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**Title: Reappraisal of the toxicity test method using the green alga *Ulva pertusa* Kjellman (Chlorophyta)**

**Running title: A novel bioassay method using a green macroalga *Ulva pertusa***

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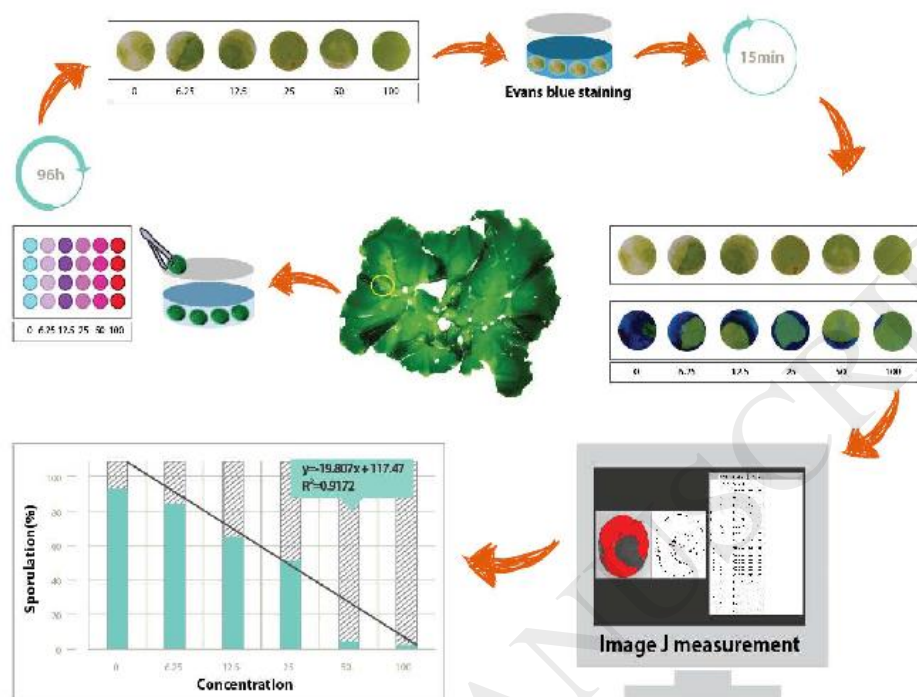
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## Graphical abstract



## [Highlights]

- The *Ulva* bioassay is a sensitive, cost-effective, and simple test method.
- The new *Ulva* test has an added value of objectiveness using staining + image analysis.
- The *Ulva* method showed consistent responses to Cu and Cd of internationally allowable ranges.
- This method is a useful tool for monitoring industrial wastewaters containing metals.

## Abstract

This study was aimed to develop an objective way of quantifying the reproductive status of the green macroalga, *Ulva pertusa* using a vital stain and programmed automated analysis (by

Image J program). The EC<sub>50</sub> values (with 95% CI), the concentrations of toxicants inducing a reduction of 50% in sporulation after 96 h exposure, from the newly developed method were similar to those obtained by the conventional method: 0.651 (0.598-0.705) mg l<sup>-1</sup> for Cd, 0.144 (0.110-0.162) mg l<sup>-1</sup> for Cu, 0.180 (0.165-0.195) mg l<sup>-1</sup> for atrazine, 0.076 (0.049-0.094) mg l<sup>-1</sup> for diuron and 30.6 (26.5-34.4) ml l<sup>-1</sup> for DMSO, respectively. When the EC<sub>50</sub> values from this study were compared to that those from literatures, the sensitivity for some toxicants was similar or higher than that of *U. fasciata* (1.930 mg l<sup>-1</sup> for germination for Cd), *U. armoricana* (0.250 mg l<sup>-1</sup> for Fv/Fm for Cu), *U. reticulata* (0.126-1.585 mg l<sup>-1</sup> for growth for Cu), and *U. intestinalis* (0.650 mg l<sup>-1</sup> for Fv/Fm for atrazine). The subjective views of the experimental performers can be eliminated using the newly developed method. The *Ulva* method gave consistent responses to Cu and Cd of internationally allowable ranges for effluents, implying that the method is a useful tool for monitoring industrial wastewaters containing these metals.

**Keywords:** Bioassay; Ecotoxicity; Image analysis; Evans Blue; *Ulva pertusa*

## 1. Introduction

Over the past few years we have developed a novel bioassay for ecological toxicity testing, the basis of which is inhibition of reproduction in the green macroalga *Ulva pertusa* by quantifying the striking change in thallus color, as reproduction progresses [1]. Since first being developed, the bioassay has been applied to more than 75 different environmental samples and the sensitivity of the *Ulva* method is similar to, and in many cases higher than, commonly available or well-established bioassay methods [2, 3]. Furthermore, the method can be considered to be both cost- and time-effective because no specialist expertise is required to carry out the bioassay and it needs only cell plates, a small volume of medium and is completed within a total time of approximately 3 h following the 96 h exposure period [2, 4].

However, despite the many merits of the test, it became apparent during extensive testing of the method with personnel having different skill levels that visual interpretation of thallus color changes is open to a degree of subjectivity that has an influence on the final results. Therefore, to overcome these problems and to ensure greater objectivity, while at the same time maintaining a degree of sensitivity similar to that of the conventional bioassay, an alternative approach for measuring thallus color change was developed. This involves a combination of a viability stain, Evans Blue, and image analysis software. In fact, the combination as such has already been used as a standard procedure in histology [5, 6]. Evans Blue is a vital stain commonly used for determining cell viability in higher plants and algae and has been used for measuring dead cells in microalgae [7], and for determining the degree of damage to *Ulva australis* tissue after antibiotic treatment [8].

While living cells exclude the Evans Blue stain, dead cells and cell debris incorporate the stain, and the reproductive areas of *Ulva* thalli should thus be stained and readily

distinguishable since reproductive cells are released, thus leaving reproductive areas empty, i.e. dead [9].

Image analysis software is helpful for viewing and measuring structures in a variety of spatial and temporal image dimensions. Image J is a public domain, Java-based image processing program developed by the National Institutes of Health in the U.S. It has been used previously to examine various morphometric characteristics of organisms [10].

This study was aimed to develop an objective way of quantifying the reproductive status of the green macroalga, *Ulva pertusa* using Evans Blue staining and Image J program.

## **2. Materials and methods**

### *2.1. Plant and Culture Conditions*

*Ulva pertusa* Kjellman was collected from sites close to Dara Harbor (Tongyeong, Geongnam) on the southern coast of Korea (34°46'15" N, 128°23'97"E). After the seaweed was transported to the laboratory in a cooler, unialgal stock cultures were maintained in plastic tanks (10 L Volume) with aerated artificial seawater medium (35 psu) prepared by dissolving commercial sea salts (Coralife; Energy Savers, CA, USA) in deionized water, KNO<sub>3</sub> (1 mM) and K<sub>2</sub>HPO<sub>4</sub> (0.1 mM) of nutrients were added to the culture. pH of the medium was adjusted to 8.0±0.2 using 1 M NaOH and 1 M HCl. Cultures were maintained at 15 °C under 30-40 µmol photons m<sup>-2</sup>s<sup>-1</sup> of white fluorescent light (FL20SS, Phillips, Netherlands) on a 12:12 h light-dark cycle.

### *2.2. Toxicity tests*

The toxicity analysis using *Ulva* was conducted following a modification of the previous protocol [2]. The major modification includes that a staining process was added and images were analyzed using by Image J (NIH Bethesda, MD, USA) instead of Motic (Moticam

Solution 2500, Motic cor., Xiamen, China) was used for image analysis. To clarify if whitened thallus color was due to completion of reproduction or due to destruction of pigments, a coverslip was placed on the bottom of each well of cell plates and was observed after the 96 h exposure to each toxicant to determine the presence/absence of released spores. For comparison, the newly developed methods with the conventional methods were summarized in Table 1.

Under optimal test conditions [2], the toxicity test was conducted by dispensing 2.5 ml growth medium into one cell as control and five different concentrations of 2.5 ml test solution into other cells in a 24-well plate (30024, SPL, Korea). A lid was placed over the cell plate(s) and then sealed with a sealing tape to prevent evaporation of the solution. Three replicate cell plates were prepared and were incubated under  $80\text{--}100\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$  of white fluorescent light at  $15\pm 1\ ^\circ\text{C}$ . After a 96 h incubation period, algal disks were harvested and their percent reproduction was measured. The test toxicant concentrations were in ranges,  $0.03125\text{--}1.0\ \text{mg l}^{-1}$  for Cd ( $\text{CdSO}_4$ ;  $1,000\ \text{mg L}^{-1}$  standard solution, Showa, Japan, CAS no. 7440-43-9),  $0.03125\text{--}0.5\ \text{mg l}^{-1}$  for Cu ( $\text{CuSO}_4$ ;  $1,000\ \text{mg L}^{-1}$  standard solution, Showa, Japan, CAS no. 7440-50-8),  $0.01563\text{--}0.25\ \text{mg l}^{-1}$  for atrazine (2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; 98.8%, Sigma-Aldrich Co., USA, CAS no. 1912-24-9),  $0.00625\text{--}0.1\ \text{mg l}^{-1}$  for diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea;  $\geq 98\%$ , Sigma-Aldrich Co., USA, CAS no. 330-54-1), and  $2.5\text{--}40.0\ \text{ml l}^{-1}$  for dimethyl sulfoxide (DMSO,  $(\text{CH}_3)_2\text{SO}$ ;  $\geq 99.9\%$ , Sigma-Aldrich Co., USA, CAS no. 67-68-5). Metals stock solutions of concentrated standards were diluted with growth medium ( $n=9$  plates, 24 disks per plate with 4 disks per concentration).

### *2.3. Evaluation of percent reproduction*

The results of the tests were evaluated as percent reproduction (%) obtained by dividing the reproductive area by whole area of disks. The formula is as follows:

$$\text{Percent reproduction (\%)} = \frac{\text{Reproductive area of disk (mm}^2\text{)}}{\text{Whole area of disk (mm}^2\text{)}} \times 100$$

After 96 h, the algal disks were withdrawn from the test medium and placed into a 2.5 ml of 0.5% (w/v) Evans Blue solution for 15 min at room temperature and subsequently washed twice with pure water to remove excess and unbound dye. Images of stained disks were then recorded using a computer assisted image camera (Moticam Solution 2500, Motic cor., Xiamen, China) and assessment of percent reproduction was carried out using Image J software (NIH Bethesda, MD, USA). Percentage sporulation of the negative controls should be higher than 75% for the validity of the test [2]. Otherwise, the test results were considered invalid.

#### *2.4. Image analysis using Image J*

The initial images of *Ulva* disks were converted into gray scale images by setting “Image-Color-Split Channels” which displays three channels of images (red, green, and blue). The green channel image was then selected, and the reproductive area (stained area) was specified using the “Image-Adjust-Threshold” setting. To obtain the threshold values for capturing the stained area, those for the whole and reproductive area were first analyzed using a total number of 134 and 137 values between the threshold value 65 and 172 for the whole area and 0 and 165 for the reproductive area, with the mean threshold value (with 95% confidence intervals; 95% CI) being 128 (110-146) and 69 (22-116), respectively. For comparisons, the threshold values were then set to 110, 128 and 146 for the whole disk area and set to 22, 69, 116 for the reproductive disk area. The best matched threshold values were then determined



by a paired sample t-test of the percent reproduction obtained from both the Evans Blue staining and the conventional method. The designated area was analyzed by setting “Analyze-Analyze particles”.

### 2.5. Statistical analysis

Analysis of variance (ANOVA) was performed to confirm significant differences in response. Multiple comparison tests by the least significant difference (LSD) were then carried out to find significant differences from the controls and between treatments. Results were then reported as  $EC_{50}$  (effective concentration at which 50% inhibition occurs) with 95% CI estimated by the linear interpolation method (ToxCalc 5.0, Tidepool Science, California, USA). A statistical comparison for significant differences of  $EC_{50}$  values to same toxicants from two method was performed using the Wilcoxon ranked sum analysis ( $p < 0.05$ ).

When the linearity test indicated no statistical significance at  $p < 0.05$ , the maximum likelihood-logit method was employed to determine  $EC_{50}$ . The coefficient of variation (CV), the standard deviation expressed as a percentage of the mean, was calculated to estimate the precision of the tests. For comparison of the two regression coefficients fitted for data from the conventional method and the new method Student t-tests were performed [11].

## 3. Results and Discussion

In the original *Ulva* bioassay, the distinction of the whitened reproductive areas is made by naked eye. The ambiguity in distinguishing the reproductive areas of the *Ulva* disks resides in the measurement of percent reproduction, which can be eliminated by the application of techniques of staining the reproductive areas (Figure 2) and subsequent automated measurement of stained areas (Figure 3). The selected threshold values from paired sample t-test were found to be 128 for the whole disk area and 69 for the reproductive area,

respectively (Figure 1).

Toxicity evaluations of five toxicants showed that there was significant decrease in percent reproduction with regard to the concentration of toxicants tested (Figure 4). The  $EC_{50}$  values (with 95% CI) of percent reproduction showed similar values in both methods ( $p < 0.05$ ). The  $EC_{50}$  values (with 95% CI) obtained by conventional method 0.740 (0.590-0.890)  $mg\ l^{-1}$  for Cd, 0.147 (0.117-0.166)  $mg\ l^{-1}$  for Cu, 0.194 (0.180-0.207)  $mg\ l^{-1}$  for atrazine, 0.053 (0.048-0.057)  $mg\ l^{-1}$  for diuron, and 26.2 (23.3-28.3)  $ml\ l^{-1}$  for DMSO. The  $EC_{50}$  values (with 95% CI) obtained by newly developed method were 0.651 (0.598-0.705)  $mg\ l^{-1}$  for Cd, 0.144 (0.110-0.162)  $mg\ l^{-1}$  for Cu, 0.180 (0.165-0.195)  $mg\ l^{-1}$  for atrazine, 0.076 (0.049-0.094) for diuron, and 30.6 (26.5-34.4)  $ml\ l^{-1}$  for DMSO (Table 2). CV values of  $EC_{50}$  from the conventional and newly developed method were 2.94% and 17.10% for Cd, 2.23% and 9.67% for Cu, 22.34% and 10.45% for atrazine, 14.95% and 7.70% for diuron, 5.01% and 7.06% for DMSO, respectively. CV values were found to vary depending on the type of toxicants tested, but to satisfy the recommended level of precision (20-40%) for reference toxicity test [12]. The relatively low CV values indicate good repeatability and stability of the methods (Table 2).

Microscopic observation of the cover slips placed on the bottom of test dishes showed that there was no spores attached on the cover slips at the highest concentration and indicated that no reproduction occurred at the highest concentration.

A statistical test of the equality of the two regression coefficients obtained from concentration-response curves of the two methods revealed that there was no statistically significant difference at  $p = 0.05$ , indicating the similarity of the extent of sensitivity of the methods. However, the conventional method is manually operated, while the new method is automated, thereby, eliminating the involvement of subjective views of the experimental performers.

The toxicity of Cd as shown by EC<sub>50</sub> values (with 95% CI) from the new method was 0.651 (0.598-0.705) mg l<sup>-1</sup> which is similar to or higher than that reported with the same species: 0.095-0.326 mg l<sup>-1</sup> for sporulation, 0.189-0.200 mg l<sup>-1</sup> for gametophyte growth (length and cell no., respectively), 0.789 mg l<sup>-1</sup> for germination [1, 2, 13], but it is lower as compared with that reported for *U. fasciata* (1.930 mg l<sup>-1</sup> for germination) [14]. The EC<sub>50</sub> values (with 95% CI) of Cu toxicity obtained from the new method were 0.144 (0.110-0.162) mg l<sup>-1</sup> which is less sensitive than some other parameters reported in the same species (0.050-0.100 mg l<sup>-1</sup> for growth, 0.023-0.027 mg l<sup>-1</sup> for gametophyte growth, 0.017 mg l<sup>-1</sup> for germination) [3]. When compared with *U. armoricana* (0.250 mg l<sup>-1</sup> for F<sub>v</sub>/F<sub>m</sub>) and *U. reticulata* (0.126-1.585 mg l<sup>-1</sup> for growth), however, *U. pertusa* sporulation measured by the new method was more sensitive than or similar to these other measured parameters [15, 16]. With the EC<sub>50</sub> values (with 95% CI) of 0.076 (0.049-0.094) mg l<sup>-1</sup> to diuron, the end-point used in this study is less sensitive than that reported previously with *U. intestinalis* (0.003 mg l<sup>-1</sup> for germling growth) and *U. pertusa* (0.010 mg l<sup>-1</sup> for motility of spores) [17, 18]. EC<sub>50</sub> values (with 95% CI) of atrazine toxicity were 0.180 (0.165-0.195) mg l<sup>-1</sup> for the new method. In a study on the green macroalga *U. intestinalis*, EC<sub>50</sub> values (with 95% CI) derived from inhibition of growth and F<sub>v</sub>/F<sub>m</sub> were 0.143 and 0.650 mg l<sup>-1</sup>, respectively [18]. In the case of the DMSO, which is increasingly used in industrial areas, the toxic effects in marine macroalgae are not well studied. In this study, the EC<sub>50</sub> values (with 95% CI) of DMSO were 26.512-34.419 ml l<sup>-1</sup>. Okumura et al. [20] reported the influence of DMSO on the growth of marine microalgae, with the range of EC<sub>50</sub> values being 3.80-23.0 mg l<sup>-1</sup>.

#### 4. Conclusions

Compared with the conventional method, the newly proposed *Ulva* test has an additional value of objectiveness in the measurement process by using staining with Evans Blue and

subsequent image analysis with a free software ‘Image J’ provided by NIH. Our EC<sub>50</sub> values of Cu toxicity (0.110-0.162 mg l<sup>-1</sup>) determined by staining and image J analysis were lower than the limit values for effluent discharge regulations of Australia (5.0 mg l<sup>-1</sup>), Canada (5.0 mg l<sup>-1</sup>), FAO (0.5 mg l<sup>-1</sup>), Germany (0.01-1.0 mg l<sup>-1</sup>), Japan (3.0 mg l<sup>-1</sup>), Korea (1.0 mg l<sup>-1</sup>) and USA (0.1-4.14 mg l<sup>-1</sup>). On the other hand, the EC<sub>50</sub> values (0.598-0.705 mg l<sup>-1</sup>) of Cd toxicity satisfied the ranges of effluent discharge regulations of Australia (1.0 mg l<sup>-1</sup>), Canada and USA (0.01-0.78 mg l<sup>-1</sup>). Since herbicides (atrazine and diuron) and DMSO have no international standard limits for effluents, only the EC<sub>50</sub> values of heavy metals (Cd, Cu) were compared with maximum permissible limits in effluents for the presence of given toxicants set by Australia, Canada, FAO, Germany, Japan, Korea and USA.

The present study has shown that the new *Ulva* method gave consistent responses to Cu and Cd of internationally allowable ranges for effluents, implying that the method is a useful tool for monitoring industrial wastewaters containing these metals. Members of the order Ulvales show a wide geographic distribution and have similar reproductive characteristics, thus making it possible to apply the present test method to other algae of these taxa, elsewhere.

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## Figure Captions

**Figure 1.** After Evans Blue staining, only the reproductive areas were stained in blue (A).

The reproductive areas were then analyzed in red based on the threshold value of 22 (the minimum value) (B), 69 (the mean value) (C) and 116 (the maximum value).

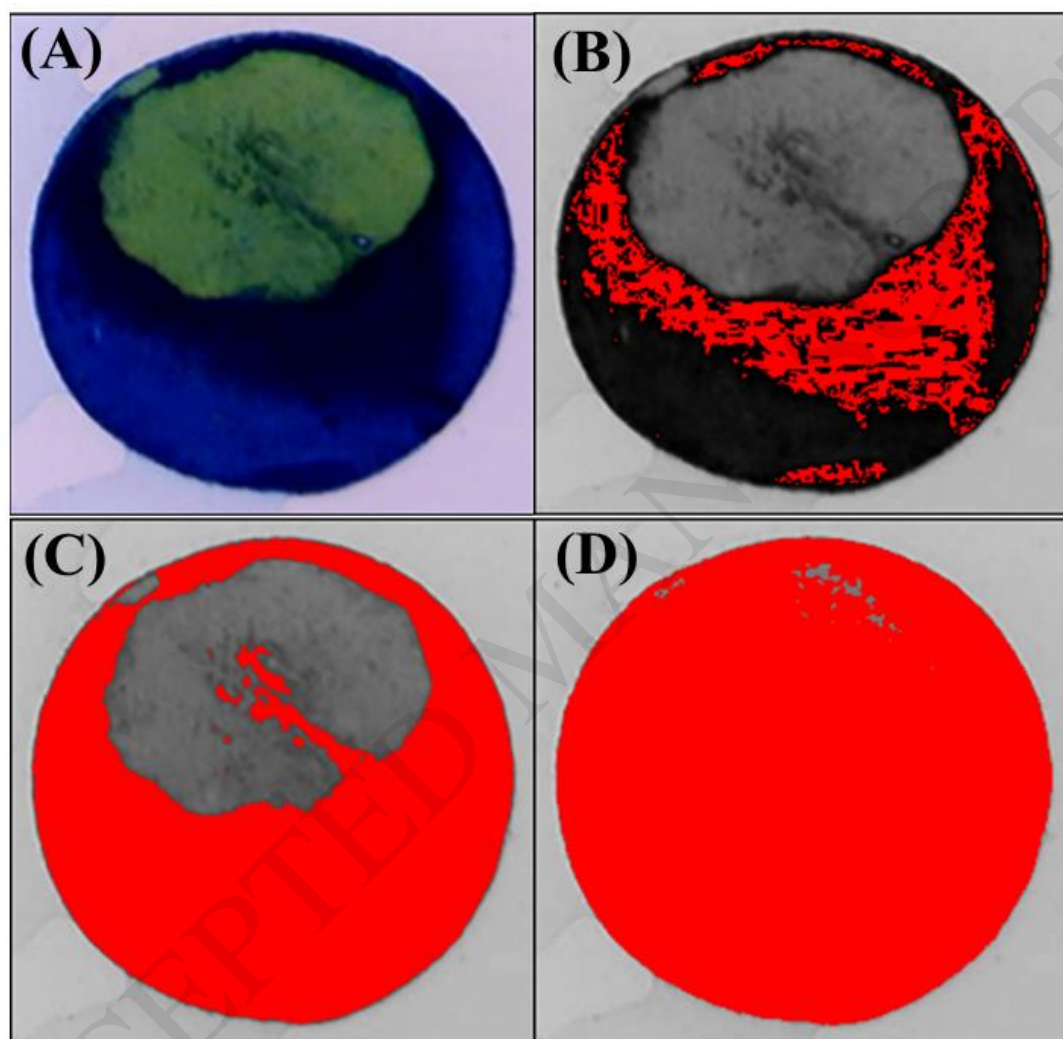
**Figure 2.** Microscopic observations of thallus cells before and after Evans Blue staining. A-B: Vegetative thallus parts before and after staining, respectively. C-D: Reproductive thallus parts before and after staining, respectively.

**Figure 3.** Indices of reproductive inhibition of *U. pertusa* exposed to different concentrations of copper (CAS no. 7440-50-8) for 96 h.

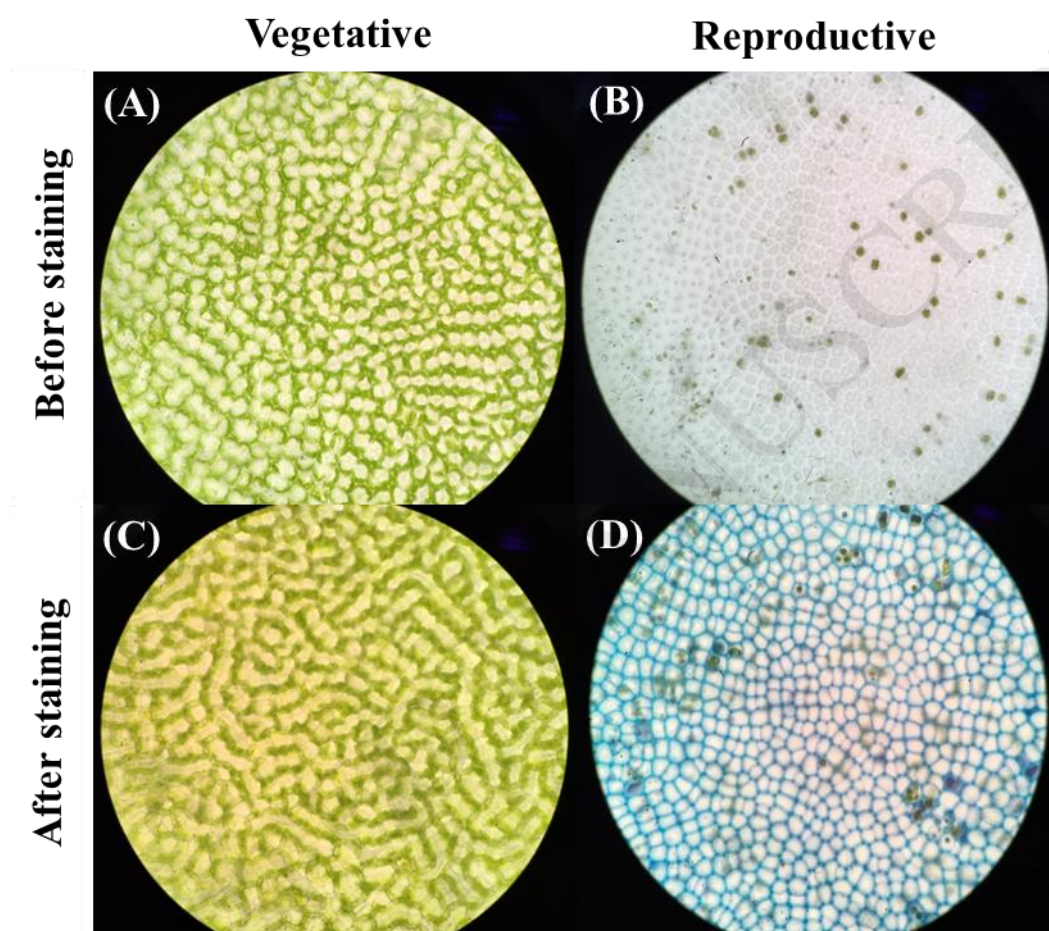
**Figure 4.** Effects of 96 h exposure to different concentrations of 5 toxicants (Cd, Cu, atrazine, diuron, DMSO) on percent reproduction from conventional and novel method. Mean and 95% CI are shown (n=9 plates, 24 disks per plate with 4 disks per concentrations). LSD means the least significant difference ( $p < 0.05$ ).









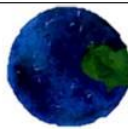
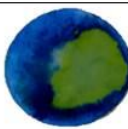
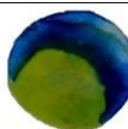
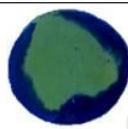
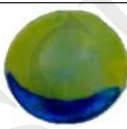
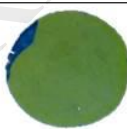
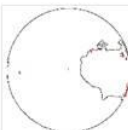





**Figure 1.** After Evans Blue staining, only the reproductive areas were stained in blue (A). The reproductive areas were then analyzed in red based on the threshold value of 22 (the minimum value) (B), 69 (the mean value) (C) and 116 (the maximum value) (D).



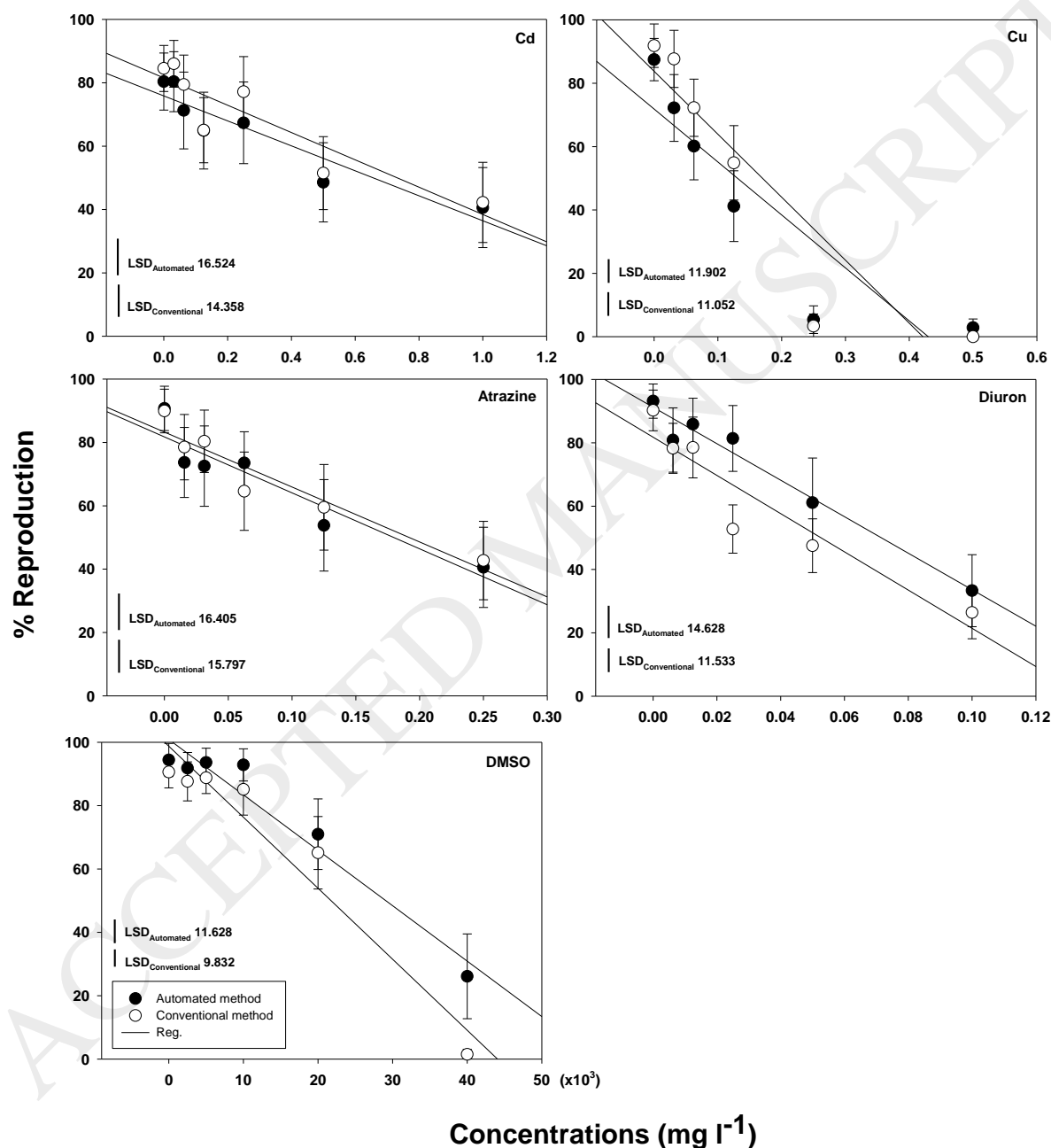
**Figure 2.** Microscopic observations of thallus cells before and after Evans Blue staining. A-C: Vegetative thallus parts before and after staining, respectively. B-D: Reproductive thallus parts before and after staining, respectively.



**Figure 3.** Indices of reproductive inhibition of *U. pertusa* exposed to different concentrations of copper ( $\text{CuSO}_4$ ; CAS no. 7440-50-8) for 96 h.

	Concentration of copper ( $\text{mg l}^{-1}$ )					
	0	0.0313	0.063	0.125	0.25	0.5
Before staining						
After staining						
Image J						
Reproduction rates (%)	89.1	78.8	78.1	55.3	14.7	1.2

**Figure 4.** Effects of 96 h exposure to different concentrations of 5 toxicants (Cd, Cu, atrazine, diuron, DMSO) on percent reproduction from conventional and novel method. Mean and 95% CI are shown (n=9 plates, 24 disks per plate with 4 disks per concentrations). LSD means the least significant difference ( $p < 0.05$ ).



**Table Legends**

**Table 1.** Summary of conventional method vs. this study

**Table 2.** Effective concentration at which 10% inhibition occurs ( $EC_{10}$ ), Effective concentration at which 50% inhibition occurs ( $EC_{50}$ ), and Coefficient of variation (CV) values for inhibition of percent reproduction in *U. pertusa* exposed to 5 toxicants. Mean and 95% CI are shown (n=9 plates, 24 disks per plate with 4 disks per concentration)

**Table 1.** Summary of conventional method vs. this study

	Conventional method	This study
Test species	<i>Ulva pertusa</i>	<i>U. pertusa</i>
Test duration	4 days (96 h)	4 days (96 h)
Temperature	15 ± 1 °C	15 ± 1 °C
Salinity	35 ± 1 psu	35 ± 1 psu
Light intensity	80-100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$	80-100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$
Photoperiod	12:12 (L:D)	12:12 (L:D)
Test vessel type	24-well plates	24-well plates
Medium	Coralife medium	Coralife medium
Test solution volume	2.5 ml	2.5 ml
Test solution pH	8.0 ± 0.2	8.0 ± 0.2
Test organism size	Ø 9.4 mm	Ø 9.4 mm
Endpoint	Percent reproduction	Percent reproduction
Distinction between reproduced and dead thallus	-	The presence and/or absence of spores settled on the cover slip
Measurement method	Naked eyes	Evans Blue staining and automated Image analysis using Image J program
Measuring type	Non-automatic	Automatic

**Table 2.** Effective concentration at which 10% inhibition occurs (EC<sub>10</sub>), Effective concentration at which 50% inhibition occurs (EC<sub>50</sub>), and Coefficient of variation (CV) values for inhibition of percent reproduction in *U. pertusa* exposed to 5 toxicants. Mean and

Toxicants	Toxicity determined by naked eyes				Toxicity determined by staining and Image J method			
	EC <sub>10</sub> (95% CI range)	CV (%)	EC <sub>50</sub> (95% CI range)	CV (%)	EC <sub>10</sub> (95% CI range)	CV (%)	EC <sub>50</sub> (95% CI range)	CV (%)
Cd (mg l <sup>-1</sup> )	0.074 (0.050-0.098)	32.34	0.620 (0.601-0.638)	2.94	0.071 (0.045-0.097)	36.37	0.724 (0.600-0.848)	17.10
Cu (mg l <sup>-1</sup> )	0.035 (0.028-0.041)	18.22	0.146 (0.143-0.149)	2.23	0.021 (0.014-0.029)	34.30	0.122 (0.110-0.134)	9.67
Atrazine (mg l <sup>-1</sup> )	0.016 (0.013-0.020)	22.62	0.262 (0.203-0.320)	22.34	0.008 (0.006-0.011)	22.82	0.204 (0.183-0.226)	10.45
Diuron (mg l <sup>-1</sup> )	0.007 (0.005-0.010)	29.92	0.056 (0.048-0.065)	14.95	0.010 (0.007-0.013)	29.47	0.069 (0.064-0.075)	7.70
DMSO (ml l <sup>-1</sup> )	11.773 (7.593-15.718)	18.43	26.226 (23.296-28.251)	5.01	13.582 (10.978-16.242)	10.77	30.603 (26.512-34.419)	7.06

95% CI are shown (n=9 plates, 24 disks per plate with 4 disks per concentration)