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An investigation into the bacterial communities present at two estuarine beaches in the South Hams, Devon, UK: the effects of oil pollution

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Abstract

Aims: To determine if there are any differences between oil-degrading:heterotroph ratios and bacterial communities from a polluted and non-polluted site.

Methods and Results: Composite samples for both water and sediment were collected from Mothecombe (polluted) and Wembury (control). Cultural enumeration and molecular analysis was performed to determine any differences between bacterial communities. No differences were observed between the bacterial communities of the two sites. Sediment samples were an order of magnitude higher in abundance compared to water samples.

Conclusion: Mothecombe appears to have recovered from oil pollution. Culture-independent studies are needed to clarify the validity of these findings.

Significance and Impact of study: This paper stresses the importance of avoiding culture-dependent methods to analyse bacterial communities. The application of ODB as indicators of oil-pollution is important, but careful consideration should be applied when selecting a control site. Previous oil pollution could increase a sites tolerance and ability to biodegrade oil due to an increase in bacterial community diversity. Direct analysis of the community, via molecular methods, is required to verify is this hypothesis is correct.

Keywords: Oil-degrading bacteria, 16S rRNA gene, bacterial community, beaches.

Introduction

Contamination via systematic and accidental spills has and continues to have a detrimental effect on coastal ecosystems. For example, the *Exxon Valdez* oil spill led to the release of 42 million tonnes of oil contaminating over 1000km of Alaskan coastline (Peterson *et al.* 2003), affecting many different organisms, via either direct hydrocarbon poisoning (Thomas *et al.* 2007) or indirect processes, such as, mechanical damage to bird wings (Lance *et al.* 2001). These ecological implications can lead to economic problems for coastal areas that rely on tourism as their sole trade, due to the reduction in marine and marine-associated wildlife.

Crude Oil is a complex mixture of hydrocarbons including linear and branched n-alkanes and polycyclic aromatic hydrocarbons (PAHs). PAHs are commonly reported as environmental pollutants of many marine organisms by forming DNA adducts, leading to mutations in the organisms DNA and ultimately the development of neoplasm's (Xue & Warshawsky 2005). Furthermore, PAHs act synergistically with sunlight (Toyooka & Ibuki 2007) increasing toxicity and compounding the problems for organisms faced with oil pollution.

Naturally occurring marine oil seeps release vast amounts of oil into the ocean. In fact, the input from these seeps alone is enough to cover the entire surface of the oceans up to twenty molecules thick (Head, *et al.*, 2006). Because of this naturally occurring phenomenon, many different groups of bacteria have been associated with hydrocarbon degradation. The identification of both obligate 'professional' and 'non professional' oil degraders (Yakimov, *et al.*, 2007) has lead to research on both biotechnological (Prieto 2007) and bioremedial applications (Prince *et al.* 2003).

The incidence of oil-degrading bacteria (ODB) can be used as an indicator of oil exposure (Leahy & Colwell 1990). However, the presence of oil-degraders in many environments, especially busy shipping channels (Wu *et al.* 2009), reduces the validity of using ODB in assessing whether or not an area has been affected by an oil spill. Therefore, it is important to determine differences in the community composition of ODB between polluted and clean sites. Furthermore, the role of bacterial communities in driving the health of ecosystems has been demonstrated, in the absence of algae, bacteria can act as a food source for zooplankton, (Sävström *et al.* 2009), which then act as a food source for organisms higher up the trophic ladder. These findings suggest bacteria are the foundations, from which many marine ecosystems develop. To unify these ideas, the comparative analysis of bacterial communities is an important initial step in identifying whether or not an ecosystem has been affected by pollution (Kostanjsek *et al.* 2005). Finally, the importance between increased microbial biodiversity and ecosystem function is becoming apparent and the utilisation of molecular techniques is allowing a comprehensive analysis of bacterial communities (de Wit 2008).

The aims of this study are 1) to determine whether there is any difference between the ratios of oil-degrading bacteria and total heterotrophic bacteria located at Mothecombe (polluted site) and Wembury (control site), 2) to investigate any long term differences between the bacterial communities present at each beach. Water and sediment samples were collected from both sites in order to analyse to bacterial composition of both sites. Both cultural enumeration and molecular profiling were carried out in order to achieve these aims.

Materials and methods

Sample Collection

Samples were collected from two estuarine beaches in the South Hams, situated on the south coast of Devon, UK. Sampling was carried out in the late autumn (average sea temp. 11°C). Mothecombe (polluted) is situated approximately 4-5 miles east of Wembury (control). At each site, composite water (2 litres) and sediments (800 grams) were taken from two stations using 500 ml wide mouth sterile plastic bottles. All samples were collected at low tide. For molecular analysis, sediment samples were shaken three times (adding 100ml each time), using synthetic sea water (Tetra instant marine sea salt).

Culture Methods

Both heterotrophic and ODB were filtered onto 0.45µm membrane pads (Millipore) using suitable dilutions based on previous data (Bradley & Waines; unpublished data). Heterotrophic bacteria were cultured on Marine salts agar (MSA), as described by (ZoBell 1941). ODB were cultured on oil agar (Walker & Colwell 1975), Arabian crude oil was used as the hydrocarbon substrate and added (sonicated) to 20 ml of the base medium. All cultures were incubated at 25°C and colony counts were performed when no more growth was detected. Cultures plates were then transferred to 4°C cold room for storage and awaited DNA extraction.

DNA Extraction from culture plates

Filter pads were removed from agar and washed with washing solution (50mM Tris/Cl pH 7.7, 25mM EDTA, 0.1% SDS, 0.1% PVP) to remove colonies. Stubborn colonies were gently scraped off and the solution was centrifuged at 13000 rpm for 5 minutes to create a pellet. 500µl of modified lysozyme (50mg/ml in TE [10mM Tris/Cl, 1mM EDTA pH8.0]) was added to pellets and incubated at 37°C for 30 mins. 35µl of lysis solution (50mM Tris/Cl pH 8, 25mM EDTA, 3% SDS, 1.2% PVP) and 400µl of warmed (60°C) extraction solution (Tris/Cl pH 8, 1mM EDTA, 0.3M Na acetate, 1.2% PVP) were further added and combined with 1ml phenol/chloroform mix, stirred gently and left on ice for 10 mins. Following centrifugation at 5000 rpm for 5 mins, the clear top layer was removed to a fresh eppendorf. Ice cold isopropanol was added to precipitate DNA and centrifuged at 5000 rpm for 5 mins. The invisible pellet was washed with 1ml 70% molecular grade ethanol via centrifugation at 5000 rpm for 5 mins. Finally, the DNA pellets were dried under vacuum for 10 mins then stored at 4°C overnight. DNA concentration and suitability for PCR was estimated using *Nanodrop* 1000 spectrophotometer.

DNA Extraction from seawater and sediment samples

2 litres of seawater and 300ml of Instant ocean sterile sea water were pre-filtered through Whatman #4 filter paper. Pre-filter seawater was then filtered onto 0.45µm membranes (Millipore), some samples needed more than one filter pad. Pads were placed onto a roller and washed in 1.5ml of washing solution (50mM Tris/Cl pH 7.7, 25mM EDTA, 0.1% SDS, 0.1% PVP) overnight. Pads were gently scraped to remove excess sediment and solutions were centrifuged for 5 minutes at 13000rpm to produce pellets. Samples needing more than one filter pad were combined and pelleted down into one sample. Two methods were used to extract DNA, QIAamp Stool Mini Kit (Qiagen) with a modified lysozyme digestion stage using 500µl of lysozyme, as previously described, and increasing the temperature in the lysis stage

to 90°C for 5 minutes. The second followed the phenol/chloroform extraction procedure as described above. Suitability for PCR was determined using Nanodrop 1000 spectrophotometer.

PCR

The primers 341F and 534R (Muyzer et al, 1993) were used to amplify 16S RNA genes via consensus sequence annealing (MWG Biotech). A touchdown PCR program was applied starting with an initial annealing temp of 65°C, dropping a degree every cycle 2nd cycle until the annealing temp equalled 55°C. A further twenty cycles was performed before a final extension at 72°C completed the process. PCR products were held at 5°C overnight until a brief analysis was undertaken using 1.5% agarose gel to determine suitability for DDGE.

Denaturing Gradient Gel Electrophoresis

The gel was composed of 8% polyacrylamide in TAE buffer (20mM Tris acetate, 10mM sodium acetate, 0.5mM EDTA) pH 8.3 containing a gradient of 40-60% denaturants (7M urea and 40% formamide). Separation was achieved using the Bio-rad Dcode universal mutation detection system. The gel was stained with SYBR GREEN (20µl in 200 ml distilled water).

Statistical Analysis

Analysis of the gels was performed using Image J software and statistical analysis carried out using Primer 6. Cluster analysis, MDS and Shannon-Wiener indices were calculated by Primer 6 as described by (Clarke, 1993).

Results

Cultural Enumeration

For oil-degraders, both beaches had an order of magnitude difference between sediment and water samples; Mothecombe 2.15×10^2 CFU g⁻¹ and 34 CFU ml⁻¹, respectively; Wembury 1.95×10^2 CFU g⁻¹ and 21 CFU ml⁻¹, respectively. A similar pattern was observed at Mothecombe for total heterotrophs (6.6×10^3 CFU g⁻¹ and 4.65×10^2 CFU ml⁻¹, respectively), however, at Wembury, bacterial counts, for both sediment and water samples, were in the same order of magnitude (7.34×10^3 CFU g⁻¹ and 1.4×10^3 CFU ml⁻¹, respectively). The ratio between oil-degraders: heterotrophs were higher in samples taken from Mothecombe (7.32% in water and 5.38% in sediment) compared to Wembury beach (1.18% in water; 4.38% in sediment). Tables 1 and 2 summarise these data.

Table 1 Colony counts and resultant ratios of ODB and heterotrophs recorded from sediment samples at both sites

Sites	ODB (CFU g ⁻¹)	Heterotrophic (CFU g ⁻¹)	Ratio (%)
Mothecombe	2.15×10^2	6.6×10^3	5.38
Wembury	1.95×10^2	7.34×10^3	4.38

Table 2 Colony counts and resultant ratios of ODB and heterotrophs recorded from water samples at both sites.

Sites	ODB (CFU ml ⁻¹)	Heterotrophic (CFU ml ⁻¹)	Ratio (%)
Mothecombe	34	4.65x10 ³	7.32
Wembury	21	1.4x10 ³	1.18

Molecular Analysis

Cultural Oil-degrading bacteria

A general pattern of banding similarity can be seen between lanes, with only minor differences between water and sediment samples. Except for one replicate, all sediment samples show two heavily stained bands (bands 3&4, Fig. 1) near the top of the gel which is absent from water samples. Within the sediment samples, Mothecombe samples have a distinct band beneath the heavily stained band, except for the anomaly. Wembury samples have a distinct band (band 9) below ubiquitous band 7. The intensity of band 6 is higher in water samples, and there is a faint band (band 8), directly underneath band 7, present only in the Wembury water samples.

The dendrogram analysis revealed that all samples are >50% similar to each other and there is clear difference between water and sediment samples with the exception of the one anomaly (Fig. 3). Interestingly, all sediment samples taken from Wembury are clustered together and two of the three Mothecombe sediment samples are clustered together (Fig. 3). 3D MDS graph analysis (Fig. 4a) shows two distinct clusters representing either sediment or water samples, again with the exception of the anomaly (MS-2). The two minor clusters within the sediment samples are also observed, with Wembury sediment samples closer to each other

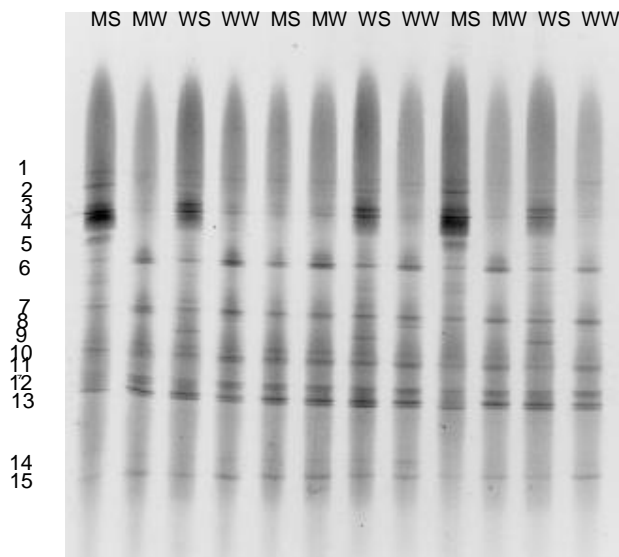


Figure 1 DGGE gel comparison of ODB located at both sites. Mothecombe sediment samples (MS), Mothecombe water samples (MW), Wembury sediment sample (WS) and Wembury water samples (WW). Numbers for bands used in analysis are numbered 1-15 on the left hand side.

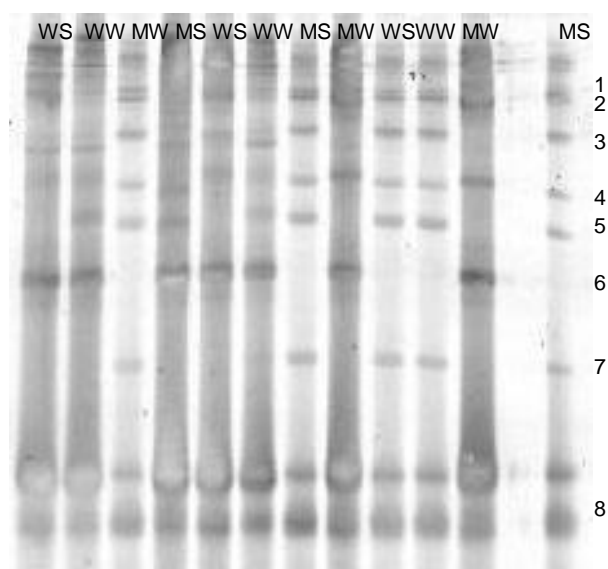


Figure 2 DDGE gel comparison for total heterotrophs at both sites. Bands that were selected for analysis are numbered 1-8 on the right hand side.

than to Mothecombe sediment samples. Shannon-Weiner indices range $H' = 1.884 - 2.554$ for all samples (Table 3).

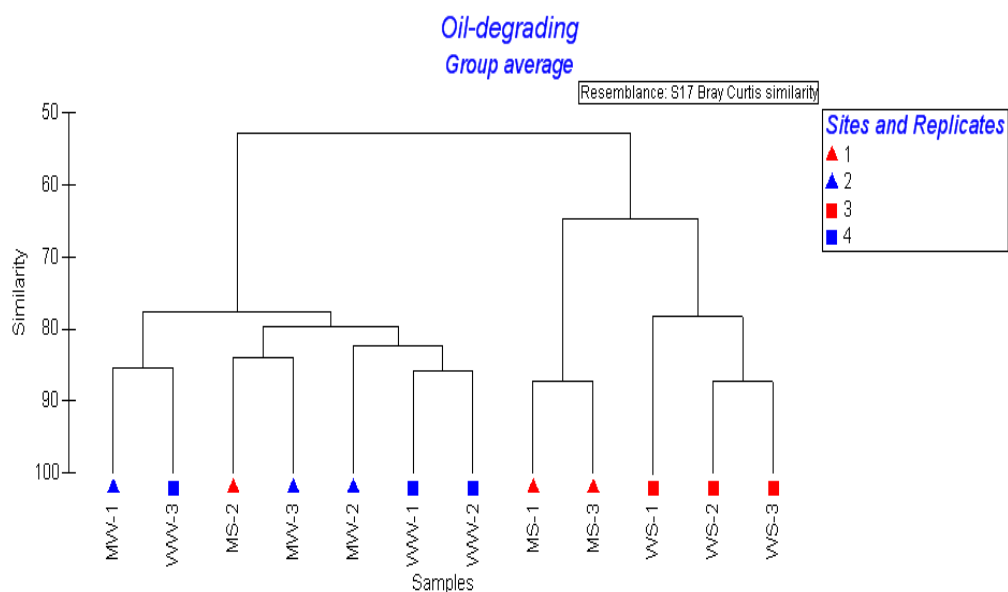


Figure 3 Dendrogram based on DGGE bands patterns using Bray-Curtis coefficients. In the key 1=MS, 2=MW, 3=WS, 4=WW.

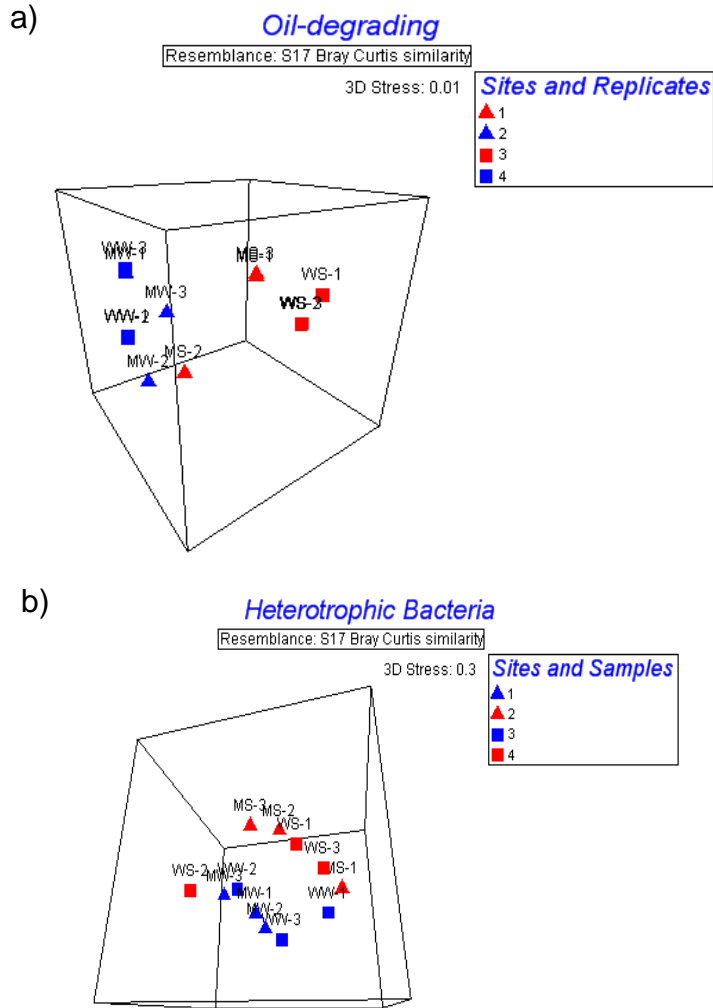


Figure 4 MDS 3D graph based using Bray-Curtis similarity coefficients comparing both sites for a) ODB b) Heterotrophs.

Cultural Heterotrophic Bacteria

No clear pattern between different samples can be observed. Heavily stained lanes show a large band roughly half way down the gel (Fig. 2), however these heavily stained lanes do not correspond to different sample sites. Band 6 only features in the lightly stained bands and band 4 predominantly features in these same bands, with the appearance in only two heavily stains bands. The bottom two bands are present in all samples.

3D MDS graph analysis again revealed a larger difference between water and sediments samples compared to inter-site differences (Fig. 4b). Again, there are two anomalies that upset this general pattern, which are WS-2 and WW-1. Shannon-Weiner indices range from $H' = 1.244-1.891$ for all samples (Table 4).

Table 3 Shannon-Weiner indices for all samples representing ODB. No. of species (S), (N) and Shannon-Weiner indices (H')

Sample	S	N	H'
MS-1	12	7956	2.112
MS-2	10	5996	2.219
MS-3	11	7138	2.09
MW-1	9	4654	2.04
MW-2	10	7491	2.206
MW-3	7	4883	1.884
WS-1	11	5437	2.213
WS-2	12	8026	2.387
WS-3	11	6805	2.31
WW-1	11	6793	2.251
WW-2	11	6688	2.254
WW-3	9	4693	1.987

Table 4 Shannon-Weiner indices for all samples representing heterotrophic bacteria

Sample	S	N	H'
MS-1	7	9956	1.703
MS-2	5	5779	1.547
MS-3	7	7146	1.765
MW-1	7	7448	1.66
MW-2	4	5451	1.306
MW-3	5	4359	1.596
WS-1	7	5892	1.891
WS-2	7	5373	1.487
WS-3	5	4428	1.647
WW-1	7	9051	1.798
WW-2	8	7256	1.798
WW-3	6	15771	1.244

Community analysis from water and sediment samples

Several DNA extraction methods resulted in unsuccessful levels of DNA and were therefore unsuitable for PCR and DGGE analysis

Discussion

For total heterotrophs, Shannon-Weiner indices revealed no difference in bacterial composition between the polluted and control site (Table 4). There appears to be just as much intra-site variation as inter-site variation in community composition. The ability of bacteria to degrade oil has been well documented (Yakimov *et al.* 2007) and the dynamics of a bacterial community in response to oil exposure has been investigated (Röling *et al.* 2002). Here, 16s rDNA analysis illustrated an initial decrease in community diversity followed by a return to initial diversity levels after 26 days during a microcosm experiment. On the basis of these results, Mothecombe, which was exposed to oil pollution, has recovered and returned to pre-oiling bacterial community diversities matching the diversity seen at the control site.

However, these results should be treated casually as they only represent the cultural bacteria present at both beaches. Furthermore, the intensity of band patterns

is a result of each individual species ability to grow on MSA plates and is therefore an unnatural representation of the true community composition. Further investigations need to ascertain the true composition of the community via direct DNA extraction from both water and sediment samples. Gentile *et al.*, stressed the importance of using 16S rRNA and 16S rDNA sequencing in determining the composition of bacterial communities at the Antarctic (Gentile *et al.* 2006). This was the primary goal of this study, but DNA extraction and PRC amplification proved difficult, this was probably an artefact of the environment from which extraction was performed. Estuarine systems have high levels of humic material which interfere with DNA extraction and purification, reducing the ability of PRC to amplify the desired DNA (Miller *et al.* 1999). Although the QIAamp stool mini kit (Qiagen) has had success in the past (Bradley and Waines, unpublished data), it failed this time to deliver both high yields and purity. The phenol chloroform method produced higher yields but in sufficient purity to achieve successful PCR, however, the technique requires high levels of skill, repetition of this method on culture colonies delivered good results and therefore provides most hope for achieving a successful DNA extraction.

The similarity between ODB at both sites is also intriguing, the high diversity witnessed at Wembury is probably a result of its close proximity to Plymouth Sound. Plymouth is an international port as well as a naval base and many fishing boats also move in and out of the area, leading to elevated levels of hydrocarbons in the water. The isolation of ODB from heavily-shipped harbours and a correlation between proximity to harbour and intensity of ODB has previously been documented (De Donno *et al.* 2008) and it is reasonable to assume that the presence of ODB at the control beach is a response to chronic trace levels of oil pollution. This subtle difference in pollution history may account for the two differences observed between the two sites DGGE profiles (Figures 1 & 2). The high number of bands retrieved from both sites may simply be a reflection of high strain diversity and not necessarily imply high genus diversity. A large scale study on the coast Kuwait revealed that the high diversity observed using 16S rDNA represented high levels of strains diversity and the predominance of only a few genera (AL-Saleh *et al.* 2009). Characterisation of the bands will indicate whether the high degree of banding reflects high genus diversity or high species/strain diversity. The participation of different genera in biodegradation of heavy oils is a result of differing substrate specificities (Mckew *et al.*, 2007) and the presence of multiple ODB enhances a community's ability to degrade heavy oil (Nakamura *et al.* 2007). It is likely that the band (band5) seen in Mothecombe sediment (Fig. 1) could reflect the presence of another genera as a result of exposure to heavy crude oil. Therefore, the effect of an oil spill may result in the presence of a more diverse ODB community, ultimately leading to a bacterial community that will be more efficient at degrading crude oil, should another accidental spill take place.

Again, the process leading to the analysis of ODB could be improved in further studies. Growing ODB on plates can have certain limitations compared to direct extraction from both water and sediment samples (Orphan *et al.* 2000). The utilisation of alk3 gene primers to detect ODB has previously provided success (Bradley & Waines, unpublished data). and should be used to determine the true composition of alkane-degrading ODB to clarify if there are no differences between the sites. Detection of PAH degrading bacteria can be achieved using primers specific for PAH dioxygenase and C230 genes, which encodes enzymes involved in

the key stages of PAH degradation (Meyer *et al.* 1999). A combination of these Primers will achieve a full comparison between the sites.

The difference in bacterial abundance and composition between water and sediment samples confers with the study conducted by (Hii *et al.* 2008). These findings are expected due to the differing abiotic conditions within each microhabitat. The fine sand grains that constitute the sediment at both beaches creates a large surface area in the sediment and increases the potential for bacteria to adhere to substrates. This results in a positive correlation between grain size and bacterial colonisation and abundance (Yamamoto & Lopez 1985). The heavy staining of bands representing sediment samples of the ODB gel suggest that hydrocarbons are present within the sediment. Long term persistence of hydrocarbons in sediments beneath mussels beds has been documented in Alaska as a result of the *Exxon Valdez* oil spill (Carls *et al.* 2001).

The long term study conducted by Bradley & Waines (unpublished data) reveals the similarity between ODB:heterotroph ratios recorded from both sites. The data presented in this present study also confers to these results where only a slight variation is witnessed (Tables 1 & 2). The long term study mentioned was initiated 2 years after the oil spill hit Mothecombe beach (Schoon 1990). These ratios support the hypothesis that the bacterial community has returned to pre-oiling levels and that this was achieved within a 2-year period. However, current culturing methods take only a small sample, can produce great variation in the results. Estuarine systems that experience a lot of freshwater in-put and large tidal fluctuations are generally more heterogeneous (Park & Park 2000). Utilisation of up-to-date technology, such as Flow-cytometry will generate more reliable results (Marie *et al.* 1996) enhancing the robustness of any future study. These techniques also allow enumeration of non-culturable bacteria again improving the quality of results.

In conclusion, Mothecombe appears to have recovered to a pre-oiling community composition and is similar to Wembury. However, the severe limitations to the methods used make this assumption crude. Further studies using the methods and technologies proposed here will help clarify the true long term effects caused by oil pollution. The use of ODB as indicators of oil pollution needs careful attention and a suitable control is imperative in deriving such conclusions from comparative studies.

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