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RESEARCH ARTICLE

A survey of *Dinophysis* spp. and their potential to cause diarrhetic shellfish poisoning in coastal waters of the United States

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Abstract

Multiple species of the genus *Dinophysis* produce diarrhetic shellfish toxins (okadaic acid and *Dinophysis* toxins, OA/DTXs analogs) and/or pectenotoxins (PTXs). Only since 2008 have DSP events (illnesses and/or shellfish harvesting closures) become recognized as a threat to human health in the United States. This study characterized 20 strains representing five species of *Dinophysis* spp. isolated from three US coastal regions that have experienced DSP events: the Northeast/Mid-Atlantic, the Gulf of Mexico, and the Pacific Northwest. Using a combination of morphometric and DNA-based evidence, seven Northeast/Mid-Atlantic isolates and four Pacific Northwest isolates were classified as *D. acuminata*, a total of four isolates from two coasts were classified as *D. norvegica*, two isolates from the Pacific Northwest coast

Abbreviations: ANOVA, One-way analysis of variance; DSP, diarrhetic shellfish poisoning; DTX1*D*, *dinophysis* toxins1; DTXs, *Dinophysis* toxins; FSW, filtered seawater; HMDS, hexamethyldisilazane; LC, HRMS, Liquid chromatography–high resolution mass spectrometry; LC-MS/MS, Liquid chromatography coupled to tandem mass spectrometry; LOD, The limit of detection; LOQ, limit of quantification; NCBI, National Center for Biotechnology Information; OA, Okadaic acid; PAR, photosynthetically active radiation; PCR, polymerase chain reactions; PSP, paralytic shellfish poisoning; PTXs, pectenotoxins; SEM, scanning electron microscopy; SPE, solid-phase extraction.

were identified as *D. fortii,* and three isolates from the Gulf of Mexico were identified as *D. ovum* and *D. caudata*. Toxin profiles of *D. acuminata* and *D. norvegica* varied by their geographical origin within the United States. Cross-regional comparison of toxin profiles was not possible with the other three species; however, within each region, distinct species-conserved profiles for isolates of *D. fortii*, *D. ovum*, and *D. caudata* were observed. Historical and recent data from various State and Tribal monitoring programs were compiled and compared, including maximum recorded cell abundances of *Dinophysis* spp., maximum concentrations of OA/DTXs recorded in commercial shellfish species, and durations of harvesting closures, to provide perspective regarding potential for DSP impacts to regional public health and shellfish industry.

KEYWORDS
diarrhetic shellfish poisoning, *Dinophysis*, harmful algal bloom, toxin profiles

INTRODUCTION

The dinoflagellate genus, *Dinophysis*, comprises more than 100 taxonomically accepted species (Gómez et al., 2011; Jensen & Daugbjerg, 2009), only 10 of which have been found to produce one or two kinds of lipophilic toxins (Reguera et al., 2014). The first group of toxins includes the closely related polyether toxins okadaic acid (OA) and *Dinophysis*toxins (DTXs), as well as their derivatives, such as esterified analogs (Yasumoto et al., 1985). Together these toxins constitute the okadaic acid group (Toyofuku, 2006), referred to in this paper collectively as OA/DTXs. The OA/DTXs are known as causative agents of diarrhetic shellfish poisoning (DSP), a human illness syndrome linked to the consumption of contaminated shellfish (Dominguez et al., 2010; Reguera et al., 2014). Prominent human symptoms of DSP are nonlethal gastrointestinal disorders such as diarrhea, nausea, vomiting, and abdominal pain, with complete recovery within three days (Barceloux, 2008).

The second group of toxins, the pectenotoxins (PTXs), are another class of lipophilic toxins produced by some species of *Dinophysis.* In mice these polyether lactones were not diarrheagenic after oral administration (Terao et al., 1986) but were lethal by intraperitoneal injection (Miles, Wilkins, Munday, et al., 2004), which led to initial regulation of these toxins in some parts of the world, including Europe. Because of the lack of adverse effects in humans who have consumed food containing PTXs, food safety authorities in several countries, including the United States, have not regulated the content of these toxins in shellfish meat. Moreover, in light of the most recent toxicological studies, the European Commission deregulated pectenotoxins in shellfish meat (European Commission, 2021). Recent studies, however, indicate that purified PTXs have the potential for ecological impacts such as exerting toxicity against various life stages of bivalves and fish (Gaillard et al., 2020; Pease et al., 2021; Rountos et al., 2019).

Outbreaks of DSP (illnesses and/or shellfish harvesting closures due to unsafe levels of OA/DTXs) caused by *Dinophysis* spp. are commonly reported in Atlantic Europe (Reguera et al., 2014; Sechet et al., 2021; Van Egmond et al., 1993), Pacific and Atlantic South America (Alcántara-Rubira et al., 2018; Díaz et al., 2022; Sunesen et al., 2021; Uribe et al., 2001) and Japan (Suzuki et al., 1997). DSP outbreaks have also been documented in Atlantic South Africa (Pitcher & Calder, 2000), New Zealand and Australia (Boundy et al., 2020; Madigan et al., 2006). In the United States, outbreaks have been rare even though *Dinophysis* spp. have been described on the west coast since the early 1900s and have been known to exist commonly in coastal waters since the mid-1900s (Horner et al., 1997; Marshall & Cohn, 1983; Steidinger et al., 2000; Wolny et al., 2020). Several studies, however, have reported an expansion and increase in the intensity of blooms of *Dinophysis* spp. in the coastal waters of Washington State (Lloyd et al., 2013; Trainer et al., 2013) and Texas (Deeds et al., 2010) as well as on the Mid-Atlantic and Northeast Atlantic coasts (Gobler et al., 2017; Tong et al., 2015; Wolny et al., 2020). This trend is concerning not only because of public health concerns associated with the potential for increased exposure to OA/DTXs but also because of the increased costs of expanding public advisory services and monitoring systems. Moreover, the economic expenses associated with potentially more frequent and prolonged closures of shellfish harvesting will rise.

The first confirmation of OA/DTXs above the regulatory level (harvesting closure and product recall) in the United States occurred along the Texas coast of the Gulf of Mexico in 2008 (Campbell et al., 2010; Deeds et al., 2010). A month-long shellfish harvesting closure was implemented after OA was detected in Eastern oysters (*Crassostrea virginica*) at levels 2–3 times above the Food and Drug

Administration (FDA) regulatory guidance level of 160 ng · g^{-1} OA equivalents in raw shellfish meat (combined free OA, DTX-1, DTX-2 and their acyl esters; equivalent to 160 μg · kg^{-1}; Deeds et al., 2010; FDA, 2020). The causative organism was identified as *Dinophysis* cf. *ovum* (Campbell et al., 2010). It was not until 3 years later, in 2011, that the first clinical report of DSP in the United States and Canada occurred on the Pacific Northwest coast. Three people became ill after consuming recreationally harvested mussels from Sequim Bay, WA that were contaminated with OA/DTXs at 2–10 times the FDA guidance level (Trainer et al., 2013), and 62 illnesses were reported in British Columbia after the consumption of cooked mussels contaminated with OA/DTXs above the action level (Taylor et al., 2013). The primary causative organism was identified as *D. acuminata*. Notable DSP events since then include a *D. acuminata* bloom associated with levels of OA/DTXs in shellfish greater than seven times the FDA guidance level in Northport Bay, NY, samples collected from either noncommercial shellfish harvesting areas or areas already closed due to PSP events (Hattenrath-Lehmann et al., 2013), and blooms of *D. norvegica* associated with levels of the novel analog dihydroDTX1 at one to six times the FDA guidance level (based on equivalent levels of DTX1) in eastern Maine, resulting in precautionary shellfish harvesting closures (Deeds et al., 2020).

There are 17 species of *Dinophysis* reported from the Pacific, Atlantic, and Gulf coasts of the United States (Campbell et al., 2010; Cembella, 1989; Dickey et al., 1992; Marshall & Cohn, 1983; Swanson et al., 2010). Among these, *D. acuminata*, *D. caudata*, *D. fortii*, *D. norvegica*, *D. ovum*, and *D. tripos* are putative or confirmed producers of OA/DTXs, with *D. acuminata*, *D. norvegica*, and *D. ovum* cited as the most common and abundant species (Marshall, 1996; Trainer et al., 2013; Wolny et al., 2020). This diversity of *Dinophysis* species and toxins in different regions of the United States has complex implications for monitoring and management of DSP outbreaks, demonstrating the need for further investigation.

Therefore, this study characterized 20 strains, representing five species of *Dinophysis* isolated from three US coastal regions, for their taxonomic and toxinological characterization, to advise monitoring and management approaches for OA/DTXs. Results are interpreted within the context of various State and Tribal monitoring and management strategies utilized since the emergence of DSP in the United States.

MATERIALS AND METHODS

Field sampling and isolation of clonal strains

Twenty *Dinophysis* strains were isolated and established as cultures from water bodies in three coastal regions of the United States: Northeast/Mid-Atlantic, Pacific Northwest, and Gulf of Mexico. Specific locations and dates of *Dinophysis* isolation are given in Table 1 and Figure 1. Water samples were collected for isolation from various depths (0–2 m) using a 20-μm plankton net or grab sample (Niskin bottle, or surface bucket sample). Individual *Dinophysis* cells were microscopically isolated from these waters using a drawn glass micropipette and transferred into separate wells of tissue culture plates (Thermo Scientific) preloaded with modified f/2-Si or L1-Si medium (Guillard, 1975; Guillard & Hargraves, 1993; Guillard & Ryther, 1962) and the ciliate *Mesodinium rubrum* (JAMR).

Culture maintenance and growth

Cultures of the kleptoplastidic, mixotrophic species of the genus *Dinophysis* were successfully established following the culture method described by Park et al. (2006), with *Mesodinium rubrum* and the cryptophyte *Teleaulax amphioxeia* as prey. The inability to successfully isolate American *Mesodinium* strains under our culture conditions led to the utilization of the Japanese prey cell line, on which all *Dinophysis* isolates fed and grew better than they did on other prey isolates, such as the Spanish or Danish *Mesodinium* and *Teleaulax* strains. *T. amphioxeia* (JATA) and *M. rubrum* (JAMR) were both isolated in 2007 from Inokushi Bay (44°38′30.8″ N, 68°23′29.4″ W) in the Oita Prefecture, Japan, as described by Nishitani et al. (2008). The *T. amphioxeia* culture was grown in f/2-Si culture media prepared from 0.22-μm filter-sterilized seawater (FSW) with a salinity of 35, obtained from Wachapreague, VA, and diluted with molecular-grade water to a 1:5 dilution ratio to achieve a salinity of 25. The *M. rubrum* culture was maintained in f/6-Si medium (salinity 25) and periodically (every 3–5 d) fed with *T. amphioxeia* at a 1:10 (predator: prey) ratio. *M. rubrum*, after its complete consumption of the cryptophyte, was used as prey for the 20 *Dinophysis* strains. To maintain cultures of *Dinophysis* in exponential growth during the experimental period, triplicate flasks of seawater (salinity 25) were inoculated with *Dinophysis* and fed every 3 d with *M. rubrum* at a ratio of 1:5 (predator: prey). All flasks were maintained in a temperature-controlled incubator at 15°C ± 1°C and provided ~100 ± 10 μmol photons · m^{-2} · s^{-1} photosynthetically active radiation (PAR) on a 14:10 light:dark (L:D) cycle. All cultures were clonal but nonaxenic.

To determine *Dinophysis* cell densities and calculate growth rates (Guillard, 1973), two aliquots from each replicate sample were fixed with unacidified Lugol's iodine solution (LabChem Inc.; 0.5%) and counted in a 1-mL Sedgwick–Rafter counting chamber at 250× magnification (Olympus CKX53 or IX50 inverted microscopes; Olympus Corp).

TABLE 1 *Dinophysis* species and strain designations, sampling locations, collection dates, and coordinates.

Coast	Species	Strain ID	Location	Date	Coordinates
Northeast/ Mid-Atlantic coast	*D. acuminata*	DANY1	Meetinghouse Creek, Peconic Estuary, NY	May 2013	40°55′42.1″ N, 72°36′49.0″ W
	D. acuminata	DAVA 01	Chesapeake Bay, Nassawadox, VA	May 2017	37°28′18.3″ N, 75°58′02.2″ W
	D. acuminata	DATC03	Nauset Marsh Estuary, Town Cove, Orleans/Eastham, MA	April 2009	41°47′31.2″ N, 69°58′49.4″ W
	D. acuminata	DAMD D2	Manklin Creek, Isle of Wight Bay, Ocean Pines, MD	May 2018	38°14′03.2″ N, 75°05′33.1″ W
	D. acuminata	DAGM01	Martha's Vineyard, MA	July 2008	41°20′30.1″ N, 70°38′30.7″ W
	D. acuminata	DINO2	Eel Pond, Woods Hole, MA	September 2006	41°31′34.3″ N, 70°40′13.4″ W
	D. acuminata	DASPM02	Nauset Marsh Estuary, Salt Pond, Eastham, MA	April 2009	41°50′07.4″ N, 69°58′19.9″ W
	D. norvegica	DNBHD3F	Gulf of Maine, Blue Hill Falls, ME	May 2018	44°38′30.8″ N, 68°23′29.4″ W
	D. norvegica	DNBHFE6	Gulf of Maine, Blue Hill Falls, ME	May 2018	44°38′30.8″ N, 68°23′29.4″ W
Pacific Northwest coast	*D. acuminata*	NWFSC 806	Budd Inlet, Olympia, WA	November 2019	47°03′00.7″ N, 122°54′19.7″ W
	D. acuminata	NWFSC 807	Budd Inlet, Olympia, WA	November 2019	47°03′00.7″ N, 122°54′19.7″ W
	D. acuminata	NWFSC 808	Budd Inlet, Olympia, WA	November 2019	47°03′00.7″ N, 122°54′19.7″ W
	D. acuminata	SB3	Sequim Bay, Sequim, WA	June 2018	48°02′14.9″ N, 123°1′29.3″ W
	D. fortii	NWFSC 803	Budd Inlet, Olympia, WA	November 2019	47°03′00.7″ N, 122°01′19.7″ W
	D. fortii	NWFSC 804	Budd Inlet, Olympia, WA	November 2019	47°03′00.7″ N, 122°54′19.7″ W
	D. norvegica	NWFSC 814	Clam Bay, Manchester, WA	February 2020	47°34′17.3″ N, 122°32′40.2″ W
	D. norvegica	NWFSC 816	Budd Inlet, Olympia, WA	May 2020	47°03′29.9″ N, 122°54′21.1″ W
Gulf of Mexico	*D. ovum*	DOSS 3195	Gulf of Mexico, Surfside Beach, TX	March 2019	28°56′11.2″ N, 95°17′45.1″ W
	D. ovum	DOSS 2206	Gulf of Mexico, Surfside Beach, TX	February 2020	28°56′11.2″ N, 95°17′45.1″ W
	D. caudata	DCSS 3191	Gulf of Mexico, Surfside Beach, TX	March 2019	28°56′11.2″ N, 95°17′45.1″ W

Microscopic identification

Aliquots of all 20 isolates were preserved in unacidified Lugol's iodine, examined using an Olympus CKX53 inverted microscope (Olympus Corp) and a Zeiss Axiovert 200 inverted microscope (Carl Zeiss), and photographed and measured using an Olympus DP73 digital camera system (Olympus America). Photos were processed using Adobe Photoshop (v. 7.0.1) to rotate or flip images for consistent orientation. In two laboratories, 20 cells from each culture were assessed following the morphometric parameters described in Wolny et al. (2020), including cell length (L) and depth (D). Biovolume was calculated based on a flattened ellipsoid shape as defined in Olenina et al. (2006), using height (h) and large diameter (d1) measurements and the estimation of small diameter (d2) according to Olenina et al. (2006).

$$Biovolume\ (\mu m^3): V = \frac{\pi}{6} \times d1 \times d2 \times h.$$

For scanning electron microscopy (SEM), 1-mL aliquots of the preserved samples were centrifuged (1000 *g*; 10 min) and then 0.5 mL of the supernatant was removed. The pellet was then resuspended by gentle vortex-mixing and 3–4 drops of the suspension was added to the top of poly-L-lysine (Sigma–Aldrich) coated coverslips (glass, 12-mm diameter) that were preplaced into individual wells of 6-well plates. The suspension was allowed to settle for 30 min before washing twice with 5 mL of Milli-Q water. An ethanol dehydration series (32%, 50%, 72%, 80%, 88%, 96%, and 100%) was then performed with a 15 min incubation time in each concentration (5 mL) before being aspirated and replaced with the next concentration. The 100% ethanol step was repeated three times before adding hexamethyldisilazane (HMDS; Sigma–Aldrich, 3–4 drops) to the top of the coverslips and allowing it to evaporate. The HMDS step was repeated once more, and the coverslips were allowed to dry completely before being glued to 13-mm aluminum SEM stubs with graphite conductive adhesive (Electron Microscopy Sciences). Stubs were dried overnight and sputter-coated with gold–palladium (Emitech Ltd.) before being viewed with a JEOL 6360LV SEM (JEOL USA Inc.). Photos were processed with Gimp (v. 2.10) to rotate or flip the images for consistent orientation of cells and insertion of scale bars.

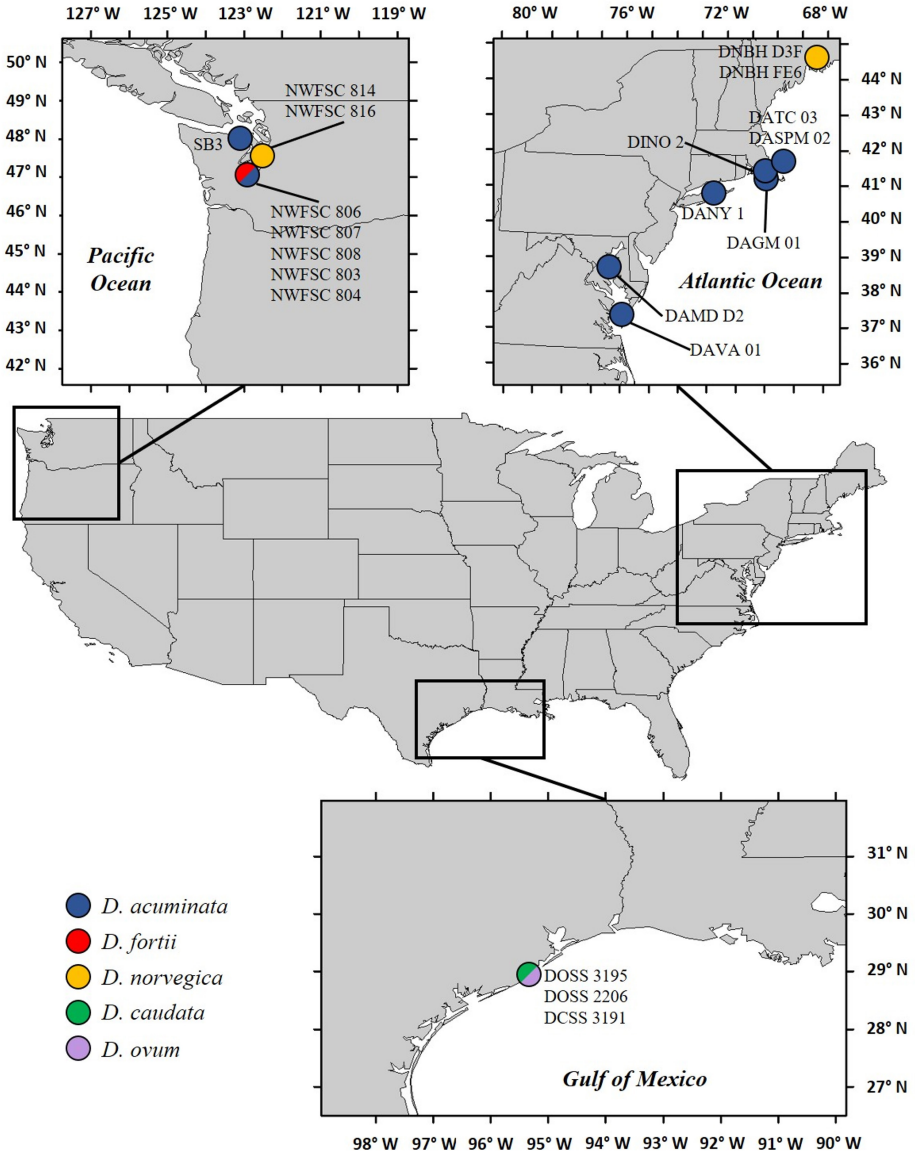

FIGURE 1 Isolation locations for the 20 *Dinophysis* strains. *Dinophysis* species are color coded.

Supplemental DNA-based identification

A subset of the isolates tentatively identified as *Dinophysis acuminata*, *D. fortii*, and *D. norvegica* based on morphological characteristics were further analyzed using DNA-based identification with the goals of: (1) confirming the classifications of *D. fortii* and *D. acuminata* isolates from the Pacific Northwest coast as separate species since these isolates shared certain morphologic characters and similar toxin profiles and (2) confirming the classification of two isolates from the Northwestern Pacific and Northeastern Atlantic coasts with distinct toxin profiles as *D. norvegica*.

Using an Olympus CKX41 inverted microscope (Olympus America), single cells from unacidified Lugol's iodine-preserved culture aliquots were isolated using mouth aspiration with drawn glass capillary tubing, then washed four to six times with molecular-grade water before being placed in either a sterile 1.5-mL microcentrifuge tube with 40 μL of molecular-grade water or a well of a Covaris ML230, 8 AFA-tube TPX sonicator strip tube (Covaris) with 20 μL of molecular-grade water, and stored at −20°C (1.5-mL microcentrifuge tubes) or 4°C (Covaris strip tubes) until further processing.

Cells were processed following Wolny et al. (2020), summarized as follows: cells were brought to a volume of 50 μL using molecular-grade water, sonicated to disrupt the cell, directly amplified using polymerase chain reactions (PCR) with three sets of primers, and Sanger-sequenced. Approximately 2,000 bases from the ITS1-LSU rRNA gene region were included. This region has been shown previously to reliably distinguish the three species in question (Handy et al., 2009; Wolny et al., 2020). Sonication was done manually

using a probe-tipped sonicator (Branson Ultrasonics Corp) with 3–5 pulses for 5 s as in Wolny et al. (2020) or with a Covaris ML230 focused ultra-sonicator. In cases where a Covaris strip tube was used instead of a 1.5-mL microcentrifuge tube and a probe-tipped sonicator, the Covaris ML230 focused ultrasonication instrument disrupted cells with a high rate of success using the following cycle protocol: %DF of 25, CPB of 50, total time of 80 s, total AFA time of 40 s, 4 repeats, AFA on for 10 s AFA off for 10 s, at 12°C with a dither of ±3 mm Y@ 20 mm/s and a plate definition of ML2330_500660 Rack 8 AFA-TUBE TPX Strip +12.7 mm offset, as suggested by Covaris technical support.

Sequence data were analyzed as in Wolny et al. (2020) using GeneiousPro (version R10.2.3). In this study, sequence data from all single cells obtained from a clonal culture were combined and edited manually before generating a consensus sequence. Consensus sequences for each culture were compared to previous data (Handy et al., 2009; Wolny et al., 2020) and to sequences from GenBank NCBI accessions.

Toxin characterization

Cultures of the 20 strains were sampled to determine the intracellular (particulate) and extracellular (dissolved) toxin content and concentrations of OA, DTX1, DTX2, dihydroDTX1, PTX2, dehydroPTX2 (putative PTX12), a putative hydroxyPTX2, and the esterified forms of OA/DTXs. A 10-mL sample of each triplicate culture of each *Dinophysis* strain was collected during exponential growth and gently centrifuged (3000 *g*, 15 min, 4°C) to separate the culture into cell pellet and supernatant. The cell pellet was resuspended in 1 mL of methanol and bath-sonicated for 15 min at 25 kHz. The supernatant was filtered (PVDF syringe filter; 0.2 μm, 13 mm) and stored at −20°C for later analysis. An aliquot (500 μL) of the cell pellet was hydrolyzed to measure total (free plus esterified) OA/DTXs, by addition of 63 μL of 2.5 M NaOH solution and heating in a water bath at 76°C for 40 min, then cooled and neutralized with 63 μL of 2.5 M HCl. The sample was then filtered (PVDF syringe filter, 0.2 μm, 13 mm) and stored at −20°C for later analysis.

Toxins in the original supernatant, containing dissolved or extracellular toxins, were extracted, concentrated, and de-salted using a 60-mg Oasis HLB solid-phase extraction (SPE) cartridge (Waters) that was previously conditioned with 3 mL of methanol followed by 3 mL of molecular-grade water. The supernatant (10 mL) was applied to the SPE cartridge and washed with 3 mL of molecular-grade water and then aspirated for 1 min to remove excess water. Toxins were eluted with 1 mL of methanol, filtered (PVDF syringe filter, 0.2 μm, 13 mm) and the eluate was stored at −20°C for later analysis.

LC–HRMS (method A)

Liquid chromatography–high resolution mass spectrometry (LC–HRMS) analysis, based on the method of Wilkins et al. (2021), was conducted in positive mode with a Q Exactive-HF Orbitrap mass spectrometer equipped with a HESI-II heated electrospray ionization interface (Thermo Fisher Scientific) with an Agilent 1200 G1312B binary pump, G1367C autosampler (tray set to 10°C), and G1316B column oven (Agilent Technologies). Analyses were performed with a Symmetry Shield RP18 column (3.5 μm, 100 × 2.1 mm; Waters) at 20°C, with mobile phases A and B of H_2O and MeCN, respectively, each of which contained formic acid (0.1% v/v). A linear gradient (0.3 mL· min-1) was used from 20% to 100% B over 21 min, held at 100% B (6 min), then returned to 20% B over 0.1 min and held at 20% B (2.9 min) to equilibrate the column (total run time 30.0 min). The flow was diverted to waste for the first 0.91 min and final 10.01 min, and the injection volume was 2 μL or 4 μL. The mass spectrometer was calibrated from *m/z* 74–1622, the spray voltage was 3.7 kV, the capillary temperature was 350°C, and the sheath and auxiliary gas flow rates were 25 units and 8 units, respectively, with MS data acquired from 2 min to 20 min. MS data was acquired in data-dependent acquisition (DDA) mode, with full-scan (FS) spectra at *m/z* 700–1450, with the resolution setting 60,000, AGC target 1×10^6, and maximum injection time (max IT) 50 ms, and the top-5 MS/MS spectra were acquired with an inclusion list, with the selection width set to *m/z* 0.7, the resolution set to 15,000, max IT to 75 ms, an AGC target of 2×10^5, and a stepped collision energy of 35, 40, and 65 eV (NH_4^+ adduct ions). The inclusion list for acquiring MS/MS spectra covered all putative PTXs and OA/DTXs observed in preliminary FS chromatograms. Data were processed using Xcalibur version 4.0 (Thermo Fisher Scientific). Elemental compositions were determined from the accurate masses and isotopomer intensities of the $[M + NH_4]^+$ and $[M + Na]^+$ adduct ions in the full-scan HRMS (Table S1 in the Supporting Information) using the NRC Molecular Formula Calculator (Feb. 2022; Mallia et al., 2019).

LC–MS/MS (method B)

Quantification of analytes was performed using a tandem quadrupole Xevo TQD ultra-performance liquid chromatography system (Waters) coupled to an electrospray ionization (ESI) source with a trapping dimension and at-column dilution (LC–MS/MS trap/ACD) following Onofrio et al. (2020). Chromatography was carried out on an Aquity BEH C18 column (130 Å, 1.7 μm, 50 × 2.1 mm, Waters). Analyses were performed under acidic chromatographic conditions in negative

(OA, DTX1, DTX2, dihydroDTX1) and positive ionization mode (PTXs) using scheduled reaction monitoring. Transitions, chromatography, and mass spectrometer conditions followed Onofrio et al. (2020). Certified reference materials for OA, DTX1, DTX2, and PTX2 (NRC-CNRC, Halifax) were used for verification of retention times and quantification by comparison to 8-point, triplicate standard curves. DihydroDTX1, PTX12, and hydroxyPTX2 were identified based on retention time, ion chromatogram for *m/z* and 3–4 transitions per analyte in positive (PTXs) or negative (dihydroDTX1) ionization mode, and quantified using DTX1 or PTX2 standard curves, as described by Deeds et al. (2020); Miles, Wilkins, Samdal, et al. (2004) and Miles et al. (2006); Table S1. The limit of detection (LOD) and limit of quantification (LOQ), respectively, were: OA, 0.26 and 0.78 ng · mL^{-1}; DTX1, 0.36 and 1.08 ng · mL^{-1}, and; PTX2, 0.92 and 2.79 ng · mL^{-1}. Peaks were integrated using TargetLynx, MassLynx version 4.2 (Waters). The intracellular toxins were normalized to the number of cells extracted or average biovolume of cells and the extracellular toxins were expressed per culture volume. Likewise, the percentage of total toxin content was calculated from the sum of the intracellular toxin content of all analogs (OA, DTX1, dihydroDTX1, PTX2, PTX12, and hydroxyPTX2) and was expressed on a per-cell basis.

Statistical analyses

One-way analysis of variance (ANOVA) with pairwise Tukey HSD multiple comparisons tests were performed on morphometric and toxin data to differentiate *Dinophysis* species. All data were normally distributed, and their variances were homogenous as determined by the Shapiro–Wilk and Bartlett tests, respectively, thus permitting the use of parametric statistical analyses. The level of significance (α) was set to 0.05 for all statistical tests performed in RStudio statistical software version 1.2.5033 (R Development Core Team, 2021). The resulting map was generated using the program "ArcGIS Pro", version 10.8.

RESULTS

Twenty monoclonal strains of *Dinophysis* were characterized using morphological and toxin analyses. Morphology, supplemented with molecular data, identified five species: *D. acuminata* (11), *D. ovum* (2), *D. norvegica* (4), *D. fortii* (2), and *D. caudata* (1) as shown in Table 1. Each strain was further characterized using both a nontargeted and targeted toxin analysis. A supplemental genetic analysis was used to confirm species identifications in select cases where morphological and/or toxin profile patterns deviated from those previously described.

Microscopic and DNA-based identification

In total, 400 cultured *Dinophysis* cells were examined using the morphometrics described by Wolny et al. (2020), including length and depth measurements and length-to-depth ratio (Table 2), as these morphologic characters, plus the overall hypotheca shape, are most commonly used to differentiate *Dinophysis* species (Larsen & Moestrup, 1992). Despite some isolates being in culture for numerous years, cell lengths, depths, and overall hypothecal shapes were within ranges reported from in situ US populations by Campbell et al. (2010), Handy et al. (2009), Tong et al. (2015), Trainer et al. (2013), and Wolny et al. (2020).

Cells identified as *Dinophysis acuminata* came from the Northeast/Mid-Atlantic and Northwest Pacific coasts. All *D. acuminata* cells were characterized by having a rounded antapical region with minimal tapering below the third sulcal rib, the greatest depth at a height (in the ventral margin) between the second and third ribs, and thin sulcal lists. Although cells from all *D. acuminata* strains displayed a great deal of morphological variability, overall, the Northeast/Mid-Atlantic strains (Figure 2a–g) were smaller ($45 \pm 2\,\mu m$ average length) and more elongated (average length-to-depth ratio $= 1.6 \pm 0.1$) compared to the strains from the Pacific Northwest coast, which had a larger and more angular antapical region (Figure 2h–k; $56 \pm 3\,\mu m$ average length and average length-to-depth ratio $= 1.4 \pm 0.1$). Strains of *D. norvegica* came from the Northeast/Mid-Atlantic and Pacific Northwest coasts. All *D. norvegica* strains were distinguished by the shape of the hypotheca, which was characterized as being angular in the antapical region with marked tapering below the third sulcal rib, the greatest depth at a height between the second and third sulcal ribs, and pronounced thecal pores. As with *D. acuminata*, *D. norvegica* strains from Northeast/Mid-Atlantic coast (Figure 2l,m) were smaller and more elongated ($54 \pm 3\,\mu m$ average length and average length-to-depth ratio $= 1.4 \pm 0.1$) than the Pacific Northwest coast strains (Figure 2 N and O; $60 \pm 2\,\mu m$ average length and average length-to-depth ratio $= 1.2 \pm 0.1$). The theca of the NWFSC 816 strain was further characterized by thick reticulate markings (Figure 2o).

Dinophysis fortii was only identified in cultures established from the Pacific Northwest coast, and *D. ovum* and *D. caudata* were only identified from cultures established from the Gulf of Mexico. These three species were easily distinguished from other *Dinophysis* species based on size and unique overall cell shape. *Dinophysis fortii* (Figure 2p,q) cells were

TABLE 2 Growth rates and morphometric measurements, including average length, depth, length-to-depth ratio, and cell volume, of 20 *Dinophysis* strains (grown at 15°C, salinity 25, 100 μmol photons · m^{-2} · s^{-1} PAR) as well as of the prey species *Mesodinium. rubrum* and *Teleaulax amphioxeia*. Data are means ± SD, $n = 20$ and $n = 3$ for morphometric and growth rate measurements, respectively.

Species	Strain ID	Length (μm)	Depth (μm)	length-to-depth ratio	Cell volume (×10^3 $μm^3$)	Growth rate (d^{-1})
D. acuminata	DANY1	47 ± 1	30 ± 1	1.6 ± 0.1	17 ± 1	0.30 ± 0.01
D. acuminata	DAVA 01	45 ± 2	29 ± 2	1.6 ± 0.1	16 ± 1	0.24 ± 0.02
D. acuminata	DATC03	45 ± 3	28 ± 2	1.6 ± 0.1	15 ± 2	0.28 ± 0.04
D. acuminata	DAGM01	43 ± 3	29 ± 2	1.5 ± 0.1	15 ± 2	0.20 ± 0.01
D. acuminata	DAMD D2	45 ± 2	29 ± 2	1.6 ± 0.1	15 ± 2	0.22 ± 0.06
D. acuminata	DINO2	44 ± 3	29 ± 2	1.5 ± 0.1	15 ± 2	0.22 ± 0.01
D. acuminata	DASPM02	43 ± 3	27 ± 3	1.6 ± 0.1	14 ± 2	0.21 ± 0.01
D. acuminata	NWFSC 806	51 ± 3	37 ± 2	1.4 ± 0.0	24 ± 3	0.10 ± 0.01
D. acuminata	NWFSC 807	61 ± 2	45 ± 3	1.3 ± 0.1	43 ± 3	0.08 ± 0.02
D. acuminata	NWFSC 808	61 ± 5	42 ± 4	1.5 ± 0.1	42 ± 7	0.07 ± 0.01
D. acuminata	SB3	51 ± 3	40 ± 5	1.5 ± 0.3	38 ± 5	0.12 ± 0.02
D. fortii	NWFSC 803	71 ± 1	53 ± 2	1.3 ± 0.0	74 ± 2	0.16 ± 0.01
D. fortii	NWFSC 804	73 ± 2	55 ± 2	1.4 ± 0.1	78 ± 5	0.13 ± 0.02
D. norvegica	NWFSC 814	55 ± 2	44 ± 3	1.3 ± 0.0	41 ± 3	0.07 ± 0.01
D. norvegica	NWFSC 816	64 ± 2	53 ± 5	1.2 ± 0.0	58 ± 8	0.10 ± 0.02
D. norvegica	DNBHD3F	53 ± 2	38 ± 2	1.4 ± 0.0	29 ± 3	0.05 ± 0.01
D. norvegica	DNBHFE6	54 ± 3	40 ± 3	1.4 ± 0.1	31 ± 4	0.06 ± 0.01
D. ovum	DOSS 3195	44 ± 3	35 ± 3	1.3 ± 0.1	18 ± 2	0.06 ± 0.01
D. ovum	DOSS 2206	44 ± 4	34 ± 3	1.3 ± 0.1	18 ± 3	0.11 ± 0.01
D. caudata	DCSS 3191	73 ± 5	37 ± 4	2.0 ± 0.1	34 ± 6	0.08 ± 0.01
M. rubrum	JAMR	29 ± 8	27 ± 8	1.0 ± 0.4	14 ± 5	1.04 ± 0.12
T. amphioxeia	JATA	5 ± 1	5 ± 1	1.0 ± 0.2	0.06 ± 0.02	0.81 ± 0.03

large (72 ± 2 μm average length) and elongated (average length-to-depth ratio = 1.4 ± 0.1), with the greatest depth at a height just below the third sulcal rib. The smallest of the *Dinophysis* species studied, *D. ovum* (Figure 2r,s; 44 ± 4 μm average length), was ovoid with little to no tapering of the antapical region (average length-to-depth ratio = 1.3 ± 0.1). *D. caudata* was the largest species studied (73 ± 5 μm average length). These cells were elongated (average length-to-depth ratio = 2.0 ± 0.1), were widest at the base of the sulcal list, and had a characteristic ventral projection below the sulcal list (Figure 2t).

For the supplemental DNA-based identification, nine of the cultures examined were newly sequenced as part of this study and were compared to each other as well as to sequences generated by Handy et al. (2009), Wolny et al. (2020), and others from NCBI. This approach allowed for the confirmation of four *D. acuminata* strains from the Pacific Northwest coast, two strains of *D. fortii* from the Pacific Northwest coast, and three strains of *D. norvegica*: two from the Pacific Northwest coast and one from the Atlantic Northeast coast.

The four strains of *Dinophysis acuminata* from Pacific Northwest coast (NWFSC 806, NWFSC 807, NWFSC 808, and SB3) were sequenced for the ITS and LSU rRNA gene region and compared to two strains of *D. fortii* (NWFSC 803 and NWFSC 804) from the same location to confirm their identities because there is overlap in both morphologic and toxigenic profiles among these strains. For these four *D. acuminata* strains, final consensus sequences were generated from 2 to 14 individual sequences, which yielded lengths ranging from 1,228 to 2,019 bases, with 91%–99.4% high quality bases, and no ambiguous bases (Table S2; Figure S1 in the Supporting Information). The final sequences were nearly identical to each other (99.85%–100%), the exceptions being a single base difference in SB3 compared to two of the four other *D. acuminata* strains tested (position 134 in Figure S1; NWFSC 808 is also missing sequence data for this position) and a gap at position 805 compared to the other three *D. acuminata* strains. There is also a base difference at position 280 for NWFSC 806 compared with two of the four other *D. acuminata* strains, with NWFSC 808 again missing sequence data at this site (Figure S1). These sequences were 3%–4% different than the two *D. fortii* strains and 0.4%–0.8% different from the *D. norvegica* sequences described below (Table S3; Figure S1 in the Supporting Information), and matched (99.8%–100% identical) other *D.*

acuminata sequences from our previous studies in the Northeast/Mid-Atlantic (Handy et al., 2009; Wolny et al., 2020) as well as those in the NCBI database. For the *D. fortii* strains, consensus sequences were created from 26 and 27 sequences, respectively. The sequences were 2,004 and 2,016 bases in length with 100% high quality bases and no ambiguities (Table S2). These two cultures had identical sequences and were most similar to, albeit seven bases different from GenBank accession MK860871, which represents an unnamed *Dinophysis* sp. described by Wolny et al. (2020). The new sequences extend representation of *D. fortii* for this gene region, as only one very short *D. fortii* sequence with two ambiguities (590 bp, accession AB355145) was available for comparison, and that sequence was a 100% match to these longer ones. The *D. fortii* strains differed from the *D. acuminata* strains as noted above and from the *D. norvegica* strains described below by 3.0%–3.3% (Table S3, Figure S1). Finally, three *D. norvegica* cultures (NWFSC 814, NWFSC 816, and DNBHD3F) were sequenced to confirm their identities because differences were observed in toxin profiles between the Pacific Northwest (NWFSC 814, NWFSC 816) and Atlantic Northeast (DNBHD3F) strains. Consensus sequences were obtained from nine to 14 individual sequences that ranged in length from 1,760 to 1,920 bases with 92.8%–97% high quality (Table S2). Of

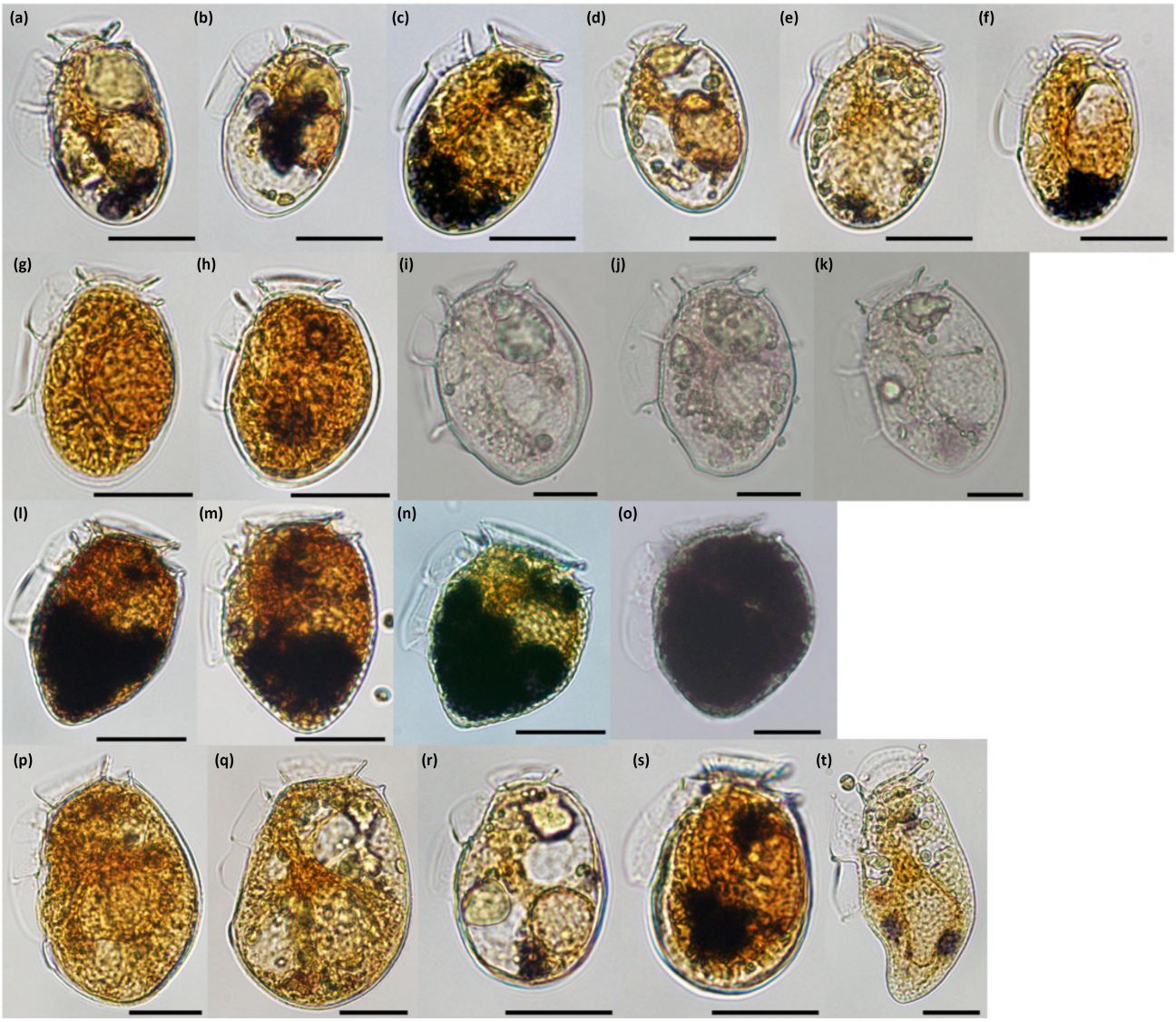

FIGURE 2 Photomicrographs (upper panels) and the corresponding scanning electron micrographs SEMs (lower panels) of the 20 *Dinophysis* strains, including D. acuminata strains DANY1 (a), DAVA 01 (b), DATC03 (c), DAMD D2 (d), DAGM01 (e), DASPM02 (f), DINO2 (g), NWFSC 806 (h), NWFSC 807 (i), NWFSC 808 (j), SB3 (k); D. norvegica strains DNBHD3F (l), DNBHFE6 (m), NWFSC 814 (n), NWFSC 816(o), D. fortii strains NWFSC 803 (p), NWFSC 804 (q); D. ovum strains DOSS 3195 (r), DOSS 2206 (s) and; D. caudata strain DCSS 3191(t). Scale bars are 20 and 10 μm for light microscope and SEM images, respectively.

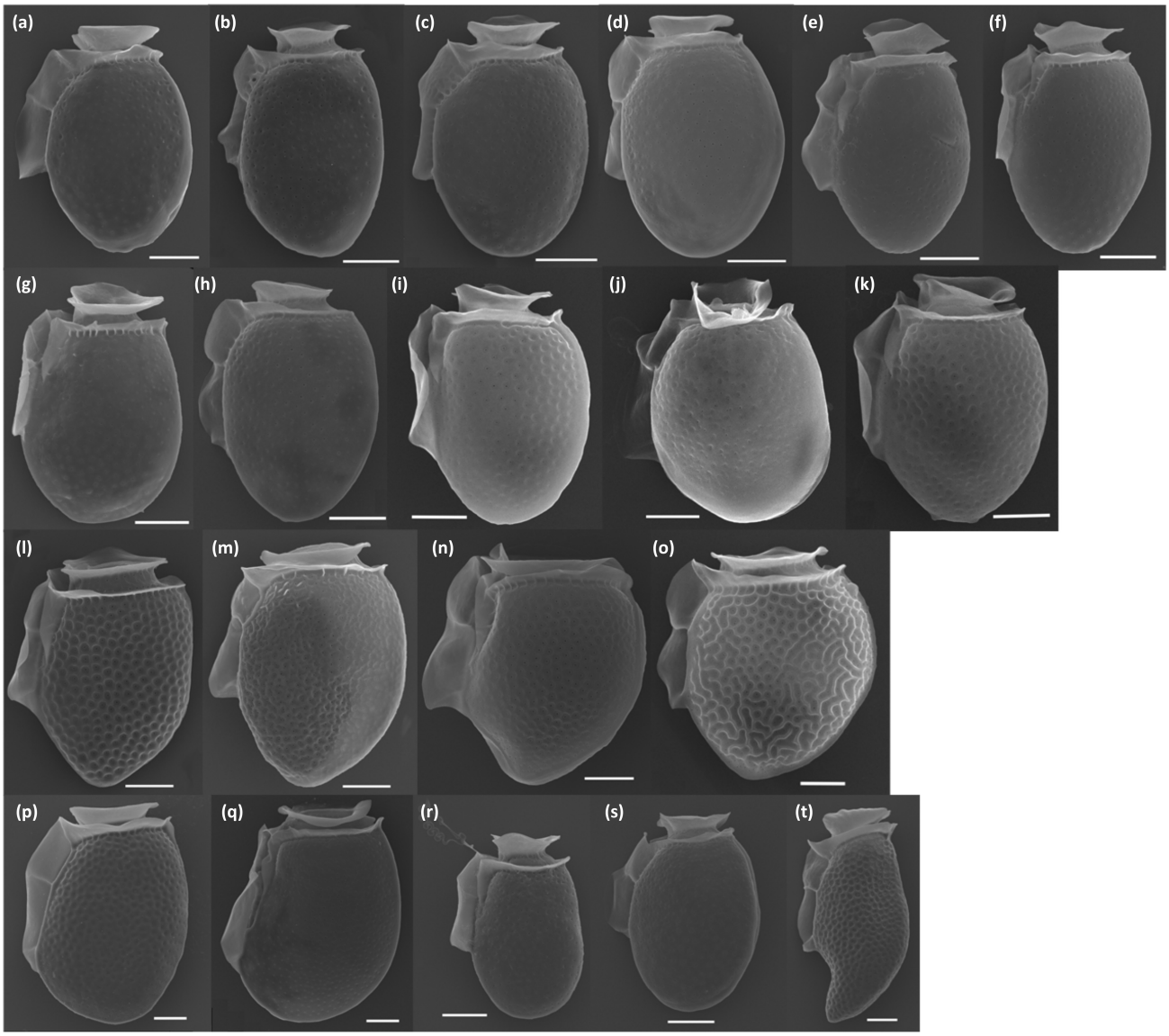

FIGURE 2 (Continued)

note, the consensus sequence for strain DNBHD3F from the Gulf of Maine (Northeast Atlantic coast) had two polymorphic bases (positions 131 and 1163; Figure S1) that were not observed in the other two strains from the Pacific Northwest coast. These were double peaks (an M and a K, ~0.1%), meaning that some sequences from strain DNBHD3F matched the NWFSC 814 and NWFSC 816 strains exactly but that others had an alternate base at that position. In addition, NWFSC 816 had a single base difference (position 559; Figure S1) from the other two *D. norvegica* strains (0.05% difference). All three were 99.7%–99.8% identical to our previous studies (Handy et al., 2009; Wolny et al., 2020) as well as to additional *D. norvegica* submissions to NCBI.

All *Dinophysis* sequences produced through this study have been deposited to GenBank with accession numbers OM939684–OM939692 (Table S2). Of note, cells identified morphologically as *D. norvegica* were the most difficult of the isolates to sequence. Introduction of ultrasonication using Covaris instrumentation increased cellular permeability and/or cellular disruption and facilitated the genetic analysis of cells that consistently failed to yield usable sequences using the more traditional probe sonication approach. Implementation of this novel methodology may help further genomic data collections by giving researchers a pathway to examine cells that may otherwise be unavailable.

Growth rate and biovolume

The growth rates of the seven *Dinophysis acuminata* strains (DANY1, DAVA 01, DATC03, DAMD D2, DAGM01, DINO2, and DASPM02) isolated from the Northeast/

Mid-Atlantic coast ranged between 0.20–0.30 per day, and the biovolumes ranged between 14–17 $\times 10^3\,\mu m^3$ (Table 2). Neither growth rates nor biovolumes were significantly different between these strains. In contrast, the four Pacific Northwest coast *D. acuminata* strains (NWFSC 806, NWFSC 807, NWFSC 808, and SB3) exhibited significantly lower growth rates (0.07–0.12 day^{-1}; one-way ANOVA, $F_{7,16}=4.14$, $p=0.008$; Tukey HSD test, $p<0.05$), and the average cellular biovolumes of these strains were significantly higher (2.4-fold) compared to the Northeast/Mid-Atlantic coast strains of *D. acuminata* (37×10^3 vs. $15\times10^3\,\mu m^3$, one-way ANOVA, $F_{7,152}=31.39$, $p=2\times10^{-16}$; Tukey HSD test, $p<0.05$; Table 2). The *D. fortii* strains (NWFSC 803 and NWFSC 804) also exhibited low growth rates (ca. 0.14 day^{-1}) and had the highest cellular biovolumes of ca. $78\times10^3\,\mu m^3$ (Table 2). The growth rates of the Gulf of Mexico strains of *D. ovum*, (DOSS 3195 and DOSS 2206) were also low, ranging between 0.06–0.11 day^{-1}, with similar biovolumes ($18\times10^3\,\mu m^3$) to the Northeast/Mid-Atlantic coast *D. acuminata* strains (one-way ANOVA, $F_{1,38}=0.39$, $p=0.53$; Tukey HSD test, $p>0.05$). The *D. caudata* strain (DCSS 3191) displayed a low growth rate (0.08 day^{-1}) with a relatively high cellular biovolume of $39\times10^3\,\mu m^3$ (Table 2). The four *D. norvegica* strains from both coasts (DNBHD3F, DNBHFE6, NWFSC 814, and NWFSC 816) also had low growth rates (0.05–0.10 day^{-1}) and relatively high cellular biovolumes ranging from 29 to $58\times10^3\,\mu m^3$, and they showed no significant variations in their growth rates; however, the cellular biovolumes of the two Pacific Northwest coast strains were significantly higher (1.7-fold) compared to the two Northeast Atlantic coast *D. norvegica* strains (Table 2).

Toxin profile and content per cell, and extracellular toxins

Toxin analysis was carried out on all 20 *Dinophysis* strains. Extracts from all strains were screened without hydrolysis by LC–HRMS (method A) to determine which OA/DTXs and PTXs were present, allowing each toxin analog to be targeted with the correct transitions and quantified using the most appropriate standard in LC–MS/MS (method B). The OA/DTXs identified by LC–HRMS were free or esterified OA, DTX1, and dihydroDTX1, and the major PTXs identified were PTX2, dehydroPTX2, and hydroxyPTX2. The dehydroPTX2 analog was tentatively identified as PTX12, previously reported only from Scandinavian *Dinophysis* blooms (Lindegarth et al., 2009; Miles, Wilkins, Samdal, et al., 2004), based on its elemental composition, elution as a pair of isomers on either side of the PTX2 peak, and the similarity of its product ion spectra to that of PTX2 (Figures S2–S4 in the Supporting Information). The identification of the putative hydroxyPTX2 analog was also based on its elemental composition, shorter retention time relative to PTX2, and similarity of its product ion spectrum to that of PTX2 (Figures S2–S4).

Intracellular toxin content and toxin profiles are shown in Table 3 and Figure 3. An aliquot of the intracellular toxin extracts underwent alkaline hydrolysis, so values represent a combination of free and esterified forms of OA/DTXs. Overall, the most abundant toxin was PTX2 (in 15 of the 20 isolates) and was the major toxin in the Pacific Northwest coast *Dinophysis fortii* and the Northeast/Mid-Atlantic coast *D. acuminata* strains, representing 83%–96% of the total cellular toxin content. OA, DTX1, and dihydroDTX1 were detected in multiple strains, but DTX2 was not found in any US strain tested in this study (Table 3). Most strains (17 of 20) produced at least one toxin of the OA/DTX group. Exceptions were *D. caudata* strain DCSS 3191 and the Pacific Northwest *D. norvegica* strains NWFSC 814 and NWFSC 816, which produced only hydroxyPTX2 and PTX2, respectively. DihydroDTX1 was only detected in *D. norvegica* isolates from the Northeast Atlantic coast.

Six distinct toxin profiles were detected among *Dinophysis* strains (Figure 3). Strains of *D. acuminata* had one of two distinct profiles that correlated with their geographical region (Figure 3). The toxin profiles of the Northeast/Mid-Atlantic coast *D. acuminata* (seven isolates) were dominated by PTX2 (mean 92%; 26–58 $pg\cdot cell^{-1}$; Table 3). DTX1 and OA were also present in low amounts (0.4–8.4 and 0.6–3.3 $pg\cdot cell^{-1}$, respectively). In contrast, the four Pacific Northwest coast *D. acuminata* strains did not produce OA and contained DTX1 as the major OA/DTX analogs, representing an average of 71% of the total toxin content (Figure 3). PTX2 was also present, but at a lower percentage of the profile (17%–44%). Pacific Northwest coast *D. acuminata* also contained up to three orders of magnitude more DTX1 than was produced by the Northeast/Mid-Atlantic coast *D. acuminata* strains (Table 3). Differences in PTX2 quota did not vary by region; thus, differences in the toxin profiles were largely driven by the relative amount of DTX1 in the Pacific Northwest coast strains (one-way ANOVA, $F_{7,16}=86.8$, $p=1.6\times10^{-11}$; Tukey HSD test, $p<0.05$).

Toxin profiles of the *Dinophysis fortii* and *D. acuminata* strains from the Pacific Northwest coast were similar, with DTX1 and PTX2 being the only toxins detected. PTX2 was the dominant toxin in the *D. fortii* profiles, accounting for 91% and 89% of the total toxin content in strains NWFSC 803 and NWFSC 804, respectively (Figure 3). Pacific Northwest coast *D. norvegica* strains NWFSC 814 and NWFSC 816 had a much different profile, producing no measured OA/DTXs and high amounts of PTX2 (155 and 182 $pg\cdot cell^{-1}$, respectively; Table 3).

TABLE 3 Intracellular and extracellular toxins from the 20 *Dinophysis* isolates: okadaic acid (OA), dinophysistoxin-1 (DTX1), dihydroDTX1, and pectenotoxin-2 (PTX2), –12 (dehydroPTX12, tentatively PTX12), and hydroxyPTX2. Data are means ± SD, $n = 3$.

		Intracellular toxin (pg · cell^{-1})						Extracellular toxin (ng · mL^{-1})				
Species	**Strain ID**	**OA**[a]	**DTX1**[a]	**DihydroDTX1**[a]	**PTX2**	**PTX12**	**Hydroxy-PTX2**	**OA**	**DTX1**	**PTX2**	**PTX12**	**Hydroxy-PTX2**
D. acuminata	DANY1	0.6 ± 0.1	0.4 ± 0.1	–	26.0 ± 0.6	–	–	0.5 ± 0.1	0.1 ± 0.0	9 ± 0	–	–
D. acuminata	DAVA 01	1.5 ± 0.2	1.3 ± 0.2	–	27 ± 6	–	–	0.3 ± 0.1	0.1 ± 0.0	7 ± 2	–	–
D. acuminata	DATC03	3.3 ± 0.5	8.4 ± 1.6	–	58 ± 6	–	–	0.7 ± 0.1	0.9 ± 0.2	10 ± 2	–	–
D. acuminata	DAGM01	2.4 ± 0.1	3.0 ± 0.4	–	46 ± 10	–	–	0.9 ± 0.4	0.3 ± 0.2	4 ± 1	–	–
D. acuminata	DAMD D2	0.7 ± 0.3	2.8 ± 1.1	–	48 ± 9	–	–	0.2 ± 0.1	0.2 ± 0.1	7 ± 1	–	–
D. acuminata	DINO2	0.7 ± 0.2	1.0 ± 0.3	–	36 ± 11	–	–	0.3 ± 0.2	–	3 ± 1	–	–
D. acuminata	DASPM02	2.9 ± 0.8	1.2 ± 0.2	–	49.0 ± 0.8	–	–	0.6 ± 0.1	0.2 ± 0.0	3 ± 1	–	–
D. acuminata	NWFSC 806	–	122.3 ± 7	–	54 ± 8	–	–	–	3.4 ± 0.9	2 ± 0	–	–
D. acuminata	NWFSC 807	–	71 ± 2	–	55 ± 6	–	–	–	1.2 ± 0.3	7 ± 1	–	–
D. acuminata	NWFSC 808	–	105 ± 5	–	22 ± 2	–	–	–	1.4 ± 0.9	6 ± 0	–	–
D. acuminata	SB3	–	57 ± 3	–	19 ± 3	–	–	–	1.3 ± 0.7	3 ± 0	–	–
D. fortii	NWFSC 803	–	22 ± 5	–	220 ± 5	–	–	–	3.9 ± 0.2	58 ± 8	–	–
D. fortii	NWFSC 804	–	31 ± 2	–	246 ± 65	–	–	–	2.1 ± 0.9	20 ± 2	–	–
D. norvegica	NWFSC 814	–	–	–	155 ± 23	–	–	–	–	15 ± 2	–	–
D. norvegica	NWFSC 816	–	–	–	182 ± 10	–	–	–	–	18 ± 1	–	–
D. norvegica	DNBHD3F	–	–	0.5 ± 0.1	–	10 ± 1	–	–	–	–	1 ± 0	–
D. norvegica	DNBHFE6	–	–	0.4 ± 0.1	–	15 ± 4	–	–	–	–	2 ± 0	–
D. ovum	DOSS 3195	44 ± 2	–	–	–	–	–	53 ± 15	–	–	–	–
D. ovum	DOSS 2206	34 ± 2	–	–	–	–	–	35 ± 4	–	–	–	–
D. caudata	DCSS 3191	–	–	–	–	–	76 ± 2	–	–	–	–	1 ± 0

[a]Intracellular and extracellular extracts underwent alkaline hydrolysis, and therefore, OA/DTXs are reported as the sum of free and esterified OA or DTX analogs. Toxin values below the limit of detection are indicated as "–."

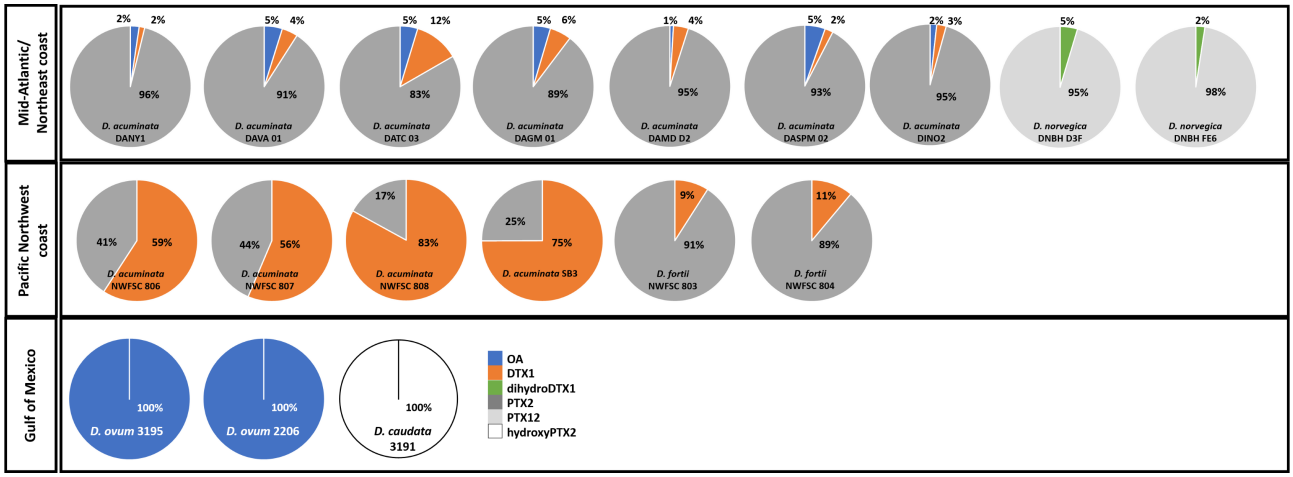

FIGURE 3 The percent composition (%) of intracellular OA, DTX1, dihydroDTX1 (free + esterified) and PTXs for each *Dinophysis* strain from the three US coastal regions. The colors indicate the toxins identified.

In contrast, the two *Dinophysis norvegica* strains isolated from the Atlantic Northeast coast, DNBHD3F and DNBHFE6, produced only dihydroDTX1 (0.5 and 0.4 pg· cell^{-1}, respectively) and putative PTX12 ([36*S, R*]-38[47]-dehydro-PTX2; 10 and 15 pg· cell^{-1}, respectively), which was unique for both OA/DTXs and PTXs among the United States isolates examined here (Tables 3 and S3). In addition, PTX12 was the dominant toxin, accounting for 95% and 97% of the total toxin content in strains DNBHD3F and DNBHFE6, respectively (Figure 3).

The highest OA content of all 20 strains was found in the two Gulf Coast *Dinophysis ovum* isolates DOSS 3195 and DOSS 2206, with OA being the only toxin detected in the intracellular (44 and 34 pg· cell^{-1}, respectively) or extracellular (53 and 35 pg· cell^{-1}, respectively) fractions and no esterified forms detected (Table S3). The OA content in *D. ovum* was, on average, two orders of magnitude greater than *D. acuminata* strains from the Northeast/Mid-Atlantic and Pacific Northwest coasts, where it only accounted for 1%–5% of the total cellular toxin content (Figure 3). Finally, the Gulf coast strain, *D. caudata*, did not produce any of the measured OA/DTXs but did contain a high amount of intracellular hydroxyPTX2 (76 pg· cell^{-1}; Table 3).

Maximum concentrations of extracellular OA/DTXs and PTX2 were produced by cultures that also had the highest intracellular content (Table 3). The highest extracellular PTX2 concentrations were found in the Pacific Northwest coast *D. fortii* strains NWFSC 803 (58 ng ·mL^{-1}) and NWFSC 804 (20 ng· mL^{-1}) and the Pacific Northwest coast *D. norvegica* strains NWFSC 814 (15 ng · mL^{-1}) and NWFSC 816 (18 ng · mL^{-1}; Table 3). The highest extracellular concentrations of DTX1 were found in *D. fortii* and the four Pacific Northwest coast *D. acuminata* strains (NWFSC 806, NWFSC 807, NWFSC 808, and SB3) with amounts ranging from 1.2 to 3.9 ng · mL^{-1} (Table 3). In addition, *D. ovum* strains (DOSS 3195 and DOSS 2206) contained the highest concentration of extracellular OA (53 and 35 ng · mL^{-1}, respectively), and were one order of magnitude greater than the average OA concentrations found in the seven Northeast/Mid-Atlantic coast *D. acuminata* strains. PTX12 was detected in the extracellular fraction in *D. norvegica* strains DNBHD3F and DNBHFE6 at concentrations of 1 ng · mL^{-1} and 2 ng · mL^{-1}, respectively; however, dihydroDTX1 was not detectable.

The percentage of toxins in esterified forms was calculated by comparing the OA/DTX profiles from the original intracellular extracts to aliquots that underwent alkaline hydrolysis. *Dinophysis fortii* and *D. acuminata* isolates (from both Northeast/Mid-Atlantic and Pacific Northwest coasts) had the highest percentages of OA/DTXs in esterified forms, ranging from 70% to 90% (Table S4 in the Supporting Information). In contrast, the two Northeast Atlantic *D. norvegica* isolates (DNBHD3F and DNBHFE6) contained mostly unesterified dihydroDTX1, with only 42% and 43% esters, respectively. However, separately prepared samples of these isolates contained only esterified forms of dihydroDTX1 by LC–HRMS, suggesting that the degree of esterification can be variable, possibly due to other factors such as the growth and feeding stage of the culture and small variations in sampling procedure. No esterified forms were found in *D. ovum* strains which produced only unesterified OA (Table S4). As described above, the two Pacific Northwest *D. norvegica* strains (NWFSC 814 and NWFSC 816), as well as the *D. caudata* strain (DCSS 3191) from the Gulf of Mexico did not contain any OA/DTXs, even after alkaline hydrolysis (Table S4).

DISCUSSION

This study characterizes the morphological features and toxin profiles of 20 strains representing five species

of *Dinophysis* isolated from three distinct coastal regions of the United States. Three of these species have been associated with shellfish harvesting closures in the United States over the past 14 years (Campbell et al., 2010; Lloyd et al., 2013; Trainer et al., 2013; Wolny et al., 2020; Table 4). Supplemental genetic analysis was used to confirm species identifications in cases where morphologic and toxin profile patterns deviated from those previously described. Using these multiple lines of evidence, isolates were identified as *D. acuminata*, *D. fortii*, *D. ovum*, *D. caudata*, or *D. norvegica*.

Species determination

In this study, as in previous studies, it was shown that large *Dinophysis acuminata* cells can appear similar morphologically to small *D. norvegica* cells in situ, particularly along with the Northeast/Mid-Atlantic coast (Wolny et al., 2020). Within this geographic region there seems to be greater morphological variability between individual cells and a greater departure from the type descriptions provided by Claparède and Lachmann (1859). Similarly, visual discrimination between in situ *D. acuminata* and a small morphotype of *D. fortii* from the Pacific Northwest coast can also be challenging. Understanding species' variability in morphology and toxigenicity helps protect public health by guiding correct species identification.

One commonly used genetic approach for dinoflagellate species identification is sequencing of ribosomal SSU/LSU rDNA and ITS regions (e.g., John et al., 2014; Litaker et al., 2009). Previous studies of *Dinophysis* have shown that these regions can identify many, but not all, of the species occurring in the United States (Handy et al., 2009; Wolny et al., 2020). In this study, ITS1-LSU sequencing confirmed the identity of *D. acuminata* and *D. fortii* from the Pacific Northwest coast, two species with statistically distinct but overlapping morphologic characters and similar toxin profiles. Sequencing of ITS1-LSU regions was also consistent with the region-specific differences in toxin profiles within the same species (*D. acuminata* and *D. norvegica* from the Northeast/Mid-Atlantic and Pacific Northwest coasts).

The *Dinophysis acuminata* complex is a grouping devised by Lassus and Bardouil (1991) to accommodate numerous small *Dinophysis* species with overlapping morphologies, including *D. acuminata*, *D. sacculus*, and *D. ovum*. Because ITS1-LSU sequences are not differentiated within the *D. acuminata* complex, *D. acuminata* and *D. ovum* isolates from this study, as well as cultured isolates and samples from field material in previous studies by our group, were discriminated according to their morphology and toxin profiles, as discussed in more detail in Wolny et al. (2020) and as done in other global regions with *Dinophysis* blooms (Fernández et al., 2019; Uchida et al., 2018). For example, Park et al. (2019) named their Korean cultures of *D. ovum* based on comparable overall cell measurements to those reported for *D. ovum* in Spanish waters by Raho et al. (2008). However, Park et al. (2019) noted that both *D. ovum*-like and *D. acuminata*-like cells, as described by Raho et al. (2008) were present in monoclonal cultures, and they suggested that morphological features cannot be used to distinguish between these species. In this study, strains of *D. ovum* were statistically distinct from *D. acuminata* based on morphometrics and produced only OA, whereas all *D. acuminata* strains produced OA and/or DTX1, and PTX2, in varying combinations depending on location (Figure 3; Table 3). Further investigation is needed, and work is ongoing to identify genome regions that reliably differentiate species within the *D. acuminata* species complex, perhaps including other morphologically similar cryptic species.

Growth rate and biovolume

Dinophysis species with larger cell volumes (i.e., *D. fortii*, *D. caudata*, *D. norvegica*, and the Pacific Northwest *D. acuminata* strains) grew about twofold slower than the smaller species evaluated in this study (Table 2; Figure 2). Differences in biovolume were substantial, with the largest *Dinophysis* isolate (i.e., *D. fortii* and *D. caudata*) being approximately three times larger than the smallest isolates (i.e., *D. acuminata* from the Northeast/Mid-Atlantic coasts and *D. ovum*).

These differences may not be apparent in situ; however, because all isolates were grown under the same culturing conditions (i.e., Mid-Atlantic water used for f/2 medium, 15°C, salinity of 25, 100 µmol photons $\cdot m^{-2} \cdot s^{-1}$ PAR, and the same ciliate and cryptophyte prey) rather than under region-specific conditions or prey-matched species that could modulate growth rate, biovolume, and toxin production (Basti et al., 2015, 2018; Fiorendino et al., 2020; Gaillard et al., 2021; García-Portela et al., 2018, 2020; Hattenrath-Lehmann et al., 2010, 2015; Hattenrath-Lehmann & Gobler, 2015). Measured growth rates were, therefore, neither optimized nor necessarily reflective of species' behavior in situ. Instead, uniform, controlled conditions were applied to obtain an initial direct comparison of the growth rates and biovolumes for the 20 isolates. Studies are underway to characterize growth and toxin production of these isolates under a variety of physicochemical conditions to better assess their potentials for bloom formation, toxin production, and ecological success in rapidly changing coastal ecosystems.

TABLE 4 Historical shellfish harvesting closures since 2002 and current guidelines for each US coastline impacted by *Dinophysis* blooms and the threat of DSP.

Region of US coastline	Dominant *Dinophysis* species during closure(s)	Years with harvesting closures due to OA/DTXs since 2002	Max. *Dinophysis* spp. cell count ($\times 10^3$ cells · L^{-1})	Max. OA/DTXs in commercial species (ng OA eq./g)	Duration of harvesting closures (d), year of closure indicated in parentheses	Commercial shellfish spp. impacted by closure(s)[a]	Conc. of *Dinophysis* spp. that trigger increased sampling or precautionary closure ($\times 10^3$ cells · L^{-1})
Long Island Sound, NY	*D. acuminata*	2011[1, STX] 2019[b]	2,120,500	1,245 (2012)	Area already closed for 30 d due to STX in shellfish (2012) ND (2019[2,b])	blue mussels, Eastern oysters, clams, scallops, whelks, conchs, moon snails	NA
Chesapeake Bay and VA/MD/DE coastal bays	*D. acuminata*	2002[^3] 2022	236,677	Traces of OA[b]	19 (2002[3,b])	Eastern oysters	10 (VA, MD), NA (DE)
Nauset Marsh Estuary, MA	*D. acuminata*	2015[b], 2019[b] 2022	>1,000	190 (2015[b]) 120 (2019[b]) 580 (2022[b])	36 (2015[b]) 10 (2019[b]) 57 (2022[b])	blue mussels, Eastern oysters, hard clams	50
Sequim Bay, WA	*D. acuminata*	2011[4,c], 2012[4], 2013, 2014, 2017, 2019, 2021	9	1,600[d] (2011) 927 (2012) 310 (2014) 1,080 (2017) 160 (2019) 390 (2021)	25 (2011) 118 (2012) 57 (2014) 69 (2017) 20 (2019) 15 (2021)	Manila clams, Pacific oysters, blue mussels, rock scallop	1
Puget Sound (all sites except Sequim Bay)	*D. acuminata*	2012, 2014, 2016, 2017, 2021	5	920 (2012) 170 (2014) 210[e] (2016) 210 (2017) 190 (2021)	98 (2012) 50 (2014) 50 (2016) 57 (2017) 14 (2021)	blue mussels, Pacific oysters, geoduck clam (gut ball)	1
Gulf of Maine, ME	*D. norvegica*	2016[5,b,g], 2018[5,b,g]	54	1,080[f] (2016[b]) 909[f] (2018[b])	16 (2016[b]) ND (2018[b])	blue mussels, Atlantic surf clams, Eastern oysters	15
Gulf of Mexico, TX coastal bays	*D. ovum*	2008[6,7,8], 2010, 2011[b], 2014[b], 2020[b]	>300	470 (2008) 280 (2010)	35 (2008) 22 (2010) 19[g] (2011[b]) 49[g] (2014[b]) 64[g] (2020[b])	eastern oysters	5

Note: References: 1 - Hattenrath-Lehmann et al. (2013); 2 – Severino, 2019; 3 - Tango et al. (2004); 4 - Trainer et al. (2013); 5 - Deeds et al. (2020); 6 - Deeds et al. (2010); 7 - Campbell et al., 2010; 8 - Swanson et al., 2010 STX indicates saxitoxin and its analogs.

ND indicates that no data are available.

NA indicates that the state's management plan for *Dinophysis* does not include a cell count trigger.

[a]Species included in these harvesting bans/closures were either because toxins were detected >160 ng OA eq./g, or because the restriction was broadly extended to all commercial species in the harvesting area based on toxin in excess of guidance levels in a sentinel species.

[b]Indicates a precautionary closure based on elevated *Dinophysis* spp. cell counts, toxin levels above guidance in sentinel species only, and/or low levels of OA/DTXs in harvestable shellfish.

[c]In 2011, 92 dozen oysters and 263 kg of Manila/little neck clams were recalled (pers. comm, Jerry Borchert, WA State Dept of Health, 4 April 2022).

[d]Based on an illness investigation (pers. comm, Jerry Borchert, WA State Dept of Health, 4 April 2022).

[e]Geoduck clam closure, toxins measured in gut ball only (pers. comm., Jerry Borchert, WA State Dept of Health, 4 April 2022).

[f]OA toxicity equivalents for dihydroDTX1 are currently not established. This value assumes equivalent potency to OA.

[g]Starting in 2011, management plan changed to precautionary closures based on cell counts above threshold, with testing in shellfish to confirm below guidance levels when needed to re-open.

Toxicity of *Dinophysis* species

The Pacific Northwest *Dinophysis acuminata* and *D. ovum* from the Gulf of Mexico contained the greatest toxin concentrations on a per-cell basis (Table 3; Figure 3). Other species contained OA/DTXs in lower concentrations (e.g., Northeast/Mid-Atlantic *D. acuminata*), including the as-yet only partially characterized congener, dihydroDTX1 (Northeast Atlantic *D. norvegica*). PTX2, putative PTX12 (dehydroPTX2), and an unidentified hydroxyPTX2 analog were also present in various isolates (Table 3 and Figure 3). PTXs are lipophilic toxins that appear to be less toxic than OA/DTXs to humans (Miles, Wilkins, Munday, et al., 2004), but which may still severely impact marine life (Gaillard et al., 2020; Pease et al., 2022).

Conservation of intracellular toxin profiles was observed within each *Dinophysis* species by region, where available, in this study (Figure 3). The region-specific toxin profiles reported here (under standardized culturing conditions) were similar to previous laboratories or field investigations, suggesting region-specific toxin profiles for this genus may be largely conserved and not highly responsive to environmental conditions. Previously characterized *D. acuminata* populations from the Northeast/Mid-Atlantic coasts also contained mostly PTX2, with lesser amounts of OA and DTX1 in both water samples and laboratory cultures (Fux et al., 2011; Hattenrath-Lehmann et al., 2013; Wolny et al., 2020). Also, in line with profiles reported here, cultures and shellfish extracts from the US Pacific Northwest coast contained only DTX1 and PTX2 (Trainer et al., 2013). On the Gulf coast of Texas, OA was the sole OA/DTX analog reported from *D. ovum*, which is thought to be the primary DSP producer in this region (Campbell et al., 2010; Deeds et al., 2010; Fux et al., 2011). In addition, *D. acuminata* complex isolates from laboratory and field studies in China (Gao et al., 2018), Japan (Kamiyama & Suzuki, 2009; Nagai et al., 2011), Norway (Miles, Wilkins, Samdal, et al., 2004) and New Zealand (Mackenzie, 2019) had comparable toxin profiles to those found in this study, including the presence of PTX2 and OA/DTXs. Exceptions exist globally, however, as blooms of *D. acuminata* in Sweden (Lindahl et al., 2007), Spain (Raho et al., 2008), and northern France (Marcaillou et al., 2001) have been associated with only OA, and *D. acuminata* isolates from Denmark (Nielsen et al., 2012), Argentina (Fabro et al., 2016), and Chile (Blanco et al., 2007; Fux et al., 2011) only contained PTX2.

Previous work by Deeds et al. (2020) detected dihydroDTX1 in two additional isolates of *Dinophysis norvegica* (DNBHFB4 and DNBHB3F) from the central coast of the Gulf of Maine on the Northeast Atlantic coast. Deeds et al. (2020) also identified PTX2 in these *D. norvegica* isolates; however, the current study tentatively identified PTX12 in isolates of *D. norvegica* (DNBHD3F and DNBHFE6; Tables 1 and 3) with no detectable PTX2. The current study used LC–HRMS to initially identify PTX12, whereas Deeds et al. (2020) used an LC–MS/MS method, which targeted PTX2 specifically. Furthermore, the two isolates tested in the study of Deeds et al. (2020) were found to have an average of 37% of the total cellular toxin quota as PTXs, whereas the *D. norvegica* strains from this study had an average of 96%, suggesting that only a fraction of the total PTXs was being measured in that initial study. Unfortunately, the two isolates studied by Deeds et al. (2020) have been lost and are no longer available to test for the presence of PTX12, but it is likely that PTX12 was misidentified as PTX2 in that study.

On a per-cell basis, Pacific Northwest coast *Dinophysis acuminata* and *D. fortii* were found to contain the highest loads of total OA/DTXs (OA, DTX1, dihydroDTX1 and their esters; one-way ANOVA, $F_{7,16}=48.31$, $p=1.4\times10^{-9}$; Tukey HSD test, $p<0.05$). The *D. ovum* strains from the Gulf of Mexico contained nearly as much toxin per cell but were smaller overall. Normalizing by biovolume, the Pacific Northwest coast *D. acuminata* and Gulf of Mexico *D. ovum* cells had the highest amounts of OA/DTXs, while *D. fortii*, the largest cells in this study, had somewhat lower OA/DTXs loads (one-way ANOVA, $F_{1,9}=287.3$, $p=3.9\times10^{-8}$; Tukey HSD test, $p<0.05$; Table S4). Growth rates for these three more toxic species, however, were relatively low under the culturing conditions when compared to the other isolates. Caution should be taken in extrapolating these growth rates to field conditions or assessing risk for a bloom or DSP, as all isolates were grown under standardized (i.e., nonoptimal) culturing conditions. It is also important to note that if phytoplankton or water samples from the field are not subjected to alkaline hydrolysis prior to toxin analysis, total OA/DTXs levels may be underestimated because esterified precursors present in most of these species will not be detected (Table S4). In agreement with the current study, several studies of *Dinophysis* cultures and blooms have indicated that a high proportion of the OA/DTXs can be in the esterified form (Deeds et al., 2020; Miles et al., 2006) as is also often the case for OA/DTX-producing *Prorocentrum* spp. (Hu et al., 1992; Kilcoyne et al., 2020; Suárez-Gómez et al., 2001).

Total PTX loads were distributed somewhat differently across the surveyed isolates. Cells from the Pacific Northwest coast *Dinophysis fortii* (PTX2) and *D. norvegica* (PTX2) isolates contained the highest quotas of intracellular and extracellular PTXs (one-way ANOVA, $F_{7,16}=32.7$, $p=2.6\times10^{-8}$; Tukey HSD test, $p<0.05$; Table 3). Except for the two Atlantic Northeast coast *D. norvegica* strains and two Pacific Northwest coast *D. acuminata* strains (NWFSC 808 and SB3), which had the lowest PTX content on a biovolume basis,

there was no difference in PTX concentration on a per-volume basis (14 isolates, one-way ANOVA, $F_{3,38}=2.6$, $p=0.07$; Tukey HSD test, $p>0.05$; Table S4).

Across PTX analogs, PTX2 was most prevalent, found in 15 of the 20 isolates, three species, and two of the three US coastal regions. The other PTXs detected, hydroxyPTX2 and PTX12, were found only in one species each, from two coastal regions (Table 3). Toxins from the PTX-group are frequently found in shellfish along with toxins from the OA-group. Since there have been no reports of human intoxication by PTXs, there are currently no regulations restricting the presence of this toxin group in shellfish in the United States (Trainer et al., 2013) or, with a recent change, the European Union (European Commission, 2021). Purified PTX2, on the other hand, has been shown in recent studies to affect the development and survival of the early life stages of a variety of aquatic organisms negatively, including the bivalves *Crassostrea virginica* (Eastern oysters; Pease et al., 2022), *C. gigas* (Pacific oysters; Gaillard et al., 2020), and the finfish *Cyprinodon variegatus* (Gaillard et al., 2020). It should also be noted that although PTX2 showed no detectable toxicity to mice via the oral route, it was very toxic when injected intraperitoneally (Miles, Wilkins, Munday, et al., 2004) and is a potent blocker of polymerization of the protein actin that is a crucial component of the cytoskeleton (Allingham et al., 2007; Hori et al., 2018). These findings suggest that exposure to PTX2 has the potential to cause significant harm to some aquatic organisms.

There have been few studies of the effects of PTX analogs other than PTX2 and its seco acid derivative; thus, the effects of these other PTXs on marine organisms require further investigation. Further work is also needed to discriminate among PTXs. For example, it is likely that PTX12 has sometimes been misidentified as PTX2 due to the similarity in structure and molecular weight for these two compounds ($[M+NH4]^+$ *m/z* 876.5 for PTX2 and $[M+NH_4]^+$ *m/z* 874.5 for PTX12) (Miles, Wilkins, Samdal, et al., 2004). Though not common across the surveyed species, PTX12 dominated the toxin profiles of the two Atlantic Northeast *Dinophysis norvegica* strains.

DSP harvesting closures

To date, DSP caused by the genus *Dinophysis* is considered a relatively new threat in the United States. Dedicated laboratory and field research on *Dinophysis* did not begin in earnest nationally until OA/DTXs were detected for the first time in the Gulf of Mexico in 2008 (Campbell et al., 2010; Deeds et al., 2010). During this event, which was linked to the presence of *D. ovum*, concentrations of OA/DTXs in shellfish (470 ng/g OA) exceeded the FDA regulatory guideline (160 ng/g OA eq.) and resulted in a 35-day closure of recreational and commercial shellfish harvesting as well as product recalls of Eastern oysters; however, no human illnesses were reported (Table 4). Four shellfish harvesting closures have since occurred along with the Gulf coastline, ranging between 19 and 64 days. Closures or precautionary closures have now occurred on all coasts of the United States, with the Pacific Northwest coastline (Puget Sound, including Sequim Bay, WA) experiencing prolonged closures, ranging between 14 and 118 days. The Northeast/Mid-Atlantic coastline has not undergone a sustained closure due to OA/DTXs, but has experienced precautionary closures, lasting 10–36 days during the 4 years since 2008, concentrated in the Long Island Sound, NY (1), Nauset Marsh Estuary, MA (2), and Gulf of Maine, MA (2). The Chesapeake Bay, VA underwent a precautionary closure for 19 days in 2002, but the Delmarva region (DE, MD, and VA) has not experienced a closure since. While the Gulf coast has the longest history of enduring DSP events, the Northwest Pacific coastline has undergone the longest closure (118 days) and been impacted for the greatest number of years (7) thus far. Overall, six closures (precautionary or prolonged) have occurred across all three coasts from 2018 to 2021, demonstrating the issue has had recent impact on commercial fisheries. Bivalve molluscan shellfish and gastropod fisheries impacted by OA/DTXs include surf clams (*Spisula solidae*), hard clams (*Mercenaria mercenaria*), Manila clams (*Venerupis philippinarum*), blue mussels (*Mytilus edulis*), Eastern and Pacific oysters (*Crassostrea gigas* and *C. virginica*), geoduck (*Panopea generosa*), scallops (*Pecten maximus*), moon snails (*Euspira heros*), conchs (*Strombus Linnaeus*), and whelks (*Buccinum undatum*; Table 4). Harvest closures have impacted these species either because toxins were detected in the edible tissues above the FDA's regulatory guidelines or because the restriction was broadly extended to all commercial species in the harvesting area based on an elevated toxin load (above FDA's guidelines) in a sentinel species.

To date, *Dinophysis* blooms in US waters have been the most concentrated along with the Northeast/Mid-Atlantic and Gulf of Mexico coastlines (Table 4). In contrast, the Pacific Northwest coast has had lower *Dinophysis* bloom densities (maximum *Dinophysis* cell count equivalent to 9×10^3 cells $\cdot$ L^{-1}; Table 4), but more frequent and prolonged closures and the highest recorded OA/DTX load in shellfish meat (1600 ng/g OA eq in shellfish meat in 2011; Trainer et al., 2013). Also, in 2011, one of the most concentrated *Dinophysis* blooms ever recorded in the US waters was detected in Long Island Sound, NY. Maximum cell concentrations of *D. acuminata* were recorded at 2.12×10^6 cells $\cdot$ L^{-1}, corresponding to OA/DTXs concentrations of 1245 ng/g OA eq. in edible shellfish meats (Table 4). In Nauset Marsh estuary a cell density of $>10^6$ cells $\cdot$ L^{-1} of *D. acuminata* was recorded during a bloom in 2015. This

was associated with toxin in sentinel shellfish exceeding FDA safety thresholds (190 ng/g OA eq. in shellfish meat; Table 4). Interestingly, elevated concentrations of *Dinophysis* spp. are not consistently linked to harmful levels of toxins in shellfish, as was seen with the precautionary closure in the Chesapeake Bay, VA, in 2002 (elevated abundance, maximum of 236×10^3 cells $\cdot$ L^{-1}, but only trace OA/DTXs in shellfish; Tango et al., 2004). Similarly, Wolny et al. (2020) reported blooms of *D. acuminata* in the MD and DE coastal bays (2015–2016) ranging from 40×10^3 $\cdot$ cells L^{-1} to 1700×10^3 cells $\cdot$ L^{-1} with toxins in shellfish reaching only 0.5–2 times the FDA's guidance level and only in noncommercial species and/or in noncommercial harvesting areas. In contrast, lower-abundance but highly toxic blooms of *Dinophysis* spp. (mainly *D. acuminata*) have occurred regularly in Puget Sound, WA, since 2011 with a maximum of 5×10^3 cells $\cdot$ L^{-1} but an average maximum toxin load 3.5 times the FDA's guideline in shellfish meat (Table 4 and references therein). In 2011, a bloom of *Dinophysis* spp. with a maximum abundance of 9×10^3 cells $\cdot$ L^{-1} from Sequim Bay, WA, resulted in the first DSP illness in the United States when three persons fell ill after eating blue mussels (*Mytilus edulis*) containing OA/DTX levels 10 times the FDA guidance level (1600 ng/g OA eq. in shellfish meat; Table 4). This incident resulted in great economic impact, including the closure of recreational and commercial shellfish harvesting sites, as well as the recall of 92 dozen oysters and 263 kg of Manila clams (*Venerupis philippinarum*; Shultz et al., 2019; Trainer et al., 2013; Table 4).

Different management strategies are currently employed among affected regions in the United States (Table 4). For example, the Northeast/Mid-Atlantic region requires a maximum cell concentration of 10–50×10^3 cells $\cdot$ L^{-1} to trigger increased water sampling or precautionary closures before analyzing the toxin concentration accumulated in the shellfish. Alternatively, when 1×10^3 *Dinophysis* cells $\cdot$ L^{-1} and 5×103 *Dinophysis* cells $\cdot$ L^{-1} are observed in water samples along with the Pacific Northwest and Gulf of Mexico coasts, respectively, the respective health departments and management authorities in these areas initiate additional sampling and/or precautionary closures. Due to differences in methods for water sampling and cell enumeration between monitoring programs, current *Dinophysis* bloom concentrations may not be comparable. Recent studies have reported high *Dinophysis* abundances in subsurface waters as thin-layer aggregates just above the pycnocline (Broullón et al., 2020; Díaz et al., 2021; Farrell et al., 2014). For instance, Díaz et al. (2021) showed that in the Puyuhuapi Fjord in Chilean Patagonia, the highest ever recorded cell abundance of *D. acuta* (664×10^3 cells $\cdot$ L^{-1}) was associated with stratification at the pycnocline (at ca. 8 m depth). Therefore, the surface-sampling method typically used in US monitoring programs may underestimate *Dinophysis* cell concentrations, particularly when they form thin layers at depth (Marshall & Egerton, 2009; Rines et al., 2010). Hence it is important to consider the vertical distribution of *Dinophysis* populations for future estimations and monitoring of blooms in the United States.

CONCLUSIONS

Overall, shellfish harvesting area closures due to *Dinophysis* blooms or accumulation of OA/DTXs in edible shellfish meat have had recent impacts to fisheries along with all three coastlines of the United States. The profiling here of geographically distinct laboratory isolates demonstrated varying dominant toxin analogs based on coasts, with surprising conservation within a species and area. Generally, *D. acuminata* and *D. fortii* from the Pacific Northwest and *D. ovum* from the Gulf of Mexico contained the most OA/DTXs per cell. The *D. acuminata* cells from the Northeast/Mid-Atlantic, generally had lower cell quotas but still have the potential to impact fisheries once higher abundances are met in situ, as seen in Hattenrath-Lehmann et al. (2013; Table 4). For *D. norvegica*, the toxicity of dihydroDTX1 relative to the currently regulated analogs OA, DTX1 and DTX2 remains to be assessed as does the wider occurrence of this congener beyond the Northeast Atlantic. Thus far, isolates of *D. norvegica* from the Pacific Northwest coast have not been found to produce dihydroDTX1. From the comparison of US harvesting closures, it is evident that FDA toxin regulatory guidelines in edible shellfish meat can be exceeded under low cell concentrations of *Dinophysis* spp., ($<10\times10^3$ cell $\cdot$ L^{-1}; Pacific Northwest coast) and high-cell concentrations do not always lead to shellfish toxicity ($>100\times10^3$ cell $\cdot$ L^{-1}; Northeast/Mid-Atlantic coast). More research is needed to assess the environmental parameters that lead to optimal growth of each species and to better understand species-specific bloom dynamics that lead to accumulation of OA/DTXs in US seafood products.

AUTHOR CONTRIBUTIONS

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. A high-level graphical image of the alignment is used to compare sequences and SNPs. Portions that are black indicate a different base relative to some other sequence in the alignment at that position. The alignment has been annotated to denote the various regions of the ribosomal genes/spacers including SSU: Small subunit, ITS1: Internal Transcribed Spacer 1, 5.8S, ITS2: Internal Transcribed Spacer 2, and LSU: Large Subunit. This alignment was generated in Geneious 10.2.3 using the MUSCLE plugin with default parameters. Alignment available upon request.

Figure S2. Left, LC–HRMS full-scan mass spectra (black lines) of PTX2 in *Dinophysis norvegica* NWFSC 814, hydroxyPTX2 in *D. caudata* DCSSL 122, and PTX12a and PTX12b in *D. norvegica* DNBH FE6. The circles show the fitted isotopic envelope *m*/*z* values and relative isotopomer intensities of the NH_4^+ (red) and Na^+ (blue) adduct ions obtained from the spectra with the NRC Molecular Formula Calculator for $C_{47}H_{70}O_{14}$ (PTX2), $C_{47}H_{70}O_{15}$ (hydroxyPTX2), or $C_{47}H_{68}O_{14}$ (PTX12). Only one viable formula was obtained for each compound. The main peak for each adduction cluster is marked with its accurate *m*/*z*, assigned formula, and mass error. Right, extracted-ion (*m*/*z* 874.4947, 876.5104, 879.4501, 881.4658, 892.5053, and 897.4607) LC–HRMS chromatograms showing the PTX2 in *D. norvegica* NWFSC 814, *D. caudata* DCSSL 122, and *D. norvegica* DNBH FE6.

Figure S3. LC–HRMS/MS product ion spectra of the ammonium adduct ions of PTX2 in *Dinophysis norvegica* NWFSC 814, hydroxyPTX2 in *D. caudata* DCSSL 122, and PTX12a and PTX12b in *D. norvegica* DNBH FE6 (see Figure S2). The fragmentation patterns establish the close similarity of hydroxyPTX2 and PTX12 to PTX2.

Figure S4. LC–HRMS/MS product ion spectra of the sodium adduct ions of PTX2 in *Dinophysis norvegica* NWFSC 814, hydroxyPTX2 in *D. caudata* DCSSL 122, and PTX12a and PTX12b in *D. norvegica* DNBH FE6 (see Figure S2). The fragmentation patterns establish the close similarity of hydroxyPTX2 and PTX12 to PTX2.

Table S1. Precursor and product ion exact masses for PTX2, PTX12, hydroxyPTX2, OA, DTX1 and 14,15-dihydroDTX1 in positive (PTXs, [M + NH4]+) or negative (OA/DTXs, [M − H]−) ionization mode.

Table S2. Sequence data quality control information for nine *Dinophysis* isolates.

Table S3. Pairwise % identity between *Dinophysis* sequences. Note that (1) ONLY sites that contain data in both sequences were compared; positions with missing data were ignored, and (2) polymorphic sites that include the base being compared to are still considered differences. (i.e., an M stands for an A or a C. If one sequence has an A and the other an M, this will be read as a difference in % identity calculation.)

Table S4. Intracellular toxin content of the 20 *Dinophysis* isolates expressed per cell and per biovolume. Data are means ± standard deviation, $n = 3$.

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