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Bioremediation of diesel oil in marine environment

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Abstract. Natural gas emissions from oil spill ensue changes to microbial consortia in oceans which might cause ecotoxicological impacts on marine life. Gas flaring, a technique in the clean-up of oil spill, is a major source of greenhouse gas emission and possess high risk of fire hazard. It is of utmost importance to avoid flaring and resort to cleaner techniques such as bioremediation. The study focuses on bioremediation of marine oil spill by indigenous bacterial consortia using beeswax as a biostimulant which supplements the limiting nutrients such as nitrate and phosphate. The experimental study was conducted by adding diesel oil in marine water with beeswax for bioremediation. The vital parameters such as dissolved oxygen, pH, diesel range organics, total microbial count, nitrate and phosphate contents were measured at intervals of 5 days. The indigenous bacteria utilized oil as carbon source and beeswax as nutrient source for growth and metabolism. The results showed 87% removal of oil content in treatment sample while only 59% reduction was achieved in the corresponding control sample. Evaporation of oil results in formation of aerosols and black carbon which can lead to climate change. The study proves that bioremediation of marine oil spill is an environmentally benign clean-up technique for oil spill which can reduce carbon emission.

1 Introduction

Black carbon produced by flaring of oil spill has strong implications for climate change. It is short-lived with strong warming potential that can cause climate change. It absorbs sunlight more than it reflects which results in faster melting of ice caps. It also accounts for a quarter of observed warming and remediating the oil spill quickly is a means of mitigating the effects of climate change. Oil can be introduced into the ocean by natural, accidental and/or intentional spills.

Bioremediation employs microbial metabolism in the optimal environment with adequate nutrients to breakdown contaminants. Bioremediation is the process that utilizes bacteria, fungi and/or green plants to naturally remove contaminants. The two approaches to bioremediation are biostimulation and bioaugmentation. Zobel (1973) states that bioaugmentation is the introduction of bacteria to a spill site to supplement the indigenous microbial population in digesting the spilled petrochemicals. The other approach is biostimulation which is the introduction of nutrients and/or other growth enhancing substances to a spill site for the growth of indigenous microbes in order to accelerate the digestion of the spilled petrochemicals. The important factors influencing the efficiency of

bioremediation are the type of microorganisms present in marine water, characteristics of the oil such as chemical composition, sorption, bio-availability, concentration and environment surrounding the spill such as moisture content, pH, temperature and nutrients.

According to the Office of Technology Assessment (OTA), mechanical methods typically recover not more than 10–15% of the oil after a major oil spill (OTA, 1990). Unless the oil spill is contained in a boom it can drift very fast. Bioremediation is a promising technology for oil spill clean-up (OTA, 1991). Many of the components of oil are readily biodegradable in nature (Atlas, 1981, 1984; Prince 1993). Bioremediation is a safe cleanup method for marine oil spills (Prince and Atlas, 2016).

Studies on the British Petroleum oil spill reported injection of approximately 500 000 tons of gaseous hydrocarbons into the ocean and the gaseous emissions constituted 40% of the total hydrocarbon discharge causing tremendous atmospheric pollution (Joye *et al.*, 2011). Many researchers have studied various aspects of the tragic oil spill of Exxon Valdez in 1989, the use of bioremediation (Atlas & Bragg 2009; Bragg *et al.* 1994; McDonnell 1992; Peterson *et al.* 2003; Pritchard *et al.* 1992) and the influence of temperature on biostimulation of marine oil spill (Coulon *et al.*, 2007).

In-situ bioremediation was found to control many undesirable impacts of amendments on environment by conventional treatment methods (Brown *et al.*, 1998; Franzetti *et al.*, 2006; Joseph, 1998; Menendez-Vega *et al.*, 2007;

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Volkering *et al.*, 1997). Presence and activity of hydrocarbon degrading microorganisms were reported along the affected shorelines (Medina-Bellver *et al.*, 2005). Researchers used fertilizers for bioremediation of marine oil spill and found it to be effective as the fertilizers rich in nitrate and phosphate provided nutrients for the oil degrading microorganisms (Gallego *et al.*, 2008; Xu *et al.*, 2005). Dominant genera observed in the marine water are *Pseudomonas* spp. and *Bacillus* spp. (Chikere *et al.*, 2016). Catechol 2, 3 dioxygenases, a group of enzymes play important role in natural attenuation processes of hydrocarbon contaminated environment (Sierra-García *et al.*, 2014). Comprehensive treatment of petroleum hydrocarbon by eventual manipulation of factors affecting bioremediation is technically and economically feasible (Adams *et al.*, 2015).

Beeswax, a natural wax produced by honeybees, is used as a biostimulant for bioremediation of hydrocarbon in the present study. One kg of beeswax can store 22 kg honey (Coggshall and Morse, 1984). India accounts for the largest beeswax production of 22 000 tons, which is 33% of the global production (Sergeeva, 2017). Beeswax can absorb oil up to 20 times its weight. It contains nitrogen, phosphorus and potassium which are used as nutrient source for the bacteria. It consists of 15% hydrocarbon, 8% free acids remaining esters. The melting point ranges from 62 °C to 64 °C and density at 15 °C ranges from 958 to 970 kg/m³. Bioaugmentation could employ organisms that degrade hydrocarbons to be encapsulated in wax to form organism-containing wax microshells which are used for removing oil spills (JUSTIA, 2018). Bioremediation can be an effective, environmentally benign means of remediating oil spill in the marine water (Mathew *et al.*, 2019).

2 Study area and sample collection

The marine sample was collected offshore of Besant Nagar beach, Chennai, India. Composite grab sampling was used to collect 10 L of sample in a clean plastic container. The site of sample collection was devoid of any immediate effluent plume from local discharge. The sample was collected during summer when the Dissolved Oxygen (DO) is lowest in order to prove the suitability of beeswax in bioremediation of marine oil spill even in the worst scenario. The DO and pH of the sample was analysed on the same day of collection. The sample was stored at 4 °C for later analysis.

3 Methodology

A lab-scale experiment to validate the effectiveness of beeswax as a biostimulant in the bioremediation of marine oil spill was carried out using diesel oil. During combustion, diesel oil produces approximately 13% more CO₂ compared to petrol.

The collected marine water sample with diesel oil and beeswax as a nutrient source was taken as the treatment and the corresponding control consisted of marine water sample with diesel oil and without beeswax. The glass beakers with sample for the control and treatment were setup

as shown in Figure 1. Six samples each was used for treatment and control. Three samples were used for the analysis of Diesel Range Organics (DRO) corresponding to 5th, 10th and 15th day and the other three samples for parameters such as pH, DO, nitrate, phosphate and microbial count. The chemical formula for diesel oil ranges from C₁₀H₂₀ to C₁₅H₂₈, DRO is a good indicator of diesel content as it corresponds to the range of hydrocarbon from C10 to C28 (API, 1994).

Initial characterisation of the sample was carried out for vital parameters. 500 mL marine sample, 15 mL diesel oil and 5 g beeswax as pellets were added to the treatment beaker while no beeswax was added to the control. Vital parameters such as DO, pH, nitrate, phosphate, and total microbial count were analysed at intervals of 5 days for 15 days. All analyses were in accordance with the American Public Health Association (APHA, 2005) standard procedure. In addition, DRO was also analysed during the same interval. DRO was analysed using Gas Chromatography-Mass Spectrometry (GC-MS). Another two samples were used for the analysis of oil content in the control and treatment at the end of the experiment to show the effective removal of diesel oil by evaporating the sample and measuring the remaining oil content using Soxhlet Extraction Method (Standard Methods American Water Works Association 5520 part D). Oil content was also analysed initially in the raw marine sample to determine the presence of any oil content. Average of three samples was obtained and represented graphically.

4 Results and discussion

The values of various parameters obtained in the experimental study is tabulated (Tab. 1). Significant reduction in DRO and oil content was observed in treatment compared to control after 15 days of experiment.

4.1 pH

The pH of the samples varied between 6.64 and 7.72 in both treatment and control (Fig. 2). The optimal pH for the growth of microorganism to degrade hydrocarbon ranges from 6 to 8 (Leahy & Colwell, 1990). The pH was within optimal range for the degradation of oil by microbes.

4.2 Dissolved oxygen

The initial DO of marine water sample was 6.5 mg/L which correlates with the DO of marine water at 35 °C. The oil degrading bacteria that utilise oil as their carbon source are mostly aerobic and require DO for cellular respiration. The DO continues to reduce as no aeration is given to both the treatment and control. The DO in treatment reduces steadily which corresponds to oxygen consumption by aerobic oleophilic bacteria signifying the degradation of oil. (Fig. 3). At the end of 15-day experiment, the DO reduced to 4.9 mg/L which is adequate for the growth of microbes. In control the DO is lower than treatment initially which indicates the high oil content that does not

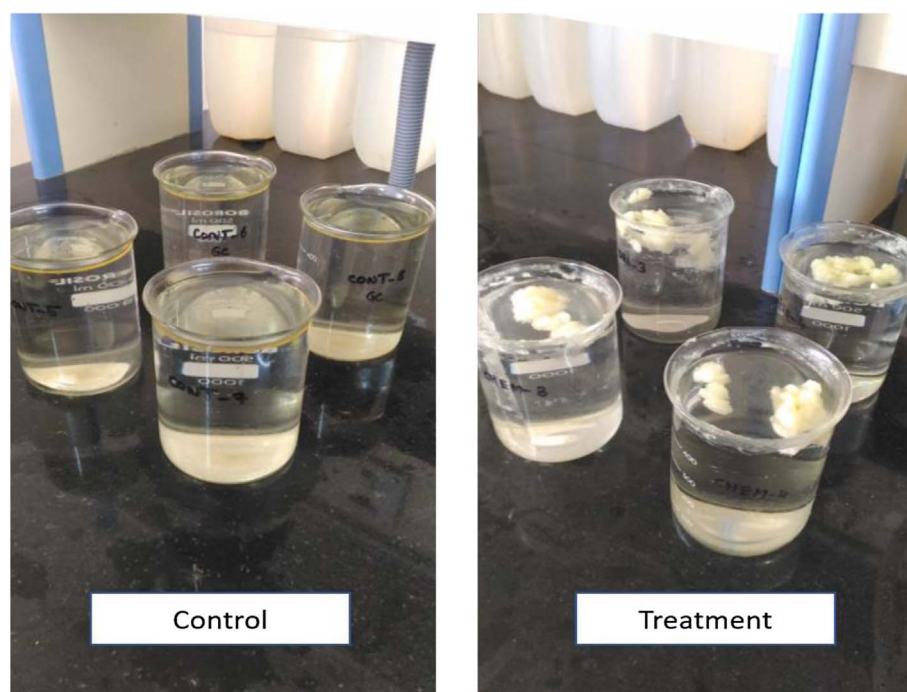


Fig. 1. Experimental Setup.

Table 1. Values of various parameters obtained in the experiment.

Parameter	Unit	Raw sample	5 th day		10 th day		15 th day	
			Control	Treated sample	Control	Treated sample	Control	Treated sample
pH		6.77	7.72	6.77	6.83	6.64	7.08	6.8
DO	mg/L	6.5	5.8	6.3	5.7	5.8	5.1	4.9
NO ₃	mg/L	1.285	1.16	1.21	1.085	BQL (LOQ:1)	BQL (LOQ:1)	BQL (LOQ:1)
PO ₄	mg/L	1.512	1.311	2.983	0.578	2.7	BQL (LOQ:0.2)	0.55
Microbial count	CFU/mL	410	120	2200	90	1900	70	1800
DRO	ppm		1361	1252	877	538	365	98
Oil content	mL	15					6.2	2

BQL: Below quantifiable limit; LOQ: Limit of Quantification.

actively degrade as in treatment, thus limits the oxygen transfer from the atmosphere to the water.

4.3 Nutrients – Nitrate and Phosphate

The beeswax added after initial characterization of the marine water sample is the source of nitrate and phosphate which are essential nutrients for the growth of indigenous microbes. The sample had an initial nitrate content of 1.285 mg/L (Fig. 4). After the addition of beeswax, the nitrate reduced significantly in the treatment within 5 days and the nitrate concentrations for 10th and 15th day were below detection limit (limit of detection: 1 mg/L). This is

due to the rapid consumption of nitrate by the proliferating indigenous microbes and the growth rate increased by 5 folds in 5 days. Later the microbial count reduced due to the deficit of nitrate. The initial concentration of phosphate in the marine water sample was 1.5 mg/L. The addition of beeswax increased the phosphate content in the treatment initially which depleted slowly as only 1 part of phosphate is consumed for every 10 parts of nitrate. It reduced after 10th day as there was no further addition of beeswax (Fig. 5). In the control, phosphate steadily reduced and was below detection limit on the 15th day (Limit of detection: 0.2 mg/L). The ratio of nitrate to phosphate required for the optimal growth of bacteria is 10:1.

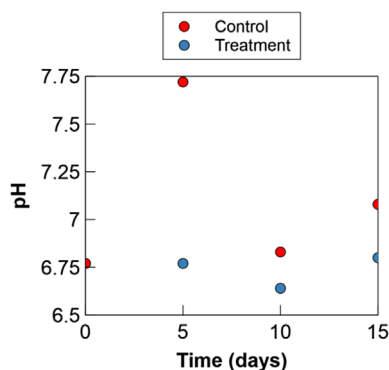


Fig. 2. Temporal variation of pH.

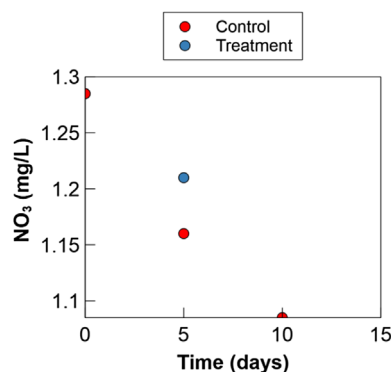


Fig. 4. Temporal variation of nitrate concentration.

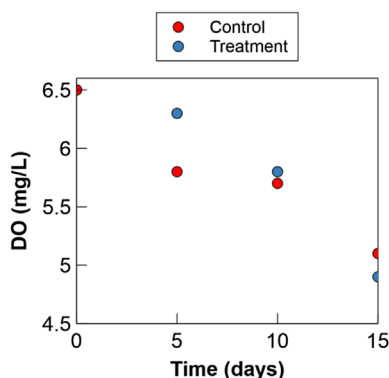


Fig. 3. Temporal variation of DO content.

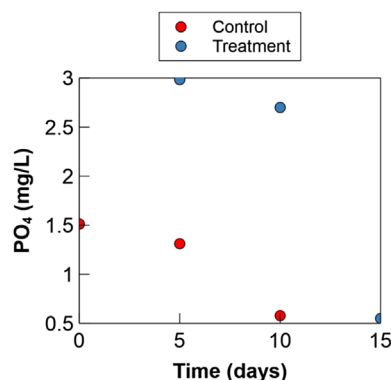


Fig. 5. Temporal variation of phosphate concentration.

4.4 Total Microbial Count

The dominant species of bacteria present in marine sample are *Bacillus* spp. and *Pseudomonas* spp. Microbial count measured as Colony Forming Units (CFU) increased from 410 to 2200 CFU/mL (Fig. 6) in the treatment which is a five-fold increase during the initial 5 days due to the abundance of nutrients from beeswax and the presence of DO in the sample but after 5th day, the microbial population reduced slowly in the treatment. On the other hand, it reduced very rapidly in the control and was 70 CFU/mL on 15th day as the nutrient source and dissolved oxygen was limiting. The nitrate and phosphate are consumed by the bacteria for their growth and metabolism. The aerobic bacteria consume DO for their cellular respiration and oil as their carbon source. The significant difference in the microbial count in treatment and control indicates the importance of nutrients for the growth of microbes which could be supplemented by addition of biostimulant such as beeswax. Hence optimal nutrient level by insitu biostimulation aids in the bioremediation of marine oil spill.

4.5 Diesel Range Organics

Diesel Range Organics (DRO) is used as a specific indicator of diesel oil in marine water. It was found to reduce significantly in the treatment to 98 ppm after 15 days whereas in

the control it reduced only to 365 ppm (Fig. 7). The reduction was more prominent in treatment due to the profuse growth of oleophilic microbes resulting from the addition of biostimulant namely beeswax leading to the bioremediation of diesel oil.

4.6 Oil content

The oil content in the initial marine water sample, measured using soxhlet extraction method, was below detection limit. 15 mL of diesel oil was added to marine water in treatment and control. The oil content reduced to 2 mL and 6.2 mL in the treatment and control respectively after 15 days of treatment (Fig. 8). The reduction in oil content was measured by evaporating the sample in a rotary evaporator. The treatment has a removal efficiency of 87% while in control only 59% oil removal was achieved. In control, the hydrocarbons in oil can undergo phase transformation by evaporation, dissolution and dispersion. It results in the formation of aerosols which could lead to smog and their presence in the atmosphere may cause global warming thus effect the air quality. Studies by Butler *et al.* (1976) showed that 25% of the crude oil was evaporated naturally within 10 days. The components of oil could accumulate in food chain thus adversely affecting the aquatic environment and human health. Hence it is important to remediate marine oil spill.

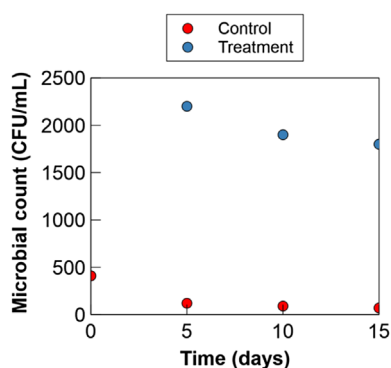


Fig. 6. Temporal variation of microbial count.

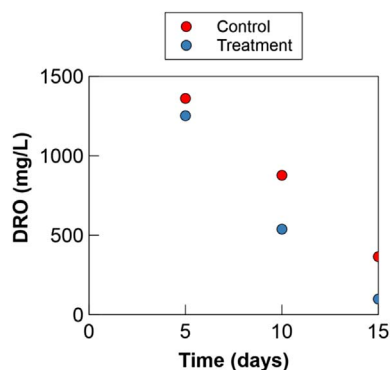


Fig. 7. Temporal variation of Diesel Range Organics.

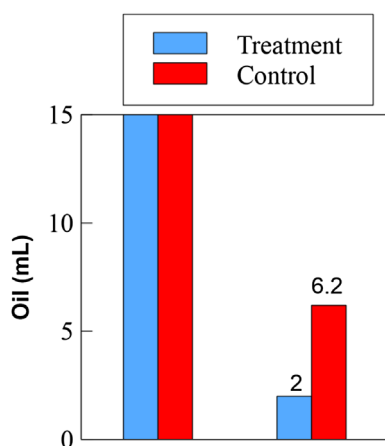


Fig. 8. Reduction in Oil Content during the experiment.

5 Conclusion

Bioremediation is an effective technique in the clean-up of petrochemical spills because it is a process of naturally converting petrochemicals into non-toxic by-products without any further local environmental disruption using microorganisms. Beeswax, a biostimulant, provides bacteria the nutrients required for its growth and metabolism. The

microbes use hydrocarbon in oil as the carbon source while dissolved oxygen is used for the cellular respiration. Nitrate and phosphate are limiting in marine environment which needs to be supplemented for the rapid growth of microbes. In the present study, the addition of beeswax resulted in five-fold increase in the microbial count by the consumption of nutrients in beeswax. The experimental study conducted on the bioremediation of marine oil spill using beeswax achieved 87% removal of oil in 15 days while only 59% was removed in control. The diesel range organics reduced more in treatment than control. The removal of oil in the control is mainly due to evaporation, dissolution and sedimentation which can cause atmospheric pollution, global warming and climate change, whereas in treatment the hydrocarbon is consumed by oleophilic microbes without producing any harmful by-products. Hence bioremediation is advantageous over conventional methods, in terms of pollution abatement, cost effectiveness, insitu and environmentally benign treatment.

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