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Abstract

Marine macroalgae are important for maintaining ecosystems in coastal areas. However, to our knowledge, only a few studies of marine macroalgae have developed protocols for assessing the toxic effects of growth inhibitors typically found in industrial wastewaters. *Sargassum horneri*, a large brown macroalga commonly found in seaweed beds in coastal Japan, can be easily collected, and its fertilized eggs can be preserved for approximately 170 days. Therefore, the present study evaluated the possibility of developing a seaweed bioassay using the germling growth of *S. horneri* to obtain toxicological data for phenol. The addition of phenol to culture media clearly inhibited the germling growth of *S. horneri*. The 14-day EC_{50} value on the basis of the specific growth rate was 58 mg phenol L^{-1}, while the 21-day EC_{50} value on the basis of the thallus area was 36 mg phenol L^{-1}. Furthermore, the no-observed-effect concentration was estimated to be 20 mg phenol L^{-1}. Renewal of culture medium did not significantly affect germling growth of *S. horneri* during our 21 days of culturing. *S. horneri* appeared to be as sensitive to phenol as other marine macroalgae reported in the previous studies. Therefore, we consider that *S. horneri* is one of the appropriate candidates of macroalgae for toxicity testing.

Keywords: macroalgae, *Sargassum horneri*, bioassay, germination, phenol, toxicity

Introduction

Toxicity tests on algae are primarily performed on microalgae using freshwater or marine species, according to conventional or newly-developed test methods (ASTM E1218-04, 2012; ISO 8692, 2012; ISO 10253, 2016; Andemichael and Lee 2016). To our knowledge, relative to studies on microalgae, only a few studies of marine macroalgae have developed protocols for assessing the toxic effects of growth inhibitors typically found in industrial wastewaters. In addition, among the standard battery of bioassay tests using microalgae and various invertebrates, not much consideration has been given to developing a seaweed bioassay for toxicants (Eklund and Kautsky 2003; Hurd et al. 2014).

A number of macroalgae have been proposed for testing single substances, effluent waters, and other complex mixtures (Hurd et al. 2014). For example, the red macroalga *Champia parvula* has been used for testing chronic reproduction in the standard ASTM E1498-92 (2012). A quantitative bioassay method for toxicants in sewage has been performed using the green macroalga *Ulva* (Han and Choi 2005; Han et al. 2008). Toxicological studies have been conducted for several toxicants using the red macroalga *Gracilaria* (Kakita and Kamishima 2006; Mendes et al. 2012; Mendes et al. 2013a; Mendes et al. 2013b; Mendes et al. 2014; Mendes et al. 2015). Because it is difficult to accurately verify mortality when measuring survival rates in macroalgae, other parameters, such as growth rate, germination, or germling growth, are often used as toxicity metrics (Hurd et al. 2014). In fact, early developmental stages, such as fertilization, germination, and germling growth, of macroalgae provide excellent experimental systems,

and those of some brown algal species have therefore been utilized for toxicity testing (Doblin & Clayton, 1995; Bidwell et al., 1998; Kevekordes & Clayton, 2000; Myers et al., 2006; Seery et al., 2006, Myers et al. 2007; Han et al. 2011).

Sargassum horneri (Sargassaceae sp.) is a large brown alga commonly inhabiting seaweed beds along the coasts of Japan and the northwestern Pacific coast. *S. horneri* is an ecologically- and economically-important seaweed that provides fishery products and food for human consumption. In addition, it is a potential bioenergy resource (Choi et al. 2008; Pang et al. 2009; Nagai et al. 2014; Miki et al. 2016a). Miki et al. (2016b) reported that fertilized eggs of *S. horneri* stored in a refrigerator for approximately 170 days grew just as successfully as eggs stored for shorter periods. This suggests that fertilized eggs of *S. horneri* could be easily used for testing toxicity. However, toxicological data for *Sargassum horneri* are lacking.

Industrial wastewater usually contains substances, such as heavy metals and synthetic organic chemicals that inhibit the growth of macroalgae. After wastewaters undergo a variety of treatments, these inhibitory pollutants are often discharged to coastal areas as part of wastewater effluent. Therefore, the effects of the various growth inhibitors in wastewater effluents on macroalgae should be elucidated before they are discharged. Wastewaters discharged from various industries, such as synthetic resins, paper and pulp, gas and coke oven plants, and coal gas and petroleum, and mine discharges have been known to contain different types of phenols. Furthermore, such phenolic compounds are often toxic and some are

known or suspected to be carcinogenic (Bazrafshan et al. 2016). Therefore, it is important to investigate the effects of phenol and phenolic compounds on the germling growth of *S. horneri*. However, little is currently known about the effects of phenols on the early growth stages of *S. horneri*. Therefore, the main objective of our study was to investigate the potential for using a seaweed bioassay test using the germling growth stages of *S. horneri* by obtaining toxicological data for that species relative to phenols. Furthermore, the necessity of culture medium renewal for batch culture of *S. horneri* during 21 days was examined.

Materials and methods

Test organism

Fertilized eggs of female *S. horneri* were collected on May 9, 2016 in a nursery greenhouse of the Sakai Ovex Co., Fukui Prefecture, Japan. The eggs were rinsed with filtered seawater, poured into 500-ml amber glass bottles filled with filtered seawater, and stored without light illumination in a refrigerator at 4°C until use following protocols by the previous studies (Nagai et al. 2014; Miki et al. 2016a; Miki et al. 2016b).

Culture medium

The basic culture medium was seawater pumped from the ocean at a depth of 320 m around the Noto Peninsula of the Ishikawa Prefecture in Japan. This seawater was filtered through a 0.45-μm-pore membrane, sterilized by autoclaving at 121°C for 20 min (SN-200, Yamato, Japan), and stored in a refrigerator at 4°C following protocols by the previous studies (Nagai et al. 2014; Miki et al. 2016a; Miki et al. 2016b).

Enriched seawater for cultivating *S. horneri* was prepared by adding Provasoli's enriched seawater (PES) medium (Provasoli 1968; Motomura 2000; Andersen et al. 2005). After confirming germling growth for 21 days using enriched seawater with various PES medium addition ratios, we determined the proper PES medium addition ratio for culturing germlings: 2% (by volume) PES medium added to 1 L of the filtered seawater. Concentrations of NH_4–N, NO_3–N, and dissolved phosphorus (D-P) in the culture media were 0.01, 11.6, and 1.05 mg L^{-1}, respectively.

Preparation of test solutions

Phenol (CAS Number: 108-95-2) was obtained from Wako Corp. (Tokyo, Japan). It is a widely-used chemical often used as a reference for biological assays (Eklund 1998; Myers et al. 2006). We confirmed that phenol concentrations at 5.65–114 mg L^{-1} were almost maintained during 20 days under the same environmental conditions we cultivated *S. horneri* (**Table 1**).

To elucidate the effects of phenol concentration on germling growth of *S. horneri* (Run1), phenol was added to the appropriately enriched culture medium at specific concentrations of 0, 20, 40, 80, and 160 mg L^{-1}. These dosing levels followed a two-fold geometric progression (Duan et al, 2017). The maximum phenol concentration was the concentration at which growth of brown macroalgae was substantially suppressed in previous studies (Eklund, 1998; Myers et al., 2006). The culture medium of all samples was renewed once after every 10 days during the 21 days we ran the batch culture in Run1. We calculated the median effective concentration (EC_{50}) values for two time periods: (1) for 14 days of culturing (14-day EC_{50}) on the basis of the specific growth rate during the logarithmic period of growth and (2) for 21 days of culturing (21-day EC_{50}) on the basis of the final thallus area in the stationary phase.

To determine the no-observed-effect concentration (NOEC) for germling growth of *S. horneri* (Run2), phenol was added to each enriched culture medium at specific concentrations of 0, 5, 10, 20, 40, and 80 mg phenol L^{-1} (a two-fold geometric progression). The maximum phenol concentration was set at the concentration obtained from our results following Run1.

To determine if culture media required renewing in Run2, we investigated the effects of culture medium renewal on the specific growth rate and on the final thallus area on the basis of the data we obtained from the three phenol concentrations that were identical in Run1 and Run2 (i.e., phenol concentrations of: 0, 20, and 40 mg L^{-1}). Based on these results, we did not need to renew the culture medium in Run2.

Macroalgal toxicity test method using germling growth of *S. horneri*

S. horneri was cultured at the germling stage in a photoincubator (EYELA LTI-700, Tokyo Rikakikai Co., Ltd., Japan) with white LED lights (SLED-F30D, Japan Global Illumination Corp., Japan) (**Figure 1**). The culture medium was poured into six-well plates. Next, one healthy, fertilized egg was transferred into each well (1 egg per well) using a pipette. The number of replicates for each phenol concentration was 12 using six-well plates. Incubation in the photoincubator began by covering the well plates with aluminum foil for 1 day. Subsequently, normal cultures were produced by uncovering the aluminum foil for 21 days. The photoincubators were maintained at 80–100 μmol photons m^{-2} s^{-1} at 20°C on a 12:12 h light:dark cycle. Light intensities were measured using a light quantum meter (MQ-200, Apogee Instruments, USA).

The method we used to evaluate *S. horneri* germling growth was similar to that used in a previous study (Miki et al. 2016b). The germling growth of *S. horneri* was observed using a stereoscopic microscope (SMZ745T, Nikon, Japan) mounted on a digital camera (DS-Fi2-L3, Nikon, Japan) over the 21-day culture period. Algal thallus area was analyzed using image analysis software (ImageJ, National Institutes of Health, USA).

Logarithmic growth phases were evaluated during a 4–14 day period because growth had a 3-day lag phase. The specific growth rate (μ) of the algal thalli was calculated using the following equation:

$$\mu = \frac{\ln A_{t_2} - \ln A_{t_1}}{t_2 - t_1} \quad (1)$$

where t_1 was the start day of the logarithmic growth phase, t_2 was the end day of the logarithmic growth phase, A_{t1} was mean thallus area (mm^2) on the start day during the logarithmic growth phase, and A_{t2} was mean thallus area (mm^2) on the final day of the logarithmic growth phase.

And, the mean thallus areas of each sample after 21 culture days were recorded as the final thallus area.

The percent inhibition ratio (I), based on μ, was calculated as follows:

$$I = \frac{\mu_c - \mu_t}{\mu_c} \times 100 \quad (2)$$

where μ_c was mean value for μ in the control and μ_t was mean value for μ in the test culture.

In addition, percent inhibition ratio, based on the final thallus area at the end of the experiment, was calculated as follows:

$$I = \frac{A_c - A_t}{A_c} \times 100 \quad (3)$$

where A_c was mean thallus area at the end of the experiment for the control culture and A_t was mean thallus area at the end of the experiment for the test culture.

Statistical procedures

Dunnett's multiple comparison tests were used to identify statistically significant differences between treatment and control groups in both RUN1 and RUN2. Differences were considered significant at $p < 0.05$. The number of replicates was 12, but some replicates that had dead, fertilized eggs were removed from the data. The 14-day EC_{50} values (based on μ) and the 21-day EC_{50} values (based on final thallus area) were calculated using a probit method; the results are shown on the logarithmic, normal probability section of the paper (JIS K 0229, 1992). Further, when there was no significant difference in μ between the experimental and control samples for the thallus area at the end of the experiment, the highest phenol concentration was determined to be the NOEC value. A comparison of means under phenol concentrations in common for RUN1 and RUN2 (phenol concentrations: 0, 20, and 40 mg L^{-1}) were performed using Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Effect of phenol concentrations on germling growth of *S. horneri* (Run1)

Figure 2 shows the growth curves for thallus areas of *S. horneri* using culture media with various phenol concentrations (0–160 mg phenol L^{-1}). **Figure 3** shows the comparison among the thallus areas for *S. horneri* after 21 days of culturing using culture media with various phenol concentrations. Fertilized eggs cultured in 0–20 mg phenol L^{-1} additions showed similar growth curves. However, germling growth

was clearly inhibited when fertilized eggs were cultured with additions of >20 mg phenol L^{-1}. **Figure 4** shows the relationship between phenol concentration and specific growth rate (a) and final thallus area at the end of the experiment (b). μ values at 0, 20, 40, 80, and 160 mg phenol L^{-1} were 0.34 ± 0.08, 0.32 ± 0.07, 0.24 ± 0.08, 0.11 ± 0.04, and 0.03 ± 0.03 day^{-1}, respectively. Thallus areas after 21 days of culturing at 0, 20, 40, 80, and 160 mg phenol L^{-1} were 12.0 ± 4.48, 9.31 ± 4.39, 7.24 ± 4.30, 1.06 ± 0.88, and 0.09 ± 0.03 mm^2, respectively. Compared with the control, additions of 40–160 mg phenol L^{-1} significantly decreased μ and thallus areas, whereas the addition of 20 mg phenol L^{-1} did not significantly decrease either value.

From these results, the 14-day EC_{50} value (based on μ) and the 21-day EC_{50} (based on thallus area at the end of the experiment) were estimated to be 58 mg phenolL^{-1} and 36 mg phenol L^{-1}, respectively. **Figure 5** shows the comparison between percent inhibition ratios (*I*) based on the specific growth rate and final thallus area after 21 days of culturing. Two endpoints toward phenol showed a little different sensitivity. It seems that longer exposures to phenol cause stronger inhibition.

NOEC value for germling growth of *S. horneri* (Run2)

Growth curves for thallus areas of *S. horneri* using culture media with various phenol concentrations. **Figure 6** shows the growth curves for thallus areas of *S. horneri* using culture media with various phenol concentrations (0–40 mg phenol L^{-1}). **Figure 7** shows the relationship between phenol concentration and

specific growth rate (a) and thallus area at the end of the experiment (b). μ values at 0, 5, 10, 20, and 40 mg phenol L^{-1} were 0.32 ± 0.06, 0.36 ± 0.04, 0.35 ± 0.04, 0.33 ± 0.05, and 0.25 ± 0.05 day^{-1}, respectively. Thallus areas after 21 days of culturing at 0, 5, 10, 20, and 40 mg phenol L^{-1} were 16.1 ± 7.15, 13.9 ± 3.75, 15.1 ± 4.91, 14.8 ± 6.28, and 8.4 ± 4.32 mm^2, respectively. Compared with the control, the addition of 40 mg phenol L^{-1} significantly decreased μ and final thallus area, whereas additions of 5, 10, and 20 mg phenol L^{-1} did not significantly decrease either value. From these experimental results, the NOEC value was estimated to be 20 mg phenol L^{-1}.

Effects of culture medium renewal on germling growth of *S. horneri* (Run 1 and Run 2)

Culture media were renewed after 10 days of culturing during the 21-day culture period in Run1, whereas the media were not renewed in Run2. **Figure 8** compares the results of Run1 and Run2 under same phenol concentrations [i.e., specific growth rate (a) and thallus area at the end of experiment (b)]. Renewal of the culture media did not significantly affect μ and thallus area, except thallus area at the 20 mg phenol L^{-1} concentration, suggesting culture medium renewal did not affect germling growth of *S. horneri*.

Discussion

Our experimental data revealed that phenol addition clearly inhibits germling growth of *S. horneri*. The 14-day EC_{50} and 21-day EC_{50} were estimated as 58 mg phenol L^{-1} and 36 mg phenol L^{-1}, respectively. Further, the NOEC value based on μ and the final thallus area was estimated to be 20 mg phenol L^{-1}. It is well known that prolonged exposure increases toxicity (Eklund and Kautsky 2003). Therefore, different toxic effects possibly appeared depending on exposure times; the longer the exposure time of phenol in batch culture, the stronger is the toxicity effect on *S. horneri*.

Previous studies have reported EC_{50} values (based on μ) for some marine microalgae. For example, EC_{50} values for *Skeletonema costatum* (diatom) was reported to be approximately 49.5–49.8 mg phenol L^{-1} (Cowgill et al. 1989), whereas EC_{50} values for the freshwater microalga *Pseudokirchneriella subcapitata* was reported to be 160 mg phenol L^{-1} (Ministry of the Environment in Japan, 2017). On the basis of our 14-day EC_{50} values for *S. horneri* (based on μ), *S. horneri* appears to react to phenol within the same order of magnitude in concentration. However, it should be considered that the exposure time for toxicity testing using *S. horneri* was much longer than that required for marine microalgae, such as *S. costatum* (i.e., 14 days for *S. horneri* vs. 72–96 h for *S. costatum*).

Several previous studies have reported the negative effect of phenol on some seaweed species using various endpoints. For example, the EC_{50} value for the development of female reproductive organs in the red alga *Ceramium strictum* was 6 mg phenol L^{-1}. The EC_{50} value for the functioning of male reproductive organs of the red alga *Ceramium strictum* was 100 mg phenol L^{-1} (Eklund and Kautsky

2003). The performance of the brown alga *Hormossira banski* germination inhibition bioassay was investigated to assess the toxicity of phenol. The EC_{50} values largely ranged from 36 to 1068 mg phenol L^{-1}. The *H.banksii* germination and growth assay was insensitive to phenol when compared with microorganism, invertebrates and vertebrates (Myers *et.al*, 2006). Relative to macroalga of previous studies, *S. horneri* seems to be relatively sensitive to phenol. Therefore, germling growth of *S. horneri* could be used to test toxicity of phenol. However, seaweed has a variety of life cycles and there is a possibility that different toxicity values may be obtained when evaluating toxicity relative to other growth endpoints. Furthermore, other reference endpoints for *S. horneri* may exhibit different EC_{50} or NOEC values. We are currently interested in evaluating other reference endpoints, such as the preservation period of fertilized eggs under various phenol concentrations (Miki et al. 2016b).

Toxicological studies using macroalgae are usually performed between one and seven days. For the brown algae, longer exposure times of up to two weeks are more frequent (Eklund and Kautsky 2003); therefore, so exposure time in our experiment was quite long (14-21 days) relative to the lengths of previous studies. Growth depends on the amount of nutrients and toxic substances provided in the growth medium. As a result, medium renewal during the culturing period is sometimes needed (Eklund and Kautsky 2003). Therefore, we investigated the effects of culture medium renewal on germling growth. In our study, we found that renewal of the culture medium did not affect the growth of *S. horneri*. This suggests that seawater enriched with 2% PES contained sufficient nutrients for germling growth of *S.*

horneri over 21 days. In addition, the loss of phenol during the experiment seemed to be minimal. However, in some cases, chemicals are easily lost during the culturing period; therefore, in such cases, the need for renewal of media should be studied in detail.

Conclusion

The addition of phenol clearly inhibited germling growth of *S. horneri*. After 21 days of culturing, the EC_{50} value (based on μ) was 58 mg phenol L^{-1}, while the EC_{50} (based on thallus coverage) was 36 mg phenol L^{-1}. Furthermore, the NOEC value was estimated to be 20 mg L^{-1} phenol. Renewal of the culture media did not affect EC_{50} values during the 21 days of culturing. *S. horneri* appears to be as sensitive to phenol as other marine macroalgae reported in the literature; therefore, it can be used for toxicity testing. In addition, *S. horneri* can be collected easily from coastal areas of Japan and along coasts of the northwest Pacific. Moreover, fertilized eggs of *S. horneri* could be preserved in a refrigerator for approximately 170 days and they demonstrate similar growth curves after long preservation times (Miki et al. 2016b). From these results, we consider that *S. horneri* is one of the appropriate candidates of macroalgae for the bioassay test.

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Table 1 Changes in phenol concentration for each sample solution during a 20-day period

	Phenol concentration [mg L^{-1}]*				
	0 day	5 day	10 day	15 day	20 day
Sample 1	5.65 ± 0.04	5.63 ± 0.09	5.6 ± 0.03	5.65 ± 0.04	5.68 ± 0.01
Sample 2	22.9 ± 0.26	22.5 ± 0.23	21.8 ± 0.04	21.3 ± 0.13	21.1 ± 0.15
Sample 3	114 ± 0.21	113 ± 1.01	109 ± 0.37	107 ± 0.17	105 ± 0.59

*Six six-well plates were used for the analysis of changes in phenol concentration during the experiment. There were three replicates for each sample. Values are presented as mean ± SD.

Figure captions

Fig 1 Photoincubators used for the culture of *S. horneri* at the germling stage

Fig 2 Growth curves for thallus areas of *S. horneri* using culture media with various phenol concentrations. Values are presented as mean ± SD. There were 12 replicates for each treatment, except for the control (11 replicates)

Fig 3 Comparison among the thallus areas for *S. horneri* after 21 days of culturing using culture media with various phenol concentrations (scale bar= 1 mm)

Fig 4 Relationship between phenol concentration and specific growth rate (a) and final thallus area at the end of the experiment (b)

Fig 5 Comparison between percent inhibition ratios (*I*) based on the specific growth rate and final thallus area after 21 days of culturing

Fig 6 Growth curves for thallus areas of *S. horneri* using culture media with various phenol concentrations. Values are presented as mean ± SD. There were 12 replicates for each treatment, except for the 10-mg L^{-1} phenol treatment (n = 10)

Fig 7 Relationship between phenol concentration and specific growth rate (a) and final thallus area at the end of the experiment (b)

Fig 8 Comparison between RUN1 and RUN2 under identical phenol concentrations: specific growth rate (a) and final thallus area (b). * Denotes significant difference between two culture media ($p < 0.05$)

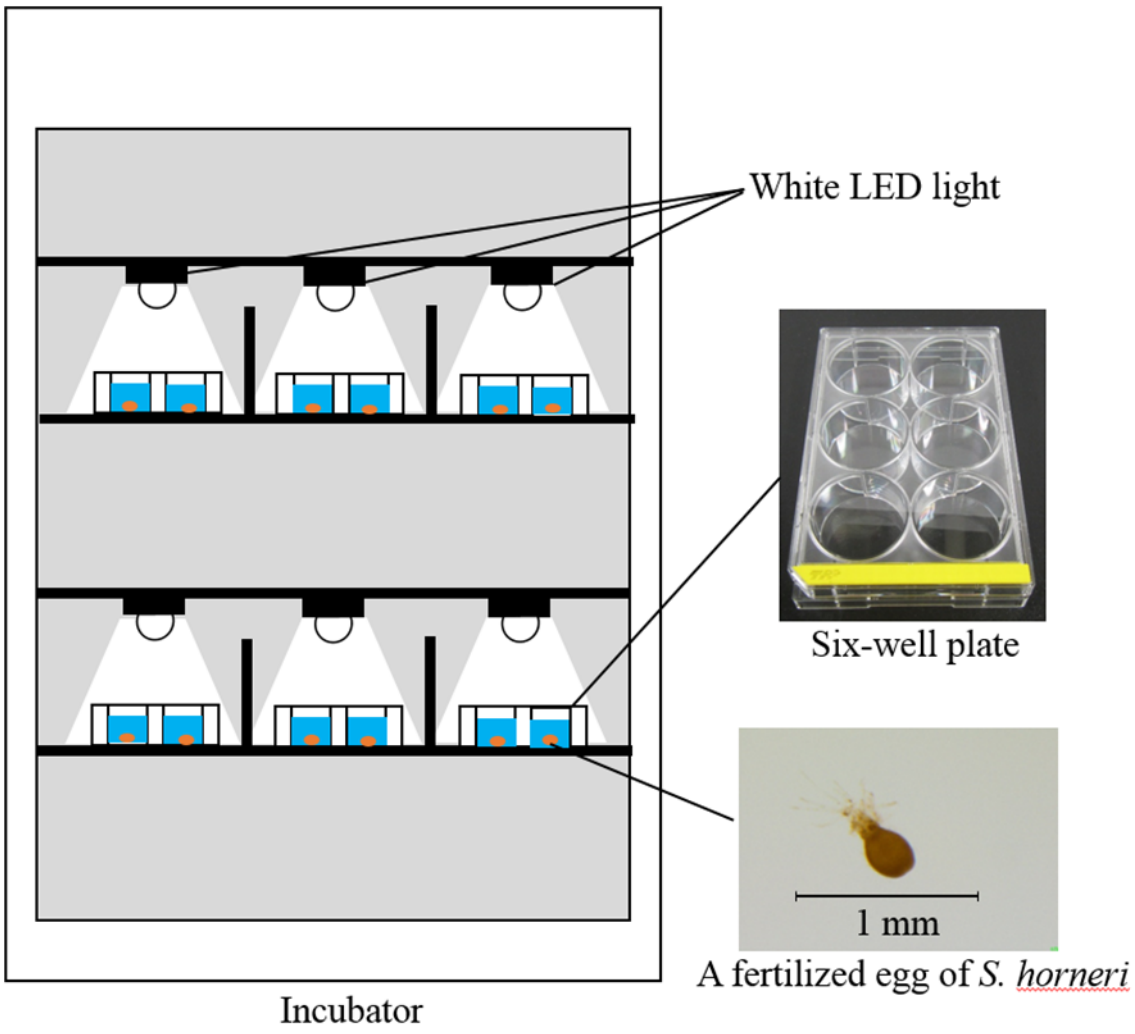

White LED light
Six-well plate
1 mm
A fertilized egg of S. horneri
Incubator

Fig. 1

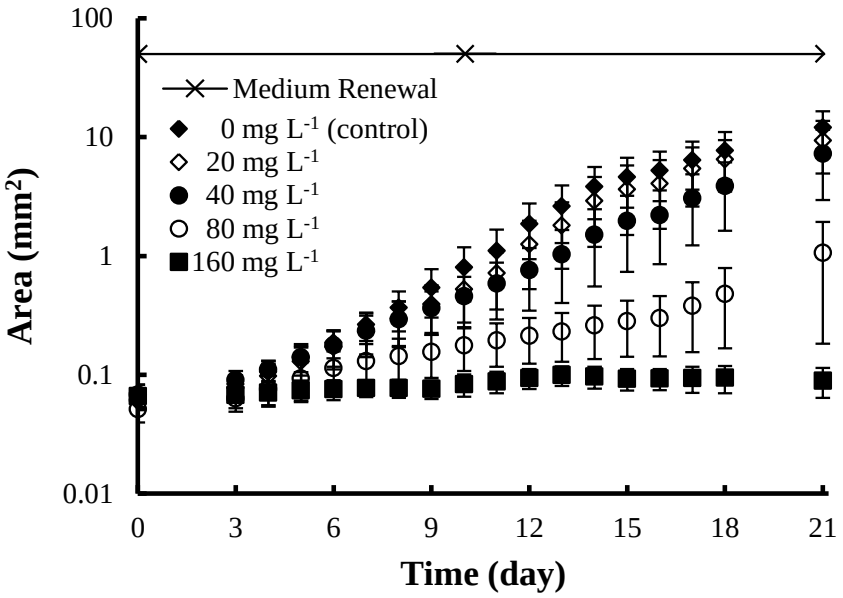

100
10
1
0.1
0.01
Area (mm²)
0
3
6
9
12
15
18
21
Time (day)
Medium Renewal
0 mg L-1 (control)
20 mg L-1
40 mg L-1
80 mg L-1
160 mg L-1

Fig. 2

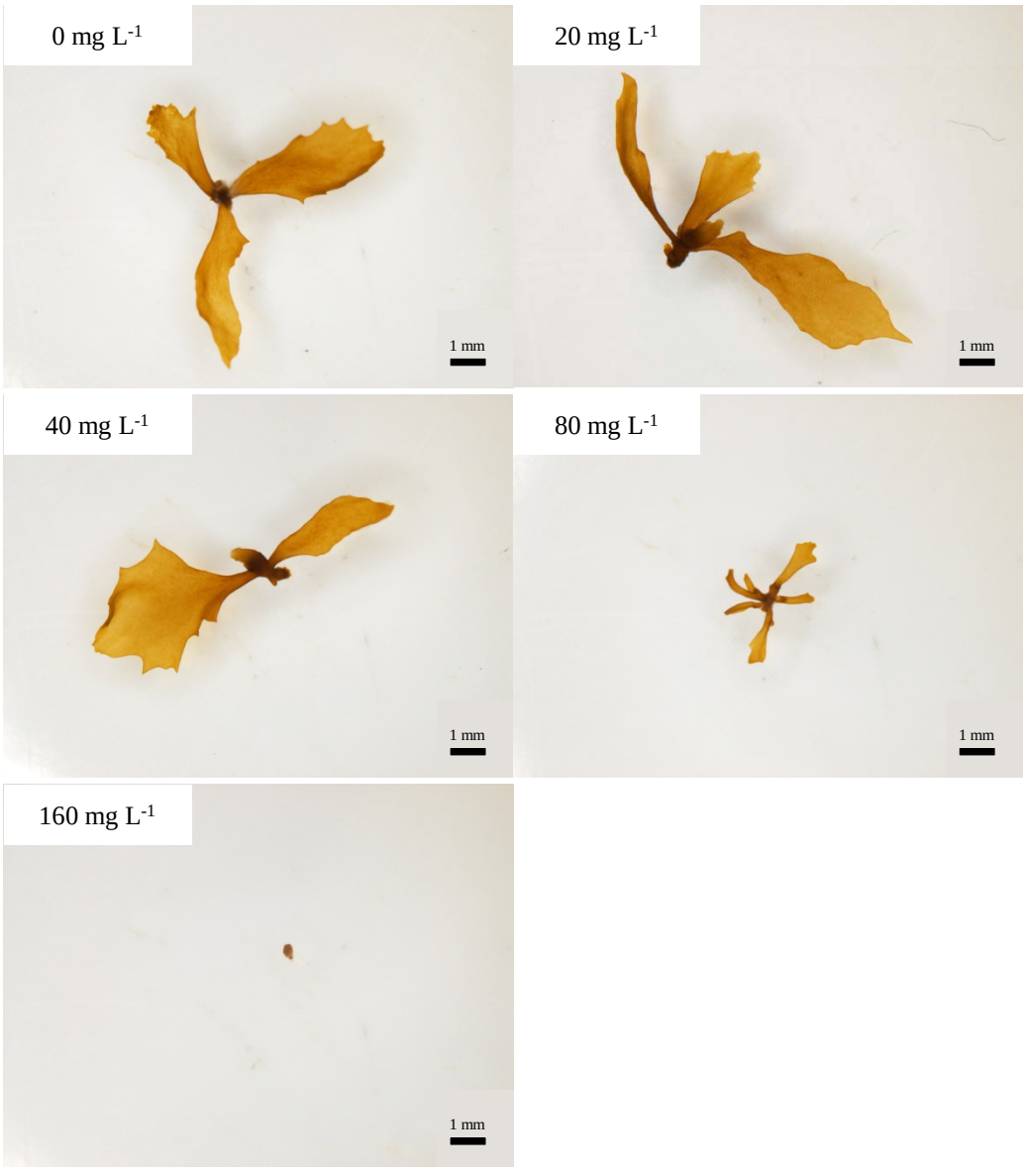
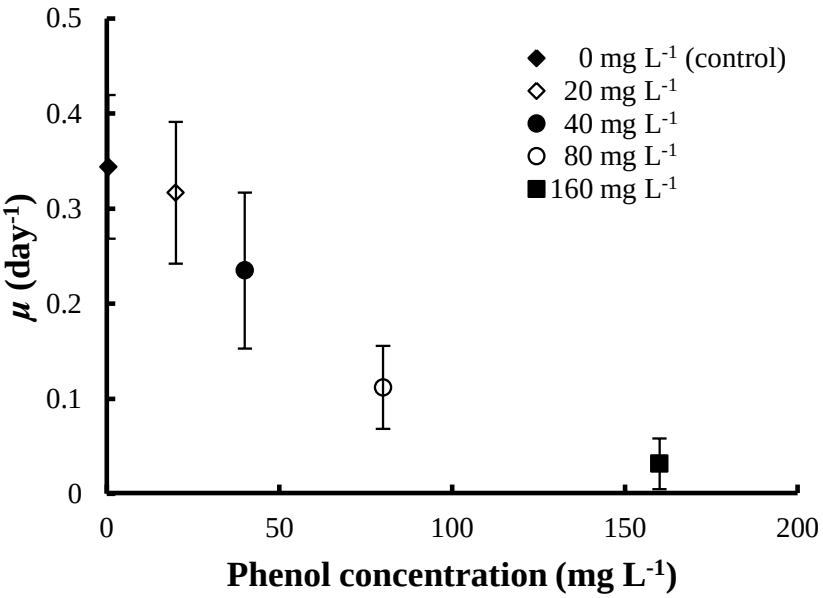


Fig. 3

(a)


0.5
0.4
0.3
0.2
0.1
0
μ (day-1)
0
50
100
150
200
Phenol concentration (mg L-1)
0 mg L-1 (control)
20 mg L-1
40 mg L-1
80 mg L-1
160 mg L-1

(b)

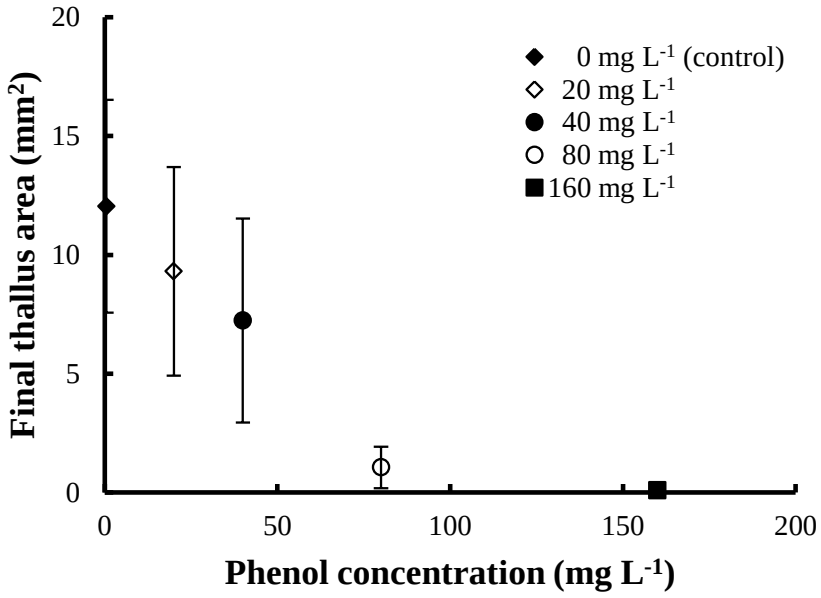

20
15
10
5
0
Final thallus area (mm2)
0
50
100
150
200
Phenol concentration (mg L-1)
0 mg L-1 (control)
20 mg L-1
40 mg L-1
80 mg L-1
160 mg L-1

Fig. 4

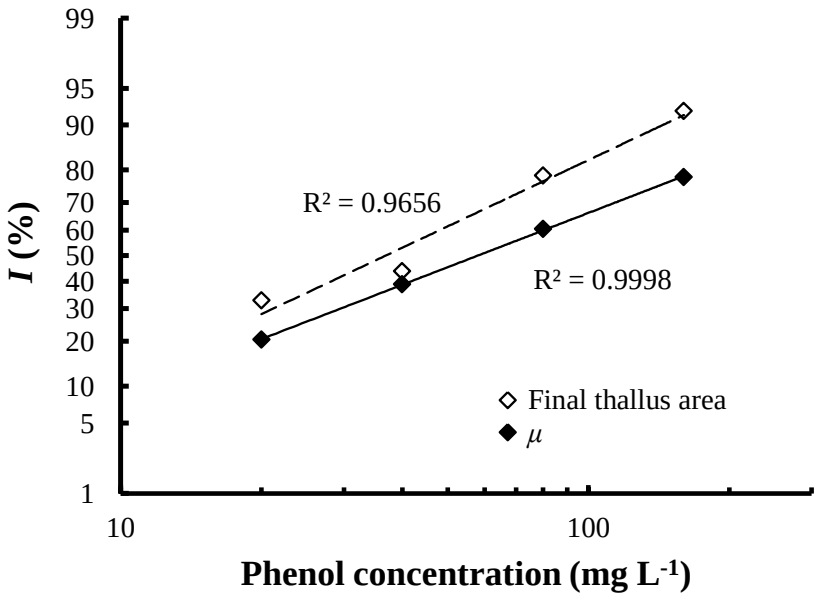

99
95
90
80
70
60
50
40
30
20
10
5
1
I (%)
R² = 0.9656
R² = 0.9998
Final thallus area
μ
10
100
Phenol concentration (mg L-1)

Fig. 5

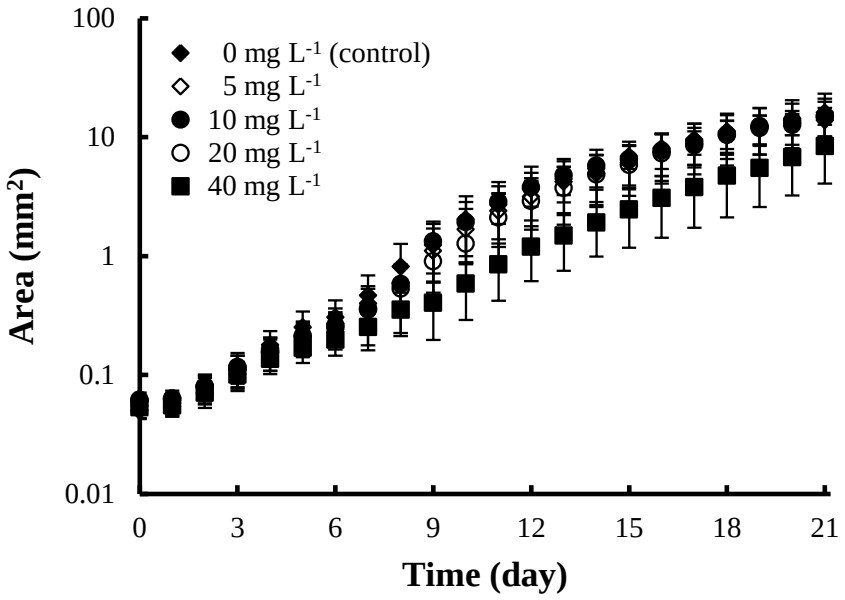
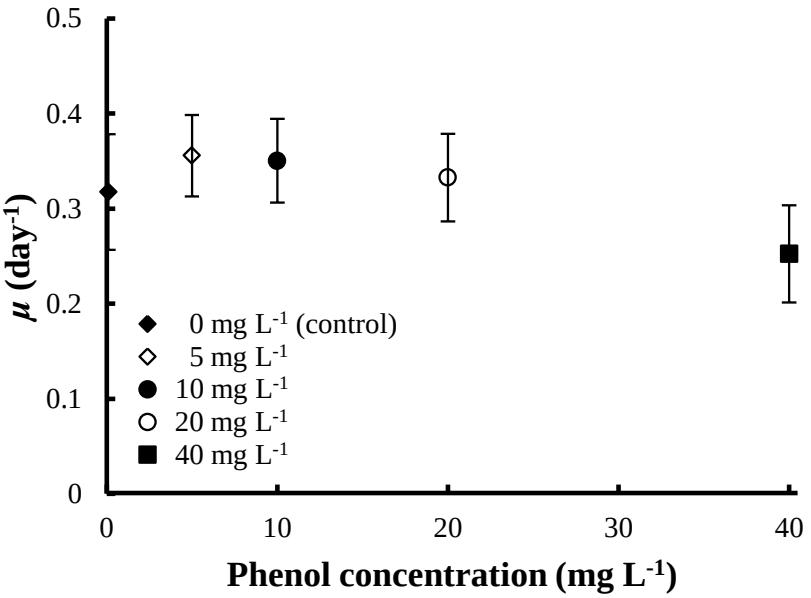

100
10
1
0.1
0.01
Area (mm²)
0 mg L-1 (control)
5 mg L-1
10 mg L-1
20 mg L-1
40 mg L-1
0
3
6
9
12
15
18
21
Time (day)

Fig. 6

(a)


0.5
0.4
0.3
0.2
0.1
0
μ (day-1)
0 mg L-1 (control)
5 mg L-1
10 mg L-1
20 mg L-1
40 mg L-1
0
10
20
30
40
Phenol concentration (mg L-1)

(b)

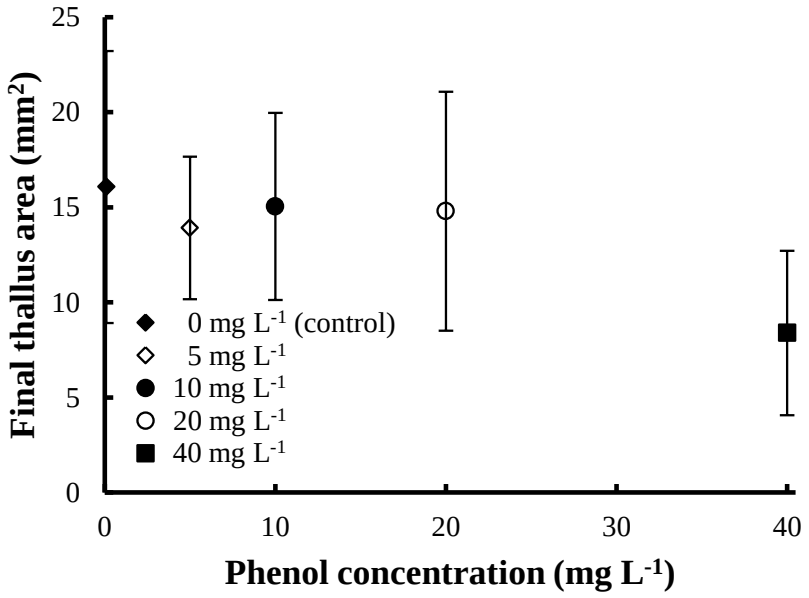
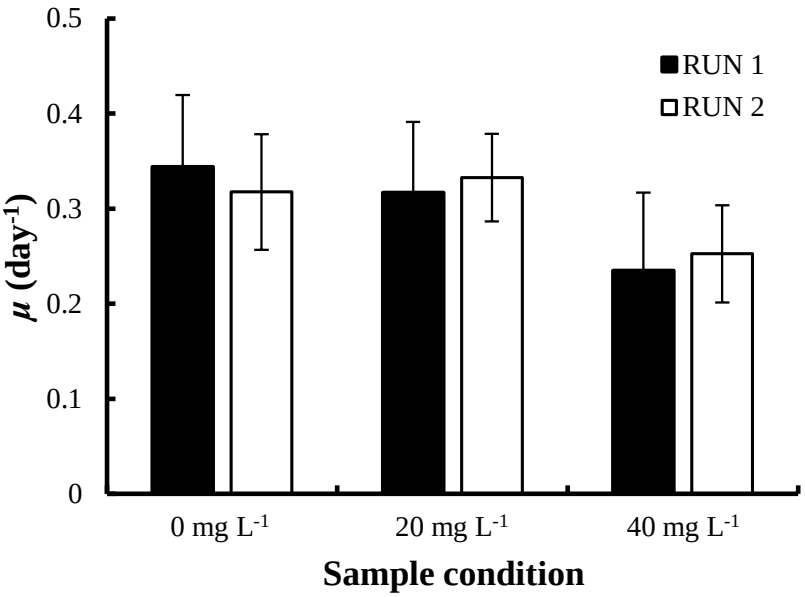

25
20
15
10
5
0
Final thallus area (mm2)
0 mg L-1 (control)
5 mg L-1
10 mg L-1
20 mg L-1
40 mg L-1
0
10
20
30
40
Phenol concentration (mg L-1)

Fig. 7

(a)


0.5
0.4
0.3
0.2
0.1
0
μ (day-1)
RUN 1
RUN 2
0 mg L-1
20 mg L-1
40 mg L-1
Sample condition

(b)

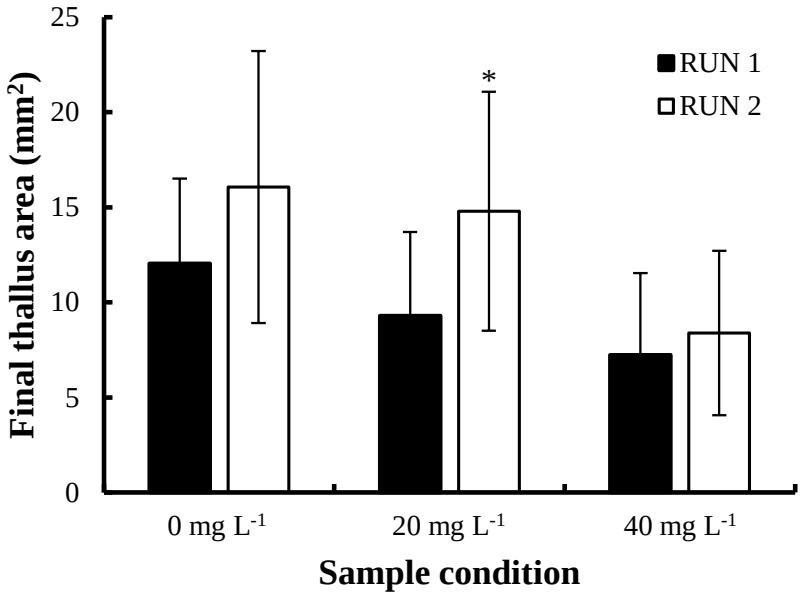

25
20
15
10
5
0
Final thallus area (mm2)
*
RUN 1
RUN 2
0 mg L-1
20 mg L-1
40 mg L-1
Sample condition

Fig. 8