

# Acute and Joint Toxicity of Twelve Substituted Benzene Compounds to *Prosilocerus akamusi* Tokunaga

## Research Article

Chuan-Wang Cao, Fang Niu, Xiao-Peng Li, Shi-Lin Ge, Zhi-Ying Wang\*

School of Forestry, Northeast Forestry University,  
Harbin 150040, China

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**Abstract:** This study investigated the toxic effects of 12 substituted benzenes exposed to *Prosilocerus akamusi* larvae singly and as mixtures. Their toxicities were quantified in terms of median effective concentration ( $EC_{50}$ ) killing 50% of the larvae. For individual substituted benzenes to 4th-instar *P. akamusi* larvae, the toxicity was in decreasing order of p-chlorophenol > nitrobenzene > phenol > 1,2-dimethylbenzene > 1,3-dimethylbenzene > chlorobenzene > p-phenylenediamine > 1,4-dimethylbenzene > m-phenylenediamine > methylbenzene > benzene > aniline. The order of toxicity among three isomers of dimethylbenzene was 1,2-dimethylbenzene > 1,3-dimethylbenzene > 1,4-dimethylbenzene while p-phenylenediamine > m-phenylenediamine. The binary substituted benzene compounds' toxicities were evaluated by toxic unit (TU), additive index (AI), mixture toxicity index (MTI) and similarity parameter index ( $\lambda$ ). The evaluation results of TU and MTI for 9 substituted benzene compounds were completely consistent while the results of AI were the same as the results of  $\lambda$  based on 24 h  $EC_{50}$  of binary substituted benzenes. The evaluation results of 10 substituted benzene compounds were consistent using TU, MTI, AI and  $\lambda$  evaluation methods. 52.63% and 47.37% of binary substituted benzene tests on *P. akamusi* larvae showed synergism and partial addition/antagonism, respectively, under mixtures of equal proportions. These results suggest that substituted benzenes indicate acute and binary joint toxicity to *P. akamusi*.

**Keywords:** Acute toxicity • Joint toxicity • Substituted benzene • *Prosilocerus akamusi*

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## 1. Introduction

Increasingly, there is need to monitor the environment for the presence of pollutants. Many important chemical compounds are derived from benzene by replacing one or more of its hydrogen atoms with another functional group. For example, benzene compound with just a hydroxyl group substituent is phenol and which with a methyl group substituent is toluene. When there is more than one substituent present on the ring, their spatial relationship becomes important, for which the arene substitution patterns ortho, meta and para were devised. For example, three isomers exist for dimethylbenzene because the two methyl groups can be placed next to each other (ortho), one position removed from each other (meta), or two positions removed from each other (para).

Benzene and substituted benzene are important pathways of water and soil contamination. In the US

alone, there are approximately 100,000 different sites that have benzene soil or groundwater contamination [1]. Benzene and substituted benzene exposure to humans are widely studied in terms of biochemical markers and molecular toxicology [2-6]. However, little is known about the acute toxicity of benzene and benzene derivatives to aquatic biota. Recently, bioassays are commonly used in environmental monitoring and depend upon well-established biological endpoints that correlate with pollutant exposure. The use of Chironomidae (midge) larvae as target organisms in toxicity tests has many advantages, including the ease of handling, their short lifespans and high sensitivity to toxicants as well as the easy laboratory maintenance of cultures [7]. Previous studies have reported the acute toxicity of contaminants to Chironomidae. These pollutants mainly focused on pesticides, heavy metals and herbicides [8,9]. In order to investigate the toxicity of basic substituted benzenes to midges, we tested the acute and joint toxicity of

\* E-mail: zhiyingwangnefu@aliyun.com

twelve common substituted benzene compounds on Chironomidae larvae, *Prosilocerus akamusi*. Our study provides pertinent information on the biological response of *P. akamusi* larvae to substituted benzenes in aquatic environments. Our results are expected to provide researchers with new insights into the assessment of substituted benzenes' effects on aquatic invertebrates.

## 2. Experimental Procedures

### 2.1 Organisms

The aquatic midges, *Prosilocerus akamusi* Tokunaga, were obtained from Harbin Dafa Market (Harbin, Heilongjiang, China), and were cultured in accordance with standard protocols with slight modification [10]. Briefly, instead of collecting and separating eggs masses, the midges were reared in mixed-age cultures. Instar determination was achieved using length estimates. Fourth-instar midges were harvested directly from the mixed-age culture and used in the bioassays.

### 2.2 Chemicals

Twelve substituted benzenes were used in this study. These test chemicals along with their trade name, chemical name, formula, chemical structure, CAS number, octanol-water-partitioning coefficient (Kow) and relative purity are listed in Table 1. All the chemicals were purchased from Sigma (St. Louis, MO). The twelve substituted benzenes were prepared in dechlorinated water (water temperature  $25 \pm 1^\circ\text{C}$  and pH 7.58) as a  $100\,000\text{ mg L}^{-1}$  stock solution in dimethylsulfoxide prior to serial dilution for use in bioassays.

### 2.3 Single toxicity experiments

The effects of substituted benzenes on *P. akamusi* were evaluated by exposing 4th-instar midges to a range of concentrations of these compounds. The acute toxicity of each compound was assayed individually using 6, 12, 24 and 48-hour static toxicity tests. Tests were conducted in 100-mL plastic cups containing 50 mL of test solution comprised of dechlorinated water as described earlier and prepared stock solution of the test compound. A stock solution of the test compound was diluted to seven concentrations. Thirty 4th-instar larvae were randomly assigned in each of the control and seven test treatments. Test plastic cups were not aerated and test solutions were not renewed during the tests. The test plastic cups were covered with clear plastic film to minimize evaporation. Tests were conducted at room temperature with photoperiod of 16 hours light to 8 hours dark. The numbers of immobilized midges were recorded at 6, 12, 24 and

48 h using a dissecting microscope ( $10\times$  magnification). Immobilization was defined as the cessation of all visible signs of movement or activity when viewed under the dissecting microscope. Each experiment was repeated using a different batch of midge larvae. An experiment was considered valid if mortality was lower than 10% in the control at the end of the test and dissolved oxygen was higher than 20% saturation.

The nominal substituted benzene concentrations used for the tests ranged from 30 to  $500\text{ mg L}^{-1}$  for chlorobenzene, nitrobenzene, 1,4-dimethylbenzene, 1,2-dimethylbenzene, 1,3-dimethylbenzene, phenol, p-chlorophenol, p-phenylenediamine and m-phenylenediamine, between 150 and  $1200\text{ mg/L}$  for methylbenzene and benzene and between 600 and  $1800\text{ mg L}^{-1}$  for aniline. These test concentrations were chosen based on earlier range finding tests.

### 2.4 Binary mixture toxicity experiments

The joint toxicity of the substituted benzenes to midges was assessed by exposing midge larvae to a mixture of the two substituted benzenes according to the Pape-Lindstrom and Lydy method with modification [11]. Briefly, the binary mixtures were combined at 1:1 of the toxic unit (TU) ratio and the 24 h effective concentrations ( $EC_{50}$ ) of binary mixtures were obtained in a similar manner as the single toxicity tests. Concurrent testing of the individual substituted benzene and the mixture was undertaken to allow direct comparison of the toxicity of the individual compounds to the mixture. The test protocol was as described earlier, with the three replicate tests analyzed separately.

### 2.5 Statistical analyses

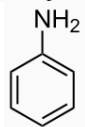
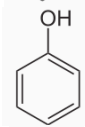
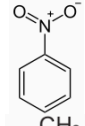
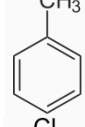
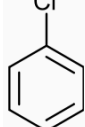
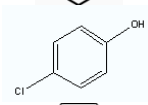
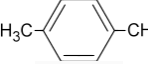
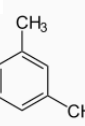
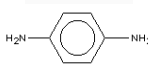
#### 2.5.1 Toxic Unit (TU)

We chose the modified toxic unit approach to model joint toxicity [12], primarily for its ease of use and understanding. In the toxic unit (TU) model, a value of 1 TU is assigned to the 50% effective concentration ( $EC_{50}$ ) value of contaminant. A sum of the TU contributed by each component describes the toxicity of a mixture as follows:

$$M = \sum_{i=1}^n TU_i = \frac{C_1}{EC_{50,1}} + \frac{C_2}{EC_{50,2}} + \dots + \frac{C_n}{EC_{50,n}}$$

$$M_0 = \frac{M}{(TU_i)_{\max}}$$

where  $C_n$  is the concentration of a chemical in a mixture and  $EC_{50n}$  is the  $EC_{50}$  for the respective component chemicals of the mixture from 1 to  $n$  [13].

| Trade name         | Chemical name          | Formula                                                       | Chemical structure                                                                   | CAS No.  | Log Kow | Purity (%) |
|--------------------|------------------------|---------------------------------------------------------------|--------------------------------------------------------------------------------------|----------|---------|------------|
| Benzene            | 1,3,5-cyclohexatriene  | C <sub>6</sub> H <sub>6</sub>                                 |    | 108-95-2 | 2.15    | 99.5%      |
| Aniline            | Aminobenzene           | C <sub>6</sub> H <sub>5</sub> NH <sub>2</sub>                 |    | 62-53-3  | 0.94    | 99.5%      |
| Phenol             | Hydroxybenzene         | C <sub>6</sub> H <sub>6</sub> O                               |    | 108-95-2 | 1.46    | 99.98%     |
| Nitrobenzol        | Nitrobenzene           | C <sub>6</sub> H <sub>5</sub> NO <sub>2</sub>                 |    | 98-95-3  | 1.86    | 99.0%      |
| Toluene            | Methylbenzene          | C <sub>7</sub> H <sub>8</sub>                                 |    | 108-88-3 | 2.73    | 99.5%      |
| benzene chloride   | Chlorobenzene          | C <sub>6</sub> H <sub>5</sub> Cl                              |   | 108-90-7 | 2.84    | 99.99%     |
| p-chlorophenol     | 4-hydroxychlorobenzene | C <sub>6</sub> H <sub>5</sub> ClO                             |  | 106-48-9 | 2.39    | 99.0%      |
| p-xylene           | 1,4-dimethylbenzene    | CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> CH <sub>3</sub> |  | 106-42-3 | 3.15    | 98.5%      |
| o-xylene           | 1,2-dimethylbenzene    | CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> CH <sub>3</sub> |  | 95-47-6  | 3.12    | 99.99%     |
| m-xylene           | 1,3-dimethylbenzene    | CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> CH <sub>3</sub> |  | 108-38-3 | 3.20    | 95%        |
| p-phenylenediamine | 1,4-benzenediamine     | C <sub>6</sub> H <sub>8</sub> N <sub>2</sub>                  |  | 106-50-3 | -0.30   | 98.5%      |
| m-phenylenediamine | 1,3-phenylenediamine   | C <sub>6</sub> H <sub>8</sub> N <sub>2</sub>                  |  | 108-45-2 | -0.42   | 99.5%      |

**Table 1.** Physical and chemical characteristics of 12 substituted benzene compounds used in toxicity tests.

CAS=Chemical Abstracts Service.

The empirically measured toxicity can then be compared to the expected toxicity predicted by M. When 50% mortality occurs at TU values lower than 1, the mixture is exhibiting greater than additive toxicity (synergism). Otherwise, when 50% mortality occurs at TU values greater than 1, the mixture is less than additive toxicity (antagonism).

### 2.5.2 Additive Index (AI)

The AI was extracted as described by Marking [12]:

$$AI = \begin{cases} 1/M - 1 & \text{if } M \leq 1 \\ 1 - M & \text{if } M > 1 \end{cases}$$

where M is the sum of the concentrations that was expressed as equal fractions of the EC<sub>50</sub> of each

component ( $M = \sum TU_i$ ). Simple addition is characterized by  $AI = 0$ ,  $AI < 0$  represents antagonism, and  $AI > 0$  indicates synergism.

### 2.5.3 Mixture Toxicity Index (MTI)

The MTI is determined using methodology originally described by K  nemann [14], according to the equation:

$$MTI = 1 - (\log M / \log M_0)$$

where  $M$  is the sum of the concentrations that was expressed as equal fractions of the  $EC_{50}$  of each component ( $M = \sum TU_i$ ).  $M_0 = M/\max(TU_i)$ . The Table 2 was employed as a measure of the mixture toxicity scale [15].

### 2.5.4 Similarity Parameter

The similarity parameter is determined using methodology originally described by Christensen and Chen [16], according to the equation:

$$\sum_{i=1}^n (TU_i)^{1/\lambda} = 1$$

where  $\lambda$  is the similarity parameter and  $TU_i$  is the toxic unit of each respective chemical in the mixture.

## 3. Results

### 3.1 Individual substituted benzene toxicity

The results from individual toxicity tests of 12 substituted benzenes are listed in Table 3. The range of 24 h  $EC_{50}$  of 12 substituted benzenes to *P. akamusi* is from 90 to 1100 mg L<sup>-1</sup>. Among the 12 substituted benzenes, p-chlorophenol was found to be the most highly toxic compound with 90.1 mg L<sup>-1</sup> of 24 h  $EC_{50}$  while aniline was least toxic to *P. akamusi* with 1069.7 mg L<sup>-1</sup> of 24 h  $EC_{50}$ . For individual substituted benzenes, the  $EC_{50}$  values gradually decreased with increasing treatment time. The 6 h  $EC_{50}$  of nitrobenzene is 4.08-fold of the

48 h  $EC_{50}$  while the 6 h  $EC_{50}$  of 1,2-dimethylbenzene is 1.28-fold of the 48 h  $EC_{50}$ . The toxicity effects of 12 substituted benzenes may come from different substituted groups on the benzene ring. For the isomers of dimethylbenzenes and nitrobenzenes, the toxicities of the isomers of dimethylbenzenes to *P. akamusi* were in order of 1,2-dimethylbenzene > 1,3-dimethylbenzene > 1,4-dimethylbenzene while the toxicities of the isomers of phenylenediamines to *P. akamusi* were in order of p-phenylenediamine > m-phenylenediamine.

### 3.2 Binary substituted benzene toxicity

Nineteen combinations of binary mixtures of substituted benzenes were found in the present study. The sum of TU ( $M$  value) contributed by binary substituted benzenes ranged from 0.600 to 1.660. The  $M$  value of methylbenzene combined with 1,4-dimethylbenzene (0.600) was lowest, showing synergism. However, the  $M$  value of nitrobenzene combined with 1,3-methylbenzene (1.660) was highest, showing a partially additive effect (Table 4). Among 19 binary substituted benzenes, the  $M$  values of ten pairs (aniline + benzene, methylbenzene + benzene, nitrobenzene + chlorobenzene, chlorobenzene + methylbenzene, chlorobenzene + benzene, nitrobenzene + methylbenzene, phenol + nitrobenzene, phenol + benzene, nitrobenzene + benzene, methylbenzene + 1,4-dimethylbenzene) were lower than 1, showing synergistic effects. Nine pairs (benzene + 1,3-dimethylbenzene, benzene + 1,2-dimethylbenzene, benzene + 1,4-dimethylbenzene, nitrobenzene + 1,3-dimethylbenzene, phenol + 1,4-dimethylbenzene, nitrobenzene + 1,4-dimethylbenzene, methylbenzene + 1,2-dimethylbenzene, nitrobenzene + 1,2-dimethylbenzene, methylbenzene + 1,3-dimethylbenzene) showed partial additive effects (Table 5). According to the study of Broderius [17], concentration addition is characterized by  $M = 1 \pm 0.2$ , where  $M < 0.8$  represents synergism and  $M > 1.2$  indicates antagonism. Thus, 31.57% of 19 substituted benzenes showed synergistic effects and additive effects, while 36.84% of these compounds showed antagonistic effects.

According to additive index evaluation, the  $AI$  of these 19 substituted benzenes was from -0.660 to 0.666. The lowest  $AI$  value (-0.660) was nitrobenzene + 1,3-methylbenzene, showing antagonistic effects, while the  $AI$  of methylbenzene + 1,4-methylbenzene (0.666) was highest, indicating synergistic effects (Table 4). 52.63% of 19 substituted benzenes showed synergistic effects while 47.37% showed antagonistic effects (Table 5).

The range of MTI was from 0.269 to 1.736. The MTI value of nitrobenzene + 1,3-methylbenzene

| MTI         | Classification for toxicity of mixtures                                                      |
|-------------|----------------------------------------------------------------------------------------------|
| MTI < 0     | Antagonism                                                                                   |
| MTI = 0     | No addition (independent action)                                                             |
| 0 < MTI < 1 | Partial addition                                                                             |
| MTI = 1     | Concentration addition (sample similar action)                                               |
| MTI > 1     | Synergism (potentiation of the toxic actions of one or more of the compounds in the mixture) |

**Table 2.** Toxicity classification of mixtures according to MTI value.

| Substituted benzene | Treatment time (h) | EC <sub>50</sub> (95% Confidence Intervals) (mg L <sup>-1</sup> ) | Slope±SE | χ <sup>2</sup> <sub>(19)</sub> |
|---------------------|--------------------|-------------------------------------------------------------------|----------|--------------------------------|
| Methylbenzene       | 6                  | 692.3 (605.9-746.9)                                               | 7.6±1.4  | 18.3                           |
|                     | 12                 | 610.6 (527.8-661.9)                                               | 10.6±2.2 | 8.9                            |
|                     | 24                 | 566.0 (481.5-614.7)                                               | 8.7±1.8  | 12.4                           |
|                     | 48                 | 531.0 (443.0-574.5)                                               | 13.0±3.5 | 12.6                           |
| Aniline             | 6                  | 1593.3 (1436.8-1840.2)                                            | 3.5±0.5  | 12.6                           |
|                     | 12                 | 1278.4 (1151.2-1359.1)                                            | 9.4±1.8  | 15.9                           |
|                     | 24                 | 1069.7 (914.6-1180.4)                                             | 6.3±1.0  | 9.4                            |
|                     | 48                 | 825.8 (637.1-950.7)                                               | 5.0±1.1  | 6.2                            |
| Phenol              | 6                  | 222.5 (178.6-264.4)                                               | 3.2±0.6  | 26.0                           |
|                     | 12                 | 184.8 (130.9-220.9)                                               | 3.9±0.7  | 43.7                           |
|                     | 24                 | 134.9 (85.5-164.1)                                                | 4.7±0.9  | 21.3                           |
|                     | 48                 | 67.7 (26.3-99.2)                                                  | 2.5±0.5  | 18.9                           |
| Chlorobenzene       | 6                  | 306.7 (232.6-356.9)                                               | 2.4±0.4  | 3.6                            |
|                     | 12                 | 228.5 (143.1-282.1)                                               | 3.7±0.9  | 6.8                            |
|                     | 24                 | 189.0 (104.5-235.8)                                               | 4.0±1.1  | 11.3                           |
|                     | 48                 | 157.9 (64.8-203.5)                                                | 4.6±1.7  | 5.2                            |
| Nitrobenzene        | 6                  | 347.8 (253.1-374.1)                                               | 2.3±0.3  | 6.4                            |
|                     | 12                 | 176.1 (139.8-203.4)                                               | 3.9±0.6  | 20.2                           |
|                     | 24                 | 106.0 (66.2-137.9)                                                | 2.8±0.5  | 14.2                           |
|                     | 48                 | 85.3 (46.9-113.8)                                                 | 3.1±0.7  | 9.9                            |
| 1,4-dimethylbenzene | 6                  | 432.0 (359.1-585.0)                                               | 3.0±0.4  | 42.9                           |
|                     | 12                 | 403.3 (367.0-449.2)                                               | 5.3±1.0  | 13.6                           |
|                     | 24                 | 305.2 (278.8-323.8)                                               | 11.6±2.0 | 21.7                           |
|                     | 48                 | 294.7 (258.5-317.3)                                               | 10.8±2.2 | 20.6                           |
| 1,2-dimethylbenzene | 6                  | 193.7 (166.0-239.5)                                               | 2.5±0.3  | 16.3                           |
|                     | 12                 | 166.8 (147.5-190.6)                                               | 3.6±0.6  | 8.8                            |
|                     | 24                 | 156.4 (133.2-175.9)                                               | 4.4±0.9  | 18.4                           |
|                     | 48                 | 127.8 (98.2-145.7)                                                | 6.3±1.5  | 3.1                            |
| 1,3-dimethylbenzene | 6                  | 337.2 (287.5-412.8)                                               | 2.1±0.3  | 19.0                           |
|                     | 12                 | 189.3 (126.0-230.7)                                               | 3.1±0.7  | 16.5                           |
|                     | 24                 | 183.7 (129.2-215.5)                                               | 4.5±1.1  | 7.4                            |
|                     | 48                 | 157.7 (80.0-185.0)                                                | 5.4±2.2  | 15.3                           |
| Benzene             | 6                  | 812.9 (700.3-891.8)                                               | 5.7±1.2  | 11.2                           |
|                     | 12                 | 777.0 (669.2-842.9)                                               | 8.6±1.8  | 10.8                           |
|                     | 24                 | 670.0 (513.7-755.9)                                               | 5.8±1.3  | 13.6                           |
|                     | 48                 | 341.5 (169.0-445.2)                                               | 3.6±0.9  | 6.6                            |
| p-chlorophenol      | 6                  | 134.2 (106.7-150.2)                                               | 6.4±1.5  | 5.4                            |
|                     | 12                 | 125.2 (109.7-135.7)                                               | 7.5±1.4  | 11.9                           |
|                     | 24                 | 90.1 (71.3-102.6)                                                 | 5.2±1.0  | 9.8                            |
|                     | 48                 | 61.8 (20.4-79.0)                                                  | 4.4±1.5  | 8.7                            |
| p-phenylenediamine  | 6                  | 460.3 (386.6-622.6)                                               | 2.3±0.4  | 4.8                            |
|                     | 12                 | 401.2 (312.9-550.3)                                               | 1.9±0.5  | 8.9                            |
|                     | 24                 | 226.5 (172.2-261.3)                                               | 4.7±0.9  | 15.7                           |
|                     | 48                 | 141.9 (104.8-171.7)                                               | 6.1±1.5  | 11.8                           |
| m-phenylenediamine  | 6                  | 673.3 (595.6-764.8)                                               | 3.9±0.6  | 3.7                            |
|                     | 12                 | 505.9 (422.8-602.9)                                               | 2.8±0.4  | 9.7                            |
|                     | 24                 | 362.7 (216.4-483.8)                                               | 2.3±0.6  | 2.3                            |
|                     | 48                 | 205.9 (132.7-253.3)                                               | 4.5±1.1  | 4.3                            |

**Table 3.** Acute toxicity of 12 substituted benzene compounds to 4<sup>th</sup>-instar *P. akamusi*.

EC<sub>50</sub> = Median Effective concentration.

| Substituted benzene                 | M     | AI     | MTI   | $\lambda$ |
|-------------------------------------|-------|--------|-------|-----------|
| benzene + 1,3-dimethylbenzene       | 1.576 | -0.576 | 0.343 | 0.095     |
| benzene + 1,2-dimethylbenzene       | 1.491 | -0.491 | 0.424 | 0.101     |
| benzene + 1,4-dimethylbenzene       | 1.293 | -0.293 | 0.629 | 0.152     |
| nitrobenzene + 1,3-dimethylbenzene  | 1.660 | -0.660 | 0.269 | 0.269     |
| phenol + 1,4-dimethylbenzene        | 1.205 | -0.205 | 0.731 | 0.707     |
| nitrobenzene + 1,4-dimethylbenzene  | 1.224 | -0.224 | 0.708 | 0.708     |
| methylbenzene + 1,2-dimethylbenzene | 1.252 | -0.252 | 0.676 | 0.733     |
| nitrobenzene + 1,2-dimethylbenzene  | 1.061 | -0.061 | 0.914 | 0.914     |
| methylbenzene + 1,3-dimethylbenzene | 1.042 | -0.042 | 0.940 | 0.990     |
| aniline + benzene                   | 0.922 | 0.084  | 1.117 | 1.117     |
| methylbenzene + benzene             | 0.886 | 0.129  | 1.175 | 1.175     |
| nitrobenzene + chlorobenzene        | 0.817 | 0.224  | 1.292 | 1.292     |
| chlorobenzene + methylbenzene       | 0.805 | 0.242  | 1.312 | 1.312     |
| chlorobenzene + benzene             | 0.775 | 0.290  | 1.368 | 1.368     |
| nitrobenzene + methylbenzene        | 0.755 | 0.324  | 1.405 | 1.405     |
| phenol + nitrobenzene               | 0.752 | 0.329  | 1.410 | 1.410     |
| phenol + benzene                    | 0.714 | 0.400  | 1.485 | 1.485     |
| nitrobenzene + benzene              | 0.663 | 0.509  | 1.593 | 1.593     |
| methylbenzene + 1,4-dimethylbenzene | 0.600 | 0.666  | 1.736 | 1.815     |

**Table 4.** Parameters of 4 evaluation methods of substituted benzene compounds.

was 0.269, showing partial addition. However, the highest MTI value (1.736) of these compounds was methylbenzene + 1,4-dimethylbenzene, showing synergism (Table 4). Among 19 substituted benzenes, 52.63% of binary substituted benzenes (aniline + benzene, methylbenzene + benzene, nitrobenzene + chlorobenzene, chlorobenzene + methylbenzene, chlorobenzene + benzene, nitrobenzene + methylbenzene, phenol + nitrobenzene, phenol + benzene, nitrobenzene + benzene, methylbenzene + 1,4-dimethylbenzene) showed synergistic effects, while 47.37% of these substituted benzenes (benzene + 1,3-dimethylbenzene, benzene + 1,2-dimethylbenzene, benzene + 1,4-dimethylbenzene, nitrobenzene + 1,3-dimethylbenzene, phenol + 1,4-dimethylbenzene, nitrobenzene + 1,4-dimethylbenzene, methylbenzene + 1,2-dimethylbenzene, nitrobenzene + 1,2-dimethylbenzene, methylbenzene + 1,3-dimethylbenzene) showed partial addition (Table 5).

Similarity parameter evaluation indicated the range of the  $\lambda$  index was 0.095 to 1.815 (Table 4). Ten binary substituted benzenes (aniline + benzene, methylbenzene + benzene, nitrobenzene + chlorobenzene, chlorobenzene + methylbenzene, chlorobenzene + benzene, nitrobenzene + methylbenzene, phenol

+ nitrobenzene, phenol + benzene, nitrobenzene + benzene, methylbenzene + 1,4-dimethylbenzene) showed synergistic joint action. Nine binary mixtures of these substituted benzenes (benzene + 1,3-dimethylbenzene, benzene + 1,2-dimethylbenzene, benzene + 1,4-dimethylbenzene, nitrobenzene + 1,3-dimethylbenzene, phenol + 1,4-dimethylbenzene, nitrobenzene + 1,4-dimethylbenzene, methylbenzene + 1,2-dimethylbenzene, nitrobenzene + 1,2-dimethylbenzene, methylbenzene + 1,3-dimethylbenzene) showed antagonistic joint action (Table 5).

## 4. Discussion

The substituted benzenes are a family of organic pollutants that will be harmful to human health by destroying water ecosystem balance. The toxicity of benzene, hydroxylbenzene (phenol), chlorobenzene, methylbenzene (toluene) and dimethylbenzene (xylene) to four chemolithotrophic bacteria (*Nitrosomonas*, *Nitrobacter*, *Thiobacillus* and *Leptothrix*) isolated from New Calabar River water were investigated by the static method of acute toxicity assessment. The results indicate

| Substituted benzene compounds       | M                | AI         | MTI              | $\lambda$  |
|-------------------------------------|------------------|------------|------------------|------------|
| benzene + 1,3-dimethylbenzene       | Partial addition | Antagonism | Partial addition | Antagonism |
| benzene + 1,2-dimethylbenzene       | Partial addition | Antagonism | Partial addition | Antagonism |
| benzene + 1,4-dimethylbenzene       | Partial addition | Antagonism | Partial addition | Antagonism |
| nitrobenzene + 1,3-dimethylbenzene  | Partial addition | Antagonism | Partial addition | Antagonism |
| phenol + 1,4-dimethylbenzene        | Partial addition | Antagonism | Partial addition | Antagonism |
| nitrobenzene + 1,4-dimethylbenzene  | Partial addition | Antagonism | Partial addition | Antagonism |
| methylbenzene + 1,2-dimethylbenzene | Partial addition | Antagonism | Partial addition | Antagonism |
| nitrobenzene + 1,2-dimethylbenzene  | Partial addition | Antagonism | Partial addition | Antagonism |
| methylbenzene + 1,3-dimethylbenzene | Partial addition | Antagonism | Partial addition | Antagonism |
| aniline + benzene                   | Synergism        | Synergism  | Synergism        | Synergism  |
| methylbenzene + benzene             | Synergism        | Synergism  | Synergism        | Synergism  |
| nitrobenzene + chlorobenzene        | Synergism        | Synergism  | Synergism        | Synergism  |
| chlorobenzene + methylbenzene       | Synergism        | Synergism  | Synergism        | Synergism  |
| chlorobenzene + benzene             | Synergism        | Synergism  | Synergism        | Synergism  |
| nitrobenzene + methylbenzene        | Synergism        | Synergism  | Synergism        | Synergism  |
| phenol + nitrobenzene               | Synergism        | Synergism  | Synergism        | Synergism  |
| phenol + benzene                    | Synergism        | Synergism  | Synergism        | Synergism  |
| nitrobenzene + benzene              | Synergism        | Synergism  | Synergism        | Synergism  |
| methylbenzene + 1,4-dimethylbenzene | Synergism        | Synergism  | Synergism        | Synergism  |

**Table 5.** Evaluation results of binary substituted benzene compounds.

that hydroxyl and chlorosubstituted derivatives of benzene may pose a greater toxicity problem to microbiota than methyl-substituted derivatives of benzene in the New Calabar River [18]. However, very little is known about the acute toxicity and mixed toxicity of these compounds to aquatic midges. The present study evaluated the single toxicity and mixed toxicity of substituted benzenes on aquatic midges, *P. akamusi*. Exposed to methylbenzene, chlorobenzene, phenol and p-chlorophenol, the *P. akamusi* larvae indicated lower motion showing anesthetization. Phenol has corrosive effects on chironomid larvae, resulting in increasing epidermis adhesion. The different toxic symptoms of chironomid larvae exposed to twelve substituted benzenes may come from different mechanisms of substituted benzenes.

Organic pollutants are divided into anesthetic toxicity pollutants and reactive toxicity pollutants [19]. Anesthetic toxicity is further divided into non-polar anesthetic toxicity and polar anesthetic toxicity. Anesthetic toxicity is basic toxicity showing low toxicity to organisms. The organic compounds with non-polar anesthetic toxicity destroy membranes but do not combine with biological macromolecules, whereas organic compounds with polar anesthetic toxicity are more toxic than organic compounds with basic toxicity [19]. Reactive toxic

organic compounds could interact with organisms and produce multiple toxicity such as covalent bonding.

The range of 24 h  $EC_{50}$  of twelve substituted benzenes is from 90 to 1100 mg L<sup>-1</sup>, showing low toxicity to 4<sup>th</sup>-instar midge larvae. However, the toxicity is higher than in fishes and crabs [20]. *P. akamusi* has high tolerance to substituted benzene pollutants, indicating high detoxifying abilities. Moreover, *P. akamusi* has high tolerance of drought because its special cuticle may prevent water evaporation [21]. According to 24h  $EC_{50}$  values and poisoning symptoms, methylbenzene, aniline, chlorobenzene, 1,2-dimethylbenzene, 1,3-dimethylbenzene and 1,4-dimethylbenzene were divided into non-polar anesthetic toxicity compounds. Some research has demonstrated that these compounds could accumulate on lipoprotein membranes, resulting in Na<sup>+</sup> channels damage on cell membranes. Phenol, p-chlorophenol and nitrobenzene have polar anesthetic toxicity. The –OH group on phenol and the –NO<sub>2</sub> on nitrobenzene produce toxicity through liposoluble action during transportation and interaction with receptors, and can further increase toxicity through binding hydrogen bonds. p-phenylenediamine and m-phenylenediamine with electrophilic NH<sub>2</sub> interact with biological macromolecules such as protein and DNA.

Based on the acute toxicity, the toxicity of groups are in decreasing order of  $-\text{NO}_2 > -\text{OH} > -\text{Cl} > -\text{CH}_3 > -\text{NH}_2$ . The toxicities of three dimethylbenzene isomers are 1,2-dimethylbenzene  $>$  1,3-dimethylbenzene  $>$  1,4-dimethylbenzene while the toxicities of phenylenediamine isomers are p-phenylenediamine  $>$  m-phenylenediamine. These results showed that atomic H of benzene substituted by different groups and different isomers of a substituted benzene resulted in different toxicity, because benzene ring structures changed by different groups produced different toxic mechanisms. Therefore, it is valuable for monitoring and evaluating water pollutants to study toxicity and toxic mechanisms of substituted benzenes to aquatic biotas.

Contaminants rarely occur in the environment as single compounds but rather as mixtures of different substances. Thus, when evaluating the environmental effects of substituted benzenes, mixtures also were considered in this study. Some researchers have reported the joint toxicity of the substituted benzene mixtures to luminescent bacteria, algae, water fleas and fish [22,23]. In this study, the joint toxicities of 19 substituted benzene mixtures were evaluated using TU, AI, MTI and  $\lambda$ . The results of TU were consistent with MTI while AI results were the same as  $\lambda$ . Nine groups (benzene + 1,3-dimethylbenzene, benzene + 1,2-dimethylbenzene, benzene + 1,4-dimethylbenzene, nitrobenzene + 1,3-dimethylbenzene, phenol + 1,4-dimethylbenzene, nitrobenzene + 1,4-dimethylbenzene, methylbenzene + 1,2-dimethylbenzene, nitrobenzene

+ 1,2-dimethylbenzene, methylbenzene + 1,3-dimethylbenzene) showed partial addition effects by TU and MTI analysis, but their binary toxicities were antagonistic joint action using AI and  $\lambda$ . These evaluation differences were from evaluation biases of each method: MTI and TU focus on joint toxicity of mixtures, while  $\lambda$  places emphasis on the deviation between joint action and additive action. Although these evaluation indexes could distinguish the joint action types, they could not differentiate the action degree. Therefore, further study on structure-activity relationships of mixtures is needed for evaluating and monitoring contaminant chemicals in water bodies.

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## Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

## References

- [1] Uzochukwu G., Proceedings of the 2007 national conference on environmental science and technology, Springer-Verlag, New York, 2009
- [2] Fustinoni S., Buratti M., Campo L., Colombi A., Consonni D., Pesatori A.C., et al., Urinary t,t-muconic acid, S-phenylmercapturic acid and benzene as biomarkers of low benzene exposure, *Chem-Biol. Interact.*, 2005, 153-154, 253-256
- [3] Baselt R., Disposition of Toxic Drugs and Chemicals in Man, 8th edition, Biomedical Publications, Foster City, CA., 2008, 144-148
- [4] Fracasso M.E., Doria D., Bartolucci G.B., Carrieri M., Lovreglio P., Ballini A., Soleo L., Tranfo, G et al. Low air levels of benzene: Correlation between biomarkers of exposure and genotoxic effects, *Toxicol. Lett.*, 2009, 192, 22-28
- [5] Eastmond D.A., Rupa D.S., Hasegawa L.S., Detection of hyperdiploidy and chromosome breakage in interphase human lymphocytes following exposure to the benzene metabolite hydroquinone using multicolor fluorescence in situ hybridization with DNA probes, *Mutat. Res.*, 2000, 322 (1), 9-20
- [6] Garte S., Taioli E., Popov T., Bolognesi C., Farmer P., Merlo F., Genetic susceptibility to benzene toxicity in humans, *J. Toxicol. Environ. Health A*, 2000, 71 (22), 1482-1489
- [7] Rakotondravelo M.L., Anderson T.D., Charlton R.E., Zhu K.Y., Sublethal effects of three pesticides on larval survivorship, growth, and macromolecule production in the aquatic midge, *Chironomus tentans* (Diptera: Chironomidae). *Arch. Environ. Con. Toxicol.*, 2006, 51, 352-359
- [8] Faria M.S., Nogueira A.J.A., Soares A.M.V.A., The use of *Chironomus riparius* larvae to assess effects of pesticides from rice fields in adjacent freshwater ecosystems, *Ecotox. Environ. Safe.*, 2007, 67, 218-226

- [9] Schuler L.J., Trimble A.J., Belden J.B., Lydy M.J., Joint toxicity of triazine herbicides and organophosphate insecticides to the midge *Chironomus tentans*. Arch. Environ. Con. Toxicol., 2005, 49, 173-177
- [10] US EPA, Standard operating procedures for laboratory cultures of *Chironomus tentans*. ERL-D-Sop CTI-015, Office of Research and Development, US Environmental Protection Agency, Duluth, MN, 1993
- [11] Pape-Lindstrom P.A., Lydy M.J., Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model, Environ. Toxicol. Chem., 1997, 16(11), 2415-2420
- [12] Marking L.L., Toxicity of chemical mixtures. In G.M.Rand and S.R. Petrocelli, eds., Fundamentals of Aquatic Toxicology: Methods and Applications, Hemisphere, New York, NY. USA, 1985, 164-176
- [13] McCarty L.S., Osburn G.W., Smith A.D., Dixon D.G., Toxicokinetic modeling of mixtures of organic chemicals. Environ. Toxicol. Chem., 1992, 11, 1037-1047
- [14] Könnemann H., Fish toxicity tests with mixtures of more than two chemicals: A proposal for a quantitative approach and experimental results, Toxicol., 1981, 19, 229-238
- [15] Lange J.H., Thomulka K.W., Use of the *Vibrio harveyi* toxicity test for evaluating mixture interactions of nitrobenzene and dinitrobenzene, Ecotox. Environ. Safe., 1997, 38, 2-12
- [16] Christensen E.R., Chen C.Y., Modeling of combined toxic effects of chemicals, In Hazard Assessment of Chemicals (C), NY: Hemisphere Publishing, 1989
- [17] Broderius S.J., Kahl M.D., Hoglund M.D., Use of joint toxic response to define the primary mode of toxic action for diverse industrial organic chemicals, Environ. Toxicol. Chem., 1995, 14, 1591-1605
- [18] Odokuma L., Oliwe O.S.I., Toxicity of substituted benzene derivatives to four chemolithotrophic bacteria isolated from the New Calabar River, Global. J. Environ. Sci., 2003, 2(2), 72-77
- [19] Blum D.J.W., Speece R.E., Determining chemical toxicity to aquatic species, Environ. Sci. Technol., 1990, 24, 284-293
- [20] Environmental Safety Assessment of Chemical Pesticides: State Environmental Protection Administration of China, Beijing: China Standards Press, 1989
- [21] Lewis M.A., The effects of mixtures and other environmental modifying factors on the toxicities of surfactants to freshwater and marine life, Water Res., 1993, 26 (8), 1013-1023
- [22] Warne M.A., Boyd E.M., Meharg A.A., Osborn D., Killham K., Lindon J.C., et al., Quantitative structure-toxicity relationships for halobenzenes in two species of bioluminescent bacteria, *Pseudomonas fluorescens* and *Vibrio fischeri*, using an atom-centered semi-empirical molecular-orbital based model, SAR QSAR Environ. Res., 1999, 10(1), 17-38
- [23] Lu G.H., Wang C., Yuan X., Zhao Y.H., QSAR study on the toxicity of substituted benzenes to the algae (*Scenedesmus obliquus*), Chemosphere, 2001, 44(3), 437-440