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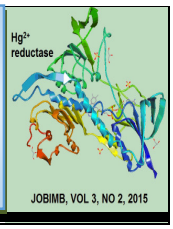

Luminescent Bacterial Testing for Monitoring Hydrocarbon Bioremediation – A Review

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ABSTRACT

Human activities in a large array of industrial and agricultural sectors produce chemical contaminants which are chiefly hydrocarbons of various types that are potentially toxic and carcinogenic to aquatic and terrestrial organisms. Globally, millions of tons of these pollutants are generated annually, and in some areas, they are released indiscriminately to the environment. In order to overcome this problem, microbiological decontamination or bioremediation has been suggested. Bioremediation has been argued to be an efficient, economic, and adaptable alternative to physicochemical remediation. However, to date, such claims of successful bioremediation are often not supported by evidence from toxicity studies. In this regard, luminescent bacteria have been employed in some hydrocarbon remediation experiments to denote reduction in toxicity. In this review, the utilization of luminescence bacteria as toxicity monitoring agent for hydrocarbon remediation is discussed.

INTRODUCTION

Rapid industrial development and the usage of pesticides in agriculture are the main factors that contribute to pollution which have threatened the natural resources [1]. Another source of pollution is heavy metal. Heavy metals pollute chemical disposal areas, firefighting training areas, landfills, and burial pits [2]. Some metals such as iron, zinc, copper and manganese are considered essential for human health but can be fatal when the amount of these metals exceeds the normal level. This may eventually cause other types of pollution, for example, water pollution and may consequently lead to severe health problems in humans as well as in other living organisms [3,4].

The toxicity of hydrocarbons, heavy metals and pesticides can be examined through bioassays [1,5–7]. In addition, bioassays can also determine the efficiency of biodegradation. Bioassays can predict and analyse the impact of heavy metals, pesticides, and biodegradation by-products on public health and ecosystem more precisely compared to chemical and analytical methods that only produce the composition of samples and analytical measurement. Nevertheless, it is the lack of an accurate definition of the term "toxicity" which is of major concern. Toxicity is a biological feedback. There are a range of toxicity measurements based on bacteria, algae and animal cells. Small mammals, fish flies, and zooplanktons also can be used to determine toxicity. A number of these toxicity measurements systems, using animals and fish larvae, are not handy and give slow response. Moreover, several toxicity measurements systems using animals may raise ethical issues. In addition, toxicants act in many ways and therefore may have different effects on living organisms as not all life-forms have the same levels and types of susceptibility and vulnerability [8]. So other than fast response, by using bioassay based on bacteria, there is much unlikely the bioassay is going to have ethical issues.

Unlike bioassay, the traditional method of monitoring pollutants in water involving standard analytical procedure such as (Gas chromatography–mass spectrometry) GC-MS or (High Performance Liquid Chromatography) HPLC cannot assess toxicity. However, as mentioned earlier, bioassays that use higher level animals such as fishes are somehow not practical because the culturing and testing of these animals are costly, laborious, and both time- and space-consuming. Due to the drawbacks of using higher level animals, bacteria, specifically bioluminescent bacteria, are used. Therefore, bioassay is an alternative to the traditional method of measuring toxicity in water, provided that lower level organisms are used.

Toxicant bioassay that uses bioluminescent bacteria in its analysis has numerous advantages. These include rapid,

economic, small test volume, and high sensitivity to a broad spectrum of toxic compounds. The toxicity assay is determined by an inhibitive mode with reduction of luminescence compared to control indicating the presence of toxicants. This paper reviews the available luminescence-based systems and their limitations. A discussion is also made on future potential improvement of the assay including the use of tropical luminescence bacteria to do away with bulky and expensive incubators much needed in the current system.

Bacterial bioluminescence

The term "bioluminescence" refers to the light emitted by living organisms. Bioluminescence is a phenomenon commonly found in marine organisms such as those classified at the lowest to the highest of the trophic levels, from bacteria to fish. For marine animals, bioluminescence helps them to locate their food and attract their mates as the luminescence emits light as glowing lures. The luminescence can also be used to protect themselves from predators. For some animals such as crustaceans and jellyfish, bioluminescence helps to hide themselves from predator through the release of clouds of light. Similar to that, counter illumination, which is another form of bioluminescence, is used by fishes and crustaceans for camouflage. In this process, the animals masquerade in bioluminescence by replacing the shade of animals using intense colour of bioluminescence. Luminescent bacteria are used by their hosts to attract preys and mates as well as to evade predators. Meanwhile, free living luminescent bacteria which especially grow on faeces cause the faeces to be eaten by fish and thus place bacteria in the fish's nutrient-rich gut [9]. Therefore, bioluminescence plays an important role in the general ecology of marine flora and fauna.

In the 1700's and the 1800's, fishermen had reported sights of shining decaying fishes and glowing seawater at night. The mystery continued when a man had found decaying human bodies that also produced light. According to [10], the phenomena could be due to the growth of saprophytic or pathogenic luminous bacteria on the decaying fishes and glowing seawater, and decaying human bodies. [11] have reported a case of a passenger on a cruise ship who was shocked to see light emitted from seawater flush lavatories. It was evident that luminescent bacteria could exist in such unusual places. This could probably be due to the suitable environment provided by their hosts and the conditions of such places. The environment and ambience suitable for the growth of luminescent bacteria will be discussed further in the next section of this paper.

There are two types of bioluminescence, namely, bacterial and intrinsic bioluminescence. Bacterial bioluminescence is a type of luminescence in organisms that have symbiotic relationship with bioluminescent bacteria. These organisms are the hosts for the bioluminescent bacteria in their internal organs such as the stomach. Meanwhile, intrinsic bioluminescence is the type of bioluminescence produced by the organisms themselves through the production of luciferase and luciferin [12]. In this paper, the focus is on the second type of bioluminescence, which is intrinsic bioluminescence.

Bacterial bioluminescent species can be found in most of the major marine phyla from bacteria to fish. For instance, comb jellies have the highest proportion of bioluminescent species. However, diatoms and arrow worms have almost no or a small number of luminescent representatives. For example, the red tide phenomenon occurs in the summer months in Southern California as a result of the increasing population of microscopic planktons known as dynoglagellates. These dynoflagellates glow in the dark when disturbed by currents or waves, resulting in a brilliant light show of turquoise glowing waves. During this red tide phenomenon, the dynoflagellates cause the levels of oxygen and light in the water to decrease. Consequently, organisms such as kelp, plankton, and fish die as the changing environment is poor and unhealthy for them to live in. This condition will cause the organic matter to decay and provide suitable environment for bacteria especially for the luminescent bacteria to grow [9].

Habitat and distribution of luminescent bacteria

The focus of this paper is the second type of bioluminescence, which is intrinsic bioluminescence. Luminous bacterium is able to produce luciferase and luciferin. Currently, four genera of luminous bacteria have been recorded. They are Vibrio, Photobacterium, Photohabdus and Shewanella. These luminous bacteria are Gram-negative, nonsporulating, chemoorganotrophic heterotrophs. Most of them are facultative aerobic. Luminous bacteria are considered to be a small part of more than 700 genera of luminous species organisms. Luminescent fish (mycophids and hatchetfish) and crustaceans (copepods, krill, and decapods) are examples of luminous organisms. These luminescent fish and crustaceans dominate in terms of biomass while bacteria and dinoflagelates dominate in terms of abundance [9]. Luminous organisms including luminescent bacteria are globally distributed in the ocean.

Luminous bacteria can be found in the seawater, sediments, and suspended particulates. They live in a variety of habitats. They exist from polar to tropical and from surface waters to the sea floor. However, luminescent bacteria can also be found in freshwater and terrestrial environment. Environmental factors such as temperature, depth, salinity, nutrient limitation, and sensitivity to photo oxidation extensively affect the distribution of luminous bacteria [10]. Even though luminescent bacteria can be found living freely in seawater, most are found to be living symbiotically. The host of luminescent bacteria is rich with nutrient and the ambience is perfect for the growth of the bacteria within the host. The host makes use of the bacterial luminescence to counter shading, escape, and avoid predation, species recognition, and reproductive advantage, as well as to attract prey. However, some luminous bacteria are obligatory symbionts. As only the host can provide optimum environment for the survival of the luminescent bacteria, these bacteria cannot be cultured *ex vivo* [13].

Moreover, luminous bacteria also can be found living in terrestrial animals as parasite of insects and marine invertebrates. Luminous bacteria found in terrestrial environment are mostly parasites of insects. They cause the infected insects to emit light. Luminescent bacteria have been observed since 1970. Other than that, luminous bacteria can also be found living as parasite in marine invertebrates as opportunistic pathogens. They enter animal's corpse through lesions caused by injury.

In addition to luminous bacteria present as parasites of marine invertebrates, these bacteria also have extremely harmful effect on commercial prawn mariculture. In 1980's, the monoculture of *Penaeus monodon* or the giant tiger prawn was developed rapidly. However, a tragedy caused by luminous bacteria led to a remarkable prevalence of diseases and decrease in the number of the animals. Luminescent vibriosis and

hepatopancreas in juveniles are diseases caused by luminous bacterial infection. These diseases cause a pathogenic state responsible for the substantial mortalities. The infection limits the growth of the animals and consequently increases the mortality rate of the infected animals. On the contrary, for vertebrate animals, luminous bacteria seldom infect these terrestrial animals.

Activity of luminescence bacteria

The benefits of bacterial luminescence have been known and will be further discussed in this paper. Although the mechanism of bioluminescence has been comprehensively studied, the reason for the production of light by these bacteria is still not well-understood [10]. Bacterial luminescence is produced when flavin mononucleotide ($FMNH_2$) and a long aliphatic aldehyde (RCHO) are oxidised by molecular oxygen catalysed by enzyme luciferase. The long aliphatic aldehyde is consumed during the reaction but is continuously synthesised by the bacteria. This process results in a persistent glow [9].

The intensity of the glow depends on a mechanism known as quorum sensing. Quorum sensing allows bacteria to sense the presence of other bacteria. This serves as a medium of communication among different bacteria for them to measure the density of their own population [14]. In this process, some small metabolic products freely diffuse across the cellular membrane and then they are excreted to the extracellular membrane. At the initial growth where the cells density and the level of autoinducers are low, low intensity of light can be detected. As bacteria grow, autoinducers will start to accumulate in confined environment, causing the intensity of light to be slightly increased. When high levels of autoinducers are present, they will activate the luminescent system in the media.

More specifically, quorum sensing is the regulatory response to the autoinducers that leads to the induction of expression of the *lux*CDABE genes [15]. In this mechanism, luciferase will be synthesised when the *lux* gene is expressed after the amount of the autoinducers has reached the threshold concentration [16]. The amount of autoinducers needed by bacteria is different from a type of bacteria to another. Similarly, the threshold amount of autoinducers is determined by the type of bacteria and the environment in which they thrive. These could explain the reason luminescent bacteria that live freely in sea water do not emit light but when they are taken to the lab and grown in the confinement flask or container, they emit light at a very high intensity [17].

Use of luminescence bacteria to monitor toxicity

Pollutants in the environment can be detected using a few methods. Chemical assay and physical parameters evaluation such as HPLC and GCMS are used to determine the effects of the pollutants produced mainly from human activities. Chemical assays play a major part in detecting pollutants nowadays. However, the cost of the equipment for chemical assays is rather high and not within the reach of many. In addition, chemical assays like HPLC and GCMS are time-consuming. A well-trained operator is also needed to operate the equipment. Considering these disadvantages, chemical assays are therefore not an appropriate alternative since the data are restricted to the concentration of the pollutants. Furthermore, aspect of toxicity is also neglected. Hence, bioassays become the logical choice as there are needs to establish the cause-effect relationships between the concentration of the contaminants and the ecological damages they cause. Moreover, bioassays can also be used to determine the potential synergistic effect of complex mixtures of chemicals [18]. To date, numerous luminescent bacteria have been isolated for the purpose of biomonitoring of toxicants (**Table** 1).

Table 1. Examples of luminescent bacteria isolated and their properties

Strain	Optimum pH	Optimum temperature (°C)	Author
Vibrio fischeri (DSMZ 7151/ NRRLB-11177)	7±0.2	20	[19]
Photobacterium leiognathi	7	18-27	[20]
Vibrio harveyi strain 525	7	27	[21]
Vibrio fischeri strain 4172	7.4		[22]
Photobacterium phosphoreum MT10204	7±0.2	30	[13]
Vibrio harveyi strain 525			[22]
Photobacterium sp. Lub-1		28	[23]
Pseudomonas fluorescens strain Shk1		RT	[15]
Vibrio logei	7±0.2	20 ± 1	[18]
Pseudomonas fluorescens ATCC-13525	6.7	37	[24]
Photobacterium phosphoreum strain 496	7	25	[25]
Photobacterium sp. strain MIE	5.5 to 7.5	24-30	[7]
Vibrio sp. isolate MZ	7.5-8.5	25-35	[26]

Biological indicators are very important these days especially in hazard assessment. Other than that, they also play an important role in determining the efficiency of remediation of hydrocarbon-polluted soils. A large array of bioassay end points are available nowadays [27]. For example, bioassays used currently are solid phase Microtox test (a bacterial test of acute toxicity), SOS-Chromotest (a bacterial test that measures genotoxicity and cytotoxicity), lettuce seed germination assay (used to assess plant germination and growth), earthworm survival assay (used to measure toxicity to soil invertebrates), and sheep red blood cell (RBC) haemolysis assay (used to represent mammalian cells) [28].

Bioassay is used to monitor any toxicity changes in bioremediation samples. This is done especially when there are complex contaminants in the samples and when the characterisation of the by-products of biodegradation has not been known. Therefore, bioassay can be considered a complement analysis to chemical assays. Reduced soil toxicity is a manifestation of a successful bioremediation. However, toxicity of the samples does not always indicate the reduction in the concentration of the target pollutants. This is very dangerous as incomplete degradation could result in the formation of toxic metabolites and in the increase of soil toxicity during the bioremediation [28].

In this paper, the main pollutants or toxicants discussed are heavy metals and hydrocarbons before and after biodegradation. Hydrocarbon pollution is currently a major problem. The effects of polltuion in the 2010 Gulf of Mexico Oil Spill were reported to be seriously detrimental to the marine lives around that polluted area. Scientists worked extremely hard to overcome or at least reduce the hazardous effects of the oil spill. The use of bacteria to degrade the oil was applied in solving the problem. This widely used method to clean the environment is the main

natural method by which the petroleum hydrocarbon contaminants could be eliminated from the surroundings [29]. In order to monitor the progress of degradation, bioassay using luminescent test can be used. This luminescent test offers a rapid, simple, and economical way to observe the degradation process.

In the case of hydrocarbon-polluted soils, the efficiency of the bioremediation procedure has been recognised by the concentration of the toxic components in polluted area. However, chemical analysis alone does not consider the bioavailability of the contaminants. This is because several compounds change into metabolites of unidentified toxicity. Hence, there is a need for bioassays to monitor environmental pollution. Clearly, bioassay has become the preferred choice [30].

Many studies have reported that oil pollution and extent of bioremediation are often determined by monitoring reduction in contaminant concentrations [27,31–33]. However, chemical data are merely not adequate to assess the biological effects because the decrease in target contaminant levels is not constantly a sign of reduced soil toxicity. Therefore, an incorporation of chemical testing for target pollutants levels and toxicity assay is suggested to observe the contamination and the process of soil remediation at polluted sites [27,34,35].

Advantages of bioluminescent bacteria bioassay

Compared to the chemical assays, bacterial assays have more advantages. Bacterial assay using luminescence bacteria is quick, susceptible, reproducible, and cost-effective, especially, the *in vivo* luminescence. In luminescence bacteria bioassay, when the toxicants or pollutants are present, the luminescence reduces. This is because the toxicity of the toxicant hinders the metabolic status of the cell due to the inhibition of the electron transport chain that is directly coupled to respiration. Therefore, it is an excellent indicator of xenobiotic toxicity. Because of this, as bacteria-based bioassays produce quick response to biological or chemical toxins, they can be used in advanced warning screening systems [36].

In addition, aquatic resource managers have to take into account the assessment of toxicological risk. To do such an assessment, the following basic water resource issues must be addressed and answered by them: What is considered as toxic? How toxic is it? Although there are quite a number of excellent and dependable toxicity bioassays to provide answers to these critical questions, Microtox, which is a toxicity bioassay that uses luminescent bacteria, has been a prominent choice. The advantages of using Microtox will be further discussed in the following section.

Bioassays using luminous bacteria are capable of assessing toxicity in water, sediments, and soils. Hence, a wide area of pollution sites can be covered by luminescence bacteria bioassays. In addition, luminous bacteria, which have perfectly adapted to their habitat, will precisely respond to unusual xenobiotics. For example, luminous bacteria isolated mainly from the marine are used widely in bioassays for detecting toxicants. An example of such bioassay is Microtox that uses *V. fisheri* isolated from the marine environment to detect toxicants such as metals, inorganic and organic solvents, and hydrocarbon-based products [37]. There are both short-term and long-term tests of toxicity using the luminescent bacteria method. Short-term tests rely on the change of light intensity whereas long-term tests are used by observing the changes in viability or growth rate [38].

In the toxicity bioassay, battery of bioassays is usually used in order to obtain precise results. This is because the level of organisms used is different from each other. Higher level animals like monkeys are rarely used in toxicity bioassays. The lower level animals such as fishes and bacteria are more widely used. Furthermore, in toxicity assessments, single test cannot substitute all other assays. This is because the sensitivity of the organisms differs significantly based on the conditions and nature of the pollutants. Hence, as discussed earlier, the toxicological profile of an environmental pollutant can be better understood. This is important especially when its impact is determined by the organisms that correspond to diverse trophic levels.

Bacteria are a key player in the majority of aquatic ecosystems. They are the most important organisms in the trophic level in terms of energy flow and nutrient cycling. Therefore, representatives of this trophic level must be included to conserve our ecosystem especially the aquatic ecosystems. Eventually, a swift, inexpensive monitoring device for toxicity of environmental pollutants, i.e., Microtox assay has been widely applied. This monitoring tool, which uses marine bacterium called *V. fischeri*, has a significantly lower coefficient of variance compared to other bioassays. This is due to the highly formalised, standardised reagents used in the tool that are less susceptible to variation [39].

Commercially available bioluminescent bacteria bioassay – Microtox

There are many toxicity bioassays available nowadays. For example, Mutatox test, Biotox Flash Test, Microtox (AZUR Environmental Ltd.) and its portable field version, Deltatox. Both Microtox and Deltatox bioassays are based on the reduction of luminescence of *V. fischeri*, the concept reviewed in this paper. Microtox has been chosen to be discussed more in this section because it has a great reputation as toxicity bioassay worldwide.

Microtox uses luminescent, Gram-negative, saprophytic marine bacteria that can be found everywhere in oceanic waters and are straightforwardly isolated and cultured from fish and marine water. The prokaryotic cells used in Microtox are taken wholly from a cloned strain of a marine bacterium, *V. fischeri* NRRL B-11177. They are isolated, cultured, and maintained by the manufacturer (SDI, Strategic Diagnostics Incorporated) [37]. Previous study has recommended that specific isolates of Vibrio (formerly taxonomically designated as *Photobacterium phosphoreum*) have been proved to be sensitive to numerous environmental contaminants.

Microtox has been named as the leading toxicity bioassay globally because of several factors. First, the protocol is entirely standardised and the materials are accessible worldwide. These bioluminescent bacteria are ready to be used because they are already lyophilised and the test solutions are also easy to handle as pre-mixing is not needed before use. Furthermore, the analyser is already wired with computer assistance, and with the help of *MicrotoxOmni* (the software for Microtox), the data are stored and can be displayed. Besides, this test uses less materials, thus reducing the costs of disposables. Furthermore, it minimises dedicated laboratory space. Other than that, due to its short exposure times, Microtox is capable of handling large sample compared to the other bioassays. Because of these

attractive features, Microtox is considered as a good environmental monitoring tool [37].

Microtox as a biomonitoring tool for hydrocarbon biodegradation

The chemistry monitoring data and the toxicity data provide a poor correlation between oil loss and sediment toxicity. Toxicity is not about what is in a sample, but it is rather about the effect of whatever is in the sample. Obviously, toxicity value, and not only concentration, is a crucial in measuring the remaining bioremediation endpoints. This is because field chemistry records have proven to be an important change in oil pollution after the biological management. However, the toxicity remains unknown. Hence, using bioassay such as Microtox, would help to determine the toxicity of the by-products [35]. In oil pollution and bioremediation, concentrations of the contaminants are often monitored without realising that chemical data alone are not sufficient to assess the biological effects because the reduction in target contaminants does not necessarily indicate reduced soil toxicity [40]. For that reason, a group of chemical analysis for the levels of target toxicant and toxicity testing to monitor the pollution and the efficiency of soil remediation at the polluted sites is suggested [7,41,42].

Moreover, Microtox is an ecotoxicological screening tool designed for the following uses: (i) to identify water toxicity; (ii) to distinguish transformation in toxicity, (iii) to determine prospect of other toxicity tests; (iv) to monitor raw drinking water contamination; (v) to determine the signs of bioterrorism, sediment and soil testing, (vi) biocide monitoring of industrial processed waters; and (vii) monitoring of remediation processes [37].

To date, there is no specific analytical method that can completely characterise the highly complex assemblage of organic compounds, i.e., petroleum hydrocarbon or oil. In order to track the destiny of spilled petroleum hydrocarbon, changes in bulk oil concentration have to be monitored, besides monitoring specific composition changes in the petroleum hydrocarbon or oil [27]. Several physical-chemical technologies such as vapour extraction, stabilisation, solidification, soil flushing, soil washing, thermal desorption, and incineration are available for the management of soil polluted with organic and dangerous materials, for example, petroleum hydrocarbons. However, for optimum performance, majority of these techniques are costly, and need continuous monitoring and control.

Unlike the physical-chemical technologies, biodegradation is an effective and inexpensive technology that remediates soils containing Polycyclic aromatic hydrocarbons (PAHs) and other hydrocarbon compounds. However, a population of microorganisms must be present to degrade the hydrocarbon compounds. Furthermore, the soil conditions must be conducive to the biodegradation of the contaminants. In bioremediation, concentrations of target contaminants are always observed in monitoring the process. However, the reduction of the concentration does not always indicate that there is a decrease in soil toxicity. During bioremediation, an increase in soil toxicity could be the result of partial degradation and development of toxic intermediary metabolites. To prove the successful process of bioremediation, an arrangement of chemical test for target pollutants levels and toxicity assay is suggested [39].

Microtox is used to evaluate the feedback of the luminous bacteria, *Vibrio fisheri* NRRL B-111 77 to chemical agents. These chemical agents include aromatic hydrocarbons in bulk water and sediments. It is a rapid and economical toxicity assay. When there is a lessening in light intensity emitted by the luminous bacteria, it is regarded as the final result deliberated by Microtox. In Microtox terminology, the EC_{50} value refers to the concentration of toxicant needed to decrease the light intensity of luminous bacteria, *Vibrio fisheri* NRRL B-111 77to 50% [41].

This assay is able to approximate the toxicity of petroleum hydrocarbon as whole. However, toxicity of the individual components in the petroleum hydrocarbon cannot be evaluated. This is due to the complex matrix of crude oil treatment by-products. Therefore, to examine contamination for organics bound to sediment and petroleum hydrocarbon toxicity in the water soluble fraction, research data produced from Microtox screening have been used. As this bioassay can be used to determine concentration criteria for oil and sludge bioremediation, United States Environmental Protection Agency (USEPA) has thus documented this technique as a toxicity assay method [39].

The Microtox method is designed for use with soil and soil-waste mixtures. To assess changes in toxicity, the method is capable of being a direct toxicity testing method. Meanwhile, for assessment of the efficacy of the bioremediation, the quantitative values are used. It has been found that the Microtox system is a monitoring device which has the potential to partially replace GC/MS analyses. These GC/MS analyses are expensive especially in a complete environmental monitoring method [43].

To sum up, in the assessment of the effectiveness of spill response and mitigation systems, luminescent bacteria bioassay can be applied as a monitoring tool by observing the quantitative changes. Besides that, this bioassay can also be applied to measure the effectiveness of bioremediation, chemical cleaning, mechanical removal, and “no treatment” treatments. Additionally, the results have established luminescent bacteria bioassay values and method detection limits for a broad assortment of dissimilar oils. They also offer extra data valuable for environmental engineers to choose if bioremediation, among other further cleanup measures, is necessary [44,45].

There is a need for tropical-climate based luminescence bacterium because the cost to analyse samples can therefore be reduced. These isolates do not need stringent temperature conditions; thus, no thermostat will be required. Hence, the test will be simpler and more economical compared to Microtox, which is a bioassay that uses bioluminescent bacterium (*V. fischeri*). This temperature factor is so essential and critical particularly in analysing many samples concurrently. Furthermore, tropical-range isolates are suitable for work in the field because of the less stringent temperature conditions such as those in Malaysia [46].

Monitoring of hydrocarbon bioremediation using bioassays

Microtox, a luminescent bacterial-based toxicity assay is one of the methods recommended by the USEPA (United States Environmental Protection Agency) as a biomonitoring tool for remediation of toxicants such as hydrocarbon sludge. Monitoring of remediation via reduction in hydrocarbon profile is not enough to indicate a decrease in toxicity as hydrocarbon

degradation is known to release soluble toxic degradation metabolites which, if not remediated completely, will cause ecotoxicological problems in the future. Hence, there is a need for ecotoxicological bioassay such as Microtox. There have been several studies conducted on hydrocarbon bioremediation monitoring using Microtox. For example, leachate from a hydrocarbon-contaminated land has been studied for its biodegradability. Total petroleum hydrocarbon (TPH)reduction and Microtox toxicity testing are two parameters monitored to indicate efficient bioremediation [33].

In one study of biodegradability of leachate from land treatment units (LTU) of hydrocarbon-contaminated soil, toxicity testing and respiratory measurement in conjunction with TPH determination was explored in the laboratory setting. The LTU was polluted with a diesel-like hydrocarbon mixture in California. Leachate was gathered from two distinct LTUs for treatability assessment and degradation extent was determined via respirometric measurement under aerobic conditions. After 161 days, merely 12% reduction in TPH concentration was noticed, indicating restricted biodegradability of the hydrocarbon components in the leachate. In the same way, Microtox toxicity studies showed no change after 130 days in agreement with the respirometric results [33].

Another study carried out on sub-Antarctic soils of the Kerguelen Islands in December 2000 which polluted with crude-oil and diesel-fuel supplemented with fertilizer showed a great increase in hydrocarbon-degrading bacteria (HDB) after the fertilizer had been added, indicating a favourable effect of the fertilizer on HDB activity and growth. The total hydrocarbon content in polluted soils was reduced to between 80 and 90% after nearly a year while the HDB counts remained high throughout the experiment. The fertilizer addition enhances n-alkanes degradation although complex hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) do not result in enhancement. Toxicity studies have shown that fertilized plots are more toxic than unfertilized plots, indicating a mixed results and that bioremediation actually produces toxic metabolites that inhibit Microtox [47].

Almutairi et al. [35] have examined the bioremediation of total petroleum hydrocarbon monitored by Microtox test and earthworm survival assays. They have observed partial hydrocarbon bioremediation and the remainder is in general more acutely toxic than fresh oil based on these bioassays. This phenomenon shows that toxic intermediary metabolites may have been produced in biodegradation. Similarly, a battery of luminescence bacterial toxicity study using luminescent bacteria such as *V. fisheri* and *V. harveyi.* [48] have used *V. fischeri* (NRRL B-111777), *V. logei*, and *Photobacterium phosphoreum* 1883 IBSO to monitor bioremediation of petroleum hydrocarbons and chlorinated aromatic hydrocarbon-contaminated soils which show that the toxicity values would increase rather than decrease after 45 days of treatment. They suggest that the increase in the content of short chain molecules during the bioremediation causes the sample to be more toxic to the luminescent bacteria.

A decrease in toxicity using the Microtox bioassay has been reported in a biostimulation study using inorganic fertilizer and bioaugmentation study using hydrocarbon-utilizing indigenous bacteria to remediate a crude oil-contaminated soil for 12 weeks. It has been observed that there is a transient increase in toxicity studies during bioremediation. It has also been proposed that hydrocarbon degradation metabolites such as the more water soluble aldehydes, which are more hydrophilic than hydrocarbons, are responsible for the increase in toxicity studies observed. When the degradation reaction is further extended, a reduction in toxicity is observed, indicating that these toxic metabolites have probably been assimilated and neutralized [49].

Another study has also reported a reduction in toxicity after biodegradation of PAH. However, this study was carried out by growing two bacterial consortia (BOS08 and C2PL05), in various hydrocarbons including low molecular weights PAH (naphthalene, anthracene and phenanthrene) and high molecular weights PAH (pyrene and perylene) at low (5-15 °C) and high (15-25 °C) temperature ranges. Researchers observed PAH-degrading bacterial populations had increased during biodegradation of PAH, the latter was determined via HPLC .They also observed a reduction in toxicity measured using the Microtox toxicity assay [50]

In the most recent study on the use of luminescent bacterium in monitoring biodegradation of hydrocarbon, *Vibrio* sp. isolate MZ, a luminescent bacterium isolated from the yellow striped scad fish (*Selaroides leptolepis*) was utilized to successfully monitor a bench scale biodegradation of 1% (w/v) sludge mixed with soil by the bacterium *Rhodococcus* sp. strain AQ5NOL2 [26]. It was also observed that in the initial phase, the toxicity to the luminescent bacterium had increased before declining after further extension of biodegradation period.

CONCLUSION

Numerous luminescent bacteria have been isolated for the purpose of toxicant monitoring. The rapid and sensitive properties of the luminescent-based monitoring system have captured the attention of researchers worldwide. In certain toxicant degradation, the bacteria toxicity assays have shown that degradation of by-products can be more toxic than that of the control samples (no degradation), and further extension of degradation periods may be needed even when instrumental analysis indicates the absence of primary hydrocarbon signals. The use of other toxicity assays may be needed to enhance the power of toxicity assay in the safekeeping of the environment. Other toxicity tests to monitor hydrocarbon degradation such as earthworm survival (to measure toxicity to soil invertebrates), seed germination (to assess plant germination and growth), Toxi-Chromotest (to measure genotoxicity and cytotoxicity), Chromotest, and red blood cell (RBC) haemolysis assay have been used and can be used as a battery of tests. A battery of tests may be needed to monitor ecotoxicological toxicity in soil samples polluted with multiple contaminants.

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