Highlights

·The NGU04 strain of *K. mikimotoi* showed potent lethal effect on abalone.

·The NGU04 strain showed much stronger haemolytic activity than other two strains.

·Bluefin tuna erythrocytes showed the highest susceptibility to the NGU04 strain-induced haemolysis.

·The direct attack by live cells is essential for the toxicity of the NGU04 strain on abalones.

· Live cell-mediated haemolytic activity is mainly responsible for the toxicity of the NGU04 strain on abalones.

Strain-dependent lethal effects on abalone and haemolytic activities of the dinoflagellate *Karenia mikimotoi*

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1 **Abstract**

2 One of the clonal strains of *Karenia mikimotoi* NGU04 isolated from Kyushu Island
3 in Japan, showed the most potent fish-killing activity in preliminary experiments. To
4 evaluate shellfish toxicity, two species of juvenile abalone were exposed to the NGU04,
5 and the toxicities were compared with those of two other *K. mikimotoi* strains. Of the two
6 abalone species tested, *N. gigantea* showed a higher sensitivity to NGU04 than the hybrid
7 abalone (*N. discus hannai* male × *N. gigantea* female) did. In comparative studies using
8 *N. gigantea*, the NGU04 exhibited stronger toxicity on abalone than other strains. The
9 cell-free culture supernatant and the ruptured cell suspension of the NGU04 showed no
10 significant lethal effects on *N. gigantea*. The NGU04 showed a stronger haemolytic
11 activity on rabbit erythrocytes than did the other two strains. Furthermore, the haemolytic
12 activities of the NGU04 strain towards erythrocytes of four fish species were also stronger
13 than those of other strains.

14

15

16 ***Keywords**: Karenia mikimotoi*; Abalone; Haemolytic activity; Fish erythrocytes; Bluefin
17 tuna

18 ***Abbreviations:*** HAB, harmful algal blooms; MS-SNF medium, microalgal medium of
19 suppressive-chelator used in sekai national fisheries research institute medium; SWM-3,
20 modified seawater medium

21

1 **1. Introduction**

2 There have been increasing concerns about the detrimental effects of harmful algal
3 blooms (HAB) or red tides on marine ecosystems and food resources, which are partly
4 related to global warming (Anderson et al., 2012; Fu et al., 2012). It has been pointed out
5 that climate changes associated with ocean warming, acidification, deoxygenation, and
6 typhoon-stimulating turbulence are affecting the aquatic ecosystem, and the occurrences
7 of HABs are intensifying in parallel with global warming (Griffith and Gobler, 2019; Liu
8 et al., 2019). For instance, eutrophication concomitant with climate change has been
9 considered as a cause of an increase in the frequency of HABs occurrence along the
10 Chinese coast (Xiao et al., 2019). Among the wide variety of marine phytoplankton
11 species, raphidophytes and dinoflagellates are the major groups causing HAB
12 (Hallegraeff, 2003; Fu et al., 2012). The marine organisms damaged by HAB vary
13 depending on the causative phytoplankton species. For example, *Chattonella marina*,
14 belonging to raphidophycean flagellate, is a well-known fish-killing species (Hishida et
15 al., 1997). In the wild or aquaculture fields, HAB due to *Chattonella* spp. have been often
16 associated with the mass mortality of fish (Landsberg, 2002). The fish-killing mechanism
17 of *Chattonella* spp. is still controversial, but the relatively high level of reactive oxygen
18 species (ROS) produced by *Chattonella* spp. are considered to be one of the important
19 toxic factors (Cho et al., 2016). The dinoflagellate *Heterocapsa circularisquama* is toxic
20 to bivalves, but no harmful effects on wild and cultured fish have been reported to date

1 (Matsuyama, 2012). According to previous studies, *H. circularisquama* displays a
2 haemolytic activity against rabbit erythrocytes in a cell-density dependent manner (Kim
3 et al., 2002), however, this dinoflagellate cannot produce ROS (Cho et al., 2016). A
4 comparable study using several strains of *H. circularisquama* suggested that its
5 haemolytic activity and bivalve toxicity are well-correlated (Kim et al., 2002; Nishiguchi
6 et al., 2016). Furthermore, *H. circularisquama* is also highly toxic to herbivorous
7 zooplankton, such as rotifers (*Brachionus plicatilis*), and it was suggested that its
8 haemolytic activity is the major responsible factor for the rotifer toxicity (Kim et al.,
9 2000). Probably a certain haemolytic agent of *H. circularisquama* may be involved in the
10 toxicity to both shellfish and rotifers. Since *C. marina*, which has no haemolytic activity,
11 showed no significant toxic effect on rotifers (Kim et al., 2019), *Chattonella* spp. and *H.*
12 *circularisquama* exhibit toxic effects on fish and shellfish through distinct species-
13 specific toxic agents, respectively. In contrast to *Chattonella* spp. and *H. circularisquama*,
14 another harmful dinoflagellate *Karenia mikimotoi* (formerly *Gyrodinium aureolum, G.* cf.
15 *aureolum, G.* type-'65, *G. nagasakiense,* and *G. mikimotoi*) is toxic to both fish and
16 shellfish (Li et al, 2017), suggesting that *K. mikimotoi* may have multiple toxic agents
17 specific to both fish and shellfish. Furthermore, *K. mikimotoi* is an unarmoured
18 dinoflagellate, also known as a eurythermal and euryhaline organism, which can arise at
19 temperatures of 4–31°C and salinity of 9–31 (Yamaguchi and Honjo, 1989; Lei and Lu,
20 2011). This species can also grow under a wide range of light intensities, ranging from 10

to 1200 μmol m^{-2} s^{-1}, and can assimilate many different nitrogen and phosphorous sources (Gentien, 1998; Richardson and Corcoran, 2015). Several incidences of HAB due to this species have been reported in western Japanese waters (Honjo, 1994; Yoshimatsu, 2008), the North Atlantic (Gentien, 1998; Davidson et al., 2009), and other coastal areas (Lu and Hodgkiss, 2004; Sun et al., 2007). The distribution of *K. mikimotoi* in various coastal areas may be attributed to its adaptability, and it is frequently associated with severe damage to both fish and shellfish. It has been reported that a bloom of *K. mikimotoi* that occurred during the summer of 2005 in Ireland was associated with fish and shellfish mortality (Mitchell and Rodger, 2007).

According to the recent review of Li et al. (2019), the toxicity of *K. mikimotoi* differs between subspecies, and significant gill disorder rather than hypoxia was observed in the fishes exposed to *K. mikimotoi*. Furthermore, it has been reported that the toxicity of *K. mikimotoi* required direct contact between fishes and intact algal cells (Li et al., 2018). Despite the wide variety of impacts on marine ecosystems and aquacultural industries worldwide, the exact toxic mechanism of *K. mikimotoi* is still unclear.

Previous studies have suggested that certain toxic factors are involved in the fish- or shellfish-killing mechanisms of *K. mikimotoi* (Arzul et al., 1994; Parrish et al., 1998; Fossat et al., 1999; Sola et al., 1999; Jenkinson and Arzul, 2001; Neely and Campbell, 2006; Mooney et al., 2007; Satake et al., 2002, 2005). Previous studies have also found that *K. mikimotoi* can generate reactive oxygen species (ROS) (Yamasaki et al., 2004),

1 with other researchers also reporting the production of ROS by *K. mikimotoi* (Gentien et
2 al., 2007). Although the role of ROS in the toxic mechanism of *K. mikimotoi* is unclear,
3 several lines of evidence suggested that the raphidophycean flagellate, *C. marina*,
4 exhibited fish-killing activity through ROS-mediated gill-tissue damage (Cho et al., 2016).
5 Hence, it is possible to speculate that *K. mikimotoi* also causes a ROS-mediated lethal
6 effect on fish, similar to *C. marina*.
7 Comparative studies with two strains of *K. mikimotoi* have demonstrated that a strain
8 with a higher haemolytic activity toward rabbit erythrocytes showed higher toxic effects
9 on marine organisms such as abalone (*Haliotus cracherodii*), shrimp (*Penaeus*
10 *semisulcatus*) (Zou et al., 2013), and rotifers (*Brachionus plicatilis*) (Zou et al., 2010) in
11 the exposure experiments at 5,000~2 x 10^4 cells mL^{-1} during 24-72 h. These findings
12 suggest that haemolytic activity is a key factor of the toxic potential of *K. mikimotoi*,
13 similar to *H. circularisquama* as described above. Regarding haemolytic activity of
14 phytoplankton species, our previous studies showed that haemolytic activity of *H.*
15 *circularisquama* was detected in both cell suspension and cell-free culture supernatant,
16 whereas the activity of *K. mikimotoi* was detected only in the cell suspension, suggesting
17 that certain haemolytic agents may be present on the cell surface of both phytoplankton
18 cells as a common feature (Cho et al., 2017). A part of the haemolytic agent may be
19 released from *H. circularisquama* cells, but not from *K. mikimotoi* cells. Since the
20 ruptured cell suspensions of both phytoplankton cells showed no significant haemolytic

1 activity, intact cell structure is a prerequisite for the effective haemolytic activity of these
2 phytoplankton cells.
3 In 2012, HAB due to high concentrations of *K. mikimotoi* occurred in the coastal areas
4 of Kyushu and Shikoku in Japan, which were associated with the mass mortality of
5 cultured fish species, including Japanese pufferfish (*Takifugu rubripes*). It has been
6 reported that the cell density of *K. mikimotoi* in the areas was high, with maximal density
7 ranges of 2×10^3-1.18×10^5 cells mL^{-1} depending on the area tested (Basti et al., 2015).
8 In our preliminary study, it was found that a strain of *K. mikimotoi* (strain NGU04)
9 isolated from the HAB area displayed a cell-density dependent (5×10^2-1×10^4 cells mL^{-1}
10) toxic effect on fish during the exposure time (0.3-4 h). Interestingly, the strain NGU04
11 produced a high level of ROS, which was almost equal to the levels of *C. marina*
12 measured at the same time (Kim et al., 2019). These findings strongly suggest that the
13 strain NGU04 might exhibit a ROS-mediated fish-killing activity. Furthermore, the strain
14 NGU04 showed that its lethal effect on rotifers is even stronger than other strains of *K.*
15 *mikimotoi* (Kim et al., 2019), suggesting that the strain NGU04 has the potential for potent
16 shellfish-killing activity as well as fish-killing activity. Since the toxic effects of the strain
17 NGU04 on rotifer was not inhibited by the addition of ROS-scavenging enzymes such as
18 superoxide dismutase and catalase, ROS-mediated rotifer toxicity might be ruled out
19 (Kim et al., 2019). These findings suggest that there is a possibility that *K. mikimotoi* may
20 exhibit multiple toxic effects on marine organisms through ROS, haemolytic toxin, and

1 still unknown other toxic factors or their combination. We examined the effects of the
2 NUG04 strain on two different species of juvenile abalone, and its haemolytic activities
3 towards the erythrocytes of rabbits and four fish species (bluefin tuna, yellowtail,
4 Japanese flounder, and red sea bream) were examined. Since abalone farming is one of
5 the major aquaculture industries in Japan, which has often been damaged by HAB due to
6 *K. mikimotoi*, two species of abalone used for aquaculture in Kyushu were selected as test
7 organisms in this study. To further evaluate the toxic features of the NGU04 strain,
8 comparative studies with the other two strains of *K. mikimotoi* with different backgrounds
9 were conducted.
10
11 **2. Materials and methods**
12
13 *2.1. Plankton culture*
14
15 A strain of *K. mikimotoi* (NGU04, clone, non-axenic) was isolated from Omura
16 Bay, Western Kyushu Island, Japan, in the late autumn of 2012, and maintained in a
17 microalgal medium containing a suppressive-chelator (MS-SNF), which had been
18 developed in the Seikai National Fisheries Research Institute, Japan. MS-SNF medium
19 was prepared with 85 mg of KNO_3 (final 840 μM), 6.24 mg of NaH_2PO_4-$2H_2O$ (final 40
20 μM), 1.11 mg of EDTA-2Na (final 3 μM), 0.421 mg of Fe(III)-EDTA (final 1 μM), 0.664

1 mg of Mn-EDTA (final 1 μM), 100 nmol of $CoCl_2$-$6H_2O$ (final 100 nM), 10 nmol of
2 H_2SeO_3 (final 10 nM), 2.84 mg of Na_2SiO_3・$9H_2O$ (final 10μM), 405 mg of $C_4H_{11}NO_3$
3 (final 405 mg L^{-1}), 95 mg of $C_4H_{11}NO_3$・HCl (final 95 mg L^{-1}), and 200μL of vitamin
4 mix S3 dissolved in 1 L of seawater. A stock solution of vitamin mix S3 is made by
5 dissolving 5 mg of thiamine HCl, 1 mg of nicotinic acid, 1 mg of calcium pantothenate,
6 0.1 mg of *p*-aminobenzoic acid, 0.01 mg of biotin, 50 mg of inositol, 0.02 mg of folic
7 acid, 30 mg of thymine in 100 mL of distilled water. The medium was dissolved in 1 L of
8 seawater and the pH was adjusted to 7.75 followed by autoclaving (75°C, 1 h). Among
9 the clonal strains of *K. mikimotoi* isolated from the HAB areas, the NGU04 strain was
10 well-adapted to laboratory culture conditions, and our preliminary study demonstrated
11 that the NGU04 strain displayed much stronger toxicity for the juvenile yellowtail
12 (*Seriola quinqueradiata*) and the rotifer *Brachionus plicatilis* than other strains. One of
13 the other two strains of *K. mikimotoi* used in this study was isolated from Suo Nada (SUO-
14 1), Japan, in 2006, and the other (NIES-2411) was obtained from the National Institute
15 for Environmental Studies (NIES) and was originally isolated from Katagami Bay, Japan,
16 in 2004. Except for NGU04, these clonal strains were cultured at 27°C in modified
17 seawater medium (SWM-3) at a salinity of 25 as described previously (Yamasaki et al.,
18 2007). The NGU04 strain was maintained under the same conditions but in MS-SNF
19 medium instead of SWM-3. All the cultivations were conducted with a 12 h:12 h
20 light:dark cycle under a cool-white fluorescent lamp (200 ± 5 μmol m^{-2} s^{-1}). To prevent

1 bacterial contamination, all the plankton culture was conducted without aeration.

2 Plankton cells were counted microscopically using a haemocytometer (Erma Inc., Tokyo,

3 Japan). For the abalone exposure experiments and haemolytic assay described below,

4 each strain of *K. mikimotoi* was used in the exponential growth phase (3.0–5.0 × 10^4 cells

5 mL^{-1}) after appropriate dilution with a medium.

6

7 *2.2. Preparation of cell-free culture supernatant and ultrasonic-ruptured cell suspension*

8 *of* K. mikimotoi

9

10 Cell-free culture supernatant was obtained from each cell suspension in the late

11 exponential growth phase by centrifugation at 5,000 × *g* for 5 min at 4°C. Microscopic

12 observation confirmed that there were no cells or cell debris in the supernatant. The

13 ruptured cell suspension was prepared by the ultrasonic treatment of each cell suspension

14 in a bath-type sonicator (XL2020, Wakenyaku Co., Ltd., Kyoto, Japan) for 60 s at 20°C.

15 Microscopic observation confirmed that all cells were ruptured by this treatment.

16 Following preparation, the cell-free culture supernatants and ruptured cell suspensions

17 were immediately used for the abalone exposure experiments and the haemolytic assays.

18

19 *2.3. Abalone exposure assay*

20

1 Juveniles of *Nordotis gigantea* (8 months old, shell width 17.2 ± 1.3 mm,
2 bodyweight 0.62 ± 0.07 g) and a hybrid of *N. discus hannai* male and *N. gigantea* female
3 (7 months old, shell width 15.6 ± 0.5 mm, bodyweight 0.35 ± 0.05 g) were provided by
4 Nagasaki Prefectural Fish Cultivation Center. The juveniles were reared in running
5 seawater at temperatures ranging from 13 to 26°C, and salinity from 33 to 34. Before the
6 experiments, the juveniles were placed in a container and kept in fresh seawater for 18 h
7 at 23 ± 1°C. Healthy individuals tightly attached to the container were used for the
8 experiments. Experiments were conducted using 200 mL beakers, each with 10 abalones,
9 and containing 100 mL of each strain of *K. mikimotoi* cell suspension with exponential
10 growth phase at varying cell concentrations (1-2 × 10^4 cells mL^{-1}), and the conditions of
11 abalone were observed for 18 h. Plankton culture medium alone was used as a control.
12 Aeration was not adopted during the experiment. The dissolved oxygen levels in the
13 exposure medium were 5.4 ± 0.5 mg L^{-1} during the experiments. The salinity and pH of
14 each exposure medium were 29 and 8.0, respectively. The experiments were carried out
15 at 23 ± 1°C under light illumination (70 ± 5 μmol m^{-2} s^{-1}). The condition of the abalones
16 was observed throughout the experiment; individuals detached from the beaker, and with
17 no response to physical stimulation, were considered dead.
18

19 *2.4. Haemolytic assay*

20

1 Rabbit blood was obtained from Nippon Bio-Test Laboratories (Tokyo, Japan). The
2 erythrocytes were washed three times with phosphate-buffered saline (PBS, pH 7.4), and
3 adjusted to a final concentration of 4% (v/v) in a plankton culture medium (modified
4 SWM-3 or MS-SNF). Triplicate 50 μL aliquots of serial two-fold dilutions of intact cell
5 suspensions of each *K. mikimotoi* strain in plankton culture medium were added to round-
6 bottom 96-well plates (Becton-Dickinson, New Jersey, USA) containing 50 μL of a 4%
7 (v/v) suspension of erythrocytes, and the plates were then gently shaken. After incubation
8 for the indicated periods at 27°C under illumination from a fluorescent lamp (200 μmol
9 $m^{-2} s^{-1}$), the plates were centrifuged at 900 × *g* for 5 min. Aliquots (50 μL) of supernatant
10 were withdrawn from the wells and transferred to flat-bottom 96-well plates (Becton-
11 Dickinson, New Jersey, USA). Released haemoglobin was determined by measuring
12 absorbance at 560 nm using a microplate reader (MPR-A4i, TOSOH Co., LTD., Tokyo,
13 Japan). Negative (zero haemolysis) and positive (100% haemolysis) controls were
14 included using erythrocytes suspended in plankton culture medium alone and in medium
15 containing 1% v/v Triton X-100, respectively.
16 For the haemolytic assay against fish erythrocytes, blood samples from red sea bream
17 (*Pagrus major*) weighing 1.8–2.0 kg, Japanese flounder (*Paralichthys olivaceus*)
18 weighing 0.9–1.1 kg, and yellowtail (*Seriola quinqueradiata*) weighing 4.5–4.8 kg were
19 obtained from the caudal blood vessel with a heparinized syringe. Blood from bluefin tuna
20 (*Thunnus orientalis*) weighing 30.0–31.0 kg was obtained via a surgical cut of the dorsal

1 aorta, and immediately transferred to a heparinized plastic container on ice. All blood
2 samples were processed as described above for rabbit erythrocytes. These aquacultured
3 fish were obtained from local dealers.

4 To analyse the haemolytic kinetics of *K. mikimotoi* strains in rabbit erythrocytes,
5 triplicate 1 mL aliquots of each strain of cell suspension were added to a 24-well plate
6 (Becton-Dickinson, New Jersey, USA). The same volume of 4% (v/v) erythrocyte
7 suspension in a plankton culture medium was added to each well, and the plate was gently
8 shaken. After the indicated periods, 100 μL aliquots of the assay mixture were withdrawn
9 and centrifuged at 900 × *g* for 5 min. Aliquots (50 μL) of the resulting supernatant were
10 then transferred to flat-bottom 96-well plates, and the released haemoglobin was
11 determined as described above.

12

13 *2.5. Statistical analysis*

14

15 Statistical analysis was performed by one-way analysis of variance (one-way
16 ANOVA) and subsequent *t*-tests using MS Excel 2010 (Microsoft, USA) program. All the
17 experiments were performed in triplicate and the differences with $p<0.05$ were
18 considered significant.

19

20 **3. Results**

1

3.1. Effect of K. mikimotoi *on the survival of juvenile abalone*

3

Although the NGU04 strain was highly toxic to both *N. gigantea* and the hybrid juvenile abalone, *N. gigantea* was more susceptible than the hybrid (Fig. 1). In particular, the difference in susceptibility between two juvenile abalone was significant at a cell density of 1×10^4 cells mL^{-1}. All *N. gigantea* individuals died after 8 and 4 h of exposure to the NGU04 strain at a cell density of 1×10^4 and 2×10^4 cells mL^{-1}, respectively (Fig. 1A). Conversely, 100% lethality was observed in the hybrid after 6 h at a cell density of 2×10^4 cells mL^{-1}, and three out of 10 hybrid juveniles survived after 18 h at a cell density of 1×10^4 cells mL^{-1} (Fig. 1B).

The toxic effect of the NGU04 strain on sensitive *N. gigantea* was compared with other *K. mikimotoi* strains (SUO-1 and NIES-2411) at a cell density of 5×10^3 cells mL^{-1}. The NGU04 strain was also highly toxic at this cell density and all individuals died within 12 h, while eight and five out of 10 abalones survived even after 18 h exposure to NIES-2411 and the SUO-1, respectively (Fig. 2). These results indicate that, among the strains tested, the NGU04 strain is highly harmful with a potentially lethal effect on abalone.

To gain insight into the toxic factors responsible for the lethal effects of the NGU04 strain on juvenile abalone, the effects of the ruptured cell suspension and cell-

free culture supernatant on *N. gigantea* were examined. As shown in Fig. 3, no dead individuals were observed after exposure to the ruptured cell suspension for 18 h. Two out of 10 juvenile abalones died after 8 h exposure to the cell-free culture supernatant, indicating that its toxic effect was substantially lower than that of the intact cell suspension.

3.2. Haemolytic activities of K. mikimotoi *on rabbit and fish erythrocytes*

The haemolytic activities of three different strains of *K. mikimotoi* against rabbit erythrocytes were examined. The NGU04 strain showed incubation time- and cell density-dependent higher haemolytic activity than that of the SUO-1 and NIES-2411 strains (Fig. 4). The activity of the NGU04 strain reached nearly maximum levels after 30 min. No significant haemolytic activity was observed in the cell-free culture supernatant and the ruptured cell suspension of the *K. mikimotoi* strains (Fig. 5).

Similar to rabbit erythrocytes, the NGU04 strain showed a much stronger haemolytic activity towards the erythrocytes of fish species tested than the other two strains (Fig. 6). Interestingly, among the fish erythrocytes tested, the bluefin tuna erythrocytes showed the highest susceptibility to the NGU04 strain.

1 **4. Discussion**

2

3 In this study, small scale exposure experiments using juvenile abalones were conducted
4 without aeration. During the exposure period (18 h), no control abalones died. Since it
5 has been reported that no death of juvenile (10-month-old) abalone (*Haliotis discus*) was
6 observed in the absence of aeration in 40 mL SWM3 medium over 24 h (Matsuyama et
7 al. 1998), abalone survival might not be seriously affected by oxygen depression under
8 the conditions used. The results demonstrated that the NGU04 strain had lethal effects on
9 juvenile abalone with different potency depending on the abalone strains. *N. gigantea* was
10 more susceptible to the NGU04 strain than the hybrid. The exact reason for the difference
11 in the susceptibility between two juveniles to the NGU04 strain is unclear, but it may be
12 associated with the altered physiological features, such as the superior feeding and growth
13 rates of the hybrid compared with the parents (Koike et al., 1988). Red tide due to *K.*
14 *mikimotoi* often causes serious damage to shellfish aquaculture, including abalone
15 (Mitchell and Rodger, 2007; Yamaguchi, 1994). Our findings suggest that abalone can
16 acquire a *K. mikimotoi*-resistant phenotype through appropriate hybridization; further
17 studies are necessary to evaluate this possibility.
18 It was also found that the NGU04 strain showed the stronger lethal effect on *N.*
19 *gigantean* than the other strains of *K. mikimotoi* (SUO-1 and NIES-2411). Similarly, our
20 previous studies reported that the toxicity of different *K. mikimotoi* strains against abalone,

1 shrimp, and rotifers was significantly different depending on the strains (Zou et al., 2010).
2 Differences in the toxicity of different *K. mikimotoi* strains toward several other animal
3 species have also been reported (Li et al., 2017; Shi et al., 2012; Silke et al., 2005).
4 Although it has been reported that *K. mikimotoi* produces an exotoxin (Gentien
5 and Arzul, 1990), the cell-free culture supernatant and the ruptured cell suspension
6 prepared from the NGU04 strain showed low or no toxicity to juvenile abalone. Non-
7 toxic effects of the cell-free culture supernatant and the ruptured cell suspension prepared
8 from the cell suspension (8-10 × 10^4 cells mL^{-1}) of a toxic strain of *K. mikimotoi* were
9 also found in the 24 h rotifer exposure study (Zou et al., 2010). A lack of toxicity in the
10 cell-free culture supernatant and the ruptured cell suspension of *K. mikimotoi* have been
11 reported in other strains of *K. mikimotoi* isolated from different areas (Li et al., 2017; Sun
12 et al., 2010; Zhang et al., 2011). These findings suggest that the intact cells are essential
13 for the toxicity of *K. mikimotoi*. Similar to our findings, it has been reported that the intact
14 algal cell is necessary for the toxic effect of *K. mikimotoi* on various marine organisms
15 such as brine shrimp, mysid shrimp and rotifer (Sun et al., 2010), and copepode, abalone,
16 prawn and fishes (Li et al., 2017). The contact-dependent toxicity of intact *K. mikimotoi*
17 cells is well described in the recent review as well (Li et al., 2019). Our recent study
18 demonstrated that the live cell suspension of the NGU04 strain (2 × 10^4 cells mL^{-1})
19 produces a high level of ROS (Kim et al., 2019). The level of ROS detected in the NGU04
20 strain was almost equivalent to that of *C. marina*, which is a well-known ROS-producing

1 species, whose ROS-mediated fish-killing mechanism has been proposed (Kim et al.,
2 2019). No significant levels of ROS were detected in the cell-free culture supernatant and
3 the ruptured cell suspension of the NGU04 strain (Kim et al., 2019), emphasizing the
4 importance of the live cell condition.

5 There was a positive correlation between the toxicity of *K. mikimotoi* towards
6 rotifers and the haemolytic activity. The SUO-1 strain, which has a potentially lethal
7 effect on rotifers, showed a potent haemolytic activity, whereas the less toxic FUK strain
8 had no significant haemolytic activity (Zou et al., 2010). The cell-free culture supernatant
9 and the ruptured cell suspension of the SUO-1 strain, which had no rotifer killing activity,
10 did not exhibit any haemolytic activity (Zou et al., 2010). Furthermore, when direct
11 contact between *K. mikimotoi* and rotifer was interrupted with a cell-impermeable
12 membrane (3 μm pores), the toxicity of the SUO-1 strain to rotifer was completely
13 inhibited (Zou et al., 2010). These findings suggest that *K. mikimotoi* may require direct
14 contact with target organisms to induce the toxic effect.

15 It seems that the haemolytic activity of the SUO-1 strain measured in this study
16 was lower than the activity reported previously (Zou et al., 2010). Although the
17 experimental conditions were not the same between the studies, the haemolytic activity
18 of the SUO-1 strain might gradually decrease during the long cultivation period under
19 laboratory conditions. Similar to the SUO-1 strain, a decrease in the fish-killing activity
20 of the *Chattonella marina* strain has been observed. A previous study showed that *C.*

1 *marina* strain isolated in 1978 (strain 78) presented a toxic effect on yellowtail in a study
2 conducted in 1989 (Ishimatsu et al., 1990), but was non-toxic to yellowtail in 1996
3 (Ishimatsu et al., 1996). Since strain 78 was cultured under laboratory conditions for more
4 than 10 years after its initial isolation, without an intervening cyst stage, toxicity might
5 have been lost during the cultivation. Hence, the duration of culture following the
6 isolation of strains from the natural field can influence their toxic potential and other
7 related activities. Since the NGU04, SUO-1, and NIES-2411 strains were isolated in 2012,
8 2006, and 2004, respectively, the higher abalone toxicity and haemolytic activity of the
9 NGU04 strain may be partly related to the relatively short period of cultivation under
10 laboratory conditions. It has been reported that cultured *K. mikimotoi* cells have less of
11 an effect on fish than natural cells (Nagai et al., 2000), and in the case of other toxic
12 dinoflagellates, the toxicity of wild populations is 10–20 times higher than that of cultured
13 populations (White 1986).
14 It has been shown that *K. mikimotoi* exhibits different extents of haemolytic activities
15 depending on the species of erythrocytes used, with horse erythrocytes being less
16 sensitive than sheep, rabbit, and cattle erythrocytes (Zou et al., 2010). Thus, *K. mikimotoi*
17 may recognize specific sites on the sensitive erythrocyte cell surface to induce haemolysis.
18 Histopathological changes in the gills and other organs of the fish and shellfish killed by
19 *K. mikimotoi* have been observed (Mitchell and Rodger, 2007). Hence, one can speculate
20 that *K. mikimotoi* might induce tissue damage leading to death through direct contact with

1 the specific target organs. The initial step of this process may be the recognition of
2 specific sites on the cell surface of target organs or cells. In general, the detrimental effect
3 of HAB on fish is significantly different between species. Large and migratory fish, such
4 as tuna and yellowtail, are highly susceptible to HAB exposure, and coastal fish tend to
5 be more tolerant of HAB toxicity (Hishida et al., 1998). Since gill tissue damage induced
6 by *K. mikimotoi* seems to be a leading cause for the fish mortality (Davidson et al., 2009;
7 Wang et al., 2001), the physiological and structural characteristics of a fish's gill tissue
8 are an important factor influencing the susceptibility to *K. mikimotoi*. The different
9 susceptibility of fish erythrocytes to *K. mikimotoi* observed in this study may be partly
10 related to the species-specific toxic mechanism. Further studies are necessary to evaluate
11 whether or not the high susceptibility of bluefin tuna erythrocytes to the NGU04 strain
12 can connect with the toxicity of this strain toward bluefin tuna.
13 Although an involvement of certain exotoxins secreted from the cells in the toxic
14 mechanism of *K. mikimotoi* cannot be completely ruled out, a recent study demonstrated
15 that *K. mikimotoi* isolated in Fujian coastal waters, in China, showed varying toxic effects
16 on several test organisms, in which the intact cells were essential (Li et al., 2017). The
17 authors of that study reported that lipophilic extracts of *K. mikimotoi* showed haemolytic
18 activity but no toxic effects on the test organisms. Hence the haemolytic assays using live
19 cells, as performed in the present study, are important for the evaluation of true toxic
20 factors of *K. mikimotoi*. Some toxins with haemolytic activity located on the cell surface

of *K. mikimotoi* may play a major role in the toxicity.

Although such toxins have not been identified yet probably due to their extremely unstable nature, the relatively quick haemolytic action of the NGU04 strain suggests that the toxin may destroy erythrocyte membrane integrity via direct attack rather than as an ion-channel blocker, which generally required prolonged incubation time to induce haemolysis. Lack of haemolytic activity and rotifer-toxicity of ruptured *K. mikimotoi* cell suspension suggest that certain toxins responsible for the haemolysis and rotifer-toxicity might disappear during the preparation of the rupture cells due to the unstable nature. Probably the cell structural integrity of *K. mikimotoi* is essential for effective toxic action. Further studies are necessary to clarify this point.

Present results together with previous findings indicate that strain NGU04 is a highly toxic *K. mikimotoi* strain with a potent haemolytic activity and a high ROS producing ability. Probably, ROS and haemolytic agents are mainly responsible for the fish- and shellfish-killing mechanisms, respectively. Further studies on the strain NGU04 may provide insight into the toxic mechanisms of *K. mikimotoi*.

5. Conclusions

K. mikimotoi has a haemolytic toxin on the cell surface, and the activities were significantly different between the strains. The strains with higher haemolytic activity

1 show stronger abalone toxicity. Analysis of cell-free culture supernatants and ruptured
2 cell suspensions, as well as comparative studies using three strains, suggest that certain
3 haemolytic toxins located on the live-cell surface of *K. mikimotoi* may directly contact
4 target organs, which may lead to the death of abalone.
5
6 **Conflict of interest**
7 The authors declare no conflict of interest.
8
9 **Author contributions**
10 D. Kim: drafting manuscript; L. Wencheng and Y. Matsuyama: culture experiments and
11 drafting materials and methods section; A. Matsuo and M. Yagi: Abalone experiments; K.
12 Cho and Y. Yamasaki: haemolysis; S. Takeshita and K. Yamaguchi: data arrangement and
13 statistical analysis; T. Oda: original concept, manuscript editing and submission.
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3

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14

Figure captions

Fig. 1. Effects of the NGU04 strain of *K. mikimotoi* on the survival of juvenile abalone (n=10), *N. gigantea* (A) and the hybrid (B). The survival of abalone in each test group was examined following exposure to NGU04 at a cell density of 1×10^4 cells mL^{-1} (●), 2×10^4 cells mL^{-1} (▲), or MS-SNF medium alone as a control (◆). Error bars represent mean ± S.D, and different letters show a significant difference ($P<0.05$).

Fig. 2. Effects of the NGU04, SUO-1, and NIES-2411 strains of *K. mikimotoi* on the survival of juvenile abalone, *N. gigantea* (n=10). The survival of abalone in each test group was examined following exposure to NGU04 (●), SUO-1 (▲), and NIES-2411 (■) at a cell density of 5×10^3 cells mL^{-1}, or medium alone as a control (◆; MS-SNF, ◇; SWM3). Error bars represent mean ± S.D, and different letters show a significant difference ($P<0.05$).

Fig. 3. Effects of the cell-free culture supernatant and the ruptured cell suspension prepared from the cell suspension (2×10^4 cells mL^{-1}) of the NGU04 strain of *K. mikimotoi* on the survival of juvenile abalone, *N. gigantea* (n=10). The survival of abalone in each test group was examined following exposure to the cell-free culture supernatant (●), the ruptured cell suspension (▲), or MS-SNF medium alone as a control (◆). Error bars represent mean ± S.D, and different letters show a significant difference ($P<0.05$).

1

2 **Fig. 4.** Incubation time- (A) and cell density-dependent (B) haemolytic activities of the

3 NGU04 (●), SUO-1 (▲), and NIES-2411 (■) strains of *K. mikimotoi* towards rabbit

4 erythrocytes. (A) Intact cell suspension of each strain of *K. mikimotoi* at a final cell

5 density of 2×10^4 cells mL^{-1} were mixed with rabbit erythrocytes and incubated for the

6 indicated periods at 26°C under illumination from a fluorescent lamp (200 µmol m^{-2} s^{-1}).

7 The extents of haemolysis were measured as described in the text. Each point represents

8 an average of triplicate measurements, and the bars indicate the standard deviation (SD).

9 (B) Intact cell suspensions of each strain of *K. mikimotoi* at the indicated final cell density

10 were mixed with rabbit erythrocytes. After 3 h incubation, the extent of haemolysis was

11 measured as described above. Each point represents the mean ± standard deviation of

12 triplicate measurements, and the asterisks indicate significant difference ($p < 0.05$) from

13 the values of other strains.

14

15 **Fig. 5.** Haemolytic activities of the intact cell suspension at a cell density of 2×10^4 cells

16 mL^{-1}, the cell-free culture supernatant, and the ruptured cell suspension prepared from the

17 same cell suspension of the NGU04 (■), SUO-1 (▧), and NIES-2411 (□) strains of *K.*

18 *mikimotoi*. Each sample was mixed with rabbit erythrocytes and incubated for 3 h at 26°C

19 under illumination from a fluorescent lamp (200 µmol m^{-2} s^{-1}), and the extents of

20 haemolysis were measured as described in Fig. 4. Each point represents the mean ±

1 standard deviation of triplicate measurements, and the asterisks indicate a significant

2 difference ($p < 0.05$) from the values of other strains.

3

4 **Fig. 6.** Haemolytic activities of the cell suspensions of the NGU04 (●),SUO-1 (▲), and

5 NIES-2411 (■) strains of *K. mikimotoi* at varying cell densities toward bluefin tuna (A),

6 red sea bream (B), Japanese flounder (C), and yellowtail (D) erythrocytes. Intact cell

7 suspension of each strain of *K. mikimotoi* was mixed with erythrocytes of each fish

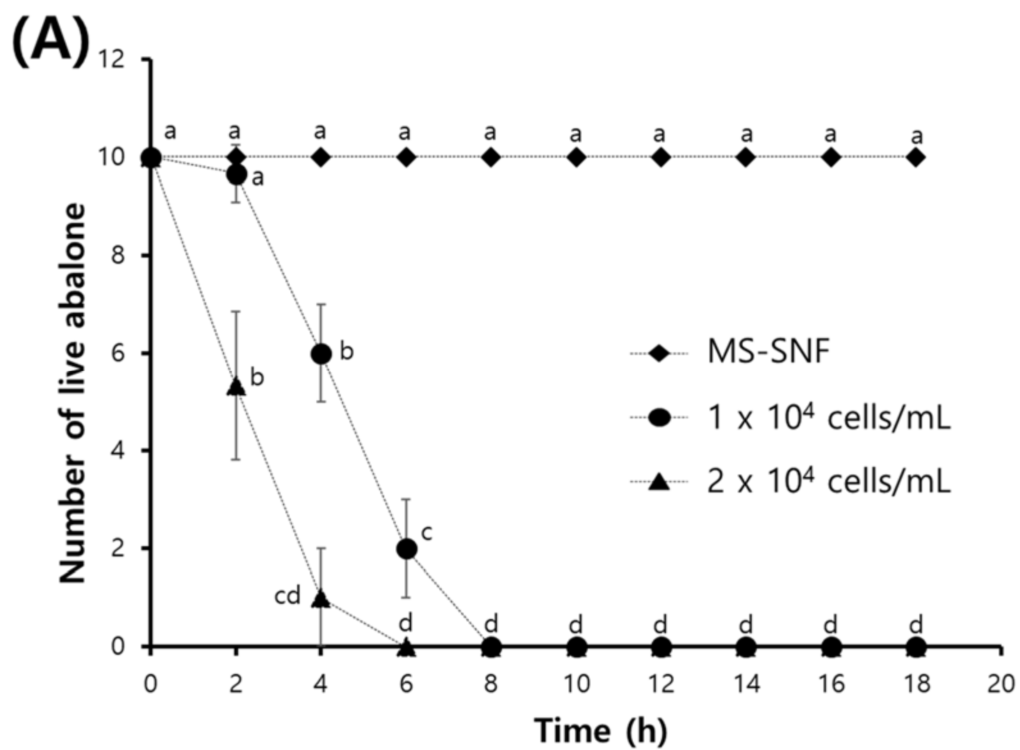
8 species and incubated for 3 h at 26°C under illumination from a fluorescent lamp (200

9 µmol m^{-2} s^{-1}), and the extents of haemolysis were measured as described in Fig. 4. Each

10 point represents the mean ± standard deviation of triplicate measurements, and the

11 asterisks indicate a significant difference ($p < 0.05$) from the values of other strains.

Fig. 1



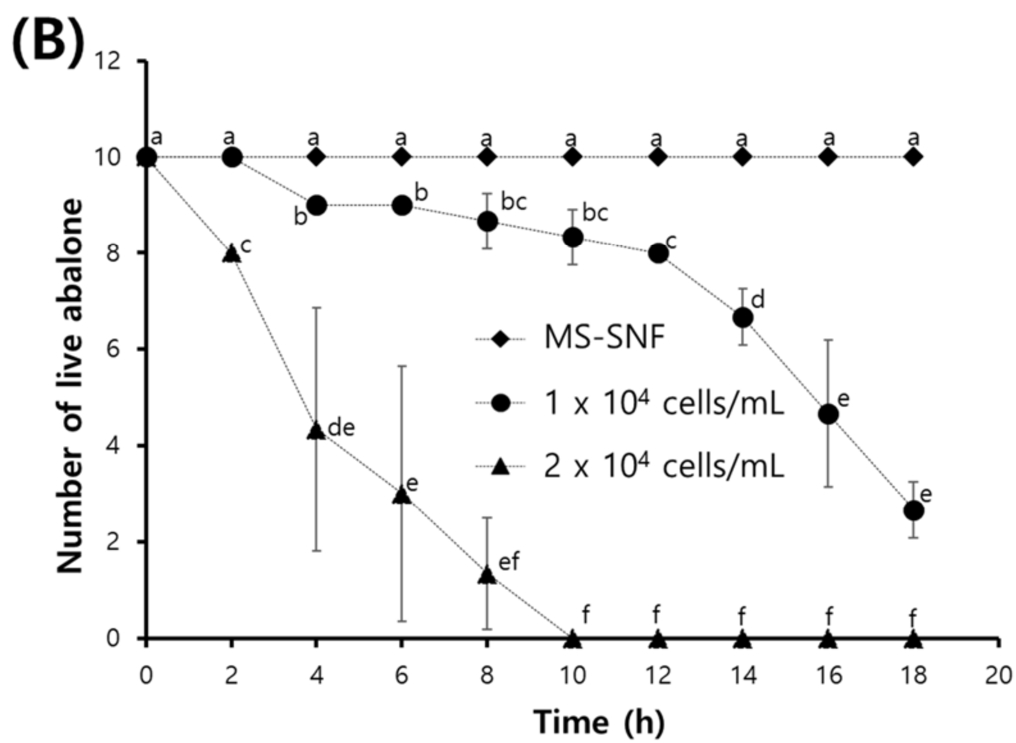


Fig. 2

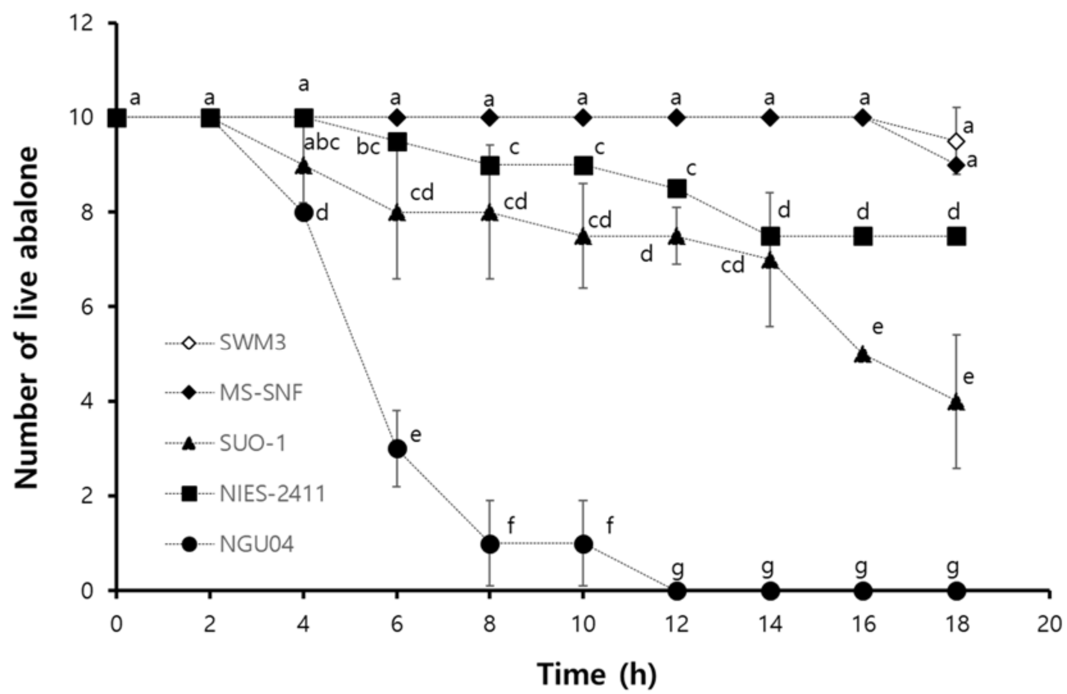


Fig. 3

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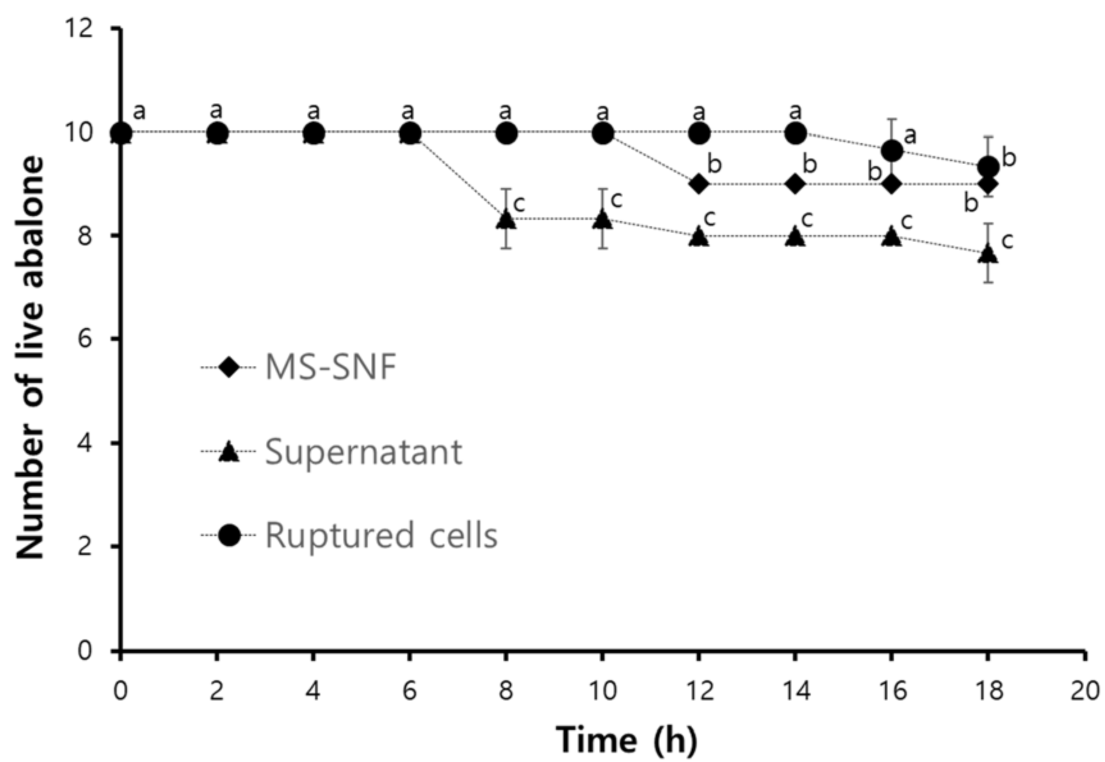


Fig. 4

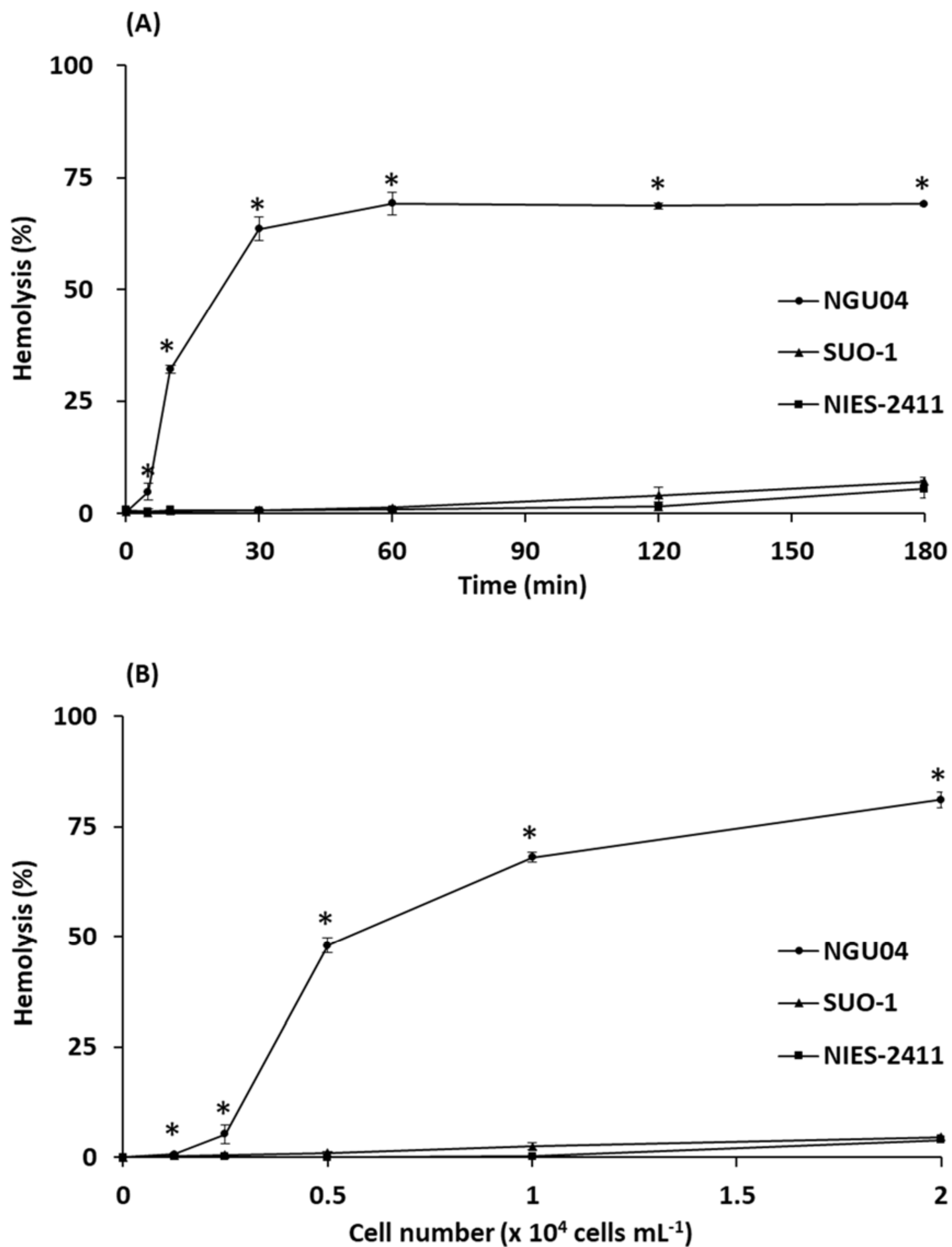


Fig. 5

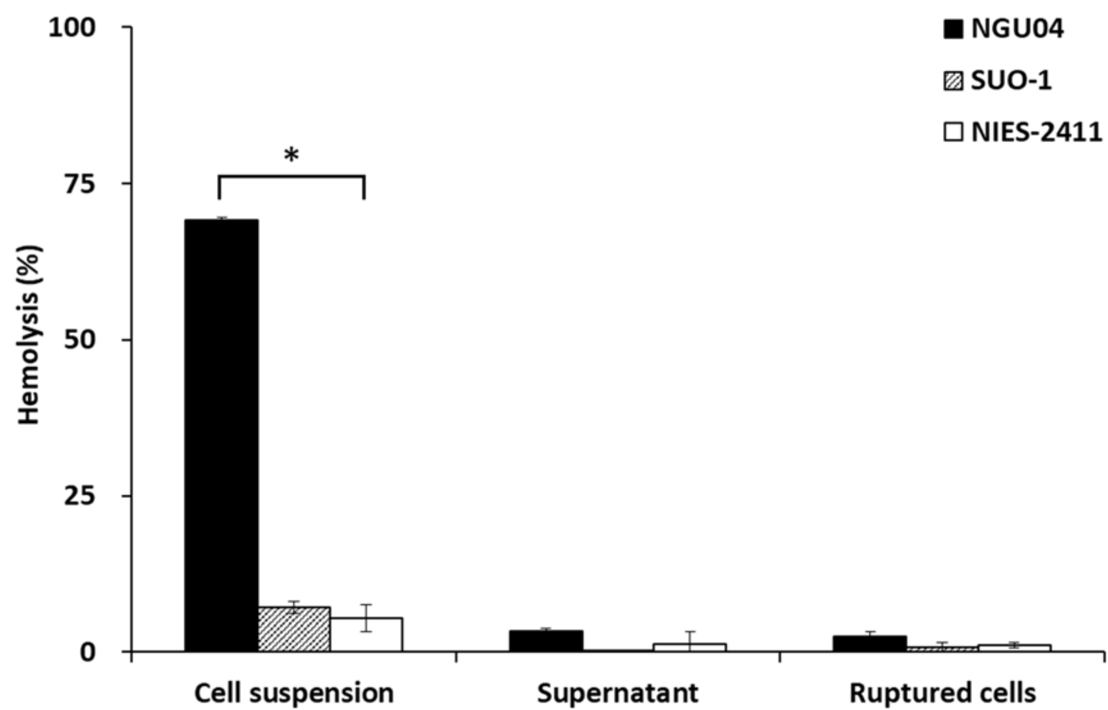
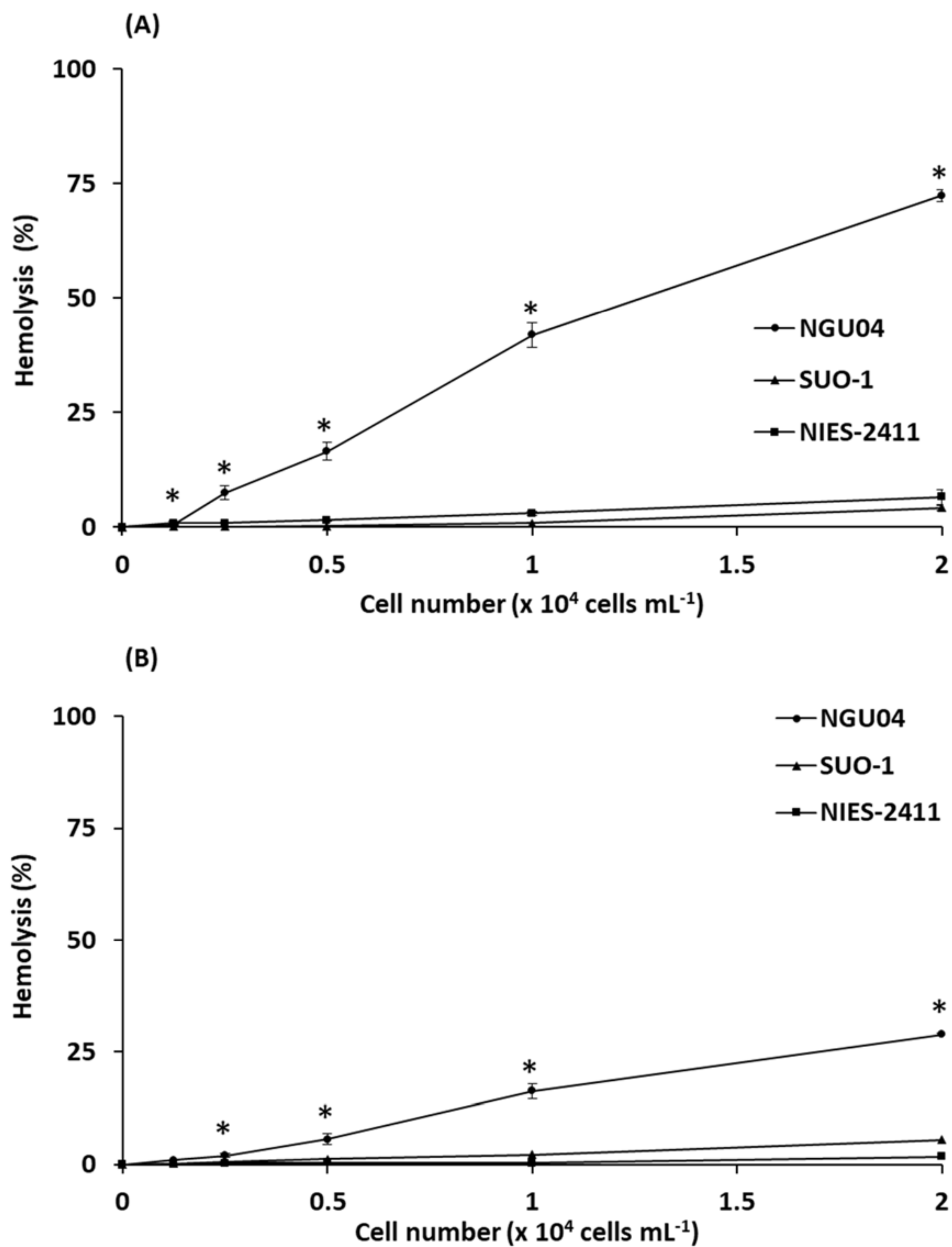
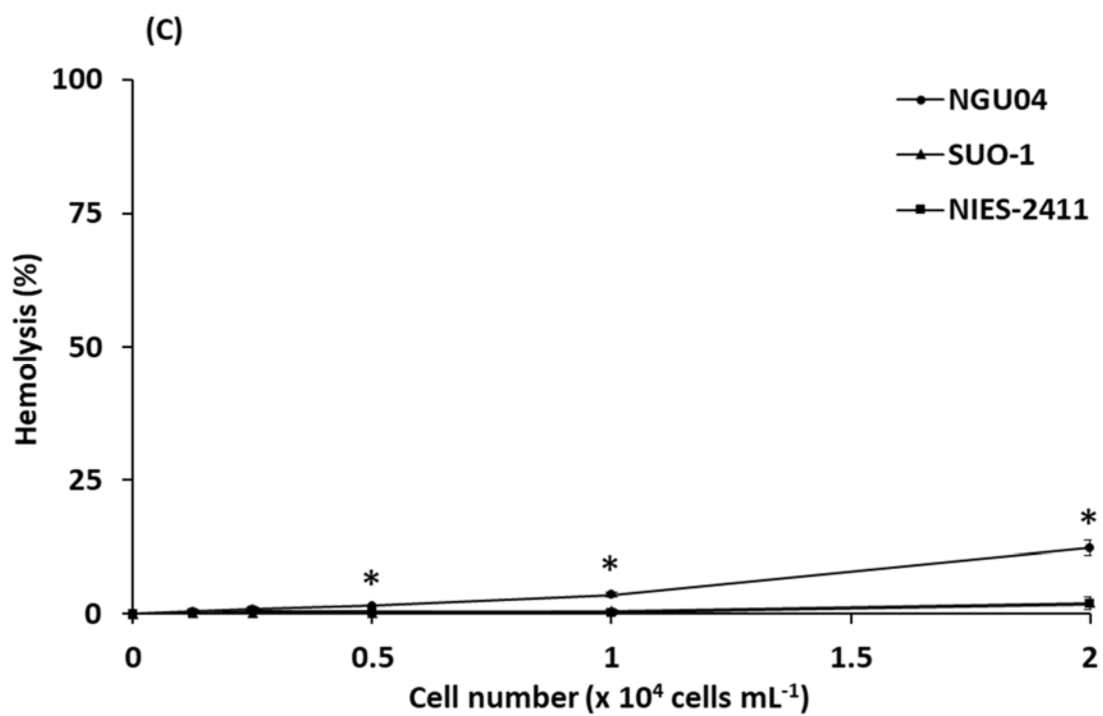
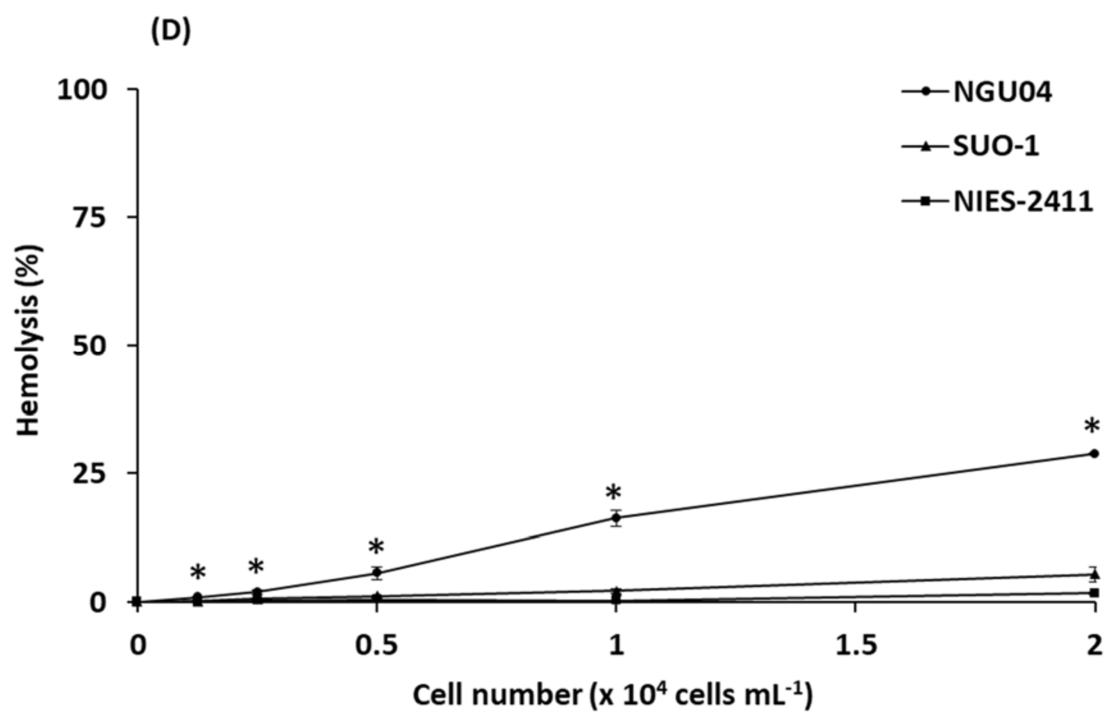


Fig. 6




(C)
100
75
50
25
0
Hemolysis (%)
NGU04
SUO-1
NIES-2411
*
*
*
0
0.5
1
1.5
2
Cell number (x 10⁴ cells mL⁻¹)


(D)
100
75
50
25
0
Hemolysis (%)
NGU04
SUO-1
NIES-2411
*
*
*
*
*
0
0.5
1
1.5
2
Cell number (x 10⁴ cells mL⁻¹)