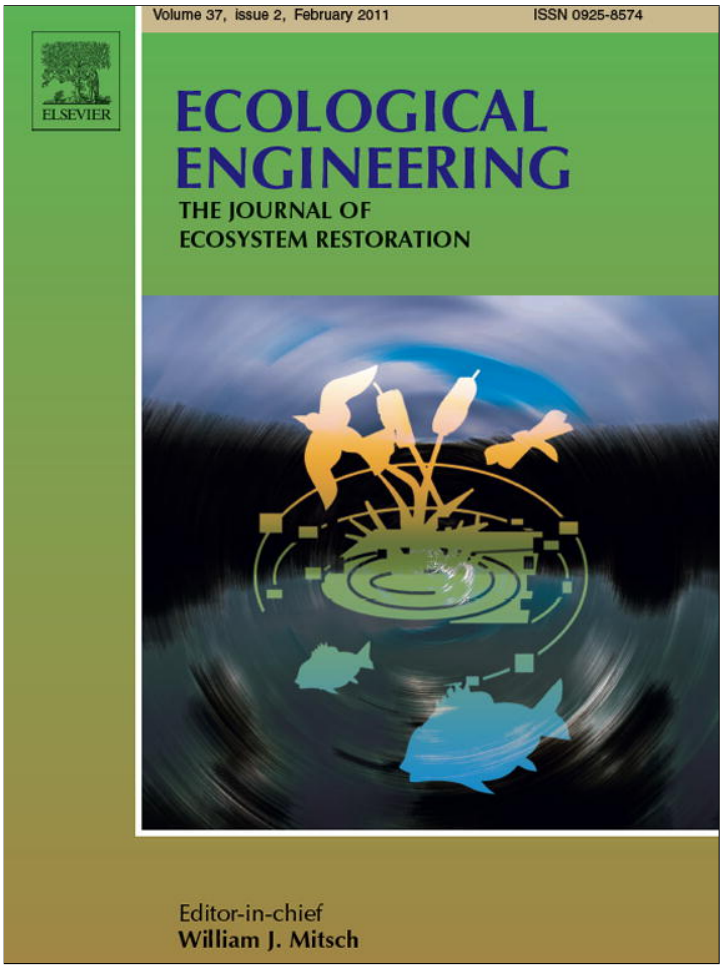

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Short communication

Anaerobic biodegradation of benzene in salt marsh sediment of the Louisiana Gulf coast

Jiou Yu [a], Rui Tao [b], Kewei Yu [b,*]

[a] Loveless Academic Magnet Program High School, Montgomery, AL 36108, USA
[b] Department of Biological and Environmental Sciences, Troy University, Troy, AL 36082, USA

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ABSTRACT

The 2010 BP oil spill became the worst environmental disaster in US history with unprecedented damages to the environment and human health. Ecosystem recovery from the oil spill largely depends on the microbial degradation of oil contaminants. In this study benzene was selected as a representative of petroleum hydrocarbons to assess its anaerobic biodegradation potential in salt marsh sediment from the Louisiana Gulf coast. The experiment was conducted in two separate phases of incubation of the sediment slurries to determine the benzene biodegradation kinetics (phase-1), and to determine if any stimulation of benzene degradation occurred after benzene exposure (phase-2). Benzene biodegradation was assumed to follow Michaelis–Menten kinetics, and the V_{max} was determined to be 1.66 mg g^{-1} $week^{-1}$ and the K_m to be 1.17 mg g^{-1}. According to this model, it would take 11 weeks for 1 m^2 of the salt marsh to degrade 1 L of benzene. Benzene degradation rates were found to have a minimum increase after the sediment had been exposed to benzene for 4 weeks, which suggests that the sediment has almost reached its natural maximum biodegradation potential.

1. Introduction

The 2010 BP oil spill in the Gulf of Mexico was unprecedented with approximately 5 million barrels of petroleum hydrocarbons released into the ocean in 87 days before the Macondo exploration well was permanently sealed (BP, 2010; NOAA, 2010). The incident became the worst environmental disaster in US history, and the environmental and human health damages from this oil spill may take decades to unveil. Coastal wetlands are sensitive to oil contamination, but traditional mechanical cleanup practices commonly show limited removal efficiency and can even cause more damages to the fragile wetland than the oil itself (Venosa and Zhu, 2003; Mitsch, 2010). Thus, bioremediation is appealing because it is less disruptive and less expensive than mechanical remediation technologies.

Both aerobic (Atlas, 1981) and anaerobic (Chakraborty and Coates, 2004) mechanisms have been studied for the biodegradation of petroleum hydrocarbons. Aerobic biodegradation has been deemed more effective due to the presence of oxygen. However, oils can be pushed into soft marine and wetland sediments by natural forces where it inhabits anaerobic environments that prolong its existence in the ecosystem. The aeration status of a system is commonly determined by its oxidation–reduction (redox) potential (Bohn, 1971). In wetland sediments, a series of redox reactions can occur with petroleum hydrocarbons and other organic matters as electron donors and various electron acceptors, in their thermodynamic order including oxygen, nitrate, manganese (IV) and iron (III), and sulfate (Van Stempvoort et al., 2004; Fan et al., 2006).

The observed toxicity of petroleum mainly comes from its lower-molecular-weight soluble components (Fuller et al., 2004), most of which belong to a class of carcinogenic polycyclic aromatic hydrocarbons (PAHs). Due to the stability of the aromatic chemical structure, PAHs have been long recognized to be recalcitrant in the natural environment. Benzene belongs to the family of monocyclic aromatic compounds (BTEX, including benzene, toluene, ethlybenzene, and xylene) known for their high toxicity and mobility in the environment.

Microbial degradation is the primary mechanism for removal of petroleum hydrocarbons in wetland sediments. In response to the 2010 BP oil spill, benzene was selected in this study as a representative of petroleum hydrocarbons to assess its biodegradation potential in Gulf coast marsh sediment. The objectives are to (1) determine the original benzene biodegradation rate in the sediment, and (2) assess the potential if benzene biodegradation capacity in the sediment can be further stimulated following exposure of the sediment to benzene. It is hypothesized that as a natural

* Corresponding author. Tel.: +1 334 808 6316; fax: +1 334 670 3662.
E-mail addresses: kyu@troy.edu, dr.kewei@gmail.com (K. Yu).

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feedback, benzene biodegradation rate may increase after the original sediment is exposed to benzene.

2. Materials and methods

2.1. Sediment sample

The sediment used for this study was sampled in May 2011, one year later after the 2010 BP oil spill, from a coastal salt marsh (N30°08.782′ W89°44.665′) on the Louisiana Gulf coast. After removing the surface vegetation, the top 30-cm of the sediment was taken in the field. The sample was stored in a refrigerator (4 °C) before the experiment. The major marsh plant species in this location were *Spartina alterniflora* and *Spartina patens*. These two species are relatively tolerant to petroleum pollution and may have the potential for the phytoremediation of oil contamination (Lin and Mendelssohn, 1998). The sediment was characterized as silty clay loam with 11% sand, 57% silt and 32% clay (Hydrometer method). The pore water of the sediment had a salinity of 12 ppt (parts per thousand), pH of 7.06 at 20 °C, nitrate 11.4 mg L^{-1}, nitrite 21.7 mg L^{-1}, and sulfate 109.6 mg L^{-1} (EPA method 300.0). The redox potential (Eh) of the sediment was measured to be +120 mV at pH 7.0. Major chemical characteristics in dry weight of the sediment were total carbon 7.2%, total nitrogen 0.43%, total potassium 2.36 g kg^{-1}, total magnesium 5.56 g kg^{-1}, total phosphorus 0.605 g kg^{-1}, total iron 19.9 g kg^{-1}, total manganese 0.123 g kg^{-1}, and total sulfur 5.43 g kg^{-1} (EPA method 200.7). The water content of the sediment was determined to be 72%.

2.2. Experimental procedure

The experiment was conducted in two separate phases of incubation of the sediment slurries to reach the 2 objectives of the study. Each phase of the incubation lasted for 4 weeks under anaerobic conditions. In phase-1, the sediment was treated with different concentrations of benzene to determine the innate constants for the benzene biodegradation kinetics (objective 1). Meanwhile, phase-1 allowed the sediment slurries to be exposed to different concentrations of benzene. The same sediment slurries from phase-1 were used in phase-2 where a single benzene concentration was applied to all the sediment slurries. The benzene degradation rates were compared among the different treatments from phase-1 to determine if any increase of benzene degradation rate occurred (objective 2).

Sediment slurry was made by using a 237-mL wide-mouth glass bottle with 40 g (wet weight) of sediment and 80 mL of diluted artificial ocean water. The artificial ocean water (36 ppt) was prepared by dissolving sea salt (Instant Ocean, Spectrum Brands Inc.) into distilled water, and was later diluted to a final salinity of 12 ppt. Thus, the sediment was incubated at the same salinity condition as in the sampling site. The bottle was covered with a screw cap lined with Teflon tape to prevent gas leakage. A hole was drilled in the center of the cap, and then a rubber stopper was inserted for future gas sampling.

In phase-1 of the study, sediment slurries were prepared according to the above procedure. Four treatments and 3 replicates were applied with a total of 12 bottles. Each bottle was then flushed with nitrogen (ultra high purity grade) for 5 min to displace headspace air and create an oxygen-free incubation environment. Benzene (>99.8% purity, analytical grade, Sigma–Aldrich Chemical Company) was added using a micro-syringe at 50 μL (treatment B), 100 μL (treatment C), and 200 μL (treatment D). The 3 bottles without the addition of benzene were considered as controls (treatment A, 0 μL). These treatments were equivalent to benzene concentrations of 0 mg g^{-1} (A), 3.91 mg g^{-1} (B), 7.83 mg g^{-1} (C), and 15.65 mg g^{-1} (D) of the sediment, respectively. Before initial measurements were taken, all sediment slurries were placed on a rotary shaker and incubated in the dark under room temperature (20 °C) for a day to allow the system to reach equilibrium. Then, 0.1 mL gas samples were taken using a Luer-lok syringe to determine the benzene concentrations in the headspace of the bottles. Benzene concentrations were monitored once a week for 4 weeks under the same incubation conditions.

At the end of phase-1 incubation, all bottles were uncovered for pH and Eh measurements. The bottles were left open for 2 days to allow benzene residue to escape. To start phase-2, the bottles were incubated under the same anaerobic condition as in phase-1. The same amount of benzene (100 μL, 7.83 mg g^{-1} equivalent) was injected into each bottle. As in phase-1, benzene concentrations in the headspace of the bottles were monitored weekly for 4 weeks after the system had reached equilibrium. At the end of the phase-2, all bottles were uncovered again for pH and Eh measurements.

2.3. Analytical methods

Benzene concentration was analyzed by using a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector (FID). A Supel-Q PLOT fused silica capillary column (30 m × 0.32 mm) was used. The oven, injector and detector temperatures were 160, 150 and 200 °C, respectively. The pH and Eh values of the sediment slurries were measured by using a pH/mV meter (Accumet AP62, Fisher Scientific) with a combination pH electrode (Fisher Scientific) and with an oxidation–reduction potential (ORP) electrode (SPER Scientific, Inc.), respectively.

2.4. Calculations and statistical analysis

The benzene degradation rates were calculated by linear regressions of the decrease in benzene concentrations over 4 weeks. All data are reported based on the dry weight of the sediment. The redox potentials were calibrated to the standard H_2 electrode by adding the correction factor (+247 mV at 20 °C) for the calomel reference to the observed instrument reading. The effect of pH on Eh was calculated according to the inverse relationship of Eh and pH as described by the Nernst equation (Bohn, 1971). Significance among the means of different treatments was determined by the *t*-test (significance level $\alpha = 0.05$). There is a statistically significant difference between the means when the calculated p value is less than 0.05.

3. Results

To verify that the experiments were under anaerobic conditions, redox potentials of the sediment slurries were measured at the end of each phase of incubation. There was no significant difference ($p > 0.05$) in Eh values among different treatments. At the end of phase-1, the Eh values were all approximately +150 mV, slightly higher than that in the field. The Eh values at the end of phase-2 decreased to approximately +10 to +50 mV (Table 1), which were lower than those at the end of phase-1 with statistical significance ($p < 0.05$).

In this study, anaerobic biodegradation of benzene is assumed to follow Michaelis–Menten kinetics:

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

where V_{max} represents the apparent maximum benzene degradation rate under the incubation conditions, K_m represents the inherent characteristic of the benzene degradation enzyme, and V

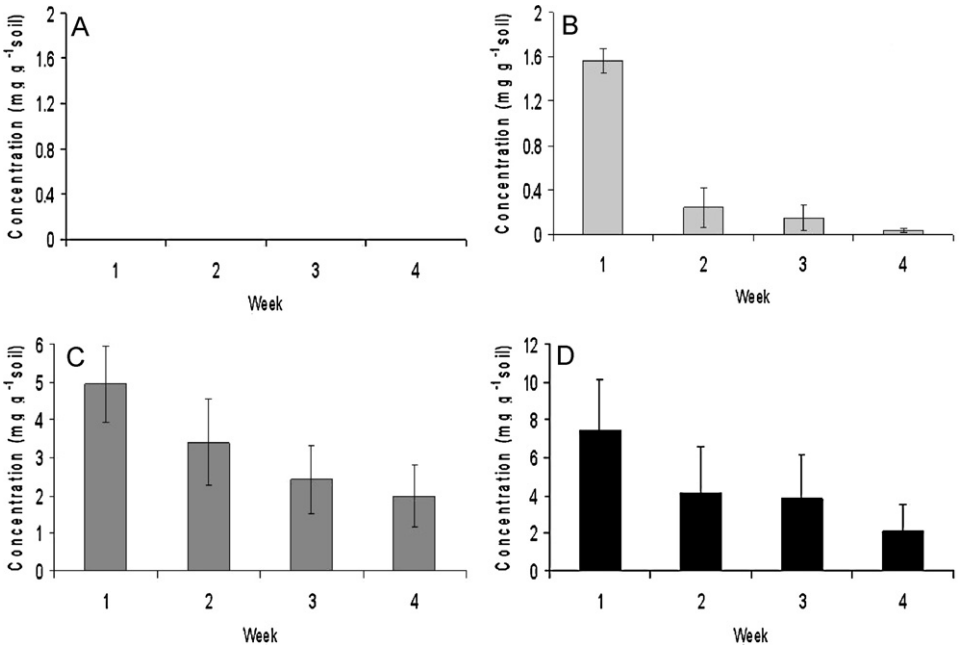

Fig. 1. Benzene degradation during phase-1 of anaerobic incubation under different treatments. Treatments included the addition of benzene at concentrations of 0 mg g^{-1} (A), 3.91 mg g^{-1} (B), 7.83 mg g^{-1} (C), and 15.65 mg g^{-1} (D) sediment. Standard deviations of the replicates ($n = 3$) are denoted by the error bars.

is the apparent benzene degradation rate under a specific benzene concentration [S].

The incubation conditions in phase-1 were more similar to those of the sampling site. Thus, benzene degradation dynamics in phase-1 was used to determine Michaelis–Menten parameters (Fig. 1). When the same amount of benzene was added in phase-2 incubation, all treatments showed a similar pattern of benzene degradation (Fig. 2). Overall, approximately 70% of the added benzene was degraded, similar to the results in phase-1 incubation when the same amount of benzene was added (Fig. 1, treatment C). Weekly measurements of benzene concentrations suggest that the degradation activity was not limited by the availability of benzene.

4. Discussion

Anaerobic biodegradation of benzene has been observed under iron reducing (Lovley et al., 1994; Coates et al., 1996; Caldwell et al., 1999), sulfate reducing (Weiner and Lovley, 1998a), and methanogenic conditions (Weiner and Lovley, 1998b). The sediment used in this study was under moderately reducing conditions

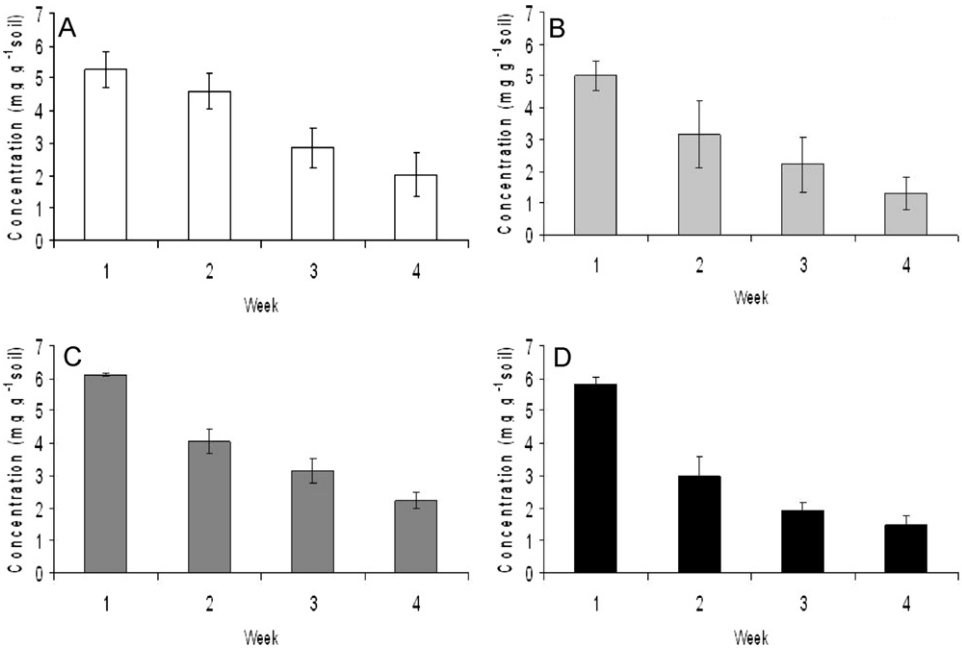

Fig. 2. Benzene degradation during phase-2 of anaerobic incubation under different treatments. After opening the bottles at the end of phase-1 of incubation, benzene was added at 7.83 mg g^{-1} sediment to all treatments (A–D as in Fig. 1). Standard deviations of the replicates ($n = 3$) are denoted by the error bars.

Table 1
Redox potentials of the sediment slurries under different treatments.

Treatment	Redox potentials (Eh, mV) at pH 7.0	
	Phase-1	Phase-2
A	+162 ± 28	+52 ± 13
B	+153 ± 2	+36 ± 13
C	+152 ± 6	+14 ± 15
D	+148 ± 9	+18 ± 1

Treatments included the addition of benzene at concentrations of 0 mg g^{-1} (A), 3.91 mg g^{-1} (B), 7.83 mg g^{-1} (C), and 15.65 mg g^{-1} (D) sediment in phase-1, and 7.83 mg g^{-1} sediment in phase-2. Both pH and Eh measurements were made at the end of each phase. Redox potentials were reported as the corresponding values at pH 7.0, according to the Nernst equation.

in the field with no presence of oxygen as indicated by the initial redox potential measurement. Redox measurement at end of the incubations verifies that the sediment remained under anaerobic conditions throughout the study (Table 1). Due to the high iron content in the sediment, the sediment slurries remained in the redox range for iron reduction (Patrick and Jugsujinda, 1992; Yu et al., 2007) after 2 months of incubation. Total iron content in the sediment was about 160 times higher than that of manganese, and thus iron likely acted as the dominant electron acceptor for benzene degradation in this study, especially in phase-2 incubation. Despite high organic matter content in the sample sediment, benzene was still readily available as the electron donor for microbial metabolisms.

To determine the constants (V_{max} and K_m) for Michaelis–Menten kinetics (Eq. (1)) of benzene biodegradation, the Lineweaver–Burk plot was used:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)$$

where V was the average benzene degradation rate, and $[S]$ was the average benzene concentration during the 4-week incubation. Each replicate of the treatments was calculated individually. The Lineweaver–Burk plot was drawn by taking the linear regression of $1/V$ against $1/[S]$ with liner regression equation of $1/V = 0.67 \times 1/[S] + 0.61$ ($R^2 = 0.83$, $n = 9$). The V_{max} was calculated to be 1.66 mg g^{-1} week^{-1}, while the K_m to be 1.17 mg g^{-1}. According to these kinetic constants, it would take 11 weeks for 1 m^2 of salt marsh to degrade 1 L of benzene (benzene concentration = 11.7 mg g^{-1} sediment with bulk density of the top 30 cm of salt marsh sediment = 0.25 g cm^{-3}, Hatton et al., 1983).

Few references are available on benzene degradation in marsh sediments. This study was likely the first attempt at characterizing the benzene biodegradation kinetics in Gulf coast salt marsh sediments. In Michaelis–Menten kinetics, the K_m value represents the affinity of the enzyme to the substrate and equals the substrate concentration at the half maximum reaction rate. The result of this study show that the K_m was 1.17 mg g^{-1}, which was 1 or 2 orders of magnitude higher than those determined in pure culture studies (Coates et al., 2001). Due to the high K_m value, changes in benzene concentration showed a non-linear decrease in treatment B (3.91 mg g^{-1}) of phase-1 incubation where benzene concentration was lower (Fig. 1). Thus, when added benzene concentrations were greatly higher than the K_m value, benzene concentrations decreased close to a linear pattern (Fig. 1C and D; Fig. 2). Under these conditions, benzene degradation occurred close to its maximum rate until benzene concentration fell close or below the K_m value.

In this study, the V_{max} for benzene degradation was found to be 1.66 mg g^{-1} week^{-1}. In phase-2 incubation, benzene degradation rates were all close to the calculated V_{max} value due to sufficient benzene availability (7.83 mg g^{-1}). This V_{max} value is crucial in

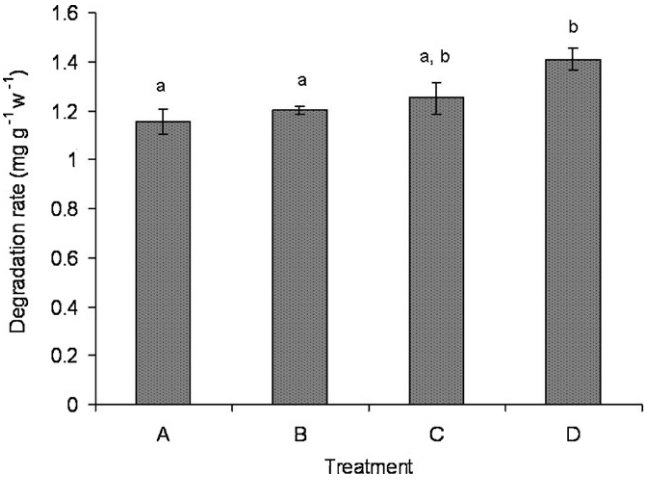

Fig. 3. Benzene degradation rates during phase-2 of anaerobic incubation under different treatments (A–D as in Fig. 2). Data represents the means of the replicates ($n = 3$) with standard deviations denoted by the error bars. Data labeled with the same letters represent no significant difference statistically ($p > 0.05$).

assessing the longevity of petroleum contaminants in coastal salt marsh sediments. The average benzene degradation rates over the 4-week period are summarized in Fig. 3. The results indicate that there was some potential for stimulation of the benzene degradation capacity during phase-1 incubation. Only when the sediment was pre-exposed to benzene at high concentrations (treatment D) did the benzene degradation rate increased significantly ($p < 0.05$) higher than the treatments with little (treatment B) or no (treatment A) exposure. Degradation rates increased by 22% between sediments exposed to high benzene concentrations (treatments D) and those with no exposure (treatment A) during phase-1 incubation. This coastal salt marsh sediment likely had a history of petroleum exposure, since there are more than 1000 natural oil seepage sites reported in the Gulf of Mexico (Etkin, 2009). The historic exposure likely explains the limited potential found in the study for increasing the benzene degradation capacity in this sediment. Even exposure to higher levels of benzene due to petroleum contamination, as in the case of the BP oil spill in 2010, can only stimulate limited increase in the degradation rate.

The apparent benzene degradation rate was much lower compared to those in pure culture studies probably due to the following reasons: (1) the high salinity of this sample sediment may significantly inhibit the degradation rate. In a study conducted on BTEX degradation of a saline enrichment culture, it was found that benzene degradation slowed nearly 4 times between non-saline sediments and the sediments exposed to 5 M NaCl (Sei and Fathepure, 2009); (2) the presence of other organic matter may compete with benzene as the electron donor. The overall degradation rate of BTEX was slowed due to the presence of added glucose or citrate that are more easily metabolized carbon sources for the microbes (Chong and Chiou, 2010); (3) the degradation rate may be inhibited by the anaerobic incubation condition. Anaerobic environments limit the number of microbial species and prevent oxygen from acting as the most favorable electron acceptor (Atlas, 1981). Future investigations on petroleum hydrocarbons degradation should focus on strategies for effectively implementing bioremediation techniques in actual field settings. To restore the ecological services of the Gulf wetland ecosystem, microbial community structures and environmental conditions must be integrated together to propose any feasible remediation techniques (Simon et al., 2004).

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