Enrichment and Isolation of Methanogens from Termite Hindgut

by Ron Anderson

Introduction

Termites are estimated to produce between 25 and 150 Tg/year of methane (Hackstein and Stumm, 1991; Kane and Breznak, 1991). They are a small but an important source of methane in the atmosphere, and they make a significant contribution to global warming.

Methane is produced by methanogens located in the termite hindgut (Breznak and Brune, 1994). Methanogens are joined by a consortium of cellulolytic protozoa and other bacteria to process wood and other materials ingested by the termite. The ingested materials are degraded into low molecular weight molecules that are used by the termite for nutrition. Protozoans provide the enzymes needed to degrade the cellulose into glucose. Glucose is then fermented to produce acetate. Acetate is the major carbon source for the termite. CO_2 and H_2 are also produced during the degradation of the cellulose to acetate.

Methanogens use CO_2 and H_2 to form methane. H_2 is provided to the methanogen by the protozoa by a process known as intra-species H_2 transfer (Brauman *et al.*, 1992; Breznak and Blum, 1991). Acetogens also use CO_2 and H_2 to produce acetate. They are in competition with the methanogens for these materials. In most situations (such as the cow rumen), methanogens out-compete the acetogens. However, in the termite gut, acetogens utilize most of the CO_2 and H_2 to produce acetate which is used by the termite for nutrition. The methane produced by the methanogens from the remaining CO_2 and H_2 is expelled into the atmosphere.

Methanogens require stringent anaerobic conditions for growth and are probably restricted to the

parts of the hindgut that are anaerobic (Bignell and Anderson, 1980; Brune *et al.*, 1995). Analysis of the hindgut by microelectrode probes has shown that there is an anaerobic zone in the shape of a tube down the center of the gut. Since the cells in the gut wall are oxygenated, the anaerobic zone is probably protected from O_2 by a mass of poorly mixed O_2 utilizing material next to the gut wall.

The epithelial cells in the hindgut are mucus free (Bignell *et al.*, 1980). Mucus would normally be expected to protect the gut wall from abrasion. Instead, spines are located in the gut wall to protect the gut wall and to assist the movement of material in the hindgut. There are structures on the outside of the gut wall that support and move the spines. The spines may be hollow and transport nutrients out of the hindgut (Bignell *et al.*, 1980).

The reactions to reduce CO_2 requires a unique cofactor know as F_{420} (Cheeseman *et al.*, 1972). F_{420} is autofluorescent. It has an absorption peak at 420 nm and fluoresces with a blue-green light. Methanogens can be located in the hindgut by observation of the blue-green light using a UV microscope (Doddema and Vogels, 1978).

Methanogens can be rods, cocci, or filaments (Breznak and Pankratz, 1977; Brune *et al.*, 1994). Their location in the gut is unknown. There are several likely locations for the methanogens in the hindgut. They may be free swimming in the gut contents. They may be intra-cellular symbionts in some of the protozoa (Giljzen *et al.*, 1991; Lee *et al.*, 1987; Odelson and Breznak, 1985; To *et al.*, 1980). They may coat the surface of filamentous bacteria located in the gut. They may be located in special tissues. Also, they may be attached to the spines from the gut wall (Breznak and Pankratz, 1977).

There are ways to stimulate methane production and promote the growth of methanogens in the

termite gut. The production of methane by methanogens can be increased by incubating the termites in an atmosphere of H_2 and CO_2 (Messer *et al.*, 1989). The growth rate of the methanogens also probably increases. In cockroaches the production of methane and the growth rate of the methanogens can probably be increased by the diet of the termite (Kaesler and Schonheit, 1989). This may also be true in termites. Feeding termites a special diet and incubating them in a CO_2 and H_2 atmosphere should also increase the density of methanogens and make isolation of methanogens easier. New methanogens may be found in the termite hindgut by this method.

The objective of this project is to isolate methanogens by an *in vivo* enrichment. A feeding study will be conducted to screen various materials for their effect on the methanogen population and methane production. The termites will be fed a diet that increases the density of methanogens in the hindgut and will be incubated in a H_2/CO_2 atmosphere to stimulate the growth of methanogens and methane production. Normal enrichment procedures will be followed to isolate methanogens from the enriched termite gut. It is hoped that this approach will allow the isolation of methanogen that have not been isolated previously. It is also expected that the location of the methanogens in the gut can be determined by microscopy. Any methanogens isolated by this procedure will be further characterized.

Materials and Methods

Termites - Specimens of the lower termite species *Reticulitermes flavipes* were collected from cardboard scraps placed under large pieces of wood in E. Leadbetter's back yard. The termites were trapped on the outer surface and in the folds of the moist cardboard scraps. Termites were collected and trapped in large groups within 24 hours of setting the cardboard traps. The termites were maintained for up to four days in the laboratory on moist cardboard and soil. Both

soldier and worker termites were collected in the traps. Only the worker termites were used for experimentation.

Enrichment Media - The enrichment medium was the anaerobic medium (AC-21) described by Breznak *et al.* (1988) modified by using the trace elements described by Greening and Leedle (1989). The medium was made and dispensed by Hungate procedures for stringent anaerobes. The antibiotics Cephalotine (20μg/ml of medium) and Clindamycine (4μg/ml of medium) were added to the medium in some experiments. In some experiments bottle plates were used. The medium was AC-21 medium with agar at a final concentration of 20g/l. In all experiments the inoculum was mixed or slurred in enough bovine rumen fluid to give a final rumen fluid concentration of 10%. The rumen fluid was filtered, centrifuged and autoclaved before use. In all cases the shake tubes or bottle plates were gassed with a H_2/CO_2 mixture (80/20 v/v) to a 30 psi. Nutrient agar was used to plate aerobic spore forming bacteria. All samples were incubated at room temperature in the dark.

Feeding Experiments - Groups of up to 15 termites were placed in divided petri dishes and stored in the dark for one or two days. The food source was a drop of solution placed at the intersection of the divider and the petri dish wall. The food sources were xylan, D-xylose, D-cellobiose, chitin, potato starch, sodium formate, sodium acetate, citric pectin and dextrose. They were used with enough water to form clear solutions or pastes (25% to 50%). Some termites were fed antibodies by wetting Whatman filter paper with dilute solutions of Cephalotine and Clindamycine. Termites for control experiments were fed moist Whatman filter paper and stored in petri dishes. After the feeding phase the termites were transferred to 10 ml GC sampling vials with a septum closure and flushed with H_2/CO_2 (80/20 v/v) for 15 seconds. After storage at room temperature the head space in the vials was sampled for analysis and the termites were removed for microscopic analysis.

Microscopy - The bacteria in the termite hindgut were observed by both phase contrast and fluorescence microscopy. A Zeiss microscope with phase contrast optics and a UV light source was utilized for both microscopic techniques. The microscope was capable of rapidly changing between the two methods. F_{420} was observed by illuminating the sample with UV light at 400 to 420 nm. The sample was observed through a low pass filter at 470 nm. It was noted that the blue color emitted by F_{420} would fade after a few minutes of exposure to UV light. It is known that F_{420} must be in oxidized state to fluoresce (Cheeseman *et al.*, 1972; Doddema and Vogels, 1978). For some observations an oxidation pretreatment was conducted by mixing the sample on the slide with a few crystals of potassium ferricyanide. This assured that the F_{420} was oxidized and enhanced the capability of observing the methanogens.

A single evaluation was made using the Nikon confocal microscope.

Head Space Gas Analysis - The methane in the head space of enrichment cultures and termite feeding studies was determined by gas chromatography using a Shimadzu GC-14A gas chromatograph with a 6' x 1/8" Supelco 80/100 Parapak R column and a flame ionization detector. The column temperature was 55^{o}C. The injector temperature and the detector temperatures were 80^{o}C. The sample injection volume was 40 µl. The carrier gas was helium, and the carrier gas inlet pressure was 1 kg/ cm^{2}. The GC method was calibrated with samples of known methane content. The data was recorded and analyzed using a Shimadzu CR501 integrator.

Enrichment Techniques - Bottle plates were used to provide a solid surface for the growth of methanogens and other hindgut bacteria. The bottle plates were inoculated with enough culture to cover the medium with culture to a depth of approximately 20 mm.

Microelectrodes - Microelectrodes were used to determine the pH, methane concentration, and the O_2 concentration in the hindgut. The microelectrodes (described by Revsbech *et al.*, 1986) were provided by L. Damgaard. The experiments were similar to the measurements described by Brune *et al.* (1995). Live termites were imbedded in 2% agarose strips formed between two glass microscope slides. To protect the microelectrodes during measurements the agarose strip was removed from the glass slides and positioned on the surface of agar medium in a plastic petri dish. The electrode was mounted on a computerized positioning device capable of accurately positioning the microelectrodes. The microelectrode was positioned to first penetrate the outer structure of the termite and then the hind gut. Measurements were made every 0.1 mm until the microelectrode penetrated the far side of the termite.

Results

Microscopic Analysis - The hindgut from the termite was removed by pulling the end of tail away from the midsection with tweezers. Usually the hindgut would separate from the stomach intact and was positioned in a straight line on a glass slide. The hindgut was then opened by dissection with a razor blade or split open by rubbing the intact hindgut between a glass slide and a cover slip. Generally the best slides were prepared by rubbing the hindgut between the slide and the cover glass. A wet mount was made using a drop of anaerobic medium. The contents of the hindgut were then observed microscopically.

The wet mounts were first observed by phase contrast microscopy. The hindgut contents consisted of bacteria, protozoans, gut wall structures, and liquid. The liquid material was of unknown composition, but it contained cellulosic matter ingested by the termite during feeding. The wide range of microorganisms including spirochetes, filamentous bacteria and cocci bacteria,

and many unidentified protozoans. The protozoans and spirochetes showed rapid motility in fresh wet mounts. After several minutes the motility would cease beginning with the microorganisms located at the edges of the wet mount. Presumably oxygen diffused into the sample from the sides and the microorganisms were killed. The high density of protozoans and other unknown materials in the hind gut made recognition of bacteria impossible. The structures of the hindgut wall were observed, and the spines attached to the gut wall were identified. Identification of other specific structures was not possible.

The microscope light source was switched from visible light to UV light with a wave length of 420 nm. The wet mounts were then observed to detect fluorescent materials. Many fluorescent materials were seen but the blue green and yellow materials were the overwhelming majority. The blue green structures were either long filamentous materials associated with the gut wall, free swimming cocci, or nonuniform materials located inside the protozoans. Figure 1 shows some of the blue green structures that were observed. The yellow materials were nonuniform blobs located either free in the hindgut liquid or inside the protozoans.

To assist interpretation of the UV microscope observations, fresh pine sawdust and cellulose fibers (Whatman filter paper) were observed. Cellulose (Whatman filter paper) did not fluoresce. Fresh saw dust from pine board fluoresced only with a yellow color similar to the color observed in the saw dust. It is assumed that the aromatic components of the lignin is the material detected by the yellow color. It was concluded that the blue green materials were methanogens.

The blue green color faded after several minutes exposure to the light source. The fading was thought to be due to changes in oxidation state of the F_{420} cofactor. Some wet mounts were pre-treated with oxidizing agent to enhance the blue green color before UV observation.

The hind gut was extensively observed. Many wet mount views were observed by both phase contrast and UV techniques by switching rapidly between the two techniques. The locations of methanogens in the mass were determined by comparing the phase contrast and UV views. Slides and a video tape were made to record the results (not included here).

A single hindgut sample was observed using confocal microscopy. The light source was a laser with an adjustable wave length (420 nm). The result of the analysis is shown in the black and white picture in Figure 2. The filamentous structures are thought to be methanogens. It should be possible to determine the color of the fluorescent material using a color camera. However, the correct combination of laser light source and camera was not available. The method was promising.. I may be able to locate the methanogens attached to the hindgut structures.

Methane Generation by Groups of Termites - The production of methane by groups of termites was determined by storing groups of three termites in vials and measuring methane in the head space of the vial after 2 to 24 hours incubation (Table 1). In some samples the head space was initially exchanged with N_2/CO_2 or H_2/CO_2 gas mixtures. The generation of methane was unaffected by N_2/CO_2 gas. The generation of methane was enhanced by exchanging the head space with a H_2/CO_2 gas mixture. The survival rate of the termites was decreased by the gas exchange of both gas mixtures.

Feeding Study - A feeding study was carried out on groups of termites to determine the effects of diet on the microorganisms in the hindgut and on the generation of methane by methanogens. The feeding study consisted of two stages. During the first stage groups of termites were fed one of several food sources for 24 hours (Table 2). During the second stage subsets of termites from stage 1 were stored in 5 ml vials and the methane content of the head space was determined. At each stage termites were dissected and the hind gut was sampled and observed.

The results of the feeding study are seen in Table 2. Termites fed cellobiose, dextrose, sodium acetate, and sodium formate died during stage 1 and were discarded. The termites fed sodium acetate and sodium formate died after ingesting the materials. Termites fed cellobiose and dextrose died by sticking to the food source. Little of these materials was ingested by the termites. Microscopic observation during stage 1 showed the number of protozoans reduced during the feeding stage.

Termites from stage 1 were sampled and tested for methane generation (Figure 2). Methane production was lower for each food source except xylose when compared to the filter paper food source or filter paper impregnated with antibiotics. For some samples the head space gas was exchanged with H_2/CO_2. In each case methane generation was increased by the exchange of head space gas. Except for the xylan food source, the combination of food source and the exchange of head space gas resulted in increased methane production.

Microscopic analysis of the termite hind guts showed that all of the protozoans were killed with the exchange of head space gasses. Motile spirochetes remained in the hind gut liquid. The amount of blue green material was greater either because there were more methanogenic bacteria or the protozoans were not blocking the observation. Slide and a video tape were prepared to document the changes in the hindgut contents.

Termite survival rates were noted during each stage of the feeding study. Few termites survived the combination of feeding and head space gas exchange (Figure 2).

These observations indicated that the microbiota in the hindgut were significantly influenced by diet and H_2. The protozoans died. The microscopic observations suggests that the methanogens

were enriched in the feeding and $H_2\backslash CO_2$ treatments.

Enrichment and Isolation - Enrichments to assist in isolation of pure cultures of methanogens was attempted (Table 3). Anaerobic enrichments were prepared from the hindgut of both fresh live termites and live termites enriched for methanogens in the feeding study. Enrichment cultures were prepared in CA-1 medium in Hungate tubes followed by multiple transfer in tubes and bottles. The tubes were gassed with H_2/CO_2 to 30 psig. The cultures were observed daily by UV microscopy to detect florescent methanogens and were tested daily for methane generation in the head space. Filamentous florescent bacteria were observed in the first culture and methane was detected in the head space. Samples were transferred to new tubes. Bottle plates were used in subsequent transfers to provide a solid surface for the growth of the filamentous bacteria. Methanogens did not grow rapidly in the subsequent transfers. While anaerobic filamentous bacteria were seen, no pure cultures were obtained.

Aerobic spore formers were found in the anaerobically enrichments. The enriched material was treated by centrifugation and pasteurization to isolate the filamentous and spore forming bacteria. Sample of both materials were streaked on nutrient agar. A pure culture of a bacillus bacteria was found from the pasteurization. Several red colonies were found on the samples from the centrifugation treatment. The colonies were restreaked and a pure culture of red rods was obtained.

Microprobe Analysis - Microprobes for pH, CH_4, and O_2 were used to evaluate the conditions inside the termite hindgut. Each probe was inserted into the hindgut of live termites by penetrating the outside of hindgut. The pH probe showed that the interior of the gut was uniformly at pH 7. Methane levels in the hindgut were less than the detection limit of the probe. The O_2 probe gave the best results (Figure 3). The analysis was repeated over several termites

and consistent results were obtained for each specimen. Stringent anaerobic conditions were found immediately after penetrating the gut wall and were maintained until the probe penetrate the other side of the hindgut. The extent of anaerobic conditions seemed to be greater than previously reported (Brune *et al.*, 1995).

Conclusions

Based on this work the following is concluded:

1. Methanogens can be observed and identified in termite hindgut by a combination of microscopic techniques including confocal, UV, and phase contrast..
2. Methanogens in live termites can be enriched by diet, antibiotics and incubation in H_2 rich atmospheric conditions.
3. Methanogens are associated with hindgut tissues and structures.
4. Most of the termite hindgut is anaerobic. Only a small annulus at the hindgut is aerobic.
5. No pure cultures of methanogens were isolated during the summer project. Pure cultures of aerobic spore forming and filamentous bacteria were isolated from the hind gut by streaking enriched cultures on nutrient agar.

Recommendations

The following projects are recommended for future courses:

1. Isolate spirochetes from the termite hindgut.
2. Isolate spore forming filamentous bacteria from the termite hindgut.
3. Isolate methanogens from the termite hindgut using *in vivo* enrichment techniques as described in this report.
4. Locate filamentous methanogens associated with the hindgut wall or spines using confocal microscopy.

Table 1. Methane Generation by Isolated Termites
(methane as a percentage of the head space gas)

Atmosphere in Head Space (Ambient Pressure)	Methane Generation after 2 hr	Methane Generation after 4 hr	Methane Generation after 24 hr	Survival after 24 hr (live/total)
Ambient Air	0.02%	0.04%	0.15%	3/3
N_2/CO_2 (80/20)	0.02%	0.04%	0.07%	1/3
H_2/CO_2 (80/20)	0.08%	0.15%	0.56%	0/3

Note: Three worker termites were isolated in 5 ml sample vials with septum closures. The head space was exchanged with the gas mixtures noted and the vials were stored at room temperature in the dark.

Table 2. Termite Feeding Study

(Methane produced by termites vs. termites fed paper only)

Food Source	Methane Generation after 24 hr Feeding	Methane Generation after 24 hr Feeding and H_2 Stimulation	Microscopic Analysis after Feeding Stage	Survival after 24 hr (live/total)	
				In Air	H_2
Whatman Filter Paper	100%	100%	No Protozoans	2/3	2/3
Whatman Filter Paper with Antibiotics	98%	92%	Some Protozoans	3/3	2/3
Cellobiose	All termites dead	after feeding stage.	-	Not tested.	
Cellulose	73%	138%	-	3/3	1/3
Chitin	57%	147%	-	3/3	1/3
Dextrose	All termites dead	after feeding stage.	-	Not tested.	
Pectin (Citrus)	38%	173%	Some Protozoans	1/3	1/3
Potato Starch	39%	138%	Some Protozoans	1/3	1/3
Sodium Acetate	All termites dead	after feeding stage.	-	Not tested.	
Sodium Formate	All termites dead	after feeding stage.	-	Not Tested	
D-Xylan	62%	103%	Some Protozoans	3/3	1/3
Xylose	116%	127%	No Protozoans	3/3	1/3

Test Procedure: Fifteen worker termites were isolated and fed the food source indicated above for 24 hours. After feeding, three termites were transferred to 5 ml vials and stored for 24 hours at room temperature. The amount of methane generated in the head space was determined by GC.

Table 3. Enrichment for Methanogens
(anaerobic enrichment in Hungate tubes)

Inoculum	Antibiotics	Rumen Fluid	Methane Generation after 4 Days	Methane Generation after 14 Days	Visual and Microscopic Observations
Fresh Gut	Yes	Yes	0.02%	0.26%	Slight turbidity - No Methanogens Present
Fresh Gut	No	Yes	0.01%	0.15%	Slight turbidity
Fresh Gut	Yes	No	0.08%	6.62%	Cloudy with Methanogens Present
Fresh Gut	No	No	<0.01%	2.05%	Cloudy with Methanogens Present
Old Gut (with pectin)	Yes	Yes	0.03%	0.67%	Slight turbidity Methanogens present

Methane concentrations reported as % of head space gas above the base Methane generation of termites fed paper.

Test Procedure: Anaerobic tubes were inoculated with hindgut material slurred in anoxic medium. The head space was exchanged with H_2/CO_2 (v/v) gas to 3 atmospheres.

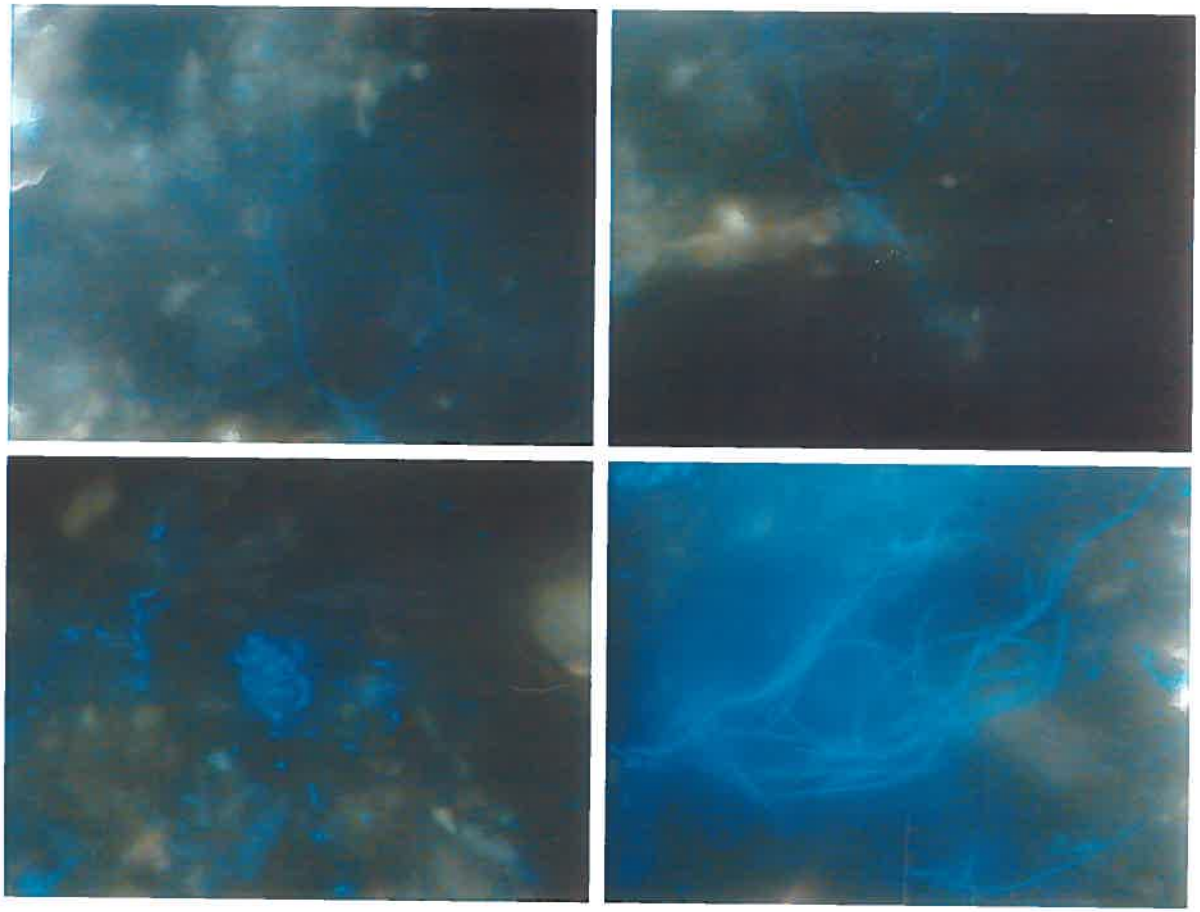


Figure 1. Photomicrographs of Methanogens in the Termite Hindgut
(UV light source at 420 nm)

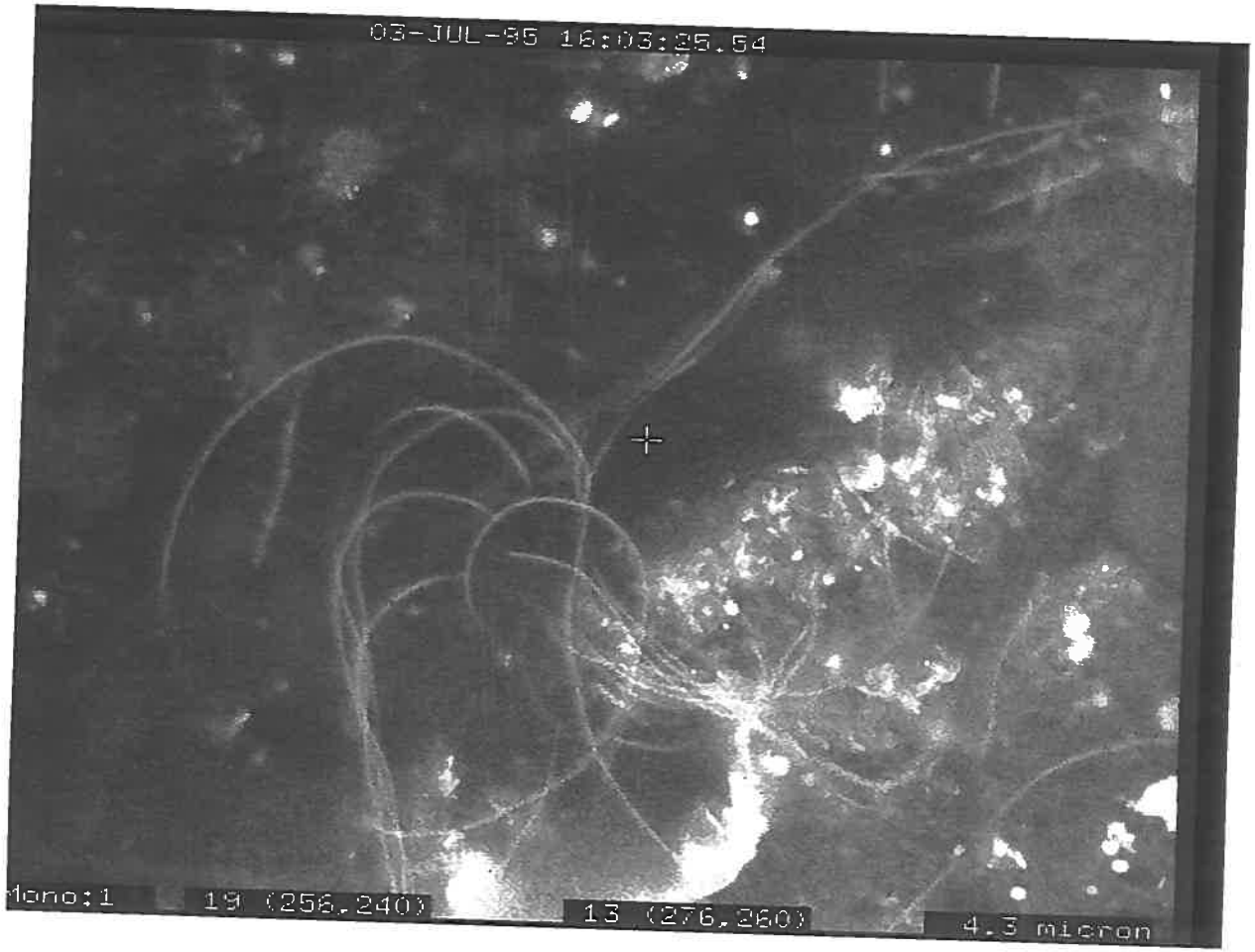
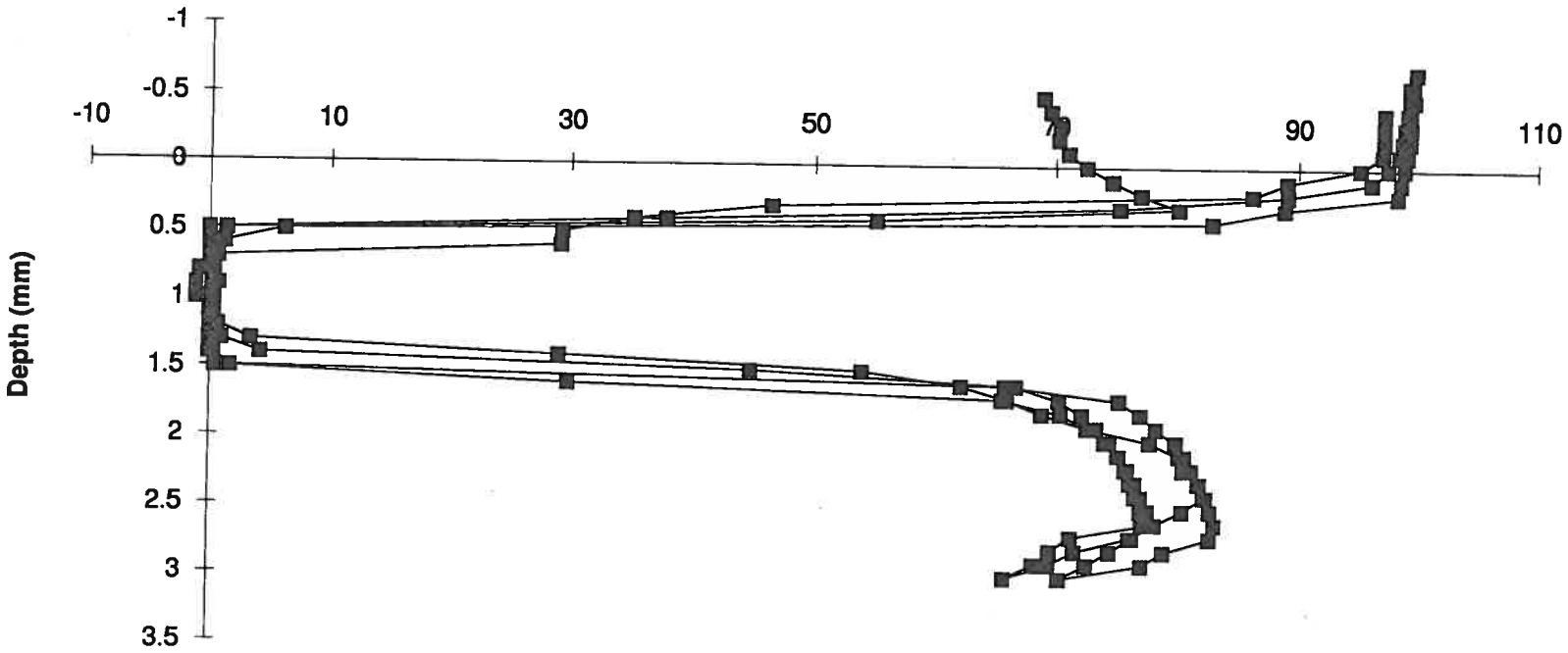


Figure 2. Confocal Photomicrograph of Methanogens in the Termite Hindgut
(UV light source at 420 nm)

Figure 3. Oxygen Tension in the Termite Hindgut

Oxygen concentration as % saturation of air.



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