knowledge about the toxicology of the different analogues is limited. A broad cross reactivity also means that samples may give a higher result, when analysed by ELISA, than with chemical methods, such as liquid chromatography-mass spectrometry (LC-MS), in which some of the analogues present may not be quantified (Samdal *et al*., 2005). No ELISA method is fully validated yet, although several attempts are being carried out at this time. When comparing results from LC-MS and ELISA, the figures from the latter are usually higher, especially at lower levels.

The major advantages of ELISA include:

- the technique is sensitive, rapid and provides a high sample throughput;
- the equipment needed is relatively cheap;
- the technique is easy to perform, can be automated, and requires minimal training.

The major disadvantages of ELISA include:

- it is impossible to distinguish between different analogues e.g. toxin profile by the ELISA;
- it requires reference material for identification and quantification.

A two day long cell-based assay, that detects E-cadherin levels, was reported to detect YTX (Pierotti *et al*., 2003), and this method was further shortened to a few hours with a slot blot procedure (Pierotti *et al*., 2007), but the method is not specific for YTX, since there are interferences with azaspiracids (Ronzitti *et al*., 2007).

4.4 Chemical methods

YTXs are polyether compounds bearing two sulfate groups and an aliphatic side chain with a conjugated diene. This chromophore allows their detection by ultraviolet (UV) detection with an absorption maximum at 230 nm (Murata *et al*., 1987).

Due to low specificity and sensitivity of UV-detection a high-performance liquid chromatography-fluorescence detection (HPLC-FLD) method has been developed which allows a detection of 100 µg YTXs/kg shellfish meat (Yasumoto and Takizawa, 1997). This method requires a derivatisation step, includes a time consuming clean up and is not validated.

For a specific as well as a sensitive detection of individual YTXs liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) techniques have been developed. The group of YTXs is routinely incorporated in multi-toxin methods for the detection of lipophilic biotoxins, that are used in some laboratories.

Due to the two sulfate groups YTXs are more polar than other lipophilic biotoxins. This results in different partitioning behaviour compared to other lipophilic toxins, and attention has to be paid to differences in recovery efficiencies in multitoxin clean-up procedures.

A problem for the analytical community is the lack of availability of standards. Up to now only YTX is provided as a certified calibrant (NNRC, Canada). This requires an indirect quantification of remaining YTXs assuming the same response as YTX during mass spectrometry (MS) detection.

One multi-toxin LC-MS/MS protocol that included some YTXs (YTX, 45-hydroxyYTX, 1a-homoYTX, carboxyYTX) has been subjected to a limited interlaboratory validation study (McNabb *et al.*, 2005). The limit of quantification (LOQ) for this method was 0.017 mg/kg shellfish. This is in line with reported LOQs from other studies (e.g. BIOTOX certification feasibility-study, EU-FP6-2003-Food-2A project no.: 514074) and this LOQ would be low enough for surveillance purposes.

Attempts to advance and validate methods for detection of YTXs by LC-MS/MS are being undertaken by the EU CRL-MB (Vigo, Spain) and were also performed under the auspices of the "BIOTOX" project. At the time of writing, these efforts have not led to an acceptable procedure yet.

The major advantages of LC-MS/MS include:

- information on the toxin profile of lipophilic biotoxins can be obtained without partitioning or any further clean-up steps;
- it is highly specific and sensitive;
- it can be automated.

The major disadvantages of LC-MS/MS include:

- it requires costly equipment and highly trained personnel;
- it requires reference material for identification and quantification.

4.5 Summary of methods

From the above brief summary of methods, it can be seen that, although currently described by EC legislation, the MBA has not been validated. Additionally, Council Directive 86/609/EEC[6] states that "*Member States may not permit the use of live animals in procedures that may cause pain, suffering distress or lasting harm if another scientific satisfactory method of obtaining the result sought and not entailing the use of live animals is reasonably and practicably available*".

At this time however, none of the methods for the determination of toxins from the YTX-group have been validated by interlaboratory studies. It is particularly important therefore, that the various methods are evaluated for their fitness for purpose.

The evidence available at this moment suggests that LC-MS/MS has the greatest potential to replace the MBA. Moreover, it is able to detect YTXs at levels below the current regulatory limit of 1 mg/kg shellfish meat. The LC-MS/MS also has the possibility for multi-toxin group

detection/quantification. However, before LC-MS/MS can be used for official purposes, validation results are needed to support their use.

5. Occurrence of YTX-group toxins

5.1 Data Collection

Following a request by the Euroepan Food Safety Authority (EFSA) for data on YTXs Germany, Italy, Norway, Portugal, Spain and United Kingdom (UK) provided data on the occurrence of YTXs in shellfish. A total of 2,881 analytical results were submitted. The number of analyses presented by the countries is relatively small and considerably different from one country to another. Table 2 shows a summary of the number of data submitted by each country including purpose of testing, analytical method applied, limit of detection (LOD) and LOQ of the methods.

Table 2: Data submissions from Member States for YTX-group toxins in the period from 2000 to 2008.

Country	Year(s) of harvesting	Number of samples	Purpose of testing [a]	Method of testing	LOD (µg/kg)	LOQ (µg/kg)
Germany	2005-2006	405	pre/post-MC	LC-MS/MS	1-10 [b]	-
Italy	2000-2005 2006-2008	220 254 [c]	pre/post-MC	HPLC LC-MS/MS	30 30	100 100
Norway	2006-2007	1433	pre-MC	LC-MS/MS	1	10
Portugal	2005-2007	512	pre-MC	ELISA	150	- [d]
Spain [e]	2005-2006	17	pre-MC	HPLC, LC-MS/MS, MBA	-	-
United Kingdom	2006-2007	40	pre-MC	LC-MS/MS	66	165
Total		**2881**				

Pre/post-MC=pre-market/post-market control samples, LOD=limit of detection, LOQ=limit of quantification

[a] Pre-market control samples are samples collected at the place of origin, before or during harvesting; post-market control samples are samples collected at the place of sale or along the distribution chain.
[b] Germany reports only LOD, and this is variable through the years and between different laboratories.
[c] The LC-MS/MS data from Italy were produced as confirmation of MBA tests, therefore their number is limited.
[d] ELISA analyses have only one determination threshold, reported as LOD.
[e] Data from Spain are statistically not relevant, therefore they were not considered in the calculations.

The submissions covered samples collected and tested during years 2005 to 2007, with the exception of a high-performance liquid chromatography (HPLC) dataset ranging between 2000 and 2005, and included pre- and post-market control samples.

Pre-market control samples (pre-MC), which are samples harvested for further processing or direct marketing as prescribed in the respective EU legislation, comprised 2477 results. Post-market control samples (post-MC), which are samples taken from the market, comprised 404 results whereas the remaining data did not come from routine shellfish monitoring. The post-

MC data (referred to samples collected at stores and supermarkets, with unknown – possibly multiple – origin) were submitted mainly from Germany (382 out of 404) while the remaining 22 were from Italy.

5.2 YTX-group toxin concentrations in shellfish

Normally the whole shellfish is consumed and therefore the occurrence data for YTXs need to be expressed in terms of whole shellfish meat. Most of the analyses were performed on whole shellfish meat. In a few samples only hepatopancreas was analysed; in this case a factor of 5 was used to convert the value to whole shellfish meat. This factor, though not representing exactly all individual molluscs, is considered to represent a good approximation.

Most of the samples reported values for four YTXs: YTX, 45-hydroxyYTX, 1a-homoYTX and 45-hydroxy-1a-homoYTX. The values were determined separately for any single analogue or in a cumulative way, depending on the analytical technique applied. Some samples, but not all, were analysed also for carboxyYTX and carboxy-1a-homoYTX. For these two analogues the animal toxicity studies are very limited and suggest a much lower toxicity than the above mentioned four variants, therefore it was decided to disregard the two carboxy-analogues in the exposure analysis.

The analysis was accordingly focused on the four analogues mentioned in Commission Regulation (EC) No 2074/2005[4]: YTX, 45-hydroxyYTX, 1a-homoYTX and 45-hydroxy-1a-homoYTX.

The conversion to YTX equivalents (YTX eq.) was performed applying a factor of 0.5 for 45-hydroxy-1a-homoYTX and a factor of 1 for all the others (see chapter 10.3).

A total of 2864 samples were initially considered for the descriptive statistical calculations. Since the development of analytical methods in the marine biotoxins area is continuous, different (and not directly comparable) methods had been used in different countries and/or at different periods of time. The submitted data were obtained with 4 different analytical methods:

1. ELISA with a LOD of 150 µg/kg
2. HPLC with a LOD of 30 µg/kg
3. LC-MS/MS with higher instrument sensitivity having a LOD variable in the range 1-10 µg/kg
4. LC-MS/MS with lower instrument sensitivity having a LOD ranging between 30 and 66 µg/kg (with most analyses at 30 µg/kg)

For the imputation of values reported below LOD or below LOQ the "bounding" approach was adopted, which consists of forcing values at the boundaries of their possible variability. The lower bound is obtained by assigning a value of zero (minimum possible value) to all the samples reported as <LOD or <LOQ. The upper bound is obtained by assigning the value of

LOD to values reported as <LOD and LOQ to values reported as <LOQ (maximum possible value).

Table 3 provides an overview of the descriptive statistics of the data grouped by analytical method and country. Samples without reported values were assigned to upper- and lower bound. When the statistical descriptors in the two approaches are the same the value is given, otherwise the lower-upper bound range is reported.

Table 3: Statistics of relevant data of YTX-group toxins in shellfish sampled in 2000 to 2008 provided by Member States.

Analytical method/Country	N	Median	Mean	P95	Maximum	% of samples not quantified	% of values >1000 µg YTX eq./kg shellfish meat
		µg YTX eq./kg shellfish meat					
ELISA							
Portugal 2005-07	512	≤150	174-260	737	1599	57.4	2.3
HPLC							
Italy 2000-05	220	705	1108-1113	3354	9620	14.5	40.0
LC-MS/MS higher instrument sensitivity							
Germany 2005-07	405	≤5	5-10	33	240	89.4	0.0
Norway 2006-07	1433	17	129	567	3707	34.8	2.8
LC-MS/MS lower instrument sensitivity							
Italy 2006-08	254	119	619-646	2328	8558	42.9	16.1
UK 2006-07	40	≤165	9-137	9-165	188	95.0	0.0

N=number of samples, P95=95th percentile, YTX eq.=YTX equivalents

For most of the data no information is available on measurement uncertainty. When a range is given it indicates the difference between using the lower or upper bound for samples below the LOD or LOQ. The upper bound is performed substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.

The concentration of YTXs in the current collection of shellfish samples from European countries ranges from "*not detected*" to 9620 µg/kg shellfish meat.

Particularly high values were reported in the Italian dataset, both HPLC and LC-MS/MS. Marine biotoxins are known to show a non-homogeneous distribution in terms of time and geographical location (Ciminiello *et al*., 1999). In this case it has to be emphasised that the dataset from Italy does not represent the whole country, but is focused on an area – the north Adriatic Sea – affected by a significant YTX contamination in recent years. This hot spot therefore represents a worst case scenario for the occurrence of YTX along the European coasts.

The percentage of samples with a non-quantified value varies to a large extent, depending on country and year of harvesting, ranging from 14.5% for Italy in the years 2000-2005 to 95.0% for UK in the years 2006-2007, with an average of 45.6% for all 2864 analysed data. The very

large proportion of non detected or non quantified values makes the UK dataset very problematic for exposure calculations, since the median, mean and 95th percentile are strongly influenced by the upper or lower bound assumption. Consequently it was decided to exclude these data from further use.

The proportion of samples exceeding the regulatory limit of 1000 µg/kg has also been reported. It varies among countries between 0% (Germany and UK) and 40% (Italy, 2000-2005 HPLC).

The overall differences in selectivity of the analytical methods, objective of the investigation, time of sample collection (pre- or post-market) and number of samples reported, makes it difficult to compare countries and to consider a single dataset as representative for the respective country. Nevertheless two rather homogeneous data sets were identified being suitable for exposure assessment:

Group 1. A set of 1838 data from Germany and Norway based on LC-MS/MS analysis with higher instrument sensitivity; 46.8% of the samples in this dataset had levels of YTXs below the LOD or LOQ;

Group 2. A set of 254 data from Italy (North Adriatic Sea) based on LC-MS/MS analysis with lower instrument sensitivity; 42.9% of the samples in this dataset had levels of YTXs below the LOD or LOQ.

Due to the different sensitivity of the analytical methods these datasets will be dealt with separately. Moreover, as can be seen in chapter 6, they also represent two different occurrence scenarios: Group 1 a low to medium contamination, Group 2 a “hot spot” (high occurrence).

For these two data upper- and lower bound values are calculated for each statistical descriptor and these values are reported as a single value when they are the same or as a range when they are different.

5.3 Difference between species

Mussels were the predominant shellfish product tested, accounting for 1779 samples of the 2092 (85%). A number of 134 samples had no species identification (6.4%). Nevertheless a few data from other species were reported and their statistical descriptors are summarised in Table 4.

Table 4: Statistical descriptors for YTX-group toxins occurrence in different shellfish.

Species	N	Total concentration of YTX-group toxins µg YTX eq. /kg shellfish meat				% of samples not quantified	% of values >1000 µg YTX eq./kg shellfish meat
		Median	Mean	P95	Maximum reported		
Group1							
Clams [b)]	36	n.a.	n.a.	n.a.	n.a.	100	0
Cockles[$ b)]	4	n.a.	n.a.	n.a.	n.a.	100	0
*Crabs**	*19*	*15*	*28-30*	*133*	*138*	*36.8*	*0 [#]*
Mussels	1552	12-13	119-120	548	3707	48.0	2.6
Others	134	≤5	5-10	34	240	91.8	0
Oysters	47	≤5	15-18	99	321	85.1	0
Scallops [b)]	46	n.a.	n.a.	n.a.	n.a.	100	0
Group2							
Clams [b)]	22	n.a.	n.a.	n.a.	n.a.	100	0
Gastropods[$,* a)]	1	n.a.	764	n.a.	n.a.	n.a.	n.a.
Mussels	227	196	689-715	2567	8558	37.9	18.1
Oysters[$ b)]	4	n.a.	n.a.	n.a.	n.a.	100	0
ALL	**2092**						

N=number of samples, YTX eq.=YTX equivalents, P95=95th percentile, n.a.= not applicable
[*] Currently not regulated
[#] Based on brown meat and not on whole flesh
[$] Samples are not enough to allow statistical calculations
[a)] Only one sample: in the column "mean" the value is reported; no statistical calculation possible.
[b)] Since no numerical result was reported, calculation for these values has not been performed.
When a range is given it indicates the difference between using the lower or upper bound for samples below the LOD or LOQ. The upper bound is performed substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.

YTXs were not detected in clams and cockles. In scallops they were detected in two samples out of 46, but at a level below the LOQ. A low level of YTXs was registered in crabs (currently not regulated) and oysters. A somewhat higher presence was recorded in mussels, with median and mean values well below the regulatory limit. The 95th percentile value was below the regulatory limit for Group 1, but much higher than the limit for Group 2.

The samples exceeding the regulatory limit of 1000 µg/kg only referred to mussels, with a proportion of 2.6% of the data in Group 1 and 18.1% of the data in Group 2 above the limit value. It is emphasised that these data are monitoring samples of which some were excluded from the market due to high test results. Consequently they offer a picture of the harvest, but not of the products offered for sale.

5.4 Influence of type of sampling and origin of the sample

The Group 1 and Group 2 datasets contained samples from local monitoring programmes including samples from both pre- and post-MC. The comparison between pre- and post-

market data in the two groups is shown in Table 5. These data were all measured by means of LC-MS/MS and represent results from 2005 to 2008.

Table 5: Statistics of LC-MS/MS data of YTX-group toxins in shellfish in Group 1 and Group 2 datasets.

Data groups	N	Total concentration of YTX-group toxins µg YTX eq./kg shellfish meat				% of samples not quantified	% of values >1000 µg YTX eq./kg shellfish meat
		Median	Mean	P95	Maximum		
Group1	1838						
pre	1456	16	127-128	561	3707	45.5	2.7
post	382	≤5	5-10	35	240	88.7	0
Group2	254						
pre	232	180	664-691	2525	8558	40.5	17.2
post	22	≤30	144-175	916	2011	81.8	4.5

N=number of samples, P95=95th percentile, YTX eq.=YTX equivalents
When a range is given it indicates the difference between using the lower or upper bound for samples below the LOD or LOQ. The upper bound is performed substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.

In Group 2 (Italy, North Adriatic Sea) the data on pre- and post-market samples refer to the same geographical area and clearly show a lower contamination in the post market samples as an effect of the screening control at the production sites. Nevertheless, 1 out of 22 post market samples has a concentration (2011 µg YTX eq./kg) above the limit value.

The samples in Group 1 are not homogeneously distributed with respect to pre-MC and post-MC: all the 1433 samples from Norway are pre-market samples, whereas 382 samples from Germany are post-market samples (origin unknown), and only 23 are pre-market samples from local production. Nevertheless, also this group shows lower levels of YTX contamination in post market samples.

The comparison of the two datasets of pre-MC samples (which related to local production) confirms the existence of significant differences in occurrence between different geographical areas.

5.5 Influence of processing

There is no information on the effect of processing (e.g. cooking) on the levels of YTXs in shellfish. However, it can be assumed that, as for other lipophilic marine biotoxins, cooking may lead to an increase in concentration of YTXs in shellfish flesh due to water loss.

6. Samples tested with both LC-MS/MS and mammalian bioassays

Not all of the 2092 samples reported in Group 1 and Group 2 were tested with both LC-MS/MS and MBA. Only for 520 samples, 269 from Norway and 251 from Italy (North Adriatic Sea) results from both LC-MS/MS and MBA were reported. Table 6 summarises the statistical descriptors for each of the subsets with respectively positive and negative MBA.

Table 6: Concentrations of YTX-group toxins measured by LC-MS/MS in samples comparatively tested by MBA.

Mouse Bioassay	N	Total concentration of YTXs µg YTX eq./kg shellfish meat				% of samples not quantified	% of values >1,000 µg YTX eq./kg shellfish meat
		Median	Mean	P95	Maximum		
Norway	**269**						
Positive	55	16	159	599	3004	43.6	1.8
Negative	214	≤10	78-79	315	3707	52.8	0.9
Italy (North Adriatic Sea)	**251**						
Positive	75	959	1636-1646	5750	8558	13.3	49.3
Negative	176	≤100	191-225	799	1647	58.0	2.3

N=number of samples, P95=95th percentile, YTX eq.=YTX equivalents
When a range is given it indicates the difference between using the lower or upper bound for samples below the LOD or LOQ. The upper bound is performed substituting "<LOD" with LOD value and "<LOQ" with LOQ value; LOD and LOQ are those defined for the specific single analysis.

The percentage of samples testing negative in the MBA but having an LC-MS/MS value higher than the regulatory limit (1000 µg YTX eq./kg shellfish meat) was 0.9% for Norway and 2.3% for Italy (North Adriatic Sea). In the dataset from Italy 13.3% of the samples testing positive in the MBA had a value below the LOD or LOQ. For the Norwegian data 43.6% of the samples testing positive in MBA had an LC-MS/MS value below LOD or LOQ. This high proportion of false positive results indicates a contribution of other lipophilic toxins, such as OA-, AZA-, PTX- or cyclic imine group toxins, or combinations thereof.Norway is still applying a protocol for the MBA with an extraction procedure that does not separate YTXs from other lipophilic toxins. This is in contrast with the protocol applied in Italy that includes an additional methanol extraction for YTXs.

It can be assumed that in countries using mammalian bioassay as screening method all bivalve molluscs showing a negative response in mammalian bioassays reach the market and will thus be consumed. From this perspective, the dietary intake of YTXs (see chapter 8) may be estimated based on the LC-MS/MS data for those samples that tested negative in the MBA, when the MBA was used as the screening method. This approach is applicable for the Italian situation. The Panel recognised, however, that in Norway the screening of shellfish for YTXs is performed with both the MBA and LC-MS/MS, and that the decision for placing shellfish on the market is always based on the LC-MS/MS results. This implies that in Norway no shellfish will reach the market with concentrations of YTXs above the current limit value of

1000 µg YTX eq./kg shellfish meat. Nevertheless, the Panel decided to use the negative MBA data from the Norwegian dataset (See Table 6) as a representation of other European countries with low to medium YTX contamination and applying the MBA as screening method.

7. Human consumption of shellfish

Limited consumption data were available for individual shellfish species across the EU. The EFSA Concise European Food Consumption Database does not yet provide sufficient information since there is no differentiation between meal sizes for fish and other seafood. Therefore, EFSA requested the Member States to provide information on shellfish consumption. Data were submitted by France, Germany, Italy, The Netherlands and UK. A compilation of the data received is presented in Table 7. The mean portion sizes for consumers only ranged between 10 g (France, bivalve molluscs) and 136 g (The Netherlands). The data from Germany, Italy and UK are within this range.

The German national food consumption survey performed by a weighing protocol in the late 1980s indicates a minimum meal size of mussels of 2 g (mainly as an ingredient in dishes), a median of 63 g, a mean of 107 g and a 95th percentile of 400 g among mussel consumers. The maximum portion size reported in this study was 1,500 g (Adolf *et al.*, 1995). The French Calipso study differentiated mussels and bivalve molluscs (Leblanc, 2006). The maximum portions for mussels (245 g) and all bivalve molluscs (415 g) varied, whereas the mean portions were similar. A survey reported by the United Kingdom indicates a mean shellfish meal size of 114 g and a maximum of 239 g (Henderson *et al*., 2002). A Dutch study reported a mean portion size of 136 g of shellfish and a maximum of 480 g. These data are for consumers only (Kistemaker *et al*., 1998). The surveys show a large variation in the percentage of the populations consuming shellfish and it is unclear whether the data are related to cooked or uncooked shellfish.

Table 7: Shellfish eating habits in France, Italy, The Netherlands, the UK, and Germany, based on national food consumption surveys.

		Number of consumers N (%)	Number of eating occasions for consumers/year	Mean portion weight (g)	95th percentile (g)	Maximum portion weight (g)	Maximum frequency
France (7 days)	INCA 1999	218/1985 (11%)	N/A	10			N/A
France (FFQ)	CALIPSO 2004 (bivalve molluscs)	962/997 (96%)	N/A	32	94	415	N/A
France (FFQ)	CALIPSO 2004 (mussels)	862/997 (86%)	N/A	22	70	245	N/A
Italy (7 days)	INN-CA 1994-96	212/1,981 (11%)	47	83		1,000	4/week
Germany (7 days)	NVS 1985-88	150/23,239 (0.6%)	171	107	400	1,500	3/week
UK (7 days)	NDNS 2000-01	212/1,631 (13%)	51	114		239	4/week
The Netherlands (2 days)	DNFCS 1997-98	47/4,285 (1.1%)	39	136	465	480	N/A

FFQ = food frequency questionnaire, 7 days = 7 day diary record, N/A = not available
INCA = Enquete Individuelle et Nationale sur les Consommations Alimentaires (Volatier, 2000).
CALIPSO = Fish and seafood consumption study and biomarker of exposure to trace elements, pollutants and omega 3 (Leblanc *et al.*, 2006)
INN-CA = Nationwide Nutritional Survey of Food Behaviour (Turrini *et al.*, 2001)
NVS = Nationale Verzehrsstudie (Adolf *et al.*, 1995)
NDNS = National Diet and Nutrition Survey (Henderson *et al.*, 2002)
DNFCS = Dutch National Food Consumption Survey (Kistemaker *et al.*, 1998)

Because YTXs have acute toxic effects, it is important to identify a high portion size rather than a long term average consumption in order to protect the health of the consumer. In the studies presented in the table above, the maximum reported sizes are in the range of 239 to 1,500 g. The CONTAM Panel noted the highest portion sizes of 1,000 g and 1,500 g, and considered it likely that the shells were included in these weight estimates. Therefore, the Panel considered the 95th percentile as a more realistic estimate of the portion size for high consumers. As shown in Table 7 the 95th percentile values range from 70-465 g, the Panel chose the figure of 400 g to be used as a high portion size in acute exposure assessments. This is in good agreement with the report of the Joint FAO/IOC/WHO *ad hoc* expert consultation on marine biotoxins (FAO/IOC/WHO, 2004a) where 380 g was reported as the highest 97.5th percentile portion size for consumers only.

8. Exposure assessment

8.1 Deterministic estimate of dietary exposure to YTX-group toxins

Based on the assumption that products tested negative in the MBA, and hence fulfil the screening criteria, reach the market, and considering the North Adriatic and Norwegian scenarios shown in Table 6 and Table 7, the dietary exposure can be estimated as in Table 8.

Table 8: Deterministic intake estimate of YTX-group toxins, based on data from Norway and Italy (North Adriatic Sea), from samples tested negative in the MBA.

	Norway	Italy North Adriatic Sea
P95 (concentration of samples tested negative in the MBA)	315 μg/kg whole shellfish meat	799 μg/kg whole shellfish meat
Exposure by eating a 400 g portion	125 μg YTX eq. per person (2.1 μg/kg b.w.)	320 μg YTX eq. per person (5.3 μg/kg b.w.)
Exposure by eating a 400 g portion containing YTXs at EU limit of 1000 μg/kg whole shellfish meat	400 μg YTX eq. per person (6.7 μg/kg b.w.)	

P95=95th percentile, YTX eq.=YTX equivalents, b.w.=body weight, MBA=Mouse bioassay, EU= European Union

These results are conservative, but not unrealistic estimates of high level YTX dietary exposure in two European countries.

8.2 Probabilistic estimate of dietary exposure to YTX-group toxins

A probabilistic estimate of dietary exposure to YTXs has been performed by a Monte Carlo simulation using the distributions of both the occurrence data (summarised in Table 6) and the data on the consumption of shellfish. Compared to the deterministic estimate the probabilistic exposure estimate provides information on the probability to exceed a specific exposure level. Because a person eating shellfish will not eat the same portion size containing the same level of toxins each time, the probabilistic calculation includes all the combinations of all different occurrence and consumption data.

For the probabilistic estimate the same concentration data obtained by the LC-MS/MS measurements of the samples tested negative in the MBA (Table 6) were used[12]. It can be

[12] All samples with quantified levels (>LOQ) of YTX -group toxins were submitted to the best fit approach of the @RISK tool, to obtain an optimal adaptation of the distribution function, as described in the following:
Italy, Risklognormal (491,66; 415,81; RiskShift (-27,234); RiskTruncate(100;)), n=74, (42% of all data)
Norway, RiskLognorm (153,68; 342,95; RiskShift (8,465);RiskTruncate(10;)), n=101 (48% of all data)
The distribution function was truncated at the respective LOQs as shown in the formula. The values below LOQ/LOD were characterised as follows: a random assignment of the values was performed using a discrete distribution [RiskDiscrete ({0;1};% <LOQ; % >LOQ) for Italy, and Norway, to reflect the number of samples at or below the LOQ and the number of samples with quantified toxin concentrations. This means that the ratio of

assumed that all bivalve molluscs showing a negative response in MBA will reach the market and will thus be consumed. From this perspective, it is not unrealistic to estimate the dietary intake of YTXs based on the LC-MS/MS data for those samples that tested negative in the MBA.

Because insufficient information is available on the distribution of portion sizes, the CONTAM Panel decided to use a triangular distribution as a simple and pragmatic approach. A triangular distribution is characterised by three values, the minimum, the most probable and the maximum. In the case of shellfish consumption a value of 0 g was used as a minimum. From the range of 10 g to 136 g reported as mean consumption figures in Table 7 the Panel chose a value of 100 g to be used as "most probable" value, although there is no evidence that it is the most frequently consumed portion. The better-documented large portion size of 400 g (see chapter 7) was used to represent the maximum.

Due to differences in the occurrence data from Italy and Norway the evaluation of dietary exposure to YTXs was performed separately for these two datasets, taking also into account the different methods applied and different values for the reported LODs and LOQs (see Table 2).

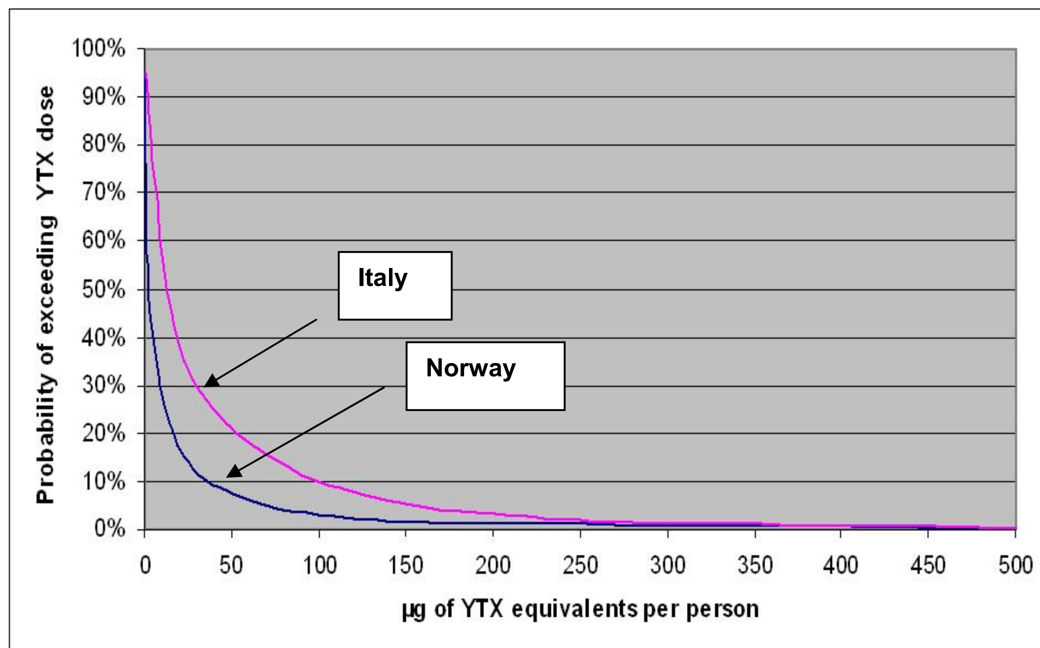

Figure 3: Probability of dietary exposure to YTX-group toxins resulting from consumption of a single portion of shellfish.

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non-quantified/quantified samples was adjusted to the different data. These latter data were simulated using a uniform distribution function [*RiskUniform (0;LOQ)*].

The resulting probabilistic dietary exposure distribution, illustrating the probability of exceeding a specific intake level of YTX equivalents when consuming a single portion of shellfish is presented in Figure 3. Due to differences in the available occurrence data the dietary exposure for Italy and Norway differs considerably. For Italy the probabilistic exposure distribution has a median of approximately 14 µg YTX equivalents per portion, a mean of approximately 40 µg YTX equivalents per portion, and a 95th percentile of 158 µg YTX equivalents per portion . The exposure distribution based on the Norwegian data has a median value of 1.9 µg YTX equivalents, a mean of 15.5 µg YTX equivalents, and a 95th percentile of approximately 88 µg of YTX equivalents per portion.

Based on the results of probabilistic dietary exposure distribution it is calculated that in Italy (North Adriatic Sea) the probability to exceed the deterministic dietary exposure of 400 µg YTX equivalents per person, corresponding to a consumption of a 400 g portion containing YTXs at the level of the current EU limit value, is 0.5%.

9. Toxicokinetics

The information on toxicokinetics of YTXs is scarce. However, in a kinetic experiment, known amounts of YTX were administered orally to mice. Urine samples were taken at hourly intervals, and the mice were killed after 6 hours and tissues were collected. Only trace amounts of YTX were found in blood, urine, and tissues. Most of the toxin was recovered from the lower intestine and faeces (Munday *et al*., 2008).

From an ongoing study in Norway (Aasen *et al*., 2008) NMRI female mice received YTX by gavage at doses of 1 and 5 mg/kg b.w., three mice per dose. Tissue samples taken at 24 hrs from stomach, small and large intestines, liver, kidney, lung, heart, thymus, spleen, brain and blood were examined for YTX by LC-MS/MS. Five mice received vehicle only, as control animals. At the highest YTX dose, the highest toxin levels were found in the ileum (242 µg/kg fresh weight), followed by colon (108 µg/kg fresh weight), jejunum (98 µg/kg fresh weight) and duodenum (74 µg/kg fresh weight). In the kidneys and spleen the levels were 41 µg/kg and 34 µg/kg, respectively. In the heart the level was only 9 µg/kg. The YTX level in the blood was estimated approximately at 8 ng/g (by ELISA). YTX in blood was almost non-detectable by LC-MS. At the YTX dose of 1 mg/kg, the levels detected were considerably lower, a maximum of about 58 µg/kg in the colon and 46 µg/kg in the ileum, and about 30 µg/kg in jejunum and duodenum. The levels in kidneys and spleen were about 10 µg/kg, while in the heart only 3 µg/kg was detected. The level of YTX in blood (by ELISA) was approximately 4 ng/g. When comparing results from LC-MS and ELISA in other organs, the figures from the latter were usually higher, especially at low levels. No YTX was found in the control animals.

In a recent study by Tubaro *et al*. (2008a), blood samples were taken from mice (n=3) 24 hours after the last gavage of YTX at 1 mg/kg/day for 7 consecutive days. The plasma concentration of YTX was analysed using direct ELISA. After correction for recovery, a

mean blood concentration of about 6 ng/mL, corresponding to 5 nM, was reported (Tubaro *et al*., 2008a).

No data on bio-transformation of YTX in experimental animals were identified.

10. Toxicity data

10.1 Mechanistic considerations

The mechanism of action of YTXs has not been determined with certainty, and the molecular processes underlying their toxicity are presently undetermined. The molecular activity of YTX has originally been investigated with reference to earlier observations that associated this class of toxins with OA group toxins, but the finding that YTX inhibits type 2A serine/threonine phosphoprotein phosphatase only at inhibitory concentrations (IC_{50} = 0.36 µg/mL)[13] that are four orders of magnitude higher than the effective doses of OA (Ogino *et al*., 1997), has provided the molecular basis to distinguish YTXs from OA and other dinophysis toxins (Ogino *et al*., 1997).

Four major molecular processes have been implicated in the mechanism of action of YTXs, comprising the modulation of calcium movements among different cellular compartments, the modulation of cellular cAMP levels, the alteration of protein disposal, and apoptosis. Very recently it was shown that YTX at 10^{-7}-10^{-6} molar concentrations inhibited beating frequency in cardiomyocytes *in vitro* (Dell'Ovo *et al*., 2008). Table 9 summarises the different molecular responses reported for YTX in different cell systems.

[13] The concentration of a substance that reduces the effect by 50%.

Table 9: Molecular responses induced by yessotoxin (YTX) in cultured cells.

Effects	Cell type and species	Concentration (M)	Time frame	Reference
Phosphodi-esterase activation	Human lymphocytes, rat mast cells, neurons	10^{-6}	seconds/minutes	Alfonso *et al.*, 2003, 2004, 2005; Pazos *et al.*, 2004, 2005, 2006
Modulation of calcium movements at several levels	Lymphocytes (human)	10^{-6}	seconds/minutes	De la Rosa *et al.*, 2001
	Immunocytes (mussel)	10^{-7}	minutes/hours	Malagoli *et al.*, 2006a
	MH1C1 (rat)	10^{-7}	seconds/minutes	Bianchi *et al.*, 2004
	Primary neuronal cells (rat)	10^{-8}	minutes/hours	Pérez-Gómez *et al.*, 2006
Calcium –dependent cAMP decrease	Lymphocytes (human)	10^{-6}	seconds/minutes	Alfonso *et al.*, 2003
Calcium –dependent cGMP decrease	Lymphocytes (human), rat mast cells	10^{-6}	seconds/minutes	Alfonso *et al.*, 2008
Altered protein disposal	MCF-7 (human) and MDCK (dog)	10^{-9}	hours	Pierotti *et al.*, 2003; Ronzitti *et al.*, 2004; Callegari and Rossini, 2008
	IPLB-LdFB (insect) and 3T3 (mouse)	10^{-8}	hours	Malagoli *et al.*, 2006b
Apoptosis and cell death	Glioma cells (rat)	10^{-6}	days	Ogino *et al.*, 1997
	HeLa S_3 (human)	10^{-10}	days	Malaguti and Rossini, 2001; Malaguti *et al.*, 2002
	BE(2)-M17 (human)	10^{-8}	days	Leira *et al.*, 2002
	Myoblasts (mouse and rat)	10^{-7}	days	Suárez Korsnes *et al.*, 2006a; 2006b; 2007
	Primary neuronal cells (rat)	10^{-8}	days	Pérez-Gómez *et al.*, 2006
	IPLB-LdFB (insect) and 3T3 (mouse)	10^{-8}	days	Malagoli *et al.*, 2006b
	Primary cultures cardiomyocytes (rat)	10^{-8}	days	Dell'Ovo *et al.*, 2008
Change in cell shape	Immunocytes (mussel)	10^{-7}	minutes/hours	Malagoli and Ottaviani, 2004

It has been shown that YTX (10^{-6} M) induces the influx of extracellular Ca^{2+} into lymphocytes, and that both L-type voltage-activated and depletion-activated calcium channels could be activated by YTX (De la Rosa *et al*., 2001). The effect of YTX on L-type voltage-activated calcium channels has been confirmed (Malagoli and Ottaviani, 2004; Malagoli *et al*., 2006a; Pérez-Gómez *et al*., 2006), but intracellular calcium does not represent the initial event leading to late cell death in YTX-treated cells (Pérez-Gómez *et al*., 2006; Dell'Ovo *et al*., 2008).

The permeability transition pore (PTP) is another component affected by YTX (Bianchi *et al*., 2004). YTX (10^{-7}-10^{-6} M) induces membrane depolarization in isolated rat liver mitochondria when micromolar calcium concentrations are present in the incubation buffer, indicating that the toxin could induce calcium uptake by mitochondria (Bianchi *et al*., 2004).

Incubation of human lymphocytes with YTX (10^{-7}-10^{-6} M) induced a decrease in cellular cAMP levels, an effect that could be due to an increase in 3',5'-cyclic nucleotide 5'-nucleotidohydrolase (PDE) activity in the cells. Indeed, Alfonso *et al*. (2003) reported that YTX increased cAMP metabolism. The fact that the effect of YTX on PDE is strictly calcium-dependent, suggests that changes in intracellular calcium could be involved in the response. It has also been reported that YTXs affect some other phosphodiesterases (Alfonso *et al*., 2004, 2005; Pazos *et al*., 2004, 2005, 2006). A change in the intracellular cAMP levels, however, was not found in primary cultures of cardiomyocytes, where 10 nM YTX induced cell death (Dell'Ovo *et al*., 2008).

The alteration of protein disposal is another molecular response induced by YTX. The toxin determines the accumulation of a fragment of the cell-cell adhesion protein E-cadherin in human breast cancer cells (Pierotti *et al*., 2003). The effect in epithelial cells is induced by very low YTX concentrations (10^{-10}-10^{-9} M) (Ronzitti *et al*., 2004). YTX does not enhance E-cadherin degradation *per se*, but interferes with its normal disposal, preventing endocytosis and complete degradation of the protein fragment produced after the initial proteolytic attacks (Callegari and Rossini, 2008). Altered cell adhesion is not detected in the first phases of YTX-induced inhibition of E-cadherin endocytosis and disposal (Ronzitti *et al*., 2004; Ronzitti and Rossini, 2008), but prolonged cell exposure to YTX results in the disruption of the E-cadherin-catenin system, altered cell-cell adhesion and inhibition of cell proliferation (Ronzitti *et al*., 2004). Such effects are also seen *in vivo* (see chapter 10.2).

Cytotoxicity and cell detachment from culture dishes were the most prominent effects originally observed upon treating cultured rat glioma cells with YTX (Ogino *et al*., 1997). The potent toxic effect of YTX has been later confirmed in other cellular systems (Malaguti and Rossini, 2001; Leira *et al*., 2002; Malaguti *et al*., 2002; Ronzitti *et al*., 2004), and evidence has been obtained that the toxin triggers cell death through apoptosis (Malaguti and Rossini, 2001; Leira *et al*., 2002; Malaguti *et al*., 2002; Malagoli *et al*., 2006b; Pérez-Gómez *et al*., 2006; Suárez Korsnes *et al*., 2006a, 2006b, 2007). Experimental data indicate that YTX activates the intrinsic pathway of apoptosis (Malaguti and Rossini, 2001; Leira *et al*., 2002;

Malaguti *et al*., 2002; Suárez Korsnes *et al*., 2006a, 2006b). Furthermore, it has been shown that YTX (10^{-7} -10^{-6} M) causes impairment in the beating activity as well as a reduction in the viability of primary cultures of rat cardiomyocytes, in a Ca^{2+}- and cAMP-independent way (Dell'Ovo *et al*., 2008).

Available knowledge and experimental data regarding the effective doses and time-frames of YTX action, as well as the different effects found in distinct cellular systems, have been explained in two ways. One hypothesis is that the low concentrations needed are due to down- or up-regulation of molecular components such as PDE, which in turn could lead to the observed long term effects (Alfonso and Alfonso, 2008). Other authors, however, proposed that YTX has two major mechanisms of action, involving high affinity ($K_D \approx 10^{-10}$ M) and low affinity ($K_D \approx 10^{-7}$ M) receptors, respectively (Ferrari *et al*., 2004; Rossini *et al*., 2006).

Further studies are needed to obtain a better understanding of the molecular mechanisms underlying the toxicity of YTXs.

10.2 Effects in laboratory animals

10.2.1 Acute toxicity

10.2.1.1 Toxicity following intraperitoneal (*i.p.*) administration

Terao *et al*. (1990) used male ICR mice (23-25 g) in a study of acute toxicity of YTX. Mice receiving *i.p.* injections of YTX at 300 µg/kg or above showed normal behaviour for the first few hours. Then, suddenly, dyspnoea set in and they died. According to Tubaro *et al*. (2003), CD-1 female mice treated with YTX are restless, and at lethal doses, dyspnoea and jumping were recorded before death. At very high doses, the mice died within 30-50 minutes after injection, while they survived for ten hours or more at doses close to the LD_{50} (512 µg/kg). Similar symptoms were described by Aune *et al*. (2002), using female NMRI mice, weighing 15-19 g. Doses used were 100, 250, 500, 750 and 1000 µg/kg (n=3). They noted shivering in mice injected the two highest doses of YTX, and just before death the mice had vigorous cramps and were jumping. No macroscopic changes are observed after lethal *i.p.* doses of YTX. Four out of five mice that died within 50-80 minutes from *i.p.* injections of YTX (2/3 at 750 µg/kg, and 3/3 at 1,000 µg/kg) showed vacuolation in the cardiac muscle, and intrecellular oedema, as examined by light microscopy (Aune *et al*., 2002). No pathological changes were seen in lung, thymus, liver, pancreas, kidney, adrenal gland, jejunum, colon and spleen.

By electron microscopy, Terao *et al*. (1990) observed severe cardiac damage in mice receiving YTX at 500 µg/kg *i.p.* Endothelial lining cells of the capillaries in the left ventricle were swollen and degenerated, and almost all cardiac muscle cells were swollen. The mitochondria became rounded and bundles of myofibrils and sarcoplasmic reticulum were separated. In a study of Aune *et al*. (2002), less severe ultrastructural changes in the heart of

mice given 1000 µg/kg of YTX *i.p.* were observed up to one hour after treatment: swelling myocardial muscle cells in the wall of the left ventricle and separation of myofibrils and mitochondria, the latter organelles being rounded in appearance. These changes were more pronounced in the vicinity of capillaries.

The apparent discrepancy in the severity of the response of the heart to YTX between the study of Terao *et al*. (1990) and Aune *et al*. (2002) is unclear. Differences in toxin purity and mice strain could be explanations.

According to Terao *et al*. (1990), no ultrastructural changes were seen in liver, pancreas, lungs, adrenals, kidneys, spleen or thymus.

Unlike YTX, di-desulfoYTX caused swelling and yellow colouring of the liver within hours of administration of toxic doses (Terao *et al*., 1990). The colouring of the livers was associated with increases in hepatic fat levels and severe fatty degeneration of the liver, with swollen mitochondria and increased phagocytic activity. Di-desulfoYTX had minimal effects on the heart, with only a slight deposition of fat in the cardiac muscle. It is interesting to note that desulfation of YTX resulted in fundamental changes in the target organ, but it is a question whether di-desulfoYTX should be included in the opinion at all since this compound has not been detected in algae or shellfish, but was produced via solvolysis of YTX. According to Ogino *et al*. (1997), the reason for studying di-desulfoYTX is the possible conversion of YTX to the desulfated analogue by intestinal microorganisms.

Since YTX provoked death showing symptoms from the nervous system (motor disco-ordination), Franchini and co-workers (2004a,b) studied the effect of a single dose of YTX (10 and 420 µg/kg b.w. given *i.p.*) on the central nervous system in Swiss CD1 mice. While the cerbral cortex was not affected at any of the doses, the higher dose caused damage to the Purkinje cells of the cerebellar cortex. Changes in intracellular calcium binding proteins and modification of the cytoskeletal components were observed. Furthermore, histopathological examination of the thymus revealed that both doses caused structural changes and apoptosis particularly in the cortico-medullar junction resulting in a reduced population of mature thymocytes. Mitoses were noted particularly in the cortex. In the duodenum the highest dose caused infiltration of blood cells and lymphocytes particularly in the Payer's patches showed signs of apoptosis. Cytokine responses in both duodenum and thymus were found at both doses.

Contrary to the findings by Franchini *et al*. (2004b), neither Aune *et al*. (2002) exposing female NMRI mice up to 1000 µg/kg b.w. *i.p.* of YTX nor Terao *et al*. (1990) exposing mice at 500 µg/kg b.w. *i.p.* observed any structural or ultra structural (Terao *et al*., 1990) changes in the thymus. These discrepancies may be due to different purities of the toxins and the use of different strains of mice.

Data on *i.p.* toxicity of YTX-group toxins were recently summarised by Munday *et al*. (2008), as shown in Table 10.

Table 10: Acute toxicity of YTX-group toxins by intraperitoneal injection in mice.

Compound	Mouse strain	Mouse sex	Parameter	µg/kg body weight	Reference
Yessotoxin	ddY	Male	LD_{50}	Between 80 and 100	Ogino *et al.*, 1997
Yessotoxin	NMRI	Female	LD_{50}	Between 500 and 750	Aune *et al.*, 2002
Yessotoxin	NMRI	Female	LD_{50}	314	Aune *et al.*, 2008
Yessotoxin	NMRI	Male	LD_{50}	412	Aune *et al.*, 2008
Yessotoxin	ICR (CD-1)	Female	LD_{50}	380	Aune *et al.*, 2008
Yessotoxin	ICR (CD-1)	Male	LD_{50}	462	Aune *et al.*, 2008
Yessotoxin	Swiss (CFW-1)	Female	LD_{50}	269	Aune *et al.*, 2008
Yessotoxin	Swiss (CFW-1)	Male	LD_{50}	328	Aune *et al.*, 2008
Yessotoxin	CD-1	Female	LD_{50}	512 (312-618)*	Tubaro *et al.*, 2003
Yessotoxin	ICR	Male	LD_{50}	300**	Terao *et al.*, 1990
Yessotoxin	Swiss albino	Female	LD_{50}	112 (96-131)*	Munday *et al.*, unpublished results
Yessotoxin	C57 Black	Female	LD_{50}	136 (112-166)*	Munday *et al.*, unpublished results
1a-HomoYTX	CD-1	Female	LD_{50}	444 (315-830)*	Tubaro *et al.*, 2003
1a-HomoYTX	not reported	not reported	Lethal dose	100	Satake *et al.*, 1997
45-HydroxyYTX	not reported	not reported	Lethal dose	~ 500	Satake *et al.*, 1997
45-Hydroxy-1a-homoYTX	CD-1	Female	Lethal dose	No deaths at 750	Tubaro *et al.*, 2003
55-CarboxyYTX	not reported	not reported	Lethal dose	~ 500	Ciminiello *et al.*, 2000a
55-Carboxy-1a-homoYTX	not reported	not reported	Lethal dose	~ 500	Ciminiello *et al.*, 2000b
45,46,47-TrinorYTX	not reported	not reported	Lethal dose	~ 220	Satake *et al.*, 1996
Di-desulfoYTX	ICR	Male	LD_{50}	301	Terao *et al.*, 1990
1-DesulfoYTX	not reported	not reported	Lethal dose	~ 500	Daiguji *et al.*, 1998
1,3-Enone isomer of heptanor-41-oxoYTX	Swiss albino	Female	Lethal dose	No deaths at 5,000	Miles *et al.*, 2004
Trihydroxylated amide of 9-methyl-41-a-homoYTX	Swiss albino	Female	Lethal dose	No deaths at 5,000	Miles *et al.*, 2005

LD_{50}= lethal dose – the dose required to kill half the members of a tested animal population
* Figures in brackets indicate 95% confidence limits.
** This figure is the LD_{50} at 3 hours after dosing. Since deaths from YTX intoxication occur at times greater than 3 hours, the true LD_{50} in this experiment is likely to lower than that indicated.

As can be seen from Table 10, the reported LD_{50} values for YTX vary considerably, from approximately 100 to between 500 and 750 µg/kg. These disparities could not be explained by use of different strains of mice only as comparisons between different strains in the same experiment only showed a variation of 1.4 fold between the least and most susceptible strain (Aune *et al*., 2008). When comparing males and females of three different strains, female mice were more susceptible than males, see Table 10 (Aune *et al*., 2008). In this experiment with three strains and both sexes, the average LD_{50} for females was 321 µg/kg, and for the males 400 µg/kg, and a total average for both sexes at 360 µg/kg. The stability of YTX and the storage conditions for the toxin used in different studies may play a more important role for the variability of the measured LD_{50} values (Aune *et al*., 2008). Recent studies indicate that YTX is unstable during long-term storage (> 6 months) in the dry state (Loader *et al*., 2007).

With the differences in reported LD_{50} of YTX, establishment of TEFs of the analogues is difficult. Also, toxicities for these have been given as “lethal dose” rather than LD_{50} in many instances. However, from Table 10, it appears that 1a-homoYTX and di-desulfoYTX are approximately as toxic as YTX, based on the given LD_{50} results reported. For analogues with information on lethal dose only, crude estimates indicate that the acute *i.p.* toxicity of 45,46,47-trinorYTX is close to that of YTX, while carboxy- and 45-hydroxyYTX appears to be slightly less toxic.

10.2.1.2 Toxicity following oral administration

In several studies no deaths were recorded in mice given YTX by gavage at 0.5 mg/kg b.w. (Terao *et al*., 1990), 1 mg/kg b.w. (Ogino *et al*., 1997), 2 mg/kg b.w. (Tubaro *et al*., 2003), 10 mg/kg b.w. (Aune *et al*., 2002). No clinical signs were observed in mice following a single oral dose of 50 mg YTX/kg b.w. (unpublished results, Munday *et al*., 2008), however, no histopathology or clinical biochemistry was done.

In a study by Aune *et al*. (2002), two experiments were undertaken, using female NMRI mice (15-19 g). In the first experiment, *i.p.* toxicity of YTX in doses between 0.1 and 1.0 mg/kg (reported in detail above) was compared with oral toxicity of YTX 1.0, 2.5, 5.0, 7.5 or 10.0 mg/kg (n=3 per group) as a single dose by gavage. One mouse per dose was sacrificed after 5 hours, the others after 24 hours. No mice died from oral exposure, even at 10 mg/kg YTX. Ten different organs were studied by light microscopy. Slight oedema was only seen in the myocardium at the two highest oral doses (7.5 and 10 mg/kg) and in one of the control mice. The changes were similar in mice given 10 mg/kg b.w. *p.o* and 1 mg/kg b.w. *i.p*. In the second experiment, mice received YTX by gavage at 2.5, 5 or 10 mg/kg (n=2). Animals surviving the treatment were killed after 1 hour. Tissues were taken from the heart and six other organs and examined by light microscopy. In addition, the myocardium was examined by electron microscopy. Results from light microscopy indicated no effects in any organs except the myocardium. By electron microscopy only slight changes in muscle cells close to the capillaries

were observed and most of the myocardium was unchanged at a dose of 2.5 mg/kg. At 5 mg/kg, moderate swelling of myocardial cells was seen near capillaries with protrusions into the capillary space. At the highest dose (10 mg/kg), swelling of myocardial cells and separation of organelles was observed, most pronounced close to the capillaries

Tubaro *et al*. (2003) studied the effects of YTX given by gavage to female CD-1 mice (18-20 g). Groups of five mice were given 1 and 2 mg/kg of YTX and observed for 24 hours. No signs of toxicity were observed in the mice, and no macroscopic changes were seen in major organs. By histopathological examination of the same organs using light microscopy, no morphological changes were seen at any of the doses. However, by using electron microscopy, analysis of the heart from mice given both doses showed presence of some alterations of myocardiocytes adjacent to capillaries. In particular, cytoplasmic protrusions of cardiac muscle cells into the capillary space were seen, along with rounding of mitochondria and packing and alterations of muscle fibres. However, the authors noted that no changes were observed in plasma lactate dehydrogenase (LDH) or creatinine phosphokinase (CK) indeces of cardiac tissue damage. According to Tubaro *et al*. (2003), no clinical signs of toxicity were observed in mice given 1 mg/kg of 1a-homoYTX or 45-hydroxyYTX, however, cytoplasmic protrusions of myocardiocytes, rounding of mitochondria and fibre modifications were reported.

In a follow up study (Tubaro *et al*., 2004), female CD-1 mice (18-20 g, n=5) were administered YTX by gavage at 2 mg/kg/day, or 1a-homoYTX or 45-hydroxy-1a-homoYTX at 1 mg/kg/day for seven days. Twenty-four hours after the last treatment, the mice were killed and submitted to necroscopic examinations. Liver, heart, lungs, kidney, spleen, stomach, duodenum, jejunum, colon, rectum, pancreas, thymus, uterus, ovaries, skeletal muscle, brain and spinal cord were sampled for histolopathological analysis by light microscopy. The heart tissue was also studied by electron microscopy. No significant differences in growth rate between treated mice and controls were observed. The necroscopic analysis and light microscopy examinations did not show any treatment-associated changes in the main organs. Electron microscopy of the heart revealed some changes in the myocardial muscle cells near capillaries, such as package of swollen mitochondria and alterations of cell boundary in mice treated with 2 mg/kg/day YTX. Similar modifications were observed in mice treated with 1a-homoYTX and 45-hydroxy-1a-homoYTX at 1 mg/kg/day. No apoptotic deoxyribonucleic acid (DNA) fragmentation was observed as a result of repeated oral exposure to YTX and the two analogues. The plasma levels of LDH and CK which are indicators of myocardial necrosis were not elevated. Neither was any apoptotic DNA fragmentation of heart muscle cells observed. According to the authors, it was noteworthy that the changes were limited to cells near the capillaries and that other parts of the myocardium were not affected. The effects observed were not more pronounced than those observed following a single administration at the same dose level.

In another study (Espenes *et al*., 2006), NMRI female mice (14 g b.w. at start) were exposed by gavage for YTX 7-times during 21 days, each dose was 1, 2.5 or 5 mg/kg (n=3). Five controls received vehicle at the same days. The mice were killed three days after the last treatment. The

heart, lung, liver, kidney, small intestine, spleen, thymus, pancreas, brain, testes and adrenals were examined by light microscopy. In addition, the myocardium was also studied by electron microscopy. No clinical symptoms were observed in any of the groups exposed to YTX. There were no differences in body weight gain between YTX-exposed mice and controls. By light microscopy, no pathological effects were observed. However, by electron microscopy, some vacuoles of uncertain significance were seen in the myocardium in mice at the highest dose of YTX (5 mg/kg, seven times). Compared with previous studies by the same authors (Aune *et al*., 2002), fewer morphological changes were seen in this study. The authors speculated whether this may be due to repair mechanisms as the changes in that study were observed up to one hour after exposure, whereas the time between treatment and examination was 3 days.

In a recent study by Tubaro *et al*. (2008a), groups of three mice (female CD-1, 18-20 g), were treated daily for seven days with YTX (1 mg/kg/day) or vehicle by gavage. Clinical signs, food consumption and body weight were recorded at 24 hours, 30 days and 90 days after the last treatment. At each time, three mice in each group were killed and blood samples and samples from the main organs were taken for histological analyses. In particular, no histopathological changes were observed in the thymus. Heart, liver, kidneys and cerebellum were also sampled for transmission electron microscopy. No mortality or other treatment-related changes, including histological or hematoclinical parameters were observed in the YTX treated mice. No ultrastructural alterations were seen in the liver, kidneys or cerebellum. However, changes in cardiac muscle cells near the capillaries (clusters of rounded mitochondria and disorganization of myofibrils) were observed 24 hours after the last treatment. These changes were also noted 30 days after treatment, but less evident compared with those seen at 24 hours. No difference in cardiac muscle cells between controls and YTX-treated mice were seen after 90 days. This indicates recovery of the ultrastructural alterations.

Tubaro and co-workers (2008b) also studied possible ultrastructural effects of YTX on skeletal muscle following oral exposure of female CD 1 mice to 1 and 2 mg/kg b.w. of YTX daily for seven days. No changes, including similar changes to those previously seen on cardiac muscle cells, were seen following YTX exposure.

Repeated oral administration of YTX to mice (1 mg/kg/day, for 7 days) was found to stabilize E-cadherin (Callegari *et al*., 2006), an effect that could be the result of the inhibition of endocytosis and degradation of E-cadherin by YTX (Callegari and Rossini, 2008). On the basis of these findings, it can be concluded that YTX alters the disposal of E-cadherin both *in vitro* and *in vivo*.

10.3 Relative potency of YTX-group toxins

There is no information available on LD_{50} of YTX-group toxins via the oral route. The majority of oral toxicity studies are done with YTX, and a few with 1a-homoYTX and 45-hydroxyYTX. In neither case have the analogues resulted in lethal effects, due to low oral toxicity. Like for

other marine biotoxins, there is more information on acute *i.p.* toxicity of YTX and several analogues. With the differences in reported LD_{50} of YTX following *i.p.* administration, establishment of TEFs of the analogues is difficult. Also, toxicities for these have been given as "lethal dose" rather than LD_{50} in many instances. It appears that 1a-homoYTX and di-desulfoYTX are approximately as toxic as YTX (Table 10). For analogues with information on lethal dose only, crude estimates indicate that the acute *i.p.* toxicity of 45,46,47-trinorYTX is close to that of YTX, while carboxy- and 45-hydroxyYTX appears to be slightly less toxic. Hence, with the exception of 45-hydroxy-1a-homoYTX it is concluded that the data available are insufficient to distinguish between YTX and its analogues with respect to *i.p.* lethality LD_{50} values and hence also to establish TEFs/relative potencies for YTX-group toxins. For 45-hydroxy-1a-homoYTX, which showed no lethality up to 750 µg/kg b.w. *i.p.*, a TEF of 0.5 is assigned. The most commonly occurring analogues have therefore been assigned the following TEFs:

YTX	1
1a-homoYTX	1
45-hydroxyYTX	1
45-hydroxy-1a-homoYTX	0.5

Relative potencies have also been studied in *in vitro* systems and the results indicate that the proposed TEFs do not underestimate the toxicity of the tested analogues (Ferrari *et al.*, 2004; Pazos *et al.*, 2005).

10.4 Chronic toxicity and carcinogenicity

No long term *in vivo* studies on YTX has been identified.

10.5 Genotoxicity

No data are available.

11. Observations in humans

There are no reports of human illness associated with YTXs.

12. Hazard characterisation

There are no long term *in vivo* toxicity studies of YTX and hence no data are available for the establishment of a tolerably daily intake (TDI). There is no data related to toxicity in humans. In

view of the potential for acute toxicity of YTX-group toxins, the CONTAM Panel decided to establish an acute reference dose (ARfD) on the basis of acute oral toxicity in mice.

YTX toxicity following oral administration was examined in a series of studies (Aune *et al*., 2002; Tubaro *et al*., 2003, 2004, 2008a; Espenes *et al*., 2006). In these studies YTX was administered by gavage in single doses up to 10 mg/kg b.w. or repeated doses up to 5 mg/kg b.w. given 7 times. Since no lethality and no clinical symptoms were observed at doses that were far above those being lethal by *i.p.* administration, it is evident that YTX is far less toxic when given by the oral route.

Following *i.p.* or *p.o.* administration, the heart has been identified as a primary target organ. By the use of light microscopy slight oedema in the myocardium was observed down to a single oral dose of 7.5 mg/kg b.w. with a no-observed-effect level (NOEL) of 5 mg/kg b.w. (Aune *et al*., 2002). In the same study by the use of electron microscopy moderate changes in myocardial muscle cells close to capillaries were already seen at 5 mg/kg b.w. with only marginal changes at 2.5 mg/kg b.w. In another study by the same group (Espenes *et al*., 2006) YTX were given in 7 repeated oral doses (1, 2.5 and 5 mg/kg b.w.) during 21 days followed by autopsy three days after the last dosing. Except for some vacuoles of uncertain significance at the highest dose, no significant changes in the heart, even in areas around capillaries were observed by electron microscopy.

In a series of studies by Tubaro *et al*. (2003, 2004, 2008a), ultrastructural changes in the myocardial muscle cells close to capillaries were observed down to single oral doses of 1 mg/kg b.w. of YTX or 1a-homoYTX and 45-hydroxyYTX. Similar changes were also observed after 7 repeated doses of 1 and 2 mg/kg b.w., respectively, in two independent studies (Tubaro *et al*. 2004, 2008a). In the latter study the mice were followed for 30 and 90 days after the last dosing and the changes were less pronounced after 30 days and were absent after 90 days, indicating that these changes were reversible. It should be noted that at doses of 2 mg/kg b.w. of YTX and below no changes were observed by light microscopy and there were no changes in plasma levels of LDH or CK, indicators of heart muscle damage. Neither was apoptosis observed in the heart muscle cells.

The small and reversible ultra-structural changes in the heart muscle appear to be treatment related, but at the lowest oral doses (1-2 mg/kg b.w.) they are only inconsistently observed by different authors. These studies have shortcomings i.e. in the reporting, and there is no information on whether the histopathological examination by light microscopy and in particular by electron microscopy was carried out in a blinded way with respect to treatment. Neither was it systematically described how the samples from the heart of each mice were examined in order to establish significant differences between the various treatment groups, (e.g. quantitative measurements of mitochondria in sections from treated and control animals). The clinical and toxicological significance of these findings are uncertain. However, cardiotoxicity of YTX at low

doses is supported by *in vitro* studies on heart muscle cells where short incubations with YTX inhibited beating activity (10^{-7}-10^{-6} M) and cell viability (10^{-8} M) (Dell'Ovo *et al*., 2008).

The Panel noted that the ultrastructural changes induced by YTX apparently are reversible and were not accompanied by any leakage of enzymes to serum, indicative of cardio-myocyte necrosis, or any apoptotic activity. Furthermore, no such myocardial changes were observed in mice orally exposed 7 times with intervals of 3 days at doses up to 5 mg/kg b.w. of YTX when electron microscopy examination was done 3 days after the last dose.

In its derivation of an ARfD the CONTAM Panel decided to use the dose of 5 mg/kg b.w. as the most robust NOAEL for acute cardiotoxicity caused by YTXs as identified by light microscopy. Considering the ultrastructural changes in the myocardium that have inconsistently been reported below this dose level, the CONTAM Panel noted that these changes may be reversible, and that they were not accompanied by leakage of enzymes to serum. There were no indications of myocardial damage as identified by light microscopy. However, because it is uncertain whether the ultrastructural changes should be considered as adverse or not, the CONTAM Panel decided to apply a factor of 2 in addition to the default uncertainty factor of 100 to establish an ARfD of 25 µg YTX equivalents/kg b.w.

13. Risk characterisation

Because YTX-group toxins have potential for acute toxic effects, the Panel concluded that the identification of a high portion size rather than a long term average consumption is of importance to assess the health risk of the consumers. It considered the 95th percentile as a realistic estimate of the portion size for high consumers, and chose the figure of 400 g to be used in acute exposure assessments.

As was shown in Table 7 consumption of a 400 g portion of shellfish meat containing YTX-group toxins at the current EU limit of 1 mg YTX eq./kg shellfish meat would result in an intake of 400 µg toxin (equivalent to 6.7 µg/kg b.w. in a 60 kg adult). This intake is below the ARfD of 25 µg YTX eq./kg b.w. (equivalent to 1500 µg YTX eq. per portion for a 60 kg adult) and consequently does not pose any health risk.

As explained in chapter 6 the Panel assumed that all shellfish samples showing a negative response in MBAs will reach the market and will thus be consumed. Therefore, the concentration data derived by LC-MS/MS for these samples (Table 6) could be used to estimate the dietary intake of YTX-group toxins.

Based on the Norwegian occurrence data consumption of a 400 g portion of shellfish meat containing YTX-group toxins at 315 µg YTX eq./kg shellfish meat corresponding to the 95th percentile of the concentration (see Table 6) would result in an intake of 125 µg YTX-group toxins (equivalent to 2.1 µg/kg b.w. in a 60 kg adult). This intake is well below the ARfD of 25

μg YTX eq./kg b.w. The same is true for the specific situation in Italy (North Adriatic sea) where consumption of a 400 g portion of shellfish containing the 95th percentile of the concentration YTX-group toxins (799 μg YTX eq./kg shellfish meat) leads to intake of 5.3 μg/kg b.w. This indicates that there is no acute health risk with respect to consumption of shellfish containing the current levels of YTX-group toxins found on the market.

The LC-MS/MS data (Table 6) show that none of the samples from the Norwegian and Italian data set that tested negative in the MBA, exceeded a value of 3.75 mg YTX eq./kg shellfish meat. Therefore the CONTAM Panel concluded that a 60 kg person, consuming a portion of 400 g of shellfish currently present on the market, would not exceed the ARfD of 25 μg/kg b.w.

The CONTAM Panel noted that, even taking into consideration all reported YTX occurrence data (MBA negative and MBA positive results) and thereby disregarding the current EU regulatory system, consumers of shellfish in Norway would not exceed the ARfD when consuming a 400 g portion. In Italy (North Adriatic Sea), the ARfD would be exceeded under these circumstances by 2.9% of the consumers.

The CONTAM Panel concluded that in order for a 60 kg adult to avoid exceeding a dose of 1500 μg YTX equivalents, corresponding to the ARfD of 25 μg YTX equivalents/kg b.w., a 400 g portion should not contain more than 3.75 mg YTX eq./kg shellfish meat. This level is above the current EU limit value of YTXs of 1 mg/kg shellfish flesh.

14. Uncertainty

The evaluation of the inherent uncertainties in the assessment of exposure to YTX-group toxins has been performed following the guidance of the Opinion of the Scientific Committee related to Uncertainties in Dietary Exposure Assessment (EFSA, 2006). In addition, the draft report on "Characterizing and Communicating Uncertainty in Exposure Assessment" which is in preparation to be published as WHO/IPCS monograph, has been considered (WHO/IPCS, 2007). According to the guidance provided by the EFSA opinion (2006) the following sources of uncertainties have been considered: Assessment objectives, exposure scenario, exposure model, and model input (parameters).

14.1 Assessment objectives

The objectives of the assessment were clearly specified in the terms of reference and the Panel prepared a risk assessment including the derivation of an ARfD, description of the different analytical methods, and an exposure assessment for the current situation. The uncertainty of the assessment objectives is considered to be negligible.

14.2 Exposure scenario

The estimate of exposure is based on measurements from only two European Countries (Norway and Italy (North Adriatic Sea)). The exposure scenario may therefore lead to overestimation of exposure when extrapolating these data to the whole European population.

Uncertainty possibly introduced by non-consideration of cooking for quantitative exposure assessment is considered to be negligible as an impact for final conclusions, because these toxins are heat stable. As the majority of the occurrence data are derived from raw shellfish and cooking leads to increased concentrations, the exposure might be somewhat underestimated.

14.3 Exposure model

The high numbers of samples having levels below LOD may introduce uncertainties to the overall estimate. However, the uncertainties regarding values below the LOD are considered to be negligible, as they do not have a major influence on the risk characterisation.

Uncertainty also arises from the fact that exposure estimates were based on occurrence data from pre-market control samples. These samples may not reflect the “real” range of occurrence of YTXs in the shellfish on the market, but may result in an overestimation of the exposure.

14.4 Model input (parameters)

Parameter uncertainty is due to using occurrence results “only” from two member states, one of which is considered to be a worst case. Although analytical methodology is assumed to deliver comparable results, appropriate calibration standards for YTX were not always available. The data were produced with non-certified calibration standards which may not be appropriate for quantification. Uncertainties regarding the analytical methodology of YTX have been considered in the opinion by taking into account the data from Norway and Italy, having major differences in LOD and LOQ.

TEFs have been used to convert the concentrations of the YTX-group toxins into YTX equivalents. As pointed out in chapter 10.3, these TEFs are based on limited *i.p.* toxicity data. However, due to the low exposure the model input parameters do not lead to major changes in the overall results.

14.5 Summary of uncertainties

In Table 11 a summary of the uncertainty evaluation is presented, highlighting the main sources of uncertainty and indicating an estimate of whether the respective source of uncertainty might have led to an over- or underestimation of the exposure or the resulting risk.

Table 11: Summary of qualitative evaluation of the impact of uncertainties on the risk assessment of the dietary exposure of YTX-group toxins.

Sources of uncertainty	Direction and magnitude
Uncertainty in analytical results	+/- [a)]
Extrapolation of occurrence data from two European Countries to whole Europe	+
Incomplete database for shellfish consumption in Europe; data only from limited number of Member States and limited data on shellfish species other than mussels	+
Influence of samples below the LOD on deterministic and probabilistic exposure estimate	+/-
Consideration of shellfish sampled for pre-market control for systematic dietary estimation of exposure	+
Influence of cooking and processing	-
Use of TEFs for estimating YTX equivalents	+
Limitation in the database for establishing the ARfD	+

a) +, ++, +++ = uncertainty with potential to cause small, medium or large over-estimation of exposure/risk
-, --, --- = uncertainty with potential to cause small, medium or large under-estimation of exposure/risk (EFSA, 2006).

The CONTAM Panel considered the impact of the uncertainties on the risk assessment of exposure to YTX-group toxins from shellfish consumption and concluded that due to the low risk involved in YTX exposure, there is minimum impact of uncertainties on the outcome of the risk assessment.

CONCLUSIONS

Hazard identification and characterisation

- Yessotoxin group toxins (YTXs) are primarily produced by the marine dinoflagellate *Protoceratium reticulatum*. They are polyether compounds, consisting of 11 contiguously transfused ether rings, an unsaturated side chain, and two sulphate esters. More than 90 YTXs are known, but only a few dozens have been fully identified. The most important YTX-group toxins are YTX, 1a-homoYTX, 45-hydroxyYTX, and 45-hydroxy-1a-homoYTX.

- The primary target organ for YTX toxicity in mice appears to be the heart, both after intraperitoneal (*i.p.*) and oral acute exposure of mice. Lethality has been shown following *i.p.*, but not after oral exposure.

- The data on *i.p.* lethality of YTX and its analogues 45-hydroxyYTX and 1a-homoYTX did not allow any distinction between their toxic potencies, and therefore they should have equal weight when summing up total YTX equivalents (YTX eq.). The toxicity of 45-hydroxy-1a-homoYTX is less, and an interim toxic equivalence factor (TEF) factor of 0.5 was used by the Panel on Contaminants in the Food Chain (CONTAM Panel).

- There are no reports on adverse effects in humans associated with YTXs.

- There are no studies on chronic effects of YTX in animals and therefore no tolerable daily intake (TDI) can be established.

- Although the oral toxicity is not well defined, the CONTAM Panel considered it appropriate to set an acute reference dose (ARfD) on the basis of cardiotoxicity in order to facilitate the risk assessment of acute exposure to YTXs.

- In its derivation of an ARfD the CONTAM Panel decided to use the dose of 5 mg/kg b.w. *p.o.* as the most robust no-observed-adverse-effect level (NOAEL) for acute cardiotoxicity caused by YTXs as identified by light microscopy. The CONTAM Panel noted that ultrastructural changes in the myocardium have inconsistently been reported below this dose level. It also noted that these changes may be reversible, and that they were not accompanied by leakage of enzymes to serum. There were no indications of myocardial damage as identified by light microscopy. However, because it is uncertain whether the ultrastructural changes should be considered as adverse or not, the CONTAM Panel decided to apply a factor of 2 in addition to the default uncertainty factor of 100 to establish an ARfD of 25 μg YTX equivalents/kg b.w.

Occurrence/Exposure

- Occurrence data on YTXs were only available from a limited number of countries.

- Levels determined by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) in samples that tested negative in the mouse bioassay (MBA) have been used for exposure assessment.

- Consumption data for shellfish are only available for a few Member States. These data do not always distinguish between shellfish species nor the type of processing. In addition, different study designs were used in the collection of the consumption data.

- From the available data, the CONTAM Panel identified the figure of 400 g as high portion size to be used in acute exposure assessments

Risk characterisation

- Consumption of a 400 g portion of shellfish meat containing YTXs at the current European Union (EU) limit of 1 mg YTX eq./kg shellfish meat would result in an intake of 400 µg toxin (equivalent to 6.7 µg/kg b.w. in a 60 kg adult). This intake is below the ARfD of 25 µg YTX eq./kg b.w (equivalent to 1500 µg YTX eq. per portion for a 60 kg adult) and consequently does not pose any health risk.

- Consumption of a 400 g portion of shellfish meat containing YTXs at 315 or 799 µg YTX eq./kg shellfish meat (corresponding to the 95th percentile of the concentration in the Norwegian and Italian data set that tested negative in the MBA, respectively) would result in an intake of 126 or 320 µg YTXs (corresponding to 2.1 or 5.3 µg YTX eq. /kg b.w. for a 60 kg adult, respectively). For both countries this intake is below the ARfD of 25 µg YTX eq./kg b.w., indicating that there is no health risk.

- The LC-MS/MS results show that none of the samples from the Norwegian and Italian data set that tested negative in the MBA, exceeded a value of 3.75 mg YTX eq./kg shellfish meat. Therefore the CONTAM Panel concluded that a 60 kg person, consuming a portion of 400 g of shellfish currently present on the market, would not exceed the ARfD of 25 µg/kg b.w.

- Taking into consideration all reported YTX occurrence data, thus both the MBA negative and MBA positive results, and thereby disregarding the current EU regulatory system, consumers of shellfish in Norway would not exceed the ARfD when consuming a 400 g portion. In Italy (North Adriatic Sea), the ARfD would be exceeded under these circumstances by 2.9% of the consumers.

- The CONTAM Panel concluded that in order for a 60 kg adult to avoid exceeding a dose of 1500 µg YTX equivalents, corresponding to the ARfD of 25 µg YTX equivalents/kg b.w., a 400 g portion of shellfish should not contain more than 3.75 mg YTX eq./kg shellfish meat. This level is above the current EU limit value of YTXs of 1 mg/kg shellfish flesh.

Methods of analysis

- The MBA is the officially prescribed reference method in the EU for the determination of YTXs. The method has shortcomings, e.g. it is not specific, not quantitative and has a high uncertainty at the level of the current regulatory limit.

- LC-MS/MS methods have the greatest potential to replace the MBA. The LC-MS/MS methods also have the possibility for multi-toxin group detection/quantification.

- Neither the MBA, nor the (bio)chemical alternative methods have been formally validated in interlaboratory studies, following recognized protocols.

RECOMMENDATIONS (INCL. KNOWLEDGE/DATA GAPS)

Hazard identification and characterisation

- There is a need for clarification of the molecular mechanism of action of YTX and its analogues and the toxicological significance of the ultrastructural changes seen in the heart.
- Further information on the oral toxicity and relative potency of individual YTX-group toxins is needed.
- Information is needed on the oral toxicity of YTXs when combined with other lipophilic toxins that often co-occur in contaminated shellfish, such as okadaic acid (OA), azaspiracids (AZAs) and pectenotoxins (PTXs).

Occurrence/Exposure

- There is a need for more detailed data on shellfish consumption including data on portion size, frequency, individual shellfish species and processing of shellfish

Methods of analysis

- Certified standards for relevant individual YTXs and certified tissue reference materials with relevant compositions and levels of YTXs are required.
- It should be investigated if reference methods can be based on performance criteria, thereby allowing the use of several methods rather than a single specific method. The feasibility of the single laboratory validation concepts should be further explored, but validation by interlaboratory trials should be the long-term objective.

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