FSA Research Project S14017:

Pilot study on the incidence of algal toxins (ASP, DSP and PSP) in Scottish Brown Crab (*Cancer pagurus*).

FINAL REPORT

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1 EXECUTIVE SUMMARY

The Brown Crab, *Cancer pagurus* is a significant fishery in Scotland, worth approximately £10M per annum. Consumption of brown crab in Norway recently resulted in a mass poisoning through the presence of diarrhetic shellfish toxins in the crab tissue that originated from marine algae toxins. Algal biotoxins including DSP are regularly detected in bivalve molluscs harvested in Scotland, but very little is known about their prevalence in crabs fished in Scottish waters. The poisoning incident in Norway prompted the Food Standards Agency Scotland (FSAS) to commission a pilot study to investigate the potential for crabs from Scottish waters to act as a vector for algal biotoxins.

The study consisted of three main components. Firstly, the crab industry in Scotland was mapped, primarily through analyses of landing data. It was determined that the nominal fishing season coincides with the biotoxin season – both being at their height in the British summer and autumn months. Over 95% of crab landings occur in only 12 ports in Scotland and these are predominantly in the West and far North-East of Scotland (including Orkney). The areas where crabs are fished also tend to coincide with areas of high biotoxin production.

Secondly, to ascertain the presence of toxin-contaminated crabs in the wild, field samples were taken during 2005 and 2006. During 2005, targeted sampling of crabs was conducted in areas that had a history of toxic events and further reactive sampling was undertaken from areas where the FSAS monitoring programme detected toxins in bivalves. The results showed that both PSP and ASP toxins were commonly detected in wild crabs (crabs with detectable PSP levels: 83% and detectable ASP levels: 35%). Within an individual sampling area, crabs sampled at the same time were highly variable with regard to the concentrations of toxins present. This was particularly true of ASP toxins. Few crabs (<5%) were found over the regulatory limit set for bivalves for either ASP or PSP toxins, however one crab was found to contain six times the regulatory limit for ASP and another over twice the regulatory limit for PSP. Despite the occurrence of a small number of toxic events during 2005 involving DSP in bivalve molluscs, DSP toxins were not detected in any of the crabs sampled during 2005.

During 2006, crabs were randomly sourced from three separate, but important, fishing areas and tested for ASP and PSP toxins. In addition, crabs were also sampled from one area where DSP toxins were detected in bivalve molluscs through the FSAS biotoxin monitoring programme. The results for 2006 were similar to 2005, in that ASP and PSP toxins were regularly detected in the crabs. Again, both ASP and PSP toxins tended to be detected at low concentrations, although high levels were occasionally detected in individual crabs. DSP toxins were also detected in crabs sampled during 2006, although at low levels.

Thirdly, laboratory feeding studies were conducted to investigate uptake of ASP and DSP toxins by crabs. These experiments were also intended to provide toxic material for the validation of testing methods used throughout the study. Although limited, these experiments demonstrated accumulation of both ASP and DSP toxins in crabs fed exclusively on toxic shellfish. One feeding experiment also provided some evidence of reduced feeding if the food source contained particularly high levels of ASP toxins.

The results of this pilot study suggested that Scottish Brown crabs could potentially be a vector for ASP, DSP and PSP when conditions are suitable. Suggestions for further studies and possible actions to help prevent the risk of contaminated crabs being placed on the market are also provided in this report.

2 INTRODUCTION

A number of species of marine micro-algae can produce biotoxins. These are usually dinoflagellates, with species of *Alexandrium* and *Dinophysis* being the most common toxin-producing dinoflagellates in Scottish waters, although species of the diatom *Pseudonitzschia* are also biotoxin producers (Smayda 2006). These toxins can accumulate in the tissues of feeding bivalve molluscs, which therefore present a significant risk to human health when consumed. The most familiar toxins that cause human intoxication have been divided into three main classes, named after the symptoms they elicit, *i.e.* diarrhetic, amnesic and paralytic shellfish poisons (DSP, ASP and PSP respectively). While the occurrence of these toxins in bivalves is well recognised and legal statutes defining maximum permissible levels are in place, it is only in recent years that other marine food-web transfer mechanisms have been identified. There is clearly a growing need to identify these potential routes and to quantify potential exposure risks. This will enable realistic human health risk assessments to be made so that mechanisms to protect consumers can be implemented where necessary.

The Food Standards Agency Scotland (FSAS) has a statutory responsibility under EU legislation (Regulation (EC) 854/2004) to monitor shellfish from classified production areas for the presence of algal toxins in Scotland. The statutory monitoring of ASP, DSP and PSP toxin groups in shellfish focuses almost exclusively on bivalve molluscs such as mussels, scallops and oysters. However, the legislation also covers a range of other invertebrates including ascidians (sea squirts) marine gastropods (snails) and echinoderms (sea urchins, sea cucumbers *etc.*). The Regulation also requires FSAS to periodically monitor shellfish harvesting waters for the presence of potentially toxic phytoplankton. There is currently no statutory responsibility for FSAS to monitor edible crustaceans (crabs, prawns and lobsters) and responsibility for ensuring that these are free from toxins rests with food business operators. Nevertheless, there are interactions between the bivalve and phytoplankton monitoring programmes and crustacean fisheries in Scotland. Currently, when levels of PSP toxins in excess of 150 μg/100g are detected in bivalve molluscs, FSAS can request samples of crustaceans to be taken for toxin testing. Discussions are then entered into with the Local Food Authorities to ascertain the need to take local action to prevent harvesting, trace product and warn local industry and consumers while the PSP toxin levels remain high. This system is based on historical practice in Scotland, and informed by limited data on the prevalence of algal biotoxins in edible crustacea harvested from Scottish waters. No similar guidance currently exists for ASP and DSP toxins.

Although bivalve shellfish have been the usual route for human poisonings relating to algal toxins in the UK, there are frequent reports from other parts of the world of humans being poisoned through other vectors, particularly fish and crabs (Ahmed *et al*. 2001; Llewellyn *et al.* 2002). Recently, there was a serious outbreak of DSP in Norway caused by contaminated Brown Crabs, *Cancer pagurus* (Castberg *et al*. 2004). It was believed that these crabs had probably been feeding on mussels that had ingested quantities of highly toxic dinoflagellates. They found DSP concentrations of between 1 and 1.5 mg kg^{-1} in the brown meat (hepatopancreas) of the crabs. *Cancer pagurus* is a major target of creel fisheries in the UK and is sold in various forms including “Cromer” crab where the viscera are used along with white meat.

The detection of potentially hazardous levels of PSP toxins and tetrodotxin (TTX) was documented in several species of crabs in Japan and Taiwan by Tsai *et al*. (1995, 1996, 1997). These authors identified the major toxic component in the crabs as being TTX, although gonyautoxins 1-4 and also saxitoxin and neosaxitoxin (PSP analogues)

were found to comprise between 10 and 60% of the total toxin load. More recently, Oikawa *et al*. (2004) highlighted a temporal and spatial link between PSP toxins in the dinoflagellate *Alexandium tamarense*, two shore crab species, *Telmessus acutidens* and *Charybdis japonica,* and their prey mussel, *Mytilus galloprovincialis.* They found that the toxin content of the crabs approached levels detected in the mussels, and that toxicity continued to occur in the crabs after it had been lost from the mussels. They also showed that there was a very large inter-individual variability in the toxicity of the crabs, with up to 30% of the animals found not to be toxic, while others contained up to 18 mouse units per gramme (mu/g) of PSP toxins.

Following this, these workers investigated the biotransformation and depuration of PSP toxins from *T. acutidens* fed mussels in the laboratory (Oikawa *et al*. 2005). This work suggested that the hepatopancreas of the crab was up to 3-fold more toxic than the mussels they were fed, demonstrating the potential for the biomagnification of PSPs from mussels and that the toxicity of the crab was proportional to the number of mussels consumed. There were also significant differences in the toxin profiles between the crab and mussel samples. The most significant of these differences with respect to risks to human health was the occurrence of saxitoxin and neosaxitoxin in the crab, but not in the mussel tissue. This was attributed to the transformation of toxins through reductive cleavage of the *O*-sulfate group of the less toxic GTXs 1/4 and 2/3 respectively. This work also showed a bi-phasic clearance of the toxins in crabs, with 50% of the toxicity being lost in the first 5 days followed by a slower phase where the toxicity was reduced to around 15% of the initial toxicity after 20 days.

Jiang *et al.* (2006) conducted feeding experiments to document the transfer and transformations of PSP toxins from the scallop *Chlamys nobilis* to the spiny lobster *Panulirus stimpsoni,* during toxin uptake and subsequent depuration. Their results showed that only the lobster's hepatopancreas contained PSP toxins. The toxin profiles observed in the hepatopancreas during toxin uptake were the same as those detected in *C. nobilis* for the first 6 days, after which N-sulfocarbamoyl toxins, which have a low toxicity, were selectively accumulated. However, during depuration the accumulated N-sulfocarbamoyl toxins were transformed into carbamate toxins, which have a higher toxicity.

Published reports demonstrating the occurrence of domoic acid (the cause of ASP) in crustacea are fewer than those for PSP toxins (Wekell et al. 1994; Ferdin *et al*., 2002; Altwein *et al.,* 1995; Vale and Sampayo, 2002), although there have been reports of domoic acid in higher animals, *e.g.* birds, fish and marine mammals (Altwein *et al*., 1995, Work *et al*., 1993, Lefebvre *et al*., 1999, Scholin *et al*., 2000). Ferdin *et al*. (2002) found levels of up to 13.4 μg g^{-1} domoic acid (DA) in sand crabs (*Emerita analoga*). This coincided with a bloom of DA-producing diatoms. DA levels in the crabs appeared to drop rapidly to below the limit of detection over approximately 3 weeks following the drop in numbers of producer cells. Vale and Sampayo (2002), found concentrations of DA up to 5.7 mg kg^{-1} in Green Crabs (*Carcinus maenas*) in Portugal.

Limited information is available regarding uptake and levels of DSP toxins in crustacea (Shummway, 1995; Vale and Sampayo, 2002; Castberg *et al*., 2004). Vale and Sampayo (2002) described an outbreak of DSP in Portugal and demonstrated the occurrence of okadaic acid and its esters in both razor clams and green crabs (*C. maenas*). They managed to analyse the remains of a meal of cooked crabs eaten by a person who subsequently became ill with DSP. The majority of okadaic acid in the mussels was in the free form, while that found in all crab samples typically comprised over 90% okadaic acid esters.

These studies show that crabs are a potential risk to humans in relation to algal toxins. From a Scottish perspective, the occurrence of an outbreak of DSP after consumption of *C. pagurus* is of particular interest, given the importance of this species in Scottish fisheries. The occurrence of DSP or ASP toxins in *C. pagurus* harvested from Scottish waters has not been documented. However, there have been previous closures of crab fisheries in the north of Scotland due to high levels of PSP toxins being found in crabs and other crustacea (Wyatt and Saborido-Rey, 1993; Tett and Edwards, 2002).

2.1 Report Objectives

The Norwegian outbreak of DSP in *C. pagurus* prompted FSA Scotland (FSAS) to fund a pilot study into the incidence of algal toxins in Scottish crabs. The purpose of this study was to provide FSA Scotland with data on the incidence of algal toxins in *C. pagurus* in Scottish waters during the 2005 and 2006 bivalve monitoring seasons to better inform the Agency of the likely risk posed by algal toxins in crabs.

The aims of this study were to obtain information on the Scottish crab fishing industry, and to obtain and analyse Brown crabs from field samples during 2005 and 2006 for the presence of algal toxins. The study also aimed to provide the FSAS with information on spatial and temporal variability of toxins in Brown crabs and, if possible, information on the uptake of toxins by *C. pagurus*.

3 MAPPING OF THE SCOTTISH CRAB FISHERY

3.1 Introduction and Data Overview

Unlike bivalve mollusc aquaculture and fisheries, crab fisheries are not routinely monitored within Scotland for toxins. The crab fishery in Scotland annually lands 8,000-10,000 tonnes of crabs (Scottish Fisheries Statistics 2004, table 20) at a value of around £10M. This compares to a total UK landing of 25,000 tonnes for all crab species and a UK figure of almost 30,000 tonnes of *Nephrops norvegicus* (langoustines). Scottish crab landings thus constitute a significant component of the UK crustacean industry.

There is limited published information on where crabs are fished in Scotland, when they are fished and how many are fished within each area. However, from the data that is available landings appear to be concentrated in ports located in the North of Scotland. Of the 6987 tonnes of crab species landed in 2004 in Scotland, 35.7% were landed in Wick, 17.6% landed in Orkney and 14.2% in all other monitored ports (Scottish Fisheries Statistics 2004, table 23). The remaining 32% is landed elsewhere in Scotland, distributed across numerous small ports. Unfortunately Brown crabs have often been categorised only as “crabs” or “other shellfish” with no recording of the actual species harvested. This broad analysis creates a degree of uncertainty in some of the available data relating to landings of Brown crabs in Scotland.

Due to the uncertainty of the actual location and size of landings of Brown crabs in Scotland, it was necessary to undertake a mapping of the Scottish crab fishing industry. This was to establish the current state of the crab fishery in Scotland and to determine if there was a crab fishing season that could coincide with the occurrence of

toxic phytoplankton blooms or the subsequent toxification of shellfish. These data would also allow the locations and sizes of landings to be more accurately determined, providing spatial as well as temporal information relating to the crab industry in Scotland.

3.2 Survey analysis

Various sources were consulted as part of the survey. The Seafish Industry Authority lists 19 companies involved in brown crab processing. The Scottish Seafood Industry Key Facts and Statistics (2004) gives a short description of Brown Crab biology, fishing gear used, fishing methods, regulations governing size and condition of crabs when landed for sale, the handling, storage and transport of live crabs, killing, boiling and extraction of the meat, and freezing, canning, hygiene in crab processing plants and nutritive value of crab meat. Industry sources showed that Scottish crabs are sold whole and as processed products. These include detached claws, white meat and brown meat products. The white meat is claw and leg meat whereas brown meat is the contents of the carapace, other than the gills, and includes the digestive tissues. The brown meat can be used either on its own or mixed with white meat as the basis of a wide range of crab products, such as pastes and pates. Research carried out by Seafood Scotland, and presented in their report Seafish (2005), indicates an expected increase in the consumption of Brown Crab products, especially in the processed form.

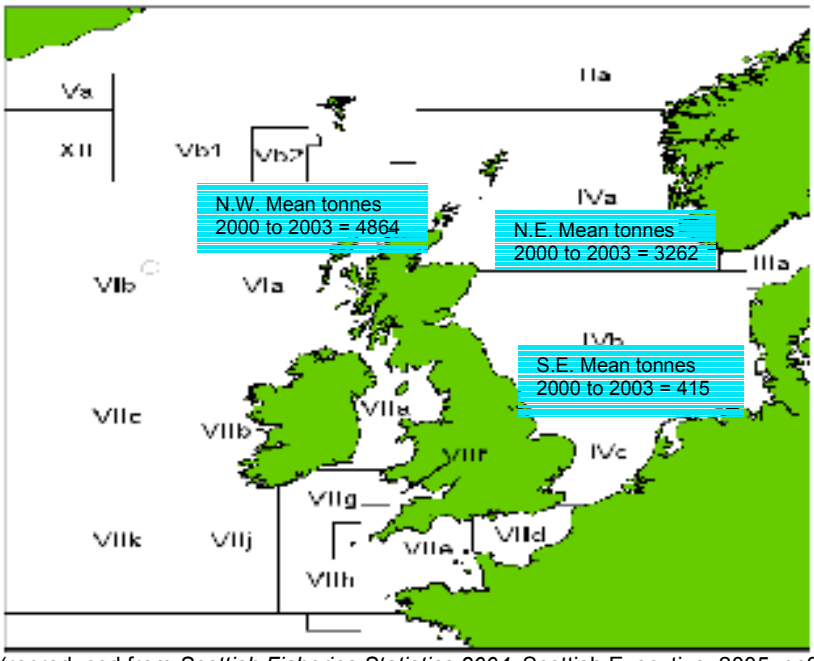
The major part of the survey was the analysis of the crab fisheries data for Scotland. The data for the spatial and temporal mapping of the Scottish crab fishery as well as tonnage landed was requested and supplied by the Scottish Government Statistics Department (Appendix 1). This comprised all declared Brown Crab landings from January 2000 to August 2004. The data for each landing included the date landed, the area fished, the area landed, the fishing vessels licence (over or under 10 meter), the fishing gear used, the live weight landed, its value and a full breakdown of the data type and parameters.

3.2.1 Spatial mapping of the Crab fishing Industry in Scotland

Survey data was analysed to attempt to map the extent of crab fishing around Scotland and the location of crab landings for all landings between 2000 and 2004.

3.2.1.1 Fishing Grounds

The waters around Scotland and the UK are divided into ICES fishing grounds, which are subsequently further subdivided into 100 rectangles. There are four main fishing grounds surrounding Scotland: VIa, VIIa, IVa and IVb. The ground VIIa had only one landing between January 2000 and August 2004 and so was not included in any analyses of the fishing grounds carried out for this report. The mean annual tonnage fished from the three most significant fishing areas (IVa, VIa and IVb) is shown in Figure 1. The annual numbers of landings of crabs from the four main fishing grounds at ports around Scotland are shown in Table 1. The South East (IVb) can be seen to be the least productive area of the three main fishing grounds.



(reproduced from *Scottish Fisheries Statistics 2004*, Scottish Executive, 2005, pp31)

Figure 1: The 4 ICES fishing grounds of the Scottish coast (VIa, VIIa, IVa and IVb) and the annual tonnage of Brown crabs caught in the 3 main fishing grounds (VIa, IVa and IVb).

Table 1: Breakdown of the number of all Brown crab landings from ICES fishing grounds around the UK.

Fishing Ground	2000	2001	2002	2003	2004*
IVb (S.E.)	326	251	299	313	238
IVa (N.E.)	798	767	712	639	407
VIa (N.W.)	861	928	793	891	544
VIIa (S.W.)	-	-	-	-	1

*only 9 months of data were available for 2004

The North East (IVa) includes Orkney and Shetland and produces a large amount of crabs annually. It is reasonable to assume that some crabs taken from this area are landed outside Scotland though it is not known how much. The North West (VIa) constitutes the largest Scottish fishing area.

3.2.1.2 Landing Ports

Between January 2000 and August 2004, Brown crab was landed at a total of 131 different ports. To allow all landing ports to be individually identified, a map of Scotland was divided into seven areas (Figure 2).

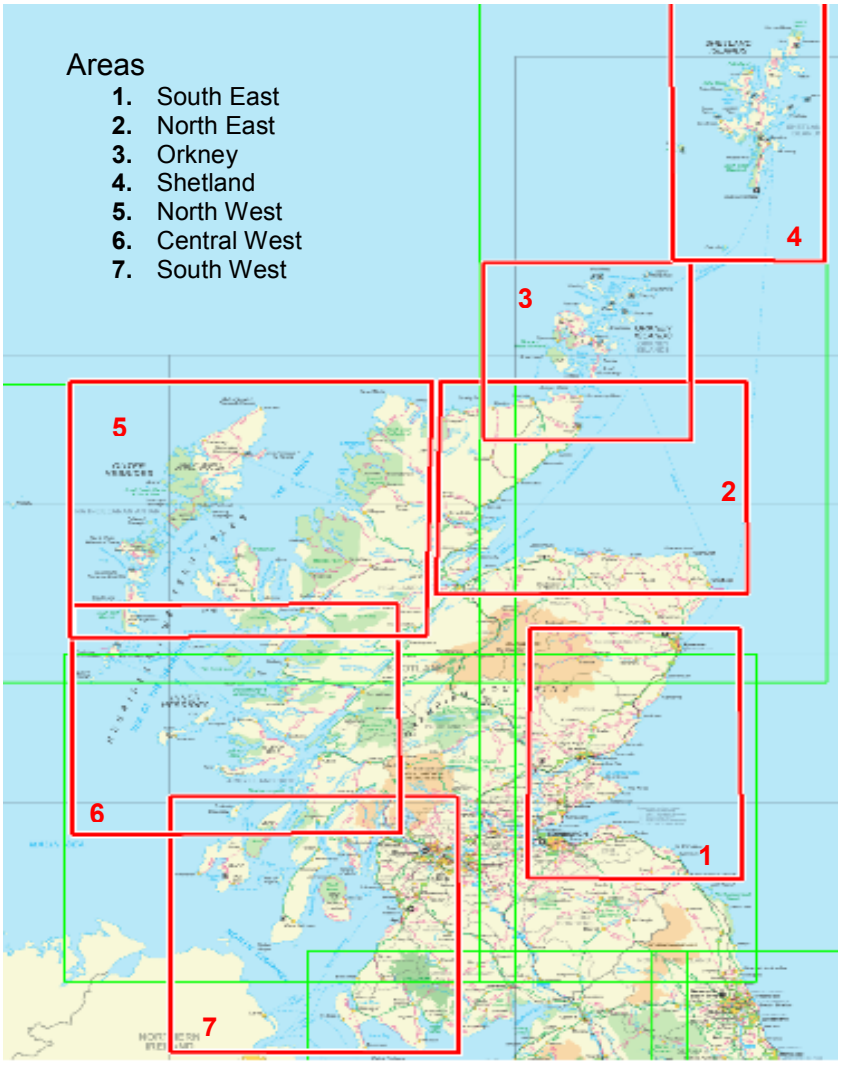


Figure 2: Map showing the geographical divisions of Scotland used in the report

The ports in each area were identified on a map and then labelled and colour coded to indicate the mean number of landings each year for the period between 2000 and 2004. Annual landings of crabs in the South East of Scotland (Area 1, Figure 3) were relatively small during this period, with less than 29 per year. Area 3 (see Figure 4), the Orkney Islands, had a high concentration of landings, indicating that within this area crab fishing is a much more significant fishery than Area 1. The North East of Scotland (Area 2, Figure 5) included a wide range of landing areas, including three of the busiest ports, Scrabster, Wick and Fraserburgh. In contrast, relatively few landings were recorded in the Shetland Islands during the same period (Area 4, Figure 6). Similarly, the South West (Area 7, Figure 7) did not have a large crab fishing industry between 2000 and 2004. The Central West area (Area 6, Figure 8) included a wide geographical distribution of landings. A large number of crabs were landed in the North West (Area 5, Figure 9) annually during 2000-2004. It should be noted that for the North West, some landing areas were often not fully clarified and occasionally overlap. This may affect the annual landing figures. For example, several areas, which have only occasionally landings on an individual basis, may be grouped together and named as a single port, thereby creating a regular annual landing. Certain of the larger ports *e.g.* Oban regularly trade with French and Spanish exporters.

Figure 3: The South East of Scotland.

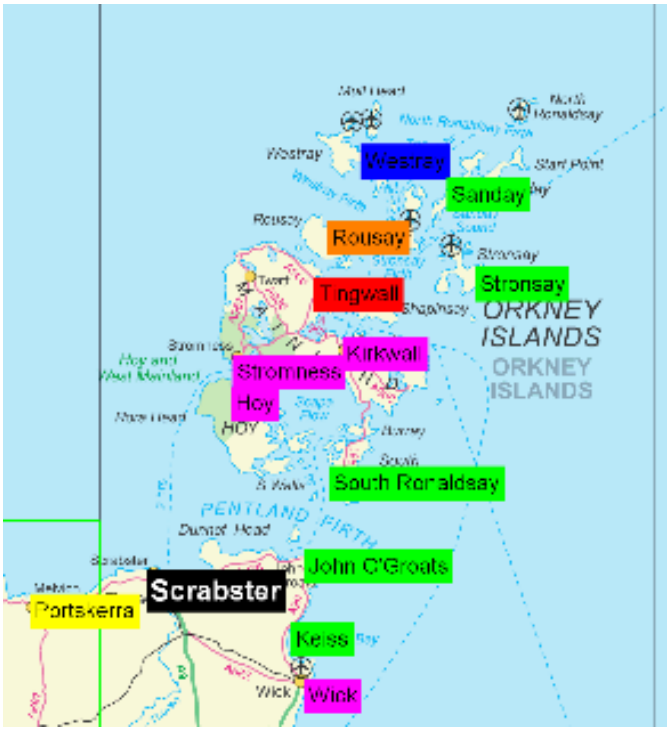


Figure 4: The Orkney Islands.

Figure 5: The North East of Scotland.

Map colour codes

- Yellow → not each year
- Orange → 1 to 15 /yr
- Green → 16 to 29 /yr
- Purple → 30 to 40 /yr
- Red → 41 to 50 /yr
- Blue → 51 to 140 /yr
- Black → >141 /yr

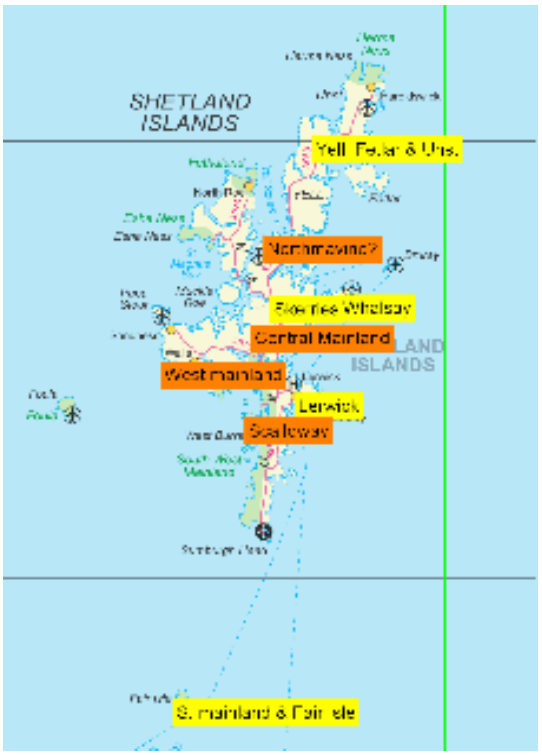


Figure 6: The Shetland Islands.

Figure 7: The South West.

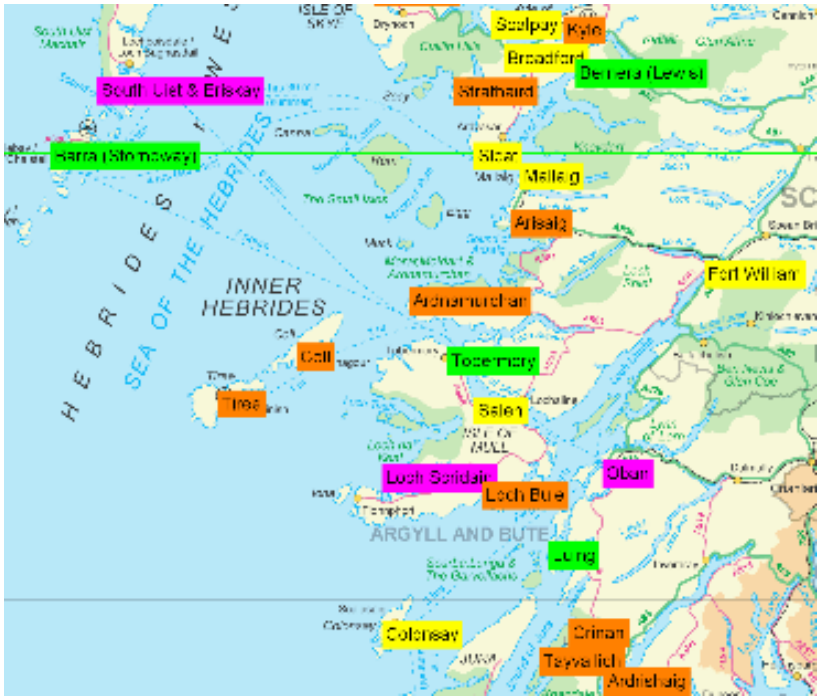
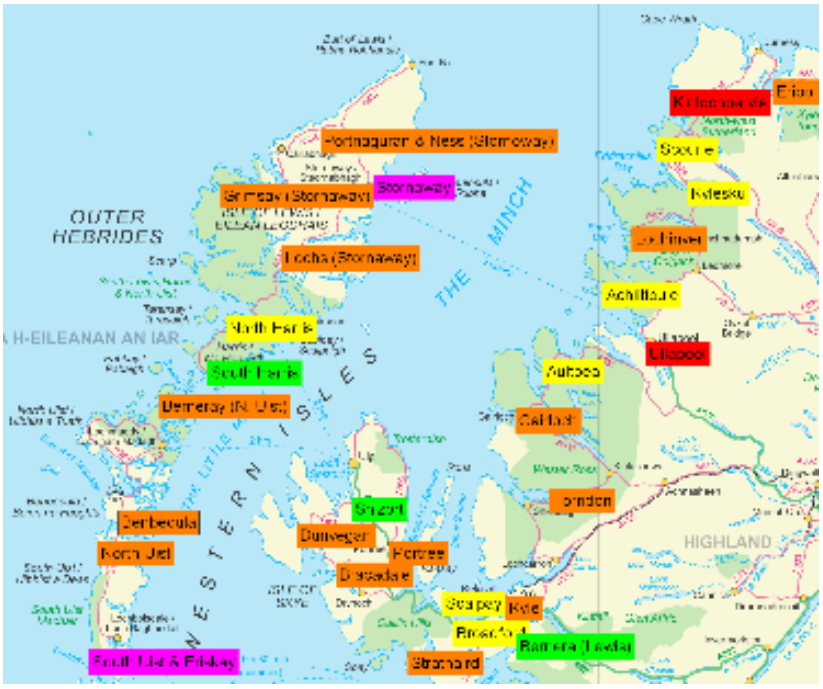


Figure 8: The Central West.

Map colour codes

- Yellow → not each year
- Orange → 1 to 15 /yr
- Green → 16 to 29 /yr
- Purple → 30 to 40 /yr
- Red → 41 to 50 /yr
- Blue → 51 to 140 /yr
- Black → >141 /yr



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- Red → 41 to 50 /yr
- Blue → 51 to 140 /yr
- Black → >141 /yr

Figure 9: The North West.

3.2.2 Analysis of landings

In 2004 there was a total of 7594 tonnes of crab landed in Scotland across the 131 ports already identified, with an estimated value of just over £8 million. The tonnage and value of crab landed across the ports for 2000 to 2003 are shown in Table 2.

Table 2: Total weight of crabs landed and total value per annum for all ports 2000-2003

	2000	2001	2002	2003
Total weight landed (tonnes)	9652	8578	7916	7840
Total Value (£)	9,743,460	9,068,746	8,662,790	8,612,458

The distribution of annual total number of landings for each of the 131 ports that recorded landings of brown crab between 2000-2004 are expressed in box plot form in Figure 10a and Figure 10b. These plots highlight that, while median landing tonnage was less than 20 tonnes per year (Figure 10a) there were some ports with weight of landings in excess of 500t per year (Figure 10b).

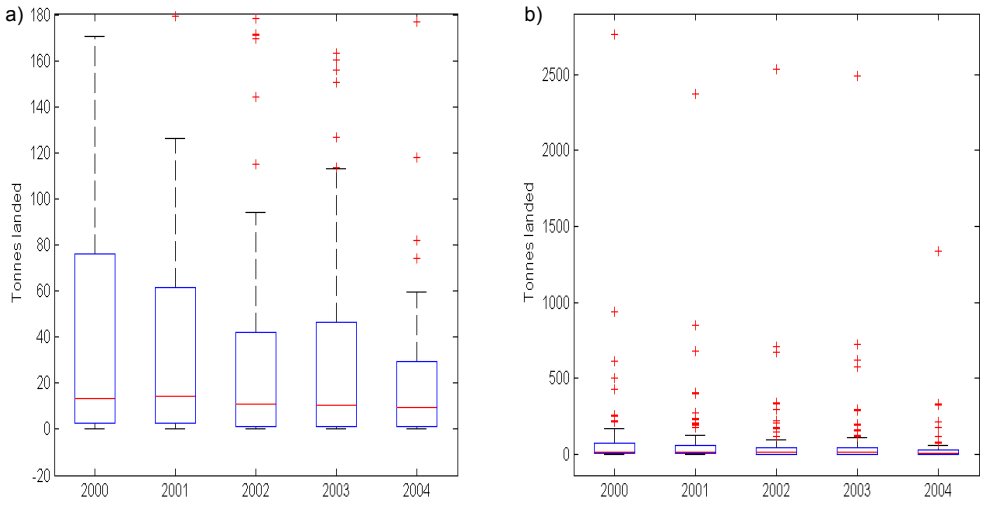


Figure 10: Boxplot showing a) the median number and b) full distribution of annual total number of landings for each of the 131 ports.

To ease analysis, the 131 ports were arbitrarily divided into groups according to mean annual landing numbers for 2000-2004 (Table 3). It should be noted that for 2004 only 9 months of crab landing data were reported which affects the overall mean values. Table 3 indicates that the majority of ports have fewer than 30 landings per year, the average being 24 ports that had no landings per year. However these ports were not always the same and only 78 of the total ports actually had one or more landings every year between 2000-2004. From the 131 Scottish ports, twelve had 30 or more landings (Appendix 1), which account for over 95% of the total landings as shown in Table 4.

Table 3: The total mean and annual landings for all 131 ports.

	Number of ports per annum					Number of ports 2000-2004	
Range of landings	**2000**	**2001**	**2002**	**2003**	**2004**	**Mean**	**%**
No landings	23	22	20	24	31	24	18.3
1 to 15 landings	60	64	72	72	70	67.6	51.6
16 to 30 landings	28	28	26	22	25	25.8	19.7
31 to 40 landings	12	8	6	5	2	6.6	5
41 to 50 landings	4	5	3	2	1	3	2.3
Over 51 landings	4	4	4	6	2	4	3.1

Table 4: A breakdown of the 12 Scottish ports (2000 to 2004), which averaged over 30 annual landings.

Port	Mean Number of Landings	Mean Weight of Landings (Tonnes)	Mean weight per landing (T)	Value
Fraserburgh	37	267	7.2	£276,956
Wick	36	163	4.5	£195,329
Kirkwall	38	78	2.1	£83,699
Stromness	37	149	3.9	£137,325
South Uist & Eriskay	36	96	2.6	£88,147
Loch Scridain (Mull)	30	611	20.3	£541,556
Oban	30	177	5.9	£198,967
Tingwall	45	259	5.7	£239,234
Ullapool	41	684	16.68	£795,474
Kinlochbervie	44	224	5.5	£241,449
Westray	52	392	7.54	£342,228
Scrabster	144	2301	15.98	£2,768,789

From Table 4 it can be seen that Scrabster is the dominant port for crab landings and that Ullapool and Loch Scridian also represent valuable crab ports. Although Loch Scridian has one of the lowest numbers of landings per year, the weight per landing, and as a result the value of those landings, indicates that it is an important port for crab landings. Although the Government Statistics (Appendix 1) indicate that vessels less than 10m in length numerically dominate the crab fishing fleet, only approximately a quarter of the annual total tonnage, for all Scotland, is actually landed by vessels under 10 m.

3.2.3 Temporal mapping of landings in Scotland

The FSA Scotland monitoring program has documented evidence of the seasonality of toxin outbreaks of marketable bivalve molluscs (Holtrop & Horgan 2005). For the present study, survey data was analyzed to establish whether crab fishing was seasonal, and, if so, whether this coincided with the occurrence of biotoxins in bivalve molluscs.

Figure 11 shows a clear seasonality for crab landings in Scotland. The pattern of increasing catches in the Spring, rising to a peak in Autumn is very similar to the peak periods for toxicity as found by the FSA Scotland monitoring programme (Holtrop & Horgan 2005). In Scotland, all three groups of toxins tend to be detected in bivalve molluscs between April and November, with peaks generally occurring in late Summer or early Autumn. The landing values shown are a monthly percentage of the annual total for that year to allow comparisons between the different years.

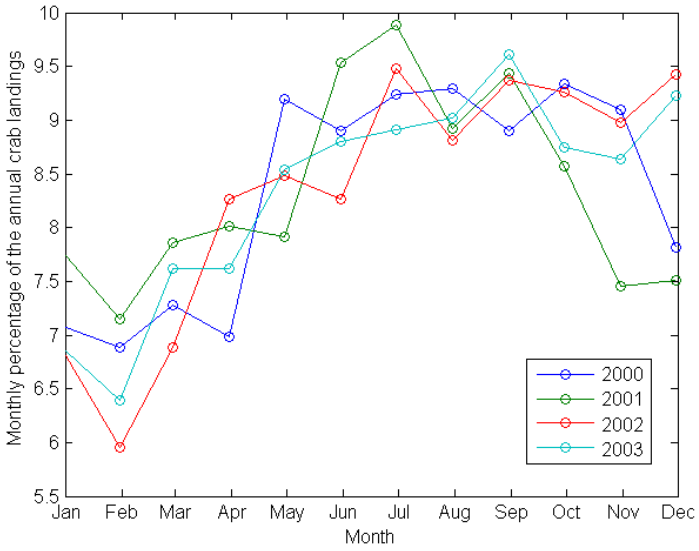


Figure 11: Total monthly landings for the years 2000 to 2003 normalized with respect to the annual tonnage landed.

3.3 Survey Discussion and Conclusions

The Scottish crab industry is predominantly a creel based fishery. Fishing effort is spread over the whole coastline and 131 different ports were officially used by vessels landing crabs between 2000 and 2004. The bulk of the fleet are vessels less than 10m in length. However, when the landing statistics are studied in more detail, it is clear that the bulk of the crabs caught in Scottish waters are fished by a relatively few large vessels, and are landed at fewer than 15 ports.

The North-East and West Coasts are the most significant fishing areas, with a particular concentration of fishing on the North-East Coast and Orkney. Scottish bivalve aquaculture is based mostly on the West Coast and in Shetland. Monitoring data from other areas tend to come from sporadic testing of “wild” sites, *i.e.* classified areas that are fished for non-aquaculture species such as cockles and razors. Thus, while there is good monitoring data available to warn the crab industry on the West Coast and Shetland if high levels of algal toxins are likely to be encountered, bivalve monitoring data to inform the crab industry working the North East, North Sea and Orkney of possible biotoxin events is likely to be sparse.

Fishing effort is at its lowest in the first three or four months of each year, then it swiftly rises to a plateau in April/May with around 9% of total annual catch being landed each month until October/November. This effort may continue until Christmas as the price of Brown crab increases (if weather conditions are suitable). The pattern of fishing effort is thus similar to the likely temporal distribution of toxins in Scotland, meaning that major blooms of toxic phytoplankton may coincide with peak crab fishing efforts.

4 DETECTION OF BIOTOXINS IN BROWN CRABS

4.1 Introduction

The main focus of the study was to ascertain the prevalence of algal toxins in *Cancer pagurus* from Scottish waters. A two-pronged approach was taken to this. To accommodate the possibility that no toxins would be found in field samples, laboratory work was planned which involved feeding crabs with toxic shellfish. This had the secondary purpose of providing material suitable for use in method validation studies. While there was some data available for DSP uptake from the Norwegian study (Catsberg *et al.* 2004) there was no comparable data for PSP or ASP toxins in *C. pagurus*. The aim of this work was therefore to confirm the hypothesis that crabs in a controlled environment can accumulate the ASP, PSP and DSP toxins.

The requirements of the crab feeding experiments changed as the results of the 2005 targeted and reactive sampling became available. The initial premise was that no toxic wild crabs would be found; so all information on toxic bio-accumulation in Brown crab tissues would be from the feeding study. However, crabs with elevated levels of PSP toxins were quickly encountered, negating the requirement for a full PSP feeding study. Initial laboratory work on toxifying crabs with PSP toxins was halted and is not described further here.

ASP toxins were also present in wild crabs but the high variability between individuals found in the wild provoked the question of linearity of toxin uptake, therefore a lab based feeding study using toxic scallops was conducted. The DSP toxins were not found in crabs sampled from Scottish waters during 2005, but due to the documented DSP presence in edible crabs in Norway, a feeding study with DSP-producing phytoplankton was carried out, to demonstrate uptake of DSP toxins in *C. pagurus*.

The main thrust of the research was, however, to determine if algal toxins could be detected in wild crabs. The 2005 sampling plan targeted areas where it was considered that there would be the highest chance of encountering crabs that contained toxins. Reactive sampling was also planned to opportunistically target areas where toxins were detected during the bivalve monitoring programme and from which crabs could be sourced. In 2006 the emphasis changed to a more random approach to acquiring samples to give a better view of the general incidence of toxins in crabs. However, this was still not a completely random survey as it focussed on three of the busiest port areas rather than a point of sale survey. Care should be taken in extrapolating from these results as the sampling focus may also have produced a bias in the results. Crabs with DSP toxins present were not detected at all in 2005 and so a reactive strategy was maintained in 2006 to maximise the likelihood of obtaining crabs that had been naturally contaminated with DSP toxins.

4.2 Methods and Materials

4.2.1 Crab Husbandry

Crabs of legal size (a minimum of 140 mm across the carapace) were collected from an area near Oban where the bivalves were toxin free (FSA shellfish monitoring program). The crabs were transferred to individual cages and were placed in 1000 L holding tanks, through which filtered seawater was circulated. Prior to feeding studies, crabs were fed non-toxic mussels until acclimatised to their surroundings. Holding tank water was changed every two days and more often if required. During water changes all the detritus of shell, uneaten food and faeces was removed and the tank cleaned. Bacterial build up from laid eggs occurred during husbandry trials so male crabs were used in all feeding studies.

4.2.2 DSP feeding study using mussels toxified with Prorocentrum lima

4.2.2.1 Trial Cultivation and Toxification of mussels (Lassus et al. 1994)

Prorocentrum lima (strain CCAP1136/12) was obtained from the Culture Collection of Algae and Protozoa (CCAP) – Scotland and cultured in 60 L bags. Approximately 150 mussels were housed in 120 L raceways supplied with filtered circulating seawater, which was changed every three days. On Day 0, 20 mussels were removed, the flesh pooled, homogenised and the concentration of DSP toxins determined. The remaining mussels were fed with *P. lima* each day. Approximately 20 L of water was removed from the circulating water in the raceway and 20 L of algae culture was added. Each subsequent day, 12 mussels were removed from the raceway and the flesh pooled, homogenised and tested for DSP toxins. This process continued for eight days when the assigned batch of *P. lima* was finished. All samples were tested for DSP toxin content using the PP2a assay (see section 4.2.5.4).

4.2.2.2 Crab DSP toxification experiment

The feeding study into DSP uptake by Brown Crabs was conducted to ensure that crabs would eat toxic mussels. Nine male crabs obtained from Oban were housed as described in Section 4.2.1. One crab was sacrificed upon arrival as a control to establish any ambient levels of DSP toxin. The remaining eight crabs were given four frozen mussels (toxicity of ~140 μg/100g OA equivalence) per day. The number of mussels eaten was recorded daily and after four days the crabs were sacrificed and individually tested for the presence of DSP toxins using the PP2a assay (Section 4.4.1.4).

4.2.3 ASP feeding study using King scallops

The availability of wild King scallops with high levels of domoic acid, the toxin responsible for causing ASP, negated the necessity of toxifying mussels. Frozen King scallops had their top shell removed, prior to being placed within individual cages, for ease of access to the internal tissues by the crabs.

Five male crabs obtained from Oban were housed as described in Section 4.2.1, with the exception that tanks were cleaned daily. Initially, the crabs were fed using King scallops with a whole animal concentration of domoic acid (DA) >250 mg/kg. However, the crabs stopped feeding after consuming one or two scallops, therefore, it was decided to attempt a feeding study with King scallops contaminated with a lower

concentration of DA. King scallops of a toxicity of ~85mg/kg were selected and fed to the five crabs. Each crab was provided with 1 scallop per /day, which was consumed by all test animals. One crab from the group was sacrificed daily and the brown meat analysed for ASP toxins using HPLC as described in 4.2.5.1.

4.2.4 Sampling Strategies for the detection of algal toxins in wild crabs

As briefly outlined earlier (Section 4.1), targeted and reactive sampling for all three toxins was carried out during 2005, while in 2006 three ports were selected to obtain crab samples (random sampling) for ASP and PSP and reactive sampling continued for DSP. Figure 12 shows the geographical areas sampled for wild Brown crabs during this study. Appendix 2 contains charts that detail the targeted, reactive and random sites for each toxin and year.

Figure 12: Map indicating the geographical locations from which Brown crabs were sampled for this project

4.2.4.1 Targeted Sampling during 2005

4.2.4.1.1 Methodology and rationale for site selection.

Historical data from the FSAS Biotoxin monitoring programme and the risk assessment for biotoxins in shellfish from classified inshore areas in Scotland (Howard, 2002, 2003; Petrie, 2004; Holtrop and Horgan 2004) was reviewed and the location of positive tests for toxic shellfish mapped to their locations.

For ASP, all test results greater than the limit of quantification were plotted. For DSP and PSP, positive results in the MBA were plotted. Application of data from the Inshore Risk Assessment (Holtrop and Horgan, 2004) was only used as a guide due to the grouping of samples from wide geographical areas into single areas for statistical purposes. Several areas were identified which had repeated toxic events during the period 2001-2004, however due to the factors outlined in Table 5, most were rejected as potential sampling sites.

Analysis of the data clearly identified that the Shetland Isles, North, West and South Skye had persistent PSP problems, with Shetland and Skye experiencing more regular annual toxic events with levels up to and exceeding regulatory limits compared to other areas. Therefore sites in these two areas were selected for PSP sample collection.

Similarly, a larger number of sites were identified as producing DSP-positive shellfish. These were: Shetland, North and West Skye, Loch Striven, Loch, Fyne, Inchard and Laxford and the Clyde estuary. Loch Striven, Loch Fyne and the Clyde area. The areas to the North and West of Skye showed similar occurrences of DSP to that of PSP in shellfish. Consequently, it was decided that samples should be taken from Shetland and West Skye for DSP also to improve sampling efficiency.

The occurrence of ASP at quantifiable concentrations in non-*Pectinidae* species is comparatively rare, with only Shetland, Loch Roag and South Skye producing a significant number of positive samples. The area around Broadford Bay in South Skye is complex, but a high proportion of ASP-containing King and Queen scallops, which are potential prey species, have been detected in this area. There is also significant creel-fishing for Brown crabs in this area making it the most suitable location for testing for ASP in *C. pagurus*. The sites selected for targeted sampling and their boundaries are shown in Appendix 2.

Table 5: Number of shellfish samples tested positive for PSP, DSP and ASP in Scotland during the period 2001-2004.

Area	Sites	Toxicity Found (# of positive tests)									Repeat Events (Toxin group)	Reason for rejection/use
		PSP			DSP			ASP				
		'01/02	'02/3	'03/4	'01/02	'02/3	'03/4	'01/02	'02/3	'03/4		
Orkney		15	0	0	11	0	0	0	0	0	-	Reject:Not repeated
Shetland		12	2	13	11	10	13	2	3	7	ASP, DSP, PSP	Reject: Wide geographical spread
Lewis	Loch Roag Complex	7	0	0	1	2	3	2	1	2	DSP, ASP	Reject: Low frequency
	Loch Liurbost	0	0	0	0	1	3	0	0	0	-	Reject: Low frequency
N. Skye	Lochs Dunvegan and Snizort	5	6	2	6	6	1	0	0	0	PSP, DSP	Use (PSP, DSP): Repeat events, defined area, known crab fishing area
W. Skye	Lochs Eishort, Harport and Slapin	3	5	2	2	8	4	0	1	1	PSP, DSP	Possible (PSP, DSP): Repeat events, defined area
S. Skye	Broadford Bay, Lochs Kishorn, Carron, Duich and Hourn	12	2	0	27	4	0	11	4	2	ASP, DSP, PSP	Use (ASP):Repeat events, defined area, known crab fishing area
	Loch Striven	0	0	3	3	9	2	0	0	0	DSP	Possible (DSP); Defined area
	Loch Fyne	0	0	0	8	5	9	0	1	0	DSP	Reject: Long loch, widespread sites
	Loch Torridon	9	0	0	6	1	0	0	1	0	DSP	Reject: low frequency
	St Abbs	3	1	0	2	1	1	0	0	0	DSP	Reject: low frequency
	Lochs Inchard and Laxford	3	0	0	20	5	1	0	0	2	DSP	Reject: reducing frequency
	Lochs Ewe and Broom	0	0	0	13	0	0	8	1	1	ASP	Reject: Low frequency
Clyde	Arran and Fairlie	0	0	0	9	4	6	0	0	0	DSP	Reject: Large area
S. Mull	I. Scridain	1	1	1	3	2	1	0	1	0	DSP	Reject: Low frequency

Local crab fishermen were identified and contacted in each of the candidate sites and asked to procure between 10 and 14 crabs. It was originally intended to sample the sites over 18 weeks to give a good probability of obtaining crabs that may have accumulated algal toxins.

However, the time taken to develop a reliable sampling plan, coupled with problems such as adverse weather conditions, resulted in difficulties in procuring samples and hence a reduced number of samples seen in Table 6.

Table 6: The 2005 Targeted sampling sites and number of crabs tested for each toxin.

Site	Number of ASP analyses	Number of DSP analyses	Number of PSP analyses	Total analyses for each site
Raasay	40	-	-	40
Loch Dunvegan	20	49	59	128
St. Abbs	-	29	-	29
Stonehaven	-	-	28	28
Total analyses for each toxin	60	78	87	

4.2.4.2 Reactive Sampling during 2005

The objective of the Reactive Sampling was to obtain wild crab samples from areas in which toxic shellfish were being detected by FSA Scotland's current statutory biotoxin monitoring programme. When shellfish were found to contain concentrations of ASP, DSP or PSP toxins approaching or above the regulatory limits, crab fishermen active in these areas were identified, contacted and arrangements were made for the collection of between 10 and 14 crabs per week for a total of 3 weeks for each toxic event.

However, few toxic events occurred during 2005 and only 70 animals for ASP, 60 animals for PSP and 20 animals for DSP were analysed as part of the reactive testing.

Table 7: The 2005 Reactive sampling sites and number of crabs tested for each toxin.

Site	Number of ASP analyses	Number of DSP analyses	Number of PSP analyses	Total analyses for each site
Tor Ness (O18)	30	0	30	60
Mull Head (O19)	30	20	30	80
Oban (SM16)	10	0	0	10
Total analyses for each toxin	70	20	60	

4.2.4.3 Random Sampling for ASP and PSP during 2006

The objective of the Random Sampling was to obtain random, independent samples of the crab population landed in Scotland during the algal toxin "season" for 2006. The results of the 'Mapping the Scottish Crab Fishery' section of this report (Section 3) were used to determine three ports (or areas) where high landings occurred. Fishmongers at these ports (or areas) were used to source crabs. The three areas chosen were Scrabster (the major port in the North East); Lochinver (to represent the far North-West) and Oban (to represent the lower part of the North West). It should be noted, however, that the crabs delivered to Integrin were not always actually from these areas. Two early samples sent to Integrin as "Oban" had actually come from Fraserburgh and

Troon. The "Oban" samples usually came from various parts of Argyll and in particular Mull. Those from Scrabster and Lochinver, however, tended to have been caught in closer proximity to the designated port.

Each month between June and October (2006) batches of between 10 and 14 crabs were requested from each port or area. The total number of crabs tested for each toxin at each port is shown in Table 8.

Table 8: The Random sampling sites for 2006 and number of crabs tested for each toxin.

Site	Number of ASP analyses	Number of PSP analyses	Total analyses for each site
Oban	50	50	100
Lochinver	50	50	100
Scrabster	50	50	100
Total analyses for each toxin	150	150	

4.2.4.4 Reactive Sampling during 2006

The primary objective of Reactive Sampling in 2006 was to obtain wild crab samples from Ronas Voe (Shetland) in which DSP toxic shellfish were detected by FSA Scotland's current statutory biotoxin monitoring programme. The FSA Scotland's toxic phytoplankton monitoring program was used to provide information on bivalve harvesting areas with high *Dinophysis* counts. This indicated the DSP toxins could potentially be detected in shellfish in Ronas Voe in the near future. Time was therefore gained to contact and make arrangements with a fisherman active in that area to supply batches of Brown crab. When DSP toxins were detected in the bivalve shellfish of Ronas Voe, batches of 12 Brown crabs were obtained fortnightly. Sampling continued until the Temporary Closure Notice was revoked in the area.

During 2006, the bivalve monitoring programme also indicated a significant increase in the number of bivalve harvesting areas affected by PSP. This was particularly evident in harvesting areas around Shetland in the Autumn (September and October), where levels of up to 413 μg/100g were detected in official control samples taken as part of the Food Standards Agency Scotland's statutory monitoring programme from Busta Voe Lee North (Shetland) on the 11th of September 06. This prompted further reactive sampling of crabs in nearby crab fishing area (waters around Ronas Voe, Vementry and Muckle Ossa). The total number of crabs tested for each toxin for the 2006 reactive sampling is shown in Table 9.

Table 9: The Reactive sampling sites for 2006 and number of crabs tested for each toxin.

Site	Number of DSP analyses	Number of PSP analyses	Total analyses for each site
Ronas Voe	50	50	100
Vementry	0	10	10
Muckle Ossa	0	10	10
Troon	0	5	5
Fraserburgh	0	5	5
Total analyses for each toxin	50	80	

4.2.4.5 Sample Transport

Crabs obtained during 2005 from fishermen were posted to Integrin live in coolboxes into which had been added frozen coolpacks. Crabs that arrived moribund or dead were discarded. The crabs obtained during 2006 were, however, immediately frozen by the fishmongers or fishermen upon receipt and then posted to Integrin in coolboxes containing frozen coolpacks. All of the 2006 samples reached Integrin still frozen.

4.2.5 Analytical methods

In all animals the brown meat was tested for the presence of ASP, PSP or DSP toxins as appropriate using a variety of methods. The brown meat comprised the homogenised contents of the carapace except for the gills and thus included all of the digestive tissues. Where high values were detected, the white muscle meat from a maximum of 3 animals was also removed and analysed for the relevant toxin. All results given are for brown meat unless otherwise stated.

4.2.5.1 HPLC for ASP toxins

Domoic acid and epidomoic acid (the cause of ASP) were detected using HPLC with Photo Diode Array (PDA) detection as laid out in Commission Decision EC/2074/2005. This method is based on the method published by Quilliam, *et al*. (1995). Domoic and epidomoic acids were extracted from 4 g of homogenised tissue matrix by adding 16 ml of 50:50 methanol:water and re-homogenising for 3 minutes. The sample was then centrifuged at 8873 x *g* for 30 mins and the supernatant removed. An aliquot of 2 ml of the supernatant was diluted with 8 ml of 50:50 methanol:water and filtered through a 0.45µm nylon filter. Solid phase extraction (SPE) was then carried out using a strong anion exchange (SAX) cartridge. The SAX cartridges were conditioned with 6 ml of methanol, 3 ml of water then 3 ml of 50:50 methanol:water. 5 ml of the filtered extract was then passed through the SAX cartridge, which was then washed with 5 ml of 50:50 methanol:water. ASP was eluted with 4 ml of 80.8 mg/ml citric acid 28 mg/ml triammonium citrate in 90:10 water:acetonitrile. From the SPE extract, an aliquot of 20 µl was then injected onto an HPLC (1 ml/min, 0.1 % v/v trifluoroacetic acid in 90:10 water:acetonitrile, column Luna C18 (2) 3 µm 4.6 x 75 mm) with UV detection (absorbance wavelength 242 nm). Calibration standards over the range 1, 25 and 250 mg/kg were prepared from certified reference standards in 50:50 methanol:water. The method can detect ASP toxins at a limit of 1 mg/kg.

4.2.5.2 Enzyme Immunoassay for PSP

PSP toxins were detected using the commercial Ridascreen Fast Saxitoxin assay (Rhone Diagnostics) as per the manufacturer's instructions. This test is a competitive enzyme-linked immunosorbent assay in which free PSP toxins and enzyme-conjugated PSP compete for anti-PSP antibody binding sites.

PSP toxins were extracted from 1 g of sample homogenate by adding 1 ml of 0.1M hydrochloric and vortex mixing for approximately 1 minute. The sample was then hydrolysed by being boiled in a water bath for 5 minutes. To ensure the samples pH was less than 4, 10 µl of 2.5M hydrochloric acid was added. The sample was then centrifuged at 3466 x *g* for 10 min and 50 µl of the supernatant was removed and added to 950 µl of buffer to make the prepared sample.

From the prepared sample, 50µl aliquots were added to individual wells on a microtiter plates coated with capture anti-IgG antibodies (which are directed against anti-PSP antibodies). To the same wells, 50 µl of enzyme conjugate (peroxidase conjugated

PSP concentrate) and 50 µl of anti-PSP antibody solution were then sequentially added. The plates were incubated at 20°C for 20min to allow equilibrium to be reached.

The excess reagents were then removed and the wells washed 3 times with 250 µl of ultra high quality water. 100 µl of substrate chromagen was added to the wells and left to incubate at 20°C for 15 minutes. During this period the bound enzyme conjugate converted the chromagen into a blue product. To stop the reaction, 100 µl of stop solution (1 N sulphuric acid) was added and absorbance read at 450 nm. The absorbance values were converted to µg/100g saxitoxin-binding equivalence.

While it was primarily designed to detect saxitoxin, the Ridascreen assay also detects other PSP toxins but with different binding affinities, which are documented in the manufacturer's literature (STX=100%, GTX2/3=70%, dcSTX=20% and neoSTX=12%). The assay reliably quantifies saxitoxin-binding equivalence down to 10 µg/100g. For the purposes of this study, this binding equivalence is assumed to be comparable to toxicity as measured by the regulatory mouse bioassay.

4.2.5.3 PSP using HPLC with Fluorescence Detection (Lawrence *et al. 2005, AOAC Method 2005.06*)

This method was employed to determine the toxin profiles of crab samples identified as having PSP toxins present by Ridascreen. PSP toxins were extracted from 5 g of shellfish homogenate by adding 3 ml of 1 % (v/v) acetic acid and boiling in a water bath for 5 minutes. The sample was then centrifuged for 10 minutes at 3600 x *g* and the supernatant removed. A further 3 ml of 0.1 % acetic acid was added to the remaining solid residue, and the process was repeated without boiling, combining the two supernatants and making up to 10 ml with water.

SPE was then carried out on this extract by passing 1 ml of extract followed by 2 ml of water onto a 3 ml 500 mg C18 cartridge (preconditioned with 6 ml methanol followed by 6 ml water) and collecting the eluent into a graduated tube. The SPE extract was adjusted to pH 6.5 with 1 M NaOH and made up to 4 ml with water.

Periodate oxidation was then carried out by adding 100 µl extract after SPE C18 cleanup to 500 µl Periodate Oxidant:(0.01 M periodic acid, 0.1 M ammonium formate, 0.1 M Na_2HPO_4 pH 8.2) The solution was left at room temperature for 1 minute; then 5 µl of concentrated acetic acid was added and vortex mixed.

50-100 µl was injected onto the HPLC at 2 ml per minute of 100-95% mobile phase A for 5 minutes then 95-30% mobile phase A for 4 minutes and back to 100% mobile phase A after another 2 minutes. Mobile phase A was 0.1 M ammonium formate pH 6 and mobile phase B was 0.1 M ammonium formate pH 6 in 5% (v/v) acetonitrile:water. The HPLC column was a Supelcosil LC-18 150 x 4.6 mm i.d. 5 µm particle size. Detection was by fluorescence excitation 340 nm emission 395 nm. Run time was 15 minutes.

Further peroxide oxidation was only deemed necessary if neo-saxitoxin, dc-neosaxitoxin, or dc-gonyautoxin2/3 were detected due to co-elution but these analogues were not detected in any of the samples.

Calibration standards for each PSP toxin were split into three groups as follows; Group 1: Saxitoxin, dc-saxitoxin, gonyautoxin-5, gonyautoxin-2/3, Group 2: Neo-saxitoxin, gonyautoxin 1/4, Group 3: dc-neosaxitoxin, dcgonyautoxin2/3. These standards were spiked over the range 3.125 to 50 ng/ml and were analysed alongside the sample extracts to produce calibration lines.

4.2.5.4 Protein phosphatase inhibition for DSP toxins

DSP toxins are heat stable lipophilic compounds that include okadaic acid (OA) and its derivatives, dinophysotoxins (DTX1, DTX2 and DTX3). DTX3 is the collective name given to esters of okadaic acid, DTX1 and DTX2. To detect and quantify the esters (DTX3) the samples were hydrolysed which converts the esters (DTX3) to OA or DTX1. The most common DSP toxins okadaic acid, DTX1 and DTX2 are phosphatase inhibitors and can be detected using a fluorescent human Protein Phosphatase 2a (PP2a) inhibition assay, adapted from that described by Mountfort *et al*. (2001) to run as a kinetic assay using an FL600 plate reader. Crab meat aliquots of 1 g were extracted by adding 4 ml of 90:10 methanol:water and homogenising for 2 minutes then centrifuging at 4500 x *g* for 10 minutes. From the resultant supernatant, 500 µl was removed and mixed with 65 µl of 2.5 M sodium hydroxide and heated at 76ºC for 40 minutes, before adding 65 µl of hydrochloric acid. The hydrolysed extract was then diluted with 495 µl of water and loaded onto a 30 mg waters Oasis HLB SPE cartridge (conditioned with 1 ml methanol and 1 ml of 40 % v/v methanol:water), and washed with 2 ml of 40 % (v/v) methanol:water followed by 1 ml of 2 % (v/v) acetic acid:water in 75 % (v/v) methanol:water. The SPE cartridge was then eluted with 1 ml of 2 % (v/v) ammonium hydroxide in 80 % (v/v) methanol:water. The eluent was dried under a stream of nitrogen at 80 ºC and resuspended in 1 ml of sample buffer. The sample, or a standard, was mixed with enzyme and buffer plus substrate in a 96-well microtitre plate, which was read continuously over a half hour period with a kinetic interval of 2.5 minutes. The plate reader software determined the concentration of samples as okadaic acid equivalents. This method quantifies phosphatase inhibiting toxins down to 10 µg OA /100g.

Due to global problems in enzyme supply this assay was unavailable in 2006. Samples taken in 2006 that were shown positive for DSP toxins by LC-MS/MS had not yet been quantified using PP2a at the time of this report.

4.2.5.5 LC-MS/MS for DSP

For the 2006 samples, LC-MS/MS was the method used to identify the presence of DSP toxins in reactive samples. Although this method will detect all of the toxin groups covered by EU legislation (Quilliam *et al*. 2001), only OA and the DTX's were analysed for this study. The method was adapted from previously published methods by Pleasance and Quilliam (1992) and Quilliam *et al.* (2001). The toxin analogues were extracted from 1g of shellfish homogenate by adding 4 ml of 90% (v/v) methanol:water and homogenising for 2 minutes. The sample was then centrifuged at 4500 x *g* for 5 minutes, then 500 µl of the supernatant was removed to which was added 66 µl of 2.5 M sodium hydroxide. This mixture was vortex mixed briefly and heated at 76ºC for 40 minutes to complete hydrolysis then 66 µl of 2.5 M hydrochloric acid was added to neutralise the extract. The extract was then evaporated to dryness at 75 ºC under a stream of nitrogen and resuspended in 100 µl of 90 % (v/v) methanol:water.

This extract was then injected onto the HPLC. Mobile phase A was 2mM ammonium formate in 50 mM formic acid and mobile phase B was 2 mM ammonium formate in 50 mM formic acid in 95 % (v/v) acetonitrile:water. A 50 x 2 mm C18(2) Luna 2.5 µm HTS column was used and the injection volume was 10 µl. A mobile phase gradient was used, consisting of 70% mobile phase A to 30% mobile phase A over 4 minutes, held for 0.5 minutes and then returning back to 70% mobile phase A at a flow rate of 0.35 ml/min. MS/MS detection was used with the following mass transitions OA and DTX2 803.5>255.1, DTX1 817.5>255.1, calibration standards over the range 1 to 100 ng/ml for DTX1 and OA were prepared from certified reference standards in blank crab extract to compensate for matrix effects. DTX2 standards were not available but no

additional peaks were observed in the OA MRM channel so quantification was not necessary.

4.2.6 Validation of Analytical Methods

Since the methods described in Section 4.4.1 had previously only been used to detect toxins in bivalve molluscs, it was necessary to carry out validation work to determine their suitability for use with the crab matrix. The methods were validated for the white and brown meat of *C. pagurus* based on the principles defined by Shah *et. al.* (1991). The basis of the validation is to show an acceptable level of toxin recovery from a sample spiked with a known level of toxin. For methods that have already been validated for other matrices, this demonstrates acceptable recovery from crab meat with minimal matrix interference to the respective assays.

4.2.6.1 ASP crab matrix validation using the UV-HPLC method

A summary of spiking studies conducted to validate the HPLC method for detection and quantification in the crab meat matrix is given in Table 10. Three aliquots from a pool of brown and white crab-meat were tested using the described method (see 4.2.5.1) to obtain a large sample containing no domoic acid (a negative sample). Negative brown and white meat was spiked with internal laboratory reference material (MDS01-141004-POOL, QCR-76b) to give a concentration of 5.2 mg/kg of domoic acid in brown meat and 5.6 mg/kg of domoic acid in white meat. Two aliquots of negative brown and white meat was spiked with Domoic Acid (98% purity, Biovectra lot: 25608) dissolved in 50:50 methanol:water to give concentrations of 75mg/kg and 175mg/kg of domoic acid in brown and white meat. These spiked samples were run according to the method (see 4.2.5.1). The qualitative results demonstrated a combined recovery of domoic acid and epi-domoic acid within 65 to 93% required of the assay. The assay's lower limit of quantification was 1 mg/kg.

Table 10: Summary of ASP recovery in spiked crab meat.

	Mean recovery as a percentage (±S.D.)	
Theoretical DA Conc'n (mg/kg)	**Brown meat**	**White meat**
0.0 (n=3)	0.0 (±0.0)	0.0 (±0.0)
5.2 (Brown) & 5.6 (White) (n=3)	88.5 (±3.3)	92.9 (±4.7)
75 (n=2)	72.0 (±12.1)	78.9 (±2.8)
175 (n=2)	65.2 (±0.1)	75.4 (±0.2)

4.2.6.2 PSP crab-matrix validation of the Ridascreen Fast Saxitoxin method

Three aliquots of pooled brown and white crab meat was tested and shown to be negative for PSP toxins. Certified reference material NRC-CRM-STX, a solution of saxitoxin dihdrochloride, at a concentration of 65 µM (STX) in hydrochloric acid of 0.003M.2HCl was added to 1 g aliquots of negative brown and negative white meat to give STX concentrations of 15.0 µg/100g, 30 µg/100g and 60.0 µg/100g. The aliquots were well mixed and tested according to the method (see 4.2.5.2). Table 11 shows the spiking studies of qualitative blank crabmeat material and the acceptable recovery of Saxitoxin (STX).

Table 11: Summary of PSP Recovery from spiked crab meat

	Mean recovery as a percentage (n=4, ±S.D.)	
Theoretical STX Conc'n (µg/100g)	**Brown meat**	**White meat**
0.0 (n=2)	0.0	0.0
15.0 (n=2)	102.8 (±9.4)	94.4 (±15.3)
30.0 (n=2)	92.8 (±11.7)	84.4 (±15.0)
60.0 (n=2)	82.8 (±5.6)	81.5 (±10.9)

4.2.6.3 DSP crab-matrix validation of the Protein Phosphatase 2A assay

Three aliquots of pooled brown and three aliquots of pooled white crab meat were tested and shown to be negative for DSP toxins. This negative brown and white crab meat was spiked with certified reference material NRC-CRM-DSP-Mus-b (13.4 µg/100g OA and 1.7 µg/100g DTX1) to give a combined OA and DTX1 concentration of 15.1 (±1.0) µg/100g in brown meat and 14.8 µg/100g in white meat. This level of DSP toxins is just below the EU regulatory level of 16 µg/100g but above the assay's lower limit of quantification of 10 µg/100g. The three aliquots were tested by the method (see 4.2.5.4) and the results showed recovery of 67.4% in brown meat and 77.3% recovery in white meat.

4.2.6.4 DSP crab-matrix validation of the LC-MS/MS method

Recovery was evaluated by adding a DSP stock solution containing 4 µg/100g each of okadaic acid (OA) and DTX1, to extracted DSP negative crab tissue (brown meat), and negative crab supernatant (n=6). The tissue was then extracted as described in the materials and methods section (see 4.2.5.5). In addition to this, a 500 µl aliquot of extract was evaporated down as described in the method, stopping prior to the addition of sodium hydroxide, to evaluate any potential toxin deterioration during hydrolysis. The hydrolysis step is necessary to convert esters into measurable okadaic acid and DTX's but may also affect quantification. The results of this investigation are summarised in Table 12. Recovery was calculated against the unhydrolysed spiked supernatant. The results clearly demonstrate that the hydrolysis process partially degrades OA and DTX1. Recovery from unhydrolysed tissue is between 42-47%. However, when this is compounded by deterioration during hydrolysis it further drops to between 27 to 38%.

Table 12: Summarised results of toxin recovery experiments

		% Recovery	
Sample	**State**	**DTX1**	**OA**
Supernatant	Unhydrolysed	100	100
	Hydrolysed	67	76
Tissue	Unhydrolysed	47	42
	Hydrolysed	27	38

The validation studies demonstrated that the methods were suitable to identify the presence of OA and DTX1 in crab matrices. The crab matrix and the necessary hydrolysis step are, however, causing recovery problems making the assay only semi-quantitative.

4.3 Results

The full raw data set can be found in Appendix 3.

4.3.1 Feeding Experiments

4.3.1.1 DSP Feeding Study using mussels toxified with *Prorocentrum lima*

Figure 13 shows mussels fed with *P. lima* rapidly became toxic in an initial trial. The end of the trial concentration in the mussels was ~140 μg/100g OA equivalence, which was outside of the limits of the standard curve of the assay. Therefore, the actual value may have exceeded this level.

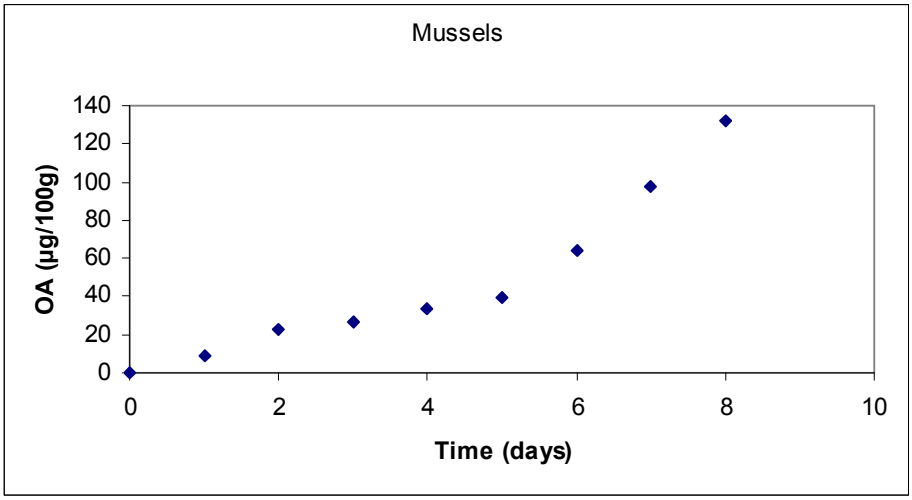


Figure 13: The results of the initial trial of DSP toxification of mussels (pooled n=12)

A trial experiment (see section 4.2.2.2) showed that crabs would eat the mussels produced in section 4.2.2.1. Three further batches of mussels were fed with *P. lima* for a total of six, seven and eight days using the method described in section 4.2.2.1. The 12 mussels from each batch were pooled, analysed and found to contain OA values up to 44 μg/100g OA equivalents (Table 13).

Table 13: Okadaic acid content of mussels produced for feeding experiment

Time mussels were fed toxic algae	n	OA (μg/100g) using LC-MS
6 days	12	39.4
7 days	12	43.5
8 days	12	44.0

4.3.1.2 Crab DSP Toxification Experiment

The number of mussels consumed by crabs was recorded over a four day feeding period and the OA concentration of the individual crabs measured at the end of this

feeding period. Elevated levels of DSP toxins were detected in the crabs after the consumption of the toxic mussels (Figure 14). In general the more mussels consumed, the higher the level of DSP toxins detected in the crabs tested (Figure 14), however the correlation was very weak (R^2=0.4).

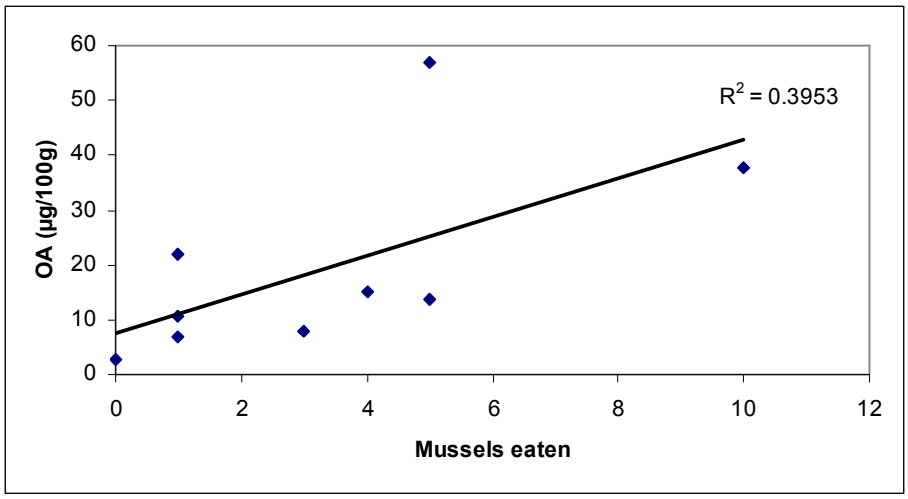


Figure 14: DSP levels (µg/100g) detected in the brown meat of single crabs, plotted against the number of mussels eaten.

4.3.1.3 ASP Feeding Study using King Scallops

The levels of domoic acid found in crabs after consumption of a number of toxic King scallops is shown in Figure 15. The levels of domoic acid appeared to increase with the number of toxic scallops consumed, however the levels of OA detected in the crab that consumed four King scallops was lower than expected.

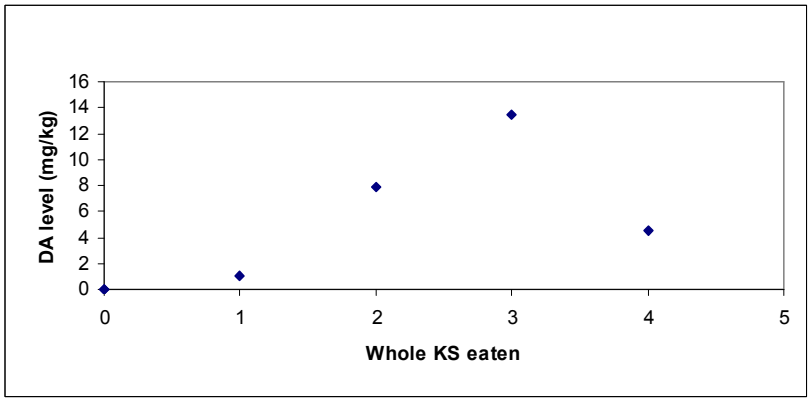


Figure 15: Levels of Domoic acid detected in brown meat of individual crabs in relation to the number of toxic whole King scallops consumed

4.3.2 Detection of algal toxins in wild crabs

4.3.2.1 ASP toxins in Targeted and Reactive samples collected during 2005, as detected by HPLC

A batch of crabs taken from near Oban at the beginning of the project, for the purposes of method development, had a putative DA peak in the brown meat that was quantified at a mean value of 25.3 mg/kg (the regulatory bivalve limit is currently 20 mg/kg). The white meat from this sample did not show any presence of DA. The DA content of this wild sample was confirmed internally by HPLC and also by an external laboratory (Michael Quilliam's group) by LC-MS/MS (Appendix 4).

The 2005 results from the targeted and reactive sampling regimes are summarised in Table 14**.** The highest values were found in the brown meat from a crab from Loch Dunvegan in October. At 121.6 mg/kg, this is just over six times the bivalve regulatory limit. The white claw meat from the three highest ASP toxic samples was tested and no ASP toxins were found (Table 15).

Table 14: 2005 targeted and reactive ASP results in mg/kg.

Sampling strategy	Sampling location	Date	n	Mean	STD	Min	Max	Median
Targeted	Raasay	17/08/05	9	7.8	10.0	0.0	29.1	2.1
		02/09/05	10	3.4	7.2	0.0	22.5	0.4
		07/09/05	10	9.7	11.8	0.0	31.1	2.8
		14/09/05	10	10.2	16.1	0.0	51.3	3.1
	Loch Dunvegan	26/08/05	10	4.7	7.0	0.0	23.8	3.3
		26/10/05	10	14.9	37.8	0.0	121.6	1.1
Reactive	Mull Head (O19)	25/10/05	10	0.9	1.0	0.0	2.4	0.6
		02/11/05	10	0.2	0.8	0.0	2.4	0.0
		11/11/05	10	0.0	0.0	0.0	0.0	0.0
	Tor Ness (O18)	24/11/05	10	0.4	1.0	0.0	3.2	0.0
		08/12/05	10	0.0	0.0	0.0	0.0	0.0
		15/12/05	10	0.2	0.3	0.0	0.8	0.0
	Oban (M16)	22/12/05	10	2.1	5.3	0.0	16.9	0.0

Table 15: The three highest ASP results in brown meat and the corresponding results in white meat (mg/kg).

Sampling location	Date and sample ID	DA in Brown meat	DA in White meat
Loch Dunvegan	FSA04-261005-10	121.6	0
Raasay	FSA04-140905-19	51.3	0
Raasay	FSA04-070905-21	31.1	0

The percentages of brown meat samples analysed for ASP that were above the regulatory limit, below the regulatory limit and below the level of quantification (LOQ) of the HPLC test are shown in Figure 16. It can be seen from this that the majority of crabs tested (65%) had levels of ASP toxins in their brown meat below the LOQ; 28% contained quantifiable ASP levels that were below the regulatory limit and 7% crabs had ASP concentrations in the brown meat above the regulatory limit of 20 mg/kg (9 out of 130).

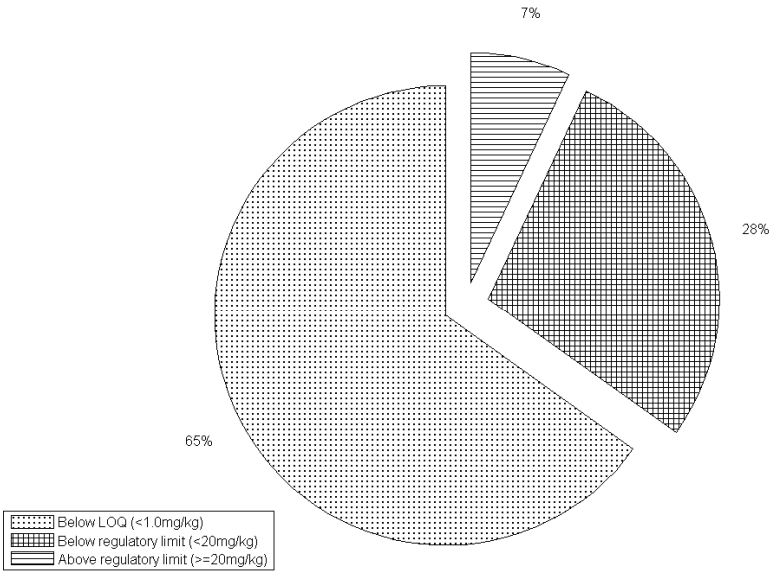


Figure 16: ASP distribution for the crab brown meat tested for the 2005 season (n=129).

4.3.2.2 ASP toxins in Random samples collected during 2006 as detected by HPLC

The mean levels of ASP detected in samples collected from the three ports Scrabster, Lochinver and Oban as part of the random sampling regime for 2006 are shown in Table 16. Mean levels of ASP detected in the brown meat were found to be below 3.5 µg/g, however only one crab out of the total 150 tested was over the regulatory limit, with a detectable level of 20.7 µg/g.

Table 16: Levels of ASP toxins (mg/kg). detected by HPLC in the brown meat of crabs collected during the 2006 random sampling.

Sampling strategy	Sampling location	Date	n	Mean	STD	Min	Max	Median
Random	Lochinver	23/06/06	10	2.1	2.6	0.0	7.5	1.1
		18/07/06	10	0.8	1.0	0.0	2.8	0.6
		23/08/06	10	1.3	1.1	0.0	2.8	1.3
		19/09/06	10	3.5	6.2	0.0	20.7	1.2
		26/10/06	10	1.5	2.1	0.0	6.2	0.8
	Scrabster	20/06/06	10	1.6	2.9	0.0	8.4	0.0
		29/07/06	10	0.8	1.0	0.0	3.5	0.9
		30/08/06	10	0.6	1.0	0.0	2.4	0.0
		28/09/06	10	0.2	0.5	0.0	1.5	0.0
		27/10/06	10	0.1	0.4	0.0	1.4	0.0
	Oban	05/06/06	10	2.1	2.2	0.0	5.9	1.8
		19/06/06	10	1.6	1.7	0.0	4.8	1.3
		21/08/06	10	0.7	1.7	0.0	5.2	0.0
		18/09/06	10	4.3	6.4	0.0	16.7	0.0
		10/11/06	10	0.8	1.2	0.0	3.0	0.0

As with the detection of ASP in samples collected during the targeted and reactive sampling during 2005, the majority (61%) of samples collected randomly during 2006 had ASP levels that were below the LOQ (1 mg/kg) for the HPLC test (Figure 17). Only 1% of brown meat samples tested for were above the regulatory limit for ASP, with the remainder of samples being between LOQ and the regulatory limit.

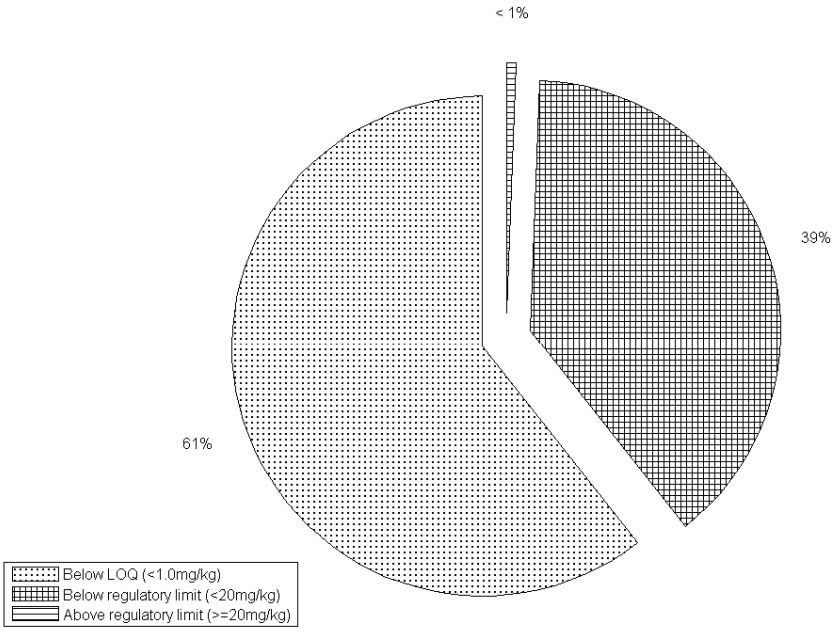


Figure 17: ASP distribution for the crab brown meat tested for the 2006 season (n=150).

The mean levels of ASP detected in the brown meat from all the individual crabs landed from Oban, Lochinver and Scrabster during 2006 were plotted per sample month (Figure 18). From this, it was determined that the highest mean toxin levels were detected in September.

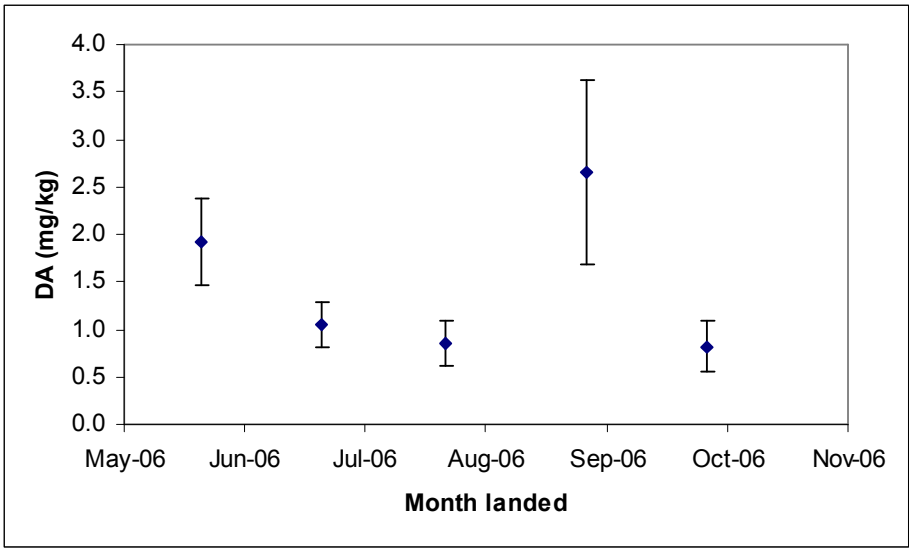


Figure 18: Mean ASP concentrations for each sampling month

Error bars represent standard error

4.3.2.3 PSP in Targeted and Reactive samples collected during 2005 as detected by the Ridascreen Fast Saxitoxin assay

The results from the targeted and reactive sampling carried out during 2005 are summarised in Table 17. There was less variation in the concentrations of PSP toxins in the brown meat compared to those detected for ASP toxins, indicating less influence of outliers and more evenly distributed data set. The greatest PSP toxin concentration detected was observed in a crab sampled reactively from Tor Ness (O18) in December, 2005.

Table 17: PSP levels (μg/100g) detected in the brown meat of crabs sampled during 2005

Sampling Stratagy	Sampling location	Date	n	Mean	STD	Min	Max	Median
Targeted	Dunvegan	02/09/05	10	16.0	2.8	11.3	20.8	15.3
		07/09/05	10	4.6	7.8	0.0	20.1	0.0
		14/09/05	9	6.7	3.9	0.0	10.0	8.4
		05/10/05	10	11.0	9.3	0.0	24.2	11.2
		13/10/05	10	17.9	15.2	0.0	57.6	13.9
		20/10/05	10	30.4	22.1	11.4	87.5	26.1
	Stonehaven	08/09/05	9	16.0	4.1	8.2	20.9	16.8
		14/09/05	9	17.7	16.6	0.0	59.0	13.2
		22/09/05	10	20.1	6.5	13.7	36.6	18.7
Reactive	Mull Head (O19)	25/10/05	10	21.3	7.1	13.7	36.0	20.0
		02/11/05	10	44.8	20.8	16.3	69.3	45.2
		11/11/05	10	26.5	16.6	10.2	60.1	18.0
	Tor Ness (O18)	24/11/05	10	55.7	31.8	18.9	120.0	42.8
		08/12/05	10	50.2	48.3	12.6	169.7	34.5
		15/12/05	10	31.9	22.0	12.0	81.5	26.9

Figure 19 shows 16% of the crabs tested were below the limit of quantification of the assay (less than 10 μg/100g) and 79% contained above LOQ but below the regulatory limit (80 μg/100g). Only a small number of crabs (4%) were found to contain levels of PSP toxins in their brown meat that were above the regulatory limit of 80 μg/100g. Over 95% of crabs sampled were therefore below the regulatory limit (percentages have been rounded up or down to nearest whole number).

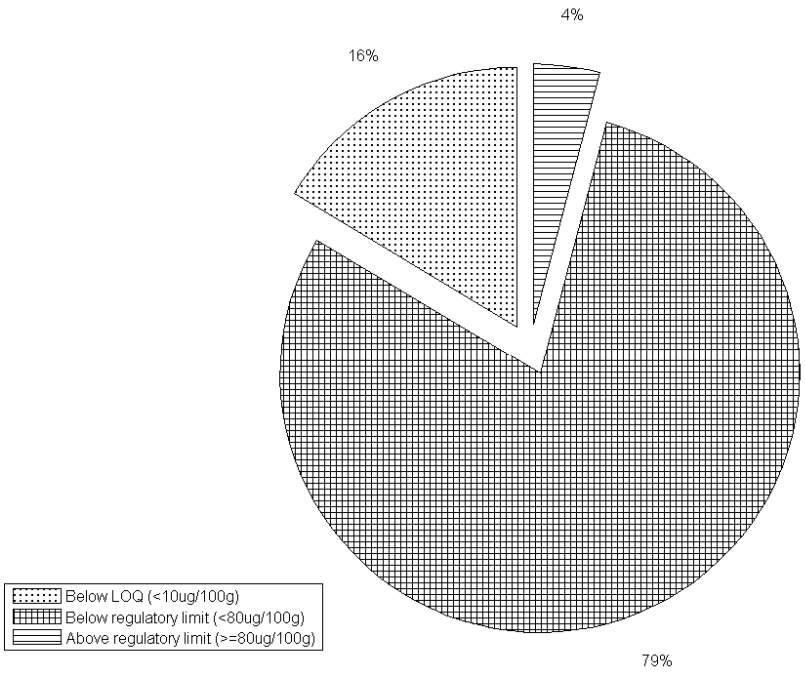


Figure 19: Distribution of 2005 PSP toxin levels for brown meat tested 2005 (n=147).

The white claw meat from three individual crabs collected from Tor Ness in Orkney during 2005 that had the highest PSP values detected in the brown meat and the white claw meat from six additional crabs with a range of concentrations of PSP toxins in the brown meat were tested for the presence of PSP toxins by Ridascreen. This analysis was carried out in triplicate. The mean of these three tests and the corresponding values of brown meat are shown in Table 18. Two out of the 9 samples contained PSP concentrations above the limit of quantification (LOQ). These results suggest that the white meat of PSP contaminated crabs can contain low levels of the toxins.

Table 18: Analysis of white meat from selected crab samples from 2005 for PSP toxins .

Original sample type	Integrin Sample ID	Area the crabs originated	STX eqv. in brown meat (μg/100g)	Mean STX eqv. in White meat (μg/100g) (±SD)
Brown meat with highest PSP levels detected	FSA04-081205-05-W	Tor Ness (Orkney)	169.7	11.4 (±2.7)
	FSA04-081205-04-W	Tor Ness (Orkney)	87.0	11.0 (±0.5)
	FSA04-081205-06-W	Tor Ness (Orkney)	60.0	9.7 (±8.6) <LOQ
Additional brown meat samples, which contained a range of detected PSP levels	FSA04-241105-03-W	Tor Ness (Orkney)	98.4	2.9 (±5.0) <LOQ
	FSA04-241105-07-W	Tor Ness (Orkney)	60.6	4.8 (±4.9) <LOQ
	FSA04-241105-08-W	Tor Ness (Orkney)	120.0	4.5 (±4.3) <LOQ
	FSA04-241105-09-W	Tor Ness (Orkney)	41.6	4.5 (±4.9) <LOQ
	FSA04-241105-10-W	Tor Ness (Orkney)	18.9	1.9 (±3.2) <LOQ
	FSA04-081205-09-W	Tor Ness (Orkney)	12.6	5.5 (±5.3) <LOQ

4.3.2.4 PSP in Random and Reactive samples collected during 2006, as detected by the Ridascreen Fast Saxitoxin assay

The levels of PSP toxin detected in brown meat from crabs sampled both randomly and reactively during 2006 are shown in Table 19. The highest levels of PSP detected in random samples were identified in crabs from Scrabster taken in the month of June. The highest level of PSP detected overall was observed in a crab obtained from Fraserburgh on the North East Coast of Scotland in July.

Table 19: PSP levels (µg/100g) in brown meat from crabs sampled during 2006

Sampling Stratagy	Sampling location	Date	n	Mean	STD	Min	Max	Median
Random	Lochinver	23/06/06	10	17.5	16.1	0.0	52.6	9.1
		18//07/06	10	3.5	7.7	0.0	22.0	0.0
		23/08/06	10	17.3	12.7	0.0	41.6	17.5
		19/09/06	10	15.7	9.8	10.2	43.0	12.6
		26/10/06	10	13.9	3.6	9.3(<LOQ)	21.1	13.8
	Scrabster	20/06/06	10	27.6	28.9	10.8	108.4	18.5
		29/07/06	10	25.9	24.0	0.0	84.1	18.2
		30/08/06	10	26.9	18.5	11.0	72.4	19.1
		28/09/06	10	17.3	5.4	11.3	27.1	17.6
		27/10/06	10	8.6	3.3	0.0	12.3	9.3
	Oban	05/06/06	10	18.4	26.5	0.0	91.5	11.9
		19/06/06	10	8.8	17.6	0.0	56.9	0.0
		21/08/06	10	9.2	8.4	0.0	26.8	8.3
		18/09/06	10	9.0	1.0	7.6(<LOQ)	10.6	8.9
		10/11/06	10	14.3	4.6	7.9(<LOQ)	20.4	14.4
Reactive	Ronas Voe	01/08/06	10	25.0	28.4	8.3(<LOQ)	100.9	12.9
		22/08/06	10	12.9	2.5	7.7(<LOQ)	15.8	13.4
		05/09/06	10	25.6	7.8	14.7	42.1	24.6
		03/10/06	10	50.8	44.2	13.3	139.0	34.0
		18/10/06	10	50.1	35.2	12.6	113.5	39.0
	Troon	07/07/06	5	6.97	3.92	0.00	9.51	8.46
	Fraserburgh	07/07/06	5	69.08	48.09	12.94	143.39	61.38
	Vementry	041006	10	43.6	26.0	12.1	101.9	37.2
	Muckle Ossa	051006	10	41.9	23.4	17.3	88.1	36.6

The random sampling distribution of PSP toxin levels detected in the brown crab meat from the 2006 is shown in Figure 20; only 2% (three crabs out of 150) were over the regulatory limit. When the random and reactive samples seen in Table 19 are combined for analysis (not shown), 5% of samples were above the regulatory limit, 67% were above LOQ but below the regulatory limit and 28% were below LOQ. This distribution is very similar to that observed in 2005 (Figure 19).

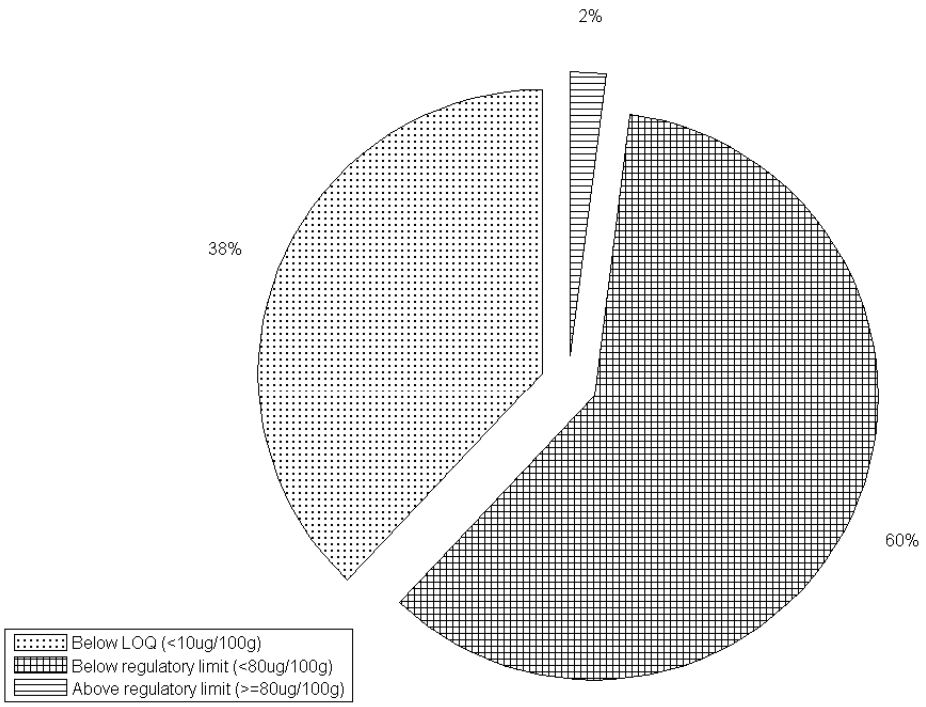
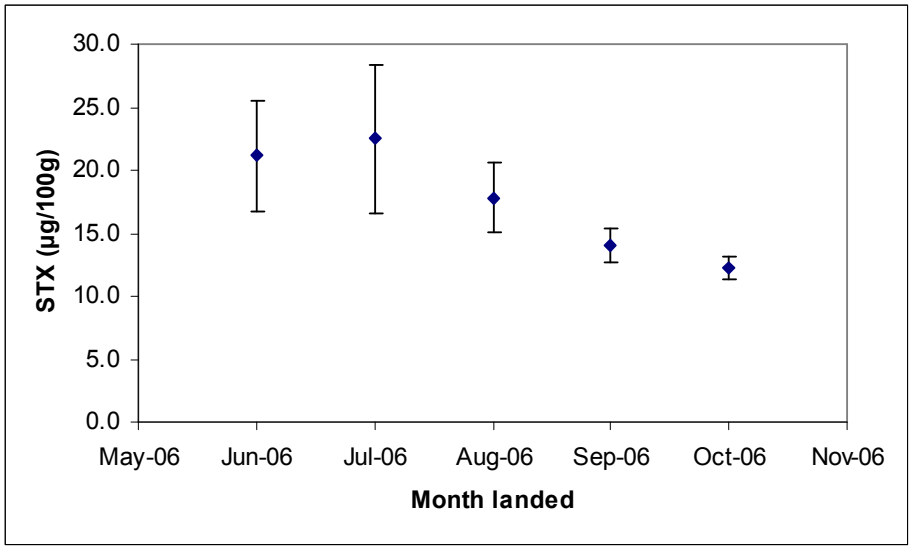


Figure 20: Pie chart of the PSP distribution for random crab samples of brown meat tested in the 2006 season (n=150).

The mean PSP concentrations detected in all the individual crabs landed from Oban, Lochinver and Scrabster per sample month were plotted (Figure 21). The graph indicates that, during the period of the study, the highest mean levels of PSP were found in crabs sampled during June and July.



Error bars represent standard error

Figure 21: Mean PSP concentration for each sampling month.

4.3.2.5 Analysis of PSP profiles by Fluorescence HPLC in samples collected during 2006

A selection of crabs previously shown to have different levels of PSP toxins (by the Ridascreen Fast Saxitoxin kit) were tested by Fluorescence HPLC. The purpose of the HPLC work was to show the peak profile rather than quantification of the toxins present as the HPLC technique is not validated for this matrix. The resultant combined toxin content (Table 20) obtained by HPLC was plotted against the Ridascreen results and a trendline fitted (Figure 22). Although the Ridascreen kit tended to give a higher reading than the HPLC, there was a correlation between the two tests, although the R^2 value was below 0.9.

Putative GTX1/4 was found in all samples, including the pooled material that had tested negative by Ridascreen. No other toxins were detected in the negative material. Saxitoxin (STX) and GTX2/3 were regularly detected in samples that were positive by Ridascreen. GTX5 was detected in only two samples. The other toxins were either not detected at all or at levels below the lowest standard (dc-STX). Figure 23 shows an example chromatogram of a crab sample extract containing several PSP toxins. The dc-STX peak had a smaller peak area than the lowest standard for dc-saxitoxin, and was therefore not reported. As with all other samples, periodate oxidation was sufficient to enable the toxin profile to be identified in this sample.

Table 20: Profile of PSP toxin analogues (µg/100g) detected by Fluorescence HPLC in selected brown meat samples collected during 2006

Sample Location	Integrin Sample ID (FSA04- No.)	GTX 1/4	GTX 2/3	STX	GTX5	DC-STX	NEO-STX	DC-NEO	DC-GTX 2/3	Total	Rida-screen
Oban	210806-POOL-02*	8.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0	0.0
	210806-POOL-01*	5.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.7	0.0
Oban	190606-04-B	4.9	0.0	15.5	0.0	0.0	0.0	0.0	0.0	20.3	56.0
	050606-03-B	7.5	0.0	14.3	0.0	0.0	0.0	0.0	0.0	21.8	91.0
	050606-09-B	5.9	4.9	0.0	0.0	0.0	0.0	0.0	0.0	10.9	21.0
Scrabster	200606-02-B	7.4	3.5	0.0	0.0	0.0	0.0	0.0	0.0	10.9	19.0
	200606-05-B	10.5	15.7	9.2	0.0	0.0	0.0	0.0	0.0	35.5	108.0
	200606-10-B	6.7	3.9	4.2	0.0	0.0	0.0	0.0	0.0	14.8	24.0
Fraserburgh	070706-06-B	17.6	4.9	35.6	3.2	0.0	0.0	0.0	0.0	61.3	143.0
	070706-07-B	3.3	3.8	17.8	6.7	0.0	0.0	0.0	0.0	31.6	48.0
	070706-10-B	8.3	13.6	9.6	0.0	0.0	0.0	0.0	0.0	31.5	61.0

** pool of 10 crabs previously shown to be negative by the Ridascreen Fast saxitoxin kit run as a control. FSA04 Number the sample ID prefix used by Integrin for these samples*

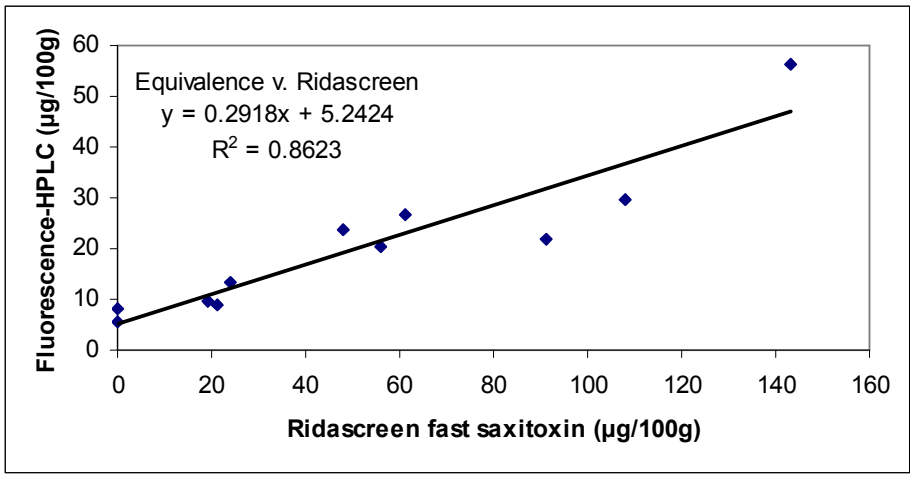


Figure 22: Comparison between the Fluorescence HPLC and Ridascreen Fast Saxitoxin kit.

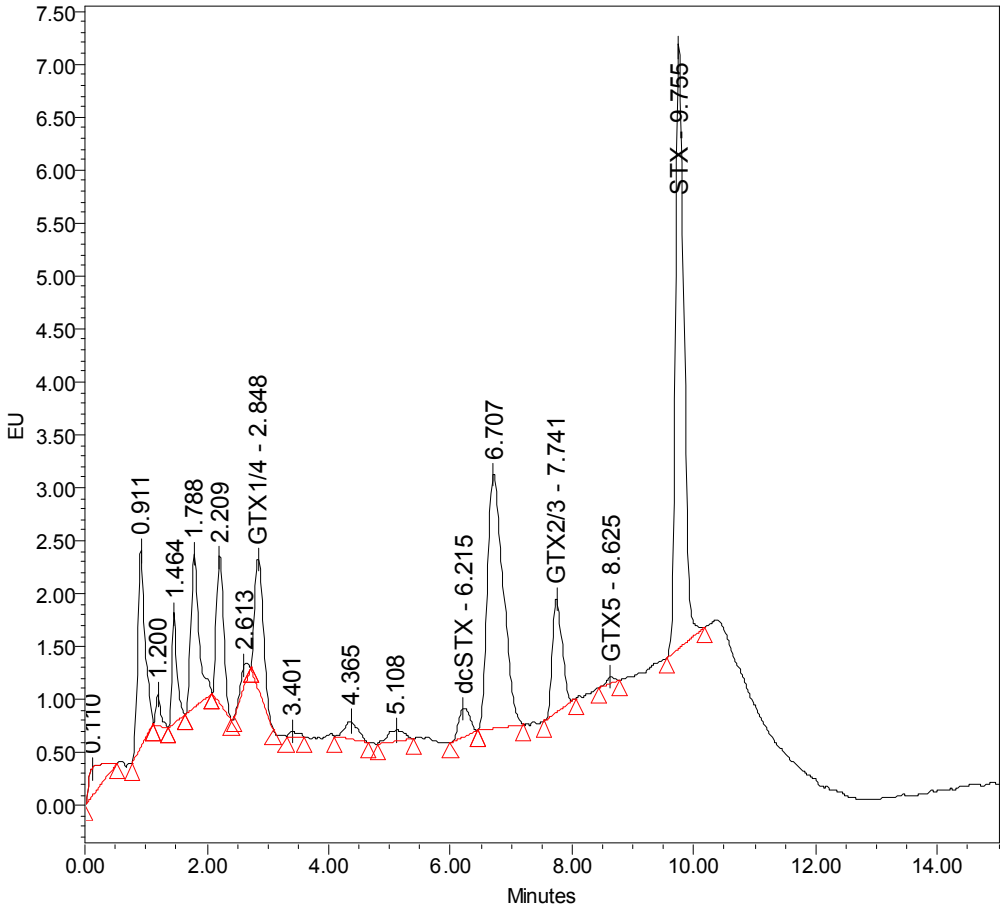


Figure 23: Example chromatogram showing several PSP toxin peaks obtained from crab sample FSA04-070706-06-B

4.3.2.6 DSP in Targeted and Reactive samples collected during 2005 as detected by PP2a

None of the samples from the 2005 targeted sites tested for DSP toxins using the PP2a assay were positive, with all 90 samples tested being below the limit of quantification (LOQ) of 10 ug/100g.

4.3.2.7 DSP in Reactive samples collected during 2006 as determined by LC-MS/MS

Out of the 50 samples tested, 11 were above LOQ (22%) for okadaic acid (OA) group toxins and about 30% contained DSP toxin analogues (OA, DTX1 and esters). Results from hydrolysed samples are shown in Table 21. The process of hydrolysis partially deteriorates OA and DTX1, totally deteriorates PTX2 and SPX1 but quantifies the dominant "DTX3" toxins (*i.e.* the esters). DTX2 toxins were not quantified because of a lack of standards.

Table 21: Mean total OA and DTX1 (µg/100g) concentrations detected in samples collected during 2006 .

Where	Sample ID	Mean	STD	Min	Max	Median
Ronas Voe	FSA04-010806-01 to 10-B	0.1	0.2	0.0	0.6	0.0
	FSA04-220806-01 to 10-B	1.3	1.7	0.0	4.7	0.7
	FSA04-050906-01 to 10-B	0.6	0.9	0.0	2.3	0.0
	FSA04-031006-01 to 10-B	0.2	0.5	0.0	1.6	0.0
	FSA04-181006-01 to 10-B	0.4	0.8	0.0	2.2	0.0

Results not corrected for recovery

Example chromatograms from the okadaic acid and DTX1 MRM channels of crab sample extracts are presented in Figure 24 and Figure 25 respectively. Clear, unambiguous detection is demonstrated in both chromatograms.

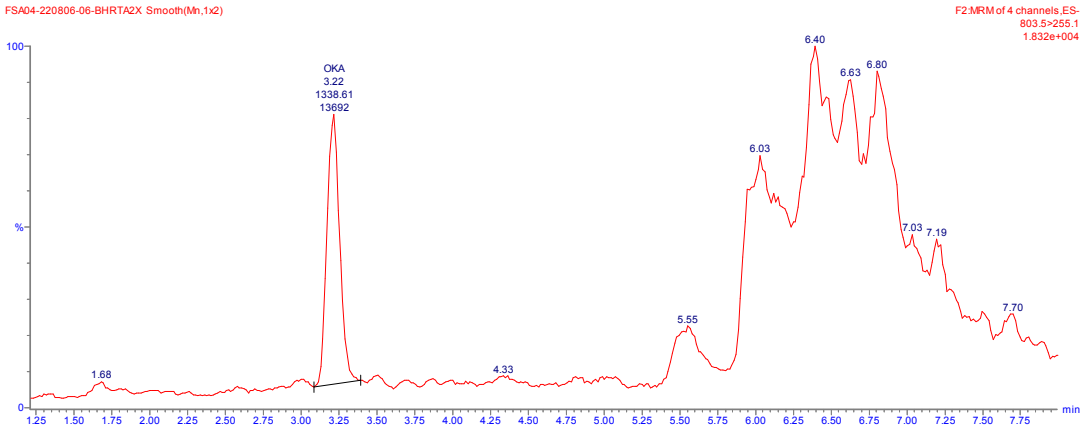


Figure 24: Example Chromatogram showing peaks from crab sample FSA04-220806-06-B with Quantifiable Levels of okadaic Acid (OA)

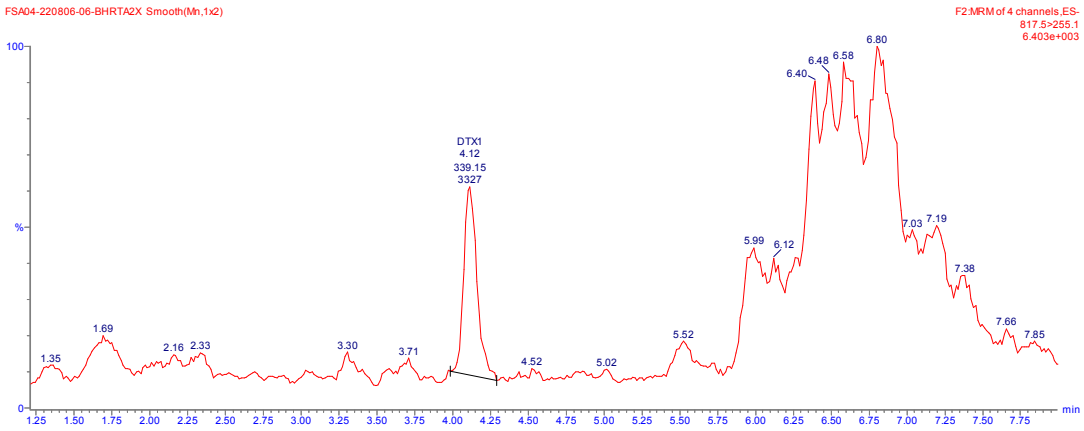


Figure 25: Example Chromatogram showing peaks from crab sample FSA04-220806-06-B with Quantifiable Levels of DTX1.

The levels of OA and DTX1 quantified by LC-MS/MS were very low. However, the validation studies suggested that the method may be underestimating these toxins and its value here is in confirmation of the presence of OA and DTX1 in the hydrolysed samples.

4.4 DISCUSSION

4.4.1 Validation of methods:

ASP toxins were quantified using the regulatory HPLC-UV method, which proved to be robust with both brown and white meat matrices (see section 4.2.6.1). The ASP samples tested during 2006 showed evidence of peak interference that was not present in the crabs tested in 2005. This interference was only evident in some samples that contained low concentrations of DA (at around 2 mg/kg or less) and may be due to the way the samples were handled between the time landed and arrival at Integrin. The effect of this interference to the overall results is however negligible, as it occurs at low levels of DA at or around the lower limit of quantification and the significant findings are those at or over the regulatory limit.

The PSP toxins could be quantified in Brown crab matrices using the Ridascreen Fast Saxitoxin assay with no matrix interferences observed. The Ridascreen is, however, an entirely quantitative method and does not provide information on which PSP toxins are present. Further analysis by fluorescence HPLC was used principally to provide information on the toxin profile. Overall, crab toxin profiles observed were dominated by STX and GTX 2/3. A peak consistent with GTX 1/4 was detected in every sample but false positive results in shellfish have been found associated with this peak, particularly when no other PSP toxin peaks are observed. Samples that were negative by Ridascreen also showed GTX 1/4 to be present by HPLC – although at levels that would be expected to be below the LOQ of the Ridascreen assay. This may also be the case with the crab tissue and therefore the presence of GTX 1/4 can only be described as putative. GTX 5 was encountered at low levels (3.2 – 6.7 µg/100g) in samples from Fraserburgh. A profile where STX and GTX 2/3 dominate will result in good quantification at all concentrations using the manufacturer's standard methodology for the Ridascreen assay as both of these toxins demonstrate strong cross-reactivity with the antibodies used in the assay (STX 100%; GTX 2/3 70% - manufacturer's data).

DSP toxins are actually a polymorphic group of unrelated, lipophilic toxin groups. Previous studies have indicated that the main toxic group of concern in Scottish waters (Smayda 2006) are the okadaic acid and dinophysis toxins, which are all phosphatase inhibitors. Therefore, the preferred assay for quantifying DSP in crabs sampled in this project was the PP2a inhibition assay. The validation work showed that this assay was suitable for use with the crab matrices. Like the Ridascreen assay, the PP2a is strictly a quantitative assay, giving no information about the toxin profile. It has a hydrolysis step to convert any okadaic acid or dinophysis toxin esters to the native toxin, so that a total toxicity is obtained.

As no PP2a inhibitors were detected during the 2005 season, and due to problems in the supply of enzymes for the PP2a assay, LC-MS/MS was used to analyse reactive samples taken during the 2006 season. This method is more sensitive than the PP2a assay and can discriminate individual toxins – including DSP toxins other than the okadaic acid and dinophysis toxins. The technique is not yet fully validated with all lipophilic toxins and with all matrices, even in shellfish, so its value in this study was mostly in terms of revealing information about toxin profile.

The validation work demonstrated that the assays used were suitable for their purpose and the crab matrices studied did not present any unusual challenges to their analysis.

4.4.2 Feeding experiments:

The toxification of mussels with the slow-growing DSP toxic algae *Prorocentrum lima* was successful: the brown crabs consumed the mussels and accumulated DSP toxins. The levels seen were higher than were encountered in wild crabs in 2006 (see 4.3.2.7) indicating that Scottish *C.pagurus* can uptake significant quantities of DSP toxins when the conditions are correct.

The ASP feeding experiment showed that ASP toxins were accumulated in Brown crab and uptake was approximately linear (Figure 15). One crab showed a non-linear relationship (*i.e.* it had accumulated less toxin despite having eaten more scallops than some of the other crabs). This can be attributed to one or all of the following: toxin level inter-variability in the scallops, different crab depuration rates or selectivity of the crab's feeding *i.e.* the crab did not eat the entire scallop. The crabs that consumed one, two and three king scallops show a linear uptake of domoic acid, which may indicate more consistency of depuration rates, scallop toxicity and feeding behaviour in these crabs.
Where DA levels in the whole scallops being fed to the crabs were >250 mg/kg, the crabs only consumed 1-2 scallops and then stopped feeding altogether. However, when the crabs were given scallops with lower whole animal DA levels (~85 mg/kg); they appeared to feed, on average, on one scallop per day for five days. This finding may indicate that crabs have a feeding preference threshold for toxic shellfish or a toxin-tolerance threshold, where high levels of domoic acid (and perhaps other toxins) may inhibit or stop feeding. It is also possible that some crabs may discriminate between the different tissues of the scallops and avoid the more toxic parts. Alternatively, some crabs may be more efficient at depurating ASP toxins than others.

4.4.3 Wild samples:

When the project was being formulated, a sampling strategy of targeting toxin "hot spots" was preferred over a random sampling approach to maximise the possibility of detecting toxins in the crabs.

The 2005 sampling started late in the toxin "season" so there was the possibility that no PSP toxins would be detected as they had already been depurated from the crabs. There were very few closures for any of the toxin groups as a result of the Food Standards Agency Scotland's statutory bivalve monitoring programme during 2005. It was therefore unexpected that the majority of crabs sampled in 2005 contained measurable levels of both ASP and PSP toxins. A large proportion of the wild crabs tested during 2005 contained ASP and PSP toxin levels well below the bivalve regulatory limits but the detection of both toxins had not been anticipated in such a large number of brown meat samples. Although some of the crabs tested during 2005 contained concentrations of PSP and ASP toxins exceeding bivalve regulatory levels (80 µg/100g and 20 mg/kg respectively), it is worth emphasising these high concentrations were only identified in the brown meat. No ASP toxins and only very low levels of PSP toxins were identified in the claw meat.

A high percentage of crabs sampled randomly during 2006 were found to contain PSP toxins in the brown meat. However, PSP levels in individual crabs were variable and only 2% of random samples were found to contain levels of PSP in the brown meat that were greater than the regulatory level set for bivalves. However, adding the reactive PSP samples to the data set resulted in a very similar picture to that obtained in 2005. Differences were observed in the PSP levels in crabs sourced from different areas (Scrabster, Lochinver and Oban) during the 2006 random sampling. Higher PSP levels were detected in the brown meat of crabs sourced from Scrabster compared with the two more southerly areas.

During 2006, less than 1% of crabs sampled randomly were found to contain levels of ASP toxins in the brown meat that exceeded the bivalve regulatory limit, compared with 7% in 2005. DSP toxins were detected using LC-MS/MS in crabs sampled reactively following a biotoxin event at Ronas Voe but only at very low levels.

This study had been prompted by the outbreak of clinical DSP in Norway that had been linked to consumption of *Cancer pagurus* so it was natural to suspect that DSP toxins would be more commonly encountered than ASP or PSP toxins. However, no DSP toxins were detected in the Scottish Brown crabs during 2005, although, as for PSP and ASP, there were very few DSP toxic events during 2005. It may be that *Dinophysis* blooms (the organism usually responsible for producing DSP toxins) are more localised than *Psuedonitzschia* or *Alexandrium* blooms (the organisms usually responsible for producing ASP and PSP toxins respectively). Large areas of Scotland are affected by ASP toxins as evidenced by their ubiquitous presence in Scottish scallops (McKenzie, 2004). Brown crabs may, therefore have access to prey items containing ASP toxins independent of actual localised blooms of *Pseudonitzschia*. PSP toxins are also widespread in occurrence around Scotland (Smayda 2006).

This hypothesis that the observed differences in ASP and PSP toxin content compared to DSP is possibly simply a result of the crabs' access to toxins, is supported by the 2006 data. In 2006 there was a dramatic increase in both DSP and PSP closures (FSAS Monitoring Programme data) and a number of product recalls. Scallops and other bivalves in the North Sea were affected by PSP toxins early in the summer and there was a general northward "drift" on the West Coast of closures due to PSP toxins as the year went on. Shetland particularly suffered, with PSP closures in place through to early winter. DSP closures occurred on the West Coast and there were actual poisonings in the UK, due to consumption of mussels from Lewis. ASP toxins, on the other hand, were not detected at levels high enough to result in any closures in bivalves other than scallops, and the usual *Pseudonitzschia* blooms were very weak (FSAS bivalve and phytoplankton Monitoring Programmes). Although the data are far from complete, there did seem to be an increase in DSP toxins, a maintenance of PSP

toxins and a decline in the prevalence of high ASP toxin levels in the crabs. This seemed to follow the changes in toxin activity seen in bivalves in 2006 compared with 2005.

However, other factors in addition to toxin availability could have affected the results. The 2005 and 2006 DSP samples were analysed using different methods. For the 2006 season, all crabs were sourced from one area, which did have DSP in the local shellfish (FSAS bivalve monitoring programme data). The first batch of crabs was obtained from Ronas Voe when the FSAS phytoplankton monitoring data documented the *Dinophysis* of toxic phytoplankton were present at 2.6 times the trigger level of 100 cells/L, although the mussels tested were negative. The Ronas Voe area was then closed to bivalve harvesting due to DSP toxins one week before the second batch of crabs was obtained and the area remained closed during the crab-sampling period. The tested crab results show the presence of DSP toxins in all batches but at low levels and there was no increase in the levels detected over time. In 2006, there was a *Dinophysis* bloom in Loch Roag (Lewis) that led to high levels of DSP toxins in cultured mussels and subsequent human poisonings, but unfortunately it was not possible to obtain crabs from this area to look for evidence of uptake of DSP toxins in the wake of the bloom.

The absence of ASP toxins in the white meat is reassuring and reflects the situation in bivalves, where the ASP toxins are generally localised in digestive tissues. The PSP results showed that low levels of PSP toxins were present in the white claw meat. Again, this is similar to the situation in bivalves where PSP toxins may be detected in muscle tissues (Sagou *et al.* 2005). As white meat is more regularly consumed than brown meat, the potential for PSP contamination in the white meat of crabs merits further investigation.

5 GENERAL DISCUSSION AND CONCLUSIONS:

The crab fishery in Scotland is an important and stable inshore fishery. It is worth approximately £10 million per year to the fishing fleet and provides for, or at least contributes to, the employment and income for a large number of vessels and crew. The UK is the world's leading supplier of this species with a 50% market share (2002/2003 average – Seafish Brown Crab Export Study, 2005). There is a healthy domestic market for this species in addition to the major export markets on the Continent.

In the UK, crabs are not generally associated with biotoxins and we have been unable to find any conclusive records of biotoxin poisonings of consumers in the UK that were caused by eating crabs. However, recent incidences of crab poisonings from Europe and the more general incidence of crab poisoning worldwide have highlighted the potential of these animals as a route for human poisonings from biotoxin producing marine micro-algae. In this study, the crab samples obtained from feeding experiments, and targeted, reactive and random sampling carried out in Scotland during 2005 and 2006 indicated that ASP and PSP toxins can occur in Brown crabs. Although only very low levels of DSP toxins were detected in wild crab samples taken in 2006, the feeding experiments demonstrated ready uptake of these toxins.

As it was difficult to ascertain what crabs had been feeding upon in the wild, it was not possible to demonstrate correlations between the toxin levels in harvested species of bivalves and in crabs sampled in the same general area. However, the widespread presence of PSP and ASP toxins in crabs suggests that Brown crabs readily take up

these toxins in their diet and that they may have difficulties in completely depurating them from their tissues. To our knowledge, this was the first record of ASP toxins in crabs sampled from UK waters.

Crabs sampled from the same place at the same time showed high variability in toxin levels, particularly for ASP. This is in line with the findings from previous studies on other species (Oikawa *et al*. 2004, Campbell *et al.* 2001). The feeding experiments carried out during the course of this study showed approximately linear uptake of toxins when crabs were fed the same diet suggesting that the large variability observed in wild crabs was probably due to individual crab feeding habits rather than differences in their biochemistry. *C. pagurus* eat what is available to scavenge but can show preferential feeding when hunting (Mascaro and Seed 2001). The highest values for domoic acid in bivalves from Scottish waters are associated with King Scallops and an obvious hypothesis is that crabs with high concentrations of ASP toxins encountered during the current study are likely to have been feeding on scallops. However, we have very little knowledge on toxin levels in most wild bivalves and other non-commercial invertebrates so there may be alternative food webs in play. There is some anecdotal evidence that scallop viscera are sometimes used to bait crab creels. Although it is actually illegal, it is worth highlighting the potential risks associated with this practice, as (depending on how long the crabs are in the creels and how much scallop viscera are available); it may result in crabs having high domoic acid levels.

The laboratory feeding experiments suggested that crabs may avoid scallops containing high levels of ASP toxins in their tissues. This may indicate a threshold of toxin tolerance, although the crabs displayed no other obvious behavioural changes that might have indicated a direct physical effect of the domoic acid on the ability to feed.

This study has shown that PSP and ASP toxins were very common at low levels in Brown crabs. The Norwegian poisoning incident suggests that, where the conditions are appropriate, crabs will also uptake DSP toxins. Only a small percentage of crabs had ASP and PSP toxin levels that were above the bivalve regulatory limits but if this percentage was extrapolated to the total fished biomass, then there is the potential for contaminated crabs entering the market. The survey of landings show that, within Scotland, crab fisheries are significantly located in areas that normally undergo toxic plankton events (Orkney and the Scottish West Coast). Peak fishing periods also coincide with likely peak toxin production periods. High levels of toxic phytoplankton in an area will probably lead to elevated toxin levels in crabs provided the crabs' prey has fed on the phytoplankton (either directly or indirectly). Individual variability in the levels of toxins present is likely to be high, but the higher the bloom activity, the higher the levels will be in the crabs generally.

The current Regulations require the statutory bivalve monitoring programme and associated phytoplankton monitoring programme to target classified shellfish production areas and consequently, does not provide full coverage of Scottish waters that may be affected by biotoxins. Of particular concern are Orkney and the far North East Coast, where there are very few classified shellfish sites but these areas include a very large crab fishery. With the suspension of the offshore scallop monitoring programme, the amount of monitoring information available to the crab industry in these areas has therefore significantly reduced.

This report clearly indicates the need for a more detailed study of the risk that marine biotoxins in crabs pose to consumers and whether any action needs to be taken by the Regulator and industry in order to mitigate any risk.

It is important, however, not to overstate either the findings of this project or the associated perceived risk. Sample sizes were modest and may not be wholly representative. This was particularly true of the 2005 data where the sampling was targeted at areas of perceived high risk. Extrapolating from the data should thus be made with caution. The bivalve regulatory levels were set based on risk assessments that assumed a portion size of 250 g. The toxicity of crabs appears almost exclusively associated with the brown meat (though PSP toxins were present at low levels in the claw meat). A portion size-study may also find that average consumption of crab brown meat is considerably less than 250 g, which would require a different concentration level to be set for crabs than for bivalves. There is little published guidance available from other authorities. The US Food and Drug Administration (FDA 2001) gives 80 µg/100g as the limit for PSP toxins in crabs, which is the same for bivalves. They, have, however a limit of 30 µg/g for ASP toxins in the viscera of Dungeness crabs (*Cancer magister*), as opposed to the bivalve limit of 20 µg/g (20 mg/kg).

The extreme individual variability from a single area is difficult to manage as it is not clear what this equates to in terms of risk. Bivalve samples are pooled so that individual variability is negated. This would be a more expensive practice with crabs and an individual portion may in any case be a single crab. However, as the brown meat is the tissue of highest risk, pooling and thoroughly mixing brown meat from many crabs may reduce the risk posed by individual crabs that have anomalously high levels of toxins due to dilution of the toxins. However, if toxin levels are generally very high in the crabs then the processed product will still have dangerously high levels of toxins associated with it. End product testing for the presence of toxins would be an effective control measure for processed brown meat products. It is much more difficult to think of an effective strategy for detecting toxins in crabs that are sold whole and avoiding fishing for crabs in areas affected by high levels of toxic phytoplankton would seem to be the only effective measure. Cooking during processing has unpredictable effects on biotoxins depending on the actual toxins present and the method of cooking employed. This area was not covered by the current study and may benefit from further investigation.

5.1.1.1 Recommendations and further work

1: The crab industry and local authorities should be made aware of the potential of crabs in Scottish waters to uptake all three groups of marine biotoxins.
2: The Agency should consider what advice to issue to local authorities and industry. Particular consideration should be given to extending the advice given in relation to PSP toxins to ASP and DSP toxins
3: The Agency should consider whether it is necessary to establish guideline levels for biotoxins in crabs and how such levels should be measured in different meat types.
4: The Agency should consider how to prevent crabs from being fished in areas where there is a danger of high levels of toxins entering crab tissues. This should be in reference to recommendation 3.
5: Discussions should be held with industry and local authorities to agree a code of best practise in relation to biotoxins in crabs.
6: The effects of processing on biotoxin levels in crabs should be determined. More detailed studies should be conducted on the distribution of toxins in specific tissues to determine possible processing strategies. The effects of cooking; canning and production of pastes etc on toxin levels should also be determined.
7: Industry should consider the need for further work to be conducted to investigate the uptake of biotoxins in other species of crabs, particularly Green Shore Crab (*Carcinus maenas*) and Velvets (*Necator puber*), to compare biotoxin uptake in these species.

8: Because of the prevalence of toxins in the brown meat rather than in the white meat, other crustaceans such as lobsters and prawns probably represent a much lower risk and need not be a priority for research as the white tail meat is what is normally consumed. Predatory species, such as lobsters, will however, probably have similar toxin contents and toxin distribution to Brown crab and thus industry norms regarding the fate of head tissues in these species should be ascertained to ensure that these are not another possible vector of biotoxins into humans.
9: COT should be made aware of this study and consider how a toxin specific risk assessment for crabs should be conducted.
10. Consideration should be given to the need to widen the geographical coverage of this study to consider the prevalence of algal biotoxin uptake in crabs in fishing areas throughout the UK

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7 APPENDICES

Appendix 1: The data supplied by the Scottish Government Statistics Department. The data comprised all declared brown crab landings from January 2000 to August 2004. Also included are the landed crab tonnage and value statistics for each year and each port and a description and the definitions of the fields.

Appendix 2: Individual maps showing the areas crabs were obtained for this study, *i.e.* the targeted, reactive and random sections for 2005 and 2006.

Appendix 3: The Database containing the date and location the crabs were sourced and the levels of toxicity each contained.

Appendix 4: Domoic acid confirmation using LC-MS/MS.

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