Evaluating the simulated toxicities of metal mixtures and hydrocarbons using the alkane degrading bioreporter *Acinetobacter baylyi* ADPWH_recA

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Abstract

Oil spillages lead to the formation of hydrocarbon and metal mixtures possessing effects on alkane-degrading bacteria that are responsible for the bioremediation of oil-contaminated soils and waters. Studies of bacterial responses to the mixture of petroleum and metal can inform appropriate strategies for bioremediation. We employed a luminescent bioreporter *Acinetobacter baylyi* ADPWH_recA with alkane degradation capability to evaluate the combined effects from heavy metals (Cd, Pb and Cu) and alkanes (dodecane, tetradecane, hexadecane and octadecane). Bioluminescent ratios of ADPWH_recA in single Cd or Pb treatments ranged from 0.25 to 1.98, indicating both genotoxicity and cytotoxicity of these two metals, while ratios <1.0 postexposure to Cu showed its cytotoxic impacts on ADPWH_recA bioreporter. Metal mixtures exhibited enhanced antagonistic effects (Ti > 4.0) determined by the Toxic Unit model. With 100 mg/L alkane, the morbidity of ADPWH-recA reduced to <20%, showing the inhibition of alkanes on Cd toxicity. Exposed to the metal mixture containing 10 mg/L Cu, the weak binding affinity of Cu with alkanes contributed to a high morbidity of > 85% in ADPWH_recA cells. This study provides a new way to understand the toxicity of mixture contaminants, which can help to optimize treatment efficiencies of bacterial remediation for oil contamination.

Keywords

Biosensor, Bioavailability, Mixture toxicity, Toxic unit model.

1. Introduction

The heavy dependence on petroleum products as major sources of energy results in serious oil spillages throughout the globe. Petroleum hydrocarbons pose serious risks to natural environments if left untreated (Liu et al., 2017; Oriomah et al., 2014). Oil contaminated sites contain a large mixture of short and long-chain aliphatic hydrocarbons like linear alkanes (n-alkanes), and a minor group of aromatic compounds like benzene (Khudur et al., 2018). Contaminants emitted from crude oil spillages can therefore affect natural systems collectively. Petroleum polluted sites are often co-contaminated by hydrocarbons and heavy metals (Biswas et al., 2015; Liu et al., 2017; Oriomah et al., 2014), typically including Cd, Pb, Cu, V and Cr (Pavlidou et al., 2010). The content of heavy metals often determines oil toxicity as they initiate the interaction with biomolecules to induce toxic impacts (Thomas et al., 2021). Heavy metals from oil contamination can inhibit the growth and survival of key microorganisms that are responsible for hydrocarbon degradation (Agnello et al., 2016), and hydrocarbons affect the mobility and transportation of heavy metals (Visca et al., 2007). The metabolism of hydrocarbons in oil leads to the release of radicals and reactive oxygen species that can damage the cellular structure of micro- and macro-organisms, thereby affecting ecological systems (Patrick-Iw et al., 2011; Ebokaiwe et al., 2013). The co-existence of heavy metal and alkane in soils can turn productive fields into wastelands (Onyejekwe et al., 2019), in waters it results in oxygen depletion, destroying biochemical interactions (Besser et al., 2009) and it increases the input of heavy metals in sediments (Zhang et al., 2020). Furthermore, the long-term exposure to crude oil can

also result in hepatocellular disruption and nephrotoxicity in human body, which threatens human health (Deng et al., 2018). This serious environmental and regulatory issue from the mixture of metals and hydrocarbons has attracted concerns from researchers (Muniz et al., 2004; Oriomah et al., 2014; Pavlidou et al., 2010; Yoo et al., 2017).

Heavy metals including Cd, Pb and Cu are often found in crude oil contaminated environments, and they are toxic to most bacterial species. Hence, we selected these three metals to combine with alkanes for toxicity evaluations in this study. The effects of Cd, Pb and Cu on microbial activities are well documented. Cd inhibits or destroys microbial enzymatic activities such as ATP production, carbon mineralization and enzymatic functions (Vig et al., 2003). The general toxicity of Pb is less than Cd, but it can alter community diversity and damage ecological functioning (Montuelle et al., 1994). Pb ions result in severe neurotoxicity in humans via food chains and webs (Tong et al., 2000; Yuan et al., 2015). Cu is an essential element for living organisms at trace levels as it provides a redox-facile ligand for metabolic enzymes (Dupont et al., 2011). However, increasing concentrations of Cu generate reactive oxygen that causes the peroxidation of lipids, oxidation of proteins, and breakdown of DNA (Busenlehner et al., 2003; Dupont et al., 2010). Due to its high affinity for thiolates, Cu can destabilize iron-sulfur clusters (Macomber and Imlay, 2009). At hydrocarbon contaminated sites, alkanes have high bioaccessibility and bioavailability, which could assist metal ions to transport inside the bacterial cell. Alkanes ranging from decane to pentacosane are main components of crude oil (Li et al., 2020), and numerous indigenous bacterial species

are found to be able to detect and utilize alkanes (Jiang et al., 2021). Alkanes and heavy metals in crude oil can form complex mixtures to affect bacterial activities that are responsible for ecological functions and contaminants removal. Previous studies show that the presence of heavy metals can significantly alter the uptake behaviours of cells towards several hydrocarbons (Tao et al., 2013; Zhang et al., 2013; Zhang et al., 2018), but few information discussing the effects of alkane-metal mixture on bacteria. We believe that in alkane-metal complexes, four alkanes can affect the toxicity of heavy metals by immobilizing or reactivating the metal ions of Cd, Pb or Cu, and in turn the three different metal ions can damage the cellular structure during the biodegradation of this complex. Therefore, we used 3 metals (Cd, Pb and Cu) and 4 alkanes (dodecane, tetradecane, hexadecane and octadecane) that generally found in crude oil to be representative and to investigate the mixture effects of heavy metals on alkane bioavailability, and the influence of alkanes on metal ion mobility.

Several biological assays have been used to evaluate toxic effects on bacteria, for example, growth rate, biomass and specific enzymatic activities (Macaskie and Dean, 1984; Pritchard and Bourquin, 1985; Bitton et al., 1992). These methods rely on the number or dry weight of cells, but not the direct response from living cells. Therefore, they can only provide the cellular mortality of environmental contaminants. However, before the cellular apoptosis, toxic effects on bacteria have already occurred. Different from those assays, the biological assay used in this study is the whole-cell bioluminescent bioreporter. This method is based on the living cells that contain a genetic fusion of luminescent genes and DNA SOS genes to respond to the toxic effects

of single or mixture contaminants, and distinguish cyto- or geno-toxicity. Our bioreporter is genetically-engineered from a soil bacterium, so it can provide stable real-time luminescent signals, which is highly valuable at practical contaminated sites. Whole-cell bioreporters can detect the toxicity of contaminants from complex environments; living cells applied in these assays can also show how environmental contaminants affect cellular activities. Therefore, the alkane-degrading and toxicity-responsive bioreporter strain, *Acinetobacter baylyi* ADPWH_recA (ADPWH_recA), was used as an indicator to evaluate the ecotoxicology of alkane-metal mixtures. ADPWH_recA is genetically engineered from a host strain of *A. baylyi* ADP1 with high alkane affinity (Li et al., 2017; Song et al., 2009). This bacterial strain is a universal soil bacterium with the ability to degrade hydrocarbons in crude oil, making it specific and sensitive to alkanes and alkane-metal mixtures. Its response to metals and alkanes directly reflects the toxic effects of crude oil contaminants. The strong capability of horizontal gene transfer makes *A. baylyi* ADP1 a robust host for genetically engineered strains. The reporter gene, *luxCDABE* gene cloned from *Photorhabdus luminescens*, is chromosomally fused with the *recA* gene that is responsible for DNA SOS in *A. baylyi* ADP1, to express biological luminescent signals. Although several toxicity bioreporters such as *Escherichia coli*, *Salmonella sp*., and *Vibrio fischeri* have been used (Ptitsyn et al., 1997; Norman et al., 2005; Rusling et al., 2007), ADPWH_recA is an ideal model strain for the qualitative and quantitative detection of both genotoxicity and cytotoxicity of many toxic compounds.

We have applied this biological assay in our study to evaluate the ecotoxicology of contaminant mixtures. The bioluminescent signals from single and multiple contaminant exposure are used to uncover the main driver of mixture eco-toxicities. From the analysis of genotoxic and cytotoxic data, the underlying effects of toxicants inside bacterial cells are characterized, and mutual interactions of metal ions mixed with alkanes in bacterial populations are analyzed using the Toxic Unit model. This modelling approach derived from Concentration Addition model allows quantitative measurements of each toxicant at different concentrations and different influence levels in a mixture (Belden et al., 2010). The Toxic Unit and Combination Index (CI)–isobologram model are both useful to interpret chemical interactions in the mixture (Chen et al., 2014). The CI-isobologram model is mainly used to determine the mutual interactions of organic compounds, and the Toxic Unit model is used for heavy metals. Furthermore, because *A. baylyi* ADPWH_recA is a universal hydrocarbon degrader, this bioassay was used to investigate the kinetic effects of various alkane molecules on heavy metal bioavailability. Unlike using biomass or growth rate as in previous studies, our study used an engineered luminescent bioreporter to distinguish cyto-/geno-toxicity of individual or mixture contaminants (Al-Anizi et al., 2014). The Toxic Unit model employed in this study visualized the mutual interactions among different metal ions and between metal ion and alkane, which could play an important role to optimize the treatment efficiency of bioremediation techniques on oil contaminated sites. In this study, our objectives are to investigate i) the toxicity of single and mixture metals; ii)

toxicity of alkane-metal mixtures; iii) the effects of alkanes on mutual interaction modes of different heavy metals.

2. Materials and methods

2.1 Bacteria strains and incubation

Acinetobacter ADPWH_recA was used as the bioreporter in this study to evaluate the toxic effects of heavy metals and hydrocarbons. Potentially genotoxic contaminants induce damage to the DNA of bacteria, and then trigger the *recA* gene to upregulate the expression of bioluminescence luxCDABE in ADPWH_recA. *Escherichia coli* JM109 served as the light-off control for the toxicity evaluation. Light off control referred to when no bioluminescence was detected from a bioreporter post-exposure to a toxicant.

For each treatment, ADPWH_recA cells were cultivated in a 50 mL sterilised centrifuge tube containing 20 mL Luria-Bertani (LB) medium supplemented with 10 mg/L kanamycin overnight at 30 ℃ and 150 rpm in a thermostatically incubator in dark. *E. coli* JM109 cells were incubated in LB medium overnight at 37 ℃ and 150 rpm in a thermostatically incubator in dark. The cells of these two strains were harvested by centrifugation at 4000 rpm for 5 min, then washed with deionized water three times and re-suspended in fresh LB medium for further use. The 1.0 litre LB medium contained 10.0 gram of tryptone, 10.0 gram of NaCl and 5.0 gram of yeast extraction was prepared in house (Sezonov et al., 2007).

2.2 Toxic effects of single metal

All the chemicals in this study were analytical grade and purchased from Sigma Aldrich (China). Three heavy metal salts, $CdCl_2$, $PbCl_2$ and $Cu(NO_3)_2$, were dissolved in deionized water to prepare the 1000 mg/L stock solution, respectively, and serially diluted to 0.01-100 mg/L for further experiments with bioluminescence measurements. Luminescent genes in ADPWH_recA were induced by Cd, Pb or Cu at 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 mg/L in LB medium at 30 ℃ and 150 rpm shaking to produce bioluminescent signals. The total volume of 200 μL was pipetted from each solution to the well of a 96-well microplate (white bottom, Nunc, Denmark) for bioluminescence detection. The bioluminescent detection was performed based on the protocol of Song et al. (Song et al., 2009), and details were provided in 2.6 section.

2.3 Kinetic analysis of bioluminescence induced by MMC

The MMC standard analysis was performed according to Song's protocol (Song et al., 2009). Mitomycin C (MMC) was added into ADPWH_recA suspension to achieve a final concentration of 0.01, 0.1, 1 and 10 mg/L. MMC served as the standard chemical for genotoxicity assessment. The corresponding bioluminescence and optical density at 600 nm (OD600) of each sample were measured every 30 min at 30 ℃ for 10 h.

2.4 Effects of single alkane

Dodecane, tetradecane, hexadecane and octadecane were individually added into deionized water supplemented with 1‰ dimethyl sulfoxide (DMSO) to prepare 10 g/L stock solution. After homogenization using 40 kHz ultrasound for 5 min, the alkane

193 stock solution was diluted to the final series concentrations of 0.1, 0.5, 1, 5, 10, 50 and
194 100 mg/L for further use. This method procedure was from Zhang's protocol (Zhang et
195 al., 2012).

196 *2.5 Combined effects of heavy metals and hydrocarbons*

197 The procedure for the measurements of mixture toxicity was based on Zhang et al's
198 protocol with certain modification (Zhang et al., 2012). The treatment for combined
199 effects of heavy metals involved mixing Cd, Pb and Cu in pairs or all together from
200 their corresponding stock solutions to reach the final concentration of 0.1, 1 and 10
201 mg/L. The effects of Cd + Pb, Cd + Cu, Pb + Cu and Cd + Pb + Cu at different
202 concentration mixtures on bacterial cells were measured via the detection of
203 bioluminescence produced from ADPWH_recA.

204 For the single heavy metal and hydrocarbon mixture treatment, Cd, Pb or Cu and C12,
205 C14, C16 or C18 alkanes were mixed to give final concentrations of 0.1, 1 or 10 mg/L
206 for heavy metal and 1, 10 or 100 mg/L for alkane. Each mixture solution contained one
207 heavy metal and one alkane chemical (Table 1).

208 For the dual/triple heavy metals and hydrocarbon mixture treatments, Cd, Pb and Cu
209 were cross-mixed at 0.1, 1 and 10 mg/L in mixtures with a sole alkane. The final
210 concentration of alkane in each mixture was 1, 10 or 100 mg/L. Each mixture solution
211 contained two or three heavy metals and one alkane (Table 1).

212 Table 1. Mixing concentrations for mixture treatments

Combined substrates: Metals: Cd, Pb, Cu Alkanes: dodecane, tetradecane, hexadecane and octadecane	Mixing concentrations: Concentration for metals in *italic*; Concentrations for alkanes in **bold**
Two metals mixture	*0.1* / *1* / *10* + *0.1* / *1* / *10*
Three metals mixture	*0.1* / *1* / *10* + *0.1* / *1* / *10* + *0.1* / *1* / *10*
Single metal and single alkane mixture	*0.1* / *1* / *10* + **1** / **10** / **100**
Two metals and single alkane mixture	*0.1* / *1* / *10* + *0.1* / *1* / *10* + **1** / **10** / **100**
Three metals and single alkane mixture	*0.1* / *1* / *10* + *0.1* / *1* / *10* + *0.1* / *1* / *10* + **1** / **10** / **100**

2.6 Bioluminescence detection

The 180 μL of ADPWH_recA suspensions and 20 μL of pure/mixed heavy metal and/or alkane solutions were transferred into each well of a 96-well microplate (white bottom, Nunc, Denmark) for bioluminescence and optical density measurement. The bioluminescence for each well was measured directly. Three biological replicates and three measurement replicates were performed for each treatment. The 96-well microplate was incubated in the dark at 30 ℃ in a thermostat incubator. After 150 rpm

shaking, optical density at 600 nm (OD600) and the bioluminescence was measured every 30 min using a microplate reader (Infinite 200Pro, TECAN, Grodig, Austria). The bioluminescent response ratio was evaluated by firstly averaging the ratio of relative bioluminescence to OD600 of samples from 240 to 600 min, and then divided by the ratio of controls (non-induced samples). A bioluminescent ratio at 1.0 indicates no toxic effects by certain chemicals, >1.0 shows genotoxicity induced by certain chemicals, <1.0 shows cytotoxicity induced by certain chemicals. Cell inhibition ratio was defined as the difference of bioluminescence of samples and toxicant-free control divided by the bioluminescence of toxicant-free control.

2.7 Analysis of binary and trinary effects of heavy metals

The statistical approach used to evaluate the combined effects of heavy metals on bacterial cells assumes each metal provokes toxicity independently (Marking and Dawson, 1975; Zeb et al., 2016). Hence, the Toxicity Unit (TU) is defined as:

$$TU = \frac{C_M}{EC50_M}$$

where C_M (mg/L) is the total concentration of the metal in the mixture, and $EC50_M$ is the concentration that produces 50% reduction in bioluminescence. For the mixture of heavy metals, the total TU is calculated as:

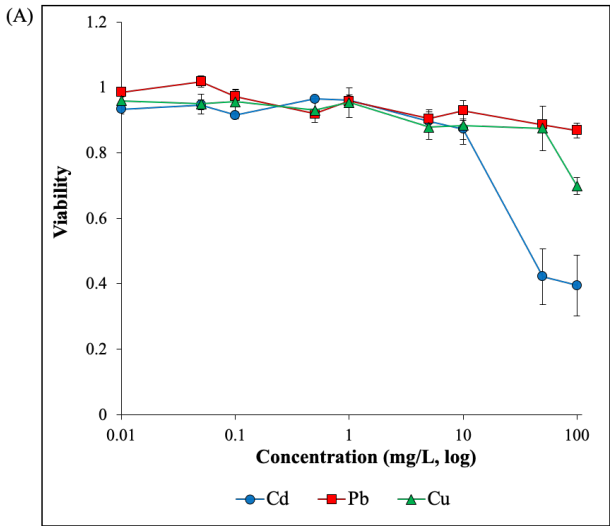
$$TU_{total} = \sum_{i=1}^{n} TU_i$$

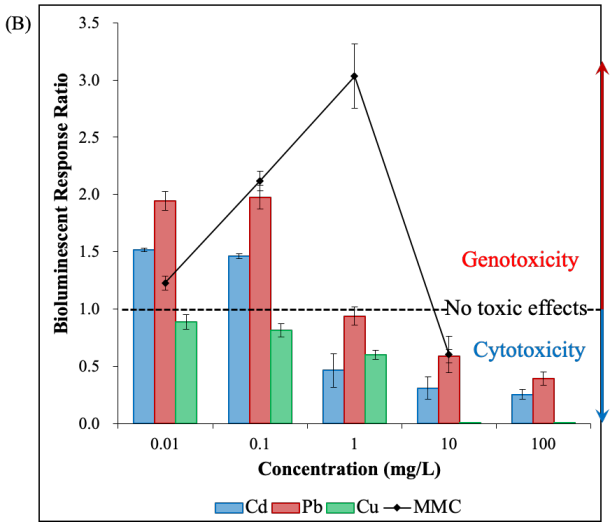
where TU_{total} represents the total toxicity unit, and TU_i is for separate compounds in the mixture. The indicator of joint toxic effect (T_i) is calculated as the TU_{total} value that

produces 0.5 of bioluminescence response ratio. If the toxicity of heavy metals is simply additive, Ti = 1.0; indicators that are <1.0 demonstrate synergistic toxicity, and indicators >1.0 demonstrate antagonistic toxicity (Gopalapillai and Hale., 2017; Belden et al., 2007; Jonker et al., 2010).

3. Results and discussion

3.1 Toxicity of single metal





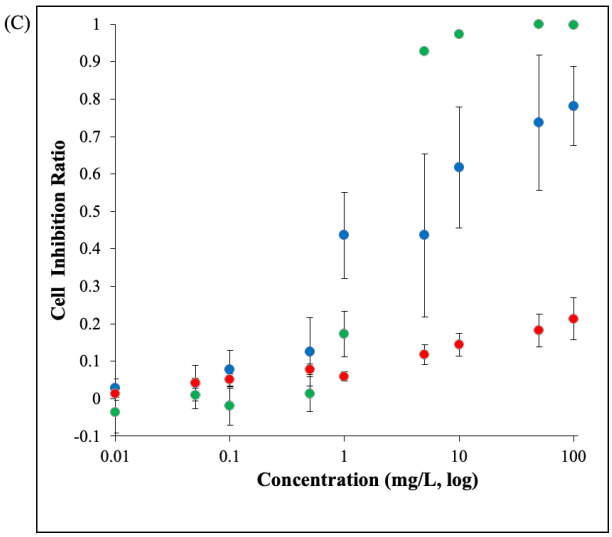


Figure 1: (A) Dose response of *Acinetobacter baylyi* ADPWH_recA exposed to Cd, Pb and Cu at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 mg/L. (B) Luminescent response from ADPWH_recA exposed to Cd, Pb and Cu at concentrations of 0.0, 0.1, 1, 10 and 100 mg/L. Bioluminescent ratio greater than 1.0 represent stronger genotoxicity of metal, and ratio smaller than 1.0 represent stronger cytotoxicity of metal. (C) Inhibitive effects of Cd, Pb and Cu on ADPWH_recA at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 mg/L. Higher ratio refers to greater cellular inhibition of metal.

The OD600 values of *Acinetobacter* ADPWH_recA exposed to Cd, Pb or Cu were shown as the viability in Figure 1A. Viability <0.8 illustrated the inactivation of bacterial growth was caused by 100 mg/L Cu, but its toxicity could be detected by a bioluminescent response ratio even down to 1 mg/L (Figure 1B). From our biological assay, > 90% of the ADPWH_recA population were damaged post-exposure to Cu ions at 5 mg/L or higher, suggesting severe cellular damage caused by this metal in bacterial cells. Consequently, a ~20 mg/L concentration of Cu was enough to affect bacterial

enzyme systems and cellular metabolism in natural waters (Wang et al., 2009; Achard-Joris et al., 2007; Stoyanov et al., 2003). The EC_{50} value of *A. baylyi* in Cu exposure was ~1.74 mg/L (Figure 1C), which is higher than for another luminescent bacteria (*Vibrio fischeri*) (Newman and Mccloskey., 1996; Wang et al., 2009; Utgikar et al., 2004), due to the Cu resistant gene cluster identified in *Acinetobacter* species (Thummeepak et al., 2020; Williams et al., 2016).

The inhibition of Cd on bacterial viability was found at 50 and 100 mg/L, producing 50% mortality (Figure 1A). However, the bioluminescent response ratio >= 1.5 indicated the genotoxic impacts of 0.01 and 0.1 mg/L Cd on ADPWH_recA, and severe cytotoxicity at 1, 10 and 100 mg/L (Figure 1B). Results in Figure 1C showed the increasing concentration of Cd caused more than 50% inhibition rate in bacterial cells due to its strong genotoxicity at low level, which is reported in previous studies (Montuelle et al., 1994; Dupont et al., 2011; Villaescusa et al., 1996). Background concentrations of Cd in the environment are much lower than for Pb and Cu (e.g. ~0.3 mg/L in soils), so its genotoxicity at these concentrations indicates it is a priority for risk assessment (Manzl et al., 2004; Pagano and Zeiger, 2010; Ochi and Ohsawa, 1983). In contrast, Pb was less toxic than Cd and Cu. The Pb ions in bacterial cells bind to the hydroxyl groups of nucleic acids, leading to the impaired functions of DNA and gene expression (Kushwaha et al., 2018). Cytotoxicity of Pb was highly dependent on its bioavailability as available metal ions bind to the functional groups of cell membrane, and Pb at ~ 100 mg/L in natural environments can precipitate at neutral pH, contributing to its slight toxic effects on bacteria (Roane., 1999).

3.2 *Bacterial responses to metal mixtures*

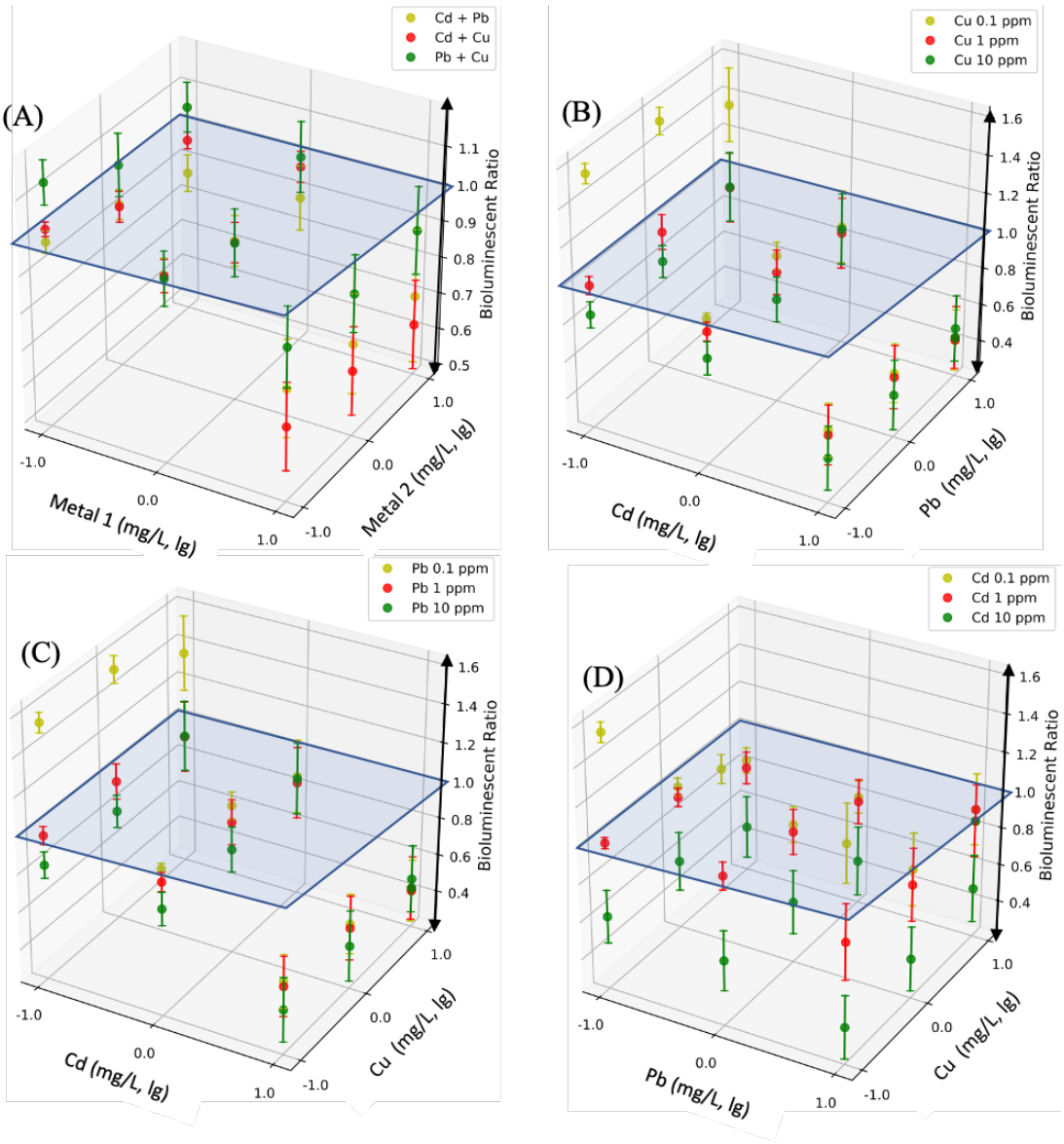


Figure 2: Toxicity of 2-metal mixture (A), and 3-metal mixture (B), (C) and (D) on bioluminescent bacteria *A. baylyi* ADPWH_recA. Two-metal mixtures consist of Cd + Pb, Cd + Cu or Pb + Cu cross-mixed at concentrations of 0.1, 1 or 10 mg/L. Three-metal mixture contains Cd, Cu and Pb at different cross-mixing concentrations of 0.1, 1 or 10 mg/L. Light blue plane at 1.0 bioluminescent ratio represents the interface of genotoxicity and cytotoxicity, from which arrow up for stronger genotoxicity and arrow

295 down for stronger cytotoxicity.

296 In addition to the study of single metal's toxicity, we applied ADPWH_recA to identify
297 the main driver of metal mixture toxicity. In bioluminescent response to all metal
298 mixtures, the toxicity indicator (Ti) ranged from 4.2 to 396 times, all greater than 1.
299 This demonstrates the antagonistic effects of metals in mixtures (Table 1). The Cd + Cu
300 mixture was the most toxic to ADPWH_recA in all 2-metal treatments. However, the
301 lowest bioluminescent ratio > 0.5 indicated less toxic impacts on bacteria compared to
302 single metals (Figure 2A). The inverse relationship of bioluminescent ratio and Cd
303 concentration indicated the dominant toxicity was from Cd rather than Cu in their
304 mixture. The exposure of Cd towards bacterial cells possibly induced the detoxification
305 systems for Cu ions, resulting in the reduced toxicity of Cu at high concentrations in
306 the mixture (Steunou et al., 2020). The presence of Cu ions can also improve the
307 tolerance towards Cd in bacteria, leading to the bioluminescent ratio > 0.5.

308 In the Cd + Pb mixture, the drop in bioluminescent ratio occurred along with the
309 increasing concentration of Cd, not Pb, demonstrating that Cd was the main toxicant
310 influencing bacterial behaviour (Figure 2A). Ti values of 4.2 for this mixture showed
311 the mutual restraint of Cd and Pb; the bioavailability of Cd may be reduced by mixing
312 with Pb (Fulladosa et al., 2005; Mowat and Bundy., 2002). As a consequence, the Cd +
313 Pb mixture did not cause strong geno- or cyto-toxicity in ADPWH_recA, shown by the
314 high bioluminescent ratio ranging from 0.87 to 1.1. This is of importance for
315 understanding the ecological toxicity of heavy metals in natural environments - the
316 interactions among diverse metal ions should be taken into consideration for

environmental risks assessments and remediation.

From bioluminescent responses of ADPWH_recA in the mixture of Cd + Pb + Cu, the Ti value was between that of Cd + Pb and Cd + Cu (Table 2), suggesting that Cu can decrease the toxicity of Cd and Pb when mixed together. In contrast, Pb and Cd increased the toxicity of the Cd + Cu and Pb + Cu mixtures, respectively. A bioluminescent ratio >1.3 could be attributed to the genotoxicity of Pb in bacterial cells with 0.1 mg/L Cd and Cu, but their increasing level suppressed this genotoxic effect (Figure 2B and 2C). At fixed Pb concentration, the slight change in bioluminescent ratio was caused by its weak toxicity in mixtures (Figure 2C). The decreasing bioluminescence with increasing Cd and Cu levels demonstrated their severe cytotoxic impacts on ADPWH_recA in the trinary metal mixture. In this study, our results visualize the possible toxic mechanisms of metal mixtures on ecological systems; metals mixed at low concentrations exhibited genotoxicity, while high-level mixing caused cytotoxicity in ADPWH_recA.

Table 2. Toxic Indicators (Ti) for eco-toxicity of metal mixtures. T_i is the TU_{total} value that is equivalent to 0.5 of the bioluminescence response ratio in metal mixtures.

Values for the Toxic Indicators of combined heavy metals (T_i)			
Cd+Pb	Cd+Cu	Pb+Cu	Cd+Pb+Cu
4.2	21	396	8.4

3.3 Toxic impacts of alkanes and single metal

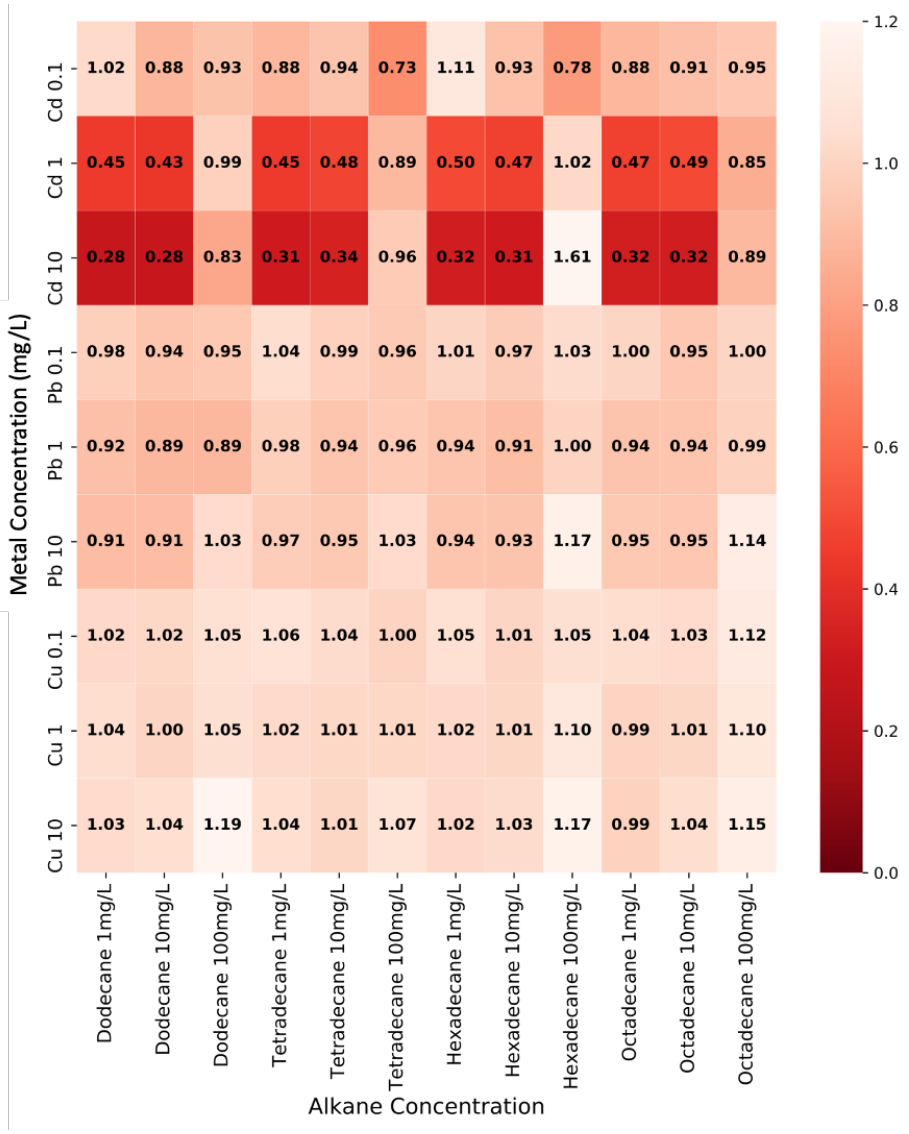


Figure 3. Heat map for the bioluminescent ratio of *A. baylyi* ADPWH_recA exposed to single metal (Cd, Pb or Cu) at 0.1, 1 and 10 mg/L in the treatment with dodecane, tetradecane, hexadecane and octadecane at 1, 10 and 100 mg/L. The intensity was grouped into five quartiles (high intensity illustrates high toxicity).

Alkanes are main components in crude oil for bacteria to deal with. Hence, the responses from bacteria exposed to alkanes are important to evaluate if this

hydrocarbon is toxic. ADPWH_recA cells can utilize linear alkanes with carbon lengths of 12 – 44 to maintain stable growth in the medium (Zhang et al., 2012). Viability > 0.85 showed the positive support from dodecane, tetradecane, hexadecane and octadecane for the growth of ADPWH_recA (ESI Figure 2). In bioluminescent response to alkanes, a ratio in the range 0.85 to 1.1 showed alkane made no geno- or cyto-toxic impacts on ADPWH_recA (ESI Figure 3). This was in agreement with previous studies showing alkanes are available carbon sources for *A. baylyi* to utilize (Ratajczak et al., 1998), and our results confirmed that dodecane, tetradecane, hexadecane and octadecane did not affect the metabolic activities of this bacterial species.

Since alkanes at the concentrations used here did not harm ADPWH_recA, it is necessary to explore their influences on the toxicity of a single or mixture metal(s). Luminescence signals of ADPWH_recA were in the range from 0.89 to 1.04 for Pb, and from 0.99 to 1.06 for Cu, showing the weak toxic effects of these two metals on bacteria with additions of 1 or 10 mg/L alkanes (Figure 3). Compared to the bioluminescent ratio of single Cu exposure in Figure 1B, the increasing ratio in Figure 3 illustrated that the reduced cytotoxicity of Cu was possibly attributed to its weakened bioavailability by binding with alkanes. This suggests that the toxicity of Cu was related to its bioavailability in the presence of alkanes. Metal bioavailability can be reduced in alkane-metal mixtures; alkanes can also stimulate bacteria to secrete extracellular surfactants to immobilize free metal ions, thereby alleviating the toxicity of heavy metals (Agnello et al., 2016).

In treatments of Cd with 1 or 10 mg/L alkanes, ratios <0.5 indicated the strong

364 cytotoxicity of Cd at 1 and 10 mg/L. However, at 100 mg/L alkanes, increases in
365 luminescent ratio (from ~0.3 to 1.0) illustrated the weakened cytotoxic impacts of Cd
366 on ADPWH_recA cells. Compared to the treatments with Cd only, treatments with 100
367 mg/L hexadecane exhibited stronger inhibition on the toxicity of Cd. The sensitive
368 response of ADPWH_recA towards hexadecane possibly strengthened its metabolic
369 activities, including generation of bioluminescent enzymes (Rosenberg et al., 1982;
370 Jung et al., 2015). During the internalization of alkane molecules, small fractions of
371 free Cd ions could be released from degraded alkane-metal complex, to cause the mild
372 genotoxic effects in ADPWH_recA, contributing to the 1.61 bioluminescent ratio in the
373 co-exposure of 10 mg/L Cd and 100 mg/L hexadecane. With decreasing alkane
374 concentrations, the bioluminescence of ADPWH_recA reduced down to 0.28 in
375 exposure to Cd, but remained at relatively stable level (~1.0) when exposed to Pb or
376 Cu. This implies that the toxic effects of heavy metals could become stronger with
377 alkane's decreasing contents at oil contaminated sites.
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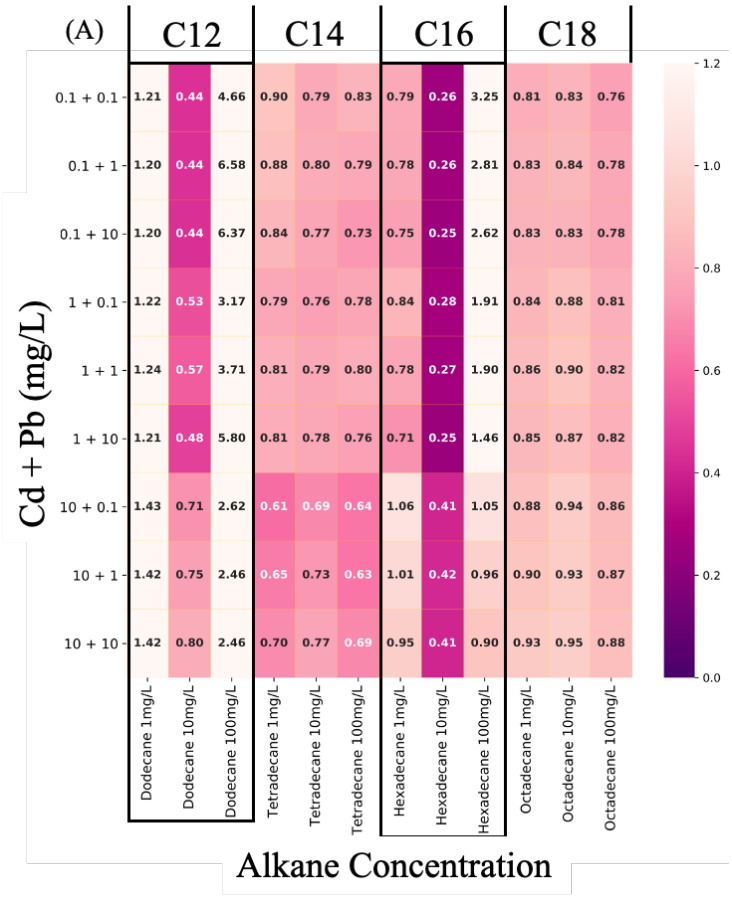
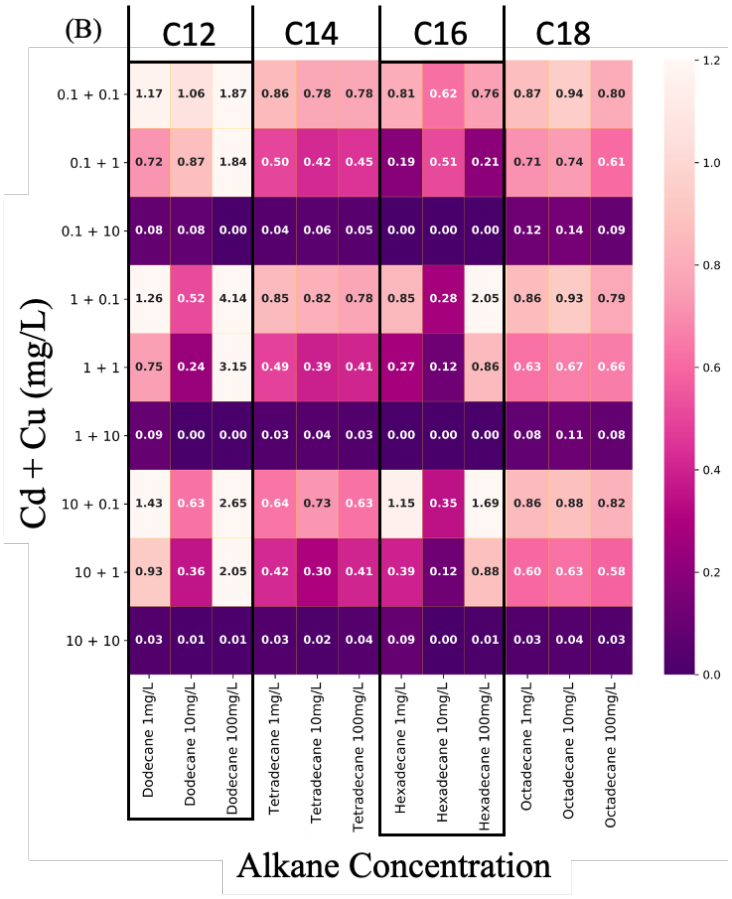
3.4 *Impacts of alkanes and metal mixtures*

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C16
C18
0.1 + 0.1
0.1 + 1
0.1 + 10
1 + 0.1
1 + 1
1 + 10
10 + 0.1
10 + 1
10 + 10
1.17 1.06 1.87 0.86 0.78 0.78 0.81 0.62 0.76 0.87 0.94 0.80
0.72 0.87 1.84 0.50 0.42 0.45 0.19 0.51 0.21 0.71 0.74 0.61
0.08 0.08 0.00 0.04 0.06 0.05 0.00 0.00 0.00 0.12 0.14 0.09
1.26 0.52 4.14 0.85 0.82 0.78 0.85 0.28 2.05 0.86 0.93 0.79
0.75 0.24 3.15 0.49 0.39 0.41 0.27 0.12 0.86 0.63 0.67 0.66
0.09 0.00 0.00 0.03 0.04 0.03 0.00 0.00 0.00 0.08 0.11 0.08
1.43 0.63 2.65 0.64 0.73 0.63 1.15 0.35 1.69 0.86 0.88 0.82
0.93 0.36 2.05 0.42 0.30 0.41 0.39 0.12 0.88 0.60 0.63 0.58
0.03 0.01 0.01 0.03 0.02 0.04 0.09 0.00 0.01 0.03 0.04 0.03
Cd + Cu (mg/L)
Dodecane 1mg/L
Dodecane 10mg/L
Dodecane 100mg/L
Tetradecane 1mg/L
Tetradecane 10mg/L
Tetradecane 100mg/L
Hexadecane 1mg/L
Hexadecane 10mg/L
Hexadecane 100mg/L
Octadecane 1mg/L
Octadecane 10mg/L
Octadecane 100mg/L
Alkane Concentration
1.2
1.0
0.8
0.6
0.4
0.2
0.0

381

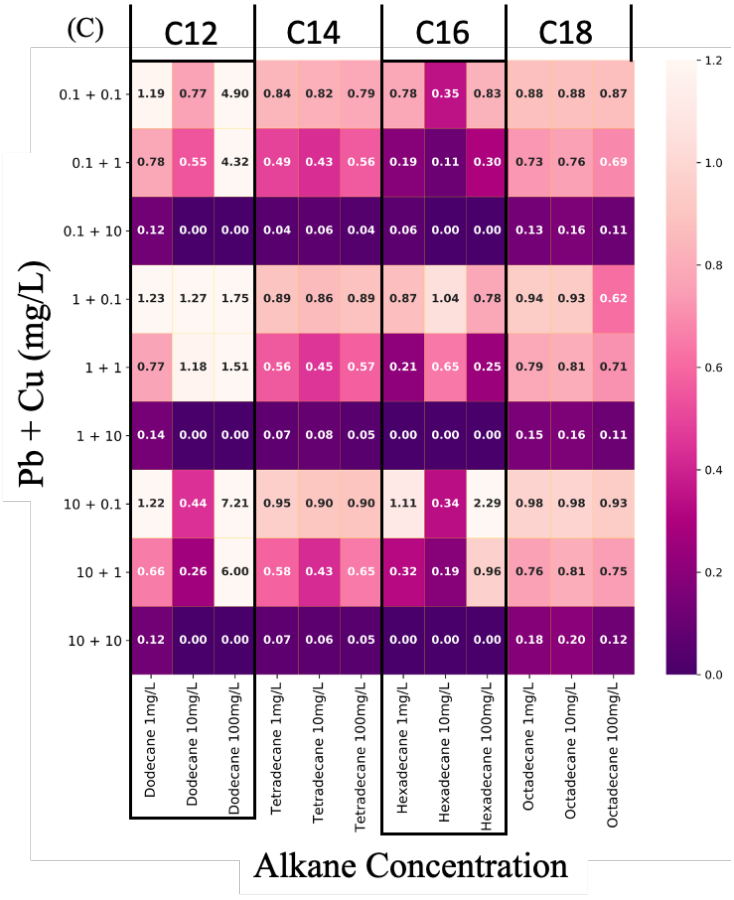
(C)
C12
C14
C16
C18
0.1 + 0.1
0.1 + 1
0.1 + 10
1 + 0.1
1 + 1
1 + 10
10 + 0.1
10 + 1
10 + 10
1.19 0.77 4.90 0.84 0.82 0.79 0.78 0.35 0.83 0.88 0.88 0.87
0.78 0.55 4.32 0.49 0.43 0.56 0.19 0.11 0.30 0.73 0.76 0.69
0.12 0.00 0.00 0.04 0.06 0.04 0.06 0.00 0.00 0.13 0.16 0.11
1.23 1.27 1.75 0.89 0.86 0.89 0.87 1.04 0.78 0.94 0.93 0.62
0.77 1.18 1.51 0.56 0.45 0.57 0.21 0.65 0.25 0.79 0.81 0.71
0.14 0.00 0.00 0.07 0.08 0.05 0.00 0.00 0.00 0.15 0.16 0.11
1.22 0.44 7.21 0.95 0.90 0.90 1.11 0.34 2.29 0.98 0.98 0.93
0.66 0.26 6.00 0.58 0.43 0.65 0.32 0.19 0.96 0.76 0.81 0.75
0.12 0.00 0.00 0.07 0.06 0.05 0.00 0.00 0.00 0.18 0.20 0.12
Pb + Cu (mg/L)
Dodecane 1mg/L
Dodecane 10mg/L
Dodecane 100mg/L
Tetradecane 1mg/L
Tetradecane 10mg/L
Tetradecane 100mg/L
Hexadecane 1mg/L
Hexadecane 10mg/L
Hexadecane 100mg/L
Octadecane 1mg/L
Octadecane 10mg/L
Octadecane 100mg/L
Alkane Concentration
1.2
1.0
0.8
0.6
0.4
0.2
0.0

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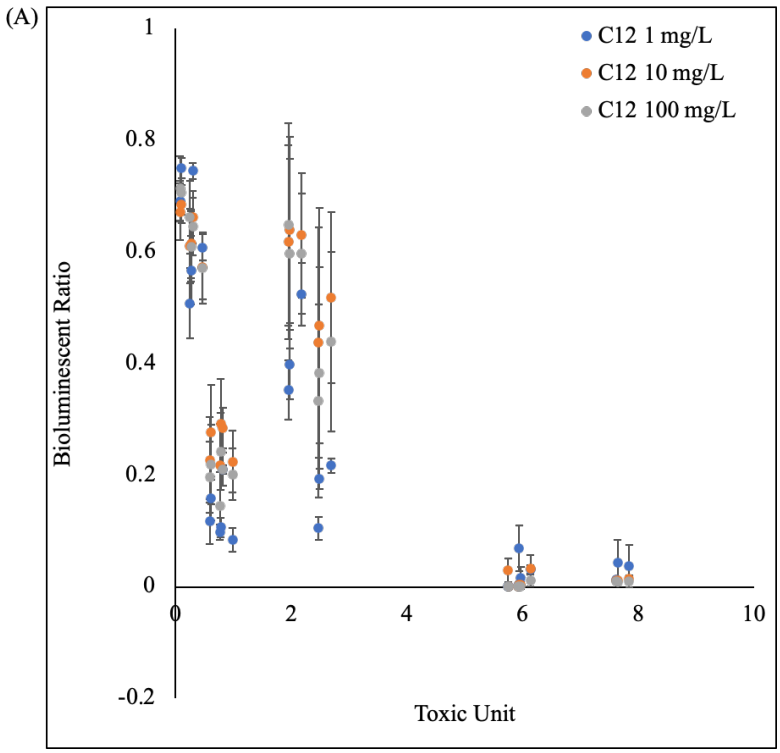
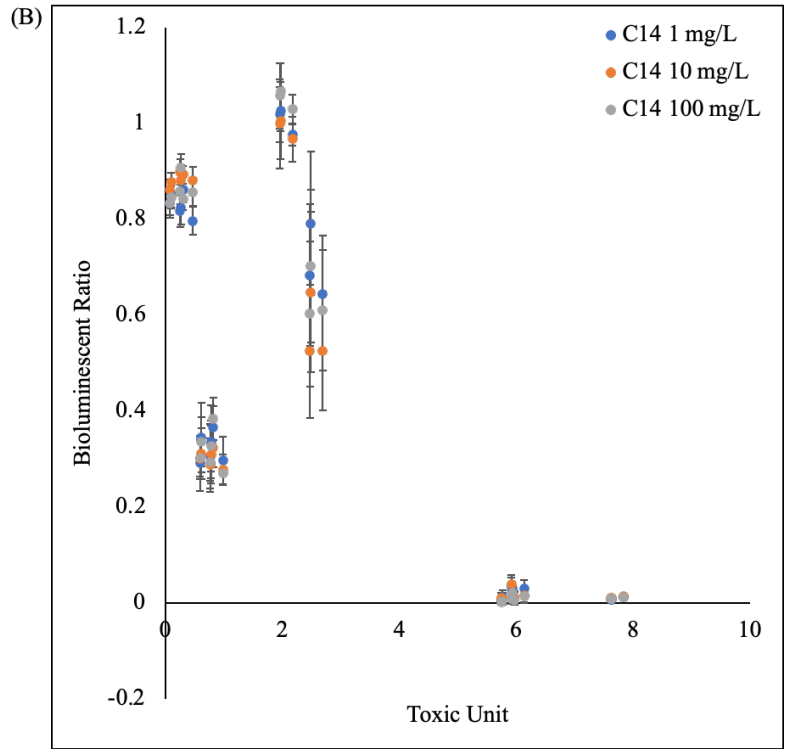
Figure 4. Heat map for bioluminescent responses of *A. baylyi* ADPWH_recA exposed to the two-metal mixture: Cd + Pb (A), Cd + Cu (B), or Pb + Cu (C) at concentrations of 0.1, 1 and 10 mg/L in the mixture with dodecane, tetradecane, hexadecane and octadecane at 1, 10 and 100 mg/L. Intensity > 1.0 represents genotoxicity of the mixture, and intensity < 1.0 represents cytotoxicity of the mixture.

To investigate the response of bacteria towards alkanes with metal mixtures, bioreporter ADPWH_recA was exposed to dodecane, tetradecane, hexadecane and octadecane with 2 or 3 metals. Dodecane enabled ADPWH_recA to produce enhanced bioluminescent signals with exposure to Cd + Pb, indicating the strong genotoxic impacts of this metal mixture on bacterial cells (Table 3 and Figure 4A). The rapid accumulation and utilization of dodecane by ADPWH_recA contributed to the release of Cd and Pb ions from metal-alkane complexes into bacterial cells (Shteinman., 2017). Therefore, metal ions inside bacteria damaged their genetic structure and triggered the expression of luminescent signals. In treatments with tetradecane, the Ti value > 3.49 demonstrated the antagonistic interactions of metal ions, and the luminescent ratio < 1.0 suggested the main damage caused by the metal-alkane complexes was cytotoxicity rather than genotoxicity. With a longer carbon chain, octadecane significantly reduced the toxicity of Cd + Pb, resulting in the luminescent ratio > 0.76 (Figure 4A). As the carbon chain length increased, the breakdown efficiencies of metal-alkane complexes by ADPWH_recA decreased, which consequently reduced the toxicity of metal ions. It is worth noting that hexadecane can lead to the genotoxicity of Cd + Pb in bacterial cells, because its attractiveness to hydrocarbon degrading bacteria contributed to the fast

accumulation of free metal ions in bacterial cells (Li et al., 2019).

In exposure to Cd + Cu or Pb + Cu mixtures, the luminescent ratio showed the toxicity mode of these two mixtures changed from genotoxicity to weakened cytotoxicity as carbon chain lengths increased (Figure 4B and 4C). However, the pattern was different when exposed to the mixture involving 10 mg/L Cu. The low bioluminescent ratio <= 0.2 shown in purple grids illustrated the severe cytotoxicity was from Cu ions (Figure 4B and 4C). Mixed with Cd or Pb, Cu was less competitive to form alkane-metal complexes (Oriomah et al., 2014), so less free Cu ion was immobilized by alkanes, resulting in its high bioavailable concentration to affect the bacterial activities. The Cu in metal-alkane mixtures was cytotoxic to ADPWH_recA, which was similar to the luminescent response post-exposure to single Cu.





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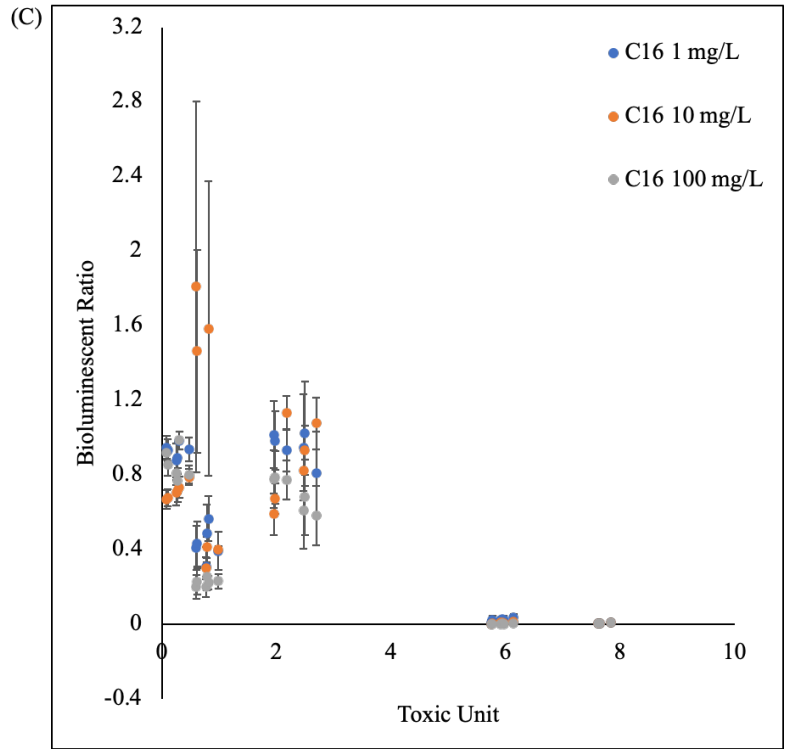

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419 Figure 5. Luminescent responses of *A. baylyi* ADPWH_recA when exposed to the three

420 metal mixture (Cd + Pb + Cu) in treatments with dodecane, C12 (A), tetradecane, C14

421 (B), hexadecane, C16 (C), and octadecane, C18 (D) at 1, 10 and 100 mg/L. Higher

Toxic Unit values on the x axis represent high concentrations of metals in mixtures; the calculation of Toxic Unit is described in section 2.7. Toxic Unit is the sum of concentration ratio, so it is unitless.

Compared to the three-metal mixture only (Figure 2B, 2C and 2D), reduced Ti values indicated the positive interactions of Cd, Pb and Cu ions with additions of alkanes on the toxic effects in ADPWH_recA (Table 3). The low bioluminescent ratio showed the toxicity of the trinary metal mixture did not respond to the concentration of alkanes, but to the carbon chain length (Figure 5). In the dodecane treatment, a bioluminescent ratio < 0.8 showed the cytotoxicity of the metal mixture on ADPWH_recA (Figure 5A). This can be attributed to the synergistic interactions of these three metals co-existence with dodecane, with Ti values < 1.0 (Table 3). In contrast, the metal mixture with tetradecane had Ti values of 2.41± 0.06 Ti, indicating the mutual inhibition of Cd, Pb and Cu in the presence of tetradecane. Bacterial activities were not significantly affected by Cd and Pb ions, but the bioluminescent ratio < 0.5 in Figure 5B showed strong cellular inhibition due to Cu at 1 and 10 mg/L. Increasing Ti values in treatments of hexadecane and octadecane demonstrated greater antagonistic effects in the interaction of Cd, Pb and Cu. Post-exposure to the metal mixture and 1 mg/L octadecane, a bioluminescent ratio of ~14 indicated high genotoxicity on ADPWH_recA, possibly caused by the enhanced mobility of Pb and Cd ions transported into bacterial cells via alkane-metal complexes (Figure 5D).

The Ti values show that the mutual inhibition among metal ions in the trinary metal mixture was enhanced by the longer-chained alkanes. Formation of alkane-metal

complexes reduced the mobility of metal ions, thereby decreasing their potential bioavailability and toxicity in bacterial cells (Biswas et al., 2015). Previous studies have reported that hydrocarbon degrading bacteria accumulate short-chain rather than long chain alkanes (Wang and Shao., 2014). Consequently, the short-chain dodecane were consumed more rapidly than octadecane. In treatments of alkanes and metal mixtures, the metal ions captured in alkane-metal complexes were released after the alkanes were degraded by the bacteria. Their utilization allowed more free ions to affect bacterial metabolism via cyto- or/and genotoxicity. Therefore, the toxicity of metal mixture was strongest in the presence of dodecane than with the other alkanes tested. Focusing on metal exposure only, the toxic effects of Cd made it the main driver in metal mixture toxicity, but its high affinity to bind with hydrocarbons weakened its effects, so that Cu became dominant in disrupting the cellular activities in bacteria. Given the specific influence of alkanes on metals, the risk assessment of binary, trinary or more metal mixtures in ecological systems requires comprehensive studies on the possible effects of organic compounds in their surrounding environments.

460 Table 3. Toxic Indicators (Ti) for eco-toxicity of metal mixtures in addition of alkanes

Metal mixture	Alkane	Ti	Toxic effects
Cd + Pb	Dodecane 1 mg/L	N.A.	Genotoxicity
	Dodecane 10 mg/L	N.A.	Not typical
	Dodecane 100 mg/L	N.A.	Genotoxicity
	Tetradecane 1 mg/L	3.49	Antagonism
	Tetradecane 10 mg/L	10.18	Antagonism
	Tetradecane 100 mg/L	4.06	Antagonism
	Hexadecane 1 mg/L	N.A.	Genotoxicity
	Hexadecane 10 mg/L	N.A.	Not typical
	Hexadecane 100 mg/L	N.A.	Genotoxicity
	Octadecane 1 mg/L	N.A.	No toxic effect
	Octadecane 10 mg/L	N.A.	No toxic effect
	Octadecane 100 mg/L	N.A.	No toxic effect
Cd + Cu	Dodecane 1 mg/L	4.17	Antagonism
	Dodecane 10 mg/L	2.07	Antagonism
	Dodecane 100 mg/L	5.59	Antagonism
	Tetradecane 1 mg/L	2.23	Antagonism
	Tetradecane 10 mg/L	1.73	Antagonism
	Tetradecane 100 mg/L	1.72	Antagonism
	Hexadecane 1 mg/L	1.93	Antagonism
	Hexadecane 10 mg/L	<0	Synergism
	Hexadecane 100 mg/L	4.07	Antagonism
	Octadecane 1 mg/L	3.05	Antagonism
	Octadecane 10 mg/L	3.34	Antagonism
	Octadecane 100 mg/L	2.78	Antagonism
Pb + Cu	Dodecane 1 mg/L	3.39	Antagonism
	Dodecane 10 mg/L	2.18	Antagonism
	Dodecane 100 mg/L	5.23	Antagonism

	Tetradecane 1 mg/L	2.21	Antagonism
	Tetradecane 10 mg/L	1.80	Antagonism
	Tetradecane 100 mg/L	2.18	Antagonism
	Hexadecane 1 mg/L	1.25	Antagonism
	Hexadecane 10 mg/L	<0	Synergism
	Hexadecane 100 mg/L	2.79	Antagonism
	Octadecane 1 mg/L	3.10	Antagonism
	Octadecane 10 mg/L	3.24	Antagonism
	Octadecane 100 mg/L	2.57	Antagonism
Cd + Pb + Cu	Dodecane 1 mg/L	<0	Synergism
	Dodecane 10 mg/L	0.63	Synergism
	Dodecane 100 mg/L	0.29	Synergism
	Tetradecane 1 mg/L	2.44	Antagonism
	Tetradecane 10 mg/L	2.34	Antagonism
	Tetradecane 100 mg/L	2.45	Antagonism
	Hexadecane 1 mg/L	3.06	Antagonism
	Hexadecane 10 mg/L	3.44	Antagonism
	Hexadecane 100 mg/L	1.84	Antagonism
	Octadecane 1 mg/L	5.58	Antagonism
	Octadecane 10 mg/L	2.65	Antagonism
	Octadecane 100 mg/L	2.39	Antagonism

[a]N.A.; Not Applicable, the Toxic Indicator Model is applicable for cytotoxic effects, but not for genotoxicity of metal mixture. [b]Not typical; metal mixture shows strong cytotoxicity at low TU, but weak cytotoxicity at high TU. [c]Antagonism; mutual inhibition of metal ions in mixture. [d]Synergism; greater effect in mixture than the sum of individual metal.

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3.5 Total versus bioavailable concentration for toxicity assessments of heavy metal – alkane co-contamination

Since the 1990s, legislative authorities have established guideline values to assess and remediate contaminated sites on the basis of total metal content (Kim et al., 2015). This could cause inaccurate estimation of potential risks, resulting in non-essential and unreasonable remediation efforts. Numerous studies have therefore advocated focussing on assessing what is bioavailable when determining risk or remediative measures (Harmsen., 2007). In oil contaminated sites, there is potential for complex interactions between the oils, metals (singly or in mixtures) and other soil constituents. This study has shown that toxicities of single or metal mixtures are not simply related to their total concentrations when present together with alkanes. This has implications for the risk-based regulatory framework.

Bioavailability is a dynamic process that is controlled by physicochemical desorption, physiological uptake and biological accumulation (Lanno et al., 2004; Peijnenburg et al., 2007). Organic matrices, including alkanes and oily phases in contaminated soils, are an important factor to modify the bioavailability of metal ions due to complexation at certain environments. As shown in Figure 1B and Figure 3, the bioluminescent ratio for Cd exposure was reduced by higher levels of alkanes, indicating decreasing toxicity of Cd ions to ADPWH_recA at the consequences of possibly reduced bioavailability of Cd. Also, in dose responses to 10 mg/L Cu, alkanes effectively reduced its toxicity, and this could be attributed to the lower bioavailability of Cu ions, thereby limiting its effects on bacteria. Toxicity of binary or trinary metal mixtures varied with alkane types, rather than the total concentration of metals in Figure 2 and Table 3, which could be attributed to the effects of alkanes on metal bioavailability. Organic matter of different types and forms can lead to specific or non-specific adsorption of metal ions and form

metal-organic complexes to further affect the toxicities of metals (Mahara et al., 2007; Wong et al., 2007; Smith., 2009). To have a better understanding of the relations between ecological toxicity and bioavailability of single metals and metal mixtures at oil contaminated sites, the bioreporter ADPWH_recA can be applied with well-established techniques for making measurements of bioavailable metals, such as the diffusive gradients in thin film (DGT) passive sampling approach, to link biotoxicity and bioavailability (Zhang and Davison., 2015).

4. Conclusions

This study used an alkane degrading bioreporter to investigate the toxic effects of single metal, single alkane, metal mixtures, and alkane-metal mixtures. The living whole-cell bioreporter can detect the toxicants from the mixture in the solution. In bioluminescent detection, the dose-toxicity curve revealed the strong genotoxicity of Cd and Pb at 0.01 and 0.1 mg/L, and cytotoxic inhibition was found for Cu at concentrations >1 mg/L. Alkanes of >= 1 mg/L can inhibit the toxicity of Pb and Cu. However, only the 100 mg/L alkane doses decreased Cd toxicity, showing that Cd is the main driver of the mixture toxicity observed in the study. In the complex mixture of alkanes with two or three heavy metals, Cd at 0.1 mg/L exhibited genotoxic effects, while Cu at 10 mg/L showed significant cytotoxicity on bacteria. The toxicity pattern of alkane-metal mixtures suggested the carbon chain length determined the interaction modes of heavy metals. We introduce a new way to understand mixture effects of metals and hydrocarbons. Further studies are required to analyse the change in bacterial genotype from different contaminated samples under diverse environmental conditions - including pH, temperature, oxygen level and nutrient concentrations, as well as to fully investigate the toxicity and bioavailability of hydrocarbons from crude oil contamination.

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