Temporal Changes in Microbial Metabolic Characteristics in Field-Scale Biopiles Composed of Aged Oil Sludge

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Received: December 2, 2013

Accepted in revised form: May 18, 2014

Abstract

Disposal of oil sludge, a hazardous waste, is currently a prevalent environmental issue. In this study, two field-scale biopiles were constructed to explore the temporal changes of microbial metabolic characteristics during the biotreatment of aged oil sludge. Bulking agent was mixed thoroughly with oily sludge to form a treated pile. The BIOLOG™ system was used to analyze the community level physiological parameters, including microbial metabolic activity, diversity, and variance. In comparison with the control, the community level physiological parameters of the treated pile were dramatically improved. Microbial metabolic activity of the treated pile was improved by 25.06% calculated from the maximums during the treatment. Microbial diversity index (Shannon index) ranges were improved from 1.64–3.02 (control pile) to 2.34–3.14 (treated pile). The numbers of petroleum-degrading bacteria and the total heterotrophic bacteria were correlated with the environmental temperature, and microbial metabolic characteristics in the treated pile revealed the distinctive carbon resources selection with the addition of cotton stalk. Temporal microbial metabolic characteristics, which have important effect on bioremediation, were revealed in this study.

Key words: aged oil sludge; bioaugmentation; biopile; community level physiological profiles; temporal change

Introduction

OIL SLUDGE IS A SOLID WASTE generated during the process of oil exploitation, transportation, and refining (Shie *et al.*, 2003). It is a mixture of crude oil, solid particles, and water. Many components of oil sludge are generally considered toxic, mutagenic, and carcinogenic (Liu *et al.*, 2009). Consequently, the release of these components from the sludge may cause extensive environmental disturbances, such as air, soil (Muratova *et al.*, 2008), surface runoff, and even ground water contamination. Furthermore, human health may be threatened in various ways (Cameotra and Singh, 2008). The disposal of oil sludge commonly consisted of stacking in the open air before the negative effects of this approach on the environment were realized. The volatile components and moisture content of oil sludge decrease during its exposure outdoors. Therefore, a large quantity of aged oil sludge was generated.

In view of the above-mentioned facts, the treatment of oil sludge has been incorporated into the agenda of environ-

mental science. In China, oil sludge has been classified as a kind of hazardous waste by the Ministry of Environmental Protection. Bioremediation is considered to be an environmental friendly and cost-effective method in the treatment of petroleum contaminated site. Biopile was a fully developed bioremediation system with piling up of materials to be treated by bioaugmentation and biostimulation (Jørgensen *et al.*, 2000). However, it was seldom used in the treatment of aged oil sludge, and there is little data showing the temporal changes in microbial metabolic characteristics among the layers of field-scale piles (Wang *et al.*, 2012). In this study, typical biopile treatment was constructed to reveal the temporal changes of microbial metabolic characteristics in the experimental period of 220 days. Microbial metabolic activity, diversity and variance, and the enumeration of culturable bacteria were chosen as parameters to describe the microbial metabolic characteristics.

Materials and Methods

Site characterization and experimental design

This field trial was developed in the Shengli oilfield (the second biggest oil field in China), Shandong Province, East China. The mean annual temperature is 12.8°C, and temperatures

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range from -3.0°C in January to 26.6°C in July. The site was an outdoor oil sludge storage pool before land clearing. Quantities of oil sludge produced by different processes of oil exploitation had been transported over the past 30 years to the site and mixed together in the pool without any treatment. The field trial was developed *in situ* after site preparation.

Aged oil sludge was dredged from the pool and mixed thoroughly. Initial samples were taken and used to analyze the physicochemical properties and microbial characteristics. The results are shown as follows: total petroleum hydrocarbons (TPHs) 17.50% (w/w), total organic carbon (TOC) 14% (w/w), hydrolyzable nitrogen 95.5 mg/kg, available phosphorus 2.74 mg/kg, moisture content 16.0% (w/w), heavy metals all conform to the national environmental quality standard for soils of China, pH 8.0, water soluble salts 6.75 g/kg. Standard methods were used in the analysis of TOC, hydrolyzable nitrogen, available phosphorus, moisture content, and pH (Lu, 2000). TPHs was analyzed according to Wang *et al.* (2010). Then, part of the sludge (about 100 m^3) was divided into two equal piles (10 m in length, 5 m in width, and 1.5 m in height), one of which were treated with different biostimulation strategies to reveal the temporal changes of microbial metabolic characteristics, and another pile existing as control.

The biostimulation strategies applied to the treated biopile consisting of local agricultural cotton stalk (about 5–7 cm in length) at a ratio of 1:3 (cotton stalk:oil sludge, v/v). A suitable moisture content of 18% to 20% (w/w) had been control in the experimental period for biodegradation requirement (Von Fahnstock *et al.*, 1998). Finally, an impermeable high-density polyethylene membrane was used to cover the two piles to prevent the dispersal of pollutants in the oil sludge and disturbances due to precipitation.

Field sampling

When samples were taken on days 0 (initial samples, October, 2010), 30 (November, 2010), 60 (December, 2010), 130 (March, 2011), and 220 (June, 2011) from each biopile, the mean daily air temperatures were 15°C , 10°C , -6°C , 8.5°C , and 28.5°C , respectively. The last four values represent typical local temperatures for the four seasons: autumn, winter, spring, and summer.

In the field sampling, each pile was divided into three equal sections along its length. Three core samples, which contained aged oil sludge from the surface (0 cm) of the pile to a depth of 80 cm, were taken randomly from each section. From each core sample, three sub-samples at depths of 0–10 cm (surface layer), 40–50 cm (middle layer), and 70–80 cm (inner layer) were obtained. Then the three sub-samples taken from the same depth and the same section were mixed together to provide a composite sample. Composite samples were stored at 4°C before subsequent analysis. The interval between field sampling and analysis in the laboratory was limited to 3 days.

Microbial community level physiological profiles

Microbial community level physiological profiles were measured by a BIOLOG system to reflect the patterns of sole-carbon-source utilization. Fresh aged oil sludge sample (10 g, dry weight equivalent) on days 0, 30, 60, and 220 was suspended in 100 mL of sterile NaCl solution (0.85%, w/v) in a glass Erlenmeyer flask. Then the flask was placed on a rotary shaker at 200 rpm for 30 min at an ambient temperature of

25°C . After settling for 20 min, the suspension was diluted 10-fold and, subsequently, $150\text{ }\mu\text{L}$ of a 100-fold diluted suspension were used to inoculate each well of a BIOLOG EcoPlate (BIOLOG, Inc., Hayward, CA) that provided 31 carbon sources located in 31 wells for microbial metabolism. The changes in optical density (OD) of each well, which are caused by the reaction of a dye during the process of incubation, indicate the microbial metabolic characteristics. The inoculated EcoPlates were incubated in the dark at 28°C for 228 h, and the OD at 590 nm was recorded using an automated BIOLOG Microplate Reader (BIOLOG, Inc.). The measuring interval varied from 12 h during the first 84 to 24 h for the remainder of the incubation period. Data were obtained at 0, 12, 24, 36, 48, 60, 72, 84, 108, 132, 156, 180, 204, and 228 h. The average well color development (AWCD) and diversity indices were calculated based on OD values according to Zak *et al.* (1994).

Enumeration of culturable bacteria

The quantities of total heterotrophic bacteria and petroleum-degrading bacteria in the aged oil sludge were assessed by the plate count method. Tryptic soy agar was used to determine the total heterotrophic bacteria. The petroleum-degrading bacteria were analyzed according to Chaîneau *et al.* (1999). Results were expressed per dry weight of sludge after the moisture content had been analyzed.

Statistical analysis

As the OD at the 72nd hour normally revealed the greatest differences in the utilization patterns in the BIOLOG plates, the diversity indices and principal component analysis were conducted based on BIOLOG data at the 72nd hour (Kong *et al.*, 2006). One-way and two-way analyses of variance were used to investigate the effects of time and layer depth on microbial metabolic activity and diversity. Statistical analyses were conducted using the SPSS 17.0 for Windows.

Results and Discussion

Microbial metabolic activity

AWCD values were calculated and marked in Fig. 1, which revealed the microbial metabolic activity in the aged oil sludge (Rodriguez and Toranzos, 2003). Logistic model was applied to fit the data points according to Nakatani *et al.* (2012). The area surrounded by fitted curve and X-axis represents the microbial metabolic activity of the corresponding sample: bigger area indicates higher microbial metabolic activity.

The equation of Logistic model applied in the fitting procedure is:

$$y = A_2 + \frac{A_1 - A_2}{1 + (X/X_0)^P}, \quad (1)$$

where A_1 is the intersection point of fitted curve and Y-axis, A_2 the asymptote ($y=A_2$) of fitted curve, x_0 the value of independent variable when $y=(A_1+A_2)/2$, P the power of fitted curve (Table 1).

According to the areas surrounded by fitted curves and X-axis shown in Fig. 1 and Table 1, the AWCD fitted curves indicated that the microbial metabolic activity in the treated pile was higher than that in the control pile.

FIG. 1. Temporal changes in average well color development (AWCD) in three layers of the two biopile. Error bars represent the standard error of the mean ($n=3$).

Samples taken from the inner layer of the treated pile ranked the order of 30 days < Initial sample < 60 days < 220 days, whereas those in the control pile ranked the order of 30 days < 60 days < Initial sample < 220 days. The four AWCD curves for the inner layer of the treated pile can be classified into two groups: there is no significant difference between the curves for the initial sample and day 30 ($p > 0.05$), whereas the curves for days 60 and 220 are obviously higher than the other two ($p < 0.05$). This suggests that the microbial metabolic activity in the inner layer of the treated pile did not improve before day 30, and it increased with the passage of time and reached a steady level before day 60. The microbial metabolic activity maintained this steady level from day 60 to 220. In the control pile, the AWCD curve corresponding to day 220 is not significantly different from that of the initial sample ($p > 0.05$).

In the middle layer, the microbial metabolic activities for day 60 in the two piles were higher than that of the initial sample and were not significantly different from that for day 220 ($p > 0.05$). The microbial metabolic activity situation in the surface layer, both in control and treated piles, improved to a steady level on day 30, and there was no obvious variation observed in the following period.

Compared with the control pile, TPHs were highly removed in the treated pile after 220 days bioremediation. TPHs removal rate in the inner, middle, and surface layer of treated pile and control pile achieved 28.9%, 32.3%, 33.5% and 20.4%, 22.5%, 26.3%, respectively. Similar relationship between microbial activity and TPHs was revealed by Sutton *et al.* (2013). The engineering strategies of excavation, agitation, and piling changed the habitat of the microorganisms in the oil sludge and caused a corresponding adjustment in microbial activity. At the early stage, the undeveloped pore structure of the oil sludge and the gravitational compaction from the upper layers caused a lack of natural convection and air diffusion into the pile, especially for the inner layer of control pile. The anaerobic environment thus created restricted microbial metabolic activity in the inner layer. In comparison, the middle layer exhibited a shorter recovery time and higher microbial metabolic activity because of its shorter convection path and better air diffusion. The surface layer of the control pile exhibited the highest AWCD curves with no noticeable recovery time because the oxygen supply was sufficient to enhance microbial metabolic activity (Li *et al.*, 2004). Air dispersion was improved and compost

TABLE 1. PARAMETERS OF FITTED CURVES FOR TWO BIOPILES

Samples	Parameters of fitted curves				R^2	Area
	A_1	A_2	x_0	P		
Treated pile inner layer						
Initial sample	0.014	0.958	77.558	3.399	0.990	136.003
30 days	0.004	0.849	75.472	3.228	0.995	120.977
60 days	0.013	1.566	70.971	4.295	0.999	237.087
220 days	0.017	1.670	66.852	3.636	0.999	256.836
Treated pile middle layer						
Initial sample	0.014	0.958	77.558	3.399	0.990	136.003
30 days	0.023	1.732	60.025	3.735	0.999	280.019
60 days	0.028	1.630	53.419	3.060	0.999	271.010
220 days	0.030	1.60	69.577	3.942	0.998	244.286
Treated pile surface layer						
Initial sample	0.014	0.958	77.558	3.399	0.990	136.003
30 days	0.012	1.573	60.973	3.273	0.999	249.289
60 days	0.019	1.482	59.563	2.776	0.999	233.449
220 days	0.017	1.657	61.580	2.745	0.999	256.673
Control pile inner layer						
Initial sample	0.014	0.958	77.558	3.399	0.990	136.003
30 days	0.06	0.140	88.659	9.565	0.562	19.813
60 days	0.007	0.583	90.958	4.717	0.920	76.945
220 days	0.005	1.068	87.641	5.137	0.962	144.678
Control pile middle layer						
Initial sample	0.014	0.958	77.558	3.399	0.990	136.003
30 days	0.024	0.467	87.101	4.829	0.946	65.184
60 days	0.021	1.189	79.335	4.183	0.973	170.108
220 days	0.022	1.352	93.076	3.204	0.994	169.738
Control pile surface layer						
Initial sample	0.014	0.958	77.559	3.399	0.990	136.003
30 days	0.024	1.531	92.602	5.279	0.985	201.712
60 days	0.012	1.514	87.974	4.316	0.999	202.258
220 days	0.020	1.597	82.949	4.687	0.998	223.911

consolidation was limited by the addition of the bulking agent, which caused an improvement in the microbial activity and the biodegradation of organic matter (Trémier *et al.*, 2009).

Microbial metabolic diversity

Shannon indices based on the BIOLOG data indicate the distribution of sole-carbon-source utilization by the microbial communities and the potential metabolic diversity of the communities (Harch *et al.*, 1997). The Richness indices indicate the amounts of carbon sources utilized by microorganisms despite the distribution or utilization degree. The two diversity indices were both calculated based on BIOLOG data at the 72nd hour. As shown in Fig. 2, in the inner layer of the control pile, the Shannon index decreased significantly before day 30 ($p < 0.05$), then recovered to a value similar to that of the initial sample on day 60 and maintained that value to the end. The suppressing effect on this metabolic diversity was also suggested by the middle layer at the early stage. However, the Shannon indices of the surface layer maintained steady levels from day 30 to 220 ($p > 0.05$).

In each layer of the treated pile, the samples taken from the last three batches reflect higher values than the initial sample except for the inner layer on day 30. Furthermore, the similarities in the Shannon indices ($p > 0.05$), which are reflected by the last three batches of samples in the middle and surface

layers, suggest that the microbial Shannon indices were all improved to a steady level on day 30.

Temporal changes in Richness indices in all three layers of each biopile are classified into two groups. Group one includes the inner layer of the treated pile, the inner and middle layers of the control pile. The Richness indices of these three layers decreased significantly on day 30 and recovered with the passage of time. The Richness indices of the other three layers fluctuated with a small amplitude. The similarity in the temporal changes was found between the Shannon and Richness indices. At the beginning of the experimental period, the suppressing effect was indicated by both indices in the inner layer of the treated pile and the inner and middle layers of the control pile. Then, the two indices recovered with the passage of time. It is inferred that the lack of oxygen and the microbial metabolic adjustment caused this interactive phenomenon. In addition, bulking agent not only improved the pore structure of the biopiles, but also acted as a slow-release nutrient for the microorganisms (Paredes *et al.*, 2002). Thus, the treated pile showed a higher microbial metabolic diversity than the control pile at different stages.

Microbial metabolic variance

As shown in Fig. 3, based on the BIOLOG data at the 72nd hour, two-dimensional principal component analysis was

FIG. 2. Temporal changes in two microbial metabolic diversity indices in three layers of two biopile samples. Error bars represent standard error of the mean ($n=3$).

applied to three batches of samples taken from the three layers of each biopile. According to the results of the principal component analysis, the first two extracted principle components corresponded to 62.58% of the total variance, which accounted for the microbial metabolic variance. The first principal component (PC 1) contributed 50.27% to the total variance, with 12.31% contributed by the second principal component (PC 2). The microbial metabolic variance was clearly reflected by the distances among sample dots in the scatter graph based on the factor scores of the first two principal components.

As the first principle component contributed 50.27% to the variance, the differentiation of microbial metabolism was mainly determined by the distance along the horizontal direction. All samples are divided into two clusters by the vertical axis (PC 1 = 0): samples taken from the treated pile are located on the right of the vertical axis; samples taken from the control pile and the initial sample are located on the left. The similarities of microbial metabolisms between the control pile and the initial sample are reflected by their positions in Fig. 3, whereas the changes in microbial metabolism are indicated by the treated pile. This shows that the microbial metabolism of

FIG. 3. Two-dimensional principal component analysis (PCA) of BIOLOG data obtained from all batches of samples. PC 1, first principal component; PC 2, second principal component.

FIG. 4. Changes in numbers of culturable petroleum-degrading bacteria and total heterotrophic bacteria among layers and biopiles during the period of bioremediation. Error bars represent the standard error of the mean ($n=3$).

the treated pile was altered by the biostimulation. The addition of nutrients might account for the variance in microbial metabolism and structure (Fede *et al.*, 2001; Grayston *et al.*, 2001).

Microbial community generally alters with the process of bioremediation. In the research on succession of bacterial community along with the removal of heavy crude oil pollutants developed by Yu *et al.* (2011), bacterial community was remarkably changed by multiple biostimulation treatments.

Samples taken from the same layer in each biopile, but from different batches are marked by an ellipse. For the control pile, three ellipses representing the inner, middle, and surface layers range from left to right along the horizontal axis, and in each layer, three samples taken at different times generally range from left to right in the time sequence. An interesting phenomenon is that the microbial metabolic characteristics of the inner and middle layers of the control pile tended to evolve to those of the next outer layer over time. For example, in comparison with the inner layer on day 30, the metabolic characteristics of the inner layer on day 60 were closer to those of the middle layer on day 30; in comparison with the middle layer on day 30, the metabolic characteristics of the middle layer on day 60 were closer to those of the surface layer on day 30. However, the treated pile did not exhibit this interesting phenomenon.

Enumeration of culturable bacteria

Quantities of bacteria in the treated pile were obviously higher than those in the control pile. This phenomenon shows that biostimulation significantly improved bacterial numbers by more than two orders of magnitude (Fig. 4).

The quantities of the two bacteria revealed similar trend. First, they increased sharply to high levels on day 30. In comparison with other research (Abioye *et al.*, 2010), the number of petroleum-degrading bacteria was of the same order of magnitude. As the temperature decreased in the winter, the quantities of both bacteria in all samples declined. However, the numbers recovered as the temperature increased in the spring. At the end of the experimental period in the summer, the numbers of both bacteria in the treated pile continued to increase, whereas some samples in the control pile showed a

slight decline. Along with the depletion of easily degradable TPHs, reduced bacterial numbers in the control pile implied extensive stagnation caused by the lack of available carbon sources at the end of the bioremediation procedure.

Conclusions

With the addition of cotton stalk, the community level physiological parameters (microbial activity and diversity) of the treated biopile were dramatically improved in comparison with the control. Relative stability throughout the experimental period and a general homogeneity across the layers were indicated by the treated biopile. In addition, an obvious suppressing effect and long recovery time of the microbial metabolic activity and diversity of the control pile were apparent in the early experimental stage. The treated biopile exhibited a shorter recovery time and higher microbial metabolic activity and diversity. Enhanced biopile could be selected for the treatment of oily sludge with the addition of cotton stalk, and the factors could also be considered that the temporal trend of petroleum-degrading bacteria and the total heterotrophic bacteria correlated with the environmental temperature.

Acknowledgments

Research was conducted by the State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing, China. This research was supported by the Young Scientists Fund of NSFC (Grant No. 40901249) and Special Environmental Research Funds of MEP for Public Service (201009015). Appreciation for site management and cooperation is gratefully expressed to Shengli oilfield.

Author Disclosure Statement

No competing financial interests exist.

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