Animal vs. Non-Animal Tests for the Monitoring of Marine Biotoxins in the EU

Point of view of the German Animal Welfare Federation

Ursula G. Sauer
Akademie für Tierschutz, D-Neubiberg

Summary
Since bivalve molluscs can contain algae biotoxins that can cause gastroenterological or even lethal neurological diseases in humans, a public health control system on marine biotoxins has been implemented in EU Directive 91/492. Currently, the reference method laid down for this purpose is the so-called "mouse bioassay" with death of the animals as an endpoint. To date, this extremely distressful animal test has not been standardised or validated, and there is scientific evidence that it is neither relevant nor reliable. Therefore different EU Member States have been striving to replace the mouse bioassay or to reduce the animal numbers and the distress for the animals. In the United Kingdom, the test is being performed with two instead of three mice, the animals are anaesthetised before injection of the mollusc extract and remain in narcosis until their death. In Germany the mouse bioassay has not been performed for many years; without restriction of consumer health safety, marine biotoxins are detected with chemical analytical test methods. The application of alternative test methods is legally required according to EU Directive 86/609 on the Protection of Laboratory Animals. Apparently there is a conflict between two equal valid EU Directives, which has to be overcome.

Zusammenfassung: Aktivitäten in der Europäischen Union zum Ersatz von Tierversuchen zur Bestimmung von Muscheltoxinen aus der Sicht des Deutschen Tierschutzbundes
Da lebende Muscheln Algengifte enthalten können, die beim Menschen Magen-Darm- oder sogar bisweilen tödlich verlaufende neurologische Erkrankungen verursachen können, ist in der diesbezüglichen EU-Richtlinie 91/492 vorgeschrieben, dass derartige Meeresfrüchte vor der Vermarktung auf ihre Unbedenklichkeit hin geprüft werden müssen. Derzeit ist hierfür als Standardmethode der so genannte „Mouse Bioassay" vorgeschrieben, mit dem Tod der Tiere als Endpunkt. Dieser äußerst belastende Tierversuch ist bislang nie standardisiert oder validiert worden und wissenschaftlichen Belegen zufolge weder aussagekräftig noch zuverlässig. In verschiedenen EU-Mitgliedsstaaten gibt es Bestrebungen, den Maustest zu ersetzen oder die Tierzahlen und die Belastung für die Tiere zu reduzieren. So wird der Versuch im Vereinigten Königreich mit zwei anstelle von drei Mäusen durchgeführt, die Tiere werden vor der Verabreichung des Muschelextraktes anästhesiert und verbleiben bis zu ihrem Tod in der Narkose. In Deutschland hingegen wird seit Jahren ohne Einschränkung des Verbraucherschutzes gänzlich auf den Maustest verzichtet, und die Toxine werden hier mit chemischen Analyseverfahren nachgewiesen. Die Vermeidung der Belastung der Tiere, Reduzierung der Versuchstierzahlen und der Einsatz tierversuchsfreier Verfahren werden in der EU-Versuchstierrichtlinie 86/609 gesetzlich vorgeschrieben. Da aber gleichzeitig der „Mouse Bioassay" in der EU-Richtlinie 91/492 als Referenzmethode verankert ist, besteht hier ein Gesetzeskonflikt, den es aufzulösen gilt, indem der Maustest EU-weit durch eine tierversuchsfreie Prüfstrategie ersetzt wird.

Keywords: shellfish toxins, diarrhetic shellfish poison (DSP), paralytic shellfish poison (PSP), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), protein phosphatase inhibition, neuroblastoma cells, mouse bioassay

1 Introduction

Bivalve molluscs filter phytoplankton, which can contain a variety of biotoxins. While these toxins are not detrimental to the molluscs, they can cause diseases in humans that range from gastroenterological symptoms to lethal neurological diseases causing respiratory paralysis. Known toxins include those from the ASP group (amnesic shellfish poison), the PSP group (paralytic shellfish poison), the DSP group (diarrhetic shellfish poison) as well as yessotoxins, pectenotoxins and azaspiracids, differing in their chemical characteristics and in the symptoms they can cause in humans. New toxins continue to be detected. Since these marine biotoxins are not inactivated or destroyed by boiling, salting or pickling, live bivalve molluscs have to be tested for safety before marketing.

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2 EU legislation on the monitoring of marine biotoxins

In the European Union, regulations on the safety testing of shellfish have been implemented in Council Directive 91/492/EEC laying down the health conditions for the production and the placing on the market of live bivalve molluscs (Commission of the European Communities, 1991). In Chapter VI of the Annex to this Directive, measures for the monitoring of production have been laid down. It is stated that a public health control system must be established that must include amongst others, a "periodic monitoring of live bivalve mollusc relaying and production areas in order to... check the possible presence of toxin-producing plankton in production and relaying waters and biotoxins in live bivalve molluscs...". This public health control system must include "laboratory tests in order to check compliance with the requirements for the end product as laid down in Chapter V of this Annex. A control system must be established to verify that the level of marine biotoxins does not exceed safety limits."

Chapter V of the Annex requires: "The total Paralytic Shellfish Poison (PSP) content in the edible parts of molluscs (the whole body or any part edible separately) must not exceed 80 microgrammes per 100 g of mollusc flesh in accordance with the biological testing method – in association if necessary with a chemical method for detection of Saxitoxin – or any other method recognized in accordance with the procedure laid down in Article 12 of this Directive. If the results are challenged, the reference method shall be the biological method. The customary biological testing methods must not give a positive result to the presence of Diarrhetic Shellfish Poison (DSP) in the edible parts of molluscs (the whole body or any part edible separately)". The level for positive results was laid down at 160 µg of okadaic acid equivalents/kg whole body or any part edible separately.

3 Test methods for the monitoring of marine biotoxins

3.1 Mouse Bioassay

The accepted biological testing methods for the monitoring of marine biotoxins are specified in Commission Decision 2002/225/EC 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 (Commission of the European Communities, 2002a). Article 5 of this Decision implements the so-called mouse bioassay to be the decisive reference method: "When the results of the analyses performed demonstrate discrepancies between the different methods, the mouse bioassay should be considered as the reference method."

In the Annex to Commission Decision 2002/225, rules are put forward on how to perform the mouse bioassay for the detection of PSP or DSP toxins respectively, differing in the test portion of the molluscs (hepatopancreas or whole body) and in the solvents used for the extraction of the biotoxins and the respective purification steps: "Three mice should be used for each test. The death of two out of three mice within 24 hours after inoculation into each of them of an extract equivalent to 5 g of hepatopancreas or 25 g whole body should be considered as a positive result for the presence of one or more of the toxins." The amount of biotoxin present in the extract is estimated in relation to the duration from injection until death of the animals.

It does undisputed that the mouse bioassay is very distressful to the animals. Already, the injection of mussel extract into the abdominal cavity is a very painful procedure. Additionally, death has been set as the endpoint of the method and it can be caused by respiratory paralysis in the presence of PSP (Dennison et al., 2002). So far the mouse bioassay has neither been standardised nor validated. Moreover, there is scientific evidence that it is neither relevant nor reliable. False positive and false negative test results are regularly observed (Park et al., 1986; see also below: Response of the German Competent Authority to the recommendations made in the Food and Veterinary Office's Mission Report; FVO, 2002b). The reason for this is that results can be affected for example by the strain of mice, their sex, age and body weight (Holland et al., 2002; Stabell et al., 1992). Additionally DSP toxins can only be detected in the mouse bioassay at concentrations that exceed the level of concern in humans, and continuous monitoring of biotoxin levels in water samples is not possible with the mouse bioassay due to its low sensitivity (Holland et al., 2002): "The dependence on mouse bioassays for marine biotoxin detection has a number of drawbacks: 1) little information is provided on toxin composition; 2) the toxicity from intra-peritoneal dosing may bear little relation to oral toxicity; 3) they can be difficult to implement for high-throughput, fast turnaround testing; 4) the use of animals for routine testing of food is increasingly unacceptable; 5) results can be variable as affected by strain of mice, non-biotoxin components in shellfish extracts and other factors; 6) the sensitivity can be marginal and therefore information is often lacking on the build-up towards toxic events or on subsequent depuration."

3.2 Alternative methods

Commission Decision 2002/225 allows for the use of alternative methods for shellfish toxin testing provided that they have been validated and that reference material enabling quantitative detection of the biotoxins is available for all toxin groups: "A series of methods such as high performance liquid chromatography (HPLC) with fluorimetric detection, liquid chromatography (LC)-mass spectrometry (MS), immunoassays and functional assays such as the phosphatase inhibition assay can be used as alternative or complementary methods to the biological testing methods, provided that either alone or combined they can detect at least the following analogues, that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection...Standards will have to be available before chemical analysis will be possible... The performance characteris-

tics of these methods should be defined after validation following an internationally agreed protocol."

As depicted in Council Decision 2002/225, currently available non-animal test methods include chemical analytical methods such as high performance liquid chromatography (HPLC) with fluorescence detection and liquid chromatography-mass spectroscopy (LC-MS). These test methods are highly specific and highly sensitive and show very good reproducibility (van Egmond et al., 2004). For the monitoring of ASP toxins, an HPLC method with fluorescence detection has been officially accepted by introduction into Council Decision 2002/226 (Commission of the European Communities, 2002b). For the detection of DSP toxins, in 2004 an HPLC method with fluorescence detection has been published as European Standard EN 14524 following validation in an interlaboratory study according to ISO general principles on assessing accuracy of measurement methods and results (EN 14524, 2004). In New Zealand, an LC/MS method has been accepted for the monitoring of DSP toxins (Truman and Stirling, 2001; Holland et al., 2002). For PSP toxins, an HPLC method with fluorescence detection also has been published as CEN standard in 2004 following validation (EN 14526, 2004). Due to lack of adequate reference material, more recently discovered biotoxins, such as yessotoxins, pectenotoxins and azaspiroids, currently can only be detected qualitatively with HPLC or LC/MS (Holland et al., 2002). As soon as reference material would be available, these test methods would also enable quantitative detection of these biotoxins.

One of the main arguments put forward by proponents of the mouse bioassay is that new toxins continue to occur and that these can only be detected in a biological test method. However, while it is true that analytical methods cannot detect previously unknown biotoxins, biological non-animal test methods are available that make use of the ability of shellfish toxins to bind specifically and reversibly to certain classes of ion channels and receptors. For instance, an MTT cell culture test method with neuroblastoma cells measures the extent to which voltage-activated sodium channels of the cells are blocked by a variety of different shellfish biotoxins, which results in cell lysis that can be detected by MTT reduction (Truman et al., 2002). For DSP detection, a functional protein phosphatase inhibition assay (Nunez and Scoging, 1997) and specific ligand receptor binding assays exist (Llewellyn et al., 2001).

Immunological methods, such as enzyme immunoassays, have also been developed (Hannah et al., 1998), but do not always show the same accuracy as the chemical analytical methods (Nunez and Scoging, 1997).

4 Monitoring of marine biotoxins in EU Member States

In each EU Member State, national reference laboratories (NRL) for the monitoring of marine biotoxins have been designated in accordance with the respective Council Decision 93/383/EEC (Commission of the European Communities, 1993). Furthermore, the laboratory of the *Ministerio de Sanidad y Consumo in Vigo*, Spain, is designated as the Community reference laboratory (CRL) for the monitoring of marine biotoxins. Amongst other duties, it is engaged in supplying "information on analytical methods and comparative testing to the national reference laboratories" and "coordinating the development of new analytical methods and informing the national reference laboratories of progress made in this area".

Due to the ethical and scientific problems related to the mouse bioassay, different EU Member States have put efforts into replacing this animal test or into reducing the animal numbers and the distress for the animals in their monitoring programmes. For instance, in the United Kingdom, the test is being performed with two instead of three mice, the animals are anaesthetised before injection of the mussel extract and remain in narcosis until their death (Dennison et al., 2002; FVO, 2002a).

In the Netherlands, live bivalve molluscs are tested for DSP with an HPLC method, whereas PSP testing is performed with a rat bioassay, which does not include death as an endpoint (FVO, 2001a). In France for reasons not related to animal protection, until 2001 oysters were not tested for marine biotoxins, since they were not included in the public health control system (FVO, 2001b).

In Germany, the mouse bioassay has not been performed at all for a number of years. Instead marine biotoxins are detected making use of chemical analytical test methods in accordance with the German Fish Hygiene Order (FischHV; Anon., 2000). Chapter 2 of Annex 3 of this regulation calls for the lipophilic biotoxins of the DSP group to be tested "either in the animal test or by means of a chemical analytical method" and the hydrophilic biotoxins of the PSP group "by means of a fluorimetric test method", with equivocal results in PSP testing to be verified by means of the animal test or HPLC test method. In Germany, the action limit for DSP toxins has been set at 400 µg/kg hepatopancreas, which is considerably lower than the respective level of concern of 160 µg of okadaic acid equivalents/kg whole body laid down by the European Commission due to the low sensitivity of the mouse bioassay.

The Food and Veterinary Office (FVO) of the Directorate General Health and Consumer Protection of the European Commission is responsible "for ensuring that Community legislation on food safety, animal health, plant health and animal welfare is properly implemented and enforced"[1]. The FVO fulfils this task mainly by carrying out inspections in Member States, the results of which are published in inspection reports. Concerning the supervision of the public health system for shellfish safety testing, the FVO annual report from 2001 states (Anon., 2002): "Taking into consideration that these products represent a relatively high food safety risk and in order to get a comparative overview of the situation in Member States, a series of missions was started in 2001" and completed in 2002 (Anon., 2003). In

[1] http://europa.eu.int/comm/food/fvo/index_en.htm

many Member States, "inappropriate control of marine biotoxins and incorrect biotoxin analysis methods" were observed (Anon, 2002 and 2003).

After their inspection of the German monitoring system on marine biotoxins, the Food and Veterinary Office commented (FVO, 2002b): "This is one of the key issues of the whole mission. In spite of the recommendation made in the report of the mission carried out in 1999... the Competent Authorities still do not use for the detection of DSP and PSP toxins the laboratory methods as laid down by Directive 91/492/EEC, i.e. biological tests. Instead, an HPLC test is used for DSP and an HPLC or LC-MS method for PSP as laid down in the FischHV." In consequence, the FVO made the following recommendations: "The action limit for DSP in the FischHV... should be brought in line with the level set by Commission Decision 2002/225/EEC... The Competent Authorities should impose on the official laboratories to use the methods recommended by the Directive and by relevant CRLs, for... biotoxins detection..." (FVO, 2002b).

The United Kingdom, the Netherlands and France also received recommendations to change their biotoxin testing programme and to reintroduce the standard mouse bioassay accordingly (FVO, 2001a; FVO, 2001b; FVO, 2002a).

5 Legal conflict in the European Union regarding the monitoring of marine biotoxins

The response of the German Competent Authority to the recommendations made in the Food and Veterinary Office's Mission Report published in an Addendum to the report (FVO, 2002b) reveals the existence of a conflict between two equal valid EU Directives: "Because of the current legal situation, use of the mouse bioassay cannot be made compulsory in Germany. Animal tests are permitted only if they are essential in the current state of the art and if the aim pursued cannot be achieved by non-animal methods. Since adequately validated alternative methods are available (HPLC for DSP and a fluorimetry and HPLC method for PSP), the mouse bioassay is not necessary. In the view of the German experts, the chemical analysis methods used in the Länder testing laboratories afford health protection at least equivalent to that of the mouse bioassay. Parts of molluscs from other Member States which had been tested with the mouse bioassay in the country of origin and classed as safe have thus repeatedly been identified as hazardous to health and withdrawn from sale using the chemical methods employed in Germany."

Thus, while Council Directive 91/492 implements the mouse bioassay as the legally binding reference method for shellfish toxin testing, on the other hand Article 7 (2) of Council Directive 86/609 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 states (Commission of the European Communities, 1986): "An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available."

According to the assessment of the German Competent Authority, this prerequisite is applicable to the monitoring of marine biotoxins with chemical analytical methods, since there is proof of evidence that these non-animal test methods offer a level of consumer protection that is at least equivalent to the one provided by the mouse bioassay. The Food and Veterinary Office, on the other hand, puts forward that in accordance with Council Decision 2002/225 the analytical methods may not be applied since reference material is not available for all groups of biotoxins (FVO, 2002b): "With regard to the chemical tests, standards or positive materials are not available to the laboratories for all of the toxic molecules in each group and the NRL identified that not all of these could be detected and/or quantified." To this, the German Competent Authority pointed out: "The NRL for marine biotoxins had noted that, while standards are not available for all individual compounds from the groups of marine biotoxins, this is not necessary for many minor components in view of their fairly low toxicity."

6 Activities to overcome the legal conflict

Ethical, legal and scientific problems speak in favour of replacing the mouse bioassay in the public health control system for marine biotoxins in live bivalve mollusc. Nevertheless, the working programme 2004[2] of the Community reference laboratory, not only lists a number of studies to develop and validate chemical analytical methods but also the working item to produce a standard operation procedure for the mouse bioassay.

Nevertheless, any further development of the mouse bioassay is to be objected. It does not stand in line with Article 23(1) of Council Directive 86/609: "The Commission and Member States should encourage research into the development and validation of alternative techniques which could provide the same level of information as that obtained in experiments using animals but which involve fewer animals or which entail less painful procedures, and shall take such other steps as they consider appropriate to encourage research in this field."

This demand has been taken up by the European Centre for the Validation of Alternative Methods (ECVAM) at the Joint Research Centre of the European Commission. In acknowledgement of the legal conflict and the ethical and scientific problems of the mouse bioassay, in June 2003, ECVAM established a Task Force on Shellfish Toxin Testing[3] that convened at the National German Centre for the Documentation and Evaluation of Alternatives to Testing in Animals (ZEBET[4]) at the Federal Institute for Risk Assessment in Berlin under the chairmanship of Barbara Grune, ZEBET. During the meeting, the problems related

[2] http://www.europa.eu.int/comm/food/food/biosafety/laboratories/biosafety_work_prog_2004_en.pdf
[3] ECVAM Newsletter July/August 2003, http://ecvam.jrc.it/index.htm
[4] ZEBET, Zentralstelle für die Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch am Bundesinstitut für Risikobewertung, Berlin

to the mouse bioassay were spelled out and substantiated, and ECVAM was urged to bring about a solution to the legal conflict between Directives 86/609 and 91/492. In consequence, a joint ECVAM/DG SANCO international workshop on non-animal test methods for shellfish toxin testing has been scheduled to take place in the winter of 2004/2005. The experts involved will be entrusted with the task to depict the state of the art of non-animal testing strategies and point to further action necessary to achieve replacement of the mouse bioassay.

At this point in time no further scientific work should be considered necessary before enforcement of a non-animal monitoring programme for ASP, DSP or PSP biotoxins. For these toxin groups, chemical analytical methods have either been officially accepted by the European Commission or have been published as European Standards. For more recently discovered biotoxins, such as yessotoxins, pectenotoxins and azaspiracids, the making available of adequate reference material seems to be the most imminent issue in order to enable quantitative detection of these toxins. However, since qualitative analysis of these toxins is already possible even without reference material, the total replacement of the mouse bioassay should not be dependent on the provision of reference material for all biotoxins, especially since the mouse bioassay in itself is not a credible quantitative test method for all groups of biotoxins.

Both from the point of view of animal welfare and on behalf of the consumers, it would be totally unacceptable if Member States that have replaced an animal test and can show that this measure has improved their public health control system would be forced to reintroduce an animal test that is ethically unacceptable and scientifically flawed. Instead, a tiered testing strategy not only making use of appropriate chemical analytical methods for the various toxin groups but also of biological *in vitro* test methods should be enforced. Such a testing strategy would meet the necessity to take into account the possible occurrence of previously unknown biotoxins in future (Llewellyn et al., 2001): "Each method possesses different virtues and it may be that a multimethod approach would harness the benefits of each method for various aspects of a shellfish testing regime."

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Correspondence to
Dr. med. vet. Ursula G. Sauer
Akademie für Tierschutz
Spechtstrasse 1
D-85579 Neubiberg
Germany
phone: +49-89-600 201 0
fax: +49-89-600 291 15
e-mail:
ursula.sauer@tierschutzakademie.de