OPTIMAL BACTERIAL DENSITY AND FERTILIZER DOSAGE FOR BIOREMEDIATION OF OIL CONTAMINATED SANDY BEACH: A CASE OF CILACAP, INDONESIA

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ABSTRACT

Bioremediation, involving biostimulation and/or bioaugmentation, is a promising method to overcome oil spills in Cilacap coastal waters. Cilacap coastal area has high risk on oil pollution. This study investigated the stimulatory effect of nitrogen concentration, bacterial density and the composition of bacterial culture in enhancing oil degradation in this area. The applications of 4 different concentrations of Slow Release Fertilizer (SRF) and 2 different densities of bacterial cells in the form of single (RCO/B/08_008) and mixed culture were employed in microcosm experiments for 28 days. The efficacy of combining bacterial culture and fertilizer application in various concentrations was also tested. Oil degradation, bacterial growth and environmental parameters were monitored periodically during the experiments. The results showed that oil degradation rate was more influenced by nutrient concentration (biostimulation) than bacterial number or culture composition (bioaugmentation) added. The efficacy of biostimulation in degrading oil was better than that of bioaugmentation. Biostimulation increased oil degradation up to 6.4 times higher than the control. The optimum of fertilizer concentration added was 7.5 mg N/g (C:N ratio of 1,000 : 75), which increased depletion rate both in biostimulation-only and the combination of biostimulation with bioaugmentation up to 6.4 and 7.5 times higher than the control, respectively. It is suggested that bioremediation of oil-contaminated sandy beach in Cilacap would be optimal by employing a combination of Slow Release Fertilizer at concentration having C/N ratio = 1,000 : 75 and RCO/B/08_008 culture at density of 0.5 x 10^8 cells/mL (100% homology with *Alcanivorax* sp. TE-9).

Keywords: Bacteria, bioremediation, coastal, fertilizer, Indonesia, oil, optimal

INTRODUCTION

Petroleum and its derived products are the major source of energy. To ensure long-term energy security for Indonesia, Pertamina (the Indonesian Oil and Gas Company) plans to expand and upgrade the refinery capacity plant in Dumai, Cilacap and Balongan. This expansion increases the pollution risk of Cilacap coastal waters due to oil spill. Therefore, there is a need to anticipate the impact of oil pollution in this area.

Bioremediation is considered to be a promising biological approach to overcome oil pollution in marine environment (Swannel *et al.* 1996; Jackson & Purdue 1999; Munawar *et al.* 2007; Xu 2010). Bioremediation is basically an enhancement of biodegradation rate (Leahy & Colwell 1990; Mrozika & Seget 2010). Biological degradation process depends on several factors such as environmental conditions (pH, temperature, dissolved oxygen/DO, degree of acclimation, accessibility of nutrients), numbers of microorganisms, types of microorganisms, cellular transport properties, chemical partitioning in growth medium and chemical structure of compounds degraded (Coartes *et al.* 2009; Lin *et al.* 2009).

To enhance the effectivity of bioremediation, those environmental factors are modified with applied nutrient supplementation

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(biostimulation) and introduction of specific competent strains or consortia of microorganisms (bioaugmentation) (Mrozika & Seget 2010; Xu & Liu 2010).

Few successes of implementing biostimulation and bioaugmentation strategies for remediating oil contaminated marine sediment in several tropical countries such as Singapore, Indonesia and India were reported (Xu *et al.* 2003; Xu *et al.* 2005; Munawar *et al.* 2007; Darmayati 2010; Darmayati *et al.* 2015; Pasumarthi & Mutnuri 2016).

Field investigation in inter-tidal foreshore environment in Singapore and Indonesia demonstrated that fertilizer (Osmocote) was able to significantly enhanced hydrocarbonoclastic bacterial growth and increased the biodegradation of aliphatics and Polycyclic Aromatic Hydrocarbon (PAH) (Xu 2010; Darmayati 2010).

Osmocote is an anorganic Slow Release Fertilizer (SRF) coated by polymeric resin and consisted of water-soluble N–P–K at concentrations of 18%, 4.8% and 8.34%, respectively. Petroganik, another fertilizer type, is organic nutrient having capability of stimulating soil microorganisms and enhancing oil degradation in oil contaminated Surabaya coastal area (Munawar *et al.* 2007).

Introduction of several selected bacteria in oil contaminated Cilacap coastal area through mesocosm study was proven to increase the rate of oil degradation up to 1.6 - 2.2 times higher than the rate of oil degradation in the control site (Darmayati *et al.* 2015).

Until now, information is lacking on the optimal bacterial density and fertilizer dosage needed to increase oil degradation rate in Indonesian coastal area. This present study was aimed to study the stimulatory effect of nitrogen concentration, bacterial density and composition of bacterial culture to accelerate oil degradation in Cilacap coastal area. Cilacap coastal area is one of the most often areas polluted by oil spills.

MATERIALS AND METHODS

Preparation of Bacterial Culture

Three isolates of oil and PAHs degrading bacteria collected from Jakarta Bay sediment were provided by Marine Microbiology Laboratory, Research Center for Oceanography, Lembaga Ilmu Pengetahuan Indonesia (LIPI/Indonesian Institute of Sciences). The isolates were RCO/B/08-008 (AB055207 *Alcanivorax* sp. TE-9, similarity 100%), RCO/B/08-004 (AM905859 *Pseudomonas balearica,* similarity 99%) and RCO/B/08-015. The first two isolates were identified using partial sequencing of 16s rRNA. The third isolate had not been sequenced yet; therefore, it only had a name code (Hatmanti & Darmayati 2009). To prepare the mix cultured inoculum, the three strains were grown separately in marine agar at 30 °C for 72 hours. Each strain was harvested using Driglasky stick and sterilized saline water, then transferred into sterilized tube. The density of each strain was calculated using measurement of Optical Density (OD) and graph which correlate OD and bacterial cell number. Dilution method using sterilized saline water was conducted to make similar density. The mixed culture was formulated by mixing all the three strain cultures in the same volume to have a density ratio of 1 : 1 : 1.

Preparation of Artificially Oil Contaminated Sediment

Oil contaminated sediment was prepared by spiking oil mousse of Arabian Light Crude Oil (ALCO) into the sample of sandy sediment and seawater collected from Cilacap coastal area. Oil mousse is a thick foamy mixture of oil and water which is formed when petroleum products are mixed with water due to the actions of wind and waves (Petropedia Inc 2017). Oil mousse used in this study was prepared by mixing Cilacap seawater and ALCO in 70 : 30 proportion using sterilized mixer for 15 x 3 minutes. Oil mousse of 4 mg was spiked into 10 g sample of Cilacap sediment to achieve target concentration of 100,000 ppm (100 mg/g) of oil-contaminated sediment.

Fertilizer Application

Gramafix® fertilizer was used in this study. This fertilizer is a granular SRF fertilizer containing macro and micro nutrients (N:P:K:Mg:C:S:Micro element = 22:7:12:2:4:3:1). This fertilizer is commonly used for agricultural purposes. This fertilizer was selected due to their good performance in previous studies on oil

Table 1 Treatment performed in each different experiment

Experiment	Treatment	Nitrogen concentration (mg/g)	Single culture density (cell/mL)	Mixed culture density (cell/mL)
Biostimulation	Control	no fertilizer and bacteria addition		
	A	1.88	-	-
	B	3.75	-	-
	C	7.50	-	-
	D	15.00	-	-
Bioaugmentation	Control	no fertilizer and bacteria addition		
	A	-	0.5×10^8	-
	B	-	1.0×10^8	-
	C	-	-	0.5×10^8
	D	-	-	1.0×10^8
Combination	Control	no fertilizer and bacteria addition		
	A	7.50	0.5×10^8	-
	B	7.50	1.0×10^8	-
	C	7.50	-	0.5×10^8
	D	7.50	-	1.0×10^8
	E	15.00	0.5×10^8	-
	F	15.00	1.0×10^8	-
	G	15.00	-	0.5×10^8
	H	15.00	-	1.0×10^8

degradation rate conducted in our laboratory (unpublished data).

There were 3 experiments conducted to determine the optimal fertilizer dosage for bioremediation i.e. biostimulation, bioaugmentation and combination between biostimulation and bioaugmentation.

In the biostimulation experiment, the applied fertilizer dosages were 1.88 mg N/g; 3.75 mg N/g; 7.50 mg N/g; and 15.00 mg N/g, respectively (Table 1). In the bioaugmentation experiment, there was no fertilizer applied (Table 1). In the combination between biostimulation and bioaugmentation experiment, the applied fertilizer dosages were 7.50 mg N/gand 15.00 mg N/g (Table 1).

Experimental Design

To study the stimulatory effect of nitrogen concentration, bacterial density and the composition of bacterial culture in accelerating oil degradation, three experiments were conducted in Completely Randomized Design (Table 1).

The treatments applied were separate ALCO-spiked sediment treatments amended with nitrogen using different fertilizer dosages and/or bacteria at different densities and compositions. One control treatment was set up as not having SRF fertilizer and no bacterial amendment.

Each microcosm in slurry form contained 15 mL seawater and 10 g oil-contaminated sediment having concentration of 100 mg/g. A microcosm is an experimental unit in a 50 mL sterilized falcon tube.

Experiments were conducted for 28 days in an incubator shaker (having shaking speed of 100 rpm) at room temperature ($28 \pm 2\,^{\circ}C$).

Measurement of oil concentration was conducted in triplicates for each treatment at each sampling time. Measurements of supporting data such as numbers of bacterial cells, environmental factors (Dissolved Oxygen/DO, pH and salinity) and nutrient (nitrogen total) were conducted without any replications in each treatment at each sampling time.

For biostimulation experiment, measurements were conducted at day-0, day-14 and day-28 after exposure. Measurements of all parameters for the bioaugmentation and combination were conducted only at day-0 and day-28 after exposure.

Analysis of Oil, Bacteria and Environmental Parameters

Oil concentration was measured from the slurry of the whole content of each microcosm. The extraction of oil was carried out using centrifugation and maceration methods with a

mixture of Dichloromethane: n-hexana (1 : 1) having proanalysis grade as a solvent. Na_2SO_4 was used to absorb the water remained in the extracted oil. Oil concentration in the slurry represented the concentration of oil in the treatment.

The numbers of bacterial cells on overlying water indicated the density of bacterial cells in porewater inside the microcosm. A detachment of bacteria was conducted by placing the microcosm in a vortex and shaking the vortex at 300 rpm for 5 minutes. A subsample was drawn from the shaken microcosm. Counting of total bacterial cells was conducted using dilution method conducted on the subsample. Acridine Orange solution (0.05%, 2.29 mL) was added into the subsample. The supernatant was then filtered using polycarbonate membrane having pore size of 0.22 μm. The polycarbonate membrane was previously submerged in Sudan Black solution to obtain a contrast background. Direct counting of total bacterial cells was conducted under epifluorescent microscope (Hobbie *et al.* 1977).

To monitor the changes of environmental condition and nitrogen concentration in this microcosm system during experiment, one set of microcosm was provided for each purpose. The microcosm set was prepared similarly with the microcosm for measuring oil concentration and counting the numbers of bacterial cells. However, the numbers of microcosms were only prepared for one replication at each sampling time in each treatment. Measurements of salinity, pH and Dissolved Oxygen (DO) were carried out for the overlying water using hand refractometer (for measuring salinity), pH meter (Horiba, Navi D-54) and DO meter (Horiba, YSI 55), respectively.

Concentration changes of soluble nitrogen (N) were measured by filtering, extracting and analyzing total nitrogen concentration for the whole content of microcosms. Persulfate digestion method was used for extraction prior to nutrient measurement (Zhu *et al.* 2001). Total nitrogen concentration was determined using HACH DR 800 direct reading colorimeter using HACH proprietary reagents.

Analytical Data

Experiments were conducted using three independent replications for oil concentration. Percentage of oil depletion was calculated from oil concentration applied in the first and the third experiments after 28 days of incubation. Oil degradation data were analyzed using One Way ANOVA. The mean of the data were compared by Duncan Multiple Range Test (DMRT) at $p \leq 0.05$. Kolmogorov-Smirnov test was used to test the normality of data, while the Levene Statistic test was used to test data heterogeneity. Statistical software used was SPSS 16.

Numbers of bacterial cells, nitrogen concentration and environmental data were provided as supporting data. These supporting data were only available for each treatment at each sampling time (without any replication). These supporting data were qualitatively analyzed.

RESULTS AND DISCUSSION

Biostimulation

Application of Slow Release Fertilizer (SRF) increased oil degradation rate in oil-contaminated coastal environment. However, the concentration of SRF should be carefully calculated to avoid a side effect in the oil-contaminated area, such as excessive algal growth. This study showed that SRF was effective to increase oil degradation. Applications of SRF at all tested N concentrations were able to increase degradation rate significantly (Table 2). Oil weight after being treated with SRF (for 28 days) ranged from 0.453 ± 0.03 to 0.815 ± 0.01 g per 10 g sediment. The remaining oil in the control was at the level of 1.185 ± 0.06 g per 10 g sediment. The percentage of oil depletion in the SRF treatment was between 44.2 ± 4.3% and 65.9 ± 3.4%. The percentage in the control treatment was only 13.3 ± 2.6%. There was statistically significant difference berween control and treatments.

Oil depletion and a numbers of bacterial cells were observed in the control. The results showed that oil degrading bacteria were available in this study site. However, the nutrients availability was insufficient for the growth of oil degrading bacteria due to the occurrence of oil spill. Nutrients concentration and hydrocarbon bioavailability are the key factors affecting oil biodegradation rates in oil-contaminated sites (Xu *et al.* 2005). Other studies proved that the growth of oil degrading bacteria and thus, oil degradation rate can be strongly increased by applying fertilizers containing inorganic N and P

Table 2 The impact of nitrogen addition at various concentrations (mg/g) on oil depletion efficiency and bacterial density during 28 days exposure; including average and standard deviations of oil content and oil depletion (n = 3), also bacterial number (n = 1)

Nitrogen concentration added (mg/g)	Oil content (g)				Oil depletion (%)
	0 d	7 d	14 d	28 d	28 d
0	1.280 ± 0.06	1.289 ± 0.02	1.270 ± 0.06	1.185 ± 0.06	13.3 ± 2.6 [e]
1.88	1.280 ± 0.06	0.984 ± 0.01	0.787 ± 0.09	0.609 ± 0.33	51.9 ± 3.4 [b]
3.75	1.280 ± 0.06	0.996 ± 0.01	0.904 ± 0.09	0.815 ± 0.01	31.0 ± 3.6 [d]
7.50	1.280 ± 0.06	1.007 ± 0.01	0.834 ± 012	0.453 ± 0.03	65.9 ± 3.4 [a]
15.00	1.280 ± 0.06	1.001 ± 0.00	0.809 ± 0.02	0.72 ± 0.03	44.2 ± 4.3 [c]
	Bacterial cells number (cells/mL)				
	0 d	7 d	14 d	28 d	
0	6.E+07	6.E+07	1.E+08	2.E+08	
1.88	2.E+08	2.E+08	3.E+08	9.E+08	
3.75	8.E+07	8.E+07	3.E+08	5.E+08	
7.50	2.E+08	2.E+08	4.E+08	1.E+09	
15.00	2.E+08	2.E+08	3.E+08	9.E+08	

Note: The same letter (a - e) in the same column showed that the difference was not statistically significant ($p < 0.05$)

(Swannell *et al.* 1996; Röling *et al.* 2002; Coulon *et al.* 2007; Darmayati 2010).

There was no guarantee that higher fertilizer dosage resulted to higher oil degradation rate. Results of this study showed that excessive dosage of fertilizer inhibited the increase of oil depletion rate and bacterial numbers. Biostimulation treatments applying fertilizer with nitrogen concentration of 1.88 and 7.50 mg/g showed an increase in oil depletion efficiency and in bacterial growth. The oil depletion efficiency increased in a range of 2.3 - 5.0 times compared to that in control. The bacterial density were in a range of 4.1 - 4.6 times higher compared to that in control (Table 2).

However, the enhancement of oil degradation was only 3.3 times when nitrogen concentration increased up to 15.0 mg/g. This result indicated that Carbon/Nitrogen (C/N) ratio of 1,000 : 75 and 1,000 : 18.8 provided better environment for the growth of oil degrading bacteria than the ratio of 1,000 : 150. Xu *et al.* (2003) mentioned that adding 0.8% of nitrogen (C/N ratio 1,000 : 33) and 1.5% of nitrogen (C/N ratio 1,000 : 61) of Slow Release Fertilizer (Osmocote) consisting of 18% nitrogen, 4.8% phosphor and 8.3% kalium (w/w) to oil contaminated sediment were sufficient to maximize the metabolic activity of biomass and to increase biodegradation of straight chain and branch chain of n-alkane, respectively.

There was high positive correlation between percentage of oil depletion and bacterial density. This was especially shown between the percentage of oil depletion obtained at day-28 and the bacterial density at day-14 (r = 0.87). Coefficient of determination of 0.77 showed that 77% of variations in the percentage of oil depletion were explained by bacterial density; while the remaining 23% of variations in percentage of oil depletion might be due to other factors such as nutrients and other environmental factors.

Until certain level, high oil concentration and high nutrient availability may increase bacterial growth. This study showed that bacterial density in microcosm with SRF application was 2.4 – 4.6 times higher than that in control at day-14 (Table 2). High bacterial density in this experiment represented high abundance of oil–degrading bacteria. Coulon *et al.* (2007) mentioned that N and P increased the abundance of oil degrading bacteria and total oil degradation. Our previous study showed also that microbial biomass increased and the growth rate of oil degrading bacteria was higher than total bacteria (Darmayati 2010). Population of oil degrading bacteria used oil as their source of energy and nutrient. Higher

number of oil degrading bacteria led to higher rate of oil depletion.

Bioaugmentation

Bioaugmentation was able to increase oil degradation, both in single or mixed culture treatment. However, bioaugmentation contributed lower increase of oil degradation compared to the increase caused by biostimulation. Significant ($p < 0.05$) increase on the percentage of oil degradation caused by bioaugmentation ranged from 28.4 ± 1.9% to 34.3 ± 6.2% (Table 3), while the increase of oil degradation percentage caused by biostimulation ranged from 44.2 ± 4.3% to 65.91 ± 3.4 % (Table 3).

This occurrence happened because the growth of oil degrading bacteria (hydrocarbonoclastic bacteria) was limited by nutrient insufficiency in the area of oil spill. C/N ratio was higher than it should be when oil spill occurred and no added nutrient administered to the oil spill area.

Increase of bacterial density in each treatment was observed, but the increase was in a low rate. The highest numbers of bacterial cells (8.2×10^8 cells/mL) was observed at day-14 after treatment in the microcosm supplemented with mixed culture. This number was only 3.61 times higher than the bacterial number in control.

There was a strong positive correlation between numbers of bacterial cells and oil degradation percentage ($r = 0.91$) in this study. Oil degradation was supported by bioaugmentation with *Alcanivorax* sp. which was much more effective than nutrient addition (McKew *et al.* 2007). Oil pollution in Thames Estuary was in less density compared to that in Cilacap coastal area. The Cilacap coastal area are heavily contaminated by oil due to industries and oil refinery establishments located in the area.

Introduction of mixed culture (*Alcanivorax* sp. TE-9, *Pseudomonas balearica* and RCO/B/08-015) and single culture (*Alcanivorax* sp.) did not give any significant effects on oil degradation rate in Cilacap coastal area. Different numbers of bacterial cells supplemented did not exhibit significant impact on oil depletion rate (Table 3). Treatment administering *Alcanivorax* sp. TE-9TE-9 having density of 1.0×10^8 cells/mL exhibited the highest oil degradation rate (34.3% in 28 days), although it was not significantly different from the other three treatments (Table 3). Cilacap coastal area had high diversity of oil degrading bacteria and various enzymes needed for oil degradation were available in this area. Oil degrading bacteria available in Cilacap coastal area were *Flexibacteraceae bacterium, Bacillus aquamaris, B. megaterium, B. pumilis, Halobacillus trueperi* and *Rhodobacteraceae bacterium* (Syakti *et al.* 2013).

Combination Treatment

Combination treatment of biostimulation and bioaugmentation exhibited the best performance of oil bioremediation. In this experiment, oil reduction was observed in the range of 13.1 ± 0.3% – 74.6 ± 3.6% at day-28 days after treatment. Bioaugmentation supplemented with SRF fertilizer having nitrogen concentration of 7.5 mg/g showed higher oil reduction than that in combination treatment supplemented with SRF fertilizer having nitrogen concentration of 15.0 mg/g (Table 4). These results occurred both in single strain or mixed culture and also in high or

Table 3 The impact of bacterial amendment on oil depletion rate (at 28 days) and bacterial abundance (at 14 days); including average and standard deviations of oil depletion (n = 3)

Treatment	Bacterial cell addition (cells/mL)	Oil depletion (%)	Bacterial cells number (cells/mL)
Control	0	13.33 ± 0.3 [a]	2.28E+08
Single Culture	0.5×10^8	28.37 ± 1.9 [b]	7.87E+08
	1.0×10^8	34.34 ± 6.2 [b]	8.22E+08
Mixed Culture	0.5×10^8	30.53 ± 4.8 [b]	7.87E+08
	1.0×10^8	27.81 ± 0.2 [b]	5.16E+08

Note: The same letter (a - b) in the same column showed that the difference was not statistically significant ($p < 0.05$)

Table 4 Comparison of oil depletion percentage (at 28 days) and bacterial abundance (at 14 days) in oil contaminated sediment exposed by different treatment of N concentration and bacterial amendment for 28 days experiment

Treatment			Parameter measured	
Fertilizer	Culture	Bacterial cell number added	Oil depletion (%)	Bacterial density (cells/ mL)
Control	-	0	13.1 ± 0.3 [a]	2.E+08
N = 7.5 mg/g	Single	0.5 x 10^8	74.6 ± 3.6 [c]	1.E+09
		1.0 x 10^8	30.2 ± 4.2 [f]	8.E+08
	Mixed	0.5 x 10^8	64.9 ± 1.0 [d]	1.E+09
		1.0 x 10^8	46.7 ± 1.5 [e]	1.E+09
N =15.0 mg/g	Single	0.5 x 10^8	24.8 ± 2.2 [c]	6.E+08
		1.0 x 10^8	38.7 ± 0.3 [b]	9.E+08
	Mixed	0.5 x 10^8	15.9 ± 3.4 [a]	6.E+08
		1.0 x 10^8	13.1 ± 0.3 [a]	6.E+08

Note: Columns marked by the same letter (a to g) are not statistically significant different ($p < 0.05$). No replications for bacterial cells number

low bacterial density. This study confirmed that C/N ratio of 1,000 : 75 was better than 1,000 : 150 for bacterial growth. Excessive nutrients concentration might suppress the growth of bacteria, whereas in combination treatments, nutrients concentration was lower, but sufficient for bacterial growth. Sufficient loading rates of nitrogen will be necessary to trigger biostimulation. According to Gibbs *et al.* (1975) approximately 4 mM of nitrogen was required to breakdown 1 mg of crude oil; phosphorus did not limit P/N ratio to a minimum of 0.02. Loading rates of nutrients below critical concentration would be a waste of resources; the same as excessive use which could promote secondary impacts such as harmful algal bloom and oxygen depletion (Bragg *et al.* 1994; Jackson & Pardue 1999).

The combination of bioaugmentation and biostimulation approach by nitrogen amendment at moderate level was proven, in the laboratory and in the field, as the best approach for reducing oil in environment (Darmayati *et al.* 2015; Pasumarthi & Mutnuri 2016). Our study in Cilacap coastal area using mesocosm approach confirmed that combination of bioaugmentation-biostimulation is the best strategy for cleaning up oil spills (Darmayati *et al.* 2015). Other studies in Goa (India) and Egypt supported this conclusion (Pasummarthi & Mutnuri 2016; El-Borai *et al.* 2016).

Enviromental Condition

Environmental conditions (pH, temperature, dissolved oxygen, degree of acclimation, accessibility of nutrients) are among important factors in biological degradation processes, besides numbers of microorganisms, types of microorganisms, cellular transport properties, chemical partitioning in growth medium and chemical structure of degraded compounds (Coartes *et al.* 2009; Lin *et al.* 2009). In this study, the impact of nutrients addition into environment could be observed obviously such as the changes of salinity and DO levels during exposures time (Fig. 1).

Salinity was shown as an important factor in bacterial growth influencing oil biodegradation in this study. The addition of SRF fertilizer increased salinity depending on the added fertilizer concentration. SRF application at 150 mg N/mL increased salinity up to 65 ppt (Fig. 1). The increase of salinity might be caused by Gramafix which contained high concentration of macronutrients (NPK), secondary macronutrients (Mg, S, Ca) and micronutrients (Zn, Fe, Cl, Mn, B, Bo, Mo). The ratio of Mg, Ca, S and micronutrient was 2:4:3:1. High content of minerals in this SRF fertilizer seemed to play an important role in increasing the catalytic activity of enzymes produced by oil degrading bacteria. Cookson Jr (1995) mentioned that Mg^{2+} and Ca^{2+}

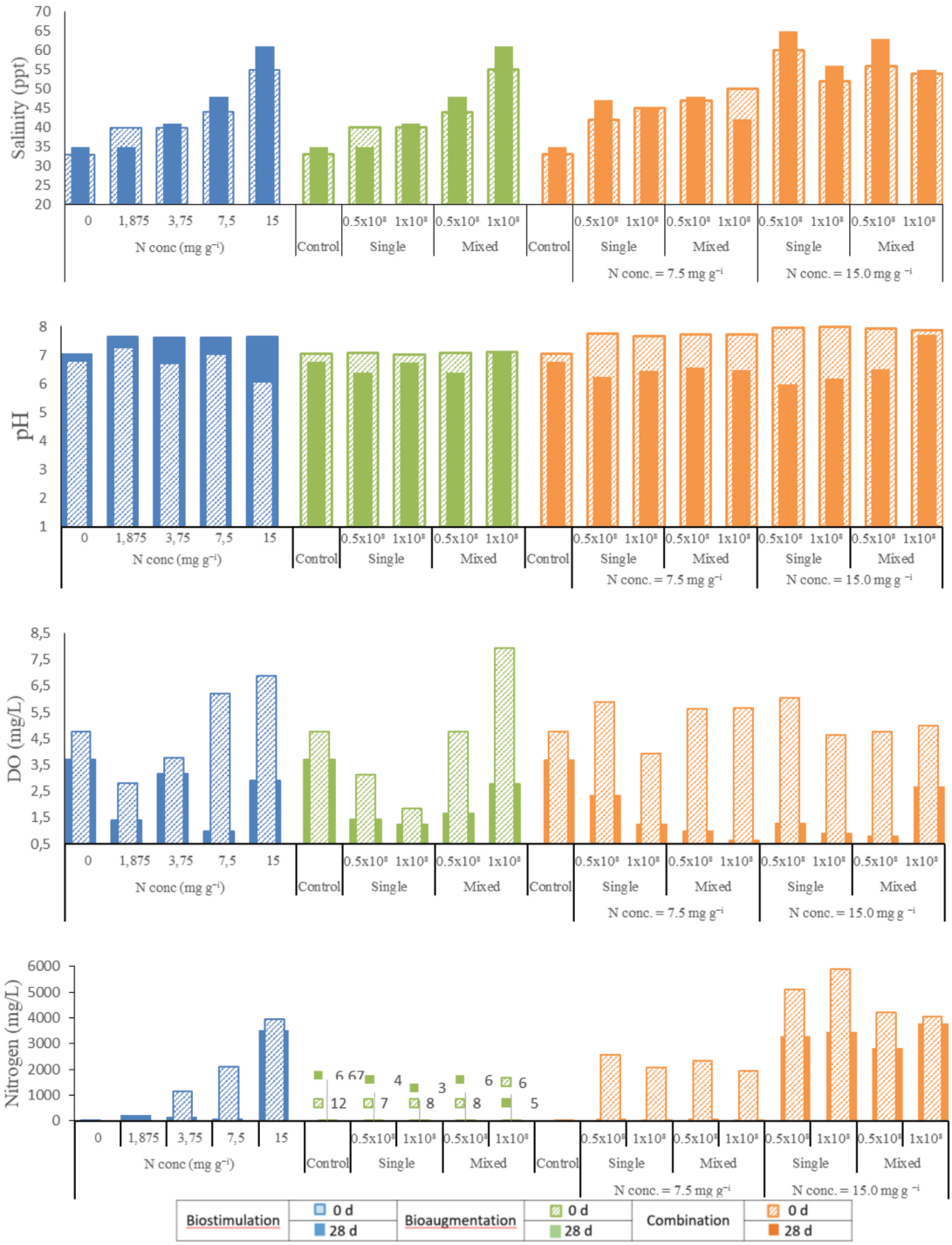

Figure 1 Environmental parameters condition at day-0 and day-28 of experiments in three different bioremediation experiments

are metallic ions that can function as a co-factor in the catalytic enzyme activity of microbes.

In this study, the range of salinity during the experiment was 32 – 65 ppt. This high level of salinity might still be tolerable by marine oil-degrading bacteria, which was shown by the bacterial growth observed at day-14 (Table 3 & 4). Other study focused on biodegradation of crude oil by a mixed bacterial community isolated from marine sediment with varying concentrations of sodium chloride (Mille *et al.* 1991). Initially, the amount of degraded oil increased with increasing salt concentration, to a maximum level of 0.4 mol/LNaCl. Thereafter, the amount of degraded oil decreased with increasing salt concentration, probably because the salt-tolerance limit of the

bacteria was reached. In this experiment, the salinity of seawater amended by fertilizer at nitrogen concentration of 7.5 mg/g sediment and 15.0 mg/g sediment increased up to 45 – 50 ppt and 52 – 65 ppt, respectively. The salinity value in biostimulation and combination of biostimulation-bioaugmentation treatments was higher than control (Fig. 1).

These experiments were conducted in the range of Dissolved Oxygen (DO) value between 0.64 – 6.05 mg/L, pH value of 5.52 – 8.19 and salinity value of 32 – 65 ppt (Fig. 1). During the experiments, DO value was always getting lower along with incubation time and increased numbers of bacteria. Dissolved Oxygen was sufficiently available to be consumed by bacteria to metabolize oil. No aeration was provided during the experiments. The oxidation of the substrate by oxygenases during the process of aerobic respiration, for which molecular oxygen is required, occurs in the initial steps of catabolism of aliphatic, cyclic and aromatic hydrocarbons by bacteria and fungi (Leahy & Colwell 1990).

pH value during the experiment was in the range of 5.56 – 7.99 which was optimal range for biodegradation process. Bioremediation treatment used in this study provided good results for reducing oil in the marine environment.

Nitrogen availability in control was in natural condition, which was in the range of 7 - 12 mg/L. Addition of fertilizer increased nitrogen availability in microcosm. However, available nitrogen observed in moderate concentration of nitrogen (7.5 mg/g) were higher than that in high concentration of nitrogen (15.0 mg/g) (Fig. 1). This might be caused by excessive nitrogen which suppressed the growth of bacteria in the environment with having nitrogen concentration of 3,943 – 5,900 ppm, so that the numbers of bacterial cells were lower and oil degradation rate was slower than the combination treatment (at nitrogen concentration of 1,940 – 2,090 ppm).

CONCLUSIONS

Oil depletion rate was influenced by the numbers of bacterial cells and nutrients concentration. Efficiency of oil degradation in biostimulation treatment was better than that in bioaugmentation treatment. However, the highest oil degradation rate was observed at a combination of bioaugmentation-biostimulation treatment. This treatment was able to increase oil degradation rate up to 7.5 times higher than that in control. In addition, the salinity range of 32 – 65 ppt caused by fertilizer addition might still be tolerable for marine oil-degrading bacteria. Bioremediation of oil contaminated sandy beach in Cilacap would be optimal by employing a combination of Slow Release Fertilizer (G) at C/N ratio of 1,000 : 75 and RCO/B/08_008 culture having bacterial density of 0.5 x 10^8 cells/mL (100% homology with *Alcanivorax* sp. TE-9).

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