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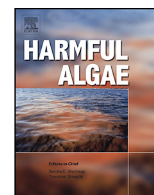

Differential inimical effects of *Alexandrium* spp. and *Karenia* spp. on cleavage, hatching, and two larval stages of Japanese pearl oyster *Pinctada fucata martensii*

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ABSTRACT

The effects of four harmful and potentially harmful dinoflagellates, *Alexandrium affine*, *Alexandrium catenella*, *Karenia mikimotoi* and *Karenia papilionacea*, on the early-life development of Japanese pearl oyster, *Pinctada fucata martensii*, were assessed. Density- and time-dependent, mild to severe effects on cleavage, hatching, D-larvae, and pre-settling larvae of pearl oysters were found. The non-PST-producer *A. affine* was highly toxic to both cleavage and hatching with potent lytic activity at a density of 2.5×10^2 cells ml^{-1}. The PST-producer *A. catenella* also affected cleavage and reduced hatching, but at 2-fold higher density with lytic activity only at the highest density tested (2×10^3 cells ml^{-1}). Cleavage was affected by *K. mikimotoi* and *K. papilionacea* at 1.5×10^4 cells ml^{-1}, but hatching was only affected by *K. mikimotoi*. Mortalities in both larval stages were not observed. D-larvae were not affected by *K. papilionacea*, but their activity decreased following exposure to *A. affine*, *A. catenella*, and mainly *K. mikimotoi*. Pre-settling larvae were more sensitive to all four harmful algae than were D-larvae. The dinoflagellate *A. catenella* had the highest effect on the activity of pre-settling larvae (10 cells ml^{-1}), followed by *K. mikimotoi* (5×10^2 cells ml^{-1}), *A. affine* (5×10^2 cells ml^{-1}), and *K. papilionacea* (1.5×10^4 cells ml^{-1}). The results of this study suggest that complex mechanisms, including paralytic shellfish toxins (PST), brevetoxins (PbTx) and a variety of lytic and membrane-disruptive toxins and/or other metabolites, could have been involved in such inimical stage-specific effects. It also highlight the threat posed by harmful algae on the recruitment of Japanese pearl oysters and potentially other bivalve species, and show that they could exert subchronic to chronic effects at realistically low cell densities, and impact bivalve populations through altered reproductive and recruitment processes, ultimately hampering both wild populations and aquaculture industries.

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1. Introduction

Harmful algal blooms (HAB) are produced by several representatives of diatoms, dinoflagellates, raphidophytes, prymnesiophytes, cyanobacteria, pelagophytes, and silicoflagellates (Anderson, 1989; Smayda, 1992; Landsberg, 2002). Owing to the increase in the occurrence, intensity, and geographical distribution of the blooms, coupled with the increased number of species added yearly to the global list of harmful algae, either as newly discovered species or as species that have been previously overlooked by monitoring programs, HAB have emerged as a worldwide concern and challenge to both science and management (Smayda, 1990; Hallegraeff, 1993; Van Dolah, 2000; Zingone and Oksfeldt Enevoldsen, 2000; Allen et al., 2006; Matsuyama and Shumway, 2009). Harmful algal blooms are often associated with human health, but ecological and sometimes ecosystematic impacts leading to severe economic losses worldwide are also common. Dozens of HAB-forming species produce potent bioactive metabolites that can be bioaccumulated and bioamplified throughout

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Table 1
Literature review of the distribution, harmful effects, and harmful compounds of the four species of dinoflagellates selected for this study.

Species	Distribution	Harmful effects	Harmful compounds	References
Alexandrium affine	Cosmopolitan	Unreported	Typically non-toxic or very low PSTs (Saxitoxins)	Anderson et al. (2012)
Alexandrium catenella	Cosmopolitan	Paralytic Shellfish Poisoning (PSP)	PSTs (Saxitoxins) Non-PSP related cytotoxins	
Karenia papilionacea	Japan, Australia, New Zealand	None known	Unknown (unconfirmed Brevetoxins), PUFAs	Haywood et al. (2004), Mooney et al. (2007), Brand et al. (2012)
Karenia mikimotoi	Cosmopolitan	Shellfish- and fish-kills	Exotoxins Glycolipids Gymnocin-A, Gymnocin-B Hemolytic ROS PUFAs	Gentien and Arzul (1990), Arzul et al. (1995), Jenkinson and Arzul (2001), Satake et al. (2002, 2005), Yamasaki et al. (2004, 2008), Brand et al. (2012)

aquatic food webs to affect terrestrial biotas and cause a number of mild to acute, sometimes lethal poisonings in humans via the ingestion of contaminated seafood, or through direct consumption of drinking waters tainted with dissolved toxins, and exposure to blooming waters and aerosols (Anderson, 1995; Falconer, 2008; Wang, 2008; Dodds et al., 2009). In aquatic environments, high biomass blooms have caused mass mortalities of a variety of aquatic organisms through predation, starvation, shading or anoxic conditions (Gainey and Shumway, 1991; Shumway, 1990; Rensel, 1993; Smayda, 1997; Landsberg, 2002). On the other hand, diverse groups of HAB species induce acute, chronic, and sublethal effects in aquatic organisms, including reptiles, mammals and seabirds, as a result of the production of biotoxins and/or other bioactive compounds with poorly understood allelopathic activities, many of which are yet to be characterized or even discovered (Shumway, 1990, 1995; Scholin et al., 2000; Shumway et al., 2003; Flewelling et al., 2005; Granéli et al., 2008; Lefebvre et al., 2010; Capper et al., 2013).

In Japan, there has been a general trend of decreasing frequency, intensity, and distribution of HAB and associated-human intoxications following the implementation of rigorous monitoring programs and the enactment of governmental regulations to control eutrophication. Several species of HAB, however, continue to bloom and cause episodic mass mortalities of both fish and shellfish (Imai et al., 2006). In addition, the geographical distribution of some *Alexandrium* spp., the causative agent of Paralytic Shellfish Poisoning (PSP), has been spreading over the past decades (Okaichi, 1997; Fukuyo et al., 2002; Imai et al., 2006). The blooms of several species of *Alexandrium* and *Karenia* fall along major bivalve cultivation areas, including those of the Japanese pearl oyster, *Pinctada fucata martensii*. Mass mortalities, physiological, behavioral, and pathological alterations have been reported since the late 1990s as acute to chronic effects of *Alexandrium* spp. and *Karenia* spp. in shellfish (Shumway and Cucci, 1987; Landsberg, 2002); however, the chronic and sublethal effects exerted on bivalve molluscs are still poorly understood, especially the effects of single and/or recurrent exposures to low-cell densities on the reproduction of bivalves.

Studies that have considered the effects of harmful algae and/or potentially harmful algae on early development of aquatic organisms are limited. In addition, the toxins and harmful compounds of both *Alexandrium* and *Karenia* species implicated in HAB events are complex (Table 1). Therefore, the effects of the non-PST-producer *Alexandrium affine*, the cosmopolitan PST-producer *Alexandrium catenella*, the fish- and shellfish-killer *Karenia mikimotoi* (reviewed in Brand et al., 2012), and the non-harmful *Karenia papilionacea* on the early-life development of Japanese pearl oysters were assessed. The effects of direct exposure to several cell densities of these HAB on cleavage, hatching, early D-larvae, and pre-settling larvae were assessed in a time- and density-dependent experimental frame. The four dinoflagellates form blooms coincident with the reproduction season of *Pinctada fucata martensii* in its three major cultivation areas in Japan (Table 2). Low densities and short exposure durations of these HAB species were chosen based on realistic densities and durations of

Table 2
Bloom maximal density range (cells ml^{-1}), bloom duration range (days), blooming periods, bloom occurrence and associated incidence of fisheries damage reported in the three main Japanese production areas of Japanese pearl oyster, *Pinctada fucata martensii*. (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).

	HAB species	Production area		
		Kyushu	Seto Inland Sea	Mie prefecture
Density	*Alexandrium affine*	2×10^2–1.8×10^4	2.67×10^3	2.6×10^3–3×10^4
	Alexandrium catenella	1.66×10^2–1.33×10^4	2.45×10^3–1.59×10^4	2.1×10^2–8×10^2
	Karenia papilionacea	ND	ND	1.37×10^3
	Karenia mikimotoi	2×10^3–1.18×10^5	9×10^3–3.1×10^5	1.17×10^3–9.16×10^3
Duration	*Alexandrium affine*	2–12	23	1
	Alexandrium catenella	7–21	7–9	1–2
	Karenia papilionacea	ND	ND	2
	Karenia mikimotoi	2–94	32–71	7–17
Period	*Alexandrium affine*	June–July	June–July	August
	Alexandrium catenella	January, May–July	April–June	January–February
	Karenia papilionacea	ND	ND	August
	Karenia mikimotoi	January, May–September, November	January–October	August–December
Occurrence (damage)	*Alexandrium affine*	2 (0)	1 (0)	2 (0)
	Alexandrium catenella	4 (0)	8 (0)	2 (0)
	Karenia papilionacea	ND	ND	1 (0)
	Karenia mikimotoi	48 (22)	160 (45)	4 (1)

ND: no data.

natural blooms that have occurred in the three production areas of pearl oysters over the past decade (Table 2).

Although the experiments were not designed to fully elucidate the toxicity process of the selected harmful algae, the mechanisms implicated in the observed inimical effects, including the overlapping effects of both characterized and uncharacterized toxins and metabolic byproducts, and the implications of such deleterious effects to the reproductive biology and aquaculture industry of the Japanese pearl oyster are discussed.

2. Materials and methods

2.1. Pearl oyster rearing

Sexually mature male and female pearl oysters, *Pinctada fucata martensii*, were reared at the K. Mikimoto & Co, Ltd. farm located in Ago Bay, Mie Prefecture, Japan. Gametes were stripped from the gonads, activated with ammonia seawater, and fertilization was carried out as described by Basti et al. (2011, 2013).

2.2. Algal cultures, molecular confirmation, and paralytic shellfish toxin analyses

The dinoflagellates *Alexandrium affine*, *Alexandrium catenella*, *Karenia papilionacea*, and *Karenia mikimotoi* were grown in autoclaved F/2 medium (Chen et al., 1969). All cultures were maintained under a 12 h light:12 h dark photoperiod, at 23 °C. Cells of the growing cultures of *A. affine*, *A. catenella*, *K. papilionacea*, and *K. mikimotoi* were harvested by centrifugation (10^4 rpm, 2 min) at room temperature (20 °C), and the DNA were extracted using 5% Chelex (Chelex 100 Molecular Biology Grade Resin, Bio-Rad Laboratories Inc., Richmond, CA) following Nagai et al. (2012). The multiplex PCR method was carried out according to Nagai (2011) to confirm *A. affine* and *A. catenella* species. Following PCR, the two *Karenia* species were also confirmed by the sub-cloning method of Nagai et al. (2008). After the cell densities were measured, 1 ml of *A. affine* and *A. catenella* culture medium were concentrated by centrifugation (3×10^3 rpm, 4 °C, 10 min). The resulting pellets were ultrasonicated at room temperature with 4-folds volume of 0.5-N acetic acid (Ravn et al., 1995), and centrifuged (10^4 rpm, 10 min). The supernatant was centrifuged (10^4 rpm, 20 °C, 10 min) through ultrafiltration membrane (YM-10, Millipore). PSP profiles were analyzed, but not quantitated, by HPLC according to the method of Oshima (1995).

2.3. Cleavage and hatching experiments

Ten minutes following fertilization, the fertilized eggs were washed with UV-treated seawater and were separately exposed in duplicate, at a density of 50 eggs ml^{-1}, to each algal species in 6-well plates (5 ml per well). The fertilized eggs were exposed to 5-cell densities of *Alexandrium affine* and *Alexandrium catenella* (0, 250, 500, 10^3, and 2×10^3), and 6-cell densities of *Karenia papilionacea* and *Karenia mikimotoi* (0, 500, 10^3, 5×10^3, 10^4, and 1.5×10^4). Following 1 h of exposure and 24 h of exposure, 1-ml aliquots were sampled and preserved in 5% buffered formalin seawater to determine the cleavage rate and the hatching rate, respectively. For cleavage rate, the percentage of 2-cell embryos indicating successful first cleavage, 4-cell embryos indicating successful second cleavage, and remaining fertilized eggs indicating arrested cleavage, were quantified and potential anomalies were determined under light microscopy. For the hatching rate, the percentages of trochophores and D-larvae were assessed and anomalies, if any, were also quantified.

2.4. D-larvae and pre-settling larvae experiments

Fertilized eggs were washed (1.0-mm pore size filtered and UV-treated seawater) then transferred to 30-L tanks at 23 °C. Larvae (27 h post-fertilization, 3 h-old D-stage) were used to assess the effects of the harmful algae on early larval stages 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 in duplicate to several densities of each algal species (same densities used for the experimental sets with fertilized eggs) in 6-well plates, at 50 larvae ml^{-1} and 25 larvae 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 re-suspend the larvae, allowed to rest for 5 min and then the percentage of actively swimming D-larvae, and actively swimming and/or crawling pre-settling larvae was directly counted under a light microscope. The cumulative morality rates were also assessed and anomalies, if observed, were quantified.

2.5. Statistical analyses

Normality (Kolmogorov–Smirnov and Shapiro–Wilk) and homoscedasticity (Bartlett's test) were checked a priori. When the parametric assumptions were met, multivariate or factorial ANOVAs were performed followed by Fisher's LSD, Newman–Keuls, or Bonferroni post hoc tests to assess the density at which the effects were significant, and *T*-test to compare the effects of *Alexandrium affine* to *Alexandrium catenella*, and *Karenia papilionacea* to *Karenia mikimotoi*. Otherwise, non-parametric Kruskal–Wallis ANOVA were conducted followed by Multiple comparisons test, Sign test, or Wilcoxon matched pairs test. Results were considered significantly different at a level of significance, $\alpha = 0.05$.

3. Results

3.1. Early-cleavage and hatching rates

Toxin analyses showed that *Alexandrium catenella* was positive for PST production (STX and GTX3) and no PST were detected from *Alexandrium affine*. The rates of early-cleavage of fertilized eggs of *Pinctada fucata martensii* exposed to the different harmful algae are shown in Fig. 1. The four dinoflagellate species had differential significant effects on cleavage. Both *Karenia mikimotoi* and *Karenia papilionacea* induced a significant decrease of the 4-cell embryos as compared to the respective controls at the highest density of 1.5×10^4 cells ml^{-1}, and *K. mikimotoi* further increased the rate of 2-cell embryos. The two *Alexandrium* species had the most marked impacts on cleavage, with inhibition of the second cleavage following exposure to *A. affine* at 2.5×10^2 cells ml^{-1} and *A. catenella* at 5×10^2 cells ml^{-1}. The division rate of fertilized eggs into 2-cell embryos was also significantly decreased following exposure to *A. affine* at 5×10^2 cells ml^{-1} and *A. catenella* at 2×10^3 cells ml^{-1}. The effects of *A. affine* were significantly higher than those of *A. catenella* (*T*-test, $P < 0.05$). In addition, *A. affine* induced a significant increase in the incidence of anomalies of the 2-cell and 4-cell embryos following exposure to 2.5×10^2 cells ml^{-1} (Fig. 2), with abnormal membrane swelling, cytoplasmic discharges, and lyses of the fertilized eggs and blastomeres (Fig. 3). These abnormalities were not observed during exposure to *A. catenella*. All *Karenia* spp. cells remained intact during the first hour of exposure; however, both *A. catenella* and *A. affine* liberated thecae and pellicle cysts were observed.

The hatching rates of D-larvae from exposed fertilized eggs were significantly affected by all algae, except *Karenia papilionacea*

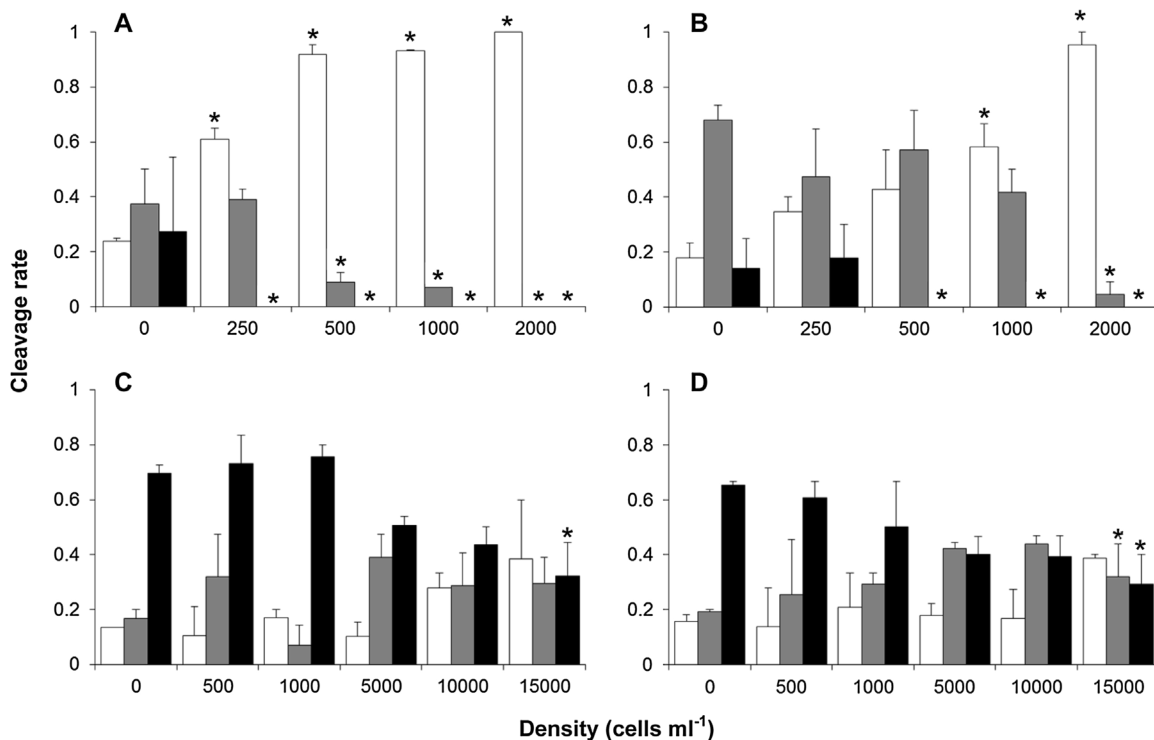

Fig. 1. Cleavage rate of *Pinctada fucata martensii* exposed to (A) *Alexandrium affine*, (B) *A. catenella*, (C) *Karenia papilionacea*, (D) *K. mikimotoi*. (□) Arrested cleavage, (■) first cleavage, (■) second cleavage, (*) Significant difference from respective control ($P < 0.05$).

(Fig. 4). A significant increase of the hatching rate into trochophores, a significant decrease of the hatching rate into D-larvae at 2.5×10^2 cells ml^{-1}, and an inhibition of the hatching into D-larvae were induced by *Alexandrium affine* at 5×10^2 cells ml^{-1}. The effects of *Alexandrium catenella* on the hatching rate were less pronounced than those of *A. affine*, yet with a significant decrease in the hatched D-larvae at 5×10^2 cells ml^{-1} and an inhibition at 2×10^3 cells ml^{-1}. At the latter cell density, both *A. affine* and *A. catenella* induced a complete inhibition of hatching and a disintegration of all stages that were no longer recognizable. Increased hatching rate into trochophores and decreased hatching rate into D-larvae were induced by *Karenia mikimotoi* at the highest density of 1.5×10^4 cells ml^{-1}. An increased rate of anomalies in the hatched trochophores was induced by *A. affine* at densities of 2.5×10^2cells ml^{-1} (Fig. 5).

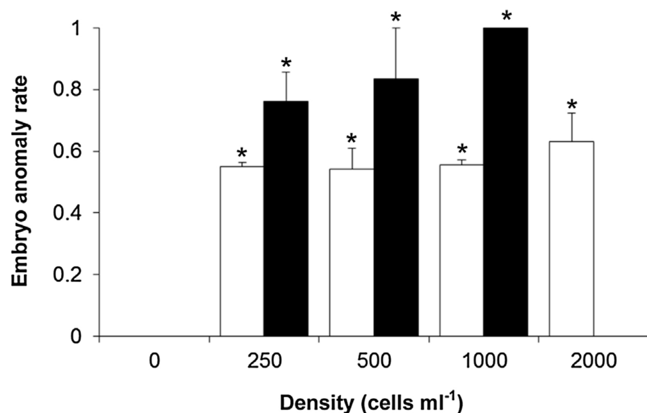

Fig. 2. Developmental anomaly of embryos of *Pinctada fucata martensii* exposed to *Alexandrium affine*. (□) Fertilized eggs, (■) 2-cell embryos. (*) Significant difference from respective control ($P < 0.05$).

3.2. Activity and mortality rates of D-larvae and pre-settling larvae

The activity rates of both D-larvae and pre-settling larvae were decreased by the presence of all algal species, except for *Karenia papilionacea* (Table 3; Fig. 6C). For D-larvae, the activity rates were all affected only by the cell densities, except in the case of *Karenia mikimotoi* for which the exposure duration also had a significant impact. For pre-settling larvae, the decrease in the activity rates was linked to both the density and the exposure duration. Significant interaction effects between the cell density and duration of exposure were found for *Alexandrium catenella*.

The activity rate of D-larvae was slightly but significantly decreased following 48 h of exposure to *Alexandrium affine* and 24 h of exposure to *Alexandrium catenella* at 2×10^3 cells ml^{-1} (Fig. 6), and both *Alexandrium* species affected the activity in a similar way (*T*-test, $P > 0.05$). Pre-settling larvae were more affected by exposure to these two species with a significant decrease in their activity rates following 24 h of exposure to *A. affine* at 2.5×10^2 cells ml^{-1}, and to *A. catenella* at as low as 10 cells ml^{-1} (Fig. 7). Comparison of the activity rate of pre-settling larvae between groups of exposure showed that for *A. affine* the activity at 10^3 cells ml^{-1} was significantly different from the other groups, and for *A. catenella* the activity rate at 10 cells ml^{-1} was significantly different from the other groups. Only exposure to *Karenia mikimotoi* resulted in decreased activity rate of D-larvae within 24 h of exposure to 5×10^3 cells ml^{-1}, but exposure to both *Karenia papilionacea* and *K. mikimotoi* resulted in decreased activity of pre-settling larvae within 24 h of exposure to 1.5×10^4 and 5×10^2 cells ml^{-1}, respectively. For both species of *Karenia*, the exposure group at 1.5×10^4 was significantly different from the others.

The mortality rates of D-larvae and pre-settling larvae exposed to *Alexandrium affine*, *Alexandrium catenella*, *Karenia papilionacea*, and *Karenia mikimotoi* were not significant.

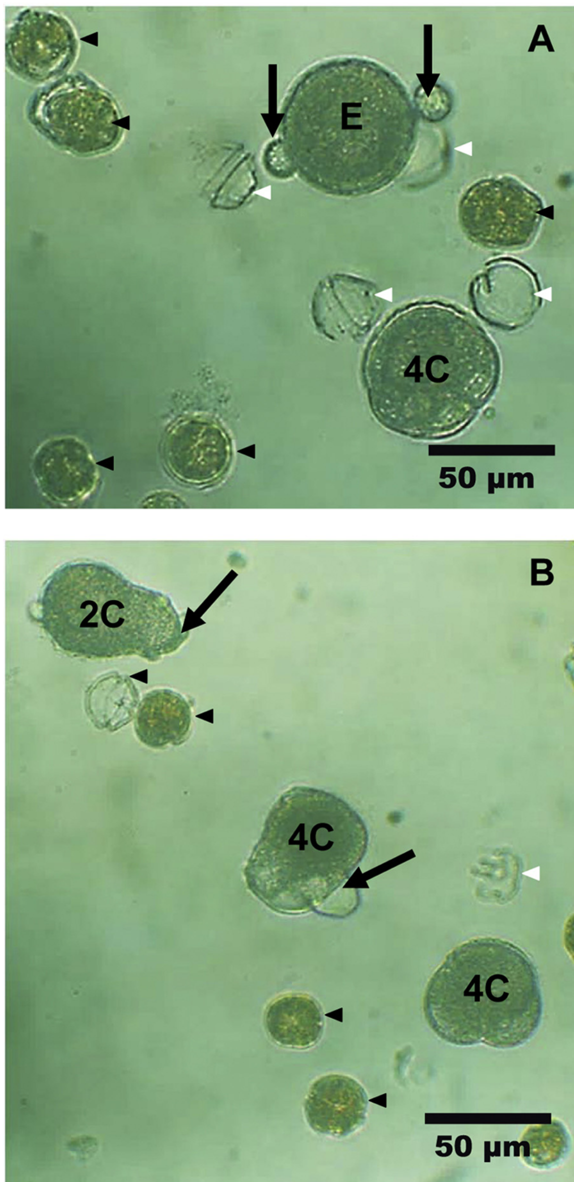

Fig. 3. Light micrographs of (A) abnormal fertilized eggs and (B) embryos of *Pinctada fucata martensii* exposed to *Alexandrium affine*. E: fertilized egg, 2C: 2-cell embryos, 4C: 4-cell embryos, white arrow heads: *A. affine* thecae, black arrow heads: *A. affine* vegetative cells, black arrows: abnormal cytoplasmic discharge.

4. Discussion

This is the first study to report: (1) harmful effects of the non-PST-producer *Alexandrium affine* on an aquatic organism at realistically low densities, with explicitly demonstrated highly potent, lytic activity toward fertilized eggs, early-embryos and hatched trochophores of a bivalve species, and with demonstrated induction of decreased motility in early-larvae and pre-settling larvae; (2) stage-specific harmful effects of the PST-producer *Alexandrium catenella* on the early-development of pearl oysters, with non-lytic activity toward dividing embryos protracting metamorphosis into D-larvae, lytic activity toward trochophores at high cell density, and non-lytic activity toward pre-settling larvae decreasing their motility at very low densities; (3) decreased cleavage and hatching of pearl oysters induced by exposure to *Karenia mikimotoi*; (4) negative effects of *Karenia papilionacea* on an aquatic organism, with mildly protracted but non-carry-on effect on early-cleavage, and decreased activity of pre-settling larvae of pearl oysters at high density.

4.1. Role of paralytic shellfish toxins, brevetoxins, and other toxins and metabolites in the inimical effects

The genus *Alexandrium* encompasses a diversity of globally distributed species, half of which are known to cause paralytic shellfish poisoning (PSP) in humans, associated with the production of paralytic shellfish toxins (PST) or cause other harmful effects (Anderson et al., 2012). In addition to the characterized intracellularly produced PST, several species of *Alexandrium* produce ichtyotoxins that are noxiuous to fish (Sugg and VanDolah, 1999; Tillmann and John, 2002; Lundholm et al., 2005), and allelochemicals which have negative effects on planktonic competitors and grazers (Fistarol et al., 2004; Arzul and Gentien, 2006; Tillmann. et al., 2007). The production of both ichtyotoxins and allelochemicals is not necessarily dependent on the production of PST (Sugg and VanDolah, 1999; Tillmann and John, 2002; Lundholm et al., 2005; Tang et al., 2007).

In the present study, the observed negative effects on early embryos, hatching rates, early D-larvae and pre-settling larvae of *Pinctada fucata martensii* confirm the PST-independent toxicity of *Alexandrium affine*. The latter induced a decreased first and second cleavage, followed by complete inhibition of the first and second cleavage resulting in decreased hatching into D-larvae with increased anomalously hatched trochophores, then complete inhibition of hatching into D-larvae at 5×10^2 cells ml^{-1}, and hatching into both trochophores and D-larvae at 2×10^3 cells ml^{-1}. More than 50–80% of the 2-cell and 4-cell embryos showed destabilized cell membranes, abnormal swellings and cytoplasmic discharges. Similar anomalies were observed for hatched trochophores at a rate of more than 40–80%. The destabilization of blastomere and trochophore cell membranes at low cell density leading to disintegration of the early-developmental stages strongly suggest that the toxins or other metabolites of *A. affine* have highly potent lytic activity targeting membrane integrity. There have been no previous reports of harmful effects of *A. affine*; however, in 1991, there have been reports of mortalities of fish and shellfish by the regular monitoring program in Nagasaki prefecture, coincident with blooms of *A. affine*. In that year, *A. affine* formed 10 blooms, 3 of which caused mortalities of Greater amberjack (*Seriola dumerlii*), yellowtail amberjack (*Seriola lalandi*), Japanese amberjack (*Seriola quinqueradiata*), red seabream (*Pagrus major*), Japanese tiger prawn (*Marsupaenaus japonicas*) and squids. The blooms of *A. affine* associated with fish and shellfish mortality occurred in June–July, lasting 5–14 days at densities ranging from 6.08×10^4 to 2.5×10^5 cells ml^{-1}. In 4 out of the 10 outbreaks of *A. affine*, the dissolved oxygen was 6.9–11.6 ppm, which excludes anoxia as a cause of death of fish and squid (Nagasaki Prefectural Institute of Fisheries, 1992).

The toxicity of *Alexandrium catenella* was exhibited upon direct exposure to freshly fertilized eggs of pearl oysters translating into a decreased rate of successful first cleavage at 2×10^3 cells ml^{-1}, and a decreased rate of successful second cleavage at 5×10^2 cells ml^{-1}, followed by a complete inhibition of cleavage at 2×10^3 cells ml^{-1}, resulting in decreased hatching into D-larvae at 5×10^2 cells ml^{-1}, and complete inhibition of hatching into both trochophores and D-larvae at 2×10^3 cells ml^{-1}. The lytic activity toward fertilized eggs, early embryonic stages and newly hatched trochophores were, however, observed only at the highest density, suggesting that *A. catenella* also produces lytic compounds, either as exudates or located on its cell membrane, seemingly less potent to early embryos and larvae of pearl oysters than those produced by *Alexandrium affine*. Matsuyama et al. (2001) reported high

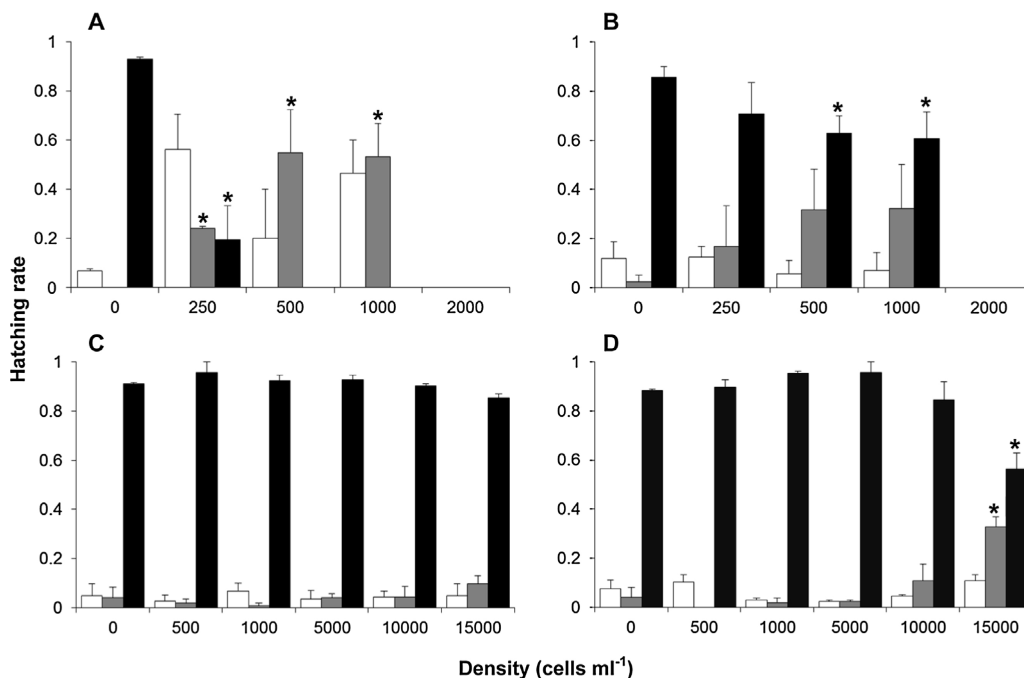

Fig. 4. Hatching rates of fertilized eggs of *Pinctada fucata martensii* exposed to (A) *Alexandrium affine*, (B) *A. catenella*, (C) *Karenia papilionacea*, and (D) *K. mikimotoi*. (□) Fertilized eggs, (■) trochophores, (■) D-larvae. (*) Significant difference from respective control ($P < 0.05$).

mortalities of torchophores of the Pacific oyster, *Crassostrea gigas*, following laboratory exposures to the PST-producer *Alexandrium tamarense* (92–96% mortality, following 8–10 h of exposure to 10^4 cells ml^{-1}) and non-PST-producer *Alexandrium taylori* (90–100% mortality, following 3–8 h of exposure to 2×10^3 cells ml^{-1}), whereas the PST-producer *Gymnodinium catenatum* had no effects on the trochophores even at the high density of 10^7 cells ml^{-1}. In addition, cell-free filtrate of both *A. tamarense* and *A. taylori* cultures exhibited less cytotoxicity toward trochophores, indicating that the cytotoxic agents were most likely located inside the cell or on the cell surface of *Alexandrium* spp. In addition, Yan et al. (2001) showed that exposure of fertilized eggs of the scallop *Chlamys farreri* to the PST-producing *A. tamarense* induced a decrease of the hatching success by 70–95% following exposure to 10^2–5×10^2 cells ml^{-1}. The same study reported that dissolved STX, *A. tamarense* cell content, and cell-free medium did not affect the hatching of scallops, but re-suspended algal cell and cell fragments did, also showing that the negative effects on egg hatching are associated with uncharacterized cytotoxic compounds located on the cell surface of *A. tamarense*, rather than exuded compounds. In more recent reports, the lytic activity of *Alexandrium* against protists seemed to act on external cell membranes (Ma et al., 2009), as was the case of lytic compounds from *A. tamarense* that increased membrane permeability to Ca^{2+} ions without specifically binding to calcium ion channels (Ma et al., 2011), and lytic compounds from three strains of *A. tamarense* complex whose toxicity were related to reactive oxygen species (ROS) and/or to secondary compounds produced by ROS-induced lipid peroxidation (Flores et al., 2012); however, cytotoxic-heatlabile exotoxin (Lush et al., 2001), hemolytic proteinaceous exotoxin (Emura et al., 2004), and a polysaccharide-based cytotoxic compound (Yamasaki et al., 2008) were recently isolated from *Alexandrium minutum*, *A. taylori*, and *A. tamarense*, respectively, showing that both exudates and cell contact are possible mechanisms for the observed deleterious effects of *Alexandrium* spp. that could be strain-specific, and both mechanisms could have been implicated in the observed effects of *A. affine* and *A. catenella* on embryos and trochophores in the present study.

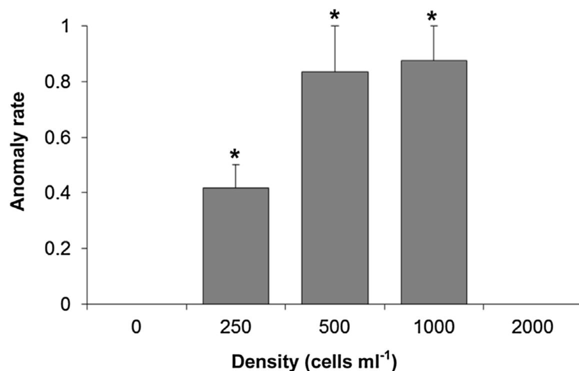

Fig. 5. Anomaly rates of trochophores from fertilized eggs of *Pinctada fucata martensii* exposed to *Alexandrium affine*. (*) Significant difference from respective control ($P < 0.05$).

In the most recently related study, *Alexandrium catenella* isolated from the Changjing River estuary, China, was shown to affect egg hatching, embryonic development and three larval stages of the Pacific oyster *Crassostrea gigas* (Mu and Li, 2013). Nonetheless, the PST production of *A. catenella* was not checked and the stage-specific effects were not clearly observed, *A. catenella* causing decreased survival, growth and settlement of D-larvae, eye-spot larvae, and umbo larvae over the density range of 3×10^2–2×10^3 cells ml^{-1} following 4 days of exposure. In our study, only the D-larval activity rate was slightly affected by exposure to both *Alexandrium affine* and *A. catenella*, with a decrease by 3% following 48 h of exposure and 6.3–10.6% following 24–48 h of exposure to 2×10^3 cells ml^{-1}, respectively. The developed shell of the D-larvae could have partially protected them from prolonged exposure to *Alexandrium* exudates through intermittent valve closures, or it could have made reduced contact with the free-swimming cells of the algae, if the lytic agents were located on *Alexandrium* cell surface. Decreased activity and mortality of scallop D-larvae following prolonged exposure (6 days) to *Alexandrium tamarense* were previously reported for *Chlamys farreri* (Yan et al., 2001). The deleterious effects of *A. tamarense* on D-larvae and eye-spot larvae of another species of scallop, *Argopecten*

Table 3
Statistical analyses of the effects of the different parameters of evaluation on the activity rates of D-larvae and pre-settling larvae exposed to the harmful algae.

	Harmful algal species	Treatments		
		Cell density	Duration of exposure	Interaction
D-larvae	*Alexandrium affine*	*	NS	NS
	Alexandrium catenella	**	NS	NS
	Karenia papilionacea	NS	NS	NS
	Karenia mikimotoi	**	**	NS
Pre-settling larvae	*Alexandrium affine*	**	**	NS
	Alexandrium catenella	**	**	*
	Karenia papilionacea	**	**	NS
	Karenia mikimotoi	**	**	NS

NS: non-significant effect.
* $P < 0.05$.
** $P < 0.01$.

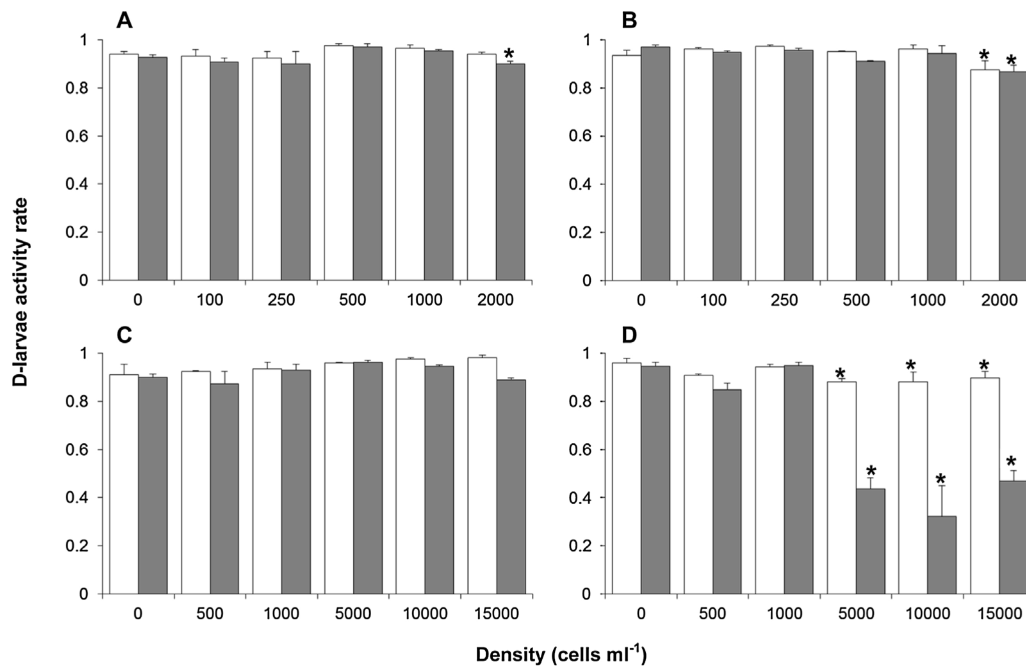

Fig. 6. Activity rate of 3 h-old D-larvae of *Pinctada fucata martensii* exposed to (A) *Alexandrium affine*, (B) *A. catenella*, (C) *Karenia papilionacea*, and (D) *K. mikimotoi*. (□) 24 h post-exposure, (■) 48 h post-exposure. (*) Significant difference from respective control ($P < 0.05$).

irradians, were confirmed in a later study that demonstrated decreased activity of both larval stages and mortalities of D-larvae at much higher densities than those of *C. farreri*. In the case of eye spot larvae of *A. irradians*, survivorship and metamorphosis were not affected even following 14 days of exposure to 10^4 cells ml^{-1}; however, the size of metamorphosed juveniles was reduced for the eye-spot larvae that were exposed to the highest cell density (Yan et al., 2003). The authors did not exclude the role that PST could have played in the effects on eye-spot larvae, although they noted that shellfish are usually not affected by PST, because the primary mechanism of neuromuscular excitability in these species is mediated via voltage-gated calcium channels. Paralytic shellfish toxins are endogenous, water-soluble, neurotoxic alkaloids including saxitoxin (STX) and its 57 described analogs, produced mainly by *Alexandrium* spp., but also by *Gymnodinium catenatum*, *Pyrodinium bahamens* and several cyanobacteria of the genera *Anabaena*, *Cylindrospermopsis*, *Aphanizomenon*, *Planktothrix* and *Lyngbya* (Weise et al., 2010; Anderson et al., 2012). Saxitoxin is the most toxic compound, followed by neosaxitoxin (NeoSTX) and gonyautoxins (GTX) 1 and 3 (Hall et al., 1990). The production of STX and GTX-3 was confirmed, although not quantified, from cells of the strain of *A. catenella* used in the present study. The presence of these cells significantly decreased the motility of pre-settling larvae following 24–89 h of exposure to 10–10^3 cells ml^{-1} by 26.6% to as much as 88%. The neurons of various shellfish species showed some level of response to STX with a varying degree of sensitivity among species (Twarog and Yamaguchi, 1974; Kao, 1993). The long-established, primary target of STX and its derivatives is the voltage-gated sodium channel (Cestele and Catterall, 2000) to which STX binds with high affinity blocking the inward flow of sodium ions into the cell eventually causing paralysis (Catterall, 2000; Llewellyn, 2006). Recent studies have shown, however, that STX has additional molecular targets including voltage-gated potassium and calcium channels, and even copper transporters (Cusick et al., 2012). Similar to its interactions with sodium channels, STX partially blocks the voltage-gated calcium channels which are the key signal transducers of electrical signaling, converting depolarization of the cell membrane to an influx of calcium ions that initiates contraction, secretion, neurotransmission, and other intracellular regulatory events (Catterall, 2000; Zakon, 2012). In addition, by binding to voltage-gated potassium channels, STX alters the control of electrical signaling in excitable cells and the regulation of ion flux and calcium transients in non-excitable cells (Wang et al., 2003; Catterall et al., 2007). Since the PST are endogenous, the decreased

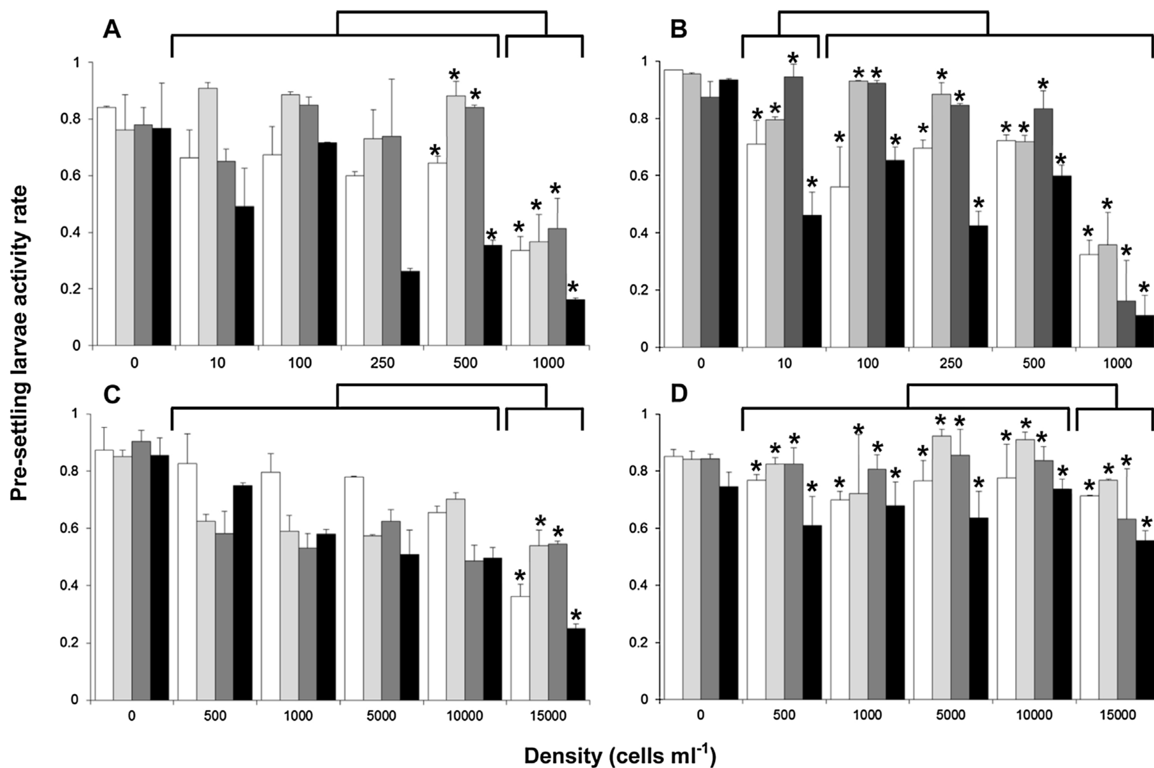

Fig. 7. Activity rate of pre-settling larvae of *Pinctada fucata martensii* exposed to (A) *Alexandrium affine*, (B) *A. catenella*, (C) *Karenia papilionacea*, and (D) *K. mikimotoi*. (□) 24 h post-exposure, (□) 48 h post-exposure, (■) 72 h post-exposure, and (■) 89 h post-exposure. (*) Significant difference from respective control ($P < 0.05$). Different clusters mark significant difference between exposure groups.

motility of pre-settling larvae at low densities could have been associated with the release of PST in larval tissues following rupture of *A. catenella* cells leading to closure of larval shells with intermittent contraction of their body and decreased motility through paralysis and/or altered cellular homeostasis, following blockage of sodium or calcium gating or modification of potassium gating. Nonetheless, these effects, if they were in action, were not potent enough to cause mortality in larvae over the ranges of density and exposure durations considered in this study. The less pronounced effects of non-PST producer *A. affine* on the activity of pre-settling larvae, and the subtle decrease in early D-larval motility observed at a 200-fold increase in *A. catenella* density in a comparable way to the effect of *A. affine*, support the role played by PST in the negative effects on pre-settling larvae; D-larvae being biologically unable to ingest cells as large as *A. catenella*. The *A. catenella* isolate from the Changjing River estuary, China, was shown to reduce survivorship of D-larvae, umbo larvae, and eye-spot larvae of the Pacific oyster, *C. gigas*, following 4 days of exposure to 6×10^2 cells ml^{-1}, 10^3 cells ml^{-1}, and 2×10^3 cells ml^{-1}, respectively (Mu and Li, 2013). The growth of both D-larvae and umbo larvae, and the settlement of eye-spot larvae were depressed following exposure to 3×10^2 cells ml^{-1}. Usually a PST-producing organism synthesizes a suite of toxins made up of several PST analogs (Wiese et al., 2010), and the production of certain suite of toxins is seemingly fixed genetically for each clonal strain of *Alexandrium*. Among the *A. tamarense* complex, to which *A. catenella* belongs, toxin profiles are very diverse among species and even strains (Burkholder and Glibert, 2006; Anderson et al., 2012) which might explain the difference in toxicity between the two strains of *A. catenella* used in this study and in Mu and Li (2013) study, as it could be related to differences in the sensitivity of both bivalve species.

In this study, both *Karenia mikimotoi* and *Karenia papilionacea* were found to affect early development of *Pinctada fucata martensii* at varying degree, with statistically higher negative overall impact of *K. mikimotoi* and higher effects on larvae than cleavage and hatching. Early cleavage was decreased by the presence of both *Karenia* species at the highest density used in the experiments; however, only *K. mikimotoi* showed a carry-on effect on the hatching success with a significant decrease in D-larvae also at the highest density. The activity of D-larvae was not affected by exposure to *K. papilionacea*, but a decreased activity in pre-settling larvae was induced within 24 h of exposure to the highest density of *K. papilionacea* (1.5×10^4 cells ml^{-1}). On the other hand, the activity of D-larvae and pre-settling larvae was significantly decreased following exposure to *K. mikimotoi* for 24 h at 5×10^3 cells ml^{-1} and 24 h at 5×10^2 cells ml^{-1}, respectively. No morphological anomalies were observed in embryos or in larvae, thereby excluding lytic activity in favor of toxicity of brevetoxins (PbTx) and/or other organic compounds, although *K. mikimotoi* is known to produce hemolysin activated via cell contact (Neely and Campbell, 2006; Zou et al., 2010). Brevetoxins (PbTx) responsible for the neurotoxic shellfish poisoning (NSP) in humans are produced by *Karenia brevis*, and other Kareniacea, including *K. papilionacea*, are suspected to produce PbTx or PbTx-like compounds (Furey et al., 2007; Ramsdell, 2008; Brand et al., 2012). The PbTx are potent lipophilic neurotoxins that bind with high affinity to voltage-gated sodium channels causing increased sodium influx and depolarization of cell membrane (Watkins et al., 2008). As discussed above for *Alexandrium catenella*, the sensitivity of pre-settling larvae to *K. papilionacea* compared to other developmental stages could have been associated with a neuromuscular toxicity of brevetoxins in larval tissue following ingestion or direct membrane uptake of brevetoxins could have occurred following disruption of *Karenia* cells in the water since PbTxs are highly soluble in lipids and can easily pass the phospholipid cell membrane (Watkins et al., 2008). D-larvae do not feed on such algal cells until they reach a larger size which explains their insensitivity to *K. papilionaceae*.

Brevetoxin-producer *K. brevis* was shown to decrease the survival of 72 h-old larvae of *Merecenaria mercenaria*, *Crassostrea gigas*, and *Argopecten irradians* following 7-day exposures to 10^3–5×10^3 cells ml^{-1} (Leverone et al., 2006). Recently, increased mortality and abnormalities of larvae resulting from 2- to 4-cell embryos exposed to whole and lysed *K. brevis* (1.5×10^3–3×10^3 cells ml^{-1}) with a concomitant decrease in larval shell length were reported for *M. mercenaria* and *C. virginica* (Rolton et al., 2014). Overall, lysed cells of *K. brevis* had a more pronounced effect on quahog and oyster larvae than did whole cells; however, the production of brevetoxins in *K. papilionacea*, and in *K. brevisulcata*, *Karenia concordia*, *Karenia cristata*, and *Karenia selliformis*, has not yet been confirmed by LC–MS (Landsberg, 2002; Haywood et al., 2004; Furey et al., 2007; Ramsdell, 2008; Brand et al., 2012) and the role PbTx could have played in the toxicity of *K. papilionacea* is therefore unclear.

While *Karenia mikimotoi* is not known to produce brevetoxins, this species elicited more pronounced effects on early developmental stages of pearl oyster *Pinctada fucata martensii* suggesting other metabolites were at play. Tang and Gobler (2012) reported insignificant low mortality (<10%) of 24 h-old larvae of *Merecenaria mercenaria* following 72 h of exposure to *K. mikimotoi* at 1.71×10^4 cells ml^{-1}. In a previous study, trochophores of *Crassostrea gigas* exposed to *K. mikimotoi* at 3×10^4 cells ml^{-1} died at as much as 99–100% within 8–10 h (Matsuyama et al., 2001). In a recent study, Shi et al. (2012) exposed juvenile larvae of Chinook salmon (*Oncorhynchus tshawytscha*), snapper (*Pagrus auratus*), sea slug, sea urchin, brine shrimp, Greenshell™ mussel (*Perna canaliculus*), Pacific oyster (*C. gigas*), and New Zealand abalone (*Haliotis iris*) to *K. mikimotoi* (negative for PbTx production), *Karenia brevis* (producer of PbTxs), and *Karenia brevisulcata* (producer of brevisulcatic acid BSXs, and *K. brevisulcata* toxins KBTs). Whereas *K. brevis* and *K. brevisulcata* were toxic to fish, *K. mikimotoi*, long-known to cause mortalities of fish- and shellfish (e.g. Pacific oyster, Japanese pearl oyster, Japanese abalone, and horned turban) in the wild (Oda, 1935), showed no acute ichtyotoxicity and affected only *H. iris* larvae. Brevetoxin-producer *K. brevis* had no effects on any of the invertebrate larvae, whereas *K. brevisulcata* was highly toxic to larvae of *P. canaliculus*, *H. iris*, and sea urchin, toxic to larvae of *C. gigas* and sea slugs, but did not affect larvae of shrimp (Shi et al., 2012). Several allelochemicals and toxic compounds have been isolated from *Karenia* spp. and the ability to produce allelopathic and lethal toxins other than brevetoxins is believed to be common to all *Karenia* species although the exact bioactivity, and complex interactions and trade-offs of these metabolites have not been fully characterized (Brand et al., 2012). Gymnocine A- and B- were isolated from *K. mikimotoi* (Satake et al., 2002, 2005) yet their toxicity to fish was weak (Silke et al., 2005) compared to the potent neuro-blocker gymnodimine produced by *Karenia selliformis* (Miles et al., 2003; MacKenzie et al., 2004; Munday et al., 2004, Munday, 2008; Molgó et al., 2007). Both *K. mikimotoi* and *K. papilionacea* were shown to produce potentially allelopathic sterols, and ichtyotoxic polyunsaturated fatty acids (PUFA) which toxicity is enhanced by the presence of reactive oxygen species (Mooney et al., 2007). The PUFA produced by *K. mikimotoi* were toxic to a variety of tissue cultures, reduced bacterial bioluminescence, were allelopathic to diatoms, cryptophytes, raphidophytes, and prasinophytes, in all cases disrupting cell membranes (Bodennec et al., 1995; Fossat et al., 1999; Sola et al., 1999; Arzul et al., 2000; Gentien et al., 2007; Chang, 2011). High levels of superoxides with allelopathic activity to other microalgae and bacteria were also found to be produced by *K. mikimotoi* (Yamasaki et al., 2004; Marshall et al., 2005a,b). A relatively newly described species, *K. papilionacea* was molecularly identified in New Zealand (Haywood et al., 2004) and thereafter from several localities as the previously known butterfly type of *K. brevis* (Steidinger et al., 1966). It is considered non-harmful, so far, and producer of PbTx and PUFAs. It could, however, produce other allelochemicals and/or toxins and secondary metabolites, and future studies should look into characterizing its toxic metabolites. At this stage of knowledge, it is difficult to make a clear cut as to which mechanism could have been implicated in the deleterious effects of *K. mikimotoi* and *K. papilionacea*. Nonetheless, it is clear that early-developmental stages of *P. fucata martensii* are more sensitive to allelochemicals than to brevetoxins.

4.2. Implications for Japanese pearl oyster reproduction and aquaculture industry

The Japanese pearl oyster has been experiencing local but severe mass mortalities since 1994 that have spread to all main pearl production areas in western Japan, namely Mie prefecture, Kyushu Island, and the Seto inland sea, reducing its total national production by two-thirds. These die-offs have been attributed to several environmental factors and pathogenic diseases, but also to recurrent HAB events (Nagai et al., 2007). Although red tides caused by the toxic dinoflagellate *Heterocapsa circularisquama* since 1998 have become the major cause of mortalities of *Pinctada fucata martensii* (Matsuyama et al., 2001; Matsuyama, 2012), the effects of other recurrent HAB species on pearl oysters, and other bivalves, have not been thoroughly explored, specifically the effects of harmful and toxic species that bloom coincidentally with the spawning season of pearl oysters.

Results presented here clearly show inimical effects on early life development of *Pinctada fucata martensii* induced by *Alexandrium affine*, considered potentially harmful, *Alexandrium catenella*, considered harmful to humans and higher trophic animals, and *Karenia papilionacea*, considered non-harmful, along with the well-known fish- and shellfish-killer *Karenia mikimotoi*. Given the fact that all four species form blooms overlapping with the spawning and larval development seasons of Japanese pearl oyster, this study demonstrates that *A. affine*, *A. catenella*, *K. mikimotoi*, and *K. papilionaeca* could adversely affect the recruitment of *P. fucata martensii*. The culture industry for Japanese pearl oysters is already challenged with unexplained diseases and mass mortalities (reviewed in Wada, 2007) and impacts on the recruitment of wild stocks would further hamper the efforts to restore the pearl production industry by reducing the genetic diversity necessary to interbreeding programs for selection of pearl oysters resistant to environmental constraints.

In the three major cultivation areas of *Pinctada fucata martensii*, blooms of *Karenia mikimotoi* reach the highest density, duration, recurrence, and associated fisheries damage as compared to the other three dinoflagellate species considered in his study. In addition, *K. mikimotoi* blooms occur during the whole spawning and larval developmental season of pearl oysters (Table 2). Therefore, the impacts that *K. mikimotoi* could have on the reproduction and recruitment of pearl oysters are expected to be the highest, especially in Kyushu and the Seto Inland Sea. In Japan, *K. mikimotoi* is an already well-known causative agent of fish- and shellfish-kills not associated with brevetoxins (Oda, 1935; Honjo et al., 1990; Ono et al., 1996; Matsuyama et al., 1998; Okaichi, 2004). There was only one report of a monospecific bloom of *Karenia papilionacea* in Mie prefecture in 2011 by the monitoring program of HAB in Japan. Many of *Karenia* blooms are composed of two to several species of *Karenia* and *K. papilionacea*, the previously known butterfly type of *Karenia brevis*, have been known to co-occur with *K. mikimotoi* (Brand et al., 2012). There is not sufficient data on *K. papilionacea* mono- and pluri-specific blooms in Japan, but its effects on the recruitment of Japanese pearl oysters seem minimal compared to *K. mikimotoi*, although its additive/synergistic effect in blooms of *K. mikimotoi* should not be excluded and

should be considered in further research on the potential impacts of simulated multispecific blooms of *Kareniacea* on the development of pearl oysters. Blooms of both *Alexandrium affine* and *Alexandrium catenella* are less frequent in all three production areas and in Mie *A. catenella* blooms during winter outside the spawning season of pearl oysters; however, a close watch should be kept on both *A. catenella* and *A. affine* since our study showed highly potent toxicity to all developmental stages and since *A. affine* did induce mortalities in the past of fish, shrimps, and squids in Kyushu.

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