Risiken erkennen – Gesundheit schützen

Mouse bioassay not suitable as a reference method for the regular analysis of algae toxins in mussels

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Like all foods that are placed on the market in the European Union, mussels and other shellfish are subject to official food control. They are examined, amongst other things, for algae toxins which are harmful for humans that can cause diarrhoea and paralysis. In order to detect these algae toxins, a chemico-physical method is routinely used in Germany. By contrast, the European Union prescribes as the reference method the mouse bioassay in which the algae toxins are detected in animal experiments.

In the following text the Federal Institute for Risk Assessment (BfR) explains why preference is given in Germany to the chemico-physical test method. Here the focus is not only on statutory animal welfare provisions but, more particularly, on scientific arguments.

The mouse bioassay has proved to be unreliable in the detection of algae toxins. Scientific studies show that the mouse bioassay does not recognise health-relevant toxins on a sufficient scale. In comparison, alternative chemico-physical methods have supplied results which have led to complaints about mussels which prevented them from reaching the market.

The results of toxin analyses in the mouse bioassay vary depending on the mouse strain, gender and weight of the animals. The results cannot be reproduced between laboratories in Member States. Furthermore, the mouse bioassay is not suitable for quantitative statements. That's why it cannot be used to monitor compliance with maximum levels for algae toxins.

BfR recommends using chemico-physical analyses like the LC/MS method as the reference method for the determination of algae toxins in mussels and only envisaging the mouse bioassay as an additional analytical step when a positive result has been obtained and further clarification is needed in the interests of consumer protection (suitability of the test results for use in court, etc.) which cannot be obtained using chemico-physical methods.

The LC/MS method is a scientific method which has been tested in line with internationally accepted criteria. It has already been recognised by the New Zealand Food Safety Authority (FSA) as an official method and successfully tested in an interlaboratory trial.

Vis a vis the European Food Safety Authority (EFSA) BfR advocates replacing the mouse bioassay with the LC/MS method as the reference method because it offers greater consumer protection and, at the same time, contributes to animal welfare.

1 Objections to the Mouse Bioassay as the method of reference in routine shellfish toxin testing

The Federal Institute for Risk Assessment (BfR) and the German National Reference Laboratory (NRL) for the Control of Marine Biotoxins wish to draw attention to the following matter for scientific discussion by the competent experts of the European Food Safety Agency (EFSA). BfR holds the opinon that application of advanced scientific methodology instead of the MBA as method for routine testing should be preferred in order to improve the protection of consumer health and to meet the principle objectives of the experimental animal welfare directives, namely the replacement, reduction and refinement of animal use. For the purpose of food control live bivalve molluscs are examined for marine biotoxins using the mouse bio-

assay (MBA). In several occasions, the MBA has failed to be sensitive enough to detect certain marine biotoxins in quantities relevant for human health.

Against this background EFSA is asked to evaluate the following scientific questions presented in order to resolve scientific concerns to the MBA as the method of reference in routine testing.

1.1 Does the MBA as the method of reference in routine shellfish toxin testing assure consumer health protection sufficiently?

European Commission Decision 2002/225/EC of 15 March, 2002, sets rules with regard to the maximum levels and the methods of analysis of marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods (Official Journal of the European Communities, L75, 62-64, 2002); in particular, it sets the maximal permissible levels for specific marine biotoxins belonging to the DSP toxin complex.

The Commission Decision permits, in principle, the use of alternative in vitro methods, such as physicochemical, functional or immunological methods, as substitutes of the stipulated Mouse Bioassay (MBA) for specific DSP toxins. However, the prerequisite for this option is the validation of such methods and the availability of an internationally accepted protocol. Article 5 explicitly states, that the MBA shall be the definitive reference method, if there are discrepancies between the results obtained by the use of different methods.

EFSA should examine if, from a scientific point of view, the MBA deserves the status of a method of reference. BfR holds the opinion that the MBA is not suitable as the definite method of reference. Especially the results of a Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs (Oslo, September 26-30 2004) are challenging the status of the MBA as a standard reference method for marine biotoxins. In an “Advance Pre-Publication Copy” new toxicity guidance levels for marine biotoxins of the DSP-group are reported. The data are shown in the following table 1.

Table 1:
FAO/IOC/WHO ad hoc hoc Expert Consultation on Biotoxins in Bivalve Molluscs(Oslo, September 26-30 2004)

Joint FAO/IOC/WHO *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs

5.9 Summary Tables of Toxicology and Methods of Analysis

Table 2: Summary data used in the derivation of the acute RfD, as well as derived and current guidance levels.

Toxin Group	LOAEL(1) NOAEL(2) µg/kg bw	Safety Factor (Human data (H) Animal data (A))	Provisional Acute RfD[a]	Derived Guidance Level/ Max Level based on consumption of 100g (1), 250g (2) and 380g (3)	Guidance Level/Max Level currently implemented in some countries[b]
AZA	0.4 (1)	10(H)	0.04 µg/kg 2.4 µg/adult	0.024 mg/kg Shellfish Meat(1) 0.0096 mg/kg SM (2) 0.0063 mg/kg SM (3)	0.16 mg/kg SM
BTX			N/A		0.8 mg/kg SM as PbTx-2
Cyclic Imines			N/A		
DA	1,000 (1)	10(H)	100 µg/kg 6mg/adult[a]	60 mg/kg SM(1) 24 mg/kg SM(2) 16 mg/kg SM(3)	20 mg/kg SM
OA	1 (1)	3(H)	0.33µg/kg 20 µg/adult[a]	0.2 mg/kg SM (1) 0.08 mg/kg SM (2) 0.05 mg/kg SM(3)	0.16 mg/kg SM
PTX			N/A		
STX	2 (1)	3(H)	0.7 µg/kg 42 µg/adult[a]	0.42 mg/kg SM(1) 0.17 mg/kg SM(2) 0.11 mg/kg SM(3)	0.8 mg/kg SM
YTX	5,000 (2)	100(A)	50 µg/kg 3 mg/adult[a]	30 mg/kg SM(1) 12 mg/kg SM(2) 8 mg/kg SM(3)	1 mg/kg SM

[a] Based on an adult bw of 60 kg.

[b] These levels are considered as standard international regulatory levels , even though some countries might have different levels

Taking into account the “Derived Guidance Levels/Max Levels” based on consumption of 250 g portions from the table the MBA would fail to detect azaspiracids, okadaic acid, dinophysistoxins and esters at the relevant concentrations, meaning that false negative results have to be expected. Although the new values for this risk assessment have not been implemented into legislation by any country yet, these toxicological data cannot be ignored from a

preventive consumer protection perspective. When the MBA is used as the definite reference method it is important to emphasise its deficits:

- Despite its status as the definite reference test, there is concern since even when this test is used there is no equivalence of results between laboratories of member states and even between repeated tests in one laboratory.

- The MBA has failed to shown good reproducibility between laboratories (McFarren 1959, LeDoux and Hall 2000, Toti et al. 1991). This has implications for the legal enforcement of limits of tolerable toxin levels in shellfish meat and the risk assessment that must underpin regulatory decisions aimed at protecting human health. It is of particular concern that Commission Decision 2002/225/EC requires adherence to maximum toxin levels for lipophilic compounds when it is unknown whether the MBA is capable of providing quantitative information on the DSP toxins involved. The MBA is definitely not considered appropriate for lipophilic toxin monitoring programmes.

  Results of the MBA for DSP and PSP toxins show high variability between animals as strain, sex, age and bodyweight can all influence the results (Stabell et al., 1992; Prakasch et al., 1971; Nagashima et al. 1991). The scientific literature describes the MBA as “impossible to be validated”. 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. (2004) criticised the low sensitivity (too many false negative findings) and the low discrimination (too many false positive findings) of the MBA as compared with chromatographic methods (i.e. LC/MS).

- **It is not possible to validate the MBA for the quantitative determination of marine biotoxins within the DSP-group.** The MBA provides only qualitative results for DSP and other lipophilic toxins. The MBA is not discriminative and the accuracy, reliability and relevance of the quantitative data for PSP-analysis are insufficient.

- There is published evidence that the sample extraction method recommended in the new guidance does not adequately extract some of the key toxins, for example yessotoxins. It is also clear from the European Food and Veterinary Office ((FVO), Grange/Ireland) reports and other sources that this extraction process is not the method used in member states. In the presence of yessotoxins (when the method extracts these compounds), or other factors such as zinc or free fatty acids, the MBA may give incorrect positive results (McCulloch et al. 1989, Park et al. 1986, Suzuki et al. 1996, Lawrence et al. 1994, Takagi et al. 1984, Stabell et al., 1991).

- The MBA has failed to be sensitive enough in some occasions and thus cannot be considered absolutely safe. Scientific literature describes several cases in which human health was compromised, although the MBA result was satisfactory (Jørgensen et al. 2004). However, alternative in vitro methods provided results that would have stopped contaminated lots from being marketed, i.e. alternative methods would have made better provision for the protection of human health (validated CEN method DIN EN 14524).

1.2 Which scientific strategies are recommended by EFSA for validation and regulatory acceptance of in vitro alternative methods for the detection of marine biotoxins?

There is a strong effort of ongoing research to develop and validate in vitro alternatives to the MBA, i.e. chromatographic methods, enzyme immunoassays, protein phosphatase inhibition

assays, ligand receptor binding assays and cell culture assays. Based on scientific literature BfR and the German NRL for the Control of Marine Biotoxins share the opinion that good progress is being made.

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 Commodity Act (Gesetz über den Verkehr mit Lebensmitteln, Tabakerzeugnissen, kosmetischen Mitteln und sonstigen Bedarfsgegenständen, Lebensmittel- und Bedarfsgegenständegesetz, - LMBG).

In 2002, Holland et al. described the new monitoring programme for marine biotoxins in New Zealand which is based on application of a 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 mg/kg and for DSP with a maximum level of 200 g/kg. Holland et al. (2002) emphasised that, in New Zealand, the suitability of the LC/MS method is currently being assessed for the detection of PSP toxins also. The German NRL for the Control of Marine Biotoxins of BfR is also working on this approach.

Irrespective of international developments and progress towards the validation of the chromatographic 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 are now available (CEN/TC 275-Published Standards.http://www.cenorm.be).

Furthermore, scientific papers have reported the development of in vitro assays as screening methods like 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.

1.3 Have robust strategies been developed for implementing the best available methods for routine shellfish toxin testing?

The current shellfish toxin testing strategies and methods should be improved to provide better protection of public health by using the best available methods. In view of the technical progress made since the shellfish toxin testing directive was inforced BfR suggests to discuss the need for an amendment of the strategies defined by current legislation. In the opinion of BfR, samples should first be examined with the regular physicochemical methods to detect contamination with marine biotoxins. The MBA should only be applied in a second step, if these tests give a positive qualitative result and further testing to ensure toxicity of the sample is necessary but cannot be achieved by other physicochemical methods.

1.4 Could the LC/MS-method be adopted as a reliable reference method after successful validation?

Worldwide there are no evaluated reference methods established for the determination of marine biotoxins in shellfish. Annually some thousend tons of mussels from New Zealand are

imported by member states of the European Union. The official method of analysis for the determination of DSP-toxins as regulated in document 2002/225/EC is a LC/MS-method, which is approved by the New Zealand Food Safety Authority and meanwhile passed a single laboratory validation according to an international protocol. In addition, this method was successfully tested in an interlaboratory study.

The European Food and Veterinary Office inspected the production- and food safety-facilities for shellfish and mussels from New Zealand in the year 2003 and stated no non-conformity in the application of the LC/MS-method with the MBA which is mandatory in Europe.

Due to the fact that some analogues of marine biotoxins of the DSP-type are not available as reference substances, alternative methods could not be validated, and, therefore, the MBA could so far not be replaced in Europe by more sophisticated methods of analysis.

However, the European Commission accepts the import of mussels from New Zealand, for DSP-content tested by a LC/MS-method.

2 References

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