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Study to investigate the effect of general anaesthesia on the paralytic shellfish poison bioassay

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1. EXECUTIVE SUMMARY

1.1 Background

Currently shellfish toxin monitoring for Paralytic Shellfish Poison (PSP) in Europe requires the use of the mouse bioassay as described in European Union Directive 91/492/EEC laying down the health conditions for the production and the placing on the market of live bivalve molluscs. The Directive stipulates that the limit for total PSP must not exceed 80 μg/100g shellfish flesh.

The extract from shellfish is injected into the unanaesthetised (conscious) mouse and the time of death is monitored. The time of death is translated into toxicity of the shellfish sample using a conversion table (Sommers table), which was created almost 70 years ago during the development of the mouse bioassay to calculate PSP toxin levels (Sommer and Meyer (1937)).

The ethical issues associated with the mouse bioassay have placed increasing pressure on regulatory bodies to develop and validate alternative analytical and *in vitro* methods for detecting PSP in shellfish. However, to date, no single chemical test has been approved to replace the mouse bioassay for the monitoring of shellfish for PSP in Europe.

The Food Standards Agency (FSA) and the Home Office both recognise that the PSP bioassay is of particular concern relative to animal welfare and therefore aim to replace this method with one which minimises animal suffering. In the absence of a validated chemical test, a refinement of the current mouse bioassay was investigated in which test animals were anaesthetised prior to being injected with toxic sample.

An initial pilot study was performed, prior to this research project, to identify a suitable method of anaesthesia. This identified that the combination of fentanyl/ fluanisone/ midazolam, via the intraperitoneal route, was the preferred method of anaesthesia to investigate the aim of this project, which is whether PSP monitoring in shellfish on anaesthetised mice was as effective as the current testing scheme, which employs conscious (unanaesthetised) mice.

The project was split into two parts:

a) **The Calibration study**. Known concentrations of saxitoxin (this is a positive reference standard for PSP and one of the components of the PSP toxin complex) were administered to both unanaesthetised and anaesthetised mice to investigate whether the response was altered by the anaesthesia. Calibration curves, relating time of death with toxicity (concentration of PSP toxin), were constructed for both unanaesthetised and anaesthetised mice. This study allowed comparison between three different methods:

1. The time of death of an unanaesthetised mouse was translated into toxin concentration using Sommers Table (current method);
2. The time of death of an unanaesthetised mouse was translated into toxin concentration using the newly developed calibration curve for unanaesthetised mice (calibration method);
3. The time of death of an anaesthetised mouse was translated into toxin concentration using the newly developed calibration curve for anaesthetised mice (anaesthesia method).

b) The monitoring sample study.
Routine shellfish samples from FSA Scotland's shellfish monitoring programme were tested on anaesthetised and unanaesthetised mice to evaluate whether the anaesthesia method could be used for routine monitoring of shellfish samples. The

majority of the shellfish samples were also analysed by high performance liquid chromatography (HPLC) to provide further information on the PSP toxin profiles of the samples tested.

1.2. General Conclusions

a) **Conclusions from the calibration study.**

- Anaesthesia caused a delay in the time of death.
- For unanaesthetised mice, not all mice died, even at high toxin concentrations.
- The time of death shows a decrease with higher toxin concentrations. The relationship between time of death and toxin concentration is different for unanaesthetised and anaesthetised mice. Therefore, two calibration curves were developed; one for unanaesthetised and one for anaesthetised mice.
- The validity of the current method in unanaesthetised mice was investigated by comparing results from the new calibration curve against those of the current method. It was found that, for high PSP concentrations, the current method used in the FSA Scotland monitoring programme could underestimate toxicity by up to 50%.
- The predicted toxin concentration was variable for all three methods (current method, calibration method and anaesthesia method). e.g. when a saxitoxin concentration of 80μg/100g shellfish was tested, the estimated toxicity ranged from 40μg/100g to 150μg/100g (where 40 is only just above the limit of detection, 80μg/100g is closure limit, and 150μg/100g is close to the toxin concentration that would cause illness in consumers). This variation was similar for all three methods.

b) **Conclusions from the monitoring sample study.**

- Although sufficient numbers of field samples were collected to allow for full assessment of the performance of anaesthesia in non-toxic and low-toxic samples, unfortunately, there were too few PSP positive samples in Scotland in 2002-2003 to allow a full statistical validation of anaesthesia in positive samples in this study.
- It was difficult to evaluate the suitability of using anaesthesia in PSP monitoring by comparison to the method currently in use, due to the inherent variability in the mouse bioassay.
- The current method and HPLC method tend to give similar monitoring toxicity estimates.
- The estimated toxicities from the calibration method and anaesthesia method tend to be higher than from the current method. Toxicities estimated from the anaesthesia method also exceed those based on the HPLC method.
- The calibration method (unanaesthetised mice) and anaesthesia method tend to give similar monitoring toxicity estimates, suggesting that use of anaesthetised mice is comparable with use of unanaesthetised mice.
- The calibration curves developed in the present study (calibration method and anaesthesia method) are likely to give more accurate toxicity estimates than the current method. This is because the newly developed calibration curves reflect current practice with respect to laboratory conditions and mouse breed, whereas the current method is based on practices that were in place almost 70 years ago.
- When the anaesthesia method is compared with the current method: when the two methods disagree on a monitoring open or monitoring closed decision, the anaesthesia method always results in monitoring closure. Results to date therefore suggest that the use of the anaesthesia method would protect the safety of the consumer.

GLOSSARY

BW	:	Body weight.
Calibration curve	:	Gives mathematical description of the relationship between time of death and toxin concentration of the sample. It is constructed from injecting mice with known quantities of toxin and observing their death times. When a monitoring sample is tested, the time of death is observed and then converted back into toxicity using the calibration curve.
CF	:	Calibration factor, used to account for variations within and between laboratories.
HPLC	:	High Performance Liquid Chromatography.
MBA	:	Mouse bioassay.
MU	:	Mouse unit, refers to toxicity level corresponding to time of death of 15 min.
ND	:	Not detected.
PSP	:	Paralytic Shellfish Poison.
Sommers Table	:	Time of death for unanaesthetised mouse is translated into monitoring toxicity using a conversion table developed by Sommer and Meyer (1937). It is the method used at present for determining monitoring toxicity as defined by legislation.
STX	:	Saxitoxin, one of the PSP components.
Tod	:	Time of death. Length of time it takes for a mouse to die following injection with toxin.

Test methods

Current method	:	The time of death of a unanaesthetised mouse is translated into toxin concentration using Sommers Table, which was developed in the 1930's.
Calibration method	:	The time of death of a unanaesthetised mouse is translated into toxin concentration using the newly developed calibration curve for unanaesthetised mice.
Anaesthesia method	:	The time of death of an anaesthetised mouse is translated into toxin concentration using the newly developed calibration curve for anaesthetised mice.
HPLC method	:	A number of different PSP toxin variants can be detected by High Performance Liquid Chromatography (HPLC). Toxin concentration was determined by quantifying the PSP variants present in each sample detected by HPLC.

2. AIMS AND OBJECTIVES OF THE INVESTIGATION

Paralytic Shellfish Poison (PSP) can be found in shellfish which feed on certain toxin producing algae. If consumed, these toxins can result in paralysis, and in severe cases cause death. Prior to shellfish being harvested for consumption, a representative sample is tested for the presence of PSP and other shellfish toxins. If the PSP level exceeds 80μg/100g shellfish flesh (the regulatory limit), the harvesting area is closed. If the level of PSP is between 40 and 80 μg/100g shellfish flesh, the rate of monitoring of shellfish from the area is increased (Table 1). The derivation of the current regulatory limit (80μg/100g) is unclear but it appears to be based on epidemiological data from previous poisoning cases rather than a toxicologically based limit. It has been reported that as little as 120μg PSP can produce moderate symptoms in humans (Shumway, 1995). Consumption of shellfish exceeding 200-500 μg of PSP can be lethal in healthy adults, while for the vulnerable (young, elderly and weak) toxicity can occur at 160 μg PSP (Prakash et al. 1971; Schantz 1970; Tennant et al. 1955). One reported case mentions severe clinical signs in a two-year old child following consumption of 100 μg of PSP (Tennant et al. 1955). Thus, the margin of safety provided by the current limit appears to be very small particularly when considering potential high level consumption (e.g. consumption of 150 g of shellfish contaminated at the regulatory limit could potentially result in poisoning).

PSP consists of a family of over 20 toxins, and currently no chemical methods have been accepted and validated by the regulatory authorities in Europe. Currently, legislation requires the mouse bioassay to be carried out as described by the Association of Analytical Chemists (AOAC, 1999), to ensure the safety of shellfish sold for human consumption as required by the European Union Directive 91/492/EEC. Shellfish extract, prepared in acid, is injected into the mouse and the animal is monitored for 20 minutes. If the mouse is still alive after 20 minutes the sample is regarded as negative, otherwise the PSP content of the sample is calculated from the observed time of death (Sommer and Meyer, 1937). The limit of detection for the current mouse bioassay protocol is approximately 30 μg/100 g of shellfish flesh (Table 1).

Although EU legislation requires PSP to be tested using the mouse bioassay, the use of laboratory mammals in toxin screening is ethically problematic. Under Directive 86/609/EEC and the Animal (Scientific Procedures) Act (1986), it is a requirement to take all steps to refine, reduce and replace the use of animals used in bioassays and scientific experiments. In order to address this conflict, a study was designed to assess the use of general anaesthesia to reduce the suffering of animals employed in the PSP bioassay.

The first stage of this investigation involved a pilot study (data not included here). This used 2 types of anaesthetic: a volatile agent (halothane) carried in either pure oxygen or in air, and an injectable agent (fentanyl/ fluanisone / midazolam) given by the intraperitoneal route. It was identified during this pilot study that the presence of anaesthesia appeared to delay the time to death by a factor of approximately 2. This delay was similar for both types of agent, but was longer when oxygen was present (probably due to the mechanism of toxicity of PSP compounds). For all methods of anaesthesia, a number of clinical signs could be observed in the anaesthetised animals that identified that toxin was present in the sample. There appeared to be no additional interaction between the injectable agent and the injected sample (e.g. related to a dilution effect) over and above that of anaesthesia per se. Since the use of an injectable agent requires no specialist equipment, uses a route of administration with which staff in testing laboratories are familiar and allows easier observation of animals, it was concluded that this was the preferable anaesthetic regime.

The use of a limited number of control animals in the pilot study demonstrated that the anaesthetic regime chosen was able to keep mice anaesthetised for at least 60 minutes. No anaesthetic deaths were observed. The combination of fentanyl/ fluanisone/ midazolam is considered a safe anaesthetic in rodents and the likelihood of a death related to the anaesthetic in healthy mice should be no more (and possibly considerably less) than 1 in 500 (pers. comm. Prof. Paul Flecknell).

The aim of the present study was to investigate whether PSP testing on anaesthetised mice was as effective as the current testing scheme where mice are not anaesthetised. The available data was then used to assess the suitability of PSP testing on anaesthetised mice as a potential replacement for the current method. The project was split into two parts:

(i) To produce a calibration study using saxitoxin standard (STX) to investigate whether anaesthesia influences the time of death of mice, and if so, to construct a new calibration curve as required; and

(ii) Carry out a statistically robust shellfish sample study where routine monitoring samples collected under the FSA Scotland 2002-2003 monitoring programme were tested on both anaesthetised and unanaesthetised mice to determine whether the anaesthesia approach could be safely adopted for routine shellfish monitoring.

3. MATERIALS AND METHODS

3.1. Bioassay protocols

a) Description of the calibration study

Female CD1 mice were purchased from Harlan, UK and acclimatised in the animal house for at least 48 hours. Mice were kept in standard polycarbonate cages (in groups of 5-6), on a sawdust bedding enriched with Des-Res (Technoplast). Room temperatures were 20-22°C and humidity between 40-80%. All animals were fed on standard CRM (BS and S) pelleted diet.

For the experimental protocol, mice were weighed using a balance (Scaltec model SBA 62) and weights recorded. Individual mice within a cage were marked using an indelible coloured marker, in order to easily identify individuals. Purified saxitoxin (diacetate salt) was obtained from the Certified Reference Materials Programme at the National Research Council (NRC), Canada. For each of ten levels of pure saxitoxin (STX, Table 2), 18 mice were injected. Half of the mice received the injectable anaesthetic combination fluanisone/fentanyl (Hypnorm, Janssen) and midazolam 5mg/ml (Hypnovel, Roche). The drugs were combined in a ratio of Hypnorm: water for injection: Hypnovel of 1:4:1 and the dose given was 0.1ml per 10g mouse bodyweight via a single intraperitoneal injection. The animals were confirmed to be insentient using a standard protocol (pinching the tail), prior to administering 1ml of the STX dose allocated, also via i.p. injection. The remaining half of the mice were unanaesthetised (conscious) when they received the dose of STX, as is current practice. Time of death was recorded for each mouse. Any mouse not dying within 20 minutes of injection (if unanaesthetised) or 45 minutes (if anaesthetised) was euthanased. The time that anaesthetised animals were kept prior to euthanasia reflected the delay in time of death, by a factor of approximately 2, observed in anaesthetised mice in the pilot study. All animals were injected by the same operator, both for toxin and anaesthetic injection, in order to reduce inter-operator variability.

The range of STX doses used was 0.2 to 1.75 μg/ml (Table 2). The lowest dose represents a level equivalent to 40 μg STX/100g shellfish flesh, and the highest equivalent to 350 μg STX/100g shellfish flesh. This range of doses was chosen to include concentrations of saxitoxin around the detection limit of the MBA to a level that can cause illness in humans. The experiment was run in two periods, in September and December 2001, with the dose of 0.336 μg STX/ml repeated to enable temporal differences to be examined. This is also the dose of STX that is normally used by the FRS Marine Laboratory for calibration. To keep in line with current practice at the FRS Marine Laboratory and recommendations by the AOAC (1999), each STX dose was tested on 9 animals.

Any clinical signs observed in the anaesthetised animals were recorded, as well as the time of the last breath, in order to see if the endpoint could be moved to an earlier time point than death.

b) Description of the monitoring sample study

The final phase of these refinement studies was to compare the results produced by animals injected with routine shellfish monitoring samples in the presence and absence of anaesthesia. This was to ensure that it would be possible to detect a range of PSP toxins present in samples gathered from Scottish waters, as well as pure saxitoxin. The study was performed in parallel with the routine sample testing performed by FRS Marine Laboratory for FSA Scotland, so that one anaesthetised and one unanaesthetised animal was injected for each sample. Samples, which produced both negative and positive results using the current MBA, were utilised.

Samples with a range of levels of toxin (ranging from not detectable to 247 μg/100 g using the current method) were tested, although the range was limited by the natural occurrence of PSP levels in the shellfish available.

It should be pointed out that the mouse bioassay protocols used in this study for both anaesthestised and unanaesthetised mice followed the method currently in use in the FSA Scotland monitoring programme for PSP, which involves the injection of a single mouse for each monitoring sample. This study did not address the precision of toxicity calculations using one mouse compared to replicate mice.
Shellfish samples were collected in Scotland during May 2002 – June 2003. Eighty-seven samples were analysed using both unanaesthetised and anaesthetised mice. The sample group comprised 43 mussel extracts, 34 extracts of whole scallop tissue, and 10 scallop gonad extracts. Initially samples were taken at random, but later, when sufficient data had been gathered for samples where the presence of PSP was not detected by the standard MBA, only samples that were suspected of containing PSP were included i.e. where samples tested during the course of routine monitoring identified that PSP toxins were present, an aliquot of the same extract was injected into an anaesthetised mouse.

Unanaesthetised and anaesthetised mice were observed for 20 and 60 minutes respectively for this phase of the research project. It was considered that observation of anaesthetised mice for around 40 minutes would be sufficient to detect PSP presence in the monitoring samples, since 20 minutes is the standard length of time that mice are observed in routine monitoring. However, the anaesthetised animals were observed for a longer period (up to 60 minutes) in case the presence of shellfish matrix had the effect of further delaying the time to death. No additional effects on time to death were apparent for the monitoring samples.
For 69 out of the 87 samples, monitoring toxicity was also determined using HPLC analysis. The HPLC technique employed in this study is described in detail in Asp *et al*. (2004). This method involves post-column oxidation of the toxins into fluorescent derivatives, and is based on the technique developed by Oshima (1995). This method allows at least 12 of the PSP toxin variants to be separated using three different mobile phases. In this study one mobile phase was employed which enabled the six carbamate PSP toxins (saxitoxin, neosaxitoxin (NEO), and Gonyautoxins 1-4 (GTX 1-4)), to be separated in a single chromatographic run. The quantities of each of the carbamate toxins in shellfish extracts were then quantified separately from peak height measurements compared with a calibration curve based on shellfish material spiked with purified toxins (STX, NEO, GTX 2/3 and GTX 2/4, purchased from NRC, Canada). These values were then used to determine the total PSP toxicity of each sample.

3.2 Statistical Approaches

The studies described above provide four methods for assessing toxicity in monitoring samples:

(i) **Current method.** The time of death in a single unanaesthetised mouse is converted to monitoring toxicity using Sommers Table (Sommer and Meyer, 1937). Sommers table provides a calibration curve in a tabular format, which was constructed over 60 years ago.

(ii) **Calibration method.** This calibration curve is based on observed time of death after administering known doses of STX to unanaesthetised animals. It is then used to convert the observed time of death into PSP toxicity for monitoring samples tested on a single unanaesthetised mouse. This method allows for investigating whether, more than sixty years on, the Sommers Table is still valid.

(iii) **Anaesthesia method.** As anaesthesia delays the time of death, a new calibration curve had to be constructed for anaesthetised mice. This calibration curve is obtained from administering known doses of STX to anaesthetised animals. For monitoring samples tested on an anaesthetised mouse, the observed time of death is then translated into PSP toxicity using this new calibration curve.

(iv) **HPLC method.** Quantification of PSP toxin variants using reversed phase High performance Liquid Chromatography with fluorescence detection. A chemical method that does not require the use of animals.

The statistical aspects of methods (i)-(iii) are discussed below.

(i) **Current method**

Sommers Table

Let toxicity be expressed as mouse units (MU), where one MU corresponds to a STX concentration (μg/ml) resulting in a death time of 15 min, following injection of 1 ml of extract into a 20 g mouse. Sommer (Sommer and Meyer, 1937) constructed a table relating observed time of death to toxicity in terms of MU (reproduced in Table 3).

As performances between and within laboratories vary, a calibration factor (CF, μg ml^{-1} MU^{-1}) is introduced. It corresponds to the STX concentration (μg/ml) that results in a death time of 15 min (= 1 MU) for a particular laboratory at a particular time. For a STX standard of known concentration (μg/ml) the death time is observed and Sommers table is used to calculate the corresponding toxicity (denoted by MU_{Som}(standard)). Then

$$CF_{Som} = \frac{\text{STX concentration of standard}}{CF_{bw} \times MU_{Som}(\text{standard})} \qquad (1)$$

The body weight correction factor CF_{bw} accounts for small mice being more sensitive to the toxin than heavy mice (as it is not always possible to use mice of 20 g). CF_{bw} = 0.93, 1.05 and 1.07 for mice weighing 18, 22 or 23 g, respectively. No correction was applied for animals weighing 19-21 g (AOAC, 1999). CF_{Som} is calculated every 4-6 weeks, and if the new CF_{Som} differs by more than 20% from the current CF, the current CF_{Som} is replaced with the new CF_{Som}. For the calibration study the CF was 0.189, whereas for the monitoring samples the CF was 0.15 (May 2002), 0.17 (August 2002, May-June 2003) and 0.189 (June-July 2002).

Estimation of PSP toxicity using Sommers Table

For a shellfish sample of unknown toxicity, the following procedure is adopted. Following injection of 1 ml of the shellfish extract into a mouse, the time of death is observed and the corresponding toxicity, denoted by MU_{Som}(sample), is calculated from Sommers table. The monitoring toxicity, expressed as μg STX/100 g shellfish, is given by

$$\text{Monitoring toxicity} = MU_{Som}(\text{sample}) \times CF_{bw} \times CF_{Som} \times 200$$

where the factor 200 (ml/100g flesh) converts STX concentration into monitoring toxicity (one μg STX/ml is equivalent to 200μg STX/100g shellfish flesh).

(ii) **Anaesthesia method**

Construction of the calibration curve

As the anaesthesia results in a delay in time to death (see Table 4), the approach above cannot be used for anaesthetised animals, as it would underestimate the monitoring toxicity. Therefore, calibration curves were constructed as follows:

For each dose, nine anaesthetised mice were injected with the toxin (the repeat of 0.336 μg/ml was treated as a separate dose). Their time of death (tod, sec) was observed and the median tod was calculated (as described in AOAC 1999). In line with previous studies (Schantz et al. 1958; Nagashima et al. 1991), a linear relationship was observed between 1/tod and logarithm (base 10) of the STX concentration (μg/ml):

$$\frac{1}{tod} = a + b \log(\text{STX}) \qquad (2)$$

Estimates of *a* and *b* were obtained from linear regression, using Genstat 6th Edition Release 6.1 (Lawes Agricultural Trust, Rothamsted, UK). The effect of period (September, December) was included in the regression but was found not to be significant.

Model (2) can be expressed in terms of MU (which is the STX concentration divided by the calibration factor) as follows:

$$\frac{1}{tod} = \frac{1}{900} + b \log(\text{MU}) \qquad (3)$$

so that

$$\text{MU}_{\text{cal}} = 10\text{^}\{\frac{900 - tod}{b \times 900 \times tod}\} \qquad (4)$$

The subscript 'cal' is used to indicate that the toxicity is derived from the calibration study (as opposed to using Sommers table). Note that equation (4) is replacing Sommers table. Also note that for a tod of 15 min (=900 sec) $\text{MU}_{\text{cal}} = 1$.

Estimation of monitoring toxicity using the calibration curve

As Sommers table is not applicable to anaesthetised mice, the calibration factor given in (1) cannot be used. To calculate the calibration factor for anaesthetised mice (CF_{cal}), the following procedure is adopted. For a STX standard of known concentration, observe the time of death following injection into an anaesthetised mouse.

Calculate the corresponding toxicity, MU_{cal}(standard), from equation (4).

Then

$$\text{CF}_{\text{cal}} = \frac{\text{STX concentration of standard}}{\text{MU}_{\text{cal}}(\text{standard})} \qquad (5)$$

CF_{cal} corresponds to the STX concentration for which, following injection of 1 ml of extract into a 20 g anaesthetised mouse, the time of death is 15 min.

For a shellfish sample of unknown toxicity the test works as follows. Observe the death time following injection of 1 ml of extract into an anaesthetised mouse.

Calculate the corresponding toxicity, MU_{cal}(sample), using equation (4). Then the monitoring toxicity (μg/100g shellfish) is given by:

$$\text{Monitoring toxicity} = MU_{cal}(\text{sample}) \times CF_{cal} \times 200$$

It was considered inappropriate to use the same body weight correction factor for awake and anaesthestised animals. However, a body weight correction factor for anaesthetised mice was not included in the above calculation since the assays involving unanaesthestised animals indicated no evidence of a relationship between death time and body weight (ranging from 18 to 22 g), with both short and long death times observed for light (18 g) as well as heavy (22 g) animals.

(iii) **Calibration method for unanaesthetised mice**

The same approach was applied to the death times from the unanaesthetised animals. Model 2 was fitted to the death times of unanaesthetised mice giving new values for a and b, and equation 4 was adjusted accordingly. The calibration factor given by (5) was also adjusted. For both unanaesthetised and anaesthetised data, no significant relationship was found between BW (ranging from 18 to 22 g) and time of death (from including log(BW/20) in regression equation (2) and fitting individual mouse data to this model).

Comparison of the four different methods for estimating monitoring PSP toxicity

MBA results that gave 'not detected' were replaced by zero (for the majority of MBA results, 31 out of 37, both awake and asleep mice gave agreement on ND. As a consequence, replacement of ND by 30, the LOD, or by 5, the average of the corresponding HPLC readings, did not alter the statistical results). For HPLC, entries that gave 'trace' (i.e. less than 10 μg/100g) were replaced by a value of 5. For each monitoring sample, toxicity was calculated according to four methods (current, calibration, anaesthesia and HPLC methods). These four methods were compared using the following two approaches, namely McNemars test and equivalence testing. Both tests allow for the differences in the measurement errors associated with each of the four methods.

McNemars test

For each pair of methods, tables were constructed showing the numbers of samples for which both methods agreed (open-open; closed-closed) or disagreed (open-closed; closed-open). To test for differences between two methods, McNemar's test was employed (Zar 1996). Each sample was analysed using both methods A and B (where A, B could be any of the four methods mentioned above). Assume we have the following table, with cells *a*, *b*, *c*, and *d* referring to the number of samples for which the corresponding methods resulted in a 'monitoring open' or 'monitoring closed' decision.

	Method B: open	Method B: closed
Method A: open	*a*	*b*
Method A: closed	*c*	*d*

Cells *b* and *c* consist of those samples for which the two methods disagree. For example, if method A is more likely to result in closure than method B, more samples would be present in cell c than in cell b (compared to method B, method A provides increased consumer safety, but potentially a loss of income to fishermen). Likewise, if

both methods are equally likely to lead to the same decision (e.g. closure of the field) then the number of disagreements should be equally distributed between *b* and *c.* McNemars test then tests whether $b / (b+c) = 0.5$. For simplicity no correction for multiple comparisons was made, and results were regarded significant if the P-value was 5% or less. Although a significant test result means that the two methods appear different, it does not provide information on the magnitude of the difference. The method described below tries to address this issue.

Equivalence testing

To investigate whether method A is equivalent to method B the graphical approach advocated by Bland and Altman (Bland and Altman 1986) was adopted. For each sample, the toxicity is estimated using both A and B, and the difference is calculated. The difference was then plotted against the average toxicity (as the true toxicity is not known, the best estimate available is the average of the two toxicities). Graphs like these give a good idea whether, on average, the difference is zero, or whether there is a relationship between toxicity-level and performance of A and B (e.g. does the difference change with toxicity level?). Furthermore, if one, or both, methods are variable, this will also show up in the graph as the differences will then show a large variation. A 95% interval, calculated as the mean difference $\pm$ 2 standard deviations, is added to the graph to indicate the range of the differences. Methods A and B are regarded equivalent if the differences fall within certain limits, which are usually specified in advance.

4. RESULTS

4.1. Performance of mouse bioassay protocols

(a) The calibration study

For the unanaesthetised mice, 7 of the 10 doses tested did not result in all of the tested animals dying (Table 4). Even for the highest dose, corresponding to a monitoring toxicity regarded as potentially lethal for human consumption, 2 out of 9 animals did not die within 20 minutes. The use of general anaesthesia delayed the time to death of mice injected with STX (Table 4, Figure 1). Anaesthetised mice took approximately twice as long to die for any given STX concentration as mice that were unanaesthetised. All anaesthetised mice showed distinctive signs of PSP intoxication (such as the tail turning blue and head nodding), that would discount death being caused by the anaesthetic itself. The possibility of using clinical signs in anaesthetised mice was investigated by analysing the time of onset of each sign against the time of death for the first 4 saxitoxin doses used (data not shown). The time that it takes for the tail to turn blue was the most reliable clinical sign, since it occurred in all anaesthetised animals injected with saxitoxin, however, the appearance of "head nod" behaviour was more closely related to time of death. Clinical signs data was also gathered for the additional saxitoxin doses, but full analysis was not completed, since there was considered to be no current additional benefit in terms of animal welfare or ease of interpreting the assay by moving to clinical signs. Further analysis could be considered if there was a strong need to reduce the duration of the anaesthetised MBA in order to facilitate a large monitoring programme.

As a rule, all anaesthetised mice died within 45 minutes. However, it was necessary to euthanise one animal during the study due to faulty injection. Therefore, this result was excluded from subsequent analyses. It should also be noted that one anaesthetised animal died relatively quickly compared to other test animals (108 seconds for 0.4 μg STX/ml). However, as it was unclear whether this result was caused by the toxin or due to other factors, it was not removed from the data.

Construction of calibration curves

As anaesthesia delays the time of death, the current approach based around Sommers table cannot be used on anaesthetised mice as it would underestimate the toxicity of monitoring samples. Therefore, new calibration curves were constructed relating the median time of death (as described in AOAC, 1999) to toxicity. For both unanaesthetised and anaesthetised animals a linear relationship was observed between the inverse median time of death and the logarithm of the toxicity (Figure 2, Table 5), with over 95% of the variation accounted for. To account for possible temporal differences between the September and December data a period effect was initially included in the linear relationship described above. This was found to be not significant and was excluded from subsequent analyses.

Performance of calibration curves

The newly constructed calibration curves (Table 5) were used to calculate the monitoring toxicity for each individual death time (Figure 3). The results indicated the following:

- For unanaesthetised animals, several of the STX doses corresponding to monitoring toxicities which exceeded field closure gave a predicted toxicity of zero (1, 2 and 2 animals for 140, 160 and 350 μg/100g monitoring toxicities, respectively). None of the anaesthetised mice gave predicted toxicities of zero.
- Although 9 animals received the same dose, there is large variation in the predicted monitoring toxicity for both unanaesthetised and anaesthetised

animals. For example, for a STX dose corresponding to 100μg/100g the predictions ranged from 85 to 135 and 67 to 180 μg/100 g for unanaesthetised and anaesthetised mice, respectively.

- For anaesthetised mice, 2 animals gave predicted toxicities more than 4-fold the corresponding STX dose of 350 μg/100g. This was believed to be due to the calibration curve being sensitive to rapid death times (less than 3 minutes). For a rapid death time, a relatively small decrease in death time results in a large increase in estimated toxicity (see also Table 5). This does not alter the decision of field closure, however, and errs on the side of consumer safety. The death times of these two animals were 142 and 155 seconds, while for the remaining animals the death times ranged from 200 – 307 seconds, corresponding to estimated monitoring toxicities of 183 – 486 μg/100g.
- Results from anaesthetised mice appear to be more variable in that they sometimes produce high toxicity estimates, but this errs on the side of consumer safety. On the other hand, although the estimates from unanaesthestised mice seem less variable, they do produce zero toxicity estimates for several of the STX concentrations, including high STX levels that could severely compromise consumer health.

Performance of current method

The current method uses Sommers table to convert death time to PSP toxicity. This conversion table was constructed almost 70 years ago, using a different strain of mice and different laboratory techniques. To check whether Sommers table is still valid, the death times of the unanaesthetised mice were also converted to PSP toxicity using Sommers table (current method). Again, the predicted PSP field toxicity was variable. Furthermore, PSP field toxicity is underestimated by up to 50% for the higher STX doses (see Figure 4). For example, for a true PSP field toxicity of 160 μg/100g, the estimated PSP field toxicity ranged from 0 – 124 μg/100g. The predicted zero field toxicities (Figure 3a, 4) correspond to unanaesthetised animals that did not die.

Body weight correction

The current method is based on mice of 20 g, and includes a correction factor for mice of different body weight. This correction in estimated PSP toxicity is up to 7% for a mouse of 23 g. The data shown in Table 4 do not show a significant relationship between body weight and time of death, with short and long times of death observed for both small (18 g) and large (22 g) animals. Furthermore, the spread in the observed time of death and hence predicted monitoring toxicity is large, in the order of 50% or more, so that inclusion of a body weight correction factor in the newly developed calibration curves is an unnecessary refinement.

(b) The monitoring sample study

In this study, 87 monitoring samples were tested using anaesthetised and unanaesthetised animals, 69 of which were analysed by HPLC also (Table 6). Two animals showed an extreme response; for one sample the anaesthetised mouse died at 2.5 min, and for another sample the unanaesthetised mouse died within 2 min. These two results have been included in the statistical analyses unless stated otherwise.

The initial aim was to test approximately 60 samples below, and 60 above the field closure limit. This allows for assessment of the agreement between unanaesthetised and anaesthetised mice to be 95% or better. Unfortunately, due to a lack of toxic samples only 9 samples above field closure limit were obtained. Similar clinical signs (tail turning blue, head nod) were observed in the anaesthetised animals injected with

monitoring samples containing PSP as were observed in anaesthetised animals injected with saxitoxin, but this data has not been analysed.

4.2. Comparison of methods

Four different estimates of monitoring toxicity are considered:

(i) **Current method:** the observed death time from a single unanaesthetised mouse is converted into monitoring toxicity using Sommers Table.
(ii) **Calibration method:** the observed death time from a single unanaesthetised mouse is converted into monitoring toxicity using the calibration curve for unanaesthetised mice, obtained from the calibration study (see Table 5).
(iii) **Anaesthesia method:** the observed death time from a single anaesthetised mouse is converted to monitoring toxicity using the calibration curve for anaesthetised mice (Table 5).
(iv) **HPLC method:** HPLC analysis is used to estimate monitoring toxicity.

Comparison of the four methods (Tables 7-8 and Figures 5-7) shows that:

- The results obtained by HPLC and the two methods based on the unanaesthetised mouse bioassay (ie the current method and the calibration method) are similar.
- The current method gives lower toxicity estimates (P=0.007, Table 7) and fewer field closures (P=0.016, Table 8b) than the calibration method.
- Although the calibration method gives somewhat lower toxicity estimates than the anaesthesia method (difference of 8 μg/100g, P=0.02), in terms of number of field closures both methods give similar results.
- The anaesthesia method gives higher toxicity estimates and more field closures than the current and HPLC methods (P < 0.016).

Variation in estimated monitoring toxicity

The variation in estimated PSP toxicity is large. When six samples, all from the same monitoring area, were tested on six anaesthetised mice, the estimated PSP toxicity ranged from 156 to 307μg/100g, with an average of 228μg/100g (Table 9). This spread is similar to that observed in the calibration study (see Figure 3b). One of the samples was tested on an unanaesthetised mouse, and its value was 124μg/100g using the current method (Sommers Table) and 226μg/100g using the calibration method (see Table 9).

Although on average the difference between two methods is no more than 14μg/100g (Table 7), for individual samples differences can be large, up to plus or minus 50μg/100g (shown in Figure 7 as horizontal lines, which indicate that 95% of the observed differences fall within these bounds). These effects were observed for mussels, scallop gonads and whole scallops (Table 6, Fig 5-7).

Monitoring closures

The toxicity measurements obtained using each of the four methods were then used to determine the effect on monitoring closures. Table 8 shows the number of samples for which the methods agree whether a site should stay open or be closed.

It should be noted that, of the 87 samples tested, only 9 samples gave an estimated toxicity exceeding 80μg/100g using the current method, while 18 samples exceeded this level using the anaesthesia method. Therefore, the following findings should be interpreted with care.

- For over 85% (ie 59 out of 69 samples) of the samples tested, all four methods agree on whether the monitoring site should stay open or be closed (based on Table 6).
- Comparison of HPLC and the current method gives the closest agreement, with only 4 out of 69 samples giving a different result.
- Results from the anaesthesia method lead to monitoring closure more often than HPLC and the current method. When there is a disagreement, the anaesthesia method results in monitoring closure.
- When for the unanaesthetised mice the current method and the calibration method are compared, the calibration method leads to monitoring closure more often (9 closures for the current method versus 16 closures for the calibration method).
- When comparing the calibration method for unanaesthetised mice to the anaesthesia method, there are no significant differences (16 and 18 monitoring closures, respectively).

5. OVERALL CONCLUSIONS

(a) Conclusions from the calibration study.

- Anaesthesia caused a delay in the time to death.
- For unanaesthetised mice, not all mice died, even at high toxin concentrations.
- The time of death shows a decrease with higher toxin concentrations. The relationship between time of death and toxin concentration is different for unanaesthetised and anaesthetised mice. Therefore, two calibration curves were developed; one for unanaesthetised and one for anaesthetised mice.
- The validity of the current method in unanaesthetised mice was investigated by comparing results from the new calibration curve against those of the current method. It was found that, for high PSP concentrations, the current method underestimates toxicity by up to 50%.
- The predicted toxin concentration was variable in all three methods (current method, calibration method and anaesthesia method). e.g. when a saxitoxin concentration of 80μg/100g shellfish was tested, the estimated toxicity ranged from 40μg/100g to 150μg/100g (where 40 is only just above the limit of detection, 80μg/100g is closure limit, and 150μg/100g is close to the toxin concentration that would cause illness in consumers). This variation was similar for all three methods.

(b) Conclusions from the monitoring sample study

- Although sufficient numbers of field samples were collected to allow for full assessment of the performance of anaesthesia in non-toxic and low-toxic samples, unfortunately, there were too few PSP positive samples in Scotland in 2002-2003 to allow a full statistical validation of anaesthesia in this study
- This study has highlighted that the estimated PSP toxicity from the mouse bioassay (using both anaesthetised and unanaesthetised mice) can be highly variable. This indicates that the mouse bioassay currently used in the FSA Scotland monitoring programme is not a good method for obtaining a precise quantitative estimate of monitoring toxicity (but may be suitable for detecting the presence or absence of high PSP concentrations).
- It was difficult to evaluate the suitability of using anaesthesia in PSP monitoring by comparison to the method currently in use, due to the inherent variability in the mouse bioassay.
- The current method and HPLC method tend to give similar monitoring toxicity estimates.
- The estimated toxicities from the calibration method and anaesthesia method tend to be higher than from the current method, and the anaesthesia estimates also exceed those of the HPLC method.
- The calibration method (unanaesthetised mice) and anaesthesia method tend to give similar monitoring toxicity estimates, suggesting that use of anaesthetised mice is compatible with use of unanaesthetised mice.
- The calibration curves developed in the present study (calibration method, anaesthesia method) give better toxicity estimates than the current method. This is because the newly developed calibration curves reflect current practice with respect to laboratory practice and mouse breed, whereas the current method is based on practices employed nearly 70 years ago.
- The results from this study indicate that, when the anaesthesia method and current method disagree on a monitoring open or monitoring closed decision, the anaesthesia method always results in monitoring closure. Results to date therefore suggest that the use of the anaesthesia method would protect the safety of the consumer.

6. RECOMMENDATIONS

The data in this report demonstrate that anaesthesia works well for negative samples (for all 69 'field open' decisions based on the anaesthesia method, the current method agreed). However, information on how anaesthesia behaves for toxic monitoring samples is limited, with only 9 toxic samples tested. To investigate this further, the following is suggested, taking into account the limited availability of toxic samples:

- If a sample tested by the current method results in a monitoring toxicity of 120 μg/100g or higher (so we can assume that, taking between animal variation into account, the underlying toxicity is at least 80μg or higher), then n=30 samples should be taken from this particular site and tested on anaesthetised mice. Ideally, this should be repeated for 4 different sites across Scotland to cover a range of toxin profiles.
- This data could be used to establish whether the agreement of the anaesthesia method with the current method is 90% or better (i.e. when the toxin level is above closure limit according to the current method, does the anaesthesia method also result in field closure?), for each toxin profile. Combining four sites (giving a total of 120 samples) would allow for assessment of the agreement between the two methods to be 97.5% or better.
- If it were not possible to obtain 30 samples for each site, data obtained for 15 samples would allow for testing the agreement between the two methods to be 80% or better for each site (i.e. each toxin profile). When combined over 4 sites, this would allow for testing an agreement of 95% or better.

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Table 1. Critical levels of PSP.

Toxicity	Comments
30 μg/100g shellfish	Limit of detection for mouse bioassay
40-80 μg/100g shellfish	Increase frequency of testing
> 80 μg/100g shellfish	Monitoring closure
160 μg	Oral dose resulting in mild clinical signs in adults[1]
200 - 500 μg	Minimum oral lethal dose in humans[1]

[1]Prakash et al. 1971; Schantz 1970; Tennant et al. 1955

Table 2. Summary of saxitoxin concentrations (μg/ml) tested, together with the corresponding monitoring toxicity (μg/100g flesh).

Period	STX	Monitoring toxicity
Sept 01	0.2	40
Dec 01	0.3	60
Sept 01	0.336	67.2
Dec 01	0.336	67.2
Dec 01	0.4	80
Dec 01	0.5	100
Sept 01	0.6	120
Dec 01	0.7	140
Dec 01	0.8	160
Sept 01	1.0	200
Dec 01	1.75	350

Table 3: Sommers Table showing conversion of time of death (min:sec) into mouse units (MU). Reproduced from Sommer and Meyer (1937).

Time	MU	Time	MU	Time	MU.	Time	MU	Time	MU	Time	MU	Time	MU
1:00	100.00	2:00	7.67	3:00	3.70	4:00	2.50	5:00	1.92	6:00	1.60	7:00	1.39
1:01	96.60	2:01	7.54	3:01	3.67	4:01	2.49	5:01	1.91	6:01	1.60	7:02	1.38
1:02	93.20	2:02	7.42	3:02	3.65	4:02	2.48	5:02	1.91	6:02	1.59	7:06	1.37
1:03	89.90	2:03	7.29	3:03	3.62	4:03	2.46	5:03	1.90	6:03	1.59	7:09	1.36
1:04	86.50	2:04	7.12	3:04	3.60	4:04	2.45	5:04	1.90	6:04	1.58	7:12	1.35
1:05	83.10	2:06	7.04	3:05	3.57	4:05	2.44	5:06	1.89	6:05	1.58	7:17	1.34
1:06	79.70	2:06	6.94	3:06	3.54	4:06	2.43	5:06	1.88	6:06	1.58	7:21	1.33
1:07	76.30	2:07	6.83	3:07	3.51	4:07	2.42	6:07	1.88	6:07	1.58	7:24	1.32
1:08	73.00	2:08	6.73	3:08	3.49	4:08	2.40	5:08	1.87	6:08	1.57	7:27	1.31
1:09	69.60	2:09	6.62	3:09	3.46	4:09	2.39	5:09	1.87	6:09	1.57	7:33	1.30
1:10	66.20	2:10	6.52	3:10	3.43	4:10	2.38	5:10	1.86	6:10	1.56	7:38	1.29
1:11	60.60	2:11	6.42	3:11	3.41	4:11	2.37	5:11	1.85	6:11	1.56	7:43	1.28
1:12	55.00	2:12	6.32	3:12	3.38	4:12	2.36	5:12	1.85	6:12	1.56	7:48	1.27
1:13	49.50	2:13	6.22	3:13	3.36	4:13	2.34	5:13	1.84	6:13	1.55	7:53	1.26
1:14	43.90	2:14	6.12	3:14	3.33	4:14	2.33	5:14	1.84	6:14	1.55	7:58	1.25
1:15	38.30	2:15	6.02	3:15	3.31	4:15	2.32	5:15	1.83	6:15	1.54	8:00	1.25
1:16	35.90	2:16	5.95	3:16	3.29	4:16	2.30	5:16	1.82	6:16	1.54	8:04	1.24
1:17	33.50	2:17	5.88	3:17	3.26	4:17	2.30	5:17	1.82	6:17	1.53	8:09	1.23
1:18	31.20	2:18	5.80	3:18	3.24	4:18	2.28	5:18	1.81	6:18	1.53	8:14	1.22
1:19	28.80	2:19	5.73	3:19	3.21	4:19	2.27	5:19	1.81	6:19	1.52	8:21	1.21
1:20	26.40	2:20	5.66	3:20	3.19	4:20	2.26	5:20	1.80	6:20	1.52	8:30	1.20
1:21	25.30	2:21	5.59	3:21	3.17	4:21	2.25	5:21	1.79	6:21	1.52	8:36	1.19
1:22	24.10	2:22	5.52	3:22	3.15	4:22	2.24	5:22	1.79	6:22	1.51	8:45	1.18
1:23	23.00	2:23	5.46	3:23	3.12	4:23	2.23	5:23	1.78	6:23	1.51	8:51	1.17
1:24	21.80	2:24	5.39	3:24	3.10	4:24	2.22	5:24	1.78	6:24	1.50	8:56	1.16
1:25	20.70	2:25	5.32	3:25	3.08	4:25	2.21	5:25	1.77	6:25	1.50	9:00	1.16
1:26	19.90	2:26	5.26	3:26	3.06	4:26	2.20	5:26	1.76	6:26	1.50	9:06	1.15
1:27	19.00	2:27	5.19	3:27	3.04	4:27	2.19	6:27	1.76	6:27	1.49	9:15	1.14
1:28	18.20	2:28	5.13	3:28	3.02	4:28	2.18	5:28	1.75	6:28	1.49	9:26	1.13
1:29	17.30	2:29	5.06	3:29	3.00	4:29	2.17	5:29	1.75	6:29	1.48	9:35	1.12
1:30	16.50	2:30	5.00	3:30	2.98	4:30	2.16	5:30	1.74	6:30	1.48	9:50	1.11
1:31	16.00	2:31	4.95	3:31	2.96	4:31	2.15	5:31	1.74	6:31	1.48	10:00	1.11
1:32	15.50	2:32	4.89	3:32	2.94	4:32	2.14	5:32	1.73	6:32	1.47	10:05	1.10
1:33	14.90	2:33	4.84	3:33	2.92	4:33	2.14	5:33	1.72	6:33	1.47	10:16	1.09
1:34	14.40	2:34	4.78	3:34	2.90	4:34	2.13	5:34	1.72	6:34	1.47	10:31	1.09
1:35	13.90	2:35	4.73	3:35	2.88	4:36	2.12	5:35	1.72	6:35	1.47	10:41	1.08
1:36	13.50	2:36	4.68	3:36	2.86	4:36	2.11	5:36	1.71	6:36	1.46	10:58	1.08
1:37	13.10	2:37	4.63	3:37	2.84	4:37	2.10	5:37	1.70	6:37	1.46	11:00	1.08
1:38	12.70	2:38	4.58	3:38	2.83	4:38	2.10	5:38	1.70	6:38	1.46	11:01	1.07
1:39	12.30	2:39	4.53	3:39	2.81	4:39	2.09	5:39	1.70	6:39	1.45	11:18	1.06
1:40	11.90	2:40	4.48	3:40	2.79	4:40	2.08	5:40	1.69	6:40	1.45	11:31	1.06
1:41	11.60	2:41	4.44	3:41	2.77	4:41	2.07	5:41	1.69	6:41	1.45	12:00	1.05
1:42	11.30	2:42	4.39	3:42	2.76	4:42	2.06	5:42	1.68	6:42	1.44	13:00	1.03
1:43	11.00	2:43	4.35	3:43	2.74	4:43	2.06	5:43	1.68	6:43	1.44	14:00	1.02
1:44	10.70	2:44	4.30	3:44	2.73	4:44	2.05	5:44	1.67	6:44	1.44	15:00	1.00
1:45	10.40	2:45	4.26	3:45	2.71	4:45	2.04	5:45	1.67	6:45	1.43	16:00	0.99
1:46	10.20	2:46	4.22	3:46	2.69	4:46	2.03	5:46	1.66	6:46	1.43	17:00	0.98
1:47	9.97	2:47	4.18	3:47	2.68	4:47	2.02	5:47	1.66	6:47	1.42	18:00	0.97
1:48	9.76	2:48	4.14	3:48	2.66	4:48	2.02	5:48	1.65	6:48	1.42	19:00	0.97
1:49	9.54	2:49	4.10	3:49	2.65	4:49	2.01	5:49	1.65	6:49	1.42	20:00	0.96
1:50	9.33	2:50	4.06	3:50	2.63	4:50	2.00	5:50	1.64	6:50	1.42	21:00	0.95
1:51	9.15	2:51	4.02	3:51	2.62	4:51	1.99	5:51	1.64	6:51	1.41	22:00	0.95
1:52	8.97	2:52	3.99	3:52	2.60	4:52	1.98	5:52	1.63	6:52	1.41	23:00	0.94
1:63	8.78	2:53	3.95	3:53	2.59	4:53	1.98	5:53	1.63	6:53	1.41	24:00	0.94
1:54	8.60	2:54	3.92	3:54	2.57	4:54	1.97	5:54	1.62	6:54	1.40	25:00	0.93
1:55	8.42	2:55	3.88	3:55	2.56	4:55	1.96	5:55	1.62	6:55	1.40	30:00	0.92
1:56	8.27	2:56	3.84	3:56	2.55	4:56	1.95	5:56	1.62	6:56	1.40	40:00	0.90
1:57	8.12	2:57	3.81	3:57	2.54	4:57	1.94	5:57	1.61	6:57	1.39	60:00	0.88
1:58	7.97	2:58	3.77	3:58	2.52	4:58	1.94	5:58	1.61	6:58	1.39		
1:59	7.82	2:59	3.74	3:59	2.51	4:59	1.93	5:59	1.60	6:59	1.39		

Table 4: Observed time of death (sec) for unanaesthetised (tod UA) and anaesthetised (tod A) mice, for 10 levels of saxitoxin (STX, μg/ml). BW: body weight (g).

STX	BW UA	tod UA	BW A	tod A	**STX**	BW UA	tod UA	BW A	tod A	**STX**	BW UA	tod UA	BW A	tod A	**STX**	BW UA	tod UA	BW A	tod A
0.2	21	369	20	845	**0.336D**[2]	19	301	21	562	**0.6**	20	225	20	374	**1**	20	180	19	259
0.2	20	418	21	1049	**0.336D**	20	311	22	633	**0.6**	20	229	20	390	**1**	18	187	21	260
0.2	21	488	20	1062	**0.336D**	20	312	21	659	**0.6**	20	242	20	393	**1**	20	191	20	270
0.2	19	492	20	1071	**0.336D**	18	312	22	692	**0.6**	19	244	21	439	**1**	21	192	20	271
0.2	20	521	19	1097	**0.336D**	21	321	18	710	**0.6**	19	250	20	489	**1**	20	193	20	305
0.2	21	ND[1]	20	1208	**0.336D**	21	333	22	718	**0.6**	21	266	18	510	**1**	20	222	19	354
0.2	20	ND	20	1256	**0.336D**	21	360	18	769	**0.6**	19	269	20	567	**1**	19	249	20	363
0.2	20	ND	21	1386	**0.336D**	22	ND	20	803	**0.6**	20	292	20	575	**1**	19	263	19	437
0.2	19	ND	20	1901	**0.336D**	22	ND	21	889	**0.6**	21	299	20	1207	**1**	18	267	19	610
0.3	20	332	20	627	**0.4**	19	250	20	108	**0.7**	21	199	20	Error[2]	**1.75**	21	137	20	142
0.3	20	343	18	684	**0.4**	22	280	20	498	**0.7**	20	205	21	269	**1.75**	21	140	19	155
0.3	22	352	19	736	**0.4**	19	295	21	553	**0.7**	19	211	22	272	**1.75**	21	160	22	200
0.3	19	394	22	753	**0.4**	19	318	21	600	**0.7**	18	214	21	302	**1.75**	18	167	21	201
0.3	21	397	22	775	**0.4**	20	346	21	608	**0.7**	20	216	20	337	**1.75**	19	174	19	215
0.3	18	398	20	788	**0.4**	21	347	22	627	**0.7**	20	219	19	342	**1.75**	21	185	20	249
0.3	22	401	21	822	**0.4**	21	ND	19	647	**0.7**	22	223	21	344	**1.75**	18	322	21	260
0.3	21	ND	21	822	**0.4**	22	ND	19	655	**0.7**	21	233	19	364	**1.75**	20	ND	18	283
0.3	20	ND	22	1209	**0.4**	21	ND	21	773	**0.7**	22	ND	18	439	**1.75**	22	ND	22	307
0.336S[2]	20	282	21	464	**0.5**	19	235	20	310	**0.8**	20	189	18	276					
0.336S	20	306	20	526	**0.5**	19	242	21	447	**0.8**	22	197	21	300					
0.336S	20	314	20	549	**0.5**	20	249	21	474	**0.8**	22	200	21	305					
0.336S	20	322	21	584	**0.5**	20	255	20	488	**0.8**	21	208	20	311					
0.336S	19	324	19	615	**0.5**	18	266	22	494	**0.8**	19	212	22	313					
0.336S	20	337	20	633	**0.5**	21	278	20	589	**0.8**	22	232	22	324					
0.336S	20	352	20	634	**0.5**	21	286	22	603	**0.8**	21	260	20	346					
0.336S	19	356	21	660	**0.5**	20	292	21	618	**0.8**	18	ND	21	352					
0.336S	20	ND	21	682	**0.5**	21	295	19	673	**0.8**	22	ND	22	366					

[1]ND: animal did not die within 20 minutes; [2]There were problems with injection of anaesthetic.

[2]S: September, D: December.

Table 5: Calibration curves, based on the relationship between time of death (tod, sec) and STX standard (μg/ml) for unanaesthetised and anaesthetised mice, using the median time of death for each dose tested (STX, μg/ml). Standard errors are given in parentheses. The corresponding formulas for calculating toxicity in mouse units (MU) for an observed death time (sec) are also given.

	Regression results	
Unanaesthetised	1/tod = 0.00500 + 0.00435 log(STX) (0.00010) (0.00027)	R^2 = 96.2%
Anaesthetised	1/tod = 0.00341 + 0.00404 log(STX) (0.00011) (0.00028)	R^2 = 95.1%
	Toxicity in terms of mouse units[1]	
Unanaesthetised	$MU_{cal} = 10^{\wedge}\{\frac{900 - tod}{3.912 \times tod}\}$	
Anaesthetised	$MU_{cal} = 10^{\wedge}\{\frac{900 - tod}{3.640 \times tod}\}$	

[1]Monitoring toxicity (μg/100g) is then calculated as $MU_{cal} \times CF \times 200$, where CF is the calibration factor, which will vary between and within laboratories. For this particular study, the CF for unanaesthetised and anaesthetised animals was 0.13 and 0.27, respectively (based on the new calibration curves).

Table 6a. Estimated monitoring toxicity (μg/100g) from testing monitoring samples. Each sample was tested on one unanaesthetised and one anaesthetised mouse. Furthermore, most of the samples were also analysed by HPLC (a blank entry denotes a sample not analysed by HPLC). Monit tox current method = monitoring toxicity estimated from Sommers table for unanaesthetised mice, Monit tox calibration = monitoring toxicity obtained from calibration curve for unanaesthetised mice, monit tox anaesthetised = monitoring toxicity obtained from calibration for anaesthetised mice, BW = body weight (g), tod = time of death (min:sec), Type = species (M=mussels, SG = scallop gonads, SW = whole scallops), ND = not detected (i.e. mouse did not die during observation time), trace = less than 10 μg/100g.

		Unanaesthetised				Anaesthetised			HPLC
Date	Type	BW	tod	Monit tox current method	Monit tox calibration	BW	tod	Monittox	Monit tox
20-May-02	M	19	ND	ND	ND	23	ND	ND	16
20-May-02	M	23	9:48	36	35	22	17:34	49	39
20-May-02	M	22	ND	ND	ND	22	ND	ND	
14-Jun-02	M	18	4:29	76	101	21	10:02	74	59
14-Jun-02	M	18	4:39	73	94	18	7:33	101	77
14-Jun-02	M	19	7:10	51	48	20	15:58	52	49
17-Jun-02	M	18	ND	ND	ND	22	ND	ND	ND
17-Jun-02	M	19	ND	ND	ND	20	ND	ND	trace
25-Jun-02	M	22	10:56	43	32	23	19:20	47	18
25-Jun-02	M	21	ND	ND	ND	21	ND	ND	16
28-Jun-02	M	20	ND	ND	ND	21	ND	ND	ND
28-Jun-02	M	23	ND	ND	ND	22	ND	ND	ND
28-Jun-02	M	21	ND	ND	ND	23	ND	ND	ND
28-Jun-02	M	23	ND	ND	ND	23	ND	ND	ND
28-Jun-02	M	20	ND	ND	ND	22	ND	ND	ND
28-Jun-02	M	22	ND	ND	ND	23	ND	ND	ND
28-Jun-02	M	21	ND	ND	ND	23	ND	ND	ND
28-Jun-02	M	21	ND	ND	ND	21	ND	ND	ND
28-Jun-02	M	23	ND	ND	ND	22	ND	ND	trace
28-Jun-02	M	22	8:18	48	41	22	13:21	58	40
11-Jul-02	M	23	ND	ND	ND	19	ND	ND	15
11-Jul-02	M	21	ND	ND	ND	20	ND	ND	12
11-Jul-02	M	21	ND	ND	ND	20	ND	ND	14
12-Jul-02	M	20	9:04	43	37	19	20:00	46	53
12-Jul-02	M	23	ND	ND	ND	18	ND	ND	ND
22-Jul-02	M	21	ND	ND	ND	21	ND	ND	18
22-Jul-02	M	23	ND	ND	ND	21	ND	ND	trace
6-Aug-02	M	23	ND	ND	ND	21	ND	ND	
6-Aug-02	M	23	ND	ND	ND	21	ND	ND	ND
6-Aug-02	M	18	7:13	43	48	21	ND	ND	ND
6-Aug-02	M	18	9:46	35	35	23	ND	ND	trace
19-May-03	M	23	3:11	124	226	21	5:00	191	
19-May-03	M	22	3:29	107	178	21	5:08	182	
19-May-03	M	23	17:58	35	23	22	19:55	46	
18-Jun-03	M	22	4:57	69	84	22	14:45	54	
18-Jun-03	M	22	4:54	70	86	21	12:28	61	
18-Jun-03	M	21	5:03	65	81	22	16:21	51	
18-Jun-03	M	22	12:00	37	29	22	19:21	47	
18-Jun-03	M	18	ND	ND	ND	21	14:35	55	

Table 6a contd.

Date	Type	Unanaesthetised				Anaesthetised			HPLC
		BW	tod	Monit tox current method	Monit tox calibration	BW	tod	Monit tox	Monit tox
18-Jun-03	M	20	5:38	58	68	22	21:00	45	
18-Jun-03	M	22	ND	ND	ND	22	14:03	56	
18-Jun-03	M	21	5:26	60	72	23	14:44	55	
18-Jun-03	M	20	5:45	57	66	21	14:16	56	
20-May-02	SG	23	ND	ND	ND	21	ND	ND	10
25-Jun-02	SG	23	ND	ND	ND	23	ND	ND	trace
25-Jun-02	SG	21	17:01	37	24	21	62:59	33	34
12-Jul-02	SG	21	ND	ND	ND	20	ND	ND	10
22-Jul-02	SG	21	ND	ND	ND	21	ND	ND	ND
6-Aug-02	SG	22	ND	ND	ND	20	ND	ND	10
6-Aug-02	SG	23	ND	ND	ND	22	ND	ND	ND
6-Aug-02	SG	22	ND	ND	ND	19	ND	ND	12
6-Aug-02	SG	18	19:13	31	22	20	11:21	66	49
6-Aug-02	SG	18	ND	ND	ND	20	ND	ND	13
20-May-02	SW	23	ND	ND	ND	23	ND	ND	trace
17-Jun-02	SW	20	5:07	71	79	21	8:50	84	35
17-Jun-02	SW	21	6:00	60	62	20	12:37	61	76
17-Jun-02	SW	21	5:43	64	66	20	11:08	67	26
25-Jun-02	SW	22	16:06	39	24	22	34:25	38	14
25-Jun-02	SW	22	7:56	50	43	22	13:54	57	52
25-Jun-02	SW	20	10:56	41	32	20	17:36	49	30
11-Jul-02	SW	20	3:32	111	172	20	6:15	131	129
11-Jul-02	SW	19	3:20	121	200	19	4:00	307	128
11-Jul-02	SW	18	3:21	111	197	19	5:41	152	77
12-Jul-02	SW	22	5:59	64	62	22	8:20	89	52
12-Jul-02	SW	23	5:59	65	62	22	14:07	56	21
12-Jul-02	SW	23	9:58	45	34	21	17:43	49	27
12-Jul-02	SW	22	12:52	42	28	21	20:38	45	10
12-Jul-02	SW	21	3:56	96	133	21	4:08	284	196
12-Jul-02	SW	21	ND	ND	ND	19	29:38	39	trace
22-Jul-02	SW	22	15:31	36	25	21	16:04	52	41
22-Jul-02	SW	22	7:36	46	45	23	13:06	59	52
22-Jul-02	SW	22	3:24	111	190	22	4:44	213	111
22-Jul-02	SW	19	ND	ND	ND	22	29:15	40	27
22-Jul-02	SW	19	7:19	46	47	21	8:21	89	88
6-Aug-02	SW	22	ND	ND	ND	19	16:15	51	20
6-Aug-02	SW	23	9:40	41	35	22	17:56	49	29
6-Aug-02	SW	22	19:40	34	22	21	15:03	54	24
6-Aug-02	SW	20	4:35	72	97	18	8:18	90	60
6-Aug-02	SW	19	12:07	36	29	20	7:00	111	50
6-Aug-02	SW	18	8:30	39	40	22	2:50	815	106
6-Aug-02	SW	18	1:59	247	1211	19	7:49	96	37
6-Aug-02	SW	19	18:59	33	22	20	ND	ND	13
6-Aug-02	SW	18	10:44	34	32	18	12:34	61	39
19-May-03	SW	22	5:27	63	71	22	7:29	102	
19-May-03	SW	22	7:03	49	49	23	16:49	50	
18-Jun-03	SW	20	5:05	64	80	22	8:20	89	
18-Jun-03	SW	20	3:30	101	176	19	5:44	150	

Table 6b. Numbers of sample results falling in <40, 40-80 and >80 μg/100g monitoring toxicity range. Based on 87 samples except for HPLC, which is based on 69 samples. See Table 6a for further explanation.

	Type	<40	40-80	>80
Current method	M	28	13	2
	SG	10	0	0
	SW	11	16	7
Calibration	M	30	6	7
	SG	10	0	0
	SW	15	10	9
Anaesthesia	M	24	16	3
	SG	9	1	0
	SW	5	14	15
HPLC	M	24	5	0
	SG	9	1	0
	SW	16	8	6

Table 7. Comparison of four methods. For each sample the difference between two methods is calculated and averaged over all samples. The range is calculated as mean difference ± 2 standard deviations, and represent bounds within which approximately 95% of the differences will lie. The p-value is from testing the mean difference against zero. The table is based on all data minus two large values (1211 for calibration method and 815 for anaesthesia method). See also Figure 7.

Comparison of methods[1]	n	Average difference	Range of differences		P-value
			Lower bound	Upper bound	
Current – Calibration	86	-7.0	-53.9	40.0	0.007
Current – Anaesthesia	86	-12.8	-92.6	67.1	0.004
Current – HPLC	69	1.5	-66.0	69.0	0.717
Calibration – Anaesthesia	85	-7.6	-66.9	51.6	0.020
Calibration – HPLC	68	2.4	-55.2	60.0	0.495
Anaesthesia – HPLC	68	13.8	-50.7	78.2	0.001

[1]Current method; unanaesthetised mouse with Sommers table, Calibration; unanaesthetised mouse in combination with calibration curve for unanaesthetised mice, Anaesthesia; anaesthetised mouse in combination with calibration curve for anaesthesia, HPLC; chemical method.

Table 8a: Comparison of four methods for estimating monitoring toxicity. Each block represents a comparison of two methods, showing the numbers of samples for which both methods agree on monitoring open (< 80μg/100g) or monitoring closed (≥ 80μg/100g). Based on 69 samples for HPLC comparisons, all other comparisons are based on 87 samples.

		Anaesthesia	
		Open	Closed
Current	Open	69	9
	Closed	0	9

		Anaesthesia	
		Open	Closed
HPLC	Open	56	7
	Closed	0	6

		Anaesthesia	
		Open	Closed
Calibration	Open	65	6
	Closed	4	12

		Current	
		Open	Closed
HPLC	Open	61	2
	Closed	2	4

		Current	
		Open	Closed
Calibration	Open	71	0
	Closed	7	9

		HPLC	
		Open	Closed
Calibration	Open	58	2
	Closed	5	4

Table 8b: P-values for method comparisons, based on McNemars test. A P-value exceeding 0.05 indicates that there is not sufficient evidence that the two methods are different

Comparison of methods[1]	P-value
Current – Anaesthesia	0.004
HPLC – Anaesthesia	0.016
Calibration – Anaesthesia	0.754
HPLC – Current	1.0
Calibration – Current	0.016
Calibration – HPLC	0.453

Table 9: Six samples collected from the same site on 19 May 2003. One sample was analysed using both an unanaesthetised and an anaesthetised mouse, the other five samples were analysed on anaesthetised mice only. For abbreviations see Table 6.

		Unanaesthetised				Anaesthetised		
Sample	Type	BW	tod	Monitoring tox current	Monitoring tox calibration	BW	tod	Monitoring tox
A	M	23	3:11	124	226	21	5:00	191
B	M					21	5:17	173
C	M					21	4:00	307
D	M					21	5:36	156
E	M					21	4:08	284
F	M					21	4:20	256

Figure 1. Observed time of death (tod, sec) for ten STX doses, nine mice per dose for a) unanaesthetised and b) anaesthetised mice. For the unanaesthetised mice, the animals that were still alive after 20 minutes are plotted against 1200 seconds, with the number of animals indicated above. Δ September 2001, x December 2001.

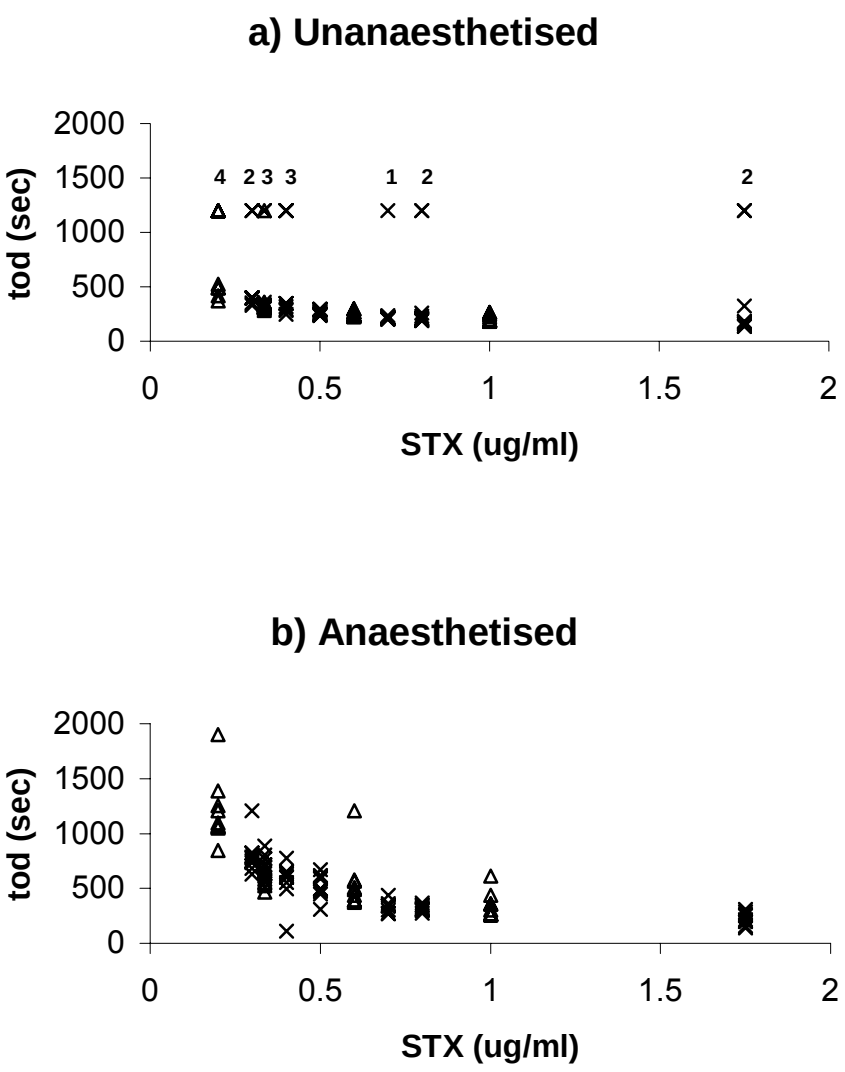


Figure 2. Calibration curve based on linear relationship between 1/tod and log(STX) where tod is the median time of death (sec) for each dose tested and STX is the saxitoxin concentration (μg/ml). See also Table 5.

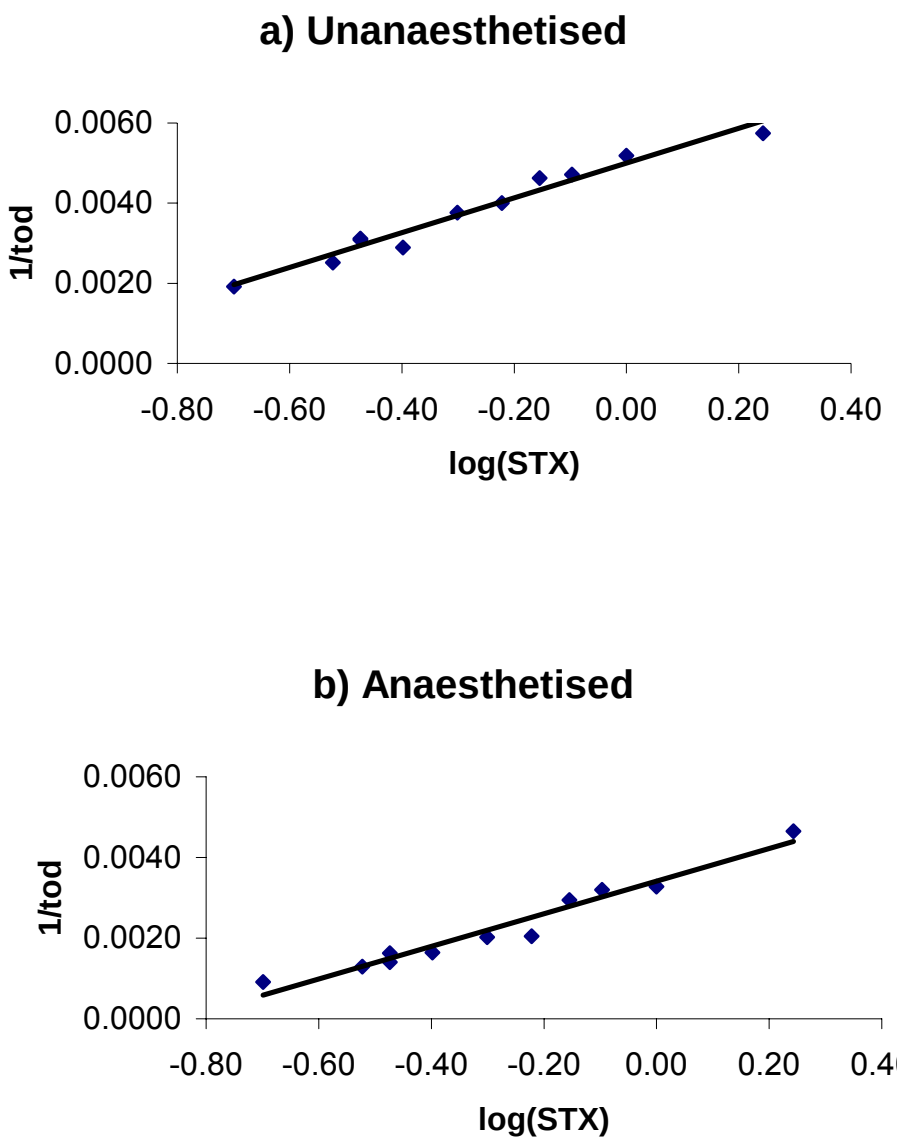
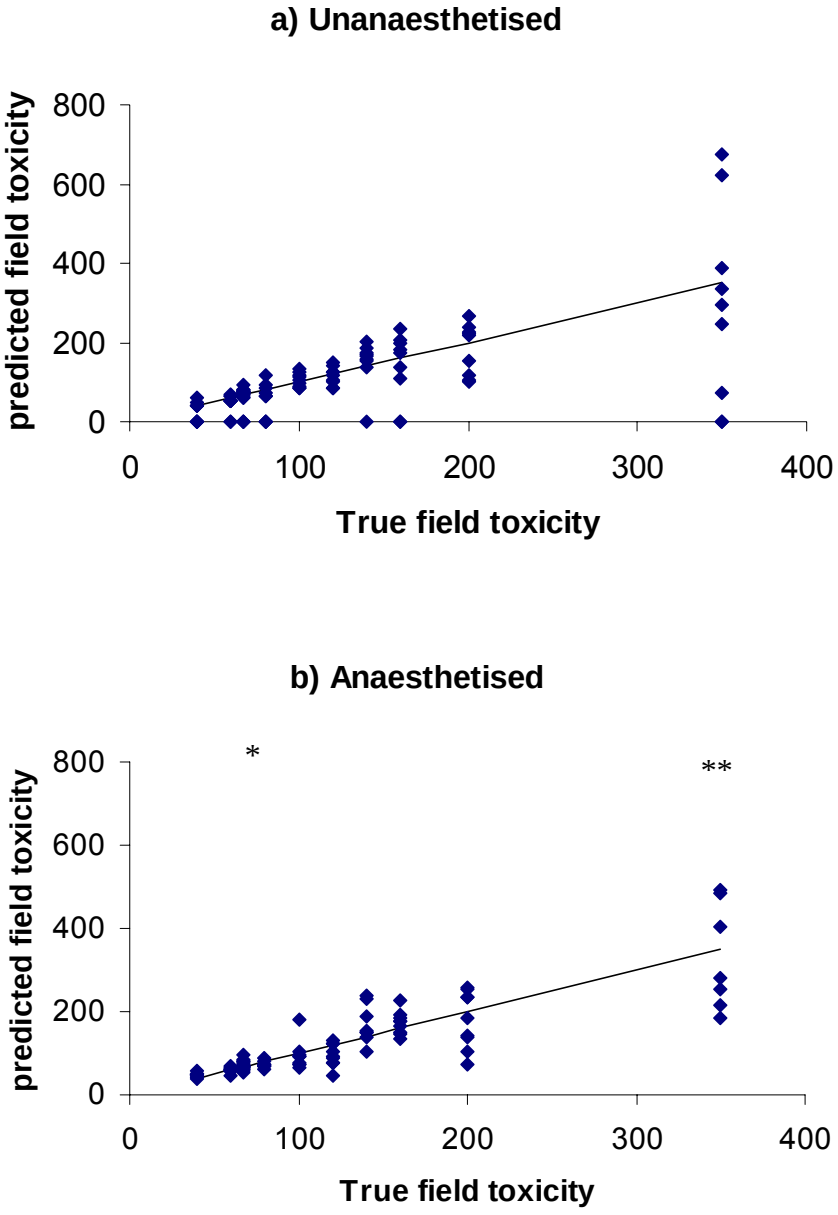


Figure 3: Predicted monitoring toxicity (μg/100g shellfish flesh) versus 'true' monitoring toxicity (STX concentration expressed as the equivalent monitoring toxicity), based on the calibration curves given in Table 5. Also indicated is the line for which predicted toxicity is equal to the true toxicity.



*For monitoring toxicity of 80μg/100g, one mouse gives predicted value of 5573 μg/100g.
**For monitoring toxicity of 350 μg/100g, two mice give values of 1127 and 1578 μg/100g.

Figure 4: Predicted monitoring toxicity (μg/100g shellfish flesh) versus 'true' monitoring toxicity, for unanaesthetised animals. The observed death time is converted to monitoring toxicity based on Sommers table (current method). Also indicated is the line for which predicted toxicity is equal to the true toxicity.

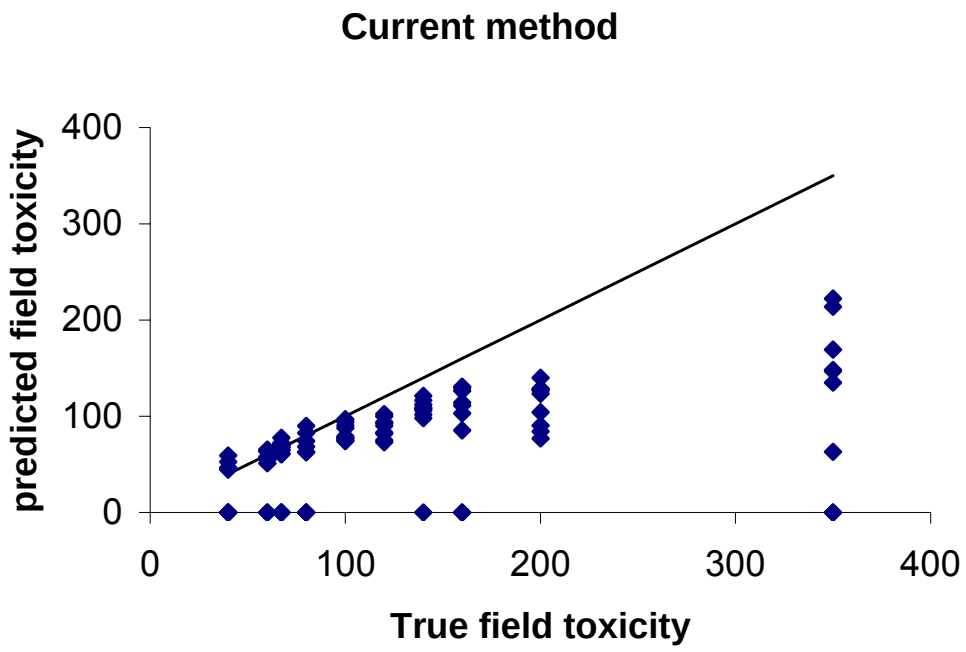


Figure 5: Bar charts for mussels (a), scallop gonads (b) and whole scallops (c) showing the estimated toxicity for each of the samples, based on unanaesthetised mice using Sommers table (current method), unanaesthetised mice using calibration curve (calibration method), anaesthetised mice and from HPLC. Not all the samples were analysed by HPLC, this is indicated by a negative bar. For whole scallops, 2 values are truncated at 400 (sample 10 with 815 μg/100g for anaesthesia method and sample 34 with 1211 μg/100 g for calibration method).

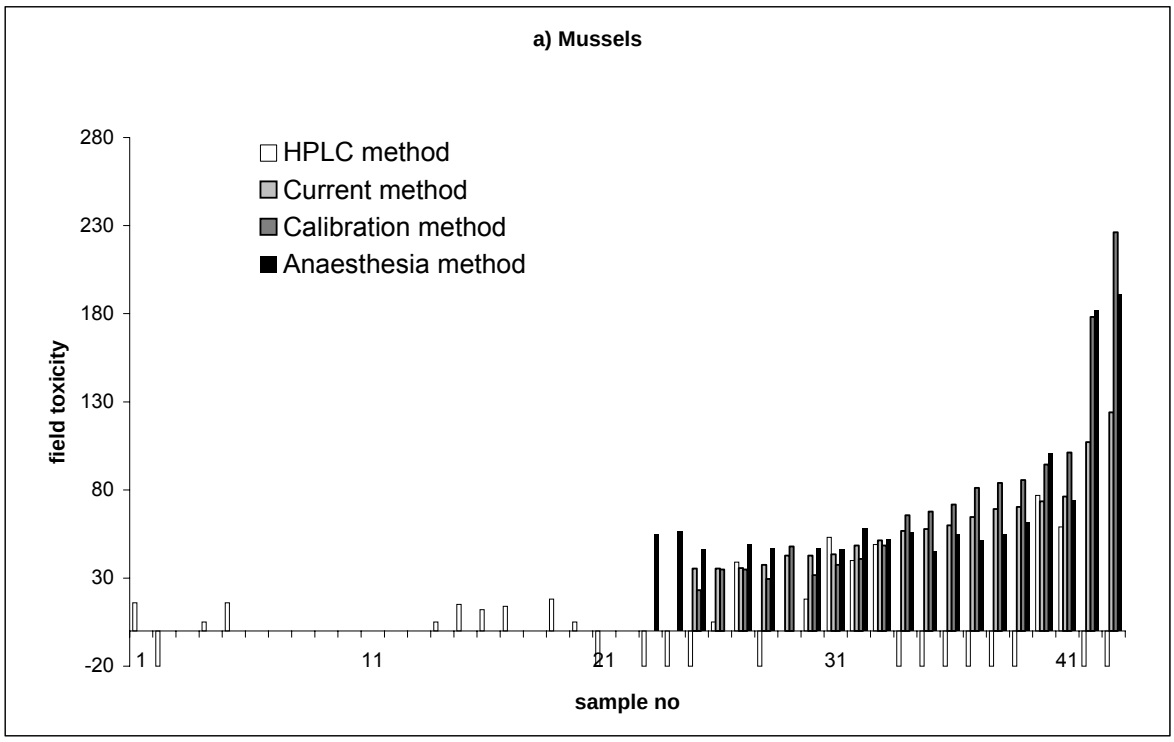
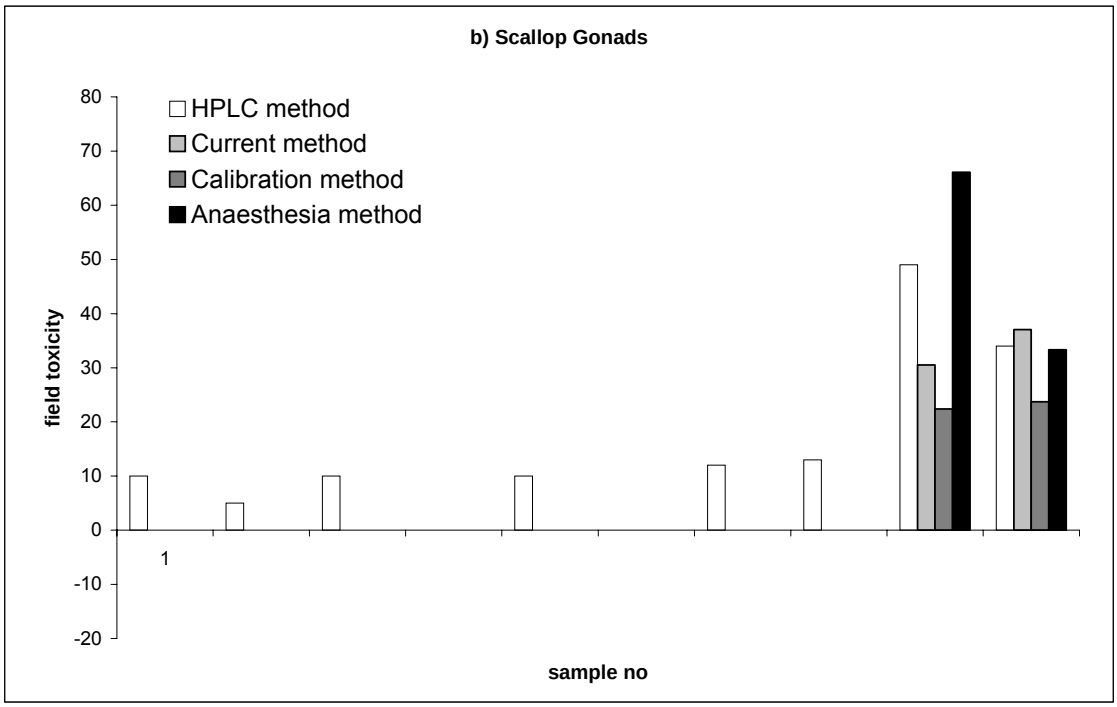


Figure 5 continued.



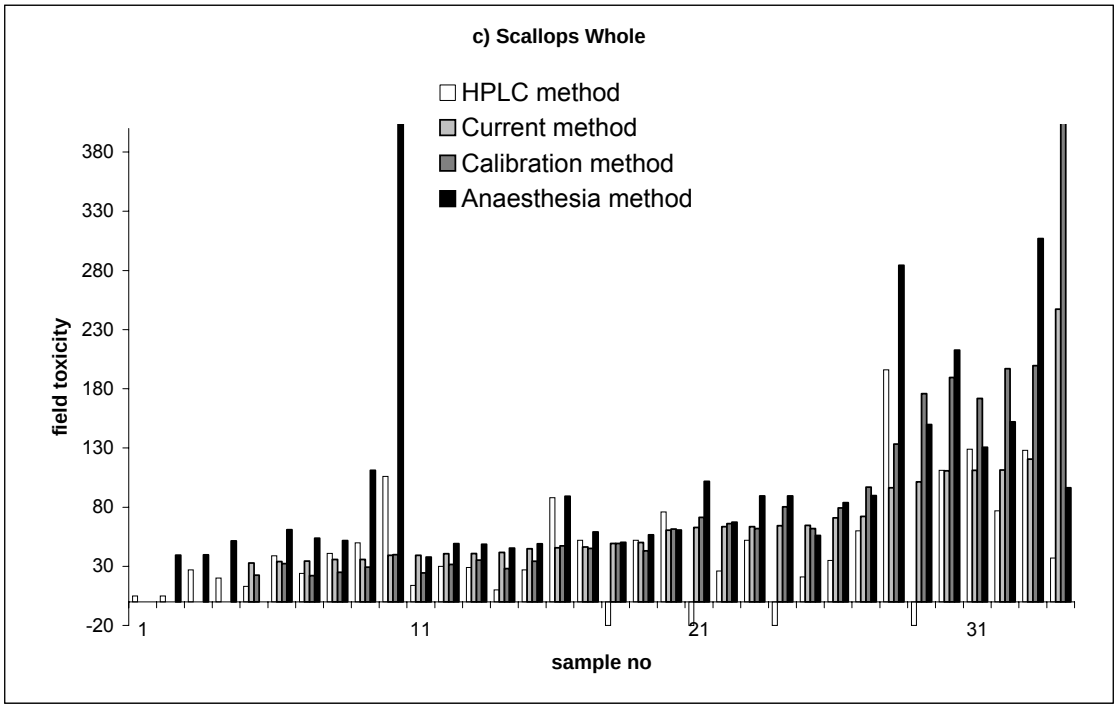


Figure 6. Comparison of four different toxicity estimates (o mussels, + whole scallops, Δ scallop gonads), based on unanaesthetised mice using Sommers table (current method), unanaesthetised mice using calibration curve (calibration method), anaesthetised mice and HPLC. Fourty-five degree line represents equality of two methods. Excludes two data points (815μg/100g for anaesthesia and 1211μg/100g for calibration method).

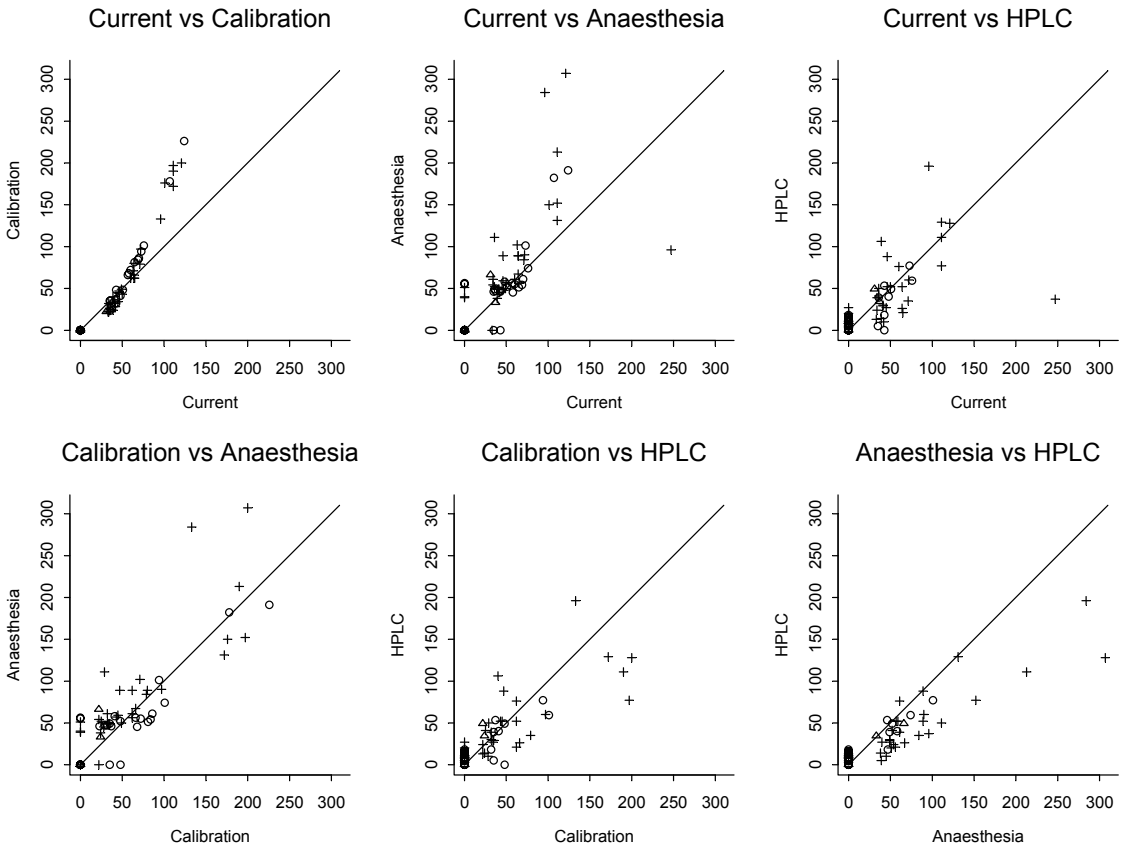


Figure 7: Comparing two methods at a time, by looking at the difference in estimated monitoring toxicity, plotted against the average toxicity (average of two methods, o mussels, + whole scallops, Δ scallop gonads). The methods compared are unanaesthetised mice using Sommers table (current method), unanaesthetised mice using calibration curve (calibration method), anaesthetised mice and HPLC. Also shown is the 95% interval for the differences. Excludes two data points (815 μg/100g for anaesthesia and 1211 μg/100g for calibration method).