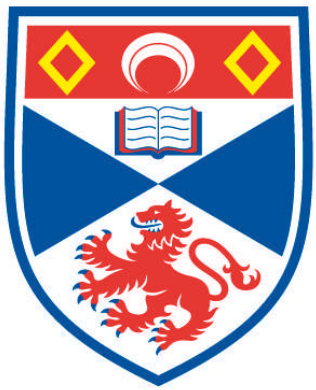
ARE TOXINS FROM HARMFUL ALGAE A FACTOR INVOLVED IN THE DECLINE OF HARBOUR SEAL POPULATIONS IN SCOTLAND?

Silje-Kristin Jensen

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Are toxins from harmful algae a factor involved in the decline of harbour seal populations in Scotland?

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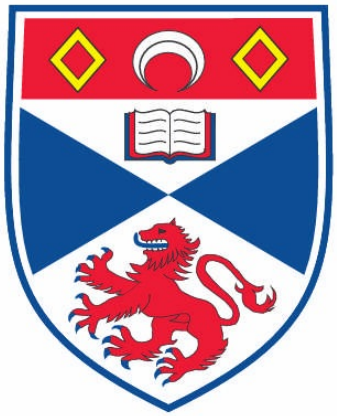

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November 2014

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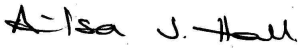
Date 10.04.15 Signature of supervisor 

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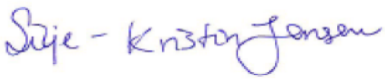
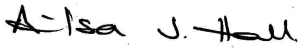
Signature of candidate 

Signature of supervisor 

"... all the water that were in the river were turned into blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river" (Exodus 7: 20-21).

Table of content

Thesis abstract

Firstly this study builds on the investigation initiated by Hall and Frame (2010), which found that Scottish harbour seals were exposed to domoic acid (DA), a potent natural neurotoxin produced by phytoplankton. Using the same sample collection technique to gather urine and faecal material from various populations around Scotland with differing population trajectories (Lonergan et al., 2007), the objective was to investigate not only exposure to DA, but also other groups of toxins such as paralytic shellfish poisoning (PSP) toxins and the lipophilic toxins okadaic acid (OA) and dinophysis toxins (DTXs). Toxins from harmful algae are thought to be potential causative factors in the on-going harbour seal decline in some regions of Scotland (Hall and Frame, 2010).

This investigation was initiated because *Pseudo-nitzschia* (which produces DA) in particular was found to be highly prevalent in the phytoplankton communities in Scotland (Fehling et al., 2004; Stobo et al., 2008) and indeed was first observed at increased concentrations at around the time the harbour seal populations were observed as declining (Lonergan et al., 2007; Stobo et al., 2008). Prior to 2000 harbour seal populations in Scotland were largely stable or increasing. In addition DA exposure has had a devastating effect on the California sea lions (CSL) from the US west coast, where morbidity and mass mortality has occurred as a result of exposure (Goldstein et al., 2008; Gulland et al., 2002; Lefebvre et al., 1999; Scholin et al., 2000). In addition to the toxin analysis in urine and faecal samples, blood samples were collected and health parameters such as white blood cell and differential cell counts were investigated. Plasma cortisol concentrations and parasite faecal egg counts were additionally investigated as parameters indicative of adrenal function and parasite burden. CSL exposed to DA have significantly lower blood cortisol levels and higher eosinophil counts (Gulland et al., 2012) so it was possible that these indicators of effects might also be seen in the harbour seals. High parasite loads are often associated with high eosinophil levels (Klion and Nutman, 2004) so these data were needed to ensure any positive

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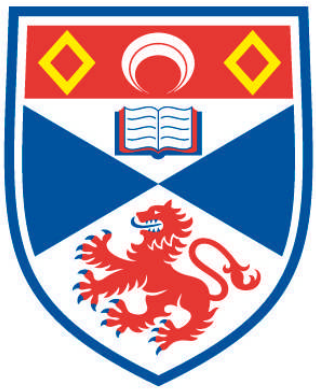

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relationships found were linked to DA and were not a consequence of parasite burdens.

The work presented in this thesis highlights the effect of exposure to the neurotoxin DA and documents that Scottish harbour seals are exposed to multiple toxins such as PSP toxins, OA and DTX-2 (Chapter 2, Chapter 4). Immunomodulatory effects of DA exposure such as lymphocytopenia and monocytosis are also reported. In general, harbour seals from the east coast and Northern Isles, where the decline in abundance has been greatest, had higher levels of DA in their excreta than animals from the west coast. The concentrations in the faeces and urine samples were generally low but time since exposure was unknown. Uptake of DA, PSP toxins, OA and DTXs in randomly selected fish from the east coast of Scotland in the Firth of Forth was investigated (Chapter 3), where benthic, flat and pelagic fish are shown to be vectors of toxin transfer and emphasis is drawn to flatfish as they seem to accumulate higher levels of toxins than the other species analysed. This indicates that harbour seals foraging off the east coast are likely to regularly encounter toxic prey that could impair their health.

In addition to live captured harbour seals, samples from dead stranded marine mammals (including cetaceans and in particular harbour porpoise) found a range of species in Scottish waters were exposed to both DA and PSP toxins (Chapter 4). A monitoring tool to rapidly determine chronic DA exposure in blood samples was published recently for DA exposed CSLs and in Chapter 5 this monitoring technique was attempted in phocid seals, and where it failed to be replicated or validated which questions its function as a DA monitoring tool. Questions regarding how quickly a marine mammal excretes DA from the body have been discussed in the literature and in Chapter 6 an experiment was set up to measure the clearance of DA by using a biomarker (Iohexol). Iohexol was successfully measured in plasma samples from captive harbour seals following oral intake, where concentration and time of the iohexol peak was identified together with the calculation of its half-life. These results indicate the approximate elimination rate of DA (and potentially other hydrophilic toxins) and can be used to better interpret urine levels of DA

measured in wild caught harbour seals. Collectively the results of this research will enable the risk posed by the ingestion of various toxins present in the Scottish marine food chain to marine mammals (particularly harbour seals but also harbour porpoise and grey seals) to be assessed.

List of figures

List of tables

List of frequently used abbreviations

ASP	Amnesic Shellfish Poisoning
CSL	California sea lions
DA	Domoic acid
DSP	Diarrhetic Shellfish Poisoning
DTX	Dinophysis-toxin
ELISA	Enzyme Linked Immunosorbent Assay
FLD	Fluorescence detection
g	Gram
HAB	Harmful Algal Bloom
UHPLC	Ultra High Performance Liquid Chromatography
Ig	Immunoglobulin
IQR	Interquartile range
Kg	Kilogram
LC-MS	Liquid chromatography–mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
Mg	Milligram
Ml	Millilitre
Ng	Nano gram
OA	Okadaic acid
PDV	Phocine Distemper Virus
PSP	Paralytic shellfish poisoning
PV	Harbour seal
SMRU	Sea Mammal Research Unit
STX	Saxitoxin
µg	Microgram
UK	United Kingdom
UME	Unusual Mortality Events
WBC	White blood cell count

Acknowledgements

During my educational training I have been told that it will be a difficult life trying to succeed as a marine biologist. I have to disagree, I have followed my heart and my dream and the experiences I have had both during my master and my PhD training, have gone beyond anything I could ever imagine or dream of. These last three years have been an unforgettable time, where I moved from Norway to Scotland, to follow my long lasting dream to pursue a study for a PhD degree. Three years of long hours in field, in the lab and in the office has come to an end. I am so proud of this work.

First and foremost I offer my sincerest gratitude to my supervisor Dr. Ailsa Hall. Thank you for supporting and being so encouraging throughout the years (before and during my PhD). I thank you for always being optimistic and smiley and for teaching me how to become a better scientist, you are a true inspiration.

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Chapter 1

1. General Introduction

1.1 Decline in the abundance of harbour seals in Scotland

Harbour (also called common) seals (*Phoca vitulina*) have the most widespread distribution of any phocid seal. There are four subspecies of harbour seals worldwide (Burg et al., 1999; Stanley et al., 1996; Westlake and O'Corry-Crowe, 2002) with the European subspecies *(Phoca vitulina vitulina)* ranging from northern France in the south, to Iceland in the west, Svalbard in the north and the Baltic Sea in the east.

The harbour seal is the smaller of the two seal species found in the UK; the other one is the larger grey seal (*Halichoerus grypus*). Harbour seals can live up to 30 years, and an adult harbour seal weighs around 80-100 kg. They have a diet that varies seasonally and differs among regions, with sandeels, gadoids, flatfish and cephalopods making up their main prey (Hall et al., 1998; Thompson et al., 1996; Wilson, 2014). The Sea Mammal Research Unit (SMRU) monitors the trends in abundance of UK harbour seal populations during their annual moult in August using aerial photography and thermal imagery to count the numbers hauled out on land. This gives a minimum population size and an index from which to determine trends. Amalgamated counts from 2007 to 2012 give a minimum estimation of 26 836 harbour seals around the British Isles (SCOS, 2013). Around 30% of the European harbour seal population is found in the UK (SCOS, 2013). In 2005 almost 50% of the British harbour seal population was found in Scotland. Today, nearly 80% of the Scottish population inhabit the islands of Orkney and Shetland, as well as the west coast of Scotland. Since 1999 the harbour seal population in eastern coastal waters around Scotland has declined significantly, especially around Orkney and Shetland. Although the Moray Firth population has been continually stable in the last few years, other harbour seal populations on the east coast are declining (Lonergan et al., 2007; SCOS, 2013). For example,

harbour seal populations on the east coast in the Tay and Eden estuaries and in the Northern Isles (Orkney and Shetland) in particular have decreased by up to 85% between 2000 and 2010, representing an average rate of decline of up to 18% per annum (SCOS, 2013). Whilst many factors have been considered as causes for these declines, one that remains of particular interest is the effect of harmful algal bloom toxins on harbour seal health and survival (Hall and Frame, 2010). This potential causal factor is therefore the focus of this thesis.

1.2 Harmful algal blooms

Phytoplankton are single-celled plants that form the base of the marine food chain. A rapid increase in phytoplankton growth is called an ‘algal bloom’, which consists of dinoflagellates or cyanobacteria (a blue-green algae), but other algae like diatoms may, under the right environmental conditions (light, nutrient, temperature, etc.), also proliferate to form blooms. There are two different seasonal blooms, the spring and autumn bloom. After winter nutrient supplies are mixed up to the surface layer, and when the spring begins, increased sea temperature and longer daylight hours become excellent conditions for phytoplankton to grow. Diatoms often dominate spring blooms.

The term Harmful Algal Blooms (HABs, also called red tides) refers to phytoplankton species that produce toxins. Around 40 species of phytoplankton are known to produce a toxin, although as many as 200 species are thought to be harmful or toxic (Hallegraeff, 1993). There are two distinct aspects to HABs: firstly, they cause hypoxia-like oxygen depletion due to either excess organic matter, which reduces oxygen dissolved in the water (Hallegraeff, 1993), that in return can cause mortality of benthic animals and fishes (Glibert et al., 2002; Granéli et al., 1989) or secondly blocking of light for subsurface communities (Landsberg, 2002). But the other main aspect of HABs is the production of toxins. Hypoxia, although potentially resulting in major fish kills, which could affect marine mammals through the mortality of their prey, will not be considered in the context of this thesis.

In certain regions in the North-East Atlantic and North Sea, there has been a noticeable increase in phytoplankton biomass (Bresnan et al., 2013), although the occurrence of phytoplankton in the water that can produce toxins does not necessarily mean that toxins are continually present, and when HABs do occur, nutrients are required for the bloom to be sustained. Eutrophication (especially caused by increased nitrogen and phosphorus) and degraded water quality is thought to be a main factor triggering such blooms (Anderson, 1989; Anderson et al., 2008; Anderson et al., 2002; Glibert and Burkholder, 2006; Heisler et al., 2008). Most of these HABs do not harm people wildlife or the environment, but some of them do. Marine algal toxins are accountable for between 50 000 - 60 000 toxic events per year. It is unclear why toxins are produced by phytoplankton, but it has been suggested that they prevent grazers from feeding on them (Bergkvist et al., 2008). With only 2 % of the world's phytoplankton taxa are known to be toxic (Smayda, 1997), HABs are reported from every part of the world and are increasing in frequency and intensity (Hallegraeff, 1993; Landsberg, 2002; Van Dolah, 2000). In Scottish waters closures due to HAB have increased since the mid 1990's (Stobo et al., 2008; Whyte et al., 2014), along with increased toxin monitoring. Today there are no clear trends regarding an increase or a decrease in toxin distribution, but there is a lot of variability between years with some groups decreasing and other toxins showing high concentration for the first time (Bresnan et al., 2013).

1.3 Routes of exposure to HAB toxins

Filter-feeding marine organisms such as shellfish and plankton-eating fish ingest these algae and act as vectors for transferring the toxin up the food chain. This can lead to mortality, throughout the food chain and pose a threat to the balance of the ecosystem. Toxic events are usually recognised by large-scale mortalities of benthic animals, fish, marine mammals and sea birds, sometimes together with human illness or death following ingestion of toxic phytoplankton either directly or via prey (Anderson and White, 1992;

Bargu et al., 2011; Bates et al., 1989; Bossart et al., 1998; DeGange and Vacca, 1989; Finucane, 1964; Fire et al., 2010b; Geraci et al., 1989; Glibert et al., 2002; Granéli et al., 1989; Gulland et al., 1998; Hernández et al., 1998; Konovalova, 1993; Konovalova, 1989; Landsberg, 2002; Lefebvre et al., 1999; O'Shea et al., 1991; Scholin et al., 2000; Sierra-Beltràn et al., 1997; Silvagni et al., 2005; Steidinger, 1983; Work et al., 1993). Although most shellfish are not affected even though they filter toxic phytoplankton, humans or marine animals consuming the shellfish may become ill.

HABs produce toxins that can cause various human illnesses depending on the nature of the toxin and cooking of the shellfish does not usually eliminate many of these toxins as they are heat stable (Isbister and Kiernan, 2005). In general they are neurotoxins but may also cause additional effects on other systems such as the respiratory system and heart. Diseases known as Paralytic Shellfish Poisoning (PSP), Diarrheic Shellfish Poisoning (DSP), Ciguatera Fish Poisoning, Amnesic Shellfish Poisoning (ASP) or Neurotoxic shellfish poisoning (NSP) are the most common outcomes in humans. Generally toxic algae are present in low concentrations having little effect on human health; toxicity therefore depends on their presence in high cell concentrations (Van Dolah, 2000).

1.4 Occurrence of HABs in Scotland

In spring and autumn, algae in the sea give rise to blooms around Scotland. Some of these result in toxins produced by harmful algae. In the European Union (EU) there are three major toxin groups that can cause significant health effects in mammalian systems (including marine mammals and humans): ASP, PSP and DSP toxins including Okadaic acid and Dinophysis Toxins.

As an EU member state, the UK is obliged to monitor the presence of marine toxins in shellfish as well as toxin-producing phytoplankton. In England and Wales, the Food Standards Agency (FSA) is the authority that enforces the legislation, and in Scotland the Food Standard Agency Scotland (FSAS) is the

national competent authority. The Official Control Biotoxin Monitoring Programme for Scotland is carried out by The Centre for Environment, Fisheries and Aquaculture Science (Cefas), and the Scottish Association for Marine Science (SAMS) are in charge of the delivery and reports on the phytoplankton aspect of the Scottish biotoxin monitoring programme (Centre for Environment, 2011; Swan and Davidson, 2011).

The most harmful toxins arise from the PSP toxin group and ever since an outbreak in the North east of England in 1968 where 78 people were admitted to the hospital displaying PSP symptoms, toxins from this group have been monitored (Ayres and Cullum, 1978). Toxins from the ASP and DSP group have also been monitored in more recent years (Gallacher et al., 2000; Turrell et al., 2007). However, toxins from the PSP group remain the most important toxin to monitor due to their potential to harm wildlife and humans and in Scottish waters almost every year shellfish farms are closed due to high concentrations of PSP toxins (exceeding the European Community regulatory limit of 80 μg STX equivalents (eq) 100 g^{-1} of shellfish flesh) (Stobo et al., 2008). Since the monitoring of DSP started in 1992 there has been an annual recording of this toxin in Scottish waters (Stobo et al., 2008). It has been known that toxins from the ASP group could have existed prior to the monitoring, that first commenced in 1998. Shellfish with DA concentrations above the regulatory limit (20 μg ASP toxins g^{-1} shellfish flesh) occurred since 1999 with a peak around year 2000 (Stobo et al., 2008). Interestingly, this is around the time when the Scottish harbour seal population in some regions also started declining (Lonergan et al., 2007)

1.5 Pseudo-nitzschia blooms, Domoic Acid and Amnesic Shellfish Poisoning

Pseudo-nitzschia is a cosmopolitan marine diatom that exhibits a range of physiological tolerances. Diatoms are microalgae encased with a siliceous cell wall (SiO2) called the frustule. Diatoms often dominate spring blooms, and due to their immobility they require turbulence to keep them in a well-mixed

water column. Under certain conditions, when the bloom has reached its peak and nutrients become limited, toxin can be produced. *Pseudo-nitzschia* is now a common phytoplankton in Scotland (Stobo et al., 2008). *Pseudo-nitzschia* seems to bloom when it can out-compete other algae in conditions when silica is available or the temperature is falling, while other algae need warmer temperatures, low salinity and calmer sea to bloom (Bates et al., 1998; Mos, 2001). Diatoms from the *P. nitzschia* group are considered to be one of the most harmful algae to marine mammal health. Not all species of *P. nitzschia* produce toxin, but those that do, produce the marine neurotoxin Domoic Acid (DA).

DA is a water-soluble compound produced by at least eight species of the genus *P. nitzschia* spp. and the microalgae *Chondria armata*. Symptoms such as memory loss caused by DA intoxication gave the associated illness the name ASP. DA causes neuronal degeneration and necrosis in specific sections of the hippocampus. DA has been found all around the world in the last decade and there have been several reports of human intoxication where *P. nitzschia* was involved (Bates et al., 1989; Cendes et al., 1995; Todd, 1993). The first DA intoxication was in 1987 when three people died and over 100 became sick from eating mussels from Prince Edward Island, Canada (Bates et al., 1989; Bates et al., 1998). Domoic acid is also one of the biggest health threats to California sea lions (*Zalophus californianus*) on the US west coast, and the effect it has depends on the amount of toxic prey the sea lions eat (Lefebvre et al., 1999). Vectors such as krill, crab, shellfish and finfish are known to filter and accumulate the toxic algae and transfer it to top predators (Landsberg, 2002).

1.6 Alexandrium blooms, Paralytic Shellfish Toxins and Paralytic Shellfish Poisoning

The microscopic marine dinoflagellate genus *Alexandrium* poses the greatest concern for top predators due to its toxicity and it is the only toxic dinoflagellate known to cause PSP found in Scottish waters. A bloom from this genus has been known to occur for several decades on the southeast

coast of Scotland (Stobo et al., 2008). There are 30 recognized species of the genus and at least eight species produce PSP toxins; (A. *acatenella*, A. *catenella*, A. *cohorticula*, A. *fundyense*, A. *ostenfeldii*, A. *minutum*, A. *tamarense* (= A. *excavatum*) and A. *tamiyavanichi*) (Anderson, 1998), where four species have been recorded in Scottish waters (A. *tamarense*, A. *minutum*, A. *ostenfeldii* and A. *tamutum*) (Collins et al., 2009). The life cycle of *Alexandrium* is important in understanding its toxicity, and consists of nine stages, which include both motile, and cyst phases. One aspect that increases its success to bloom is the cyst phase, and its ability to reside on the bottom sediment when growth conditions are unfavourable as seen in Fig. 1.1 from Anderson et al., (1996).

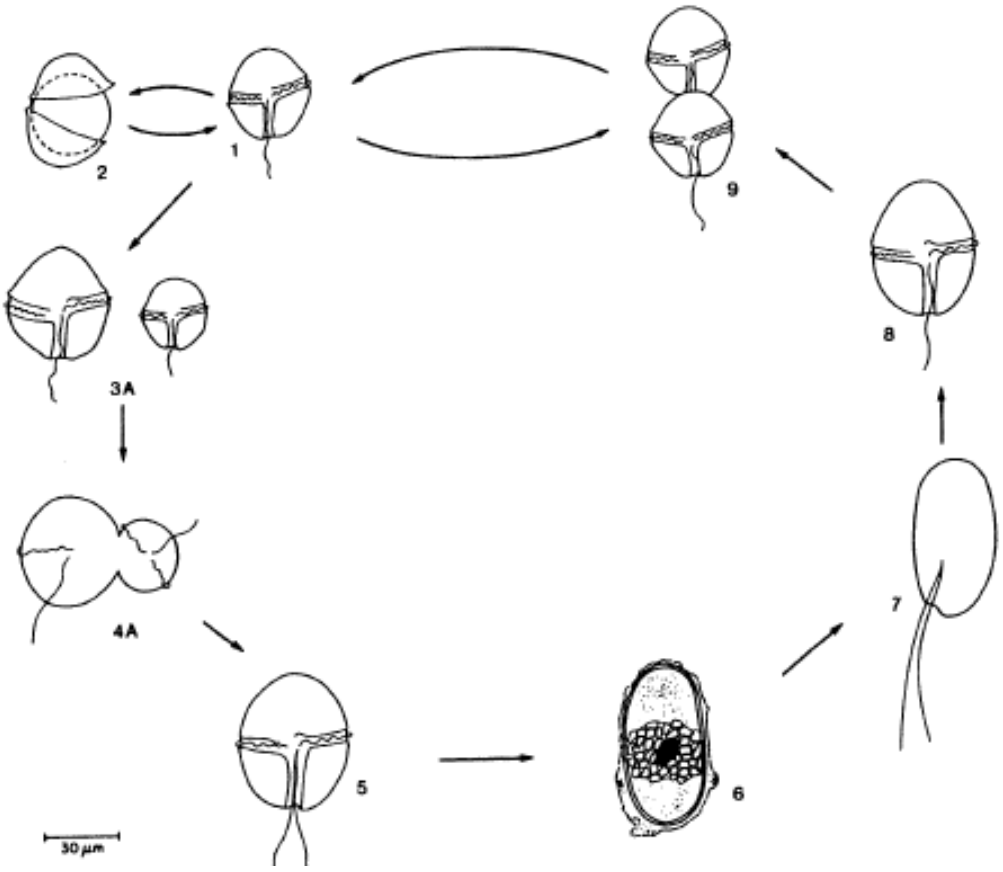

Figure 1.1: Life cycle diagram of *Alexandrium tamarense*. Stages are identified as follows: (1) vegetative, motile cell; (2) temporary or pellicle cyst; (3) anisogamous "female" and "male" gametes; (4) fusing gametes; (5) swimming zygote or planozygote; (6) resting cyst or hypnozygote; (7 & 8) motile, germinated cell or planomeiocyte; and (9) pair of vegetative cells following division. Adapted from Anderson et al.,(Anderson et al., 1996).

The motile stage of the cells develops by binary fission and blooms occur when conditions are favourable. If conditions are poor (e.g. low temperature or changes in salinity) the motile species can go into a ‘’temporary cyst’’ which allows them to avoid unfavourable short-term fluctuations in the ocean (Anderson, 1998). Higher levels of toxicity have been associated with a limitation of phosphorous or other nutrients (John and Flynn, 2000). In Scotland *Alexandrium* produces toxins whilst in the planktonic phase of the life cycle (Wyatt and Jenkinson, 1997).

PSP toxins are neurotoxins that block sodium channels in nerve axons interfering with signal transmission, causing paralysis or death. Toxic *Alexandrium* species normally produce more than one PSP toxin derivative that can cause the devastating PSP. Blue mussels (*Mytilus edulis*) are normally used as a sentinel for monitoring these toxins as they are relatively insensitive to PSP toxins and will keep grazing when other shellfish stop (Bricelj et al., 1990). However in Scotland King Scallops (*Pecten maximus*) are used for this monitoring. Historically there have only been a few cases of PSP events in Scottish waters (Table 1.1) where shellfish production had to be closed due to the presence of PSP toxins, but in Orkney there have been several events of PSP over the years (Töbe et al., 2001). In 1968, 80 % of the Shags (*Phalacrocorax aristotelis*) on the Farne Islands on the northeast coast of England, were killed (Coulson et al., 1968). Fish are reported to also carry STX and therefore cause sickness when ingested, where the fish gut is usually the toxic part (Adnan, 1984; Nakamura et al., 1984).

1.7 Dinophysis spp. blooms, lipophilic toxins and Diarrhetic Shellfish Poisoning

Dinophysis spp. are normally a temperate genus with over 200 species, six of which can be found in Scottish waters (Hart et al., 2007; Larsen and Moestrup, 1992). *Dinophysis* spp. seems to puzzle taxonomists since they form morphotypes, which compromise taxonomic identification. *Dinophysis* spp. appear throughout Scottish coastal waters in relatively low numbers and are indigenous to offshore waters and sea lochs.

Dinophysis spp. are known to produce several derivatives of toxins known as dinophysistoxin (DTX1-4) together with okadaic acid (OA) (James et al., 1999; Landsberg, 2002). Okadaic acid is a lipophilic toxin that causes DSP associated with the algae genera from the planktonic dinoflagellate *Dinophysis* and the epibenthic dinoflagellate *Prorocentrum*. Shellfish (primarily bivalve filter-feeding molluscs) consumption of toxins in northern Europe and subsequent DSP illness has been recorded from 10 countries. Monitoring programs try to minimize the impact of DSP, mostly to protect public health and although there are no known mortalities recorded due to DSP, monitoring the toxin has increased because it is becoming a threat in Scottish waters (Table 1.1). In laboratory animals, these toxins cause epithelial damage and fluid accumulation in the gastrointestinal tract. These substances have also been shown to be tumour promoters (Fujiki et al., 1988; Suganuma et al., 1988b). OA and some of its derivatives are potent inhibitors of protein phosphatases, which play many roles in cellular function. The effect that DSP has on marine organisms is, however, poorly understood.

Table 1.1: Overview over the main Harmful algal bloom (HAB) species in Scotland, the toxic syndrome, effect of toxic ingestion, location of outbreaks, intensity of the outbreak and the pharmacologic target.

Organism	**Syndrome**	**Effect**	**Where (effect)**	**Intensity of problem**	**Pharma-cologic target**
Pseudo-nitzschia spp.	ASP	DA intoxication of shellfish and ASP danger to humans	Islay to east of Aberdeen	Endemic, causing long-duration shell-fishery closure	Glutamate receptors
Alexandrium spp.	PSP	STX intoxication of shellfish and PSP danger to humans	Mostly east coast to Northern Isles	Endemic; a common cause of shell-fishery closure	Voltage dependent sodium channel Site 1

Dinophysis spp.	DSP	OA intoxication of shellfish and DSP danger to humans	Widespread	Occasional cause of shell- fishery closure	Serine/threonine protein phosphatases

1.8 Effect of toxins on marine mammals

Marine mammals feed on prey, often shared with humans including various species of fish, cephalopods and shellfish which makes marine mammals good sentinels for likely effect of toxins in seafood (Goldstein et al., 2008).

1.8.1 Domoic acid

In laboratory studies mice, rats and monkeys have been reported with symptomatic responses after exposure to DA, where the more common symptoms were scratching, tremors and seizures (Iverson et al., 1989; Scallet et al., 1993; Tryphonas et al., 1990). For birds and mammals natural exposure to DA causes symptoms such as, seizures, vomiting, amnesia, behavioural abnormalities and in the worst cases coma and death follows (Fehling et al., 2004; Goldstein et al., 2008; Gulland et al., 1998; Scholin et al., 2000; Tryphonas et al., 1990).

1.8.2 Paralytic Shellfish Poisoning toxins

Ingesting contaminated mussels or fish containing toxins (PSP toxins) can cause a potentially lethal disease, PSP. Clinically this is characterized by symptoms such as ataxia, tingling, numbness, drowsiness, incoherence, gastrointestinal malfunction and muscular weakness (Andrinolo et al., 1999). Respiratory depression is the most severe symptom where death can rapidly occur (Clark, 1968; Coulson et al., 1968).

1.8.3 Lipophilic and other toxins

DSP is the most common intoxication caused by contaminated shellfish in Europe, where OA and DTX are common lipophilic toxins causing gastro abdominal disturbances (Gerssen et al., 2009). After ingestion, OA binds to protein phosphatase receptors which build up phosphorylated proteins which can then lead to clinical symptoms of DSP like fatigue, weakness, cramps, vomiting, abdominal pain and diarrhoea (Larsen and Moestrup; Yasumoto et al., 1984).

Brevetoxin (PbTxs) are neurotoxins produced by the dinoflagellate *Karenia brevis*. Toxic blooms from this genus are known to cause fish mortalities and death of marine mammals (Flewelling et al., 2005). PbTxs have not been detected in Scottish waters and will not be tested for in this thesis (Cook et al., 2010).

1.8.4 Acute effects of toxin exposure

Acute effects, defined as when an organism is suddenly exposed to a substance, are characterised by the onset of severe symptoms that develop quickly and can lead to a health crisis. Symptoms usually subside when exposure stops. Acute toxic exposures are known to have dramatic effects in humans as was evidenced on Prince Edward Island after humans consumed toxic mussels (Perl et al., 1990). Behaviour, pathological or physiological changes often occur when animals are exposed to high concentrations of toxin, exposure can also lead to mortality (Landsberg, 2002). In the US following a HAB event California sea lions may be found stranded alive, showing neurological signs like ataxia, head weaving, seizures or coma indicating DA toxicosis (Gulland et al., 2002). During a mass stranding in 1998, and an even larger stranding event in 2002 together with strandings in 2000, 2001 and 2005 almost a third of the females experienced prenatal reproductive failure (Goldstein et al., 2008; Gulland et al., 1998). Foetuses are in danger of repeated direct exposure through the amniotic fluid from pregnant females that have ingested DA. Experimental exposure to DA in laboratory

animals has shown abnormal development of brain neurons which is likely to occur at the completion of neurodevelopment of the foetus (Ramsdell and Zabka, 2008). In a study in mice by Ryan et al., (2005), gene expression was shown to be affected following acute DA exposure. Experimental studies in monkeys confirms that clinical signs together with brain lesions occur which may also inflict excitotoxic central nervous system damage (Tryphonas et al., 1990).

In a Finnish lake, a cyanobacterial (*Anabaena lemmermannii*) bloom occurred between 2002 and 2003, where symptoms such as eye irritation, fever, skin rash and abdominal pain were reported in children after they had been swimming in the lake and had been exposed to the bloom. These symptoms do not represent typical PSP symptoms, but rather acute effect due to a toxic bloom (Rapala et al., 2005). There have been no reported mortalities in humans caused by drinking contaminated water with PSP toxins. However, in Australia 14 sheep died after drinking contaminated water with the neurotoxic *Anabaena circinalis* which produces saxitoxin (Negri et al., 1995). Acute cases of PSP can be hard to track because of the lack of post mortem indications of organ injury due to the respiratory failure effect PSP can have. In Scotland, there have been six cases of poisoning of the neurotoxin anatoxin-a in dogs where they had either swum in a contaminated loch, or walked along the shore. All the dogs showed nervous symptoms of acute PSP. The neurotoxin was isolated from the dogs' stomach (Gunn et al., 1992). In 1990 a PSP epidemic occurred in Guatemala where 26 people died from eating clams contaminated with saxitoxin (Rodrigue et al., 1990).

Diarrhetic Shellfish Poisoning is a milder seafood poisoning where the main acute symptoms are gastrointestinal signs like diarrhoea and vomiting. Generally the symptoms resolve within two to three days (Dawson and Holmes, 1999; Lange et al., 1989). Stranded manatees (*Trichechus manatus latirostris*) and green sea turtles (*Chelonia mydas*) were found exposed to OA in Florida, US between 2003 and 2006 (Capper et al., 2013), although the exposure was not the cause of death, questions regarding if these toxins can compromise their health did arise from these findings. To date OA and DTXs

have not been shown to have any negative effect on marine mammal health, but OA was detected in an unusual mortality event affecting bottlenose dolphins (*Tursiops truncatus*) in Texas (Fire et al., 2010b).

1.8.5 Chronic effects of toxin exposure

Chronic effects are often characterized by continuous or repeated exposure (days, months or years) to a low concentration of a substance. Symptoms will not always be obvious and recent observations have outlined a chronic disease in California sea lions where epilepsy and unusual behaviours are seen (Goldstein et al., 2008; Ramsdell and Gulland, 2014). This chronic disease may be a result of acute exposure but is diagnosed in animals outside a large HAB event. Animals strand individually rather than as part of a mass mortality event and show hippocampal atrophy with necrosis in the amygdala and sometimes the olfactory bulb (Ramsdell and Gulland, 2014). The lack of information about low dose exposure to toxins from harmful algae is of concern. However, a recent long term, low level DA exposure study in zebra fish (Hiolski et al., 2014) found that gene transcription involved in neurological function and development was altered. Although the gene transcription changes were not consistent across all exposure levels, the most notable finding was that central nervous system mitochondrial function, based on respiration rates and protein content, was affected. The authors concluded that this might indicate fundamental cellular level impacts from repeated low exposure (at levels below those that induce seizures or behavioural changes) that could have chronic health implications. Existing monitoring programs prevent acute toxin poisoning in humans, but due to very limited knowledge about the chronic effects, little is being done to monitor toxins on a wider temporal or spatial scale. The recent study by Hiolski et al., (2014) does suggest that there may be harmful impacts caused by long-term and low-level exposure and as the frequency of harmful algal blooms increases, the likelihood of marine mammals being indirectly exposed (e.g. via low toxic level prey) to toxins increases. When organisms are exposed to lower level, repeated toxin contact, the toxin may not be excreted completely and

repeated exposures without total depuration may, over time, exceed the threshold of toxicity and cause chronic effects (Goldstein et al., 2008; Landsberg, 2002).

These low dose, or repeated exposure levels are not well described and experimental results are varied, but effects have been found to be more common in adult animals (Scallet et al., 1993; Sobotka et al., 1996). Recent observations have described chronic disease in juvenile California sea lions linked to DA ingestion possibly through milk (Goldstein et al., 2008; Maucher and Ramsdell, 2005). The numbers of stranded California sea lions are increasing, and especially the proportion suffering from chronic effects. California sea lions have shown neurological signs after DA exposure such as sporadic seizures, and unusual behaviours and pathological changes have been documented (Goldstein et al., 2008). Low doses by systemic injection of DA (in rats) have been shown to produce clinical signs like seizures, fatigue, lack of appetite, muscular twitching and behavioural changes together with mild lesions and brain gliosis (Goldstein et al., 2008; Scallet et al., 1993; Sobotka et al., 1996). How relevant these findings are for low level oral exposure is difficult to assess.

Although saxitoxins have only shown acute effects in mammals, it seems that prior exposure to non-lethal doses of PSP toxins can lower the susceptibility of rats to lethal doses as noted by Parkas et. al. (1971) . However no other information on sub chronic or chronic effects of PSP toxins exists for animals.

As mentioned earlier DSP is known to be a short-term acute disease, but interestingly, there is increasing evidence suggesting the role of OA-like polyether toxins as tumour promoters in skin and stomach in both humans and wildlife (Landsberg, 2002; Suganuma et al., 1988b). Thus again, low-level chronic exposure should induce cellular changes that have long-term effects on individual fitness.

1.9 Aims of this study

Exposure to toxins from harmful algae has caused devastating health effects and mortality in marine mammals in several places around the world (Doucette et al., 2006; Doucette et al., 2012; Gulland, 2000; Gulland et al., 2012; Hernández et al., 1998; Scholin et al., 2000). Because of these observed effects, there has been an interest in studying these toxins and their involvement in the decline of the harbour seal populations in Scotland. Currently it is not known why the population is declining so rapidly (SCOS, 2013), particularly after a period of stability in many regions, but after 2000 a change may have occurred which led to the populations in Orkney, Shetland and on the east coast in particular, to decline. Interestingly, the timing fits when DA in Scottish waters reached its peak and have also occurred over the regulatory limit on an annual basis since then in Scotland (Stobo et al., 2008).

If the Scottish harbour seals are exposed to a range of toxins this study will provide information on the effect this may have on their health and what type of prey are likely to be the most important vectors, how quickly the toxins are cleared by the seals (i.e. the depuration rate or half-life) and how any exposure in harbour seals compares to other top predators such as cetaceans and grey seals. The results from this thesis will help with the conservation and management of this now vulnerable population.

1.10 Thesis structure

The main goal of this thesis was to investigate the exposure of harbour seals to toxins from harmful algae in Scotland and investigate their transfer through the marine food chain to these and other top predators.

The first objective was to investigate multiple algal toxin exposure to harbour seals (Chapter 2). Chapter 2 measures toxins in various matrices (largely faeces and urine) for the major groups of toxins currently identified in Scottish waters (mainly DA, PSP toxins and lipophilic toxins) to determine the types and quantities of toxins the harbour seals are exposed to and relate the levels

found in the excreta to health parameters in individuals, particularly immune and endocrine endpoints. Anonymous faecal samples collected from harbour seal haul-out locations were gathered on a regular basis during spring, summer and autumn to better understand the temporal and spatial variation in the toxin exposure.

The second objective was to investigate randomly selected fish from the east coast of Scotland to determine the concentration of toxins accumulated by the fish and what prey species are likely to be of most importance as vectors (Chapter 3). The results will then be compared to the diet of harbour seals from the same region, recently studied in a PhD project by Wilson (2014).

The third objective was to examine toxin exposure in stranded marine mammals in collaboration with the Scottish Marine Animal Stranding Scheme (Chapter 4). Faecal material, urine and other body fluids (such as thoracic and amniotic fluid) are collected during the post mortem examination of dead marine mammals. This enabled me to compare the levels found in harbour seals with other top predators foraging in the same region but perhaps in different locations and feeding on different prey.

The fourth objective was to assess a method to study the historic exposure of Scottish harbour seals to DA by using archived harbour seal blood samples (Chapter 5). This method ostensibly detects a naturally occurring, DA specific antibody produced when an animal is chronically exposed to DA. The objective of Chapter 5 was firstly to verity the method published for California sea lions to see if this could also be detected in harbour and grey seal samples and to study chronic DA exposure and to determine whether exposure rates have increased over the same time frame as the decline in abundance.

The fifth objective was to assess how quickly DA clears from the harbour seals by measuring a substrate (iohexol) that has been shown to clear out of the body at the same rate as DA (Chapter 6). The results of this study will

then allow me to more readily interpret, particularly the urinary results, from the live captured harbour seals.

Chapter 2

2. Exposure of Harbour Seals to multiple Algal Toxins in Scotland

2.1 Introduction

2.1.1 Domoic acid in marine mammals

Pseudo-nitzschia diatoms are a common part of the phytoplankton community in Scotland, but only some *Pseudo-nitzschia* species produce DA (Fehling et al., 2006). Monitoring for DA in shellfish production areas in Scotland started in 1998 and it has since been detected in Scottish shellfish above the regulatory limit (20 mg DA/kg of shellfish meat) (Stobo et al., 2008; Tett and Edwards, 2002). Exposure to DA by top predators such as seals can occur in two ways; acute and chronic exposure. Acute exposure can happen when a rapid absorption of large amounts of DA is involved. Such acute exposure can cause severe impacts such as seizures, coma reproductive failure and death (Geraci et al., 1989; Goldstein et al., 2008; Goldstein et al., 2009; Scholin et al., 2000), while heart disease, chronic epilepsy and reproductive failure can come from repeated more long-term (chronic) exposure to DA (Brodie et al., 2006; Goldstein et al., 2008; Goldstein et al., 2009; Gulland et al., 2002; Lefebvre et al., 1999; Scholin et al., 2000; Silvagni et al., 2005). In humans DA triggers the condition ASP and can cause neuronal degeneration and necrosis in specific parts of the brain. After the outbreak of illness in humans caused by the consumption of DA toxic blue mussels in Canada in 1987 the regulatory level was established for human consumption (Bates et al., 1989). In the US, acutely DA exposed California sea lions may show neurological signs such as ataxia, head weaving, seizures or coma which together indicate DA toxicosis (Gulland et al., 2002). Haematological parameter changes have also been documented in CSLs following DA toxicosis, but the physiological mechanism underlying this is not well understood (Gulland et al., 2002). For

example, Gulland et al., (2012) found that eosinophil counts were significantly higher in CSLs showing clinical signs of DA toxicity, and this was also reported for bottlenose dolphins in the northern Gulf of Mexico (Schwacke et al., 2010). Studies have suggested that pregnant CSL females may have higher exposure due to sequestration of the toxins in the amniotic fluid and that DA causes reproductive failure (Brodie et al., 2006; Goldstein et al., 2008; Goldstein et al., 2009). In addition, endocrinological changes have also been documented when CSLs are exposed to DA as it also affects the adrenal gland, lowering the production of cortisol (Gulland et al., 2012; Gulland et al., 2002).

2.1.2 PSP toxins in marine mammals

PSP toxins and its derivatives produced by the dinoflagellate *Alexandrium* pose the greatest potential concern for seals in Scotland due to their highly lethal effects. With a lack of chronic toxicity data on PSP toxins the European Food and Standards Authority derived an oral acute reference dose (ARfD) of 0.5 µg STX equivalents/kg b.w. in humans (Alexander et al., 2009). Canids, considered to be evolutionarily and physiologically similar to seals, have a lethal dose of 180 – 200 µg STX/kg b.w. (McFarren et al., 1961). Mice have an acute oral LD_{50} dose of 263 µg STX/kg, humans have a minimum oral dose of 7–16 µg of STX/kg body weight (Levin, 1992; Schantz et al., 1975), but mortal cases in humans linked to PSP poisoning have been observed in humans with oral doses of between 500 – 12400 µg STX/kg body weight (Meyer, 1953). Since the outbreak of PSP in the UK in 1968 where 80 % of the Shags on the Farne Islands on the northeast coast of England, were killed (Coulson et al., 1968), a programme has been set up to monitor the Scottish coast for the presence of *Alexandrium* in the vicinity of shellfish aquaculture sites. Concurrently, shellfish were also checked for the presence of PSP toxins using the mouse bioassay (MBA). Although toxic events have been very sporadic in Scottish waters, no known human cases of PSP have been reported (Swan and Davidson, 2011). PSP toxins has also been found in a range of organisms such as fish and benthic invertebrates (Landsberg, 2002)

even though *Alexandrium* were absent (Sakamoto et al., 1992). Toxic *Alexandrium* normally produce more than one PSP toxin derivative including STX, gonyautoxin I~VIII, neosaxitoxin (NEO) and decarbamoyl toxins, all of which can cause PSP. The biochemical production of the neurotoxins from the PSP toxin family is complex. PSP toxins bind to the voltage-gated sodium channels in the brain, blocking the flow of ions across the cell membrane. This process inhibits nerve and muscle cells to send electrical signals, which prevents normal cellular function, and this causes paralysis. PSP toxins can also bind to the potassium channel where it modifies the channel gating and reduces the potassium conductance (Cusick and Sayler, 2013; Narahashi et al., 1967). Respiratory depression is the most severe symptom of PSP, following which death can rapidly occur. Evidence of STX exposure in the endangered North Atlantic right whale (*Eubalaena glacialis*) (Doucette et al., 2006) occurred in 2001 where STX was considered to be a contributing factor in the failure of the population to recover from decline. Saxitoxin was also considered to be involved in the sudden unusual mortality event of the Mediterranean monk seals (*Monachus monachus*) off western Sahara in 1997 (Hernández et al., 1998) and humpback whales (*Megaptera novaeangliae*) off Cape Cod Bay, USA in 2001 (Geraci et al., 1989).

2.1.3 Lipophilic toxins in marine mammals

Another group of toxins that causes illness are the lipophilic toxins such as Okadaic acid and its derivatives, the Dinophysis toxins (DTXs). These lipophilic toxins cause the human illness known as DSP and the toxins are produced by the dinoflagellate *Dinophysis* spp. (Larsen and Moestrup, 1992; Yasumoto et al., 1984). Okadaic acid and DTXs are two lipophilic toxins groups that inhibit the serine/theorine protein phosphatases PP1 and PP2A and builds up phosphorylated proteins, the protein phosphatases are essential in regulating the physiological functions such as growth and division in a cell. Chronic exposure to OA and DTXs can result in skin and stomach tumour development and when injected into the brains of rodents it induces neuronal damage reminiscent of that seen in Alzheimer's disease (Fujiki and

Suganuma, 2009; Suganuma et al., 1988a). In laboratory animals, these toxins cause epithelial damage and fluid accumulation in the gastrointestinal tract and can in extreme cases cause death. A maximum tolerated level of these toxins have been set at 160 µg OA equivalents /kg shellfish meat (European Commission (2002)). Regular monitoring of these toxins in shellfish started in 1997 and occasional shellfish closures occur (Tett and Edwards, 2002). To date OA and DTXs have not shown to have any negative effect on marine mammal health, but OA was detected in an unusual mortality event among bottlenose dolphin in Texas (Fire et al., 2010b). Low levels of OA was also detected in stranded manatees in Florida between 2003 and 2006 (Capper et al., 2013).

2.1.4 Multiple toxin exposure in marine mammals

In Sarasota Bay, Florida, USA bottlenose dolphins have been exposed to multiple toxins (brevetoxin and/or DA) on an annual basis since 2000 (Twiner et al., 2011). North Atlantic right whales have been found to be exposed to both DA and STX via consumption of contaminated copepods (*Calanus finmarchicus*) (Leandro et al., 2010) which is thought to have had an effect on their reproduction. However, information on exposure of marine mammals to multiple toxins is sparse. Acute and chronic exposure to individual toxins from harmful algae thus have the potential to compromise an animals' fitness but the potential synergistic effects of multiple toxin exposure, particularly over a long period of time has not been assessed which thus raises questions about the combined health effects. Immunosuppression is thought to be one of the effects that can come from multiple toxin exposure (Twiner et al., 2011). Dragunow et al., (2005) discovered that OA toxicity increases (*in vitro*) in the presence of a toxin produced by the dinoflagellate *Karenia* called gymnodimine. This demonstrates that being exposed to multiple toxins could potentially have a synergetic effect.

2.1.5 Objectives of this chapter

Thus this study (a) builds on the earlier work by Hall and Frame (2010) to investigate the temporal and spatial exposure of harbour seals in Scotland to DA (b) investigates exposure to other potentially lethal toxins including the PSP toxins as well as those that could cause serious health effects (OA and the DTXs) in urine and faecal material from live-captured harbour seals. In addition scat collected from haul out sites provides an indication on the spatial scale of the toxin exposure (c) determines the occurrence of combined exposure to multiple toxins in these populations to a level that might affect their health, population dynamics and therefore cause a decline in abundance (d) investigates the response of the harbour seals to the toxins by examining several health parameters, and (e) examines the levels of the main toxins in potential prey vectors to determine any link between the concentrations and differing degrees of exposure in declining compared to stable harbour seal populations.

2.2 Methods

2.2.1 Sample collection

2.2.1.1 Live captured harbour seals

Urine, faeces and blood samples were collected from live-captured harbour seals (Table 2.1, n =111) between 2008 and 2013, from west Scotland central and south hereby named ‘west coast’. Samples were also collected from the North coast, Orkney and Shetland are hereby named ‘Northern Isles’ and samples from Moray Firth and southern east coast are hereby named ‘east coast’ (Fig. 2.1, West, North and East) with the aim of detecting and quantifying a range of harmful algal toxins (DA, STX and lipophilic toxins, Fig. 2.1, Table 2.1). The harbour seals populations have different abundance trends where the west coast population is stable or increasing in certain areas, while in the Northern Isles and the east coast the population of harbour seals are declining. The harbour seal samples were collected in relation to

capture-release studies carried out by the SMRU under the UK Animal (Scientific Procedures) Act 1986, Project and Personal Licenses. Once captured, all the seals were weighed and anaesthetised using 0.05 mg kg $^{-1}$ Zoletil100 (Virbac, France) intravenously using the extradural vein. Blood samples were collected from the same vein and stored in plain and heparinised blood vacuum tubes (Vacutainers, Becton Dickinson Ltd, UK). A subsample was taken out from the heparinised tube for further haematology work. Each sample was then centrifuged (3,000 x g, 10 min) and serum or plasma was extracted and subsequently stored at -20°C for health analysis. For DA analysis urine was identified as one of the major routes of DA excretion (Suzuki and Hierlihy, 1993), hence urine samples were collected using a catheter. Furthermore faecal samples were also collected manually using a modified plastic pipette when possible.

Table 2.1: Number of live captured harbour seals by region, season (spring = Mar, Apr, May; summer = Jun, Jul, Aug; autumn = Sept, Oct) and matrix (faeces, urine or serum).

Region	Season	Faeces (*Sex*)	Urine (*Sex*)	Serum
East coast	Spring	7 (*2 F*, *5 M*)	6 (*1 F*, *5 M*)	7
	Summer	4 (*1 F*, *3 M*)	10 (*4 F*, *6 M*)	10
	Autumn	9 (*5 F*, *4 M*)	19 (*11 F*, *8 M*)	19
Northern Isles	Spring	n.a	n.a	n.a
	Summer	10 (*9 F*, *1 M*)	21 (*16 F*, *5 M*)	21
	Autumn	15 (*8 F*, *7 M*)	17 (*7 F*, *10 M*)	17
West coast	Spring	16 (*9 F*, *7 M*)	23 (*10 F*, *13 M*)	23
	Summer	4 (*4 F*, *0 M*)	14 (*10 F*, *4 M*)	14
	Autumn	n.a	n.a	n.a

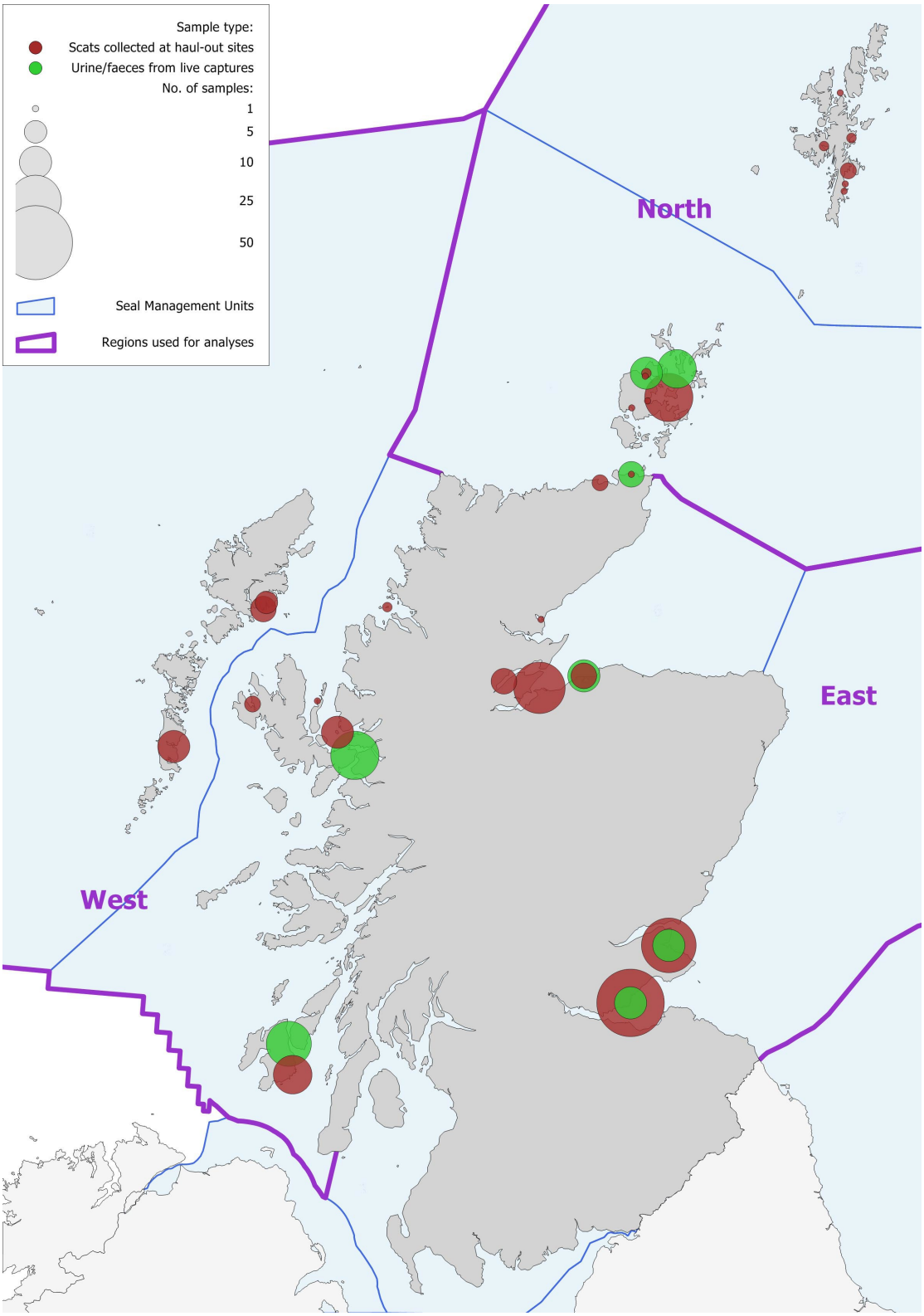

Figure 2.1: The collection sites of harbour seal faecal and/or urine samples. The size of the circles reflects the sample size, green circles indicate samples from live captured and stranded harbour seals, and brown circles indicate anonymous faecal samples collected at haul-out sites. The purple boundaries and labels represent the regions used for the analysis.

2.2.1.2 Haulout collection of anonymous faecal samples

Faecal samples were collected from selected sites around Scotland (Fig. 2.1) in spring and autumn between 2010 and 2013 (with a few samples in 2003 and 2008) at harbour seal haul-out sites. Samples were collected at low tide into individual plastic bags and stored at -20 °C until extraction and analysis. Grey seals also sometimes use some of these haul-out sites, therefore sub-samples of the faeces from the mixed sites were stored for DNA extraction and species identification (carried out in collaboration with Xelect, St. Andrews. UK).

2.2.2 Otolith identification

Any remaining faecal material was sieved and the fish otoliths were removed and identified to fish species using a reference collection and other available guides (this identification was carried out in collaboration with Mr. John Watkins) (Breiby, 1985; Brodeur and Center, 1979; Härkönen, 1986).

2.2.3 Domoic acid analysis

2.2.3.1 Domoic acid extraction

Faecal samples were extracted and analysed based on the method published by Lefebvre (1999). The samples were defrosted and a 4 g subsample was homogenized and extracted using 50 % methanol in a 1:4 ratio. Samples were then centrifuged at 3000 x *g* for 30 min, the supernatant was filtered through a 0.45 µm syringe filter (Millipore Corp., Bedford, MA) and collected for Solid Phase Extraction (SPE), using Strong Anion Exchange (SAX) columns (Supelco, UK). Between 2-3 ml of filtrate were passed through the 3 ml SPE column that was pre-conditioned with 6 ml of methanol followed by 3 ml of water then by 3 ml of methanol:water (1:1). The cartridge was not allowed to get dry at any point during the procedure. Two ml of the sample supernatant was loaded into the cartridge slowly (ca. 1 drop/s). The column was then washed with 5 ml 10 % acetonitrile. Finally 5 ml of 0.5 N sodium

chloride in 10 % acetonitrile was used to elute the DA into a 15 ml Falcon tube. The sample was stored in a fridge and analysed within a month of extraction (Lefebvre et al., 1999). Urine samples were used directly for the Amnesic Shellfish Poisoning Enzyme-Linked Immunosorbent Assay (ASP ELISA assay kits, Biosense, Norway) method.

2.2.3.2 ASP ELISA analysis

For DA confirmation the Amnestic Shellfish Poisoning Enzyme-Linked Immunosorbent Assay (ASP ELISA assay kits, Biosense, Norway), has been found to be a reliable, sensitive and rapid method for detecting DA in various matrices in the marine environment (Garthwaite et al., 2001). The ELISA assay has no cross-reactivity to non-toxic, structural analogues like kainic acid. This direct competitive ELISA was used to measure DA concentrations in urine and toxin extracted and SPE filtered faecal samples. The assay is based on detection of DA by polyclonal anti-DA antibodies, where free DA in the sample (e.g. urine or faeces) competes for binding to the polyclonal antibodies with DA-conjugated protein coated on the plastic wells. A colour-producing enzyme is added and the bound (antibody) proportion is measured through the colour intensity using a microplate reader at wavelength of 450 nm. The limit of quantification (LOQ) for the ASP ELISA was set to 0.004 µl/ml (urine) and 0.020 µg/g (faeces). The intra-assay CV was 0.04 (n = 10) and an inter-assay CV was 0.12 (n = 6).

2.2.3.3 Instrumentation for DA analysis using UHPLC-MS/MS

For confirmation of the results obtained using the ASP ELISA a method based on the use of ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was used to quantify DA and was carried out at Marine Scotland Science, Aberdeen (Fig. 2.2). A hybrid triple quadruple linear ion trap mass spectrometer (3200 QTRAP, ABSciex) fitted with a TurboIonSpay® Ion source was coupled to a 1290 Infinity UHPLC system (Agilent) comprising a 1290 Infinity binary pump, a 1290 Infinity thermostated

column compartment and a 1290 Infinity auto-sampler. Elution of DA was achieved using a kinetex C18 column (100 mm × 2.1 mm, 1.7 µm – Phenomenex) using an isocratic elution (12 % B). Mobile phase A was 100% aqueous and mobile phase B was 95% acetonitrile, both containing 2 mM ammonium formate and 50 mM formic acid. With a flow rate set up at 0.3 ml/min and a column oven maintained at 20º C throughout the analysis, the run time per sample was six minutes. The injection volume for both standards and sample extracts was 5 µl. The mass spectrometer was used in multiple reaction-monitoring (MRM) mode and three specific transitions, one for quantitation and the other two for confirmation, were monitored for DA. The monitored transitions and source parameters are detailed in Table 2.2 and 2.3. LOQ was selected using the lowest standard that had a peak, and the noise and the signal intensity was visually quantified. The signal to noise ratio (S/N) was then calculated. At the end the LOQ was proportionally calculated. The limit of detection for DA (LOD) was set to 0.004 µg/g and limit of quantification (LOQ) to 0.025 µg/g.

Table 2.2: Mass spectrometer optimised parameters for the considered DA transitions

	Transition	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
DA	**T1**	310	266.3	-50	-3	-10	-20	-3
	T2	310	222.3	-50	-3	-10	-25	-2
	T3	310	160.2	-40	-4	-10	-30	-1

Q: quadrupole
m/z: mass/charge
DP: declustering potential
V: voltage
EP: entrance potential
CEP: collision cell entrance potential
CE: collision energy potential
CXP collision cell exit potential

Table 2.3: Source/Gas optimised parameters

	CUR	IS	TEM	GS1	GS2	CAD
Source /Gas	10	-4500	450	55	50	Medium

CUR: Curtain gas voltage
IS: electro spray voltage
TEM: source temperature
GS1: nebuliser gas pressure

GS2: heater gas pressure
CAD: collisional activated dissociation voltage

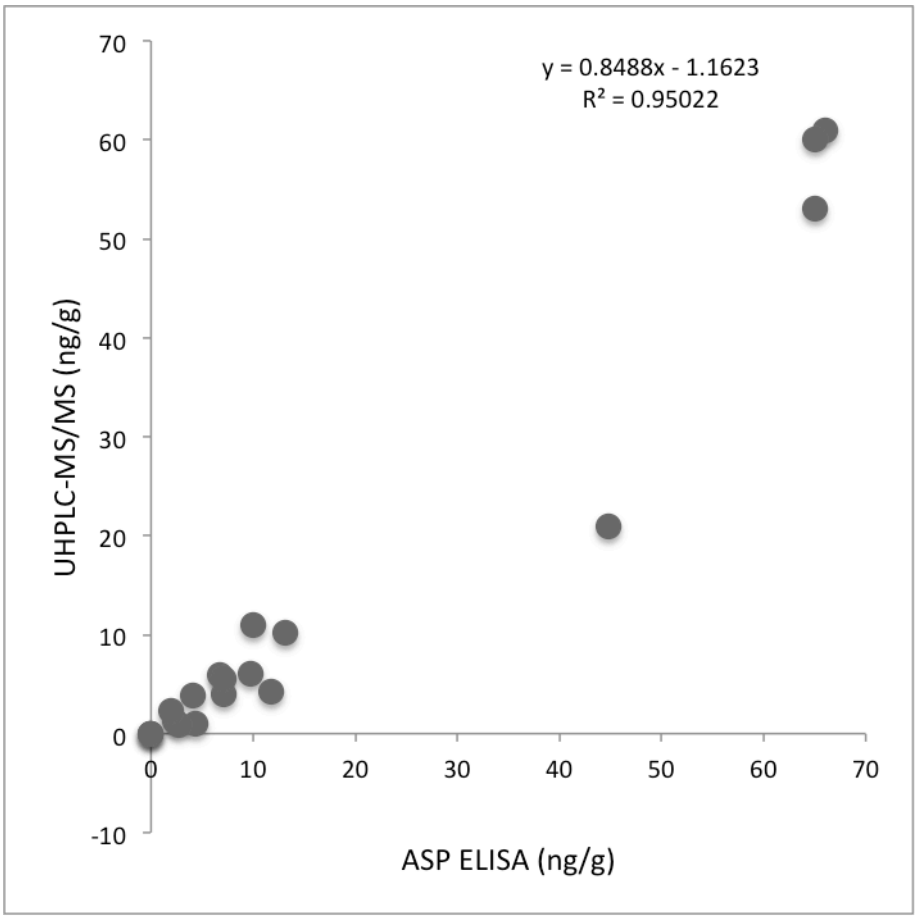

Figure 2.2: Relationship between DA concentrations (ng/g) in faecal samples from the ASP ELISA kit and the analysis using the UHPLC-MS/MS.

2.2.4 PSP toxin analysis

2.2.4.1 PSP toxin extraction

For faecal extraction a 4 g subsample was homogenized and then mixed with in a 1:1 ratio of 0.1 M hydrochloric acid and boiled between 2-5 min while stirring. The mixture was allowed to cool and then centrifuged for 10 min at 3500 x *g*. The supernatant was passed through a 0.45 µm filter and stored at 4° C until protein precipitation.

2.2.4.2 Protein precipitation for PSP toxin analysis

An aliquot of 500 µl sample extract was pipetted into a 2.5 ml eppendorf tube where 2.5 µl of 30 % tricarboxylic acid (TCA) was added. The sample was briefly vortexed before it was centrifuged for 5 min at 14 000 x *g*. To bring the pH in between 2-4, 35 µl and 1.0 M NaOH was added to the sample. The sample was then briefly vortexed and centrifuged for 5 min at 14000 x *g*. The supernatant was filtered through a 0.2 µm filter at 10 000 x *g* for 3 min. The filtered sample was transferred into a vial ready for HPLC analysis.

2.2.4.3 Urine sample preparation

PSP toxin was analysed in urine samples based on the method by Garcia et al., (2005) where 2 ml urine was mixed with 200 µl of 0.5M acetic acid and stored in a 2-5 ml eppendorf tube at 4° C until analysis. The sample was transferred into vials before analysis with the HPLC.

2.2.4.4 Instrumentation for PSP toxin analysis using HPLC

High Performance Liquid Chromatography (HPLC) with fluorescence detection (FLD), which is designed to separate compounds that are dissolved in solution with the purpose of identifying, quantifying and purifying the individual components, was used for evaluating PSP toxin levels. This method is very specific and selective, and can separate substances that fluoresce (PSP toxins wavelength: excitation: 330 nm, emission: 390 nm) it has been used for detecting individual concentration and total PSP toxicity of shellfish samples (Van de Riet et al., 2009). The instrument components (a-d) used in this analysis were different to those described by Van de Riet et al., (2009) as follows:

a) *LC system* - A Shimadzu (Tokyo, Japan) Prominence UFLCXR liquid chromatographic system equipped with a CBM-20A communication bus module, a LC-20AD XR binary pump, a DGU-20A5R degassing unit, a SIL-20AC XR auto-sampler, and a CTO-20AC column oven.

b) *Post-column reaction system.* An Eppendorf (Stevenage, UK) post-column reaction module comprising a FH-40 reactor heater and a TC-50 temperature controller capable of maintaining temperature at 85ºC with reagents delivered by two LC-20AD pumps and a DGU-20A3R degassing unit.

c) *Reaction coil.* - Supelco teflon tube (Sigma-Aldrich, Gillingham, UK) with a total volume of 2.4 ml.

d) *Fluorescence detector.* - Jasco FP-2020 Plus fluorescence detector

e) LC columns. – 1. Agilent Zorbax BonusRP, 3.5 µm, 4.6 x 150 mm (Agilent Technologies). 2. Thermo BetaBasic 8, 5 µm, 4.6 x 250 mm (Fisher Scientific, Nepean, ON, Canada). To confirm some of the results obtained with the PCOx method, extracts, which were found to contain PSP toxins, were investigated further. A pre-column oxidation HPLC-FLD method (AOAC, 2005) refined and validated for UK shellfish samples (Turner et al., 2008) was used to validate the results obtained from the first method of analysis. Samples were analysed following reverse-phase SPE clean up and both periodate and peroxide oxidation, with additional analysis of unoxidised extracts being used to compensate for the presence of naturally fluorescent matrix co-extractives. The results from the validation can be seen in Fig. 2.3. Only results from the Marine Scotland Science analysis will be used in this chapter. The LOD and LOQ for the PSP toxins are described in Table 2.4 and for this study a calculation of total toxicity was calculated according to Quilliam (2007) and these numbers are used in all the figures and models.

Table 2.4: Table showing the limit of detection (LOD) and the limit of quantification (LOQ) for the different PSP toxins, the units are expressed in µg STX diHCl eq/kg

PSP toxin	LOD	LOQ
Saxitoxin (STX)	0.67	2.00
decarbamoyl saxitoxin (dcSTX)	4.33	13.00
Neosaxitoxin (NEO)	15.30	46.00
Gonyautoxins (GTX) -2	0.67	2.00
GTX-3	0.67	2.00
dcGTX-2	0.33	1.00
dcGTX-3	0.67	2.00
GTX-5	0.33	1.00
GTX-1	5.67	17.00
GTX-4	6.00	18.00

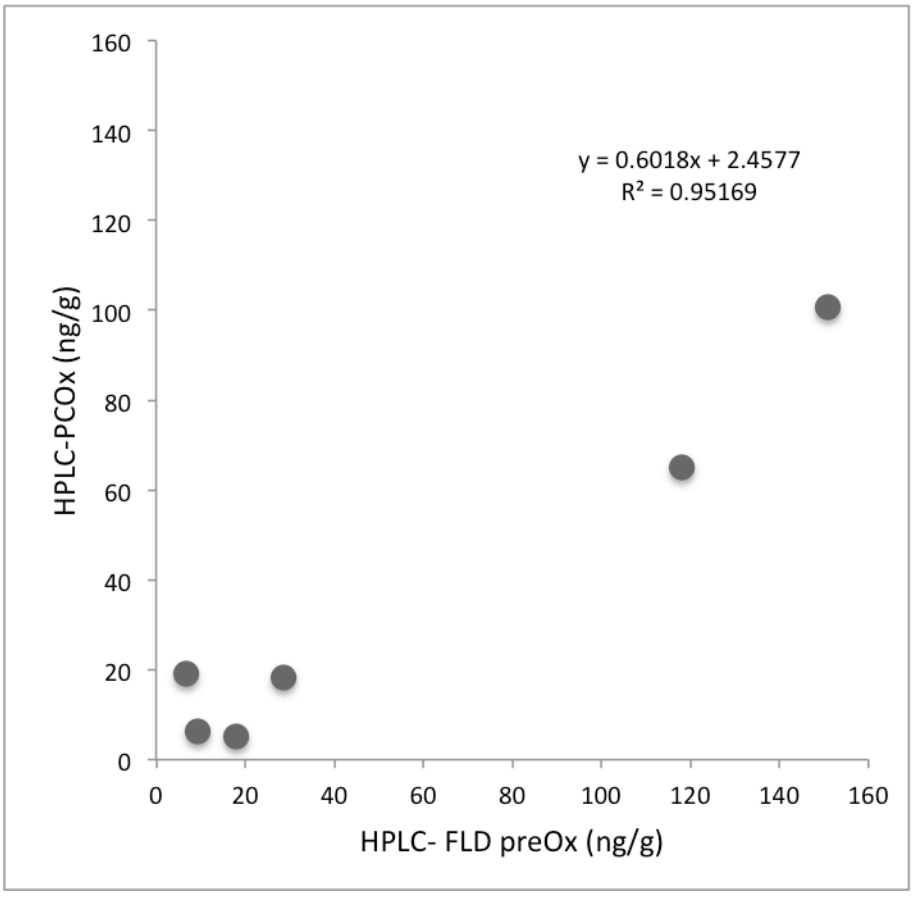

Figure 2.3: Validation of PSP toxins using the method HPLC-FLD analysed at Marine Scotland Science (MSS) compared with pre-column oxidation HPLC-FLD analysed at The Centre for Environment, Fisheries and Aquaculture Science (CEFAS).

2.2.5 Lipophilic toxin analysis

2.2.5.1 Sample extraction

For the lipophilic toxins a 2 g faecal sample was placed in a 50 mL disposable centrifuge tube. The extraction solvent (8 ml, 80% v/v methanol-water) was added using a calibrated pipette and the sample and solvent were blended using a homogenizer. The sample was then centrifuged (3000 x *g* for 12 min) and a 2 ml proportion of the supernatant extract was filtered (0.2 µm cellulose acetate syringe filter, Nalgene syringe filter, Thermo Scientific, Weltham, MA, USA) and stored in an amber vial glass in the freezer until hydrolysis. An aliquot of 150 µl sample extract (non-hydrolysed) was used directly for LC/MS analysis.

2.2.5.2 Hydrolysis of Esterified Forms of OA, DTX-1, and DTX-2

In order to detect OA, DTX-1 and 2 in their esterified forms the sample extracts were hydrolysed. This was carried out on an aliquot of 150 µl sample extract mixed with NaOH solution (18.5 ml, 2.5M) and the sample was heated in a water bath for 40 min at 76° C. The sample was allowed to cool down before the reaction was stopped with HCl (18.5 ml, 2.5M). The sample was filtered through a 0.2 µm cellulose acetate filter and collected for analysis. The hydrolysed samples were qualitatively detected by comparing if an > 10 % increase of the analyte peak (compared to the non-hydrolysed sample) had occurred before hydrolysis.

2.2.5.3 Instrumentation for lipophilic toxin analysis using LC/MS

Separations were carried out with an Agilent 1200 series LC system (Agilent Technologies, West Lothian, UK) consisting of a binary pump G1312A an auto-sampler G1367B, a temperature controlled compartment G1316A and a degasser G1379B. The detector was an Applied Biosystems API 150EX (Warrington, UK) mass spectrometer with a TurboIonspray® (Applied Biosystems) atmospheric pressure ionization interface. To divert the post

column flow of the mobile phase to waste a switching valve (Valco, Schenkon, Switzerland) was used for the initial 2 min of both the LC/MS multiple toxin and LC/MS hydrolysed extract analysis. For the multiple toxin and hydrolysed extract analyses the flow rates, injection volumes, and column temperatures were 0.25 ml/min, 5 ml, and 25° C. The mass spectrometer was operated in both ionization modes (positive and negative) and selected ion monitoring (SIM) acquisition modes. The LOD was > 0.006 µg/g and LOQ was > 0.02 µg/g for both OA and DTX-2.

2.2.6 Health parameters in Harbour seals

2.2.6.1 Total white blood cell counts

For total white blood cells (WBC), 10 µl whole blood was diluted 1:11 with Baar's fluid (mixture of 0.25 g saponin, 3.5 g sodium citrate, and 1 ml formalin, 0.1 g brilliant creysil blue). The diluted blood was allowed to settle for 10-15 min to lyse the red blood cells. The diluted blood was then filled into the haemocytometer chambers (Double Improved Neubauer, Philip Harris Scientific Ltd., Glasgow, UK) where the white blood cells were counted manually in duplicate using a light 40x magnification. Total WBC was calculated from the mean of the duplicates x 10 x 1000 / 10^6 = total white cells x 10^6/ml. This value is the cell concentration in the original suspension.

2.2.6.2 Differential white cell count

A thin smear of blood was made onto a glass slide, air dried and stored at room temperature for further analysis. The dried blood smears were stained with Leishman's stain (VWR International Ltd., Leicestershire, England, UK) first in neat Leishman's stain containing methanol that will fix the cells for 5 min then equal quantities of distilled water and Leishman's stain for 5 min, finally the smear was fixed for 2 min in deionised water. Examination of the stained smears was performed under a light microscope using 100x magnification, oil immersion. WBC differentials, including monocytes,

lymphocytes, neutrophils, and eosinophil were identified and 200 cells counted manually. The proportion of each cell type was then converted to an absolute quantity using the total white blood cell counts.

2.2.6.3 Faecal egg count

Faecal eggs were counted to assess the level of parasite infection by calculating the number of internal parasite eggs per gram of faeces. This was carried out because a parasite infection is known to increase the eosinophil cell count, a blood parameter also affected by exposure to DA. Only fresh faeces from live captured harbour seals were used and faeces were weighed and diluted at 1:4 with water saturated with sodium chloride. The faeces were passed through a sieve and mixed before a sample was taken and transferred to a McMaster egg counting slide. The sample was allowed to settle for 30 sec before being counted under a light microscope at 20x magnification.

2.2.6.4 Cortisol quantification

For the quantification of cortisol in the plasma samples, a commercially available solid phase enzyme-linked immunosorbent assay (ELISA) kit was used (DRG Cortisol ELISA EIA-1887) by DRG diagnostics (Marburg – Germany). The kit is based on the principle of competitive binding and allows the measurement of cortisol in unextracted plasma and serum samples. The levels were measured according to the ELISA kit instructions with a standard curve ranging between 0 and 800 ng/ml. This work was carried out in collaboration with a laboratory technician at the Sea Mammal Research Unit.

2.2.7 Faecal DNA extraction and analysis

2.2.7.1 DNA extraction (isolation of DNA from faeces)

DNA was isolated using a kit from QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions where DNA binds

specifically to the QIAamp silica-gel membrane while contaminants pass through. In brief a sample between 180-220 mg is weighed up and lysed in an optimized buffer, an inhibitEx tablet is added to absorb inhibitors. The sample is then allowed to digest proteins and proteinase K is used to ensure high yield of all types of DNA. Alcohol is added then the sample DNA is allowed to bind to the QIAamp spin column after it is washed and the DNA is eluted with a low salt buffer from the spin column to a 1.5 ml eppendorf tube where the pure DNA is ready for quantification.

2.2.7.2 DNA quantification carried out and written by Xelect, St. Andrews, UK

The DNA samples had been previously extracted from seal scats collected from field locations and frozen. Attempts were made to quantify the DNA content of the samples by spectrophotometry and agarose gel electrophoresis. Due to the highly degraded nature and the relatively low abundance of DNA from scat samples, quantification results were generally less than 2 ng/µl and abundant fragment size was below 100bp. This is unsurprising, given the nature of the samples. Trial amplifications revealed that despite the state of the DNA it was still possible to amplify all 3 PCR targets. Purified DNA was obtained from tissue samples of one female grey seal and one male harbour seal for use as positive controls for species and sex. Taqman primers and FAM labelled probes were synthesized according to Matejusova et al., (2010). One assay targeted the Y chromosome sequence ‘SRY’ and successful amplification of this sequence was used to indicate a male. The harbour seal assay was designed to target a sequence unique to harbour seals and the HG assay was unique to the grey seal. Amplification of at least one of these species assays was required to confirm that a negative male was actually a female, rather than a failed sample or assay. Additionally, an Internal Positive Control (IPC) was used to detect PCR inhibition within the DNA samples. This involves a DNA template and VIC labelled PCR assay being spiked into the samples. A positive reaction would always be expected with the IPC assay unless the sample contained PCR inhibitors. A ROX Dye was also added to each well to determine and standardise mechanical and

optical variation between wells. Each plate also contained 4 No Template Control (NTC) reactions. These are full PCR reactions but with distilled water in the place of 2 DNA sample. Any PCR amplification on these reactions indicates DNA contamination of the PCR reagents. QPCR was performed using Agilent Brilliant III Ultrafast qPCR mastermix, following manufacturers instructions, samples were sent for analysis to Xelect Ltd, Scottish Oceans Institute, St Andrews, UK. Thermal cycling conditions were as follows; 10 minutes at 95°C followed by 50 cycles of 10 seconds at 95°C and 1 minute at 60°C. FAM, VIC and ROX fluorescence data was collected at the end of the 1 minute step. Ct values, the number of cycles required for the recorded fluorescence to pass a set threshold, were generated using baseline-corrected raw fluorescence (dR) threshold set at 1000, using the MX4000 v3.0 adaptive baseline algorithm. Any reaction that crossed the fluorescence threshold before the completion of 50 cycles was considered to be positive. ROX data was used to normalise the FAM and VIC values, to correct for mechanical variation between wells.

2.2.8 Data analysis

The harbour seals were divided into three different regions, the Northern Isles (including the north coast of mainland Scotland), the west coast and the east coast (Fig. 2.1, Marked in map with: “North”, “West” and “East”). Investigation of regional, annual and sex related variation was carried out in relation to toxin exposure. The year 2011 was removed from the analysis as only one sample was collected that year. The dataset was analysed as presence/absence in regards to the LOQ, as DA measured in urine has been shown to be a difficult matrix to analyse with ELISA (Seubert et al., 2014). In addition, bearing in mind that time since exposure or uptake is not known, generalized linear models were used to select the models that best fitted the data with toxin concentration (in urine or faeces) as the dependent variable and sex, region and month (categorical) as the independent factors. Only models that had enough data are presented. Each toxin was tested in turn before the effect of combined exposure of multiple toxins was investigated. To specify the

relationship between toxin concentration and the haematological parameters, linear models were used. Toxin concentration was used as the dependent variable and white cell count, cortisol or faecal egg count as the independent variables. The faecal egg counts were treated as a confounding variable in relation to the eosinophil count. Because of outliers in the dataset some of the data was transformed to a logarithmic scale in the plots and models. Akaike's information criterion (AIC) was used to rank the models. All analyses were performed using R software (R Development Core Team 2007).

2.3 Results

2.3.1 Domoic acid in live captured harbour seals

Between 2008 and 2013 162 individual harbour seals were caught and samples collected for the analysis of toxins from harmful algae (Table 2.5).

Table 2.5: Percentage positive (>LOQ) for DA among live captured harbour seal samples, represented by toxin as well as season where spring = Mar, Apr, May; autumn = Sept, Oct, Nov; winter = Dec, Jan, Feb (sample size are in brackets).

Region	Season	DA urine	DA faeces	PSP toxin faeces	OA faeces	DTX-2 faeces
East coast	Spring Summer Autumn	88.9 (9) 100.0 (10) 71.4 (21)	11.1 (9) 25.0 (8) 36.6 (11)	42.9 (7) 0.0 (1) 25.0 (4)	0.0 (7) 0.0 (2) 100.0 (3)	85.7 (7) 0.0 (2) 33.3 (3)
Northern Isles	Spring Summer Autumn	0.0 (1) 100.0 (21) 30.0 (20)	n.a 45.5 (22) 0.0 (17)	n.a n.a 25.0 (8)	n.a n.a 20.0 (5)	n.a n.a 0.0 (5)
West coast	Spring Summer Autumn	29.6 (27) 100.0 (14) n.a	4.8 (21) 9.1 (11) 0.0 (1)	45.0 (20) n.a n.a	33.3 (6) n.a n.a	16.7 (6) n.a n.a

2.3.1.1 DA in urine samples

Between 2008-2010 all of the urine samples (n = 50) from the live captured harbour seals analysed for DA were found to contain quantifiable levels (ASP ELISA LOQ > 0.004 µg/ml) of DA. The year with highest DA concentration

measured in urine was 2008 where the mean was 4.57 µg/ml and the 75% quantile: 2.53 µg/ml. This year (2008) was significantly different to the other years (Fig. 2.4, Table 2.6, model 1, $p < 0.01$, the figure shows the concentration plotted on a $\log_{10}$ scale due to the skewed distribution of the data). Between 2012-2013 (n = 71) only 44.29 % was found to contain quantifiable levels of DA.

Across all years, the east coast region had a significantly higher average DA concentration (Table 2.6, model 2, $p = 0.02$, mean: 2.99 µg/ml and 75 % quantile 1.82 µg/ml) than the Northern Isles (mean: 0.97 µg/ml and 75 % quantile 0.65 µg/ml) and the west coast (mean: 0.41 µg/ml, 75 % quantile 0.36 µg/ml) (Fig. 2.5). Seals showing urinary exposure to DA were more likely to be females (Table 2.6, model 3, $p = 0.02$, mean: 2.40 µg/ml, 75 % quantile: 1.43 µg/ml) than males (mean: 0.34 µg/ml, 75 % quantile: 0.13 µg/ml). A seasonal trend was observed where all urine sampled from June to September had concentrations of DA > LOQ (Fig. 2.6), whereas May and October had significantly lower concentrations than the rest of the year (Table 2.6, model 4, $p < 0.01$). The highest DA measured in harbour seal urine was from a female caught on the east coast in September 2008 with 63.17 µg/ml. Models including other interaction terms (year and region, sex and month, or the four-way interaction with year, region, sex and month) did not improve the fit of the model ($P > 0.46$, AIC > 148.6).

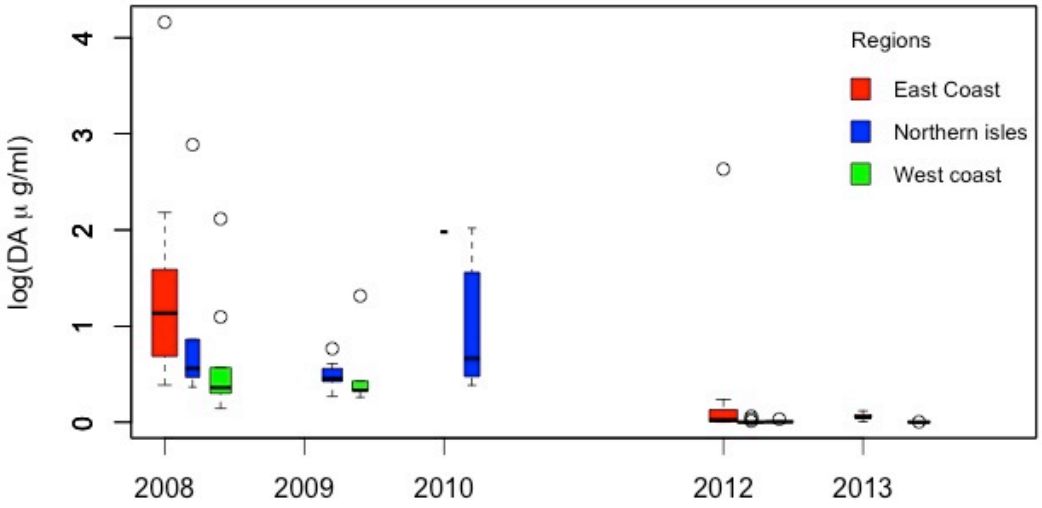

Figure 2.4: Concentration of DA in urine (µg/ml) by year, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x inter quartile range (IQR) of DA and the black horizontal line indicates the median of the data set, separate circles are outliers. (DA range: 0.00 – 63.17 µg/ml).

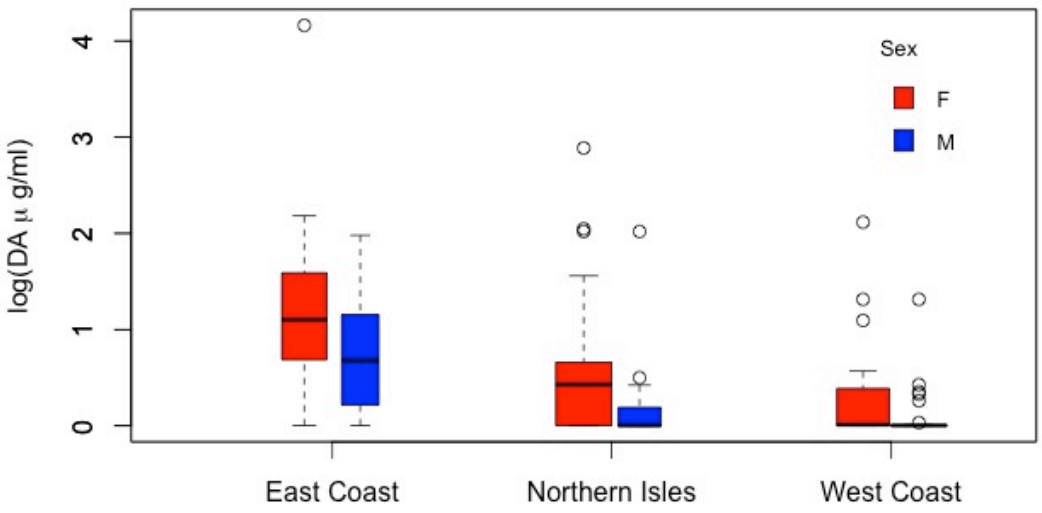

Figure 2.5: Concentration of DA in urine (µg/ml) by regions, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different sex' sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0.00 – 63.17 µg/ml).

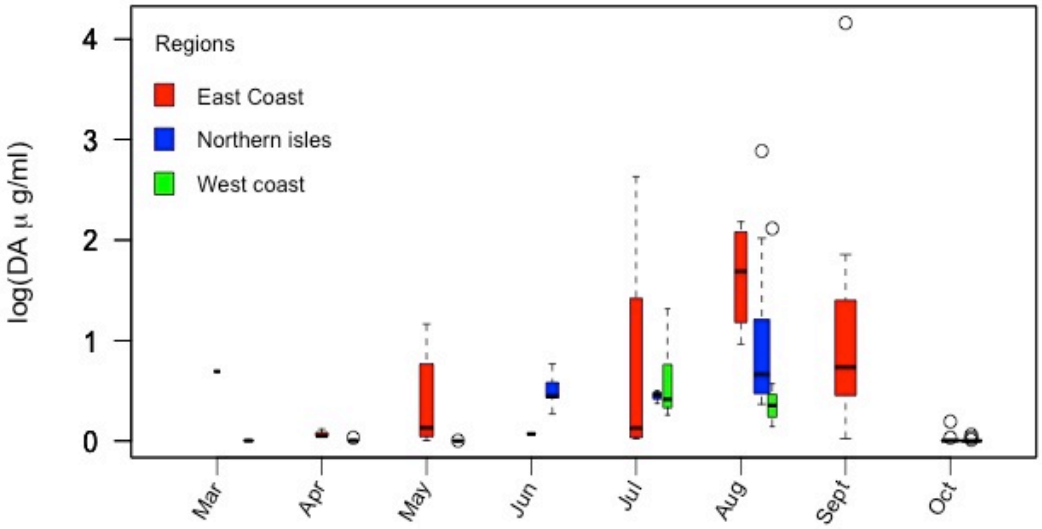

Figure 2.6: Concentration of DA in urine (µg/ml) by month, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0.00 – 63.17 µg/ml).

Table 2.6: Regression models for DA concentration in urine samples.

Model	Variable	Residual deviance	df fitted	df	P value	AIC
1.	Year	100.5	119	1	<0.01	104.5
2.	Region	146.0	118	2	0.02	152.0
3.	Sex	147.9	119	2	0.02	151.9
4.	Month	77.1	113	7	<0.01	93.1

The table shows results from live captured harbour seals with separate variables: year, region, sex and month of the year. Alternative models were explored using forward-selection with the three variables and interaction terms among them. Res deviance, degrees of freedom (df), p-value and AIC are shown for each model. Model without explanatory variables, res dev = 153.6/120 df.

2.3.1.2 DA in faecal samples

Between 2008-2010, 29.55 % of the faecal samples (n = 44) collected from the live captured harbour seals analysed for DA were found to contain levels above the LOQ (> 0.020 µg/g, Fig. 2.7). From 2012-2013 (n = 53), only 11.32 % was documented with quantifiable levels of DA. There was a significant difference between the DA concentrations throughout the years, with 2008 (Table 2.7, model 1, p = 0.04, mean: 1.60 µg/g, 75 % quantile: 1.47 µg/g) being the year with the highest proportion of DA positive faecal samples while in 2013 all samples (n = 16) were negative. There was a significant spatial difference in DA exposure where the east coast (mean: 0.79 µg/g, 75 % quantile: 0.03 µg/g) and the Northern Isles (mean 0.90 µg/g, 75 % quantile: 0.29 µg/g) had a higher proportion of positive faeces samples (> LOQ) than the west coast (Table 2.7, model 2, mean; 0.14 µg/g, 75 % quantile: < LOQ). There was no difference between the sex and months (Table 2.7, model 3, p = 0.54 and model 4, p = 0.51) for DA exposure (Fig. 2.8 and 2.9). The DA proportion positive differed throughout the years between the regions and in 2012 all samples from the Northern Isles (n = 17) were negative for DA (Table 2.7, model 5, p = 0.01). There was a significant interaction between region and sex where on the east coast there was more DA positive males than females (Table 2.7, model 6, p < 0.04). Other interaction terms (year and sex, year and month, region and month or the four-way interaction with year, region, sex and month) did not improve the fit of the model (*P* > 0.91, AIC > 103.8). The highest DA concentration in faeces was measured in the Northern Isles in 2008 where a harbour seal female had 25.87 µg/g.

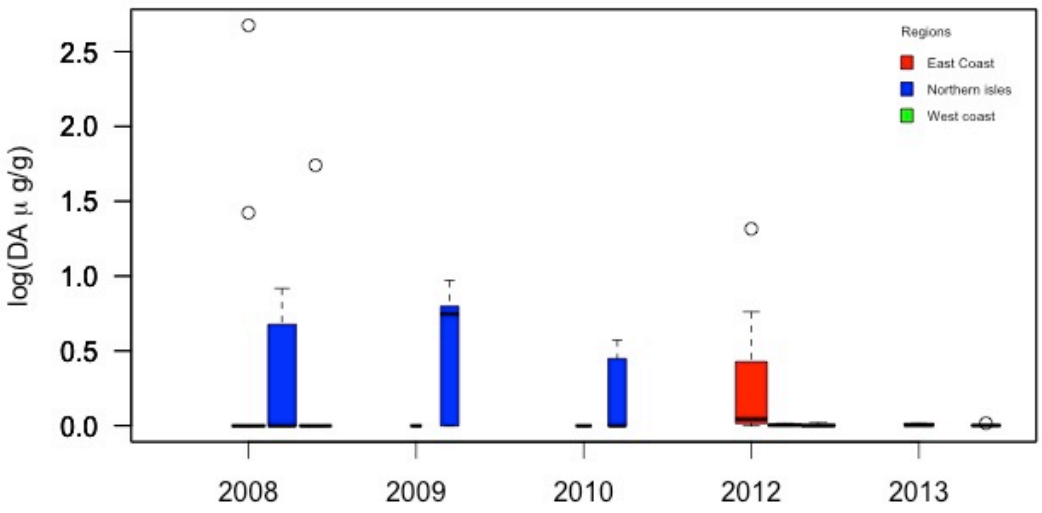

Figure 2.7: Concentration of DA in faeces (µg/g) by year, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0.00 – 25.87 µg/g).

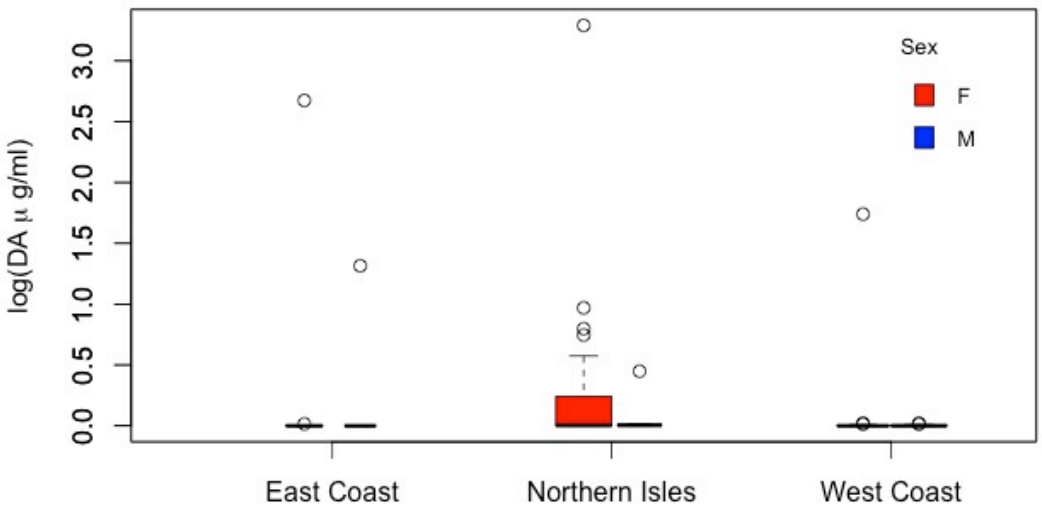

Figure 2.8: Concentration of DA in faeces (µg/ml) by region, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different sex' sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0.00 – 25.87 µg/g).

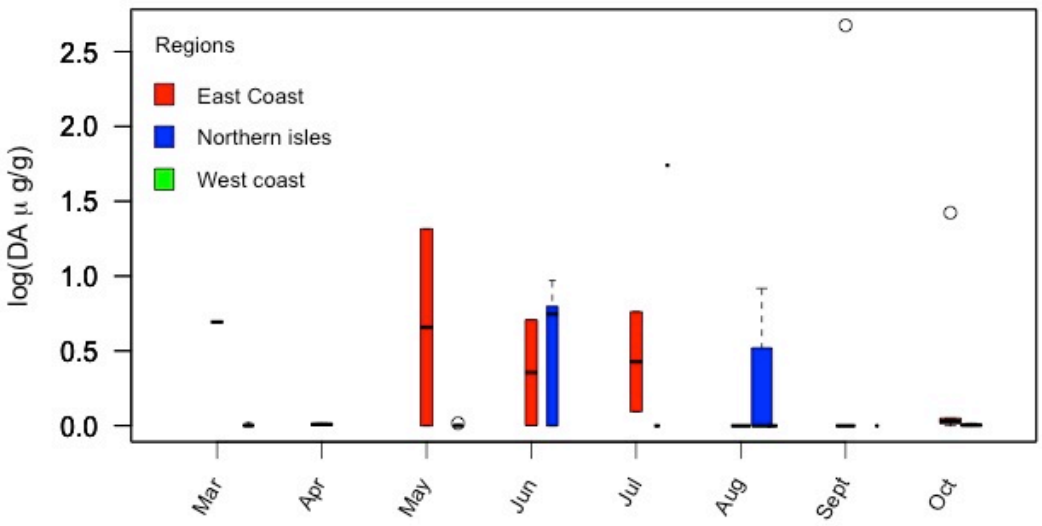

Figure 2.9: Concentration of DA in faeces (µg/g) by month, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0.00 – 25.87 µg/g).

Table 2.7: Regression models for DA concentration in faecal samples.

Model	Variable	Residual deviance	df fitted	df	P value	AIC
1.	Year	91.1	95	1	0.04	95.1
2.	Region	89.7	94	2	0.05	95.7
3.	Sex	95.6	95	1	0.54	99.6
4.	Month	81.8	89	7	0.51	97.8
5.	Year x Area	79.4	91	5	0.01	91.4
6.	Region x Sex	84.85	91	5	0.04	96.9

The table shows results from live captured harbour seals with separate variables and interactions between them, these include: year, region, sex and month of the year. Alternative models were explored using forward-selection with the three variables and interaction terms among them. Res deviance, degrees of freedom (df), p-value and AIC are shown for each model. Model without explanatory variables, res dev = 95.96/96 df.

My analysis of DA in urine or faecal samples showed that the harbour seals exposed to DA were significantly more likely ($p = 0.02$) to belong to populations from the east coast, where 77.08 % (n = 48) were found to contain detectable levels of DA, or from the Northern Isles where 71.67 % (n = 60) were > LOQ as compared with the west coast where a total of 53.70 % (n = 54) were > LOQ. The results also indicate that females 77.78 % (n = 81) were significantly more likely ($p < 0.01$) to be exposed to DA than males 56.79 % (n = 81). A positive correlation was found between DA in faecal samples and DA in urine (Fig. 2.10, $p < 0.05$).

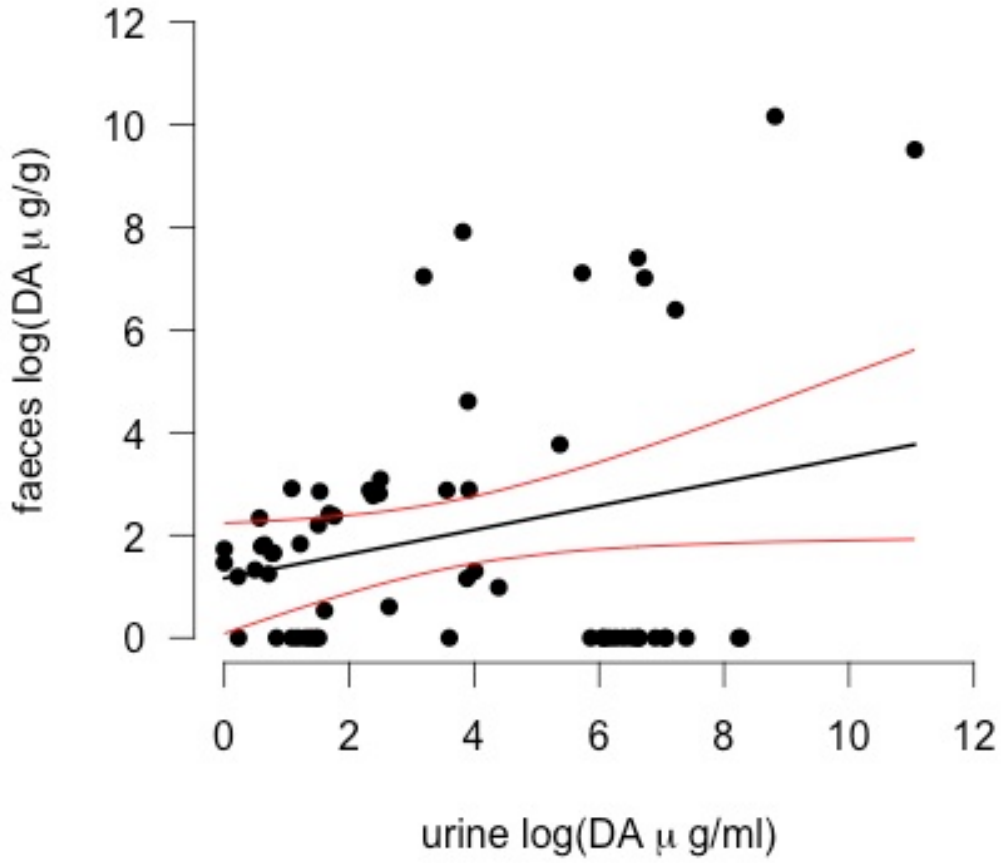

Figure 2.10: The relationship between DA concentrations in faeces plotted against DA concentration in urine plotted on a logarithmic scale, from live captured harbour seals with 95% confidence interval of the linear regression line.

2.3.2 Domoic acid in anonymous faecal samples

Of the 180 anonymous faecal samples (faeces collected from haul-out sites previously frequented by harbour seals) collected 63.33 %, were > LOQ for DA (Table 2.8).

Table 2.8: Percentage positive (>LOQ) for DA among anonymous haul-out faecal samples, represented by toxin as well as season where spring = Mar, Apr, May; autumn = Sept, Oct, Nov; winter = Dec, Jan, Feb (sample size are in brackets).

Region	Season	DA faeces	PSP toxin faeces	OA faeces	DTX-2 faeces
East coast	Spring Summer Autumn	75.0 (16) 43.2 (44) 78.3 (23)	0.0 (6) 75.0 (20) 73.3 (15)	57.1 (7) 40.9 (22) 6.3 (15)	14.3 (7) 4.5 (22) 31.3 (16)
Northern Isles	Spring Summer Autumn	45.5 (22) 75.0 (15) 75.0 (4)	66.7 (6) n.a 100.0 (2)	16.7 (6) n.a 0.0 (4)	0.0 (6) n.a 25.0 (4)
West coast	Spring Summer Autumn	100.0 (17) 59.0 (39) n.a	n.a n.a n.a	n.a n.a n.a	n.a n.a n.a

There was a significant temporal difference with 2010 being a year with the highest proportion positive (mean: 7.36 µg/g, 75 % quantile: 0.90 µg/g, Table 2.9, model 1, $p = 0.01$, Fig. 2.11). There was no significant spatial difference in the proportion positive (Table 2.9, model 2, $p = 0.14$, Fig. 2.12), but the east coast (mean: 6.10 µg/g, 75 % quantile: 0.90 µg/g) and Northern Isles (mean 3.42 µg/g, 75 % quantile: 2.61 µg/g) had a higher mean DA concentration than the west coast (mean: 2.15 µg/g, 75 % quantile: 1.84 µg/g). The highest DA concentration measured was on the east coast at 100.46 µg/g DA. There was also a significant difference between the months of DA exposure where June and May had a lower proportion positive for DA than the rest of the year (Table 2.9, model 3, $p < 0.01$, Fig. 2.13). The best model was achieved when the interaction between region and month was fitted to the data (Table 2.9, model 5, AIC: 219.3), where the Northern Isles in June were significantly different than the other months and regions with a higher proportion of samples with DA above the LOQ. Other interaction terms or the three-way interaction with year, region and month was not possible to model due to insufficient data.

Table 2.9: Regression models analysing DA concentration in anonymous faecal samples.

Model	Variable	Residual deviance	df fitted	df	P value	AIC
1.	Year	225.1	178	1	<0.01	229.1
2.	Region	234.2	177	2	0.14	240.2
3.	Month	214.9	173	6	<0.01	228.9
4.	Year x Region	227.6	175	4	<0.01	227.6
5.	Region x Month	185.3	163	13	<0.01	219.3

The table shows results from separate variables and interactions between them, these include: year, region and month. Alternative models were explored using forward-selection with the three variables and interaction terms among them. Res deviance, degrees of freedom (df), p-value and AIC are shown for each model. Model without explanatory variables, res dev = 236.6/179 df.

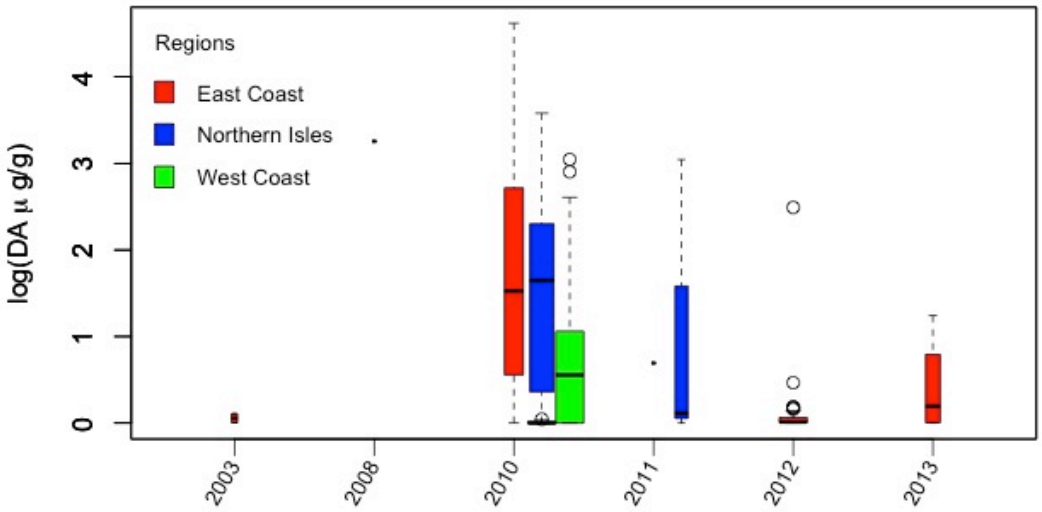

Figure 2.11: Concentration of DA anonymous faecal samples (µg/g) by year, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0.00 – 100.46 µg/g).

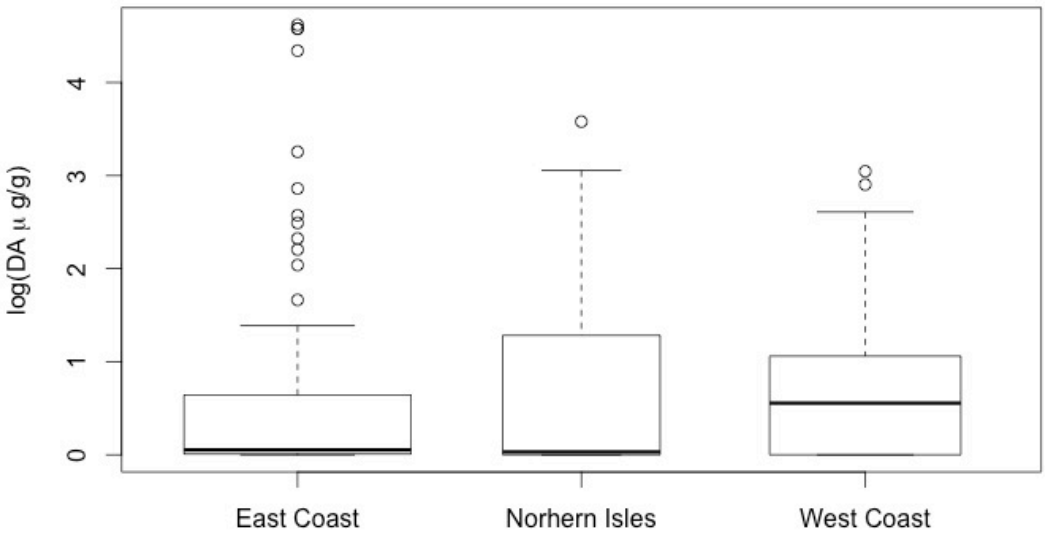

Figure 2.12: Concentration of DA in anonymous faecal samples (µg/g) by region, plotted on a logarithmic scale from live captured harbour seals. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0.00 – 100.46 µg/g).

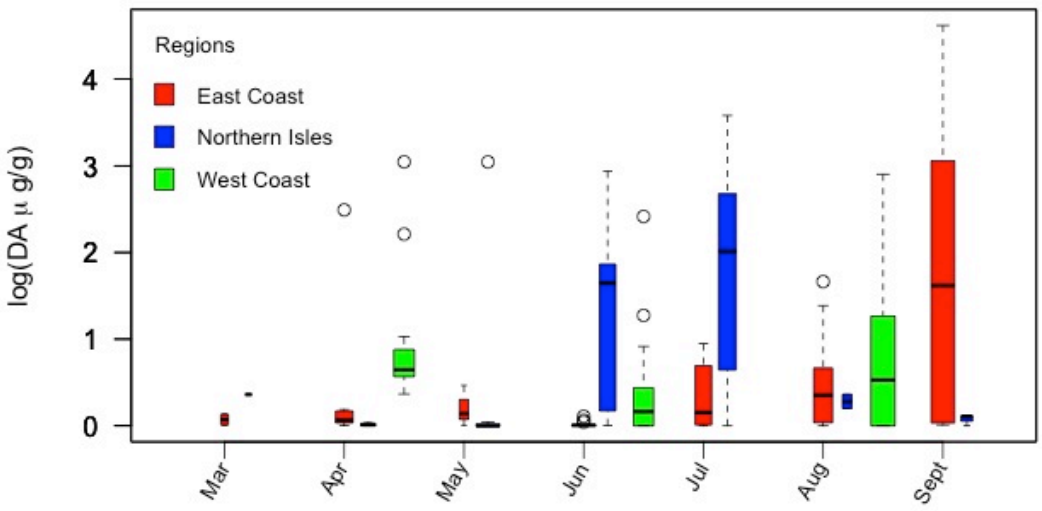

Figure 2.13: Concentration of DA in anonymous faecal samples (µg/g) by month, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0.00 – 100.46 µg/g).

2.3.3 PSP toxins from live captured harbour seals

Results using the HPLC-PCOx method and with confirmation using pre-column oxidation LC-FLD methods established the presence of PSP toxins in 16 (39.02 %) faecal samples of the 41 live captured harbour seal tested, where 25.00 % (n= 28) were found exposed in 2012 and 69.23 % (n = 13) were found exposed in 2013 (Fig. 2.14). Five (38.46 %) out of thirteen live captured harbour seals from the east coast were found with PSP toxins in their faeces. Two (25.00 %) out of eight seals tested from the Northern Isles were found with PSP toxins and nine (45.00 %) out of 20 seals tested from the west coast were found exposed to PSP toxins. There was no significant difference between the regions (Table 2.10, model 1, p = 0.47). The west coast had the highest mean of 30.67 STX µg eq/kg (75 % quantile: 8.00 STX µg eq/kg) followed by the east coast with 16.08 STX µg eq/kg (75 % quantile:

4.00 STX µg eq/kg) and the Northern Isles 10.88 STX µg eq/kg (75 % quantile: 10.50 STX µg eq/kg). Although May (Fig. 2.15) was the month with the highest exposure of PSP toxins, this was not significant, but 2013 had the highest concentration of the two years (Table 2.10, model 2, p = 0.53, Fig. 2.15, mean 65.7 STX µg eq/kg, 75 % quantile: 53.00 STX µg eq/kg). None of the urine samples had traces of PSP toxins (n = 31).

Table 2.10: Regression models analysing PSP toxins concentration in faecal samples.

Model	Variable	Residual deviance	df fitted	df	P value	AIC
1.	Region	49.41	36	2	0.47	55.41
2.	Month	44.42	34	4	0.16	54.42

The table shows results from live captured harbour seals faecal samples (model 1 and 2) with separate variables, these include: region and month of the year. Because of a low sample number, interaction models were not available.

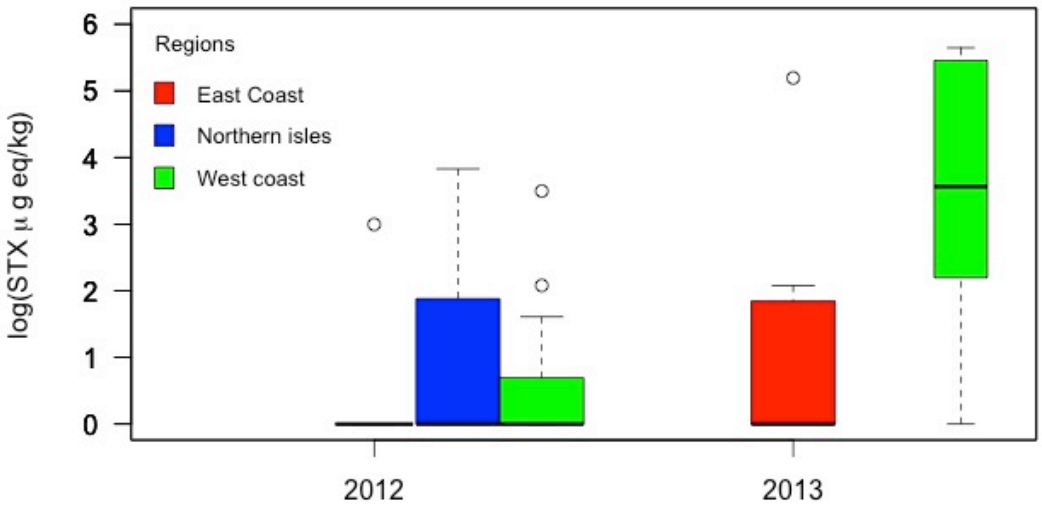

Figure 2.14: Concentration of PSP toxins in faecal samples by year, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of PSP toxins and the black horizontal line indicates the median of the data set, separate circles are outliers (PSP toxin range: 0.00 - 282.00 STX µg eq/kg).

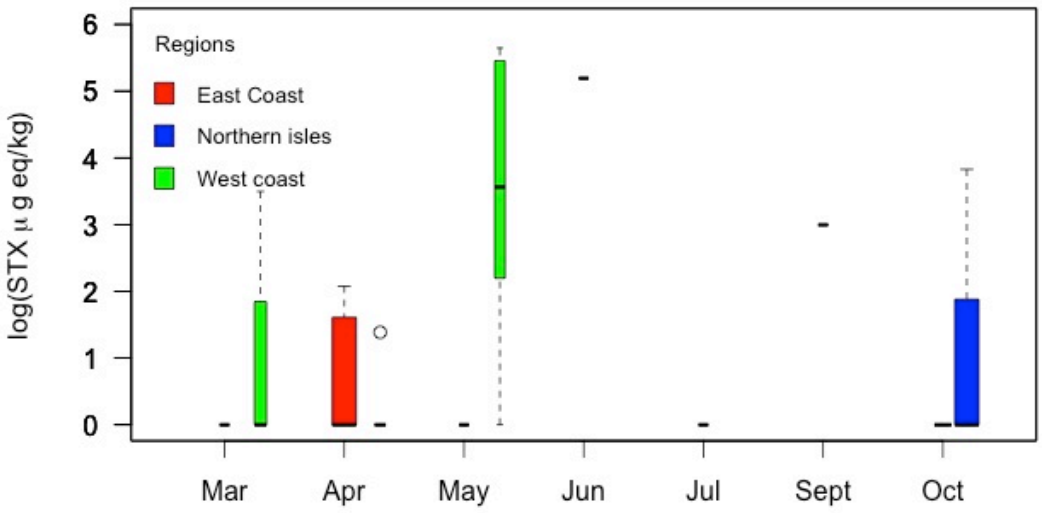

Figure 2.15: Concentration of PSP toxins in faecal samples by month, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of PSP toxins and the black horizontal line indicates the median of the data set, separate circles are outliers (PSP toxin range: 0.00 - 282.00 STX µg eq/kg).

2.3.4 PSP toxins in anonymous harbour seal faecal sample

Two archived faecal samples from 2003 were analysed for this study, and faecal samples from 2012 and 2013 were collected from haul-out sites for this study of PSP toxins (n = 46). One of the seal faecal samples from 2003 was found to contain PSP toxins. For 2012, 54.5 % was found with levels > LOQ (mean: 44.89 STX µg eq/kg, 75 % quantile: 44.50 STX µg eq/kg) and for 2013 76.92 % was > LOQ (mean: 26.15 STX µg eq/kg, 75 % quantile: 19.00 STX µg eq/kg) (Fig. 2.16). Anonymous faecal samples were only collected from harbour seals on the east coast (n = 42) and in the Northern Isles (n = 7). On the east coast 59.52 % were found to be > LOQ for PSP toxins and in the Northern Isles 71.43 % were > LOQ. There was no significant difference between the two regions sampled (Table 2.11, model 1, p = 0.55) or between the months (Table 2.11, model 2, p = 0.99, Fig. 2.17). Unfortunately no samples were available for PSP toxins analysis from the west coast of Scotland.

Table 2.11: Regression models analysing PSP toxins concentration in anonymous faecal samples

Model	Variable	Residual deviance	*df* fitted	*df*	P value	AIC
1.	Region	65.07	47	1	0.55	69.07
2.	Month	45.77	42	5	0.99	57.77

The table shows results from anonymous harbour seals faecal samples with separate variables, these include: region and month of the year. Because of a low sample number, interaction models were not available.

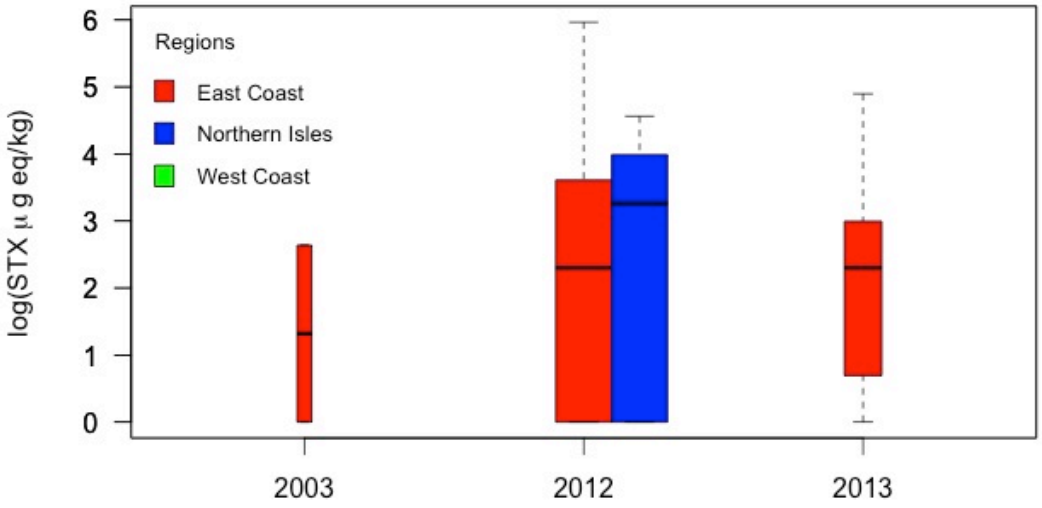

Figure 2.16: Concentration of PSP toxins in anonymous faecal samples by year, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of PSP toxins and the black horizontal line indicates the median of the data set, separate circles are outliers (PSP toxin range: 0.00 - 389.00 STX µg eq/kg).

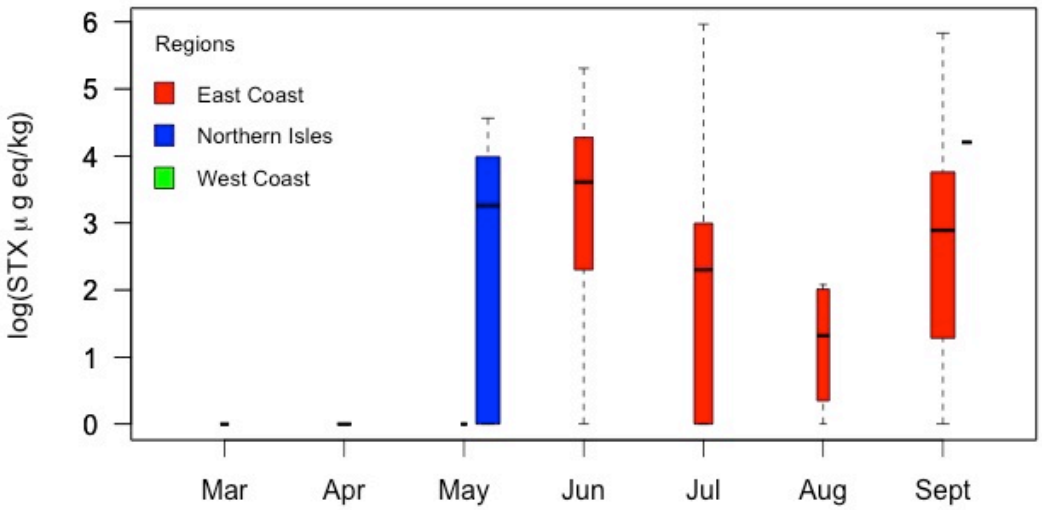

Figure 2.17: Concentration of PSP toxins in anonymous faecal samples by month, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of PSP toxins and the black horizontal line indicates the median of the data set, separate circles are outliers (PSP toxin range: 0.00 - 389.00 STX µg eq/kg).

2.3.5 Lipophilic toxins in live captured harbour seals

Six out of the 23 live-captured harbour seals tested had been exposed to OA and eight seals had been exposed to DTX2. Of the seals exposed to OA, three were captured on the east coast, one in the Northern Isles and three on the west coast (Table 2.5, 2.12, Fig. 2.18). The highest concentration of OA was measured on the east coast in a live captured harbour seal with a faecal sample at 0.065 µg/g. The sample size was not sufficient for statistical inference, but May and October appeared to be important months of OA toxicity (Fig. 2.19).

Table 2.12: Lipophilic toxins percentage positive (%) in faecal samples from live captured harbour seals presented annually from 2003 to 2013 (sample sizes are in brackets).

OA / *DTX-2* in faeces	**2008**	**2012**	**2013**
All regions	OA: 100.0 (1) DTX-2: 0.0 (1)	OA: 33.3 (9) DTX-2: 11.1 (9)	OA: 15.4 (13) DTX-2: 53.8 (13)
East coast	OA: 100.0 (1) DTX-2: 0.0 (1)	OA: 50.0 (4) DTX-2: 25.0 (4)	OA: 0.0 (7) DTX-2: 85.7 (7)
Northern Isles	OA: n.a DTX-2: n.a	OA: 20.0 (5) DTX-2: 0.0 (5)	OA: n.a DTX-2: n.a
West coast	OA: n.a DTX-2: n.a	OA: n.a DTX-2: *n.a*	OA: 33.3 (6) DTX-2: 16.7 (6)

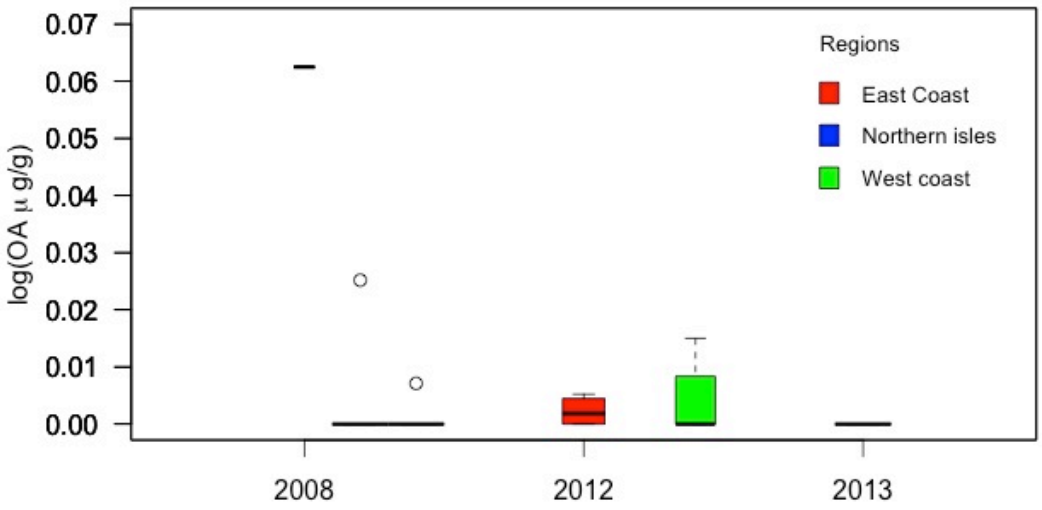

Figure 2.18: Concentration of OA in faecal samples by year, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of OA and the black horizontal line indicates the median of the data set, separate circles are outliers (OA range: 0.00 – 0.06 µg/g).

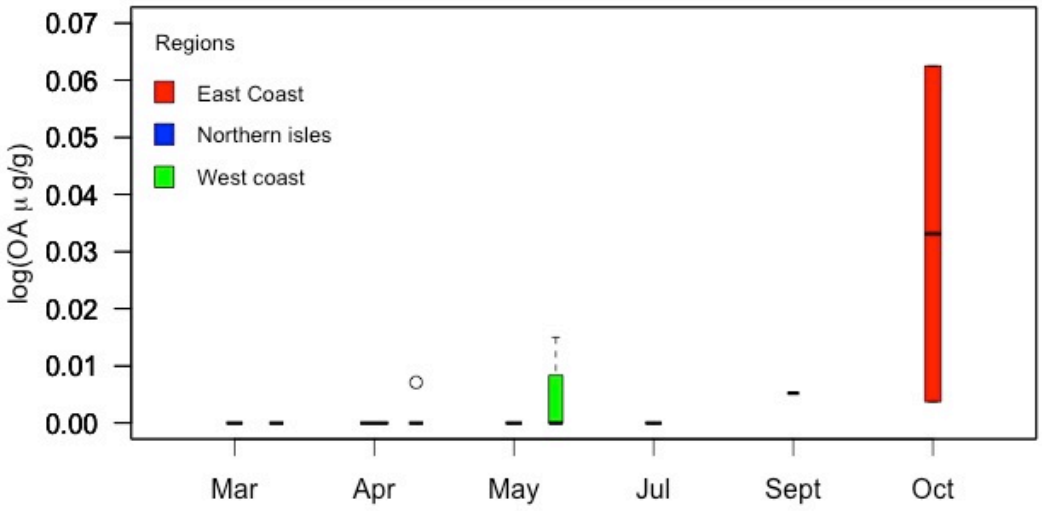

Figure 2.19: Concentration of OA in faecal samples by month, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of OA and the black horizontal line indicates the median of the data set, separate circles are outliers (OA range: 0.00 – 0.06 µg/g).

Of the six seals exposed to DTX-2, seven were found on the east coast and one on the west coast (Table 2.12, Fig. 2.20). The highest sample measured of DTX-2 was found on the east coast with 2.01 µg/g. The sample size was not sufficient for statistical inference, but April appeared to be an important month of DTX-2 toxicity (Fig. 2.21). Two of the six positive seals exposed to DTX-2 had according to the Bristol stool chart (Lewis and Heaton, 1997) a type 7 category indicating a loose/runny stool, watery with no solid pieces.

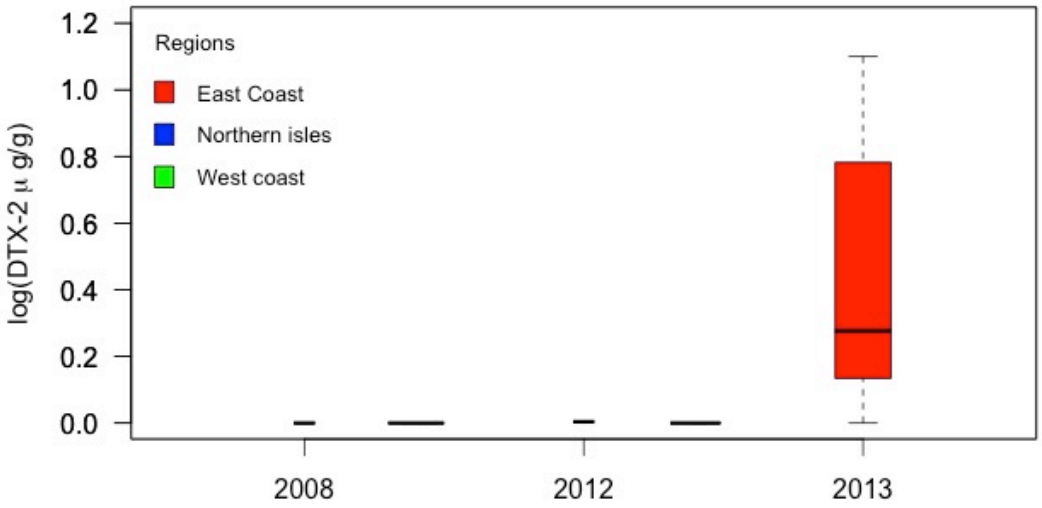

Figure 2.20: Concentration of DTX-2 in faecal samples by year, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DTX-2 and the black horizontal line indicates the median of the data set, separate circles are outliers (DTX-2 range: 0.00-2.01 µg/g).

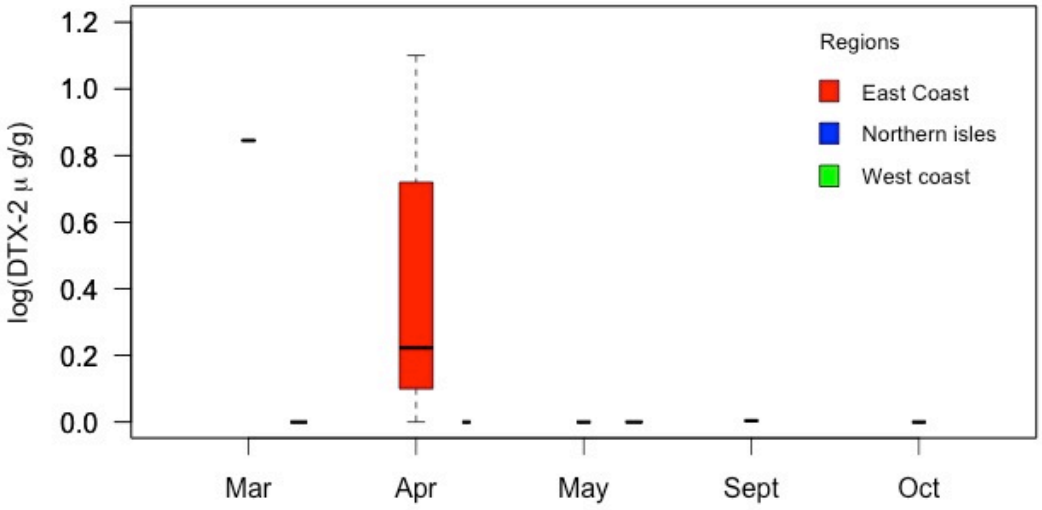

Figure 2.21: Concentration of DTX-2 in faecal samples by month, plotted on a logarithmic scale from live captured harbour seals. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DTX-2 and the black horizontal line indicates the median of the data set, separate circles are outliers (DTX-2 range: 0.00-2.01 µg/g).

2.3.6 Lipophilic toxins in anonymous faecal samples

Of the anonymous harbour seal faecal samples 15 out of 55 (27.3 %) were found exposed to OA, 14 of these seals were from the east coast, and one from the Northern Isles (Table 2.13). The highest sample was measured from the east coast in 2012 with OA levels at 0.029 µg/g (Fig. 2.22). The other lipophilic toxin found in the anonymous faecal samples was DTX-2, where eight out of 55 samples were exposed to DTX-2. Seven of these samples were found on the east coast and one in the Northern Isles (Table 2.13) and the highest faecal sample measured was on the east coast in 2012 with 0.014 µg/g (Fig. 2.24). The sample size for both toxins were not sufficient for statistical inference, for OA toxicity (Fig. 2.23) and for DTX-2 (Fig. 2.25).

Table 2.13: Lipophilic toxins percentage positive (%) in anonymous faecal samples presented annually from 2003 to 2013 (sample sizes are in brackets).

OA / DTX-2 in faeces	**Toxins**	**2003**	**2011**	**2012**	**2013**
All regions	**OA** **DTX-2**	0.0 (2) 0.0 (2)	0.0 (4) 25.0 (4)	31.4 (35) 20.0 (35)	28.6 (14) 0.0 (14)
East coast	**OA** **DTX-2**	0.0 (2) 0.0 (2)	n.a n.a	34.5 (29) 24.1 (29)	28.6 (14) 0.0 (14)
Northern Isles	**OA** **DTX-2**	0.0 (2) 0.0 (2)	0.0 (4) 25.0 (4)	16.7 (6) 0.0 (6)	n.a n.a
West coast	**OA** **DTX-2**	n.a n.a	n.a n.a	n.a n.a	n.a n.a

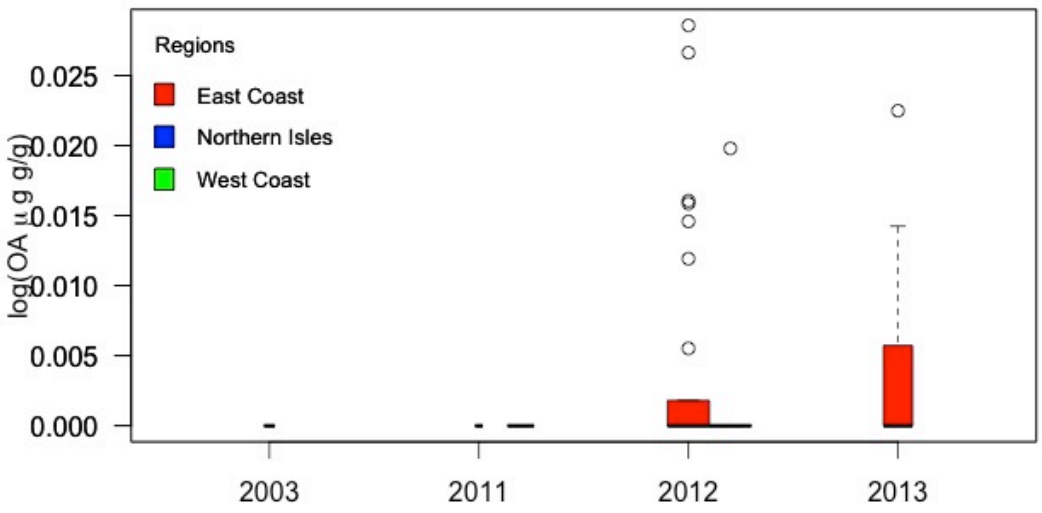

Figure 2.22: Concentration of OA in anonymous faecal samples by year, plotted on a logarithmic scale. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of OA and the black horizontal line indicates the median of the data set, separate circles are outliers (OA range: 0.00 – 0.03 µg/g).

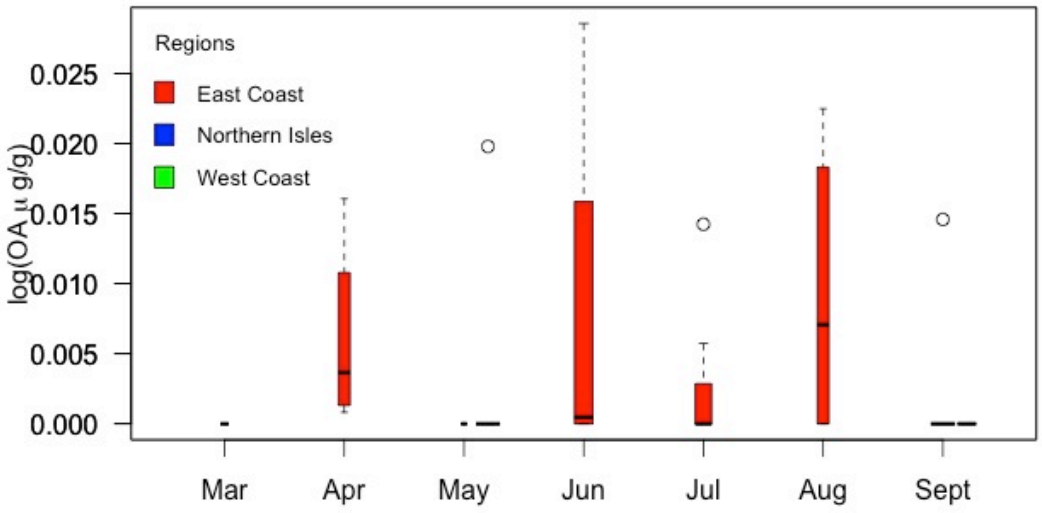

Figure 2.23: Concentration of OA in anonymous faecal samples by month, plotted on a logarithmic scale. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of OA and the black horizontal line indicates the median of the data set, separate circles are outliers (OA range: 0.00 – 0.03 µg/g).

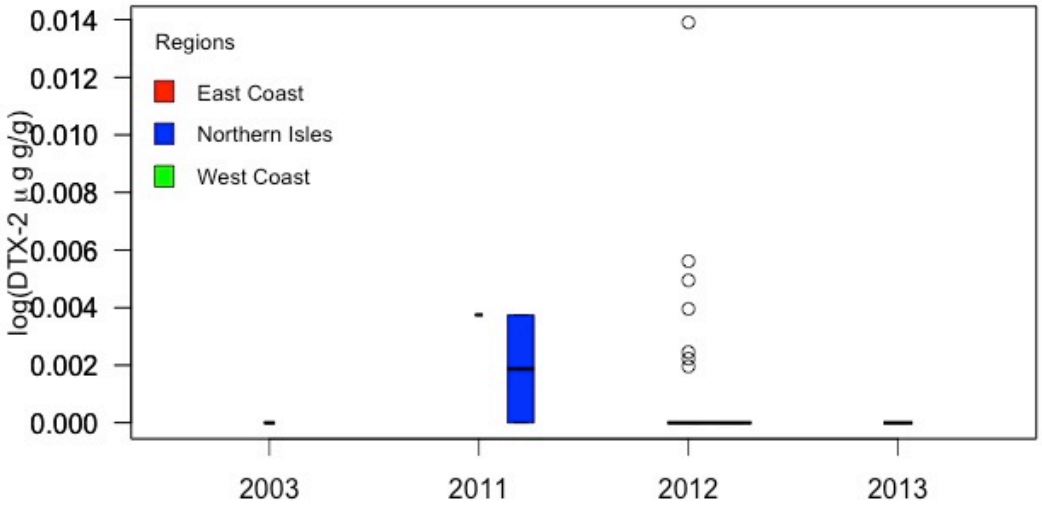

Figure 2.24: Concentration of DTX-2 in anonymous faecal samples by year, plotted on a logarithmic scale. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DTX-2 and the black horizontal line indicates the median of the data set, separate circles are outliers (DTX-2 range: 0.00 – 0.01 µg/g).

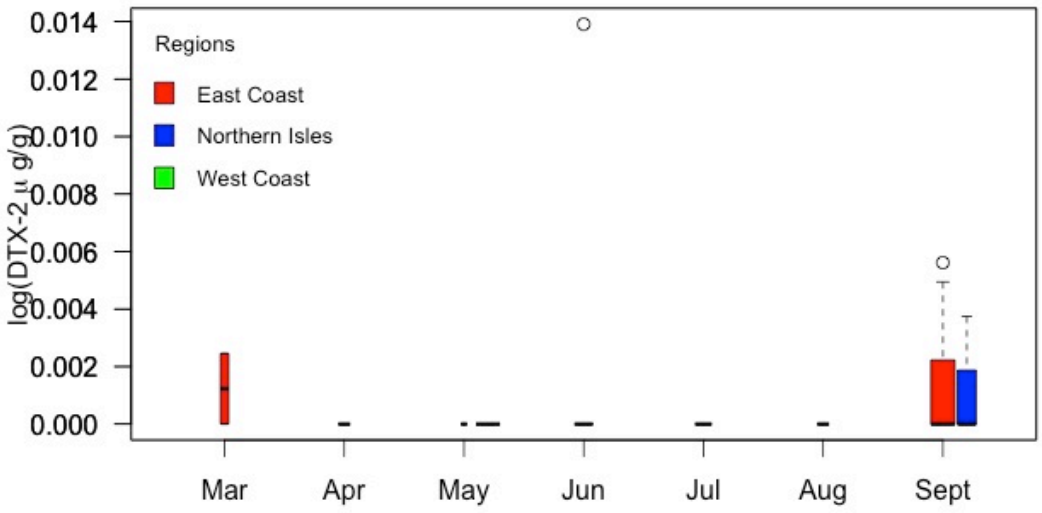

Figure 2.25: Concentration of DTX-2 in anonymous faecal samples by month, plotted on a logarithmic scale. The colours represent the different regions sampled. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DTX-2 and the black horizontal line indicates the median of the data set, separate circles are outliers (DTX-2 range: 0.00 – 0.01 µg/g).

2.3.7 Health parameters

2.3.7.1 Haematological parameters in live captured harbour seals

All live captured harbour seals handled in this study were in good visible health with no apparent abnormal neurological signs. However, among animals with DA exposure above the LOQ in urine, a significant negative relationship was found between blood lymphocyte counts and urinary DA concentration (Fig. 2.26, Table 2.14). In addition, a significant positive relationship was found between blood monocyte counts and urinary DA concentration (Fig. 2.27, Table 2.14). The same trends were seen for harbour seals positive for DA exposure in faecal samples (Fig. 2.28 and 2.29, Table 2.15) as faecal and urinary DA levels were correlated. Although some of the other leukocyte counts came out significant in the regression model (Table 2.15), these were not considered to be biologically important. A full overview of mean, median and range for neutrophil, lymphocyte, monocyte and eosinophil count can be found in Table 2.16. Faecal parasite egg counts

(nematodes) were carried out to control for any eosinophilia associated with parasite infection but no relationship was seen. Plasma cortisol concentration had no significant relationship with any of the haematological parameters. No relationships were found between the any of the haematological parameters and PSP toxins exposure.

Table 2.14: Regression models for DA concentration in urine samples and white cell count.

Model	Variable	Residual deviance	*df* fitted	*df*	P value	AIC
1.	Neutrophils	12.49	57	1	<0.001	81.81
2.	Lymphocytes	166.20	98	1	<0.001	260.48
3.	Eosinophils	31.52	61	1	0.08	141.16
4.	Monocytes	77.82	98	1	<0.001	224.51

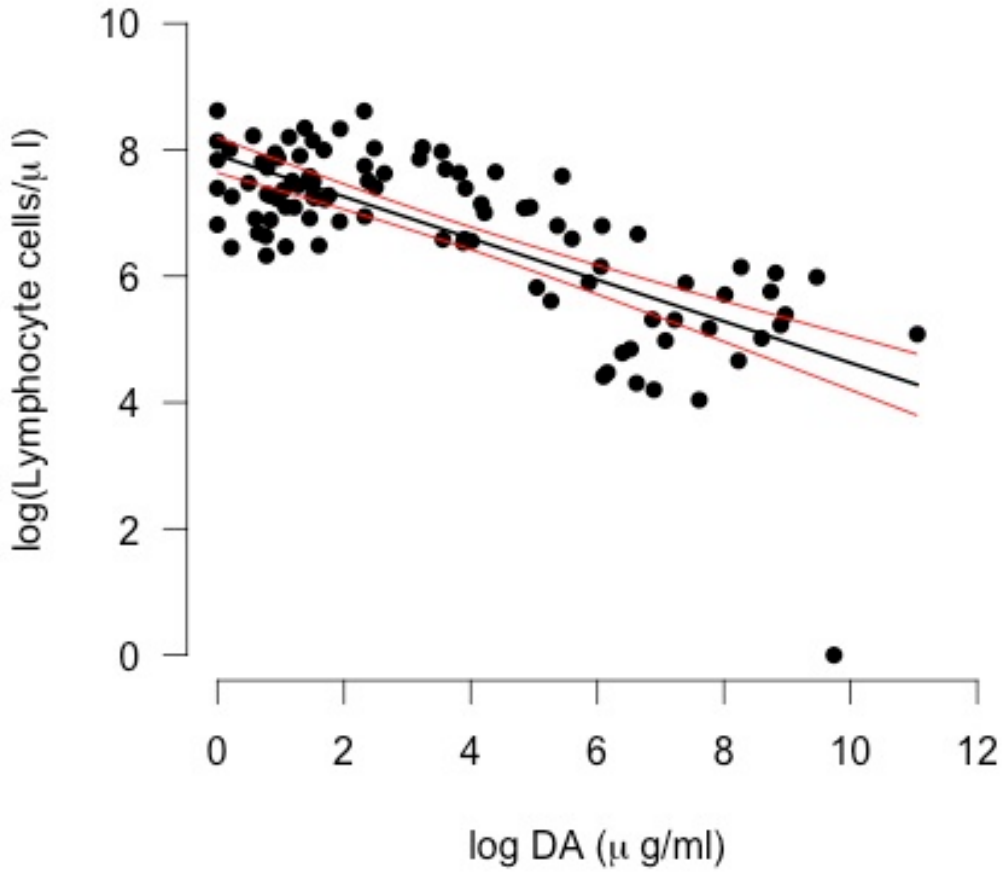

Figure 2.26: Concentration of DA in urine samples from live captured harbour seals plotted on a logarithmic scale compared with lymphocytes cell/ μ l count in live captured harbour seals with 95% confidence interval of the linear regression line.

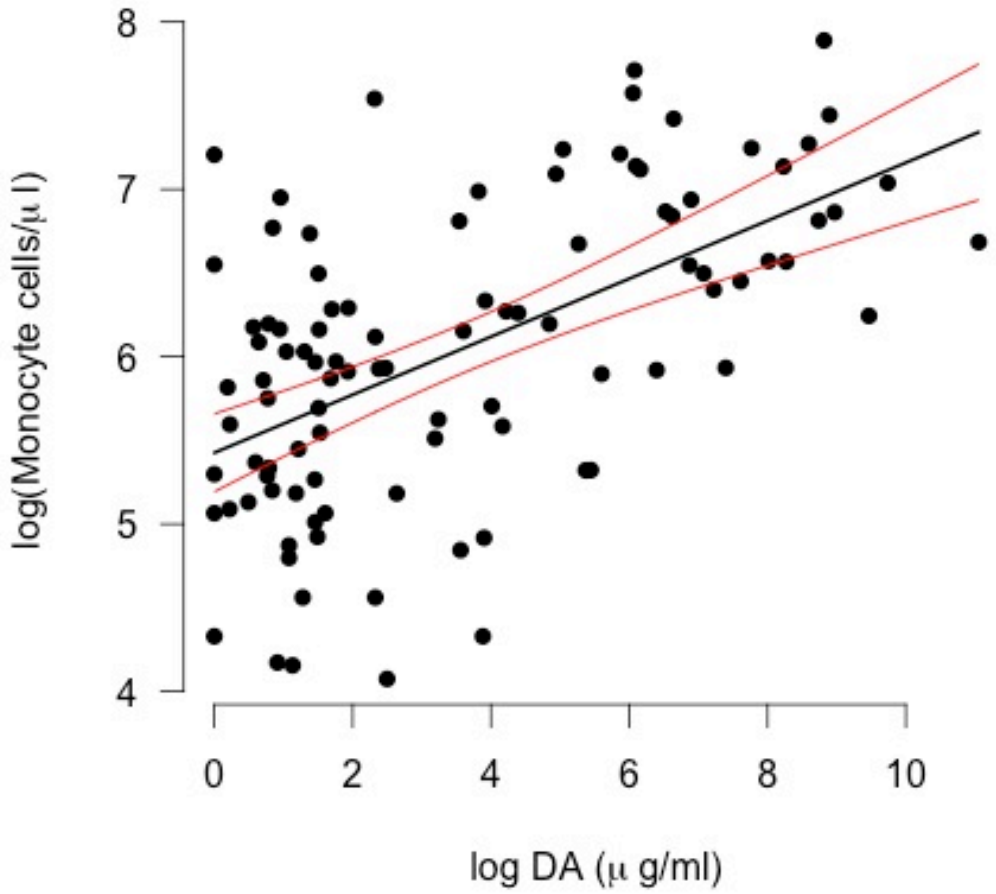

Figure 2.27: Correlation of concentration of DA in urine samples from live captured harbour seals plotted on a logarithmic scale and monocyte cell/ μ l count in live captured harbour seals with 95% confidence interval of the linear regression line.

Table 2.15: Regression models for DA concentration in faecal samples and white cell count

Model	Variable	Residual deviance	*df* fitted	*df*	P value	AIC
1.	Neutrophils	3.64	11	1	0.4	26.34
2.	Lymphocytes	35.87	47	1	<0.01	113.7
3.	Eosinophils	5.70	13	1	0.9	34.08
4.	Monocytes	5.67	11	1	<0.001	31.87

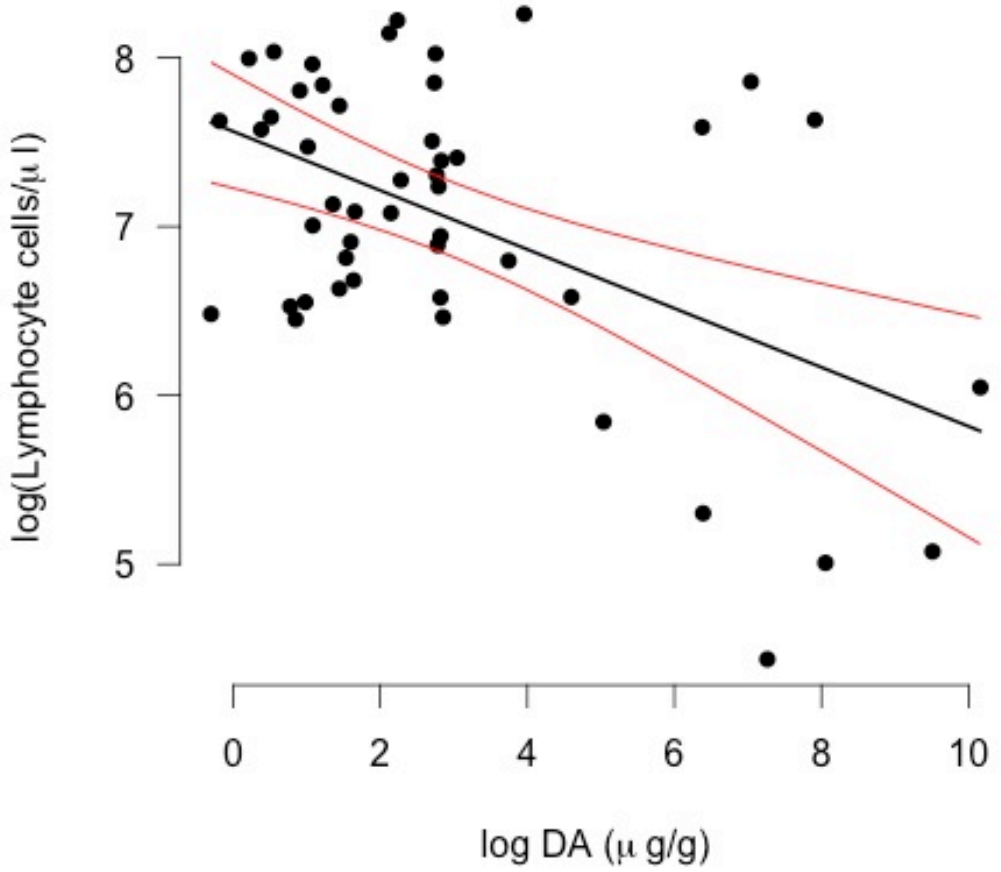

Figure 2.28: Correlation of concentration of DA in faeces samples from live captured harbour seals plotted on a logarithmic scale and lymphocytes cell/ μ l count in live captured harbour seals with 95% confidence interval of the linear regression line.

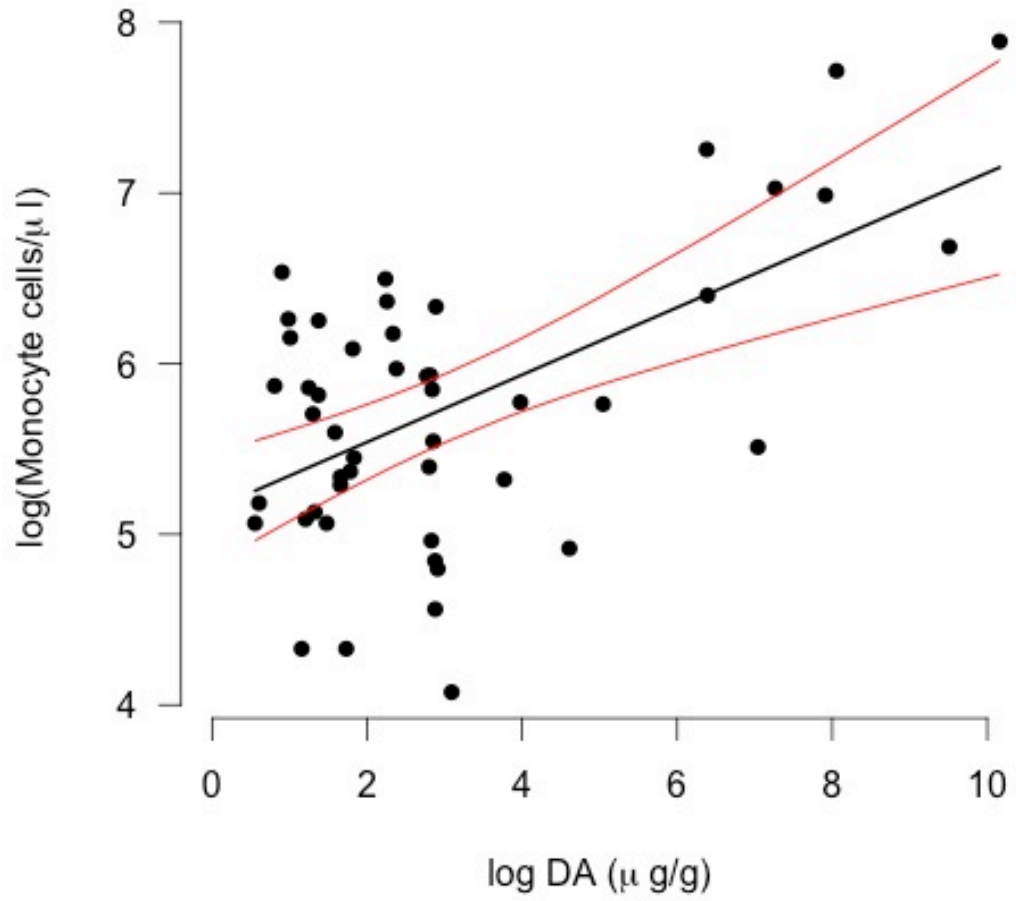

Figure 2.29: Correlation of concentration of DA in faeces samples from live captured harbour seals plotted on a logarithmic scale and monocyte cell/ μ l count in live captured harbour seals with 95% confidence interval of the linear regression line.

Table 2.16: The table displays the mean, median and range for neutrophil, lymphocyte, monocyte and eosinophil count (x 10^6 cells/ml) and plasma cortisol concentration (ng/ml) for live captured harbour seals. Groups were divided into not exposed to DA (DA urine <LOQ µg/ml, DA faeces <LOQ µg/g), and exposed to DA (DA urine >LOQ µg/ml, DA faeces >LOQ µg/g). *Value differs significantly ($p < 0.05$) from the other classification group.

Variable	Matrices	Mean ± SD	Median	Range	Reference range lower*	Reference range upper**
Neutrophil count	< LOQ DA urine > LOQ DA urine* < LOQ DA faeces > LOQ DA faeces	3080 ± 1399 2460 ± 1290 2511 ± 1237 2151 ± 1580	2850 2148 2071 1656	941-7887 769-5821 858-7887 808-6732	1120-2173	9300-11050
Lymphocyte count	< LOQ DA urine	1926 ± 1117	1655 720	557-5534 0-5501	440-1458	4794-6000

	> LOQ DA urine* < LOQ DA faeces > LOQ DA faeces	1076 ± 1140 1277 ± 1056 1163 ± 1177	982 722	73-4208 85-3861		
Monocyte count	< LOQ DA urine > LOQ DA urine* < LOQ DA faeces > LOQ DA faeces*	349 ± 283 779 ± 574 571 ± 453 864 ± 828	244 602 391 602	64-1348 59-2668 76-2232 59-2668	0-96	1170-2397
Eosinophil count	< LOQ DA urine > LOQ DA urine < LOQ DA faeces > LOQ DA faeces	645 ± 403 760 ± 517 640 ± 384 791 ± 564	601 671 561 690	157-2199 112-2297 146-1786 254-2359	0-464	2875-5157
Plasma cortisol	< LOQ DA urine > LOQ DA urine* < LOQ DA faeces > LOQ DA faeces	454 ± 247 248 ± 170 326 ± 232 172 ± 37	426 189 226 181	111-800 66-800 66-800 117-220		

* 90% CI on lower threshold ** 90% CI on upper threshold adapted from Greig et al. (2010)

2.3.8 Multiple toxin exposure

2.3.8.1 Exposure to two or more toxins and relationship with white blood cell counts in live captured harbour seals

Screening for multiple toxins first started in 2012 for live captured harbour seals with only one sample screened for multiple toxins from 2008. This is the first reported study where a marine mammal has shown to be exposed to more than two toxins from harmful algae at the same time (full overview see Table 2.17). There were two samples that indicated exposure to all four toxins at once, one was sampled on the east coast and the other was collected on the west coast (Table 2.17). There were no indications of any relationship with white blood cell counts in the live captured harbour seals exposed to two or more different toxins.

Table 2.17: Overview over percentage exposed to different toxins (urine and faeces) from live captured harbour seals with respect to toxin(s) and region, sample size in brackets.

Toxin(s)	East cost	Northern Isles	West coast
DA	77.1 (48)	71.7 (60)	53.7 (54)
STX	38.5 (13)	25.0 (8)	45.0 (20)
OA	25.0 (12)	20.0 (5)	33.3 (6)
DTX-2	58.3 (12)	0.0 (5)	0.0 (6)
DA+STX	25.0 (16)	25.0 (8)	25.0 (20)
DA+OA	16.7 (12)	20.0 (5)	33.3 (6)
DA+DTX-2	63.6 (11)	0.0 (5)	0.0 (6)
STX+OA	11.1 (9)	0.0 (5)	25.0 (4)
STX+DTX-2	37.5 (8)	0.0 (5)	0.0 (4)
OA+DTX-2	8.3 (12)	0.0 (5)	0.0 (6)
DA+STX+OA	11.1 (9)	0.0 (5)	25.0 (4)
DA+STX+DTX-2	33.3 (9)	0.0 (5)	0.0 (4)
DA+OA+DTX-2	10.0 (10)	0.0 (4)	0.0 (6)
STX+OA+DTX-2	11.1 (9)	0.0 (5)	0.0 (4)
DA+STX+OA+DTX-2	11.1 (9)	0.0 (5)	25.0 (4)

2.3.8.2 Exposure to two or more toxins and relationship with circulating cortisol concentrations in live captured harbour seals

For harbour seals exposed to two toxins at once there was a no clinical significant difference in combination with plasma cortisol, nor was there any trend in the high or low plasma cortisol for the seals exposed to three toxins.

2.3.8.3 Exposure to multiple toxins in anonymous faecal samples

Screening for multiple toxins was carried out in the anonymous faecal samples from 2012, the results are displayed in Table 2.18. One faecal sample was found to contain 3 toxins (STX, OA and DTX-2) and it was sampled on the east coast (Table 2.18).

Table 2.18: Overview of anonymous faecal samples from harbour seals with respect to toxin(s) and region, (sample size are shown in brackets).

Toxin(s)	East cost	Northern Isles	West coast
DA	89.3 (84)	70.3 (41)	71.4 (56)
STX	63.4 (41)	75.0 (8)	n.a
OA	31.1 (45)	10.0 (10)	n.a
DTX-2	15.6 (45)	10.0 (10)	n.a
DA+STX	30.8 (26)	66.7 (3)	n.a
DA+OA	31.3 (16)	0.0 (5)	n.a
DA+DTX-2	15.0 (20)	0.0 (5)	n.a
STX+OA	8.3 (24)	0.0 (7)	n.a
STX+DTX-2	29.2 (24)	0.0 (7)	n.a
OA+DTX-2	2.3 (44)	0.0 (9)	n.a
DA+STX+OA	0.0 (11)	0.0 (4)	n.a
DA+STX+DTX-2	0.0 (10)	0.0 (4)	n.a
DA+OA+DTX-2	0.0 (20)	0.0 (4)	n.a
STX+OA+DTX-2	5.9 (17)	0.0 (3)	n.a
DA+STX+OA+DTX-2	0.0 (14)	0.0 (4)	n.a

2.3.9 Prey identification

A total of 1986 otoliths were recovered from the 70 samples of faecal material collected from both live captured and anonymous harbour seals samples (full overview see Table 2.19) and were used for prey species identification. Of the total number of faecal samples; 38 faecal samples were from the east coast, 19 from the Northern Isles and 10 from the west coast. Seventeen species of prey were identified while three prey species remained unidentified. Otolith identification gives an interesting insight into the seals' diet, indirectly showing

how algal toxins can be transferred to the seals through the food web (see Chapter 3 for additional information).

The three highest DA contaminated faecal samples where otoliths were recovered came from harbour seals from the east coast sampled between July and August 2013. These faecal samples had otoliths from (listed in order from percentage frequency of occurrence): Plaice (*Pleuronectes*), Dab (*Limanda limanda*), Whiting (*Merlangius merlangus*), Long rough dab (*Hippoglossoides platessoides*), Sandeel (*Ammodytes spp*) and Cod (*Gadus morhua*).

Of the faecal material collected containing fish otoliths, the three highest PSP contaminated faecal samples were from harbour seals from the east coast and the west coast. The two east coast faecal samples were collected in June and July 2013 while the west coast faecal sample was collected in May 2013. On the east coast the harbour seals had been eating (listed in order from percentage frequency of occurrence): plaice, dab and unidentified flat fishes, while the only otolith identified from the west coast faecal harbour seal sample came from a poor cod (*Trisopterus minutus*).

There were only three faecal samples where OA was measured and from which otoliths were recovered, two of these faecal samples were from the east coast and the seals had been foraging on plaice, dab, flounder and other unidentified flatfish together with whiting and one was from Orkney where the seal had been foraging on sandeel and mackerel (*Scomber scombrus*).

For DTX-2 there was six faecal samples positive for DTX-2 where otoliths were recovered, the three highest DTX-2 contaminated faecal samples were collected on the east coast and Orkney, where the seals on the east coast had been foraging on Goby (*Gobiidae*) and whiting and the last sample collected from Orkney had been foraging on sandeels.

Table 2.19: Overview of otoliths collected from live captured and anonymous harbour seal faecal samples, total number and by region.

Species	Total	East coast	Northern Isles	West coast
Atlantic cod (Gadus morhua)	17	7	10	0
Dab (*Limanda limanda*)	668	664	1	3
Common dragonet (*Callionymus lyra*)	1	1	0	0
Eelpout (*Zoarces viviparous*)	4	1	1	2
Flounder *(Platichthys flesus*)	16	16	0	0
Goby (*Gobiidae*)	28	27	1	0
Lemon sole (*Microstomus kitt*)	3	0	3	0
Long rough dab (*Hippoglossoides platessoides*)	7	7	0	0
Mackerel (*Scomber scombrus*)	1	0	1	0
Norway pout (*Trisopterus esmarkii*)	10	0	7	3
Plaice (*Pleuronectes*)	642	634	7	3
Poor cod (*Trisopterus minutus*)	65	1	4	60
Rockling (none specific)	1	0	1	0
Saithe (*Pollachius virens*)	2	0	1	1
Sandeel (*Ammodytes marinus*)	440	285	115	40
Whiting (*Merlangius merlangus*)	13	13	0	0
Wrasse (non specific)	1	0	1	0
Unidentified flatfish	64	62	2	0
Unidentified roundfish	2	1	1	0
Unidentified trisopteran	1	0	1	0

2.4 Discussion

2.4.1 Domoic acid

There has been a temporal change in DA exposure in harbour seals before and after 2012. All the urine samples between 2008-2010 contained quantifiable amounts of DA, while only 43.6% of the harbour seal urine samples had DA levels above the LOQ in 2012-2013. This trend, with a high proportion of positive samples in 2008-2010 compared to 2012-2013, was also seen in faecal samples from both live captured harbour seals and anonymous faecal samples for which 2008 and 2010 were years with quantifiable levels of DA. These findings can be related to the annual variability in *Pseudo-nitzschia* blooms in Scotland (Fehling et al., 2006). Phytoplankton monitoring data from 2006-2013 obtained from the Scottish Association for Marine Science (SAMS) revealed three large *Pseudo-nitzschia* blooms (> 100 000 cells per litre) on the east coast in the last 10 years; two in 2008 and one in 2010 (Stubbs et al., 2013). In Orkney there has been one large *P. nitzschia* spp. bloom during the study period, this occurred in 2010. Phytoplankton monitoring data obtained from the west coast of Scotland is hard to generalize and is more detailed due to a larger concentration of monitoring sites. However, there are large *Pseudo-nitzschia* blooms every year, which vary by location, but there is little information on how widespread these blooms are and light microscope identification cannot differentiate between toxic and non-toxic species of *Pseudo-nitzschia*. For example: on the Isle of Harris, at Loch Stockinish (Fig. 2.30), *P. nitzschia* (> 100 000 cells per litre) blooms have been detected every year since 2006, except in 2007 and 2011. Loch Laxford (Fig. 2.34) on the northwest coast reported *Pseudo-nitzschia* blooms (> 100 000 cells per litre) in 2006, 2007, 2009 and 2010. Further south Loch Torridon, just north of Isle of Skye has had blooms > 100 000 cells per litre every year since 2006 with the exception of 2012.

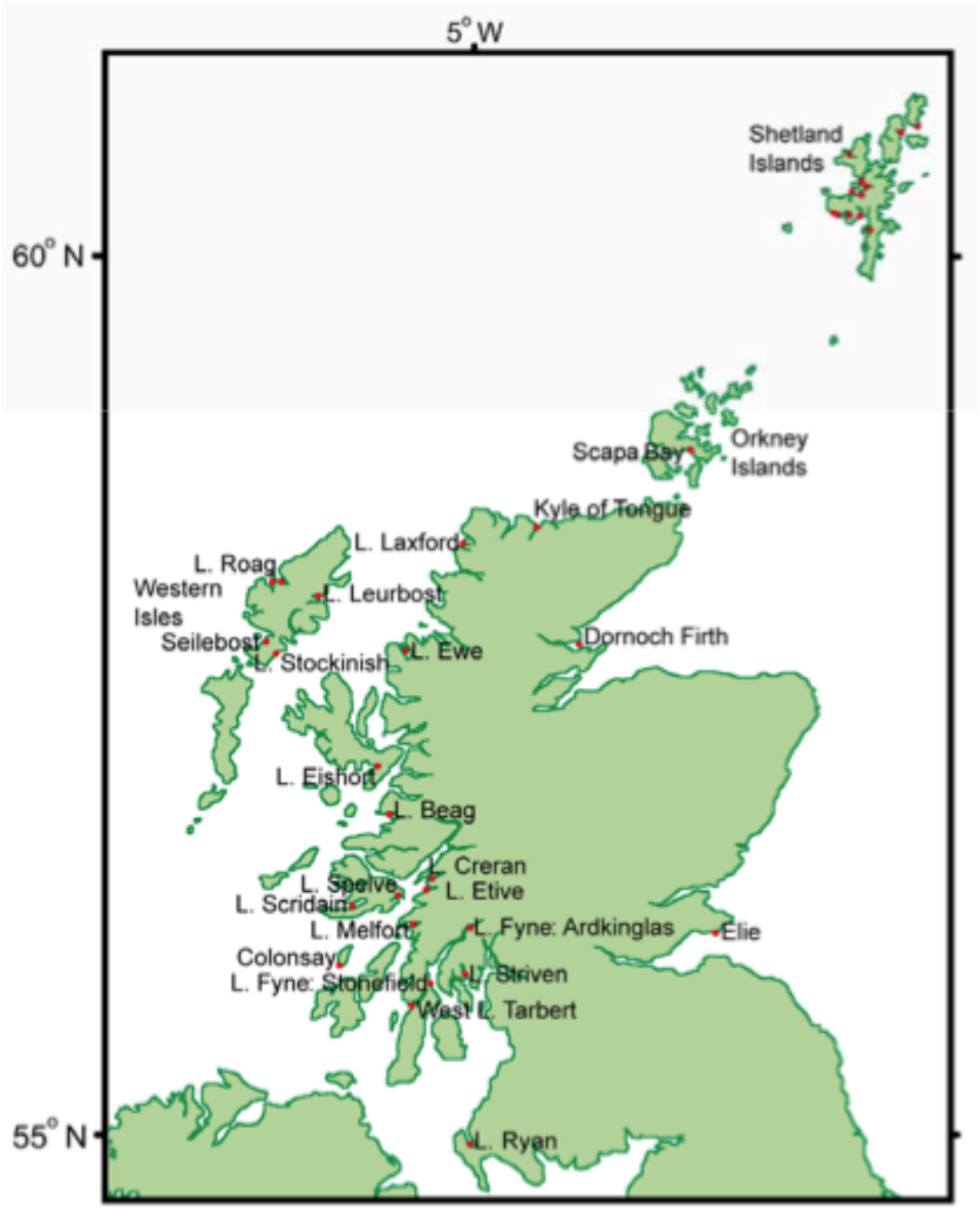

Figure 2.30: Coastal phytoplankton sampling sites contributing to the Scottish Phytoplankton Monitoring Programme, from Swan and Davidson (2011).

These monitoring data indicates the degree of variation on the west coast and it is important to stress that these blooms are monitored in sheltered areas where shellfish farms are established (Swan and Davidson, 2010), and are therefore regions that are not necessarily representative of seal foraging areas. Harbour seals have been found to forage up to 100 km from their haulout site, although on the west coast the movement usually remains within

25 km of the coast (Cunningham et al., 2009). Because of the phytoplankton monitoring locations, offshore blooms are often undetected. In addition there is a time lag between the phytoplankton blooms and the accumulation of the toxin in tissues, and based on the length of the harbour seals foraging trips which can last up to two weeks (Sharples et al., 2012), although the duration of the trips are usually a couple of days (Sharples et al., 2012). DA, a stable amino acid (Johannessen, 2000), can potentially stay in the food chain for weeks to months before it is depurated. This means that the harbour seals could potentially be exposed to DA contaminated prey for a prolonged time period, even weeks after *Pseudo-nitzschia* blooms die out. In addition DA has been shown to be present in the sediment for months and years (Sekula-Wood et al., 2011).

Goldstein et al., (2008) reported that female CLSs due to their life history constraints were five times more likely to experience a rapid absorption of DA or an 'acute' toxicity. In this study, a sex difference was seen in the Scottish harbour seals as more females had a higher concentration of DA in their excreta compared to males. This difference in DA could be explained by different foraging strategies between females and males, as females have been found to forage further out from their haul-out sites than males (Cunningham et al., 2009) or differences in prey choice between the sexes.

A study of Pacific harbour seals (*Phoca vitulina richardii*) found DA was detected in 65 % (n = 26) of the urine sampled (DA range 0.4 – 10 ng/ml), and in faecal samples (DA range: 0.002 – 2.9 µg/g). Two live-stranded harbour seals were found, off the California coast with symptoms indicative of DA toxicosis (McHuron et al., 2013). The live captured Scottish harbour seals had DA concentrations in urine higher than the levels reported for CLSs suffering from 'acute' DA toxicity (DA range: 0.01-3.72 µg/ml (Goldstein et al., 2008)) and the Pacific harbour seals, although none of the live captured Scottish harbour seals were found with seizures upon capture. For faecal samples the DA concentration was similar to those reported for CSLs experiencing 'chronic' disease (DA range: 1.6-4.15 µg/g: (Goldstein et al., 2008)). However, since we do not know when the seals were exposed to DA we cannot predict

how high the DA levels may have been. From the DA results reported here it is possible that Scottish harbour seals are experiencing a prolonged or 'chronic' exposure to DA as we are not seeing any stranding of seizing harbour seals, an indication of a recent and more 'acute' DA toxicity. From the literature we know the estimated half-life for DA in serum samples is approximately 20 min for rats (Suzuki and Hierlihy, 1993) and 2 hours for monkeys (Truelove and Iverson, 1994). DA is usually excreted from urine within two days (Bejarano et al., 2007) and it is thought to take a few days up to a week for DA to clear in faecal material (Iverson et al., 1989). Based on the DA biomarker clearance study (Chapter 6), we have an indication of the elimination half-life of the DA biomarker (3 hours) and how quickly DA is cleared out of a harbour seals body (within 10 hours). This means that the harbour seals that are out foraging for days will be in the risk of being continuously exposed to DA.

This study reports potential immunomodulatory effects of DA exposure and suggests lymphocytopenia and monocytosis. This has also been suggested in other marine mammal species (Gulland et al., 2002; Levin et al., 2010; Pulido, 2008). DA has shown to have a direct effect on both T-cells and monocytes *in vitro* (Levin et al., 2008) leading to the development of neuroinflammation and neurodegeneration. CSLs affected with DA toxicosis have been shown to develop eosinophilia (Gulland et al., 2012), an outcome also recorded in bottlenose dolphins from the northern Gulf of Mexico (Schwacke et al., 2010). This was not seen in the live captured Scottish harbour seals. Interestingly, the study revealed a negative relationship between increased DA concentration in urine and faeces and lymphocyte counts while a positive relationship between increased DA concentration in faeces and urine and monocyte counts. Abnormalities in leukocyte counts have been associated with exposure to DA. For example, neutrophilia has been associated with DA exposure, where a human patient was admitted to hospital (Perl et al., 1990) showing neurological signs associated with DA such as confusion and disorientation. Although eosinophilia was not seen in this study, different species might react differently to toxin exposure. The precise immunomodulatory effects of DA in this species are unclear, and the

consequences of these low lymphocyte counts may be an increased risk of infection while an increased monocyte count can be an indicator of an on-going infection.

Otoliths recovered in the faeces are used to determine the diet of harbour seals (Sharples et al., 2012; Tollit and Thompson, 1996), indicating what the seals have been feeding on and in this study, these fish prey species represent potential vectors for the trophic transfer of DA. Otolith identification does not give a complete description of the harbour seal diet, as some fish might be underrepresented because of complete digestion in the gut or fragile otoliths (e.g. mackerel) (Silva and Neilson, 1985). However, the highest levels of DA measured in fish (see Chapter 3 for details about the results) matched the otoliths that were identified in harbour seals exposed to DA from the east coast where the greatest decline in harbour seal abundance is occurring. Species of diet importance to harbour seals includes plaice, dab, long rough dab, whiting and sandeels.

2.4.2 PSP toxins

Saxitoxin and other PSP toxins have caused temporary closure of a number of shellfish production areas on the Scottish east coast and in Orkney (Howard, 1995-1998) during HAB episodes. PSP toxins are known to accumulate in marine fish can be the cause of sea birds and marine mammal deaths (Landsberg, 2002; Shumway, 1990). Lethal doses of PSP toxins vary with species (Levin, 1992; Meyer, 1953; Schantz et al., 1975).

Only four of the 30 recognised species belonging to the genus *Alexandrium* have been reported to occur in Scottish coastal waters (Swan and Davidson, 2011). Data from the Scottish phytoplankton monitoring program indicated that large *Alexandrium* blooms (> 500 cells per litre) occurred on the east coast of Scotland in 2008, 2009, 2012 and 2013 (Stubbs et al., 2013; Swan and Davidson, 2010, 2011). In Orkney there have been numerous *Alexandrium* blooms, where in 2006, 2007 2009 and 2011 water samples

contained >2000 cells per litre. On the west coast there were several larger *Alexandrium* blooms in 2013 (> 500 cells per litre). Interestingly, over half of the live captured harbour seals found exposed to PSP toxins (55.6 %) were captured on the west coast in May 2013. Although active screening of PSP toxins in the live captured harbour seals first started in 2012, both 2012 and 2013 were years in which harbour seals were found exposed to PSP toxins, suggesting PSP toxins should continue to be monitored in harbour seals. Blooms of *Alexandrium* occur on the east coast every year and although not all of them produce toxins, if PSP toxins are found in shellfish harvesting is suspended until the PSP toxins have fallen below maximum permitted levels. Although only acute effects of PSP toxins have been reported in mammals such as seals, prior exposure to non-lethal doses of saxitoxin may in fact make animals less susceptible to lethal doses as humans who eat low level of PSP toxins appear to be less susceptible to develop PSP (Prakash et al., 1972). In a study by Kvitek et al. (1991) captive sea otters (*Enhydra lutris*) were shown to display selective feeding when given the choice of low-dose and high-dose of toxic prey. If harbour seals are exposed to PSP toxins on a regular basis they may too potentially become less susceptible to the toxin, but not enough information is known about the harbour seal acute and chronic PSP toxin exposure in Scotland to make any assumptions on how this would potentially affect the population.

Similar to the uptake of DA by harbour seals, plaice, dab and other flat fish species, are likely to be of particular importance as vectors for PSP toxins (see Chapter 3 for PSP toxin information in fish). These fish are benthic feeders and are most likely exposed to PSP toxins through their diet of zooplankton (White, 1981b) and other invertebrates (Landsberg, 2002). Finding of exposure of PSP toxins in Scottish harbour seals raise questions about monthly and annual exposure and because of the devastating effect these toxins have shown to have (e.g. respiration, peripheral heat conservation) (Geraci et al., 1989) exposure could potentially lead to affect feeding, behaviour and recuse fitness.

2.4.3 Lipophilic toxins

This study is the first to document exposure to OA and DTX-2 in harbour seals. The finding of two harbour seals exposed to DTX-2 with loose/runny faeces is the first anecdotal evidence that DTX-2 could cause gastrointestinal problems in a marine mammal.

Shellfish (primarily bivalve filter-feeding molluscs) consumption of lipophilic toxins is severe in northern Europe. Monitoring programs are trying to minimize the effect of these toxins, mostly to protect public health and although there are no known human mortalities recorded of exposure to lipophilic toxins, monitoring has increased because it is becoming a health threat in Scottish waters. *Dinophysis* spp. appear throughout Scottish coastal waters in relatively low numbers and are indigenous to offshore waters and sea lochs. Data from 2006-2013 from the phytoplankton monitoring program reveals the biggest bloom of *Dinophysis* spp. occurred on the east coast in 2007 (> 50 000 cells per litre) and although Orkney and the west coast experience *Dinophysis* spp. blooms over the trigger limit (> 100 cells per litre) usually every spring and autumn, the largest bloom in Scotland was the one recorded in 2007. OA and DTX-2 are toxins that cause DSP with symptoms like fatigue, weakness, cramps, vomiting, abdominal pain and diarrhoea (Larsen and Moestrup, 1992; Yasumoto et al., 1984). According to the scientific panel on contaminants in the food chain (Panel, 2008) they found that OA, DTX-1 and DTX-2 were among the most toxic compounds in the Okadaic acid toxin group (Panel, 2008).

The effect the lipophilic toxins have on marine organisms is poorly understood and there are only two records of marine mammals exposed to these toxins. Firstly in a study by Fire et al., (2010b) where they found 3 out of 8 bottlenose dolphins exposed to OA, with levels ranging between 0.003-0.010 µg/g. Secondly in a study by Capper et al., (2013) who found OA in the lower gastrointestinal tract of a manatee (0.016 µg/g). In the same study they found one turtle to have traces of OA (0.009 µg/g).

The otoliths recovered from the faecal samples positive for OA and DTX-2 also gives an indication of the potential vectors. On the east coast flatfish together with whiting and goby seem to be of importance while in Orkney sandeel otoliths were found in the OA positive samples. On the west coast Norway eel pout and eelpout appear to be important vectors for OA and DTX-2 exposure.

2.4.4 Multiple toxin exposure

The current study does not document any clinically important effects of multiple toxin exposure. This could be due to a low sample size that was analysed for multiple toxins. To date there is little knowledge about the effect of multiple toxin exposure, but it is thought that several toxicants together can enhance the effects of individual substances (H.T. Hogberg pers. comm.).

There were four faecal samples indicating animals were exposed to DA and STX, one from the east coast, one from Orkney and two from the west coast where the one from the east coast had been foraging on plaice and founder. The otoliths recovered from the faecal sample collected in Orkney revealed that sandeel had been the choice of prey while the two faecal samples collected from the west coast had otoliths from poor cod. There were three faecal samples indicating animals were exposed to DA and DTX-2, two of the samples were collected on the east coast where the seals had been foraging on goby and whiting and one was collected on the west coast where the seals had been foraging on eelpout. There was only one sample where otoliths were recovered with DA, STX, OA and DTX-2 contamination, this sample was collected on the east coast and the seal had been foraging on dab, whiting, plaice, founder and unidentified flatfish which is the dominated group of fish harbour seals are known to forage on in this region (Wilson, 2014).

A few studies have reported multiple toxin exposure in marine mammals, such as the co-occurrence of OA and brevetoxin in bottlenose dolphins that was found stranded along the Texas coast (Fire et al., 2010b), co-occurrence of

brevetoxin and DA was reported in bottlenose dolphins from Sarasota Bay, Florida over a ten-year period from 1999-2009 (Twiner et al., 2011; Twiner et al., 2012). A study by Capper et al., (2013) reported multiple toxin exposure (brevetoxins, OA, STX and *Lyngbya* toxins) in stranded manatees. The western North Atlantic population of right whale population was found exposed to DA and PSP toxins between 2001 and 2006 in the Bay of Fundy, Great South Channel, Roseway Basin, and Cape Cod Bay although no associated effects was reported (Doucette et al., 2012).

2.4.5 Dose estimation

Oral dose estimations can be carried out by calculating how much toxin the seals are likely to ingest on a daily basis. For example, in a study by Härkönen and Heide-Jørgensen (1991) they estimated an average of 4 680 kcal per seal as their daily energy requirement which corresponds to ingestion of ca. 3.7-4.2 kg fish per seal per day. Since the toxins occur in the viscera of the fish and from the samples in this study, based on available literature, I estimated the viscera to be approximately 20% of the fish total mass (Horn et al., 2005), a seal would ingest about 0.8 kg viscera per day. An adult harbour seal weighs about 80 kg (total body mass) and is approximately 60 kg (lean body mass) (Reilly and Fedak, 1991) and if it consumed fish with the highest DA detected in the viscera (plaice; 117.4 µg/g) an adult harbour seal would ingest ~1.6 mg DA/kg (lean body mass)/day (lethal oral dose 2.71 mg DA kg^{-1} adapted from Bejarano et al. (2007)). Similarly, for PSP toxins if a harbour seal ingested the highest PSP toxins detected in the fish identified from the faecal material (dab: 1021 µg STX eq/kg), it would ingest ~13.6 µg STX eq per kg of lean body mass/day. Some PSP analouges might be more toxic than others, and it has been reported that an oral dose between 180-200 µg/kg b.w would kill a dog (Mons et al., 1998). Whilst these levels are well below the lethal levels reported for various mammalian species and laboratory animal models, seals feed in bouts, with a mean foraging trip duration of between 1 to 5 days (Sharples et al., 2012). Therefore there may be days when this estimated mean daily oral dose is greatly exceeded. Further

detailed pharmacokinetic modelling incorporating oral dose estimates, the half-life of the toxins in seals and their feeding behaviour is required to estimate the true impact of these levels on the health and survivorship of harbour seals.

Estimating exposure and inferring potential lethal effects from the urine and faecal concentrations of toxins in live captured animals could be biased. Sick seals may not haul-out and if they do they may haul-out alone and therefore not be accessible for capture due to the impacts of the toxins. Therefore spatial exposure was investigated by collecting anonymous faecal samples at a wider range of harbour seal haul-out sites. This provides a better indication of the temporal and spatial variation in exposure although it will not provide information about the health of the animals. As mentioned above, harbour seals forage at sea for an average of between 1-5 days at a time (Sharples et al., 2012), and will therefore also defecate at sea. This means that the anonymous/random faecal samples, may only come from seals that had foraged closer to the shore but those feeding farther offshore may have depurated the toxins by the time they return to haul out, so their levels might go undetected. Although there is little knowledge about the assimilation efficiency of DA in harbour seals, there is data on the clearance rate of DA in monkeys and rats (Suzuki and Hierlihy, 1993; Truelove and Iverson, 1994) where DA, which is primarily excreted in urine, remains in the tissues and urine for a few hours after ingestion of toxins, whilst excretion rates through faeces may take much longer (clearance in harbour seals is estimated in Chapter 6).

2.4.6 Scottish harbour seal decline and the link with algal toxins

This study demonstrates the link between exposure and prey consumption, with evidence that the harbour seals are exposed to DA on an annual basis and that there are regional differences with the east coast being a potential hot spot as higher DA levels are found in this region. By contrast, harbour seals sampled on the west coast had significantly lower DA levels. However,

the west coast regions sampled in this study were limited so further locations need to be included in future studies to determine whether this pattern persists. Results from Chapter 3 documents toxin levels in the Scottish harbour seal fish prey on the east coast of Scotland where the biggest decline is occurring. PSP toxin uptake was first studied in Scottish harbour seals in 2012 and the data from 2012 and 2013 seem to suggest an absence of regional differences. There is a lack of information regarding harbour seals exposure prior to 2012 and in addition, the lack of seal carcasses from the west coast in particular makes regional comparisons difficult.

The decline in Scottish harbour seals was first detected in the counts of seals hauled out during their annual moult in August around 2000. In Orkney and the North Coast (mainland) the population has declined at an annual rate of ~13 %, while the Shetland harbour seal population has declined less rapidly (30 % since 2000) (SCOS, 2013). The west coast and the Western Isles harbour seal populations appear to be stable over the same period. The Moray Firth population on the east coast had declined, but more recent population counts suggest that it may now be stable (SCOS, 2013). The greatest decline has been seen in the Firth of Tay population on the east coast where the average rate of the annual decline is ~18 % and the population size has decreased by 85 % since 2000 (SCOS, 2013). Although several factors have been considered responsible for the sharp harbour seal population decrease, there is not a clear single factor, which would explain the decline. The most likely explanation probably includes a combination of factors, such as shooting and trauma (Bexton et al., 2012; Matthiopoulos et al., 2014). Toxins from harmful algae were first monitored in harbour seals by Hall and Frame (2010) who found the Scottish harbour seals to be exposed to DA. Seeing that DA has caused increased mortality or reduced reproductive success in CSLs (Goldstein et al., 2008) the hypothesis was to further investigate if this is occurring in Scottish harbour seals.

Although there is little information about the occurrence of toxin-producing algal blooms in offshore areas where the seals are foraging, this study clearly demonstrates that the toxins are taken up by the prey species consumed by

the seals at levels that have the potential to cause harmful and lethal effects that would perturb the population dynamics of these species.

2.5 Conclusions

Here I show that the exposure of Scottish harbour seals to DA, PSP toxins and the lipophilic toxins OA and DTX-2 is likely to occur through their consumption of contaminated fish prey such as plaice, dab, long rough dab, whiting, and cod rather than through the secondary ingestion of toxins in the water column or sediment. Harbour seals on the east coast of Scotland had over three times higher DA concentrations in their urine compared to those from the Northern Isles and the west coast and although there is a lack of anonymous faecal samples from the west coast there is an indication that there are higher DA concentration values on the east coast. Of some note is the fact that the east coast is the region with the greatest population decline. Although the DA levels in urine reported in this study are similar to those measured in the urine of acutely poisoned CSLs, urinary levels do not appear to be representative of acute toxicity as the live captured harbour seals all appeared healthy upon capture. This is because neither study was able to determine exactly when the toxins had been consumed, making any direct comparisons unreliable. The results in this study highlight a likely chronic rather than acute exposure of Scottish harbour seals to DA and PSP toxins, where long-term effects are not yet fully understood. Although no overt health effects were observed in the live captured animals as assessment was only possible for a few minutes around capture and adrenaline and other stress responses let alone sedation from the process of capture may obliterate subtle neurological signs. This study suggests immunomodulatory effects of DA exposure including lymphocytopenia and monocytosis, which may suggest risks for the health and survival of exposed harbour seals.

Chapter 3

3. Potential fish vectors for toxins from harmful algae in SE Scotland

3.1 Introduction

3.1.1 Harmful algal blooms and marine organisms

The term “harmful algal bloom” or HAB is often used to describe visible blooms of certain types of marine algae that produce toxins. There is a growing interest in measuring the progression of toxins from the producing organism (phytoplankton) throughout the food chain as both HABs and their toxic products are known to cause fish deaths, render shellfish poisonous and cause detrimental health effects and mortality in humans, birds and marine mammals through trophic and predator-prey interactions (Anderson and White, 1992; Coulson et al., 1968; Landsberg, 2002; Lefebvre et al., 1999; Scholin et al., 2000; Sierra-Beltràn et al., 1997). HABs can also cause important economic losses, especially for aquaculture and fisheries (Hoagland et al., 2002; Perl et al., 1990; Scholin et al., 2000; Sierra-Beltràn et al., 1997; White, 1981b) thus understanding the key drivers of toxin distribution and uptake through coastal ecosystems is of interest to conservation managers.

3.1.2 Direct exposure to algal toxins

Direct exposure to toxins from harmful algae occurs if organisms ingest or inhale (e.g. filter-feeding, predation) the algae and its associated toxin (Landsberg, 2002). Key vectors such as zooplankton, sponges and shellfish together with planktivorous fish that directly feed on algae, filter the water and absorb algae and toxins into their somatic tissues or viscera (Landsberg, 2002; Lefebvre et al., 1999; Teegarden et al., 2001). Of the marine mammals that are exposed to HAB toxins, manatees are directly exposed through their

main food sources, turtle grass and eelgrass where the toxin is found in the epiphytes on the seagrass, but also in the seagrass blades and rhizomes (Capper et al., 2013). Benthic organisms can be exposed through consumption of toxic cells or absorption of toxins that sink to the bottom as some toxic algae have sedimentary cysts or resting stages as part of their lifecycle (Anderson et al., 1996; Landsberg, 2002). Cysts from *Alexandrium* have shown to be 1000 times more toxic in their cyst stage than in their vegetative cell stage (Dale et al., 1978).

3.1.3 Indirect exposure to algal toxins

Trophic transfer of toxins from harmful algae occurs when an organism consumes another organism lower down the food chain that has been exposed directly to the toxic algae. Such indirect exposure thus occurs following the consumption of contaminated prey from planktivorous fish and squid, carnivorous molluscs and crustaceans (Deeds et al., 2008; Landsberg, 2002; Lefebvre et al., 1999; Turner and Tester, 1997). This is the mechanism by which toxins are then transferred from lower organisms to top predators such as marine mammals. In addition there is also an indication that some toxins can persist in the food chain long after the toxin produced phytoplankton bloom has ended (Sekula-Wood et al., 2009). These are the pathways that can move algal toxins from the bottom to the top of the food chain where the entry point can be through zooplankton that feed directly on toxic algae. Although top predators may be exposed to toxins directly, trophic transfer of toxins is probably the most important pathway for higher trophic levels to be affected by toxins from harmful algae.

3.1.4 Domoic acid in marine organisms

Pseudo-nitzschia is a widespread marine diatom that exhibits a range of physiological tolerances. Diatoms often dominate spring blooms, and due to their immobility they require turbulence to keep them in a well-mixed water column. There are several factors that have been shown to trigger the

production of domoic acid (DA) in *Pseudo-nitzschia*. Under certain environmental conditions, when the blooms have reached their peak and nutrients become limited, the diatoms can become stressed and this can be a factor to trigger toxin production (Bates et al., 1998; Takagi, 1993). *Pseudo-nitzschia* is a common diatom in the phytoplankton community in Scotland (Stobo et al., 2008) and they can out-compete other algae in conditions when silica is available or the temperature is falling, while other algae need warmer temperatures, low salinities and calmer seas to bloom (Bates et al., 1998; Mos, 2001). Not all species of *P. nitzschia* produce toxin, but those that do, produce the neurotoxin domoic acid (DA). In addition DA is an amino acid and a glutamate agonist that interferes with neurotransmission in the brain and causes damage to the hippocampal area by interfering with the ions and depolarizing the neurons. This causes neuronal swelling and the nerve cells can go through an apoptosis. These nerve cells are important for memory storage and learning and there have been several reports of humans and animals exposed to DA where *P. nitzschia* was involved (Bates et al., 1989; Costa et al., 2005b; Fire et al., 2009; Gulland et al., 1998; Hall and Frame, 2010). DA is one of the biggest health threats to CSLs, and the effect it has depends on the amount of toxic prey the sea lions ingest through their diet. Vectors such as krill, crab, shellfish and finfish are known to filter or ingest and accumulate the algae (Landsberg, 2002; Lefebvre et al., 1999). North Atlantic right whales have been found to be exposed to DA throughout the spring, summer and autumn months in 2005-2006, most likely through ingestion of a contaminated copepod vector (Leandro et al., 2010). A study in Western Greenland discovered that three Calanus species can retain DA after 10 hrs of depuration in filtered sea water and act as potential vectors of DA (Tammilehto et al., 2012). In Scotland, data from Marine Scotland Science revealed the presence of DA in copepods at the Stonehaven coastal monitoring site on the northeast coast of Scotland. A maximum of 0.02 ng DA per copepod was detected which coincided with cell densities of *P. nitzschia spp*. of approximately 150,000 cells/L (K. Cook pers. comm.). There are several reports of fish as vectors for DA, such as the northern anchovy (*Engraulis mordax*) and Mackerel (Altwein et al.; Lefebvre et al., 1999; McGinness et al., 1995; Sierra-Beltràn et al., 1997; Work et al., 1993) where

toxicity in fish is restricted to the gut content although brain and muscles in anchovies have shown low DA uptake (Lefebvre et al., 2001). This shows that foraging strategy can influence whether or not a top predator is exposed to DA. No data currently exists on toxins in fish from Scottish waters.

3.1.5 PSP toxins in marine organisms

The microscopic marine dinoflagellate genus *Alexandrium* poses the greatest concern for top predators, due to its toxicity and it is the only genus with toxic dinoflagellate known to produce PSP toxins and other members of its family. PSP toxins can cause the human illness PSP which has largely been associated with the consumption of contaminated mussels (Shumway, 1990). A regular bloom from this genus has been known to occur for several decades on the southeast coast of Scotland, but PSP events are rare and after an outbreak in 1968 where 80 % of the Shags on the Farne Islands on the northeast coast of England, were killed after eating contaminated sandeels (Clark, 1968; Coulson et al., 1968), the Scottish coast has been monitored for *Alexandrium* and PSP toxins. Most research studies have been focused on species that are of economical value such as shellfish and PSP toxins have been documented in both bivalves and gastropods (Shumway, 1990). Zooplankton has been identified as a vector for higher trophic levels as they transport PSP toxins through the food chain (Teegarden et al., 2001; Turner, 2014; White, 1981b). Archived samples of copepods collected during summer 2008 in coastal waters near Stonehaven, North-east of Scotland, were analysed for PSP using HPLC-FLD (J.P. Lacaze, pers. comm.). Toxicity results were found to be negative, which fits with the low cell abundance of *Alexandrium* (below 60 cells/L) present at the time of sampling.

Today there are numerous reports of lethal and sub lethal occurrences involving exposure to PSP toxins (Fire et al., 2012; Landsberg, 2002; Landsberg et al., 2006) and laboratory experiments confirms similar sensitivity of PSP toxins to fish as seen in homeotherms (White, 1981a). In 1976 in the Bay of Fundy in Canada, mortality of the Atlantic herring (*Clupea harengus*

harengus) was documented in connection with an *Alexandrium tamarense* bloom and PSP toxin production, the vector was reported to be the planktonic herbivore *Limacina retroversa* (White, 1977). Zooplankton have also been documented to bioaccumulate PSP toxins (Teegarden and Cembella, 1996). In 1984 fish mortality was reported from the Faroe Islands in connection with a bloom of *Gonyaulax excavate* (Mortensen, 1985). The Mediterranean monk seal was exposed to PSP toxins in 1997 likely through contaminated fish and STXs were a likely factor in the mass mortality of the population inhabiting the Mauritanian coast of Africa (Hernández et al., 1998). Other animals such as the endangered North Atlantic right whales population inhabiting the Bay of Fundy and adjacent New England waters are exposed to PSP toxins on an annual basis and questions were raised after this discovery if PSP toxins are compromising their health and reproductive status (Doucette et al., 2012; Durbin et al., 2002).

3.1.6 Lipophilic toxins in marine organisms

Some dinoflagellates from the genus *Dinophysis* produce toxins that can cause the human illness DSP (Yasumoto et al., 1984). OA and DTX-2 are two structural derivatives in the DSP toxin group (Yasumoto et al., 1984), which are the most abundant along European coasts (Aune and Yndestad, 1993). Despite OA being considered a more harmless toxin than DA and PSP toxins, it presents a potential threat as a tumour promoter (Fujiki et al., 1991). DTXs have also shown to induce tumours (Fujiki et al., 1988) on mice skin and is, together with OA, a potential threat to health. Shellfish (primarily bivalve filter-feeding molluscs) consumption is large in northern Europe and DSP illness has been recorded from several countries (Dale et al., 1993; Kat, 1983; Krogh et al., 1985). Exposure to OA was confirmed in a human after eating razor clams (*Solen marginatus*) and green crabs (*Carcinus maenas*) where a level of 16 μg/100 g was found in the edible parts. There is little information about these toxins in fish, to my knowledge the only report of natural OA in fish was from a barracuda (*Sphyraena barracuda*) although the toxin confirmation has never been verified (Gamboa et al., 1990; Landsberg, 2002). In a molecular

development study by Escoffier et al., (2007) the development of medaka fish (*Oryzias latipes*) embryos was studied where they found that OA disturbs embryo development which can impact fish recruitment. However, in general very little is known about the natural exposure of these lipophilic toxins and their effect on marine organisms.

3.1.7 Trophic transfer of harmful algal toxins

Reports and studies indicate that zooplankton, krill and fish are important vectors for transferring harmful algae toxin up the food chain (Bargu et al., 2002; Landsberg, 2002; Maneiro et al., 2000). In addition to fish and zooplankton; uptake has been documented in crabs, lobsters, gastropods (sea-snails, sea-slugs and limpets), bivalves (mussels) and echinoderms (starfishes and sea-urchins). This provides evidence of toxin exposure to edible benthic communities (Landsberg, 2002; Silva et al., 2013). Shellfish is the most common vector for transferring toxins to humans, and although fish have been shown to accumulate toxins, they do not appear (except from a few exceptions) to accumulate in the flesh of the fish, only in the gut (viscera). Bretz et al., (2002) suggested using species other than shellfish for toxin monitoring such as sand crabs as they have shown to be a comparable indicator species to mussels. However, the extent and pace that these toxins are transferred through the Scottish food chain is virtually unknown, particularly outside the shellfish production regions.

In this thesis I show that Scottish harbour seals are exposed to DA, PSP toxins and OA together with DTXs (Chapter 2). The aim of this chapter was therefore to investigate the potential routes of trophic transfer to the Scottish harbour seals main prey, together with a random selection of by-caught fish species collected on the east coast of Scotland by determining the levels of DA, PSP toxins and lipophilic toxins (OA and DTXs) in the viscera of the fish. This information will enable me to estimate the ingested concentrations of the various toxins by the harbour seals from the east coast.

3.2 Methods

3.2.1 Fish collection

Fish were opportunistically collected from local fishermen in Pittenweem in the summer and autumn of 2012 and throughout the calendar year of 2013 (except Jan, Feb and Apr 13) from the fishing grounds in the Firth of Forth (Fig. 3.1). By caught fish were collected based on availability during the seasons and varied in length from 5 cm to 50 cm. Note that not all species were caught every month. Of notable importance was that the fish were not collected during any HAB blooms in the region. A scientific cruise in the Firth of Forth hosted by Marine Scotland Science collected flatfish for toxin analysis in the autumn of 2012 (Fig. 3.1). The viscera (fish gut) were removed from pools of 2 or more fish (depending on size) to provide a sample of ~50 g (stomach prioritized) and stored at -20 °C until further analysis. Out of interest, one cod liver was divided up and analysed for all three, toxin groups as humans in several countries consume cod liver, particularly in the form of cod liver oil.

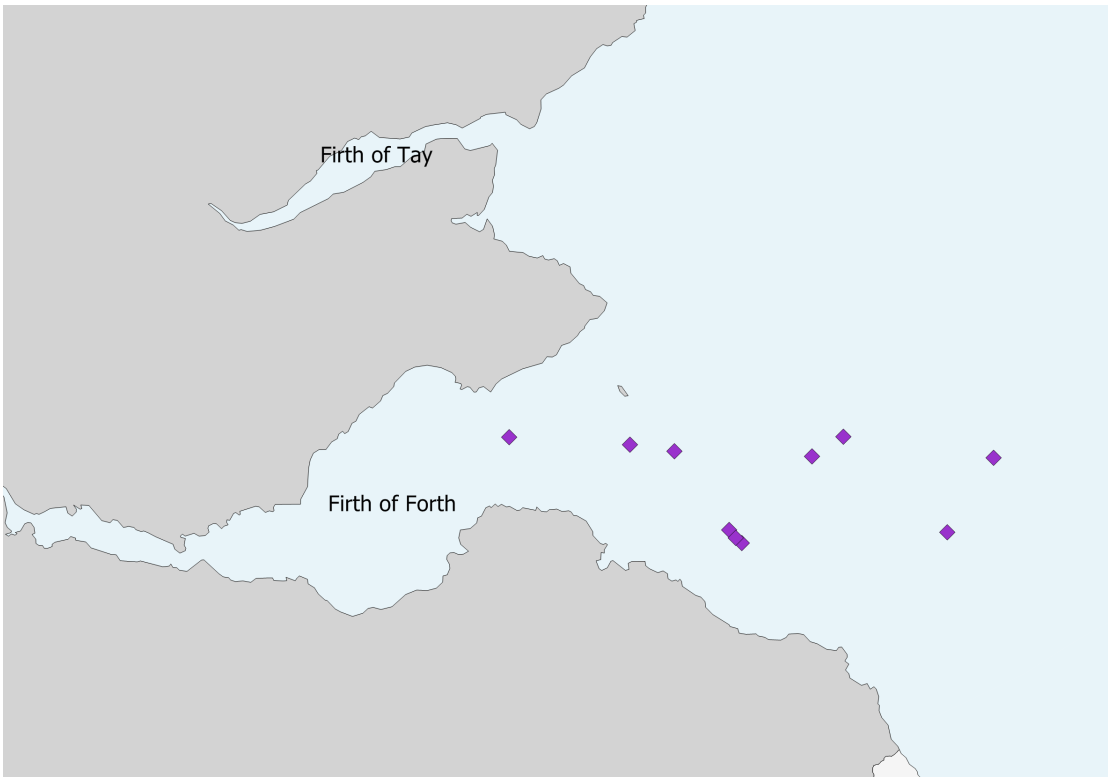

Figure 3.1: This map shows the sampling sites (purple squares) of fish samples collected off the southeast coast of Scotland between 2012-2013.

3.2.2 DA extraction and quantification

For extracting DA, no less than 50 g of viscera was blended in a mixer and a 4 g sub sample removed. DA was extracted following the procedure given in Chapter 2, section 2.2.3.1; Sample extraction. Detection and quantification of DA in the extracts was done using the same instrumentation as described in Chapter 2, section 2.2.3.3; Instrumentation. The limit of detection (LOD) was set to 7.00 ng/g and limit of quantification (LOQ) to 25.00 ng/g.

3.2.3 PSP toxin extraction and quantification

For PSP toxin extraction, a 4 g aliquot fish viscera subsample was extracted following the same method described in chapter 2 section; 2.2.4.1 Sample extraction and protein was precipitated following the method in chapter 2, section; 2.2.4.2 Protein precipitation. PSP toxins were measured and quantified using the instrumentation described in Chapter 2 section; 2.2.4.4 Instrumentation for PSP toxin analysis using HPLC. The LOQ for the PSP

toxins are described section 2.2.4.4 Instrumentation for PSP toxin analysis using HPLC.

3.2.4 OA and DTXs extraction and quantification

To extract the lipophilic toxins the fish viscera were homogenised and 5 g subsampled. Lipophilic toxins were extracted following the same method as described in chapter 2, section 2.2.5.1 Sample extraction and 2.2.5.2 Hydrolysis of Esterified Forms of OA, DTX-1, and DTX-2. The extracts were measured and quantified using the instrumentation described in chapter 2; section 2.2.5.3 Instrumentation. The LOD was put to 6.00 ng/g and LOQ of 20.00 ng/g for both OA and DTX-2.

3.2.5 Data analysis

The data for this Chapter is presented in ng/g to match the current literature and the conversion from ng to µg/g is to divide by 1000. Data exploration using boxplots were made for each fish species group. This enabled me to compare levels in each fish species to one another. Regression models were used to select the models that best fitted the data with toxin concentration of the toxin as the dependent variable and month (factor) as the independent variable. Akaike's information criterion (AIC) was used to rank the models. The AIC is a useful measure that balances goodness of fit of the model against model complexity (Akaike, 1974). All analyses were performed using R software (R Development Core Team 2007).

3.3 Results

3.3.1 Domoic acid detection in fish

Over the 2-year study period on the east coast a total of 26 species and 215 individually pooled samples were analysed for DA. Of the samples analysed for DA 41.4 % was found >LOQ (n = 89) which included 18 different species

of fish. DA ranged from 50 ng/g to 117 400 ng/g (Table 3.1). The highest sample was measured in a plaice (*Pleuronectes*, Fig. 3.2 and 3.3) suggesting these flatfish in particular may be important vectors of DA. Other flatfish exposed to DA included species such as dab (Fig. 3.2 and 3.3), lemon sole (*Microstomus kitt*, Fig. 3.2 and 3.3), long rough dab (Fig. 3.2 and 3.3) and brill (*Scopthalmus rhombus*, Fig. 3.2 and 3.3). Of the benthic fish species exposed to DA, these included; gurnard (*Chelidonichthys*, Fig. 3.4), sandeel (Fig. 3.4) and sculpin (*Cottidae*, Fig. 3.4). The pelagic fishes exposed to DA included bib (*Trisopterus luscus*, Fig. 3.5 and 3.6), cod; Fig. 3.5 and 3.6), haddock (*Melanogrammus aeglefinus*, Fig 5 and 6), herring (*Clupea harengus*, Fig. 3.5 and 3.6), horse mackerel (*Trachurus trachurus*, Fig. 3.5 and 3.6), mackerel (Fig. 3.5 and 3.6), pollock (*Pollachius pollachius*, 3.5 and 3.6), saithe (*Pollachius virens*, Fig. 3.5 and 3.6), sprat (*Sprattus sprattus,* Fig. 3.5 and 3.6), and whiting (*Merlangius merlangus*, Fig. 3.5 and 3.6). For a full overview of the species and range levels for DA see Table 3.1. The cod liver analysed did not contain any detectable DA. With all fish groups (flatfish, benthic and pelagic) analysed together, there was a seasonal difference in DA uptake where May, June and November had a lower monthly mean than the other months (Table 3.2, model 1, $p < 0.01$ and Table 3.3). There was no seasonal difference with the fish group split into flatfish, benthic and pelagic (Table 3.2, model 2, 3 and 4).

Table 3.1: Overview over the fish sampled and analysed for the different toxins, here represented by min and max toxin concentration (sample size in brackets).

Species	DA range ng/g	STX range mg STX diHCl eq/kg	DTXs range ng/g	OA range: (LOQ >20 ng/g)
Bearded rockling (non specific)	0 (3)	0.2-15.37 (2)	0 (2)	0 (2)
Bib (*Trisopterus luscus*)	0 - 70 (2)	0.3 (1)	0 (1)	0 (1)
Brill (*Scopthalmus rhombus*)	80 (1)	0.01 (1)	0 (1)	0 (1)
Cod (*Gadus morhua*)	0 – 2655 (12)	0 – 0.09 (12)	0 (10)	0 (10)
Dab (*Limanda limanda*)	0 – 5075 (31)	0 – 1.02 (29)	4.98 (29)	0 – 31.6 (29)

Dogfish (*Scyliorhinus canicula*)	0 (1)	0.01 (1)	0 (1)	0 (1)
Dover Sole (*Solea solea*)	0 (1)	n.a	0 (1)	0 (1)
Flounder (*Platichthys flesus*)	0 (1)	n.a	n.a	n.a
Gurnard (non specific)	0 – 31 (7)	0 – 23 (4)	0 (5)	0 (5)
Haddock (*Melanogrammus aeglefinus*)	215 – 811 (2)	0.06 – 0.70 (2)	0 (2)	0 (2)
Hake (*Merluccius merluccius*)	0 (4)	0 – 0.65 (4)	0 (4)	0 (4)
Herring (*Clupea harengus*)	0 – 114 (5)	0 – 0.05 (3)	0 (2)	0 (2)
Horse Mackerel (*Trachurus trachurus*)	75 – 680 (2)	0.05 – 0.35 (2)	0 (2)	0 (2)
Lemon Sole (*Microstomus kitt*)	0 - 5580 (29)	0 – 21.2 (26)	2.5-7.45 (21)	0 – 97.0 (21)
Long rough dab (*Hippoglossoides platessoides*)	0 – 1800 (24)	0 – 0.75 (20)	0 (14)	0 – 16.0 (14)
Mackerel (*Scomber scombrus*)	80 – 14265 (9)	0 – 2.2 (8)	0 (10)	0 (10)
Monk fish (*Lophius* sp.)	0 (1)	0 (1)	0 (1)	0 (1)
Plaice (*Pleuronectes*)	0 – 117400 (34)	0 – 0.76 (33)	1.73-3.13 (31)	0 (31)
Pollack (*Pollachius pollachius*)	80 (1)	0.03 (1)	0 (1)	0 (1)
Red mullet (*Mullus Surmuletus*)	0 (1)	0.07 (1)	0 (1)	0 (1)
Saithe (*Pollachius virens*)	0 – 275 (3)	0 – 0.1 (3)	0 (3)	0 (3)
Sandeel (non specific)	0 – 5 (9)	0 (5)	0 (5)	0 (5)
Sculpin (Cottidae)	0 – 135 (10)	0 – 0.39 (8)	0 (8)	0 (8)
Sprat (*Sprattus sprattus*)	17 (1)	0 (1)	0 (1)	0 (1)
Tusk (*Brosme brosme*)	0 (2)	0 (1)	0 (1)	0 (1)
Whiting (*Merlangius merlangus*)	0 – 270 (18)	0 – 0.36 (15)	0 (16)	0 – 35.5 (16)

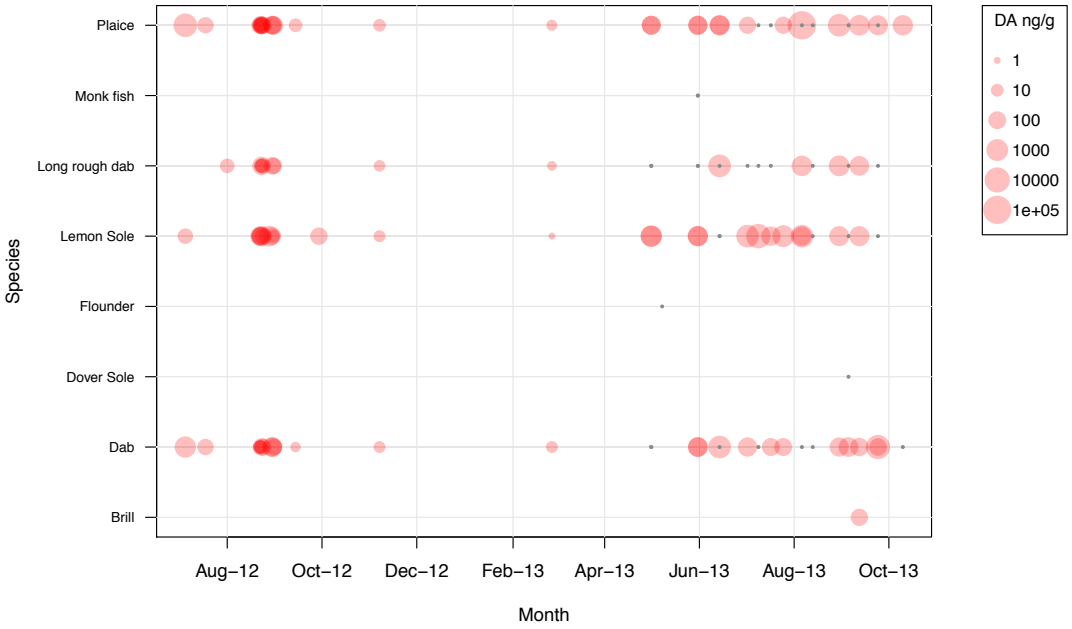

Figure 3.2: DA concentrations in flatfish species sampled in 2012 and 2013, circle size (small to large) represents concentration (low to high), the concentration level can be found in the legend. The small grey dots represent sample effort with no toxin found.

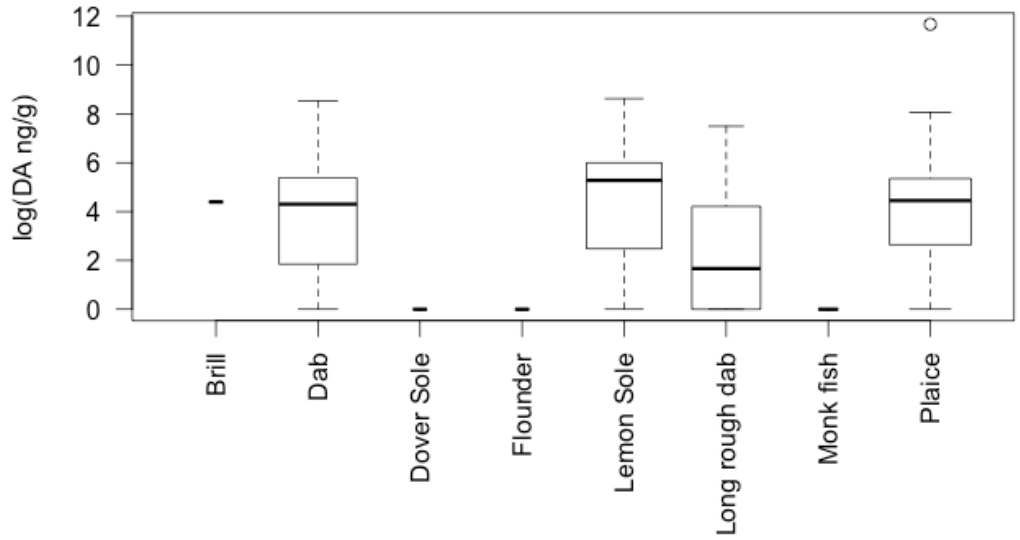

Figure 3.3: Concentration of DA in flatfish (ng/g), by species, plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0 – 117400 ng/g).

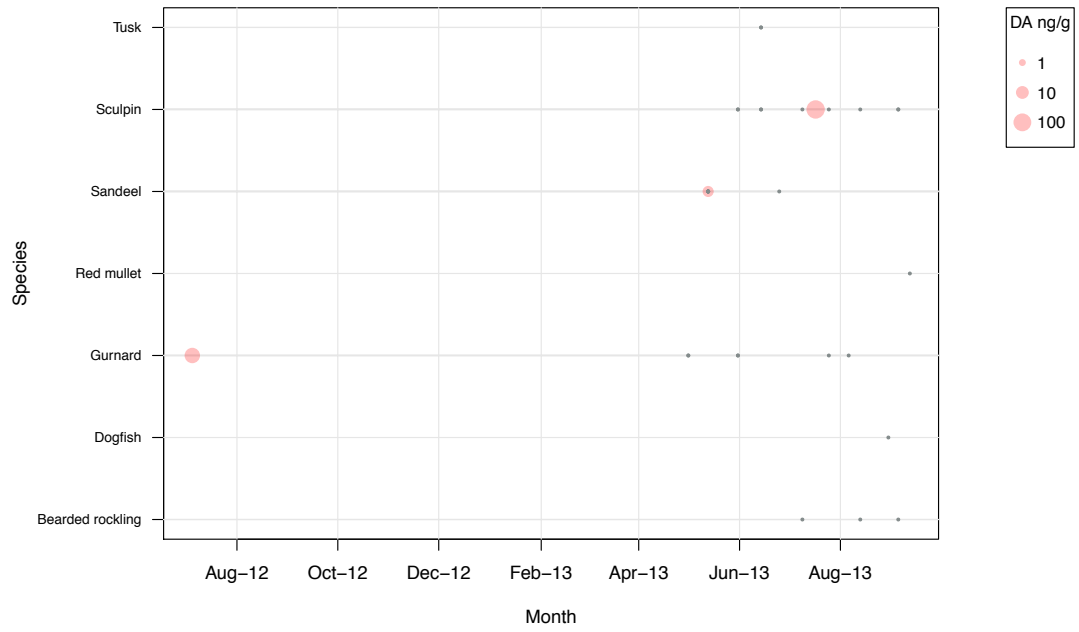

Figure 3.4: DA concentration in benthic species sampled in 2012 and 2013, circle size (small to large) represents concentration (low to high), the concentration level can be found in the legend. The small grey dots represent sample effort with no toxin found. DA range: 0 – 135 ng/g.

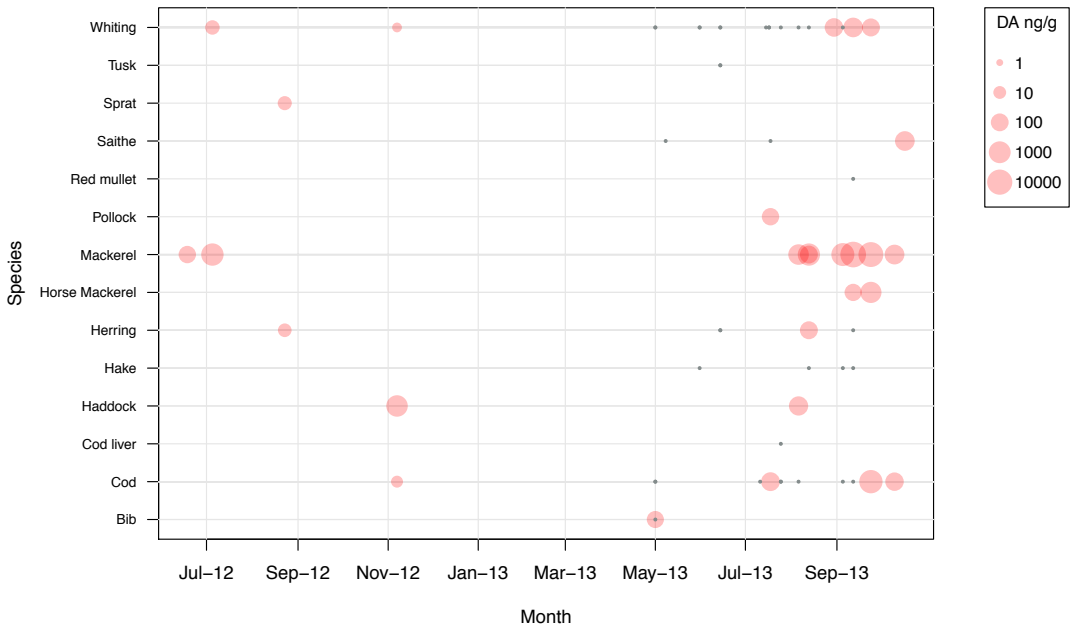

Figure 3.5: DA concentration in pelagic fish species sampled in 2013, circle size (small to big) represents concentration (low to high), and the concentration level can be found in the legend. The small grey dots represent sample effort with no toxin found. Range: 0 – 14265 ng/g.

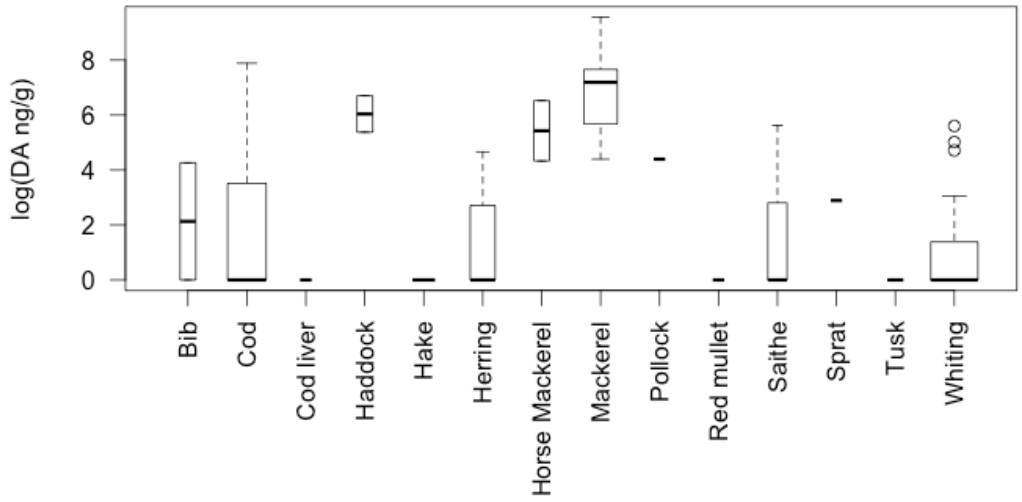

Figure 3.6: Concentration of DA in pelagic fish (ng/g) by species, plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers (DA range: 0 – 14265 ng/g).

Table 3.2: Regression models for DA concentration measured in fish.

Model	Variable	Residual deviance	*df* fitted	*df*	P value	AIC
1.	Month (all groups)	269.36	7	214	<0.01	285.36
2.	Month (flatfish)	156.52	7	122	>0.11	172.52
3.	Month (benthic)	5.41	4	32	>0.99	15.41
4.	Month (pelagic)	67.47	6	62	>0.06	81.47

Table 3.3: Toxin concentrations in all fish species that were positive (> LOQ) are represented (sample sizes are in brackets).

Month	DA ng/g	STX mg eq/kg	OA (ng/g)	DTX-2 (ng/g)
Feb	0.00 (4)	0 (3)	0 (3)	0 (3)
May	26.19 (42)	27.7 (18)	5.88 (17)	0 (17)
Jun	27.78 (18)	15.38 (13)	0 (9)	0 (9)
Jul	48.72 (34)	47.5 (40)	8.11 (37)	0 (37)
Aug	61.90 (63)	43.08 (65)	12.5 (56)	0 (56)
Sep	48.65 (37)	81.82 (33)	8.33 (36)	0 (36)
Oct	80.00 (5)	40.00 (5)	0 (5)	0 (5)
Nov	14.29 (7)	42.86 (7)	14.29 (7)	0 (7)

3.3.2 PSP toxin detection in fish

For the STXs 175 individually pooled samples were selected this included 24 species of fish. The fish with the highest PSP toxin measured was a lemon

sole with 21.2 mg STX eq/kg (Fig. 3.7 and 3.8). Other flatfish exposed to PSP toxins included species such as: dab (Fig. 3.7 and 3.8), long rough dab (Fig. 3.7 and 3.8), plaice (Fig. 3.7 and 3.8) and brill (Fig. 3.7 and 3.8). The benthic fish exposed to PSP toxins included fish species such as; bearded rockling (Fig. 3.9 and 3.10), dogfish (*Scyliorhinus canicula*, Fig. 3.9 and 3.10), gurnard (Fig. 3.9 and 3.10) and sculpin (Fig. 3.9 and 3.10). The more pelagic fish species exposed to PSP toxins included bib (Fig. 3.11 and 3.12), cod (Fig. 3.11 and 3.12), haddock (Fig. 3.11 and 3.12), hake (Fig. 3.11 and 3.12), herring (Fig. 3.11 and 3.12), horse mackerel (Fig. 3.11 and 3.12), mackerel (Fig. 3.11 and 3.12), pollock (Fig. 3.11 and 3.12), red mullet (*Mullus Surmuletus*, Fig. 3.11 and 3.12), saithe (Fig. 3.11 and 3.12), and whiting (Fig. 3.11 and 3.12). For a full overview over the range levels in the different species see Table 3.1. The cod liver contained 0.017 mg STX eq/kg suggesting PSP toxins do accumulate separately in the liver. There was a seasonal difference where September had a higher concentration of PSP toxins than the rest of the months (Table 3.3 and Table 3.4, model 1, $p < 0.01$). For the flatfish group there was a seasonal difference where September was the month with higher concentration than the rest (Table 3.4, model 2, $p < 0.01$), there was no seasonal difference between the benthic or the pelagic fish species (Table 3.4, model 3 and 4, $p > 0.05$).

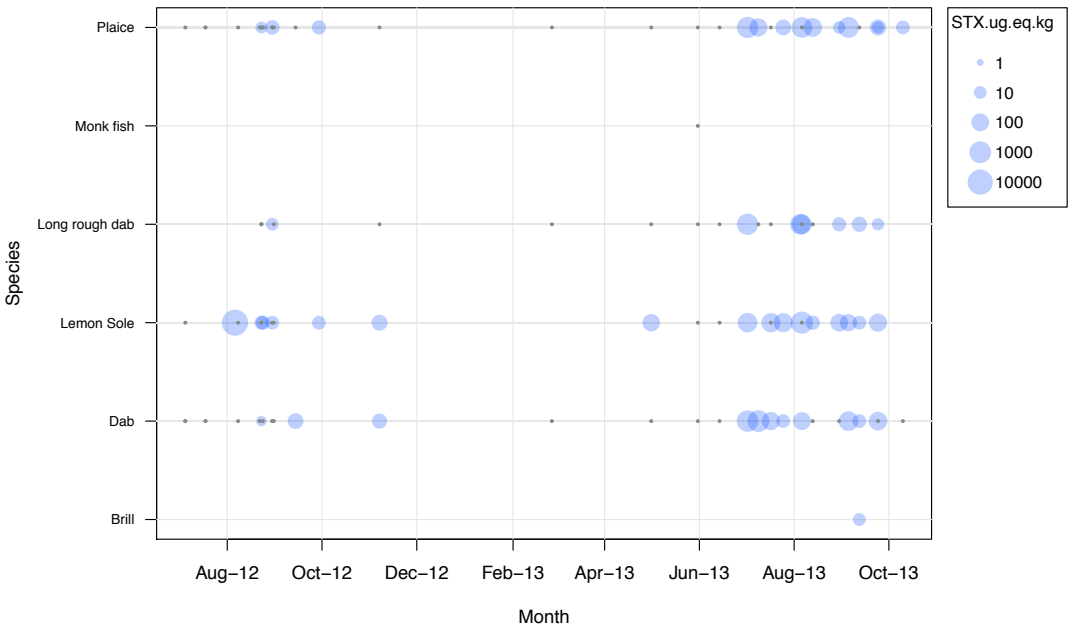

Figure 3.7: PSP toxin concentration in flatfish species sampled in 2012 and 2013 for, circle size (small to big) represent concentration (low to high), the concentration level can be found in the legend. The small grey dots represent sample effort with no toxin found.

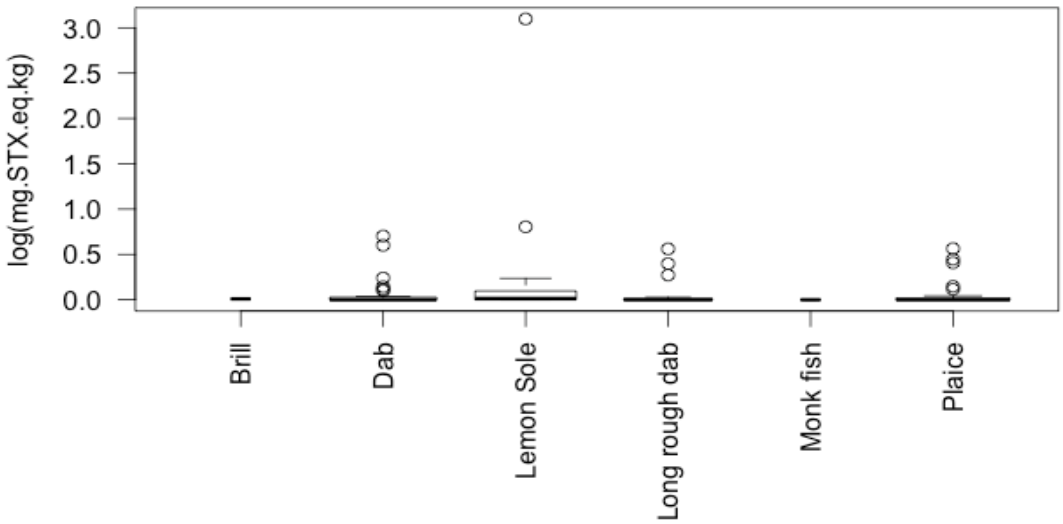

Figure 3.8: Concentration of PSP toxins in flatfish (mg STX eq/kg) by species, plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of PSP toxins and the black horizontal line indicates the median of the data set, separate circles are outliers (STX range: 0 – 21.15 mg STX eq/kg).

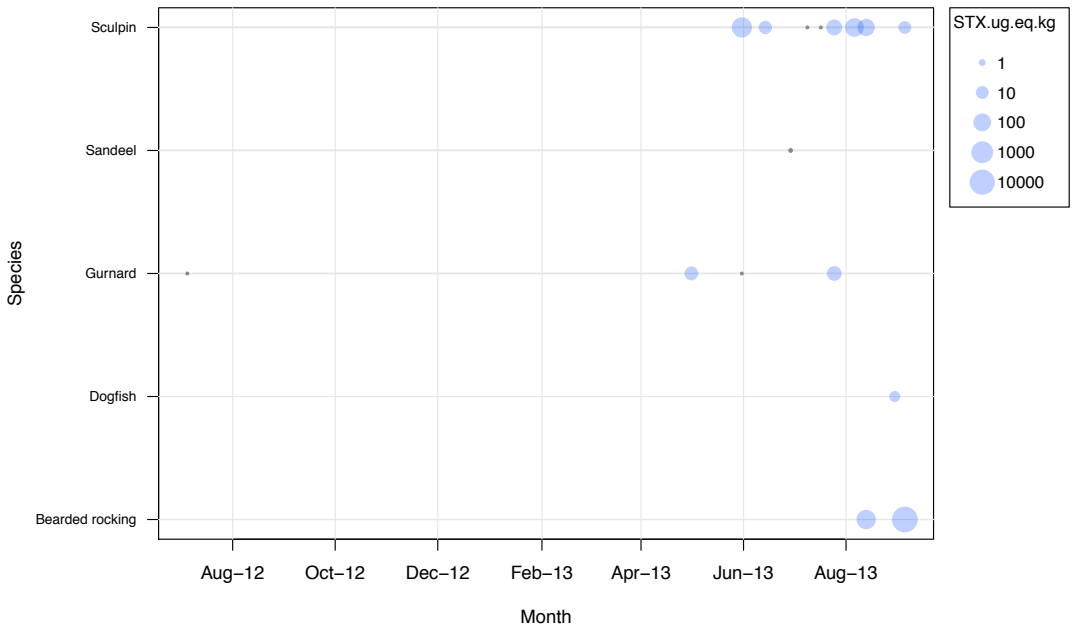

Figure 3.9: PSP toxin concentration in benthic fish species sampled in 2012 and 2013 for, circle size (small to big) represent concentration (low to high), the concentration level can be found in the legend. The small grey dots represent sample effort with no toxin found.

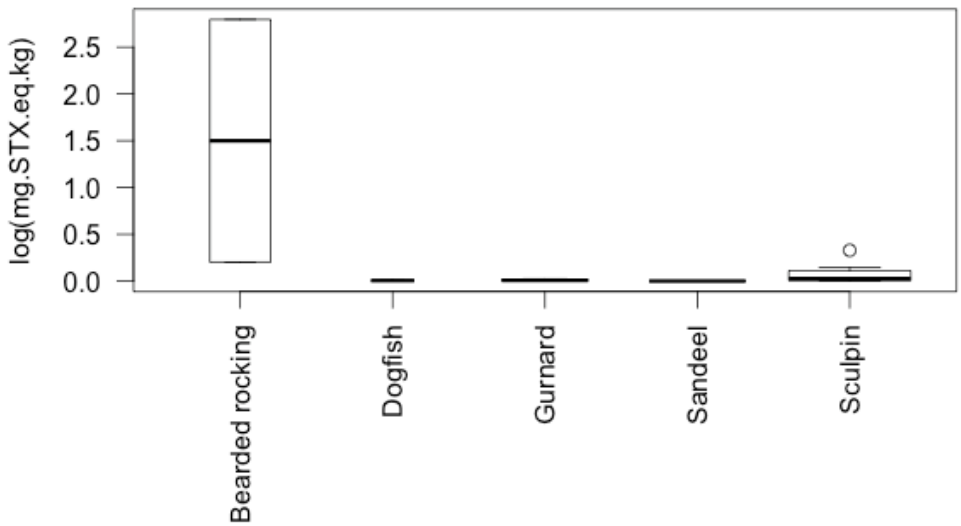

Figure 3.10: Concentration of PSP toxins in benthic fish (mg STX diHCl eq/kg) by species, plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of PSP toxins and the black horizontal line indicates the median of the data set, separate circles are outliers (STX range: 0 – 15.39 mg STX eq/kg).

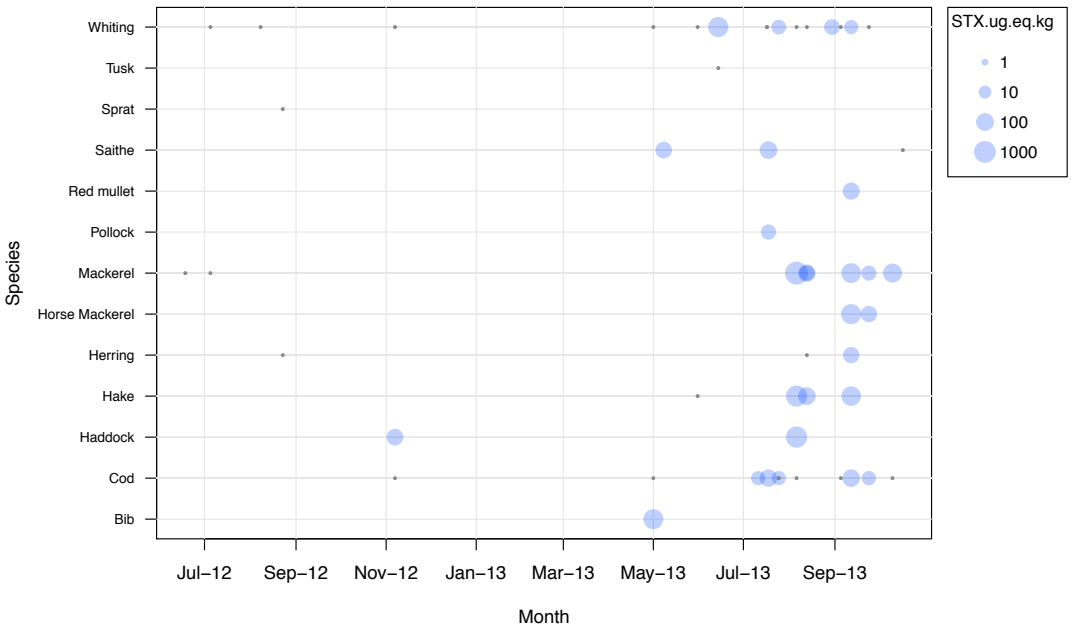

Figure 3.11: PSP toxin concentration in pelagic fish species sampled in 2012 and 2013, where circle size (small to big) represent concentration (low to high), the concentration level can be found in the legend. The small grey dots represent sample effort with no toxin found.

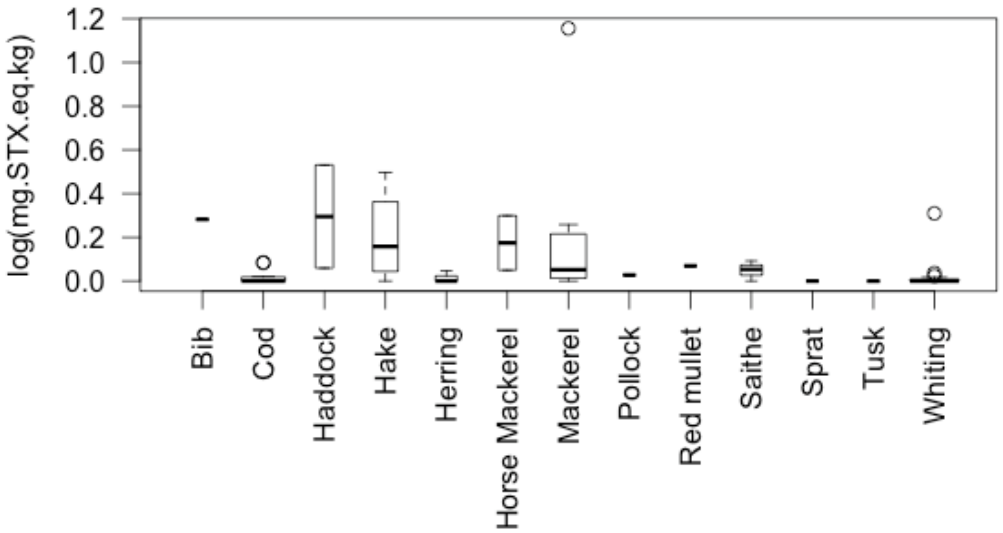

Figure 3.12: Concentration of PSP toxins in pelagic fish (mg STX eq/kg) by species, plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of PSP toxins and the black horizontal line indicates the median of the data set, separate circles are outliers (STX range: 0 – 2.18 mg STX eq/kg).

Table 3.4: Regression models for PSP toxin in fish (all groups and by group).

Model	Variable	Residual deviance	*df* fitted	*df*	P value	AIC
1.	Month (all groups)	224.23	7	183	<0.01	240.23
2.	Month (flatfish)	124.17	7	109	<0.01	140.17
3.	Month (benthic)	16.29	4	21	>0.05	26.29
4.	Month (pelagic)	52.29	6	42	>0.29	66.29

3.3.3 Lipophilic toxin detection in fish

A total of 170 individually pooled samples were analysed for OA and DTXs, this included 25 species of fish. There was a significant difference between the months of the year (Table 3.5, model 1, $p < 0.05$), where May had a significant lower percentage positive OA concentration than the other months, interestingly all of the samples collected in November were > LOQ. When investigating if there was a seasonal difference between the fish groups (flatfish, benthic and pelagic) there was no seasonal difference for OA concentration and between the flatfish and pelagic group (Table 3.5, model 2 and 3, $p > 0.06$). All of the benthic fish species were negative for OA.

For DTX-2 there was no seasonal difference (Table 3.5, model 4, $p > 0.05$) and there were not enough data to run models on the individual fish groups. Lemon sole was the fish species with the highest OA concentration at 97.0 ng/g (Fig. 3.13 and 3.14), and the only fish with DTX-2 concentration (> LOD) at 7.5 ng/g. Other flatfish exposed to OA included dab, dover sole (*Solea solea*), long rough dab and plaice (Fig. 3.13 and 3.14). For the benthic fish exposed to OA, these species included bearded rockling (non specific) and gurnard. There was six species of pelagic fish exposed to OA, these included cod, haddock, hake (*Merluccius merluccius*), horse mackerel, mackerel and whiting (all Fig. 3.15 and 3.16). Of the flatfish exposed to DTX-2 (but levels were < LOQ), the species included were: dab and plaice. Although the levels

were not quantified, the results still indicate DTX-2 exposure to these fish species. In addition, the cod liver sample did not contain detectable levels of OA or DTXs. There was no DTX-1 found in any of the fish samples.

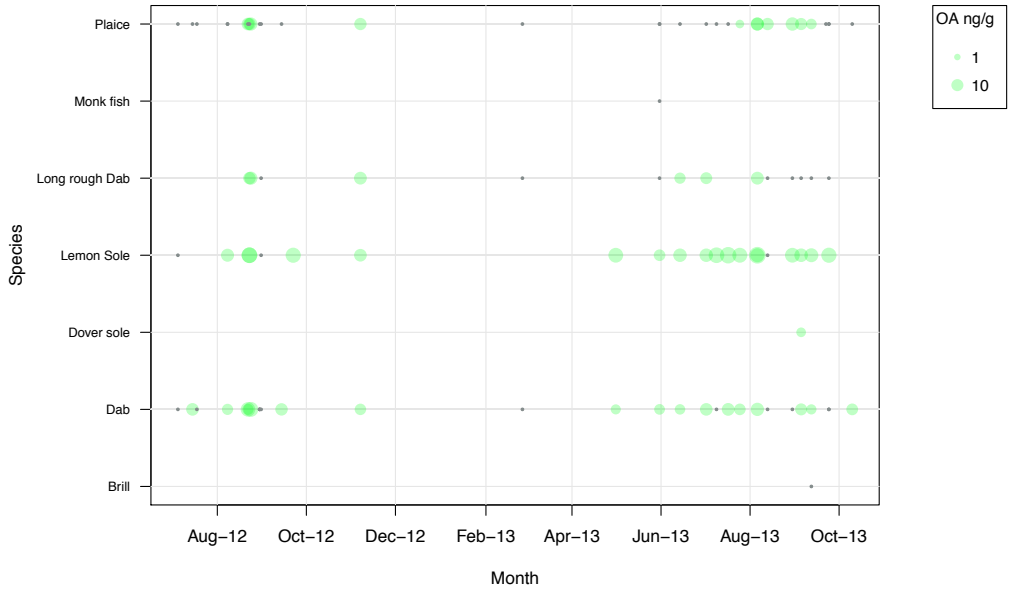

Figure 3.13: OA concentration in flatfish fish species sampled in 2012 and 2013, where circle size (small to large) represent concentration (low to high), the concentration level can be found in the legend.

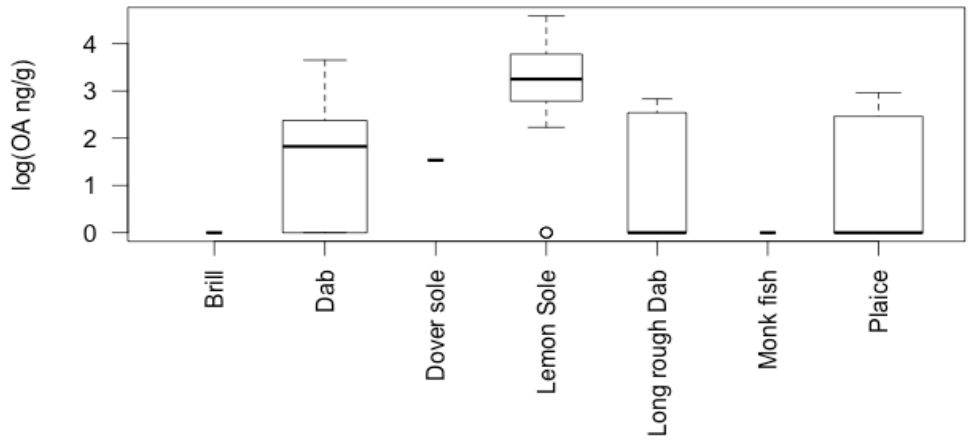

Figure 3.14: Concentration of OA in flatfish (ng/g) by species, plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of OA and the black horizontal line indicates the median of the data set, separate circles are outliers (OA range: 0 – 97 ng/g).

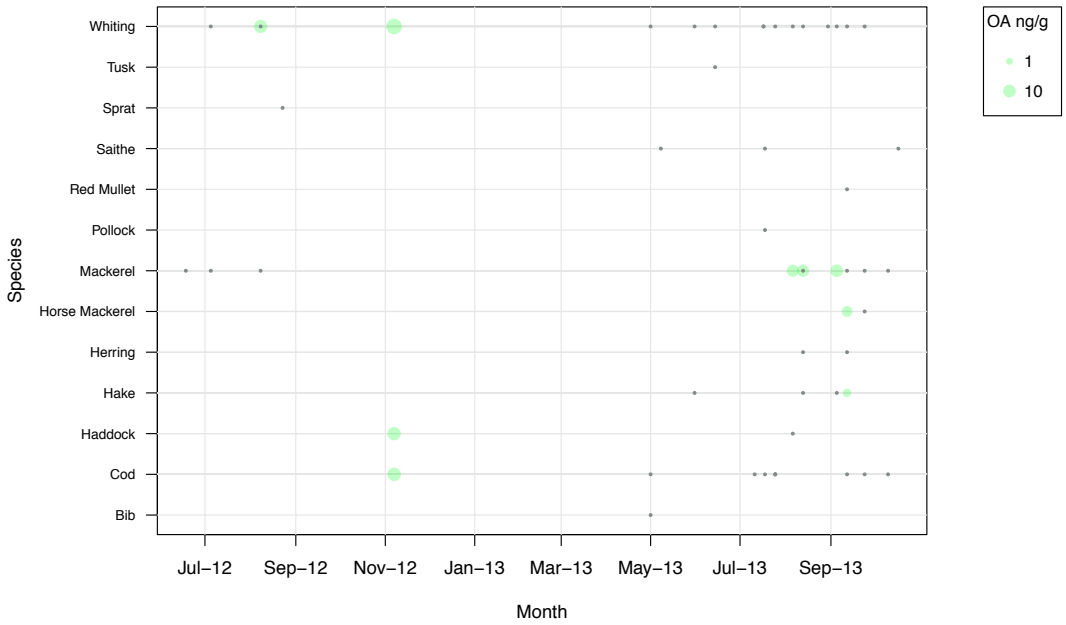

Figure 3.15: OA concentration in pelagic fish species sampled in 2012 and 2013, where circle size (small to large) represent concentration (low to high), the concentration level can be found in the legend.

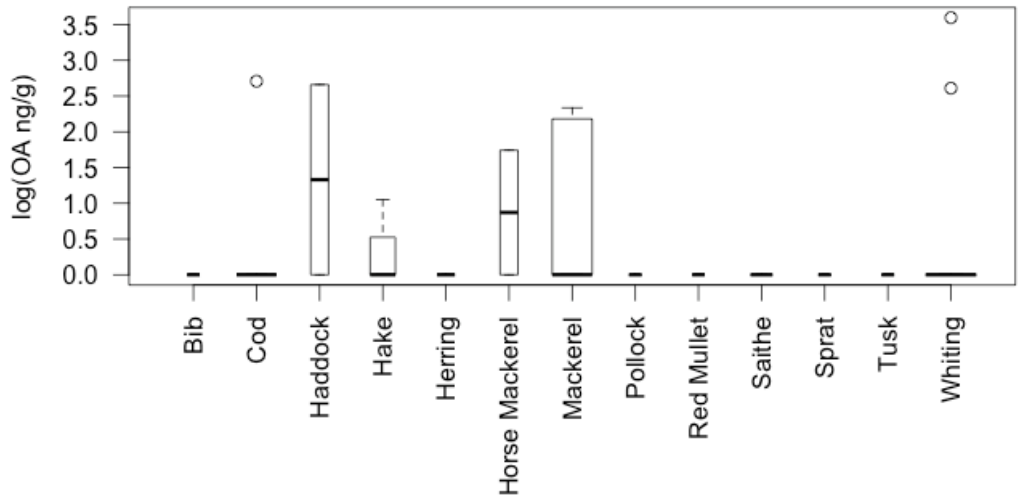

Figure 3.16: Concentration of OA in pelagic fish (ng/g) by species, plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of OA and the black horizontal line indicates the median of the data set, separate circles are outliers (OA range: 0 – 36 ng/g).

Table 3.5: Regression model for OA and DTX-2 concentration in fish (all groups and by flatfish and pelagic fish).

Model	Variable	Residual deviance	*df* fitted	*df*	P value	AIC
1. (OA)	Month (all groups)	186.93	7	169	<0.01	202.93
2. (OA)	Month (flatfish)	117.33	7	92	>0.06	133.33
3. (OA)	Month (pelagic)	27.53	6	51	>0.17	41.53
4. (DTX)	Month (all groups)	9.19	7	169	>0.99	25.20

3.4 Discussion

3.4.1 Harmful algae toxins in fish from the Firth of Forth, Scotland

The trophic transfer of toxins from harmful algae through the food chain to higher predators has been documented in many places around the world (for review see (Landsberg, 2002)), but in Scotland there is a lack of information on the pervasiveness of these toxins in the food chain. Detection of DA, PSP toxins and the lipophilic toxins (OA and DTX-2) confirms that fish on the east coast of Scotland are ingesting toxins throughout the year even when the HABs responsible for their production are not present. This chapter documents the presence of these toxins in pelagic and benthic fish species capable of contaminating fish eating predators such as, but not exclusively (see Chapter 4), Scottish harbour seals.

3.4.2 Domoic acid in fish

Phytoplankton monitoring data from 2006-2013 reveals that *Pseudo-nitzschia* is present annually on the east coast from April to September, with an occasionally occurrence in March and October (Stubbs et al., 2013). Although blooms of *Pseudo-nitzschia* occurs, toxin are not always produced as not all strains are toxic (Stubbs et al., 2013; Swan and Davidson, 2011). This study documents that the highest DA concentrations were found in benthic fish species such as plaice, lemon sole, dab and long rough dab. Of the pelagic fishes the highest DA concentration measured was in cod and mackerel. Although I lack fish data from January, March, April and December I document that DA was detected from the remaining months where fish was sampled. This suggests that there is possible a slow depuration rate of DA in these fish, or they are exposed to DA across a wide range of seasons and thus almost at any time of the year, even if it is not present in sentinel shellfish. It has been suggested that the sediment could potentially be a reservoir of DA and thus act as a constant source to be taken up the benthic feeding organisms. This would not be detected in rope-cultured shellfish from toxin monitoring sites (Gallacher et al., 2000). An example of benthic

organisms that are exposed to DA is the filter feeding worm (*Urechis caupo*), where the worm was found with DA levels as high as 700 µg/g DA^{-1} (Goldberg, 2003). The authors also discovered that the deposit feeding olive snail (*Olivella biplicata*) had DA levels at 2 µg/g^{-1} DA. Other benthic species that have been found exposed to DA are estuarine bivalves that reside in mud and sand (Vale and Sampayo (2001). Another example is a study by Kaniou-Grigoriadou (2005) where they found high concentration of DA in the sand and rock burrowing venus clam (*Venus verrucosa*). Collectively these studies provide evidence that benthic invertebrates are probably very important initiators in the movement of DA from the benthos to the next level in the chain.

Moving up the food chain from benthic invertebrates to fish, limited data exists on the natural exposure and toxic effect of DA in fish. Northern anchovy from the US west coast have been reported to contain up to 40 mg/g DA in the muscle (Lefebvre et al., 2001; Lefebvre et al., 1999; Work et al., 1993). Mackerel have also been shown to act as a vector of DA transfer with levels up to 142.9 µg/g (Sierra-Beltràn et al., 1997). In a study by Lefebvre et al., (2001) they demonstrated that anchovies could experience neuroexcitotoxicity after DA exposure, where findings of DA in the brain tissue and flesh raised concerns. The authors also suggested that anchovies would act as an easy prey target, due to the fact that sinking dead anchovies could be a potential pathway of DA transfer to benthic communities. Sardines (*Sardina pilchardus*) and Atlantic horse mackerel were studied by Vale and Sampayo (2001) where DA was found in the gut (range: 11.8 – 492.4 µg/g), muscle (range: 0.4 – 3.5 µg/g) and brain (range: 0.2 – 1.7 µg/g) of sardines.

DA exposure to fish eating predators have been reported in CSLs from the US west coast, where morbidity and mortality has occurred as a result of DA exposure from contaminated anchovies (Gulland, 2000; Gulland et al., 2002; Lefebvre et al., 1999; Scholin et al., 2000). Humpback whales have also been found to be exposed to DA through eating contaminated anchovies and sardines (Lefebvre et al., 2002b). In Scotland there has been one report of DA

in cetaceans, where a dead minke whale (*Balaenoptera acutorostrata*) was found to have taken up DA following a large *Pseudo-nitzschia* bloom although it was not established whether this was the cause of death (Turrell et al., 2006).

From the results discussed in chapter 2, the most important potential vectors of DA transfer to Scottish harbour seals from the identification of otoliths in the harbour seals scats were benthic fish such as plaice, dab and sandeels together with more pelagic fish such as whiting and cod was found in seals exposed to DA. These are specific for the east coast region. Results from a PhD study confirms that the diet of the harbour seal on the east coast is dominated by flatfish such as plaice but that sandeel together with the pelagic fishes such as cod and whiting are also important in the diet (Wilson, 2014).

3.4.3 PSP toxin in fish

Monitoring data from the Scottish phytoplankton monitoring group reveals that *Alexandrium* blooms have been detected from 2006-2013 on the east coast annually from April to June, with more sporadic occurrences from July to August. Rarely is *Alexandrium* detected from September to March (Stubbs et al., 2013; Swan and Davidson, 2011). PSP toxins were found in all the months fish were sampled and analysed. As for the DA results, there is a lack of data from January, March, April and December. However, my results do suggest that STX can be accumulated throughout the year even when a bloom is not present. The life cycle of *Alexandrium* is important, and consists of nine stages, which includes both motile and cyst phases. One aspect that increases *Alexandrium* success in producing blooms is the cyst phase, and its ability to remain in the bottom sediment when growth conditions are unfavourable. The motile stage of the cells develops by binary fission and blooms occur when conditions are favourable. If conditions are poor (e.g. low temperature or changes in salinity) the motile species can go into a ‘’temporary cyst’’ which allows them to avoid unfavourable short-term fluctuations in the ocean (Anderson, 1998).

In Norway, mortality of farmed salmon has been related to STX during a bloom of *Alexandrium tamarense* (http://www.sintef.no/Fiskeri-og-havbruk-AS/Marine-ressursteknologi/Barekraftig-produksjon/Algeovervakning/Gift-algeoppblomstring-i-Nord-Norge/). Oral administration of PSP toxin in both flounder, salmon and cod confirms that fish are susceptible to STX (White, 1981a). Fish feeding directly on PSP toxin producing algae or on contaminated zooplankton are in the risk of becoming intoxicated (White, 1984). In addition, uptake of PSP toxins dissolved in sea water through the gills, has also been suggested (Montoya et al., 1996).

Atlantic mackerel is an important vector as it accumulates PSP toxins in the liver (Castonguay et al., 1997). Information on the median lethal concentration (LC_{50}) of PSP toxins in water is very limited and the oral median lethal dose (LD_{50}) for PSP toxins varies among laboratory species such as cats (Andrinolo et al., 1999), and in studies with salmon and cod it usually varies between 400 and 1000 µg STX eq/kg (White, 1981a). There is a lack of precision in lethal oral dose in humans, but an acute reference dose in the region of 0.3-0.8 μ g/kg bw has been suggested (Anon, 2006). In a study by Chen and Chou (2001) extract from toxic algae was tested on milkfish (*Chanos chanos*) where they discovered the LC_{50} to be 870 µg STX eq/L. In a study of fish larval stages Levfebvre et al., (2004) discovered that exposure to dissolved PSP toxins can cause morphological abnormalities and short term exposure can negatively impact the larval survival. However, accumulation of PSP toxins in the edible muscle of fish is not likely to occur (Bakke and Horsberg, 2010). Other studies on the effect of PSP toxins in marine organisms was carried out by Lenz et al. (2014) where they discovered voltage-gated sodium channel genes in marine copepods, where they concluded that ingested toxic *Alexandrium* could potentially affect the organism (Lenz et al., 2014). As mentioned earlier in this chapter PSP toxins have been suggested to be a factor in the population failure of the endangered North Atlantic right whale population where the primary prey *C. finmarchicus* is likely to act as the vector. Levels as high as 0.95 µg STX eq./g

in faeces samples were documented (Doucette et al., 2006; Doucette et al., 2012).

3.4.4 Lipophilic toxins in fish

Data from the Scottish phytoplankton monitoring program reveals annual occurrences of *Dinophysis* sp. on the east coast (Stubbs et al., 2013; Swan and Davidson, 2010, 2011), where on average there are about two months of the year when cell counts are above the trigger level (> 100 cells/l). These are usually in the summer months of June and July. Detection of Dinophysis cells generally starts in April and ends usually at the end of the summer (August/September). Fish analysed for OA and DTXs occurred throughout the year except in January, March, April and December. OA occurred every month sampled except February whilst DTX-2 occurred only in the spring, summer and autumn months from May to August. These results suggest both OA and DTX-2 are taken up by several species of fish and can be found in the viscera of the fish from early spring to late autumn. There are no specific studies of lipophilic toxins in fish but OA has been studied in zooplankton where it had a potential lethal effect on the larvae (*Artemia franciscana*) (Demaret et al., 1995). Tintinnids, which are small planktonic ciliates (microzooplankton) have been suggested to play an important role in the transfer of OA to higher trophic levels (Maneiro et al., 2000).

3.5 Conclusion

I have found that a wide variety of fish species caught on the in the Firth of Forth on the east coast of Scotland from the area around Firth of Forth contain measureable levels of all three toxins of interest; DA, PSP toxins and the lipophilic toxins OA and DTX-2. The toxins were not necessarily only found during the seasons of peak phytoplankton blooms, in the spring and autumn but were found throughout the year. This study therefore documents that several species of fish are potential vectors for these algal toxins to higher trophic levels, which suggests that a wide variety of susceptible top predators could be affected, particularly those that forage in the coastal zone.

Linking these findings to the recent diet studies in the Scottish harbour seals shows that flatfish are the most important group of fish on the east coast. The fact that flatfish dominated the diet of the seals in the Tay and Eden estuaries (Wilson, 2014) fits well with my findings and leads to the conclusion that this population could be at particular risk of exposure to all three groups of toxins. Interestingly, this is the area where the biggest decline of harbour seals is occurring (Lonergan et al., 2007) and is clearly of particular importance as the decline in this region could indeed be explained by ingestion of these toxins through fish, although no regional comparison is possible in this study. Taken in conjunction with the evidence presented in Chapter 2, where the proportion of positive samples and concentrations in the urine and faeces were, in general, highest in the harbour seals from the east coast, these findings suggest toxin exposure cannot be ruled out as a cause of the decline in this region (although there is a lack of comparable faecal samples from the west coast where the populations are stable).

Given the impact that these toxins could cause they could pose a serious threat not only to the harbour seals, but also other fish eating predators as several cetaceans are also exposed, particularly to DA, as seen in Chapter 4. These results are key for future risk assessment models in order to estimate and model the individual and population level effects of toxin exposure through reconstructing the different diets, foraging strategies and uptake of these in the various top predators.

Chapter 4

4. Toxins from harmful algae in stranded marine mammals in Scotland

4.1 Introduction

4.1.1 Marine mammal strandings

Stranding of a marine mammal means that an animal, for various reasons, has washed ashore, and been left helpless if it is still alive but often the stranding is of a dead animal (Geraci, 2005). In addition to single strandings, mass stranding events can occur and these are defined as beaching of more than two marine mammals (usually alive) (Geraci et al., 1999). Mass strandings can draw lots of attention from the public. Mass strandings can occur in species that for some reason come into unfamiliar areas, such as shallow or in-shore regions (Geraci, 2005), but may also be due to a widespread infection or toxic event such as a large HAB event. There are several factors that are thought to cause marine mammals to strand, such as oceanographic events, tidal movements, extreme weather, prey or illness or injury of an animal. Many cetaceans come inshore perhaps through curiosity or to feed, but few strand. Those that do strand alive often become trapped and ground due to the tides (Smith et al., 1985). Other cetaceans may strand due to noises in the ocean, and some whalers use this phenomenon, using sharp sounds to drive dolphins ashore (Kuiken and Hartmann, 1991). Direct effects of human activity (e.g. navy exercises using sonar) has increased the attention towards stranding of marine mammals such as beaked whales (Fernandez et al., 2004). Generally the reason for the stranding of a mammal is hard to identify and all unusual mortality events are referred to as Unusual Mortality Events (UMEs).

In Europe UMEs or major die offs have occurred in harbour seal populations where two epidemics caused by phocine distemper virus (PDV) (Osterhaus and Vedder, 1988) have been reported. The first occurred in 1988 where 60 % of the North Sea harbour seal population was killed (Härkönen et al., 2006) and the second outbreak was in the summer of 2002 where thousands of dead seals were found (Härkönen et al., 2006; Jensen et al., 2002). Outside of these mass events, seals that strand in Scotland are mainly harbour seals and grey seals both of which are indigenous to Scottish waters although occasional vagrant species, particularly from the Arctic are also reported. In 2012 grey seals were the most common seal species to be reported stranded, representing 26.2 % of the total strandings reported to the Scottish Marine Animal Stranding Scheme (n = 126) whereas harbour seals represented only 8.3 % (n = 39) of the total stranding in 2012 (Brownlow and Davison, 2012). This possibly represents a difference due to the difference in population size and many of the grey seals reported are pups of the year in which high natural mortality occurs. Between 1992 and 1995 there was a peak in grey seal strandings in the UK which may have been due to increased shooting (Brownlow and Davison, 2012). However, one of the most common cause of death for harbour seals in Scotland in particular have in the last couple of years been trauma due to spiral lesions (Bexton et al., 2012; Brownlow and Davison, 2012). These traumatic deaths are probably due to interactions between seals and vessels operating ducted propellers, research into this cause of mortality is continuing.

Of the cetaceans, the most commonly stranded species in 2012 and indeed in previous years has been the harbour porpoise (*Phocoena phocoena*) representing 14 % (n = 68) of the total stranding in 2012. Two harbour porpoises were found the same year with spiral lesions (Brownlow and Davison, 2012). In addition to these single dead strandings, two live mass strandings of the Long-finned pilot whales (*Globicephala melas*) have occurred recently in Scotland, the first occurred in 2011 in Kyle of Durness on the north coast of Scotland and the other in 2012 on the east coast near Pittenweem. These are rare occurrences, but are events that bring a great deal of public interest.

The causes of many marine mammal strandings cannot be explained, but toxins from harmful algae can be linked to some of them. Mass strandings due to toxic uptake have been suggested as a cause of death in Mediterranean monk seals, manatees and CSL (Capper et al., 2013; Gulland, 2000; Gulland et al., 1998; Hernández et al., 1998; Scholin et al., 2000).

4.1.2 Domoic acid and potential vectors in stranded marine mammals

Among marine mammals, domoic acid (DA), a neurotoxin produced by the diatom *Pseudo-nitzschia,* has caused mortality events since 1998 particularly in CSL from the consumption of contaminated planktivorous fish (Lefebvre et al., 1999; Scholin et al., 2000). Pacific harbour seals have been found with clinical signs indicative of acute DA toxicosis (McHuron et al., 2013). DA has also been considered to have a potentially fatal outcome in the North Atlantic right whale population (Leandro et al., 2010). DA was detected in the intestinal content and faeces during an UME of bottlenose dolphins (Fire et al., 2010b) although it was unclear what role the toxin had in the stranding event. Interestingly, in a study by Fire et al., (2009) they discovered that the offshore feeders; the pygmy sperm whales (*Kogia breviceps*) and the dwarf sperm whales (*Kogia sima*) stranded along the U.S. Atlantic coast from 1997 to 2008 were exposed to DA, highlighting the importance of toxin investigation in the cetacean food web. Cetacean mortality, especially long-beaked common dolphins (*Delphinus capensis*) have been associated with DA producing *Pseudo-nitzschia* blooms in southern California (De La Riva et al., 2009)

In Scotland there is a lack of information on how widespread DA is among marine mammals, but the toxic diatoms are regularly found in Scottish water and harbour and grey seals are exposed ((Hall and Frame, 2010), Chapter 2, S. Tarrant unpublished data). From Chapter 3 I found DA is accumulated in several groups of fish from SE Scotland, particularly DA seems to accumulate in flatfish. This vector may clearly not be restricted to just the SE coast that I

have focused on in this study, but may also be important in other areas, as evidenced from the widespread finding of toxins in harbour seals throughout Scotland (see Chapter 2). Other species such as crustaceans and cephalopods can act as vectors (Lefebvre et al., 2002a) of trophic toxin transfer to marine mammals (Fire et al., 2009; Lefebvre et al., 2002b; Scholin et al., 2000), but none of these species has been investigated in Scottish waters. Long-finned pilot whales together with dwarf sperm whales are known to feed on cephalopods (Santos et al., 2006) and DA levels have been reported in the cephalopods digestive glands (Costa et al., 2005a). Other cetaceans such as the white-beaked dolphin (*Lagenorhynchus albirostris*) and the Atlantic white-sided dolphin (*Lagenorhynchus acutus*) feed on clupeids (e.g. herring), small mackerel, gadids (e.g. Atlantic cod and haddock and oceanic cephalopods (Das et al., 2000; Kinze et al., 1997)) which are all prey species in which toxins have been detected (see Chapter 3).

4.1.3 Other toxins in stranded marine mammals

From July to May 1997 around 117 Mediterranean monk seals died and neurotoxins from the PSP toxins group were found in the water and in some of the seals (Hernández et al., 1998). Okadaic acid (OA) was detected in stomach and intestinal contents of bottlenose dolphin (Fire et al., 2010b), a toxin known to cause gastrointestinal problems. In the same study DA and brevetoxin was detected in faecal material from a bottlenose dolphin, the study raises the question of the effect multiple toxin exposure can have in stranding events (Fire et al., 2010b). In a study by Capper et al. (2013) they discovered low concentrations of multiple toxins (OA and PSP toxins) in stranded manatees on Florida's shoreline between December 2003 and February 2006.

The overall aim of this chapter was therefore to investigate toxins from harmful algae in dead stranded fish eating top predators from around the Scottish coast. I focus mainly on DA to compare the results to the levels found in live captured harbour seals. Samples from dead stranded animals may give

a unique opportunity to investigate exposure in a wide variety of different species and to potentially assess any health effects, although the carcasses are often too decomposed for full necropsies to be carried out and cause of death to be established.

4.2 Methods

4.2.1 Sample collection from stranded marine mammals

In collaboration with the Scottish Marine Mammal Stranding Scheme (SMASS), Scottish Rural University College, Inverness, faeces, urine and other body fluids (such as amniotic fluid) was collected from a variety of stranded marine mammal species (Fig. 4.1). Between 2008-2013 a total of 166 samples from 136 individuals (includes 15 species) were analysed for toxins from harmful algae (Table 4.1). Phocids included in this study were harbour seals and grey seals. Cetaceans investigated in this study (hereafter referred to as “other cetacean”) included: long-finned pilot whales, Atlantic white-sided dolphin, bottlenose dolphin, common dolphin (*Delphinus delphis*), harbour porpoise, dwarf sperm whale, minke whale (*Balaenoptera acutorostrata*), sei whale (*Balaenoptera borealis*), Sowerby’s beaked whale (*Mesoplodon bidens*), sperm whale (*Physeter macrocephalus*), striped dolphin (*Stenella coeruleoalba*), white-beaked dolphin and unidentified pelagic delphinid (this includes one of the following: common dolphin, striped dolphin, white-beaked dolphin, Atlantic white-sided dolphin, Risso’s dolphin (*Grampus griseus*), long finned pilot whale or killer whale (*Orcinus orca*)).

The samples represented 87 urine and 43 faecal samples together with: 15 liver tissues, seven pericardial fluids, three stomach contents, three thoracic fluids, three amniotic fluids, two gastric fluids, two kidney tissues and one unidentified body fluid. Based on the amount of material available DA was prioritized to be extracted and then the other toxins of interest in this study, the PSP and lipophilic toxins (only faecal material).

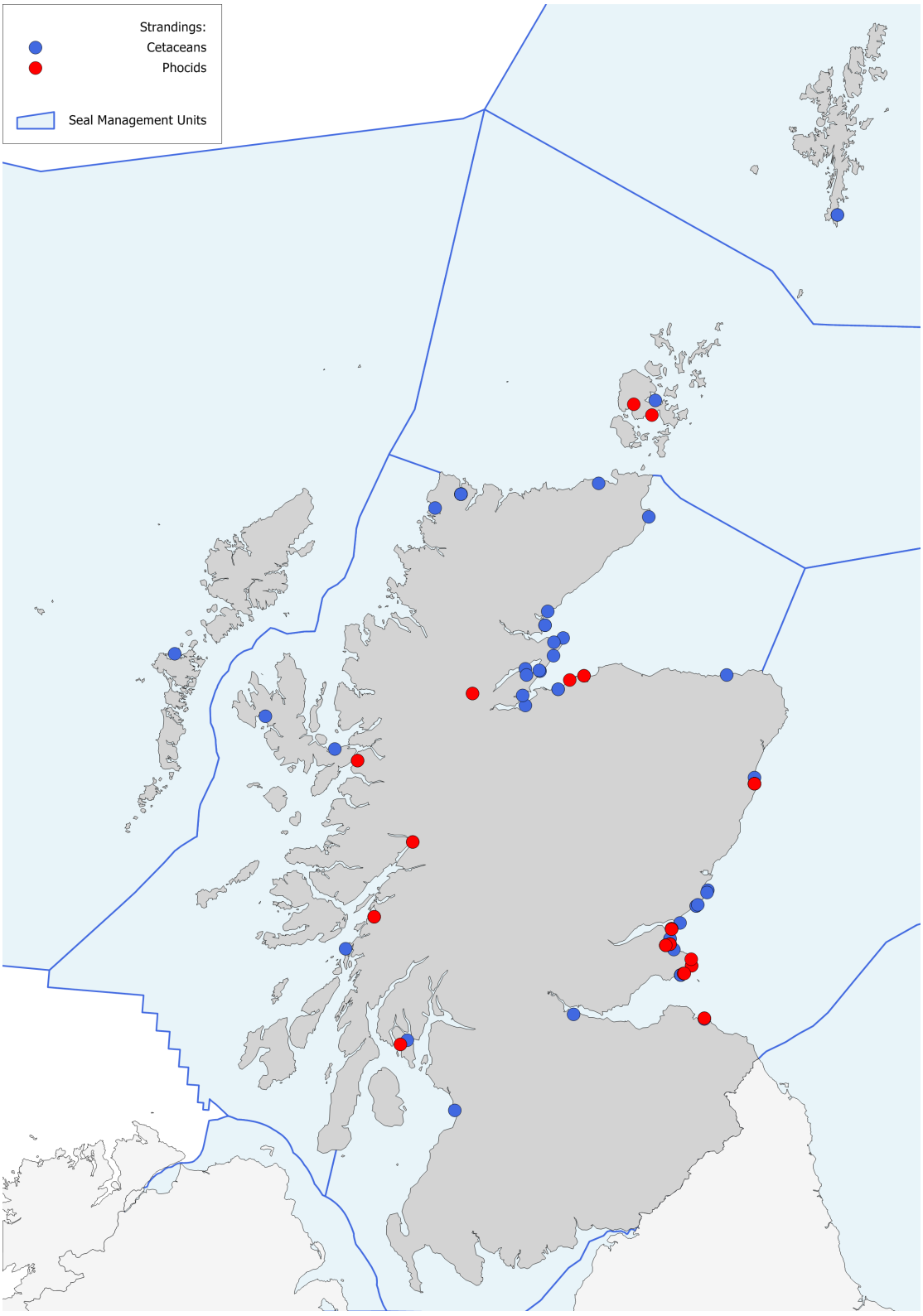

Figure 4.1: Stranding locations of cetaceans and phocids in Scotland between 2008-2013, from which samples were obtained for biotoxin analyses.

4.2.2 DA extraction and quantification

For extracting DA, 2-4 g of faecal material was homogenized and DA was extracted following the procedure in Chapter 2, section; 2.2.3.1 “Sample extraction”. Urine samples together with body fluids such as amniotic fluid, pericardial fluid and thoracic fluid were used directly in the assay. The samples were analysed using the same ASP Enzyme Linked Immunosorbent Assay (ELISA) method described in Chapter 2, section 2.2.3.2. The DA assay has been widely used to detect DA in various matrices including shellfish tissue and urine and faeces from marine mammals (Kleivdal et al., 2007b; Lefebvre et al., 1999). The LOQ is described in Chapter 2, section 2.2.3.2.

4.2.3 STX extraction and quantification

For STX extraction 2-4 g faecal material was extracted following the same method described in Chapter 2 section; 2.2.4.1 “Sample extraction” and protein was precipitated following the method in Chapter 2, section; 2.2.4.2 “Protein precipitation”. STX was measured and quantified using the instrumentation described in Chapter 2 section; 2.2.3.5 “Instrumentation”. The LOQ is described in Chapter 2, section 2.2.3.5.

4.2.4 OA and DTXs extraction and quantification

To extract the lipophilic toxins faecal material were homogenised and 2 g subsampled, lipophilic toxins were extracted following the same method as described in chapter 2, section 2.2.5.1 “Sample extraction” and 2.2.5.2 “Hydrolysis of Esterified Forms of OA, DTX-1, and DTX-2”. The extracts were measured and quantified using the instrumentation described in Chapter 2; section 2.2.5.3 “Instrumentation”. The LOQ is described in Chapter 2, section 2.2.5.3.

4.3 Results

4.3.1 Toxin exposure in stranded harbour seals

A total of 17 stranded harbour seals were tested for DA in urine where ten (58.82 %) were above the LOQ. Eight of these samples stranded along the east coast of Scotland where six (75.0 %) were above LOQ. Four samples were collected around the Northern Isles where two (50 %) were above the LOQ. For the west coast, five harbour seal urine samples were analysed and 20 % was above LOQ. The mean for the urine samples tested 0.0834 µg/ml (75 % quantile: 0.0352 µg/ml) and the highest urine sample measured for DA was from the Highlands with levels of 0.627 µg/ml (Fig. 4.2).

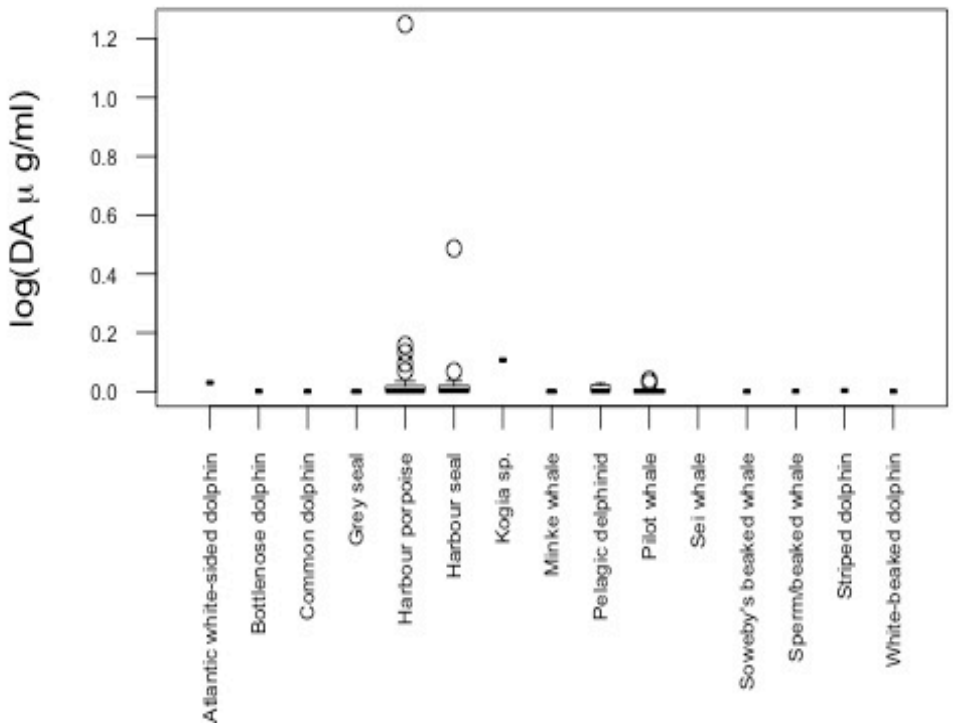

Figure 4.2: Concentration of DA in urine (µg/ml) by species plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers

For the faecal samples fifteen samples were analysed for DA, where 13 of these came from the east coast, one from the Northern Isles and one from the west coast. Eleven samples were > LOQ (73.3 %). From the east coast 76.9 % (n = 13) were above the LOQ. One of the faecal samples collected on the Northern Isles was > LOQ, and none from the west coast were above the LOQ. The mean for the faecal samples measured was 0.169 µg/g (75% quantile: 0.232 µg/g) and the highest faecal sample was collected from the east coast with 0.519 µg/g (Fig. 4.3). However, in a general linear model, with concentration as the dependent variable and area as a factor, there was no significant relationship between DA concentration in urine (Table 4.1, model 1) and the region sampled. It was not possible to run any statistical models on the other matrices for harbour seals due to lack of data. Three females were found on the east coast dead with a foetus and neonatal exposure (measured in the thoracic, gastric or amniotic fluid together with faeces) occurred in all three cases. DA was detected in the mother and foetus with levels from 0.006 to 0.164 µg/g DA in the faecal samples and in thoracic fluid (< LOD, 0.004 µg/ml) and amniotic fluid (0.022 – 0.023 µg/ml) and gastric fluid (< LOD 0.0004 µg/ml). DA was detected at low levels < LOD in pericardial fluid in a harbour seal from the east coast.

Table 4.1: Regression model for dead stranded harbour seals seen by area for urine samples.

Model	Variable	Residual deviance	*df* fitted	*df*	P value	AIC
1.	Area (urine)	19.55	2	16	0.4	25.55

PSP toxin was detected in 2/4 of the harbour seal faecal samples tested, three of the samples were collected on the east coast and one was collected on the west coast. The two samples with PSP toxin were collected from the east coast with levels > LOQ at 12.00 – 18.00 µg STX eq/kg. Two harbour seal faecal samples was analysed for OA and DTX-2, one from the east coast and the other from the west coast. None of these faecal samples had any traces of OA or DTX-2.

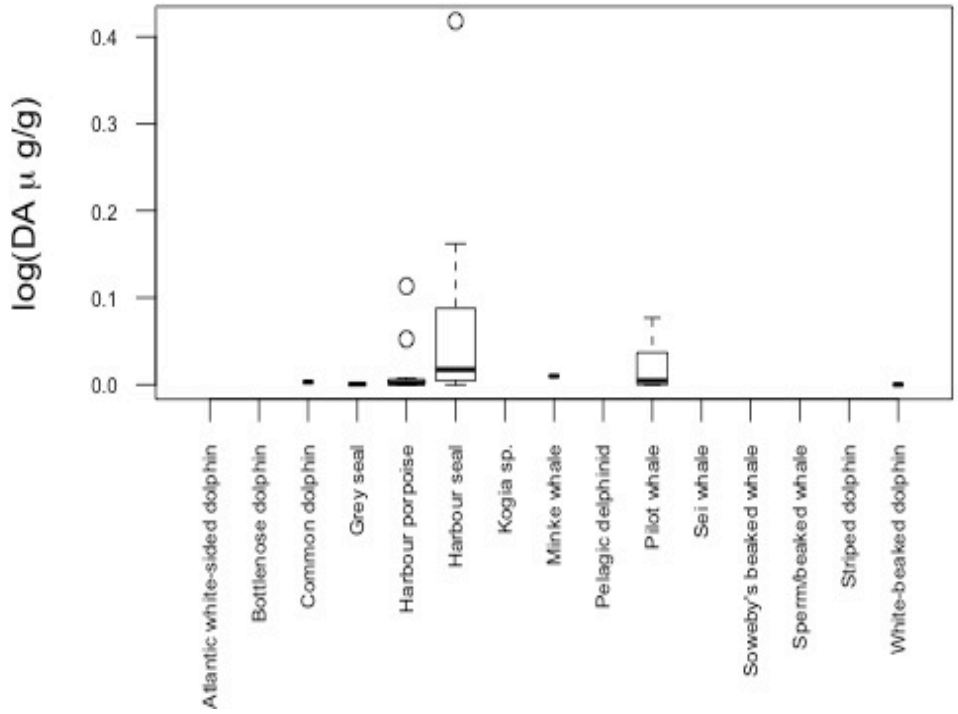

Figure 4.3: Concentration of DA in faeces (µg/g) by species plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers.

4.3.2 Toxin exposure in stranded grey seals

DA was detected, but below the LOQ in three of the nine (33.3 %) faecal samples tested from grey seals (Fig. 4.3). Only two urine samples were available for analysis for DA where the level found was again below the LOQ (Fig. 4.2). Three pericardial fluid samples were available for DA analysis and two of the samples (66.7 %) were positive for DA concentration with levels above the LOQ at 0.030 µg/ml (Table 4.2).

Two faecal samples from grey seals were analysed for PSP toxins and both were collected on the east coast. PSP toxins were detected in one of the two faecal samples analysed for the toxin with levels of 40 µg STX eq/kg. The same two faecal samples from the east coast were analysed for lipophilic toxins, where the one was found with DTX-2 levels of 0.002 µg/g.

4.3.3 Toxin exposure in stranded cetaceans

There was one urine sample from an Atlantic white-sided dolphin with DA concentration of 0.031 µg/g (Fig. 4.2). One bottlenose dolphin urine sample was analysed for DA from the west coast, but the result was < LOQ. One striped dolphin urine sample was found < LOQ with DA levels at 0.003 µg/g and two samples from white beaked dolphins were analysed for DA where both were < LOQ (Table 4.2, Fig. 4.2). The fourth dolphin species analysed for DA in both urine and faeces from the east coast, was the common dolphin again with levels < LOQ (Table 4.2, Fig. 4.2).

Thirty-one harbour porpoises was analysed for DA where fourteen urine samples were > LOQ for DA (DA range: 0.002 – 2.487 µg/ml, Table 4.2, Fig. 4.2) and 13 of these 14 were found on the east coast and one was collected from the Northern Isles. Among the faecal samples 4/6 porpoises were found > LOQ (range: 0.006 – 0.120 µg/g, Table 4.2, Fig. 4.3), all of the samples were collected from the east coast.

There was one whale from the family *Kogia* spp. where a urine sample was > LOQ with levels at 0.113 µg/ml DA (Table 4.2, Fig. 4.2), this whale was sampled on the west coast. Two minke whales were sampled and analysed for DA where two urine samples were < LOQ (DA: 0.0008-0.002 µg/ml), but one faecal sample was > LOQ with a concentration of 0.0099 µg/g (Table 4.2, Fig. 4.2). The faecal sample was also analysed for OA and PSP toxins where OA levels at 0.04 µg/g was found and no PSP toxins were found in the minke whale, this positive whale was sampled on the Northern Isles. Seven pelagic delphinids were sampled for DA, where urine samples revealed concentrations > LOQ for DA from three of the samples (DA range: 0.190 – 0.275 µg/ml, Fig. 4.2), two of these samples were collected on the west coast and the third was collected from the Northern Isles.

Of the 17 samples from the mass stranding of long-finned pilot whale in Kyle of Durness, two out of seven urine samples was > LOQ for DA (DA range: 0.004 – 0.031 µg/g, Table 4.2, Fig. 4.2). Fourteen liver samples were tested

for DA and one was found with levels of 0.164 µg/g. Two stomach samples were analysed for DA where DA was detected with levels of between 0.017 – 0.046 µg//g. One kidney samples was tested for DA where levels were < LOQ. Of the 21 long-finned pilot whales stranded in Pittenweem, thirteen urine samples were tested for DA where 5 (38.5 %) was found above the LOQ (DA range: 0.004 – 0.041 µg/ml, Table 4.2, Fig. 4.2). Three out of eight faecal samples from the Pittenweem stranding were > LOQ (37.5 %, Table 4.2, Fig. 4.3), DA ranges: 0.013 – 0.080 µg/g.

One pericardial fluid sample from a Sei whale was tested for DA and was found < LOQ, the whale was sampled at the east coast (Table 4.2). One urine sample from a Sowerby's beaked whale sampled on the west coast was analysed for DA and was < LOQ, as was a urine sample from a sperm whale collected on the east coast (Table 4.2).

Table 4.2: DA concentrations in stranded marine mammal species by matrix: urine, faeces, tissue (liver or kidney), stomach contents and other. Concentrations mean and (range) are expressed in µg/g or µg/ml. LOQ: limit of quantification, na: no sample available

Species	*n*	n (>LOQ)	Urine (µg/ml)	Faeces (µg/g)	Liver/kidney (µg/g)	Stomach (µg/g)	Other* (µg/ml)
Atlantic white-sided dolphin	1	1	0.031	na	na	na	na
Bottlenose dolphin	1	1	0.001	na	na	na	na
Common dolphin	1	0	<LOQ	<LOQ	na	na	na
Grey seal	9	2	<LOQ	<LOQ	na	na	0.020 (0.030)
Harbour porpoise	41	15	0.097 (0.0002-2.488)	0.015 (0.002-0.120)	na	na	0.030
Harbour seal	28	14	0.083 (0.003-0.627)	0.105 (0.005-0.519)	na	na	0.0156 (0.004-0.020)
Dwarf sperm whale	1	1	na	0.113	na	na	na
Minke whale	2	1	<LOQ	0.010	na	na	na

Pelagic delphinid	7	3	0.011 (0.002-0.027)	na	na	na	na
Long-finned pilot whale	33	8	0.019 (0.00004-0.041)	0.005 (0.003-0.080)	0.012 (0.00005-0.164) (liver) 0.0002 (kidney)	0.032 (0.018-0.046)	na
Sei whale	1	0	na	na	na	na	na
Sowerby's beaked whale	1	0	na	0.6	na	na	na
Sperm whale	1	0	na	<LOQ	na	na	na
Striped dolphin	1	0	na	nd	na	na	na
White-beaked dolphin	2	0	<LOQ	nd	na	na	na

*Including: pericardial fluid, gastric fluid, thoracic fluid and amniotic fluid

4.4 Discussion

DA has a rapid renal clearance and poor fat solubility, which makes DA exposure estimation very difficult and the concentrations in the various matrices difficult to interpret beyond identifying that animals have taken up the toxin. DA only remains in the tissues and urine for a few hours after ingestion of toxic prey and studies in monkey and rats shows that DA is primarily excreted in urine (Suzuki and Hierlihy, 1993; Tryphonas et al., 1990). DA clearance rate through a rats kidney is approximately 9 ml/min/kg body weight and it has a half-life of only 20 min (Suzuki and Hierlihy, 1993; Truelove and Iverson, 1994). Clearly the time lapse between exposure and stranding was not known nor is how much urine or faeces was voided before the individual seals or whales died. Some of the samples were also collected days after the stranding so natural degradation of DA might have occurred (Bouillon et al., 2006).

4.4.1 DA in stranded harbour and grey seals

The finding of DA in harbour seals and grey seals were not surprising as these are species that have been documented to be exposed to DA in Scotland (Chapter 2, S. Tarrant unpublished results). DA was found in low levels in both harbour and grey seals and the toxin is not thought to be the cause of their deaths as no lesions consistent with either acute of chronic DA toxicity in any of the seal brains submitted for histopathology between 2011-2014 was found (J. Baily pers. comm). However only a very few brains were available for histopathology, given that most carcasses, particularly harbour seals were too decomposed for complete histology.

The mean levels in the dead stranded harbour seal faecal samples collected (mean: 0.077 µg/g, 75 % quantile: 0.093 µg/g, Fig. 4.4) compared with those found in the live captured animals were much lower (mean: 0.591 ng/g, 95 % quantile: 0.016 µg/g, Fig. 4.4) and were significantly lower than in the anonymous faecal samples from the harbour seal haul out sites (mean: 4.238 µg/g, 75 % quantile: 1.520 µg/g, Fig. 4.4).

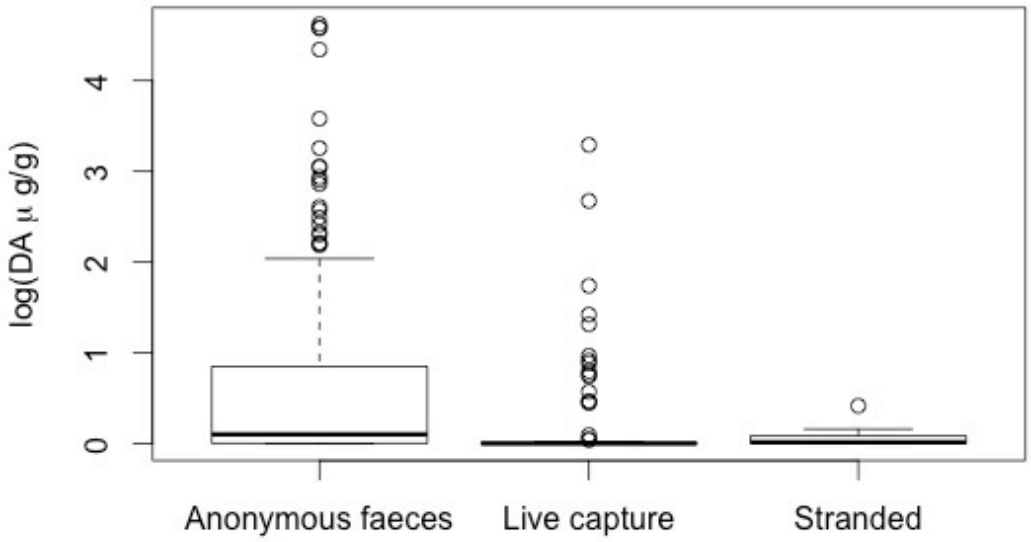

Figure 4.4: Concentration of DA in faeces (µg/g) by live captured, stranded and anonymous faecal samples from harbour seals, plotted on a logarithmic scale. The width of the box is proportional to the square root of the samples sizes and the box goes from the first quartile to the third quartile, whiskers represent the lowest and highest values still within 1.5 x IQR of DA and the black horizontal line indicates the median of the data set, separate circles are outliers.

Three pericardial fluid samples were analysed in grey seals where two were found with DA > LOQ and to my knowledge this is the first evidence that DA is detected in pericardial fluid. DA has been shown to be cardiotoxic as the heart is also one of the most affected organs in sea lions that died following DA intoxication (Gulland et al., 2002). In southern sea otters (*Enhydra lutris nereis*), DA has been looked upon as a risk factor in association with myocarditis and dilated cardiomyopathy (Kreuder et al., 2005).

Neonatal exposure occurred in three cases where three dead female harbour seals were found on the east coast with a foetus and in all three cases DA was detected in the thoracic, gastric or amniotic fluid together with faecal samples from the foetus. Neonatal animals have been shown to be more susceptible to DA than adults (Brodie et al., 2006; Goldstein et al., 2009), possibly from insufficient renal clearance (Xi et al., 1997). Amniotic fluid has been shown to have a poor elimination of DA, where 8 days after stranding DA was still detectable in the amniotic fluid of a CSL (Brodie et al., 2006). In addition, studies suggest that DA also recirculates as pregnant rats takes

longer to eliminate DA, exposing the foetus to DA for a prolonged period (Maucher et al., 2012; Maucher and Ramsdell, 2005). In a study by McHuron et al., (2013) they found DA both in the stomach contents of stranded harbour seals (1.4 ng/g = 0.001 µg/g) and in the milk (2.2 ng/ml = 0.002 µg/g). This study together with McHuron et al., (2013) shows that DA can be detected in several fluids and matrices in phocids and although little is known about the metabolism of DA in phocids (see Chapter 6 for more information about DA clearance rate) it is likely that it, like other mammals, is rapidly excreted in the urine and faeces and is not metabolised given it is detectable as the parent molecule in these matrices (Chapter 2). The DA levels measured in urine and faecal samples collected from grey seals were < LOQ except in two cases where grey seals were shot in June and levels measured in the pericardial fluid were quantifiable.

4.4.2 DA in stranded cetaceans

This study provides evidence of DA exposure in several top predators, not only the harbour seals that are the focus of this thesis. Out of the 12 cetaceans studied six species had DA levels > LOQ, although there was a low sample size for most cetaceans in this study. DA was detected in another five species indicating exposure although the levels were < LOQ. DA has been detected in cetaceans in several places around the world, such as during an UME in gray whales (*Eschrichtius robustus*) that occurred between 1999 and 2000 (Gulland et al., 2005). During the UME grey whales stranded on the west coast of North America and DA was detected in serum, urine and faecal samples in a juvenile whale with levels in the urine and faeces of 1.6 and 0.52 µg DA/ml substrate, respectively (Gulland et al., 2005). Conclusions from this study was that the juvenile grey whale probably died from DA intoxication although toxic doses for cetaceans have not been established (Truelove and Iverson, 1994).

Toxins from harmful algae in stranded marine mammals were also reported by Fire et al., (2009), where they analysed several species of cetacean which included: the fin whale (*Balaenoptera physalus*), common dolphin, northern

right whale, pygmy killer whale (*Feresa attenuata*), Atlantic white-sided dolphin, humpback whale (*Megaptera novaeangliae*), Blainville's beaked whale (*Mesoplodon densirostris*), melon-headed whale (*Peponocephala electra*), harbour porpoise, Stenella sp., bottlenose dolphin and Cuvier's beaked whale (*Ziphius cavirostris*). None of these cetaceans were found exposed to DA or OA. In the same study stranded whales from the family *Kogia* spp., were documented with DA exposure (Fire et al., 2009). The whale from the family *Kogia* spp. in this study (dwarf sperm whale) was found exposed to DA with comparable levels to the study by Fire et al., (2009). In addition, a stranded minke whale was found after a severe harmful algal bloom in southern California where DA was detected in gastric fluid and faeces (DA: 258 µg/g) of the whale and otoliths from northern anchovy were recovered from the whales stomach (Fire et al., 2010a).

The finding of DA in offshore feeders such as the dwarf sperm whale, sperm whales and long-finned pilot whale may be indicative of offshore blooms that are not currently being monitored, as all monitoring is inshore in Scotland. Although the majority of these samples were negative this is the first time DA has been detected in such a wide range of cetaceans in Scotland and questions, which prey are responsible for transferring the toxins to offshore species and how their foraging patterns and strategies may make them vulnerable to exposure. Thus, the lack of toxin analysis from potential offshore vectors is an obstacle to definitively explaining how the offshore feeders are exposed, but from Chapter 3 I demonstrate that inshore both pelagic and benthic fish species take up all three groups of toxins examined in this study.

This study examines a range of different matrixes for the presence of DA, such as stomach contents, kidney, liver and different bodily fluids in which DA was detected (> LOQ) and these include amniotic, thoracic and pericardial fluid as well as liver and stomach contents. In a study by Gulland et al., (2000) they analysed for DA in stranded CSLs kidney, stomach washings, cerebrospinal fluid and brain samples and no DA was found in these samples. Interestingly, stranded bottlenose dolphins have been found exposed to DA with levels in the stomach content of 10 ng/g (0.010 µg/g), intestinal content

39 ng/g (0.039 µg/g) and faecal material 9 ng/g (0.009 µg/g) (Fire et al., 2010b). This study shows that DA can be detected in various bodily fluids, many of which are worth investigating during a stranding event.

The cause of death for the cetaceans were varied (pneumonia, physical trauma, starvation, live stranding or an bacterial infection), these were discussed and ruled out as an indirect cause of death correlated with the toxin exposure. In general although some of the seal and cetaceans samples were found > LOQ, the levels were so low that acute high level DA toxicosis could probably be ruled out as a cause of the stranding.

4.4.3 Other toxins in marine mammals

PSP toxins were detected in two harbour seal faecal samples, one harbour porpoise and one grey seal sample. The levels were low and are not thought to be the cause of their stranding. However, this is the first evidence that PSP toxins can be detected in grey seals and harbour porpoise. PSP toxins have been found in harbour seals from Scotland (see chapter 2).

OA was detected in bottlenose dolphins in a study by Fire et al., (2010b) with levels in intestinal contents reaching 10 ng/g (0.010 µg/g). With only two samples analysed for OA, this toxin was detected in faecal samples > LOQ from a minke whale. This is the first evidence of OA in cetaceans in Scottish waters. DTX-2 was analysed for in two harbour seal faecal samples and two grey seal faecal samples, where one of the grey seal samples was collected at Tentsmuir on the east coast. This shows that not only harbour seals are exposed to multiple toxins, but also the larger grey seal.

4.5 Conclusions

Due to the fact that toxic doses for cetaceans have not been established, as well as the condition of the samples collected from animals stranded for an unspecified length of time, it is difficult to interpret what the DA levels in the samples represent for the health or cause of death of the animal. Sick animals

could feed in new areas and on species that they don't normally consume, exposing them to novel agents such as these toxins. Malnourished animals may not have been feeding and this is likely to affect the results.

Understanding the role and distribution of DA and other harmful algal toxins in the inshore and offshore food chain is important in order to understand the risk that these pose but it is clear that the range and distribution of toxins merits further investigation in future. Results from this study seem to suggest that there is a difference in the exposure to DA from the inshore feeders such as the harbour seal and the harbour porpoise with higher levels of DA, while the offshore feeders such as the sperm whale and the long-finned pilot whale have lower concentrations of DA.

Future research should focus on offshore monitoring of phytoplankton species and their abundance and the DA exposure of vector prey items consumed by offshore feeders. Information about potential vectors can be analysed from otoliths and other hard parts collected in faecal samples and stomach contents. Potential synergistic effects of multiple toxins exposure are thought to lead to reduced fitness so studies on the effect of these toxins, especially in cetaceans are required.

Chapter 5

5. Evidence for a biomarker of domoic acid exposure in phocid seals is equivocal

5.1 Introduction

5.1.1 Overview of the immune system in marine mammals

Immunology in marine mammals is still a developing discipline and an understanding of the functioning of the immune system at the species level is important for studying and determining the general health of both individuals and populations. The immune system is highly complex and can be affected by many different extrinsic factors, such as stress, toxins and pollutants and intrinsic factors such as age and life history stage. It can also be used as a "biomarker system" for studying the impact of these effects and factors at various levels from individuals to populations (Vos et al., 2003), but this requires knowledge of what is 'normal' for a given species. The immune system is composed of two major parts: the innate (non-specific) immune system and the acquired (specific) immune system. When an organism is exposed to a foreign substance (known as an antigen), such as a microbe or a protein, the innate immune system is the first line of defence, where cells and proteins are ready to mobilize and fight antigens at the place of infection such as by phagocytosis. If an antigen is able to overcome the innate immune defence the acquired immune system starts working by proliferating, activating and creating mechanisms for eliminating antigens.

Immunoglobulins (Ig or antibodies) and lymphocytes comprise the major part of the acquired immune system and are responsible for long-term protection in case of re-exposure to the same or a similar antigen. In mammalian serum there are five immunoglobulin classes of antibody molecules: IgG, IgM, IgA and of less importance IgE and IgD. Lymphocytes play an important role and

are divided into B and T-lymphocytes. B-lymphocytes (humoral immunity) produce antibodies and each B-lymphocyte is programmed to make one specific antibody. When a B-lymphocyte comes across an antigen it gives rise to larger plasma cells where each plasma cell produces antibodies. When an antibody and an antigen interlink, the B-lymphocytes are incapable of entering the cell so destruction of these target cells is left to the T-lymphocytes (cell-mediated immunity) (Vos et al., 2003). T-lymphocytes are programmed to remember, recognize and respond to antigens. T-lymphocytes have two roles in the immune defence: the first involves direction and regulation of other immune response pathways, and the second is to directly attack antigens (Paul, 1993). Of particular interest to this study are the antibodies and many have been partly purified and described in several marine mammal species (Boyden and Gemeroy, 1950; Cavagnolo, 1979; King et al., 1993; Travis and Sanders, 1972). Antibodies are made up of two important components, light chain and heavy chain proteins, these form the base of the antibody which form a Y-shaped structure. The tip of the Y shaped form is unique for each antibody structure (Nash and Mach, 1971).

5.1.2 Immunoglobulin Classes in Pinnipeds

As early as the 1970's attempts were made to classify and characterize pinniped immunoglobulins from sera (Nash and Mach, 1971) where they concluded that proteins in pinnipeds were homologous to human immunoglobulins IgG, IgA and IgM. IgG are the smallest and most common antibodies found. In general IgG is produced in a delayed response to an infection and can be detected in the body for a longer period with a half life of 25.8 days (Mankarious et al., 1988) and interestingly the amount of IgG in pinnipeds seem to be significantly higher compared to terrestrial carnivores. IgA is a neutralizing antibody, which prevents invading pathogens and act as a first line defence against antigens, it is the second most common immunoglobulin in serum. IgM forms a large part of the antibodies and is the first class of antibodies produced by foetuses and when an antibody response occurs (King et al., 1994; Paul, 1993).

5.1.3 Low level (chronic) domoic acid exposure in pinnipeds

Domoic acid is a naturally occurring neurotoxic amino acid produced by microscopic marine algae such as the diatom *Pseudo-nitzschia*. High-level exposure through contaminated fish has had a significant impact on marine mammals (Lefebvre et al., 1999). Although much less is known about low-level or chronic exposure to DA, CSLs seem to experience heart disease, chronic epilepsy and reproductive failure that could be a result of longer-term exposure to the algal toxin (Gulland et al., 2002; Lefebvre et al., 1999; Scholin et al., 2000; Silvagni et al., 2005). A study by Hiolski et al., (2014) discovered that low-level repeated exposure to DA can alter gene transcription in zebrafish (*Danio rerio*, AB strain), and impair the mitochondrial function in the brain. Pacific harbour seals were found to be exposed to DA and had brain lesions consistent with DA toxicosis (McHuron et al., 2013), however it was unclear whether the seals in the study were experiencing acute or chronic exposure to DA.

Thus distinguishing between acute and chronic exposure is important but is not possible from concentrations in excreta alone. Urine and faecal samples dated back to 2008 indicate that Scottish harbour seals are probably now exposed to DA on an annual basis (Chapter 2 and (Hall and Frame, 2010)). The levels found in both the urine and faecal samples are in the lower range of those found in the chronically exposed CSLs, but higher than concentrations found in Pacific harbour seals. It is unclear how exposure levels relate to acute or chronic DA toxicosis in Scottish harbour seals, but it seems likely that populations are experiencing a chronic low-level, repeated exposure to DA (see Chapter 2). It is therefore important to have an efficient and rapid diagnostic test to assess toxin exposure, particularly for predicting what effects might be seen at the population level as the frequency and intensity of HABs seem to be increasing world-wide (Hallegraeff, 1993).

5.1.4 Domoic acid specific natural antibody

Lefebvre et al., (2012) recently published a study where they reported a method to detect chronic, low-level exposure to DA. They found that a novel DA-specific immunoglobulin G (IgG, here referred to as DA-specific antibody) response could be used as an indicator for this category of exposure. This DA-specific antibody was first discovered in zebrafish that was used as a vertebrate model in the experiment. The fish were exposed to DA via intracoelomic (IC) injection two to four times every month (over a nine month period). After 18 weeks an immune response occurred in the fish, after the DA-specific antibody was seen to increase although the zebrafish seemed healthy. Since the finding of the DA-specific antibody, samples from naturally DA exposed wild CSLs were examined and the DA specific antibody was found in their serum samples. The results of the zebrafish experiment indicated that chronic low-level exposure to DA does not build resistance to the effects of DA, as would be expected if the antibodies were functioning as perhaps expected and as they do in response to pathogen exposure. Instead chronic exposure increased neurologic sensitivity to DA as the chronically exposed zebrafish (i.e. those that developed DA specific antibodies) were found to be three times more sensitive to DA than the naively exposed zebra fish (Lefebvre et al., 2012). If this effect is consistent across taxa, it has very important implications for determining, understanding and predicting the effect of low-level chronic DA exposure in harbour seals and indeed other piscivorous marine mammals. It was therefore important for me to replicate this method using samples from Scottish harbour seals that have been collected and archived at SMRU since before the start of the observed decline in abundance.

5.1.5 Enzyme linked immunosorbent assays (ELISA)

Enzyme linked immunosorbent assays (ELISA) are highly sensitive immunoassays that can be used to detect antibodies and other proteins in blood samples (Butler, 2000). They can be designed to detect and quantify peptides, proteins, bacterial antigens, hormones and antibodies. In the assay

an antigen must be immobilized to a solid surface and then exposed to a specific monoclonal or polyclonal antibody conjugated with an enzyme. Enzyme-labelled antibodies have been used for many years for detecting antigens (Butler, 2000) and the assay quantifies the amount of antigen in a sample by assessing the conjugated enzyme activity through incubation with a substrate that produces a measurable product, such as optical density (Butler, 2000). There are generally three main methods that form the types of ELISAs, indirect ELISA, direct ELISA and sandwich ELISA. Indirect ELISAs (or iELISA) involve two binding processes where the primary antibody is incubated with the antigen involved, followed by incubation of a labelled secondary antibody (Crowther, 1995; Crowther and Walker, 2009). A direct ELISA assay involves adsorption of an antigen to a plastic plate where a protein (normally bovine serum albumin) is added to the plate to block out additional binding sites on the plate. There is no secondary antibody and the primary antibody is linked to the detection enzyme (Crowther and Walker, 2009). The most sensitive ELISA method is the sandwich ELISA and has the name because the method quantifies the antigens, which is captured between two layers of antibodies (the capture antibody and the detection antibody) (Crowther and Walker, 2009).

5.1.6 Aims of study

The objective of this study was therefore to use the iELISA assay modified and suggested by Lefebvre et al., (2012) to investigate if Scottish harbour seals had been historically exposed to DA by using archived serum samples. I wanted to determine if there is a pattern of antibody development around the same time as the harbour seal decline was detected (around year 2000). The results would provide further information on the potential link between the decline and the uptake of DA, both temporally and spatially. The main aims of this study were therefore to firstly verify and replicate the published method by using sera from some of the same individuals as in the Lefebvre et al., (2012) study and secondly apply the approach to phocid seals, particularly focusing on harbour seals with the aim of determining whether exposure rates

(estimated from the prevalence of these DA antibodies in the samples) have increased over the same time frame as the decline in abundance. Samples were also available from the other phocid species found in UK waters, the grey seal. Investigation of the presence of these DA specific antibodies in grey seals will also be examined, as current evidence suggest they are less exposed to DA (S. Tarrant unpublished data). Finally, commercially available dog serum from laboratory animals was included as additional quality control samples.

5.2 Method

5.2.1 CSL and dog serum samples

Three California sea lion serum samples were sent from the Marine Mammal Centre, Sausalito, CA, US to the SMRU, UK laboratory. These three samples were identical to the samples in the study published by Lefebvre et al., (2012) and would be used to replicate and verify the method.

Unfortunately, I did not obtain the identical control samples (sea lion serum from healthy sea lions born and raised in captivity at Sealife Park Hawaii) used in the Lefebvre et al., (2012) study for method validation control. Therefore, other negative controls were substituted such as commercially available dog reference serum (from the Laboratory of Cellular Physiology, National Heart Institute, National Institutes of Health, United States Public Health Service, Bethesda, Maryland) stored at -20 °C was used as a quality control as this serum is collected from pathogen free laboratory animals that would not have been exposed to DA.

5.2.2 Phocid serum samples

Serum samples from harbour and grey seals collected by the Sea Mammal Research Unit under the UK Animal (Scientific Procedures) Act 1986, Project and Personal Licenses were available for evaluation.

5.2.3 Negative controls - IgG removal and dilution curve

In the Lefebvre et al., (2012) study they detected a DA specific antibody of subclass IgG, so in order to validate the method and provide important negative controls, both serum albumin and IgG were removed from the respective serum samples from the different species in the study. This was also carried out to test for matrix effects to see if there was anything (except antibodies), in the serum samples that could interfere with the modified assay. Removal was carried out using the Thermo Scientific Pierce® Albumin/IgG Removal Kit (product nr. 89875) following the manufacturer's instructions. The kit contains a resin mixture of immobilized cibacron blue that binds to the serum albumin and protein A that binds to many species and subclasses of IgG including harbour seals (Swart et al., 1995). The filtrate, containing sample with albumin and IgG removed was used for immediate iELISA analysis, this providing a useful negative control. In addition to the IgG removal samples, an antibody serum dilution curve was carried out with a heavy chain secondary antibody in order to quantify the magnitude of any change in absorbance ratio for the different species tested. This would also assist in assessing whether serum dilution would indicate a change in the assay response. Dilution curves was not reported in the Lefebvre et al., (2012) study.

5.2.4 Indirect ELISAs for Domoic acid-Specific Antibody Detection in serum

This method had been modified using the ASP ELISA assay from Biosense, (ASP ELISA assay kits, Biosense, Bergen, Norway), where detection of DA-specific sea lion IgG was investigated by using the DA conjugated 96-wells plate (Fig. 5.1). Serum is added to the well and any DA-specific antibodies present in the serum are allowed to bind to the DA conjugated to the plate well. After washing, a 1:5 000 and a 1:10 000 dilution of two different horseradish peroxidase (HRP) labelled anti-canine IgG's (heavy chain and light chain: Bethyl Laboratories Inc., Montgomery, Texas, USA) which are known to cross-react with (Capers, 2006; Colvocoresses, 2004) were tested to determine whether there was any difference in binding between these two

detecting antibodies. The HRP labelled anti-canine IgG was incubated for 90 min to allow it to bind to the DA specific antibody in the serum. The plate was washed for a second time to remove any unbound HRP labelled anti-canine IgG. Tetramethylbenzidine (TMB) was added which acts as a visualizing reagent designed to react to the bound HRP labelled anti-canine IgG (Fig. 5.2). The plate was left to incubate in the dark for 30 min and the optical density was measured in an automatic microplate reader at 450 nm (OD_{450}) (Lefebvre et al., 2012). Absorbance ratios were calculated to compare the results with the Lefebvre et al., (2012) publication, this was carried out following the published method where the absorbance of the serum sample (X) was divided by the mean control serum absorbance plus three times its standard deviation (SD).

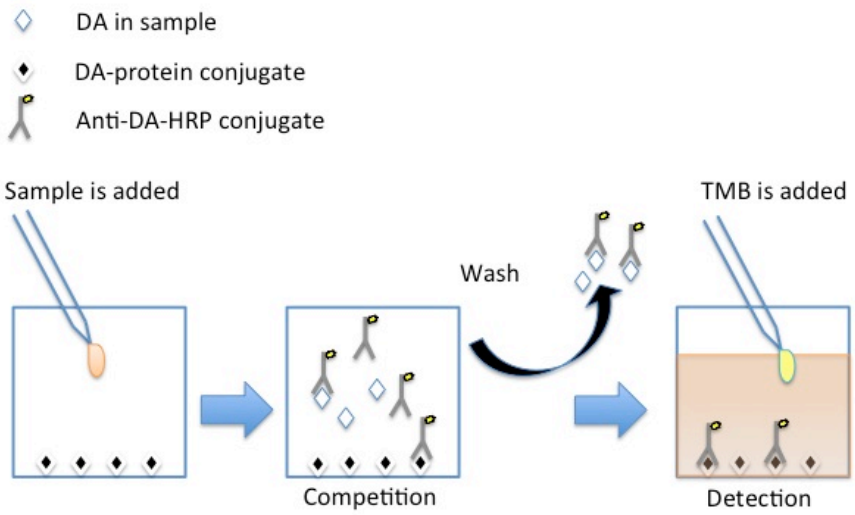

Figure 5.1: Assay summary for the original ASP ELISA DA (Biosense) detection method. Adapted and modified from the Biosense protocol.

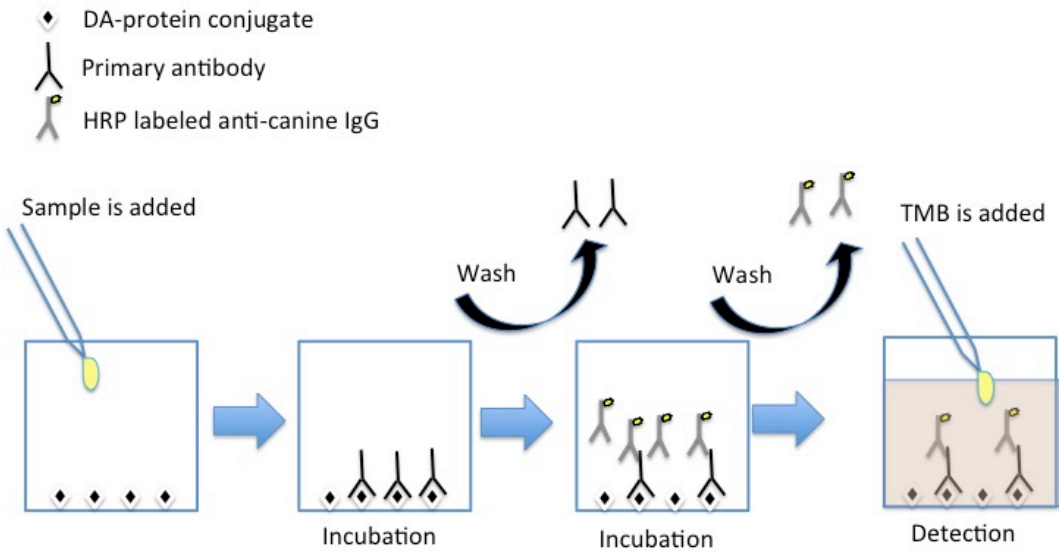

Figure 5.2: Summary of the modified assay which is an Indirect ELISAs (iELISA) for Domoic acid-Specific Antibody detection in pinniped serum, modified from the ASP ELISA as shown in Figure 5.1.

5.2.5 Blockage of nonspecific binding sites

A separate trial was carried out to remove any interfering binding proteins that might be present in the serum sample by using a bovine serum albumin buffer (BSA) (Sigma-Aldrich, Lot #41K1645). BSA is known for its stability and lack of interference with biological reactions and are thought to prevent nonspecific antibody binding by hindering hydrophobic interaction between proteins and ionic interactions (Renshaw, 2007).

5.3 Results

5.3.1 Validation of iELISA assay (CSL and reference dog serum)

For the validation of the published method a trial was carried out according to the publication by Lefebvre et. al., (2012) using CSL serum samples. The first trial revealed a nineteen times higher absorbance ratio in all the three replicates provided by The Marine Mammal Center than published by Lefevbre et al., (2012) (mean of duplicates, Table 5.1). Thus, some

modification was made to the method in an effort to reduce these ratios. To block out any interfering proteins, a BSA buffer was added. The buffer reduced the optical density and the absorbance ratio decreased –by up to 92.6 % to 1.44 which is a comparable ratio to those that had been published (see all in Table 5.1). Then the total IgG and albumin were removed to provide a negative sample. Removing the IgG decreased the absorbance ratio by another 11.1 % (Table 5.1), however according to Lefebvre et al., (2012) if the absorbance ratio is >1 the sample is still positive for DA.

To rule out binding or interference with the HRP labelled anti-canine antibodies I first ran the assay with the anti-canine antibodies at 1:5 000 and 1:10 000 alone with no serum added. These secondary antibodies had no interference with the modified assay as the absorbance ratio was close to zero (Table 5.2).

BSA buffer was also tested in the modified assay, alone and together with the HRP labelled anti-canine antibody. It did not interfere with the modified assay, as the absorbance ratio was very low and similar to the results obtained from the blanks 0.21 ± 0.2 (Table 5.2). The anti-canine IgG or the BSA buffer did not bind to the plate-well and can both be ruled out as potential interference factors, in terms of falsely increasing the absorbance ratio.

The reference dog serum had the highest absorbance ratio of all the serum tested > 18.29 (Table 5.1). From the results it appears that when the HRP labelled anti-canine IgG was diluted to 1:10 000 the absorbance decreased slightly and when the IgG was removed completely the absorbance decreased nineteen times (Table 5.1).

5.3.2 Trial of DA-specific antibody detection in phocid serum samples

To further investigate true DA-specific antibody presence in the samples of interest to this research, serum from harbour seal pups and harbour seal adults together with grey seal pups was assayed following the same method as published by Lefebvre et al., (2012) at a 1:5 000 and 1:10 000 dilutions of

the HRP labelled anti-canine IgGs. The harbour seal pup serum revealed a high absorbance ratio > 5.45 (Table 5.3), where Lefebvre stated that values >1 indicate presence of DA-specific antibodies. The grey seals had a lower absorbance ratio than the harbour seals < 2.53 (Table 5.3), but according to Lefebvre criteria, with detection of DA-specific antibodies.

5.3.3 Negative control trial

Mean absorbance ratio among the CSLs control samples (all serum tested stripped of IgG and albumin) was 0.18 ± 0.2 (SD). These samples were used as a control to see if any additional proteins in the serum or the buffer were interacting with the ASP ELISA kit components or binding to the plate-well. Additional blanks (n = 9) were treated with all the kits components, but had no added serum and revealed a mean absorbance ratio of 0.23 ± 3 (SD) (Table 5.2). Both the control sample and the blanks did not bind or interfere with the plate-well or assay. Hyperbolic serum dilution curves measuring levels from high to low was expected and documented in the CSL and harbour seal serum (Table 5.4, Fig 5.3), although the grey seal serum had a flatter curve which was unexpected.

Table 5.1: Absorbance ratio of serum samples of California Sea Lion (CSL) tested for DA specific antibodies using an indirect enzyme-linked immunosorbent assay (iELISA) at 1:5 000 and 1:10 000 dilution of the secondary antibody. Absorbance Ratio = sample serum absorbance - (mean control serum absorbance+3SD); Values >1 indicate presence of DA-specific antibody.

CSL **- ID #**	1:5 000 ***with BSA **IgG removed**	1:10 000 ***with BSA**
10046 (Lefebvre)	1.23	n.a
10046 (replicate)	19.44 / 1.44* / 1.16**	12.65 / 0.39*
10047 (Lefebvre)	1.72	n.a
10047 (replicate)	19.12 / 1.38* / 1.14**	17.30 / 0.33*
9759 (Lefebvre)	9.88	n.a
9759 (replicate)	19.72 / 1.27**	n.a
Reference dog	19.94 / 1.80**	18.29

Table 5.2: Absorbance ratio bovine serum albumin (BSA), HRP labelled anti-canine IgG and blanks tested for DA specific antibodies via an indirect enzyme-linked immunosorbent assay (iELISA) at 1:5 000 and 1:10 000 dilution. Absorbance Ratio = sample serum absorbance - (mean control serum absorbance+3SD); Values >1 indicate presence of DA-specific antibody.

Other	1:5 000 ****IgG removed**	1:10 000	Blank 1 / 2 / 3
HRP anti-canine IgG light chain	0.21	0.22	
HRP anti-canine IgG heavy chain	0.21	0.21	
BSA buffer	0.22	n.a	
BSA buffer + HRP anti-canine IgG	0.33	n.a	
Blank 1 / 2 / 3 4 / 5 / 6 7 / 8 / 9			0.23 / 0.22 / 0.21 0.21 / 0.21 / 0.23 0.21 / 0.21 / 0.23
BSA	0.23	n.a	

Table 5.3: Absorbance ratio of serum samples of harbour seals (PV) and Grey seals (Hg) and reference dog serum tested for DA specific antibodies via an indirect enzyme-linked immunosorbent assay (iELISA) at 1:5 000 and 1:10 000 dilutions. Absorbance Ratio = sample serum absorbance - (mean control serum absorbance+3SD); Values >1 indicate presence of DA-specific antibody

Harbour seal (Pv) Grey seal (Hg) **- ID #**	1:5 000 ***with BSA **IgG removed**	1:10 000 ***with BSA**
Pv		
11718	11.82	5.74 / 2.10*
60266	11.17	1.20 / 0.90*
76481	5.45 / 2.30* / 1.89**	n.a
Hg		
58737/2	2.61 / 1.56* / 0.72**	1.77
58753/4	2.53 / 1.45* / 0.57**	1.72

Table 5.4: Absorbance ratio of serum samples diluted from 1:1 to 1:16 from CSL, PV and Hg

Sample	Dilution curve				
	1:1	1:2	1:4	1:8	1:16
Grey seal	0.68	0.54	0.50	0.69	0.73
Harbour seal	2.20	1.62	1.23	0.92	0.83
California sea lion	1.60	1.14	0.98	0.77	0.64

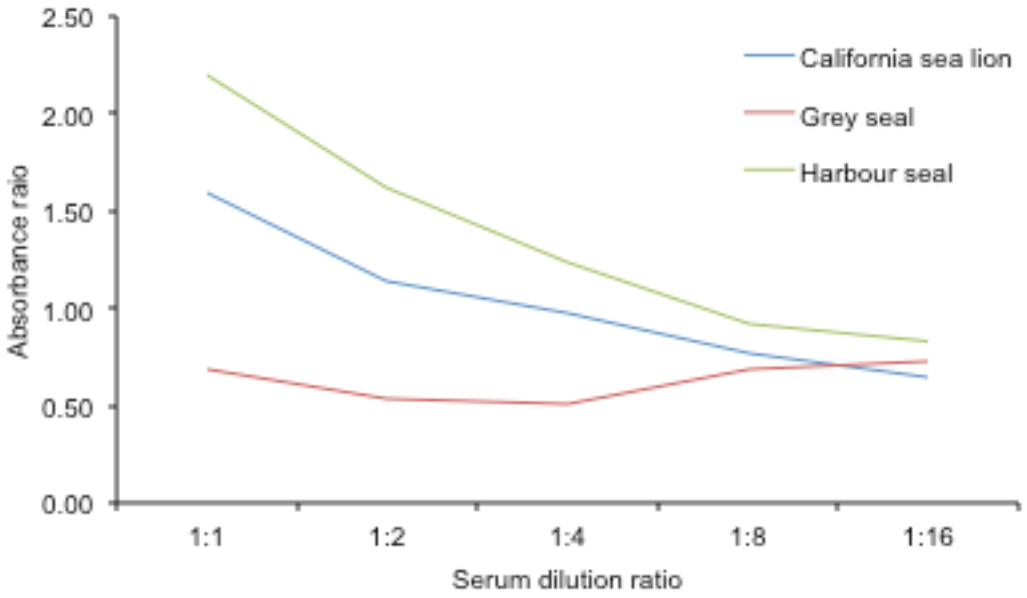

Figure 5.3: Dilution curve from 1:1 to 1:16 of the selected species tested for the magnitude of change in absorbance ratios.

5.4 Discussion

5.4.1 Failure to replicate iELISA results using CSL and dog reference serum

From the results of this study we were unable to replicate and validate the findings of Lefebvre et al., (2012) using the same serum samples. This could be for a number of reasons:

1) The samples sent from TMMC were contaminated or serum proteins and binding sites were damaged due to defrosting during the transportation process
2) The BSA might have blocked out some the specific binding sites when testing the CSL serum which would give another absorbance ratio
3) After removing all the IgG from the sample, the absorbance ratio was almost the same as when adding the BSA to the assay, indicating there was no binding of IgG (or other proteins) when adding BSA which lead to the conclusion that the assay is not detecting IgG

Although these reasons could interfere with the assay, evidence from this study suggests that the modified assay is not detecting DA specific antibodies.

The study by Lefebvre et al., (2012) found evidence, through whole-genome microarray profiling and up regulation of immune-relevant genes, that zebra fish undergo an immune response to the sub-acute repetitive DA dose regime carried out in the study. Domoic acid is a small water-soluble amino acid and a neurotoxin. It is doubtful that a small amino acid such as DA alone could trigger the humoral immune system to produce antibodies (Lefebvre et al., 2012) and it has been suggested that DA could be binding to a larger protein and thus has the potential to be antigenic. It has been shown that specific humoral immune responses do occur against small haptens (small molecules that can elicit an immune response when attached to a larger carrier protein) that are similar in size to DA when linked to a serum protein, or a larger carrier protein for immunogenicity (Yoshida et al., 1970). Haptens have the property of antigenicity but not immunogenicity.

The ASP Biosense assay is designed to detect free DA in a sample, and free DA is known to occur in serum (Truelove and Iverson, 1994). Free DA in serum could potentially interfere with the modified assay and interact with the DA specific antibodies (if indeed they have been generated). In the Lefebvre et al., (2012) study they had three CSLs with no clinical signs of DA-induced excitotoxicity (e.g. seizures), this is the only evidence used to suggest that these CSLs were experiencing a low level chronic DA exposure. It has been suggested that most of the free DA in monkeys is cleared out of their serum after 30 min (Truelove and Iverson, 1994), but no studies have been carried out in CSL to measure this. From the results presented in Chapter 6 DA is cleared by harbour seals after 10 hours. If this were the case for CSLs there is a possibility that free DA is present in some of the serum samples, as there is no evidence from the published study that excludes any potential free DA in the CSL serum samples. There is a possibility that if the DA specific antibodies were binding to the DA conjugate on the ASP ELISA plate well then the DA specific antibodies would compete with any free DA in the serum

and bind to either of them. If a binding of free DA in the serum and DA specific antibodies occurred, these would go undetected, as they would be washed out in the washing step and thus free DA could potentially interfere with the modified assay. Although this doesn't explain why I didn't get the same result by using the same samples, and although I continued to test this assay with other samples I were sceptical about this reliability. The reference dog serum had the highest optical density measured of all samples in this study. Reasons why will be discussed further in the discussion.

5.4.2 DA specific antibodies in phocid seals

One of the harbour seal serum samples was collected from an animal in Rødsand in Denmark, an area where toxic phytoplankton blooms are uncommon (Anderson et al., 2001). This serum sample was selected as a possible harbour seal 'control' due to the low toxic activity in the region and therefore the expectation that it would be negative. In addition, harbour seal pup serum samples from Lismore on the west coast of Scotland were tested. These animals might also be expected to be naïve as they were all neonates. According to Lefebvre et al., (2012), an immune response towards DA starts after approximately 18 weeks of exposure and DA specific antibody production increases after 24 weeks. Harbour seal females give birth and nurse their pups on land and fast during the beginning of the lactation period (Thompson et al., 1994) and although maternal transfer of DA is known to occur (Maucher and Ramsdell, 2005; Rust et al., 2014), the west coast is an area where the seals are unlikely to be chronically exposed to DA (See Chapter 2). It would be very unlikely for these pups to have developed DA specific antibodies as they are weaned after four weeks and would be sampled within this time period when they only get milk from their mother. Studies of antibodies transfer from mother to milk has been carried out in several marine mammals for example: northern fur seal and harbour seal pups, where they found a slow increase of IgG during the first four months of the pups life (Cavagnolo, 1979; Ross et al., 1993). We can therefore assume that potential DA specific antibodies would be unlikely transferred from mother

to pup in a high volume. Grey seal serum samples were chosen as another phocid control sample as they have shown to have low levels of DA (Tarrant et al. 2014 unpublished results) and would again be unlikely to have developed DA specific antibodies. However, these assumptions are based on limited knowledge of actual exposure and if the CSL samples were validated and the dog serum had been negative then I would have concluded that these samples also contained DA-specific antibodies. The serum dilution curve confirms some antibody binding, as the binding decreased with the dilution concentration.

5.4.3 Validation of iELISA assay

The ASP ELISA assay from Biosense is not designed to detect DA specific antibodies, the assay is designed to detect free DA in shellfish samples (Kleivdal et al., 2007a), and has been used to detect free DA in urine and faecal samples from marine mammals although urine samples have shown to be a difficult matrix to analyse with high coefficient of variation probably due to the salt content (Seubert et al., 2014). The ASP ELISA plate-well contains conjugated DA, proteins and blocker (C. Miles, ASP ELISA developer pers. comm.).

Since all our serum samples had a higher absorbance ratio than was measured in CSLs, it is more likely that a non-specific IgG is binding to the conjugated DA, the protein or the blocker coated on the plate-well. There is no evidence in the Lefebvre et al., (2012) study that they removed any non-specific IgG antibodies from the samples they analysed.

The HRP labelled anti-canine IgG is designed to bind to dog IgG, but it is known to bind to IgG from other species (Capers, 2006; Colvocoresses, 2004) and this study confirmed there was no interference in terms of higher absorbance ratio from this detecting antibody for both the heavy and light chain variants. The HRP labelled anti-canine IgG has an enzyme label, where a colour is produced after incubation with a substrate. The optical density is

measured using a spectrophotometer, and a reaction is only possible if the HRP labelled anti-canine IgG has bound to an antibody on the plate-well. If these antibodies were DA specific a reaction would not occur in the reference dog serum. The reason for the high absorbance in the reference dog serum is likely due to the fact that the HRP labelled anti-canine IgG has a higher affinity to the dog than any other species since it is the homologous species.

5.4.4 Evidence of method failure

1) The reference dog serum would not have been exposed to DA, therefore finding a high absorbance ratio measured in the serum indicates binding of non-specific IgG to the DA conjugate coated on the plate-well. This was confirmed when all the IgG was removed from the dog sera and the absorbance ratio decreased nineteen times to ~1.8.

2) The BSA buffer is designed to block any non-specific protein binding sites and although it decreased the absorbance ratio (for example in the CSL sera from 19.44 to a comparable level of 1.44, where Lefebvre et al., (2012) reported the same sample to have an absorbance ratio of 1.16) it does not confirm that DA-specific antibodies are the antibodies binding. This highlighted the need to include critical controls to ensure the true binding of any naturally occurring DA-specific antibodies.

3) As mentioned in the Methods section, the blanks were treated with all the ASP ELISA kit components and the steps of the assay followed as for the other samples but the blank wells did not include any serum. The blanks would indicate if anything was interfering from the kit. Since the absorbance ratio was low between 0.23 and 0.21, this implied no interference from the kit components.

4) The HRP-labelled anti-canine IgG was tested alone and with the BSA buffer to rule out any binding to the DA conjugate coated on the plate-well.

Both these tests revealed a low absorbance ratio also around 0.2, which would indicate no binding.

5.5 Conclusions

Results from this study reveal that if DA specific antibodies are present the modified assay method published by Lefebvre et al., (2012) is either difficult to replicate and validate or it is detecting an unrelated protein (or proteins) present in canids and phocids. One interpretation of the findings reported here is that they indicate binding to a non-specific IgG. There seem to be some fundamental issues with the assay design. At this stage it would not be recommended that this modified method be used without further research to analyse serum samples for DA-specific antibodies in pinnipeds. It could be the case that DA specific antibodies are only possible (at this stage) to measure in serum of zebrafish, which was the model used for the Lefebvre et al., (2012) study. Future studies should concentrate on using purified IgG from known exposed animals and develop a method that specifically can detect any DA specific antibodies.

Chapter 6

6. Biomarker for domoic acid clearance in harbour seals

6.1 Domoic acid clearance rate in harbour seals

6.1.1 Introduction

Pharmco- or toxicokinetics (PK or TK) is the study of the absorption, distribution and metabolism of substances (drugs or toxins) in the body. This discipline therefore involves studying the time course of a drug or toxin in various body compartments (Hedaya, 2012). Pharmacokinetics generally models the doses of therapeutic drugs so they will be efficient and produce the desired response and not be given at doses high enough to cause toxicity. Understanding the movement of drugs or toxins and how they are metabolised by the body involves two aspects a) empirical data from time-course dosing studies and b) mathematical models, which estimate various kinetic parameters, allowing researchers to determine the pathways and physiological impacts the drugs or toxins may have. In the first stage, the dosing studies, most PK studies use intravenous dosing (either with the drug itself or a surrogate marker compound), although oral dosing and other routes can also be used. Often the route of exposure for the drug or toxin of interest is chosen but for oral dosing some information about the bioavailability (i.e. the fraction of the drug or toxin that is absorbed or stored by the body and how much is just eliminated unchanged) of the compound is required. The metabolism and elimination rates can then be estimated by collecting blood or urine samples at timed intervals from when the compound was administered and measuring the amount of the compound present in those samples. Using plasma or serum samples in dosing studies is the most common way of

investigating a drug in PK models, because whole blood with the different cellular elements becomes too complex (Hedaya, 2012).

6.1.2 Pharmacokinetic parameters

There are several key parameters that are required in the second stage of the process, the PK models, and these are:

- Maximum drug or toxin concentration (C_{max})
- Time of maximum concentration (T_{max})
- Elimination rate constant (k_e)
- Systemic clearance (Cl)
- Elimination half-life ($T_{1/2}$)
- Area under the drug/toxin concentration versus time curve (AUC)
- Volume of distribution (V_d)
- Bioavailability (F)

After the administration of a drug or marker compound, a maximum concentration (C_{max}) is reached in the circulation, the time the compound takes for the concentration to be reached is referred to as T_{max}. The elimination rate constant (k_e) is a description of a compound that follows first-order kinetics (elimination of the compound is directly proportional its concentration) (per unit of time). The elimination rate constant is related to two other parameters, clearance (Cl) which measures the time it takes for the drug or compound to be excreted from the body (per unit time) and the half-life ($T_{1/2}$) describes the time it takes for a drug to decrease by 50 % of the current concentration, most drugs have several half-lives. Pharmacokinetic modelling is performed by either non-compartmental or compartmental methods. A non-compartmental model requires fewer assumptions and calculates the exposure to a drug by estimating the area under the drug concentration versus time curve (AUC), the AUC (Fig. 6.1) is used to calculate the overall clearance of a drug or compound (Buxton, 2006). Bioavailability (F) describes the rate and extent to which a drug enters the body and becomes available at the site of action. Bioavailability ranges in value from 0 – 100 % which

corresponds to the proportion that has reached the systemic circulation (Hacker et al., 2009). Drugs that are given intravenously (iv) are the most efficient and have a bioavailability of 100 %, while other administrated doses weather it is orally, subcutaneous, intramuscular, nasal, inhaled, sublingual or transdermal the bioavailability ranges between 0 – 100 %.

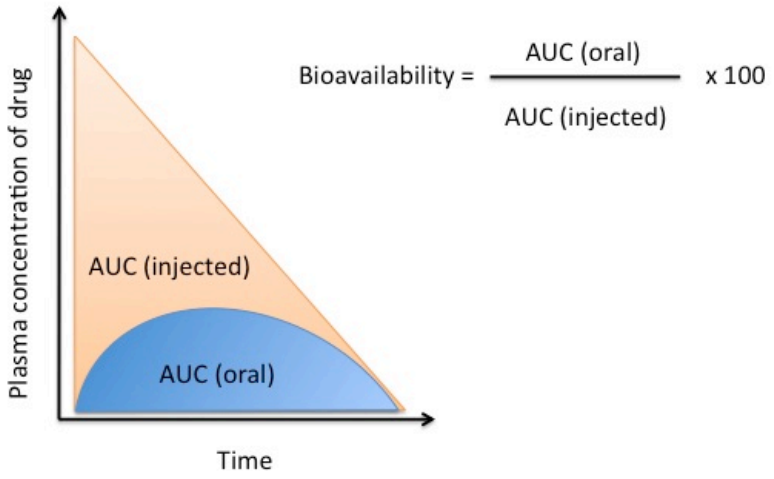

Figure 6.1: Determination of bioavailability of a drug, adapted from Hedaya (2012).

To calculate the bioavailability parameter following an oral administration of a drug, the rate of drug concentration (AUC) after an oral dose is compared with an intravenously (iv) dose (Equation 1).

$$\text{Equation 1: } F = \frac{AUC(oral)}{AUC\ (iv)}$$

When an animal is exposed to a drug or a toxin before the previous exposure (dose) is completely removed, accumulation occurs. This means that the resulting concentration will be higher. Steady state (Fig. 6.2) is an important parameter and can be defined as when the rate of drug or toxin input is equal to the rate of drug elimination, this depends on the half-life of the drug or toxin and the longer the half-life the longer it takes to reach steady state (Hedaya, 2012).

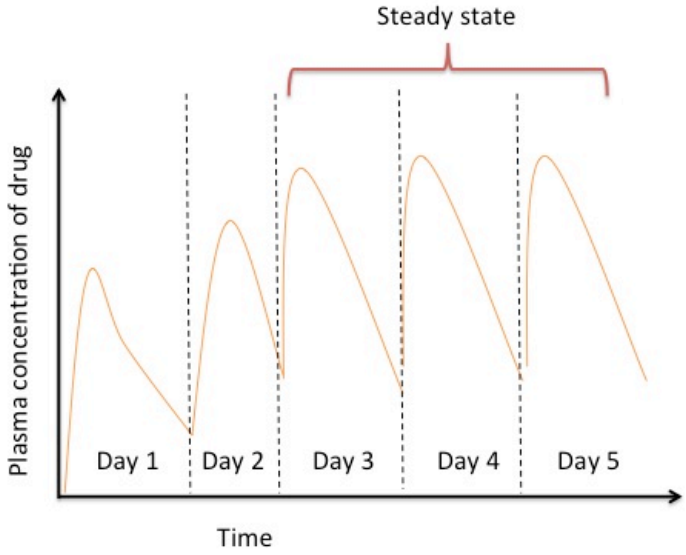

Figure 6.2: Illustration of an example of how long it takes for a drug to reach steady state, adapted from Hedaya (2012).

6.1.3 Domoic acid toxic mechanisms and clearance rates

Domoic acid (DA) is an amino acid and a glutamate agonist that interferes with neurotransmission in the brain by binding to receptors in the central nervous system and causing damage or cell death in the hippocampal area. Thus because the neurons affected by DA are located in the hippocampus, affected people experience short-term memory loss, which is one of the most noticeable symptoms of DA poisoning (Perl et al., 1990). Interestingly in the study by Perl et al., (1990) no DA was detected in the blood of the people affected by the 1987 Canadian DA toxin event which raised questions regarding the clearance rate of DA. Normal kidney function plays an important role in the clearance of DA and it was documented in a study by Preston and Hynie (1991) that after a nephrectomy in rats, DA serum and brain levels were elevated compared to rats with intact kidneys. Studies of rats and monkeys indicate that DA has a rapid renal clearance (approximately 9 ml/min/kg body weight) and has a half-life of 20 min (Suzuki and Hierlihy, 1993; Truelove and Iverson, 1994). Inulin has been used to measure the clearance rate of DA, because it is not metabolized, secreted or reabsorbed in the body (Stitzer and

Martinez-Maldonado, 1978; Suzuki and Hierlihy, 1993). Iohexol, a nonradioactive contrast medium used as a marker for renal and plasma clearance, has been shown to be comparable to inulin as it is excreted intact by the kidneys (Brown and O'reilly, 1991; Gaspari et al., 1995). Iohexol has had an increasing interest in the scientific community and is now commonly used and has been validated in cats and dogs as a measure of glomerular filtration rate (GFR) (Brown et al., 1996; Gleadhill and Michell, 1996; Goy-Thollot et al., 2006). Because iohexol has a low affinity for serum proteins and iodinated compounds are stable in plasma samples there is no major metabolism, deiodination or biotransformation. This means it can be substituted for inulin in both kidney function tests and to understand the pharmacokinetics of similar compounds, such as DA and potentially other hydrophilic toxins. Iohexol is also excreted though the urine unchanged with an average half life (in rats and dogs) of 74 min (Mützel and Speck, 1980).

Numerous outbreaks of toxicity worldwide, and results of this study (chapters 2 and 3) indicate that DA moves up the marine food chain (Landsberg, 2002). The effect DA has depends on the amount of toxic prey and therefore the dose of DA consumed by predators (Gulland, 2000; Lefebvre et al., 1999). Because no information exists on comparative pharmacokinetic parameters for marine mammals, the present study was the first step in investigating how quickly the DA biomarker iohexol is metabolised, cleared and excreted by healthy harbour seals. By collecting serial plasma samples from captive animals and measuring how long iohexol is detectable in the serum following an oral exposure I can determine basic pharmacokinetic parameters, particularly AUC and elimination half-life. This is of importance in the interpretation of the DA concentration levels measured in the urine of live captured and dead stranded harbour seals (Chapter 2), and to model the individual and population level risks and consequences of DA uptake to harbour seals in future.

6.2 Method

6.2.1 Harbour seals

Five male captive harbour seals were housed at the Sea Mammal Research Unit individually in continuous flowing salt-water tanks. These seals were captured in the Eden estuary on the east coast of Scotland, using hand held nets under the SMRU Home Office Animal (Scientific Procedures), Act 1986 Project Licence. Four harbour seals were adult males and one was a sub-adult male. The four adult males had a body weights from approximately 80 – 100 kg while the sub-adult male weighed 50 kg (Table 6.1). The harbour seals were clinically healthy and were feeding daily before the study started. The seals were not given food 8 hours prior to the start of the experiment.

Table 6.1: Harbour seals used in this study by name, sex (male or female), developmental stage (pup, sub-adult, adult) and mass (kg).

Harbour seal individual	Sex	Developmental stage	Mass (kg)
Mac	M	Adult	80.6
Xav	M	Adult	82.8
Vern	M	Adult	86.4
Uri	M	Adult	101.4
Tim	M	Sub-adult	49.6

6.2.2 Immobilization

Harbour seals were immobilized using a combination of midazolam (Hypnovel; Roche, Welwyn Garden City, UK) 10mg/2ml solution, 0.3ml/10kg IM as a premed and 0.1ml/10kg IV (to control tremors) and ketamine (Ketaset 100mg/ml solution, 0.1ml/10kg IV). While anesthetized, an Instech Solomon CBAS C70 7Fr heparin coated PU round tip catheter was inserted using a Dispomedica 8Fr peel away sheath introducer into the extradural vein. The catheter was kept from coagulating at the luer with heparinized saline (10 U heparin/ml 0.9% NaCl) at each sampling.

6.2.3 Trial 1

The first procedure involved one harbour seal (Mac). After the seal was immobilized, iohexol (Omnipaque^{TM} 350, 350 mg/mL, Amersham Health, Cork, Ireland) was administered through a feeding tube at a dose rate of 2 ml/kg (lean body mass, total dose: 140.0 ml). The feeding tube was flushed with 150ml water before removal. Time zero was set at the end of the administered Iohexol. Two heparinized blood samples (10 ml) were obtained from the catheter at 0, 30, 120, 600 and 1440 min after injection. The animal was observed during and after the procedure for any adverse reactions to iohexol. Urine samples were collected at 0 and 1440 min by urinary catheterisation. Blood samples were centrifuged at 2700 g for 12 min and plasma was removed and stored at -20 °C until analysis. Because of the analytical problems that occurred, only plasma samples were analysed in this study.

6.2.4 Trial 2a

Based on the results from trial 1, a decision was made to increase the blood sampling intervals to 10, 20, 30, 60, 120, 600 and 1440 min after administration of iohexol (total dose; 130 ml), and this was carried out with the second harbour seal (Xav). This trial was carried out twice on the same seal, leaving the seal to recover for a week in between the trials.

6.2.5 Trial 2b

After encountering some problems with the method for analysing iohexol using UV spectrophotometry (see section 6.2.6), the samples from the second set of trials were analysed using LC/MS/MS. Thus based on the results from the UHPLC-MS/MS analysis (see 6.2.5 Analysis of iohexol using the UHPLC-MS/MS method) a further trial was planned, this time involving three individual harbour seals (Verne, Uri and Tim). After immobilization (as above) iohexol was administered again orally (total doses as follow; Verne: 172.8 ml, Uri:

202.8 ml, Tim: 161.2 ml) and blood was collected in the same way as described in section 6.2.3 Trial 1. Collection time for blood samples was more frequent being every hour from 1 to 10 h.

6.2.6 Analysis of iohexol as iodine using a UV spectrophotometric method

6.2.6.1 Sample preparation and analysis method trial 1 and 2

Initially, for the first trial, the blood samples were analysed following a colorimetric method published by Bäck et al. (1988) where iohexol is deiodinated by hydrolysis of alkaline and iodine release by the ceric arsenite reaction. Before the analysis a standard (50 µl serum from a non-trial exposed seal) + 50 µl iohexol) and a blank (50 µl serum) is prepared. The samples were added to polystyrene tubes where 5 ml of 1 mol/l NAOH is added. The sample were vortexed and incubated for 2 hrs in 90 °C. After incubation 50 µl were transferred into a micro centrifuge tube where 1 ml of the working solution (5 parts 1 mol/l sulphuric acid with 1 part bromide/bromate solution and 4 parts arsenite solution) are added to each sample. Then at timed intervals 50 µl ceric ammonium sulphate were added to the sample mixed and left to incubate for 20 min. The mix was then transferred into micro cuvettes and read using a UV-Visible spectrophotometer at 410 nm. The iodine concentrations were calculated using the equation in the publication by Bäck et al. (1988).

$$C = Cstand \times \log(Ablank / Asample) / \log(Ablank / Astand).$$

Where: Cstand = concentration of the standard
Ablank = absorbance blank
Asample = absorbance sample
Astand = absorbance standard

A standard curve was made to validate the assay and allow determination of the quantity of iodine in the samples. This iodine detection method is based

on a method first described by Sandell and Kolthoff (1934) and relies on the iodine as a reducing agent for cerium. The production of iodine in the reaction is reduced by trivalent arsenic (Bäck et al., 1988).

6.2.7 Analysis of iohexol using the UHPLC-MS/MS

6.2.7.1 Sample preparation for trial 1, 2 and 3

Because of the failure of the analytical method (section 6.2.4 Analysis of iohexol as iodine using a UV spectrophotometric method) it was decided to use an alternative method to directly analyse the iohexhol in the plasma samples. This method could not be carried out at SMRU so the plasma samples were stored at -20^{o}C following collection for transfer to the Marine Science Scotland laboratory for analysis.

The sample preparation was based on a method published by Lee et al. (2006). Before the analysis 100 µl of sample aliquot + 100 µL of acetonitrile in 2 mL Eppendorf was vortexed twice for 5 sec. The sample was then centrifuged for 4 min at 14 000 rpm. The sample supernatant was then filtered through a 0.2 µm centrifugal filter. The filtered sample was then centrifuged for 2 min at 14 000 rpm and again filtered using a 0.2 µm centrifugal filter. Filtered samples were transferred into 350 µL amber vials for UHPLC-MS/MS analysis.

6.2.7.2 Analytical method UHPLC-MS/MS

For the calibration a spiking solution (SS): 26.5 µL of iohexol 350 (Omnipaque) + 9970 µL of distilled water was used (Fig 6.3). Plasma samples were analysed following the method published by Lee et al. (2006) with some modifications and was performed at the Marine Scotland Science Laboratory in Aberdeen.

The method involving the use of ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was modified

and optimised for the specific detection of iohexol. A 3200 QTRAP mass spectrometer (ABSciex) was coupled with a 1290 Infinity UHPLC system (Agilent), comprising a 1290 Infinity binary pump, a 1290 Infinity Thermostated column compartment and a 1290 Infinity auto-sampler. Separation of the substrate was achieved using an XBridge column (C18 – 3.5 µm – 150 * 3 mm) using an isocratic elution (30% B). Mobile phase A was 100% aqueous and mobile phase B was 95% acetonitrile, both containing 2mM ammonium formate and 50 mM formic acid. With a flow rate was set up at 0.4 ml/min and a column oven maintained at 25 ºC throughout the analysis, the analysis run time was eight minutes. The injection volume for both standards and sample extracts was 5 µL. The mass spectrometer was used in multiple reaction-monitoring (MRM) mode and three specific transitions, one for quantitation and the other two for confirmation, were monitored for iohexol. The monitored transitions and source parameters are detailed in Tables 6.2 and 6.3.

Table 6.2: Mass spectrometer optimised parameters for the considered iohexol transition

	Transition	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Iohexol	T1	822.02	804.1	176	6	34	31	8
	T2	822.02	375.2				87	4
	T3	822.02	603				35	6

DP: declustering potential

EP: entrance potential

CEP: collision cell entrance potential

CE: collision energy potential

CXP: collision cell exit potential

Table 6.3: Source optimised parameters

	CUR (V)	IS (V)	TEM (ºC)	GS1 (psi)	GS2 (psi)	CAD (V)
Source /Gas	10	5200	300	20	15	9

CUR: curtain gas voltage

IS: electro spray voltage

TEM: source temperature

GS1: nebuliser gas pressure

GS2: heater gas pressure

CAD: collisional activated dissociation voltage

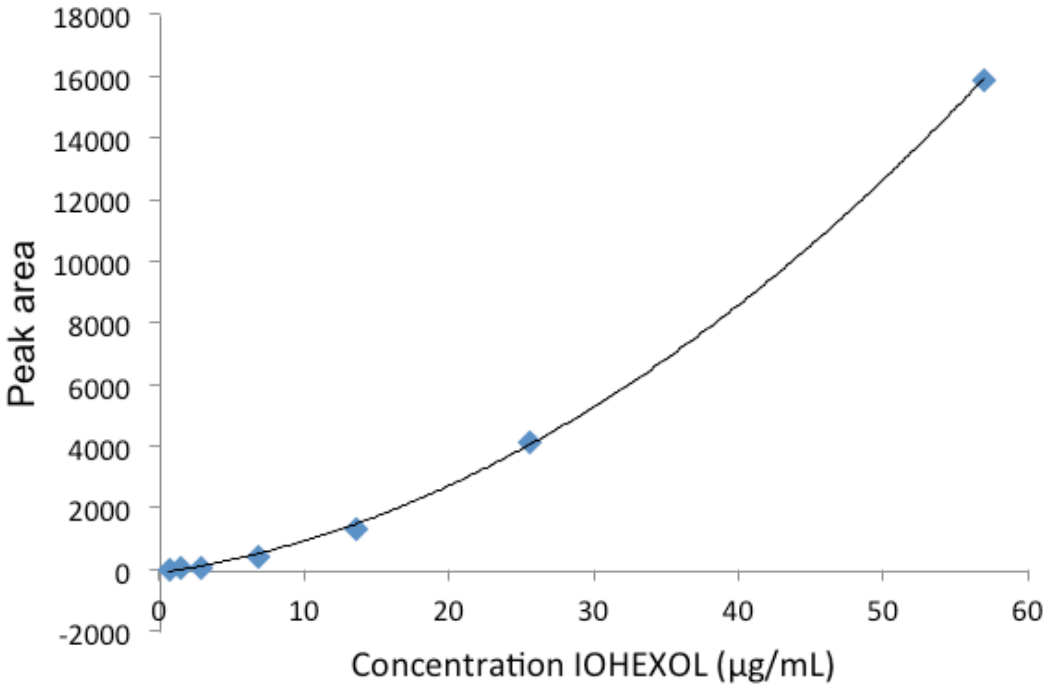

Figure 6.3: Calibration curve: because linear regression did not give the best fit (R^2~0.9800 at best), a polynomial regression fit was used.

6.2.8 Pharmacokinetic models

Because the seals are orally exposed to toxins this study was designed to give an oral dose of the marker compound, iohexol and measure the iodine (using UV spectrophotometry) or iohexol (using UHPLC-MS/MS) content in

plasma samples collected serially over a 1-12 h time period. To evaluate the pharmacokinetics of iohexol there are several models that could be considered. The most frequently used are the non-, one- and two compartmental models. In a non-compartmental model the elimination of a drug follows a first order process and is often used by clinicians to calculate half-life (Fig. 6.4). In a one compartmental model it is assumed that the drug is being distributed instantaneously throughout the body (Fig 6.4) (Hacker et al., 2009). In a two compartmental model it is assumed that the drug is distributed into two distinct compartments, which represent the central and peripheral compartment (Fig. 6.4) (Hacker et al., 2009).

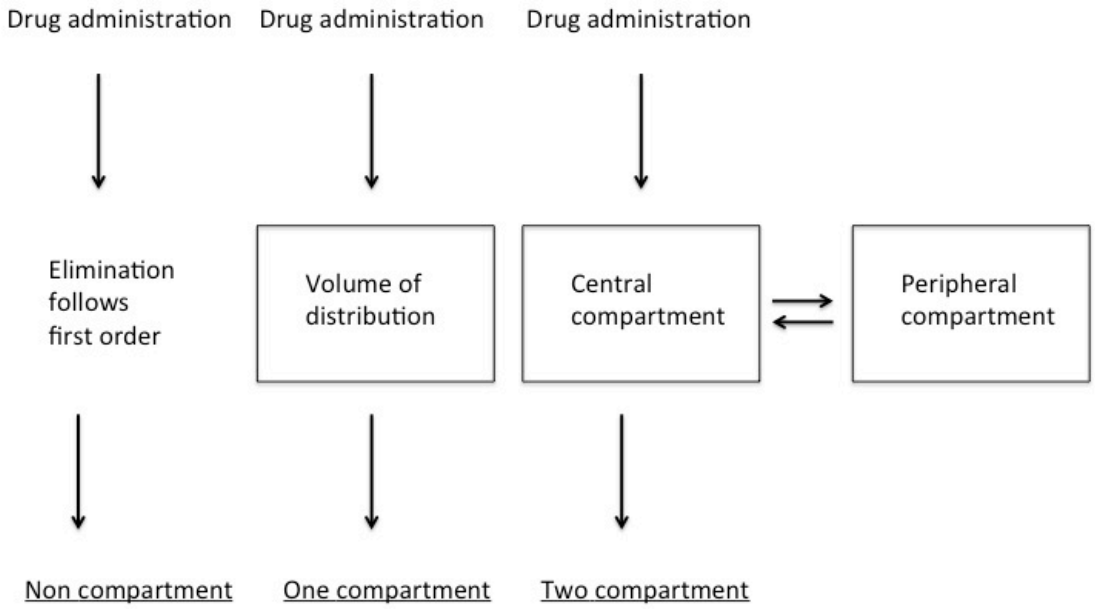

Figure 6.4: Models to be considered when designing a drug or toxin clearance study, adapted from Hedaya (2012).

For this study a non-compartmental analysis was used where the area under the concentration time curve or AUC, T_{max}, C_{max}, and elimination half-life was calculated using the WinNonlin programme (6.3.0.395, Core Version 04Jun2007, Pharsight, Mountain View, CA). Bioavailability and total clearance was not possible to calculate because the lack of an intravenous administered dose in addition to the oral dose data. The AUC, which represents the total iohexol exposure over time, was calculated from the concentration in the

plasma (Cp) versus time of sampling. The most common approach is a numerical approximation method called the trapezoidal rule (Equation 2).

$$\text{Equation 2: } AUC = \frac{Cn+Cn-1}{2}(t_n - t_{n-1})$$

The elimination half-life ($t_{1/2}$) is given by (Equation 3):

$$\text{Equation 3: } t\frac{1}{2} = 0.693 * MRT$$

Where 0.693 is the natural logarithm of 2 and MRT is the mean residence time, which is calculated by the non-compartmental model using the WinNonlin programme (Reed et al., 2009).

6.3 Results

6.3.1 Harbour seal reaction to iohexol

The harbour seals in all studies (Mac, Xav, Verne, Uri and Tim) completed the study without any untoward effect of the compound administrated. No observed changes in behaviour after the study were completed.

6.3.2 Results using a UV spectrophotometric method

The results from first set of samples were promising (Fig. 6.5, black line) as a peak of iodine was measured in the UV spectrophotometer after 30 min since the iohexol was administered. However, in a replicate analysis the curve flattened out around 30 min and a peak was first detected only after 10 hrs since administration of the iohexol (Fig. 6.5, red line).

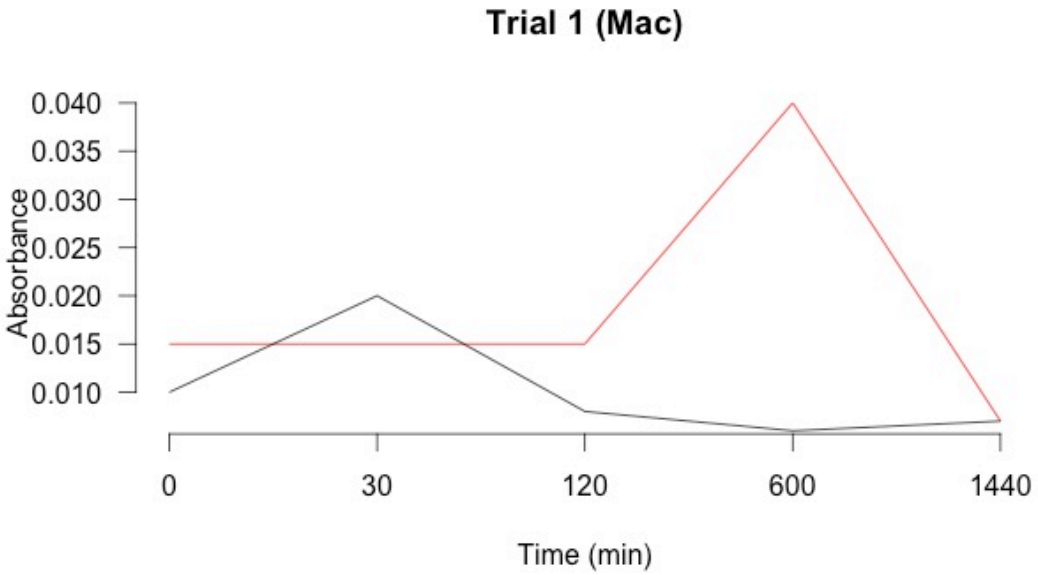

Figure 6.5: The iodine absorbance result from trial 1 by using UV spectrophotometer with the iodine absorbance measured in the serum from the harbour seal “Mac” vs the serum sample time, black line is the first analysis and the red line is the replicate.

For the second trial the blood-sampling interval was increased to investigate if there was a peak between 10 and 60 min. There was no real peak identified in the second trial (Fig. 6.6, black and red line).

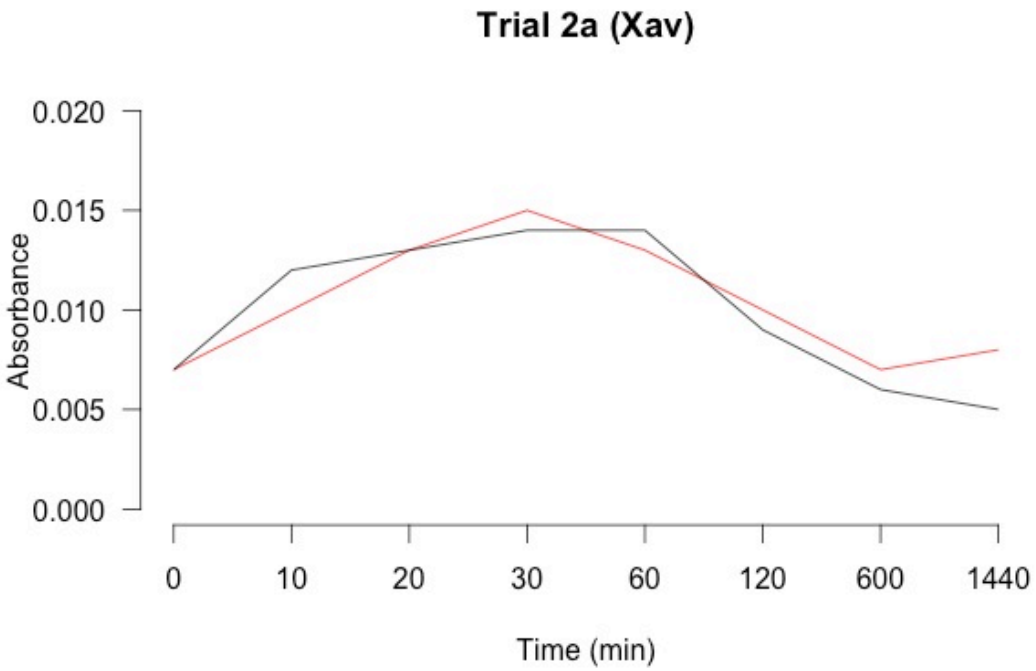

Figure 6.6: The iodine absorbance result from trial 2a by using UV spectrophotometer with the iodine absorbance measured in the serum from the harbour seal “Xav” vs the serum sample time, black line is the first analysis and the red line is the replicate.

In the replicate trial there was a peak at 20 min (Fig. 6.7, black line) but when this sample was run as a duplicate, two peaks were identified, the first at 10 min and the second at 30 min (Fig. 6.7, red line).

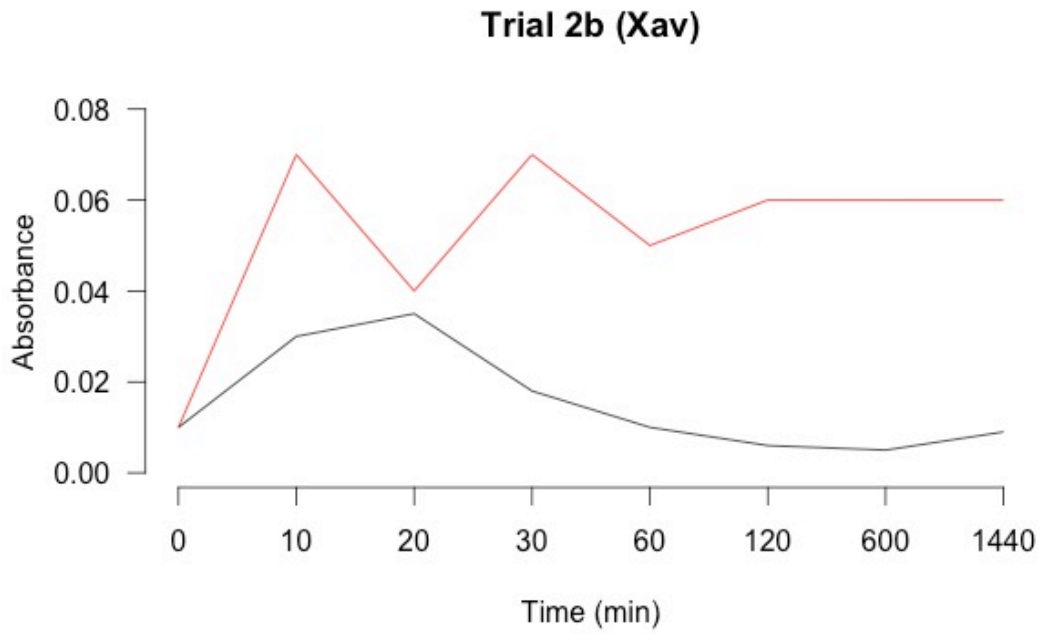

Figure 6.7: The iodine absorbance result from trial 2b by using the UV spectophotometric method with the iodine absorbance measured in the serum from the harbour seal “Xav” vs the serum sample time, black line is the first analysis and the red line is the replicate.

After the first and second trial a standard curve was analysed to examine if the method was working optimally and to determine the relationship between concentration of iodine and absorbance. Several trials of the standard curve were carried out, but unfortunately the results were not consistent or successful, so that no increase in absorbance was seen with increasing concentration of iohexol (Fig, 6.8). This indicated some fundamental failure of the method and invalidated the results obtained above.

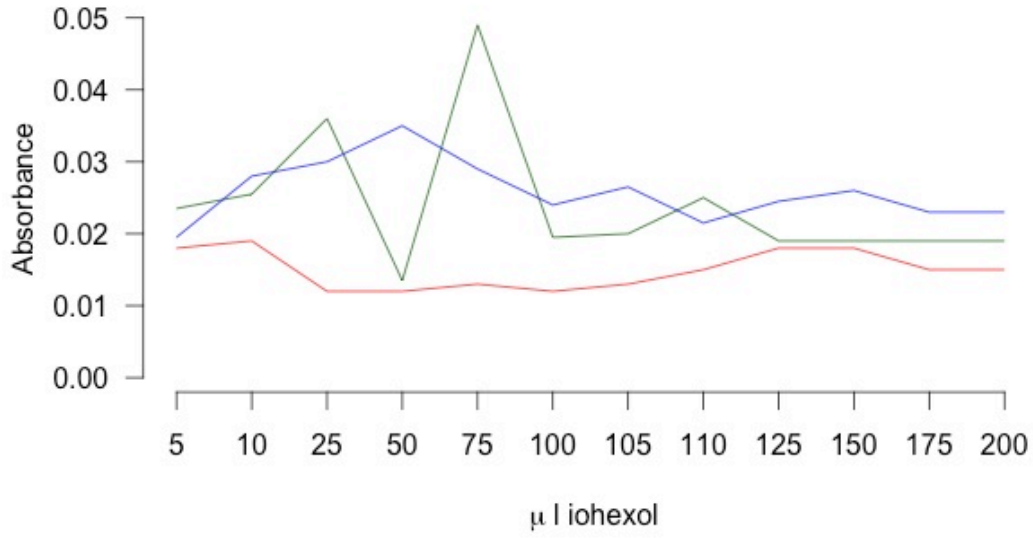

Figure 6.8: Three trials (blue, red and green line) of a standard dilution curve for iodine concentration where the absorbance is measured based on the iodine concentration in the iohexol.

6.3.3 Analytical method UHPLC-MS/MS

Due to the inconsistency of the UV-spectrophotometric method, the plasma samples were then analysed using an UHPLC-MS/MS. The method was successful in measuring iohexol concentrations in the plasma (Fig. 6.9 and Fig. 6.10).

6.3.2.4 Pharmacokinetic models

The dose rate chosen was based on studies in dogs (Klenner et al., 2009) and because harbour seals have very large fat stores, the dose was calculated on a lean body mass basis so as to be comparable. Omnipaque 350 contains 755mg/ml of iohexol thus the administered dose was 755mg/mL * 2mL/kg (lean body mass) = 1510mg/kg.

The result from the UHPLC-LC/MS/MS method are shown in Table 6.4 and 6.6 for the five harbour seals (Mac, Xav, Verne, Uri and Tim). Because the sample time points were much fewer for the first three trials, the additional pharmacokinetic parameters were only calculated for the last three seals

trialled. Thus the Cp by time and AUCs calculated for Verne, Uri and Tim are given in Table 6.7.

Table 6.4: Table showing the different pharmacokinetic parameters C_{max} and T_{max} for both harbour seals Mac and Xav in trial 1 and 2 a and b

Time (min)	Cp (Mac)	Cp (Xav 2a)	Cp (Xav 2b)
0	0	0	0
10	-	0.14	0
20	-	2.07	0
30	0	2.92	0
60	-	4.06	0.19
120	7.65	8.47	5.49
600	1.07	0	0.44
1440	0	0	0

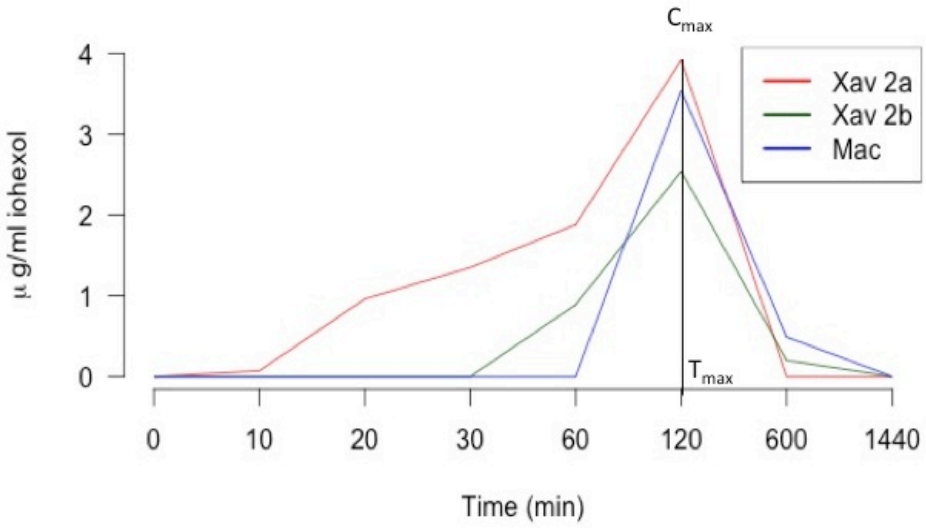

Figure 6.9: Graph showing the concentration of iohexol measured in plasma of the two different harbour seals, Mac and Xav (2a and b) where C_{max} , T_{max} is indicated on the graph.

Table 6.5: Table showing the different pharmacokinetic parameters C_{max} , and T_{max} for the two different harbour seals Mac and Xav in trial 1 and 2 a and b.

Seal	C_{max}	T_{max} (min)
Mac	3.5	120
Xav 2a	3.9	120
Xav 2b	2.5	120

Table 6.6: Table showing the measured iodone concentration (Cp) in plasma (μg/ml) and the time the sample was collected for the three harbour seals Verne, Uri and Tim.

Time (min)	Cp (Verne)	Cp (Uri)	Cp (Tim)
0	0.00	0.00	0.00
60	15.51	17.87	26.81
120	30.28	24.81	38.70
180	51.44	32.92	34.72
240	54.44	32.92	30.69
300	44.91	36.90	23.89
360	27.45	37.13	18.24
420	19.91	36.76	17.64
480	16.67	32.31	20.37
540	13.10	27.64	26.62
600	12.50	22.92	16.06

Table 6.7: Table showing the AUC for the three harbour seals using the linear rule Verne. Uri and Tim.

Time	AUC (Verne)	AUC (Uri)	AUC (Tim)
0	0	0	0
60	16.75	19.3	28.95
120	66.2	65.4	99.7
180	154.45	127.75	179
240	268.8	198.85	249.65
300	376.1	274.25	308.6
360	454.25	354.2	354.1
420	505.4	434	392.85
480	544.9	508.6	433.9
540	577.05	573.35	484.65
600	604.7	627.95	530.75

A single kinetic profile may be well summarized by the peak concentration C_{max}, the time when the peak occurred T_{max}, the elimination half life, $t_{1/2}$ and the AUC. T_{max} and C_{max} are obtained from the Cp/time plot for each seal as shown in Fig. 6.10 and Table 6.8, elimination half-life ($t_{1/2}$) is shown in Table 6.8.

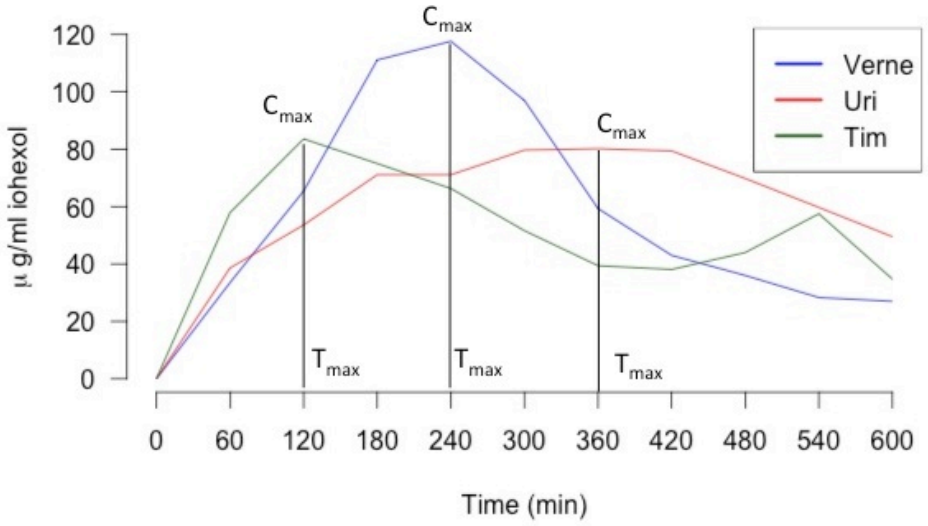

Figure 6.10: Graph showing the concentration of iohexol measured in plasma of the three different harbour seals, Verne, Uri and Tim where C_{max} , T_{max} is indicated on the graph.

Table 6.8: Table showing the different pharmacokinetic parameters C_{max} , T_{max} and elimination half-life ($t_{1/2}$) for the three different harbour seals, Verne, Uri and Tim.

Seal	C_{max}	T_{max} (min)	Elimination half-life (min, $t_{1/2}$)
Verne	117.6	240	191.4
Uri	80.2	360	197.4
Tim	83.6	120	226.8
Average ± SD	93.8 ± 20.68	240 ± 120	205.2 ± 19.2

6.4 Discussion

Exposure to DA has occurred for many years in marine mammals (Fire et al., 2009; Hall and Frame, 2010; Lefebvre et al., 1999; McHuron et al., 2013; Scholin et al., 2000), and continues to pose a threat to their health and survival. Therefore there is an increasing interest in understanding how quickly DA is cleared out of the body.

In this study, elimination of the DA biomarker iohexol was in part adequately described by the equation of a non-compartmental model, although total clearance rate and bioavailability could not be calculated in this study. Iohexol is biphasic meaning it has a rapid distribution and slower elimination. This study documents the maximum drug or toxin concentration (C_{max}), the time of

maximum concentration (T_{max}) and the elimination half-life ($t_{1/2}$) of iohexol. The elimination half-life (measured in min), which is the time period required for the concentration of the drug or toxin to be reduced by one-half was in this study measured to be on average 205.2 (3.42 h) ± 19.2. In a study by Truelove et al. (1994), rats had a mean half-life (min) of 21.0 (0.35 h) ± 3.2 and in monkeys a mean (min) of 16.0 (0.27 h). In addition to DA having a short half-life in both rats and monkeys (Suzuki and Hierlihy, 1993; Truelove and Iverson, 1994), iohexol was measured to be below detection limits after four hours of exposure. Iohexol clearance rate have been measured in dogs to have a half-life of 173 (2.88 h) ± 53 minutes (Collignon et al., 2012), and are physiologically more similar to harbour seals than rat and monkey. This is very similar to the elimination half-life I estimated for the harbour seals.

Establishing a single pharmacokinetic reference value for estimating the elimination of DA would be incorrect as there can be several reasons why there is a difference between the pharmacokinetic parameters between the harbour seals in this study. These differences can be age-related, affected by mass and body size and by diet, hydration status and fitness. From the first and second (2a and 2b) trials (Mac and Xav 2a and b) no true peak (C_{max}) of iohexol was identified as it occurred between 120 and 600 min.

In the third trial, which involved the harbour seals Verne, Uri and Tim the peak was identified on average at 240 ± 120 min. Both Verne and Uri had a T_{max} that occurred later than Tim and both of these seals were adult harbour seals (> 5 years) while Tim was a sub-adult (2-4 years) as estimated from their mass and the mass/age relationships for UK harbour seals (SMRU unpublished data). Age could potentially be a factor that could affect the elimination of iohexol (Hoek et al., 2007). Since the iohexol is used for measuring renal failure, kidney function, hydration status together with body size could play a role at slowing down the clearance in harbour seals with a lower renal function (Gaspari et al., 1995; Perl et al., 1990; Suzuki and Hierlihy, 1993).

Blood is a difficult matrix to use for estimating exposure to DA because it is quickly eliminated, as demonstrated by the results of this study. Although we were not able to continue to collect blood samples beyond 10 h, to the point of complete disappearance of iohexol, the declining Cp suggested that this would have occurred within the next 2-3 hours. Because of this rapid removal of DA from the blood, samples such as urine and faeces have been collected (Chapter 2 and Chapter 4) in order to measure DA exposure in marine mammals. DA is primarily eliminated in the urine following absorption (Suzuki and Hierlihy, 1993)} which makes this the preferred matrix for determining the time course of exposure. Maucher and Ramsdell (2005) measured DA in 3 body fluids; urine, plasma and milk and after a 1.0 mg/kg intraperitoneal exposure in lactating rats they found that most of the DA is excreted through the urine and that the 1-hr values were 3 orders of magnitude greater than in the plasma and that the plasma had 10 times higher DA concentrations than milk. This clearly indicates that most systemic DA is cleared quickly through the kidneys. There have been several models for DA clearance studies in mice, rats and cynomolgus monkeys where symptoms such as tremors, seizures and scratching have been reported after varying doses of DA exposure (Iverson et al., 1989; Tasker et al., 1991; Tryphonas et al., 1990) and CSL with DA toxicosis exhibit similar symptoms such as ataxia, head weaving, scratching, decreased responsiveness and seizures (Gulland et al., 2002).

Future studies will therefore need to focus on a) determining how often harbour seals urinate (which could be determined from captive experiments) b) measuring the clearance of iohexol after an intravenous injection to determine bioavailability and c) collection and analysis of urine preferably on an hourly basis at the same time as the intravenous experiment. This experiment would need to ensure that the bladder is empty at the start. More sophisticated models involving a two compartmental model could then be used for calculating the total clearance, as well as the urinary excretion rate of the DA biomarker iohexol. This knowledge could then be used to back-calculate DA exposure levels for different harbour seal haulout sites around Scotland, where average foraging distances are different (ranging in the UK

from < 10 to > 60 km, (Sharples et al., 2012). These data could then be added into a risk assessment model to evaluate if DA is indeed a likely factor causing the decline in the harbour seal population in Scotland.

Chapter 7

7. General discussion

7.1 Thesis aim

This thesis was motivated by questions regarding the cause or causes of the harbour seal decline in Scotland, focussing on the role that toxins from harmful algae may play. These were:

1. Are the Scottish harbour seals exposed to multiple toxins in Scotland and what are the potential health effects of this exposure?
2. What are the key prey species involved in the trophic transfer of toxins to harbour seals?
3. Are there other fish eating predators exposed to toxins in Scotland, or is it only occurring in Scottish phocids?
4. For how long has harbour seals in Scotland been chronically exposed to domoic acid (DA)? Can the recent discovery that CSLs have natural antibodies to DA (Lefebvre et al., 2012) be used to investigate historic DA exposure in archived blood samples from harbour seals in Scotland?
5. Following exposure to DA, how quickly are the harbour seals excreting the toxin, compared to other model species?
6. Can exposure to toxins from harmful algae be a factor responsible for the harbour seal population decline in some regions of Scotland?

7.2 The exposure of Scottish harbour seals to multiple toxins

DA produced by the diatom *Pseudo-nitzschia* has become an interesting potential factor in the decline in abundance (in some regions) of the Scottish harbour seals (Hall and Frame, 2010). Results from this study show that in addition to their continued exposure to DA, at least on a relatively annual

basis over the last 6 years, Scottish harbour seals are additionally exposed to several other toxins that have the potential to cause substantial morbidity and mortality. In particular I found they were ingesting PSP toxins, a critical finding given their very lethal nature (García et al., 2004; Hernández et al., 1998; Long et al., 1990).

Although the levels measured in the faecal samples collected were low, harbour seals are at risk of dying if exposure exceeds a threshold dose (Bates et al., 1989). There are many members of the PSP toxin family and it is a complicated toxin to study, with extraction methods and toxin quantification still under development. PSP toxins could potentially have a devastating effect on the harbour seal population and indeed PSP toxins were the cause of the mass mortality in the monk seal populations of the western Sahara (Hernández et al., 1998). In addition to causing mass mortality, PSP toxins can exhibit sub-lethal health effects that are hard to demonstrate in free-living animals as they can inhibit critical body processes such as respiration (Mons et al., 1998).

Although there is no information on the effect of PSP toxins in harbour seals, several reasons exist that could increase the susceptibility of a seal to these PSP toxins, than for example for humans; (1) seals have blubber (composed of adipocytes), which the water-soluble PSP toxins cannot access/perfuse because of their hydrophilic nature, (making the toxin more concentrated in the body tissues and organs). (2) The marine mammalian dive reflex which optimizes respiration by peripheral vasoconstriction where blood is shunted away from non-critical tissues and directed to the vital organs such as the heart and brain, which limits access to other organs such as the kidney and liver (Geraci et al., 1989). (3) The respiratory systems sensitivity to anaesthetic agents in pinnipeds (reports of complications due to anaesthesia involve apnoea, bradycardia, extended recovery, hypo- and hyperthermia and in worse case death occurs (Gales, 1989; Hammond and Elsner, 1977; McDonell, 1972; Sinnett et al., 1981)), due to their physiologic and anatomical adaptation (i.e. cardiovascular, respiratory and thermoregulatory) to diving (Gales, 1989; McDonell, 1972; Ridgway and Simpson, 1969).

The third group of toxins investigated in Chapter 2 was the lipophilic toxins such as OA and DTXs. These toxins have not been widely studied in marine mammals and only a few papers in the literature exist indicating exposure in bottlenose dolphins in Texas, USA (Fire et al., 2010b) and in manatees in Florida (Capper et al., 2013). Investigation of exposure to both OA and DTX-2 confirmed that Scottish harbour seals are exposed to not only DA and PSP toxins, but to three different toxin groups. I also observed DTX-2 in relation to a couple of possible cases of gastroenteritis, which may be indicative of poor digestion (Chapter 2). Although these toxins cause the most common shellfish illness DSP in humans, knowledge of its presence and location in the food chain is sparse, and to my knowledge only one case of natural OA exposed fish exists (Gamboa et al., 1990).

There was significant regional and temporal variation, particularly in exposure and uptake of DA in harbour seals for which I had most data. Due to the small sample size, I was not able to investigate the regional and temporal variation in the other toxins I focussed on (PSP toxins, OA and DTXs) so clearly further work on this is needed on these toxin groups in the future. It is interesting that all the urine samples collected and analysed between 2008 and 2010 for the live captured harbour seals contained quantifiable amounts of DA. This trend was also seen for faecal samples both for the live captured and anonymous in the years from 2008 to 2010. Annual variation in the *Pseudo-nitzschia* spp. blooms in Scotland can explain this observed DA trend (Fehling et al., 2006). Another explanatory factor for the DA variability from the west coast to the east coast can be explained by the hydrographical conditions as studies have shown that DA levels can be higher in areas where the salinity is higher compared to less saline areas (Doucette et al., 2008). The ecosystem from the west coast is quite similar in many ways to the east coast (Baxter, 2011), where for example the coastal waters around the east coast and the west coast of Scotland are affected by fresh water from numerous small rivers (Baxter, 2011). Although limited detailed knowledge exists on the detailed hydrographical parameters (such as temperature and salinity) in the areas where the harbour seals are foraging, studies indicate that the west coast

shows a lower and more variable salinity content than the east coast (Baxter, 2011), and this is probably due to the increased flow rate and fresh water input from the rivers along the west coast.

Difference in sampling time in relation to excretion rate could also explain the lower levels measured on the west coast. It would be interesting to investigate offshore hydrographical parameters (such as wind stress, heat fluxes, temperature and current) in addition to phytoplankton blooms in the areas where the harbour seals are foraging. Regional differences in foraging duration and travelling time to the different haul out sites (Sharples et al., 2012) need to be taken into account when comparing toxin levels in excreta from seals inhabiting the different regions. Regionally comparison should therefore be made with caution, as I still don't know what the toxin levels represent in the harbour seals given that I don't know the time of toxin ingestion. However, further inferences could be made from modelling approaches (see below). Even though the proportion of positive samples was higher on the east coast, the significantly lower concentration on the west coast is of particular interest in relation to the preferred prey consumed. On the west coast the harbour seals prefer to forage on gadoid fish (Wilson, 2014) compared to seals on the east coast that forage more intensively on flatfish and sandeels (Sharples et al., 2009; Wilson, 2014). Flatfish has in this study been highlighted as an important vector of toxin transfer and changes in harbour seal diet can thus have major consequences for exposure. Although caution is taken when interpreting the toxin levels measured in the harbour seals throughout this study, I have found a higher toxin concentration levels for the majority of samples collected (urine and faeces in live captured animals and faecal samples from anonymous animals) in the areas where the population is in decline (east coast and Northern Isles). This is important information and linking these findings to the preferred prey and potential vectors of toxin transfer, I conclude that toxins from harmful algae remain a potentially significant factor in the harbour seal population decline.

Due to the lack of sample quantity to measure all toxins in all samples and the limited resources available for this study, analysis for DA was prioritised. This enabled me to investigate the relationship between DA in faecal and urine samples and certain basic immune parameters that could be measured in the field or on return to the laboratory. Of particular interest were the white blood cell levels (Levin et al., 2005) and particularly the eosinophil counts, which are significantly elevated in CSL with acute and chronic DA toxicosis (Gulland et al., 2012; Schwacke et al., 2010; Twiner et al., 2011). Exposure to DA has been reported to have other immunological effects such as increased T-lymphocyte proliferation and can in turn decrease host resistance (Levin et al., 2010). The immunomodulatory effects, possibly as a result of DA exposure reported in this study (Chapter 2), were lower lymphocyte cell counts. (lymphocytopenia) related to an increase in urinary and faecal DA concentrations. In addition effects on the monocyte cell counts (monocytosis) were also documented where an increase in urinary or faecal DA concentration was related to an increase in cell counts. Lymphocytopenia can increase the risk of infection, and interestingly monocytosis often occurs in response to a chronic inflammation or infection, indicating these two effects could be linked. The yearly reoccurrence of both lymphocytopenia and monocytosis may indicate that this effect is related to chronic DA exposure. These correlations with white blood cell counts have not been documented in other exposed species before so this could be a species-specific effect. However, the implication for the health of the individuals and the levels of exposure that may elicit this response requires much further investigation. Comparing the mean lymphocyte cell counts in this study to wild-caught harbour seals from California, US indicated that the mean lies around the lower threshold of the normal range (Greig et al., 2010), which confirms lymphocytopenia. In addition, when comparing the monocyte cell counts to the wild-caught harbour seals from California, the mean lies closer to the upper threshold for the normal rage (Greig et al., 2010), indicating a monocytosis. Assuming these reference ranges are appropriate for use in UK harbour seals, I can conclude that the changes in white cell counts may be clinically significant.

A reduction in lymphocyte counts can be indicative of chronic stress, (which also increases the endogenous production of glucocorticoids (Duncan et al., 1994)), chronic infection or malignancy. Diseases caused by parasite infections (Kelly et al., 2005) or viral infection (Bossart et al., 1990) can often be accompanied by lymphocytopenia and immunosuppression, which can result in opportunistic infections (Ramaekers et al., 1975). Results of this study may indicate an immune suppression associated with DA but I was not able to investigate for any bacterial or viral infections. Natural killer (NK) cells are one of three types of lymphocytes and play a large role in defending the host from virally infected cells and tumours and reduced NK cells were found when harbour seals were given fish contaminated with persistent organic pollutants (Ross et al., 1996). Given the susceptibility of harbour seals to viral infections such as phocine distemper virus (Osterhaus and Vedder, 1988), this could be an important lymphocyte sub-set to investigate and identify further in relation to DA uptake or the other toxin groups studied in this thesis.

An increase in monocytes can occur if the body is exposed to an acute bacterial, protozoal or a viral infection (Duncan et al., 1994) and can also occur during chronic inflammatory conditions. Monocytosis was documented in bottlenose dolphins recovering from morbillivirus bronchopneumonia (Reidarson et al., 1998). To my knowledge there is only one case described in the marine mammal literature where lymphocytopenia and monocytosis occurred simultaneously, in a CSL suffering from adenovirus (Dierauf et al., 1981).

I was able to show that harbour seals are exposed to multiple toxins from harmful algae but did not have sufficient samples to relate these combined levels in individual animals with health parameters. It may be that if the occurrences of toxins in prey are positively correlated (so that fish with high levels of DA also have high levels of the other toxins) then some of the relationship seen could be due to the synergistic or additive effects of these additional toxins. Results from Chapter 3 document that of the flatfish species; plaice, lemon sole and dab are the three species that accumulate the highest levels of all three groups of toxins. Multiple toxin exposure has been

documented in bottlenose dolphins from Texas, US where OA and DA was detected at low levels in the tissues (Fire et al., 2010b), although no associated health effects were reported. In a study by Dragunow et al., (2005) they suggested that *in vitro* toxicity of OA is increasing in the presence of the algal toxin gymnodimine (produced by dinoflagellates from the genus *Karenia*). Further investigations into multiple toxins both at lethal and at the sub-lethal levels are required. DA bind to glutamate receptors in numerous organs suggesting exposure can not only have neurological effects, but other perhaps more subtle health effects, which indicates that studies of the occurrence and abundance of glutamate receptors in other organs in harbour seals (such as the adrenal glands (Gendron and Morley, 2005; Kristensen, 1993)) should be investigated.

Cortisol levels in the blood were also an important blood parameter in the CSL studies, where results documented that levels are significantly lower in CSL with DA toxicosis (Gulland et al., 2012). The lack of evidence for lower levels of cortisol or higher levels of eosinophils suggests that the Scottish harbour seals captured and sampled in this study were not suffering from acute or chronic toxicosis. Although the levels measured in the harbour seals urine and faecal samples were comparable to the levels reported for the CSL taken into rehabilitation at the Marine Mammal Centre in California (Goldstein et al., 2008), the highest level in the prey collected during the large DA mortality events on the US west coast were 223 µg/g compared to the highest level measured on the east coast of 117 µg/g which represent a 90.6 % higher level than the prey collected on the east coast of Scotland (but see below for further discussion on levels in prey).

I would conclude that this indicates that in the surviving seals I was able to study in the various regions exposure is perhaps more likely to be low level and chronic in nature. I did not observe seals showing neurological signs and these have not been reported to SMRU by observers or through the seal rehabilitation centres during my PhD time. However in February 2009 there were some unusual events where harbour seal pups were found in fields, on pavements, golf courses and on roads along the east coast (C. Seddon pers.

comm.). The phytoplankton monitoring didn't start until March that year so it is difficult to say if this unusual behaviour event could be linked with exposure to HAB. There was also an observation on a seizing grey seal in July 2011 (A. and M. Reeve pers. comm.), phytoplankton monitoring document bloom over the trigger limit > 50 000 cells per litre. Although these events are rare it is quite possible though that those with the most severe symptoms and health effects from exposure above the threshold for effects do not make it back to the haul out site after foraging. Thus, given that these conclusions were drawn from studying the survivors it was important to investigate exposure levels in the same matrices and pathological effects in any carcasses that washed ashore and were the subject of post mortem examination by the Scottish Marine Animal Stranding Scheme (SMASS).

7.3 Domoic acid in dead stranded marine mammals

Dead marine mammals were sampled and the levels compared to those seen in the live captured animals. The 28 dead harbour seals that were available did not have significantly higher levels than the live animals and the causes of death attributed to these individuals, where available were not linked to toxin exposure. However, the sample size of seals available for this study was small and may not represent the dominant causes of mortality for seals in these regions.

Due to the amount of decomposition, detailed pathology and histology was not always possible for these animals. Limited conclusions can be drawn although the ultimate cause of death was not thought to be toxin related it is still possible that toxins could have been involved either as a direct or an indirect cause of death. Impacts on organs such as the heart (Goldstein et al., 2008) and brain (Goldstein et al., 2008) were not investigated and neither were sequelae (Teitelbaum et al., 1990) that could be related to toxins other than DA, particularly PSP toxins and this aspect should therefore be prioritised in future. Intrauterine DA exposed mice showed hippocampal damage and neuronal death occurred in the offspring (Dakshinamurti et al.,

1993), it would be interesting investigate any possible DA effects in pups of harbour seals that are exposed to DA in utero.

All freshly dead harbour seals should have brain and heart tissue taken for histology. Brains should be serially sectioned and examinations should be carried out to investigate for any gross abnormalities. Lungs should be checked if they are congested as this was a consistent feature of the Monk seals' mortalities (Harwood). Both lung and brains should be analysed for PSP toxins. However, the occurrence of fresh harbour seal carcasses washing ashore around the Scottish coast is very limited (Brownlow and Davison, 2012). Unless the animals die close to shore they do not appear to strand. For example, despite the very high mortality that must have occurred on the east coast given the very significant decline in numbers (~90% since 2000, (SCOS, 2013)), the magnitude and speed of the decline cannot be explained only by effects on fecundity but must be due to increased mortality as well (N. Hanson, pers. comm.) yet very few dead seals are reported as stranded in this region. This is also unlikely to be due to reporting bias as the SMASS have good coverage in this area and it is highly populated with many dog walkers and other members of the public using most of the coastline for recreation.

Samples of faeces and urine from other marine mammals, particularly cetaceans, that strand around the UK coast were also analysed to compare with the results for the harbour seals and to determine if other coastal species particularly harbour porpoises had higher levels or prevalence of exposure compared to more offshore species. Results from this investigation document exposure of both the coastal and offshore cetaceans where the harbour porpoise (inshore species) had a significantly higher level than the offshore feeders. The DA levels measured in harbour seals and the harbour porpoise were the highest of the stranded marine mammals, but interestingly were lower than those that had been measured in the live captured harbour seals. Stranding of harbour porpoises was documented in relation to DA in 2002, 2005, 2007 and 2010 in California, US (Wilkin et al., 2012) with levels ten times higher than what is reported in this thesis. The DA levels measured in

grey seals (that are known to generally forage further off the coast than harbour seals (Thompson et al., 1996) were below the LOQ except in two cases where grey seals were shot in June on the east coast and levels measured in the pericardial fluid were quantifiable and comparable to fluid levels that had been measured in harbour seals.

Interestingly DA levels measured in the faecal samples from the pilot whales were the highest of the offshore feeders and although no diet information exists on these samples, pilot whales are known to forage on cephalopods (Desportes and Mouritsen, 1993). Cephalopods have been found to act as vectors of DA (Costa et al., 2005b) although levels in these prey have not been investigated around the Scottish coast.

7.4 Previous exposure to domoic acid in harbour seals

In order to find out if DA exposure in particular is a recent or historic phenomenon and to find out if the appearance of markers of exposure appeared in the population at about the same time as the decline in harbour seal abundance for the regions of interest (east coast and Northern Isles), an aim of this study was to use the recent discovery that acute and chronically exposed CSL generate antibodies against DA (Lefebvre et al., 2012). The archived set of blood samples collected from harbour seals over the last 20 years or so available at SMRU provided an exceptional resource for this purpose. However, I was unable to replicate or validate the method published by Lefebvre et al., (2012) despite the availability of identical samples from CSL used in the paper. The reasons for this are unclear but several trials were carried out, using different samples from phocid seals and most importantly using commercially available dog serum, which would not contain antibodies against DA. In addition to the high absorbance ratios found in samples that should have been negative, given they were from pups or regions without DA suggests some fundamental problem with this approach. False positive results were being obtained, as the method may be detecting proteins other than anti-DA antibodies. However, the results presented by

Lefebvre et al., (2012) were compelling so further investigation are needed before this approach is completely dismissed. For example extraction and isolation of the specific antibodies from the blood samples could be carried out using the DA antibodies produced by Biosense (Norway) using a different format such as a dot-blot or Western blot assays (Pryor, 2001). Although most commercial manufacturers only supply antibodies as part of a kit format, they may be persuaded to supply a small amount for evaluation purposes.

Monoclonal antibodies have been produced to detect DA (Kawatsu et al., 1999), but designing an antigen where DA is bound to a larger carrier molecule would be interesting to carry out and generating monoclonal antibodies that are DA specific and would only bind to a specific epitope on the designed DA antigen. Conducting laboratory animal experiments where an animal is inoculated with the antigen (carrying the specific epitope) and investigations to see if production of antibodies occurs. Further development an assay that is able to detect these antibodies might refine the approach to determine the presence of naturally occurring DA-specific antibodies in the serum of exposed animals.

If DA specific antibodies exist they would most likely bind to any free DA in the blood and make it more difficult to excrete via the kidney, at it would keep circulating in the body. It would be interesting to look for DA conjugates in exposed animals where free and covalently bound (to a carrier molecule) toxin was investigated. Enzymatic proteolysis or immunohistochemistry of tissues in exposed animals could be investigated in order to see if it gives ELISA-detectable DA-containing protein fragments. Another experiment would be to investigate for the presence of DA-specific antibodies using labelled DA (such as a fluorescent tag), which can be detected using a fluorescence polarization immunoassay.

7.5 Vectors for toxin exposure to harbour seals

The vectors for exposure are clearly the seals' prey but it is important to determine which species are the primary vectors, particularly for future risk assessment studies. I was able to collect and analyse fish samples throughout the spring, summer and autumn, which were discards from different fishermen on the east coast of Scotland. I found that all three groups of toxins were detected in fish collected on the east coast. DA and PSP toxins were detected in all three groups of fish (pelagic, benthic and flatfish), while the lipophilic toxins were only detected in one pelagic fish and several flatfishes. Interestingly DTX-2 was only detected in flatfish, suggesting this might be the major vector group.

Pseudo-nitzschia are not motile and they are relatively heavy due to the silica within their cell walls, which can cause the algae to sink to the seabed if the water turbulence is low (Swan and Davidson, 2011). Studies have been carried out to prove that DA may persist long after a *Pseudo-nitzschia* spp. bloom has ended and that DA can act as an important vector in the deep-water food chains (Sekula-Wood et al., 2009). Further evidence of DA contamination to benthic organisms are proven by Kvitek et al., (2008) where they discovered DA in four feeding groups; filter feeders, predator, scavengers and deposit feeders.

Harbour seals mainly forage on benthic prey in certain areas, within 20-45km of the coast (Thompson et al., 1994; Tollit and Thompson, 1996; Wilson, 2014). Although toxin exposure in fish was only investigated on the east coast using fish discards collected from the North Sea, it appears that benthic demersal flatfish accumulate the highest amount of toxin. This was also the conclusion of a study of DA in benthic and benthopelagic fish species in Monterey Bay, California, where DA were significantly higher in benthic fish when there were few toxic cells present in the water surface (Vigilant and Silver, 2007).

The results from Chapter 3 shows that fish on the east coast are exposed to a range of toxins and continuing this type of screening/investigation is highly recommended and should be conducted throughout the Northern Isles and the west coast region. Investigation of benthic organisms would be an additional priority to understand the trophic linkages between top predators, fish and the benthic invertebrates.

This dataset has provided the first comprehensive data on concentrations of toxins in fish viscera in from a small area in the UK and has been of interest to the UK Food Standards Agency who are responsible for understanding potential human health risks. Of some note was that the data were not obtained during any HAB event and therefore probably represent the minimum exposure. Despite this, the concentrations were log-normally distributed where some fish had relatively high levels of toxins. These data will therefore be invaluable in a risk assessment model which could be seasonally structured so that model simulations could take account of the fact that toxin exposure may be higher during the summer than at other times of the year.

Further work on exposure through analysis of whole fish rather than just fish viscera as although the viscera contain by far the largest proportion of the toxins, some may be found in the brain of fish (Lefebvre et al., 2002a; Vale and Sampayo, 2001). This would improve the accuracy of the oral exposure levels and dose estimates. In addition to studying fish prey from other regions, improving information about levels of toxins in the most important prey such as sandeels, which were underrepresented in this study would be a importance. Also prey should be collected from the specific harbour seal (and grey seal for comparison) foraging regions rather than from the fishermen, they should be obtained from all regions, particularly the west coast and should be particularly analysed during HAB events. This information would help with estimating more accurately how much toxin the harbour seals could be exposed to in the different regions in Scotland.

7.6 Estimating the elimination of toxins in harbour seals

The data on the concentrations of toxins in fish provide a minimum estimate for exposure estimation but in order to determine how likely animals are to consume sufficient toxin for it to produce adverse health effects more information about how the animals metabolise and excrete the toxins is required and particularly the time course for excretion. Harbour seals feed in bouts so they may not consume sufficient toxic prey in one bout to exceed the effect threshold. However, if the feeding bouts were sufficiently close together, the effect of repeated oral exposure in relation to excretion rate and whether animals reach steady state would increase. The experiment I carried out in captive animals using iohexol as a marker for DA excretion estimation found that the elimination half-life is approximately 3.5 hours, and the concentration peak occurred at around 4 after ingestion. All the DA is therefore likely to be eliminated by 10-12 hours.

However, in order to complete the pharmacokinetic model, further data are needed by studying the plasma iohexol time course following an intravenous dose and by studying the iohexol concentration in urine samples. Additional information about the urination rate and if the bladder empties entirely every time urination occurs (in harbour seals) would also improve the accuracy of the elimination estimates. This additional data would enable me to complete the clearance calculation. This information would be used to construct a more realistic two compartmental pharmacokinetic model. It would also be interesting to investigate if the same clearance pattern occurs in CSL that have very high exposures during large bloom events and are known to experience morbidity and mortality from DA exposure.

Investigation of a biomarker for PSP toxins and the lipophilic toxins is also required to understand the clearance of these toxins. In addition experiments to then understand the clearance rate of multiple toxins combined would be important to investigate. It is possible that these toxins interact with each other and this could affect the clearance, peak concentration and half-life of each one.

7.7 Risk assessment studies

The studies in this thesis have investigated the exposure, uptake, effect and excretion of toxins in harbour seals and compared the results to levels in other marine mammals found stranded in UK waters. The results will be critical in the development of appropriate risk assessment models. For example, data on the levels of toxins in individual harbour seals could be combined with the data collected on the individuals' movements.

Some of the animals studied were also equipped with SMRU GPS/GSM tags and when the data are available, it could be used to establish the individual animals' fine scale foraging pattern (assuming its future behaviour is comparable to its previous behaviour), region of exposure and the temporal and spatial factors that dictate the concentrations found in the excreta when the animals were captured at the haulout site. This individual based approach could then be combined into a specific individual based population model describing the impact that the toxins may have on the population abundance. Simulations would take into account the type and amount of prey consumed (based on recent investigations into the diet of harbour seals (Wilson, 2014)) and the dose of toxin assimilated by a number of individuals in a population, in an approach similar to that used for the CSL (Bejarano et al., 2007).

Combined with dose-response data derived from what is known about lethal levels for each toxin in turn (and in combination) from the literature on animals and humans (Andrinolo et al., 2002; Negri et al., 1995; Schantz et al., 1975), the effect that this exposure would have on the probability of survival for each individual in the population would be estimated. When the number of individuals remaining in the population at the end of each year is combined, the effect on the population abundance and growth rate would be assessed and compared with the observed population trends.

This study has provided much of the empirical data needed for assessing the risks to the harbour seals in Scotland from the potentially lethal effects of

toxins from harmful algae. I have shown that DA and PSP toxins in particular could be likely factors involved in the decline, where further investigation is needed. Combining the results from the individual studies, specifically on multiple exposure, concentrations in prey and elimination rates into a risk assessment framework will be the next step in determining the true probability that they are one of the major drivers. Nevertheless, I have also shown that positive relationships between specific white blood cells and concentrations of DA in the urine and faeces may suggest sub-lethal health effects that, combined with other factors such as infectious disease or nutritional stress, may cause mortality as an indirect effect of toxin uptake.

7.8 Method development

In the recent years there has been discussion on the validity of using urine as a monitoring matrix for quantifying DA because of the salt content which is a result of the mechanism for regulating salt balance (Seubert et al., 2014), and comparisons with different analytical techniques should interpreted with care. Further investigations are needed in sample preparation and development of a method that can reliably measure DA in urine samples. Similarly, for the faecal samples it is difficult to draw conclusions from comparing the results from one study to another due to differences in equipment, analytical materials, and technical capability. Limit of detection and quantification are needed in these types of studies and it is important to communicate this in research papers.

A problem with studying multiple toxins is the amount of sample that are needed for extracting the toxins. Methods that can reduce the amount of sample needed to extract the different toxins are required, this way monitoring of multiple toxins could be more readily conducted. Preferably a method that does not rely on mass spectrometry as these instruments are usually very expensive and the method requires large samples due to the long clean-up processes. The ASP ELISA method developed by Biosense is an excellent

low cost method that can easily be applied in basic laboratories which was well correlated with the results obtained by UHPLC/MS-MS.

7.9 Conclusions

It is not possible at present to conclude that the results of my thesis can for certain explain any of the current regional harbour seal declines, as I am missing perhaps the most compelling evidence of dead stranded harbour seals which died of toxicosis. However, there are several interesting conclusions that can be drawn from the current results. Choice of prey, which could be driven by regional or seasonal changes in availability, in the harbour seals habitat will play an important role in determining exposure level. When comparing the levels across the years DA monitoring there has been a general decrease (i.e. since 2008). It could be that the toxin investigations began only after a peak in environmental levels and that we are now seeing a general decline. However, continued surveillance of levels in predators and prey will indicate if this general pattern continues.

This is the first evidence that harbour seals are exposed to DA, PSP toxins and lipophilic toxins and thus the first time harbour seals are reported taking up three different toxin groups simultaneously, which in turn makes toxin exposure investigation in this population more complex. Lymphocytopenia and monocytosis are associated with exposure to DA, but the individual and population level effects of these changes are not yet well understood and certainly warrant further investigation.

Evidence of trophic transfer of toxins on the east coast largely appear to be driven by flatfish, as they accumulate higher concentration of all three groups of toxins. Evidence of toxin exposure to other fish eating predators was found with DA concentrations being higher among the inshore than the offshore feeders. Future close inter-disciplinary collaborations between algologists, chemists, ecologists and epidemiologists are needed to achieve a better

understanding of the dynamics and distribution of these toxin groups and their potential health effects in marine mammals and fish around Scotland.

Presentations and publications arising from this thesis

Presentations

Dec. 11 **The 19th biannual conference for the International Society for Marine Mammalogy. Tampa, Florida.** Detection of anti-Brucella spp. antibodies in Weddell seal, Antarctic fur seal and Southern Elephant seal. **S-K Jensen**, Nymo I.H, Forcada J, Hall A, Godfroid J.

Feb. 12 **UK Student Chapter for Marine Mammalogy, St. Andrews, UK.** Detection of anti-Brucella spp. antibodies in Weddell seal, Antarctic fur seal and Southern Elephant seal

Jan.13 **UK Student Chapter for Marine Mammalogy, Oban, UK.** Exposure of Scottish harbour seals (*Phoca vitulina*) to biotoxin from harmful algae in Scotland

Oct.13 **Guest speaker at Glasgow Pathology, Glasgow.** Title: Marine mammals from the Arctic to the Antarctic with a stop in Scotland.

Dec. 13 **Biannual conference on biology of marine mammals, Dunedun, NZ.** Title: Is toxin from harmful algae the cause of the Scottish harbour seal decline?

Andrew Brownlow, Johanna Bailey, Mark Dagleish, Rob Deaville, Geoff Foster, **Silje-Kirsten Jensen**, Ailsa Hall, Eva Krupp, Robin Law, Barbara Moriarty-Pearson, Rod Penrose, Paul Jepson. *Pilot error? Assessing the role of disease in a pilot whale mass stranding event.* Poster Presentation **Centenary celebration conference for the Natural History Museum's celebration of: A Century of Strandings 20/09/13.**

Scientific publications arising from this thesis

Detection and effects of harmful algal toxins in Scottish harbour seals and potential links to population decline. Silje-Kristin Jensen, Jean-Pierre Lacaze, Guillaume Hermann, Joanna Kershaw, Andrew Brownlow, Andrew Turner, Ailsa Hall (doi:10.1016/j.toxicon.2015.02.002). Toxicon.

Investigation into the long-finned pilot whale mass stranding event, Pittenweem, Fife, 2nd September 2012. Report to Marine Scotland November 2013. Andrew Brownlow, Johanna Baily, Mark Dagleish, Nick Davison, Rob Deaville, Geoff Foster, **Silje-Kirstin Jensen**, Paul Jepson, Eva Krupp, Robin Law, Barry McGovern, Maria Morell, Rod Penrose, Matt Perkins, Fiona Read

Investigation into the long-finned pilot whale mass stranding event, Kyle of Durness, 22nd July 2011. Report to Marine Scotland October 2012. Andrew Brownlow, Johanna Baily, Mark Dagleish, Rob Deaville, Geoff Foster, **Silje-Kirstin Jensen**, Eva Krupp, Robin Law, Rod Penrose, Matt Perkins, Paul Jepson

Non-scientific publications arising from his thesis

Shellfish poisoning in Scottish harbour seals (2014). Article for the mammal society, Mammal News Spring edition. Page: 20-21.

Who's poisoning my food? (2014). Article for the Orkney Bulletin, Autumn 2014

Scientific publications published during thesis

West Greenland harbour porpoises assayed for antibodies against Toxoplasma gondii: false positives with the direct agglutination method. (2014). M-A Blanchet, J Godfroid, E M Breines, M-P Heide-Jørgensen, N H Nielsen, I Hasselmeier, M Iversen, **S-K Jensen**, K Åsbakk. Vol. 108: 181–186, 2014. doi: 10.3354/dao02715

***Brucella* antibody seroprevalence in Antarctic fur seal (*Arctocephalus gazella*), Weddell seal (*Leptonychotes weddellii*) and southern elephant seal (*Mirounga leonina*).** (2013). **S-K Jensen**, I.H. Nymo. J. Forcada, A. Hall and J. Godfroid. Diseases of aquatic organisms. Vol. 105: 175–181. *doi: 10.3354/dao02633*

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