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Combined Phytotoxicity of Fluorene, Fluoranthrene or Phenanthrene in Anthracene-Contaminated Soil to Crop Seedling Growth

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Abstract

The phytotoxicity of different combinations of phenanthrene, fluorene, and fluoranthrene in anthracene-contaminated soil to seedling growth was studied. Seeds of sticky rice and water morning glory were planted in soil contaminated, with anthracene alone, a mixture of anthracene with each polycyclic aromatic hydrocarbon (PAH), and various combinations of PAHs. The results showed that anthracene + fluorene and anthracene + fluoranthene were more toxic to the growth of sticky rice seedlings than anthracene alone or anthracene + phenanthrene. However, all combinations of anthracene with other PAHs were toxic to water morning glory seedlings. When there were 3 - 4 PAHs present together, anthracene + fluoranthene + fluorene was the most toxic combination for shoot length, root length, and fresh weight of both plants, but not for dry weight. Responses of dicot and monocot to the toxicity of PAH co-contamination were different when anthracene was present alone or with other PAHs.

Keywords: Co-contamination, polycyclic aromatic hydrocarbons, toxicity testing

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of recalcitrant compounds in the environment containing at least 2 benzene rings. Incomplete combustion of organic materials, accidental spillage of crude oils, effluent or sludge from creosote treatment facilities, and discharges of petroleum hydrocarbon products are the main sources of PAH contamination. PAHs are recalcitrant contaminants because they have the potential to accumulate in living organisms and soils. PAHs are mutagenic and carcinogenic to animals, and could be bioaccumulated and biomagnified along the food chain [1-3].

Anthracene is one of the PAH compounds generally found in wood-preserving areas [4] and it was identified in the USEPA list as a pollutant that should be quickly removed from the environment [5]. Anthracene phytotoxicity has been reported by Wieczorek and Wieczorek [6]; the foliar application of anthracene at 280 μg resulted in the rate of photosynthesis in *Lactuca sativa* and *Raphanus sativus* decreasing by approximately 20 % [6]. Toxicity of anthracene has been reported in another plant by inhibition of photosynthetic activity and electron transport inhibition in duckweed [7].

Even though anthracene is not a human carcinogen, it was identified as the fastest photomodified hydrocarbon and a highly phototoxic compound [6]. In petroleum- or PAH-contaminated soil, many PAHs are normally found together, including anthracene, fluorene, fluoranthene and phenanthrene. The ratio and concentration of each PAH depends on the source of contamination. Each PAH mentioned above has been reported to be phytotoxic. However, the phytotoxicity of anthracene combined with other PAH, a compound generally found in contaminated environments, is not known. In this study, fluorene, fluoranthene and phenanthrene, which are commonly co-contaminated with anthracene [8,9], were selected to test their phytotoxicity to important monocot and dicot crops in Thailand when combined with anthracene. Water morning glory (*Ipomoea aquatica* Forssk.), and sticky rice (*Oryza sativa* L. 'RD6') were selected for this study. Rice has been studied for its capacity to enhance hexachlorocyclohexane biodegradation in its rhizosphere and to accumulate phenanthrene in its root [10,11], whereas water morning glory has been reported to accumulate heavy metal in its biomass [12]. The combined phytotoxicity of these PAHs at different total concentrations and different plant species was compared.

Materials and methods

Preparation of PAH-spiked soil

Soil with no previous history of PAH contamination was collected from Kookaew Temple, Kantharawichai District, Mahasarakham Province, Thailand. The soil was sent to Central Laboratory (Thailand) Co., Ltd., Khonkaen, Thailand, for physical and chemical characterization. The soil used possessed a clay texture, pH of 8.1, 2.4 % (dry) organic matter content, 109.5 μs cm^{-1} (0.11 ds/m) electrical conductivity, 0.29 % total nitrogen, and 58.38 mg kg^{-1} available phosphorus.

Phenanthrene (Sigma-Aldrich, purity 98 %), fluorene (Sigma-Aldrich, purity 98 %), fluoranthrene (Sigma-Aldrich, purity 99 %), and anthracene (Fluka, purity 98 %) were weighed separately and dissolved in acetone. Each PAH solution was spiked into soil to give final concentrations at 0, 2, 20, 200 and 400 mg kg^{-1} dry soil in a 1:1 ratio of each contaminant. These concentrations covered the range for each PAH contaminated sites. After thorough mixing, the soil was dried at room temperature (28 - 30 °C) inside a fume hood to allow the solvent to evaporate. The soil was subdivided into 50 g dry soil portions in 120 ml plastic planting containers that water could not penetrate. Control soil samples received only acetone. The soil samples were moistened with distilled water to a level of 65 % of water holding capacity before use.

Phytotoxicity assay

Phytotoxicity assays were performed according to Chouychai *et al*. [13]. Seeds of sticky rice 'RD6' (collected from a farm in Roi-Et Province) and water morning glory (commercial seeds of the Chia Tai Group, Bangkok, Thailand) was immersed in distilled water for 3 h. The experiment was performed in triplicate, and ten seeds were inoculated per replicate in plastic containers and kept at 30 °C in a room that received natural sunlight. The soil was watered twice per day to maintain humidity and soil moisture content. After 10 days, seeds that germinated in each treatment were counted. Twenty plants were randomly removed to measure their fresh weight, dried weight, shoot length, and root length. The data were shown as mean ± standard deviation and were used to calculate the EC20 and additive index.

Statistical analysis

Two-way ANOVA, followed by LSD testing, was used to examine the toxicity of individual PAHs and PAH mixtures to plants. Estimation of concentration that caused a 20 % reduction of growth (EC20) was determined using probit analysis, and the additive index was calculated following Chen and Lu [14] and Wang *et al*. [15].

Results and discussion

Combined phytotoxicity of anthracene with another PAH

The presence of 400 mg/kg anthracene decreased the shoot length of rice seedlings, but did not affect root length. In the presence of anthracene together with fluorene or fluoranthene, the shoot and root length of rice decreased significantly when compared with seedlings growing in anthracene or anthracene + phenanthrene contaminated soil at all total concentrations (**Figures 1A** and **1C**). The presence of all PAH mixtures greater than 20 mg/kg decreased the shoot length of water morning glory significantly, but there was no differentiation between the various toxicities of combined PAH (**Figure 1B**). At 400 mg/kg total concentration, all PAH mixtures also significantly decreased the root length of the water morning glory. Anthracene + phenanthrene was least toxic to the root length of the water morning glory (**Figure 1D**).

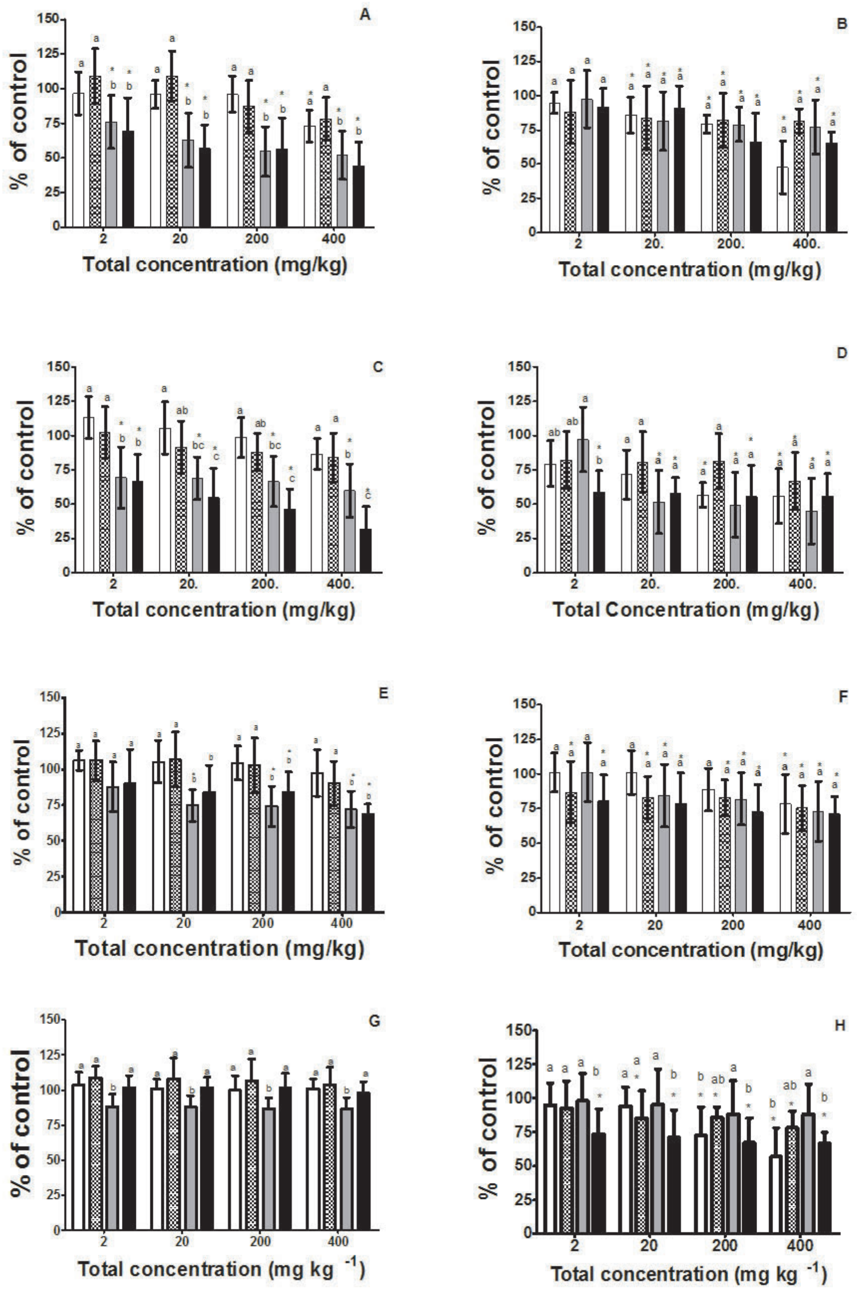

Figure 1 Growth of seedling growing in PAH contaminated soil with different total concentrations. Symbol: ☐ anthracene, ▩ anthracene + phenanthrene, ▢ anthracene + fluoranthene, ■ anthracene + fluorene; different lowercase letter showed significant difference from other PAHs at the same total concentration. *Significant difference from control of each compound. **A**: Shoot length of sticky rice, **B**: Shoot length of water morning glory, **C**: Root length of sticky rice, **D**: Root length of water morning glory, **E**: Fresh weight of sticky rice, **F**: Fresh weight of water morning glory, **G**: dried weight of sticky rice, **H**: dried weight of water morning glory.

Only anthracene + fluoranthene and anthracene + fluorene at concentrations higher than 200 mg/kg significantly decreased the fresh weight of rice seedlings (**Figure 1E**). However, only anthracene + fluoranthene affected the dry weight of rice seedlings when compared with other PAH mixtures (**Figure 1G**). When the total concentration of PAH mixtures was increased to 200 mg//kg, the anthracene-only treatment also decreased the dry weight of water morning glory seedlings (**Figure 1H**).

For sticky rice, there were synergistic effects between anthracene and fluorene or fluoranthene to root length and fresh weight. However, the interaction between anthracene and phenanthrene was antagonistic to root length and had no effect on fresh weight. This synergy was identified for the water morning glory's fresh weight, but only anthracene and fluorene had a synergistic effect on the water morning glory's root length (**Table 1**).

Table 1 EC20 and additive index of each compound to each plant species.

PAHs mixtures	Sticky rice (days)				Water morning glory (days)			
	Root length	Additive index	Fresh weight	Additive index	Root length	Additive index	Fresh weight	Additive index
ANT	>400	-	>400	-	2.64	-	327.9	-
FLT*	118.7	-	>400	-	288.3	-	264.2	-
FLU*	203.8	-	>400	-	246.6	-	144.3	-
PHE*	184.9	-	>400	-	208.7	-	148.1	-
ANT + FLU	0.14	0.0005	93.9	0.23	0.01	0.002	4.69	0.02
ANT + FLT	0.01	0.00001	15.3	0.04	6.69	1.28	134.8	0.46
ANT + PHE	>400	1.58	>400	1	14.7	2.82	256.6	1.26
(ANT + PHE) + FLU	0.01	0.00004	>400	1	11.5	0.41	>400	2.16
(ANT + PHE) + FLT	26.7	0.14	>400	1	59.3	2.12	286.7	1.62
(ANT + FLT) + FLU	4.9	245.0	124.3	4.22	95.5	7.33	184.2	1.32
Total 4 PAHs	23.2		>400		257.5		288.7	

*Data shown for additive index calculated only. Symbol: ANT = anthracene, FLU = fluorene, FLT = fluoranthene, PHE = phenanthrene

Anthracene has been reported to be toxic to many plant species, such as duckweed [7], lettuce, and radish [6]. However, in this study, anthracene was toxic to shoot and root growth of water morning glory, but not toxic to sticky rice. When other PAHs were present together, anthracene + fluorene was the most toxic to shoot and root length in sticky rice, but was the most toxic to weight in the water morning glory. This result indicated that there may be different toxic mechanisms between dicot and monocot plants and for different PAHs compounds.

The study of the metabolism of PAHs in rice and water morning glory has rarely been conducted. The chlorophyll content of rice seedling cultivar Yangdao 4 exposed to phenanthrene and pyrene-contaminated soil was decreased, and the superoxide dismutase (SOD) activity of these seedlings increased [16]. Anthracene exposed hydroponically and uptake in *Festuca arundinacea* was metabolited to be anthrone and antraquinone [17]. Some studies have revealed that PAHs can be metabolized within the plant cell, and the differences in the mechanism of PAH metabolism in dicots and monocots have been reported by many. For example, tomatoes can metabolize 15 % of fluoranthene, but lettuce and wheat can metabolize only 6 and 9 %, respectively [18]. Additionally, the distribution of radioactivity in 2 types of plant cells after exposure to radiolabelled benzo[*a*]pyrene was different. Soybean cells retained 16.2 % of radioactivity as polar metabolites, but wheat cells retained 48.6 % of the radioactivity as unchanged benzo[*a*]pyrene [19]. The PAH metabolism in water morning glory was not known, but there was a report that this plant species could accumulate 4 PAHs in this study in plant tissue [20].

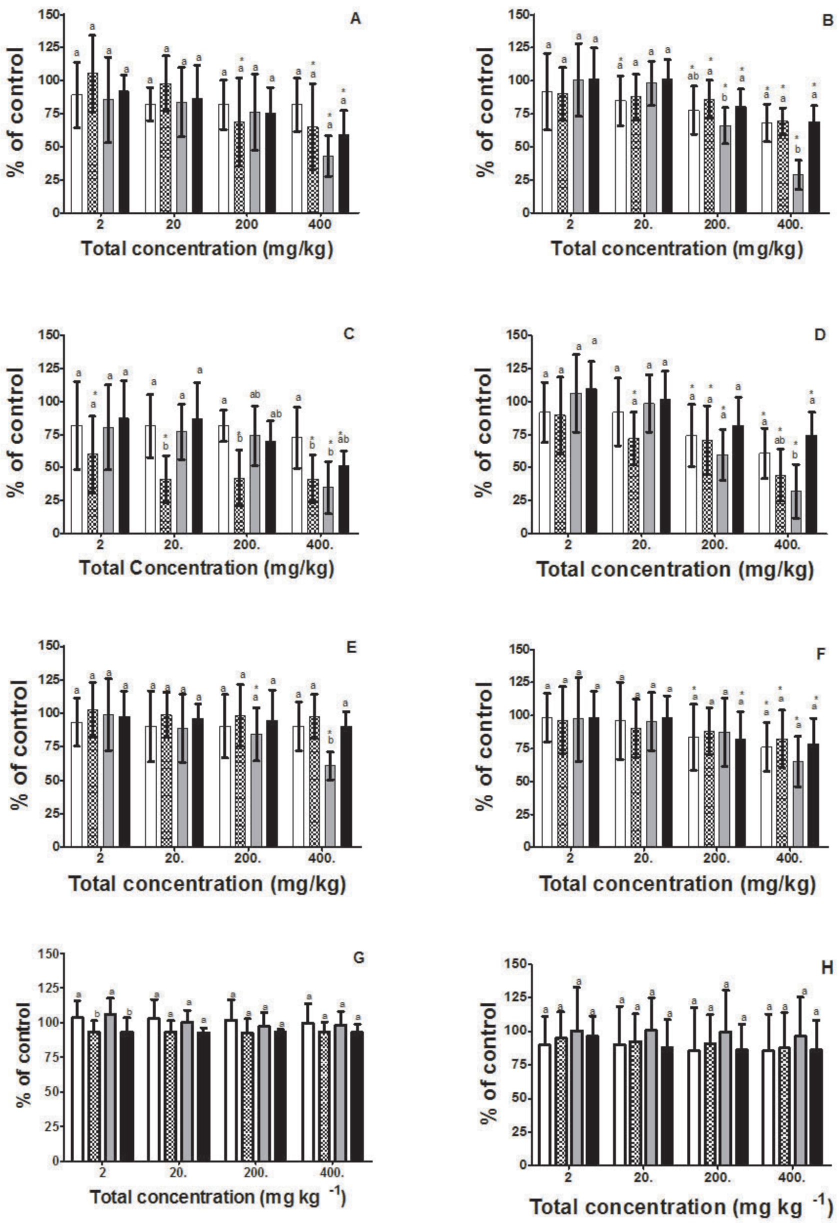

Figure 2 Growth of seedling growing in PAH contaminated soil with different total concentration. Symbol: ☐ anthracene + phenanthrene + fluoranthene, ▩ anthracene + phenanthrene + fluorene, ▢ anthracene + fluoranthene + fluorene, ■ total 4 PAHs; different lowercase letter showed significant difference from other PAHs at the same total concentration. *Significant difference from control of each compound. **A**: Shoot length of sticky rice, **B**: Shoot length of water morning glory, **C**: Root length of sticky rice, **D**: Root length of water morning glory, **E**: Fresh weight of sticky rice, **F**: Fresh weight of water morning glory, **G**: dried weight of sticky rice, **H**: dried weight of water morning glory.

Combined phytotoxicity of anthracene with 2 or 3 other PAHs

The combined phytotoxicity of anthracene and other PAHs did not affect the shoot length of sticky rice (**Figure 2A**). The presence of anthracene with phenanthrene and fluorene was the most toxic to root length of rice at total concentrations ranging from 2 - 200 mg/kg (**Figure 2C**). When total concentration was 400 mg/kg, both anthracene+phenanthrene+fluorene and anthracene+fluorene+fluoranthene decreased the root length of rice significantly. The presence of anthracene with fluorene and fluoranthene was the most toxic to the shoot and root length of water morning glory (**Figures 2B** and **2D**). The presence of anthracene with fluorene and fluoranthene was the most toxic to the fresh weight of the sticky rice seedlings at a total concentration of 400 mg/kg. This combination was more toxic than the presence of 4 PAHs together at the same total concentration (**Figure 2E**). For the water morning glory seedlings, the fresh weight was decreased significantly when the total concentration was 400 mg/kg, but there were no differences among the PAH combinations (**Figure 2F**). The presence of anthracene with other PAHs at the highest total concentration did not affect the dried weight of either plant type (**Figures 2G** and **2H**).

When anthracene and phenanthrene were present in the soil, the addition of fluorene or fluoranthene increased the synergistic effect on the sticky rice's root length, but there was an antagonistic effect between PAHs in the absence of phenanthrene. Only fluorene increased the synergistic effect of anthracene and phenanthrene on the water morning glory's root length. However, the synergistic effect on fresh weight was not detected (**Table 1**).

The toxicity of anthracene with 3 other PAHs to the shoot and root length of rice was not different from the toxicity of anthracene with 2 other PAHs. Only anthracene+phenanthrene+fluorene decreased the root length of rice more than a combination of all the PAHs. Anthracene with 3 other PAHs was not toxic to the fresh weight and dry weight of sticky rice seedlings. Additionally, this combination did not affect the dry weight of water morning glory seedlings. However, the presence of 4 PAHs at a total concentration of 200 mg/kg significantly decreased the fresh weight of water morning glory seedlings, but this decrease was not different from the toxicity of anthracene with 2 other PAHs.

The elongation of shoot and root in plants involves several endogenous hormones, such as auxin, cytokinin, gibberellin, ethylene and abscisic acid. Seed germination requires a balance between gibberellin and abscisic acid level [21]. PAHs also have been reported to disrupt endogenous hormone levels. For example, the presence of fluoranthene was reported to induce abscisic acid and ethylene levels in pea plants and then induce formation of lysigenous intercellular space [22,23]. In our study, the presence of fluorene or fluoranthene with anthracene decreased the shoot and root length of sticky rice more than anthracene with phenanthrene or anthracene alone. It is possible that fluorene and fluoranthene may disrupt endogenous hormone levels, especially abscisic acid and ethylene, in sticky rice more than the other 2 PAHs, and that this disruption affects shoot and root elongation. The additive index showed that there were synergistic effects between anthracene alone or with phenanthrene and fluorene or fluoranthene in sticky rice (**Table 1**). However, all tested PAHs were toxic to the shoot and root length of water morning glory. Anthracene with fluorene and fluoranthene seemed to be the most toxic.

When organic pollutants present together in the environment, the interaction between each organic pollutant and test organism will be synergistic or antagonistic, depending on the species, experimental condition, ratio and type of each pollutant. Additionally, synergistic interactions often occur between different toxic mechanisms of each pollutant [14,15]. In our study, the interaction of anthracene with fluorene was synergistic to both plant's growth. There have been reports that anthracene disrupted photosynthesis in plants [7], but that fluorene disrupted water accumulation in plants [24]. This finding may be the reason behind the synergistic effect between these compounds. The mechanism underlying the physiological levels of these combined toxins should be studied in greater detail.

In this study, the combined toxicity of anthracene with other PAHs was investigated only during the seedling growth period. The young seedlings were exposed during the early period to pollutants in soil [13]. Poor growth of seedlings was not a good indicator for use in phytoremediation or the yield in the next period. The bioaccumulation of each PAH was not measured in this study. However, the accumulation of PAHs in plants after exposure for 1 week were reported in *Salicornia fragilis* [25] and rice seedlings grown in 72 mg/kg phenanthrene-contaminated soil for eight weeks could accumulate phenanthrene as 0.05 and 0.5 mg/kg in shoot and root, respectively [26]. The current limitation of the complete description of PAH toxicity pathways in crops means that future studies are needed to dissect the complex mechanism of PAH phytotoxicity. The combined effect of PAHs on phytoaccumulation and phytostimulation capacity will be performed in future experiments.

Conclusions

The phytotoxicity of different combinations of phenanthrene, fluorene, and fluoranthrene in anthracene-contaminated soil to seedling growth were different. Anthracene + fluorene and anthracene + fluoranthene were more toxic to growth of sticky rice seedlings than anthracene alone or anthracene + phenanthrene. However, all combinations of anthracene with other PAHs were toxic to water morning glory seedlings. When there were 3 - 4 PAHs present together, anthracene + fluoranthene + fluorene was the most toxic combination to shoot length, root length, and fresh weight of both plants, but not for dry weight. The responses of dicot and monocot to toxicity of PAH co-contamination were different when anthracene was present alone or with other PAHs.

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