



## Abstract

New behavioral indexes including combined index (CI), distribution index (DI), toxic index (TI), and altitude index (AI) for a biological early warning system (BEWS) were developed and evaluated using *Daphnia magna* in this study. The sensitivity and stability of each index were compared to evaluate the performance of the indexes through a real-time exposure test with a synthetic copper solution. The applicability of the CI to the field sample was evaluated through an effluent exposure test. The proportional relationship between toxicity level and magnitude of response was much lower in the effluent due to the complexity of water than in the copper solution. The results showed that the CI was most sensitive among the three indexes, while the DI was confirmed as the most useful index among the individual indexes. The combined index (CI) shows not only sensitivity but also stability in normal conditions below the statistically significant threshold ( $p < 0.01$ ), whereas the individual indexes displayed unstable index values in normal conditions ( $p > 0.01$ ). The CI improved performance of the BEWS in terms of sensitivity and stability, and it was confirmed as the higher correlation coefficient between the magnitude of the index and the toxicity level of the water sample.

## 1 Introduction

Because there is a need for the early detection of water contamination, research has focused on a biological early warning system (BEWS) over the last few decades (Butterworth et al., 2001; Kieu et al., 2001). In order to apply the system to regional environmental conditions and achieve different monitoring aims, BEWS has been diversified through the employment of various test organisms such as water fleas, mussels, algae, and fish. Among these organisms, *Daphnia magna*, which is a species of water flea, is widely used because of its usefulness in biomonitoring and high sensitivity to toxic chemicals (Butterworth et al., 2001). Because of its high sensitivity, behavioral changes in living *Daphnia magna* are a powerful real-time indicator in the detection of toxicants

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in the warning system. Different types of behavioral parameters have been developed for various kinds of monitoring systems; the monitoring systems of Knie, Kerren Umwelttechnik, and BBE Moldaenke employ swimming activity, phototactic behavior and swimming speed, and so on (Baillieul et al., 1998; Kieu et al., 2001). When *Daphnia magna* faces a change in water quality or a toxic condition in the BEWS, its behavior changes markedly, and the level of abnormality is determined from the recorded behavioral data. If the toxic level calculated from the measured data via the specific algorithm increases to above an intentionally fixed threshold, an alarm is triggered by a BEWS (Butterworth et al., 2001).

Even if the design of BEWS is appropriate for its goal, the possibility exists that the system will trigger false alarms during its practical operation. Problems can be also caused by uncontrolled variables such as improperly defined thresholds or machine error (Butterworth et al., 2001). In addition to these problems, inherent limitation exists due to behavioral deviations in living test organisms during the operation period and between individual organisms. To minimize the possibility of false alarms caused by these biological variations, the simultaneous utilization of a sufficient number of behavioral parameters is needed to supplement the weakness of individual parameters. The *Daphnia*-Toximeter, produced by BBE Moldaenke, is an example of a system that reduces the frequency of false alarms by simultaneously employing various parameters: velocity, fractal dimension, swimming height, distance between water fleas, and the number of moving water fleas (Baillieul et al., 1998; Michels et al., 1998; Lechelt et al., 2000; Jeon et al., 2008).

In a previous study, Jeon et al. (2008) developed a new BEWS, which is equipped with a grid counter device (GC) that identifies the position of the test organism at the grid level instead of at the pixel level. In addition, this system adopted a new algorithm for the alarm function, achieving not only cost effectiveness but also high performance. Accordingly, this study was conducted to enhance the power of the system developed by Jeon et al. (2008). The objectives of the present study are as follows: (1) develop a new, reasonable behavioral index that can be applied to a GC device-equipped BEWS;

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(2) improve the performance of the BEWS by applying various parameters at the same time. To reach these goals, a new type of index called the distribution Index (DI) was developed from newly suggested behavioral parameters, and the altitude Index (AI) was additionally generated by modifying the swimming height parameter of the *Daphnia* Toximeter (Lechelt et al., 2000). Finally, the combined index (CI) was derived by combining three behavioral indexes, including the toxic index (TI) that was developed by Jeon et al. The CI performance was evaluated by real-time exposure tests using synthetic water and effluent.

## 2 Materials and methods

### 2.1 System design and organism preparation

This study employed a BEWS equipped with a grid counter device. Compositions of the system were the same as in the previous study (Jeon et al., 2008), except that the number of chambers was adjusted from six to two. All methods for culturing *Daphnia magna* were conducted according to the USEPA guidelines (Weber, 1993). A constant temperature of  $22.5 \pm 1$  °C and 16 h of light with 8 h of dark were maintained during the culturing and the exposure test. A synthesized freshwater was prepared within the pH range of  $8.0 \pm 0.2$ , with hardness of  $170 \pm 10$  mg L<sup>-1</sup> and alkalinity of  $110 \pm 10$  mg L<sup>-1</sup> as CaCO<sub>3</sub> (MgSO<sub>4</sub>,  $9.98 \times 10^{-4}$  M; CaSO<sub>4</sub>,  $6.98 \times 10^{-4}$  M; NaHCO<sub>3</sub>,  $2.28 \times 10^{-3}$  M; KCl,  $1.07 \times 10^{-4}$  M).

### 2.2 Design of indexes

#### 2.2.1 Distribution index

The DI is a newly developed index generated from the swimming range parameter. This behavioral parameter is based on the homeostasis of *Daphnia's* swimming range. It is well known that the movement of *Daphnia* is predominantly affected by the degree

of light intensity and food abundance (Young et al., 1993). Hence, if the food distribution and light intensity are maintained regularly, the swimming range will be consistent. Previous studies of the behavior of *Daphnia* observed typical changes in activity mass resulting from the stimulus of environmental condition change (Butterworth et al., 2001; Shimizu et al., 2002). This finding motivated our study because activity mass is proportional to movement range. The previous research found that stimulus expressed as avoidance of chemicals or retardation by adverse effects affects the swimming activity, making it greater or lesser than in normal conditions. This means that the movement range could be widened or narrowed, according to the type of behavior elicited by the stimulus. Additionally, spinning behavior, which is an inherent habit of *Daphnia* in stressful conditions (Dodson et al., 1994), could also contribute to dramatic changes in movement ranges. We, therefore, assumed that the range of swimming distribution could be differentiated from normal conditions according to these activity patterns – wide, narrow, and extremely narrow ranges – and developed a new behavioral index based on this assumption.

In this study, with the GC device, a rectangular chamber containing water fleas was divided into 100 parts. Each section was marked for the location of water fleas according to the combination of numbers on the x- and y-axes; the sections were numbered from 0 to 99 (Fig. 1a). A camera was used to record the spatial data of individual water fleas, and the information was transferred to a computer. When a water flea migrated to another section, a new section number was recorded in the same manner. The data of the locations of the water fleas were shown in detail. The frequencies of appearance at each section were obtained at one minute intervals (Fig. 1b), and the distribution level (DL) was calculated (Fig. 1c). If the distribution range of the water fleas was concentrated in a specific, narrow region of the chambers, only a few sections showed a high frequency of appearance, so the value of DL increased. On the other hand, if each section showed a similar frequency of appearance, it implied that the water fleas moved in a wide range around the test chamber, and the value of DL decreased.

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the toxicant stimulates *Daphnia*, the average swimming position moves to an abnormal height, which makes the AI differ from normal conditions.

We generated the AI using a grid counter device. As mentioned in the case of the DI, each chamber was divided into 100 parts, and each section was numbered. The altitude of *Daphnia magna* was thus recognized according to section numbers from 0 to 9 along the y-axis from the top to the bottom, as shown in Fig. 2a. The method used to record the altitude was the same as the method used with DI. The average altitude, called altitude level (AL), was calculated from the recorded altitudes (Fig. 2b). The equation for AL is

$$AL = \mu_{Alt} \quad (3)$$

where  $\mu_{Alt}$  is the average of every observed altitude for 1 min. After this AL calculation, the  $Za$  for AL and the  $\log(1/p)$  for  $Za$  using a student's t-test were derived in the same way as for DI. Finally, the AI was produced in real time by adding a step to reduce noise at an interval of 1 min.

### 2.2.3 Toxic index and combined index

The TI previously developed by Jeon et al. (2008) was also employed in this study for the derivation of the CI and the comparison with the new indexes. All calculation methods were identical to those in that previous study except for the transformation procedure. The raw data on swimming distance were directly used to calculate  $Za$  in this study. First, the number of events counted (NC) was measured to indicate the swimming distance of *Daphnia* for 1 min, and the deviation ratio ( $Za$ ) for NC was calculated to detect whether a considerable change in NC had occurred.  $Za$  were compared between the normal condition and the toxic condition using a student's t-test, and the  $\log(1/P)$  was derived. The TI was then calculated with a moving average using the same methods as used for the DI and AI at 1 min intervals. Finally, three parameters were combined into the CI by averaging the values of DI, AI, and TI. This CI was derived every 1 min and was compared to the DI, AI, and TI through a real-time exposure test.

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## 2.3 Experiment methods

### 2.3.1 Preparation for tests

The experiment was composed of two different real-time exposure tests. The copper exposure test was conducted on synthetic water samples, and the effluent exposure test used effluents sampled from three separate regions. The performance of the indexes was compared, and the applicability of the CI, which combined three indexes, to the field sample was evaluated using the results of the copper and effluent exposure tests. The age of *Daphnia magna* used in the tests ranged from 96 to 120 h for the investigation of behavioral changes. If the test organism is too young, it could be excessively sensitive, or it could die soon after exposure to a toxic chemical. The test organisms were selected from one stock culture to reduce variance in the behavioral character among individuals and the third brood neonates were used. Every test was conducted without food; the absence of food does not affect the behavior of *Daphnia magna* (Demeester, 1992).

In the copper exposure test, copper ( $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ ) was used as the representative toxic chemical because of *Daphnia magna*'s high sensitivity to it and its wide use in various industries. Notably, South Korea uses  $400 \text{ Gg Cu yr}^{-1}$  of copper annually and is the third highest copper-producing country in Asia, making copper an important toxicant and thus relevant for use in this study (Graedel et al., 2004). Before the test, a preliminary acute toxicity test for determining LC50 of copper was conducted over 24-h using *Daphnia magna* in a static condition. Because 24-h LC50 is typically chosen as the toxic level of one toxic unit (TU), the determination of a middle concentration of 75 ppb for the main test was based on the 24-h LC50 value of the pre-test (54.371 to 70.717 ppb). The minimum concentration of 18.75 ppb was determined as the basis for the lowest observed effect concentration (12.5 ppb) in the pre-test to investigate the effects at different concentrations both above and below 24-h LC50. In the main test, we prepared copper solution daily with hard water at concentrations of 0 ppb, 18.75 ppb, 37.5 ppb, 75 ppb, 150 ppb, and 300 ppb. These concentrations are relevant

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for the BEWS study; it was reported that copper was present in a range of 0.2–11 ppb in the Yeongsan River in South Korea (Kim et al., 2011). The copper exposure tests were triplicated for every test set.

The effluent exposure test sampled three different effluents from Iksan, Yesan, and Yeosu. The toxicity level of each effluent was respectively confirmed as 0 TU, 50.50 TU, and 1.23 TU by the preliminary 24-h whole effluent toxicity test. The non-toxic effluent from Iksan was used for demonstrating the stability of parameters. Others, including the diluted samples, were used to estimate the early warning capability of parameters in the field water samples. The effluent from Yesan was diluted to 0.3, 0.7, 1, 1.6, 2.6, 3.3, 3.4, and 4.3 TU. The main contaminants in the sample waters from Yesan and Yeosu were not revealed; however, the characteristics of the two water samples were quite different. The sample from Yesan was of fresh water and that from Yeosu was of salt water (Kim et al., 2009).

### 2.3.2 Test procedures

The exposure test was conducted for 180 min. Before the exposure, we carried out a 120 min feeding time, 180 min adaptation time, and 180 min unexposed period. During the feeding time, we fed *Daphnia magna* a mixture of Pseudokirchneriella subcapitata and yeast, CEROPHYLL, and trout chow (YCT). Because the copper exposure test was conducted in the absence of food, *Daphnia magna* needed a full feeding. To avoid the effect of environmental conditions such as light, temperature, and food level, we did not support any nutritive element and maintained constant light and temperature conditions in the isolated monitoring room after feeding (Dodson, 1989). In the next step, the adaptation time, *Daphnia magna* were transferred into two test chambers; each chamber contained 10 *Daphnia magna*. *Daphnia magna* were allowed to swim in the chamber with water flux for 180 min, because an adaption period of at least 2 h is required for *Daphnia magna* to obtain a constant swimming pattern by acclimating to water flow after being transferred (Wolf et al., 1998; Untersteiner et al., 2003). In the subsequent step, the unexposed period, the behaviors of *Daphnia* were recorded for

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180 min. These data were used as a control when the student's t-test was conducted with the data on the exposed period. In the last step, the exposure period, the influent sample to chambers was replaced with a copper solution or effluent, and this condition was maintained for 180 min. Every pump was stopped during the replacement period.

5 Data obtained for 30 min before and after stopping the pump was excluded to avoid exceptional behavioral changes caused by this procedure. Because the detection time of toxicant exposure by BEWS is not lengthy, we limited the maximum exposure time to 180 min to evaluate whether our system could detect differences in behavior in the early phase of contamination.

## 10 2.4 Data analysis

A student's t-test was utilized to compare differences between the behavioral index data during the unexposed and exposed periods. Matlab R2009b, Microsoft Excel 2010, and Sigmaplot 10.0 were used for every data treatment introduced in the above sections, including the student's t-test and graph illustration.

## 15 3 Results and discussion

### 3.1 Copper exposure test

The results of the indexes in the normal condition (0 ppb of copper) are expressed as graphs in Fig. 3. All individual indexes (DI, AI, and TI) displayed statistically significant differences in index values ( $p < 0.01$ , index  $> 2$ ) in the uncontaminated condition. In contrast, the result of the CI was stable at below 2, a threshold implying a probability of 0.01 % for significant behavioral difference. This result proves the usefulness of combining indexes at the same time in terms of data stability. The raw data from the copper exposure test are summarized in Fig. S1 in the Supplement. Although fluctuations in the time series data are apparent, all indexes show the increasing tendency with increased concentration. For the quantitative evaluation of the power of the indexes to

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reflect changes in toxicity levels, the averages of every calculated index value during exposure time at each concentration were derived as a representative value. Then, linear regressions between the copper concentration and the average values were conducted, as shown in Fig. 4. A comparison of the correlation coefficients ( $r^2$ ) showed that the CI had the highest one and the DI showed the highest value among the individual indexes. This result means that the CI is most capable of performing accurate early water contamination warning, and the DI best reflects the differences in toxicity levels among the individual indexes derived from the three different behavioral parameters.

### 3.2 Effluent exposure test

The results of the CI in the real-time effluent exposure test are expressed as graphs in Fig. 5, where (a) and (b) display the results of the non-toxic effluent from Iksan and the linear regression between the average of the CI and TU of different effluents, respectively. The exposure to non-toxic effluent shows stable index values during the test period at below 2 ( $p > 0.01$ , index  $< 2$ ), which proves the stability of the CI. In (b), which shows the results of tests using effluent from Yeosu and Yesan, the correlation is much weaker than that in the copper exposure test, with a huge deviation. This result was expected from the deviation of data from the copper exposure test. These fluctuating data confirmed that the system is insufficiently powerful to detect precise toxicity levels of the field samples without replication. The raw data from the effluent exposure tests are summarized in Fig. S2 in the Supplement. This result shows that data from the Yeosu effluent have a higher index value than even the water sample containing a higher number of toxic units, which implies that the different characteristics of water samples can affect the magnitude of index values.

### 3.3 Discussion

In this study, we suggested a new concept of behavioral parameters related to changes in swimming range. Using this new parameter, the DI and CI were derived, and the



between regional water characteristic and the behavior of test organisms are required at the monitoring site where the system is set.

The conclusions derived from this study are as follows.

1. The DI derived from the new behavioral parameter shows the best performance among the three indexes employed.
2. The CI improved performance of the BEWS in terms of sensitivity and stability, and it was confirmed as the higher correlation coefficient between the magnitude of the index and the toxicity level of the water sample.
3. Large deviations in the index data and unexpected changes in behavioral responses to water samples containing different characteristics occurred in the present study; therefore, further studies to enhance the accuracy and applicability of BEWSs in the field are needed.

**Supplementary material related to this article is available online at:**

**[http://www.drink-water-eng-sci-discuss.net/6/39/2013/](http://www.drink-water-eng-sci-discuss.net/6/39/2013/dwesd-6-39-2013-supplement.pdf)**

**[dwesd-6-39-2013-supplement.pdf](http://www.drink-water-eng-sci-discuss.net/6/39/2013/dwesd-6-39-2013-supplement.pdf).**

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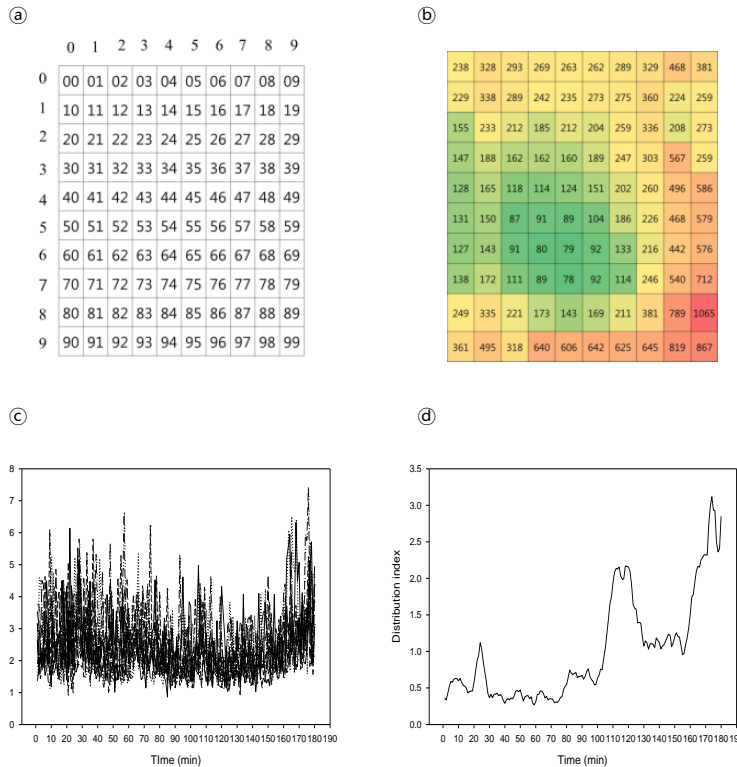
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**Fig. 1.** Procedure for generating the distribution index. **(a)** Numbering sections 00 to 99 for recognition of each section. **(b)** Calculation of appearance frequency of *Daphnia magna* at each section; this image was colored to easily view the frequency level of occupation (Red: highest, Yellow: medium, Green: lowest). **(c)** Plots of 10 distribution levels derived from 10 *Daphnia magna*. **(d)** A Plot of a DI derived from 10 distribution levels.

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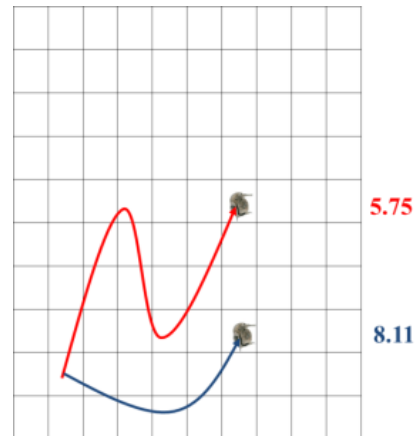
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a)

0	0	0	0	0	0	0	0	0	0
1	1	1	1	1	1	1	1	1	1
2	2	2	2	2	2	2	2	2	2
3	3	3	3	3	3	3	3	3	3
4	4	4	4	4	4	4	4	4	4
5	5	5	5	5	5	5	5	5	5
6	6	6	6	6	6	6	6	6	6
7	7	7	7	7	7	7	7	7	7
8	8	8	8	8	8	8	8	8	8
9	9	9	9	9	9	9	9	9	9

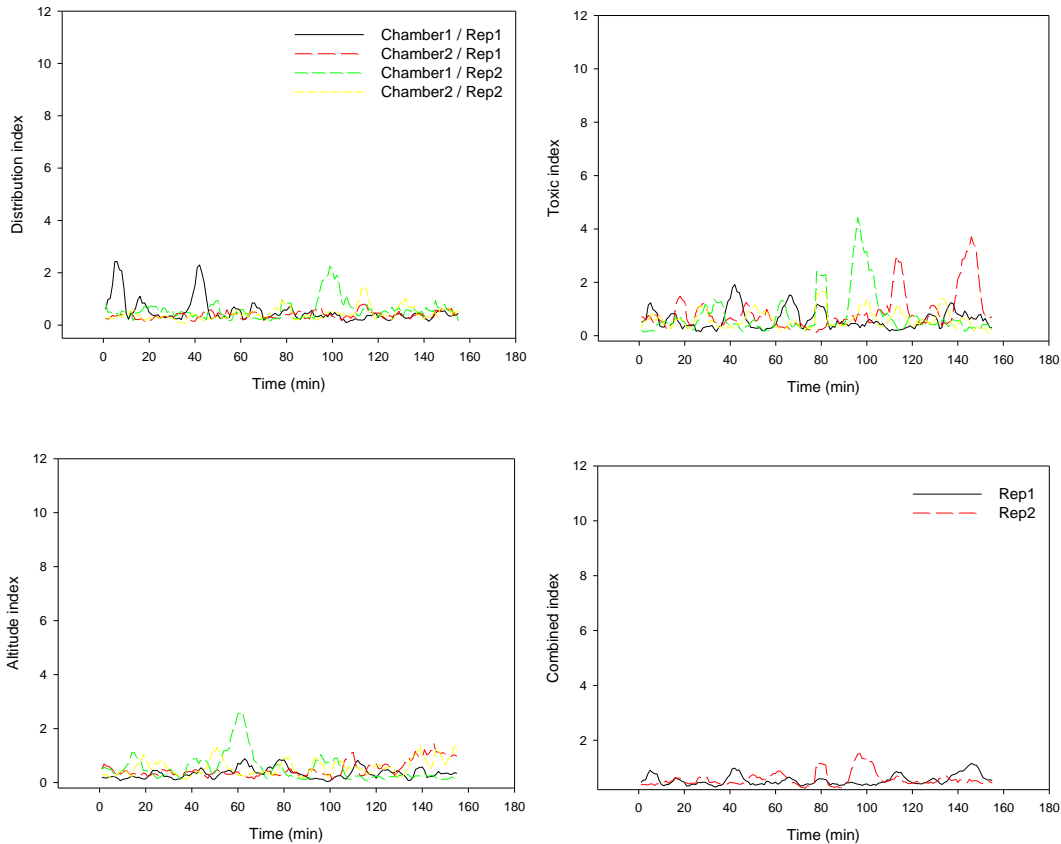
b)



**Fig. 2.** Recognition of swimming altitude of *Daphnia magna*. **(a)** Numbering sections 0 to 9 for recognition of each altitude. **(b)** Example of altitude recognition; the average altitudes of *Daphnia magna* colored red and blue are calculated.

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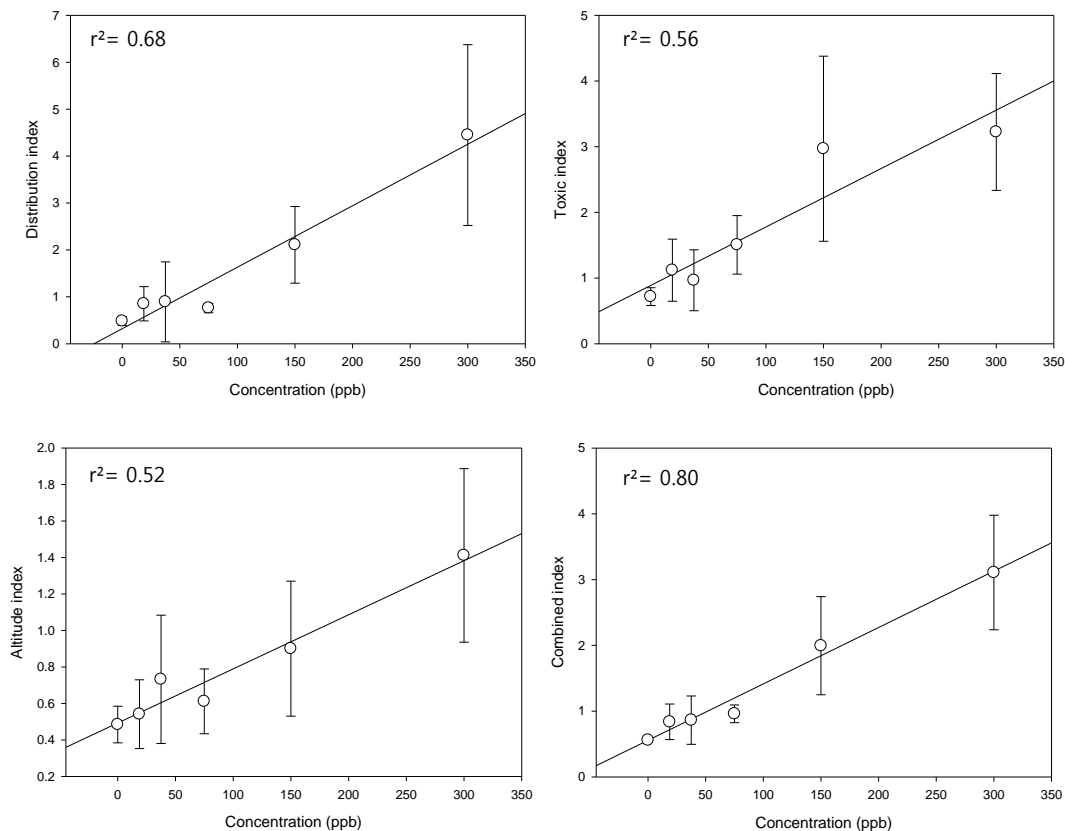
**Fig. 3.** Results of indexes (DI, TI, AI, and CI) in normal condition. The Rep# refers replication number of test.

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**Fig. 4.** Linear regressions between representing value (average) of index and copper concentration. The line is regression line and  $r^2$  is correlation coefficient value.

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