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Life cycle, physiology, ecology and red tide occurrences of the fish-killing raphidophyte *Chattonella*

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ABSTRACT

The marine fish-killing raphidophytes of the genus *Chattonella* currently consist of five species, i.e. *C. antiqua*, *C. marina*, *C. minima*, *C. ovata* and *C. subsalasa*. The distribution of *Chattonella* species was confirmed in tropical, subtropical and temperate regions in the world accompanying mass mortalities of fishes in nature and in aquaculture. The fish-killing mechanisms are still unclear, but suffocation is the ultimate cause of fish death. Increasing evidence is pointing towards the generation of reactive oxygen species (ROS, e.g. superoxide), which are responsible for the gill tissue injury and mucus production that leads to death of fishes. A taxonomic revision was proposed based on morphology and genetic diversity that *Chattonella antiqua* and *Chattonella ovata* should be varieties of *Chattonella marina* possessing nomenclatural priority. Optimum temperatures for growth are 25 °C for *C. antiqua* and *C. marina*, 25–30 °C for *C. ovata* and 20–30 °C for *Chattonella subsalsa*. Adequate ranges of salinity for growth were about 20–30 for *Chattonella* species. *Chattonella* cells generally divide once a day. Laboratory culture experiments with artificial synthetic medium demonstrated that *C. antiqua*, *C. marina* and *C. ovata* used only Fe chelated with EDTA for growth, although tested diatoms and dinoflagellates used rather many kinds of chelated Fe. A suitable concentration of humic acid supplied with iron also had enhancing effects on the growth of *C. antiqua*. Diel vertical migration was observed in *Chattonella*, and the cells reached 7.5 m deep at night in the case of *C. antiqua* demonstrated by a mesocosm experiment in the Seto Inland Sea. *Chattonella* species have diplontic life history and have haploid cyst stage in their life cycle. Encystment was observed through formation of pre-encystment small cells after the depletion of nitrogen, and the small cells sink to the sea bottom to complete cyst formation by attachment to the solid surface such as diatom frustules and sand grains. Newly formed cysts are in the state of spontaneous dormancy and they need cold temperature period of four months or longer for maturation (acquisition of germination ability). Cysts germinate in early summer and resultant vegetative cells play an important role as seed populations in blooming in the summer season. However, relatively small part of cyst populations actually germinate from bottom sediments, and success of red tide formation is dependent on the growth in water columns. Since red tides of *Chattonella* were observed when diatoms were scarce in seawater, diatoms appear to have a key for the predominance of *Chattonella* in water columns. Diatom resting stages in sediments need light for germination/rejuvenation, whereas *Chattonella* cysts can germinate even in the dark, implying the selective germination of *Chattonella* cysts at the sea bottom under calm oceanographic conditions which contribute to bloom formation of *Chattonella*. As a mechanism of red tide occurrences of *Chattonella* in coastal sea, "diatom resting hypothesis" was presented. Biological control using diatoms is proposed through the germination/rejuvenation of resting stages suspending from bottom sediments to euphotic layer by sediment perturbation with submarine tractors or fishing trawling gears. Since diatoms have much higher growth rates, and newly joined diatom vegetative cells grow faster and prevent occurrence of *Chattonella* red tides as a result. As another prevention strategy for *Chattonella* red tides, algicidal bacteria inhabiting in seaweed beds and seagrass beds are presented. Co-culture of fish and seaweeds in aquaculture areas, and the developments of seaweed- and seagrass-beds would be practical and ultimately environment-friendly strategies for the prevention of harmful red tides of *Chattonella* by virtue of natural algicidal bacteria supplied from seaweeds and leaves of seagrass.

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1. Introduction

Marine phytoflagellates belonging to the genus *Chattonella* (Raphidophyceae) contain notorious fish-killing species that cause severe damage to fish farming (amount of billion yen for one case of red tide in Japan), especially to yellowtail *Seriola quinqueradiata* cultures during the summer season (Okaichi, 1997; Imai et al., 2006b). The worst record of fisheries damage was given by *Chattonella antiqua* in Harima-Nada, the Seto Inland Sea of Japan, in the summer of 1972 to cultured yellowtail; i.e. fish-kill of 14.2 million individuals worth 7.1 billion yen (about 90 million US dollar). The distribution of *Chattonella* in the world is shown in Fig. 1 (modification of Edvardsen and Imai, 2006). The first record of massive bloom of *Chattonella* (*C. marina*, reported as a new species of *Hornellia marina*) and mass mortality of fish was reported from Malabar Coast, India (Subrahmanyan, 1954). Noxious red tides of *Chattonella* (*C. antiqua*, *C. marina* and *C. subsalsa*) causing massive fish kills have been reported thereafter in Japan, Korea (Kim et al., 2007), China (Tseng et al., 1993), India (Jugnu and Kripa, 2009), USA (south-east coast, California) (Tomas, 1998; Lewitus et al., 2008), and South Australia (Hallegraeff et al., 1998). In recent years of 21st century, *Chattonella ovata* had also started to form harmful red tides and to give fishery damages by fish-killing (Songhui and Hodgkiss, 2001; Guarado et al., 2004; Takatsuji et al., 2005; Hiroishi et al., 2005; Yamaguchi et al., 2008b). The inhabitation of *Chattonella* has also been confirmed in tropical, subtropical and temperate regions of southeast and east Asia, New Zealand, Brazil, Europe (North Sea and Mediterranean Sea), Egypt, etc. (Vrieling et al., 1995; Imai et al., 1998; Orlova et al., 1998; Tomas, 1998; Rhodes et al., 2001; Reifel et al., 2002; Band-Schmidt et al., 2004; Edvardsen and Imai, 2006; Zhang et al., 2006; Mikhail, 2007). In case of Japan, empty cysts of *Chattonella* can be commonly detected with the primulin-staining method (Yamaguchi et al., 1995) from deep sediments of 16th century (Ichimi et al., 2005). Therefore, *Chattonella* spp. are considered to be hidden flora (Smayda, 2002) that inhabit at low cell density as back ground flora, and they became dominant along with eutrophication of the coastal sea such as the Seto Inland Sea (Imai et al., 2006b).

2. Morphology and taxonomy

The species belonging to the genus *Chattonella* are unicellular, golden-brown (fucoxanthin-containing), naked flagellates possessing a forwardly directed anterior flagellum with tubular hairs (tripartite tubular mastigonemes) and a smooth trailing flagellum from a more or less pronounced flagellar groove. The genus *Chattonella* is a member of the Class Raphidophycese, Phylum Heterokontophyta (Chromophyta, Ochrophyta) in stramenopiles.

In the past, seven species were described in the genus *Chattonella* as shown in Fig. 2 (Ono and Takano, 1980; Hara and Chihara, 1982; Hara et al., 1994; Hallegraeff and Hara, 1995). Recent analyses of LSU rDNA demonstrated that *Chattonella globasa* Hara et Chihara is a naked type of *Dictyocha fibula* var. *stapedia* (Dictyochophyceae, Heterokontophyta) (Takano et al., 2007). Observations of ultrastructure and sequence analyses of 18S rDNA also revealed that *Chattonella verruculosa* Hara et Chihara is belonging to Dictyochophyceae and newly established a genus *Pseudochattonella*, currently having *P. verruculosa* and *P. farcimen* (Hosoi-Tanabe et al., 2007; Edvardsen et al., 2007). Accordingly, the genus *Chattonella* has five species at present, i.e. *Chattonella antiqua* (Hada) Ono 1980 (biosynonym of *Hemieutreptia antiqua* Hada 1974), *Chattonella marina* (Subrahmanyan) Hara et Chihara 1982 (biosynonym of *Hornellia marina* Subrahmanyan, 1954), *Chattonella minima* Hara et Chihara 1994, *Chattonella ovata* Hara et Chihara 1994 and *Chattonella subsalsa* Biecheler 1936.

Table 1 shows identification key to these five species of *Chattonella* on the basis of morphological characteristics (Imai, 2009, modified from Hara and Chihara, 1987; Hara et al., 1994). In *C. minima*, the size is somewhat smaller than *C. marina* but the morphology is almost the same as *C. marina,* although the number of chromosomes at the metaphase was 90–110 for *C. minima*, about 50 for *C. marina* and about 29 for *C. antiqua* (Hara et al., 1994). Therefore, discrimination is practically unfeasible between *C. marina* and *C. minima* with common microscopy. Detailed investigations are needed on chromosomes of *Chattonella* species. Currently, four species are recognizable for live materials with normal light microscopy (Fig. 3).

Recently, Demura et al. (2009) proposed a taxonomic revision of *C. antiqua*, *C. marina* and *C. ovata* based on morphological characteristics and genetic diversity such as nuclear ITS rDNA regions, chloroplast rbcL gene, mitochondrion COI gene and selected microsatellite region. As a result of the comparison of 104 strains, the morphological characters for the three species formed a continuum of variation, and their genetic divergence was at the intraspecies level when compared with other heterokontophytes. Consequently,

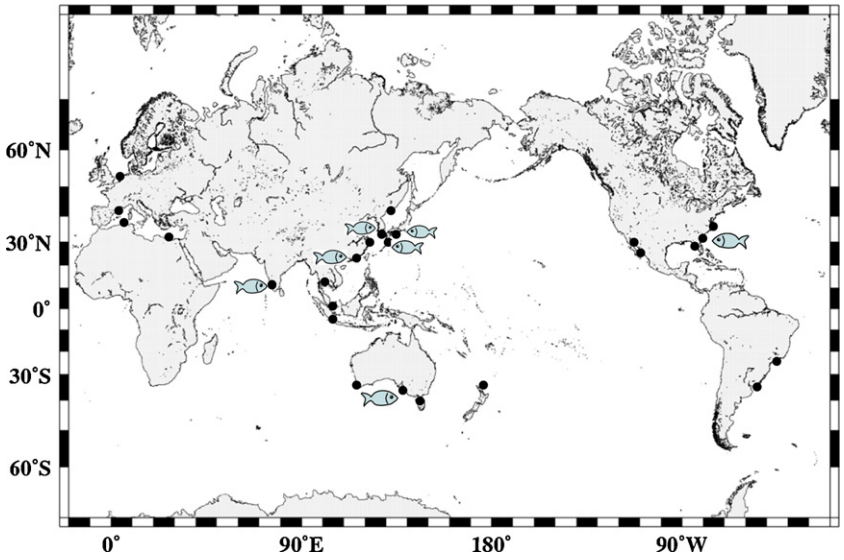

Fig. 1. Global distribution (closed circles) of *Chattonella* (*C. antiqua, C. marina, C. ovata* and *C. subsalsa*). Fish icons indicate sites where mass mortalities of fish have occurred. After Edvardsen and Imai (2006) modified.

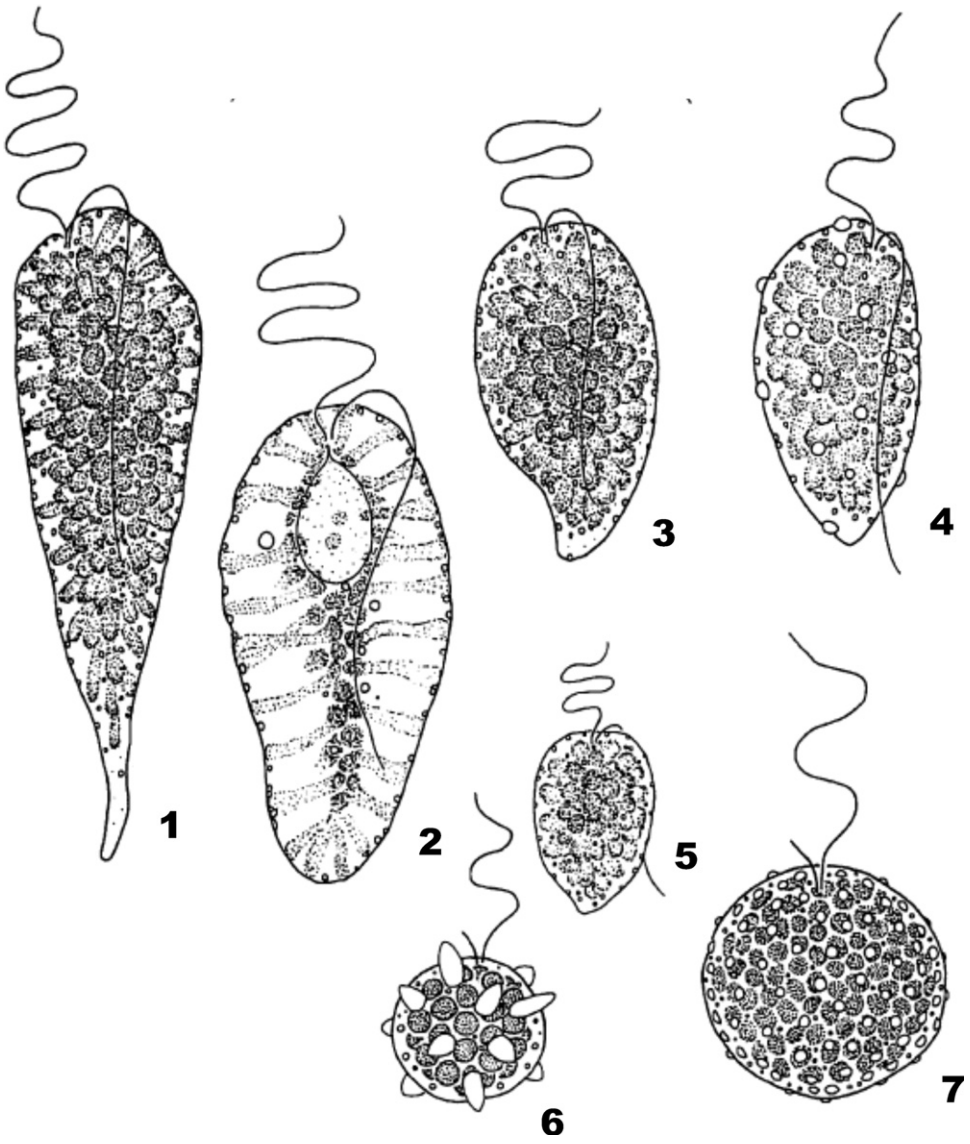

Fig. 2. Five species of *Chattonella* (Raphidophyceae) (1–5) and two species of Dictyochophyceae formerly belonging to *Chattonella* (6 and 7). *Chattonella antiqua* (1), *C. ovata* (2), *C. marina* (3), *C. subsalsa* (4), *C. minima* (5), *Pseudochattonella verruculosa* (6), *Dictyocha fibula* var. *stapedia* (7).
After Hara and Chihara (1987) and Hallegraeff and Hara (1995) modified.

they presented an idea that *C. antiqua* and *C. ovata* should be varieties of one species of *C. marina* with nomenclatural priority. These three species may be called as "marina complex". In the first place, Hara et al. (1994) established the species *C. marina* without direct comparative observations of the strains from Japan with that from India (type locality). Demura et al. (2009) also made no investigations about the Indian strains of so-called *C. marina*. Comparative studies by direct observations (especially strains of India) are basically needed in the future for the correct taxonomy of *Chattonella* species.

Table 1
Identification of *Chattonella* species on the basis of morphology.

1 a. Mucocysts present	2
1 b. Mucocysts absent	3
2 Cells elongated, with pointed posterior end	*C. subsalasa*
3 a. Cells ovate	*C. ovata*
3 b. Cells not ovate, with pointed posterior end	4
4 a. Cells 20–50 μm long	*C. minima*
4 b. Cells 35–70 μm long	*C. marina*
4 c. Cells 50–130 μm long, with a posterior tail	*C. antiqua*

Modification of Hara and Chihara (1987), Hara et al. (1994) and Hallegraeff and Hara (1995).

3. Ecophysiology

3.1. Growth physiology

A massive increase in cell number is essential for occurrences of fish-killing red tides of *Chattonella*. The vegetative cells of *C. antiqua* and *C. marina* multiply by binary fission. Therefore, to understand the mechanisms of red tide occurrences it is important to investigate the effects of environmental factors on the growth of *C. antiqua* and *C. marina*. Irradiance, water temperature, salinity, and nutrients are the most significant factors affecting the growth of *Chattonella*. Biological factors are also important such as competition with autotrophic plankton, grazing by zooplankton and protists, infection, parasitism, bacterial attack, etc. Nutrient competition and bacterial predation are discussed later.

Cell division in *C. antiqua* and *C. marina* can be synchronously induced under light-dark regimes, and division occurs in the dark

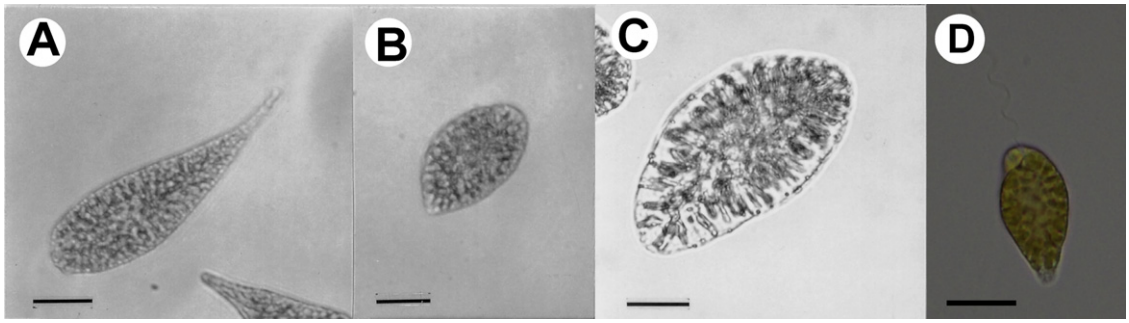

Fig. 3. Photomicrographs of *Chattonella antiqua* (A), *C. marina* (B), *C. ovata* (C) and *C. subsalsa* (D). Scale bar 20 μm.

period (Nemoto and Furuya, 1985; Ono, 1988). Growth of *C. antiqua* and *C. marina* is observed at an irradiance of 30 μmol m^{-2} s^{-1} or more, with saturation at 110 μmol m^{-2} s^{-1} or more (Fig. 4) (Nakamura and Watanabe, 1983a; Yamaguchi et al., 1991). The maximal growth rates of both species are about 1 division day^{-1} (Yamaguchi et al., 1991). The maximal growth rates of the two strains of *C. ovata* were 2.09 divisions day^{-1} and 1.49 divisions day^{-1} and half saturation constants (K_s) were 178 μmol m^{-2} s^{-1} and 87.5 μmol m^{-2} s^{-1} with growth saturation at over 300 μmol m^{-2} s^{-1} (Fig. 5) (Yamaguchi et al., 2010). These values of parameters for the growth of *C. ovata* are much larger than those of *C. antiqua* and *C. marina* (Yamaguchi et al., 1991). A strain of *C. marina* from Australia showed growth saturation at light intensity of 400 μmol m^{-2} s^{-1} or more, suggesting an adaptation to high light intensity environments (Marshall and Hallegraeff, 1999). In *C. subsalsa* (Fig. 6), μmax growth constants and the half saturation for light were 1.26 divisions day^{-1} and 69 μmol m^{-2} s^{-1} (Zhang et al., 2006), resembling the values for *C. antiqua* (μ_{max} = 1.34 divisions day^{-1}, K_s = 42 μmol m^{-2} s^{-1}) and *C. marina* (μ_{max} = 1.39 divisions day^{-1}, K_s = 63 μmol m^{-2} s^{-1}) (Yamaguchi et al., 1991).

The growth responses of *C. antiqua* and *C. marina* were examined at 30 combinations of different temperatures (10–30 °C) and salinities (10–35 psu, practical salinity units) under a saturating light intensity of 120 μmol m^{-2} s^{-1} (Yamaguchi et al., 1991). Both species grew at temperatures from 15° to 30 °C and salinity from 10 to 35 psu (Fig. 7). The maximal growth rates of *C. antiqua* and *C. marina* were obtained with the combination of 25 °C and 25 psu, and 25 °C and 20 psu, respectively. The most suitable temperature and salinity for *C. antiqua* are identical to those obtained by Nakamura and Watanabe (1983a). Yamochi (1984)

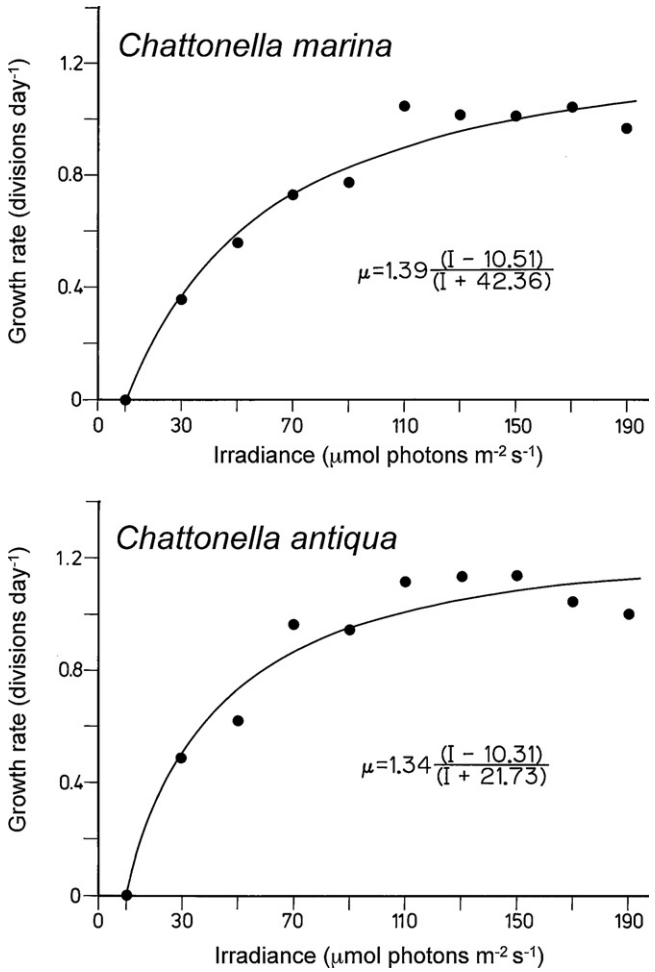

Fig. 4. Growth rates of *Chattonella marina* and *C. antiqua* as a function of irradiance. After Yamaguchi et al. (1991).

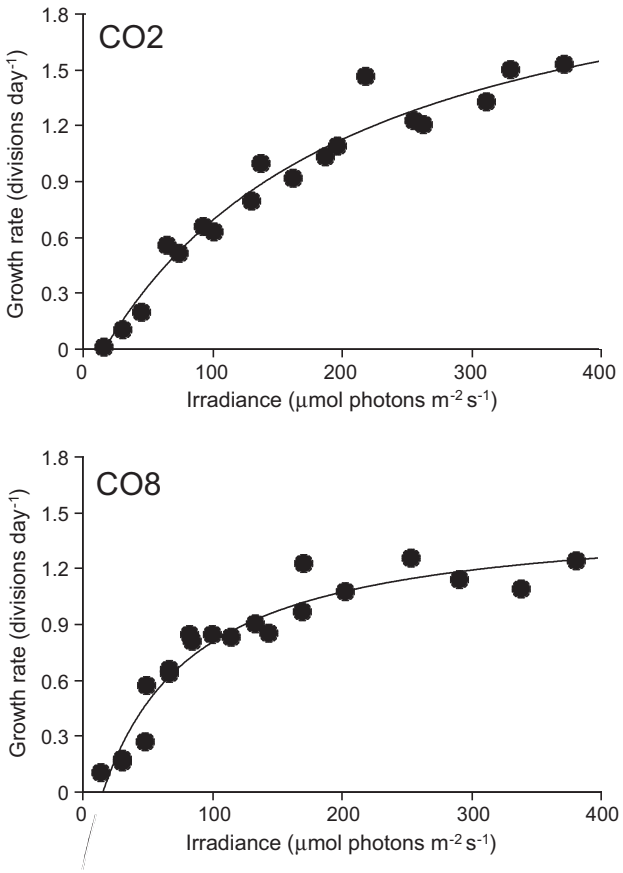

Fig. 5. Effect of irradiance on growth of *Chattonella ovata* (strains CO2 and CO8). After Yamaguchi et al. (2010).

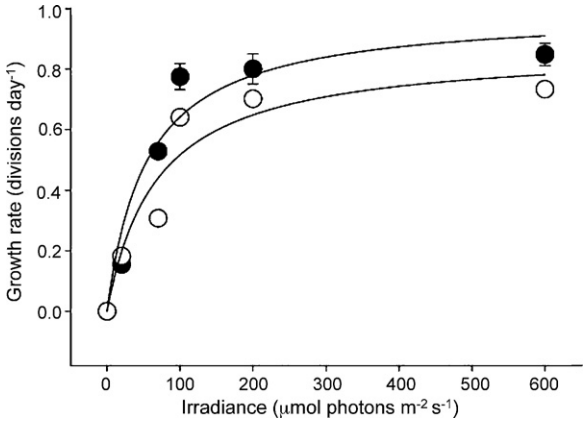

Fig. 6. Growth rates of *Heterosigma akashiwo* (filled circles) and *Chattonella subsalsa* (open circles) as a function of light intensity. Error bars, standard deviation. After Zhang et al. (2006).

reported that survival ranges were 13–31 °C for both species of *Chattonella*. In Harima-Nada in the Seto Inland Sea, vegetative cells of *C. antiqua* and *C. marina* were observed in the temperature ranges of 19.2–28.8 °C and 18.8–28.0 °C, respectively (Yoshimatsu and Ono, 1986). On the contrary, Australian strains of *C. marina* grow at a temperature of 10 °C, suggesting an adaptation to lower temperature conditions than Japanese waters (Marshall and Hallegraeff, 1999). The three strains of *C. ovata* from the Seto Inland Sea tolerate a wide range of temperature (15–32.5 °C) and salinity (10–35 psu) with a suitable range of over 20 °C and salinity of over 20 psu (Fig. 8) (Yamaguchi et al., 2010). The maximal growth rates were achieved in the combination 25–30 °C and salinity of 25–30 psu, and these values appear to be higher than those for *C. antiqua* and *C. marina*. In the Seto Inland Sea, *C. ovata* has been detected at temperatures of 15.8–31.3 °C and salinity of 18.4–32.02 psu. Appearances of high cell densities were observed in summer at temperatures of 26.0–29.5 °C and salinity of 31.47–31.97 psu. Optimum conditions for *C. ovata* appear to be higher temperatures than those for *C. antiqua* and *C. marina*. *C. subsalsa* shows relatively uniform optimum growth rates between 20 and 30 °C, significantly reduced rates at 10 and 16 °C, and no growth at 4 °C (Zhang et al., 2006). The growth of *C. subsalsa* was observed under salinity conditions of 5psu and higher in laboratory experiments. Occurrences of *C. subsalsa* have been observed at temperatures of 17–33 °C and salinities of 6–36 psu, with blooming ranges of 24–31 °C and 11–28 psu in the Delaware Inland Bay, USA (Fig. 9). Blooms of *Chattonella* species generally occurred during summer season. The water temperature drops to below survival range during winter, so vegetative cells of *Chattonella* cannot overwinter in the water column. Therefore, they need cysts in the life history for overwintering and persisting existence in the coastal sea of temperate areas.

Growth and uptake kinetics of nitrate and phosphate were examined in detail for *C. antiqua*. According to Nakamura and Watanabe (1983c), the uptake kinetics of *C. antiqua* followed the Michaelis–Menten equation, and the half-saturation constants (K_s) were 3.0 μM for nitrate and 1.9 μM for phosphate, respectively. *C. antiqua* could take up these nutrients in the dark at rates almost equal (83–93%) to that at light. Table 2 shows kinetic constants for growth of *C. antiqua*, *C. ovata* and *C. subsalsa*. The half-saturation constants for the growth of *C. antiqua* for nitrate and phosphate were calculated to be 1.0 μM and 0.11 μM, respectively (Nakamura et al., 1988). K_s values of nitrogen (8.98 μM for nitrate) and phosphorus (0.84 μM for phosphate) for *C. subsalsa* are rather higher than those of *C. antiqua*. It is thought partly because the strains of *C. subsalsa* were originally isolated from extremely eutrophicated water of Delaware Inland Bays, USA.

Nutrient requirements have been investigated for *C. antiqua*. This species uses nitrate, ammonium, and urea, but not amino acids (glycine, alanine, and glutamate) as nitrogen sources (Nakamura and Watanabe, 1983b). However, high concentration of ammonium (>150 μM) is toxic to *C. antiqua*. *C. ovata* uses only inorganic nitrogen, and not urea (Yamaguchi et al., 2008a). Inorganic phosphate, and occasionally glycerophosphate, can serve as phosphorus sources for *C. antiqua* (Iwaski, 1973; Nakamura and Watanabe, 1983c). *C. ovata* is capable of using ATP and ADP for growth as phosphorus source in addition to inorganic phosphorus (Yamaguchi et al., 2008a).

Iron is an essential trace metal for the growth of *C. antiqua* (Iwaski, 1973; Nakamura and Watanabe, 1983c; Okaichi and Montani, 2004). Bioassay experiments using *C. antiqua* revealed that iron is one of the growth limiting factors in seawater of the Yatsushiro Sea (Shikata et al., 2011). Laboratory culture experiments were carried out targeting *Chattonella* using a newly

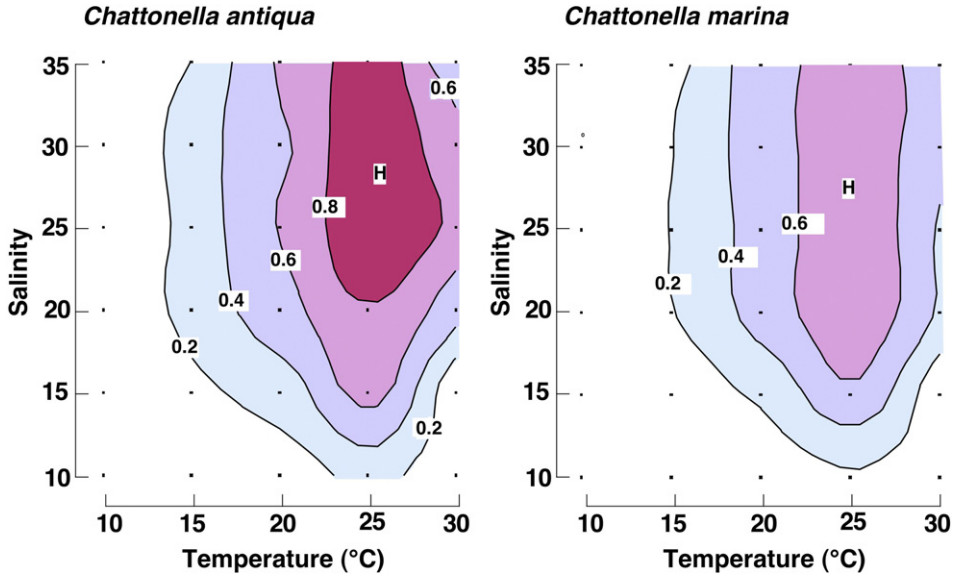

Fig. 7. Response surface contours of the growth rates of *Chattonella antiqua* and *C. marina* as functions of temperature and salinity. After Yamaguchi et al. (1991) modified.

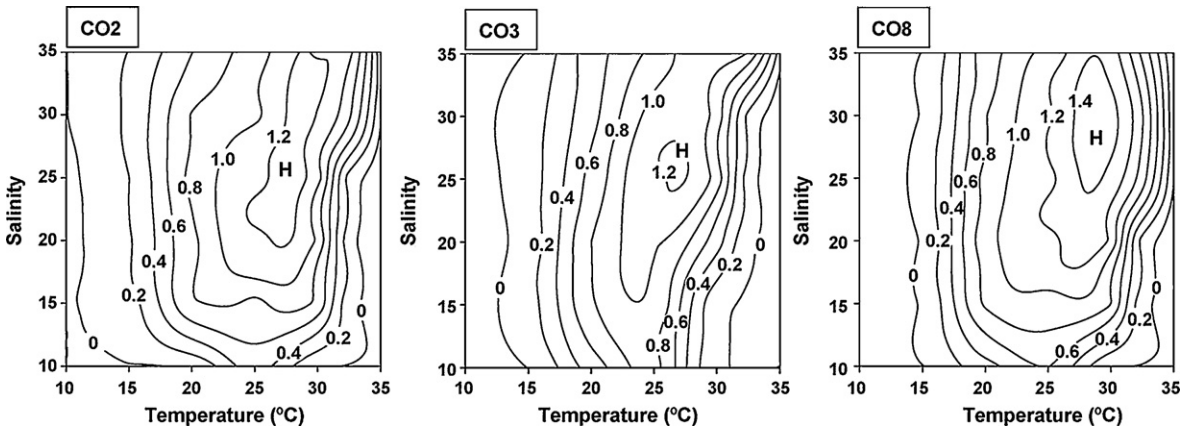

Fig. 8. Response surface contours of the growth rates of three strains of *Chattonella ovata* as functions of temperature and salinity.
After Yamaguchi et al. (2010).

developed artificial synthetic medium (Imai et al., 2004) with different Fe species (Naito et al., 2005a,b, 2008). *C. antiqua*, *C. marina* and *C. ovata* used only Fe chelated with EDTA for growth, but not other chelated Fe (Fig. 10). Other diatoms and dinoflagellates used rather many kinds of chelated Fe. A suitable concentration of humic acid supplied with iron also had enhancing effects on the growth of *C. antiqua* (Fukuzaki et al., 2011). It is suggested that the composition and concentration of humic substances possibly affects the bloom developments of *C. antiqua* by controlling the iron availability. *Chattonella* spp. revealed rather narrow range of iron utilization despite they form red tides in coastal seas. This is an enigma of *Chattonella* red tides.

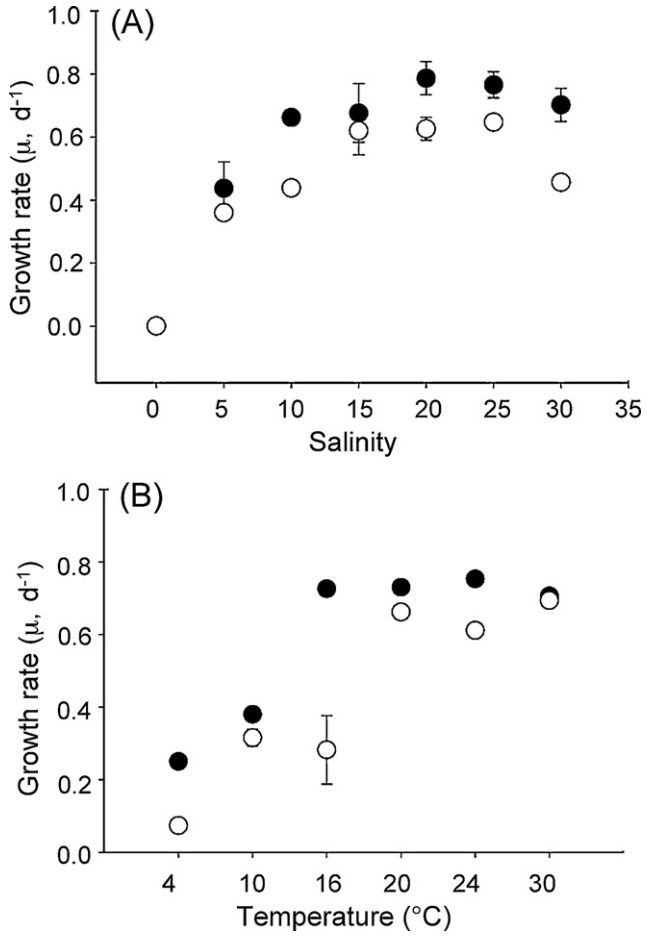

Fig. 9. Growth rates of *Heterosigma akashiwo* (filled circles) and *Chattonella subsalsa* (open circles) as a function of salinity (A) and temperature (B). Error bars, standard deviation.
After Zhang et al. (2006).

In *Chattonella antiqua*, the polyamines of spermidine, caldopentamine and homocaldopentamine were detected (Nishibori and Nishijima, 2004; Nishibori et al., 2009). The concentrations of spermidine (a species of polyamine) showed a marked increase during the period of exponential growth phase and a linear correlation was observed between the free spermidine contents of the cell and the growth rates (Nishibori and Nishijima, 2004). In another aspect of other micronutrients, *C. antiqua* requires vitamin B_{12} for growth, but it is rare for vitamin B_{12} to be a limiting factor in coastal waters (Nishijima and Hata, 1986).

The mixotrophy of *C. ovata* and *C. subsalsa* was demonstrated using fluorescent-labeled bacteria and the cyabobacterium *Synechococcus* sp. in laboratory experiments (Seong et al., 2006; Jeong et al., 2010). The mixotrophy may support the formation of red tides in relatively nutrient poor water areas.

The warning level of cell density is 100 cells ml^{-1} for *C. antiqua* in the Seto Inland Sea. This level means high probability of beginning of fish-kill in aquaculture in the sea. When a *C. antiqua* bloom exceeds this level, a warning is emitted to fishermen and authorized people from each local governments related to fisheries, and fishermen take countermeasure such as stopping of feeding, moving of pen cages (vertically and/or horizontally). In order to evaluate the degree of danger and harm of *C. antiqua*, the warning level of cell density was converted to equivalent level of

Table 2
Kinetic constants for growth of *Chattonella antiqua*, *C. ovata* and *C. subsalasa*. Data are from Nakamura (1985), Nakamura et al. (1988), Zhang et al. (2006), and Yamaguchi et al. (2008a). μ_{max}, maximal growth rate; K_s, half saturation constant; q_0, minimum cell quota; ND, no data.

	PO_4^{3-}	NO_3^-	NH_4^+
C. antiqua			
μ_{max} (divisions day^{-1})	1.41	1.41	1.07
K_s (μM)	0.11	1.00	0.23
q_0 (pmol)	0.62	7.8	7.7
C. ovata			
μ_{max} (divisions day^{-1})	1.25	1.14	ND
K_s (μM)	ND	ND	ND
q_0 (pmol)	0.48	5.5	ND
C. subsalsa			
μ_{max} (divisions day^{-1})	0.81	0.87	0.84
K_s (μM)	0.84	8.98	1.46
q_0 (pmol)	ND	ND	ND

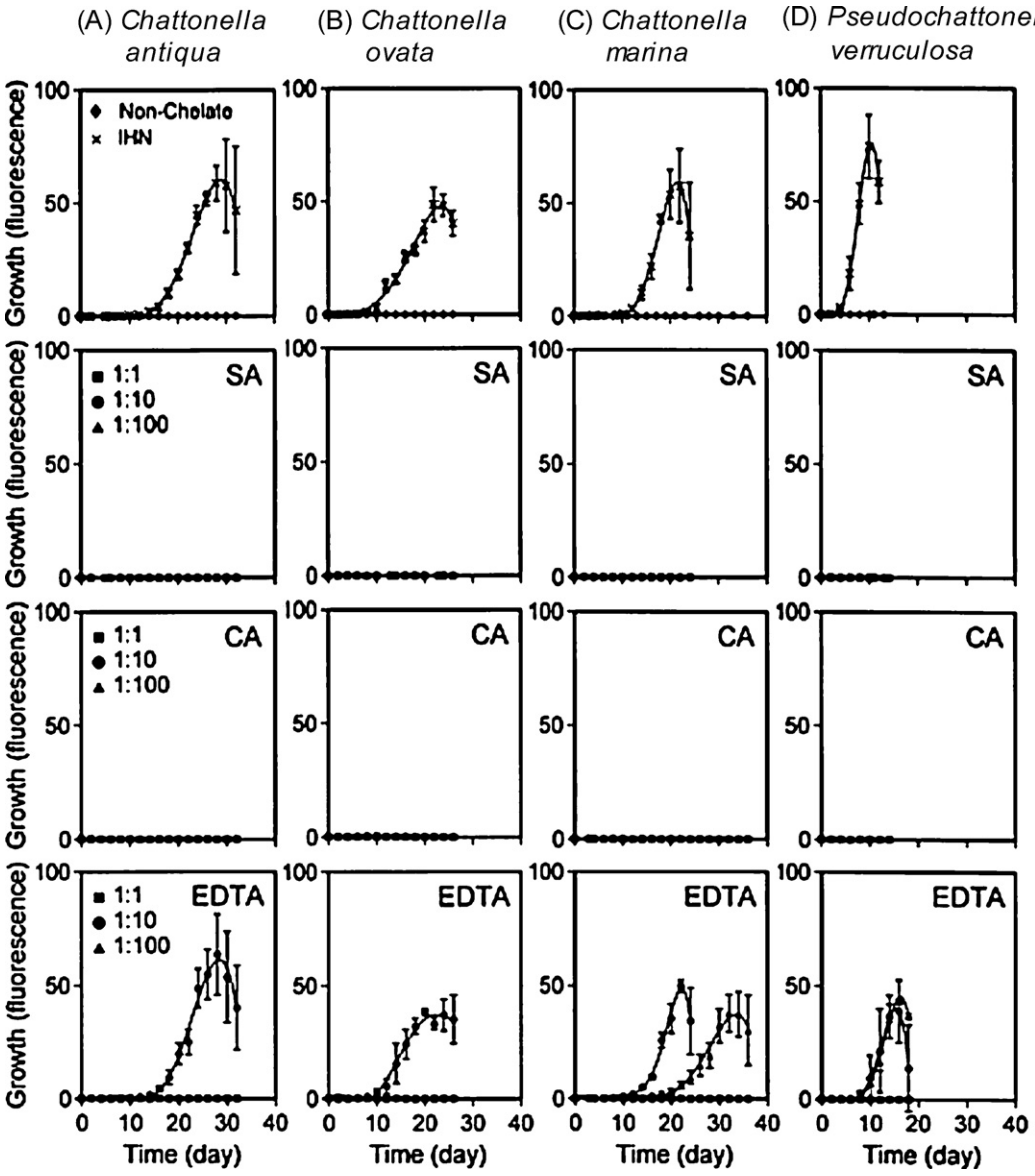

Fig. 10. Growth curves of the three species of *Chattonella* (Raphidophyceae) and *Pseudochattonella verruculosa* (Dictyochophyceae) in cultures supplied with iron salicylate (SA), iron citrate (CA), iron ethylenediaminetetraacetate (EDTA), $FeCl_3$ (non-chelate) and in the modified IHN-medium. Growth curves are shown for (A) *Chattonella antiqua*, (B) *C. ovata*, (C) *C. marina* and (D) *Pseudochattonella verruculosa*. Data of fluorescence represent mean and standard deviation.
After Naito et al. (2005a) modified.

inorganic nutrients of nitrogen and phosphorus based on minimum cell quota for N (q_0^N) and P (q_0^P) (Imai et al., 2006b). *C. antiqua* can easily reach the warning level by consuming only small amounts of N (0.78 μM) and P (0.062 μM). *C. marina* and *C. ovata* have fish-kill activities with similar cell level as *C. antiqua*. Therefore, species of *Chattonella* (*C. antiqua*, *C. marina* and *C. ovata*) are regarded to be extremely dangerous harmful red tide organism. Information is needed on the warning level of cell density that *C. subsalsa* cause fish-kill in relevant water areas.

3.2. Diel vertical migration

It is well known that natural populations of *Chattonella* species perform diel vertical migration in coastal embayments during blooming. They usually reach the depth of 10 m or shallower at night, but sometimes 20 m and deeper under some conditions and cause killing of yellowtail rearing in "big pen cage" with depth of 20 m or deeper employed to avoid high cell density layer (<10 m) in usual blooming case (Imai, 2010b). It is necessary to exactly know the depth to which *Chattonella* motile cells themselves reach at night without effects of tidal currents.

Diel vertical migration and nocturnal uptake of P and N by *C. antiqua* in the nutrient-rich lower layer was demonstrated in a 1.5-m-tall axenic culture tank, in which nutrients were vertically stratified analogous to nutrients observed in a natural *Chattonella* red tide (Watanabe et al., 1991). Watanabe et al. (1995) used an *in situ* mesocosm to show that *C. antiqua* undergoes vertical migration at night to reach deep water that has ample nutrients, and that a bloom of *C. antiqua* occurred when there was a stable nutricline at a shallow depth within the range of vertical migration of *C. antiqua*. Mesocosm experiments were conducted at a cove off the Ieshima Islands in northern Harima-Nada, from 21 July to 14 August 1989. The mesocosm was 18 m deep, 5-m diameter and had a volume of ~350 m^3. Immediately after enclosure on 21 July, N and P were enriched throughout the water column. *C. antiqua* was present at 2 cells ml^{-1} (at 5 m at 09:00 h on 8 August). From 9

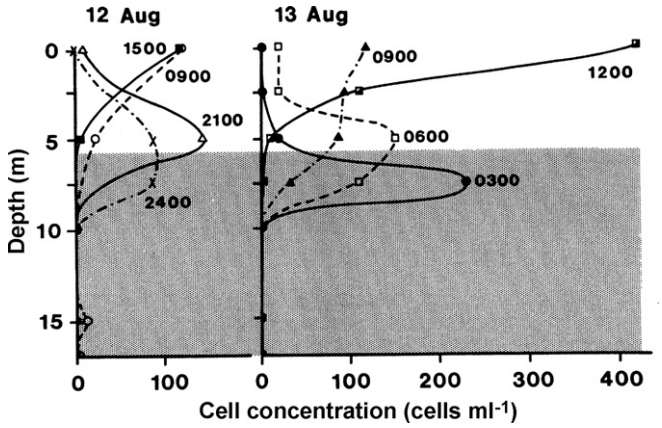

Fig. 11. Diel vertical migration of *Chattonella antiqua* observed in the mesocosm between 12 and 13 August 1989. Artificial nutrient enrichment was conducted in the shaded zone (6–18 m).
After Watanabe et al. (1995).

to 13 August, the population of *C. antiqua* at the surface (at 09:00 h) increased from 8 to 116 cells ml^{-1}, and there was a *C. antiqua* red tide.

Between 15:00 and 18:00 h on 12 August, *C. antiqua* cells began to migrate downward (Fig. 11). At 03:00 h on 13 August, most cells (229 cells ml^{-1}) migrated to a depth of 7.5 m, which was within the nutrient-rich zone. Between 03:00 and 06:00 h, the cells began to migrate upward, and at 12:00 h, most cells reached the surface (421 cells ml^{-1}). The migration speed (as total group velocity) was calculated to be ~0.8 m h^{-1}, with the same upward and downward migration speeds. This *in situ* mesocosm study showed that oceanographic conditions leading the marine ecosystem in the Seto Inland Sea towards a *C. antiqua* red tide involved a complex combination of environmental factors: excess of N and P compared with Si, initial bottom temperature of 20–22 °C (optimum for excystment to inoculate *C. antiqua*), surface temperature of 25–27 °C (optimum for growth of *C. antiqua*), stable shallow nutrient stratification, absence of copper toxicity, and diel vertical migration by *C. antiqua* (Watanabe et al., 1995).

In the case of *C. subsalsa*, the vertical migration was observed utilizing quantitative real-time PCR used to enumerate each species in a mixed bloom of four species (*C. subsalsa*, *Heterosigma akashiwo*, *Fibrocapsa japonica* and *Pseudochattonella verruculosa*) in the Delaware Inland Bays, USA (Handy et al., 2005). *C. subsalsa* vertically migrated in both a uniform well-mixed water column and under stratified conditions together with other raphidophyte species.

3.3. Fish-killing mechanism

Fish-killing events have been reported for *C. antiqua, C. marina, C. ovata* and *C. subsalasa* so far. The fish-killing mechanisms of *Chattonella* spp. are still unclear, but suffocation is the ultimate cause of fish death. The physical clogging of gills by *Chattonella* cells and mucus excretion was earlier proposed as the cause of killing of fish (Matsusato and Kobayashi, 1974). Gill damage by polyunsaturated fatty acids as hemolytic substance was also suspected as a cause of fish-kill (Okaichi, 1980; Shimada et al., 1983). HPLC evidence for production of brevetoxin (neurotoxin) was presented (Onoue and Nozawa, 1989; Endo et al., 1992; Khan et al., 1996). Keppler et al. (2006) reported sublethal effects of short-term exposure to *C. subsalsa* on the eastern oyster *Crassostrea virginica* based on a hypothesis that *C. subsalsa* toxicity is related to brevetoxin production. However, further confirmation is still required using mass spectrometry to relate the toxicity (neurotoxins) to *Chattonella* species (Hallegraeff and Hara, 1995). Recent increasing evidence is pointing towards the generation of reactive oxygen species (ROS, e.g. superoxide), which are responsible for the gill tissue injury and mucus production that leads to death of fish (Tanaka et al., 1994; Ishimatsu et al., 1996; Marshall et al., 2005). Ishimatsu et al. (1996) found that the strain of *C. marina* with no productivity of superoxide did not kill yellowtail at cell density of 4000 cells ml^{-1} (enough density for fish-kill by ordinary *Chattonella*) in laboratory experiments. ROS of *Chattonella* stimulated the production and excretion of mucus of gill tissue, and heavily produced mucus blocked the lamellar water channels in yellowtail, involving in death by suffocation (Hishida et al., 1997). And further, it is reported that mucus from gills of yellowtail enhanced the production of ROS by *Chattonella* (Shimada et al., 1993; Tanaka et al., 1994; Oda et al., 1998). Marshall et al. (2003) proposed a synergistic role of ROS and free fatty acids during fish death by *C. marina*. Kim and Oda (2010) suggested that cell surface structure of *C. marina*, glycocalyx (Yokote and Honjo, 1985), has NADPH-dependent O_2^- generation system. They further suggested that the continuous accumulation of discharged glycocalyx on the gill surface occurs during *C. marina* exposure, which may be responsible for the ROS-mediated severe gill tissue damage leading to fish death. Thus, ROS appear to play a key role in involving fish-kill by *Chattonella*.

4. Life history of *Chattonella*

As vegetative cells cannot overwinter in water columns in coastal sea of temperate coastal areas due to too low temperature for survival, species of *Chattonella* produce cysts in their life cycles (Imai and Itoh, 1986, 1988; Yamaguchi et al., 2008b). The function of cysts is pointed out as (i) a source of the recurrent occurrences of microalgal species, (ii) a vector for expansion of species distribution, (iii) a resistant cell against unfavorable environmental conditions for survival, (iv) a chance of recombination of genetic information for species possessing sexual reproduction process for cyst formation (Wall, 1971; Anderson et al., 1983; Fukuyo, 2003). Cyst formation usually terminates blooming of microalgae due to stopping cell multiplication through cyst producing process, and this is also the case for *Chattonella*.

4.1. Cell division

Species of raphidophycean flagellates ordinarily multiply with a longitudinal cell division. In the case of *Chattonella antiqua*, cell division takes place during darkness. Light irradiation is essential for nuclear DNA replication in *C. antiqua*, and the timing of the replication is dependent upon the timing of the onset of the last irradiation (Nemoto et al., 1987). After the nuclear division, cell division commences from the anterior end of the cell. Following the formation of new gullets from where flagella emerge, a longitudinal furrow extends about the long axis of the cell. The two daughter cells attach through only a small protoplasmic constriction at the final stage of cell division, and then they each split into two cells that finish the cell division. *C. marina* and *C. ovata* show the same manner of cell division as *C. antiqua*.

4.2. Morphology of cysts

At present, in species belonging to *Chattonella*, natural cysts have been identified in *C. antiqua*, *C. marina* and *C. ovata* from sediments collected from the Seto Inland Sea, Japan (Imai and Itoh, 1986, 1988; Yamaguchi et al., 2008b). In the case of *C. subsalasa*, although the morphology of cysts has not been identified yet, those cysts were detected and estimated by using of quantitative real-time PCR from the bottom sediments of Delaware's Inland Bays, USA (Portune et al., 2009).

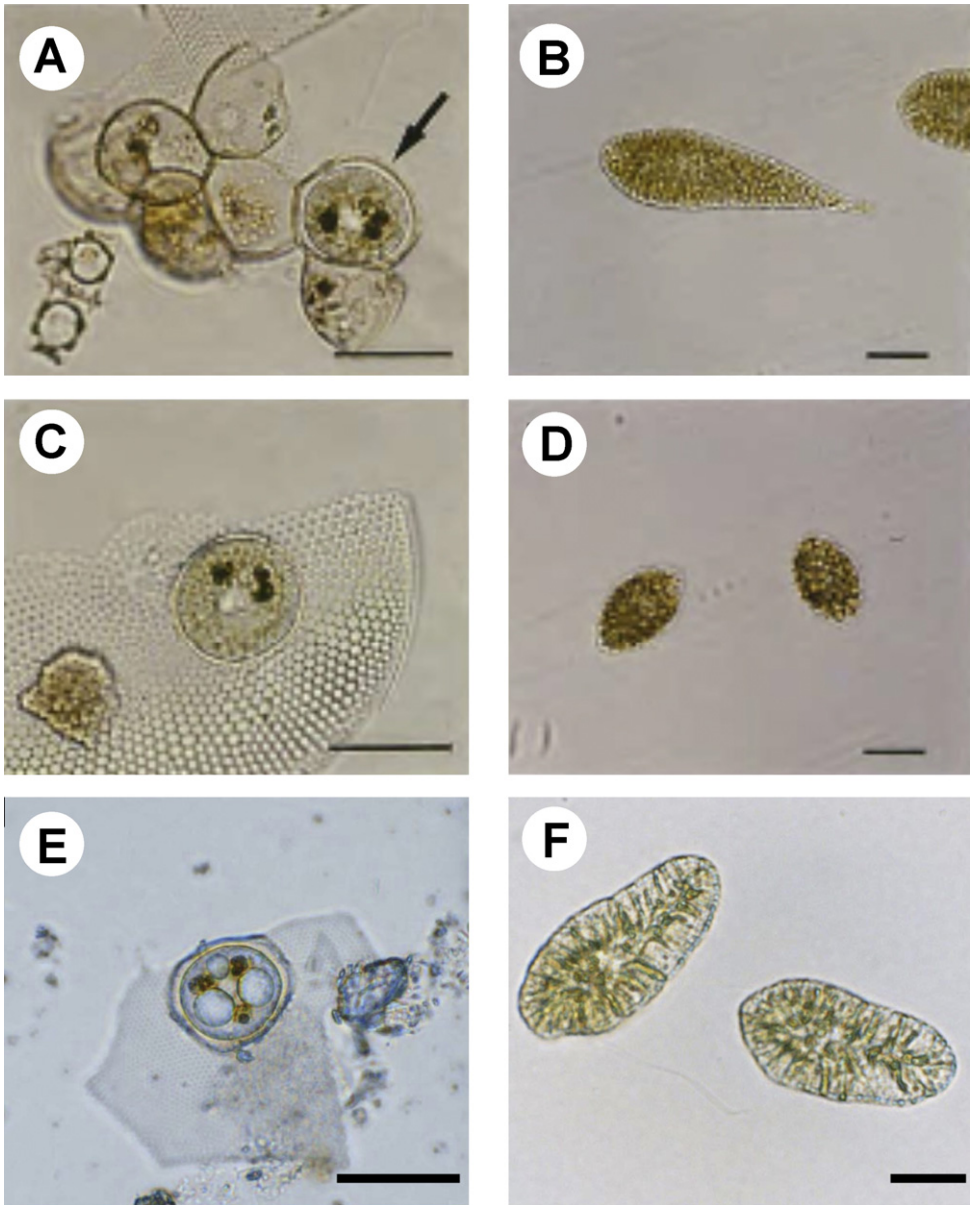

Fig. 12. Natural cysts and vegetative cells of *Chattonella antiqua* (A and B), *C. marina* (C and D) and *C. ovata* (E and F). Scale bar, 30 μm. After Imai and Itoh (1988) and Yamaguchi et al. (2008b).

Fig. 12 shows light micrographs of natural cysts of *C. antiqua, C. marina* and *C. ovata* found in sediments of the Seto Inland Sea. No distinct differences were noticed in morphology among the cysts of the three species as is the case of cysts of the toxic dinoflagellate *Alexandrium tamarense* and *Alexandrium catenella* (Fukuyo et al., 1982). Therefore, incubation of cysts and cultivation of germinated vegetative cells are required for the discrimination of the cysts of the three species (Imai and Itoh, 1988; Yamaguchi et al., 2008b). The live cysts of *C. antiqua, C. marina* and *C. ovata* reveal the following morphological characteristics: (i) they are mostly hemispherical with a diameter of 25–35 μm and a height of 15–25 μm, (ii) they usually adhere to solid surfaces such as diatom frustules, sand grains, etc., (iii) they are yellow-greenish to brownish in color, (iv) they have several spots of dark brown or black materials, (v) they have many chloroplasts, smaller in size than those of vegetative cells, visible with epifluorescence microscopy under blue-light excitation (Fig. 13), (vi) they are uninucleate, (vii) they have no ornamentations on the surface of the cyst wall (Imai and Itoh, 1986, 1988; Yamaguchi et al., 2008b). Solitary cysts and clusters of several cysts (occasionally more than 10) including empty ones were observed. The cysts of *Chattonella* have a structure for future germination, and a circular opening with a diameter of about 7 μm was observed on the wall of an empty cyst after germination (Fig. 14).

Under adequate conditions for germination (22 °C), the cysts of *Chattonella* with germinability start to germinate, and vegetative cells after germination were abundantly observed during the period from the 4th to 6th day of incubation (Imai et al., 1984a). The cysts of *Chattonella* favor darkness and low light conditions rather than high irradiance for the germination (Imai et al., 1984a; Imai, 1995; Ichimi et al., 2003). *Chattonella* cysts can germinate under low dissolved oxygen concentrations as compared with some dinoflagellate cysts (Montani et al., 1995). One cell excysts from one cyst. Vegetative cells of *Chattonella* newly germinated within 24 h, are similar in size and color to the cysts, and they enlarge later to the size of common vegetative cells and begin multiplication by cell division. The cysts are markedly smaller than vegetative cells in *C. antiqua*, *C. marina* and *C. ovata* (Fig. 12).

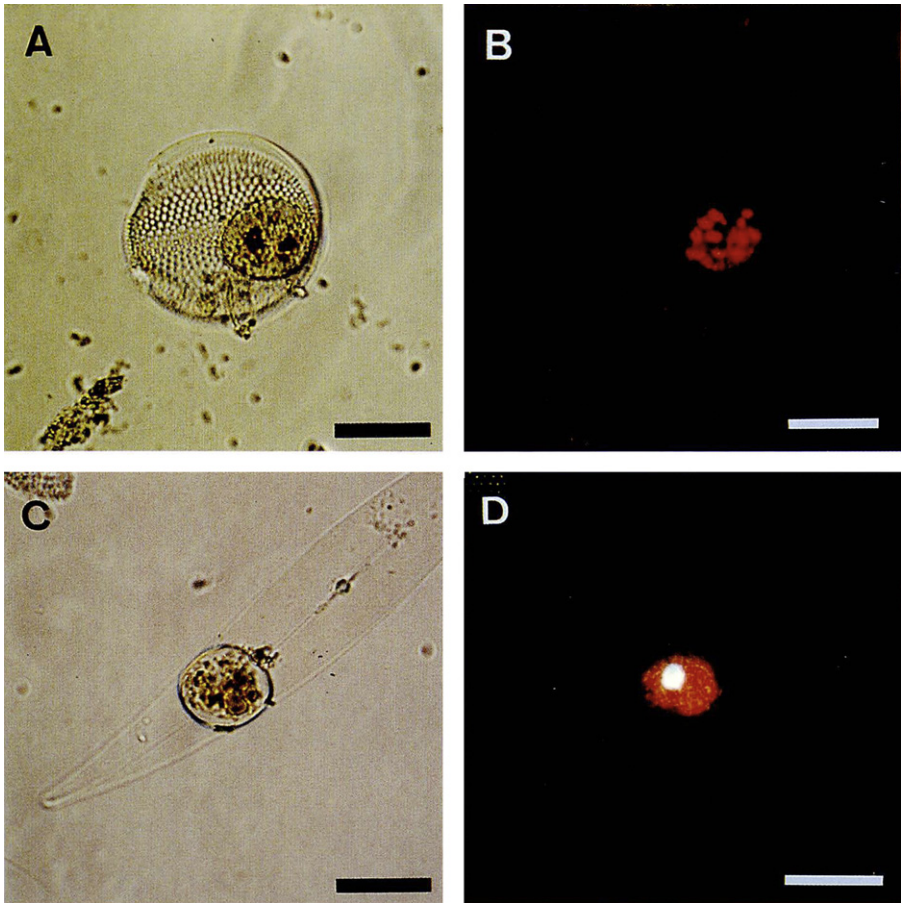

Fig. 13. Normal light and epifluorescence micrographs of cysts of *Chattonella*. Scale bar, 30 μm. (A) A cyst observed under normal light, (B) the same cyst as in (A), observed under blue-light excitation, (C) a cyst observed under normal light after fixation with glutaraldehyde and staining with DAPI (4′6-diamidino-2-phenylindole) and (D) the same cyst as in (C) observed under violet excitation. Blue white fluorescence of nucleus (diameter of about 8 μm) is visible (D).
After Imai and Itoh (1988).

4.3. Conditions for cyst formation

In *C. marina*, cyst formation was observed in culture under laboratory conditions (Imai, 1989). Nitrogen limitation was effective in inducing cyst formation. After the incubation in a nitrogen-limited medium at 25 °C with ca. 150 μmol m^{-2} s^{-1} on a 14 h light, 10 h dark photo-cycle, pre-encystment small cells similar to cysts in size and color were observed (Fig. 15), and thereafter cultures were subjected to low light intensities (ca. 15 μmol m^{-2} s^{-1} or below). Cysts were successfully formed on glass beads, added for attachment to complete cyst formation. The cysts formed in culture displayed morphological characteristics quite similar to those natural cysts observed in sediments (Fig. 16). Germination of cysts produced in culture was confirmed under adequate conditions (22 °C, ca. 50 μmol m^{-2} s^{-1} on a 14 h light, 10 h dark photo-cycle) after storage at 11 °C in the dark for more than four months. In *C. antiqua*, cyst formation was also observed in culture under similar conditions to those described above (Imai, 1989; Nakamura et al., 1990).

The following process is presumed for the cyst formation in *C. antiqua, C. marina* and *C. ovata* in the Seto Inland Sea on the basis of the above results. Initially, vegetative cells grow in surface water and thereafter experience nutrient depletion. This acts as a trigger inducing cyst formation, and pre-encystment small cells are formed. The pre-encystment small cells sink to the sea bottom (Imai et al., 1993b) and adhere to solid surfaces such as diatom frustules and sand grains, where cyst forming cells experience low light intensity, essential for the completion of cyst formation (Imai, 1989). Consequently, the combination of factors such as nutrient depletion, adherence to solid surfaces, and low light intensity (or darkness) is needed for cyst formation. In fact, at the final stage of a *Chattonella* red tide (mainly by *C. antiqua*) observed in northern Hiroshima Bay in the late summer of 1990, it was confirmed by the direct count method using epifluorescence microscopy that many cysts are newly formed and newly supplied to the sea bottom there (Imai et al., 1993b). Nakamura and Umemori (1991) reported that the optimum range of temperature is 21.6–26.6 °C for the cyst formation of *C. antiqua* in Harima-Nada, the Seto Inland Sea.

4.4. Life history

The life history of *Chattonella* had long been an enigma before the discovery of cysts. The morphology of cysts of *Chattonella* (*C. antiqua, C. marina* and *C. ovata*) has been identified and the conditions for cyst formation are also clarified. Yamaguchi and Imai (1994) determined the nuclear DNA contents at various stages (vegetative cells, pre-encystment small cells, cysts produced in culture, natural cysts, small cells just after the germination within 24 h) in the life history of *C. antiqua* and *C. marina* by means of an epifluorescence microscopy-based fluorometry system after staining with DNA-specific fluorochrome 4′6-diamidino-2-phenylindole (DAPI) (Yamaguchi, 1992), and found that vegetative cells of both *C. antiqua* and *C. marina* were diploid and that their cysts were haploid, i.e. they have a diplontic life history (Fig. 17).

Vegetative cells of *C. antiqua* and *C. marina* ordinarily multiply by asexual binary cell fission, as mentioned before. The nucleus of

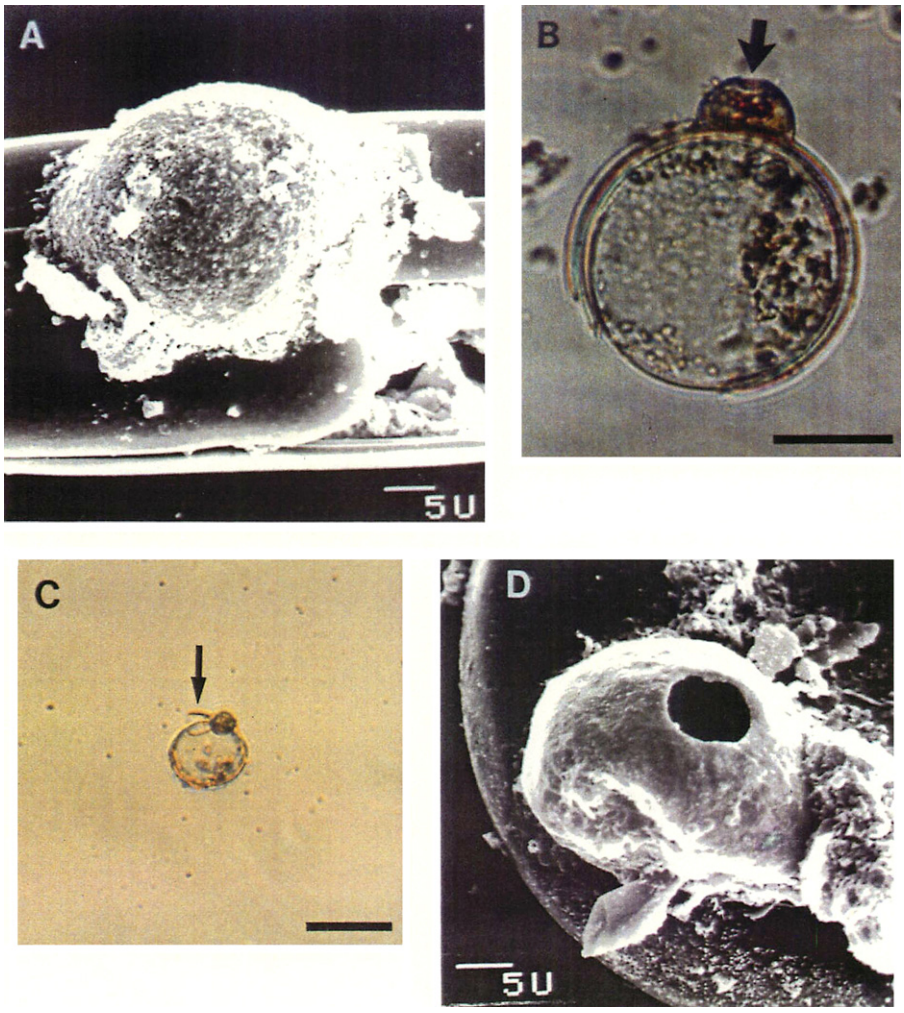

Fig. 14. Scanning electron micrographs and light micrographs of *Chattonella* cysts. Scale bar, 30 μm. (A) Scanning electron micrograph of a cyst adhered to pennate diatom frustule. (B) Light micrograph of the lateral view of cyst adhered to centric diatom frustule, showing a particular structure for germination (arrow). (C) Light micrograph of an empty cyst after the germination, with an opening and a lid (arrow). (D) Scanning electron micrograph of an empty cyst adhered to centric diatom frustule, with a circular opening with diameter of about 7 μm.
After Imai and Itoh (1988).

the G1 cell of *C. antiqua* was oblong, about 14 μm long by 10 μm wide, and that of the G2 + M cells was similar in appearance but larger, about 23 μm long by 15 μm wide (Yamaguchi and Imai, 1994). Assuming the DNA content of the vegetative cells of *C. antiqua* in G1 phase to be 2C, G2 + M cells were 4C. Interestingly, the DNA contents of the pre-encystment small cells and cysts (both natural and artificially produced) were 1C. Therefore, the meiosis in *C. antiqua* could be indicated by the transition from the 2–4C to

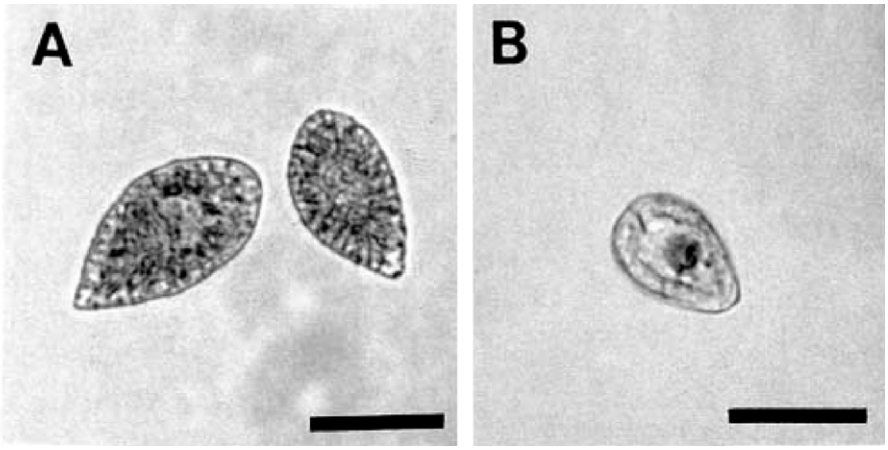

Fig. 15. Common vegetative cells (A) and a pre-encystment small cell of *Chattonella marina* observed in cyst formation medium after 10 days incubation. Scale bar, 30 μm.
After Imai (1989).

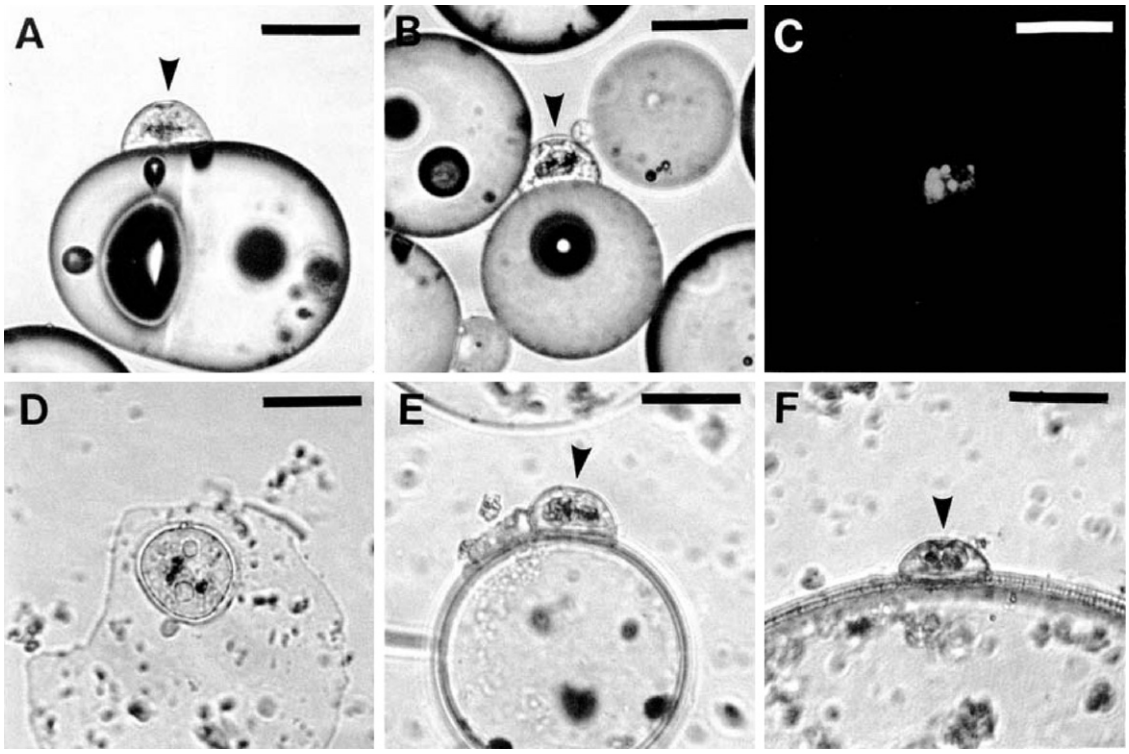

Fig. 16. Cysts of *Chattonella marina* formed in culture (A–C) and naturally observed *Chattonella* cysts in sediments (D–F). Scale bar, 30 μm. A structure for germination is indicated by an arrow head for each cyst. Scale bar, 30 μm. (A and B) Lateral view of formed cysts adhered to glass bead. (C) Epifluorescence image of cyst in (B) observed under blue-light excitation showing autofluorescence of chloroplasts. (D) Dorsal view of a natural cyst adhered to a fragment of diatom frustule. (E and F) Lateral view of natural cysts adhered to centric diatom frustule.
After Imai (1989).

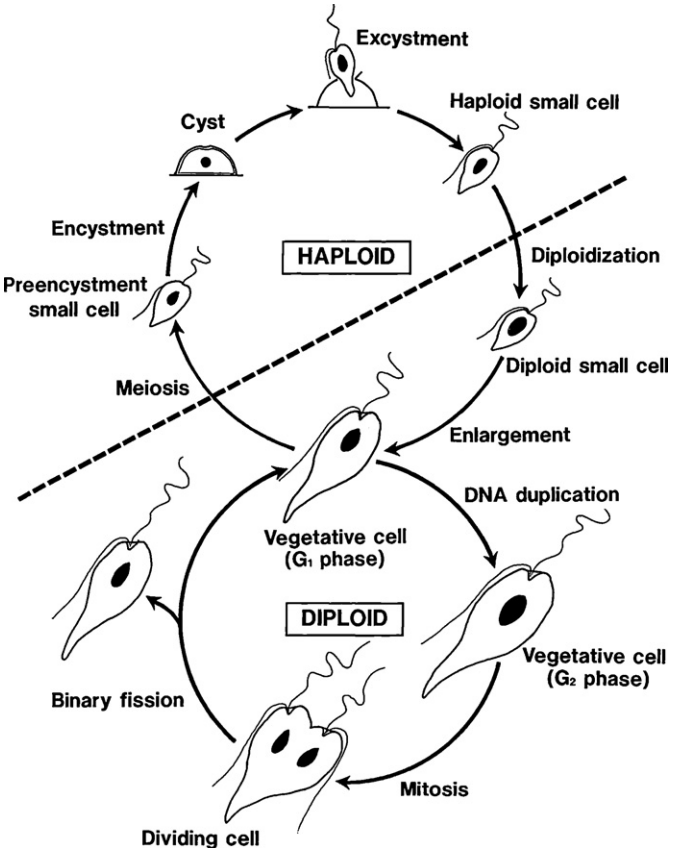

Fig. 17. Schematic representation of the life history of *Chattonella antiqua* and *C. marina* based on DNA microfluorometry.
After Yamaguchi and Imai (1994).

the 1C category in the pre-encystment small cell stage. The DNA contents of newly excysted cells, measured within 24 h of germination, revealed a DNA level from 1C to 2C. The difference 1C and 2C cells was only in nuclear size. These facts strongly suggest that DNA diploidization occurs shortly before the cells enlarge into normal vegetative cells without cell fusion.

In *C. marina*, DNA amounts of the nucleus of vegetative cells was almost equal to *C. antiqua*, and the changes in nuclear DNA content that occur during the life cycle was the same as the changes in *C. antiqua*. *C. ovata* also produces pre-encystment small cells in culture under the same conditions for the experiments of *C. antiqua* and *C. marina* (Imai, unpublished data). Although analysis of DNA contents was not carried out, it is presumed that *C. ovata* has also a diplontic life history. Since there is no ploidy study on *C. subsalsa* yet (Portune et al., 2009), it is needed to investigate the life history of *C. subsalsa*.

5. Bloom ecology

5.1. Annual life cycle

Red tides of *Chattonella* are caused by the motile, planktonic stage in their life history during the summer season. They have a cyst stage for overwintering, and the cysts play a major role in the total ecology of the neritic species of *Chattonella* by serving several important functions, as is the case in some toxic dinoflagellates (Wall, 1971; Fukuyo et al., 1982; Anderson et al., 1983; Dale, 1983). Cysts settle to bottom sediments to overwinter and thereby ensure the persistent existence of the species in the same area such as the Seto Inland Sea, and the germination of cysts provides the inoculum into overlying water columns for blooms in the summer season there.

Water temperatures seasonally fluctuate in temperate waters such as the Seto Inland Sea. Temperatures of around 10 °C or below

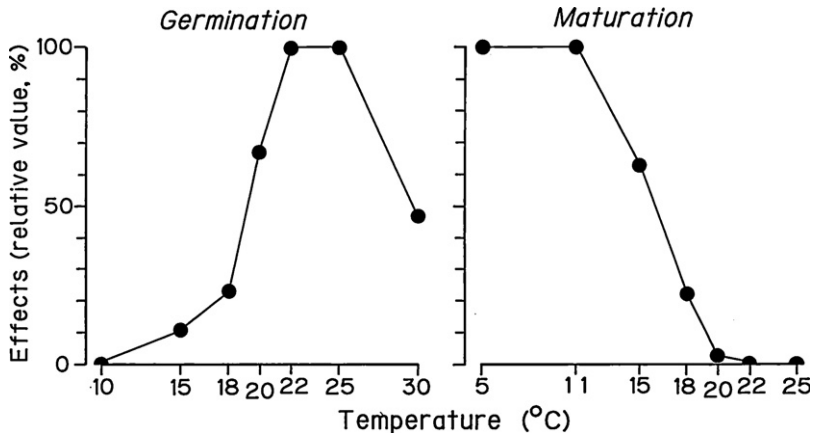

Fig. 18. Effects of incubation temperature on the germination of *Chattonella* cysts, and of storage temperature on maturation (acquisition of germination ability) of the cysts in sediments. The ordinates indicate the percentagae of maximum value obtained with experiments.
After Imai et al. (1991a).

are usual in winter and of 25 °C or higher in summer. According to field observations and laboratory experiments using sediment samples collected from the Seto Inland Sea, it was confirmed that the temperature is a crucial factor affecting the physiology (dormancy, maturation, germination, etc.) of cysts of *Chattonella* (Imai and Itoh, 1987; Imai et al., 1989, 1991a, 1998; Yamaguchi et al., 2008b).

Fig. 18 shows the effects of incubation temperature on the germination of mature cysts of *Chattonella*, and of storage temperature on the maturation (acquisition of the germination ability) of dormant cysts in sediments (Imai et al., 1989, 1991a). The germination of *Chattonella* cysts was not possible at 10 °C, very low at 15 °C and 18 °C, while it increased at 20 °C with maxima at 22 °C and 25 °C, and then decreased markedly at 30 °C. For maturation of immature dormant *Chattonella* cysts, low storage temperature of 11 °C or below for more than four months is needed, whereas no maturation is observed at 20 °C or higher. Storage temperatures of 15 °C and 18 °C are critical for the maturation, viz. a part of cyst populations mature and acquire germination ability. Concerning dormancy of mature cysts (loss of the germination ability), the effects of temperature were furthermore investigated (Imai et al., 1989). When mature cysts of *Chattonella* in sediments are stored at low temperature of 11 °C or below, the germinability is maintained (Fig. 19). They gradually lost the germination ability at 15 °C and 18 °C during storage, and did so rapidly at 20 °C or higher. Thus, the cysts of *Chattonella* have a temperature window for vigorous germination corresponding to the bottom temperature in early summer in the Seto Inland Sea

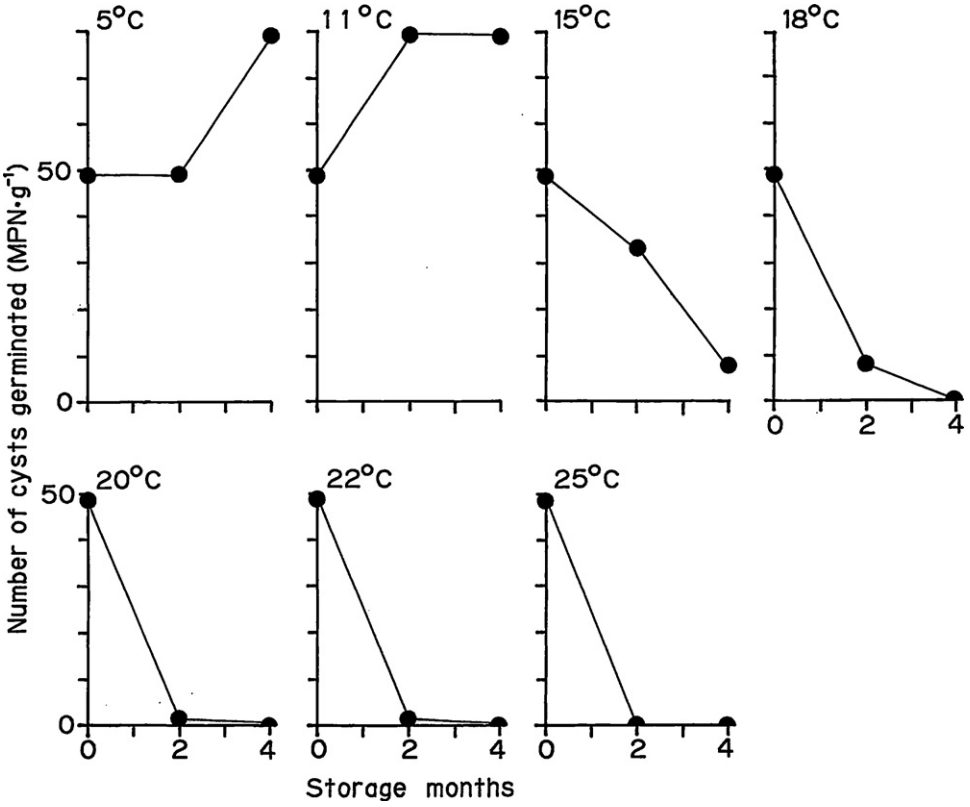

Fig. 19. Effects of different storage temperatures on dormancy induction in the cysts of *Chattonella* in sediments.
After Imai et al. (1989).

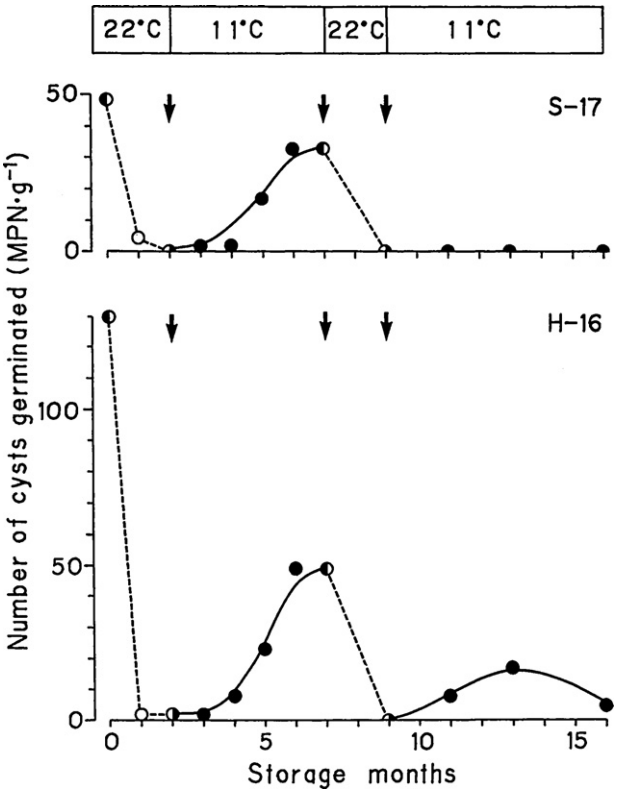

Fig. 20. Effects of shifts of storage temperature on dormancy and maturation in *Chattonella* cysts in sediments. Arrows indicate the point of time of temperature shifts.
After Imai et al. (1989).

(20 °C or higher). On the other hand, immature dormant *Chattonella* cysts require a period of cold winter temperatures for more than four months as a mandatory period for maturation, indicating that the dormancy of *Chattonella* cysts can be regarded as genetically regulated spontaneous dormancy, as is the case in some higher plant seeds.

Fig. 20 demonstrates the effects of shifts of storage temperature on dormancy and maturation of *Chattonella* (*C. antiqua* and *C. marina*) cysts in sediments (Imai et al., 1989). The cysts with germination capacity lost it rapidly within two months when stored at 22 °C. After the lowering the storage temperature to 11 °C, these cysts gradually recovered germinability. Some of the cysts in the sediment of H-16 (Harima-Nada, eastern Seto Inland Sea) were able to recover the germination ability after the twice losing of the ability. The results imply that *Chattonella* cysts buried underneath the sediment surface cannot germinate even at optimal temperature conditions for germination and they are induced into the secondary dormancy to lose germination capacity. The cysts in the state of secondary dormancy experience the second winter, and those cysts again acquire the germination ability by low temperature during winter season and they also play a significant role in summer blooms as seeding populations.

Using freshly collected bottom sediments from Suo-Nada, the Seto Inland Sea, the seasonality of germination ability was investigated for *Chattonella* (*C. antiqua* and *C. marina*) cysts using the extinction dilution method (MPN method) (Imai et al., 1984b; Imai and Itoh, 1987). The cysts of *Chattonella* revealed a marked seasonality in the germination ability (Fig. 21). It was weak from autumn to early winter, strengthened during winter gradually up to a high level, which was maintained between spring and early summer, and again decreased rapidly during summer season. Based on these field data and the temperature characteristics of germination and dormancy in the cysts, the annual life cycle of *Chattonella*, including vegetative and dormant phase, is summarized in Fig. 22 (Imai and Itoh, 1987; Imai et al., 1991a, 1998; Imai, 2004). In the Seto Inland Sea, vegetative cells of *C. antiqua*, *C. marina* and *C. ovata* are basically observed from June to September and occasionally cause red tides, mainly in July and August. These vegetative cells originate from the germination of cysts in the bottom sediments in early summer when the bottom water temperature reaches an adequate level of around 20 °C (Imai et al., 1984a; Imai, 1990). They multiply asexually in summer and produce pre-encystment small cells under unfavorable conditions such as nutrient depletion. These small cells then sink to the sea bottom (Imai et al., 1993b), and cyst formation is completed there after the adhesion to solid surfaces such as diatom frustules and sand grains. The cysts of *Chattonella* spend a period of spontaneous (genetically regulated) dormancy there until the following spring. They never germinate in autumn, even when the bottom water temperature descends to the optimal range for germination, 20–22 °C. The maturation of cyst progresses during winter season. In spring, many cysts complete the period of spontaneous dormancy and already stand by and acquire the ability of germination. From spring to early summer, however, they must spend a period of

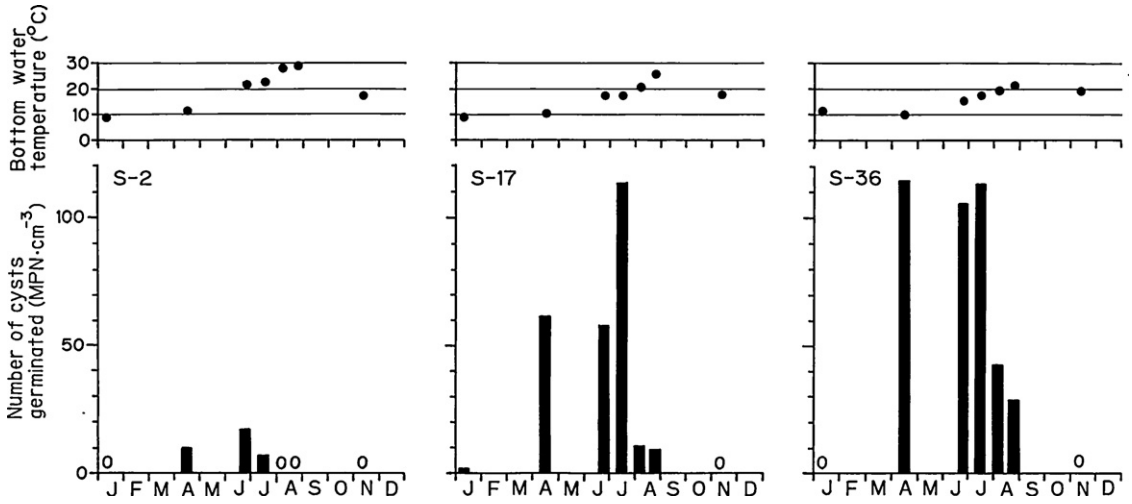

Fig. 21. Seasonal changes in germination ability of *Chattonella* cysts from fresh sediment samples collected at three stations in Suo-Nada, the Seto Inland Sea, in 1985. Cyst germination was measured with the extinction dilution method (Imai et al., 1984b). The changes of bottom water temperatures (1 m above the bottom) are also shown. After Imai and Itoh (1987).

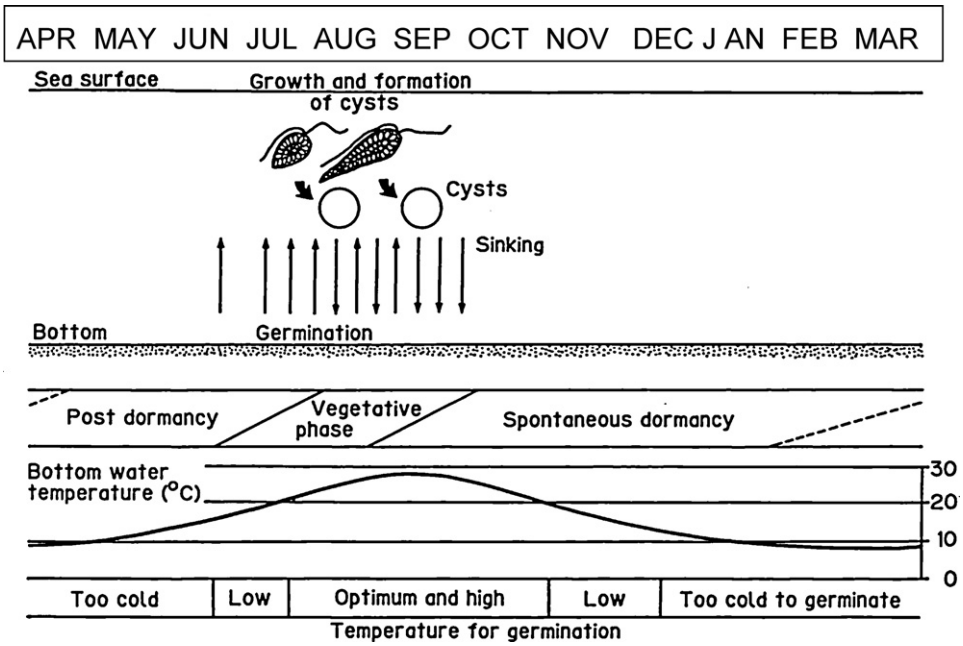

Fig. 22. Schematic representation of the annual life cycle of *Chattonella* in the Seto Inland Sea including vegetative and cyst phases. The seasonal fluctuation of bottom water temperature is also shown.
After Imai and Itoh (1987).

post-dormancy, a kind of enforced dormancy, due to the temperature too low for germination. Vegetative cells appear in the overlying water column thereafter through the germination of cysts. Most of the cyst populations, however, remain in sediments without germination, and they are carried over to the next year, or year to year, via the secondary dormancy which is induced by high temperatures (~22 °C) during the summer season (Imai et al., 1989). Secondary dormancy has already been reported in the resting spores of the diatom *Eunotia soleirolii* (von Stosch and Fecher, 1979). The cysts of *Chattonella* thus can survive without germination for more than two years, indicating that *Chattonella* can form red tides after years with no occurrences of red tides.

As *C. antiqua*, *C. marina* and *C. ovata* are observed basically in the summer season, they spend most of their life as cysts in sediments. In summary, the annual life cycle of *Chattonella* is considered to be well adapted to the seasonal temperature fluctuations in temperate coastal waters such as the Seto Inland Sea. Moreover, the alternation between benthic and planktonic stages is presumably unconstrained in coastal shallow areas, because most part of those sea such as the Seto Inland Sea are shallower than 50 m (Imai and Itoh, 1987; Imai et al., 1991a,b, 1998).

In the Delaware's Inland Bay, Zhang et al. (2006) observed *C. subsalasa* in the temperature range between 17 and 33 °C, with blooms occurring at temperatures between 24 and 31 °C. The cysts of *C. subsalsa* were detected in sediments of the bays by using of the quantitative real-time PCR (Portune et al., 2009). Considering the population dynamics of *C. subsalsa*, cysts probably play an important role in overwintering in the Delaware Bays. It is thought that investigations are urgently needed for *C. subsalsa*.

5.2. Cyst distribution and bloom dynamics of Chattonella in the Seto Inland Sea

Live cysts of *Chattonella* (*C. antiqua* and *C. marina*) can be enumerated by the direct count technique with epifluorescence microscopy by virtue of the autofluorescence of chloroplasts under the observation with blue light excitation as shown in Fig. 13 (Imai and Itoh, 1988; Imai, 1990). Fig. 23 depicts the distribution of the direct counts of live cysts of *Chattonella* in sediments of Suo-Nada, western Seto Inland Sea, in the springs of 1986 and 1987. Densities of the cysts in sediment samples ranged from 0 to 787 cm^{-3} wet sediment (mean 125) in March of 1986, and from 0 to 490 cm^{-3} wet sediment (mean 91) in June of 1987. Spatial distributions of cysts showed similar patterns in both years, i.e. a highly accumulated area was noticed in the eastern-central part of Suo-Nada. However, the high density areas (seed beds) of *Chattonella* cysts do not always coincide with those of vegetative cells during the summer red-tide season (Imai et al., 1986, 1998; Imai, 1990). For example, in Suo-Nada, the red tides are generally found in the western-southern coastal shallow areas (Imai et al., 1986; Imai, 1990). The accumulation mechanisms of cysts are probably different from those of vegetative cells. In the eastern Seto Inland Sea, including Harima-Nada where severe damage to cultured yellowtail fisheries have been caused by red tides of *Chattonella*, the distribution of live cysts of *Chattonella* in sediments were investigated in 1988 and 1989 (Itakura et al., 1991). The cysts were widely detected in the whole area (0–1503 cysts cm^{-3} wet sediment). It was suggested that the abundance of vegetative cells in the preceding year in water column affects the proportion of viable cysts to the total live cysts in the following year.

The dynamics of cysts and vegetative cells of *Chattonella* (*C. antiqua* and *C. marina*) were investigated together with the environmental factors in Suo-Nada. A conceptual model of the process of *Chattonella* red tides in Suo-Nada is presented in Fig. 24 (Imai et al., 1986). The cysts of *Chattonella* presumably start to germinate from the coastal shallow area where the bottom water temperature is always relatively higher than the deeper area and reaches the optimum level (ca. 20 °C) for germination in early June. The inoculated vegetative populations appear as a result of the germination of cysts in the southwestern coastal shallow area in spite of the lower densities of cysts, and multiply there in surface waters. As the bottom water temperature gradually rises from the coastal shallow area to deeper area, the germination of *Chattonella* cysts presumably continues for a rather long period. In midsummer, the cysts in deeper water areas also start to germinate, and new populations start to grow in the surface waters. Accumulation

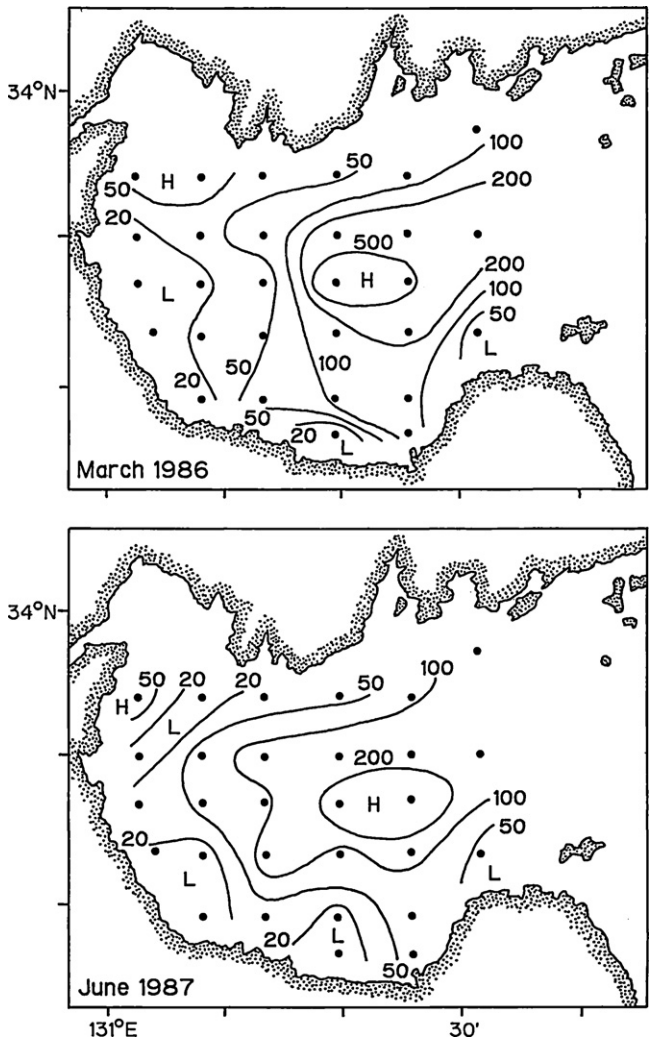

Fig. 23. Distribution of *Chattonella* cysts in Suo-Nada enumerated by the direct count method using epifluorescence microscopy detecting chloroplast autofluorescence under blue light excitation. Numerals indicate the number of cysts per cubic centimeter wet sediment.
After Imai (1990).

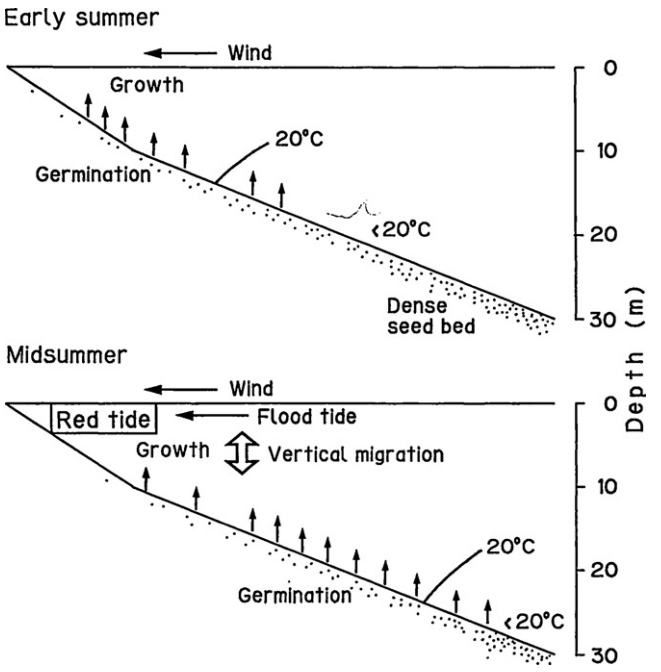

Fig. 24. A schematic representation of the outbreak of *Chattonella* red tide in the south-western coastal area of Suo-Nada, western Seto Inland Sea.
After Imai et al. (1986).

of vegetative cells by winds blowing to the coastal area from offshore in daytime probably play an important role in supporting the population density at a higher level, because motile *Chattonella* cells vertically migrate to the upper layer during daytime (Watanabe et al., 1995).

The mean density (top 3-cm depth) of *Chattonella* cysts is about 100 cm^{-3} wet sediment, being equivalent to about 300 cysts cm^{-2} in sea bottom. Even when a burst germination occurred all at once and provided a large one-time input of vegetative populations early in the bloom, it results in an increase of only 1 cell ml^{-1} or lower in the water column of the upper 5-m layer. Consequently, vegetative growth after inoculation by the germination of cysts is crucially essential for the formation of *Chattonella* red tides. In the case of the *Chattonella* red tide formed in Suo-Nada in the summer of 1987, the temporal changes in the total live cysts and those with the germination ability in freshly collected sediment samples (top l-cm depth) were investigated (Fig. 25), and it is confirmed that the number of cysts possessing the germination ability did not change significantly between late June (before the red tide) and mid July (in the middle stage of red tide), although the number of vegetative cells in the water column drastically increased by about two orders of magnitude from late June to mid July (Imai, 1990; Imai et al., 1998). This fact implies that the number of cysts that actually germinated during the summer red-tide season was small, and that environmental factors affecting the processes after the germination such as vegetative growth, advection, mortality, competition, and encystment, are important for red-tide formation and sequences thereafter, as Anderson et al. (1983) and Imai et al. (1991a,b, 1998) have suggested. After the bloom of *Chattonella*, the density of total live cysts dramatically increased in bottom sediments all over area of Suo-Nada (Fig. 25). The same pattern of increase in cyst densities in sediments after a occurrence of red tide was also confirmed by the *C. antiqua* bloom occurred in Hiroshima Bay, the Seto Inland Sea, in 1990 (Imai et al., 1993a). In this case, the highest densities of the pre-encystment small cells were observed in deeper layers (Fig. 26). Since cyst formation of *C. marina* took place more frequently under the low light intensity conditions or darkness in laboratory experiments (Imai, 1989), the above field observation indicates a significant involvement of negative phototaxis during the course of encystment.

5.3. Diatom resting hypothesis as mechanisms of Chattonella red tide occurrences

When vegetative cells of *Chattonella* appear in the water columns after the germination of cysts at sea bottom in early summer, environmental factors such as nutrients and competitors (mainly diatoms) may crucially affect the development of *Chattonella* populations thereafter. A hypothesis is presented on the occurrence mechanisms of *Chattonella* red tide in Fig. 27 (Imai, 1995; Imai et al., 1998).

It is empirically known that *Chattonella* red tides have been observed when diatoms are scarce (the order of 10^2 cells ml^{-1} or fewer) in surface water (e.g. Yoshimatsu and Ono, 1986; Montani et al., 1989; Nakamura et al., 1989; Imai, 1990; Shikata et al., 2010, 2011; Onitsuka et al., 2011). Moreover, *Chattonella* species often form red tides even in the presence of rather high concentrations of silicate together with the ample amounts of phosphate and nitrate in which the growth of diatoms may not be limited (Montani et al., 1989; Nakamura et al., 1989).

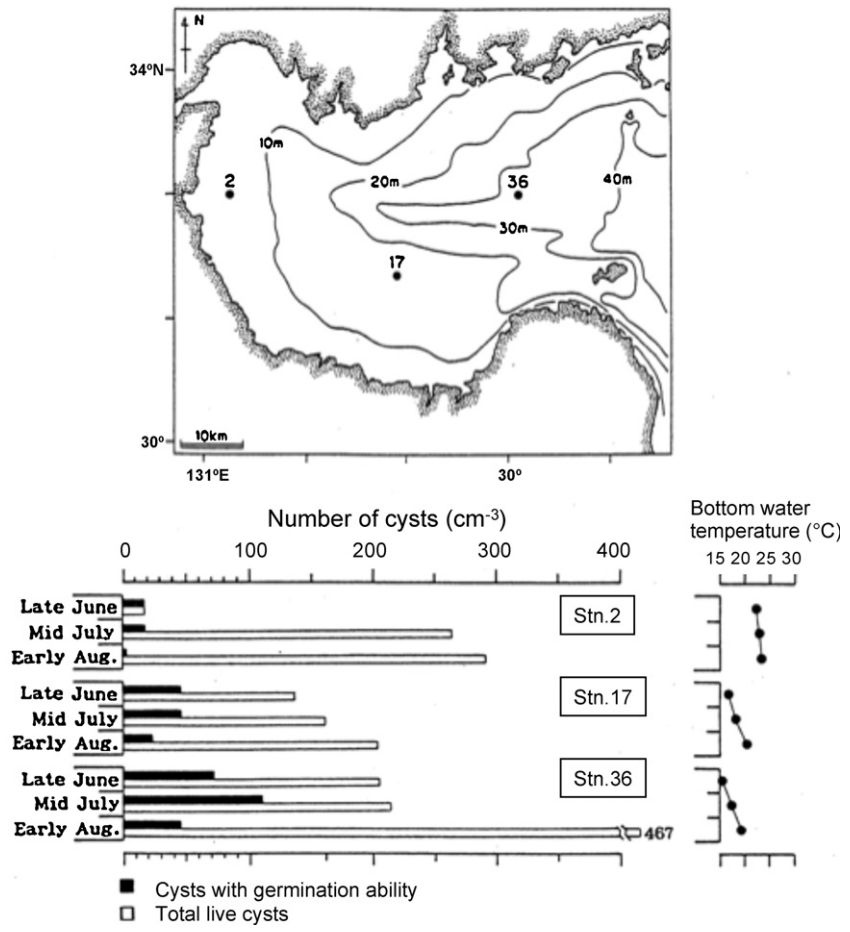

Fig. 25. Temporal changes in the total live cysts of *Chattonella* and those with germination ability in freshly collected sediment samples at three stations in Suo-Nada during the summer of 1987. The changes of bottom water temperature are also shown.
After Imai (1990) and Imai et al. (1998).

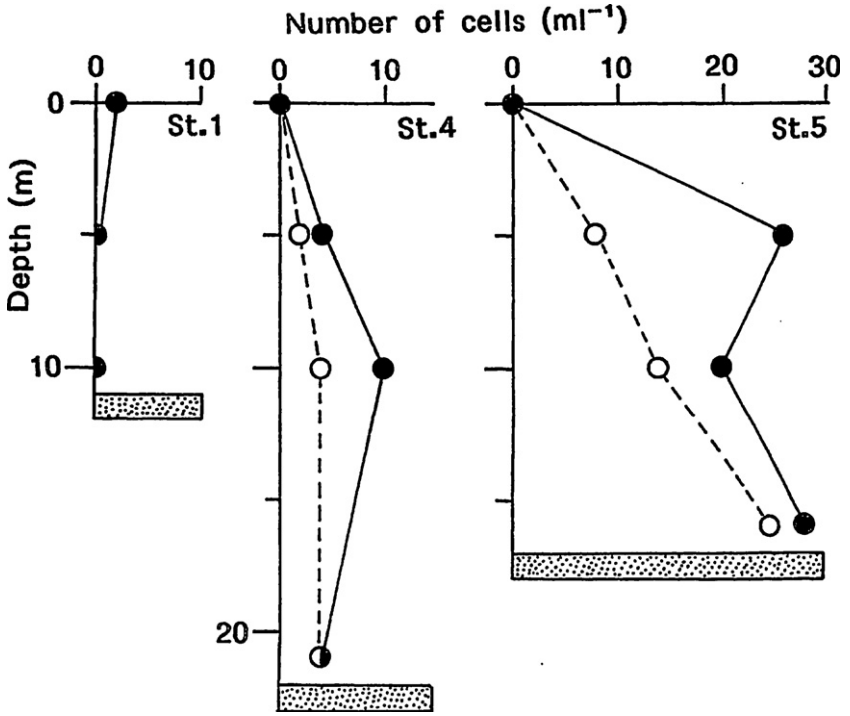

Fig. 26. Vertical profiles of *Chattonella* cell densities of total cells (filled circles) and pre-encystment small cells (open circles) in northern Hiroshima Bay on 10 September 1990.
After Imai et al. (1993a).

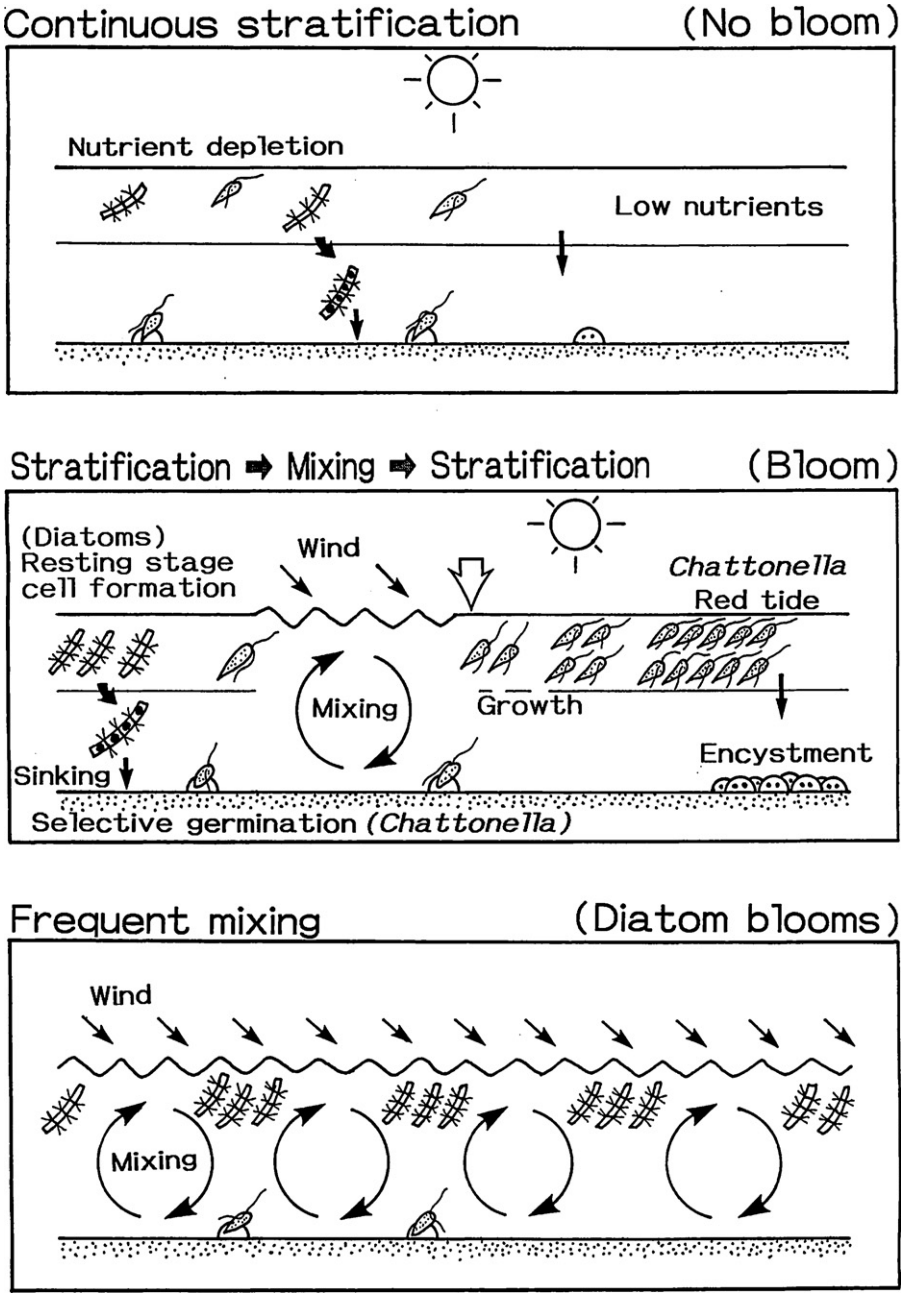

Fig. 27. A schematic representation of the "Daitom Resting Hypothesis" for the scenario on the occurrences of *Chattonella* red tides in the Seto Inland Sea in the summer season.
After Imai (1995) and Imai et al. (1998).

As competitors for nutrients, the diatoms (mainly Centrales) theoretically dominate over *Chattonella* because diatoms usually have higher growth rates under the same environmental conditions (Eppley, 1977; Yamaguchi, 1994; Shikata et al., 2010). However, the diatoms form resting stage cells under conditions of nutrient limitation especially of nitrogen (Davis et al., 1980; French and Hargraves, 1980; Hargraves and French, 1983; Garrison, 1984; Smetacek, 1985; Itakura et al., 1993). Itakura and Imai (1994) found that resting spores of *Chaetoceros* spp. were observed more frequently in areas at low ambient DIN concentrations (<1 μM) in Harima-Nada, eastern Seto Inland Sea. Resting stage cells rapidly sink to the sea bottom, and exist at densities of 10^3–10^6 g^{-1} wet sediment (Imai et al., 1990; Itakura et al., 1997, 1999; Itakura, 2000). Accordingly, the cell densities of diatoms can be reduced in water columns via resting stage cell formation and sinking, after a strong stratification and the resultant exhaustion of nutrients by diatoms in the surface layer. The diatom resting stage cells cannot generally germinate and/or rejuvenate at low light intensities or in the dark (Hollibaugh et al., 1981; Hargraves and French, 1983; Garrison, 1984; Imai et al., 1996), whereas *Chattonella* cysts can germinate in the dark (Imai et al., 1984a; Imai, 1995; Ichimi et al., 2003). This allows the continuous and selective seeding (inoculation) of *Chattonella* populations to the surface water. The diatoms which survive in surface water might be in a physiologically less active state induced by nutrient depletion (Kuwata and Takahashi, 1990), and downsized diatom cells beyond the limit have no way to survive in water columns. Moreover, the capacity for vertical migration could aid in the growth and accumulation of *Chattonella* populations in a stratified water column with a shallow nutricline (thermocline) (Watanabe et al., 1995; Shikata et al., 2011).

Because of the decrease of diatoms in the surface water (euphotic layer), *Chattonella* spp. obtain a competitive advantage, and thus can become dominant despite lower growth rates of at most 1 division day^{-1} than diatoms, after mixing events with supply of nutrients to the surface layer and/or inflowing river water. Yanagi (1989) previously pointed out by the analysis of weather and marine conditions that mixing events by strong winds frequently contribute to the occurrences of *Chattonella* red tides in the south part of Harima-Nada, the Seto Inland Sea of Japan. When the nitrogen concentration increased 2 μM by a mixing event and *C. antiqua* utilized it exclusively, the cell increase was estimated to be 260 cells ml^{-1} based on the minimum cell quota (q_0) of *C. antiqua* ($q_0^N = 7.7$ pmol $cell^{-1}$, $q_0^P = 0.6$ pmol $cell^{-1}$) (Nakamura, 1985). *C. antiqua* can theoretically reach this level from 2 cells ml^{-1} within one week with a growth rate of 1 division day^{-1}. The timely mixing event and/or inflow river water (=nutrient supply to the euphotic surface layer), which must occur after an appropriate stratification accompanying the nutrient exhaustion and sinking and/or inactivation of diatoms, is thought to be essential for the formation of *Chattonella* red tides. The existence of primary vegetative populations (1 cell ml^{-1} or more) of *Chattonella* seeded from the germination of cysts and grown thereafter is of course the necessary requirement for this scenario.

6. Biological control of red tides

Since the fisheries damages by *Chattonella* (especially *C. antiqua*, *C. marina* and *C. ovata*) are huge amounts of tremendous magnitude, it is urgently needed to develop countermeasures against the red tides in order to reduce negative impacts to aquaculture. Physical and chemical methods had been proposed but no means except for clay scattering were adopted practically due to unpredictable negative effects to coastal ecosystems. Biological control would be promising because impacts to ecosystems are hoped to be milder and more environment-friendly when inhabiting organisms are utilized.

6.1. Utilization of diatoms

Diatoms are very common primary producers in marine ecosystems. If harmless diatoms can be applied, it will be an ideal countermeasure for harmful red tides. As shown in Fig. 28, sediment perturbation is proposed as a strategy for controlling occurrences of noxious red tides of *Chattonella* utilizing diatoms in shallow coastal areas (Imai, 2010a). Sediment perturbation would suspend the diatom resting stage cells releasing from bottom sediments to water columns. As diatom resting-stages need light (at least 5–10 μmol photons m^{-2} s^{-1}) for germination and/or rejuvenation (Imai et al., 1996; Itakura, 2000), released and suspended diatom resting stage cells will hopefully germinate and/or rejuvenate after reaching the euphotic layer. Resultant vegetative cells will rapidly multiply as competitors with very high growth rates to overwhelm the populations of harmful flagellates such as *Chattonella* in water columns. Sediment perturbation using submarine tractor is commonly employed for the improvements of sea bottom environments in coastal areas such as intensive aquaculture areas and eutrophicated embayments in Japan. There is no report about the arising of bad effects after the practical application of sediment perturbation in the sea so far. This strategy is thought to be very environment-friendly, and give almost no risk to coastal ecosystems.

6.2. Utilization of algicidal bacteria

The biological control of red tides by using of grazers such as copepods, bivalves and ciliates had been examined, but the results were minimal because of the huge scale of red tides (Shirota, 1989). On the other hand, microorganisms such as viruses, bacteria and heterotrophic dinoflagellates appear to be promising control agents against red tides, as they can be abundant in marine ecosystems, proliferate rapidly, and sometimes are host-specific (Nakamura et al., 1992; SCOR-IOC, 1998). Here possibility of algicidal bacteria is discussed below mainly focusing on *Chattonella* red tides.

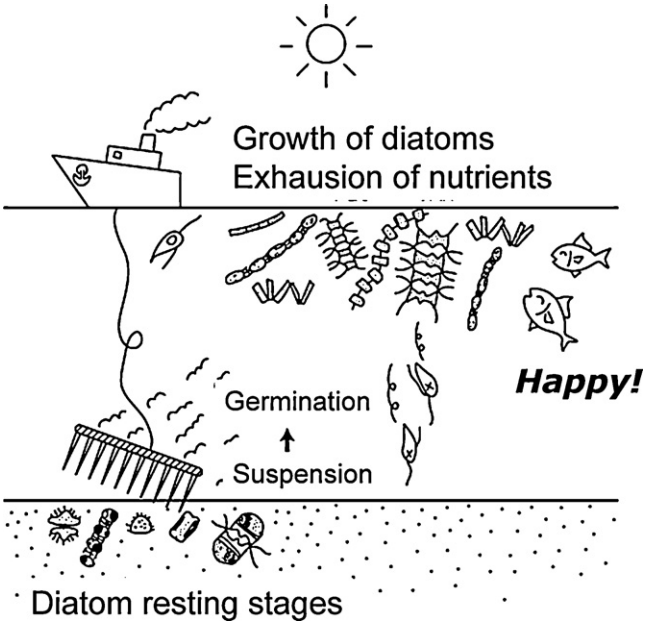

Fig. 28. Sediment perturbation as a proposed prevention strategy for *Chattonella* red tides in coastal sea. Bottom sediments containing numerous diatom resting stages are suspended to euphotic layers by sediment perturbation with submarine tractor or trawling fishing gears, and germinated and/or rejuvenated diatom vegetative cells multiply vigorously and predominate in water columns, resulting in decay of *Chattonella* populations by virtue of nutrient exhausion.
After Imai (2010a).

During the last two decades, algicidal bacteria have been identified in marine coastal ecosystems of the world and have received attention concerning termination of noxious red tides (Imai et al., 1993b; Doucette et al., 1998; Sakata, 2000; Yoshinaga, 2002; Mayali and Azam, 2004; Salomon and Imai, 2006). A bacterium *Cytophaga* sp. J18/M01 that kills *C. antiqua* was isolated from Harima-Nada, the Seto Inland Sea in the summer of 1990 (Imai et al., 1991b, 1993b). When even one cell of this bacterium was applied to a culture of *C. antiqua*, all the cells in culture vessel (~300 ml) were entirely killed within a few days (Fig. 29). Population dynamics of *Chattonella* and the algicidal bacterium *Cytophaga* sp. J18/M01 were investigated by using of the indirect fluorescent antibody technique at a station in northern Harima-Nada (Imai et al., 2001). This bacterium increased accompanying the decline of *Chattonella* populations (Fig. 30), indicating the contribution of algicidal bacteria to rapid terminations of red tides in the coastal seas such as the Seto Inland Sea. The bacterial strains of the same species of *Cytophaga* sp. AA8-2 and AA8-3 were also isolated from Ago Bay, Japan, in the summer of 1995 (Imai et al., 1999). Sohn et al. (2004) reported an algicidal bacterium *Kordia algicida* OT-1 isolated from a red tide in Masan Bay, Korea, and this bacterium showed the same sequences of 16S rRNA gene as *Cytophaga* sp. AA8-2 and AA8-3 (Mayali, 2007). Therefore, the algicidal bacteria *Cytophaga* sp. J18/M01, AA8-2, AA8-3 and *K. algicida* OT-1 appear to inhabit in coastal waters ubiquitously in the world (Yooseph et al., 2010).

Many strains of algicidal bacteria have actually been isolated from various sites of coastal seas of the world (Yoshinaga, 2002;

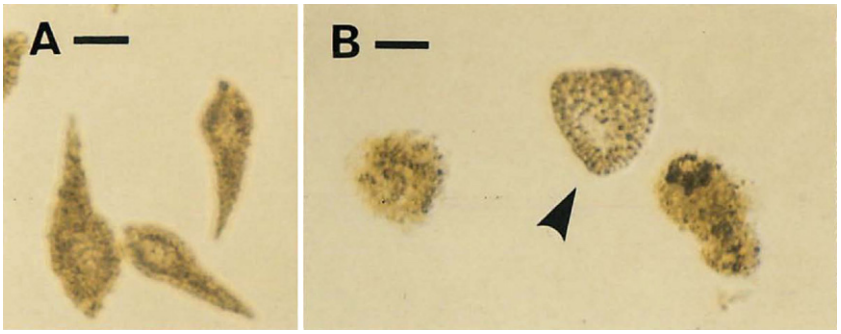

Fig. 29. Algicidal process of the bacterium strain J18/M01 against *Chattonella antiqua*. Scale bar, 30 μm. (A) Normal vegetative cells. (B) A deformed cell (arrow head) and destroyed cells.
After Imai et al. (1991b).

Mayali and Azam, 2004). These bacteria were classified phylogenetically by using of database of SSU rDNA, and many algicidal bacteria were found to be new species. Most of algicidal bacteria are categorized into three groups, α- and γ-proteobacteria (mainly the genera *Alteromonas* and *Pseudoalteromonas*), and *Cytophaga/Flexibacter/Bacteroides* (CFB) group (mainly the genus *Cytophaga*) (Yoshinaga, 2002; Mayali and Azam, 2004; Imai et al., 2006a).

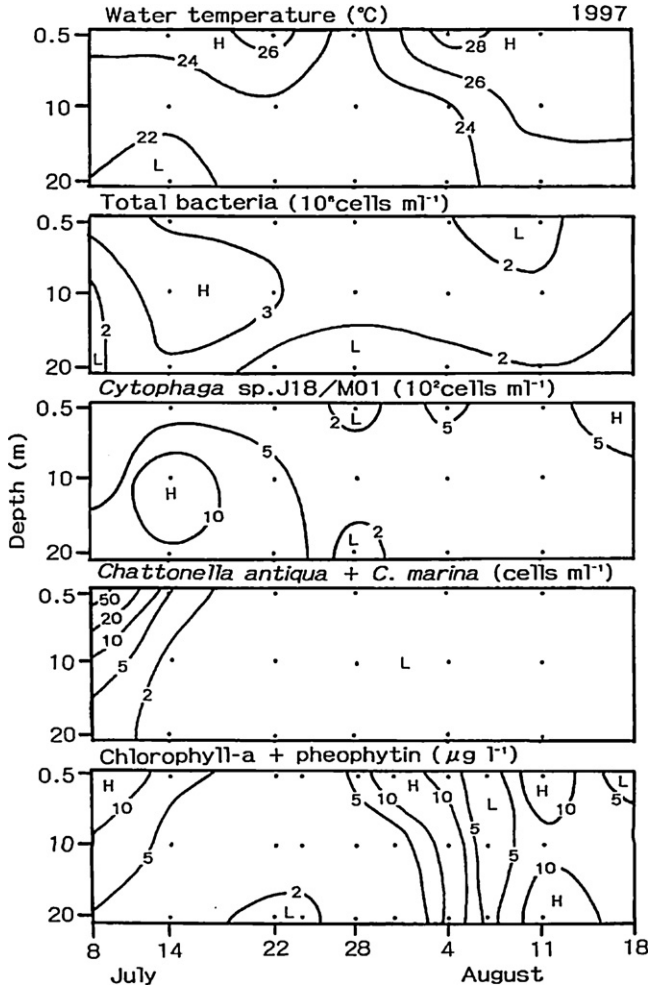

Fig. 30. Changes in vertical profiles of water temperature, total bacteria, algicidal bacterium *Cytophaga* sp. J18/M01, *Chattonella* (*C. antiqua* and *C. marina*) and phytoplankton biomass (chlorophyll *a* + pheophytin) at Stn. NH3 (34°42.8′N, 134°41′E) in northern Harima-Nada, the Seto Inland Sea, during the summer of 1997. The bacterium was monitored with indirect fluorescence assay using antibodies.
After Imai et al. (2001).

6.3. Seaweed and seagrass beds: possible prevention strategies for red tides

As an unexpected aspect of ecology of algicidal bacteria, it was found that huge number of algicidal bacteria attached onto the surface of seaweeds such as *Sargassum* spp., *Ulva* sp. (Chlorophyta) and *Gelidium* sp. (Rhodophyta) (Imai et al., 2002). Maximum number of killers about 10^5–10^6 g^{-1} (wet weight of seaweeds) was detected for *Karenia mikimotoi*, *F. japonica* and *H. akashiwo*. Killer bacteria against *Chattonella* were also detected at the density of the order of 10^3 g^{-1} wet weight. Algicidal bacteria were abundant in seawater collected at seaweed beds in Osaka Bay (the Seto Inland Sea) and Obama Bay (the Sea of Japan) despite no bloom occurrences by relevant microalgal species. Algicidal bacteria were actually isolated from the surface of *Ulva* sp. and *Gelidium* sp. and surrounding seawater (Imai et al., 2006a). These facts indicate a potential of seaweed beds and surrounding seawater for preventing red tide occurrences by the killing function of algicidal bacteria continually released from the surface of seaweeds.

Based on these findings, we can here propose a new prevention strategy of red tides by using of macroalgae (seaweeds) in aquaculture area. Co-culturing of seaweeds such as *Gelidium* sp. and/or *Ulva* sp. and fish such as red sea bream (*Pagrus major*) or yellowtail is proposed to be effective in cage cultures (Imai et al., 2002). Many algicidal bacteria will be continually released from the surface of macroalgae to seawater, and contribute to reduce cell density of phytoplankton including harmful species. Consequently, these bacteria presumably play an important role in preventing the occurrences of noxious red tides. This strategy may be effective in enclosed and small-scale inlets. The most excellent merit of this strategy is that seaweeds give no negative images to fishermen of aquaculture and common consumers. Moreover, *Ulva* sp. is actually being utilized as supplementary foods for rearing red sea breams in some cage cultures of Mie and Ehime Prefectures in Japan.

In a seagrass (*Zostera marina*) bed located in Osaka Bay, the Seto Inland Sea, the existence of algicidal bacteria was investigated to evaluate significance as a source of algicidal bacteria against harmful algal blooms (Imai et al., 2009). The density of algicidal bacteria to each HA species was found to be undetected level (for *H. akashiwo*) to 6.43×10^7 g^{-1} (wet weight) (for *K. mikimotoi*) from the seagrass leaf (Table 3). No microalgal bloom was detected in the seagrass bed during studying period. The killer bacteria against the dinoflagellate *K. mikimotoi* were most abundant, and followed by those against *Cochlodinium polykrikoides*. *C. antiqua* killer bacteria were 9.19×10^6 g^{-1} wet weight leaf. The number of total algicidal bacteria that killed at least one harmful species was 9.12×10^7 g^{-1} (wet weight), and that of the total viable (colony-forming) bacteria was 3.03×10^8 g^{-1} (wet weight), surprisingly indicating that high percentage (30.1%) of isolated bacteria

Table 3
Abundance of algicidal bacteria against 5 species of harmful algal bloom (HAB) detected from the surface of seagrass (*Zostera marina*) leaf and from the seawater of the seagrass bed (particle-associated [PAB] and free-living [FLB] fractions) collected on July 13, 2006.

Target HAB species	Algicidal bacteria		
	Zostera leaf ($\times 10^6$ g^{-1} wet leaf)	Sea water ($\times 10^3$ ml^{-1})	
		PAB	FLB
Chattonella antiqua	9.19	4.8	0
Heterosigma akashiwo	0	2.4	0
Heterocapsa circularisquama	9.19	0	0
Karenia mikimotoi	64.3	2.4	0
Cochlodinium polykrikoides	27.6	2.4	0

possessed algicidal activity from seagrass leaves. In the seawater sample collected at the *Zostera* bed, algicidal bacteria against each harmful species were detected from particle-associated fraction (>3 μm), from undetected level (for *Heterocapsa circularisquama*) to 4.8×10^3 ml^{-1} (for *C. antiqua*). No algicidal bacteria were detected from free-living fraction. In the surface water collected from Harima-Nada, most of algicidal bacteria were detected in the particulate fraction larger than 3 μm (Park et al., 2010). The number of viable bacteria was 6.45×10^4 ml^{-1} in PAB (Particle associated bacteria) and 1.42×10^3 ml^{-1} in FLB (Free-living bacteria), and the number of total algicidal bacteria was 7.19×10^3 ml^{-1} in seawater of the *Zostera* bed. Algicidal bacteria occupied rather high percentage of PAB (11.1%) and the total viable bacteria (10.9%) in seawater of seagrass bed. Hence, it is strongly suggested that algicidal bacteria in *Zostera* beds vigorously kill red tide forming species and these bacteria would play a potentially important role in preventing harmful red tide occurrences in coastal areas.

In coastal areas of Japan such as the Seto Inland Sea, seagrass beds had been lost to 1/4 by reclamations during the era of high-speed economic growth (1960s and 1970s), which would imply the loss of the potential ability for preventing red tide occurrences. On the other hand, eutrophication had intensively progressed in this period, indicating the promotion of red tide occurrences.

As prevention strategies for red tides, artificial restoration and/or developments of seaweed and seagrass beds can be proposed as shown in Fig. 31. Here we newly make an additional proposal of artificial restoration of seagrass beds for preventing red tide occurrences in coastal areas. When we artificially develop and restore the natural seaweed and seagrass beds under the large-scale plan, these newly recovered areas presumably function as tools to prevent the occurrences of harmful algal blooms by virtue of the released algicidal bacteria. And further, seaweed and seagrass beds also serve as purifying grounds of seawater by absorption of inorganic nutrients and nursery grounds for important fishery resources such as fish and invertebrates, and these areas are important components for promoting "Sato-Umi" (Yanagi, 2008) plan for sustainable fisheries in coastal areas. These strategies are environment-friendly and would contribute as ultimate counter measures for red tides. It appears to be worth investigating and discussing about the implementation of artificial developments or restoration of aquatic plant beds around HAB occurring areas in the near future.

7. Future research

Though several issues for future research have been pointed out in several sections, some important problems are pointed out here.

The taxonomy of the species of *Chattonella* appears to be somewhat confused at present. The species *C. minima* was described based on only one culture strain. The discrimination between *C. minima* and *C. marina* is substantially impossible, although the number of chromosome is 90–110 for *C. minima*, about 50 for *C. marina* and 29 for *C. antiqua* (Hara et al., 1994). It is needed to examine chromosome numbers of isolated strains of *C. marina* as many as possible and try to find *C. minima* strains. The second problem in taxonomy is a lack of comparison between the strains of *C. marina* from Japan and India. Considering the cyst physiology of maturation (acquisition of germination ability) needing cold winter season in Japanese *C. marina*, Indian strains of *C. marina* should be compared about the cyst physiology as well as morphological characteristics.

C. subsalsa has relatively shorter history as HAB species than other *Chattonella* species. The investigations are not enough to understand the HAB occurrences of this species, especially on morphology (identification of natural cysts), physiology and ecology of cysts, e.g. effects of environmental factors such as temperature, salinity, irradiance, oxygen, etc. on dormancy,

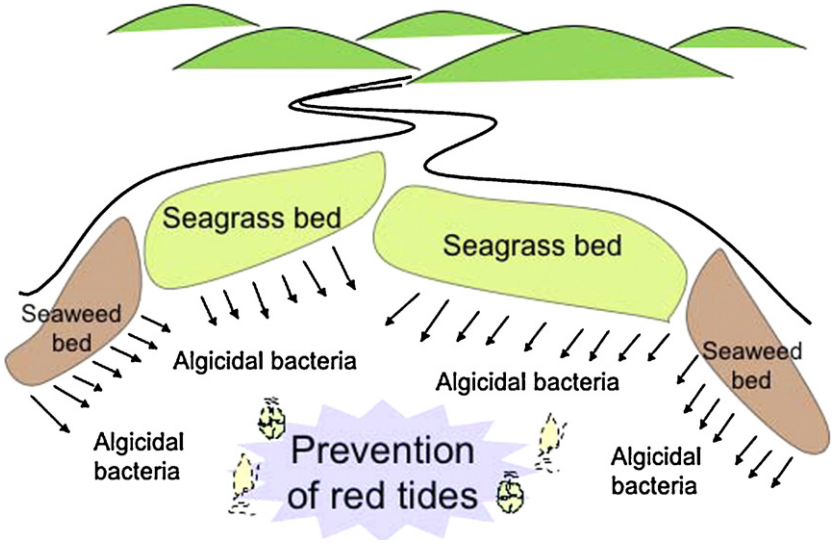

Fig. 31. Schematic representation of strategies for preventing red tide occurrences by restoration and/or development of seaweed- and seagrass beds in coastal areas. Algicidal bacteria would be supplied to seawater and prevent red tide occurrences by virtue of controlling phytoplankton populations within some moderate levels.
After Imai et al. (2009).

maturation, germination and survival of cysts. It is important to grasp the role of cysts in bloom dynamics of *C. subsalsa*. Surveying cyst distribution of *C. subsalasa* in different coastal areas gives information on a potential for HAB occurrences by this species in relevant areas. A pioneer study was carried out for the toxic dinoflagellate *A. tamarense* (Anderson et al., 1982).

As biological agents for controlling occurrences of *Chattonella* red tides, the nutrient competitors diatoms and algicidal bacteria were proposed. Diatoms can be applied by simple bottom sediment perturbation through germination/rejuvenation of resting stage cells suspended into euphotic layer using submarine tractor and/or trawling fishing gear. Algicidal bacteria will be supplied from seaweed and seagrass beds. The application of algicidal bacteria is started to examine by a project of Agriculture, Forestry and Fisheries Ministry of Japan from this year (fiscal year 2011) as an environment-friendly tool for *Chattonella* red tides because *Chattonella* red tides are continually causing severe fish-kill damages in aquaculture areas of Japanese coasts such as Yatsushiro Sea in Kyusyu almost every year.

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