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Temporal variation of caspase 3-like protein activity in cultures of the harmful dinoflagellates *Karenia brevis* and *Karenia mikimotoi*.

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3 **Temporal variation of caspase 3-like protein activity in cultures of the**
4 **harmful dinoflagellates *Karenia brevis* and *Karenia mikimotoi***
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27 **KEYWORDS** : caspase 3-like protein activity, cell death, harmful dinoflagellate, *Karenia*
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Abstract

To better understand the role of caspase-like proteins in the cellular activities of harmful dinoflagellates, cultures of *Karenia brevis* and *Karenia mikimotoi* were monitored over a 56-d period. The cellular abundance, maximum photochemical efficiency of photosystem II (F_v/F_m), percentage of dead cells (revealed by SYTOX-labelling of nuclei of cells with a compromised membrane) and the variation in caspase 3-like protein activity were monitored every seven days. During the cultures' late decline phase, DNA degradation was also confirmed in *Karenia* cells using a TUNEL assay. *Karenia brevis* reached its maximal cellular abundance after three weeks then declined to its lowest value on day 56. In cells of *K. brevis* the caspase 3-like activity likely served some housekeeping functions as demonstrated by the constitutive level of activity observed throughout the whole experiment (~ 3,000 to 6,664 RFU/h/mgprotein). The increase in activity which occurred on days 21 and 28 (~ 38,000 RFU/h/mgprotein on day 28) coincided with the appearance cyst-like structures in the cultures and suggests a role in the encystment progression of *K. brevis*. For *K. mikimotoi*, the highest cellular abundance was reached on day 42 after which it declined to reach its lowest value, along with the highest percentage of dead cells, on day 56. The caspase 3-like protein activity (< 1,300 RFU/h/mgprotein) was elevated at the beginning of the experiment (on days 0, 7, and 14) and just before the decline in cellular abundance (on days 35 and 42) and likely played some housekeeping functions as well as a role in the death of the cells. This study demonstrates the involvement of caspase-3 like protein activity in a variety of processes in cells of the harmful algae *K. brevis* and *K. mikimotoi*.

INTRODUCTION

The excessive growth of specific microalgae and cyanobacteria are of concern since a number of them can produce potent toxins. *Karenia brevis* and *K. mikimotoi* are unarmoured marine dinoflagellates which can form Harmful Algal Blooms (HABs). *Karenia brevis* is a producer of brevetoxins and is responsible for the occurrence of red tides in the Gulf of Mexico where it is well known to cause fish and marine mammal mortalities (Walsh *et al.*, 2006). *Karenia mikimotoi* is common in the waters around Japan and in the Northeast Atlantic region where blooms of this species have been associated with fish and shellfish kills (Gentien, 1998). Because of the economic impacts of HABs and their effects on the marine biota and humans, there have been many studies on how environmental factors influence their initiation and the production of toxins (Cembella and John, 2006; Granéli and Flynn, 2006; Vargo, 2009). Less attention has however been devoted to the study of the factors leading to the demise of HABs. Among some of the different factors known to play a role in the termination of HABs are water mass advection, cyst formation, nutrient limitation, grazing, cell lysis due to lytic bacteria or viruses and cell death (Vargo, 2009).

The study of cell death processes in microalgae is still in its infancy and is virtually nonexistent in harmful microalgae (Veldhuis and Brussard, 2006). However, several other species of microalgae have been proposed to undergo programmed cell death (PCD), necrosis (Dunn *et al.*, 2002) as well as a range of intermediate cell death modes (Franklin and Berges, 2004; Franklin *et al.*, 2006; Jiménez *et al.*, 2009). Because it results in the complete loss of the organism, the occurrence of PCD in unicellular cells seems counterintuitive. It is believed that phytoplankton undergo PCD as a strategy to relieve a population of nutrient stress, to remove ageing and/or damaged cells, or to limit the multiplication of viruses during infection (reviewed by Bidle and Falkowski, 2004). PCD could also be used to maximise the biological fitness of the community (reviewed by Bidle and Falkowski, 2004), a strategy suggesting that cells have awareness of the state of their conspecifics/predators and a potential for signalling (Toth *et al.*, 2004). PCD is an autocatalytic process that may lead to the apoptosis of the cell, a process characterized by specific morphological and biochemical requirements such as condensation and margination of the chromatin and nuclear fragmentation (Kerr *et al.*, 1972). This process is orchestrated by caspases, a family of cysteine proteases with unique substrate specificities (Kurokawa and Kornbluth, 2009). Alternatively, necrosis is described as a passive and unprogrammed type of cell death characterised by a loss of membrane integrity,

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3 cell swelling and lysis of the cell. In contrast to PCD, necrosis has typically been defined as a
4 caspase-independent event, but some evidence suggests that this is not always the case (Van
5 Cruchten and Van den Broeck, 2002). It is particularly challenging to determine how cell
6 death operates in unicellular organisms given the literature's natural bias for multicellular
7 organisms and mammalian cells (Franklin *et al.*, 2006).
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14 In mammalian cells, caspases are divided into two groups: initiator caspases (e.g.
15 caspase-2, -8, -9, -10) which regulate and activate other caspases; and executioner caspases
16 (e.g. caspase-3, -6, -7) which act downstream of the cascade after being activated by
17 upstream caspases (Kurokawa and Kornbluth, 2009). Although caspases have never been
18 identified in microalgae, antibodies raised against human caspases have been shown to cross-
19 react with extracts of, among others, marine cyanobacteria and chlorophytes (Segovia *et al.*,
20 2003; Berman-Frank *et al.*, 2004; Moharikar *et al.*, 2006; Zuppini *et al.*, 2007). Additionally,
21 caspase activity assays performed using artificial substrates (designed to be specific for
22 mammalian caspases) showed increases in caspase-like protein activities associated with the
23 occurrence of death in a variety of microalgal species under stress conditions (Berman-Frank
24 *et al.*, 2004; Segovia and Berges, 2005; Ross *et al.*, 2006; Zuppini *et al.*, 2007; Bidle and
25 Bender, 2008; Darehshouri *et al.*, 2008; Affenzeller *et al.*, 2009; Bouchard and Yamasaki,
26 2009; Jiménez *et al.*, 2009). Finally, the use of mammalian caspase inhibitors has been shown
27 to prevent the occurrence of death in diatoms, dinoflagellates and chlorophytes (Vardi *et al.*,
28 1999; Bidle and Bender, 2008; Segovia and Berges, 2009). The presence of variant forms of
29 caspases, called metacaspases (Uren *et al.*, 2000), in protists suggests they could be involved
30 in the PCD process of phytoplankton. However, because metacaspases possess a substrate
31 affinity different from that of caspases (Vercammen *et al.*, 2004; Watanabe and Lam, 2005),
32 enzymatic activities measured in protists (and in this study) using the classical aspartate-
33 containing caspase substrates do not provide any indication of the implication of
34 metacaspases in PCD. Indeed, the actual identity of the proteases responsible for these
35 caspase-like activities still remains to be clearly determined (Bonneau *et al.*, 2008).
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55 Despite the suggested implication of caspase-like proteins in the death of unicellular
56 algae, the actual role of caspase-like proteases in microalgae remains unclear. In mammalian
57 cells, cell death events have been shown to occur with or without the involvement of caspases
58 (Leist and Jäättelä, 2001) and these enzymes have been proposed to have alternative cellular
59 functions independent of cell death (Los *et al.*, 2001; Yi and Yuan, 2009). In microalgae,
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3 caspase-like protein activities have been proposed to be constitutive and to have some
4 housekeeping functions (Segovia and Berges, 2005) and, increases in caspase-like protein
5 activity have also been reported in cells of *Dunaliella viridis* undergoing cell death events
6 (e.g. autophagic-like and paraptotic-like deaths) which normally do not require the activation
7 of caspase-like proteases to proceed (Jiménez *et al.*, 2009). Clearly, the implication of
8 caspase-like protein activities in microalgal processes remains to be clarified. Most of the
9 studies performed so far with microalgae have assessed the variations of caspase-like protein
10 activities under stressful conditions. To obtain a better understanding of the role of caspase-
11 like protein activities in microalgal processes, it is important to determine their basal level in
12 microalgae grown in cultures under relatively stress-free conditions. The aim of this study
13 therefore was to monitor the variation of caspase 3-like protein activity along with other
14 physiological variables over more than 50 days in batch cultures of the toxic species of
15 dinoflagellate, *Karenia brevis* and *K. mikimotoi*.
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METHOD

Dinoflagellate cultures and sampling procedure

Two cultures of the harmful dinoflagellates, *Karenia brevis* (CCMP 2228) and *K. mikimotoi* (PLY 705) were grown in 500 ml volumes of L1-Si medium (Guillard and Hargraves, 1993). Cells were grown under a 12 h:12 h light : dark cycle at an irradiance of $\sim 30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Following the respective culture collection's recommendations, *K. brevis* and *K. mikimotoi* were grown at a temperature of 25 °C and 15 °C, respectively. During the dark phase of day 27, a fault with the incubator set at 25 °C caused the temperature to gradually decline until it reached 18 °C the following morning. The temperature was back to 25 °C at the time of sampling *K. brevis* on day 28 (at 12:00). Four independent culture flasks were used as replicates for each of the two species considered in this study. Sampling was performed weekly over a 56-d period (excluding the 49th day for *K. brevis*) and consistently performed at the same time, i.e., 5 h into the light period. The initial cellular abundance in each culture flask was between 100-300 cells mL⁻¹.

Cellular abundance, cyst enumeration and growth rate determination

Cell counts were determined on 1-mL of Lugol's fixed samples (kept at 4 °C) using a Sedgwick-Rafter chamber and a Nikon microscope (Eclipse E200). The growth rate (μ) was calculated as $\mu = \ln(N_T / N_0) / (\Delta t)$ where N_0 is the population cell density at the beginning of a time interval, N_T the cell density at the end of the time interval and Δt the length of the time interval ($t_T - t_0$). The number of doublings per day (k) was calculated as $k = \mu / \ln 2$ and the doubling time (T_2) calculated as $T_2 = \ln 2 / \mu$. Cyst-like structures were also observed in *K. brevis* samples. These cyst-like structures were enumerated using the same method described above for the cellular abundance. The total cellular abundance reported for *K. brevis* excludes the cyst-like structures. Although a resting cyst stage has never been documented for *K. brevis*, the presence of a sexual cycle is evident from the existence of planozygotes (Walker 1982; Steidinger *et al.*, 1998; Van Dolah *et al.*, 2009). The cyst-like structures observed in the present study are similar to asexual temporary or ecdysal cysts which have been reported for stressed dinoflagellates and for *K. brevis* upon exposure to algicidal bacteria (Roth *et al.*, 2008).

Photosynthetic efficiency – F_v/F_m

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3 To obtain an indication of the physiological condition of the microalgal cells (Kolber and
4 Falkowski, 1993), the maximum photochemical efficiency of photosystem II (F_v/F_m) was
5 determined by using a fluorescence induction and relaxation (FIRe) fluorometer (Satlantic).
6 In order to allow for the relaxation of non-photochemical quenching, the cells were dark-
7 acclimated for 20 min at the growth temperature. F_v/F_m was determined as $(F_m - F_0)/F_m$ where
8 F_0 is the minimal fluorescence when all the reaction centres are open and F_m , the maximal
9 fluorescence measured when all of the reaction centres are closed.
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17 **Cell death – SYTOX-labelling**

18 Cell death was quantified using SYTOX-green (Invitrogen), a nucleic acid-specific mortal
19 stain that only passes across the plasma membrane of dead cells, staining the nucleus brilliant
20 green under blue light illumination. Since SYTOX-green only penetrates cells with a
21 compromised membrane it provides an indication of a late stage in cell death. It is important
22 to mention that SYTOX does not label cysts or lysed cells. Stain concentration and
23 incubation time were optimized prior to use and assays were validated using heat-killed cells
24 (heating at 65 °C for 1h) to verify that stain uptake was proportional to the quantity of dead
25 cells. SYTOX-green working stocks were prepared in Milli Q water to a final concentration
26 of 50 µM. After the addition of SYTOX-green to a final concentration of 0.5 µM, 1.5 mL-
27 samples of algal culture were incubated in the dark, at room temperature for 1 h. SYTOX-
28 positive cells were counted using a Zeiss epifluorescence microscope (Ex: 465-495 nm; Em:
29 515-555 nm) and a glass Sedgwick-Rafter chamber. For each sample, the abundance of
30 SYTOX-positive cells present in four to eight rows of the chamber (50 µl/row) was
31 determined. To estimate the percentage of dead cells, the concentration of SYTOX-positive
32 cells was divided by the concentration of Lugol's fixed cells and multiplied by 100. SYTOX-
33 positive cells were photographed using a Zeiss epifluorescence microscope equipped with a
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51 **Protein extraction and quantification**

52 Ten mL samples of each culture were centrifuged (Rotanta 460 R, Hettich Zentrifugen,
53 Germany) at 4 °C for 10 min (3800g). The supernatant was then carefully removed and the
54 pellets containing the algal cells snap frozen at -80 °C. Pellets were then suspended in 200 µl
55 of extraction buffer (100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, pH 7.4) and sonicated
56 on ice using a microtip sonicator (Vibra-Cell, Sonics and Materials, Inc.) until just thawed
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3 and refrozen immediately. This freezing/sonification cycle was repeated three times to ensure
4 maximum extraction of proteins. To remove insoluble material and unbroken cells, a
5 centrifugation (3800g, 5 min) was performed and the supernatant, containing the protein
6 extract, collected and transferred to a clean microtube. The Bio-Rad Protein Assay was used
7 to determine the total protein concentration of samples using bovine serum albumin as a
8 standard protein. The absorbance (750 nm) of the samples was measured using a microplate
9 reader (FLUOstar OPTIMA, BMG LABTECH, Germany).

17 **Caspase 3-like protein activity determination**

18 Caspase 3-like protein activity was determined using the Enzchek® Caspase-3 Assay Kit #2
19 (Invitrogen). Cell extracts (50 µl) were incubated with the caspase substrate Z-DEVD-R110
20 (25 µM, final concentration) for 3.5 h in the dark, at room temperature. The appearance of
21 fluorescent rhodamine 110 (R-110) upon enzymatic cleavage of the non-fluorescent substrate
22 Z-DEVD-R110 was subsequently assayed using a microplate reader (Ex: 485 nm; Em: 520
23 nm). Negative controls (samples without the Z-DEVD-R110 substrate) and substrate-only
24 controls (mixture of activity buffer and Z-DEVD-R110 substrate to determine the
25 background fluorescence of the substrate) were also carried out. Pre-incubating the cell
26 extracts for 20 min with the reversible caspase inhibitor Ac-DEVD-CHO (20 µM, final
27 concentration) prior to the substrate addition confirmed that the fluorescence signal observed
28 with the extracts of *Karenia* cells was due to the activity of caspase 3-like proteases.
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41 **DNA fragmentation – TUNEL labelling**

42 Nuclear DNA fragmentation was assessed using the *In situ* Cell Death Detection Kit,
43 Fluorescein (Roche diagnostics). This terminal deoxynucleotidyl transferase-mediated dUTP
44 nick end labelling (TUNEL) assay labels free 3-OH ends of DNA and is used to detect
45 fragmented DNA and to visualise apoptotic cells. Due to the limited culture volume and the
46 length of the experiment, this assay was only performed once, i.e., when the cultures had
47 reached the late decline phase as it was initially hypothesised that the caspase 3-like activity
48 would be highest during this phase. Twenty-five mL of 50-d old cultured cells of *K. brevis*
49 and *K. mikimotoi* were fixed with 0.1 % glutaraldehyde, centrifuged for 15 min at 1500g at
50 4°C. The pellet containing the cells was permeabilized using 0.1% Triton X-100 in PBS for
51 15 min on ice. Cells were subsequently washed twice with PBS and labelled following the
52 manufacturers' instructions. Samples were resuspended in PBS and the green fluorescence
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3 observed with a Zeiss epifluorescence microscope (Ex: 450-490 nm; Em: > 520 nm).
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5 Negative controls were treated as described above but labelled only with the label solution
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7 (without terminal transferase). Positive controls consisted of permeabilized cells pre-treated
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9 with DNase I (Sigma) for 15 min prior to labelling with the TUNEL reaction mixture. All of
10
11 the cells present in the positive control samples of *K. brevis* and *K. mikimotoi* were stained
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13 with the label solution.
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16 **Statistical analyses**

17 To assess the differences for the caspase 3-like protein activity in the presence or absence of
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19 caspase inhibitor (Fig. 3A-B), repeated measures analyses of variance (rm ANOVAs)
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21 (Minitab 15.1.0.0, Minitab Inc.) were performed with time as the repeated factor. When
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23 significant differences were found, a t-test was performed for each specific sampling day
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25 (except for day 56 for *K. mikimotoi* due to the lack of replication for the inhibitor treatment).
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28 **RESULTS**

29 **Cellular abundance, growth rates, cyst-like structure abundance and physiological state** 30 **of *Karenia* cells**

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32 The cellular abundance of *K. brevis* increased from ~ 300 cells mL⁻¹ to more than 7,500 cells
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34 mL⁻¹ in 21 days (Fig. 1A). On days 28 and 35, the abundance was ~ 4,000 cells mL⁻¹ and after
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36 day 35, the abundance gradually decreased until it reached 390 cells mL⁻¹ on the last day of
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38 sampling (Fig. 1A). The growth rate calculated for this species was 0.18 d⁻¹ with a doubling
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40 time of 3.8 days (Table I). *Karenia mikimotoi* was characterised by a longer lag phase and
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42 slower growth rate compared to *K. brevis*. The cellular abundance only peaked on day 42
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44 (1,443 cells mL⁻¹) after which it declined to reach 212 cells mL⁻¹ on the last day of sampling
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46 (Fig. 1A). The growth rate determined for *K. mikimotoi* was 0.07 d⁻¹ and the doubling time
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48 9.6 days (Table I).
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53 The number of cyst-like structures present in *K. brevis* samples (Fig. 2B inset) started
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55 to increase on day 21 and reached its highest values on day 28 (1,200 cyst-like structures mL⁻¹
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57). Their abundance subsequently declined to reach less than 200 cyst-like structures mL⁻¹ on
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59 day 56 (Fig. 1B).
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The maximum photochemical efficiency of photosystem II, F_v/F_m , which generally provides an indication of the physiological state of the cells varied between 0.50 and 0.70 for the two studied species (Fig. 1C). For *K. brevis*, F_v/F_m values increased from an initial value of 0.55 to a value of 0.65 on day 21 (Fig. 1C). Values subsequently remained relatively high and varied between 0.59 (day 35) and 0.64 (Fig. 1C). F_v/F_m values for *K. mikimotoi*, increased from an initial 0.50 to reach 0.70 on day 14, and then remained between 0.66 and 0.69 for the rest of the sampling period (Fig. 1C).

Mortality – SYTOX-positive cells

The DNA marker SYTOX-green was used to discriminate between live cells (SYTOX-negative cells) and cells with a compromised cell membrane (dead cells = SYTOX-positive cells). The abundance of SYTOX-positive cells for *K. brevis* reached a maximum value on day 21 with 1,273 cells mL⁻¹ (Fig. 2A), then decreased but remained around 300 cells mL⁻¹ up to day 35 before reaching values lower than 110 cells mL⁻¹ for days 42 and 56 (Fig. 2A). The abundance of SYTOX-positive cells remained variable for *K. mikimotoi* cells with peaks of ~ 40 cells mL⁻¹ observed on days 21 and 42 (Fig. 2B). The highest value occurred on day 56 with 86 cells mL⁻¹ (Fig. 2B).

The percentage of SYTOX-positive *K. brevis* cells remained below 10 % up to day 14 (Fig. 2C) and, except for the transient decline observed between days 28 and 35, values subsequently remained near 15 % until the end of the experiment when it increased to 20 % (day 56) (Fig. 2C). The transient decline in the percentage of SYTOX-positive cells observed between days 28 and 35 coincided with the increase in the abundance of cyst-like structures (Fig. 1B). The low initial percentage of SYTOX-positive *K. mikimotoi* cells increased to reach 12 % on day 14, then subsequently decreased to near zero on day 28 (Fig. 2C), i.e., when the cells were actually growing (Fig. 1A), and increased again on the last day of sampling to reach 44 % (Fig. 2C). Insets in Figures 2A and B show bright field micrographs of *K. brevis* and *K. mikimotoi*, respectively and cells positively labelled with the SYTOX-green marker. These pigment-containing cells were considered dead because their nucleus fluoresced bright green when excited with blue light.

Caspase 3-like protein activity and effect of the caspase inhibitor

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3 In *K. brevis* cells, a constitutive level of caspase 3-like protein activity was observed
4 throughout the experiment (Fig. 3A). Initial values (on days 0, 7, and 14) were slightly below
5 4,000 RFU/h/mg protein and the subsequent increase in activity on days 21 and 28 (~ 38,000
6 RFU/h/mg protein on day 28) coincided with the increase in the abundance of cyst-like
7 structures. On days 35, 42 and 56, the activity remained between 3,140 and 6,664
8 RFU/h/mgprotein (Fig. 3A). For *K. mikimotoi*, the caspase 3-like protein activity varied
9 between 1,000 and 1,300 on days 0, 7 and 14 (Fig. 3B). On days 21 and 28 the activity
10 decreased to near detection limits before increasing again on days 35 and 42 (~ 656 and 500
11 RFU/h/mg protein, respectively), i.e., just before the decline in cellular abundance. Values on
12 days 49 and 56 were also near detection limits (Fig. 3B).
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22 Table II presents the results of the repeated-measures ANOVAs which were
23 performed to verify the presence of significant differences between the caspase 3-like activity
24 measured in the presence and the absence of the inhibitor. Pre-treating the cell extracts with
25 the reversible caspase inhibitor Ac-DEVD-CHO (20 μ M, final concentration) before the
26 caspase substrate supplementation efficiently prevented the increase in caspase 3-like protein
27 activity (Fig. 3AB; Table II). The inhibitor was very effective in both *K. brevis* and *K.*
28 *mikimotoi* cells for which it inhibited, on average, 97.6 % and 92.8 %, respectively, of the
29 caspase-like protein activity (Fig. 3A-B, Table II).
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38 DNA fragmentation

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40 Using a TUNEL assay, the presence of DNA degradation was assessed in cells of *Karenia*
41 which had reached the cultures' decline phase. Figures 4B and F display bright field
42 micrographs of *K. brevis* and *K. mikimotoi*, respectively, while figures 4A and E show the
43 same samples with the localized incorporation of the TUNEL fluorescent label, which is
44 indicative of DNA fragmentation in both species of *Karenia*. Although the total number of
45 TUNEL-positive cells was not counted, the majority (~ 85 %) of *K. brevis* and *K. mikimotoi*
46 cells contained degraded DNA. Results obtained for the positive control treatment, which
47 involved the pre-treatment of the samples with DNase (Fig. 4C-G), are comparable to those
48 obtained with *Karenia* cells from the late decline phase (Fig. 4A-E) and therefore confirm the
49 positive-TUNEL labelling in both species of *Karenia*. The negative controls show the
50 absence of incorporation of the TUNEL fluorescent label (Fig. 4D-H).
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DISCUSSION

Understanding the factors controlling the growth, division and death processes in protists such as dinoflagellates is complex due to the unique nature of these organisms.

Dinoflagellates usually reproduce vegetatively but they can also reproduce sexually, some possessing a thick cellulosic cell wall while others do not, and they can exploit different nutritional modes. The chromosomal structure of dinoflagellates is also unique as they possess a special form of nucleus in which the chromosomes are almost permanently condensed throughout the cell cycle (reviewed by Segovia, 2008). The type of cell death occurring in dinoflagellates and other microalgae is thought to be species-specific and/or to be dependent on a triggering factor (Segovia, 2008; Jiménez *et al.*, 2009). In mammalian cells, high levels of caspase activation have been proposed to mediate apoptosis while the limited activation of the same enzymes may play a role in other cellular processes such as cell proliferation, cell differentiation, DNA repair, etc. (Los *et al.*, 2001; Algeciras-Schimmich *et al.*, 2002; Abraham and Shaham, 2004; Launay *et al.*, 2005). Caspase-like activities measured so far in diverse species of microalgae under stress have generally been proposed to play a role in the occurrence of an active cell death process similar to PCD in metazoans (Bidle and Falkowski, 2004). Recently, however, a form of PCD has been shown to be a caspase-like independent event (Affenzeller *et al.*, 2009) while cell death modes other than PCD have been shown to require the involvement of caspase 3-like protein activity to proceed (Jiménez *et al.*, 2009). It is essential to obtain a better understanding of the role of caspase-like proteins in the cellular activities of microalgae. The aim of this study was to follow the temporal variation of the caspase 3-like protein activity in two cultures of harmful dinoflagellates and to verify its possible implication in the cellular processes of *Karenia* spp.

With a reported optimal growth temperature between 22 and 28 °C (Vargo, 2009), cells of *K. brevis* grew well during the first three weeks of the experiment. The growth rate determined for *K. brevis* (0.18 d^{-1}) was in agreement with that reported in the literature for the same species ($0.17\text{-}0.36 \text{ d}^{-1}$; Magaña and Villareal, 2006). The percentage of dead *K. brevis* cells which remained between 3 and 20 % during the whole experimental period, suggests that a fraction of the *K. brevis* population was constantly undergoing cell death. This observation is in agreement with the constitutive level of caspase 3-like protein activity which was measured throughout the experiment in cells of *K. brevis*. This constitutive level

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3 of caspase 3-like protein activity could well have served some housekeeping functions
4 (Segovia *et al.*, 2003) and/or played a role in the regulation of the cell cycle (Algeciras-
5 Schimnich *et al.*, 2002). The caspase 3-like protein activity generally remained between 3,
6 000 and 6,665 RFU/h/mg protein throughout the whole sampling period except on days 21
7 and 28 when it increased to reach its highest value (~ 38, 000 RFU/h/mg protein on day 28).
8 This increase in activity matched the increase of the abundance of cyst-like structures
9 observed on the same days. This suggests a role of caspase-3 like proteases in the cell
10 cycle/encystment progression of *K. brevis* and provides evidence for their involvement in
11 processes other than cell death (Algeciras-Schimnich *et al.*, 2002). Combined together the
12 formation of cyst-like structures and the death of *K. brevis* cells (revealed by SYTOX-
13 labelling) do not entirely explain the decline in cellular abundance which occurred after day
14 28 and therefore suggests that a fraction of the community may have undergone lysis and
15 escaped the SYTOX-labelling.
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28 The appearance of cyst-like structures in cultures of *K. brevis* can cautiously be
29 attributed to the cells entering the stationary phase. However, the presence of such a high
30 abundance of cyst-like structures in other cultures of *K. brevis* which were grown under the
31 same experimental conditions (i.e., 25 °C, 12:12: L:D cycle at 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) has
32 not previously been observed in our laboratory nor reported in the literature. These cyst-like
33 structures are similar to asexual temporary or ecdysal cysts documented previously for other
34 dinoflagellates under stress conditions (Blanco, 1995), such as cold temperatures (Hardeland,
35 1994). The formation of cyst-like structures has also been reported for *K. brevis* upon
36 exposure to bacteria (Roth *et al.*, 2008). Whether or not the transient decline in temperature,
37 which occurred on day 27 inside the incubator, contributed to the increased formation of cyst-
38 like structures is unclear but remains a plausible explanation.
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50 Compared to *K. brevis*, lower cellular abundance was reached in *K. mikimotoi*
51 cultures and the growth rate determined for these cells (0.07 d^{-1}) was at the lower end of the
52 values reported in the literature (~ 0.07 to 0.15 d^{-1} ; Garcia and Purdie, 1992; Staehr and
53 Cullen, 2003). The limited growth is not likely to be attributed to the growth temperature
54 selected (15 °C) since *K. mikimotoi* is generally grown at temperatures ranging between 11
55 and 16 °C (as recommended by CCMP). The caspase 3-like protein activity in cells of *K.*
56 *mikimotoi* was not as elevated as in cells of *K. brevis* (Fig. 3AB) but a clear constitutive level
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3 of activity was observed at the beginning of the experiment (values > 1, 000 RFU/h/mg
4 protein on days 0, 7 and 14). This activity may have provided some housekeeping functions
5 and/or played a role in the death of the “less vigorous” *K. mikimotoi* cells as suggested by the
6 increase of the percentage of dead cells (~ 10 % on days 14 and 21) which followed the
7 caspase 3-like protein activation (on days 0, 7 and 14). *Karenia mikimotoi* is difficult to grow,
8 and known to be sensitive to agitation (Gentien *et al.*, 2007). To explain the limited growth
9 observed in cultures of *K. mikimotoi*, Gentien *et al.* (2007) proposed an autotoxicity
10 mechanism which involves the release of an exotoxin (18 : 5n3 fatty acid) from *K. mikimotoi*
11 cells. We suggest that the mechanical stress endured by the cells during subculture and/or the
12 inoculation of old cells may have facilitated the release of toxins from *K. mikimotoi* cells and
13 influenced their growth. Although particular care was taken not to handle the culture flasks
14 between the 7-d sampling intervals, this exotoxin could still have leaked from old/stressed
15 cells and acted as a growth deterrent during the initial part of the study. Interestingly, the
16 subsequent increase, albeit slow, in *K. mikimotoi* cellular abundance matched the increase in
17 the physiological state (F_v/F_m) of the cells, and declines in both the caspase 3-like protein
18 activity (on days 21 and 28) and the percentage of dead cells (on days 28 and 35). The
19 subsequent increase in caspase 3-like protein activity (on days 35 and 42 with values of 656
20 and 500 RFU/h/mg protein, respectively) observed prior to the decline in cellular abundance
21 suggests a role of caspase 3-like enzymes in the death process of *K. mikimotoi*.
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39 The high efficiency with which the inhibitor Ac-DEVD-CHO precluded the increase
40 in caspase 3-like protein activity in both species of *Karenia* strengthens the involvement of
41 caspase-like enzymes, and not of other generic proteases, in the caspase-like activity
42 detected, although the identity of the actual proteases involved in the process is still unclear.
43 The caspase-3 like protein activity measured in species of *Karenia* either had some
44 housekeeping functions, played a role in the formation of cyst-like structures (for *K. brevis*)
45 or in cell death. While the implication of caspase-like protein activity in cyst formation has
46 never been demonstrated before, the existence of a caspase-mediated pathway leading to cell
47 death has been observed in a variety of microalgae. Bidle and Bender (2008) reported an
48 increase in caspase-like activities in an aging culture of the diatom *Thalassiosira pseudonana*
49 while the occurrence of PCD, with the participation of cysteine proteases, was similarly
50 proposed to explain the sudden termination of a bloom of the freshwater dinoflagellate
51 *Peridinium gatunense* (Vardi *et al.*, 1999). Because the type of cell death in microalgae can
52 be species-specific and influenced by a triggering factor, it is challenging to identify a
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3 process common to diverse species of microalgae (Segovia 2008; Jiménez *et al.*, 2009;
4 Affenzeller *et al.*, 2009). In cells of the green alga *Micraterias denticulate*, the occurrence of
5 PCD, associated with an increase in caspase-like activity, has been observed when cells were
6 exposed to H₂O₂ (Darehshouri *et al.*, 2008) while a caspase-like independent PCD was
7 reported for the same species upon exposure to salt stress (Affenzeller *et al.*, 2009). Similarly,
8 Jiménez *et al.* (2009) reported the occurrence of a variety of cell death events in the
9 chlorophyte *Dunaliella viridis* depending on the type of environmental stressors tested. In
10 their study, the increase in caspase-like activity was surprisingly shown to mediate not only
11 apoptosis but also other cell death programmes (Jiménez *et al.*, 2009). Whether harmful
12 dinoflagellates display an ability to undergo a programmed-form of cell death is unclear but
13 seems plausible considering the results obtained from other studies performed with species of
14 non-toxic dinoflagellates (Vardi *et al.*, 1999) and coral symbiotic dinoflagellates (Dunn *et al.*,
15 2002).

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28 DNA degradation, a generally well accepted marker of apoptosis, can be detected
29 using a TUNEL assay, among other techniques. This assay however has been reported to lack
30 specificity because it can label some forms of non-apoptotic DNA fragmentation (Labat-
31 Moleur *et al.*, 1998). Nevertheless, the positive labelling of degraded DNA was observed in
32 senescent cells of *Dunaliella viridis* (Jiménez *et al.*, 2009). Zuppini *et al.* (2007) also reported
33 increases in caspase 3-like activity, chromatin condensation, DNA fragmentation, cell
34 shrinkage and detachment of the plasma membrane and therefore the occurrence of PCD in
35 heat-stressed cells of *Chlorella saccharophila* (Zuppini *et al.*, 2007). Increases in caspase-like
36 activities also coincided with an increase in chromatin margination and DNA fragmentation
37 in darkened cells of *Dunaliella tertiolecta* (Segovia *et al.*, 2003) while the occurrence of
38 PCD, defined as the induction of cytoplasmic shrinkage and DNA fragmentation, was
39 proposed for the dinoflagellate *Peridinium gatunense* upon CO₂ limitation and oxidative
40 stress (H₂O₂) (Vardi *et al.*, 1999). In the present study, a TUNEL assay was used to determine
41 the presence of degraded DNA in cultures of *Karenia* which had reached the late decline
42 phase. The presence of degraded DNA in cells of *K. brevis* was associated with a constitutive
43 level of caspase 3-like activity (at least on days 42 and 56) while the presence of degraded
44 DNA in cells of *K. mikimotoi* followed the activation of caspase 3-like proteases observed on
45 days 35 and 42. While these data are insufficient to claim the occurrence of a specific type of
46 cell death they certainly provide evidence of the cells' deteriorating condition.
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In the present study, cells of both *K. brevis* and *K. mikimotoi* displayed high F_v/F_m values which are consistent with values previously reported in the literature (Schaeffer *et al.*, 2007; 2009). However, the maintenance of high F_v/F_m values even during the decline phase of the cultures is unexpected and surprising since F_v/F_m is commonly used as an indicator of the physiological state of cells, which responds mostly to nutrient limitation and high irradiance (Kolber and Falkowski, 1993). Because large proportions of dead “but intact and pigment-containing” cells are now known to be present in phytoplankton communities (Veldhuis *et al.*, 2001; Agustí 2004), Franklin *et al.* (2009) assessed the effect of dead cells on the apparent photosystem II efficiency of a variety of microalgae. Their results demonstrated that the presence of photosynthetically non-functional, pigment-containing dead cells had little effect on the photosynthetic efficiency of a number of phytoplankton species, including dinoflagellates. In their study, the efficiency of photosystem II (F_v'/F_m') of the dinoflagellate *Amphidinium carterae* decreased by only 20 % in the presence of 75 % of photosynthetically non-functional cells. Similarly, the presence of 50 % of photosynthetically non-functional cells barely influenced the initial efficiency of photosystem II which was measured in the absence of dead cells. These results are very similar to those obtained in the present study and are in accordance with other results obtained in the field which showed that mass microalgal mortality can be accompanied by relatively constant PSII efficiency (Dijkman and Kromkamp, 2006).

Besides the report of increased caspase 3-like activity in stressed cells of toxic cyanobacteria *Microcystis aeruginosa* (Ross *et al.*, 2006), to the best of our knowledge, caspase-like protein activities have never been reported for species of harmful microalgae. We report variations in caspase 3-like protein activity in cultures of the harmful dinoflagellates and suggest that it can have housekeeping functions, play a role in the death of *Karenia* cells and in the formation of cyst-like structures (in *K. brevis*). Further research is needed to determine the variation of caspase 3-like protein activity in harmful dinoflagellates under stress and to verify if an active caspase-mediated cell death pathway could contribute to the decline of *Karenia* blooms *in situ*.

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Table I. Comparisons of growth variables for the two dinoflagellate species studied.

	Maximal cell abundance (MCA; cells mL⁻¹)	Day MCA was reached	Growth rate (μ; day⁻¹)	Division (k; day⁻¹)	Doubling time (T₂; days)
<i>K. brevis</i>	7646	21	0.18	0.26	3.8
<i>K. mikimotoi</i>	1443	42	0.07	0.10	9.6

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Table II. Results from repeated-measures ANOVAs. The source of variation is partitioned into individual effects of the factor “Treatment” and the repeated measure “Time”, and the interaction between these two factors (Treatment * Time). df = degree of freedom; MS = mean square; F = Fisher’s distribution value; P = probability value

Species	Factors	df	MS	F	P
<i>K. brevis</i>	Treatment	1	3.6 x 10 ⁶	254.86	0.000
	Time	4	1.2 x 10 ⁶	85.53	0.000
	Treatment*Time	4	1.1 x 10 ⁶	78.15	0.000
	Error	30	1.4 x 10 ⁴		
<i>K. mikimotoi</i>	Treatment	1	3.4 x 10 ⁶	174.87	0.000
	Time	3	5.8 x 10 ⁵	29.97	0.000
	Treatment*Time	3	6.0 x 10 ⁵	30.69	0.000
	Error	24	1.9 x 10 ⁴		

FIGURE DESCRIPTION

- Figure 1.** Cellular abundance of *K. brevis* (left y-axis) and of *K. mikimotoi* (right y-axis) (A). Abundance of cyst-like structures for *K. brevis* (B) and optimal quantum yield of photosystem II fluorescence (F_v/F_m) for *K. brevis* and *K. mikimotoi* (C). Inset in (B) shows a healthy Lugol-stained *K. brevis* cell (left panel) and a cyst-like structure (right panel). Scale = 25 μm . Each point is the average of samples from four separate flasks \pm SE.
- Figure 2.** Abundance of SYTOX-positive cells for *K. brevis* (A) and *K. mikimotoi* (B). Note the different scales between A and B. Micrographs of *K. brevis* (A inset) and *K. mikimotoi* (B inset) taken under the bright field (left panel) and blue light fluorescence micrographs of cells with a compromised membrane (dead cells; right panel). Cells were stained with the fluorescence probe SYTOX-green. Scale = 20 μm . Percentage of SYTOX-positive cells for *K. brevis* and *K. mikimotoi* (C). Dashed lines show the highest values reached for *K. brevis* (short dashes) and for *K. mikimotoi* (long dashes). Each point is the average of samples from four separate flasks \pm SE.
- Figure 3.** Temporal variation of caspase 3-like protein activity in *K. brevis* (A) and *K. mikimotoi* (B) cultures. Note the different scales between A and B. The white symbols represent the activity of caspase 3-like enzyme measured in the presence of the caspase inhibitor Ac-DEVD-CHO (20 μM). Each point is the average of samples from four (only one sample for the inhibitor treatment at day 56 for *K. mikimotoi*) separate flasks \pm SE. Significant differences at $p \leq 0.05$ are indicated by an asterisk. RFU = relative fluorescence units.
- Figure 4.** TUNEL assay results for *K. brevis* (A-D) and *K. mikimotoi* (E-H). Fluorescence micrographs of *Karenia* cells stained with the TUNEL marker (A, E), of the same cells taken under the bright field (B, F), of cells pre-treated with DNase (positive controls) (C, G) and of cells stained only with the label solution, i.e., without terminal transferase (negative controls) (D, H). Scale = 25 μm .

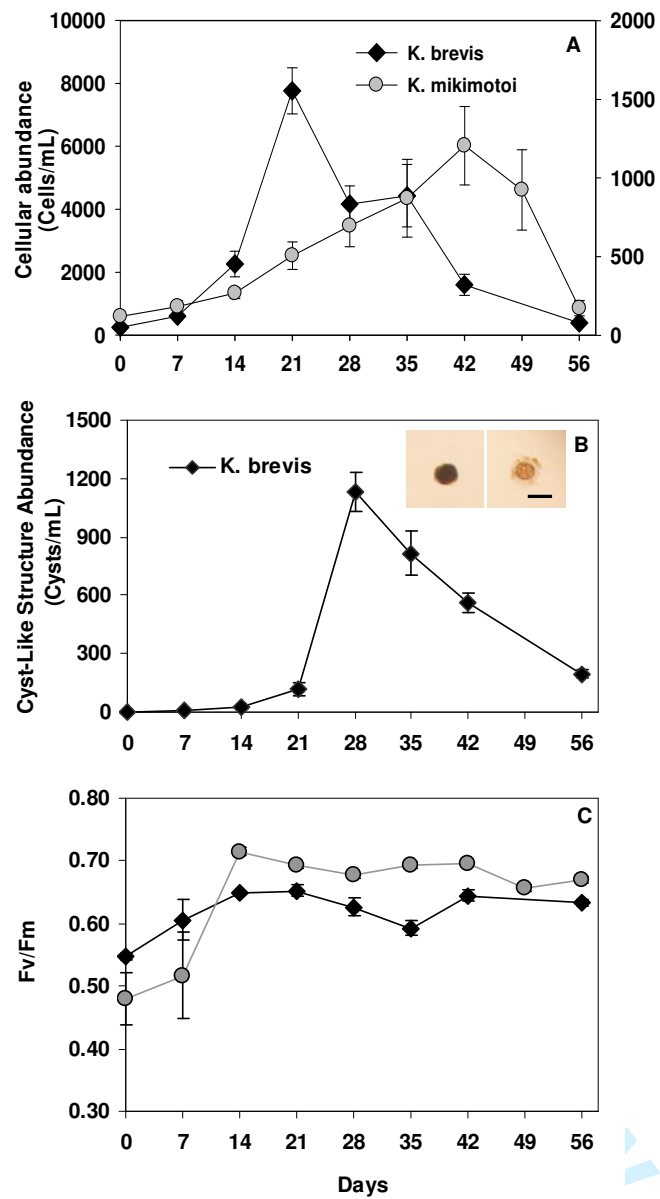


Figure 1.

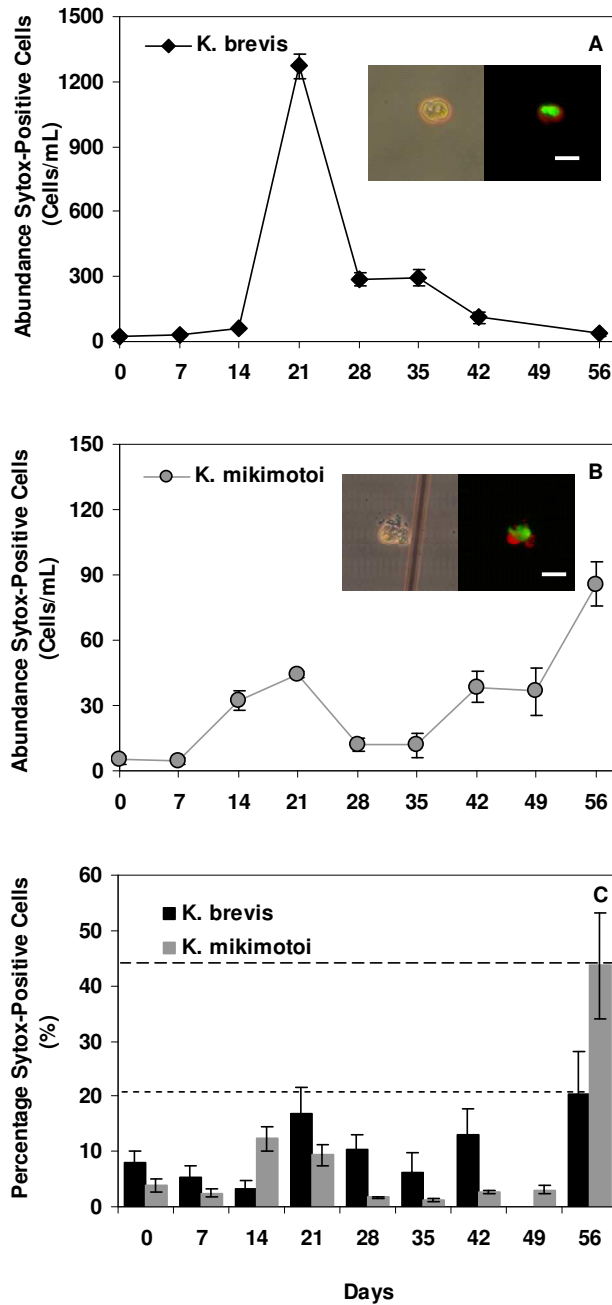


Figure 2.

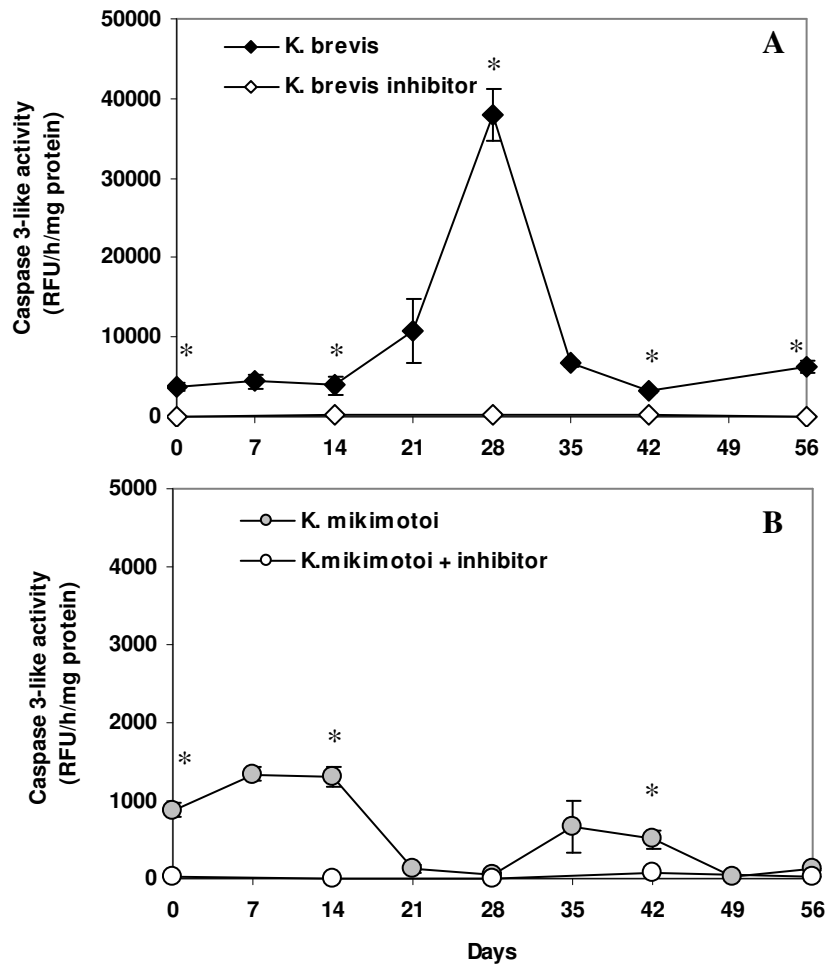


Figure 3.

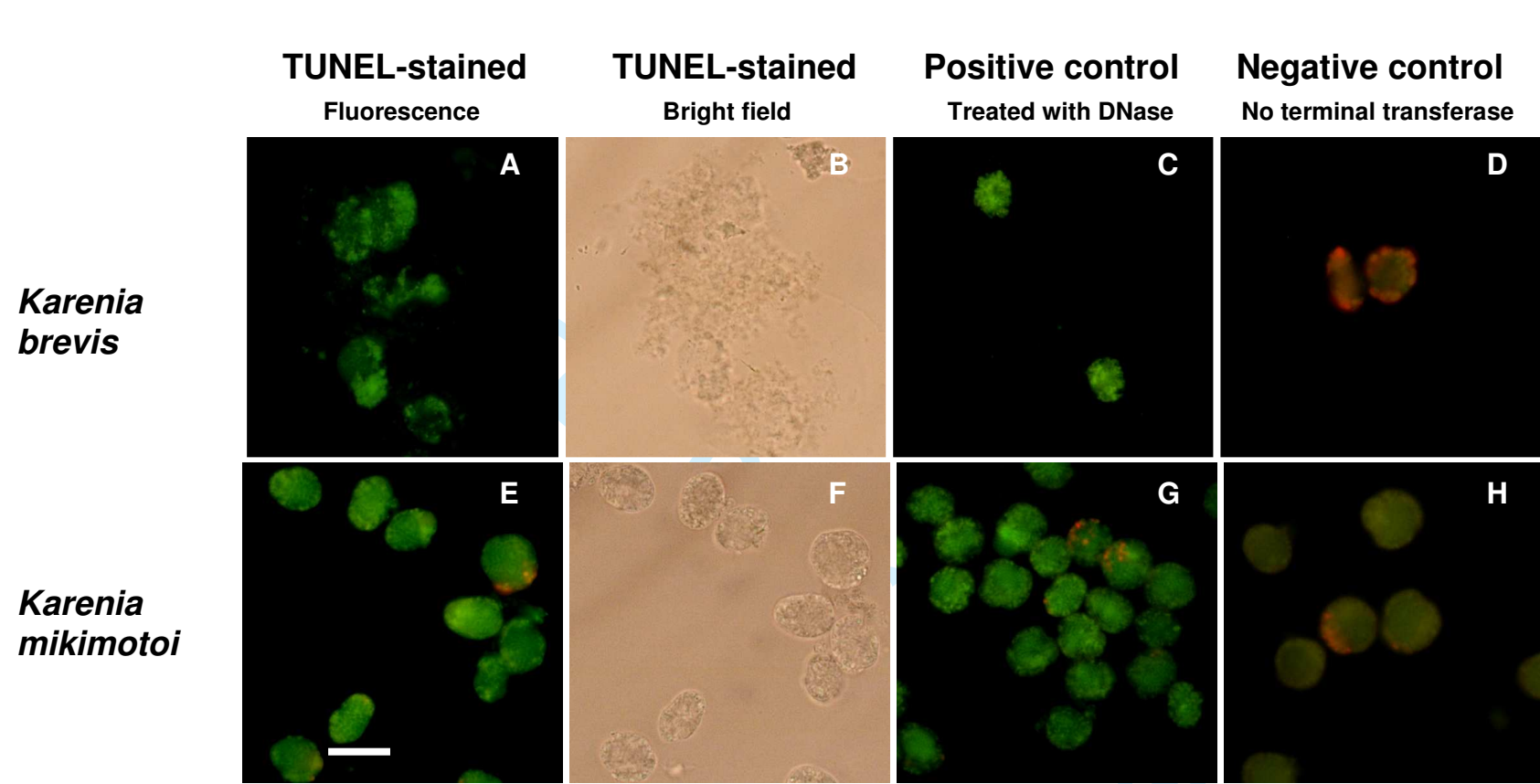


Figure 4.