

CHARACTERIZATION OF *DINOPHYSIS* SPP. (DINOPHYCEAE, DINOPHYSALES) FROM THE MID-ATLANTIC REGION OF THE UNITED STATES¹

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Due to the increasing prevalence of *Dinophysis* spp. and their toxins on every US coast in recent years, the need to identify and monitor for problematic *Dinophysis* populations has become apparent. Here, we present morphological analyses, using light and scanning electron microscopy, and rDNA sequence analysis, using a ~2-kb sequence of ribosomal ITS1, 5.8S, ITS2, and LSU DNA, of *Dinophysis* collected in mid-Atlantic estuarine and coastal waters from Virginia to New Jersey to better characterize local populations. In addition, we analyzed for diarrhetic shellfish poisoning (DSP) toxins in water and shellfish samples collected during blooms using liquid-chromatography tandem mass spectrometry and an in vitro protein phosphatase inhibition assay

and compared this data to a toxin profile generated from a mid-Atlantic *Dinophysis* culture. Three distinct morphospecies were documented in mid-Atlantic surface waters: *D. acuminata*, *D. norvegica*, and a “small *Dinophysis* sp.” that was morphologically distinct based on multivariate analysis of morphometric data but was genetically consistent with *D. acuminata*. While mid-Atlantic *D. acuminata* could not be distinguished from the other species in the *D. acuminata*-complex (*D. ovum* from the Gulf of Mexico and *D. sacculus* from the western Mediterranean Sea) using the molecular markers chosen, it could be distinguished based on morphometrics. Okadaic acid, dinophysistoxin 1, and pectenotoxin 2 were found in filtered water and shellfish samples during *Dinophysis* blooms in the mid-Atlantic region, as well as in a locally isolated *D. acuminata* culture. However, DSP toxins exceeded regulatory guidance concentrations only a few times during the study period and only in noncommercial shellfish samples.

Key index words: diarrhetic shellfish poisoning; dinoflagellate; *Dinophysis*; dinophysistoxin;

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environmental clones; harmful algal bloom; okadaic acid; phylogeny

Abbreviations: ACL, anterior cingular list; dNTP, deoxyribonucleotide triphosphates; DSP, diarrhetic shellfish poisoning; DTX, dinophysistoxin; GTR, general time reversible; HAB, harmful algal bloom; LC-MS/MS, liquid-chromatography tandem mass spectrometry; LSL, left sulcal list; NCBI, National Center for Biotechnology Information; OA, okadaic acid; PCL, posterior cingular list; PTX, pectenotoxin; REGWF, Ryan-Einot-Gabriel-Welsh F test; UPGMA, unweighted pair group method and arithmetic mean

Approximately one dozen species within the dinoflagellate genus *Dinophysis* are capable of producing diarrhetic shellfish poisoning (DSP) toxins (okadaic acid [OA], dinophysistoxins [DTX1 and DTX2], and their derivatives) with at least 10 of these species documented to reach densities sufficient to present threats to both human health and the shellfish harvesting and aquaculture industries (Reguera et al. 2011, 2014). *Dinophysis* species and their toxins have been extensively studied in Europe and Japan where they have been recognized as problematic since the 1970s (Yasumoto et al. 1978). Only since 2008 have *Dinophysis* blooms in the United States been recognized as harmful due to the presence of DSP toxins in shellfish in excess of the 0.16 ppm OA eq. regulatory guidance concentration above which shellfish are not permitted to be harvested (NSSP 2017). In that year, OA detected in eastern oysters (*Crassostrea virginica*) along the Texas coast of the Gulf of Mexico resulted in the first confirmed shellfish harvesting closure in the United States due to DSP toxins (Deeds et al. 2010) with the causative organism identified initially as *Dinophysis* cf. *ovum* (Campbell et al. 2010). Subsequently, OA and dinophysistoxin 1 (DTX1) were reported in shellfish concurrent with blooms of *D. acuminata* in Long Island Sound, New York (Hattenrath-Lehmann et al. 2013), and Puget Sound, Washington (Lloyd et al. 2013, Trainer et al. 2013). More recently, blooms of *D. acuminata* and *D. norvegica* have been implicated in DSP-related shellfish harvesting closures along the Massachusetts and Maine coasts, respectively (M. Brosnahan, J. Deeds, unpub. data).

Locally, multiple species of *Dinophysis* have been reported in the mid-Atlantic and Chesapeake Bay regions since the 1960s (Table S1 in the Supporting Information). However, these various reports detailing which species of *Dinophysis* are present and/or predominant are complicated by the morphological variation between many small *Dinophysis* species (<50 μm) and historic identifications being made solely based on cell morphology, generally using light microscopy. Over the past two decades, there has been increasing awareness that the morphological

variation among these small *Dinophysis* species is much greater than what was indicated when the species were first described. Accordingly, a majority of the historic species identifications must be considered tentative rather than definitive. Furthermore, none of these previous reports included the measurement of DSP toxins, which were not identified until the early 1980s (Murata et al. 1982). In a series of cruises conducted in offshore waters along the eastern coast of the United States between 1964 and 1974, Marshall (1969, 1976, 1978) documented 17 different *Dinophysis* species. In 1978, Marshall and Cohn (1981) conducted a survey along the northeastern seaboard and reported six different *Dinophysis* species along the Delaware and New Jersey coastlines: *D. acuminata*, *D. caudata*, *D. fortii*, *D. norvegica*, *D. ovum* and *D. tripos*, with *D. fortii* occurring most frequently and in the highest concentration (512 cells \cdot L⁻¹). In a separate series of surveys conducted between 1977 and 1980 at the mouth of the Chesapeake Bay, within the Chesapeake Bay plume, and adjacent Atlantic waters, 13 different species of *Dinophysis* were reported, with *D. fortii* and *D. ovum* occurring most frequently (Marshall 1980, 1982, Marshall et al. 1981). The Chesapeake Bay Program established routine phytoplankton monitoring in Maryland (1984–2010) and Virginia (1985–present) with samples collected at fixed stations throughout Chesapeake Bay and its major tributaries on a monthly or twice monthly basis (see Lacouture et al. 2006 for a program synopsis and <https://www.chesapeakebay.net/what/data> for database attributes). Phytoplankton data collected in this region between 1984 and 2004 indicated the presence of 16 different *Dinophysis* species but the authors noted that occurrences of species capable of producing toxins were rare (Marshall 1996, Marshall et al. 2005, 2009).

Dinophysis acuminata was first reported within the Chesapeake Bay during a cruise conducted in the spring of 1978 (Marshall 1980). While this species was recorded in offshore waters of the mid-Atlantic on multiple occasions prior to this, it was not recorded routinely within the Chesapeake Bay until the mid-1980s (Marshall 1984, 1985, Marshall and Egerton 2009). Between 1999 and 2016, *D. acuminata* has been recorded yearly throughout the Virginia portion of the Chesapeake Bay, occurring in 13% of 2,756 samples at an average concentration of 403 cells \cdot L⁻¹ (CBP 2019). The first *Dinophysis* bloom reported during this 40-year period occurred in 2002 when Marshall et al. (2004) documented a large bloom (peak cell concentration of 236,000 cells \cdot L⁻¹), reported as *D. acuminata* with various morphologies, in the Potomac and Rappahannock rivers and their subestuaries. This bloom led to a precautionary shellfish harvesting closure, but only trace concentrations of OA were detected (Tango et al. 2004).

Within the Chesapeake Bay (Marshall et al. 2005, 2009) and the Maryland and Delaware coastal bays (P. Tango, W. Butler, E. Whereat, unpub. data),

most small- to medium-sized ovoid *Dinophysis* morphospecies were reported as *D. acuminata* based on light microscopy observations. The identification of *D. acuminata* using light microscopy has been problematic due to morphologic overlap with several other species, *D. ovum* and *D. sacculus* in particular. Thus, Lassus and Bardouil (1991) categorized these species as the “*Dinophysis acuminata*-complex.” As initial work in this study, ribosomal DNA (rDNA) sequence analyses of individual cells from two different *Dinophysis* blooms in the Maryland Coastal Bays in 2012 indicated a match with publicly available sequences for both *D. acuminata* and *D. sacculus*, the latter being a species known to bloom mainly along the European Atlantic, Mediterranean, and Adriatic coasts (Zingone et al. 1998, Ninčević-Gladan et al. 2008, Kacem et al. 2009) and not in the mid-Atlantic region (although it was noted in low abundance, along with *D. ovum*, in historical reports). Further, the *Dinophysis* sequences from these 2012 bloom samples were found to be genetically distinct from sequences of *D. cf. acuminata* collected offshore of the Chesapeake Bay, along the Virginia-North Carolina border in 2007 (Handy et al. 2009).

The increasing presence of potentially toxigenic *Dinophysis* species throughout mid-Atlantic waters highlights the need to better characterize the species diversity of local populations and to assess the prevalence of DSP toxins. Here, we present side-by-side morphological analyses, using both light and scanning electron microscopy, and a rDNA sequence analysis, using a ~2-kb sequence of ribosomal ITS1, 5.8S, ITS2, and LSU DNA, on *Dinophysis* morphospecies collected in mid-Atlantic estuarine and coastal waters from Virginia to New Jersey between 2013 and 2016. For comparative purposes, we have included data for *D. acuminata* and *D. norvegica* from Nova Scotia, Canada, *D. sacculus* from the western Mediterranean Sea, and *D. ovum* from the Texas coast of the Gulf of Mexico. In addition, we analyzed for the presence and composition of DSP toxins in water and shellfish samples collected between 2010 and 2016 during regional blooms of *Dinophysis* and compared findings to the toxin profile of a mid-Atlantic isolate of *D. acuminata*.

MATERIALS AND METHODS

Plankton samples.

Sample collection: Water samples collected between 2013 and 2016 throughout the mid-Atlantic region were used for this study (Fig. 1; Table S2 in the Supporting Information). In the Maryland portion of the Chesapeake Bay, the Maryland Coastal Bays, and offshore waters, whole water surface (0.5–1 m) grab phytoplankton samples (1 L) were collected and fixed with unacidified Lugol’s iodine (#LC156725, LabChem Inc., Zelienople, PA, USA) during routine sampling by the Maryland Department of Natural Resources (MD DNR) and Maryland Department of Environment (MDE). Additional Lugol’s preserved phytoplankton samples from *Dinophysis* bloom events or from areas of interest were collected by Old Dominion University (ODU), the Virginia Department of Health (VDH), the

National Research Council Canada (NRCC), the Citizen Monitoring Program of the University of Delaware (UD), New Jersey Department of Environmental Protection (NJ DEP), Virginia Institute of Marine Science (VIMS), University of Maryland (UMD), and Texas A & M University (TAMU).

Field sample examination: Lugol’s preserved samples were gently inverted and a 3-mL aliquot was withdrawn and settled for 1 h in a Lab-Tek Chamber Slide™ (#155379, Nalge Nunc International, Rochester, NY, USA). The entire chamber was scanned at 100× magnification using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Thornwood, NY, USA) to enumerate *Dinophysis* spp. Species identifications were made by examining cell features at 400× magnification. Samples with *Dinophysis* concentrations of ≥ 1000 cells · L⁻¹ were considered for further morphologic analysis.

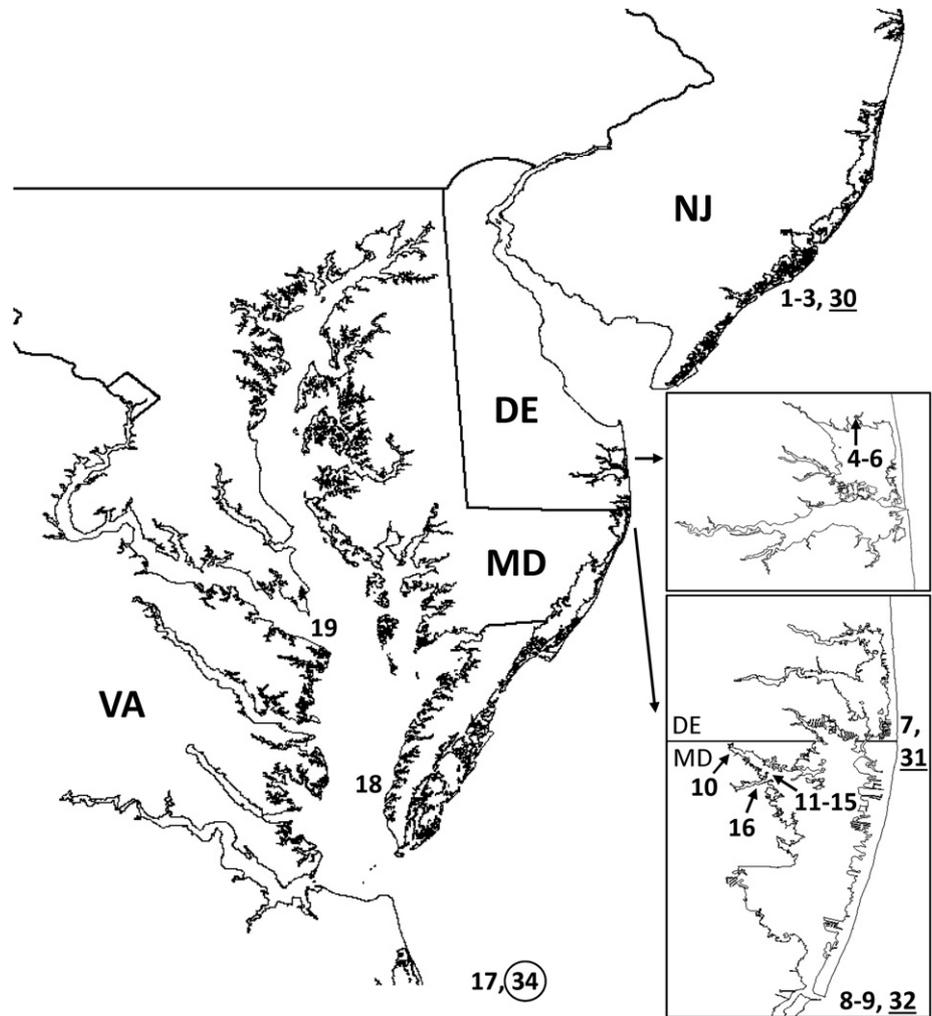
Morphologic analysis.

Light microscopy: From Lugol’s preserved field samples, one *Dinophysis acuminata* clonal isolate from Delaware, USA, and one *D. ovum* clonal isolate from Texas, USA (see details below), cells were visualized through a Zeiss Axiovert 200 inverted microscope and individually picked using drawn glass tubing and mouth aspiration. Each cell was washed two to six times with deionized water, photographed and measured using an Olympus DP73 digital camera system (Olympus America, Center Valley, PA, USA), placed into a sterile 1.5-mL microfuge tube containing approximately 40 μ L of deionized water, and frozen at -20°C for rDNA sequence analysis. A minimum of 20 cells from each geographic location was photographed and measured, with a minimum of 10 cells from each geographic location isolated for rDNA sequence analysis. Cell measurements followed the morphometric parameters described in Tong et al. (2015), which included cell body length, cell body depth, left sulcal list length (LSL), anterior circular list width (ACL), posterior circular list width (PCL), and total length to depth ratio (L:D). Morphometric data were analyzed using a univariate ANOVA to identify significant differences between *Dinophysis* morphospecies with post hoc REGWF multiple comparison tests at an Alpha of 0.05 using IBM SPSS v. 20.

Scanning electron microscopy: Field samples with sufficient *Dinophysis* cells were prepared for scanning electron microscopy by gravity filtering Lugol’s preserved material (added dropwise onto a 0.45- μ m polycarbonate membrane until material was visible) in a filtering apparatus as described in Truby (1997). The Lugol’s solution was rinsed out with three to six washes of deionized water. Cleaned cells were dehydrated in an ethanol dehydration series of three rinses each of 30%, 50%, 70%, and 100% EtOH. Filters were then mounted with carbon tape to aluminum stubs, air-dried, sputter-coated with ionized gold, and viewed and photographed on a LEO 435VP scanning electron microscope (LEO Electron Microscopy, Cambridge, UK) operated at 15 kV. For samples in which there was not a sufficient number of *Dinophysis* cells or there were a greater number of cells of species not of interest, *Dinophysis* cells were collected with a drawn glass pipette and mouth aspiration. These cells were placed into 15-mL centrifuge tubes with deionized water and then each 15-mL sample was processed as above after gravity filtering onto a polycarbonate membrane.

Molecular analysis. Extraction and PCR: Individual cells frozen in microfuge tubes were thawed and sonicated using a probe-tipped sonicator (Branson Sonifier 150; Branson Ultrasonics Corp, Danbury, CT, USA) set to a power level of 5. The sonicator probe was immersed in the sample, and three to five pulses of sonication were used over 5 s. The probe was washed between each sample with Eliminase (Decon Laboratories; King of Prussia, PA, USA) or a 1:10 bleach solution, rinsed with deionized water, and wiped dry. Samples without cells were sonicated and used as negative controls.

FIG. 1. Mid-Atlantic collection sites for *Dinophysis* samples analyzed both morphologically and genetically (corresponding to Figs. 4–6). Inset panels: Top – Northern Delaware Inland Bays, Bottom – Southern Delaware Inland Bays and northern Maryland Coastal Bays. Nonunderlined numbers indicate *D. acuminata* morphospecies (either regular alone or regular and small cell forms) were identified at these locations. Underlined numbers indicate *D. norvegica* was identified at these locations. Circled number indicates collection site of *Dinophysis* sp., previously identified as *D. cf. acuminata* in Handy et al. (2009). See Table S2 for collection site GPS coordinates.



Sonicated single cells had enough material for three separate polymerase chain reactions (PCRs) targeting a large portion of the ribosomal gene (~2 kb of the ITS1, 5.8S, ITS2, and LSU). These regions had been shown previously to be phylogenetically informative for the Dinophysiales group (Handy et al. 2009). From isolated single-cell samples, rDNA regions were amplified using three pairs of primers: Dino1662F/25R (Yamaguchi and Horiguchi 2005, Handy et al. 2008), 25F/LSUR2 (Kogame et al. 1999, Takano and Horiguchi 2006), and OrnITSF/OrnLSUR1 (Handy et al. 2009). A full list of both PCR and sequencing primers can be found in Table S3 in the Supporting Information. PCRs were run in 20 μ L volumes containing the following: 7.14 μ L of 10% trehalose, 2.23 μ L of molecular-grade water, 1.43 μ L of 10 \times PCR reaction buffer (Invitrogen, Carlsbad, CA, USA), 0.714 μ L of 50 mM MgCl₂, 0.143 μ L of each primer (10 μ M), 0.071 μ L of 10 mM deoxyribonucleotide triphosphates (dNTPs; New England BioLabs, Ipswich, MA, USA), and 0.07 μ L of Platinum Taq (Invitrogen), with 8 μ L of sonicated sample.

Cycling conditions were 95°C for 2 min, followed by 40 cycles of the following: 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. This was followed with a 72°C step for 5 min, after which the reactions were held at 4°C. To verify amplification, products were analyzed using precast 1.2% E-gel agarose gels according to the manufacturer's protocols with the E-Base Integrated power supply (Invitrogen). Gels were run for 15 min at 60–70 V (constant voltage) and then

visualized using a Gel Doc 2000 gel documentation system (Bio-Rad, Hercules, CA, USA). Successfully amplified products were purified by adding 2 μ L of Exosap-IT to 5 μ L of PCR product and incubating at 37°C for 15 min, followed by 15 min at 80°C.

Sequencing. Each cleaned PCR product was sequenced using 0.25 μ L of BigDye Terminator v. 3.1 (Applied Biosystems, Foster City, CA, USA), 1.875 μ L of 5 \times sequencing buffer, 5 μ L of 10% trehalose, 1 μ L of 10 μ M primer, and 0.875 μ L of molecular-grade water, for a total of 9 μ L, to which 1 μ L of purified PCR product was added. Sequencing products were purified as described by Handy et al. (2011) using a Performa DTR V3 96-well short plate (#89939; Edge Bio, Gaithersburg, MD, USA) and sequenced at least bidirectionally on an ABI 3500 instrument (Applied Biosystems). Specifically, amplicons from Dino1662F/25R1 were sequenced with the primers Dino1662F, 25R1, 25F; amplicons from 25F/LSUR2 with primers 25F, LSUR2, and D3A (Takano and Horiguchi 2006); and amplicons from OrnITSF/OrnLSUR1 with primers OrnITSF/OrnLSUR1.

Dinophysis ovum sequencing. Initial attempts to sequence preserved cells of *Dinophysis ovum* collected during the 2008 bloom in Port Aransas, Texas, following the procedure described above failed. As a substitute, a sequence for *D. ovum* processed previously at TAMU from samples collected during the 2008 bloom event was used. Bloom samples processed at TAMU were fixed with acidified Lugol's (10 g of I₂,

20 g of KI, 20 mL glacial acetic acid, 200 mL deionized water) and stored at 4°C until analysis (within 6 months). Samples were destained with sodium thiosulfate, and then, individual cells were isolated as described above. Direct PCR amplification was performed ($n = 18$) following the method of Henrichs et al. (2008). Primers amplifying the ITS region (ITS1, ITS4; White et al. 1990) were used in the PCR (50 μ L reaction volume). PCR products were visualized on a 2% agarose gel and positive bands were extracted for purification. Purified PCR products were sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems), and the resulting sequences were edited using Sequencher v. 4.2 (Genecodes, Ann Arbor, MI, USA). An additional *D. ovum* sequence was generated from a single-cell picked from a new clonal culture (DoSS 3195) established at TAMU from a March 2019 bloom at Freeport, Texas. The culture was isolated and maintained as described below for the mid-Atlantic *D. acuminata* culture and sequenced as described above for all other single-cell samples used in this study.

Postsequence processing. Sequence data were processed in GeneiousPro v. 10.2.3 (Drummond et al. 2010). The sequences were assembled and manually trimmed for quality, assembled into contigs (default settings), manually edited, and then converted to consensus sequences. Consensus sequences were generated for each sample (single cell) in this study. For manual editing, traces were examined to be confident that the base calls were correct from both the sequencer and GeneiousPro analyses. In some cases, if sequence quality was poor (e.g., at the end of a sequence read) for one sequence but of better quality for the other, bases were changed to reflect the higher quality sequence. For this analysis, all of the sequences were within the same genus, and therefore, hypervariable regions were not excluded as all sequences could be aligned with few gaps. General sequence identity was examined using BLAST to search the National Center for Biotechnology Information (NCBI) nonredundant database (Altschul et al. 1990), using nucleotide BLAST with the nr/nt database and blastn.

For species identification, an alignment was constructed first using the “muscle alignment” function in GeneiousPro (Edgar 2004). It included the successfully sequenced cells from this study, seven Dinophysiacean sequences published in Handy et al. (2009) from offshore of the Chesapeake Bay, including *Dinophysis* sp., *D. caudata*, *D. cf. acuminata*, and *Phalacroma rapa*, used as an outgroup. Ten other representative sequences including *D. acuminata*, *D. norvegica*, and *D. acuta* were obtained from GenBank. The alignment was trimmed based on the *P. rapa* sequence to include only the ITS1, 5.8S, ITS2, and LSU portions of the ribosomal operon. This alignment (including some gaps) was 1,976 bases in length with sequences ranging from 674 to 1,924 bases. Note: In the case of the *D. ovum* ITS sequence generated previously at TAMU and several of the additional sequences obtained from GenBank for other *Dinophysis* species, sequences were shorter than most of those generated here and in Handy et al. (2009) (i.e., *D. ovum* from TAMU 680 bp, *D. norvegica* 910 bp, and *D. sacculus/D. acuminata* ~1,300 bp). While these sequences did not cover the full ITS1-LSU section of the ribosomal genes, they were still found to be useful for differentiating between most *Dinophysis* species (detailed further in Results), so they were included in the analysis. In contrast, another *Dinophysis* species, *D. fortii* (accession AB355145), was compared with sequences generated here, but was not included in the phylogenetic analyses because of its short length (590 bp) and position in the ITS region.

Phylogenetic-based species identification: The program MrModeltest v. 2 (Nylander 2004) was used to determine that the GTR model (general time reversible with no rate variation) was the best fit model for this alignment through

GeneiousPro (Drummond et al. 2010). The alignment was analyzed three ways using *Phalacroma rapa* (accession EU780655, Handy et al. 2009) as an outgroup. These included an unweighted pair group method and arithmetic mean (UPGMA) consensus tree (1,000 replicates) with a Jukes-Cantor genetic distance model (Jukes and Cantor 1969), a maximum-likelihood analysis using PhyML (100 bootstraps, GTR model, fixed proportion of invariable sites set to 0, number of substitution rate categories set to 1, according to Guindon et al. 2010), and a Bayesian inference using MrBayes 3.2.6 (GTR model, equal rate variation, 1,100,000 chain length, subsampling frequency of 200, 4 heated chains, a burn-in length of 100,000, a heated chain temperature of 0.2, a random seed of 29,701 and unconstrained branch lengths, according to Huelsenbeck and Ronquist 2001).

Toxin analysis. To assess the presence and/or relative composition of the DSP toxins OA, dinophysistoxin 1 (DTX1), and dinophysistoxin 2 (DTX2) in mid-Atlantic shellfish and water, various shellfish and water samples collected by the MD DNR, MDE, NJ DEP, VDH, UD, the Delaware Department of Natural Resources and Environmental Conservation (DNREC), and the University of Maryland Eastern Shore (UMES) between 2010 and 2016 were analyzed at FDA using liquid-chromatography tandem mass spectrometry (LC-MS/MS) and/or a commercial *in vitro* bioassay for DSP-like activity based on protein phosphatase inhibition. The LC-MS/MS testing was performed using an Acquity ultra-performance liquid chromatography system (Waters Corporation, Manchester, UK) coupled to a Sciex QTrap 5500 mass spectrometer equipped with a Turbo V ionization source (SCIEX, Framingham, MA, USA). All other method details followed the protocol “LC-MS/MS Method for the Detection of DSP toxins in Shellfish” that was recently adopted by the Interstate Shellfish Sanitation Conference (ISSC) for use in the US National Shellfish Sanitation Program (NSSP) (available at: <http://www.issc.org/Data/Sites/1/media/00-2017biennia/Meeting/-taskforcei2017/17-103-supporting-documentation.pdf>; accessed April 1, 2019). *In vitro* protein phosphatase inhibition assays (#PN520025, Abraxis, Warminster, PA, USA) were performed according to the manufacturer’s instructions with the exception that 2 g of shellfish homogenate was extracted in 20 mL of MeOH, performed according to the LC-MS/MS protocol, thereby harmonizing the extraction protocols between the two methods. For all shellfish, samples were typically shucked fresh and sent to FDA frozen, and, unless otherwise noted, 10–12 individuals for each species were composited by homogenization. All shellfish samples were subjected to alkaline hydrolysis to measure total DSP toxins (free+esterified), as is required for regulatory shellfish monitoring in the United States (US FDA 2011). For water samples, 250–1000 mL of bloom water was passed through a 47-mm type GF/F filter (Whatman, Sigma-Aldrich, St. Louis, MO, USA) under mild vacuum, immediately frozen, and sent to the FDA for analysis. For analysis, filters were sonicated thoroughly (i.e., until the filters were completely broken down to loose glass fibers) in 5 mL of MeOH, on ice, using a model W-225R Cell Disruptor (Heat Systems – Ultrasonics, Inc., Plainview, NY, USA) equipped with a micro-tip and set to the maximum with a 50% duty cycle. After centrifugation and decanting, the glass pulp was extracted a second time with an additional 5 mL of MeOH. Due to the low concentrations of toxins typically found in these samples, filtered water samples were only tested by LC-MS/MS.

Characterization of a laboratory culture of mid-Atlantic Dinophysis. **Isolation and culture maintenance.** A clonal isolate of *Dinophysis acuminata* (DADE01) was established in culture at VIMS in May 2015 from surface water collected from Torquay Canal, Delaware (Fig. 1; Table S2), following the single-cell

isolation methods described by Tong et al. (2011). At the time of water collection, the salinity was 24.8, water temperature was 23.6°C, and the abundance of *D. acuminata* was 142,000 cells · L⁻¹ as determined by the UD Citizen Monitoring Program. During isolation and maintenance of the culture, *D. acuminata* were fed *Mesodinium rubrum* which had been previously raised on *Tealeulax amphioxiea* isolated from Japan (Nishitani et al. 2008) following the protocols of Park et al. (2006) as modified by Hackett et al. (2009). The dinoflagellate, ciliate, and cryptophyte were grown in modified f/6-Si medium (Anderson et al. 1994) at 15°C in dim light (65 μmol photons · m⁻² · s⁻¹) under a 14:10 h light-dark photocycle.

To assess the morphology and toxigenicity of the DADE01 culture, cells were inoculated into fresh medium at 200 cells · mL⁻¹, fed *Mesodinium rubrum* at a 1:10 ratio of predator to prey, and monitored every 3 d for the complete consumption of *M. rubrum* by examining and enumerating 1-mL subsamples in a Sedgewick-Rafter counting cell at 100× using an Olympus CX31 (Olympus America) light microscope. Three days after all ciliate prey were consumed (i.e., during late exponential growth of the dinoflagellate), the culture was harvested for morphometric and molecular analyses (15 mL of culture preserved with unacidified Lugol's solution [#87-2795, Carolina Biological Supply Co., Burlington, NC, USA]) and toxin analysis (120,000 cells in 24 mL of culture). The former was shipped to MD DNR for microscopic analysis and the latter remained at VIMS for in-house toxin analysis.

Toxin analysis of the DADE01 culture: The 24 mL of culture, consisting of both cells and medium, was bath sonicated (Fisher Ultrasonic Cleaner, Model FS30H) at room temperature for 15 min and loaded onto an Oasis HLB 60 mg cartridge (Waters Corporation, Millford, MA, USA) that was previously equilibrated with 3 mL of MeOH and 3 mL of Milli-Q water. The cartridge was then washed with 6 mL of Milli-Q water, blown dry, and eluted with 1 mL of 100% MeOH into a glass 1.5-mL high recovery LC vial and stored at -20°C until analyzed. Just prior to analysis by LC-MS/MS following the methods of Smith et al. (2018), a portion of the sample underwent alkaline hydrolysis to measure total DSP toxins (free+esterified according to the ISSC shellfish LC-MS/MS method as above). Both extracts were analyzed using a Dionex UltiMate 3000 liquid chromatography system (Thermo Scientific Dionex, Waltham, MA, USA) coupled with an AB 4000 mass spectrometer with electrospray ionization (SCIEX).

RESULTS

Morphological analysis. In all, 322 individually picked *Dinophysis* cells were examined using the six metrics outlined in Tong et al. (2015). From all samples analyzed, five primary morphospecies were identified, measured, and photographed from 24 preserved water samples, a clonal culture of *D. acuminata* (DADE01) established from Torquay Canal, Delaware, and a clonal culture of *D. ovum* (DoSS 3195) established from Freeport, Texas. Multiple cells representing each morphospecies were isolated for single-cell rDNA sequence analysis. These cells included individuals exhibiting morphological variations noted within descriptions for each morphospecies (i.e., cells with and without antapical protrusions). All cells identified morphologically as *D. sacculus* were from the Mediterranean Sea, from samples collected at Port Andratx, Mallorca, Spain

(September 2009) or Port de Villefranche, France (April 2010). All cells identified morphologically as *D. ovum* were either from a field sample collected in the Gulf of Mexico near Port Aransas, Texas in February 2008 or from a culture established from Freeport, Texas in March 2019. Samples collected from February through August during 2013 to 2016 from mid-Atlantic waters had one or more of three distinct morphospecies present. These morphospecies were identified as *D. acuminata*, *D. norvegica*, and "small *Dinophysis* sp." These same three morphospecies were also observed in samples from West Jeddore, Nova Scotia, Canada. Table 1 provides the range and average measurement of the six metrics used to evaluate the *Dinophysis acuminata*-complex organisms examined from field material in this study (the three morphospecies observed in the mid-Atlantic region, *D. ovum* from the Gulf of Mexico, USA, and *D. sacculus* from the western Mediterranean Sea). When the six metrics were considered together, they provided evidence that these five morphospecies could be separated from each other using light and electron microscopy.

The morphospecies identified as *Dinophysis acuminata* (Fig. 2a, Fig. S1, a and b in the Supporting Information) from the mid-Atlantic and Nova Scotian regions, including culture DADE01 from Torquay Canal, Delaware, was comprised of cells with a straight to convex dorsal surface. The posterior end was rounded with some degree of tapering of the hypotheca beginning at the third sulcal rib. From field material, we determined that the average cell length was 45.09 μm and depth was 31.15 μm. The L:D ratio was 1.46. Thecal markings ranged from cells with numerous, deep pores showing areolation to cells with markedly few, shallow pores. The sulcal lists of *D. acuminata* were thin and flexuous compared to other *Dinophysis* species in this study. In only a few instances were reticulate markings observed on either the sulcal or cingular lists. Some cells identified as *D. acuminata* presented with one to multiple protrusions at the base of the cell as illustrated by Jørgensen (1899), Dodge (1982), and Zingone et al. (1998).

The morphospecies identified as *Dinophysis norvegica* (Fig. 2b, Fig. S1, c and d) from the mid-Atlantic and Nova Scotian regions was characterized by a robust triangular shaped body. The ventral and dorsal surfaces were convex along the area of the ribs and tapered below the third sulcal rib to a broad point. The maximum cell depth occurred along the middle of the cell, typically in the area of the second and third sulcal ribs. The cells had an average length of 58.00 μm and depth of 43.36 μm. The L:D ratio was 1.34. The theca of *D. norvegica* was heavily rugose and consistently presented with numerous deep, areolated pores. The cingular lists of *D. norvegica* were thin and small relative to the size and shape of the cell, whereas the sulcal lists were larger and proportional to the size of the cell. Sulcal lists were usually

TABLE 1. Range and average measurements for the six morphometrics used to evaluate the morphology of mid-Atlantic *Dinophysis* species and comparative *Dinophysis* species (in bold).

Species	n	Cell length			Cell depth			L:D ratio		
		avg.	min.	max.	avg.	min.	max.	avg.	min.	max.
<i>D. acuminata</i>	156	45.09	37.23	58.92	31.15	21.03	42.48	1.46	1.16	2.02
<i>D. norvegica</i>	59	58.00	46.35	70.51	43.36	32.58	51.49	1.34	1.20	1.78
small <i>D. acuminata</i>	19	37.15	20.17	41.59	25.71	23.68	30.47	1.46	1.35	1.68
<i>D. ovum</i>	33	44.11	37.89	49.14	35.54	28.83	40.21	1.25	1.12	1.57
<i>D. sacculus</i>	35	53.98	49.69	62.04	34.11	28.31	44.18	1.60	1.35	1.83

Species	n	Left sulcal list length			Anterior cingular list width			Posterior cingular list width		
		avg.	min.	max.	avg.	min.	max.	avg.	min.	max.
<i>D. acuminata</i>	156	26.36	16.84	35.66	12.24	7.83	18.35	18.33	13.11	26.24
<i>D. norvegica</i>	59	38.21	30.58	44.04	24.47	17.02	30.70	31.28	22.84	38.99
small <i>D. acuminata</i>	19	21.09	13.67	24.60	10.90	8.21	12.85	16.03	12.00	18.77
<i>D. ovum</i>	33	26.38	22.39	30.76	13.93	10.79	18.77	21.33	15.78	26.75
<i>D. sacculus</i>	35	33.66	24.95	38.77	14.62	12.11	16.63	20.88	17.39	23.63

reticulate. One to numerous protrusions were observed at the antapical end of some cells.

In the mid-Atlantic and Nova Scotian regions, the morphospecies identified as “small *Dinophysis* sp.” had a distinctly tapered antapical region (Fig. 2c, Fig. S1, e, and f). Cells had slightly convex dorsal and ventral surfaces with the maximum width occurring between the second and third sulcal rib. The posterior end of the cell tapered to a broad point. Antapical protrusions were not noted on any cells observed ($n = 19$). The cells had an average length of 37.09 μm and depth of 26.69 μm . The L:D ratio was 1.38. The theca of the “small *Dinophysis* sp.” was

heavily rugose and presented with numerous, deep areolated pores, much in the same fashion as *D. norvegica* (Fig. 2b). The cingular and sulcal lists were thin and not well-developed.

The morphospecies identified as *Dinophysis sacculus* (Fig. 2d, Fig. S2, a and b in the Supporting Information) from the western Mediterranean Sea was the largest of the cells in the *D. acuminata*-complex. Zingone et al. (1998) described *D. sacculus* as having an elongate shape more representative of a rectangle compared to the ovoid shapes of *D. acuminata* and *D. ovum*. In cells observed during this study, the ventral surface was slightly to markedly convex and the

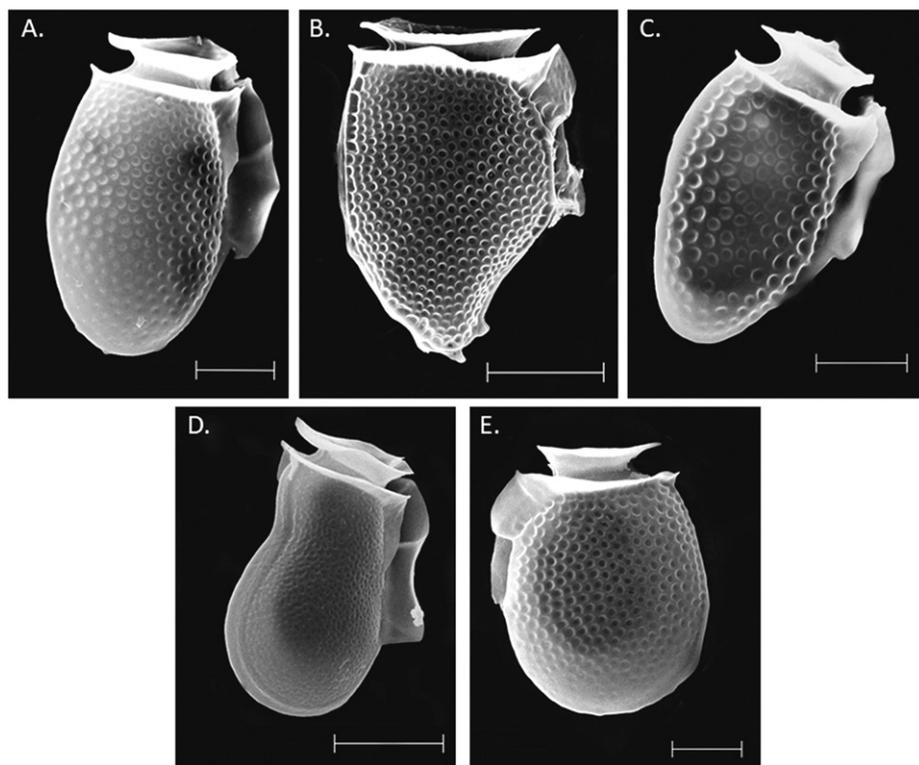


FIG. 2. Scanning electron micrographs (SEM) of *Dinophysis* morphospecies. (a) *D. acuminata* from the St. Martin River, MD, scale bar = 20 μm ; (b) *D. norvegica* from offshore of the DE/MD border, scale bar = 10 μm ; (c) small cell form of *D. acuminata* from offshore Ocean City Inlet, MD, scale bar = 20 μm ; (d) *D. sacculus* from the Mediterranean Sea at Port Andratx, Mallorca, Spain, scale bar = 20 μm ; (e) *D. ovum* from the Gulf of Mexico at Port Aransas, TX, scale bar = 10 μm .

dorsal surface was slightly to markedly concave, particularly in the region adjacent to the second sulcal rib. The hypotheca of *D. sacculus* did not taper and terminated with a rounded shape in line with the cell or slightly arced giving a kidney bean appearance. The cells had an average length of 53.98 μm and depth of 34.11 μm . The L:D ratio was 1.60. The theca of *D. sacculus* was relatively smooth compared to the other *Dinophysis* species as the pores were shallow depressions. The cingular and sulcal lists of *D. sacculus* were well developed and ornamented with fine reticulate markings. Antapical protrusions were not seen in any specimens examined ($n = 35$).

The morphospecies identified as *Dinophysis ovum* (Fig. 2e, Fig. S2, c and d) from the Texas coast of the Gulf of Mexico, as well as culture DoSS 3195 from this same location, had a convex dorsal surface and a more or less convex ventral surface. The posterior end of the cell was round with no tapering, giving the cell a robust oval shape. From the field material, we determined that the average cell length was 44.11 μm and depth was 35.54 μm . The L:D ratio was 1.25. The theca of *D. ovum* was heavily rugose and consistently presented with numerous, deep areolated pores. The sulcal and cingular lists of *D. ovum* were thin and small relative to the size and shape of the cell. The posterior cingular list was particularly under-developed. Reticulate markings were present on the sulcal lists, and few cells showed antapical protrusions.

The ability to use the metrics outlined by Tong et al. (2015) to speciate these *Dinophysis* was consistent with statistically significant differences in morphometric characteristics. Univariate ANOVA on morphometric data, with post hoc REGWF multiple comparisons tests, indicated that species within the *Dinophysis acuminata*-complex could be separated from each other using a series of morphometrics (Fig. 3). Of all the morphospecies considered in this study, *D. norvegica* was significantly larger, with greater LSL length, ACL width, PCL width, cell length, and cell depth. Morphologically, *D. acuminata* was also significantly different from *D. sacculus*, based on all of the metrics measured. The L:D ratio, a measurement that broadly represents hypothecal plate shape, was significantly different between *D. acuminata*, *D. ovum*, *D. sacculus*, and *D. norvegica*.

When focusing on cells identified as *Dinophysis acuminata*, there was a considerable amount of variability in cell morphology, with significant differences in traits between geographic populations. Cells from Nova Scotia, Canada, were significantly larger, with greater LSL length, ACL width, PCL width, and cell length than cells from the mid-Atlantic region (Fig. S3 in the Supporting Information). There was greater within population variability and considerable overlap in morphological features between populations from the mid-Atlantic, without clear morphometric differentiation between New Jersey, Delaware, Maryland, and Virginia populations.

Dinophysis acuminata, *D. norvegica*, and “small *Dinophysis* sp.” observed in mid-Atlantic waters during the study period were not distributed evenly. In offshore surface samples collected from Delaware to Virginia ($n = 20$), *D. acuminata* and “small *Dinophysis* sp.” were observed together in 19 samples, with 13% of the total *Dinophysis* population being the small cell morphotype. Only one sample from this set also contained *D. norvegica*. The single *Dinophysis* bloom that occurred in New Jersey waters during the study period was collected in Barnegat Bay, but occurred during an unusual upwelling event that pushed offshore water into the bay (B. Heddendorf, pers. comm.). This bloom contained all three morphospecies, with 85% *D. acuminata*, 11% “small *Dinophysis* sp.”, and 4% *D. norvegica*. For samples collected in the Maryland Coastal Bays ($n = 39$), the *Dinophysis* population was represented by the *D. acuminata* morphospecies alone, with no small cell morphotypes or *D. norvegica* observed. In a time series of samples collected during a *Dinophysis* bloom in the Delaware Inland Bays in 2015 ($n = 7$), *D. acuminata* only was observed during the first half of the bloom while low amounts (4%–5%) of the small cell morphotype were also observed during bloom peak and decline. For samples collected in the Maryland portion of the Chesapeake Bay ($n = 16$) and in Virginia waters at the mouth of the Chesapeake Bay ($n = 2$), the *Dinophysis* population was only represented by the *D. acuminata* morphospecies. In the comparative sample collected from West Jeddore on the east coast of Nova Scotia, Canada in August 2016, all three morphospecies were observed at a ratio of 44% *D. norvegica*, 40% *D. acuminata*, and 16% “small *Dinophysis* sp.”. It should be noted that all samples analyzed in this study were collected from surface waters. Future studies will focus on the relative abundance of *Dinophysis* spp. at various depths, particularly in offshore waters.

Molecular analysis of mid-Atlantic and related Dinophysis spp. Out of the 322 individual cells observed for the morphologic analysis, 201, representing the various morphospecies, were single-cell isolated, photographed, and used for rDNA sequencing. Of these, 37 single-cell samples (18% of the total) produced usable DNA sequences representing *Dinophysis acuminata*-complex morphospecies from the mid-Atlantic, the western Mediterranean Sea and Nova Scotia, Canada (Figs. 4 and 5; Table S2). Also included were cells from a laboratory culture of *D. acuminata* (DADE01; Fig. 4, cells 21–23) established from the Delaware Inland Bays and cells re-isolated from a water sample collected in 2007 from outside the mouth of the Chesapeake Bay that contained *D. cf. acuminata* as described in Handy et al. (2009; Fig. 5, cell 34). All *D. ovum* morphospecies cells, collected from the 2008 bloom in the Gulf of Mexico near Port Aransas, Texas, and analyzed morphologically during this study, failed to produce usable sequences. As substitutes, a sequence from a *D.*

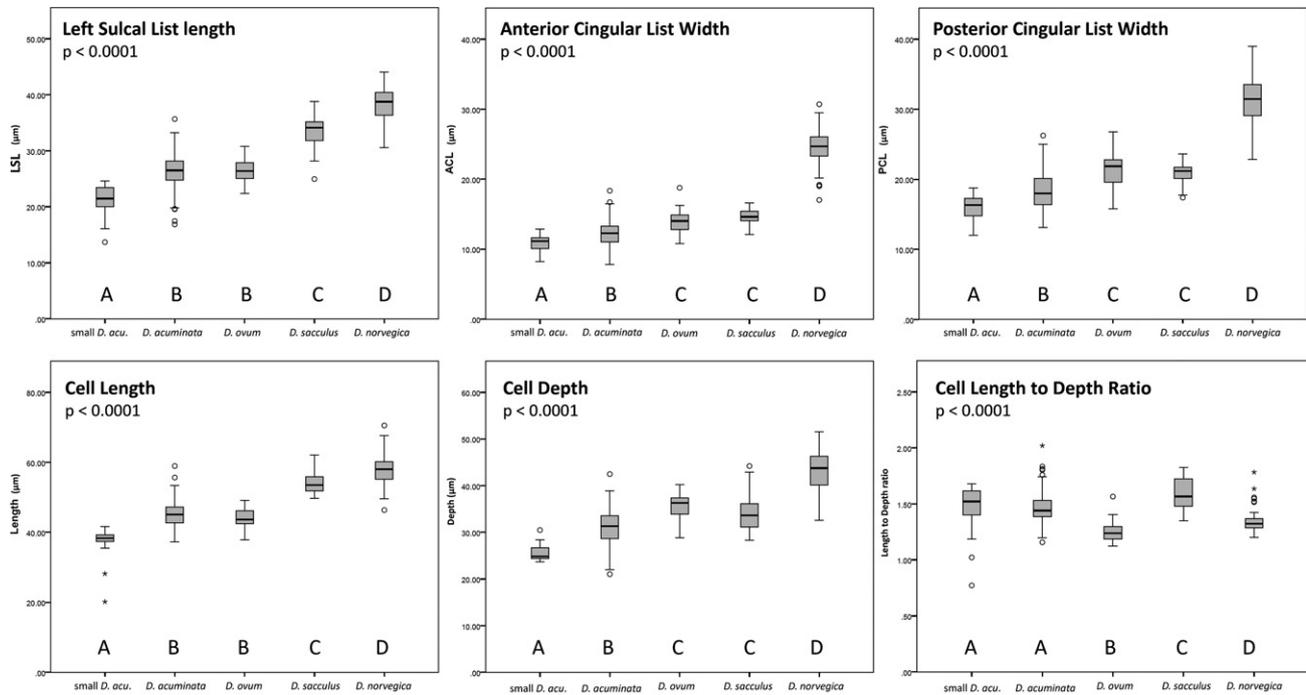


FIG. 3. Box and whisker plots of morphometrics examined in *Dinophysis* spp. Upper and lower limits of the box represent the 1st to 3rd quartiles, with bold horizontal lines indicating the median. Whiskers represent the range of 95% of the data. Circles and asterisks are outliers outside the 95% percentile, with asterisks indicating the value is more than 3x the value within the box. Letters (A–D) represent the results of the post-hoc REGWF multiple comparison test. Groups with the same letter are not significantly different from each other.

ovum cell from a culture established from Freeport, Texas, in 2019 (DoSS 3195; Fig. 5, cell 35) and a sequence generated previously at TAMU using material from the 2008 *D. ovum* bloom (single-cell sample not photographed) were used. Cells from laboratory cultures were used in the molecular analysis only. While these cells were examined microscopically for visual consistency with cells from field material, they were not used in the multivariate analysis due to potential differences in cell morphology that are known to be artifacts of laboratory culturing. The DNA sequences from cells identified morphologically as *D. acuminata*, “small *Dinophysis* sp.”, *D. sacculus* (from the Mediterranean Sea), and *D. ovum* (from the Gulf of Mexico) were identical. When compared in their entirety to sequences available in GenBank via BLAST the closest pairwise matches were to sequences of *D. acuminata* and *D. sacculus*. Sequence data from cells collected during this study that were identified morphologically as *D. norvegica* matched publicly available *D. norvegica* sequences. The *D. norvegica* sequences (Fig. 5, cell 32 as an example) were 99.3% (pairwise) similar to those from *D. acuminata*-complex (Fig. 4, cell 21 as an example), which corresponded to six base differences in the ITS1, zero in the 5.8S, three bases in the ITS2, and three bases in the LSU. The two cells re-isolated from the 2007 offshore Chesapeake Bay sample documented in Handy et al. (2009) that sequenced successfully were

distinct from each other. One matched *D. acuminata*-complex sequences from this study, as well as other publicly available sequences for *D. acuminata* and *D. sacculus* (Fig. 4, cell 17), while the other matched previous sequences for *D. cf. acuminata* (Fig. 5, cell 34).

Using these 38 sequences, along with additional published sequences for *Dinophysis acuminata*, *D. acuta*, *D. caudata*, *D. norvegica*, *D. sacculus*, and *Phalacroma rapa* (as an outgroup), a consensus tree was generated from three different analyses: UPGMA, maximum-likelihood, and Bayesian (Fig. 6). Five clades were recovered corresponding to (1) the *D. acuminata* complex, including the “small *Dinophysis* sp.”, (2) *D. norvegica*, (3) *D. acuta*, (4) a clade containing *Dinophysis* sp. cells (identified as *D. cf. acuminata* in Handy 2009), and (5) *D. caudata*. The support values for this tree were generally consistent between the analyses. Another regionally important *Dinophysis* species, *D. fortii*, was excluded from the analysis because the available sequence (accession AB355145) significantly reduced phylogenetic resolution, likely because of the length (590 bp) and location within the ITS region. When this sequence was compared to *D. acuminata*, *D. ovum*, and *D. sacculus* sequences, there was only a 92% match. The *D. fortii* sequence most closely aligned with clade 4, but was still distinct from the *Dinophysis* sp. within that clade (data not shown). Therefore, using these methods,

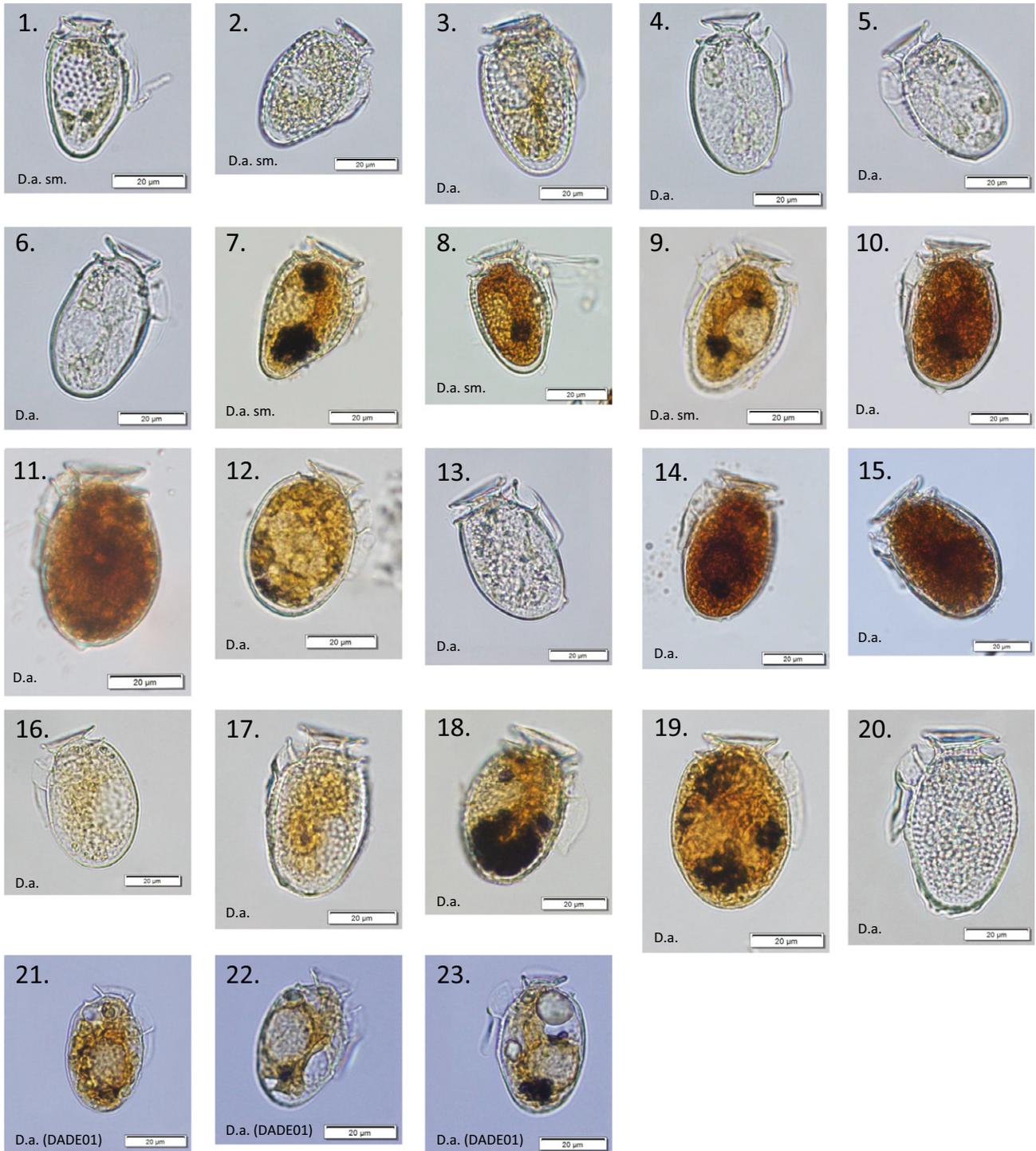


FIG. 4. Light micrographs of single-cell samples of *Dinophysis acuminata* (both regular [D.a.] and small cell forms [D.a. sm.]) corresponding to rDNA data presented in Fig. 6. *First Row.* Cell 1 (small cell form), Cell 2 (small cell form), and Cell 3 – Barnegat Bay, NJ; Cell 4 and Cell 5 – Torquay Canal, DE. *Second Row.* Cell 6, Torquay Canal, DE; Cell 7 (small cell form), offshore DE/MD border; Cell 8 (small cell form) and Cell 9 (small cell form), offshore Ocean City Inlet, MD; Cell 10, Bishopville Prong, MD. *Third Row.* Cell 11, Cell 12, Cell 13, Cell 14 and Cell 15, St. Martin River, MD. *Fourth Row.* Cell 16, Manklin Creek, MD; Cell 17, outside mouth of Chesapeake Bay at VA/NC boarder; Cell 18, Cherrystone, VA; Cell 19, Potomac River, VA; Cell 20 West Jeddore, Nova Scotia, Canada. *Fifth Row.* Cell 21, Cell 22 and Cell 23, *D. acuminata* culture DADE01 isolated from Torquay Canal, DE. Additional collection details are provided in Table S2. [Color figure can be viewed at wileyonlinelibrary.com]

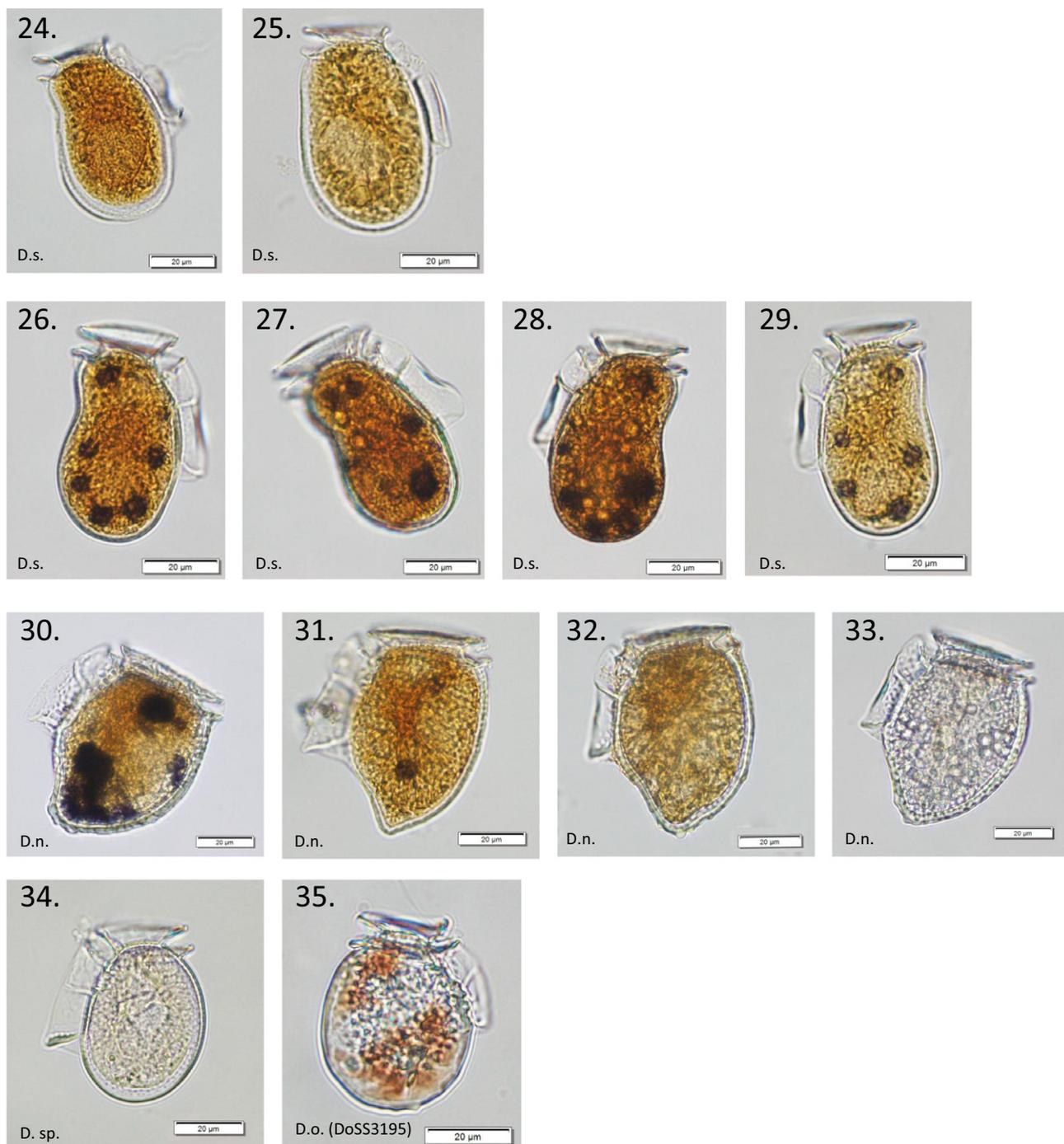


FIG. 5. Light micrographs of single-cell samples of *Dinophysis* sp. (*D. sp.*), *D. norvegica* (*D.n.*), *D. ovum* (*D.o.*), and *D. sacculus* (*D.s.*) corresponding to rDNA data presented in Figure 6. *First Row:* *D. sacculus*; Cell 24 and Cell 25, Port D'arse, France. *Second Row:* *D. sacculus*; Cell 26, Cell 27, Cell 28 and Cell 29, Port d'Andratx, Spain. *Third Row:* *D. norvegica*; Cell 30, Barnegat Bay, NJ; Cell 31, offshore DE/MD border; Cell 32, offshore Ocean City Inlet, MD; Cell 33, West Jeddore, Nova Scotia, Canada. *Fourth Row:* *Dinophysis* sp. (previously *D. cf. acuminata* in Handy et al. 2009); Cell 34, outside mouth of Chesapeake Bay on VA/NC border and *D. ovum*; Cell 35, culture (DoSS3195) isolated from the Freeport, TX coast of the Gulf of Mexico. Additional collection location details are provided in Table S2. [Color figure can be viewed at wileyonlinelibrary.com]

D. norvegica, *D. caudata*, *D. acuta*, and *D. fortii* can be reliably resolved from species of the *D. acuminata*-complex. The *D. acuminata*-complex species (*D. acuminata*, *D. ovum*, and *D. sacculus*) and the

“small *Dinophysis* sp.” analyzed in this study, however, could not be resolved.

Toxin analysis. One of the earliest *Dinophysis* blooms ($12,000 \text{ cells} \cdot \text{L}^{-1}$) examined for toxicity in

Maryland occurred in Manklin Creek in May 2010 (Fig. 1). The LC-MS/MS analysis of a 600 mL filtered water sample detected the presence of OA and DTX1 (at a ratio of 54% OA:46% DTX1) as well as pectenotoxin 2 (PTX2), for the first time in this region. In January 2012, another bloom ($77,000 \text{ cells} \cdot \text{L}^{-1}$) occurred in the Bishopville Prong of the St. Martin River (Fig. 1). A single shellfish sample (three composited specimens of the eastern oyster *C. virginica*) confirmed the presence of OA and DTX1 in MD shellfish for the first time (0.05 ppm OA eq. in shellfish meat at a ratio of 78% OA:22% DTX1). In this sample, PTX2 was also detected at 0.08 ppm. PTXs are not routinely analyzed for in regulatory shellfish samples in the United States as they have not been shown to cause gastroenteritis in humans and are not regulated (US FDA 2011). In April and May 2012, another bloom occurred in the Bishopville Prong of the St. Martin River ($68,000 \text{ cells} \cdot \text{L}^{-1}$). This time, Atlantic ribbed mussels (*Geukensia demissa*), a more abundant but noncommercial species, were collected throughout the bloom period and showed a maximum toxin concentration of 0.20 ppm OA eq. in shellfish meat (range 0.023–0.20 ppm; 69% OA:31% DTX1 $\pm 1\%$; $n = 3$). This is the highest DSP toxin concentration recorded to date in Maryland. In 2013, multiple species of shellfish were sampled in the northern Maryland and southern Delaware coastal bay system (Fig. 1) by MD DNR, MDE, and DNREC to assess the prevalence of DSP toxins in the areas where commercial shellfish harvesting occurs. A more detailed account of this study will be presented elsewhere, but in total, 100 samples were analyzed with 60 having detectable concentrations of DSP toxins. The range of total DSP toxins found was 0.01–0.13 ppm OA eq. with an average toxin profile of 59% OA :41% DTX1 $\pm 12\%$ ($n = 60$). Between April and May 2015, an extensive bloom of *D. acuminata* occurred in the St. Martin River and its tributaries, Manklin Creek and Bishopville Prong, located in the Isle of Wight section of the Maryland Coastal Bays (Fig. 1). Maximum cell densities ranged from 2,600 to 42,000 $\text{cells} \cdot \text{L}^{-1}$, depending on the location, while maximum concentrations of DSP toxins only reached 0.10 ppm OA eq. in noncommercial Atlantic ribbed mussels (Fig. 7).

Besides the single Bishopville Prong ribbed mussel sample from 2012, the only other location within the study area to exceed regulatory guidance concentrations in shellfish for DSP toxins was Torquay Canal, a dead-end lagoon off Bald Eagle Creek in northern Rehoboth Bay, one of the three Delaware Inland Bays (Fig. 1). This shallow, poorly flushed, stratified system, outside of approved shellfish harvesting waters, is routinely impacted by seasonal anoxia and fish kills, and has been shown to contain multiple HAB species, including prolonged, high abundance blooms of *Dinophysis acuminata* (Luther et al. 2004, Ma et al. 2006, this study). In May 2013, oysters tested from

this location contained 0.17 ppm OA eq. with a composition of 66% OA:34% DTX1. Prolonged (1–2 month) blooms of *D. acuminata* occurred in this system in both 2015 and 2016, reaching maximum cell densities of 645,000 and 1,740,000 $\text{cells} \cdot \text{L}^{-1}$ and maximum toxin concentrations in oysters of 0.35 and 0.33 ppm OA eq., respectively (Fig. 8). To date, DSP toxins have not been found in any commercial shellfish species in open harvesting areas in excess of regulatory guidance concentrations within the study region.

Toxin composition was also analyzed in the DADE01 culture of *Dinophysis acuminata* from Torquay Canal, Delaware. OA, DTX1, and PTX2 were detected in the extract of combined *Dinophysis* cells and medium. Once subjected to alkaline hydrolysis, the toxin concentrations of OA and DTX1 increased by at least an order of magnitude (6.0–132.4 ppm and 3.3–35.7 ppm for OA and DTX1, respectively, in the combined cells and culture media), indicating the extensive presence of esterified toxin derivatives (Fig. S4 in the Supporting Information). After alkaline hydrolysis, the percent composition of the toxin profile was 55% OA, 15% DTX1, and 31% PTX2 with a ratio of 65% OA:35% DTX1 prior to hydrolysis and 79% OA:21% DTX1 after hydrolysis. Overall, the esterified toxins made up 95% and 90% of the total OA and DTX1 pools in the culture, respectively.

DISCUSSION

Diarrhetic shellfish poisoning toxins were first detected in the Gulf of Mexico in 1990 (Dickey et al. 1992), but the first DSP shellfish harvesting closure in this region, due to *Dinophysis ovum*, did not occur until 2008 (Campbell et al. 2010, Deeds et al. 2010). Similar closures have now occurred in 2011 in Washington State, due to blooms comprised of *D. acuminata*, *D. fortii*, and *D. norvegica* (Lloyd et al. 2013, Trainer et al. 2013), in 2015 in Massachusetts, due to *D. acuminata* (M. Brosnahan, unpub. data), and in 2016 in Maine, due to *D. norvegica* (J. Deeds and W. Stutts, unpub. data). Although there have been no confirmed illnesses or shellfish harvesting closures in other regions of the US to date, DSP toxins above regulatory guidance levels have also been reported in noncommercial shellfish samples from New York (Hattenrath-Lehmann et al. 2013) and California (Schultz et al. 2019). With this increase in the prevalence of various *Dinophysis* spp. and their toxins on every US coast in recent years, the need to identify and monitor problematic *Dinophysis* populations has become apparent. With the exception of the Gulf of Mexico and the central Gulf of Maine, the most commonly reported *Dinophysis* species associated with the presence of DSP toxins in shellfish in the United States is *D. acuminata* (Hattenrath-Lehmann et al. 2013, Trainer et al. 2013, Schultz et al. 2019), but worldwide,

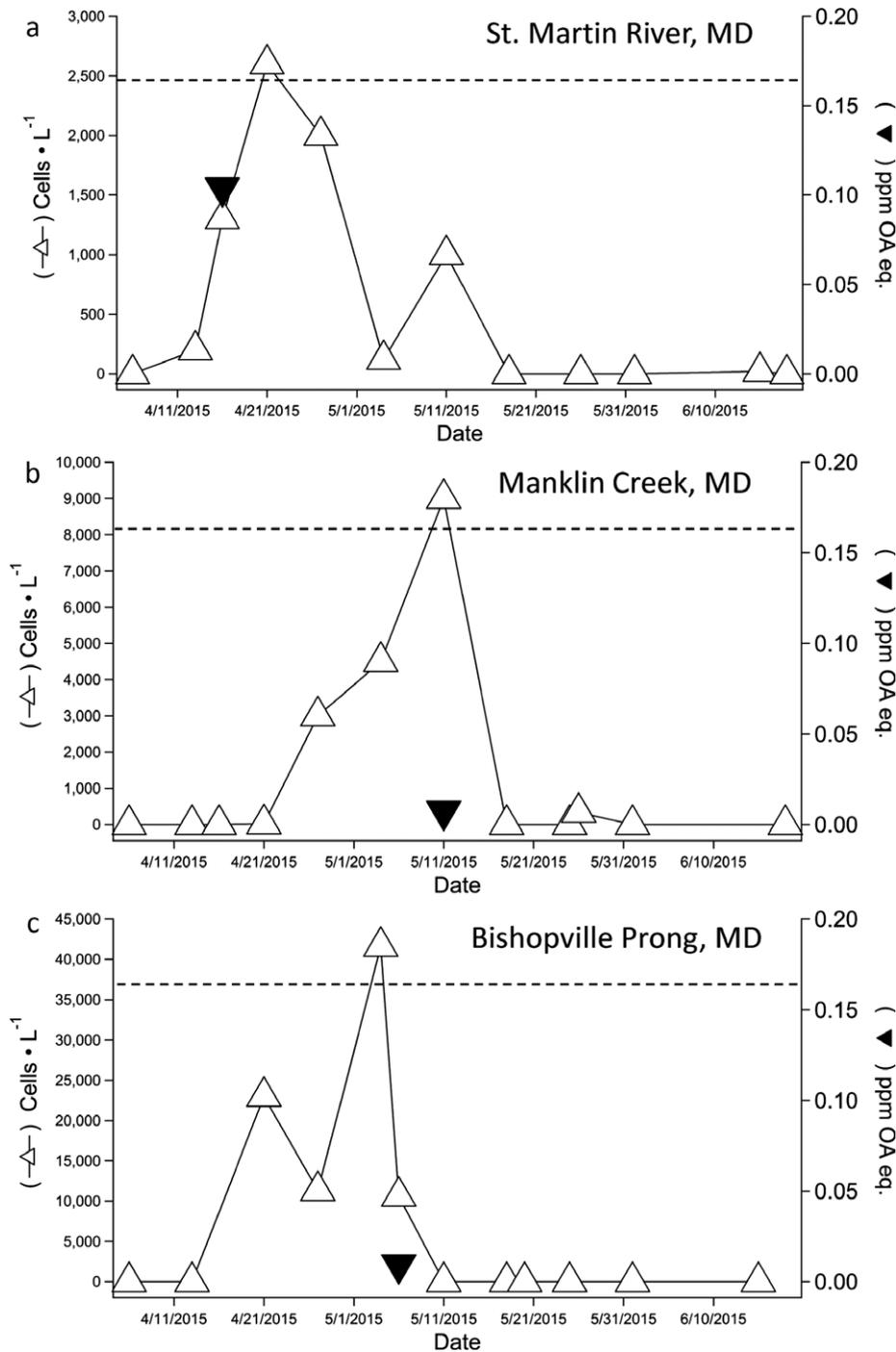


FIG. 7. *Dinophysis acuminata* cell concentrations (open triangles) and DSP toxin concentrations (closed triangles), as determined by protein phosphatase inhibition assay, in Atlantic ribbed mussels (*Geukensia demissa*) collected from the Maryland Coastal Bays in 2015. (a) St. Martin River. (b) Manklin Creek. (c) Bishopville Prong. Dashed line indicates 0.16 ppm OA eq. regulatory guidance concentration for DSP toxins in shellfish.

risks for DSP contamination in shellfish of this region.

History of the Dinophysis acuminata-complex. The morphologic characters commonly used to diagnose *Dinophysis* species are the shape, relative size, and ornamentation of the hypothecal plates (Dodge

1982, Zingone et al. 1998). Distinguishing *Dinophysis* species using only light or scanning electron microscopy can be problematic due to morphological variability of the overall cell shape and hypothetical plate ornamentation within each species. This is particularly true for *D. acuminata*, *D. ovum*, and *D. sacculus*,

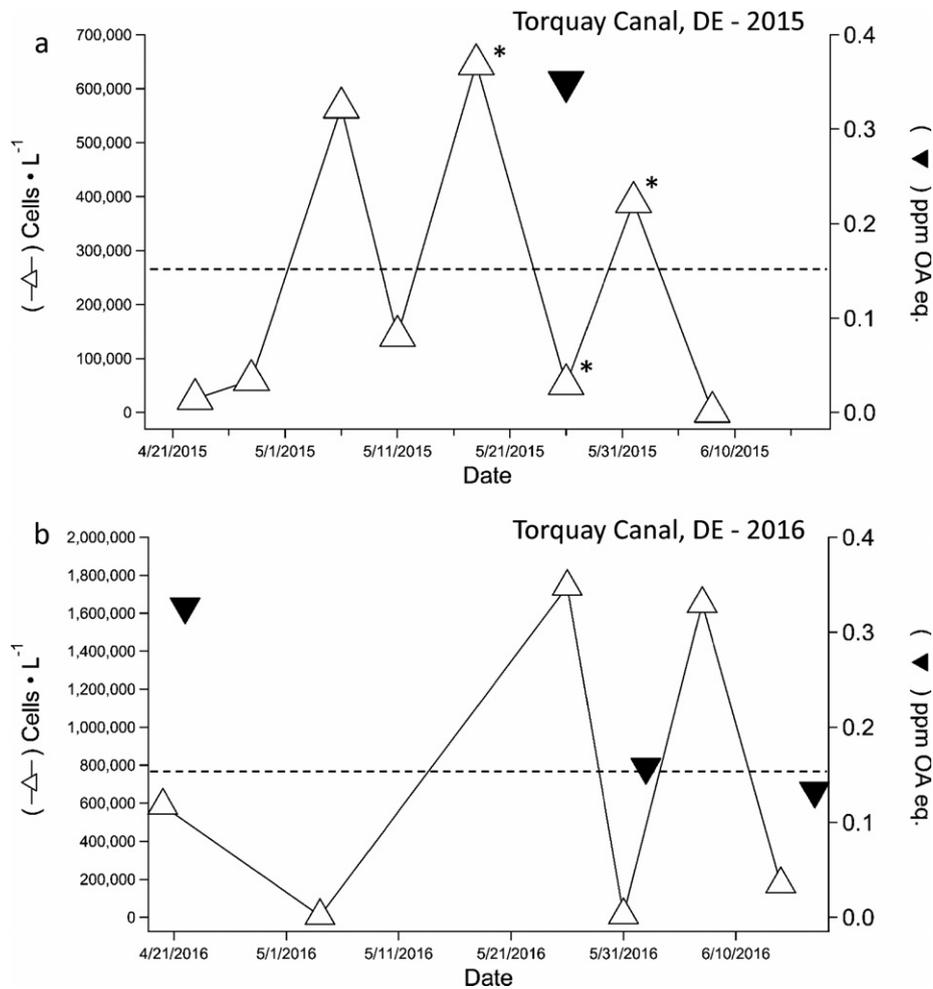


FIG. 8. *Dinophysis acuminata* cell concentrations (open triangles) and DSP toxin concentrations (closed triangles), as determined by protein phosphatase inhibition assay, in eastern oysters (*Crassostrea virginica*) collected from Torquay Canal in the Delaware Inland Bays. (a) 2015. (b) 2016. Dashed line indicates 0.16 ppm OA eq. regulatory guidance concentration for DSP toxins in shellfish. "*" indicates presence of the small cell form of *D. acuminata* (4%–5% of the total population).

with recognition of the morphological overlap occurring between these species dating back to the work of Kofoid and Skogsberg (1928) who summarized and illustrated most known Dinophysiales. Lasso and Bardouil (1991) grouped *D. acuminata*, *D. ovum*, and *D. sacculus*, along with an unidentified small *Dinophysis* sp., in the "*Dinophysis acuminata*-complex" and noted that *D. norvegica* co-occurred with species in this complex in the French waters of their study area. Bravo et al. (1995) added *D. pavillardii* to the complex and Koukaras and Nikolaidis (2004) indicated that *D. okamurai* and *D. recurva* should also be included in this group. Edvardsen et al. (2003) stated that species delineation between *D. acuminata* and *D. norvegica* using morphometrics and genetics was not always apparent and suggested that species hybrids existed in Norwegian waters.

In the late 1800s the species categorized within the *Dinophysis acuminata*-complex were initially described and illustrated with varying degrees of

morphological details (see Claperède and Lachmann 1859 for *D. acuminata*, first identified along the Norwegian coast of the North Sea, Stein 1883, for *D. sacculus*, first identified from the Northern Adriatic Sea, and Schütt 1895 for *D. ovum*, first identified from European Atlantic waters). There has been taxonomic and nomenclatural confusion surrounding these and other small *Dinophysis* species since, as detailed by Zingone et al. (1998; see Fig. 3), Bravo et al. (1995), and Raho et al. (2008). Subsequently, Reguera et al. (2014) cautioned against *D. acuminata* reports not accompanied by supporting information. The investigation into the morphology of *D. sacculus*, and comparison to *D. acuminata*, conducted by Zingone et al. (1998) concluded that despite the morphological variation in *D. sacculus* found in the Mediterranean Sea and along the European Atlantic coast it could be reliably separated from the morphologically variable *D. acuminata* found in European Atlantic waters from

Portugal northwards to Norway by examining the overall shape of the hypothecal plates. Zingone et al. (1998) went on to state that *D. sacculus* and *D. pavillardii* were synonymous and the name *D. sacculus* should be retained based on nomenclatural priority. However, this work did not present any genetic analyses. In contrast, Guillou et al. (2002) published the first sequences for both *D. acuminata* and *D. sacculus*, among other *Dinophysis* species, from French waters but no morphological descriptions or illustrations of the organisms were made available for comparison. Raho et al. (2008) presented a morphological and DNA-based comparison of *D. acuminata* and *D. ovum*, but *D. sacculus* was not included. Adding to the difficulties of speciating *Dinophysis* is the low inter-species variability in nuclear genes (LSU and ITS regions) and mitochondrial genes (*cox1* and *cob*) as numerous authors have reported (Edvardsen et al. 2003, Raho et al. 2008, 2013, Park et al. 2019).

Morphological analysis. In an effort to determine what *Dinophysis* species were responsible for forming blooms in the mid-Atlantic region of the US we examined *Dinophysis* morphospecies from Virginia to New Jersey. Cells in field material from the mid-Atlantic region were compared to cells collected from the Texas coast of the Gulf of Mexico, Nova Scotia, Canada, and the Mediterranean waters of Spain and France. Our analysis indicated that the mid-Atlantic *D. acuminata* cells were smaller and more morphologically variable, similar to those described as *D. acuminata* from the Atlantic European coast by Zingone et al. (1998), compared to *D. acuminata* found in Nova Scotia, Canada, which is more similar to the original description of the species by Claperède and Lachmann (1859). There were distinct morphological differences between *D. acuminata* and *D. sacculus* found in the western Mediterranean Sea and *D. ovum* found in the Gulf of Mexico waters along Texas. The *D. sacculus* cells were characterized by elongated hypothecal plates with a distinct concave region adjacent to the second sulcal rib whereas *D. ovum* cells exhibited a robust oval shape that lacked tapering of the heavily rugose hypothecal plates. We determined that in the mid-Atlantic region *D. acuminata* and *D. norvegica* were the predominant species and could be morphologically distinguished from each other based on overall cell size and hypothecal plate shape. Additionally, *D. norvegica* only co-occurred occasionally with *D. acuminata* in offshore surface waters, whereas *D. acuminata* alone formed blooms in the coastal bays of Delaware, Maryland, and Virginia. These findings differ from the historic work of Marshall (1969, 1976, 1978, 1980, 1982, 1984, 1985), Marshall and Cohn (1981), and Marshall et al. (1981) who noted multiple *Dinophysis* species to be present in low abundance with *D. fortii* as the dominant species in the mid-Atlantic region. It should be noted that Marshall and Egerton (2009)

also reported an apparent increase in the presence of *D. acuminata* within the lower Chesapeake Bay compared to the earlier surveys. Similar shifts in the *Dinophysis* community were reported along the Norwegian and Sardinian coasts by Naustvoll et al. (2012) and Bazzoni et al. (2015), respectively, through their analyses of 25-year time-series data sets. Naustvoll et al. (2012) did not observe a relationship between nutrients and *Dinophysis* and hypothesized changes in the *Dinophysis* community may be influenced by shifts in the prey community it relies on. Additionally, dinoflagellate parasites, such as *Amoebophrya* and *Parvilucifera*, are known to infect and impact some *Dinophysis* populations (Park et al. 2004, Chambouvet et al. 2008, Alves-de-Souza et al. 2012). The relationship between the mid-Atlantic *Dinophysis* population and nutrients, prey, and species-specificity between host and parasite will be the focus of future studies.

Molecular analysis. Using a ~2 kb portion of the ribosomal operon, including ITS1, 5.8S, ITS2, and LSU regions, shown previously to be phylogenetically informative for the *Dinophysiales* group (Handy et al. 2009), we determined that this region is useful in distinguishing many of the known DSP-causing species of *Dinophysis*, including *D. acuta*, *D. norvegica*, and *D. fortii* (based on comparison of base pair differences) from the *D. acuminata*-complex species. However, this region was not useful in distinguishing the three *D. acuminata*-complex species from each other. While previous studies had suggested that ribosomal genes may be uninformative for this group, no study to our knowledge had analyzed all three morphologically confirmed species simultaneously and with such a large portion of the ribosomal operon. Park et al. (2019) recently found that the mitochondrial marker *cox1*, reported previously to be diagnostic for this group (Raho et al. 2008), could not differentiate isolates of *D. acuminata* and *D. ovum* from Korean waters. Future studies will focus on the use of alternative gene regions to differentiate these distinct morphospecies.

Identity of the mid-Atlantic “Small *Dinophysis* sp.” Small cells are known to occur in *Dinophysis* blooms and their role is suspected to be part of the sexual life cycle (Escalera and Reguera 2008, Raho et al. 2008) or a rapid physiological response to changing environmental conditions (Reguera and González-Gil 2001). In some instances, small cell forms have been synonymized with the large forms (e.g., *Dinophysis skagii*=*Dinophysis acuminata*; Lassus et al. 2016). Lassus and Bardouil (1991) noted that the small *Dinophysis* sp. (see Lassus and Bardouil 1991; Fig. 2c), which co-occurred with *Dinophysis acuminata*-complex species during blooms along the French coast, was morphologically distinct enough to be described as its own species. We have noted a similar morphological variant within our mid-Atlantic populations of *Dinophysis*. Statistical analysis of the metrics used to examine cell morphology in this

study separated the “small *Dinophysis* sp.” from the other morphospecies, however our rDNA sequence analysis concluded that while it was distinct from *D. norvegica*, it could not be separated from the other species in the *Dinophysis acuminata*-complex. The small cell morphotype was observed most frequently in offshore samples of the mid-Atlantic region and was not routinely observed during *D. acuminata* blooms in the coastal bays, except in 2015 during a prolonged, high cell concentration bloom in Torquay Canal, Delaware, when it was noted in low abundance at the peak and termination of the bloom (Fig. 8). Similarly, Park et al. (2019) reported the presence of small cell forms in a Korean *D. acuminata* culture after the culture had been held for months without prey. Together with our rDNA sequence analysis, this suggests the small cell morphotype present in our samples is a life-history stage of *D. acuminata* but further work will be required to confirm this hypothesis.

Toxin analysis. In the mid-Atlantic region of the US, OA and DTX1 were found to be the only DSP toxins present in both bloom water and shellfish, ranging in relative proportion from 54:46% to 78:22%. It should be noted that PTX2 was also found in both water and shellfish but was not monitored for routinely as it does not contribute to DSP and is not regulated in the United States. These same toxins, in similar relative proportion, were also found in a clonal culture of *Dinophysis acuminata* (DADE01) established from a bloom that occurred in the Torquay Canal area of the Delaware Inland Bays in 2015. The toxins and relative proportions found in this study are similar to those reported for *D. acuminata* blooms in New York (Hattenrath-Lehmann et al. 2013) and for several laboratory cultures isolated from Massachusetts waters (Fux et al. 2011). These were also the primary lipophilic toxins shown to be present in the only report available for a laboratory culture of *D. sacculus* (Riobó et al. 2013). In the Puget Sound region on the US west coast, DTX1 and PTX2 were initially reported to be the only *Dinophysis*-associated lipophilic toxins present (Trainer et al. 2013) but more recently, low concentrations of OA have also been found both in water and shellfish (Kim et al. 2017; J. Deeds and W. Stutts, unpub. data). In Puget Sound and the surrounding coastal waters of Washington State, *D. acuminata* is considered to be the primary DSP toxin-producing species, but additional toxic species, such as *D. fortii* and *D. norvegica*, are also known to be present (Trainer et al. 2013). At this time, it is unclear which species are producing which toxins in this region. In contrast, lipophilic shellfish toxins in the Gulf of Mexico coastal waters of Texas are composed only of OA produced by *D. ovum* (Campbell et al. 2010, Deeds et al. 2010, Fux et al. 2011). Therefore, within the *D. acuminata*-complex, toxin analysis does appear to be useful in separating *D. ovum* from *D. acuminata* and *D. sacculus*, at least in

the United States, but not in separating *D. acuminata* from *D. sacculus*. The other *Dinophysis* species found in mid-Atlantic waters, *D. norvegica*, often reported to co-occur with *D. acuminata*-complex species, has not occurred in numbers sufficient to determine the DSP toxins it produces in this region. Monospecific blooms of *D. norvegica* that occurred in the summers of 2016 and 2018 along the central Maine coast indicated the presence of DSP-like toxins that are distinct from OA, DTX1, and DTX2 in both water and shellfish (J. Deeds and W. Stutts, unpub. data). It remains to be determined if these same compounds are also produced by *D. norvegica* in the mid-Atlantic or Puget Sound regions.

In the mid-Atlantic region, DSP toxins have not exceeded the regulatory guidance concentration of 0.16 ppm OA eq. in any commercial shellfish samples tested to date. The precautionary shellfish harvesting closure that occurred on the Potomac River in 2002 was initiated based on elevated cell concentrations ($236,000 \text{ cells} \cdot \text{L}^{-1}$) but subsequent testing of shellfish found only trace concentrations of DSP toxins (Marshall et al. 2004, Tango et al. 2004). Since testing began in 2010, DSP toxins have exceeded guidance concentrations in noncommercial shellfish only a few times and only in tributaries of the Maryland and Delaware coastal bays. One of the first *Dinophysis*-associated shellfish harvesting closures on the US east coast occurred in Massachusetts in 2015 due to a bloom of *D. acuminata* (M. Brosnahan, pers. comm.). During this bloom, DSP toxins in sentinel blue mussels (*Mytilus edulis*) barely exceeded regulatory guidance concentrations (max. 0.19 ppm OA eq.), with OA and DTX1 being present at a ratio of $67:33 \pm 3\%$ ($n = 6$), respectively (J. Deeds and W. Stutts, unpub. data). It is not known why similar blooms of *D. acuminata* in New York waters have resulted in substantially higher concentrations of DSP toxins (ranging from 0.96-1.24 ppm OA eq.) in multiple shellfish species (Hattenrath-Lehmann et al. 2013).

SUMMARY AND CONCLUSIONS

Increases in toxigenic *Dinophysis* blooms throughout the United States, including the mid-Atlantic region, highlight the need to better define the speciation of these populations. We examined cells for six morphometrics using light and scanning electron microscopy, conducted molecular analyses on a ~2-kb rDNA sequence, and assessed toxin profile and concentration using LC-MS/MS and in vitro protein phosphatase inhibition assays to characterize the mid-Atlantic population of *Dinophysis*. Our study has identified regular and small cell forms of *D. acuminata* as the predominant *Dinophysis* species in both inshore and offshore surface waters in this region. To better understand the role of *D. acuminata* small cell forms in the life cycle of this species, its presence and relative abundance should be

documented when conducting monitoring for *Dinophysis* and DSP toxins. Also present in the mid-Atlantic region is *D. norvegica*, a species recently implicated in DSP events in Maine. Due to the low abundance of cells in samples available during this study, the toxicity of *D. norvegica* was not determined but will be considered in future research efforts. While a shift in the predominant species of *Dinophysis* within a population is not unprecedented (see Naustvoll et al. 2012 and Bazzoni et al. 2015) our findings do differ from the regional phytoplankton surveys conducted by Marshall between 1964 and 1985. These earlier surveys reported a community dominated by *D. fortii*, with only an occasional presence of *D. acuminata*-complex species. It is possible that additional *Dinophysis* species are present in deeper offshore waters that were not sampled extensively during this study as our focus was to assess *Dinophysis* abundance in areas that could potentially impact human health due to the accumulation of toxins in shellfish. Future work will focus on sampling additional offshore areas and depths within the water column to further compare current observations to historical works. Additionally, the roles that nutrients, predator-prey interactions, and parasitic infections play in supporting a *D. acuminata*-dominated population need to be investigated.

ADDITIONAL FINDINGS FROM THIS STUDY

One of the initial reasons for this study was that cells isolated from both the winter and spring 2012 *Dinophysis* blooms in the Bishopville Prong of the St. Martin River, Maryland, were morphologically consistent with *D. acuminata* by light microscopy but were genetically distinct from cells identified as *D. cf. acuminata* during a previous study in the region (see Fig. 3 and Fig. S1, n and o in Handy et al. 2009). This prompted us to confirm which species of *Dinophysis* were present in mid-Atlantic waters using multiple techniques and also to re-evaluate the identity of cells from that earlier study. The new morphologic analysis and rDNA sequence re-analysis of additional cells identified previously as *D. cf. acuminata* concluded that this cell was originally misidentified and does not fit within the *Dinophysis acuminata*-complex based on our analytics. Genetically, these cells are more closely related to, but minimally distinct (2 base pairs different) from, *D. fortii* based on the only available sequence (accession AB355145; 590 bp). These cells, with a pear-shaped body, were found outside the mouth of the Chesapeake Bay in waters on the Virginia/North Carolina border in the fall of 2007 (Figs. 1 and 5 [cell 34]; Table S2). The cells are larger than *D. acuminata* and *D. ovum* and have hypothecal plates that are shaped differently from the similarly sized *D. sacculus*. Cells ($n = 10$) measured 51.90 μm in length and 43.73 μm in depth and had well-developed and highly reticulate cingular and sulcal lists,

with an average LSL length of 33.25 μm . Cells of this type have not been observed again in any regional samples collected since that date (T. Egerton, J. Wolny, unpub. data) nor were they documented previously (H. Marshall, pers. comm.). During the fall of 2007, an unusual export event occurred from the Gulf of Mexico which transported Gulf waters through the Florida Straits and up the Atlantic Coast (Walsh et al. 2009) and cells typically seen in the Gulf of Mexico were reported on Florida's east coast (Wolny et al. 2015). We hypothesize that these *Dinophysis* sp. cells were transported to the area in this Gulf water mass. The identity of this species has not been determined; however, it should not be considered *D. cf. acuminata*. The sequence information deposited previously in GenBank (accessions EU780640 and FJ477081) has been updated accordingly.

This paper honors the past and continuing contributions of Dr. Harold G. Marshall (Morgan Professor Emeritus, Old Dominion University) and his life-long commitment to documenting phytoplankton trends in the Chesapeake Bay region, all while teaching students the skills and passion necessary to do the same.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Micrographs of mid-Atlantic *Dinophysis* morphospecies. *Dinophysis acuminata* from the St. Martin River, MD (a) LM and (b) SEM, scale bars = 20 μm ; *D. norvegica* from offshore of the DE/MD border (c) LM, scale bar = 20 μm , and (d) SEM, scale bar = 10 μm ; and small cell form of *D. acuminata* from offshore Ocean City Inlet, MD (e) LM and (f) SEM, scale bars = 20 μm .

Figure S2. Micrographs of comparative *Dinophysis acuminata*-complex species. *Dinophysis sacculus* from the Mediterranean Sea at Port Andratx, Mallorca, Spain (a) LM and (b) SEM, scale bars = 20 μm ; and *D. ovum* from the Gulf of

Mexico at Port Aransas, TX (c) LM, scale bar = 20 μm and (d) SEM, scale bar = 10 μm .

Figure S3. Box and whisker plots of morphometrics examined in *Dinophysis acuminata* cells from six geographic regions. Upper and lower limits of the box represent the 1st to 3rd quartiles, with bold horizontal lines indicating the median. Whiskers represent the range of 95% of the data. Circles and asterisks are outliers outside the 95% percentile, with asterisks indicating the value is more than 3x the value within the box. Letters (A–D) represent the results of the post-hoc REGWF multiple comparison test. Groups that contain the same letter are not significantly different from each other.

Figure S4. Concentrations of the DSP toxins OA and DTX1 before (free) and after (free+esterified) alkaline hydrolysis of the *Dinophysis acuminata* clonal culture isolate DADE01 (dissolved+particulate toxin), relative to PTX2. The latter is degraded upon alkaline hydrolysis.

Table S1. List of *Dinophysis* species reported in the mid-Atlantic and Chesapeake Bay regions by Marshall (1969, 1976, 1978, 1980, 1982, 1996), Marshall and Cohn (1981) and Marshall et al. (2005, 2009). Names in parenthesis are the currently accepted nomenclature.

Table S2. Locations of single-cell samples and field study sites used in the analysis of *Dinophysis* spp. in the mid-Atlantic region. Numbers in the first column correspond to locations on the map in Figure 1, cells depicted in Figures 4 and 5, and phylogenetic data presented in Figure 6. “#” indicates sequence data was generated at TAMU without a corresponding photograph of the single-cell sample.

Table S3. PCR and sequencing primers used in this study to analyze mid-Atlantic *Dinophysis* and comparative *Dinophysis* species. P: PCR; S: sequencing.