

Communication

Brevetoxin-Producing Spherical Cells Present in *Karenia brevis* Bloom: Evidence of Morphological Plasticity?

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Abstract: Spherical cells were detected in low salinity waters during a bloom of *Karenia brevis* in Alabama coastal waters. These balls resembled *K. brevis* in size and organelle appearance, contained similar concentration of brevetoxin, and occurred during ongoing *K. brevis* bloom. Based on the environmental conditions in which these cells were observed, we speculate that a rapid drop in salinity triggered the sphere formation in *K. brevis*. Brevetoxin concentrations were comparable between surface water samples containing typical and atypical cells ranging from 1 to 10 ng/mL brevetoxin-3 equivalents. Accurate identification and quantification of cell abundance in the water column is essential for routine monitoring of coastal waters, so misidentification of these spherical cells may result in significant underestimation of cell densities, and consequently, brevetoxin level. These potential discrepancies may negatively impact the quality of regulatory decisions and their impact on shellfish harvest area closures. We demonstrate that traditional monitoring based on microscopy alone is not sufficient for brevetoxin detection, and supporting toxin data is necessary to evaluate potential risk.

Keywords: *Karenia brevis*; morphology; plasticity; sphere-shape cell; salinity; cryptic species; regulatory monitoring; shellfish harvest area

Karenia brevis is a marine dinoflagellate which naturally produces a suite of neurotoxins known as brevetoxins. In humans, brevetoxins can cause respiratory distress following exposure to aerosols, gastrointestinal distress, and mild neurological symptoms following consumption of contaminated shellfish. In addition to potential human health effects, blooms of *K. brevis* can cause significant economic and environmental impacts. Monitoring *K. brevis* abundance is well established in Gulf of Mexico waters with blooms reported frequently in Florida and Texas, and more recently in the northern Gulf of Mexico [1]. Routine monitoring efforts rely on light microscopy, and the characteristic cellular morphology is a cornerstone of *Karenia* identification. Likewise, accurate quantification of cell abundance in the water column is essential for regulatory monitoring in coastal waters. For *K. brevis*, a threshold of 5000 cells per liter has been recommended by the U.S. National Shellfish Sanitation Program [2] to close shellfish harvest areas in U.S. waters. This guideline relies on the accurate identification and enumeration of *K. brevis* cells and initiates monitoring of waters and shellfish meats for the presence of brevetoxins.

The morphology of the unarmored dinoflagellate *K. brevis* has been described in detail elsewhere (e.g., *Gymnodinium breve* in [3], *K. brevis* in [4]). A typical description of *K. brevis* addresses the cell shape as dorso-ventrally flattened, ventrally concave, with a straight apical groove. Cells are wider than they are long (20–40 μm), with a round nucleus located in the posterior left quadrant, and

consist of peripheral chloroplasts. Two flagella are located at the equatorial cingulum and descend approximately two times the length of the cell. The last characteristic defining *K. brevis* is the sulcus that extends to the epicone. As with many phytoplankton species, other factors including geographic location, salinity preference, toxin profile, pigment profile, and even behavior (e.g., swimming behavior, bloom dynamics) can be key to accurate identification. The salinity optimum for *K. brevis* growth and proliferation has been reported as 30–35 ppt; however, blooms have been detected in salinities as low as 5 ppt in northern Gulf of Mexico [5]. In cultures, *Karenia* spp. tolerate salinities between 17.5 to 45 ppt depending on the clone [5]. In terms of photo-pigments, *K. brevis* contains gyroxanthin, high levels of fucoxanthin, and other accessory pigments, rather than carotenoid peridinin, which is utilized by other dinoflagellates [6]. The shape of the cell and the location and shape of the nucleus are the most distinctive characteristics of *Karenia* spp. and are often the deciding attributes for *K. brevis* identification. The unique shape of *K. brevis* allows identification even at relatively low resolution, an advantageous characteristic for manual and automated counting (e.g., FlowCam particle analysis, Imaging flow cytobot). In addition, the majority of preservation techniques do not significantly alter the cell shape, which increases taxonomic confidence based solely on shape.

During blooms of *K. brevis* in Alabama coastal waters (late fall 2015) we conducted several opportunistic cruises. During two of these offshore sampling events (11/30/15 and 12/1/15), we collected 23 surface water samples in transects southeast of Dauphin Island and westward parallel to the AL and MS coastline, respectively (Figure 1). Subsamples were immediately preserved for phytoplankton identification in both Lugols and glutaraldehyde using standard techniques and remaining non-preserved whole water allowed preparation samples for subsequent brevetoxin analysis (particulate toxin levels from water filtered on GF/F (Whatman), dissolved toxin from filtrate, and total toxin from non-filtered seawater). Preserved and non-preserved cells were identified and enumerated by light microscopy (OM900 inverted light microscope at 400 $\times$, Meiji Techno America, CA, USA). During these sampling events, we identified not only *K. brevis* and *K. mikimotoi* (a smaller species of equal length and width but with an oblong nucleus), but also encountered spherical cells of what we believe to be atypical *K. brevis*. These “balls” contained many characteristic identifiers of *K. brevis*, including the production of brevetoxins. By microscopy, the cells were of similar volume, possessing a large visible, spherical nucleus, and chloroplasts that were similar in shape and color to regular *K. brevis* cells found in adjacent samples (Figure 2). The typical *K. brevis* cells exhibited higher variability in cell size with mean length of $24.6 \pm 1.5 \mu\text{m}$ and mean width of $28.3 \pm 2.9 \mu\text{m}$ ($n = 20$). Spherical cells showed very consistent size with a mean width (diameter) of $22.4 \pm 0.8 \mu\text{m}$ ($n = 20$). We were not able to measure the volume of the cells accurately by light microscopy, so we were not able to statistically determine relationships with volume; however, the volume of the sphere and typical *K. brevis* were similar (Figure 3). Brevetoxin (PbTx) concentrations in the field collected samples (particulate, dissolved, total) were quantified using enzyme linked immunosorbent assay (ELISA; Abraxis, NJ, USA), a specific and highly sensitive method that cross reacts with several PbTX congeners (i.e., PbTX-3 100%; deoxyPbTX-2 133%; PbTX-5 127%; PbTX-2 102%; PbTX-9 83%; PbTX-6 13%; and PbTX-1 5%) but does not cross react with other shellfish toxins such as saxitoxins or domoic acid (Abraxis), or the functionally related ciguatoxins (also tested during this study). The analyses revealed that field samples containing the spherical cells contained PbTx's, ranging from 3 to 7 ng/ml PbTx-3 equivalents. This was comparable to water samples containing cells of typical *K. brevis* morphology but without spherical cells which had toxin concentrations ranging from 1 to 10 ng/mL PbTx-3 equivalents.

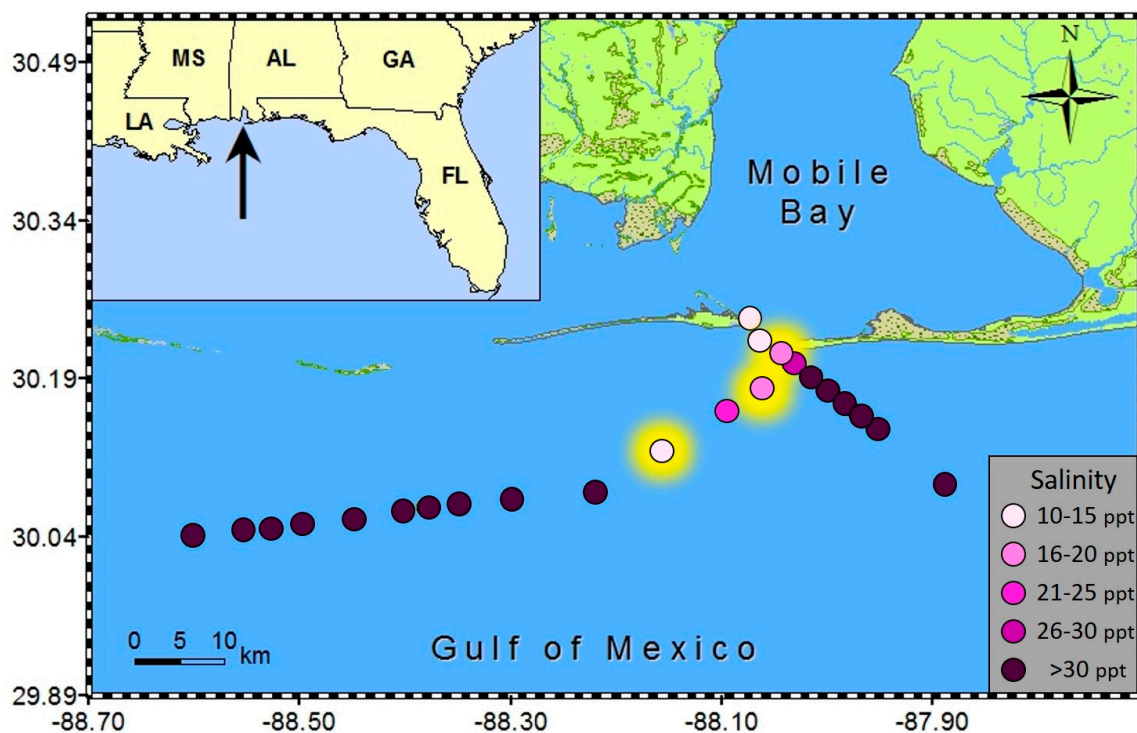


Figure 1. Locations of sampling points during 11/30/15 and 12/1/15 cruises. The yellow halo indicates presence of “balls”. Two of the most northern samples showed low salinity, but no *Karenia* cells (and consequently no “balls”) were detected in those samples.

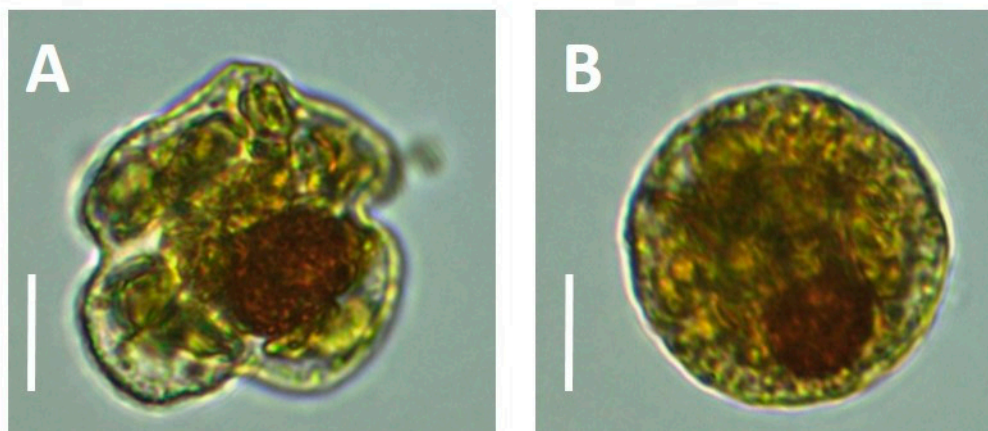


Figure 2. Morphological plasticity of *Karenia brevis* observed in a single sample collected in Alabama coastal waters (11/30/15). The characteristic *K. brevis* morphology (A) was observed at low density along with a much higher density of spherical cells (B) in low salinity offshore water (16.5 ppt in the sample used for this figure). Scale is 10 μm.

Salinity was below 20 ppt in all three samples in which spherical shaped cells were encountered. Specifically, the salinities were 16.5 ppt, 14.9 ppt, and 17.9 ppt, while surrounding samples in the same transects ranged from 21.6 to 38 ppt (Figure 4). Patchy surface salinity and relatively rapid rates of change are common in coastal Alabama waters [7]. There were two samples at very low salinities (10.7 and 13.3 ppt) that did not contain *Karenia* “balls”; however, these samples were also lacking any regular *Karenia* cells that would serve as a necessary precursor for “ball” formation (Figure 4). These two water samples contained typical brackish species of phytoplankton. The most dominant dinoflagellate was *Prorocentrum minimum* and *Heterocapsa rotundata*; both species are associated with freshwater discharge and are typical in coastal waters. These dinoflagellate species are not known to

produce brevetoxins which were supported by negative PbTX tests. Since water temperatures were consistent across sampling sites in the transects, and brevetoxins were confirmed in samples containing only the “balls”, we hypothesize that the spherical cells could be a rapid morphological response to low salinity stress of *K. brevis*, which was blooming in the vicinity and confirmed in adjacent high salinity waters. This is supported by the lack of spheres in water samples collected in adjacent high salinity waters; however we acknowledge that other factors could also be involved in this response and should be evaluated in detail in future culture studies.

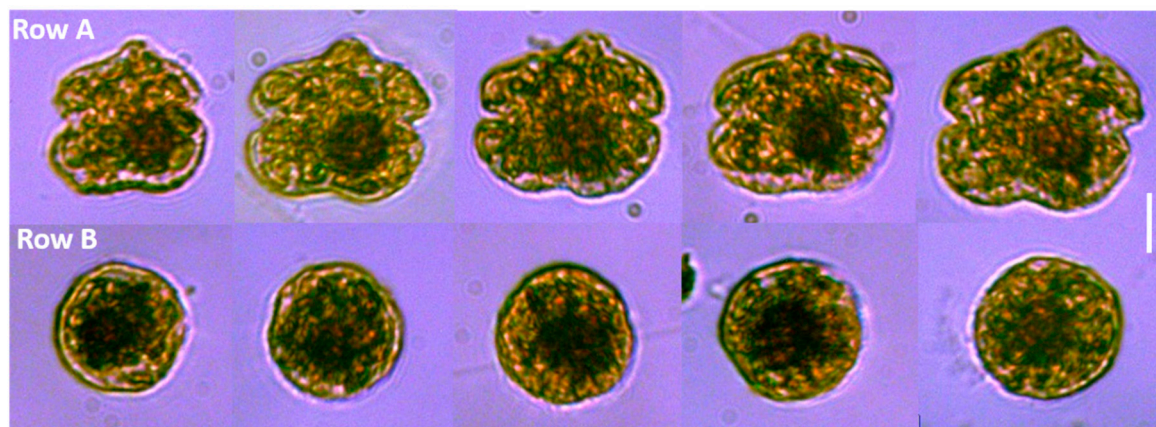


Figure 3. Morphological plasticity of *Karenia brevis* observed in a single sample collected in Alabama coastal waters (12/1/15). At least 20 cells were measured (showing only a subset of 5 cells for each category). The typical *K. brevis* morphology (Row A) exhibited higher variability in cell size with mean length of $24.6 \pm 1.5 \mu\text{m}$ and mean width of $28.3 \pm 2.9 \mu\text{m}$. Spherical cells (Row B) showed very consistent size with mean width (diameter) of $22.4 \pm 0.8 \mu\text{m}$. Scale is $10 \mu\text{m}$.

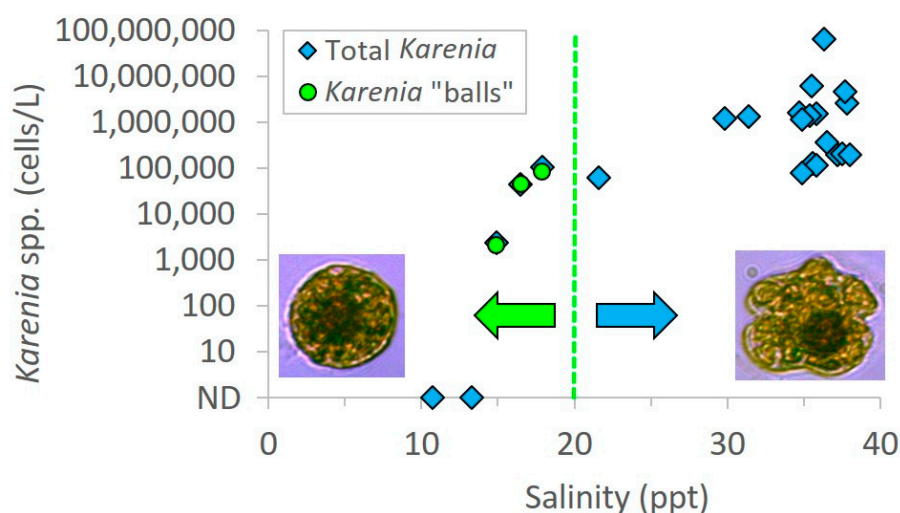


Figure 4. Relationship between *Karenia* abundance and salinity during offshore field sampling. Spherical cells thought to be *K. brevis* were observed only at salinities below 20 ppt. There were two samples at very low salinities, but no *Karenia* cells (and consequently no “balls”) were detected in those samples. ND = not detected.

In the samples containing spheres, 90–100% of cells were in the sphere stage, with only a small portion of the cells maintaining the “traditional” *K. brevis* morphology. Importantly, when present, these “balls” were found in non-preserved, Lugol’s preserved, and glutaraldehyde preserved samples, indicating that the preservation method did not induce sphere formation from these field samples. Since brevetoxins were confirmed in samples containing only spheres (in total, particulate, and dissolved partitions), there were morphological similarities between typical *K. brevis* and spherical

cells, and spheres were detected in waters adjacent to *K. brevis* blooms, we speculate that a rapid drop in salinity caused by freshwater plumes from the Mobile Bay estuary triggered a physiological response and *Karenia* cells entered an “emergency shut down” sphere stage, similar to first steps of dinoflagellate cyst development. For *Karenia* cells to go through this dramatic physiological change, significant re-organization of the cytoskeletal proteins would be involved. This brings two additional questions to this possible phenomenon, (1) what is the benefit to this response (e.g., increased resistance or survivability)? and (2) is this process reversible when cells return to optimal environmental conditions (i.e., akin to a temporary pellicle cyst)? Unfortunately, we are unable to answer these questions at this time, but future efforts with cultured isolates may enhance our understanding of these processes.

Sphere-shaped *K. brevis* has been previously reported in culture experiments and were associated with aging cultures [8], reproductive cells [9], a cyst-like stage [10], and with cell desiccation under the microscope [10]. However, morphological changes identified in field samples have not been well documented, and may represent an important anomaly in *K. brevis* bloom dynamics, toxin production by cells, or growth cycle. Experienced taxonomists have communicated seeing these effects following salinity drop below 25 ppt (e.g., Wolny, 2015 pers. comm.) but these observations have not been reported in the literature. Brown et al. [5] highlighted that the salinity optima of *K. brevis* is highly dependent on cultured clones, but in natural waters *K. brevis* generally prefers salinities above 30 ppt. It is expected that increased osmotic pressure as a result of low salinity would cause the cell volume to increase, and several *Karenia* toxicity studies have documented significant changes in cell volume as a response to a salinity drop. For example, Sunda et al. [11] detected an initial increase in cell volume by 17% when *K. brevis* cells acclimated at 35 ppt, and were then exposed to a salinity of 27 ppt, but a morphological change was not reported.

Cysts of *Karenia* have been hypothesized by several investigators. Resting cysts have been identified in samples from sediment on the West Florida Shelf in 2010 [12]. Temporary, thin wall cysts of *Karenia mikimotoi* were recorded under nitrogen starvation in the laboratory [13]. A true dormant dinoflagellate cyst has been characterized as having a very thick cell wall, lack of pigments/chloroplasts, and being packed with stored energy compounds that have no color (e.g., sugars and lipids) [13]. The spherical cells that we have reported here were not consistent with this, containing a relatively thin cell wall and packed with chloroplasts (Figure 2), which is more consistent with temporary cysts. Persson et al. [9] reported on *K. brevis* cells changing from a traditional shape to a sphere within one minute following cold shock from 20 °C to 15 °C. This was interpreted as pellicle cysts of late zygotes. Our samples of the *K. brevis* bloom that was ongoing at the time of our sampling transects (as evidenced by our clear microscopic identification; see Figure 2) were collected in cold winter waters of the northern Gulf of Mexico where surface water temperatures were approximately 13 °C ± 1 °C. The *Karenia* cells blooming in these waters were therefore exposed to temperatures below their optima. However, all samples were collected within a similar temperature range (±1 °C) and no temperature gradients were observed. Samples containing “balls” were only observed in the low salinity patches during our sampling transects and were confirmed to produce PbTXs, hence our suggestion of the salinity hypothesis for ball formation of *K. brevis*. It appears that *K. brevis* can be morphologically very plastic, and a variety of environmental stressors may be able to trigger these gross cell changes [9,13]. More data is required to determine the exact mechanisms and environmental parameters required to induce these morphological effects in *K. brevis*. We hope that the data and observations presented here may stimulate further investigations of this kind by phytoplankton ecologists.

The absence of a theca allows some morphological flexibility in all unarmored dinoflagellates, however sphere-shaped cells of *K. brevis* are not expected during traditional monitoring. *K. brevis* has a distinctive cytoskeleton structure with unique microtubule arrangement and a sterol rich membrane that contributes to cell flexibility [14]. This unusual potential for flexibility is often overlooked since *K. brevis* is known to be very fragile and can rupture easily with turbulence, resulting in the aerosolization of brevetoxins [15]. To date, there have been twelve different *Karenia* species described

and many of these have been reported as brevetoxin producers [4,16], so accurate identification of cells and toxins is extremely important in the assessment of risk. In areas where toxin profiles of *Karenia* have been established, intrageneric, and, or, intraspecific molecular probes (e.g., [15]) may provide a useful companion technique for unambiguous identification, regardless of morphology. Molecular identification was attempted in isolated cells from preserved samples during this study, but samples were unfortunately too degraded to amplify. Additional studies using advanced methods are ongoing to sequence and clone these spherical cells. Interestingly, some raphidophytes (*Chattonella antiqua*, *Chattonella marina*, *Heterosigma akashiwo*, and *Fibrocapsa japonica*) also produce brevetoxin-like compounds [17,18], however none of these species were detected during this study. Since only dinoflagellates were present and only *Karenia* spp. have been shown to produce PbTXs to date, confirmation of brevetoxins in field collected samples (as was conducted in this study) is also an important piece of confirmatory evidence for identification. Some unarmored dinoflagellates in order Brachidinales have been studied because of possible phylogenetic connection to *Karenia* but have not been shown to produce PbTXs [18]. Two genera in this order (*Brachidinium* and *Asterodinium*) have been reported to have high morphological versatility with projected body extensions hypothesized as an adaptation to environmental conditions [18]. Together with our observations, these studies support our hypothesis of morphological plasticity as a response to adverse conditions in some dinoflagellate species.

Morphological plasticity of *K. brevis* could make species identification in field samples considerably more difficult. While taxonomists may be able to correctly identify sphere-shaped cells as *K. brevis* by microscopy (particularly when coupled with specific brevetoxin analyses), many monitoring programs are time-intensive and thereby enhanced by junior scientists, volunteers, and citizen scientists who may not be aware of these possible modifications or have toxin detection methods readily available. With no clear records of these potential morphological changes currently in the literature, this could be easily overlooked. However, if the dinoflagellate balls described in this study are in fact a morph of *K. brevis*, this may result in a significant underestimation of bloom density. While many factors are involved in brevetoxin bioaccumulation in shellfish (e.g., time, temperature, salinity, flow, feeding rate), *K. brevis* density above 5000 cells per liter of seawater has been adopted as the current closure threshold for all U.S. waters. At this very low cell density and with only a small subsample for microscopic evaluation, a potential underestimate of *K. brevis* cells could cause delays in precautionary harvest closures.

Author Contributions: L.N. performed taxonomic identifications, data analyses, data interpretation, and figure preparations. A.R. designed the sampling plan and collected and prepared samples, conducted brevetoxin analyses, was involved in data interpretation, and provided funding and resources for completion of this work. This article was written as equal partnership.

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