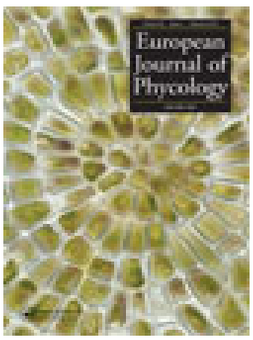

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Extremely high level of reactive oxygen species (ROS) production in a newly isolated strain of the dinoflagellate *Karenia mikimotoi*

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ABSTRACT

We found that the newly isolated dinoflagellate strain *Karenia mikimotoi* NGU04 generates O_2^- at a level almost equal to that of the raphidophycean flagellate *Chattonella marina*, and much higher than those of other strains of *K. mikimotoi*. Fluorescence microscopy observation of strain NGU04 suggested that ROS such as O_2^- and H_2O_2 are generated in certain intracellular compartments. As in *C. marina*, a significant increase in O_2^- generation was observed in the presence of lectins suggesting that *K. mikimotoi* has a signalling system that can respond to extracellular stimuli. The NGU04 strain showed a greater lethal effect on rotifers than other strains, but the toxicity was not inhibited by ROS-scavenging enzymes. Our findings indicate that different strains of *K. mikimotoi* can generate different amounts of ROS depending on the strain, but certain toxic factors other than ROS may be responsible for rotifer toxicity.

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KEYWORDS Algae; harmful dinoflagellate; hydrogen peroxide; *Karenia mikimotoi*; reactive oxygen species (ROS); rotifer toxicity

Introduction

Harmful algal blooms (HABs) are serious threats for marine ecosystems and aquaculture industries, and can cause enormous damage to marine food resources worldwide. *Karenia mikimotoi* (formerly *Gyrodinium aureolum*, *G.* cf. *aureolum*, *G.* type-'65, *G. nagasakiense* and *G. mikimotoi*) is known as a causative dinoflagellate of HABs worldwide (Hansen *et al.*, 2000). HABs resulting from *K. mikimotoi* have been reported in Western Japanese coastal waters (Honjo, 1994; Yoshimatsu, 2008), the North Atlantic (Gentien, 1998; Davidson *et al.*, 2009) and other coastal areas (Lu & Hodgkiss, 2004; Li *et al.*, 2017). Red tides caused by *K. mikimotoi* in Japan have led to massive killing of fish (Honjo, 1994) and shellfish (Yamaguchi, 1994). Since the mid-1960s, when *K. mikimotoi* blooms first occurred (Iizuka & Irie, 1966), mortality of various fish and invertebrate species caused by *K. mikimotoi* has been reported in Europe, Australia, Japan, South America and North Africa (Landsberg, 2002). Regarding the toxic mechanisms of *K. mikimotoi*, it has been reported that *K. mikimotoi* produces various toxic agents such as low molecular weight haemolytic toxins (Yasumoto *et al.*, 1990; Jenkinson & Arzul, 2001; Neely & Campbell, 2006; Mooney *et al.*, 2007), cytotoxic polyethers (Satake *et al.*, 2002, 2005) and reactive oxygen species (ROS) (Yamasaki *et al.*, 2004; Gentien *et al.*, 2007). Widdows *et al.* (1979) and Matsuyama *et al.* (1999) showed that *G. mikimotoi* strongly inhibited the filtration rate of bivalves. Sellem *et al.* (2000) demonstrated that the 18:5n3 fatty acid produced by *G.* cf. *mikimotoi* exhibited detrimental effects on the sea urchin *Paracentrotus lividus*. Mitchell & Rodger (2007) reported that *K. mikimotoi* blooms were associated with fish and shellfish mortality, and they also found histopathological changes in the gills, gastrointestinal tracts and livers of the fish and shellfish killed by *K. mikimotoi*.

Herbivorous zooplankton, such as rotifers copepods, have been used to elucidate toxic mechanisms of HAB species (Wang *et al.*, 2005; Zhenxing *et al.*, 2006; Estrada *et al.*, 2008), and several dinoflagellates exhibited lethal effects on the rotifer *Brachionus plicatilis* (Abe & Hirayama, 1979; Kim *et al.*, 2000*a*). We previously found that rotifers are highly sensitive to *K. mikimotoi* (Zou *et al.*, 2010), and a comparative study between two strains of *K. mikimotoi* isolated from different localities in Japan demonstrated that the rotifer toxicity of *K. mikimotoi* varied significantly depending on the strain (Zou *et al.*, 2010).

In 2012, HABs of *K. mikimotoi* occurred in coastal areas of Kyushu Island in Japan and were associated with mass mortalities of various cultured fish species,

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including Japanese pufferfish (*Takifugu rubripes*). We have been studying the fish-killing mechanism of HAB species, especially *Chattonella marina*, focusing on the relationship between ROS generation and ichthyotoxic potential, so the potent fish-killing activity of the newly isolated *K. mikimotoi* prompted us to examine the ROS generation of this strain in this study. To evaluate ROS as a potential toxic factor of the *K. mikimotoi* strain, rotifer exposure experiments were performed in the presence or absence of ROS-scavenging enzymes.

Materials and methods

Plankton culture

A *Karenia mikimotoi* strain (NGU04, clone, non-axenic) was isolated from Omura Bay, Western Kyushu Island, Japan, in late autumn 2012, and maintained in MS-SNF medium (Table 1). From this area, 12 clonal strains of *K. mikimotoi* were established. Among the strains, NGU04 was well adapted to laboratory culture conditions, and was able to grow continuously as described later. In the preliminary study, NGU04 showed potent lethal toxicity against juvenile yellowtail (*Seriola quinqueradiata*) as well as substantial toxicity to a rotifer (*B. plicatilis*), seeming even more toxic than some other *K. mikimotoi* strains. One of the other two strains of *K. mikimotoi* used in this study was isolated from Suo Nada (SUO-1), Japan, in 2006, and another (strain 2411) was obtained from the National Institute for Environmental Studies (NIES), originally isolated from Katagami Bay, Japan, in 2004. *Chattonella marina*, which was isolated from Kagoshima, Japan, in 1985, and is known to produce high levels of ROS (Oda *et al.*, 1997), was provided by Kagoshima Prefectural Fisheries Experimental Station, and since then it has been maintained in our laboratory. These clonal strains, except NGU04, were maintained at 27°C in 100 ml flasks containing 50–60 ml of modified seawater medium (SWM-3) at a salinity of 25 (Yamasaki *et al.*, 2007), which was used as the growth medium. The NGU04 strain was maintained under the same conditions in MS-SNF medium instead of SWM-3 medium. The cultures were kept under a 12:12 h photoperiod using a cool-white fluorescent lamp (200 ± 5 µmol photons m^{-2} s^{-1}). Culture cell numbers were counted microscopically using a haemocytometer (Erma Inc., Tokyo, Japan). The rotifer *B. plicatilis* was originally provided by Dr A. Hagiwara (Faculty of Fisheries, Nagasaki University, Japan) and was cultured with *Nannochloropsis oculata*, as described previously (Kim *et al.*, 2000*a*).

Table 1. Microalgal medium with suppressive-chelator used in Seikai National Fisheries research institute (MS-SNF) for *Karenia mikimotoi* NGU04 strain. The medium was dissolved in 1 l of seawater and the pH was adjusted to 7.75 followed by autoclaving (75°C, 1 h).

Compound	Final concentration	Concentration in seawater (l^{-1})
KNO_3	840 µM	85 mg
NaH_2PO_4-$2H_2O$	40 µM	6.24 mg
EDTA-2Na	3 µM	1.11 mg
Fe(III)-EDTA	1 µM	0.421 mg
Mn-EDTA	1 µM	0.664 mg
$CoCl_2$-$6H_2O$	100 nM	100 nM
H_2SeO_3	10 nM	10 nM
Na_2SiO_3· $9H_2O$	10 µM	2.84mg
$C_4H_{11}NO_3$	405 mg l^{-1}	405 mg
$C_4H_{11}NO_3$· HCl	95 mg l^{-1}	95 mg
Vitamin mix S3*	200 µl	200 µl

*Composition of Vitamin mix S3: Thiamine HCl, 5 mg; Nicotinic acid, 1 mg; Calcium pantothenate, 1 mg; *p*-Aminobenzoic acid, 0.1 mg; Biotin, 0.01 mg; Inositol, 50 mg; Folic acid, 0.02 mg.

Measurement of superoxide anion (O_2^-)

For the detection of O_2^-, we employed a chemiluminescence (CL) assay using L-012 (Fujifilm Wako Pure Chemical Industry, Co., Ltd, Osaka, Japan), a highly sensitive CL probe, as described previously (Kadomura *et al.*, 2006; Kim *et al.*, 2009). Reaction mixtures consisted of, in the order of addition, 80 µl of each flagellate cell suspension, 10 µl of superoxide dismutase (SOD: Cu, Zn-SOD) solution (final 100 U ml^{-1}) or growth medium, and 10 µl of L-012 (final 100 µg ml^{-1}). SOD is an enzyme which catalyses the dismutation reaction of superoxide to hydrogen peroxide. To confirm superoxide generation, we used SOD. If the CL response was inhibited or reduced by SOD, it is supporting evidence for superoxide generation. The CL response of the growth medium alone was measured as background. During 30 s incubation, the CL response of each sample was recorded by a Mithras LB940 plate recorder (Berthold Technologies GmbH and Co. KG, Bad Wildbad, Germany). All CL measurements were conducted in triplicate at 27°C using 96-well microplates. To examine the effects of lectins, 10 µl of varying concentrations of each lectin solution in the plankton growth medium was added to the plankton cells, and then 10 µl of L-012 (final 100 µg ml^{-1}) was added to start the measurement of the CL response. Concanavalin A (Con A) and wheatgerm agglutinin (WGA) were obtained from Sigma Chemical Co. (St. Louis, Missouri). Castor bean haemagglutinin (CBH) was purified from castor bean as previously described (Mise *et al.*, 1977).

Measurement of hydrogen peroxide (H_2O_2)

H_2O_2 was detected by a *p*-hydroxyphenylacetate (PHPA) assay (Hyslop & Sklar, 1984) at 27°C. After the addition of PHPA (final 1 mM) and horseradish peroxidase (final 100 µg ml^{-1}) to the cell suspension in the medium, the change in fluorescence intensity during 30 min of incubation was measured using a fluorescence spectrophotometer (Hitachi Model 650-60, Hitachi, Tokyo, Japan) at

an excitation wavelength of 317 nm and an emission wavelength of 400 nm, in the presence or absence of catalase (final 200 U ml^{-1}). Catalase converts H_2O_2 to H_2O and O_2. Thus, the catalase-inhibited increase in fluorescence intensity is considered as actual H_2O_2. The concentration of H_2O_2 was estimated using a standard curve of H_2O_2 in cell-free medium. The standard solution of H_2O_2 in the medium was prepared from reagent-grade H_2O_2 (Santoku Chemical Industries Co. Ltd, Japan). Under these assay conditions, the increase in fluorescence was proportional to the concentration of H_2O_2.

Fluorescence microscopy observation

Flagellate cells were incubated with 2-methyl-6(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) (final 100 µM) for 15 min at 27°C. After incubation, the flagellate cells were immediately observed under a fluorescence microscope (Keyence BZ-X710). ROS production in flagellate cells was also examined by 5-(and -6)-carboxy-2,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (final 10 µM), which is a cell membrane-permeant agent that is trapped intracellularly after cleavage by cellular esterases (Czupryna & Tsourkas, 2011; Zhou *et al.*, 2015). Fluorescence produced by the oxidation of 2′,7′-dichlorodihydrofluorescein (DCF) with ROS including H_2O_2 was observed using a fluorescence microscope (Keyence BZ-X710) at 27°C (Mahadev *et al.*, 2001).

Rotifer exposure assay

The tests were conducted in 48-well plates (Becton-Dickinson Co., Ltd, Franklin Lakes, New Jersey, USA). To each well containing 900 µl of *K. mikimotoi* cell suspension (2×10^4 cells ml^{-1}), 10–12 rotifers in 100 µl of culture medium were added. As a negative control, rotifers were incubated in 1 ml of the plankton culture medium alone. The plates were incubated at 27°C under plankton culture conditions. The number of dead rotifers was counted every hour by stereomicroscopic observation over the course of the incubation. For the NGU04 strain, MS-SNF medium was used instead of SWM-3 medium. Three wells were used for each test. To examine the potential involvement of ROS in *K. mikimotoi* lethality to rotifers, toxicity tests were conducted in the presence of SOD (final 100 U ml^{-1}), which catalyses the conversion of superoxide to hydrogen peroxide, and catalase (final 200 U ml^{-1}), which then catalyses the decomposition of hydrogen peroxide to water and oxygen.

Statistical analysis

All the experiments were performed in triplicate and data were expressed as the mean ± standard deviation. Data were analysed with a paired Student's *t*-test to evaluate significant differences. $p < 0.05$ was considered statistically significant.

Results

Comparison of superoxide anion (O_2^-) generation among the three K. mikimotoi *strains and* C. marina

An immediate CL response was observed in the *K. mikimotoi* strain NGU04 after the addition of L-012 (Fig. 1). In the presence of SOD (final 100 U ml^{-1}), the response was inhibited to an almost background level of the growth medium alone (Fig. 1A), suggesting that the CL response reflects the O_2^- level generated by *K. mikimotoi* cells. Surprisingly, the CL response induced by the NGU04 strain was nearly equivalent to that of *C. marina*, which is known to produce high levels of ROS and to induce a potent L-012-mediated CL response (Fig. 1D). Consistent with the previous tendency, the chemiluminescence responses of the other two *K. mikimotoi*, strains isolated in different years at different locations were almost at trace levels (Fig. 1B, C). Morphological observation showed that strain NGU04 is flattened, and has a straight apical groove and oval nucleus located on the left side. These features are identical to another *K. mikimotoi* strain, therefore, they are clearly the same species. Nevertheless, the present results indicated that the level of ROS generated by the NGU04 strain is exceptionally high, and may be the highest for dinoflagellates reported to date.

Similar to *C. marina*, increased CL responses were observed in the NGU04 strain after the addition of lectins such as Con A which recognizes glucose and

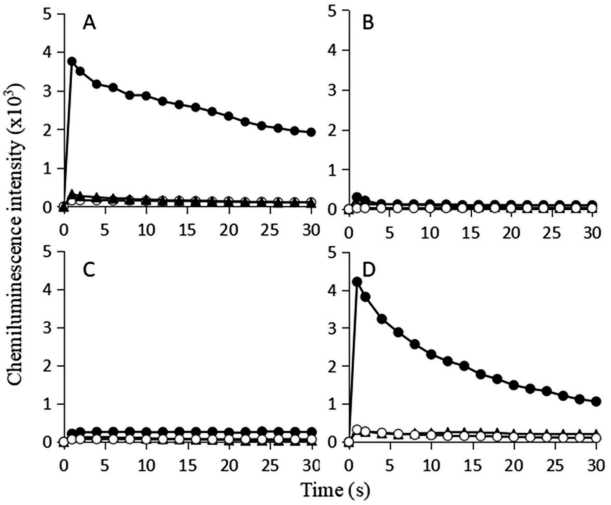


Fig. 1. L-012-dependent chemiluminescence responses of *Karenia mikimotoi* and *Chattonella marina*. (**A**) NGU04, (**B**) SUO-1 and (**C**) 2411 strains of *K. mikimotoi* (final 2×10^4 cells ml^{-1}), and (**D**) *C. marina* (final 2×10^4 cells ml^{-1}) were subjected to chemiluminescence analysis in the presence (○) or absence (●) of SOD (final 100 U ml^{-1}). Background luminescence (▲) of the growth medium alone was measured at the same time.

mannose (Fig. 2A). The effect of Con A was concentration dependent, and the maximum stimulatory effect was observed at 25 μg ml^{-1}. The other lectins WGA and CBH, which have specificity to galactosamine and galactose, respectively, also showed stimulatory effects in a concentration-dependent manner. The relationship between the peak value of CL responses of the NGU04 strain and concentrations of lectins showed different profiles depending on the lectins (Fig. 2B). The lectin-response profiles of the *K. mikimotoi* NGU04 strain differed from those of *C. marina* examined under the same conditions (Fig. 2C).

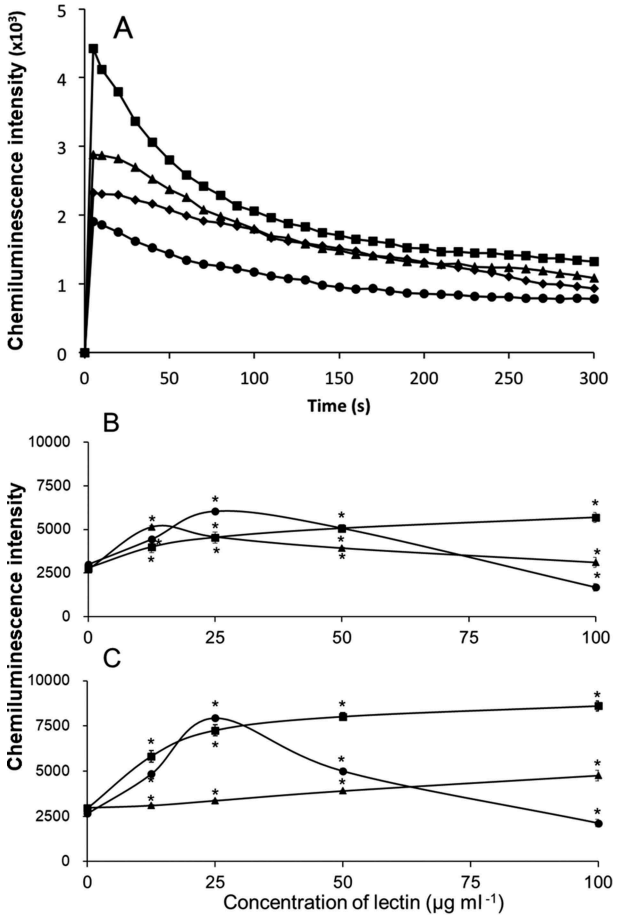


Fig. 2. Effects of lectins on the chemiluminescence responses of the NGU04 strain of *Karenia mikimotoi* and of *Chattonella marina*. (**A**) After simultaneous addition of L-012 (final 100 μg ml^{-1}) and Con A at final concentration of 0 (●), 25 (■), 50 (▲) and 100 μg ml^{-1} (◆) to the cell suspension (final 2 × 10^4 cells ml^{-1}) of the *K. mikimotoi* NGU04 strain, chemiluminescence responses during the first 300 s were measured. (**B**) After simultaneous addition of L-012 (final 100 μg ml^{-1}) and indicated final concentrations of Con A (●), WGA (▲) or CBH (■) to the cell suspension of the *K. mikimotoi* NGU04 strain, the peak value of each chemiluminescence response was measured and plotted against each lectin concentration. (**C**) Effects of Con A (●), WGA (▲), or CBH (■) on the chemiluminescence responses in *C. marina* (final 2 × 10^4 cells ml^{-1}) examined as described above for NGU04 strain of *K. mikimotoi*. (B, C) Points indicate the mean of triplicate measurements, bars indicate ± standard deviation. Asterisks indicate significant differences between with and without lectins ($p < 0.05$).

Detection of hydrogen peroxide (H_2O_2) in the three K. mikimotoi *strains*

To further study ROS production by *K. mikimotoi*, we employed a fluorescence assay using PHPA for the detection of H_2O_2. A high level of H_2O_2 was detected in the NGU04 strain, which almost completely disappeared in the presence of catalase, confirming that the assay is specific for H_2O_2 (Fig. 3A). However, no significant H_2O_2 was detected in the other two strains of *K. mikimotoi* (Fig. 3B, C).

Fluorescence microscopic observation of the three K. mikimotoi *strains using two ROS-detecting probes*

As shown in Fig. 4, a dot-like fluorescence was observed on the cell surface of the NGU04 strain after incubation with the MCLA probe, whereas no significant fluorescence was observed in the other strains of *K. mikimotoi*. These results are consistent with those obtained in the CL response analysis (Fig. 1). Since we previously found that the MCLA-dependent CL response of *C. marina* was significantly inhibited by SOD (Kim *et al.*, 2007), it is considered that the MCLA-mediated CL response in strain NGU04 was mainly due to superoxide. Furthermore, we investigated ROS generation in *K. mikimotoi* using CM-H2DCFDA, which is another fluorescent probe for detecting ROS including

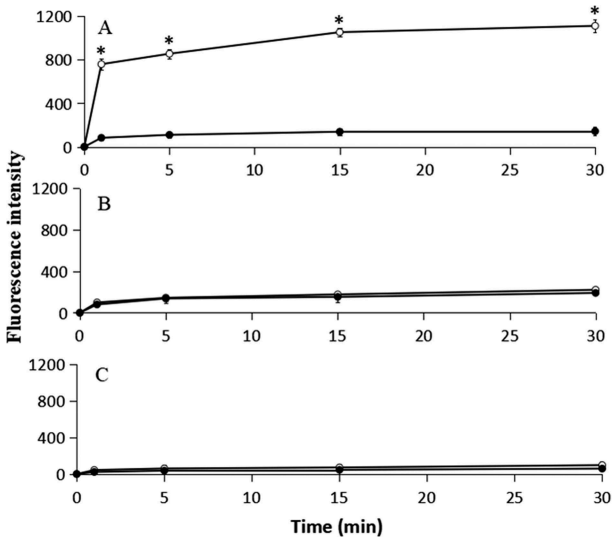
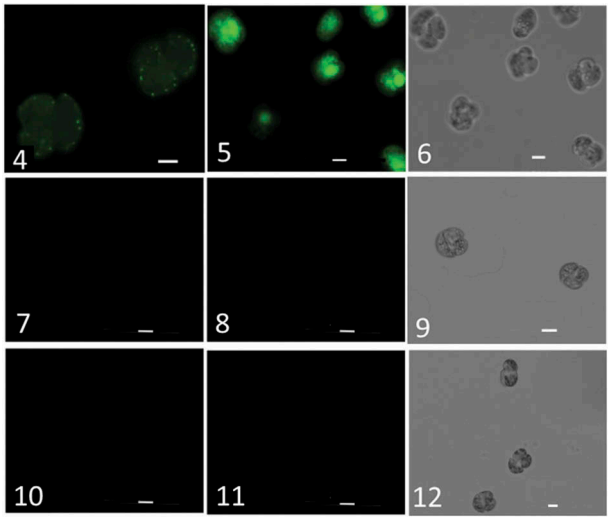
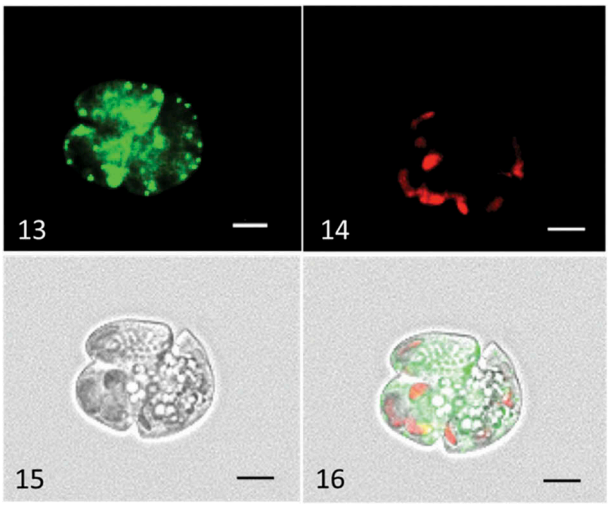


Fig. 3. Detection of H_2O_2 in the cell suspensions of (**A**) NGU04, (**B**) SUO-1 and (**C**) 2411 strains of *Karenia mikimotoi*. The cell suspensions (final 2 × 10^4 cells ml^{-1}) were subjected to the H_2O_2 detection assay in the presence (●) or absence (○) of catalase (final 200 U ml^{-1}) as described in the text. Points indicate the mean of triplicate measurements, bars indicate ± standard deviation. Asterisks indicate significant differences with and without catalase ($p < 0.05$).



Figs 4–12. Fluorescence microscopy observations of the NGU04 (Figs 4, 5, 6), SUO-1 (Figs 7, 8, 9) and 2411 (Figs 10, 11, 12) strains of *Karenia mikimotoi* after incubation with MCLA (Figs 4, 7, 10) as a specific fluorescent probe for O_2^- or CM-H_2DCFDA (Figs 5, 8, 11) as a fluorescent probe for H_2O_2. The cells were observed under phase-contrast (Figs 6, 9, 12). Bars indicate 5 µm.



Figs 13–16. Enlarged photographs of a cell of the *Karenia mikimotoi* NGU04 strain. **Fig. 13**. MCLA-derived fluorescence. **Fig. 14**. Intrinsic fluorescence derived from chlorophyll. **Fig. 15**. Phase-contrast observation. **Fig. 16**. Picture combining the three observations. Bars indicate 5 µm.

H_2O_2. Only the NGU04 strain showed intense fluorescence in the intracellular region after incubation with CM-H2DCFDA (Fig. 5). The enlarged picture of a cell of the NGU04 strain observed with MCLA (Fig. 13) clearly indicated that the location of MCLA-derived fluorescence was distinguishable from intrinsic chlorophyll fluorescence (Fig. 14). These fluorescence microscopic observations also suggest that the NGU04 strain has an extremely potent ability to generate ROS compared with the other strains of *K. mikimotoi*.

Effects of the three K. mikimotoi *strains on the rotifer* B. plicatilis

The NGU04 strain showed greater rotifer toxicity than the other *K. mikimotoi* strains, and all the rotifers exposed to NGU04 died within 2 h (Fig. 17A). However, some of the rotifers exposed to other strains

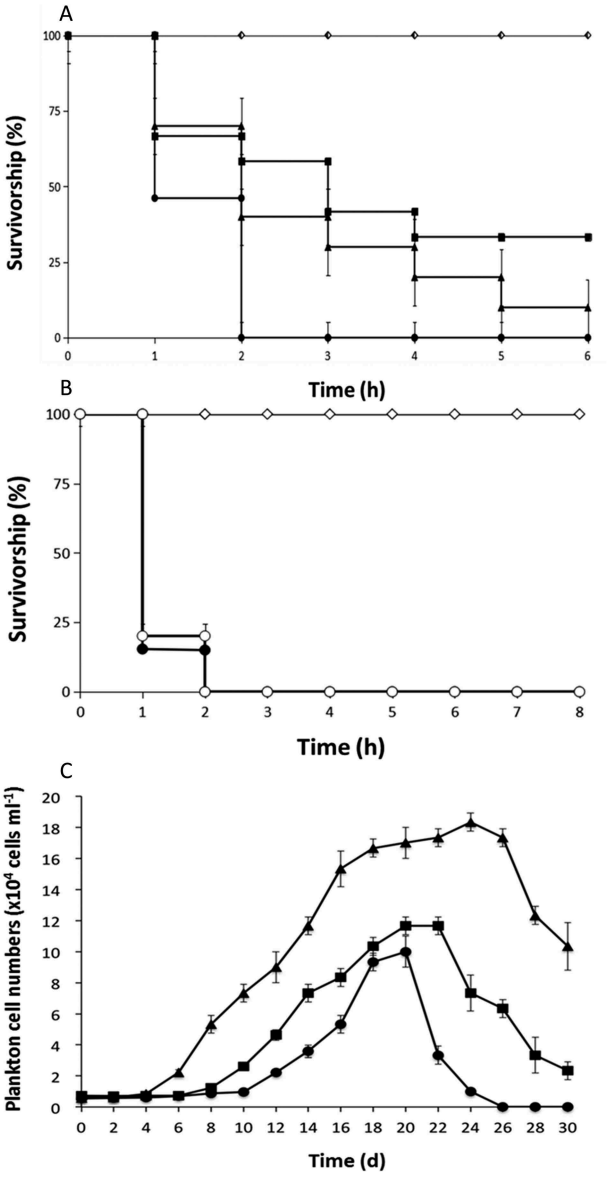


Fig. 17. (**A**) Effects of the NGU04, SUO-1 and 2411 strains of *Karenia mikimotoi* or *Chattonella marina* on the rotifer *Brachionus plicatilis*. After exposure to NGU04 (●), SUO-1 (▲), 2411 (■), *C. marina* (◆), or medium alone as control (◇), the viabilities of rotifers in the test groups were examined. (**B**) Effect of SOD and catalase on the toxicity of the NGU04 strain of *K. mikimotoi* against the rotifer. After exposure to NGU04 in the presence (○) or absence (●) of SOD (100 U ml^{-1}) and catalase (200 U ml^{-1}), or medium alone as control (◇), the viabilities of rotifers in the test groups were examined. (**C**) Growth curves of the NGU04 (●), SUO-1 (▲) and 2411 (■) strains of *K. mikimotoi*. After starting the culture of the strains at initial cell density of 1.3–1.5 × 10^4 cells ml^{-1}, the cell number of each culture was counted every 2 days. Points indicate the mean of triplicate measurements, bars indicate ± standard deviation.

still survived even after 6 h. *C. marina* showed no toxic effect on the rotifer as previously reported (Zou *et al.*, 2010). Furthermore, the lethal effect of the NGU04 strain on the rotifer was not inhibited by the addition of SOD and catalase (Fig. 17B).

Growth curves of the three strains of K. mikimotoi

The growth curves of three strains of *K. mikimotoi* were compared. Each culture of the three strains was started at an initial cell density of 1.3–1.5 × 10^4 cells ml^{-1}. After 14 days, all the cultures reached the exponential growth phase, and the cell density of NGU04, SUO-1 and 2411 strains was 3.5, 11.7 and 7.3 × 10^4 cells ml^{-1}, respectively (Fig. 17C). The maximum cell densities of NGU04, SUO-1 and 2411 strains were 10.0, 18.6 and 11.6 × 10^4 cells ml^{-1}, respectively (Fig. 17C). These results suggest that the growth rates of the three strains of *K. mikimotoi* follow the order SUO-1>2411>NGU04.

Discussion

In 2004, we reported that a strain of *K. mikimotoi* isolated from the Yatsushiro Sea, Japan, produced ROS, but the levels of ROS detected in the strain were significantly lower than those of *C. marina* (Yamasaki *et al.*, 2004). Since then we have tried to detect ROS production in several strains of *K. mikimotoi* and other dinoflagellates (Cho *et al.*, 2017), but the detected ROS levels were quite low or even negligible, as reported previously. In this study, we found that the strain *K. mikimotoi* (NGU04) isolated in 2012 produces ROS at levels almost as great as those of *C. marina* examined under the same experimental conditions. To the best of our knowledge, this is the first study to detect such a high level of ROS in *K. mikimotoi* cells. Regarding the biological significance of ROS generation by marine microalgae, the red tide phytoplankton species *Heterosigma carterae*, which also belongs to the raphidophycean flagellates, has been reported to produce ROS, and showed a ROS-mediated toxic effect on rainbow trout (Yang *et al.*, 1995). ROS generation of other raphidophycean flagellates, such as *Fibrocapsa japonica* (Oda *et al.*, 1997; Pezzolesi *et al.*, 2010) and *Olisthodiscus luteus* (Kim *et al.*, 1999), have been reported. These findings suggest that ROS production is a common biological feature of raphidophycean flagellates, although there are many other algal and plant species that can produce ROS (Roberty *et al.*, 2014; Smirnoff & Arnaud, 2019). When comparative studies were conducted under the same experimental conditions, *Chattonella* tended to show a much higher level of ROS than other species, despite the ROS levels in raphidophycean flagellates being significantly different between species, strains of the same species, growth conditions and assay methods. Although the exact ROS generation mechanism of red tide phytoplankton, including *K. mikimotoi* and *C. marina*, remains unclear, our previous study demonstrated that ROS generation by *C. marina* was increased by extracellular stimuli, such as lectins, which can recognize cell surface-specific carbohydrate chains and bind to them (Nakamura *et al.*, 1998; Oda *et al.*, 1998). It has been demonstrated that some lectins can induce various cellular signalling processes after binding to the cell surface carbohydrate moieties, including the stimulation of ROS generation in human leukocytes (Cohen *et al.* 1980; Kayashima *et al.* 1980). Hence, the unicellular microalga *C. marina* might have intracellular signalling pathways which can respond to lectin stimuli and thus lead to increased ROS generation. Similar to *C. marina*, the generation of ROS by the *K. mikimotoi* NGU04 strain was also increased in the presence of lectins such as Con A, WGA and CBH. The lectin-response profiles of the strain NGU04 differed from those of *C. marina* when examined under the same conditions (Fig. 2C). This may be due to the different cell surface structures, especially the lectin-binding sites, between the raphidophycean flagellate *C. marina* and the dinoflagellate *K. marina*, although the detailed underlying mechanisms of lectin-induced increase in ROS generation remain unclear.

In human leukocytes, it is well known that a membrane protein, NADPH oxidase, is a major enzyme system responsible for ROS generation (Smirnov *et al.*, 2014). Furthermore, our previous immunoblotting study using antibodies raised against the human neutrophil NADPH oxidase large subunit (gp91phox) suggested that *C. marina* has a gp91phox homologous protein as a cell-surface-located protein (Kim *et al.*, 2000*b*). Since the ROS level of the strain NGU04 was also increased in the presence of lectins, this strain of *K. mikimotoi* might have a superoxide-producing enzyme system similar to *C. marina*. Further studies are necessary to clarify the exact mechanism of ROS generation in *K. mikimotoi*. For such a study, our NGU04 strain discovered in this study is useful, and an immunoblotting analysis similar to that used in the *C. marina* study described above may provide an insight into the ROS generation mechanism of *K. mikimotoi*.

Since O_2^- can be converted to H_2O_2 through enzymatic or spontaneous processes, depending on the biological circumstances, it is generally considered that H_2O_2 detected in certain biological systems is derived from primarily produced O_2^-. However, in the case of *Chattonella*, several lines of evidence suggest that the underlying mechanisms of O_2^- and H_2O_2 generation are different and that each system operates independently in the cells (Kim *et al.*, 2007). As in *Chattonella*, it has been reported that the marine phytoplankton *Hymenomonas carterae* (Haptophyta) produces extracellular H_2O_2 without the involvement of

O_2^- generation (Palenik *et al.*, 1987). To further study ROS production by *K. mikimotoi*, we employed a fluorescence assay using PHPA for the detection of H_2O_2, and found that a significantly high level of H_2O_2 was detected in the NGU04 strain, which almost completely disappeared in the presence of catalase, confirming that the assay is specific for H_2O_2 (Fig. 3A). However, no significant H_2O_2 was detected in the other two strains of *K. mikimotoi* (Fig. 3B, C). These results indicate that the NGU04 strain has an extremely high ability to produce both O_2^- and H_2O_2, although the association between O_2^- and H_2O_2 generation systems remain unclear. The fluorescence microscopy observations also suggest that the NGU04 strain has a potent ability to generate ROS compared with the other strains of *K. mikimotoi*. Since the images of the cells of the NGU04 strain obtained by fluorescence microscopic observation were quite different depending on the probes used (Figs 4, 5), one can speculate that different reactive oxygen species are produced in different intracellular locations in the cells. Further studies with highly specific fluorescence probes may provide insight into these points.

We have previously reported that the rotifer, *B. plicatilis*, was highly sensitive to *K. mikimotoi*, and that the lethal effects of *K. mikimotoi* on the rotifer differed depending on the strains tested (Zou *et al.*, 2010). We found that NGU04 strain showed greater rotifer toxicity than the other *K. mikimotoi* strains. Furthermore, the lethal effect of the NGU04 strain on the rotifer was not inhibited by the addition of SOD and catalase. These results suggest that the NGU04 strain may potentially be more harmful than others, but ROS might not be the major toxic factor against the rotifer. This notion may also be supported by the fact that *C. marina* showed no toxic effect on the rotifer.

In addition to the possible involvement of ROS in the fish-killing mechanism of *Chattonella*, which is still controversial, the molecular mechanism of ROS generation and its biological significance in unicellular microalgae such as *Chattonella* and *Karenia* are interesting subjects. It has been reported that O_2^- generation in *C. marina* is primarily controlled through photosynthesis, whereas H_2O_2 production by *H. akashiwo* occurs through a metabolic pathway not directly linked to photosynthesis (Marshall *et al.*, 2002). Our previous study suggested that ROS produced in *C. marina* plays an important role in their own growth, especially in cell division (Oda *et al.*, 1995). Here, we showed that the SUO-1 strain has better growth characteristics than the others, and the growth rate of the NGU04 strain was relatively lower than the others. These results suggest that ROS generation may not be positively related to the growth rate in *K. mikimotoi*.

Red tides caused by *K. mikimotoi* have been associated with mass mortality of fish species (Honjo, 1994; Mitchell & Rodger, 2007; Madhu *et al.*, 2011). In our preliminary study, it was confirmed that the strain NGU04 showed potent lethal toxicity against juvenile yellowtail (data not shown). In the present study, we could not identify ROS as a toxic factor. However, ROS are known to exhibit various physiological impacts on biological systems, such as an increase in vascular permeability, perturbation of membrane transport systems (Halliwell & Gutteridge, 1984; Dean, 1987) and induction of mucus secretion (LaMont, 1989). We are planning to investigate the effects of the NGU04 strain on fish species under laboratory conditions in our future study, in terms of the possible involvement of ROS.

In the present study, a strain of the dinoflagellate *Karenia mikimotoi* (strain NGU04) isolated in 2012, in Japan, showed extremely high ROS generation activity at levels almost equal to the raphidophycean flagellate *Chattonella marina*. This is the first report of a *K. mikimotoi* strain with such high ROS-producing activity. Since the ROS levels of other *K. mikimotoi* strains were almost negligible, ROS generation of *K. mikimotoi* varies significantly between strains. Fluorescence microscopy observation using specific ROS suggested that O_2^- and H_2O_2 are generated in different intracellular compartments in *K. mikimotoi*. The NGU04 strain showed greater toxicity to the rotifer than the other two strains, but the results suggested that ROS is not a main toxic factor against the rotifer.

Author contributions

D. Kim: drafting manuscript; L. Wencheng and Y. Matsuyama: culture experiments and drafting materials and methods section; K. Cho and Y. Yamasaki: ROS analysis; S. Takeshita and K. Yamaguchi: data arrangement and statistical analysis; T. Oda: original concept, manuscript editing and submission.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Abe, T. & Hirayama, K. (1979). Lethal effect of *Gymnodinium* sp. on the rotifer, *Brachionus plicatilis*

[aquatic invertebrates]. *Bulletin of the Faculty of Fisheries Nagasaki University*, **46**: 1–6 (in Japanese, with English abstract).

Cho, K., Kasaoka, T., Ueno, M., Basti, L., Yamasaki, Y., Kim, D. & Oda, T. (2017). Haemolytic activity and reactive oxygen species production of four harmful algal bloom species. *European Journal of Phycology*, **52**: 311–319.

Cohen, M.S., Metcalf, J.A. & Root, R.K. (1980). Regulation of oxygen metabolism in human granulocytes: relationship between stimulus binding and oxidative response using plant lectins as probes. *Blood*, **55**: 1003–1010.

Czupryna, J. & Tsourkas, A. (2011). Firefly luciferase and rluc8 exhibit differential sensitivity to oxidative stress in apoptotic cells. *PloS ONE*, **6**: 1–12.

Davidson, K., Miller, P., Wilding, T.A., Shutler, J., Bresnan, E., Kennington, K. & Swan, S. (2009). A large and prolonged bloom of *Karenia mikimotoi* in Scottish waters in 2006. *Harmful Algae*, **8**: 349–361.

Dean, R.T. (1987). Free radicals, membrane damage and cell-mediated cytolysis. *British Journal of Cancer. Supplement*, **8**: 39–45.

Estrada, M., Sala, M.M., van Lenning, K., Alcaraz, M., Felipe, J. & Veldhuis, M.J.W. (2008). Biological interactions in enclosed plankton communities including *Alexandrium catenella* and copepods: role of phosphorus. *Journal of Experimental Marine Biology and Ecology*, **355**: 1–11.

Gentien, P. (1998). Bloom dynamics and ecophysiology of the *Gymnodinium mikimotoi* species complex. In *Physiological Ecology of Harmful Algal Blooms* (Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M., editors), 155–173. Springer, Berlin.

Gentien, P., Lunven, M., Lazure, P., Youenou, A. & Crassous, M. P. (2007). Motility and autotoxicity in *Karenia mikimotoi* (Dinophyceae). *Philosophical Transactions of the Royal Society B: Biological Sciences*, **362**: 1937–1946.

Halliwell, B. & Gutteridge, J. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal*, **219**: 1–14.

Hansen, G., Daugbjerg, N. & Henriksen, P. (2000). Comparative study of *Gymnodinium mikimotoi* and *Gymnodinium aureolum*, comb. nov.(= *Gyrodinium aureolum*) based on morphology, pigment composition, and molecular data. *Journal of Phycology*, **36**: 394–410.

Honjo, T. (1994). The biology and prediction of representative red tides associated with fish kills in Japan. *Reviews in Fisheries Science*, **2**: 225–253.

Hyslop, P.A. & Sklar, L. A. (1984). A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes. *Analytical Biochemistry*, **141**: 280–286.

Iizuka, S. & Irie, H. (1966). The hydrographic conditions and the fisheries damages by the red tide occurred in Omura Bay in summer 1965. II. *Bulletin of the Faculty of Fisheries. Nagasaki University*, **21**: 67–101.

Jenkinson, I.R. & Arzul, G. (2001). Mitigation by cysteine compounds of rheotoxicity, cytotoxicity and fish mortality caused by the dinoflagellates, *Gymnodinium mikimotoi* and *G.* cf. *maguelonnense*. In *Harmful Algal Blooms 2000* (Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. & Lewis, R.J., editors), 71–84. IOC, Paris.

Kadomura, K., Nakashima, T., Kurachi, M., Yamaguchi, K. & Oda, T. (2006). Production of reactive oxygen species (ROS) by devil stinger (*Inimicus japonicus*) during embryogenesis. *Fish and Shellfish Immunology*, **21**: 209–214.

Kayashima, K., Onoue, K., Nakagawa, A. & Minakami, S. (1980). Superoxide anion-generating activities of macrophages as studied by using cytochalasin E and lectins as synergistic stimulants for superoxide release. *Microbiology and Immunology*, **24**: 449–461.

Kim, D., Nakamura, A., Okamoto, T., Komatsu, N., Oda, T., Ishimatsu, A. & Muramatsu, T. (1999). Toxic potential of the raphidophyte *Olisthodiscus luteus*: mediation by reactive oxygen species. *Journal of Plankton Research*, **21**: 1017–1027.

Kim, D., Sato, Y., Oda, T., Muramatsu, T., Matsuyama, Y. & Honjo, T. (2000a). Specific toxic effect of dinoflagellate *Heterocapsa circularisquama* on the rotifer *Brachionus plicatilis*. *Bioscience, Biotechnology, and Biochemistry*, **64**: 2719–2722.

Kim, D., Nakashima, T., Okamoto, T., Komatsu, N., Oda, T., Iida, T., Ishimatsu, A. & Muramatsu, T. (2000b). Mechanism of superoxide anion generation in the toxic red tide phytoplankton *Chattonella marina*: possible involvement of NAD(P)H oxidase. *Biochimica et Biophysica Acta*, **1524**: 220–227.

Kim, D., Nakashima, T., Matsuyama, Y., Niwano, Y., Yamaguchi, K. & Oda, T. (2007). Presence of the distinct systems responsible for superoxide anion and hydrogen peroxide generation in red tide phytoplankton *Chattonella marina* and *Chattonella ovata*. *Journal of Plankton Research*, **29**: 241–247.

Kim, D., Yamasaki, Y., Yamatogi, T., Yamaguchi, K., Matsuyama, Y., Kang, Y.S., Lee, Y. & Oda, T. (2009). The possibility of reactive oxygen species (ROS)-independent toxic effects of *Cochlodinium polykrikoides* on damselfish (*Chromis caerulea*). *Bioscience, Biotechnology, and Biochemistry*, **73**: 613–618.

Landsberg, J.H. (2002). The effects of harmful algal blooms on aquatic organisms. *Reviews in Fisheries Science*, **10**: 113–390.

LaMont, J.T. (1989). Oxygen radicals stimulate gallbladder glycoprotein secretion. In *Mucus and Related Topics* (Chantler, E. & Ratcliffe, N.A., editors), 273–278. The Company of Biologists, Cambridge.

Li, X., Yan, T., Lin, J., Yu, R. & Zhou, M. (2017). Detrimental impacts of the dinoflagellate *Karenia mikimotoi* in Fujian coastal waters on typical marine organisms. *Harmful Algae*, **61**: 1–12.

Lu, S. & Hodgkiss, I.J. (2004). Harmful algal bloom causative collected from Hong Kong waters. *Hydrobiologia*, **512**: 231–238.

Madhu, N.V., Reny, P.D., Paul, M., Ullas, N. & Resmi, P. (2011). Occurrence of red tide caused by *Karenia mikimotoi* (toxic dinoflagellate) in the Southwest coast of India. *Indian Journal of Geo-Marine Sciences*, **40**: 821–825.

Mahadev, K., Zilbering, A., Zhu, L. & Goldstein, B. J. (2001). Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. *Journal of Biological Chemistry*, **276**: 21938–21942.

Marshall, J.A., Hovenden, M., Oda, T. & Hallegraeff, G.M. (2002). Photosynthesis does influence superoxide production in the ichthyotoxic alga *Chattonella marina* (Raphidophyceae). *Journal of Plankton Research*, **24**: 1231–1236.

Matsuyama, Y., Uchida, T. & Honjo, T. (1999). Effects of harmful dinoflagellates, *Gymnodinium mikimotoi* and *Heterocapsa circularisquama*, red-tide on filtering rate of bivalve molluscs. *Fisheries Science*, **65**: 248–253.

Mitchell, S., & Rodger, H. (2007). Pathology of wild and cultured fish affected by a *Karenia mikimotoi* bloom in Ireland, 2005. *Bulletin – European Association of Fish Pathologists*, **27**: 39–42.

Mise, T., Funatsu, G., Ishiguro, M. & Funatsu, M. (1977). Isolation and characterization of ricin E from castor beans. *Agricultural and Biological Chemistry*, **41**: 2041–2046.

Mooney, B.D., Nichols, P.D., De Salas, M.F. & Hallegraeff, G.M. (2007). Lipid, fatty acid, and sterol composition of eight species of Kareniaceae (Dinophyta): chemotaxonomy and putative lipid phycotoxins. *Journal of Phycology*, **43**: 101–111.

Nakamura, A., Okamoto, T., Komatsu, N., Ooka, S., Oda, T., Ishimatsu, A. & Muramatsu, T. (1998). Fish mucus stimulates the generation of superoxide anion by *Chattonella marina* and *Heterosigma akashiwo*. *Fisheries Science*, **64**: 866–869.

Neely, T. & Campbell, L. (2006). A modified assay to determine hemolytic toxin variability among *Karenia* clones isolated from the Gulf of Mexico. *Harmful Algae*, **5**: 592–598.

Oda, T., Moritomi, J., Kawano, I., Hamaguchi, S., Ishimatsu, A. & Muramatsu, T. (1995). Catalase-and superoxide dismutase-induced morphological changes and growth inhibition in the red tide phytoplankton *Chattonella marina*. *Bioscience, Biotechnology, and Biochemistry*, **59**: 2044–2048.

Oda, T., Nakamura, A., Shikayama, M., Kawano, I., Ishimatsu, A. & Muramatsu, T. (1997). Generation of reactive oxygen species by raphidophycean phytoplankton. *Bioscience, Biotechnology, and Biochemistry*, **61**: 1658–1662.

Oda, T., Nakamura, A., Okamoto, T., Ishimatsu, A. & Muramatsu, T. (1998). Lectin-induced enhancement of superoxide anion production by red tide phytoplankton. *Marine Biology*, **131**: 383–390.

Palenik, B., Zafiriou, O.C. & Morel, F.M.M. (1987). Hydrogen peroxide production by a marine phytoplankter. *Limnology and Oceanography*, **32**: 1365–1369.

Pezzolesi, L., Cucchiari, E., Guerrini, F., Pasteris, A., Galletti, P., Tagliavini, E., Totti, C. & Pistocchi, R. (2010). Toxicity evaluation of *Fibrocapsa japonica* from the Northern Adriatic Sea through a chemical and toxicological approach. *Harmful Algae*, **9**: 504–514.

Roberty, S., Bailleul, B., Berne, N., Franck, F. & Cardol, P. (2014). PSI Mehler reaction is the main alternative photosynthetic electron pathway in *Symbiodinium* sp., symbiotic dinoflagellates of cnidarians. *New Phytologist*, **204**: 81–91.

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 Letters*, **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 Letters*, **46**: 3537–3540.

Sellem, F., Pesando, D., Bodennec, G., El Abed, A. & Girard, J.P. (2000). Toxic effects of *Gymnodinium* cf. *mikimotoi* unsaturated fatty acids to gametes and embryos of the sea urchin *Paracentrotus lividus*. *Water Research*, **34**: 550–556.

Smirnoff, N. & Arnaud, D. (2019). Hydrogen peroxide metabolism and functions in plants. *New Phytologist*, **221**: 1197–1214.

Smirnov, A., Daily, K.P. & Criss, A.K. (2014). Assembly of NADPH oxidase in human neutrophils is modulated by the opacity-associated protein expression state of *Neisseria gonorrhoeae*. *Infection and Immunity*, **82**: 1036–1044.

Wang, L., Yan, T., Yu, R. & Zhou, M. (2005). Experimental study on the impact of dinoflagellate *Alexandrium* species on populations of the rotifer *Brachionus plicatilis*. *Harmful Algae*, **4**: 371–382.

Widdows, J., Fieth, P. & Worrall, C.M. (1979). Relationships between seston, available food and feeding activity in the common mussel *Mytilus edulis*. *Marine Biology*, **50**: 195–207.

Yang, C.Z., Albright, L.J. & Yousif, A.N. (1995). Oxygen-radical-mediated effects of the toxic phytoplankter *Heterosigma carterae* on juvenile rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms*, **23**: 101–108.

Yamaguchi, M. (1994). Physiological ecology of the red tide flagellate *Gymnodinium nagasakiense* (Dinophyceae) – mechanism of the red tide occurrence and its prediction. *Bulletin of Nansei National Fisheries Research Institute*, **27**: 251–394.

Yamasaki, Y., Kim, D.I., Matsuyama, Y., Oda, T. & Honjo, T. (2004). Production of superoxide anion and hydrogen peroxide by the red tide dinoflagellate *Karenia mikimotoi*. *Journal of Bioscience and Bioengineering*, **97**: 212–215.

Yamasaki, Y., Nagasoe, S., Matsubara, T., Shikata, T., Shimasaki, Y., Oshima, Y. & Honjo, T. (2007). Allelopathic interactions between the bacillariophyte *Skeletonema costatum* and the raphidophyte *Heterosigma akashiwo*. *Marine Ecology Progress Series*, **339**: 83–92.

Yasumoto, T., Underdal, B., Aune, T., Hormazabal, V. Skulberg, O.M. & Oshima, Y. (1990). Screening for hemolytic and ichthyotoxic components of *Chrysochromulina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters. In *Toxic Marine Phytoplankton* (Graneli, E., Sundstrom, B., Edler, L. & Anderson, D.M., editors), 436–440. Elsevier, New York.

Yoshimatsu, S. (2008). Long-term variation in phytoplankton in southern part of Harimanada. *Bulletin of the Plankton Society of Japan*, **55**: 41–44.

Zhenxing, W., Yinglin, Z., Mingyuan, Z., Zongling, W. & Dan, W. (2006). Effects of toxic *Alexandrium* species on the survival and feeding rates of brine shrimp, *Artemia salina*. *Acta Ecologica Sinica*, **26**: 3942–3947.

Zhou, Q., Liu, C., Liu, W., Zhang, H., Zhang, R., Liu, J., Zhang, J., Xu, C., Liu, L., Huang, S. & Chen, L. (2015). Rotenone induction of hydrogen peroxide inhibits mTOR-mediated S6K1 and 4E-BP1/eIF4E pathways, leading to neuronal apoptosis. *Toxicological Sciences*, **143**: 81–96.

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.