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Optimisation and dose responses of bioluminescent bacterial biosensors induced with target hydrocarbons

Optimización y dosis respuestas de biosensores bacterianos bioluminiscentes inducidos con hidrocarburos objetivos

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Abstract

Routine analytical methods are constrained in the speed of application, sample throughput and inability to determine the right bioavailable loading of pollutants. Microbial biosensor technology resolved these constraints by offering the most rapid, sensitive, reliable and cost-effective technology, especially in a bioavailable context. This study describes the growth characterisation, optimisation and induction bioassay of three different *lux*-marked biosensors, thus testing their responses to doses of target hydrocarbons (naphthalene, toluene, Isopropylbenzene) and solution of mixed hydrocarbons. These biosensors, *Pseudomonas fluorescence* HK44, *Escherichia coli* HMS174 and *Pseudomonas putida* TVA8 harbours *lux*CDABE reporter genes coupled to induction by hydrocarbons. Biosensors harvested at optimal exponential phase and induced with hydrocarbon using the optimised assay conditions are highly sensitive and responsive to their inducers in a proportionate dose-dependent manner. The established dose responses of these catabolic biosensors signify the prospect of extrapolation for estimating the genuine contamination loading of pollutants for environmental relevance. However, several factors may contribute to the quenching effect at high concentration of inducers. Robust responsiveness to mixed hydrocarbon solution has been also realised accentuating its feasibility in analysing real environmental samples containing heterogenous pollutants. This study emphasises the suitability of bioluminescent bacterial biosensors for pollutants analysis and notably the detection of soluble bioavailable fractions of diverse hydrocarbons, hence, serves as a reliable bioindicator of hydrocarbon pollution in an environment. Even so, the real value of biosensors is for a suite of ecologically justified biosensors to be applied in complementary combinations with other focused analytical or chemical methods for broad and resourceful inference.

Keywords: Biosensors, bioreporters, *lux*CDABE, bioavailability, naphthalene, toluene, isopropylbenzene, hydrocarbon.

Resumen

Los métodos analíticos de rutina están limitados a la velocidad de aplicación, al número de muestras y a la incapacidad para determinar la correcta carga de contaminantes biodisponibles. La tecnología de biosensores resolvió estas limitaciones al ofrecer tecnología más rápida, sensible, confiable y económica, especialmente en un contexto biodisponible. Este estudio describe la caracterización del crecimiento y la optimización de tres diferentes biosensores *lux*-marked y sus bioensayos de inducción, poniendo a prueba sus respuestas a dosis de hidrocarburos objetivos (naftaleno, tolueno, Isopropilbenceno) y a la solución de hidrocarburos mixtos. Estos biosensores, *Pseudomonas fluorescence* HK44, *Escherichia coli* HMS174 and *Pseudomonas putida* TVA8, albergan genes reporteros *lux*CDABE asociados a la inducción por hidrocarburos. Los biosensores cosechados en la óptima fase exponencial e inducidos con hidrocarburos usando las condiciones de ensayo optimizadas, son altamente sensibles y responden a sus inductores en un estado proporcional dependiente de la dosis. Las dosis respuestas establecidas de estos biosensores catabólicos indica la posibilidad de extrapolar para estimar la carga de contaminación genuina de agentes contaminantes para el cuidado del medioambiente. Sin embargo, muchos factores pueden contribuir al efecto inhibidor a altas concentración de inductores. La robusta capacidad de respuesta a la solución mixta de hidrocarburos también se ha obtenido acentuando su viabilidad en el análisis de muestras ambientales reales que contienen contaminantes heterogéneos. Este estudio destaca la idoneidad de los biosensores bacterianos bioluminiscentes para el análisis de agentes contaminantes y, en particular, la detección de fracciones solubles biodisponibles de diversos hidrocarburos; por ello, estos sirven como un confiable bioindicador de la contaminación por hidrocarburos en el medioambiente. Aún así, el valor real de los biosensores es que un conjunto de biosensores ecológicamente justificados se aplique en combinaciones complementarias con otros métodos analíticos o químicos específicos para una inferencia amplia y hábil.

Palabras clave: Biosensores, biorreportadores, *lux*CDABE, biodisponibilidad, naftaleno, tolueno, Isopropilbenceno, hidrocarburo.

1 Introduction

Hydrocarbon pollutants such as volatile organic compounds (VOCs) (e.g., BTEX) and polycyclic aromatic hydrocarbons (PAHs) are widespread in diverse environments (Medina-Moreno *et al*., 2014; Meney *et al*., 1998) and are of great health concern mostly due to their potential toxicity, mutagenicity and carcinogenicity (Cisneros-de la Cueva *et al*., 2016; Estelmann *et al*., 2015). The ubiquitous nature of these pollutants is resulted from environmental pollution mostly natural and anthropogenic sources (Ambrosoli *et al*., 2005; Zakaria *et al*., 2020; Ibrahim *et al*., 2020).

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Studies have used varieties of routine conventional analytical and chemical techniques for detecting a wide range of pollutants, which were proven highly efficient in identifying and quantifying countless chemical pollutants, but not without limitations (Kleemann and Meckenstock, 2011; Poster *et al*., 2006; Berset *et al*., 1999). Besides, few studies showed the use of developed immunoassays for PAHs detection in environmental samples (Knopp *et al*., 2000). Now, the advent of biosensor technology has enabled researchers to value a rapid, sensitive and cost-effective approach for diagnosing pollutants in environmental samples as chiefly realised in the microbial biosensor application. Increasingly, genetically engineered microorganisms (GEMs) are being constructed to be applied in environmental diagnostics (Liu *et al*., 2019; Godwill, 2014; Unge *et al*., 1998). A significance in its application is the fact that it can conveniently resolve the constraints of other techniques and uniquely determine the soluble bioavailable fractions of hydrocarbons of concern (Paton *et al*., 2006; Heitzer *et al*., 1994). Bacterial biosensors (also termed bioreporters) combine a sensing element responsible for detecting the analyte and a reporter element, which allows for quantification of the signal (Mendoza-Madrigal *et al*., 2013; Köhler *et al*., 2000). Specifically, bioluminescent bioreporters are originally constructed as whole-cell bacterial biosensors responding to specific chemicals or physical agents in their environment via visible light produced (Yeh and Ai, 2019; Van Der Meer, 2006). One of the most commonly used reporter proteins for optical detection in microbial systems is the bacterial luciferase (from *V. fischeri*) primarily for luminescence. Here, the bioluminescence-based biosensors, the *lux* genes (commonly *lux*CDABE) introduced into the environmentally relevant bacteria, are rapid, reproducible and sensitive to a wide range of inorganic and organic contaminants (Paton *et al*., 1997; Steinberg *et al*., 1995). These luciferase genes (*lux*CDABE) have been previously used in several studies in plasmid construction as highly sensitive reporters for detecting specific hydrocarbons (King *et al*., 1990) and mercuric ions (Selifonova and Eaton, 1996), and for environmental stress monitoring. (Van Dyk *et al*., 1994). The study of Rogowsky *et al*. (1987) recorded that the *lux*A and *lux*B genes encode the α and β subunits of the luciferase, which convert a long-chain aldehyde substrate to the corresponding acid with the emission of light. Other *lux* genes such as *luxC*, *luxD*, and *luxE* genes encode a reductase, transferase, and a synthetase, respectively, which constitute the fatty acid reductase complex in charge of synthesising luciferase substrate.

Biosensors are genetically modified luminescent bacteria that provide a rapid, easily measurable response in the presence of relevant toxic compounds; thus, the bioluminescence is said to offer advantages of quicker response times and higher short-term sensitivity in the range of seconds to minutes (Woutersen *et al*., 2011) and has been acknowledged to be an excellent reporter mechanism for microbial biosensors (Atlas *et al*., 1992; Meighen, 1988). An inducible bacterial bioluminescent system shows increasing bioluminescence after exposure to a specific compound (termed the 'lights on system') (Woutersen *et al*., 2011). Here, promoter and reporter genes may be inserted from other bacteria to give an optimal response to the compounds of interest, serving as a bioavailability estimating tool for specific contaminants (Bhattacharyya *et al*., 2003). While the genetically engineered bacteria with constitutive expression normally have a high expression of luminescence that decreases under toxic conditions (termed the 'lights off system'), the biosensor response is not compound-specific as they are constitutively marked. A case of constitutive expression is notable in the *Vibrio fischeri*, a natural bioluminescent bacterium often used to detect acute cytotoxicity (Woutersen *et al*., 2011).

Biosensors based on luminescent bacteria have evidenced in various studies to be a valuable additional tool for pollutant detection, monitoring and ecotoxicity testing mostly for environmental relevance (Paton *et al*., 2006; Bhattacharyya *et al*., 2005). Additionally, the public and regulatory divergence in the use of actual pollutant concentration as a measure of toxicity for environmental significance has further strengthened the application of bacterial biosensor to resolve the bioavailable fraction of pollutants. This further buttress its relevance and usage for ecotoxicity testing, bioremediation and monitoring (Steinberg *et al*., 1995). An increasing number of luminescent biosensors harbouring the bacterial luciferase gene (*lux*CDABE) have been constructed and described (Weitz *et al*., 2001; Applegate *et al*., 1998), which were mostly applied for rapid hydrocarbon compound detection, toxicity testing and environmental monitoring coupled with bioremediation techniques (Huseein, 2016; Woutersen *et al*., 2011; Close *et al*., 2009). However, the biosensors required the finest growth characterisation, as well as assay optimisation to enable maximum induction geared towards optimal application. The

goal of this study stressed the dose responses of three *lux*-marked bacterial biosensors to target analytes for genuine extrapolative application in diagnosis of hydrocarbon contaminants uniquely in bioavailable context for environmental relevance. Therefore, this study initially examined and described the biosensor's growth characterisation, which then focused on the optimisation of the three different *lux*-marked bacterial biosensors and their induction bioassay for assessing their dose responses to the target inducers and mixed hydrocarbons. These biosensor's relevance for the rapid diagnoses of the bioavailable fractions of hydrocarbons or target analytes in environmental samples is also discussed in this study.

2 Materials and methods

2.1 Bioluminescent bacteria strains and lux-marked properties

Three different *lux*-marked bacterial biosensor strains (*Pseudomonas fluorescence* HK44, *Pseudomonas putida* TVA8 and *Escherichia coli* HMS174) were used for this study, which are inducible bioreporter systems that can sense a target hydrocarbon or related compounds. The previous study by King *et al*. (1990) developed a biosensor assay involving *P. fluorescens* HK44 mainly for specific and single compound detection where the biosensor made use of a genetically modified *P. fluorescens* strain, which produced bioluminescence in the presence of naphthalene. This strain (*P. fluorescens* HK44) harboured a fusion of the promoter of its own *nah*G gene for naphthalene degradation to the *lux*CDABE gene. The *P. putida* TVA8 was constructed by introducing the tod-*lux*CDABE gene fusion into the chromosome of *P. putida* F1, a bacterium capable of biodegradation of toluene (Applegate *et al*., 1998). Other studies by Shingleton *et al*. (2001;1998) further confirmed *P. putida* TVA8 as a benzene, toluene, ethylbenzene and xylenes (BTEX) degrader. Plasmid pOS25 in *E. coli* HMS174 was constructed by the fusion of regulatory region ipbRo/pA9 of the isopropylbenzene catabolic operon of pRE4 (originally from *P. putida* RE204) to upstream of *lux*CDABE genes from *V. fischeri* (Eaton and Timmis, 1986). The *E. coli* HMS174 harbouring this plasmid (pOS25) was then used by Selifonova and Eaton (1986) to study the regulation of the ipb operon. They also investigated the suitability of the ipbRo/pA9-*lux*CDABE reporter for detecting isopropylbenzene (IPB), other derivatives and other hydrocarbon mixtures mainly petroleum-derived products and coal tar creosote. A range of different *lux*-marked bioluminescent bacterial biosensors used in this study are described in Table 1.

2.2 Hydrocarbon in water preparation

The stock preparation of hydrocarbon in water substrate for the induction and dose-response bioassay was achieved by adding a known amount (in gram) of hydrocarbon (naphthalene, toluene and IPB/Cumene) to a known volume of double deionised water (ddH_2O) in a sterile substrate bottle. Magnetic stirring for 5 hours in a closed substrate bottled was achieved and further shaken at room temperature in the shaker at 250 rpm for 12 to 48 hours depending on the substrate solubility in water. This ensured adequate dissolution of hydrocarbons until the hydrocarbon crystals became very much indistinct (for naphthalene and IPB). The stock solution of prepared hydrocarbon in water was then filter sterilised using a 0.22 μm Millipore filter and preserved in a sterile airtight substrate bottle at room temperature. Subsequent dilution of the stock solutions with double-deionised water produced a range of standard hydrocarbon solutions used for various bioassays.

Table 1. List of three different *lux*-marked bioluminescent bacterial biosensors used in this study with their respective target analytes/inducers.

Biosensor	**Plasmid[a]/ Transposon[b]**	**Expression**	**Analyte**	**References**
P. fluorescens HK44	pUTK21	nahG-*lux*CDABE	Naphthalene	King *et al*., 1990
P. putida TVA8	putida F1	Mini-Tn5Kmtod-*lux*	Toluene	Applegate *et al*., 1998
E. coli HMS174	pOS25	ipbRo/pA'-*lux*CDABE	IPB/Cumene	Selifonova and Eaton, 1996

a = Plasmid for *P. fluorescens* HK44 and *E. coli* HMS174, while b = Transposon for *P. putida* TVA8.

2.3 Bacterial biosensor growth conditions

All three bacterial biosensor strains were grown in Luria Bertani (LB) medium containing appropriate antibiotics (14 mg/L Tetracycline for *P. fluorescens* HK44, and 50 mg/L Kanamycin for *P. putida* TVA8 and *E. coli* HMS174). The prepared stock concentration of these antibiotics was initially sterilised by microfiltration using the 0.22 μm syringe aided microfilter (Millipore) into sterile Eppendorf tubes, which was then wrapped in an aluminium foil (only Tetracycline) and stored at 4 ºC for fresh use only. The LB antibiotic plates (LB Tetracycline or Kanamycin plates) were prepared by adding an appropriate and required amount of antibiotics to sterilised LB agar medium at ~50 ºC.

2.4 Growth characterisation and optimisation of biosensors

Prior to the actual induction bioassay, the three bacterial biosensors were initially characterised to establish their growth patterns and optimised to improve their sensitivity to different target hydrocarbons such as naphthalene, toluene and isopropylbenzene (IPB). Growth curves for the biosensors were determined by the measurement of optical density (OD_{550} nm) and luminescence. These bacterial biosensors were inoculated in LB broth medium containing appropriate antibiotics (Tetracycline for *P. fluorescens* HK44, and Kanamycin for *P. putida* TVA8 and *E. coli* HMS174), incubated at 30 ºC and 200 rpm on a shaking incubator for overnight incubation (14 hours). One mL of the overnight culture was re-inoculated into a triplicate flask of antibiotic-containing LB broth, which was then incubated at 30 ºC and 200 rpm on a shaking incubator for the batch growth characterisation. Optical density at 550 nm wavelength and luminescence (in RLU) were measured after every 30 minutes for the 14 hours batch culture growth. The OD_{550} nm was measured on a Cecil spectrophotometer for overnight and batch culture, where samples with OD values greater than 0.8 were double diluted with LB broth and reading was re-taken.

Luminescence measurements were made in relative light units (RLU) using a portable Jade luminometer after mixing together 100 μL of cell suspension and 900 μL of standard solution of hydrocarbons (15 mg/L of naphthalene, 25 mg/L of toluene and IPB each) in a luminometer cuvette and incubated in a rotary shaker for 60 minutes at 25 ºC and 200 rpm.

2.5 Biosensor bioassay optimisation

The biosensor induction bioassay was optimised to determine the suitable duration of induction with target analyte since this affects the sensitivity and performance of biosensors as well as the light output. Here, the biosensors harvested mostly at the exponential phase were induced with their target hydrocarbons, followed by incubation at 25 ºC and 200 rpm but with varying induction time interval. All assays were performed in triplicates by inoculating the flasks containing 200 mL LB broth medium and appropriate antibiotics with 1 mL of overnight culture, then incubated at 30 ºC and 200 rpm on a shaker for the batch incubation. OD at 550 nm wavelength was measured on a Cecil spectrophotometer for overnight and batch culture to monitor the growth of bacterial biosensors. Cells from the batch were harvested at maximum exponential growth phase (maximum period obtained from the biosensor growth characterisation) and 100 μL of the cells were induced with 900 μL of their respective target standard hydrocarbon solutions in a cuvette. Luminescence was measured with Jade luminometer after 30, 60, 90 and 120 minutes of induction in the rotary incubator at 25 ºC and 200 rpm. The optimal induction periods and light output was determined and recorded for the three biosensors namely *P. fluorescence* HK44, *E. coli* HMS174 and *P. putida* TVA8.

2.6 Biosensor dose response to target hydrocarbons

To determine the dose response of the three *lux*-marked bacterial biosensors, these biosensors were induced with varied concentration of standard hydrocarbon solutions using the optimised parameters derived from optimisation experiments. *P. fluorescens* HK44 was inductively exposed to standard naphthalene solutions with varying concentrations of 10, 15 and 30 mg/L then incubated at 25 ºC and 200 rpm in the rotary shaker for 60 minutes induction period before luminescence was measured. *P. putida* TVA8 was induced with toluene standard solutions with differed concentrations of 10, 25 and 50 mg/L and incubated for 90 minutes while the *E. coli* HMS174 was exposed to IPB standard solutions with varied concentrations of 15, 25 and 50 mg/L and 60 minutes of induction was allowed in the rotary shaker incubator at 25 ºC and 200 rpm before the measurement of

light output in RLU. All the induction bioassays were carried out (in triplicates) in a cuvette with 100 μL harvested biosensor cell (at exponential phase from the batch culture) and 900 μL of the standard solutions of target hydrocarbons. Besides, induction with ddH_2O was used as blank (0 mg/L of target hydrocarbons) to check for background luminescence.

2.7 *Biosensor induction with mixed hydrocarbons*

In an attempt to mimic a genuine hydrocarbon contaminated sample such as groundwater, a mixture of hydrocarbons water sample was prepared, which contained 15 mg/L naphthalene, 25 mg/L toluene and 25 mg/L isopropyl benzene in ddH_2O. The response of the three *lux*-marked bacterial biosensors was investigated when exposed to the prepared mixed hydrocarbon solution. Prior to the induction bioassay, the resulting mixed hydrocarbon solution regarded as stock concentration was diluted with ddH_2O to 15, 25 and 50% (v/v) while ddH_2O was used as blank (0% (v/v)). The three biosensors were exposed to the standard solutions of mixed hydrocarbons (15, 25 and 50% (v/v)) using the optimised induction bioassay and parameters to investigate their response to the mixed hydrocarbon water sample. As a re-statement of induction bioassay, 100 μL of harvested biosensor cell (at exponential phase from the batch culture) was mixed with 900 μL of the standard solutions of mixed hydrocarbons (0, 15, 25 and 50% (v/v)) in a cuvette then incubated in a rotary shaker at 25 ºC and 200 rpm. The optimal induction period of 60 minutes was allowed in the shaking incubator for *P. fluorescens* HK44 and *E. coli* HMS174, contrary to 90 minutes induction time for *P. putida* TVA8. After that luminescence was measured and recorded in RLU.

2.8 *Data analysis*

The experiments were performed in triplicates and data generated were used to calculate the standard deviations where the resulting standard error (SE) were represented by error bars in the sigma plot figures. Student's t-test analysis at α = 0.05 level and ANOVA was used where applicable to check the results for significance where applicable.

3 Results and discussion

3.1 *Description of biosensor growth characteristics*

Luminescence throughout the 14 hours batch growth was measured for the three biosensors induced with target hydrocarbons as explained in the methods. The mini-growth curves in Figure 1 established growth characteristics of *P. fluorescens* HK44, *P. putida* TVA8 and *E. coli* HMS174 from 10 to 14 hours of growth study while measuring induction properties (OD and luminescence) at 30 minutes intervals. *P. fluorescens* HK44 and *P. putida* TVA8 exhibited the usual bacterial growth curve.

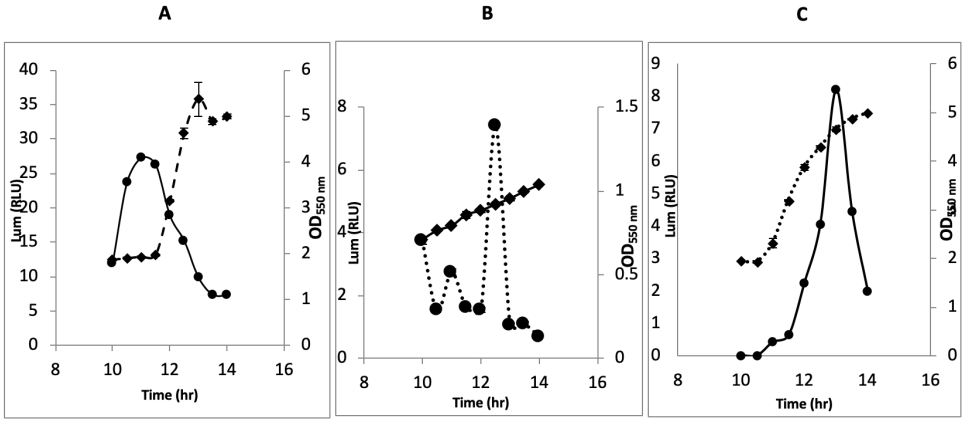

Fig. 1. Mini-growth curve for the three biosensors grown in LB medium: A= *P. fluorescens* HK44 (solid line represents RLU, dash line represents OD), B= *E. coli* HMS174 (round dotted line represents RLU, solid line represents OD), C= *P. putida* TVA8 (straight line represents RLU, round dotted line represents OD). Error bars represent the standard error of mean bioluminescence and OD of three replicates.

However, there was a difference at the time when maximum luminescence was reached, and the OD values obtained for the batch growth. The maximum luminescence for *P. fluorescens* HK44 was reached at 11 hours batch growth producing mean luminescence of 27.3 RLUs and OD_{550} of 1.93. The luminescence increased between 10 to 11 hours of growth with a corresponding increase in OD, while the stationary phase proceeded immediately after 11 hours to decline phase for *P. fluorescens* HK44. The *P. putida* TVA8 exhibited a prolonged lag phase (up to ten and half hours), afterwards proceeded to exponential growth phase until a peak was attained. This signifies that bioluminescence rose sharply and peaked at exactly the 13^{th} hour of growth before declining sharply afterwards. The *E. coli* HMS174 showed a distinguished trimodal pattern of growth where maximum luminescence was observed after 12 hours of growth (~12.5 hours) with a corresponding increase in OD during the growth phase recorded from 10 to 14 hours. The three biosensors were characterised by a prolonged lag phase for at least 8 or 9 hours of growth, while the exponential phase gradually proceeded afterwards for *P. fluorescens* HK44 and *E. coli* HMS174. However, *P. putida* TVA8 remained in the lag phase until 10 hours of batch growth as seen in Figure 1. The growth characterisation of the three *lux*-marked bacterial biosensors revealed that the maximum bioluminescence and induction of biosensor responsiveness occurred between 10 to 13 hours of batch growth for the three biosensors, where *P. fluorescens* HK44, *P. putida* TVA8 and *E. coli* HMS174 reached their maximum bioluminescence at hour 11, 13 and 12.5, respectively.

3.2 Optimisation of induction bioassay and biosensor harvest

The biosensor's sensitivity and response to target hydrocarbons were investigated by optimising the biosensor harvest in terms of response to inducers and the status of ecophysiology. This provided the optimum induction period for various biosensors to achieve the most sensitive and optimal dose responses to their respective inducers. Table 2 shows parameters obtained from the bioassays and biosensors optimisation for increased biosensor sensitivity and optimum responsiveness.

From Table 2, *P. fluorescence* HK44 harvested at 11 hours of growth responded optimally to induction by 15 mg/L of naphthalene when induced for 60 minutes. Meanwhile, the *P. putida* TVA8 exhibited maximum induction with 25 mg/L toluene at 90 minutes induction period using culture optimally harvested at 13 hours of growth. Lastly, *E. coli* HMS174 harvested at 12.5 hours of growth also responded optimally to induction by 25 mg/L of isopropylbenzene after the induction period for 60 minutes.

3.3 Biosensor's response to hydrocarbons and dose response curve

The operating range often signifies the minimum concentration of hydrocarbon detectable by a biosensor and the maximum concentrations of hydrocarbons tolerated by a specific biosensor. The dose-response curves relate the responsiveness of biosensors when induced with different concentrations (standard solutions) of targeted hydrocarbons. The dose-response curve in Figure 2 displays that the target analyte doses or concentrations that yield maximum luminescence (100%) after induction. *P. fluorescence* HK44 produced highest luminescence with 15 mg/L of naphthalene, and induction with 30 mg/L of naphthalene produced the least light output.

Meanwhile, an induction with the ddH_2O (0 mg/L Naphthalene) produced no luminescence. Here, luminescence (in RLU) was represented in percentage where the maximum luminescence obtained from all induction bioassays was designated as 100%, the minimum was the least light output derived from an induction bioassay, while 0% luminescence represent no luminescence as mostly seen in the case when using ddH_2O as blank.

Table 2. Assay and biosensor optimisation.

Biosensor	Inducers	Concentration	Optimum harvest time	Induction time
P. fluorescence HK44	naphthalene	15	11	60
P. putida TVA8	toluene	25	13	90
E. coli HMS174	isopropylbenzene	25	12.5	60

The *P. putida* TVA8 responded optimally when induced with 25 mg/L of toluene producing the maximum light output while a higher dose of the target analyte (50 mg/L of toluene) yielded the least luminescence. The *E. coli* HMS174 also yielded maximum luminescence with 25 mg/L of IPB, while the highest concentration of IPB caused the least or no luminescence. Statistical inference established that all the three biosensors had a significant higher luminescence for the optimally induced concentration of the target hydrocarbons than the blank control containing no target analytes. The dose-response study of these biosensors showed that the target analytes (naphthalene, toluene and IPB) with the highest concentration produced the least luminescence after induction assay. The response of *P. fluorescence* HK44 to 15 mg/L naphthalene and *P. fluorescens* TVA8 to 25 mg/L toluene resulted in significant induction relative to the control (ddH_2O).

3.4 Biosensor's response to mixed hydrocarbons

The three *lux*-marked biosensors were significantly induced, thus producing detectable light output when exposed to the prepared mixture of hydrocarbon solution (consisting of 15 mg/L naphthalene, 25 mg/L toluene and 25 mg/L isopropylbenzene). In Figure 3, 25% (v/v) concentration of the mixed hydrocarbons solution caused maximum induction of *P. fluorescence* HK44 while the *P. putida* TVA8 was maximally induced at 50% (v/v) concentration of the mixed hydrocarbon signifying a proportional increase in light output when concentration increases. For the *E. coli* HMS174, only 15% (v/v) concentration of the mixed hydrocarbons solution yielded the highest light output; however, this luminescence decreased as the concentration of mixed hydrocarbons increases to 25% (v/v) and 50% (v/v). Figure 3 revealed that the three bioluminescence-based bacterial biosensors were highly responsive across different concentrations of the mixed hydrocarbon solution and the optimal responses were significant compared to that of the blank control (ddH_2O) with no hydrocarbon contaminants.

Aside from the use of conventional analytical methods for analysing environmental pollutants, the whole-cell bacterial biosensors also represent a convenient testing method for pollutants quantification and measurement of contaminants' bioavailability as routine analytical techniques often fail to provide information about the soluble bioavailable fractions of contaminants in environmental samples (Hynninen *et al*., 2010; Hynninen and Virta, 2010). Also, the cost and turn-around time in analysing enormous samples within a short period is another constraint. Consequently, an alternative to chemical and analytical methods in terms of assessing bioavailability, bioreporter (biosensor) was proposed as a sensor for soil and water contaminants (Close *et al*., 2009). Conveniently, the microbial biosensor technology has resolved these constraints of other methods through the provision of a rapid, sensitive and cost-effective method of analysing environmental samples. Among many instances, Paton *et al*. (2009) applied a luminescence-based biosensor for assessing naphthalene biodegradation in soils from a manufactured gas plant. Other studies by Woutersen *et al*. (2011), Paton *et al*. (1995) and Tauriainen *et al*. (1999) have also shown its usefulness have no bound by demonstrating its applicability in freshwater monitoring. Specifically, Woutersen *et al*. (2011) evaluated the suitability of luminescent bacteria for online detection and monitoring of toxic compounds in drinking water and its sources, denotes that luminescence is the detection method of choice for online monitoring of water, sensitivity and fast response times. However, in deriving an efficient usage and exploiting a high-performance biosensor for environmental relevance, it is mostly important to establish the finest characterisation and optimisation of these biosensors and assays to assess their ecophysiology and responsiveness. This generally enables an optimal assessment of the pollutant loading in contaminated samples. Therefore, this study investigates the biosensors and bioassay optimisation, as well as dose responses of three *lux*-marked bacterial biosensors to their targeted and mixed hydrocarbons.

The characterisation of the three bioluminescent bacterial biosensors describes their unique growth patterns, detects the strain's peak of the exponential phase and the optimum biosensor harvest time for efficient induction bioassay (Table 2). In this study, the optimisation of the biosensor's performance in terms of sensitivity, response speed and optimum induction period was achieved, which consequently improved the performances of the biosensors when the optimised parameters were applied for the induction bioassay.

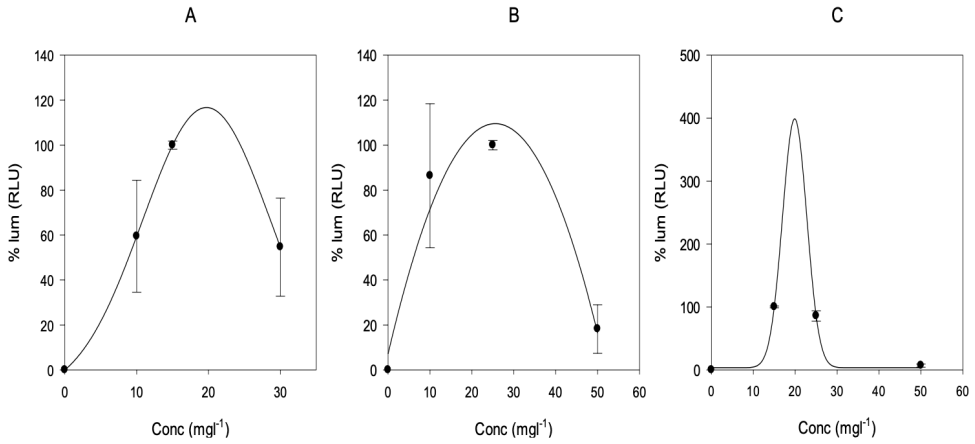

Fig. 2. Dose-response curves of the three *lux*-marked biosensors (A = *P. fluorescens* HK44, B = *P. putida* TVA8, C = *E. coli* HMS174) induced with target hydrocarbons (*P. fluorescens* HK44 induced with naphthalene, *P. putida* TVA8 induced with toluene, and *E. coli* HMS174 induced with isopropylbenzene of varying concentrations in mg l^{-1}). Results are mean of three replicates bioluminescence measurements (RLU) and error bars represent the standard error (SE).

It is noteworthy to state that most sensitive and optimal dose responses were attained with bacterial strain harvested and used for induction at their maximum exponential phase. Weitz *et al*. (2001) also reported the highest sensitivity of luminescent biosensor using cells in late exponential growth. However, it must be acknowledged that other countable factors such as genetic construct, host strains, medium compositions and amounts of bacteria per measurement may affect the sensitivities and induction coefficients of these biosensors (Tauriainen *et al*., 1999). A study by Woutersen *et al*. (2011) confirmed that most *lux* strains are sensitive with detection limits which range from milligrams per litre to micrograms per litre, and that the high sensitivities are often with compound-specific strains. Thus, this present study in a way justifies the inductive expression of a bioluminescent bacterial biosensor and corroborates that assay and biosensor optimisation can increase the sensitivity and biosensors' response to their target hydrocarbons.

The dose-response curves (Figure 2) portray a proportional increase in luminescence when the concentration of the target hydrocarbons increases. This implies that the responsiveness of the three tested biosensors to their target analyte was dose-dependent, and further validated the hydrocarbon specificity of these biosensors. The results of the biosensor dose response (Figure 2) hinted that biosensors can distinguish the bioavailable fraction to enable a meaningful dose-response extrapolation for the assay especially for predicting contaminant loading in the environmental samples (Harkins *et al*., 2004). Previous work by Hussein *et al*. (2016) applied this for the diagnostic determination of naphthalene and toluene in refined oil product-contaminated water samples, enabling the prediction and appraisal of the soluble bioavailable fractions of these hydrocarbons in the contaminated water samples (Kerosene, diesel and motor oil-contaminated water samples). O'Neill *et al*. (2003) significantly estimated PAH concentration in contaminated marine sediment induced with *P. fluorescens* HK44. This inferred that catabolic biosensor could serve as a viable tool for estimating bioavailability of specific contaminants (Bhattacharyya *et al*., 2005).

In the dose-response curve (Figure 2), aside from the fact that the level of biosensor induction attained is related to inducer's concentrations, there was a characteristic decline in light output produced mostly with the highest hydrocarbon concentrations (50 mg/L) after the peak luminescence was reached, denoting that the highest dose of target analytes produced the least luminescence for the three biosensors. This may be attributed to the cell toxicity arising from the toxic concentration of pollutant resulting in the quenching effect of the biosensors as also related by Trogl *et al*. (2007). The same fate was experienced by Selifonova and Eaton (1996) where the response to IPB increased as its concentration increased (from 1 to 100 μM), then decreased by as much as 99% at 1 mM IPB higher concentration.

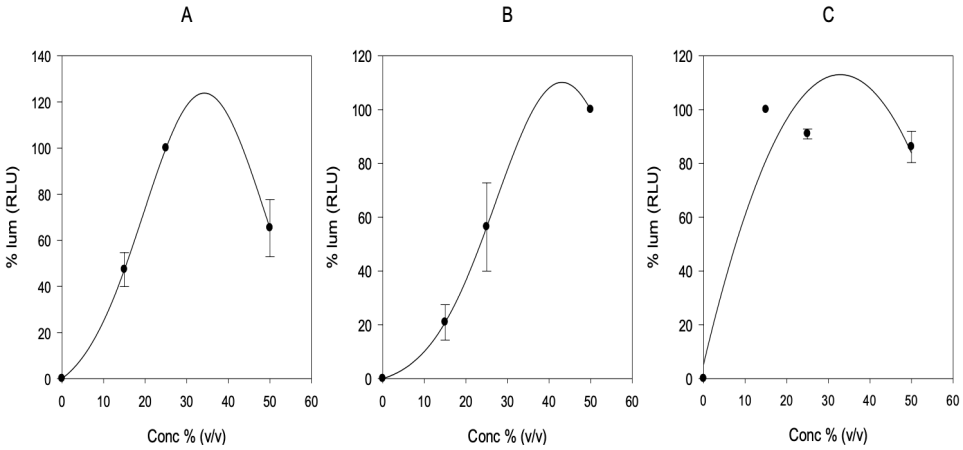

Fig. 3. Responses of the three biosensors (A = *P. fluorescens* HK44, B = *P. putida* TVA8, C = *E. coli* HMS174) to varied concentration (% (v/v)) of mixed hydrocarbon solution (containing 15 mg l^{-1} naphthalene, 25 mg l^{-1} toluene and 25 mg l^{-1} isopropylbenzene). Results designate mean of three replicates bioluminescence (RLU) and the standard error is represented by the error bars.

This was also attributed to the toxicity of IPB and other hydrocarbons to bacterial cells due to alteration of membrane structure and function (Sikkema *et al*. 1995), as well as limited water solubilities at high concentration that decreases its value as an inducer of ipb-*lux* (Selifonova and Eaton, 1996). Also, the reduced rate of luciferase synthesis, biosensor's interaction with toxic chemicals or admixtures, limited presence of reaction substrate (such as O_2, aldehyde, reduced flavin mononucleotide, NADPH, and ATP), intracellular dilution by cellular growth and intrinsic stability of the luciferase enzyme could contribute to the decrease of the bioluminescence response by bioreporters (Heitzer *et al*., 1994: Trogl *et al*., 2007; Heitzer *et al*., 1998). In a case of no luminescence, Paton *et al*. (1995) attributed this to an insufficient mass of the soluble fraction of compound being assimilated through the cell membrane. The three catabolic biosensors were strongly induced by prepared contaminated water sample containing mixed hydrocarbons, responding differently to the same inducer of mixed hydrocarbons (Figure 3). This necessarily pointed at its applicability for the analysis of environmental samples with mixed contaminants as demonstrated by several studies (Hussein *et al*., 2016; Heitzer *et al*., 1998). Additionally, Woutersen *et al*. (2011) buttressed that bacterial *lux* strains sensing specific pollutants have an edge to also respond to mixtures of contaminants inducing the same effect, which could be used for the sum effect, including compounds that are yet identified by chemical method.

The work of King *et al*. (1990) reported that *P. fluorescens* HK44 emits light not only in the presence of naphthalene, but also other substituted analogues, emphasising that it can emit light when catabolising naphthalene and salicylate as it contains the plasmid pUTK21 with a *nah*G-*lux*CDABE gene fusion. In Figure 3, it was deduced that *E. coli* HMS174 produced the highest luminescence at 15% (v/v) concentration of the mixed hydrocarbon solution, while *P. fluorescens* HK44 and *P. putida* TVA8 yielded the maximum observable light output at 25% and 50% (v/v) concentration of mixed hydrocarbons, respectively. This may suggest that an increased combined effect of the mixed hydrocarbons might have caused maximum luminescence by *E. coli* HMS174 at low concentration (15% (v/v)) of the mixed hydrocarbon solution, as Selifonova and Eaton (1996) reported the possibility of identifying other variety of gratuitous inducers of the ipb operon by *E. coli* HMS174 aside its target analyte. Heitzer *et al*. (1998) also reported similar response where a different combined mixture of organic solvents resulted in either additive, intermediate or synergistic effect on the light output.

On a second thought, the ability of these *lux*-marked biosensors to sense and response to non-specific compounds related or substituted analogues could explain the reason for the overestimation of target analytes especially in real environmental samples containing heterogeneous contaminants since previous studies have overestimated the presence

of hydrocarbons in environmental samples contrary to the analytical values. O'Neill *et al*. (2003) significantly overestimated polycyclic aromatic hydrocarbon concentration in contaminated marine sediment induced with *P. fluorescens* HK44. Besides, Hussein (2012) predicted an elevated concentration compared to the actual values in the application of bioluminescent biosensor for diagnosing hydrocarbons in contaminated groundwater, where similar biosensors used were significantly responsive across a several ranges of groundwater samples producing maximum bioluminescence in response to groundwater concentration as low as 0.5% (v/v). However, prior to these studies, Heitzer *et al*. (1998) associated the detection of the overrated concentration of naphthalene in JP-4 complex mixture to membrane perturbation.

In comparison, Trogl *et al*. (2007) clarified that an ideal analytical procedure is selective, responding only to the target compound and ignoring other admixtures. However, this is not the standard behaviour for most bioreporters. On this note, it must be acknowledged that the overestimation of hydrocarbon conversely to their actual values may suggest the limitation of biosensor technology though depending on which perspective it is being perceived. Overall, bioluminescence-based biosensors, which *lux* genes are introduced into the environmentally relevant bacteria, are rapid, reproducible and sensitive to a wide range of inorganic and organic contaminant (Paton *et al*. 1997; Steinberg *et al*. 1995); they mostly serve as a tool for estimating bioavailability of specific contaminants (Bhattacharyya *et al*. 2005).

Conclusions

In this study, the characterisation of the biosensors has allowed for the understanding of the biosensor's growth characteristics to enable harvest at optimum conditions in their finest state. Further optimisation of biosensors and induction bioassay produced the most sensitive and highly responsive biosensors when exposed to various inducers. Aside from the significant response of the three *lux*-marked biosensors to the mixed hydrocarbons signifying their viability in analysing real environmental samples, these bioreporters produced a dose-dependent response to their respective target hydrocarbons, which could enable a meaningful dose-response extrapolation for biosensor assay primarily for estimating contaminant loading in the environmental samples. While the microbial biosensor application cannot replace conventional analytical methods for the analysis of pollutants in environmental samples, it provides a better option for the detection and analysis of bioavailability (soluble bioavailable fractions) of various hydrocarbons, and importantly serves as bioindicator of hydrocarbon pollution in an environment. This suggests that biosensor application can resolve the constraints of other analytical methods, thus may be applied complimentarily in combination with robust chemical or analytical methods for an efficient all-around purpose including monitoring of environment and bioremediation processes.

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