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Development of a chemically defined artificial medium for marine red tide-causing raphidophycean flagellates

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Abstract: Most species of marine red tide raphidophycean flagellates have shown poor or no growth in chemically defined artificial media such as ASP_7. We investigated the development of a new artificial medium for the red tide raphidophycean flagellates *Chattonella antiqua, C. marina, C. ovata, C. verruculosa* and *Fibrocapsa japonica*. First, preliminary enrichment experiments were conducted on 10 elements which are common in natural seawater but are not contained in the artificial basal medium of modified ASP_7. Those were tested at the concentrations present in natural seawater. The preliminary experiments revealed that molybdenum and iodine affect the growth of these harmful flagellates. Detailed enrichment experiments for molybdenum and iodine were then carried out, and 2 patterns were basically confirmed for the requirements of molybdenum and iodine. *C. antiqua* required molybdenum for growth, and the best growth was achieved by the simultaneous addition of molybdenum and iodine to the basal medium. *C. verruculosa* grew excellently by the sole enrichment of molybdenum in the basal medium. In the cases of *C. marina, C. ovata* and *F. japonica*, the sole enrichment of iodine in the basal medium gave comparably positive effects on growth compared to the simultaneous addition of molybdenum and iodine. In conclusion, a chemically-defined artificial medium (IHN-medium) was developed for these 5 species of raphidophytes by the simultaneous enrichment of molybdenum and iodine to the basal medium. This IHN-medium has also allowed the growth of a broad spectrum of other phytoplankton belonging to such groups as Dinophyta, Heterokontophyta (Bacillariophyceae and Raphidophytceae), Cryptophyta, Chlorophyta, Euglenophyta and Haptophyta.

Key words: *Chattonella, Fibrocapsa*, artificial media, molybdenum, iodine

Introduction

There are many phytoplankton species (184–268) that cause red tides in eutrophicated coastal waters around the world (Sournia 1995). Red tides of some microalgal species especially belonging to the Raphidophyceae and Dinophyceae, have frequently caused damages to fisheries through mass mortalities of cultured fish and bivalves (Okaichi 1997; Imai et al. 1998; Fukuyo et al. 2002). Consequently, to understand the mechanisms leading to red tide occurrences is important for establishing countermeasures to reduce the negative impacts of these red tides.

Red tides are generally the result of massive growth of phytoplankton to unusually high levels of cell densities with accompanying water discoloration. Accordingly, the nutritional conditions of the seawater supporting the growth of these phytoplankton play an important role in red tide occurrences. It is a fundamentally important issue when clarifying the outbreak mechanisms of red tides, to understand the nutritional physiology such as the nutritional requirements of red tide-causing microalgae and their growth kinetics (Iwasaki 1973, 1979; Nakamura & Watanabe 1983a, b). Such examinations are commonly designed and carried out through culture experiments in the laboratory.

Most species of marine phytoplankton in culture collections have been maintained in natural seawater enriched with major inorganic nutrients, vitamins and trace metals. However, variations in the quality of the seawater used as the base of the culture media pose problems in maintaining

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certain delicate species for longer periods (Harrison et al. 1980). On the other hand, chemically-defined artificial media are an essential base for nutritional experiments on marine phytoplankton, because it is necessary to control the composition and concentrations of nutrients such as major inorganic nutrients (N, P), vitamins, other organic matters, trace metals and chelators. However, culturable species of phytoplankton are generally fewer in chemically-defined artificial media than in seawater-based media.

Most species of fish-killing marine raphidophycean flagellates have been unable to be grown in chemically-defined artificial media, except for *Chattonella antiqua* (Hada) Ono (strain Ho-1) in the H-medium to some extent (Nakamura & Watanabe 1983a, b, c). Therefore, there is a paucity of information on the nutritional physiology of raphidophycean flagellates. To obtain information concerning the nutritional physiology of harmful raphidophycean species is an important and urgent assignment. In the present study, we investigated the development of a chemically-defined artificial medium (IHN-medium) allowing good growth of the marine red tide-causing raphidophycean flagellates, *C. antiqua*, *C. marina* (Subrahmanyan) Hara et Chihara, *C. ovata* Hara et Chihara, *C. verruculosa* Hara et Chihara and *Fibrocapsa japonica* Toriumi et Takano. Furthermore, we also report good growth of other marine phytoplankton species in the new artificial medium developed in this study.

Materials and Methods

Organisms

Five species of Raphidophyceae, *C. antiqua* (NIES-1), *C. marina* (MS-3-P), *C. ovata* (HA-93), *C. verruculosa* (ver-3-P) and *F. japonica* (HA-84) were used in this study. These are all axenic clone cultures. The sterility check was carried out with fluorescence staining using 4'6-diamidino-2-phenylindole (DAPI) and epifluorescence microscopy (Imai 1987). These clones have been maintained in the modified SWM-3 culture medium, an enriched seawater medium after a modification of the original recipe of Chen et al. (1969) by excluding soil and liver extracts (Itoh & Imai 1987) and supplementing Na_2SeO_3 (Imai et al. 1996).

Preliminary experiments

These raphidophycean species grow well in seawater-based media such as the modified SWM-3 medium, but they have shown poor growth or no growth in artificial media such as the modified ASP_7 (Table 1) which was modified by substituting $NaH_2PO_4 \cdot 2H_2O$ for K_2 glycerophosphate and Fe-EDTA for $FeCl_3$, and supplementing Na_2SeO_3 to the original recipe (Pintner & Provasoli 1958). Based on this observation, it was considered that some essential elements are lacking in the artificial media.

First, we selected 10 elements that are common in natural seawater but are not contained in the artificial basal medium of modified ASP_7 (Table 1) according to the elemental composition of seawater (Goldberg 1965). The list of the 10 elements selected and their concentrations in natural seawater are shown in Table 2.

Stock cultures of raphidophyte species grown in the modified SWM-3 medium were diluted to 1/50 with the artificial basal medium, and 5-ml diluted cultures were inoculated into sterile screw-capped test tubes (15-mm diameter, 150-mm length). Glass-distilled demineralized water

Table 1. Composition of the artificial basal medium (modified ASP_7).

Substance		Substance	
NaCl	0.43 M	P-I metals (in 10 ml)	
KCl	9.4 mM	H_3BO_3	1 mmol
$MgSO_4 \cdot 7H_2O$	37 mM	$MnCl_2 \cdot 4H_2O$	3.5×10^{-2} mmol
$CaCl_2 \cdot 2H_2O$	7.5 mM	$ZnCl_2$	4.0×10^{-3} mmol
$NaNO_3$	0.59 mM	$CoCl_2 \cdot 6H_2O$	1.0×10^{-4} mmol
$NaH_2PO_4 \cdot 2H_2O$	65 μM	$CuCl_2 \cdot 2H_2O$	1.0×10^{-6} mmol
$Na_2SiO_3 \cdot 9H_2O$	0.33 mM		
Na_2-EDTA	30 μM	S-3 vitamins (in 2 ml)	
Fe-EDTA	2 μM	B_1-HCl	0.5 mg
Na_2SeO_3	2 nM	Ca-Pantothenate	0.1 mg
Tris	4.1 mM	Nicotinic acid	0.1 mg
NTA	0.37 mM	*p*-Aminobenzoic acid	10 μg
		Biotin	1 μg
P-I metals	10 ml	Inositol	5 mg
S-3 vitamins	2 ml	Folic acid	2 μg
		Thymine	3 mg
Distilled and purified water up to	1000 ml	Vitamin B_{12}	1 μg
pH	7.8		

Table 2. Abundance of selected elements in seawater and those concentrations employed in the preliminary spike experiments for growth of raphidophycean flagellates. Concentrations of elements in seawater refer to Goldberg (1965).

Element	Symbol	Concentration in seawater (nM)	Compound	Concentration (nM) for experiment
Lithium	Li	26100	LiCl	26000
Fluorine	F	68400	NH_4F	68000
Aluminium	Al	74	$AlCl_3 \cdot 6H_2O$	74
Vanadium	V	49	VCl_3	49
Nickel	Ni	29	$NiCl_2 \cdot 6H_2O$	29
Bromine	Br	837500	KBr	837000
Rubidium	Rb	1400	RbCl	1400
Strontium	Sr	91300	$SrCl_2$	91000
Molybdenum	Mo	104	$Na_2MoO_4 \cdot 2H_2O$	100
Iodine	I	472	KI	472

Table 3. Results of the preliminary spike experiments for growth of raphidophycean flagellates. No symbols indicate no tests.

Species	10E*	6E**	4E***	I+F	Mo+Ni	Mo+I	I	F	Mo	Ni
C. verruculosa	++	++	++	+	++		−	−	++	−
C. antiqua						++				
C. marina			++				+			
C. ovata						++	+			
F. japonica			++		−		++			

++: clear growth enhancement (comparable growth to that in SWM3)
+: slight growth enhancement (recognizable growth)
−: no growth enhancement
* 10E: Li, F, Al, V, Ni, Br, Rb, Sr, Mo, I
** 6E: F, Al, V, Ni, Mo, I
*** 4E: F, Ni, Mo, I

(Milli-Q system) was employed for the preparation of the artificial media. All reagents were of the highest purity available. The elements for the examination were added singly or in combination to these test tubes at the concentrations reported in seawater (Goldberg 1965), which are presented in Table 2. The test tube cultures were incubated at a temperature of 20°C with a light intensity of ca 100 μmol photons $m^{-2} s^{-1}$ under a photo-cycle of 14 h light and 10 h darkness. The growth yield in each test tube was compared with that in the control tube (no addition of the elements in Table 2) by observation with the naked eye. Concerning the elements that gave positive growth effects, transfer growth experiments were carried out in order to characterize the positive effects of these elements.

Transfer growth experiments

According to the results of the preliminary experiments, the addition of molybdenum (Na_2MoO_4) and iodine (KI) showed positive effects on the growth of raphidophytes in the artificial seawater medium (Table 3). Consequently 4 kinds of artificial media were prepared by the addition of molybdenum and/or iodine to the artificial basal medium, i.e. basal medium+Mo, basal medium+I, basal medium+Mo+I, and basal medium (no addition of molybdenum or iodine). These media were sterilized using 60-ml polycarbonate square-shaped bottles (Nalgene Co.) by autoclaving (121°C, 15 min). For the transfer growth experiments, sterile polystyrene test tubes (13-mm diameter, 100-mm length) with screw caps were used (Fisher Brand Co.).

The stock culture of each raphidophyte species grown in the modified SWM-3 medium was transferred at 1/50 quantities into the each of the 4 artificial media supplemented with molybdenum and/or iodine as described above. These first transfer cultures were dispensed into the sterile polystyrene test tubes in triplicate for each medium and species. The total volume in each test tube was 5 ml. The cultures were incubated under the same conditions described above. The growth in the tubes was monitored by *in vivo* fluorescence (Brand & Guillard 1981; Yamaguchi et al. 1991; Imai et al. 1993) using a fluorometer (Turner Designs Co., 10-AU005). In the late logarithmic growth phase, the next transfer was carried out for each experimental medium and species with the inoculation at 1/50 quantities. Based on the results of 3 or 4 transfer growth experiments, the stimulatory effects of molybdenum and iodine were evaluated on the growth of *C. antiqua*, *C. marina*, *C. ovata*, *C. verruculosa* and *F. japonica*.

Results

The results of the preliminary experiment showed positive effects of the addition of molybdenum (Na_2MoO_4) and/or iodine (KI) on the growth of the 5 species of raphidophytes in the artificial seawater medium (Table 3). Transfer experiments were consequently carried out and Fig. 1 illustrates the effects on growth in *C. antiqua*, *C. marina* and *C. ovata*. In *C. antiqua*, the growth was limited in the basal medium (no addition of molybdenum and iodine) and basal medium+I at the 2nd transfer and thereafter. On the other hand, *C. antiqua* showed excellent growth in the basal medium+Mo+I at the 2nd and 3rd transfers. In basal medium+Mo, *C. antiqua* was able to grow to about half the final densities observed in basal medium+Mo+I. *C. marina* and *C. ovata* also showed excellent growth in basal medium+Mo+I, even at the 4th transfer (Fig. 1). The effect of the addition of iodine was comparable to that of the full addition (Mo+I) for the growth of these 2 species. The sole addition of molybdenum had a lesser effect than that of iodine, but gave a positive effect to some degree compared to the control. *C. marina* and *C. ovata* showed some growth even at the 4th transfer in the basal medium (no addition of molybdenum and iodine) though cell yields were low.

In the case of *C. verruculosa* (Fig. 2), a high growth yield was achieved by the sole addition of molybdenum, showing comparable growth to that in the basal medium with full addition (Mo+I). This species was unable to grow in the artificial media with the sole addition of iodine and no addition at the 3rd transfer.

In the case of *F. japonica* (Fig. 3), the sole addition of iodine to the artificial basal medium had a comparable effect on growth to that of the full addition (Mo+I). The addition of molybdenum had a less positive effect than iodine addition, and *F. japonica* also showed some growth even at the 4th transfer in the basal medium (no addition of molybdenum and iodine) though cell yields were low.

The maximum cell yields were compared to evaluate more clearly the effects of the addition of molybdenum and/or iodine to the basal medium upon growth of the 5 species of raphidophycean flagellates (Fig. 4). Two patterns were confirmed. First, *C. antiqua* and *C. verruculosa* re-

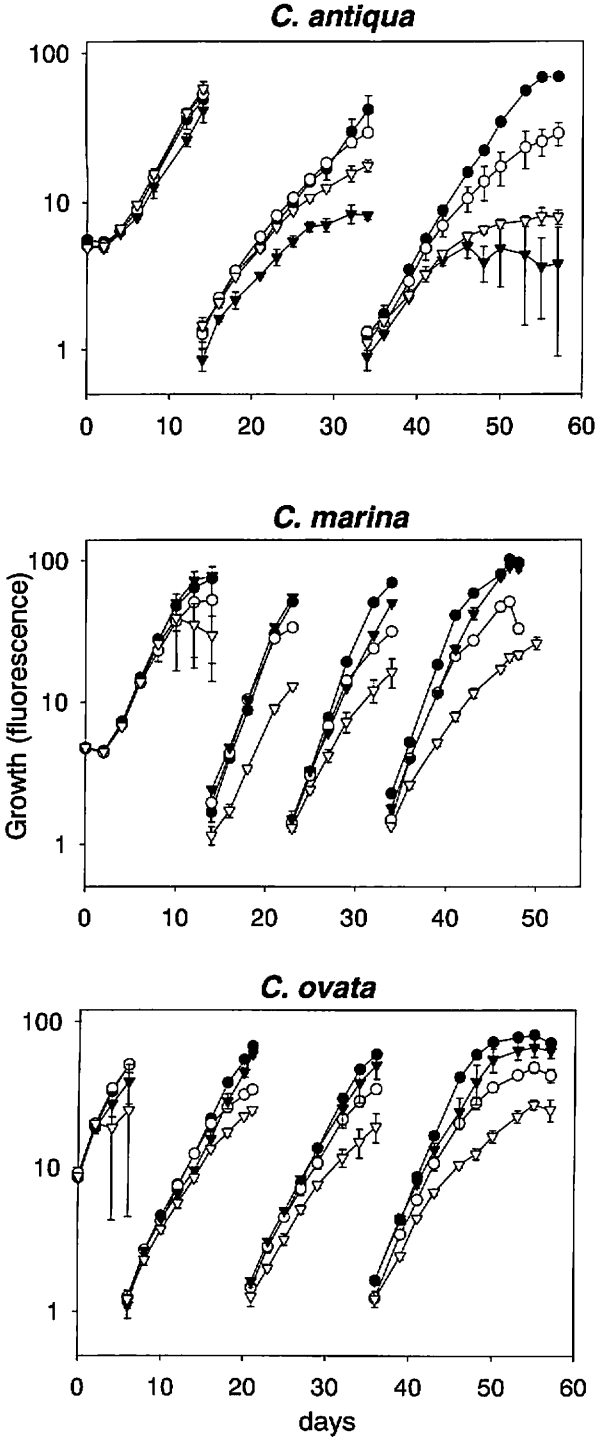

Fig. 1. Growth of *C. antiqua*, *C. marina* and *C. ovata* over 3 or 4 tranfers in the artificial basal medium enriched with both molybdenum and iodine (●), with molybdenum (○), with iodine (▼), and with no enrichment (control, ▽). Error bars: ±1 SD and $n=3$.

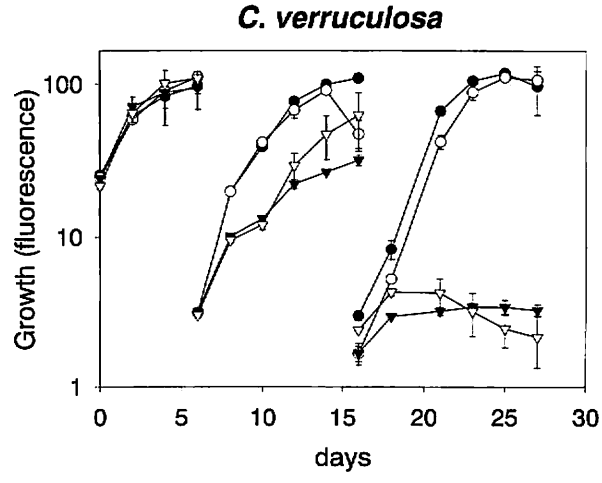

Fig. 2. Growth of *C. verruculosa* over 3 tranfers in the artificial basal medium enriched with both molybdenum and iodine (●), with molybdenum (○), with iodine (▼), and with no enrichment (control, ▽). Error bars: ±1 SD and $n=3$.

quired molybdenum for growth. In particular, the sole addition of molybdenum to the artificial basal medium was observed to have a significantly positive effect on the growth of *C. verruculosa*. *C. antiqua* exhibited better growth when molybdenum and iodine were added simultaneously, rather than for the sole addition of molybdenum to the basal medium. In the cases of *C. marina*, *C. ovata* and *F. japonica*, the second pattern, the sole addition of iodine gave a comparatively stimulatory effect on growth compared to the simultaneous addition of iodine and molybdenum. However, the sole addition of molybdenum also gave positive effects to some extents on the growth of these 3 species as compared with no addition.

The cell densities of the maximum cell yields shown in Fig. 4 were estimated based on the relationships between cell number and *in vivo* fluorescence (data not shown). The mean fluorescence values of the maximum cell yields for the contents of the polystyrene test tubes at the last transfer in the basal medium+Mo+I were 70 for *C. antiqua*, 103 for *C. marina*, 81 for *C. ovata*, 117 for *C. verruculosa*, and 32 for *F. japonica*, respectively. According to these values, the cell densities were estimated to be 2.8×10^4 cells ml^{-1} for *C. antiqua*, 4.1×10^4 cells ml^{-1} for *C. marina*, 2.0×10^4 cells ml^{-1} for *C. ovata*, 1.1×10^6 cells ml^{-1} for *C. verruculosa* and 5.4×10^4 cells ml^{-1} for *F. japonica*, respectively.

When iodine was not added to the artificial medium, the color of the cultures became paler and more greenish in *C. antiqua*, *C. marina*, *C. ovata*, and *F. japonica*. They also showed the same trend in cell color in the basal medium with no addition of molybdenum and iodine.

Based on the above results, a chemically-defined medium was developed for growth of 4 species of *Chattonella* and *F. japonica* (IHN-medium, Table 4). These raphidophycean flagellates also showed normal morphology in the IHN-medium as verified by microscopic observations.

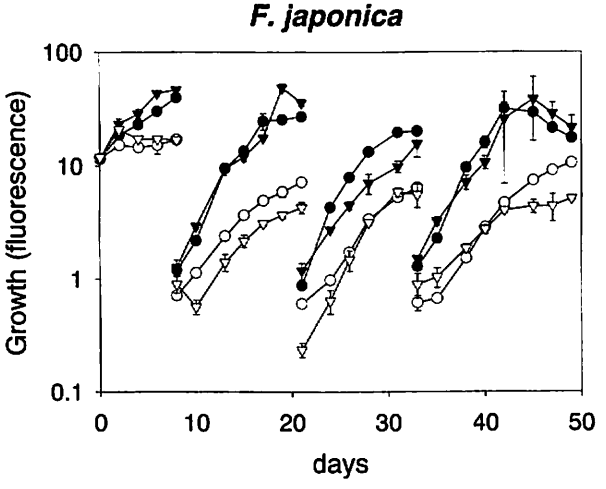

Fig. 3. Growth of *F. japonica* over 4 tranfers in the artificial basal medium enriched with both molybdenum and iodine (●), with molybdenum (○), with iodine (▼), and with no enrichment (control, ▽). Error bars: ±1 SD and $n=3$.

Discussion

The class Raphidophyceae comprises a relatively small number of species. However, marine species of this taxon, such as *C. antiqua*, *C. marina*, *C. verruculosa*, *F. japonica* and *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara, are well known to cause harmful red tides accompanying mass mortalities of fish in temperate and subtropical areas such as Japan, south-east Asia, southern Australia, Brazil, USA, and the North Sea (Okaichi 1997; Nehring 1998; Tomas 1998; Marshall & Hallegraeff 1999; Imai 2000; Backer-Hansen et al. 2001; Tiffany et al. 2001). Therefore, there is an urgent and compelling need to understand the mechanisms leading to the occurrence of red tides caused by harmful raphidophytes. However, there is an ex-

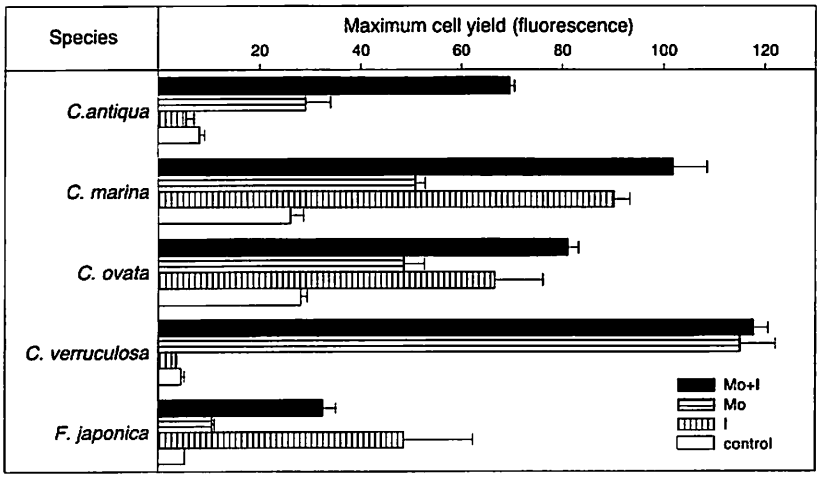

Fig. 4. Comparison of maximum cell yields of *C. antiqua*, *C. marina*, *C. ovata*, *C. verruculosa* and *F. japonica* during growth at th last transfer in the artificial basal medium enriched with both molybdenum and iodine (Mo+I, closed column), with molybdenum (Mo, horizontally striped column), with iodine (I, vertically striped column), and with no enrichment (control, open column).

Table 4. Composition of the IHN-medium, chemically-defined artificial seawater medium developed for red tide raphidophycean flagellates. For preparation of the IHN-medium, KI and $Na_2MoO_4 \cdot 2H_2O$ (☆) are added to the basal medium. See Table 1 for the compositions of P-I metals and S-3 vitamins.

Substance		Substance	
NaCl	0.43 M	Na_2SeO_3	2 nM
KCl	9.4 mM	☆ KI	0.472 μM
$MgSO_4 \cdot 7H_2O$	37 mM	☆ $Na_2MoO_4 \cdot 2H_2O$	0.1 μM
$CaCl_2 \cdot 2H_2O$	7.5 mM	Tris	4.1 mM
$NaNO_3$	0.59 mM	NTA	0.37 mM
$NaH_2PO_4 \cdot 2H_2O$	65 μM		
$Na_2SiO_3 \cdot 9H_2O$	0.33 mM	P-I metals	10 ml
Na_2-EDTA	30 μM	S-3 vitamins	2 ml
Fe-EDTA	2 μM		
		Distilled and purified water up to	1000 ml
		pH	7.8

treme paucity of studies on the effects of minor elements, such as trace metals and organic substances, on the growth of important raphidophyte species. In the present study, a chemically-defined artificial seawater medium for the growth of harmful raphidophyte species was developed (IHN-medium, Table 4). The maximum growth yields of these species in the IHN-medium (Fig. 4) were almost comparable to those gained in the seawater-based medium (modified SWM-3), though *F. japonica* exhibited a slightly lower value in the IHN-medium. We have obtained maximum cell yields (fluorescence values) of 64–82 for *C. antiqua*, 81–100 for *C. marina*, 51–57 for *C. ovata*, 79–89 for *C. verruculosa* and 100–130 for *F. japonica*, respectively, in the modified SWM-3 medium in polystyrene test tubes in other experiments. Hence, detailed studies on the effects of such trace elements are now feasible through use of this artificial medium.

Nakamura et al. (1983a, b) studied the growth of *C. antiqua* (Ho-1) using an artificial medium (H-medium). The H-medium contains basically the same elements as the basal medium (Table 1) of this study except for addition of $Na_2MoO_4 \cdot 2H_2O$. *C. antiqua* was able to grow to some degree in Mo-supplemented basal medium, and more abundantly in the artificial basal medium simultaneously supplemented with both molybdenum and iodine (Figs. 1, 4). We were able to develop a more suitable artificial medium for *C. antiqua* (IHN-medium) by supplementing the basal medium with $Na_2MoO_4 \cdot 2H_2O$ and KI simultaneously at the concentrations found in ambient seawater.

Molybdenum is known to be necessary for functioning of the nitrate reductase in algae (Fogg 1974; Morris 1974). A requirement for molybdenum has also been reported for nitrogen fixation by cyanobacteria (Fogg 1974) and in the assimilation of nitrate nitrogen by *Scenedesmus obliquus* (Turpin) Kützing (Arnon et al. 1955). Iodine commonly exists in seawater, and it is implausible for iodine to be a factor limiting the growth of phytoplankton in natural seawater. However, an iodine requirement has been reported for the growth in some species of brown algae (Pedersén 1969; Woolery & Lewin 1973) and red algae (von Stosch 1964; Iwasaki 1967) in laboratory culture. Iodine is thus an essential inorganic nutrient for some kinds of algae (O'Kelley 1974). When iodine was not added to the artificial medium, cell color became paler and more greenish in *C. antiqua*, *C. marina*, *C. ovata*, and *F. japonica*, indicating a decrease in the cellular content of fucoxanthine, which is a common and abundant brown-colored carotenoid of brown color in brown algae and marine raphidophytes (Fiksdahl et al. 1984; Chihara 1997). It is supposed that iodine may be related to the fucoxanthine synthesis process. To our knowledge, this is the first report on the positive effects of iodine on the growth of unicellular microalgae.

The IHN-medium contains molybdenum and iodine at the concentrations present in natural seawater (Tables 2, 4). It remains unknown as to whether the IHN-medium has the optimum concentrations of iodine and molybdenum for the growth of these raphidophycean flagellates. This is a question to be examined in the future.

From the present experimental results, 2 patterns were confirmed for the requirements of molybdenum and iodine for the growth of red tide raphidophycean flagellates (Fig. 4). Particularly in *C. verruculosa*, molybdenum was crucially essential for growth (Figs. 2, 4), and this pattern differed significantly from that for the other raphidophytes examined. According to analyses of 18S rRNA gene sequence and ultrastructure, it has recently been found that *C. verruculosa* does not belong to the Raphidophyceae but rather to the Dictyochophyceae within the Heterokontophyta (Fukaya et al. 2001, Abstract A27 of the 25th Annual Meeting of the Japanese Society of Phycology). The present result concerning the molybdenum requirement of *C. verruculosa* might reflect this fact.

Multiple recipes for chemically-defined artificial media have been developed for marine phytoplankton (Provasoli et

al. 1957; Pintner & Provasoli 1958; Gates & Wilson 1960; Iwasaki 1967; Morel et al. 1979; Harrison et al. 1980, 1988; Nakamura & Watanabe 1983a; Keller et al. 1987). Except for the ASP_{12} and ASP_{12} I media (Provasoli 1964; Iwasaki 1967), iodine is not contained in those artificial media. Furthermore, some of these artificial media do not contain molybdenum. The IHN-medium (Table 4) developed in this study provides both iodine and molybdenum, and moreover it also contains selenium (Se) which has been pointed out to be essential for many phytoplankton species including red tide-causing and toxic species (Keller et al. 1987; Price et al. 1987; Harrison et al. 1988; Ishimaru et al. 1989; Cosper et al. 1993; Imai et al. 1996; Boyer & Brand 1998; Doblin et al. 1999). In our laboratory, the 5 species of raphidophytes used in this study have been continually and successfully transferred and grown in the IHN-medium for more than 2 years, and the following marine phytoplankton have also been maintained for more than 2 years in good condition; *H. akashiwo* (Raphidophyceae, Heterokontophyta), *Karenia mikimotoi* (Miyake et Kominami ex Oda) Hansen et Moestrup (formerly *Gymnodinium mikimotoi* Miyake et Kominami ex Oda and *G. nagasakisense* Takayama et Adachi, Dinophyta), *Heterocapsa circularisquama* Horiguchi, *H. triquetra* (Ehrenberg) Stein (Dinophyta), *Ditylum brightwellii* (West) Grunow ex van Heurck (Bacillariophyceae, Heterokontophyta), *Rhodomonas ovalis* Nygaard (Cryptophyta), *Eutreptiella gymnastica* Throndsen (Euglenophyta), *Oltmannsiellopsis viridis* (Hargraves et Steele) Chihara et Inoue (Chlorophyta), and *Cricosphaera roscoffensis* (Dangeard) Gayral et Fresnel (Haptophyta). Therefore the present IHN-medium is considered to be an excellent broad spectrum artificial medium for cultivating various marine phytoplankton species in the laboratory. In addition, this medium is easy to prepare and is autoclavable without precipitates. It is hoped that this IHN-medium will work effectively for investigations on the nutritional physiology of phytoplankton, including harmful species, and contribute to understanding the mechanisms leading to the occurrence of harmful algal blooms.

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