PROJECT REPORT No. OS38

OILSEED RAPEMEAL AS A NOVEL BIOSUPPORT FOR COMBINED ABSORPTION AND BIODEGRADATION OF SPILLED OIL

SEPTEMBER 1999
Price £4.00

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by

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This is the final report of a three month project which started in October 1998. The work was funded by a grant of £8,755 from HGCA (Project No. 1777).

The Home-Grown Cereals Authority (HGCA) has provided funding for this project but has not conducted the research or written this report. While the authors have worked on the best information available to them, neither HGCA nor the authors shall in any event be liable for any loss, damage or injury howsoever suffered directly or indirectly in relation to the report or the research on which it is based.

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SUMMARY

Two varieties of oilseed rapemeal were used to investigate the feasibility of this industrial by-producct being used as a support for oil degrading bacterial consortia – Glucosinolate containing oilseed rapemeal (GORM) and glucosinolate free oilseed rapemeal (ORM). Research was carried out on the effect rapemeal has on the ability of oil degrading bacteria to degrade a variety of hydrocarbons under seawater conditions. This was done using the bacterial consortia naturally occurring in ORM & GORM and consortium MPD-7 (isolated from Cormorant North Sea Crude Oil by Maria Piedad Diaz).

Both ORM and GORM were able to enhance the growth of oil degrading bacteria, with maximum populations occurring after only 4 days. Those growing in the absence of rapemeal reached maximum populations after 14 days incubation, although consortium MPD-7 remained constantly low throughout. GORM proved much more efficient at growth enhancement than ORM, with populations rising >700% compared to 40% for the latter. Degradation experiments using crude oil and a heavy distillate cut showed little or no evidence of enhancement of degradation with either ORM or GORM present. This was due to rapid and extensive hydrocarbon loss (70-90%) by other means rather rapemeal's bacterial biodegradation e.g evaporation.

Diesel was subject to extensive bacterial biodegradation in the presence of GORM, during the trial run using a glass stopper instead of a foam bung (reductions of 50% & 70% occurring after only 1 & 3 days, respectively).

Although further research is required to back up these results, it has been indicated rapemeal , especially GORM, is extremely efficient at enhancing the rate and extent of hydrocarbon biodegradation by oil degrading bacteria and thus, has great potential to be used in bioremediation.

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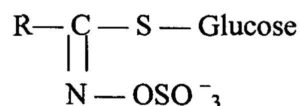
CHAPTER 1 Introduction

1.1 Background to oilseed rapemeal.

Oilseed rapemeal (ORM) is a industial by-product of oilseed rape, a crop processed for its oil. Generally entering its crushing and processing process at around 40% oil content, this is reduced to between 1%-10% oil content depending on the extraction route taken. Rapemeal itself consists of mainly protein and fibre, but also contains complex carbohydrates, minerals and vitamins. Anatomically, the meal consists of 2 major components : oil extracted embryo's and the hulls, the latter component containing much less protein but more fibre than the fomer component.

Another important component of rapemeal are its glucosinolates, these being sulfur contaning compounds naturally found in some plants and with the basic structure :



with variations occuring in the nature of the side chain (6). Glucosinolates are found in high concentrations in the meal fraction of the seed and have a number of properties ranging from the production of isothiocyanates (typical mustard oils) and thiocynates from hydolysis, both these having been shown to possess both antifungal and antimicrobial effects (6,9), to the deterence of feeding insects and other herbivores/pests.

1.2 **Current use of oilseed rapemeal.**

Due to its high protein content, rapeseed meal is currently utilised as an inclusion in a number of animal feeds, such as for pigs, poultry and cattle. For this to occur, rapemeal must possess a low glucosinolate content as these compounds have been attributed to a number of problems including – increases in thyroid size, liver weight and low palatability in pigs due to their pungent breakdown products, liver heamorrhage in poultry and goitrogenic effects in bulls. Due to these complications, glucosinolates have now been breed out of oilseed rape, converting GORM (glucosinolate containing oilseed rapemeal) to double 0 ORM (0-glucosinolates & 0-Arasic Acid).

Because the Blair House aggreement states only 1 million tons of rapemeal feed by-products can be produced a year and the large backlog of meal already existing, other uses must be made for this by-product. One solution would be to ensure such meal is used for non-feed purposes.

1.3 **Potential uses of oilseed rapemeal.**

A range of potential uses have been suggested for rapemeal including fuel, animal bedding, compost, fertiliser, microbial culture media, biodagradable plastics and adhesives for wood most of which need to be investigated before they can be commercially exploited (6).

One such suggetion for ORM combines its properties of an absorbant material with its fertiliser characteristics. Previous studies have shown rapemeal to be a cost effective

absorbant for oil and other hydrocarbons and results indicated it to be as good as, if not better, absorbant for wt oil absorbed per g absorbant than other commercially used absorbants such as Oil-dry. This, along with the fact that rapemeal possesses a high nutrient content i.e N, P & K and other essential nutrients required by microorganisms for growth ,suggests this product has great potential to be used in the bioremediation of spilled oil and the vast quantities of other hydrocarbons which are polluting our environment, allowing:

A) Enhanced absorbtion of hydrocarbons
B) Enhancement of the growth of hydrocarbon-utilising bacteria and therefore degradation of hydrocarbons – acting as a support and providing the essential nutrients which are otherwise unavailable.

1.4 Hydrocarbons polluting the environment.

Pollution from hydrocarbons can occur in a number of ways. Whether accidently or operationally it may be encountered in both marine and inland environments and from a number of different sources.

One of the most predominant types of hydrocarbon pollution is that from crude oil - a mixture of hydrocarbons containing 4-26 carbon atoms in the molecules (8). The widespread use of oil and its fractional cuts has inevitably resulted in its discharge to the environment and led to it becoming an increasingly menacing pollutant of our oceans (11). The world demand for oil is > 3200×10^6 $t.a^{-1}$ which represents ~ 39% of the worlds commercial energy demands (10). Of this ~ 3.25×10^6 $t.a^{-1}$ reaches our oceans from sources such as: ballest water from Tanker Operations, fuel and cargo oil dumped during Dry Docking, Tanker Accidents, waste water from Coastal Refineries and production water and

drilling muds + cuttings from Offshore Production Platforms (8). Although each of these may contribute only a minor amount of pollution, if they occur near land they may have devastating effects on the coastal ecosystem.

In comparison to air and water, land (or soil) is more variable and complex in composition and functions as a sink for pollutants, filtering out and retarding the passage of chemicals to groundwater (10). Its known soil can receive a diverse range of pollutants from the atmosphere, waste diposal practises but most of all from industrial and urban uses of land. Such pollutants also include hydrocarbons, ranging from saturated alkanes- CH_4, C_2H_6, C_3H_8 through to branched alkanes up to $C_{76}H_{154}$ and aromatic hydrocarbons.
Generally, there are 3 major sources of this pollution :

A) Fuel storage & Distribution – leaking underground storage tanks and spillages from distribution depots, along with road accidents release large amounts of diesel and petrol
B) Disposal of used lubricating oils – domestic gardens, land around garages and farmland maybe severely polluted with lubricating oils which contain Polcyclic Aromatic Hydrocarbons (PAH's).
C) Leakage of solvents from industrial sites – Hydrocarbon solvents are used widely in industry for cleaning metals and other components and maybe released along with other hydrocarbons in wastewater.

Such types of pollution often finds its way into rivers. These, along with petrol washed off roads and atmospheric fallout from exhausts, eventually find there way into drains and sewers. Finally entering our streams and rivers, this route contributes to about 25% of all petroleum products entering our oceans and is the greatest source of marine pollution for oil

,being classed as Municipal & Industrial run-off (1).

1.5 Bioremediation of hydrocarbon pollution.

A variety of techniques are currently used in the clean up hydrocarbons, especially in the marine environment following an oil spill. These range from the active addition of dispersents or burning of oil at sea, to elevating scrapers, front-end loaders and vacuum trucks which all actively remove oil directly from the shoreline or high/low pressure flushing which washes off oil to recovery points (7). Such techniques are well known to require large numbers of cleaners and many hours of work.

Studies on the fate of hydrocarbons in the environment has formed the basis for bioremediation, a relatively new technique, in which the rates of hydrocarbon biodegradation are accelerated by the active addition of hydrocarbon-utilising bacteria or addition of fertilizers, providing adequate nutrients to the natural microorganisms present in the polluted area (3).

1.6 Hydrocarbon-utilising bacteria.

Hydrocarbon-utilising bacteria are those which have the ability to utilise hydrocarbons as sole sources of energy and carbon and a diverse group of bacteria have been shown to have this ability with *Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter* and *Brevibacterium* being just a few.
Its been shown the initial steps in the biodegredation of hydrocarbons involves oxidation the substrate by oxygenases (3). Alkanes are subsequently converted to carboxylic acids, futher biodegraded, via β-oxidation, to Acetate. Aromatics are generally hydroxylated to form cis-

Diols, whose rings are cleaved to form Catechols. These are subsequently degraded to intermediates of the tricarboxylic acid cycle. Complete biodegradation forms the non-toxic products of CO_2 + H_2O aswell as cell biomass.

Hydrocarbon-utilising bacteria are widely distributed in the marine, freshwater and soil habitats, also naturally occuring in oilseed rape (2). In the marine environment such bacteria are widely spread throughout our oceans, in a ubiquitous manner especially following an oil spill. When such an incident occurs in the environment, the proportion of oil-degrading bacteria increases rapidly rising from $<1\% \rightarrow 10\%$ of the total bacterial population.
The majority of oil which is spilt in the sea eventually undergoes microbial degradation, but at a rate that's too slow to provide immediate relief in massive pollution incidents (4). This could be attributed to low counts of hydrocarbon-degrading bacteria, toxicity of some compounds or suboptimal temperatures. On the otherhand, numerous studies have been carried out on the degradation of petroleum by marine bacteria and have shown that the nutrients N & P are a major limiting factor, especially in the sea where levels are naturally low (4). This along with the fact that low molecular weight alkanes and aromatics- benzene and toluene are readily biodegraded, whilst more complex structures such as branched alkanes and Polycyclic Aromatic Hydrocarbons (PAH's) are much more resistant, play a major role in the rate of degradation.

1.7 Aims

Suggestions have been made to plough oilseed rapemeal into fields, landfill sites or beaches where a large amount of hydrocarbon pollution may occur. Here it has the potential to not only add more hydrocarbon-utilising bacteria to that environment, but act as a

support to these microorganisms providing the essential nutrients required to enhance bacterial growth and thus the rate of hydrocarbon degradation.

Therefore, the objectives to this project were :

1. Examine the effect of ORM on the ability of oil degrading bacteria to degrade oil under Seawater conditions.
2. Measure the rate of oil degradation using an ORM/bacteria mixture.

Chapter 2 Materials and Methods

2.1 Determination of the effect of rapemeal on oil-utilising bacterial populations.

2.1.1 Preparation of fresh water and marine BHB & Oil agar plates.

Populations of hydrocarbon degrading bacteria were determined using BHB agar plates, containing a layer of oil as a carbon source. These were made up in both fresh water and marine conditions.

Fresh water plates were prepared in the following way: 3.27g BHB agar & 10g agar Bacteriological were dissolved in 1000ml of distilled water. This was then autoclaved at 121^0C for 15 minutes allowing sterility. After standing for 10 minutes, the molten agar was poured into agar plates and a drop of sterile PIPER Oil added forming a film upon the agar surface. These were then left to naturally cool and set before being overturned to remove condensation.

Marine plates were prepared in the same way as that for fresh water plates, only 30g NaCl was added to the BHB and Bacteriological agar mixture before autoclaving at 121^0C for 15 minutes took place.

2.1.2 Culture of Maria's Consortium.

Preparation and inoculation of the growth medium- Marine Broth

Maria's consortium is a mixture of halotolerant bacteria isolated from Cormorant North sea crude oil and which obtain energy via the utilisation of hydrocarbons . Previously cultured by Maria this consortium was named MPD-7.

These bacteria are just one of the types of bacteria to be used in this experiment, determining the potential of ORM & GORM to be used as a support to oil degrading bacteria by measuring the increase in bacterial population numbers.

Marine Broth was prepared by dissolving 7.48g Marine Broth in 200ml distilled water. Sterilization was carried out by autoclaving at 121^0C for 15 minutes, after which a period natural cooling ensued.

Inoculation was carried out using 2ml of cell culture from the already existing cultures held in refridgerated conditions where they remained in a stationary phase. Bacteria were cultured aerobically in pre-sterilized 250ml conical flasks for 24hrs at 31^0C with shaking. Cultures were then transferred to refridgerated conditions were they remained until required for inoculation.

2.1.3 Preparation of ORM & GORM bacteria

1g ORM was suspended in 10ml of 121°C (15mins) autoclave sterilized seawater and bacterial cells extracted by continual shaking at 230rpm for 20 minutes. Following settlement of ORM, 1ml of suspended cells were washed once and finally resuspended in 1ml of sterile seawater, this being used for inoculation in the experiment.
This technique was also used to isolate 1ml of GORM bacterial cells.

Following isolation and preparation of all bacteria and media, 250ml sterile conical flasks were set up as shown in table 1. Flasks then underwent continual shaking at 160 rpm and 25°C.

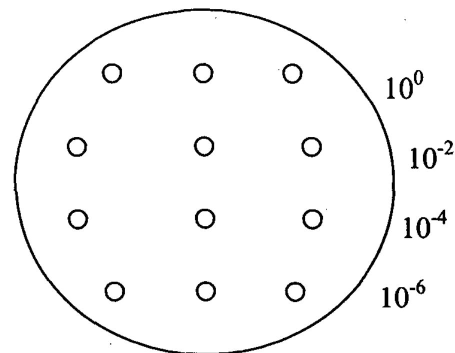
Table 1. Contents of 250ml conical flasks used in bacterial population experiment under seawater conditions. ST- Sterile (autoclaved 121°C, 15mins), T- 'TOTAL' Allwyn crude oil, MPD7- Maria's consortium

Flask	Contents of flask
A1 A2	Sand (40g), Seawater (50ml)-ST, Crude Oil (0.5ml)-T, ORM+Bac. (1g) " " " "
B1 B2	Sand (40g), Seawater (50ml)-ST, Crude Oil (0.5ml)-T, ORM+Bac. (1g), MPD7 (1ml) " " " "
C1 C2	Sand (40g), Seawater (50ml)-ST, Crude Oil (0.5ml)-T, MPD7 (1ml). " " " "
D1 D2	Sand (40g), Seawater (50ml)-ST, Crude Oil (0.5ml)-T, ORM Bac. (1ml) " " " "
E1 E2	Sand (40g), Seawater (50ml)-ST, Crude Oil (0.5ml)-T,GORM+Bac.(1g) " " " "
F1 F2	Sand (40g), Seawater (50ml)-ST, Crude Oil (0.5ml)-T,GORM+Bac.(1g) MPD7 (1ml) " " " "
G1 G2	Sand (40g), Seawater (50ml)-ST, Crude Oil (0.5ml)-T, GORM Bac(1ml) " " " "
H1 H2	Sand (40g), Seawater (50ml)-ST, Crude Oil (0.5ml)-T. "CONTROL" " " " "

2.1.4 Determination of variations in bacterial populations.

Conical flasks were removed from the shaker and rapemeal allowed to settle. 200µl of cell suspension was removed from each flask and made into a sequence of dilutions, ranging from $10^0 \rightarrow 10^{-6}$, using sterile seawater. Dilutions were then plated out on BHB+ salt + Piper oil agar plates as 20µl spots, in triplicate, and left at 30°C for 3 days to grow.This arrangement is shown in Figure 1.

Figure 1. Diagram of a BHB agar + salt + oil culture plate used to grow bacterial colonies. Inoculations were made in triplicate at each dilution using 20µl spots and plates were left to grow at 30°C for 3 days. This was repeated for each flask.



Flask "X"

Bacterial populations were calculated from colony counts. Plates were inspected using a stereo microscope and bacterial dilutions with between 20-200 colonies were used. Averages were calculated and converted into colony forming units per millimeter of flask medium (CFU/ml).

2.2 Determination of the effect of rapemeal on the degradation ability of oil-utilising bacteria.

2.2.1 Preparation of calibration curves.

Various amounts of sterile 'Total' crude oil were weighed out into 25ml volumetric flasks, using a 9 digit electronic scale. Flasks were made up to 25ml using Carbon Tetrechloride (CCL_4) and vigorously shaken to dissolve all oil. Flasks were then analysed for oil content using a Perkin-Elmer 599 Infrared spectrophotometer. Peak heights were measured from graphs drawn by the spectrophotometer and placed on a calibration graph against oil concentration (mg/l).

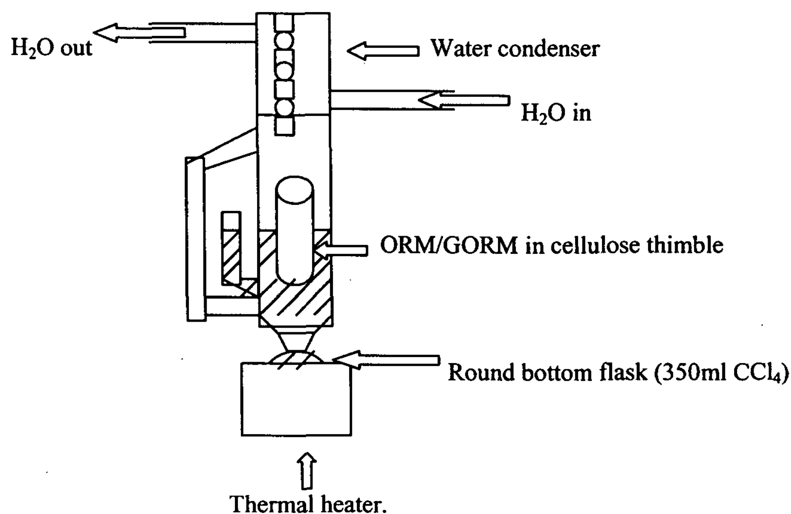
This method was repeated to produce calibration curves for a heavy distillate cut and diesel, both to be used as added degradation hydrocarbons and vegetable oil, used to calculate the residual oil content of ORM & GORM.

2.2.2 Extraction of residual oil from ORM & GORM.

To allow an accurate analysis of the rate of degradation of added hydrocarbons, all residual oil present in ORM & GORM was removed. 500g of ORM/GORM was ground up to a similar size, using a mortar and pestle and placed into 3 cellulose extraction thimbles. Oil extraction was undertaken in 3 Soxhlet extractors each with 350ml of CCl_4 in the round bottomed flasks, as shown in figure 2.

Heated by an electrothermal heater this continued for 2 hours for ORM and 3 hours for GORM.

Figure 2. Diagram of one of the Soxhlet extractors. 350ml of CCl_4 was heated and its vapours rose up the outside arm into the main chamber. Here it condensed and dripped through the ORM/GORM packaged in a thimble, extracting residual oil. Levels of CCl_4 + oil rose to the height of the inner arm before draining back into the round bottomed flask.



Following extraction of residual oil from ORM/GORM, the mixture of oil + CCL_4 was used to determine residual oil content as a %. 500µl of oil + CCL_4 was pipetted into a 25ml volumetric flask and made up to 25ml with new CCL_4. This was then analyzed for a peak height from the I.R. spectrophotometer and an oil concentration (mg/l) obtained from a calibration curve.

Flasks to be used for the degradation experiment were set up in the following way. All contents were added to 250ml wide necked conical flasks, sterilized by autoclaving at 121^0C for 15 minutes.

Table 2. Table showing the contents of the flasks used in the degradation experiment. 5 copies of each condition were set up, as 5 readings of oil content were measured. ST- Sterile (autoclaved 121°C, 15mins), T- 'Total' Alwyn crude oil 1PP- 1 drop from a Pasteur Pipette.

Flask	Contents
A1→A5	Seawater-ST (50ml), ORM + Bacteria (10g), Crude oil-T (1PP).
B1→B5	Seawater-ST (50ml), ORM Bacteria (10g worth), Crude oil-T (1PP).
C1→C5	Seawater-ST (50ml), GORM + Bacteria (10g), Crude oil-T (1PP).
D1→D5	Seawater-ST (50ml), GORM Bacteria (10g worth), Crude oil-T (1PP).
E1→E5	Seawater-ST (50ml), Crude oil-T (1PP). 'Control'

Seeing as residual oil extraction also probably removed natural bacteria from ORM and GORM, bacteria was added to the appropriate flasks by placing the equivalent amount ORM/GORM into sterile seawater-10g in 50ml seawater, per flask. Cells were extracted by placing the suspended rapemeal on a rotary shaker at 230rpm for 2 hours. An extra amount of seawater was then added to the rapemeal, equivalent to the amount absorbed by the seeds themselves (GORM absorbs its own weight of seawater, ORM absorbs 3 times its own weight of seawater).

Rapemeal was allowed to settle, whilst seawater and bacterial suspension was removed and 50ml added to each flask, apart from those of the control. All flasks were placed on a rotary shaker at 125rpm at room temperature.

This method was repeated for the heavy distillate cut and diesel except, fewer readings were taken on both these hydrocarbons and fewer conditions were set up for the diesel.

2.2.3 Determination of the rate and extent of oil degradation.

For each day of measurement, a whole flask of each condition was sacrificed. 25ml of CCl_4 was added to each flask and vigorously shaken to dissolve all added hydrocarbons present. Along with this, 50ml and 150ml of sterile seawater was added to GORM and ORM flasks respectively, to allow distinct separation and prevent blockage of the separating flasks. Flask contents were then poured into a separating flask and allowed to settle into a 2 phase system – 'Seawater + ORM/GORM' phase above a denser 'CCl_4 + hydrocarbon' phase.

The 'CCl_4 + hydrocarbon' phases were then separated off into 25ml volumetric flasks and made up to 25ml with fresh CCl_4. Hydrocarbon contents were determined using the I.R. spectrophotometer, where peak heights obtained were measured on a calibration curve and converted to concentrations (mg/l).

All values were plotted against time (days).

Chapter 3 RESULTS

3.1 Measurement of bacterial populations indicating the effect of oilseed rapemeal on the ability of oil-utilising bacteria to degrade oil under seawater conditions.

A measurement of bacterial populations, via colony counts, was carried out to indicate the effect of oilseed rapemeal (ORM/GORM) on oil-utilsing, on the basis of the higher the rate and extent of bacterial growth the higher the theoretical rate and extent of oil degradation occurring. 'TOTAL' Allwyn crude oil (a light oil) was selected as a carbon and energy source during the experiment, whilst bacteria naturally occurring in ORM & GORM and consortia MPD-7 (grown by Maria Piedad Diaz -work unpublished) were used for inoculation.

Results of enhancement of oil-utilising bacterial populations are summarized in figures 3 & 4 respectively. Rapid increases in bacterial populations occurred immediately in the presence of GORM, this being > 700% following inoculations with GORM bacteria and with both GORM bacteria & consortium MPD-7. Following this initial increase over a 4-day period, populations cultured in GORM vastly fell to initial levels, afterwhich a shallow but continual reduction occurred for the remainder of the experiment.

Initial populations of ORM bacteria were higher than those of GORM (Figure 3), although unlike before a somewhat smaller increase of only ~ $5x10^8 \rightarrow 7x10^8$ CFU/ml (~ 40%) occurred in the initial period of 4 days. This was for flasks inoculated with ORM bacteria + MPD-7, whilst a continual reduction in bacterial populations occurred in the other ORM setup throughout the 18 day experiment.

Bacterial populations increased by similar amounts without the presence of ORM & GORM although increases were much less rapid, occurring over 14 rather than 4 days respectively

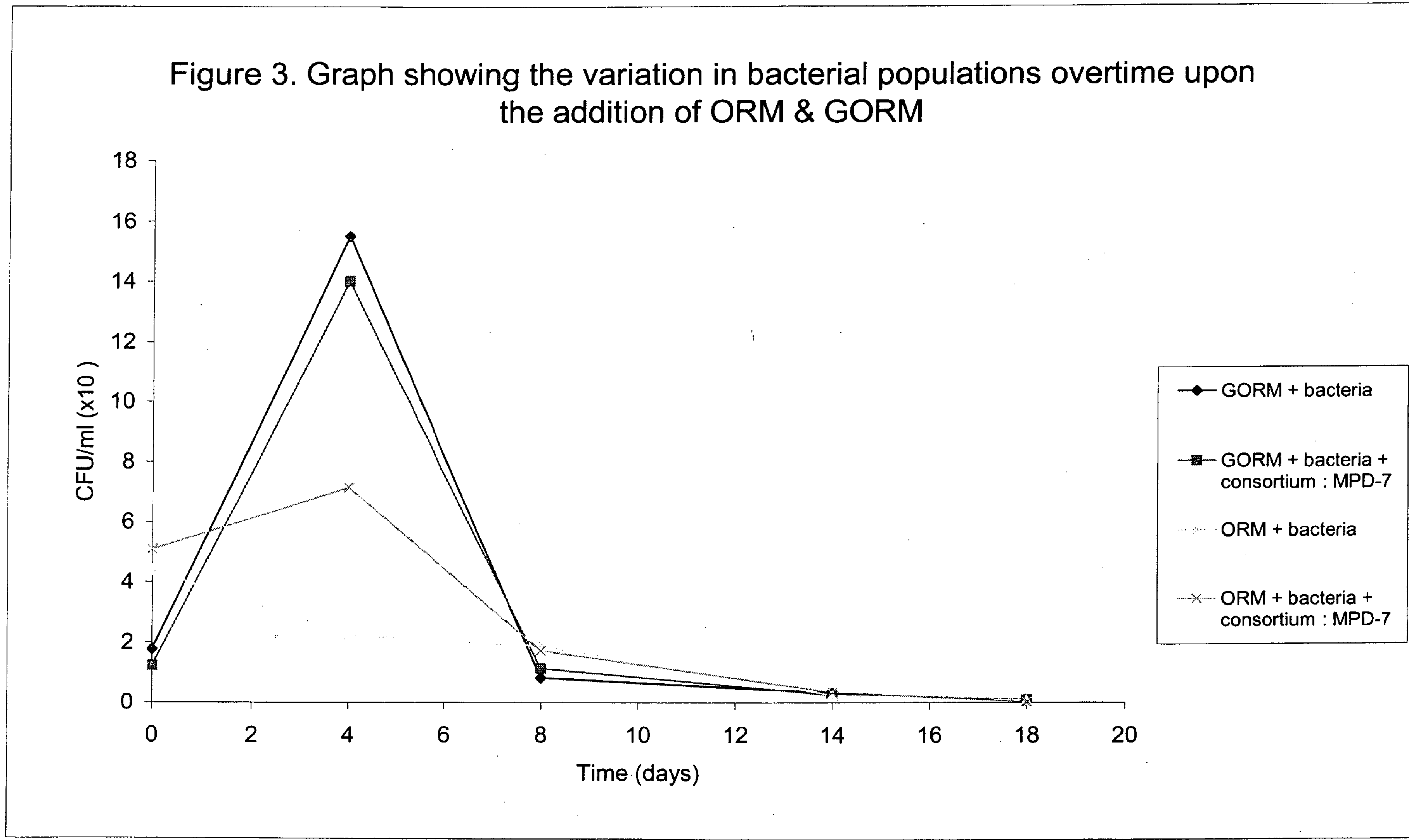


Figure 3. Graph showing the variation in bacterial populations overtime upon the addition of ORM & GORM
CFU/ml (x10)
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
Time (days)
GORM + bacteria
GORM + bacteria + consortium : MPD-7
ORM + bacteria
ORM + bacteria + consortium : MPD-7

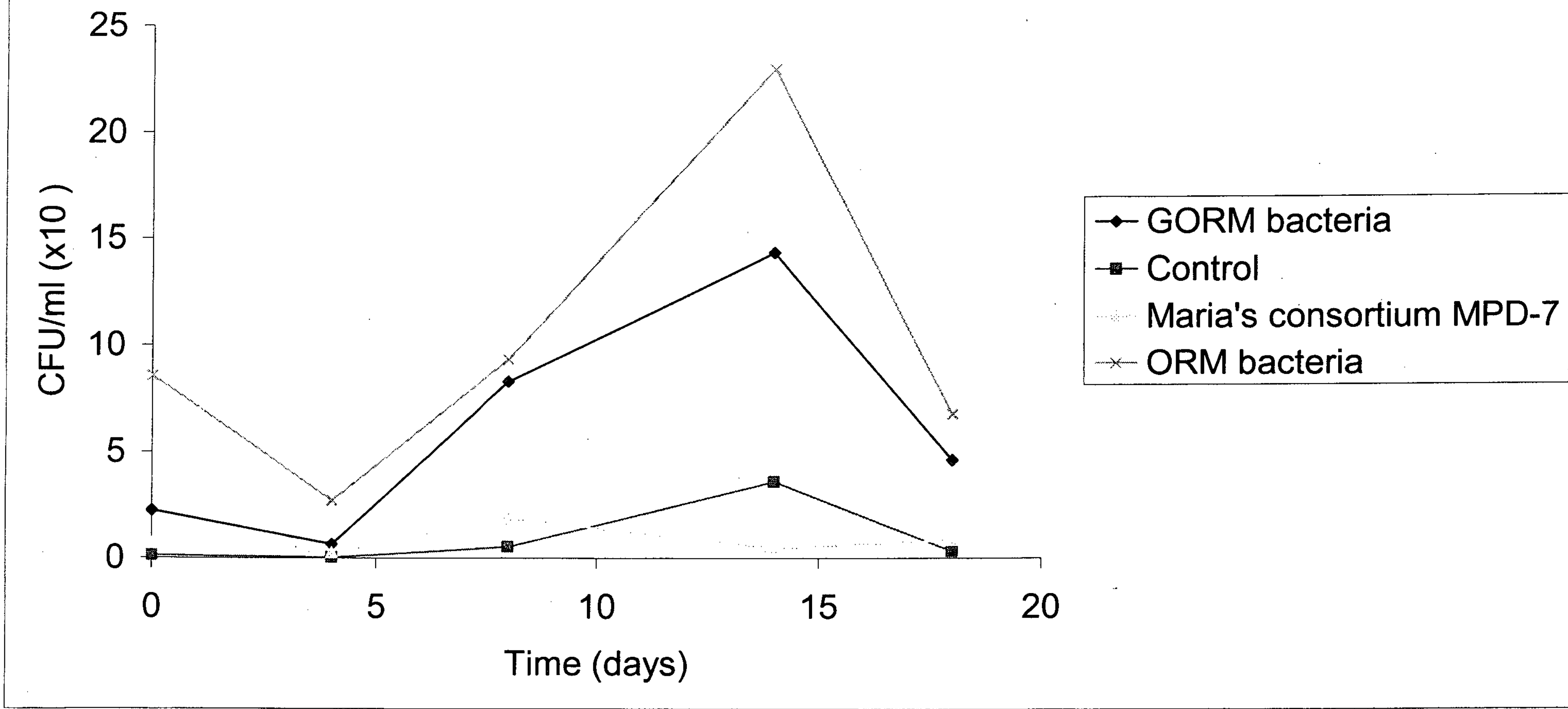


Figure 4. Graph showing the variation in bacterial populations overtime of GORM bacteria, ORM bacteria & Maria's consortium : MPD-7
CFU/ml (x10)
25
20
15
10
5
0
0
5
10
15
20
Time (days)
GORM bacteria
Control
Maria's consortium MPD-7
ORM bacteria

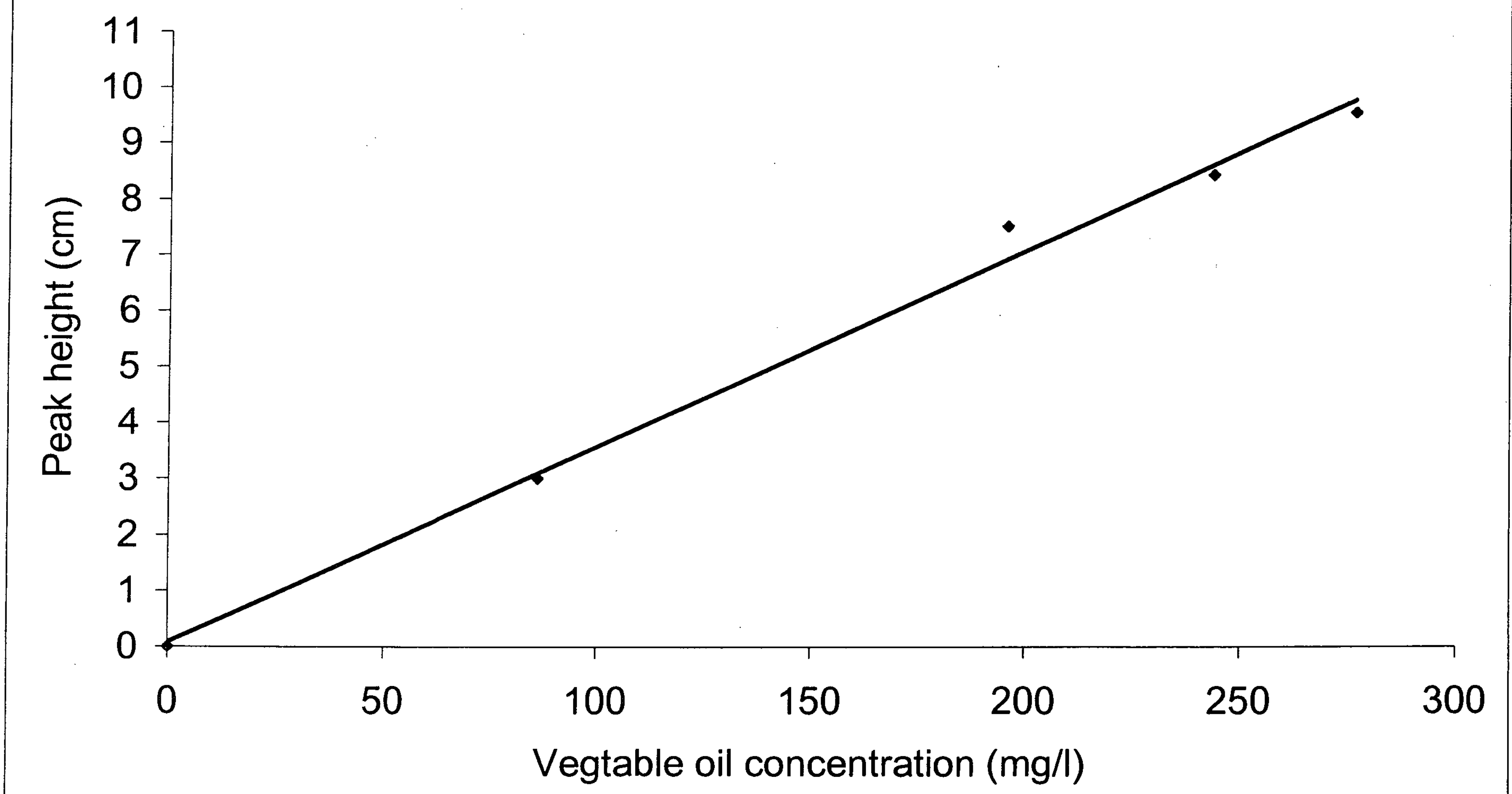


Figure 5. Calibration graph of peak height (cm) against vegtable oil concentration (mg/l)

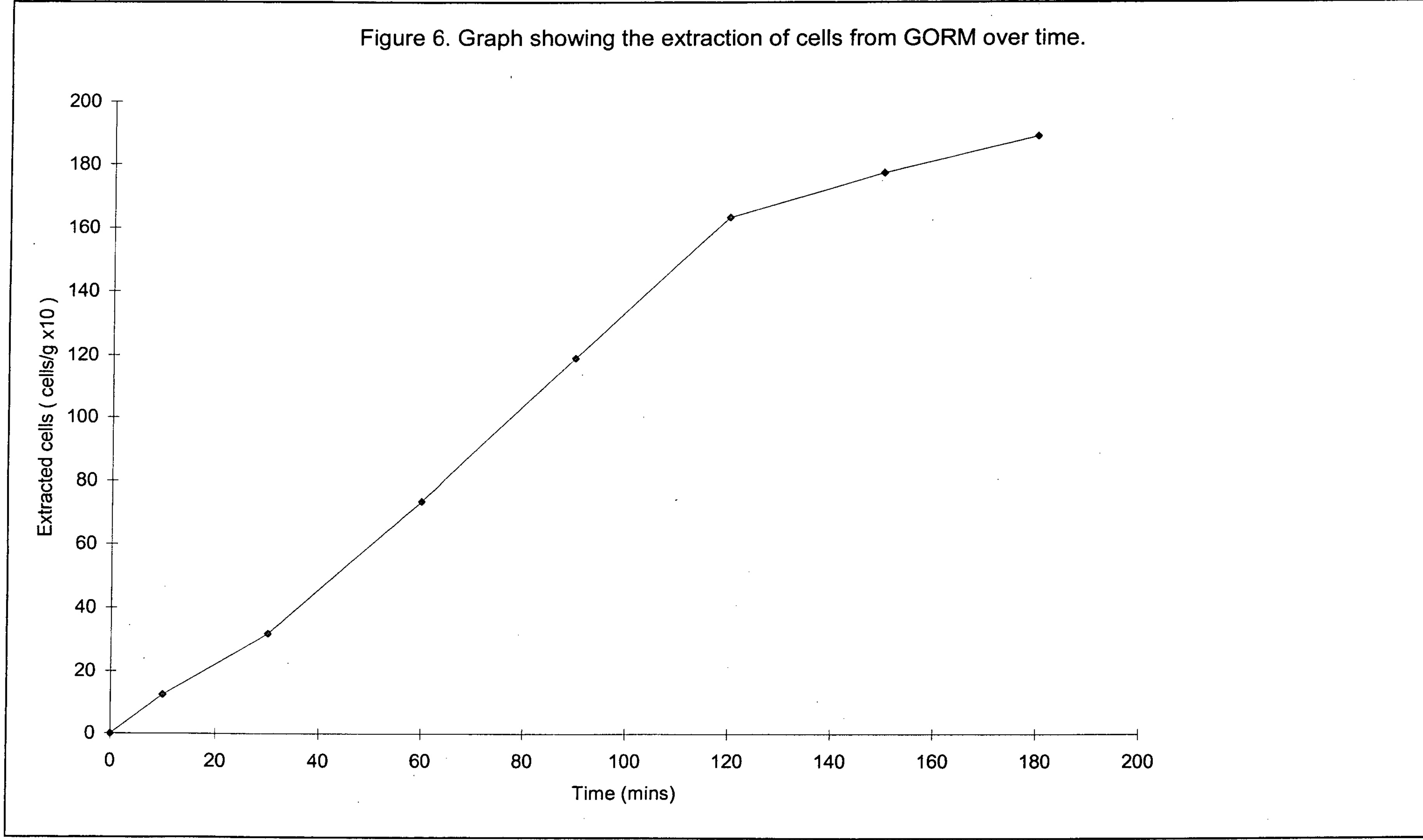


Figure 6. Graph showing the extraction of cells from GORM over time.

(Figure 4). Populations were some 2 orders of magnitude lower than those shown in figure 3 for all 4 conditions throughout the experiment. Whilst both both ORM & GORM bacterial populations vastly increased, utilising the available carbon and energy source, consortium MPD-7 and the sediment bacteria present in the control remained low and quite constant, indicating no enhancement of growth.

Both the rate and extent of growth of oil-utilising bacteria was vastly higher with the presence of rapemeal compared to its absence, although results indicate GORM enhances growth much more efficiently than ORM.

3.2 Measurement of biodegredation of crude oil in the presence of ORM/GORM indicating its effect on their oil-utilising bacteria, under seawater conditions.

Besides measuring the variation in bacterial population sizes as an indication of the effect of rapemeal on the ability of oil degrading bacteria to degrade oil, the overall extent of oil biodegradation was measured using an I.R-Spectrophotometer. Prior to this, the residual oil content of ORM and GORM was calculated (8.0% & 1.3% in ORM and GORM respectively) from the calibration curve produced (Figure 5).

3.2.1 Degradation of 'TOTAL' Allwyn crude oil.

Figures 8&9 and Table 3 show the results of biodegradation of 'TOTAL' Allwyn crude oil by ORM and GORM bacteria, with and without the presence of ORM/GORM itself, under seawater conditions.

Similar to the bacterial population experiment, rapid reductions in oil concentrations took place, within 3 days of inoculation, this occuring in all flasks. Initial oil concentrations in ORM flasks were up to x2.5 higher than in GORM flasks, indicating the 8% residual

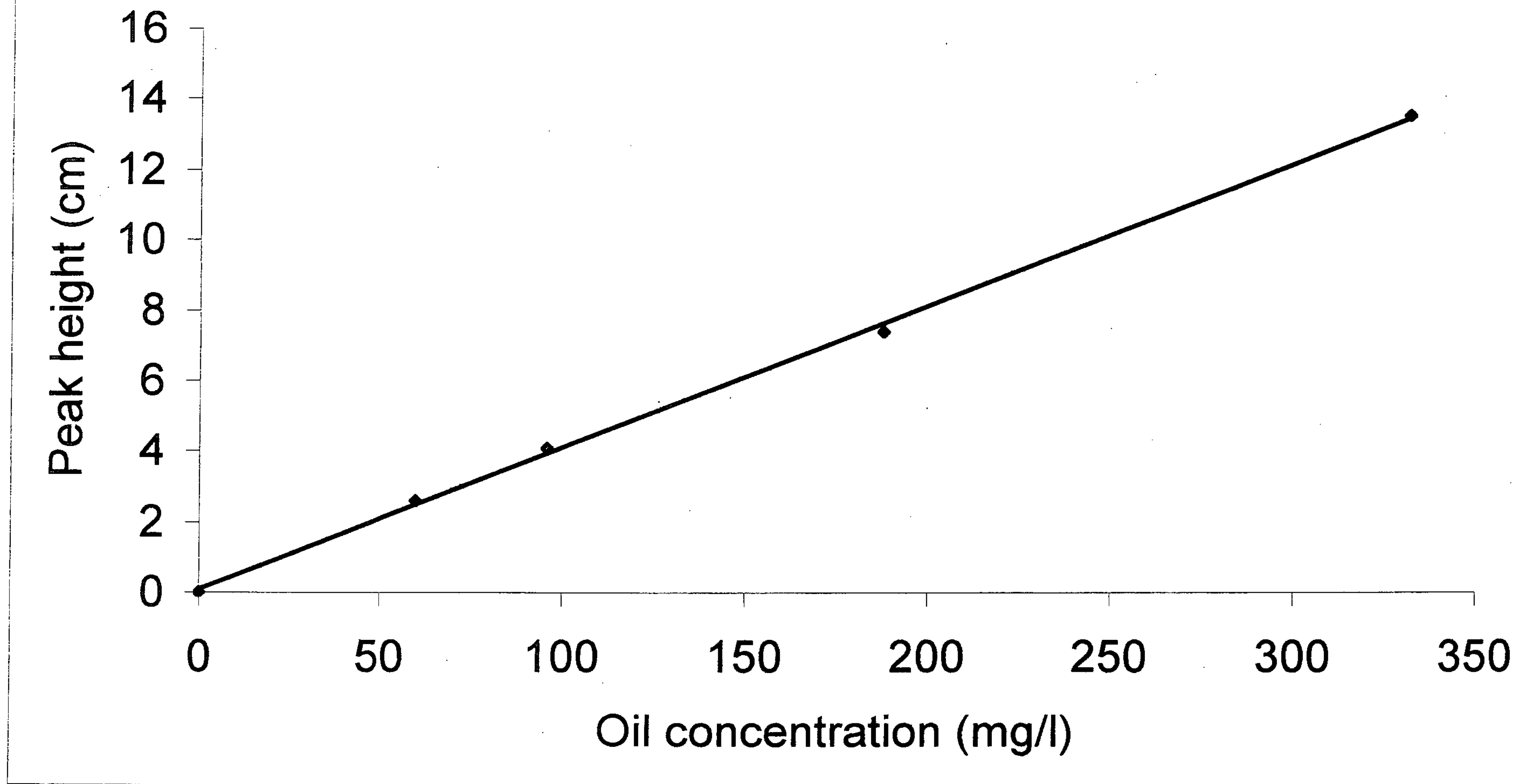


Figure 7. Calibration graph of peak height (cm) against crude oil concentration (mg/l)
Peak height (cm)
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
Oil concentration (mg/l)

Table 3 Reductions in 'TOTAL' crude oil concentrations following the addition of the naturally occurring hydrocarbon degrading bacteria of ORM and GORM.
Flasks were set up to contain sterile seawater (50ml),'TOTAL' crude oil (20mg) and variations in the presence/absence of ORM & GORM as follows:
A - ORM + natural bacteria (10g), B - Natural ORM bacteria (10g worth),
C - GORM + natural bacteria (10g), D - Natural GORM bacteria (10g worth),
E - Control (no added bacteria)

Flask	Day	Peak Height (cm)	Oil conc. (mg/l)	Reduction in oil Conc. (%)
A1	0	10.6	251.07	0
A2	3	4.6	110.48	60.00
A3	7	3.0	71.77	71.41
A4	10	1.9	45.16	82.01
A5	12	1.2	27.42	89.08
B1	0	11.8	283.87	0
B2	3	8.0	192.74	32.10
B3	7	2.9	69.35	75.57
B4	10	0.2	8.87	96.88
B5	12	0.2	8.13	97.14
C1	0	4.3	101.35	0
C2	3	1.1	25.81	74.53
C3	7	1.0	23.39	76.92
C4	10	1.4	29.03	71.36
C5	12	0.6	12.90	87.27
D1	0	4.7	108.87	0
D2	3	1.3	29.03	73.34
D3	7	1.1	25.00	77.04
D4	10	0.6	12.90	88.15
E1	0	4.9	117.74	0
E2	3	0.8	16.13	86.30
E3	7	1.2	29.35	75.07
E4	10	1.3	33.06	71.92
E5	12	1.3	30.65	73.97

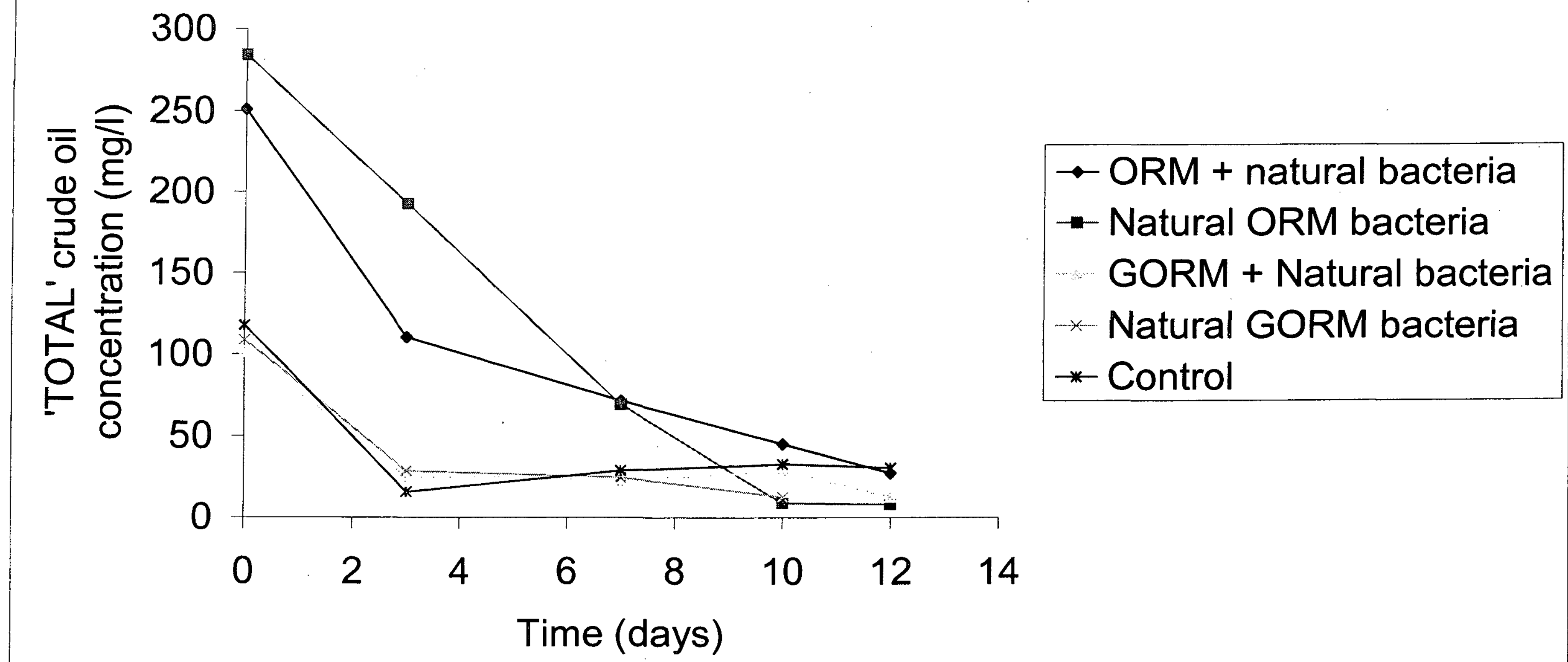


Figure 8. Graph showing the bacterial degradation of 'TOTAL' crude oil overtime, with and without the presence of ORM & GORM.
'TOTAL' crude oil concentration (mg/l)
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
Time (days)
ORM + natural bacteria
Natural ORM bacteria
GORM + Natural bacteria
Natural GORM bacteria
Control

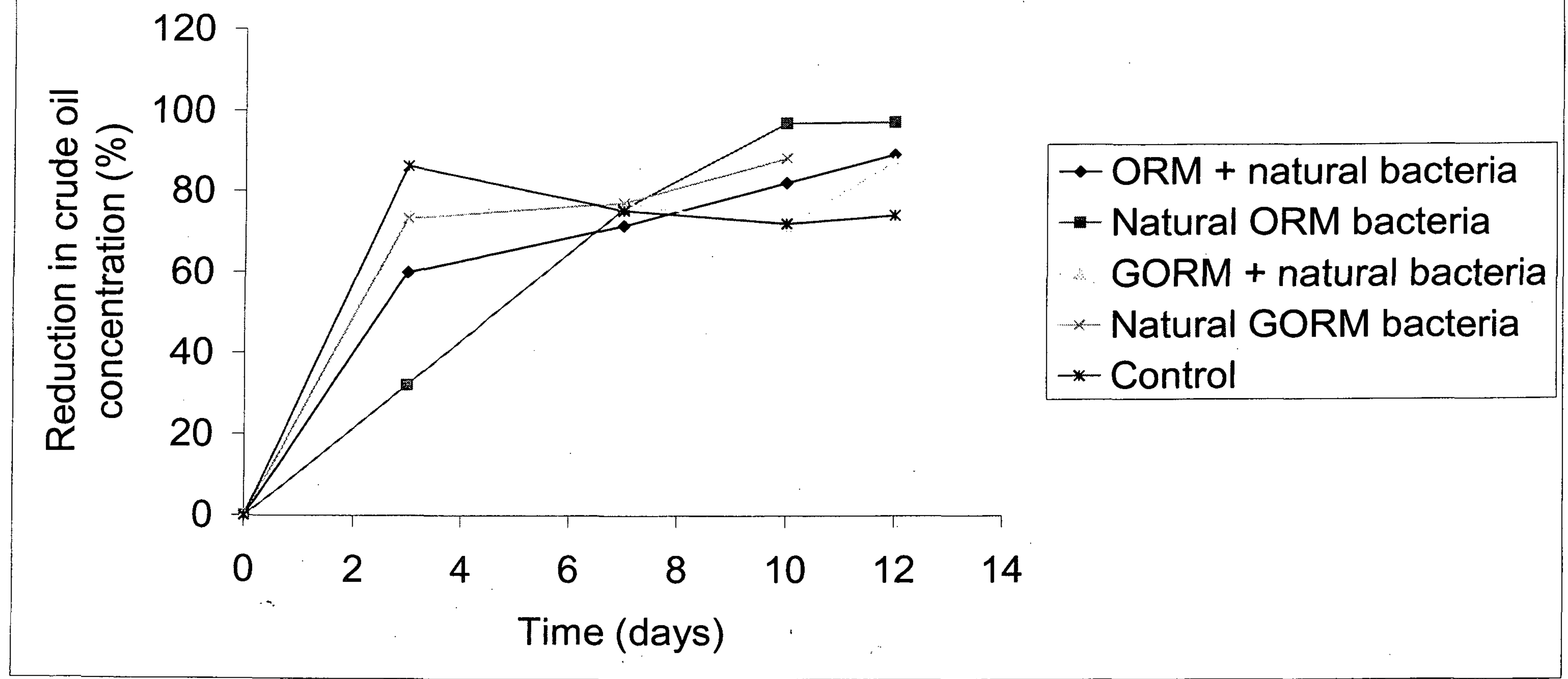


Figure 9. Graph showing the variation in 'TOTAL' crude oil concentrations overtime, from hydrocarbon degrading bacteria of ORM & GORM.

content of ORM is a major factor complicating degradation results. The initial rate of oil degradation in the presence of ORM was double that without its presence (60% & 32% after 3 days respectively), this continuing to fall steeply for the remainder of the experiment to a final 89% & 97% on day 12 (Table 3). In contrast, oil reduction in flasks with and without the presence of GORM had a slightly higher initial rate of reduction up to day 3, afterwhich reductions were vastly reduced-oil concentrations remaining fairly constant in both up until day 12 (falling only a further 12% & 15% during this period respectively).

Throughout the experiment, variations in crude oil concentrations in the control remained similar to those occuring in the GORM flasks (Figure 8), indicating not only residual oil content of GORM having no effect on biodegradation measurements but mainly, initial crude oil losses in these flasks is due to some other reason rather than ORM/GORM bacterial biodegradation. Possible reasons include seawater contamination or evaporation. These results give little indication of bacterial biodegradation of 'TOTAL' Allwyn crude oil being enhanced by oilseed rapemeal or whether ORM is more/less efficient at degradation enhancement than GORM.

3.2.2 Degradation of a heavy distillate cut.

The measurement of biodegradation of hydrocarbons was repeated using a heavy distillate cut, on the basis it contains heavier less volatile hydrocarbons which have higher boiling points, therefore would overcome the possibilty of excessive oil loss from evaporation. Similar to the crude oil experiment, no lag period was evident before rapid and vast hydrocarbon reduction took place (ranging from 97-99%), this time occuring after only 1 day on inoculation (Figures 11&12, Table 4). Distillate concentrations remained quite constant,following this, till the end of the incubation period in all cases (Figure 11). Flasks containing just ORM bacteria showed the presence of oil concentrations that were to large

too read off the equivalent calibration curve (Figure 10), thus results are not shown on graph. This is undoubtedly due to the high residual oil content (8%) present.

As was the case with crude oil, the variations in distillate concentrations within the control were similar to those of the bacterially inoculated flasks - with an initial reduction of 97% followed by a constant period till day 5 (final reductions being 99.7%, respectively). This again indicates hydrocarbons have been lost from someother means rather than bacterial biodegradation by ORM & GORM bacteria and results similarly give no indication of whether degradation has been enhanced, or whether ORM or GORM is more efficient at biodegradation enhancement.

3.2.3 Degradation of diesel.

Bacterial biodegradation of diesel was measured as this is another typical hydrocarbon which regularly pollutes our environment, thus the potential of rapemeal to enhance its degradation was determined. Due to the unexpected losses of the heavy distillate cut, a variation in the methodology was carried out in that a glass stopper was used to seal the flask, rather than a foam bung. This hopefully would prevent major losses by evaporation, and the results obtained for this experiment are from a trial run of this procedure.

The results of the trial run of degradation of diesel, using a glass stopper, are summarized in Figures 14&15. Concentrations of diesel in the control remained quite constant over the 3 day experiment with levels falling by ~ 4% after 1 day, followed by a small rise of ~ 10% (Figure 15). Initial losses occurred probably due to volatility and it would be expected the low molecular weight alkanes in diesel to continue evaporate to a level of around 10-15% reductions over the 3 day incubation. A rise occurred in diesel concentrations due to human error-small variations in the amount of diesel added by the Pasteur Pipette.

In contrast to this, diesel in the presence of GORM and its bacteria was subjected to extensive biodegradation (losing 50% & 70% after only 1& 3 days, respectively). Although futher experiments are required, comparing rates and extents of diesel degradation with GORM and its bacteria to that of just GORM bacteria, ORM and its bacteria and just ORM bacteria, it would seem bacterial biodegradation of diesel, and maybe other hydrocarbons used in this project (crude oil + heavy distillate cut) is greatly enhanced by the presence of oilseed rapemeal (GORM).

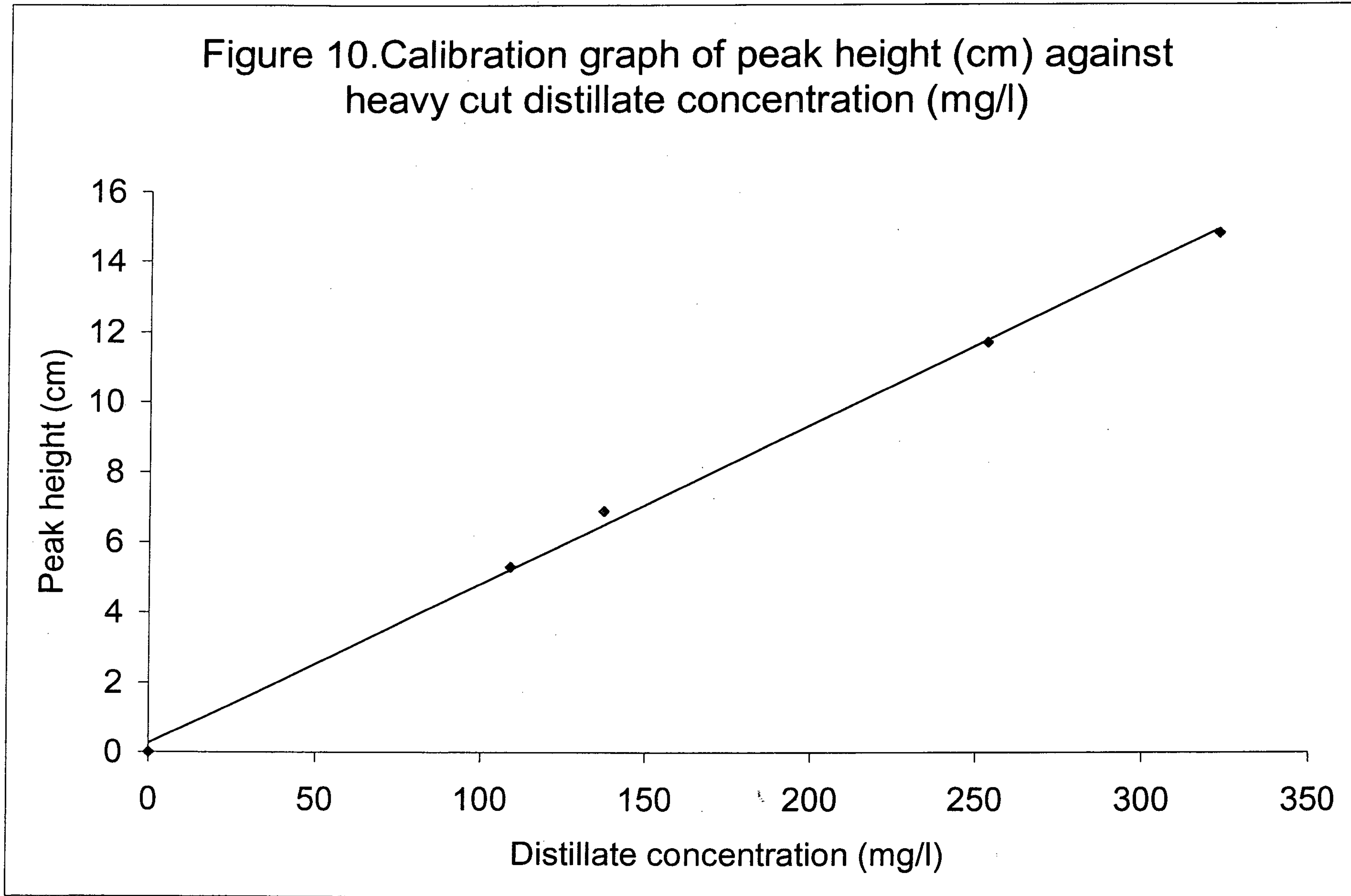


Figure 10.Calibration graph of peak height (cm) against heavy cut distillate concentration (mg/l)
Peak height (cm)
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
Distillate concentration (mg/l)

Table 4 Reductions in heavy distillate cut concentrations following addition of the naturally occurring hydrocarbon degrading bacteria of ORM and GORM.
Flasks were set up to contain sterile seawater (50ml), Heavy distillate cut (mg) and the following of ORM or GORM :
A – ORM + natural bacteria (10g), B – Natural ORM bacteria (10g worth)
C – GORM + natural bacteria (10g), D – Natural GORM bacteria (10g worth)
E – Control (no added bacteria). THTM – To High To Measure.

Flask	Day	Peak Height (cm)	Distillate conc. (mg/l)	Reduction in Distillate conc. (%)
A1	0	9.6	202.05	0
A2	1	0.3	1.6	99.21
A3	4	1.1	20.55	89.83
A4	5	0.8	10.27	94.92
B1	0	14.6	313.35	0
B2	1	62.5	THTM	THTM
B3	4	49.0	THTM	THTM
B4	5	55.2	THTM	THTM
C1	0	11.1	236.30	0
C2	1	0.2	1.12	99.53
C3	4	0.4	4.28	98.12
C4	5	0.3	1.71	99.28
D1	0	13.0	277.39	0
D2	1	0.6	6.85	97.33
D3	4	1.2	20.55	92.59
D4	5	1.0	16.27	94.13
E1	0	13.6	290.23	0
E2	1	0.6	7.71	97.34
E3	4	0.2	0.85	99.71
E4	5	0.2	0.90	99.69

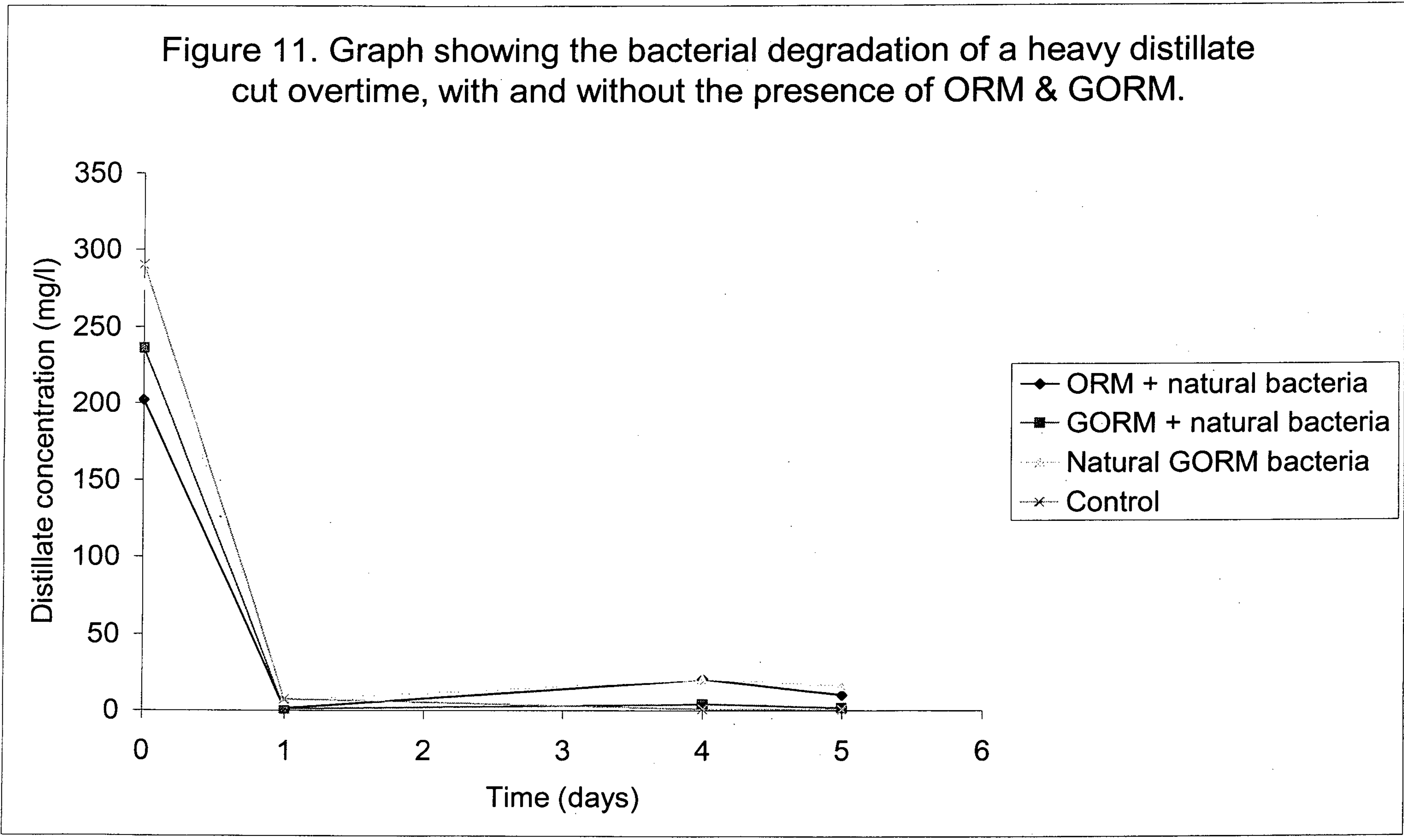
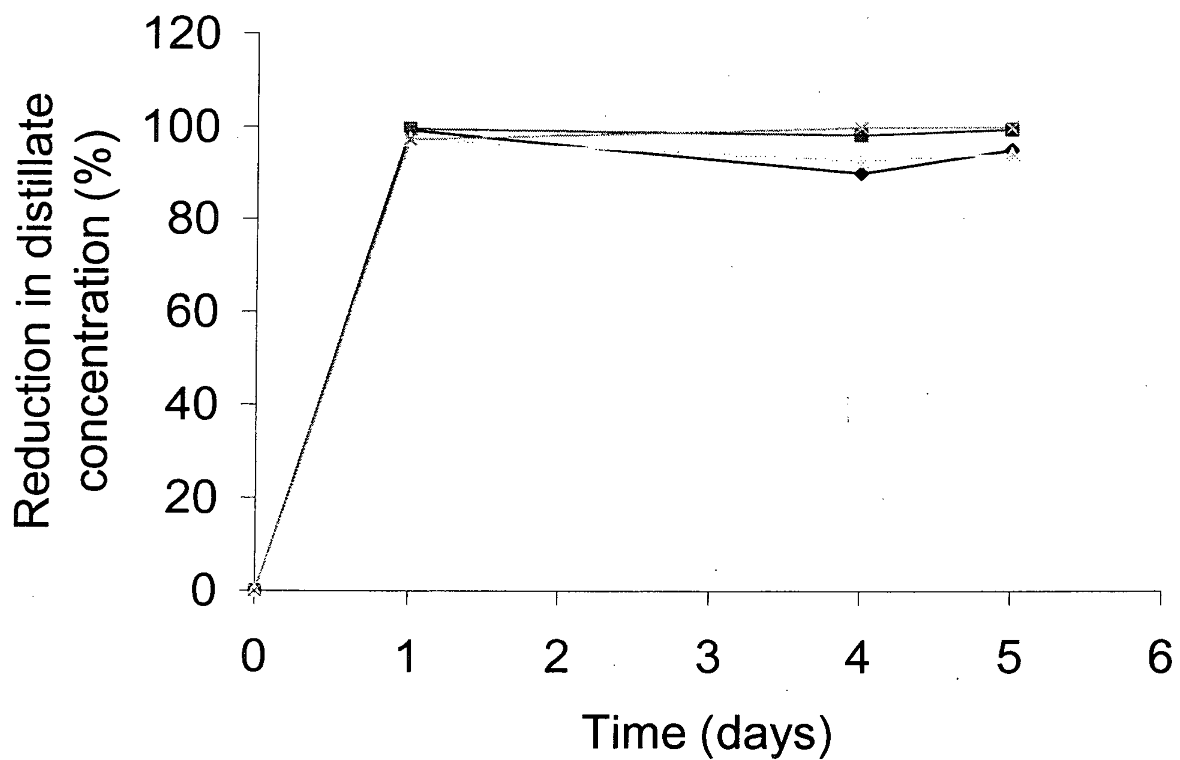
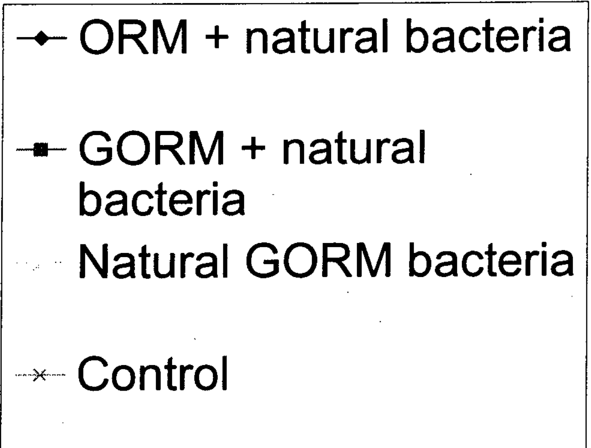


Figure 11. Graph showing the bacterial degradation of a heavy distillate cut overtime, with and without the presence of ORM & GORM.

Figure 12. Graph showing the variation in heavy distillate cut concentrations overtime from hydrocarbon degrading bacteria of ORM & GORM.

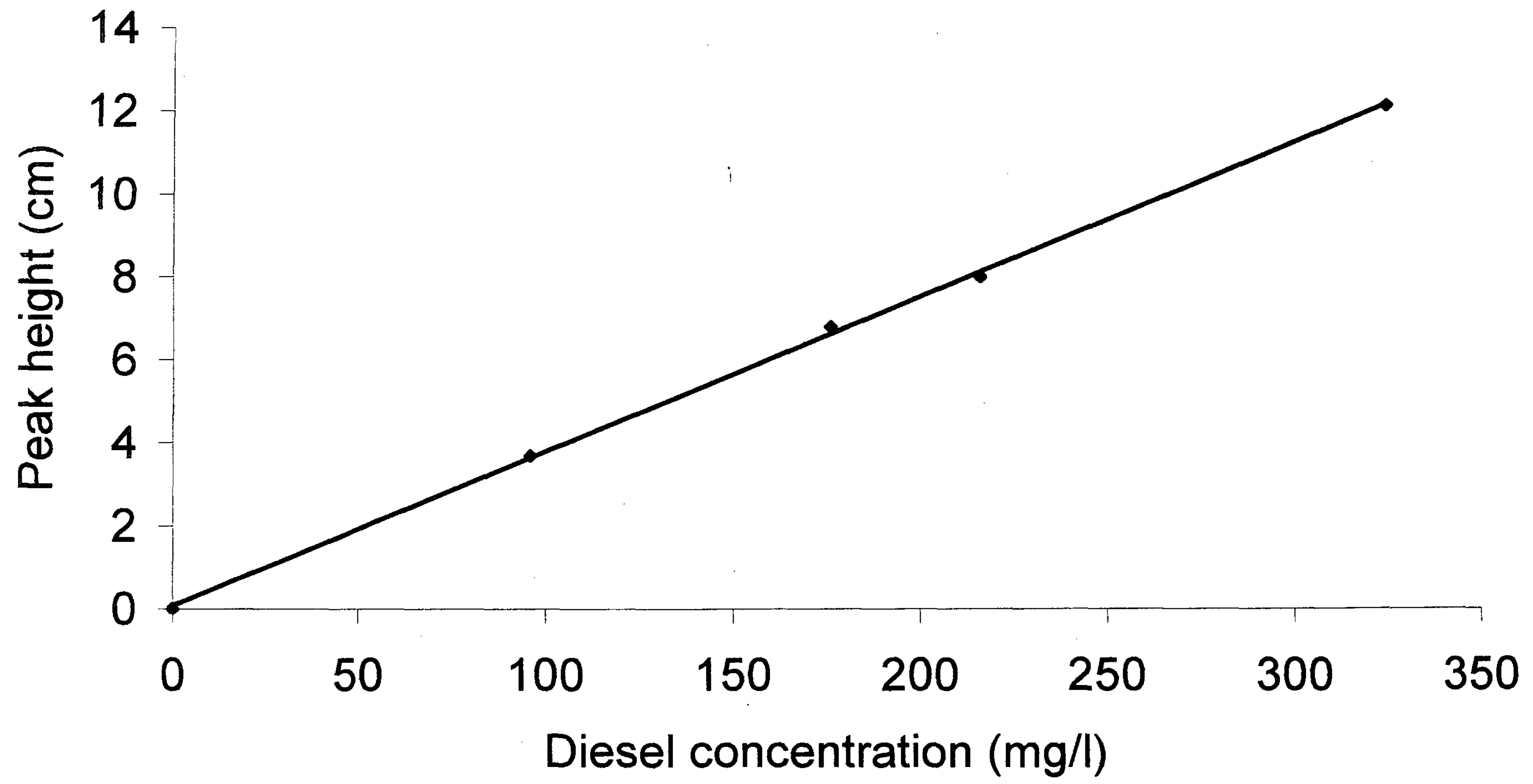


Figure 13. Calibration graph of peak height (cm) against Diesel concentration (mg/l)
Peak height (cm)
0
2
4
6
8
10
12
14
0
50
100
150
200
250
300
350
Diesel concentration (mg/l)

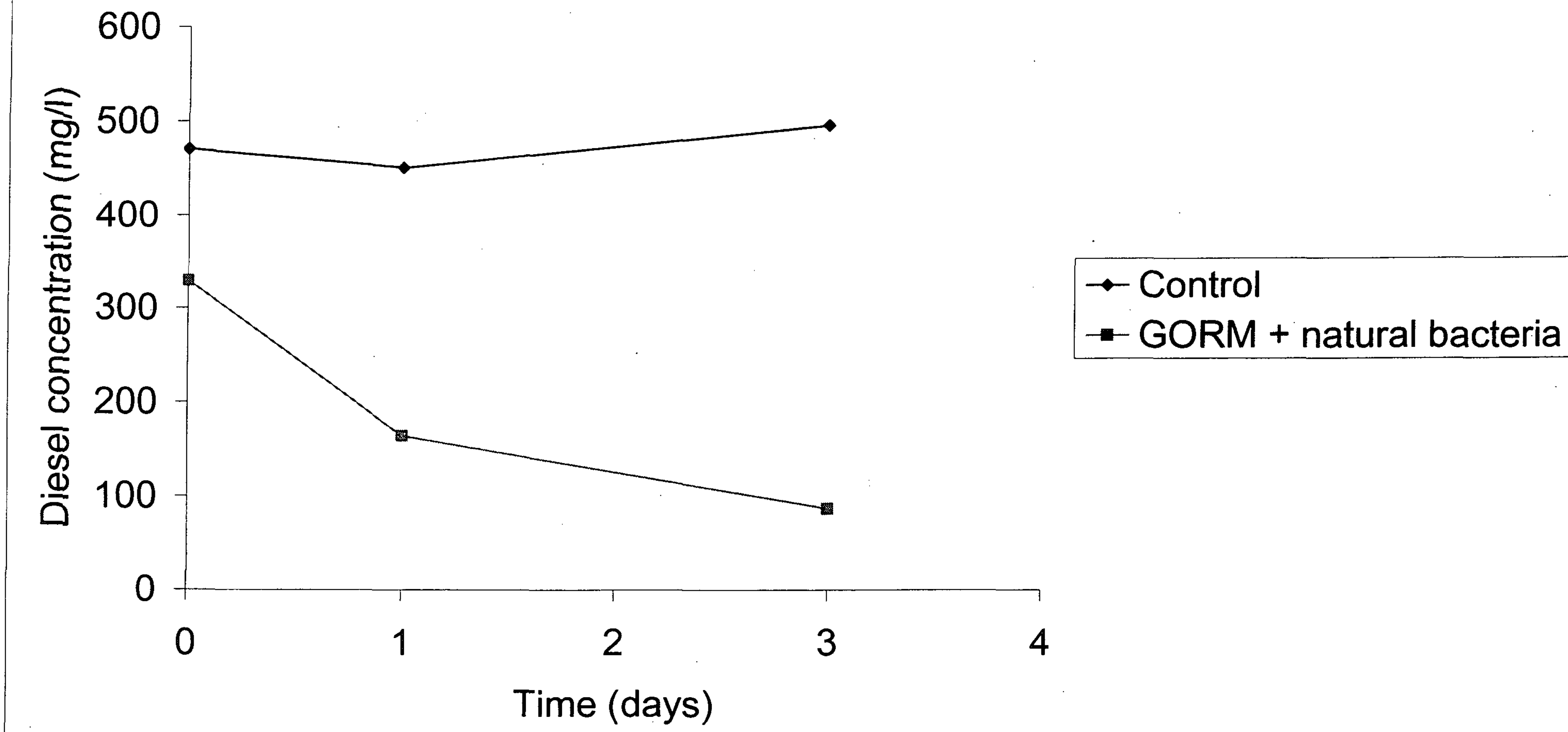


Figure 14. Graph showing the bacterial degradation of diesel in the presence of GORM.

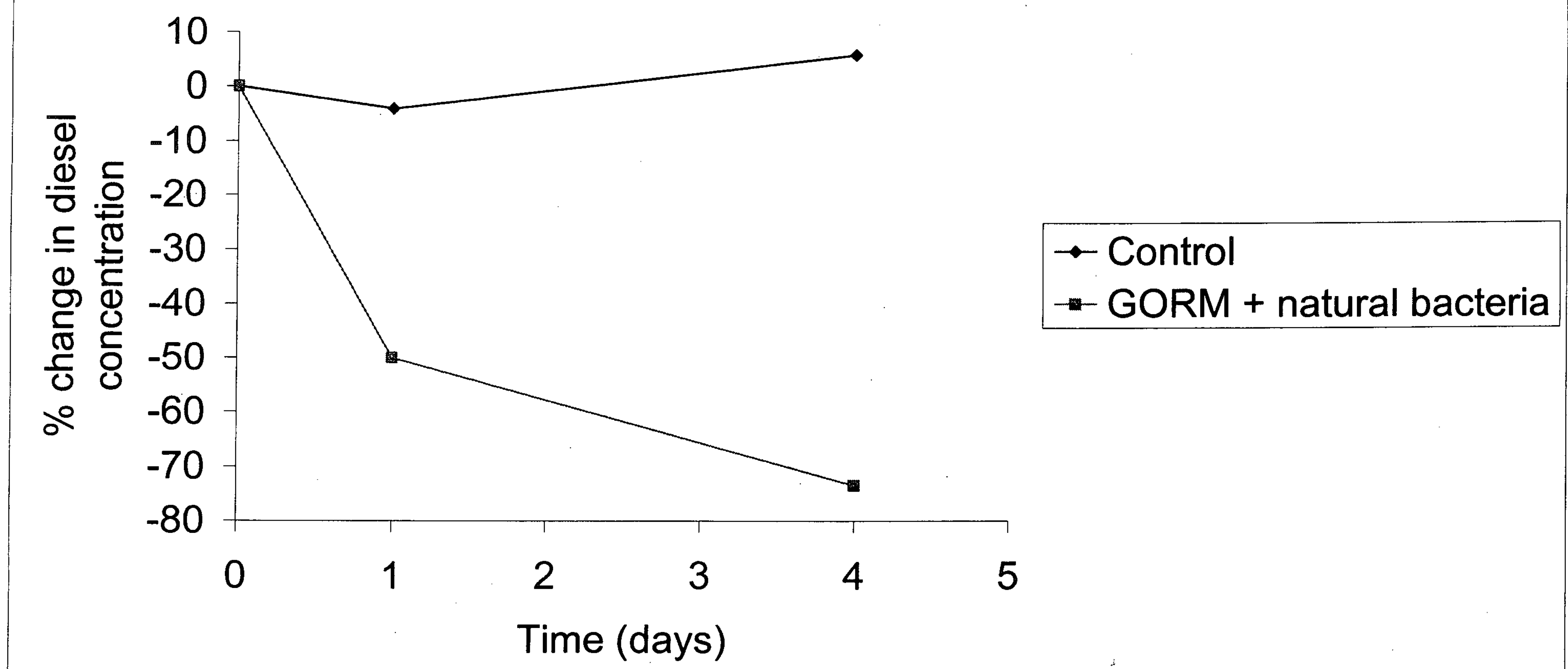


Figure 15. Graph showing the variation in diesel content overtime.
% change in diesel concentration
10
0
-10
-20
-30
-40
-50
-60
-70
-80
0
1
2
3
4
5
Time (days)
Control
GORM + natural bacteria

Chapter 4 Discussion

Petroleum and petroleum products as pollutants occupy an intermediary position and being natural products, seldom enter the biosphere in large quantities except as a consequence of man's activities (5). Its therefore not surprising that marine and other aquatic ecosystems are poorly equipped to handle large influxes of hydrocarbons. As physical removal or burning of accidently spilled oil is seldom feasible and dsipersion or sinking may adversely affect marine life, artificially stimulated biodegradation is being consideredas an alternative (4). Already having been shown to be an effective absorbant material for oil (6), the micro-environment it could provide and nutrients it possesses (N & P) indicate oilseed rapemeal has a large potential to be used in bioremediation.

4.1 The effect of rapemeal on populations of oil-utilising bacteria

The capacity of the oil-utilising bacteria in glucosinolate containing rapemeal (GORM) to increase in population size to such an extent, over such a short period of time, indicates GORM is extremely efficient at enhancing bacterial growth.This, in theory, would indicate similar conclusions regarding oil biodegradation, since these bacteria used only the added oil as a carbon and energy source. Similar conclusions could be said about glucosinolate-free rapemeal (ORM), although it appears clear rapemeal without glucosinolates is much less efficient at enhancing growth than that which does. This could be due to such things as glucosinolates acting as a limiting factor for bacterial growth, or, the consortium of oil-utilising bacteria present in GORM being different to that in ORM and environmental conditions being more favourable to the former. Previous studies have already determined there is a much greater number of bacteria present in ORM than in GORM, this explaining the higher population sizes (day 0) of the former shown in figures 3&4 (Edna Hanna-work

unpublished). On the otherhand, GORM is said to have a richer consortium of oil-utilising bacteria present.

The rapid fall in populations of bacteria in the presence of GORM (Figure 3) was most probably due to the carbon source (0.5ml crude oil) being rapidly utilised by the high numbers of bacteria, this inturn causing rapid death. Although it requires further research to determine this, if true, it would suggest in theory with a continual carbon source present, such as from an oil spill or another type of hydrocarbon pollution, the rapid increase in oil-utilising bacteria populations would continue, in turn allowing rapid oil degradation and environmental recovery. Other possible reasons for this rapid fall include :

a) Rapid immobilization of bacteria onto the rapemeal after a 2-4 day lag-period – suggesting a potential efficiency of immobilization of oil degrading bacteria.
b) Continual evaporation of seawater caused an increase in the amount of sediment and rapemeal extracted and plated out compared to bacteria.
c) Aggrevation (shaking) was insufficient, allowing conditions to become anoxic – O_2 availability being a limiting factor in oil degradation and bacterial growth.
d) N & P became limiting factors in the flasks again from the high numbers of bacteria.

and could also explain the reductions in ORM flasks, although further research would be required to determine exactly which, if any, occurred.

Previous studies by Atlas et al (1972) have shown that degradation of Sweden crude oil occurred after a lag-period of 2-4 days, followed by a rapid degradation period of ~ 12-13 days after incubation. These results agree with the growth of ORM and GORM bacteria (shown in figure 4) – a lag-period of 4 days followed by rapid growth up to 14 days, afterwhich death occurred probably due to complete oil degradation or nutrient limitation.

As shown in figures 3&4, the numbers of bacteria is some 2 orders of magnitude lower in the latter than in the former, respectively. Figure 6 shows the extraction of bacterial cells from rapemeal overtime, via shaking, and indicates extraction should occur for 2-3 hours. In contrast, extraction took place for only 30 minutes for the results shown in figure 4 – explaining this dilemma.

4.2 The effect of rapemeal on bacterial biodegradation of hydrocarbons.

All methods of direct measurement of crude oil degradation are subject to limitations and are able to give only relative values (5). Although the I.R-Spectrophotometer is a sensitive device, obtaining a measurement of the amount of oil present by the number of C—H bonds it detects, care must be taken using it. Firstly it gives no indication as to which hydrocarbons are degraded to what extent in a complex mixture such as crude oil. Also, care must be taken when comparing results of two separate hydrocarbon mixture as variations in mixtures containing aliphatics (n-alkanes) may seem high, due to their high C—H contant, compared to mixtures of aromatics which have much lower C—H contents.

The rapid reductions in 'TOTAL' Allwyn crude oil concentrations which occurred in all flasks, including the control, suggests oil has been lost by some other means rather than natural bacterial biodegradation (figure 8). Primary suggestions included natural bacteria present in the Allwyn oil carrying out vast degradation once in ideal conditions i.e. seawater + nutrients, or, the seawater itself being contaminated with oil-utilising bacteria. Both of these proved negative, with no colonies appearing when both were plated out (30^0C for 3 days). This left evaporation as a possible explanation, although further research using gas chromatography may determine this possibility.

Percentage wise, oil in the presence of GORM bacteria was reduced to a greater extent than that in the presence of ORM bacteria (figure 9). This is probably related to the large

amount of residual oil (8%) present in the ORM flasks, rather than a more efficient enhancement of degradation by GORM. Degradation in the ORM flasks was rapid and remained rapid throughout the 12 day experiment. This is expected as its known residual oil (esters) are much more readily degraded by bacteria than petroleum hydrocarbons, enabling continual and vast reductions over the whole 12 days. In contrast to this, degradation in GORM flasks became a minimum from day 3 onwards. This was because most crude oil was initially lost, whilst little residual was available for futher degradation.

One indication of bacterial degradation enhancement by rapemeal is shown in figure 8. Here initial reductions of oil concentration in the presence of ORM is around x2 that without the presence of ORM. This could be due to the enhanced nutrients available from ORM to these bacteria and the fact that immobilization onto rapemeal would create a micro-environment for these bacteria, possibly creating ideal environmental conditions for oil biodegradation.

Both crude oil and heavy distillate results of hydrocarbon biodegradation give little or no indication of bacterial enhancement with respect to utilisation. Heavy distillate cut results again showed a major loss of hydrocarbons, after only 1 day, although unexpectedly to a greater extent than the crude oil. Similarly, platings of seawater and distillate cut gave no indication of contamination by oil-utilising bacteria, with no colonies being observed after 3 days growth.

The ability of the diesel concentration (control-figure 14) to remain quite constant over the 3 day experiment indicates that the glass stopper was efficient as a seal for the flasks, preventing the escape of volatile hydrocarbons and that evaporation is a strong explantion for the vast reductions of crude oil and heavy distillate over such a short incubation period.

The small drop of ~ 4% diesel over the first day is again due to volitility, but of a more respectable rate and agree's with work by Atlas et al (1972) who studied bacterial degradation of petroleum and measured volatility losses of 32-36% over 8 days using Sweden crude oil. Care must be taken in comparing these losses as there could be a large difference in the composition of low mwt (volatile) : high mwt (less volatile) hydrocarbons between the two petroleum produts.

In contrast to this, diesel in the presence of GORM and its bacteria was degraded by 50% and 73% after 1 and 3 days, respectively. This suggests biodegradation has been greatly enhanced by this rapemeal and is further supported by the fact that this degradation rate is a lot quicker than that obtained by Atlas et al. (5) - where they showed biodegradation to occur to a similar extent (60-70%) after 14 days. Similar extents of biodegradation of petroleum in seawater occurred when these two researched N & P being the limiting factor of bacterial degradation (4), but again rates were slower with ~ 70% being lost over 18 days in ideal N : P ratio's.

Initial concentrations of diesel (day 0) are lower in the presence of GORM compared to that of the control. This is probably due to the fact that the mixing process required to dissolve all added hydrocarbons in the flask in CCl_4, ultimately produced an emulsion of CCl_4 when rapemeal was present – this being due to surfactants on the rapemeal itself. A settling period was then required to allow the emulsion to break-up, reforming the 2 phase system – CCl_4 + oil below rapemeal + seawater. Although this eventually occurred, there was never a 100% emulsion break-up, inevitably causing a small proportion of oil to be lost.

Although these last set of diesel results seem promising, futher research is required to determine the enhancement of biodegradation by rapemeal. Comparing degradation of diesel in the presence of GORM to that in the presence of ORM, just ORM bacteria and just GORM bacteria would need to be carried out. Along with this,experiments of crude oil and

heavy distillate degradation would need to be repeated not only using a glass stopper as a seal instead of a foam bung, but adding another environmental condition to the experiment of unsterilised seawater and washing extracted ORM and GORM bacteria before inoculation, to remove all the problems encountered in this project related to the presence residual oil.

The possible use of hydrocarbon-utilising microorganisms for accelerated degradation of hydrocarbon pollution is receiving serious consideration (5) and the use of rapemeal as an enhancer seems promising. Its already known that mixed enrichments, such as that in rapemeal, can degrade highly complex substrates, such as petroleum, more efficiently than a single microorganism. Due to the fact that practical use of enrichments of unknown composition is likely to encounter licensing difficulties, because of its potential side effects on marine life, the bacteria which make up the enrichment in rapemeal would have to be accurately determined before any commercial use in bioremediation could be applied.

Chapter 5 Conclusions

The study of oilseed rapemeal and its effect on the ability of oil degrading bacteria to degrade oil has produced the following conclusions :

- Both ORM & GORM are able to enhance the growth of oil degradaing bacteria, probably by providing a micro-environment and nutrients which are otherwise unavailable.
- GORM is much more efficient at growth enhancement than ORM.
- Results from the crude oil and heavy distillate experiments gave no indication of bacterial biodegradation enhancement, due to hydrocarbon losses by other means rather than bacterial utilisation.
- Degradation of diesel seemed greatly enhanced by GORM, although further comparisons are required to determine this.
- If results similar to those of the bacterial growth & diesel degradation experiment were obtained in further experiments, this would suggest oilseed rapemeal to have great potential to be used in bioremediation.

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