The impact of washing the edible parts (adductor and gonad) on paralytic shellfish poisoning (PSP) toxin concentrations in the King scallop *Pecten maximus*

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FOOD STANDARDS AGENCY

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1. INTRODUCTION

Previous studies funded by Food Standards Agency Scotland (FSAS -Project code S02011) and by the EC (FPV TALISMAN – Project code 70849) identified that over 99% of domoic acid (DA) (the biotoxin responsible for amnesic shellfish poisoning, ASP) associated with contaminated king scallops (*Pecten maximus*) could be eliminated from the edible parts through effective shucking (removal of the hepatopancreas, mantle and gills). Further studies conducted at the Scottish Association for Marine Science (SAMS) showed the importance of flushing out the loop of gut that runs through the gonad (Campbell et al., 2003), in reducing DA burden. Shucking has since been recognised as an important process in controlling the risk of ASP from king scallops, and is now incorporated in the HACCP plans of most UK scallop processors.

Data are lacking on the distribution of diarrhetic shellfish poisoning (DSP) and paralytic shellfish poisoning (PSP) toxins in king scallops, and the efficacy of shucking for removal of these biotoxins has not been scientifically demonstrated. This information is required to determine appropriate levels of End Product Testing (EPT) for shucked king scallops, and therefore help to protect public health by ensuring only safe product is placed on the market.

Workpackage 4 of the EC funded project SPIES-DETOX (Collective Research Project 0302790-2) ran concurrently with the present study and examined shucking and washing practice to optimise removal of ASP and PSP toxins from the edible tissues of king scallops. The SPIES-DETOX project examined scallops from two separate wild populations which had naturally acquired PSP toxins. The aim of the present FSAS study is to provide complementary data using king scallops artificially toxified by feeding them with a PSP toxin producing algal culture. This strategy was adopted to avoid reliance on obtaining sufficient naturally toxified material in the time frame and to provide supplementary material to enable the distribution of PSP toxins in the tissues of king scallops to be verified as well as to examine toxin removal through shucking and washing practices previously validated for DA.

2. FEED TRIAL FF I: TOXIFICATION (PSP) OF KING SCALLOPS BY FEEDING CULTURED *ALEXANDRIUM* SP

Materials and Methods

Algal culture

The purpose of this short trial was to confirm the PSP toxin burden in scallops could be increased by feeding them cultures of a toxin producing *Alexandrium tamarense* strain as supplied by Fisheries Research Services (FRS) (original designation 04/197/A1, isolated from Stonehaven; currently maintained at SAMS as CCAP 1119/17: Collins et al. in press).

The *Alexandrium* cells were cultured in L1 medium, at a temperature of 16 $^{\circ}$C and in a 12:12 h light: dark regime. When the cultures reached densities of $\geq$ 6000 cells ml^{-1} they were routinely sub-cultured. The total culture volume was increased approximately seven days prior to feeding to the scallops by sub-culturing one part of a dense culture to two parts freshly prepared sterile L1 medium to a maximum volume of 1 l. Sequential sub-cultures were initiated on alternate days for over a week to ensure that a sufficient number of dense cultures were available for each feed.

Toxin production in the cultures was confirmed using the Jellet Rapid Test (JRT) for PSP toxins and by liquid chromatography-tandem mass spectrometry (LC-MS-MS) analysis at FRS.

Recirculation system

A recirculating system of total capacity 40l consisting of three shallow tanks and a reservoir housing a submersible pump (AquaMedic 'Oceanrunner' model OR2500 producing a flow rate of approximately 12 l min^{-1}) was set up for feeding batches of scallops the cultured *A. tamarense*. Seawater was supplied from the Firth of Lorn via sub-sand filters and the laboratory pumping station. In the recirculating system each tank inlet was fitted with an open ended tube or manometer so that the flow rates could be equalized (Figure 1).

Scallops closely graded to the minimum landing size (110 mm shell height) were hand collected by divers (07 November 2007), from a local population in the Sound of Mull close to the entrance to Loch Aline. Nine scallops, three per replicate tank, were introduced to the system on the 03 December 2007 and allowed to acclimatise. A period of feeding with non-toxin producing microalgae, *Pavlova lutheri*, commenced to determine if the flow rates and conditions in the tanks were such that the scallops would feed freely. Microalgal cell ingestion rates showed the scallops were actively feeding, reducing cell levels in the system from 58,000 cells ml^{-1} to undetectable quantities in less than 2 h.

Scallop feeding regime

The scallops were fed the cultured *A. tamarense* over a 10 day period from 10 to 19 December 2007. *A. tamarense* was added on 11 occasions beginning at two day intervals and ending with three feeds on the penultimate day (Table 1). Prior to adding each feed, the seawater in the system was drained off and replaced with fresh seawater. Each tank was drained to a depth of 25 mm so that the scallops did not dry out and become unduly stressed. Faecal material was removed by siphon as the seawater was changed. The seawater drained from the system was treated with bleach and subsequently poured into the drains leading to a soakaway on the foreshore to avoid any possibility of introducing viable toxic *Alexandrium* cells to the local seawater.

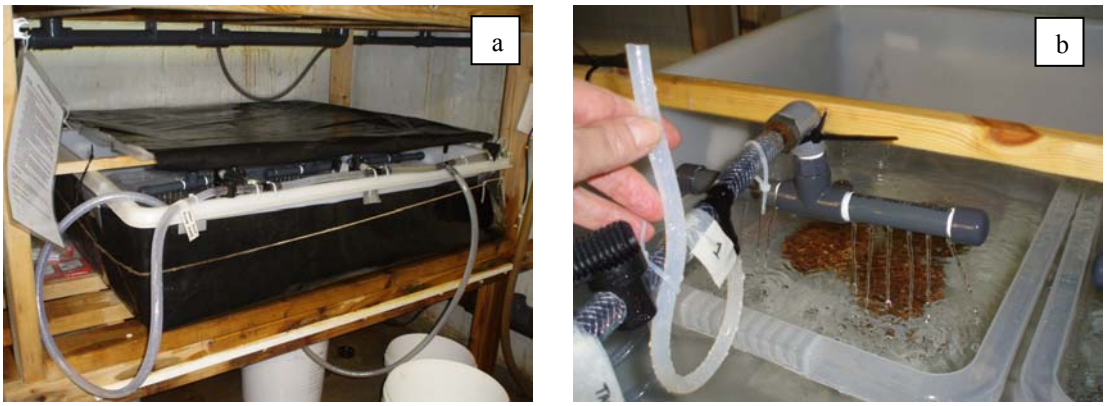

Figure 1a) the covered tank housing the three replicate recirculating systems, b) one of the three replicate tanks in the system showing the spray bar for seawater delivery and manometer

At each feed the volume of the toxin producing culture was noted and a subsample counted to provide an estimate of the total number of cells. The *A. tamarense* cells were then added to the reservoir tank.

Sub-samples of 1 ml were taken from each of the three scallop tanks approximately 5 min after adding the culture to the recirculating system reservoir to confirm the circulating cell concentration and that there was an equal delivery of the toxic cells to each tank.

Individual scallops were identified by a number written on the shell and the animals were rearranged before each feed so a consistent order was maintained from the tank inlet to the tank rear in an attempt to standardise exposure to the algae.

Controls
A sub-sample of scallops, from the same population, sacrificed at the start of the experiment, served as controls.

Scallop processing
Once the feeding phase was completed the scallops were removed, shell width and height recorded (using a ruler), shucked and the tissues separated into the edible parts (adductor and roe) and all other soft tissue (OT). OT consisted of mantle, gut including the hepatopancreas (HP) and the ligament trimmed off the adductor. The shucking process was standardized thus: all scallop tissues were placed on four layers of 'WypAll' blue, absorbent, paper tissue (Kimberly-Clark product MJT 005), and allowed to drain prior to being weighed and stored at -20 $^{\circ}$C. The shucking knife, drip tray, forceps, and hands were rinsed under running tap water in between handling each individual.

A significant amount of information on the anatomical distribution of the PSP toxin in scallops was collected from field populations examined in 2007 as part of the SPIES-DETOX project; as this trial was intended merely to confirm artificial toxification, only the OT was analysed as this is where the bulk of the toxin lies, see Discussion section below.

Table 1. Feeding Trial FF I (day and cell density in 1 l volume) for preliminary toxification trial.

Day (date)	Cell density (cells ml$^{-1)}$ added		Temperature (maximum recorded)
	***P. lutheri* (non-toxic)**	***A. tamarense* (toxic)**	
1 (03.12.07)	4.50 x 10^9		13..0
2			13.0
3	3.80 x 10^9		12.5
4	6.35 x 10^9		14.0
5	none		10.5
6	none		-
7	none		-
8(10.12.07)	none	4.66 x 10^6	12.0
9	none	3.28 x 10^6	12.0
10	none	3.76 x 10^6	13.0
11	none	5.07 x 10^6	13.8
12	none	3.45 x 10^6	13.5
13	none	no feed	-
14	none	no feed	-
15	none	3.59 x 10^6	10.0
"	none	2.54 x 10^6	10.0
16	none	3.28 x 10^6	-
17	none	3.12 x 10^6	10.8
"	none	2.74 x 10^6	10.8
"	none	3.44 x 10^6	10.5
18	End, sacrifice scallops and store tissues.		

PSP detection (all experiments)

Tissues for analysis were stored frozen prior to analysis. On thawing they were macerated with a Polytron PT 2100 homogeniser. Up to 10 g of homogenised tissue was weighed into 50 ml plastic centrifuge tubes and an equal weight of 0.1 M HCl added using a Sartorius ISO 9001 top loading balance reading to two decimal places. The R-Biopharm Ridascreen Fast PSP ELISA assay method for tissue preparation and analysis was followed. The tissue was heated in a water bath at 100 ºC for five minutes with frequent vortexing then centrifuged at 3500 to 4500 rpm for 15 minutes. Supernatant was stored frozen. Prior to analysis supernatant preparations were acidified with 5 N HCl and diluted with test kit buffer, if required, to bring the sample within the range of the PSP toxin standards supplied with the kit.

R-Biopharm state the Ridascreen Fast PSP detection kit cross reactivity as: saxitoxin 100%; decarbamoyl saxitoxin 20%; gonyautoxin 2 and 3, 70%; and neosaxitoxin 12%. The limit of detection is 50 ppb. Note: the carbamate toxins (saxitoxin STX, neosaxitoxin NEO and the gonyautoxins GTX 1, 2, 3 and 4) are reportedly the most potent (Bricelj and Shumway, 1998), and the N-sulfocarbamoyl toxins (B and C) are the least potent, the decarbamoyl (dc) toxins having intermediate specific toxicities.

All PSP toxin concentrations are expressed as µg 100 g^{-1} shellfish tissue to allow ready comparison with the regulatory action limit of 80 µg $100g^{-1}$.

LC-MS-MS analysis at FRS

Prior to analysis, sample extracts were cleaned-up using C18 cartridges. Extracts were diluted with acetonitrile to give acetonitrile:water (80:20 v/v) and loaded onto pre-conditioned ZIC-HILIC SPE cartridges. A wash of 1 ml acetonitrile:water (80:20 v/v) with 0.1% formic acid was used with PSP toxins eluted with 3 ml acetonitrile:water (10:90 v/v) with 0.1% formic acid. Extracts (5 µl) were analysed using a ZIC-HILIC analytical column with MS/MS detection and quantification, as detailed below. Correction was made for any ion enhancement or suppression from the shellfish matrix via the use of standard addition.

PSP toxins were determined using a 3200 QTrap® hybrid quadrupole-linear ion trap mass spectrometer (LC-MS-MS) equipped with atmospheric pressure ionisation fitted with an electrospray ionisation source (turbo V® Source). The instrument was supplied by Applied Biosystems, Warrington, UK. The MS/MS was coupled to an Agilent 1200 series LC system comprising of a degasser, binary pump, column oven and autosampler.

Multiple reaction monitoring (MRM) transitions and instrument settings used are given in Turrell et al. (in press) MS/MS analysis was split into two separate data acquisition periods. Period one was used for detection of GTX1 to 5, dcGTX2, dcGTX3, C1 and C2 and period two for STX, NEO, dcSTX and dcNEO. For periods one and two, different ionspray voltages (5250 and 5500 V, respectively) were used and the probe gas temperature (450 ºC), nebuliser and auxiliary gas velocities (60 and 40 l min^{-1}, respectively) were the same for both periods.

A ZIC-HILIC column (150 x 2.1 mm, 3.5 µm particle size, Sequant, Sweden) attached to a ZIC-HILIC guard column (14 x 1.0 mm) was used for the separation of PSP toxins.

A mobile phase consisting of two eluents, A (100% deionised water) and B (95% (v/v) acetonitrile), both containing 2 mM ammonium formate and 3.6 mM formic acid and a flow rate of 0.2 ml min^{-1} was used for all analyses. The column temperature was 30 ºC and injection volume was 5 µl with toxin separation performed under gradient conditions (initially 50% A, 50% to 85% A over 15 min, hold 5 min, re-equilibrate 15 min).

Statistical analysis (all experiments)
Statistical analysis of the data was performed using Minitab version 14 or the Data Analysis function in Excel 2002 (106874.6845) SP3.

Results

The toxin producing strain of *A. tamarense* was cultured successfully in 1 l volumes in the modified culture regime. Cell densities in the 1 l cultures added to the scallop tanks averaged 3.5 x 10^6 cells l^{-1}. The cultures gave a positive result for PSP toxins when tested using the JRT.

The recirculating system worked well, the scallops did not exhibit any stress behaviours and fed freely on the non-toxic and toxin producing cultures. Some bivalve species have been reported to exhibit stress and cease feeding in the presence of PSP toxins (Bricelj and Shumway, 1998).

LC-MS analysis

Using LC-MS-MS, the *A. tamarense* culture was shown to produce an array of PSP toxins with the major toxins being the N-sulfocarbamoyl C2 toxin and the potent carbamate toxins NEO, STX, GTX-4 and GTX-3. Lower concentrations of corresponding epimeric C and GTX toxins were observed (Table 2). Trace levels of GTX-5 and the decarbamoyl toxins, dcGTX-3 and dcGTX-2, were detected in one culture, but other decarbamoyl toxins were not detected.

Table 2. LC-MS-MS analysis of toxin profile for *Alexandrium* 04/197/A1 (CCAP 1119/17)

		Toxin (nM) [a]												
Culture ID	**Flask ID**	**STX**	**dcSTX**	**NEO**	**GTX1**	**GTX4**	**GTX2**	**GTX3**	**dcGTX-2**	**dcGTX-3**	**GTX-5**	**dcNEO**	**C1**	**C2**
04/197/A1	SAMS	120	nd [b]	1106	85	270	9	22	3	2	4	nd	167	512
		Toxin (fg $cell^{-1}$)												
Culture ID	**Flask ID**	**STX**	**dcSTX**	**NEO**	**GTX1**	**GTX 4**	**GTX2**	**GTX3**	**dcGTX-2**	**dcGTX-3**	**GTX-5**	**dcNEO**	**C1**	**C2**
04/197/A1	SAMS	144	nd	1395	140	444	14	34	4	3	5	nd	318	974

[a] STX, saxitoxin; dcSTX, decarbamoylSTX; NEO, Neosaxitoxin; GTX1, gonyautoxin-1; GTX4, gonyautoxin-4; GTX2, gonyautoxin-2; GTX3, gonyautoxin-3; dcGTX2, decaramoylGTX-2; dcGTX3, decaramoylGTX-3; GTX5, gonyautoxin-5; dcNEO, decarbamoylNEO
[b] nd; not detected

Ridascreen Fast PSP analysis.
Analysis of the OT from the control scallops (sub-sampled from the collected population before the feeding experiment started) showed the feeding regime had significantly increased ($P < 0.001$) the mean PSP burden, by two and a half times from a mean of 89.3 to 223.3 μg 100 g^{-1} tissue. The residual level of PSP in the control scallops' OT is presumably a result of PSP events the previous summer. Given that the Ridascreen Fast PSP kit detects only 12% of the available NEO and does not detect GTX-4, the PSP toxin concentrations reported here may be underestimating the total toxicity since the LC-MS-MS analysis detected nearly ten times as much NEO as STX in the cultured toxin producing algae fed to these scallops (Table 2).

3. FEED TRIAL FF II: EFFICACY OF SHUCKING AND WASHING TECHNIQUES TO REMOVE PSP FROM THE EDIBLE TISSUES OF *PECTEN MAXIMUS*

Materials and Methods

Scallop feeding regime
A second feeding trial commenced on March 17th 2008, using the system as described above. On this occasion the scallops, from the same source population and held in SAMS sea water aquaria since collection on 30 January 2008, were stocked at a higher density of six individuals per replicate tank. As previously the scallops, which were hand collected by divers, were closely graded to the minimum landing size (110 mm shell height) in an attempt to minimize variation in toxin accumulation. The feeding regime was intensified to account for the increased stocking density and also with the aim of achieving greater levels of toxification. The scallops were fed a total of 4 l of cultured *A. tamarense* day^{-1} and the cultures were also of higher cell densities that in the previous trial (approximately 6,000 cells ml^{-1}). Using the more intense feeding regime it was noted that small quantities of *A. tamarense* could be seen accumulating in the reservoir tank and so an air stone was added to help keep the toxic cells in suspension and hence available to the scallops.

Scallop processing
Once the feeding element of the experiment was completed the scallops were sacrificed and processed according to one of the three washing protocols used for the field populations examined in May 2007 (SPIES-DETOX). This regime was selected to allow a direct comparison of the field and lab studies.

Shell width and height were recorded (using a ruler) and the tissues separated into adductor, roe and all other tissues (OT). OT included the ligament trimmed off the adductor. As previously the shucking process was standardized. All scallop tissues were placed on four layers of 'WypAll' blue, absorbent, paper tissue (Kimberly-Clark product MJT 005), and allowed to drain prior to storage in bags or tubes at -20 °C. The shucking knife, drip tray, forceps, macerator components and hands were rinsed clean under running tap water in between handling each individual.

Effect of washing the edible parts:
0 wash: Six scallops (two from each tank) were measured (shell height and width) and shucked. The tissues were divided into gonad, adductor and OT and bagged or tubed for storage without any washing.

10 minute wash: Six scallops (two from each tank), were measured and shucked. On this occasion the tissues were shucked as 'roe-on adductors' and OT. With the roe-on product, the roe remains attached to the adductor along with the kidneys. The roe-on products were then placed in a stainless steel colander, sitting in a plastic mixing bowl, and left to soak in running tap water for 10 min. At 0, 5 and 10 min the colander was agitated for 30 sec, so the roe-on product was lightly tossed in the stream of tap water and the bowl beneath the colander emptied. After 10 minutes the product was blotted on 4 WypAll layers, the gonad and adductor separated, weighed, and bagged or tubed and stored frozen (-20 °C).

30 minute wash: A further six scallops (two from each tank) were then processed in the same fashion but with 30 minutes soaking in running tap water, with agitation (for 30 sec) and bowl-emptying every 10 minutes. The tissues were stored frozen.

* NOTE As the roe-on product was washed as a batch of three it was not always possible to link gonad and adductor data to the corresponding OT PSP value within that batch.

Controls: Prior to the start of the feeding experiment six scallops were sacrificed and their tissues shucked and prepared to allow the determination of the anatomical distribution of the toxin: the gonad was washed using a wash bottle of distilled water (MilliQ) whilst rubbed between finger and thumb to test for the removal of surface mucous. A 1 ml disposable pipette was introduced to the entrance of the gonadal gut loop and used to flush out the content. The adductor was similarly rinsed in distilled water until it looked clean.

All tissues were analysed using the R-Biopharm Ridascreen Fast PSP kit as described above.

Results:

Toxin distribution and level in control scallops
PSP toxins were detected predominantly in the OT (78.9%), with lesser amounts in the gonad (14.5%) and adductor muscle (6.6%). The mean PSP level in the OT was 75.4 µg 100 g $^{-1}$ SD = 23.7. Mean toxicity of the edible parts of the control group were adductor 6.4 µg 100 g $^{-1}$, SD = 3.6 and gonad 13.8 µg 100 g $^{-1}$, SD = 4.5, n = 6 in each case (Figure 2a). The mean gonad weight was 4.8 g SD = 2.0.

Toxification
The analysis of the other tissue (OT) including the hepatopancreas showed that toxification was achieved, the overall mean level was 166.2 µg 100 g, $^{-1}$, SD = 61.2 (n = 18) compared to the controls (mean 75.4 µg 100 g $^{-1}$ SD = 23.7, n = 6) and there was no significant difference in the mean OT toxicity of the three groups of scallops whose edible tissues were then subjected to the different washing regimes (Figure 2b).

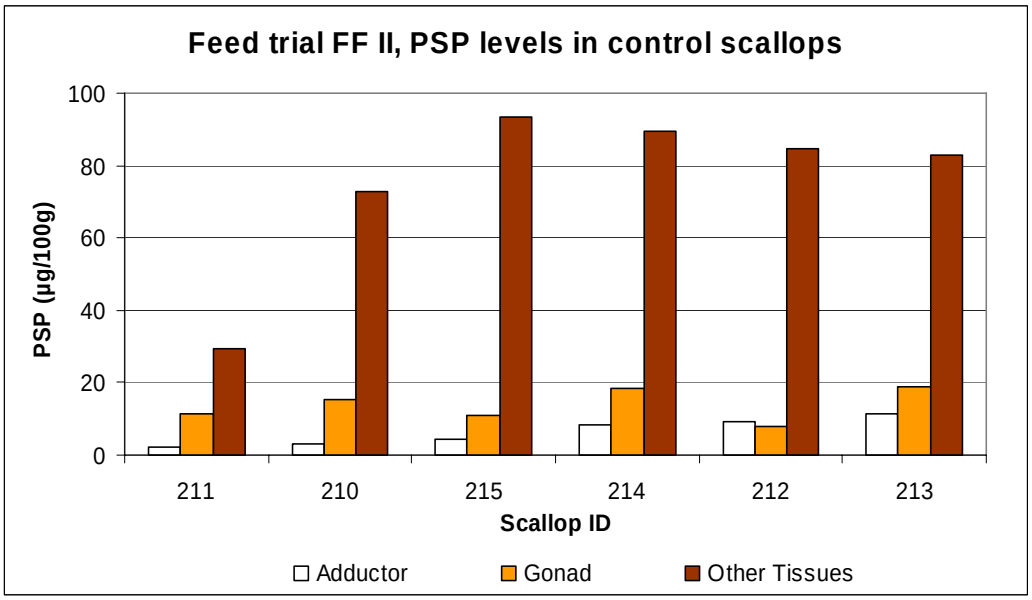

Figure 2a. Anatomical distribution of PSP concentration ranked by ascending adductor levels in six FF II individual scallops (controls) from a sub-sample of the population prior to feeding toxin producing *Alexandrium*.

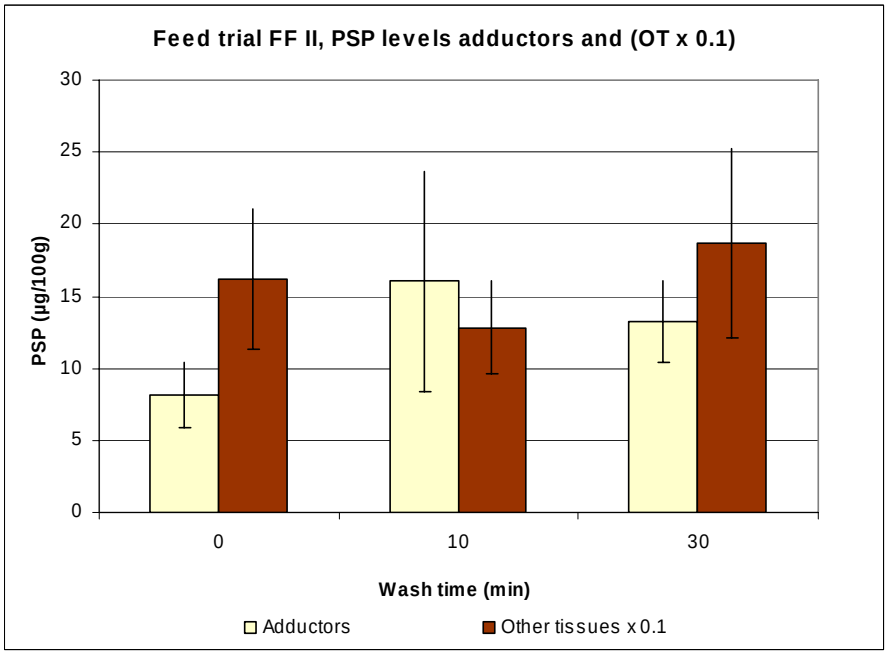

Figure 2b. Mean PSP toxin concentrations in FF II artificially toxified scallops: in the adductors which were exposed to different washing regimes (0, 10 or 30 min) and in other tissues, OT (no wash treatment, values reduced to 10%). Error bars = 95% confidence limits, n = 6 in each case

Effect of washing the edible parts:
The mean toxicity for gonads in the three treatments were 0 wash 109.5 µg 100 g $^{-1}$ (SD = 25.11), 10 min wash 122.92 µg 100 g $^{-1}$ (SD = 26.97) and 30 min wash 71.98 µg 100 g $^{-1}$ (SD = 15.30). There was no significant difference between the toxin levels of the 0 wash and 10 min wash treatments, but there was significantly less toxin in the gonads from the 30 min wash treatment. A regression analysis showed a significant loss of toxin with increased washing time (R Square value 0.354, $P < 0.05$) Figure 3. The mean gonad weight (all treatments) was 4.31 g SD = 1.77. There was no significant loss of adductor toxicity with increased washing time (Figure 2b).

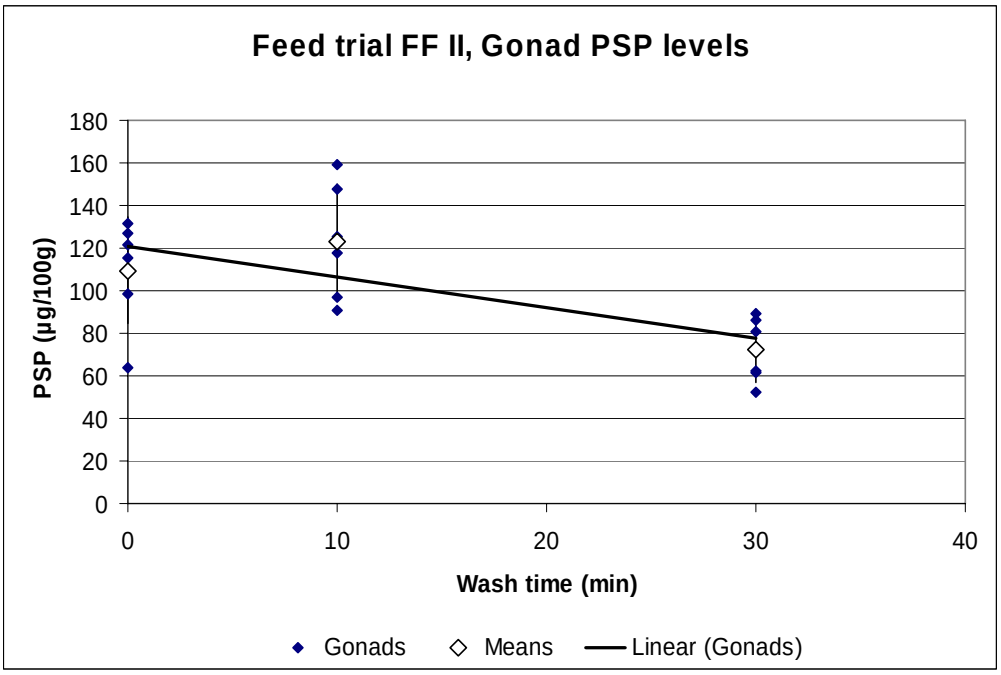

Figure 3. Gonad toxicity (µg 100 g $^{-1}$ tissue) for FF II artificially toxified scallop gonads washed for either 0, 10 or 30 min

LC-MS-MS analysis
PSP toxins were not detected in the adductor or gonad of the control scallops using LC-MS-MS.
STX was present in the OT of the control scallops (Table 3, samples FF2/9 and FF2/10 each comprising three scallops combined) but there were considerably greater concentrations of NEO (Table 3).

In the gonad of the artificially contaminated scallops fed the toxin producing culture, PSP was primarily present as STX, NEO, GTX-3 and C1. Low sample numbers precludes statistical analysis but from a basic examination of the data there was no evidence of washing (0 wash compared with 30 min wash) reducing the toxin levels in the gonad. In the OT of the experimental scallops, the PSP was represented by STX, higher than two orders of magnitude over that found in the controls, NEO was present, as was GTX-1, -3 and -4. GTX-4 was present at a relatively high level not seen in the

controls. The presence of NEO and GTX-4 suggest that the PSP levels reported using Ridascreen Fast PSP method may be underestimates.

4. FEED TRIAL FF III: EFFICACY OF SHUCKING AND WASHING TECHNIQUES TO REMOVE PSP FROM THE EDIBLE TISSUES OF *PECTEN MAXIMUS*: ANATOMICAL DISTRIBUTION OF TOXIN AND THE EFFECT OF CONTAMINATION BY HEPATOPANCREAS.

Materials and Methods

Scallop feeding regime

The third feeding trial commenced on April 24th 2008, using the same holding system as described above. The king scallops, collected locally from the Sound of Kerrera by divers and held in SAMS outdoor raceway aquaria, were again stocked at a density of six individuals in each of the three replicate tanks of the recirculation system. The scallops were by necessity sourced from a different location to those in the previous trial as the Sound of Mull scallops in the SAMS raceways had recently spawned prolifically and the corresponding loss of gonad biomass rendered them unsuitable for use in the experiment.

The scallops were closely graded to the minimum landing size (110 mm shell height) in an attempt to minimize variation in toxin accumulation. They were fed a total of 4 l of cultured *Alexandrium* day^{-1} and the cultures were of high cell densities (approximately 6,000 cells ml^{-1}). To prevent any *Alexandrium* cells accumulating in the reservoir tank an air stone was again used to help keep the algae in suspension and available to the scallops.

Scallop processing

Once the feeding element of the experiment was completed the scallops were sacrificed and processed to allow a more in-depth study of the anatomical distribution of the PSP toxin in the gonad and also to allow for a study on the impact of gross contamination of the edible tissues by the contents of the hepatopancreas during shucking. Prior to the start of the feeding experiment six scallops were sacrificed and their tissues prepared for analysis as controls. At the end of the feeding trial scallops were selected at random from the three replicate tanks, measured (shell height and width) and prepared as a 'roe-on' product.

Controls

The tissues from the control scallops were shucked and prepared in such a way as to allow the determination of the anatomical distribution of the toxin: the gonad was washed using a wash bottle of distilled water (MilliQ) whilst rubbed between finger and thumb to test for the removal of surface mucous. A 1 ml disposable pipette was introduced to the entrance of the gonadal gut loop and used to flush out the content. The adductor was similarly rinsed in distilled water until it looked clean.

Contamination solution

The hepatopancreas (HP) was then removed from each of the 18 scallops. These were separately weighed, pooled and homogenised and the resulting 42.5 g of homogenized tissue diluted to 10% by the addition of distilled water to prepare sufficient volume of a HP contamination solution.

Washing and sampling procedures

A sub-sample of adductor muscle (approximately 25%) was removed after careful surface washing (30 sec) of this tissue in fresh distilled water. A tag was inserted through the remaining (roe-on) adductor

muscle to enable tracking of individual tissues through the following washing treatments where six roe-on adductors including kidneys were washed together. The objective being to generate before and after results for the same adductor tissue in an attempt to identify potential sources of variation in the data set. The gonads were not sub-sampled to test for increased toxification before and after soaking in the HP solution, as unlike the adductor, the gonad is a membrane bound organ and sub-sampling would disrupt its integrity.

Contamination

After removing the adductor sub-sample, all 18 roe-on tissues were contaminated by submerging in HP solution for 12 minutes with occasional agitation. The objective was to mimic poor shucking practice where edible tissues are allowed to come into contact with gut-content on the shucking table, and also to try and produce a standardized level of toxicity in the tissues, reducing individual variation prior to washing treatment.

The roe-on tissues were divided into three groups of six. A '0 wash' set of roe-on tissues was prepared by applying a two or three second quick rinse of tap water to remove visible HP solution contamination. Two further sets of six were washed in a colander and under running tap water as described previously for 10 and 30 minutes respectively. The roe-on tissues were bagged and frozen (-20 ºC) prior to analysis. For a summary of the treatments see Table 4.

Sub-sampling of gonad and kidney tissues

A loop of gut runs through the scallop gonad. While frozen, a subsample of gonad tissue remote from the gut-loop and not therefore containing any gut content was carefully removed to an eppendorf tube. Approximately equal proportions of testicular and ovarian tissues were removed. When thawed the remaining gonad was cut free from the adductor, any black parts of the hepato-pancreas were cut away and the kidneys separated into an eppendorf tube. The objective was to determine if the PSP toxin burden of the gonad was primarily associated with the gut-loop or associated with gonad tissue itself, and to determine any PSP toxin contribution from the kidney.

All tissues were weighed and refrozen (-20 °C) prior to PSP extraction and toxin assay. The tissues were analysed using the R-Biopharm Ridascreen Fast PSP kit as described above.

Table 4. Summary of experimental process, feed trial FF III.

Scallops	Tissue	Sample / scallop	N	N (samples)
PRE-TOXIFCATION CONTROL (6)	Adductor	1	6	6
				-
	Gonad containing gut loop	1	6	6
	Gonad tissue sub-sample (no gut loop)	1	6	6
	Kidney	2 (as one)	6	6
	Other tissue (OT) including hepatopancreas (HP)	1	6	6
TOXIFIED (18)	Adductor sub-samples: all 18 roe-on meats washed as for 'anatomical distribution' and adductor sub-sample removed from each	1	18	18
H-P dissected out of all toxified scallops, pooled, homogenised for contamination solution		1 pooled	18	(sub-sampled)
	H-P solution sub-samples taken to verify toxicity and check homogeneity			3
Edible tissues (roe-on) contaminated and then washed for 0, 10 or 30 min				
	Adductor, contaminated, washed 0, 10 , 30	1	18	18
	Gonad (less sub-sample) containing gut loop, contaminated, washed 0, 10, 30	1	18	18
	Gonad sub-sample tissue (no gut loop), contaminated, washed 30	1	6	6
	Kidneys (both)	1	6	6
	OT minus HP: 6 INDIVIDUALS	1	18	6
		Sample total		115

Results

Toxin distribution and level in control scallops

The anatomical distribution of the toxin in the control scallops showed it was predominantly in the OT (71%), with lesser amounts in the gonad (15%), and adductor muscle (14%) (Figure 4 and see also Table 6 for toxin ratios). The mean PSP burden of the adductor, gonad and OT in the controls was 6.67 μg 100 g $^{-1}$ SD = 1.36, 7.49 μg 100 g $^{-1}$ SD = 3.54 and 35.4 μg 100 g $^{-1}$ SD = 7.41 respectively, n = 6. The mean gonad weight was 4.5g SD = 2.0.

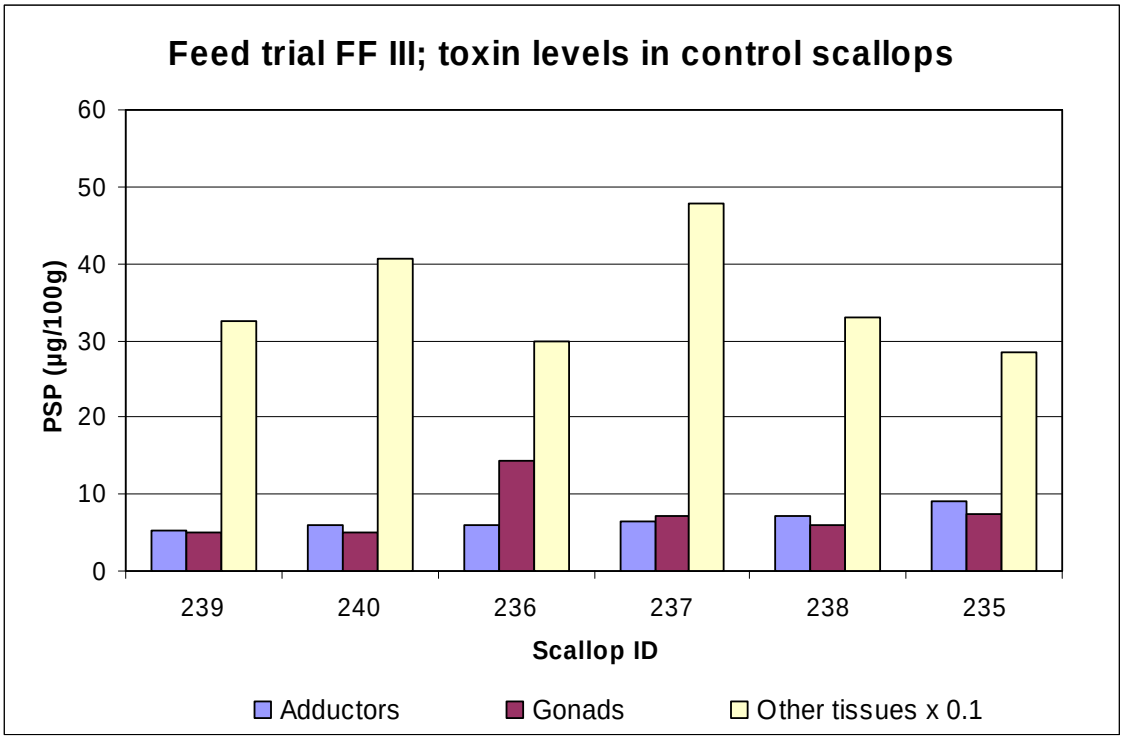

Figure 4. Anatomical distribution of PSP toxin ranked by adductor levels in six FF III individual scallops (controls) sub-sampled from the population prior to feeding toxin producing *A. tamarense.* OT concentration values reduced to 10%.

Toxification

After feeding the king scallops with *A. tamarense*, analysis of the other tissue (OT) including the hepatopancreas showed that toxification was achieved, the mean level from three sub-samples of the OT of the 18 toxified scallops was estimated as 144.6 µg 100 g $^{-1}$ * compared to the controls which had a mean of mean 35.4 µg 100 g $^{-1}$ SD 7.4, n = 6. [*note this value is calculated from the separate HP and (OT minus HP) values]. Three sub-samples of the HP contamination solution were tested and the mean PSP toxin concentration was 204.5 µg 100 g $^{-1}$.

Impact of contamination and effect of washing

The adductor muscles were in effect toxified twice, once by feeding the live scallops the toxin producing algae and once by immersion in the HP contamination solution (for three quarters of each adductor tissue only, after shucking out the edible parts). As a result of feeding the scallops, the adductor muscles in the experimental animals acquired between 4 to 6 times the PSP toxin concentrations observed in the original control animals (Figure 5). Because of the high level of variation in the amount of PSP acquired by individuals in the same treatment the data do not show that soaking the adductors in the HP contamination solution significantly increased the PSP contamination. No significant differences in the PSP levels were noted in the contaminated (soaked) adductors washed for either 0, 10 or 30 min. However it should be noted that washing did not reduce adductor PSP toxin concentrations to those of the original scallop batch (controls) i.e. the PSP toxins acquired from feeding of toxic algae were not removed (Figure 5).

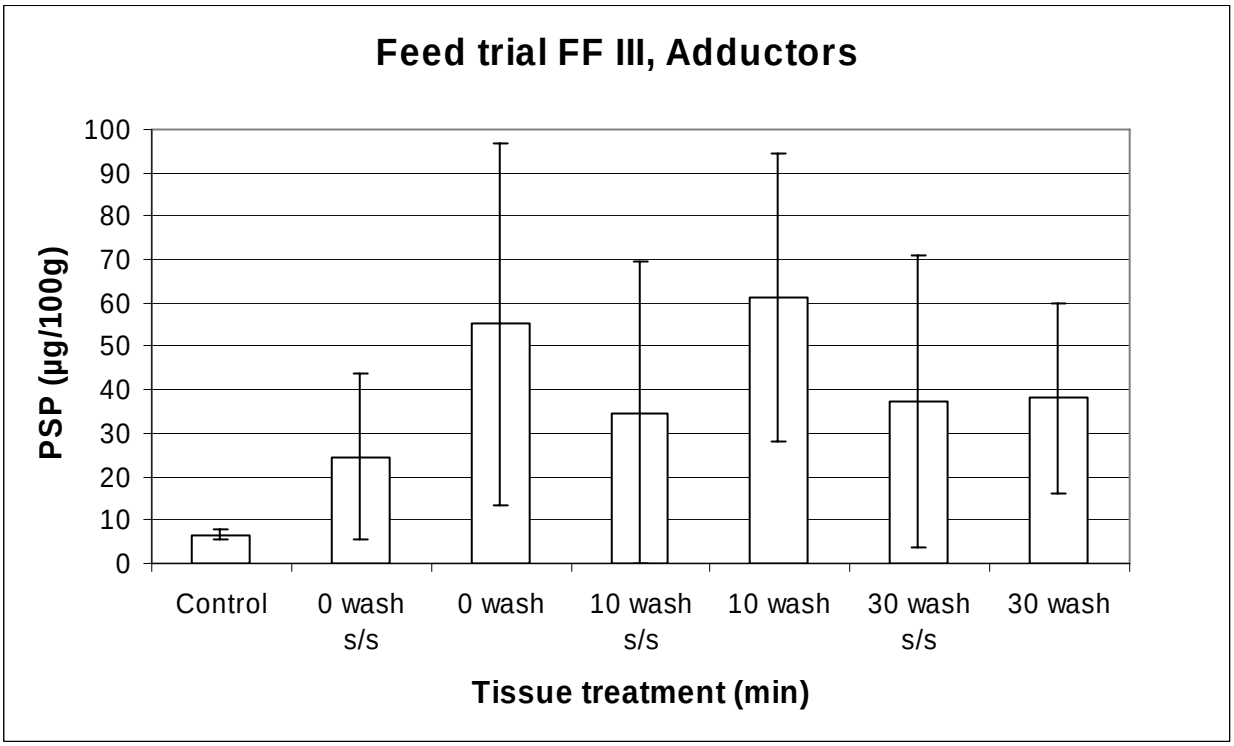

Figure 5. Mean PSP toxin concentrations in FF III adductor tissues from control scallops (unfed), adductors from remaining scallops fed toxic algae (subsequently soaked in a hepatopancreas contamination solution then subjected to a 0, 10 or 30 minute wash) and s/s as their corresponding adductor subsamples removed prior to washing treatment. Error bars = 95% Confidence limits, n = 6 in each case

There was no correlation between pre- and post soaking PSP levels in the adductor tissue i.e. the sub-samples with the highest PSP levels did not produce the adductors with the highest levels post-soaking (Figure 6). The adductor sub-sampling and tagging method did not help to elucidate sources of variation in the data set. Two sub-sampled and three (different) soaked adductors had PSP levels greater than the action level of 80 µg 100 g $^{-1}$. The levels of PSP in the adductors from toxified scallops in this trial (e.g. 30 min wash sub-sample, not soaked, mean 37.2 µg 100 g $^{-1}$) were higher than those found in scallops from a wild population studied in 2007 which had far greater OT toxicity (Scapa, see discussion, anatomical preparation 12.5 µg 100 g $^{-1}$).

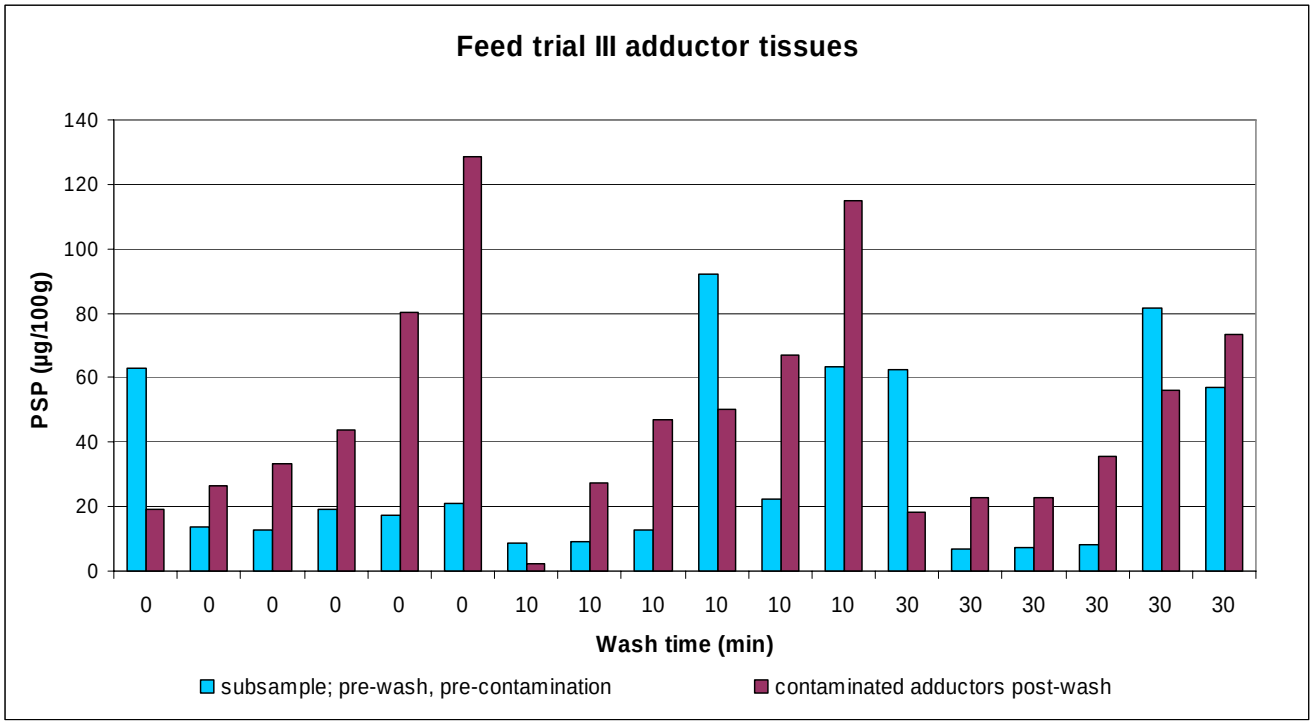

Figure 6. PSP concentrations in individual FF III scallop adductors prior to (as sub-sample) and post soaking in a contamination solution of macerated, toxic scallop hepatopancreas. Paired results ranked by ascending order per wash group of post wash tissues. The first 10 min, post-wash value was below the detectable limit (5 µg 100g^{-1} shellfish flesh) and is accorded a nominal value of 1 µg 100 g $^{-1}$

The toxification treatments (feeding the king scallops toxin producing algae and soaking the whole gonad in the HP contamination solution) produced a significant increase in the PSP toxin concentrations in the gonads (Figure 7). Mean levels increased from 7.5 µg 100 g $^{-1}$ SD = 2.8 to 83.7 µg 100 g $^{-1}$ SD = 8.9. Washing the gonads did not reduce the contamination with PSP toxins. However, notably, in the sub-sampled gonad tissue collected away from the gut-loop (Figure 7, samples 30 s/s), there were significantly less PSP toxins detected (mean level 11.7 µg 100 g $^{-1}$ SD = 2.7) suggesting that the level of PSP in the gonad is associated with the contents of the loop of the gut running through the gonad.

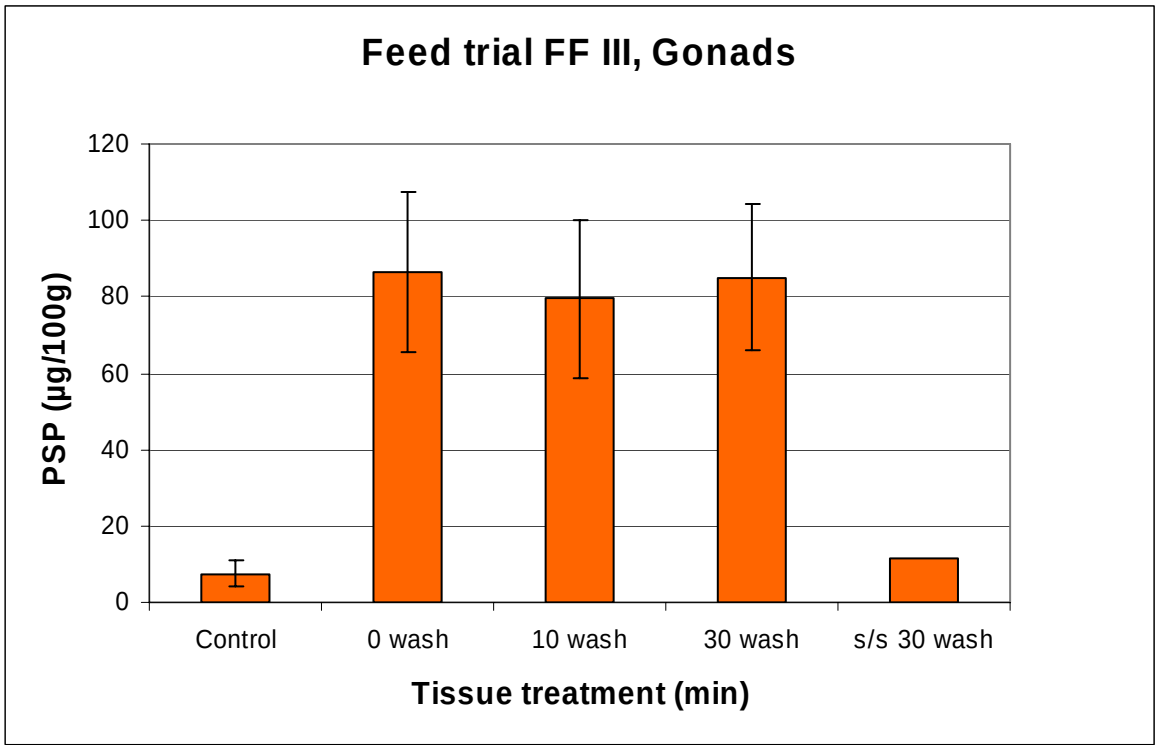

Figure 7. Mean PSP concentrations in whole gonad (i.e. including gut loop) from FF III control scallops (unfed), scallops fed toxic algae and the gonad soaked in a hepatopancreas contamination solution and subjected to a 0, 10 or 30 minute wash and a sub-sample of gonad tissue containing no gut-loop from the 30 min wash treatment (30 s/s). Error bars = 95% confidence limits, n = 6 in each case.

The kidneys were found to contain significantly higher levels of PSP per gramme than the whole gonad. Mean kidney toxicity was 165.4 µg 100 g $^{-1}$ SD = 6.5. The kidneys constitute a relatively small amount of tissue; in these samples comprising around 4.5% of the gonad weight (mean kidney weight 0.2 g SD = 0.07 compared with mean gonad weight of 4.2 g SD = 2.0), although it should be noted the gonads of these scallops were not well developed and therefore not at their maximum size.

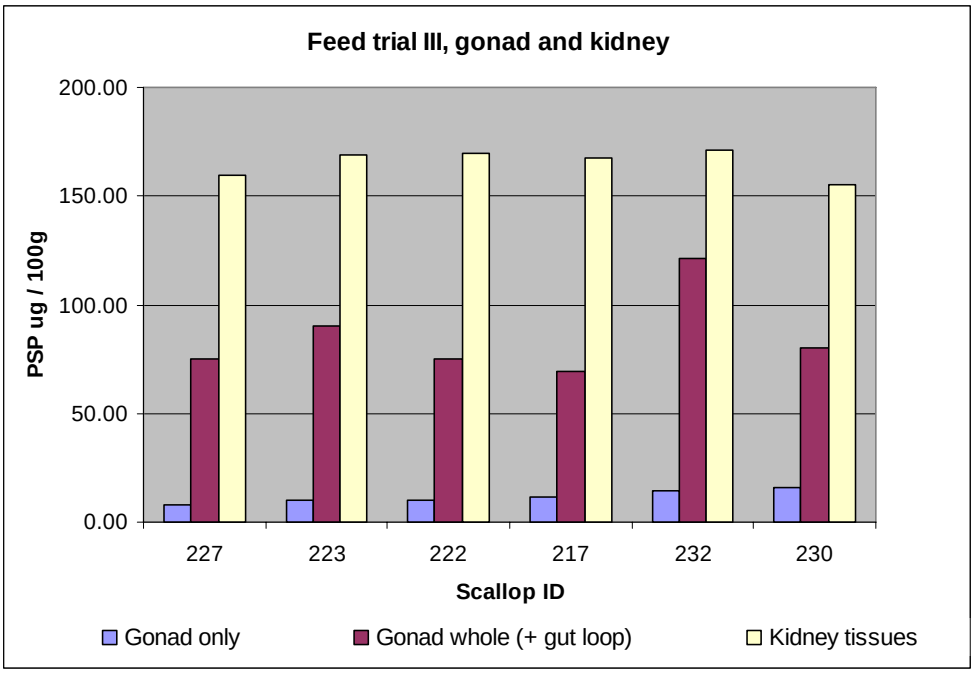

Figure 8. Comparison of PSP toxins in gonad tissue without gut-loop, the remaining gonad tissue with gut loop and kidney tissues, for each of six experimental FF III scallops toxified by feeding cultured *Alexandrium*. Gonads and kidneys, attached to adductor, soaked in a hepatopancreas contamination solution and subsequently washed for 30 minutes.

LC-MS-MS analysis

Low sample numbers preclude statistical analysis, but descriptively, the LC-MS data from Feed Trial III (Table 5) generally support the observations from the Ridascreen analysis in so far as gonad with gut-loop has higher PSP toxin concentrations (present as STX) than gonad sub-samples containing no gut loop. Also the kidneys show high concentrations of PSP toxin, present primarily as STX and NEO, GTX-2 and GTX-3. Moreover the adductors appear to acquire toxin (as STX) after soaking in HP solution. The other tissues (mantle, and gill, gut without HP) have a toxin content, present as STX.

NEO was not detected in the gonads of the scallops artificially toxified in feed trial FF III, but it was present in the gonads of the artificially toxified scallops from feed trial FF II. These scallops were fed the same strain of *Alexandrium* which had a relatively high level of NEO when tested. The presence of NEO in the kidney particularly suggests the Ridascreen method may be underestimating the toxicity of this tissue.

5. DISCUSSION

The scallops used in Feed Trial FF II were sourced in the Sound of Mull, close to the entrance of Loch Aline and FF III animals from the Sound of Kerrera. The collection sites were separated by an approximate distance of 10 km. Overall toxicity in the edible parts of these populations was low at the time of collection, but had a somewhat different anatomical distribution to the other field populations examined as part of the SPIES-DETOX project (Kelly et al. 2008), in that a higher percentage of the total toxin burden was found in the gonads and adductor muscle relative to the OT (Table 6 toxin ratios). In the control scallops for these experiments the PSP levels in the OT of the control scallops represented 79% and 71% of the toxin burden (trial FF II and FF III respectively) whereas for the Loch Laxford and Scapa (Orkney) field populations the OT values were 92% and 94% of the toxin burden (Kelly et al. 2008). Importantly it should be noted that the Mull and Kerrera populations were collected late in 2007 or early in 2008, i.e. outwith the season of naturally occurring blooms of PSP-producing algae. The Laxford and Scapa populations were sampled as close to the height of a naturally occurring bloom as possible. Table 6 summarises results obtained for both these wild populations together with scallops force fed in these experiments.

It is difficult to make assumptions as to whether the observed difference in the anatomical distribution is also a factor of the time elapsed since the toxic algae were ingested. Samples collected for the Phytoplankton monitoring programme indicated the presence of *Alexandrium* sp. cells in the seawater immediately prior to the collections of the Laxford and Scapa field populations but these were not collected from exactly the same locations, or at the same time as the shellfish; the laboratory populations in this study were sacrificed immediately after feeding finished. Toxin distribution is also influenced by the interconnected factors of season / reproductive state (gonad physiology) and gonad weight. The gonads of the Scapa scallops were substantially larger than those from the other populations. Gonad means calculated for all measured scallops were: Scapa 18.9 g (SD 7.8, n = 50) Laxford 10.0 g (SD 4.5, n = 60) FF II 3.5 g (SD 2.1, n = 48) FF III 4.3 (SD 1.8, n = 15). The toxin profile of the *Alexandrium sp.* affecting the wild populations (Loch Laxford and Scapa) and the Sound of Mull and Kerrera scallops prior to their collection, is not known.

The laboratory populations in these experiments were fed at a rate which created a toxic cell density in the recirculation system of 150 cells ml^{-1} four times daily. Overall the toxicity created in the laboratory populations by feeding the *Alexandrium* sp. cultures was similar to that naturally acquired by the Loch Laxford population but less than the 350 µg 100 g^{-1} reported by Bougrier et al (2001) in *P. maximus* fed a monospecific culture of *A. minutum* for 10 to 15 days at a continuous, maintained density of 120 cells ml^{-1}. PSP tissue distribution data are summarized in Table 6 along with wild population data. The concentration of PSP toxins detected in the king scallops from Scapa was a result of an *Alexandrium* sp. bloom recorded at 6,060 cells l^{-1}, the second highest cell density recorded by the FSAS commissioned Phytoplankton Monitoring Programme, in any of the 48 locations surveyed across Scotland, in 2007. The only larger bloom (9,180 cells l^{-1}) was recorded from Cliff Sound, south-west Shetland in late July, although as there were no associated PSP toxins detected in shellfish with this bloom, the majority of the algae may have been the non-toxic western-European strain of *A. tamarense* (Swan and Davidson, 2008; Collins et al. in press).

It is logical to assume soaking in the HP solution added toxicity to the adductors but the small (n = 6) data set on adductor tissues does not show that statistically due to the high level of variation of toxin

concentrations between individual scallops. The gonads were not sub-sampled to test for increased toxification before and after soaking in the HP solution as, unlike the adductor, the gonad is a membrane bound organ and sub-sampling would disrupt its integrity. When considering the entire data set (0, 10 and 30 min) in feed trial FF II, there was an indication that PSP toxins decreased in the gonad with increased washing time (up to 30 min), although there was no significant difference in toxin concentration following a 10 minute wash. There was no indication of loss of PSP toxins in the gonad with increased washing time in feed trial FF III. PSP toxin contamination of the edible meats, either from contact with the HP and its contents on shucking, or from feeding on toxic cells, was not substantially removed even by prolonged washing. Careful shucking to remove and separate the hepatopancreas sac intact remains advisable.

Sub-samples of gonad tissue only (i.e. no gut loop) had, on average, eight times less PSP than the corresponding whole gonad. Therefore, contamination from the gut loop contributes a large part of the gonad toxicity. The finding that there is some toxin in the gonad tissue itself, i.e. that tissue collected away from the gut loop, supports a similar observation made on the sea scallop *Placopecten magellanicus* by Cembella et al. (1993). In feed trial FF III, the toxin in the gonad was not reduced by washing for up to 30 minutes. This is in contrast with findings on the ASP toxin in *P. maximus* gonad, which although also associated with the gut loop, decreased substantially and in direct correlation with increased washing time (Campbell et al. 2003) and with careful flushing of the gut loop as used in the 'anatomical determination' method here. The PSP toxin may be differently bound, adhering to the gut lining or be intracellular.

The mean PSP concentration in the kidneys as detected by the Ridascreen in feed trial FF III were 165 μg 100 g^{-1} which is higher than the estimated level in the OT (145 μg 100 g^{-1}). All the tested kidneys had been subjected to the 30 minute wash treatment. Even though the kidney is a small organ (mean 0.2 g, n = 6), clearly the careful trimming of these tissues from a roe-on product would be good practice where any PSP contamination was suspected. The presence of NEO in the kidney as demonstrated by LC-MS-MS (Table 5) suggests that the Ridascreen determination is an underestimate. It is worth noting that LC-MS-MS analysis of the OT of the Scapa scallop population (data from SPIES-DETOX, Kelly et al. 2008) also showed the presence of NEO, and GTX-1 and GTX-4 in addition to the other toxins detected by the Ridascreen, indicating that the production of these toxins is not peculiar to the cultured strain of *Alexandrium* used in this trial.

RECOMMENDATIONS:

1) Washing the edible parts (adductor and roe) can on occasion (feed trial FF II) reduce PSP toxin concentrations. It does not, however, eliminate PSP toxin levels as effectively or to the same extent compared with the ASP toxin, domoic acid. Although washing cannot be used to reliably reduce contamination of the edible parts to below the safety limit of 80 μg 100 g^{-1} **washing the edible parts by soaking in running tap water, after shucking remains a recommendation.**

2) PSP toxin contamination of the edible parts by rupture of the hepatopancreas may not be removed by 'normal' or standard washing practices (i.e. < 20 minutes) **When PSP contamination is a possibility effective shucking practice should avoid rupture of the hepatopancreas and consequent contamination of the edible parts on the shucking table or during handling from spilt gut fluid, knives etc.**

3) Relative to the other tissues **higher levels of PSP toxins are associated with the kidneys and also the gut loop that runs through the gonad**. (The kidneys are the brown tissue attaching the adductor to the roe). The washing protocol should assist in flushing the gut-loop of the gonad e.g. tossing batches of roe-on product under running water and changing washing water rather than static soaking. **Kidney should be carefully trimmed from the roe-on product.**

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Table 3: PSP analysis by LC-MS-MS: July 2008. Feed trial FF II, comparison of PSP levels in adductor, gonad and other tissues (OT) in control and artificially toxified scallops. Each LC-MS sample comprised tissues from three scallops, tissue identity in second column. Figures in nM concentrations. Controls are scallops sacrificed prior to start of feeding experiment,

Sample	Sample Description	Wash	STX	dcSTX	NEO	dcNEO	GTX-1	GTX-2	GTX-3	GTX-4	GTX-5	dcGTX-2	dcGTX-3	C1	C2
FF2/1	adductors 210,211,212	Controls	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF2/2	adductors 213,214,215	Controls	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF2/3	gonads 210,211,212	Controls	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF2/4	gonads 213,214,215	Controls	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF2/5	gonads 193,194,198	0 wash	**94.6**	0.0	**462.4**	0.0	0.0	**8.3**	**115.6**	0.0	0.0	0.0	0.0	0.0	**64.4**
FF2/6	gonads 203,204,209	0 wash	**330.4**	0.0	**717.1**	0.0	0.0	**24.1**	**145.4**	**7.6**	0.0	0.0	0.0	0.0	**193.3**
FF2/7	gonads 197,199,200	30 wash	**135.5**	0.0	**138.8**	0.0	0.0	0.0	**62.3**	0.0	0.0	0.0	0.0	0.0	0.0
FF2/8	gonads 205,207,216	30 wash	**63.3**	0.0	**199.2**	0.0	0.0	0.0	**31.8**	**15.5**	0.0	0.0	0.0	0.0	**64.3**
FF2/9	other tissue 210,211,212	Controls	**5.7**	0.0	**214.6**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF2/10	other tissue 213,214,215	Controls	**6.6**	0.0	**303.5**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
FF2/11	other tissue 193,194,198	0 wash	**153.8**	0.0	**349.3**	0.0	**31.6**	0.0	**47.0**	**197.2**	0.0	0.0	0.0	**179.4**	**970.2**
FF2/12	other tissue 203,204,209	0 wash	**183.1**	0.0	**491.5**	0.0	**14.0**	**14.6**	**30.5**	**168.3**	0.0	0.0	**7.2**	**219.6**	**1478.4**
FF2/13	other tissue 197,199,200	30 wash	**110.2**	0.0	**510.0**	0.0	**20.0**	**7.8**	**45.3**	**213.4**	0.0	0.0	**5.6**	**404.5**	**979.8**
FF2/14	other tissue 205,207,216	30 wash	**237.7**	0.0	**399.2**	0.0	**26.4**	0.0	**52.0**	**177.1**	0.0	0.0	**2.3**	**272.2**	**1209**

Table 5: PSP toxin analysis by LC-MS-MS: July 2008. Feed trial FF III, comparison of PSP toxin concentrations in gonad, adductor, kidney, hepatopancreas and other tissue of artificially toxified scallops. Sub-samples of adductor are those toxified by feeding only (no hepatopancreas solution soak). Sub-samples of gonad contain no gut loop. In this case the OT is mantle, gill and gut minus the hepatopancreas. Each LC-MS-MS sample is comprised of tissues from three scallops, tissue identity in second column. Figures in nM concentrations

Sample	Sample Description	Wash	STX	dcSTX	NEO	dcNEO	GTX -1	GTX-2	GTX-3	GTX-4	GT X-5	dcGTX -2	dcGTX-3	C1	C2
FF3/1	gonads 227,230,232	30 wash	**217.4**	0.0	0.0	0.0	0.0	**19.2**	**55.8**	**11.4**	0.0	0.0	0.0	0.0	0.0
FF3/2	other tissue w/o HP 227,230,232	30 wash	**142.9**	0.0	0.0	0.0	0.0	0.0	0.0	**13.8**	0.0	0.0	0.0	0.0	**184.2**
FF3/3	adductors 227,230,232	30 wash	**70.9**	0.0	0.0	0.0	0.0	0.0	**13.8**	0.0	0.0	0.0	0.0	0.0	0.0
FF3/4	adductors sub sample 227,230,232	no HP soak, 30 wash	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF3/5	gonads subsample 227,230,232	no gut-loop	**58.52**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF3/6	gonads subsample 217,222,223	no gut-loop	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FF3/7	kidneys 227,230,232	30 wash	**3672.9**	0.0	**568.26**	0.0	0.0	**249.48**	**517.44**	**25.41**	0.0	0.0	0.0	0.0	0.0
FF3/8	kidneys, 217,222,223	30 wash	**6429.6**	0.0	**2492.88**	0.0	0.0	**233.12**	**513.24**	0.0	0.0	0.0	0.0	0.0	0.0
FF3/9	Hepato-pancreas soup	All	**579.0**	0.0	**496.8**	0.0	**40.2**	**16.8**	**60.0**	**207.0**	0.0	0.0	**10.8**	**366.6**	**324.0**

Table 6. Summary comparison of data for PSP toxin concentrations in tissues of wild scallops and all force feeding experiments together with mean gonad wet weights. Mean PSP values rounded up, as µg/100g, highlighted in grey shading. PSP levels determined using R-Biopharm Ridascreen Fast PSP assay. Wild and pre-feeding data from analysis of scallops prepared for determination of the anatomical distribution of the toxin (minimum washing – careful, distilled water, surface rinse). Post toxic feeding data from '0' wash edible tissues (quick, tap water, surface rinse). Wild, naturally toxic scallops obtained from Loch Laxford, May 2007 and Scapa, Orkney, July 2007, data from Kelly et al. 2008. Animals subsequently experimentally toxified by force feeding were sourced from Sound of Mull December 2007 (FF I), Sound of Kerrera January 2008 (FF II) and Sound of Kerrera April 2008 (FF III). Toxin ratio means are only calculated from PSP concentrations in contiguous tissues not subjected to 10 and 30 min wash treatments. Other tissues were not subjected to any treatments and therefore mean values can include results from wash treatment animals where applicable and so *n* is larger.

Samples			PSP µg/100g						
			Wild or Pre-feeding (FF controls)				Post toxic feeding		
			adductor	gonad	gonad wt. (g)	other tissues	adductor	gonad	other tissues
Wild samples (SPIES-DETOX)	Laxford May 2007	mean	10	17	7.0	239	*n/a*	*n/a*	*n/a*
		(SD, N)	*(1, 6)*	*(5, 6)*	*(3.5, 12)*	*(153,24)*	*n/a*	*n/a*	*n/a*
		toxin ratios (%)	1 (2%)	2 (4%)		44 (94%)	*n/a*	*n/a*	*n/a*
	Scapa July 2007	mean	13	70	20.0	912	*n/a*	*n/a*	*n/a*
		(SD, N)	*(2, 6)*	*(2, 6)*	*(7.3, 11)*	*(176, 24)*	*n/a*	*n/a*	*n/a*
		toxin ratios (%)	1 (1%)	5 (7%)		70 (92%)	*n/a*	*n/a*	*n/a*
Feeding Study samples	FF I Dec. 2007	mean	*no sample*	*no sample*	*2.3*	109	*no sample*	*no sample*	223
		(SD, N)	*no sample*	*no sample*	*(2.5, 9)*	*(22, 6)*	*no sample*	*no sample*	*(38, 9)*
		toxin ratios (%)	n/a	n/a		n/a	n/a	n/a	n/a
	FF II Jan. 2008	mean	6	14	3.5	75	8	109	180
		(SD, N)	*(4, 6)*	*(4, 6)*	*(2.1, 48)*	*(24, 6)*	*(2, 6)*	*(25, 6)*	*(106,18)*
		toxin ratios (%)	1 (7%)	2 (14%)		12 (79%)	1	14	28
	FF III April 2008	mean	7	7	4.5	35	25*	87	145**
		(SD, N)	*(1, 6)*	*(4, 6)*	*(1.3, 5)*	*(7, 6)*	*(19, 6)**	*(21, 6)*	*(n/a, 18***)*
		toxin ratios (%)	1 (14%)	1 (15%)		5 (71%)	1	2	6
*from adductor subsamples; **estimated value; ***pooled H-P tissues									