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The toxin goniodomin, produced by *Alexandrium* spp., is identical to goniodomin A

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ABSTRACT

In 1968 Burkholder and associates (*J. Antibiot. (Tokyo)* **1968**, *21*, 659–664) isolated the antifungal toxin goniodomin from an unidentified Puerto Rican dinoflagellate and partially characterized its structure. Subsequently, a metabolite of *Alexandrium hiranoi* was isolated by Murakami et al. from a bloom in Japan and its structure was established (*Tetrahedron Lett.* **1988**, *29*, 1149–1152). The Japanese substance had strong similarities to Burkholder's but due to uncertainty as to whether it was identical or only similar, Murakami named his toxin goniodomin A. A detailed study of this question now provides compelling evidence that Burkholder's goniodomin is identical to goniodomin A. Morphological characterization of the dinoflagellate suggests that it was the genus *Alexandrium* but insufficient evidence is available to make a definite identification of the species. This is the only report of goniodomin in the Caribbean region.

1. Introduction

The renowned microbiologist Paul R. Burkholder (Horsfall, 1975), while searching the Caribbean Sea for pharmacologically useful marine natural products, isolated a novel metabolite from the bloom of an unknown algal species, which he harvested in southwestern Puerto Rico (Burkholder et al., 1960, 1967). At the outset of these studies, Burkholder had already achieved remarkable success at locating pharmacologically valuable, anti-bacterial natural products, having discovered the potent antibacterial chloramphenicol. Parke-Davis Company carried out commercial development of this structurally unique antibiotic just in time to save the lives of countless soldiers infected with typhus during the Korean War. Many other anti-bacterial agents were discovered during that period but anti-fungal agents were more challenging. As a consequence, Burkholder was using an anti-fungal assay to search for useful natural products. Goniodomin was the product of this search.

Starting with a kilogram of wet cells, Burkholder and coworkers were able to purify 250 mg of goniodomin (Sharma et al., 1968) using an isolation procedure directed by activity against *Candida albicans*. Preliminary characterization of the compound was carried out but they had insufficient material to establish the structure with the primitive spectroscopic instrumentation available at that time. A few years after he collected cellular material from this massive bloom, an industrial accident occurred that eradicated living organisms from the waters (Cintrón et al., 1970). Life eventually returned but Burkholder's organism has never been observed again.

Two decades after Burkholder's report, Murakami and associates, working with a bloom of a dinoflagellate in a rock pool at Jōgashima Island in Japan, isolated and identified a substance with similar properties (Murakami et al., 1988). They identified the dinoflagellate as *Goniodoma pseudogoniaulax*, which is now considered synonymous with *Alexandrium pseudogonyaulax*, but the organism was later found to be *A. hiranoi* (Kita and Fukuyo, 1988; Murakami et al., 1998), a morphologically similar species. Unfortunately, Murakami and co-workers were unable to obtain a comparison sample of Burkholder's goniodomin. Burkholder had passed away in 1972 (Horsfall, 1975) and none of his samples or other materials still existed. Murakami et al. (1988) named their compound goniodomin A (GDA) due to uncertainty as to whether it was truly identical or only similar to Burkholder's goniodomin. The absolute configuration of GDA (**1**) was established later (Takeda, 2008; Takeda et al., 2008). Two additional *Alexandrium* species, *A. monilatum* (J. F. Howell) Balech, 1985 and *A. pseudogonyaulax* (Biecheler) Horiguchi ex Kita & Fukuyo, 1992, have since been found to produce GDA (MacKenzie et al., 2004; Bravo et al., 2006; Hsia et al., 2006; Hsia, 2008; Zmerli Triki et al., 2018). Blooms of the three species have been

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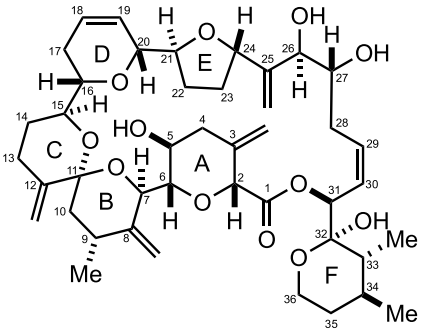

Fig. 1. Goniodomin A (**1**).

observed in estuarine waters in many locations around the world including the Eastern Pacific, North, Central and South Americas, the European North Sea and North Africa although never in the Caribbean Sea (Hsia, 2008; Hsia et al., 2006; Anderson et al., 2012; Krock et al., 2018; Kremp et al., 2019). Herein we present evidence that Burkholder's goniodomin is identical to GDA isolated from *A. monilatum* in the York River, VA (which is identical to GDA previously isolated from *Alexandrium* spp. in other locations (Hsia, 2008; Hsia et al., 2006; Takeda, 2008; Takeda et al., 2008; Zmerli Triki et al., 2018; Krock et al., 2018; Kremp et al., 2019) (Fig. 1).

2. Results and discussion

2.1. Isolation

In the isolation procedure reported by Sharma et al. (1968), the toxin was extracted with methanol and then further concentrated by partitioning between water and ethyl acetate. A detailed description of an open-column chromatographic separation using silica gel was provided. The first step employed elution with benzene, which removed substantial quantities of fast moving orange and yellow pigments. Further elution with ethyl ether immediately yielded first green and then orange pigments with goniodomin eluting mainly under the green pigments. Treatment of the goniodomin-rich fractions with ethyl ether caused the goniodomin to crystallize away from the pigments. Recrystallization from ethyl acetate-hexane followed by aqueous methanol gave analytically pure material.

During isolation of GDA from York River, VA, USA blooms of *A. monilatum*, chromatographic behavior was observed that was similar to that reported by Sharma et al. for goniodomin (1968), including the copious contamination by pigments. Attempts to remove the pigments by rechromatography on silica gel were unsuccessful even when elution was retarded by using ethyl ether-benzene mixtures. Treatment of enriched fractions with cold ethyl ether, as had been observed by Sharma et al. (1968), caused the GDA to crystallize, leaving a green pigment in solution. Additional trituration with ethyl ether yielded crystalline material that was only slightly discolored. A second form of goniodomin, termed goniodomin B (GDB), has been reported (Espiña et al., 2016). GDA isolated by the Sharma procedure is not contaminated by GDB which runs significantly slower than GDA on silica gel and more rapidly than GDA on C18 (Takeda, 2008).

2.2. Melting and solubility characteristics

Sharma et al. reported a melting point of 199 °C for goniodomin (1968). They observed the compound to be freely soluble in chloroform, benzene and methanol but sparingly soluble in ethyl ether and hexane (Sharma et al., 1968). Neither Murakami nor others reported a melting point for GDA (Murakami et al., 1988, 1998; Takeda, 2008; Takeda et al., 2008; Hsia, 2008; Hsia et al., 2006; Zmerli Triki et al., 2016). In fact, Takeda reported that GDA is a non-crystalline substance, with the implication being that there is some structural difference between Burkholder's goniodomin and GDA (Takeda, 2008). In the present study, GDA derived from *A. monilatum* was found to be crystalline when triturated with ethyl ether, giving microcrystals, m.p. 199.5 – 200 °C. It had solubility characteristics similar to Sharma's isolate, including the remarkably low solubility in ethyl ether.

2.3. Determination of empirical formula

Sharma et al. (1968) obtained an elemental analysis for goniodomin that was consistent with a formula of $C_{43}H_{60}O_{12}$ (Calcd: C 67.18 %, H 7.81 %; Found: C 66.58 %, H 7.71 %) but a high resolution mass spectrum of goniodomin indicated an empirical formula of $C_{43}H_{58}O_{11}$ (Calcd: *m/z* 750.3975; Observed: *m/z* 750.3938). They rationalized this difference as one water of hydration in the crystal rather than the mass spectrometer failing to produce a detectable molecular ion at *m/z* 768. Electron impact ionization, employed on instruments in that era, was notorious for causing loss of water from molecular ions of alcohols (Budzikiewicz et al., 1967, Chapt. 2).

Murakami et al. (1988) obtained an elemental analysis on GDA, which supported a $C_{43}H_{60}O_{12}$ empirical formula. This was confirmed by a secondary ion mass spectrum (SIMS) using protonated diethanolamine, which produced a parent ion at *m/z* 874 ($C_{43}H_{60}O_{12}$ + DEA + H^+) for the diethanolamine salt (Murakami et al., 1988). SIMS is a softer ionization method and frequently gives molecular ions in cases where electron impact gives dehydration products.

In the present study, electron impact ionization gave a weak molecular ion consistent with $C_{43}H_{60}O_{12}$ (calc *m/z* 768.4, obs *m/z* 768.4) and a stronger ion at *m/z* 750. With electrospray ionization, a high-resolution spectrometer produced intense cationated species establishing the $C_{43}H_{60}O_{12}$ empirical formula ($C_{43}H_{60}O_{12}$ + NH_4^+: calc *m/z* 786.4423, obs *m/z* 786.4411; $C_{43}H_{60}O_{12}$ + K^+: calc *m/z* 807.3716, obs *m/z* 807.3696).

2.4. Acetylation

Sharma et al. (1968) prepared the diacetyl derivative of goniodomin by treatment with acetic anhydride and pyridine. The empirical formula of the diacetate, established by elemental analysis as $C_{47}H_{64}O_{14}$ (Calcd: C 66.73 %, H 7.43 %, CH_3C = O 10.44 %; Found: C 66.23 %, H 7.89 %, CH_3C = O 10.43 %), indicates that the true empirical formula of goniodomin should be $C_{43}H_{60}O_{12}$, in conflict with their $C_{43}H_{58}O_{11}$ assignment. Murakami et al. (1988) also prepared the diacetate derivative of GDA. SIMS gave a molecular ion for the diethanolamine salt at *m/z* 958 ($C_{47}H_{64}O_{14}$ + DEA + H^+). Therefore, GDA is at least isomeric with, if not identical to, Burkholder's goniodomin. Using NMR, Murakami et al. (1988) established that acetylation had occurred on the hydroxyl groups on carbons 26 and 27, based on downfield shifts of H26 and H27 of the diacetate derivative relative to the spectrum of GDA. GDA has additional hydroxyl groups on C5 and C32. Steric hindrance makes them poor candidates for acetylation.

2.5. Infrared spectra

The infrared spectrum of Burkholder's goniodomin (Fig. 2a) showed a carbonyl stretching band near 1750 cm^{-1} and a broad stretching band for alcoholic hydroxyl groups between 3300 cm^{-1} and 3500 cm^{-1} (Sharma et al., 1968). The 1750 cm^{-1} band might reflect the presence of an ester or a ketone. The band was not eradicated by treatment with sodium borohydride leading Sharma et al. (1968) to conclude it reflected the presence of an ester or lactone. It should be noted that the lack of an intense OH stretching band near 3265 cm^{-1} in the infrared spectrum did not support water being present in the sample. Murakami et al. (1988) reported hydroxyl and ester groups for GDA at 3430 cm^{-1} and 1760 cm^{-1}, respectively. The infrared spectrum of GDA from *A. monilatum* (see Fig. 2b) confirms Sharma's and Murakami's reported

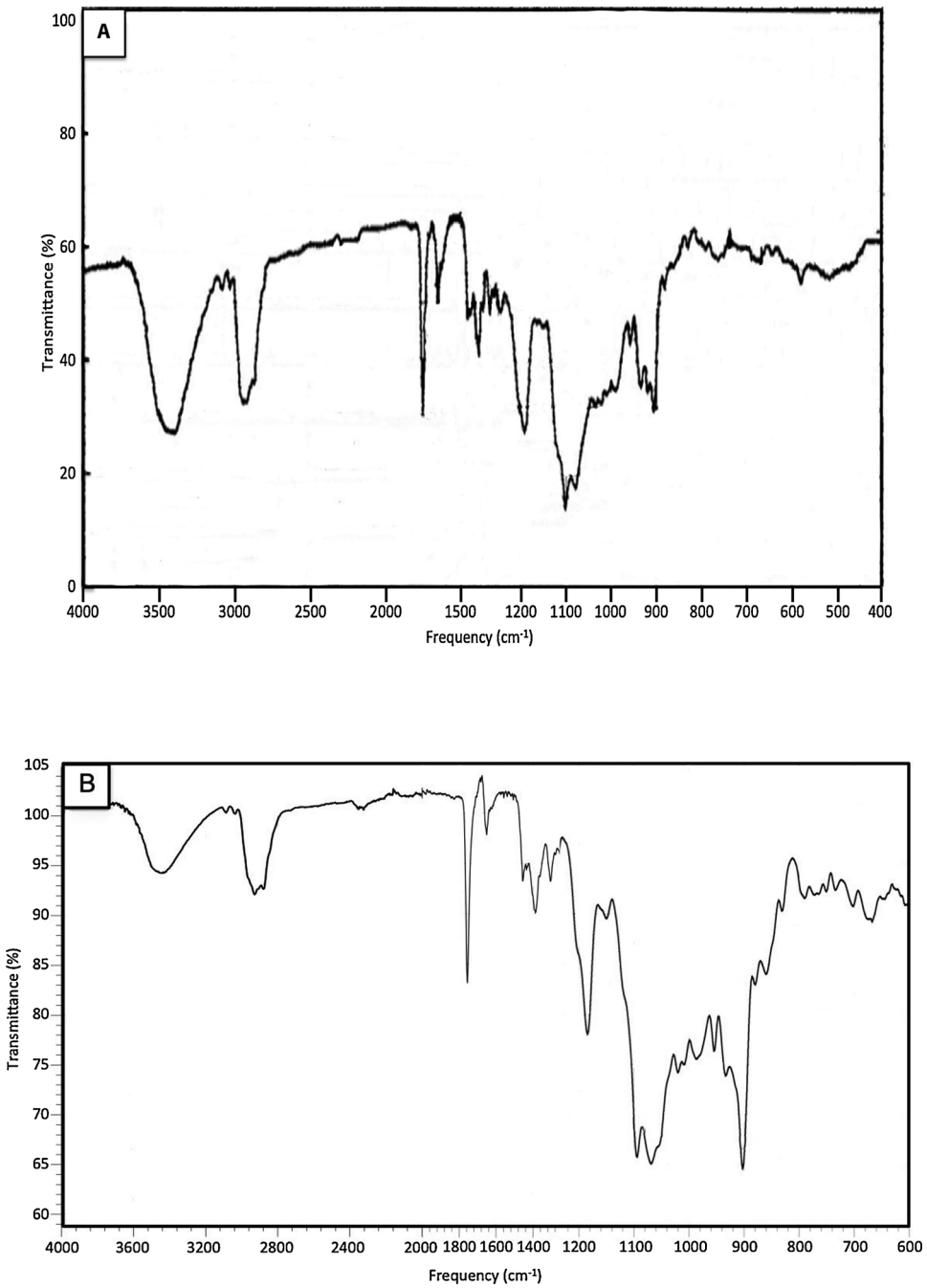

Fig. 2. Infrared spectra. A) Goniodomin (KBr pellet), figure adapted from Sharma et al. (1968). B) GDA (neat solid) from York River *A. monilatum*.

signals for the hydroxyl and ester groups and has absorption signals in the fingerprint region (1500 − 700 cm^{-1}) that correspond well to those reported by Sharma.

Sharma et al. (1968) reported strong hydroxyl infrared absorptions for the diacetate derivative of goniodomin at 3510 and 3575 cm^{-1}, suggesting that not all of the hydroxyl groups had been acetylated. Negligible water absorption near 3265 cm^{-1}, further supported the conclusion that the empirical formula of goniodomin is $C_{43}H_{60}O_{12}$ not $C_{43}H_{58}O_{11}$. They also observed absorption bands near 3450, 1760 and 1655 cm^{-1}. Murakami et al. (1988) reported that the infrared spectrum of the diacetate derivative of GDA (film) contained major absorption bands at 3450, 1760, 1740 and 1655 cm^{-1}.

2.6. ^{1}H NMR spectrum

The proton spectrum (Fig. 3a) of goniodomin, was a key element of the characterization data published by Sharma et al. (1968). NMR spectroscopy was in its infancy in the 1960's. Spectra were acquired on field-swept, continuous wave spectrometers at frequencies no greater than 100 MHz. Instrumental limitations led to low sensitivity and poor line shapes. Sensitivity could be increased by raising the r.f. power, but with risk of saturating singlets and other narrow lines thereby degrading the accuracy of integration. Additionally, high concentrations of analyte were needed which further degraded spectral quality. Nevertheless, Sharma's NMR spectrum of goniodomin revealed important details of the structure of goniodomin and now provides strong evidence for goniodomin being identical to GDA (Sharma et al., 1968).

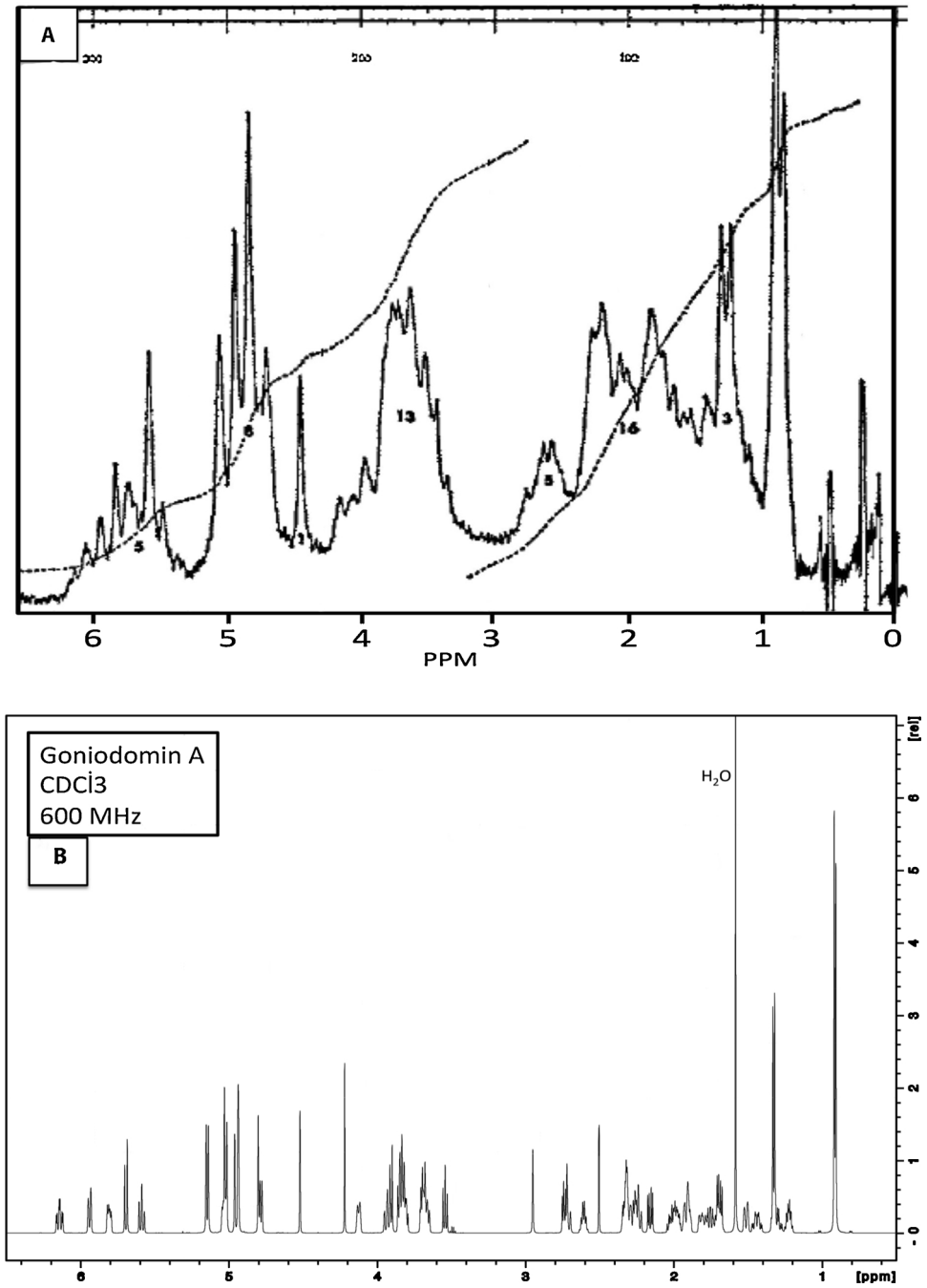

Fig. 3. NMR spectra. A) ^{1}H NMR spectrum of goniodomin (100 MHz, $CDCl_3$, D_2O exchanged). Adapted from Sharma et al. (1968). B) ^{1}H NMR spectrum of GDA (600 MHz, $CDCl_3$).

For comparison, a 600 MHz spectrum of GDA ($CDCl_3$) isolated from an *A. monilatum* bloom in Virginia is shown in Fig. 3b. The spectrum was acquired with a modern, digitally pulsed Fourier transform spectrometer. In addition, a 600 MHz ^{1}H spectrum of GDA acquired using a C_6D_6 solution is included in the Supplementary Material (Fig. S1). Comparison was also made with previously reported spectra for GDA in $CDCl_3$, CD_2Cl_2 and C_6D_6 (Table S1, Murakami et al., 1988; Hsia, 2008; Takeda et al., 2008), establishing that the GDA obtained from York River, VA was identical to material isolated from *A. hiranoi* (Japan) and *A. monilatum* (Gulf of Mexico).

Hydroxyl groups at 4.1 and 4.45 ppm were assigned on the basis of exchange with D_2O. In the upfield region of the spectrum of goniodomin (Fig. 3a) are a pair of overlapping methyl doublets at 0.93 ppm and a third one at 1.25 ppm all of which are also seen in GDA. The doublets at 0.93 have been assigned by Hsia et al. (2006) to the methyl groups on C34 and C35 of GDA and the doublet at 1.25 ppm to the methyl group on C9. For goniodomin, a group of signals between 5.3 and 6.3 ppm integrated for five protons. Sharma et al. (1968) correctly assigned four of the hydrogen atoms as a pair of cis olefins with vicinal coupling constants of 12 Hz. For GDA, Hsia et al. assigned these olefinic signals as H18-H19 and H29-H30 (5.81–5.97 and 5.59–6.16 ppm, respectively). The remaining signal in that cluster can now be assigned to H31, the lactone CH—O, observed by Hsia et al. (2006) at 5.71 ppm. The H31 signal is unusually far downfield due to deshielding by the double bond. The region between 4.3 and 5.3 ppm in the spectrum of goniodomin contains a singlet at 4.45 ppm, which can be assigned as H2 (4.53 ppm observed for GDA). Sharma et al. (1968) attributed the remaining four peaks to six protons but it is likely the four peaks are

due to eight protons rather than six. Peak intensities may have been suppressed by use of elevated r.f. power. The eight signals in this region have been assigned by Hsia et al. (2006) to the $=CH_2$ groups at positions 3, 8, 12 and 25. Sharma et al. (1968) assigned the unresolved group of signals between 3.0 and 4.3 ppm as 13 hydrogen atoms on oxygen-substituted carbon atoms. There are, in fact, 10 of them in that region. The three methyl groups plus 23 other hydrogen atoms on unsubstituted methylenes and methines lie between 0.7 and 3.0 ppm. Neither goniodomin nor GDA has any aromatic or other hydrogen atoms that would fall downfield of 6.3 ppm. Overall, after making allowances for the differences in the NMR spectrometers that were used for these studies conducted 50 years apart, good correspondence is observed between the ^1H spectra of goniodomin and GDA.

2.7. Fragmentation ions in the mass spectrum

Sharma et al. (1968) reported electron impact fragmentation data for goniodomin. Electron impact mass spectra arise from molecules that have been brought into the vapor state by heating under high vacuum. Electrons colliding with these molecules cause loss of an electron yielding cation-radicals from which 1) daughter cation-radicals can be formed by loss of neutral fragments or 2) paired free radical and cationic species can be formed by cleavage of carbon-carbon bonds. In both cases the daughter species can undergo further fragmentation. These cation-radicals and cations contain a wealth of structural information but interpretation of mass spectra of macrolides or other cyclic compounds is challenging because two bonds must be broken to create fragments (Schulz et al., 2017). The complexity of this situation is increased by the initially formed cation radical frequently losing water molecules to create multiple families of fragments. With GDA the presence of a double bond adjacent to C31 makes the alkyl-O linkage of the lactone vulnerable to cleavage under the high temperatures involved in electron impact ionization. Loss of a water molecule from the initially formed cation radical ($\mathbf{1}^{+\cdot}$) yields isomeric cation radicals **2** and **3** (Fig. 4). Alkyl-O cleavage of **2** and **3** yields carboxylic acid cation-radicals **4** and **5**, respectively, which are still the same mass (*m/z* 750) as **2** and **3**. Alternative structural assignments for **4** and **5** would include allenes formed by McLafferty rearrangement of **2** and **3** and oxiranes created by the 32-hydroxyl group participating in the alkyl-O cleavage reaction. Cation-radicals **4** and **5** can then undergo a cascade of further fragmentations.

Table 1 lists ions reported by Sharma et al. (1968) for goniodomin along with a similar list of major fragment ions observed in the electron impact spectrum of GDA. Although the two fragment lists were created using different samples on different generation instruments (an AEI MS9™ from the early 1960s for goniodomin versus a much newer Waters GCT Premier™ spectrometer for GDA), 90 % of the ions reported by Sharma et al. (1968) for goniodomin are present in the spectrum of GDA. Proposed structures for the charged species are presented in Fig. 4. Significantly, goniodomin quantitatively lost one water molecule in the MS9™ spectrum such that the parent cation-radical (*m/z* 768) could not be detected whereas *m/z* 768 was observed as a weak (~10 % intensity) ion in the spectrum from the Waters spectrometer. Unassigned ions in the two spectra may result from fragmentation pathways involving initial cleavages at other sites or from impurities present in the samples. It should be noted, though, that electrospray spectra of the GDA failed to show the plethora of low-mass peaks. It should also be noted that low-mass ions of uncertain origin are often observed but investigators frequently do not report or discuss them (for example, see Krock et al., 2018).

Analysis of the fragment ions leads to the conclusion that the *m/z* 750 ion is structurally heterogeneous involving loss of water from several sites, one being the 5-hydroxyl group. Fig. 4 shows postulated structures for cation-radicals **2** and **3** resulting from water loss involving the 5- and 27-hydroxyl groups, respectively. These cation-radicals can undergo loss of additional water molecules. The alkyl-O linkage is hypothesized to be the main site of first cleavage. Cation radicals **4** and **5** can undergo decarboxylation by McLafferty rearrangements to yield decarboxylation products **6** and **7**, respectively. Observation of the *m/z* 706 cation-radicals **6** and/or **7** provides support for this hypothesis. Subsequent loss of two water molecules occurs to give *m/z* 688 and 670 cation-radicals.

For both goniodomin and GDA, cation-radicals **4** and **5** undergo preferential fragmentations of the carbon chains at the junctions between heterocyclic rings. These sites are favored by the resulting cations and radicals being stabilized by the heterocyclic oxygen atoms. Cleavage of **4** occurs at the C6-C7 bond (a) to give cationic species **8** (*m/z* 611). Cation-radical **5** cleaves at the C18-C19 bond (b) to give cationic species **10** (*m/z* 319) and **11** (*m/z* 431). Cation-radical **5** also undergoes cleavage of the C15-C16 bond to give cation **12** (*m/z* 401). Cleavage of the C21-C22 bond of **5** yields cation **11**. The remaining fragment ions observed at masses greater than 300 are formed by processes consistent with those detailed in Fig. 4. Overall, for the higher masses the correspondence between goniodomin and GDA is excellent.

Below *m/z* 300 the fragmentation patterns of goniodomin and GDA diverge. The explanation for this is more likely instrumental than structural. GDA shows numerous fragments that are not readily assigned. The melting point and NMR spectra of the GDA (see Fig. 3b and Supplementary Material) indicate excellent purity. Minor impurities might produce intense fragment peaks but it is more likely that the unassigned fragments are arising by pathways not initiated by formation of seco acids. Support for this conclusion arises from comparison of the electron impact spectrum with one acquired by electrospray ionization where small fragment ions were observed in the MS-MS CID spectrum of the NH_4^+ adduct (data not shown). Krock et al. (2018) saw similar fragment ions in the MS-MS CID spectrum of the ammonium adduct of **1**.

In the spectrum of goniodomin, prominent low molecular weight fragment ions were observed at *m/z* 139 and *m/z* 113. Structure **12** can be proposed for the *m/z* 139 and closely related structure **13** for *m/z* 113, both derived from Ring A. The *m/z* 139 ion is not observed with significant intensity in the electron impact spectrum of GDA obtained in the present study but the *m/z* 113 peak is the most intense signal in that spectrum. It should be pointed out, however, that a strong *m/z* 139 ion is observed in the fragmentation of the NH_4^+ adduct of GDA using electrospray introduction on a Waters triple quadrupole spectrometer (Personal communication from Juliette Smith, VIMS).

2.8. Antifungal activity

Both goniodomin and GDA have been reported to have antifungal activity. Sharma et al. (1968) employed growth inhibition of *C. albicans* to guide the isolation of goniodomin. Murakami et al. (1988) reported that GDA showed antifungal activity against *C. albicans* and *Mortierella ramannianus*. The York River VA GDA was submitted to CO-ADD (Community for Open Antimicrobial Discovery) at the University of Queensland for a quantitative assessment of anti-fungal activity. CO-ADD evaluated antifungal activity against *C. albicans* (ATCC 90028) and *Cryptococcus neoformans* (ATCC 208821) using fluconazole, a modern day azole-class antifungal agent, as the positive control. Working with a definition of activity being >80 % inhibition of growth, The York River, VA GDA was deemed to be inactive as an anti-fungal agent. They also tested antibacterial activity against *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Acitobacter baumannii* (ATCC 19606), and *Pseudomonas aeruginosa* (ATCC 27853) using colistin as the positive control and against *Staphylococcus aureus* (ATCC 43300) using vancomycin as the positive control. In no case was antibacterial activity observed for GDA using the same definition of activity. Further detail is provided in the Supporting Information.

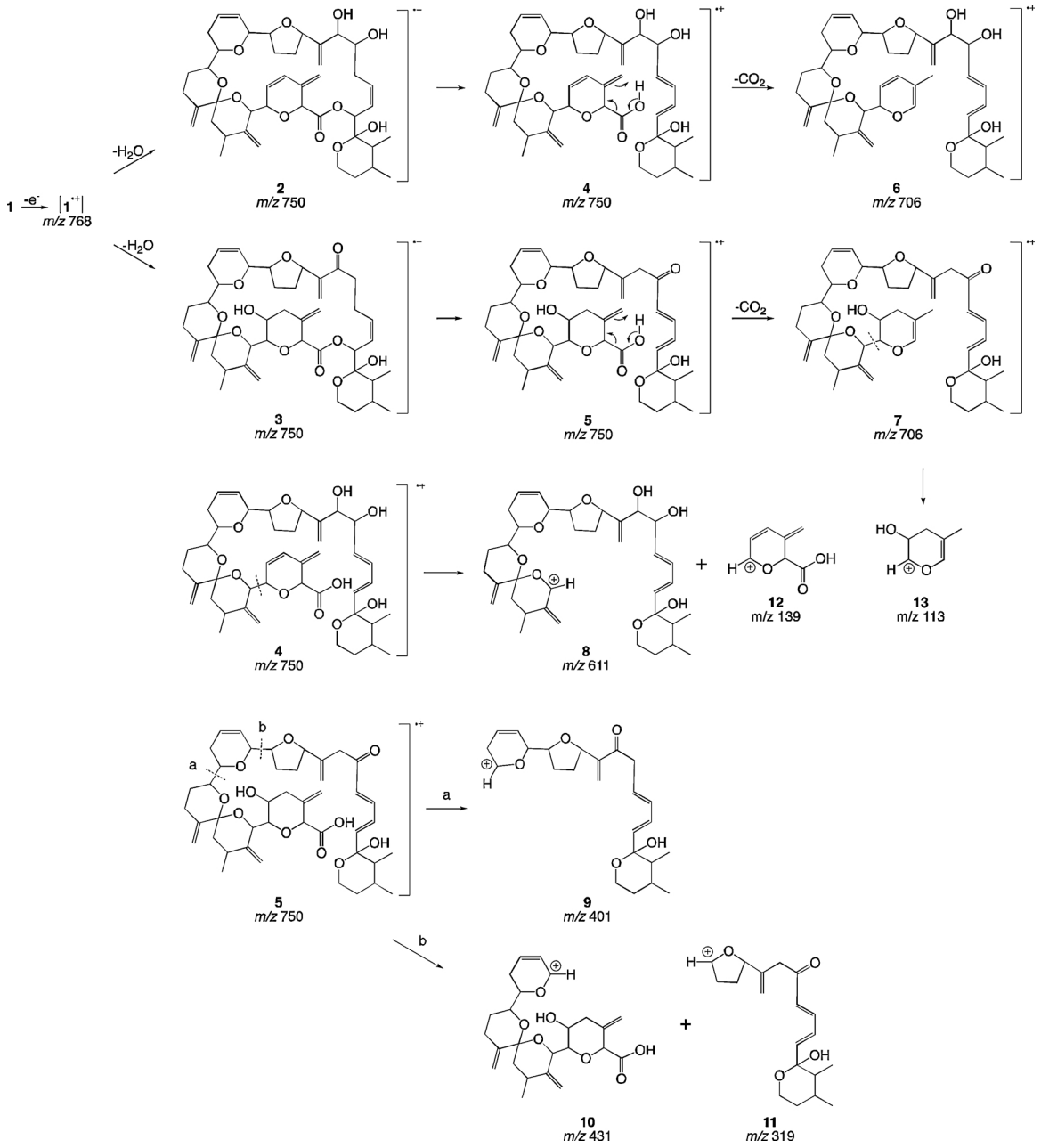

Fig. 4. Proposed fragmentation pathway for **1**.

2.9. Producing organism

Silva (1965 and 1969) made a partial analysis of Burkholder's *Goniodoma* sp. working with samples provided by Burkholder and ruled out *Alexandrium* (*Goniodoma*) *pseudogonyaulax* and *A. ostenfeldii,* as well as *Fragilidium heterolobum* and settled on calling it simply *Goniodoma* sp. In the 50 years since Burkholder and Silva did their studies, two additional *Alexandrium* species (*A. hiranoi*, and *A. monilatum*) that produce GDA have been identified (Hsia et al., 2006; Zmerli Triki et al., 2016). *Alexandrium monilatum* is unique among the goniodomin producers for blooms forming long chains of cells (Connell and Cross, 1950; Howell, 1953). These chains frequently contain 40 or more cells. With cells averaging diameters of 50 microns, the chains can be as much as 2 mm in length and would have been readily observed. In Burkholder's reports there is no mention of chains being observed (Burkholder et al., 1967; Sharma et al., 1968), which effectively eliminates *A. monilatum* from consideration. The possibility exists that the dinoflagellate that Burkholder and Silva studied is a species not known to produce goniodomin. Of particular note would be *A. taylori* Balech, 1994 which based on phylogenetic and morphological characteristics is closely related to *A. hiranoi* and *A. pseudogonyaulax* (Penna et al., 2008; Leaw et al., 2005; Kim et al., 2005; John et al., 2014) but there is no report of *A. taylori* producing GDA. In fact, Lim et al. have reported that *A. taylori* produces gonyautoxin GTX4 and related paralytic shellfish poisoning toxins (Lim et al., 2005). Thus, we are left with *A. hiranoi* as the most likely identity of Burkholder's dinoflagellate unless it is one of the numerous poorly characterized *Alexandrium* spp. (Anderson et al., 2012) or one that is totally unknown. We conclude that the body of evidence published by Burkholder and Silva is insufficient to make a definitive identification of the species by modern day standards for morphological identification (Steidinger and Tangen, 1997).

In an attempt to make an assignment by molecular methods, sediment samples were obtained from the site near Magüeyes Island where Burkholder had collected the *Goniodoma* cells from which the

Table 1
Fragment ions observed in electron impact mass spectra of goniodomin and GDA.

m/z	Goniodomin[a]	GDA[b]	Empirical Formula	Structural Assignment
768	Not Obs.	Obs.	$C_{43}H_{60}O_{12}^{+\cdot}$	**1**$^{+\cdot}$
750	Obs.	Obs.	$C_{43}H_{58}O_{11}^{+\cdot}$	**2-5**
732	Obs.	Obs.	$C_{43}H_{56}O_{10}^{+\cdot}$	**2-5** - H_2O
714	Obs.	Obs.	$C_{43}H_{54}O_{9}^{+\cdot}$	**2-5** - $2H_2O$
706	Obs.	Obs.	$C_{42}H_{58}O_{9}^{+\cdot}$	**6** and **7**
688	Obs.	Obs.	$C_{42}H_{56}O_{8}^{+\cdot}$	**6** and **7** - H_2O
670	Obs.	Obs.	$C_{42}H_{54}O_{7}^{+\cdot}$	**6** and **7** - $2H_2O$
611	Obs.	Obs.	$C_{24}H_{31}O_{7}^{+}$	**8**
593	Obs.	Obs.	$C_{24}H_{29}O_{6}^{+}$	**8** - H_2O
575	Obs.	Obs.	$C_{24}H_{27}O_{5}^{+}$	**8** - $2H_2O$
563	Obs.	Not Obs.	Unassigned	
557	Obs.	Obs.	$C_{24}H_{25}O_{4}^{+}$	**8** - $3H_2O$
539	Obs.	Obs.	$C_{24}H_{23}O_{3}^{+}$	**8** - $4H_2O$
501	Not Obs.	Obs.	Unassigned	
431	Obs.	Obs.	$C_{24}H_{31}O_{7}^{+}$	**10**
413	Obs.	Obs.	$C_{24}H_{29}O_{6}^{+}$	**10** - H_2O
401	Obs.	Obs.	$C_{24}H_{33}O_{5}^{+}$	**9**
387	Obs.	Obs.	$C_{23}H_{31}O_{5}^{+}$	**10** - CO_2
383	Obs.	Obs.	$C_{24}H_{31}O_{4}^{+}$	**9** - H_2O
369	Obs.	Obs.	$C_{23}H_{29}O_{4}^{+}$	**10** - CO_2 - H_2O
335	Not Obs.	Obs.	Unassigned	
319	Obs.	Obs.	$C_{19}H_{27}O_{4}^{+}$	**11**
301	Obs.	Obs.	$C_{19}H_{25}O_{3}^{+}$	**11** - H_2O
283	Obs.	Obs.	$C_{19}H_{23}O_{2}^{+}$	**11** - $2H_2O$
275	Not Obs.	Obs.	$C_{19}H_{21}O^{+}$	**11** - $3H_2O$
149	Not Obs.	Obs.	$C_{8}H_{7}O_{3}^{+}$	Phthalate?
139	Obs.	Not Obs.	$C_{7}H_{7}O_{3}^{+}$	**12**
113	Obs.	Obs.	$C_{6}H_{9}O_{2}^{+}$	**13**

[a] Sharma et al., 1968.
[b] Present work. Selected masses are presented below *m/z* 300; full spectrum is in the Supplementary Material.

goniodomin had been isolated. With the hope that the sediment might still contain viable cysts, efforts were made to resurrect an active culture but without success. Attention was then turned to examination of DNA that might still be present. DNA was extracted from the sediment but PCR amplification using SSU primers specific for *A. monilatum* was unsuccessful. This was followed up with degenerate LSU primers designed to amplify many different *Alexandrium* species including *monilatum, hiranoi* and *pseudogonyaulax*. This also failed to reveal the presence of any candidate dinoflagellates. The failure to observe species of *Alexandrium* was not due to inhibition of enzymatic activity of the polymerase by substances in the sediment samples because amplification of non-targeted DNA fragments was observed. It is our conclusion that no assignment of the *Alexandrium* species can be made at this time.

3. Conclusions

In all probability Burkholder's goniodomin is GDA rather than an isomer of it. Of particular note in this regard are their melting points, their chromatographic behavior, their low solubility in ethyl ether and in particular their infrared, NMR and mass spectra. In retrospect, Burkholder might have been able to complete the structural studies of goniodomin if he had turned to X-ray crystallography rather than relying on other types of spectroscopy and more classical methodology since at the time of his investigations the structures of other natural products of comparable complexity had been established by crystallography, e.g., vitamin B_{12}. (Hodgkin et al., 1955).

Burkholder may have believed the Puerto Rican goniodomin to be identical to the toxin formed by algal blooms observed along the southeastern U.S. coast. In the introduction to the 1968 publication (Sharma et al., 1968), Burkholder put the Puerto Rican bloom in perspective by citing a report by Connell and Cross (1950) of blooms in Galveston Bay, TX of a species of *Gonyaulax* (subsequently renamed *Alexandrium*) and another by Howell (1953) of blooms of *G. monilata* (subsequently renamed *A. monilatum*) on the east coast of Florida. In both of those cases, the toxin is now presumed to have been GDA (Hsia et al., 2006; Zmerli Triki et al., 2016). One significant difference between the organism producing Burkholder's goniodomin and those described by Howell and by Connell and Cross is that they prominently discussed chain formation in their publications.

A central question is why other blooms of goniodomin-producing dinoflagellates have not been observed in the Caribbean region. The dearth of research-oriented regional universities and marine laboratories employing investigators with interests in blooms of harmful algae, suggests that the failure to observe these blooms is due to a deficiency in monitoring rather than an absence of occurrence.

Goniodomin produced in the Caribbean is also identical to goniodomin A produced by *A. monilatum* in the York River, VA, USA. We recommend the name goniodomin A be retained rather than reverting to Burkholder's goniodomin designation because the related substance, goniodomin B (GDB), has been isolated from *A. hiranoi* (Takeda, 2008; Espiña) and *A. pseudogonyaulax* (Krock et al., 2018). Future studies of goniodomin-producing *Alexandrium* species will reveal additional related compounds formed by enzymatic and non-enzymatic reactions of GDA.

4. Materials and methods

4.1. Instrumental methods

NMR spectra of GDA were acquired using a Bruker AV-III-600™ spectrometer equipped with a 5 mm cryoprobe and operating at 600 MHz located in the Small Molecule NMR Facility at Vanderbilt University. The electron impact mass spectrum was acquired on a Waters GCT Premier™ spectrometer with solid sample being introduced via the direct insertion probe and the electrospray spectrum was acquired on a Waters Synapt™ G2-Si spectrometer, both at the Univ. of Illinois School of Chemical Sciences. The infrared spectrum was acquired in the Chemistry Department at William & Mary using a solid sample on a Shimazu IRTracer-100™ spectrometer with a MIRacle 10™ ATR attachment.

4.2. Isolation of GDA from A. monilatum

Cells were collected via plankton net from a 2013 bloom in the York River, VA and concentrated by centrifugation to yield a moist paste, which was stored at −80 °C to disrupt the cells and then lyophilized to dryness *in vacuo* without heat. Dried cells (50 *g*) were extracted five times with MeOH aided by bath sonication; the extract was separated from cell debris by filtration. The cell debris was air-dried and ground with mortar and pestle to yield a fine green powder, which was extracted once more with methanol. The combined extracts were concentrated, taken up in benzene and allowed to stand overnight at room temperature causing a viscous mass to collect. The supernatant was collected and the residue was further washed with benzene and then with ethyl ether to fully extract GDA. The combined extracts were evaporated to yield 7.3 *g* of dark mobile residue.

Guided by the chromatographic procedure of Sharma et al. (1968), the residue was loaded on a chromatography column that had been packed with 90 g of flash silica gel under benzene (Still et al., 1978). The column was eluted first with 700 ml of benzene, to remove fast running lipid constituents. Further elution was carried out with ethyl ether (700 ml). The presence of GDA was monitored by TLC. Initial fractions were green, which transitioned to red as elution progressed. The first green fractions eluted in mixtures of benzene and ethyl ether and contained little GDA. The subsequent fractions, extending from the green fractions into the red, were rich in GDA which tailed off as the elution of red pigments progressed. The goniodomin-rich fragments were pooled and concentrated to yield 2.8 g of highly enriched

material, which was rechromatographed on 100 g of flash silica gel packed under benzene. The column was eluted isocratically with 1:1 (v:v) benzene-ethyl ether with the eluent being collected in ~10 ml fractions: 1–5 colorless, 6 – 18 green pigment #1, 19–25 green pigment #2, 26–41 red pigment #1, and 42–48 red pigment #2. The column was then washed with 440 ml of ethyl ether. TLC evaluation indicated that fractions 30–34 contained GDA but it was heavily contaminated with pigments. Fractions 35–45 contained the best material; these fractions were pooled and after trituration with ethyl ether yielded 74 mg of high quality GDA. Fractions 46–48 contained small amounts of GDA. Fractions 30–34 and 46–48 were combined with the ethyl ether fraction and evaporated. The residue (165 mg) was reprocessed to yield another ~35 mg of highly enriched GDA after trituration with cold ethyl ether. The combined material was largely free of pigments and other impurities as demonstrated by ^{1}H NMR (See Figs. 3b and S1) and other spectra. It should be noted that individual blooms vary widely in content of pigments and of GDA.

4.3. Analysis of GDA

TLC was carried out on Merck silica gel 60 F_{254} 2.5 × 10 cm analytical plates. Elution with 1:3 (v:v) benzene:ethyl acetate gave an R_f of ~0.60. GDA is not fluorescent and does not quench the fluorescent pigment on the plate; it can be viewed with iodine or with phosphomolybdic acid, the later being more sensitive. HPLC analysis was carried out using C18 reverse-phase columns eluted with acetonitrile-water gradients and monitored by end absorption at 200 nm. Goniodomin B elutes from C18 columns significantly earlier than GDA and has a lower R_f on silica gel TLC plates.

GDA undergoes facile degradation under acidic and basic conditions. Slow degradation is observed when stored in methanol and in other solvents if they pick up water. Particular care must be taken to avoid acidic contaminants, which are frequently present in ethyl acetate and chloroform. For this reason, C_6D_6 is preferable to $CDCl_3$ as an NMR solvent. A further benefit is that the spectral dispersion is better in C_6D_6 than in $CDCl_3$. When acquiring electrospray mass spectra, addition of formic acid or other acids should be avoided but addition of sodium, potassium and ammonium ions yields intense adduct ions.

4.4. Antimicrobial activity

Analyses were carried out by CO-ADD at the University of Queensland, Australia. Fungi strains were cultured for 3 days on Yeast Extract-Peptone Dextrose agar at 30 °C. A yeast suspension of 1×10^6 to 5×10^6 cells/mL (as determined by absorbance at 530 nm) was prepared from five colonies. These stock suspensions were diluted with Yeast Nitrogen Base broth to a final concentration of 2.5×10^3 CFU/mL. Then, 45 µL of the fungal suspension was added to each well of the compound-containing plates. Plates were covered and incubated at 35 °C for 24 h without shaking.

Growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm, while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm, after the addition of resazurin (0.001 % final concentration) and incubation at 35 °C for additional 2 h. The absorbance was measured using a Biotek Synergy HTX plate reader. The percentage of growth inhibition was calculated for each well, using the negative control (media only) and positive control (fungi without inhibitors) on the same plate. Samples with inhibition value less than 80 % were classed as inactive. Additional details are provided in the Supporting Information.

4.5. Sediment analysis

An attempt was made to identify genetically the *Alexandrium* species from sediment samples collected by Dr. Nicolaos Schizas (University of Puerto Rico, Mayagüez, Puerto Rico) from the site of Burkholder's bloom. DNA was extracted from the sediments using the PowerLyzer PowerSoil DNA Isolation kit (Qiagen) following the manufacturer's protocol with bead beating using the FastPrep™-24 (MP Biomedicals) instrument. Extracted DNA was eluted with 100 µL of the kit's elution reagent, and stored at -20 °C until further analysis. Standard PCR was performed to confirm amplifiability of the DNA using general ITS primers A and B (Goggin, 1994) as previously described (Moss et al., 2008). In addition, the DNA was screened in an *A. monilatum*-specific Taqman qPCR assay (Amon2_500F: 5'-TGAAAGGTAAGGTGCCTGTG-3', Amon2_658R: 5'GCAGAAACATGTTGCCAAAG 3', Amon2_Probe: 5'-TGCAAGCACAAGCAACCCAGC-3') with primers at a concentration of 0.9 µM using the TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) following the manufacturer's protocol. Standard PCR was attempted using *Alexandrium* genus-specific primers (Alexspp-3: 5'-TTTCAGCCTTGCGACCATAC, Alexspp-5: 5'TTAAAGACGGACTACT GCG-3'). Each 25 µl reaction contained 1 µl (50–100 ng) of genomic DNA, PCR buffer at 1x concentration, $MgCl_2$ at both 1 mM and 1.5 mM, nucleotides at 0.2 mM each, primers at 0.8 µM each, and 0.06 U/µl of Taq DNA polymerase (Life Technologies). The thermal cycling protocol included denaturation at 94 °C for 4 min; 40 amplification cycles with denaturation at 94 °C for 30 s, annealing at a gradient between 52 °C and 5 °C for 30 s, and extension at 65 °C for 45 s; followed by a 65 °C final extension for 5 min. PCR products were electrophoresed on 1.2 % agarose gels, stained with ethidium bromide and visualized on a UV transilluminator. Images were digitally recorded. Later, combinations of primers were employed that had previously been used (Vandersea et al., 2017) to amplify rRNA gene regions of different *Alexandrium* species. A nested PCR strategy was used where the amplification product of the Alex1600 F/LSU-B was used as the template for PCR amplification using the Alex1600 F/Alex5.8SR and the LSU-A/AlexLSU-R1 primer sets (Lenears et al., 1989). Amplification products of the expected size were cloned and sequenced as previously described (Moss et al., 2008) on a 3500 Genetic Analyzer (Applied Biosystems).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2019.101707.

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