

1       **New naphthalene whole-cell bioreporter for the monitoring and**  
2       **assessment of polycyclic aromatic hydrocarbons contaminated**  
3       **groundwater**

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## Abstract

A novel naphthalene bioreporter was designed and constructed in this work to assess the bioavailable naphthalene. A new vector pWH1274\_Nah was constructed by Gibson isothermal assembly fused with 9-kb naphthalene-degrading gene NahAD and cloned into the salicylate bioreporter *Acinetobacter* ADPWH\_lux as the host. The ADPWH\_Nah bioreporter could effectively metabolize naphthalene, respond to the central metabolite salicylate and evaluate the bioavailable naphthalene in natural water samples. This whole-cell bioreporter was sensitive and behaved positively bioluminescence in presence of salicylate from 0.5  $\mu$ M to 100  $\mu$ M, whereas its high specificity kept its silence to other PAHs (pyrene, anthracene and phenanthrene). The bioluminescence response was quantitatively measured within 4 hours exposure to naphthalene and the model simulation further proved that the bioluminescence was activated by salicylate after naphthalene metabolism dynamics. The ADPWH\_Nah bioreporter also achieved the rapid evaluation of the bioavailable naphthalene in sixteen PAHs contaminated groundwater samples after the chemical spill accident in China, showing high consistency with chemical analysis. The engineered *Acinetobacter* variant had significant advantages in fast detection in the laboratory and potentially *in situ*. The state-of-the-art concept of cloning PAHs degrading pathway in salicylate bioreporter host opened the door for the construction and assembly of high throughput PAHs bioreporter array, suitable for risk assessment and contamination management at organic contaminated sites.

**Keywords:** Naphthalene; whole-cell bioreporter; *Acinetobacter baylyi*; Gibson cloning; polycyclic aromatic hydrocarbons (PAHs)

## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), a group of persistent organic pollutants, exist widely in subsurface environments (Wilcke, 2000). PAHs have been designated by the US Environmental Protection Agency (US EPA) as priority pollutants due to their high carcinogenicity and toxicity to humans and animals (Boffetta et al., 1997). Naphthalene is a classical member of PAHs with high carcinogenicity, drawing great concerns due to its high solubility in water and high volatility (Valdman et al., 2004a). As one of the most widespread xenobiotic pollutants, the detection and natural attenuation of naphthalene are the main challenges in PAHs contamination management (Valdman et al., 2004b).

Chemical analysis is the main approach for naphthalene measurement in environmental samples. Gas chromatography - mass spectrometry (GC/MS) (Potter and Pawliszyn, 1994) and High-performance liquid chromatography (HPLC) (Oliferova et al., 2005) are the most applied techniques, though they are expensive and require laborious pre-treatment in environmental monitoring (Zhang et al., 2013). Recent years, the raising attentions on genetically engineered whole-cell bioreporters attribute to their highly sensitivity, low-cost, time-efficiency and easy operation (Song et al., 2009). More importantly, whole-cell bioreporter is able to assess the bioavailability and toxicity of contaminants *in situ* (Kohlmeier et al., 2008; Deepthike et al., 2009; Tecon et al., 2009). They are also possible for further real-time or online measurement of environmental contamination and evaluation of their ecological impacts (Elad et al., 2011; Chen et al., 2013). It is therefore viewed as a supplementary technique to chemical analysis for environmental risk assessment. Numerous whole-cell bioreporters have been reported to sense crude oil and PAHs, such as n-alkane (Zhang et al., 2012a), BTEX (Keane et al., 2008; Kuncova et al., 2011), naphthalene (King et al., 1990; Trogl et al., 2012) and phenanthrene (Shin et al., 2011). Particularly for naphthalene, *Pseudomonas fluorescens* HK44 is the mostly investigated naphthalene bioreporter (King et al., 1990; Trogl et al., 2007). Not only directly applied in wastewater monitoring (Valdman et al., 2004a), *Pseudomonas fluorescens* HK44 is also able to achieve online naphthalene detection (Valdman et al., 2004b) and PAHs degradation assessment (Paton et al., 2009), or immobilized in gel to maintain its stability and long-term storage (Trogl et al., 2005).

Nevertheless, there is still lack of applicable whole-cell bioreporter for many other

PAHs. It is attributed to the construction principle that the reporter genes are fused to the promoters of degradation gene (or operon) with the biological signaling chains coupled to fluorescent or bioluminescent proteins (Belkin, 2003; van der Meer et al., 2004). For instance, the conventional methods for naphthalene bioreporter construction followed the fusion of *lux* or *gfp* reporter gene in the operon encoding naphthalene metabolism (like *nahR*) (Shin, 2010), hosted by indigenous naphthalene-degrading microbes (like *Pseudomonas fluorescens*) (King et al., 1990) or genetic-engineering model strains (like *Escherichia coli*) (Mitchell and Gu, 2005), thereby allowing the expression of biological signals during the naphthalene metabolism (Ripp et al., 2000). The construction of each bioreporter is unique and laborious for the specific PAHs molecule. Most PAHs metabolisms follow the salicylate pathway, including naphthalene (Yen and Serdar, 1988; Harwood and Parales, 1996; Chen and Aitken, 1999; Johri et al., 1999; Loh and Yu, 2000), and salicylate behaves as an important signaling metabolite for whole-cell bioreporter. By cloning naphthalene-degrading operons into the salicylate bioreporter host, the detection of metabolic salicylate can quantify the existence of the parent naphthalene. This concept is then suitable to construct series of PAHs whole-cell bioreporters for multi sensing array.

In this study, a novel type of naphthalene bioreporter was constructed and applied for the measurement of groundwater naphthalene contamination. Gibson isothermal assembly (Gibson et al., 2009) was introduced for the assembly of a recombinant naphthalene-degrading circuit, pWH1274\_Nah, with capability of transferring naphthalene to the central metabolite salicylate. The new bioreporter ADPWH\_Nah was constructed by cloning the pWH1274\_NahAD vector in host *Acinetobacter* ADPWH\_lux (Huang et al., 2005), the *salAR* and *luxCDABE* operon of which was inducible by salicylate. Converting naphthalene to salicylate by the expression of *nahAD* operon on the vector, the ADPWH\_Nah bioreporter was able to quantitatively respond to naphthalene and assess the bioavailable naphthalene in water samples. The naphthalene degrading pWH1274\_Nah vector can be replaced by other circuits with respective PAHs metabolic operon to achieve the biological detection of targeting PAHs. Our work presents a routine and simple method for the construction of various whole-cell bioreporters responsive to different PAHs molecules, showing its wide applicable prospective in water monitoring and assessment.

## 2. Materials and methods

### 2.1 Bacterial strains, plasmids and culture media

The bacterial strains and plasmids are listed in Table 1. Unless otherwise stated, all the chemicals are analytical grade reagents from Sigma Aldrich (USA). The cultivation medium for ADPWH\_lux was Luria-Bertani (LB, Thermo Scientific, USA). ADPWH\_Nah and ADPWH\_1274 was cultivated and induced in LB medium supplemented with 100 µg/mL ampicillin (LBA100), and the cultivation medium for *E. coli* DH5α (with pWH1274 or pWH1274\_NaAD) was LB medium with 300 µg/mL (LBA300). Minimal medium with 20 mM sodium succinate (MMS) was used for the induction of bioreporter strains, containing 2.5 g Na<sub>2</sub>HPO<sub>4</sub>, 2.5 g KH<sub>2</sub>PO<sub>4</sub>, 1.0 g NH<sub>4</sub>Cl, 0.1 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 µL saturated CaCl<sub>2</sub>, 10 µL saturated FeSO<sub>4</sub>, 1 mL Bauchop & Elsdén solution and 20 mM sodium succinate in 1.0 L deionized water. Minimal medium agar (1.4%, MMA) was supplemented with naphthalene crystals as the sole carbon source for the selection of positive *Acinetobacter* clone with pWH1274\_Nah vector.

### 2.2 Gibson isothermal assembly for pWH1274\_Nah vector

The Gibson isothermal assembly and construction of naphthalene whole-cell bioreporter ADPWH\_Nah were briefly illustrated in **Figure 1**. Our targeting vector pWH1274\_NahAD was assembled by the *nahAD* operon (9 kb, **Figure 1A**) and pWH1274 plasmid (6 kb, **Figure 1A**), with the one-step *in vitro* Gibson recombination method which can assemble DNA products as large as 900 kb (Gibson et al., 2009). Plasmids pDTG1 (Dennis and Zylstra, 2004) was extracted from *Pseudomonas putida* NCIB9816 (Cane and Williams, 1982) as the template for *nahAD* operon. The primers (Sangon Biotech, China) for polymerase chain reaction (PCR) and Gibson isothermal assembly were listed in **Table 2**. The two pairs of primers were designed and synthesized with 30+ bp overlap (**Figure 1B**). pWH1274 and NahAD operon were separately amplified by PCR using the primer pairs NahA\_for/NahD\_rev and 1274\_for/1274\_rev (**Table 2**). The PCR system (50 µL) contained 1 µL DNA template, 1×reaction buffer (Fermentas, USA), 0.2 mM each deoxynucleoside triphosphate (Fermentas, USA), 0.2 µM each primer and 2.5 units Dream Taq DNA polymerase (Fermentas, USA). The reaction was performed with initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 sec, 56°C

for 30 sec and 72°C for 5 min, and a final additional extension at 72°C for 10 min. The amplified products were isolated by 1% agarose gel and further purified with QIAquick gel extraction kit (Qiagen, Germany) according to manufacturer's instructions. The two fragments were processed and fused together using a T5 exonuclease, Taq DNA ligase and high fidelity Phusion polymerase, incubated at 50°C for 12 h (**Figure 1B**). After transformation via heat shock, the positive clone (DH5 $\alpha$ \_pWH1274\_Nah) was selected on LBA300 agar plate and the plasmid pWH1274\_Nah was extracted from DH5 $\alpha$ \_pWH1274\_Nah cells.

### 2.3 Transformation of pWH1274 and pWH1274\_NahD vector in ADPWH\_lux

The competent cells of ADPWH\_lux were prepared as following: after grown in LB medium at 30°C overnight with shaking at 150 rpm, the 100  $\mu$ L cell suspension was harvested by centrifugation at 3000 rpm for 10 min at 4°C, washed and resuspended in 1 mL 10% glycerol. One microliter pWH1274\_Nah vector was electro-transformed into 50  $\mu$ L competent ADPWH\_lux cells. Subsequently, the cells were transferred into 500  $\mu$ L of SOC medium and incubated at 30°C for 2 h. The cell suspension was then spread on MMA with naphthalene crystals for the selection of positive transformants capable of metabolizing naphthalene. The plasmid pWH1274 was also electro-transformed into *Acinetobacter* ADPWH\_lux as the negative control with similar selection process mentioned above, except for the LBA300 agar plate as the final selection medium. The confirmation of successful pWH1274\_Nah vector transformation and function was conducted by the metabolism of naphthalene and PCR amplification with NahA\_for/NahD\_rev primer pairs.

### 2.4 Bioluminescence induction of ADPWH\_Nah by PAHs

Each of the 100 mM PAHs stock solution was prepared by dissolving a specific weight of PAHs (naphthalene 128.2 mg, pyrene 202.3 mg, toluene 92.1 mg, anthracene 178.2 mg, phenanthrene 178.2 mg, sodium salicylate 160.1 mg and sodium benzoate 144.1 mg) in 10 mL dimethyl sulfoxide (DMSO, for naphthalene, pyrene, toluene, anthracene and phenanthrene) or deionized water (for sodium salicylate and sodium benzoate). The PAHs induction solution was made by series dilution to the final concentration of 0.5, 1, 5, 10, 20, 50 and 100  $\mu$ M with deionized water. After inoculation in LBA300 medium at 30°C overnight, 1 mL ADPWH\_Nah, ADPWH\_1274 and ADPWH\_lux cell suspensions were harvested by centrifugation at

3000 rpm for 10 min at 4°C, washed twice and resuspended in MMS medium of the same volume (Zhang et al., 2013). The 20 µL of bioreporter suspension and 180 µL PAHs solution was transferred into each well of a 96-well black optical-flat microplate (Corning Costa, USA). The bioluminescence and absorbance at 600 nm wavelength (OD<sub>600</sub>) were detected by the Synergy 2 Multi-Detection Microplate Reader (BioTek Instrument, USA). The measurement was conducted every 10 min for 5 h and the incubation temperature was 30°C. All the detections were carried out in triplicates.

## 2.5 *Naphthalene and n-alkane detection in PAHs contaminated groundwater*

A total of sixteen real groundwater samples were collected from a PAHs contaminated site in China (**Figure 2** and the locations were listed in **Table S1** of Supplementary Material) on 14<sup>th</sup> July 2014. The site was historically contaminated by the chemical spills from *China Petroleum Lanzhou Petrochemical Company*, consequently causing the *Lanzhou Tap Water Crisis* on 11<sup>th</sup> April 2014. Ten shallow groundwater samples (SW01, SW03, SW04, SW05, SW06, SW07, SW08, SW09, SW11 and SW15) were taken at 4.5 m depth and the other six deep groundwater samples (DW02, DW05, DW07, DW08, DW09 and DW11) were collected at 8.0 m depth. The groundwater samples were stored at 4°C until further assessment (within 72 h). The chemical analysis of PAHs and n-alkane contamination was GC/MS following USEPA methods (Zhang et al., 2013) and the results were listed in **Table S2**. The 2 mL of groundwater sample was homogenized with 40 kHz ultrasound for 300 s and mixed well by vortexing for 10 s before bioreporter detection. The ADPWH\_Nah and ADPWH\_alk (Zhang et al., 2012a) bioreporter strains were cultivated in LBA300 and LB medium overnight at 30°C respectively, followed by centrifugation harvest (3000 rpm for 10 min) and resuspended in MMS medium. The 20 µL of bioreporter suspension and 180 µL groundwater sample was transferred into the 96-well black optical-flat microplate, following the same bioluminescent measurement procedure mentioned above.

## 2.6 *Quantitative model for ADPWH\_Nah's response to naphthalene*

From the mathematical gene regulation model for the bioreporter response to specific chemicals (Zhang et al., 2012b; Al-Anizi et al., 2014), the direct induction of *salAR* operation in ADPWH\_lux by salicylate is expressed by the following Equation (1).

$$\alpha_{[S]} = \alpha_{m[0]} + \alpha_m \cdot \frac{[S]}{K_I^{-1} + [S]} \quad (1)$$

Here,  $[S]$  represents the salicylate concentration in bioreporter cells ( $\text{cell}^{-1}$ ) and  $K_I$  refers to the specific inducer binding rate of SalR regulator to salicylate molecule.  $\alpha_{m[0]}$  ( $\text{s}^{-1} \cdot \text{cell}^{-1}$ ) is the transcription rate of *salAR* operon baseline expression in the absence of the inducer, and  $\alpha_m$  represents the maximal transcription rate with saturated salicylate induction. Considering the metabolization rate from naphthalene to salicylate ( $m_{N-S}$ ) by NahAD enzymes, the salicylate concentration is the formula of naphthalene concentration ( $[N]$ ,  $\text{cell}^{-1}$ ) and Equation (2) then describes the quantitative gene expression of *salAR* operon induced by naphthalene.

$$\alpha_{[S]} = \alpha_{m[0]} + \alpha_m \cdot \frac{[N]}{(m_{N-S} \cdot K_I)^{-1} + [N]} \quad (2)$$

## 2.7 Data analysis

The distributions of n-alkane and naphthalene in groundwater samples were interpolated by Kriging method, analyzed and plotted by Surfer 8.0 (Golden Software). The SPSS package (Version 11.0) was used for statistical analysis and  $p$  value  $< 0.05$  was considered to indicate statistical significance. Brown-Forsythe and Shapiro-Wilk test was conducted for the data equality and normality, and the null hypothesis was rejected for  $p$  value less than 0.05.

## 3. Results and discussions

### 3.1 Genetic information of naphthalene bioreporter ADPWH\_Nah

Plasmid pWH1274 is a widely used shuttle vector for *Escherichia coli* and *Acinetobacter calcoaceticus* (Hunger et al., 1990). The *nahAD* operon encoding naphthalene metabolism was amplified by PCR from the pDTG1 plasmid in *Pseudomonas putida* NCIB9816 (**Table 1**) (Cane and Williams, 1982). To improve the naphthalene metabolization to salicylate, the *nahAD* was cloned into pWH1274 with the constitutive promoter  $P_{tet}$  (Hansen and Sorensen, 2000) via the Gibson isothermal assembly (Gibson et al., 2009) (**Figure 1**). The pWH1274 and pWH1274\_Nah vectors were then transformed into the salicylate bioreporter, ADPWH\_lux, as the negative control (ADPWH\_1274) and positive bioreporter strain (ADPWH\_Nah) for naphthalene sensing.

The existence of *nahAD* operon in ADPWH\_Nah was confirmed by PCR with the



primer pairs NahD\_rev/NahA\_for (**Table 2**). Only ADPWH\_Nah had the positive band of *nahAD* fragment with the expected 9 kb size, and the results were negative for ADPWH\_lux and ADPWH\_1274 (**Figure S1**). ADPWH\_Nah could also grow in minimal medium with naphthalene as the sole carbon source, showing the functions of NahAD enzymes metabolizing naphthalene to salicylate. The salicylate molecules further activated the  $P_{sal}$  promoter and triggered the expression of *salAR* operon and *luxCDABE* gene, giving bioluminescent signals for the detection.

### 3.2 Naphthalene induction kinetics of ADPWH\_Nah

In the absence of the naphthalene, ADPWH\_Nah, ADPWH\_1274 and ADPWH\_lux had similar bioluminescent baseline from 50 RLU (relative light units) to 200 RLU (**Figure 3**). Exposed to 50  $\mu$ M naphthalene, only ADPWH\_Nah showed rapid and positive response within 5 minutes and the dramatic bioluminescent increasing lasted for 2 hours and subsequently stabilized at the high level of 2,500 to 3,100 RLU (**Figure 3**). No response was observed for ADPWH\_1274 and ADPWH\_lux to naphthalene, indicating that naphthalene did not activate the *salAR* operon and *luxCDABE* gene. The results proved the functions of *nahAD* operon for naphthalene metabolism, and the  $P_{tet}$  was a strong promoter for *nahAD* expression and encoding, compared to the *nahR* regulator (Mitchell and Gu, 2005).

From the response of ADPWH\_Nah to different naphthalene concentrations (**Figure 4**), the detection limit of ADPWH\_Nah was 1  $\mu$ M and the quantification time was 1 hour. The 5  $\mu$ M naphthalene induction was significantly higher than the negative control within only 5 minutes after induction. The rapid and sensitive response of ADPWH\_Nah suggested its potential as a real-time naphthalene whole-cell bioreporter. Throughout the induction, no significant bioreporter growth suppression was observed for each naphthalene concentration (**Figure S2**), and naphthalene therefore showed no impact on the growth and activities of ADPWH\_Nah bioreporter. The increasing bioluminescence signal occurred in the first 2 h and the stable response time was identified from 200 min to 240 min. The positive relationship between ADPWH\_Nah's bioluminescence and naphthalene concentration also demonstrated the feasibility that ADPWH\_Nah could be used as the analytical bioreporter to quantify naphthalene in aqueous phase.

ADPWH\_Nah has high naphthalene response specificity, as illustrated in **Figure 5**.

With similar responsive pattern to sodium salicylate and sodium benzoate, the three strains had the same mechanisms of *salAR* operon activation by salicylate or benzoate (Zhang et al., 2012b). In the presence of pyrene, toluene, anthracene and phenanthrene of different concentrations, ADPWH\_Nah did not show significant response (**Figure 5**) (Huang et al., 2005). The results indicated that ADPWH\_Nah specifically responded to naphthalene in natural environment with limited interference by other PAHs. With the response of the negative control ADPWH\_1274 to salicylate and benzoate (**Figure S3**), their disturbance can be rectified to determine the actual naphthalene concentration.

### 3.3 The quantitative response of ADPWH\_Nah to naphthalene

**Figure 6** further illustrated the quantitative response of ADPWH\_Nah to different concentrations of naphthalene, sodium salicylate and sodium benzoate. From the average bioluminescence response ratio between 200 and 240 min (stable response period), the mathematical model successfully predicted the behaviour of ADPWH\_Nah and the calculation fitted well with the experimental data. The linear relationship between bioluminescent response and naphthalene was found when naphthalene concentration ranged from 1  $\mu\text{M}$  to 50  $\mu\text{M}$ . At higher concentration, the bioluminescent intensity of ADPWH\_Nah for naphthalene became saturated, whereas the response ratio for salicylate kept increasing. It was explained by the limited rates of NahAD enzymes to metabolize naphthalene into salicylate at high concentration.

Compared to the response of ADPWH\_lux to various PAHs chemicals (Zhang et al., 2012b), the inductive ADPWH\_Nah had similar gene expression rate and specific inducer binding rate, as listed in **Table 3**. The gene expression rate of ADPWH\_Nah to naphthalene was  $130.4 \text{ s}^{-1} \cdot \text{cell}^{-1}$ , similar to that of ADPWH\_lux to salicylate ( $124.2 \text{ s}^{-1} \cdot \text{cell}^{-1}$ ), indicating the same promoter activation of *salAR* operon and *luxCDABE* gene. The specific inducer binding rate  $K_I$  of ADPWH\_Nah was 4310, significantly lower than 23,255 (the binding rate of salicylate to *salR* regulon in ADPWH\_lux). The  $\alpha_m$  of ADPWH\_Nah's response to naphthalene was  $53.2 \text{ s}^{-1} \cdot \text{cell}^{-1}$ , much lower than that of ADPWH\_Nah's response to salicylate, whereas its naphthalene  $K_I$  (75,301) was much higher than the value in salicylate induction (5,181). The results suggested the insufficient metabolization rate from naphthalene to salicylate ( $m_{N-S}$ , 0.069 from calculation), consequently resulting in the low available salicylate to promote the *salAR* operon. ADPWH\_Nah and ADPWH\_lux had similar gene expression rate (46.4

s<sup>-1</sup>·cell<sup>-1</sup> and 40.4 s<sup>-1</sup>·cell<sup>-1</sup> respectively) and specific inducer binding rate (745 and 632 respectively), further confirming the expression of *salAR* operon in ADPWH\_Nah was stimulated by salicylate, not benzoate, as in ADPWH\_lux (Zhang et al., 2012b). It is the direct evidence for the *nahAD* encoding naphthalene metabolism via salicylate pathway, instead of benzoate, consistent with previous research (Yen and Serdar, 1988; Loh and Yu, 2000).

#### 3.4 The response of ADPWH\_Nah to PAHs contaminated groundwater

The distribution of naphthalene and n-alkane in the real PAHs contaminated groundwater samples, via both bioreporter and GC/MS analysis, showed the reliability and feasibility of whole-cell bioreporter in environmental monitoring and bioavailability assessment. At 4.5 m depth, significant total n-alkane contamination ranged from 210 µg/L to 980,200 µg/L (**Figure 7B**) and the total naphthalene concentration was from 6.0 µg/L to 63.4 µg/L (**Figure 7D**). The highest total naphthalene contamination was found at SW07 (63.0 µg/L) and SW03 (42.3 µg/L). As for bioavailable naphthalene (**Figure 7C**), SW03 had the highest contamination (64.0 µg/L) and there was extremely low level of naphthalene bioavailability in SW07 (3.0 µg/L). Since SW03 was the latest contaminated point where the tap water contamination was found, it is proposed that the majority of PAHs were bioavailable after recent contamination. For SW07 with long contamination history, the bioavailable fraction of PAHs or alkane had been metabolized by indigenous microbes and there was low naphthalene bioavailability. SW03 also had the highest bioavailable and total n-alkane content (**Figure 7A**, 515,338 µg/L and 980,200 µg/L respectively). At 8.0 m depth, similar spatial distribution of naphthalene and n-alkane contaminants was identified (**Figure 7**). Alkane was detectable in all the sampling points (198 µg/L at DW09 to 306,310 µg/L at DW08) and DW08 had the highest bioavailable n-alkane contamination (69,209 µg/L). Accordingly, the highest bioavailable and total naphthalene (33.0 µg/L and 66.5 µg/L respectively) was observed at DW08 point, indicating the vertical transportation of PAHs contaminants from surface soil (SW07) to deeper soil (DW07) after long term chemical spills.

Positive relationship (Pearson coefficient was 0.548 and *p*-value<0.01) was found between the bioavailable naphthalene (bioreporter data) and total naphthalene (GC/MS data). Bioavailable and total n-alkane concentrations were also highly correlated (Pearson coefficient was 0.805 and *p*-value<0.01). The results suggested

that biological monitoring via whole-cell bioreporter (ADPWH\_Nah and ADPWH\_alk) was comparable to chemical analysis. The positive relationship between bioavailable n-alkane and naphthalene (Pearson coefficient was 0.853 and  $p$ -value<0.01) further suggested the same contamination sources for the groundwater at different depths, as the historical chemical spills in *China Petroleum Lanzhou Petrochemical Company*. More interestingly, the bioavailability-to-total ratio gave more information on the chemical spill history of the contaminated sites. From Sticher's work of bioreporter assessment on the bioavailable alkane in groundwater (Sticher et al., 1997), numerous contaminant compounds were present at the contaminated sites and bioreporter only monitored the inducible fraction of the total targeting molecules. The high catabolic gene expression, responsible to bioremediation capacities, were linked to the inducible substrates instead of their total amounts (Wilson et al., 1999). In this study, the recent contamination had high bioavailable and inducible fraction of the organic pollutants. Bioremediation was therefore a suitable approach for contamination mitigation. Oppositely, the PAHs or alkane at the sites with long contamination history had low bioavailability, and chemical/physical remediation approaches were then suggested due to less bioremediation potential. Given the rapid detection time (less than 5 hours) and low sample volume requirement (180  $\mu$ L), whole-cell bioreporter was a supplementary tool to environmental assessment for decision making and site management (Zhang et al., 2013).

### 3.5 Multi whole-cell bioreporter array for high throughput PAHs assessment

Some multi whole-cell bioreporter arrays have been applied for hydrocarbons (Tecon et al., 2010), and there is still no such high throughput PAHs bioreporter array. From the conventional approach of bioreporter construction, the laborious and unique cloning work for the specific PAHs molecule is not suitable for the routine approaches. The performance of different bioreporter strains is also affected by respective environmental factors, as carbon source or temperature, and the whole-cell bioreporter array therefore suffers from the variations for calibration and standardization. With the salicylate bioreporter ADPWH\_lux, the clone of ring-hydroxylating dioxygenase (RHD) genes (Cebron et al., 2008) with stronger expressed promoter helps the assembly of multi whole-cell bioreporter array for various PAHs molecules. The *phnAaBAcAdDHGCF* operon from *Acidovorax* NA3 (Singleton et al., 2009) is

capable of metabolizing phenanthrene via the salicylate pathway (Pinyakong et al., 2000). Other *nidDBAC* in *Mycobacterium vanbaalenni* PYR-1 (Kim et al., 2006) is also responsible for pyrene and mineralization respectively, viewed as the candidate for the corresponding circuit component for whole-cell bioreporter. Via mining the new functional genes (Wang et al., 2010), more metabolic building blocks can be characterized for more specific whole-cell bioreporters construction, based on the salicylate metabolite pathway and response, and high throughput PAHs detection.

#### **4. Conclusions**

This study developed a new whole-cell bioreporter, ADPWH\_Nah, for the rapid detection of bioavailable naphthalene in environmental samples. The bioreporter has high specificity to sense naphthalene and successfully evaluates the spatial distribution of bioavailable naphthalene in groundwater after *Lanzhou Tap Water Crisis*. The findings also suggest a new concept for multiple PAHs whole-cell bioreporter construction, metabolizing PAHs by introducing functional operon into sensible salicylate, to assemble bioreporter array for rapid and high throughput environmental monitoring.

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## 6. Table

**Table 1** Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Reference
<b>Bacteria</b>		
<i>Escherichia coli</i> DH5α	High efficient competent cells.	Tiagen, China
<i>Pseudomonas putida</i> NCIB9816	Naphthalene degrader with NahAD operon (9 kb) for naphthalene metabolism.	(Cane and Williams, 1982)
<i>Escherichia coli</i> DH5α_pWH1274_Nah	<i>Escherichia</i> cells with pWH1274_Nah vector.	This study
ADPWH_lux	<i>Acinetobacter</i> bioreporter responsive to salicylate. A promoterless <i>luxCDABE</i> from pSB417 was inserted between <i>sala</i> and <i>salR</i> genes in the chromosome of ADP1.	(Huang et al., 2005)
ADPWH_Nah	<i>Acinetobacter</i> bioreporter responsive to naphthalene. The pWH1274_Nah vector exited in ADPWH_lux.	This study
ADPWH_1274	<i>Acinetobacter</i> bioreporter as the negative control of ADPWH_Nah. The pWH1274 vector exited in ADPWH_lux.	This study
<b>Plasmids</b>		
pDTG1	Plasmid with the promoterless NahAD operon (9 kb) from <i>Pseudomonas putida</i> NCIB9816.	(Dennis and Zylstra, 2004)
pWH1274	<i>Escherichia coli</i> and <i>Acinetobacter baylyi</i> shuttle plasmid (6 kb), containing P <sub>tet</sub> constitutive promoter and <i>EcoRV</i> restriction site for cloning. Ampicillin is used as antibiotic selection.	(Hunger et al., 1990)
pWH1274_Nah	NahAD operon cloned into the <i>EcoRV</i> site of pWH1274 vector.	This study

542 **Table 2.** Primers used in this study.

Primers	Sequence (5'→3')
NahA_for	AGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGCG GGATATTGACATATAACGTCGTATTACG
NahD_rev	GCACGCCATAGTGACTGGCGATGCTGTCGGAATGGA CGATACGATCAGGTCAACCACTTATATC
1274_for	ATCGTCCATTCCGACAGCATCGCC
1274_rev	ATCCCGCAAGAGGCCCGGCAGTAC

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**Table 3.** The model parameters of ADPWH\_lux and ADPWH\_Nah response to various PAHs.

Inducer	Bioreporter strains			
	Gene expression rate ( $s^{-1} \cdot cell^{-1}$ )		Specific inducer binding rate $K_I$	
	ADPWH_lux	ADPWH_Nah	ADPWH_lux	ADPWH_Nah
Salicylate	124.2	130.4	23255	5181
Naphthalene	-	53.2	-	75301
Benzoate	46.4	40.4	745	632

## 7. Figure

**Figure 1.** Schematics of ADPWH\_Nah construction and response mechanisms. (A) PCR amplification of pDTG1 (9 kb) for naphthalene degrading operon NahAD and pWH1274 (6 kb) plasmid for transformation vector. (B) Gibson isothermal assembly for pWH1274\_NaAD vector. (C) Naphthalene metabolism and the induction of *salAR* operon by central metabolite salicylate.

**Figure 2.** Contaminated sites and sampling points. Lanzhou city was located in northwest China (A) and the contaminated site was in the west city area and close to the Yellow River (B). Ten groundwater samples were collected 4.5 m depth (C) and six groundwater samples were collected at 8.0 m depth (D).

**Figure 3.** Dynamic bioluminescence response of ADPWH\_Nah and ADPWH\_1274 to naphthalene inducer. The relative bioluminescence response to negative control (DMSO-MMS) ranged from 50 RLU to 200 RLU for ADPWH\_Nah, ADPWH\_1274 and ADPWH\_lux. In the presence of 50  $\mu$ M naphthalene in DMSO-MMS, the highest bioluminescent signal of ADPWH\_Nah was  $3077 \pm 62$  RLU, significantly higher than that of ADPWH\_1274 ( $119 \pm 3$  RLU) and ADPWH\_lux ( $131 \pm 19$  RLU).

**Figure 4.** Quantitative response of ADPWH\_Nah to series of naphthalene concentrations. Kinetic responsive curve of ADPWH\_Nah in the presence of 0  $\mu$ M, 1  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, 50  $\mu$ M and 100  $\mu$ M naphthalene.

**Figure 5.** The response of ADPWH\_Nah (A), ADPWH\_1274 (B) and ADPWH\_lux (C) to various PAHs chemicals. The three whole cell bioreporters had the same responsive pattern to sodium salicylate and sodium benzoate, illustrating their similar stimulation mechanisms of *salAR* operon activation by salicylate or benzoate. ADPWH\_Nah showed the unique positive response to naphthalene, but no response in the presence of pyrene, toluene, anthracene and phenanthrene.

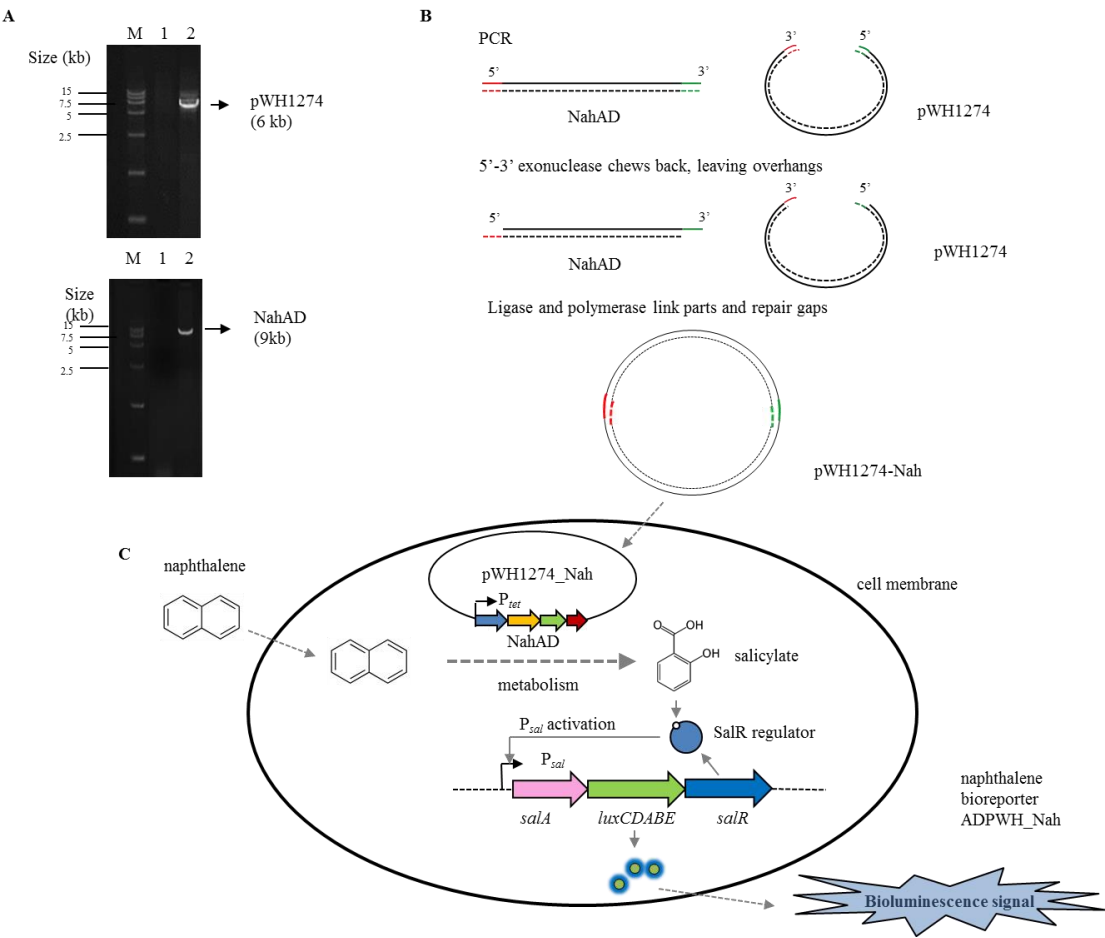
**Figure 6.** The model simulation of ADPWH\_Nah's response to different concentrations of naphthalene, sodium salicylate and sodium benzoate. The experimental data were the average bioluminescence response ratio of ADPWH\_Nah between 200 and 240 min induction.

**Figure 7.** N-alkane and naphthalene contamination in groundwater. The bioavailable n-alkane (A) and naphthalene (C) were analyzed by whole-cell bioreporter

ADPWH\_alk and ADPWH\_Nah respectively, and calculated from the model simulation. The total n-alkane (B) and naphthalene (D) were obtained by GC/MS. The contaminants distribution was plotted by Surfer 8.0 (Golden Software). Positive relationship was found between bioavailable and total n-alkane (Pearson coefficient is 0.805 and  $p$ -value<0.05), and bioavailable and total naphthalene (Pearson coefficient is 0.548 and  $p$ -value<0.05).



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Figure 1

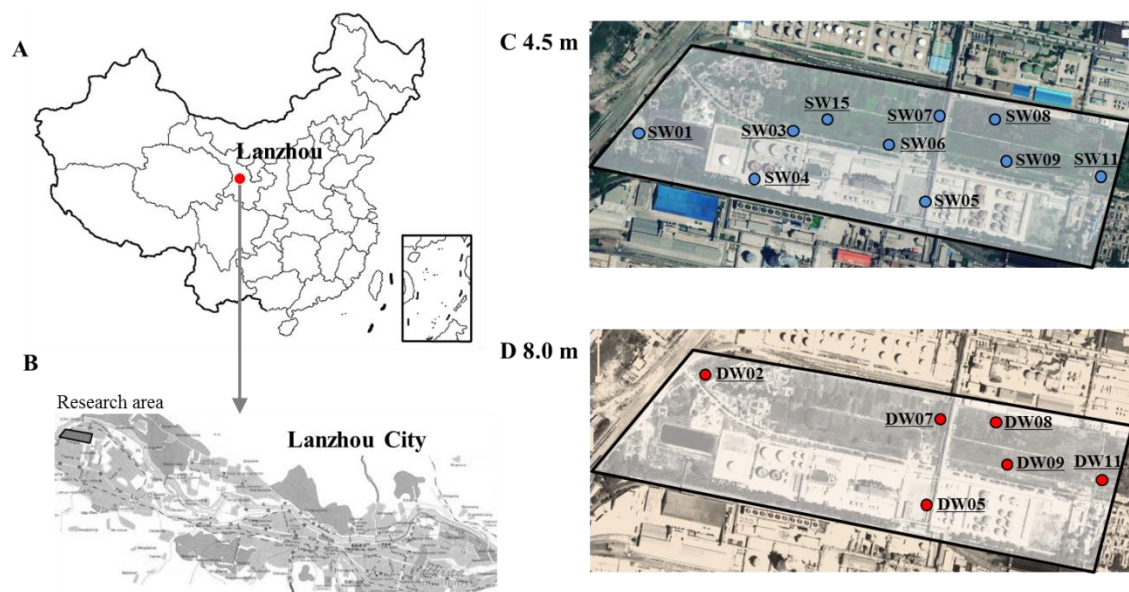


Figure 2

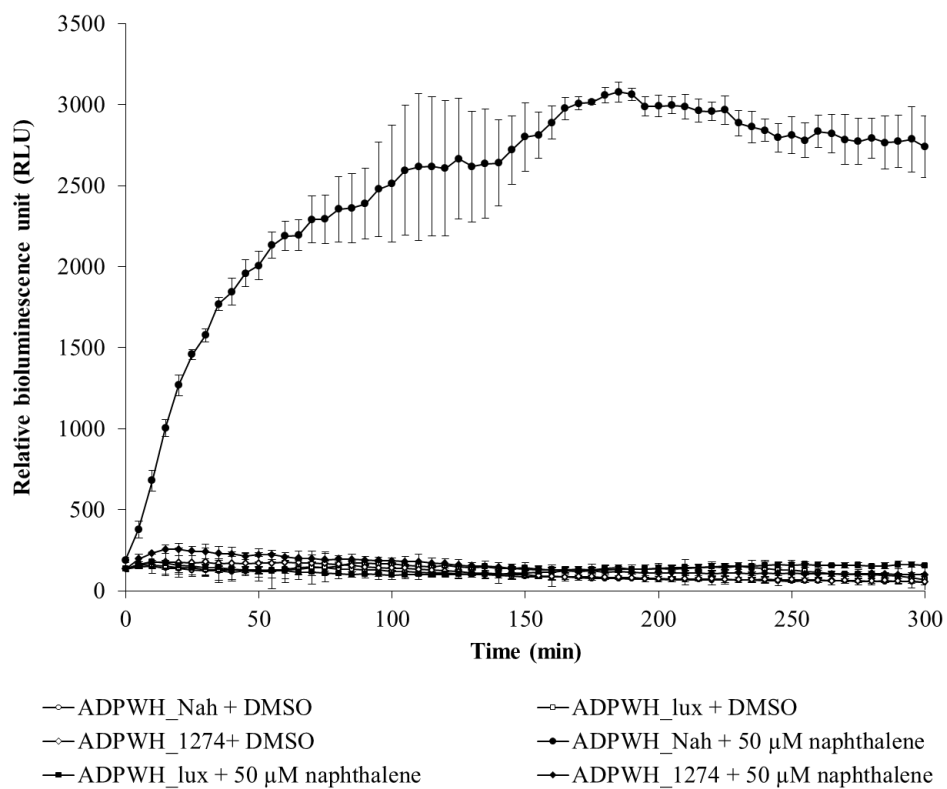


Figure 3

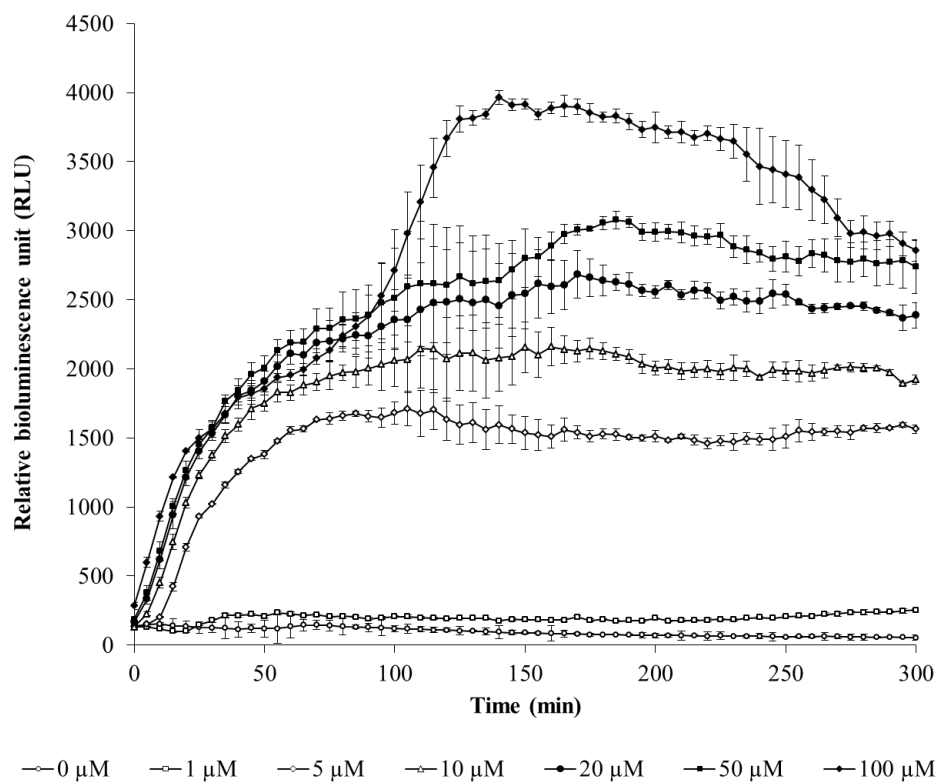
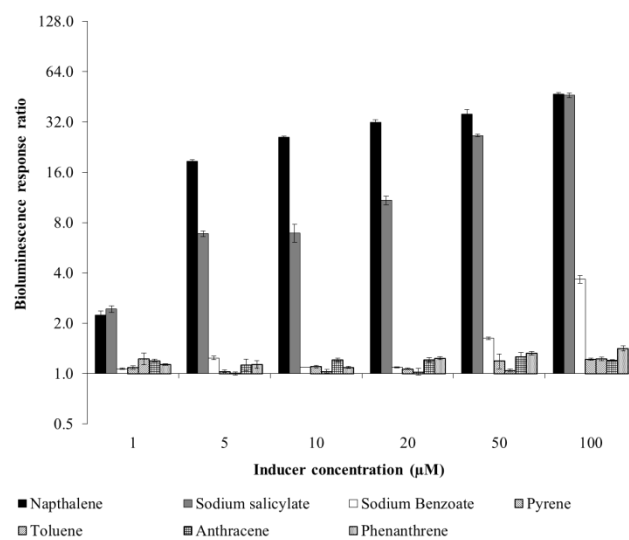
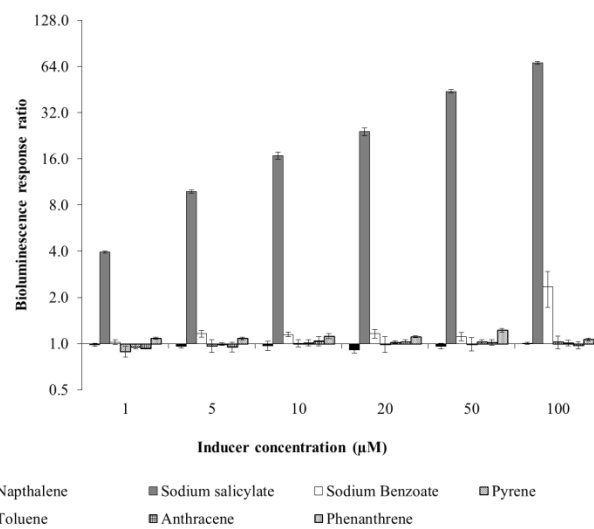


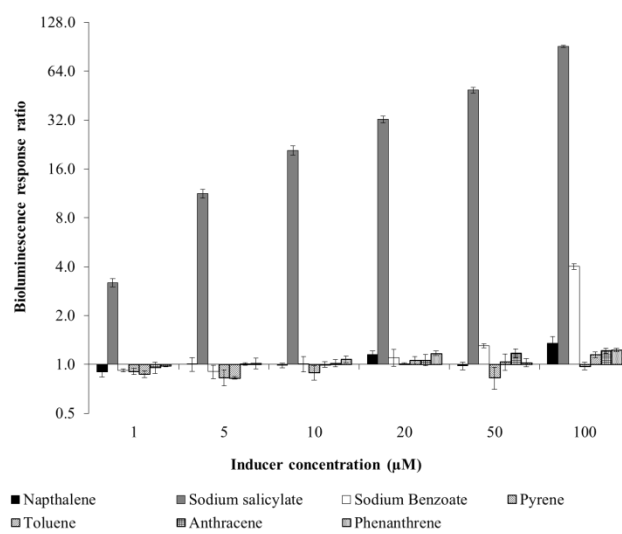
Figure 4



(A)



(B)



(C)

Figure 5

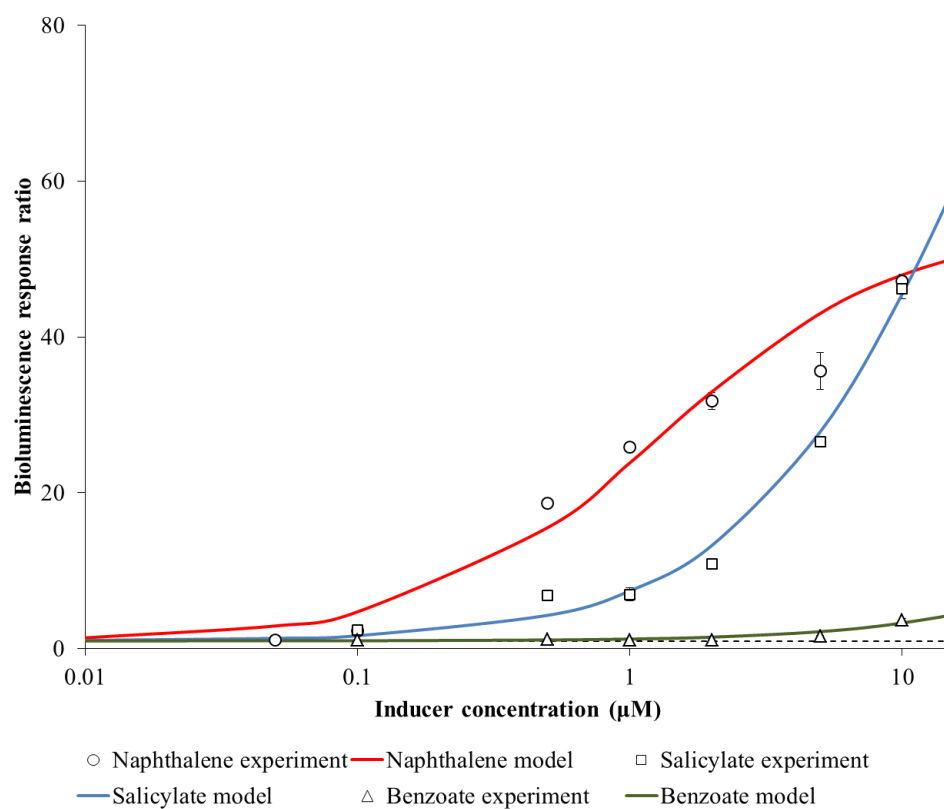


Figure 6

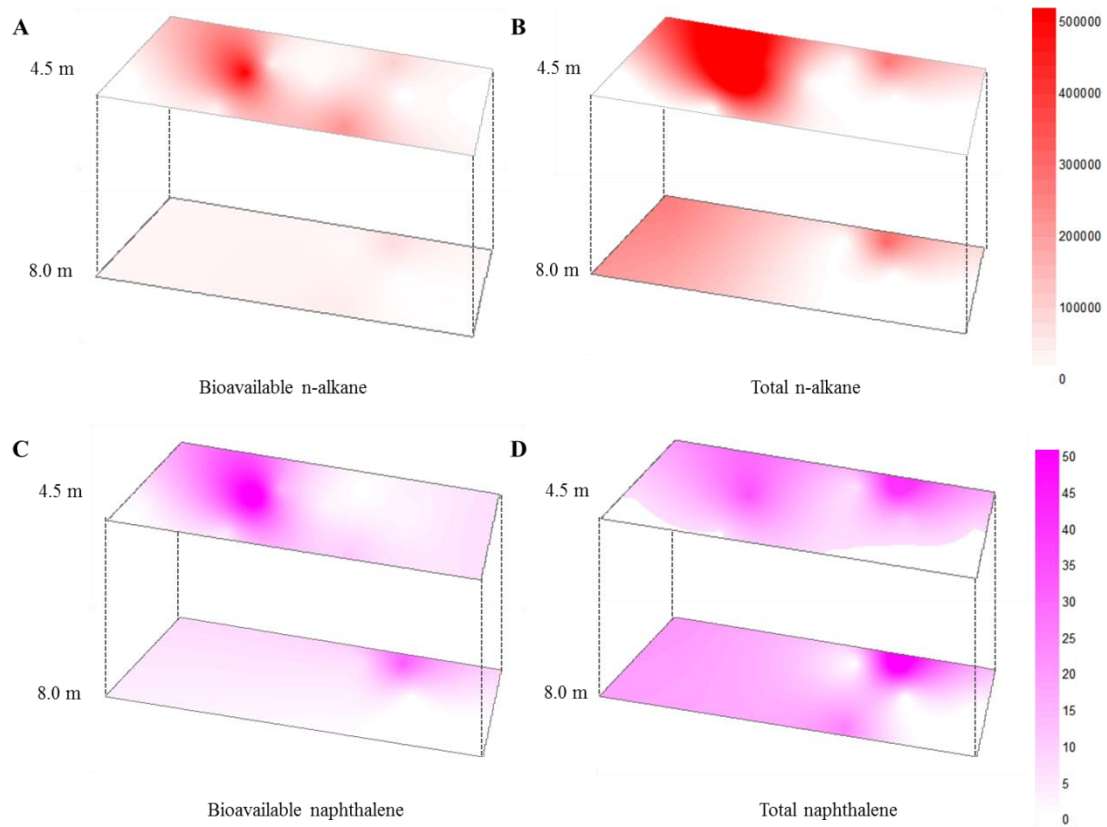


Figure 7