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Lethal effects of ichthyotoxic raphidophytes, *Chattonella marina*, *C. antiqua*, and *Heterosigma akashiwo*, on post-embryonic stages of the Japanese pearl oyster, *Pinctada fucata martensii*

Leila Basti [a,*], Kiyohito Nagai [b], Jiyoji Go [b], Sho Okano [b], Tatsuya Oda [c], Yuji Tanaka [a], Satoshi Nagai [d]

[a] Department of Ocean Sciences, Tokyo University of Marine Science and Technology, Minato, Tokyo 108-8477, Japan
[b] K. Mikimoto & Co. Ltd., Osaki Hazako, Hamajima-Cho, Shima, Mie 517-0403, Japan
[c] Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Nagasaki, Nagasaki 852-8521, Japan
[d] Metagenomics Research Group, National Research Institute of Fisheries Science, Fisheries Research Agency, Fukuura, Kanazawa, Yokohama, Kanagawa 236-8684, Japan

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ABSTRACT

The inimical effects of the ichthyotoxic harmful algal bloom (HAB)-forming raphidophytes *Heterosigma akashiwo*, *Chattonella marina*, and *Chattonella antiqua* on the early-life stages of the Japanese pearl oyster *Pinctada fucata martensii* were studied. Fertilized eggs and developing embryos were not affected following exposure to the harmful raphidophytes; however, all three algal species severely affected trochophores and D-larvae, early-stage D-larvae, and late-stage pre-settling larvae. Exposure to *C. marina* (5×10^2 cells ml^{-1}), *C. antiqua* (10^3 cells ml^{-1}), and *H. akashiwo* (5×10^3 cells ml^{-1}) resulted in decreased success of metamorphosis to the trochophore stage. A complete inhibition of trochophore metamorphosis was observed following exposure to *C. antiqua* at 5×10^3 cells ml^{-1} and *C. marina* at 8×10^3 cells ml^{-1}. In all experiments, more than 80% of newly formed trochophores were anomalous, and in the case of exposure to *H. akashiwo* at 10^5 cells ml^{-1} more than 70% of D-larvae were anomalous. The activity rates of D-larvae (1-day-old) were significantly reduced following exposure to *C. antiqua* (8×10^3 cells ml^{-1}, 24 h), *C. marina* (8×10^3 cells ml^{-1}, 24 h), and *H. akashiwo* (10^4 cells ml^{-1}, 24 h). The activity rates of pre-settling larvae (21-day-old) were also significantly reduced following exposure to *C. antiqua* (10^3 cells ml^{-1}, 24 h), *C. marina* (8×10^3 cells ml^{-1}, 24 h), and *H. akashiwo* (5×10^4 cells ml^{-1}, 24 h). Significant mortalities of both larval stages were induced by all three raphidophytes, with higher mortality rates registered for pre-settling larvae than D-larvae, especially following exposure to *C. marina* (5×10^2–8×10^3 cells ml^{-1}, 48–86 h) and *C. antiqua* (10^3–8×10^3 cells ml^{-1}, 72–86 h). Contact between raphidophyte cells and newly metamorphosed trochophores and D-larvae, 1-day-old D-larvae, and 21-day-old larvae resulted in microscopic changes in the raphidophytes, and then, in the motile early-life stages of pearl oysters. Upon contact and physical disturbance of their cells by larval cilia, *H. akashiwo*, *C. marina* and *C. antiqua* became immotile and shed their glycocalyx. The trochophores and larvae were observed trapped in a conglomerate of glycocalyx and mucus, most probably a mixture of larval mucous and raphidophyte tricosyts and mucocytes. All motile stages of pearl oyster larvae showed a typical escape behavior translating into increased swimming in an effort to release themselves from the sticky mucous traps. The larvae subsequently became exhausted, entrapped in more heavy mucous, lost their larval cilia, sank, become immotile, and died. Although other toxic mediators could have been involved, the results of the present study indicate that all three raphidophytes were harmful only for motile stages of pearl oysters, and that the physical disturbance of their cells upon contact with the ciliary structures of pearl oyster larvae initiated the harmful mechanism. The present study is the first report of lethal effects of harmful *Chattonella* spp. towards larvae of a bivalve mollusc. Blooms of *H. akashiwo*, *C. antiqua* and *C. marina* occur in all major cultivation areas of *P. fucata martensii* during the developmental period of their larvae. Therefore, exposure of the motile early-life stages of Japanese pearl oysters could adversely affect their population recruitment. In addition, the present study shows that

* Corresponding author. Tel.: +81 03 5463 0732; fax: +81 03 5463 0732.
E-mail address: lbasti1@kaiyodai.ac.jp (L. Basti).

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further research with early-life development of pearl oysters and other bivalves could contribute to improving the understanding of the controversial harmful mechanisms of raphidophytes in marine organisms.

1. Introduction

Species of the genera *Chattonella*, *Heterosigma*, and *Fibrocapsa* are cosmopolitan, unicellular, bloom-forming raphidophyceans associated with mass mortality of wild and cultured fish in coastal waters worldwide (Imai et al., 1998). The raphidophytes *Fibrocapsa japonica*, *Heterosigma akashiwo*, and all five species described in the genus *Chattonella* (*C. marina*, *C. antiqua*, *C. subsalsa*, *C. minima*, and *C. ovata*) have formed blooms in Japanese coastal waters with associated fish-kills and serious economic impacts on aquaculture (Yamaguchi, 2008; Imai and Yamaguchi, 2012). Both *C. antiqua* and *C. marina* frequently bloomed and caused mass mortality of fish in the 1970s and 1980s. Their blooms decreased at the beginning of the 1990s, and during 2000s new records of fish-killing were associated with blooms of *C. ovata* (Imai et al., 2006; Yamaguchi et al., 2008). Although in the 1970s, fish-killing events were reported for *F. japonica* in the Seto Inland Sea (Iwasaki, 1971; Okaichi, 1972, 1989; Toriumi and Takano, 1973; Cho et al., 1999), harmful raphidophyte blooms have mainly been associated with *C. marina* and *C. antiqua*; *H. akashiwo* forms recurrent blooms, but rarely causes fish-kills (Imai et al., 2006).

The mechanism of ichthyotoxicity of raphiophytes has been a long-standing controversy. Mechanical clogging of the gills resulting in suffocation (Sakai et al., 1986; Ishimatsu et al., 1990, 1991, 1996; Tsuchiyama et al., 1992; Hishida et al., 1997), production of brevetoxin-like neurotoxin (Onoue et al., 1990; Khan et al., 1995, 1996a, 2008; Furey et al., 2007), of a hemolytic cytotoxin resulting in necrosis of gill cells, and compromised ability of oxygen transport in the blood (Oda et al., 1992; Nakamura et al., 1998; Marshall et al., 2003; Tang and Au, 2004; de Boer et al., 2004, 2009; Kuroda et al., 2005), of reactive oxygen species (ROS) resulting in oxidative stress and lipid peroxidation (Shimada et al., 1989, 1991, 1993; Tanaka et al., 1992, 1994; Oda et al., 1992, 1994, 1995, 1997, 1998; Kim et al., 1999; Kim and Oda, 2010), and the secretion of bioactive exudates and polyunsaturated fatty acids (Marshall et al., 2003; Twiner et al., 2004) have all been proposed as toxic mechanisms, acting separately or in a more complex synergy.

The effects of harmful raphidophytes on invertebrates have received little attention compared to the extensive literature on the effects on fish, in spite of reports of mass mortalities during blooms of *Chattonella* spp. and *Heterosigma akashiwo*. In Japan, mortalities of shrimp (species not specified), short-neck (Manila) clam *Ruditapes philippinarum*, ark clam *Anadara* (*Scapharca*) *kagoshimensis*, Gazami crab *Portunus trituberculatus*, octopus (species not specified), and Japanese Kuruma shrimp *Marsupaeneus japonicus* were documented around the island of Kyushu during blooms of *H. akashiwo*, *Chattonella antiqua*, and mixed blooms of *Chattonella marina* and *Chattonella antiqua* (Kyushu Sea Fisheries Coordination Office, 2004–2013). Lysosomal destabilization in the hepatopancreas of adult eastern oysters (*Crassostrea virginica*) was reported following experimental exposure to *H. akashiwo*, *Chattonellasubsalsa*, and *Fibrocapsa japonica*, indicating that raphidophytes compromise the health of oysters (Keppler et al., 2005, 2006). In another study, exposure of adult green-lipped mussel (*Perna viridis*) to *C. marina* had no effect on antioxidant enzyme activity nor on lipid peroxidation (LPO) suggesting that despite the high levels of hydrogen peroxide (H_2O_2) produced by *C. marina* compared to other raphidophyte species, ROS-associated damage is unlikely to be the toxicological mechanism in adult mussels. The authors concluded that levels of H_2O_2 were below the threshold levels to trigger antioxidant responses and LPO in mussels (Kwok et al., 2012).

The effects of harmful raphidophytes on the reproduction and early-life development of invertebrates, notably bivalve mollucs, have also been very little explored. Most of the studies that have assessed the effects of experimental exposure to harmful algal blooms (HAB) on the early-life development of bivalves have focused on the interactions between harmful dinoflagellates and early-stage larvae (e.g. Yan et al., 2003; Stoecker et al., 2008; Tang and Gobler, 2009, 2012; Basti et al., 2011, 2013, 2015a). In the earliest study, *Heterosigma akashiwo* (5×10^4 cells ml^{-1}, 8–10 h exposure) and *Chattonella antiqua* (10^4 cells ml^{-1}, 8–10 h exposure) had no effect on trochophores of the Pacific oyster *Chattonella gigas* (Matsuyama et al., 2001). Insignificant mortalities of <10%, and <20% of 24-h-old larvae of northern quahog, *Mercenaria mercenaria*, were reported following exposure to *Fibrocapsa japonica* (1.32×10^4 cells ml^{-1}, 3 day-exposure) and *Chattonella marina* (1.07×10^4 cells ml^{-1}, 3 day-exposure), respectively (Tang and Gobler, 2009). Nonetheless, fertilized eggs, 24-h-old larvae, and eye-spot larvae of the bay scallop (*Argopecten irradians*) were affected following exposure to *H. akashiwo* (Wang et al., 2006). The exposure to *H. akashiwo* resulted in decreased metamorphosis of fertilized eggs (5×10^4 cells ml^{-1}, 9–15 h exposure), decreased to inhibited metamorphosis of eggs (10^4–2×10^4 cells ml^{-1}, 24 h exposure), and increased inactivity and mortality of 24-h-old larvae and eye-spot larvae (10^5–2×10^5 cells ml^{-1}, 48–96 h exposure). In a recent preliminary study, 4-cell embryos of Japanese pearl oyster *Pinctada fucata martensii* developed from fertilized eggs exposed to *H. akashiwo* (5×10^4 cells ml^{-1}), *C. marina* (10^4 cells ml^{-1}) or *C. antiqua* (10^4 cells ml^{-1}) for 60 min showed no sign of toxicity to early cleavage, and the D-larvae were also not affected following 24 h of exposure (Basti et al., 2015b). The recurrence and persistence of blooms of harmful raphidophytes in all cultivation areas of the Japanese pearl oyster during the reproductive period of *P. fucata* (discussed further below) compelled further detailed study of the effects of *H. akashiwo*, *C. marina*, and *C. antiqua* on early-life development of the pearl oysters.

The effects of exposures to the three harmful raphidophyte species on fertilized eggs, embryos, early D-larval stage and pre-settling larvae were monitored at several cell densities and over extended exposure durations. The time taken for embryonic and larval development in bivalve molluscs is species-specific and temperature-dependent. At fertilization, Japanese pearl oyster egg undergoes meiotic division with the expulsion of two polar bodies, indicating successful fertilization. Cell division, or cleavage, begins and within 2 h 40 min at 23 °C, the developing embryo from the cleavage of one initial single cell reaches the morula stage, which is a collection of small cells termed blastomeres in a spherical zone arising from the cleavage of the zygote inside the pellucid zone. Within 4 h 40 min to 7 h post-fertilization, the embryo reaches the gastrula stage during which cleavage ends and cells begin to differentiate in a process called gastrulation to give rise to the future organs of the larvae. Within 8 h post-fertilization, the embryo differentiate into a motile larval stage, or trochophore; a spherical to pear-shaped larvae girdled by a ring of cilia or

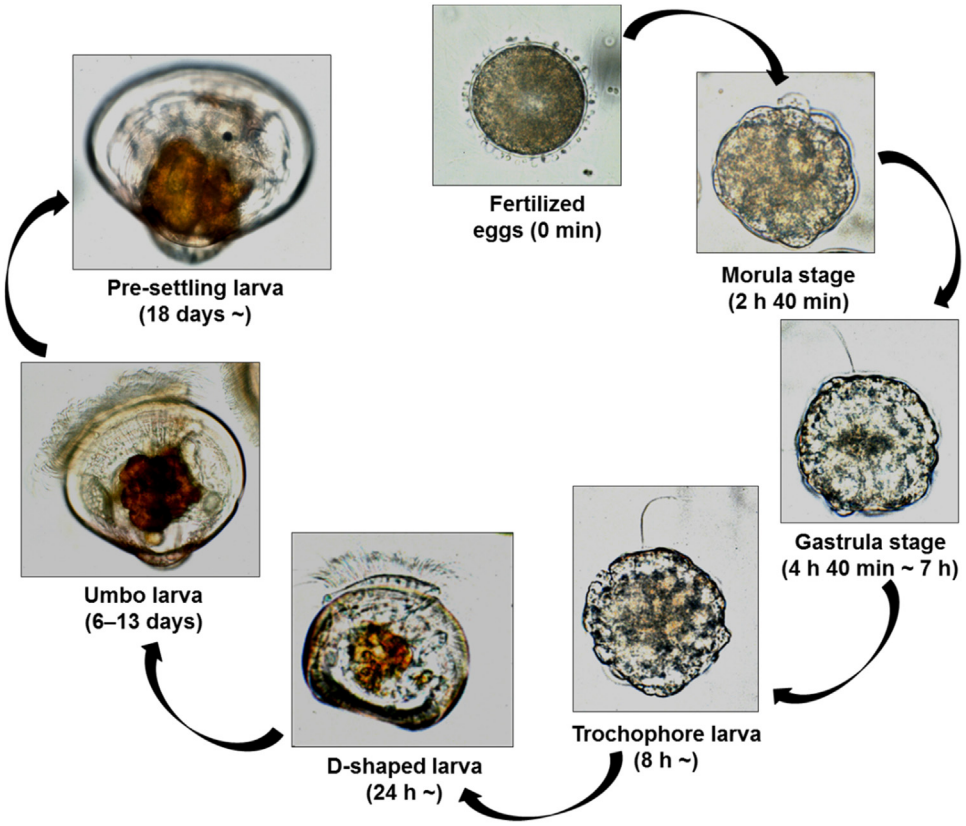

Fig. 1. Early-life development of Japanese pearl oyster *Pincatda fucata martensii*.

prototroch. Trochophores also exhibit a sensory plate, an apical tuft of cilia and an ocellus or a simple eye and have already developed the mouth, stomach, anus, differentiating and partially calcifying shell, and other structures including those involved in homeostasis. Within 24 h, trochophores transform into a straight-hinged D-shaped larvae with fully calcified shell that encloses complete the body of the larva. In D-larvae, the prototroch has transformed into a ciliary velum that insures swimming, feeding and respiration. Larvae continue to develop into eye-spot or umbo larvae within 6 to 13 days, and reach the pre-settling larvae starting from 18 days post-fertilization before settlement, completion of metamorphosis, and then beginning of post-larval development (Fig. 1).

2. Materials and methods

2.1. Pearl oyster rearing and algal culture

Gametes were stripped from gonads of sexually mature pearl oysters *Pinctada fucata martensii* reared at the K. Mikimoto & Co, Ltd. farm located in Ago Bay, Mie Prefecture, Japan. Following activation with ammonia seawater, artificial fertilization was carried out as described in Basti et al. (2011). The raphidophytes *Heterosigma akashiwo*, *Chattonella marina*, and *Chattonella antiqua* were isolated from Ago Bay (2013) and cultured in autoclaved enriched f/2 medium (Chen et al., 1969). All algae were cultured under a 12 h Light:12 h dark photoperiod, at 23 °C.

2.2. Experimental exposures

The exposure experiments were carried out using the experimental design of Basti et al. (2015b). Briefly, freshly fertilized eggs were exposed in duplicate to the harmful raphidophytes, at a density of 50 eggs ml^{-1}, in 6-well plates (5 mL per well). Embryonic development was followed for 7 h, up to the gastrula stage. To determine effects on hatching success, the percentages of fertilized eggs, trochophores and D-larvae were assessed and anomalies were quantified, after 24 h of exposure to the algae.

In another set of experiments, fertilized eggs from the same pool of genitors were washed (1.0-mm pore size filtered and UV-treated seawater) then transferred to 30-L tanks, at 23 °C. Newly developed D-larvae (3-h-old) were used to assess the effects of the harmful raphidophytes on early larvae of Japanese pearl oysters. The 21-day-old pre-settling larvae were commercially grown and provided by K. Mikimoto and Co. Ltd. Both D-larvae and pre-settling larvae were exposed to several densities of each algal species in 6-well plates, at 50 larva ml^{-1} and 25 larva ml^{-1}, respectively (total volume per well = 10 ml). The activity rate of D-larvae and pre-settling larvae were assessed *in vivo* following 24 h and 48 h of exposure, and 24 h, 48 h, 72 h, and 89 h of exposure, respectively. At each assessment time, the well plates were gently agitated to resuspend the larvae, allowed to rest for 5 min and then the number of actively swimming larvae was counted under a light/phase contrast microscope.

2.3. Statistical analyses

Parametric (multivariate and factorial ANOVA) and non-parametric (Kruskal–Wallis ANOVA) analyses were performed followed by post hoc tests (Newman–Keuls or Sign and Wilcoxon tests). Results were considered significantly different at a level of significance, $\alpha = 0.05$.

3. Results

3.1. Effects on fertilized eggs, embryogenesis, and hatching

Fertilized eggs and developing embryos were not affected by exposure to the three species of raphidophytes; however, when

Table 1
Development and anomaly rates of fertilized eggs of *Pinctada fucata martensii* exposed to the harmful raphidopytes, and anomaly rates in resulting trochophores and D-larvae.

Raphidophyte species	Density (cell ml^{-1})	Development rate		Anomaly rate	
		Trochophore	D-larvae	Trochophore	D-larvae
Chattonella antiqua	0	0.07 ± 0.03	0.93 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
	5×10^2	0.09 ± 0.04	0.79 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
	10^3	0.14 ± 0.06	0.79 ± 0.06	0.84 ± 0.17 *	0.00 ± 0.00
	5×10^3	0.95 ± 0.05 *	0.00 ± 0.00 *	1.00 ± 0.00 *	0.00 ± 0.00
	8×10^3	0.00 ± 0.00 *	0.00 ± 0.00 *	1.00 ± 0.00 *	0.00 ± 0.00
Chattonella marina	0	0.00 ± 0.00	0.87 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
	5×10^2	0.39 ± 0.06	0.49 ± 0.01	0.87 ± 0.02 *	0.00 ± 0.00
	10^3	0.76 ± 0.19 *	0.07 ± 0.07	1.00 ± 0.00 *	0.00 ± 0.00
	5×10^3	0.94 ± 0.03 *	0.04 ± 0.01	1.00 ± 0.00 *	0.00 ± 0.00
	8×10^3	0.89 ± 0.10 *	0.00 ± 0.00	1.00 ± 0.00 *	0.00 ± 0.00
Heterosigma akashiwo	0	0.00 ± 0.00	0.94 ± 0.06	0.00 ± 0.00	0.04 ± 0.04
	5×10^3	0.11 ± 0.04	0.87 ± 0.06 *	0.83 ± 0.17 *	0.12 ± 0.03
	10^4	0.14 ± 0.02 *	0.84 ± 0.01 *	0.90 ± 0.10 *	0.11 ± 0.01
	5×10^4	0.13 ± 0.11 *	0.82 ± 0.15 *	0.95 ± 0.04 *	0.11 ± 0.05
	8×10^5	0.48 ± 0.01 *	0.43 ± 0.01 *	0.94 ± 0.06 *	0.79 ± 0.02 *

* Significant difference from respective control ($P < 0.05$).

embryos started metamorphosing into motile trochophores, cell density-dependent inimical effects started to occur (Table 1). The success of metamorphosis into D-larvae was significantly reduced for fertilized eggs exposed to *Chattonella marina*, *Chattonella antiqua*, and *Heterosigma akashiwo* at 5×10^2 cells ml^{-1}, 5×10^3 cells ml^{-1}, and 5×10^3 cells ml^{-1}, respectively. Following 24 h of exposure, the developed trochophores exposed to *C. antiqua* (5×10^3 cells ml^{-1}) and to *C. marina* (8×10^3 cells ml^{-1}) failed to transform into D-larvae. More than 80% of the trochophores from fertilized eggs exposed to *C. antiqua* at 1×10^3 cells ml^{-1}, *C. marina* at 5×10^2 cells ml^{-1}, and *H. akashiwo* at 10^3 cells ml^{-1} were anomalous (Table 1). Also, in the case of fertilized eggs exposed to *C. antiqua* at 5×10^3 cells ml^{-1} and *C. marina* at 10^3 cells ml^{-1}, all trochophores were anomalous. In the case of fertilized eggs exposed to *H. akashiwo*, 11–78% of the D-larvae were anomalous; however, the anomaly rate was significant for only the highest cell density of 10^5 cells ml^{-1}.

All three species of raphidophytes ceased swimming when they came in contact with the motile stages of pearl oysters, both early-trochophores and early-D larval stages, and discharged their glycocalyx. This typical behavior was followed by the appearance of sticky mucus-like traps surrounding the newly developed trochophores and D-larvae hampering their motility. The ciliary structures of both trochophores and D-larvae continued to beat irregularly, but ultimately detachment of the cilia and lytic loss of cytoplasmic masses occurred (Fig. 2).

3.2. Effects of direct exposure on D-larvae and pre-settling larvae

The effects of direct exposures of D-larvae and pre-settling larvae to the three species of raphidophytes on the activity rates and mortality rates are shown in Figs. 3–5 and in Tables 1 and 2. Significant cell-density dependent reductions of the activities of both larval stages were induced by all 3 raphidophytes. The duration of exposure played a significant role in the negative effects on the activity rates of pre-settling larvae in the case of exposures to *Heterosigma akashiwo* and *Chattonella antiqua*, and interactive effects between cell density and duration of exposure were significant in the experimental exposure of pre-settling larvae to *C. antiqua* (Table 2).

The activities of D-larvae decreased following 24 h of exposure to *Chattonella antiqua* and *Chattonella marina* at 8×10^3 cells ml^{-1}, and *Heterosigma akashiwo* at 10^4 cells ml^{-1} (Fig. 3). The activity of the D-larvae significantly decreased by more than 60% following 48 h of exposure to 8×10^3 cells ml^{-1} of *C. marina*, and the reduction was significantly higher than the one induced by *C. antiqua*. The activities of pre-settling larvae decreased significantly following 24 h of exposure to *C. antiqua*, *C. marina*, and *H. akashiwo* at 10^3 cells ml^{-1}, 8×10^3 cells ml^{-1}, and 5×10^4 cells ml^{-1}, respectively (Fig. 4). Comparisons between the activity rates of exposed groups of pre-settling larvae showed a significant difference between the group exposed to 10^2 cells ml^{-1} and the other groups exposed to *C. antiqua*. In the case of *C. marina*, the activity rate of the group of pre-settling larvae exposed to 8×10^3 cells ml^{-1} was significantly different from the others. In the case of *H. akashiwo*, the activity rates of the groups exposed to 5×10^4 cells ml^{-1} and 10^5 cells ml^{-1} were significantly different from the activities of other groups, the activity rates of which were in turn significantly different among each other.

Exposure to the three raphidophytes caused a significant increase in the mortality rates of both early D-larvae and pre-settling larvae (Table 2). The mortalities of D-larvae exposed to *Heterosigma akashiwo* and *Chattonella marina* were cell density-dependent, whereas the mortalities of D-larvae exposed to *Chattonella antiqua* were dependent on the duration of exposure. In the case of pre-settling larvae, both the cell density and the duration of exposure had significant effects on the mortalities of the larvae exposed to *C. marina* and *C. antiqua*, whereas the mortalities of those larvae exposed to *H. akashiwo* were dependent on the duration of exposure. Additionally, cell density and duration of exposure played a synergistic effect in the mortality of pre-settling larvae exposed to *C. antiqua*.

Mortalities of D-larvae, although significant, were less than 6% in the exposure experiments to *Heterosigma akashiwo* and *Chattonella antiqua*. They reached almost 35% of mortality following 48 h of exposure to *Chattonella marina* at 8×10^3 cells ml^{-1} (Table 3). Higher mortalities were recorded for pre-settling larvae exposed to all three raphidophytes than for D-larvae (Fig. 5). Exposure to *Chattonella* spp. induced a significant increase of mortalities of pre-settling larvae following 72 h of exposure to *C. antiqua* at 10^3 cells ml^{-1}, and following 48 h of exposure to *C. marina* at 5×10^2 cells ml^{-1}. The highest mortalities of 38% and 34% were registered following 89 h of exposure to *C. marina* and *C. antiqua* at 8×10^3 cells ml^{-1}, and overall there was a significant difference in the mortalities of pre-settling larvae among the two species of *Chattonella*. For both experiments involving exposures to *Chattonella* spp., the mortality of pre-settling larvae exposed to 8×10^3 cells ml^{-1} were significantly

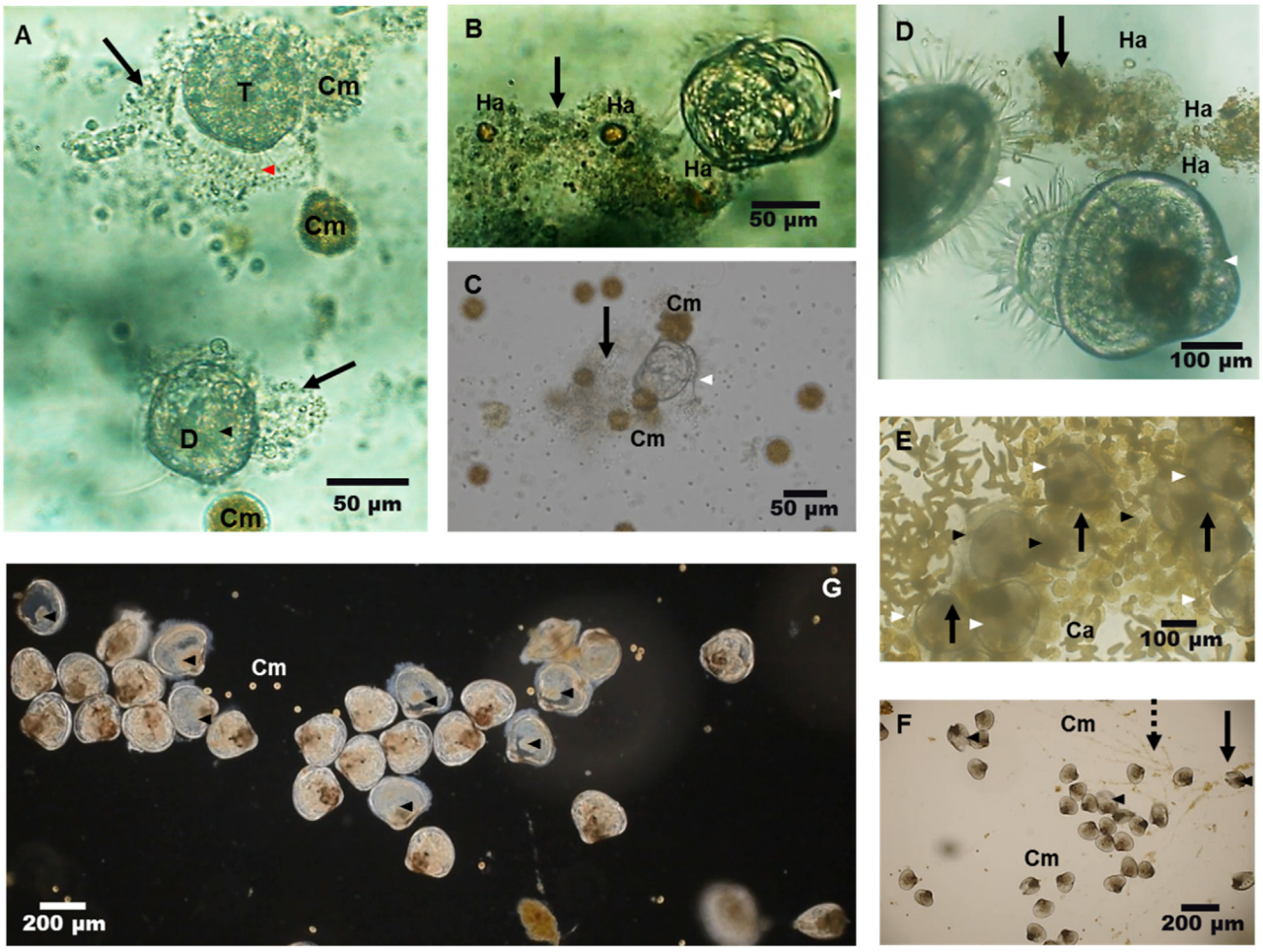

Fig. 2. Light micrographs of abnormal trochophores and D-larvae of *Pinctada fucata martensii* exposed to the harmful raphidophytes. Late trochophore and early D-larvae newly metamorphosed from fertilized eggs of pearl oysters exposed to *Chattonella marina* (A). D-larvae trapped in mucus during exposure to *Heterosigma akashiwo* (B) and *Chattonella marina* (C). Pre-settling larvae trapped in mucus during exposure to *Heterosigma akashiwo* (D), *Chattonella antiqua* (F), and *Chattonella marina* (F, G). D: D-larvae, T: trochophore, Ha: *H. akashiwo* cells, Ca: *C. antiqua* cells, Cm: *C. marina* cells. Black arrows: mucus traps, dashed-arrows: mucus strings, red arrow head: abnormal velum with physical damage, white arrow head: live larvae trapped in mucus, black arrow heads: dead larvae trapped in mucus.

different from the other groups of exposures. In the case of exposure to *H. akashiwo*, the highest mortality rate of 14% was recorded following 89 h of exposure to 10^5 cells ml^{-1} (Fig. 5).

Similar to trochophores and D-larvae developed from exposed fertilized eggs, both 3-h old D-larvae and 21-day-old pre-settling larvae directly exposed to the raphidophytes were observed surrounded by sticky mucus-like traps after the three raphidophyte species came into contact with the larvae, liberated their glycocalix, and became immotile (Fig. 2).

4. Discussion

The present study is the first report of toxic effects of two *Chattonella* species on the early-life development of a bivalve mollusc. In previous studies, exposure to *Chattonella antiqua* did not affect trochophores of the Pacific oyster *Crassostrea gigas* (Matsuyama et al., 2001), and exposure to *C. marina* did not affect 24-h-old D-stage larvae of the northern quahog *Mercenaria mercenaria* (Tang and Gobler, 2012). Harmful effects of *Heterosigma akashiwo* on early-life development of the bay scallop *Argopecten irrandians* reported in one previous study (Wang et al., 2006) are also shown in the present study for Japanese pearl oysters.

Although the experiments performed in the present study do not fully elucidate the complex mechanism involved in the harmful effects of raphidophytes, it was demonstrated that exposure to *Heterosigma akashiwo*, *Chattonella marina* and *Chattonella antiqua* affected only the motile stages of the early-life development of the Japanese pearl oyster. Increased anomalies of trochophores, and decreased motility and survivorship of D-larvae (3-h-old) and pre-settling larvae (21-day-old) were caused by exposures to the three raphidophyte species. The toxicity mechanism of harmful raphidophytes remains unclear. It is known that *H. akashiwo* and *Chattonella* spp. produce a variety of harmful byproducts, and the inimical effects reported in the present study could have been the result of a synergistic interaction between several extracellular, cell surface-located, and intracellular toxic metabolites, activated following physical disturbance of the cell surface of raphidophytes.

The production of brevetoxin-like phycotoxins by raphidophytes, in both cultures and bloom waters, was proposed as a toxic mechanism responsible for fish death (Onoue et al., 1990; Endo et al., 1992; Khan et al., 1996b, 1997), and for sublethal effects in adult eastern oysters *Crassostrea virginica* (Keppler et al., 2006). Another species of harmful algae notorious for the production of brevetoxins is *Karenia brevis*. This naked dinoflagellate forms recurrent red tides in the Gulf of Mexico associated with outbreaks of neurotoxic shellfish poisoning (NSP) and episodic mortalities of aquatic organisms including birds and mammals. Recently, *K. brevis* has been reported to affect the early-life development of the eastern oyster *C. virginica*, and the northern quahog *Mercenaria*

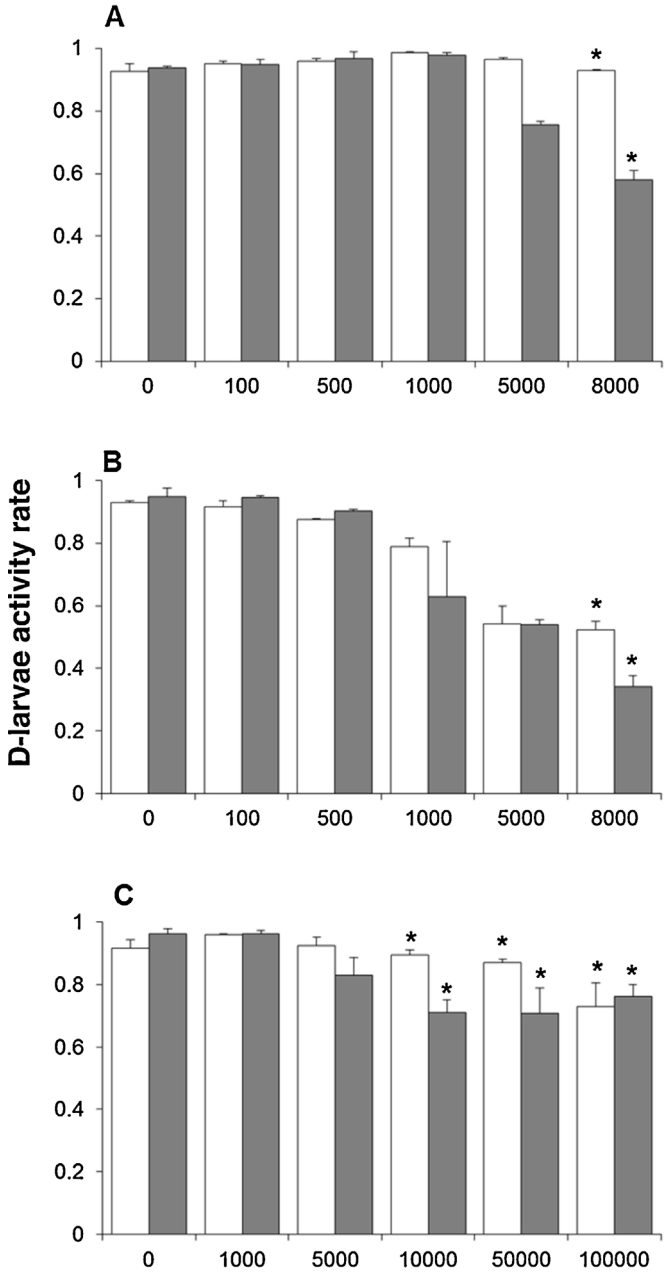

Fig. 3. Activity rate of 3h-old D-larvae of *Pinctada fucata martensii* exposed to (A) *Chattonella antiqua*, (B) *Chattonella marina*, and (C) *Heterosigma akashiwo*. (*) Significant difference from respective control ($P < 0.05$). (□) 24 h post-exposure, (■) 48 h post-exposure.

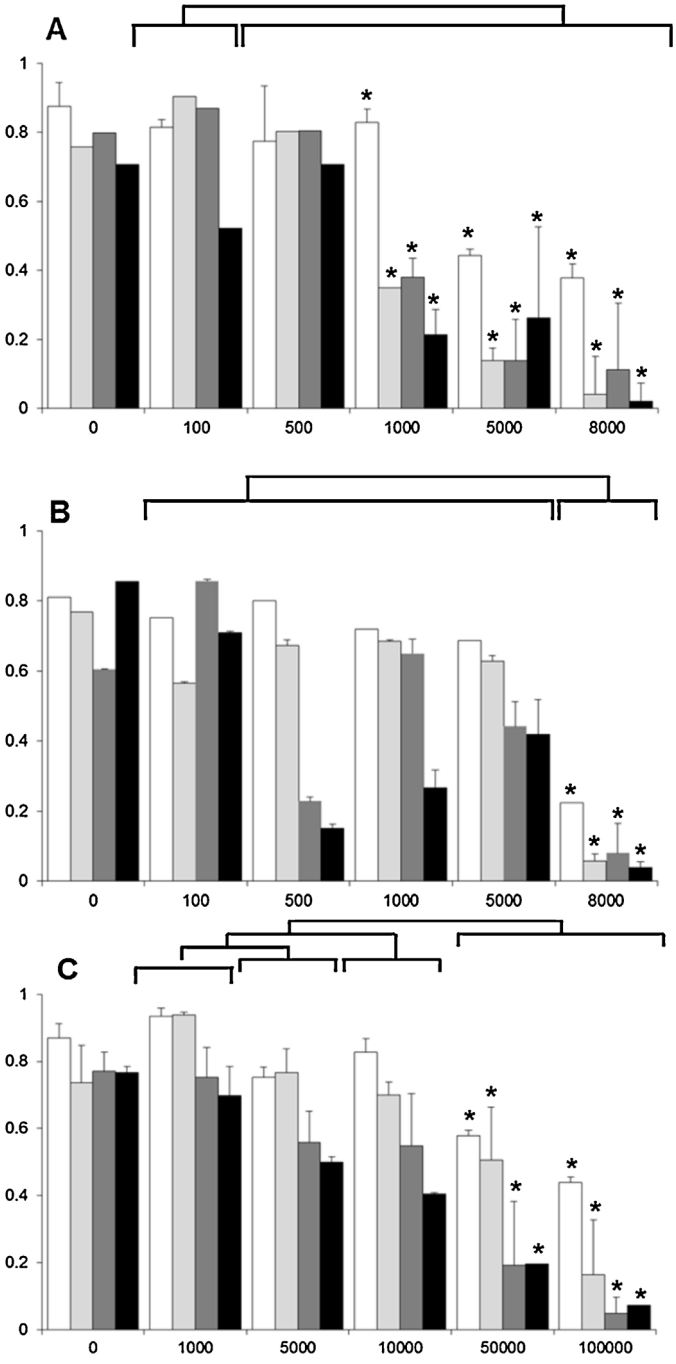

Fig. 4. Activity rate of pre-setting larvae of *Pinctada fucata martensii* exposed to (A) *Chattonella antiqua*, (B) *Chattonella marina*, and (C) *Heterosigma akashiwo*. (*) Significant difference from respective control ($P < 0.05$). (□) 24 h post-exposure, (■) 48 h post-exposure, (■) 72 h post-exposure, and (■) 89 h post-exposure. Different clusters mark significant difference between exposure groups.

mercenaria (Rolton et al., 2014, 2015). The negative effects, however, occurred mainly during embryogenesis and early development, and were more pronounced with lysed cells than with whole intact cells. The authors concluded that, in addition to brevetoxins, other unidentified bioactive compounds could have been associated with the negative effects of *K. brevis*. In the present study, fertilized eggs and developing embryos of pearl oysters were not affected following exposure to any of the harmful raphidophyte species. Only when embryos reached the motile trochophore stage did the inimical effects occur, concomitant with lysis of the fragile raphidophyte cells. Recent laboratory experiments revealed that exposure to *H. akashiwo* and to its extract, HaTx, caused paralysis in the brine shrimp, *Artemia salina* (Astuya et al., 2015). Furthermore, HaTx demonstrated cellular and neural toxic properties in mammalian cells that were mediated by a fast and reversible blockade of voltage-dependent sodium channels (VDSCs) similar to the tetrodotoxin (TTX) action, suggesting that the extract HaTx is closely related to brevenals, the brevetoxin antagonists (Astuya et al., 2015). Lysate of raphidophytes liberated in the experimental medium, containing reactive compounds including brevetoxins or their antagonists, could explain the toxicity in larval stages, following either excitation or blockade of VDSCs and alteration in sodium homeostasis. Nonetheless, the production of brevetoxin-like compounds by harmful raphidophytes still awaits further confirmation with LC-MS analyses, and several authors have demonstrated low concentrations or even the

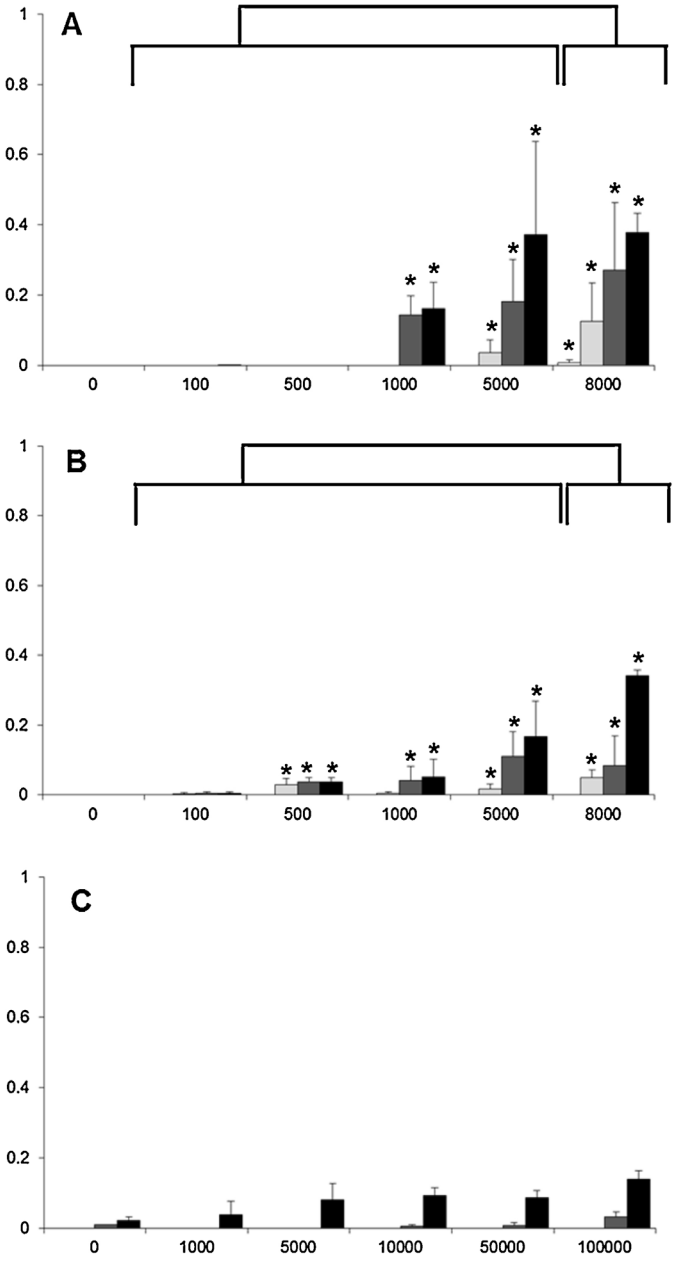

Fig. 5. Mortality rate of pre-setting larvae of *Pinctada fucata martensii* exposed to (A) *Chattonella antiqua*, (B) *Chattonella marina*, and(C) *Heterosigma akashiwo*. (*) Significant difference from respective control ($P < 0.05$). (□) 24 h post-exposure, (■) 48 h post-exposure, (■) 72 h post-exposure, and (■) 89 h post-exposure. Different clusters mark significant difference between exposure groups.

lack of these neurotoxin-like compounds in raphidophytes (Khan et al., 1996b; Lewitus and Holland, 2003; de Boer et al., 2012).

Following the initial cell contact between pearl oyster larvae and raphidophytes, the cells of the latter liberated their cell surface, the glycocalyx, which induced entrapment of larvae in heavy mucus, seemingly a mixture of sticky glycocalyx and larval mucus. The same process was observed during experimental exposures of D-larvae and pre-settling larvae which showed typical escape behavior translating into active swimming before succumbing to exhaustion, and sinking to the bottom of the experimental wells. Suffocation via physical clogging of fish gills by *Chattonella* and *Heterosigma akashiwo* cells and mucus secretion was the earliest proposed cause of fish-killing mechanisms (Matsusato and Kobayashi, 1974). It is still unclear whether the mucus is produced solely by fish gills or is a mixture of fish gill mucus and cell secretions by the raphidophyte, which seems the most plausible explanation in the present study. Death of oyster larvae occurred at a later stage, generally within 2–6 h and 24–48 h following entrapment of trochophores, and D-larvae and pre-settling larvae in the mucus, respectively. These results suggest that other toxic substances were perhaps at play. The generation of reactive oxygen species (ROS), including superoxide and hydroxide radicals, was demonstrated for harmful raphidophytes, and an increasing body of literature has demonstrated that ROS production causes injuries and induces hypersecretion of mucous in fish gills leading to death, following epithelial lifting, cell necrosis, and alteration of chloride cells resulting in massive mucus production in the gills and alterations of physiological responses such as hypoxia and subsequent anoxia (Tanaka et al., 1994; Ishimatsu et al., 1996; Oda et al., 1997, 1998; Kim et al., 2000, 2007; Twiner et al., 2001; Kuroda et al., 2005; Marshall et al., 2005; Jugnu and Kripa, 2009). The glycocalyx, or cell surface structure of raphidophytes, particularly in *Chattonella marina* was shown to generate NADPH-dependent superoxide ions, $.O_2^-$, and is easily discharged when physically stimulated (Kim and Oda, 2010). Among several harmful and ichthyotoxic HAB species tested, the greatest producers of superoxide are raphidophytes of the genus *Chattonella* (Marshall et al., 2005), which in the present study had the strongest harmful effects on larvae of pearl oysters. In addition, free fatty acids (FFAs) acting as hemolytic and hemo-agglutinating compounds were also suspected in the early 1990s (Okaichi, 1980; Shimada et al., 1983). The synergistic role of ROS and FFAs in fish-kill mechanisms has been shown for *C. marina* (Marshall et al., 2003). In a more recent study, the quantitative role of ROS, FFAs and characterized phycotoxins were compared among seven species of HAB associated with fish-kills: the raphidophytes *Fibrocapsa japonica*, *Chattonella marina* and *H. akashiwo*, the toxic dinoflagellates *Alexandrium catenella* (paralytic shellfish toxins, PSP) and *Karlodinium veneficum* (karlotoxins, KmTxs), the haptophyte *Prymnesium parvum* (prymnesins), and the harmful dinoflagellate *Karenia mikimotoi*. The *in vitro* fish gill assay revealed that

Table 2
Statistical analyses (Multivariate ANOVA, Factorial ANOVA, Kruskal–Wallis ANOVA) of the effects of the different parameters of evaluation on the activity and mortality rates of D-larvae (DL) and pre-settling larvae (PL) exposed to the harmful raphidophytes, *Heterosigma akashiwo*, *Chattonella marina*, and *Chattonella antiqua*.

	Harmful algal species	Activity			Mortality		
		Treatments			Treatments		
		Cell density	Duration	Interaction	Cell density	Duration	Interaction
DL	*Heterosigma akashiwo*	**	NS	NS	*	NS	NS
	Chattonella marina	**	NS	NS	**	NS	NS
	Chattonella antiqua	*	NS	NS	NS	**	NS
PL	*Heterosigma akashiwo*	**	**	NS	NS	*	NS
	Chattonella marina	**	NS	NS	*	**	NS
	Chattonella antiqua	**	**	*	**	*	*

NS: non significant effect, (*) $P < 0.05$, (**) $P < 0.01$.

Table 3
Mortality rates of D-stage larvae of *Pinctada fucata martensii* exposed to the harmful raphidophytes, *Heterosigma akashiwo*, *Chattonella marina*, and *Chattonella antiqua*.

Harmful algal species	Density (cell ml^{-1})	D-larvae	
		24 h post-exposure	48 h post-exposure
Chattonella antiqua	0	0.006 ± 0.002	0.012 ± 0.001
	10^2	0.007 ± 0.005	0.009 ± 0.001
	5×10^2	0.003 ± 0.001	0.005 ± 0.005
	10^3	0.003 ± 0.001	0.005 ± 0.003
	5×10^3	0.003 ± 0.001	0.024 ± 0.002
	8×10^3	0.002 ± 0.002	0.058 ± 0.017
Chattonella marina	0	0.021 ± 0.017	0.004 ± 0.001
	10^2	0.027 ± 0.008	0.028 ± 0.001
	5×10^2	0.043 ± 0.009	0.056 ± 0.018
	10^3	0.061 ± 0.023	0.097 ± 0.067
	5×10^3	0.034 ± 0.015	0.151 ± 0.001
	8×10^3	0.055 ± 0.007*	0.341 ± 0.231*
Heterosigma akashiwo	0	0.008 ± 0.006	0.006 ± 0.001
	10^3	0.014 ± 0.001	0.005 ± 0.004
	5×10^3	0.017 ± 0.005	0.008 ± 0.001
	10^4	0.010 ± 0.011	0.019 ± 0.010
	5×10^4	0.016 ± 0.011*	0.030 ± 0.013*
	8×10^5	0.039 ± 0.003*	0.43 ± 0.003*

* Significant difference from respective control ($P < 0.05$).

the two strains of *C. marina*, isolated from Australia and Japan, were the most toxic and the major producer of superoxide radicals; however, these ROS may also have caused synergistic effects with fatty acids of *C. marina* producing other toxic compounds through lipid peroxidation. In the case of *F. japonica* and *H. akashiwo*, other uncharacterized toxic compounds with a lipid component were involved (Dorantes-Aranda et al., 2015). The production of nitric oxide (NO) substances in the perikaryon of *C. antiqua* and in the cytoplasm of *C. marina* was demonstrated, and their involvement in fish-kills, potentially acting in synergy with ROS, was also proposed (Shimada et al., 1986; Kim et al., 2006). Nitric oxide is known to enhance mucus secretion in gastric mucus-cell fraction (Brown et al., 1993) as well as to oxidize hemoglobin into methemoglobin resulting in hypoxia (Vedel et al., 1998; Lee et al., 2003; Kim et al., 2006). The involvement of ROS, NO and FFAs in the hypersection of mucus and subsequent death of larvae of pearl oysters should be further explored.

The involvement of cell-contact in the toxicity of HAB to aquatic organisms has been described for several dinoflagellate species, notably in the allelopathic effects they exert on other competing phytoplankton species and potential grazers (Uchida et al., 1996; Yamasaki et al., 2011). In the case of the highly potent shellfish-killing dinoflagellate, *Heterocapsa circularisquama*, cell contact with early-life stages of bivalve molluscs was established as a primordial step in the initiation of broad cytotoxicity in gametes, embryos, and larvae (Matsuyama, 2003; Matsuyama et al., 2001; Basti et al., 2011, 2013). The main difference with raphidophytes is that *H. circulasiquama* liberates its outer cell wall when in contact with early developmental stages of bivalves, becomes inactive, and transforms into intact temporary cyst without lyses. Similarly, in a recent study, the dinoflagellates *Alexandrium catenella*, *A. affine*, *K. mikimotoi* and *K. papilionacea* were all found to affect early life stages of Japanese pearl oyster differentially, without lyses (Basti et al., 2015b). In raphidophytes, however, allelopathy was described only recently, when *C. marina* was found to decrease growth and survivorship of another HAB-forming dinoflagellate, *Gymnodinium catenatum* (Fernández-Herrera et al., 2016). In their study, cell-to-cell contact between *C. marina* and *G. catenatum* induced many changes in the latter, including loss of motility, swelling, loss of flagella, enlargement of the nucleus, transformation into temporary cysts, higher percentage of longer chains, and lysis. They suspected that tricosyts and mucocyts of *C. marina*, that are easily liberated under stress situations, could have been associated with the high lethality in the direct cell-to-cell contact with *G. catenatum*, in comparison with the milder yet still potent effect of culture filtrates. The involvement of tricosysts and mucocyts of raphidophytes as a defense or attack mechanism against other species has never been documented prior to that study (Yamaguchi et al., 2011) except for the qualitative description of the role of tricosysts of *F. japonica* as mechanical

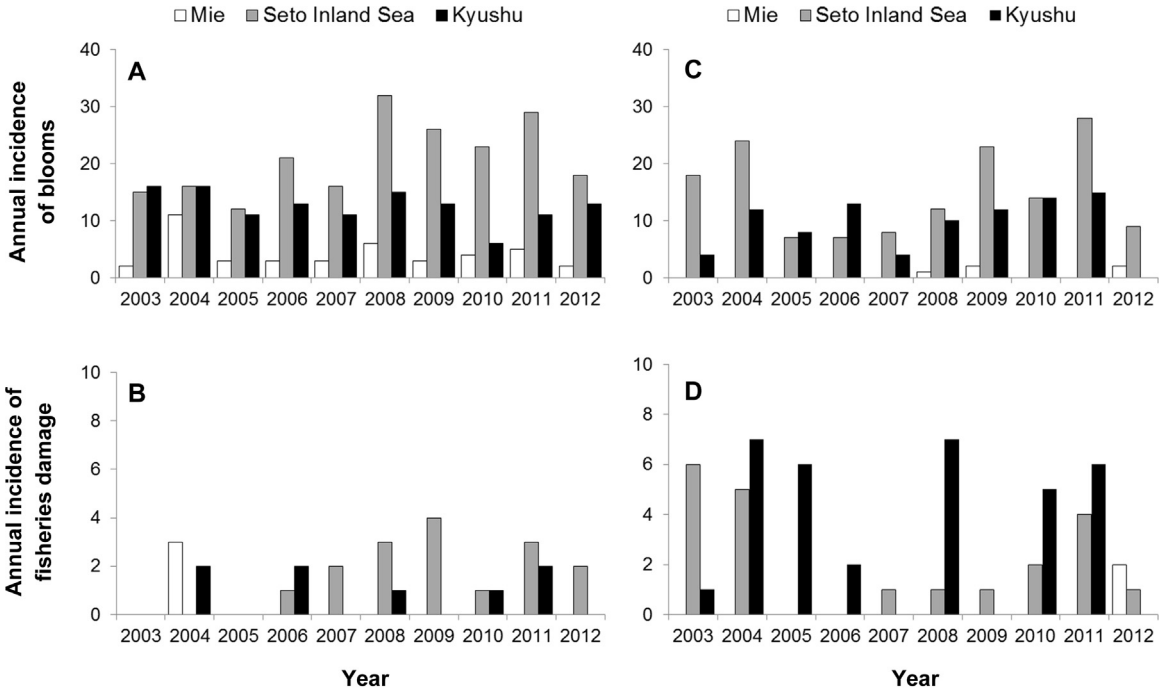

Fig. 6. Decadal annual incidence of blooms (A, C) and decadal annual incidence of fisheries damage (B, D) caused by harmful raphidophytes *Heterosigma akashiwo* (A, B), and *Chattonella marina* and *Chattonella antiqua* (C, D) in Western Japan. (Data compiled after the annual reports on harmful algae of the Seto Inland Sea Fisheries Coordination Office, Kyushu Sea Fisheries Coordination Office, and Fisheries Research Division of Mie Prefectural Science and Technology Promotion Center, 2004–2013).

deterrents of the two heterotrophic dinoflagellate grazers *Obelia rotunda* and *Oxyrrhis marina* (Tillmann and Reckermann, 2002), and the involvement of exudate such as ROS were also proposed as the mechanism of allelopathic effects of *C. marina*.

In western Japan, *Heterosigma akashiwo*, *Chattonella marina* and *Chattonella antiqua* bloom annually in all major cultivation areas of the Japanese pearl oyster, namely Mie Prefecture, the Seto Inland Sea, and Kyushu Island (Fig. 6). In 2003–2012, the highest annual incidence of blooms of *H. akashiwo* occurred in the Seto Inland Sea (208 blooms) followed by Kyushu Island (125 blooms) and Mie Prefecture (42 blooms). Fish- and shellfish-kills associated with blooms of *H. akashiwo* accounted for less than 8% of the total incidence of blooms in all cultivation areas in Japan. Blooms of *C. marina* and *C. antiqua*, which are quite often reported in mixture due to difficulties in distinguishing the two species with conventional morphological identification in monitoring programs, occurred mainly in the Seto Inland Sea (150 blooms) followed by Kyushu (92 blooms). In both areas, mass mortality of fish and shellfish accounted for 40% of the total incidence of the blooms. Blooms of *Chattonella* spp. in Mie prefecture are relatively very recent. The first bloom of *C. marina* in Mie prefecture was reported in 2008, followed by 2 blooms in 2009. In 2012, *C. marina* bloomed again twice, one time in mixture with *H. circularisquama* (cell densities: 3.7×10^3 cells ml^{-1} for *C. marina* and 9.4×10^2 cells ml^{-1} for *H. circularisquama*) and another time in mixture with both *H. circularisquama* and *K. mikimotoi* (cell densities: 8.0×10^2 cells ml^{-1} for *C. marina*, 1.7×10^4 cells ml^{-1} for *H. circularisquama*, and 1.7×10^3 for *K. mikimotoi*). In both cases, mass mortality of Japanese pearl oyster was reported; however, the role played by co-occurring *H. circularisquama* and *K. mikimotoi* in the shellfish-kills is not clear and further investigations are needed to understand the effects of mixed harmful blooms on aquatic organisms in western Japan.

The cell densities of blooms of *Heterosigma akashiwo*, and *Chattonella marina*/*Chattonella antiqua* ranged from 9×10^4 cells ml^{-1}–1.67×10^6, and 10^2 to 2.7×10^5 cells ml^{-1}, respectively, which are several orders of magnitude higher than the maximal densities used in the present study. In addition, all three raphidophytes form blooms in all three major cultivation areas coincident with the spawning period of the Japanese pearl oyster, and persist from a few consecutive days to more than 2 months. In the present study, the effects of the harmful raphidophytes on larvae of Japanese pearl oysters occurred at realistically low cell densities and short durations of exposure. Subsequently, all three raphidophyte species, especially *C. antiqua* and *C. marina*, could affect larval development and population recruitment of Japanese pearl oysters, and hamper the pearl culture industry. In previous studies, several other recurrent bloom-forming harmful algae were reported to cause mild to severe inimical effects on the early life development of Japanese pearl oyster (Basti et al., 2011, 2013, 2015a,b) and further research is required to study in more detail the impacts of harmful algal blooms on the early-life development of Japanese pearl oyster and other commercially important bivalve species. Particularly, the effects of mono- and multi-specific blooms of harmful algae on the early-life stages of bivalves should be explored.

As in other species of harmful algae, complex toxins and metabolites could have been involved in the lethal effects of *C. marina*, *C. antiqua* and *H. akashiwo* on larvae of Japanese pearl oysters. Nonetheless, this is the first study to emphasis the role played by the glycocalyx-mucus traps in the overall effects of the raphidophytes on bivalve larvae, and the role of physical disturbance of the glycocalyx in initiating the lethal effects. In addition to all the potential synergistic actors (ROS, NO, brevetoxins and brevenals, FFAs), such traps could have induced energetic exhaustion of larvae that would have enhanced the effects of the other stressors, if there were any. The lethal effects of all three raphidophyte species, which form recurrent and long-lasting blooms reaching high cell densities in the wild, need clarification.

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