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The 3 "R"S approach to marine biotoxin testing in the UK

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Abstract:
The current reliance on the use of the Mouse Bioassay to detect shellfish biotoxins in national shellfish safety programmes in Europe means that there are regulatory aspects that potentially conflict between food hygiene and animal experimentation legislation. In the United Kingdom, the use of animals for any scientific purpose requires authorisation under the Animals (Scientific Procedures) Act 1986. Such use must consider fully all aspects of the 3 "R"s, including the use of reasonably practicable alternatives as they become available, minimising animal numbers on protocols and ensuring methodology minimises potential suffering. Laboratories within the UK have investigated a number of approaches to address the 3 "R"s in the shellfish biotoxin monitoring programme. These include pre-screening methods, reduction of duration of test and the number of animals used for each sample and the use of anaesthesia. A number of issues exist for regulators in considering whether and when alternative or refined strategies can and should be required to be used for such testing programmes. These include criteria for validation and when the validation process can be considered to have been completed. Collaboration and good communication between regulators, laboratories and the industry to resolve such issues is essential.

Keywords: mouse bioassay, biotoxins, 3Rs, PSP, DSP

Introduction:

A variety of biotoxins can accumulate in the flesh of bivalve molluscs which feed on phytoplankton species and thus enter the food chain. Consuming contaminated shellfish can cause illness and, in extreme cases, can lead to death. Statutory testing of shellfish destined for human consumption for biotoxins is therefore required under European and UK Food Safety Regulations to ensure the safety of consumers. These regulations set acceptable maximum levels for common toxin groups found in European waters, including Diarrhetic (DSP) and Paralytic Shellfish Poisons (PSP). The reference method currently specified by European Food Safety legislation for Official Control testing for PSP and DSP are mouse bioassays (MBAs). The use of bioassays is regulated by legislation governing the use of animals in experimental procedures both at a European and a national level.

Legislative requirements:

The control of shellfish biotoxin testing in Europe and the UK is summarised in Fig. 1. The reliance on the use of MBAs to detect shellfish biotoxins in national shellfish safety programmes means that effective interaction is essential between those responsible for implementing the 2 strands of legislation, with there being the potential for significant conflict due to inconsistencies in/ differing interpretations of the content of such complex legislation, as seen in Table 1.

The use of the "3 Rs" in marine biotoxin testing in the UK:

In the UK, performance of any animal test requires specific licences granted under the Animals (Scientific Procedures) Act 1986. This Act embodies the principles of the "3 Rs" (Replacement, Reduction and Refinement) as defined by Russell and Burch [1959]. The Act specifies that animals may not be used in experiments if there are other reasonably practical alternative non-animal methods available (Section 5.5 a) and that procedures must use the minimum number of animals and cause the least pain, suffering, distress or lasting harm, whilst producing satisfactory results (Section 5.5 b). A number of approaches have been used within UK monitoring laboratories to try and address these principles with respect to the MBA.

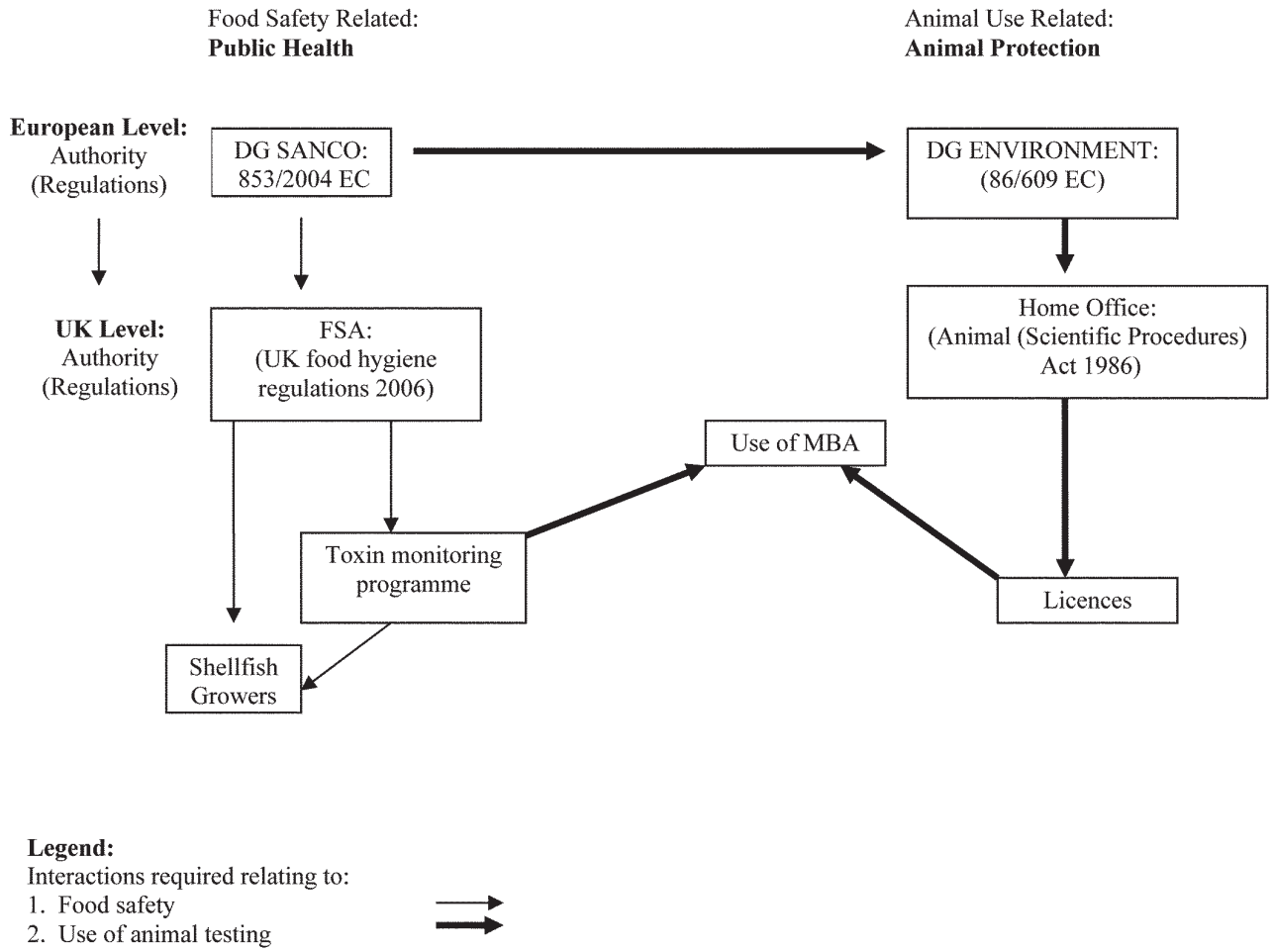

Fig. 1: Diagrammatic representation of Control of Shellfish Biotoxin Testing

Initial advances involved refinement and reduction, and more recently moves have been made towards replacement.

Standard MBA methodology:

PSP Method (AOAC 1995)

- 3 mice per sample
- 1ml* of extract, pH 2-4 injected i/p
- Use further animals for dilution steps
- Death within 60 minutes = positive sample
- **Median** death time gives toxin level of sample
- Death by respiratory paralysis

DSP method (Yasumoto 1978)

- 3 mice per sample
- 1ml* of extract injected i/p
- Death of **2 out of 3** animals within 24 hours = positive result
- Result not quantitative
- Reason for death of animal unknown (body weight loss of 12% even for animals injected with negative samples)

*1ml is 5x recommended volume for weight of mouse used (Karl-Heinz 2001)

A significant number of issues relating to the validity of both the MBAs exist in the literature (Hess 2006). These concerns include: sex/ strain/ weight effects on results (Nagashima 1991, Stabell 1992, Park 1986), within and between laboratory variability (Holtrop 2006, Le Doux 2000, McFarren 1959, Prakash 1971), questions with respect to the appropriateness of currently specified regulatory levels (Anon 2005, Aune 2002, Miles 2005, Miles 2006), the ability of (in particular) the DSP MBA to detect all the toxins of relevance in the group and the occurrence of factors in naturally occurring samples that cause interference with the results (e.g. Aune 1998, Lawrence 1994, Suzuki 1996). Alternative non-animal methods have the potential to be significantly more accurate than biological methods and are likely to provide a far higher level of sensitivity and precision for quantification. There is the potential for significant suffering for the animals used in MBAs using the standard methodologies, including responses characteristic of pain (Roughan 2001) seen after injection and the mode of death. Welfare concerns related to these tests have led to a number of changes to the methods used in UK testing laboratories in an attempt to reduce suffering.

Possible modifications to PSP methodology:
Refinement

1. Use highest pH within allowed range (pH 4). Adjustment of pH up to four has been shown not to affect stability of the toxin or effectiveness of the assay [Indrasena 2000], but significantly reduces potential discomfort for the animal due to the logarithmic nature of the pH scale (a small change in pH value gives large effect in reducing acidity). Currently used in UK to reduce level of suffering.

2. Shorten duration of assay to ≤ 20 minutes. The detection level of the MBA is around 350-400 µg PSP/kg shellfish flesh, whilst the current regulatory limit is 800 µg PSP/kg shellfish flesh. A mouse injected with a sample containing most detectable levels of toxin will have died within 20 minutes with deaths between 20-60 minutes confined to levels near this detection limit [Dennison 2002]. A 20 minute assay therefore significantly reduces the time for animal suffering without risk of non-detection of samples containing toxin levels which would require closure of shellfish areas. Currently used in UK to reduce duration of suffering.

3. Possible use of anaesthesia (Holtrop 2006) – this lengthens the assay duration but is feasible with calibration, as calibrated outcomes were similar with and without anaesthesia, but requires the use of a pre-screen to reduce sample numbers tested for practical use in a high throughput monitoring programme. Currently, the use of anaesthesia for the PSP bioassay is being reconsidered in the UK, as pre-screen available. It may be introduced if a full replacement cannot be achieved soon.

Reduction

1. Optimise the frequency & location of sites tested using risk assessment principles

2. Use 2 mice per sample. As previously stated, the AOAC based method uses the median death time of 3 animals to calculate the level of toxin in a sample. However, using the shorter death time given using two animals provides at least as safe a result (in terms of public health protection), as taking the median death time from three animals. Currently, 2 animals are used per assay in UK. This has reduced animal numbers by >30%.

Replacement

The AOAC has recently accredited the Lawrence HPLC method (AOAC Official Method 2005.06 Paralytic Shellfish Poisoning Toxins in Shellfish [Lawrence 2005]) and this method has been accepted into the European Food hygiene Regulations. However, there has been little apparent change within Europe in the levels of use of the MBA, due it seems, at least in part, to difficulties within some laboratories in implementing the Lawrence method at a practical level. In the UK, a partial replacement to the use of animals has been achieved by the introduction of a pre-screen. Between January 2005 and May 2006 Jellet Rapid Test for PSP™ was used to screen out negative samples in the Scottish testing programme. This was replaced with a modified version of the Lawrence HPLC method [Algoet 2007] for pre-screening throughout the UK from late 2006. Such pre-screening reduces the need for the MBA by between approximately 65 and 90% depending on levels of PSP present.

Possible Modifications to DSP methodology:
Refinement

1. Use clinical signs predictive of death: (cyanosis of extremities, hypothermia, lack of elicited responses and prostration). Pathognomic clinical signs are currently used in UK to reduce the level of suffering.

2. Possible reduction of assay duration, made possible by the use of clinical endpoints. A reduced duration assay is currently in use in the UK to reduce duration of suffering.

Reduction

1. Optimise frequency & location of sites tested using risk assessment principles

2. Use 2 mice per sample, based on mathematical principles:

Using 3 mice/ sample possible results are:

Definitive = 3 mice dead (+ve) OR 3 mice alive (-ve)
Mixed = 2 mice dead + 1 alive (+ve) OR 2 mice alive + 1 dead (-ve)

Using 2 mice/ sample possible results are:

Definitive = 2 mice dead (+ve) OR 2 mice alive (-ve)
Indeterminate = 1 dead + 1 alive (?)

There are then 2 approaches for indeterminate results:

1. Precautionary principle (assume toxin is present as 1 mouse has died) = close field
2. Inject 3rd mouse: will then have 2 + 1 "mixed" result for decision (slight delay in obtaining result)

The UK currently uses a "2 + 1" approach to the number of mice use, giving >30% reduction in animal numbers.

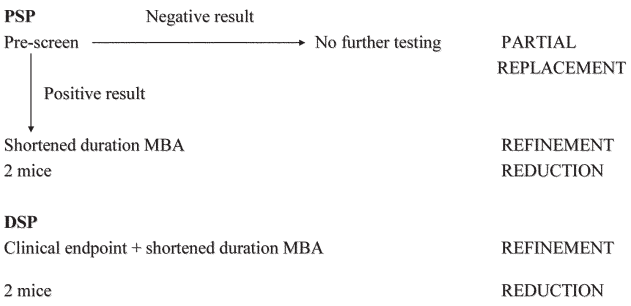

Fig. 2: Current use of MBAs in UK toxin testing programme

Table 1: Main areas of potential conflict between 2 strands of legislation

Content of Regulation EC 85/2004	Content of Directive 86/609/EEC
Use of biological reference methods	Use must be made of reasonably practicable alternatives
Fixed protocols- animal numbers and duration of monitoring are specified with little flexibility for change	All protocols must cause least impact possible on animals (including minimising animal numbers, reducing test durations and using clinical endpoints)
Endpoint is death without intervention	Animals undergoing severe pain or distress which can not be alleviated MUST be killed immediately.

Replacement:

None currently used in the regulatory monitoring programme in the UK.

Summary of current UK testing strategy/use of MBAs in UK:

This is summarised in Fig. 2.

Problems with Progressing Replacement Strategies:

Alternative non-animal methods have the potential to be significantly more accurate than biological methods and are likely to provide a far higher level of sensitivity and precision for quantification. However, as well as issues relating to a lack of certified reference materials, which has been emphasised as a significant hurdle in moving to alternatives by many, including the European Committee on the Validation of Alternative Methods (ECVAM) expert group on shellfish biotoxins [Hess 2006], there appears to be a lack of consensus as to when methods can be considered as validated. Under Directive 86/609/EEC there is a clear system for acceptance of alternatives via ECVAM. By comparison, although EC Regulation 854/2004 indicates methods may be progressed via approval of Standing Veterinary Committee (SVC), there is little clarity in procedure/ requirements for submission. Progressing alternatives to date has proved extremely slow, despite a variety of promising methods and approaches being developed that could potentially replace animal testing either completely or in part [e.g. review Hess 2006, Jellet 2002, Lawrence 2004, Lawrence 2005, Mackintosh 2002, McNabb 2005].

Complete replacement of the PSP bioassay seems possible in the near future using HPLC technology (see above) if regulators and laboratories push for its use.

However, a number of significant issues remain to the replacement of the DSP bioassay. The relevance to human health of intraperitoneal (i.p.) toxicity in mice of some lipophilic compounds that cause positive results in this assay, in particular yessotoxins, has yet to be proven and it has been suggested that some of the compounds currently specified in the Food Hygiene Regulation should be removed [Anon 2005]. Equally, the sensitivity of mice to other compounds in the lipophilic group is poor depending on which extraction method is chosen. Failure of methods to extract certain compounds is one reason that it is a fallacy that the MBA will detect all new toxins when used for routine monitoring. An example of this was the failure to pick up Amnesic Shellfish Poisoning (ASP) in Irish shellfish which contained levels of toxin that caused human illness. It is usual toxicological practice to use the route of human exposure to a toxin when testing, both for relevance and presence. As such, the i.p. route used in the MBA would seem to be highly questionable.

Due to the complexity of the lipophilic toxin group, and the different mode of action of the compounds within it, a flexibility in approach is needed, which may include the use of pre-screens, use of more than one method used for routine monitoring, or acceptance of a testing strategy using marker compounds or naturally incurred materials as reference compounds. It should be a priority for regulators to set and agree pragmatic criteria for method validation, bearing in mind that screening and reference methods may have differing validation criteria. Since the DSP MBA is not itself a validated method, and is known to suffer from a significant level of both false positive and negative results validation of new methods against it would appear inappropriate, a view shared by the ECVAM working group [Hess 2006].

LCMS is the most promising alternative methodology. Methods currently exist that cover all the toxins required to be monitored in the Directive (McNabb 2005, Stobo 2005), but more standards are required for fully quantitative methods. However, it is important to note that the lack of quantification and reliability of the current MBA methodology means that an alternative method that is only partially quantitative may still provide significant improvement in accuracy/ reliability for provision of monitoring for public health. An LCMS method for replacement has been developed [McNabb 2005] and used successfully for over 3 years in New Zealand for monitoring samples.

Summary:

Much can be achieved to reduce the suffering of animals and numbers required to monitor for biotoxins by constructive dialogue between different regulators, scientists and industry. Currently reliance on MBA means there is significant potential for conflict between elements of food safety and animal experimentation legislation at an EC, and therefore national, level. This, and the need to clarify validation

requirements, necessitates urgent resolution within Europe. In order to make better provision for public health, it is important that a transparent mechanism to introduce improved techniques be agreed by all. It appears hopeful that complete replacement of the MBA for PSP should soon be possible. However, until it is recognised by some scientists and food safety regulators that the currently used, non-validated, MBA can not be considered a bioreactor for detection of any toxic compound within monitoring programmes, replacement of the MBA for DSP will remain problematic.

References:

Algoet, M., Luque-Perez, E., Rowland, S., Hatfield, R., Norton, D., Philo, M., Lees, D., 2007. A practical and alternative use of the AOAC HPLC method: the qualitative screening of shellfish samples for paralytic shellfish poisoning toxins. Presentation at 6th International Conference Molluscan Shellfish Safety, 18-23 March, Blenheim, New Zealand.

AOAC 1995. Supplement March 1997. Paralytic Shellfish Poison. Biological method. Final action. In: Hellrich, K. (eds), Official Methods of Analysis. 16th Edition, pp. 22-23, sec 35.1.37. Association of Official Analytical Chemists, Arlington, Virginia, USA.

Anon 2005. Report of the Join FAO/ IOC/ WHO ad hoc Expert Consultation on Biotoxins in Molluscan Bivalves (Oslo, Norway, 26-30 September 2204), 31pp. Rome, Italy: Food and Agricultural Organisation. Website http://www.fao.org/es/esn/food/risk_biotoxin_en.stm

Aune, T., Ramstad, H., Heidenreich, B., Landsverk, T., Waaler, T., Egaas, E., Julshamm, K. 1998. Zinc accumulation in oysters giving mouse deaths in paralytic shellfish poisoning bioassay. The Journal of Shellfish Research, 17:1243-1246.

Aune, T., Sorby, R., Yasumoto,. T., Rasmstad, H., Landsverk, T., 2002. Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. Toxicon 40: 77-82.

Dennison, N., Turriff, J., Kinnear, S., Petrie, J. 2002. Refinement of the Mouse Bioassays for Diarrhetic (DSP) and Paralytic Shellfish Poisons (PSP). Poster presentation. Annual Scand-LAS Symposium, Gardermoen, Norway.

Hess, P., Grune, B., Anderson, D. B., Aune, T., Botana, L. M., Caricato, P., Van Egmond, H. P., Halder, M., Hall, S., Lawrence, J. F., Moffat, C., Polettie, R., Richmond, J., Rossini, G. P., Seamer, C., Vilageliu, J. S., 2006. Three Rs Approaches in Marine Biotoxin Testing. ATLA 34: 193-224.

Holtrop, G., Petrie, J., McElhiney, J., Dennison N. 2006. Can General Anaesthesia be used for the Paralytic Shellfish Poison bioassay. Toxicon 47: 336-347.

Jellet, J. F., Roberts, R. L., Laycock, M. V., Quilliam, M. A., Barrett, R. E., 2002. Detection of paralytic shellfish poisoning (PSP) toxins in shellfish tissue using MIST Alert, a new rapid test, in parallel with the regulatory AOAC mouse bioassay. Toxicon 40: 1407-1425.

Karl-Heinz, D., Hall, R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J.-M., Van de Vorstenbosch, C., 2001. A Good practice Guide to the Administration of Substances and Removal of Blood Including Routes and Volumes. J. Appl. Toxicol. 21: 15-23.

Lawrence, J. F., Chadha, R. K., Ratnayake, W. M. N., Truelove, J. F. 1994. An incident of elevated levels of unsaturated free fatty acids in mussel extracts from Nova Scotia and their toxic effect in mice after intraperitoneal injection. Natural Toxins 2:318-321.

Lawrence, J.F., Niedzwiadek, B., Menard, C. 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J. of AOAC Internat. 88 (6): 1714-1732.

Le Doux, M., Hall. S., 2000. Proficiency testing of eight French laboratories in using the AOAC mouse bioassay for paralytic shellfish poisoning: Inter-laboratory collaborative study. Journal of AOAC International 83: 306-310.

McFarren, E. F., 1959. Report on collaborative studies of the bioassay for paralytic shellfish poison. Journal of AOAC International 42: 263-271.

McNabb, P., Selwood A., I., Holland P., T., 2005. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. Journal of AOAC International 88 (3): 761-772.

Miles, C. O., Samdal, L. A., Aasen, J. A. G., Jensen, D. J., Quilliam, M. A., Petersen, D., Briggs, L. M., Wilkins, A. L., Rise, F., Cooney, J. M., MacKenzie, A. L., 2005. Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. Harmful Algae 4: 1075-1091.

Miles, C. O. Wilkins, A. L., Munday, J. S., Munday, R., Hawkes, A. D., Jensen, D. J., Cooney, J. M., Beuzenberg, V. , 2006. Production of 7-epi-pectenotoxin-2 seco acid and assessment of its acute toxicity to mice. J. Agric. Food. Chem. 54: 1530-1534.

Nagashima, Y., Noguchi, T. Kawabata, T., Hashimoto, K. 1991. Dose-death time curve of paralytic shellfish poisons in ddY strain mice. Nippon Suison Gakkaishi 57: 699-704.

Park, D. L., Adams, W. N., Graham, S. L., Jackson, R. C., 1986. Variability in the mouse bioassay for determination of paralytic shellfish poisoning toxins. Journal of the AOAC International 69: 547-550.

Prakash, A., Medcof, J. C., Tennant, A. D., 1971. Paralytic shellfish poisoning in eastern Canada. Bulletin of the Fisheries Research Board of Canada 177: 1-87.

Roughan J. V., Flecknell P. A.,. 2001. Behavioural effects of laparotomy and analgesic effects of ketoprofen and carprofen in rats. Pain **90:** 65-74.

Russell, W. M. S., Burch, R. L., 1959. The principles of humane experimental technique (special edition, UFAW 1992). Universities Federation for Animal Welfare, Potters Bar, Hertfordshire.

Suzuki, T., Yoshizawa, R., Kawamura T, Yamasaki M., 1996. Interference of Free Fatty Acids from the Hepatopancreas of mussels with the Mouse Bioassay for Shellfish Toxins. Lipids 31(6):641-645.

Stabell, O.B., Steffenak, I., Aune, T., 1992. An evaluation of the mouse bioassay applied to extracts of 'diarrhetic' shellfish toxins. Food Chem. Toxicol. 30: 139-144.

Yasumoto T., Oshima, Y., Yamaguchi, M., 1978. "Occurrence of a new type of shellfish poisoning in the Tohoku district" Bull. Jpn. Soc. Sci. Fish. 44: 1249-1255.