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Short Communication

The Requirement of Selenium for the Growth of Marine Coccolithophorids, *Emiliania huxleyi, Gephyrocapsa oceanica* and *Helladosphaera* sp. (Prymnesiophyceae)

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Marine coccolithophorids, *Emiliania huxleyi, Gephyrocapsa oceanica* and *Helladosphaera* sp. were found to require selenium for their growth. The optimum concentrations were 1–10 nM for SeO_2 and SeO_3^{2-} and above 1 μM for SeO_4^{2-}. During the depletion of selenium algal growth was strongly suppressed accompanied with the decrease in net photosynthesis.

Key words: Artificial seawater — Coccolithophorids — *Emiliania huxleyi* — *Gephyrocapsa oceanica* — *Helladosphaera* — Selenium-depletion — Selenium-requirement.

A unicellular calcifying alga, coccolithophorid, *Emiliania huxleyi* (Prymnesiophyceae) fixes inorganic carbon mainly by two reactions, namely photosynthesis and intracellular calcification. The calcification reaction produces calcium carbonate crystals known as coccoliths, which are excreted and placed to cover the cell surface. The alga is known to form huge blooms in the ocean and is consequently expected to affect the air-sea interchange of CO_2. The growth of the alga and the production of coccoliths are affected by the status of nutrients, such as nitrogen and phosphorus, in the laboratory culture (Paasche 1998). In addition to the major nutrients, microelements were also shown to affect the growth of coccolithophorids. For example, the enrichment of iron to the ocean increased the population of phytoplankton including prymnesiophytes and enhanced the fixation of the atmospheric CO_2 in the field experiments of IronEX II (Coale et al. 1996).

In many studies with *Emiliania huxleyi*, many kinds of artificial media have been used for its laboratory culture. Those culture media, for example, were Droop's artificial seawater 'S32' (Paasche 1964), seawater enriched with synthetic f/2 medium of Guillard (1975), fresh Erd-Schreiber's seawater (Rowson et al. 1986), ASP-2 medium of Provasoli et al. (1957) and K-medium of Keller et al. (1987). The present authors have used natural seawater enriched with ESM (Okaichi et al. 1982) for the laboratory culture of several species of coccolithophorids (Sekino and Shiraiwa 1994, Shiraiwa et al. 1994). It, however, is sometimes inconvenient for researchers who live in inland area to obtain natural seawater and is difficult to get good quality of soil extracts with the same compositions. Therefore, it is necessary to use a complete synthetic medium so that uncertain element affecting algal growth is not contained. Physiological and biochemical studies in coccolithophorids have been strongly limited to one species, *Emiliania huxleyi*. This may be due to the difficulties of the isolation of various species of cells from the ocean and the subsequent laboratory culture of coccolithophorids, except *Emiliania*. In this study we therefore tried to find adequate culture media for the laboratory culture of coccolithophorids. As a consequence, we found that the addition of selenium is necessary for the culture of coccolithophorids and the soil extract in ESM can be replaced with selenium.

The marine coccolithophorids, *Emiliania huxleyi* collected in the South Pacific Ocean in 1990, *Gephyrocapsa oceanica* and *Helladosphaera* sp. collected in the Aomori-bay at northern Japan in 1990 were generous gifts of Drs. I. Inouye and M. Kawachi of University of Tsukuba, Japan. For the stock culture, those algae were grown in natural seawater medium enriched with ESM that contains soil extracts (Okaichi et al. 1982) at 20°C under weak light (24 $\mu mol\ m^{-2}\ s^{-1}$) with the regime of 16-h light and 8-h darkness. Natural seawater and soil were collected from the Japan Sea at Kakuda coast and the F1-layer in beech woods at Tainai, Kurokawa, Niigata, Japan. Soil extracts were made by boiling of 1 kg dried soil with 1 liter of deionized and distilled water in an autoclave and then by filtering the extracts through Toyo No. 2 filter. The ESM enrichments contained 120 mg $NaNO_3$, 5 mg K_2HPO_4, 0.259 mg Fe-EDTA, 0.332 mg Mn-EDTA, 0.1 mg thiamin-HCl, 0.001 mg biotin, 0.001 mg vitamin B12 and 1 g Tris in a liter medium and also 10 ml of the soil extracts were added separately. The other microenrichments used in this study were Keller's K-medium and Guillard's f/2-medium

Abbreviations: G, the doubling time; k_g, the growth constant; PCV, packed cell volume.

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(Sigma, St. Louis, U.S.A.) and Provasoli's enriched seawater (PES) (Starr and Zeikus 1993).

Cells were harvested by centrifugation from the stock culture and the resultant pellet was suspended in the fresh medium of artificial seawater, Marine Art SF (Senju Pharmaceutical Co., Osaka, Japan) of which the pH was adjusted with 8.3 mM Tris to 8.2. After incubating cells for 3 d for starvation of micronutrients, small amount of algal cells were inoculated into fresh medium in a L-shape tube (TAITEC, Tokyo, Japan) to attain a cell density of approximately 0.05 ml packed cell volume (PCV) per liter. Cultures in experiments were performed under the regime of 16-h light and 8-h darkness. The temperature and the light intensity were 25°C and 70–100 μmol m^{-2} s^{-1}, respectively. The suspensions were shaken by hand once a day on an occasion of the measurement of optical density. The artificial seawater used in the preculture for starvation was Marine Art SF that contains 21.9 g NaCl, 10.0 g $MgCl_2 \cdot 6H_2O$, 1.4 g $CaCl_2 \cdot 6H_2O$, 3.7 g Na_2SO_4, 0.63 g KCl, 0.18 g $NaHCO_3$, 90 mg KBr, 36 mg $Na_2B_4O_7 \cdot 10H_2O$, 13 mg $SrCl_2$, 3 mg NaF, 1 mg LiCl, 70 μg KI, 0.8 μg $MnCl_2 \cdot 4H_2O$, 0.2 μg $CoCl_2 \cdot 6H_2O$, 8 μg $AlCl_3 \cdot 6H_2O$, 5 μg $FeCl_3 \cdot 6H_2O$, 0.2 μg $Na_2WO_4 \cdot 2H_2O$ and 20 μg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in a liter.

Change in cell concentration during growth was monitored by measuring optical density at 750 nm (OD_{750}) using a spectrophotometer (Spectronic 20A, Shimadzu-Bosch & Romb, Kyoto, Japan) and the values were transformed to PCVs using a calibration curve. The growth constant (k_g · day^{-1}) and the doubling time (day) were calculated at the logarithmic growth phase using the equations of 24 $(t_2 - t_1)^{-1} \log (N_2 \cdot N_1^{-1})$ and $(\log 2)\ k_g^{-1}$, respectively, where t_1 and t_2 represent culture times (h) at the beginning and the end of the logarithmic phase, respectively, and N_1 and N_2 represent PCVs at time t_1 and t_2, respectively. The growth rate at the linear growth phase was expressed as (ml PCV) $liter^{-1}$ day^{-1}. The number of cells was determined under a microscope using a Thoma's hemocytometer.

To determine the activity of photosynthesis and respiration, algal cells were washed twice with the artificial seawater that contained 50 mM Tricine-NaOH buffer (pH 8.0) and then suspended in the seawater at a cell density of 1 ml PCV $liter^{-1}$. Changes in the concentration of O_2 were measured by a Clark-type O_2 electrode (Rank Bros., London, England). The concentration of total dissolved inorganic carbons (DIC), temperature and light intensity were 20 mM, 25°C and 350 μmol m^{-2} s^{-1}, respectively. Concentration of chlorophyll was determined according to Jeffrey and Humphrey (1975).

Emiliania huxleyi hardly grew in natural seawater and artificial seawater, Marine Art SF, without addition of ESM (Fig. 1). As the increase in turbidity of algal suspension was obtained in light, but hardly in darkness, growth curve was graphed with only the illumination period instead of total time of culture on the abscissa in Fig. 1. The high rate of algal growth was observed only when natural seawater or Marine Art SF was enriched with ESM. However, soil extracts were not required when natural seawater was used as the basal medium. The algal growth was dependent on the amount of soil extracts below a half concentration of control and was not affected by the removal of vitamins. On the other hand, the addition of five times higher amount of ESM suppressed algal growth to 60% of control (data not shown).

Table 1 Relationship between the composition of microelements in various media and the growth of a coccolithophorid, *Emiliania huxleyi*

	Microenrichments			
	ESM	K	f/2	PES
Microelement (nM)				
Mn	750	900	910	810
Fe	620	10,000	15,000	1,200
Co	0	50	42	17
Cu	0	10	40	0
Zn	0	80	77	150
Se	0	10	0	0
Mo	0	30	25	0
Soil extracts	Added	none	none	none
Growth parameter				
Growth constant (k_g day^{-1})	0.54	0.54	0.18	0.19
G (day)	0.55	0.55	1.62	1.58
Linear-growth rate [(ml PCV) $liter^{-1}$ day^{-1}]	0.939	0.559	0.016	0.010

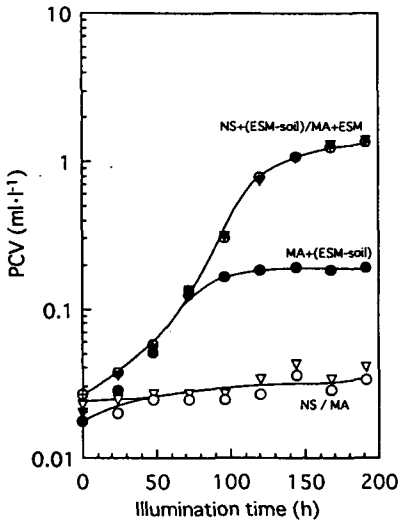

Fig. 1 Effect of the removal of soil extracts from various culture media on the growth of *Emiliania huxleyi*. Cells had been grown in the ESM-deficient media for 3 d and then inoculated into respective media. NS, natural seawater; MA, Marine Art SF; ESM and (ESM−soil), ESM with and without soil extracts, respectively.

The high rate of growth of *Emiliania huxleyi* was also observed in the artificial seawater of Marine Art SF that was enriched with either ESM or K-medium, but not with f/2 or PES (Table 1). As either selenium or soil extracts were contained in the effective media, selenium was expected to be highly required for the growth of *Emiliania huxleyi*. To confirm the results, cells grown in the complete, artificial seawater-ESM-medium were transferred to the medium without soil extracts in the presence or absence of 10 nM sodium selenite (Fig. 2). In the first culture algal growth ceased after 90 h in the medium without selenite, whereas the growth continued further in control (Fig. 2A). When a tiny portion of the Se-deficient culture was inoculated into the fresh medium, *Emiliania huxleyi* did not grow at all in the absence of selenite, but showed a normal growth in the presence of 10 nM sodium selenite (Fig. 2F). These results clearly proved that soil extracts can be replaced by selenium for the growth of coccolithophorids in the artificial seawater with ESM.

The concentration of chlorophyll and the rate of net O_2 evolution in light had been maintained nearly constant in the first culture, irrespective of the presence or absence of selenium in the medium (Fig. 2B, C). On the other hand, both gross photosynthesis and dark respiration were greatly increased in the selenium-depleted culture. In the second culture, those increased rates were gradually diminished in the presence of selenium and consequently came back to the previous levels of the cells grown in the selenium-sufficient medium (Fig. 2I, J). This also accompanied with the decrease in the activity of gross photosynthesis.

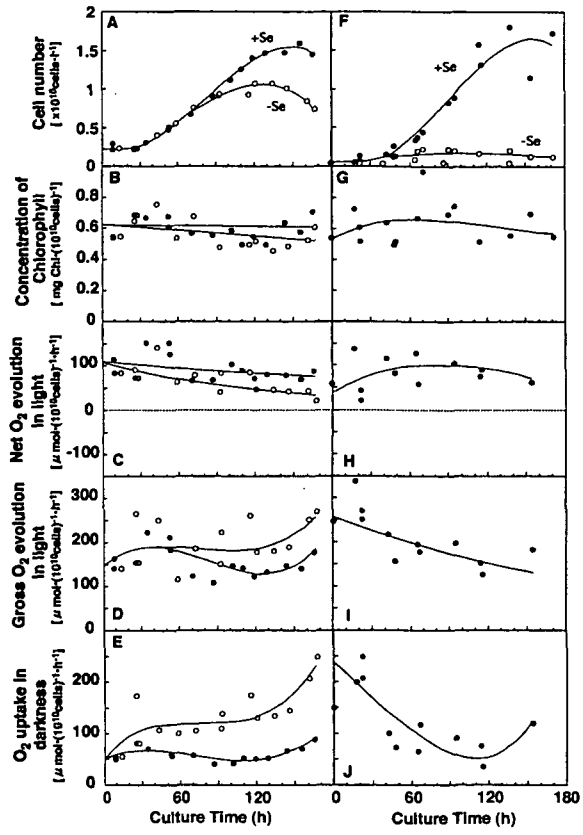

Fig. 2 Changes in cell number (A and F), the concentration of chlorophyll (B and G), net photosynthesis (C and H), gross photosynthesis (D and I) and dark respiration (E and J) during the first (A–E) and the second (F–J) cultures of *Emiliania huxleyi*. Cells grown for 170 h in the selenium-deficient culture in the first culture were inoculated into the second culture. ● and ○, in the presence or absence of 10 nM selenite, respectively. Lines were drawn according to a curve-fit functions of Deltagraph, ver. 4.0 (Deltapoint Inc., Monterey, CA, U.S.A.).

Three compounds of selenium (selenium dioxide, sodium selenite and sodium selenate) were tested at various concentrations for the growth of three kinds of coccolithophorids, *Emiliania huxleyi, Gephyrocapsa oceanica* and *Helladosphaera* sp. (Fig. 3). Both selenium dioxide and sodium selenite were effective widely between 1 nM and 1 μM for *Emiliania huxleyi*, but those were rather toxic above 1 μM. However, selenate showed no positive effect at such low concentrations but was highly effective above 1 μM. Similar results were also obtained in *Gephyrocapsa oceanica* and *Helladosphaera* sp., although the latter was more sensitive to sodium selenite and selenium dioxide above 10 nM (Fig. 3B, C). Interference with the sulfur-metabolism may be one of the possibilities for this toxicity in coccolithophorids, as reported in *Cricosphaera* (Boisson et al. 1995).

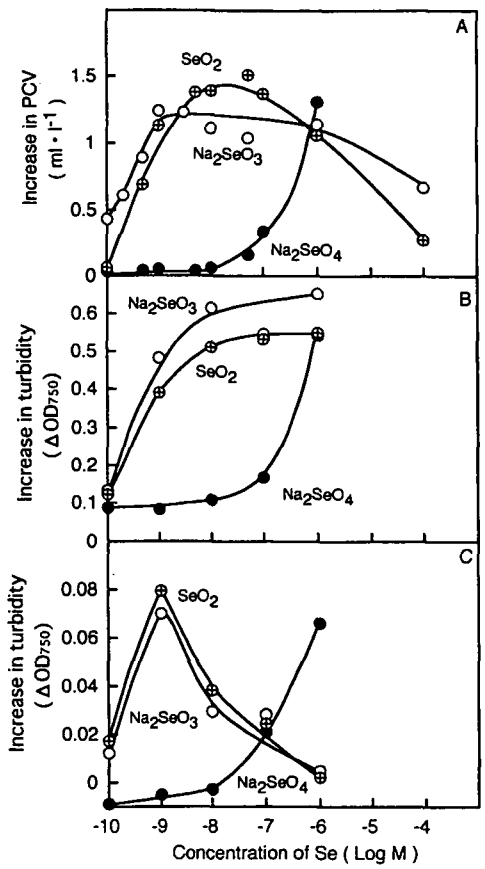

Fig. 3 Relationship between the growth of *Emiliania huxleyi* (A), *Gephyrocapsa oceanica* (B) and *Helladosphaera* sp. (C) and the concentrations of various chemical forms of selenium during culture until the stationary growth phase. ●, sodium selenate; ○, sodium selenite; ⊕, selenium dioxide.

Selenium is a trace metal that exists naturally in both seawater and fresh water and is artificially released from copper refineries. Typical open oceanic concentration of selenium was estimated to be 0.1 μg liter^{-1} (1.27 nM) in the forms of selenite and selenate ions (Raymont 1980). This concentration may support the algal growth (Fig. 3). This is the reason why the addition of soil extracts or selenium was not required in natural seawater (Fig. 1). Other micronutrients contained in ESM such as iron and manganese are strongly limited in oceanic water, since those oceanic concentrations are 2 and 0.02–0.1 μg liter^{-1}, respectively (Raymont 1980). Actually, the limitation of iron in oceanic water was proven by the experiments of the fertilization of iron (IronEXII) which showed the enhancement of algal population after fertilization (Coale et al. 1996).

Selenium is known to be involved in selenomethionine and selenocysteine and also to be essential for selenoenzymes such as glutathione peroxidase (Boisson et al. 1995). The requirement or the function of selenium has been reported in other microalgae such as a diatom *Thalassiosira pseudonana* (Price et al. 1987, Price and Harrison 1988), and haptophyte algae *Cricosphaera elongata* (Boisson et al. 1995) and *Chrysochromulina breviturrita* (Wehr and Brown 1985). In a unicellular green alga, *Chlamydomonas reinhardtii*, selenium is known to be an inducer of glutathione peroxidase. The activity of the enzyme attained at 360 μmol NADPH oxidized (mg Chl)$^{-1}$ h^{-1}, and the enzyme functions for the reduction of the harmful effects by hydrogen peroxide under low CO_2 and strong light conditions (Yokota et al. 1988, Shigeoka et al. 1991). *Emiliania huxleyi*, however, was shown to possess only very little activity of glutathione peroxidase [19 μmol NADPH oxidized (mg Chl)$^{-1}$ h^{-1}] even when the alga was grown in a selenium-sufficient medium (data not shown). In contrast to *Chlamydomonas reinhardtii*, coccolithophorids could not grow at all after the depletion of selenium (Fig. 2). Physiological importance of selenium and the functions of selenoenzymes in coccolithophorids are remained for further investigation.

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