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Microbial methane oxidation assessment and characterisation in bench-scale landfill bioreactors

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CHAPTER 2.2.

PH, bacterial populations and methane emission observations on three different nutrient amendment types in landfill soil: a preliminary study

ABSTRACT

Three bioreactor sets (A, B and C) were constructed to investigate the affects of nutrient addition to soil on methane (CH_4) emissions from the waste. Set A, had no soil nutrient additions. Set B contained nitrate and phosphate additions to soil at concentrations of 50 µM and 150 µM, respectively. Set C had dried sewage cake added to soil serving as a readily available nutrient source. Gas chromatography and pH measurements were performed on all 3 anaerobic bioreactors. Tryptone, yeast, glucose (TYG) media was used for general aerobic bacterial counts from leachate and soil. Nitrate Mineral Salts (NMS) medium was used to enrich for methanotrophs from soil. Scanning electron microscopy (SEM) was used to observe the morphologies of bacterial populations associated with leachate and soil from the 3 bioreactor sets. Gas chromatography revealed that the anaerobic bioreactor of Set B was the most efficient at CH_4 production, initiating in week 2 (8 % CH_4). Set A anaerobic bioreactor was the least efficient at CH_4 production where CH_4 was only observed in week 7. By then the pH in the Set A anaerobic bioreactor had decreased to 5 and CH_4 levels were at 0.4 %. pH deviations to acidic levels were not observed in Sets B or C where CH_4 was produced till week 6. Leachate dilutions of 40 ml per week were concluded at being efficient at limiting leachate pH fluctuations. No significant differences in the bacterial counts between TYG and NMS plates were observed for soil, indicating heterotrophic bacterial species growth on NMS media. Leachate bacterial counts were above 6 log cfu/ml for all bioreactor sets. SEM of leachate from the 3 anaerobic bioreactors showed rods, cocci and EPS material presence. Scanning electron microscopy of soil revealed presumed debris (soil) covered rods and coccoid-shaped cells.

INTRODUCTION

This was a pilot study involving the use of bench-scale bioreactors to simulate landfill processes. Usually under field conditions, bacteria experience reduced levels of nutrients (Amann, *et al,* 1999) which are often not accounted for when isolating bacteria in the laboratory. This leads to instances where bacteria have an overabundance of essential nutrients which do not facilitate the selection of important landfill bacterial consortium members. It is estimated that only 1 % of environmental bacteria are cultivable (Amann *et al.* 1999; Hanson and Hanson, 1996) and 'overfeeding' of bacteria may be responsible for the poor number of successful isolations of environmental bacteria. Methanotroph bacteria tend to constitute only 1 % of soil bacteria (Hanson and Hanson, 1996; Wise *et al*. 1999; Wise *et al.* 2001) and culturing difficulties in the laboratory could be attributed to incorrect media concentrations. For culture purposes, enrichment procedures can yield a wider variety of methanotroph strains (Wise *et al.* 1999). However, low success rates with regard to culturing of these bacteria have stimulated the use of molecular methods for taxonomic purposes in methanotroph research (Murrell and Radajewski, 2000).

Methanotrophs receive their carbon from the CH_4 they oxidise. Nitrogen (in the preferred form of nitrate) and Phosphorus (in the form of phosphate) sources tend to be limiting in the environment, as these elements are most widely used by every bacterial, fungal and plant species in soil. These elements are crucial to basic cell function and energy generation. However, too high concentrations of either element can also inhibit methanotroph growth. Bussmann *et al.* (2004) reported in their studies of forest soil methanotrophs that phosphate concentrations did not inhibit methanotroph growth.

However, nitrate tended to be inhibitory at concentrations higher that 50 µM whereby CH_4 oxidation was seen to cease completely at levels above this concentration (Bussmann *et al*. 2004). It has been proposed that inhibition is caused by competition for the active site of the methane monooxygenase enzyme by nitrate, that catalyses CH_4 oxidation reactions (Hanson, 1996; Mancinelli, 1997; Murrell and Radajewski, 2001; Bodelier and Laanbroek, 2004).

Biofilm formation is favoured in the environment and in landfills the wastes serve as the adhesion surfaces for waste degrading bacteria. In this nutrient deficient environment (nitrogen and phosphorus sources), the bacterial organisation into biofilms enhances scarce nutrient and water accumulation necessary for waste degradation reactions. Scanning electron microscopy (SEM) serves as a visual tool for the morphological analysis of waste degrading bacteria and dehydrated forms of possible exopolysaccharide (EPS) material in the leachate.

In this study, 3 bioreactor sets were constructed and commissioned as bench-scale landfill simulators. Set A, served as control with no nutrient additions to the soil. Set B soil contained nitrate and phosphate at concentrations of 50 µM and 150 µM respectively (Bussmann *et al.* 2004). Set C soil contained dried sewage cake as an example of a readily available source of nutrients.

The aim of this experiment was to set up a pilot study to observe changes in parameters relating to CH_4 emissions and bacterial populations and the possible effect these

parameters may have on different soil nutrient additions on the CH_4 oxidation capabilities of methanotrophs in the soil. SEM was employed for morphological observation of soil and leachate samples.

MATERIALS AND METHODS

Bioreactor design

Three sets of bioreactors were constructed and set up as previously described (Chapter 2.1). Anaerobic bioreactor waste components were kept at the same proportions to each other whilst the aerobic soil bioreactors differed in phosphate (PO_4) and nitrate (NO_3) sources and the operating temperature was changed from 37 ºC to 25 ºC. Set A soil bioreactor (Figure 2.2.1 A) contained no supplementary nutrient additions (simulating current landfill field conditions) and was observed over a 12 week period. Set A anaerobic bioreactor leachate was diluted at 10 ml per week for the first 7 weeks, and thereafter at 40 ml per week until week 12. Set B soil bioreactor (Figure 2.2.1 B) contained 50 µM NO_3 + 150 µM PO_4 additions to the soil while Set C soil bioreactor (Figure 2.2.1 C) contained dried sewage cake added to the soil. The dried sewage cake was obtained from Goudkoppies Wastewater Treatment Plant (Johannesburg, South Africa) and was estimated from the Treatment plants data to contain 50g/kg N and 8 g/kg P. Sets B and C were run over a 6-week period and leachate dilution in the anaerobic bioreactors for each of these sets was at 40 ml per week.

pH analyses

pH analyses on leachate samples were done weekly using pH indicator strips (Merck).

Gas Chromatography

Samples were taken and analysed as described in Chapter 2.1. Samples were taken twice weekly in order to determine the precise onset of methanogenesis.

Bacterial sampling and culturing

Sampling and culturing procedures for leachate and soil samples were as described in Chapter 2.1. For methanotroph culturing a pre-enrichment step in NMS media prior to plating was performed. All soil NMS media plates were incubated in an air atmosphere supplemented with 90 %: 10 % CH_4: CO_2 atmosphere at 25º C. Soil TYG plates were also incubated at this temperature with the CH_4: CO_2 atmosphere emitted. Leachate TYG plates (incubated at 37º C), soil TYG plates and NMS media plates were counted 7 days after incubation, at weekly intervals.

Scanning Electron Microscopy (SEM)

Leachate and soil samples were sampled at week 1 from all bioreactor sets for a general morphological comparison between samples. Aqueous solutions of leachate and soil slurries were filtered onto 0.2 micron membranes and fixed in 3 % gluteraldehyde overnight. Samples were then dehydrated in alcohol and prepared for SEM according to methods in Lindsay and von Holy, (1999). Samples were then viewed using a Jeol 840 scanning electron microscope.

RESULTS

Waste morphology

Figure 2.2.2 shows the MSW (contents in anaerobic bioreactors) after degradation experiments from bioreactor sets A (12 weeks), B (6 weeks) and C (6 weeks), respectively. Organic waste such as meat and vegetables degraded to pulp (Figure 2.2.2 I). By contrast, paper and plastic (Figure 2.2.2 II and III) did not degrade after each bioreactor experiment.

pH

Figure 2.2.3 shows the pH profiles of leachate samples from the anaerobic bioreactor of Set A over 12 weeks (Figure 2.2.3 A), compared to Sets B and C over 6 weeks (Figure 2.2.3 B). For Set A anaerobic bioreactor the pH dropped to 6 in week 6 and further decreases were observed in subsequent weeks (Figure 2.2.3 A). An increase in pH to 7 occurred in week 11 followed by a decrease again to pH 6 in week 12. pH levels for Sets B and C rose steadily to a final pH of 8.5 in week 6 (Figure 2.2.3 B), and never decreased below pH 6.

Gas Chromatography

Set A anaerobic bioreactor (Figure 2.2.4 A) initiated CH_4 production at day 51 (0.4 % CH_4), Set B at day 17 (0.4 % CH_4) and Set C at day 31 (3 % CH_4, Figure 2.2.4 B). CO_2 production for Set A initiated at day 26, Set B at day 15 and Set C at day 35 (Figure 2.2.4 B). Set A anaerobic bioreactor showed reduced levels of biogas production compared to

Sets B and C, which were also observed to produce CH_4 earlier than Set A in their respective runs at days 17 and 31, respectively. Set B anaerobic bioreactor was the highest CH_4 producer at 8 % CH_4 on day 21.

Bacterial counts

i) Leachate

Counts for aerobic bacteria in leachate were lower at pH 7 for Set A (6.2 log cfu/ml in week 2, Figure 2.2.5 A) compared to Sets B (6.8 log cfu/ml in week 2) and C (6.7 log cfu/ml in week 2, Figure 2.2.5 B). However, in weeks 7-11 for Set A, counts were observed to be higher than those of weeks 2-5 for Set A (Figure 2.2.5 A).

ii) Soil

Counts for aerobic soil bacteria on TYG agar were highest in Set A (8.5 log cfu/ml in week 2 in weeks 1 – 4, Figure 2.2.6 A) compared to Sets B (7.5 log) and C (6.9 log) (Figure 2.2.6 B). In comparison Set B log cfu/ml values were higher than those of Set C (Figure 2.2.6 B).

Counts for potential methanotrophs on NMS plates showed marginal differences in bacterial counts between aerobic bioreactors for Sets A, B and C (Figure 2.2.7 A and B), across a 6-week period. In comparison, TYG counts were higher than NMS counts for all sets, in this study (Figure 2.2.6 and Figure 2.2.7). Bacterial counts on NMS media for Set A soil did not differ drastically over a 12-week period (Figure 2.2.7 A).

Methanotroph confirmation

Bacteria isolated from NMS plates as potential methanotrophs, were streaked onto SONA for confirmation. All isolates subsequently grew on SONA and were therefore not methanotroph isolates.

SEM

Scanning electron micrographs of leachate sampled from anaerobic bioreactors A, B and C revealed rod and coccoid-shaped bacterial forms (Figure 2.2.8). In addition, 'web-like' strands of material, which may have been dehydrated EPS, were observed in micrographs of leachate sampled from bioreactor Set A (Figure 2.2.8 A-C) and for leachate sampled from Set C (Figure 2.2.8 G-I). No distinguishable 'web-like' strands were observed for leachate from Set B (Figure 2.2.8 D-F).

Similarly, scanning electron micrographs of soil from aerobic bioreactors A, B and C showed rod and coccoid-shaped bacterial forms associated with soil debris (Figure 2.2.9).

DISCUSSION

In this study bioreactor Set A aerobic soil served as the control as no nutrients were added to the soil and thus simulated current landfill field conditions. In this set, the anaerobic bioreactor, representing the anaerobic waste component, showed pH deviations to acidic levels, decreasing as low as pH 5. This pH shift inhibited methanogenesis in this study and low CH_4 levels (0.4 %) were observed. In this bioreactor set, leachate dilution

was at 10 ml per week for the first 7 weeks of the run. Due to the acidic pH obtained previously, 50 ml per week anaerobic water was added to this bioreactor from weeks 8 – 9, followed by 300 ml in week 10 in an attempt to raise the pH level. The addition of 50 ml anaerobic water did not alter the pH. In week 11, the pH rose to 7, which was attributed to the addition of 300 ml anaerobic water. However, the pH of the bioreactor decreased again to 6 in week 12 (Figure 2.2.3 A). It appears that souring of the anaerobic bioreactor was too severe, rendering methanogens unproductive and incapable of immediate recovery. Low gas levels supported this conclusion with CH_4 levels not exceeding 0.4 %. CO_2 levels were also seen to decrease from day 36 (Figure 2.2.4 A). Set A anaerobic bioreactor was run for twice the amount of time (12 weeks) compared to Sets B and C which only ran for 6 weeks each. Therefore, the final leachate volume that was recycled to Set A anaerobic bioreactor was higher than that recycled to Sets B and C. This would increase the levels of acidic products in Set A anaerobic bioreactor, which may explain why pH levels were frequently recorded at acidic pH as well as the souring of this anaerobic bioreactor. Bacterial colony numbers in the leachate were however still regarded as high for Set A (7.1 log cfu/ml in week 11, Figure 2.2.5 A) and we attributed this to the isolation of acidogens present in the leachate as these bacteria and their by-products would have predominated under the acidic conditions (Karakashev *et al*, 2005)).

Set B anaerobic bioreactor initiated methanogenesis as early as week 2 and it was also the only bioreactor to consistently produce CH_4 over 6 weeks, peaking at 8 % CH_4 on day 21 (Figure 2.2.4 B). pH levels rose sharply from 6 in week 1 to 8 in week 2 (Figure 2.2.3 B). This pH level was maintained throughout the experiment and methanogenesis continued

at under this pH condition, preventing the build-up of acidic by-products. Bacterial counts for leachate from Set B were higher (a range of 6.8 – 7.0 log cfu/ml for weeks 2 – 5, Figure 2.2.5 B) compared to than that of Set A (6.2 – 6.0 log cfu/ ml) where a lack of inhibitory compounds in Set B allowed bacterial populations to proliferate.

Set C anaerobic bioreactor initiated methanogenesis in week 5 (Figure 2.2.4 B), two weeks later than Set B but still earlier than Set A. CH_4 levels peaked at 3 % on day 31 whilst CO_2 was only recorded once on day 36 for Set C. pH levels were also consistently maintained at neutral (Figure 2.2.3 B). Bacterial counts from leachate for Set C (6.7 – 7.0 log cfu/ml for weeks 2-5, Figure 2.2.5 B) were similar to those of Set B and therefore also higher that those of Set A. Maintenance of optimum pH levels once again allowed bacterial populations to proliferate without the build-up of inhibitory compounds. It can therefore be concluded that leachate dilution was necessary and effective at pH maintenance at neutrality in order to allow for methanogenesis to proliferate. Dilution volumes of 40 ml per week were important at elevating pH levels from 6 to 7 as early as week 2 for Sets B and C as this factor allowed slow growing methanogenic bacterial populations to establish at a much more rapid rate. This in effect prevented inhibitions to methanogen population growth and maintenance, which may indicate that in order to allow methanogenesis to establish, leachate recycling needs to be delayed. This would prevent the early build-up of acidic by-products, which creates an acidified anaerobic digester, as shown in Set A.

However, methanogenesis was at a lower rate in Set C compared to set B (3 % versus 8 % CH_4, Figure 2.2.4 B) and methanogenesis onset occurred later in Set C compared to Set B. This therefore indicated that Set B anaerobic bioreactor was more efficient of the 3 sets at waste biodegradation and CH_4 production, in this study. The faster onset of methanogenesis in Set B anaerobic bioreactor could be due to differences in soil nutrient compositions between bioreactor sets, as all other parameters were the same between sets.

In this study, overall higher bacterial counts were obtained on TYG agar compared to NMS agar for all bioreactor sets. TYG is a general growth medium and thus may have selected for general soil populations whereas NMS has been previously used to select for CH_4 utilising bacteria (Hanson, 1996). However, testing of presumptive methanotrophs, isolated on NMS agar, on SONA plates showed that all isolates grew and so were disregarded as being methanotrophs. As a result, those isolates that grew on the NMS media plates were suggested to be part of the general soil bacterial population. From this it may also be suggested that general heterotrophic soil bacteria were able to grow under CH_4 incubation conditions on NMS medium but were false positives for methanotrophs. Single strength NMS media concentration was used in this study (as in Chapter 2.1), and results suggested that a higher concentration, e.g. triple strength, may enrich for a wider variety of methanotrophs. Wise *et al* (1999) were successful at methanotroph isolation using a 0.2 X strength NMS media, however, it was decided that a higher strength medium concentration may enrich for rarer methanotroph strains. A higher medium concentration was suggested as the lower strength medium did not appear to be able to

select for methanotrophs. Concerning the environmental conditions used in this study, a comparative study with a single and triple strength NMS medium was used in further experiments.

SEM of leachate from Sets A, B and C showed similar populations (rods and cocci) and Set A showed the presence of 'web-like material' (Figure 2.2.8) which we attribute to EPS strand formation (Lindsay and von Holy, 1999). The presence of EPS material indicates the presence of the biofilm form, which is necessary for efficient waste degradation. Bacteria associated with waste degradation in landfills are also generally rod or coccoid-shaped cells e.g. *Methanobacteriales* species which are filamentous rods and *Methanococcales* species which are coccoid shaped (Karakashev, 2005). Thus, it may be suggested that the bacteria observed in SEM micrographs of leachate from the different bioreactor sets showing similar rod and coccoid-shaped morphologies were part of the degrading bacterial populations in the leachate. Similarly, rod and coccoid-shaped cells were also observed associated with soil particles in SEM micrographs of soil sampled from all the bioreactor sets. These micrographs demonstrated visually the in situ associations between soil particles and bacteria. Sessitsch *et al.* (2001) detected rod shaped *Acidobacterium* species in fertilized soil, which may be similar to the dried sewage cake amended soil that was used in Set C in this study.

CONCLUSIONS

This study revealed the optimum parameters associated with the commissioning of 3 different bioreactor sets, at 3 different soil nutrient conditions. A 40 ml dilution per week of leachate in the anaerobic bioreactors was needed to prevent sharp decreases in pH to acidic levels as shown by a lack of pH deviation by anaerobic bioreactor sets B and C. Set B was shown to be the most efficient waste digester with methanogenesis initiating in week 2, the earliest of all 3 bioreactor sets. Preliminary investigations of Sets B and C pointed to the fact that changes in CH_4 emissions could be due to the different soil nutrient additions. CH_4 oxidation data are needed from the aerobic reactors to assess the effectiveness of the bioreactor system and to assess the hypothesis that CH_4 oxidation drives CH_4 formation. Further investigations may show whether different nutrient additions affect CH_4 generation times and if Set B had the most efficient nutrient concentrations for future possible field applications. Single strength NMS growth medium may not effectively enrich for methanotrophs, as other heterotrophic bacteria were isolated under CH_4. This suggested the need for different NMS medium concentrations in further studies. SEM proved useful in morphologically assessing bacteria associated with waste degradation and soil and visually showed that this study was a satisfactory simulation of landfill conditions.