[BIO10] Treatment of Tapis A oil-contaminated sediment by using a dual-stage biodegradation

Hii Yii-Siang and Ah Theem, Law

Institute of Oceanography, Kolej Universiti Sains dan Teknologi Malaysia, 21030 Mengabang Telipot, Terengganu.

Introduction

Malaysian marine environment is vulnerable to oil pollution, especially in the Straits of Malacca (Law et al., 2002). Busy maritime transports, active oil explorations and increases industrial activities are the primary factors that lead to oil pollution in the aquatic environment. When oil intruded into the environment, sediment acts as the final "sink" for the pollutants. Oily sediment may pose long term hazardous effects on a wide spectrum of organisms. Toxicity of oil on other aquatic organisms was widely reported (Law, 1997; Perkin et al., 2003). In fact, hydrocarbons in the surface sediment is categorized as one the contaminants that threaten human safety (Wezel and Vegter, 2001).

Disposal of the oil-contaminated sediment present a difficult situation because improper handling of the oily materials may cause oil pollution elsewhere. Up to this point of time, techniques for removing oil and hydrocarbons pollution are mostly targeted on the paraffin fraction which is less toxic but caused severe amenity issue (Clark, 1997). There is no sufficient technique for removing hydrocarbons that deposited on the marine sediment. Incineration, solvent extraction and secure landfill are the common approaches for combating oily sediment, however these technique are either too destructive or too costly for implementation. Biodegradation is one of the important pathways for decomposing oil-contaminated sediment in the natural environment. The rate of the oil-biodegradation is generally slower then other approaches especially when there are alternate source of organic-carbon presents in the environment (Oh et al., 2002). Although certain approaches such as addition of slow-releasing fertilizers, chemical inducer and solvent have successfully boosted biodegradation of some recalcitrant hydrocarbons in the oily sediment (Suchanek et al., 2000), output of these techniques are still inconsistence and unpredictable (Eweis et al., 1998). There are rarely applications of biological treatment at industrial scale nowadays. Hence, this study aims to develop a cheaper and efficient treatment which could improve biodegradation of oil in the aquatic sediment. Basically, efficiency of a biodegradation treatment is depending on the ability of bacterial inoculants and the supplementation of optimal conditions for the microbial activities. This study used a consortium of bacteria to improve efficiency of the inoculants and a dual stage treatment to maintain sustainability of the optimal conditions for the microbes.

Materials and methods

Bacterial seeding

A bacterial consortium which consisted of AR3, OG1 and OG2 were used in the study. The bacterial consortium is more efficient in degrading oil in the sediment compared to single inoculant. **AR3** is a motile, aerobic chemoorganotroph; gram negative marine bacterium with a size of 1.5 x 0.5 μm. AR3 was isolated from Port Dickson coastal environment and it noble oil degrading ability was reported by Law and Teo (1997). **OG1** and **OG2** are marine bacteria isolated from the coastal environment of Port Klang and Marang respectively. Both OG1 and OG2 are motile, gram negative bacteria with the ability to withstand 1500 mg L^{-1} Tapis A crude oil. However, these bacteria are not able to acquire crude oil as their source of carbon. According to the identification keys described by Bridson (1990), AR3 is classified as *Pseudomonas* while OG1 and OG2 are taxonomically identified as *Pseudomonas* and *Vibrio* type of bacteria respectively.

Marine Sediment and Medium

Marine sediment used in this study was collected from Malaysian coastal environment. The sediment was pre-cleaned, sieved (<200 μm) and burnt in muffle furnace at 600˚C for 2 hours to remove organic

fraction of the sediment. Synthetic seawater medium of Law and Button (1977) at pH 8 and salinity 30 $g.L^{-1}$ was used in this study. The liquid medium was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. Vitamin mixture B_1, B_{12} and d-Biotin was added into the liquid medium by using sterilized syringe after autoclaving. Solid medium used in this study was prepared by using sterilized marine agar 2216 (Difco Lab). Malaysian Tapis A crude oil was obtained from ESSO. Tapis A crude oil is a high quality medium light crude oil with low sulfur content.

Efficiency of inoculants

Efficiency of a single bacterium inoculant, AR3 and a bacterial consortium that consisted of AR3, OG1 and OG2 in reducing hydrocarbons in sediment was compared. The experiment was conducted by inoculating the working inoculant into 100 g of 100 mg kg^{-1} crude oil contaminated sediment. 300 mL of sterilized synthetic medium of Law and Button (1977) was added into a 1 L flask. Later, 2 x 10^8 cell g^{-1} of AR3 was inoculated into the sample for single bacterium; while, 2 x 10^8 cell g^{-1} of each AR3, OG1 and OG2 were inoculated into the oil contaminated sediment for evaluating oil-biodegradation of the bacterial consortium in sediment. The experiments were conducted under optimal condition at 28°C, pH 8.0, and 30 ppt. The flask was stirred at 250 rpm for 10 days.

Single-stage biodegradation

Single-stage biodegradation (Conventional treatment) was conducted in a 1 L sterilized flask. 100 g of treated organic free sediment was added into the flasks and the sediment was contaminated by using Tapis A crude oil to make up a concentration of 1000 mg kg^{-1}. The flask was sterilized by autoclaving at 121°C, 15 psi for 15 minutes. Later, 300 mL of sterilized medium was added into the flasks. Bacterial consortium that consisted of AR3, OG1 and OG2 was inoculated into the flask at 1.3 x 10^8 cell g^{-1} respectively. The experiments were maintained at optimal condition for 20 days as described above. Bacterial population and hydrocarbons in sediment were determined every two days.

Dual-stage biodegradation

Generally, dual-stage biodegradation is the combination of two conventional treatments. First stage treatment was similar to that described in the single-stage treatment. A break down was proposed in the dual-stage treatment when there is a sign of slow down observed in the experiment (≈ 7 days). After the first stage break down, water content in the sediment was removed and the sediment was flushed by using 100 mL of sterilized seawater for 3 times. Fresh sterilized medium was added into the sediment and a new batch of bacteria was inoculated into the sediment at 1.3 x 10^8 cell g^{-1}. The experiment was continued for the remaining time.

Three-stage biodegradation

Three stage biodegradation in the study was an attempt to improve dual stage biodegradation. Three-stage treatment is basically the combination of three single stage treatments. A breakdown was introduced twice in the three stage biodegradation whenever a slow down sign was observed in the biodegradation (day 7 and 14). The breakdown step was similar to the dual stage treatment which involved sediment flushing and re-inoculation of bacteria into the sediment.

Bacterial population in sediment

Total bacteria population in the sediment was assessed by using phospholipids quantization method as described by Findlay et al (1989). Where as, the composition of different bacterial species was assessed by using plate count method (APHA, 1998). AR3, OG1 and OG2 are differentiated by using their particular colony. Generally, OG2 could be easily differentiated by its distinctive yellowish colony. OG1's colony could be differentiated by its mucoid and irregular colony and its undulate margin. Colony formed by AR3 is rather lobate and relatively less slimy compared to OG1. Population of the specific bacterium could be estimated by considering its percentage of compositions and the total bacterial population in the sediment.

Determination of hydrocarbon in sediment

The level of hydrocarbon in sediment was traced quantitative and qualitatively as described in the standard method of UNEP

(1992). Briefly, sediment sample was extracted by using methanol/KOH saponification. The extracts was cleaned and fractionized by using silica/alumina column. Sulfur content in the sample was removed by using activated copper column. Quantitative analysis was conducted by using fluorescence spectrophotometric method where the concentration of crude oil in sediment was measured at excitation 310 nm and emission 360 nm. Where as, qualitative analysis on the saturated hydrocarbons (C_{12}-C_{32}) and 16 EPA's priority aromatic compounds were conducted by using GC-FID (Agilent, HP 6890) with MSD (Shimadzu, QP 5000) confirmation.

Bioassay of P. monodon larvae

The compartment tank technique developed by Law and Shazili (1995) was followed in the study. Each compartment was kept with one *P. monodon* post larvae. There were 30 *P. monodon* post larvae 25 in each experimental tank. Aged seawater was continuously pumped into the compartment tank at 30 mL.hour^{-1}. Small aeration was slowly pumped into each compartment to maintain dissolved oxygen at above 5 mg O_2.L^{-1}. The experiments were conducted at room temperature 28±0.5 °C for 45 days. Only life *Artemia* nauplii at density of 2.5 individual mL^{-1} of culture water were fed to the *P. monodon* post larvae through out the experiment and artemia left over were removed 2 hours after feeding. The testing organisms were fed twice a day and water quality parameters such as, salinity, pH, dissolved oxygen, temperature, ammonium, nitrite and total alkalinity were monitored once every 5 days. Survival and weight of the *P. monodon* post larvae was recorded at an interval of 5 day.

Results and discussion

Biodegradation presents an important pathway for decomposing hazardous compounds in the natural environment. It involves a series of biochemical processes that alter the pollutants and thus removing the compound from the environment. The whole process of the biodegradation is rarely completed by a single bacterium. Results of this study reveal that, incorporation of AR3, OG1 and OG2 into a bacterial consortium improved biodegradation of oil in the sediment. When the bacteria are inoculated in consortium, AR3 will act as fusing agent for aromatic ring and hydrocarbons in the consortium because, specific enzyme to fuse aromatic and C-H bonding of the hydrocarbons. AR3 will transform hydrocarbons into aldehyde and fatty acid; which are the common compounds that incorporated into tricarboxylic acid cycle (TCA) of bacterial metabolism processes. When hydrocarbons were transformed into aldehyde and fatty acid, most of the metabolites were quickly assimilated by OG1 and OG2. As a result, AR3 was encouraged to degrade more hydrocarbons in order to gain sufficient energy and materials to sustain its metabolism. Meaning, these reactions are boosting oil-biodegradation in the sediment.

Biodegradation of hydrocarbons by using the bacterial consortium showed a significant improvement in hydrocarbons degradation in the sediment ($p<0.05$). Results revealed that, 58.4 mg.kg^{-1} hydrocarbons were removed by AR3 at the maximum rate of 0.608 mg kg^{-1} h^{-1}. Bacterial consortium was able to remove 68.0 mg kg^{-1} at a maximum rate of 0.768 mg kg^{-1} h^{-1}. Qualitative wise, inoculation of bacteria in consortium also performed higher degradation of the priority hydrocarbons compounds listed by USEPA. Figure 1 shows PAHs contained in the sediment treated with single inoculant and bacterial consortium. Higher degree of hydrocarbons degradation was found in both saturated and aromatic compounds. Results reveal that, aromatic hydrocarbons such as phenanthrene, fluorine, acenapthene and acenaphthylene were degraded at greater extent by using the bacterial consortium. Moreover, recalcitrant compounds with 5 aromatic rings and above are also displayed an encouraging trend of degradation.

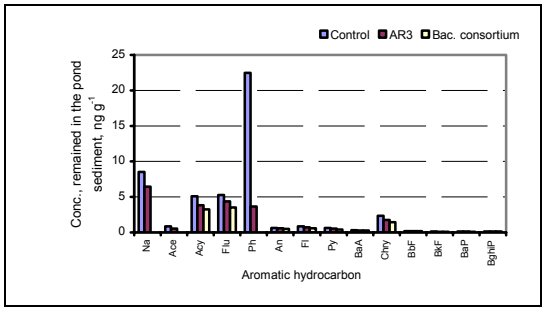


FIGURE 1 **A**romatic hydrocarbons remained in sediment after microbial degradation

When the bacteria are working in a consortium, competition among the bacteria is inevitable. The common factors that prompt bacterial competition are space and nutrients for growth. As the population of bacterial increased during the process of biodegradation, interspecies competition becomes intense and it will lead to retardation on hydrocarbons biodegradation. Some inhibitors or toxic products such as, aminoglycosides may be produced by bacteria to inhibit other bacteria growth under stress conditions. Accumulation of secondary metabolite in the sediment also caused retardation on other bacteria activities. Negative interaction among the bacteria was widely reported (Burgess *et al.*, 1999). In order to overcome the limitations, multiple stage treatment is proposed to enhance biodegradation of hydrocarbons. Actually, multiple stages treatment is a combination of an "interval breaks" and "stimulation" process. Biodegradation of hydrocarbons is given a "break" when there is a sign of degradation retardation. The sediment will be washed and flushed before re-application of new bacterial inoculants. It is anticipated that, the level of toxic byproducts and other inhibiting secondary metabolites accumulated in the sediment will be removed and the sediment will be "re-conditioned" for the subsequent stage of treatment. Results from the experiment revealed that, multiple stages biodegradation was able to improve hydrocarbons and organic carbon degradation. Figure 2 show the efficiency of multiple stages biodegradation in reducing hydrocarbons in 1500 mg kg^{-1} Tapis A crude oil contaminated sediment. The figure shows that dual stage and three stages biodegradation is more efficient compared to single stage biodegradation. Statistical analysis reveals that, there is no significant improvement of three-stage biodegradation compared to dual-stage biodegradation. A conventional single stage biodegradation was able to remove 665±33.2 mg kg^{-1} of Tapis A crude where as, dual stage biodegradation was able to remove 894.7±40.4 mg kg^{-1} Tapis A crude oil from a 1500 mg kg^{-1} crude oil-contaminated sediment. Three stage treatments was attempted to further enhance crude oil degradation, it removed 866.3±31.3 mg kg^{-1} of Tapis A crude oil from 1500 mg kg^{-1} crude oil contaminated sediment. Yet, the degradation efficiency was no significant improvement ($p>0.05$). The amount of hydrocarbons in the sediment did not declined significantly. It is believed that, those hydrocarbons residues left in the sediment were recalcitrant fraction of oil which resisted bacterial degradation. Thus, dual-stage treatment was sufficient for triggering optimal biodegradation of hydrocarbons in sediment (Figure 3).

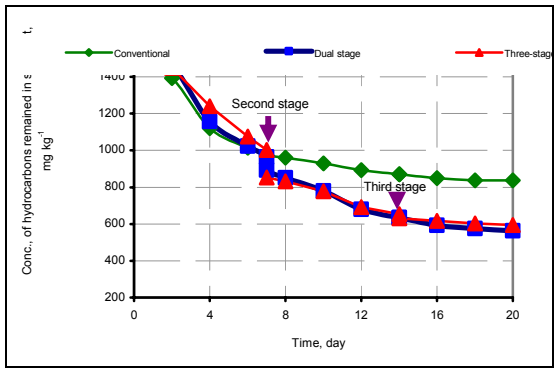
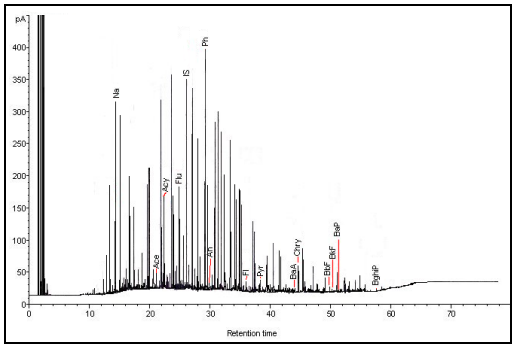
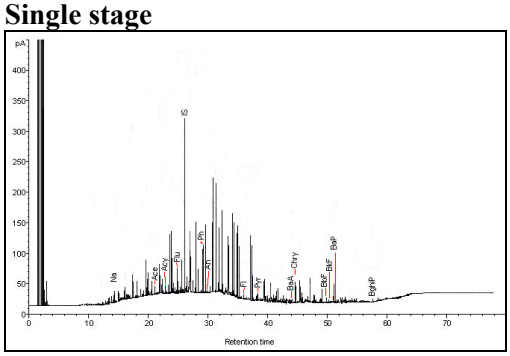


FIGURE 2 Conventional, dual stage and a three stage treatment of hydrocarbons in sediment

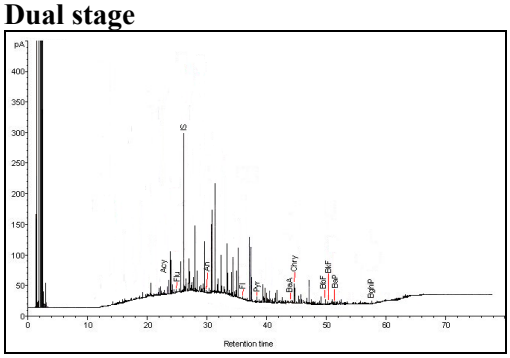
Control



Single stage



Dual stage



Three stage

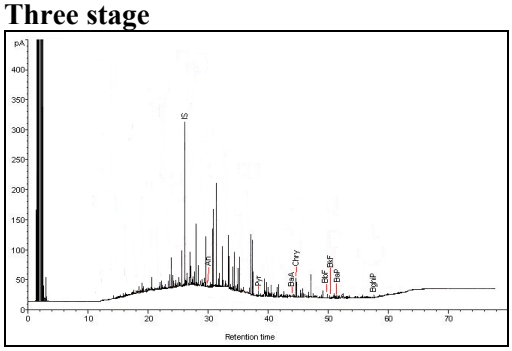


FIGURE 3 Aromatic compounds content in the oil-contaminated sediment after treated with single, dual and three stage treatments. X axis indicates retention time while Y axis indicates detector responses

AR3 was dominated in the sediment during the first stage degradation where the level of hydrocarbons decreased drastically. During the second-stage treatment, the organic degrading bacteria; OG1 and OG2 grew rapidly. This may be deal to the reduction of carcinogenic hydrocarbons content in the sediment and OG1 and OG2 are believed to utilize the metabolite produced by AR3 during the degradation of hydrocarbons in the sediment. Results of these experiments supported the hypothesis that, AR3, OG1 and OG2 might have symbiotic relationship among each other.

Presently, there are no formal guideline and water quality standards for the safety level of hydrocarbons in the sediment. It is important to evaluate safety of the treated sediment by using biological approach. Bioassay assessment was conducted using post larvae of *P. monodon* in order to validate effectiveness of the dual stage treatment on removing hydrocarbons pollution in the pond sediment. Results reveal that, crude oil pollution in aquaculture sediment is fatal. *P. monodon* post larvae show 100% mortality within 15 days in the pond sediment that containing 1500 mg kg^{-1} Tapis A crude oil. Single stage treatment of oil in sediment improved the sediment conditions. Maximum growth rate of the *P. monodon* post larvae in water in contact with single stage treated sediment was 0.54±0.02 g day^{-1}. Dual stage biodegradation was more capable to eliminate adverse effects of crude oil in the oil polluted sediment. Maximum growth rate of the P. monodon larvae kept in water in contact with dual stage treated sediment was 0.76±0.03 g day^{-1}. Statistical analysis revealed that ($P>0.05$), there is no significant difference between the growth rates of *P. monodon* post larvae maintained in dual stage treated sediment and the control experiment. This study summarized that, application of the bacterial in consortium was able to improve hydrocarbons degradation. It is believed that, there is a symbiotic relationship between oil-degrading bacteria and organic degrading bacteria. Application of multiple stages treatment was able to improve biodegradation of hydrocarbons in the sediment. Success of the technique indicates feasibility to in-cooperate the dual-stage into other biological treatment for combating oil pollution at industrial scale.

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References

American Public Health Association (APHA). (1998). Standard methods for the examination of water and wastewater (20th ed.). APHA. Washinton D.C. pp 1220

Bridson, E. Y. (1990). The oxoid manual 6th edition. Unipath Ltd., England

Burgess, J. G., E. M. Jordan, M. Bregu, A. Mearns-Spragg, K. G. Boyd. (1999). Microbial antagonism: a neglected avenue of natural products research. *Journal of Biotechnology* 70 (1999): 27–32

Clark, R. B. (1997). Marine pollution (4th edition). Oxford University Press. New York. 215p

Eweis, J. B., S. J. Ergas, D. P. Y. Chang and E. D. Schroeder. (1998). Bioremediation principles. McGraw-Hill International Co. Singapore. 296p

Findlay, R. H., M. K. Gary and W. Les. (1989). Efficacy of phospholipid analysis in determining microbial biomass in sediments. *Appl. Environ. Microbiol.* 55(11): 2888-2893

Law A. T., Y. S. Hii, K. J. Jong, and M. L Mok,. (2002). JICA/UPM Malacca Straits Expedition No. 4. Petroleum hydrocarbons, nitrogen and phosphorous distribution. In Tropical marine environment: Charting strategies for the Millenium. F. M. Yusoff, M. Shariff, H. M. Ibrahim, S. G. Tan & S. Y. Tai (eds), pp 281-301. Malacca Straits Research and development centre (MASDEC), Universiti Putra Malaysia, Serdang, Malaysia.

Law, A. T. (1997). Acute and chronic toxicity of the water soluble fraction (WSF) of a Malaysia crude oil to *Penaeu monodon* post larvae. *In* : Asean Marine Environment Management: Quality criteria and monitoring for aquatic life and human health protection. Watson, D., K. S. Ong and G. Vigers (eds.). Proceeding of the Asean-Canada Technical Conference On Marine Science, Penang. Malaysia.

Law, A. T. and D. K. Button. (1977). Multiple-carbon-source limited growth kinetics of a marine coryform bacterium. *Journal of bacteriology*. Vol 5: 162-167

Law, A. T. and K. S. Teo. (1997). Oil biodegradation in the Straits of Malacca: Phenanthrene degradation by AR-3. *J Mar Biotechnol.* 5: 162 – 167

Oh, Young-sook, Doo-seup Sim and San-jin Kim. (2001). Effects of nutrients on crude oil biodegradation in the upper intertidal zone. Marine pollution bulletin, Vol. 42(12):1367-1372

Perkins, R. A., S. Rhoton, C. Behr-Andres. (2003). Toxicity of dispersed and undispersed, fresh and weathered oil to larvae of a cold-water species, Tanner crab (C. bairdi), and standard warm-water test species. *Cold Regions Science and Technology* 36 (2003) 129– 140

Suchanek, M. Jan Kostal, Katerina Demnerova, Blanka Kralova. (2000). Use of sodium dodecyl sulphate for stimulation of biodegradation of n-alkanes without residual contamination by surfactant. *International biodeterioration and biodegradation.* Vol., 45 (2000): 27-33

UNEP (United Nations Environmental Programme). (1992). Determination of petroleum hydrocarbons in sediment. Reference methods for marine pollution studies No. 20. UNEP/IAEA/IOC, New York. 75p

Wezel, A. V. and J. Vegter. (2001). Human health and ecological consideration in contaminated land management. *In* Proceeding of CLARINET (Contaminated land rehabilitation network for environmental technologies) conference: Sustainable management of contaminated land. Federal Environmental agency (ed.). Pp 61-67