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Selenium requirements for growth of the red tide dinoflagellates *Heterocapsa circularisquama, H. triquetra* and *Karenia mikimotoi*

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Abstract

Trace elements are known to restrict phytoplankton growth in the sea. Selenium is one of important trace elements and known to be an essential element for certain harmful algae. In this study, we investigated selenium requirements for the growth of harmful algal boom forming three dinoflagellates, *Heterocapsa circularisquama, H. triquetra* and *Karenia mikimotoi* about two chemical forms of inorganic selenium (selenite and selenate) using an artificial synthetic medium of remodified ASP7. These three dinoflagellate species required selenium for growth as a form of selenite, but could not grow under a form of selenate. Morphological changes with selenite depletion like selenium requiring centric diatom were not detected so far by microscopic observation.

Key words: Red tide, Dinoflagellate, *Hetrocapsa circularisquama, Heterocapsa triquetra, Karenia mikimotoi*, Selenium requirement, Selenite, Selenate, Growth

Introduction

Since 1960's, occurrences of red tides in eutrophicated coastal areas have been recognized as a serious problem (Okaichi, 1997). Noxious red tides have caused mass mortalities of cultured fish and bivalves so far (Honjo, 2000; Imai et al., 2006). In order to predict and reduce negative impacts of red tides, it is essential to understand the occurrence mechanisms of the red tide events.

As factors for harmful microalgal species to develop blooms to the level of red tides (water discoloration), major environmental factors such as water temperature, salinity, light, macro-nutrients and micro-nutrients are significant. Especially, nutritional factors are crucial. Many laboratory experiments and field studies on macro-nutrients (mainly nitrogen and phosphorus) have been carried out for several decades, but macro-nutrients can not always explain mechanisms of the red tide occurrences (Boyer and Brand, 1998; Sunda, 2006). Planktonic algal species have diverse nutrient requirements, and micro-nutrients for example trace metals (iron, manganese, zinc, molybdenum, selenium, etc.) (Wells et al., 1991; Boyer and Brand, 1998; Sunda, 2006) and B group vitamins (Nishijima and Hata, 1986; Granéli and Risinger, 1994; Uchida et al., 2001) have been considered as supplemental but crucial factors influencing outbreaks of red tides. However, there is a paucity of investigations on trace metals as a factor affecting the growth of red tide algae due to the lack of suitable artificial media for culture experiments allowing the growth of targeted algal species.

Among the trace metal elements, selenium has been regarded as a major limiting factor for the growth of phytoplankton depending on the plankton species in the sea (Harrison et al., 1988). Selenium is an essential part of the enzyme glutathione peroxidases that works detoxification of hydrogenperoxides (Overbaugh and Fall, 1982), and is incorporated into various amino acids and proteins in marine algae (Bottino et al., 1984). Selenium is also indispensable in cell division and antioxidant of membrane system (Doucette et al., 1987).

Selenium has various forms in the coastal water, such as selenite, selenate, elemental selenium and organic selenide (Cutter, 1989; Takayanagi et al., 1989; Cutter and Cutter, 2004), and in particulate matters (Doblin et al., 2006). The selenite is usually available for most part of selenium requiring species of phytoplankton (Harrison et al., 1988; Imai et al., 1996, 2004; Doblin et al., 1999), while the selenate requirement is limited in some species (Wehr and Brown, 1985). Red tide-form-

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ing microalgae such as the dictyochophyte *Pseudochattonella verruculosa* (formerly *Chattonella verruculosa*) (Imai et al., 1996) and the pelagophyte *Aureococcus anophagefferens* (Cosper et al., 1993) are known to require selenium for their growth. Studies on selenium requirements concerning harmful dinoflagellates were done about somewhat limited species such as *Karenia mikimotoi* (formerly *Gymnodinium nagasakiense*) (Ishimaru et al., 1989), *Pyrodinium bahamense* (Usup and Azanza, 1998), *Gymnodinium catenatum, Alexandrium minutum* (Doblin et al., 1999), and *Cochlodinium polykrikoides* (Lee, 2008) so far. However, selenium requirements remain unknown for rather many other dinoflagellate species.

In the present study, we examined the requirements of two chemical forms of inorganic selenium (selenite and selenate) for the growth of the three important red tide dinoflagellates *Heterocapsa circularisquama, H. triquetra* and *K. mikimotoi*.

Materials and methods

Three species of red tide dinoflagellates, *H. circularisquama* (HU9433, isolated from Uranouchi Inlet of Kochi Prefecture), *H. triquetra* (NM-98, from Maizuru Bay of Kyoto Prefecture) and *K. mikimotoi* (G303, from Suo-Nada of the western Seto Inland Sea), were used in this study. These were axenic and were maintained in a seawater-based medium of modified SWM-3 (Chen et al., 1969 ; Imai et al., 1996). The experimental artificial medium (remodified ASP7, Table 1) was prepared based on the artificial synthetic medium of modified ASP7 (Provasoli et al., 1957). To examine the requirements of selenium on the growth of red tide organisms, we prepared three kinds of experimental media based on the remodified ASP7 that contain 2nM of selenate (as Na_2SeO_4), or 2 nM of selenite (as Na_2SeO_3), or no selenium. The ultra pure water was used for the preparation of remodified ASP7. All the chemical reagents for stock solutions were the extra pure reagents. The experimental media were sterilized at 121°C for 15 minutes and stocked in 50 ml glass bottles with screw cap until experiment. All the glassware used in experiments were washed with 3 M HCl and combusted at 450°C for 1 hour.

The culture of each species was maintained in 5-ml modified SWM-3 medium in experimental test tubes (13×120 mm) with polypropylene snapped cap. The maintained culture of each species in the modified SWM-3 at the late exponential growth phase was inoculated into the each experimental remodified ASP7 medium (5 ml) in experimental test tubes (13×120 mm) with 1/20 volume. Incubation was made at a temperature of 20°C with light intensity of ca. 50 mol photons m^{-2} s^{-1} under the 14 hr light : 10 hr dark photo-cycle. Each experiment was conducted with run of 5 replicate test tubes. The growth was monitored every other day at the time after 6 to 8 hr of the beginning of light period using a fluorometer (Turner Design Co. 10-AU005) with *in vivo* chlorophyll *a* fluorescence (Brand et al., 1981 ; Imai et al., 1993). Test tubes were mixed carefully before every measurement. Experimental cultures of each species in the late exponential phase were again inoculated into the new same experimental

Table 1. Composition of the basal medium of remodified ASP7. Treatment of addition of selenite (Na_2SeO_3) or selenate (Na_2SeO_4), or no addition was made to the basal medium for culture experiments.

Substance	Amount	Substance	Amount
NaCl	25 g	Vitamin B_1-HCl	0.5 mg
KCl	0.7 g	Ca-Pantothenate	0.1 mg
$MgSO_4 \cdot 7H_2O$	9 g	Nicotinic acid	0.1 mg
$CaCl_2 \cdot 2H_2O$	1.1 g	p-Aminobenzonic acid	10 μg
$NaNO_3$	0.59 mmol	Biotin	1 μg
$NaH_2PO_4 \cdot 2H_2O$	65 μmol	Inositol	5 mg
$Na_2SiO_3 \cdot 9H_2O$	0.33 mmol	Folic acid	2 μg
Na_2-EDTA	30 μmol	Thymine	3 mg
Fe-EDTA	2 μmol	Vitamin B_{12}	1 μg
Na_2SeO_3	2 nmol	Tris	0.5 g
H_3BO_3	1 mmol	NTA	70 mg
$MnCl_2 \cdot 4H_2O$	35 μmol		
$ZnCl_2$	4 μmol		
$CoCl_2 \cdot 6H_2O$	0.1 μmol	Pure water Up to	1,000 ml
$CuCl_2 \cdot 2H_2O$	1 nmol	pH	7.8

medium with 1/20 volume. This is the second transferred growth experiment. Transfer growth experiments were carried out three to five times, and the selenium requirement was judged according to the growth. In the third transfer experiment of *K. mikimotoi*, 2 nM of selenite was added to three tubes of experimental series of selenate addition and no selenium addition in order to confirm the effectiveness of selenite addition.

Growth experiments were further conducted using sterilized polystyrene test tubes (13×100 mm, gamma radiation treatment; Fisher Brand Co.) under the conditions of no elution of selenium from any glassware during the course of culture experiments. Precultures were made in the remodified ASP7 medium with no addition of selenite for *H. circularisquama* and with addition of 0.05 nM selenite for *H. triquetra*. At the stationary growth phase of preculture, *H. circularisquama* was inoculated into the experimental remodiified ASP7 medium with selenite (1 nM) and no selenium (addition of 1/20 volume and initial cell density of about 2000 cells ml^{-1}). In the case of *H. triquetra*, 1/30 volume was inoculated from preculture at the stationary growth phase to the same experimental media of *H. circularisquama*. The initial cell density was about 3600 cells ml^{-1}. Growth (*in vivo* fluorescence) of both species was monitored using a fluorometer as mentioned above.

Results

Figure 1 depicts the growth of *H. circularisquama* and *H. triquetra* in the modified SWM-3 medium and the remodified ASP7 with selenate, selenite, and no selenium during the transferring experiments. In the modified SWM-3 culture medium, both *H. circularisquama* and *H. triquetra* always showed good growth over the five times transferring experiment. In the remodified ASP7 culture media, the both *Heterocapsa* species exhibited the best growth in the selenite-addition treatment, which was comparable to the result obtained in the SWM-3 medium. On the other hand, in the remodified ASP7 media with selenate and no selenium, they showed approximately half growth as compared to the growth in the selenite added artificial medium. Consequently, the addition of selenite obviously enhanced the growth of *H. circularisquama* and *H. triquetra*.

The growth patterns of *K. mikimoti* were also the same one as the former two *Heterocapsa* species (Fig. 2). However, more amplified effects of selenite deficiency was observed in this species than two *Heterocapsa* species. After 46 days of the start of incubation experiment (the 10th day after the third transfer), addition of 2 nM selenite was made into three tubes out of

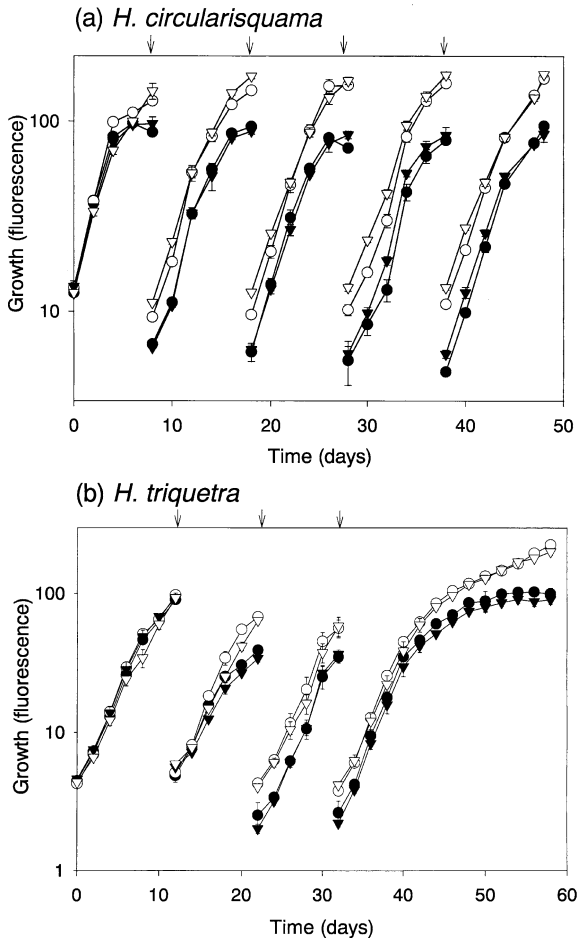

Fig. 1. Growth of (a) *Heterocapsa circularisquama* and (b) *H. triquetra* in the modified SWM-3 medium (based on natural seawater) (▽), remodiified ASP7 (chemically defined medium based on artificial seawater) with selenate (+Se VI) (●), with selenite (+Se IV) (○), and without selenium (−Se) (▼). Arrows indicate the timing of transfers. Error bars; SD ($n=5$)

five replicates of the selenate and no selenium treatments in order to know the positive effects of selenite for the growth of *K. mikimotoi*. The growth in selenite-supplemented tubes then evidently restored as is the case of original selenite treatment. Consequently, the effect of selenite on the growth enhancement of *K. mikimotoi* was clearly demonstrated.

Results of growth experiments using polystyrene tubes with no elution of Se during the course of culture experiments are shown in Fig. 3 for *H. circularisquama* (a) and *H. triquetra* (b). The growth of *H. circularisquama* was not observed for five days of incubation and then began dying, undoubtedly demonstrating the requirement of selenite for the growth and survival. The result for *H. triquetra* also revealed the markedly suppressed growth as compared with selenite addition (1 nM). Slight growth, much lower than that in glass

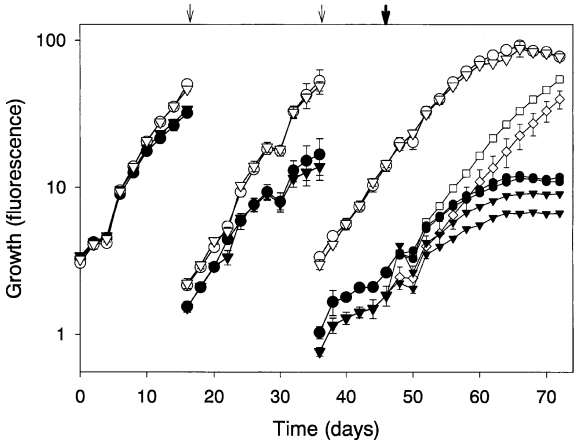

Fig. 2. Growth of *Karenia mikimotoi* in the modified SWM-3(▽), remodified ASP7 with selenate (+Se VI) (●), with selenite (+Se IV) (○), and without selenium (−Se) (▼). In the last transfer series, 2 nM of selenite (final concentration) was added to the tubes of +SeVI (□) and −Se (◇) in 3 subcultures. Narrow arrows indicate the timing of transfer, and the bold arrow indicates the addition of 2nM of selenite. Error bars; SD ($n=5$ before day 46, $n=3$ thereafter)

test tubes, might be due to some carrying-over of Se from preculture to experimental culture.

Discussion

In this study, it was revealed that selenite gave positive effects on the growth of the three red tide dinoflagellates *H. circularisquama*, *H. triquetra* and *K. mikimoti*. Previously, *K. mikimoti* was reported to require selenium in the form of selenite for the growth (Ishimaru et al., 1989). The present study demonstrated that *K. mikimoti* could not utilize selenate for the growth. Among the marine phytoplankton including harmful species, there are rather many species requiring selenium for growth (Harrison et al., 1988; Imai et al., 1996; Usup and Azanza, 1998; Doblin, et al., 1999; Lee, 2008). The selenate utilization in the phytoplankton is rare and only known in one freshwater species *Chrysochromulina breviturrita* belonging to Haptophyceae (Wehr and Brown, 1985).

The general chemical form of selenium required by natural assemblages of phytoplankton appears to be selenite (Wrench and Measures, 1982). In the seawater, selenium exists in various chemical forms and the total dissolved selenium concentrations in the coastal water are commonly ranging from 0.6 to 4.5 nM (Sugimura et al., 1976; Cutter, 1989). However, dissolved selenite concentration is generally less than 1 nM (Koike et al., 1993), and in some cases its concentration decreases down to 0.02 nM (Wrench and Measures, 1982). Seasonal changes of dissolved selenite concentrations in the seawater are commonly affected by the consumption by phytoplankton (Wrench and Measures, 1982).

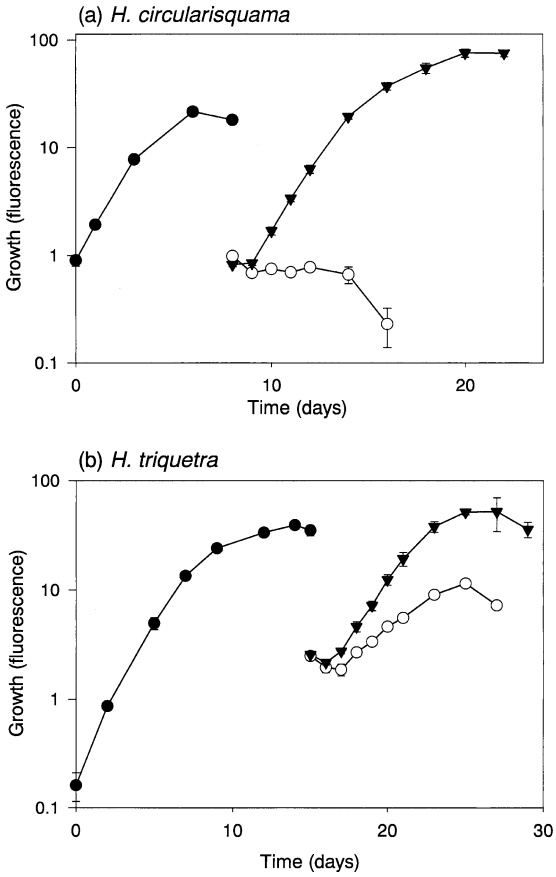

Fig. 3. Growth of (a) *Heterocapsa circularisquama* and (b) *H. triquetra* in remodiified ASP7 with selenite (+1nM Se IV: ▼) and without selenium (−Se: ○) using polystyrene tubes with no elution of Se. Precultures (●) were made in the remodified ASP7 medium with no addition of selenite for *H. circularisquama* and with addition of 0.05 nM selenite for *H. triquetra*. Error bars; SD ($n=5$)

In the media treated with selenate and no selenium in glass test tubes, the cell growth was observed to some extent in all three dinoflagellates (Figs. 1 and 2). These results might be attributed to some selenium contamination during the course of experiment. All glassware for experiments was washed carefully to prevent any contaminations. However, it is considered that selenium contaminations occurred through the elution from the glassware for storage of the culture medium and from the glass test tubes used for incubation experiments. The results of Fig. 3 obtained with the experiments using polystyrene tubes indisputably proved the selenium requirements for growth of both *Heterocapsa* species. And elution of Se from glass test tubes was also strongly suggested by the results of Fig. 3. The experiments should be performed by using of

polystyrene tubes or so.

No morphological changes were noticed for the cells of three dinoflagellates in this study as a result of selenium deficiency. In the centric diatom *Thalassiosira pseudonana*, selenium was required for the growth (Price et al., 1987) and selenium deficiency induced cell elongation for the obstruction of mitotic and cytokinetic component in the cell division (Doucette et al., 1987). The decrease of chlorophyll quota per cell by the selenium limitation was reported in a study of the toxic dinoflagellate *Gymnodinium catenatum* (Doblin et al., 1999). Accordingly, there is a possibility that the similar decrease of pigment quota might occur in these three dinoflagellates, although the photosynthetic pigment quota per cell was not measured in this study. Further comparative studies are necessary to examine chlorophyll *a* quota under the conditions of selenium limitation and repletion.

To reveal the role of selenium in the dynamics of the red tide phytoplankton in coastal environments, kinetic studies would be basically needed. Selenium requirements for the growth about some red tide phytoplankton species have been reported so far. Concerning the relationship between selenium concentrations in the coastal water and the outbreaks of red tides, only a study was carried out in Tanabe Bay of Wakayama Prefecture, Japan, about *K. mikimotoi* (Koike et al., 1993) to our knowledge. However, the frequency of measurements was not enough to understand the role of selenium for red tide occurrences. More detailed and frequent observations would be essential in the time course of bloom occurrences.

Since *H. circularisquama* has been recurrently causing severe fishery damages to aquacultures of bivalves in the coasts of western Japan (Matsuyama et al., 2001) and most recently in Kamoko of Sadogashima Island in Sea of Japan, Niigata Prefecture, it is important to investigate the effects of selenium as a regulating factor in the occurrences of red tides of this species as well as that of macro-nutrients through the measurements of algal growth potentials (AGP) in the seawater of some coastal areas of bivalve aquacultures.

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