Analysis of Marine Biotoxins

Approaches to Validation and Regulatory Acceptance of Alternative Methods to the Mouse Bioassay as method of Reference

Position Paper Nr. 013/2005 of the BfR of April 07, 2005

Contents

1. Objective of the position paper – Summary of objections to the mouse bioassay as a method of reference for the analysis of marine biotoxins

Germany holds the opinion that suitable physicochemical methods for the detection of marine biotoxins are available, which assure consumer protection and which, when applied, will dispense with animal experiments being used as the reference method. These comprise scientific methods which have been validated in compliance with international stipulations, e.g. a number of methods have been published as standards by the European Committee for Standardization (CEN).

In German government laboratories, no animal experiments have been carried out for the purposes of detecting marine biotoxins since the late 1980s. Routinely, only in vitro testing methods have been used; so far, without compromising consumer health.

This situation complies both with the high standard set for consumer protection in Europe and with the requirements of the European Directive on the protection of animals used for experimental and other scientific purposes as well as the German Animal Welfare Act.[1] [2]

Germany's position was again criticised by the EU-Commission on occasion of the inspection tour by the Food and Veterinary Office of the European Commission (FVO) in February, 2002. Germany was summoned to employ the biological testing methods stipulated by EU legislation.[3] In its commentary to the FVO report Germany explained its position in detail.[4]

[1] Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provision of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/WG) (Official Journal of the European Communities No. L358 of 18 December 1986).

[2] Tierschutzgesetz in der Bekanntmachung der Neufassung vom 25. Mai 1998 (BGBL I S. 1105) - Animal Welfare Act of 25 May 1998 (German Federal Legal Gazette I p. 1105).

[3] Bericht über einen Kontrollbesuch des Lebensmittel- und Veterinäramtes in Deutschland, 18.- 28. Februar 2002, Bewertung der Durchführung der Richtlinie 91/492/EWG des Rates (lebende Muscheln) und Richtlinie 91/493/EWG des Rates (Fischereierzeugnisse). http://europa.eu.int/comm/food/fs/inspections/vi/reports/germany/vi_rep_germ_8588-2002sum_de.pdf
Report Extract in Respect of a Food and Veterinary Office Mission to Germany from 18 to 28 February 2002 regarding the Implementation of Council Directive 91/492/EEC (Live Bivalve Molluscs) and Council Directive 91/493/EEC (Fishery Products). http://europa.eu.int/comm/food/fs/inspections/vi/reports/germany/vi_rep_germ_8588-2002sum_en.pdf

Germany made particular reference to Article 7 Paragraph (2) of European Animal Protection Legislation (Council Directive 86/609/EEC). This article stipulates that within the European Union animal experiments are not to be carried out if a scientifically satisfactory, justifiable and practical alternative, which does not require the use of animals, is available. The text of the explanation says: ”As....alternative and adequately validated testing procedures are available, it is not necessary – also on the basis of the legal requirements of EU Commission Decision 2002/225/EC of 15 March, 2002, with its instructions for implementation of Council Directive 91/492/EEC - to conduct the Mouse Bioassay”.

In preparation for the FVO inspection scheduled for the year 2004, a meeting of experts was held on 19 May, 2004, at the German Federal Institute for Risk Assessment in Berlin to discuss the activities of the national reference laboratories for shellfish toxins and control of bacterial and viral contamination of shellfish. The representatives of the test laboratories of the German States again pointed out the deficiencies of the Mouse Bioassay and referred to the use of in vitro testing methods to routinely determine the content of marine algal toxins in shellfish and shellfish products. In the course of the discussion it was established that despite the lack of certified standard substances for several marine algal toxins, the physico-chemical analytical procedures have proved to be superior to the Mouse-Bioassay and to represent the more appropriate methods for safeguarding consumer protection. Nevertheless, the food safety monitoring authority stressed the urgent need for action in order to provide the option to conduct the Mouse Bioassay in borderline cases, preferably, in the National Reference Laboratory.

2. Overview of current legal regulations in the EU and in Germany

In the following section, the German and European legislation relevant to the discussion is briefly explained.

Council Directive 91/492/EEC of 15 July 1991 laying down the health conditions for the production and the placing on the market of live bivalve molluscs (Official Journal of the European Commission No. L 268/1).

Council Directive 91/492/EEC laying down the health conditions for the production and the placing on the market of live bivalve molluscs stipulates that, for the purposes of food safety monitoring, biological testing methods, i.e. Mouse Bioassays, are to be employed for the determination of marine biotoxins belonging to the PSP (Paralytic Shellfish Poison) and DSP (Diarrhoeic Shellfish Poison) toxin groups (Annex, Chapter V, Nos. 6 and 7). In addition, other procedures such as chemical methods or other methods approved in the selection requirements (Article 12) are provided only for PSP. Also, in case of disagreement, the Mouse Bioassay should be used as the definitive reference test. The regulation for maximum levels for PSP remains effective (see Table 1).

Regulation on the health safety requirements for fishing products and for live bivalve molluscs (Fish Hygiene Act – FischHV) in the edition of 8 July 2000 (1998 (German Federal Legal Gazette, BGBl., I p. 819) last amended by Article 1 of the Regulation of 2 April 2003 (1998 (German Federal Legal Gazette, BGBl., I p. 478)

When implementing Council Directive 91/492/EEC into national law (FischHV), Germany has deviated from the EU regulations relating to application and legal status of biological testing

[4] Comments of Competent Authority, DG (SANCO)/8588/202, Comments to the Draft Report of the FVO Mission to Germany from 18 to 28 February 2002. http://www.europa.ei.int/comm/food/fs/inspections/vi/reports/germany/index_en.html

methods: For the testing for toxins of the PSP and DSP groups, the Bioassay may be used, but it can be substituted by physicochemical methods (Annex 3, Chapter 2).

Commission Decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods. (Official Journal of the European Commission No. L 75/62)

As a consequence of the Decision 2002/225/EC of 15 March 2002, physicochemical testing procedures are allowed for all marine biotoxins as an alternative to animal experiments. A prerequisite is, however, the validation of these test procedures in compliance with an internationally accepted protocol (Annex to Decision, last sentence). Such validation procedures have not yet been conducted for all methods, which is partially due to the current lack of reference materials.

Commission Decision 2002/225/EC defines the maximum levels for the toxins previously comprising the DSP group, i.e. Okadaic Acid (OA) and Dinophysis Toxins (DTX), as well as for Yessotoxins (YTX), Pectenotoxins (PTX) and Azaspiracids (AZA) (see Table 1). A further consequence of Decision 2002/225/EC is the rearrangement of the DSP group which now comprises all these compounds.

Article 5 of Decision 2002/225/EC explicitly states that, in case of discrepancies between the results obtained by the use of different methods, the Mouse Bioassay should still be considered as the definitive reference test for all of the marine biotoxins specified herein.

Commission Decision 2002/226/EC of 15 March 2002 establishing special health checks for the harvesting and processing of certain bivalve molluscs with a level of amnesic shellfish poison (ASP) exceeding the limit laid down by Council Directive 91/492/EEC (Annex Chapter V No. 7a) (Official Journal of the European Commission No. L 75/65)

Decision 2002/226/EC lays down the procedure to be used for specified shellfish, in which the content of Domoic Acid is exceeding the authorised maximum level of 20 mg per kg of tissue by up to 250 mg/kg. The content of toxins of the ASP group (Domoic Acid) is determined by employing HPLC methods.

Council Decision of 14 June 1993 on reference laboratories for the monitoring of marine biotoxins (93/383/EEC) (Official Journal of the European Commission No. L166/31)

Decision 93/383 EEC stipulates among other things that in every Member State a national reference laboratory shall be designated to ensure effective supervision procedures for the testing for marine biotoxins and to coordinate the conduct of the necessary analyses within the national laboratories.

In order to guarantee uniformity in the test procedures for marine biotoxins within the Community, a Community Reference Laboratory shall be designated. Furthermore, Decision 93/383/EEC stipulates that, apart from co-ordinating the application of test procedures, the exploration of new analytical methods and informing the national reference laboratories about any progress made shall also be part of its responsibilities.

Regulation (EC) No. 853/2004 (specific hygiene rules for food of animal origin) Annex III Section VII Chapter V No. 2
Regulation (EC) No. 854/2004 (supervision) Annex II Chapter II

In 2004, the Community Legislature passed the so-called ”EU Hygiene Package” of regulations, which shall bring together and replace the existing hygiene regulations for the food sector currently contained in numerous individual guidelines. Regulation (EC) No. 853/2004 (Hygiene) Annex III Section VII Chapter V No. 2 lays down the maximum levels for ASP, PSP and DSP. Article 11, referring to Article 12 of the same regulation, entails authorisation to specify approved analysing methods for marine biotoxins. Regulation (EC) No.854/2004 (Supervision) Annex II Chapter II gives the monitoring authorities in the Member States the mandate to examine live molluscs for the presence of marine biotoxins. The so-called ”EU Hygiene Package” shall come into effect on 1 January, 2006.

Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (Official Journal of the European Commission No. L 358)

Article 7 of Directive 86/609/EEC lays down that experiments using animals are not allowed if, in order to achieve the desired objective, a scientifically acceptable, justifiable and practical alternative is available, which does not require the use of animals.

Article 23 of the Directive places the Commission and the Member States under the obligation to promote the development and validation of alternative methods.

German Animal Welfare Act as announced on 25 May 1998 (German Federal Legal Gazette, BGBL., I p. 1105, corr. p.1818) - Tierschutzgesetz in der Fassung der Bekanntmachung vom 25 Mai 1988 (BGBl. I S. 1105, ber. S. 1818)

The content of Section §7 Paragraph 2 of the German Animal Welfare Act corresponds to Article 7 of Directive 86/609/EEC.

3. Overview of test procedures for marine biotoxins

3.1 Outline of state-of-the-art analyses, including toxin groups, definition of maximum levels , reference methods, reference materials

In Table 1, the toxin groups ASP, DSP, Yessotoxins, Pectenotoxins, Azaspiracids and PSP are allocated the current maximum levels applicable, the prescribed reference methods, the availability of analytical methods and reference materials.

For the **ASP group** the same maximum levels apply within the EU and in Germany. The HPLC method is used and quantitative determination in vitro is assured. Reference materials for Domoic Acids are available.

In contrast, the European Directive stipulates a maximum level of 800 µg/kg hepatopancreas based on the Mouse Bioassay which is less sensitive **for the determination of Okadaic Acid and Dinophysis toxins**; this is significantly above the limit laid down in the German Fish Hygiene Act. With the Mouse Bioassay it is not possible to obtain quantitative results. The death of the test animals is the methodical endpoint determined (see Table 2). The Mouse Bioassay constitutes the ”reference method” in Europe. In Germany, a maximum

level of 400 µg/kg hepato-pancreas for toxins of the DSP group was laid down in order to safeguard consumer protection. This level is easily monitored using the HPLC method which permits quantitative determination. Reference materials for the DSP group are available. The Mouse Bioassay is, therefore, not used in Germany.

In case of the **Yessotoxin, Pectenotoxin and Azaspiracid** toxin groups the maximum levels apply according to Decision 2002/225/EC. These toxins can only be distinguished with sufficient accuracy when multiple Mouse Bioassays are conducted. The reason is that Yessotoxin, for example, is of significantly lower oral toxicity than the other compounds, but it is of equal potency in the Mouse Bioassay. There are already qualitative in vitro detection methods for all the toxins listed. The analytical methods can produce quantitative results as soon as reference materials become available.

For determination of compounds of the **PSP group** the European Directive and the German Fish Hygiene Act lay down identical maximum levels. The Mouse Bioassay is a semi-quantitative procedure. In Germany, the substances which are regarded as the toxicologically relevant lead compounds for the PSP toxin group are determined quantitatively using in vitro methods (HPLC). Reference materials for these compounds are commercially available.

3.2 Comparison between Mouse Bioassay and alternative methods (short description, endpoint of procedure, advantages, disadvantages)

Table 2 shows the currently available or published methods in summarised form, i.e. Mouse Bioassay procedures, chromatographic methods, enzyme immunoassays, protein phosphatase inhibition assays, ligand receptor binding assays and cell culture assays.

The brief description includes the methodical endpoint of the procedure. The present state of development becomes apparent when advantages and disadvantages are compared. For each method selected literature is listed in a bibliography (see Annex).

The application of the **Mouse Bioassay** as a method of reference for the detection of marine biotoxins is criticised in scientific terms as well as in terms of animal protection legislation. The conflict with the animal welfare legislation has already been detailed in Point 1 (Objective of the position paper): The test inflicts considerable suffering to the animals. Death of the animals is the methodical endpoint. The scientific literature describes the Mouse Bioassay as "impossible to validate". The test results show great variability between laboratories. Gender, weight and strain of the mice influence the outcome of the test. At the 5th International Conference on Molluscan Shellfish Safety (June 2004), McNabb et al. [5] , for example, criticised the low sensitivity (too many false negative findings) and the low discrimination (too many false positive findings) of the Mouse Bioassay as compared with chromatographic methods (i.e. LC/MS).

As early as 1985, Yasumoto et al. reported the **chromatographic detection** of tetrodotoxin (i.e. the toxin of the globe fish, Spheroides) using HPLC. In 1989, a fluorimetrical procedure to determine the content of PSP algal toxin in shellfish and shellfish products was published in the Official Collection of Test Procedures in accordance with §35 German Food and

[5] McNabb, P., Holland, P., Ginkel van R., Selwood, A.,: Using Liquid Chromatrography Spectrometry (LCMS) to manage Shellfish Harvesting and Protect Public Health. Book of Abstracts, 5th International Conference on Molluscan Shellfish Safety, 14th-18th June 2004, National University of Ireland, Galway.

Commodity Act (Lebensmittel- und Bedarfsgegenständegesetz LMBG)[6]. In 2002, Holland et al. described the new monitoring programme for marine biotoxins in New Zealand which is based on application of the LC-MS method. Prerequisite for the monitoring programme was completion of the validation of the in vitro method for ASP with a maximum level of 20 µg/100g and for DSP with a maximum level of 20 µg/100g. Holland et al. (2002) emphasise that, in New Zealand, the suitability of the LC/MS method is currently being assessed for the detection of PSP toxins also. The NRL for the control of marine biotoxins of the BfR is also working on this approach.

Irrespective of international developments and progress toward the validation of the chomatographic procedures, the activities of CEN are of particular importance for the regulatory acceptance of in vitro methods in Europe. In October 2004, the European standard DIN EN 14524 "Foodstuffs – determination of okadaic acid and dinophysis toxin in mussels – HPLC method with solid phase extraction clean-up" was published. The European standards EN 14176:2003 for the determination of domoic acid in mussels by HPLC and EN 14526:2004 for the determination of saxitoxin in mussels by HPLC were adopted by CEN and imminent publication has been announced.[7]

Furthermore, scientific papers have reported the development of **Enzyme Immunoassays, Protein Phosphatase Inhibition Assays, Ligand-Receptor Binding Assays and Cell Culture Assays**. There are still no standard protocols for these methods, i.e. the development of these methods has not yet been completed. Enzyme immunoassays offer, for example, rapid detection with high sample throughput. Further support for these methods should be encouraged because of scientific requirements and the provisions of Article 23 of the Animal Protection Directive 86/609/EEC.

3.3 Situation within the EU and at the international level

Table 3 lists the test procedures for the determination of marine biotoxins currently in use in different countries for the official monitoring of marine biotoxins in shellfish and crustaceans[8]. It is self-understood that this table must be constantly supplemented and updated. This overview shows that, internationally, testing for ASP toxins is making exclusive use of the in vitro method based on chromatographic procedures. However, the situation is very heterogeneous internationally in regard to the determination of **DSP toxins** such as **Okadaic Acid and Dinophysis Toxins, Yessotoxins, Pectenotoxins and Azaspiracids** as well as for **PSP toxins**.

In New Zealand and Germany, DSP toxins are determined by exclusive use of chromatographic procedures. In contrast, in Belgium, France, Spain and the United Kingdom, the Mouse Bioassay is the only means of testing for these toxins. However, attention must be drawn to the fact that, after the FVO inspection in 2002, the U.K. was reprimanded for incorrect use of the official method[9] since the U.K. uses a reduced number of mice in the Mouse Bioassay. Dennison et al. (2002) reported on this modification at the 4th World Congress on

[6] Gesetz über den Verkehr mit Lebensmitzteln, Tabakerzeugnissen, kosmetischen Mitteln und sonstigen Bedarfsgegenständen (Lebensmittel- und Bedarfsgegenständegesetz – LMBG) in der Fassung der Bekanntmachung vom 9. September 1997 (BGBl. I S. 2296) - Food and Commodity Act of 9 September 1997 (Federal Law Gazette Part I, p. 2296).
[7] CEN/TC 275-Published Standards. http://www.cenorm.be
[8] Minutes of VI meeting of EU-NRLs on Marine Biotoxins, 21-22 November 2003, Vigo/Spain.
[9] Report of the inspection tour of the United Kingdom by the Food and Veterinary Organisation, 08-17 July 2002, Assessment of the implementation of Council Directive 91/492/EEC (live molluscs) and Council Directive 91/493/EEE (fishing products). http://europa.eu.int/comm/food/fs/inspections/vi/reports/united_kingdom/vi_sum_unik_8614-2002_de.pdf

"Alternatives and Animal Use in the Life Sciences".[10] In Ireland and Norway testing involves parallel use of the Mouse Bioassay and chromatographic procedures. The test laboratories in Austria use the HPLC method; the Mouse Bioassay is only applied in cases where HPLC gives positive results. The Netherlands use the Rat Bioassay as well as chromatographic procedures. Italy applies the Mouse Bioassay; however, if reference substances are available for Okadaic Acid (OA) and Dinophysis Toxins (DTX), Yessotoxins (YTX) and PSP toxins, then the HPLC procedure is used. Portugal uses ELISA for screening for and LC/MS for determination of OA and DTX. Indicator species from particular harvesting areas are tested with the Mouse Bioassay. PTX is determined using LC/MS and Yessotoxins are tested for with the Mouse Bioassay in the case that cell density of the algal species, Protoceratium reticulatum, in the water exceeds 1000 cells per litre. In Sweden, HPLC is used for the DSP and PSP groups, whereas the Norwegian laboratories apply the Mouse Bioassay on samples coming from Sweden. Denmark determines DSP levels using the Mouse Bioassay and confirms the findings using LC/MS/MS (Liquid Chromatography-Tandem Mass Spectrometry). The Mouse Bioassay is also applied to identify PSP. The presence of PTX, YTX and AZAs is detected qualitatively using LC/MS/MS. In Finland, animal experimentation for the determination of DSP is forbidden. Greece determines OA, DTX-1, DTX-2, ASP and PSP by means of the HPLC method. However, a different source reports that the Mouse Bioassay is also applied for the detection of DSP, AZAs and PSP.

In its annual report for 2002, the FVO concluded that, in general, the control and determination of marine biotoxins in the member states are performed incorrectly, i.e. not in compliance with the legal requirements (see point 2).[11]

As already mentioned under point 3.2, relevant international expert publications have reported about the reliability of in vitro detection methods, which can be used as alternatives to animal experiments. Along with biochemical detection methods, analytical techniques are gaining recognition which permit sensitive detection of marine biotoxins as well as a high degree of specificity for the investigated substances. The most significant among these methods is the use of mass spectrometry combined with liquid chromatography (LC/MS; LC/MS/MS). Numerous drafts for methods have already been published, which would facilitate immediate replacement of the Mouse Bioassay. However, Decision 2002/225/EC (Annex) requires validation of alternative methods by use of reference materials for all the individual compounds specified. This will prevent substitution of the Mouse Bioassay as the routine or reference method for years to come; whereas the Mouse Bioassay, on the other hand, has never been subjected to such stringent assessment criteria.

In July 2004, an EU working group which was set up as a result of a decision of the representatives from the European NRLs convened for the first time. Its aim is to select a suitable method for the detection of the toxins of the DSP group and to validate it even without reference standards being available for all substances. This step has already been completed[12] in New Zealand as is shown in Table 3.

[10] Dennison, N., Zuur, G., Petrie, J., Turrif, J., Kinnear, S., and Bourke D.: Pilot Investigation of the Use of General Anesthesia to Refine the Statutory Mouse Bioassay for Paralytic Shellfish Poison. Abstract, Proceedings, Fourth World Congress on Alternatives and Animal Use in the Life Sciences, August 11-15 (2002) New Orleans, USA.

[11] Food and Veterinary Office: Annual Report 2002, pp 21-22. http://europa.eu.int/comm/food/fvo/annualreports/final_2002_en.pdf

[12] Holland, P.T., McNabb, P., Selwood, A., Page, T., Bell, K., Mackenzie, L.: Marine biotoxin monitoring of New Zealand shellfish – a new mangement programme based on LC-MS. Proc. 2nd Int. Conference on Harmful Algae Management and Mittigation, Nov. 2001, Qingdao, China. S. Hall and YL. Zou eds., in press 2002.

3.4 Need for action to be taken towards validation and administrative acceptance of alternative methods

The commentaries on current testing methods and the comparison between the Mouse Bioassay and in vitro testing methods point to the urgent need for action in order to achieve legal acceptance of chromatographic methods for the determination of marine biotoxins of the DSP and PSP toxin groups and the supply of reference substances for the European reference laboratories.

Developments at the international level should also be taken into consideration. One of the important results of the International Conference on Molluscan Shellfish Safety (Ireland, June 2004) concern the discussion about the forthcoming deregulation of toxin classification. Recent investigations of the toxicity of the toxin groups ”Yessotoxins, YTX” and ”Pectenotoxine, PTX” have shown that comparable doses, when applied orally, have significantly lower, if any, toxic effects in the test animals. Intraperitoneal administration has led to a considerable overestimation of the toxic potential. It can be expected that in the future it will not be necessary to determine all individual standards. This possibility to resolve the problem of the validation of physicochemical methods was discussed at length at the meeting of the EU-LC/MS Working Group on July 6, 2004, in Vigo (Spain).

In addition to the recognition of validated in vitro methods (HPLC), support for the validation of other in vitro procedures should be increased since application of more methodical endpoints for toxin determination would provide additional confirmation of the findings. The cell culture assays, for instance, would thus facilitate biological proof of the cytotoxicity of toxins.

The need for action to achieve validation and recognition of physicochemical testing procedures for the analysis of the different toxin groups is summarised in Table 4. On the basis of current publications, the development and validation of the in vitro methods for **the DSP and PSP toxin groups** are considered to be completed. Regulatory acceptance of these validated in vitro methods is recommended as the next step.

On the basis of the scientific literature, the use of the Mouse Bioassay as a method of reference for marine biotoxins is no longer considered appropriate.

Yasumoto et al. have published the Mouse Bioassay in 1971.[13] At the time, this test procedure represented the state-of-the-art and was of high scientific value. Since then, however, extensive critical investigations concerning the method's reliability and accuracy have been conducted and published. The conclusions are that it is not possible to validate the Mouse Bioassay and that it is not sensitive enough for DSP toxins. The level of consumer protection provided is insufficient due to false negative results. The Mouse Bioassay was shown to be completely inappropriate for the monitoring of shellfish toxins in fishing grounds. During the past 30 years, reliable physicochemical methods have been developed, evaluated and applied in parallel to the Mouse Bioassay.

4. Proposal of the BfR

On the basis of the criticism of the Mouse Bioassay as a method of reference for the detection of marine biotoxins and, in particular, the reports in the international literature, and in pre-

[13] Yasumoto, T., Hashimoto, Y., Bagnis, R., Randall, JE., Banner AH.: Toxicity of the surgeonfishes. Bull Jap Soc Sci Fish 37, 724-734 (1971).

paration of the FVO inspection visit in November 2004, the BfR suggests that the BMVEL submits the following proposals to the Commission:

1. Amendment of the relevant legislation with the result that samples are first examined with the available physicochemical methods to detect contamination with marine biotoxins. If the tests give a positive qualitative result and if further testing to ensure consumer protection (usability of test results in legal proceedings etc) is necessary but cannot be achieved by physicochemical methods, then the Mouse Bioassay shall be employed as method of reference according to Art. 5 of Decision 2002/225/EC.

   The BfR agrees with the use of animal experiments only in exceptional justified cases conducted in the interest of protecting consumer health and suggests to designate a laboratory to perform these tests.

2. Provision of financial support in accordance with Article 23 Paragraph 1 of Directive 86/609/EEC to guarantee validation and legal recognition of physicochemical methods, in particular, for the supply of toxin standards and reference materials necessary for this purpose.

3. The EU Commission should be asked to consult its legal department since in the present case Community legislation relating to Consumer Protection is in conflict with that relating to Animal Protection, and the necessary weighing up seems to require clarification of certain aspects of Community legislation.

4. The EU Commission should examine if, from a scientific point of view, the Mouse Bioassay deserves the status of a method of reference; in particular, when taking into consideration the criteria listed in Commission Decision of 12 August 2002 implementing Council Directive 97/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC) (Official Journal of the European Communities No. L 221).[14] The BfR holds the opinion that the Mouse Bioassay is, in principle, not suitable as a method of reference.

[14] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2000/657/EC) (Official Journal of the European Communities No. L 221).

Table 1: Overview of analytical procedures currently available for marine biotoxins; as of September 2004

Group of Toxins	Adopted Maximum Level		Reference Method		Physicochemical Analytical Methods		Possibility to validate for the Detection of Lead Compounds		Available Reference Substances
	EU 91/492/EEC Directive **2002/225/EG** Decision **2002/226/EG** Decision 853/2004 Ordinance	**Germany Fish Hygiene Act (Fisch-Hygiene-Verordnung, FH-VO)**	**EU**	**quantitative analytical procedure**		**quantitativeanalytical procedure***	**Mouse Bioassay**	**Analytical Methods**	
ASP Group (Amnesic Shellfish Poison) Domoic acid and isomers	20 mg/kg shellfish tissue	20 mg/kg shellfish tissue	HPLC	yes	HPLC LC/MS	yes yes	no	HPLC - yes LC/MS - yes	domoic acid
DSP Group (Diarrhetic Shellfish Poison) Okadaic acid (OA) and Dinophysis toxins (DTX)	800 µg/kg hepatopancreas corresponds to 160 µg/kg homogenised shellfish tissue	400 µg/kg hepatopancreas; corresponds to 80 µg/kg shellfish tissue	Mouse Bioassay	no	HPLC LC/MS LC/MS/MS	yes yes yes	no	HPLC – yes LC/MS – yes LC/MS/MS – yes	okadaic acid DTX1
Yessotoxins (YTX) 45 OH YTX, Homo YTX, 45 Homo OH YTX	1 mg/kg shellfish tissue		Mouse Bioassay	no	HPLC LC/MS LC/MS/MS	yes* yes* yes*	no	HPLC – yes* LC/MS – yes* LC/MS/MS – yes*	none
Pectenotoxins (PTX) PTX-1, PTX-2	160 µg/kg shellfish tissue		Mouse Bioassay	no	HPLC/FD LC/MS	yes* yes*	no	HPLC/FD – yes* LC/MS – yes*	none
Azaspiracids (AZA) AZA-1, AZA-2, AZA-3	160 µg/kg shellfish tissue		Mouse Bioassay	no	LC/MS LC/MS/MS	yes* yes*	no	LC/MS – yes* LC/MS/MS – yes*	none
PSP Group (Paralytic Shellfish Poison) STX, NEO-STX and others	800 µg/kg shellfish tissue	800 µg/kg shellfish tissue	Mouse Bioassay	semi-quantitative	HPLC/FD	yes	no	yes	STX, NEO-STX and other compounds

quantitative analytical procedures* = when certified reference materials become available; but, at present, qualitative procedures

Table 2: Overview of detection methods for marine biotoxins; as of September 2004

Detection Method	Short Description and endpoint determined	Advantages	Disadvantages
Mouse Bioassay see Bibliography (1)	• 3 mice are used. • Proof considered positive when death of 2 out of 3 mice occurs within 24 hours (5 hours) following administration of shellfish tissue extract. • Method endpoint is the death of test animals.	• Biological test system, which is used to directly demonstrate the presence of unknown toxins.	• Test entails severe suffering of test animals. Death of animals is method endpoint. • Conflict with Animal Welfare Directive. • Limit of detection at 800 µg/kg shellfish hepato-pancreas for DSP; not very sensitive • Test cannot be validated. Large variation in results between laboratories. Sex, weight and strain of mice influence the test results.
Analytical Methods • High Performance Liquid Chromatography (HPLC) with Fluorimetric and UV Detection (HPLC/FLD; HPLC/DAD) • Liquid Chromatography - Mass Spectrometry (LC-MS) • Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) • Capillary Electrophoresis see Bibliography (2)	• Detection with analytical measuring instruments. • Separation on the basis of the physicochemical properties of molecules and comparison with reference standards.	• Rapid accurate identification and quantitative determination of toxins possible; suitable for detection of all marine toxins. • More sensitive than Mouse Bioassay • High selectivity; suitable for monitoring as preventive measure. • LC-MS is validated according to international standards, cf. Holland et al. (2002). • HPLC methods have been validated (CEN; §35-LMBG; AOAC) • HPLC/FD and LC-MS already mentioned in 2002/225/EC as alternatives to the Mouse Bioassay.	• Reference materials not available for all toxins. • In general, structurally unrelated, unknown toxins cannot be detected.

Detection Method	Short Description and endpoint determined	Advantages	Disadvantages
Enzyme Immunoassays see Bibliography (3)	• Immunological detection by binding to specific antibodies. • Endpoint determined is the amount of bound antibodies.	• Rapid detection possible with high sample throughput. • More sensitive than Mouse Bioassay. • Already mentioned in 2002/225/EC as alternative to the Mouse Bioassay.	• For each group of toxin a different specific test is required. • Cross reactions with toxins of the same group are possible. • At present, no internationally validated standard protocol (SOP) available.
Protein Phosphatase Inhibition Assays see Bibliography (4)	• Biochemical assay. • Endpoint determined is the inhibition of specific enzymes (i.e. protein phosphatases 1 und 2a) by DSP toxins.	• Rapid detection possible with high sample throughput. • Good comparability with chromatographic methods and Mouse Bioassay . • More sensitive than Mouse Bioassay; more sensitive than ELISA. • Already mentioned in 2002/225/EC as alternative to the Mouse Bioassay.	• Suitable only for the detection of DSP toxins. • At present, no internationally validated standard protocol (SOP) available.
Ligand Receptor Binding Tests see Bibliography (5)	• Biochemical assay. • Endpoint determined is the displacement of radioactively labelled toxin standard from the specific receptor binding sites at the cell membrane.	• Very specific; suitable to detect all marine toxins. • Routine detection possible with high sample throughput. • More sensitive than Mouse Bioassay • Good comparability with chromatographic methods and Mouse Bioassay .	• For each kind of toxin a different specific test is required. • At present, no internationally validated standard protocol (SOP) available.

Detection Method	Short Description and endpoint determined	Advantages	Disadvantages
Cell Culture Tests see Bibliography (6)	• Cell biological proof of the cytotoxicity of the toxins. • Endpoint determined is the growth of cells in culture, e.g. a mouse neuroblastoma cell line. Determination by vital stain or bio-chemical test (MTT test).	• Very specific; suitable to detect many marine toxins. • More sensitive than Mouse Bioassay . • Rapid detection possible with high sample throughput.	• Different assays are required for PSP and DSP toxins. • Only total toxicity is determined without accurate identification of individual toxins. • At present, no internationally validated standard protocol (SOP) available.

Table 3

Test Procedures for the official control of marine biotoxins in shellfish and crustaceans employed in EU Member States and Overseas							
	Marine Biotoxins	**OA + DTX** Okadaic acid and Dinophysis toxins	**AZA** Azaspira-cids	**PTX** Pectenoto-xins	**YTX** Yessoto-xins	**PSP** Paralysis inducing shellfish toxins	**ASP** Amnesia inducing shellfish toxins
Country							
Belgium		MBA	MBA	MBA	MBA	MBA	HPLC
Denmark		MBA ver LC/MS/MS	qual LC/MS/MS	qual LC/MS/MS	qual LC/MS/MS	MBA	HPLC
Germany		HPLC-FLD "LC/MS; LC/MS/MS"	qual LC/MS/MS	qual LC/MS/MS	qual LC/MS/MS	HPLC-FLD*	HPLC
Finland		MBA to test for DSP prohibited in Finland				k.A	HPLC
France		MBA	MBA	MBA	MBA	MBA	HPLC
Greece		MBA HPLC-FLD	MBA n.a.	n.a.	n.a.	MBA HPLC-FLD	HPLC
Ireland		MBA + LC/MS	MBA + LC/MS LC/MS	MBA + LC/MS	MBA + LC/MS	MBA	HPLC
Italy		MBA part. HPLC	MBA	MBA	MBA part. HPLC	MBA part. HPLC	HPLC
Luxem-burg		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
The Nether-lands		RBA "LC/MS; HPLC-FLD"	RBA	n.a.	n.a.	HPLC-FLD HPLC-FLD*	HPLC
Austria		HPLC-FLD MBA	HPLC MBA	HPLC MBA	HPLC MBA	HPLC-FLD MBA	HPLC
Portugal	Imports from Non-EU	"ELISA; LC/MS" MBA MBA	LC/MS	LC/MS	MBA**	HPLC-FLD MBA	HPLC HPLC
Sweden		HPLC MBA(NOR)	HPLC MBA(NOR)	HPLC MBA(NOR)	HPLC MBA(NOR)	HPLC MBA(NOR)	n.a.
Spain		MBA	MBA	MBA	MBA	MBA	HPLC
United Kingdom		MBA	MBA	MBA	MBA	MBA	HPLC
Norway		MBA + LC/MS	LC/MS	LC/MS	MBA + LC/MS	HPLC-FLD	HPLC
New Zealand		LC/MS	LC/MS	LC/MS	LC/MS	MBA	HPLC

n.a.: at present, no details available
*: MBA as last resort
**: only if Proceratium reticulatum > 1000 cells/L
ver: confirmation with
(NOR): conducted in Norway
part.: HPLC, if reference standards are available
MBA: Mouse Bioassay
RBA: Rat Bioassay
HPLC: High Performance Liquid Chromatography
FLD: Fluorescence Detection
"LC/MS: Liquid Chromatography/Mass Spectrometry;"
LC/MS/MS: Liquid Chromatography/Tandem Mass Spectrometry
MBA + LC/MS: comparison between measurements

Table 4: Need for action towards validation and official acceptance of physicochemical procedures for analytical detection of the different toxin groups

- **Development, Validation**
- **Official Acceptance**

Group of Toxins	**Need for action towards validation and official acceptance of physicochemical procedures for analytical detection of the different toxin groups**	
	Development, Validation	Official Acceptance
ASP Group (Amnesic Shellfish Poison) Domoic acid and isomers	Development and Validation have already been completed	HPLC Method has already been officially accepted in EU (2002/226/EC)
DSP Group (Diarrhetic Shellfish Poison) Okadaic acid (OA) and Dinophysis toxins (DTX)	Development and Validation have already been completed • see DIN EN 14524-2004 “Foodstuffs – Determination of okadaic acid in mussels - HPLC method using purification by solid phase extraction, derivatization and fluorimetric detection"; German Version EN 14524:2004 (Date of publication: October 2004) • see Holland et. al. (2002)	Official Acceptance of alternative methods in EU subject to detection of specified analogous compounds, see 2002/225/EEC in New Zealand for LC-MS
Yessotoxins (YTX)		Official Acceptance of alternative methods in EU subject to detection of specified analogous compounds, see 2002/225/EEC
Pectenotoxins (PTX)		Official Acceptance of alternative methods in EU subject to detection of specified analogous compounds, see 2002/225/EEC
Azaspiracids (AZA)		Official Acceptance of alternative methods in EU subject to detection of specified analogous compounds, see 2002/225/EEC
PSP Group (Paralytic Shellfish Poison)	Development and Validation have already been completed • see EN 14524-2004 “Foodstuffs – Determination of saxitoxin in mussels - HPLC method using pre-column derivatization with peroxide or periodate oxidation"	Official Acceptance by EU pending Cf. announcement made in April, 2004, see Protocol Notes by ECVAM of 6.4.2004

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