Hochschule Coburg
Studiengang Bioanalytik

Method improvement for phycotoxin extraction of marine mammals´ tissues and body fluids

Bachelorarbeit

Zur Erlangung des akademischen Grades
Bachelor of Science (B.Sc.)

Vorgelegt von: Lena Kittel
Gutachter: Prof. Dr. Aileen Funke

Ausgabedatum: 01.04.2022
Abgabedatum: 30.06.2022

Acknowledgment

I would like to thank Dr. Bernd Krock and his work group at the Alfred Wegener Institute for the opportunity to write my bachelor's thesis there and for all the support I have received. A special thank goes to Dr. Marina Arregui for being a mentor and friend. She has supported and taught me a lot for the time being.

List of contents

I. List of figures

II. List of tables

III. List of Abbreviations

AZA.....Azaspiracids
DA.....Domoic acid
ELISA.....enzyme-linked immunosorbent assay
ESI.....electrospray ionization
FAO.....Food and Agriculture Organization
FLD.....fluorescence detector
GYM.....Gymnodimine
HAB.....harmful algal blooms
HPLC.....high-performance liquid chromatography
IOC.....Intergovernmental Oceanographic Commission of UNESCO
LC-MS/MS.....liquid chromatography coupled to tandem mass spectrometry
LoD.....limit of detection
m/z.....mass to charge value
OA.....Okadaic acid
PSP.....paralytic shellfish poisoning
PTX.....Pectenotoxins
SPE.....solid-phase extraction
SPX.....Spirolides
SRM.....selective reaction monitoring
TIC.....total ion chromatogram
UHPLC.....Ultra-high performance liquid chromatography
WHO.....World Health Organization

Well-known abbreviations in general use and international abbreviations of the SI-System are not included.

1. Abstract

Phycotoxins are natural metabolites produced by several species of microalgae. Under favourable environmental conditions such as light, nutrients, temperature and salinity, those algae species can grow exponentially and form dense aggregations. These aggregations are called “harmful algal blooms” (HAB) when harmful to the environment, aquatic life, and human health. In the last decades, anthropogenic activities such as fertilization, extensive fishing, sea travel and increased production of greenhouse gases have led to an increase in HAB events.

Microalgae are on the base of the marine food web and, thus, are ingested by organisms at higher trophic levels, leading to transference and accumulation of phycotoxins along the food web, ending up in top predators like marine mammals, sea birds and even humans. In sea birds and marine mammals, several mass-mortality events have been associated with phycotoxins. In humans, different illnesses have been described due to the consumption of contaminated seafood, some of them causing severe gastrointestinal and neurological symptoms. Therefore, further investigations and constant monitoring of phycotoxin transference along the food web are necessary to protect biodiversity and secure food safety and human health.

Marine mammals show great potential as sentinel species to study the presence and consequences of phycotoxin consumption as they are, in most cases, at the top of the marine food web, they have long life spans and have a wide range of distribution, including coastal ecosystems which are thought to be more affected by HAB. Moreover, they consume similar trophic resources to humans, confirming their potential as sentinels to assess the negative effects of HAB and phycotoxins on public health.

The aim of this work was to examine different types of marine mammal tissues and body fluids for the presence of phycotoxins and, thus, implement an effective extraction protocol. The samples were collected opportunistically from stranded animals on the north and central Argentinian coast in 2018. Marine mammals’ tissues and body fluids are complex matrices that have proven to be difficult to extract. Therefore, different trials, including different extraction solvents and clean-up steps, among others, have been tested to improve the initial method draft based on the literature. Furthermore, toxin recovery with solid-phase extraction was carefully assessed. As a result, two final extraction protocols have been proposed for the phycotoxin analysis in marine mammals, which are supposed to assure a sufficient phycotoxin recovery if present in the marine mammal samples. However, during the method development, no phycotoxins could be measured in the analysed marine mammal samples; therefore, some previously planned trials could not be executed. In the future, more experiments should be carried out using marine mammal samples containing phycotoxins to evaluate further steps of the extraction protocols.

2. State of the art

The oceans are a huge habitat for many species of plants and animals. However, oceans' health is threatened by the interference of humans. Anthropogenic activities are the cause of several emerging problems, such as overfishing, pollution and climate change. Moreover, they can favor events like the blooming of algae species to an alarming extent. For the sake of human health and species diversity, ocean health should be carefully monitored and preserved, and for that, marine mammals constitute good bioindicators to address environmental and human health concerns.

The following sections will provide information about blooms of harmful algae, the different classes of toxins they produce and the consequences for environmental and human health. Also, a brief overview of the classification system of marine mammals and the impact of toxins on them will be given. Finally, methods for measuring algal toxins will be addressed.

2.1 Harmful algal blooms

Marine phytoplankton are microscopic cells or colonies that float in the water. They are essential components of the marine ecosystem as primary oxygen and biomass producers. Phytoplankton species belong to the domain of eukaryote and eubacteria and are categorized by their size as picophytoplankton, typically 0.5-0.8 µm in diameter, nanophytoplankton approximately 0.8 µm in diameter to over 5 µm in length, and microphytoplankton with diameters between 2 and 100 µm (Geider et al., 2014). The most important group for this bachelor´s thesis is microphytoplankton or microalgae, which includes species of diatoms, dinoflagellates and cyanobacteria that can produce potent biotoxins (Hallegraeff et al., 2004).

From the taxonomic point of view, cyanobacteria cannot be considered algae because of their procaryotic nature (Shimizu, 1993). However, since other microalgae species emerged initially from cyanobacteria (Martin & Kowallik, 1999) and they share many characteristics with algae, like photosynthetic activity, authors (e.g., Shimizu, 1993) include them in the category of microalgae.

Under favorable conditions of light, nutrients, salinity and temperature (Geider et al., 2014), microalgae can proliferate exponentially and form dense aggregations with up to millions of cells per litre known as "blooms". Some blooms can cause oxygen depletion, gill damage or massive biotoxin production, negatively impacting aquacultures, fisheries, and environmental and human health. In these cases, these blooms received the name "harmful algal blooms (HAB)" (Hallegraeff, 2004).

2.2 Phycotoxins

Phycotoxins are secondary metabolites produced by several microalgae species to protect themselves against natural predators (Campbell, 1985). Since these microalgae are at the bottom of the marine trophic web, they constitute a food source for zooplankton, filter-feeding invertebrates, planktivorous fish and algal grazers, which also constitute the prey of marine organisms at higher trophic levels (Burkholder et al., 2018). That way, toxic phytoplankton can be transferred and bioaccumulated along the food web, ending up in top predators like marine mammals (Fire et al., 2011; La Riva et al., 2009), sea birds (Gayoso & Fulco, 2006), and humans (Perl et al., 1990). Moreover, the different classes of phycotoxins have structural variants, most of them still unknown, produced by the phytoplankton or the metabolism of the organisms that ingest them, that may differ from the original toxin in their toxicity (Jaime et al., 2007).

In the last decades, the incidence and intensity of HAB have increased (van Dolah, 2000; Hallegraeff, 2004) due to different factors related to anthropogenic activities like global

warming (Gobler, 2020), water eutrophication (Parsons & Dortch, 2002) reduction of predators due to intensive fishing (Geider et al., 2014), transportation of resting algal cysts in the ballast water of ships (Hamer et al., 2001), or translocations of shellfish stocks (Nagai et al., 2007).

Different classification systems have been proposed to categorize phycotoxins. One way is by clustering them according to the symptoms they cause in the human body when ingested with seafood. There are six different poisonings described due to the consumption of contaminated seafood: “paralytic shellfish poisoning”, “neurotoxic shellfish poisoning”, “ciguatera fish poisoning”, “diarrhetic shellfish poisoning”, “amnesic shellfish poisoning”, and “azaspiracid poisoning” (reviewed in Daranas et al., 2001).

“Paralytic shellfish poisoning” is caused by saxitoxin and its’ variants, a phycotoxin group produced by various dinoflagellate species of the genera *Alexandrium, Gymnodinium* and *Pyrodinium* (van Dolah et al., 2002). This toxin binds with high affinity to the voltage-gated sodium channels, blocking them. Symptoms include tingling or numbness around the perioral area, muscular weakness, floating sensation, progressive loss of ventilatory efficiency and, in extreme cases, death due to respiratory paralysis (Kao, 1993).

The “neurotoxic shellfish poisoning” is caused by brevetoxins mainly produced by the dinoflagellate *Karenia brevis*. When entering the human organism, they bind with high affinity to site five on the voltage-dependent sodium channel so that the voltage sensitivity of the channel gets altered. This binding results in an inappropriate channel opening and inhibits channel inactivation. The symptoms include nausea, tingling and numbness of the perioral area, severe muscular aches, loss of motor control and seizures (van Dolah et al., 2002).

People infected by “ciguatera fish poisoning” usually show symptoms of gastrointestinal inflammation that lead to severe dehydration and weakness, followed by cardiovascular and neurological symptoms. Features that separate this poisoning from others are severe pruritus, temperature reversal and tingling and numbness of the extremities (Ragelis, 1984). The responsible toxins are ciguatoxins, produced by dinoflagellates of the genus *Gambierdiscus*. Their chemical structure, pharmacological target and clinical signs are similar to brevetoxins; however, ciguatoxins show a greater toxic potential, resulting in long-term neurotoxic symptoms that might include tachycardia, hypertension, paralysis, or even death (Fire & van Dolah, 2012).

“Diarrhetic shellfish poisoning” received that name due to the dominant symptoms of diarrhea, nausea, vomiting and abdominal pain in affected people (Yasumoto et al., 1978). The diarrheic toxins causing these symptoms are okadaic acid and its’ analogs, especially dinophysistoxins, produced by the dinoflagellate species *Dinophysis fortii*, amongst others (Yasumoto et al., 1985). These toxins also have tumor-promoting activity (Fujiki et al., 1988). The mode of action of okadaic acid is by inhibiting serine/threonine protein phosphatases 1 and 2A (Bialojan & Takai, 1988). In the past, two other toxin groups called pectenotoxins and yessotoxins were initially associated with diarrhetic shellfish poisoning. However, despite being acutely toxic, they do not induce diarrhea, the characteristic symptom of the disease, and for that reason, they have been excluded from this group of toxins (Aune & Yndestad, 1993).

“Amnesic shellfish poisoning" is characterized by vomiting, diarrhea, confusion, memory loss, disorientation, and coma induction in extreme cases. The causative toxin is domoic acid, produced by some diatom species of the genus *Pseudo-nitzschia* (Wright et al., 1989). Domoic acid acts as an analog of glutamate and, therefore, is a potent glutamate receptor agonist (van Dolah et al., 2002).

The major causative agents of “azaspiracid poisoning” are azaspiracids (Satake et al., 1998), which are produced by dinoflagellates of the genera *Azadinium* (Tillmann et al., 2009) and *Amphidoma* (Krock et al., 2012). Symptoms of affected people are similar to the “diarrhetic shellfish poisoning” and include nausea, vomiting, severe diarrhea and stomach cramps (McMahon & Silke, 1996). The mode of action of this group of toxins is not fully understood yet. However, it has been demonstrated that they upregulate ATP-regulated anion channels leading to a regulatory volume decrease of the cell (Boente-Juncal et al., 2021).

Since this toxin classification based on human symptoms was not useful for scientific purposes, another classification system based on the chemical structure of phycotoxins was proposed by the joint Food and Agriculture Organization (FAO), the World Health Organization (WHO), and the Intergovernmental Oceanographic Commission of UNESCO (IOC) Expert Consultation on biotoxins in molluscan bivalves in 2004. Therefore, they agreed to specify eight distinct groups of toxins: the azaspiracid group, the brevetoxin group, the cyclic imines group, the domoic acid group, the okadaic acid group, the pectenotoxin group, the saxitoxin group and the yessotoxin group. This classification system is useful for later analysis since the chemical structure determines whether the toxins can be distinguished as lipophilic or hydrophilic. Fat-soluble or lipophilic toxins, which are dissolvable in organic solvents, include azaspiracids, brevetoxins, cyclic imines, okadaic acid, pectenotoxins, and yessotoxins. On the other hand, water-soluble or hydrophilic toxins include toxins that can be solved in polar solvents, like water, such as saxitoxins and domoic acid (FAO/WHO/IOC, 2004; Joint FAO/WHO, 2016).

2.3 Marine mammals and phycotoxins

Marine mammals are defined in the Marine Mammal Protection Act as mammals that are “morphologically adapted to the marine environment” or “primarily inhabit(ing) the marine environment (Marine Mammal Commission, 2007)”. They belong to three different orders: order Carnivora, which includes marine otters, polar bears and pinnipeds like seals, sea lions and walruses; order Sirenia, composed of manatees and dugongs (Berta et al., 2015); and the superorder Cetartiodactyla, which includes the infraorder Cetacea, represented by cetaceans. This infraorder is composed of two superfamilies: Mysticeti (baleen whales) and Odontoceti (toothed whales) (Richardson et al., 1995; World Register of Marine Species [WoRMS], 2022). Some marine mammal species are fully aquatic, like cetaceans and sirenians, whereas others also spend some time on land or ice, like pinnipeds, sea otters and polar bears (Berta et al., 2015).

Marine mammal species are linked to phytoplankton as the primary producer by various trophic levels, except for sirenians, which feed directly on primary producers (Berta et al., 2015). Baleen whales mainly feed on zooplankton (Würsig et al., 2017) which is one trophic level above phytoplankton (Trites, 2019), whereas pinnipeds and odontocetes feed on preys that are five or more trophic levels above primary producers. Moreover, the distribution of marine organisms at higher trophic levels is related to the geographical patterns of primary productivity. Since primary production is highest in coastal upwelling regions, moderate in coastal regions and lowest in the central gyres of ocean basins, most marine organisms, including marine mammals, are found in coastal areas and zones of upwelling (Berta et al., 2015).

Different sources show that a connection between marine mammal mortality events and the bloom of harmful microalgae exists. According to a review from Broadwater et al. (2018), 31 mass mortality events of marine mammals with the involvement of HAB toxins happened globally between 1946 and 2015. Marine mammals can get in touch with phycotoxins primarily by ingestion, but inhalation of aerosolized toxins is also possible (Broadwater et al., 2018). Copepods have been shown to ingest toxic microalgae and accumulate toxins in their gut system

and perhaps other tissues (Turriff et al., 1995). In 2017, D'Agostino et al. investigated the transfer of domoic acid through the trophic web. They proved that copepods, a zooplanktonic prey species of the southern right whale, act as vectors for the transfer of domoic acid to the whales (D'Agostino et al., 2017). In 1998, over 400 California sea lions died during the same time a toxic diatom bloom occurred. Phycotoxins produced by this algae were detected in planktivorous fish and sea lion body fluids (Scholin et al., 2000).

Another example is the stranding of over 2200 marine mammals, mainly sea lions and dolphins, in 2002 in Southern California. This event happened in temporal and geographical correlation to seasonal blooms of the algal species *Pseudo-nitzschia* spp., known as a producer of the neurotoxin domoic acid. Also, some of the animals that were alive when stranding showed severe neurological symptoms (La Riva et al., 2009). In 2008, a massive stranding of over 100 bottlenose dolphins in Texas coastal waters was temporarily and spatially coincident with the HAB of different toxin-producing species. Phycotoxin analysis performed on the gastrointestinal contents collected at necropsies revealed the presence of domoic acid, brevetoxins and okadaic acid, thus, indicating a correlation between the HAB and the mortality event (Fire et al., 2011).

The purpose of investigating the presence and impact of phycotoxins on marine mammals is because they are at the top of the marine food web and, therefore, can serve as sentinels for the health of the marine environment. Also, many species have long life spans and are long-term coastal residents. Moreover, they consume similar trophic resources to humans, and thus, the negative effects of HAB and phycotoxins on public health can be assessed (Bossart, 2011). Monitoring only seafood like mussels might be misleading since they contain only trace amounts of phycotoxins, whereas higher amounts can be found in higher trophic levels due to accumulation (Scholin et al., 2000).

2.4 Methods of phycotoxin determination

In the European Union, there are different reference methods for determining phycotoxins. The detection methods can be separated into biological methods such as the mouse bioassay and the enzyme-linked immunosorbent assay (ELISA) and analytical detection methods such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography (HPLC) coupled to a fluorescence detector (FLD) (Bundesinstitut für Risikobewertung, 2022). Biological methods are limited to detecting the presence of a specific group of toxins, being unable to identify or quantify it (Vilariño et al., 2014). On the contrary, highly precise and sensitive analytical methods like HPLC-FLD and LC-MS/MS allow multiple toxins to be identified and quantified in a single run (Quilliam, 2004). HPLC-FLD and LC-MS/MS are the methods routinely used for phycotoxin analysis at the Alfred Wegener Institute, where this work was done, and thus, will be explained in more detail.

Both methods use HPLC to separate the different toxins first. For that, a small amount of the sample gets injected into a liquid stream, or mobile phase, and carried at high pressure through a column or stationary phase. The mobile phase usually contains a mixture of different solvents suitable for the analyte. Also, the column can vary in packing material, length and pore size. Separation of the mixture's components is achieved due to their physicochemical interactions with the stationary phase, which causes analytes to elute at different times, so-called retention times (Hanai, 1999). Ultra-high performance liquid chromatography (UHPLC) is an advanced method of HPLC, using the same principle but with shorter columns packed with smaller particles and using higher pressure, resulting in an improved resolution, sensitivity and efficiency (Taleuzzaman et al., 2015).

In this work, three liquid chromatography modes are used: reversed-phase chromatography, ion-pair reversed-phase chromatography and hydrophilic interaction chromatography. Reversed-phase chromatography is used to separate non-polar compounds working with an aqueous organic mobile phase and an organic stationary phase, usually silica with bonded fatty acid chains. Non-polar compounds will be stronger retained in the stationary phase than polar ones. By decreasing the polarity of the mobile phase, compounds elute. The same procedure can be used to separate polar compounds in a non-polar phase by adding ion-pair reagents. Ion-pair reagents form uncharged adducts with charged ions that can be separated in the non-polar stationary phase (Worsfold et al., 2005). Hydrophilic interaction liquid chromatography works with a modified polar stationary phase and an aqueous organic mobile phase. Polar compounds partition between the organic mobile phase and polar stationary phase and get retained. Compounds get eluted by increasing the aqueous proportion of the mobile phase (Buszewski & Noga, 2012).

In the next step, the separated analytes are detected through a mass spectrometer or fluorescence detector. In HPLC-FLD, the toxins must be derivatized to become fluorescent and detectable (Hanai, 1999). For that, after separating the analytes in the HPLC system, the sample enters a post-column derivatization system that oxidizes the toxins and converts them into fluorescent products before reaching the detector (Sullivan & Wekell, 1984).

For the mass spectrometric analysis, the separated compounds need to be ionized. Therefore, the sample enters the ionization chamber. In mass spectrometry, several ionization techniques are available, but in tandem, the low energy electrospray ionization (ESI) is mainly used to obtain unfragmented primary ions, where the column eluate passes through a charged and heated metal tube. At the end of the tube, charged droplets, rapidly decreasing in size, are sprayed out; and ions are formed while the solvent gets vaporized. Through electrical lenses, the charged beam of ions gets focussed and carried in the vacuum system of the MS. Next, the stream enters an analyser that scans for ions with a specific mass to charge value (*m/z*). Only the selected *m/z* can reach the detector and trigger a signal. The analyser used in this work is the quadrupole, which uses four opposite charged rods with radio frequencies to separate and focus the ions (McMaster, 2005).

For the tandem mass spectrometry, a triple quadrupole is applied to induce fragmentation of the original precursor ions. Therefore, the first quadrupole is used for scanning and separating the precursor ions, and the second is used as a collision cell fragmenting the ions with a heavy gas molecule. The third quadrupole again scans and separates the produced fragments. Finally, results are presented in a three-dimensional array of signal strength versus time versus *m/z* information. The MS can be used to search for known toxins in a sample, for quantification and structural analysis of compounds (McMaster, 2005).

3. Aim of the work

Phycotoxins can be a serious threat to human and animal welfare. Therefore, it is crucial to understand their structure and analogs, their impact on sea dwellers, and their transformation and accumulation up the marine trophic chain. Among marine organisms, marine mammals are sentinel species due to their long life spans, large-habitat occupancy, and their high position in the marine trophic chain. Consequently, they can serve as indicators of threats to public health and the environment and thus, can be used to study the effects of HAB and phycotoxins on human and ocean health.

This bachelor thesis has been part of a postdoctoral project aiming to identify and quantify the phycotoxins that marine mammals are exposed to in the Argentinian Sea. Several HAB events have been reported in this region, including toxic dinoflagellates and diatom species (Esteves et al., 1992; Ferrario et al., 1999; Krock et al., 2015). However, the impact of phycotoxins on marine mammals has not been thoroughly investigated. Not many studies concerned with this topic are currently available, and there is still no European standardized protocol for investigating phycotoxins in marine mammals using HPLC-FLD and LC-MS/MS. Most previous studies published on phycotoxins in this group of animals have used immunochemical (ELISA) or biological methods (mouse bioassay), only capable of detecting classes of toxins but not identifying or quantifying them. Since working with marine mammals' tissues and fluids is challenging and often constrains sample availability, developing improved protocols that allow the extraction of toxins, minimizing the amount of sample used and reducing matrix effects of these complex samples is essential. Furthermore, the group at AWI where this project was conducted has considerable experience in determining phycotoxins and unknown variants in phyto- and zooplankton but lacks experience working with marine mammal samples.

Considering the aspects mentioned above, this bachelor's thesis focused on developing a suitable method adapted to the facilities and equipment available for extracting phycotoxins from various marine mammals' tissue and body fluid samples. Several trials were conducted considering different centrifugation speeds, extraction solvents, disruption methods and clean-up steps. Finally, two protocols to effectively extract phycotoxins in marine mammals' tissues and body fluids were proposed.

4. Material

This bachelor's thesis aims to measure phycotoxins in marine mammals' tissues and body fluids and, therefore, to develop an effective extraction method. This section will detail the material, including standards and samples, chemicals, equipment, and software used to measure marine mammal samples and conduct various trials.

4.1 Standards and samples from previous projects

For conducting phycotoxin measurements, standards from saxitoxin and its' analogs, also called "Paralytic Shellfish Poisoning" toxins (PSP toxins), and lipophilic toxin standards were purchased from the Certified Reference Material Programme of the Institute of Marine Biosciences, National Research Council, Canada (Table 1). Furthermore, extracts from phytoplankton from past project samples, already analysed and known to contain toxins, were also used for this bachelor's thesis.

Table 1: List of all phycotoxins standards used for this bachelor's thesis. The toxins are divided into two groups according to their chemical structure: lipophilic and hydrophilic.

Project number/ standard	**Lipophilic/hydrophilic**
Domoic acid (DA)	Lipophilic
Okadaic acid (OA)	
Pectenotoxins (PTX)	
Gymnodimine (GYM)	
Spirolides (SPX)	
Azaspiracids (AZA)	
PSP toxins	Hydrophilic

4.2 Area of marine mammal sample collection

The marine mammal samples analysed in this project were collected in 2018 along the Argentinian coast. The study area comprises the north and centre of Patagonia from the north of the San Matías Gulf to the south of the San Jorge Gulf (Figure 1).

Figure 1: Map of South America. Highlighted are San Matías Gulf (Golfo San Matías) and San Jorge Gulf (Golfo San Jorge) (Google Maps, 2022). Samples from stranded marine mammals were collected in 2018 from the area within both Gulfs.

4.3 Marine mammal samples

Marine mammals´ samples were collected from stranded animals, lyophilized (Operon FDU-8606, Korea) to remove the water, and sent to the AWI, where they were kept at -20 °C until extractions were performed. Part of the samples received was used in the present work, including tissue and body fluid samples from three species of marine mammals: two cetaceans (one odontocete and one mysticete) and one pinniped (Table 2).

Table 2: Marine mammals' tissue and body fluid samples that were analysed as part of this work. The table provides information on the species, animal code and type of tissue or body fluid examined.

Species	Animal code	Tissue/ Body fluid
Delphinus delphis (common dolphin)	Dd 100	Stomach content
Delphinus delphis (common dolphin)	Dd 89-I	Stomach content
Delphinus delphis (common dolphin)	Dd 97-I	Stomach content
Delphinus delphis (common dolphin)	Dd 109	Feces
Eubalaena australis (Southern right whale)	BFA 27	Kidney
		Feces
Eubalaena australis (Southern right whale)	BFA 28	Liver
		Feces
Eubalaena australis (Southern right whale)	BFA 24	Feces
Eubalaena australis (Southern right whale)	BFA 25	Feces
Otaria flavescens (South American sea lion)	OF 1421	Urine
		Stomach content

4.4 Chemicals and supplies

Several reactives and consumable lab materials were used in this work. Table 3 provides information about each specific reactive or material and manufacturer details.

Table 3: List of chemicals and supplies used for this work. The list contains information about each specific substance or material and the manufacturer.

Chemicals/ Supplies	Manufacturer
Deionized water system: Milli-Q IQ 7000	Merck Millipore
Hydrochloric acid	Merck Millipore
Methanol	AppliChem
Acetic acid	Merck Millipore
Ammonia	Merck Millipore
Acetonitrile HPLC Gradient	Carl Roth GmbH
Acetonitrile UHPLC Gradient	Biosolve B.V.
Formic acid	Chemsolute
Octanesulfonic acid	Sigma Aldrich
Heptanesulfonic acid	Sigma Aldrich
Ammonium phosphate	Sigma Aldrich
Ammonium formate	Fluka™ Analytical
Tetrahydrofuran	Carl Roth GmbH
Phosphoric acid	Fluka™ Analytical
Pipette tips	Starlab International GmbH
Starguard sensitive powder-free nitrile gloves	Starlab International GmbH
5 ml Eppendorf Tubes	Eppendorf AG
2 ml HPLC vials	Agilent Technologies
Aluminum caps	Fisher Scientific
PTFE caps	Agilent Technologies
Spin filter: Ultrafree® - MC Filter Unit Inserts 0.45 µm Durapore® HV; Ultrafree® Tubes	Merck Millipore
pH indicator paper pH 0-14	Whatman™
Superclean LC-18 1 ml tubes	Supelco®
Superclean ENVI-Carb 3 ml tubes	Supelco®
Superclean LC-Si 3 ml tubes	Supelco®
Lysing matrix D	Thermo Savant

4.5 Equipment

For the determination of phycotoxins in marine mammals' tissues and body fluids, and other samples, a collection of devices was used (Table 4).

Table 4: List of the equipment used to measure phycotoxins in marine mammals' samples and information about each manufacturer.

Instrument	Manufacturer
Centrifuge 5424 R	Eppendorf AG
Centrifuge 5810 R	Eppendorf AG
LC-MS/MS (lipophilic phycotoxins): - HPLC System 1100 Series - API 4000 QTrap triple quadrupole mass spectrometer	Agilent Technologies Sciex
LC-MS/MS (hydrophilic phycotoxins): XEVO TQ-XS tandem quadrupole atmospheric pressure mass spectrometer coupled to an ACQUITY UHPLC system	Waters Cooperation
HPLC-FLD: - HPLC System 1100 Series - Post-column derivation system: Pinnacle PCX	Agilent Technologies Pickering Laboratories
Eppendorf Pipettes	Eppendorf AG
Ultrasonic bath: Sonorex Digitec DT	Bandelin electronic GmbH & Co. KG
Vortexer: Vortex-genie 2®	Scientific Industries
Visiprep 24^{TM} DL	Supelco®
Bio101 FastPrep instrument	Thermo Savant

4.6 Software

For further analysis with the extracted marine mammals' tissues and body fluids samples and other samples measured, specific equipment software and general used software programmes were used to acquire and process data (Table 5).

Table 5: Software programmes used for data analysis in this work. The list contains specific information about the names and the providers of the software programmes.

Software	Provider
Analyst 1.4.2	Sciex
MassLynx	Waters Corporation
OpenLab CDS Chem Station C01.10	Agilent Technologies
Excel	Microsoft Corporation

5. Methods

Different steps were carried out to extract and detect phycotoxins from marine mammals' tissues and body fluids. This section provides information about the methodological aspects of this work. Included are the initial protocol draft for extracting marine mammals' samples as well as solid-phase extraction (SPE) protocols and phycotoxin detection procedures with the LC-MS/MS and the HPLC-FLD. As the method development will be part of this work, more details, especially concerning the extraction protocol, can be found in chapter 6.

5.1 Initial protocol draft

As there is no standard operation protocol for extracting phycotoxins from marine mammals' tissues and body fluids, the development of an extraction protocol will be one objective of the bachelor's thesis. From studying research papers concerned with the extraction of phycotoxins from marine sea dwellers' tissue, the following protocol draft was proposed, adapted from Blay et al. (2011); Luckas et al. (2015); Turner et al. (2014):

1. Weigh tissue into an Eppendorf tube.
2. Extract with suitable extraction solvents for lipophilic and hydrophilic toxins, respectively. Adjust the volume to the amount of tissue.
3. Mix the samples with a vortexer.
4. Disrupt the cells of the tissue matrix by putting the sample tubes in an ultrasonic bath for 5 min to release phycotoxins into the solvent.
5. Centrifuge the samples and transfer the supernatant into a new Eppendorf tube.
6. Spin-filter the samples before transferring them into an HPLC vial for later measurements.
7. Store the samples in the freezer at -20 °C.

5.2 Method development

Considering the challenges encountered during the extraction process, different trials concerning the centrifugation speed, the extraction solvent, the cell disruption method, and the matrix clean-up were conducted. In this section, the methods used to perform the different trials were summarized.

5.2.1 Sample weight and solvent volumes

Due to a limited amount of lyophilized samples, the use of high quantities like 2 g as described when working with shellfish tissue (e.g., Quilliam, 2004) or between 1 and 4 g as conducted when working with marine mammals tissue (e.g., Lefebvre et al., 2016) was not possible. However, it should be highlighted that most phycotoxin extraction protocols are developed for fresh samples from sea dwellers. Since mammalian cells contain approximately 75 % water (Cole & Eastoe, 1988), the 0.5 g of lyophilized sample used in the present work will be equivalent to 2 g of fresh tissue.

The volumes of solvent added were adjusted to the weight of the tissue sample. Most authors use a volume of 1:4 ratios of sample to extraction solvent (e.g., Lefebvre et al., 1999) or 1:5 (e.g., Fire et al., 2010; Quilliam, 2004). Therefore, 2 ml of solvent was considered adequate for extracting.

5.2.2 Extraction solvents

Due to their structure, PSP toxins are hydrophilic (FAO/WHO/IOC, 2004). Thus, extractions have been performed using hydrochloric acid (e.g., Luckas et al., 2015; Turner et al., 2014) or acetic acid in aqueous solutions (Turner et al., 2018) and thus, under acidic conditions. Following Luckas et al. (2015), it was decided to use 0.2 M hydrochloric acid for extracting PSP toxins.

The lipophilic nature of azaspiracids, brevetoxins, cyclic imines, okadaic acid, pectenotoxins, and yessotoxins, according to their structure (FAO/WHO/IOC, 2004), requires an organic solvent for their extraction. Compared with literature, methanol was widely used (e.g., Blay et al., 2011; Luckas et al., 2015; Quilliam, 2004). Some authors used an 80 % aqueous methanol solution (e.g., Quilliam, 2004), others a 90 % aqueous methanol solution (e.g., Luckas et al., 2015) or 100 % methanol for extractions (e.g., D'Agostino et al., 2017; EURLMB, 2015). As the availability of the samples was limited, domoic acid was also included in the extraction procedure for lipophilic phycotoxins, as many authors extract it with 50 % aqueous methanol (e.g., Fire et al., 2010; Luckas et al., 2015). For that, an extraction solvent of 80 % aqueous methanol was used to recover lipophilic phycotoxins and domoic acid together. As DA has been included in lipophilic extractions, it will be referred to as lipophilic in the following sections.

As described in the chromatographic results, the separation of signals for urine, fecal, and stomach content samples from Dd 109, BFA 28, OF 1421, Dd 89, Dd 97, and BFA 27 extracted with 0.2 M hydrochloric acid could not be achieved. Low pH values were thought to be the cause. Thus, pH paper test stripes were used to measure the pH of these samples. Later, 0.25 % ammonia was added to the samples to increase the pH and avoid extreme acidic values. Also, all previously extracted samples with hydrochloric acid were reextracted with 1 % aqueous acetic acid, a weaker acid having higher pH values (between 2 and 3) than hydrochloric acid, for comparing chromatographic results.

5.2.3 Extraction steps

According to Luckas et al. (2015), all sample tissues and fluids were extracted three times, assuring good recovery of most of the phycotoxins that may be present in the sample. After using 2 ml extraction solvent for the initial extraction, 1 ml was used for the second and third re-extraction. Afterward, the three supernatants obtained per sample were combined.

Marine mammal samples known to contain phycotoxins would be needed to determine if three extractions are appropriate, too many or not enough. Thus, the concentration of phycotoxins must be measured separately after each extraction step to calculate the number of necessary steps for high extraction efficiency. Unfortunately, no trials or results could be presented for this section due to the absence of phycotoxins in the marine mammals' tissue samples analysed for this work.

5.2.4 Sample clean-up

Some samples of body fluids, especially feces of marine mammals, were problematic due to highly turbid extracts following the original protocol draft (see Section 5.1) for PSP toxin extraction, causing repeated blockage of the spin-filter and, in worse cases blocking the HPLC column. For that reason, four modifications to the initial protocol were proposed to obtain cleaner samples.

A) Centrifugation speed

Initially, based on standardized protocols (Luckas et al., 2015), samples were centrifuged at 2500 x g for 5 minutes in the first two extraction steps and at the maximum centrifugation speed for the centrifuge used, which was 4000 x g, for 10 minutes in the third and last extraction. Centrifugation at a higher speed was proposed to increase the separation between tissue matrix and extract. Baleen whale fecal samples BFA 24, BFA 25, and BFA 28, which did not show clear separation of the phases and presented a turbid supernatant after centrifuging, were chosen for that. These samples were centrifuged at 10000 x g following the work performed by Lefebvre et al. (2016) with marine mammal fecal samples for the same time slots as performed before in each extraction step.

B) Saturated sodium chloride solution

Another approach for obtaining a better separation and clear supernatants was adding saturated sodium chloride solution to the sample and centrifuge afterward, as performed by Sayfritz et al. (2008). As a first try, 0.5 ml of saturated sodium chloride solution was added to one whale fecal sample (BFA 28) extracted with hydrochloric acid and centrifuged.

C) Cell disruption method

Following the protocol of Luckas et al. (2015), ultrasonication for 5 min was used for each extraction step to disrupt the cells. As an alternative cell disruption method, homogenizing with lysing matrix was suggested as conducted by other authors (e.g., D'Agostino et al., 2017). A trial between ultrasonication and homogenizing was carried out to observe visual differences. Therefore, four aliquots, 0.25 g sample each, of a whale fecal sample (BFA 28) were extracted three times in FastPrep tubes using either 1 ml lipophilic or hydrophilic solvent for the first extraction and 0.5 ml solvent for the next two re-extractions. For the cell disruption comparison, two samples, one extracted with hydrophilic and one with lipophilic solvents, were put in an ultrasonic bath for 5 min for each extraction step. The other two were homogenized with added lysing matrix by reciprocal shaking for 45 s in the Bio101 FastPrep instrument at a speed of 6.5 m/s for each extraction step.

D) Solid-phase extraction

As conducted by many authors, solid-phase extraction seemed to be a useful tool for cleaning up samples with a complex matrix (e.g., Nielsen et al., 2016; Quilliam et al., 1995). Therefore, as a first try, a C18 SPE cartridge was used after extracting three marine mammal fecal and stomach content samples (BFA 27, Dd 89, Dd 97) with hydrochloric acid for PSP toxin analysisand combining the supernatants.

5.2.5 SPE trials

As the initial trial with C18 SPE gave good results concerning sample clean-up, further tests with different cartridges for PSPs and lipophilic toxins were considered. Dependent on the cartridge sorbent and the chemical structure of the measured toxins, some were used for retaining and concentrating toxins and others for retaining matrix compounds. A mixture of known toxin groups was used in the trials to see differences in toxin concentrations before and after the SPE.

5.2.5.1 Lipophilic toxins

Based on the literature, a SPE with a silica cartridge (Wang et al., 2015) to retain hydrophilic matrix compounds or a SPE with a C18 cartridge (Quilliam, 2004) to retain lipophilic toxins were considered for lipophilic toxins. Trials were carried out with old extracts from phytoplankton samples containing a mixture of toxin groups: DA, cyclic imines such as SPX and GYM (FAO/WHO/IOC, 2004), AZA, PTX and OA. The old extracts were pooled together, evaporated and resuspended in 2 ml 80 % aqueous methanol, creating a new sample further referred to as the initial sample. 900 µL of the initial sample were used for each cartridge, leaving 200 µL as an aliquot of the initial sample.

Protocol for the Superclean LC-18 (1 ml) (adapted from Quilliam, 2004):

1. Condition the cartridge with one cartridge load of 100 % methanol followed by one cartridge load of deionized water. Elute both to the level of top frit. Discard to waste.
2. Add the sample extract to the cartridge and elute to the top frit. Discard to waste.
3. Wash the cartridge with one load of 20 % aqueous methanol and elute to dryness. Discard to waste.

4. Elute the cartridge with 2 ml of 80 % aqueous methanol and elute to dryness. Collect the extract in a clean tube.

Protocol for the Superclean LC-Si (3 ml) (adapted from the manufacturer's protocol):

1. Condition the cartridge with one cartridge load of 100 % methanol. Elute to the level of top frit and discard to waste.
2. Add the sample extract to the cartridge and elute to the top frit. Collect in a clean tube.
3. Wash the cartridge with 2 ml of 80 % aqueous methanol and elute to dryness. Collect in the same tube as the sample extract.

5.2.5.2 PSP toxins

For PSP toxins, the authors used a SPE with a carbon cartridge (Turner et al., 2018) to retain PSP toxins and a SPE with a C18 cartridge (Turner et al., 2014) to retain lipophilic matrix compounds. Trials with both cartridges were carried out by using old extracts from phytoplankton samples known to contain PSP toxins and a PSP toxin standard mix. After pooling all together and evaporating, toxins were dissolved in 2 ml 1 % aqueous acetic acid. After creating this new sample, further referred to as the initial sample, 900 µL were used for each cartridge, leaving an aliquot of 200 µL of the initial sample.

Protocol for the Superclean LC-18 (1 ml) (adapted from the manufacturer's protocol):

1. Condition the cartridge with one cartridge load of 100 % methanol followed by one cartridge load of deionized water. Elute both to the level of top frit and discard to waste.
2. Add the sample extract to the cartridge and elute to the top frit. Collect in a clean tube.
3. Wash the cartridge with 2 ml of 1 % aqueous acetic acid and elute to dryness. Collect in the same tube as the sample extract.

Protocol for Superclean ENVI-carb (3 ml) (adapted from Turner et al., 2018):

1. Condition the cartridge with one cartridge load of 20 % aqueous acetonitrile with 1 % acetic acid followed by one cartridge load of a 0.025 % ammonia solution. Elute both to the level of top frit. Discard to waste.
2. Add the sample extract to the cartridge and elute to the top frit. Discard to waste.
3. Wash the cartridge with 1.75 ml of deionized water and elute to dryness. Discard to waste.
4. Elute the cartridge with 2 ml of 20 % aqueous acetonitrile with 1 % acetic acid and elute to dryness. Collect the extract in a clean tube.

5.2.5.3 Post-analysis of SPE cartridges

Once toxin concentrations of the samples after the SPE trials were measured and compared to their concentrations in the initial sample, toxin recoveries of each toxin group after the SPE clean-up were obtained. To improve the procedure for each SPE cartridge, different solutions were tried for eluting retained toxins.

First, the waste of the C18 cartridge used to clean up lipophilic toxins collected after adding the sample to the cartridge and washing it with 20 % aqueous methanol was analysed. After that, the same cartridge was washed with 2 ml of 100 % methanol, 50% aqueous methanol and 50 % aqueous methanol with 0.3 M acetic acid. Finally, each solution was eluted to dryness and collected in separate HPLC vials. The same procedure was conducted for the silica cartridge

used to clean up lipophilic toxins, except that no waste containing potential toxins was generated in this case.

The collected waste, obtained after adding the sample and washing it with deionized water, was analysed to evaluate the carbon cartridge efficiency in retaining PSP toxins. After reviewing the results, no further eluents were applied. For further improvements with the C18 cartridge, the same cartridge was washed twice with 2 ml 1 % aqueous acetic acid each and collected separately in HPLC vials. Also, 2 ml of deionized water was applied to the cartridge and collected in an HPLC vial.

5.3 LC-MS/MS

LC-MS/MS was used to measure all different classes of phycotoxins. Lipophilic toxins were measured with the Sciex API 4000 QTrap triple quadrupole mass spectrometer coupled with an Agilent 1100 Series HPLC System. Table 6 lists all the HPLC parameters when measuring lipophilic phycotoxin samples with LC-MS/MS.

Table 6: HPLC parameters for lipophilic phycotoxin analysis with LC-MS/MS.

Column	HyperClone 3 µm BDS C8 130 A 50 mm x 2mm	
Temperature	20 °C	
Flow	0.2 ml/min	
Injection volume	5 µL	
Run time	19 min gradient	
Eluent A	950 ml water 50 ml buffer (50 mM formic acid, 2 mM ammonium formate)	
Eluent B	950 ml acetonitrile 50 ml buffer (50 mM formic acid, 2 mM ammonium formate)	
LC-gradient		
Time/min	**A [%]**	**B [%]**
10.00	0	100
16.00	0	100
19.00	95	5
Stop		

MS parameters for measuring lipophilic phycotoxin samples with LC-MS/MS are detailed in Table 7.

Table 7: MS parameters for lipophilic phycotoxin analysis with LC-MS/MS.

Capillary	5.5 kV
Curtain gas	20 psi
Source temperature	275 °C
Declustering Potential	50 V
Entrance Potential	10 V
Collision Cell Exit Potential	15 V
Collision gas flow	medium
Collision	20 eV – 55 eV

PSP toxins were measured with the ACQUITY UHPLC system coupled to the Waters XEVO TQ-XS tandem quadrupole atmospheric pressure mass spectrometer. Table 8 includes all UHPLC parameters for detecting PSP toxins with LC-MS/MS.

Table 8: UHPLC parameters for PSP toxin measurements with LC-MS/MS.

Column	Acquity UHPLC Glycan BEH Amide 130A 1,7µm 2,1x150mm	
Temperature	60 °C	
Flow	0.4 – 0.8 ml/min	
Injection volume	1 µL	
Run time	11 min gradient	
Eluent A	500 ml water 300 µL ammonium hydroxide 75 µL formic acid	
Eluent B	700 ml acetonitrile 300 ml water 100 µL formic acid	
LC-gradient		
Time/min	**A [%]**	**B [%]**
5.00	2	98
7.50	2	98
9.00	50	50
9.50	50	50
10.00	2	98
10.60	2	98
10.61	2	98
11.00	2	98
Stop		

Table 9 lists all MS parameters used when measuring PSP toxins with LC-MS/MS.

Table 9: MS parameters used for PSP toxin measurements with LC-MS/MS.

Capillary	0.5 kV
Cone	10 V
Source temperature	150 °C
Desolvation temperature	600 °C
Desolvation flow rate	1000 l/h
Cone	150 l/h
Nebuliser	7.0 bar
Collision gas flow	0.15 ml/min
Collision	20 eV

The LC-MS/MS is able to detect phycotoxins. The selective reaction monitoring (SRM) was used in both LC-MS/MS systems for toxin identification. SRM is a targeted mass spectrometry technique that scans only for specific molecule fragments obtained from the original molecule, also called transitions (Hoffmann, 2013).

The data acquisition was carried out with specific software: the MassLynx software for Waters XEVO TQ-XS MS and the Analyst software for the SCIEX API 4000 QTrap triple quadrupole MS.

First, the software was used to determine the peak areas of the standards. Then, based on known transitions for the standards and the retention times at which they appeared, peak identification, and thus absolute areas, were obtained for the samples analysed. Afterward, based on the standard peak area and its known concentration, concentrations of each class of toxins were determined in the samples analysed using Excel software.

Also, the detection limit (LoD) for each toxin class was calculated based on standard peak heights and baseline noise values to define the signal-to-noise ratio. In the next step, the known concentration of the standard was multiplied by three and divided by the signal-to-noise ratio to obtain an adequate LoD. A peak was only accepted if it was at least three times higher than the baseline noise. All calculations were conducted using Excel.

5.4 HPLC-FLD

To measure PSP toxins, the HPLC-FLD Agilent LC1100-FLD G1321A was also used. The parameters used in HPLC were given as listed in Table 10.

Table 10: Parameters used for the HPLC when measuring PSP toxins with the HPLC-FLD.

Column	Phenomenex Luna C18, 5µ, 250 x 4.6 mm	
Temperature	20 °C	
Flow	1 ml/min	
Injection volume	20 µL	
Run time	45 min gradient	
Eluent A	6 mM octanesulfonic acid 6 mM heptanesulfonic acid 40 mM ammonium phosphate 0,75 % tetrahydrofurane	
Eluent B	13 mM octanesulfonic acid 50 mM phosphoric acid + ammonia 15 % acetonitrile 1,5 % tetrahydrofuran	
LC-gradient		
Time/min	**A [%]**	**B [%]**
0.00	100	0
15.00	100	0
16.00	0	100
35.00	0	100
36.00	100	0
45.00	100	0
Stop		

Table 11 provides all necessary parameters for fluorescence with the HPLC-FLD.

Table 11: Parameters used for the fluorescence detection of PSP toxins measured with the HPLC-FLD.

Derivatization	Every 0.4 ml/min
Oxidants	10 mM periodic acid 550 mM ammonia 0.75 M nitric acid
Excitation wave length	333 nm
Emission wave length	395 nm

The HPLC-FLD can detect signals of fluorescent compounds derived from PSP toxins. To identify toxins, PSP toxin standards were measured, and retention times were compared to standards. Also, for quantification, six PSP standard mixes containing PSP toxins from lower to higher concentrations were measured in each run. Next, a software program named OpenLab CDS Chem Station C01.10 was used for further calculations. This software determines the absolute areas from toxins in the standard and the samples. Sample concentrations were calculated using the equations obtained from standard calibration curves.

The calculations of the LoDs for each PSP toxin were conducted as described in Section 5.3.

6. Results

The main objective of this bachelor's thesis was to develop an effective extraction protocol for the determination of phycotoxins in marine mammal tissues and body fluids. As part of the method development, different trials were conducted concerning the centrifugation speed, the extraction solvent, the cell disruption method, and the matrix clean-up. This chapter presents all results obtained from the trials for attaining the final extraction protocol.

6.1 Sample weight and solvent volumes

The solvent volume considered (2 ml) for the first extraction thoroughly wet and covered the 0.5 g of lyophilized samples from marine mammals' tissues and fluids, as shown in Figure 2. Even a layer of solvent was observed swimming over the sample, guaranteeing enough solvent volume. In the re-extractions, the pellet obtained after centrifuging in the previous extraction was well mixed, with a spoon, within 1 ml of the same solvent, and the result showed a wet and well-mixed matrix with a thin layer of solvent over it.

Figure 2: Marine mammals' tissue samples with extraction solvent in 5 ml Eppendorf tubes. The image shows the first extraction step, where 2ml of solvent was used to cover 0.5 g of the tissue samples from Dd 100, BFA 27 and BFA 28.

6.2 Extraction solvents

For measuring PSP toxins in a number of marine mammal samples extracted with 0.2 M aqueous hydrochloric acid solution with LC-MS/MS, signals appeared within the first minutes, specifically between minutes 2 and 5, as shown in in the total ion chromatogram (TIC) of one urine sample of a sea lion (OF 1421) represented in Figure 3A. The samples' signals shifted to the beginning of the chromatograms compared to the TIC of the PSP toxin standard mix, diluted in 1 % aqueous acetic acid and measured in the same run as the samples (Figure 3B), prevented the identification of any toxin, if present.

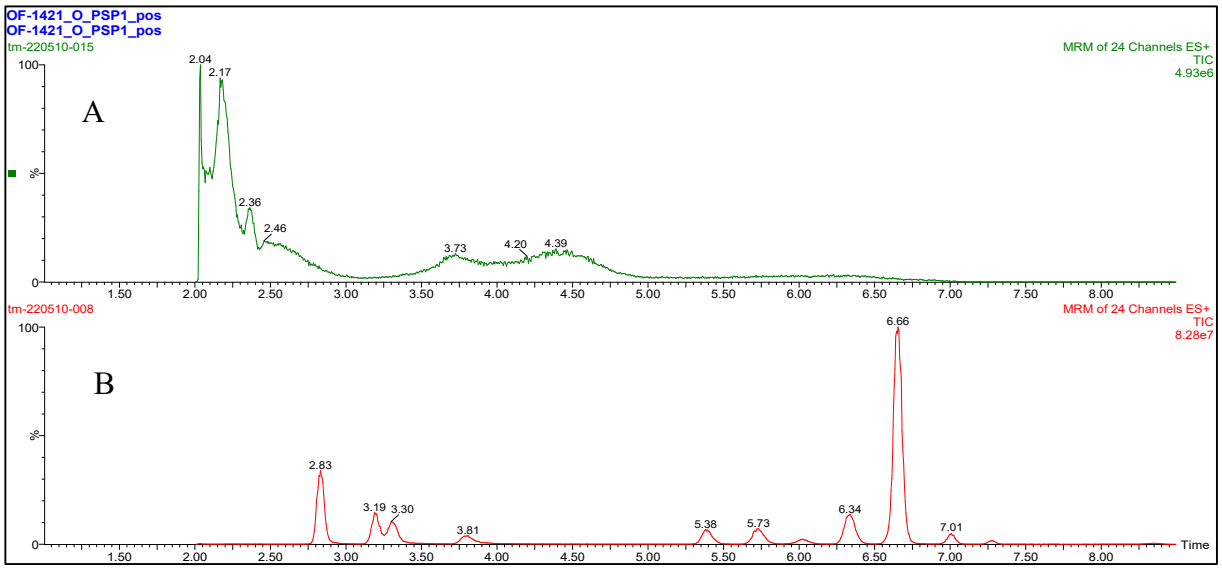

Figure 3: TICs of the urine sample from a sea lion (OF 1421) extracted with O.2 M aqueous hydrochloric acid solution (A) and from PSP toxin standards in 1% acetic acid (B) measured with LC-MS/MS. Sample peaks appear to shift to the front of the chromatogram compared to the standard peaks.

Adding ammonia to the samples extracted with 0.2 M aqueous hydrochloric acid to achieve better signal separation increased the pH of all samples so that pH values in a range between 4 and 9 were achieved (Figure 4B). Figure 4 shows one urine sample of a sea lion (OF 1421) as an example of the process of basifying the extracts obtained from marine mammals' body fluids samples extracted with 0.2 aqueous hydrochloric acid. The pH value before was 0 (A), and after adding 130 µL 0.25 % ammonia 4 (B). Those values were obtained by comparing the colour pattern of the used pH paper test stripes with the pH value colour scale (C).

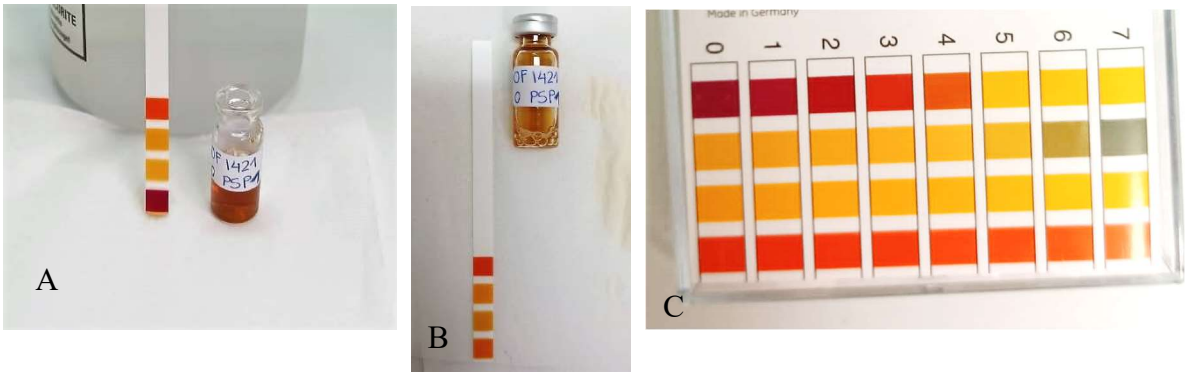

Figure 4: The pH measurement before and after adding ammonium to the urine sample of a sea lion (OF 1421) extracted with hydrochloric acid. A) pH of the sample before adding ammonia, B) pH of the sample after adding 130 µL of ammonia, and C) scale showing the p pH values belonging to different color patterns. Using the scale the pH-value of the sample is 0 before adding ammonia, and 4 afterwards.

The different body fluids needed different amounts of ammonia to increase the pH value (

Table *12*).

Table 12: Volumes of ammonia added for each type of body fluid. Three body fluids (urine, stomach content and feces) were extracted with hydrochloric acid and used for measuring PSP toxins. Different volumes of ammonia were added to basify the samples. Especially the stomach content samples needed more ammonia to show an increase in pH than the other body fluids.

Body fluids	**Volume of ammonia [µL]**
Urine	130
Stomach content	145
Feces	130

Once pH values were increased, samples were remeasured with LC-MS/MS. The shift of peaks to the front of the TIC observed in the samples extracted with 0.2 M hydrochloric acid was no longer observed after the increase of the pH by adding ammonia. Figure 5 illustrates this in the urine sample from a sea lion (OF 1421) extracted with hydrochloric acid. Compared with the TIC of the PSP toxin standard (Figure 5C), the detected signals after adding ammonia were no longer shifted (Figure 5A) compared to the TIC of the same sample without ammonia (Figure 5B).

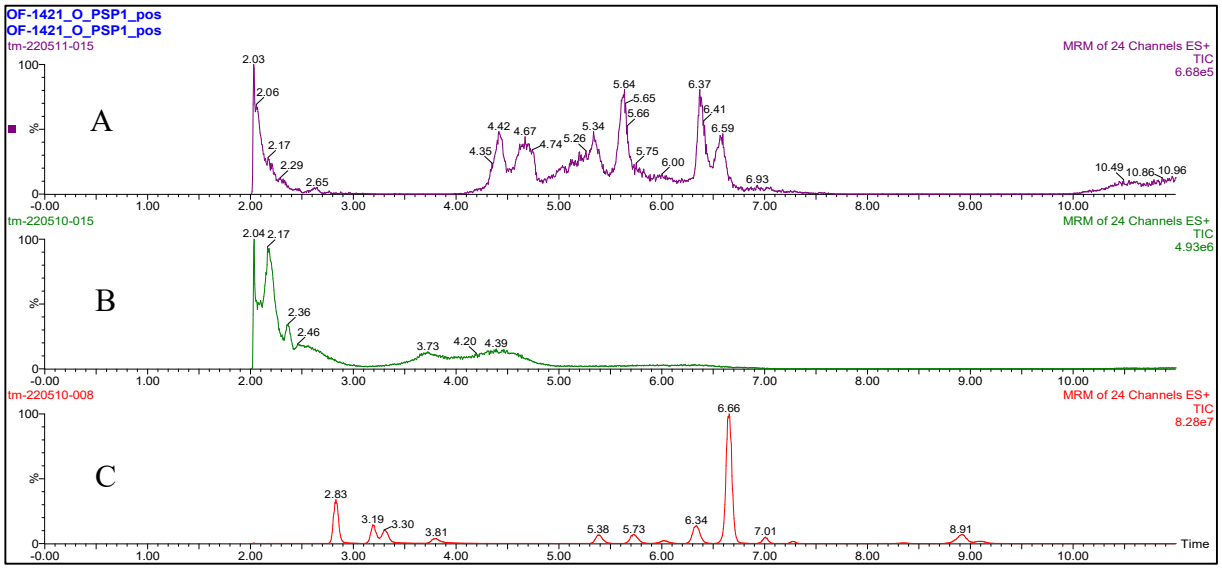

Figure 5: TICs of a urine sample from a sea lion (OF 1421) extracted with 0.2 M aqueous hydrochloric acid with the added ammonia (A) and without ammonia (B) compared to the TIC of the PSP toxin standard mix (C). Peaks in TIC from the sample without ammonia were shifted to the front compared to the TIC from the standard. With the added ammonia, the peaks in the TIC of the sample looked not shifted anymore.

The same samples were extracted again with 1% aqueous acetic acid. The pH value of the 1 % aqueous acetic acid solution was between 2 and 3, measured with pH paper test stripes. When measuring the samples extracted with acetic acid with LC-MS/MS, signals did not appear as shifted as when extracted with hydrochloric acid compared to the standard mix. However, signals appeared earlier than the standard and the sample extracted with hydrochloric acid after adding ammonia.

As an example, Figure 6 shows the TICs of a urine sample of a sea lion (OF 1421), extracted with 1 % aqueous acetic acid (Figure 6A) and 0.2 M aqueous hydrochloric acid (Figure 6B). Peaks in the TIC of the urine sample extracted with 1 % aqueous acetic acid are less shifted than in the TIC of the same sample extracted with 0.2 M aqueous hydrochloric acid. However,

they appear earlier than in the TIC of the standards (Figure 6C) and the TIC of the same sample extracted with hydrochloric acid and the added ammonia in Figure 5A.

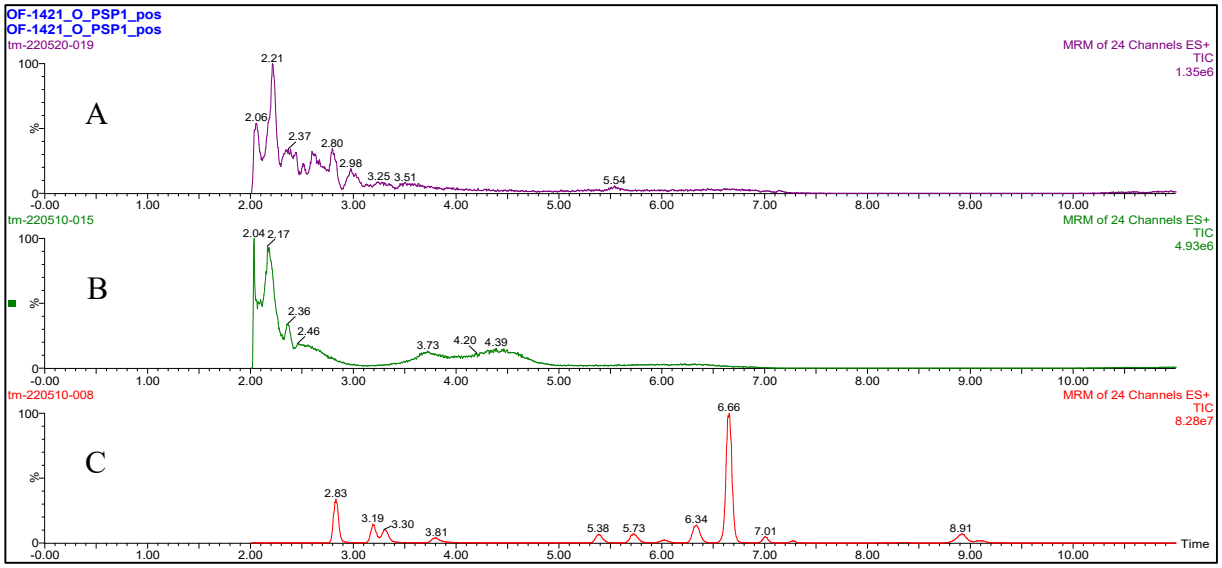

Figure 6: TICs from a urine sample of a sea lion (OF 1421) extracted with different solvents and measured with LC-MS/MS together with the PSP toxin standards. Chromatogram A shows the TIC of the sample extracted with acetic acid, chromatogram B the TIC of the sample extracted with hydrochloric acid and chromatogram C shows the TIC of the standard of PSP toxins. Comparing the first two chromatograms, signals in chromatogram A look less shifted to the front as in chromatogram B. Compared with chromatogram C, signals of the sample appear different and all in the first 5 min.

When the same samples were analysed with HPLC-FLD, the pH value did not influence measurements in any case. As an example, the sea lion urine sample´s chromatograms (OF 1421) extracted with 0.2 M aqueous hydrochloric acid with (B) and without (A) ammonia are shown in Figure 7. In both chromatograms, all fluorescent signals look similar, appearing at the same retention time and with a certain intensity. However, when comparing both samples with the PSP standard mix, signals were not matching the known peaks of different toxins present in the standard (Figure 7C). Thus, it was concluded that no toxins could be detected in the urine sample. The same results applied to the other samples analysed.

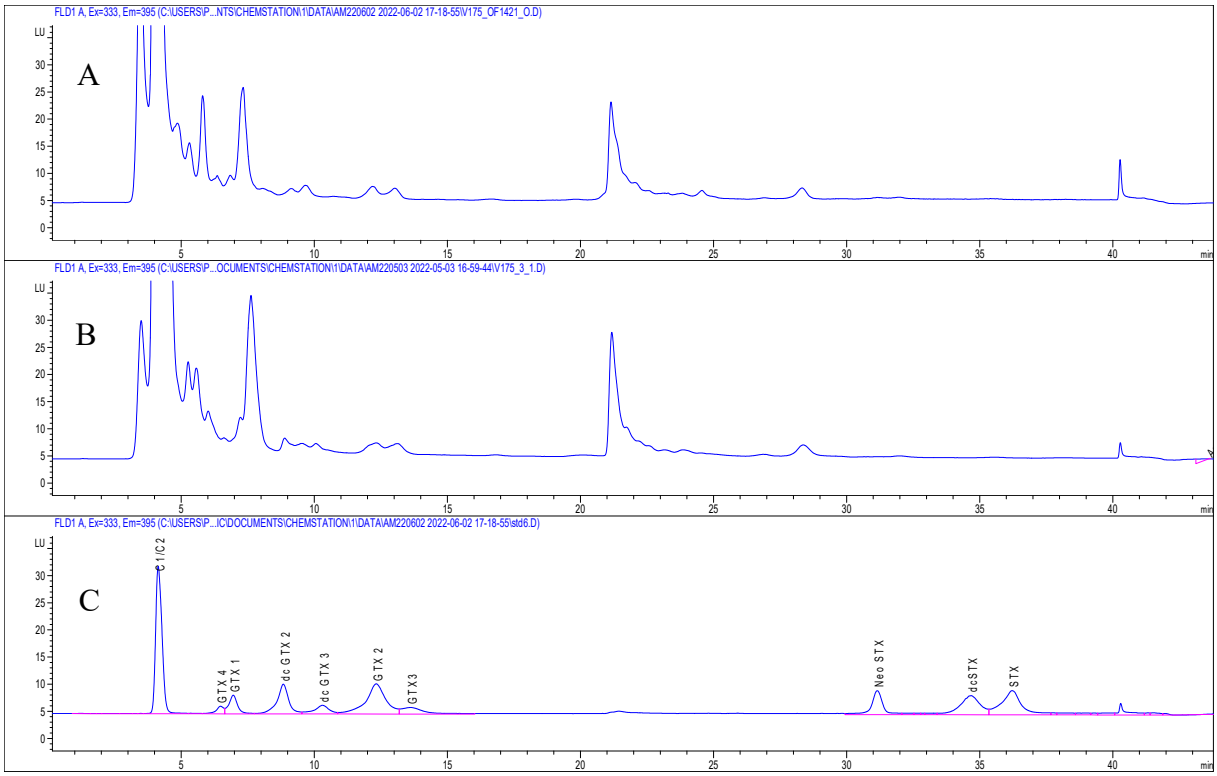

Figure 7: HPLC-FID TICs of the sea lion urine sample (OF 1421) under different pH values. A) pH value before adding ammonia (pH = 0); B) pH value after adding ammonia (pH = 4), and C) standard with PSP toxins in 1% aqueous acetic acid. Graphs showed that 1) no peak shift was observed under different pH conditions, and 2) none of those "potential peaks" correspond to PSP toxins, as they do not match the known PSP peaks in the standard peak.

6.3 Sample clean-up

Some samples, especially fecal samples extracted with hydrophilic solvents for PSP toxin detection, resulted in highly turbid extracts. Moreover, only part of the particles sediment to the bottom to form the pellet, while the rest formed another layer above the solution, preventing the collection of the supernatant, where, if present, toxins are expected to be dissolved. For example, Figure 8 shows the appearance of the fecal sample of one baleen whale (BFA 28) in 1% acetic acid after centrifuging. The formation of three layers can be observed: The bottom and top layers contain solid sample compounds, keeping the very turbid-looking supernatant in between.

Thus, three different methods were conducted to enhance the separation between matrix compounds and extracts: increasing the centrifugation speed, adding highly saturated sodium chloride solution to the sample, and finally, trying an alternative cell disruption method.

Figure 8: Tube with a whale fecal sample (BFA 28) after extraction with 1 % aqueous acetic acid and centrifugation. The separation between sample matrix and extract solvent was insufficient as the supernatant is very turbid. The formation of three layers was observed: both top and bottom layers containing solid compounds and the liquid part in the middle.

A) Centrifugation speed

The sample separation with a higher centrifugation speed showed no improvement compared to the lower centrifugation speed, as shown in Figure 9. In addition, the supernatant remained very turbid, showing that an optimal separation between solvent and particles was not achieved with higher speed. Whale fecal sample BFA 28, previously shown in Figure 8, continued showing three-layer formation with the supernatant in the middle (right tube in Figure 9). However, negative aspects of higher centrifugation speeds were not observed either.

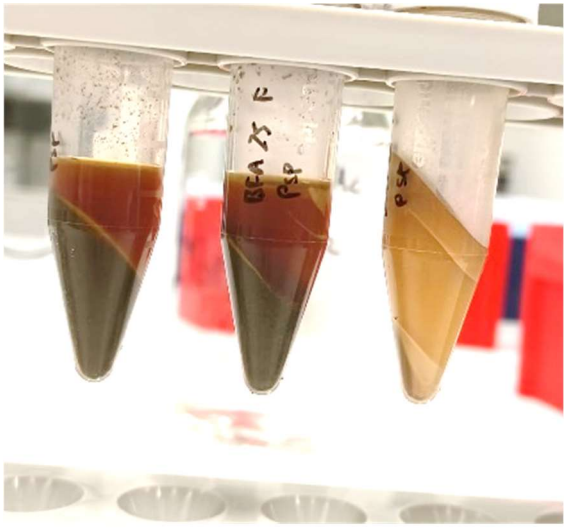

Figure 9: 5 ml-Eppendorf tubes with three different whale fecal samples (left to right: BFA 24, BFA 25, BFA 28) extracted with 1% aqueous acetic acid for PSP toxin extraction after centrifuging with 10000 x g.The separation between sample matrix and solvent seemed to have not increased as the supernatants are still very turbid. Three-layer continued to be formed for BFA 28, with two solid layers in the top and bottom and the supernatant in the middle.

B) Saturated sodium chloride solution

Figure 10 shows a whale fecal sample (BFA 28) extracted with 1 % aqueous acetic acid after adding saturated sodium solution and centrifugation. The separation seemed to work better as the supernatant looked clearer than before, although the three-layer formation still appears as in Figure 8.

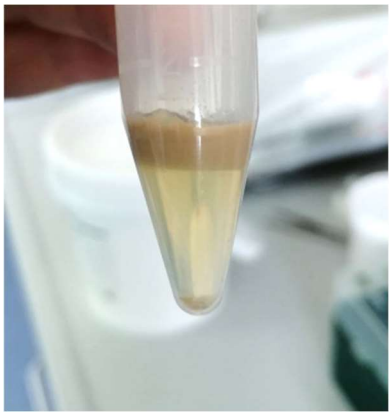

Figure 10: Tube with whale fecal sample (BFA 28) extracted with 1 % aqueous acetic acid after adding saturated sodium solution and centrifuging. The separation between sample matrix and solvent seemed to be improved as the supernatant looked clear. However, a formation of three different layers with the supernatant in the middle appeared.

However, after pooling together the three supernatants obtained throughout the three extraction procedures and reducing the solvent volume under a gentle stream of nitrogen to fit in 2 ml vials, a white residue with a crystalline structure could be seen at the bottom of the tube (Figure 11).

Figure 11: Tube with the extract of a whale fecal sample (BFA 28) after evaporation, extracted with 1 % aqueous acetic acid with added saturated sodium solution. A white, crystalline residue can be observed at the bottom of the tube.

C) Cell disruption method

Samples extracted with 80% aqueous methanol for lipophilic toxins analysis showed that the sonicated extracts, obtained after pooling the re-extractions together (right tube, Figure 12), looked very clear. On the contrary, in the joined homogenized extracts (left tube, Figure 12), turbidity appeared in the lower third of the tube.

Figure 12: Tubes with the extracts of a whale fecal sample (BFA 28) extracted with 80% aqueous methanol using homogenization with lysing matrix (left tube) and sonication (right tube). A clearer supernatant was obtained after sonication.

For PSP toxin analysis, pooled extracts from whale fecal samples (BFA 28) in 1% acetic acid obtained after sonication (left tube, Figure 13) and homogenization (right tube, Figure 13) showed turbidity in both cases. Visually, the sonicated sample seemed slightly less turbid (Figure 13).

Figure 13: Tubes with the extracts of a whale fecal sample (BFA 28) extracted with 1% acetic acid using sonication (left tube) and homogenization (right tube) with lysing matrix. Both extracts looked very turbid, although the sonicated one looked slightly clearer than the homogenized extract.

D) Solid-phase extraction

HPLC-FLD chromatographic results showed a clear reduction in the baseline noise after SPE clean-up in all samples. For example, Figure 14 shows a comparison between the stomach content sample from a dolphin (Dd 97) with (B) and without (A) the SPE clean-up step. When no SPE was applied, a high baseline noise appeared in the chromatogram. On the contrary, a nice and flat baseline was observed in the chromatogram of samples after SPE cleaning.

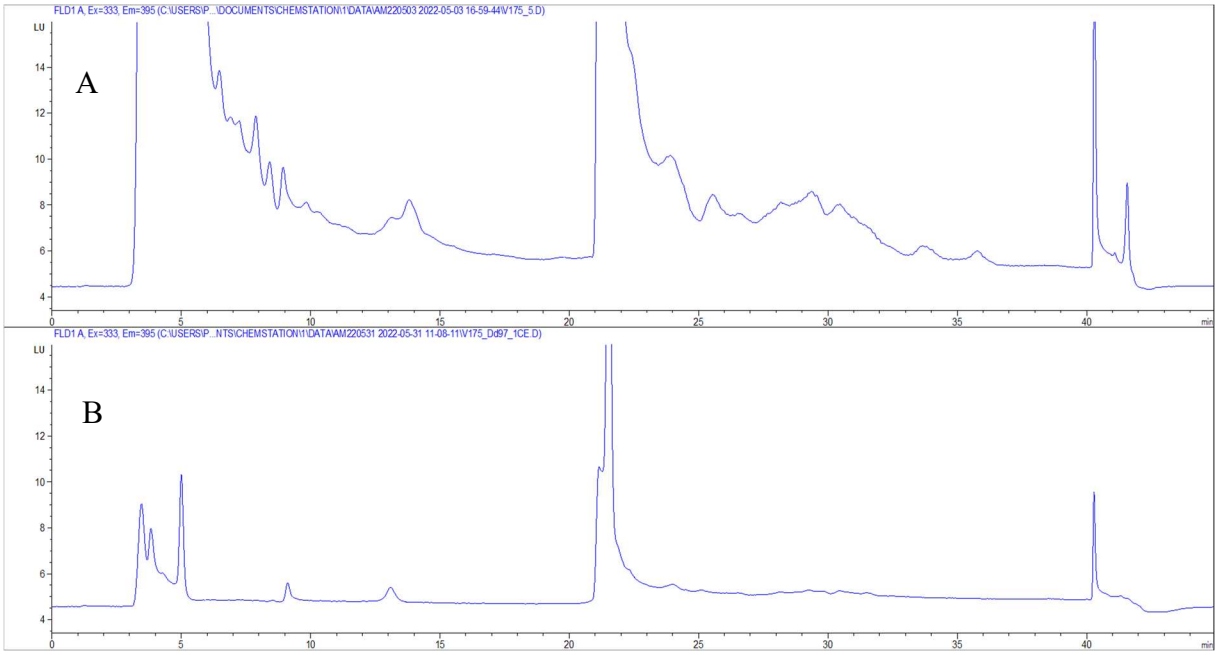

Figure 14: HPLC-FLD chromatograms comparing the baselines from the stomach content sample of a dolphin (Dd 97) in 1% acetic acid with (B) and without (A) SPE clean-up step. A reduction in the baseline noise was observed after SPE with the C18 cartridge.

6.4 Trial between silica cartridges and C18 cartridges for lipophilic phycotoxins

A clear difference could be observed when comparing the toxin recoveries from all used lipophilic toxin groups for the silica and the C18 cartridge (Figure 15). Recovery rates for toxin groups after the SPE with the C18 cartridge are significantly lower than for the silica cartridge, whereas DA and OA were not recovered. Compared to the initial sample, the highest recovery with 45 % was obtained for PTX, followed by AZA with 29 %. Recoveries for GYM and SPX are both below 15 %. Contrary, all toxin recoveries for the different toxin groups after the SPE with the silica cartridge are higher than 60 % in relation to the initial sample, except for domoic acid with 11 % recovery. GYM has the second-lowest recovery with 62 %, whereas recovery rates above 100 % were found for AZA and OA. Detailed information about recovered toxin groups and their recovery proportion compared to the initial sample is shown in Table 14 in the Appendix (Chapter 9). Also, the different toxins LoDs considered are presented in Table 19 in the Appendix section.

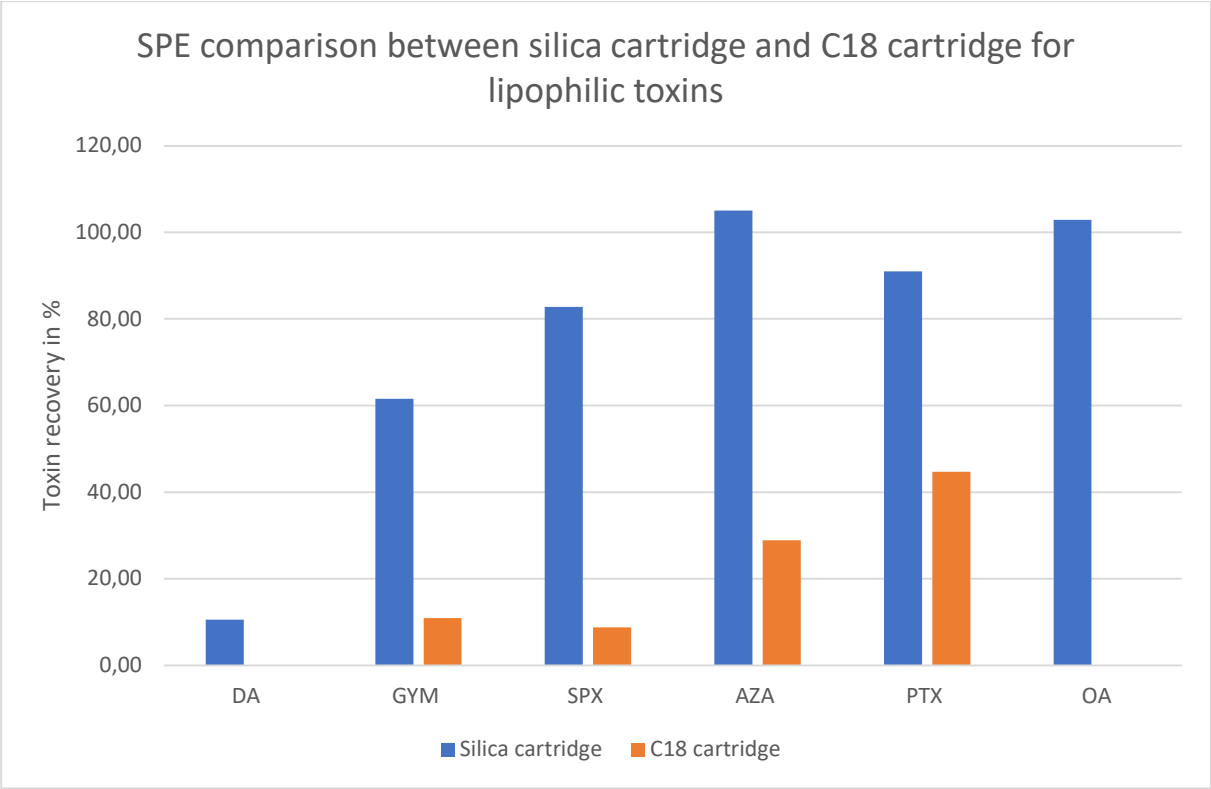

Figure 15: Toxin recovery after the SPE clean-up with a silica cartridge and C18 cartridge for samples containing lipophilic toxins. Six toxin classes were represented on the x-axis: DA, GYM, SPX, AZA, PTX and OA. Y-values were expressed as percentages to the initial sample. Coloured in blue are the toxins recovered from the silica cartridge, and in orange are the ones recovered from the C18 cartridge. Toxin recoveries were higher for silica cartridges than for C18 cartridges. For silica cartridges, the recovery of each toxin group was higher than 60 %, except for DA. For C18 cartridges, all toxin recoveries were lower than 50 %, and OA and DA were not recovered at all.

6.4.1 Post-analysis of SPE cartridges for lipophilic toxins

For the C18 cartridge, four classes of toxins were found when analysing the waste (Figure 16). Concentrations between 31 % and 46 % compared to the initial sample were obtained for DA, SPX and PTX. For GYM, only 13 % could be found. After washing the cartridge with different solvents, some retained toxins were eluted (Figure 16). High amounts of OA and AZA were found with recovery rates over 100 % after eluting with 100 % methanol. The 50 % aqueous methanol fractions with and without 0.3 M acetic acid did not elute any toxin group. In the Appendix (Chapter 9), detailed information is given referred to toxin concentrations (Table 15) and toxin percentages in relation to the initial sample (Table 16) present in the waste and eluent fractions from the C18 cartridge for lipophilic toxin analysis.

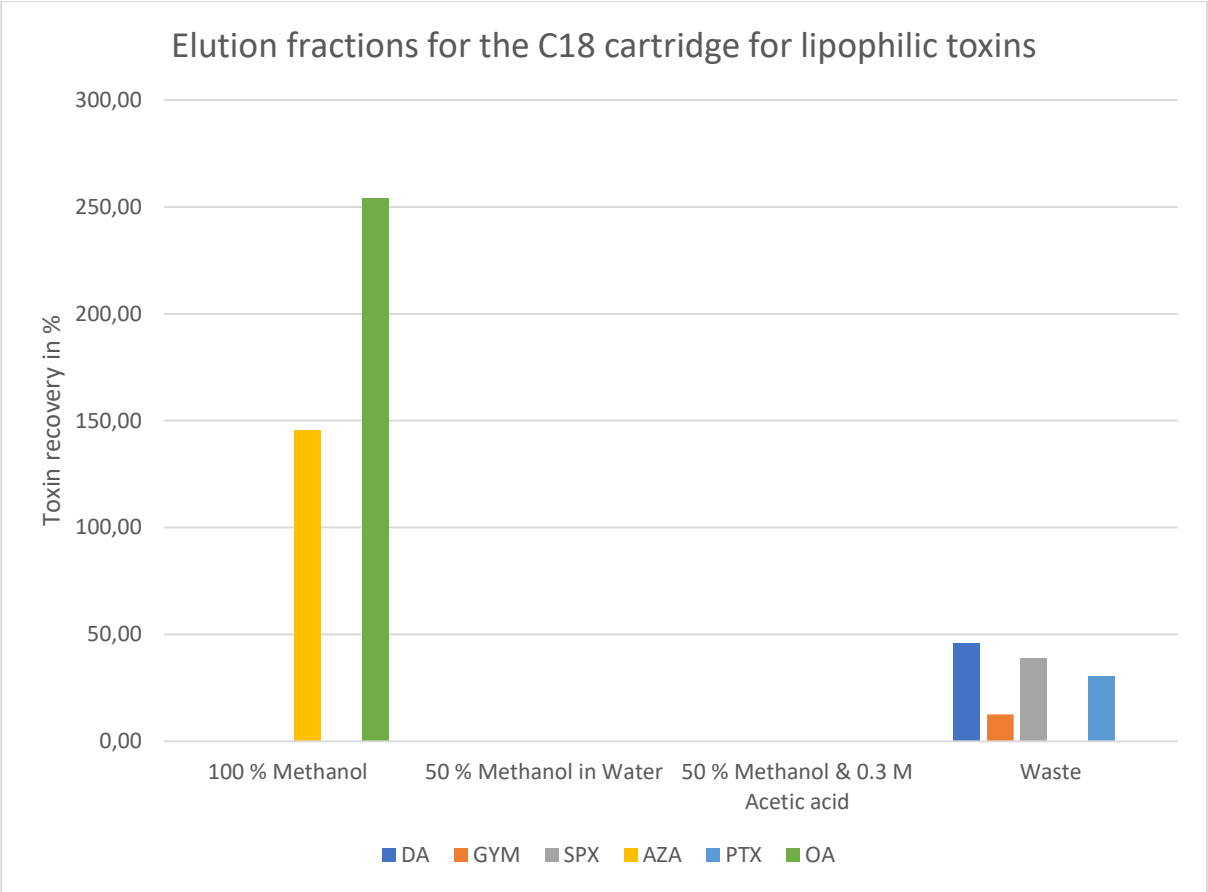

Figure 16: Elution fractions of the SPE C18 cartridge used for lipophilic toxins. Three different elution fractions, which are 100 % methanol, 50 % aqueous methanol and 50 % aqueous methanol with 0.3 M acetic acid, were used to elute retained toxins and analysed together with the waste. Toxin recovery of each fraction for the toxin groups DA, GYM, SPX, AZA, PTX and OA is expressed as a percentage in relation to the initial sample. Intermediate amounts of SPX, GYM, PTX and DA were eluted in the waste, and more than 100 % of OA and AZA were eluted in the 100 % methanol fraction. Both 50 % aqueous methanol fractions did not elute any toxins.

After applying three different solutions to the silica cartridge to elute retained toxins, only retained amounts of GYM could get eluted (Figure 17). Only 1 % of GYM was obtained in the 100 % methanol fraction, whereas approximately 35 % was found in the 50 % aqueous methanol with 0.3 M acetic acid fraction. No toxin groups were eluted with the 50 % aqueous methanol fraction. In the Appendix (Chapter 9), detailed information is given referred to toxin concentrations (Table 17) and toxin percentages in relation to the initial sample (Table 18) present in the eluent fractions from the silica cartridge for lipophilic toxin analysis. The different toxin LoDs considered for both cartridges are presented in Table 19 in the Appendix section.

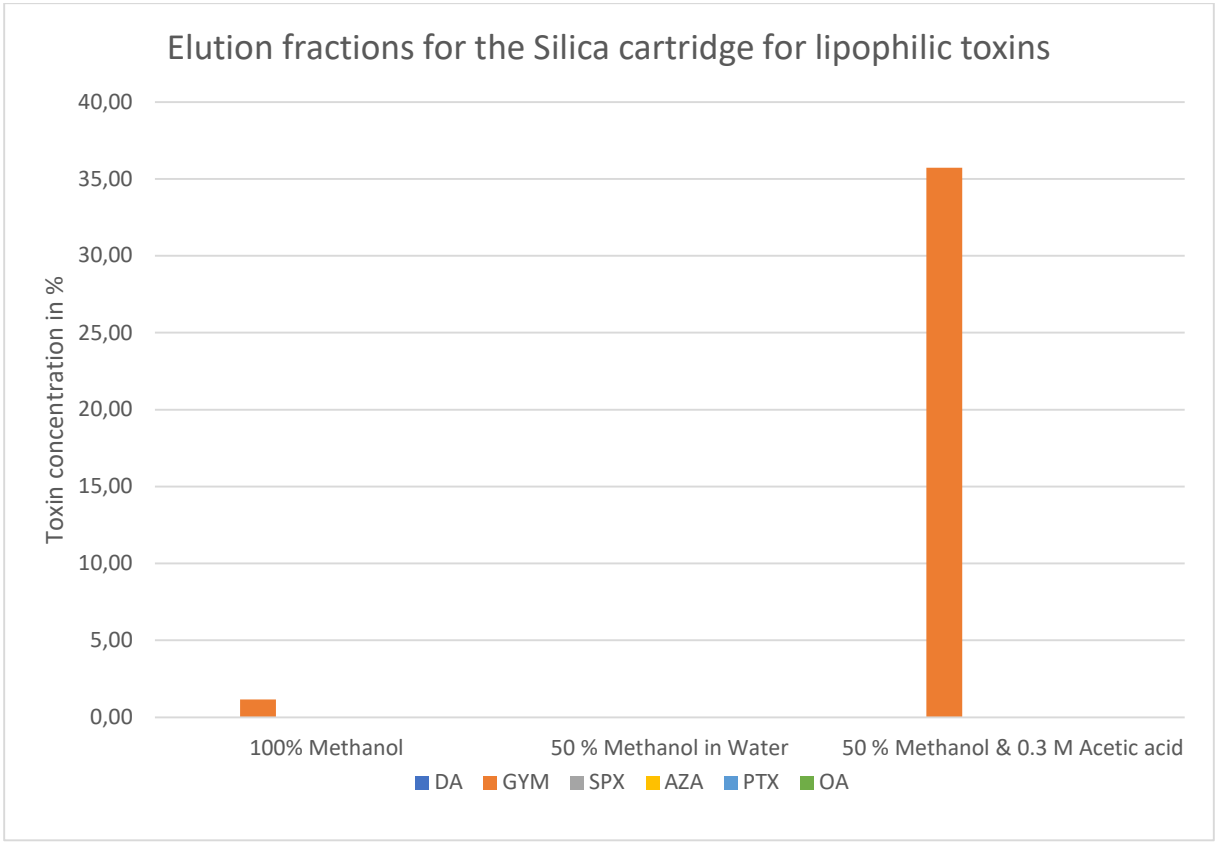

Figure 17: Elution fractions of the SPE silica cartridge used for lipophilic toxins. Three fractions were used to elute retained toxins of the cartridge: 100 % methanol, 50 % aqueous methanol and 50 % aqueous methanol with 0.3 M acetic acid. Toxin recoveries in the eluted fractions were measured of the toxin groups DA, GYM, SPX, AZA, PTX and OA. The recovery of each toxin group was expressed as a percentage of the initial sample. Only GYM could be detected in the different eluents with trace amounts of 1 % in 100 % methanol and 36 % in the 50 % aqueous methanol with 0.3 M acetic acid fraction.

6.5 Trial between carbon cartridges and C18 cartridges for PSP toxins

When comparing PSP toxin recoveries for the carbon cartridge and the C18 cartridge, higher recovery rates for five out of nine toxins were found for the carbon cartridge (Figure 18). For the carbon cartridge, the highest recovery rates were obtained for GTX 1 and GTX 2 with over 89 %. Concentrations relative to the initial sample for dc GTX 2, GTX 3, GTX 4 and C1/C2 ranged between 57 % and 75 %. Finally, the toxin recoveries of dc GTX 3, STX and NEO from carbon cartridges were below 26 %.

For the C18 cartridge, recoveries for all PSP toxins were moderate, within the 35 % and 70 % range. The lowest recovery was obtained for GTX 4 with 38 % and the highest recovery for dc GTX 2 with 69 % (Figure 18).

In the Appendix (Chapter 9), Table 20 contains toxin concentrations in the initial sample (no SPE clean-up) and after carbon or C18 SPE clean-up. Recovery percentages obtained after carbon or C18 SPE are also shown in the table. The different PSP toxin LoDs considered appear in Table 22 in the Appendix.

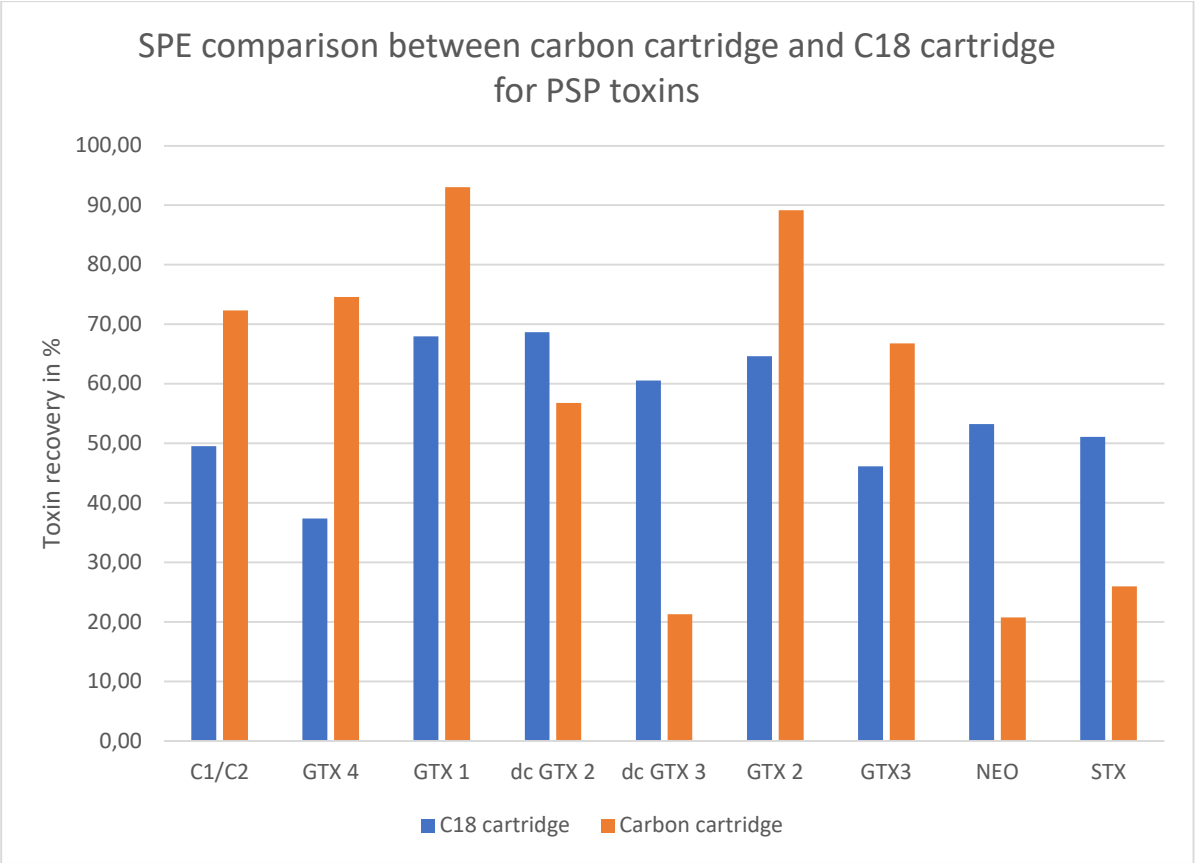

Figure 18: Toxin recovery after using SPE carbon and C18 cartridges to clean up samples for PSP toxin extractions. The recovery of all nine PSP toxins is expressed as percentages in relation to the initial sample. Toxin recoveries from the C18 cartridge are colored in blue and from the carbon cartridge in orange. For five out of nine toxins, recoveries are higher with the carbon cartridge than with the C18 cartridge. Except for dc GTX 3, STX and NEO, toxin recoveries are over 50 % with the carbon cartridge. Toxin recoveries with the C18 cartridge are between 35 % and 70 %.

6.5.1 Post-analysis of SPE cartridges for PSP toxins

Eight classes of toxins were identified in the waste collected from the carbon cartridge during SPE cleaning (Figure 19). Amounts of over 45 % in relation to the initial sample were obtained for dc GTX 2, dc GTX 3, NEO and STX. A smaller amount, less than 20 % in relation to the initial sample, was recovered for C1/C2, GTX 2, GTX 3 and GTX 4. No GTX 1 could be detected in the waste (Figure 19). Detailed information concerning toxin concentrations and recovery percentages compared to the initial sample are shown in Table 21 in the Appendix (Chapter 9). The PSP toxins LoDs considered appeared in Table 22 also in the Appendix.

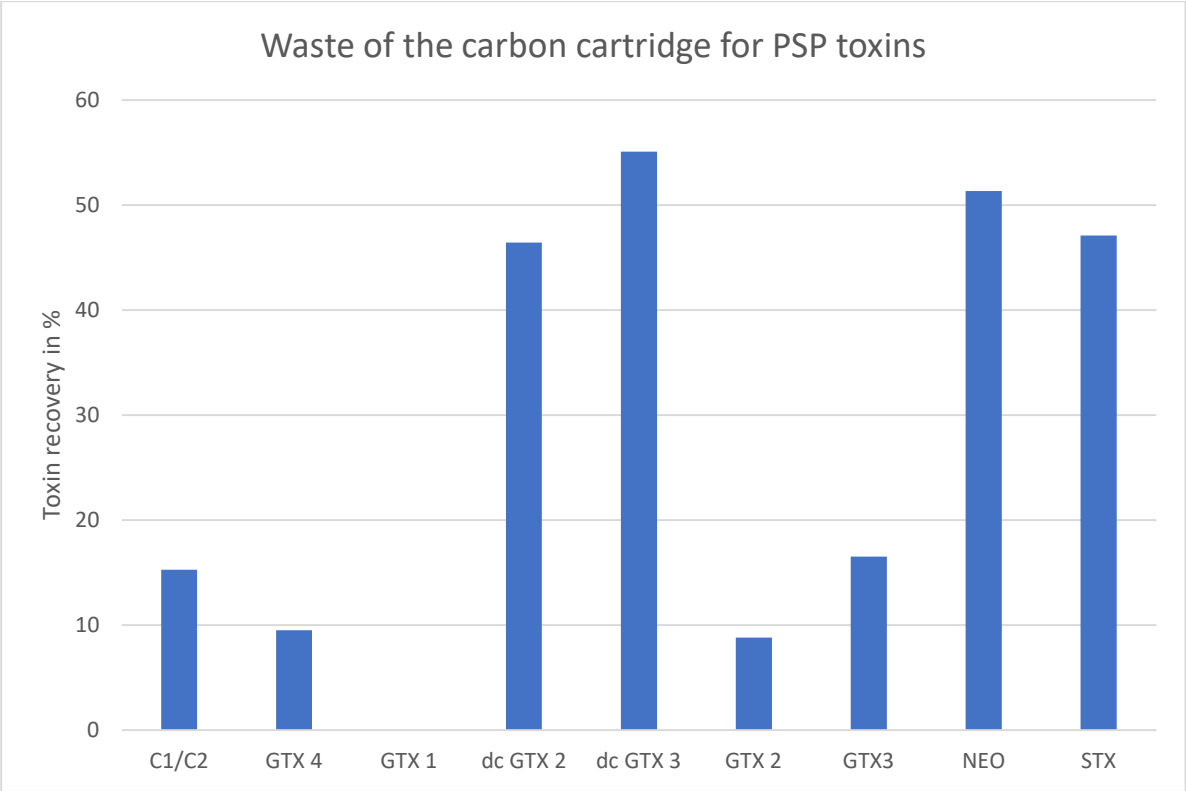

Figure 19: Toxin recovery in the waste of the SPE carbon cartridge used for PSP toxins. Toxin recovery is expressed as percentages in relation to the initial sample. High amounts of dc GTX 2, dc GTX 3, NEO and STX with recovery rates over 45 % were found in the waste, whereas smaller amounts below 20 % of C1/C2, GTX 4, GTX 2 and GTX 3 could be detected.

For the C18 cartridge, no PSP toxins were detected with HPLC-FLD after applying two more volumes of washing solvent and one volume of deionized water.

6.6 Toxin detection

As part of the methodology development, several marine mammals' samples were analysed with HPLC-FLD and LC-MS/MS to determine the presence of lipophilic or hydrophilic toxins. Toxins could not be detected in any of the 12 samples analysed belonging to nine individuals from three different species, as resumed in Table 13.

Table 13: List of Marine mammals' tissue and body fluids samples determined for phycotoxins. The list contains information about each specific animal's tissue or body fluids. No toxins were detected in the analysed samples.

Animal	Tissue/ Body fluid	Toxins detected
Dd 100	Stomach content	Non detected
Dd 89-I	Stomach content	Non detected
Dd 97-I	Stomach content	Non detected
Dd 109	Feces	Non detected
BFA 27	Kidney	Non detected
BFA 27	Feces	Non detected
BFA 28	Liver	Non detected
BFA 28	Feces	Non detected
BFA 24	Feces	Non detected
BFA 25	Feces	Non detected
OF 1421	Urine	Non detected
OF 1421	Stomach content	Non detected

6.7 Final extraction protocol for lipophilic toxins

After assessing the results of the trials detailed in this chapter, an improved extraction protocol for lipophilic toxins was proposed, modified from the initial protocol draft (Section 5.1).

1. Weigh 0.5 g of lyophilized tissue into an Eppendorf tube.
2. Extract with 2 ml 80 % aqueous methanol.
3. Mix the samples with a vortexer.
4. Sonicate the sample for 5 min.
5. Centrifuge the samples using Centrifuge 5810 R at 4000 x g for 5 min and transfer the supernatant into a new tube.
6. Reextract the sample with 1 ml 80 % aqueous methanol.
7. Repeat steps 3 – 5 and pool the supernatants together.
8. Reextract the sample again with 1 ml 80 % aqueous methanol and repeat steps 3 and 4.
9. Centrifuge the samples using Centrifuge 5810 R at 4000 x g for 10 min and pool the supernatants together.
10. Use SPE to clean the sample.
11. Spin-filter the samples with a 0.45 µm pore-sized filter using Centrifuge 5424 R for 1 min at 15000 x g before transferring them into an HPLC vial for later measurements.
12. Store the samples in the freezer at -20 °C.

6.8 Final extraction protocol for PSP toxins

Results from the trials detailed in the sections above were used to conclude with a modified extraction protocol compared to the initial one (Section 5.1) for PSP toxins.

1. Weigh 0.5 g of lyophilized tissue into an Eppendorf tube.
2. Extract with 2 ml 1 % aqueous acetic acid.
3. Mix the samples with a vortexer.
4. Sonicate the sample for 5 min.
5. Centrifuge the samples using Centrifuge 5810 R at 4000 x g for 5 min and transfer the supernatant into a new tube.
6. Reextract the sample with 1 ml 1 % aqueous acetic acid.
7. Repeat steps 3 – 5 and pool the supernatants together.
8. Reextract the sample again with 1 ml 1 % aqueous acetic acid and repeat steps 3 and 4.
9. Centrifuge the samples using Centrifuge 5810 R at 4000 x g for 10 min and pool the supernatants together.
10. Use a SPE with a carbon cartridge to clean the sample.
11. Condition the cartridge with one cartridge load of 20 % aqueous acetonitrile with 1 % acetic acid followed by one cartridge load of a 0.025 % ammonia solution. Elute both to the level of top frit. Discard to waste.
12. Add the sample extract to the cartridge and elute to the top frit. Discard to waste.
13. Elute the cartridge with 2 ml of 20 % aqueous acetonitrile with 1 % acetic acid and elute to dryness. Collect the extract in a clean tube.
14. Spin-filter the samples with a 0.45 µm pore-sized filter using Centrifuge 5424 R for 1 min at 15000 x g before transferring them into an HPLC vial for later measurements.
15. Store the samples in the freezer at -20 °C.

7. Discussion

Marine mammals can be used as bioindicators to assess the effects of phycotoxin ingestion on human health. To detect phycotoxins in marine mammals' tissues and body fluids, an effective extraction protocol is of great importance. The phycotoxin extraction of shellfish samples has already been described by many authors since it is a crucial part of monitoring seafood safety (EU 2019/627, 2019). However, the matrix of marine mammal samples is very complex due to their dietary intake, and few studies are available. Therefore, this bachelor's thesis aims to develop and improve the extraction and detection of phycotoxins in marine mammals' samples by carrying out various trials. In this chapter, results from the trials will be discussed and put in context with literature to evaluate each step of the final extraction protocols proposed.

7.1 Extraction procedure: Solvents, volumes and extraction steps

Finding the right amount of tissue and solvent volume is essential for achieving a good toxin recovery without wasting the sample matrix or highly diluting the extract. Since none of the analysed marine mammal samples were found to contain phycotoxins, no further assessment concerning solvent and tissue ratios for obtaining a good toxin recovery could be performed. However, considering the tissue weights and solvent volumes used by other authors working with marine mammals samples (e.g., Fire et al., 2010; Lefebvre et al., 2016), toxin recoveries were expected to be similar.

Concerning re-extractions, the fact that toxins could not be detected in the marine mammal tissues and fluids analysed in this work prevents conclusions about the efficiency of three re-extractions or the necessity to increase or decrease their number. However, according to Quilliam et al. (1995), extracting the mussel tissue sample residue three times results in a toxin recovery of almost 100 % compared to the 98% recovery obtained in a single-step extraction. Although 2 % toxin-loss seems acceptable compared to the more time-consuming three-step extraction, phycotoxins may be present in small concentrations in some individuals' tissue or body fluid samples, requiring recovery percentages as high as possible to overcome detection limits.

Different extraction solvents are required considering the structure and physicochemical properties of the different toxin classes. For lipophilic toxins, an organic solvent is necessary, being methanol widely used in different dilutions. Some authors used a dilution of 90:10 (v/v) methanol-water (Luckas et al., 2015), whereas others used 100 % methanol for lipophilic toxin extractions (e.g., D'Agostino et al., 2017, EURLMB, 2015). Some authors like Quilliam (2004) proposed to use a methanol volume of 80 % in water to reduce the amount of lipids in the sample matrix. Considering that many marine mammal tissues and fluids are expected to contain considerable lipid percentages (e.g., blubber, stomach content) due to the carnivorous diet and ingestion of lipid-rich prey, most species follow (Berta et al., 2015; Würsig et al., 2017), the 80 % methanol-water solution was viewed as a good option.

Moreover, domoic acid is a water-soluble toxin (FAO/WHO/IOC, 2004) usually extracted separately from other toxin classes with 50 % methanol in water (e.g., Fire et al., 2010, Luckas et al., 2015). Also, other authors included domoic acid in the extraction process of PSP toxins with 0.1 M hydrochloric acid (Blay et al., 2011). The sample limitation when working with marine mammals made it seem advantageous to extract domoic acid with other toxin classes from the same sample simultaneously. In this work, 80% methanol was used to extract domoic acid together with lipophilic toxins. However, domoic acid was not detected in the samples analysed. It is uncertain if this could be related to using the wrong proportion solvent with 80 % methanol instead of 50 % or because no domoic acid was present in the samples analysed.

For PSP toxins, two extraction solvents have traditionally been considered in the literature. Hydrochloric acid in an aqueous solution is recommended by the AOAC (1990) and hence, widely used. However, it should be considered that hydrochloric acid can promote toxin conversion into more toxic analogs due to its extremely low pH value (Ben-Gigirey et al., 2012, Vale et al., 2008). Acetic acid in an aqueous solution is the other extraction solvent for PSP toxins. It is a weaker acid than hydrochloric acid that can be used to study toxin profiles, as it leaves the toxin structure unaffected (Nutrition Division, 2004). In the present work, hydrochloric acid in an aqueous solution was considered initially as toxin conversion was anticipated to have already happened as part of metabolic activities, including contact with hydrochloric acid in gastric digestion, of different marine organisms along the marine trophic web until reaching marine mammals (Campbell & Reece, 2011). However, shifted signals, compared to standard peaks obtained with the UHPLC-MS/MS equipment, led to reconsidering the extraction solvent. It seemed as if no retention of the sample compounds occurred. Considering that the HILIC system usually operates with a gradient system, starting with a low polarity organic solvent and eluting with a high polar solvent (Buszewski & Noga, 2012), the signal shift could be explained due to an increase in solvent polarity due to the low pH value of the sample after injection, preventing polar compounds from being retained on the column. Extremely low pH values between 0 and 2 were confirmed for body fluid samples of marine mammals extracted with aqueous hydrochloric acid.

Adding ammonia to basify the samples and avoid signal shift to the front of the chromatograms gave good results, supporting the previous explanation. Moreover, it should be mentioned that different ammonia quantities had to be added based on the tissue or fluid analysed. For example, stomach content needed a higher concentration of ammonia, probably due to the mammal stomach's acidic environment caused by hydrochloric acid production (Campbell & Reece, 2011).

The results described made consider 1 % aqueous acetic acid as the extraction solvent, in agreement with previous authors (e.g., Turner et al., 2018). Acetic acid is less acidic than hydrochloric acid, with a pH value between 2 and 3 for 1% aqueous acetic acid solutions. Nevertheless, signals in the TIC continued looking shifted compared to the standards and the samples extracted with hydrochloric acid with added ammonia, suggesting that the sample pH might still be too low. In accordance, Dell'Aversano et al. (2005), who validated HILIC-MS for the analysis of PSP toxins, described a change in retention times and worse peak separation with decreasing pH values of the mobile phase. Moreover, the manufacturer guidelines for the UHPLC column used in this work specified that solvent pH values should be between 3 and 8 to obtain reliable results (Waters Corporation, 2015).

With HPLC-FLD, no shift in signal occurred independently of samples extracted with 0.2 M hydrochloric acid or 1% acetic acid. According to the manufacturer, the pH stability of the HPLC column is in a range between 1.5-8; thus, the column is not so sensitive to pH changes.

Working with HPLC-FLD provides excellent results, but the method is more time-consuming and complex and requires many clean-ups, derivatizations and analytical runs for each sample than the LC-MS/MS (Turner et al., 2019). Therefore, to be able to use LC-MS/MS, equipment´s methodological development should be carried out to trust the results obtained.

7.2 Clean-up of marine mammal samples

Due to the complex matrixes of marine mammals' body fluids, highly turbid extracts were obtained. In general, biological samples contain a variety of complex components (e.g., salts, proteins, lipids) that can interfere with analysis (Peng et al., 2016). Moreover, the heterogeneous and carnivorous diet of most marine mammal species may lead to a variety of prey compounds

in their body fluid samples from digestion (Berta et al., 2015; Würsig et al., 2017). Also, body fluid matrixes can be affected by the time of sample collection and be even more complex when samples are collected hours after the death of the stranded animals (Wang et al., 2012). Crude sample extracts are problematic when measuring with HPLC-FLD or LC-MS/MS since they might cause high noise signals, preventing or underestimating toxin peaks (Matuszewski et al., 2003). Also, matrix effects of crude extracts can lead to shifts in retention times, complicating toxin identification (Dell'Aversano et al., 2005) or clogging the HPLC columns.

One of the most complex matrices to extract in this work was whale fecal samples for PSP toxins identification, as the separation between solvent and sample matrix was insufficient after centrifuging in most cases. Usually, cell fragments sediment in the bottom, whereas the less dense solvent containing the dissolved toxins is in the top as a supernatant (Kurreck et al., 2022). However, when extracting the fecal sample of BFA 28 with 1 % aqueous acetic acid, a three-layer formation was observed, most likely caused by the same matrix compounds responsible for turbidity in the fecal sample extracts. These problems did not occur during the extraction with lipophilic solvents. An explanation might be that the fecal samples contained high amounts of lipids that would easily dissolve in methanolic solvents but not in hydrophilic solvents, where they can form emulsions in the water solvent (Picket, 2008). Baleen whales primarily feed on zooplankton (Würsig et al., 2017), and zooplankton species consist of approximately 84 % lipids (Michaud & Taggart, 2007). In addition, a study on fecal samples from North Atlantic right whales carried out by Swaim et al. (2009) concluded that their feces consists of lipids almost entirely. Taking all this into account, it seems plausible that lipids are responsible, at least partially, for difficult matrix separation. Hence, different clean-up methods were tested to solve the problem of turbid samples.

The sediment rate was expected to increase with higher centrifugation speeds (Worsfold et al., 2005). However, the extracts' clearance did not improve with higher centrifugation speeds, indicating that dissolved proteins or other contaminants were still in the extract. Reasons might be that the sedimentation of these particles requires a higher centrifugation speed above 10000 x g or a longer centrifugation time (> 10 min). Therefore, more trials should be conducted with whale fecal samples and less problematic tissue or body fluid matrixes, as the latest might benefit from a higher centrifugation speed.

The clean-up step based on adding saturated sodium chloride solution to the samples extracted with acetic acid solutions visually improved the sample matrix and extract separation. The rationale is that sodium chloride increases the ion strength of the aqueous solvent reducing the solubility of lower polarity compounds (Worsfold et al., 2005). However, when the solvent volume gets reduced, the sodium chloride precipitates and forms a solid residue at the bottom of the tube. Salts are hazardous to the HPLC system as they may be precipitated and cause irreversible damage to the column (Rabel, 1980). Moreover, the influence of salt on the detection of phycotoxins is uncertain. For example, high salt concentrations were described to suppress the ionization of some PSP toxin analogs in the LC-MS/MS (Dell'Aversano et al., 2005). On the contrary, this clean-up step was conducted by Sayfritz et al. (2008) before, using a ratio of 1:11 (v:v) 5% sodium salt in solvent without claiming problems for the equipment. Thus, reducing the added sodium chloride concentrations might be considered in future trials in highly turbid fecal samples extracted with hydrophilic solvents; however, extreme caution should be taken to avoid damaging the expensive and sensitive chromatographic equipment.

Another approach to improve disruption and obtain less turbid extracts might be changing the cell disruption method . For example, Lefebvre et al. (2016) used different approaches to disrupt different marine mammal samples. A homogenizer was used for fecal samples and stomach and intestinal content, whereas sonication was applied for urine, serum and other body fluids.

Although turbidity of the extracts for both methods was still high when comparing each for both lipophilic and PSP toxins, sonication seemed to produce slightly clearer extracts than homogenizing and was therefore kept in the initial extraction protocol draft. Furthermore, the efficiency of both methods in terms of toxin recovery should be evaluated. Powell et al. (2002) compared DA recovery rates for homogenizing sand crab samples with and without a subsequent sonication step. They showed that after sonication, recovery rates increased by about 40 % compared to the non-sonicated homogenates resulting in a total recovery of 97 ± 2.9 % DA. Also, parameters like time and power intensities for sonication impact the amounts of phycotoxin released. For example, sonicating cyanobacteria at 20 kHz for 5 min resulted in high amounts of toxins, whereas toxin concentrations decreased with sonication periods over 10 min (Rajasekhar et al., 2012). Based on the aspects mentioned above and visual assessment, sonication seemed to be a better choice for disrupting the marine mammal matrixes than homogenizing with lysing matrix. However, further trials with marine mammal samples containing phycotoxins should be carried out.

SPE is another clean-up technique used to remove interferents present in the matrix, generating a solution containing primarily analytes. Two strategies can be used to achieve this: 1) the analytes are retained in the cartridge while matrix components break through the column, or 2) the analytes of interest are not retained and elute while the matrix components get trapped in the cartridge (Cochran et al., 2019). Results after SPE clean-up of marine mammals samples produced chromatograms with reduced baseline noises, proving its efficiency in removing matrix effects before sample injection. Also, this indicates that high amounts of lipophilic matrix compounds were previously enriched in the sample extract, potentially causing blockage of spin-filters and the HPLC column. Another advantage when using cartridges that retain the target analytes is that by reducing the extract volume in the elution step, analytes can be concentrated prior to analysis. Due to the positive results for SPE, this step was implemented for further extractions as a final clean-up of the extract.

7.3 SPE trials

To secure that toxin loss is reduced to a minimum during the SPE clean-up, the toxin recovery of different cartridges for lipophilic and PSP toxins needed to be assessed.

The C18 cartridge used to clean up lipophilic toxins was expected to retain lipophilic toxins due to the lipophilic sorbent. However, the recovery rates of most toxins (e.g., GYM, SPX, AZA and PTX) were below 50 % in relation to the initial toxin concentration, whereas others like DA and OA were not recovered at all. The deficient recovery obtained could be a matter of an insufficient elution volume or inadequate eluent selection. Moreover, four toxin groups, DA, GYM, SPX, and PTX, were detected in the waste after the washing step with 20 % aqueous methanol, indicating that this solvent was inadequate for washing the column. In the case of DA, the elution of this toxin with the washing step is very likely due to its hydrophilic nature. Also, the initial 80 % aqueous methanol solvent might have caused the toxin loss. To improve toxin recovery when using SPE C18 cartridges, a good option would be to reduce the organic solvent to less than 50 % for lipophilic toxins to retain on the cartridge, which is why authors like Nielsen et al. (2016) dilute methanol-rich extracts with deionized water.

Post-elution steps were performed as the sum of percentages for the elution solvent, and the waste was not 100% for all toxin classes; even some toxins, like OA and DA, were not present at all. The high recovery of AZA and OA when 100 % methanol was used as an eluent is in agreement with their observed late retention times in the chromatographic separation indicating a higher affinity for the column due to a higher lipophilic nature. Since DA is more hydrophilic than the other five toxin groups that were measured, the retained 50 % DA was expected to elute with 50 % aqueous methanol with or without acetic acid. However, neither DA nor other

toxin groups eluted with these solvents. Thus, a more hydrophilic solvent with less than 20 % methanol content might be needed to elute the retained DA. No SPX or PTX were recovered with the different eluents tried.

The hydrophilic silica cartridge was expected to retain hydrophilic matrix compounds and let the lipophilic toxins run through the column. For that reason, high toxin recoveries of over 80 % were found for SPX, AZA, PTX and OA. However, lower recovery percentages were obtained for GYM (62%) and very low values for DA (11%). Almost 50% of GYM unrecovered was eluted with 50% aqueous methanol, suggesting that GYM may require a less lipophilic eluent for total recovery. Their partial hydrophilic nature will also explain why GYM is found in the waste of the C18 cartridge, which was washed with a 20 % aqueous methanol solution. However, it should be highlighted that trace amounts of GYM also elute with 100 % methanol. Those differences in the polarity of GYM could be due to structural differences in analogs of GYM leading to differences in the protonation or deprotonation of the molecule functional groups (Harju et al., 2016). For DA, their highly hydrophilic character suggests that most of it was retained in the hydrophilic cartridge. The tried eluent solutions seemed to be not as hydrophilic as required for eluting DA, preventing better recoveries, in agreement with results obtained from the C18 cartridge.

The recovery rates of more than 100 % for AZA and OA observed in the clean-up with the silica cartridge and in the 100 % methanol fraction with the C18 cartridge exceeding the initial toxin concentration can be a matter of reducing matrix effects. Matrix compounds can suppress the electrospray ionization process of the toxins in the MS leading to an underestimation of toxin concentrations, as reported in Matuszewski et al. (2003). Therefore, recovery rates of over 100 % for AZA and OA in 100 % methanol solution could be explained due to a reduction in matrix compounds after SPE clean-up and, thus, an enhanced toxin ionization process.

In conclusion, toxin recovery of lipophilic toxins with both cartridges is not sufficient, although results generally seem better when using the silica cartridge. Since DA has been reported previously in whale fecal samples in the study area (D'Agostino et al., 2017), obtaining a good toxin recovery is essential to draw further conclusions on toxin enrichment in marine mammals. Further trials concerning all mentioned aspects, including different cartridges, washing solvents and eluents, should be conducted to implement the best SPE procedure for lipophilic toxins in the final extraction protocol.

For PSP toxins, satisfactory recoveries between 65 % and 95 % were obtained for C1/C2, GTX 4, GTX 1, GTX 2 and GTX 3 with carbon SPE. On the contrary, low quantities with less than 60 % recovery were measured for dc GTX 2, dc GTX 3, NEO and STX. The remaining quantities were found in the washing step waste. When the recovery and the waste percentages were considered together, they approached the initial concentrations for all toxins. Since PSP toxins are hydrophilic, it is most likely that the deionized water used as the washing solvent eluted substantial quantities of the more polar toxins STX, NEO, dc GTX 3 and dc GTX 2 that retained longer on the chromatographic column but also considerable amounts of the other PSP toxins. Therefore, erasing the washing step or assessing other washing solutions would allow toxin recoveries close to 100 %. It should be mentioned that the carbon SPE procedure was performed following the EU standardized extraction protocol guidelines for PSP toxins in shellfish (Turner et al., 2018). As the EU protocol is established to detect phycotoxins in seafood, phycotoxin detection above certain percentages might be sufficient to ensure food safety. However, when working with marine mammal samples, achieving the highest recovery percentages is necessary to detect toxins that might be present in low concentrations.

For SPE with C18 columns, PSP toxins were not expected to be retained in the cartridge due to its' lipophilic sorbent. However, toxin recoveries were only moderate, with recovery rates between 35 % and 70 %, indicating that high quantities of PSP toxins were retained in the cartridge. Possible explanations could be that the 2 ml volume of washing solution was too low or did not match the required polarity for eluting PSP toxins. However, no further toxins were recovered after increasing the extraction solvent volume or washing the cartridge with deionized water.

In conclusion, the carbon SPE method without the washing step with deionized water described in Section 5.2.5.2 was implemented in the enhanced PSP extraction protocol.

7.4 Analysed marine mammal samples

There are different possible explanations for not obtaining phycotoxins in the marine mammal samples analysed. First, a correlation between exposure to phycotoxins and enrichment in tissue and body fluids of the stranded animal is not guaranteed, especially for hydrophilic toxins that might be easily eliminated throughout fluids out of the body. In agreement with this, laboratory studies conducted with rats showed that DA could be eliminated through urine in less than 24 hours (Suzuki & Hierlihy, 1993). Moreover, no HAB have been reported for the time and area of routinely collected samples, and thus, the presence of toxins in the marine mammal samples is not assured.

Secondly, choosing a suitable extraction solvent is a critical aspect. Not detecting any toxin signals might be a matter of using the wrong solvent and obtaining no or very low signals under the threshold. For example, Sayfritz et al. (2008) obtained significantly higher PSP toxin concentrations when extracting shellfish samples with 80:20 (v/v) acetonitrile: water with 0.1 % formic acid compared to hydrochloric acid. Also, as mentioned before, the proper solvent for domoic acid should be assessed since most authors extract it separately from the other toxins with 50 % aqueous methanol (e.g., Luckas et al., 2015). Furthermore, some toxin analogs can differ in their chemical features from the original toxin, for example, the okadaic acid analog DTX-4 is water-soluble instead of lipophilic (Hu et al., 1995). Also, using a solvent volume that is too high could cause phycotoxin signals to be under the LoD. In contrast, using too little solvent might make the extraction of phycotoxins insufficient. Furthermore, assessing how many re-extraction steps are needed for a full toxin recovery is important, as extracting three times might not be enough.

Moreover, some of the marine mammal samples analysed underwent SPE clean-up trials with silica (lipophilic toxins) and C18 (PSP toxins) before trials with old samples and old standards, together with post-recovery trials were conducted. Considering that toxins, in various percentages, were demonstrated to remain in the cartridge or got lost in the waste, this could have been the case for the marine mammal samples analysed, especially if very low phycotoxin concentrations were present.

Finally, considering that all marine mammal samples analysed in this work were part of method development, re-analysis of them, following the improved extraction protocols proposed should be considered. Moreover, further improvements and modifications should be tested and, if positive, implemented in the proposed protocols.

7.5 Future aspects

Further future improvements to the extraction protocol for different phycotoxin classes are planned. For that, it is essential to count on marine mammal tissue and fluids that contain phycotoxins. The absence of toxins in any of the marine mammal samples analysed for this work impeded some of the trials initially considered and made it necessary to work with old

samples from other marine organisms for some trials. Collaborating with other research institutions to receive intoxicated marine mammal samples could be a solution. Once marine mammal tissues and fluids with toxins are available, the three-step extraction should be assessed to guarantee high toxin recoveries. Also, sonication should be evaluated as a proper disruption method to release toxins present within mammal cells into the solvent matrix and, if necessary, enhanced with other methods.

Finally, further improvements could be achieved for SPE clean-up procedures. For example, C18 cartridges might be a good option to concentrate lipophilic toxins, similar to carbon cartridges for PSP toxins; however, the procedure performed was inadequate. Diluting the samples before SPE clean-up or using other eluents might increase toxin recovery and determine if C18 cartridges are a good option to clean samples for lipophilic toxin analysis.

8. Conclusions

The present work was focused on the development of methodological improvements for phycotoxin extraction in marine mammal tissue and body fluids. As a result, the following conclusions were drawn:

1. For PSP toxin analysis, hydrochloric acid and acetic acid in aqueous solutions have proven to be adequate extraction solvents for measurements with the HPLC-FLD.
2. Low pH values for the PSP extraction solutions caused signal shifts preventing peak identification with the LC-MS/MS. Thus, methodological improvement with the LC-MS/MS equipment should be undertaken to obtain proper results.
3. Preliminary results suggest that sonication is a better disruption method than lysing-matrix homogenization for marine mammal tissues and fluids.
4. Solid-phase extraction clean-up has proven to efficiently remove matrix interferences from marine mammals' body fluids for phycotoxin analysis.
5. SPE carbon cartridges resulted in high recovery ranges for most PSP toxins. However, the washing step should be eliminated to obtain recoveries close to initial concentrations.
6. SPE silica cartridges generated a better recovery than C18 cartridges for most lipophilic toxins. To improve toxin recovery, washing the cartridge with 50 % aqueous methanol should be implemented in the protocol. On the contrary, recovery for domoic acid was insufficient and should be further evaluated.

9. Appendix

For the SPE trials with lipophilic toxins, the initial sample and the sample after the SPE clean-up with the silica and the C18 cartridges were measured with LC-MS/MS. The lipophilic toxins detected in the initial sample were: DA, GYM, STX, OA, PTX and AZA. Toxin concentrations were calculated as described in Section 5.4. In the following step, toxin concentrations of the initial sample were used to calculate toxin recoveries after the SPE clean-up. STX and GYM showed toxin peaks at multiple transitions. To simplify results, toxin concentrations of the same group were summed up (Table 14).

Table 14: Toxin concentrations of lipophilic toxins detected with LC-MS/MS in the initial sample, after the SPE with the silica cartridge (SPE Si) and the C18 cartridge (SPE C18), as well as the toxin recovery after the SPE clean-up expressed in percentages related to the initial toxin concentration. Each toxin class is listed with its transitions and retention times (RT). Toxin concentrations of GYM and SPX with different transitions were summed up.

Toxin	Transition	RT [min]	Toxin Mix [pg/µL]	SPE Si [pg/µL]	Recovery [%]	SPE C18 [pg/µL]	Recovery [%]
DA	312/266	7.63	64.50	6.82	10.57	0.00	0.00
GYM	508/490	9.14	2507.16	1699.14	67.77	185.67	7.41
GYM	522/504	9.36	184.81	158.74	85.89	15.70	8.50
GYM	524/506	8.88	20.06	14.53	72.43	2.42	12.06
GYM	524/506	9.36	23800.00	14440.00	60.67	2680.00	11.26
GYM total			26512.03	16312.41	61.53	2883.79	10.88
SPX	692/164	9.65	124.79	103.70	83.11	12.02	9.63
SPX	692/164	11.16	12.31	9.80	79.63	0.00	0.00
SPX total			137.09	113.50	82.79	12.02	8.77
AZA	842/824	12.57	4.20	4.42	105.05	1.21	28.89
PTX	876/213	12.09	50.37	45.81	90.95	22.50	44.67
OA	946/223	14.59	1935.00	1990.00	102.84	0.00-	0.00

After analysing the waste of the C18 cartridge, lipophilic toxin concentrations could be measured. Also, lipophilic toxins were detected when washing the cartridge with different eluents such as 100 % methanol, 50 % aqueous methanol and 50 % aqueous methanol with 0.3 M acetic acid (Table 15).

Table 15: Lipophilic toxin concentrations detected with LC-MS/MS in the waste of the C18 cartridge and after washing the cartridge with different eluents. The cartridge was washed with 100 % methanol, 50 % aqueous methanol and 50 % aqueous methanol with 0.3 M acetic acid. Each toxin class is listed with its specific transition and retention time. Toxin concentrations of GYM and SPX appearing at different transitions were summed up. RT stands for retention time, MeOH for methanol and HAc for acetic acid.

Toxin	Transition	RT [min]]	100% MeOH [pg/µL]	50 % MeOH [pg/µL]	50 % MeOH +0.3 M HAc [pg/µL]	Waste [pg/µL]
DA	312/266	7.63	0.00	0.00	0.00	29.60
GYM	508/490	9.14	3.84	0.00	0.00	1117.48
GYM	522/504	9.36	0.00	0.00	0.00	12.61
GYM	524/506	8.88	0.00	0.00	0.00	3.15
GYM	524/506	9.36	0.00	0.00	0.00	2200.00
GYM total			3.84	0.00		3333.24
SPX	692/164	9.65	0.00	0.00	0.00	53.28
SPX	692/164	11.16	0.00	0.00	0.00	0.00
SPX total			0.00	0.00	0.00	53.28
AZA	842/824	12.57	6.11	0.00	0.00	0.00
PTX	876/213	12.09	0.00	0.00	0.00	15.37
OA	946/223	14.59	4915.00	0.00	0.00	0.00

Lipophilic toxin concentrations measured with LC-MS/MS in the waste and in the different elution fractions of the C18 column were used to calculate the toxin recovery in relation to the initial toxin concentration (Table 16).

Table 16: Lipophilic toxin recovery expressed in percentages related to the initial toxin concentration detected with LC-MS/MS in the waste of the C18 cartridge and after washing the cartridges with different eluents. The eluents used were 100 % methanol, 50 % aqueous methanol and 50 % aqueous methanol with 0.3 M acetic acid. Each toxin class is presented with its transition and retention time. Total concentrations of GYM and SPX appearing at different transitions were used to calculate their recovery in total. MeOH stands for methanol, HAc for acetic acid and RT for retention time.

Toxin	Transition	RT [min]]	100% MeOH [%]	50 % MeOH [%]	50 % MeOH + 0.3 M HAc [%]	Waste [%]
DA	312/266	7.63	0.00	0.00	0.00	45.89
GYM	508/490	9.14	0.15	0.00	0.00	44.57
GYM	522/504	9.36	0.00	0.00	0.00	6.82
GYM	524/506	8.88	0.00	0.00	0.00	15.71
GYM	524/506	9.36	0.00	0.00	0.00	9.24
GYM total			0.01	0.00	0.00	12.57
SPX	692/164	9.65	0.00	0.00	0.00	42.69
SPX	692/164	11.16	0.00	0.00	0.00	0.00
SPX total			0.00	0.00	0.00	38.86
AZA	842/824	12.57	145.45	0.00	0.00	0.00
PTX	876/213	12.09	0.00	0.00	0.00	30.51
OA	946/223	14.59	254.01	0.00	0.00	0.00

After washing the silica cartridge with different eluents, the toxin concentrations in each fraction were measured (Table 17). The eluents used were 100 % methanol, 50 % aqueous methanol and 50 % aqueous methanol with 0.3 M acetic acid.

Table 17: Lipophilic toxin concentrations detected with LC-MS/MS after washing the SPE silica cartridge with different eluents. For washing, 100 % methanol, 50 % aqueous methanol and 50 % aqueous methanol with acetic acid were used. Each toxin class is listed with transition and retention time. The concentrations of GYM and SPX at different transitions were summed up. RT stands for retention time, MeOH for methanol and HAc for acetic acid.

Toxin	Transition	RT [min]	100% MeOH [pg/µL]	50 % MeOH [pg/µL]	50 % MeOH + 0.3 M HAc [pg/µL]
DA	312/266	7.63	0.00	0.00	0.00
GYM	508/490	9.14	303.72	0.00	0.00
GYM	522/504	9.36	0.00	0.00	83.95
GYM	524/506	8.88	0.00	0.00	8.02
GYM	524/506	9.36	0.00	0.00	9380.00
GYM total			303.72	0.00	9471.98
SPX	692/164	9.65	0.00	0.00	0.00
SPX	692/164	11.16	0.00	0.00	0.00
SPX total			0.00	0.00	0.00
AZA	842/824	12.57	0.00	0.00	0.00
PTX	876/213	12.09	0.00	0.00	0.00
OA	946/223	14.59	0.00	0.00	0.00

With the measured lipophilic toxin concentrations in each eluent fraction of the silica cartridge, toxin recovery could be calculated in relation to the initial toxin concentrations (Table 18).

Table 18: Recoveries for lipophilic toxins expressed as percentages in relation to the initial concentration of the toxin mix measured with LC-MS/MS after washing the silica cartridge with different eluents. For washing the cartridge, 100 % methanol, 50 % aqueous methanol and 50 % aqueous methanol with 0.3 M acetic acid were used. Each toxin is listed with its transition and retention time. Total toxin recovery for GYM and SPX were calculated with the summed-up concentrations of each at different transitions. RT stands for retention time, MeOH for methanol and HAc for acetic acid.

Toxin	Transition	RT [min]	100% MeOH [%]	50 % MeOH [%]	50 % MeOH + 0.3 M HAc [%]
DA	312/266	7.63	0.00	0.00	0.00
GYM	508/490	9.14	12.11	0.00	0.00
GYM	522/504	9.36	0.00	0.00	45.43
GYM	524/506	8.88	0.00	0.00	40.00
GYM	524/506	9.36	0.00	0.00	39.41
GYM total			1.15	0.00	35.73
SPX	692/164	9.65	0.00	0.00	0.00
SPX	692/164	11.16	0.00	0.00	0.00
SPX total			0.00	0.00	0.00
AZA	842/824	12.57	0.00	0.00	0.00
PTX	876/213	12.09	0.00	0.00	0.00
OA	946/223	14.59	0.00	0.00	0.00

Standard concentrations and their signal-to-noise ratios were used to calculate the LoD of each lipophilic toxin measured with LC-MS/MS (Table 19).

Table 19: LoDs of each lipophilic toxin standard used for the SPE trials. The parameters used to calculate the LoD comprise the standard concentrations and the signal-to-noise ratios (S/N).

Standard	Concentration [pg/µL]	S/N	LoD [pg/µL]
AZA	100.00	329.55	0.91
GYM A	50.00	103.33	1.45
SPX	100.00	42.07	7.13
PTX 2	500.00	103.57	14.48
DA	100.00	69.00	4.35
OA	500.00	6.58	227.85

PSP toxin concentrations were measured with HPLC-FLD before and after the SPE clean-up with a C18 and carbon cartridges. Toxin recovery after the SPE was calculated in relation to the initial toxin concentration (Table 20).

Table 20: Toxin concentrations of PSP toxins measured with HPLC-FLD before and after the SPE clean-up with C18 (SPE C18) and carbon cartridges (SPE Carb) together with the toxin recoveries after the SPE clean-up expressed as percentages in relation to the initial toxin concentrations. Each toxin is listed with the retention time (RT).

Toxin	RT	Toxin mix [pg/uL]	SPE C18 [pg/uL]	Recovery [%]	SPE Carb [pg/uL]	Recovery [%]
C1/C2	4.3	12356.26	6120.48	49.53	8934.14	72.30
GTX 4	6.7	607.74	227.26	37.39	453.14	74.56
GTX 1	7.2	488.06	331.68	67.96	454.12	93.05
dc GTX 2	8.9	22.06	15.15	68.67	12.52	56.76
dc GTX 3	10.6	30.33	18.36	60.54	6.46	21.30
GTX 2	12.6	1665.49	1076.89	64.66	1484.65	89.14
GTX3	14.1	5165.13	2382.68	46.13	3448.31	66.76
NEO	31.7	479.86	255.57	53.26	99.51	20.74
STX	38	815.91	416.73	51.08	211.75	25.95

PSP toxins were measured in the waste of the carbon cartridge with HPLC-FLD, and toxin recovery was calculated afterward compared to the initial sample (Table 21).

Table 21: Toxin concentration in the waste of the carbon cartridge measured with HPLC-FLD and toxin recovery expressed as percentages in relation to the initial toxin concentration before the SPE clean-up. Each toxin is listed with the retention time (RT).

Toxin	RT	Waste [pg/uL]	Recovery [%]
C1/C2	4.3	1887.45	15.28
GTX 4	6.7	57.69	9.49
GTX 1	7.2	0.00	0.00
dc GTX 2	8.9	10.24	46.43
dc GTX 3	10.6	16.71	55.10
GTX 2	12.6	146.41	8.79
GTX3	14.1	853.55	16.53
NEO	31.7	246.31	51.33
STX	38	384.34	47.11

The LoDs of the PSP toxin standards used for quantification were calculated with their concentrations and signal-to-noise ratios (Table 22).

Table 22: LoD of each PSP toxin standard calculated with the concentrations and signal-to-noise (S/N) ratios measured with HPLC-FLD.

Toxin	Concentration [pg/µL]	S/N	LoD [pg/uL]
C1/2	64.5	20.3	9.53
GTX 4	27.4	0.89	92.36
GTX 1	102.5	2.8	109.82
dc-GTX2	8.05	5.3	4.56
dc-GTX 3	2.25	1.6	4.22
GTX 2	8.2	4.6	5.35
GTX 3	2.7	1.3	6.23
NEO	51	3.3	46.36
STX	7.5	3.7	6.08

10. Eidesstattliche Erklärung

HOCHSCHULE COBURG

Fakultät Angewandte Naturwissenschaften

Name: Lena Kittel

Eidesstattliche Erklärung des/der Studierenden über die Selbstständigkeit der Arbeit nach 35 Abs. 7 RaPo

Hiermit versichere ich, dass ich die Arbeit selbstständig verfasst habe und keine weiteren außer den angeführten Quellen und Hilfsmitteln benutzt habe. Alle wörtlichen und sinngemäßen Zitate sind als solche gekennzeichnet.
Ich versichere, dass meine Projektarbeit bis jetzt bei keiner anderen Stelle veröffentlicht oder anderweitig für Prüfungszwecke vorgelegt wurde.

.....
Unterschrift

Bremerhaven, 30.06.2022

_____
Ort, Datum

11. References

AOAC. (1990). *Official methods of analysis of the Association of Official Analytical Chemists* (15th. ed., Vol. 2). AOAC.

Aune, T [Tore], & Yndestad, M. (1993). Diarrhetic shellfish poisoning. In I. R. Falconer (Ed.), *Algal toxins in seafood and drinking water* (pp. 87–104).

Ben-Gigirey, B., Rodríguez-Velasco, M. L., Otero, A., Vieites, J. M., & Cabado, A. G. (2012). A comparative study for PSP toxins quantification by using MBA and HPLC official methods in shellfish. *Toxicon: Official Journal of the International Society on Toxinology*, *60*(5), 864–873. https://doi.org/10.1016/j.toxicon.2012.05.022

Berta, A., Sumich, J. L., & Kovacs, K. M. (2015). *Marine mammals: Evolutionary biology* (3rd ed.). Elsevier Science & Technology.

Bialojan, C., & Takai, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *The Biochemical Journal*, *256*(1), 283–290. https://doi.org/10.1042/bj2560283

Blay, P., Hui, J. P. M., Chang, J., & Melanson, J. E. (2011). Screening for multiple classes of marine biotoxins by liquid chromatography-high-resolution mass spectrometry. *Analytical and Bioanalytical Chemistry*, *400*(2), 577–585. https://doi.org/10.1007/s00216-011-4772-2

Boente-Juncal, A., Raposo-García, S., Louzao, M. C., Vale, C., & Botana, L. M. (2021). Targeting chloride ion channels: New insights into the mechanism of action of the marine toxin azaspiracid. *Chemical Research in Toxicology*, *34*(3), 865–879. https://doi.org/10.1021/acs.chemrestox.0c00494

Bossart, G. D. (2011). Marine mammals as sentinel species for oceans and human health. *Veterinary Pathology*, *48*(3), 676–690. https://doi.org/10.1177/0300985810388525

Broadwater, M. H., van Dolah, F. M., & Fire, S. E. (2018). Vulnerabilities of marine mammels to harmful algal blooms. In J. M. Burkholder, S. E. Shumway, & S. L. Morton (Eds.), *Harmful algal blooms: A compendium desk reference* (pp. 191–222). Wiley Blackwell.

Bundesinstitut für Risikobewertung. (2022, May 20). *Bewertung von marinen Biotoxinen in Lebensmitteln*. https://www.bfr.bund.de/de/bewertung_von_marinen_biotoxinen_in_lebensmitteln-62066.html

Burkholder, J. M., Shumway, S. E., & Morton, S. L. (Eds.). (2018). *Harmful algal blooms: A compendium desk reference*. Wiley Blackwell. https://doi.org/10.1002/9781118994672

Buszewski, B., & Noga, S. (2012). Hydrophilic interaction liquid chromatography (HILIC) - a powerful separation technique. *Analytical and Bioanalytical Chemistry*, *402*(1), 231–247. https://doi.org/10.1007/s00216-011-5308-5

Campbell, I. M. (1985). Secondary metabolism and microbial physiology. In *Advances in Microbial Physiology. Advances in Microbial Physiology Volume 25* (Vol. 25, pp. 1–60). Elsevier. https://doi.org/10.1016/s0065-2911(08)60290-8

Campbell, N. A., & Reece, J. B. (2011). *Biologie* (8., akt. Aufl. [Nachdr.]. *Pearson Studium - Biologie*. Pearson Studium. https://swbplus.bsz-bw.de/bsz333360648cov.htm

Cochran, J. K., Bokuniewicz, H. J., & Yager, P. L. (Eds.). (2019). *Encyclopedia of ocean sciences* (Third edition). Academic Press imprint of Elsevier. https://doi.org/Kirk

Cole, A. S., & Eastoe, J. E. (1988). *Biochemistry and oral biology* (Second edition). Wright.

D'Agostino, V. C., Degrati, M., Sastre, V., Santinelli, N., Krock, B., Krohn, T., Dans, S. L., & Hoffmeyer, M. S. (2017). Domoic acid in a marine pelagic food web: Exposure of southern right whales *Eubalaena australis* to domoic acid on the Península Valdés

calving ground, Argentina. *Harmful Algae*, *68*, 248–257. https://doi.org/10.1016/j.hal.2017.09.001

Daranas, A. H., Norte, M., & Fernández, J. J. (2001). Toxic marine microalgae. *Toxicon*, *39*(8), 1101–1132. https://doi.org/10.1016/s0041-0101(00)00255-5

Dell'Aversano, C., Hess, P., & Quilliam, M. A [Michael A.] (2005). Hydrophilic interaction liquid chromatography-mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins. *Journal of Chromatography. A*, *1081*(2), 190–201. https://doi.org/10.1016/j.chroma.2005.05.056

Esteves, J. L., Santinelli, N., Sastre, V., Díaz, R., & Rivas, O. (1992). A toxic dinoflagellate bloom and PSP production associated with upwelling in Golfo Nuevo, Patagonia, Argentina. *Hydrobiologia*, *242*(2), 115–122. https://doi.org/10.1007/BF00018067

EURLMB (2015). *EU-harmonised standard operating procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS*.

Commission implementing regulation (EU) 2019/627 of 15 March 2019, European commission (Ed.) – Official Journal of the European Union (2019).

FAO/WHO/IOC. (2004). *Report of the joint FAO/IOC/WHO ad hoc expert consultation on biotoxins in bivalve molluscs.*

Ferrario, M. E., Sar, E. A., & Castaños, C. (1999). Potentially toxic species of the diatom genus *Pseudo-nitzschia* in Argentinian coastal waters. *Nova Hedwigia*, *68*(1-2), 131–147. https://doi.org/10.1127/nova.hedwigia/68/1999/131

Fire, S. E., & van Dolah, F. M. (2012). Marine Biotoxins: Emergence of harmful algal blooms as health threats to marine wildlife. In A. A. Aguirre, R. S. Ostfeld, & P. Daszak (Eds.), *New directions in conservation medicine: Applied cases of ecological health* (pp. 374–389). Oxford University Press Inc.

Fire, S. E., Wang, Z., Berman, M., Langlois, G. W., Morton, S. L., Sekula-Wood, E., & Benitez-Nelson, C. R. (2010). Trophic transfer of the harmful algal toxin domoic acid as a cause of death in a minke whale (*Balaenoptera acutorostrata*) stranding in southern California. *Aquatic Mammals*, *36*(4), 342–350. https://doi.org/10.1578/AM.36.4.2010.342

Fire, S. E., Wang, Z., Byrd, M., Whitehead, H. R., Paternoster, J., & Morton, S. L. (2011). Co-occurrence of multiple classes of harmful algal toxins in bottlenose dolphins (*Tursiops truncatus*) stranding during an unusual mortality event in Texas, USA. *Harmful Algae*, *10*(3), 330–336. https://doi.org/10.1016/j.hal.2010.12.001

Fujiki, H., Suganuma, M., Suguri, H., Yoshizawa, S., Takagi, K., Uda, N., Wakamatsu, K., Yamada, K., Murata, M., & Yasumoto, T. (1988). Diarrhetic shellfish toxin, dinophysistoxin-1, is a potent tumor promoter on mouse skin. *Japanese Journal of Cancer Research : Gann*, *79*(10), 1089–1093. https://doi.org/10.1111/j.1349-7006.1988.tb01531.x

Gayoso, A. M., & Fulco, V. K. (2006). Occurrence patterns of *Alexandrium tamarense* (Lebour) Balech populations in the Golfo Nuevo (Patagonia, Argentina), with observations on ventral pore occurrence in natural and cultured cells. *Harmful Algae*, *5*(3), 233–241. https://doi.org/10.1016/j.hal.2004.12.010

Geider, R. J., Moore, C. M., & Suggett, D. J. (2014). Ecology of marine phytoplankton. In R. K. Monson (Ed.), *Ecology and the Environment* (pp. 483–531). Springer New York. https://doi.org/10.1007/978-1-4614-7501-9_23

Gobler, C. J. (2020). Climate change and harmful algal blooms: Insights and perspective. *Harmful Algae*, *91*, 101731. https://doi.org/10.1016/j.hal.2019.101731

Google Maps. (2022). *Location of Golfo San Matías and Golfo San Jorge*.

Hallegraeff, G. M. (2004). Harmful algal blooms: A global overview. In G. M. Hallegraeff, D. M. Anderson, & A. D. Cembella (Eds.), *Monographs on oceanographic*

methodology: 11, Ed. 2. Manual on harmful marine microalgae (2nd ed., pp. 25–50). UNESCO Publishing.

Hallegraeff, G. M., Anderson, D. M., & Cembella, A. D. (Eds.). (2004). *Monographs on oceanographic methodology: 11, Ed. 2. Manual on harmful marine microalgae* (2nd, rev. ed.). UNESCO Publishing.

Hamer, J. P., Lucas, I. A. N., & McCollln, T. A. (2001). Harmful dinoflagellate resting cysts in ships' ballast tank sediments: potential for introduction into English and Welsh waters. *Phycologia*, *40*(3), 246–255. https://doi.org/10.2216/i0031-8884-40-3-246.1

Hanai, T. T. (1999). *Hplc: A practical guide* (1st ed.). *RSC Chromatography Monographs: v.6*. Royal Society of Chemistry. https://ebookcentral.proquest.com/lib/kxp/detail.action?docID=1185618

Harju, K., Koskela, H., Kremp, A., Suikkanen, S., La Iglesia, P. de, Miles, C. O., Krock, B., & Vanninen, P. (2016). Identification of gymnodimine D and presence of gymnodimine variants in the dinoflagellate *Alexandrium ostenfeldii* from the Baltic Sea. *Toxicon: Official Journal of the International Society on Toxinology*, *112*, 68–76. https://doi.org/10.1016/j.toxicon.2016.01.064

Hoffmann, E. (2013). *Mass Spectrometry: Principles and Applications* (3rd ed.). John Wiley & Sons Incorporated. https://ebookcentral.proquest.com/lib/kxp/detail.action?docID=326409 https://doi.org/Edmond

Hu, T., Curtis, J. M., Walter, J. A., & Wright, J. L. C. (1995). Identification of DTX-4, a new water-soluble phosphatase inhibitor from the toxic dinoflagellate *Prorocentrum lima*. *Journal of the Chemical Society, Chemical Communications*(5), 597. https://doi.org/10.1039/c39950000597

Jaime, E., Gerdts, G., & Luckas, B. (2007). In vitro transformation of PSP toxins by different shellfish tissues. *Harmful Algae*, *6*(3), 308–316. https://doi.org/10.1016/j.hal.2006.04.003

Joint FAO/WHO. (2016). *Toxicity equivalence factors for marine biotoxins associated with bivalve molluscs.*

Kao, C. Y. (1993). Paralytic Shellfish Poisoning. In I. R. Falconer (Ed.), *Algal toxins in seafood and drinking water* (pp. 75–86).

Krock, B., Borel, C. M., Barrera, F., Tillmann, U., Fabro, E., Almandoz, G. O., Ferrario, M. E., Garzón Cardona, J. E., Koch, B. P., Alonso, C., & Lara, R. (2015). Analysis of the hydrographic conditions and cyst beds in the San Jorge Gulf, Argentina, that favor dinoflagellate population development including toxigenic species and their toxins. *Journal of Marine Systems*, *148*, 86–100. https://doi.org/10.1016/j.jmarsys.2015.01.006

Krock, B., Tillmann, U., Voß, D., Koch, B. P., Salas, R., Witt, M., Potvin, E., & Jeong, H. J. (2012). New azaspiracids in *Amphidomataceae* (Dinophyceae). *Toxicon*, *60*(5), 830–839. https://doi.org/10.1016/j.toxicon.2012.05.007

Kurreck, J., Engels, J. W., & Lottspeich, F. (Eds.). (2022). *Bioanalytik* (4. Aufl. 2022). Springer Berlin Heidelberg. https://doi.org/Jens

La Riva, G. T. de, Johnson, C. K., Gulland, F. M. D., Langlois, G. W., Heyning, J. E., Rowles, T. K., & Mazet, J. A. K. (2009). Association of an unusual marine mammal mortality event with *Pseudo-nitzschia spp.* Blooms along the southern California coastline. *Journal of Wildlife Diseases*, *45*(1), 109–121. https://doi.org/10.7589/0090-3558-45.1.109

Lefebvre, K. A., Powell, C. L [Christine L.], Busman, M [Mark], Doucette, G. J [Gregory J.], Moeller, P. D. R., Silver, J. B., Miller, P. E [Peter E.], Hughes, M. P., Singaram, S., Silver, M. W., & Tjeerdema, R. S. (1999). Detection of domoic acid in northern

anchovies and California sea lions associated with an unusual mortality event. *Natural Toxins*, *7*(3), 85–92. https://doi.org/10.1002/(SICI)1522-7189(199905/06)7:3<85::AID-NT39>3.0.CO;2-Q

Lefebvre, K. A., Quakenbush, L., Frame, E., Huntington, K. B., Sheffield, G., Stimmelmayr, R., Bryan, A., Kendrick, P., Ziel, H., Goldstein, T., Snyder, J. A., Gelatt, T., Gulland, F., Dickerson, B., & Gill, V. (2016). Prevalence of algal toxins in Alaskan marine mammals foraging in a changing arctic and subarctic environment. *Harmful Algae*, *55*, 13–24. https://doi.org/10.1016/j.hal.2016.01.007

Luckas, B., Erler, K., & Krock, B. (2015). Analysis of marine biotoxins using LC-MS/MS. In D. B. Stengel & S. Connan (Eds.), *SpringerLink Bücher: Vol. 1308. Natural products from marine algae: Methods and Protocols* (pp. 277–298). Humana Press.

Marine Mammal Commission. (2007). *The marine mammal protection act of 1972 as amended.*

Martin, W., & Kowallik, K. (1999). Annotated English translation of Mereschkowsky's 1905 paper 'Über Natur und Ursprung der Chromatophoren im Pflanzenreiche'. *European Journal of Phycology*, *34*(3), 287–295. https://doi.org/10.1080/09670269910001736342

Matuszewski, B. K., Constanzer, M. L., & Chavez-Eng, C. M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, *75*(13), 3019–3030. https://doi.org/10.1021/ac020361s

McMahon, T., & Silke, J. (1996). Winter toxicity of unknown aetiology in mussels, *14*, 2.

McMaster, M. C. (2005). *Lc/ms: A practical user's guide*. Wiley Interscience. http://www.loc.gov/catdir/enhancements/fy0619/2004063820-b.html

Michaud, J., & Taggart, C. T. (2007). Lipid and gross energy content of North Atlantic right whale food, *Calanus finmarchicus*, in the Bay of Fundy. *Endangered Species Research*, *3*, 77–94. https://doi.org/10.3354/esr003077

Nagai, S., Lian, C., Yamaguchi, S., Hamaguchi, M., Matsuyama, Y., Itakura, S., Shimada, H., Kaga, S., Yamauchi, H., Sonda, Y., Nishikawa, T., Kim, C.-H., & Hogetsu, T. (2007). Microsatellite markers reveal population genetic structure of the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae) in Japanese coastal waters. *Journal of Phycology*, *43*(1), 43–54. https://doi.org/10.1111/j.1529-8817.2006.00304.x

Nielsen, L. T., Hansen, P. J., Krock, B., & Vismann, B. (2016). Accumulation, transformation and breakdown of DSP toxins from the toxic dinoflagellate *Dinophysis acuta* in blue mussels, *Mytilus edulis*. *Toxicon : Official Journal of the International Society on Toxinology*, *117*, 84–93. https://doi.org/10.1016/j.toxicon.2016.03.021

Nutrition Division. (2004). *Marine biotoxins*. *FAO food and nutrition paper: Vol. 80*. Food and Agriculture Organization of the United Nations.

Parsons, M. L., & Dortch, Q. (2002). Sedimentological evidence of an increase in *Pseudo-nitzschia* (Bacillariophyceae)abundance in response to coastal eutrophication. *Limnology and Oceanography*, *47*(2), 551–558. https://doi.org/10.4319/lo.2002.47.2.0551

Peng, J., Tang, F., Zhou, R., Xie, X., Li, S., Xie, F., Yu, P., & Mu, L. (2016). New techniques of on-line biological sample processing and their application in the field of biopharmaceutical analysis. *Acta Pharmaceutica Sinica. B*, *6*(6), 540–551. https://doi.org/10.1016/j.apsb.2016.05.016

Perl, T. M., Bédard, L., Kosatsky, T., Hockin, J. C., Todd, E. C., & Remis, R. S. (1990). An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *The New England Journal of Medicine*, *322*(25), 1775–1780. https://doi.org/10.1056/NEJM199006213222504

Picket, J. P. (Ed.). (2008). *American heritage student science dictionary*. Houghton Mifflin.

Powell, C. L [Christine L.], Ferdin, M. E., Busman, M [Mark], Kvitek, R. G., & Doucette, G. J [Gregory J.] (2002). Development of a protocol for determination of domoic acid in the sand crab (*Emerita analoga*): a possible new indicator species. *Toxicon*, *40*(5), 485–492. https://doi.org/10.1016/S0041-0101(01)00236-7

Quilliam, M. A [M. A.]. (2004). Chemical methods for lipophilic shellfish toxins. In G. M. Hallegraeff, D. M. Anderson, & A. D. Cembella (Eds.), *Monographs on oceanographic methodology: 11, Ed. 2. Manual on harmful marine microalgae* (2nd ed., pp. 211–246). UNESCO Publishing.

Quilliam, M. A [M. A.], Xie, M., & Hardstaff, W. R. (1995). Rapid extraction and cleanup for liquid chromatographic determination of domoic acid in unsalted seafood. *Journal of AOAC INTERNATIONAL*, *78*(2), 543–554. https://doi.org/10.1093/jaoac/78.2.543

Rabel, F. M. (1980). Use and Maintenance of Microparticle High Performance Liquid Chromatography Columns. *Journal of Chromatographic Science*, *18*(9), 394–408. https://doi.org/10.1093/chromsci/18.9.394

Ragelis, E. P. (1984). Ciguatera seafood poisoning. In E. P. Ragelis (Ed.), *ACS Symposium Series. Seafood Toxins* (Vol. 262, pp. 25–36). American Chemical Society. https://doi.org/10.1021/bk-1984-0262.ch003

Rajasekhar, P., Fan, L., Nguyen, T., & Roddick, F. A. (2012). Impact of sonication at 20 kHz on *Microcystis aeruginosa*, *Anabaena circinalis* and *Chlorella sp*. *Water Research*, *46*(5), 1473–1481. https://doi.org/10.1016/j.watres.2011.11.017

Richardson, W. J., Greene Jr., C. R., Malme, C. I., & Thomson, D. H. (1995). *Marine mammals and noise* [Nachdr.]. Academic Press.

Satake, M., Ofuji, K., Naoki, H., James, K. J., Furey, A., McMahon, T., Silke, J., & Yasumoto, T. (1998). Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. *Journal of the American Chemical Society*, *120*(38), 9967–9968. https://doi.org/10.1021/ja981413r

Sayfritz, S. J., Aasen, J. A. B., & Aune, T [T.] (2008). Determination of paralytic shellfish poisoning toxins in Norwegian shellfish by liquid chromatography with fluorescence and tandem mass spectrometry detection. *Toxicon*, *52*(2), 330–340. https://doi.org/10.1016/j.toxicon.2008.06.001

Scholin, C. A., Gulland, F [F.], Doucette, G. J [G. J.], Benson, S., Busman, M [M.], Chavez, F. P., Cordaro, J., DeLong, R., Vogelaere, A. de, Harvey, J., Haulena, M., Lefebvre, K., Lipscomb, T., Loscutoff, S., Lowenstine, L. J., Marin, R., Miller, P. E [P. E.], McLellan, W. A., Moeller, P. D., . . . van Dolah, F. M. (2000). Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature*, *403*(6765), 80–84. https://doi.org/10.1038/47481

Shimizu, Y. (1993). Microalgal metabolites. *Chemical Reviews*, *93*(5), 1685–1698. https://doi.org/10.1021/cr00021a002

Sullivan, J., & Wekell, M. M. (1984). Determination of paralytic shellfish poisoning toxins by high pressure liquid chromatography. In E. P. Ragelis (Ed.), *ACS Symposium Series. Seafood Toxins* (Vol. 262, pp. 197–205). American Chemical Society. https://doi.org/10.1021/bk-1984-0262.ch018

Suzuki, C., & Hierlihy, S. L. (1993). Renal clearance of domoic acid in the rat. *Food and Chemical Toxicology*, *31*(10), 701–706. https://doi.org/10.1016/0278-6915(93)90140-T

Swaim, Z. T., Westgate, A. J., Koopman, H. N., Rolland, R. M., & Kraus, S. D. (2009). Metabolism of ingested lipids by North Atlantic right whales. *Endangered Species Research*, *6*, 259–271. https://doi.org/10.3354/esr00163

Taleuzzaman, M., Ali, S., Gilani, S., Imam, S., & Hafeez, A. (2015). *Ultra performance liquid chromatography (UPLC)-a review* (Austin Journal of Analytical and Pharmaceutical Chemistry).

Tillmann, U., Elbrächter, M., Krock, B., John, U., & Cembella, A. (2009). *Azadinium spinosum* gen. et sp. nov. (Dinophyceae) identified as a primary producer of azaspiracid toxins. *European Journal of Phycology*, *44*(1), 63–79. https://doi.org/10.1080/09670260802578534

Trites, A. W. (2019). Marine mammal trophic levels and trophic interactions. In J. K. Cochran, H. J. Bokuniewicz, & P. L. Yager (Eds.), *Encyclopedia of ocean sciences* (pp. 589–594). Academic Press imprint of Elsevier. https://doi.org/10.1016/B978-0-12-409548-9.11618-5

Turner, A. D., Boundy, M., & Harwood, T. (2018). *Determination of paralytic shellfish poisoning toxins and tetrodotoxin by UHPLC-HILIC-MS/MS: Guidance Protocol v8.*

Turner, A. D., Boundy, M., Harwood, T., & Hird, S. (2019). *Determination of paralytic shellfish toxins and tetrodotoxins in shellfish by ultra-high performance hydrophilic-interaction liquid chromatography-tandem quadrupole mass spectrometry.* Waters Corporation. https://doi.org/10.13140/RG.2.2.34343.09127

Turner, A. D., Tarnovius, S., & Goya, A. B. (2014). Paralytic shellfish toxins in the marine gastropods *Zidona dufresnei* and *Adelomelon beckii* from Argentina: Toxicity and Toxin Profiles. *Journal of Shellfish Research*, *33*(2), 519–530. https://doi.org/10.2983/035.033.0221

Turriff, N., Runge, J. A., & Cembella, A. D [A. D.] (1995). Toxin accumulation and feeding behaviour of the planktonic copepod *Calanus finmarchicus* exposed to the red-tide dinoflagellate *Alexandrium excavatum*. *Marine Biology*, *123*(1), 55–64. https://doi.org/10.1007/BF00350323

Vale, C., Alfonso, A., Vieytes, M. R., Romarís, X. M., Arévalo, F., Botana, A. M., & Botana, L. M. (2008). In vitro and in vivo evaluation of paralytic shellfish poisoning toxin potency and the influence of the pH of extraction. *Analytical Chemistry*, *80*(5), 1770–1776. https://doi.org/10.1021/ac7022266

van Dolah, F. M. (2000). Marine algal toxins: Origins, health effects, and their increased occurrence. *Environmental Health Perspectives*, *108 Suppl 1*, 133–141. https://doi.org/10.1289/ehp.00108s1133

van Dolah, F. M., Doucette, G. J [Gregory J.], Gulland, F., Rowles, T., & Bossart, G. D. (2002). Impacts of algal toxins on marine mammals. In J. Vos, T. O,ÄôShea, M. Fournier, & G. D. Bossart (Eds.), *New Perspectives: Toxicology and the Environment. Toxicology of marine mammals* (Vol. 20026034). CRC Press. https://doi.org/10.1201/9780203165577.ch10

Vilariño, N., Fraga, M., & Rodríguez, L. P. (2014). Functional and receptor-based assays for marine toxins. In L. M. Botana (Ed.), *Food Science and Technology Ser. Seafood and freshwater toxins: Pharmacology, Physiology, and Detection, Third Edition* (3rd ed., pp. 333–347). Taylor and Francis.

Wang, Z., Broadwater, M. H., & Ramsdell, J. S. (2015). Analysis of diarrhetic shellfish poisoning toxins and pectenotoxin-2 in the bottlenose dolphin (*Tursiops truncatus*) by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography. A*, *1416*, 22–30. https://doi.org/10.1016/j.chroma.2015.08.066

Wang, Z., Maucher-Fuquay, J., Fire, S. E., Mikulski, C. M., Haynes, B., Doucette, G. J [Gregory J.], & Ramsdell, J. S. (2012). Optimization of solid-phase extraction and liquid chromatography-tandem mass spectrometry for the determination of domoic acid in seawater, phytoplankton, and mammalian fluids and tissues. *Analytica Chimica Acta*, *715*, 71–79. https://doi.org/10.1016/j.aca.2011.12.013

Waters Corporation. (2015). *ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm Columns and Glycan Performance Test Standards.*

World Register of Marine Species. (2022, June 9). *Cetacea*. https://www.marinespecies.org/aphia.php?p=taxdetails&id=2688

Worsfold, P., Townshend, A., & Poole, C. (Eds.). (2005). *Encyclopedia of analytical science*. Elsevier/Acad. Press.

Wright, J. L. C., Boyd, R. K., Freitas, A. S. W. de, Falk, M., Foxall, R. A., Jamieson, W. D., Laycock, M. V., McCulloch, A. W., McInnes, A. G., Odense, P., Pathak, V. P., Quilliam, M. A [M. A.], Ragan, M. A., Sim, P. G., Thibault, P., Walter, J. A., Gilgan, M., Richard, D. J. A., & Dewar, D. (1989). Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. *Canadian Journal of Chemistry*, *67*(3), 481–490. https://doi.org/10.1139/v89-075

Würsig, B., Thewissen, J. G. M., & Kovacs, K. M. (Eds.). (2017). *Encyclopedia of Marine Mammals* (3rd ed.). Elsevier Science. https://ebookcentral.proquest.com/lib/kxp/detail.action?docID=5154042

Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G. K., & Clardy, J. (1985). Diarrhetic shellfish toxins. *Tetrahedron*, *41*(6), 1019–1025. https://doi.org/10.1016/S0040-4020(01)96469-5

Yasumoto, T., Oshima, Y., & Yamaguchi, M. (1978). Occurrence of a new type of shellfish poisoning in the Tohoku district. *NIPPON SUISAN GAKKAISHI*, *44*(11), 1249–1255. https://doi.org/10.2331/suisan.44.1249