Distribution of DSP toxins in King Scallops (*Pectin maximus*) and the effects of current shucking practises on toxin concentrations in edible tissues

FINAL REPORT

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1 Summary

King Scallops (*Pecten maximus*) were fed *Prorocentrum lima*, a producer of lipophilic toxins responsible for diarrhetic shellfish poisoning (DSP), in order to artificially contaminate the scallops with Okadaic acid (OA) and dinophysis toxin (DTX). The distribution of the toxins within the different tissue components of the scallops was determined using a PP2A inhibition assay. The effect of different washing techniques on the concentrations and distribution of the toxins was also examined. The highest DSP toxin concentrations were detected in the remainder sub group samples comprising gut, mantle and other visceral tissues. Concentrations of OA in all the adductor muscle only samples were not quantifiable as they were below the limit of quantification (100 µg OA equ/ kg) for PP2A inhibition assay .Results for the Gonad only tissue group revealed highly variable levels of toxicity, and over 70% of individual gonads contained concentrations of OA and DTX 1 and their esters which exceeded regulatory limits. In all samples the presence of OA and DTX1 esters was demonstrated by an increase in DSP toxin concentration after hydrolysis. LC-MS/MS analysis of the algal culture used in feeding experiments determined the ratio of OA to DTX1 to be 2:1 within non hydrolysed samples and 4:1 following hydrolysis, indicating the presence of OA and DTX esters. With LC-MS/MS analysis detectable levels of DTX1 and an increase in OA concentration of over 95% was found in King Scallops subjected to alkaline hydrolysis. This demonstrated the importance of the hydrolysis step in determining true OA/DTX concentrations in contaminated shellfish. The experiments showed that shucking the king scallops reduced the OA and DTX concentrations to below the limit of quantification of the PP2A inhibition assay (10 µg OA equ /100 g).

2 Introduction

It is well documented that almost all (>95%) of the biotoxin responsible for Amnesic Shellfish Poisoning (ASP), Domoic acid (DA), in King Scallops (*Pecten maximus*) is associated with the hepatopancreas, mantle and gills and that the risk of poisoning from this toxin group can be reduced by removing these tissues prior to consumption (McKenzie *et al*, 2002). This technique is referred to as shucking and it is now recognised as an important process in controlling the risk of ASP from King Scallops. As a result it is now incorporated in the HACCP plans of most UK scallop processors as per Seafish guidelines (Seasider Scallops Ltd, HACCP plan for Chilled Shucked Scallops, 2007). These guidelines outline the appropriate method of shucking the King Scallops starting with deshelling and removal of the scallop meat which comprises the adductor muscle and roe only. A preliminary washing step involving a quick rinse of the scallop meat is undertaken using a plastic colander, followed by a critical inspection and trimming of any remaining 'black material' and viscera if required. The muscle and gonad are then vigorously washed for 2 minutes under flowing water, drained and packed.

To date, shucking has been assumed to be equally effective in removing the algal biotoxin groups responsible for Paralytic Shellfish Poisoning (PSP) and Diarrhetic Shellfish Poisoning (DSP). However, whilst there is a wealth of information available on the distribution of ASP toxins in King Scallops and their removal through shucking practices (Campbell D. A. *et al* 2001 and 2003) there is a lack of equivalent scientific evidence relating to PSP and DSP. This information is required to determine whether current shucking practices used for ASP also provide a sufficient safeguard to protect public health from PSP and DSP toxins. The Food Standards Agency Scotland (FSAS) has funded a study through Workpackage 4 of the EC

funded project SPIES-DETOX, which aims to optimise shucking and washing practices for removal of ASP and PSP biotoxins. This study aims to complement the work already undertaken on ASP and PSP by determining the distribution of DSP toxins in King Scallops, and the effectiveness of current shucking protocols in removing these toxins from the edible tissues.

The DSP toxins are a diverse group of lipophilic toxins. This study focused on the phosphatase inhibiting lipophilic toxins: Okadaic acid (OA) and dinophysis toxins (DTXs) as these are the most common cause of DSP in Scottish shellfish (Stobo L.A. *et al* (2008), Food Standards Agency S14033, Marine Biotoxins-Shellfish Poisons- AE15Shellfish.pdf). Due to the seasonal nature of DSP contamination, and difficulties associated with obtaining naturally contaminated shellfish from the field during the course of this study, artificially toxified scallops were employed as the basis of the experimental programme. Natural contamination of shellfish with these toxins is usually associated with *Dinophysis* blooms. However, these dinoflagellates are difficult to culture under laboratory conditions and therefore a more tractable dinoflagellate, *Prorocentrum lima (P.lima)* was used in this study. Toxic strains of these algae which primarily produce OA and DTXs are commercially available and are routinely cultured in large volumes at this laboratory.

In this study a series of experiments using King Scallops toxified with *P.lima* were designed to determine 1) the distribution of OA and DTX toxins within different scallop tissues and 2) the potential of shucking practise to reduce OA and DTX concentrations in the processed product which comprises the adductor muscle and gonad. Ester derivatives of OA and DTXs are known to occur within shellfish tissues during contamination (Mountfort *et al*, 2001) and the concentrations of these esters can be determined by the inclusion of alkaline hydrolysis step in the preparation of the samples (Mountfort *et al.*, 2001). Within the study, samples were tested with and without this hydrolysis step in order to determine the amount of esterification of OA and DTXs in the different tissues.

3 Objectives

The current study aimed to provide Food Standards Agency Scotland (FSAS) with a firm understanding of the tissue distribution of DSP toxins in King Scallops and of the likely effects of shucking practises currently employed by the industry on the levels of toxicity in processed shellfish. It is intended that the data provided by this study will be used by the FSAS to supplement existing guidance on shucking and thus help to minimise the risk of consumers being exposed to DSP toxins in processed King Scallops.

<u>The specific objectives of this study were:</u>

1: To determine the optimal conditions for the artificial toxification of King Scallops with OA and DTX toxins.

2: To determine the distribution and modification of OA and DTX toxins in contaminated King Scallops.

3: To determine the likely effects of shucking practises currently employed by King Scallop processors on OA and DTX concentrations in end product.

These objectives were addressed in three separate experimental modules.

4 Materials and methods

4.1 Animal husbandry

King Scallops for use in the study were dive-caught from waters local to Integrin's Barcaldine Laboratory. On delivery the scallops were allowed to acclimatise in large holding tanks before being moved to 120L experimental raceways situated in the controlled temperature (CT) facilities at Integrin. The temperature of the water in these raceways was kept chilled at 10-12 degrees centigrade using a LKB Bromma, 2219 multi-temp II chiller unit. The room temperature in the CT facilities remained between 18-20 degrees centigrade throughout the studies. Figure 1 shows the raceway arrangement used to house scallops during all the feeding studies.

Figure 1. Raceway experimental tanks arranged in CT room with King Scallops.

4.2 Algal culturing and feeding experiments

Algae for use in the feeding studies were grown within Integrin's CT facility in Oban. Practices employed for the feeding experiments were as described previously in FSAS project S14017 which examined the uptake of mussel DSP biotoxins by crabs; *Cancer pagurus* (Vincent *et al*. 2007). Briefly, *P.lima* (PL2V from The Culture Collection of Algae and Protozoa (CCAP); Appendix 1) was grown in staggered cultures (Figure 2). One litre cultures were scaled up in increasing volumes and culture bags with a final volume of 60 litres provided sufficient toxic algae for each experiment. *P.lima* are slow growing algae and culture cycles take approximately three weeks (from inoculum to on-growing or harvesting). See Appendix 2 for details of media and culture conditions. The cultures were fed to the scallops using the raceway system (Figure 1). The target concentrations of toxin used in this study were based on whole animal concentrations and were in excess of 500 µg OA equ/ kg of shellfish flesh (the regulatory limit for OA and DTX toxins in shellfish is 160 µg OA equ/ kg).

Figure 2. Algal cultivation in carboys

4.3 Processing of King Scallops

King Scallops were processed in accordance with Seafish guidelines excluding the 2 minute wash step that follows inspection and trimming. Where the whole animal was required, shells were opened using a sharp knife and the contents were removed into a plastic colander, washed briefly under running water and prepared for testing. When specific tissue groups were required the following method was employed: hold the shell between the fingers and thumb, with the flat side of the shell against the thumb, draw a sharp knife down the shell to release the adductor muscle from the shell. Discard the flat side of the shell leaving the whole scallop in the other side. Make a clean cut across the foot of the scallop taking care not to puncture the hepatopancreas (gut sack). Cut away the gill and mantle and remove the adductor muscle and gonad from the other tissue. Wash the muscle and gonad briefly under running water. On a clean surface inspect the muscle and gonad to ensure all 'black material' (alimentary track) has been removed and no remnants of visceral tissues remain, trim if required. Wash the muscle and gonad briefly under running water. Prepare for testing.

Due to the epiphytic nature of *P.lima* the algae prefers to adhere to and grow on surfaces (Foden *et al*, 2005). Consequently, feeding trials resulted in the outer part of the shells becoming coated with algal particles. In order to ensure the scallop tissue was not contaminated by abundant highly toxic algal particles, the outer scallop shells were cleaned and rinsed prior to shucking in all the experimental modules.

4.4 DSP toxin analysis

The strain of *P.lima* employed in this study produces predominantly OA and its esters as well as smaller quantities of DTX1 (Bravo *et al*, 2001). These toxins are protein phosphatase inhibitors and a UKAS accredited in-house protein phosphatase (PP2A) inhibition assay was used to detect and measure the OA and DTX 1 present in the algal and shellfish samples. This sensitive assay allows large numbers of samples to be screened quickly and easily. Preliminary experiments determined the concentration of DSP toxins per algal particle at approximately 1.6 pg OA equ /cell. Further details are given in Appendix 3.

The PP2A inhibition assay parameters were:

Limit of Quantification (LOQ): 100 µg OA and DTXs 1-3 OA equ/kg

Limit of Detection (LOD): 50 µg OA and DTXs 1-3 OA equ/kg

The method works by measuring the amount of inhibition of human PP2A in the presence of Okadaic acid or DTX toxins. Within a certain range the inhibition of serine/ threonine protein phosphatase type 2A is proportional to the concentration of OA and DTX's in solution. Inhibition of this enzyme by OA standards of known concentration allows generation of a standard curve against which the OA and DTXs in extracts from the shellfish samples can be quantified in total OA equivalents. Fluorescent protein phosphatase inhibition assay procedures are based on those of Mountfort *et al*, (2001). The assay was adapted to run as a kinetic assay using the FL600 plate reader. All assay components, other than enzyme and buffer, are combined in a single buffer solution, prepared in large volumes and stored frozen. The final simplified assay allows the rapid testing of up to 16 samples in a 96-well microtitre plate through the mixing in each well of enzyme, sample or standard, and buffer with substrate. The assay is then read over a 30 minute period and the concentration of samples is automatically determined by the plate reader software. The assay also includes a hydrolysis step which converts the DTX3 toxins (a group of esters of OA which are unable to inhibit PP2A) in the sample into free OA and DTX 1. This step is required to allow quantification of total toxicity (esters + free toxins) using the inhibition assay. For an overall risk assessment of shellfish tissue it is therefore necessary to test both non-hydrolysed and hydrolysed samples in order to determine the proportion of esters and the total OA and DTX concentrations present in the tissues (Mountfort *et al*, 2001).

The PP2A method can quantify between 100-300 µg OA equ/kg shellfish tissue. In order to quantify the concentration of DSP as accurately as possible, all samples were tested in duplicate dilutions: neat and 1:3, as per the standard method routinely used in the laboratory. However, in samples showing extremely high levels of inhibition (equivalent to more than 3000 µg OA equ /kg) it was necessary to increase the dilution, in order to obtain an inhibition measurement within the range of quantitation. The OA and DTX concentrations obtained for the samples tested at higher dilutions are less accurate than concentrations obtained for samples not subjected to elevated dilutions. However, the results for samples within a specific tissue group are comparative.

The PP2A assay is quantitative but not capable of providing information on toxin profiles. In order to provide a clearer understanding of the toxin profiles in samples, LC-MS analysis using an HPLC Waters 2695 Separations module, with aqueous / organic mobile phase gradient coupled with a Bruker High Capacity Trap Ion Trap detector was performed (Appendix 4). Scans

in negative mode and an OA calibration curve range of 0 to 200 µg/100 g (prepared in methanol) were used. DTX1 reference material was not available and therefore the peak areas for DTX1 transitions were analysed and compared. The analysis determined the proportion of OA and DTX1 in both the King scallops and the *P.lima* algal samples, with and without hydrolysis. Comparison of the determined toxin profiles was then carried out. The differences indicated the amount of esterification which had occurred within the samples.

4.5 PSP and ASP toxin analysis

PSP and ASP analyses were carried out on a sample group of scallops from each batch used in this study to determine any prior toxin contamination. The standard Integrin methods used were High Performance Liquid Chromatography (HPLC) with Photo Diode Array (PDA) detection for ASP (Quilliam *et al*, 1995, Takemoto *et al*, 1958, Peng *et al*, 1994) and the commercial Ridascreen Fast PSP SC ELISA kit (Usleber *et al*, 1997, Kasuga *et al*, 1996, Egmond *et al*, 1994), which was performed according to the manufacturer's instructions. These methods are both UKAS accredited for use at Integrin in a range of shellfish including King Scallops. For more details on these methods see www.Integrin.co.uk.

5 Experimental module 1-To determine the optimal conditions for the artificial toxification of King Scallops with OA and DTX toxins

5.1 Methodology

The first module was designed to assess the optimal conditions required to toxify the scallops to an appropriate DSP concentration, i.e. greater than 500µg OA equ. /kg of tissue.

Following delivery to the laboratory, scallops were allowed to acclimatise for 7 or 8 hours, then transferred into raceway tanks. Between 30-35 scallops were placed in each raceway. Three control scallops were removed immediately and tested for background concentrations of ASP, DSP and PSP using the methods described. A buffer tank fed filtered seawater to the raceways.

Algal cultures at 18-25 days old were used for the feed. Cell counts were determined using a Sedgewick Rafter to calculate the concentration of algal particles per millilitre in the cultures prior to feeding. The average count per 20 litre culture was 1.1×10^4 cells/ml. The feed volume was calculated based on the total volume of the system, approximately 450 litres, consisting of 3 raceways at approximately 120 litres each, plus 1 buffer tank at approximately 90 litres. An approximate 70% water change was carried out each day. This prevented any build up of nitrites or ammonia from the scallops which would have lead to poor water condition and may have been harmful to the scallops. Between 10 – 12 litres of algae was added to the buffer tank once per day following the water change. This gave an algal concentration of approximately 2.5×10^5 cells/litre of water in each raceway. Three scallops were removed from each raceway at day 1, and five scallops were removed from each raceway at day 4, 7, 9, 11 and 14 post feeding. These were tested as whole individual scallops for OA/DTX using the PP2A inhibition method. As

described above, the outer part of the King Scallop shells were cleaned to remove all adherent algae that could potentially affect the measurement of toxin concentrations. Once the required level of toxicity (>500µg OA equ/ kg) was achieved, the toxification was stopped. Remaining scallops were removed from raceways and frozen in shells. Raceways were cleaned in preparation for module 2. Algal cultivation was continued by inoculation of further carboys to ensure 100-120 litres of algal would be available. Appendix 2 describes the culture medium and conditions of growth.

5.2 Results

The results for the control samples (time zero) tested for ASP, DSP and PSP are shown in Table 1. All samples tested were below the limit of quantification for DSP and ASP toxins. Results for PSP analysis were quantifiable however they were all very close to the limit of quantification which is 10 µg PSP /100g.

Table 1. Background concentrations of ASP, DSP and PSP in control whole scallop samples (time zero)

Control samples	ASP -µg/g	PSP -µg PSP /100g	DSP - µg OA equivalents/kg
M1C1	<LOQ (0.00)	11.70	<LOQ (26.7)
M1C2	<LOQ (0.00)	11.70	<LOQ (23.4)
M1C3	<LOQ (0.00)	12.30	<LOQ (24.5)

The toxin concentrations of the King Scallops removed from raceways on days 1, 4, 7, 9, 11 and 14 as determined by PP2A inhibition assay are given in Table 2. After 10 days of feeding with *P. lima*, the PP2A assay analysis indicated that whole King Scallops had reached a mean concentration of DSP greater than 800 µg OA equivalents/kg (5 x the regulatory limit of 160 µg OA equ/ kg; Figure 3). Each scallop was tested individually and high variability in the concentrations of DSP was observed between individuals with standard deviations ranging from 18 to 42. This level of variation was in agreement with other work involving Pacific oysters and King Scallops (Bougrier *et al*, 2003). The experiment determined the optimal conditions for contamination of King Scallops with DSP toxins. This information was applied in the two following modules.

Table 2. Mean concentration of Okadaic acid and DTXs obtained over 14 day intoxication period.

Date	Days post feeding	Number of scallops analysed	Mean concentration of toxin (µg OA equ./kg	Standard Deviations
31-Mar-08	0	3	21.7 (<LOQ)	1.5
01-Apr-08	1	9	53.7 (<LOQ)	47.7
04-Apr-08	4	15	316.2	181.0
07-Apr-08	7	15	461.2	313.4
09-Apr-08	9	15	551.9	249.1
11-Apr-08	11	15	895.8	421.5
14-Apr-08	14	15	795.8	389.3

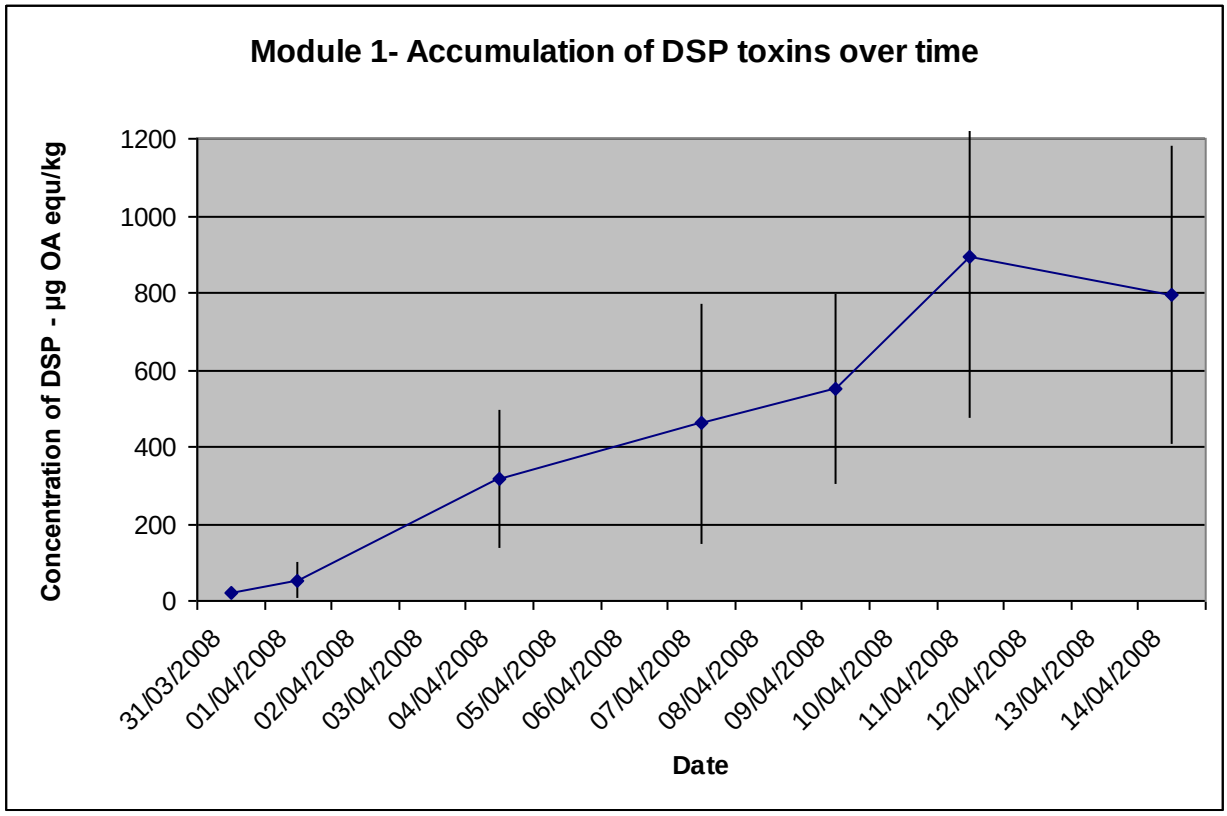


Figure 3. Accumulation of DSP toxins in King Scallops over 14 day period. Mean concentration of DSP obtained from PP2A inhibition assay measured in µg OA equ/100g of tissue. The standard deviation (given on Table 2) for each sample group is shown.

5.3 Conclusion

Using an estimated algal feed concentration of $2.5X10^4$ cells/ml for 10 days within the total raceway system resulted in mean concentrations of DSP contamination greater than 500 µg OA equ/kg of tissue, as determined by standard PP2A inhibition assay protocol. The whole aquaculture system required daily water changes and was maintained at 10-12 degrees centigrade.

6 Experimental module 2-To determine the distribution and modification of OA and DTX toxins in contaminated King Scallops

6.1 Methodology

This experiment was designed to investigate the toxin distribution in the different tissue compartments of King Scallops and compare these with the profile of toxin found within the feed algae. This experiment employed a new set of 115 scallops, which were allowed to acclimatise following delivery. Of these, 10 were removed at time zero as control samples and tested for ASP, PSP and DSP as described previously. From Module 1 the optimal time period for toxification to a suitable concentration (<500 µg OA equ /kg) was 10 days, and the *P.lima* feeding programme was planned on this basis. As per module 1, each algal culture 60 litre bag or 20 litre carboy was counted to assess the number of cells per millilitre. A set volume, based on the algal concentration within the culture, was then added to the buffer tank. This maintained an average concentration of algae within the raceways of 2.5×10^5 cells/ml. Scallops were tested at day 7 to check that OA/DTX toxins were being accumulated.

At day 10 feeding was stopped and the scallops were removed from the raceway. The scallops were split into 3 groups: 1 group with 15 scallops and 2 groups made up of 30 scallops. After the 10 day feeding period, these were processed and tested as follows:

Group 1-Toxin concentrations in whole King Scallops (15 scallops): Each scallop was prepared whole for toxin analysis and an aliquot of the whole tissue preparation was tested for DSP using PP2A inhibition assay including alkaline hydrolysis. This information was obtained to verify that the DSP within the scallops had reached the required concentration. A second set of aliquots was prepared for analysis by LC-MS /MS, with and without alkaline hydrolysis in order to verify the toxin profile.

For comparison with the King Scallops, five samples of the *P.lima* algal culture were also prepared for analysis by LC-MS/MS. This enabled the ratio of OA and DTX1 used for King Scallop contamination to be determined. In addition, using this data the amount and type of toxin transformations occurring in King Scallop tissues could be estimated.

Group 2- Distribution of OA/DTX toxins in adductor muscle, gonad, and remaining tissues (30 scallops): Each scallop was shucked into three tissue subgroups: Muscle only, Gonad only and Remainder (which consisted of the gut, mantle and other visceral tissue), resulting in 30 individual tissue samples for each group. The Muscle and the Gonad groups were tested for DSP using PP2A inhibition assay with and without alkaline hydrolysis. The Remainder tissue group was tested for DSP using PP2A inhibition assay including only alkaline hydrolysis.

Group 3 – Toxin concentrations in shucked end product (muscle + gonad) (30 scallops): Thirty scallops were shucked as described previously in the methods section. Each scallop was shucked into two tissue subgroups – Muscle + Gonad together (to represent roe-on product), and Remainder tissues, resulting in 30 samples per tissue group. The tissues were tested for DSP using PP2A inhibition assay and all samples were hydrolysed to determine total toxicity.

Raceways were cleaned in preparation for module 3 and algal cultivation was continued as described previously.

6.2 Results

All control scallops collected prior to feeding with *P.lima* were found to be below the limit of quantification for DSP toxins. Quantifiable concentrations of ASP and PSP were detected in the control scallops however this was considered unlikely to influence the study. The concentrations of ASP, PSP and DSP toxins detected in these control samples are shown in Table 3.

Table 3. Background concentration of ASP, DSP and PSP in control whole scallop samples (time zero).

Sample ID – control samples	**ASP -µg/g**	**DSP - µg OA equivalents /kg**	**PSP -µg PSP /100g**
M2C1	15.90	LOQ (33.1)	LOQ (<2.5)
M2C2	11.80	LOQ (31.5)	22.75
M2C3	10.30	LOQ (35.4)	14.68
M2C4	11.20	LOQ (35.4)	LOQ (<2.5)
M2C5	11.70	LOQ (36.0)	LOQ (<2.5)
M2C6	11.50	LOQ (27.9)	29.40
M2C7	12.50	LOQ (26.2)	22.16
M2C8	8.00	LOQ (28.9)	18.68
M2C9	10.30	LOQ (28.9)	19.80
M2C10	8.70	LOQ (27.9)	LOQ (<2.5)

6.2.1 Group 1-Toxin concentrations in whole King Scallops (15 scallops) including determination of toxin profile using LC-MS/MS analysis

6.2.1.1 PP2A analysis

After feeding for 10 days, each of the 15 whole King Scallops had reached DSP toxin concentrations in excess of 800 µg OA equ/ kg by PP2A assay. The mean concentration for the group was more than 10 x the regulatory limit by PP2A assay at 1.7025 mg OA equ/ kg (Figure 4).

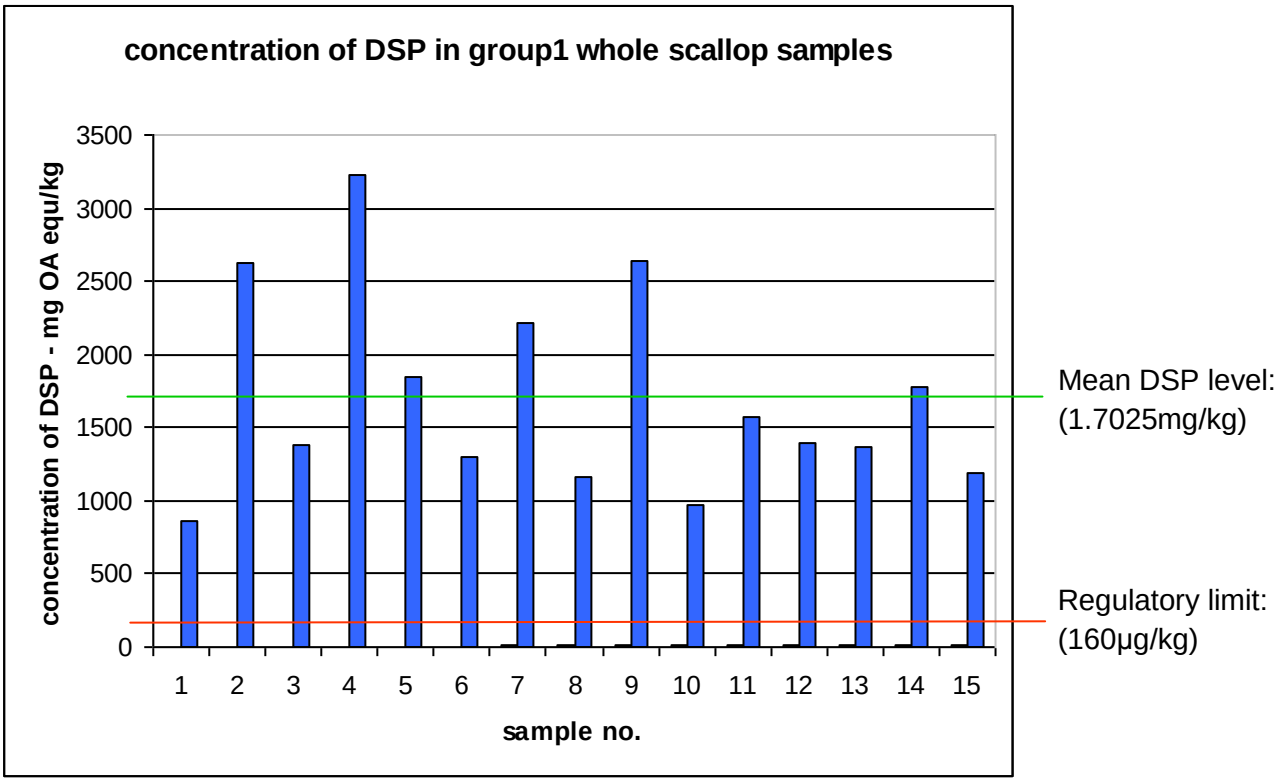


Figure 4. Concentration of DSP toxins in whole King Scallops obtained by PP2A inhibtion assay after 10 days of toxification – values given in µg OA equ/ kg tissue. Regulatory limit of 160 µg OA equ/ kg and mean DSP concentration of 1702.5 µg OA equ/ kg are shown.

As in Module 1 the variation in toxin concentrations measured by PP2A between individuals was very high (%CV >40). This data is presented in Table 4. Standard deviation and %CV values are also presented to highlight the high level of variation within the group of samples.

Table 4. Individual concentration values of DSP toxins -measured as µg OA equ / kg tissue obtained for the whole King Scallops samples using PP2A inhibition analysis method.

Sample	DSP concentration
1	861.3
2	2627.4
3	1384.6
4	3223.2
5	1842.2
6	1302.9
7	2221.3
8	1164
9	2642.1
10	977
11	1571.9
12	1389
13	1366.8
14	1777.7
15	1186.8
Mean	1702.5
StDev	687.8
%CV	**40.40**

6.2.1.2 LC-MS/MS analysis

A duplicate set of whole King Scallop samples was also analysed by LC-MS/MS to determine the ratio of OA and DTX1 with and without hydrolysis (see Appendix 4 for full method details). Reference standards were not available for DTX1, therefore profile information is based on a comparison of peak areas (Table 5). The variability between individual samples was high at %CV >35%, with more variability found within the unhydrolysed sample sets, %CV >60%. Figure 5 and Figure 6 show the relative proportion of OA and DTX1 based on peak areas obtained by LC-MS/MS analysis for unhydrolysed and hydrolysed samples.

Table 5. Peak area values obtained for OA and DTX1 from LC-MS/MS analysis in King Scallop samples with and without alkaline hydrolysis

Sample number	DTX1 - unhydrolysed	OA – unhydrolysed	DTX1 - hydrolysed	OA - hydrolysed
1	Not detected	7861130	905759	10520531
2	887730	3800296	6752328	12405673
3	2395133	16215253	3954169	21058628
4	2245871	4925575	5778984	25398684
5	6625090	36360130	4629727	18970387
6	5724733	10689154	2766629	11154812
7	2256228	6227689	4565591	18387348
8	3297506	22856835	1905745	8375038
9	1481062	2922906	6583659	29692061
10	Not detected	4678143	4801755	21833680
11	3018179	8809391	6073019	23751348
12	1338910	10671797	4511945	19312719
13	1209394	21333404	6602437	31285690
14	1811189	6812745	3896464	20178365
15	3757742	21205916	7420001	35433941
Mean	**2772982**	**12358024**	**4743214**	**20517260**
St dev	**1736708**	**9423752**	**1871057**	**7875249**
%CV	**62.6**	**76.3**	**39.4**	**38.4**

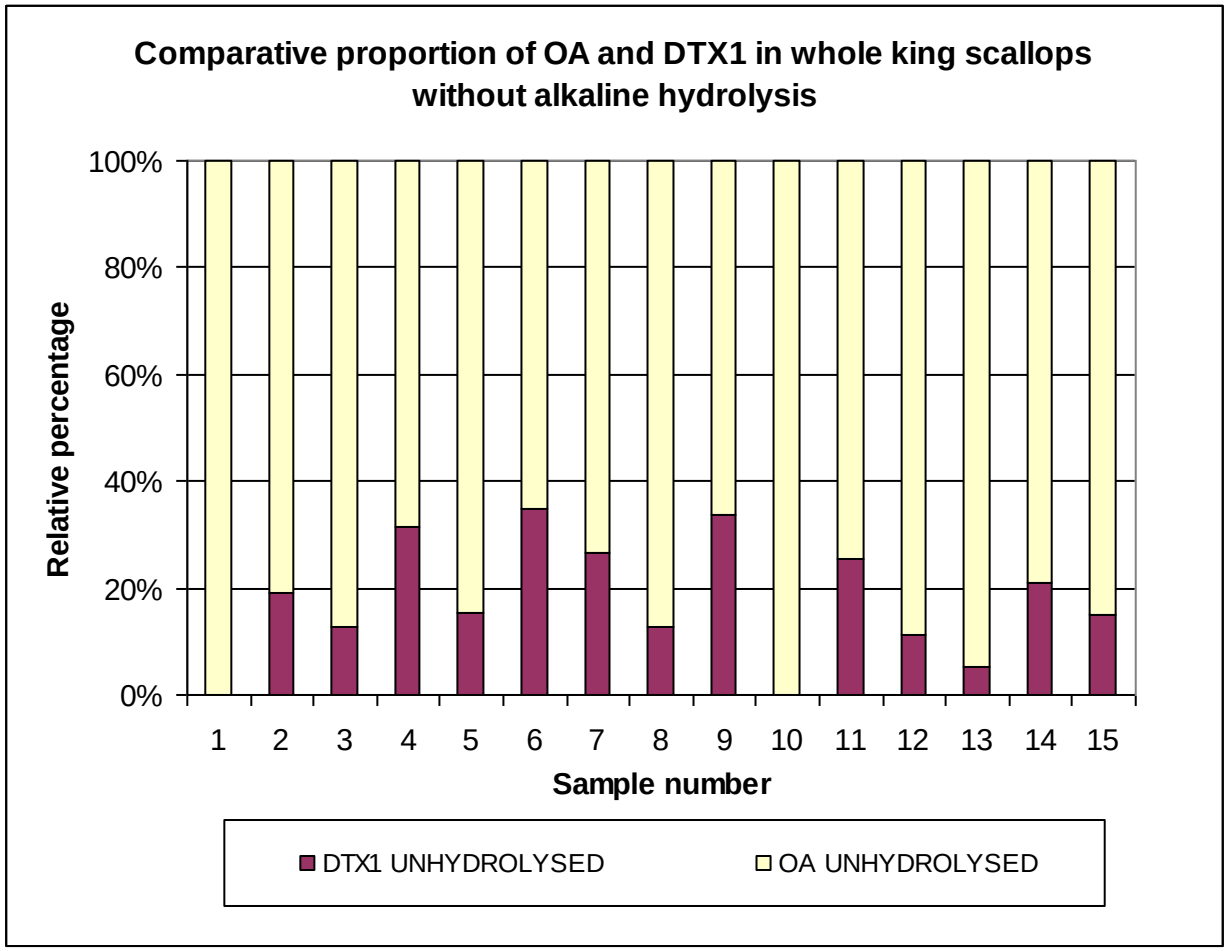


Figure 5. Comparative proportion of OA and DTX1 based on peak area from LC-MS/MS analysis in whole King Scallop samples without alkaline hydrolysis.

The ratios of OA and DTX 1 peak areas within the unhydrolysed King Scallop samples was highly variable between individual samples (Figure 5), indicating that there was no relationship between the two toxins within this sample group. Sample 1 and sample 10 did not have detectable levels of DTX1 when analysed without hydrolysis.

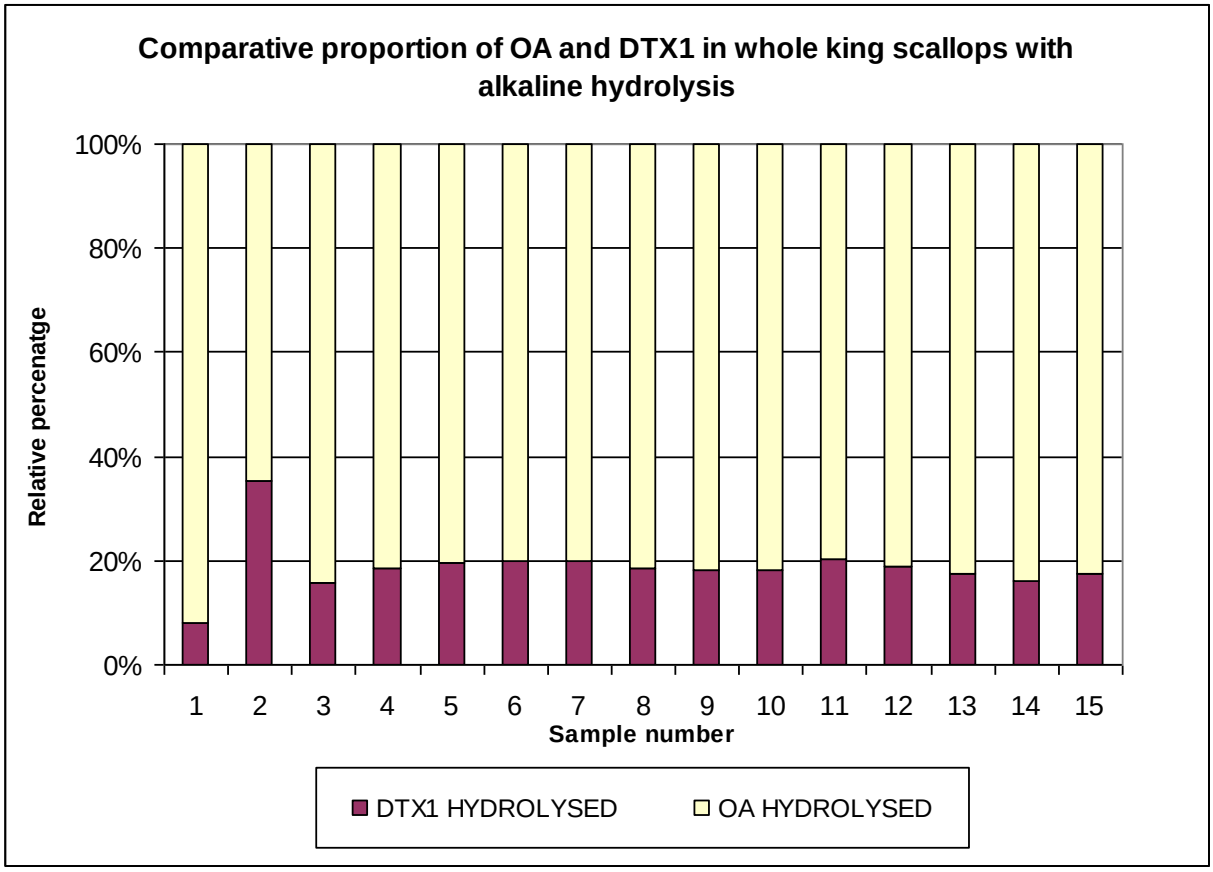


Figure 6. Comparative proportion of OA and DTX1 based on peak area from LC-MS/MS analysis in whole King Scallop samples with alkaline hydrolysis.

After hydrolysis a consistent relationship was found in 13 of the 15 samples with the ratio of OA/DTX1 at approximately 4.5:1 (Figure 6).

Table 6. Percentage of esterification determined by comparison of the concentration of OA only in µg/kg of tissue obtained by LC-MS/MS analysis in the same whole King Scallop sample treated with and with out alkaline hydrolysis in the preparation procedure.

Sample number	OA µg/kg tissue- unhydrolysed	OA µg/kg tissue - hydrolysed	Percentage of esterified OA in each sample
1	34.9	533	93.45
2	71.3	696.6	89.77
3	77.4	1123	93.10
4	99.9	1241.8	91.96
5	180	914.6	80.32
6	49.3	516.9	90.46
7	133	885	84.97
8	111.2	375.4	70.36
9	48.9	1460.3	96.65
10	18.7	530.2	96.47
11	79.5	579	86.27
12	49.2	466	89.44
13	103.5	770.7	86.57
14	34.9	533	87.88
15	71.3	696.6	88.26
Mean	**8.13**	**76.38**	**88.40**
St dev	**4.17**	**31.80**	**6.61**
%CV	**51.36**	**41.64**	**7.47**

The LC-MS/MS analysis determined that 70% to 96% of the OA had been esterified in the 15 King Scallop samples tested (Table 6 –mean value of 88.4%). This is in agreement with other studies on shellfish toxification with DSP (Vale *et al*, 2002, MacKenzie *et al*, 2002). This data indicated that the amount of esterification of OA only was consistent between scallop samples, %CV = 7.5%.

The OA only concentration values shown in Table 6 indicate that there would be a potential to obtain false negative results for samples which have a high concentration of esterified toxins, if analysis was carried out without hydrolysis. The mean OA only value obtained for the unhydrolysed samples was 81 µg/kg compared to the mean value for the same group of samples after hydrolysis which was 764 µg/kg.

Table 7. Percentage of esterification determined by comparison of the peak areas obtained for DTX1 by LC-MS/MS analysis in the same whole King Scallop sample treated with and without alkaline hydrolysis in the preparation procedure.

SAMPLE NO.	DTX 1 -unhydrolysed	DTX1 - hydrolysed	Percentage Esterification of DTX1
1	NOT DETECTED	9963349	NA
2	887730	16205587	94.5
3	2395133	43495859	94.5
4	2245871	11557968	80.6
5	6625090	46297270	85.7
6	5724733	27666290	79.3
7	2256228	9131182	75.3
8	3297506	19057450	82.7
9	1481062	13167318	88.8
10	NOT DETECTED	24008775	NA
11	3018179	15182547.5	80.1
12	1338910	22559725	94.1
13	1209394	33012185	96.3
14	1811189	9741160	81.4
15	3757742	37100005	89.9
Mean	2772982.1	22543111.4	86.4
Stdev	1736708.3	12450670.7	7.0
%CV	62.6	55.2	8.1

The amount of esterification of DTX1 within the samples was determined by comparison of the DTX1 peak areas obtained for the whole King Scallop samples with and without hydrolysis. The results indicated that esterification of DTX1 had taken place in the 15 king scallops, ranging from 75 to 96 %, with a mean of 86.4% (Table 7). This was comparable to the results obtained for OA. The amount of DTX1 esterification was not significantly different between individual scallop samples (% CV >10) and these results were in agreement with other studies conducted on bivalves (Vale *et al*, 2002, MacKenzie *et al*, 2002).

6.2.1.3 LC-MS/MS analysis of algal samples

The mean ratio of OA to DTX1 within algal samples was 1.4:1 when analysed without alkaline hydrolysis (Figure 7). When hydrolysis was carried out the proportion of OA in the algal samples increased resulting in a mean ratio of 4.7:1, OA to DTX1 (Figure 8), which is similar to that observed in the contaminated King Scallop samples that were analysed following hydrolysis.

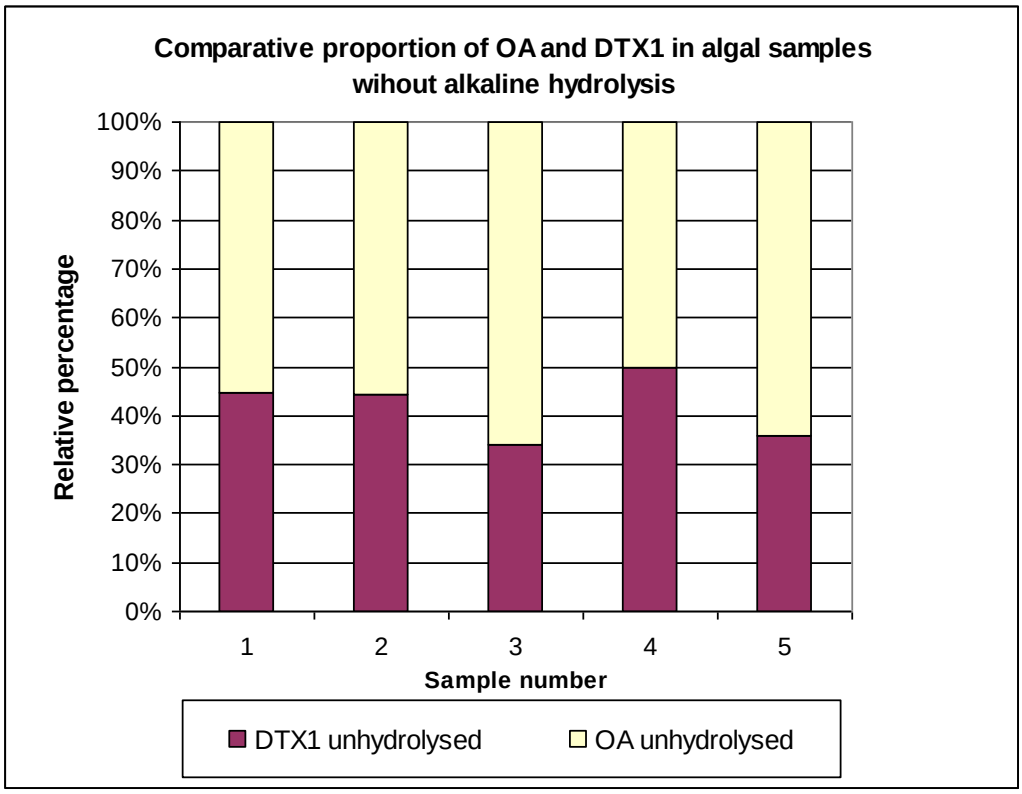


Figure7. Comparison of peak areas obtained for Okadaic acid and DTX1 for unhydrolysed algal samples, from LC-MS/MS analysis

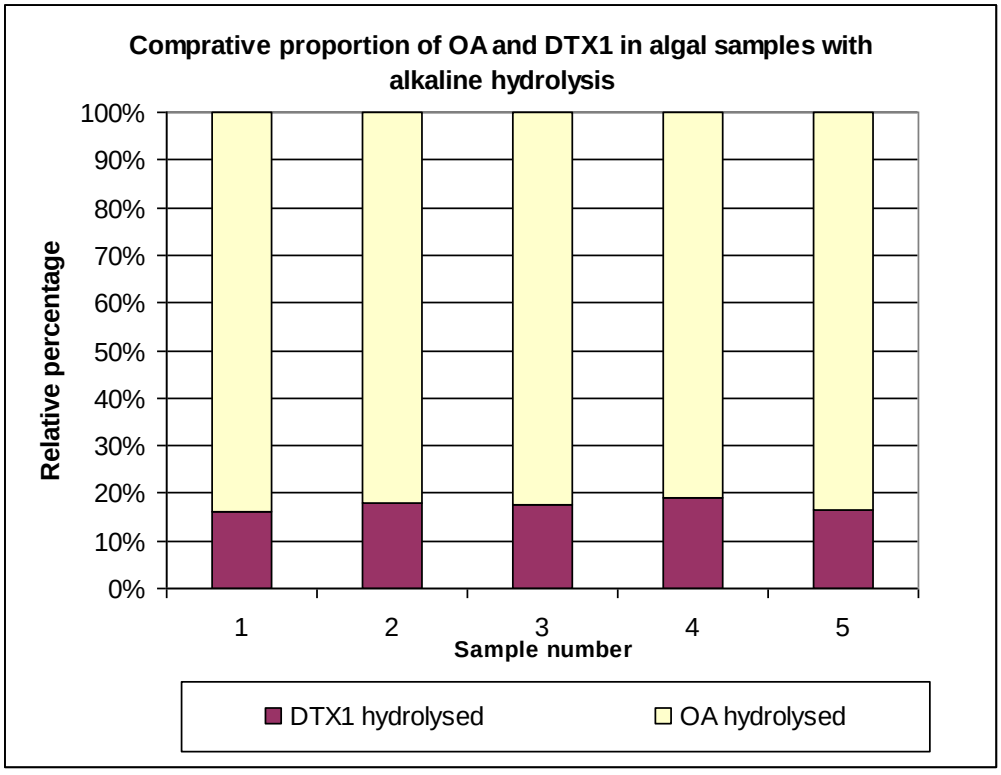


Figure 8. Comparison of peak areas obtained for Okadaic acid and DTX1 for hydrolysed algal samples, from LC-MS/MS analysis.

6.2.1.4 Conclusion

The LC-MS/MS analysis indicated that toxin concentrations in whole King Scallops were highly variable after the contamination period of 10 days. The ratio of OA to DTX1 within the unhydrolysed scallops was inconsistent between individuals. The proportion of OA varied between 65% and 100% in relation to DTX1 within the individual scallops. In the algal samples the proportion of OA in relation to DTX1 (based on peak areas) varied between 50% and 65%, indicating a greater proportion of DTX1 in the profile than was found in the King Scallops. The results obtained without hydrolysis of the samples indicated that there was no relationship between the profile of the feed algae and the profile within the individual King Scallops. A consistent OA to DTX1 ratio of approximately 80% : 20% was found in over 86% of the King Scallops after hydrolysis. The same ratio of OA to DTX1 was observed in the hydrolysed algal samples tested by LC-MS/MS analysis. The hydrolysed toxin profiles of the *P.lima* culture used within the study and the King Scallops tested were comparable. Further testing of a larger number of *P.lima* cultures and King Scallop samples would be required to investigate the toxin profiles fully.

6.2.2 Group 2- Distribution of OA/DTX toxins in adductor muscle, gonad, and remaining tissues (30 scallops)

6.2.2.1 PP2A analysis

When the separate tissue groups were examined from the individual scallops in Group 2 it was found that the toxin concentrations varied significantly in the Gonad (%CV >95%) and Remainder tissues (%CV >35%). Variation in toxin concentrations was not observed in muscle tissue as the toxin concentration was below the limit of quantification (100 µg OA equ/ kg) in all samples (Table 8). The highest toxin concentrations were found in the Remainder tissue samples. In addition, three hydrolysed Gonad samples contained toxin at concentrations > 1mg OA equ/ kg, and one of these (Scallop 16) contained toxin concentrations exceeding 1.91 mg OA equ/ kg.

Table 8. Results for Group 2 of Module 2 -showing the DSP concentrations obtained for the different tissue subgroups using PP2A inhibition assay.

	Concentration of Okadaic acid and DTXs (-µg OA equ/ kg)				
Sample	**Muscle – unhydrolysed**	**Muscle – hydrolysed**	**Gonad - unhydrolysed**	**Gonad - hydrolysed**	**Remainder –hydrolysed**
1	32.3	49.7	50.3	144.9	1163.8
2	29.2	36	49.6	226.3	3581.9
3	26.2	33.9	45.1	183.2	4588.9
4	23.9	49.2	49.7	140	1079.8
5	0	36.2	53.3	163.8	3190.2
6	28	46.6	56.2	242.1	3263.2
7	22.4	40.2	45.3	194.5	2309.8
8	24.1	53.2	50.1	148.4	3871.6
9	20.9	57.6	51.1	215.1	4393.2
10	22.6	40.9	51.3	272.6	3155.2
11	29.4	60.5	57.1	557.6	4336.3
12	32.8	50.7	64	657.4	5685.4
13	26.4	55.2	101.4	823.7	3788.6
14	27.3	48.2	53.5	229.8	3623.6
15	27.9	53.4	45.4	151.8	2409.2
16	28.6	61.9	334.3	1910.5	4003.4
17	21.9	35.3	147.3	159.6	3664
18	27.3	39.7	205.8	908.2	6936.3
19	29.1	46.1	188.9	678.9	5205.2
20	26.2	36.3	148.3	176.9	2395.9
21	19.8	68.1	199.8	688.3	3341.8
22	25.5	53	718.7	1131.2	5350.1
23	33.9	37.9	622.1	1062.1	2363.8
24	33	53.7	206.9	769.9	3769.9
25	33.9	43.2	76.6	186	1997.9
26	35.9	37.2	46.5	56.2	3413.4
27	27.5	42.1	159.2	426	5660.6
28	32.7	47	51.7	89.1	2939.1
29	33.4	43.4	67.4	215.9	2939.9
30	19.2	38.2	60.2	155.7	3391.4
Mean	26.7	46.5	135.2	432.2	3593.8
St. dev	6.8	8.9	162.2	417.3	1316.1
%CV	253.6	192.2	1199.5	965.5	366.2
Mean % esterification-Muscle		40.93	**Mean % esterification-Gonad**	64.37	
	stdev	18.79	**Stdev**	21.49	
	%CV	45.90	**%CV**	33.39	

The mean DSP concentration for the muscle group was below 100 µg OA equ / kg with and without hydrolysis. The unhydrolysed Gonad tissue samples had a mean value of 135.2 µg OA equ / kg which is above LOQ but below the regulatory limit (160 µg OA equ / kg). However, the coefficient of variation (% CV) was extremely high >110% indicating significant variation between individual samples(raw data presented in Appendix 6).

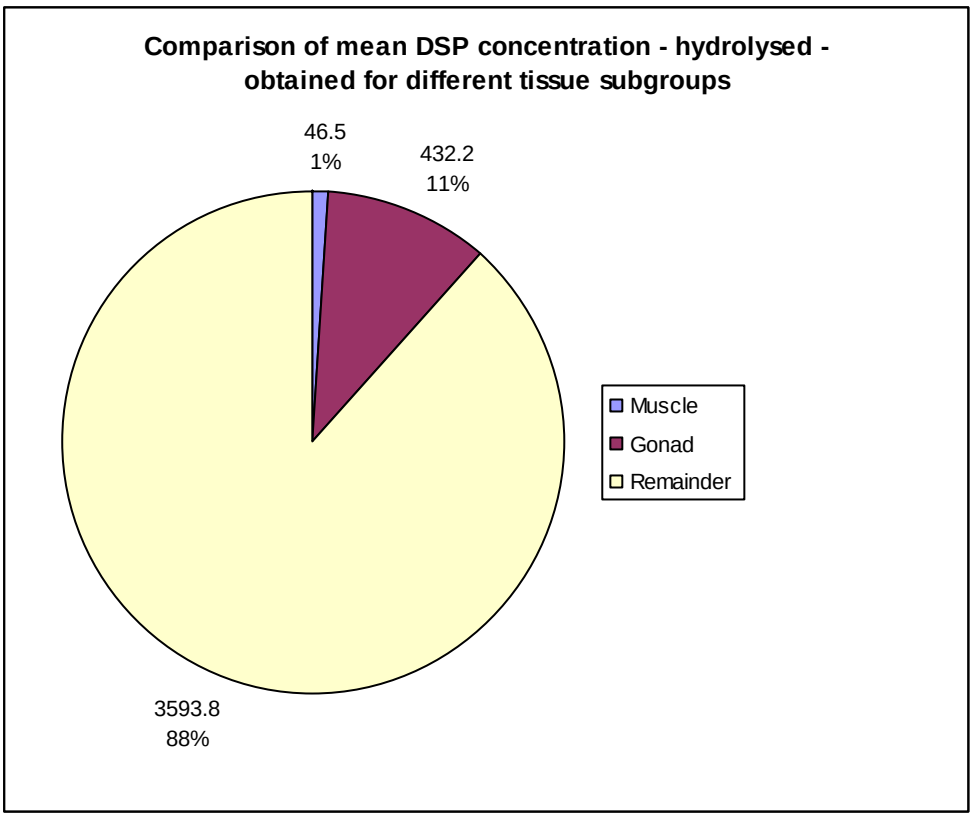


Figure 9. Comparison of the mean DSP concentrations obtained for the three sub groups by PP2A inhibition analysis, also expressed as relative percentages.

On average, the Muscle tissue contained only 1% of the total DSP toxin responsible for contamination of a whole King Scallop. The Gonad tissue contained 11% of the total DSP toxin in a toxified scallop and the Remainder tissue contained 88%. These findings were based on mean DSP concentrations (µg OA equ /kg) obtained using PP2A inhibition assay (Figure 9).

The mean DSP concentration for the Gonad tissue group was almost three times greater in the hydrolysed samples compared with unhydrolysed samples. However, significant variation in DSP concentration was observed between individuals within both hydrolysed and unhydrolysed Gonad tissue samples (CV >95%). An increase in DSP concentration of ≥ 65% after alkaline hydrolysis was observed in 20 of the 30 Gonad samples analysed using the PP2A inhibition assay. However the percentage increase between samples was variable (%CV=33.4%). Within the Muscle group a mean increase in DSP of 40.9% after alkaline hydrolysis was observed. However, significant variation in DSP concentration between samples was also observed (%CV of 45.9%) in this tissue group.

6.2.2.2 Conclusion

The mean DSP concentration values obtained for all Muscle tissue samples, unhydrolysed or hydrolysed, were below the limit of quantification (LOQ). Two out of 30 hydrolysed Gonad tissue samples contained toxin below the limit of quantification of the assay **(**100 µg OA equ

/kg). The toxin concentrations of all other hydrolysed gonad tissue samples were above or very close to the regulatory limit of 160 µg OA equ /kg. Over 85% of the DSP toxin within the King Scallops tested was contained within the Remainder tissue.

6.2.3 Group 3 – Toxin concentrations in shucked end product (muscle + gonad) (30 scallops)

6.2.3.1 PP2A analysis

All samples from group 3 were analysed using a PP2A inhibition assay which included an alkaline hydrolysis step (Figure 10) in order to assess the total toxicity of each tissue group. The analyses showed that 93% of the shucked muscle and gonad combined tissue samples were below the limit of quantification of the assay (100 µg OA equ / kg). The highest toxin concentrations detected in shucked muscle and gonad tissues were; Sample 11, which was quantified at 164.8 µg OA equ/ kg (the only sample of shucked product containing OA concentrations that were higher than the regulatory limit) and sample 28, which was quantified at 110.0 µg OA equ/ kg (Figure 11). These samples were also found to contain 2.8774 mg OA equ/ kg and 1.7533 mg OA equ/ kg respectively in their Remainder tissue. All 30 Remainder tissue samples were greater than 1 mg OA equ/ kg with a mean value of 3.5754 mg OA equ/ kg and range of 1.3777 mg OA equ/ kg to 5.9790 mg OA equ/ kg (full results included in Appendix 6).

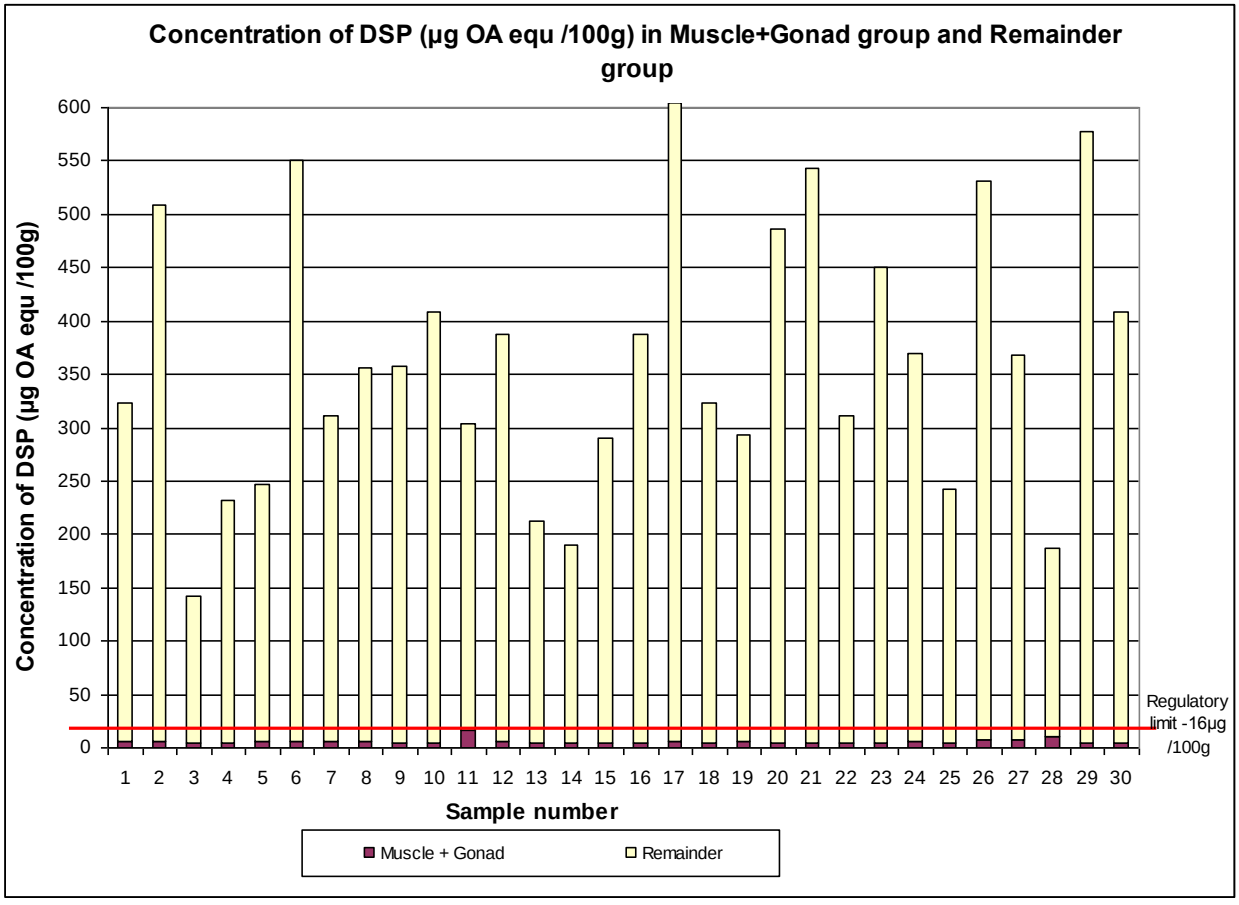


Figure 10. The concentration of Okadaic acid and DTXs found in the Muscle and Gonad combined tissue and the Remainder tissue samples from Group 3 of Module 2 by PP2A inhibition assay including alkaline hydrolysis

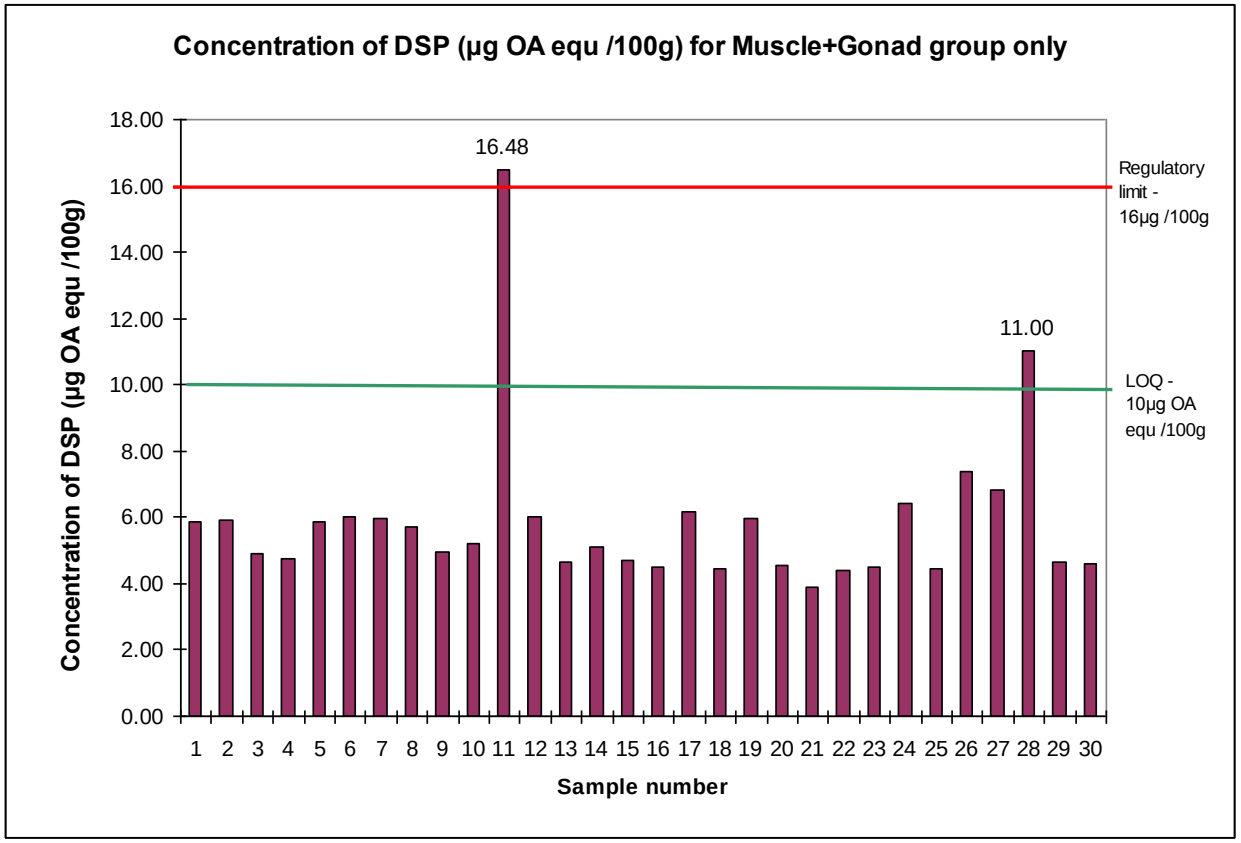


Figure 11. Concentration of DSP (µg OA equ/ 100g) in Muscle + Gonad only group obtained by PP2A inhibition assay with alkaline hydrolysis

6.2.3.2 Conclusion

The highest concentrations of DSP toxins were found in the Remainder tissue of the King Scallops. Quantifiable DSP toxin concentrations were found in only 6% (2 out of 30) of the end product tissue sample group (Muscle and Gonad combined).

Although DSP toxins had been found in the Gonad only tissue of Group 2, the diluting effect of the muscle tissue when combined with the gonad was sufficient to reduce the concentration of DSP toxins in the end product tissue group (Muscle + Gonad) to below LOQ in more than 93% of samples. All the corresponding Remainder tissue samples were found to be >1mg OA equ/ kg. However, no relationship was found in the ratio of OA and DTXs between Remainder tissue and Muscle + Gonad combined tissue (Figure 10).

7 Experimental Module 3 - To determine the likely effects of shucking practices currently employed by King Scallop processors on OA and DTX toxin concentrations in end product

7.1 Methodology

This experiment aimed to investigate the effects of different washing treatments of shucked scallops on the concentrations of DSP toxins remaining in the marketed end product (i.e. adductor muscle plus gonad). For these experiments a batch of 115 scallops was obtained and allowed to acclimatise as described previously. From this batch 10 scallops were removed immediately and tested for ASP, DSP and PSP background concentrations. The remaining scallops were distributed evenly between 3 raceways. Toxic *P.lima* algae were used to feed the scallops as per the previous modules. After the 10-day feeding period, 5 scallops were removed from each raceway (15 scallops in total) and tested to ensure that the scallops contained DSP toxin at concentrations greater than 500 µg OA equ /kg. Subsequently, 60 scallops were removed and split into 3 groups of 20 individuals. Following shucking, each group was treated according to the procedure detailed in the King Scallops, section 4.3, from Module 1.

Group 1 (control) – No washing

Group 2 – 1 minute rinse under running water

Group 3 – 5 minute vigorous rinse under tap.

Scallops in all groups were shucked to leave a combined muscle and gonad sample (resulting in 20 samples per wash group), and all visible alimentary tracks were removed. These scallops represented correctly shucked, roe-on end products. The appropriate wash treatment was conducted for each group as described above. The shucked samples were homogenised and divided into aliquots for testing with the PP2a inhibition assay.

Results were analysed by ANOVA to investigate variation in DSP toxin concentrations between the three groups.

7.2 Results

All pre-feeding control samples (time zero) were shown to be below the limit of quantification for DSP measured by PP2A inhibition assay. Table 9 shows the detected concentrations of each of the three toxins analysed. The concentrations of ASP toxins in the control samples were much higher than found in other modules. As the project was undertaken in the autumn, a possible cause was that the scallops being used had been exposed to toxic algal blooms over the summer period (Campbell *et al,* 2001). The presence of these toxins was considered unlikely to affect the study.

Table 9. Background concentrations of ASP, DSP and PSP in control whole scallop samples (time zero).

Sample ID	ASP -µg/g	DSP - µg OA equivalents /kg	PSP -µg PSP /100g
M3C1	41.47	LOQ (38.6)	20.70
M3C2	34.83	LOQ (43.1)	19.50
M3C3	48.45	LOQ (41.8)	23.30
M3C4	37.18	LOQ (42.8)	LOQ (<2.5)
M3C5	41.35	LOQ (37.7)	LOQ (<2.5)
M3C6	47.78	LOQ (42.4)	LOQ (<2.5)
M3C7	57.66	LOQ (42.9)	LOQ (<2.5)
M3C8	34.88	LOQ (42.8)	LOQ (<2.5)
M3C9	41.25	LOQ (38.1)	LOQ (<2.5)
M3C10	45.97	LOQ (40.4)	LOQ (<2.5)

After 10 days of feeding with *P.lima* the scallops had all reached a sufficient level of DSP toxification (Table 10), where the mean toxin concentration per whole scallop was much higher than the statutory limit for DSP, at 1.81 mg OA equ/ kg. No significant difference (P= 0.663, unpaired t test) was found between the mean concentrations of DSP toxin in scallops from this Module (3) and Module 2 (previously shown in Table 4) at 95% confidence interval.

Table 10. Concentration of Okadaic acid and DTXs obtained for toxified whole samples before remaining samples were split and shucked to muscle and gonad.

Sample ID	Concentration of Okadaic acid -µg OA equ/ kg
1	637.9
2	1781.0
3	2526.2
4	2965.1
5	1053.6
6	1162.4
7	1950.0
8	1010.2
9	2473.7
10	1751.9
11	1837.9
12	2243.9
13	2639.3
14	1578.2
15	1565.2
Mean value	**1811.8**
St.dev	**671.2**
%CV	**37.05**

Concentrations of DSP toxins in all samples of the combined muscle and gonad shucked tissue within all three wash treatment groups were below the limit of quantification for the PP2A inhibition assay (Table 11).

Table 11. Concentration of DSP (µg OA equ/kg) obtained for King Scallops treated with different wash procedures after shucking to muscle and gonad end product.

	DSP concentration in µg OA equ/ kg as obtained by PP2A inhibition assay.		
	Group 1 (control)	**Group 2 - 1 min wash**	**Group 3 – 5 min wash**
	41.6	40.5	44.0
	43.3	36.4	45.8
	49.1	33.1	46.6
	47.6	46.8	41.5
	41.3	35.7	44.1
	46.3	41.9	48.4
	50.7	39.4	45.3
	45.9	44.1	38.7
	53.9	40.7	43.4
	48.0	48.8	46.0
	40.3	42.5	47.8
	41.5	40.8	40.8
	45.3	43.3	20.6
	42.2	45.9	39.5
	36.5	45.2	37.3
	38.1	38.5	41.0
	41.6	43.2	49.4
	40.0	45.0	47.8
	40.3	47.0	38.3
	41.2	40.3	40.0
Mean	43.7	4.1.9	42.3
St dev	4.5	04.1	6.3
% CV	10.21	9.67	14.85

An ANOVA based on the 3 sets of results, showed that there was no significant difference between any of the wash treatment groups (see Figure 12). There was > 40x more variation within a group than between the groups.

Groups	Count	Sum	Average	Variance
Group 1	20	874.742	43.7371	1.99237
Group 2	20	838.977	41.94885	1.64666
Group 3	20	846.289	42.31445	3.94643

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.357029	2	0.178514	0.706013	0.497873	3.158843
Within Groups	14.41238	57	0.252849			
Total	14.76941	59				

Figure 12. Anova of the three wash treatment groups: single factor

7.3 Conclusion

Within the study whole King Scallops were found to contain very high concentrations of DSP toxin (>1mg OA equ /kg). However, when shucked to Muscle and Gonad end product (a roe-on product) the concentrations of DSP toxin were below the regulatory limit in all the samples. This indicated that the risk of DSP from consumption of the King Scallop end product was low. No significant differences in DSP toxin concentrations were found between different washing treatments of the Muscle and Gonad end product.

8 Discussion

- When all tissue sub-groups from Module 2 were combined it was observed that significantly high concentrations of DSP toxins were present in the Remainder tissue groups (Appendix 6, Figure 6.1). As this tissue group contains the digestive organs of the bivalve it is probable that undigested algal particles contributed to the elevated concentrations. The Remainder tissue contains the mantle and gills which may also have contained trapped algal particles, resulting in the detection of higher concentrations of DSP toxin in these groups.

- Within the hydrolysed Gonad only group, in Module 2, some samples had very high DSP concentrations. More than 70% of samples were above the regulatory limit. However the results were extremely variable between samples where the CV was greater than 95%. This may be as a result of a small section of alimentary track inside the gonad, the contents of which are likely to vary between individuals.

- No quantifiable DSP toxin contamination was found in the adducter muscle tissue samples throughout the study.

- Since this study employed artificially toxified King Scallops that were subjected to toxic algae for a short contact period, further investigation is required to assess the long-term effects of toxin accumulation in scallops. It is possible that different tissue distribution profiles of toxins within the King Scallops may occur when they are subjected to longer

periods of exposure to toxic algae or to natural algal toxin events. A natural event would potentially enable investigation of the occurrence of other lipophilic toxins such as Pectenotoxin or Yessotoxin. This was not possible within the scope of this study.

9 Recommendations

- The findings of this study indicate that there is no obvious risk of DSP from King Scallop (*Pecten maximus*) combined muscle and gonad end product. This is caveated by the fact that the study was a set of laboratory experiments under specific conditions. The investigations were limited by the sample size, short exposure period to toxic algae, low biomass gonads due to time of year (post spawning), artificially engineered DSP contamination with only one strain of DSP producing algae and individual sample analysis for DSP toxin concentrations.

- Shucking should always be carried out in accordance with the Seafish HACCP guidelines. Specific attention should be paid to the trimming and inspection step, to ensure all visible signs of the alimentary tract associated with the muscle section are removed and the shucked end product is rinsed under running water.

- Alkaline hydrolysis should always be carried out in order to assess the overall risk of DSP within the King Scallops.

- Further investigation is required i) to assess the toxin distribution after contamination for a longer time period; ii) to compare these findings with DSP positive King Scallops which have been naturally contaminated; iii) to examine the distribution and concentration of DSP toxin at a pre-spawning stage when the biomass of the gonad specifically would be much greater than in the tested samples.

10 Acknowledgements

The authors wish to thank Loch Fyne Sea Farms for the supply of King Scallops, Dr John Leftley, Dr JD McKenzie, the FSAS for funding and all lab staff that contributed to the study.

11 Appendices

11.1 Appendix 1

Prorocentrum lima (Ehrenberg) Dodge (1975)
CCAP 1136/11
Isolator: Bravo
Origin: Marine; Vigo, Spain
Culture: Medium K; B; sub
Other: CCMP685; PL2V; toxic strain

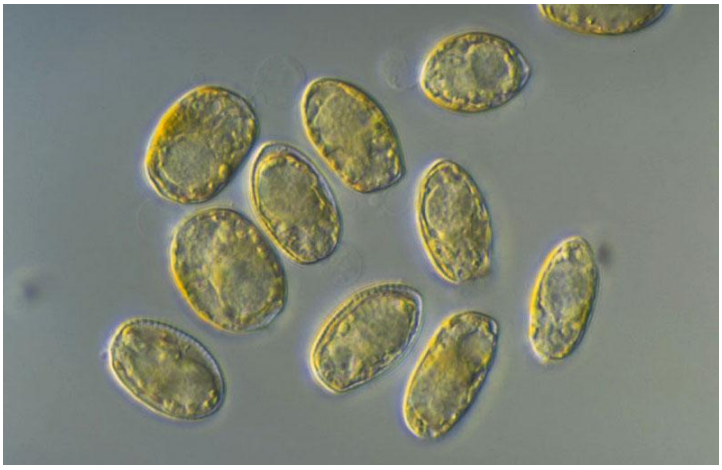


Figure 13. *Prorocentrum lima* (c/o Dr J. Leftley).

11.2 Appendix 2

Culture conditions for *P.lima*.

The medium used is f/2, which is an enriched seawater. Refer to link below- http://www.ccap.ac.uk/media/documents/f2.pdf

The algae were grown at 18-20 deg C. Salinity of the seawater was between 30-34 parts per thousand. The light was continuous and warm white fluorescent.

11.3 Appendix 3

Analysis of the *P.lima* with PP2a inhibition assay to determine Okadaic acid and DTX concentration per cell.

Methodology

P.lima samples were removed and cell counts performed. 2 x 50 ml samples of algae were centrifuged at 8000rpm and all the supernatant was decanted off. The pellets were combined and extracted with 10 mL of 90 % methanol by homogenising for 2 minutes, then centrifuged for 10 minutes. Aliquots of this stock algal solution were then diluted as follows

- Dilution 1: 100 µl of stock supernatant with 900 µl of water - sample A
- Dilution 2: 100 µl of supernatant with 4900 µl of water - sample B
- Dilution 3: 50 µl of supernatant with 4950 µl of water - sample C
- Dilution 4: 10 µl of supernatant with 4990 µl of water - sample D

Step		**Dilution factor**
Extraction in 10ml of 90% MeOH		1:10
Additional dilution	Sample A	1:10
	Sample B	1:50
	Sample C	1:100
	Sample D	1:500

1ml was taken from each dilution and extracted by solid phase extraction (SPE) and recovered in 1 ml of assay buffer. The samples were then assayed as per the PP2A inhibition assay.

Results

Low enzyme activity of 8.7% was obtained for the sample A (Dilution 1). This activity indicated an approximate concentration of OA and DTXs greater than 7 ng OA equ. /ml.

Very high activity of 91 % was obtained for sample B (Dilution 2). This indicated an OA and DTXs concentration of less than 2 ng OA equ. /mL, however this is below the limit of detection for the assay. Samples C and D – Dilution 3 and 4 - also had very high activity values, >91%.

Algal cell count

The cell count for the initial algal culture was 4.3×10^3 cells/ml, therefore 100ml of culture contained 4.3×10^5 cells.

Conclusion

The results indicated that the *P.lima* culture was producing OA and DTX s detectable by the PP2A enzyme inhibition assay. A concentration range of 7-10 ng OA equ. /ml was obtained. No further dilutions were analysed. Sample A had a cell concentration of 4300 cells/ml. Therefore concentration of DSP is approximately 1.6 pg [OA equ] per cell.

11.4 Appendix 4

Methodology for the investigation of Okadaic acid and Dinophysis toxin profiles in contaminated King scallops and *P.lima* algae using LC-MS/MS.

15 king scallop samples and 5 algal culture samples were extracted with 90% methanol, and divided into 2 sets. One set was hydrolysed as per PP2A inhibition assay protocol and the duplicate set was left unhydrolysed. The samples were dried down and resuspended in methanol for analysis by LC-MS/MS

The samples were run using an HPLC Waters 2695 Separations module, under the following conditions:

- Flow rate: 0.35ml/min

- Column temperature 50 ^{o} C

- Injection Volume: 20 µl

- Autosampler temperature: 4 ^{o} C
- Mobile phases used were:

A: Aqueous phase: 2mM Ammonium Formate, 50 mM Formic Acid

B: 95% ACN, 5% water, 2mM Ammonium Formate, 50mM Formic acid.

Gradient applied:

Time	**A**	**B**
0	60	40
0.5	60	40
8	5	95
10	5	95
10.1	60	40
15	60	40

The Mass Spectrometer (Bruker High Capacity Trap ion trap MS) with electrospray ionization in the negative mode conditions were:
- Scan range: m/z 750 - 950
- Nebuliser gas: 40 psi
- Dry gas: 10 L/min
- Dry temperature: 365^{o}C

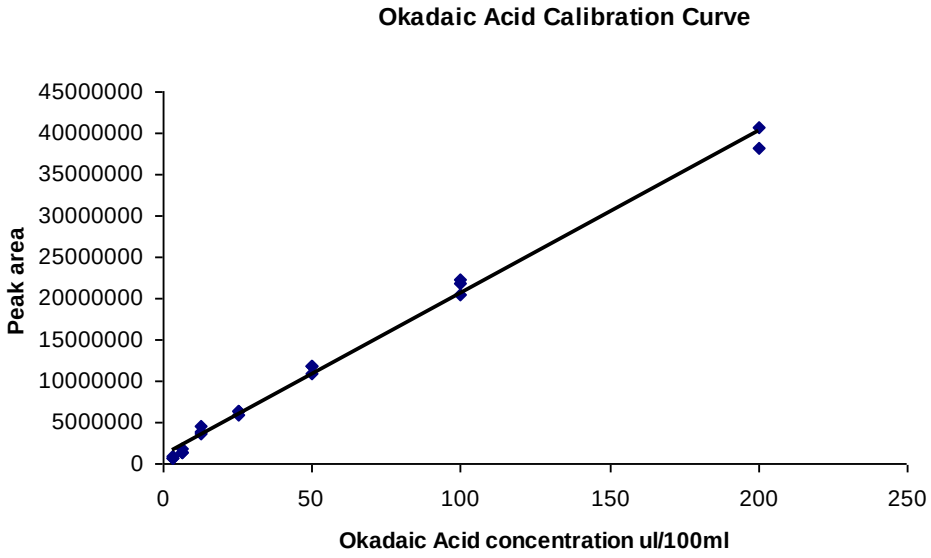
Smart Parameter Settings were used:
- Target mass m/z 803
- Compound stability 100%
- Trap drive level 100%

Auto MS2 was conducted using the most abundant ion with a fragmentation amplitude of 1V, isolation width 2m/z and scan range m/z 100 – 950.

The calibration curve range used was from 0 to 200 µg OA /100g (prepared in methanol)

Calibration curve obtained:

Calibration curve obtained:



11.5 Appendix 5

Recorded mass for all samples tested within project S14043

All weights given are in grams

Module 1 :

Group 1: Whole Scallops

ID	Day 0	Day 1	Day 4	Day 7	Day 9	Day 11	Day 14
1	43.09	43.70	62.87	65.92	37.44	48.59	60.99
2	53.81	60.13	56.64	63.26	52.87	53.69	60.65
3	22.24	58.05	63.05	65.23	60.76	58.78	47.19
4		62.89	60.88	59.97	59.34	65.55	61.20
5		87.32	65.79	52.49	65.10	56.49	54.51
6		75.36	57.89	*	57.01	62.42	69.34
7		71.49	53.17	*	59.66	55.77	58.37
8		58.78	52.19	*	55.45	52.59	51.10
9		59.70	51.12	*	74.67	53.71	62.80
10			59.03	*	67.92	58.89	64.13
11			54.87	*	68.76	62.75	53.19
12			58.48	*	65.27	56.38	60.29
13			59.78	*	60.85	58.72	55.79
14			62.19	*	62.33	47.07	72.71
15			47.45	*	61.10	57.43	65.56
mean	**39.71**	**64.16**	**57.69**	**61.37**	**60.57**	**56.59**	**59.85**
st dev	**16.05**	**12.44**	**5.11**	**5.48**	**8.46**	**5.04**	**6.80**
% CV	**40.42**	**19.39**	**8.86**	**8.92**	**13.96**	**8.91**	**11.36**

* weight data lost due to computer failure

Module 2:
All weights given are in grams

Group 1

ID	Whole scallop
1	55.34
2	63.06
3	59.32
4	63.42
5	70.45
6	61.87
7	60.16
8	73.28
9	55.34
10	64.13
11	59.37
12	56.52
13	74.25
14	66.62
15	62.97
mean	63.07
Stdev	5.96
% CV	9.45

Group 2

ID	Muscle only	Gonad only	Remainder
1	24.45	5.62	47.31
2	32.88	4.18	26.24
3	18.06	4.43	21.54
4	20.05	6.7	24.16
5	23.86	7.66	23.19
6	22.86	1.31	26.2
7	21.74	2.18	23.63
8	25.23	5.38	22.96
9	25.34	3.37	24.58
10	30.37	5.08	25.39
11	21.38	1.87	14.05
12	21.18	3.52	14.37
13	23.98	1.78	14.61
14	24.23	5.19	15.43
15	26.06	7.39	13.42
16	22.36	1.44	18.21
17	21.61	7.82	12.71
18	26.95	1.46	16.97
19	20.83	2.2	14.05
20	24.05	7.66	15.6
21	23.73	1.88	15.71
22	27.48	2.55	15.58

23	24.83	2.11	14.53
24	24.66	3.71	19.02
25	29.92	4.23	17.12
26	21.89	6.08	12.63
27	24.21	0.8	15.36
28	28.18	5.05	16.51
29	22.14	2.26	13.24
30	23.28	2.04	14.2
Mean	24.26	3.90	18.95
Stdev	3.21	2.17	6.99
% CV	13.23	55.74	36.89

Group 3

ID	**Muscle + Gonad combined**	**Remainder**
1	28.398	21.568
2	29.728	21.99
3	30.94	19.66
4	28.01	19.99
5	25.85	21.17
6	33.32	25.06
7	25.23	20.17
8	25.7	19.21
9	30.04	20.09
10	22.43	20.85
11	24.44	23.51
12	28.59	22.28
13	23.13	17.76
14	24.15	18.41
15	27.95	14.14
16	33.43	18.35
17	26.01	15.21
18	31.03	16.91
19	29.82	15.89
20	31.61	16.29
21	25.95	15.5
22	27.48	17.4
23	29.27	15.65
24	36.2	14.97
25	26.6	20.61
26	28.61	16.37
27	31.18	15.6
28	23.39	13.25
29	31.21	17.21
30	27.91	17.89
Mean	28.25	18.43
Stdev	3.32	2.93
% CV	11.74	15.88

Module 3:

All weights given are in grams

Group 1

ID	Whole Scallops
1	57.17
2	51.97
3	43.54
4	47.42
5	43.34
6	42.76
7	40.29
8	39.47
9	42.18
10	41.57
11	41.78
12	46.14
13	45.37
14	43.46
15	57.54
Mean	45.60
Stdev	5.48
% CV	12.02

Group 2

	Muscle + Gonad combined		
ID	**No wash**	**1min**	**5 min**
1	21.55	28.22	30.29
2	26.53	27.09	27.77
3	27.61	27.77	28.30
4	22.02	22.40	28.29
5	23.97	30.27	36.02
6	21.98	28.83	27.19
7	22.52	29.01	29.90
8	29.10	27.91	22.97
9	27.67	23.07	29.62
10	23.88	26.10	34.15
11	26.61	29.29	25.53
12	22.37	29.56	33.05
13	22.75	28.47	29.73
14	25.33	30.31	23.11
15	29.61	26.07	24.31
16	31.41	25.58	24.25
17	22.96	31.77	27.89
18	28.13	32.24	26.10
19	26.42	25.46	26.01
20	27.50	29.21	24.41
Mean	**28.35**	**27.93**	**27.94**
Stdev	**10.92**	**2.58**	**3.60**
% CV	**38.53**	**9.23**	**12.89**

11.6 Appendix 6

Raw data for Study S14036

For the following tables:

ASP concentration measured as µg Domoic acid and Epidomoic acid / g, obtained by HPLC-UV detection

PSP concentration measured as µg Saxitoxin equ / 100g obtained by proprietary ELISA kit

DSP concentration measured as µg Okadaic acid equ / kg obtained by PP2A inhibition assay

Module 1:

DSP concentration obtained by PP2A inhibition assay measured in µg Okadaic acid equ /kg of tissue.

ID	Date of scallop sampling						
	31-Mar-08	01-Apr-08	04-Apr-08	07-Apr-08	09-Apr-08	11-Apr-08	14-Apr-08
1	23.3	26.6	258.7	472.5	276.5	1416.1	1222.1
2	20.4	168	491	271.2	1160	357.5	1477.9
3	21.4	28	64.4	853.7	600.8	517.7	673.2
4		43.2	122.9	411.7	705.3	1123.3	391.7
5		92.1	484.7	199	357.8	1646.7	722.8
6		24.9	366.1	108.1	734.5	661.5	1156.5
7		38.3	508.9	608.8	303.7	1367.7	1183.6
8		25.8	345	135.9	410	1067.1	1005
9		36	692.9	110.6	465.6	943.3	240.7
10			314.5	439.1	373.6	401.5	403.9
11			398.1	928.9	655.3	996.2	929.4
12			175.7	1139.2	752.4	1106.8	872.9
13			101.5	239.4	185.2	999.3	914.1
14			115	438.5	623	204.2	603
15			303.8	562.1	674.5	628.4	139.6
Mean	21.7	53.7	316.2	461.2	551.9	895.8	795.8
st dev	1.5	47.7	181	313.4	249.1	421.5	389.3
% CV	**6.76**	**88.89**	**57.23**	**67.96**	**45.14**	**47.06**	**48.93**

Module 2

Group 2 – DSP toxin concentrations from 30 scallops split into 3 tissue sub groups and analysed by PP2a inhibition assay

	Muscle		Gonad		Remainder
ID	**Un-hydrolysed**	**Hydrolysed**	**Un-hydrolysed**	**Hydrolysed**	**Hydrolysed**
1	32.3	49.7	50.3	144.9	1163.8
2	29.2	36	49.6	226.3	3581.9
3	26.2	33.9	45.1	183.2	4588.9
4	23.9	49.2	49.7	140	1079.8
5	0	36.2	53.3	163.8	3190.2
6	28	46.6	56.2	242.1	3263.2
7	22.4	40.2	45.3	194.5	2309.8
8	24.1	53.2	50.1	148.4	3871.6
9	20.9	57.6	51.1	215.1	4393.2
10	22.6	40.9	51.3	272.6	3155.2
11	29.4	60.5	57.1	557.6	4336.3
12	32.8	50.7	64	657.4	5685.4
13	26.4	55.2	101.4	823.7	3788.6
14	27.3	48.2	53.5	229.8	3623.6
15	27.9	53.4	45.4	151.8	2409.2
16	28.6	61.9	334.3	1910.5	4003.4
17	21.9	35.3	147.3	159.6	3664
18	27.3	39.7	205.8	908.2	6936.3
19	29.1	46.1	188.9	678.9	5205.2
20	26.2	36.3	148.3	176.9	2395.9
21	19.8	68.1	199.8	688.3	3341.8
22	25.5	53	718.7	1131.2	5350.1
23	33.9	37.9	622.1	1062.1	2363.8
24	33	53.7	206.9	769.9	3769.9
25	33.9	43.2	76.6	186	1997.9
26	35.9	37.2	46.5	56.2	3413.4
27	27.5	42.1	159.2	426	5660.6
28	32.7	47	51.7	89.1	2939.1
29	33.4	43.4	67.4	215.9	2939.9
30	19.2	38.2	60.2	155.7	3391.4
mean	26.7	46.5	135.2	432.2	3593.8
stdev	6.8	8.9	162.2	417.3	1316.1
%CV	**25.36**	**19.22**	**119.95**	**96.55**	**36.62**

Group 3 - DSP toxin concentrations from 30 scallops split into 2 tissue sub groups and analysed by PP2a inhibition assay

	Muscle + Gonad combined	Remainder
ID	**Hydrolysed**	**Hydrolysed**
1	58.5	3168.2
2	59.1	5021.2
3	49.1	1377.7
4	47.7	2266.6
5	58.7	2403.9
6	60.2	5440.6
7	59.7	3053.6
8	57	3497.3
9	49.8	3528.8
10	52	4036.5
11	164.8	2877.4
12	60	3811.1
13	46.7	2083.6
14	51.2	1845.6
15	47.2	2862
16	44.9	3829.6
17	61.9	5979
18	44.7	3193.6
19	59.9	2879.8
20	45.6	4821.8
21	38.8	5386.1
22	44.1	3068.8
23	45.1	4457.7
24	64.3	3629.2
25	44.4	2375.2
26	73.6	5234.6
27	68.5	3609.8
28	110	1753.3
29	46.4	5724.6
30	45.8	4043.6
mean	58.7	3575.4
stdev	24.1	1247.2
%CV	**41.04**	**34.88**

Modules 1 & 2:
Comparison of mean DSP concentration (OA equ. µg/kg) in different tissue groups

ID	**Mean DSP concentration - hydrolysed**
Muscle	46.5
Gonad	432.2
Remainder	3593.8
Muscle + Gonad	**58.7**
Remainder	**3575.4**

Module 3

Combined concentrations of DSP toxins from all tissue groups from Module 2

The mean concentrations of DSP toxins, as determined using the PP2A assay, in all the tissue sub groups from all three sub-groups as examined in Module 2 were calculated. From this, it was observed that significantly high concentrations of DSP toxins were present in the Remainder tissue groups (Figure 13)

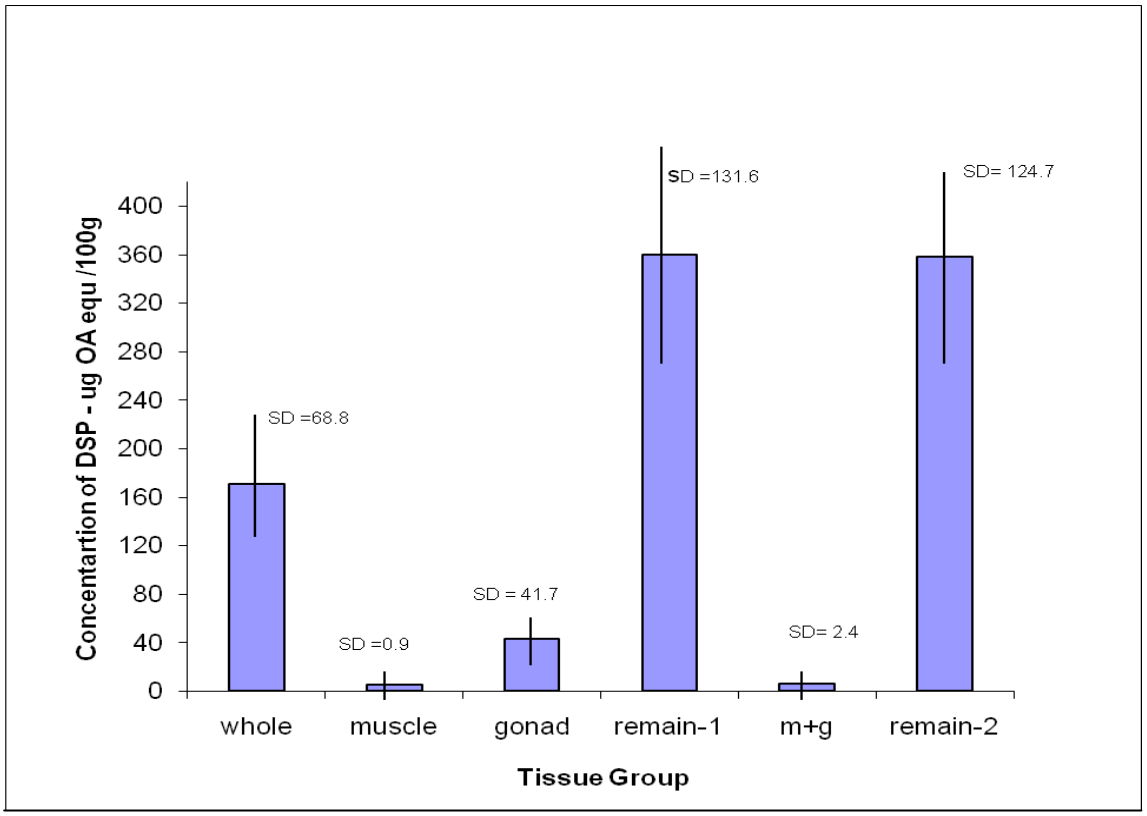
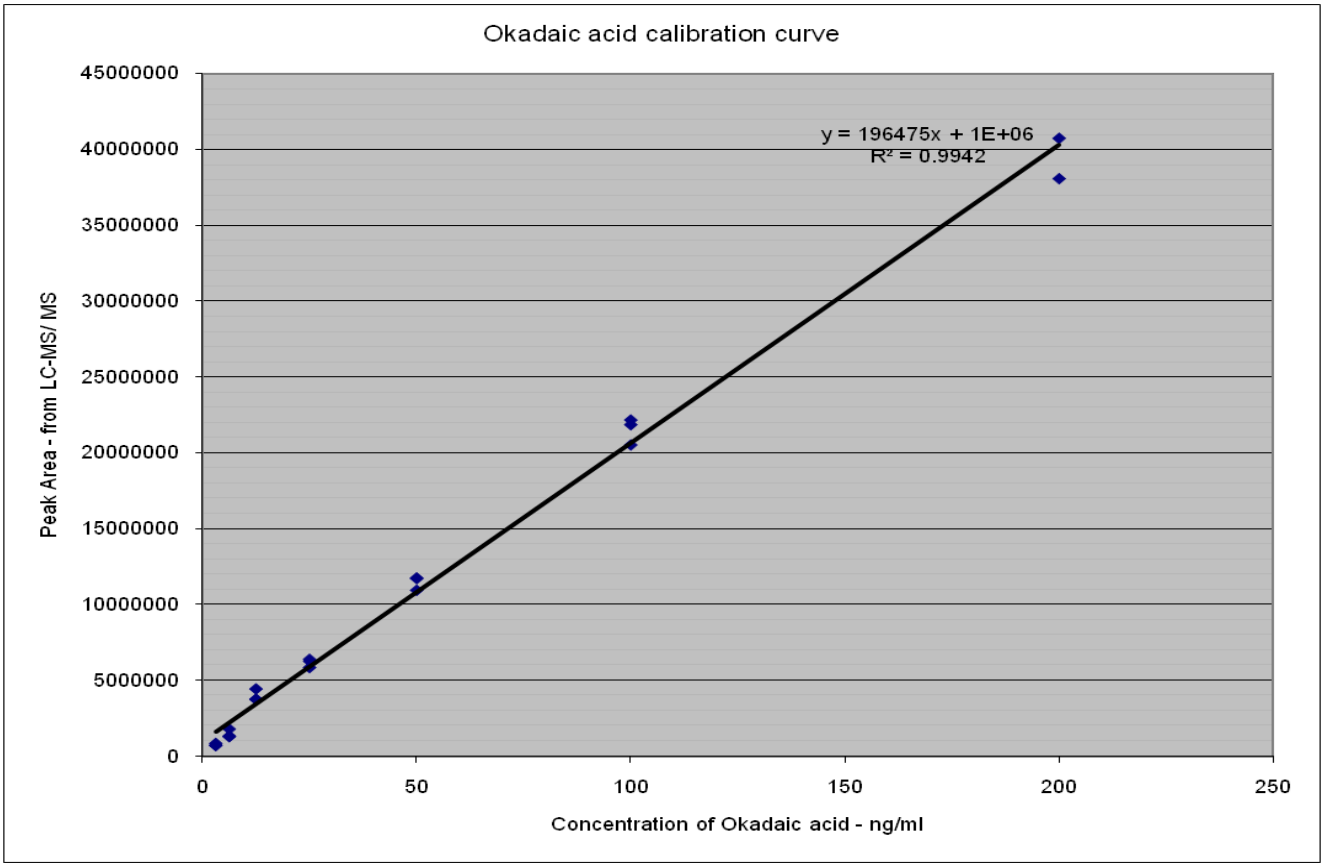


Figure 14. Comparison of mean DSP concentration in all tissue subgroups from Module 2 measured as µg OA equ / 100g tissue by PP2A inhibition assay with alkaline hydrolysis, with variation between individuals shown as standard deviation (SD) values.

11.7 Appendix 7

LC-MS-MS data for whole king scallop and algal samples

Module 2



LC-MS analysis of whole king scallop unhydrolysed samples

	OkAc NHy	N-Hy Dilution(µl)	ng/ml	ng/ml(500(µl)	µg/kgtissue
S1	7861130	100	34.92	6.98	34.9
S2	3800296	500	14.25	14.25	71.3
S3	16215253	100	77.44	15.49	77.4
S4	4925575	500	19.98	19.98	99.9
S5	36360130	100	179.97	35.99	180.0
S6	10689154	100	49.31	9.86	49.3
S7	6227689	500	26.61	26.61	133.0
S8	22856835	100	111.24	22.25	111.2
S9	2922906	500	9.79	9.79	48.9
S10	4678143	100	18.72	3.74	18.7
S11	8809391	200	39.75	15.90	79.5
S12	10671797	100	49.23	9.85	49.2
S13	21333404	100	103.49	20.70	103.5
S14	6812745	200	29.59	11.83	59.2
S15	21205916	100	102.84	20.57	102.8

LC-MS analysis of whole King scallop hydrolysed samples

	OkAc HY	HY Dilution(µl)	ng/ml	ng/ml(500(µl)	µg/kg tissue
S1	10520531	1100	48.46	106.60	533.0
S2	12405673	1200	58.05	139.32	696.6
S3	21058628	1100	102.09	224.60	1123.0
S4	25398684	1000	124.18	248.36	1241.8
S5	18970387	1000	91.46	182.93	914.6
S6	11154812	1000	51.69	103.37	516.9
S7	18387348	1000	88.50	176.99	885.0
S8	8375038	1000	37.54	75.07	375.4
S9	29692061	1000	146.03	292.07	1460.3
S10	21833680	500	106.04	106.04	530.2
S11	23751348	500	115.80	115.80	579.0
S12	19312719	500	93.21	93.21	466.0
S13	31285690	500	154.15	154.15	770.7
S14	20178365	500	97.61	97.61	488.1
S15	35433941	500	175.26	175.26	876.3

LC-MS analysis of Algal samples – unhydrolysed and hydrolysed

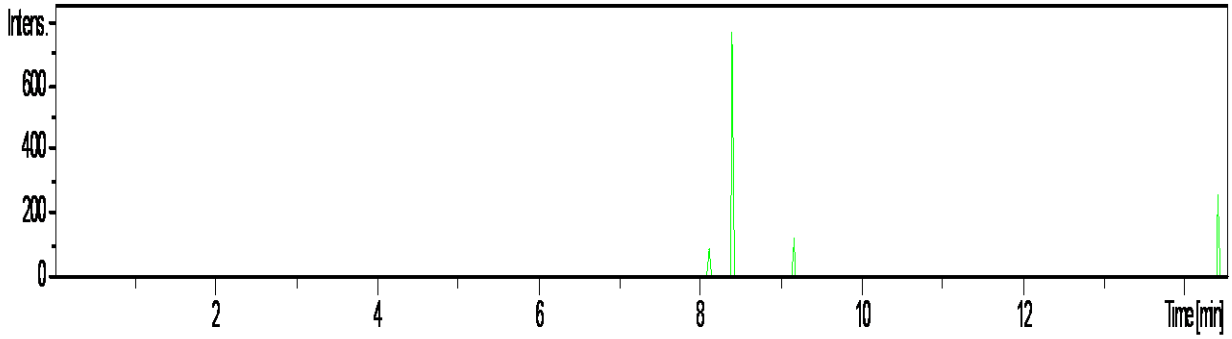
Algae	OkAc N-Hy	N-Hy Dilution(µl)	ng/ml	ng/ml(500(µl)	µg/100mL
S1	18731766	300	90.25	54.15	1.08
S2	19248202	300	92.88	55.73	1.11
S3	18021335	300	86.63	51.98	1.04
S4	20776261	300	100.66	60.39	1.21
S5	18388140	300	88.50	53.10	1.06

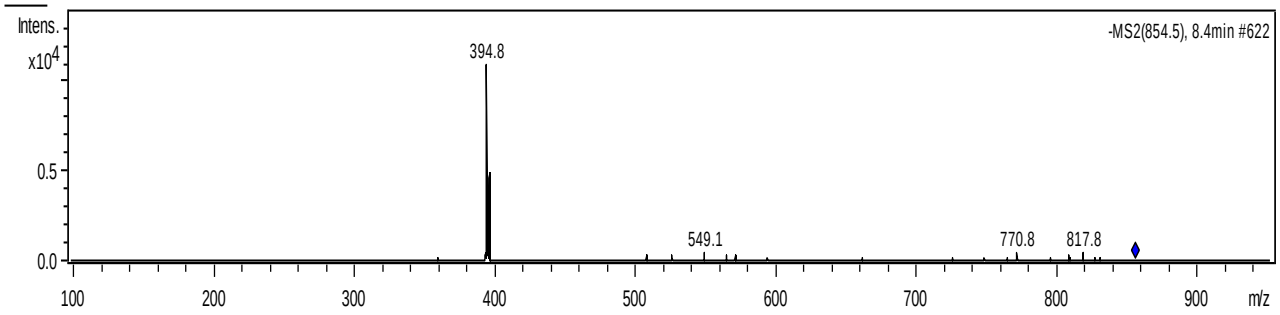
Algae	OkAc HY	HY Dilution(µl)	ng/ml	ng/ml(500(µl)	µg/100mL
S1	37869286	2000	187.65	750.62	15.01
S2	35036957	2500	173.24	866.19	17.32
S3	28030066	3000	137.58	825.45	16.51
S4	33395071	2500	164.88	824.41	16.49
S5	30467287	2500	149.98	749.90	15.00

11.8 Appendix 8

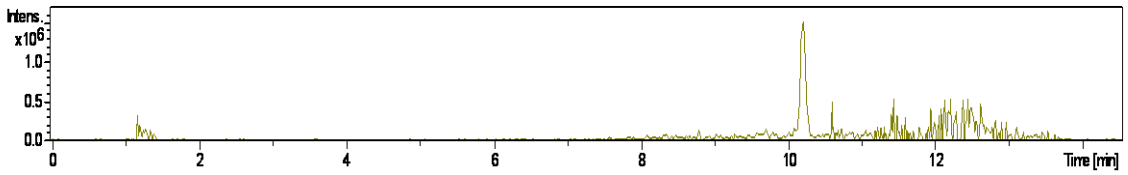
Chromatograms and MS raw data

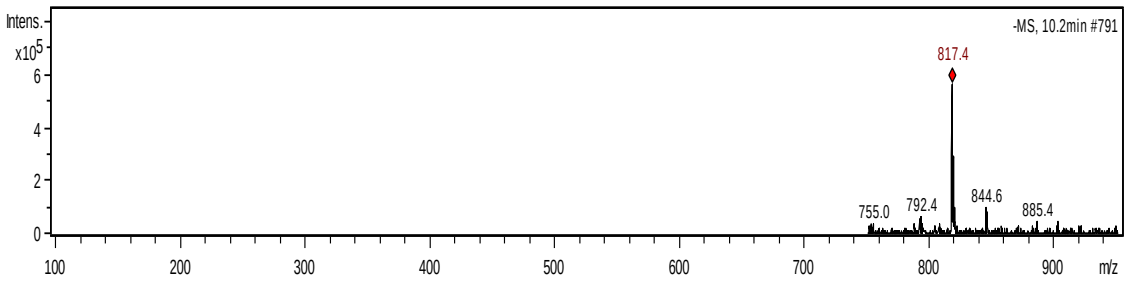
CHROMATOGRAM FROM STD 1 (200NG OA)



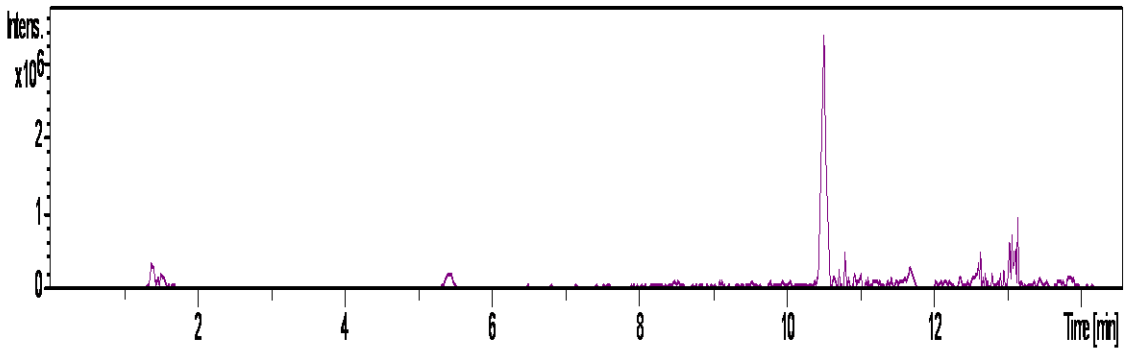


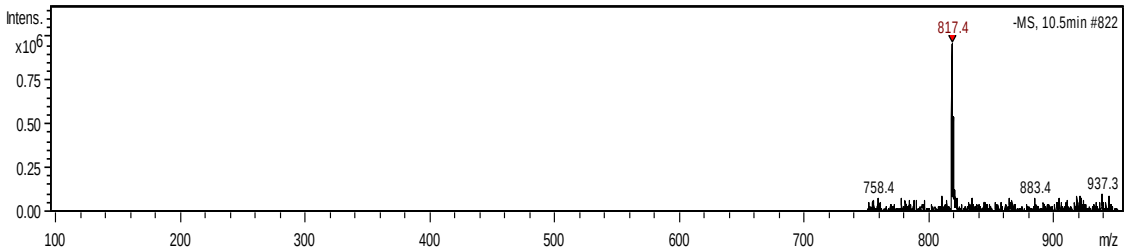
ALGAE HYDROLIZED



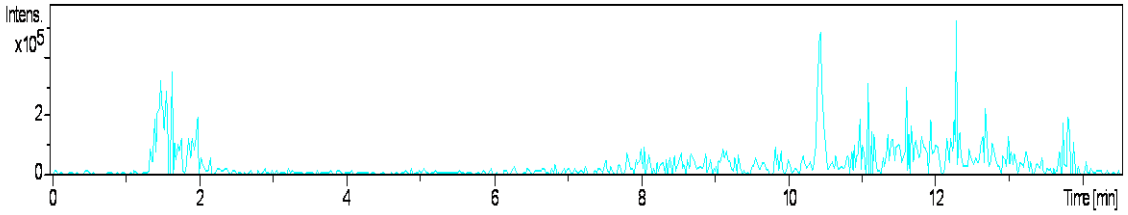


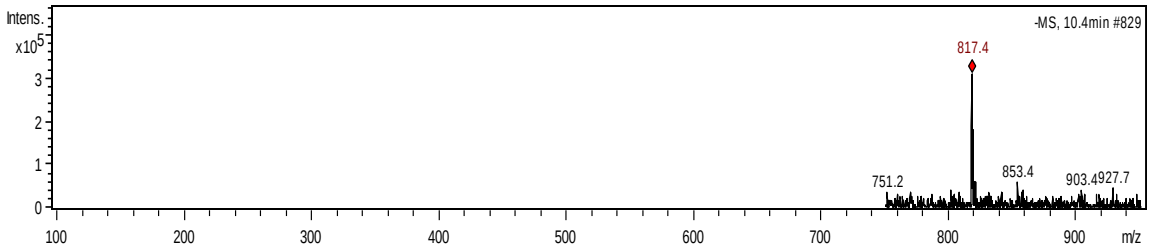
ALGAE NON HYDROLIZED





KING SCALLOPS HYDROLIZED





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