# EVALUATION OF POLYCYLIC AROMATIC HYDROCARBON-INDUCED TOXICITY IN VIETNAMESE SETTLED DUST: COMBINATION OF INSTRUMENTAL ANALYSIS AND *IN VITRO* BIOASSAYS

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**Introduction:**

Polycyclic aromatic hydrocarbons (PAHs) and a diversity of their derivatives are among the most widespread and abundant organic pollutants, and have received increasing attention because of their high toxicity, including both carcinogenic and non-cancer effects on human and animals. Seven unsubstituted PAHs such as benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), chrysene (Chr), dibenz[a,h]anthracene (DA), and indeno[1,2,3-c,d]pyrene (IP) were identified as probable human carcinogens by the US EPA. Other high-molecular-weight (HMW) PAHs (e.g., dibenzopyrenes) and some methylated PAHs (Me-PAHs) like 7,12-dimethylbenz[a]anthracene (DMBA) and 3-methylcholanthrene (3-Me-Cho) were reported to have higher carcinogenic potencies. To our knowledge, the majority of previous studies have focused on 16 priority parent PAHs (or 16 EPA PAHs) which lead to uncomprehensive evaluation of total toxicity of PAH mixtures and underestimation of their potential risks. As PAHs and their derivatives are considered as potential ligands of aryl hydrocarbon receptor (AhR), the use of Dioxin Response Chemically Activated LUciferase gene eXpression (DR-CALUX®) assays for screening PAH-induced toxicity has been reported elsewhere[1-4]. However, this approach has some limitations that make difficulties in selecting appropriate sample preparation methods and affect accuracy of the assays for PAH analysis[5]. In addition, many PAHs can be metabolized by the cells in DR-CALUX assays. Therefore, an alternative reporter gene assay namely PAH-CALUX® has been introduced and exhibited promising applicability for comprehensive detection of carcinogenic potency of PAH-related compounds in complex mixtures[5,6].

In Vietnam, potential emission sources of PAHs and their derivatives have been attributed to urbanization-industrialization processes such as increasing traffic intensity, domestic and industrial fuel combustion, and improper waste processing activities[7-11]. Tuyen et al. (2014) reported relatively high potential carcinogenic risk of road dust contaminated with PAHs and Me-PAHs for children and adults in Hanoi metropolitan area[7,8]. Our recent study on PAHs and Me-PAHs in workplace dust from an informal end-of-life vehicle (ELV) processing area in northern Vietnam also indicated a possible cancer risk for dismantling workers under the worst exposure scenarios[11]. It should be noted that the cancer risk estimated in these studies was based on concentrations of limited individual compounds with toxic equivalency factors (TEFs) relative to BaP. In the present study, we applied a novel approach combining instrumental analysis and *in vitro* bioassays (i.e., PAH-CALUX) for the determination of PAHs and Me-PAHs, and their contributions to total PAH-induced toxicity in Vietnamese settled dust. The effect of extract clean-up methods on the assay results was also discussed.

**Materials and methods:**

Settled dust samples were collected during 2015–2016 in some areas in northern Vietnam, comprising informal end-of-life vehicle (ELV) dismantling workshops (ELV-1, -2, -3), urban houses (UH-1, -2, -3), urban roads (UR-1, -2, -3), and industrial roads (IR-1, -2, -3). The dust samples with particle size < 100 μm were extracted with acetone/hexane (1:1, v/v) and toluene by using a focused ultrasound processor. Nineteen PAHs and fifteen Me-PAHs were measured by using a gas chromatograph equipped with a quadrupole mass spectrometer at electron impact ionization mode. Detailed information on chemical and instrumental analysis of PAHs and Me-PAHs was described in our previous study[11]. Based on instrumental results, theoretical BaP equivalents (BaP-EQs) in samples were calculated by using different TEF schemes: (1) TEFs proposed by Nisbet and LaGoy (1992)[12]; (2) relative carcinogenic potencies (RCPs) proposed by Larsen and Larsen (1998)[13]; and (3) relative potencies in PAH-CALUX (REPs) established by Pieterse et al. (2013)[5]. For bioassays, crude extracts were devided and purified separately by using two methods: (1) multilayer silica gel columns containing 44% $H_2SO_4$-impregnated silica gel and activated silica gel, with elution solvent as hexane (hereinafter referred to as "persistent-PAHs"; and (2) activated silica gel columns with elution solvent as dichloromethane/hexane (1:3, v/v) (hereinafter referred to as "total-PAHs"). The eluates were evaporated under gentle nitrogen stream and reconstituted in dimethyl sulfoxide. The BaP equivalents (BaP-EQs) in samples were measured by using PAH-CALUX assays with a rat hepatoma H4IIE cell line (BioDetection Systems B.V.), and expressed as ng BaP-EQ $g^{-1}$. The culture conditions and assay protocols were described elsewhere[5,6].

**Results and discussion:**

**Concentrations, profiles, and emission sources of PAHs and Me-PAHs in settled dust.** Concentrations of PAHs ($\Sigma_{19}$PAHs) and Me-PAHs ($\Sigma_{15}$Me-PAHs) in the settled dust samples of this study ranged from 510 to 4800 (median 1200) ng $g^{-1}$ and from 110 to 4400 (median 320) ng $g^{-1}$, respectively. Levels of PAHs in the ELV samples were about 2 to 7 times higher than those detected in urban house dust, however, the difference in PAH concentrations between the categories (i.e., workplace, house, and road dust) was not statistically significant. Meanwhile, Me-PAHs were more abundant in the ELV dust at levels much higher than those in the samples from other areas. In general, concentrations of PAHs in Vietnamese settled dust were within the low to moderate range in the worldwide comparison[14,15]. The ratios of Me-PAHs and PAHs in the ELV dust (average 0.84, range 0.50 to 1.5) and urban house dust (0.33, 0.22–0.44) were greater than the values calculated for urban and industrial road dust (0.16, 0.070–0.35). High-molecular-weight (HMW) PAHs with 4 rings or more dominated in almost all the samples, accounting for 57% to 89% (average 73%) of $\Sigma_{19}$PAHs. The most predominant unsubstituted PAHs were phenanthrene (Phe), pyrene (Pyr), fluoranthene (Flt), chrysene (Chr), and benzo[g,h,i]perylene (BP). Me-PAHs with 2 or 3 rings such as methylnaphthalenes, methylanthracenes, and methylphenanthrenes were relatively abundant in the ELV, urban house, and industrial road dust, whereas proportions of HMW Me-PAHs such as methylbenz[a]anthracenes were higher in the urban road dust. The accumulation profiles and diagnostic ratios of PAHs and Me-PAHs in these settled dust samples have revealed combustion processes (e.g., fuel combustion in vehicles, industrial coal combustion, and other domestic combustion) as principal emission sources of PAH-related compounds in Vietnam. Besides, petrogenic origin of PAHs and Me-PAHs was detected in the ELV sites, which have been likely due to the leaks of petroleum products and their derivatives (e.g., crude oils, liquid fuels, lubricants, etc.) used in vehicles and engines during dismantling processes.

**Estimation of carcinogenic potency of PAHs in settled dust.** As mentioned above, several PAHs and their derivatives are classified as human carcinogens, and the carcinogenic potency of a PAH mixture is usually expressed as BaP-EQs under different TEF and REP schemes. The theoretical BaP-EQs in our settled dust samples ranged from 45 to 850 (median 210) ng $g^{-1}$, from 33 to 550 (median 120) ng $g^{-1}$, and from 370 to 3800 (median 770) ng $g^{-1}$ by using TEFs[12], RCPs[13], and REPs[5], respectively (Fig. 1).

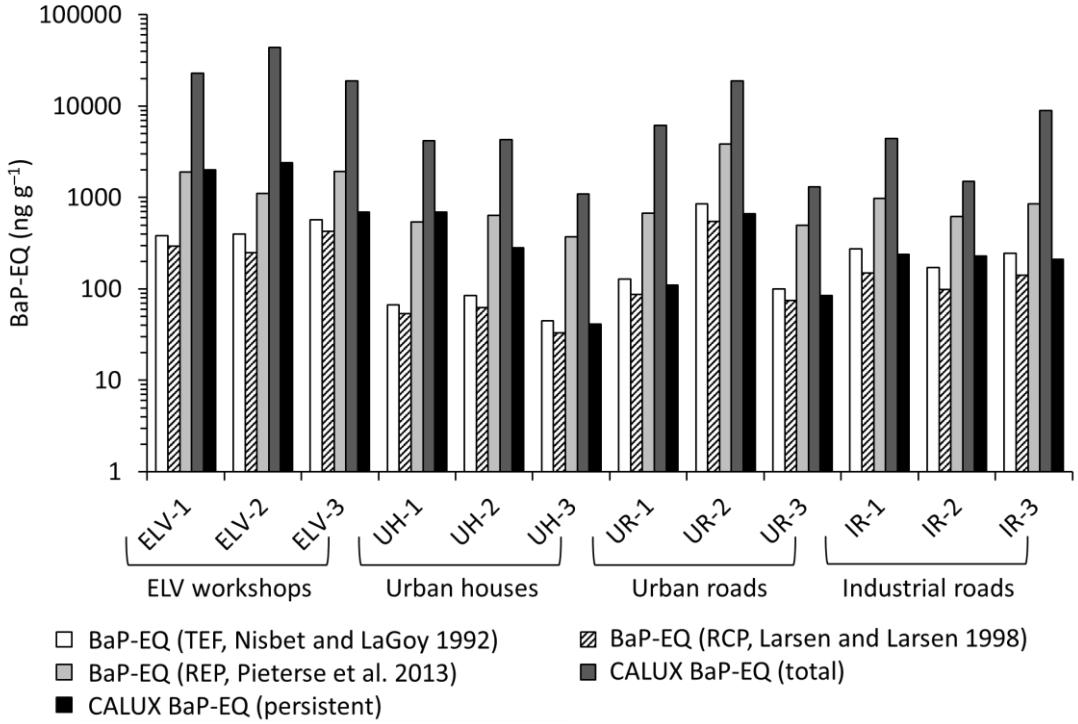


**Fig. 1.** Carcinogenic potency relative to BaP (ng BaP-EQ $g^{-1}$) in settled dust from northern Vietnam

In TEF- and RCP-BaP-EQs, BaP and DA were the most dominant contributors because of their higher TEFs (1 and 5, respectively) and potencies (1 and 1.1, respectively) as compared with other compounds. These two compounds accounted for 84% ± 6.0% and 72% ± 11% of TEF-BaP-EQs and RCP-BaP-EQs, respectively. For REP-BaP-EQs, average contributions of PAHs decreased in the order: Bb/jF (46% ± 6.0%) > BkF (17% ± 3.0%) > Chr (14% ± 6.0%) > IP (11% ± 3.0%) > BaP (9% ± 4.0%) > DA (3% ± 1.0%). This pattern was mainly due to the big differences between REPs of BbF, BjF, BkF, Chr, and IP, and their TEFs or RCPs proposed earlier. For

example, REPs of BbF (5.0), BjF (1.3), BkF (3.7), Chr (0.8), and IP (1.3) were about one to two orders of magnitude higher than their TEFs and RCPs (0.01 to 0.1). Despite of these differences, we found strongly positive correlations between TEF-, RCP-, REP-BaP-EQs, and $\Sigma_{19}$PAHs (Pearson's $r > 0.900$, $p < 0.001$). The significant associations between these overall values and concentrations of several individual compounds such as BaA, Chr, Bb/j/kF, BaP, DA, IP, and BP were also observed ($r > 0.800$, $p < 0.005$), implying the importance of pyrogenic originated HMW PAHs in terms of emission loads and toxic contributions.

**PAH-induced toxicity in settled dust derived by PAH-CALUX.** In order to obtain more comprehensive information on the total PAH-induced toxicity in the settled dust, we applied PAH-CALUX assays to dust extracts treated by two different clean-up methods. The CALUX BaP-EQs in our dust samples ranged from 41 to 2400 (median 260) ng BaP-EQ $g^{-1}$ for acidic silica gel-treated fractions (persistent-BaP-EQs), and from 1100 to 44,000 (median 5300) ng BaP-EQ $g^{-1}$ for activated silica gel-treated fractions (total-BaP-EQs). It is clear that treating dust extracts by $H_2SO_4$-impregnated silica gel can reduce 84% to 98% of carcinogenic potencies caused by PAHs in our samples. For the whole dataset, the total-BaP-EQs were relatively matched with TEF-BaP-EQs and RCP-BaP-EQs ($r > 0.610$, $p < 0.05$), but not REP-BaP-EQs ($p > 0.05$). The theoretical TEF-, RCP-, and REP-BaP-EQs accounted for 4.0% ± 3.1%, 2.6% ± 2.6%, and 19% ± 13% of total-BaP-EQs, indicating considerable contributions of "unidentified" compounds. Interestingly, $\Sigma_{19}$PAHs and $\Sigma_{15}$Me-PAHs were not correlated ($p > 0.05$), probably due to their different origins from pyrogenic and petrogenic sources, respectively; but both $\Sigma_{19}$PAHs and $\Sigma_{15}$Me-PAHs were highly associated with total- and persistent-BaP-EQs ($r > 0.900$, $p < 0.001$). This finding suggests that not only unsubstituted PAHs but also their derivatives (e.g., Me-PAHs) can contribute to total PAH-related toxicity. Unfortunately, TEFs and REPs of the most of Me-PAHs investigated in this study have not been well characterized.

The gaps between theoretical and bioassay-derived (i.e., PAH-CALUX) results are probably due to several factors. Firstly, there are many inconsistencies in the methods that used to derive TEFs and RCPs (based on compilation of data from both *in vivo* and *in vitro* toxicity studies on experimental animals)[12,13], and REPs (based on estimation the ratios between $EC_{50}$ of a potent PAH carcinogen and BaP by using *in vitro* bioassays with a rat hepatoma cell line)[5,6]. Various limitations of TEF schemes have been listed by Pieterse et al. (2013), for instance, the deviations of test systems (e.g., species-specific differences, administration ways, and scoring criteria), the misconsideration of multiple factors other than AhR transactivation, and the differences in pharmacokinetics[5]. Although the REP values were systematically estimated and showed better matching performance in predicting total PAH-induced toxicity than TEF schemes, the effectiveness of REP-based prediction seems to be higher in synthetic mixtures rather than in complex matrices[5]. Therefore, additional comprehensive and detailed studies should be conducted in order to provide more relevant REP values.

Secondly, it is obvious that instrumental analysis can only characterize a subset of PAH-related compounds, leading to underestimation of theoretical BaP-EQs. A great number of PAHs and their derivatives and transformation products existed in the environment could significantly contribute to the total PAH-related toxicity. More analytical attention should be paid to HMW PAHs with high carcinogenic potency (e.g., benzo[c]fluorene, dibenzo[a,h/a,l/a,i]pyrene, benzo[j/l]aceanthrylene, etc.), Me-PAHs (e.g., DMBA, 3-Me-Cho, 5-Me-Chr, etc.), halogenated PAHs (e.g., polychlorinated/brominated/mix-halogenated naphthalenes), nitro-PAHs, and other heterocyclic PAHs.

In addition, the synergetic and antagonistic effects among PAHs, and between PAHs and other interfering compounds, should be considered. Some PAHs such as anthracene, Phe, Flt, and Pyr that are thought as weak or non-AhR ligands and as inhibitors of carcinogenic PAHs in the assays. However, this effect can be ignored because concentrations of these antagonists were in the same order of magnitude as BaP, which were much lower than required levels for a significant signal reduction[5]. Meanwhile, the additive effects caused by typical AhR ligands as dioxin-like compounds (DLCs) seem to be important. The interferences of PAHs in DR-CALUX assays have been investigated elsewhere and such effects can be masked by using acidic silica gel and carbon columns in clean-up steps[16], whereas the impacts of DLCs on PAH-CALUX assays and elimination methods have not been reported. The CALUX-REP of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was estimated to be 5.0 that is equal to the value derived for the highest potent compound as BbF[5]. By analyzing acid-resistant fractions, we have found that persistent AhR agonists can contribute 2.0% to 16% of total-BaP-EQs. Further studies are needed to provide appropriate standard operation procedures of sample preparation and data processing for PAH-CALUX assays.

**Implications for cancer risk assessment.** We have estimated incremental lifetime cancer risk (ILCR) of dust-bound PAHs for residents in the investigated areas by using similar approach applied in our previous studies[7,8,11]. Three major exposure pathways such as ingestion, inhalation, and dermal contact were considered. The total ILCR of carcinogenic PAHs (based on theoretical TEF-BaP-EQs) in dust estimated for adults and children ranged from $4.5 \times 10^{-7}$ to $8.6 \times 10^{-6}$ (median $2.1 \times 10^{-6}$) and from $2.6 \times 10^{-7}$ to $5.0 \times 10^{-6}$ (median $1.2 \times 10^{-6}$), respectively. Meanwhile, the values derived from PAH-CALUX assays (total-BaP-EQs) were within the ranges

of $1.1 \times 10^{-5}$ to $4.4 \times 10^{-4}$ (median $5.3 \times 10^{-5}$) and of $6.5 \times 10^{-6}$ to $2.6 \times 10^{-4}$ (median $3.1 \times 10^{-5}$) for adults and children, respectively. In general, the cancer risk estimations in this study were considered acceptable (within the range of $10^{-6}$ to $10^{-4}$ according to the US Environmental Protection Agency). However, possible cancer risk (ILCR > $10^{-4}$) was estimated for dismantling workers and children in the ELV workshops under the worst exposure scenarios utilizing CALUX results. These findings have indicated that effect-based bioanalytical tools (e.g., PAH-CALUX assays) can provide more relevant data with lower possibility of underestimation for PAH-related cancer risk assessment, as compared with conventional instrumental analysis.

**Conclusions:**

In this study, a novel approach combining instrumental analysis and *in vitro* bioassays has been applied to determine the occurrence, profiles, emission sources, and carcinogenic toxicity of unsubstituted and methylated PAHs in settled dust samples from Vietnam. PAH-induced toxicity in the dust derived by using PAH-CALUX assays was compared with theoretical values calculated by absolute concentrations of selected compounds using different schemes of toxic equivalency factors and relative potencies. Our preliminary results have indicated significant contributions of unknown/unidentified compounds such as HMW PAHs and a variety of their derivatives, to total carcinogenic potency in Vietnamese settled dust. More intensive investigations on PAH-CALUX assays should be performed, for example, to estimate relative potencies for other potent carcinogens, to characterize and minimize negative factors influencing assay results, and to enhance applicability of this method to a wider spectrum of field samples.

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**References:**

1. Hamers T, van Schaardenburg MD, Felzel EC, et al. (2000) *Sci. Total Environ.* 262: 159-174.
2. Machala M, Vondracek J, Blaha L, et al. (2001) *Mut. Res.* 497: 49-62.
3. Ciganek M, Neca J, Adamec V, et al. (2004) *Sci. Total Environ.* 334-335: 141-148.
4. Bekki K, Takigama H, Suzuki G, et al. (2009) *J. Health Sci.* 55(4): 601-610.
5. Pieterse B, Felzel E, Winter R, et al. (2013) *Environ. Sci. Technol.* 47: 11651-11659.
6. Pieterse B, Winter R, Felzel E, et al. (2010) *Organohalogen Compounds* 72: 661-664.
7. Tuyen LH, Tue NM, Suzuki G, et al. (2014) *Sci. Total Environ.* 491-492: 246-254.
8. Tuyen LH, Tue NM, Takahashi S, et al. (2014) *Environ. Pollut.* 194: 272-280.
9. Wang Q, Miyake Y, Amagai T, et al. (2016) *J. Water Environ. Technol.* 14: 166-176.
10. Anh HQ, Tran TM, Thuy NTT, et al. (2019) *Chemosphere* 224: 428-436.
11. Anh HQ, Tue NM, Tuyen LH, et al. (2019) *Sci. Total Environ.* 672: 468-478.
12. Nisbet ICT, LaGoy PK (1992) *Regul. Toxicol. Pharmacol.* 16: 290-300.
13. Larsen JC, Larsen PB (1998) http://dx.doi.org/10.1039/9781847550095-00033.
14. Ma Y, Harrad S (2015) *Environ. Int.* 84: 7-16.
15. Majumdar D, Rajaram B, Meshram S, et al. (2016) *Int. J. Environ. Sci. Technol.* 14: 397-420.
16. Windal I, Denison MS, Birnbaum LS, et al. (2005) *Environ. Sci. Technol.* 39: 7357-7364.