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Toshimitsu Onduka [1]*, Rei Somiya [1], Takahiro Yamagishi [2], Takeshi Hano [1], Hiroshi Yamamoto [2], Kazuhiko Mochida [1]

[1] Hatsukaichi Branch, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan

[2] Health and Environmental Risk Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

*Corresponding author: Toshimitsu Onduka, Ph.D.

Email: onduka@affrc.go.jp; ORCID ID: 0000-0002-2852-4848

Acknowledgments

The authors are grateful to Dr. Goro Yoshida (Nagasaki Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency) for his guidance on culturing wakame (*Undaria pinnatifida)* and Ms. Mika Momosaki and Ms. Yuko Makimoto (Hatsukaichi Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency) for their assistance. We thank Editage (www.editage.com) for English language editing. This study was supported by the Environment Research and Technology Development Fund (JPMEERF20185003) of the Environmental Restoration and Conservation Agency of Japan.

Abstract

Macroalgae are one of the main producers in marine environments. However, only a few toxicity test methods have been established that use reference strains of macroalgae to evaluate the effects of chemicals on the growth and reproduction of macroalgae to monitor water quality. We selected reference strains of Chlorophyta, *Ulva aragoënsis*; Phaeophyceae, *Ectocarpus siliculosus*; and wakame, *Undaria pinnatifida*, as test species to establish a microplate-based method to investigate the toxicity of potassium dichromate, 3,5-dichlorophenol, and two common herbicides (diuron and simazine). We determined the growth of the three macroalgae in their early life stages and during the sporangia formation stage in *E. siliculosus* under laboratory conditions. We observed that the growth and sporangia formation in these algae were impaired in a dose-dependent manner. Additionally, we investigated the sensitivity of these macroalgae by comparing the toxicity values of toxicants used in this study with those obtained from a database. Compared to other microalgae and plant species, macroalgae showed a relatively high sensitivity to organic compounds, including herbicides. Growth tests using *U. aragoënsis* and *E. siliculosus* produced reliable results at 0–32 and 25–32 practical salinity units (PSU), respectively. The tests established in this study could test the toxicity of chemical substances in macroalgae and are thus expected to contribute to a better understanding of the environmental risks of chemical substances on aquatic biota. The tests could be applied to all effluent toxicity tests used for the management of seawater and brackish water quality.

Keywords

Ectocarpus siliculosus, *Ulva aragoënsis*, *Undaria pinnatifida*, Potassium dichromate, 3,5-Dichlorophenol, Herbicides

Introduction

Bioassays are valuable tools that reveal the biological effects of chemicals or wastewater on water quality monitoring and ecological risk assessment (Norton et al. 1992; Escher and Leusch 2011). To conserve ecosystem and species diversity, monitoring the effects of chemicals on representative species in major taxonomic groups such as *Raphidocelis subcapitata*, *Desmodesmus subspicatus*, *Phaeodactylum tricornutum*, and *Navicula pelliculosa* in algae; *Anabaena flos-aquae* and *Synechococcus leopoliensis* in cyanobacteria; *Daphnia magna* in crustacea; and *Danio rerio*, *Pimephales promelas*, *Cyprinus carpio*, *Oryzias latipes*, *Poecilia reticulata*, *Lepomis macrochirus*, and *Oncorhynchus mykiss* in fish is strongly recommended (Fairman et al. 1998; OECD 2004, 2011, 2019). The whole effluent toxicity (WET) testing, which uses bioassays to comprehensively control wastewater, has also been established and is used for wastewater management in the United States of America, Canada, and the European Union (Norberg-King et al. 2018).

Macroalgae are one of the main producers in marine environments (Mann 1973), and some species of macroalgae are eaten worldwide, but mainly in Asia (Kılınç et al. 2013). Monitoring the effects of chemicals on the representative macroalgae species would lead to better ecosystem conservation. Several studies have investigated toxicity testing methods based on the settlement and growth of zoospores of the Phaeophyceae macroalga, *Fucus vesiculosus* (Girling et al. 2015); germination, germ tube elongation, photosynthesis, and chlorophyll fluorescence of immobilized gametophytes of *Undaria pinnatifida* (Park et al. 2016; Lee et al. 2019a, 2020; Wang et al. 2019); photosynthesis and carbon concentrating mechanisms (CCMs) of sugar kelp *Saccharina latissima* (Johansson et al. 2012); dry weight biomass of *Ectocarpus siliculosus* (Softcheck 2021); settlement and growth of zoospores of Chlorophyta macroalgae, *Ulva fasciata* (Hooten and Carr 1998), *Ulva lactuca* (Wendt et al. 2013), and *Ulva intestinalis* (Girling et al. 2015); and the sporulation of *Ulva pertusa* measured using image analysis (Han and Choi 2005; Han et al. 2009). However, the reproducibility of these methods is poorer than that using pure culture strains because these test methods involve *Ulva* and sugar kelp collected from the environment, and the quality of test algae differs often. Some toxicity tests based on the settlement and growth of zoospores and elongation of germ tubes have been reported, but the tests were time-consuming, because the area, length, and cell number of the germinating bodies or the number of zoospore settlements need to be measured under a microscope in at least 10 individuals in each concentration group, using growth as a measurement parameter (Girling et

al. 2015; Hooten and Carr 1998; Park et al. 2016; Wang et al. 2019). Furthermore, training the personnel to conduct these experiments takes a long period of time. There is also a possibility of differences in test results obtained by different testers due to human errors. Tests based on photosynthesis and CCMs are easy, high-throughput tests using pulse amplitude-modulated fluorometry, which is suitable for screening the toxicity of many chemicals or wastewater samples. However, these tests are not ideal for chemical risk assessment based on growth or reproduction because their effects do not depend on the macroalgal biomass.

A few toxicity test methods using macroalgae, including Phaeophyceae macroalga, giant kelp, *Macrocystis pyrifera* (Chapman et al. 1995), Florideophyceae macroalga, and *Champia parvula* (United States Environmental Protection Agency [US EPA] 2002) have been established. To the best of our knowledge, the test method using *C. parvula* (US EPA 2002) is the only established toxicity test method using macroalgae to evaluate the effects of chemicals and wastewater on the reproduction of macroalgae to monitor water quality. Toxicity test methods with reference strains such as *E. siliculosus*, a model organism of Phaeophyceae (Peters et al. 2004; Coelho et al. 2012), are preferable. The physiological ecology, appropriate culture conditions, and genetic information of *E. siliculosus* have been well documented (Charrier et al. 2008), making it easy to design experiments, develop new methods, and thoroughly discuss the test results using this species. Since a culture strain is more homogeneous than the alga collected from the environment, the test using a culture strain can be conducted at any time and is considered more reproducible than the tests using algae collected from the environment. The applicability of the tests under a wide range of salinities would allow these methods to be used as water quality monitoring tools, such as WET testing to evaluate the toxicity of wastewater and environmental waters. However, no methods have been established for testing macroalgae that can be tested over a wide range of salinities using culture strains.

The primary aim of this study was to establish a test method using a culture strain of *Ulva aragoënsis* (previously known as *Ulva flexuosa* [Chlorophyta], cultured for seaweed laver in Japan; Shimada et al. 2018), *E. siliculosus*, and *U. pinnatifida*. We tested the toxicity of potassium dichromate ($K_2Cr_2O_7$) and 3,5-dichlorophenol (3,5-DCP), which are common reference substances mentioned in inhibition test guidelines (Test guideline 201, OECD 2011), as well as two common herbicides diuron and simazine, on macroalgae in the early life stage, which is when they have relatively high sensitivity to environmental changes and toxicants. Additionally, we investigated the effect of salinity on the accuracy

of toxicity tests to determine the salinity range of test methods that can be used to monitor water quality, such as WET testing. The secondary aim of this study was to develop a chronic toxicity analysis method using Phaeophyceae because there are no published studies on reproductive toxicity tests using macroalgae. We evaluated the toxicity of the chemicals on sporangia formation in *E. siliculosus*. Moreover, we investigated the sensitivity of these macroalgae compared to that of other algae and plant species based on the acute and chronic toxicity values obtained in this study and those obtained from the Ecotox database (https://cfpub.epa.gov/ecotox/).

Materials and methods

Chemicals

Potassium dichromate ($K_2Cr_2O_7$; molecular weight [MW] = 294.18; purity > 99.5%), 3,5-dichlorophenol (3,5-DCP; MW = 163.0; purity > 98.0%), simazine (6-chloro-2-N,4-N-diethyl-1,3,5-triazine-2,4-diamine; MW = 201.66; purity > 99.0%), liquid chromatography mass spectrometry (LC-MS) grade methanol, and ultrapure water were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea; MW = 233.09; purity > 98%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Stock solutions of $K_2Cr_2O_7$ and 3,5-DCP were prepared by diluting with Milli-Q water from a Milli-Q water purification system (Elix essential UV3 equipped with synergy UV, Merck, Darmstadt, Germany). Stock solutions of simazine and diuron were prepared by diluting with dimethyl sulfoxide (DMSO; product code: D4540, Sigma-Aldrich Japan, Tokyo, Japan). These stock solutions were used in the toxicity tests, as described in the "Growth inhibition tests" and "Sporangia formation inhibition test" sections.

Provasoli's enriched seawater (PES) medium was prepared by adding 20 mL of PES stock solution (Provasoli 1966) to 1 L of natural seawater filtered through sand, activated carbon, and finally, a glass-fiber cartridge filter (47 mm diameter; 1.2 μm pore size GF/C filter; Whatman, Cytiva, Tokyo, Japan), hereafter referred to as "filtered seawater," (Practical Salinity Unit [PSU]: 31 ± 1 [mean ± standard error]). The PES medium made from artificial seawater, M-PES medium, was prepared by adding 20 mL of PES stock solution to 1 L of artificial seawater, Marine Art SF-1 (Tomita Pharmaceutical Co. Ltd., Naruto, Tokushima, Japan) filtered through a GF/C filter. Provasoli's enriched medium (PESI) was prepared by

adding 20 mL of PESI stock solution (Provasoli 1966) to 1 L of filtered seawater. Before preparing the media, filtered seawater and Marine Art SF-1 were sterilized using a bottle top filter (0.2 μm pore size; 595-4520; Thermo Fisher Scientific K. K., Tokyo, Japan), and PES and PESI stock solutions were sterilized at 121 °C for 20 min in an autoclave (STH307FA, Advantec Toyo Kaisya, Ltd., Tokyo, Japan). The stock solutions and the DMSO (for the solvent control) were diluted 10,000 times with PES medium, M-PES medium, and PESI medium to prepare the test solutions for *E. siliculosus*, *U. aragoënsis*, and *U. pinnatifida*, respectively. M-PES and PES media test solutions for the freshwater tolerance test were prepared by diluting 20 mL of PES stock solution in 1 L of Milli-Q water.

Test organisms

Ulva aragoënsis strain KU-1532 (= HOK-90, Fig. 1a) and *E. siliculosus* strain KU-1372 (= Esil 32 male, Fig. 1b) were obtained from the Kobe University Macro-Algal Culture Collection (Kobe, Hyogo, Japan). The KU-1372 strain is a male gametophyte with a fully sequenced genome and is widely used in the fields of physiology and molecular biology. The male and female gametophytes of *U. pinnatifida* were obtained from Dr. Goro Yoshida at the Fisheries Technology Institute, Japan Fisheries Research and Education Agency.

The *E. siliculosus* and *U. aragoënsis* strains were cultured in a Petri dish (diameter = 90 mm; depth = 20 mm, AS ONE corp., Osaka, Japan) with PES medium (Provasoli 1966), under a 12:12 h light:dark cycle (with a light intensity of 58 ± 2 μmol/m^2/s) at 12.7 ± 0.1 °C and 14.0 ± 0.1 °C, respectively, in a temperature-controlled growth chamber (MLR-350; Sanyo, Osaka, Japan, LH-241S; Nippon Medical and Chemical Instruments Co. Ltd., Osaka, Japan). To collect the zoospores, *U. aragoënsis* was matured at 19.2 ± 1.8 °C for a few days under the same light:dark cycle conditions and finely cut with scissors in PES medium and filtered through a nylon mesh filter with a pore size of 10 μm (pluriStrainer; Funakoshi Co., Ltd., Tokyo, Japan). The zoospores in the filtrate were counted using a hemocytometer (Bürker-Türk; AS ONE Corp., Osaka, Japan) under an inverted microscope (IX71; Olympus, Tokyo, Japan) and used for growth inhibition tests as described in the "Growth inhibition tests" section. Cultured *E. siliculosus* were finely cut with scissors in the PES medium and filtered through nylon mesh filters with pore sizes of 10 μm and 40 μm for toxicity analysis; plumules of the sporophytes on the 10 μm mesh were resuspended in the PES medium and counted with a counting chamber (MPC-200; Matsunami Glass Ind., Ltd., Osaka, Japan)

under the inverted microscope and used for the growth inhibition tests as described in the "Growth inhibition tests" section. Approximately 200–400 plumules of sporophytes were transferred to a 24-well plate (305047; Falcon, Corning, NY, USA) that contained 2.5 mL of the PES medium in each well and incubated for 10 to 14 days under the same growth conditions as *E. siliculosus* (12:12 h light: dark cycle with a light intensity of 58 ± 2 μmol/m^2/s at 12.7 ± 0.1 °C and 14.0 ± 0.1 °C). Sporophytes were observed under the inverted microscope, picked before maturation, and used for sporangia formation inhibition tests, as described in the "Sporangia formation inhibition test" section.

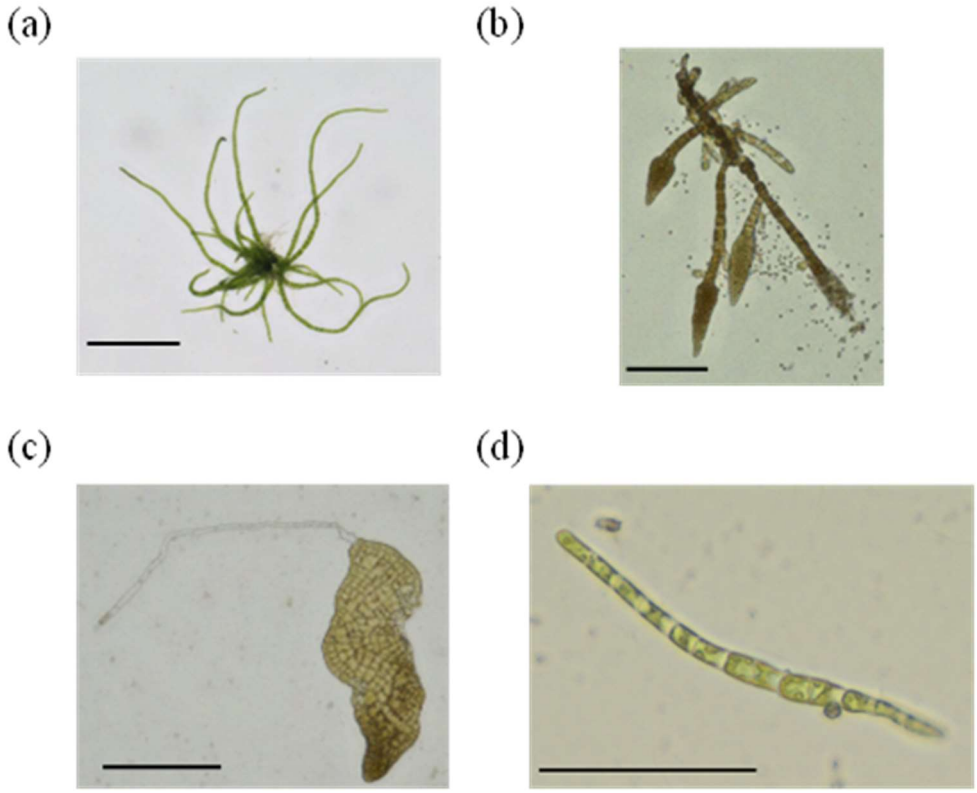

Fig. 1 Morphological examples of sporophytes of (a) *Ulva aragoënsis*, (b) *Ectocarpus siliculosus*, and (c) *Undaria pinnatifida*, and (d) germinating body of *U. aragoënsis*. Scale bars represent 500 μm in a and 100 μm in b–d.

The male and female gametophytes of *U. pinnatifida* were cultured in aerated flasks with the PESI medium (Provasoli 1966) under a 15:9 h light: dark cycle at 21.0 ± 0.5 °C in a temperature-controlled growth chamber (LH-241S; Nippon Medical and Chemical Instruments Co., Ltd.). The culture method

described by Niwa (2016) was followed to collect the plumules of *U. pinnatifida* sporophytes (Fig. 1c). The PESI medium was used according to culture conditions as follows: 100 mL of the PESI medium and 0.1 g of male and female gametophytes of *U. pinnatifida* were homogenized using a blender (7012HBC, Waring; Conair Corporation, Stamford, CT, USA) at 20,000 rpm for 40 s, and the homogenate was diluted 10 times with the PESI medium. The diluted homogenate was cultured in a beaker covered with aluminum foil under a 9:15 h light: dark cycle (with a light intensity of 56 ± 2 μmol/m^2/s) at 14.2 ± 0.0 °C for over three weeks in a temperature-controlled growth chamber (LH-241S; Nippon Medical and Chemical Instruments Co., Ltd.). The culture solution was filtered through nylon mesh filters with pore sizes of 10 μm and 40 μm. The plumules of the sporophytes on the 10 μm mesh filter were resuspended in the PESI medium and counted using the counting chamber under the inverted microscope and used for growth inhibition tests, as described in the "Growth inhibition tests" section.

Growth inhibition tests

Zoospores of *U. aragoënsis* and plumules of sporophytes of *E. siliculosus* and *U. pinnatifida* were used for the tests. The experimental conditions of the test methods are presented in Table 1. Growth inhibition tests were performed as previously described (Hooten and Carr 1998; Wendt et al. 2013), with slight modifications. We conducted the tests in 24-well plates using nominal $K_2Cr_2O_7$, 3,5-DCP, diuron, and simazine concentrations (Table S1). The growth inhibition tests on *E. siliculosus* and *U. aragoënsis* were performed using serial dilutions of the PES medium and M-PES medium (0, 5, 10, 20, 40, and 100% of PES or M-PES medium, for the freshwater tolerance test). The tests were conducted in a temperature-controlled growth chamber. The environmental conditions for the testing period (toxicity tests) are listed in Table S2.

After the tests were performed, a cover glass (φ13 mm, c1100; Matsunami Glass Ind., Ltd.) was placed in the well if the height distribution of the cultured algae was large and over 10 pictures were taken at different focal lengths to obtain fully focused images. The algae were observed and photographed automatically in all the wells using the batch capture and optical modes of the microscope (BZ-X810; Keyence Corporation, Osaka, Japan). Fully focused wide-area images of each well were obtained using the BZ-X analyzer software (Keyence Corporation).

Table 1 Experimental conditions of the test methods using marine macroalgae

Test method	Growth inhibition test			Sporangia formation inhibition test
Test species	*Ulva aragoënsis*	*Undaria pinnatifida*	*Ectocarpus siliculosus*	*Ectocarpus siliculosus*
Test container	24-well plate	24-well plate	24-well plate	12-well plate
Volume of test solution	2.5 mL/well	2.5 mL/well	2.5 mL/well	1.5 mL/well
Repetition number	4	4	4	10–17
Test chemicals	$K_2Cr_2O_7$, 3,5-DCP, diuron, simazine	$K_2Cr_2O_7$, 3,5-DCP	$K_2Cr_2O_7$, 3,5-DCP, diuron, simazine	$K_2Cr_2O_7$, 3,5-DCP, diuron, simazine
Test medium	M-PES medium	PESI medium	PES medium	PES medium
Set temperature	20 °C	15 °C	13 °C	13 °C
Light (L): dark (D) cycle	12:12 h	9:15 h	12:12 h	12:12 h
Test period	3 days	7 days	7 days or 10 days	3 days
Initial biomass (per well)	1.0×10^5 of zoospore	About 200–300 of plumules	About 50–150 of shed macroalgae	One grown macroalga from shed macroalgae for 10 days
Factors	Cell number, length, area, and perimeter	Area	Area	Number of sporangia
Number of observed individuals (per well)	>10	5	10	1–2

$K_2Cr_2O_7$, Potassium dichromate; 3,5-DCP, 3,5-dichlorophenol.

The area, length, circumference, and cell number of over 10 germinating bodies of *U. aragoënsis* (Fig. 1d), five plumules of sporophytes of *U. pinnatifida*, and 10 plumules of sporophytes of *E. siliculosus* per well were measured after the images were corrected for unevenly illuminated background with the subtract background feature and binarized using the ImageJ software 1.53c (Schneider et al. 2012).

Sporangia formation inhibition test

The sporangia formation inhibition tests were performed on the sporophytes of the *E. siliculosus* before they reached the maturation stage. The tests were performed under the test conditions in Table 1 using a series of nominal $K_2Cr_2O_7$, 3,5-DCP, diuron, and simazine concentrations as depicted in Table S3. The tests were conducted in a temperature-controlled growth chamber. Environmental conditions during the toxicity testing period are listed in Table S2. After the tests were performed, the number of sporangia in each mature sporophyte (10–17) of the algae was counted using an inverted fluorescence phase contrast microscope (BZ-X810, Keyence Corporation).

Chemical analysis

The test solutions were sampled at the beginning and end of the growth, and sporangia formation inhibition tests were used to determine the actual toxicant concentrations during the experiment. Simazine, 3,5-DCP, and diuron concentrations in the test solutions were analyzed using solid-phase extraction followed by LC-MS. For 3,5-DCP analysis, an Oasis HLB plus LP cartridge (186000132; Nihon Waters K. K, Tokyo, Japan) was conditioned with 10 mL of methanol followed by 10 mL of Milli-Q water. The water sample was filtered using a PTFE syringe filter unit (pore size 0.45 μm; Membrane Solution, Ltd., Tokyo, Japan), and 8 mL of the sample was used for 3,5-DCP analysis. The cartridge was then washed with 10 mL Milli-Q water and air dried for 1 h. The analytes were eluted in a test tube with 10 mL of methanol, and 100 μL of atrazine-$^{13}C_3$ (1 mg/L in methanol) was spiked as an internal standard and the analytes were further analyzed. Simazine and diuron were extracted from the test solutions using MonoSpin® C18 (GL Sciences Incorporated, Tokyo, Japan), according to the manufacturer's instructions. The toxicants were extracted from the cartridge in 0.5 mL of methanol, 5 μL of atrazine-$13C_3$ (1 mg/L, in 5 mL methanol solution) was spiked as an internal standard, and the toxicants were subjected to further analyses.

The actual toxicant concentrations were determined using an LC-30AD Prominence system

(Shimadzu, Kyoto, Japan). The toxicants were separated on an Inertsil ODS-4 column (2.1 mm i.d. × 50 mm, 2 μm, GL Science Incorporated) equipped with an Inertsil ODS-4 guard cartridge (2.1 mm i.d. × 10 mm, 2 μm, GL Science Incorporated). The mobile phase comprised 5 mM ammonium acetate (solution A) and 5 mM ammonium acetate in methanol (solution B), and the flow rate was 0.2 mL/min with the following gradient: 60–95% B for 3 min, 95% B for 3 min, and 60% B for 4 min to return to the initial condition. The injection volume was 5 μL. Electrospray mass spectrometry analysis was performed for the toxicants (LC-MS-8030; Shimadzu), and analytes were ionized through electrospray ionization in the negative-ion mode. LC-MS was performed in multiple reaction monitoring modes. Analyte-specific detection parameters are presented in Table S4.

Seawater recovery rate and method quantification limit (MQL) were determined for the test chemicals, except $K_2Cr_2O_7$, in the samples as follows: seawater for the recovery test was spiked with specified amounts of 3,5-DCP, diuron, and simazine and received a pretreatment similar to that of the exposed test samples (3,5-DCP: 10 μg/L; diuron and simazine: 0.5 μg/L). The recovery test was performed seven times, and standard deviations regarding the analyte concentrations and mean recovery rates were determined. The standard deviations multiplied by 10 were taken as MQLs for the analytes. The standard deviations for 3,5-DCP, diuron, and simazine were 0.31, 0.04, and 0.14 μg/L, respectively. Thus, the MQLs of 3,5-DCP, diuron, and simazine were 3, 0.4, and 2 μg/L, and their recovery rates were 105%, 100%, and 90%, respectively. The $K_2Cr_2O_7$ analysis was performed as follows: the test solution of $K_2Cr_2O_7$ was filtered using a PTFE syringe filter unit and diluted with ultrapure water. The actual toxicant concentrations of $K_2Cr_2O_7$ were analyzed using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 8800; Agilent Technologies Japan, Ltd., Tokyo, Japan). The geometric mean values of the toxicant concentrations were used to estimate the 1% effective concentration (EC_1), 10% effective concentration (EC_{10}), 50% effective concentration (EC_{50}), lowest observed effect concentrations (LOECs), and no observed effect concentrations (NOECs).

Comparison of the sensitivity of the macroalgal species

Acute toxicity data, such as EC_{50} or median lethal concentration (LC_{50}), and chronic toxicity data, such as NOECs, were obtained in this study and from the Ecotox database (https://cfpub.epa.gov/ecotox/). The Ecotox database was also used to retrieve the toxicity effects of test chemicals on the growth, population,

reproduction, and mortality of selected plant and algal species, which were compared with the findings of our study. These indicators closely represented growth and reproduction investigated in our study. The data on toxicity values for the test period of less than one day were excluded because this period was not long enough for these indicators to observe the effect of exposure to the pesticide. Where multiple toxicity data based on differences in factors and exposure periods were reported in a previously published study, the minimum toxicity values were selected to compare highly sensitive indicators (Tables S5–9). The geometric mean was used for comparison of the sensitivity of the species for which the toxicity values had been previously reported.

Data analysis

The values of EC_1, EC_{10} and EC_{50} were estimated using R v 4.1.2 (R Core Team 2022) with the drc (two-parameter logit-log analysis) packages (Ritz et al. 2015), and then multiple comparison tests were performed using R v 4.1.2 (R Core Team 2022) with the multcomp (Dunnett's test) packages (Hothorn et al. 2008; Ritz et al. 2015). The area, length, circumference, and cell number of *U. aragoënsis* germinating bodies, plumules of *E. siliculosus*, and plumules of *U. pinnatifida* were analyzed using Dunnett's tests in R v 4.1.2 (R Core Team 2022). Differences between treatment groups were considered significant at $p < 0.05$.

Results

Effects of test chemicals in the growth inhibition test

In the tests using *U. aragoënsis*, the actual measured concentrations of $K_2Cr_2O_7$, 3,5-DCP, diuron, and simazine were 106 ± 1%, 108 ± 5%, 102 ± 3%, and 80 ± 4% of the targeted nominal concentrations, respectively (Table S1). In the growth inhibition tests using *U. aragoënsis*, the average cell number, length, area, and perimeter were over eight cells, 180 μm, 4000 μm^2, and 700 μm, respectively, in the control groups (Fig. 2, S1). The cell number, length, area, and perimeter of the germinating bodies decreased along with the concentration of toxicants (Fig. 2, S1). Cell number and length in the 500 μg/L 3,5-DCP exposure group and length in the 250 and 500 μg/L $K_2Cr_2O_7$ exposure groups were higher than those in the control groups. The toxicity values, such as EC_1, EC_{10}, EC_{50}, and NOEC, were almost similar despite the difference in the indicators (Table 2).

Table 2 Toxicity values of the growth inhibition tests and the sporangia formation inhibition tests using macroalgae

Species	Endpoint	Effect measurement	Count (n)	Test period	$K_2Cr_2O_7$ (μg Cr/L)[a]						
					NOEC	LOEC	EC_1	EC_{10}	EC_{50}	b	e
Ectocarpus siliculosus	Growth	Area	>10	7-d	900	1800	230 (140–310)	890 (730–1050)	3100 (2800–3400)	-1.762	3097
			>10	10-d	450	910	72 (35–110)	340 (250–420)	1400 (1200–1500)	-1.553	1383
	Reproduction	Sporangia formation	10-14	3-d	430	940	0.51 (-1.6–2.7)	36 (-32–100)	1800 (760–2800)	-0.5645	1774
Ulva aragoënsis	Growth	Cell count	10	3-d	260	520	58 (11–106)	250 (150–350)	950 (780–1100)	-1.643	952.3
		Length	10	3-d	520	1100	63 (3.2–120)	360 (190–520)	1800 (1400–2200)	-1.376	1763
		Area	10	3-d	520	1100	45 (1.6–88)	280 (150–420)	1600 (1200–100)	-1.295	1552
		Perimeter	10	3-d	520	1100	54 (8.3–100)	350 (200–490)	1900 (1500–2200)	-1.291	1894
Undaria pinnatifida	Growth	Area	5	7-d	<2000	2000	100 (43–160)	910 (640–1200)	6900 (6200–7700)	-1.085	6938
Species	Endpoint	Effect measurement	Count (n)	Test period	3,5-DCP (μg/L)						
					NOEC	LOEC	EC_1	EC_{10}	EC_{50}	b	e
Ectocarpus siliculosus	Growth	Area	>10	7-d	240	670	52 (20–83)	240 (170–320)	1000 (830–1200)	-1.545	1008
			>10	10-d	140	550	180 (140–230)	370 (330–410)	690 (650–730)	-3.484	688.7
	Reproduction	Sporangia formation	10-14	3-d	450	860	185 (53–320)	400 (260–540)	810 (710–920)	-3.104	814.3
Ulva aragoënsis	Growth	Cell count	10	3-d	1100	2400	150 (40–270)	680 (430–930)	2600 (2200–3100)	-1.618	2647
		Length	10	3-d	1100	2400	100 (14–190)	600 (340–870)	3000 (2500–3500)	-1.370	3004
		Area	10	3-d	450	1100	42 (4.1–80)	280 (150–420)	1600 (1300–2000)	-1.254	1640
		Perimeter	10	3-d	450	1100	41 (4.9–76)	300 (160–440)	1900 (1600–2300)	-1.190	1928
Undaria pinnatifida	Growth	Area	5	7-d	80	180	15 (6.1–25)	87 (58–120)	420 (360–490)	-1.391	421.2
Species	Endpoint	Effect measurement	Count (n)	Test period	Diuron (μg/L)						
					NOEC	LOEC	EC_1	EC_{10}	EC_{50}	b	e
Ectocarpus siliculosus	Growth	Area	>10	7-d	3.8	8.2	0.67 (-0.02–1.34)	2.1 (1.0–3.2)	6.0 (4.8–7.2)	-2.096	5.993
	Reproduction	Sporangia formation	10-14	3-d	1.8	4.3	0.000067 (-0.0048–0.00062)	0.026 (-0.068–0.12)	6.0 (0.39–12)	-0.4030	5.967
Ulva aragoënsis	Growth	Area	10	3-d	2.2	3.8	0.15 (0.01–0.28)	1.3 (0.72–1.81)	9.1 (7.3–10.9)	-1.112	9109
Species	Endpoint	Effect measurement	Count (n)	Test period	Simazine (μg/L)						
					NOEC	LOEC	EC_1	EC_{10}	EC_{50}	b	e
Ectocarpus siliculosus	Growth	Area	>10	7-d	35	83	0.34 (-0.71–1.4)	5.7 (-3.9–15.3)	75 (41–110)	-0.8509	75.34
	Reproduction	Sporangia formation	10-14	3-d	110	220	0.44 (-0.89–1.8)	14 (-6.0–33)	320 (190–450)	-0.6985	317.5
Ulva aragoënsis	Growth	Area	10	3-d	16	33	1.3 (-0.01–2.6)	12 (6.7–17.3)	91 (71–110)	-1.085	90.97

The toxicity values were rounded to two significant digits. Data in parentheses represent the 95% confidence interval. $K_2Cr_2O_7$, potassium dichromate; 3,5-DCP, 3,5-dichlorophenol; NOEC, no observed effect concentration; LOEC, lowest observed effect concentration; EC_1, 1% effective concentration; EC_{10}, 50% effective concentration; EC_{50}, 50% effective concentration; b and e, the estimated parameter of log-logistic method (two parameters); a, the concentration is shown as chromium concentration.

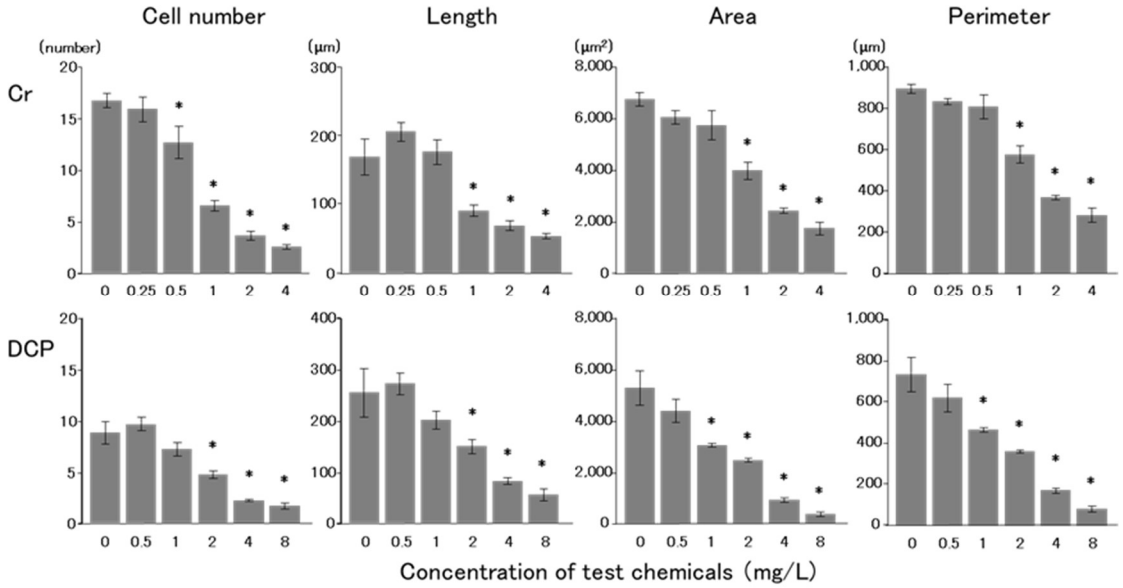

Fig. 2 Effect of potassium dichromate and 3,5-dichlorophenol on cell number, length, area, and perimeter of the germinating body generated from the zoospore of *Ulva aragoënsis* for 3 days. Cr and DCP indicate potassium dichromate and 3,5-dichlorophenol, respectively. Asterisk (*) indicates a significant difference ($p < 0.05$) from the control. The error bar indicates the standard error (N = 4).

The calculated EC_{50} values based on the area were the lowest for diuron, followed by those for simazine, $K_2Cr_2O_7$, and 3,5-DCP (Table 2; EC_{50} values: 9.1, 91, 1600, and 1600 μg/L, respectively). The NOEC value of each chemical tested was between 0.5 and 2 times the EC_{10}.

In the tests using *E. siliculosus*, the actual measured concentrations of $K_2Cr_2O_7$, 3,5-DCP, diuron, and simazine were 90 ± 1%, 66 ± 10%, 102 ± 2%, and 74 ± 3% of the targeted nominal concentrations, respectively. The recovery rate of $K_2Cr_2O_7$ after seven days (7-d exposure test) was similar to that after 10 days (10-d exposure test), but the recovery rates of 3,5-DCP in the 7-d exposure test were higher than those in the 10-d exposure test (Table S1). The average area of the plumule was over 4000 μm^2 and 30,000 μm^2 in the control group in the 7-d and 10-d tests, respectively. The plumule areas in the 250 and 500 μg/L $K_2Cr_2O_7$ and 3,5-DCP treatments in the 7-d exposure groups and the 250 and 500 μg/L 3,5-DCP treatments in the 10-d exposure groups were higher than those of the control groups (Fig. S2). The area of the plumule decreased with increasing toxicant concentration (Fig. S2), and toxicity values such as EC_1, EC_{10}, EC_{50}, and NOEC obtained in the 7-d and 10-d tests were similar (Table 2). The EC_{50} of diuron in the 7-d test was

the lowest (6.0 μg/L), followed by those of simazine (75 μg/L) and 3,5-DCP (1000 μg/L), and that of $K_2Cr_2O_7$ (3100 μg/L) was the highest (Table 2). The NOEC values of each chemical tested were in the desired range (half to twice that of EC_{10}), except for those of 3,5-DCP during the 10-d exposure test and simazine during the 7-d exposure test.

In tests using *U. pinnatifida*, the actual measured concentrations of $K_2Cr_2O_7$ and 3,5-DCP were 93 ± 4% and 58 ± 16% of the targeted nominal concentrations, respectively (Table S1). The average area obtained was over 180 μm^2 during the 7-d tests (Fig. S3). The area of the plumule decreased with increasing toxicant concentration (Fig. S3); the EC_{50} of 3,5-DCP (420 μg/L) was lower than that of $K_2Cr_2O_7$ (6900 μg/L) (Table 2). The NOEC value of 3,5-DCP was in the desired range (half to twice that of EC_{10}), but the NOEC value of $K_2Cr_2O_7$ was higher than twice that of EC_{10}.

Effects of test chemicals in the sporangia formation inhibition test

The actual measured concentrations of $K_2Cr_2O_7$, 3,5-DCP, diuron, and simazine were 88 ± 2%, 95 ± 4%, 100 ± 3%, and 105 ± 4% of the targeted nominal concentrations, respectively (Table S3). The average number of sporangia formed exceeded 14 in the 3-d tests (Fig. 3), and the sporangia found were mostly plurilocular. The number of sporangia formed decreased with increasing chemical concentrations (Fig. 3). The EC_{50} of diuron was the lowest (6.0 μg/L), followed by those of simazine (320 μg/L) and 3,5-DCP (810 μg/L), and that of $K_2Cr_2O_7$ (1800 μg/L) was the highest (Table 2). The NOEC value of 3,5-DCP was in the desired range (half to twice that of EC_{10}), but the NOEC values of $K_2Cr_2O_7$, diuron, and simazine were more than twice that of EC_{10}.

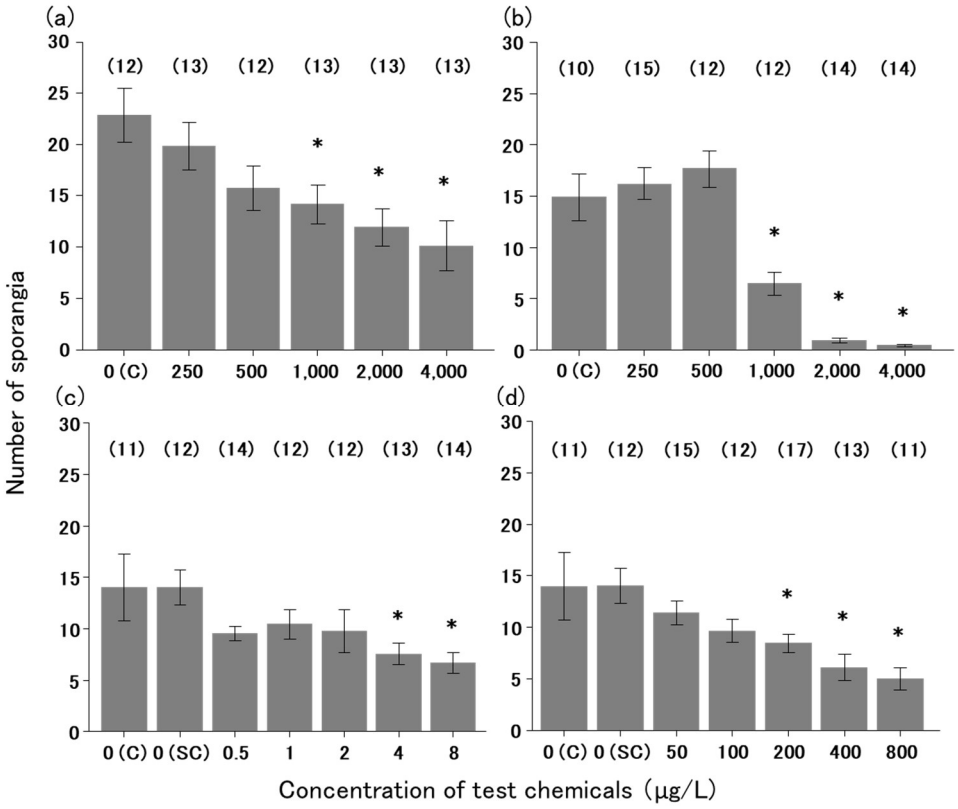

Fig. 3 Effect of (a) potassium dichromate, (b) 3,5-dichlorophenol, (c) diuron, and (d) simazine on sporangia formation in *Ectocarpus siliculosus* for 3 days. C and SC indicate the control group and solvent control group, respectively. Asterisk (*) indicates a significant difference ($p < 0.05$) from the control. The error bar indicates the standard error. The number of replicates in each group is shown in parentheses.

Freshwater tolerance test

Ulva aragoënsis grew in 0 to 32 PSU conditions in both PES and M-PES media. The germinating bodies in the 7–14 PSU group in the PES medium and the 4–14 PSU groups in the M-PES medium were larger than those in the control group (Fig. 4a, b). The area of *E. siliculosus* increased depending on PSU; the area of *E. siliculosus* less than 18 PSU in the PES medium and less than 20 PSU in the M-PES medium was significantly lower ($p < 0.05$) than that in the control groups (Fig. 4c, d).

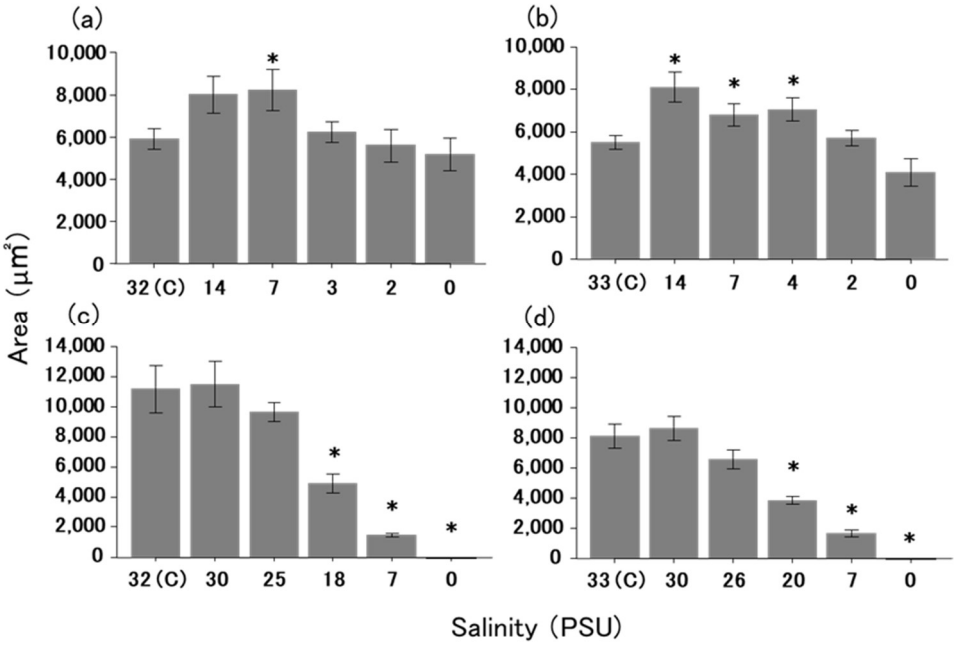

Fig. 4 Effect of salinity on the area of (a, b) the germinated zoospore of *Ulva aragoënsis* for 3 days, (c, d) the *Ectocarpus siliculosus* biomass for 7 days, using PES medium (a, c) and M-PES medium (b, d). C indicates the control group. Asterisk (*) indicates a significant difference ($p < 0.05$) from the control. The error bar indicates the standard error (N = 4).

Discussion

This study demonstrated that typical toxicants such as 3,5-DCP, $K_2Cr_2O_7$, diuron, and simazine affected the growth of Chlorophyta (*U. aragoënsis*) and Phaeophyceae macroalgae (*E. siliculosus* and *U. pinnatifida*) in a concentration-dependent manner. These results indicate that the test methods employed in this study are reliable for analyzing the toxicity of the chemical substances in the test media.

Growth inhibition tests using Ulva collected from the environment have been reported by various researchers (Hooten and Carr 1998; Wendt et al. 2013; Girling et al. 2015). However, this study is the first to establish a test using a pure culture of *U. aragoënsis*. We investigated four types of endpoints, including the cell number, length, area, and perimeter of germinating bodies in the growth inhibition test with *U. aragoënsis;* the sensitivities based on these parameters were similar (Fig. 2, Table 2), and the NOEC value of each chemical tested was in the desired range (half to twice that of EC_{10}) (Table 2). These results suggest that all four indicators were available to test toxicity. This implies that the toxic effects can be examined

simply by observing the number of germinated cells under a microscope or by automatically measuring the area, length, and perimeter of the germinating bodies through image analysis. The diuron EC_{50} value for *U. aragoënsis* in this study (9.1 μg/L for germling growth) was similar to that reported for *U. intestinalis* (3 μg/L) for germling growth (Girling et al. 2015) and lower than that reported for *U. pertusa* (76 μg/L) for reproductive inhibition (Lee et al. 2019b). These results demonstrated that Ulva spores at the germination stage are more sensitive to diuron than those at the reproductive stage.

The reported growth inhibition tests using *E. siliculosus* were 14-d toxicity tests based on the growth rate, and high variability was observed in the growth rates based on the dry weight data in the test (Softcheck 2021). This study examined the growth of *E. siliculosus* (using the area of the plumule as an indicator) in 7-d and 10-d tests, and the difference in toxicity values was investigated. The toxicity values were similar regardless of the difference in the duration of the tests (Table 2). These results suggest that the 7-d test is sufficient to assess the toxicity of chemicals based on the growth of the test organism. The NOEC value of each chemical tested ranged from half to twice that of EC_{10} (Table 2). These results indicate that the plumule variability in the area in this study is suppressed, and the area of the plumule is a suitable indicator of toxicity.

Previous studies reported that gametophyte suspensions of *U. pinnatifida* could be used for the high-throughput toxicity test method (Park et al. 2016; Lee et al. 2019a, 2020; Wang et al. 2019). As the plumule of *U. pinnatifida* is more sensitive to salinity changes than the gametophytes (Baba 2008), it is assumed that the plumule is more highly sensitive to chemicals than the gametophytes. To the best of our knowledge, this is the first study to verify the test using *U. pinnatifida* sporophytes. In the growth inhibition tests described in the "Growth inhibition tests" section, the area of the germinating body or plumule was calculated by analyzing the microscope images using image analysis software (Fig. 3). Standardization of imaging and image analysis methods could minimize personal errors and facilitate the establishment of a high-throughput test system. Although the EC_{50} value was obtained using this method, it was difficult to detect NOECs precisely because of the large variation in the growth of the spore bodies in some cultures (data not shown). To reduce the variation between species, it is necessary to examine the strains of *U. pinnatifida* gametophytes or increase the number of repetitions of the test to narrow the estimation range of the population mean and make it easier to obtain statistically significant differences.

A few test methods have been reported that examine the effect of toxicants on macroalgae

reproduction, such as sexual reproduction of the Florideophyceae *C. parvula* (US EPA 2002) and asexual replication of the Chlorophyta *U. pertusa* (Han and Choi 2005; Han et al. 2009; Lee et al. 2019b). Our study is the first to apply the sporangia formation inhibition test to Phaeophyceae *E. siliculosus*. Our findings demonstrated that typical toxicants such as 3,5-DCP, $K_2Cr_2O_7$, diuron, and simazine affected the formation of sporangia in *E. siliculosus* at certain concentrations (Fig. 3), indicating the potential use of sporangia formation in toxicity tests. The sporangia formation test was similarly sensitive to the growth inhibition test for common reference substances and herbicides (Table 2). The sporangia formation test, which can detect effects on gametogenesis, may be more effective in investigating the effects of teratogenic chemicals, such as heavy metals and pharmaceuticals (Quinn et al. 2009; Ali et al. 2013). Although the EC_{50} values were obtained, the NOEC values for each chemical tested (except for 3,5-DCP) were higher than those of EC_{10} (Table 2). This ambiguity could be attributed to the high variability in the number of sporangia. To improve the issues found in the test, it is essential to identify strains that form sporangia stably post-culture.

The toxicity values for macroalgae obtained here were compared with the reported toxicity values for algae and plants in the toxicity database (Fig. 5, Tables S6–9). The toxicity of $K_2Cr_2O_7$ for the macroalgae species tested ranged from median values to over 90 percentile values of toxicity values for plants and algal species (Fig. 5), indicating that these species have relatively low sensitivity compared to other plants and algal species. *Undaria pinnatifida* had a relatively lower sensitivity to $K_2Cr_2O_7$ among the macroalgae examined in this study (Table 2). The alginate in seaweeds binds well to heavy metals because it contains many carboxyl groups (Fourest and Volesky 1997). Many large brown algae contain alginate (Afonso et al. 2019) and *U. pinnatifida* between 20% and 50% of its dry weight (Skriptsova et al. 2004). These data indicate that the sensitivity to $K_2Cr_2O_7$ may be low owing to the alginate content of the macroalgae. The toxicity values of 3,5-DCP for the macroalgae species were lower than the median values of toxicity values for plants and algal species, except for the NOEC value for *U. aragoënsis* (Fig. 5), indicating relatively high sensitivity compared to that of other plants and algal species. Toxic reactive oxygen species have been reported to cause toxicity of chlorophenols, including 3,5-DCP (Igbinosa et al. 2013). Reactive oxygen species are removed in algae by enzymes such as superoxide dismutase and catalase, as well as by pigments such as carotenes and flavonoids (Karuppanapandian et al. 2011). Differences in the function of these enzymes and the pigment content in the test organisms are likely to

contribute to their toxic effects. The toxicity values of diuron and simazine were lower than the median values of toxicity values for plants and algal species, except for the NOEC value of diuron for *E. siliculosus*, indicating relatively high sensitivity of the macroalgae to these photosynthetic inhibitors (Fig. 5). Therefore, the microplate-based bioassay method for macroalgae established in this study was validated. These test methods demonstrated high sensitivity and reproducibility and may be added to the methods routinely used for the toxicity evaluation of hazardous chemicals.

The freshwater tolerance of *U. aragoënsis* and *E. siliculosus* was also tested. The growth of *U. aragoënsis* was either similar to or greater than that of the control group (32 or 33 PSU, salinity in the PES or M-PES media) in the salinity range tested (0 to 33 PSU; Fig. 4a, b). The growth of *E. siliculosus* was either similar to or greater than that of the control group (32 or 33 PSU, salinity in the PES or M-PES media) in the salinity range tested (25 to 33 PSU; Fig. 4c, d). Therefore, the above salinity levels can be tested, but they need to be adjusted when performing WET tests. These tests can be used in WET testing to evaluate the toxicity of wastewater and environmental waters.

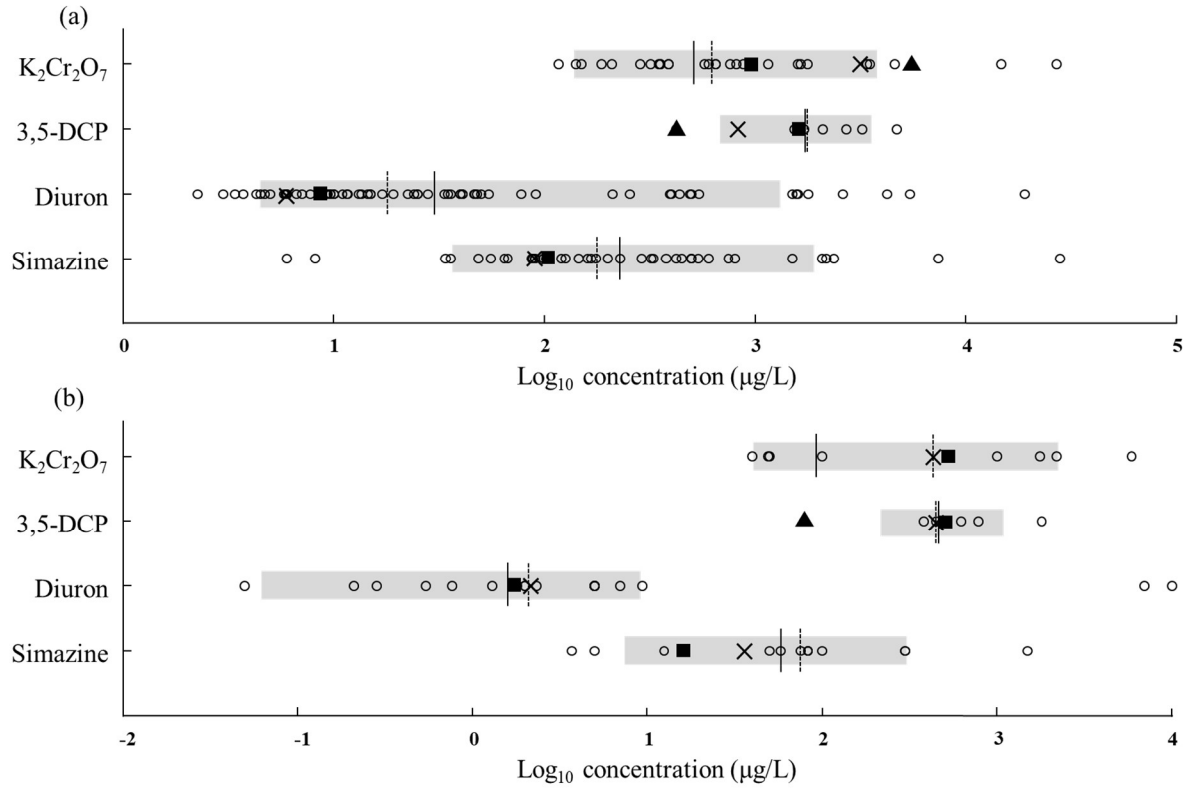

Fig. 5 Acute (a) and chronic (b) toxicity of 3,5-dichlorophenol (3,5-DCP), potassium dichromate ($K_2Cr_2O_7$), diuron, and simazine for plants and algal species. Crosses, solid squares, and solid triangles represent the toxicity values for *Ectocarpus siliculosus*, *Ulva aragoënsis*, and *Undaria pinnatifida*, respectively, in the present study, while the other symbols represent the toxicity values from Tables S6–9. Acute toxicity values below 1 μg/L and chronic toxicity values below 0.01 μg/L were not indicated. Solid and dashed lines indicate the average and median values, respectively. Grey shading indicates the ranges of the 10 to 90 percentile values.

Conclusions

This study examined the toxic effects of four typical toxicants on three species of macroalgae and demonstrated that all the methods tested could be used in toxicity tests. These growth inhibition tests can be upgraded to a high-throughput analysis system that offers high reproducibility in tests that consider growth as an indicator through standardization of imaging and image analysis methods. This study also demonstrated that the growth inhibition test using *U. aragoënsis* and *E. siliculosus* could be performed at varying levels of salinity, indicating that the tests can be applied during WET testing of seawater and brackish water. Additionally, the macroalgae showed relatively high sensitivity to organic compounds, including herbicides, compared to other microalgal and plant species. This study established a highly sensitive test system applicable to toxicity effect tests and WET tests on seawater and brackish water. These toxicity tests are expected to contribute to a better understanding of the environmental risks of chemical substances and aid in environmental administration.

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Statements and declarations

Funding

This work was supported by the Environment Research and Technology Development Fund (Grant number JPMEERF20185003) of the Environmental Restoration and Conservation Agency of Japan.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

The study was conceptualized by TO and KM. The data was curated and analyzed by TO and RS. HY and KM acquired the funds for the study. TO, RS, and TY were involved in the experimental investigations. The methodology to be adopted was decided upon by TO and RS. HY and KM were responsible for project administration and supervision. TO and RS participated in the validation of the tests. TO prepared the original draft and was also involved in the review and editing along with RS, TY, TH, HY, and KM.

Data availability

The datasets generated or analyzed in this study can be procured on request from the corresponding author.