

Using bubble respirometer to monitor microbial activity in passive bioventing system

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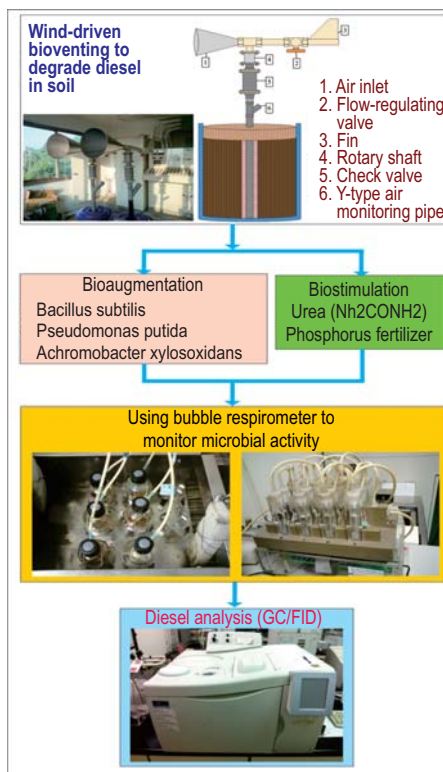
Abstract

Aim : The study aimed to investigate the relationship between the diesel degradation efficiency in soil and microbial activity during the wind-driven bioventing remediation. The study also aimed to compare the indicators of cumulative oxygen uptake and 24-hr oxygen uptake rate with the analysis results of diesel concentration.

Methodology: A self-made wind-driven bioventing equipment was used as passive bioventing device. In the wind-driven bioventing pilot study for highly diesel contaminated soil (approximately 20,000 mg kg⁻¹), the bubble respirometer collocated with the bioslurry method was used to monitor the microbial activity in soil. The diesel concentration was analyzed by GC/FID.

Results: The result of respiration assays showed that the microbial activities of *Bacillus subtilis* and *Pseudomonas putida*, which are capable of degrading diesel, could be consecutively bioaugmented for two weeks, and that of *Achromobacter xylosoxidans* could be consecutively bioaugmented for three weeks after adding individual microorganism. While appropriate amount of nutrients were added in the pilot, the cumulative oxygen uptake in the *Pseudomonas putida* set elevated from 219 mg-O₂ to 328 mg-O₂ in one week that inferred the microbial activity of *Pseudomonas putida* and which could be elevated rapidly in a highly diesel contaminated environment. However, the 24-hour oxygen uptake rate in the *Achromobacter xylosoxidans* set could be maintained in the first three weeks between 4.8 mg-O₂ hr⁻¹ and 5.0 mg-O₂ hr⁻¹ that illustrated the microbial activity of *Achromobacter xylosoxidans*, which was the highest among three microorganisms in the highly diesel contaminated environment.

Interpration: The research results demonstrated a positive correlation between the diesel degradation efficiency and 24-hour average oxygen uptake rate of microorganisms during the experiment, suggesting that the 24-hour average oxygen uptake rate could be used as an indicator of microbial activity.



Introduction

The bioventing method converts vadose zone contaminated soil into a ventilated environment by supplying oxygen and nutrients to facilitate the growth of aerobic microorganisms and to sustain microbial activity which mainly degrade petroleum contamination (Brown *et al.*, 1996). The venting not only removes petroleum contaminants by physical means, but also enhances microbial activity and promotes biodegradation of petroleum contaminants (Texas Research Institute, 1980). Both biostimulation and bioaugmentation technologies operate either through addition of nutrients or enhanced aeration (biostimulation), or through addition of microorganism (bioaugmentation). As such these approaches seek to ensure successful remediation of the site by enhancing the microbial activity under appropriate environmental conditions (Boopathy, 2000). Nine bacterial strains including *Bacillus subtilis* were isolated from the polluted stream which could degrade crude oil (Adebusoye *et al.*, 2007). Szulc *et al.* (2014) demonstrated that bioaugmentation of *Pseudomonas putida* contributed to the highest diesel oil biodegradation efficiency, even the benefits of bioaugmentation was found in a diesel-polluted clay-rich soil (Masy *et al.*, 2014).

Pseudomonas putida and *Pseudomonas fluorescens* were selected as a coculture to degrade benzene, toluene, ethylbenzene, and xylenes under various growth conditions (Shim, 2005). The literature also illustrated that addition of nutrients were necessary to enhance biodegradation of oil pollutant (Kim *et al.*, 2005). On the other hand, excessive nutrient concentrations can also inhibit the biodegradation activity (Chaillan *et al.*, 2006; Chaîneau *et al.*, 2005). Urea is usually added as a nitrogen source for cellular growth, but it can also serve as an electron acceptor (Leeson and Hinchee, 1997). Literature also indicates that the application of biosolids that contain nutrients, organic matters, and microorganisms had positive effect on the enhancement of biodegradation rate of toluene and naphthalene in the contaminated soil (Chang *et al.*, 2009). Nutrient introduction (biostimulation) promoted a positive effect on microbial population for the microbial consortium consisting *Achromobacter xylosoxidans* (Colla *et al.*, 2014). The results also indicate that the edaphic community structure and dynamics were different according to the treatments employed. However, carbon dioxide evolution demonstrated insignificant difference in soil microbial activity between biostimulation and bioaugmentation treatments.

Microorganisms in soil consume oxygen and produce carbon dioxide during respiration and the rate has proved to have correlation with the degradation rate of hydrocarbon (Cookson, 1995; Plaza, 2005). By monitoring the amount of oxygen uptake or titrating absorbed carbon dioxide in the potassium hydroxide (KOH) solution, the microbial activity and the degradation efficiency can be evaluated (Balba *et al.*, 1998). Cumulative

oxygen uptake and oxygen uptake rate are often used as indicators of respiration monitoring (Chang *et al.*, 2009). Relationship between oxygen uptake and microbial activity on the biodegradation of diesel was studied by Nwankwo *et al.* (2014). The amount of oxygen consumed during microbial activities was computed and the rate of dissolved oxygen concentration was calculated, the specific oxygen uptake rate was high, while high diesel concentration existed in the soil.

In view of the above, the present study was carried out to investigate the effect on microbial activity in diesel-contaminated soil after adding microorganisms and nutrients in passive bioventing pilot. Study also aimed to compare the indicators of cumulative oxygen uptake and 24-hr oxygen uptake rate with the analysis results of diesel concentration by using GC/FID. By means of evaluating a correlation between oxygen uptake and diesel degradation, and constructing an appropriate indicator of microbial activity, which can be utilized for an efficient and effective monitoring of microbial activity at diesel contaminated sites.

Materials and Methods

Passive bioventing equipment : It is a self-made wind driven bioventing equipment, introducing oxygen-containing air from atmosphere into the vadose zone. In appearance, it has a trumpet-shaped inlet to inhale air from the environment, a fin at the end to automatically aim at the wind direction, and a flow-regulating valve to adjust the amount of air. It is fixed underground casing by ropes and clips (Fig. 1).

Construction of bioventing pilot sets: Five bioventing pilot sets for highly diesel contaminated soil (approximately 20,000 mg kg⁻¹) were built in this research. Soil was obtained from a contaminated site in Taichung, Taiwan. On the basis of soil texture analysis and element analysis, the soil is concluded as was sandy loam with a C:N:P ratio of 1000:10:1. After one month of insulation, the oxygen concentration of these pilot sets was brought down to

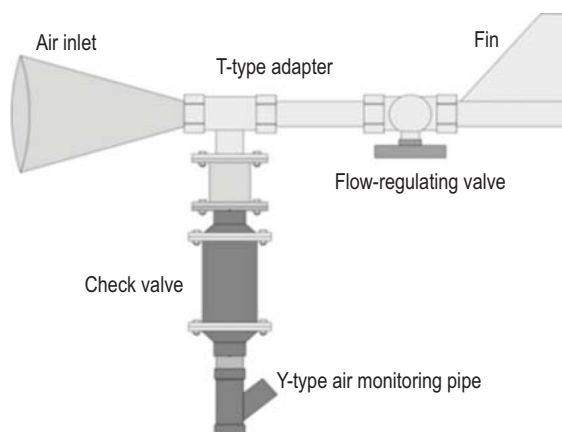


Fig. 1 : Sketch diagram of passive bioventing equipment

10% and wind-driven bioventing systems were then established for the experiment. The man-made wind field was created by adjustable fans and the amount of air introduced into vadose zone was adjusted according to the requirements of the experimental set up. In order to investigate the effects of microorganism and nutrients, soil in the pilot sets were collected weekly to monitor microbial activities, and to analyze the diesel concentration.

Batch respiration experiment of bioslurry method : Bioslurry method increases the chances of interactions between microorganisms and diesel in the soil. According to the results of microbial respiration activity monitored by bioslurry method, the respiration rate was higher by the bioslurry method within four weeks, and the variability was the lowest with a soil:water ratio of 1:3 (Aspray, 2007). Hence, the same ratio was followed in this research. In order to stimulate a diesel-degradation, a bacterium strain was isolated from a diesel-polluted site, and nutrients source and operation conditions were optimized (Dahalan *et al.*, 2014). Based on the results of element analyses, 200 mM urea (NH_2CONH_2) was added as nitrogen supplement, and 8.06 mM Centrum junior with extra calcium (from Pfizer) was added as phosphor and microelement supplement into bioslurry to conform to the C:N:P ratio of 100:10:1, as suggested in a earlier report

(Cookson, 1995). In addition, a test tube with 6N KOH solution for absorbing carbon dioxide generated from the experiment was installed. After assembly, the bottle lid with a washer was placed and the bottle was fixed in a thermostat water bath set at 32°C. The needle was stabbed into the bottle lid in a tilted manner. The pressure of oxygen cylinder was set at 2.2 to 2.5 psi, and the stirrer was set at a proper speed. The monitoring software of the bubble respirometer was then launched to record the oxygen uptake of microorganisms in soil. Recording for each batch was conducted for five days. In order to exclude the non-biological oxygen uptake, the background oxygen uptake was measured by a reaction bottle filled with deionization water only. These background values were deducted from each batch measuring data.

Microorganisms capable of degrading diesel : One pilot set was used as a blank without air supplement, and the others were ventilated with enough oxygen into soil zone by an artificial field of wind speed 0.6 m/s in passive bioventing equipments. Local bacterial, strains *Bacillus subtilis*, *Achromobacter xylosoxidans* and *Pseudomonas putida*, capable of degrading diesel, were domesticated into experimental pilot sets. To promote adaption to highly diesel contaminated soil, microorganisms were incubated

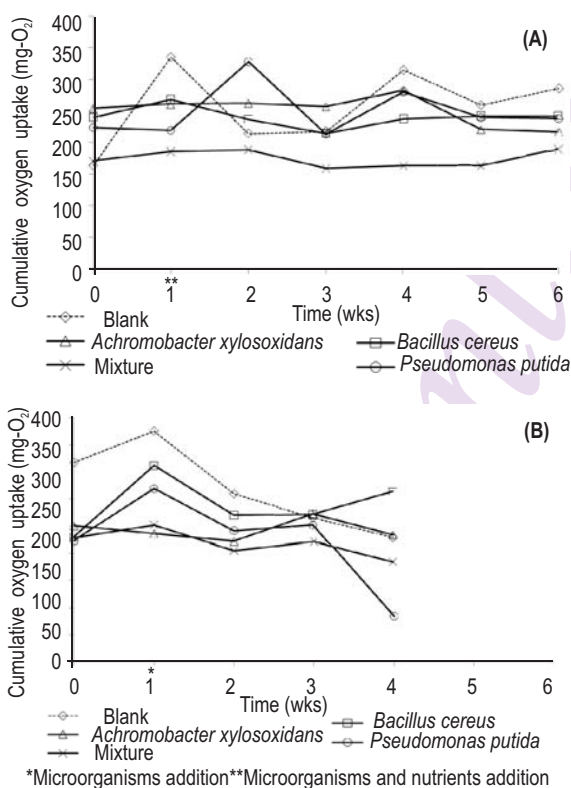


Fig. 2 : The cumulative oxygen uptake of bioventing pilot sets with and without the addition of nutrients. (A) With the addition of microorganisms only; (B) With the addition of microorganisms and nutrients

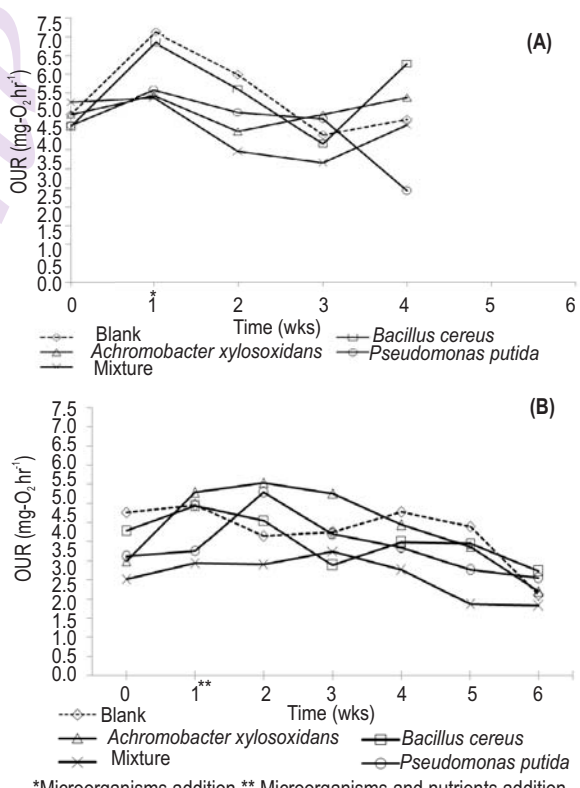


Fig. 3 : The 24-hr average oxygen uptake of bioventing pilot sets with and without the addition of nutrients. (A) With the addition of microorganisms only; (B) With the addition of microorganisms and nutrients

in liquid Lysogeny broth culture medium and then domesticated into pilot sets. In the following experiments, sufficient nutrients were also provided into pilot sets to create an environment suitable for microbial degradation. Data from experimental pilot sets were compared with the data from blank to evaluate the variability in microbial activity.

Results and Discussion

Except for the blank pilot, the other four pilot sets with various microorganisms were ventilated for four weeks by passive bioventing equipments to supply enough oxygen during the first phase. In the second phase, the pilot sets were added with various microorganisms and nutrients and were ventilated for six weeks. The nutrients added were formulated by mixing urea (NH_2CONH_2) solution and commercial phosphorus fertilizer to conform to the C:N:P ratio of 100:10:1. The results and correlations of cumulative oxygen uptake, oxygen uptake rate and diesel degradation trend are discussed as follows.

Without adding nutrients into the bioventing pilot sets, soil samples were taken at week 0 (the start of the pilot experiment) for 5-day batch biological respiration experiment by bubble

respirometer as background data of pilot sets without adding microorganisms. At week 1, the microorganisms capable of degrading diesel, were added. Pilot sets were named by the microorganisms added, such as *Bacillus subtilis* set, *Achromobacter xylosoxidans* set, *Pseudomonas putida* set, and pilot with mixture of three microorganisms was named as mixture set. The cumulative oxygen uptake of these four sets at week 0 were approximately between 200 mg- O_2 and 250 mg- O_2 in 5-day batch experiment, while the cumulative oxygen uptake of blank was higher, 368 mg- O_2 . After one week of the addition of microorganisms, the cumulative oxygen uptakes of *Bacillus subtilis* set and *Pseudomonas putida* set were substantially elevated, 362 mg- O_2 and 319 mg- O_2 , respectively, suggesting that liquid Lysogeny broth medium could substantially increase the microbial activity. At week 2, the microbial cumulative oxygen uptakes were found decreasing, probably because of the exhaustion of Lysogeny broth medium. At week 3, the cumulative oxygen uptakes of the microorganism sets were kept at the same level or slightly elevated. At week 4, the cumulative oxygen uptake of *Bacillus subtilis* set increased to 314 mg- O_2 , while in other sets it decreased. The above findings suggest that *Bacillus subtilis* set was well-adapted to higher diesel contaminated environment without addition of nutrients (Fig. 2A).

In bioventing pilot sets with addition of microorganisms and nutrients during second phase, the cumulative oxygen uptake in the *Bacillus subtilis* set was found to be elevated from 239 mg- O_2 to 268 mg- O_2 at week 1, and then maintained its level of 237 mg- O_2 until week 4. The cumulative oxygen uptake in the *Achromobacter xylosoxidans* set was consistent from week 1 to week 4 at 282 mg- O_2 and thereafter it decreased. The cumulative oxygen uptake in *Pseudomonas putida* set elevated from 219 mg- O_2 to 328 mg- O_2 at week 2 and thereafter gradually decreased. The cumulative oxygen uptake in the mixture set was found between 160 mg- O_2 and 190 mg- O_2 in six weeks (Fig. 2B). The above findings suggest that the microbial activity in the *Pseudomonas putida* set in a highly diesel contaminated environment was elevated due to addition of nutrients. Nevertheless, the results of cumulative oxygen uptake indicated insignificant difference in soil microbial activity between biostimulation and bioaugmentation, the finding results are in conformity with the findings of Colla et al. (2014) on carbon dioxide evolution demonstrated insignificant difference between biostimulation and bioaugmentation treatments.

Although the cumulative oxygen uptake was optimized in a 5-day respiration experiment, however, a short term indication is needed at remediation site to determine the current microbial activity. Therefore, variability of microbial oxygen uptake rate within 24 hours of each respiration experiment was calculated to find the microbial activity trend, which is quantitatively shown as the arithmetic mean.

After one week, in a experiment of bioventing system without nutrients into the pilot sets, the 24-hr average oxygen

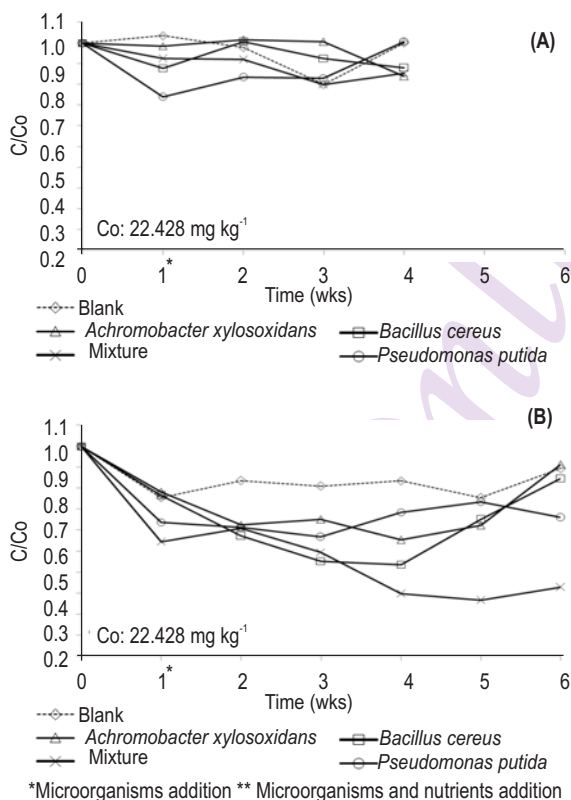


Fig. 4: The relative diesel concentration of bioventing pilot sets without and with the addition of nutrients. (A) With the addition of microorganisms only; (B) With the addition of microorganisms and nutrients

uptake rate of *Bacillus subtilis*, *Achromobacter xylosoxidans* and *Pseudomonas putida* sets were found to be elevated from 4.1 mg-O₂ hr⁻¹ to 6.4 mg-O₂ hr⁻¹, from 4.4 mg-O₂ hr⁻¹ to 4.9 mg-O₂ hr⁻¹ and from 4.1 mg-O₂ hr⁻¹ to 5.1 mg-O₂ hr⁻¹, respectively. These results indicate that microorganisms utilized nutrients from the liquid Lysogeny broth medium. At week 2, due to depletion of the nutrient level, there was decrease in all the oxygen uptake rates. At week 3, the oxygen uptake rate in the *Achromobacter xylosoxidans* set increased from 4.0 mg-O₂ hr⁻¹ to 4.4 mg-O₂ hr⁻¹. While at week 4, in case of *Bacillus subtilis* set, it increased from 3.6 mg-O₂ hr⁻¹ to 5.8 mg-O₂ hr⁻¹, while in the mixture set, it increased from 3.1 mg-O₂ hr⁻¹ to 4.2 mg-O₂ hr⁻¹, respectively. These findings suggest that it took three weeks for microorganisms to gradually increase the activity in the highly diesel contaminated environment without nutrients. The 24-hr average oxygen uptake rate in the *Bacillus subtilis* set was the higher compared to other sets (Fig. 3A).

In case of bioventing pilot sets with nutrients, a 24-hr average oxygen uptake rate of *Bacillus subtilis* set was measured as 4.4 mg-O₂ hr⁻¹ at week 1, thereafter, it gradually decreased at a rate 2.9 mg-O₂ hr⁻¹ to 3.5 mg-O₂ hr⁻¹ until week 4. The oxygen uptake rate in the *Achromobacter xylosoxidans* set was steady during first three weeks between 4.8 mg-O₂ hr⁻¹ and 5.0 mg-O₂ hr⁻¹ and then it gradually decreased. The oxygen uptake rate in the *Pseudomonas putida* set increased to 4.8 mg-O₂ hr⁻¹ in the first two weeks, and then gradually decreased. The oxygen uptake rate of mixture set was in the range of 2.8 mg-O₂ hr⁻¹ and 3.2 mg-O₂ hr⁻¹ in four weeks. From these results, it can be inferred that the microbial activity in the *Achromobacter xylosoxidans* set in the highly diesel contaminated environment was the highest on the addition of nutrients (Fig. 3B). In the bioventing pilot sets without nutrients, the relative diesel concentration (C/C₀) was low at week 1, similar to the microbial activity trend. These results suggest that nutrients in the liquid Lysogeny broth medium might have facilitated the microbial degradation of diesel contamination. However, after depletion of nutrients, degradation stopped, since there was insufficient nutrient in the soil to facilitate microbial degradation. The relative diesel concentration at week 3 and 4 was still up to 0.8 (Fig. 4A).

When the nutrients were added into the bioventing system, the diesel degradation trend in *Bacillus subtilis* set continued for three weeks, and the relative diesel concentration was 0.45. Whereas in the case of *Achromobacter xylosoxidans* set, diesel degradation continued for two weeks and the relative diesel concentration was 0.62. In *Pseudomonas putida* set, diesel degradation trend remained for only one week, while relative diesel concentration was 0.63. In the case of mixture set, diesel degradation trend continued for four weeks and the relative diesel concentration reached 0.30 (Fig. 4B). These results are consistent with the conclusion of Kim *et al.* (2009). According to relative diesel concentration trend of bioventing pilot sets without and with the addition of nutrients in Fig. 4, adding nutrients were

necessary to enhance biodegradation of oil pollutant.

The results of the present study concluded that the microbial activity in three sets containing microorganisms increased while relative diesel concentration decreased within four weeks of experimental set up. The finding results are in conformity with the findings of Balba *et al.* (1998) on the degradation rates for hydrocarbons. In addition, the oxygen uptake rate was higher while high diesel concentration existed in soil, which is consistent with the result of Nwankwo *et al.* (2014). Based on the results of the respiration experiment, it can be inferred that the 24-hr average oxygen uptake rate was more appropriate measure of biological activity trend as compared to cumulative oxygen uptake, and 24-hr average oxygen uptake rate could serve as an efficient and effective indicator to monitor microbial activity at the diesel contaminated sites.

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