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Development of a LC-MS/MS method for the quantification of goniodomins A and B and its application to *Alexandrium pseudogonyaulax* strains and plankton field samples of Danish coastal waters

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ABSTRACT

An external standard of goniodomin A (GDA) was prepared from a strain of *Alexandrium pseudogonyaulax* originating from New Zealand and its chemical structure was confirmed by nuclear magnetic resonance (NMR) spectroscopy. Using the GDA standard, an ultra-performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) method in selected reaction monitoring (SRM) mode was developed for separation and quantification of GDA. This method was successfully applied to planktonic field samples collected during an oceanographic expedition conducted with *R/V* Uthörn along the Danish west coast, Limfjord and Kattegat in June 2016. In addition, this method was used to characterize goniodomin (GD) profiles of 17 *A. pseudogonyaulax* strains from the coastal North Sea and from Limfjord. Highest GDA levels were found in Limfjord (up to 590 ng NT^{-1} m^{-1}), but GDA was also detected in the North Sea appearing at the latitude of Sylt Island northwards and in Kattegat from the eastern mouth of Limfjord down to the Kiel Bight, but at lower abundances than within Limfjord. This is the first reported detection of GDA in planktonic field samples. Chemical analysis of 17 strains of *A. pseudogonyaulax* revealed that all strains were producers of GDA (5–35 pg $cell^{-1}$) as well as in most cases minor amounts (0.01–0.07 pg $cell^{-1}$, expressed as GDA equivalents) of goniodomin B (GDB).

1. Introduction

Alexandrium pseudogonyaulax is a dinoflagellate with a global distribution (MacKenzie et al., 2004; Bravo et al., 2006; Zmerli Triki et al., 2014). Recently, *A. pseudogonyaulax* has been demonstrated to produce goniodomin A (GDA) (Zmerli Triki et al., 2016), a biologically active metabolite associated with marine invertebrate mortalities (Harding et al., 2009). GDA was first reported to be produced by *Goniodoma* sp. in Puerto Rico and its antibiotic property was described (Sharma et al., 1968). At that time the structure of GDA was not fully elucidated. Twenty years later GDA, was isolated from a bloom of *A. hiranoi* (the strain was initially described as *Goniodoma pseudogonyaulax*, but later correctly identified to represent the new species *A. hiranoi*, Kita and Fukuyo, 1988) and described as a macrocyclic polyketide (Murakami et al., 1988) (Fig. 1A). It has now been revealed that GDA is a metabolite not only of *A. hiranoi* but also *A. monilatum* (Hsia et al., 2006), and *A. pseudogonyaulax* (Zmerli Triki et al., 2016). It has been demonstrated that GDA possesses a range of biological effects including antifungal activity (Murakami et al., 1988), cell division inhibition of sea urchin eggs (Murakami et al., 1988), antiangiogenic activity (Abe et al., 2002), and perhaps most significantly in terms of its adverse effects on marine ecosystems, it has been associated with mortality in aquatic invertebrates (Harding et al., 2009). Not much is known about the transfer of GDA in food webs, but GDA has been shown to accumulate in the marine snail *Rapana venosa* in controlled exposure experiments (Harding et al., 2009). The effects of human consumption of intoxicated shellfish of this metabolite and its possible ichthyotoxic effects remain unknown. Recently a variant of goniodomin, goniodomin B (GDB) was found, which has a slightly modified ring architecture and a shortened length of the macrocycle (Espiña et al., 2016) (Fig. 1B).

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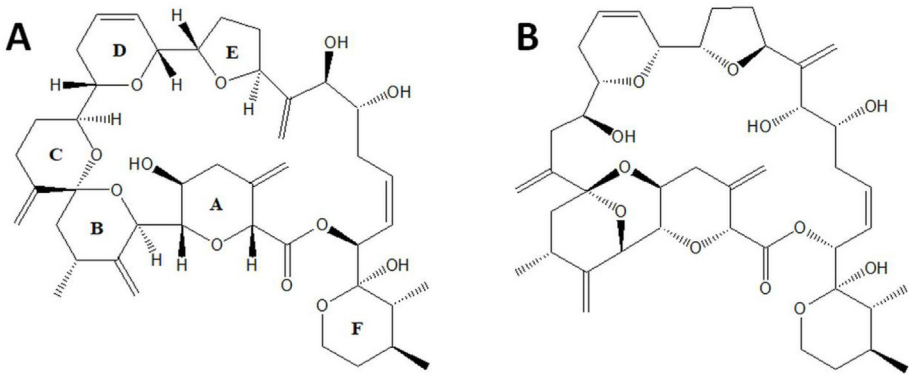

Fig. 1. Chemical structures of GDA (A) and GDB (B).

To the best of our knowledge there is no liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the determination of GD. Also no tandem mass spectrometric data of GDA and GDB have been reported in the literature. For this reason, the aim of this study was to characterize GDA by tandem mass spectrometry and to develop a liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the detection and quantification of GDA and GDB in planktonic samples.

2. Methods

2.1. Isolation and structural elucidation of GDA

HPLC grade acetonitrile (ACN), methanol (MeOH), hexane, and isopropanol (iPrOH) used in the extraction procedure and HPLC purification were purchased from Sigma-Aldrich (Schnelldorf, Germany). Water (H_2O) was purified from a Milli-Q system, Millipore (Bedford, MA, USA). NMR solvents were purchased from Sigma-Aldrich.

Small scale preliminary analysis by LC-MS of *A. pseudogonyaulax* culture extracts revealed an abundance of GDA in culture media, as well as within the cell pellets. Due to the decomposition of intracellular GDA, a GDA quantitative standard was isolated exclusively from the supernatant of centrifuged *A. pseudogonyaulax* (strain CAWD-138) cultures. CAWD-138 was acquired from Cawthron Institute Culture Collection for Micro-Algae and Protozoa, New Zealand. 10 L batch cultures of *A. pseudogonyaulax* were maintained at 15 °C with an irradiance of 250 mmol photons m^{-2} s^{-1} in f/2 media made with pasteurised seawater with a salinity of 30; a total of 60 L were produced. The algal cultures were harvested at the beginning of stationary phase with an average cell count of 2463 cells mL^{-1}. Cell concentrations were determined by light microscopy. Cultures were harvested via centrifugation using an Avanti J-26 XP continuous centrifuge fitted with a JCF-Z rotor, Beckman Coulter (Brea, CA, USA). Cultures were centrifuged with a feed rate of 40 mL min^{-1} at 1100 × *g* and 15 °C and the supernatant was extracted using Diaion HP20SS polymeric reversed phase sorbent, Mitsubishi Kasei Kogyo Co., Ltd. (Tokyo, Japan) equilibrated with MeOH, packed in a 25 g SNAP cartridge, Biotage (Uppsala, Sweden) and eluted on a Biotage Isolera One flash chromatography purification system. Cultures were harvested in 2 × 10 L batches with a sorbent to supernatant ratio of approximately 1.6 g L^{-1}. Sorbent was extracted immediately using a step wise gradient of MeOH in H_2O, with 20% increments in MeOH composition, and a starting composition of 20% MeOH. 250 mL fractions (8 column volumes) were manually collected. GDA eluted in 100% MeOH.

Fractions from all cultures containing GDA were pooled and fractionated on a semi-preparative Waters 600 HPLC (Waters Corporation, Milford, MA) equipped with a Luna II C_{18} (250 mm × 10 mm, 5 µm, 100 Å, Phenomenex, Torrance, CA, USA) column coupled with a Waters 996 photodiode array detector. Separation was achieved using a H_2O:ACN gradient from 40 to 90% ACN over 15 min with a flow rate of 5 mL min^{-1}. Multiple 150 µL injection volumes were manually fractionated with collection every 2–5% increments in ACN composition. GDA eluted between 80% and 83% ACN. For structural confirmation this purified sample was subjected to analysis by NMR spectroscopy on an 18.8 T Bruker Ascendan magnet with an Avance III HD console, Bruker Corporation (Billerica, MA, USA) equipped with a Bruker TCI cryoprobe. The sample was dissolved in C_6D_6 (> 99.8% atom).

Due to minor impurities final purification for a quantitative sample was undertaken in normal phase HPLC. This was achieved on a Dionex Ultimate 3000 equipped with a Waters XBridge BEH Amide column (5 µm, 130 Å, 10 mm × 150 mm) coupled to a Corona Veo RS, Thermo Fisher Scientific (Waltham, MA, USA) charged aerosol detector (CAD). A multistep hexane:iPrOH gradient from 0-0-5-15-30-90% over 0-5-15-20-22.5–27 min was used for the separation and fractionation was monitored by CAD using an HPLC effluent splitter, Analytical Scientific Instruments, Inc. (Richmond, CA, USA) with an approximate 1:25 split. A flow rate of 2.7 mL min^{-1} was used and a column compartment temperature of 30 °C. GDA eluted at 26.1 min. Purification resulted in 1.0 mg GDA. Purification was confirmed by NMR spectroscopy using the same Bruker instrument in CD_3OD (≥99.8% atom).

2.2. UPLC-MS/MS method development for GDA/B

2.2.1. Chromatographic separation

Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm, Waters, Eschborn, Germany) equipped with an in-line 0.2 µm Acquity filter. The flow rate was 0.6 mL min^{-1}, and the injection volume was 0.1 µL. Three different elution systems were tested (García-Altares et al., 2013):

1) *Mobile phases in acidic conditions (*pH *2)* Mobile A consisted of 2 mM ammonium formate and 50 mM formic acid in Milli-Q water. Mobile B consisted of 2 mM ammonium formate and 50 mM formic acid in ACN/Milli-Q water (95:5, v/v).
2) *Mobile phases in close to neutral conditions (*pH *6.8)* Mobile A consisted of 5 mM ammonium acetate in Milli-Q water. Mobile B consisted of 5 mM ammonium acetate in ACN/Milli-Q water (95:5, v/v).
3) *Mobile phases in slightly alkaline conditions (*pH *7.9)* Mobile A consisted of 5 mM ammonium bicarbonate in Milli-Q water. Mobile B consisted of 5 mM ammonium bicarbonate in ACN/Milli-Q water (95:5, v/v).

After optimization of the elution system, the ammonium bicarbonate system (3) was selected. Chromatographic separation was performed by gradient elution, starting with 10% B for 0.5 min, and increasing to 100% B in 3.5 min followed by 0.5 min isocratic elution at 100% B, return to initial conditions during 0.1 min and 1 min column equilibration time at 10% B.

2.2.2. Mass spectrometry

An ACQUITY UPLC System coupled to a Xevo TQ-S mass spectrometer equipped with a Z-Spray source, Waters, was used. The mass spectrometer was operated with the following optimized source-dependent parameters: collision energy 25 eV, source temperature 150 °C, desolvation temperature 500 °C, desolvation gas flow 796 L h^{-1} N_2, cone voltage 35 V, cone gas flow 146 L h^{-1} N_2, collision gas flow 0.14 mL min^{-1}. The mass spectrometers were operated in the selected reaction monitoring (SRM) in the positive mode analyzing two mass transitions for both, GDA and GDB: one for quantification and another for confirmation. The transitions employed were GDA (*m/z* 786.5 → 733.5, *m/z* 786.5 → 607.5), and GDB (*m/z* 722.5 → 719.5, *m/z* 722.5 → 607.5).

2.3. Tandem mass spectrometric characterization of GDA

Experiments were performed on a triple quadrupole mass spectrometer (API 4000 QTrap, Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray interface, coupled to a liquid chromatograph, model 1100, Agilent (Waldbronn, Germany). The liquid chromatograph included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A). Separation of lipophilic toxins was performed after injection of 10 µL sample by reversed-phase chromatography on a C8 phase. The analytical column (50 × 2 mm) was packed with 3 µm Hypersil BDS 120 Å, Phenomenex (Aschaffenburg, Germany) and maintained at 20 °C. The flow rate was 0.2 mL min^{-1} and gradient elution was performed with two eluents, where eluent A was water and eluent B was acetonitrile/water (95:5 v/v), both containing 2.0 mM ammonium formate and 50 mM formic acid. Initial conditions were 12 min column equilibration with 5% B, followed by a linear gradient to 100% B in 10 min and isocratic elution until 15 min with 100% B. The system was then returned in 3 min to initial conditions (total run time: 30 min).

Full scan experiments of *A. pseudogonyaulax* extracts were performed in the mass range from *m/z* 720 to *m/z* 820 in the positive ion mode. Mass spectrometric parameters were as follows: curtain gas: 20 psi, CAD: medium, ion spray voltage: 5500 V, temperature: 275 °C, nebulizer gas: 50 psi, auxiliary gas: 50 psi, interface heater: on, declustering potential: 50 V.

Collision induced dissociation (CID) product ion spectra were recorded for the sodium adduct ion ($[M+Na]^+$) of GDA (*m/z* 791) and for its ammonium adduct ion ($[M+NH_4]^+$; *m/z* 786). CID spectra were recorded on the same instrument in the enhanced product ion (EPI) modus in the mass range from *m/z* 150 to 800 and in positive ionization and unit resolution mode. The following parameters were applied: collision energy spread: 10 V, collision energy: 55 V. All other mass spectrometric parameters were as described above for the full scan experiment.

2.4. Expedition field sampling 2016

An oceanographic expedition with the German *R/V* Uthörn was performed from Bremerhaven, Germany along the Danish West coast up to the western inlet of Limfjord and further to Løgstør, Denmark (Fig. 2) between 13th June and 18th June 2016. Between 19th June and 23rd June several arms of Limfjord were sampled in daily excursions from Løgstør. The expedition continued from Løgstør to Kattegat, Great Belt, Kieler Förde and Kiel Canal back to Bremerhaven from 24th to 29th June 2016. A total of 44 stations were sampled (Fig. 2).

At each station a 20 µm phytoplankton net (438-030, Hydro-Bios, Kiel, Germany) was used to get one or two vertical net tows with the lower depth depending on the total water depth.

Net tow concentrates were taken up in a defined volume (usually 1 L) with filtered sea water. 50 mL aliquots of the net tow concentrates were taken for cell isolations and 18 mL aliquots were fixed with paraformaldehyde (1% final concentration) for microscopic inspection. The rest of the net tow concentrates were filtered over a filter array with 200, 50 and 20 µm sieves. Plankton of each size fraction were rinsed with filtered sea water into 50 mL centrifugation tubes, Sarstedt (Nümbrecht, Germany) and adjusted to 45 mL. All aliquots were transferred into 15 mL centrifugation tubes, Sarstedt, and centrifuged for 15 min at 3220 × *g* and 4 °C. Supernatants were removed and pellets were re-suspended in 1 mL 0.03 M methanol, respectively, and transferred to FastPrep tubes containing 0.9 g lysing matrix D, Thermo-Savant (Illkirch, France). Samples were homogenized by reciprocal shaking for 45 s at maximal speed (6.5 m s^{-1}) in a FastPrep instrument, Thermo-Savant, and subsequently centrifuged for 15 min (10 °C, 16,100 × *g*). Supernatants were transferred into spin filters, Ultra-free, Milllipore, and filtered by centrifugation for 30 s at 10 °C and 5000 × *g*. Filtrates were transferred to HPLC vials and stored at −20 °C until analysis.

2.5. Strain isolation, culture conditions, cell harvest

Single cells of *Alexandrium pseudogonyaulax* were isolated by micropipette from live net tow concentrates under a stereomicroscope, M5A, Wild (Heerbrugg, Switzerland). Single cells were transferred into individual wells of 96-well tissue culture plates (Trasadingen, Switzerland) each containing 250 µL of K-medium (Keller et al., 1987), prepared from 0.2 µm sterile-filtered seawater from the sampling location. Plates were incubated at 15 °C under dim light (ca. 30 µmol photons m^{-2} s^{-1}) in a controlled environment growth chamber, Model MIR 252, Sanyo Biomedical, (Wood Dale, USA). After 3–4 weeks, clonal isolates were transferred to 24-well tissue culture plates. Finally, 17 growing strains were used as inoculum for batch cultures in 65 mL polystyrene cell culture flasks and were maintained thereafter at 15 °C under a photon flux density of 50 µmol m^{-2} s^{-1} on a 16:8 h light:dark photocycle in a temperature-controlled growth chamber. All strains were identified as *A. pseudogonyalax* by light microscopy and LSU rDNA sequencing as reported in detail elsewhere (Kremp et al., in prep).

For toxin analysis all strains were grown in 200 mL Erlenmeyer flasks under standard culture conditions. Cell concentrations from exponentially growing cultures (at cell densities ranging from 400 to 4000 cells mL^{-1}) were determined by settling Lugol's iodine-fixed samples and counting > 600 cells under an inverted microscope. Cell pellets were harvested by centrifugation, Eppendorf 5810R (Hamburg, Germany) at 3220 × *g* for 10 min and were extracted for lipophilic toxins with 500 µL methanol and for paralytic shellfish toxins (PST) with 500 µL 0.03 M acetic acid, respectively, by reciprocal shaking at 6.5 m s^{-1} with 0.9 g lysing matrix D, Thermo Savant, in a Bio101 FastPrep instrument, Thermo Savant, or 45 s. Extracts were then centrifuged, Eppendorf 5415 R, at 16,100 × *g* at 4 °C for 15 min. Each supernatant was transferred to a 0.45-mm pore-size spin-filter, Millipore Ultrafree, and centrifuged for 30 s at 800 × *g*, and the resulting filtrate being transferred into an UPLC autosampler vial for UPLC–MS/MS analysis.

2.6. PST measurements

All *A. pseudogonyaulax* strains were tested for paralytic shellfish toxins (PST) by ion pair chromatography, post-column derivatization and fluorescence detection as previously described (Krock et al., 2007). Limits of detection (LOD) were defined as signal-to-noise ratio (S/N) = 3. As positive controls external standards of C1/2, GTX1/4, GTX2/3, B1, NEO and STX (Certified Reference Material programme of IMB-NRC, Halifax, NS, Canada) were run.

2.7. Measurements of lipophilic phycotoxins

All *A. pseudogonyaulax* strains also were tested for lipophilic phycotoxins as detailed in Krock et al. (2008) including cycloimines as the

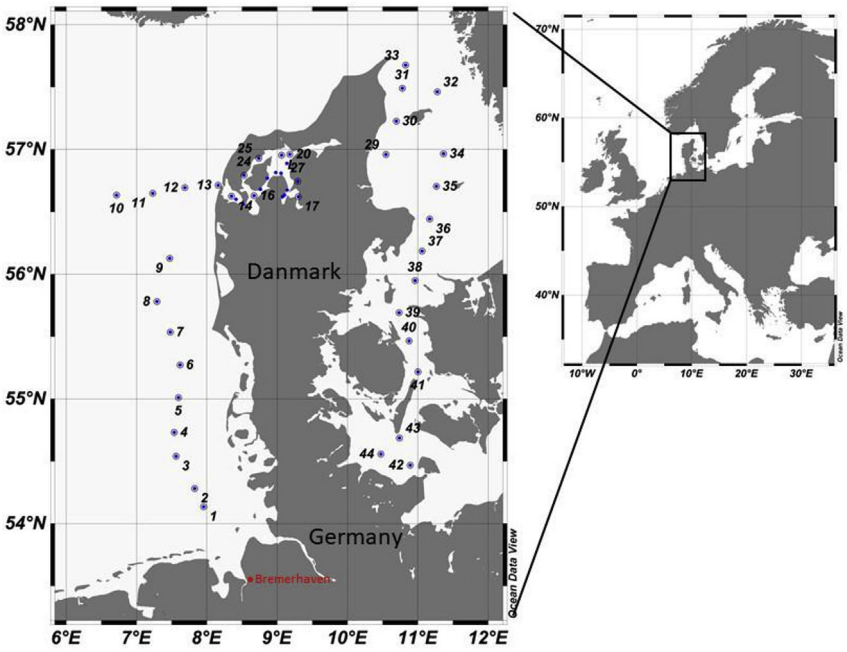

Fig. 2. Geographical map of the expedition transect and sampling stations.

genus *Alexandrium* is known to produce these toxin classes as well.

2.8. GDA/B measurements

The calibration solutions for GDA (10, 50, 100, 500 and 1000 pg μL^{-1}) were prepared and used to generate an external calibration curve. External calibration was used to quantify GDA/B concentration. Quantification was performed by calculating the absolute peak areas of *m/z* 786.5 → 733.5 for GDA and *m/z* 722.5 → 719.5 for GDB, respectively. GDB values were calibrated against the GDA calibration curve and expressed as GDA equivalents due to unavailability of a GDB standard.

3. Results

3.1. GDA standard

Structural confirmation of GDA purified from *A. pseudogonyaulax* culture supernatant was achieved with dqf-COSY, HMBC, NOESY, and HSQC experiments (Supporting Figs. S1–S4). A comparison of measured chemical shifts with those published by Takeda et al. (2008) confirmed the structural conclusion (Table 1). The final purification of a quantitative standard of GDA was confirmed by one dimensional proton NMR in CD_3OD (Supporting Fig. S5).

3.2. Mass spectrometric characterization of GDA

A full scan experiment of an *A. pseudogonyaulax* extract revealed that the most abundant pseudo-molecular ions of GDA formed under the applied conditions were the sodium adduct (*m/z* 791) at 1,200,000 cps and the ammonium adduct (*m/z* 786) at 500,000 cps (Fig. 3). The much higher abundance of the sodium adduct in comparison to the ammonium adduct clearly indicated that the sodium adduct was the preferred ion species. Consequently, the CID spectrum of the most abundant pseudo-molecular ion of GDA (*m/z* 791) was recorded and resulted in a spectrum will several water losses from the pseudo-molecular ion (*m/z* 773 and 755) and a few smaller fragments (*m/z* 609, 565, 413 and 357) (Fig. 4). In addition to the water losses, also an elimination of *m/z* 44 (*m/z* 747) followed by several water losses (*m/z* 729, 711 and 693) was observed. Despite of the well-defined CID spectrum of GDA, the ion yield with less than 16,000 cps for the most abundant ion was relatively low and prompted the search for conditions with higher ion yield. For this reason, the CID spectrum of the second most abundant pseudo-molecular ion of GDA, the ammonium adduct *m/z* 786 was recorded. The CID spectrum of *m/z* 786 was characterized by few well-defined fragments in the upper mass range and numerous not well resolved fragments in the mass range from *m/z* 150 to 450 (Fig. 5). The highest fragment was formed by the elimination of *m/z* 17 (ammonia) resulting in *m/z* 769 followed by several water losses (*m/z* 751, 733, 715 and 697). In addition to this ion cluster, a second fragment with *m/z* 607 followed by two water losses was observed. It is noteworthy that in contrast to *m/z* 791 there was no elimination of *m/z* 44 observed in the CID spectrum of *m/z* 786. However, the intensity of the most abundant fragment of the ammonium adduct was more than one order of magnitude higher than in the CID spectrum of the sodium adduct. In order to achieve maximum sensitivity for the detection of GDA, the ammonium adduct was selected for the development of the LC-MS/MS method.

3.3. UPLC-MS/MS method for GDA/B detection

Baseline separation of GDA and GDB was achieved with acidic, neutral and slightly alkaline mobile phases. However, peaks of GDA and GDB were narrower in the alkaline ammonium bicarbonate and the acidic ammonium formate system than peaks under the neutral ammonium acetate system. But the sensitivities of GDA and GDB were higher in the alkaline than in the acidic elution system. For this reason, the alkaline ammonium bicarbonate mobile phase was selected as the chromatographic system. Finally, several mobile phase gradients were tested for optimal peak separation, which resulted in the method described above in the method section.

In tandem mass spectrometry the collision energy is the mass spectrometric parameter with the highest impact on analyte sensitivity. During method development collision energies of 10, 15, 20, 25, 30, 40 and 50 eV were investigated. GDA/B sensitivities increased with increasing the collision energy from 10 to 25 eV, but then decreased with increasing the collision energy from 25 to 50. Therefore, 25 eV was selected as the optimal collision energy. Extracted ion LC-MS/MS

Table 1
Table of chemical shifts of goniodomin A in C_6D_6 purified from CAWD-138 in comparison to the shifts of goniodomin A in C_6D_6 published by Takeda et al. (2008). Carbon number assignment is in accordance with Takeda et al. (2008).

Position	Takeda et al.		CAWD-138		Position	Takeda et al.		CAWD-138	
	δ_H	δ_C	δ_C	δ_H		δ_H	δ_C	δ_H	δ_C
1		168.6		168.7	19	6.23	129.7	6.27	129.7
2	4.21	76.4	4.19	76.50	20	4.37	76.8	4.41	76.9
3		140.7		140.8	21	4.01	82.0	4.04	82.0
3 = CHa	4.99	112.0	5.00	112.0	22a	2.25	30.7	2.28	30.7
3 = CHb	4.76		4.76		22b	2.25		2.28	
4a	2.29	41.4	2.31	41.4	23a	1.57	31.8	1.56	31.8
4b	2.78		2.80		23b	2.14		2.13	
5	4.10	70.9	4.15	71.0	24	5.19	79.8	5.24	79.8
5-OH	4.31		3.49		25		147.9		148.0
6	3.62	80.8	3.65	80.9	25 = CHa	5.06	113.2	5.06	113.2
7	5.09	73.6	5.11	73.7	25 = CHb	5.04		5.04	
8		149.2		149.3	26	4.05	81.4	4.07	81.4
8 = CHa	5.15	108.0	5.15	108.0	26-OH			3.81	
8 = CHb	4.92		4.94		27	3.91	73.3	3.96	73.4
9	2.53	34.5	2.54	34.5	27-OH	2.89			
9-Me	1.38	20.8	1.42	20.8	28a	2.96	32.7	2.99	32.8
10a	2.11	44.3	2.11	44.4	28b	2.10		2.12	
10b	1.71		1.74		29	6.42	136.2	6.47	136.3
11		100.4		100.4	30	5.84	123.7	5.88	123.7
12		150.4		150.5	31	5.93	73.9	5.96	74.0
12 = CHa	4.92	110.4	4.93	110.5	32		97.7		97.7
12 = CHb	4.68		4.68		32-OH	2.35		2.40	
13a	1.99	27.7	1.97	27.8	33	1.30	41.5	1.31	41.6
13b	1.97		1.97		33-Me	0.97	13.0	0.99	13.0
14a	1.22	25.5	1.19	25.6	34	1.67	31.1	1.68	31.2
14b	1.38		1.37		34-Me	0.74	20.1	0.75	20.2
15	3.69	76.4	3.71	76.5	35a	1.13	34.6	1.13	34.6
16	3.80	77.0	3.83	77.0	35b	1.16		1.16	
17a	1.55	27.7	1.55	27.8	36a	3.88	60.5	3.90	60.6
17b	1.50		1.48		36b	3.54		3.56	
18	5.63	123.7	5.65	123.7					

chromatograms of a GDA standard solution and an GD profile of *A. pseudogonyaulax* strain X-LF19-F5 isolated from Limfjord containing GDA and GDB are shown in Fig. 6.

Calibration curves based on peak areas and peak heights were linear for GDA in the range from 10 to 1000 pg μL^{-1}. The calibration equation obtained from peak areas (A) and the corresponding concentrations (C) in the range of 10–1000 pg μL^{-1} were A = 108.08C + 112.25 (R^2 = 0.9980) and A = 122.17C + 168.62 (R^2 = 0.9986) on two subsequent days. The limit of detection (LOD) of GDA (S/N = 3) was determined as 2.34 pg μL^{-1} for the UPLC-MS/MS analysis.

3.4. Goniodomin cell quotas of *A. pseudogonyaulax* strains

All 17 strains of *A. pseudogonyaulax* isolated from Limfjord were proven to be GDA producers with GDA cell quotas ranging from 5 to 35 pg $cell^{-1}$ (Fig. 7). In addition, eleven strains also produced minor

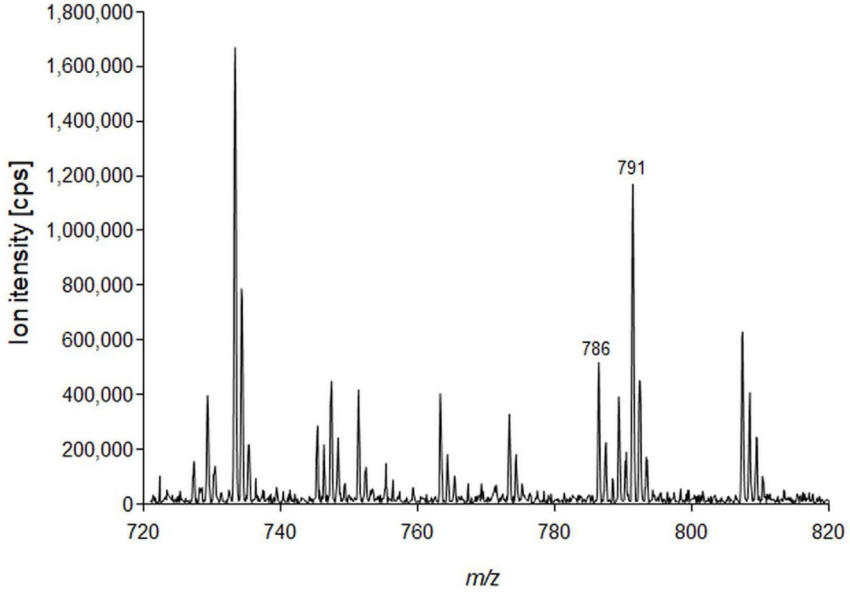

Fig. 3. Full scan spectrum in the mass range m/z 720–800 at 13.3 min of an A. pseudogonyaulax extract (peak of GDA).

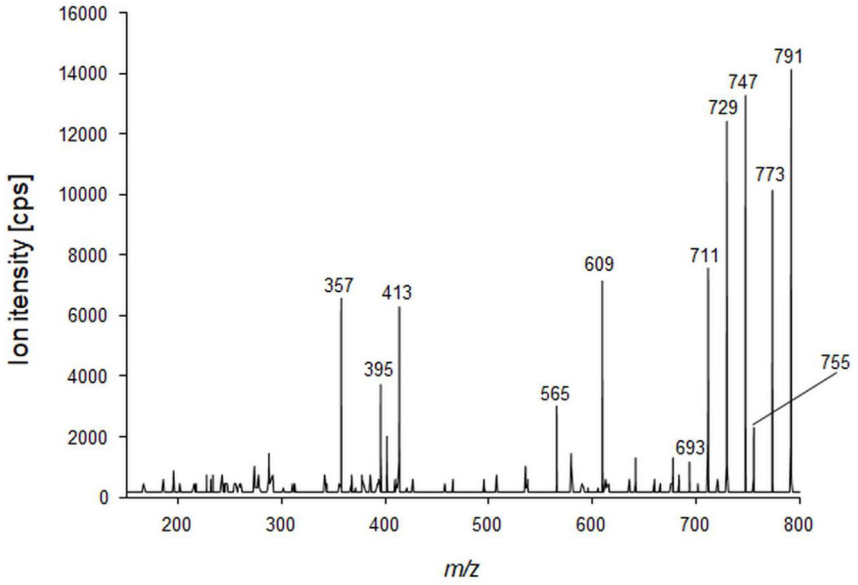

Fig. 4. Collision induced dissociation (CID) spectrum of m/z 791 (sodium adduct of GDA) in the mass range m/z 150–800.

amounts of GDB ranging between 0.01 and 0.07 pg cell^{-1}, which was roughly 0.2% of the major compound GDA. All stains were also tested for paralytic shellfish poisoning toxins (PST), but no PST could be detected. Detection limits were estimated individually for each toxin group on a per cell basis and depended on extracted cell numbers.

LOD of C1/2 ranged between 61 and 519 fg cell^{-1}, of GTX1/4 between 800 and 6730 fg cell^{-1}, of GTX2/3 between 43 and 361 fg cell^{-1}, of B1 and NEO between 230 and 1930 fg cell^{-1} and of STX between 47 and 400 fg cell^{-1}. None of the strains contained any cycloimines that are known to be produced by some species of the genus *Alexandrium*.

3.5. GDA distribution in plankton field samples

GDA was detected in all three areas sampled during the expedition: North Sea, Limfjord and Kattegat (Fig. 1). The occurrence of GDA in the 20–50 µm plankton fraction was very patchy throughout the entire expedition, but highest GDA levels were detected in Limfjord (up to 590 ng NT^{-1} m^{-1}), whereas GDA in the North Sea and the Kattegat in no case reached 100 ng NT^{-1} m^{-1} (Fig. 8). GDA was not detected in the German Bight, but appeared north of Sylt Island to the western mouth of Limfjord. GDA was hardly detected in the Kattegat, but appeared further south in the Great Belt and Kiel Bight area. Further survey information can be found in the PANGAEA data base (Krock et al., 2017).

4. Discussion

Bulk production, isolation and structural confirmation of GDA allowed for the preparation of analytical standard solutions for LC-MS method development and quantification of GD. Only little has been published on chemical analysis of GD. In 1988, Murakami et al. (1988) was the first to publish the structure of GDA elucidated by ^{1}H and ^{13}C-NMR spectroscopy. Eighteen years later the chemical structure of GDA was confirmed by NMR in a different species and high resolution mass

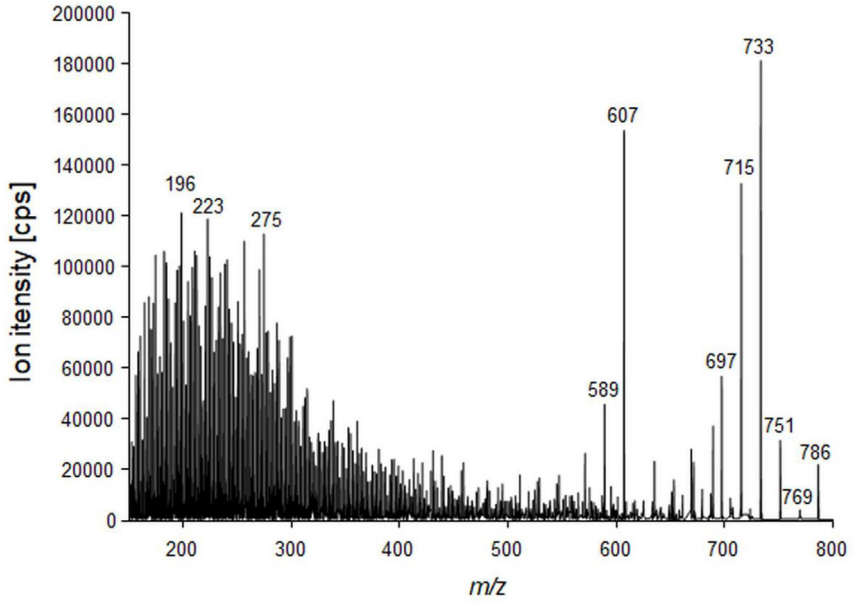

Fig. 5. CID spectrum of m/z 786 (ammonium adduct of GDA) in the mass range m/z 150–800.

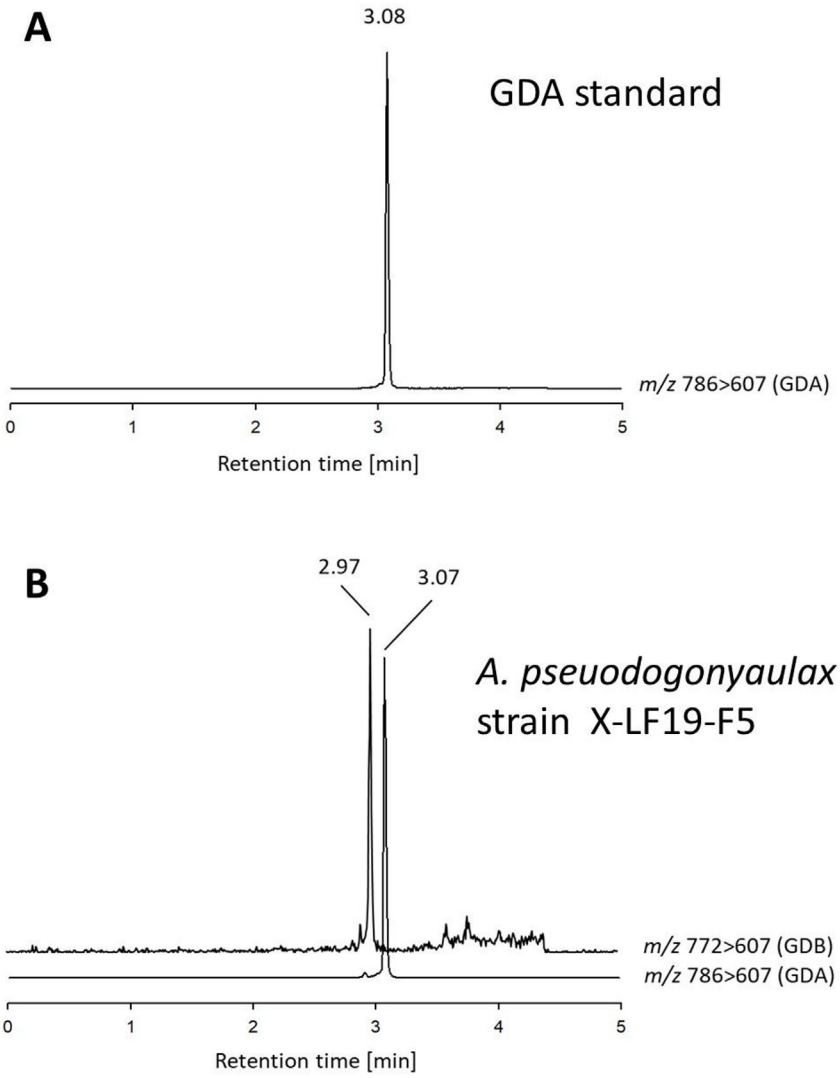

Fig. 6. Ion trace chromatograms of A) a GDA standard solution and B) A. pseudogonyaulax strain X-LF19-F5 containing GDA and GDB.

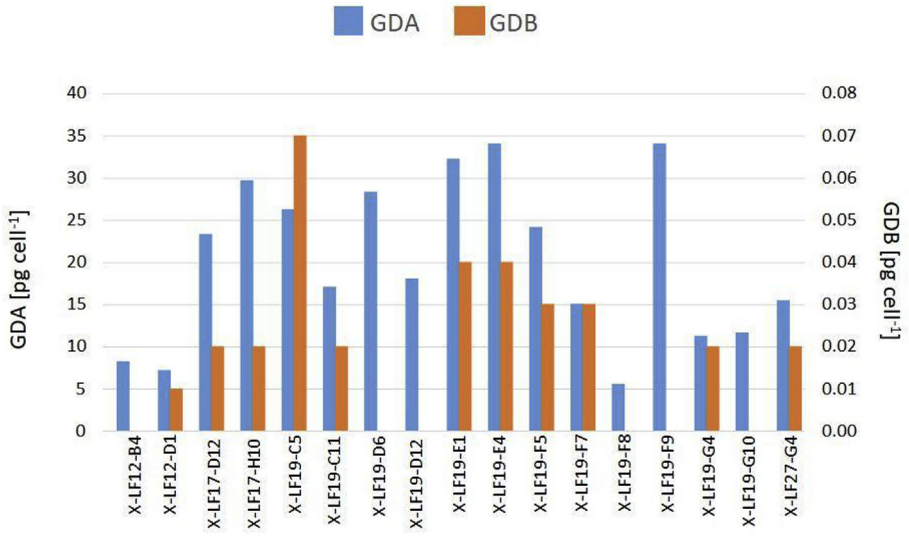

Fig. 7. Goniodomin profiles of 17 A. pseudogonyaulax strains. Left vertical axis show GDA cell quotas (blue bars) and right vertical axis shows GDB cell quotas (orange bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

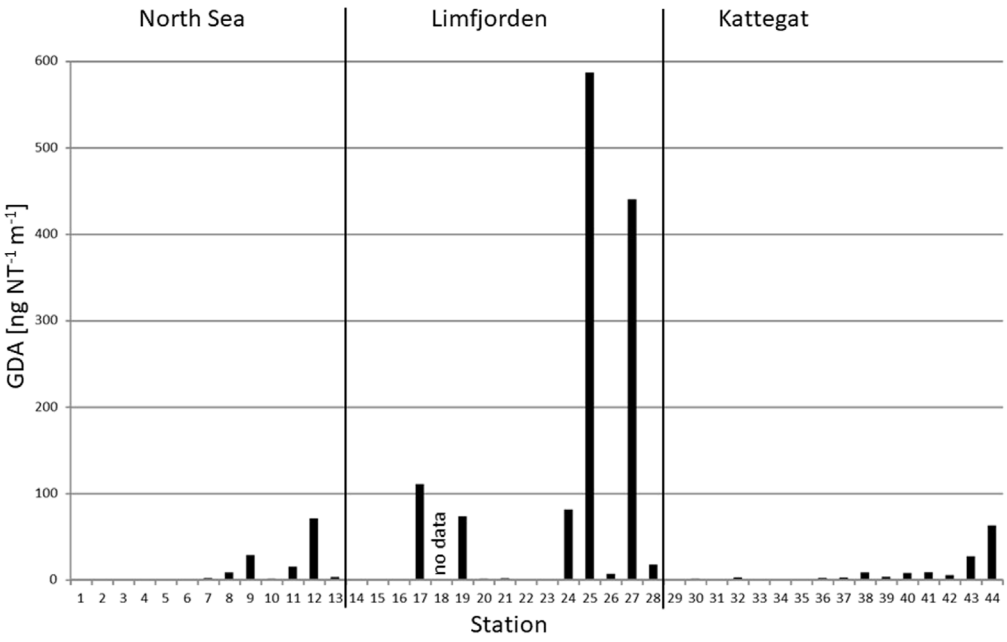

Fig. 8. GDA abundances in the 20–50 µm size fraction of vertical net tows during the expedition. As most sampling stations in Limfjord were shallower than the standard 20 m sampling depth and accordingly different net tow depths were applied across sampling stations, GDA abundances were normalized to ng per net tow per meter of water column (ng NT^{-1} m^{-1}).

spectra of GDA were published (Hsia et al., 2006). Very recently GDA was reported by single quadrupole mass spectrometry in a strain of *A. pseudogonyaulax*, which was isolated after incubation of resting cysts from Bizerte Lagoon, Tunisia (Zmerli Triki et al., 2016). In the present study, a description of a quantification method for GDA and GDB in plankton field samples and cultures of GDA/B producing organisms by UPLC coupled triple quadrupole (QqQ) in the SRM mass spectrometry (MS) mode is presented. In addition, CID spectra of the sodium adduct (Fig. 4) and the ammonium adduct of GDA (Fig. 5) are presented for identification of this compound as commercial GD standards are still not available. In terms of identification, the sodium adduct *m/z* 791 may be more useful due to the well-defined fragments (Fig. 5), however, it is noteworthy that despite the numerous fragments formed by the ammonium adduct *m/z* 786, the total ion yield of the most abundant fragment, *m/z* 733 with almost 200,000 cps (Fig. 4), is more than an order of magnitude higher than the most abundant fragment of the sodium adduct with 14,000 cps. For this reason, methods for the detection and quantification of GD based on fragmentation of the ammonium adduct are likely to be more sensitive than those using transition of the sodium adduct. An interesting difference in the fragmentation of both adducts is that CO_2 is eliminated only from the sodium adduct *m/z* 791 resulting in the fragment *m/z* 755 (Fig. 4), but the elimination of CO_2 is not observed in the case of the ammonium adduct *m/z* 786. This characteristic fragmentation may be used as an additional tool for identification of GD in unknown samples.

Goniodomins are structurally similar to pectenotoxins (PTX). Both toxin groups share several chemical features: Both toxins groups are macrocyclic lactones, i.e. macrocycles are formed by an intramolecular ester bond. Both macrocycles consist of five (GD) or six (PTX) tetrahydrofuryl and tetrahydropyranyl rings and a six carbon atom long aliphatic moiety with different substitutions and a conjugated unsaturation in the case of PTX. In addition, both groups possess an exocyclic tetrahydropyranyl ring (Fig. 9). These structural similarities are also reflected in the mass spectrometric behaviour of both toxins classes that both are most sensitively detected as ammonium adducts (discussion above, Quilliam et al., 2001, Krock et al., 2008). Furthermore, as most polyketides, CID spectra of GD and PTX are characterized by several water losses from their pseudo-molecular ions. In addition, CID spectra of both groups are very fragment rich, especially in the mass range below *m/z* 500 (Fig. 5, Suzuki et al., 2009), a feature in which these spectra differ from the CID spectra of many polyketide marine biotoxins.

In general, little is known about the mechanisms of toxicity of GD and PTX, but due to their structural similarity also similar modes of action can be assumed. In fact, PTX have been shown to be hepatotoxic (Terao et al., 1986) and besides its antibiotic activity (Sharma et al., 1968), GD were recently shown to be cytotoxic and to target hepatocytes (Espiña et al., 2016). These data indicate that there may be common pharmacokinetic mechanisms between both toxin groups. Certainly more research is needed for the assessment of potential health risks that may be caused by the consumption of GD contaminated seafood.

Even though GD have been known for 30 years, virtually nothing is known about their occurrence in plankton field samples. This is probably due to the lack of commercially available standards for compound identification and quantitation and suitable detection methods. In addition, GD (in contrast to PTX) are not regulated and there is no legislative need to monitor GD in seafood. In order to demonstrate the applicability of the developed LC-MS/MS method for the detection of GD in the field, plankton samples of an expedition into Danish waters (Fig. 2) were analysed where potentially GD-producing *A. pseudogonyaulax* had been reported before (Jakobsen et al., 2015). GDA was detected at many stations throughout the entire transect with the highest GDA abundances in Limfjord, which connects the North Sea in the west with the Kattegat in the east. Limfjord is a very shallow embayment so that the vertical depths of plankton net tows were variable among stations depending on the water depth at each station. To enable comparisons between net tows of different stations toxin values were normalized to net tow meter (NT^{-1} m^{-1}). There are no data for comparison, but GDA levels as high 550 ng NT^{-1} m^{-1} were not reached by any other phycotoxin during this expedition. Assuming a mean GDA cell quota of 20 pg $cell^{-1}$ and water volume of 125 L sampled per meter water depth, this would correspond to a maximum *A. pseudogonyaulax* cell density of 220 cells L^{-1} in Limfjord in summer 2016.

In addition, the strains of *A. pseudogonyaulax* that were isolated during the expedition were analysed for the presence of GD, paralytic shellfish poisoning (PSP) toxins and other lipophilic toxins known to be produced by the genus *Alexandrium*, but no toxins other than GD were detected. The absence of other phycotoxins in *A. pseudogonyaulax* highlights the fact this species does not produce any of the phycotoxins typically known for the genus, but only produces GD, at least in the North Sea region. The observed GDA cell quotas in the range about

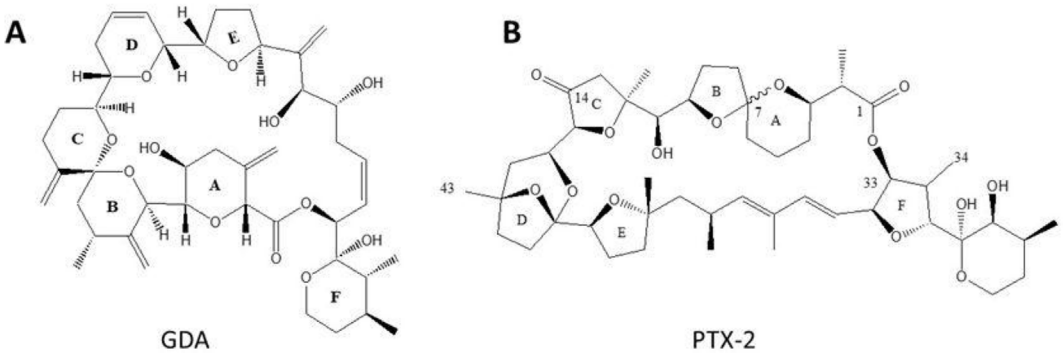

Fig. 9. Chemical structures of A) goniodomin A (GDA) and B) pectenotoxins-2 (PTX-2).

20 pg cell^{-1} are the first report of GD content in GD-producing species and no data for comparison are available yet. But the cellular GD content of the tested *A. pseudogonyaulax* strains is comparable to the toxin content of other toxic phytoplankton of comparable size (Tillmann et al., 2009; Martens et al., 2016). In most strains also GDB was detected at two orders of magnitude lower levels than GDA (Fig. 8). GDB levels were relatively close to the limit of detection, which on a per cell basis depends on the available biomass and thus varies among samples. This means that the lack of detection in the strains without detectable GDB does not necessarily mean that these strains did not produce GDB at low levels.

5. Conclusions

This work has performed the first quantification of GDA in plankton field samples as demonstrated on an expedition to the North Sea, Limfjord and the Baltic Sea in 2016. Although the highest abundances of GDA were found in the Limfjord area, GDA was also detected in a number of samples collected in the North Sea and the western Baltic Sea, albeit at significantly lower concentrations. These results suggest that *A. pseudogonyaulax* may become a problematic species in the North Sea and especially in Scandinavian waters, and due to the ability of GDA to bioaccumulate in marine species and its association with invertebrate mortalities (Harding et al., 2009), it may have adverse consequences on the aquaculture industry in the area. To date there no reports of human intoxications caused be GD, but still little is known about the toxicity of GD to vertebrates including humans or to the environment. The lack of reports of shellfish poisoning events associated with GD may also be due to the fact that *A. pseudogonyaulax* was not an abundant species in the investigated area. Climate change and anthropogenic activities may favour proliferation of this species and we suggest to monitor GDA levels in plankton and bivalves for commercial use as a precautionary measure until a risk assessment on the exposure of shellfish consumers to GD will be available as a solid basis for potential regulation of this toxin class.

Ethical statement

The authors declare to follow the ethics outlined in the Elsevier “ethics in research and publication brochure”.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2018.10.007.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.toxicon.2018.10.007.

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