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Ecotoxicity Assessment of Potassium Hydrogen Phthalate and Verification of Standard Reference Toxicity Test Method Using Potassium Hydrogen Phthalate

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ABSTRACT

Phthalates are animal carcinogens. Potassium hydrogen phthalate (KHP), which has the least complicated structure among phthalates, is used for the analysis of total organic carbon and formaldehyde. However, its toxicity has not been confirmed. A 24-hour acute toxicity test was performed using *Daphnia magna*, a water flea used to evaluate aquatic toxicity owing to its high sensitivity. The lowest observed effect concentration of KHP was found to be 240 mg/L. The effects of phosphorus, nitrogen, and Cr(6+), which are able to be discharged along with KHP, were also confirmed using tests. At 240 mg/L KHP, toxicity increased as phosphorus, nitrogen, and Cr(6+) increased. In addition, tests were performed to confirm the half maximal effective concentration of KHP. Through 10 test repetitions, the average ecotoxicity value was found to be 0.3, the average half maximal effective concentration was 327.75 mg/L, and the coefficient of variation (%) was 3.16%; because the latter value is lower than 25%, which is what is generally suggested for the water pollution standard method, the reproducibility of the tests is sufficient to replace the existing standard reference toxicity test that uses potassium dichromate. In addition, the half maximum effective concentration of potassium hydrogen phthalate is approximately 218 times more than that of potassium dichromate; therefore, toxicity is relatively low. In conclusion, KHP is a feasible alternative to the highly toxic potassium dichromate for performing the standard reference toxicity test.

Keywords: Acute toxicity, *Daphnia magna*, Ecotoxicity, Half maximum effective concentration, Lowest observed effect concentration, Potassium hydrogen phthalate

Introduction

Phthalates increase the flexibility and elasticity of plastics and are extensively applied in medical devices and toys, among other products. However Phthalates are animal carcinogens (Shea, 2003). Di-2-ethylhexyl phthalate is classified by the International Agency for Research on Cancer (IARC, 2022) as a possible carcinogen (Group 2B). Introduction of products containing phthalates into water may affect aquatic organisms. Furthermore, caution

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is required in the use of phthalates to prevent exposure to humans. Incidentally, potassium hydrogen phthalate (KHP), which has the least complicated structure among phthalates, is used for the analysis of total organic carbon (TOC) and formaldehyde. Currently, waste fluid containing KHP can be discharged into aquatic ecosystems after treatment within the emission-standards set by the water pollution prevention facility, but its toxicity to the aquatic environment has not been confirmed. In the present study, a toxicity test was carried out using *Daphnia magna*, a sensitive water flea, in accordance with the water pollution standard method—acute toxicity test method using *D. magna* (ES 04704.1b).

Water fleas are crustaceans that live in freshwater. Their carapace is mainly composed of chitin, a polysaccharide, and their lifespan and size vary depending on the species (Ebert, 2005). The appearance of a female of the species *D. magna* is shown in Fig. 1 (NIER, 2014). This species is used to perform ecotoxicity tests according to the water pollution standard method (ES 04704.1b). It ranges in size from <0.5 mm to >6 mm, and in average lifespan from 40 days at 25°C to 56 days at 20°C (Ebert, 2005). The reproduction of *D. magna* is divided into parthenogenesis and sexual reproduction. When it is cultured in the recommended environment: ES 04704.1b, it produces female neonates. Upon maturity, the female neonates only produce females through parthenogenesis. At 20°C, they lay 6 to 10 eggs at a time, averaging 57 eggs over 50 days. However, if the culture environment is unsuitable, or the number of mature females exceeds 10 per 1 L of culture medium, a male is born and mates with a female who produces resting eggs (NIER, 2014). Because only female neonates should be used for the ecotoxicity test, maintaining an appropriate culture environment is important. To standardize experiments, the Ministry of Environment of the Republic of Korea has mandated that laboratories source *D. magna* from the National Institute of Environmental Research.

Fig. 1. Appearance of female *Daphnia magna.* Data from NIER. Guidelines for Ecotoxicity Test•Operation; 2014.

In the present study, i first exposed *D. magna* to KHP to confirm the lowest observed effect concentration (LOEC), which is the lowest concentration at which an effect is observed within 24 hours.

Second, the study attempted to confirm, at the LOEC of KHP, changes in toxicity based on the concentrations of other important factors. When wastewater containing KHP is treated and discharged, it can spread through river flow and wind up in lakes. In Korea, Ambient Water Quality Standard factors for Lakes include potential of hydrogen (pH), phosphorus (P), nitrogen (N), suspended solid (SS), dissolved oxygen (DO), Chlorophyll-a, *Escherichia coli*, chemical oxygen demand (COD), and TOC (ME, 2015). Therefore, the present study attempted to confirm the increase in the 24-hours acute toxicity at KHP's LOEC according to P and N concentration, and the increase in the 24-hours acute toxicity at KHP's LOEC according to the concentration of Cr(6+) constituting $K_2Cr_2O_7$ used in the standard reference toxicity test. The pH, SS, DO, Chlorophyll-a and *E. coli* were excluded because it was difficult to prepare specific concentrations. In addition, COD and TOC are organic substance concentration indicators, but are disregarded because KHP is an organic substance.

Third, the present study attempted to calculate the half maximal effective concentration and ecotoxicity of KHP. Finally, the study aimed to verify the standard reference toxicity test method using KHP by confirming its precision.

Materials and Methods

Water fleas

D. magna were sourced from the National Institute of Environmental Research, Korea, as mandated by the Ministry of Environment of the Republic of Korea.

Culture medium

To ensure the survival of *D. magna*, the culture medium was prepared in accordance with ES 04704.1b. Then 2.4 g $MgSO_4$, 3.84 g $NaHCO_3$, and 0.16 g KCl were accurately weighed and put in a 1 L beaker, dissolved with purified water, and stirred with a magnet (Solution 1). In a separate 1 L beaker, 2.4 g of $CaSO_4 \bullet 2H_2O$ was accurately, dissolved in purified water, and stirred with a magnet for 4 hours (Solution 2). Solutions 1 and 2 were added to a 20 L bottle, and it was topped up with purified water. The prepared 20 L solution was aerated for 1 day. The prepared culture medium should have at least 3.0 mg/L of DO, pH of 7.6-8.0, hardness of 160-180 as $CaCO_3$ mg/L, and alkalinity of 110-120 as $CaCO_3$ mg/L. The above items were measured at least once a week as shown in

Table 1. Culture medium analysis target ingredients and methods

Test item	Analysis method
pH	Water pollution standard method (ES 04306.1c)
DO	Water pollution standard method (ES 04308.2c)
Temperature	Water pollution standard method (ES 04307.1b)
Hardness	Drinking water quality testing standards (ES 05301.1b)
Alkalinity	Determination of alkalinity (KS I ISO9963-1)

pH, potential of hydrogen; DO, dissolved oxygen.

Fig. 2. An incubator for *Daphnia magna*.

Table 1, and confirmed to have no anomaly. Every Monday, Wednesday, and Friday, the *D. magna* individuals were transferred to a newly prepared culture medium using a dropper.

Culture condition and food

In accordance with ES 04704.1b, the culture temperature of *D. magna* was set to 20±2°C, and the illumination range to 500-1,000 Lux. The daily photoperiod was set to 16 hours for light and 8 hours for darkness. An incubator that can maintain temperature, light, and photoperiod was purchased to culture the *D. magna* (Fig. 2). For food, green algae (*Chlorella vulgaris*) and a mixture of Yeast, Cerophyll, and trout chow (YCT) were purchased from a food manufacturer (Chemtopia); a certificate stating the cell concentration of *C. vulgaris* (approximately 1.5×10^8 cells/mL) was provided.

Table 2. Feeding amount per 50 neonates according to the culture day

Culture day	*Chlorella vulgaris* (mL)	YCT (mL)
2nd day	0.3 mL/2 L	0.3 mL/2 L
3rd day	0.4 mL/2 L	0.4 mL/2 L
4th day	0.6 mL/2 L	0.6 mL/2 L
5th day	0.9 mL/2 L	0.9 mL/2 L
6th day	1.3 mL/2 L	1.3 mL/2 L
7th day	1.7 mL/2 L	1.7 mL/2 L
8th day	2 mL/2 L	2 mL/2 L

YCT, yeast, cerophyll, and trout chow.

Table 3. Feeding amount after separation of 10 adult *Daphnia magna* per 1 L

Culture day	*Chlorella vulgaris* (mL)	YCT (mL)
9th day	0.4 mL/1 L	0.4 mL/1 L
10th day	0.5 mL/1 L	0.5 mL/1 L
11th day	0.6 mL/1 L	0.6 mL/1 L
12th day	0.7 mL/1 L	0.7 mL/1 L
13th day after	0.75 mL/1 L	0.75 mL/1 L

YCT, yeast, cerophyll, and trout chow.

Subculture

Fifty one-day-old *D. magna* neonates were placed in 2 L of culture medium using a dropper; the culture was labeled '2nd culture day'. Subculture was prepared under the culture condition described above. Food was supplied daily at a gradually increasing rate according to the culture day (Table 2). On days when food could not be supplied, such as holidays, the feeding amount per culture day was summed up and supplied on the preceding day. For example, in case it was not possible to supply food on the day after the 2nd culture day, 0.7 (0.3+0.4) mL of *C. vulgaris* and YCT would be supplied on the 2nd culture day itself. On the 9th day of culture, 10 adult *D. magna* were transferred using a dropper to 1 L of culture medium. It is important to limit *D. magna* mature females to ≤10 per culture medium, as the toxicity test should only be carried out using female neonates. After separation, feed was supplied according to growth (Table 3). When the amount of food supplied exceeds that required for the growth of *D. magna*, *C. vulgaris* may attach to their body (Fig. 3) and inhibit their swimming. Therefore, the *D. magna* were fed strictly according to the guidelines presented in Tables 2 and 3. After the 12th culture day, 50 neonates born from mature female *D. magna* were placed in 2 L of culture medium and cultured until they became adults. This process of subculture was repeated.

Fig. 3. *Chlorella vulgaris* attached to the *Daphnia magna*'s body surface.

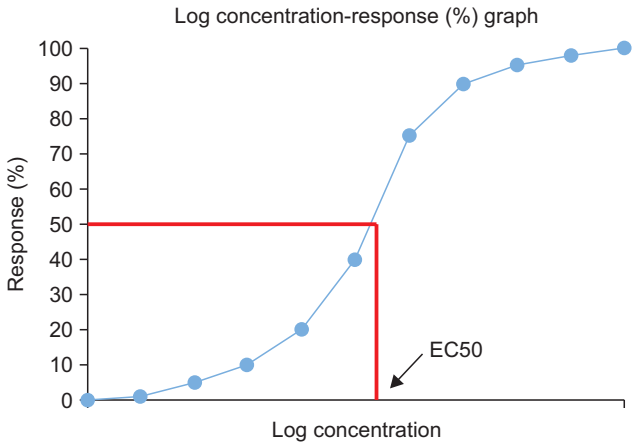

Fig. 4. An example of function graph about the log value of the concentration-the response (%).

Table 4. Acute toxicity test condition

Photoperiod	Light:Dark =16 h:8 h
Illuminance	500-1,000 Lux
Exposure time	24 h
Temperature	20±2°C
Number of repetitions per concentration	4
Number of neonates per concentration	20
Sample volume	50 mL

Acute toxicity test

The acute toxicity test evaluates toxicity that occurs shortly after exposure of the test substance to test animals (MFDS, 2021). The test period of an acute toxicity test using *D. magna* (ES04704.1b) is 24 hours.

The test procedure was as follows. The day before the toxicity test, all neonates were removed from beakers containing more than 12-day-old mature females using a dropper. On the day of the test, newly born neonates that are approximately less than 24 hours old were transferred to 1 L of culture medium using a dropper 2 hours before the toxicity test. The neonates were then supplied with *C. vulgaris* and YCT. An hour before the test, the test solutions were prepared. Then test solution was poured into four 50 mL beakers. Afterwards, 5 of the neonates that were less than 24 hours old were transferred to a beaker using a dropper. Therefore, 20 neonates were exposed to the same test solution. After completing the transfer, the beakers containing the neonates were kept under the conditions presented in Table 4 without feeding. Exactly 24 hours later, the toxicity test results were collected. From the low concentration test solution beakers to high concentration test solution beakers, the beakers were knocked gently and observations were made for 15 seconds. The number of immobilized or dead neonates was determined. Immobilization means the inability to swim regardless of antenna or appendage movement (ES 04704.1b). Unlike chemical analysis, evaluation results may vary depending on the health of the test organism (NIER, 2014).

EC50

EC50 is the half maximal effective concentration, where half of the test subjects are affected within 24 hours (NIER, 2014). A low value indicates high toxicity. The EC50 is calculated using a statistical method (ES 04704.1b). First, a function curve of the log values of the test substance concentration, that is, the response (%) of test subjects, should be created. Numerous biological response curves commonly assume a sigmoidal shape (Gadagkar & Call, 2015), and an example of the function graph is shown in Fig. 4. Then, from the sigmoid curve, the concentration at which half of the test subjects are affected can be determined (Indrayanto *et al*., 2021).

The test procedure for EC50 confirmation was as follows. The day before the toxicity test, all neonates were removed from beakers containing more than 12-day-old female *D. magna* using a dropper. On the day of the test, approximately 130 newly born neonates (less than 24 hours old) were transferred to 1 L of culture medium using a dropper 2 hours before the toxicity test, then supplied with *C. vulgaris* and YCT. One hour before the test, the test solution (100%) was diluted with culture medium to prepare 6.25%, 12.5%, 25%, and 50% dilution solutions. Then each dilution solution was poured into four 50 mL beakers, respectively. Finally, test solution was poured into four 50 mL beakers. The less than 24 hours old neonates were transferred to beakers (5 neonates per beaker) using a dropper as shown in Fig. 5. After complet-

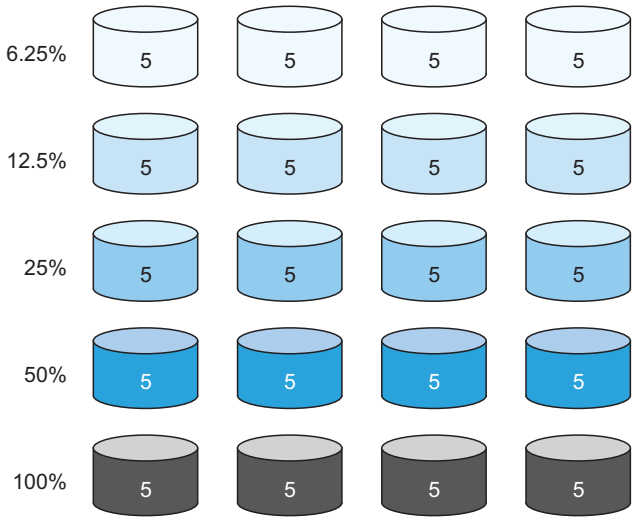

Fig. 5. Schematic diagram of dilution ratio and number of specimens. Data from NIER. Guidelines for Ecotoxicity Test•Operation; 2014.

ing the transfer, the beakers containing the neonates were left under the conditions presented in Table 4, without feeding. Exactly 24 hours later, the toxicity test results were collected. All the dilution solution and test solution beakers were gently knocked and observed for 15 seconds. The number of affected neonates was determined. To calculate EC50 using the statistical method, less than 10 neonates must be affected in the 6.25% dilution solution, and all the neonates must be affected in the test solution (100%). If 11 to 19 of the neonates are affected in the test solution (100%), the toxicity test should be retested by preparing an additional 75% dilution solution (ES 04704.1b). The Probit or Trimmed Spearman Karber (TSK) methods were used (ES 04704.1b) to calculate the EC50. The Probit method was used when there was more than one data instance of 1-99% affected rate, excluding 0% and 100%. The TSK method was used when there was one or less data instance of 1-99% affected rate, excluding 0% and 100%. The affected rate (%)=the number of affected neonates per concentration/the total number of exposed neonates per concentration×100 (NIER, 2014). For example, if 4 out of 20 neonates were affected at 25% dilution solution, the affected rate (%) is calculated as 4/20×100 and is 20%. When the number of affected neonates according to concentrations is fed to the Probit method program or TSK method program, the EC50 value of the substance is calculated.

Toxic unit (TU)

TU is an indicator of ecotoxicity and can be calculated as TU=100/EC50 according to the water pollution standards method (ES 04704.1b). A high value indicates high toxicity. As shown in Table 5, the TU of effluent from the wastewater discharge facility (clean areas) and the public wastewater treatment facility (I, II, III, IV areas) in Korea should be ≤1 (ME, 2022).

Table 5. Korea's ecotoxicity emission standard

Division	Area	Acceptance standard (TU)
Wastewater discharging facility	Clean area	1≥
Public wastewater treatment facility	I, II, III, IV	1≥

TU, toxic unit.

Table 6. TOC analyzer condition for potassium hydrogen phthalate

TOC analyzer 2100s (Analytik-jena, Germany)
Combustion tube: outer diameter 16 mm
Combustion temperatur: 800°C
Injection volume: 500 μL
Detector: NDIR

TOC, total organic carbon; NDIR, non dispersive infra red.

Table 7. QC standard and calculated values

QC item	Standard	Calculated value
Limit of quantification (mg/L)	≤0.3	0.2557
Accuracy (%)	80-120	113.3
Precision (%)	±20	3.4

QC, quality control.

TOC analysis

After preparing a test solution using KHP, the accuracy of the sample's concentration was measured through TOC analysis under the conditions presented in Table 6. According to the water pollution standard method (ES 04311.1c), TOC analysis comprises TC-IC and NPOC methods. The TC-IC method calculates the amount of organic carbon by measuring total carbon and inorganic carbon and subtracting inorganic carbon from total carbon. The NPOC method measures the remaining organic carbon after completely removing inorganic carbon by aeration upon sample acidification. In ES 04311.1c, the TC-IC method should be used if the inorganic carbon ratio in total carbon is less than 50%, and the NPOC method if this ratio exceeds 50%. The calibration curve for sample quantification was created by diluting total carbon standard solution and inorganic carbon standard solution procured from SCP Science. The calibration curve ranged from 0.3 mg/L to 30 mg/L. The solutions were measured by appropriately diluting them with purified water so that

their quantitative analysis value could be included in the calibration range. The measured value was calculated by multiplying the quantitative analysis value with a dilution multiple.

Internal quality control

To ensure the accuracy of the prepared KHP sample, internal quality control was required to first check the performance of the TOC analyzer. The accuracy/precision verification test of the analytical instrument was performed according to ES 04311.1c. Seven 0.3 mg/L TOC samples were prepared and measured to calculate the limit of quantification. Four samples of 1 mg/L TOC were prepared and measured to calculate accuracy/precision. Then the limits of quantification, accuracy, and precision were calculated. As the calculated values met the standards required by the ES 04311.1c (Table 7), the accuracy/precision of the instrument and the skill of the analyst were considered verified.

Standard reference toxicity test

In accordance with ES 04704.1b, a standard reference toxicity test using potassium dichromate ($K_2Cr_2O_7$) must be carried out once a month to confirm *D. magna* health and sensitivity to toxicity. First, 1 g of $K_2Cr_2O_7$ was placed in a 1 L volumetric flask and dissolved in purified water to prepare 1,000 mg/L of $K_2Cr_2O_7$. Because 2 to 5 mg/L of $K_2Cr_2O_7$ is recommended for the test solution (NIER, 2014), 3 mL of 1,000 mg/L of $K_2Cr_2O_7$ was aliquoted into a 1 L volumetric flask and diluted with the culture medium. After use as a test solution, it was diluted to 6.25%, 12.5%, 25%, and 50% dilution solutions using the culture medium. The toxicity test was carried out under the conditions presented in Table 4, without feeding, and the EC50 of $K_2Cr_2O_7$ was calculated. The EC50 control chart of the standard reference toxicity test performed monthly from 22.1.12 to 22.6.3 is shown in Fig. 6. The coefficient of variation (%) representing the reproducibility or precision of the tests is calculated by dividing the standard deviation by the average and multiplying by 100. The coefficient of variation (%) of the test repeated 6 times in the laboratory was 10.42%. Although there is no satisfaction standard for the coefficient of variation (%) of EC50, the coefficient of variation (%) standard generally suggested by the water pollution standard method (NIER, 2022) for ions, heavy metals, and organic matter is ≤ 25%. Since the coefficient of variation (%) of EC50 is less than 25%, it can be said that the precision is good. In addition, the suitable EC50 range of $K_2Cr_2O_7$ is 0.9 to 2.1 mg/L (ES 04704.1b); therefore, the sensitivity to toxicity was maintained at an appropriate level.

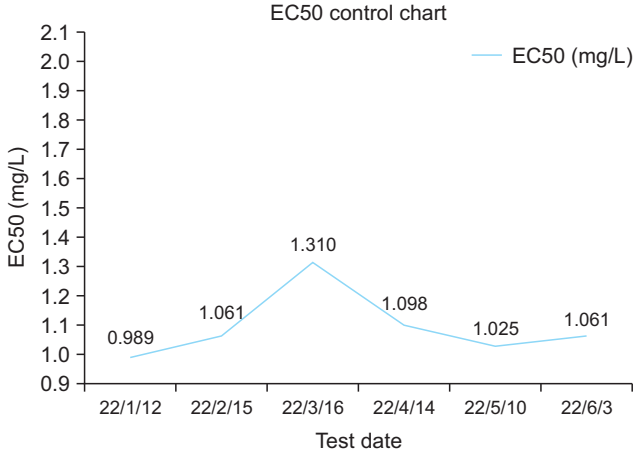

Fig. 6. Standard reference toxicity test EC50 control chart.

Confirmation test for LOEC of KHP

In the exposure-response test, LOEC refers to the minimum concentration at which an effect is observed on a test subject for the first time (NEIR, 2021). The 24 hours-LOEC of KHP is the KHP's minimum concentration at which the test subject is observed to be affected for the first time within 24 hours. To confirm the LOEC of KHP, 2.125 g of potassium hydrogen phthalate procured from Supelco Inc. (Bellefonte, PA, USA), was accurately measured and put in a 1 L flask, dissolved in purified water to prepare 1,000 mg/L KHP, and used as the test solution. Afterwards, 1,000 mg/L KHP was aliquoted step by step and diluted with the culture medium. To measure the accuracy of the prepared KHP sample, TOC was measured under the conditions presented in Table 6. The *D. magna* at the start of this study was called n generation (n gen), and on the day of TOC analysis, neonates from the n generation mature female were used for the toxicity test under the conditions presented in Table 4.

Confirmation test for correlation between chemical factors and acute toxicity at the LOEC of KHP

This test was performed after determining the LOEC of KHP. First, 2.125 g of KHP from Supelco Inc. was accurately measured, placed in a 1 L flask, dissolved in purified water to prepare 1,000 mg/L KHP, and used as the test solution. The test solution was diluted with the culture medium to prepare a concentration corresponding to the KHP LOEC.

P-acute toxicity test at KHP LOEC

When preparing the P standard stock solution according to the Water Pollution Standard Method (ES 04350.1b), 4.39 g of potassium dihydrogen phosphate from Merck Inc. was accurately weighed and placed in a 100 mL flask, and then dissolved in purified water (10,000 mg/L of P). The 2-day-test EC50 of potassium dihydrogen phosphate for *D. magna* has been reported to be 100 mg/L or more

(ECHA, 2022). The P 10,000 mg/L solution was added to the solution corresponding to the KHP's LOEC to include the EC50. KHP's LOEC-P 20 mg/L, KHP's LOEC-P 40 mg/L, KHP's LOEC-P 80 mg/L, KHP's LOEC-P 100 mg/L, KHP's LOEC-P 200 mg/L, KHP's LOEC-P 500 mg/L samples were prepared. Samples were analyzed three times according to the water pollution standard method (ES 04350.1b). Acute toxicity tests were carried out under the conditions presented in Table 4.

N-acute toxicity test at KHP LOEC

When preparing a N standard stock solution according to the Water Pollution Standard Method (ES 04350.1b), 7.218 g of potassium nitrate from Wako Inc. was accurately weighed and put in a 100 mL flask, then dissolved in purified water (10,000 mg/L of N). The 1-day-test EC50 of potassium nitrate for *D. magna* has been reported to be 490 mg/L (Dowden & Bennett, 1965; EPA, 2022). The N 10,000 mg/L solution was added to the solution corresponding to KHP LOEC to include the EC50. KHP's LOEC-N 20 mg/L, KHP's LOEC-N 40 mg/L, KHP's LOEC-N 80 mg/L, KHP's LOEC-N 100 mg/L, KHP's LOEC-N 200 mg/L, KHP's LOEC-N 500 mg/L samples were prepared. Samples were analyzed three times according to the water pollution standard method (ES 04350.1b) and acute toxicity tests were carried out under the conditions presented in Table 4.

Cr(6+)-acute toxicity test at KHP LOEC

First, 2 mL of 1,000 mg/L hexavalent chromium standard solution, procured from Accustandard Inc., was placed in a 100 mL flask and purified water added to make 20 mg/L of Cr(6+). Because the 1-day-test EC50 of Cr(6+) for *D. magna* has been reported to range from 0.10 to 0.36 mg/L (Choi *et al*., 1999), 20 mg/L solution of Cr(6+) was added to the solution corresponding to the KHP LOEC to include the EC50. KHP's LOEC-Cr(6+) 0.04 mg/L, KHP's LOEC-Cr(6+) 0.08 mg/L, KHP's LOEC-Cr(6+) 0.1 mg/L, KHP's LOEC-Cr(6+) 0.2 mg/L, KHP's LOEC-Cr(6+) 0.4 mg/L samples were prepared and analyzed according to the water pollution standard method (ES 04415.2c). Then acute toxicity tests were carried out under the conditions presented in Table 4.

Confirmation test for EC50 and TU values of KHP

Preparation of test solution and quantitative analysis of KHP

For EC50 to be calculated using a statistical method, less than 10 neonates should be affected in the 6.25% dilution solution, and all neonates must be affected in the test solution (100%). This test was performed after confirming the LOEC of KHP. A solution close to the LOEC of KHP was set to 25% dilution solution of the EC50 test. First, 6.25%, 12.5%, 25%, and 50% dilution solutions, and test solution (100%) were prepared using KHP from Supelco Inc. To measure the accuracy of the prepared KHP samples, TOC was measured under the conditions presented in Table 6.

Measurement of EC50 and TU of KHP

The day before the toxicity test, all neonates were removed from the beakers containing more than 12-day-old mature females using a dropper. On the day of the test, approximately 130 neonates that were less than 24 hours-old were transferred to 1 L of culture medium using a dropper, and then supplied with *C. vulgaris* and YCT. Then each prepared dilution solution were poured into four 50 mL beakers. Finally, the prepared test solution was poured into four 50 mL beakers. Every test was performed at 3 pm. Neonates that were less than 24 h-old were transferred to beakers (5 neonates per beaker) using a dropper as shown in Fig. 5. After completing the transfer, the beakers containing the neonates were left under the conditions presented in Table 4, without feeding.

Results

LOEC of KHP

First, 1,000 mg/L KHP was aliquoted step by step and diluted with the culture medium. The first test was performed to identify an approximate range and was diluted to 50, 100, 200, 300, 500 mg/L. The TOC analysis and toxicity test results are presented in Table 8. Accuracy is expressed as % by dividing measured KHP concentration by Prepared KHP concentration and multiplying by 100. As the accuracy (%) suggested by ES 04311.1c ranges within 80-120%, the accuracy can be said to be significant. Because the affected neonates increased sharply between KHP 200 mg/L and 300 mg/L, the 24-hours

Table 8. KHP LOEC sample (50, 100, 200, 300, 500, 1,000 mg/L) measurement result

Preparsed KHP Conc (mg/L)	Measured KHP Conc (mg/L)	Accuracy (%) (=measured Conc/prepared Conc×100)	22.3.08 n generation mature female's affected neonate number
KHP 50	54.1	108.2	0
KHP 100	97.2	97.2	0
KHP 200	215.3	107.7	0
KHP 300	269.3	89.8	11
KHP 500	485.3	97.1	20
KHP 1000	937.3	93.7	20

KHP, potassium hydrogen phthalate; LOEC, lowest observed effect concentration; Conc, concentration.

LOEC was estimated to range between 220 and 260 mg/L. KHP 1,000 mg/L was diluted with the culture medium to prepare 220, 230, 240, 250, and 260 mg/L samples, and KHP was measured under the conditions presented Table 6 to confirm the accuracy of the prepared KHP samples. On the day of the TOC instrument analysis, neonates from the n generation mature females were used to perform the toxicity test under the conditions presented in Table 4. As shown Table 9, the TOC analysis and toxicity test results showed that the number of affected neonates was 1 at 240 mg/L, 1 at 250 mg/L, and 2 at 260 mg/L. To confirm the reproducibility of the tests, additional experiments were carried out using neonates from the n+1 and n+2 generations of mature females. The instrument analysis and toxicity test results are shown in Tables 10 and 11. The concentration at which neonate begins to be affected in repeated Toxicity tests is 240 mg/L, so KHP of 240 mg/L can be said to be a 24-hours LOEC for *D. magna*.

Table 9. KHP LOEC sample (220, 230, 240, 250, 260 mg/L) measurement result

Prepared KHP Conc (mg/L)	Measured KHP Conc (mg/L)	Accuracy (%) (=measured Conc/prepared Conc× 100)	22.3.10 n generation mature female's affected neonate number
KHP 220	215.2	97.8	0
KHP 230	223.3	97.1	0
KHP 240	233.2	97.2	1
KHP 250	237.8	95.1	1
KHP 260	245.5	94.4	2

KHP, potassium hydrogen phthalate; LOEC, lowest observed effect concentration; Conc, concentration.

Correlation between chemical factors and acute toxicity in LOEC of KHP

First, 240 mL of the test solution was aliquoted, placed in a 1 L flask, and dissolved in a culture medium to prepare 240 mg/L KHP. Then KHP was analyzed with a TOC analyzer, and the results are presented in Table 12. Meanwhile, each factor was added to 240 mg/L KHP, and tests were performed to determine the effect of factors. Correlation confirmation tests about 240 mg/L KHP-Cr(6+) were performed on April 21st, April 23rd, May 3rd, and May 19th. Correlation confirmation tests on 240 mg/L KHP-P, 240 mg/L KHP-N were performed on April 21st, May 5th, and May 16th. Samples were prepared newly whenever the test was performed. Instrumental analysis was carried out using ion chromatography and UV spectrophotometer that had completed internal quality control. On the same day as that of the instrument analysis,

Table 10. KHP LOEC sample (220, 230, 240, 250, 260 mg/L) instrument analysis result

Prepared KHP Conc (mg/L)	22.3.10 measured KHP Conc	22.3.21 measured KHP Conc	22.4.05 measured KHP Conc	Measured KHP Conc average	Accuracy (%) (=measured Conc average/prepared Conc×100)
KHP 220	215.2	215.4	213.4	214.7	97.6
KHP 230	223.3	222.5	223.1	223.0	96.9
KHP 240	233.2	231.0	226.4	230.2	95.9
KHP 250	237.8	240.2	242.2	240.1	96.0
KHP 260	245.5	251.6	250.3	249.1	95.8

KHP, potassium hydrogen phthalate; LOEC, lowest observed effect concentration; Conc, concentration.

Table 11. KHP LOEC sample (220, 230, 240, 250, 260 mg/L) toxicity result

KHP Conc (mg/L)	22.3.10 n gen mature female's affected neonate number	22.3.21 n+1 gen mature female's affected neonate number	22.4.05 n+2 gen mature female's affected neonate number
KHP 220	0	0	0
KHP 230	0	0	0
KHP 240	1	0	1
KHP 250	1	1	2
KHP 260	2	2	4

KHP, potassium hydrogen phthalate; LOEC, lowest observed effect concentration; Conc, concentration.

toxicity tests were performed under the conditions presented in Table 4. The accuracy (%) of prepared samples and toxicity test results were confirmed the following day.

P-acute toxicity test results at KHP LOEC

Samples were analyzed three times according to the water pollution standard method (ES 04350.1b) and the accuracy (%) of the average value for each concentration was found to be included in the 90-110% range. Neonates born from n+3 generation mature female were exposed to P-KHP 240 mg/L samples on April 21st and those born from the n+4 generation mature female were exposed to P-KHP 240 mg/L samples on May 5th. Finally, neonates born from the n+5 generation mature female were exposed to P-KHP 240 mg/L samples on May 16th, and the results of the three repeated toxicity tests are shown in Table 13. The average value of the number of affected neonates in three repeated tests for each concentration was rounded up to the first decimal place and expressed as an integer.

Table 12. Prepared KHP 240 mg/L sample instrument analysis result

Measured date	Prepared KHP Conc (mg/L)	Measured KHP Conc (mg/L)	Accuracy (%) (=measured Conc /prepared Conc×100)
4.21	KHP 240	235.8	98.3
4.23	KHP 240	233.1	97.1
5.03	KHP 240	232.6	96.9
5.05	KHP 240	229.8	95.8
5.16	KHP 240	234.0	97.5
5.19	KHP 240	227.3	94.7

KHP, potassium hydrogen phthalate; Conc, concentration.

N-acute toxicity test result at KHP's LOEC

Samples were analyzed three times according to the water pollution standard method (ES 04350.1b) and the accuracy (%) of the average value for each concentration was found to be included in 90-110% accuracy level. Neonates born from the n+3 generation of mature females were exposed to N-KHP 240 mg/L samples on April 21st and those born from the n+4 generation of mature females were exposed to N-KHP 240 mg/L samples on May 5th. Finally, neonates born from the n+5 generation of mature female were exposed to N-KHP 240 mg/L samples on May 16th, and the results of the three repeated toxicity tests are shown in Table 14. The average value of the number of affected neonates in three repeated tests for each concentration was rounded up to the first decimal place and expressed as an integer.

Cr(6+)-acute toxicity test result at KHP's LOEC

On April 21st, samples were prepared to KHP 240 mg/L-Cr(6+) 0.04 mg/L, KHP 240 mg/L-Cr(6+) 0.08 mg/L, KHP 240 mg/L-Cr(6+) 0.1 mg/L, KHP 240 mg/L-Cr(6+)

Table 13. Number of affected neonate according to P concentration at KHP 240 mg/L

Date	Number of affected neonate					
	KHP 240 mg/ L+P 20 mg/L	KHP 240 mg/ L+P 40 mg/L	KHP 240 mg/ L+P 80 mg/L	KHP 240 mg/ L+P 100 mg/L	KHP 240 mg/ L+P 200 mg/L	KHP 240 mg/ L+P 500 mg/L
22.4.21	0	0	8	16	20	20
22.5.05	0	0	5	13	20	20
22.5.16	0	0	6	15	20	20
Average	0	0	6	15	20	20

P, phosphorus; KHP, potassium hydrogen phthalate.

Table 14. Number of affected neonate according to N concentration at KHP 240 mg/L

Date	Number of affected neonate					
	KHP 240 mg/ L+N 20 mg/L	KHP 240 mg/ L+N 40 mg/L	KHP 240 mg/ L+N 80 mg/L	KHP 240 mg/ L+N 100 mg/L	KHP 240 mg/ L+N 200 mg/L	KHP 240 mg/ L+N 500 mg/L
22.4.21	0	8	11	14	16	20
22.5.05	0	4	8	14	19	20
22.5.16	0	7	9	13	18	20
Average	0	6	9	14	18	20

N, nitrogen; KHP, potassium hydrogen phthalate.

Table 15. Number of affected neonate according to Cr(6+) Conc (0.04, 0.08, 0.1, 0.2, 0.4 mg/L) at KHP 240 mg/L

Date	Number of affected neonate				
	KHP 240 mg/L+ Cr(6+) 0.04 mg/L	KHP 240 mg/L+ Cr(6+) 0.08 mg/L	KHP 240 mg/L+ Cr(6+) 0.1 mg/L	KHP 240 mg/L+ Cr(6+) 0.2 mg/L	KHP 240 mg/L+ Cr(6+) 0.4 mg/L
22.04.21	20	20	20	20	20

Conc, concentration; KHP, potassium hydrogen phthalate.

Table 16. Number of affected neonate according to Cr(6+) Conc (0.002, 0.004, 0.006, 0.008, 0.01 0.02 mg/L) at KHP 240 mg/L

Date	Number of affected neonate					
	KHP 240 mg/ L+ Cr(6+) 0.002 mg/L	KHP 240 mg/ L+ Cr(6+) 0.004 mg/L	KHP 240 mg/ L+ Cr(6+) 0.006 mg/L	KHP 240 mg/ L+ Cr(6+) 0.008 mg/L	KHP 240 mg/ L+ Cr(6+) 0.01 mg/L	KHP 240 mg/ L+ Cr(6+) 0.02 mg/L
22.04.23	0	0	4	11	15	20
22.05.03	0	0	3	12	15	20
22.05.19	0	0	3	13	17	20
Average	0	0	3	12	16	20

Conc, concentration; KHP, potassium hydrogen phthalate.

0.2 mg/L, KHP 240 mg/L-Cr(6+) 0.4 mg/L, and their accuracy (%) was confirmed to range within 90–110 % through instrumental analysis (ES 04415.2c). In addition, a toxicity test was carried out using neonates born from the n+3 generation of mature female. As for the toxicity test result, all neonates were affected in all concentration ranges as shown in Table 15. Considering this, a 1 mg/L solution of Cr(6+) was prepared, and KHP 240 mg/L-Cr(6+) 0.002 mg/L, KHP 240 mg/L-Cr(6+) 0.004 mg/L, KHP 240 mg/L-Cr(6+) 0.006 mg/L, KHP 240 mg/L-Cr(6+) 0.008 mg/L, KHP 240 mg/L-Cr(6+) 0.01 mg/L and KHP 240 mg/L-Cr(6+) 0.02 mg/L samples were prepared by adding 1 mg/L solution of Cr(6+) to 240 mg/L of KHP. Since the Cr(6+) concentration of the samples was less than 0.04 mg/L, which is the limit of quantification (ES 04415.2c), instrumental analysis was not performed. Neonates born from the n+3, n+4, and n+5 generations of mature females were exposed to Cr(6+)-KHP 240 mg/L samples on April 23rd, May 3rd, and May 19th, respectively. The results of the three repeated toxicity tests are shown in Table 16.

The average value of the number of affected neonates in the three repeated tests for each concentration was rounded up to the first decimal place and expressed as an integer. To visually evaluate the concentration-response relationship based on the results, linear regression graphs using the least squares method were created. Concentration-affected neonate number data, which is concentration below the maximum concentration at which all *D. magna* were not affected and that exceeding the minimum concentration at which all *D. magna* died, were disregarded. For the P concentration-response graph, 40, 80, 100, 200 mg/L–affected neonate data were used. For the N concentration-response graph, 20, 40, 80, 100, 200, 500 mg/L–affected neonate data were used. For the Cr(6+) concentration-response graph, 0.004, 0.006, 0.008, 0.01, 0.02 mg/L–affected neonate data were used. Through tests across three generations, regression analysis graphs of the average number of affected neonates according to the concentration were created, and are shown in Fig. 7. The 24-hours acute toxicity tended to increase as the concentrations of P, N, and Cr(6+) increased at 240 mg/L KHP. In the case of P, the affected neonates appeared at 240 mg/L KHP+80 mg/L P; therefore, the threshold was the highest. In the case of N, the affected neonates appeared at 240 mg/L KHP+40 mg/L N. In the case of Cr(6+), affected neonates appeared at 240 mg/L KHP+0.006 mg/L Cr(6+); therefore, the threshold was the lowest. As a result of regression analysis of the relationship between toxicity and the concentration of P, N and Cr(6+) at 240 mg/L KHP, the equations were y=0.1205x–2.4029, y=0.0339x+5.8548, y=1188.1x–1.2062, respectively. Furthermore, the correlation coefficient (R value), a value that primarily determines the existence of a relationship between the signal and analyte concentration, was 0.915639, 0.800832, and 0.870997, respectively. The closer the R value is to 1, the better (MFDS, 2015; NIER, 2011). Therefore, the correlation between toxicity and P concentration at 240 mg/L KHP is best applied to the linear regression equation.

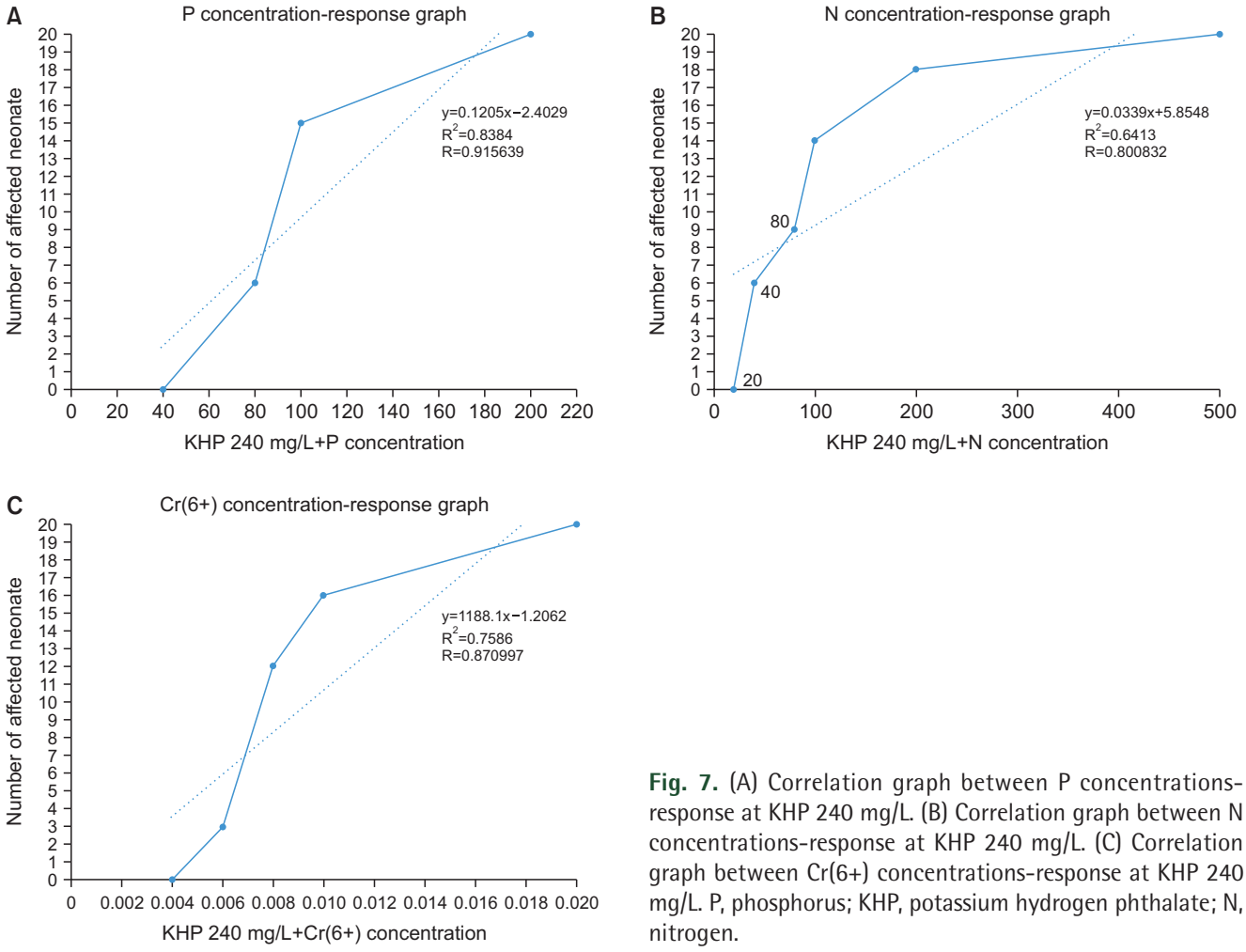

Fig. 7. (A) Correlation graph between P concentrations-response at KHP 240 mg/L. (B) Correlation graph between N concentrations-response at KHP 240 mg/L. (C) Correlation graph between Cr(6+) concentrations-response at KHP 240 mg/L. P, phosphorus; KHP, potassium hydrogen phthalate; N, nitrogen.

Table 17. Prepared KHP EC50 25% sample instrument analysis result

Date	Sample	Prepared KHP Conc (mg/L)	Measured KHP Conc (mg/L)	Accuracy (%) (=measured Conc /prepared Conc×100)
22.3.18	250 mg/L (25%)	250	261.0	104.4
22.3.22	250 mg/L (25%)	250	247.0	98.8
22.4.05	250 mg/L (25%)	250	246.0	98.4
22.4.08	250 mg/L (25%)	250	229.0	91.6
22.4.22	250 mg/L (25%)	250	248.2	99.3
22.4.23	250 mg/L (25%)	250	258.2	103.3
22.5.03	250 mg/L (25%)	250	237.3	94.9
22.5.05	250 mg/L (25%)	250	249.3	99.7
22.5.16	250 mg/L (25%)	250	239.9	96.0
22.5.19	250 mg/L (25%)	250	240.5	96.2

KHP, potassium hydrogen phthalate; Conc, concentration.

EC50 and TU of KHP

Quantitative analysis result of KHP

The LOEC of KHP was confirmed to be 240 mg/L. The KHP 250 mg/L was set to 25% dilution solution of EC50 test. First, 2.125 g of Supelco Inc.'s potassium hydrogen phthalate was accurately measured and placed in a 1 L flask and then dissolved in purified water to prepare 1,000 mg/L as KHP. This was considered the test solution (100%), the test solution was diluted to 6.25%, 12.5%, 25%, and 50% step by step using the culture medium to prepare 62.5, 125, 250, and 500 mg/L as KHP. The analysis results of a 25% dilution solution (250 mg/L), where the 24-h acute toxicity of *D. magna* was observed, are as shown in Table 17.

EC50 and TU KHP

The first test was performed on day 22.3.18 (Fig. 8). Exactly 24 hours later, the toxicity test results were collected. From the 6.25% dilution solution (KHP 62.5 mg/L) beakers to the test solution (KHP 1,000 mg/L) beakers, the beakers were gently knocked and observed for 15 seconds, and the number of affected neonates was determined. One neonate was affected at 250 mg/L, and all 20 neonates were affected at 500 and 1,000 mg/L. The calculated KHP's EC50 and KHP's TU were 341.51 mg/L and 0.3, respectively. Therefore, the test and dilution solutions used in the toxicity test were suitable for the KHP's EC50 confirmation test. To confirm reproducibility, from 22.3.18 to 22.5.19, tests were carried out using the neonates from n+1 generation mature females to those of the n+5 generation mature females. The tests were performed across five generations, and the tests on neonates born from the same generation of mature females were performed twice. The TU values were expressed to

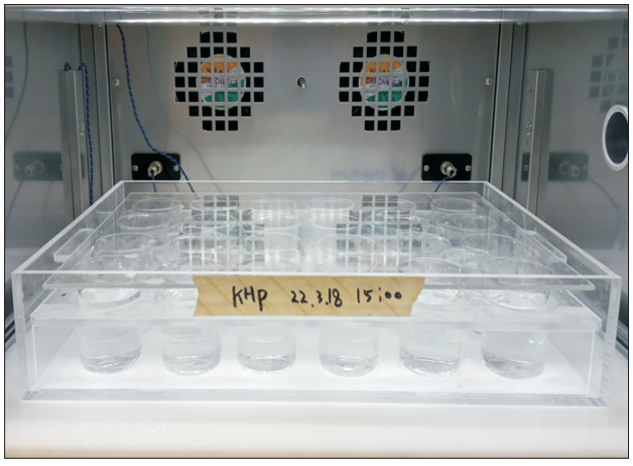

Fig. 8. The toxicity test for confirming EC50 of KHP (22.3.18). KHP, potassium hydrogen phthalate.

Table 18. KHP toxicity test results calculated by TSK program

Date	Test subject	Number of affected unit						EC50 (mg/L)	TU
		Control	6.25% dilute	12.5% dilute	25% dilute	50% dilute	100%		
22.3.18	n+1 generation mature female's neonate	0	0	0	1	20	20	341.51	0.3
22.3.22	n+1 generation mature female's neonate	0	0	0	2	20	20	329.88	0.3
22.4.05	n+2 generation mature female's neonate	0	0	0	2	20	20	329.88	0.3
22.4.08	n+2 generation mature female's neonate	0	0	0	1	20	20	341.51	0.3
22.4.22	n+3 generation mature female's neonate	0	0	0	4	20	20	307.79	0.3
22.4.23	n+3 generation mature female's neonate	0	0	0	2	20	20	329.88	0.3
22.5.03	n+4 generation mature female's neonate	0	0	0	2	20	20	329.88	0.3
22.5.05	n+4 generation mature female's neonate	0	0	0	3	20	20	318.64	0.3
22.5.16	n+5 generation mature female's neonate	0	0	0	2	20	20	329.88	0.3
22.5.19	n+5 generation mature female's neonate	0	0	0	3	20	20	318.64	0.3
	Average							327.75	0.3
	Standard deviation							10.36	0.01
	Coefficient of variation (%)							3.16	3.21

KHP, potassium hydrogen phthalate; TSK, Trimmed Spearman Karber; TU, toxic unit.

one decimal place according to ES 04704.1b regulations, and the measurement results are presented in Table 18. The average values of EC50 and TU calculated through 10 repeated tests were 327.75 mg/L and 0.3, respectively. The coefficient of variation (%) of the KHP EC50 value calculated using 10 repeated tests was 3.16%.

Discussion

In this study, 24-hours acute toxicity tests for KHP were carried out using *D. magna*, and the following study results were obtained.

Through a 24-hours acute toxicity test, the LOEC of KHP was found to be 240 mg/L. The 24-hours acute toxicity tended to increase as the concentration of P, N, and Cr(6+) increased at 240 mg/L KHP, at which point EC50 is expected to be lower and ecotoxicity (TU) to be higher. In addition, a regression analysis was performed on the relationship between acute toxicity and P, N, and Cr(6+) concentrations at 240 mg/L KHP.

Because the slope of the linear regression equation (6+Cr concentration—the number of affected neonates) was the largest, the increase in toxicity according to the Cr(6+) concentration exceeded that of P and N.

The KHP EC50 value was found to be determined by the number of affected neonates observed at 250 mg/L KHP. The average EC50 and TU values of KHP were calculated through 10 repeated tests to be 327.75 mg/L and 0.3, respectively. The coefficient of variation (%) of KHP's EC50 value calculated through 10 repeated tests was 3.16%. The coefficient of variation (%) of the EC50 value calculated in the standard reference toxicity tests using potassium dichromate performed monthly from January 2022 to June 2022 in this laboratory was 10.42%. The reproducibility can be said to be good because the coefficient of variation (%) of KHP's EC50 and the coefficient of variation (%) of potassium dichromate's EC50 are well below the 25%. The EC50 of KHP is approximately 218 times higher than that of potassium dichromate, which is 1.5 mg/L (average values of 0.9 mg/L and 2.1 mg/L), and the toxicity of KHP is lower. In conclusion, KHP can be used as an alternative to the highly toxic potassium dichromate designated as a hazardous chemical for the standard reference toxicity.

However, the limitation of this study is as follows. The discharged wastewater does not simply contain P, N, and Cr(6+), but contains many other substances, so there are many variables for 24-hours acute toxicity at 240 mg/L KHP. Therefore, additional studies for many other substances are needed.

In addition, the *D. magna* cultured in this laboratory were in good health, and the coefficient of variation (%) of the KHP's EC50 was 3.16%, which was suitable for reproducibility. However, there are no KHP EC50 measurement values for *D. magna* cultured in other laboratories, and no toxicity tests on unhealthy *D. magna*.

Therefore, additional experiments should be performed to adopt the standard toxicity test method using KHP and the Ministry of Environment which is in charge of the test standards should set the EC50 range of the KHP through the review of additional experiments.

Conflict of Interest

The author declares that (s)he has no competing interests.

Acknowledgments

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