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MASTER THESIS

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Studies on the stability of the phycotoxin goniodomin A and its conversion products in organic extractants and in the culture medium

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Abbreviations

APCI ..... *atmospheric pressure chemical ionization*
API ..... *atmospheric pressure ionization*
CID ..... *collision induced decay*
EPI ..... *enhanced product ion scan*
ESI ..... *electrospray ionization*
GD ..... *goniodomin*
GDA ..... *goniodomin A*
PFD ..... *photon flux density*
R/V ..... *research vessel*
RP ..... *reverse phase*
-sa ..... *seco acid*
SPE ..... *solid phase extraction*
SRM ..... *selected reaction monitoring*

Abstract

The aim of the present work was to determine conditions for a reliable analysis of the potentially ichthyotoxic phycotoxin goniodomin A (GDA) by LC-MS/MS. For this purpose, the question under which conditions GDA is chemically stable should be answered.

In view of this, stability studies were performed on GDA using organic and aqueous solvents.

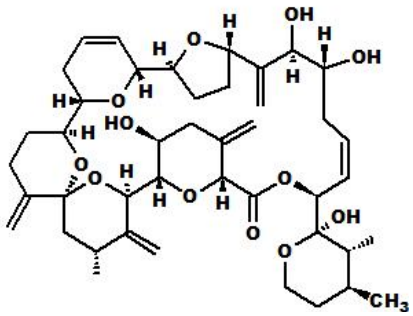
In addition, similar stability analyses of the congeners GDB/C and analyses with cell extracts of GD producing dinoflagellate species were performed. Different extractants and extraction methods were tested for the gain of GDA from dinoflagellate cells. Selected reaction monitoring (SRM) was used for detection and identification of the compounds accompanying GDA, which was also supported by fragmentation analyses when required. In the course of this work, the analytical system was optimized with respect to the eluent, transitions and adduct ions. The different methods were applied to biological samples from dinoflagellate cell cultures and to field samples from Danish and German coastal waters.

The results of the stability analyses showed that GDA is stable in organic solvents but rapidly converts to GDA seco acid in presence of water. To avoid this reaction when extracting natural GDA from cells, it may be useful to process samples directly and to harvest the cells by vacuum filtration. For the analysis of GDs by LC-MS/MS, it is also advisable to use an alkaline eluent, since an acidic eluent favors the interconversion of GDA/B/C and thus distorts the analytical results. With the help of this finding, it was possible to revise the results of a field study among others.

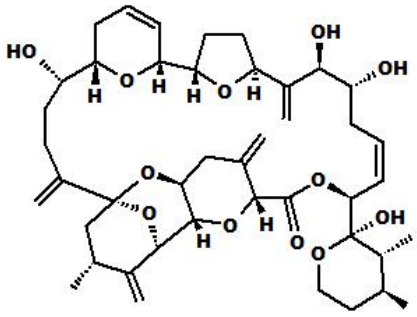
1 Introduction

Goniodomin A (GDA) is a polyether macrolide which is produced by dinoflagellates of the genus *Alexandrium*. The first goniodomin was described in 1968 as an antifungal substance isolated from a Puerto Rican dinoflagellate referred to as *Goniodoma*[1]. The identity of the organism was not determined at that time, but according to the morphological description made at the time of its discovery, it is assumed to be a member of the genus *Alexandrium*[2]. Recently, Harris et al. found that the goniodomin in the 1968 initial description is GDA[2]. GDA was described as such in 1988 when it was isolated in Japan from the rock pool organism *Alexandrium hiranoi* [3]. In the early 2000s, many attempts were made towards the elucidation of the structure and absolute configuration of GDA in order to enable the total synthesis of GDA. In 2015, recurrent blooms of a GD producing dinoflagellate, *Alexandrium monilatum*, became an issue in parts of the Gulf of Mexico and brought GD research back into focus. One year later, the existence of an isobaric congener was proposed that was named GDB. In 2018, Krock et al. published the development of an LC-MS/MS method for the quantification of GDA and GDB in which they misinterpreted a substance with a molecular weight of 754 Da for GDB (768 Da)[4] due to a wrong structure of GDB published by Espiña et al.[5] The lower molecular mass component was later identified by Harris et al. to be a desmethyl-GDA.

GDA



GDB



34-desmethyl-GDA

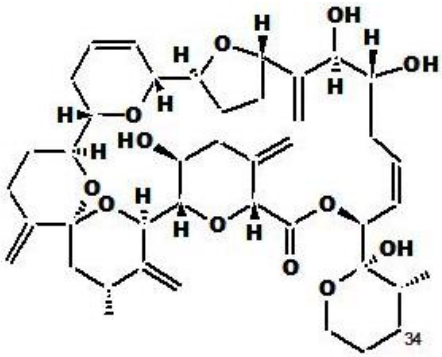


Figure 1-1: Structures of GDA, GDB and 34-desmethyl-GDA

As current research has shown, there exist many goniodomin-like substances that could be isolated from *Alexandrium* cells. Recently, the existence of two novel goniodomins was proposed. The structure proposals will be published shortly[6]. The goniodomins have been named goniodomin C (GDC) and goniodomin D (GDD).

GDC

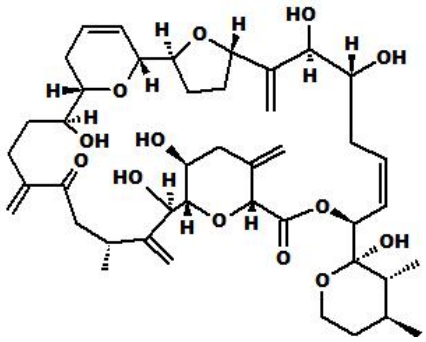


Figure 1-2: Structure of GDC

Moreover, the existence of a seco acid of GDA was confirmed and the mechanism of formation was proposed. The reaction can take place under aqueous alkaline conditions. The intramolecular ester bond (lactone) in the GD molecule is subjected to hydrolysis from which a hydroxycarboxylic acid (seco acid) results (see Figure 1-3). Seco acids of other GDs have not been identified yet. There might be a broad spectrum of conversion products and potential congeners that are to be reported about in near future.

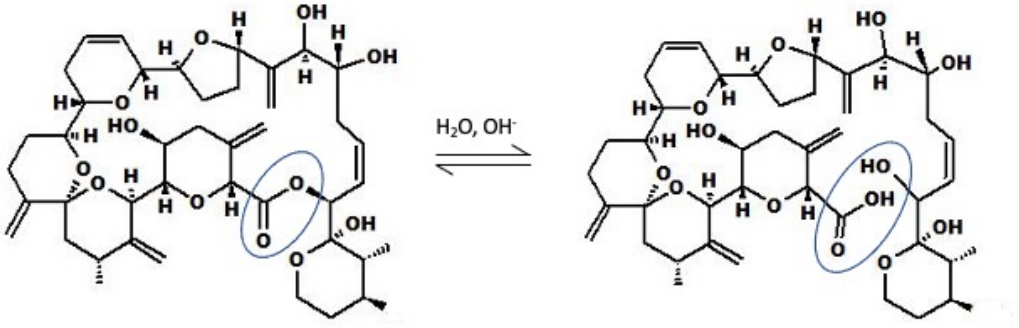


Figure 1-3: Formation of GDA seco acid (hydroxycarboxylic acid) by hydrolytic cleavage of the intramolecular ester bond in GDA (lactone)

GDA is a poorly studied substance but there are indications that interconversion between GDA and its congeners GDB and GDC is possible. A study is to be published soon. So far, it is also not yet clear whether GDB and GDC are metabolic products or arise as artifacts. These two toxins will also be addressed briefly in the present work.

Early on it was found that GDA exerts an antifungal effect[1]. Efforts were made to investigate the physiological effects of this toxin ranging from morphological changes in rat liver and thymus[7] to conformational changes of actin and conditioned by that, the activation of actomyosin ATPase[8]. Cytotoxicity studies with GDA on mammalian noncontractile cell models revealed a cytotoxic effect that was reflected in membrane polarization and actin cytoskeleton disturbance.[5] Furthermore, it was considered a potential cytostatic due to its antiangiogenic activity in endothelial cells[9]. Although studies indicated cytotoxicity of GDA, effects on human health are currently unknown[10] and might not be relevant because there are no suitable entry points for the toxin.

In contrast, the ecological relevance of GD producing *Alexandrium* species should be stressed. Blooms these species have frequently been associated with death of fish and marine invertebrates. For example, a bloom of *Alexandrium monilatum* in York River (USA) was causally linked to death of veined rapa whelk, an invasive gastropod, and the high amount of GDA found in whelk tissue lead to the conclusion that the toxicity of this dinoflagellate is connected to its goniodomin production[11].

Red tides caused by GD-producing species might also become an economical issue when aquacultures and touristic regions are affected. In times of global climate change, massive growth and widespread distribution of harmful microalgae is an increasing phenomenon. The near-global spread of the producer organisms may also pose a greater problem in the future.

This thesis aims to optimize the handling of samples containing GDs in the laboratory for the best possible preservation of the sample composition. For this purpose, stability studies of goniodomin standards and cell extracts will be performed under feasible laboratory conditions. A further focus will be on how GDs behave in marine environments. Various experimental approaches are taken to understand the transformation of the toxin in its natural environment and to include chemical as well as biological factors. Additionally, the four dinoflagellate species known to produce GDA will be presented and their extra- and intracellular goniodomin profiles will be compared.

Field samples from German and Danish coastal waters and Limfjord will be analyzed for their GD content to gain an overview about the natural distribution of goniodomins and to emphasize the gain in currency of goniodomin research. The last chapters will include an optimization of the analytic conditions used for goniodomin determination and quantification. Finally, the optimized conditions will be applied to a stability study with the three congeners GDA/B/C.

1.1 Stability of GDA in organic extractants

There is evidence that GDA can undergo a manifold of conversion reactions besides the conversion to GDB and GDC but the conditions that favor these reactions have not been elucidated yet.

To study GDA, it is necessary to determine conditions under which it is stable for at least the duration of extraction and determination. Establishing such conditions enables a range of experiments that would otherwise be susceptible to falsification by substances that arise from chemical conversion of GDA. One example is experiments aimed at determining the toxicological properties of the pure substance GDA. Likewise, it is necessary to establish stable GDA standards in order to enable routine toxin screening of environmental samples that also allows quantification of GDA.

Based on this, further studies could deal with the ecological relevance of GDA, for example whether there are other goniodomins formed besides GDA and in which quantities, whether they are physiologically relevant and able to distribute in the oceans. It might also be important to know, whether transformation products are already formed in the organism or they occur after release due to lysis of the dinoflagellate cells or exudation into seawater. In this context, the analysis of natural toxin profiles of GD-producing dinoflagellates seems to be reasonable. In order to reflect these profiles as unbiased as possible, it is necessary to critically analyze all aspects of the extraction process, sample analysis and storage. Also the cultivation conditions might have a potential influence. But this particular aspect will not be topic of this thesis.

The overall objective in this thesis is to determine conditions that preserve the original composition of the sample during extraction and storage. In addition, it should be elucidated, which conversion products result from inadequate storage and how fast they develop.

In a first experiment, commonly used extraction conditions will be tested with cells of *Alexandrium hiranoi* to gain an overview of the stability of goniodomins in these extracts and possible conversion products. The commonly used method includes the lysis of cells by reciprocal shaking with ceramic beads in anhydrous methanol and the consecutive removal of macromolecular cell constituents.

As an alternative to methanol, acetone will be tested, which is also a commonly used extractant for lipophilic phycotoxins. The effect of the extraction agent on the sample composition will be tested and additionally, the efficiency of the two different organic solvents can be compared.

There is evidence that conversion products of GDA are formed as a result of hydrolysis. Accordingly, they occur primarily in the presence of water. Besides, unusually high or low pH values might favor conversion reactions. In principle, however, they can be excluded in a natural environment and by the choice of extraction agent. In contrast, the presence of water cannot always be excluded.

Biological sample material in particular retains a certain amount of residual water that can be stored between and within cells. This could promote conversion reactions of the toxin released from damaged cells before and during extraction. For this reason, an additional treatment will be introduced that includes drying the cell pellets prior to extraction. The extraction then will take place together with the treatments containing residual water to allow for a comparison of the methods

1.2 Stability of GDA in aqueous environment

Following stability experiments of GDA in organic solvents, the objective in the subsequent chapter is to assess the chemical behavior of GDA in aqueous environment. This is particularly relevant for evaluating the ecological relevance of goniodomins, as GDA can be released into seawater by exudation or rupture of dinoflagellate cells.

The following experiments are aimed to determine how stable GDA is in seawater and whether transformation products are formed that may be of relevance to a toxic effect of GD-producing dinoflagellates. It is already known that the lactone bond GDA can be subject to hydrolysis under aqueous and alkaline conditions (see Figure 1-3). Therefore, seco acids and possibly other hydrophilic conversion products are expected to be formed in the appropriate environments. Besides the identification of potential conversion products formed from GDA under aqueous conditions, a further aim is to study the kinetics of a potential conversion process.

Chemical analytical studies are an important supplement to biological toxicity assays. The combination of both is necessary to eventually answer the question of whether GDA itself exhibits a toxic effect in the aqueous environment after release from cells of *Alexandrium* spp. or whether there are other related substances that should be considered for future toxicity assays with marine organisms. In view of this, three different sample types will be analyzed:

First of all, the GD content of field samples from the Danish Limfjord will be evaluated. The samples in question were collected with SPATT bags (“solid phase adsorption toxin tracking”). These are resin filled pouches for passive adsorption of dissolved polyether toxins[12] which are suitable to collect GDA and its congeners released into seawater. SPATTs are a valuable tool to analyze the distribution and transformation of phycotoxins in their natural environment.

The second experiment will also study GDA released from cells but in this case the samples are taken from a monoclonal culture that was grown under defined conditions. The culture supernatant of an *A. pseudogonyaulax* strain that was also isolated from Limfjord will be analyzed for its goniodomin content. Compared with the corresponding field samples, the culture might provide a less complex

environment and it will show the effect of the dinoflagellates on their own, as it excludes interfering substances like toxins or other small biomolecules from other marine organisms. By this simplification, it might not reflect the actual natural situation in a marine environment but it can still be compared with it.

The third part of this experimental series will treat the pure substance GDA and its behavior in culture medium based on autoclaved sea water as a model without any biological influence. In parallel, the effect of pure water on the toxin will be investigated by uptake of the substance in deionized water. Samples will be measured over a certain period of time in order to analyze the time course of a potential conversion of GDA to more hydrophilic substances. As a control, the time-dependent behavior of GDA in anhydrous methanol will be monitored.

1.3 Toxin profiles of GD producers

Since cells of the species *A. hiranoi* and *A. pseudogonyaulax* have already been subject of experiments in the previous chapters, the *Alexandrium* species known to produce GDA are to be discussed in the following. The species will be introduced and the question briefly addressed in 1.1, which GDs are metabolites of the organism and which are formed as transformation products after release into seawater, is to be discussed, also with regard to the ecological relevance of GDA and GDs in general.

Currently there are four species of *Alexandrium* known to produce goniodomins, with one identified as such only one year ago (2020). When the first goniodomin was described in Puerto Rico, no precise information on the producer species was provided. The 1968 publication referred to the species as *Goniodoma sp.*. The genus name was used as eponym for the discovered toxin. Until today, the identity of the organism could not be clarified. According to the morphological description made at the time of its discovery, it was assumed to be a member of the genus *Alexandrium*.[2]

In 1985, a dinoflagellate species blooming during summer time in rockpools of the Japanese Pacific coast was the first species identified to produce GDA. Initially it was erroneously identified as *Goniodoma pseudogoniaulax* that is today known as *Alexandrium pseudogonyaulax*. Three years later it was identified to be a different species and was named *Alexandrium hiranoi*. [3]

In 2006, the chain-forming dinoflagellate *Alexandrium monilatum* was identified as GDA producer.[13] The species was first described in the 1950s as causative organism of frequently occurring red tides that have been associated with fish and invertebrate mortalities.[14] Those were assumed to be linked to its GDA production.[15] *A. monilatum* is native to American waters. Among other regions it is frequently observed in the Gulf of Mexico.[15]

In March 2016, an *Alexandrium pseudogonyaulax* strain isolated from Bizerte Lagoon, Tunisia, was reported to produce GDA as well.[16] Phylogenetic studies presented in this publication revealed the cosmopolitan character of *A. pseudogonyaulax*. Studies on the occurrence of this species in the North Sea proposed that it might develop dominance of the *Alexandrium* population in waters of Northern Europe.[17]

Recently, also a fourth species, *Alexandrium taylorii,* was shown to produce GDA.[18] The species has been reported in the Mediterranean Sea[19], Indonesia[20], Malaysia[21] and Japan[22]. *Alexandrium taylorii* is responsible for recurrent dense blooms in the Mediterranean but it has not been causatively linked to fish kills yet.[18] As all four species belong to one phylogenetic cluster, it is to be assumed that potential other members of this group might also produce goniodomins.[18] A known fifth member of this phylogenetic group, *Alexandrium satoanum*, has not been tested for goniodomins yet.[18]

In this chapter the goniodomin profiles of the species *A. hiranoi*, *A. monilatum*, *A. pseudogonyaulax* and a Mediterranean strain of *A. taylorii* (AY7T) will be analyzed with HPLC-MS/MS through selected reaction monitoring of all known goniodomins. The intracellular profiles will be evaluated by analysis of cell extracts. The extracellular profiles will be established based on the goniodomin content determined in the culture supernatants. For comparison of the individual species, the toxin concentrations will be related to the cell count and specific differences in the profiles will be highlighted.

1.4 Analysis of *A. pseudogonyaulax* samples from Limfjord

Previous experiments with biological material were largely based on cell cultures in which the sample material was generated under controlled conditions. Since with these samples natural conditions are only reflected to a limited extent, it is obligatory to analyze natural samples (field samples) in addition. In the course of the experimental series described in 1.2, SPATTs have already been studied as field samples taken in course of a survey in the Limfjord. This expedition will now be discussed in more detail in order to illustrate the increasingly important role of a GD producing species in northern European waters.

In the course of studies concerning the distribution of *A. pseudogonyaulax* in German and Danish coastal waters and the evaluation of a future hazard potential of this species, a field survey was conducted in the Limfjord and also in parts of the adjacent waters of North Sea and Baltic Sea (see Figure 1-4). The Limfjord is a shallow brackish water sound in Denmark that separates North Jutland

from the Jutland Peninsula. It is connected to the North Sea in the West and the Kattegat in the East. It has a maximum depth of 28 m[23] and is an important production area for mussels in Denmark[24].

An earlier study conducted in 2016 was aimed at the relationship of salinity and the distribution of *Alexandrium* species and their toxins in North Sea estuaries and the Baltic Sea.[17] This study revealed that there are important drivers other than salinity, such as nutrient input and habitat conditions, that affect the composition of the population. This was reflected by long-term monitoring data revealing that *A. pseudogonyaulax,* a species that had previously been associated with marine environments with higher salinities, gained dominance over the *Alexandrium* population in the brackish Limfjord in recent years. It was proposed that the species might expand further into the Baltic Sea.

The sampling areas of 2016 were resampled in 2020 to confirm these trends, also in light of an improved quantification method for goniodomins.[4]

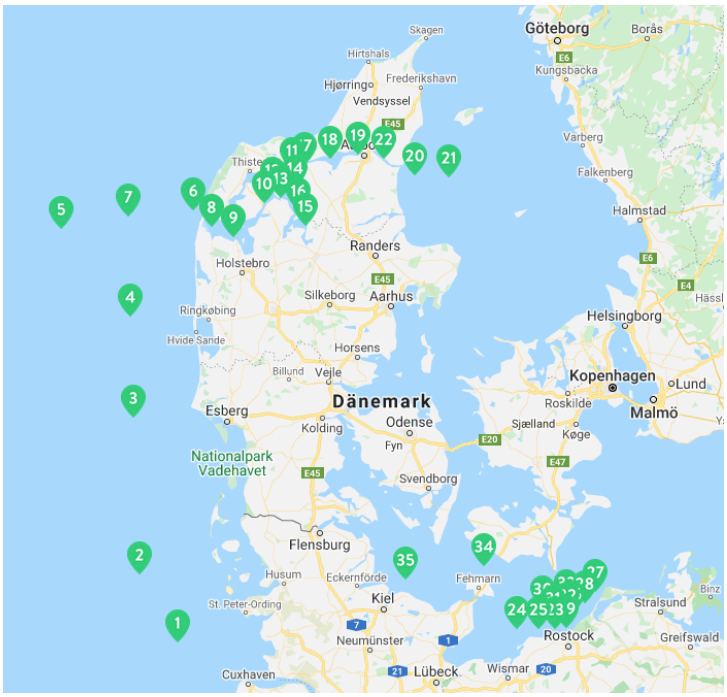


Figure 1-4: Sampling stations during the field survey with R/V Uthörn 2020 (map created with itilog)

1.5 Influence of analytic conditions on the composition of goniodomin samples

In previous analyses, observations were made which suggest that the apparent sample composition could be influenced by the analytical system. In the stability experiments with GDA (see 3.1 and 3.2), a certain amount of the congeners GDB and GDC was detected immediately after preparation of the samples that did not change within the period analyzed. This was assumed as an indication that the conversion is due to the analytical system and does not take place in the uninfluenced sample.

This assumption was supported by the finding that low amounts of GDB and GDC were always present in freshly prepared GDA standards. The identities of the congeners were confirmed by means of the corresponding standards. Moreover, all three congeners were detected in these standards.

Since the purity of the standards was assured by the provider, contamination could be excluded in principle so that it might be reasonable to consider the analytic conditions as a potential cause of a spontaneous interconversion of GDA, B and C.

In LC-MS/MS, the analyte comes into contact with a mobile phase, which has the purpose to elute the analyte from the chromatography column and to facilitate ionization by the additives it contains. It is possible that, depending on the pH and additives of the mobile phase and the duration of contact, chemical transformations of the analyte may either be supported or be suppressed. Therefore, the choice of the measurement system could result in artifact signals suggesting the presence of a toxin that is actually not part of the original sample state.

In order to verify this hypothesis, freshly prepared standards of GDA, GDB and GDC of confirmed purity will be analyzed using two different eluent systems. It is assumed that the eluent pH might have an influence on the interconversion of GDA, B and C. For this reason, the optimization involves the comparison of an alkaline eluent with the acidic eluent used in the previous analyses.

Furthermore, the use of different adduct ions may contribute to an improved MS/MS analysis of single goniodomins as they are assumed to have different affinities for certain adduct ions. Therefore, the SRM method will be supplemented with the sodium adducts of known goniodomins so that a comparison of ammonium versus sodium adducts will be possible. Thus, four combinations of eluent and adduct ion will be tested. If a signal occurs in all systems, it can be assumed that the corresponding substance is not an eluent artifact.

Next, the systems will be applied to biological samples to test whether the results of the optimization are confirmed in these more complex matrices. In course of this, samples of experiments conducted previously will be re-measured in order to determine whether the earlier results were falsified by the presumably unsuitable system and what the actual GD composition of these samples is.

1.6 Re-assessment of the stability of GDA/B/C

After successful optimization of the measurement system manifested in the suppression of the formation of artifact signals, the stability of GDA, GDB and GDC in methanol and aqueous solvents will be revisited. Up to this point, little attention has been paid to the stability of GDB and GDC due to the limited material available and as the main focus of this thesis is on GDA.

It will be analyzed whether there is a difference in the chemical behavior of GDA compared with the earlier analyses performed with the unoptimized method and which conversion products actually arise under aqueous conditions.

Additionally, the actual congeners GDB and GDC will be analyzed for their behavior in the different solvents and the question is to be answered, whether there arise different conversion products than those of GDA. Apart from the measurement conditions, the procedure of sample preparation and storage will be identical to the stability experiment with pure GDA described in 1.2.

2 Methods

2.1 Cultivation of algae

2.1.1 Preparation of culture medium

K medium (Keller et al., 1987)[25] was used as culture medium for all strains. Instead of artificial seawater as described in the original recipe, North Sea water was used for preparation of the culture medium. 4 L of North Sea water were autoclaved and cooled down to ambient temperature prior to further use. The following stock solutions were utilized for preparation of the medium.

Stock solutions

Table 2-1: Stock solutions for preparation of K medium

Compound	Stock solution concentration	Final molar concentration
$NaNO_3$ (Applichem)	75 g/L dH_2O	8.82 x 10^{-4}
NH_4Cl (Merck)	2.67 g/L dH_2O	5.00 x 10^{-5}
$NaH_2PO_4 \times H_2O$ (Merck)	5 g/L dH_2O	3.60 x 10^{-5}
H_2SeO_3 (Sigma Aldrich)	12.5 mg/L dH_2O	1.00 x 10^{-8}
Tris-HCl (pH = 7.2) (Sigma Aldrich)	121.1 g/L dH_2O	1.00 x 10^{-3}

K trace metals stock solution

Table 2-2: Ingredients of the K trace metals stock solution for preparation of K medium

Compound	Stock concentration	Molar concentration
$Na_2EDTA \times 2\ H_2O$	41.6 g/L	1.12 x 10^{-4}
$FeCl_3 \times 6H_2O$	3.15 g/L	1.17 x 10^{-5}
$Na_2MoO_4 \times 2H_2O$	6.3 g/L	2.60 x 10^{-8}
$ZnSO_4 \times 7H_2O$	22 g/L	7.65 x 10^{-8}
$CoCl_2 \times 6H_2O$	10 g/L	4.20 x 10^{-8}
$MnCl_2 \times 4H_2O$	180 g/L	9.10 x 10^{-7}
$CuSO_4 \times 5H_2O$	9.8 g/L	3.92 x 10^{-8}

F/2 vitamin solution

Table 2-3: Ingredients of the vitamin stock solution for preparation of K medium

Compound	Stock solution concentration	Final molar concentration
Vitamin B_{12}	1 g/L	3.69×10^{-10}
Biotin	0.1 g/L	2.05×10^{-9}
Thiamine, HCl		2.96×10^{-7}

The trace metal solution was heated on a magnetic stirrer to 60 - 70 °C before use. In the following table the volumes of components added to the sterile seawater are listed.

Table 2-4: Volumes of K medium components

Volume	Medium component
4 mL	$NaNO_3$
4 mL	NH_4Cl
4 mL	$NaH_2PO_4 \times H_2O$
0.4 mL	H_2SeO_3
4 mL	Tris-base (pH 7.2)
4 mL	K trace metal mix
2 mL	F/2 vitamin solution

After adding the components, the pH was set to 8.2 with 1 N HCl. The final medium was filtered with a 0.1 µm Supor® membrane at 200 mbar and stored in a dark place at room temperature until further use.

2.1.2 Stability of GDA in organic extractants

For this experiment, a stock culture of *Alexandrium hiranoi* with a total cell count of 791,000 was prepared. This particular strain was chosen because it provided the highest GDA yields of all available strains. Five subcultures were prepared in large-scale plastic flasks in 500 mL of sterile K medium each with initial cell densities of 75, 100, 125, 250 and 500 cells per mL.

In order to prepare a dilution series, 1 L of a dilution with 500,000 cells was produced from 158 mL of stock culture and the corresponding volume of K medium. From this dilution, 500 mL (K1), 250 mL (K2), 125 mL (K3) and 125 mL (K4) were successively taken. The culture flasks were thoroughly inverted to obtain homogenous cell densities. Cultures K2-K4 were replenished with sterile K medium to 500 mL each. A fifth culture with a density of 75 cells per mL was prepared from 12 mL of the remaining stock culture. The flask was filled up with K medium to 500 mL. The dinoflagellates were cultivated at a temperature of 20 °C on a 16 h light : 8 h dark photo-cycle and a photon flux density (PFD) of 100 µE.

Table 2-5: Dilutions used for long-term culture of Alexandrium hiranoi

Culture vessel	mL of K medium added	Cell count	Cell density (cells/mL)
K1		250,000	500
K2	250	125,000	250
K3	375	75,000	125
K4	375	50,000	100
K5			75

2.2 Extraction of goniodomins from algal cells

2.2.1 Extraction of cells (*A. hiranoi*) for a stability assay in organic extractants

Prior to harvesting, from each culture a 500 µL sample was taken for fixation with Lugol's iodine solution and light microscopy to determine the cell count per culture. The culture with the highest cell density was selected for the experiment. This was culture K2 with a density of 2328 cells/mL. Cultures K4 and K5 were discarded due to poor growth. The other two flasks were cultivated further for other experiments.

To harvest the cells, the cell culture flask was inverted and the content was transferred to a 1 L glass bottle. To determine the cell loss caused by liquid transfer, a further counting sample was taken. A cell density of 2069 cells/mL was determined but it was found to be sufficient for this experiment. The culture volume was divided into twelve aliquots. Cells were harvested by centrifugation at 4,000 rcf for 15 min (Eppendorf 5424 R, Hamburg, Germany). After centrifugation, the supernatants were decanted and combined in one flask, from which a 500 µL sample was taken to test for cell loss. The cell pellets were resuspended in remaining culture medium and transferred to twelve 2 mL cryotubes. After centrifugation at 12,000 rcf for 15 min (Eppendorf 5810 R, Hamburg, Germany) and removal of residual water, the pellets were stored at -80 °C. Half of the cell pellets were lyophilized overnight prior to the extraction process (Thermo Savant).

For extraction, about 0.9 g of lysing matrix D (MP Biomedicals, Eschwege, Germany) and 250 µL either methanol or acetone (HPLC grade) were added to each pellet. The cells were ruptured for 45 s at a speed of 6.5 m/s in a FastPrep-24 instrument (MP Biomedicals). Cell debris was removed from the extracts by centrifugation for 15 min at 16,100 rcf (Eppendorf 5810 R) and consecutive centrifugation of supernatants for 1 min at 10,000 rcf through spin-filters with a pore size of 0.45 µm (Millipore Ultrafree). The filtrate was transferred to HPLC micro-sampling vials with screw tops and PTFE septa. The cell pellets were treated a second time with 250 µL of extraction solvent and again subjected to the described extraction procedure. Between analyses, the vials were equipped with new caps and were stored in the refrigerator at a temperature of 4 °C. During analysis, the autosampler temperatures were set to 10 °C by default. At the beginning of the experiment, the samples were measured daily. After a period of ten days the intervals between analyses were extended since during the first measurements there were no significant changes observed. The concentrations for each time point were calculated based on the sum of peak areas of all MS/MS transitions. For each treatment, the mean concentration value of the three samples was calculated. Quantification was performed by use of an external GDA standard with a concentration of 500 pg/µL.

2.2.2 Extraction of cells of four species of *Alexandrium* for determination of their GD profiles

Samples of all species except *A. hiranoi* were already present as frozen cell pellets and supernatants, produced in a similar way as described earlier. Cells of *A. hiranoi* were harvested by centrifugation (4,000 rcf, 15 min). The culture supernatants were decanted and frozen at -20 °C. The cell pellets were resuspended and transferred to 2 mL cryotubes. After centrifugation (15 min at 16,100 rcf) and removal of the supernatant, the cells were added a defined volume of methanol (see Table 2-6) and ruptured with lysing matrix D in a FastPrep-24 instrument (MP Biomedicals). The extracts were centrifuged for 15 min at 16,100 rcf (Eppendorf 5810 R) and spin-filtrated for 1 min at 10,000 rcf. For LC-MS/MS analyses, the final extracts were transferred to HPLC micro-sampling vials. The cell pellets were again treated with the same volume of methanol and the extraction steps were repeated.

Table 2-6: Cell count, culture volume and volume of extraction solvent

	Cell count	**Culture volume [mL]**	**Extraction volume [µL]**
A. hiranoi	103,450	50	2x 250
A. pseudogonyaulax	677,400	250	2x 250
A. taylorii	319,500	300	2x 250
A. monilatum	115,050	50	2x 250

2.3 LC-MS/MS Analyses

2.3.1 Mass spectrometers and coupled chromatography systems

Two different LC-MS/MS systems were utilized in the course of the experimental work for this thesis for reasons of comparability in the laboratory surrounding.

API 4000 Q TRAP® LC/MS/MS System

The API 4000™ (Sciex, Darmstadt, Germany) is a hybrid triple quadrupole LIT mass spectrometer that can be equipped either with an ESI source (TurbolonSpray® probe) or an APCI probe. For all experiments described, the ESI source was used. The instrument was coupled to an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) and sample separation was performed using a HyperClone 3 µm BDS C_8 130 Å 50 x 2 mm column (Phenomenex®, Aschaffenburg, Germany).

Data evaluation was performed using the inherent Analyst software. Separation of sample components was achieved with an acidic eluent and gradient elution. Mobile phase A consisted of deionized water with 50 mM formic acid and 2 mM ammonium formate for detection of GD ammonium adducts. Mobile phase B consisted of 50 mM formic acid and 2 mM ammonium formate in 100% acetonitrile. A gradient was applied to the HPLC method. After an initial equilibration at 20% B for 12 min, B was increased to 100% in 4 min. Analytes were eluted at 100% B for 12 min, after which the initial conditions were re-established within 0.5 min, followed by 2 min column equilibration at 20% B. The flow rate was set to 0.2 mL/min and the total run time was 30 minutes consisting of 12 min equilibration time and 18 min gradient run.

Table 2-7: Gradient applied in the HPLC (API4000)

Run time [min]	%A (aqueous)	%B (organic)
12 (equilibration)	80	20
4	0	100
16	0	100
18	80	20

Waters ACQUITY Xevo TQ-XS UPLC/MS System

The Waters Xevo TQ-XS is a tandem quadrupole MS system equipped with a ZSpray™ dual-orthogonal API source. It is coupled to an ACQUITY UPLC® system (Waters, Eschborn, Germany) equipped with a Purospher STAR RP-18 endcapped (2 µm) column (Merck Millipore, Darmstadt, Germany). For system control, data acquisition and evaluation, the included MassLynx® software was employed.
Separation of sample components was achieved with an acidic eluent. Mobile phase A consisted of 0.2% formic acid and 0.004 % NH_4OH in deionized water. The organic eluent (mobile phase B) was composed of acetonitrile and 5% H_2O. A gradient elution was applied, starting with 5% B for 1.5 min and rising to 100% B in 3.5 min with consecutive isocratic elution at 100% B for 3 min, return to the initial conditions within 0.5 min and 1 min column equilibration at 5% B. The UPLC was operated at a flow rate of 0.6 mL/min and the total run time per sample was 8 minutes.

Table 2-8: Gradient applied in the UPLC (Xevo TQ-XS)

Run time [min]	%A (aqueous)	%B (organic)
Initial	95	5
1.5	95	5
3.5	0	100
6.5	0	100
7.0	95	5
8.0	95	5

2.3.2 MS analyses

Transitions in selected reaction monitoring (SRM)

For SRM analyses, the following transitions of single ammonium adducts of goniodomins were used.

Table 2-9: Selected Reaction Monitoring - transitions of ammonium adducts

Precursor ion (*m/z*)	Product ion (*m/z*)	Toxin
772.5	607.5	34-desmethyl-GDA
772.5	719.5	34-desmethyl -GDA
786.5	607.5	GDA, GDB (Quantifier)
786.5	733.5	GDA, GDB (Qualifier)
790.5	719.5	Undescribed
790.5	737.5	Undescribed
800.5	719.5	Undescribed
800.5	729.5	Undescribed
802.5	749.5	Undescribed
804.5	607.5	GDC, GDA/B seco acid
804.5	751.5	GDC, GDA/B seco acid
814.5	607.5	Undescribed
814.5	743.5	Undescribed
822.5	733.5	GDC seco acid
858.5	733.5	Undescribed

Fragmentation analysis

Analysis on the identity of GDC in SPATT extracts

Fragmentation analyses were carried out in Enhanced Product Ion mode (EPI) for *m/z* 804 with a collision energy of 40 eV and for *m/z* 809 with a collision energy of 55 eV. In both cases, no collision energy spread was used. For all other parameters the presets of the instrument were used. The mass range was set to *m/z* 100-820.

Fragmentation analysis of *m/z* 802

Fragmentation analyses were performed for identification of an undescribed substance with *m/z* 802. The collision energy was set to 40 eV and there was no collision energy spread used. An *m/z* range of 100-820 was scanned for fragment ions.

2.4 Field samples

2.4.1 SPATT bags

During the Limfjord survey, SPATT bags were positioned at the outflow of a FerryBox. The bags were equipped with the adsorbent DIAION® HP20 (Supelco, Darmstadt, Germany). For extraction, the SPATT bags were washed with deionized water and dried at 50 °C in a drying oven overnight. The resin was transferred from the bags to 50 mL conical tubes. The samples were shaken in 30 mL MeOH overnight. The following day, the methanol was separated from the resin using chromatography columns. The resin was added to the column and rinsed with 25 mL methanol. The extract was rotary evaporated to approximately 1 mL, transferred to Eppendorf tubes and reduced to dryness under a nitrogen stream. The dry residue was resuspended in 400 µL methanol, transferred to a spin filter and centrifuged for 1 min. The sample was transferred to HPLC vials for LC-MS/MS analysis.

2.4.2 Net haul extracts

The samples were taken during an oceanographic expedition with R/V Uthörn in fall 2020. They were collected along the German West coast, the Danish Limfjord and the Western Baltic Sea with vertical net hauls with 20 µm mesh phytoplankton net (model 438030, HYDRO-BIOS, Kiel, Germany). Plankton concentrates were filtered through a three-stage gauze filter with mesh sizes 200, 50 and 20 µm. Material from each mesh was transferred to conical tubes in small volumes of filtered seawater and was pelleted by centrifugation. The supernatants were removed and the cell pellets were stored at -20 °C until extraction. Part of the cell pellets were already extracted on board the research vessel, the remaining were extracted in December 2020. For extraction, 0.9 g lysing matrix D (ThermoSavant, Illkirch, France) and 1 mL methanol were added (1.5 mL at station 14, 200 µm fraction). Cells were homogenized by reciprocal shaking at 6.5 m/s for 45 s (FastPrep-24 5G, MP Biomedicals, Eschwege, Germany) and subsequently centrifuged for 15 min at 16,100 rcf. The supernatants were filtered through centrifugation filters (1 min, 10,000 rcf). Filtrates were immediately transferred to HPLC-vials for analysis.

2.5 Analysis of culture supernatant

Solid phase extraction (SPE)

In a preliminary experiment, direct injection of culture supernatant did not permit detection of goniodomins. Therefore, the GD content of all culture supernatants analyzed in this thesis was concentrated using C_{18} SPE cartridges (Supelco). The procedure was similar for all supernatants with the only variations in culture volume and final volume of the SPE extract. As an example, the preparation of the supernatant analyzed in 3.2.2 is described here. It was obtained from a culture of an *Alexandrium pseudogonyaulax* isolate strain from Limfjord (X-LF-12-D1) with a density of 1278 cells/mL. The 250 mL supernatant had been stored at -20 °C for almost five months prior to further processing. The SPE cartridge was conditioned with 2 mL MeOH and equilibrated with 2 mL deionized H_2O prior to sample application. After completed application of the supernatant, the cartridge was washed with 10 mL deionized H_2O for removal of salts. The retained GDs were eluted with MeOH in five fractions of 5 mL each (generally: a tenth of the culture volume). The resulting fractions were collected and concentrated to a final volume of 250 µL each in a rotary evaporator

(Heidolph instruments, Schwabach, Germany). The fraction that eluted first was centrifuged through a spin filter to remove macromolecules. The fractions were transferred to HPLC vials for analysis.

2.6 Stability studies of GDA in culture medium and pure water

1.65 µg solid GDA was dissolved in 1 mL deionized water or culture medium, respectively. To improve the solubility of the relatively lipophilic solid in the aqueous solvents, it was first dissolved in 100 µL methanol and then 900 µL aqueous solvent was added. From each sample, 500 µL was taken and transferred to a fresh HPLC vial as a replicate. Samples were stored at room temperature and measured once a day for ten days. Analyses were performed with the API4000 instrument. An automated valve switch was added to the HPLC method to prevent the salt fraction from entering the mass spectrometer.

2.7 Optimization of the LC-MS/MS system for analysis of GDs

Two eluents were compared, an acidic eluent that had been used so far an alkaline eluent with 252.5 µL NH_4OH (25%) in ACN/H_2O (9/1) and the equivalent in 100% water. The eluent was tested successfully with both MS instruments. The existing MS methods were supplemented with sodium adducts of GDA, GDB, GDC and GDA seco acid. With help of fragmentation analyses with the corresponding goniodomin standards, the most abundant fragments were identified and chosen as quantifiers. Reliable fragments with lower abundance were chosen as qualifiers. Furthermore, the collision energy for these transitions was adjusted to 55 eV because the Na adducts of GDs appeared to be more stable than the NH_4 adducts. The following transitions were added to the SRM methods. Additionally, the transitions of the NH_4 adducts in the SRM method of the Xevo TQ-XS instrument were supplemented with a different quantifier that was not detected with the API4000 (see Table 2-11).

Table 2-10: Transitions used for detection and quantification of sodium adducts of goniodomins (both MS instruments)

	Precursor	**Quantifier**	**Qualifier**	**Collision energy**
	m/z	***m/z***	***m/z***	**[eV]**
GDA/B	791.5	413.3	747.5	55
GDC/GDA seco acid	809.5	765.5	747.5	55

Table 2-11: Optimized transitions used for detection and quantification of ammonium adducts of goniodomins (Xevo TQ-XS)

	Precursor *m/z*	Quantifier *m/z*	Qualifier *m/z*	Collision energy [eV]
34-desmethyl-GDA	772.5	125.4	607.5	40
9-desmethyl-GDA (putative)	772.5	139.5	593.3	40
GDA/GDB	786.5	139.5	607.5	40
GDC/GDA seco acid	804.5	139.5	751.5	40
***m/z* 790**	790.5	147.3	737.5	40
***m/z* 802**	802.5	139.5	749.5	40
***m/z* 814**	814.5	139.5	607.5	40
GDC seco acid	822.5	139.5	733.5	40

2.8 Comparison of two extraction methods

Two different extraction methods were to be compared with regard to the content of GDA seco acid in cell pellets of *Alexandrium monilatum*. For this experiment, a frozen pellet of *A. monilatum* with a cell count of 139,250 that had been stored for 3 months was processed in comparison to a fresh culture of 300 mL of the same strain with a cell count of 442,520.

Cells of the fresh culture were harvested at a density of 1702 cells/mL. Half of the culture (150 mL) was applied to a 47 mm glass vacuum filter (GF/F circular, Whatman). Cells were drawn dry at 200 mbar for 20 min. For extraction, the filter was transferred to a 50 mL tube and covered with 5 mL methanol for 30 min. Subsequently, the filter was processed with ultrasound (7 cycles at medium power). The extraction process was repeated once. Next, the liquid extracts were decanted into 5 mL conical tubes and reduced under a nitrogen stream. The residual extracts (750 µL) were united and spin-filtrated, next they were transferred to a HPLC vial for analysis.

The other half of the culture was divided among three 50 mL tubes. Cells were harvested by centrifugation. The pellets were resuspended in residual water and united. After centrifugation and removal of residual water, cells were ruptured and extracted in 2x 500 µL methanol. The frozen pellet was extracted the same way.

2.9 Preparation of treatments for re-assessment of the stability of GDA/B/C

Treatments were prepared from the appropriate solid substance immediately prior to the first measurement in the series. For preparation of the GDA treatments, 1.65 µg of crystalline GDA was dissolved in 200 µL methanol. Two treatments were prepared with a concentration of 1.65 ng/µL each. 100 µL stock solution was added 400 µL deionized water or K medium, respectively.

Of GDB two vials were available, each containing 1.59 µg. The solid was dissolved in 200 µL methanol and added 800 µL of the aqueous solvent. As control, a fresh GDB standard with a concentration of 3.18 ng/µL was prepared in methanol. The GDC treatments were prepared using 12.6 µg of the solid. The material was dissolved in 300 µL and for preparation of the aqueous treatments, each 100 µL was transferred to a fresh vial and added 900 µL deionized water or K medium. The original vial was added 900 µL methanol. The final concentration was 4.2 ng/µL.

The samples were measured once daily for ten days and were stored at ambient temperature between analyses. Analyses were performed with the Xevo TQ-XS instrument with the alkaline eluent and the adjusted SRM method described in 2.7.

3 Results

3.1 Stability of GDA in organic extractants

3.1.1 Time course of GD concentrations

Due to strong fluctuations of the concentration values measured over time, not the absolute concentration values were plotted over time but instead the logarithmic ratios of peak areas of the goniodomins detected in the differently treated cell extracts of *A. hiranoi* were visualized.

API4000 HPLC-MS/MS

Two diagrams were created for a clearer presentation of the progressions of all GDA:GD ratios. The first diagram shows the ratios of GDA to GDs that are already identified and were found in the extract. In the second diagram, the ratios of GDA peak area to the peak areas of undescribed substances likely to be related to GDA are shown. The ratio GDA:GDA seco acid was inverted since this curve in some cases overlapped with the GDA:GDB curve.

The ratios of the peak areas of goniodomins detected in analyses with the API4000 instrument stayed essentially constant over the entire time period analyzed. The ratios were similar between the different treatments with exception of treatment 4 (acetone extracts of dried cell pellets) in which the ratio GDA seco acid to GDA was slightly higher than in the other treatments.

In the second diagram there were stronger fluctuations of the peak ratios which were pronounced in the substances that were detected in lower amounts (*m/z* 790 and *m/z* 814). In treatment 4 (acetone extracts of dried cell pellets) the curves were less well separated due to generally lesser precision at lower concentrations. Nevertheless, the values were in good accordance with those of the other three treatments.

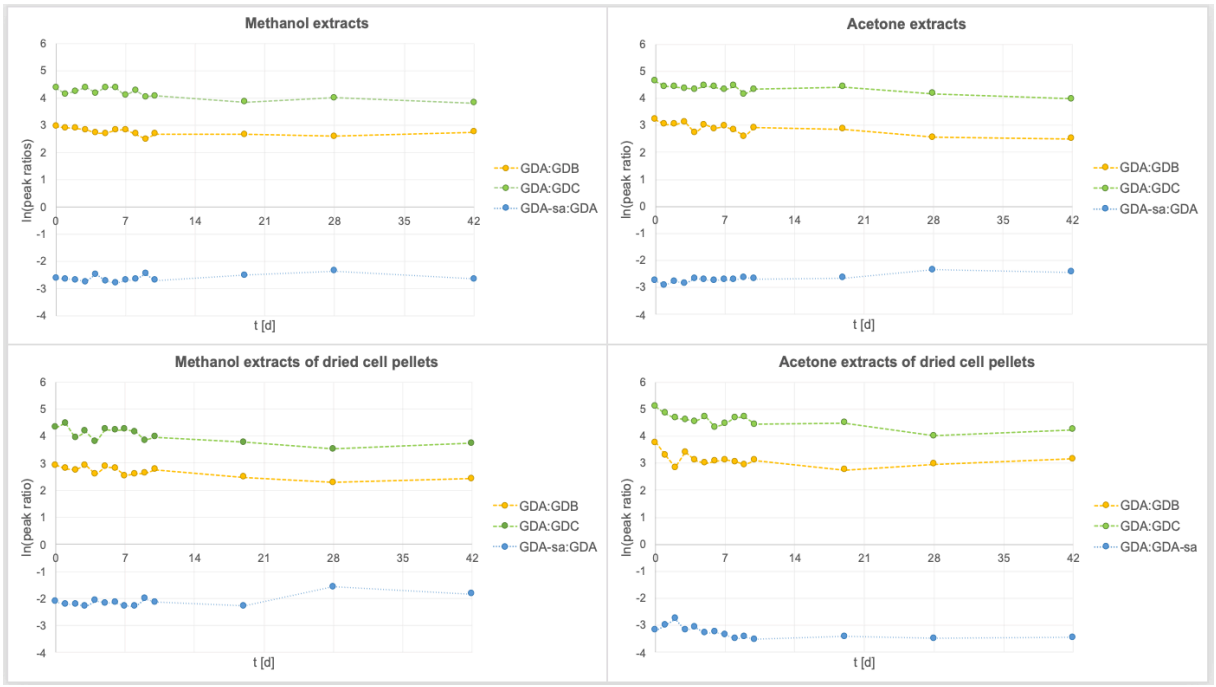


Figure 3-1: Time course of GDA:GD peak ratios over a period of six weeks as determined with the API4000 instrument

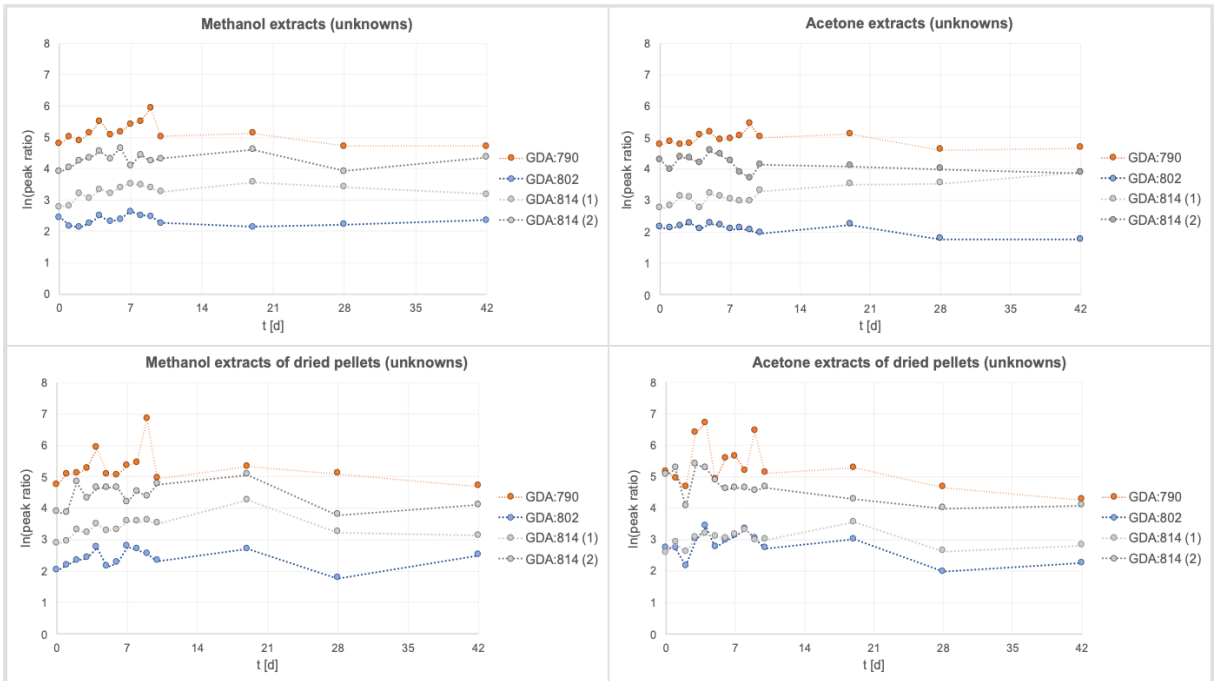


Figure 3-2: Time course of GDA:GD peak ratios for undescribed GDs as determined with the API4000 instrument

Despite the fluctuations of the absolute concentration values, the mostly constant time course of peak ratios suggests the assumption that the concentrations in the four treatments did not change over time. For this reason, the mean values of the GDA concentrations over six weeks were calculated for each treatment. The values and standard deviations are depicted in Figure 3-3.

Treatments 1, 2 and 3 had somewhat similar GDA concentrations. The minimum concentration was determined for the methanol extracts of dried cell pellets (2.18 ng/µL).

The acetone extracts had the highest concentration of all treatments (2.61 ng/µL). For the methanol extracts, an average GDA concentration of 2.30 ng/µL was calculated.

Remarkably, the acetone extracts of dried cell pellets had a much lower mean GDA concentration of only 1.06 ng/µL. The fluctuation of the concentration values measured with the API4000 instrument was reflected in the standard deviation values ranging from 0.25 (treatment 4) to 0.50 (treatment 2).

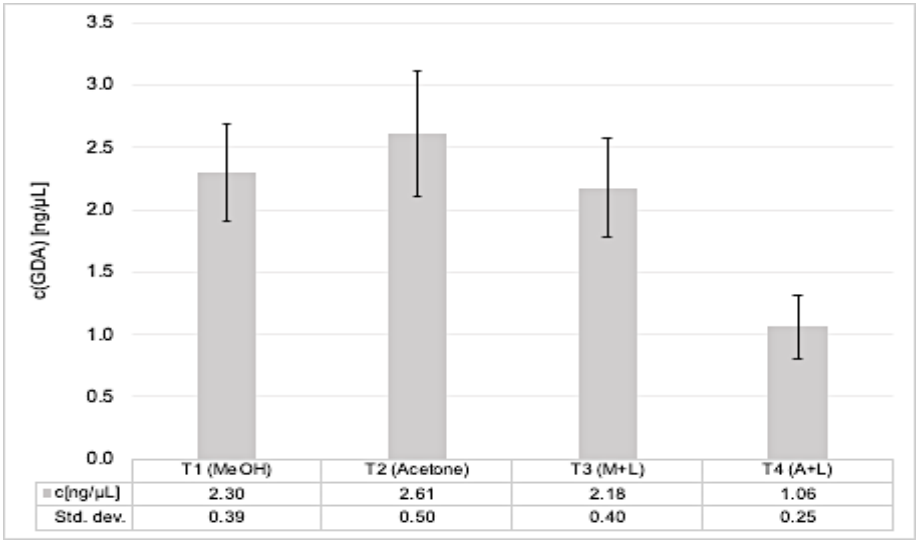


Figure 3-3: Mean values of GDA concentrations in organic solvents (API4000). Abbreviations: M+L = methanol extracts of lyophilized cell pellets; A+L = acetone extracts of lyophilized cell pellets

Xevo TQ-XS UPLC-MS/MS

In parallel, the samples were analyzed with a Xevo TQ-XS UPLC-MS/MS instrument to allow a comparison of the two MS instruments with regard to the detection of goniodomins and a confirmation of the determined results based on the dual measurement. The observation that the peak ratios stayed constant was confirmed by these analyses but the ratios determined were different: The relative amounts of GDC and GDA seco acid were comparatively smaller whereas the relative quantity of GDB was about the same or slightly higher than in the analysis with the API4000. For almost all substances the peak ratios were approximately identical. Like in the analysis with the other MS/MS instrument, however, the acetone extracts of dried pellets showed a smaller ratio of GDA seco acid to GDA, i.e. a lower relative amount of GDA seco acid than in the other treatments.

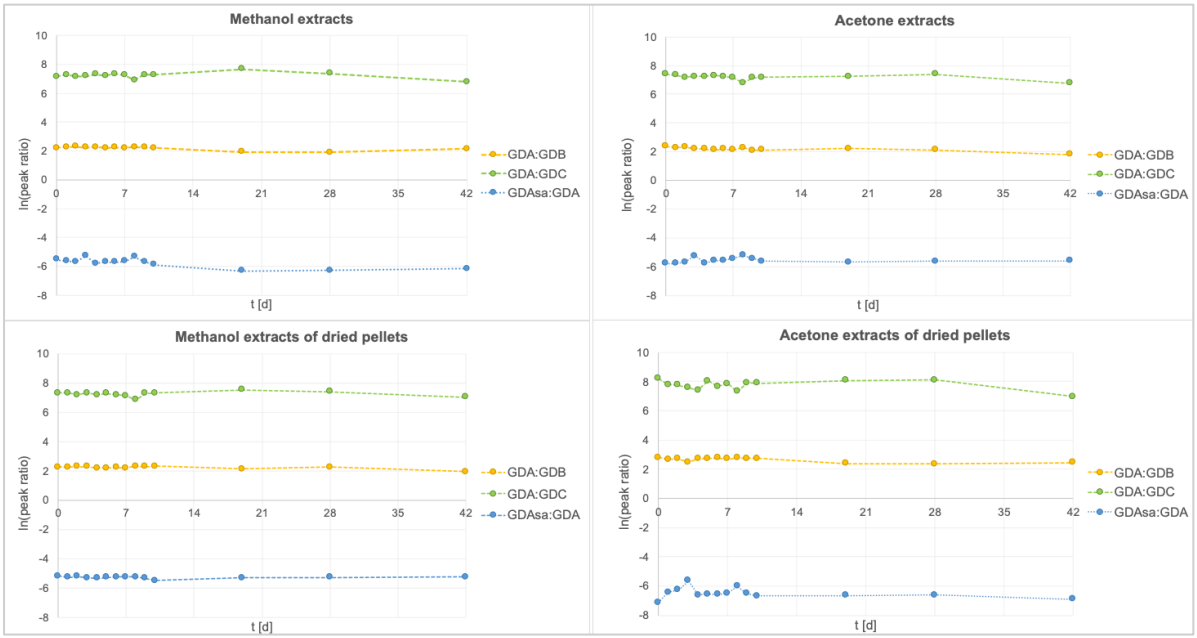


Figure 3-4: Time course of GDA:GD peak ratios over six weeks as determined with the Xevo TQ-XS instrument

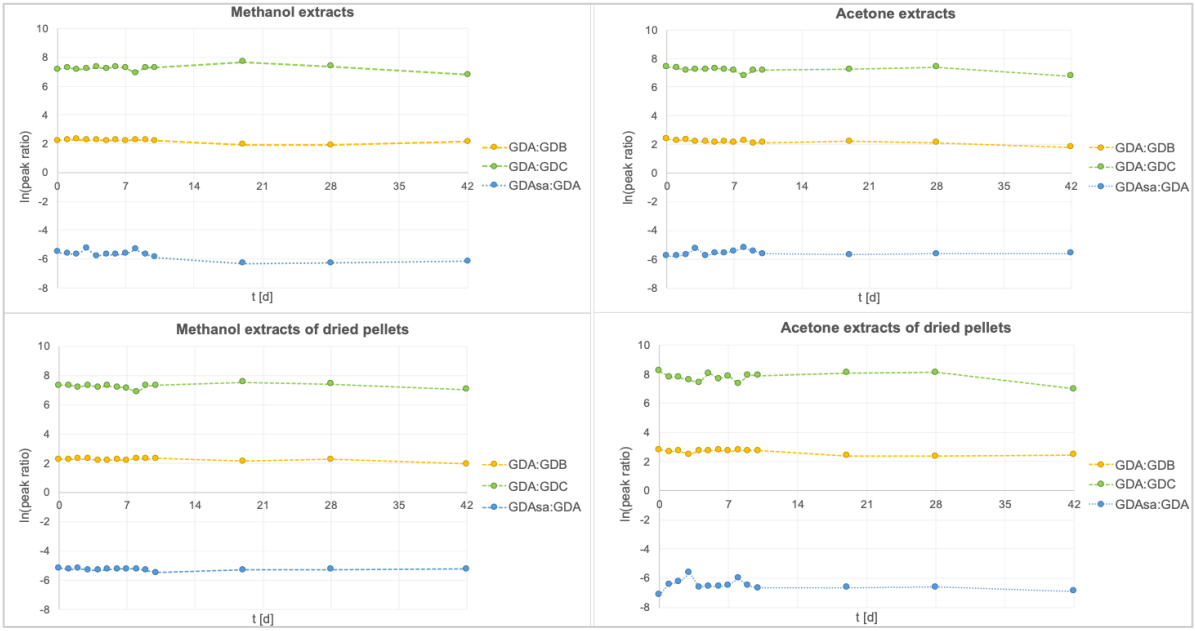


Figure 3-5: Time course of GDA:GD peak ratios for undescribed GDs as determined with the Xevo TQ-XS instrument

The mean concentration values determined with the Xevo TQ-XS instrument were on average about 0.8 ng/µL higher than the values obtained with the API4000. Treatments 1 and 3 differed only slightly in terms of their determined concentrations, whereas treatment 2 had the highest determined concentration at a level of 3.44 ng/µL. The mean concentration calculated for treatment 4 was 1.06 ng/µL and therefore well below that of the other three treatments. The standard deviations in all four treatments were slightly higher than in the analyses on the API4000. The measured concentration

values of treatment 4 had the lowest standard deviation (0.42) and those of treatment 2 had the highest (0.69).

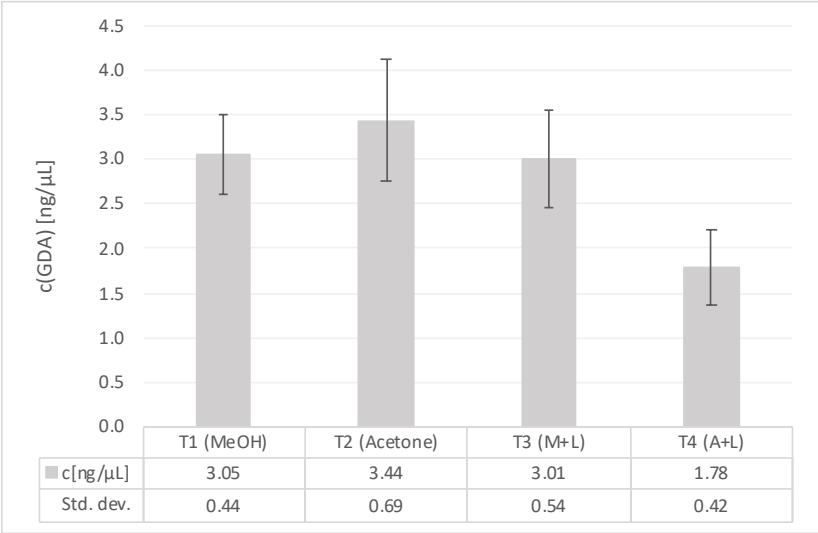


Figure 3-6: Mean values of GDA concentrations in organic solvents (Xevo TQ-XS)

3.1.2 Sample composition

The extracts of *Alexandrium hiranoi* were analyzed with a SRM method consisting of 15 transitions of known goniodomins and substances with a fragmentation pattern that suggests a structural relationship to GDA. In total, there were nine different components detected of which GDA was the main component by far. Of the eight minor compounds detected, there were each four knowns and four unknowns. Transitions and retention times of all goniodomins detected in the extracts of *A. hiranoi* are listed in the table below.

Table 3-1: Transitions and retention times of goniodomins detected in extracts of A. hiranoi

Name	***m/z* Parent**	***m/z* Daughter**	**RT [min] HPLC system**	**RT [min] UPLC system**
GDA	786.5	607.5	8.0	3.5
GDB	786.5	607.5	7.8	3.4
GDC	804.5	751.5	7.0	3.0
GDA-sa	804.5	751.5	7.2	3.2
GDC-sa	822.5	733.5	6.9	2.8
Undescribed	790.5	737.5	8.0	3.5
Undescribed	802.5	749.5	7.8	3.4
Undescribed	814.5	607.5	8.0	3.5
Undescribed	814.5	607.5	7.8	3.4

API4000

The major compound of all treatments was found to be GDA, making up 70% to 80% of the total GD pool. The largest share was determined in the two treatments in which acetone was used as extraction agent (T2 and T4). As minor components, GDB (4-5%), GDC (1%) and the seco acids of GDA (3-8%) and GDC (<1%) were detected.

The minor component with the largest share in the total composition was an undescribed conversion product with the transition *m/z* 802>749 (6-9%). This substance eluted at about the same time as GDB and could also be found as $^{13}C_2$-isotope at the transition *m/z* 804>751. Furthermore, a pair of signals with the transition *m/z* 814>607 with retention times identical to those of GDA and GDB was detected. The substance with *m/z* 814 coeluting with GDB is labelled with (2) throughout this work. Of the undescribed goniodomins, the one with the transition *m/z* 790>737 and a retention time similar to that of GDA had the lowest abundance of 0.5% in all treatments. This could possibly be a $^{13}C_4$-isotope of GDA. The least abundant compound was GDC seco acid with abundances between 0.1% and 0.4%.

The abundances of all detected sample constituents measured over a ten-day period were averaged and are shown for each treatment in Table 3-2. There were no major differences found between the abundances of the first three treatments. Remarkably, GDA seco acid was found throughout all treatments. It was even detected in an especially high amount in the methanol extracts that were gained from dried pellets (T3).

Both GDC and GDC seco acid were slightly more abundant in the methanol treatments (T1 and T3) than in the acetone treatments (T2 and T4) but they were generally detected in only very low amounts. In the acetone extracts of lyophilized cell pellets (T4), the percentage of GDA was particularly high whereas most of the other substances detected had a lower abundance than in the other three treatments. There were two exceptions, namely the substances with *m/z* 814 and *m/z* 790, which eluted simultaneously with GDA. The substance with *m/z* 790 appeared with the same abundance in all treatments.

Table 3-2: Averaged abundances of sample constituents detected with the API4000

	T1 (MeOH)	T2 (Acetone)	T3 (M+D)	T4 (A+D)
GDA	73%	74%	70%	80%
***m/z* 802**	8.8%	8.8%	9.0%	5.5%
GDA-sa	5.1%	4.8%	7.9%	3.3%
GDB	4.6%	3.9%	4.5%	3.5%
***m/z* 814**	3.9%	3.7%	3.5%	4.9%
***m/z* 814 (2)**	1.3%	1.1%	1.2%	0.8%
GDC	1.1%	0.9%	1.1%	0.8%
***m/z* 790**	0.5%	0.5%	0.5%	0.5%
GDC-sa	0.4%	0.1%	0.4%	0.1%

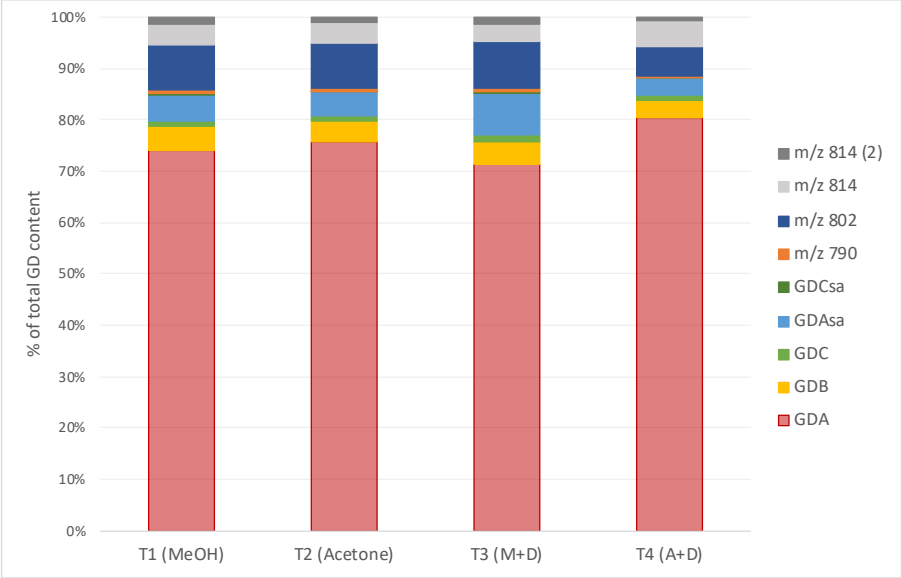


Figure 3-7: Averaged abundances of sample constituents detected with the API4000

Xevo TQ-XS

In the analyses with the Xevo TQ-XS instrument, a higher proportion of GDA in the total sample was determined. The proportion was identical (86%) in all treatments except for the acetone extracts of dried cell pellets (T4), in which it was slightly higher at 91%. GDB accounted for the second largest share of the total sample with about 9% for the methanol extracts (T1 and T3) and the acetone extract (T2) and 6% in the acetone extract that was prepared from lyophilized cells (T4). The substance with *m/z* 802 accounted for the third largest proportion (1.7%-3.4%). In general, the minor components made up a much smaller proportion of the total sample in the analyses with this instrument. GDA-sa was found with an abundance of only 0.2%-0.4%. The acetone extracts of dried cell pellets had a higher

percentage of GDA and *m/z* 814 than all the other treatments and all four treatments had the same proportion of the substance with *m/z* 790.

Table 3-3: Averaged abundances of sample constituents detected with the Xevo TQ-XS

	T1 (MeOH)	T2 (Acetone)	T3 (M+D)	T4 (A+D)
GDA	86%	86%	86%	91%
GDB	9.1%	9.4%	9.1%	6.1%
***m/z* 802**	3.3%	3.4%	3.2%	1.7%
***m/z* 814**	0.7%	0.7%	0.7%	0.9%
GDA-sa	0.3%	0.3%	0.4%	0.2%
***m/z* 814 (2)**	0.3%	0.3%	0.3%	0.2%
***m/z* 790**	0.2%	0.2%	0.2%	0.2%
GDC	0.1%	0.1%	0.1%	0.0%
GDC-sa	0.0%	0.0%	0.1%	0.0%

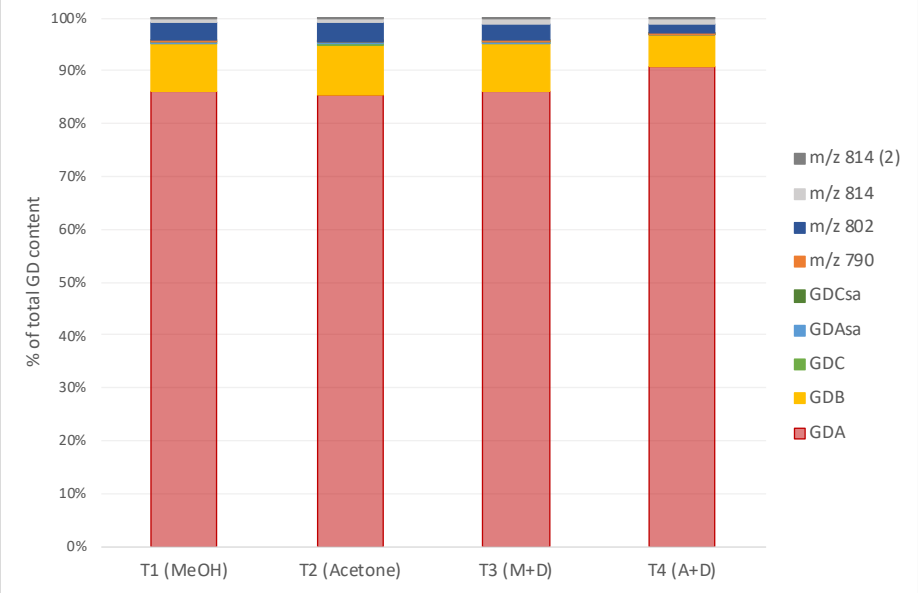


Figure 3-8: Averaged abundances of sample constituents detected with the Xevo TQ-XS

3.2 Stability of GDA in aqueous environment

3.2.1 SPATT bags

The SPATTs contained only a little amount of GDA and only in the replicate samples 278 and 279, which were taken in the Western Baltic Sea. GDB, GDC and desmethyl-GDA were not detected at all.

In all extracts, a substance was found which, like GDC, was characterized by fragmentation from *m/z* 804 to 751 and eluted only marginally earlier from the C_8 column. However, the identity of GDC could not be confirmed when the samples were subjected to fragmentation analyses and compared with GDC standards (see following pages). As major components of the SPATT extract, seco acids of GDA and GDC were detected. In sample 278, the concentration of GDA seco acid exceeded the others by far. Moreover, an undescribed substance with *m/z* 802 and a retention time of 7.8 min was detected in most of the samples. A minor compound with *m/z* 790 was detected at a retention time of 6.7 min. Of all SPATTs, the samples taken in the Baltic Sea (278 and 279) contained the largest total amount of goniodomins whereas in the Kattegat the least were detected.

The concentration values in ng/µL are listed below in Table 3-4. Concentrations were calculated based on the peak area of an external GDA standard with a concentration of 1 ng/µL. Seco acids are abbreviated with the suffix "-sa".

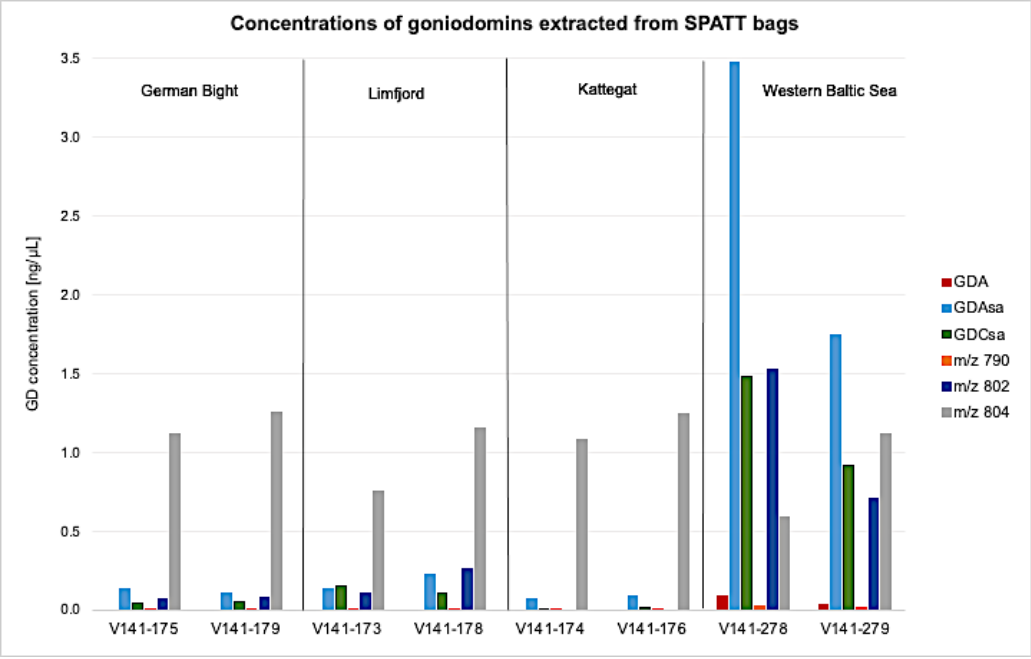


Figure 3-9: Concentrations of goniodomins extracted from SPATT bags

Table 3-4: Retention times and concentrations of all toxins (in ng/µL) detected in SPATT extracts (API4000 instrument)

Toxin	GDA	GDB	GDC	GDA-sa	GDC-sa	*m/z* 790	*m/z* 802	*m/z* 804
RT [min]	8.0	7.8	7.0	7.3	6.9	6.7	7.8	7.1
V141-130	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29
V141-173	0.00	0.00	0.00	0.13	0.15	0.00	0.11	0.76
V141-174	0.00	0.00	0.00	0.07	0.01	0,01	0.00	1.08
V141-175	0.00	0.00	0.00	0.14	0.05	0.01	0.07	1.12
V141-176	0.00	0.00	0.00	0.09	0.02	0.01	0.00	1.25
V141-178	0.00	0.00	0.00	0.23	0.11	0.01	0.26	1.16
V141-179	0.00	0.00	0.00	0.11	0.06	0.01	0.08	1.26
V141-278	0.10	0.00	0.00	3.48	1.48	0.03	1.52	0.59
V141-279	0.04	0.00	0.00	1.74	0.92	0.02	0.71	1.12

Fragmentation analyses were carried out to yield information about the identity of the substance with *m/z* 804 that eluted at about the same time as GDC. It was questioned that the detected substance was GDC, as it had never been detected in aqueous environments before. The samples were compared with a GDC standard and an *A. pseudogonyaulax* extract in which GDC had been detected in earlier analyses. The extract originating from the same survey as the SPATT bags was chosen for comparison in order to cope for potential retention shifts due to the sample matrix. Both ammonium and sodium adducts of GDC were analyzed. As the spectra of all SPATT extracts were similar, the sample with the highest ion yield was selected for comparison with a GDC standard with a concentration of 2 ng/µL.
In the SPATT extract (Figure 3-11), the prominent fragment *m/z* 751 was found as well as the precursor *m/z* 804, which was not detectable in the GDC standard (Figure 3-10), indicating that the NH_4 adduct of actual GDC is less stable than the precursor of the putative GDC in the SPATT extract. The intensities of the fragment *m/z* 751 differed considerably, with twice the intensity achieved in the sample (about 3.0e4) than in the GDC standard (about 1.5e4). The fragment *m/z* 607 did not occur in this spectrum. This particular fragment is an important indicator of the F ring cleavage, a characteristic fragmentation reaction of goniodomins. In the spectrum of the SPATT extract, a fragment with *m/z* 337 stands out, which was not found in the spectrum of the GDC standard. It is also notable that GDC eluted almost 0.1 min later than the undescribed *m/z* 804 detected in the SPATT extracts. This strengthens the evidence that the two analytes cannot be the same substance. To further confirm the disparity of the two substances, the spectra of the Na adducts (*m/z* 809) were compared. From these it is also evident that the substances cannot be identical. Here it is even more obvious, because the spectra do not match except for the first two fragments. Despite the overall higher ion yield in the spectrum of the SPATT extract, the fragment intensities are so indistinct that they can almost be treated as noise.
Similar to the spectrum of the NH_4 adduct, a single clearly protruding fragment (*m/z* 199) can be seen that was not found in the CID spectrum of the GDC standard.

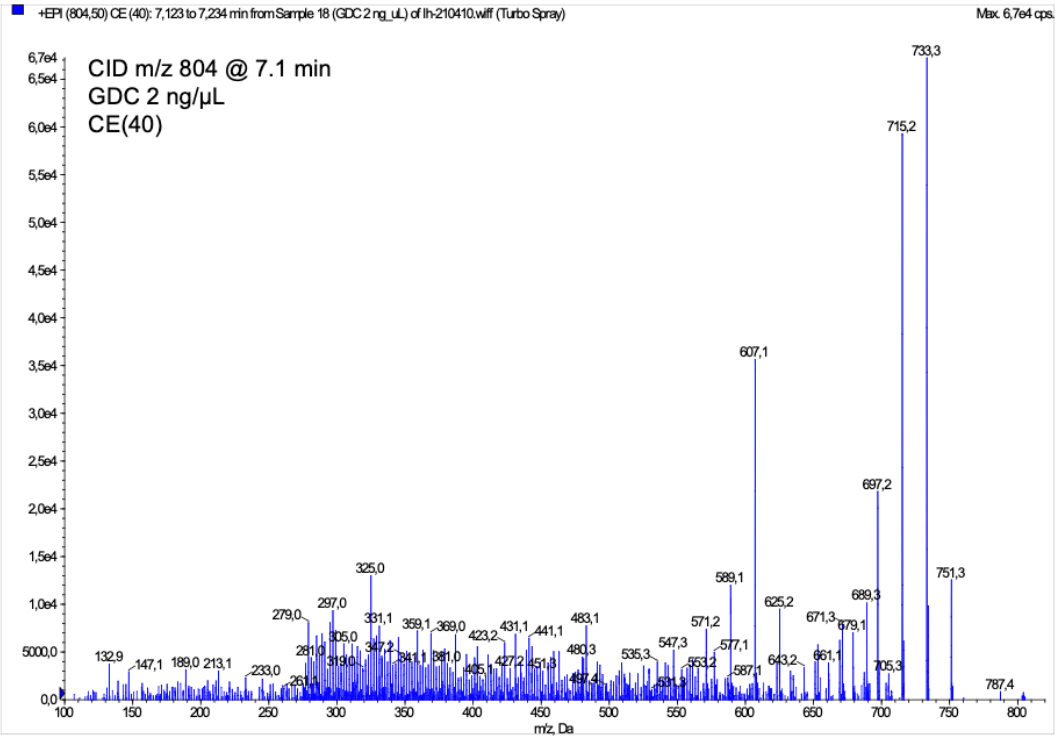


Figure 3-10: CID of GDC $[M+NH_4]^+$ (std. conc. 2 ng/µL)

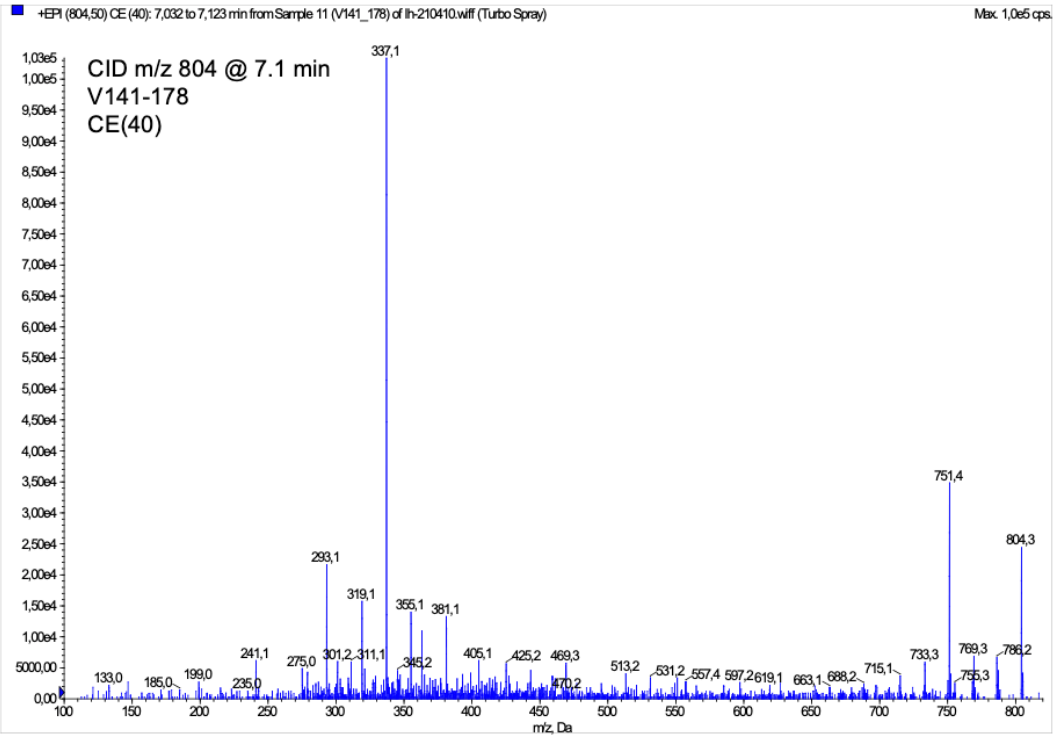


Figure 3-11: CID of m/z *804 in SPATT extract V141-178*

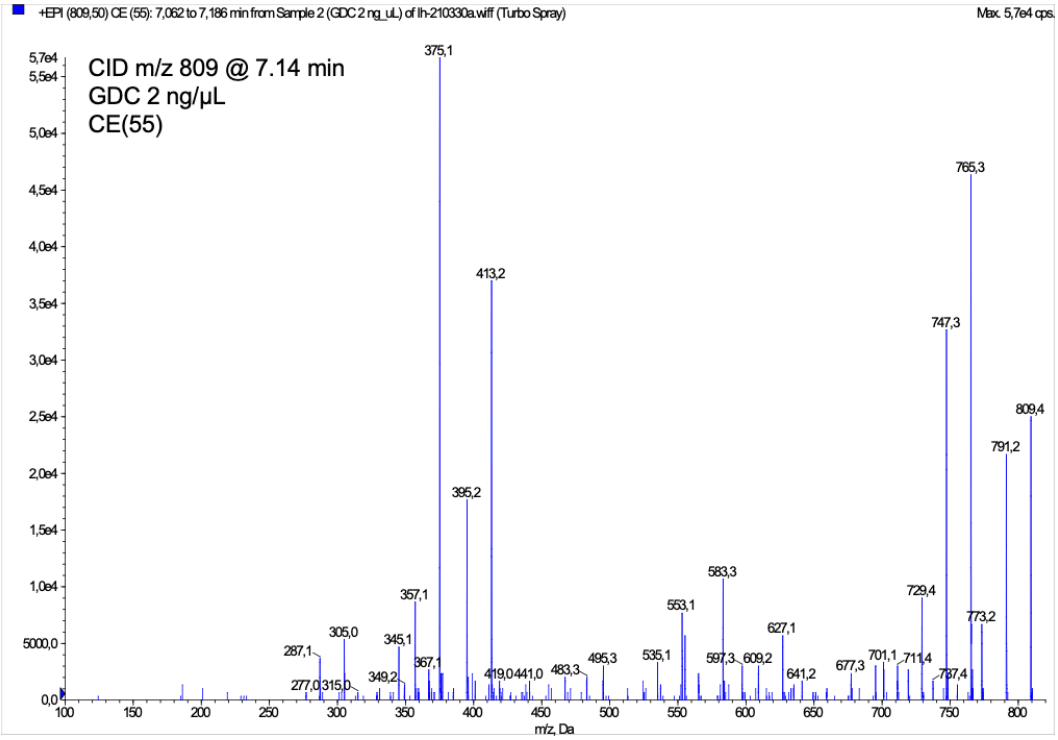


Figure 3-12: CID of GDC $[M+Na]^+$ (std. conc. 2 ng/µL)

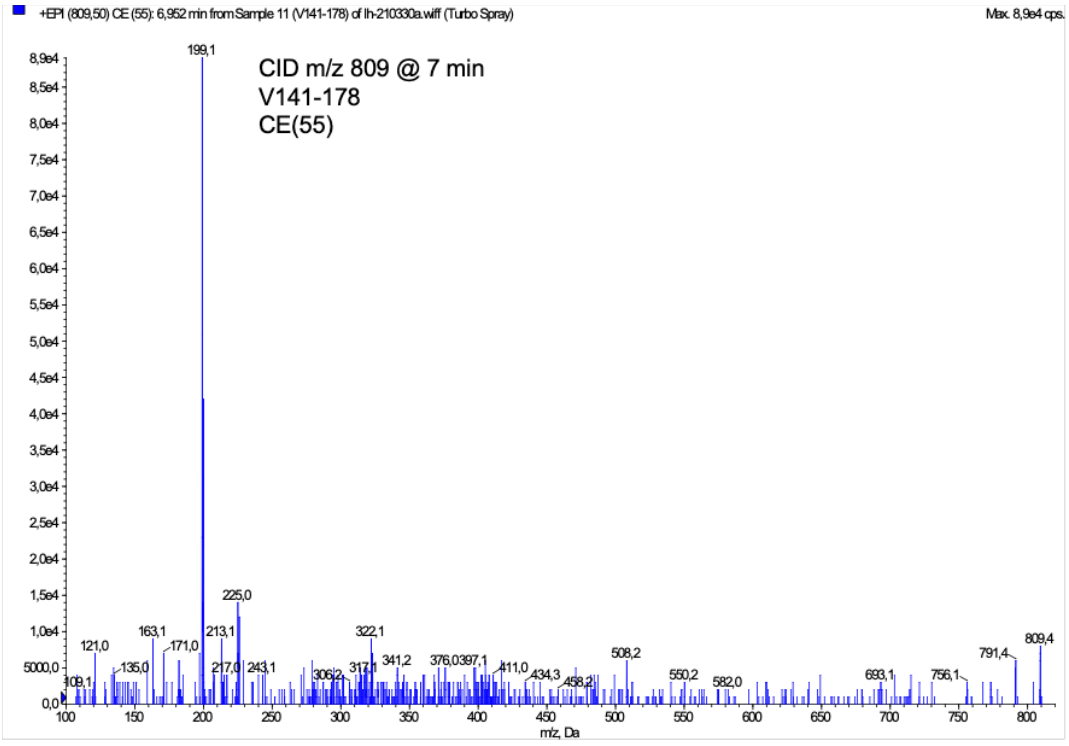


Figure 3-13: CID of m/z *809 in SPATT extract V141-178*

3.2.2 Culture supernatant of *Alexandrium pseudogonyaulax*

In the culture supernatant of the *Alexandrium pseudogonyaulax* isolate originating from the Limfjord, GDA seco acid was determined as the main component with a cell quota of 2.58 pg/cell. Generally, the seco acids accounted for the major goniodomin proportion in the culture supernatant. Although GDC seco acid was the second most abundant substance, it occurred at a concentration only slightly higher than GDA. The amount of GDA was determined to be 0.26 pg/cell. The fourth and least compound to be found was the undescribed substance with the transition *m/z* 802>749 that had already appeared in previous measurements. GDB and GDC were not detected at all and generally, no other compounds were found.

The extracellular goniodomin profile of *A. pseudogonyaulax* indicates that the formation of seco acids is favored in an aqueous and slightly alkaline environment whereas the corresponding lactones are the predominant species in organic and anhydrous solvents. Nevertheless, GDA could still be detected in small amounts in the culture supernatant.

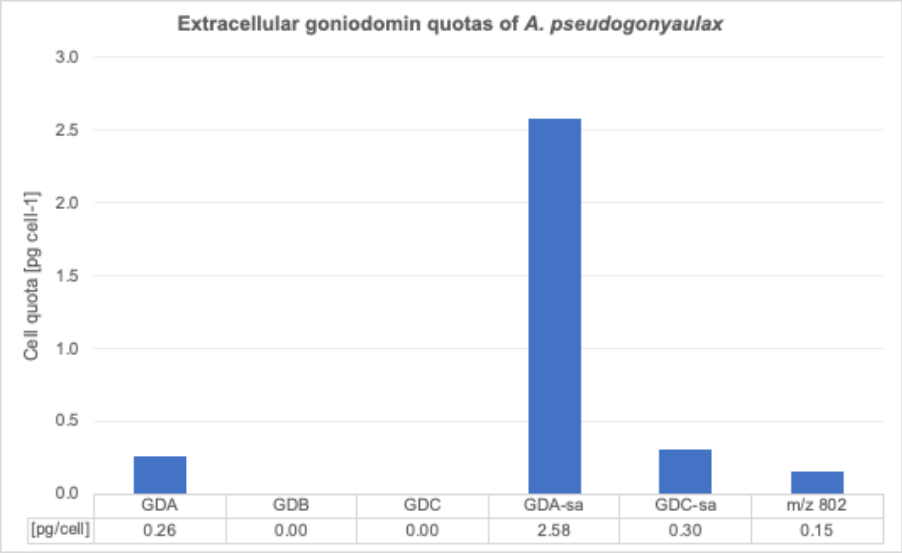


Figure 3-14: Extracellular goniodomin quotas of A. pseudogonyaulax *(strain isolated from Limfjord)*

3.2.3 Stability of GDA in culture medium and water

Degradation of GDA

The GDA concentration in the methanolic GDA standard remained approximately constant over the entire measurement period. In contrast, GDA concentrations in the two aqueous treatments decreased visibly, as can be seen in Figure 3-15. The initial concentrations were above 1 ng/µL and fell to below 0.25 ng/µL, less than a quarter of the initial concentration, within ten days. The initial concentration of GDA in methanol was notably higher. The GDA concentration in pure water was apparently slightly higher than the concentration in K medium, throughout the whole time-span of 10 days. The concentration in K medium appeared to decrease more rapidly. From day 6 onwards, the concentration curves of the aqueous treatments seemed to run approximately parallel. Assuming exponential degradation of GDA, the degradation curves can be approximated by the following equations:

Degradation of GDA in deionized water:

$f(x) = -0.424 \ln(x) + 1.1684$ $R^2 = 0.9634$

Degradation of GDA in K medium:

$f(x) = -0.474 \ln(x) + 1.1205$ $R^2 = 0.9871$

According to these equations, the half-life of GDA in deionized water is approximately 2.5 days and in K medium it is 2 days, implying a slightly faster degradation in K medium than in deionized water.

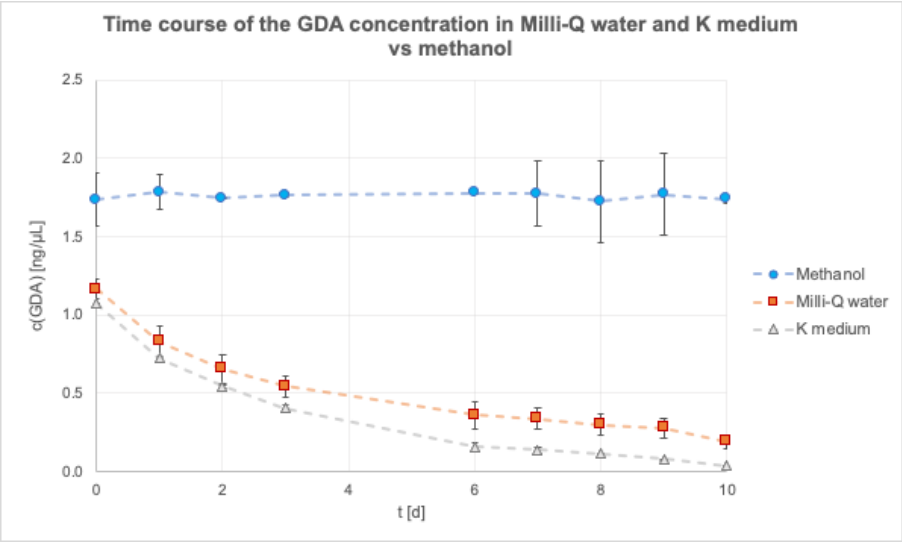


Figure 3-15: Time course of the GDA concentration in deionized water, K medium and methanol

Formation of conversion products

At the day of sample preparation (t_0), GDA was the substance with the highest concentration. At this time point, conversion products had already formed: GDB, GDC, GDA seco acid and GDC seco acid with concentrations below 400 pg/µL. From the beginning of the stability experiment, the concentrations of the seco acids were notably increasing. On the first day, the concentration of GDA seco acid already exceeded that of GDA. GDC seco acid formed rather slowly and exceeded the concentration of GDA on the fifth day. Eventually, the seco acids were the predominant species in the aqueous samples, which is in accordance with the GD profile in the culture supernatant of *A. pseudogonyaulax* in 3.2.2. The concentrations of GDB and GDC were initially low and dropped further below 50 pg/µL. In the culture medium, the concentration of GDB even fell below the detection limit. The time courses of the two aqueous treatments were relatively similar with the major difference that concentrations in deionized water were tending to be higher. However, the eventual concentration of GDA seco acid was higher in culture medium than in water yet conversely, the concentration of GDC seco acid appeared to be higher in water. The GDA concentration of the methanolic standard remained constant. GDB and GDC were the only conversion products detected in the control. The concentrations were approximately identical to those of the aqueous treatments and appeared to remain largely constant over the period analyzed.

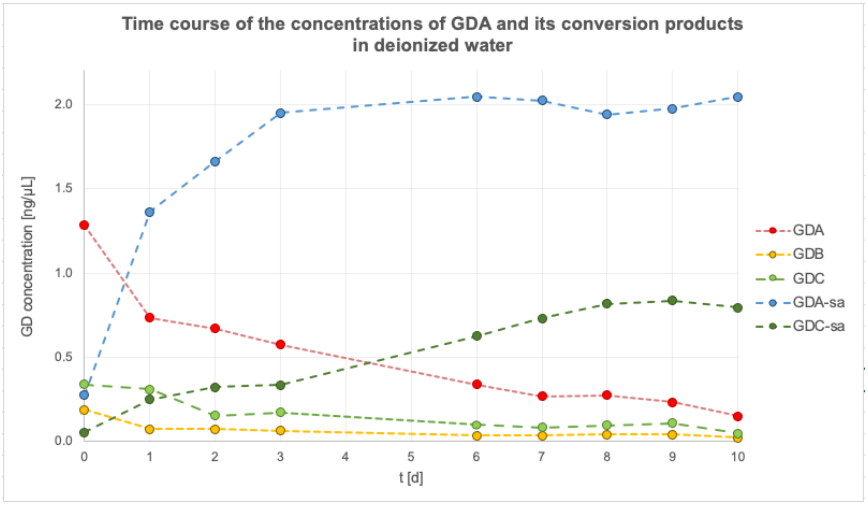


Figure 3-16: Time course of the concentration of GDA and its conversion products in deionized water

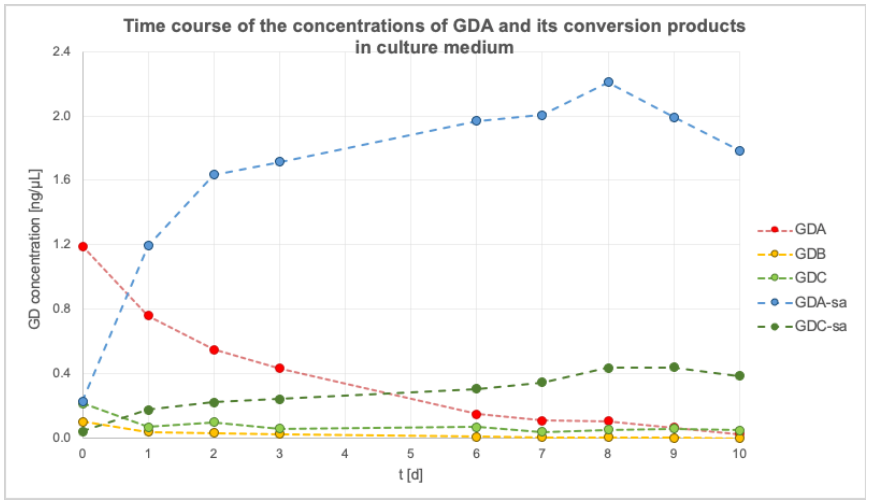


Figure 3-17: Time course of the concentration of GDA and its conversion products in culture medium

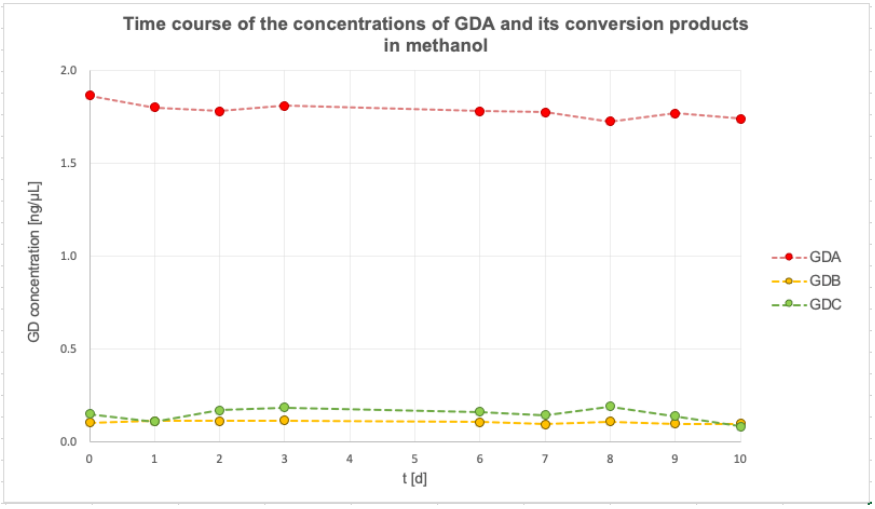


Figure 3-18: Time course of the concentration of GDA and its conversion products in methanol (control)

For a better visualization of the conversion process, the logarithmic ratios of GD concentrations were calculated. A switch from positive to negative values or vice versa indicates a reversal of the concentration ratio. The ratio of GDA to GDB and GDC concentrations remained constant in deionized water, indicating an equally fast degradation of the three substances. On day 10, in K medium, the GDB concentration fell below the detection limit so that it was impossible to calculate a concentration ratio. Until this time point, the concentration ratios remained constant as well. In both treatments the x-axis intercept of the GDA:GDA-sa ratio curves is located between day 0 and 1.

The x-axis intercept of the two GDA:GDC-sa ratio curves can be estimated to be located between day 4 and 5. Apparently, the curves of the seco acids run in parallel.

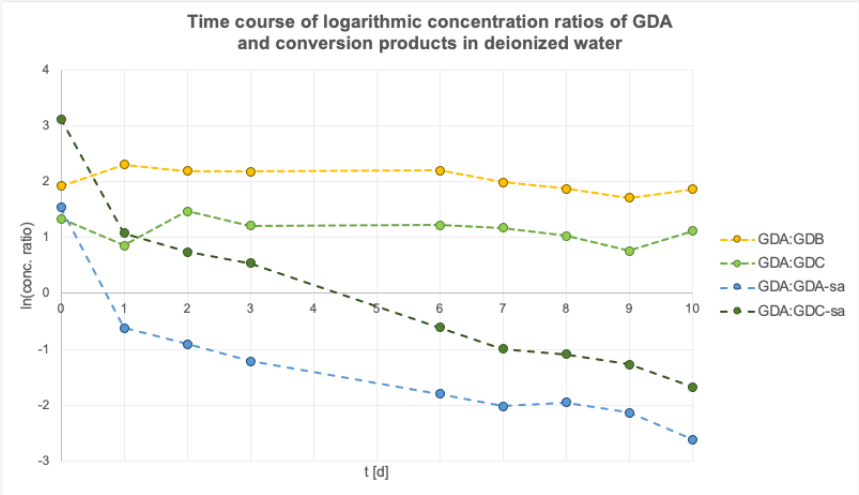


Figure 3-19: Time course of logarithmic concentration ratios of GDA and conversion products in deionized water

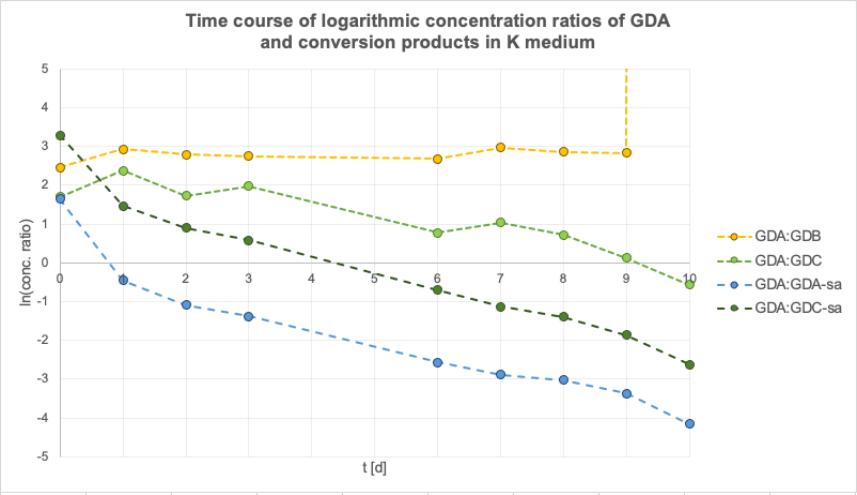


Figure 3-20: Time course of logarithmic concentration ratios of GDA and conversion products in culture medium

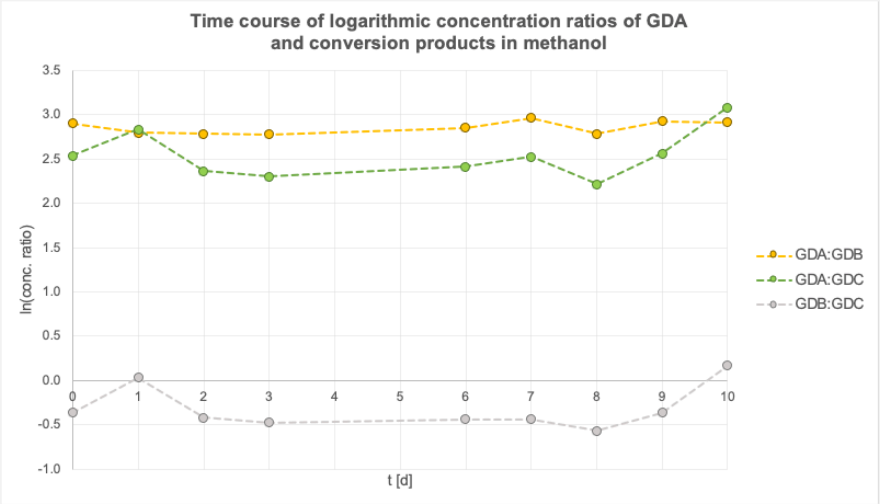


Figure 3-21: Time course of logarithmic concentration ratios of GDA and conversion products in methanol

The ratios of GDA/B/C in the methanolic standard illustrate that the concentrations of all three substances are constant, just like it was found in the experiment conducted previously with the extracts of *A. hiranoi*. The slight fluctuation of the ratio GDA:GDC runs in parallel to that of GDB:GDC and is therefore only due to the course of the GDC concentration.

3.3 GD producer species and their toxin profiles

3.3.1 *Alexandrium hiranoi*

The major component of the cell content of *Alexandrium hiranoi* was GDA with a cell quota of approximately 12 pg/cell. Minor compounds with the highest abundance were *m/z* 802 (1 pg/cell) and GDA seco acid (0.84 pg/cell). For GDB, a cell quota of 0.61 pg/cell was determined and for GDC only 0.15 pg/cell. A pair of signals with *m/z* 814 was detected with retention times exactly resembling those of GDA and GDB. The GDA analog had a cell quota three times higher than the GDB analog. The least abundant compound was GDC seco acid with 0.01 pg/cell. The seco acids and *m/z* 802 were possibly artifacts resulting from the presence of residual water in the cell pellet. The culture supernatant contained only traces of GDA. GDB and C were not detected. The main component was GDA seco acid with an amount of approximately 9 pg/cell. The substance with *m/z* 802 appeared with a cell quota of about 2 pg/cell and for GDC seco acid, a cell quota of 0.79 pg/cell was determined. The sum of amounts of toxins detected in the culture supernatant is nearly identical with the GDA amount isolated from the cells.

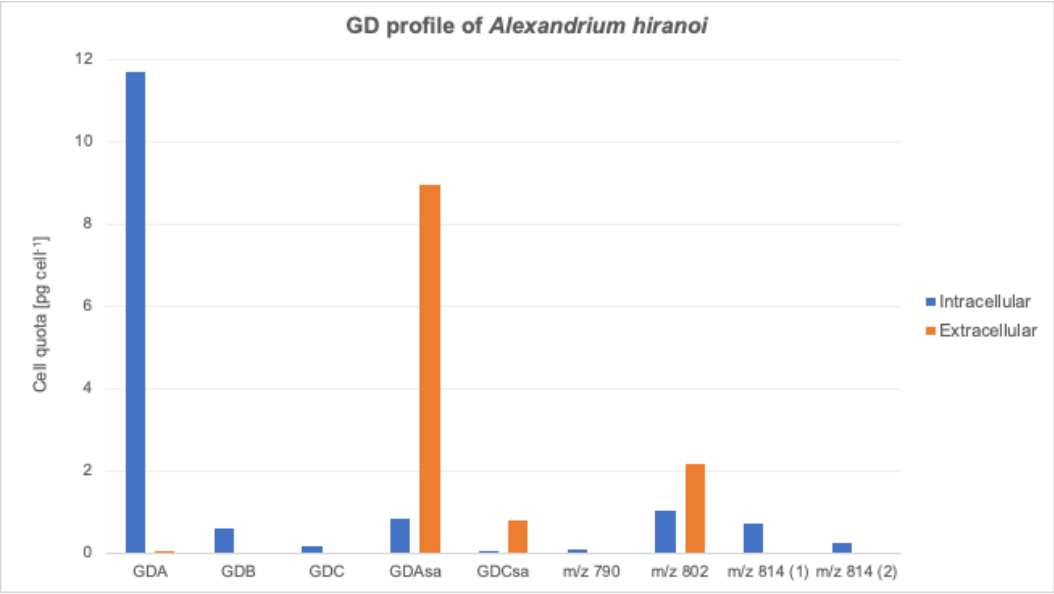


Figure 3-22: Extra- vs. intracellular GD profile of Alexandrium hiranoi

Table 3-5: Extra- vs. intracellular GD profile of Alexandrium hiranoi

	Retention time [min]	Intracellular GD quota [pg/cell]	Extracellular GD quota [pg/cell]
GDA	8.0	11.68	0.03
GDB	7.8	0.61	n.d.
GDC	7.1	0.15	n.d.
GDA-sa	7.3	0.84	8.94
GDC-sa	6.9	0.01	0.79
***m/z* 790**	8.0	0.10	n.d.
***m/z* 802**	7.8	1.01	2.19
***m/z* 814**	8.0	0.72	n.d.
***m/z* 814 (2)**	7.8	0.24	n.d.

3.3.2 *Alexandrium pseudogonyaulax*

The profile of *Alexandrium pseudogonyaulax* is quite similar to that of *Alexandrium hiranoi*, with the major difference that 34-desmethyl-GDA is formed by the cells and that the amounts of produced goniodomins are generally lower. However, the amount of 34-desmethyl-GDA was low with a cell quota of 0.01 pg/cell. The culture supernatant contained little GDA (0.26 pg/cell). As main extracellular component GDA seco acid was detected. GDC seco acid and *m/z* 802 were present as well, the amount of GDC seco acid (0.30 pg/cell) exceeding that of *m/z* 802 (0.15 pg/cell) by double.

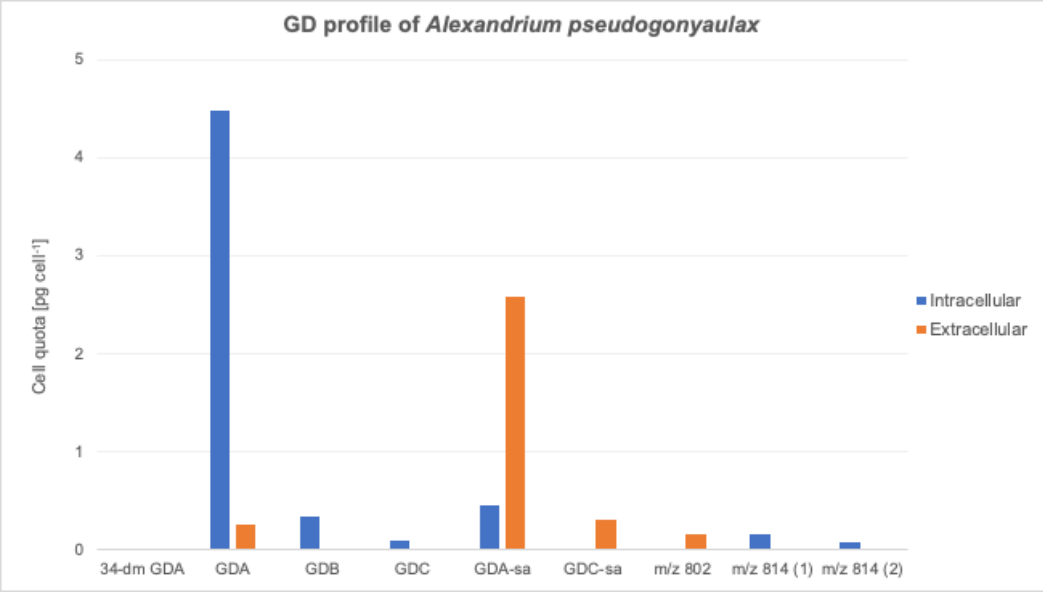


Figure 3-23: Extra- vs. intracellular GD profile of Alexandrium pseudogonyaulax

Table 3-6: Extra- vs. intracellular GD profile of Alexandrium pseudogonyaulax

	Retention time [min]	Intracellular GD quota [pg/cell]	Extracellular GD quota [pg/cell]
GDA	8.0	4.48	0.26
GDB	7.8	0.35	n.d.
GDC	7.1	0.10	n.d.
GDA-sa	7.3	0.46	2.58
GDC-sa	6.9	0.01	0.30
34-dm GDA	7.8	0.01	n.d.
***m/z* 802**	7.8	n.d.	0.15
***m/z* 814**	8.0	0.16	n.d.
***m/z* 814 (2)**	7.8	0.09	n.d.

3.3.3 *Alexandrium taylorii (*AY7T)

The Mediterranean strain of *Alexandrium taylorii* revealed presence of a putative 9-desmethyl-GDA. With 34-desmethyl-GDA it shares the retention time but can be distinguished by the fragmentation pattern that suggests a location of the demethylated site outside the F ring.

In cells of *A. taylorii* AY7T, 34-desmethyl-GDA was not detected. The cells showed a relatively high content of GDA seco acid compared with the other species. The extracellular toxin portion was again dominated by GDA seco acid. The amount of *m/z* 802 slightly exceeded that of GDC seco acid.

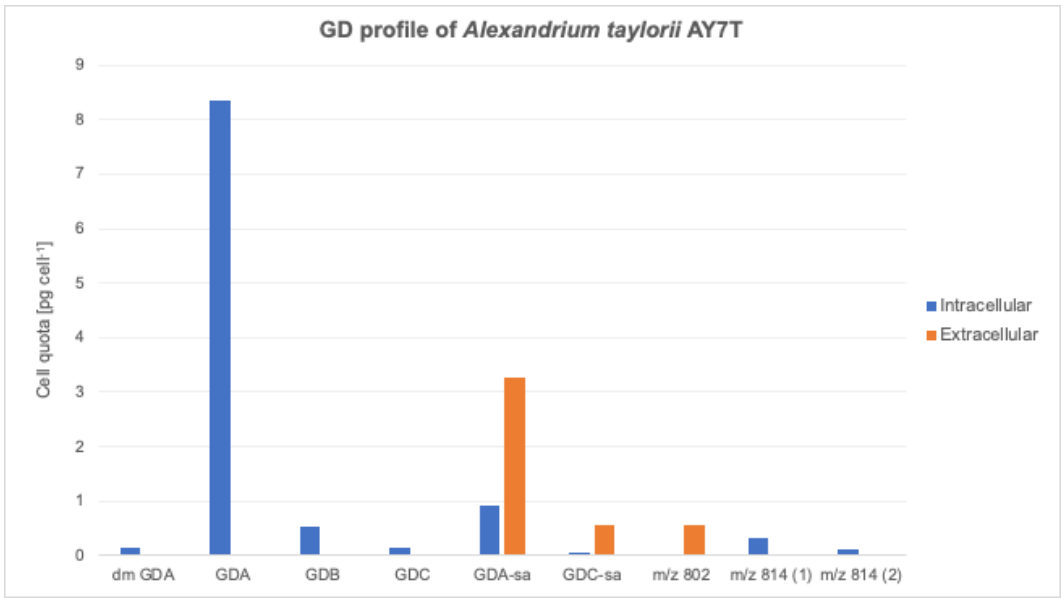


Figure 3-24: Extra- vs. intracellular GD profile of Alexandrium taylorii *AY7T*

Table 3-7: Extra- vs. intracellular GD profile of Alexandrium taylorii *AY7T*

	Retention time [min]	Intracellular GD quota [pg/cell]	Extracellular GD quota [pg/cell]
GDA	8.0	8.35	n.d.
GDB	7.8	0.52	n.d.
GDC	7.1	0.16	n.d.
GDA-sa	7.3	0.92	3.26
GDC-sa	6.9	0.06	0.55
dm GDA	7.8	0.15	n.d.
***m/z* 802**	7.8	n.d.	0.57
***m/z* 814**	8.0	0.32	n.d.
***m/z* 814 (2)**	7.8	0.11	n.d.

3.3.4 *Alexandrium monilatum*

The species native to the Gulf of Mexico, Central and South American waters shows a completely different goniodomin profile compared with the other three species. It is immediately noticeable that the cell quotas are much higher than those of the other species, as the maximum quota is almost 30 pg/cell. The next highest amounts were found in the extracts of *Alexandrium hiranoi*, reaching about 12 pg/cell at maximum.

Surprisingly, the main component of the cell extract was GDC of which a cell quota of 28 pg/cell was determined. It was shortly followed by GDA with a quota of 27 pg/cell. Even GDB was detected at an unusually high level of 20 pg/cell. Apparently, *Alexandrium monilatum* additionally produces the 34-desmethyl variant of GDA. Like in the other species, desmethyl-GDA is produced only in low amounts (0.12 pg/cell). The other desmethyl variant was not detected. A new undescribed substance with *m/z* 790 was detected at a retention time similar to that of GDC. It appeared in the extract as well as in the supernatant, but there in smaller quantity.

The composition of the culture supernatant was also unusual with *m/z* 802 being far the most abundant substance with a cell quota of about 12 pg/cell. GDC seco acid appeared in a higher abundance than GDA seco acid, which seems logical in view of the high GDC amount detected in the cell extract. Furthermore, GDA was detected in the supernatant but at a level of only 0.66 pg/cell.

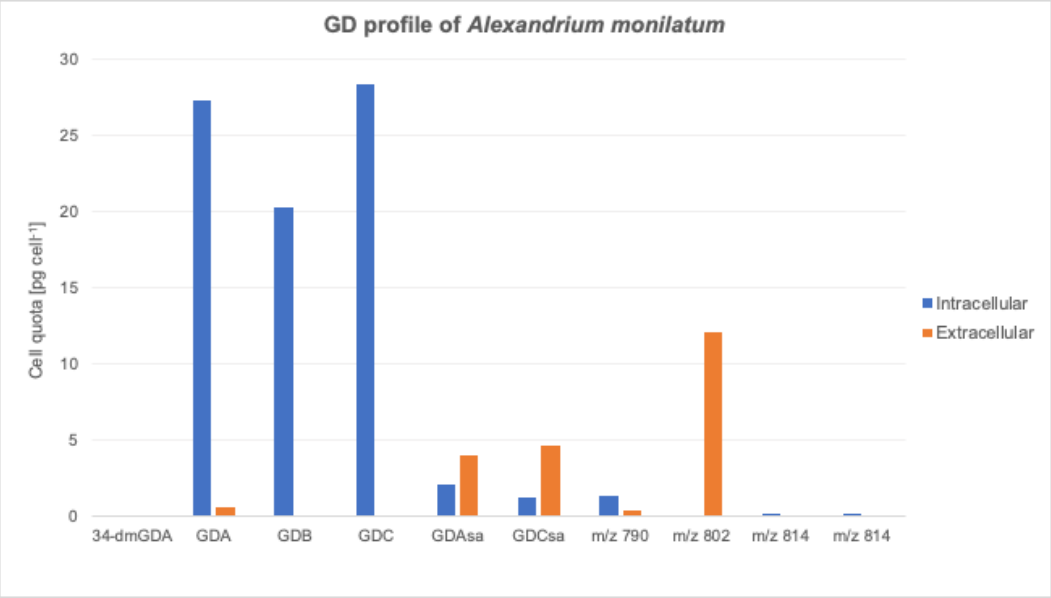


Figure 3-25: Extra- vs. intracellular GD profile of Alexandrium monilatum

Table 3-8: Extra- vs. intracellular GD profile of Alexandrium monilatum

	Retention time [min]	**Intracellular GD quota [pg/cell]**	**Extracellular GD quota [pg/cell]**
GDA	8.0	27.29	0.66
GDB	7.8	20.30	0.07
GDC	7.1	28.40	n.d.
GDA-sa	7.3	2.09	3.98
GDC-sa	6.9	1.30	4.66
34-dmGDA	7.8	0.12	n.d.
***m/z* 790**	7.1	1.36	0.39
***m/z* 802**	7.8	0.01	12.09
***m/z* 814**	8.0	0.25	n.d.
***m/z* 814 (2)**	7.8	0.23	n.d.

3.3.5 Comparison of the four species

In summary, the GDA cell quotas of the four species were quite variable. Of all species analyzed, *Alexandrium monilatum* had the highest GDA cell quota by far, and GDA was not even the main component of the intracellular toxin content. The species with the next highest cell quota at a level of approximately 12 pg/cell was *A. hiranoi*, followed by the Mediterranean strain *A. taylorii* AY7T with

about 9 pg/cell. The lowest cell quota was determined for *A. pseudogonyaulax* with roughly a seventh of that of *A. monilatum*.

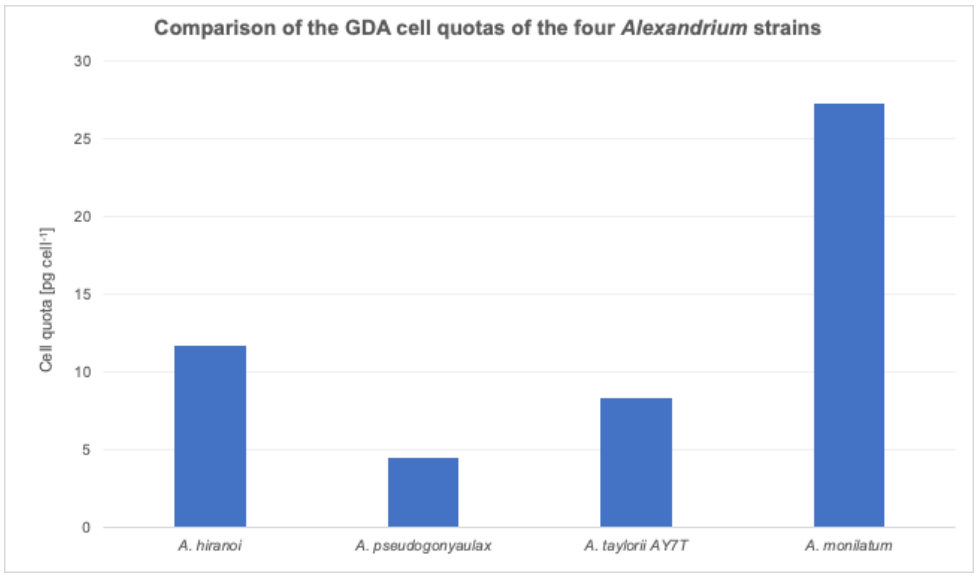


Figure 3-26: Comparison of the intracellular GDA cell quotas of the four Alexandrium *strains studied*

With exception of *A. monilatum*, the proportions of the individual toxins detected in the cell extracts of all species are relatively similar with GDA accounting for about 76-79% of the total toxin content, GDB ranging from 4-6% and GDC with 1-1.8%. The main differences are in the type of desmethyl-GDA congener and the presence of a substance with *m/z* 802 and one with *m/z* 790 in the extract of *A. hiranoi*. The cells of *A. monilatum* are a special case, with 35% GDC, 33.5% GDA and 25% GDB as main components.

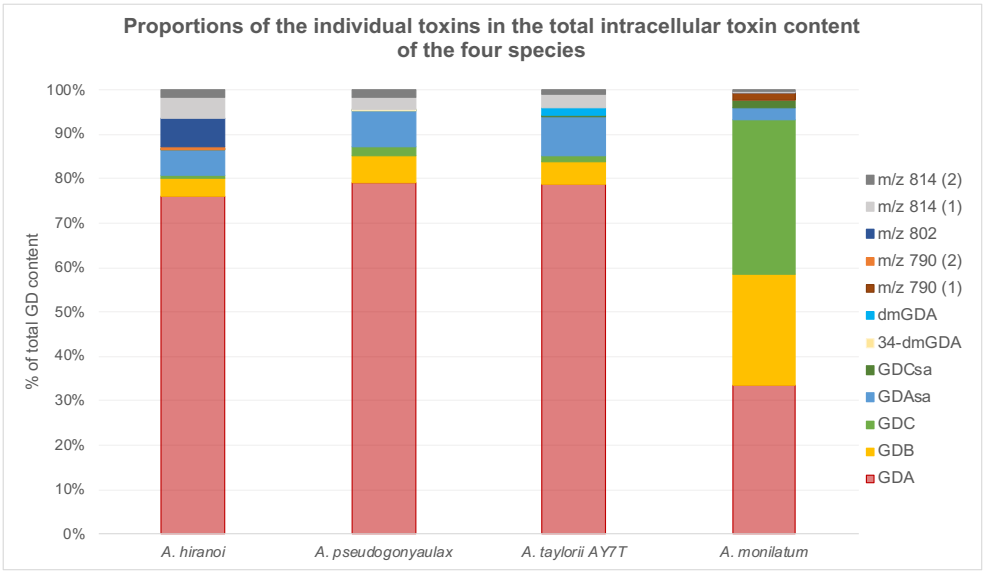


Figure 3-27: Proportions of the individual toxins in the intracellular toxin content of the four species

In analogy to the content of the lactone in the cell extracts, the percentage of GDA seco acid in the culture supernatant was similar for all species (74-78%) except *A. monilatum* (18%). The percentages of the other toxins are more variable with the species, especially *m/z* 802 and GDA, which is in case of *A. taylorii* AY7T completely absent and in case of *A. pseudogonyaulax* accounts for 7% of the total extracellular toxin portion. The large proportion of *m/z* 802 (56%) in the culture supernatant of *A. monilatum* is worth a more detailed analysis.

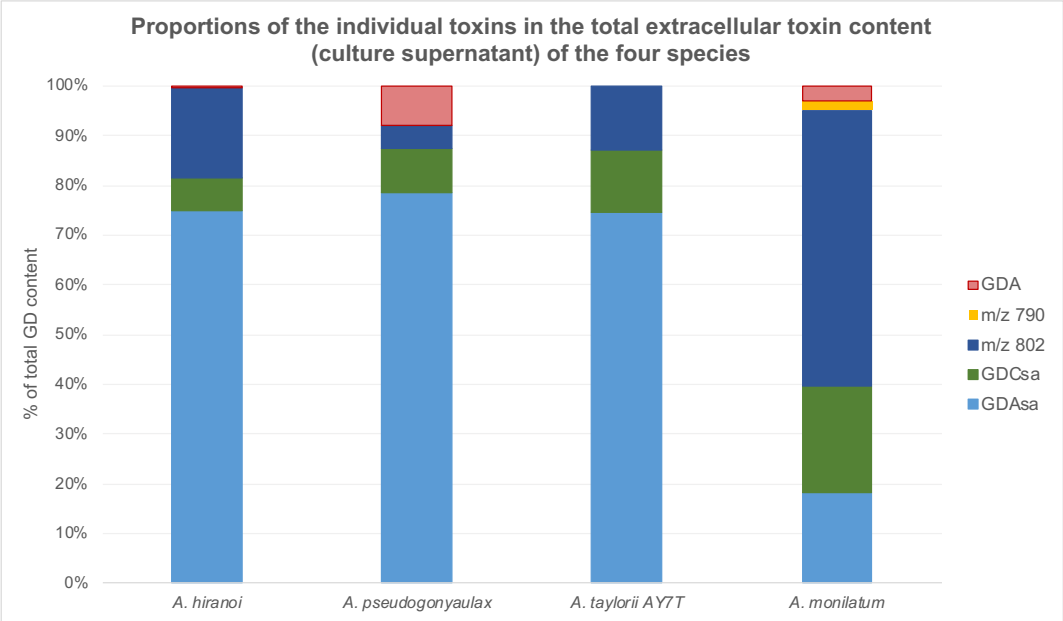


Figure 3-28: Proportions of the individual toxins in the extracellular toxin content of the four species

Table 3-9: Proportions of the individual toxins [%] in the intra- and extracellular toxin content of the four species

	A. hiranoi		*A. pseudogonyaulax*		*A. taylorii* AY7T		*A. monilatum*	
	Intra	Extra	Intra	Extra	Intra	Extra	Intra	Extra
GDA	76.0	0.3	79.2	7.9	78.9	n.d.	33.6	3.0
GDB	4.0	n.d.	6.2	n.d.	4.8	n.d.	25.0	n.d.
GDC	1.0	n.d.	1.8	n.d.	1.5	n.d.	35.0	n.d.
GDA-sa	5.5	74.8	8.1	78.4	8.7	74.4	2.6	18.3
GDC-sa	0.1	6.6	0.2	9.1	0.6	12.6	1.6	21.4
34-dmGDA	n.d.	n.d.	0.2	n.d.	n.d.	n.d.	0.2	n.d.
9-dmGDA	n.d.	n.d.	n.d.	n.d.	1.4	n.d.	n.d.	n.d.
m/z 790 (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.7	1.8
m/z 790 (2)	0.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
m/z 802	6.6	18.3	n.d.	4.6	n.d.	13.0	0.0	55.5
m/z 814 (1)	4.7	n.d.	2.8	n.d.	3.0	n.d.	0.3	n.d.
m/z 814 (2)	1.6	n.d.	1.6	n.d.	1.0	n.d.	0.3	n.d.

3.3.6 Fragmentation analyses of newly detected substances

The supernatant of *A. monilatum* was subjected to fragmentation analysis of the precursor *m/z* 802, which was the predominant substance detected in the sample.

CID spectrum of *m/z* 802

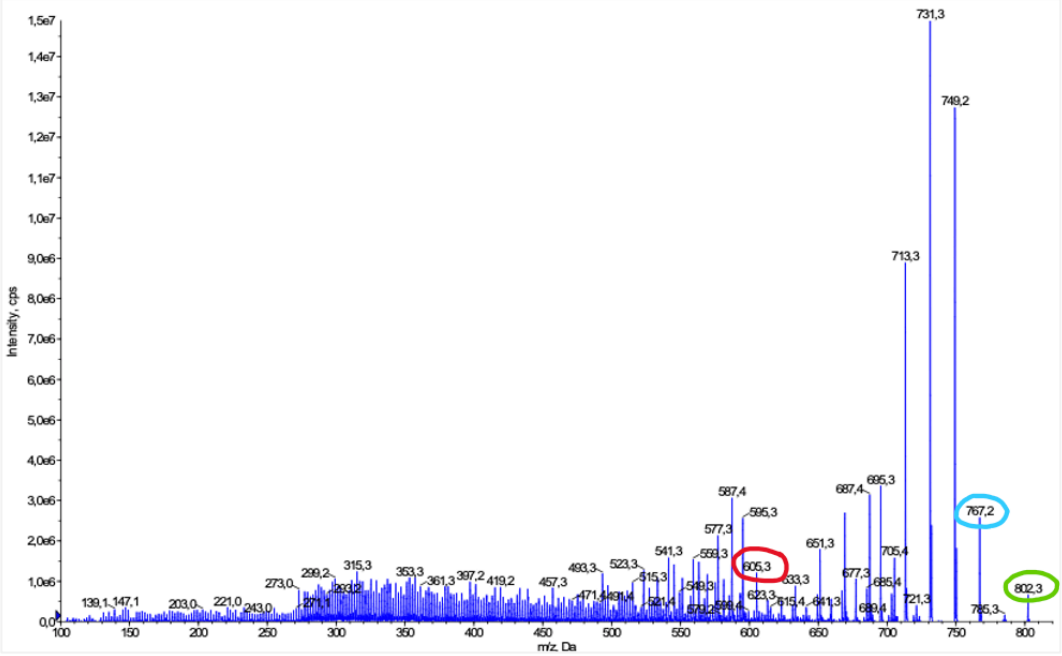


Figure 3-29: CID spectrum of m/z *802 from the supernatant of A. monilatum*

Apparently, the ion *m/z* 802 is the ammonium adduct of a substance with a molecular weight of 784, which is two mass units less than GDC and GDA seco acid. The corresponding single proton adduct $[M+H]^+$ = 785 appears in the spectrum as a low signal marked as 785.3. The fragmentation pattern strongly resembles that of GDA seco acid because of the appearance of the fragment *m/z* 767 (analog: *m/z* 769) and the presence of the precursor ion, just with a negative mass shift of 2 Da. Furthermore, the fragment resulting from cleavage of the F ring (*m/z* 605) does not appear pronounced, just as it is the case with the spectrum of GDA seco acid. GDC, however, preferentially undergoes cleavage of the F ring as radical, resulting in the positively charged residue with *m/z* 607. For comparison, the spectra of GDA seco acid and GDC are shown below.

CID spectrum of GDA seco acid (*m/z* 804)

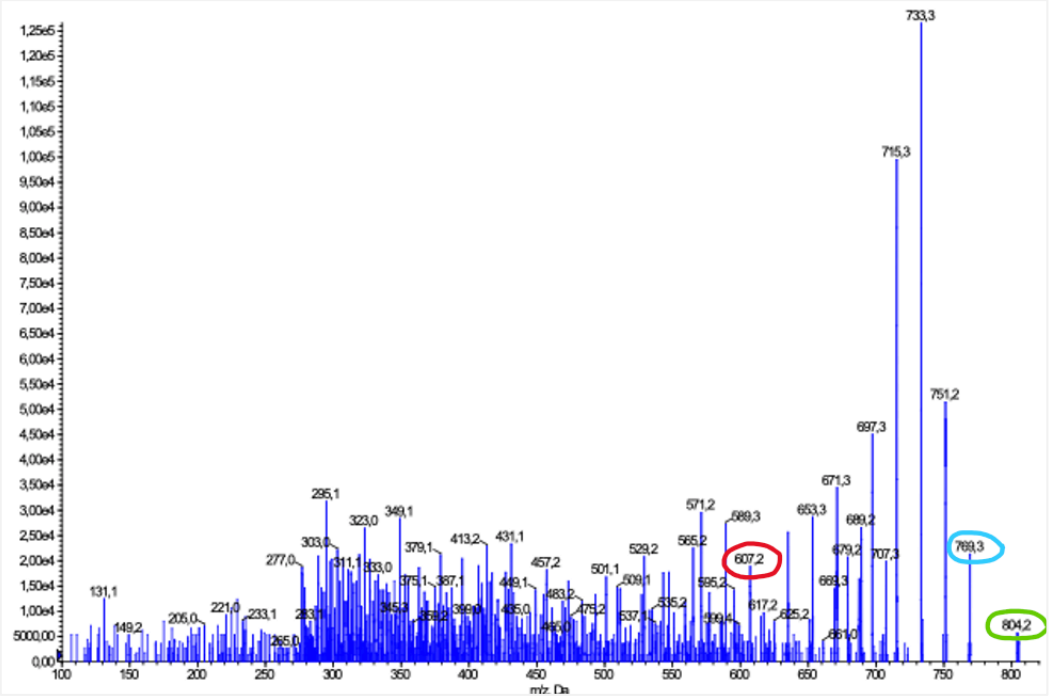


Figure 3-30: CID spectrum of GDA seco acid $[M+NH_4]^+$ (std conc. 10 ng/µL)

CID spectrum of GDC (*m/z* 804)

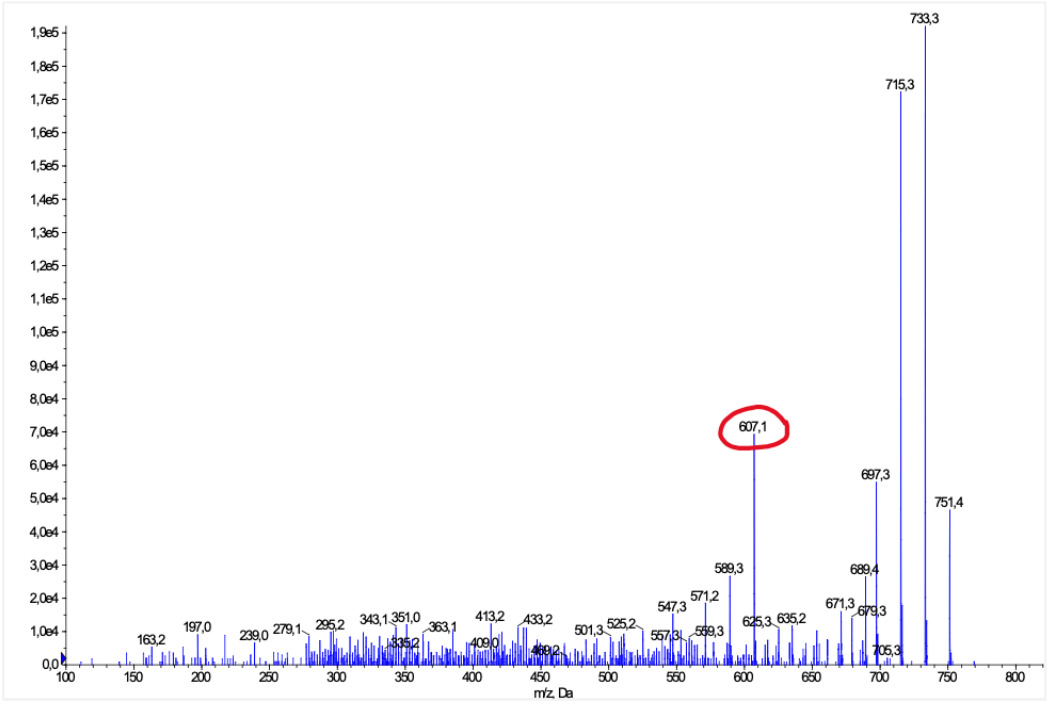


Figure 3-31: CID spectrum of GDC $[M+NH_4]^+$ (std. conc. 2 ng/µL)

3.4 Analysis of *A. pseudogonyaulax* samples from Limfjord

The GD amounts determined in the net haul extracts were normalized to ng/net tow/m in order to compensate for the differences in water depths from which the samples were taken. Because the total biomass gained with the mesh array was divided into three aliquots for different experiments, the amount of GDs was multiplied with factor 3. GDs were detected in almost all sampling stations. The main sites (stations 10-17) were located in the Limfjord. The maximum GDA amount was detected at station 14 that was situated in the Bay of Bjørnsholm. The GDA amount determined in the at this station was at a level of about 1.5 µg/NT/m. Throughout all stations at which GDs were detected, GDA was the main component by far. It was usually accompanied by a smaller quantity of GDB and even less GDC. In addition, with GDA seco acid and low amounts of GDC seco acid two products of GD lactone hydrolysis were detected. Furthermore, three undescribed substances were detected: *m/z* 802 and two signals with *m/z* 814 that have been mentioned earlier. The quantity of *m/z* 802 was in the majority of cases between those of GDB and GDC and the substances with *m/z* 814 were detected only in samples with a GDA amount higher than 300 ng/NT/m. The least amounts of goniodomins were detected in the North Sea (stations 1-7), the Kattegat (stations 20-22) and the Bay of Lübeck (stations 23-28). Finally, at the last four stations of the expedition, which were located in the Bay of Kiel, elevated GD concentration values were detected. In the samples no desmethyl-GDA species was found.

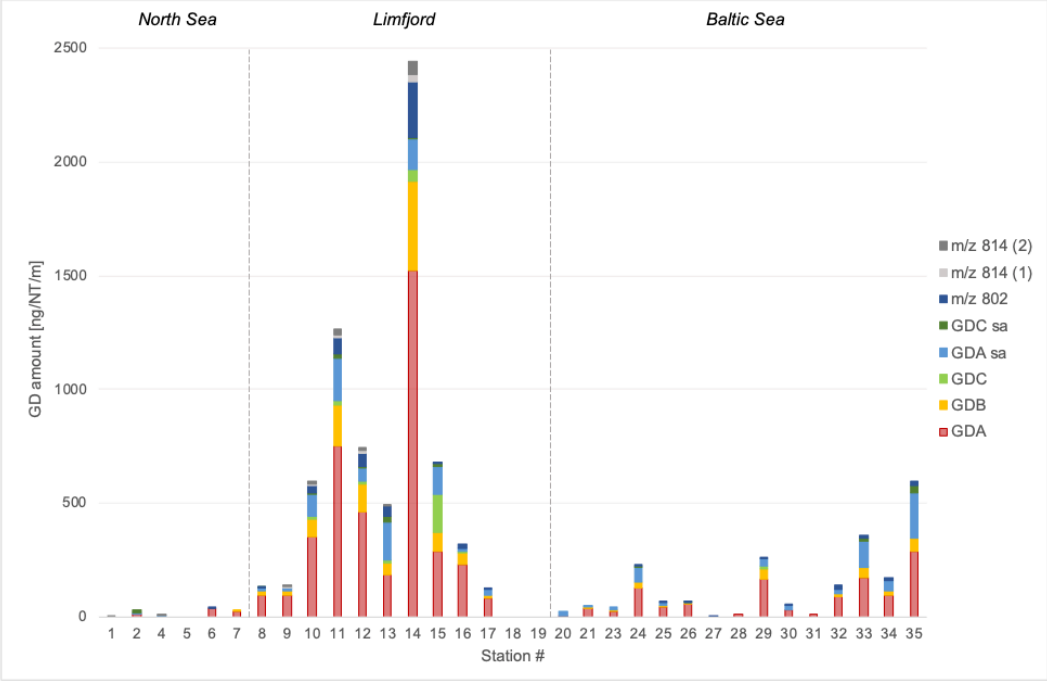


Figure 3-32: Total goniodomin content of the R/V Uthörn expedition 2020 in ng/net tow/m

The quantitative ratio of GDA to GDB and GDA to GDC, respectively, was similar in all samples, provided that the concentrations of GDB and GDC did not fall below the detection limit. For other GDs detected in these samples, the variability of the ratios was higher.

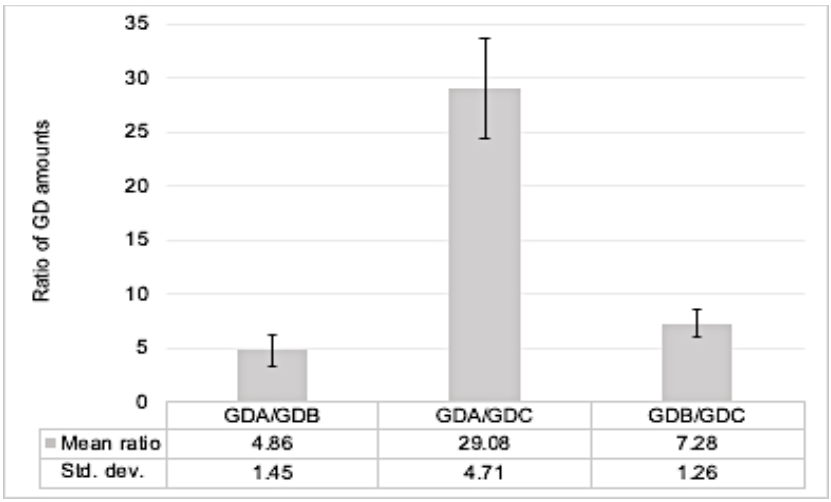


Figure 3-33: Mean ratio of goniodomin amounts detected in net haul extracts

3.4.1 20 µm mesh fraction

The 20 µm mesh fraction accounted for the largest portion of goniodomins. This was expected, as this mesh size retains cells of *A. pseudogonyaulax* which except for gametes usually have a diameter larger than 30 µm.[26] In this GD fraction the substance with *m/z* 802 was not present but instead, the fraction fully accounted for the amount of *m/z* 814 determined in the total cell mass. GDA was the main compound of all samples, followed either by GDB or GDA seco acid.

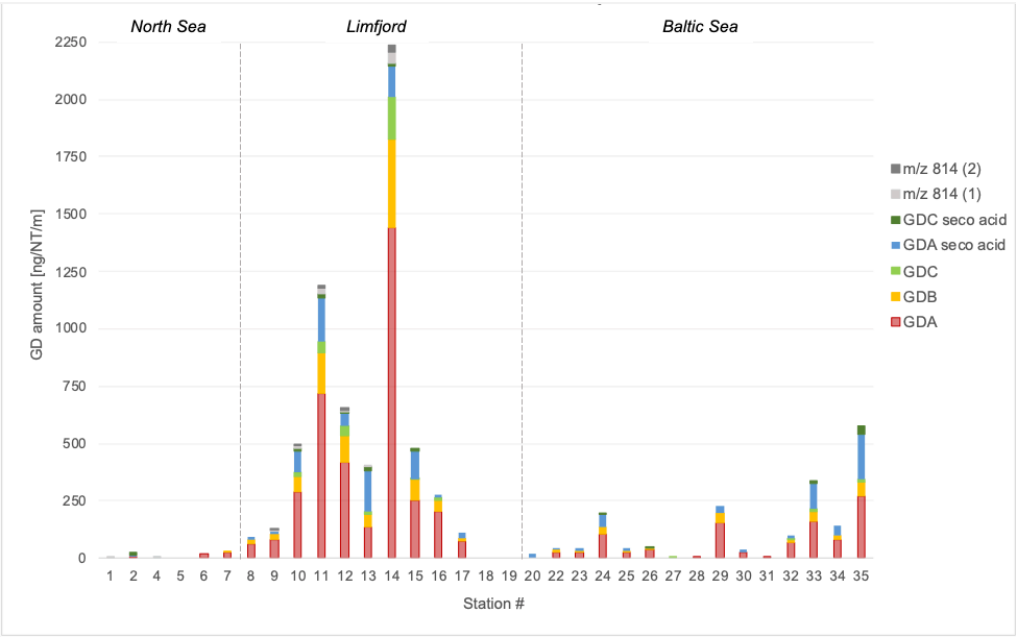


Figure 3-34: Goniodomin content of the 20 µm mesh [ng/net tow/m]

The ratios of amounts of GDA/B/C detected in the 20 µm fraction were similar for all samples and are in good accordance with the ratios determined for the total GD content of the field samples.

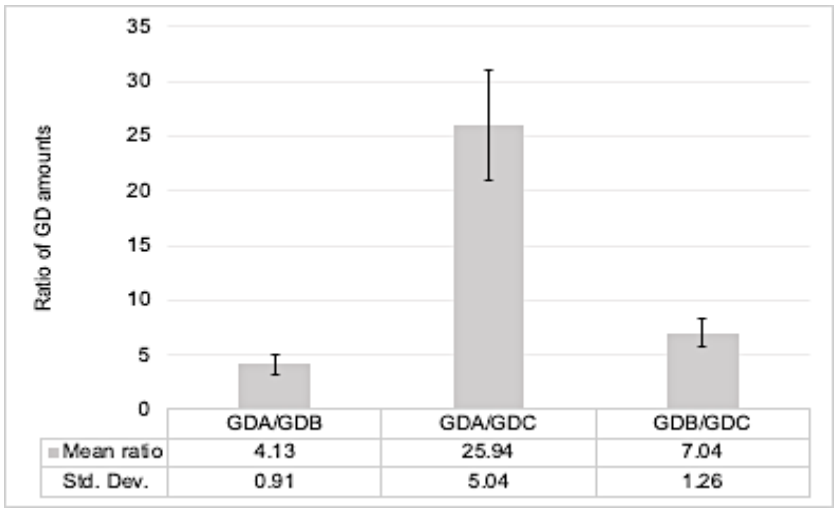


Figure 3-35: Mean ratio of goniodomin amounts detected in the 20 µm fraction

3.4.2 50 µm mesh fraction

As can be recognized by the scale, the total amounts of goniodomins found in the 50 µm mesh fraction were considerably lower than in the 20 µm fraction, with the GDA amount reaching a maximum level of less than 50 ng/NT/m. In these samples, no GDC nor any seco acid was detected. Instead, occasionally low amounts of GDB occurred at the Limfjord sampling stations and *m/z* 802 that appeared at levels almost identical to GDA between stations 11 and 14.

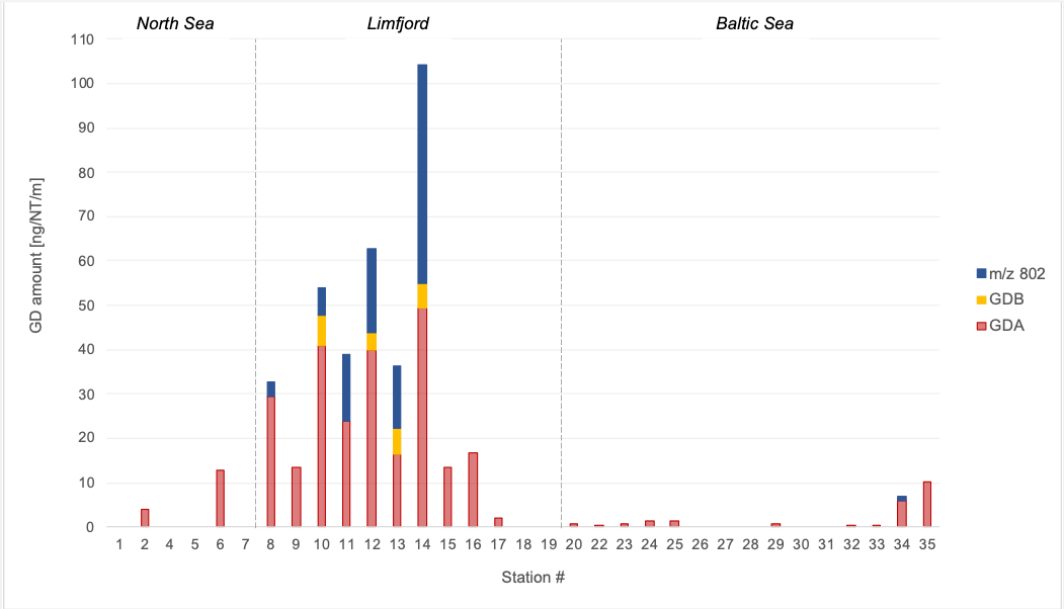


Figure 3-36: Goniodomin content of the 50 µm mesh [ng/net tow/m]

3.4.3 200 µm mesh fraction

The goniodomin levels in the fraction sampled with the largest mesh size were expectedly the lowest with a maximum GDA amount of about 30 ng/NT/m at station 14. The percentage of hydrophilic conversion products was higher than in the smaller mesh sizes. GDA was determined as the main component of all samples but *m/z* 802 and GDA seco acid could be detected with comparatively high amounts. GDA seco acid only occurred in samples from the Baltic Sea (stations 24 to 34) whereas *m/z* 802 was found in almost all sampled regions. In the samples 24-26 and 29-33, which were collected in the Baltic Sea, a higher amount of GDs than in the 50 µm mesh fraction at these stations was determined.

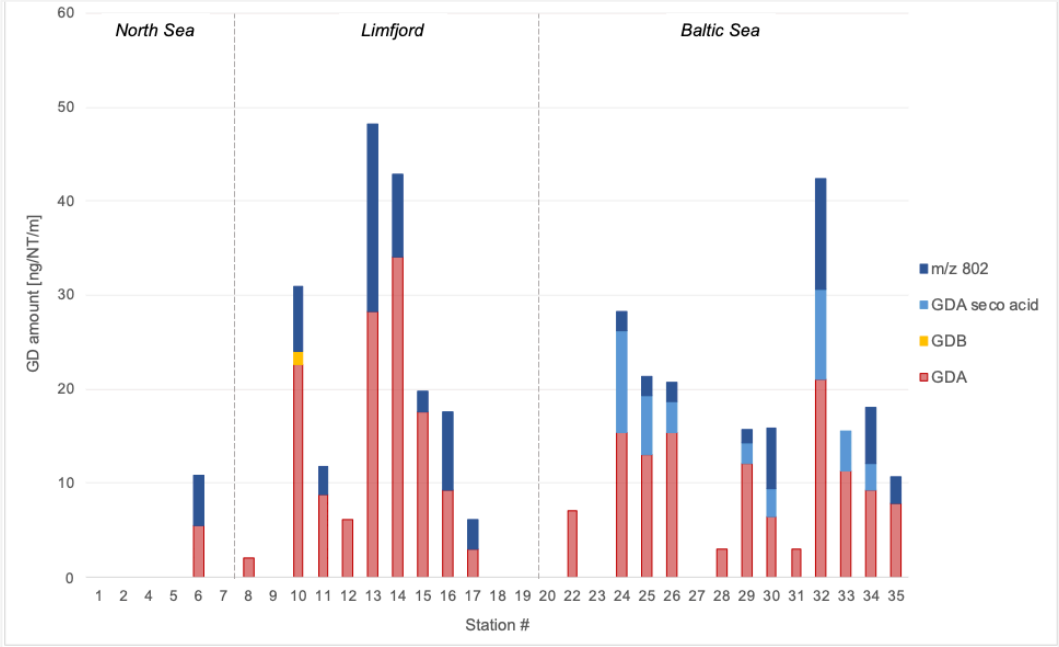


Figure 3-37: Goniodomin content of the 200 µm mesh [ng/net tow/m]

3.4.4 Lipophilic toxins of other toxigenic species

The amounts of other lipophilic toxins detected during the field survey were noticeably lower than the GD amounts. Pectenotoxin-2 (PTX-2), which is structurally related to goniodomins, was found frequently but only in low amounts, at maximum about 4 ng/NT/m. Furthermore, spirolides, okadaic acid, yessotoxin and gymnodimines were detected.

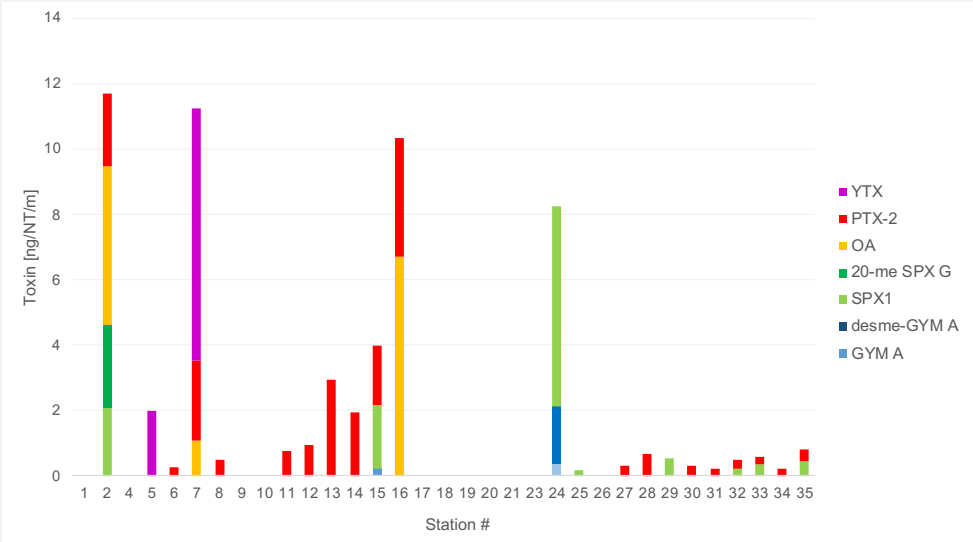


Figure 3-38: Total amount of lipophilic toxins of other toxigenic species detected in the net haul extracts (diagram provided by Dr. Bernd Krock, AWI)

3.4.5 Correlation of cell count and GD content

The population of *Alexandrium* spp. was significantly dominated by *A. pseudogonyaulax* (>99%). Only in some cases, cells of *A. ostenfeldii* and *A. margalefii* could be identified, two strains of which no GD production is known. Instead, *A. ostenfeldii* is known as a producer of neurotoxic cycloimines[27] and has occasionally been reported to produce paralytic shellfish toxins[28] or both[29]. Because of the obvious dominance of *A. pseudogonyaulax*, the total *Alexandrium* cell count was approximated to be equal to the number of cells of *A. pseudogonyaulax*. The total cell count per station was correlated with the total goniodomin content per station and a good agreement was noted (R^2 = 0.8027).

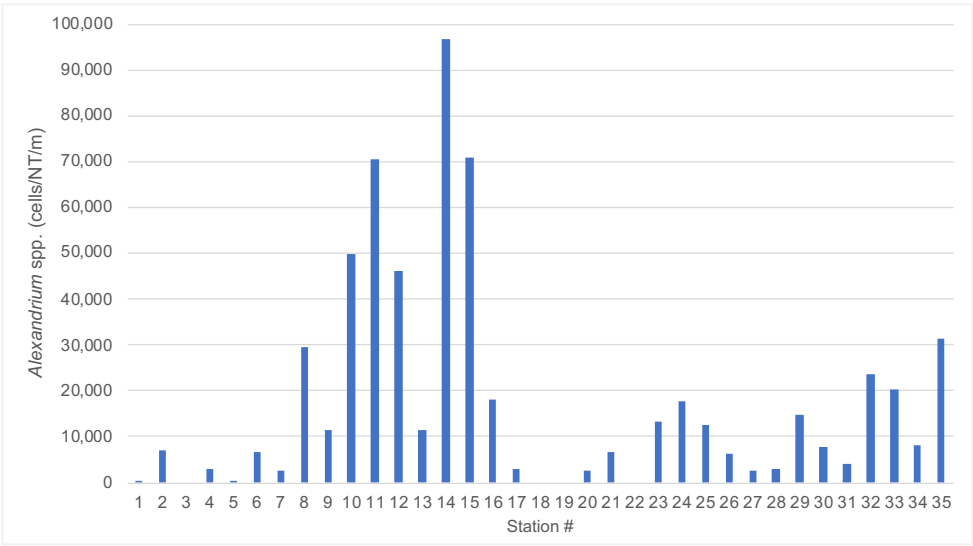


Figure 3-39: Cell count of Alexandrium cells per net tow (data provided by Dr. Urban Tillmann, AWI)

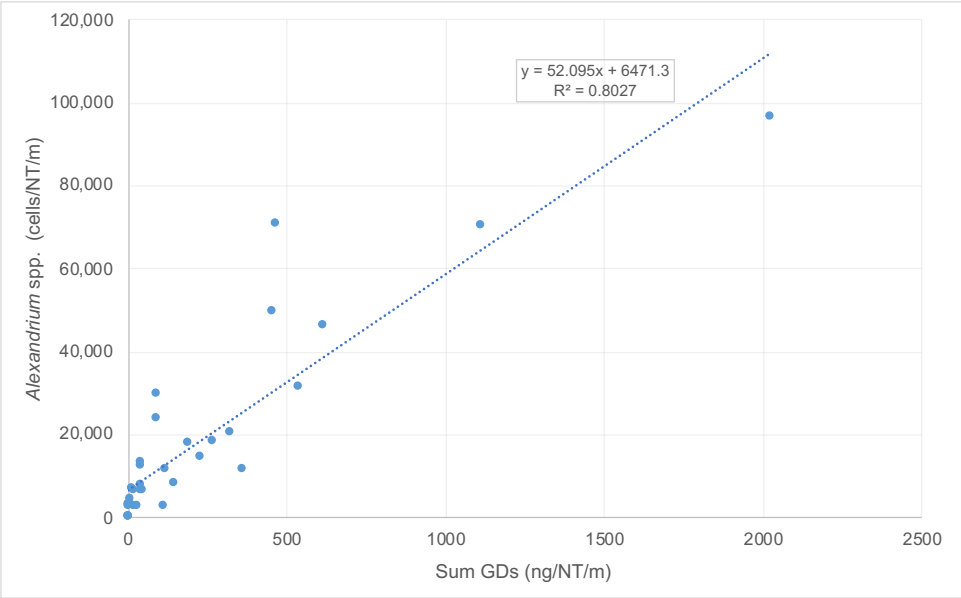


Figure 3-40: Correlation of Alexandrium *cell count and total GD content (data provided by Dr. Urban Tillmann, AWI)*

3.5 Optimization of the analytic system

3.5.1 Influence of the eluent on chromatographic separation

The retention times of essentially all GDs were shifted relating to chromatography with the acidic eluent, with GDC being the only exception. GDA, GDB and 34-desmethyl-GDA eluted later whereas the seco acids of GDA and GDC eluted earlier than with acidic chromatographic separation. The use of an alkaline eluent also supported a considerably better separation of GDC and GDA seco acid than with the acidic eluent. Additionally, a better separation was achieved for GDB and 34-desmethyl-GDA.

Table 3-10: Differences of GD retention times depending on the eluent pH

Toxin	RT [min] acidic	RT [min] alkaline
GDA	3.52	3.83
GDB	3.41	3.51
GDC	3.00	2.98
GDA seco acid	3.20	2.20
GDC seco acid	2.78	1.65
34-desmethyl-GDA	3.42	3.74

3.5.2 Influence of the analytic system on molecular response

The molecular responses of the three congeners GDA/B/C were calculated as the ratio of the sum of peak areas detected in the standard to the standard concentration. For a better comparison of the four response values per substance, the ratio to the lowest response was formed for each value.
In all three substances, the lowest response was determined for the combination of Na adduct and alkaline eluent. Generally, the sodium adducts had significantly lower responses compared with the corresponding ammonium adducts. The largest difference was obtained between the two adduct ions of GDA which indicated a clear preference of ammonium adducts over sodium adducts. For GDB and GDC, the ratios of these responses were considerably lower. In case of GDA and GDB, the ammonium adducts were best measured with the alkaline eluent whereas the ammonium adduct of GDC yielded the highest response when the acidic eluent was used. The ratios of ammonium adduct responses with acidic versus alkaline eluents were about 1:2 or 2:1 in GDC, respectively. The response ratio of the sodium adducts was the same for all three substances (4:1).

Table 3-11: Molecular responses of GDA/B/C depending on adduct ion and eluent pH

GDA	**NH_4**	**Na**	**NH_4**	**Na**
Acidic	3159585	27329	484	4
Alkaline	4903962	6522	737	1

GDB	**NH_4**	**Na**	**NH_4**	**Na**
Acidic	602808	29904	87	4
Alkaline	1241163	6924	179	1

GDC	**NH_4**	**Na**	**NH_4**	**Na**
Acidic	52249	2928	70	4
Alkaline	22089	749	29	1

3.5.3 Influence of the analytic system on conversion behavior

Goniodomin A

Looking at the four combinations of adduct ion and eluent pH (Figure 3-41), it can be seen that both factors play an essential role in the detection of GDA, B and C. There is a noteworthy difference between acidic and alkaline systems. Apparently, the two systems with alkaline eluents suppress the formation of conversion products in the standard, except for very small amounts of GDB, which can be detected as ammonium adduct. Instead, when using the acidic eluent, a high proportion of the conversion products GDB and GDC is observed.

The results also show that there are substantial differences between the two adduct ions used. When sodium is chosen as adduct, GDC predominates in the standard instead of GDA, whereas as ammonium adduct it accounts for the smallest fraction of all three congeners in the standard. According to that, GDC has a higher affinity to sodium ions than GDA and GDB.

Since the formation of GDC appears to be suppressed when the alkaline eluent is used, it principally does not matter which of the two adducts is used. However, because of the detection of small amounts of GDB when the ammonium adduct is measured, the use of the sodium adduct may be prioritized when analyzing GDA.

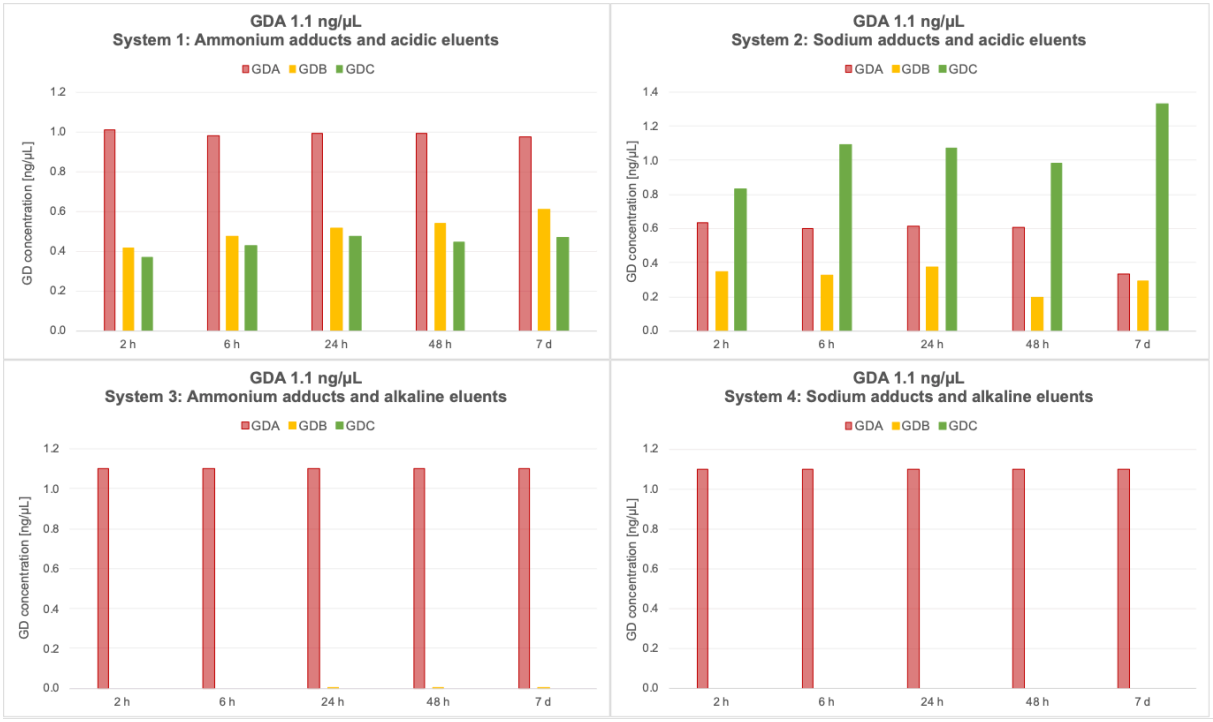


Figure 3-41: Analysis of a GDA standard (1.1 ng/µL) with variations of eluent pH and adduct ion

Goniodomin B

The four systems also had different effects on the analysis of a GDB standard. Again, the acidic eluent appeared to be predominantly unsuitable, since GDC could be detected here as by-product. This was particularly evident when measuring the sodium adduct, due to the higher affinity for Na^+ ions already mentioned. GDA appeared in the GDB standard exclusively as ammonium adduct but did hardly impair the quantification of GDB, due to the low deceptive concentration.

The best results were obtained by means of the alkaline eluent and quantification with the sodium adduct of GDB. With this combination, the formation of GDA and GDB was almost completely suppressed. Despite the different proportions of conversion products in the four eluent-adduct combinations, GDB could in principle be quantified correctly with all of them. The only exception was the combination of ammonium adducts and acidic eluents, where comparatively larger deviations of the measured GDB concentration from the actual concentration were found.

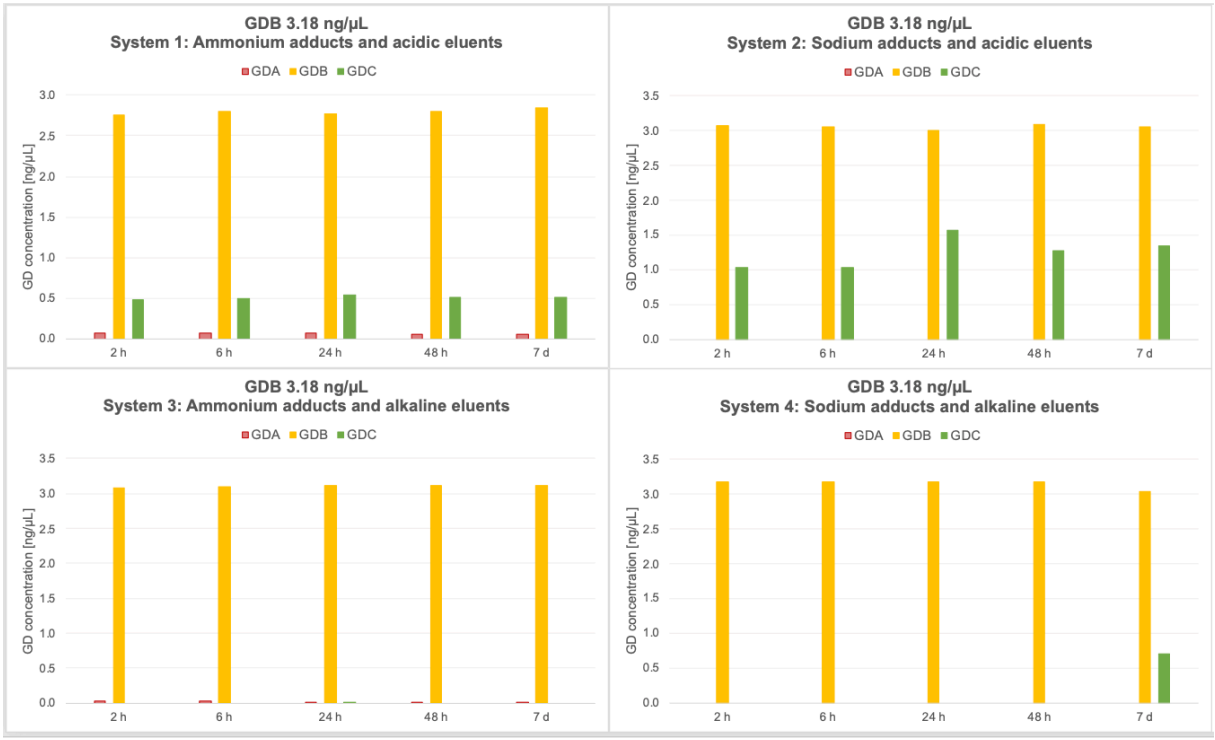


Figure 3-42: Analysis of a GDB standard (3.18 ng/µL) with variations of eluent pH and adduct ion

Goniodomin C

In the case of GDC, the differences between the four systems are particularly pronounced.
The concentration values determined for the GDC standard had an obviously higher deviation in all of the analytic systems except for system 4. For example, the measured concentration values of GDC when using the combination of ammonium adducts and acidic eluent were less than half the actual concentration of the standard. Additionally, the concentration values even decreased apparently over time when ammonium adducts were measured. In contrast, the concentrations calculated for GDA and GDB in the respective standards deviated only slightly from the actual concentrations but they stayed constant over the analyzed time period of 7 days.
Looking at the previous results, it would have been expected that GDC would be best measured with acidic eluents and as a sodium adduct. In fact, however, the only combination with which the correct concentration values could be determined is system 4 with sodium adducts and alkaline eluents.

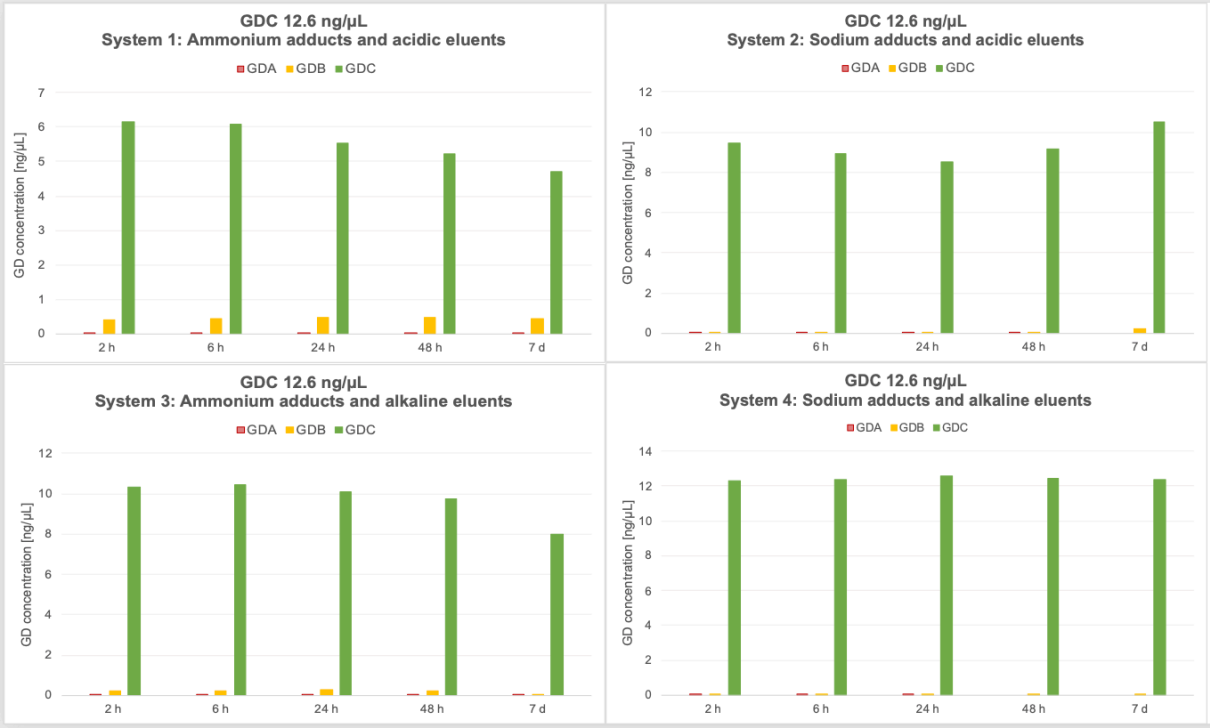


Figure 3-43: Analysis of a GDC standard (12.6 ng/µL) with variations of eluent pH and adduct ion

3.5.4 Adjustment of the SRM method (Xevo TQ-XS)

In SRM, the base peak is usually used for quantification to achieve maximum sensitivity. A frequent fragmentation reaction of goniodomins results in loss of the F ring. The transition from parent ion to the fragment *m/z* 607 resulting from this reaction had been frequently used for quantification but was not the preferred fragmentation reaction in case of the seco acids (see also 3.3.6). In a publication from 2020, the transition *m/z* 786>139 was used for quantification of GDA with a Xevo TQ-MS instrument.[30] The fragment ion *m/z* 139 arises from the F ring and had never been observed in CID spectra obtained with the API4000. Surprisingly, when product ion scans (Daughter Scans) of a range of goniodomins were performed with a Xevo TQ-XS, the fragment was not only found but also proved to be the base peak. The spectra of GDA, B and C are shown below.

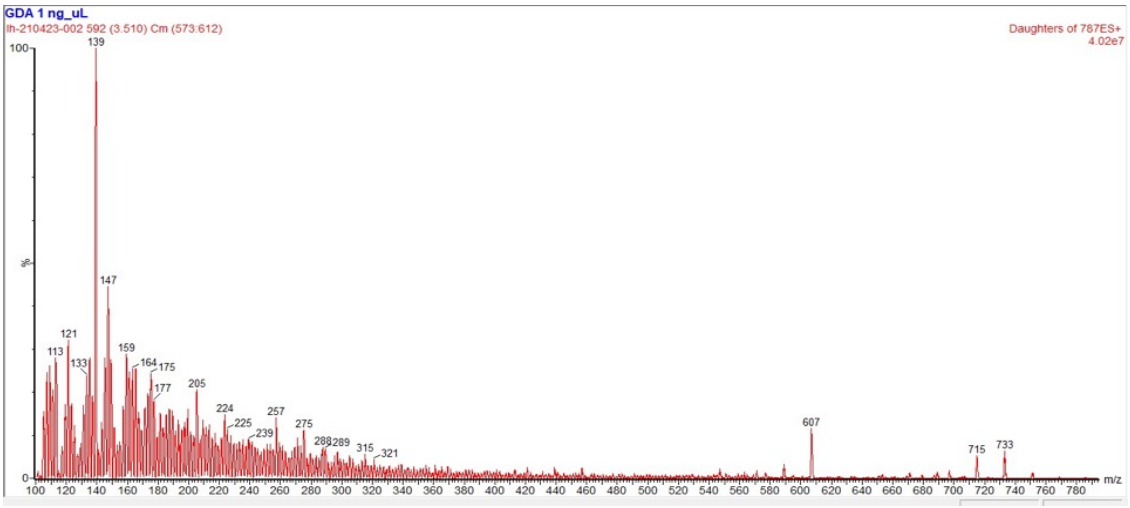


Figure 3-44: CID of GDA $[M+NH_4]^+$ (std. conc. 1 ng/µL)

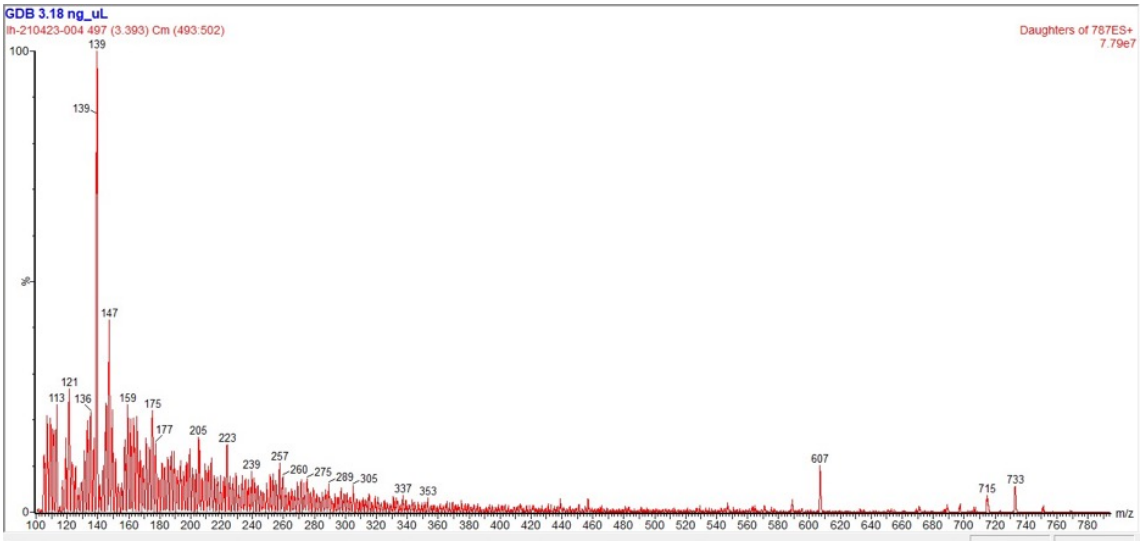


Figure 3-45: CID of GDB $[M+NH_4]^+$ (std. conc. 3.18 ng/µL)

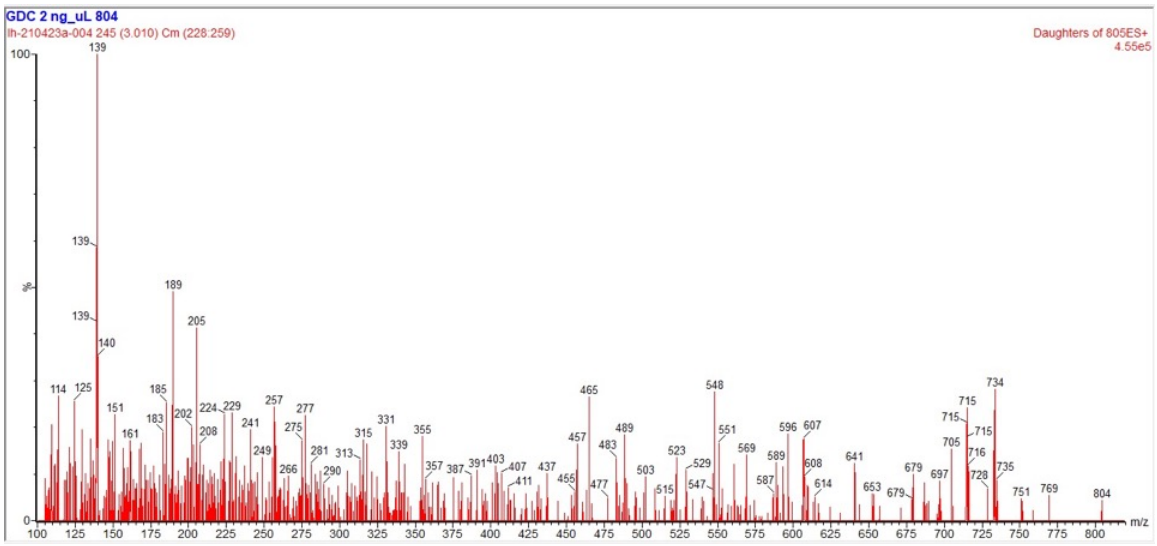


Figure 3-46: CID of GDC $[M+NH_4]^+$ (std. conc. 2 ng/µL)

The overall fragmentation of the GDs was rather scarce with the fragment *m/z* 139 protruding. The fragment was tested in SRM mode in comparison with the previously used transitions and was found to yield a good response so that it was added to the SRM method for future analyses. The 34-desmethyl species of GDA expectedly yielded a fragment *m/z* 125 that was added to the SRM method as well. The second most intense fragment was kept as qualifier fragment.

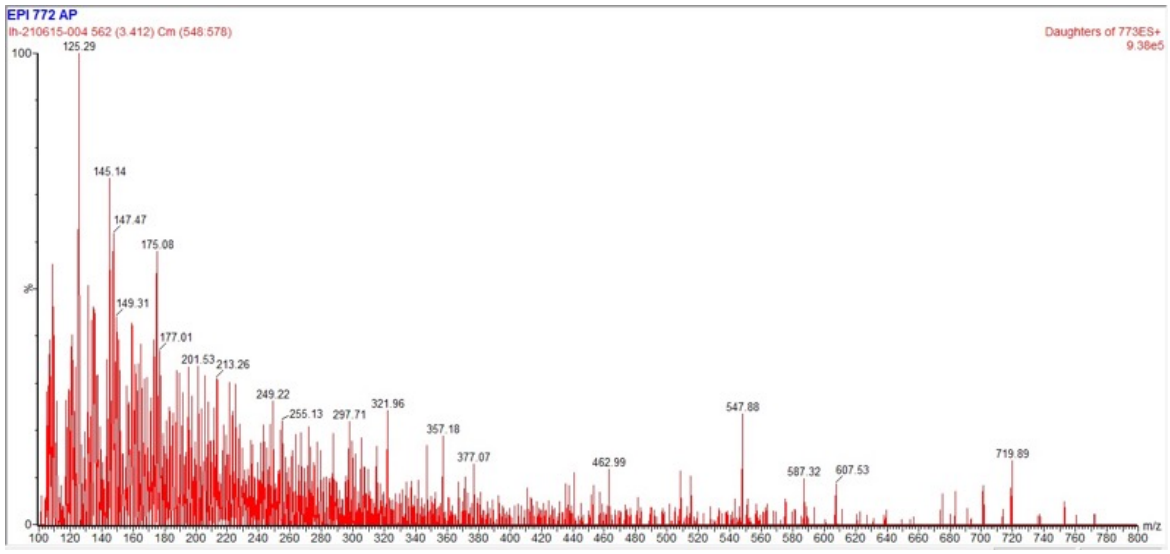


Figure 3-47: CID of 34-desmethyl-GDA $[M+NH_4]^+$ in a methanol extract of A. pseudogonyaulax

3.5.5 Re-assessment of previous analyses with the optimized system

The results of 3.5.3 suggest that LC-based analyses of GD samples are likely distorted by use of acidic eluents. Because of that, analyses conducted prior to optimization were repeated in order to determine the actual GD content of the samples.

Stability of GDA in organic extractants

Since a repetition of the stability experiment described in 3.1 was not possible, the original samples were subjected to analysis with the four eluent/adduct ion systems in order to determine how types and amounts of GDs detected vary depending on treatment and analytic system, how the treatments actually differ and whether there have been any conversion products formed during storage at 4 °C.

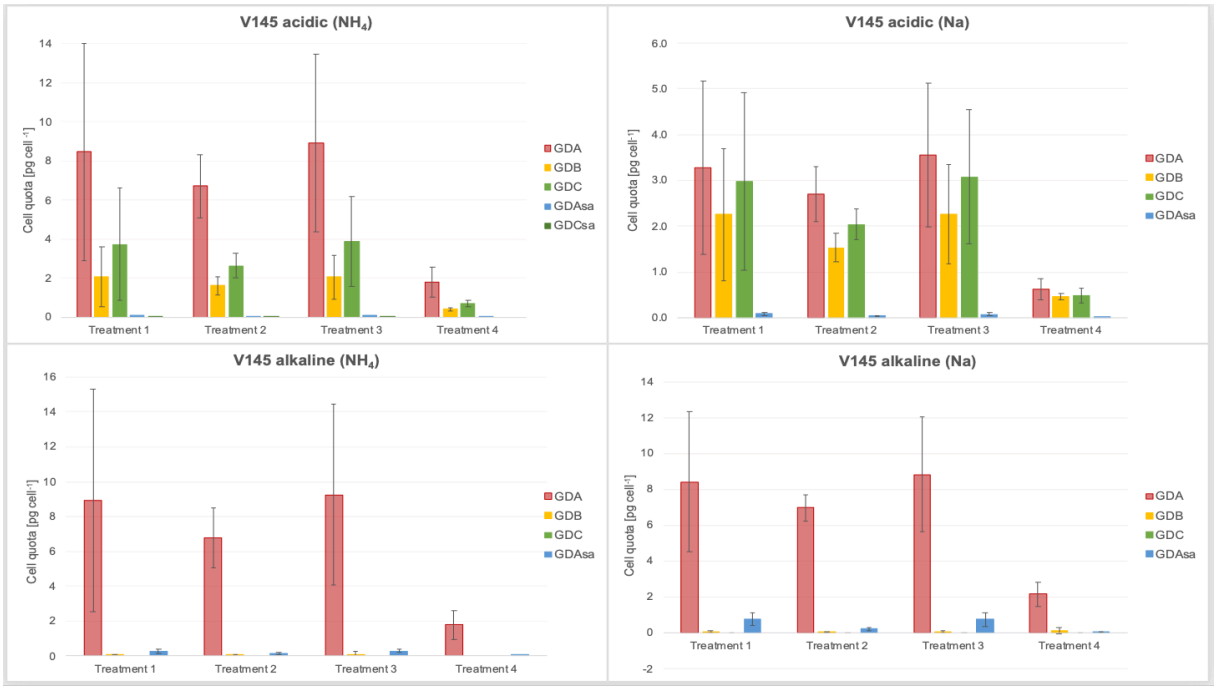


Figure 3-48: Re-assessment of the V145 samples (stability of GDA from cells of A. hiranoi *in organic extractants)*

Essentially, the same observation could be made like in the GDA standard analyzed before: The occurrence of GDC was only observed when acidic eluents were used. In this case also a quite large amount of GDB was formed. The alkaline eluents, however, provided a probably more trustworthy profile in which GDA was the main component by far and only traces of GDA seco acid as well as GDB were detected. Since GDB even occurred as Na adduct, it can be assumed that it was either already produced by cells of *Alexandrium hiranoi* or that it formed from GDA during storage in the organic

solvents. In the course of this chapter, cell profiles of *A. hiranoi* will be revisited among those of the other three GD producers. The cell quotas determined with the four systems were essentially the same with exception for system 2 (acidic eluent, Na adducts), which appears to be unsuitable for quantification anyway because of the favored conversion of GDA to GDB and GDC. Again, the most suitable option appears to be the combination of alkaline eluents with transitions of Na adducts.

Below the percentages of GDs that were determined with sodium adducts and alkaline eluents are depicted. The percentage of GDA was at about 90% in all treatments. Apparently, the amount of GDA seco acid is slightly less in the treatments that included a drying process (T2 and T4). In treatment 4 (acetone extract of dried cell pellets), GDB was formed in higher proportion. In the methanol extracts, the percentages of the single goniodomins are nearly identical. Nevertheless, the standard deviations of the separate treatments were quite high so that further experiments would be necessary to confirm these findings.

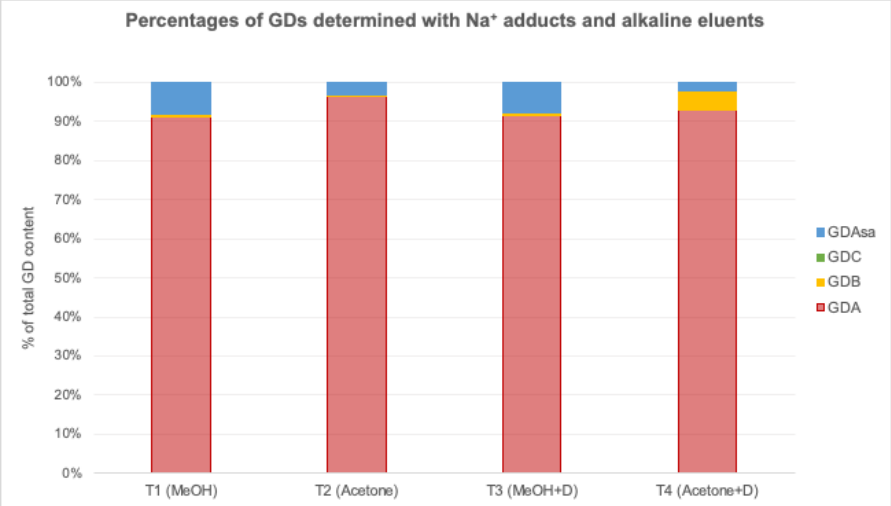


Figure 3-49: Percentages of GDs determined in four types of A. hiranoi *extracts (Na adducts, alkaline eluent)*

A. pseudogonyaulax **samples from Limfjord**

A re-assessment of the net haul extracts from the field survey with R/V Uthörn revealed that the presence of GDB and GDC is obviously an artifact of the eluent used, just like in the samples analyzed earlier. The extracts appeared to contain only GDA and GDA seco acid. Since the samples were measured with a preliminary SRM method containing only the transitions of GDA/B/C, GDA seco acid and GDC seco acid, the determination of other sample constituents was not possible. Between the two systems based on the alkaline eluent, a difference in the amount of GDA seco acid could be observed, indicating favored formation of sodium adducts of GDA seco acid compared with GDA, which has a higher affinity to ammonium ions.

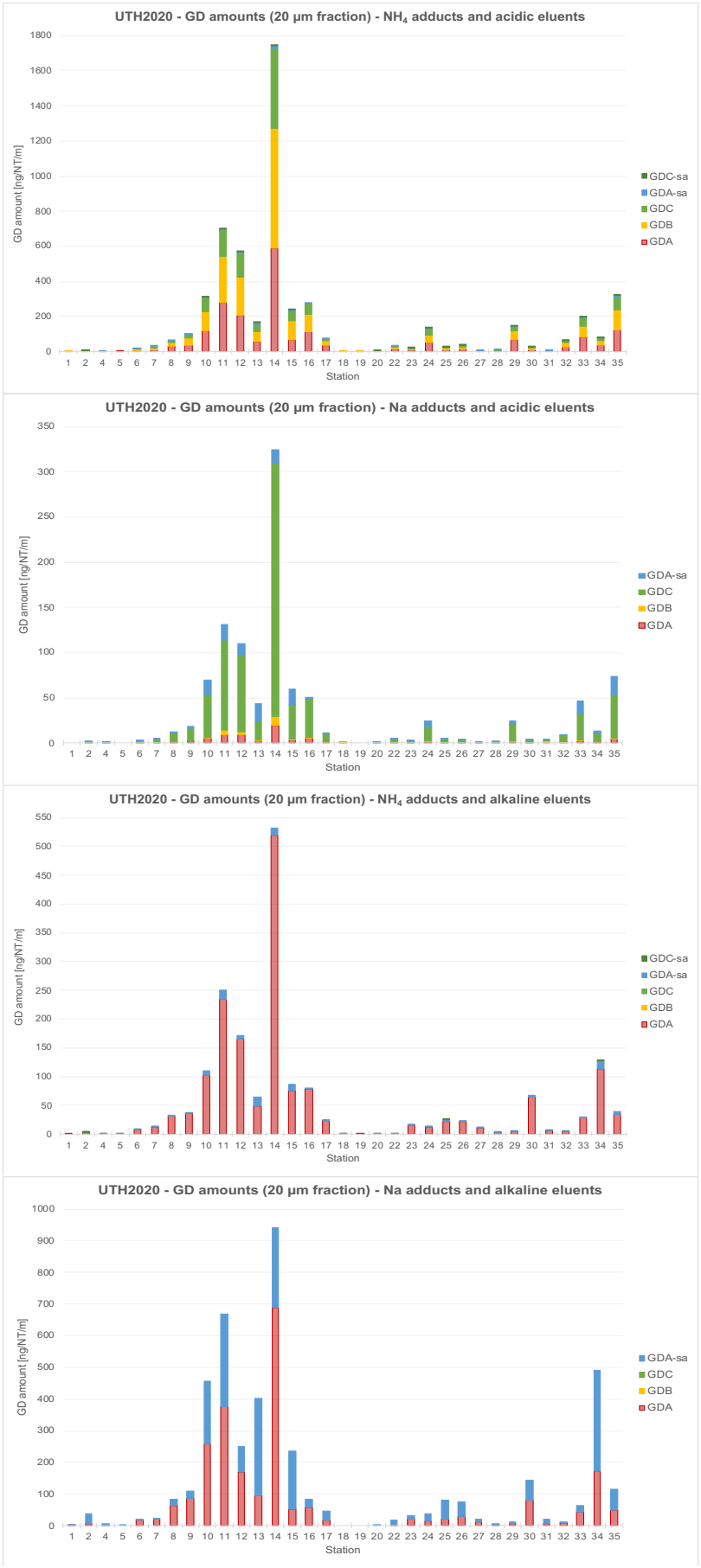


Figure 3-50: Comparison of R/V Uthörn net haul extracts with four systems (20 µm mesh)

Re-assessment of *Alexandrium* GD profiles under optimized chromatographic conditions

The dinoflagellate cell extracts did not contain any GDB nor GDC, except for those of *A. monilatum*, containing at least 9 pg GDB per cell. Among GDA and its desmethyl species that are characteristic for *A. pseudogonyaulax* and the Mediterranean *A. taylorii*, small amounts of GDA seco acid were detected. These are probably artifacts created by residual water. Only in cell extracts of *A. monilatum*, GDA seco acid was detected in an unusually high amount of 14 pg/cell. As minor components, GDC seco acid and 34-desmethyl-GDA were detected at a level of 0.04 and 0.05 pg/cell, respectively.

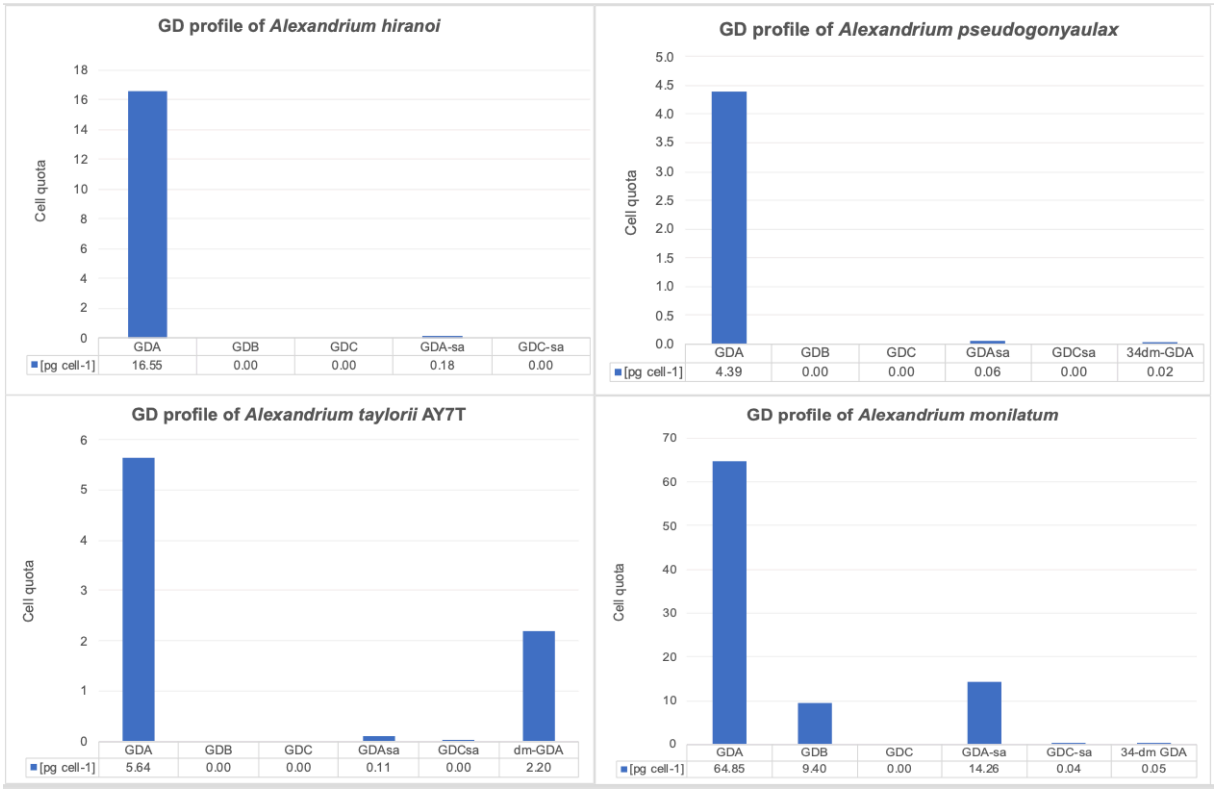


Figure 3-51: Revisited GD profiles of cells of the four Alexandrium *species*

Figure 3-52 shows the proportions of the individual goniodomins that were found in the cell extracts of the four *Alexandrium* species. Here it becomes even clearer that the proportion of GDA seco acid in the cell extract of *A. monilatum* was higher than in the extracts of the other species. In *A. monilatum* it was found to be about 16%, whereas in the other three species, the percentage was about only 1%. In the cell extract of *A. taylorii*, putative 9-desmethyl-GDA represented almost a third of the total goniodomin content, whereas the amount of the other desmethyl congener, 34-desmethyl-GDA in cells of *A. pseudogonyaulax* and *A. monilatum* was limited to less than 1%.

As mentioned earlier, GDB was found in the cell extract of *A. monilatum*. It accounted for the third largest share of the total GD content detected in this extract. Just as GDA seco acid, this might be only

an artifact but not a substance produced by the species itself. In contrast to GDA seco acid, which probably arises during storage or extraction, the occurrence of GDB might be due to insufficiently suppressed formation in contact with the mobile phase.

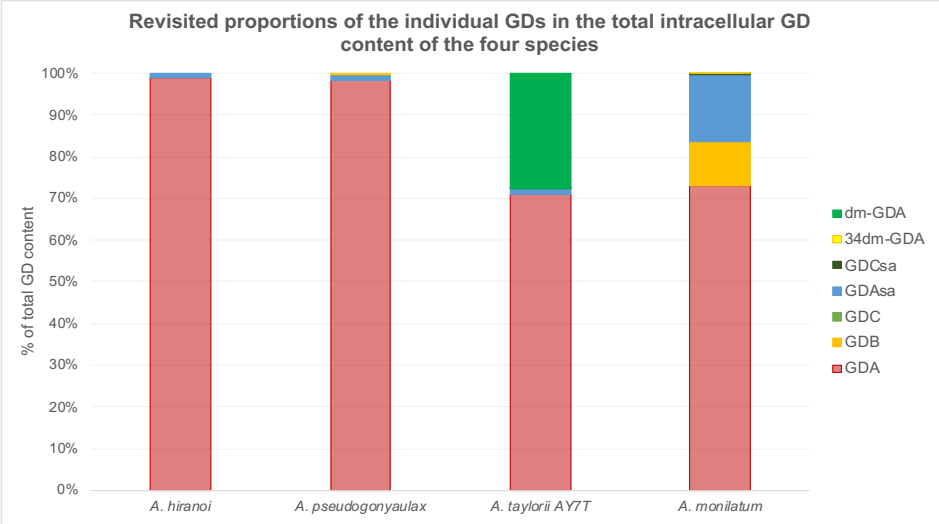


Figure 3-52: Revisited proportions of individual GDs in the intracellular GD content of the four Alexandrium *species*

The GDA cell quotas generally differed slightly between analyses with acidic and alkaline pH but the order of species with highest to lowest GDA cell quota stayed the same. For *A. monilatum* the GDA cell quota determined was more than twice as high as in the previous analysis with the acidic eluent. The sum of the goniodomin concentrations, however, was in the same order of magnitude.

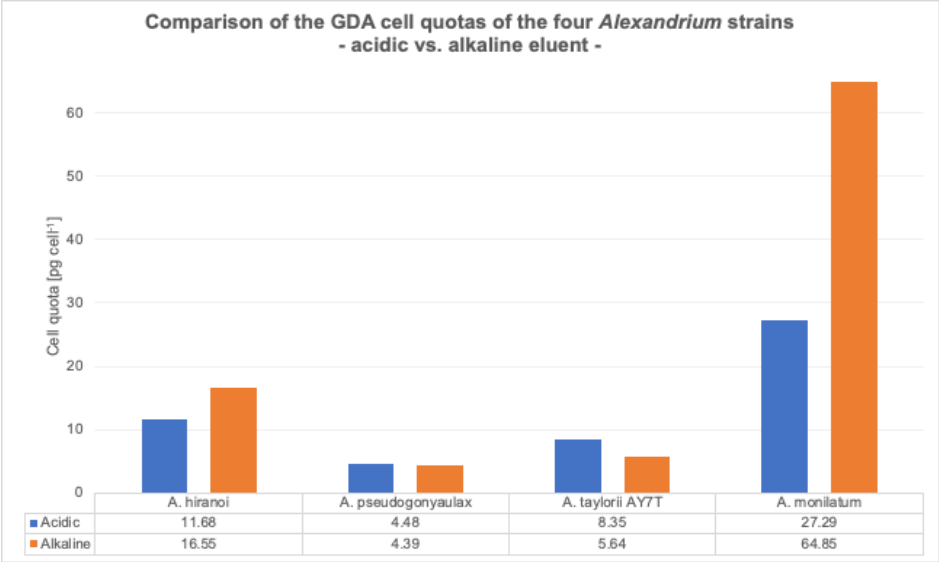


Figure 3-53: Comparison of the GDA cell quotas determined with the acidic vs. the alkaline eluent

3.5.6 Differences in the composition of GD samples measured with two LC-MS/MS instruments

Exemplary measurements were performed on two different sets of samples to allow comparison between the two LC-MS/MS instruments with different contact times of the sample with the acidic mobile phase. It was expected that the application of two LC methods of different run times to the same sample leads to different results on behalf of the amount of conversion products.

Specifically, it is expected that fewer transformation products, or at least lower concentrations of them, will occur when using the method with the shorter contact time. One UPLC run of the Xevo TQ-XS instrument is 10 min shorter than the HPLC run of the API4000 instrument.

Apparently, the expectation was confirmed: In both sample sets analyzed, the percentage of GDA achieved with the Xevo TQ-XS was actually higher than in the API whereas in the analyses performed with the latter instrument, the percentage of conversion products was higher. The major part appears to have transformed to GDA seco acid and to *m/z* 802, respectively. As the only exception, GDB was determined with higher percentage in the Xevo TQ-XS analyses and it accounted for the largest part of conversion products determined with this method.

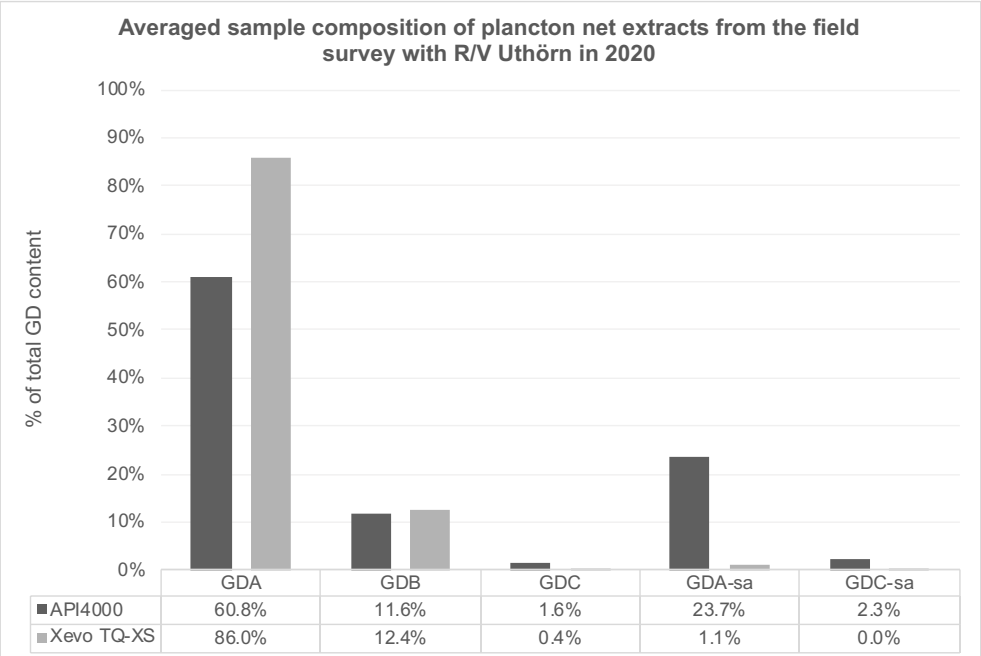
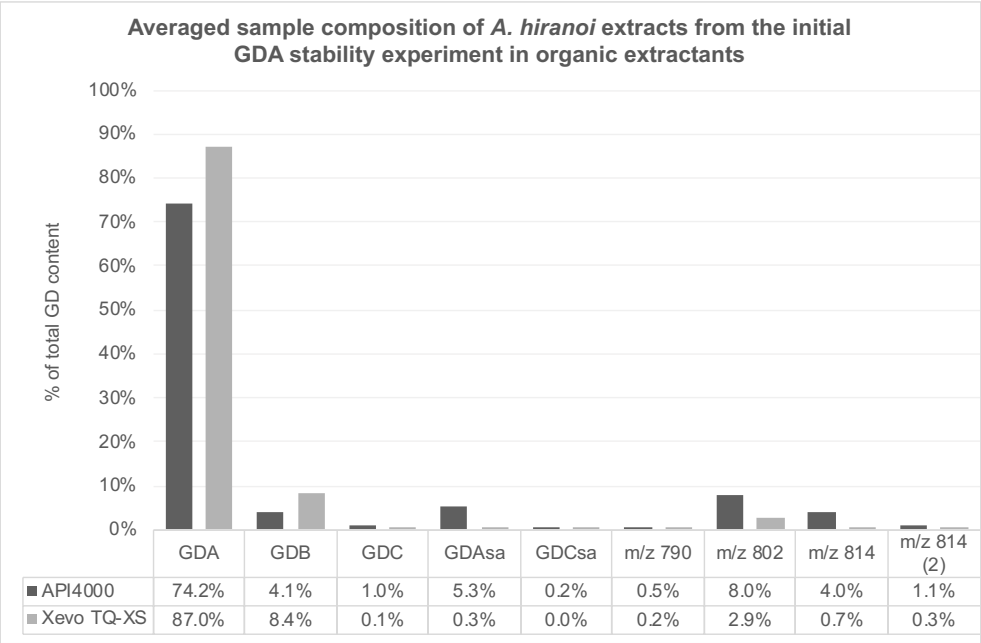


Figure 3-54: Comparison of the averaged sample composition of plankton net extracts from the R/V Uthörn field survey 2020



	GDA	GDB	GDC	GDAsa	GDCsa	m/z 790	m/z 802	m/z 814	m/z 814 (2)
API4000	74.2%	4.1%	1.0%	5.3%	0.2%	0.5%	8.0%	4.0%	1.1%
Xevo TQ-XS	87.0%	8.4%	0.1%	0.3%	0.0%	0.2%	2.9%	0.7%	0.3%

Figure 3-55: Comparison of the averaged composition of A. hiranoi extracts from the initial stability experiment

3.5.7 The influence of the extraction method on formation of GDA seco acid

When re-assessing the intracellular GD profile of *A. monilatum* previously in this chapter, GDA seco acid was detected at an unusually high concentration. The formation of GDA seco acid by hydrolysis of the lactone bond in GDA is favored in aqueous samples and at alkaline pH values. In cell extracts the seco acid is normally present in small amounts and probably arises as an extraction artifact. Cell pellets obtained from centrifugation generally contain considerable residues of water. If the cells cannot be processed immediately, they are usually frozen at -20 °C. A potential issue here is the formation of ice crystals, which favors cell lysis and thus also the exposure of the cell contents to the aqueous environment.

Long-term storage could promote conversion reactions even at these low temperatures. To verify this effect, cell extracts produced directly from a culture and extracts of frozen cell pellets were compared. In addition, a different extraction method was tested on a fresh culture against the production of pellets by centrifugation and cell rupture with lysing matrix. The alternative method included filtration of the culture content and successive drying of the filtered cell mass, extraction with methanol and cell rupture with ultrasound (see 2.8). To exclude the possibility of a misinterpretation of a signal as GDA seco acid, the detected GDA seco acid was compared with standards and samples using two different MS/MS instruments. A smoothing algorithm of the respective MS software was applied to the extracted ion chromatograms in order to enable the comparison of the retention times. Analyses

with the Xevo TQ-XS revealed identical retention times (3.17 min) for the GDA seco acid standard and the substances detected in both *A. monilatum* cell extract and culture supernatant.

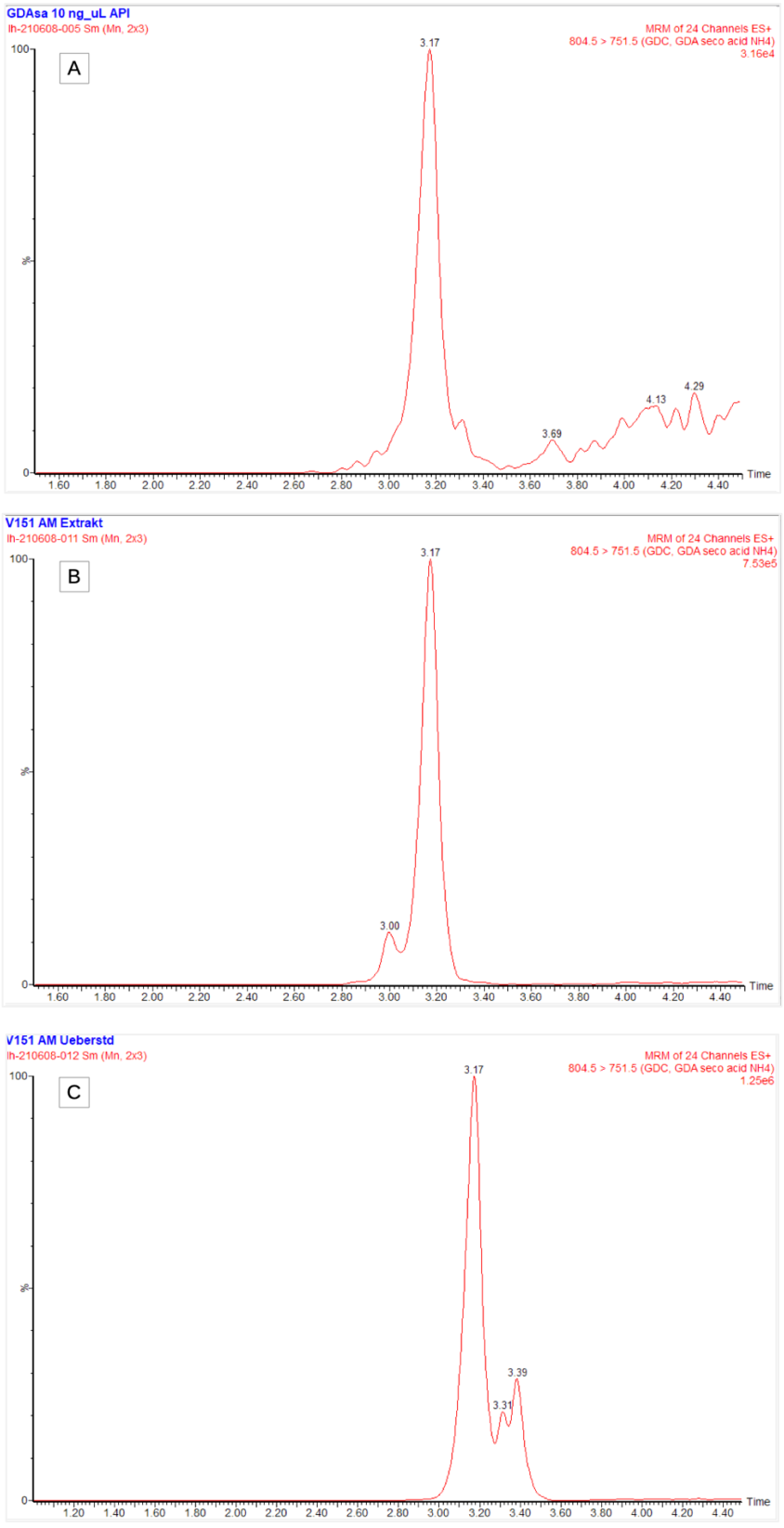


Figure 3-56: Extracted ion chromatograms m/z *804>751 of samples containing GDA seco acid measured with Xevo TQ-XS. A: GDA-sa Std. 10 ng/µL. B: Methanol extract of* A. monilatum*. C: SPE extract of* A. monilatum *supernatant*

In analyses with the API4000 the retention times of the samples as well as the standards differed slightly in the second decimal place, which might have been due to slight variability between the single measurements and a low data acquisition rate. Nevertheless, it can be assumed that the substance detected in the extract of *A. monilatum* is GDA seco acid.

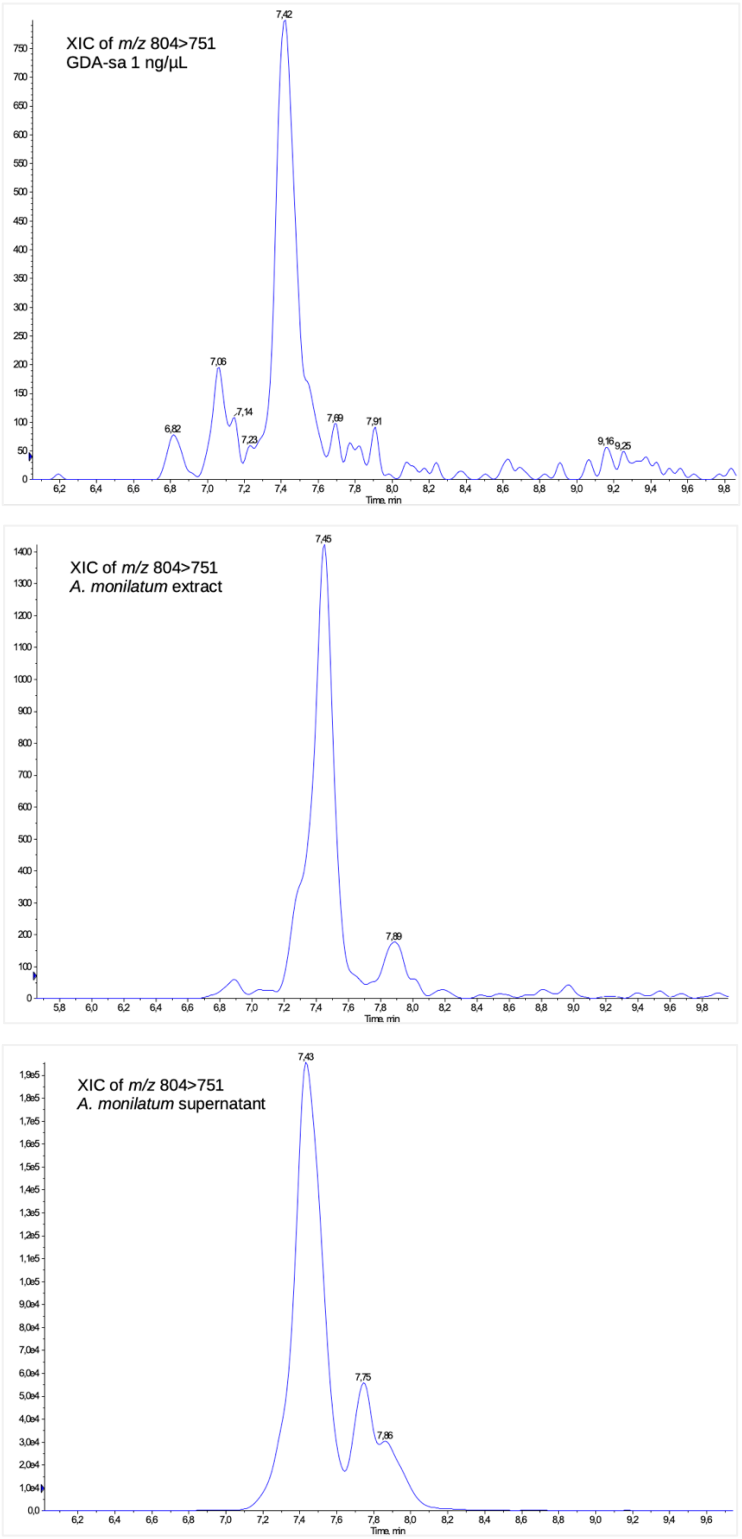


Figure 3-57: Extracted ion chromatograms m/z *804>751 of GDA seco acid standards and samples of* A. monilatum *measured with API4000*

The alternative extraction method including filtration and ultrasonication of the cells had the largest share of GDA compared with the extracts gained by the conventional method. Only little GDB and GDA seco acid were detected. The other two extracts differed slightly in terms of the proportion of all GDs detected. While the percentages of GDB and GDC in the extract of the frozen pellet were less, the percentage of GDA seco acid was higher than in the extract of the fresh culture. In both extracts, 34-desmethyl-GDA had the same share of the total goniodomin content. All in all, the alternative method showed the lowest amount of conversion products and, in particular, the formation of GDA seco acid was reduced.

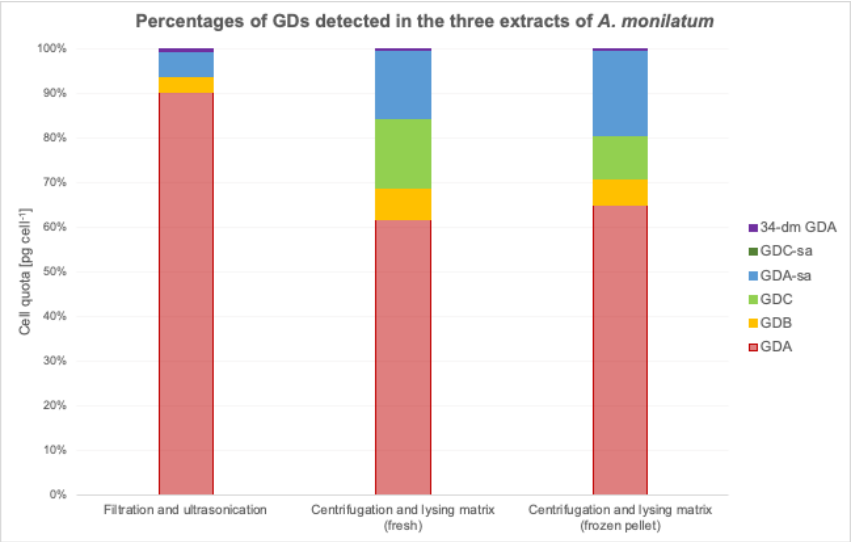


Figure 3-58: Percentages of GDs detected in the extracts of A. monilatum

Based on the absolute values of cell quotas, however, it is not possible to say definitely whether this method is actually the better one. The three extracts showed different GDA cell quotas with the extract gained with the alternative method having the lowest, whereas the cell quotas of 34-desmethyl-GDA were at about the same level. Furthermore, the GDA cell quotas were considerably lower than those determined in the GD profile of *A. monilatum* before (see 3.3.4) although they were exactly the same strain. The occurrence of GDC seems to be due to the extraction method since the extracts gained after filtration showed no GDC at all. Apparently, it does not appear as artifact due to the eluent used but also as extraction artifact. Unlike those of other species, the extracts of *A. monilatum* had an unusually high GDA concentration between 4 and 5 ng/µL so that the concentration of conversion products likely exceeds the limit of detection.

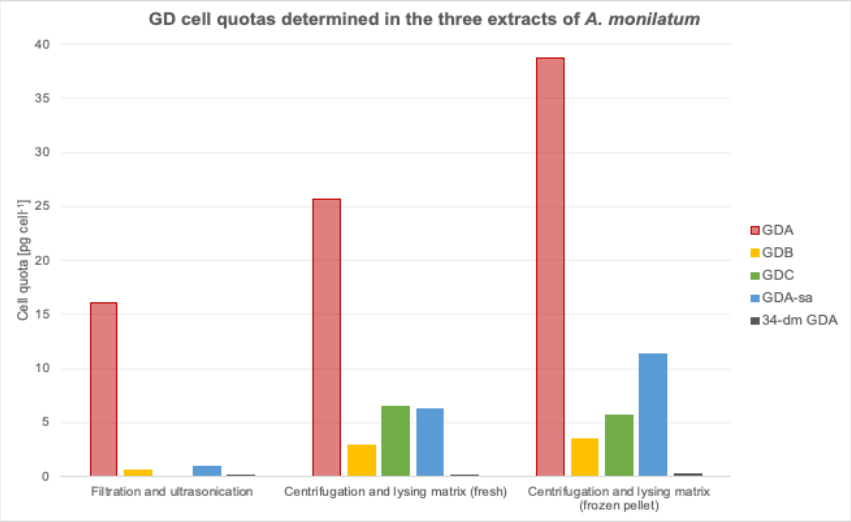


Figure 3-59: GD cell quotas determined in the extracts of A. monilatum

3.6 Re-assessment of the stability of GDA/B/C

3.6.1 Goniodomin A

The concentration of GDA in methanol remained constant over ten days (Figure 3-60). Furthermore, low amounts of *m/z* 814 (at the retention time of GDA) and GDA seco acid were detected with constant concentrations, indicating a formation from GDA due to the eluent pH or other analytic factors. The concentration of GDA was slightly lower than the actual concentration of the standard due to quantitative inclusion of *m/z* 814 which accounted for about one tenth of the sample composition. In general, the findings on the stability of GDA in methanol do not differ largely from those before, except for the fact that neither GDB nor GDC was formed in the period of ten days.

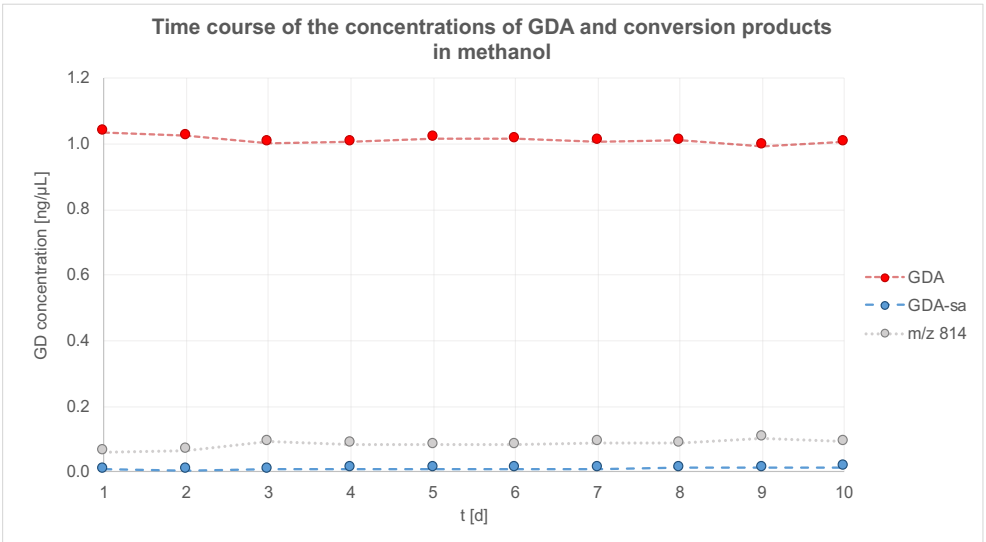


Figure 3-60: Time course of concentrations of GDA and conversion products in methanol

In deionized water, a decline of the GDA concentration over time could be observed (see Figure 3-61) like in the stability experiments with GDA in aqueous solvents performed previously. However, the conversion of GDA to GDA seco acid appeared to progress more slowly and the GDA concentration did not fall to such low levels as the concentration curve obtained with the acidic eluent in 3.2.3. This implies a slower reaction kinetic due to the use of the alkaline eluent. Furthermore, in contrast to the earlier results, no GDC seco acid was determined in the aqueous GDA treatment. As a further putative conversion product, *m/z* 814 was found to occur in a constant ratio to GDA but its amount relative to GDA was lower than in the methanol treatment. Consequently, it is conceivable that this substance arises from GDA during the analytic process.

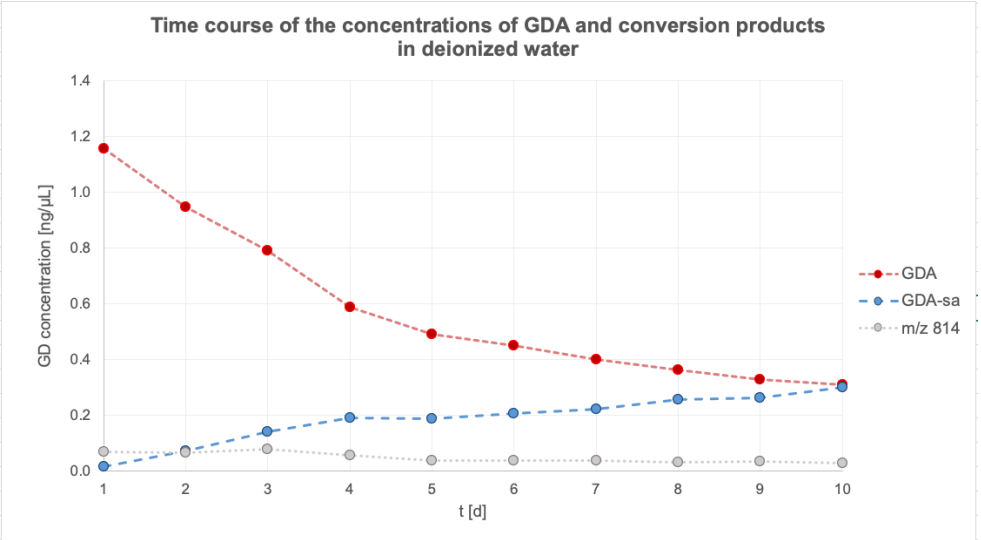


Figure 3-61: Time course of concentrations of GDA and conversion products in deionized water

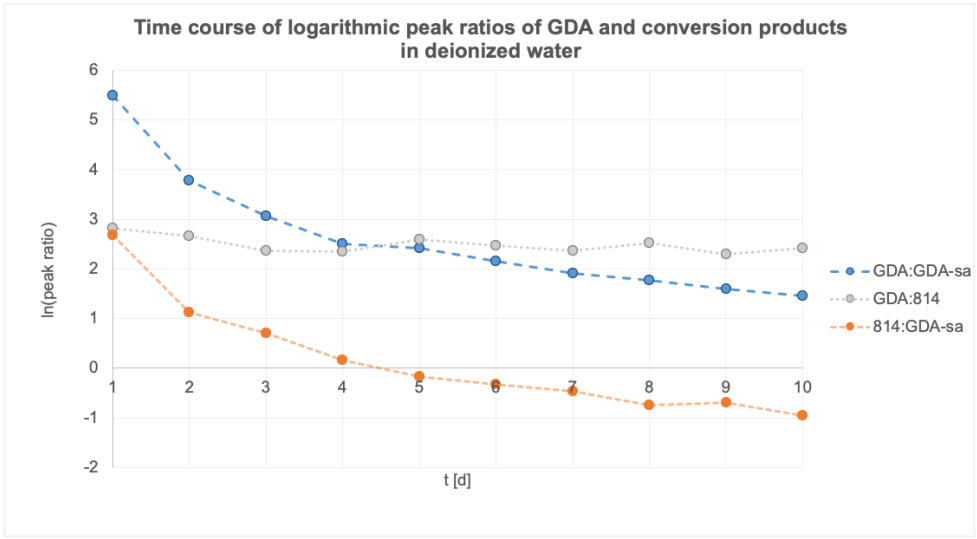


Figure 3-62: Time course of logarithmic peak ratios of GDA and conversion products in deionized water

In K medium (Figure 3-63), the progress of the concentration curves was different from that obtained in deionized water, which was manifested in an apparently slower degradation of GDA and consequently a less pronounced increase in the concentration of GDA seco acid. The initial concentration of GDA was lower than in the other two treatments and the concentration curve had no intercept with the concentration curve of GDA seco acid. The absolute difference of GDA concentrations between day 1 and 10 was 0.65 ng/µL whereas the difference in deionized water was higher with about 0.8 ng/µL. The results of the stability experiment in 3.2.3 were reversed regarding the kinetics of degradation depending on the solvent. Generally, the half-life of GDA in both treatments appears to be shifted to higher values compared with the previous stability experiment. The amount of *m/z* 814 relative to GDA was decreased in comparison to its amount in deionized water.

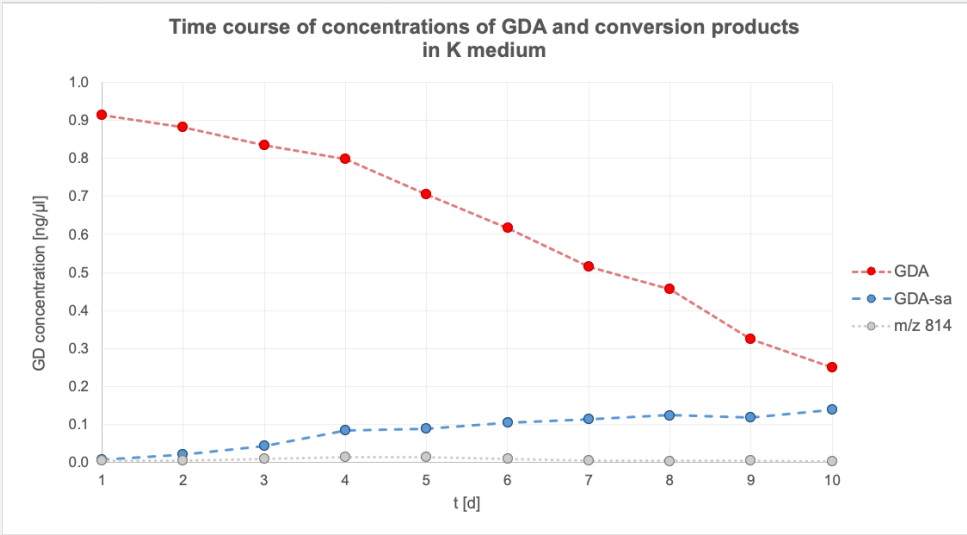


Figure 3-63: Time course of the concentrations of GDA and conversion products in K medium

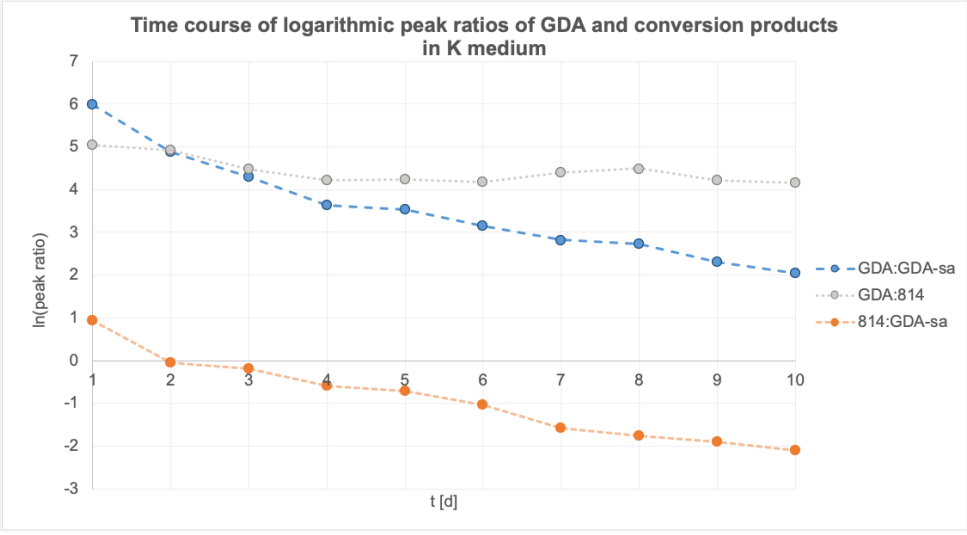


Figure 3-64: Time course of logarithmic peak ratios of GDA and conversion products in K medium

3.6.2 Goniodomin B

Despite slight fluctuations, the time course of the GDB concentration in methanol suggests that GDB is stable in methanol over the ten-day period analyzed. Due to the quantification, the fluctuations of the GDB concentration run symmetrically to those in the concentration of *m/z* 814, which shares the retention time with GDB and is therefore, as well as due to the relatively constant ratio to the concentration of GDB, possibly also a measurement artifact. A very low concentration of GDA seco acid was also detected. It is presumably not formed from GDB but from GDA, which is also present here as a potential artifact. Since the presence of GDA in the GDB standard could not be completely suppressed when the analytic method was optimized, the occurrence of GDA in this standard may not necessarily be due to a transformation in the sample.

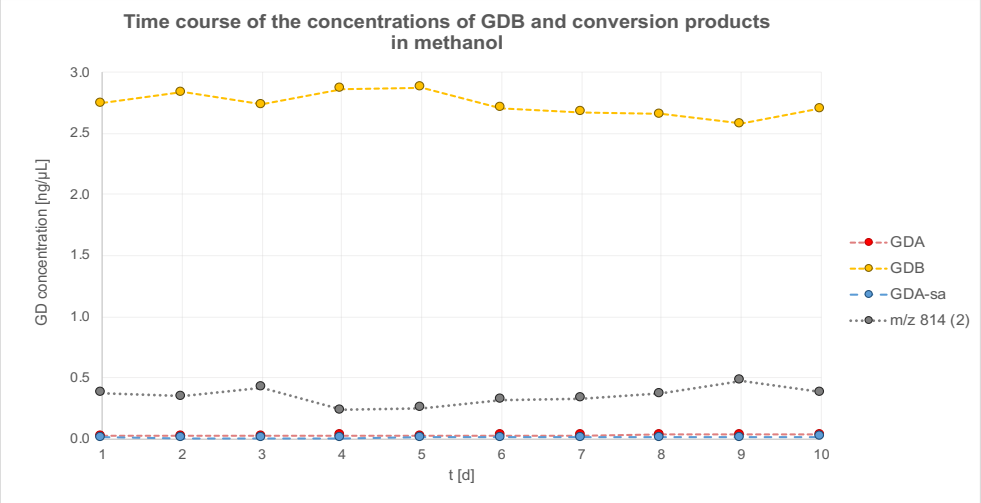


Figure 3-65: Time course of concentrations of GDB and conversion products in methanol

In deionized water a slight decrease of the GDB concentration over time was observed with a parallel increase of the concentration of GDA seco acid. The logarithmic peak ratios are shown in Figure 3-67. The initial concentration of GDB was already low with only 0.75 ng/µL and decreased by about 0.35 ng/µL within ten days. The conversion products present in this sample were the same as already observed in the methanol standard with a constant ratio of GDB to GDA and GDB to the substance with *m/z* 814, confirming their occurrence due to conversion during the analytic process. As it has been observed in the treatments of GDA, the earlier eluting analog of *m/z* 814 was also detected in a lower relative amount to GDB in deionized water compared to methanol.

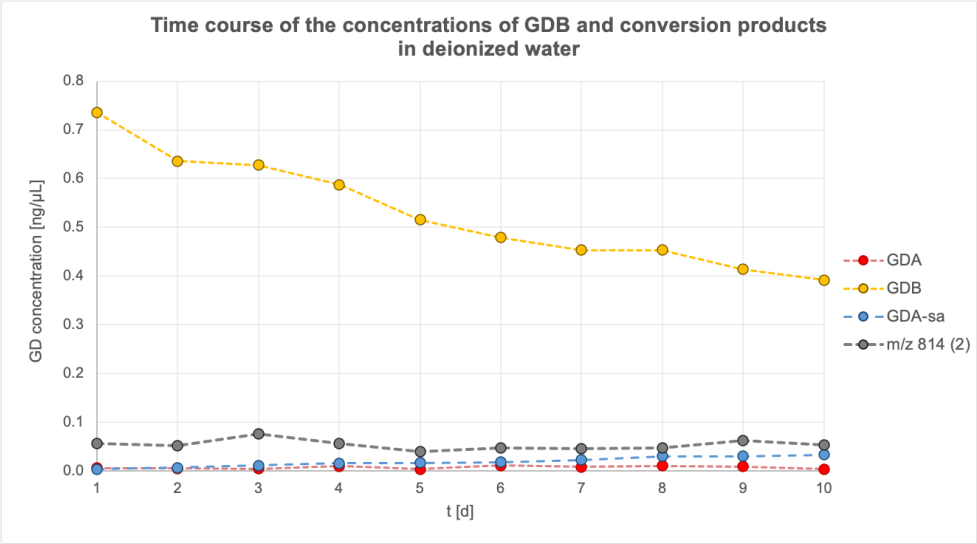


Figure 3-66: Time course of the concentrations of GDB and conversion products in deionized water

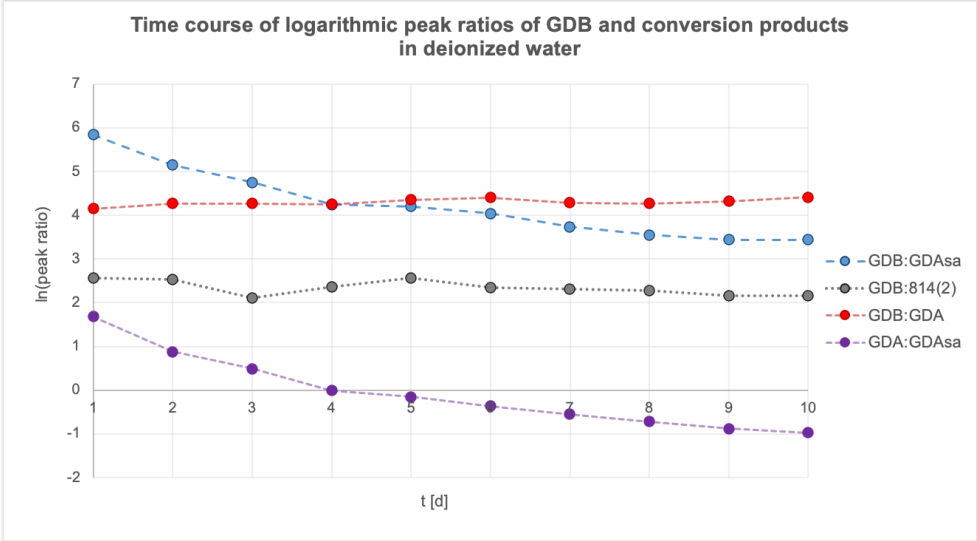


Figure 3-67: Time course of logarithmic peak ratios of GDB and conversion products in deionized water

The behavior of GDB in K medium versus deionized water was largely contrary to that of GDA.

The difference between initial and final concentration of GDB and therefore the slope of the concentration curve was larger than in deionized water. The initial concentration was almost 1 ng/µL higher than in the deionized treatment whereas the three detected conversion products GDA, GDA seco acid and *m/z* 814 emerged with comparatively lower concentrations. This is reflected in the irregular course of the logarithmic peak ratios. Nevertheless, the ratio of GDB to GDA and *m/z* 814, respectively, was be assumed to be approximately constant whereas in parallel, the concentration of GDA seco acid slightly increased.

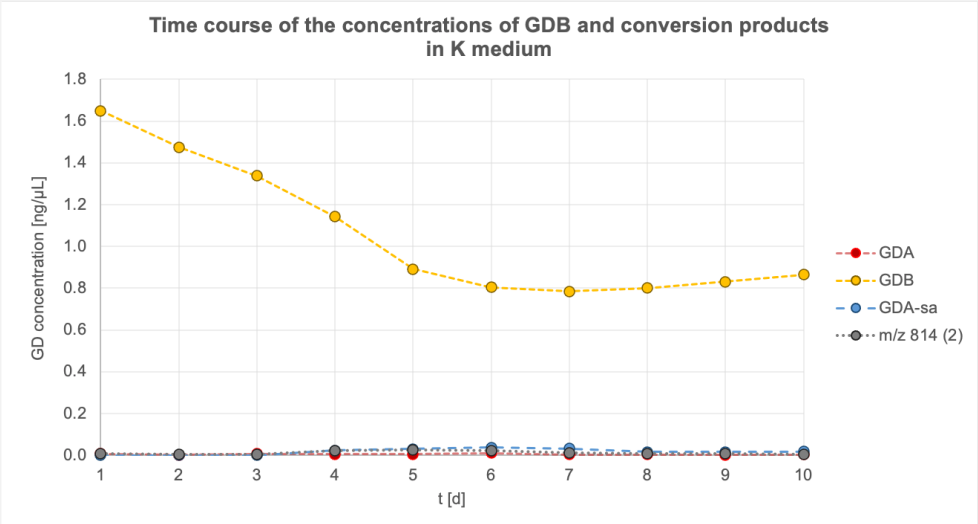


Figure 3-68: Time course of the concentrations of GDB and conversion products in K medium

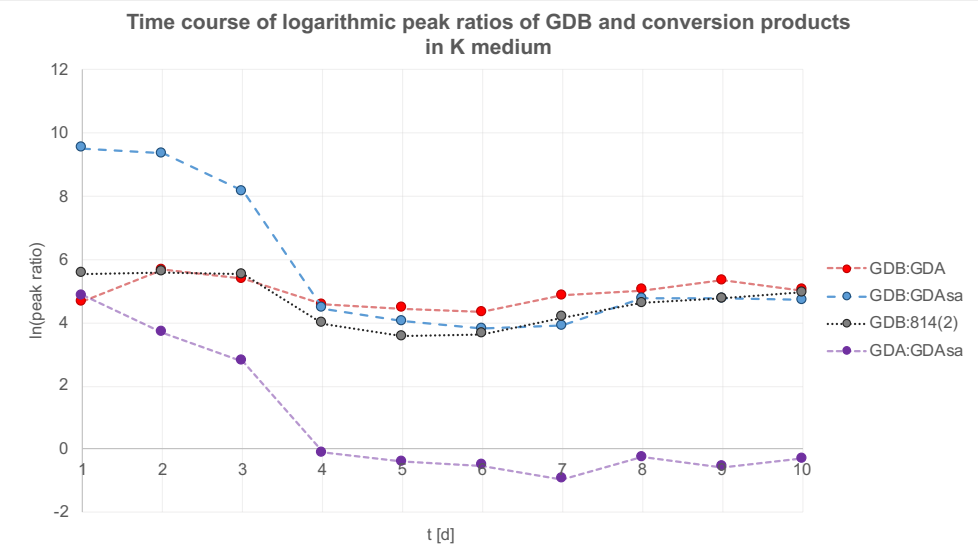


Figure 3-69: Time course of logarithmic peak ratios of GDB and conversion products in K medium

3.6.3 Goniodomin C

As the only of the three congeners, GDC appears to be rather unstable in methanol at room temperature. The initial concentration of GDC in the methanol standard was determined to be 1.69 ng/µL and decreased to 0.61 ng/µL in the course of nine days. In parallel, there was no significant formation of conversion products observed. GDA and GDB as well as GDA seco acid were detected in trace amounts and their concentrations did not increase over time.

Apparently, the peak ratios of GDC to the congeners were at a relatively low level indicating a small difference in the peak areas. With this respect, it should be noted that the responses of GDC versus the congeners GDA/B are considerably different (see also Table 3-11), which is expressed in the fact that a similar peak area corresponds to a concentration in the ng/µL range in the case of GDC and in the pg/µL range in the case of GDA/B. Therefore, the results obtained for GDA and GDB are likely to be afflicted with errors.

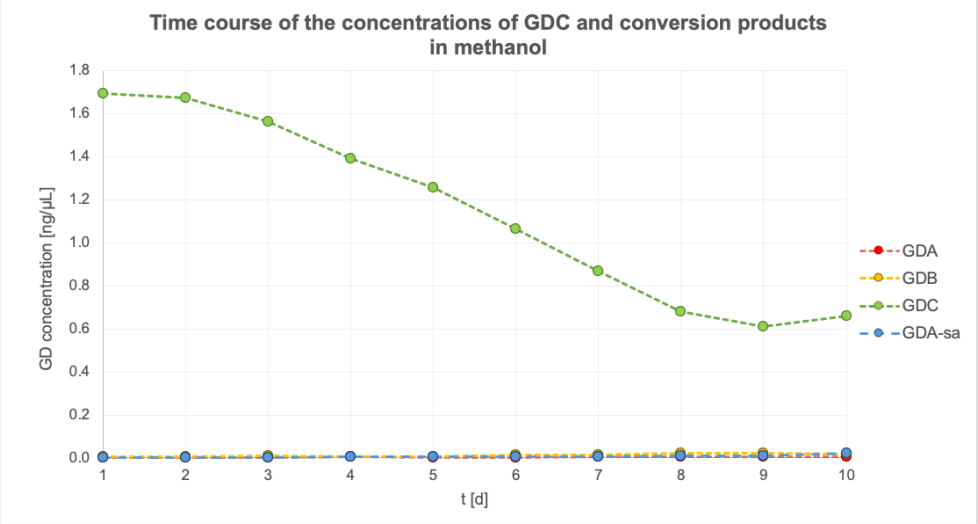


Figure 3-70: Time course of concentrations of GDC and conversion products in methanol

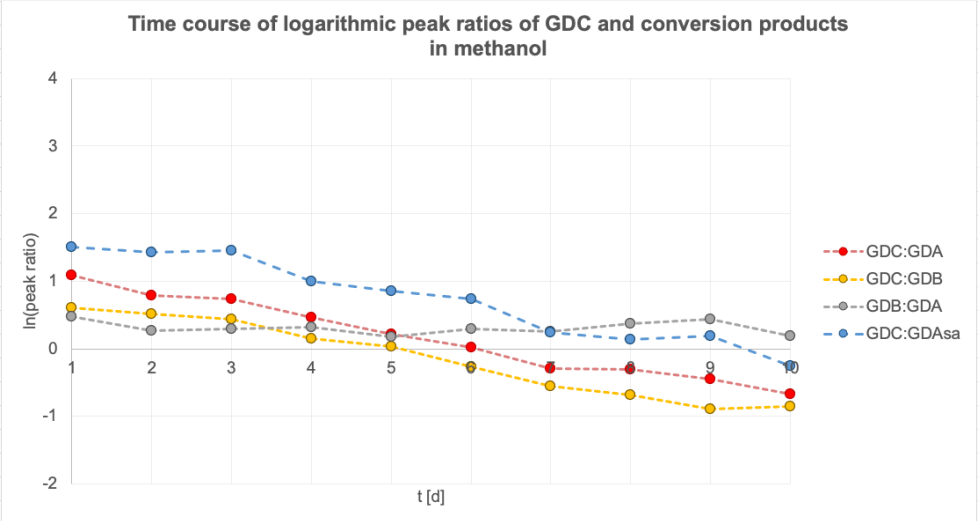


Figure 3-71: Time course of logarithmic peak ratios of GDC and conversion products in methanol

The stability of GDC did not improve when deionized water was used as a solvent. In contrast, the concentration rapidly decreased in the course of three days and at day 4, GDC could not be detected anymore. Additionally, the initial concentration was already lower than in methanol. Despite the obvious degradation of GDC, no conversion products could be identified except for the very low concentrations of GDA, GDB and GDA seco acid. The ratio of GDB and GDA was approximately constant and decreased relatively to GDA seco acid.

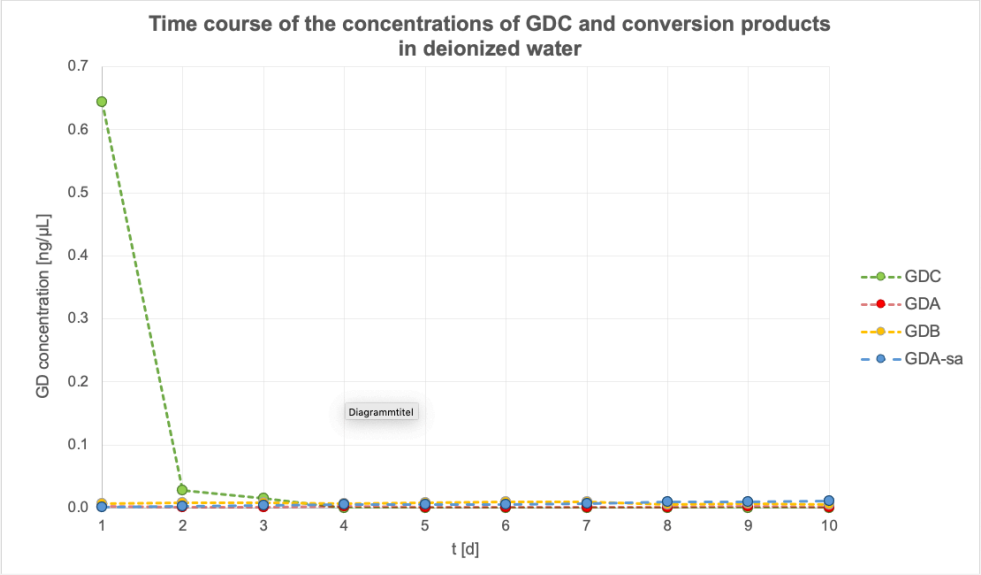


Figure 3-72: Time course of the concentrations of GDC and conversion products in deionized water

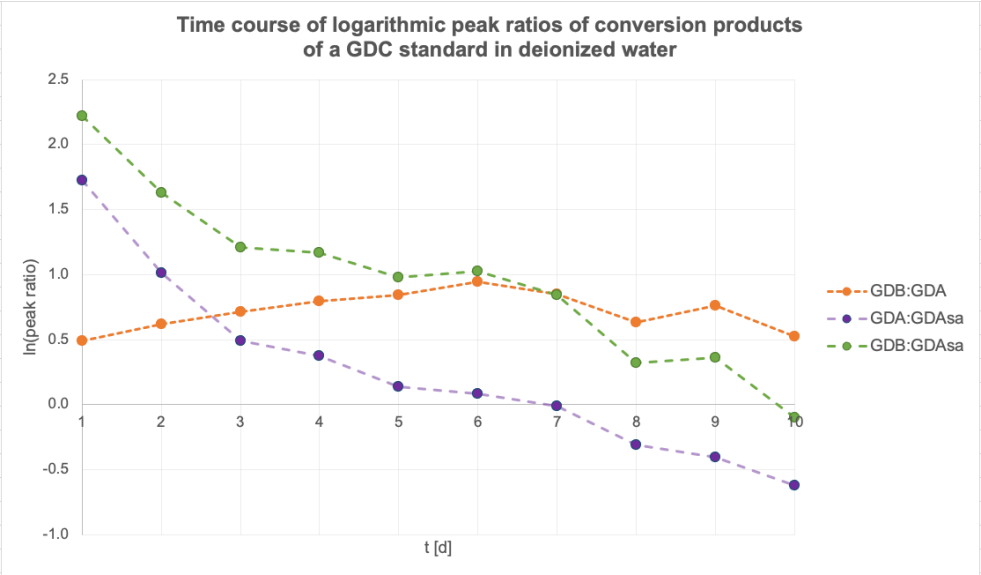


Figure 3-73: Time course of logarithmic peak ratios of conversion products of a GDC standard in deionized water

In K medium, GDC was not detected at all, indicating either more rapid degradation than in deionized water or problems with the detection of this substance. Nevertheless, small concentrations of GDA, GDB and GDA seco acid could be detected that should have their origin in GDC. The concentrations are depicted on a pg/µL scale in Figure 3-74. As observed in the respective aqueous treatments of GDA and GDB before, the concentration in the GDC standard in K medium decreased over time. Due to the low GDA concentration, GDA seco acid hardly exceeded the detection limit and was detected only between days 4 and 8.

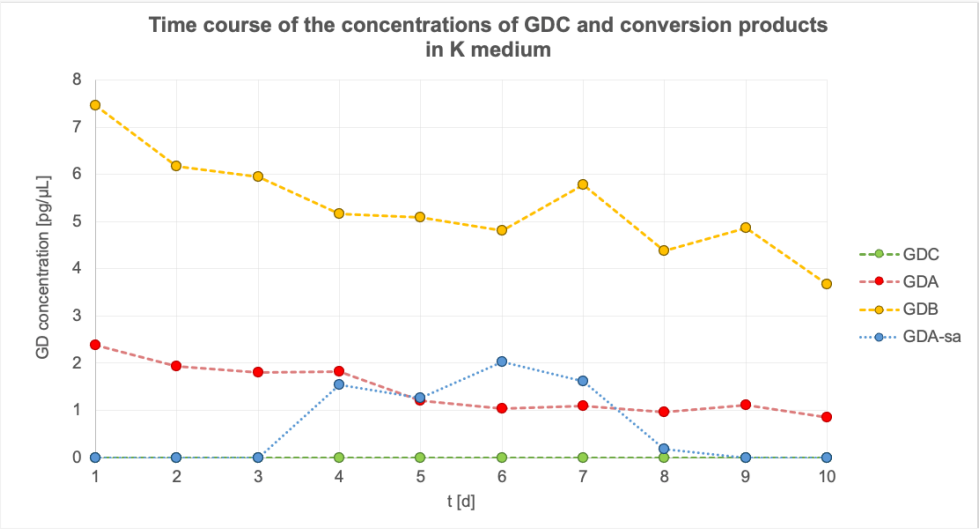


Figure 3-74: Time course of the concentrations of GDC and conversion products in K medium

4 Discussion

4.1 Stability of GDA in organic extractants

The observed time courses suggested that the sample constitution of cell extracts of *A. hiranoi* does not change over a period of at least 14 days when stored in methanol or acetone at 10 °C.

The application of a drying method to the frozen cells did not lead to a clear improvement with regard to the formation of conversion products. This was confirmed by use of two different LC-MS/MS systems. It may be more appropriate to use a drying method that does not require the cells to be frozen.

The concentration values determined with the two LC-MS/MS instruments were different.

The values obtained with the Xevo TQ-XS were on average 0.8 ng/µL higher than those of the API4000. The relative concentrations differed as well. In the analyses with the Xevo TQ-XS, the general share of conversion products in relation to GDA was lower (9-14 %) than that in the API4000 analyses (20-30%). But remarkably, the percentage of GDB measured with the Xevo instrument was higher with 6-9% versus 3.5-4.6%. The differences may result from the unequal response and ionization efficiency of the GDA congeners. Furthermore, the different chromatographic systems with slightly different eluents may contribute to differences in the formation of conversion products during chromatographic separation. The time courses of GDA in the different treatments measured with the two instruments are depicted in the appendix (6.2). The parallel progression of the fluctuations observed in the four treatments indicates technical variability within the measurements., i.e. a fluctuation in response. This was particularly evident in the case of the API4000, which is the older of the two instruments and also had a defective degasser. After two weeks, an obvious increase of the GDA concentration was observed. The concentrations of the other GDs detected increased in parallel. This supports the assumption that the increase in concentration probably resulted from evaporation processes due to the relatively long data acquisition times at a temperature of 10 °C. Particularly the analyses with the API4000 may have contributed to evaporation as the data acquisition time was half an hour per sample.

Between the individual treatments there were no major differences, except for the fact that acetone extracts prepared from dried cell pellets had much lower concentration values, which could be explained by mixing effects: Despite thorough mixing, it is possible that the last treatment contained a lower proportion of cell material because the cells were still actively motile at the time of harvesting and, accordingly, the concentration values happened to be lower. A solvent-related effect can be ruled out because the acetone treatment without drying step had quite high GDA concentration values,

which were even higher than those of the methanol treatment. It therefore appears to be a pure mixing effect.

The data suggest that both methanol and acetone are suitable solvents for the extraction of goniodomins from dinoflagellate cells. A storage temperature of 4-10 °C seems to be sufficient in order to preserve the goniodomin content. The effect of higher temperatures was not analyzed but the process of cell rupture generates heat which could also have an influence on the sample constitution. But the fact that intact GDA was detected and the structurally related pectenotoxins are heat-stable[31] supports the suggestion that brief exposure to heat during the extraction process does not affect the stability of GDA. Moreover, there were no experiments performed with frozen samples but it is expected that lower temperatures also reduce the reaction rate of potential conversion processes. Nevertheless, if samples are to be stored in the refrigerator, the vial lids should be tightly closed to prevent evaporation of the organic solvent. In this experiment, screw top vials were used because they presumably close tighter than crimp caps and can be replaced more quickly between measurements.

A considerable amount of conversion products was present right at the beginning, which could indicate that a transformation had already taken place before the initial measurement. The conversion products could either have originated as extraction artifacts or they may have already been formed in the cells. During the extraction process, conversion could also occur upon contact with certain cellular components.

By the fact that the concentration values of GDA did not change significantly over time, it can be assumed that it is provisionally stable in both methanol and acetone. This was also confirmed by the subsequent stability experiment in which the pure substance was dissolved in methanol as a control for the time-dependent degradation of GDA in aqueous solvents.

The share of conversion products in the total intracellular goniodomin content was relatively low at a level of 20%. The original toxin composition could not be inferred from the data because apparently, a conversion had already occurred. In future experiments this could possibly be remedied by other extraction methods and analytical techniques. At the time of this experiment, little pure material was available, so that It was necessary to use biological material instead. Later, however, such an experiment was also performed using standards of GDA, GDB and GDC.

4.2 Stability of GDA in aqueous environment

The results of all experiments indicate that GDA is not stable in water and quickly transforms to GDA seco acid and, somehow, to GDC seco acid. The transformation process of GDA to GDA seco acid is already known; a study will be published in near future. A seco acid is formed from the hydrolytic cleavage of a lactone and is especially favored under alkaline conditions. Slightly alkaline conditions are given in case of K medium, which was set to a pH of 8. Nevertheless, an equal formation was observed in deionized water.

Even though the concentration had dropped considerably within ten days, there was still a small residual of GDA detected in culture supernatants as well as in SPATT bags. It did not completely disappear within ten days, even at ambient temperature. The presence of GDA in supernatants that were stored frozen for months before analysis indicates that GDA may be actually still present in sea water under favorable conditions but it is clearly not the proportionally largest component remaining after conversion has taken place. A much greater influence also with regard to toxic effects should have the more water-soluble components GDA seco acid and GDC seco acid and possibly *m/z* 802.

Lysis of *Alexandrium* cells and release of GDA might happen at different times, triggered by fresh water influx and mechanical stimuli. Differences in its spatial distribution in sea water might be an important aspect to consider. It could be possible that in blooms a certain amount of GDA is present in water before it degrades. This amount might be sufficient to carry out physiological effects on shellfish and finfish. Focus of cytotoxicity studies should also be on GDA seco acid which is obviously the major conversion product that can be found in aqueous environment.

On days four and five, an incorrect position of the six-port switching valve in the HPLC caused the sample to enter the waste instead of the mass spectrometer. Because of that, no data are available for those particular days. The lines in this interval are only interpolated and do not reflect real measured data. All in all, however, this should not affect the curves.

4.3 GD producer strains and their toxin profiles

Cells were usually harvested during the exponential growth phase so that a certain degree of comparability was given. Differences in the profiles that are due to varying amounts of GD conversion products might be caused by the different storage times of the cell pellets prior to the extraction process.

Pellets of *A. hiranoi* and *A. monilatum* were processed three days after harvest of the cells whereas *A. taylorii* AY7T and *A. pseudogonyaulax* had been stored at -20 °C for about five months. This may be reflected in the slightly higher percentage of GDA seco acid compared to the *A. hiranoi* and *A. monilatum* extracts. Besides the large differences in GDA cell quotas determined in the four species, the differences in desmethyl-GDA species are noteworthy as well. So far, only 34-desmethyl- GDA that was described by Harris et al. was known to be produced by cells of *A. pseudogonyaulax* but apparently a different desmethyl species is existent that is produced by the Mediterranean *A. taylorii.* It is assumed to be 9-desmethyl-GDA. Interestingly, in contrast a Japanese strain of this species was shown to produce 34-desmethyl-GDA but the results have not been published yet. This underlines that there might be a variability not only in cell quotas but also in toxin species between different strains of a species. Because of that the results obtained in this chapter should not be understood as the cell quotas of a whole species but should only be taken as an approximation and a qualitative comparison of the toxins produced by the strains analyzed. The differences in desmethyl-GDA species might for example be interesting for studies on the secondary metabolism of these species.

There has been a range of undescribed GDs detected: *m/z* 790 occurred either at the retention time of GDA or at the retention time of GDC but the latter only in *A. monilatum*. The pair of signals with *m/z* 814 at the exact retention times of GDA and GDB was also noteworthy but they cannot be easily explained by formation of adducts based on GDA and GDB. Except for *m/z* 802, none of the undescribed substances were found in the culture supernatants so that they are either real cellular components, isotopes or extraction artifacts. The results of fragmentation analysis of *m/z* 802 indicated that this substance is related to GDA seco acid. The extensive occurrence in the supernatant but not in the cell extract suggests a hydrophilic character of the substance and its appearance due to hydrolytic effects of the extracellular surrounding. The substance, which differs from GDA seco acid in the spectrum only by a mass difference of 2 Da, could possibly result from a dehydrogenation or from exchange of a carbonyl group by a methylene group. This could also explain the shift in the retention time due to a shift from higher to lower polarity of the substance. The identification of these potential toxins might be subject to further studies but it might be challenging as they only appear in low amounts that are not sufficient for structural analyses via NMR.

4.4 Analysis of *A. pseudogonyaulax* samples from Limfjord

As the data obtained from the field survey suggest, *A. pseudogonyaulax* has increasing impact on the *Alexandrium* population in the sampling areas. *A. pseudogonyaulax* was determined as the predominant species in all samples taken and in parallel, goniodomins made up the largest proportion of the lipophilic toxins detected in all sampling stations. Additionally, a good correlation was determined between the cell count of *A. pseudogonyaulax* and the total GD concentration per sample. GDA was the major compound of the extracts. The similar ratios of GDB and GDC to GDA indicate that they are no true metabolites of the species but that they arise as artifacts. The toxin content determined in the 20 µm fraction resembled that of the extract of *A. pseudogonyaulax* isolate from the Limfjord except for a lack of 34-desmethyl-GDA in the field samples. Presumably the amounts of the desmethyl species in these samples were below the detection limit of the API instrument. Remarkably, *m/z* 802 occurred only in the two fractions with a larger mesh size. Likewise, in the cell extract of *A. pseudogonyaulax* this substance was not detected. Generally, the larger fractions contained a higher proportion of hydrophilic substances that could be caused by uptake and metabolization of *A. pseudogonyaulax* cells by predators or lysed cells in aggregates or similar.

Slightly elevated amounts of GDs were detected in the Baltic Sea 200 µm fraction compared with 50 µm fraction which is unusual and was supposed to be due to aggregate formation, also due to the presence of jellyfish which clogged the filters. Another clogging event occurred at station 3 due to a dense bloom of *Noctiluca*, a bioluminescent dinoflagellate species. Because of that, at this station no extracts were prepared.

The amount of toxins has increased compared with the results of the previous expedition in 2016 and for the first time, GDs were detected in the North Sea close to Helgoland. In the Limfjord, the concentrations of nutrients and biomass were increased compared with North and Baltic Sea while in parallel the amount of GDs as well as the abundance of *A. pseudogonyaulax* was increased. Further expeditions will certainly provide valuable insights into the trends by inclusion of the results into the long-term monitoring.

4.5 Influence of analytic condition on the composition of GD samples

4.5.1 Optimization of the analytic system

Obviously, the eluent used has a strong influence on the apparent composition of goniodomin samples. Based on the results of the experiments performed in this chapter it is recommended to use alkaline instead of acidic eluents and to adapt the MS methods to the affinities of the single goniodomins to different adduct cations. As Harris et al. found (results are to be published soon), the conversion of GDA to GDB and GDC occurs readily with proton acids. Consequently, it is likely that the use of formic acid as an eluent additive, as was the case in the acidic eluent used in this work, promotes the interconversion of the three congeners. When the alkaline eluent is used, the reaction rate of the conversion process seems to be reduced.

The observation that GDA favorably forms ammonium adducts whereas GDB and GDC preferably form sodium adducts is consistent with findings of Harris et al.. They also analyzed the affinity of the three congeners to other cations and found that potassium ions are suitable adducts for GDA and lithium ions for GDB and GDC. This might be an interesting approach for further optimizations of the MS/MS method but it may require additional optimizations of the LC method. Proton adducts are not suitable for MS/MS analyses of GDs because they rarely occur in CID fragmentation and only when ammonium adducts of GDA and GDB are subjected to fragmentation.

A general problem of the existing method is the decrease in molecular response of GDs when sodium adducts are used for quantification. This is especially evident in case of GDC. Consequently, the detection limits for the substances are also relatively high, which could partly complicate their identification.

To enable reliable analysis of as many substances as possible, the existing methods must be optimized further. This includes the identification of the sodium adducts or other alkali metal ion adducts of other undescribed GDs and to evaluate whether they are suitable for quantification of the respective substances, i.e. whether they are preferably formed in comparison to ammonium adducts. Furthermore, the contact time of the sample with the mobile phase can be assumed to have an influence on the sample composition and if it is possible to achieve a good separation, a gradient shorter in time could be developed additionally.

4.5.2 The influence of the extraction method on formation of GDA seco acid

The data suggest that the extraction method used has a certain influence on the formation of GDA seco acid, indicating that it is rather an extraction artifact than a cellular component. The extraction method including filtration and ultrasonication of the cells showed reduced formation of GDA seco acid and other conversion products so that the extraction method can be considered an important driver of the conversion besides the analytic process. As expected, the frozen cell pellet had a higher percentage of GDA seco acid than the extract gained from the fresh culture. In order to maintain the original sample state for quantitative analyses, it may be reasonable to extract the cell pellet as soon as possible and to avoid freezing and long-term storage of the unprocessed cell pellets. If it is not possible to process them directly, it could be reasonable to dry the cell pellets before freezing them.
The differences in the GDA cell quota of the three extracts might be due to different growth phases of the cultures, losses of cell material during the harvesting procedure, an unequal distribution of cells between the different pellets of the culture or incomplete extraction.

4.6 Re-assessment of the stability of GDA/B/C

In this experiment, the stability of GDA in methanol and the tendency to degrade in aqueous solvents were once again confirmed. Even when stored at room temperature, no conversion to GDB and GDC occurred in the methanolic standard within the sampled interval. The curves of GDA in deionized water were clearly flattened compared to those of previous measurements performed with the API4000 instrument and with the acidic eluent. One possible cause could be the different detector sensitivity of the two MS instruments used for analysis. Due to different molecular responses of the analyzed substances that might also differ between the two instruments, the comparability of the results is limited. In addition, it is possible that the analysis is disturbed by the simultaneous formation of GDA seco acid due to the eluent pH and the solvent-dependent conversion. Conversely, it would have been expected that the curves take on a greater incline as a result. It could possibly help to use an eluent with neutral pH and the addition of ammonium ions to eliminate this factor. The previous results indicate that GDA seco acid preferably forms sodium adducts. The MS method containing both ammonium and sodium adducts might therefore facilitate a shift of the abundances to sodium adducts, provided that a sufficiently high amount of sodium ions is available.
GDB also proved to be provisionally stable in methanol. In aqueous solvents, it also showed a time-dependent decrease in concentration that was apparently more pronounced in K medium than in

deionized water. The handling of GDC seems to be more problematic because it appears to be instable in methanol and in aqueous surrounding.

Contrary to what was anticipated, no specific conversion products of GDB and GDC were found. It is possible that they are not yet covered by the existing SRM method. This could be remedied by full scans which was not realized in this work due to time constraints.

There were several issues with the analysis of goniodomins in aqueous solvents. Particularly GDC was hardly detectable. GDC is actually the most hydrophilic compound of the three congeners but appeared to be not stable in any of the used solvents and in case of K medium, it was not detected at all. In principle, it is not surprising that GDC was not found in the aqueous treatments, since it had already never been found in culture supernatants when measured with the acidic eluent. However, the formation of GDC from GDA/B requires the addition of a water molecule, which contrasts with instability in the presence of water. Earlier, a GDC standard prepared for an experiment that was not directly related to this work was subjected to a full scan over the range of *m/z* 800-820 but it did not result in detection of GDC or any other relevant substances in the sample. As a possible explanation for the difficult detection of GDC, it is conceivable that the molecules adhere to the walls of the sample vial and are thus not available for analysis. It is also possible that there was an uneven distribution caused by the transfer of GDC between the sample vials during preparation of the treatments or that the substance did not completely dissolve. Due to the inlet switch used it is also possible that GDC seco acid and potentially other hydrophilic substances resulting from a conversion of GDC elute before the start of the inlet and therefore do not reach the mass analyzer, but are directed into the waste instead. One way to assess this would be to measure the corresponding samples in deionized water only, as it would then be possible to pass the sample directly into the mass spectrometer. In this case, however, no comparison with the chemical behavior in K medium is possible, at least not by means of ESI, which is susceptible to interference from salts.

The analysis of the three substances in K medium yielded results that are difficult to exploit. Apparently, the use of K medium has an adverse effect on the simultaneous analysis of sodium and ammonium adducts. Sodium adducts could be preferably formed due to the high salt content of the medium. The quantification of ammonium adducts is thereby impeded. Furthermore, it is possible that salt input to the mass analyzer still occurs to a small extent despite adjustment of the inlet method. This could also result in variability of the response and a suppression of the signal.

5 Conclusions

The focus of this work was to determine conditions under which goniodomin A is stable. To this end, various aspects of algal cell extraction, storage of goniodomin containing samples, and their analysis were investigated.

It was shown that GDA can be stored in anhydrous organic solvents – acetone and methanol – at a temperature of 4 °C for a period of six weeks. Additionally it was found that the toxin can be stored at room temperature for at least ten days. Storage in the freezer has not been investigated, but it is assumed that this is equally possible, since the lower temperature reduces the kinetics of a potential conversion reaction. In contrast, it is problematic when GDA comes into contact with water, as this promotes a rapid conversion to GDA seco acid. GDA is susceptible to this reaction because its lactone bond can undergo hydrolytic cleavage to form the corresponding hydroxycarboxylic acid. This reaction also occurs when GDA is extracted from cells of *Alexandrium* due to water entrapment between cells. Attempts were made to prevent this by lyophilization of cell pellets prior to extraction, but this proved unsuccessful. Another drying method tested was filtration over glass fibers via vacuum, which resulted in a considerably lower percentage of GDA seco acid compared to extraction without prior drying of the cells. However, the yield of GDA was somewhat lower, which is why this method should be critically reviewed again.

It was also found that in LC-MS/MS analysis of goniodomins, the pH of the eluent has a crucial influence on the interconversion of GDA/B/C and that the choice of adduct ion can contribute to suppression of interfering signals. If an acidic eluent is used, a conversion of GDA to GDB and further reaction to GDC probably takes place during the chromatographic process. This conversion distorts results of GD analyses, which on the one hand complicates the quantification of GDs and on the other hand may lead to incorrect assumptions regarding the GD profiles of producer species or the composition of field samples. The use of an alkaline eluent resulted in a significant reduction of conversion in GD samples and also suggested that GDB and GDC are artifacts rather than actual metabolites of *Alexandrium* species.

The analysis of four strains of the *Alexandrium* species known to produce GDA revealed differences in the production of desmethyl-GDA species and in the cell quotas of GDA. The species *Alexandrium monilatum* was found to have by far the highest intracellular GDA concentrations. For all the species analyzed, GDA was determined to be the major component of the cellular GD content. In addition, small amounts of other previously undescribed GDs were found, for which it could not be determined

yet whether they are artifacts or true cellular components. A particular ecological relevance of these substances can be excluded in principle, especially since they were not or only hardly detected in the aqueous culture supernatants of the species.
In the culture supernatants as well as in SPATT bag extracts used to identify dissolved toxins in waters of North Sea and Baltic Sea, GDA seco acid was present as the major component in good agreement with the chemical behavior of pure GDA in culture medium.

In the course of the evaluation of an expedition with R/V Uthörn in the Danish Limfjord and adjacent parts of the North Sea and the Baltic Sea, samples were examined in which cells of *Alexandrium pseudogonyaulax* were predominant. It was found that in these samples GDA and to a lesser extent its hydrolysis products constituted the major part of the samples. Furthermore, the toxin levels determined were in good correlation with the cell number of *A. pseudogonyaulax* in the samples. *A. pseudogonyaulax* is the only known GD producer in North and Baltic Seas to date. As it increasingly dominates the *Alexandrium* population in these waters, monitoring programs as well as an ongoing dissertation are focusing on this species. They will show whether this trend will continue and determine the potential threat from this species.

6 Appendix

6.1 Material

Table 6-1: Laboratory devices and disposables used throughout the experimental work for this thesis

Description	Model	Manufacturer
Bench-top bead beating lysis system (homogenizer)	FastPrep®-24 Classic	MP Biomedicals GmbH D-37269 Eschwege
Centrifuges	eppendorf Centrifuge 5424 R eppendorf Centrifuge 5810 R	Eppendorf AG D-22331 Hamburg
Evaporator with metal block thermostat	Liebisch™	Gebr. Liebisch GmbH & Co. KG D-33649 Bielefeld
Lab Water Purification system	Milli-Q® IQ 7000 Ultrapure Lab Water System with Q-POD	Merck KGaA D-64293 Darmstadt
Pipettes	Eppendorf Research 10-100 µL 100-1000 µL 500-5000 µL	Eppendorf AG D-22331 Hamburg
Pipette tips	Quality Pipette Tips 200 µL (yellow), 100-1000 µL (blue) epT.I.P.S. dualfilter 5000 µL	Sarstedt AG & Co. D-51588 Nuembrecht Eppendorf AG D-22331 Hamburg
Vortex mixer	VORTEX-GENIE™ 2	Scientific Industries, Inc. 11716 Bohemia, NY
Tubes	15 mL 17 x 120 mm High clarity PP conical tube 50 mL 114 x 28 mm PP Safe-lock Tubes 1.5 mL Microtube 2 mL PP	Falcon Corning Science México S.A. de C.V. Sarstedt AG & Co. D-51588 Nuembrecht Eppendorf AG D-22331 Hamburg Sarstedt AG & Co. D-51588 Nuembrecht
Filter unit inserts	ULTRAFREE®-MC 0.45 µm DURAPORE ® HV	Merck Millipore Ltd. Tullagreen, Carrigtwohill Co. CORK Ireland
Lysing matrix	Lysing Matrix D Bulk	MP Biomedicals GmbH D-37269 Eschwege

Description	Model	Manufacturer
Glassware	Schott Duran 50-5000 mL 1000 mL (for culture medium) Volumetric cylinder BLAUBRAND® 50 mL, 500 mL, 1000 mL	SCHOTT AG D-95666 Mitterteich OMNILAB-LABORZENTRUM GmbH & Co. KG D-28359 Bremen
HPLC vials	Clear, screw top, micro sampling Screw, 2 mL, clear	Agilent Technologies Santa Clara, CA 95051 US
Vial caps	9 mm blue screw, PTFE/RS 11 mm crimp, PTFE/S (aluminium)	Agilent Technologies Santa Clara, CA 95051 US
Ultrasound homogenizer	Sonopuls HD 2070	BANDELIN electronic GmbH & Co. KG D-12207 Berlin
Rotary evaporator **Vacuum pump** **Cryostat**	Laborota 4002-Control Rotavac Vario	Heidolph Instruments GmbH & Co. KG D-91126 Schwabach Colora Messtechnik GmbH D-73547 Lorch
Vacuum manifold **SPE cartridge**	VISIPREP-24™ DL SUPELCLEAN LC-18 6 mL	Supelco Park Bellefonte, PA 16823-0048 USA
Vacuum pump	Ilmvac™ LVS	ILMVAC GmbH D-98693 Ilmenau
Heating bath	IKA® HBR4 digital	IKA®-Werke D-79219 Staufen (Breisgau)
Vacuum pump	KNF Type N035AN.18	KNF Neuberger D-79112 Freiburg (Breisgau)
Glass vacuum filter holder	16306/7/16/16 includes: Glass funnel Clamp Filter support Silicone O-ring Glass base	Sartorius stedim biotech D-37079 Goettingen
Glass microfiber filters	GF/F 46 mm circles (0.7 µm)	Whatman International Ltd Maidstone England

6.2 Additional graphs

6.2.1 Time course of GDA concentration in organic extractants – API4000

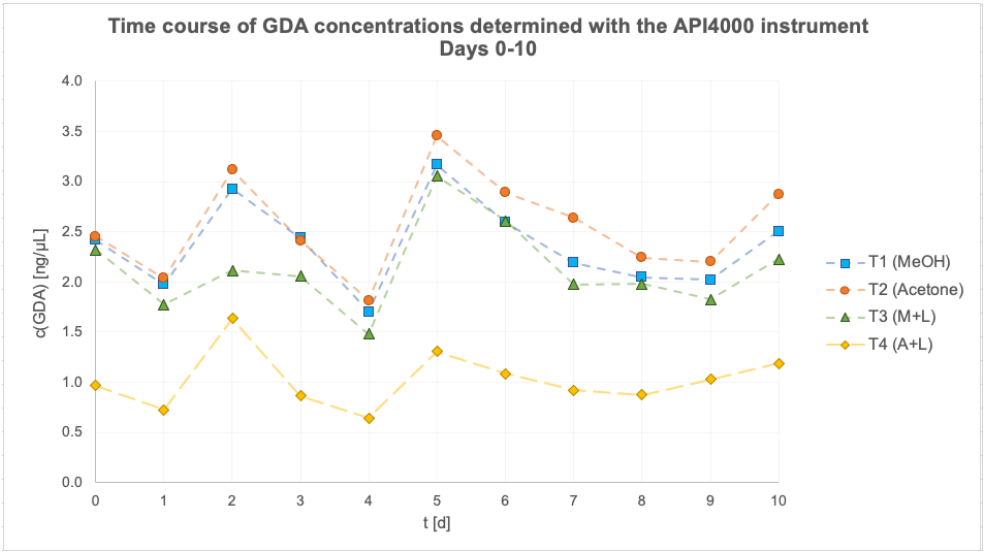


Figure 6-1: Time course of GDA concentration determined with the API4000 instrument (Days 0-10)

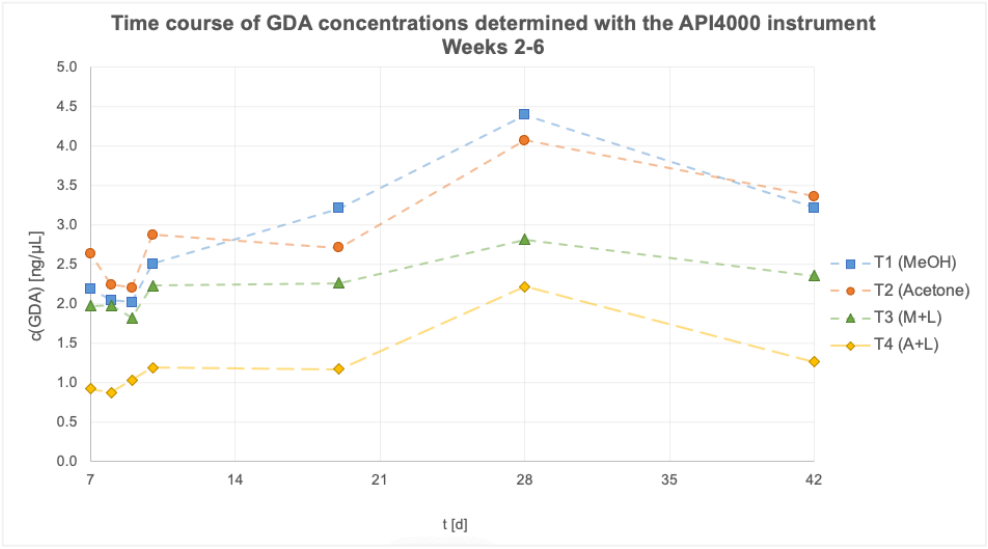


Figure 6-2: Time course of GDA concentration determined with the API4000 instrument (Weeks 2-6)

6.2.2 Time course of GDA concentration in organic extractants – Xevo TQ-XS

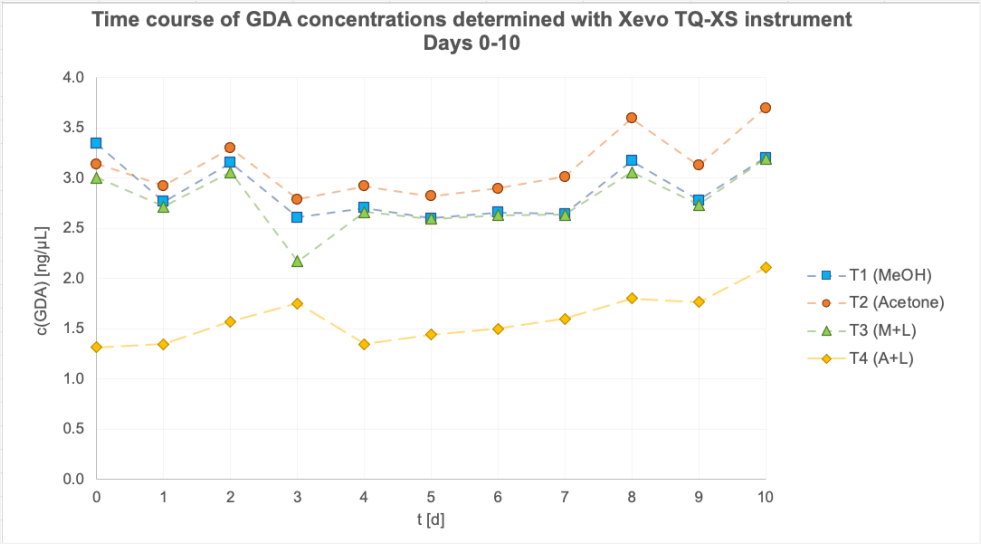


Figure 6-3: Time course of GDA concentration determined with the Xevo TQ-XS instrument (Days 0-10)

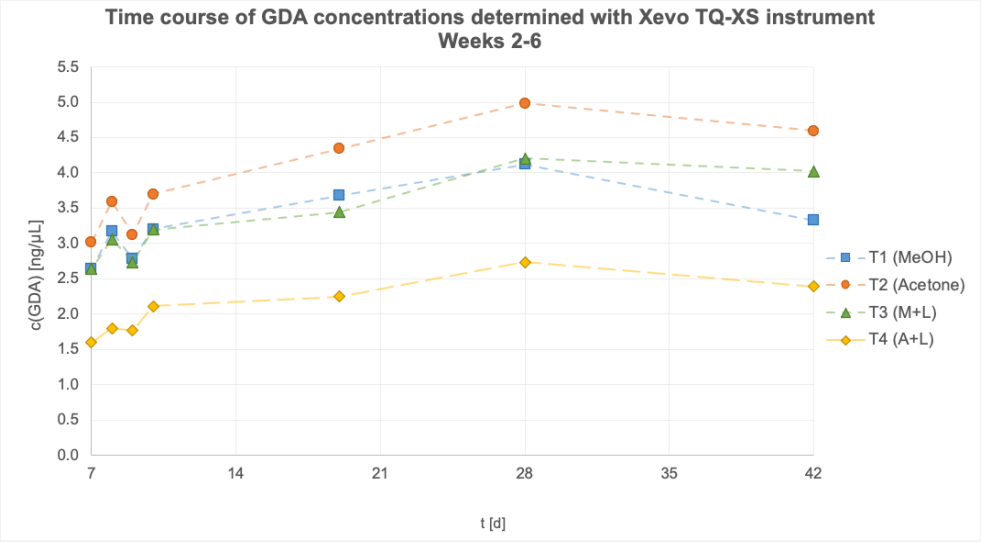


Figure 6-4: Time course of GDA concentration determined with the Xevo TQ-XS instrument (Weeks 2-6)

6.3 References

1. Sharma, G. M., Michaels, L. & Burkholder, P. R. GONIODOMIN, A NEW ANTIBIOTIC FROM A DINOFLAGELLATE. *J. Antibiot.* **21**, 659–664 (1968).

2. Harris, C. M. *et al.* The toxin goniodomin, produced by Alexandrium spp., is identical to goniodomin A. *Harmful Algae* **92**, 101707 (2020).

3. Murakami, M., Makabe, K., Yamaguchi, K., Konosu, S. & Wälchli, M. R. Goniodomin a, a novel polyether macrolide from the dinoflagellate goniodoma pseudogoniaulax. *Tetrahedron Letters* **29**, 1149–1152 (1988).

4. Krock, B. *et al.* 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. *Toxicon* **155**, 51–60 (2018).

5. Espiña, B. *et al.* Cytotoxicity of goniodomin A and B in non contractile cells. *Toxicology Letters* **250–251**, 10–20 (2016).

6. Harris, Constance M. *et al.* Alkali Metal and Acid-Catalyzed Interconversion of Goniodomin A with Congeners B and C. *Journal of Natural Products (under review)*.

7. Terao, K., Ito, E., Murakami, M. & Yamaguchi, K. Histopathological studies on experimental marine toxin poisoning—III. Morphological changes in the liver and thymus of male ICR mice induced by goniodomin A, isolated from the dinoflagellate Goniodoma pseudogoniaulax. *Toxicon* **27**, 269–271 (1989).

8. Furukawa, K. *et al.* Goniodomin A induces modulation of actomyosin ATPase activity mediated through conformational change of actin. *Journal of Biological Chemistry* **268**, 26026–26031 (1993).

9. Abe, M. *et al.* Goniodomin A, an antifungal polyether macrolide, exhibits antiangiogenic activities via inhibition of actin reorganization in endothelial cells. *Journal of Cellular Physiology* **190**, 109–116 (2002).

10. Anderson, D. M. *et al.* The globally distributed genus Alexandrium: Multifaceted roles in marine ecosystems and impacts on human health. *Harmful Algae* **14**, 10–35 (2012).

11. Harding, J. M., Mann, R., Moeller, P. & Hsia, M. S. Mortality of the Veined Rapa Whelk, Rapana venosa, in Relation to a Bloom of Alexandrium monilatum in the York River, United States. *shre* **28**, 363–367 (2009).

12. MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P. & Selwood, A. Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. *Toxicon* **44**, 901–918 (2004).

13. Hsia, M. H. *et al.* Production of goniodomin A by the planktonic, chain-forming dinoflagellate Alexandrium monilatum (Howell) Balech isolated from the Gulf Coast of the United States. *Harmful Algae* **5**, 290–299 (2006).

14. Howell, J. F. Gonyaulax monilata, sp. nov., the Causative Dinoflagellate of a Red Tide on the East Coast of Florida in August-September, 1951. *Transactions of the American Microscopical Society* **72**, 153–156 (1953).

15. May, S. P. *et al.* Effects of the toxic dinoflagellate Alexandrium monilatum on survival, grazing and behavioral response of three ecologically important bivalve molluscs. *Harmful Algae* **9**, 281–293 (2010).

16. Zmerli Triki, H., Laabir, M., Moeller, P., Chomérat, N. & Kéfi Daly-Yahia, O. First report of goniodomin A production by the dinoflagellate Alexandrium pseudogonyaulax developing in southern Mediterranean (Bizerte

Lagoon, Tunisia). *Toxicon* **111**, 91–99 (2016).

17. Kremp, A. *et al.* Distributions of three Alexandrium species and their toxins across a salinity gradient suggest an increasing impact of GDA producing A. pseudogonyaulax in shallow brackish waters of Northern Europe. *Harmful algae* **87**, 101622 (2019).

18. Tillmann, U., Krock, B., Wietkamp, S. & Beran, A. A Mediterranean Alexandrium taylorii (Dinophyceae) Strain Produces Goniodomin A and Lytic Compounds but Not Paralytic Shellfish Toxins. *Toxins* **12**, 564 (2020).

19. Giacobbe, M. G. *et al.* Recurrent high-biomass blooms of Alexandrium taylorii (Dinophyceae), a HAB species expanding in the Mediterranean. in *Biodiversity in Enclosed Seas and Artificial Marine Habitats* (eds. Relini, G. & Ryland, J.) 125–133 (Springer Netherlands, 2007). doi:10.1007/978-1-4020-6156-1_11.

20. MATSUYAMA, Y. Effects of harmful algae on the early planktonic larvae of the oyster, Crassostrea gigas. *Harmful Algal Blooms 2000* (2001).

21. Lim, P. T., Usup, G., Leaw, C. P. & Ogata, T. First report of Alexandrium taylori and Alexandrium peruvianum (Dinophyceae) in Malaysia waters. *Harmful Algae* **4**, 391–400 (2005).

22. Emura, A., Matsuyama, Y. & Oda, T. Evidence for the production of a novel proteinaceous hemolytic exotoxin by dinoflagellate Alexandrium taylori. *Harmful Algae* **3**, 29–37 (2004).

23. Hovedsiden. https://web.archive.org/web/20040806064004/http://www.limfjord.dk/om_fjord.htm (2004).

24. Jørgensen, K. & Andersen, P. RELATION BETWEEN THE CONCENTRATION OF DINOPHYSIS ACUMINATA AND DIARRHEIC SHELLFISH POISONING TOXINS IN BLUE MUSSELS (MYTILUS EDULIS) DURING A TOXIC EPISODE IN THE LIMFJORD (DENMARK), 2006. *shre* **26**, 1081–1087 (2007).

25. Keller, M. D., Selvin, R. C., Claus, W. & Guillard, R. R. L. Media for the Culture of Oceanic Ultraphytoplankton1,2. *Journal of Phycology* **23**, 633–638 (1987).

26. Zmerli Triki, H., Laabir, M. & Kéfi Daly-Yahia, O. Life history, excystment features, and growth characteristics of the Mediterranean harmful dinoflagellate Alexandrium pseudogonyaulax. *Journal of Phycology* **51**, 980–989 (2015).

27. Cembella, A. D., Lewis, N. I. & Quilliam, M. A. The marine dinoflagellate Alexandrium ostenfeldii (Dinophyceae) as the causative organism of spirolide shellfish toxins. *Phycologia* **39**, 67–74 (2000).

28. Hansen, P. J., Cembella, A. D. & Moestrup, Ø. The Marine Dinoflagellate Alexandrium Ostenfeldii: Paralytic Shellfish Toxin Concentration, Composition, and Toxicity to a Tintinnid Ciliate1. *Journal of Phycology* **28**, 597–603 (1992).

29. Van de Waal, D. B. *et al.* Characterization of multiple isolates from an Alexandrium ostenfeldii bloom in The Netherlands. *Harmful Algae* **49**, 94–104 (2015).

30. Onofrio, M. D., Mallet, C. R., Place, A. R. & Smith, J. L. A Screening Tool for the Direct Analysis of Marine and Freshwater Phycotoxins in Organic SPATT Extracts from the Chesapeake Bay. *Toxins* **12**, 322 (2020).

31. Marine biotoxins in shellfish – Pectenotoxin group. *EFSA Journal* **7**, 1109 (2009).

Declaration of Originality

I confirm that the submitted thesis is original work and was written by me without further assistance. Appropriate credit has been given where reference has been made to the work of others.

Butzbach, 28.10.21

Place, date

Signature