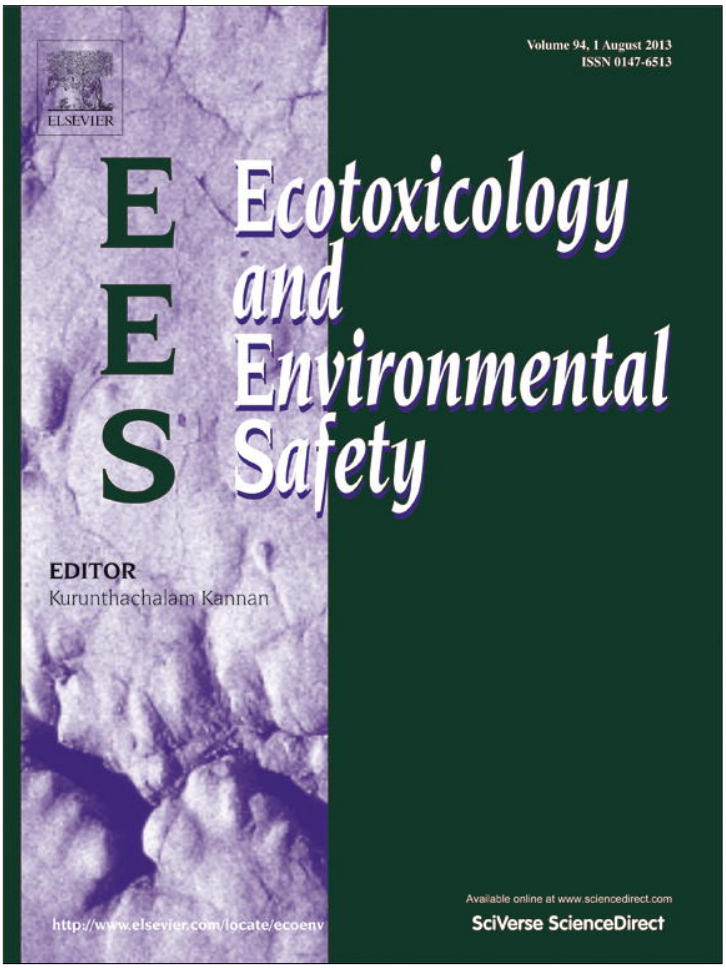

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Toxicity cutoff of aromatic hydrocarbons for luminescence inhibition of *Vibrio fischeri*

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ABSTRACT

Effects of individual petroleum hydrocarbons on the luminescence inhibition of *Vibrio fischeri* were evaluated according to a standard protocol to develop a quantitative structure–activity relationship and identify the apparent toxicity cutoff. Eighteen aromatic hydrocarbons, including benzene and its derivatives and polycyclic aromatic hydrocarbons (PAHs), were chosen as model compounds with their log K_{ow} values between 2.7 and 6.4. The obtained values of 50 percent luminescence inhibition (EC_{50}) showed a good linear correlation with log K_{ow} up to ~5. However, toxic effects were not observed for more hydrophobic chemicals with log K_{ow} value > 5. The calculated chemical activities that caused EC_{50} were mostly between 0.01 and 0.1. This agrees with an earlier hypothesis concerning a chemical activity resulting the critical membrane concentration of aromatic hydrocarbons. The highest chemical activities for aromatic hydrocarbons with log K_{ow} value > 5 or melting point > 100 °C are < 0.01 when they are spiked at their water solubility level according to the standard test protocol; this occurs for two primary reasons: (1) partitioning between organism and the test solution and (2) decreasing fugacity ratio with increasing melting point. Accordingly, luminescence inhibition by petroleum hydrocarbons is well explained by the baseline toxicity model. However, the apparent toxicity cutoff observed for single chemicals is not necessarily valid in a complex mixture, because baseline toxicity is regarded concentration additive.

1. Introduction

Luminescence inhibition of *Vibrio fischeri* has been widely used as a quick and convenient bioassay for the evaluation of adverse effects caused by chemical contaminants in the environment (e.g., Grote et al., 2005; Isidori et al., 2003; Jacobs et al., 1993; Johnson and Long, 1998; Kudlak et al., 2012; Ren and Frymier, 2002). Due to its high reproducibility and low cost, this method is one of the standardized tests used to evaluate the toxicities of environmental samples (ISO, 1998) and identify toxic fractions in environmental mixtures such as wastewater effluent (Svendsen et al., 2005), contaminated groundwater (Hartnik et al., 2007), landfill leachates (Isidori et al., 2003), and soil/sediment extracts (Brack et al., 1999; Grote et al., 2005; Kammann et al., 2005) via bioassay-directed fractionation.

Although the toxic mode-of-actions that lead to luminescence inhibition have not been fully elucidated, it is regarded as a good ecotoxicological end-point for the baseline toxicity often caused by mixtures of chemicals including petroleum hydrocarbons (Cronin and Schultz, 1997; Jaworska and Schultz, 1994; Vighi et al., 2009; Wei et al., 2004). For example, bioluminescence inhibition correlates well with the total polycyclic aromatic hydrocarbons (PAHs) level (Kudlak et al., 2012) and alkylated benzenes and PAHs were identified in the active fractions (Hartnik et al., 2007), suggesting that apparent luminescence inhibition could be caused by the narcotic effects of aromatic hydrocarbons. Effective concentrations for 50 percent luminescence inhibition (EC_{50}) have been reported for various organic chemicals (El-Alawi et al., 2002a; Gutiérrez et al., 2002; Hartnik et al., 2007; Jacobs et al., 1993; Johnson and Long, 1998; Parvez et al., 2008; Ren and Frymier, 2002; Wei et al., 2004) and quantitative structure–activity relationships (QSARs) have been developed based on those values (Cronin and Schultz, 1997; Parvez et al., 2008; Ren and Frymier, 2002; Vighi et al., 2009; Wei et al., 2004). However, EC_{50} values for individual chemicals have rarely been reported for very hydrophobic organic chemicals with their log K_{ow} greater than 5.0, and the values that

Abbreviations: a_{EC50}, chemical activity that causes EC_{50}; a_h, highest chemical activity in the test system; a^S/a^L, fugacity ratio; C_{free}, freely dissolved concentration; C_{nom}, nominal concentration; PAHs, polycyclic aromatic hydrocarbons; QSARs, quantitative structure–activity relationships; S, aqueous solubility; S^L, sub-cooled liquid solbility, ΔS_m, melting entropy; S^S, solubility of a solid; T_m, melting temperature.

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have been reported for these chemicals are not very reliable because they often exceeded their water solubility. For example, the EC_{50} for benzo[*a*]pyrene was reported to be 8.11 mg/L (Jacobs et al., 1993) and 7.93 mg/L (El-Alawi et al., 2002a), whereas its aqueous solubility is only 0.00147 mg/L (Kwon and Kwon, 2012). The significant difference between the reported nominal concentration and aqueous solubility might be because of partitioning between the organism and medium, sorption to dissolved phases or onto walls, etc. (Heringa et al., 2004). Although individual data on highly hydrophobic hydrocarbons are unreliable, effective fractions extracted from field samples that cause luminescence inhibition often contain hydrophobic hydrocarbons such as 3- and 4-ring PAHs (Grote et al., 2005; Hartnik et al., 2007; Jacobs et al., 1993). Thus, there is a gap between the results for environmental mixtures and individual chemicals.

As postulated in literature, highly hydrophobic organic chemicals do not exert a baseline toxicity because of high cohesive energy of a pure solid, which reduces their solubility, and precipitation of the pure chemical starts to occur below the concentration level at which the toxic effect could occur (e.g., Mayer and Reichenberg, 2006). Although the toxicity cutoff might not be directly related with the log K_{ow} values of chemicals, the log K_{ow} cutoff is often discussed because log K_{ow} increases with increasing molecular size and melting point for homologous chemicals and is a widely used simple descriptor for QSARs for many toxicity-related properties. However, the applicability domain of the proposed QSARs that use log K_{ow} has not been clearly defined in previous studies.

In this study, we collected literature experimental data on the luminescence inhibition of *V. fischeri* and performed additional experiments using 18 benzene derivatives and PAHs with log K_{ow} values between 2.7 and 6.4 in order to derive a QSAR and identify apparent toxicity cutoff. The relationship between log ($1/EC_{50}$) and log K_{ow} is described using the spiked nominal concentration and calculated free concentration. The applicability domain of the proposed equation is defined with consideration of the solubility of the chemicals present and the chemical activity that initiates baseline toxicity.

2. Theory

Acute luminescence inhibition of *V. fischeri* is often regarded as non-specific toxicity (Jaworska and Schultz, 1994), i.e., toxic response occurs because the amount of xenobiotic chemicals accumulated in the plasma membrane exceeds a tolerable limit. The critical mass showing narcotic effects is often called the critical body burden or critical body residue (e.g., Escher and Hermens, 2002; van Wezel et al., 1996). The chemical exchange between the freely dissolved species in the solution and those absorbed by small dispersed particles such as dissolved organic matter and cells may be regarded to be relatively fast compared to the time scale of toxicity tests (Schlautman and Morgan, 1993); therefore, phase equilibrium between the cell membrane and solution may be assumed to occur relatively fast unless the chemical is significantly metabolized. For most narcotic chemicals, chemical transformation is not likely to occur within the short experimental time, which is typically 15 min.

Under these assumptions, the toxic concentration (EC_{50} in this assay) is related to the hydrophobicity of a chemical often denoted by its log K_{ow} value used as a surrogate for the equilibrium partition coefficient between lipid membrane of *V. fischeri* and water. Assuming that the free energy of the transfer from 1-octanol to water is linearly related with that from the biota to water and the observed toxicity is a result of the accumulation of xenobiotics in the body, the following relation (Eq. (1)) is used to predict ecotoxicity from hydrophobicity of a chemical, (Schwarzenbach et al., 2003):

$$\log(1/EC_{50}) = a \log K_{ow} + b \quad (1)$$

where *a* and *b* are empirically determined constants. However, the applicability of Eq. (1) for highly hydrophobic chemicals with their log $K_{ow} > 5$ for the luminescence inhibition of *V. fischeri* is unclear because their effects have either not been observed (Brown et al., 2001) or EC_{50} values exceeding their water solubility have been reported (El-Alawi et al., 2002a; Jacobs et al., 1993). This may be explained by the insufficient membrane concentration caused by the decrease in bioavailability via sorption, uptake by organisms and/or by reduced chemical activity of the introduced chemical species because of their higher melting points that accompany higher log K_{ow} values (Mayer and Holmstrup, 2008; Mayer and Reichenberg, 2006). In their pure state under ambient condition, many hydrophobic chemicals are solids and their dissolution into water or cell membranes is considered to occur via two conceptual steps: (1) melting of the solid to a sub-cooled liquid and (2) mixing with surrounding solvent molecules, i.e., water or membrane bilayers. The decreased solubility of a solid is approximated by the following equation (Yalkowsky, 1979):

$$\log_{10}\frac{a^S}{a^L} = \log_{10}\frac{S^S}{S^L} = \frac{\Delta S_m}{2.303R}\left(1-\frac{T_m}{T}\right) \quad (2)$$

where *a* and *S* denote the chemical activity and solubility of the chemical and subscripts *S* and *L* represent solid and sub-cooled liquid, respectively, ΔS_m is the melting entropy, *R* is the gas constant (8.314 J mol^{-1} K^{-1}), and T_m and *T* are the melting point and temperature in *K*, respectively. Baseline toxicity initiates when the chemical activity is approximately between 0.01 and 0.1 (Bobra et al., 1983; Mayer and Holmstrup, 2008; Mayer and Reichenberg, 2006). Because the reference state for the chemical activity of an organic solid is sub-cooled liquid, a^L is unity. Thus, the maximum chemical activity of an organic solid can be calculated using Eq. (2). Those chemicals with maximum chemical activities that are lower than the critical amount causing non-specific effects would not exert any apparent effects.

3. Materials and methods

3.1. Materials

Analytical grade toluene (99.9 percent), *o*-, *m*-, and *p*-xylenes (99.5 percent each), naphthalene (99 percent), 2-methylnaphthalene (97 percent), 1-ethylnaphthalene (97 percent), 2-ethylnaphthalene (99 percent), fluorene (99 percent), fluoranthene (99 percent), phenanthrene (97 percent), anthracene (99 percent), pyrene (99 percent), dibenzothiophene (98 percent), and dimethyl sulfoxide (DMSO; 99.5 percent) were purchased from Sigma-Aldrich (St Louis, MO, USA). Analytical grade acenaphthene, benz[*a*]anthracene, and benzo[*a*]pyrene were purchased from Supelco (Bellefonte, PA, USA). Analytical standard 9-methylanthracene (99 percent) was purchased from Fluka (Buchs, Switzerland).

3.2. Data collection and evaluation

The EC_{50} values for the luminescence inhibition of *V. fischeri* for benzene and its derivatives, PAHs and alkylated PAHs were collected from literature (El-Alawi et al., 2002a, 2002b; Gutiérrez et al., 2002; Hartnik et al., 2007; Jacobs et al., 1993; Johnson and Long, 1998; Parvez et al., 2008; Ren and Frymier, 2002; Wei et al., 2004). For quantitative comparison and the development of QSARs, only values obtained from 15 min tests using the same experimental protocol were chosen (Hartnik et al., 2007; Jacobs et al., 1993) and reported EC_{50} values that were greater than the water solubility (Table 1) were excluded.

The freely dissolved concentration in the test medium (C_{free}) was calculated assuming that the introduced chemicals distribute only in the solution and lipid-phase of the organism and phase equilibrium is reached instantaneously:

$$C_{free} = \frac{1}{1 + K_{lipw} f_{lip} (M_{V.\ fischeri}/V_w)} C_{nom} \quad (3)$$

where K_{lipw} is the lipid–water partition coefficient, f_{lip} is the lipid content of *V. fischeri*, $M_{V.\ fischeri}$ is the mass of *V. fischeri* used in the assay, and C_{nom} is the

Table 1
Molecular weight (MW), melting point (T_m), water solubility (S), melting entropy (ΔS_m), fugacity ratio (a^S/a^L), log K_{ow}, log K_{lipw}, and measured luminescence inhibition from this study and literature.

Compound	MW (g/mol)	T_m[a] (K)	S (mg/L at 25 °C)	ΔS_m[h] (J/mol K)	a^S/a^L	log K_{ow}[k]	log K_{lipw}	Luminescence inhibition					
								This study			Literature		
								$EC_{50,nom}$ (mg/L)	$EC_{50,free}$ (mg/L)	a_{EC50}	$EC_{50,nom}$ (mg/L)	$EC_{50,free}$ (mg/L)	a_{EC50}
Benzene	78	278.5	1789[b]	35.40	1.000	2.13	2.27[m]				102.78[p]	102.436	0.057
Toluene	92	178.1	580[b]	37.15	1.000	2.73	2.88[m]	13.823(±0.904)	13.639(±0.892)	0.0235(±0.0015)			
Ethylbenzene	106	178.0	187[b]	51.43	1.000	3.15	3.30[m]				9.69[p]	9.353	0.050
o-Xylene	106	247.8	221[b]	54.90	1.000	3.12	3.27[m]	3.743(±0.068)	3.622(±0.065)	0.0164(±0.0003)			
m-Xylene	106	225.2	160[b]	51.40	1.000	3.20	3.35[m]	2.637(±0.158)	2.534(±0.152)	0.0158(±0.0009)			
p-Xylene	106	286.3	214[b]	59.77	1.000	3.15	2.71[n]	4.227(±0.557)	4.188(±0.552)	0.0196(±0.0026)			
Naphthalene	128	353.3	30.6[c]	54.39	0.297	3.37	3.09[n]	0.530(±0.025)	0.519(±0.025)	0.0051(±0.0002)	0.68[q]	0.665	0.007
Acenaphthylene	152	364.8	16.1[d]	30.30	0.441	4.07[l]	3.83[m]				0.31[q]	0.277	0.005
Acenaphthene	154	366.4	4.16[d]	59.83	0.191	3.92	4.08[m]	0.440(±0.017)	0.362(±0.014)	0.0173(±0.0007)	0.74[q]	0.609	0.029
Fluorene	166	387.8	1.57[c]	48.53	0.172	4.18	4.34[m]	0.727(±0.094)	0.521(±0.067)	0.0533(±0.0069)	3.23[q]	2.316	0.237
Phenanthrene	178	372.2	0.82[c]	45.19	0.258	4.46	5.05[o]	0.197(±0.052)	0.065(±0.017)	0.0208(±0.0055)	0.53[q]	0.176	0.056
Anthracene	178	488.8	0.044[c]	58.58	0.011	4.54	5.28[o]	ND			33.40[q]	7.550	1.682
Fluoranthene	202	383.2	0.199[d]	49.37	0.183	5.22	5.62[o]	0.373(±0.064)	0.049(±0.007)	0.0455(±0.0067)	2.02[q]	0.238	0.223
Pyrene	202	423.6	0.086[c]	40.17	0.130	5.18	5.74[o]	ND					
Benz[*a*]anthracene	228	433.5	0.0168[d]	49.20	0.068	5.91	6.46[o]	ND			0.26[q]	0.005	0.020
Chrysene	228	528.5	0.0007[c]	49.21	0.010	5.61	6.46[o]				1.49[q]	0.028	0.231
Benzo[*a*]pyrene	252	454.1	0.00147[c]	38.20	0.090	6.40	7.35[o]	ND			8.11[q]	0.020	0.949
Dibenzothiophene	184	371.2	1.47[e]	56.42[i]	0.188	4.38	4.54[m]	0.227(±0.022)	0.139(±0.013)	0.0179(±0.0017)	0.12[p]	0.074	0.009
1-Methylnaphthalene	142	242.6	31.7[b]	49.30	1.000	3.86	4.03[m]				0.50[p]	0.419	0.009
2-Methylnaphthalene	142	307.6	21.5[c]	58.87	0.796	3.86	4.16[m]	0.373(±0.045)	0.296(±0.036)	0.0110(±0.0013)	0.30[p]	0.238	0.013
1-Ethylnaphthalene	156	259.1	11.58[b]	56.5[j]	1.000	4.40	4.56[m]	0.177(±0.009)	0.107(±0.005)	0.0092(±0.0005)			
2-Ethylnaphthalene	156	265.6	7.97[f]	56.5[j]	1.000	4.38	4.54[m]	0.093(±0.003)	0.057(±0.002)	0.0072(±0.0003)			
9-Methylanthracene	192	354.5	0.261[g]	56.5[j]	0.276	5.07	5.24[m]	ND					

[a] Lide (2010).
[b] Wasik et al. (1983).
[c] Kwon and Kwon (2012).
[d] Walters and Luthy (1984).
[e] Means et al. (1980).
[f] Eganhouse and Calder (1976).
[g] Mackay and Shiu (1977).
[h] Chickos et al. (1999).
[i] Coon et al. (1988).
[j] Estimated value using Walden rule (Schüürmann et al., 2007).
[k] Suggested values by Sangster Research Laboratory.
[l] Paasivirta et al. (1999).
[m] Endo et al. (2011).
[n] Tcaciuc et al. (2012).
[o] van der Heijden and Jonker (2009).
[p] Hartnik et al. (2007.
[q] Jacobs et al. (1993).

concentration of the chemical spiked in the medium. Typical fatty acids content in bacterial cells is 0.01 and phospholipid content in *Escherichia coli* is 0.02 (Alberts et al., 2002). Although the lipid content of *V. fischeri* has not been documented in literature, we assumed f_{lip} of 0.01 because it is known to have lipid content (Zhao et al., 1998). For chemicals without reported K_{lipw} values, K_{lipw} were calculated using the following relation (Endo et al., 2011):

$$\log K_{lipw} = 1.01 \log K_{ow} + 0.12 \quad (4)$$

The chemical activity of a solute is defined as the ratio of C_{free} to its sub-cooled liquid solubility:

$$a = \frac{C_{free}}{S^L} \quad (5)$$

Because the luminescence inhibition test is conducted in 15 min, the nominal concentration should be lower than water solubility to avoid potential formation of microcrystals. The highest attainable chemical activity when the nominal concentration does not exceed the water solubility (a_h) was calculated by combining Eqs. (2), (3), and (5), as follows:

$$a_h = \frac{exp[(\Delta S_m/R)(1-T_m/T)]}{1 + K_{lipw} f_{lip} (M_{V.\ fischeri}/V_w)} \cdot \frac{C_{nom,h}}{S^s} \quad (6)$$

where $C_{nom,h}$ is the highest allowable concentration of the chemical spiked in the medium, i.e., the water solubility.

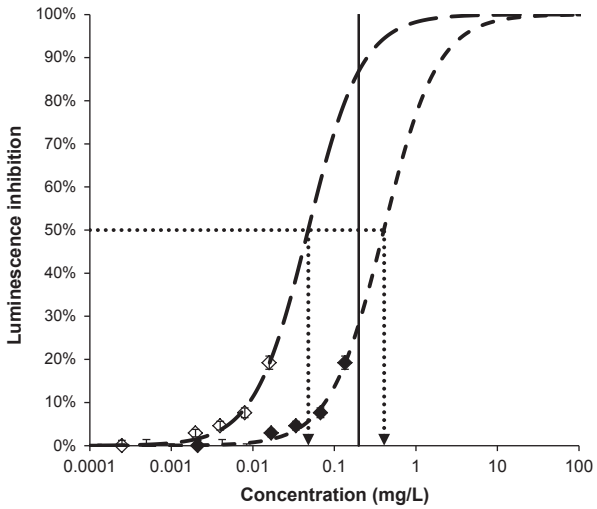

Fig. 1. Luminescence inhibition of *Vibrio fischeri* by fluoranthene. Filled diamonds represent values measured using nominal concentrations and open diamonds represent the free concentration calculated using Eq. (3). Vertical bars denote the standard errors of three measurements. Sigmoidal curves used for the derivation of EC_{50} values are represented by lines composed of short- and long-dashes for nominal and free concentrations, respectively. Dotted lines indicate extrapolation.

3.3. Luminescence inhibition test

The luminescence inhibition of *V. fischeri* was measured using Microtox® Model 500 system (Strategic Diagnostics Inc., Newark, DE, USA). Freeze-dried *V. fischeri* was purchased from Strategic Diagnostics Inc. Stock DMSO solutions of the individual chemicals were added to deionized water (max. DMSO content 0.1 percent v/v) up to approximately 90 percent of their aqueous solubility. The highest nominal concentration in the test medium after the addition of saline diluent (2 percent NaCl, purchased from Strategic Diagnostics Inc.) was approximately 45 percent of the aqueous solubility. Luminescence inhibition was measured according to the manufacturer's protocol after 5 and 15 min of exposure. EC_{50} values were derived using Microtox® Omni software (Strategic Diagnostics Inc.). The results after 15 min of exposure were used to derive the QSAR.

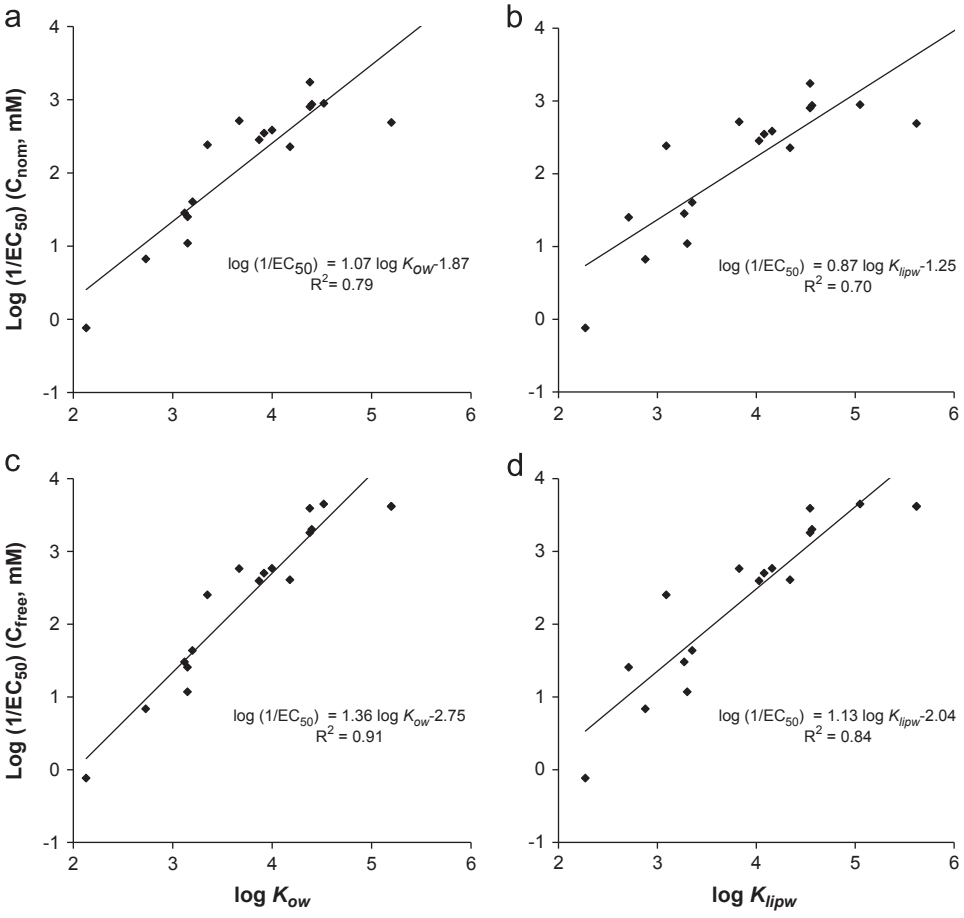

Fig. 2. Relationships between log ($1/EC_{50}$) and log K_{ow} or log K_{lipw}. Nominal concentrations ($EC_{50,nom}$) were used in (a) and (b) and calculated free concentrations ($EC_{50,free}$) were used in (c) and (d). Lines represent best-fit linear equations.

4. Results and discussion

4.1. Development of a structure–activity relationship

Table 1 presents the melting point (T_m), aqueous solubility (S), melting entropy (ΔS_m), fugacity ratio (a^S/a^L), log K_{ow}, log K_{lipw}, and experimental 15 min EC_{50} values measured in this study and collected from literature for all aromatic hydrocarbons evaluated. The measured EC_{50} values in this study agree well with literature values for chemicals with log K_{ow} values less than 5.0 except for anthracene, which has a very high melting point (Lide, 2010) because of its molecular symmetry. The EC_{50} values for anthracene, pyrene, benz[a]anthracene, benzo[a]pyrene, and 9-methylanthracene could not be determined even at the highest nominal concentration used. However, the EC_{50} values for anthracene, chrysene, benz[a]anthracene, and benzo[a]pyrene reported by Jacobs et al. (1993) significantly exceeded their water solubility. The calculated free concentrations at EC_{50} for anthracene, chrysene and benzo[a]pyrene were higher than their water solubility even accounting for the solubility enhancement due to the addition of DMSO (Kwon and Kwon, 2012). Thus, these values were not used for the derivation of a QSAR.

For fluoranthene, luminescence inhibition was observed to be less than 50 percent at the highest dose used (0.135 mg/L); therefore, the EC_{50} value was derived via extrapolation (Fig. 1). The $EC_{50,free}$ that was calculated using Eq. (3) (0.0479 mg/L) is sufficiently lower than the solubility (0.199 mg/L) (Walters and Luthy, 1984), which implies that inhibition is likely to occur when the maximum dose is higher than its aqueous solubility.

For the derivation of a QSAR, EC_{50} values for 13 aromatic hydrocarbons and four compounds from literature (Hartnik et al., 2007; Jacobs et al., 1993) were used ($n=17$). Fig. 2 shows the changes in log ($1/EC_{50}$) with increasing log K_{ow} or log K_{lipw} using both $EC_{50,nom}$ (Fig. 2a and b) and $EC_{50,free}$ (Fig. 2c and d). As shown in Fig. 2, luminescence inhibition of *V. fischeri* showed good linearity with respect to log K_{ow} or log K_{lipw}, especially when the calculated free concentration was used as a dose-metric.

4.2. Toxicity cutoff

The minimum chemical activity of a single chemical that initiates baseline toxicity was proposed to be ~0.01 and above in earlier studies (Bobra et al., 1983; Mayer and Holmstrup, 2008; Mayer and Reichenberg, 2006). Fig. 3 shows the calculated chemical activity that causes EC_{50} (a_{EC50}) versus log K_{ow} for the chemicals in this study. Most a_{EC50} values were between 0.01 and 0.1 except for those for naphthalene and ethylnaphthalenes, which

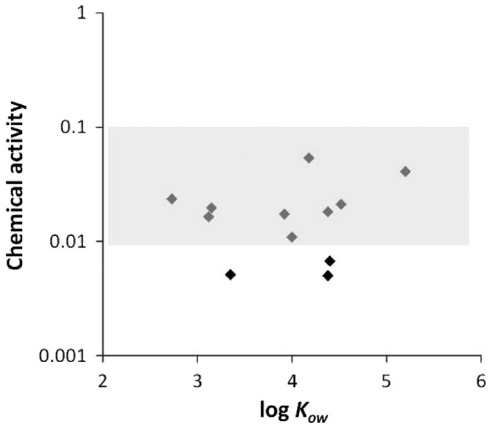

Fig. 3. Calculated chemical activities of individual chemicals tested in this study at the EC_{50} values for luminescence inhibition of *V. fischeri*. The shaded area indicates the proposed range of chemical activities that cause baseline toxicity.

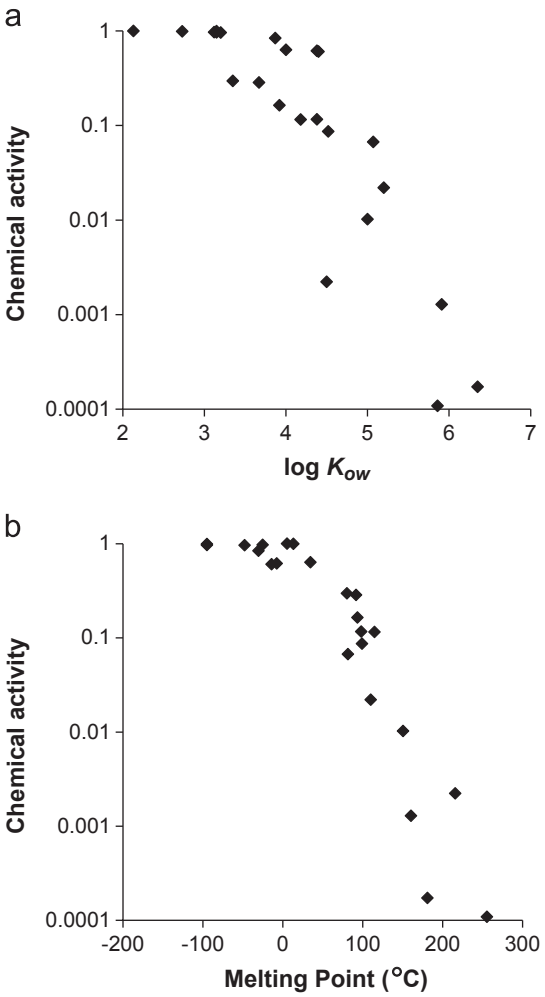

Fig. 4. Estimated highest chemical activities in the test system of chemicals initially introduced to the test solution at their aqueous solubility with respect to (a) log K_{ow} and (b) melting point (T_m).

were also within a factor of two from the proposed range of chemical activity. This shows that the luminescence inhibition of *V. fischeri* by aromatic hydrocarbons could be explained by baseline toxicity.

As stated above, the fugacity ratio indicates the maximum chemical activity that a pure organic chemical crystal can exert. The fugacity ratios of anthracene and chrysene are 0.011 and 0.010 (Table 1), which are close to the suggested borderline of the minimum chemical activity that results in apparent narcotic effects. The highest dose applied in this study was approximately one half of the aqueous solubility because of dilution. The free concentration in the test system should be much lower because of equilibrium partitioning between *V. fischeri* and medium. This explains why they did not show apparent luminescence inhibition within the range investigated.

Fig. 4a and b describes the estimated highest chemical activities for single chemicals in the test system when the nominal concentration of each chemical is equal to its aqueous solubility with increasing log K_{ow} and melting point, respectively. According to the hypothesis that baseline toxicity becomes apparent above a chemical activity of 0.01, PAHs with log K_{ow} values greater than 5 or melting points greater than about 100 °C are not likely to show apparent toxicity as a single chemical for the luminescence inhibition of *V. fischeri*. Thus, it is likely that the proposed linear equation between log ($1/EC_{50}$) and log K_{ow} is restricted to log K_{ow}

value below about 5 when the test is performed according to the standard protocol. Passive dosing could be an alternative approach to quantify the effects of individual chemicals (Brown et al., 2001; Kwon et al., 2009; Smith et al., 2013), and will due to the short test duration require pre-equilibration of the media or very fast passive dosing kinetics (e.g., Smith et al., 2010).

As discussed earlier, partitioning between the organism and test solution could be important for highly hydrophobic chemicals in the *V. fischeri* luminescence inhibition when their nominal dose is below their aqueous solubility. This would explain the observed toxicity cutoff for highly hydrophobic chemicals. However, the potential additive effects of highly hydrophobic chemicals present in a mixture may also be important. Recently, Smith et al. (2013) showed using the *Daphnia magna* immobilization test that mixtures of PAHs that had no or negligible acute toxicity as a single chemical resulted in significant toxicity because individual solid chemicals dissolve up to their solubility, leading to increased exposure. Thus, highly hydrophobic chemicals that did not show apparent toxicity for luminescence inhibition may contribute to the overall toxicity when they are present in mixtures.

Acknowledgments

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