The Effect of Biostimulation and Biostimulation-Bioaugmentation on Biodegradation of Oil-Pollution on Sandy Beaches Using Mesocosms

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Abstract
To investigate a suitable biological remediation approaches for anticipating oil spills in Cilacap sandy beach (Indonesia), some alternative strategies using biostimulation and a combination of biostimulation-bioaugmentation have been evaluated in intertidal near shore Cilacap, Indonesia. The purpose of the study was to compare the efficacy of biostimulation using slow release fertilizer (SRF) only, combination of biostimulation-single strain bioaugmentation, and combination of biostimulation-consortium bioaugmentation, to enhance oil degradation. The experiment was conducted using sediment polluted 100,000 ppm Arabian Light Crude Oil in a mesocosm system for 90 days. The parameters measured were oil depletion, bacterial growth, and changes in environmental conditions. The results showed that the affectivity on oil depletion of biostimulation-bioaugmentation combination was observed faster and higher than biostimulation only. At the 16th day application, the biostimulation with the added consortium and single strain treatment, increased oil depletion percentage by 2.2 and 1.6 times that of the control, respectively. For a longer period of treatment, both of combination treatments showed similar efficacy in degrading oil contamination in sandy beach. It is proposed that combination of biostimulation-bioaugmentation with the consortium is relatively better alternative for combating oil-pollution for a short period.

Keywords
Biostimulation; Bioaugmentation; Oil Spill; Oil Degradation; Sandy Beach; Mesocosm

1 Introduction
The need for reliable and efficient oil spill cleanup techniques seems to be inevitable as the demand for liquid petroleum increases. Bioremediation can be considered as one of the best alternative as it is, an effective and economical solution method to remove crude oil from a contaminated marine environment in an integrated environmental restoration effort (Philp et al., 2005). There are two main approaches to oil spill bioremediation which are bioaugmentation and biostimulation. Bioaugmentation involves the addition of oil degrading bacteria to supplement the existing microbial population. Biostimulation involves the addition of nutrients, or growth-enhancing co-substrates, which improve habitat quality to stimulate the growth of indigenous oil degraders (Lee and Merlin, 1999).

Some biostimulation studies have proved that the growth of oil-degrading bacteria and oil degradation can be strongly enhanced by fertilization with inorganic Nitrogen and Phosphorous (Bragg et al., 1994; Swannell et al., 1996; Röling et al., 2002). A previous study on the Indonesian coast showed that the organic nutrient (Petroganik) stimulates soil microorganisms in an oil-polluted environment (Munawar et al., 2007) and Osmocote (slow release fertilizer) enhanced oil-degradation in Seribu Island (Darmayati, 2010). To develop bioremediation techniques for Indonesian coastal areas, a selection of alternative inexpensive slow release fertilizers made from local products for the purpose of enhancing oil degradation has been conducted in the laboratory (unpublished data).

Bioaugmentation has been considered as a potential strategy for oil bioremediation since the 1970s, although hydrocarbon-degrading microorganisms are also widespread in nature (Zhu et al., 2001). The efficacy of microorganism addition to oil-polluted environments is still debatable. Some of them have
shown the capability to degrade petroleum in the laboratory and under controlled conditions (Aldrett et al., 1997; Raghaven and Vivekanandam, 1999), and other study showed no significant result in enhancing biodegradation rate (Simon et al., 2004). Selected oil-degrading bacteria from Jakarta Bay have been shown to enhance oil degradation in the laboratory (Darmayati, 2009; Hatmanti et al., 2009; Teramoto et al., 2011). However, the efficacy of bacterial application (bioaugmentation) in the field was still questionable for Indonesian marine waters. Laboratory assays cannot always be extrapolated to the field unless there are parallel larger-scale outdoor experiments. Mesocosm experiments are a logical intermediate step between in-vitro findings and full-scale application (Santas et al., 1999).

Until now, there has been almost no implementation of bioremediation techniques at oil-polluted sites on the coast of Indonesia. Therefore, we investigated suitable biological remediation approaches for anticipating oil spills in Indonesian sandy beach. Cilacap has been used as an experimental site due to being one of the areas where most oil spills occurred. Based on data from various sources, during the period 1989-2011, there were 16 cases of oil spills occurred in Cilacap area. These were 12 cases due to tanker accidents and 4 cases due to leaked pipes (Mauludiyah, 2012). A locally produced Slow Release Fertilizer and indigenous bacteria (Alcanivorax sp. TE9, Pseudomonas balearica st 101 and RCO/B/08015) from Indonesian oil-polluted waters have been used for this experiment. The purpose of the study was to compare the efficacy of using fertilizer only (F biostimulation), a combination of a fertilizer and a single strain (FSC biostimulation-single strain bioaugmentation), also a combination of a fertilizer and a consortium (FCC biostimulation-consortium bioaugmentation) for degrading oil in a mesocosm experiment.

2 Results
2.1 Oil degradation
Cilacap sandy beach exhibited a natural ability to clean itself from oil spilled at the level of 100.000 mg/kg (Figure 1). During a 90 day experiment, the oil has decreased about 60% (60.31± 3.93 %). The oil-depletion percentage increased almost 10 % higher than the control (69.21±2.36%) when biostimulation only (F) was applied, at a rate of 68 gr fertilizer/kg wet weight sediment (15 g N/kg). However, biostimulation (7.5 g N/kg wet weight sediment) which was combined with bioaugmentation (single strain or consortium) showed a better performance with a degradation percentage at the level of 18.5 - 20 % higher than control (78.51 ± 3.86% - 80.08 ± 2.6 %). At the 3 months observation, there was no significant difference between the application of biostimulation plus the single strain (FSC) and biostimulation plus the consortium (FCC) in the terms of the oil-degradation. Though, the addition of consortium resulted in greater oil depletion than single strain treatment at the 16th days sampling (Figure 1). The FCC and FSC treatment increased oil degradation by 2.2 and 1.6 times that of the control, respectively.

The affectivity of biostimulation-bioaugmentation combination appeared faster and higher than biostimulation only on oil degradation. It was showed by the value of decay rate which was expressed as k value (Table 1) and the percentage of oil depletion (Figure 1). The efficacy of biostimulation-bioaugmentation combination treatment was observed significantly different from control 16 days after treatment (Figure 1). There k values at the 16th days were higher than others which were 0.050 day⁻¹ and 0.085 day⁻¹ for single strain and consortium, respectively. Biostimulation only treatment reached the significantly difference with control at the 90th days after treatment. It was when k value of control

![Figure 1 Oil degradation percentage over time for the mean of each treatment](http://ijms.biopublisher.ca)
was 0.01 day\(^{-1}\) and biostimulation was 0.13 day\(^{-1}\). The efficacy of biostimulation - only treatment was lower than combination treatment. However, it was higher than natural bio attenuation (control).

Table 1 K-value in each treatments at different time periods of experiment, \(n = 3\)

| No. | Treatment              | K-value at certain periods of time (day\(^{-1}\)) |
|-----|------------------------|-----------------------------------------------|
|     |                        | 0-8 days | 0-16 days | 0-23 days | 0-90 days |
| 1   | Control (C)            | 0.014    | 0.025     | 0.029     | 0.010     |
| 2   | Fertilizer (F)         | 0.028    | 0.021     | 0.036     | 0.013     |
| 3   | Fertilizer + Single strain (FSC) | 0.034 | 0.050 | 0.051 | 0.017 |
| 4   | Fertilizer + Consortium (FCC) | 0.038 | 0.085 | 0.059 | 0.015 |

2.2 Microbial growth

Microbial population density in all treatments varied during the experiment (Figure 2). Total cell count in the control sediment (1.98–15.61 x 10\(^7\) cells/ g ) over time of the experiment was lower than other treatments. The total number of cells in F, FSC and FCC were in the range of 3.98–29.09 x 10\(^7\) cells/ g ; 3.35–43.53 x 10\(^7\) cells/ g and 3.65–38.80 x 10\(^7\) cells/ g. A lag phase was observed in all treatments on the first 3 days after treatment, except in the control. An exponential phase for different treatments occurred at different time periods. The exponential phase of the FSC and FCC treatments were observed between 8-16 days, whereas it occurred at between 16–23 days in the fertilizer-only (F) treatment. In the control, it was not so clear, it may have been around 16–30 days.

The growth of bacterial cells was concomitant with the loss of oil concentration (Figure 2). There was a strong negative correlation between oil concentration and population density. The value of coefficient

![Figure 2](relation_of_oil_depletion_and_cell_numbers.png)

Figure 2 Relation of oil depletion (%) and bacterial cell numbers in each treatments during the 90 day mesocosm experiment. Treatments were C is oiled sediment only, F is oiled sediment with fertilizer (15 g N/kg) , FSC is oiled sediment amended with Fertilizer (7.5 g N/kg) plus single strain (2.5 L, 8 x 10\(^8\) cells/mL) and FCC is oiled sediment amended with Fertilizer (7.5 g N/kg) plus consortium (2.5 L, 8 x 10\(^8\)/mL)
correlation in different treatments ranged between -0.65 – -0.93. After 23-60 days of treatment, the rate of oil degradation dropped. Then, population growth became almost stagnant at 60-90 days after treatment and entered the decline phase.

2.3 Environmental conditions
The experiment was conducted in the dynamic environment of an estuary (Figure 3). Pore water conditions inside and outside mesocosm were different. Oxygen availability, pH and redox potential in the pore water outside the mesocosm were higher than inside the mesocosm (Table 2). In situ data during the experiment was available for surface seawater temperature inside the mesocosm which was in the range of 25.2 – 29.30°C (27.25 ± 0.83°C).

Table 2 Pore water quality over time experiment inside and outside mesocosm

| Parameter          | Inside mesocosm (n =72) | Outside mesocosm (n = 6) |
|--------------------|-------------------------|--------------------------|
|                    | Range       | Mean ± SD               | Range       | Mean ± SD               |
| DO (mg/L)          | 0.17 – 3.24 | 0.74 ± 0.59             | 2.24 – 4.95 | 4.16 ± 1.11             |
| Salinity (ppt)     | 13 – 56     | 35 ± 7.40               | 27 – 34     | 30.2 ± 2.58             |
| pH                 | 6.25 – 7.3  | 6.62 ± 0.17             | 6.61 – 7.39 | 6.93 ± 0.32             |
| ORP ( mV)          | (-518) –151 | (-124.438) ± 180.85     | 22 – 120    | 65.25 ± 40.65           |

Figure 3 Pore water quality in each treatments over time mesocosm experiment. (n=3 for each sampling time.). Parameters measured were A (salinity), B (Redox potential, C (pH) and D (Dissolved Oxygen). The treatments included C (oiled sediment only); F (oiled sediment + fertilizer 15 g N/kg); FSC (oiled sediment + fertilizer 7.5 g/kg + single strain 2.5L, 8 x 10^8 cell/mL), and FCC (oiled sediment + fertilizer 7.5 g/kg + consortium 2.5L, 8 x 10^8 cell/mL).

The availability of oxygen in pore water declined during the experiment, not only in the bioremediation treatment mesocosm, but also in the control mesocosm, although, the depletion rate in the control was lower than in the treatment (Figure 3 A). The decrease of DO in the treatments were significant until 8 days after treatment, after which it was stable to the value < 0.5 mg/L until the end of experiment. The lowest average DO value was 0.30 mg/L which was observed in the FSC treatment 90 days after incubation, whereas the highest average value, which was 0.90 mg/L, occured in the fertilizer-only treatment.
on the day treatment was applied (0 d).

Redox potential in the control and treatments during the experiment decreased until the end of the experiment (Figure 3 B). Measurement of environment quality for 0 day was conducted 9 hours after application of treatment. The effect of oxygen usage by microbes during 9 hours before measurement was expressed in the difference of redox potential value in the control and the 3 other treatments. In the control, the redox potential was higher than 100 mV during early stage of experiment (0 – 16 days after treatment). Due to technical error, no data was available by the 23rd day. From 60-90 days after treatment, the ORP value was negative until the end of the experiment ((-160) – (-449) mV). In all bioremediation treatments, the value at day 0, 9 hours after the application, was already lower than the control (23 - 50 mV). From the 3rd day after treatment until the end, the ORP value was declining. The lowest value was observed in FSC treatment (-455 mV) at the 90th day.

Salinity varied but pH value was relatively stable during the experiment (Figure 3 C and D). Salinity outside and in the control mesocosm was lower than in the treatments which were in the range of 27 - 34 ppt and 13 – 56 ppt, respectively. The lowest salinity was observed on the 90th day after treatment when it rained for about 6 hours before the sampling time. Higher salinity in all treatments were detected when fertilizer was applied on day 0 and days 16 of the experiment. The range value on days 0 and 16 of the treatment were 40 – 45 ppt and 39 – 46 ppt. On the other sampling days, salinity returned to almost normal conditions with an average range of 33.1 – 33.7 ppt in each treatment. pH value fluctuated within a very small range during the experiment (Figure 3D). The range and mean value of pH in the control and treatment was 6.5 – 7.3 and 6.25 – 6.8, respectively.

3 Discussions

The primary goal of the present study was to investigate suitable biological remediation approaches for anticipating oil spills in Cilacap sandy beach (Indonesia). It has been conducted by comparing four bioremediation strategies. This present study indicated that biostimulation, or a combination of biostimulation-bioaugmentation, is promising for enhancing oil-degradation when oil spills occur on the Cilacap coast. It was showed by the oil depletion percentage (Figure 1) and the value of the decay rate with biostimulation only, and with combinations of fertilizer and microbes, which were always higher than the control (Table 1), except for the biostimulation-only treatment in the early stage.

It was interesting to note that the Cilacap coastal environment was able to degrade crude oil naturally at a high level of 60.31 ± 3.93 % over 3 months for a pollution level of 100 g/kg oil in the sediment (Figure 1). The result is high compared with other previous studies in Indonesia, California and Hongkong (unpublished data, Bento et al., 2005). This high intrinsic capability may be caused by several factors, such as environmental conditions, oil characteristics and the availability of oil-degrading microbes in this coastal area. Environment condition can be showed at outside mesocosm data (Table 2). Arabian light crude oil was predominant oil used by Cilacap oil refinery unit (IBP, 2009). Therefore, the site exposed prior to this oil and the native microbes might have capability to degrade the contaminant. The presence of PAH-degrading bacteria in the sediment in mangrove swamps in this area has been reported (Syakti et al., 2008). However, it can be predicted that the limits of nutrient and microbial availability will occur when more than 100.000 mg/kg oil is spilled. It was proved by the increase of oil depletion rate when the addition of nutrient only and nutrient plus microbes applied in this present study. Therefore, bioremediation technology can be applied to make oil-clean up faster in Cilacap coastal.

Biostimulation, by adding slow release fertilizer Gramafix at a nominal concentration of 7.5 gr N/kg sediment or 15g N/ kg sediment, enhanced oil depletion. This result supports many previous works indicated that the capability of indigenous bacteria can be enhanced by addition of nutrients (exp. slow release fertilizer) (Xu et al., 2003; Xu et al., 2005; Darmayati, 2010). The fertilizer impacted on the increase of biomass which was followed by a decrease in DO that is consumed by bacteria and a depletion of oil in the sediment (Figure 2 and Figure 3). Numbers of bacterial cells in bioremediation treatments were higher than the control during experiment. In the present work, there was a strong positive correlation between the total cell number and
the oil depletion percentage \( (r = 0.63) \). In our previous study, the density of oil-degrading bacteria growth over 2 months on an oil-polluted sandy beach amended with slow release fertilizer (Osmocote) had increased to 34.8% of the total bacterial population (Darmayati, 2010). This showed that, in oil-polluted sediment, the higher the total number of bacterial cells, the higher the availability of oil-degrading bacteria.

The amendment of fertilizer 15 g/kg sediment might be too much for the level of oil pollution 100 g oil/kg sediment. The depletion rate and bacterial numbers of biostimulation only treatment was lower than the combination treatment which applied as low as 7.5 kg N/kg (Table 1; Figure 2), although, the initial number of bacterial cells in biostimulation only and the combination treatments was similar (Figure 2). This may have been caused by the Carbon/Nitrogen (C/N) ratio of 1000 : 75 which provided better environment for oil degrading bacteria than the ratio of 1000 : 150. Xu et al. (2003) found out that an addition 0.8% and 1.5% of slow-release fertilizer, Osmocote consisting of 18, 4.8, and 8.3% NPK (w/w) to oil polluted sediment was sufficient to maximize the metabolic activity of biomass and biodegradation of straight chain and branch chain n-alkane, respectively. This equates to a C/N ratio of 1000 : 33 and 1000 : 61 which they say is sufficient to maximize the metabolic activity of biomass and biodegradation of straight chain and branch chain n-alkane, respectively. An excessive nutrient concentration may suppress the growth of bacteria, whereas in combination treatments, nutrient concentration was lower but still enough for bacterial growth. Sufficient loading rates of nitrogen will be necessary if biostimulation is to occur. According to Gibbs et al., (1975) approximately 4mM of nitrogen was required to breakdown 1 mg of crude oil and phosphorus was not become limiting down to a minimum P/N ratio of 0.02. Loading rates below the critical concentration will be a waste of resources as would excessive use which could also promote secondary impacts such as harmful algal bloom and oxygen depletion (Bragg et al., 1994; Jackson and Pardue, 1999).

Slow release fertilizer use (Gramafix) provided not only NPK in the ratio of 22:7:12, but also Mg, Ca, S and micro nutrients in the ratio of 2:4:3:1. These minerals seemed to play an important role in boosting the catalytic activity of enzymes produced by oil degrading bacteria. Cookson and John (1995) mentioned that Mg\( ^{2+} \) and Ca\( ^{2+} \) are metallic ions that can function as a cofactor in the catalytic enzyme activity of microbes. Salinity was increased significantly up to 46 ppt when this SRF was applied to oil-contaminated sediment (Figure 3D). This may also have been caused by the addition of numerous minerals from Gramafix. This high level of salinity may still be under the tolerable level for marine oil-degrading bacteria. It can be shown by the growth observed until 23 days after treatment (Figure 2 B,C, and D). Mille et al., (1998) investigated the biodegradation of crude oil by a mixed bacterial community isolated from marine sediment with varying concentrations of sodium chloride. Initially the amount of oil degraded increased with increasing salt concentration, to a maximum level of 0.4 mol/l NaCl. Thereafter the amount of oil degraded decreased with increasing salt concentration, probably as the salt-tolerance limit of the bacteria was reached.

In the present study, the combination of biostimulation-bioaugmentation performed better than biostimulation only. Result of this study indicated that oil degradation rate was faster and the number of bacterial was higher during 90 days experiment in both combination treatments. It may caused by synergistic activity of exogenous and indigenous oil degrading bacteria and stimulation by sufficient concentration of nutrient provided from application of slow release fertilizer (Gramafix) at 7.5 g N/kg. Availability of carbon and energy source and favorable environmental conditions, namely temperature, salinity, oxygen and nutrients, may also support the growth. In bioaugmentation, the addition of oil-degrading bacteria boosts biodegradation rate, whereas in biostimulation, the growth of indigenous hydrocarbon degraders is stimulated by the addition of nutrients (mainly N and P) or other growth limiting nutrients (Nikolopoulou and Kalogerakis, 2010). Nikolopoulou et al., (2013) also mentioned that the success of oil spill bioremediation depends on the establishment and maintenance of physical, chemical and biological conditions that favor enhanced oil biodegradation rates in the marine environment. This result was incoherent with previous work (Nikolopoulou et al., 2013; Ueno et al., 2007; Stallwood, 2005).
Exogenous effective microbes which pre-adapted with the projected pollutant and intended environment may increase metabolic capability of indigenous bacteria in oil polluted area. In this study, a combination of Gramafix and synthetic consortium of selected three oil degrading bacteria collected from Jakarta Bay and pre-adapted to Cilacap coastal environment was able to degrade 74% oil after 2 weeks inoculation. It was more effective than a combination of Gramafix and a single strain amendment. It might be caused by consortium have more diverse enzyme that increase their capability. Westlake (1982) noted that no single microbial species has the enzymatic ability to metabolize more than two or three classes of compounds typically found in a crude oil. A consortium composed of many different bacterial species is thus required to degrade a crude oil spill significantly. This result was in agreement with other previous study (Baek et al., 2007; Mittal and Singh, 2009; Subaphol et al., 2006).

The consortium formulated in this study was consisted of Alcanivorax sp. TE9, Pseudomonas balearica st 101 and RCO/B/08015. Except RCO/B/08015 strain that has not been identified yet, the genus of other two strains have been known as oil degrading bacteria (Yakimov et al., 2005; Rolling et al., 2002; Darmayati et al., 2008). The combination of bioaugmentation and inoculation consortium showed rapid degradation until the first 16 days. It is a common pattern observed for the combination treatment. Therefore, it is proposed that combination of biostimulation - bioaugmentation with the consortium is relatively better alternative for combating oil-pollution on sandy beaches for a short period. Further study to find method for maintaining a good rate of oil degradation is needed to be done.

4 Materials and Methods

4.1 Site description
This research was conducted at a shoreline along the mouth of the Donan river estuary in the Cilacap region, Indonesia, between August – October 2011. The site was estuarine which has semi diurnal tidal pattern. In a downstream area was located Cilacap industrial estate, harbor for oil refinery and other industries which potentially contribute oil to the environment.

4.2 Sediment preparation and Spiking
Seashore sand uncontaminated by oil was collected (surface layer : 0 – 15 cm) from the north coast of Nusakambangan Island (Figure 1). Prior to spiking, the sandy sediment was air dried for 24 hr and then homogenized by sieving to 8 mm. Moisture content was determined through oven drying at 105°C for 24h. Total oil concentration was determined gravimetrically (US EPA,1999).

For experimental work, Arabian Light crude oil (Table 3) was applied by sprayer into sediment to provide 100,000 ppm of oil-polluted sediment and then homogenized thoroughly. Oil-polluted sediment was put outdoor for 5 days to allow weathering. Oiled sediment was prepared in volumes of 0.45 m³ for 12 plots. Each box, filled with 0.0375 m³ of oil-polluted sediment, was 50 x 50 x 15 cm in dimension. Tilling up to 15 cm depth in each plot was conducted once a week throughout the experiment (90 days).

4.3 Slow release fertilizer
Fertilizer used in the study was local production SRF (Gramafix®, PT.CV. Sinar Kencana, Bandung,
Indonesia). Gramafix is a granular type of SRF which contain macro and micro nutrient (N:P:K:Mg:C:S:Micro element = 22:7:12:2:4:3:1) and used for agricultural purpose. This was selected based on the result of microcosm study in our laboratory previously (Darmayati et al., 2014). Nutrient was added in the appropriate amount to have a final concentration equivalent to a C: N molar ratio of 100:15 for biostimulation only treatment and 100:7.5 for combination SRF plus single strain/consortium.

4.4 Bacterial culture preparation

Three selected strain cultures were obtained from the Research Center for Oceanography Culture Collection, Indonesian Institute of Sciences. These were Alcanivora sp. TE9, Pseudomonas balearica st 101 and RCO/B/08015, collected from Jakarta ports. They have capability to degrade Poly Aromatic Hydrocarbons (PAHs) and the first two have been determined by 16sRNA partial sequencing (Darmayati, 2009; Hatmanti and Darmayati, 2009). According to preliminary observation, Alcanivorax sp. TE9 and consortium B (a mixture of the three strains) showed the best performance (unpublished data).

Stock cultures from nutrient agar were transferred onto marine agar plates, then transferred to the adaptation media. Pre-acclimated bacteria conducted using media contain sterilized Cilacap seawater which was amended with NH₄NO₃ (500 mg/L), KH₂PO₄ (100 mg/L), yeast extract 0.01% (w/v) and Arabian Light crude oil 10% (w/w). The cultures were transferred in the log time to the fresh media with a higher volume. The volume of the media was increased in stages: 250 mL, 500 mL, 1.0 L and 12 L.

4.5 Experimental design

A controlled application of petroleum hydrocarbon on a sandy beach was used to test the efficacy of bioremediation strategies for enhancing the oil-biodegradation process. The strategies were, the addition of fertilizer only (F), single strain culture + fertilizer (FSC) and consortium + fertilizer (FCC). Intrinsic remediation was used as a control (C). Each treatment was conducted in triplicate. Tilling was done once a week to add oxygen and to homogenize the sediment.

A randomized complete block design with three blocks was established, with three plots assigned per block. Plot elevation, and location relative to the Donan River, served as criteria. Treatments were assigned randomly to plots within a block such that each block would have all the three potential treatments. The plots assigned were presented in Fig.4. Three blocks were established, the distance between blocks was 6 meters and between plots (treatments) was 2 meter (Xu et al., 2005)

Figure 4 Plot lay out on the inter-tidal foreshore of Donan river bank on a randomized complete block design

4.6 Mesocosm design and construction

Mesocosms measuring 50 x 50 x 170 cm were constructed with 4 bamboo walls coated in transparent plastic (Figure 5). The top and bottom were open to allow seawater in and out from the bottom, also sunlight in for photo oxidation from above. The enclosure structures (plots) were inserted 30 cm deep into the ground. The upper parts of the constructions were 140 cm in height above ground to prevent the oil from spilling into the environment at high tides. There was a 25 cm strip of clean sediment between the treated sediment and the inside wall of each enclosure, and also a clean base of 40 cm was provided to eliminate any impact from background oil in the surrounding area. This was done by removing 100 x 100 x 55 cm
of sediment in situ, and replacing it with clean sandy sediment from Nusakambangan Island, some of which had been treated (polluted) with oil. 50 x 50 x 15 cm of the oil-polluted sediment was inserted into the enclosure mesocosm boxes which lay on a 100 x 100 x 40 cm uncontaminated sand base from Nusakambangan Island.

In the middle of each plot a pore water sampler was constructed. It was made from a hose protected by PVC paralon with small holes in 5 – 10 cm from the end which allowed filtered pore seawater from treated sediment to be collected. It was immersed to a depth of 10 cm during the experiment.

4.7 Sampling Strategy
Periodical sampling was conducted 0, 3, 8, 16, 23, 60 and 90 days after treatments. Sampling were conducted before high tide (water height was about 5 cm inside each plot). The samples were sediment and pore water. Composite sediment samples for oil and microbial analysis were collected from 5 sampling points in each plots. The samples were then separated into an oil-free glass (100 gram) for oil analysis and a 50 mL sterile falcon tube for microbial analysis. All samples were transported to the laboratory in a cool box with ice inside.

Pore water samples for environment analysis were collected carefully by syringe to avoid oxygen contamination and transferred into 50 mL airtight bottles. All samples were transported to the laboratory in a cool box with ice inside.

4.8 Sample Analysis

4.8.1 Oil Analysis
Oil concentration was determined gravimetrically (US EPA, 1999). Samples were prepared by putting a 2 g subsample of homogenized sediment into a glass tube. Extraction was conducted by using the maceration method with a mixture of Dichloromethane : n-hexana (1:1) in proanalysis grade as a solvent. Na₂SO₄ was used to absorb the water remained in samples. The performance of oil biodegradation expected on an oiled shoreline can be estimated based on the first-order kinetic models. The first order relationship was expressed as: 
\[ C_t = C_0 e^{-kt} \]
where \( C_0 \) is initial oil concentration at day 0, \( C_t \) is oil concentration in time \( t \), \( k \) is the biodegradation rate coefficient. In this case, we used as depletion (decay) rate coefficient.

4.8.2 Microbial analyses
Subsample sediments from each plot were processed for enumeration of total cell bacteria. They were prepared by mixing 1 g wet weight of sediment into dilution water containing 9 mL seawater sterile. Then, this solution was placed in vortex at 300 rpm for 15 minutes to detach the bacteria from the sediment. Dilution of this subsample sediment was transferred into Acridine Orange solution, then filtered by polycarbonate membrane (0.22 µm) that submerged previously on sudan black solution. Direct counting under epifluorescent microscope was used for enumerating total cell bacteria (Hobbie et al., 1977).

4.8.3 Environmental parameters
To monitor changes in the sediment’s environmental conditions during the experiment, measurements of salinity, pH, Dissolved Oxygen (DO) and Oxidation-reduction potential (ORP) were carried out immediately after sampling by using a hand refractometer, pH meter (Horiba, Navi D-54), DO meter (Horiba, YSI 55) and ORP meter (Horiba, Navi D-52), respectively.

4.9 Statistical analysis of data
The experiment was conducted using three independent replicates. Data were subjected to analysis of variance and the averages were compared by Duncan multiple range test at \( p \leq 0.05 \). All statistical analysis was performed using SPSS 16. The rate of oil degradation and relation with other parameters measured were assessed by simple regression and correlation analysis.

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Author’s contributions
YD carried out the design and execution of the study, performed the statistical analysis and drafted the manuscript. HSS conceived of the study, participated in the design and coordination, and helped to refine the manuscript. TP and DAS participated in the design of the study, supervised the implementation of study and helped to refine the manuscript participated in the design of the study, supervised and
coordinated the implementation of study in the field and helped to refine the manuscript.

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