

Development of a method to assess the ichthyotoxicity of the harmful marine microalgae *Karenia* spp. using gill cell cultures from red sea bream (*Pagrus major*)

Nobuyuki Ohkubo · Yuji Tomaru ·
Haruo Yamaguchi · Saho Kitatsuji ·
Kazuhiko Mochida

Received: 21 December 2016 / Accepted: 19 June 2017 / Published online: 11 July 2017
© Springer Science+Business Media B.V. 2017

Abstract The present study reports the development of a method to investigate ichthyotoxicity of harmful marine microalgae using cultured red sea bream (*Pagrus major*) gill cells. The cultured gill cells formed adherent 1–2 layers on the bottom of the culture plate and could tolerate seawater exposure for 4 h without significant alteration in cell survival. The microalgae *Karenia mikimotoi*, *Karenia papilionacea*, *K. papilionacea* phylotype-I, and *Heterosigma akashiwo* were cultured, then directly exposed to gill cells. After *K. mikimotoi* and *K. papilionacea* phylotype-I exposure, live cell coverage was significantly lower than in the cells exposed to a seawater-based medium (control cells; $P < 0.05$). Toxicity of *K. mikimotoi* cells was weakened when cells were ruptured, and was almost inexistent when the algal cells were removed from the culture by filtration. Significant cytotoxicity was detected in the concentrated ruptured cells, and in the concentrated of ruptured cells after freezing and thawing though cytotoxicity was weakened; whereas, cytotoxicity almost disappeared after heat treatment. In addition, examination of the distribution of toxic substances from the ruptured cells showed that cytotoxicity

mainly occurred in the fraction with the resuspended pellet after centrifugation at 3000×g.

Keywords Cytotoxicity · *Karenia mikimotoi* · *Karenia papilionacea* · Marine fish gill cells

Introduction

Harmful algal blooms (HABs) occasionally occur off southern and western Japan, causing death of farmed fish and shellfish and leading to great losses to fisheries. HABs have been reported in several other countries; therefore, an understanding of how HABs kill fish and shellfish and the development of effective mitigation technologies are urgently needed. Ishimatsu et al. (1996) hypothesized that reactive oxygen species (ROS) released from *Chattonella* spp. are involved in fish kills. However, the mechanisms involved are still not completely understood, and little information is available on the fish-killing mechanisms of other species of HAB-causing plankton, particularly *Karenia mikimotoi*, which mainly blooms in Japan, Korea, and Europe (Brand et al. 2012; Imai et al. 2006). Elucidating HAB fish-killing mechanisms is difficult, because it requires repeated-exposure tests under controlled conditions using HAB plankton. It is impossible to predict when HABs will occur, and cultured HAB sometimes do not exhibit any ichthyotoxicity (Mooney et al. 2010).

Recently, cytotoxic assays of harmful marine microalgae using mammalian cell lines (Katsuo et al. 2007) and fish embryo cell lines (Skjelbred et al. 2011)

N. Ohkubo (✉) · Y. Tomaru · S. Kitatsuji · K. Mochida
National Research Institute of Fisheries and Environment of
Inland Sea, Fisheries Research and Education Agency, 2-17-5
Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan
e-mail: nobuyuki@affrc.go.jp

H. Yamaguchi
Faculty of Agriculture, Kochi University, Nankoku, Kochi
783-8502, Japan

have been performed. Cell culture assays have several advantages over in vivo techniques, including a reduction in the number of experimental fish, faster results, simplicity, cost-effectiveness, and specificity. Moreover, Tanneberger et al. (2013) reported good agreement between in vivo and in vitro methods of comparing the effects of toxic chemicals by using a fish gill cell line-based toxicity assay.

The gill is thought to be the organ that incurs maximum damage by *K. mikimotoi* (Mitchell and Rodger, 2007), with gill epithelia being the primary uptake sites of water-borne contaminants. Thus, it is important to reveal the damage in gill epithelia of marine fish caused by *Karenia*. Previously, gill cell cultures have mainly been performed using rainbow trout (*Oncorhynchus mykiss*) (Bols et al. 1994; Pärt et al. 1993). Recently, Dorantes-Aranda et al. (2011; 2015) used rainbow trout gill cell line to test the cytotoxicity of harmful marine microalgae; however, these gill cells are not sufficiently saline-tolerant and cannot be directly exposed to toxic microalgae in seawater for more than 3 h. Exposing cultured gill cells to cultured HAB cells that are ichthyotoxic, in a highly reproducible manner, would constitute a new development in HAB ichthyotoxicity research. Therefore, the establishment of an evaluation method using cultured gill cells of a marine species that represents fish-farming mortalities in Japan due to HABs should facilitate elucidation of the mechanisms involved in HAB-related gill epithelial damage.

In the present study, we developed a gill cell culture from the red sea bream (*Pagrus major*), a popular farmed marine fish in Japan, to study the ichthyotoxicity of the harmful marine microalgae *K. mikimotoi*. This dinoflagellate has significantly affected fish farming, including that of *P. major* (Fisheries Agency, Japan 2013); more recently, other *Karenia* species (i.e., *K. papilionacea* and *K. papilionacea* phytotype-I; Yamaguchi et al. 2016), and *Heterosigma akashiwo* have formed blooms in southern and western Japanese waters, creating serious concerns for fish farmers (Imai et al. 2006).

Materials and methods

Fish

Red sea breams (30–180 g) were provided by a commercial fish farm (A-marine Kindai, Wakayama, Japan). They were kept in 60-L tanks with a filtered seawater

flow-through system at 20 °C and under a 14 h light:10 h dark cycle for 4–12 months. The fish were fed twice a day with commercial food (Otohime EP 0 and 2, Marubeni Nisshin Feed, Tokyo, Japan). A total of eight fishes were used in this study. Procedures for animal care and use were in accordance with the guidelines for animal experimentation at National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency.

Cell culture medium and chemicals

Leibovitz's L-15 culture medium with L-glutamine and fetal bovine serum (FBS) was obtained from Gibco Life Technology (Carlsbad, CA, USA). A mixture of penicillin-streptomycin-amphotericin B and gentamicin were obtained from Nacalai Tesque (Kyoto, Japan). Bacitracin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ampicillin, nystatin, metronidazole, chloramphenicol, and collagenase were obtained from Wako Pure Chemical Industries (Osaka, Japan). Dispase II was obtained from Boehringer Ingelheim (Ingelheim, Germany).

Preparation of gill cell culture

Gill cells were isolated in a laminar flow hood using sterile techniques according to Bols et al. (1994), with some modifications. The fish were stunned by a blow to the head, blood was sampled using a syringe, and fish were decapitated. The gills were dissected out and all eight gill arches were excised with sterile instruments, cleaned, and placed in ice-cold physiological saline solution for marine fish (Hirano et al. 1971), supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.1 µg/mL amphotericin B, 400 µg/mL gentamicin, 10 IU/mL bacitracin, 20 IU/mL ampicillin, 50 IU/mL nystatin, 125 µg/mL metronidazole, and 10 µg/mL chloramphenicol. Gill arches were gently pressed using a cell scraper for approximately 15 min to remove blood cells in ice-cold physiological saline solution for marine fish supplemented with antibiotics and fungicide (composition as described). The gill filaments were then excised from the arches, transferred to physiological saline solution for marine fish containing 2 mg/mL collagenase and 1.2 mg/mL dispase II in a 50-mL centrifuge tube, and the lamellae were cut into small pieces. After a 2-h incubation at 25 °C with slow shaking (approximately 60 rpm), cell suspensions were

filtered twice through stainless steel mesh (75 and 50 μm), subsequently transferred to a 50-mL centrifuge tube, and 30 mL of Leibovitz's L-15 culture medium supplemented with 20 mM NaCl, 10% FBS, 20 IU/mL penicillin, 20 $\mu\text{g}/\text{mL}$ streptomycin, and 40 $\mu\text{g}/\text{mL}$ gentamicin (pH 7.6) was added. The contents were centrifuged for 10 min at $500\times g$ at 15 °C. The pellet was resuspended in the L-15 culture medium and centrifuged again in the same manner. After washing, cells were resuspended in the L-15 culture medium as described above, plated at 1×10^5 cells/ cm^2 in 12- or 24-well culture plates (BD Falcon, USA), and then maintained in an incubator at 20 °C in the dark. Cell counts were obtained using a hemocytometer. Twenty-four hours after seeding, non-adherent cells were removed by changing the basal culture medium (Leibovitz's L-15 culture medium supplemented with 20 mM NaCl, 10% FBS, 20 IU/mL penicillin, and 20 $\mu\text{g}/\text{mL}$ streptomycin; pH 7.6). The medium was renewed two times per week.

Subcultivation was conducted from 2 weeks (at confluence) to 2 months after establishing the primary cultures. Cells were rinsed with phosphate-buffered saline solution and detached using 0.1% trypsin and 0.02% EDTA for 5 min at 10 °C. Then, 500 μL of the basal culture medium was added and mixed. A volume of 100 μL of detached cells was transferred into a 24-well culture plates, and 500 μL of the basal culture medium added.

Electron microscopy

Gill cells were grown on tissue culture coverslips (Nunc Thermanox coverslip, Thermo Scientific, USA) placed at the bottom of the culture plate wells. The culture medium was discarded and the coverslips with the attached gill cells were rinsed twice with Hank's balanced salt solution (Gibco Life Technology, Carlsbad, CA, USA). The gill cells were then fixed in 0.5 mL of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at 25 °C. They were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h at 25 °C. The cultures were then dehydrated through a graded ethanol series, transferred to *t*-butyl-alcohol, then freeze dried. After drying, the coverslips were mounted on stubs, sputter coated with Pt for 4 min (JEC-3000FC, JOEL, Tokyo, Japan) and examined using scanning electron microscopy (JSM-6510LV, JOEL).

Preparation of microalgae cultures

The toxic microalgae, *K. mikimotoi* strain KmURN6Y (Km, originally collected from Uranouchi Bay, Japan in 2009 and isolated by H. Yamaguchi), *K. papilionacea* strains KpURN1Y (Kp1) and KpURN9Y (Kp9) (collected from Uranouchi Bay, Japan in 2009 and isolated by H. Yamaguchi), and *K. papilionacea* phylotype-I strains KspNOM1H (Ksp1) and KspNOM6H (Ksp6) (collected from Nomi Bay, Japan in 2012 and isolated by H. Yamaguchi), were obtained from Kochi University. The *H. akashiwo* strain NIES-6 (originally collected from Osaka, Japan in 1979 and isolated by M. Watanabe) was obtained from the National Institute for Environmental Studies, Japan. *K. mikimotoi* and *H. akashiwo* were maintained in SWM-3 medium enriched with 2 nM Na_2SeO_3 (Imai et al. 1996), and *K. papilionacea* and *K. papilionacea* phylotype-I were maintained in Daigo IMK medium (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan). Salinity of seawater used for media preparation was adjusted to 30. Cultures were maintained at 20 °C, and at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (cool white fluorescent lamps) under a 12/12 h light/dark cycle.

Intact-cell suspensions at concentrations of 5×10^4 cells/mL were prepared from cultures in the late exponential growth phase to the stationary phase. They were taken directly from the culture and diluted as needed.

Preparation of ruptured cells and the cell-free medium of the *K. mikimotoi* culture

Ruptured cells and a cell-free medium were prepared from the stationary phase of a *K. mikimotoi* culture, in order to understand the mechanisms of toxicity associated with this species. The ruptured-cell suspension was prepared as follows: 25 mL of the *K. mikimotoi* culture (7×10^4 cells/mL) was centrifuged at $2200\times g$ for 5 min at 4 °C. Then, the supernatant was discarded and the pellet was homogenized for 1 min on ice with a glass homogenizer, resuspended in 25 mL of new SWM-3 medium (equivalent cell density of 7×10^4 cells/mL), and used as the ruptured-cell suspension. *K. mikimotoi* cultures (7×10^4 cells/mL) were filtered through a polycarbonate membrane (Isopore, Merk, Darmstadt, Germany) with a 3- μm pore size (gravity filtration), and the filtrate was used as the cell-free medium.

Preparation of concentrated ruptured cells of the *K. mikimotoi* culture

A 50 mL aliquot of the *K. mikimotoi* culture (2×10^4 cells/mL) was centrifuged at $2200 \times g$ for 5 min at 4 °C. The supernatant was then discarded and the pellet was homogenized for 1 min on ice with a glass homogenizer, resuspended in 5 mL of new SWM-3 medium, and used as a 10X concentration of ruptured-cell suspension (equivalent cell density of 2×10^5 cells/mL). For a preliminary analysis of the localization of toxic substance(s), the concentrated ruptured-cell suspension was fractionated by centrifugation. A 2.0 mL aliquot of the 10X-concentrated ruptured-cell suspension was centrifuged at $3000 \times g$ for 5 min at 4 °C, and the pellet was resuspended in 2.0 mL of new SWM-3. The supernatant was then centrifuged at $10,000 \times g$ for 5 min at 4 °C, and the pellet was resuspended in 2.0 mL of new SWM-3. The supernatant and resuspended pellets were then used for the cytotoxicity assay. A 5X concentration of the ruptured-cell suspension (equivalent cell density of 1×10^5 cells/mL) was prepared as a positive control by adding the same volume of SWM-3 medium to the 10X-concentrated ruptured-cell suspension. To elucidate the characteristics of the toxic substance(s), the 5X concentration of ruptured-cell suspension was heated for 5 min at 100 °C, or frozen for 15 min at -80 °C and thawed at 20 °C and gill cell viability was assayed.

A 50 mL aliquot of the *K. mikimotoi* culture (7×10^4 cells/mL) was centrifuged at $2200 \times g$ for 5 min at 4 °C. The supernatant was then discarded and the pellet was homogenized for 1 min on ice with a glass homogenizer, resuspended in 5 mL of new SWM-3 medium, and used as a 10X concentration of ruptured-cell suspension (equivalent cell density of 7×10^5 cells/mL). 5X and 2.5X concentration of the ruptured-cell suspension (equivalent cell density of 3.5×10^5 and 1.8×10^5 cells/mL, respectively) were prepared by adding SWM-3 medium to the 10X-concentrated ruptured-cell suspension.

Gill cell tolerance to seawater

To determine whether the gill cells (seeded as described above) remained viable after direct exposure to seawater-based medium, they were exposed to SWM-3 medium for 4 or 6 h, and IMK medium for 4 h. Cell viability was determined using a LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA,

USA). Once exposure was complete, the medium was discarded and the gill cells were rinsed with a 1/2 concentration artificial seawater (Herbst 1904) (adjusted to pH 7.5 with HEPES). Physiological saline solution containing the LIVE/DEAD® dye (2 μM calcein AM and 4 μM EthD-1) was then added to all the wells, and the cells were incubated for 30 min at 20 °C in the dark.

Fluorescence images of live gill cells in the center of each well were recorded using a color digital camera (DP 71, Olympus, Tokyo, Japan) mounted on the microscope (IX71N-22FL/PH, Olympus, Tokyo, Japan). The area occupied by the surviving gill cells was then determined using image analysis software (ImageJ; U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>), and was compared between the experimental group and a control group, which was cultured only with the basal culture medium.

Assay for testing the toxicity of harmful microalgae

Gill cells at the confluent stage were subcultured twice to minimize the possibility of a lack of variation in the primary-cultured gill cells, the medium was discarded, and 0.4 mL of cultured marine microalgae was directly added to each well of the 24-well culture plates. After adding the microalgae, the gill cells were incubated for 3.5 h at 20 °C in the dark. Once exposure was complete, the medium was discarded and the gill cells were rinsed with 1/2 concentration artificial seawater. LIVE/DEAD® dye was then added, as described above.

Fluorescence images of live gill cells in the center of each well were recorded using a color digital camera mounted on the microscope. The area occupied by the surviving gill cells was then determined using image analysis software, as described above, and was compared between the experimental group and a control group, which was exposed with the seawater-based culture medium.

Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) to identify significant differences between the treatments, and a Bonferroni correction was applied for multiple tests. If the data were heteroscedastic, a Kruskal-Wallis test and a posteriori Steel's test were performed. To test the relationship between the concentration of ruptured-cell suspension of the *K. mikimotoi* culture and the live cell coverage of gill cells culture, we

used the Jonckheere-Terpstra test. Differences were regarded as significant at $P < 0.05$. Microsoft Excel add-in software Excel Tokuei 2012 for Windows (SSRI Inc., Tokyo, Japan) was used for all statistical analyses.

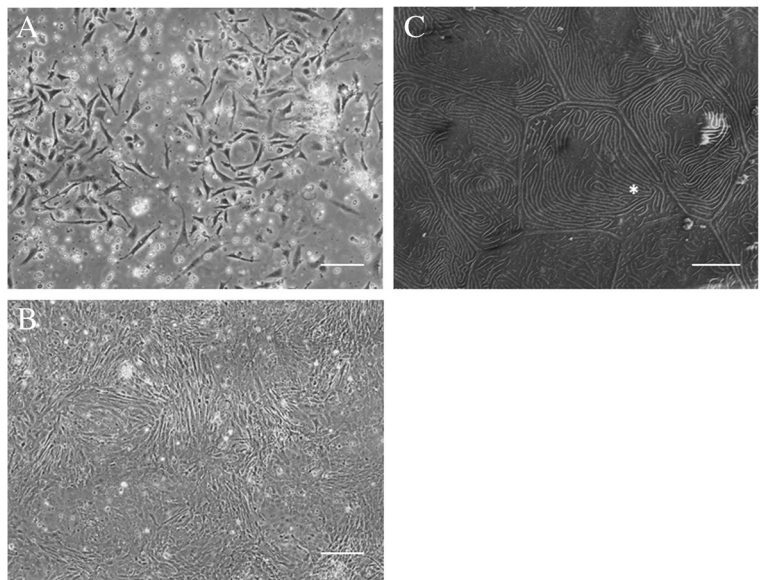
Results

Gill cell culture

The primary-cultured gill cells had an irregular, spindle shape, and formed an adherent monolayer on the bottom of the culture plate, but red blood cells were also present (Fig. 1a). The epithelial-like cells appeared dark under a phase-contrast microscope. At confluence, most of the plates were covered with 1–2 layers of polygonal-shaped epithelial-like cells within 2–3 weeks (Fig. 1b), and both flattened and elongated cells were observed. Scanning electron micrographs of the cell surface showed a typical characteristic of epithelial cells of fish gill filament such as microridges (Fig. 1c), and the cell surface was similar to that of intact secondary lamella from red sea bream gills (Hashimoto et al. 1987). Some cells lacked microridges and had smooth surfaces.

The confluent cell layers could be maintained for months in the basal culture medium, and the cells could be successfully detached and subcultured by trypsin treatment. The cultured cells were confirmed to subculture five passages.

Fig. 1 Photomicrograph of primary-cultured gill cells from red sea bream (*Pagrus major*). **a** Cells at day 5 after seeding; scale bar, 100 μm . **b** Cells at day 18 after seeding; scale bar, 200 μm . The majority of the bottom of the culture plate was covered with 1–2 layers of cells at confluence. **c** Scanning electron micrograph of the surface of the gill cells; scale bar, 10 μm . The cell in the center displays a typical pattern of microridges characteristic of epithelial cells of fish gill filament (*asterisk*)



Tolerance of the gill cells to seawater

The cultured gill cells were saltwater-tolerant; $98.3 \pm 0.4\%$ (mean \pm SE) of the SWM-3-treated plates (Fig. 2a) and $97.2 \pm 0.7\%$ of the IMK-treated plates (Fig. 2b) was covered with live cells for 4 h at 20 $^{\circ}\text{C}$, and no significant differences were observed between the L-15 culture medium. Although significantly different to that of the control ($P = 0.045$), $96.1 \pm 1.0\%$ of the cells of the SWM-3-treated plates still survived for 6 h (Fig. 2a). Therefore, red sea bream gill cells could resist direct exposure to a seawater-based medium for at least 4 h.

Exposure to *K. mikimotoi*

Exposure to *K. mikimotoi* caused a high percentage of gill cells to detach from the bottom of the culture plate, and the area of live cells significantly decreased ($P < 0.0001$) compared to that of the control treatment (SWM-3 media) (Fig. 3). *K. mikimotoi* cells adhered to gill cells on the bottom of the culture plate and strongly fluoresced so we evaluated the survival ratio of gill cells by the live cell coverage. Live cell coverage was only $17.6 \pm 5.4\%$ (Fig. 4a) on the plate following *K. mikimotoi* exposure, compared to $97.0 \pm 0.5\%$ of control cells exposed to SWM-3.

The area of live cells decreased to $61.0 \pm 3.6\%$ of the plate after exposure to ruptured *K. mikimotoi* cells (Figs.

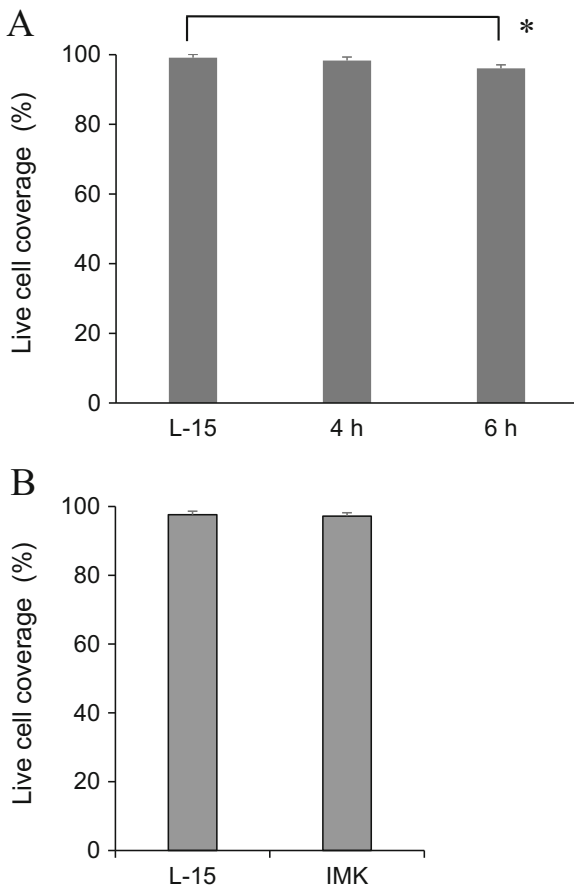


Fig. 2 Effect of seawater-based medium exposure on cultured gill cell viability. Cells were exposed to SWM-3 medium for 4 or 6 h (**a**), and IMK medium for 4 h (**b**). Values represent means \pm SEs of live cell coverage (%) in triplicate wells. L-15: Leibovitz's L-15 culture medium; IMK: Daigo IMK medium

3d and 4a) and was significantly different to that of the SWM-3 treatment ($P < 0.0001$). However, no significant differences were observed between the effect of the filtrate (cell-free medium) ($88.5 \pm 4.6\%$) and the SWM-3 treatment (Fig. 4a).

Cytotoxicity was detected after the concentrated ruptured cells of *K. mikimotoi* had been centrifuged at $3000\times g$, which significantly decreased the live gill cell coverage to $53.7 \pm 12.0\%$ ($P = 0.0350$) (Fig. 4b). However, after the concentrated ruptured cells of *K. mikimotoi* had been re-centrifuged at $10,000\times g$, the live gill cell coverage of the fraction did not significantly decrease ($P = 0.9864$), although some gill cell damage was observed in the supernatant ($P = 0.0350$) (Fig. 4b). Significant cytotoxicity was also detected in the 5X concentration of ruptured cells (positive control) ($P = 0.0350$). In addition, the live gill cell coverage

significantly decreased with increasing concentrations of ruptured cells ($P < 0.001$) (Fig. 4c).

Significant cytotoxicity was also detected in the 5X concentration of ruptured cells after freezing and thawing ($P = 0.0189$) though cytotoxicity was weakened; and cytotoxicity almost disappeared after heat treatment ($P = 0.7972$) (Fig. 4d).

Exposure to *K. papilionacea* phylotype-I, *K. papilionacea*, and *H. akashiwo*

Cytotoxicity was also detected as a result of exposure to *K. papilionacea* phylotype-I, which significantly decreased the live gill cell coverage compared to the IMK media (Fig. 5c). Live gill cell coverage decreased to $44.7 \pm 2.9\%$ after Ksp1 exposure ($P = 0.0002$) and to $79.3 \pm 3.2\%$ after Ksp6 exposure ($P < 0.0001$). No significant difference was detected in live cell coverage between media containing *K. papilionacea* phylotype-I (Ksp 1) cells removed by filtration and the IMK medium. Live gill cell coverage did not decrease significantly after *K. papilionacea* ($P = 0.3316$) or *H. akashiwo* exposure ($P = 0.1351$) (Fig. 5b or a).

Discussion

Morphology of cultured red sea bream gill cells was similar to that of a rainbow trout gill cell line, RTgill-W1 (Bols et al. 1994), and previous observations of primary-cultured fish gill cells (Avella and Ehrenfeld 1997; Bui and Kelly 2015; Leguen et al. 2007; Zhou et al. 2005). Moreover, these cells exhibited seawater tolerance using conventional plates. Using red sea bream gill cells is unnecessary to acclimate the cells to seawater, thereby making it possible to directly expose the cells to harmful marine microalgae. To our knowledge, this is the first study that has developed an ichthyotoxicity assay for HABs using the cultured gill cells of a fully marine fish (oceanodromous).

Exposure to *K. mikimotoi* caused gill cells to detach from the bottom of the microplate (Fig. 3c). Detached epithelia (epithelial cells lift) have been previously observed in the gills of marine fish exposed to *K. mikimotoi* (Mitchell and Rodger, 2007). The cytotoxicity of the *K. mikimotoi* ruptured-cell suspension was lower than that of the *K. mikimotoi* intact-cell suspension, and was almost non-existent in the cell-free medium (Fig. 4a). Cytotoxicity of the concentrated ruptured-

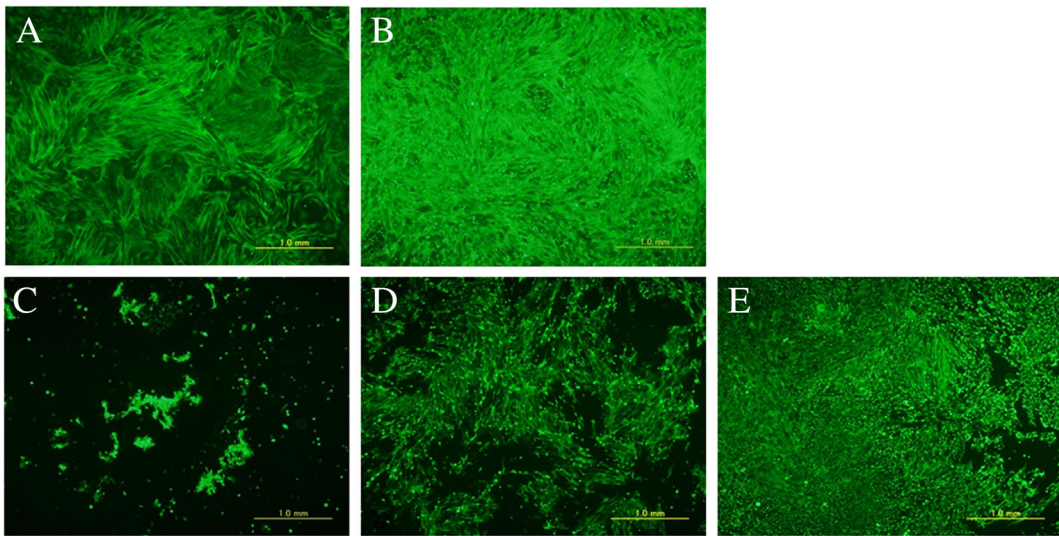


Fig. 3 Fluorescence images of cultured gill cells after exposure to different algal components of *Karenia mikimotoi*. **a** Leibovitz's L-15 culture medium. **b** SWM-3 medium. **c** Intact cells. **d** Ruptured cells. **e** Cell-free medium

cell suspension was mainly detected in the pellet that had been resuspended after centrifugation at 3000×g (Fig. 4b). The cytotoxicity of this suspension remained

after freezing though cytotoxicity was weakened but almost disappeared after heating (Fig. 4d). Therefore, *K. mikimotoi* cytotoxicity may be caused by labile

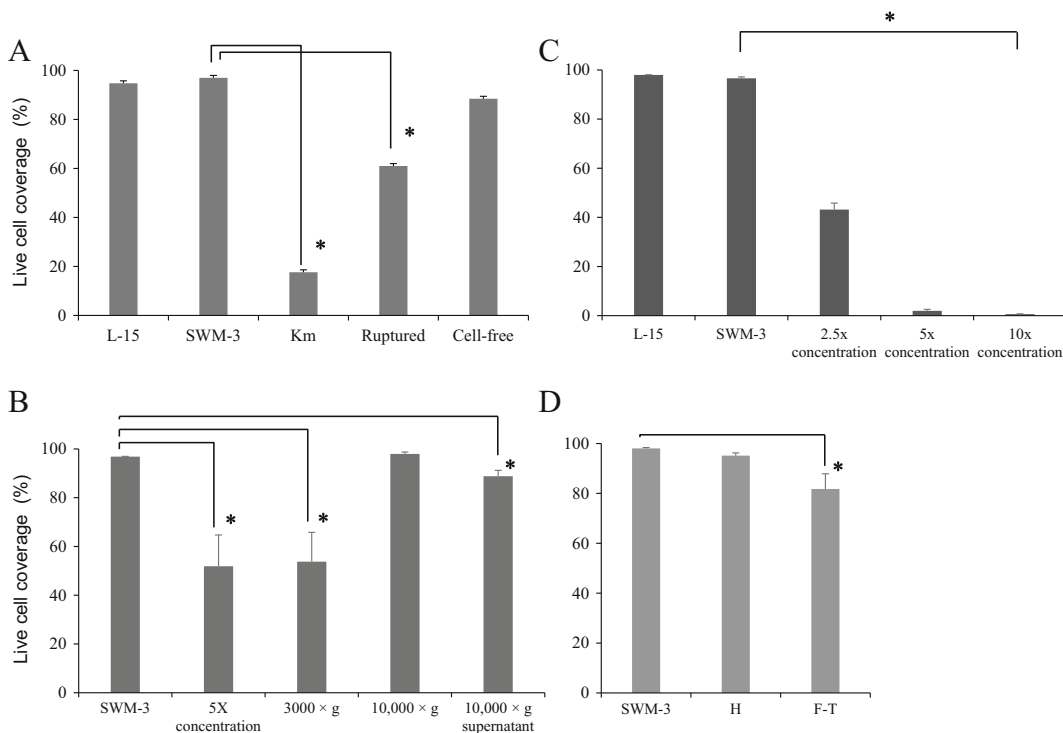


Fig. 4 Gill cell bioassay using three different algal components of *Karenia mikimotoi* (**a**). Effect of a concentrated *K. mikimotoi* ruptured-cell suspension after centrifugation (**b**), different concentrations of ruptured-cell suspension (**c**), and those after heating or freezing/thawing (**d**) on gill cell viability. Km: intact cells of

K. mikimotoi; Ruptured: ruptured cells; Cell-free: cell-free medium; SWM-3: SWM-3 medium; H: heating; F-T: freezing and thawing. Values represent means ± SEs of live cell coverage (%) in triplicate wells. Asterisks indicate significant differences ($P < 0.05$) in exposure to SWM-3 medium

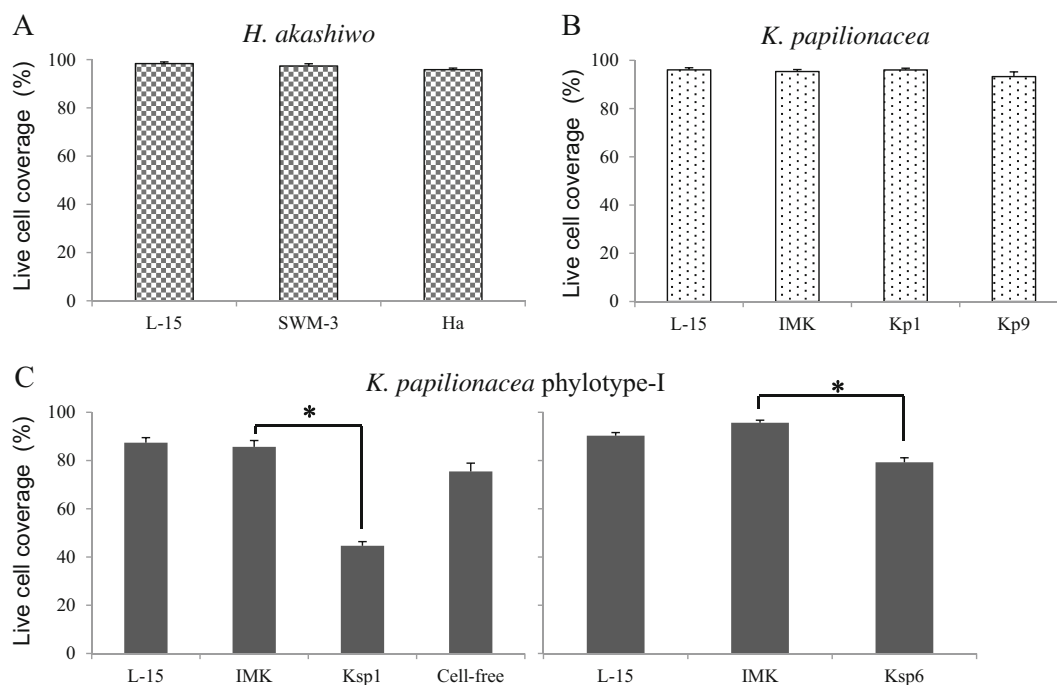


Fig. 5 Effect of different algal strains on gill cell viability. **a** Ha: *Heterosigma akashiwo*. **b** Kp1 and Kp9: *Karenia papilionacea*. **c** Ksp1 and Ksp6: *K. papilionacea* phylotype-I. Cell-free: cell-free medium; L-15: Leibovitz's L-15 culture medium; SWM-3: SWM-

3 medium; IMK: Daigo IMK medium. Values represent means \pm SEs of live cell coverage (%) in triplicate wells. Asterisks indicate significant differences ($P < 0.05$) in exposure to a seawater-based medium

substances that are transmitted by direct contact between *K. mikimotoi* cells and gill cells. Zou et al. (2010) reported that *K. mikimotoi* cells attack the membranes of mammalian erythrocytes and rotifers by direct cell-to-cell contact. However, they did not observe any toxicity in *K. mikimotoi* ruptured-cell suspensions (Zou et al. 2010); in contrast, toxicity in *K. mikimotoi* ruptured-cell suspensions was observed in the present study. This difference may be due to the instability of the toxicant, or differences between cultured gill cells, rotifers, and erythrocytes in assay sensitivity. Although several hypotheses have been proposed regarding the mechanism involved in *K. mikimotoi* toxicity, no conclusive evidence has been found (Mooney et al. 2009). Gymnocin, which is a cytotoxic polyether, has been isolated from *K. mikimotoi* (Satake et al. 2002, 2005), but is weakly toxic to fish and has extremely low solubility in water. It has also been proposed that polyunsaturated fatty acids and/or palmitic acid in the membranes of *Karenia* spp. are toxic (Mooney et al. 2011). Ishimatsu et al. (1996) hypothesized that ROS released by *Chattonella* cells stimulate fish gills to secrete mucus that traps the *Chattonella* cells, thereby destroying the gas exchange

capability of the gills by diverting the respiratory water current away from the lamellae. However, ROS production by *K. mikimotoi* is 10-fold lower than that of *Chattonella* (Mooney et al. 2011; Yamasaki et al. 2004), and ROS production by *K. mikimotoi* has been shown to have no effect on rainbow trout gill cells (Mooney et al. 2011). Therefore, it seems unlikely that ROS production by *K. mikimotoi* is involved in fish killing.

K. papilionacea and *H. akashiwo* were not toxic to gill cells (Fig. 5b and a), which supports the results of previous studies that also showed that the two HAB-causing species were not very toxic to fish (Brand et al. 2012; Imai et al. 2006). On the other hand, *K. papilionacea* phylotype-I showed toxicity to gill cells (Fig. 5c), but not in the cell-free medium preparation, similar to *K. mikimotoi*. It has recently been reported that the two strains of *K. papilionacea* (originally collected from New Zealand and USA.) produce very low concentrations of brevetoxin, a neurotoxic compound that is also produced by *Karenia brevis* (Fowler et al. 2015), and characterized this dinoflagellate as a potentially toxic species. Thus, it is important to continue

studying *K. papilionacea* phylotypes found in Japanese waters.

In conclusion, the toxicity of harmful marine microalgae on gill epithelial cells of marine fish was assessed using a simple and effective method. To our knowledge, this is the first study that has developed a toxicity assay for HAB species using cultured gill cells of an oceanodromous fish. Using this gill cell assay method, in our future studies, we plan to identify the cytotoxic substances responsible for ichthyotoxicity of *K. mikimotoi*, and to elucidate the effects of environmental conditions on the level of toxicity of this microalga.

Acknowledgements We are grateful to Dr. Shigeru Itakura (Fisheries Agency of Japan), Dr. Osamu Kurata (Nippon Veterinary and Life Science University), and Ms. Chiaki Hiramoto (National Research Institute of Fisheries and Environment of Inland Sea). This study was supported in part by a grant-in-aid from the Fisheries Agency of Japan.

References

- Avella M, Ehrenfeld J (1997) Fish gill respiratory cells in culture: a new model for Cl^- -secreting epithelia. *J Membr Biol* 156:87–97
- Bols N, Barlian A, Chirinotrejo M, Caldwell S, Goegan P, Lee L (1994) Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills. *J Fish Dis* 17:601–611
- Brand L, Campbell L, Bresnan E (2012) *Karenia*: The biology and ecology of a toxic genus. *Harmful Algae* 14:156–178
- Bui P, Kelly SP (2015) Claudins in a primary cultured puffer fish (*Tetraodon nigroviridis*) gill epithelium model alter in response to acute seawater exposure. *Comp Biochem Physiol A* 189:91–101
- Dorantes-Aranda J, Waite T, Godrant A, Rose A, Tovar C, Woods G, Hallegraef G (2011) Novel application of a fish gill cell line assay to assess ichthyotoxicity of harmful marine microalgae. *Harmful Algae* 10:366–373
- Dorantes-Aranda J, Seger A, Mardones J, Nichols P, Hallegraef G (2015) Progress in understanding algal bloom-mediated fish kills: the role of superoxide radicals, phycotoxins and fatty acids. *Plos one*. doi:10.1371/journal.pone.0133549
- Fisheries Agency (2013) Red Tides in the Seto Inland Sea. Fisheries Agency, Japan. <http://www.jfa.maff.go.jp/setouti/akasio/gepou/pdf/24nenpou-1.pdf> (in Japanese) Accessed 7 April 2017
- Fowler N, Tomas C, Baden D, Campbell L, Bourdelais A (2015) Chemical analysis of *Karenia papilionacea*. *Toxicon* 101: 85–91
- Hashimoto T, Suzuki Y, Sugimura M, Atoji Y (1987) Ultrastructure of gills and pseudobranchs of red sea bream. *Res Bull Fac Agr Gifu Univ* 52:173–181 (in Japanese with English abstract)
- Herbst C (1904) Concerning the necessary inorganic substances for the development of sea urchin larvae, their role and their defensibility. III. Part. The role of essential inorganic material. *Arch Entwick Org* 17:306–520
- Hirano T, Johnson D, Bern H (1971) Control of water movement in flounder urinary bladder by prolactin. *Nature* 230:469–471
- Imai I, Itakura S, Matsuyama Y, Yamaguchi M (1996) Selenium requirement for growth of a novel red tide flagellate *Chattonella verruculosa* (Raphidophyceae) in culture. *Fish Sci* 62:834–835
- Imai I, Yamaguchi M, Hori Y (2006) Eutrophication and occurrences of harmful algal blooms in the Seto Inland Sea, Japan. *Plankton Benthos Res* 1:71–84
- Ishimatsu A, Sameshima M, Tamura A, Oda T (1996) Histological analysis of the mechanisms of *Chattonella*-induced hypoxemia in yellowtail. *Fish Sci* 62:50–58
- Katsuo D, Kima D, Yamaguchi K, Matsuyama Y, Oda T (2007) A new simple screening method for the detection of cytotoxic substances produced by harmful red tide phytoplankton. *Harmful Algae* 6:790–798
- Leguen I, Cauty C, Odjo N, Corlu A, Pruneta P (2007) Trout gill cells in primary culture on solid and permeable supports. *Comp Biochem Physiol A* 148:903–921
- Mitchell S, Rodger H (2007) Pathology of wild and cultured fish affected by a *Karenia mikimotoi* bloom in Ireland, 2005. *Bull Eur Ass Fish Pathol* 27:39–42
- Mooney B, de Salas M, Hallegraef G, Place A (2009) Survey for karlotoxin production in 15 species of gymnodinioid dinoflagellates (Kareniaceae, Dinophyta). *J Phycol* 45:164–175
- Mooney B, Hallegraef G, Place A (2010) Ichthyotoxicity of four species of gymnodinioid dinoflagellates (Kareniaceae, Dinophyta) and purified karlotoxins to larval sheepshead minnow. *Harmful Algae* 9:557–562
- Mooney B, Dorantes-Aranda J, Place A, Hallegraef G (2011) Ichthyotoxicity of gymnodinioid dinoflagellates: PUFA and superoxide effects in sheepshead minnow larvae and rainbow trout gill cells. *Mar Ecol Prog Ser* 426:213–224
- Pärt P, Norrgren L, Bergstrom E, Sjoberg P (1993) Primary cultures of epithelial cells from rainbow trout gills. *J Exp Biol* 175:219–232
- Satake M, Shoji M, Oshima Y, Naoki H, Fujita T, Yasumoto T (2002) Gymnocin-A, a cytotoxic polyether from the notorious red tide dinoflagellate, *Gymnodinium mikimotoi*. *Tetrahedron Lett* 43:5829–5832
- Satake M, Tanaka Y, Ishikura Y, Oshima Y, Naoki H, Yasumoto T (2005) Gymnocin-B with the largest contiguous polyether rings from the red tide dinoflagellate, *Karenia* (formerly *Gymnodinium*) *mikimotoi*. *Tetrahedron Lett* 46:3537–3540
- Skjelbred B, Horsberg TE, Tollefsen KE, Andersen T, Edvardsen B (2011) Toxicity of the ichthyotoxic marine flagellate *Pseudochattonella* (Dictyochophyceae, Heterokonta) assessed by six bioassays. *Harmful Algae* 10:144–154
- Tanneberger K, Knobel M, Busser F, Sinnige T, Hermens J, Schirmer K (2013) Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environ Sci Technol* 47: 1110–1119

- Yamaguchi H, Hirano T, Yoshimatsu T, Tanimoto Y, Matsumoto T, Suzuki S, Hayashi Y, Urabe A, Miyamura K, Sakamoto S, Yamaguchi M, Tomaru Y (2016) Occurrence of *Karenia papilionacea* (Dinophyceae) and its novel sister phylotype in Japanese coastal waters. *Harmful Algae* 57:59–68
- Yamasaki Y, Kim D, Matsuyama Y, Oda T, Honjo T (2004) Production of superoxide anion and hydrogen peroxide by the red tide dinoflagellate *Karenia mikimotoi*. *J Biosci Bioeng* 97:212–215
- Zhou B, Liu W, Wu R, Lam P (2005) Cultured gill epithelial cells from tilapia (*Oreochromis niloticus*): a new in vitro assay for toxicants. *Aquat Toxicol* 71:61–72
- Zou Y, Yamasaki Y, Matsuyama Y, Yamaguchi K, Honjo T, Oda T (2010) Possible involvement of hemolytic activity in the contact-dependent lethal effects of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*. *Harmful Algae* 9:367–373