarctic crusts. Within each mat gradients of light, temperature, pH, and many compounds are formed by environmental conditions as well as by individual populations within the community.

My project entailed observing the patterns of succession in a microbial mat from the Sippewissett Marsh, MA after a disturbance event. In the Sippewissett Marsh microbial mats are frequently covered by layers of sand brought in during tidal inflows. Sand grains are a major component of the microbial mats in this area. These periodic disturbances do not disrupt the community structure but alter the gradients within the mat forcing the bacterial populations to migrate up to the new mat surface. Within each microbial mat, populations that may appear to comprise a minor portion of the community may play a major role during succession as a colonizer (Ferris et al, 1997). One part of my project was to observe the sequence of colonization of the new sand though T-RFLP analysis, spectral determination of pigments, microscopy and microelectrode profiles. The second portion of my project entailed the determination of how saturating levels of nitrate and ammonia effect this successional process and the community structure as a whole. Nitrate and ammonia gradients in microbial mats are important factors for determining community structure (Paerl H.W. et.al 2000). The disruption of these gradients by elevating external nitrate and ammonia concentrations should induce changes in the mats diversity and structure and possibly provide a excellent environment for denitrifiers or other bacteria to bloom.

Materials and Methods

Sample and Experimental Conditions

Samples were collected from the Sippewissett Marsh in Woods Hole, MA.Two adjacent 20 X 20cm sections were removed and placed in plastic pans of equal dimensions. The mats were undercut to a depth of about 4cm.The mats only half filled

the pans leaving the remainder of the pans to be filled later with seawater. The mats were then transported to the laboratory at the Marine Biological Laboratory (MBL). The mats were placed 60cm under a 60-Watt bulb without a light-dark cycle.

Sand was collected from Stoney Beach in Woods Hole, Massachusetts. The sand was rinsed, autoclaved and then allowed to dry at 80°C. The sand was placed on the top of both of the mats to an approximate depth of 7mm. The was initially placed on the mats to a depth of 3mm but because of visible greening of the sand by the first evening of the study more sand was placed on top to its final depth. Also I feared that a 3mm sand layer would not be thick enough to effectively observe mat migrations.

Seawater pumped into the Loeb building was used to hydrate the mats through a constant drip system. The seawater possessed a salinity of about 30‰, a pH of 7.8, and concentrations of nitrate, ammonia and sulfide of 2.15μM, 12.64μM, and 0M respectively. One mat received seawater directly from the pipes. The second mat received seawater with sodium nitrate and ammonia chloride added to final concentrations of ~2mM and ~200μM respectively. The seawater was then sterilized to minimize any possibility of growth in the media that is not from the mat itself and placed in a sterile carboy to dispense the seawater.

Cores Extraction

Cores 8mm in diameter were taken for use in pigment extraction and DNA extraction. Each core was segmented into 3mm cross sections from the top of the mat. For the cores taken after the sand was added the sand disturbance layer was taken as one cross section which ranged from 5-7mm in depth. Cores were taken on day 0, day 3, day 6, day 10, and day 11. After the removal of each core sterile sand was placed back into the mat to diminish any effects caused by coring.

Pigment Analysis

Each 3mm cross section was placed in a 2ml screw cap tube with 500μl of dH_2O and ~1g of 0.1mm glass beads. The tubes were shaken in a bead beater for 2 minutes. Tubes were spun multiple times at 12,000 rpm for 2 min until only a small or no pellet formed. 200μl of the supernatant was added to 800μl of dH_2O. Each sample was run on a

Beckman spectrophotometer where the absorbance spectrum between 400 and 1050nm could be calculated.

T-RFLP Analysis

Total genomic DNA was extracted from each 3mm cross section using the Mo Bio Ultra Clean Soil DNA Kit. A segment of the 16s rRNA gene sequence was amplified by PCR using the universal bacterial primers 8 Forward – HEX (5' [5-HEX] AGA GTT TGA TCC TGG CTC AG 3') and 14 Reverse (5' GGT TAC CTT GTT ACG ACT T 3'). The 8F primer possessed a 5' terminal HEX tag for detection. The PCR conditions were as follows:

1X 95°C for 5 minutes

30X
- 95°C for 30 seconds
- 55°C for 30 seconds
- 72°C for 1 minute

200ng of each PCR reaction was digested with RsaI at 37°C for 1.5 hours and then inactivated at 80°C for 30 minutes. The digested DNA was then shipped off to the sequencing facility at the Marine Biological Laboratory. The sequence facility performed the T-RFLP electrophoresis and band detection. Analysis of the banding pattern was done using the program GeneScan Version 3.1 from Applied Biosystems.

Analysis of the banding patterns was done using the TAP database for identifying specific terminal restriction fragment sizes located at http://www.cme.msu.edu/RDP/trflp/#program (T.L. Marsh et. al, 2000).

Microelectrode Profiles

The microelectrodes used in this experiment were generously lent to the Microbial Diversity Course by Dr. Pieter Visscher at the University of Connecticut. Profiles were generated for both pH and sulfide levels at increments of 1mm.

Results

Macroscopic Analysis

About ten hours after the sand was placed on both mats, cyanobacteria could be easily seen turning the fresh sand green. By day three the –N mat showed more growth by cyanobacteria on top of the sand than the +N mat. Also by this time a bloom of bi-flagellated eukaryotes were observed in the media of the +N mat. On the fifth day of the experiment the +N mat's surface began to turn white due to the presence of long white filaments (The description of these filaments is presented below). The white filamentous growth progressed until the tenth day when the cyanobacteria were observed to be increasing in volume. On day twelve purple bacteria could be seen through the top layer of the +N mat by a distinct reddish tint. This same process was observed in the –N mat but only in areas where cores were removed and the mat had begun to grow into the newly disturbed area. The rest of the +N mat was almost completely green after day eight.

Microscopic Analysis

Sand disturbance zone: The initial colonizers were different for each mat with *Lyngbya* largely present in the +N mat and *Microcoleus* in the –N mat. This changed by day three where *Microcoleus* had replaced much of the *Lyngbya* in the +N mat. Some *Leptolyngbya* and *Pseudoanabaena* were also observed in both mats but population sizes did not change dramatically over time. The white filaments that began to form on day 3 appeared to consist of *Lyngbya* filaments draped in granules. These granules may have been composed of sulfur granules, which would be consistent with the high levels of sulfide in the mat (microscopic and microelectrode analysis), but this observation was not verified. SYBR Green staining of the filament did reveal large amounts of rod shaped bacteria in contact with the sheath material, (Figure 1) but they did not appear to be associated with the granules (Figure 2). Microelectrode profiling, see below, showed the seawater above the mat to be highly alkaline with a pH of 9.8. This could induce the precipitation of calcium carbonate which could then stick to the cyanobacterial sheaths.

However, this assumption is merely speculation at this time. Raising the pH of a sample would help in clarify this issue.

The reddening of the mat on day twelve was due a bloom of purple bacteria of various morphologies. Those that were easily discernable were rod and cocci shaped with internal sulfur granules. Neither bacterial type was motile.

Lower Mat: For the original mat underneath the sand disturbance very little was discernable using microscopy. Therefore only the most distinctive bacteria were noted in my observations. The red layer, which is indicative of purple sulfur bacteria, appeared to be largely composed of coccoid bacteria that divide in two planes to forms sheets of cells. The majority of these colonies were observed to never grow larger than four cells. They also seemed to possess internal sulfur granules based on their diffraction pattern under phase contrast microscopy. The small size of the cells made it difficult to detect multiple granules in one cell. In both mats these cells were observed throughout the mat by the end of the experiment on day fifteen. However, the population in the +N mat was much greater than in the –N mat.

Throughout the span of the experiment a possible type of green algae was found in the pink layer of both mats when the layer was present. They were large colonial structures with about 8-10 cells in a congregate with an outer round sheath. It is also possible that these cells are instead chloroplasts. They are also non-motile and never observed outside the red layer of the mat. Its' presence in the purple sulfur layer shows that it is at least tolerant to high sulfide concentrations if not requires the presence of sulfide to survive.

Microelectrode Analysis

Both mats showed a varied degree of population heterogeneity so two profiles were constructed for each mat. One profile was taken in an area of high cyanobacteria density and the other in an area of low cyanobacteria density.

For all sites sulfide levels remained relatively low until a couple of millimeters beneath the surface of the mat (Figure 3-6). The sites with the most cyanobacteria, irrespective of the mat, showed a dramatic increase in sulfide levels below 3mm depth

(Figure 3,5). For the cyanobacteria replete areas the sulfide began to increase below 2mm depth (Figure 4,6). This increase in sulfide concentration mostly likely marks the oxic-anoxic barrier in the mat. The presence of the cyanobacteria may be effecting the concentration of sulfide near the mat surface by generating oxygen and pushing the oxic-anoxic layer further down into the mat.

The profiles in the +N mats also show another increase in sulfide concentration between 8 and 10mm depth (Figure 5,6). This increase possible marks a large population of sulfate reducing bacteria only present in the +N mat.

T-RFLP Analysis

The analysis of the banding patterns from the T-RFLP gel was largely inconclusive. Many of the band sizes were unknown to the TAP database. Many other bands corresponded to a multitude of bacterial species that prevented accurate identification.

There were very few similarities observed between time points in either mat. It appears as if each mat is constantly turning over, replacing bacterial populations over time. Two bands of 308 and 225bp were only present in the top two sections of the +N mat on day six as well as in the top two sections in the –N mat on day ten. This might indicate that these are transitional species that bloomed earlier in the successional process because of the elevated nitrogen concentrations. Another band at 171bp is present throughout the +N mat on the sixth day but only present in the bottom two sections of the –N mat on the same day. This may indicate the loss or lessening of a gradient present in both mats. The band correlated with the bacterium *Kineoporia rhizophila. K. rhizophila* can digest complex polysaccharides and incorporate inorganic nitrogen (Korn-wndisch and Kutzner, 1991). Various Vibrio species were detected throughout all of the mat samples. Other bacteria that were detected belonged to the genera of *Nitrosopira, Eubacterium, Microbacterium*, and *Brevibacterium. Nitrosospira* is an ammonia oxidizer and *Eubacterium moniliforme* is a nitrate reducer found in soils. However, *Microbacterium* and *Brevibacterium* are only found in rotting cheese and meat. This result is most likely due to the limited TAP database.

It is also important to note that no phototrophic bacteria were detected using T-RFLP. This either was due to the limited TAP database or that most cyanobacteria do not possess a RsaI restriction site in the amplified region of the 16s rRNA gene sequence. In order to correct this, different restriction enzymes could be used or even cyanobacterial and purple bacterial specific primers.

Spectral Analysis

The spectral data was largely incomprehensible. No major patterns could be accurately discerned within wither mat. This may have been due to the heterogeneity present in both mats. Differences between time points and mats may only be due to the location in the mat the core was removed from and not an actual change in community structure.

Bacteriochlorophyll a and b as well as chlorophyll a were detected in all cores. Chlorophyll a, the reaction center pigment in cyanobacteria, decreased in intensity with depth. Also the amount of bacteriochlorophyll a and b in the upper layers of the mats increased with time which is consistent with microscopic observations.

A peak at 960nm was also observed in various core sections without a defined pattern. This absorbance peak could not be determined. I am presently attempting to unravel this mystery.

I do not believe that using the detection of pigments is an accurate way to monitor population migrations in microbial mats. This method does not take into account cell counts and pigment amounts per cell. Therefore it can only be used as a qualitative assay for the presence or absence of a certain pigment.

Conclusions and Future Directions

The only conclusive result was that saturating levels of nitrate and ammonia effected the community structure and successional process of a microbial mat after a disturbance. The lack of any replication brings doubt into all of the patterns I was able to discern. In order to accurately describe the process of microbial mat succession many replicates would have to be taken and analyzed due to the extensive community

heterogeneity. This was seen on the macroscopic level as two cores taken from the same mat at the same time had very different banding patterns. Other dramatic macroscopic observations like the bloom of purple sulfur bacteria and the white cyanobacterial filiaments on the +N mat showed that under laboratory conditions elevated levels of nitrogen compounds do effect community structure and succession. Unfortunately I was unable to accurately define these changes on the molecular level.

The –N mat was not an effective control and I did not analyze it for that purpose. The process of relocating a mat to laboratory conditions severely altered the environment for the microbial mat. Many inhibitory and beneficial factors are removed which would alter the community dramatically. Future experiments should incorporate laboratory experiments with an additional mat as a control that does not receive the sand disturbance. Alternatively this experiment could be run out in the marsh. Under these conditions more of the environmental factors effecting the natural mat communities would be taken into account but the feasibility of the experiment would be more difficult.

Acknowledgements

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Figure 1. Image of the white filaments taken under a Zeiss confocal imaging microscope at 20X under blue light. Small congregates of bacteria are easily seen attached the cyanobacterial filaments.

Figure 1

Figure 2. Images were taken on at 40X magnification under phase contrast. The right image corresponds to the sample stained with SYBR Green stain under blue light fluorescence. Arrows indicate individual bacteria and do not appear to be associated with the particulate matter.

Figure 2

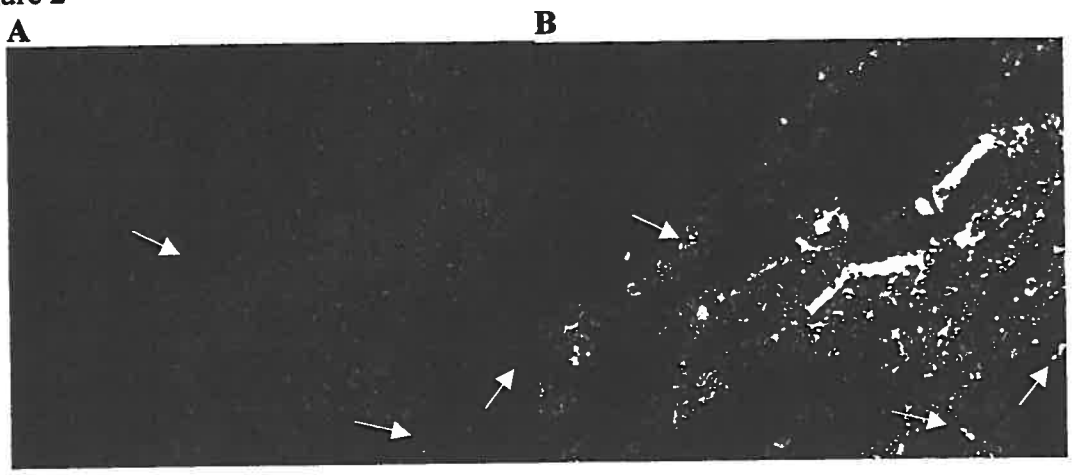
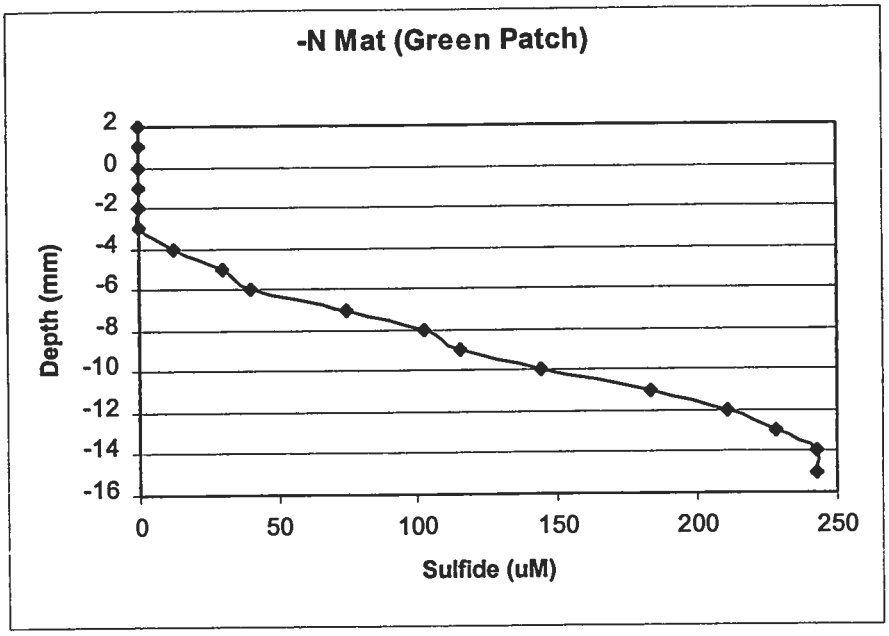


Figure 3-6. All profiles were taken on day five after the addition of sand. 0mm depth represents the mat surface. Negative values represent distance into the mat.

Figure 3A.



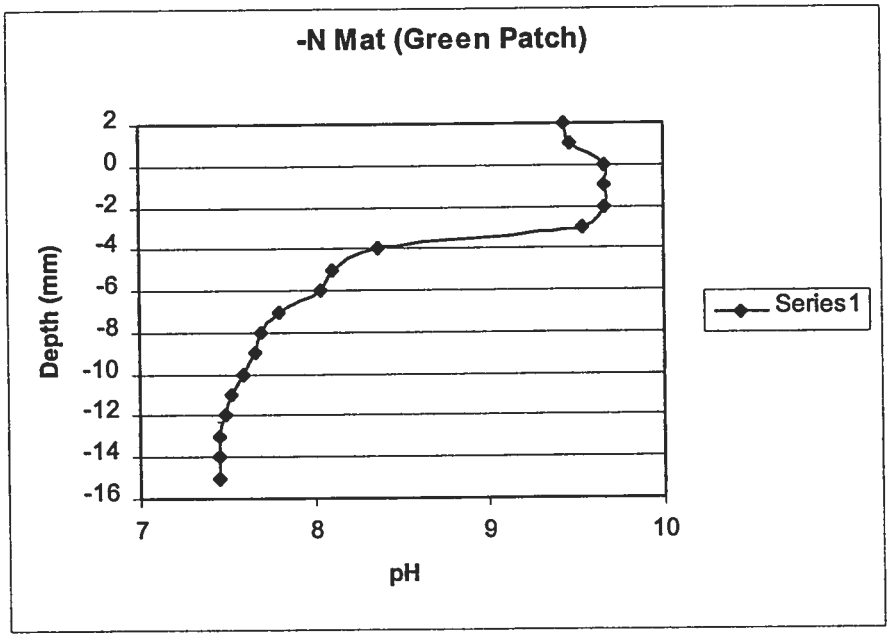
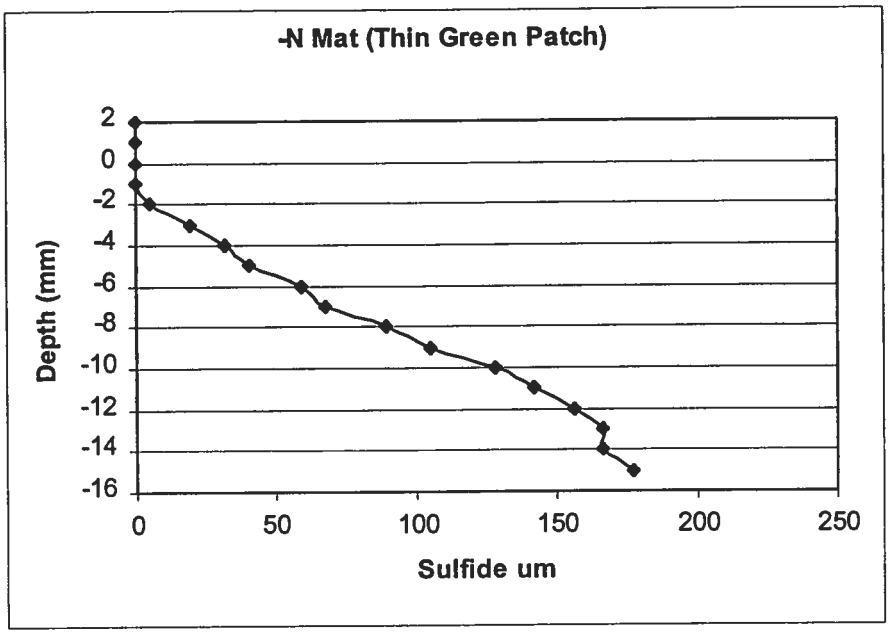


Figure 3B.

Figure 4A.



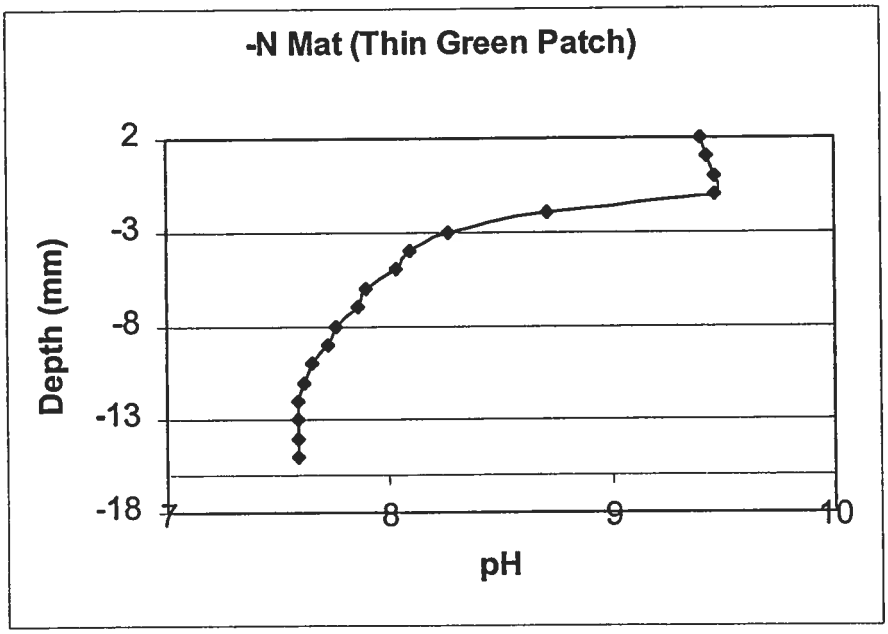
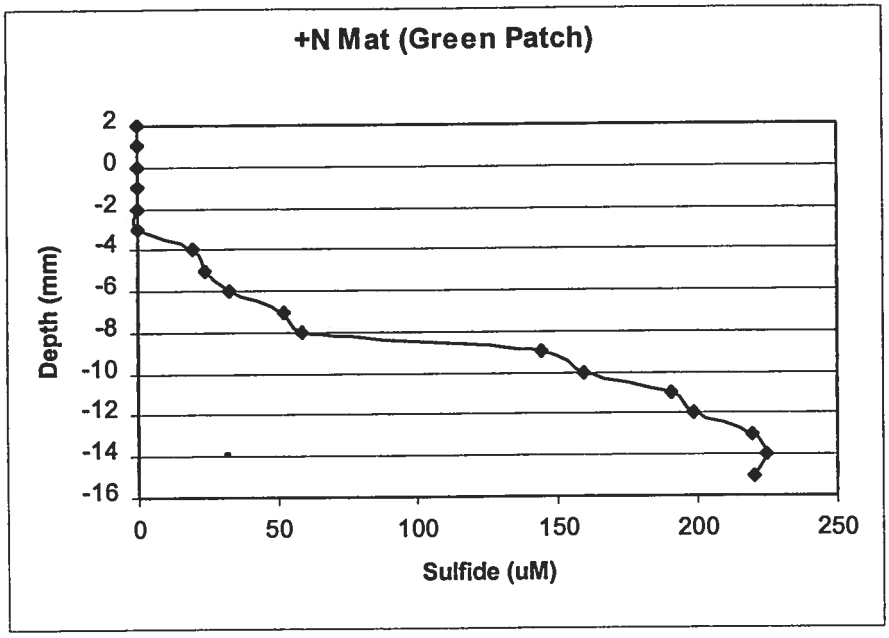


Figure 4B.

Figure 5A.



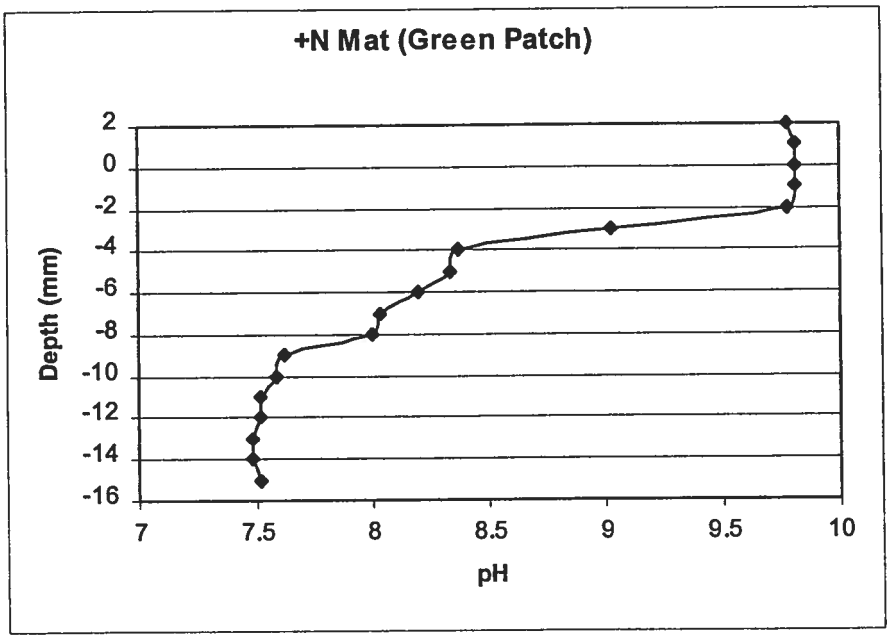
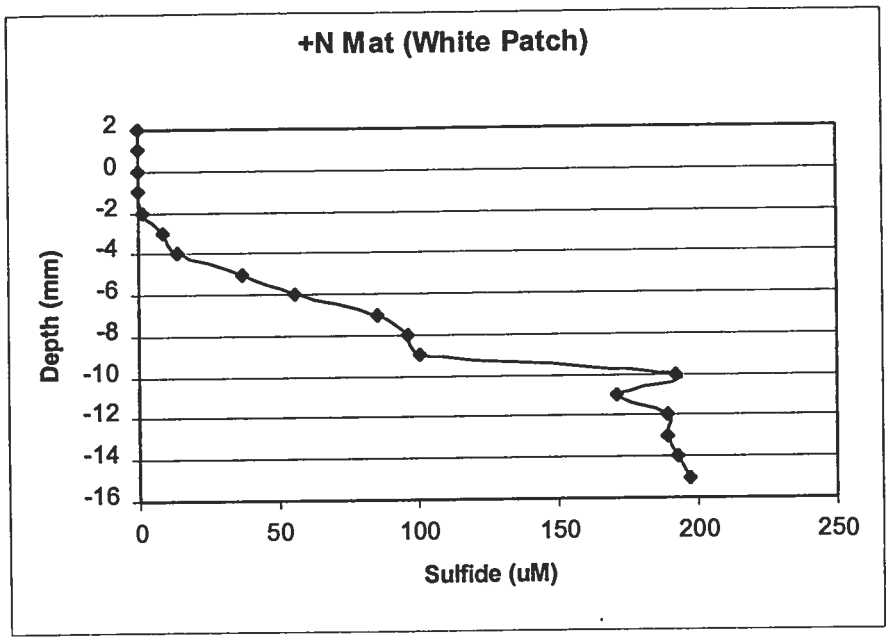


Figure 5B.

Figure 6A.



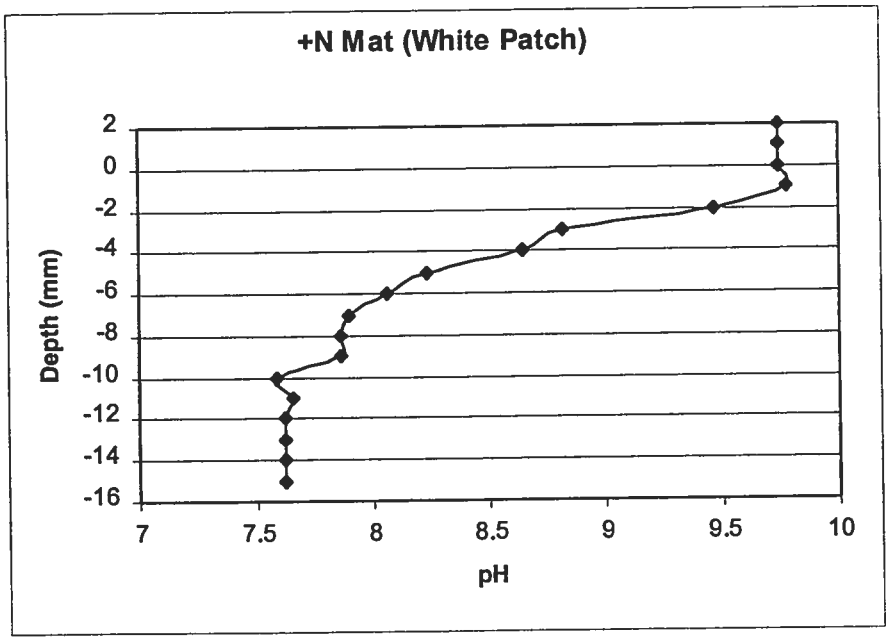


Figure 6B.