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Effects of Preservation Period of Fertilized Eggs and High Concentrations of Nitrogen in Nutrient Sources on Germling Growth of *Sargassum horneri*

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Abstract

The effects of high concentrations of nitrogen sources on the germling growth of *Sargassum horneri* were investigated for the restoration of coastal barren ground using fertilization materials containing high concentrations of nitrogen. Moreover, the safekeeping period of fertilized eggs of *S. horneri* was studied to elucidate performance stability as an appropriate method using fertilized eggs. The fertilized eggs of *S. horneri* that had been preserved in a refrigerator for approximately 170 days were able to grow and demonstrate the same growth curve as those that had been preserved for shorter periods. This demonstrates that our culture method can be applied to examine the effects of nitrogen sources on the germling growth of *S. horneri.* The addition of over 2 mg-N L^{-1} of ammonium (NH_4-N) or nitrite (NO_2-N) clearly inhibited growth, and the addition of 50 mg-N L^{-1} of NH_4-N or NO_2-N had lethal effects on the germling growth of *S. horneri.* The addition of 1 mg-N L^{-1} of NH_4-N or NO_2-N did not clearly promote or inhibit growth. The addition of 50 mg-N $L^{-1}$of nitrate (NO_3-N) did not inhibit growth. It is expected that the effects of NH_4-N or NO_2-N on seaweed growth depends on the concentration level, growth stage of the seaweed, and seaweed species. On the basis of our results, nitrogen fertilizers that contain high concentrations of some nitrogen sources should be carefully considered before they are applied to restore barren ground in nutrient deficient coastal areas.

Keywords: *Sargassum horneri*, Fertilized eggs, Nitrogen fertilizer, High concentration, Growth inhibition, Toxicity

Introduction

Seaweed beds generally comprise macroalgae, such as those belonging to Sargassaceae and Laminariaceae. Seaweed beds are important areas of primary production in coastal areas. They supply food for herbivorous animals and living space for fishery products, which have an important role in ecosystem stability. However, seaweed beds in Japan have rapidly declined, a phenomenon known as “isoyake” in Japanese. Generally, “isoyake” is considered to be caused by a combination of factors, including a rise in seawater temperature, depletion of seaweeds by herbivorous marine animals, and effects of nutrient deficiencies in seawater (Choi et al. 2008; Fujita et al. 2010).

One solution to improve nutrient deficiency in seaweed beds is the application of artificial fertilizers, which has been tried in many areas in Japan (Fujita et al. 2010). Sewage treatment effluent, industrial drainage treatment effluent, and deep seawater (DSW), which have high concentrations of nutrients, are proposed as concrete liquid fertilizers (Fujita et al. 2010; Ueki et al. 2012). In addition, solid fertilizers, such as humus material and steel slag, have been used to increase seaweed bed reproduction (Yamamoto et al. 2006; Yamamoto et al. 2010; Ueki et al. 2011; Kato et al. 2015).

There are many studies that discuss the relationship between seaweed growth and nitrogen concentrations. Many seaweed species can simultaneously use ammonium (NH_4-N) and nitrate (NO_3-N), although very few reports discuss nitrite (NO_2-N). The uptake of NH_4-N is generally higher than NO_3-N at ecologically relevant concentrations. NH_4-N does not appear to be toxic to most seaweeds because ammonium additions of 100–500 μM yield normal uptake rates (Phillips et al. 2004; Hurd et al. 2014).

However, most of previous studies did not consider fertilizer applications to nutrients deficient ocean areas. The nitrogen concentration of seawater is much lower than that of some fertilizers, such as sewage treatment effluent. In addition, the effects of such high concentrations of different nitrogen sources on the early stage growth of Sargassaceae sp. are still unknown.

Sargassum horneri (Sargassaceae sp.) is a large brown alga common in seaweed beds in Japan. *S. horneri* is an important seaweed from the viewpoint of marine resources, such as fishery products, human food source, and a potential bioenergy resource. The steady disappearance of this alga has resulted in the study of artificial culture methods using indoor tanks (Pang et al. 2009).

The main objective of this study was to investigate the effects of high concentrations of nitrogen sources on the germling growth of *S. horneri*, under the assumption that the algae ground reproduction uses fertilization materials that contain high concentrations of nitrogen sources. Furthermore, the safekeeping period of fertilized eggs of *S. horneri* was investigated for a culture test to elucidate the effects of the safekeeping period on early stage growth.

Materials and methods

Sargassum horneri

Fertilized eggs from female *S. horneri* plants were collected from Fukui Prefecture, Japan, in April 2013 and 2014. Once gathered, the fertilized eggs were rinsed with filtrated seawater, poured into amber glass bottles (500 mL) filled with filtrated seawater, and stored without light illumination in a refrigerator at 4°C until use (Nagai et al. 2014; Miki et al. 2015).

Culture medium and analysis of seawater

The seawater used for the *S. horneri* culture medium was taken from ocean waters at a depth of 320 m off the Noto Peninsula, north of the Ishikawa Prefecture. The seawater was filtrated through a membrane filter (0.45 μm pore size) into the glass bottles that had been sterilized by autoclaving at 121°C for 20 min

(SN-200, Yamato, Japan) and stored in a refrigerator at 4°C until ready for use (Nagai et al. 2014; Miki et al. 2015).

Enriched seawater was prepared by adding 20 mL PES medium (Provasoli, 1968; Motomura 2000) to 1 L of filtrated seawater, which was used as the control culture medium for cultivating *S. horneri* (control 1). To elucidate the effects of NH_4-N addition on the growth of *S. horneri*, NH_4Cl was added to the control medium at specific concentrations of 1, 2, 5, 10, and 50 mg-N L^{-1}. To elucidate the effects of NO_2-N addition on the growth of *S. horneri*, $NaNO_2$ was added to the control medium at specific concentrations of 1, 2, 5, 10, and 50 mg-N L^{-1}. The nutrient quality of each culture medium is shown in Table 1.

And, to compare the effects of NH_4-N and NO_3-N additions on the growth of *S. horneri*, enriched seawater without nitrogen addition was prepared by using nitrogen deficient PES medium (control 2). NH_4Cl and $NaNO_3$ were added to the nitrogen deficient control medium at specific concentrations of 1, 10, and 50 mg-N L^{-1} respectively. The nutrient quality of each culture medium is shown in Table 2.

The filtered seawater quality was analyzed to determine the pH, inorganic nitrogen, phosphate, and iron contents. The pH was measured using a pH meter (HM-25R, DKK-TOA Corp., Japan). Nitrogen and phosphate concentrations were determined by a colorimetric method using an auto analyzer (Auto Analyzer, TRAACS2000, Bran Luebbe, Germany). Fe concentration was determined using a trace iron analyzer (FEA-07, Kimoto Electric Corp., Japan) based on luminol chemiluminescence (Miki et al. 2015).

S. horneri culture experiments using fertilized eggs

Figure 1 shows the photoincubator (EYELA LTI-700, Tokyo Rikakikai Co., Ltd., Japan) with LED lights (LDR14N-W, Toshiba, Japan) used for the culture of *S. horneri* at the germling stage. A 30-mL portion of the culture medium was poured in culture dishes (diameter = 8.7 cm) into which healthy, fertilized eggs

were transferred using a pipet (15 eggs per dish). The culture dishes were covered with aluminum foil, preserved in the photoincubator for one day, and then cultured without aeration for 21 days in the photoincubator. Culture media was not exchanged during the culture periods. 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 germling growth of *S. horneri* was observed using a stereoscopic microscope (SMZ745T, Nikon, Japan) mounted with a digital camera (DS-Fi2-L3, Nikon, Japan) over the 21-day period. The area of the algae thalli was analyzed by image analysis software (ImageJ, National Institutes of Health, USA).

The specific growth rate (μ) of the algae 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 is the initial time (day) of the logarithmic growth phase, t_2 is the final time (day) during the logarithmic growth phase, A_{t1} is the thalli area (mm^2) on the initial day during the logarithmic growth phase, and A_{t2} is the thalli area (mm^2) on the final day during the logarithmic growth phase. The percent inhibition based on a specific growth rate was calculated as follows:

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

where μ_c is the specific growth rate of the control culture and μ_t is the specific growth rate of the test culture. The percent inhibition based on the final thalli area was calculated as follows:

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

where A_c is the final thalli area of the control culture and A_t is the final thalli area of the test culture.

Differences between average final areas of the algae thalli after 21 culture days and the specific growth rates (μ) at the logarithmic growth phase were evaluated using a one-sided Student's t-test. Results where $p < 0.05$ were considered significant. The number of replicates per culture dish was fifteen, but some dead fertilized eggs during the culture periods were removed from the data.

Results

Effect of preservation period of fertilized eggs on the germling growth

Fertilized eggs that were preserved for 3, 129, 167, and 208 days in a refrigerator were found to grow and increase in the thallus area. Figure 2 shows the growth curves of thalli areas using fertilized eggs that were preserved for different lengths of time and Table 3 shows specific growth rates and final thalli areas.

Fertilized eggs of *S. horneri* preserved for different lengths of time showed almost the same growth curves. Average specific growth rates for fertilized eggs preserved for 3, 129, 167, and 208 days were 0.32, 0.31, 0.33, and 0.31 day^{-1}, respectively. When compared with the average specific growth rate of 0.32 day^{-1} preserved for 3 days, there were no significant differences in average growth rates between the four groups ($p > 0.05$). The average thallus area after 21 days' culture following a preservation period of 3, 129, 167, and 208 days were 7.70, 7.35, 7.78, and 6.09 mm^2, respectively. When compared with the average thallus area of 7.70 mm^2 preserved for 3 days, there were no significant differences between the groups with the exception of the 208 day preservation period ($p = 0.02$).

We concluded that fertilized eggs preserved for about 170 days at least could be used for examining the effects of nitrogen sources on germling growth of *S. horneri.* Such an easy preservation method of *S. horneri* fertilized eggs using a refrigerator is critical because the seasonal period for obtaining fertilized eggs from ocean seaweeds is limited.

Effect of NH_4-N concentrations on germling growth of *S. horneri*

Figure 3 shows the growth curves of the thalli areas using different NH_4-N concentrations in the culture media and Figure 4 shows the comparison of the final thalli areas after 21 days' culture. All fertilized eggs,

except those in the 50 mg-N L^{-1} of NH_4-N culture, showed growth. The eggs added to the 50 mg-N L^{-1} of NH_4-N culture showed only a black phase after one day of culture after which they did not grow at all.

Average specific growth rates associated with NH_4-N concentrations of 1, 2, 5, and 10 mg-N L^{-1} were 0.38, 0.26, 0.26, and 0.26 day^{-1}, respectively. When compared with the average specific growth rate of 0.35 day^{-1} at 0 mg-N L^{-1} addition of NH_4-N (control 1), NH_4-N additions of 2, 5, and 10 mg-N L^{-1} significantly decreased the specific growth rates ($p < 0.05$). The 1 mg-N L^{-1} addition of NH_4-N did not show any negative effects and only slightly increased the average specific growth rate.

The average thalli areas after 21 days' culture at NH_4-N concentrations of 1, 2, 5, and 10 mg-N L^{-1} were 8.36, 3.60, 2.86, and 2.91 mm^2, respectively. When compared with the average thallus area of 8.13 mm^2 at 0 mg-N L^{-1} addition of NH_4-N (control 1), NH_4-N additions of 2, 5, and 10 mg-N L^{-1} significantly decreased the thalli areas ($p < 0.05$). The addition of 1 mg-N L^{-1} of NH_4-N did not decrease the final thallus area ($p = 0.40$).

Effect of NO_2-N concentrations on germling growth of *S. horneri*

Figure 5 shows the growth curves of the thalli areas using different NO_2-N concentrations in the culture media and Figure 6 shows the comparison of the final thalli areas after 21 days' culture. All fertilized eggs cultured in 1–50 mg-N L^{-1} of NO_2-N showed growth. Average specific growth rates associated with NO_2-N concentrations of 1, 2, 5, 10, and 50 mg-N L^{-1} were 0.32, 0.27, 0.30, 0.25, and 0.12 day^{-1}, respectively. When compared with the average specific growth rate of 0.36 day^{-1} at 0 mg-N L^{-1} addition of NO_2-N (control 1), NO_2-N additions from 1 to 10 mg-N L^{-1} significantly decreased specific growth rates ($p < 0.05$).

The average thalli areas after 21 days' culture at NO_2-N concentrations of 1, 2, 5, and 10 mg-N L^{-1} were 6.31, 3.90, 3.70, 3.38, and 0.35 mm^2, respectively. When compared with the average thallus area of

5.54 mm^2 at 0 mg-N L^{-1} addition of NO_2-N (control 1), NO_2-N additions of 2, 5, and 10 mg-N L^{-1} significantly decreased the final thalli areas ($p < 0.05$). The 1 mg-N L^{-1} addition of NO_2-N did not decrease the final thallus area ($p = 0.31$).

Figure 7 shows a comparison of the inhibition ratios between NH_4-N and NO_2-N concentrations (A, Inhibition ratio based on specific growth rate; B, Inhibition ratio based on final thalli areas). It is clear that a high concentration of NH_4-N or NO_2-N added to the seawater over 2 mg-N L^{-1} inhibited the germling growth of *S. horneri*.

Comparison of the effects of NO_3-N and NH_4-N additions on germling growth of *S. horneri*

Figure 8 shows the growth curves of the thalli areas using different NO_3-N and NH_4-N concentrations added in the culture media and Figure 9 shows the comparison of the average specific growth rates between NO_3-N and NH_4-N concentrations. All fertilized eggs cultured in 0.3–50.3 mg-N L^{-1} of NO_3-N showed stable growth. Average specific growth rates associated with NO_3-N concentrations of 1.3, 10.3, and 50.3 mg-N L^{-1} were 0.32, 0.33, and 0.39 day^{-1}, respectively. When compared with the average specific growth rate of 0.30 day^{-1} at 0.3 mg-N L^{-1} of NO_3-N (control 2), NO_3-N concentrations of 1.3,10.3 and 50.3 mg-N L^{-1} significantly increased specific growth rates ($p < 0.05$).

In contrast, all fertilized eggs cultured in 1-50mg-N L^{-1} of NH_4-N showed unstable growth. Especially, the eggs added to the 50 mg-N L^{-1} of NH_4-N did not grow at all. Average specific growth rates associated with NH_4-N concentrations of 1, 10, and 50 mg-N L^{-1} were 0.28, 0.24, and 0 day^{-1}, respectively. When compared with the average specific growth rate of 0.30 day^{-1} at 0 mg-N L^{-1} addition of NH_4-N (control 2), NH_4-N concentration of 10 mg-N L^{-1} significantly decreased specific growth rate ($p < 0.05$). Effect of NH_4-N concentration of 1 mg-N L^{-1} on specific growth rate was not clear ($p = 0.28$).

Discussion

A number of previous studies reported that many seaweed species use NH_4-N and NO_3-N at the same time and ammonium does not seem to be toxic since ammonium additions of 100–500 μM (NH_4-N concentrations from 1.4 to 7.0 mg-N L^{-1}) maintains normal uptake (Hurd et al. 2014). The presence of NH_4-N inhibited NO_3-N uptake by mature plants but not by the germlings of *Fucus distichus* (Thomas et al. 1985). NH_4Cl and $NaNO_3$ rich media (50 μM of N) accelerated *Ulva lactuca* growth to a maximum specific growth rate of 16.4 ± 0.18% per day and 9.4 ± 0.18% per day, respectively (Ale et al. 2011). Contrary to these previous studies, the addition of more than 2 mg-N L^{-1} of NH_4-N clearly inhibited the growth of *S. horneri* at the germling stage. It was not clear that addition of 1 mg-N L^{-1} of NH_4-N promote or inhibit the growth. We estimated an EC_{50} of NH_4-N to be between 1 and 50 mg-N L^{-1}, and the addition of 50 mg-N L^{-1} was lethal to germling growth. It is well known that total ammonia exists in aqueous solutions as NH_3 (unionized ammonia gas) and NH_4^+ (ammonium ion). NH_3 has higher toxicity than NH_4^+ but ammonia exits primarily as NH_4^+ in natural seawaters, and the negative effect of NH_3 is negligible (Rich.1973). However, at high concentrations of ammonia in seawaters, the negative effect of NH_3 to fertilized eggs of *S. horneri* may become to be not negligible. These results suggest that the effect of NH_4-N on seaweed greatly varies according to the NH_4-N concentration, the growth stage, and the seaweed species. Some industrial treated wastewater often contains high concentrations of NH_4-N (Miki et al. 1994). Therefore, when such industrial treated wastewater is applied to the sea as nitrogen fertilizer and when the dilution effects are small, NH_4-N may inhibit the growth of *S. horneri* at the germling stage.

Compared with NH_4-N, NO_2-N is usually unstable in the environment because it is easily oxidized to NO_3-N in aerobic conditions by microorganisms. Therefore, most studies have focused on NH_4-N or NO_3-N and there are few studies on high concentrations of NO_2-N (Machiguchi et.al 1985; Hurd et al. 2014). However, some industrial treated wastewater has often high concentrations of NO_2-N (Alleman

1985; Balmele et al. 1992; Miki et al. 1994) and our results showed that an addition of more than 2 mg-N L^{-1} of NO_2-N clearly inhibited the growth of *S. horneri* at the germling stage. Further, the addition of 1 mg-N L^{-1} of NO_2-N did not inhibit the growth, and the addition of 50 mg-N L^{-1} of NO_2-N did not result in the death of fertilized eggs, but showed a high inhibition ratio. It is clear that a high concentration of NO_2-N has a negative effect on the germling growth, similar to a high concentration of NH_4-N.

Compared with NH_4-N and NO_2-N, our experimental data showed that an addition of 50 mg-N L^{-1} of NO_3-N did not inhibit the germling growth of *S. horneri* at all and increased the growth rate. When high concentrations of nitrogen fertilizer, such as treated wastewater, are directly supplied to nitrogen deficient ocean areas, the conversion of NH_4-N or NO_2-N to NO_3-N by a nitrification reaction process will lead to decrease the possibility of the negative effect on the germling growth of *S. horneri*. Much attention should be paid to the nitrogen sources and the concentration level in nitrogen fertilizer before the application in nitrogen deficient coastal areas.

Conclusion

In the present work, the effects of high concentrations of NH_4-N, NO_2-N and NO_3-N on the germling growth of *S. horneri* were evaluated by the culture test method, using fertilized eggs that had been preserved for long periods of time. The fertilized eggs that had been preserved for about 170 days were able to grow and showed the same growth curve as those with shorter preservation periods. This result confirmed that such a culture method can be applied to examine the effects of high concentrations of nitrogen sources on the germling growth of *S. horneri*. The addition of over 2 mg-N L^{-1} of NH_4-N or NO_2-N clearly inhibited the germling growth and the addition of 50 mg-N L^{-1} had lethal effects on the germling growth of *S. horneri*. The addition of 1 mg-N L^{-1} of NH_4-N or NO_2-N did not clearly promote or inhibit the germling growth. It is expected that the effects of NH_4-N or NO_2-N on seaweed germling

growth depend on the nitrogen concentration and the growth stage of the seaweed. Compared with NH_4-N and NO_2-N, the addition of 50 mg-N L^{-1} of NO_3-N did not inhibit germling growth and increased the germling growth rate of *S. horneri*. Nitrogen fertilizers containing high concentrations of some nitrogen sources should be carefully considered before they are applied to restore barren ground in nitrogen deficient coastal areas.

Acknowledgments

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Table 1 Nutrient quality of each culture medium

	NH_4−N (mg L^{-1})	NO_2−N (mg L^{-1})	NO_3−N (mg L^{-1})	D−P (mg L^{-1})	Fe (μg L^{-1})
Control 1*	<0.01	<0.01	11.6	1.04	550
NH_4−N addition	1, 2, 5,10, 50	<0.01	11.6	1.04	550
NO_2−N addition	<0.01	1, 2, 5,10, 50	11.6	1.04	550

*Enriched seawater

Table 2 Nutrient quality of each culture medium

	NH_4−N (mg L^{-1})	NO_2−N (mg L^{-1})	NO_3−N (mg L^{-1})	D−P (mg L^{-1})	Fe (μg L^{-1})
Control 2**	<0.01	<0.01	0.3	1.04	550
NH_4−N addition	1, 10, 50	<0.01	0.3	1.04	550
NO_3−N addition	<0.01	<0.01	1.3,10.3,50.3	1.04	550

**Enriched seawater without nitrogen addition

Table 3 Specific growth rate and final thallus area of *S. horneri*

Preservation period (day)	μ * (day^{-1})	Area of thalli after 21 days of culturing * (mm^2)	Logarithmic growth phase (day)
3	0.32 ± 0.07	7.70 ± 1.70	3 - 12
129	0.31 ± 0.05	7.35 ± 2.83	3 - 12
167	0.33 ± 0.03	7.78 ± 2.85	3 - 12
208	0.31 ± 0.03	6.09 ± 1.91	3 - 12

*Values are mean ± SD. The number of replicates was n = 15 for 3, 129 and 167 day of preservation period. The number of replicates was n = 11 for 208 day of preservation period

Figure captions:

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

Fig. 2 Growth curves of thalli areas for *S. horneri* using fertilized eggs preserved for different lengths of time. Values are mean ± SD. The number of replicates was n = 15 for 3, 129 and 167 day of preservation period. The number of replicates was n = 11 for 208 day of preservation period

Fig. 3 Growth curves of thalli areas for *S. horneri* using the culture media with different NH_4-N concentrations. Values are mean ± SD. The number of replicates was n = 15 for 0 and 10 mg-N L^{-1} of NH_4-N. The number of replicates was n = 14 for 1, 2, and 5 mg-N L^{-1} of NH_4-N

Fig. 4 Comparison among final thalli areas for *S. horneri* after 21 days' culture using culture media with different NH_4-N concentrations (Scale bar: 1 mm)

Fig. 5 Growth curves of thalli areas for *S. horneri* using culture media with different NO_2-N concentrations. Values are mean ± SD. The number of replicates was n = 15 for 0, 1, 2, and 10 mg-N L^{-1} of NO_2-N. The number of replicates was n = 14 for 50 mg-N L^{-1} of NO_2-N. The number of replicates was n = 12 for 5 mg-N L^{-1} of NO_2-N.

Fig. 6 Comparison among final thalli areas for *S. horneri* after 21 days' culture using culture media with different NO_2-N concentrations (Scale bar: 1 mm)

Fig. 7 Comparison of the inhibition ratio between NH_4-N and NO_2-N concentrations (A, Inhibition ratio based on the specific growth rate; B, Inhibition ratio based on the final thalli area)

Fig.8 Growth curves of the thalli areas for *S. horneri* using different NO_3-N and NH_4-N concentrations added in the culture media. Values are mean ± SD. The number of replicates was n = 15 for 0 and 1 mg-N L^{-1} of NH_4-N. The number of replicates was n = 14 for 10 mg-N L^{-1} of NH_4-N. The number of replicates was n = 15 for 0, 1, 10, 50 mg-N L^{-1} of NO_3-N

Fig. 9 Comparison of the specific growth rates between NH_4-N and NO_3-N concentrations.

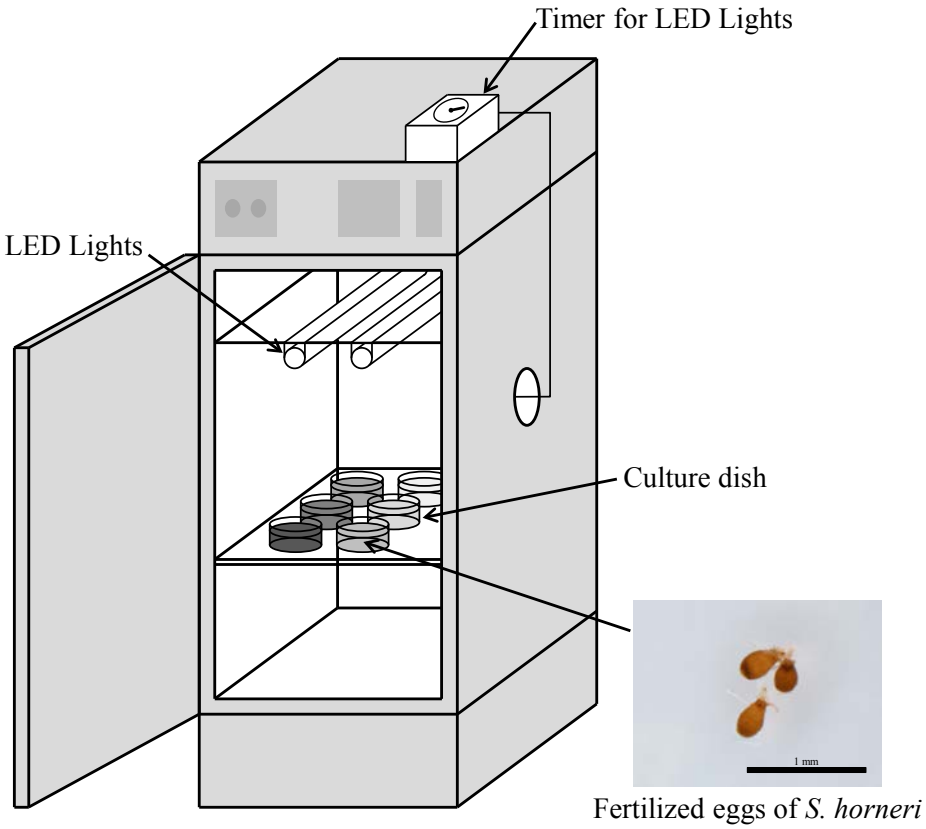

Timer for LED Lights
LED Lights
Culture dish
1 mm
Fertilized eggs of S. horneri

Fig. 1

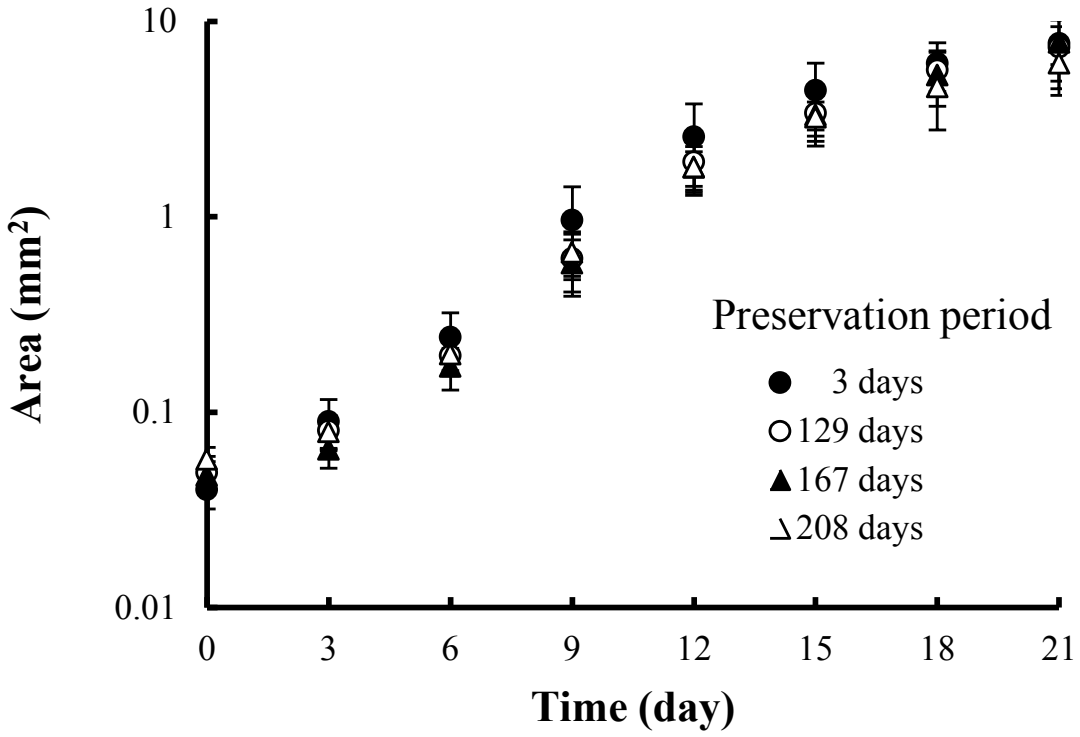

10
1
0.1
0.01
Area (mm2)
0
3
6
9
12
15
18
21
Time (day)
Preservation period
3 days
129 days
167 days
208 days

Fig. 2

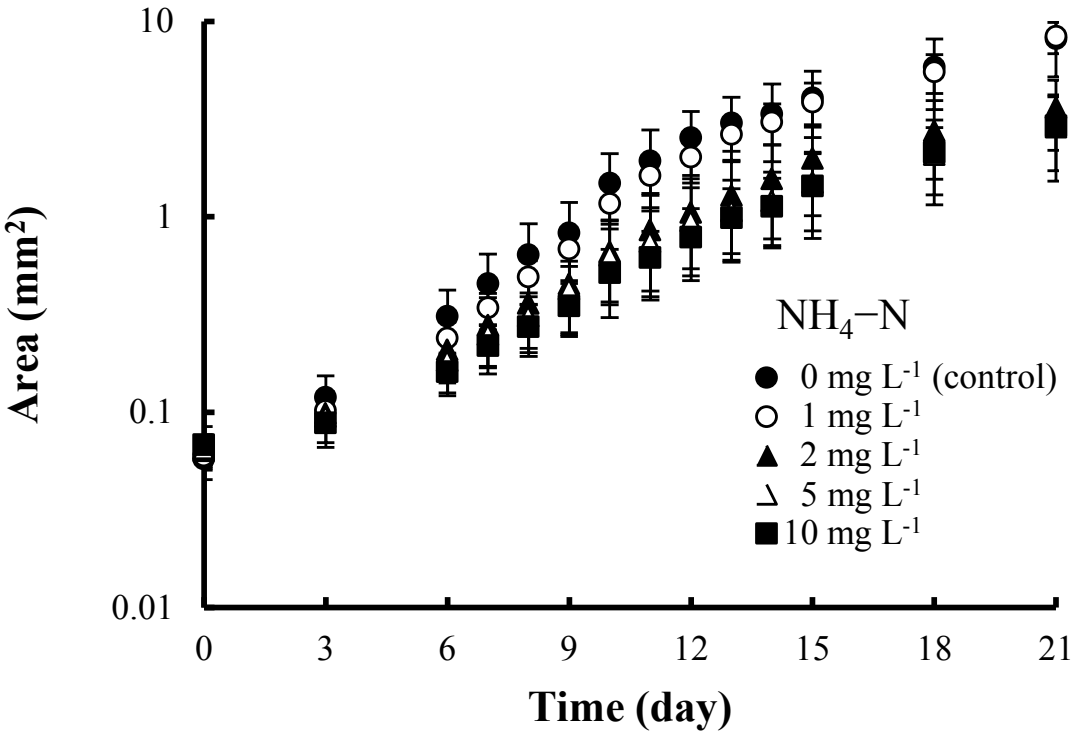

10
1
0.1
0.01
Area (mm2)
0
3
6
9
12
15
18
21
Time (day)
NH4−N
0 mg L-1 (control)
1 mg L-1
2 mg L-1
5 mg L-1
10 mg L-1

Fig. 3

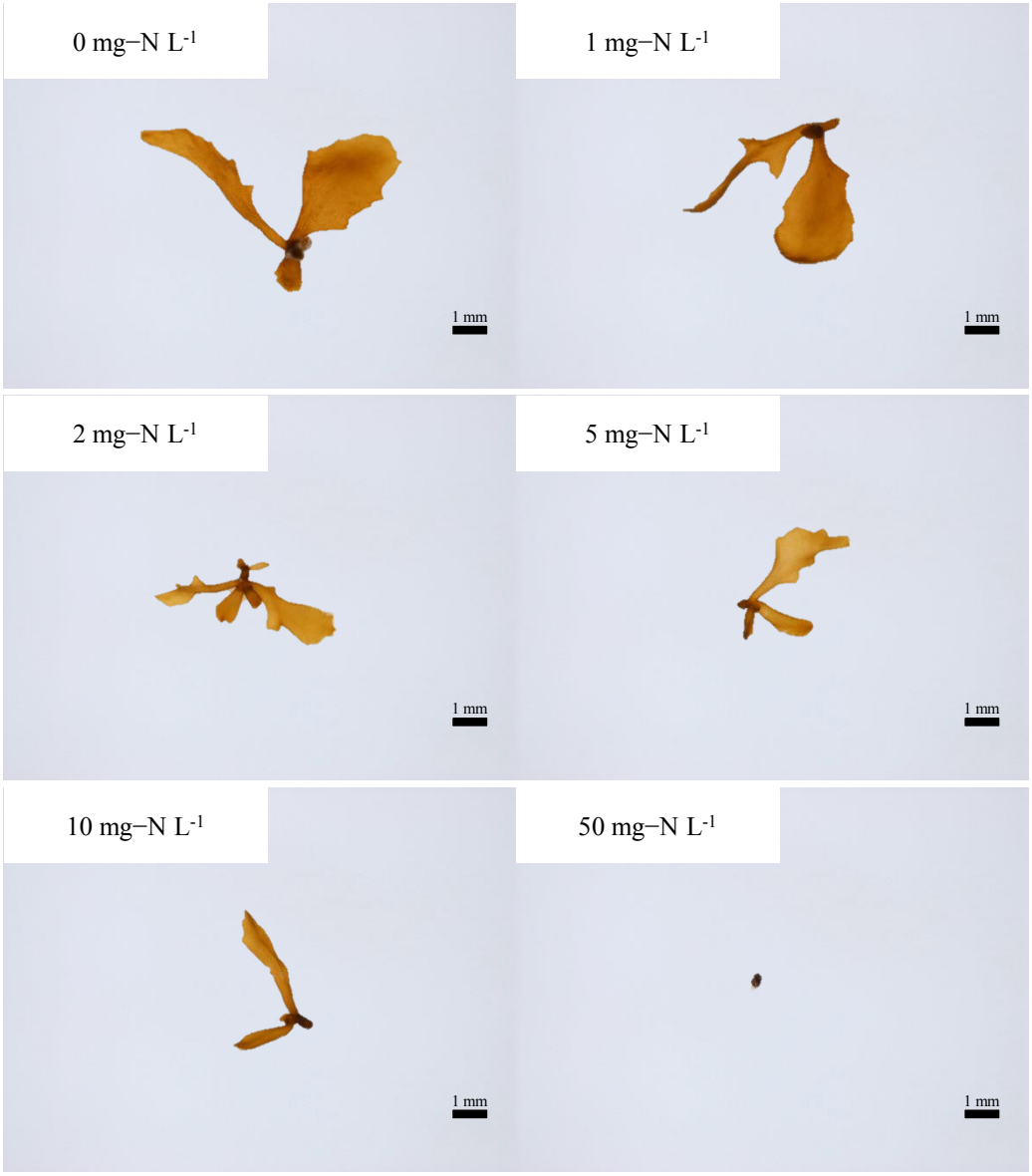


Fig. 4

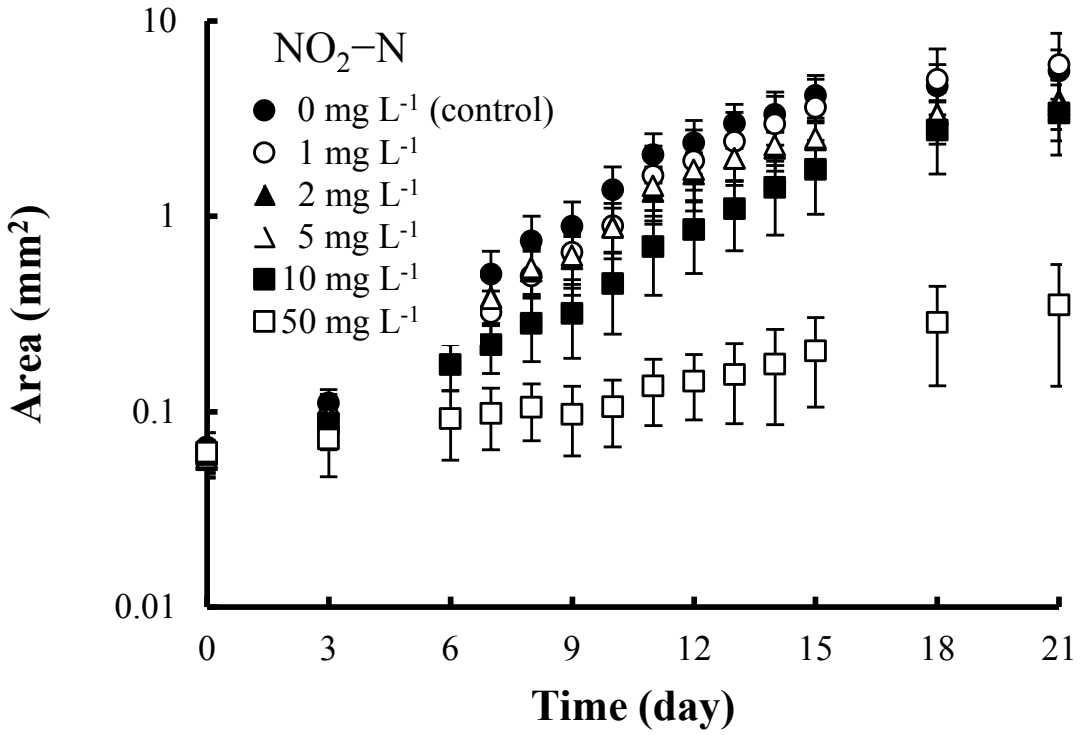

10
1
0.1
0.01
Area (mm²)
NO2−N
0 mg L-1 (control)
1 mg L-1
2 mg L-1
5 mg L-1
10 mg L-1
50 mg L-1
0
3
6
9
12
15
18
21
Time (day)

Fig. 5

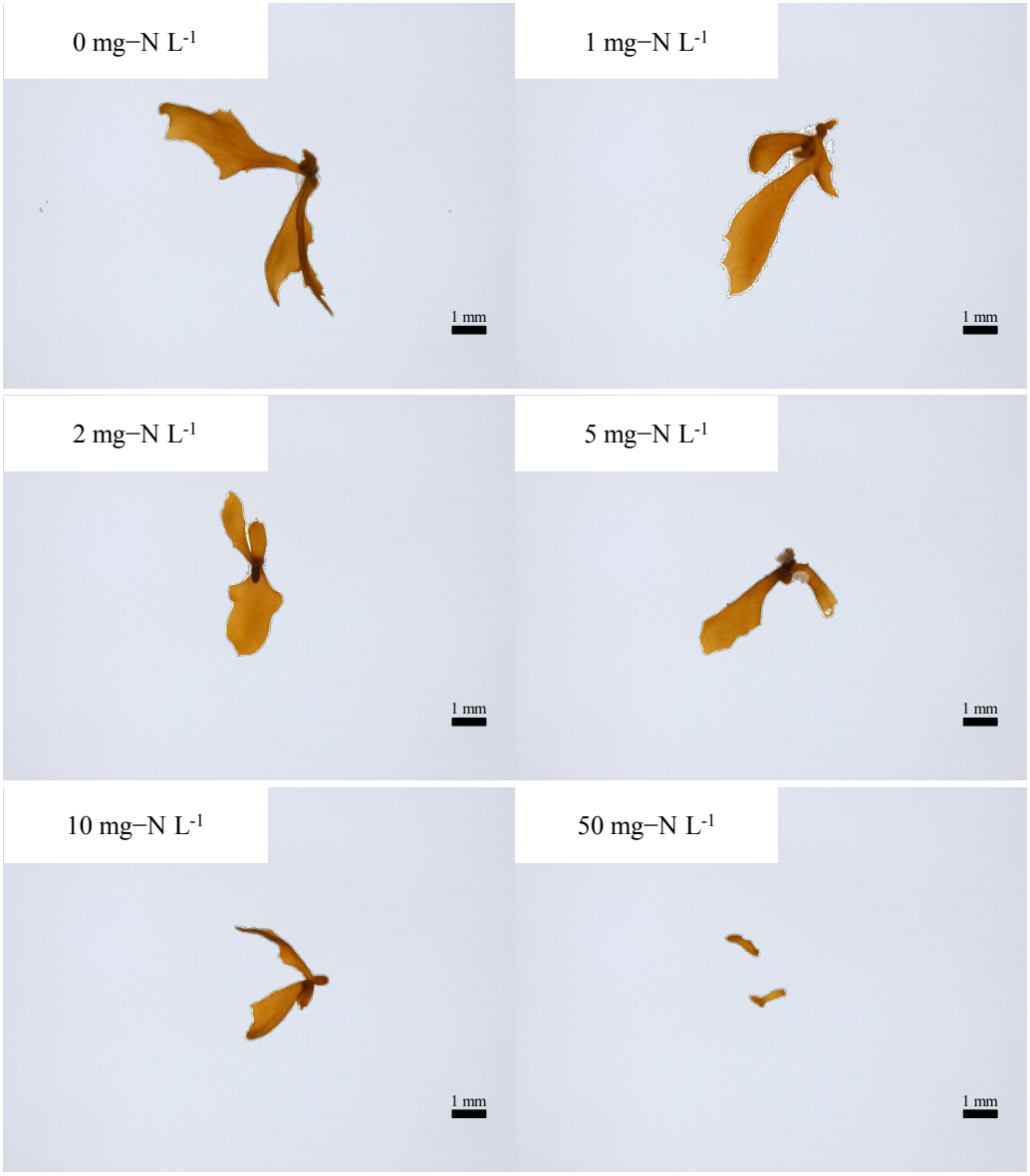
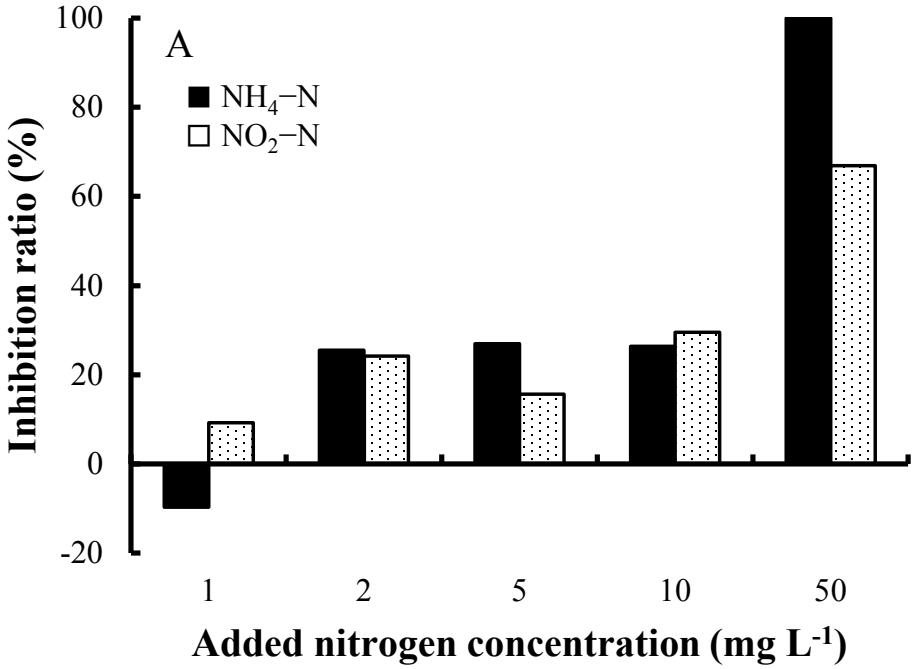


Fig. 6


A
NH4–N
NO2–N
Inhibition ratio (%)
100
80
60
40
20
0
-20
1
2
5
10
50
Added nitrogen concentration (mg L-1)

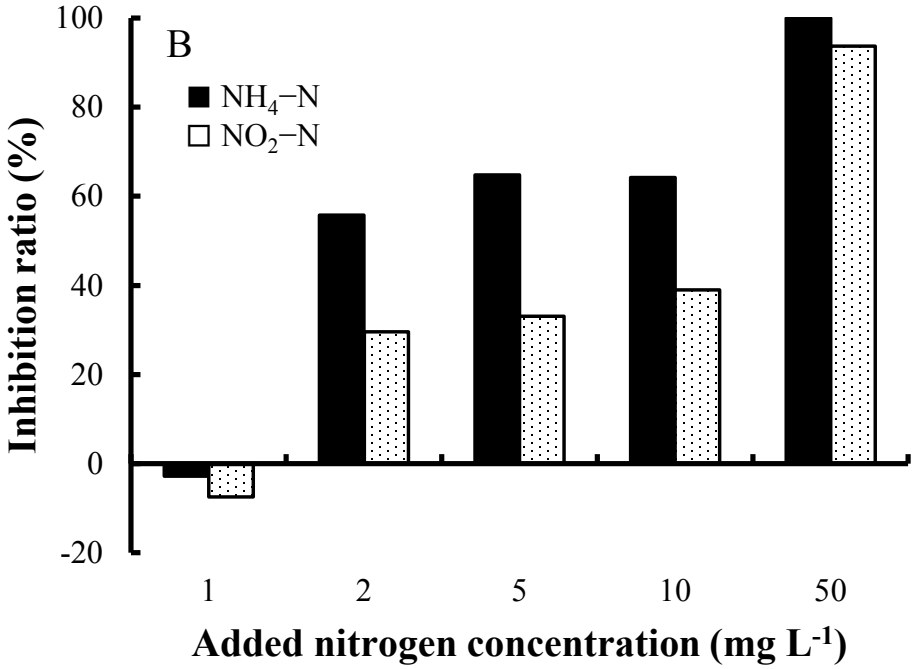

B
NH4–N
NO2–N
Inhibition ratio (%)
100
80
60
40
20
0
-20
1
2
5
10
50
Added nitrogen concentration (mg L-1)

Fig. 7

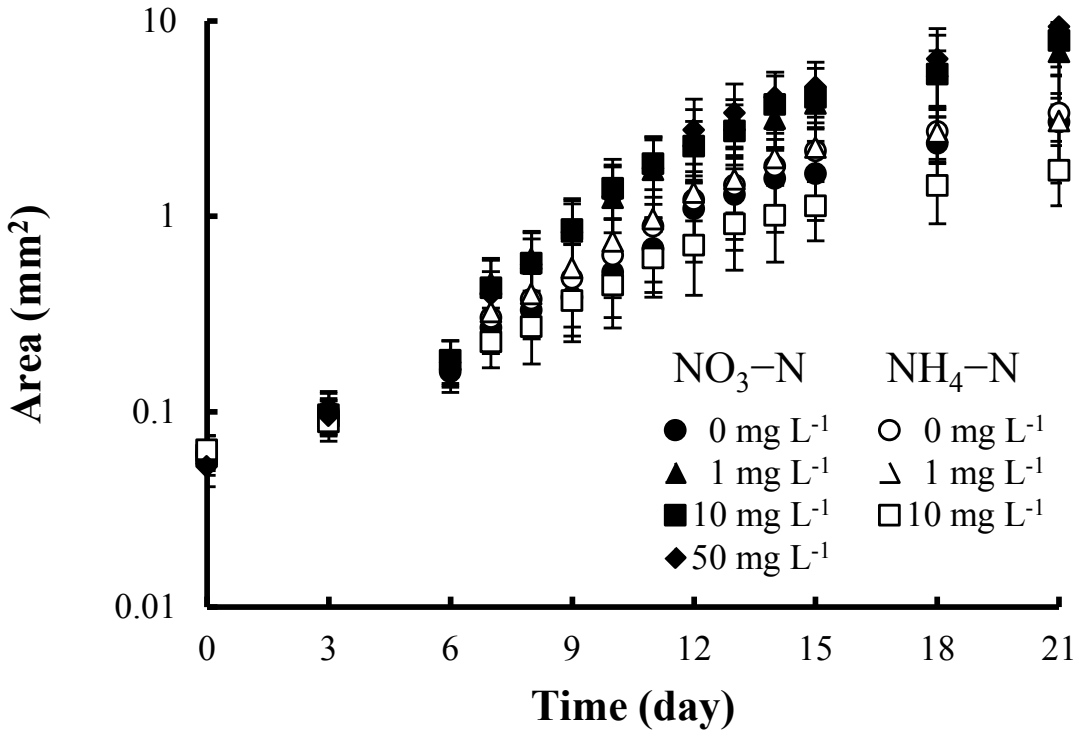

10
1
0.1
0.01
Area (mm²)
0
3
6
9
12
15
18
21
Time (day)
NO3−N
NH4−N
0 mg L-1
1 mg L-1
10 mg L-1
50 mg L-1
0 mg L-1
1 mg L-1
10 mg L-1

Fig. 8

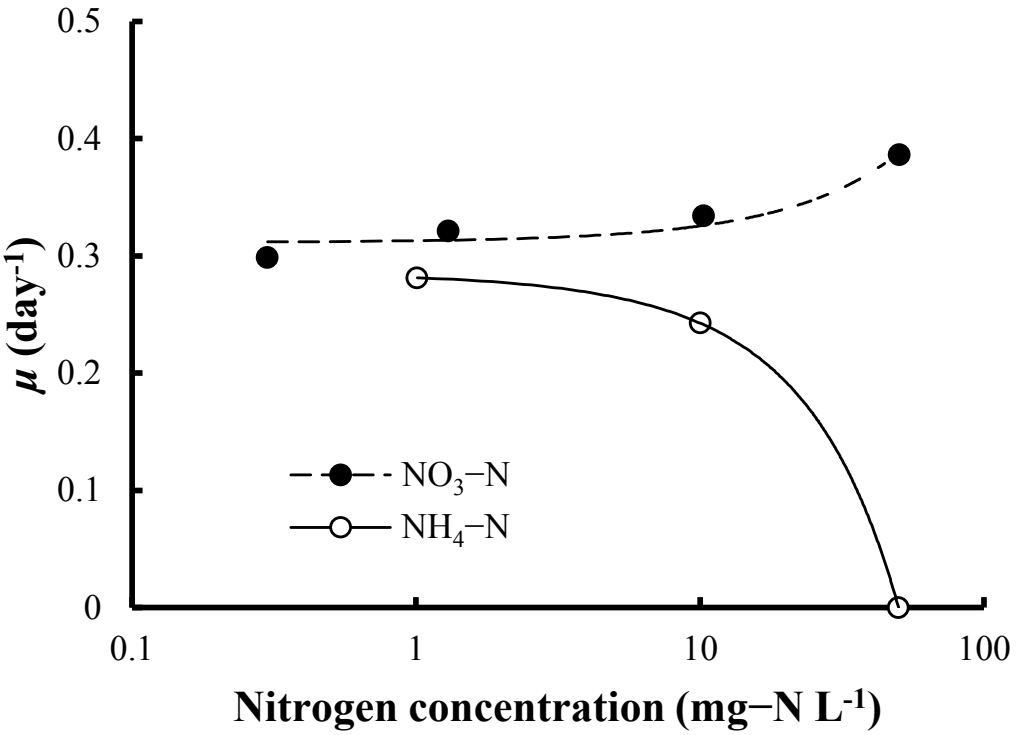

0.5
0.4
0.3
0.2
0.1
0
μ (day-1)
NO3−N
NH4−N
0.1
1
10
100
Nitrogen concentration (mg−N L-1)

Fig. 9