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Oil degrading and heterotrophic bacteria composition in the oil-spilled affected mangrove forest sediment in Mactan Island, Central Philippines

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Abstract

Oil spill increases the amount of hydrocarbons in marine ecosystems that disrupts the ecological balance with severe economic consequences. This study was conducted to assess the microbial composition in the sediments of oil affected areas in Mactan Island, Cebu eight months after the incident. Four sites designated as S1 (low) , S2 (mid) and S3 (high) representing oil spill contamination gradient were established. A reference site (Ref) was selected. Sediment samples from each site were collected for microbial count enumeration (heterotrophic and oil degrading bacteria) using the spread plate technique and the MPN method respectively. An increasing pattern of heterotrophic bacteria and decreasing trend of oil degrading bacteria counts were observed along S1, S2 and S3. Results revealed the heterotrophic bacteria count in all sites did not vary significantly. In addition, Ref exhibited highest and lowest count for heterotrophic and oil degrading bacteria respectively whereas S3 and S2 displayed highest in density for the oil degrading bacteria. The proliferation of oil degrading microbe implies the possible presence of oil residues including those PAH components that gets more toxic through time.

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Introduction

Oil spill increases the amount of hydrocarbons in marine ecosystems that disrupts the ecological balance with severe economic consequences. In August 2013, M/V Saint Thomas Aquinas collided with M/V Sulpicio Express Siete at 1.9 km from the coastal area of Talisay, Cebu. The incident resulted to the leakage of 120 000 L bunker fuel, 20 000 L diesel fuel and 20 000 L lube oil. The southwest monsoon wind carried leaked oil to the coastal areas in thesouthern parts of Mactan Island. This affected the mangrove areas, aquaculture ponds and intertidal areas which may result to disruption of ecological processes and economic loss. Recent studies suggestthat microorganisms are the chief agents for the biodegradation of polyaromatic hydrocarbons (PAHs) in aquatic and terrestrial environments (Alexander *et al.,* 1982; Swanell and Head, 1994). These microbes feed on oil and refined oil products, gaining energy for growth and reproduction by breaking down the hydrocarbons. Bacteria and yeast appear to be the dominant degraders in aquatic ecosystems. It is widely accepted that no single microbial species will completely degrade any particular oil (Cooney and Summers, 1976). Buried oil persists for a long time in deeper sediment layers where there is deficient oxygen and only anaerobic microbes can survive. Thus, the recovery or active process of oil degradation is a function of microbial interaction and density.

Hence, this study was conducted to assess the microbial composition in sediments of oil spilled areas and compare it with a reference site. Specifically this study aimed to: (1) measure the microbial count of heterotrophic and oil degrading bacteria present in the sediment; (2) characterize the bacteria in terms of morphology and biochemical test, and (3) compare the relative density and relative composition of the oil spill affected sites with that of the reference site.

Materials and methods

Study Sites

Three study sites from oil spill affected areas, designated as S1 (10°15'2.24"N 123°57'33.43"E), S2 (10°17'9.93"N 123°55'19.05"E) and S3 (10°15'12.85"N 123°55'9.55"E), were selected based on the extent of the oil spill impact (Fig. 1). These were the coastal barangays of Alegria, Calawisan and Day-as, which were identified to have a low, mid and high oil spill impact, respectively. The 3 sites are characterized by reforested mangrove community dominated by *Rhizopora* sp. Patches of *Avicennia* and *Sonneratia* species were also evident in all sites. Even without the oil spill incident, this part of Mactan Island is also at high risk of chronic exposure from small-scale oil and grease contamination. The decommissioned old oil depot and the Mactan Channel that is heavy in maritime activities are located less than a km away from Calawisan's intertidal mangrove forest. A large tract of the reef backwater in the area has been reforested with mangrove saplings while the majority of the reef flat depressions are home to seagrass and seaweed beds. This side of Mactan Island is also fringed with coral reef. Since the most visible impact in terms of the presence of oil is on mangrove sediment, the study focused primarily on the microbial component of this habitat. The reference site, (Ref, 9°45'48.05"N 123°32'28.86"E) which is about 97 km away from the oil spilled areas was designated in Dalaguete, Cebu. The natural growth mangrove in the reference site is a fringing type and is composed primarily of *Sonneratia* and *Avicennia sp.* while the reforested mangrove is planted with *Rhizophora mucronata*. The reforested mangrove is already larger in area coverage compared to the natural growth. The sediment is sandly to silty loam in texture. The area is not away from any heavy navigational activities.

Sample Collection and Processing

Sediment samples at about 0-5 cm in depth were collected using a sterile spatula and transferred into a sterile falcon tubes. Sediment samples were collected from each of the 3 sampling quadrats along a transect. These were mixed as the composite sediment samples and served as the source of inocula for microbial enumeration. There were 3 transects done per site. All samples were kept in a container with ice and transported into the laboratory for

further processing and analysis. About 5 g of sediment from each sample were suspended in 45 mL of 0.1% Tween 80. The mixtures were subjected to shaking at 200 rpm for 30 min. Serial dilutions were made with sterile saline solution (0.85%).The spread plate technique was employed to enumerate heterotrophic bacteria (HB) counts using a Marine Agar. Three replicates were prepared from each category and incubated at 29°C for 48 hours. Hydrocarbon-degrading bacteria (DB) were estimated by serial dilution in tubes with 10 mL of mineral medium (MM) with crude oil using the MPN method. The mineral medium composition per 100ml was

prepared as follows: 0.45g K_2HPO_4; 0.02g $MgSO_4 .7 H_2O$; 0.01g $CaCl_2$; 0.01g NaCl; 0.0002g $FeCl_3$; 0.01g$(NH_4)_2SO_4$ and 5 ml crude oil. Filtered-sterilized crude oil was aseptically added as the carbon source. The media was then inoculated and incubated for 7 days at 29°C. After incubation, 50 mL aliquots from growth-positive tubes were inoculated in MM plus crude oil and incubated for further 7 days at 29°C. Sub-samples (1 mL) from the MPN tubes were taken and plated onto Tryptic Soy Agar (Difco). Isolates were described as petroleum degraders after growth in media with crude oil as the sole carbon source.
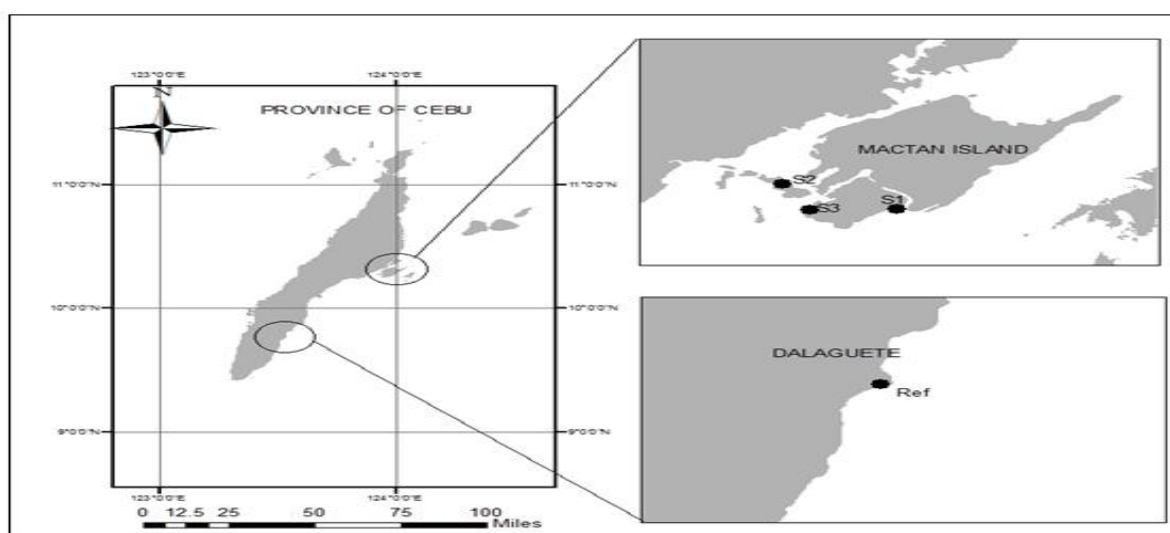
Fig. 1. Map of the sampling sites.

Data Analysis

Descriptive statistics was employed to determine the mean, standard deviation, standard error of the mean (SEM), the range and the distribution of the data. All averaged values were reported as mean ± SEM. Levene's test was employed to test the homogeneity of the variance. If the variance was homogenous, comparison was carried out using ANOVA test to determine if there was a significant difference within groups, otherwise a robust test (i.e. Welch test) was used. Multiple comparison was also employed to determine if there was a statistical difference between groups; posthoc tukey multiple comparison was used if the variance was homogenous, otherwise the Tamhane's multiple comparison test. All statistical analyses were done with 95% confidence interval.

Results

Microbial Count

The results of the microscopic analysis of the bacterial smear from the composite samples showed cocci and bacilli bacterial morphology from S1, S2, S3 and Ref. Correspondingly, the bacterial smears tested positive for catalase and oxidase biochemical reactions. Further, gram stain test imparted negative for most of the bacterial samples.

The results of the microbial count from Ref contained the greatest number of heterotrophic bacteria and the least number of oil degrading bacteria (Fig. 2). There is no significant difference($p \geq 0.05$) for the heterotrophic bacteria count in all sites. However the microbial count for the Oil degrading bacteria in S1,

S2 and S3 sites differ significantly with each other (p=0.031).

The total bacterial count (Fig. 2) in Ref is significantly higher compared to S1, S2 and S3. However there is no significant difference (p≥0.05) between the S2 and S3 sites.
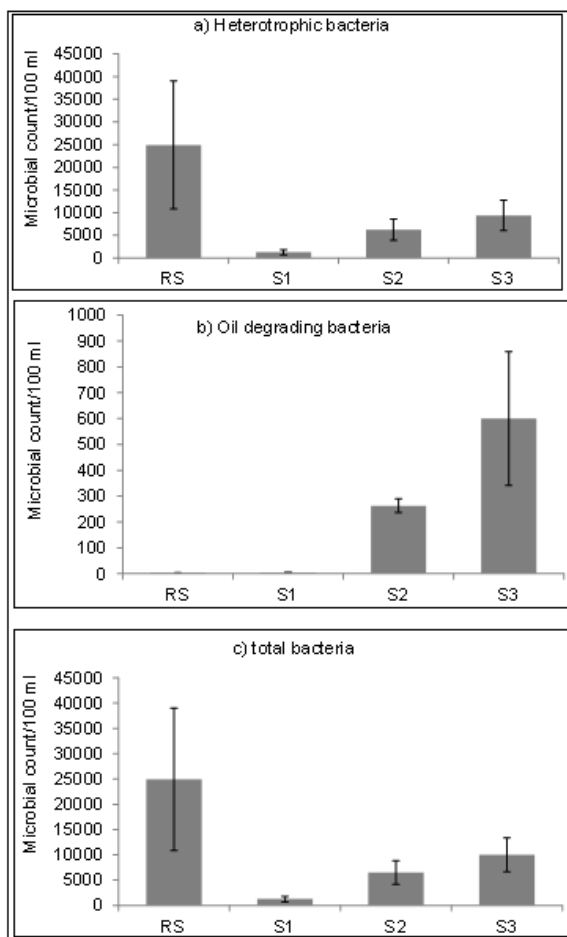
Fig. 2. Microbial Count/100 ml of a) heterotrophic bacteria b) oil degrading bacteria and c) total bacterial count.

Relative Density and Composition

There is greater density in terms of the heterotrophic bacteria in Ref and in S1 compared to S2 and S3. In contrast, the results from the oil degrading bacteria showed highest density in S3 and S2 sites respectively compared with S1 and Ref (Fig.3).

The relative composition for the oil degrading bacteria is higher in S3 and S2 sites compared with S1 and Ref respectively (Fig. 4). Contrastingly, Ref and S1 showed greater abundance for the heterotrophic
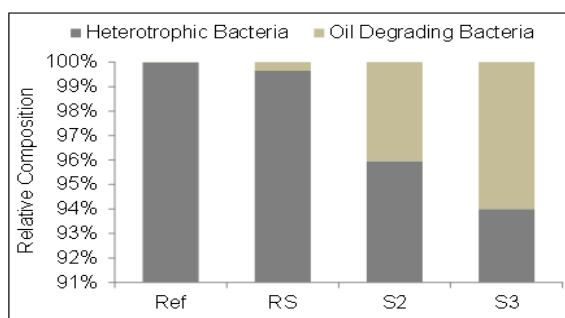
bacteria.

Discussion

There is no single bacterium capable of degrading all the oil components. We observed that there were differences in the density of heterotrophic bacteria and oil degrading bacteria between the different sites and the reference site. Oil degrading bacteria or hydrocarbon degrading bacteria are ubiquitous in areas when after an oil spill has occurred. The influx of oil causes the immediate increase in the number of hydrocarbon degrading populations. The degradation of both crude and refined oils seems to involve a consortium of microorganisms, including both eukaryotic and prokaryotic forms. The most common genera known to be responsible for oil degradation comprise mainly *Nocardia, Pseudomonas, Acinetobacter, Flavobacterium, Micrococcus, Arthrobacter, Corynebacterium, Achromobacter, Rhodococcus, Alcaligenes, Mycobacterium, Bacillus, Aspergillus, Mucor, Fusarium, Penicillium, Rhodotorula, Candida* and *Sporobolomyces*, (Atlas, 1981; Bossert and Bartha, 1984; Atlas and Bartha, 1992; Sarkhoh *et al.,* 1990). Several studies have also demonstrated that there was an increase in oil degrading bacteria in sediments (Venosa *et al.,* 1996) after the contamination of oil. Only *Acinetobacter* species was found in the sediments from the oil affected sites. This is partly due to the selective medium which only catered for the growth of *Acinetobacter* species. Results implied that the sites are still contaminated with PAHs. The study of Mcnaughton *et al.,* (1999) showed that microbial communities within contaminated ecosystems were found to be dominated by competitive organisms that utilize available nutrients and thrive on toxic elements. Further, they noted that the presence of these toxic chemicals lead to the increase of the complexity of microbial communities which shifted from hydrocarbon degrading to heterotrophic bacteria. Consequently, this would explain the higher density of oil degrading bacteria in the oil affected sites. The absence of oil degrading bacteria in the reference site would further validate that they will proliferate suitably under high PAH concentration.

Isaac *et al.* (2013) cited that microbial communities found in PAH-contaminated soils are dominated by the microorganisms that can utilize them as carbon and energy source.
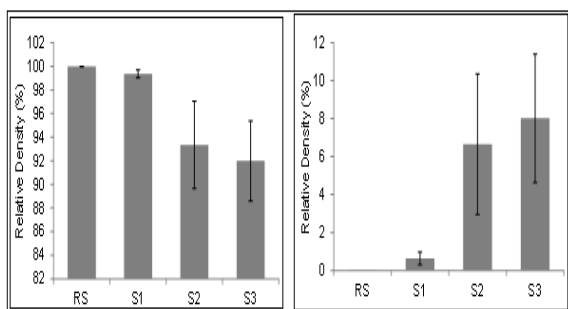
Fig. 3. Relative density of a) heterotrophic bacteria b) oil degrading bacteria.

The results reaffirmed the drastic change in the microbial community structure as observed after the British petroleum oil spill in the Gulf of Mexico. Pre-oil spill samples contained microorganisms that exhibit high levels of interactions. The biochemical tests revealed that the bacterial species tested positive for catalase and oxidase. Morphologically, these species appeared in cocci or bacilli form. The Gram stain test rendered red to pinkish color, indicating a negative result. These were the common characteristics exhibited by both heterotrophic and oil degrading bacteria.

Fig. 4. Relative composition of heterotrophic and oil degrading bacteria.

Recommendation

Further investigations should be conducted to understand the microbial successions in oil spill polluted sites to address the bioremediation of these areas. Assessment of the kind of oil residues present in the study sites should also be considered to determine the relationship of the kind of oil degrading bacteria that are responsible for the kind of degradable oil residues. In addition, characterization of the microbial load will be beneficial to indicate the possible long term effects of the oil spill not only in coastal communities but in other areas in general.

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