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Master's thesis

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ABSTRACT

There are ongoing developments to enable treatment of oil based drill cuttings with subsequent deposition offshore rather than the current practice to transport and treat such material on land. A sample from a step in the development of a microwave based treatment method has been tested in the present study. Microcosm and DGGE experiments were conducted to assess and compare the adverse effects of microwave treated drill cuttings with untreated oil based muds on macrofauna and microbial communities. Sediment sample from Boknafjord at a depth of 134 m were transferred into cores and treated with drill cuttings in an average layer of 11 mm, the adverse effect was measured on macrofauna diversity, biomass, number of species and microbial survival rate. The results showed that the treated cuttings were significantly less affected on the macrofauna number of species than the OBM sample, evidenced by statistical analysis, but less significantly by biomass for the macrofauna, and without significant difference with regards to macrofauna diversity or microbial communities. The cause of the effects for the oil based drill cuttings can be due to the presence of organics while for the microwave treated drill cuttings observations of effects that were different from the control sediment might alternatively have been caused by heavy metals. This latter can be argued for by the fact that the microwave treated drill cuttings was in more fine grained particles than the control sediment which could have made the metals more bioavailable for the macrofauna and microbial communities. This hypothesis needs to be further examined to be conclusive.

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Thanks.

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ABBREVIATIONS

Anova – Analysis of variance

APS – Ammonium persulfate

bp – Base pairs

B – Biomass

CN – Control

DGGE – Denaturing gradient gel electrophoresis

F01 & F02 – Control sediments

F12 & F14 – Sediment + Microwave treated drill cuttings

F10, F16, & F20 – Sediment + Oil based drilling muds

F13 – Negative control

GGBS – Ground granulated blast furnace slag

H' – Shannon-Wiener diversity index

H_0 – Null hypothesis

LL – Lower layer

N – Number of species (macrofauna) in a core

OC – Organic carbon

OCDC – Oil contaminated drill cuttings

OBF – Oil based fluids

OBM – Oil based drilling muds

PAH – Polycyclic aromatic hydrocarbons

PCB – Polychlorinated biphenyls

PFA – Pulverized fuel ash

PCR – Polymerase chain reaction

ROV – Remotely operated under water vehicle

S10 – Microwave treated drill cuttings

SBM – Synthetic based drilling muds

SD – Sediment

SP – Significance probability

TAE – Tris-acetate EDTA

TPH – Total petroleum hydrocarbon

TN – Total Nitrogen

TCC – Thermo-mechanical cuttings cleaner

USEPA – United states environmental protection agency

UPL – Upper layer

WBM– Water based drilling muds

1.0 INTRODUCTION

1.1 Background of the Thesis and Problem Formulation

The oil and gas reserves in the world have been exploited by man since in the 1800s. Regulations and monitoring programs are essential for countries producing oil to assess the potentially harmful effects it has to the marine environment and to humans in a broad picture (Kingston, 1992).

Oil based drilling fluids replaced water based drilling fluids because of the better results in drilling operations but drilling processes have mostly gone back to the use of water based fluids due to the observed harmful effect oil based fluids has on the environment (Olsgard and Gray, 1995, Ball et al., 2012). Workers safety issues, environmental effects and exposure of marine organisms to polycyclic aromatic hydrocarbons (PAH) are just some disadvantages of the use of oil based fluids (Ball et al., 2012).

Barite ($BaS0_4$) which is a major constituents of drilling mud is discharged into the ocean after use. Barite contains heavy metals, and the quality of the barite is distinguished by the concentration of the heavy metals in it. Barites are known by their insolubilities, high density and a high sedimentation rate and hence a good indication of dispersion of drill cuttings. All the above properties make drill cuttings affects the sediment living benthos. Sediment contamination and its effects on sediment communities has been a major focus for monitoring. (Olsgard and Gray, 1995)

International organizations and environmental agencies have recognized that the detrimental effects of the contamination of biota cannot be shown through risk assessment which is based on chemical analysis alone. Therefore the biological consequences of the presence of pollutant must be considered in the assessment of the quality of the environment (Gray, 1992, Bayne, 1988).

It is clear and evident that considerable amount of drill cuttings when disposed in the ocean settle on the sediment which causes contamination, may cause changes in sediment community structure and functioning; decrease in biomass, reduced level of dissolved oxygen and less diversity in benthos community (Trannum et al., 2016, Schaanning et al., 2008).

1.2 Purpose of the thesis

The main objective of this thesis is to evaluate if there are significant toxic or community effects of using micro wave treated and untreated oil-based drill cuttings on a faunal and microbial community. This will be assessed by laboratory experiments with treated drill cuttings using the microwave treated method compared to experiments with untreated cuttings.

2.0 THEORY

In this chapter drill cuttings and drilling mud are explained and the different methods of treatment drilling waste presented.

2.1 Drill cuttings and drilling mud

Just what is drill cuttings and drilling mud? It is important to define some of the basic terms in drilling and what is being discharged to the marine environment. "Drill cuttings are broken bits of crushed rock gotten from the grinding action of the drill bit as it penetrates the earth for geotechnical or mineral exploration"(Neff, 2005). Oil contaminated drill cuttings (OCDC) emerges from the drilling activities in the exploration and extraction of oil and gas. The drilling of wells requires the use of “drilling muds” (drilling fluids) (Shang et al., 2005).

Drilling mud which can also be called drilling fluids which can be defined as "a heavy viscous suspension of solids (ex. clay, barite) in liquids (ex. water, oil) containing chemical additives as required to modify its properties, used in drilling operations to carry rock cuttings to the surface and also to cool/lubricate the drill bit"(Neff, 2005). Drilling fluids are progressively pushed down the well through the hollow string and then recovered through the well annulus carrying the rock phase that is extracted from the well. These acts to cool and lubricate the drill bit, provide hydraulic power, maintain the stability of the well-bore, and transfer the drill cuttings back to the platform (Ball et al., 2012). As the drill bit grinds rocks into drill cuttings, these cuttings become entangled within the fluid flow and are carried to the platform where the cuttings are separated from the fluids and other contaminants so that the mud can be re-used in the operation (Ball et al., 2012).

The drill cuttings and the mud are treated with special devices to separate the cuttings from the mud (Denoyelle et al., 2012). When the drill cuttings and the drill mud are successfully separated the drill cuttings are discharged to the sea, it usually contains trace to considerable amounts of drilling mud after separation (Hess et al., 2013). The ocean floor was considered to be an endless dumping ground not until the 1970s and 1980s, where it was evident that OBFs(Oil Based Fluids) and drill cuttings have undesirable effect on the local ecology (Ball et al., 2012). Drill cuttings pose a waste management problem to the petroleum industry as a result of the quantity produced and their content of both organic and inorganic contaminants such as petroleum hydrocarbons, polychlorinated biphenyls PCB, and heavy

metals. The contaminant present in drill cuttings depend on the chemistry of the drilling fluid and the composition of the formation rock (Leonard and Stegemann, 2010).

Drilling mud can be divided into three types: oil-based drilling mud (OBM), synthetic-based drilling mud (SBM) and water-based drilling mud (WBM)(Hess et al., 2013). WBM are considered to be less harmful to the marine environment, and is therefore used the most but recent studies have proven otherwise therefore should be used with caution (Trannum et al., 2010). WBM's consists of either fresh or salt water, the water contains a weighting agent (normally barite), clay or organic polymers and inorganic salts, inert solids and organic additives(Neff, 2005). The principal constituent of drilling mud is barite, About 90% of it is usually discharged after use (Olsgard and Gray, 1995). It is insoluble and settles on the seabed, therefore the effect of drill cuttings is more likely to be found on bottom-living communities(Olsgard and Gray, 1995). Although it is considered nontoxic to organisms, it can be present in the water column for a considerable period of time, and the fine-grained particles of the mud may therefore lead to the spreading of barite with ocean currents(Neff, 2005). OBM's mostly consist of a base oil(diesel oil or mineral oil), barite, clays, emulsifiers, water, calcium chloride, lime and other additives (Dardir and Abdou, 2013).

2.2 Drill Cuttings Release

When drill cuttings and water-based drilling muds are released to the marine environment, the largest particles and solids form a plume and that settles quickly accumulates on the sea floor. The fine-grained particles drift with prevailing currents away from where it was discharged and are diluted in receiving waters (Neff, 2005). For drill cuttings and water-based drilling mud discharged at or near the sea surface, the pollutants are diluted in the water column and settle as thin layers over a wider area of the sea floor. On the other hand, if discharged near the sea floor, they will accumulate and form high piles near the discharge pipes (Neff, 2005).

(Breuer et al., 1999) stated that about 75% of the drill cuttings discharged into the environment forms piles, and the rest is spread over large areas of the seafloor. It shows that thick layers of drill cuttings in the proximity of the wells, and a thinning wedge of drill cutting deposits further away from the well. Water based drill cutting piles on the seafloor are

characterized by poorly sorted variable mixture of clay, impoverished benthic communities associated with them.

In Table 1 an overview of the reported waste volumes is given:

Table 1: Development in amount of drilling waste, slop and oil contaminated mass given in tons (Ormeloh, 2014).

	2006	**2007**	**2008**	**2009**	**2010**	**2011**
Drilling waste onshore	100920	119396	129984	131348	231741	259010
Drilling waste injected	112638	103622	100927	93368	54376	25169
Slop treated onshore	7875	6783	8642	12695	19451	34338
Oil contaminated waste	1436	2399	2526	2479	2260	3266
Total	222869	232200	242079	239890	307828	321783

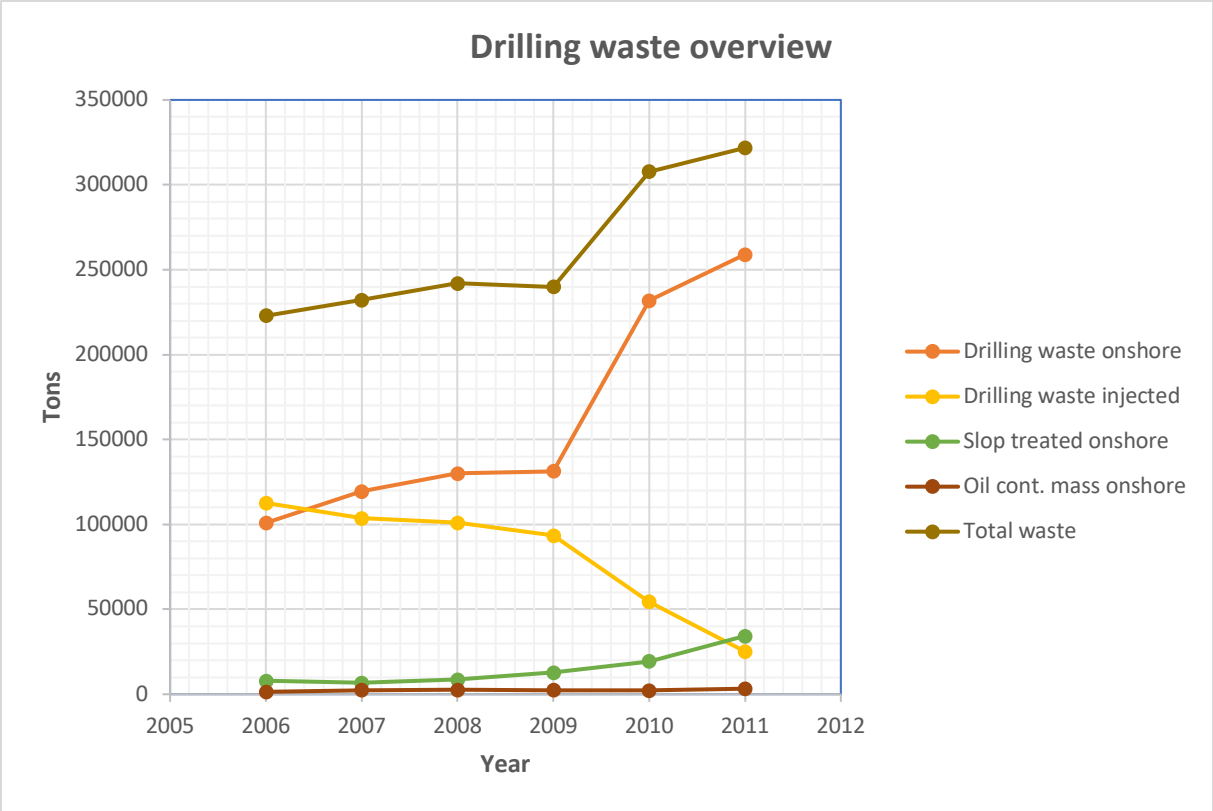

Figure 1: Amount of drilling waste, slop and oil contaminated mass (Ormeloh, 2014).

When the drill cuttings are deposited on the seafloor, they become more cemented and more resistant to erosion (Trannum et al., 2011). The physical features (size and shape) of drill cutting piles depend on the platform construction and location of the well, the rate at which the drill cuttings is being discharged, the kind of mud used in the drilling and the ocean currents affecting the sediments and cuttings deposited in the area (Trannum et al., 2011). All drill cutting piles differ each representing a mixture of contaminants, sediment composition and benthic community, and each pile is affected by the local hydrographic regime (Breuer et al., 1999).

2.3 Drill Cuttings Treatment Methods

2.3.1 Thermal treatment

Thermal desorption of drill cuttings was introduced to the oil industry in the early mid 1990's, following the successful treatment of contaminated soils from industrial activities. Since then, thermal desorption has evolved into an acceptable technology for treatment of

drilling wastes from both from onshore and offshore operations. Offshore discharge of oil based drilling waste without treatment is not acceptable because of the environmental impacts. (Murray et al., 2008)

In thermal desorption, energy is added to a body which leads to an increase in the temperature of the body which is above the boiling point of the volatile compounds in the body. By cooling the vapours, the volatile compounds can be collected and fractionated. In oil-based drilling waste the main constituents of the volatile compounds are the base oils and the water from the drilling fluid. This method has been used to separate drilling waste into reusable base oil and the residual oil content of the solids meeting environmental standards for disposal, thermal desorption has proven to be both environmentally and commercially acceptable. (Murray et al., 2008)

2.3.2 Thermo-mechanical Cuttings Cleaner treatment (TCC)

The TCC is a thermal desorption unit which separates the incoming waste into water, oil and solids (Thermtech, 2014). There has been emphasis on the weight of the module, since having a heavy module can add much weight to the platform, therefore the weight is not allowed to exceed the lift capacity of typical offshore cranes, TCC units are used both on-, and offshore to treat oil contaminated drill cuttings in several countries (Kirkness and Garrick, 2008).

The main use of the thermo-mechanical cuttings cleaner (TCC) is to convert hazardous oily cuttings into useful products. TCC facilities are only available onshore in Norway. However, offshore TCC units will in due time be introduced, negating the need for transport of cuttings to shore. Cuttings are allowed disposed to sea when no toxic fluids are attached. TCC separation is accomplished by generating temperatures of 240°C to 300°C sufficient for evaporation of oil and water. (Bilstad et al., 2013)

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TCC modifies the kinetic energy supplied by a drive unit into thermal energy through the development of friction in the mill (Thermtech, 2014). The drive unit is rotating the shaft on which hammer arms are mounted, the shaft is situated in a process chamber in which the waste is pumped, through fast rotation of the hammer arms the waste fed into the mill will be pressed towards the inner wall and heat is generated due to the friction between the waste particles (Murray et al., 2008).

The implied intense agitation in the process has two advantages (Murray et al., 2008). The retention time is greatly reduced since the solids are grinded and diffusion distance for the oil is reduced. This helps in relieving it of the capillary forces that binds the oil to the solid, Secondly compared to the atmospheric boiling point, the oil can be vaporized at a lower temperature since the laminar oil vapour layer around the oily solids is reduced and the surrounding vapour is dominated by super-heated water vapour (Kirkness and Garrick, 2008).

The recovered oil quality is depending on temperatures developed in the mill and processing time before the oil is leaving the system. If one of them is too high, the oil can be degraded. In the TCC the oil is subjected to high temperatures only for a couple of seconds before it is evaporated and leaves the system. In comparison to other thermal desorption technologies the TCC process temperature is moderate and the evaporation more gentle resulting in a high quality of the recovered oil. It is important to mention that the Flash Point of the oil is not altered by the treatment and will be the same in the recovered oil. This is important since oil with a lower flash point is more easily ignited. Therefore a decrease in the Flash point might have prevented the re-use of the recovered oil (Thermtech, 2014).

Mesocosm and bottle slurry experiments conducted by (Trannum et al., 2016) to assess and compare the effects of thermally treated drill cuttings (TCC) versus water based drilling mud (WBM) on benthic communities showed that in TCC treatment $CaCO_{3(s)}$ was

precipitated, this is produced by $Ca(OH)_{2(s)}$ in TCC. This caused the biodegradation product $\sum CO_2$ to be absorbed. The biodegradation product was released in WBM treatment. There was mass mortality and reduction in macro faunal biomass in TCC treatments but did not have an impact on faunal in WBM exposure. The adverse effect caused by TCC was conceivably by high alkaline conditions induced by CaO(s).

2.3.3 Microwave Heating

Microwave heating can treat oil contaminated drill cuttings (OCDC) such that the residual oil levels can be reduced below the current environmental discharge limits. A number of factors affects the amount of contaminant being extracted. These include microwave power, treatment time, sample mass and moisture content. In traditional heat processing, heat is transported to a material through conduction, convection and radiation. In microwave heating energy is delivered to a body through molecular interaction of the electromagnetic field. Here the internal temperature distribution of a material through subject to conventional heating is limited by its thermal conductivity (Shang et al., 2005).

There are three classifications for the behaviour conduct of components upon interaction with microwave field:

1. Transparent (low dielectric loss material): Microwaves pass through them with less absorption
2. Opaque (conductors): Microwave are reflected by the objects and do not pass through them
3. Absorbing (high dielectric loss material): Based on the strength of the electric field and dielectric loss factor the absorbance of the microwave energy is based on these(Meredith, 1998).

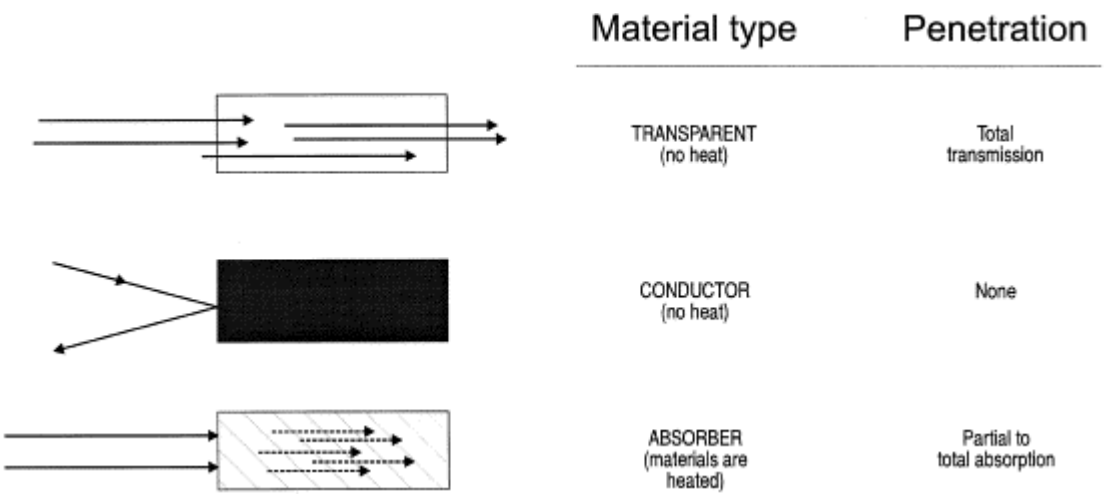


Figure 2: Interaction of microwave with materials (Haque, 1999).

Microwave analysis has some specific leverage in the treatment of materials which contain a mixture of absorbers and transparent components. Microwave energy is absorbed by the substances with a high dielectric loss while passing through the low loss transparent material, resulting in selective heating. In this case, significant energy savings are possible, since the dielectric material can be heated without heating the entire matrix (Clark et al., 2000).

Microwave energy also have some potential in mineral treatment and metal recovery operations such as heating, drying, carbothermic reduction of oxide minerals, leaching, roasting, smelting, pre-treatment of refractory gold ore and concentrate, spent carbon regeneration and waste management. However challenges remain to be overcome through a fundamental understanding of microwave interaction with minerals (Haque, 1999).

2.3.4 Stabilization/Solidification

Solidification refers to the approach that encapsulates the pollutant in a monolithic solid of high structural integrity. The encapsulation may be of fine waste particles (micro-encapsulation) or of a large block or container of wastes (macro-encapsulation), creation of a durable solid to encapsulate contaminants. In solidification it does not necessarily mean a chemical reaction between the wastes and the solidifying reagents but may mechanically glue the waste into the monolith. Pollutant movement is barred by vastly decreasing the surface

area exposed to leaching and/or by isolating the wastes within an impervious encapsulate. Stabilization refers to those approaches that lower the hazard potential of a pollutant by converting the contaminants into their least soluble, mobile or toxic form (Ball et al., 2012).

Stabilization and solidification are processes that combine, thereby changing both their physical and chemical structure, to ensure that the contaminants will remain in the matrix even if the monolith deteriorates (Ball et al., 2012). Stabilization/solidification is a generally endorsed waste management technology, which the USEPA (united states environmental protection agency) identified as the most outstanding demonstrated available technology for over 50 US Resource Conservation and Recovery Act—listed hazardous wastes (Chandler et al., 1997).

Stabilization and solidification is an inexpensive technology that can be applied quickly. The energy requirement is low if industrial by-product binders, such as pulverized fuel ash (PFA) or ground granulated blast furnace slag (GGBS) are used (Wiles, 1987). Depending on the nature of the resulting stabilization and solidification output, there is the possibility of reusing it as a construction material (Chandler et al., 1997). However its efficacy in the treatment of waste containing large amounts of organic compounds is debatable because of the harmful effects that organic compounds can have on the hydration of binders (Trussell and Spence, 1994).

Moreover, there is little possibility for the chemical uptake of organic contaminants into hydration products. Any incapacitation of organic contaminants will be as a result of the physical entrapment in the matrix porosity. Sorption, such as non-polar (insoluble) compounds are more likely to be retained by the solid, whereas polar (soluble) compounds will remain leachable. Limited publication exists on the use of stabilization and solidification in the analysis of drill cuttings (Leonard and Stegemann, 2010). In a work where petroleum drill cuttings were analyzed with cement, lime and pulverized fuel ash (PFA) which gives a better and improved unconfined compressive strength the leaching of chlorides was not noticed (Tuncan et al., 2000).

2.3.5 Incineration

Incineration describes the oxidation or combustion of organic components of waste. One example is the use of rotary kilns where the drilling waste is treated at temperatures between 1200°C and 1500°C resulting in a material which is less harmful (Ifeadi and MNSE,

2004). On the contrary, incineration is not suitable for the treatment of inorganic components of waste like metals which will only oxidize and leave the process as ash or vapor, the ash needs to be disposed in a prudent manner while the metals can be removed from the vapors by air pollution control equipment prior to discharge, it is considered to be a robust treatment for drilling cuttings (Ormeloh, 2014).

However, "slurrified cuttings" that shall be incinerated will require additional energy supply. On top of that it is a very energy intensive treatment option where only a part of the heat energy can be recovered for other purposes and a high amount of CO_2 and NOx is generated (Thermtech, 2014).

2.3.6 Bioremediation

Bioremediation can be defined as any process that make use of microorganism such as fungi, bacteria etc to biologically degrade contaminated soil and waste into non-toxic residue. This is a natural process where organics are being degraded naturally into a non-toxic form. It's been reported that the process of bioremediation generate some greenhouse gases such as methane from the anaerobic processes of the microorganism (Diplock et al., 2009).

Biosurfactants are microbial products that can reduce surface tension at air–water interface these are preferred than the chemically induced surfactants because biosurfactants easily produced from renewable resources, less toxic, good biodegradability, excellent surface activity, great environmental compatibility, and high activity at extreme environmental conditions (Urum and Pekdemir, 2004).

As a biological process, the degree of bioremediation is dependent upon the environment in which bioremediation takes place, the composition of the organic contents to be degraded and the type of treatment used. Bioremediation can be a fast process (requiring weeks for completion) when conditions are favorable or be a relatively slow process, requiring months or years to reach the desired result. Environmental factors such as nutrient availability, micro-organisms present in the soil and aerobic conditions are all factors that play a role in achieving bioremediation (Vidali, 2001).

Oil contaminated drill cuttings and crude oil contaminated soil were firstly characterized using gas chromatography and mass spectrometry, to identify the crude present. The contaminated soil and oil contaminated drill cuttings were treated by enhanced biodegradation in industrialized scaled experiments. After a year of bioremediation process,

the removal of hydrocarbons reached by biodegradation an extent of 60% but there was an increase in the concentration of N-urea which has high detrimental effects on the hydrocarbon degrading fungal populations due to the production of toxic concentration of ammonia gas by nitrification. The saturated hydrocarbons were extensively assimilated; aromatic hydrocarbons were less degraded than saturated (Chaillan et al., 2006).

Roughly, 83% of oil-based drill cuttings can be removed when washed with a rhamnolipid (produced by *Pseudomonas aeruginosa*) when subjected to bioremediation in a stainless-steel box with sawdust as a bulking agent. These depends on a number of factors such as washing time, stirring speed, rhamnolipid concentration and liquid/solid ratio the organics reduced from 85,000 mg/kg to 12,000mg/kg after 120days. (Yan et al., 2011)

Studies done by (Kogbara et al., 2016) compared the effectiveness of bio augmentation, bio stimulation as well as the combination of both supplemented with phytoremediation in the degradation of drill cuttings. The bioremediation processes were treated with three different options, option A was treated with bio augmentation supplemented with phytoremediation, option B was treated with bio stimulation and bio augmentation supplemented with phytoremediation, option C was treated with bio stimulation supplemented with phytoremediation, and option O had drill cuttings and soil mixture served as control. Fertilizer tillage and water was added to option B mushroom substrate and elephant grass was used to option A and C respectively. The samples were observed for total petroleum hydrocarbon (TPH), organic carbon (OC), total nitrogen (TN), pH, metal concentration and fungal count. After 56 days, an initial concentration of 4,114 milligram per kilogram for TPH reduced by 5.5%, 68.3%, 75.6%, and 48% in option O, A, B and C respectively. Bio stimulation and bio augmentation supplemented with phytoremediation is more effective in the degradation of TBH. The treatment options reduced metal concentrations ranging from 0% to 16% only with an initial low metal concentration.

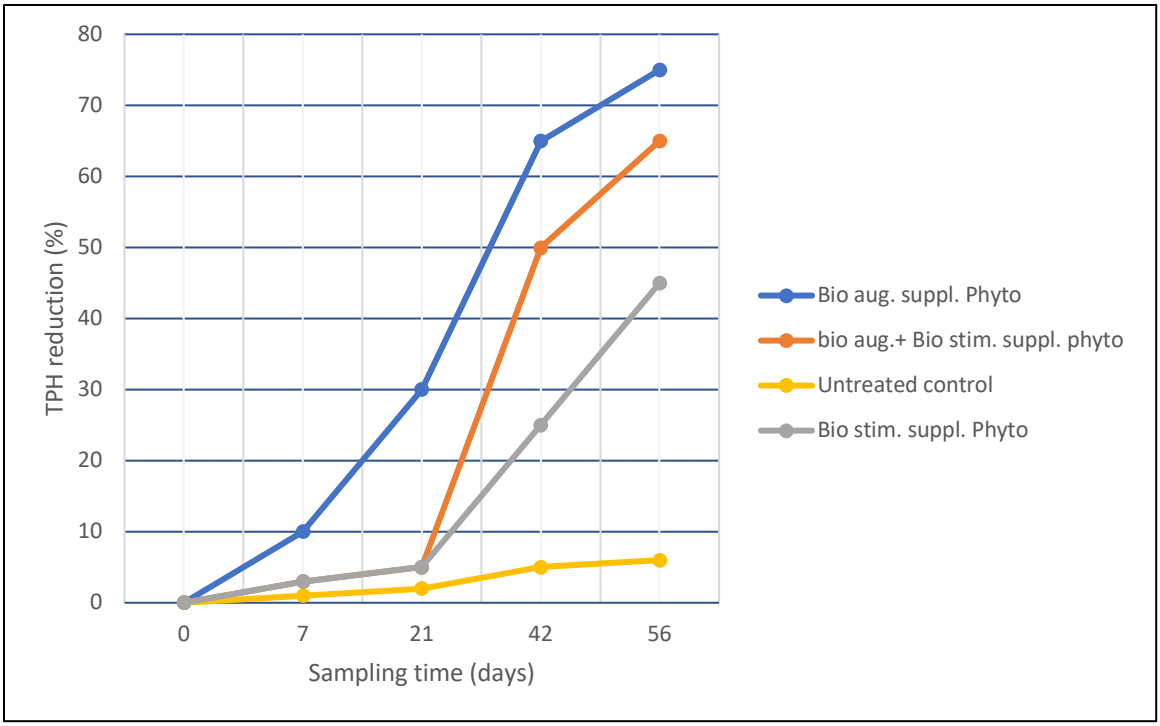


Figure 3: Percentage of TPH reduction in different treatments (Kogbara et al., 2016).

2.4 Effect of Drill cuttings and Drill fluid on Community

It is imperative to check the effect of treated drill cuttings and drill fluids fauna and the ecosystem. Water-based fluids are generally considered safe for discharge or assumed to have little effects on the benthos. Some studies have been done to investigate the physical disturbance water-based drill cuttings have on benthic ecosystems. When natural sediment particles and water-based drill cuttings were added to a benthic community for a period of six months, some changes were observed such as significant reduction in number of taxa, abundance, biomass and diversity of macrofauna with increasing thickness of drill cuttings, which was not observed for the natural sediment particles. It also increased the rate of oxygen consumption and also penetration of oxygen (Trannum et al., 2010).

Some studies were conducted to test the effect of discharged drill cuttings on macrozoobenthos around several locations in the Dutch sector of the North Sea since 1985. 11 surveys were done in sites contaminated with oil-based fluids (OBFs) and 4 surveys on water-based fluids (WBFs), the densities of the species were inspected for consistent spatial abundance of individual species around the sites. 15 of the species that were abundant before the contamination showed reduced abundance near the OBFs sites, these species are now important bio-indicators in the Dutch sector of the North Sea. There were no noticeable

changes close to the WBFs sites. The opportunist polychaeta *Capitella capitata* which is used to measure quality and presence of organics showed in increased number near OBFs locations (Daan et al., 1994).

Impacts of oil industry activities and pollution have commonly been measured by assessing changes in species assemblages of micro fauna in response to pollution (Gray et al., 1990). Drill cuttings and drilling fluids can induce stress on prokaryotes (Griffin and Calder, 1977).

Prokaryotic cells such as bacteria can be used to bio-monitor how good and efficient the treatment of "clean-cut" treatment is, as an index of community and ecological activity disturbances. By measuring the CO_2 production and the isotope of CO_2 of phospholipid fatty acids the bioavailability of hydrocarbons in the oil contaminated drill cuttings can be determined (Main, 2015).

2.5 Impacts on benthic fauna and foraminifera

Due to environmental laws and regulations the discharges from offshore petroleum activities to the marine environment have been greatly reduced as compared to the situation 15 years back (Hess et al., 2013). Even though these laws and regulations have decrease the spread of pollutant and greatly improve water quality, the reduced and controlled discharge of contaminated drill cuttings into the sea still have impact on the marine environment (Hess et al., 2013). Earlier work have shown that benthic fauna are affected and have their composition changed by the discharge of drill cuttings into the sea (Hess et al., 2013).

Benthic faunas can be affected in three ways; by covering and burying the organism directly, by affecting the surrounding organisms due to the discharged substances, and when the microbes degrade the organic compounds in the drill cuttings leading to a depleted level of dissolved oxygen (Ball et al., 2012). The most affected areas are identified by a lower diversity of fauna and the appearance and dominance of opportunistic species (Schaanning et al., 2008). The nutrients in the area can serve as food for the opportunistic species, the opportunistic species might thrive in the habitat due to reduced competition and less predation (Alve, 1995). The diversity of fauna might be the same further away from the discharge area (Davies et al., 1984).

Even after drill cuttings deposits have ceased the affected faunas have shown that drill cuttings had a negative effect on the benthic community effects after several years (Olsgard

and Gray, 1995). The benthic communities living in the sediment can be harmed if the concentrations of toxins are high (Frontalini and Coccioni, 2011).

2.6 Extraction and purification of DNA from soil samples

Most scientist have agreed that majority of the bacteria in environmental samples cannot be cultured using only classical cultural techniques (Amann et al., 1995). The ratio (proportion) of bacteria that can be cultured from natural habitats ranges from 0.001% in seawater to 0.3% in soil (Roose-Amsaleg et al., 2001). Hence, there is a need to study microbial communities using culture-independent methods. These methods, instead of relying on obtaining pure cultures first, require only the extraction and analysis of biomolecular components (e.g. DNA, RNA, proteins, lipids) of the cells. Most commonly, DNA-based methods are used.

There are two methods of extracting DNA: cell extraction methods and cell lysis methods (Torsvik et al., 1990, Holben et al., 1988, Steffan and Atlas, 1988). Cell extraction isolation depends on the separation of microbial cells from their environmental matrix before lysis to release DNA (Roose-Amsaleg et al., 2001). Some major constraints of the cell extraction method is that it requires a lot of time to process and only a few samples can be processed concurrently (Steffan and Atlas, 1988). Direct cell lysis is preferred than the cell extraction methods because of their better recovery (Roose-Amsaleg et al., 2001).

The yields with classical cell extraction range from 30-50% for various peat soils, 20-30% for clay-loam or forest soils (Steffan and Atlas, 1988, Bakken and Lindahl, 1995). The major drawback of direct cell lysis is that the method also extracts impurities such as humic and fulvic acids which are inhibitors to polymerase chain reaction (PCR) (Steffan and Atlas, 1988, Tsai and Olson, 1992, Tebbe and Vahjen, 1993) therefore purification is required (Roose-Amsaleg et al., 2001). Figure 4 shows the steps of direct cell lysis and cell extraction methods.

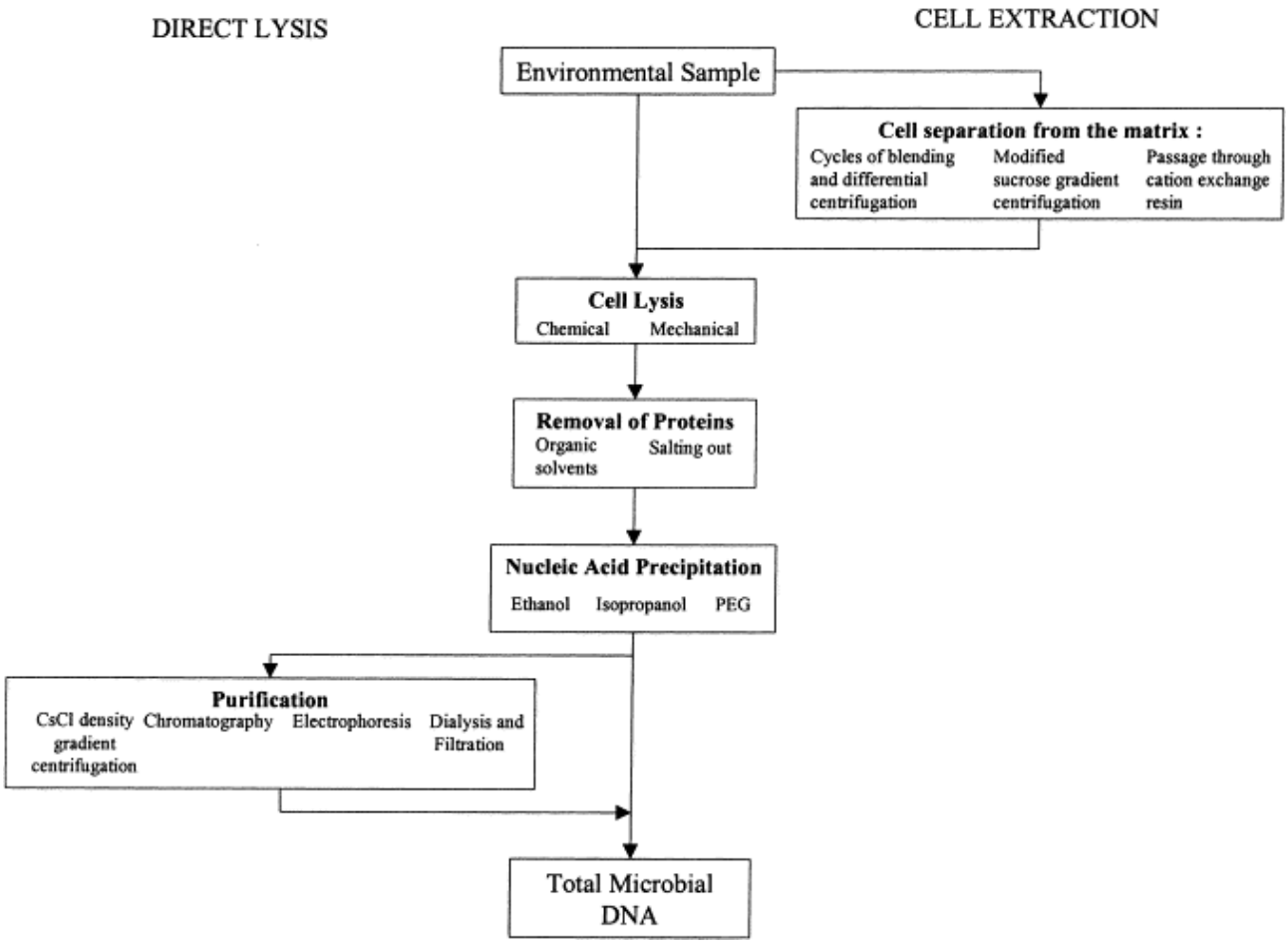


Figure 4: Protocols for extraction and purification of microbial DNA from environmental samples (Roose-Amsaleg et al., 2001).

Cell lysis methods aims to release the DNA by breaking the cell wall and membrane of the microorganism (Roose-Amsaleg et al., 2001). It is challenging to purify microbial DNA from soil than from other environments such as water (Pan et al., 2013). Humic acids is difficult to remove , making DNA purification a demanding process (Tebbe and Vahjen, 1993).

A lot of work have been done on the purification of DNA using electrophoresis, notably to remove the impurity humic acid (Pitcher et al., 1989, Hilger and Myrold, 1991, Van Elsas et al., 1991). Low melting agarose gel is used more than standard agarose because low meting gels containing DNA can be melted at low temperatures (30-40°C) and can be used for polymerase chain reaction (PCR) without any further step (Herrick et al., 1993, Young et al., 1993).

2.7 Polymerase chain reaction (PCR)

PCR is a technique used in amplifying one or a few copies of double stranded DNA (Bessesen et al., 1990, Bej et al., 1990). The application of PCR amplification have found increasing interest in environmental microbiology (Josephson et al., 1991, Bej et al., 1991). The potential of small amount of target organisms in environmental samples being detected makes this technique significant (Josephson et al., 1991). A typical PCR process consists of 20-40 cycles in which temperature changes, the applied temperature at each step and the length of time applied on each cycle depends on different parameters e.g. enzymes used for the synthesis, melting temperature of primers (Badr, 2008).

PCR first starts with initialization which does a heat activation by hot-start PCR for DNA polymerase (Khanna and Stotzky, 1990). This occurs at an optimum temperature of 98°C, depending on how thermostable the DNA polymerase is, it is held for 1-10 minutes (Badr, 2008). The next is denaturation which is the first regular cycling step (Khanna and Stotzky, 1990). It occurs at a temperature of 94-98°C for 20-30 seconds (Roose-Amsaleg et al., 2001). In this step the double stranded DNA is melted by breaking the hydrogen bonds between complementary bases producing single stranded DNA molecules (Pan et al., 2013). These has to be for a short period so as not to break the phosphodiester bonds (Steffan and Atlas, 1988). Annealing comes after denaturation which basically means hybridization of the primers on the two separated DNA strands (Roose-Amsaleg et al., 2001). Primers are small fragments of DNA, which initiates DNA polymerase (Pan et al., 2013). They start initiation always at the 3' of the parent strand. Annealing takes place at a lower temperature 50-65°C for 35-45 seconds (Muyzer and Smalla, 1998). It is very important to determine a proper temperature in this step because efficiency and specificity are greatly affected by annealing temperature (Roose-Amsaleg et al., 2001). This temperature has to be low enough to allow hybridization of the primer to the strand, with high temperature the primer might not bind at all (Badr, 2008). The next step which is extension is to make a complete copy of template, the DNA nucleotides binds with the primer (Muyzer et al., 1993). An enzyme taq polymerase binds the primer with DNA nucleotides to make the new copy of DNA (Badr, 2008).

At the end of 30 cycles one billion copies of a single DNA can be duplicated (Badr, 2008). The temperature used depends on the DNA polymerase used (Holben et al., 1988). The process of denaturation, annealing and elongation constitute a single cycle, a lot of cycles is required to amplify the DNA target to millions of copies (Chien et al., 1976). Next is final

elongation which occurs at a temperature of 70-74°C for 5-15 minutes, this ensures any single stranded DNA is fully elongated (Holben et al., 1988). The final step is to cool down the reaction to 4-15°C for an indefinite time (Badr, 2008).

2.8 Denaturing gradient gel electrophoresis (DGGE)

This is a technique used to separate DNA fragments of the same size with different sequences based on their melting characteristics (Muyzer and Smalla, 1998). DNA fragments of the same length but with different sequences can be separated (Muyzer and Smalla, 1998, Fischer and Lerman, 1979). The separation is based on the reduced electrophoretic movement of a partly melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturant (Muyzer and Smalla, 1998, Pan et al., 2013).

DNA fragments have their melting temperature based on their nucleotide sequence, their melting occurs in a distinct fashion so called *melting domains:* a change of a helical to a partly melted molecule occurs and the movement of the molecule stops when the domain with the lowest melting temperature reaches its melting point (Muyzer and Smalla, 1998, Muyzer et al., 1993). GC (guanine-cytosine) bindings are stronger than AT bindings (adenine-thymine) because GC bindings have 3 hydrogen bonds while AT bindings have 2 hydrogen bonds therefore GC rich sequences have a high melting point (Muyzer et al., 1993). 50% of sequence variants in DNA fragments of up to 500bp can be detected by using DGGE (Myers et al., 1985). DGGE uses an extra sequence called GC clamps which prevents the complete separation of the DNA strand, these clamps can be used to increase the detection of sequence variants of DNA fragments close to 100% (Muyzer and Smalla, 1998, Myers et al., 1985, Sheffield et al., 1989).

DGGE uses a denaturant gradient made up of urea and formamide which triggers the DNA to denature. DNA bands in DGGE can be viewed using ethidium bromide, SYBR Green I is used in place of ethidium bromide as it is discovered to be carcinogenic. Before DGGE analysis of DNA fragments it is important to know the melting behavior of the DNA fragments. (Muyzer and Smalla, 1998)

PCR products obtained from different bacteria present in a sample and then subsequently separated by DGGE, the result obtained gives patterns of bands, for which the

bands represent the number of predominant members in the in the microbial communities (Muyzer et al., 1993, Muyzer and Smalla, 1998).

Stratified water column of Mariager fjord in Denmark was studied using DGGE analysis of 16s rDNA fragment to examine the presence and action of sulfate reducing bacteria (Teske et al., 1996) the idea was that amplified PCR product would establish the presence of different bacteria populations i.e. biodiversity, the bacteria population that is active is attained after the amplification of ribosomal DNA (rDNA) (Muyzer and Smalla, 1998). DGGE can be used to study community changes by using many samples taken at different time intervals, this makes the technique a powerful means for monitoring community behavior after environmental changes (Myers et al., 1985). The separation of DNA fragments which have certain amount of sequence variation have been demonstrated that it is not always possible (Vallaeys et al., 1997, Muyzer and Smalla, 1998).

3.0 MATERIALS AND METHODS

The experiment included four treatments, S10 (microwave treated drill cuttings), OBM (oil-based cuttings), SD (sediment) and CN (control). OBM drill cuttings were delivered by Norwegian Technology AS as mud, S10 were microwave treated OBM samples of the same batch delivered by the Norwegian Technology AS as dry matter, SD was collected on the 14th November 2017, from Boknafjord at a water depth of 134 meters.

3.1 Experimental set up

3.1.1 Rinsing of S10 sample

The S10 sample was rinsed to reduce the concentration of the treated cuttings added to the benthic community. It is practical to reduce the concentration of S10 cuttings as the benthic community do not come in contact with very high concentrations of cuttings in the environment. An apparatus designed by Dr. Leon Moodley was used to achieve this.

Figure 5: S10 rinsing.

As shown in figure 5 the large cylinder is filled with unfiltered sea water, S10 drill cuttings are carefully poured from the top of the cylinder and allowed to sediment in 4 hours. These is done in four cycles.

This is an inexpensive method to reduce the concentration of the S10 cuttings before adding to the benthic community. A better but expensive way is by pouring large amount of drill cuttings in the ocean then using an ROV (remotely operated under water vehicle) to collect the sediment in the area (Alberty et al., 1997, Breuer et al., 2002).

On the 19th of February 2018, 90 grams of S10 cuttings and sea water was homogenized in a beaker with the aid of a spoon and poured into the cylinder. After 20 minutes, the thickness of S10 sediment at the bottom was approximately 2mm, this shows a high sedimentation rate of S10. After 4 hours the S10 sediment at the bottom is carefully removed undisturbed first by opening a valve at the bottom of the cylinder this removes all the supernatant then transferred into a beaker and poured into the cylinder to complete a cycle. Some of the supernatant is saved for salinity, pH measurements and filter paper test, the pH and salinity are measured after ever cycle. S10 sample was then stored at 8°C prior to the addition on sediment.

The salinity of the sea water and supernatant was measured with a refractometer. The salinity of sea water used, and the supernatant was constant throughout at 34‰.

Table 2: pH of sea water and supernatant after rinsing

	Sea water pH	S10 pH
1st rinse	8.176	8.102
	8.138	8.100
Average	**8.157**	**8.101**
2nd rinse	8.142	8.122
	8.133	8.133
Average	**8.138**	**8.128**
3rd rinse	8.159	8.111
	8.186	8.104

Average	**8.173**	**8.108**
4th rinse	8.139	8.115
	8.165	8.107
Average	**8.152**	**8.111**

Table 2 shows the pH of sea water measured before every rinse and the supernatant pH after the rinse. There was an average increase of 0.043 between the pH of sea water and the supernatant after every rinse.

Sediment is lost during the rinsing, it is important to know the rate at which sediment is lost after every rinse. After every rinse 100ml of the supernatant was collected in two different flasks and poured on a Whatman® GF/F 47mm Ø filter. The filters were kept overnight in an oven at 100°C and their respective weights measured.

W1a: weight of filter 1 before drying (grams)

W1b: weight of filter 1 after drying (grams)

W2a: weight of filter 2 before drying (grams)

W2b: weight of filter 2 after drying (grams)

Wrt: rate of loss of sediment in the rinse (gram per milliliters)

Table 3: Rate of sediment loss and weights of filters

	W1a	**W1b**	**W2a**	**W2b**	**Wrt $*10^{-2}$**
1st rinse	0.1301	0.1546	0.1309	0.1595	0.0270
2nd rinse	0.1316	0.1614	0.1305	0.1576	0.0280
3rd rinse	0.1304	0.1608	0.1330	0.1554	0.0260
4th rinse	0.1304	0.1570	0.1309	0.1580	0.0270

The weight of the filter is subtracted from the weight of the filter after drying to get the weight of the sediment. An average weight of sediment loss is calculated after every rinse, the average is divided with the volume of water used (100 ml) to get the rate at which sediment is lost after every rinse. An average of 0.027 g/ml sediment was lost after every rinse of the S10 drill cuttings.

3.2 Treatment and addition of test material

Cores with SD with an average thickness 11mm labelled F1, F02, F10, F12, F14, F16, and F20 are kept cool (8°C), almost dark and supplied with unfiltered sea water with a flowrate of 55-60 ml/min. Benthic communities can be maintained under these conditions for several months (Schaanning et al., 2008).

15 ml of rinsed S10 drill cuttings was added to F12 and F14 each on the 22nd February 2018, and 15 ml of OBM was added each to F10, F16, and F20 on the 23rd February 2018. All treatments were left undisturbed for a day before the connection of unfiltered sea water was reconnected. F01 and F02 represent CN (control) with SD without any addition/treatment.

3.3 Termination Period

On the 21st of March 2018, approximately a month of incubation of the treatments the supply of unfiltered sea water was stopped and the water in the cores was siphoned out carefully so as not to "disturb" the treatment. First water was removed with a syringe and then with the aid of a tissue so as not to lose sediment, figure 6 shows before water was siphoned out and figure 7 shows after water was removed. The two-distinct layer which can be seen in both figure 6 and 7 is carefully separated with the aid of a spoon and labelled upper layer (UPL) and lower layer (LL) for each core, one reason for separating the cores into layers is to detect the penetrating rates of the treatments in the sediment. Each layer was homogenized properly, then approximately 250 mg was weighed for DNA extraction, polymerase chain reaction (PCR), and denaturing gradient gel electrophoresis (DGGE) this was stored at -80°C prior to the experiments. The remaining sediment after 250 mg was removed was used for faunal analysis.

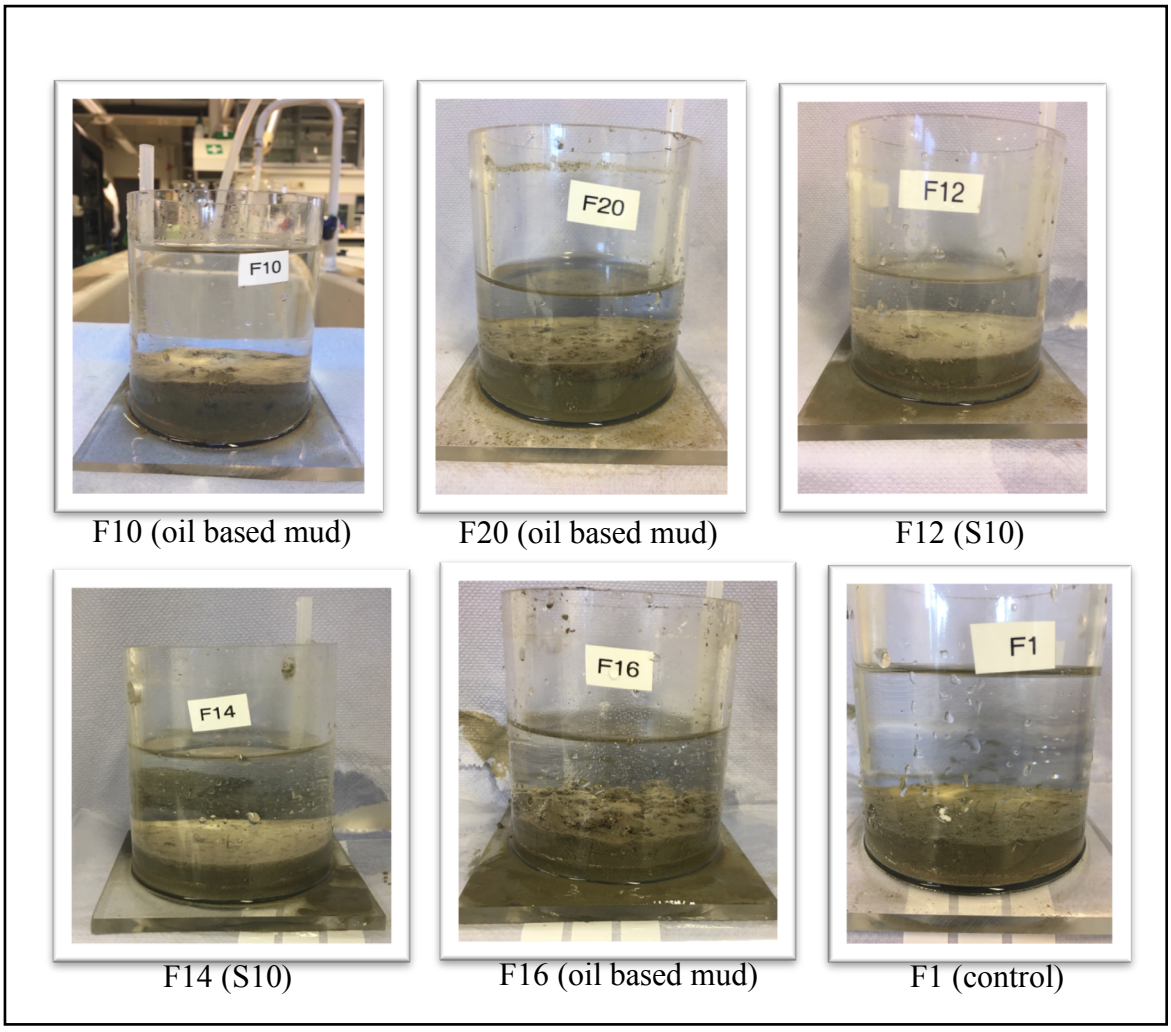
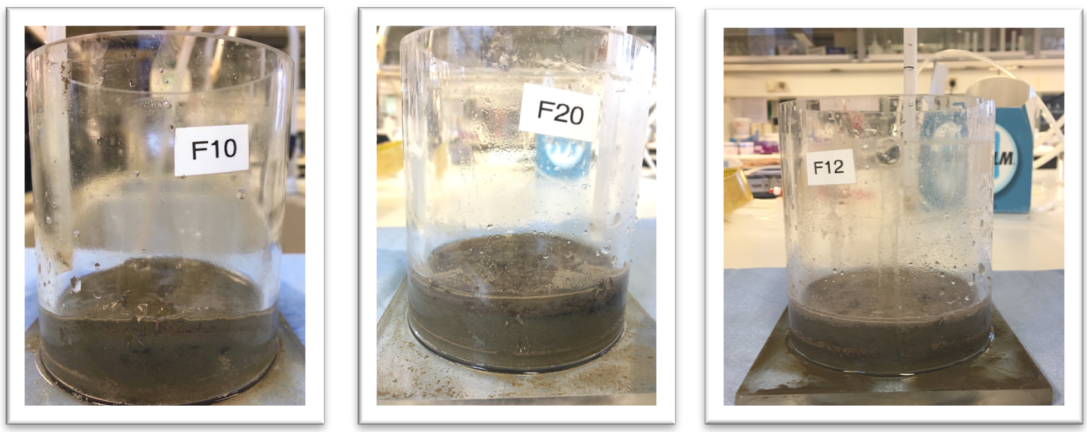


Figure 6: Treatment CORE's with sea water showing two distinct layers.



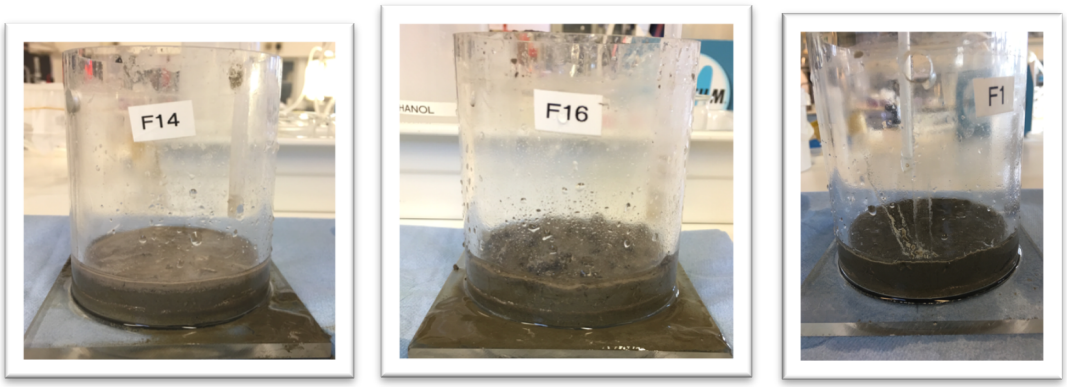


Figure 7: Treatment CORE's without sea water showing two distinct layers.

As water was removed all the OBM treatments had a distinct "oily" smell, there were visible black areas/patches in all the OBM treatment. This are anoxic regions created as a result of the treatment. The reason could be as a result of OBM cuttings adding organics in the sediment which increases the rate of biodegradation by the microorganisms and hence increases the rate at which oxygen is consumed or could also be as a result of less bioturbation from the animals which were affected. This can also be sulphate reducing bacteria oxidizing carbon and producing a precipitate ferrous sulphide (FeS) as a result of the degradable organics present in the OBM treatment (Schaanning et al., 2008).

On the other hand, all S10 treatments did not have any visible dark areas/patches. They however had a distinct "chemical" smell and changed the sediment color to light grey.

3.4 PCR-DGGE analysis of sediment bacterial communities

3.4.1 DNA Extraction

The DNeasy PowerLyzer PowerSoil® kit was used for the extraction of DNA from 250mg of sediment samples which were stored at -80°C until processing. All the procedures were followed according to the manufacturer's instructions up until the elution step, where 100 µl Milli-Q water was used instead of C6 solution. After extraction the concentration and purity of each sample was measured using a spectrophotometer (Nanodrop, Thermos Fisher). Integrity of the total community DNA was then assessed using a 2% of agarose gel. This was made from 1 gram of agarose in 50 ml running buffer (Tris-Acetate EDTA) to further evaluate the concentration of DNA in the samples.

A commercially available 50× concentrate of TAE (VWR) was used to prepare 1× TAE (40 mM Tris, 20 mM Acetate and 1 mM EDTA, pH 8.6) by diluting it with Milli-Q water.

3.4.2 Agarose gel electrophoresis

3.4.2.1 Preparation of 2% agarose gel

- 1 gram of agarose is measured in an Erlenmeyer flask.
- 50 ml of TAE buffer is poured into the agarose containing Erlenmeyer flask.
- The mixture is heated in a microwave until all the agarose is completely dissolved and allowed to cool to a temperature approximately 60°C.
- 5 µl of gel green is added into the Erlenmeyer flask.
- The flask is swirled gently to mix.
- The agarose is poured into the gel casting tray with the comb in place and allowed to polymerize for at least 30 minutes.

3.4.2.2 Preparation of DNA for the gel electrophoresis chamber

- TAE buffer is poured into the gel electrophoresis chamber making sure the level is above the agarose gel but below the maximum; the comb is removed gently making sure the gel does not break.
- On the first well and last well on the agarose gel 6 µl of Trackit® 100bp DNA ladder (Invitrogen) is carefully loaded with the aid of a pipette.
- 3 µl of Orange G loading dye (6X, VWR) buffer with 5 µl of sample DNA each is loaded into the well making a total of 14 wells with the first and last ladder wells.
- The agarose gel is run for 90 minutes at 60 volts in the gel electrophoresis machine as shown in figure 8.
- The gel image was taken using a BioRad GelDoc XR+ system and is shown in figure 13.

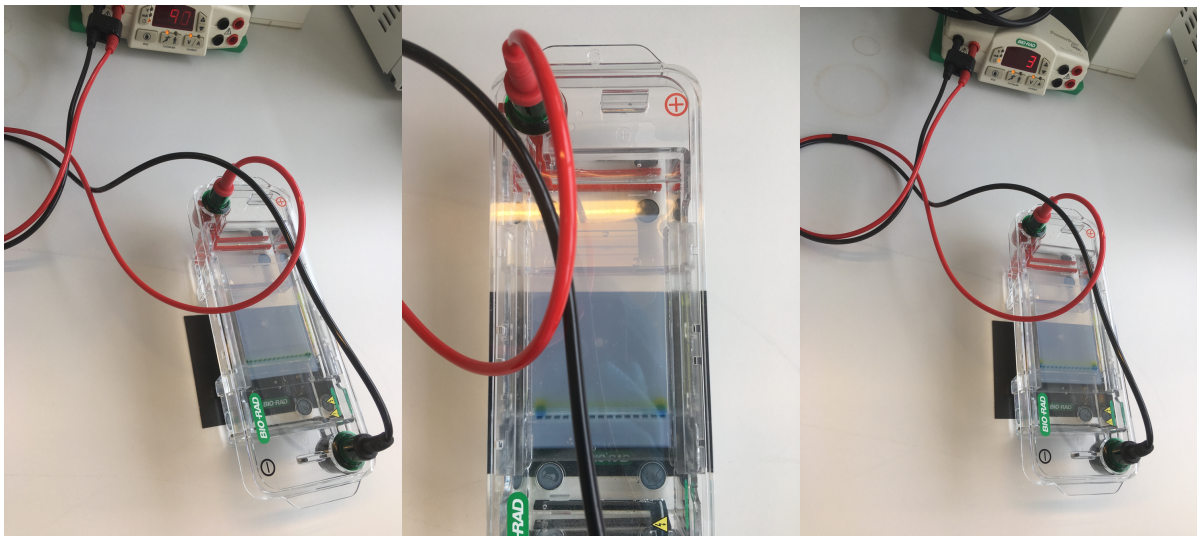


Figure 8: Agarose gel in gel electrophoresis chamber run at 60 volts for 90 minutes.

3.4.3 Polymerase chain reaction (PCR)

PCR reaction setup

Chemicals used with volume	
Dream taq hot start green PCR master mix	25 µl
Forward primer 341fGC	5 µl
Reverse primer 907r	5 µl
Template DNA	1 or 2 µl
Molecular grade water (Sigma Aldrich).	Up to 50 µl

A negative control (F13) was added;

F13: 25µl Dream taq hot start green PCR master mix + 5 µl of 341fGC forward primer + 5 µl 907r reverse primer + 15 µl milli-Q water

Table 4: PCR program

Step	Temperature (°C)	Time (minutes)	No of cycles
Initial denaturation	95	3.00	

Denaturation	95	0.50	30 cycles
Annealing	56	0.75	
Extension	72	1.00	
Final extension	72	15.00	

The PCR mix was run in the thermal cycler (Applied Biosystems) using the PCR program shown in table 4. Agarose gel was made using the procedure already described and placed in the gel electrophoresis machine containing TAE buffer to check the PCR products. In the first and last well, 6 µl of Trackit™ DNA ladder was loaded with the aid of a pipette, 5 µl of PCR mix sample are loaded into the wells making 15 wells in total with the negative control. It is not required to add loading buffer to the PCR mix samples as the Dream taq hot start green PCR master mix contains loading buffer. The gel electrophoresis machine was run for 90 minutes at 60 volts, the gel image was taken using a BioRad GelDoc XR+ system as seen in figure 14.

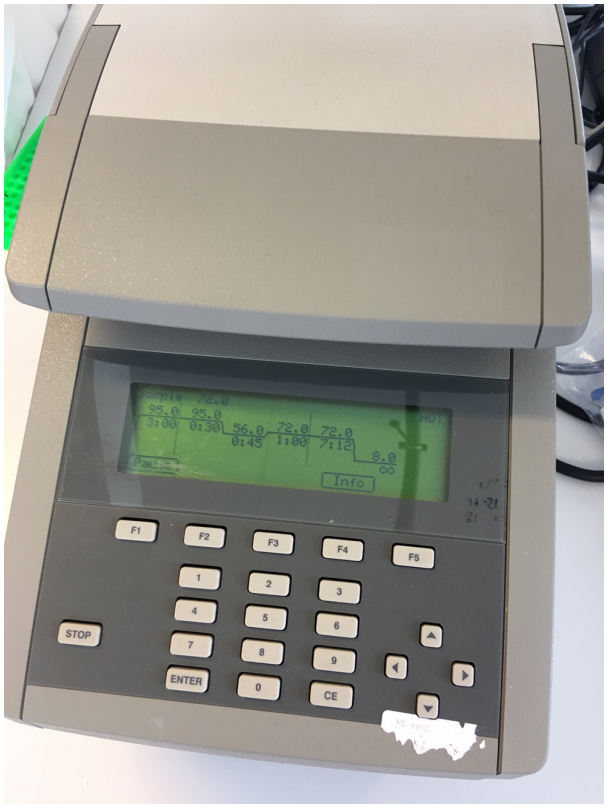


Figure 9: Thermal cycler showing PCR program.

3.4.4 Denaturing gradient gel electrophoresis (DGGE)

A commercially available 50× concentrated TAE buffer (VWR) was diluted using milli-Q water to obtain 7 L of 1× TAE running buffer.

3.4.4.1 Preparation of stock solutions

To make 50 ml of 8% acrylamide gel, the chemicals listed in Table 5 were mixed.

Table 5: Reagents added to make stock solutions

	0% Stock Solution	**80% Stock Solution**
Urea	-	16.8 grams
Acrylamide bis	10 ml	10 ml
50 × TAE buffer	1 ml	1 ml
Deionized formamide	-	16 ml
H_20 (milli-Q)	Fill greiner tube to 50 ml	Fill greiner tube to 50 ml

- 50 ml greiner tubes and aluminum foil were used, aluminum foil is used to cover the tubes to protect from light.
- Urea is first dissolved with approximately 5 ml of milli-Q water and vortexed to make sure all the urea is dissolved.
- Other reagents are added except milli-Q water which is added last. The mixture is a vortexed again to make sure all reagents are dissolved.
- Milli-Q water is added to the greiner tubes to make a total volume of 50 ml.

3.4.4.2 Preparation of working solutions from stock solutions

Reagents used:

0% stock solution

80% stock solution

N,N,N',N'-Tetramethylethy (TEMED)

Ammonium Persulfate (APS) 10%

Table 6: Reagents added to make working solutions

	20% working solution	**80% working solution**
0% stock solution	9 ml	-
80% stock solution	3 ml	12 ml
TEMED	24 µl	240 µl
APS 10%	75 µl	75 µl

- The reagents are mixed with APS as the last so as not to start the polymerization process (Zwart and Bok, 2004).

3.4.4.3 Assembling gel chamber

- The glass plates are cleaned with detergent (e.g. decon 90) to remove impurities.
- The gel chamber is assembled as shown in figure 10

Figure 10: Assembly of gel chamber with pump and stirring bean.

3.4.4.4 Casting the gel

- The 80% working solution is poured in the right leg of the stirring bean and the 0% working solution is poured on the left leg of the stirring bean.
- The pump is turned on starting from a low flowrate making sure the two working solutions is properly mixed as it flows into the gel chamber through the tubing.
- The gel chamber fills slowly, the flow is stopped when the level of acrylamide reaches approximately 0.5cm below the comb level (Zwart and Bok, 2004).
- Milli-Q water is used to rinse the tubing so as to avoid damaging the tubing through blockage after polymerization of acrylamide solution.
- The comb is placed on the gel chamber, a mixture of 5 ml of 0 %, 5 µl of TEMED, 50 µl APS is loaded into the gel chamber with the aid of a pipette.
- The gel chamber is covered with aluminum foil to protect from light and allowed to polymerize for 2 hours.

3.4.4.5 Heating of TAE buffer

- The gel electrophoresis tank is filled until with the 7 L mark with TAE buffer.

- The TAE buffer is heated to 60°C as shown in figure 11

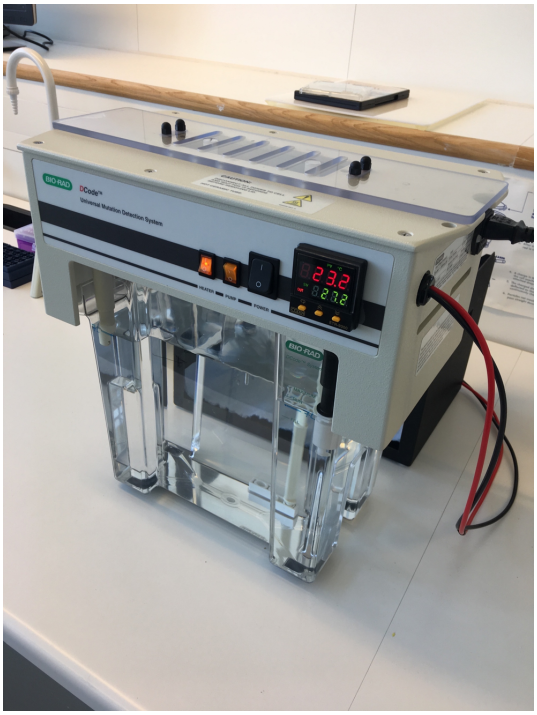


Figure 11: TAE buffer heated to 60°C in electrophoresis machine.

3.4.4.6 Running gel in electrophoresis machine

- The gel chamber with the solidified gel is placed into the gel electrophoresis machine.
- With the aid of a pipette the wells are flushed with TAE buffer to remove excess acrylamide solution in the wells.
- 30 µl of the PCR mix samples are loaded in the wells with 20 µl of the negative control as the last well.
- The gel electrophoresis machine is run for 20 minutes without turning the pump on, and then for 18 hours at 60 V with the pump turned on.
- As can be seen in figure 9 the samples in the well start migrating on the gel showing three different colors.

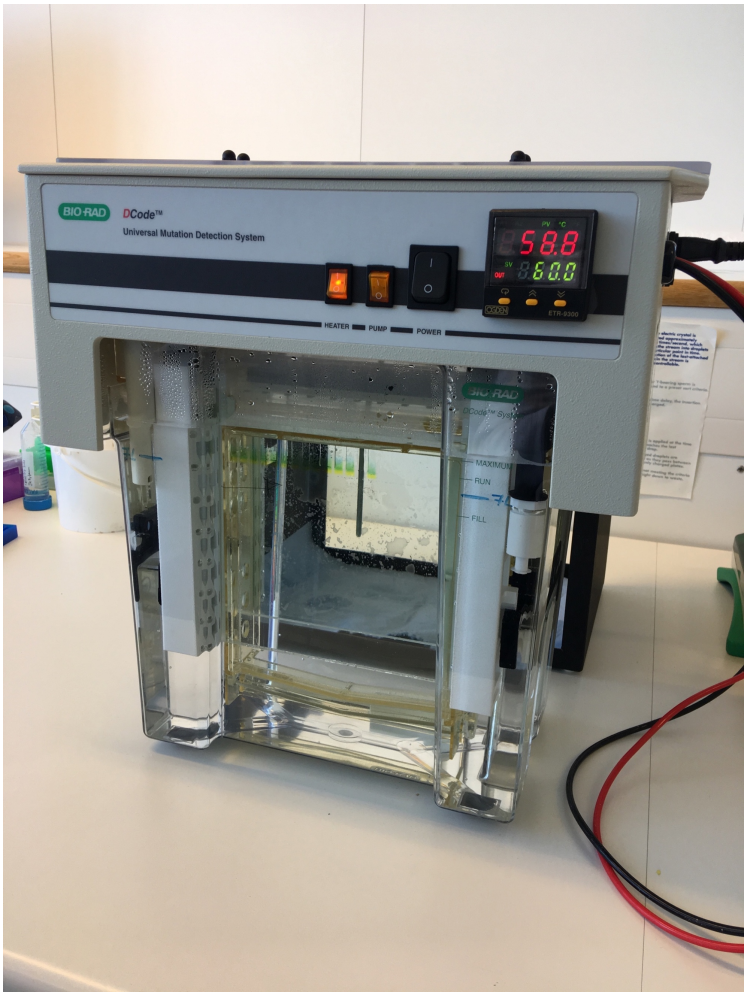


Figure 12: Gel electrophoresis machined with gel chamber loaded with PCR mix.

3.4.4.7 Staining of gel

Reagents used:

GelRed 10,000 × (VWR)

TAE buffer

- 100 µl of GelRed was mixed in 1 L of TAE buffer.
- The gel is kept in the mixture to stain it for 1 hour. The gel image was taken using a BioRad GelDoc XR+ system as seen in figure 12

3.4.5 Macrofaunal analysis

The sediment in all the cores were washed through a 63 mm sieve for retrieval of macrofauna. The sieve residues were checked under a microscope to retrieve macrofauna, the macrofauna were sorted into main taxonomic groups such as mollusca, polychaeta and crustacean. The organisms were identified into the closest taxon possible. The total number of macrofauna in all cores were noted as the number of species (N) in that particular core, foraminifera were not picked out and does not make up the biomass. The wet weight of the macrofauna (biomass) was measured for all cores. The wet weight is with the carbon shells of animals such as bivalves, and then the Shannon-Wiener diversity index (H') was also calculated for each core using the formula:

$$\mathbf{H'(i)} = \{-\sum \left(\frac{\boldsymbol{n}}{\boldsymbol{Nj}}\right) * \boldsymbol{LN}\left(\frac{\boldsymbol{n}}{\boldsymbol{Nj}}\right)\}$$

Where H'(i) is the Shannon-Wiener diversity index for the i^{th} specie.

n is the number of that specie (i) in the core

Nj is the total number of species in the core

The Shannon-Wiener diversity index (H') is given by

H' = H'(1) + H'(2) + H'(3)……H'(i)

H'(1) is the Shannon-Wiener diversity index for 1^{st} specie in the core

H'(2) is the Shannon-Wiener diversity index for 2^{nd} specie in the core

H'(3) is the Shannon-Wiener diversity index for 3^{rd} specie in the core

H'(i) is the Shannon-Wiener diversity index for i^{th} specie in the core

The Shannon-Wiener diversity index which is the negative sum of each operational taxonomic units also gives a measure of diversity and evenness in a community (Hill et al., 2003). The Shannon-Wiener diversity index will be more accurate if all the species in the community is sampled (Hill et al., 2003). In this study foraminifera were not picked, does not make up the biomass and was not used in calculating the Shannon-Wiener diversity index (H'). However, the values gotten from this is still valuable to indicate results.

3.4.6 Anova (analysis of variance) analysis

Statistical comparisons were done by One-way ANOVA with Post Hoc tests: Scheffe and Tukey HSD using the IBM SPSS Statistical software version 25. These statistics were performed on the number of species, biomass and Shannon-Wiener index of macrofauna and DGGE survival rate of the microbial communities.

4.0 RESULTS

This chapter presents the results obtained from the extraction of DNA from the sediment sample, the PCR result from the DNA, the DGGE result from the PCR products and the faunal responses.

4.1 DNA Extraction

Table 7: Concentrations of samples DNA.

	Sample	Concentration ng/µL	260/280	260/230
Control	F1 UPL	12.0	1.74	2.32
	F1 LL	1.70	0.91	1.40
S10	F12 UPL	13.5	1.60	1.03
	F12 LL	3.0	1.20	1.66
	F14 UPL	2.5	1.23	0.88
	F14 LL	14.1	1.56	0.82
OBM	F16 UPL	8.1	1.39	0.75
	F16 LL	5.7	1.11	1.53
	F20 UPL	6.6	1.59	1.63
	F20 LL	14.3	1.63	0.97
	F10 UPL	13.1	1.37	0.73
	F10 LL	2.4	1.23	0.04

Table 7 gives the concentration and the ratio of the absorbance of the nucleic acids. The purity of the sample DNA analyzed by ultra-violent absorption $A_{260/280}$ and $A_{260/230}$, $A_{260/280}$ is used to assess the purity of nucleic. A 260/280 ratio approximately 1.8 is generally accepted as pure DNA and a ratio of approximately 2.0 is considered as pure RNA, protein impurity in sample reduces the ratio for DNA (Mackey and Chomczynski, 1997). 260/230 ratios lack protein sensitivity in DNA (nucleic acid), a 260/230 ratio of 2.00 has 100% nucleic acid and 0% protein, a 260/230 ratio of 1.94 has 70% nucleic acid and 30% protein (Mackey and Chomczynski, 1997). An interesting trend in the concentration of DNA as shown in table 7 is the upper layers

of the cores are more concentrated in DNA than the lower layers with the exception of core 14 and core 20.

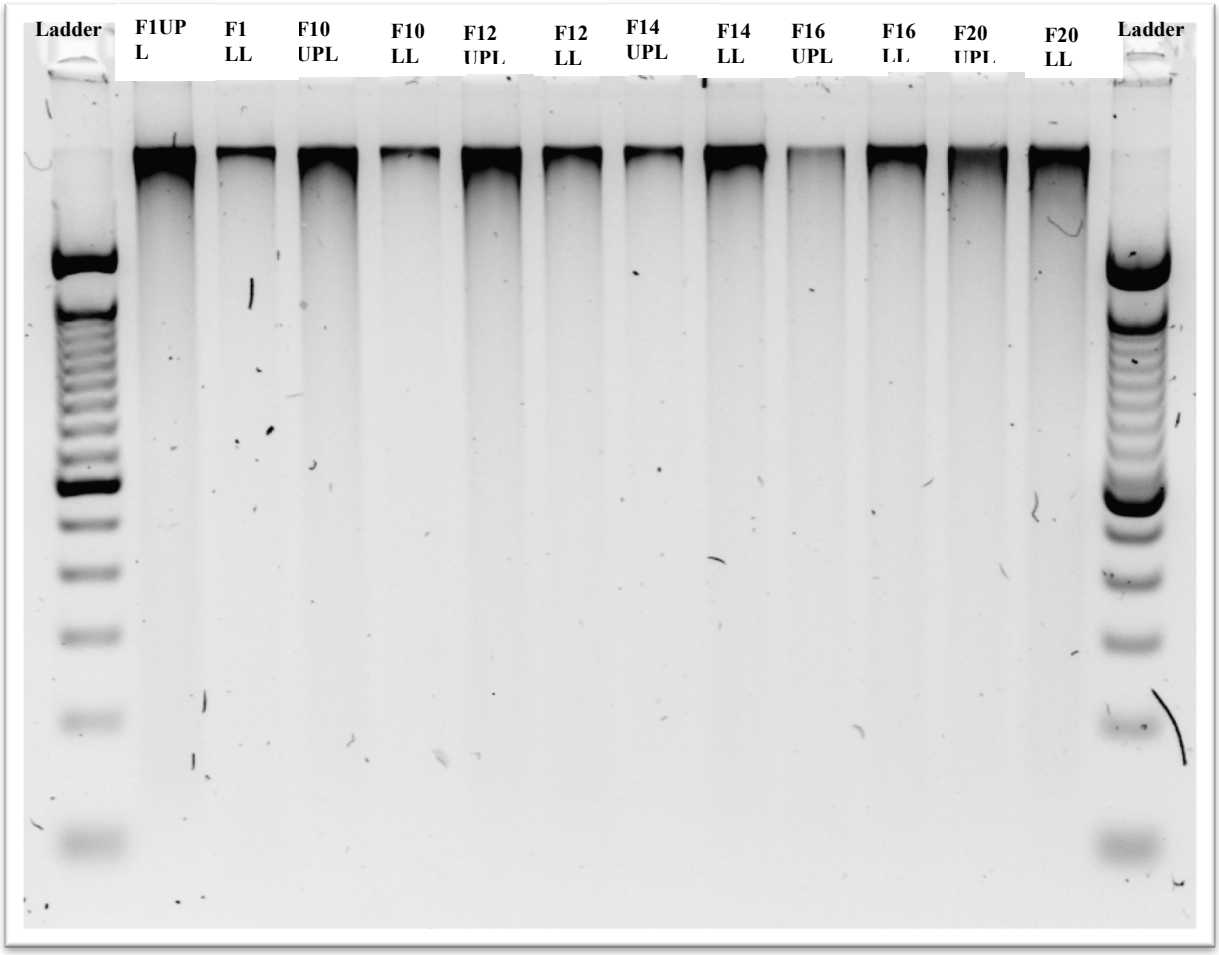


Figure 13: Agarose gel (2%) electrophoresis of DNA samples.

The quality of the extracted DNA was assessed by agarose gel (2%) electrophoresis and the gel image taken using a BioRad GelDoc XR+ system as seen in figure 13 The image shows that the concentration of the 12 samples is high enough for polymerase chain reaction.

Table 8: Concentration, Template DNA, and Milli-Q water added to PCR mix.

Sample	Concentration (ng/µl)	Template DNA added (µl)	Milli-Q water added (µl)

F1 UPL	12.0	1	14
F1 LL	1.70	2	13
F10 UPL	13.1	1	14
F10 LL	2.4	2	13
F12 UPL	13.5	1	14
F12 LL	3.0	2	13
F14 UPL	2.5	2	13
F14 LL	14.1	1	14
F16 UPL	8.1	1	14
F16 LL	5.7	1	14
F20 UPL	6.6	1	14
F20 LL	14.3	1	14

From table 8 samples with concentration above 5 ng/µl, 1 µl of the template DNA is used for the PCR mix. Samples with concentrations below 5 ng/µl, 2 µl of the template DNA is used for the PCR mix. A total volume of 50 µl is required for PCR mix so depending on what volume of template DNA added the remaining volume makes molecular grade water (Sigma Aldrich).

4.2 PCR reaction

The DNA from all the sediment samples were successfully amplified using the forward primer 341fGC and reverse primer 907r. The amplified fragments from all the sediment samples were about 560 bp (figure 14). A negative control (F13) was added. It is good for no band to appear for the negative control shown in figure 14 as it shows that the samples are not contaminated.

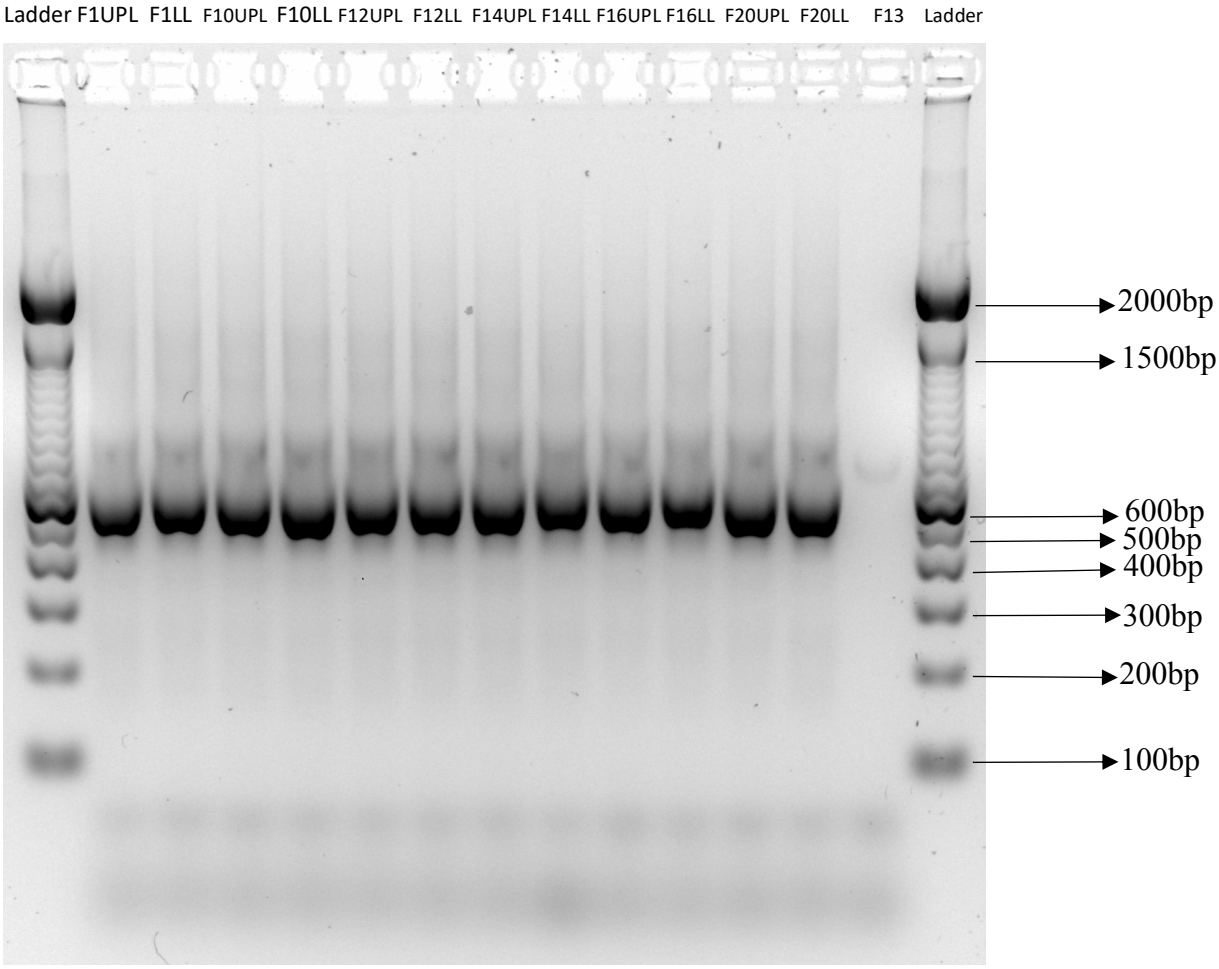


Figure 14: Agarose gel (2%) electrophoresis of PCR amplified products.

4.3 DGGE analysis

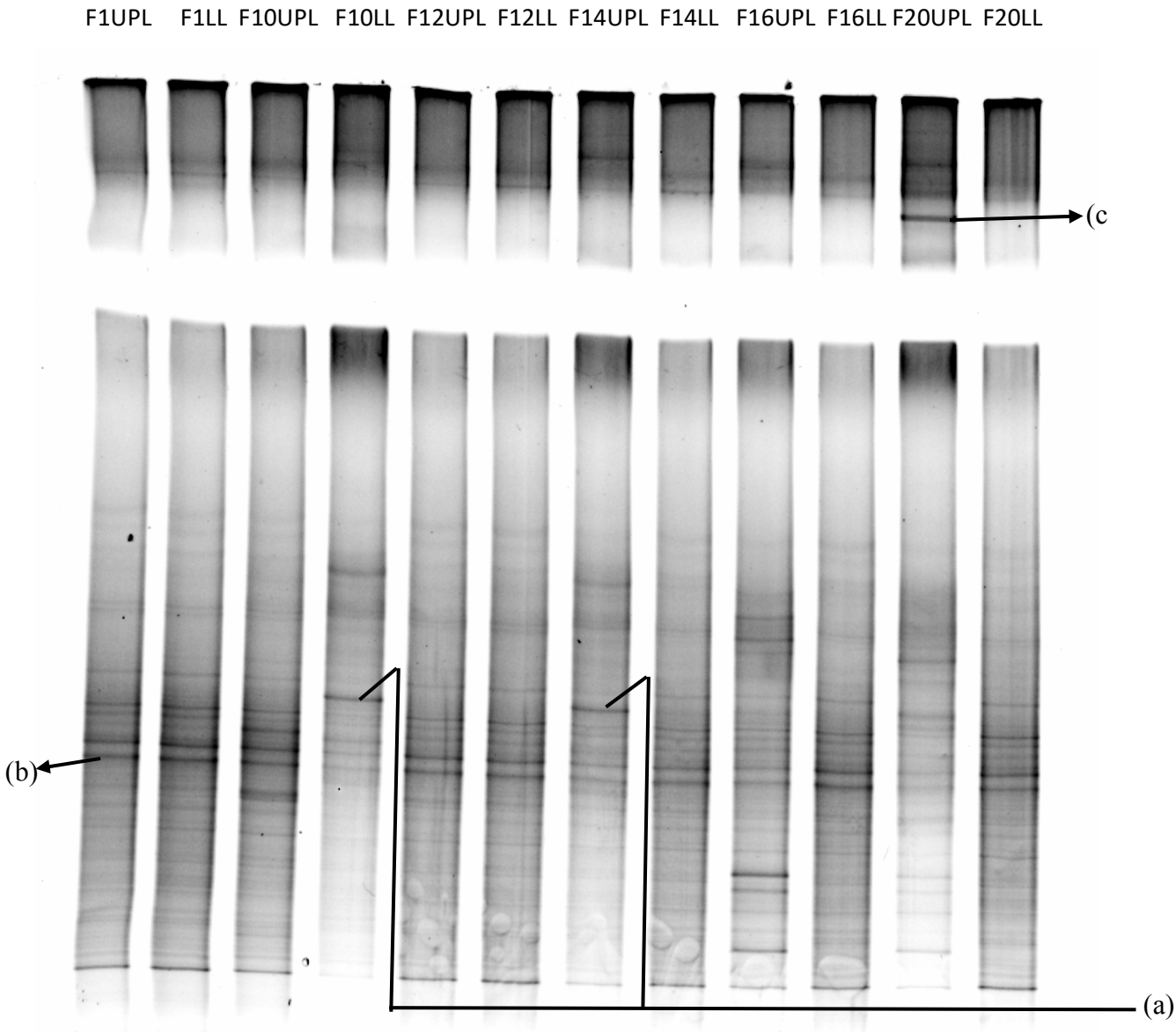


Figure 15: DGGE profiles of ribosomal DNA fragments obtained after amplification of DNA extracted from Boknafjord sediment sample.

The DGGE analysis of sediment samples showed individual banding patterns (figure 15). This represents the different dominant microbial communities present in each sample. The DGGE banding patterns were evaluated by counting the total number of bands shown by each sample, assuming that each band represent a bacteria specie. The highest microbial diversity was found in the control sediment, this representing the sample in which nothing was added. The lowest microbial diversity index was found in the oil-based mud (F20 UPL).

The mean number of the dominant microbial community is evaluated for the control, S10 and OBM. The percentage survival rates and mortalities of the bacteria community was calculated subsequently.

Table 9: Percentage survival rate and mortality of bacterial community of the control, OBM and S10 treatments with the mean number of dominant bands

	Mean No. of dominant bands	survival rate of bacteria communities (%)	Mortality of bacteria communities (%)
control	31.0	100	0
OBM	20.0	64.5	32.8
S10	20.8	67.2	35.5

After counting the number of dominant bands for all the cores as shown in table 9 the mean number of dominant bands were calculated for the control, OBM and S10. Assuming the control had 100 % of the dominant bacteria species therefore with 100 % survival rate and 0 % mortality of the bacteria community. Comparing this to the mean number of dominant bands for OBM and S10 the survival rate and mortality of the bacteria community is then calculated. The survival rate of the control, OBM and S10 shown in a bar chart in figure 16

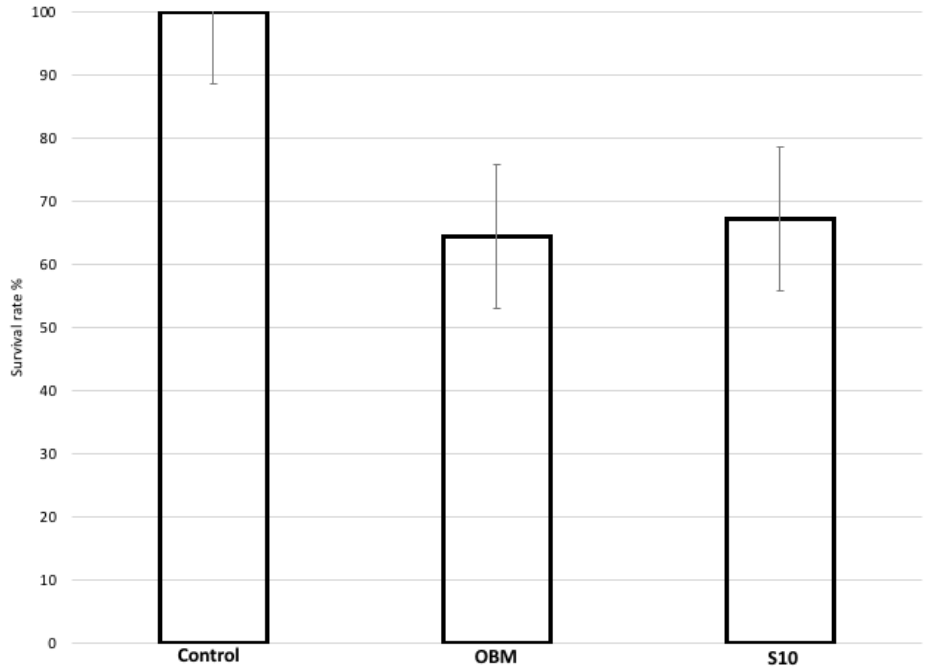


Figure 16: Effects to the exposure of oil-based drill cuttings (OBM) and thermally treated drill cuttings (S10) on sediment sample with respect to the reference cell (100%). Mean and standard deviation bars are shown.

From figure 16 the survival rate of bacteria communities for S10 and OBM are similar and different from the control. The survival rate of bacteria community in S10 is slightly higher than the survival rate of bacteria community in OBM.

4.4 Faunal responses

Table 10: Number of species (N), biomass, Shannon-Wiener diversity index (H') OBM: oil-based mud, S10: thermally treated drill cuttings

	Sample	N	Biomass (g)	H'
Control	F1 UPL	18	0.78	1.38
	F1 LL	21	1.18	1.55
	F2 UPL	17	0.72	1.36

	F2 LL	19	0.81	1.39
OBM	F10 UPL	3	0.13	0.64
	F10 LL	4	0.15	0.69
	F16 UPL	2	0.09	0.69
	F16 LL	1	0.04	0
	F20 UPL	3	0.15	0.64
	F20 LL	3	0.12	1.10
S10	F12 UPL	6	0.23	1.33
	F12 LL	7	0.38	1.08
	F14 UPL	5	0.35	0.67
	F14 LL	7	0.45	0.85

The number of taxa (N) without foraminifera, biomass (B) and Shannon-Wiener biodiversity index are presented in table 10. The biomass measure is with the carbon shells of the organisms. There was a faunal reduction in S10 and OBM treatments in the number of species (N) and biomass (B) when compared to the control as shown in figure 17 and figure 18. A noteworthy trend is that all the treatment cores containing OBM had high mortality of bivalves. Bivalves are noted to be very sensitive to OBM treatment (see in discussion). Polychaeta seem to thrive in OBM treatments. A second control F2 was not used in the DGGE experiments but was introduced in the macrofauna analysis to get a mean number of species, biomass and diversity index for the control.

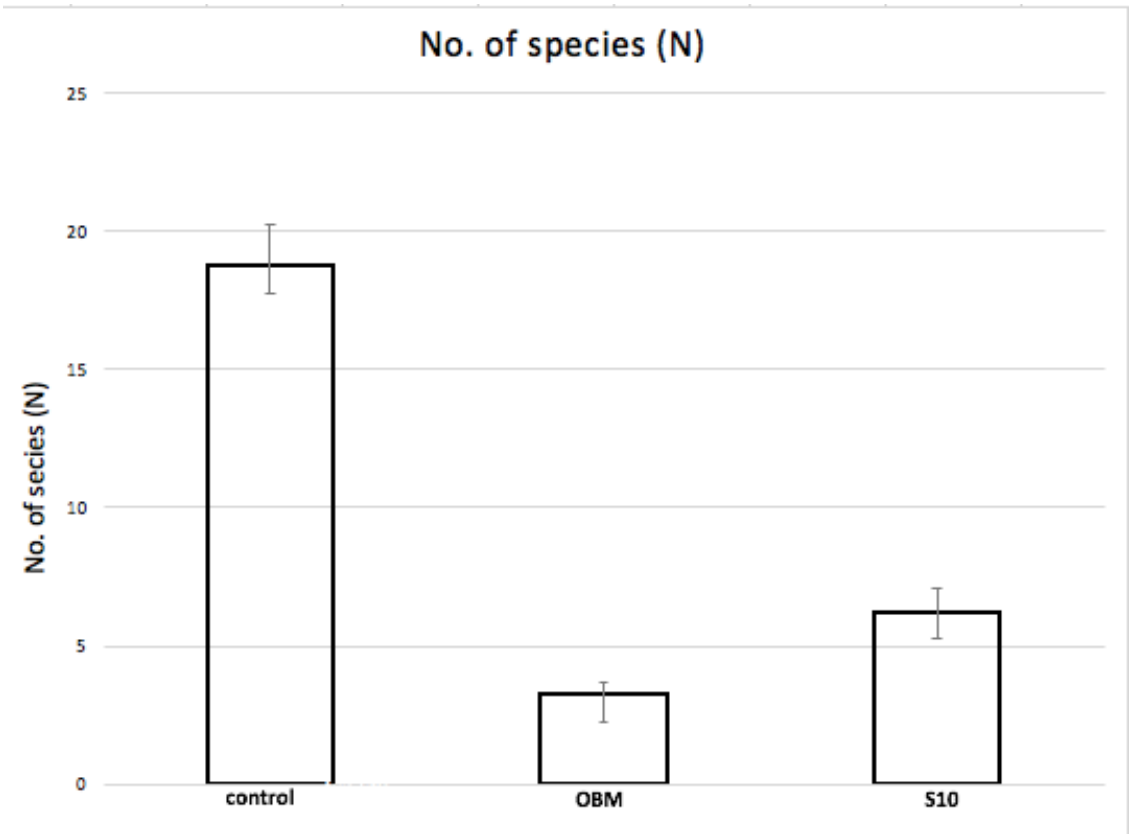


Figure 17: Mean number of species with the standard deviation. OBM: oil-based mud, S10: thermally treated cuttings.

Figure 17 shows the number of species in the macrofauna in control, OBM and S10 treatments. OBM had the least number of species while S10 had a higher number of species. The control had a higher number of species when compared to both S10 and OBM treatments. This is in agreement with the DGGE results where the survival rate of the bacterial communities in the control sediment was similarly different from the survival rate of the OBM and S10 treatments.

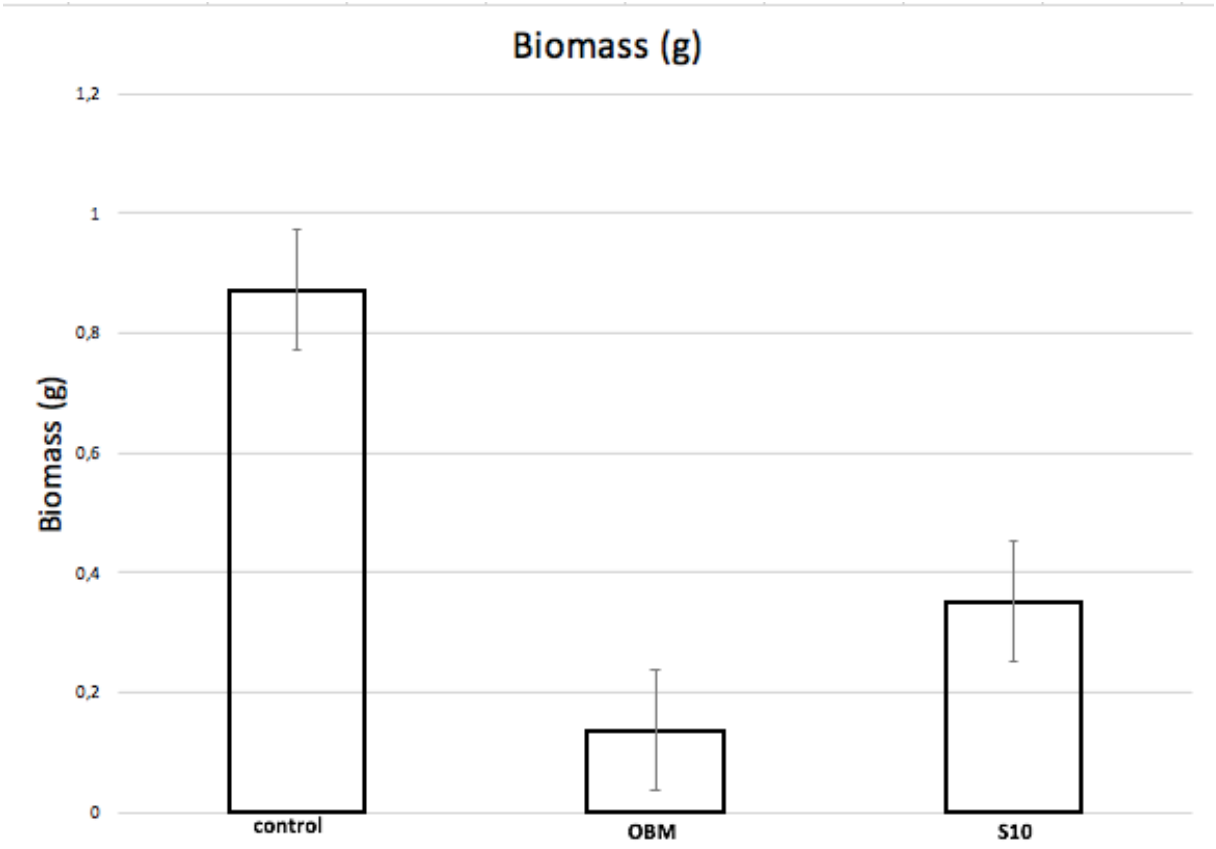


Figure 18: Mean biomass with the standard deviation. OBM: oil-based mud, S10: thermally treated cuttings.

Figure 18 shows the biomass in grams of the macrofauna in the control, OBM and S10 treatments. There were differences in the biomass of the macrofauna in control, S10 and OBM similarly to the number of species in fig 4.5. OBM had also regarding biomass the lowest mean value, but the difference OBM and S10 seems less clear than number of species due to higher variance.

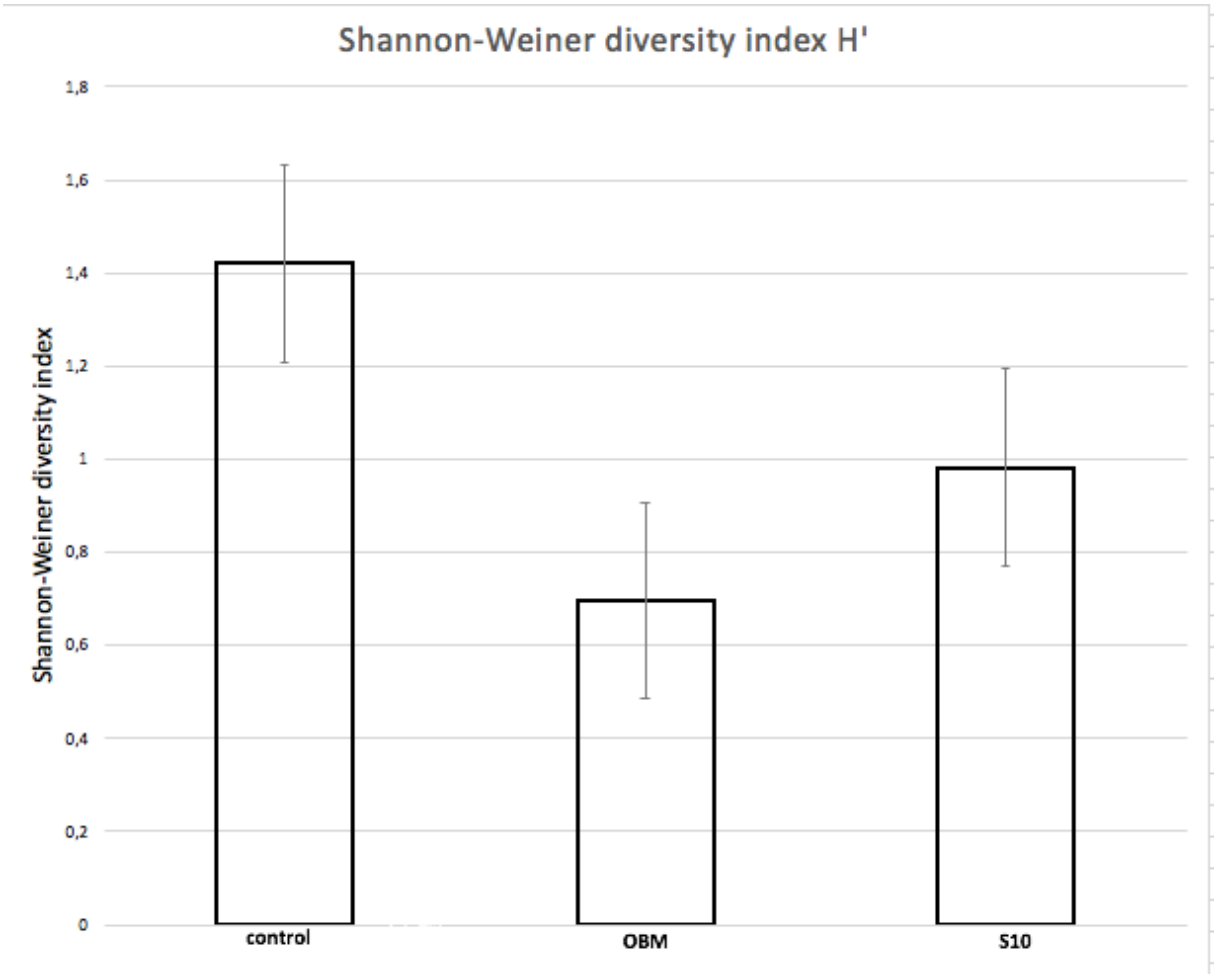


Figure 19: Mean Shannon-Wiener diversity index (H') with the standard deviation. OBM: oil-based mud, S10: thermally treated cuttings.

Figure 19 shows the Shannon-Wiener diversity index (H') of the macrofauna in the control, OBM and S10 treatments. As with the number of species and biomass the diversity of the species in the control was different from the diversity of species in the OBM and S10 treatment, and the diversity in OBM was lower than S10. However, the variance in all were higher and the differences less in the number of species and biomass.

The Shannon-Wiener diversity index will be more accurate and less of error if all the species in a community is sampled (Hill et al., 2003). In this study foraminifera were not picked, does not make up the biomass and was not used in calculating the Shannon-Wiener diversity index (H'). However, the values obtained from this is still valuable to indicate results in the experimental context of the present study.

Table 11: Summary of statistical test results in comparisons of means by 'One way Anova' (Scheffe)

Organisms, Variables and Treatments	Probability of significance	
Microorganisms: Survival rate of microbial community	Control	S10
OBM	0,009	0,916
S10	0,008	
Fauna Number of species		
	Control	S10
OBM	0,000	0,017
S10	0,000	
Biomass	Control	S10
OBM	0,000	0,123
S10	0,001	
Shannon-Wiener diversity index	Control	S10
OBM	0,008	0,309
S10	0,094	

Key: ($p<0.05$) = Significantly different ($p>0.05$) = Not Significantly different

Significantly different at 5% level

Not Significantly different

One-way Anova analyses were applied to the different treatments. Table 11 shows the summary of these statistical test results comparing the means of the different treatments pair-wise in the two organism groups (macrofauna and bacteria community) and the variables

tested. The probability figures in table 11 shows the Scheffe Post Hoc analysis results. Tukey results were similar and did not indicate any other levels of significance (not shown). In most cases the treatments (OBM and S10) gave results that were significantly different from the control. The results showed that the two cuttings treatments were significantly different in one variable (number of species) not significantly different at 5% level in the other two.

5.0 DISCUSSION

The objective of this thesis is to evaluate if there are significant toxic or community effects of using microwave treated drill cuttings (S10) and untreated oil based drill cuttings (OBM) on a faunal and microcosm community.

Toxicity experiments were carried out in a community microcosm where S10 and OBM drill cuttings were added to cores with sediment. Faunal and DGGE analyses were carried out.

5.1 Macrofauna impacts

Bivalves have been used as indicator organisms of environmental pollution as a result of oil based mud (Daan et al., 1994), demonstrating these organisms sensitivity to oil based mud discharges. They experienced adverse effect, high sensitivity and decreased abundance with increase in concentration of oil based mud cuttings (Daan et al., 1994). Bivalves in this study showed high sensitivity and high mortality to the oil based mud treatment. Organic enrichment have been suggested by (Addy et al., 1984) to be the major stressor of oil based mud on macrofauna. After the termination period in this study, black layers/spots were seen in the OBM treatments. These are anoxic regions caused by the degradation of organics on the OBM cuttings which leads to an increase in the consumption of oxygen by the organisms. Studies by (Daan et al., 1994) and (Eagle and Rees, 1973) reported the opportunistic specie *Capitella capitata* occurring in increased numbers and thriving in organic rich sediments being a "fast colonizer" in OBM polluted sediment. In this study *Capitella capitata* (polychaeta) thrived in the OBM polluted sediments, and more so than S10 treatment.

S10 (microwave treated drill cuttings) have a discharge limit of oil levels of oil contaminated drill cuttings (OCDC) at 1 % (w/w) set by the environmental regulations (Shang et al., 2006). Therefore, for a properly treated microwave drill cuttings (S10), there will be reduced organics present. It should be at a concentration that is not toxic to macrofauna. The bivalves which have a high mortality rate in the oil based mud (OBM) did not have the same mortality rate in the S10 treatment. It can be hypothesized that the reduced concentration of organics in S10 treatments made the bivalves to have lower mortality than those in the OBM. The null hypothesis is that there is no difference between S10 and OBM results (see further below).

(Schindler, 1987) suggested the decrease in diversity, biomass, in the number of species and increase in diversity and dominance by opportunistic species in oil contaminated

fields were caused by a stressor. Results obtained by (Gray et al., 1990) showed that within 500 m to 1000 m of discharge of oil contaminated drill cuttings (OCDC) there is a decrease in diversity, number of species and increase in dominance of opportunistic species as some of the responses.

The macrofaunal analysis in this study showed a reduction in the number of species, biomass and diversity in both OBM and S10 treatments. One of the cause of adverse effect of OBM treatment to macrofauna can be attributed to the presence of organics on the cuttings. (Shang et al., 2006) reported that the minimum concentration of oil in microwave treated drill cuttings should be below 1 % (w/w) this standard was set by the environmental legislation. What then caused the decrease in the number of species, biomass and diversity of macrofaunal in S10 treatment? pH has been noted by scientists as one of the crucial factors in determining prokaryotic diversity and also a stressor (Chong et al., 2010). In this study pH of the S10 supernatant during rinsing and the sea water have an average difference of 0.047 from the control. This small difference in pH indicates that there must be something else present in S10 which reduces the diversity, number of species and biomass of macrofauna. It can be due to the heavy metals. (Randrianarimanana, 2014) obtained some interesting results while working on treated and untreated drilling waste on *Daphnia magna*. Treated TCC (thermochemical cutting cleaner) showed an analytical increase in the concentration of heavy metals with the exception of mercury and increase in toxicity to *Daphnia magna*. A common reason for both these apparent increases in analytical metal concentrations and toxicity can be an increase in the surface area when it is in fine grained particles as TCC treatment causes and it being dissolved in water. This could have caused both bio-and analytical and availability of metals. In the present study the difference in metal concentrations between the control sediment and S10 is not known. However, the S10 will naturally contain metals and had it was finer grained than the control sediment it may therefore have caused a higher bioavailability of metals to the macrofauna. A possibly higher metal concentration and possible increase in the bioavailability to the macrofauna may be the cause that lead to a reduction in the number of species, diversity and biomass in the S10 treatments compared to the controls.

Figure 17, 18 and 19 shows that the number of species, biomass, and the diversity of macrofauna when OBM and S10 treatments were added have different results from the control. The variance analysis (anova) shows which treatments were significantly different from the control.

The anova of the macrofauna for the number of species, Shannon-Wiener diversity index and biomass as shown in table 11 is used to test statistically the differences between the control, OBM and S10 are significantly different or not significantly different. In the null hypothesis (H_0) formulated the control is not different form the other OBM and S10 treatments and the treatments themselves are not different from each other. The null hypothesis (H_0) is rejected (saying the two compared variables are probably not the same). If the significance probability (SP) was less than 5%.

In comparison the number of species between OBM and control, the SP was 0% (table 11). Thus, it can be concluded to reject the H_0, and that the OBM and the control are different with high probability. The SP of 0% is the probability of being wrong in this conclusion. The same goes for S10 and control with an SP of 0% we can reject the H_0 also. However, OBM and S10 have an SP value of 1.7% for the number of species, but still below the significance level of 5%, so therefore we can reject the H_0 at the 5% level of comparison also (with 1.7% probability of being wrong). In conclusion, for the number of species (figure 17) the statistical analysis shows that the control, OBM and S10 are all significantly different.

For the biomass, H_0 for OBM and S10 not being different from the control can both be rejected (Table 11). In the comparison of OBM and S10 for the biomass of the macrofauna SP was 12.3%. If the null hypothesis (H_0) is rejected it will be 12.3% probability of being wrong, which is above the set acceptance level of 5%. Thus, it is not statistically significant at this level, but with a higher uncertainty the results may be interpreted as a non-significant indication for a difference in the biomass of OBM and S10.

The Shannon-Wiener diversity index (Shang et al., 2006) for the macrofauna showed an interesting pattern. The control and OBM comparison has an SP of 0.8% and the H_0 can be rejected, supporting a conclusion that they are significantly different. But the S10 is not significantly different from the control (SP of 9.4%, Table 11). Still the analysis shows that OBM and S10 are not significantly different (SP of 30.9%), leaving the overall conclusion somewhat ambiguous.

5.2 DGGE

(Taketani et al., 2010) examined the hypothesis that microbial communities in the mangrove sediments behave differently to the disturbance of a hydrocarbon pollution. Results after PCR amplification and DGGE showed that the bacterial communities have a reduced number of bands when compared with the control in response to the oil pollution.

In another studies by (Brakstad et al., 2008) microbial communities associated with the arctic fjord ice were polluted with petroleum oils and evaluated. Sample cores with oil contaminated ice and clean ice without any contamination were analyzed using PCR-amplified bacterial 16S gene fragments by denaturing gradient gel electrophoresis. The results showed that the bacterial communities in oil contaminated ice produced fewer bands than communities in clean ice. In conclusion they stated that the microbial communities were adversely affected by oil contamination. The bacterial number were stimulated, the diversity was decreased, and the abundance reduced of a few genera.

DNA was extracted by (Nakatsu et al., 2000) from six soils from an agroecosystem in Norway and USA. The microbial communities were separated using PCR-amplified 16S rDNA and then amplified using DGGE. One of the soil was contaminated with PAH (polycyclic aromatic hydrocarbons). All the soil samples showed a great bacterial diversity except the soil contaminated with PAH, which showed a reduced bacterial diversity.

In the present study all the OBM treatments showed a reduced number of bands when compared to the control. This is in tandem with literature of how detrimental OBM is to the microbial communities, reducing them in diversity and abundance.

Microwave treated drill cuttings have below 1% (w/w) of oil after treatment (Shang et al., 2006). Therefore, one of the stressor contributing to the reduced diversity, number of bands and low abundance of the microbial communities in the S10 treatment may alternatively can be attributed to the heavy metals. (Randrianarimanana, 2014) showed that after treatment of TCC the heavy metal concentration increases with the exception of mercury. A possible increase in concentration and/or bioavailability of metals may increase the toxicity effect on the microbial communities.

(Kandeler et al., 1996) notice that reduced biomass, number of bands and enzyme activities appeared with increase in metal concentration, acting as a pollutant to the microbial communities. This suggest that even with the considerable reduced organics in the microwave

treated drill cuttings (S10), it may still be as detrimental to the microbial communities as the oil based muds (OBM) due to the presence of metals.

Table 11 shows the mean number of dominant bands, survival rate and mortality of the bacterial community for the control, OBM and S10. The mean number of bands in the control for the bacterial communities is assumed to be 100% survival rate and 0% mortality. The number of bands for the OBM and S10 is used to calculate the survival rate and mortality for the S10 and OBM (see appendix for detailed calculation). Figure 16 shows a bar chart representing the survival rate of the control, OBM and S10. Table 11 shows the statistical test by one way anova for the survival rate of the microbial communities.

The null hypothesis (H_0) states the OBM, control and S10 are not different with respect to the microbial communities. If we decide to reject the H0 and conclude that the OBM is different from the control it will be 0.9% probability of being wrong. Since this is lower than 5% we can reject the null hypothesis and conclude that the OBM is not equal to the control in the microbial communities. Similarly, for the S10 and the control with a SP of 0.8% (Table 11) it can be concluded that the S10 treatment is not equal to the control in the microbial community. But for the S10 and OBM treatments rejecting the H0 means it will be 91.6% probability of being wrong, and in other words the null hypothesis stating that the OBM and S10 treatments in the microbial communities are the not different must be accepted.

We can see that the slight indications that the S10 treatments showed less effects than the untreated drill cuttings (OBM) (Fig. 4.4) is not supported statistically).

Further details regarding the results on DGGE of the microbial communities:

The DNA obtained from the sediment sample was pure (with an ultra-violent absorption ratio of $A_{260/280}$ approximately 1.8) and intact (high molecular weight bands on agarose gel) (Mackey and Chomczynski, 1997). After PCR amplification of extracted DNA agarose gel (2%) electrophoresis confirmed that the product size was expected, fragments of DNA were just above 560 bp (figure 14). This is in accordance with works by (Myers et al., 1985) that only small base-pairs of about 500 bp can be separated by DGGE.

After DGGE of PCR-amplified 16S rDNA fragments the DGGE pattern for the upper and lower layer of the control (F1 UPL and F1 LL) shows the same number of predominant members in the microbial communities present in both control samples. Most of the dominant members of the microbial communities in the control is also seen in the two thermally treated cuttings (F12 UPL, F12 LL, F14 UPL, and F14 LL). A band (a) which only appears in F14 UPL and

F10 LL which is not seen in the control can be as a result of less predation from lager animals, presence of heavy metals or nutrients added by OBM or the thermally treated drill cuttings which made the microbial communities flourish. All the samples with OBM treatment (F20, F10, and F16) show an individual banding different from the control sediment. This gives information on how OBM treatment changes the microbial diversity. This can also be as a result of the OBM treatment making the dominant community less dominant and reducing the population. An example is the bacteria specie (b) which is a dominating one in the control sediment and S10 exposure which also appears in OBM treatments but not dominating showing a reduction in population. An explanation to this may be that the taxa is sensitive and responds to OBM treatment.

6.0 CONCLUSION

The objective of this study is to evaluate if there are any significant toxic or community effects of using the microwave treated drill cuttings (S10) and the untreated oil based drill cuttings (OBM) on a fauna and microcosm community.

There were clear indications that both the microwave treated drill cuttings and oil based drill cuttings treatments had community effects on the macrofauna and microbial communities. The cause of the effects for the oil based drill cuttings can be due to the presence of organics while for the microwave treated drill cuttings it might alternatively have been caused by heavy metals. This latter can be argued for by the fact that the microwave treated drill cuttings was in more fine grained particles than the control sediment which could have made the metals more bioavailable for the macrofauna and microbial communities. However, this needs further investigation to conclude about.

The number of species in the macrofauna analysis showed a statistically significant difference between the microwave treated cuttings and the untreated oil based mud, with the microwave treated samples being less affected. The same was indicated in the biomass of the macrofauna, but at a non-significant statistical level. The diversity of the macrofauna showed an ambiguous result. Overall, the effects on macrofauna was less in the microwave treated than in the untreated oil based mud, but it was not significantly evidenced in all analyzed variables. As with the microbial community's metal toxicity may also have influenced the results of the macrofauna, however this needs separate studies to conclude about.

7.0 FURTHER RECOMMENDATIONS

This study focused only on the analysis of microbial communities and macrofauna impact after the addition of S10 and OBM drill cuttings to a natural sediment. It does not give information about the complexities of the dynamics of the microbial communities and macrofauna, such as the influence of metals, and environmental factors, such as seasonal variations, variations in contamination level, thickness of sedimented cuttings, etc. Advice on further studies should examine different samples over a long period of time to get more information about the complexities of the microbial communities and the macrofauna.

REFERENCES

ADDY, J., HARTLEY, J. & TIBBETTS, P. 1984. Ecological effects of low toxicity oil-based mud drilling in the Beatrice oilfield. *Marine Pollution Bulletin,* 15**,** 429-436.

ALBERTY, M. W., HAFLE, M. E. & MINGE, J. C. Mechanisms of shallow waterflows and drilling practices for intervention. Offshore Technology Conference, 1997. Offshore Technology Conference.

ALVE, E. 1995. Benthic foraminiferal responses to estuarine pollution: a review. *Journal of Foraminiferal Research,* 25**,** 190-203.

AMANN, R. I., LUDWIG, W. & SCHLEIFER, K.-H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological reviews,* 59**,** 143-169.

BADR, A. Molecular approaches to plant systematic and evolution. Proceedings of Egyptian-Swedish Symposium commemorating Vivi Taeckholm's 110th Birthday, 2008. 127-167.

BAKKEN, L. & LINDAHL, V. 1995. Recovery of bacterial cells from soil. *Nucleic acids in the environment.* Springer.

BALL, A. S., STEWART, R. J. & SCHLIEPHAKE, K. 2012. A review of the current options for the treatment and safe disposal of drill cuttings. *Waste Management & Research,* 30**,** 457-473.

BAYNE, B. 1988. Measuring the biological effects of pollution: the mussel watch approach. *Water Pollution Research and Control Brighton.* Elsevier.

BEJ, A. K., MAHBUBANI, M., DICESARE, J. & ATLAS, R. 1991. Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. *Applied and Environmental Microbiology,* 57**,** 3529-3534.

BEJ, A. K., STEFFAN, R. J., DICESARE, J., HAFF, L. & ATLAS, R. M. 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. *Applied and Environmental Microbiology,* 56**,** 307-314.

BESSESEN, M. T., LUO, Q., ROTBART, H. A., BLASER, M. J. & ELLISON, R. 1990. Detection of Listeria monocytogenes by using the polymerase chain reaction. *Applied and environmental microbiology,* 56**,** 2930-2932.

BILSTAD, T., STENBERG, E. S., JENSEN, B., LARSEN, T. & TOFT, M. 2013. Offshore drilled cuttings management. *AGH Drilling, Oil, Gas,* Vol. 30**,** 47-50.

BRAKSTAD, O. G., NONSTAD, I., FAKSNESS, L.-G. & BRANDVIK, P. J. 2008. Responses of microbial communities in Arctic sea ice after contamination by crude petroleum oil. *Microbial ecology,* 55**,** 540-552.

BREUER, E., HOWE, J., SHIMMIELD, G., CUMMINGS, D. & CARROLL, J. 1999. Contaminant Leaching from Drill Cuttings Piles of the Northern and Central North Sea: A Review. *Center for coastal & marine sciences. 49p*.

BREUER, E., PEPPE, O. & SHIMMIELD, G. 2002. A novel approach for the study of North Sea drill cuttings accumulations: The combined use of an ROV and benthic lander for in situ measurements. *Underwater Technology,* 25**,** 77-82.

CHAILLAN, F., CHAÎNEAU, C. H., POINT, V., SALIOT, A. & OUDOT, J. 2006. Factors inhibiting bioremediation of soil contaminated with weathered oils and drill cuttings. *Environmental Pollution,* 144**,** 255-265.

CHANDLER, A. J., EIGHMY, T. T. & HARTL, J. 1997. Solidification and stabilization. Studies in Environmental Science. 57**,** 763–790.

CHIEN, A., EDGAR, D. B. & TRELA, J. M. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. *Journal of bacteriology,* 127**,** 1550-1557.

CHONG, C. W., PEARCE, D. A., CONVEY, P., TAN, G. A., WONG, R. C. & TAN, I. K. 2010. High levels of spatial heterogeneity in the biodiversity of soil prokaryotes on Signy Island, Antarctica. *Soil Biology and Biochemistry,* 42**,** 601-610.

CLARK, D. E., FOLZ, D. C. & WEST, J. K. 2000. Processing materials with microwave energy. *Materials Science and Engineering: A,* 287**,** 153-158.

DAAN, R., MULDER, M. & VAN LEEUWEN, A. 1994. Differential sensitivity of macrozoobenthic species to discharges of oil-contaminated drill cuttings in the North Sea. *Netherlands Journal of Sea Research,* 33**,** 113-127.

DARDIR, M. & ABDOU, M. 2013. Ether-based muds show promise for replacing some oil-based muds. *Petroleum Science and Technology,* 31**,** 2335-2347.

DAVIES, J., ADDY, J., BLACKMAN, R., BLANCHARD, J., FERBRACHE, J., MOORE, D., SOMERVILLE, H., WHITEHEAD, A. & WILKINSON, T. 1984. Environmental effects of the use of oil-based drilling muds in the North Sea. *Marine Pollution Bulletin,* 15**,** 363-370.

DENOYELLE, M., GESLIN, E., JORISSEN, F. J., CAZES, L. & GALGANI, F. 2012. Innovative use of foraminifera in ecotoxicology: A marine chronic bioassay for testing potential toxicity of drilling muds. *Ecological indicators,* 12**,** 17-25.

DIPLOCK, E. E., MARDLIN, D. P., KILLHAM, K. S. & PATIN, G. I. 2009. Predicting Bioremediation of Hydrocarbons: Laboratory to field scale.

EAGLE, R. & REES, E. 1973. Indicator species—a case for caution. *Marine Pollution Bulletin,* 4**,** 25.

FISCHER, S. G. & LERMAN, L. S. 1979. Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. *Cell,* 16**,** 191-200.

FRONTALINI, F. & COCCIONI, R. 2011. Benthic foraminifera as bioindicators of pollution: a review of Italian research over the last three decades. *Revue de micropaléontologie,* 54**,** 115-127.

GRAY, J. S. 1992. Biological and ecological effects of marine pollutants and their detection. *Marine Pollution Bulletin,* 25**,** 48-50.

GRAY, J. S., CLARKE, K., WARWICK, R. & HOBBS, G. 1990. Detection of initial effects of pollution on marine benthos: an example from the Ekofisk and Eldfisk oilfields, North Sea. *Marine Ecology Progress Series,* 66**,** 285-1990.

GRIFFIN, L. F. & CALDER, J. A. 1977. Toxic effect of water-soluble fractions of crude, refined, and weathered oils on the growth of a marine bacterium. *Applied and environmental microbiology,* 33**,** 1092-1096.

HAQUE, K. E. 1999. Microwave energy for mineral treatment processes—a brief review. *International Journal of Mineral Processing,* 57**,** 1-24.

HERRICK, J. B., MADSEN, E., BATT, C. & GHIORSE, W. 1993. Polymerase chain reaction amplification of naphthalene-catabolic and 16S rRNA gene sequences from indigenous sediment bacteria. *Applied and Environmental Microbiology,* 59**,** 687-694.

HESS, S., ALVE, E., TRANNUM, H. C. & NORLING, K. 2013. Benthic foraminiferal responses to water-based drill cuttings and natural sediment burial: results from a mesocosm experiment. *Marine Micropaleontology,* 101**,** 1-9.

HILGER, A. & MYROLD, D. 1991. Method for extraction of Frankia DNA from soil. *Agriculture, Ecosystems & Environment,* 34**,** 107-113.

HILL, T. C., WALSH, K. A., HARRIS, J. A. & MOFFETT, B. F. 2003. Using ecological diversity measures with bacterial communities. *FEMS microbiology ecology,* 43**,** 1-11.

HOLBEN, W. E., JANSSON, J. K., CHELM, B. K. & TIEDJE, J. M. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Applied and Environmental Microbiology,* 54**,** 703-711.

IFEADI, C. & MNSE, F. The treatment of drill cuttings using dispersion by chemical reaction (DCR). A paper prepared for presentation at the DPR Health, Safety & Environment (HSE) International Conference on Oil and Gas Industry in Port Harcourt, Nigeria, 2004.
JOSEPHSON, K., PILLAI, S., WAY, J., GERBA, C. & PEPPER, I. 1991. Fecal coliforms in soil detected by polymerase chain reaction and DNA-DNA hybridizations. *Soil Science Society of America Journal,* 55**,** 1326-1332.
KANDELER, F., KAMPICHLER, C. & HORAK, O. 1996. Influence of heavy metals on the functional diversity of soil microbial communities. *Biology and fertility of soils,* 23**,** 299-306.
KHANNA, M. & STOTZKY, G. Transformation by DNA bound on clay minerals: implications for gene transfer in soil and other habitats, abstr. Q-191. Abstr. 90th Annu. Meet. Am. Soc. Microbiol, 1990. 320.
KINGSTON, P. 1992. Impact of offshore oil production installations on the benthos of the North Sea. *ICES Journal of Marine Science,* 49**,** 45-53.
KIRKNESS, A. & GARRICK, D. 2008. Treatment of Nonaqueous-Fluid-Contaminated Drill Cuttings-Raising Environmental and Safety Standards.
KOGBARA, R. B., OGAR, I., OKPARANMA, R. N. & AYOTAMUNO, J. M. 2016. Treatment of petroleum drill cuttings using bioaugmentation and biostimulation supplemented with phytoremediation. *Journal of Environmental Science and Health, Part A,* 51**,** 714-721.
LEONARD, S. A. & STEGEMANN, J. A. 2010. Stabilization/solidification of petroleum drill cuttings. *Journal of Hazardous Materials,* 174**,** 463-472.
MACKEY, K. & CHOMCZYNSKI, P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques,* 22**,** 474-481.
MAIN, C. E. 2015. *On the fate of plumes from deep-sea oil well blowouts, and their effects on sediment communities.* University of Southampton.
MEREDITH, R. 1998. *Engineers Handbook of Industrial Microwave Heating,* London.
MURRAY, A. J., KAPILA, M., FERRARI, G., DEGOUY, D., ESPAGNE, B. J.-L. & HANDGRAAF, P. 2008. Friction-Based Thermal Desorption Technology: Kashagan Development Project Meets Environmental Compliance in Drill-Cuttings Treatment and Disposal. Society of Petroleum Engineers.
MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain

reaction-amplified genes coding for 16S rRNA. *Applied and environmental microbiology,* 59**,** 695-700.

MUYZER, G. & SMALLA, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek,* 73**,** 127-141.

MYERS, R. M., FISCHER, S. G., LERMAN, L. S. & MANIATIS, T. 1985. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic acids research,* 13**,** 3131-3145.

NAKATSU, C. H., TORSVIK, V. & ØVREÅS, L. 2000. Soil Community Analysis Using DGGE of 16S rDNA Polymerase Chain Reaction Products Indiana Agric. Exp. Stn. Purdue Univ.# 16101. *Soil Science Society of America Journal,* 64**,** 1382-1388.

NEFF, J. M. Composition, environmental fates, and biological effect of water based drilling muds and cuttings discharged to the marine environment: A synthesis and annotated bibliography. Report prepared for the Petroleum Environmental Research Forum (PERF). Washington DC: American Petroleum Institute, 2005.

OLSGARD, F. & GRAY, J. S. 1995. A comprehensive analysis of the effects of offshore oil and gas exploration and production on the benthic communities of the Norwegian continental shelf. *Marine Ecology Progress Series***,** 277-306.

ORMELOH, J. 2014. Thermomechanical cuttings cleaner–qualification for offshore treatment of oil contaminated cuttings on the norwegian continental shelf and martin linge case study.

PAN, Q., WANG, F., ZHANG, Y., CAI, M., HE, J. & YANG, H. 2013. Denaturing gradient gel electrophoresis fingerprinting of soil bacteria in the vicinity of the Chinese Great Wall Station, King George Island, Antarctica. *Journal of Environmental Sciences,* 25**,** 1649-1655.

PITCHER, D., SAUNDERS, N. & OWEN, R. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letters in applied microbiology,* 8**,** 151-156.

RANDRIANARIMANANA, J. V. 2014. *Study of treated and untreated drilling waste using a bioassay approach by*

determination of the mobility inhibition of Daphnia magna. masters, University of Stavanger.

ROOSE-AMSALEG, C. L., GARNIER-SILLAM, E. & HARRY, M. 2001. Extraction and purification of microbial DNA from soil and sediment samples. *Applied Soil Ecology,* 18**,** 47-60.

SCHAANNING, M. T., TRANNUM, H. C., ØXNEVAD, S., CARROLL, J. & BAKKE, T. 2008. Effects of drill cuttings on biogeochemical fluxes and macrobenthos of marine sediments. *Journal of Experimental Marine Biology and Ecology,* 361**,** 49-57.

SCHINDLER, D. W. 1987. Detecting ecosystem responses to anthropogenic stress. *Canadian Journal of Fisheries and Aquatic Sciences,* 44**,** s6-s25.

SHANG, H., SNAPE, C., KINGMAN, S. & ROBINSON, J. 2006. Microwave treatment of oil-contaminated North Sea drill cuttings in a high power multimode cavity. *Separation and purification technology,* 49**,** 84-90.

SHANG, H., SNAPE, C. E., KINGMAN, S. W. & ROBINSON, J. P. 2005. Treatment of Oil-Contaminated Drill Cuttings by Microwave Heating in a High-Power Single-Mode Cavity. *Industrial & Engineering Chemistry Research,* 44**,** 6837-6844.

SHEFFIELD, V. C., COX, D. R., LERMAN, L. S. & MYERS, R. M. 1989. Attachment of a 40-base-pair G+ C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proceedings of the National Academy of Sciences,* 86**,** 232-236.

STEFFAN, R. J. & ATLAS, R. M. 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. *Applied and Environmental Microbiology,* 54**,** 2185-2191.

TAKETANI, R. G., FRANCO, N. O., ROSADO, A. S. & VAN ELSAS, J. D. 2010. Microbial community response to a simulated hydrocarbon spill in mangrove sediments. *The Journal of Microbiology,* 48**,** 7-15.

TEBBE, C. C. & VAHJEN, W. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Applied and environmental microbiology,* 59**,** 2657-2665.

TESKE, A., WAWER, C., MUYZER, G. & RAMSING, N. B. 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Applied and Environmental Microbiology,* 62**,** 1405-1415.

THERMTECH. 2014 *The Thermomechanical Cuttings Cleaner* [Online]. Bergen. Available: http://thermtech.no/home/the_tcc/ [Accessed].

TORSVIK, V., GOKSØYR, J. & DAAE, F. L. 1990. High diversity in DNA of soil bacteria. *Applied and environmental microbiology,* 56**,** 782-787.

TRANNUM, H. C., NILSSON, H. C., SCHAANNING, M. T. & ØXNEVAD, S. 2010. Effects of sedimentation from water-based drill cuttings and natural sediment on benthic macrofaunal community structure and ecosystem processes. *Journal of Experimental Marine Biology and Ecology,* 383**,** 111-121.

TRANNUM, H. C., SCHAANNING, M. T., JOHANSEN, J. T., MOODLEY, L., WESTERLUND, S. & BAUSSANT, T. 2016. Mesocosm study with thermally treated (TCC) and water-based drill cuttings (WBM).

TRANNUM, H. C., SETVIK, Å., NORLING, K. & NILSSON, H. C. 2011. Rapid macrofaunal colonization of water-based drill cuttings on different sediments. *Marine pollution bulletin,* 62**,** 2145-2156.

TRUSSELL, S. & SPENCE, R. 1994. A review of solidification/stabilization interferences. *Waste management,* 14**,** 507-519.

TSAI, Y.-L. & OLSON, B. H. 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Applied and Environmental Microbiology,* 58**,** 754-757.

TUNCAN, A., TUNCAN, M. & KOYUNCU, H. 2000. Use of petroleum-contaminated drilling wastes as sub-base material for road construction. *Waste Management and Research,* 18**,** 489-505.

URUM, K. & PEKDEMIR, T. 2004. Evaluation of biosurfactants for crude oil contaminated soil washing Chemosphere.

VALLAEYS, T., TOPP, E., MUYZER, G., MACHERET, V., LAGUERRE, G., RIGAUD, A. & SOULAS, G. 1997. Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiology Ecology,* 24**,** 279-285.

VAN ELSAS, J., VAN OVERBEEK, L. & FOUCHIER, R. 1991. A specific marker, pat, for studying the fate of introduced bacteria and their DNA in soil using a combination of detection techniques. *Plant and Soil,* 138**,** 49-60.

VIDALI, M. 2001. Bioremediation: An overview. Pure and Applied Chemistry.

WILES, C. C. 1987. A review of solidification/stabilization technology. *Journal of hazardous materials,* 14**,** 5-21.

YAN, P., LU, M., GUAN, Y., ZHANG, W. & ZHANG, Z. 2011. Remediation of oil-based drill cuttings through a biosurfactant-based washing followed by a biodegradation treatment. *Bioresource Technology,* 102**,** 10252-10259.

YOUNG, C. C., BURGHOFF, R. L., KEIM, L. G., MINAK-BERNERO, V., LUTE, J. R. & HINTON, S. M. 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Applied and environmental microbiology,* 59**,** 1972-1974.

ZWART, G. & BOK, J. 2004. Protocol DGGE. *Netherlands Inst Ecol*.

APPENDIX A-Sediment lost during rinsing

W: weight of filter paper

1st rinse

W of filter paper 1: 0.1301 g

W of filter paper 2: 0.1309 g

W of filter paper 1 after drying: 0.1546 g

W of filter paper 2 after drying: 0.1595 g

Weight of sediment lost on filter 1:

(0.1546 -0.1301) g = 0.0245 g

Weight of sediment lost on filter 2:

(0.1595-0.1301) g = 0.0286 g

Average weight of sediment lost on 1st rinse

= (0.0245+0.0286)/2 g

= 0.0531/2

=0.02655 g

The rate at which sediment was lost in the 1st rinse:

0.02655g/100ml = $0.02655*10^{-2}$ g/ml

2nd rinse

W of filter paper 1: 0.1316 g

W of filter paper 2: 0.1305 g

W of filter paper 1 after drying: 0.1614 g

W of filter paper 2 after drying: 0.1576 g

Weight of sediment lost on filter 1:

(0.1614 -0.1316) g = 0.0298 g

Weight of sediment lost on filter 2:

(0.1576-0.1305) g = 0.0271 g

Average weight of sediment lost on 1st rinse

= (0.0298+0.0271)/2 g

= 0.0569 /2

=0.02845 g

The rate at which sediment was lost in the 2nd rinse:

0.02845g/100ml = 0.02845*10^{-2} g/ml

3rd rinse

W of filter paper 1: 0.1304 g

W of filter paper 2: 0.1330 g

W of filter paper 1 after drying: 0.1608 g

W of filter paper 2 after drying: 0.1554 g

Weight of sediment lost on filter 1:

(0.1608 -0.1304) g = 0.0304 g

Weight of sediment lost on filter 2:

(0.1554-0.1330) g = 0.0271 g

Average weight of sediment lost on 1st rinse

= (0.0304+0.0224)/2 g

=0.0264 g

The rate at which sediment was lost in the 3rd rinse:

0.0264g/100ml = 0.0264*10^{-2} g/ml

4th rinse

W of filter paper 1: 0.1304 g

W of filter paper 2: 0.1309 g

W of filter paper 1 after drying: 0.1570 g

W of filter paper 2 after drying: 0.1580 g

Weight of sediment lost on filter 1:

(0.1570 -0.1304) g = 0.0266 g

Weight of sediment lost on filter 2:

(0.1580-0.1304) g = 0.0271 g

Average weight of sediment lost on 4th rinse

= (0.0266+0.0271)/2 g

= 0.02685 g

The rate at which sediment was lost in the 4th rinse:

0.02685g/100ml = 0.02685*10^{-2} g/ml

Average rate of sediment lost = (0.02655+0.0285+0.0264+0.02685) *10^{-2} g/ml

= 0.1083/4 = 0.0271 g/ml

APPENDIX B-DGGE

Acquisition Information

Imager	Gel Doc™ XR+
Exposure Time (sec)	6.408 (Auto - Faint Bands)
Dark Type	Referenced
Ref. Bkgd. Time (sec)	20
Flat Field	Applied (Lens)
Serial Number	721BR14274
Software Version	5.2.1
Application	SYBR Green
Excitation Source	UV Trans illumination
Emission Filter	Standard Filter

Image Information

Acquisition Date	09.05.2018 10.17.08
User Name	Millennium
Image Area (mm)	X: 109.1 Y: 96.8
Pixel Size (um)	X: 137.9 Y: 137.9

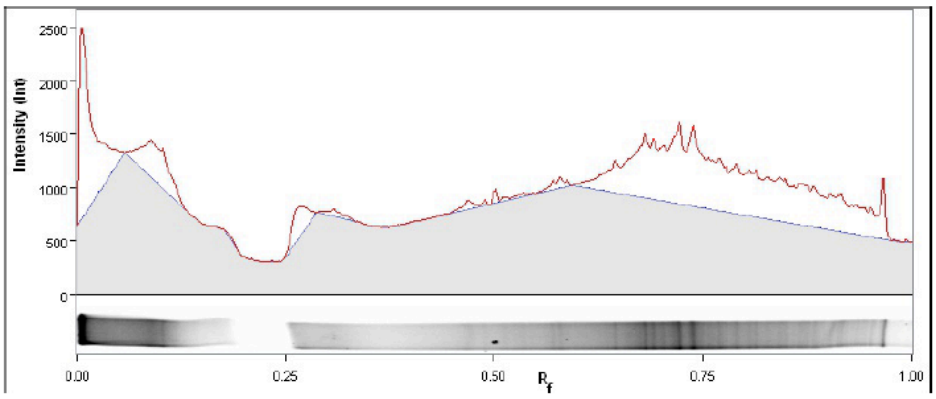
Data Range (Int)	9 - 4095

Analysis Settings

Detection	Lane detection: Manually created lanes Lane Background Subtraction: Lane background subtracted with disk size: 10 Lane width: Variable

Lane And Band Analysis

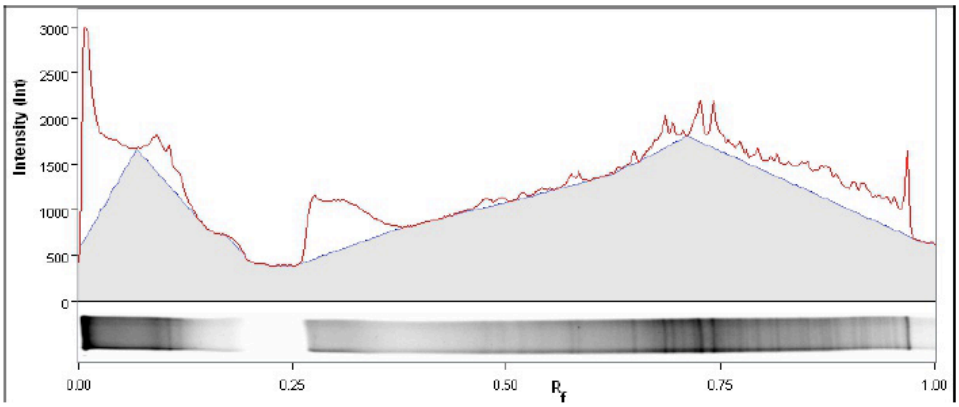
Lane 1



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	9.79 mm

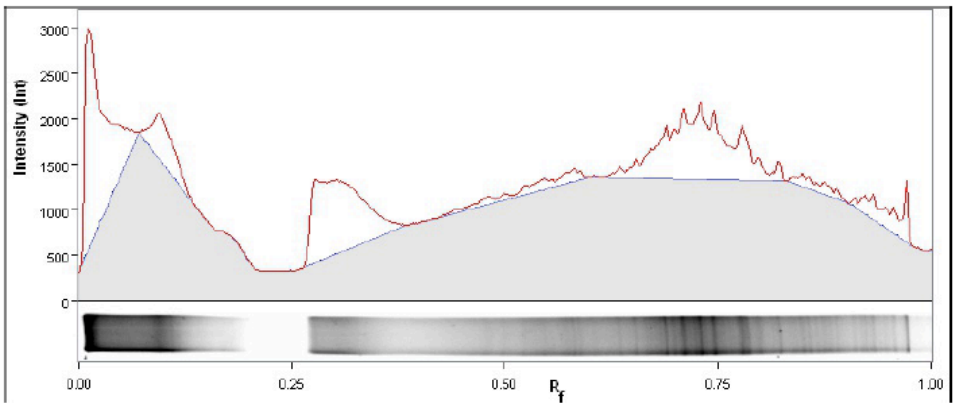
Lane 2



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	8.55 mm

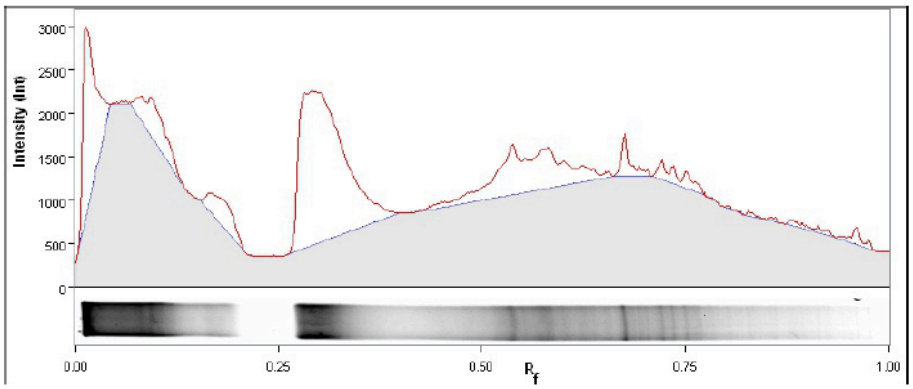
Lane 3



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	7.45 mm

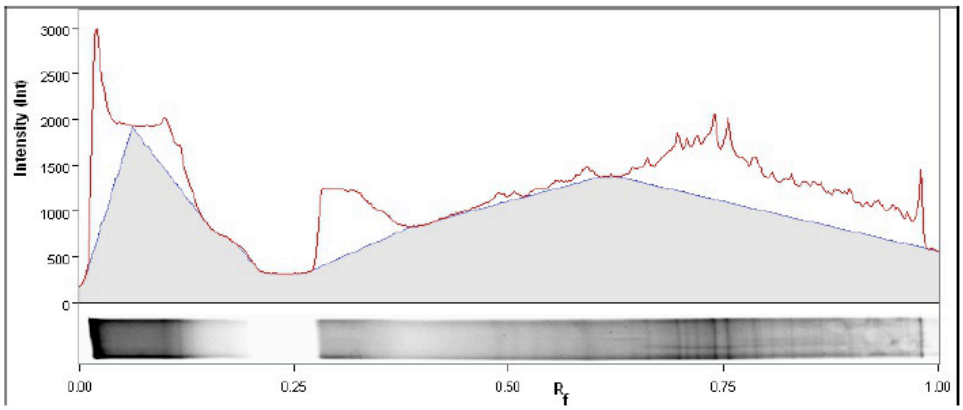
Lane 4



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	7.86 mm

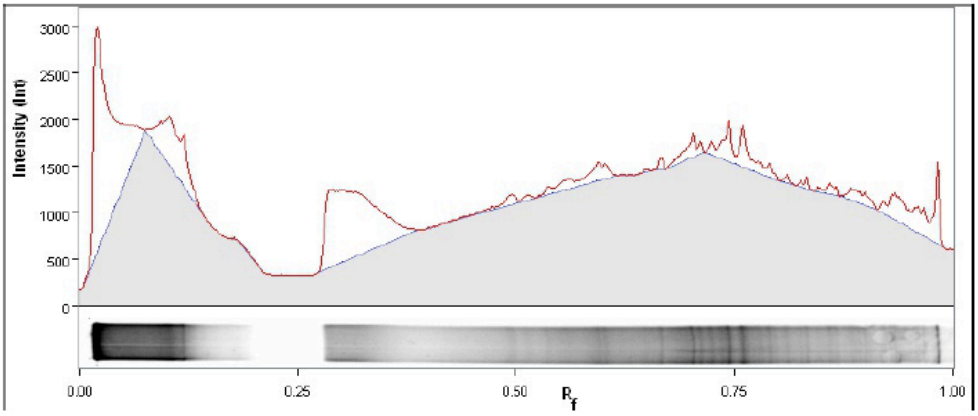
Lane 5



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	7.17 mm

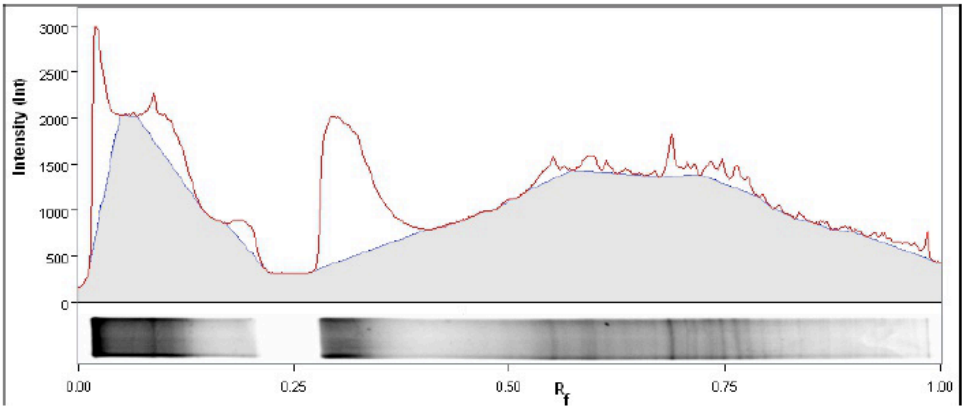
Lane 6



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	7.72 mm

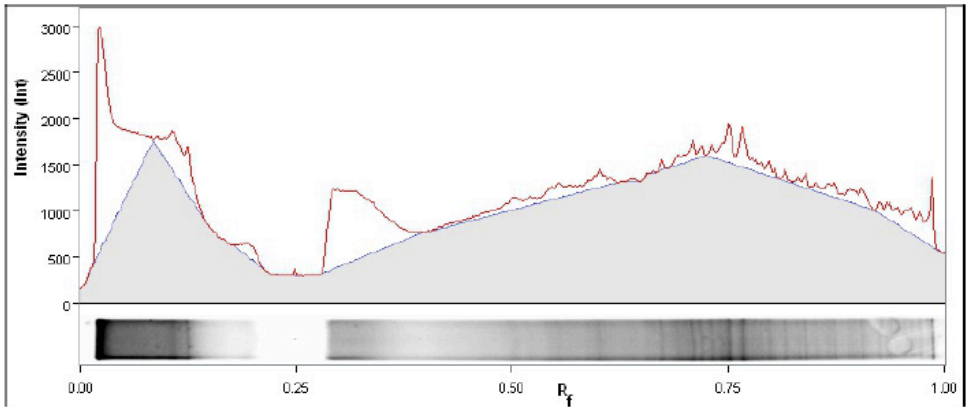
Lane 7



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	7.17 mm

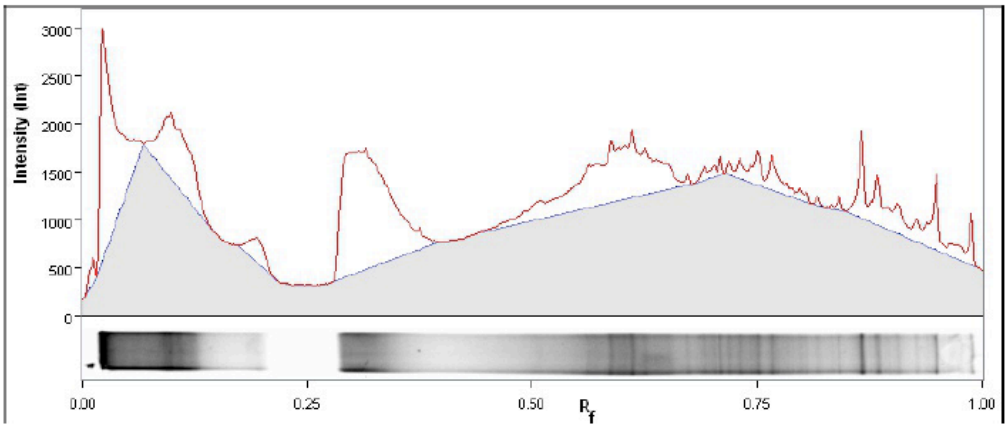
Lane 8



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	6.90 mm

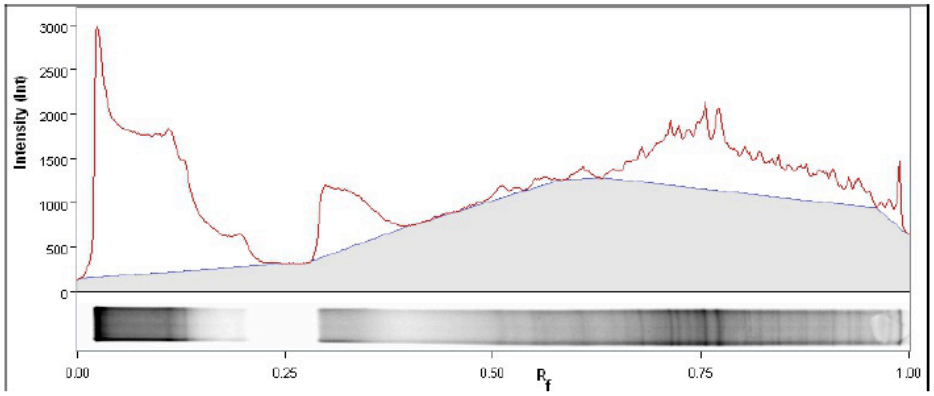
Lane 9



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	7.45 mm

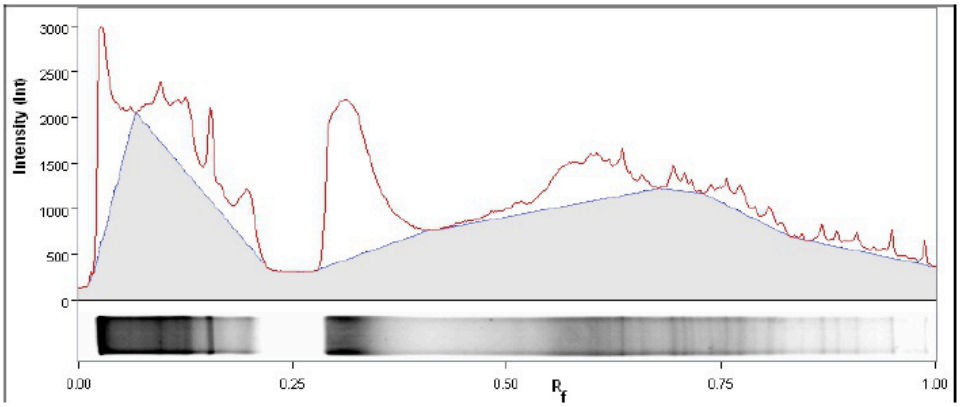
Lane 10



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	7.86 mm

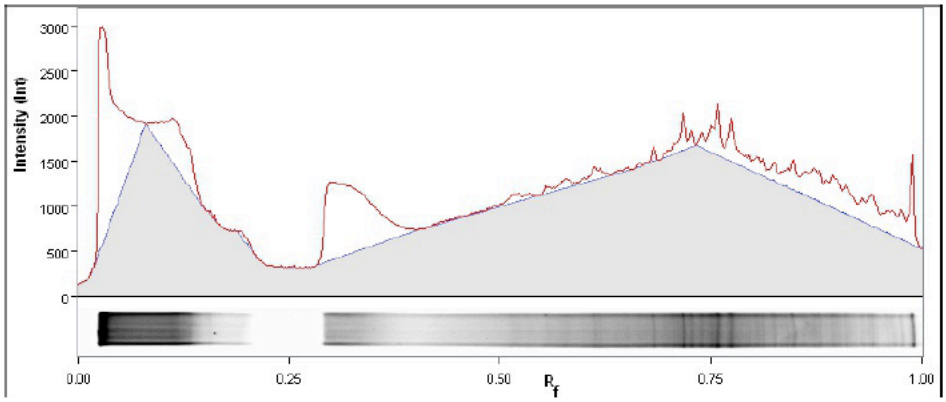
Lane 11



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	7.45 mm

Lane 12



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %

Lane Background	Lane background subtracted with disk size: 10
Lane Width	8.41 mm

APPENDIX C-Macrofauna

F1 UPL

species	number (n)	shannon index	shannon index calculations
Nematodes	4	=n1	-0,334239422
Polycheata	5	=n2	-0,355814957
Bivalves	5	=n3	-0,355814957
Sipunculida	4	=n4	-0,334239422
Sum	**18**	**=N**	**-1,380108757**
		H' =	**1.380108757**

F1 LL

species	number (n)	shannon index	shannon index calculations
Nematodes	5	=n1	-0,341686792
Polycheata	4	=n2	-0,315852967
Bivalves	4	=n3	-0,315852967
Sipunculida	6	=n4	-0,357932277
Ophiuroidea	2	=n5	-0,223940501
sum	**21**	**=N**	**-1,555265503**
		H'	**1.55265503**

	F10 UPL		
species	number (n)	shannon index	shannon index calculations
Nematodes	1	=n1	-0,366204096
Polycheata	2	=n2	-0,270310072
Bivalves			
Sipunculida			
Sum	**3**	**=N**	**-0,636514168**
		H'	**0.636514168**

		F10 LL	
species	number (n)	shannon index	shannon index calculations
Polycheata	1	=n1	-0,34657359
Nematodes	1	=n2	-0,34657359
Bivalves			
Sipunculida		=n3	
Sum	**2**	**=N**	**-0,693147181**
		H'	**0.69314781**

	F12 UPL		
species	number (n)	shannon index	shannon index calculations
Nematodes	2	=n1	-0,366204096
Polycheata	2	=n2	-0,366204096
Bivalves	1	=n3	-0,298626578
Sipunculida	1	=n4	-0,298626578
Sum	**6**	**=N**	**-1,329661349**
		H'	**1.329661349**

	F12 LL		
species	number (n)	shannon index	shannon index calculations
Nematodes	2	=n1	-0,357932277
Polycheata	2	=n2	-0,357932277
Bivalves			
Sipunculida	3	=n3	-0,363127654
Sum	**7**	**=N**	**-1,078992208**
		H'	**1.078992208**

	F14 UPL		
species	number (n)	shanno	shannon index calculations
Nematodes		=n1	
Polycheata	2	=n2	-0,366516293
Bivalves	3	=n3	-0,306495374
Sipunculida			
Sum	**5**		**-0,673011667**
		H'	0.673011667

	F14 LL		
species	number (n)	shannon index	shannon index calculations
Nematodes	1	=n1	-0,298626578
Polycheata	2	=n2	-0,366204096
Bivalves	1	=n3	-0,298626578
Sipunculida	2	=n4	-0,366204096
Sum	**6**	**=N**	**-1,329661349**
		H'	**1.329661349**

	F16 UPL		
species	number (n)	shannon index	shannon index calculations
Nematodes	1	=n1	-0,34657359
Polycheata	1	=n2	-0,34657359
Bivalves		=n3	
Sipunculida		=n4	
Sum	**2**	**=N**	**-0,693147181**
		H'	**0.693147181**

	F16 LL		
species	number (n)	shannon index	shannon index calculations
Polycheata	1	=n1	0
Nematodes		=n2	
Bivalves		=n3	
Sipunculida		=n4	
Sum	**1**	**=N**	**0**
		H'	**0**

	F20 UPL		
species	number (n)	shannon index	shannon index calculations
Nematodes	1	=n1	-0,366204096
Polycheata	2	=n2	-0,270310072
Bivalves		=n3	
Sipunculida		=n4	
Sum	**3**	**=N**	**-0,636514168**
		H'	**0.636514168**

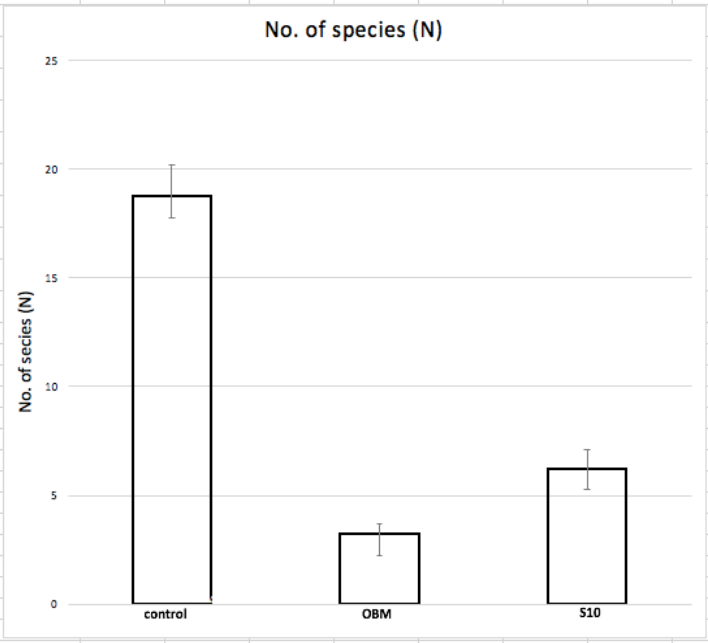
	F20 LL		
species	number (n)	shannon index	shannon index calculations
Nematodes	1	=n1	-0,366204096
Polycheata	1	=n2	-0,366204096
Bivalves		=n3	
Sipunculida	1	=n4	-0,366204096
Sum	**3**	**=N**	**-1,098612289**
		H'	1.098612289

	F2 UPL		
species	number (n)	shannon index	shannon index calculations
Nematodes	4	=n1	-0,340451525
Polycheata	4	=n2	-0,340451525
Bivalves	5	=n3	-0,35993395
Sipunculida	4	=n4	-0,340451525
Sum	**17**	**=N**	**-1,381288527**
		H'	**1.38**

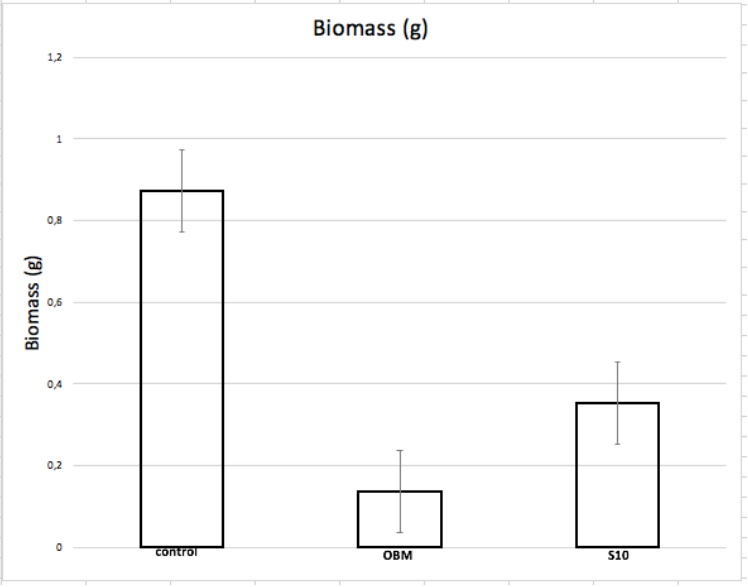
	F2 LL		
species	**number (n)**	**shannon index**	**shannon index calculations**
Nematodes	4	=n1	-0,328030446
Polycheata	5	=n2	-0,35131607
Bivalves	5	=n3	-0,35131607
Sipunculida	5	=n4	-0,35131607
Sum	**19**	**=N**	**-1,381978656**
			1.381978656

Appendix C 2-Macrofauna Analysis

	control	OBM	S10
	18	3	6
	21	4	7
	17	3	5
	19	3	7
mean	18,75	3,25	6,25
standard deviation	1,479	0,4330127	0,8292



	control	OBM	S10
	0,78	0,13	0,23
	1,18	0,15	0,38
	0,72	0,15	0,35
	0,81	0,12	0,45
MEAN	0,8725	0,1375	0,3525
STANDARD DEVIATION	0,2828	0,015	0,0918



APPENDIX D-Macrofauna survival rate

Control mean number of bands: 31

OBM mean number of bands: 20

S10 mean number of bands: 20.83

Assumption:

survival rate of the microbial communities in control= 100%

Mortality of microbial communities in control: 0%

Taking 31 as the total number of bands

Survival rate of microbial communities in OBM = 20/31 *100%

= 64.52%

Survival rate of microbial communities in S10 = 20.83/31 * 100%

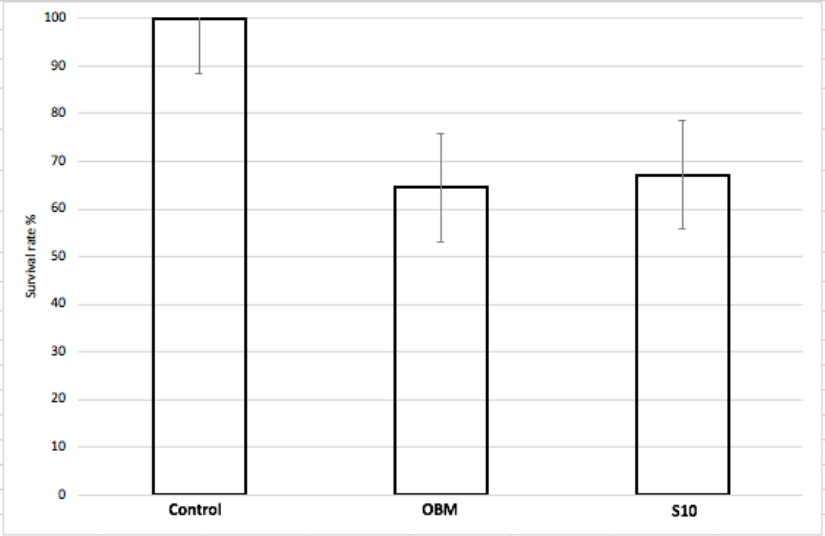
=67.20%

Mortality of microbial communities in OBM treatment = 100-64.52 = 35.48%

Mortality of microbial communities in S10 treatment = 100-67.20 = 32.80%

SURVIVAL RATE

	S10	OBM	control
	17	19	31
	16	20	31
	27	19	
	21	22	
	22		
	22		
AVG	20,8333333	20	31
percentage	67.2	64.52	100
stdev	3,97072621	1,41	0



APPENDIX E-Analysis of Variance (Anova)

treatment	code	Survival
control	1	31
control	1	31
OBM	2	17
OBM	2	16
OBM	2	27
OBM	2	21
OBM	2	22
OBM	2	22
S10	3	19
S10	3	20
S10	3	19
S10	3	22

Survival rate Anova

Dependent Variable:

Scheffe

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1	2	10,167*	2,507	0,009	2,85	17,48
	3	11,000*	2,659	0,008	3,24	18,76
2	1	-10,167*	2,507	0,009	-17,48	-2,85
	3	0,833	1,982	0,916	-4,95	6,62
3	1	-11,000*	2,659	0,008	-18,76	-3,24
	2	-0,833	1,982	0,916	-6,62	4,95

*. The mean difference is significant at the 0.05 level.

treatment	code	Number of sp
control	1	18
control	1	21
control	1	17
control	1	19
OBM	2	3
OBM	2	4
OBM	2	3
OBM	2	3
S10	3	6
S10	3	7
S10	3	5
S10	3	7

Number of species Anova

Multiple Comparisons

Dependent Variable:

Scheffe

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1	2	15,500*	0,825	0,000	13,09	17,91
	3	12,500*	0,825	0,000	10,09	14,91
2	1	-15,500*	0,825	0,000	-17,91	-13,09
	3	-3,000*	0,825	0,017	-5,41	-0,59
3	1	-12,500*	0,825	0,000	-14,91	-10,09
	2	3,000*	0,825	0,017	0,59	5,41

*. The mean difference is significant at the 0.05 level.

treatment	code	Biomass
control	1	0,78
control	1	1,18
control	1	0,72
control	1	0,81
OBM	2	0,13
OBM	2	0,15
OBM	2	0,15
OBM	2	0,12
S10	3	0,23
S10	3	0,38
S10	3	0,35
S10	3	0,45

Biomass Anova

Multiple Comparisons

Dependent Variable:

Scheffe

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1	2	,73500*	0,09316	0,000	0,4632	1,0068
	3	,52000*	0,09316	0,001	0,2482	0,7918
2	1	-,73500*	0,09316	0,000	-1,0068	-0,4632
	3	-0,21500	0,09316	0,123	-0,4868	0,0568
3	1	-,52000*	0,09316	0,001	-0,7918	-0,2482
	2	0,21500	0,09316	0,123	-0,0568	0,4868

*. The mean difference is significant at the 0.05 level.

Shannon-Wiener diversity index Anova

treatment	code	Shannon Wiener Index
control	1	1,38
control	1	1,55
control	1	1,36
control	1	1,39
OBM	2	0,64
OBM	2	0,35
OBM	2	1,1
OBM	2	0,69
S10	3	1,33
S10	3	1,08
S10	3	0,67
S10	3	0,85

Multiple Comparisons

Dependent Variable:

Scheffe

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	000000000*	0,175526984313587	0,008	0,212864431534518	1,237135568465480
	3	##########	0,175526984313587	0,094	-0,074635568465483	0,949635568465482
2	1	000000000*	0,175526984313587	0,008	-1,237135568465480	-0,212864431534518
	3	##########	0,175526984313587	0,309	-0,799635568465482	0,224635568465482
3	1	##########	0,175526984313587	0,094	-0,949635568465482	0,074635568465483
	2	##########	0,175526984313587	0,309	-0,224635568465482	0,799635568465482

*. The mean difference is significant at the 0.05 level.