

GENERAL REFEREE REPORTS

Committee on Natural Toxins and Food Allergens

Marine and Freshwater Toxins

JAMES M. HUNGERFORD

U.S. Food and Drug Administration, Seafood Products Research Center, 22201 23rd Dr SE, Bothell, WA 98021, Tel: 425-483-4894, Fax: 425-483-4996, E-mail: james.Hungerford@fda.gov

Summary

In a very busy and exciting year, 2005 included First Action approval of a much needed official method for paralytic shellfish toxins and multiple international toxin symposia highlighted by groundbreaking research. These are the first-year milestones and activities of the Marine and Freshwater Toxins Task Force and Analytical Community. Inaugurated in 2004 and described in detail in last year's General Referee Report (1) this international toxins group has grown to 150 members from many regions and countries. Perhaps most important they are now making important and global contributions to food safety and to providing alternatives to animal-based assays. Official Method **2005.06** was first approved in late 2004 by the Task Force and subsequently Official First Action in 2005 (2) by the Methods Committee on Natural Toxins and Food Allergens and the Official Methods Board. This nonproprietary method (3) is a precolumn oxidation, liquid chromatographic method that makes good use of fluorescence detection to provide high sensitivity detection of the saxitoxins. It has also proven to be rugged enough for regulatory use and the highest level of validation. As pointed out in the report of method principle investigator and Study Director James Lawrence, approval of **2005.06** now provides the first official alternative to the mouse bioassay after many decades of shellfish monitoring.

This past year in April 2005 the group also held their first international conference, "Marine and Freshwater Toxins Analysis: 1st Joint Symposium and AOAC Task Force Meeting," in Baiona, Spain. The 4-day conference consisted of research and stakeholder presentations and symposium-integrated subgroup sessions on ciguatoxins, saxitoxin assays and liquid chromatography (LC) methods for saxitoxins and domoic acids, okadaic acids and azaspiracids, and yessotoxins. Many of these subgroups were recently formed in 2005 and are working towards their goals of producing officially validated analytical methods. (Abstracts from the Baiona 2005 meeting cited in this report can be found in the online version of the conference abstract book in the *Files and Folders* section of the Marine and Freshwater Toxins online community at www.aoac.org.) An active topic for discussion in Baiona and subsequent Task Force activities was the expert consultation for Codex which met in Oslo, Norway in 2004

(previously described and cited in last year's GR report, ref. 1). The consultation group's executive summary report (http://www.fao.org/es/ESN/food/risk_biotoxin_en.stm) describes suggested changes in action levels as well as methods, method validation, and other issues.

September 2005 saw the AOAC Task Force efforts further supported by another symposium, "Marine and Freshwater Toxins: Quality Methods for Food Safety and International Trade," at the AOAC INTERNATIONAL Annual Conference in Orlando, Florida. The multidisciplinary talks at this full day symposium ranged from ciguatoxins to cyanobacterial toxins, and spanned toxicology, biochemistry, molecular biology and analytical chemistry. Again, the symposium preceded Task Force meetings. Toxin subgroups, including a new group on cyanobacterial toxins, met for engaging and productive subgroup discussions. All of these activities were preceded by a Wiley Award symposium for Task Force member Mike Quilliam of NRC Canada. These talks, presented at a half-day symposium on the first day of the Annual Meeting, focused on Quilliam's work with LC tandem mass spectrometry (LC/MS/MS) and certified reference standards and materials, and included related presentations by some of his many research collaborators.

To maintain flow and continuity between symposia and between Task Force meetings, the group now uses new electronic discussion forums. Individual subgroup areas, under the Marine and Freshwater Toxins Task Force, comprise this online community. First introduced by AOAC INTERNATIONAL in early 2005, these new resources are being used to distribute information and to supplement the in-person subgroup meetings and electronic mail in the group's validation efforts.

Toxins, Topics, and Implications

Regarding this year's reports, some of the existing Topic Advisor and Study Director Topics now have revised titles. In some cases, topics were renamed simply to better reflect the emphasis on a particular assay type. For example, Don Richard now reports on *Animal Bioassays for Phycotoxins*. In this case the change was made to improve clarity. Other topic name changes are more fundamental in nature as discussed below.

Henceforth toxin names such as saxitoxins will be used rather than paralytic shellfish poisoning (PSP) toxins. These changes are not mere subtleties but actually reflect a change in how we view the toxins and methods for detecting them. They relate to our understanding of the toxins, their vectors, their associated human illnesses and, ultimately, choice of detection methods. Misconceptions have sometimes been embedded in the illness-and-vector-associated names that we have used over the years. PSP refers to an illness caused by the

saxitoxins in shellfish and similarly, domoic acid is associated with amnesic shellfish poisoning (ASP), and yet it is now known that these toxins and others such as the brevetoxins (*see* Brevetoxins, Immunological Methods report below) are also found in many other organisms in marine food webs. In some cases these are not only the usual molluscan shellfish but finfish used for human consumption. Outbreaks, such as paralytic symptoms caused by saxitoxins (STXs) in pufferfish (4, 5) have been associated with these vectors in areas such as Florida where STXs were not previously a health threat. In contrast to the old adage, what we don't know can hurt us.

How we regard the various toxin groups and associated illnesses not only relates to considerations of risk, it also brings this discussion to the crucial question of what constitutes a member of an illness-associated group of toxins. Outdated information misleads and complicates our choice of analytical methodology. One of the most important topic title changes in this regard concerns analytical methods for the toxins associated with diarrhetic shellfish poisoning (DSP). Discussions of DSP-implicated toxins now relate primarily to okadaic acid, the dinophysins toxins (DTXs), and their associated esterified forms (collectively, the okadaic acid family of toxins). At the same time, however, pectenotoxins (PTXs) are sometimes included among DSP toxins even though they are far less toxic. Not so long ago, even the yessotoxins (YTXs are not diarrhetic) were included among the diarrhetic shellfish toxins. Such misleading nomenclature has played a role in complicating efforts to provide alternatives to the mouse bioassay for this previously ill-defined, confusing and misnamed mix of DSP toxins. At the other end of the spectrum, the azaspiracids (AZAs; *see* report of Ambrose Furey) although far less widespread in their distribution at the time of writing, are strongly diarrhetic. The azaspiracids are also very different from the okadaic acids in their mechanism of action. Such mechanistic differences are critical with regard to applicability of functional assays, since both public health and the mechanism of detection are tied up in the specific toxicological mechanism. For all of these reasons, the specific term okadaic acid rather than DSP will instead be used in topic titles.

Although not as toxic as the saxitoxins or the ciguatoxins, the okadaic acids and the azaspiracids toxins have great importance with regard to shellfish, seafood safety, and international trade. In the context of the Task Force methods selection criteria (http://www.aoac.org/marine_toxins/analyt_criteria.htm) they cause considerable economic and human health impact. These toxins are also the targets of upcoming method validation efforts. In addition to validation of existing methods, development of new methodology continues (6). Both validation and methods development efforts are discussed in more detail below by Topic Advisors.

In related developments, new Topic Advisor appointments have also been made to provide more complete topical and methodological coverage of the toxins. This year Topic Advisor Philipp Hess (Ireland Marine Institute, Galway, Ireland) reports on methods development and validation

efforts in okadaic acids by LC, and toxicologist Aurelia Tubaro (University of Trieste, Italy) reports on yessotoxins. Tubaro is also chair of a subgroup on this topic and her report reflects on both toxicological and analytical aspects of this controversial family of compounds. As part of our efforts to more completely cover the cyanobacterial toxins, Ambrose Furey (Cork Institute, Bishopstown, Cork, Ireland) will also report on the anatoxins in addition to his other topic, the azaspiracids).

Additional changes will be suggested by the General Referee for 2006. It is thought, for example, that 2 crucial aspects of toxin method validation should be addressed. These include the production of reference toxins and reference materials (ultimately, certified and readily available) and the quality, continuity, and continuing availability of biological and biochemical materials used in toxin assays. The former impacts all forms of toxin detection methods and the latter is a crucial but underemphasized requirement for the success of assays ranging from immunoassays to binding assays and the various functional assays based on mechanisms of toxicity.

Finally, this year's report includes, in some cases, annotations from subgroup activities of the Marine and Freshwater Toxins Task Force to enhance the information given in the reports below. In this same vein, readers are encouraged to join in subgroup discussions both online and in Task Force meetings.

Selected Study Director and Topic Advisor Reports

Anatoxins.—Topic Advisor Ambrose Furey, PROTEOBIO, Mass Spectrometry Centre for Proteomics and Biotoxin Research, and All-Ireland Safefood Biotoxin Research Network Co-ordinator (Food Safety Promotion Board), Cork Institute of Technology, Department of Chemistry, Bishopstown, Cork, Ireland, Tel: 353-21-4326701, Fax: 353-21-4345191, E-mail: afurey@cit.ie. Furey reports that anatoxins continue to be the target of intense research. Anatoxin-a (AN) and its analog homoanatoxin-a (HMAN) are neurotoxic alkaloids produced by several cyanobacteria genera, including *Anabaena* and *Planktothrix* (formerly *Oscillatoria*) spp. (7–10) that contaminate lakes, reservoirs and rivers. AN is a low molecular weight bicyclic secondary amine and is a potent nicotinic agonist that acts as a post-synaptic, depolarizing, neuromuscular blocking agent (11). Fatal intoxications due to AN have included cattle in Finland (12) and dogs in Scotland and Ireland (13, 14) These toxic incidents are frequently dramatic due to the rapidity of onset of toxic symptoms, which include muscle fasciculation and convulsions, resulting in death within minutes due to respiratory failure (11, 15). AN and HMAN are unstable and are converted to epoxy- and dihydro-degradation products, processes that are dependent on a number of environmental parameters, especially pH and light (16, 17).

Most early studies of freshwaters contaminated by AN, have been conducted using LC-UV (18), but this method does not detect AN degradation products. A highly sensitive LC method with fluorimetric detection, using the 4-fluoro-

7-nitro-2,1,3-benzoxadiazole (NBD-F) derivatization reagent, was developed for the simultaneous determination of AN, HMAN, and their degradation products (14, 17). Gas chromatography (GC) has also been used to determine derivatized AN in freshwaters using both MS and electron capture detection (19–23). A number of LC/MS methods have been used for the determination of AN in cyanobacteria (24–27), food supplements (28), and fish muscle (29).

Forensic investigations of suspected AN poisonings are often troubled by problems in detecting this toxin in biological and environmental matrixes due to its rapid decay (half-life <1 day; 16). Another major impediment in the investigation of suspected AN poisonings is the lack of commercial availability standards; currently there are no certified standards or reference materials for AN, HMAN, and their epoxy- and dihydro degradation products.

Another serious difficulty which may especially hamper forensic or in vivo investigations of suspected AN poisonings is the presence of the amino acid phenylalanine (Phe), which not only shows a similar chromatographic elution profile but possesses the same nominal mass as AN, and also produces isobaric fragment ions in its MS/MS spectra. The death of a young adult in the United States in July 2002, following exposure to lake water, was ascribed in the coroner's report to AN poisoning. This conclusion was reached based mainly on evidence of the identification of AN using LC single quadrupole MS (LC/MS). This identification has subsequently been shown to be incorrect and was, in fact, the result of the inability of LC/MS to discriminate between Phe and AN (30). In September 2003, an investigation of the fatal intoxication of 2 dogs in a lake in eastern France revealed the presence of AN in benthic cyanobacteria (*Planktothrix*), found along the shoreline. However, the large quantity of Phe present prevented confirmation and quantification of AN in the stomach and intestine contents of the dogs (31).

In the Advisor's Laboratory a detailed study was carried out on the mass fragmentation pathways for AN and HMAN using a quadrupole ion-trap (QIT) instrument and this has led to the development of sensitive LC/MS methods for their detection in forensic samples (32). Further studies were extended to include the AN degradation products; hybrid quadrupole time-of-flight (QqTOF) MS was used to confirm formulae assignments of the product ions using high mass accuracy data. Nano electrospray ionization (ESI) is a valuable source that enables detailed MS studies to be carried out on very small sample quantities. This is particularly relevant to AN studies because only picogram-microgram quantities of the anatoxins are found in forensic samples. QIT MS complements QTOF MS data by permitting the elucidation of fragmentation pathways, and these methods were applied to the predominant degradation products of AN and HMAN (33). Research was also carried out in the Advisor's Laboratory to develop LC/MS/MS methodologies

to avoid the mis-identification of AN in forensic investigations of acute neurotoxic poisonings (34) due to the presence of the Phe.

Because of the prevalence of AN in freshwater sources worldwide and owing to the potent, toxic nature of this compound, many research teams are devising strategies for the analysis of potable waters and cyanobacterial scum. A hydrophilic interaction LC with electrospray mass spectrometry (HILIC-MS) multitoxin method for the analysis of AN, cylindrospermopsin, deoxycylindrospermopsin, saxitoxin and analogs and microcystin-LR was recently developed by Dell'Aversano et al. (27). Analysis of algal samples required no sample cleanup or preconcentration step. A solid-phase microextraction (SPME) has been developed by Ghassempour et al. (35), which reports a linear range of 50–10 000 ng/mL, with a detection limit of 11.2 ng using a polyaniline coated fiber. Dagnino and Schripsema (36) have developed a quantitative proton nuclear magnetic resonance (NMR) method for AN with verification by GC/MS, which is suitable for analyzing microgram quantities of AN in very dilute samples. A toxicological study examining the nicotine effects on the activity of mice exposed prenatally to AN has demonstrated that there are no enduring effects observed in exposed mice following nicotine challenge, nor did mice exhibit changes in baseline levels of motor activity (37). Research in 2 alkaline Kenyan crater lakes, Lake Sonachi and Lake Simbi, has found for the first time that the cyanobacterium *Arthrospira fusiformis* is a producer of microcystins and AN (38). Codd et al. (39), examined the properties and occurrence of AN in order to establish the pertinent factors which need to be considered when developing strategies to protect human health. The paper is a useful reference for regulators recommending a protocol based on a set of logical steps to minimize risk to consumers: (1) situation assessment; (2) assessment of priorities for action; (3) identification of control points; (4) economic appraisal of technically feasible options; (5) environmental appraisal of options; (6) selection of options; (7) development of a plan of action; (8) implementation of plan of action; (9) assessment of effectiveness; and (10) modification of the plan accordingly.

Animal Bioassays for Marine Toxins.—Topic Advisor Donald J.A. Richard, New Brunswick, E1C 4L8, Canada, Tel: 506-855-4824, E-mail: donr@nb.sympatico.ca, or donrichard@phycotoxins.ca. The possibility of reduced animal use gained momentum with the June 2005 acceptance by AOAC of the Lawrence method (3) for PSP toxins. This method becomes the first officially approved alternative to the mouse bioassay since it became an Official First Action in 1959; several other investigations into alternatives have also been published. The applicability of in vitro methods as a screening tool was investigated in California (40). In general, and for California conditions, the authors felt that the use of

any of the 3 methods (RIDASCREEN[®], MIST Alert[™], now Jellett PSP Rapid Test; and the neuroblastoma bioassay) would not compromise public health. It was also noted that no method could replace the timeliness of the mouse bioassay for decision-making purposes and that for a laboratory with high throughput, it was still the least expensive option. Using a post-column HPLC-fluorescence detection (FLD) method (41), but modifying the sample preparation, Asp et al. (42) concluded that their procedure could safely substitute for the mouse bioassay. However, a successful interlaboratory, interspecies validation trial will probably be required prior to wider acceptance. Although not directly related to the mouse bioassay, Van Egmond et al. (43) confirmed the applicability of the Lawrence method to European shellfish by incorporating strict guidelines on methodology. Their 3 previous studies showed coefficients of variation ranging from 35 to 62% for decarbamoylsaxitoxin in sharp contrast to the 8% variation in their most recent study. The next few years will no doubt encompass revolutionary changes in the way PSP toxins are analyzed and managed within the laboratory environment. Now is an opportune time to revisit all aspects of PSP management, including methods and equivalency, regulatory limits and risk assessment. Some of the studies are already under way. A strategic resolution on the value of improving the existing mouse bioassay method is essential. Unless it can be replaced in its entirety, and within the foreseeable future, the evaluation and incorporation of several improvements regarding accuracy, precision and overall animal reduction should be pursued.

Yotsu-Yamashita, Topic Advisor for Tetrodotoxins, has kindly provided the first English translation of the mouse bioassay method for the detection of tetrodotoxins (TTXs) in pufferfish meat (44), which is accepted for official use in Japan. This procedure is provided in her topic report.

Azaspiracids.—Topic Advisor Ambrose Furey, PROTEOBIO, Mass Spectrometry Centre for Proteomics and Biotxin Research, and All-Ireland Safefood Biotxin Research Network Co-ordinator (Food Safety Promotion Board), Cork Institute of Technology, Department of Chemistry, Bishopstown, Cork, Ireland, Tel: 353-21-4326701, Fax: 353-21-4345191, E-mail: afurey@cit.ie. Azaspiracids (AZAs) are a group of polyether toxins that cause food poisoning in humans (45). These toxins, produced by marine dinoflagellates, accumulate in filter-feeding shellfish, especially mussels (46). Furey reports that the biotoxin field of AZAs remains an important topic of research; current areas of interest include LC/MS method development, organic synthesis of AZAs, studies into the mechanism of action, and the toxicological effects of the AZA toxins.

In 1998, Japanese and Irish researchers isolated AZA1 from shellfish which had been implicated in human intoxications, and this work resulted in the declaration of a

new toxin syndrome which was named Azaspiracid Poisoning (AZP; 47). However, uncertainties persisted regarding the structure of AZA1; an attempted total synthesis of AZA1 by Nicolaou et al. (48–51) led to a proposed revision of the assigned structure.

Furey and his research team at PROTEOBIO, CIT, Cork, developed sensitive LC-ESI-MSⁿ methods for the determination of the major AZAs (AZA1-3) and their hydroxyl analogs (AZA4, AZA5, AZA7, AZA8, AZA9, AZA10, and AZA11). The major MSⁿ fragmentation pathways in the hydroxylated analogs were elucidated using hydrogen/deuterium (H/D) exchange experiments. A rapid and sensitive LC/MS³ method was developed using unique parent and product ion combinations, involving the ion resulting from the fragmentation of the A-ring; this work facilitated the development of a reliable quantitative LC/MS³ method capable of determining all 11 known analogs of AZA (52).

Hess et al. (53) recently presented results obtained over a 3 year period where AZAs accumulate primarily in the hepatopancreas of mussels. This is contrary to results obtained by James et al. (54) whose extensive studies demonstrated that AZA dissipates into all shellfish tissue compartments and who therefore recommends that the meat must be tested in any workable testing regime to protect human health (45, 54, 55). Hess et al. (53) found that cooking AZA-contaminated mussels concentrated AZA levels 2-fold through the loss of water during the cooking process. These observations and discrepancies have serious implications for the validation of methods to be used in monitoring and for deciding upon a regulatory level to ensure human safety. A study to determine the most efficient extraction solvent for determining levels of AZA in shellfish tissue also revealed that duplicate extraction of samples with 100% methanol was the most efficient approach (53). *Note from General Referee:* These issues should be sorted out prior to validation of methods for the AZAs.

Cytotoxicity tests conducted to study the effects of AZA1 on neuroblastoma cells were performed by Roman et al. (56). The results demonstrated that AZA1 disrupts the cytoskeletal structure, inducing a time- and dose-dependent decrease in F-actin pools. A link between F-actin changes and diarrhetic activity has been suggested, and this may explain the severe gastrointestinal disturbance induced in cases of AZA intoxication. AZA1 causes a significant increase in $[Ca^{2+}]_i$ levels in lymphocytes. Control of $[Ca^{2+}]_i$ is a critical component of cellular homeostasis for all cell types. Elevation of $[Ca^{2+}]_i$ levels can lead to cell death. These studies may form the basis for the development of a screening test to determine the presence of AZA in shellfish extracts.

Alfonso et al. (57) has determined that AZA4, a low abundance hydroxyl analog of AZA3 found in AZA-contaminated shellfish tissue, has a distinctly different

mode of biological action from that of the other known analogs of AZA. Changes in intracellular calcium concentration [Ca^{2+}]; are one of the main ways in which information is transferred between cells; when Ca^{2+} signalling is stimulated in the cell, the levels of cytosolic Ca^{2+} increase. AZA4 appears to inhibit Ca^{2+} entry in human T lymphocytes. Experiments conducted with fluorescence digital microscopy indicated that although AZA4 did not modify cytosolic Ca^{2+} in resting cells, it did appear to inhibit increases in Ca^{2+} levels induced by the drug thapsigargin (Tg). When AZA was added to cells along with nickel, a known Ca^{2+} inhibitor, the results were additive. AZA4 was also shown to inhibit maitotoxin (MTX)-stimulated Ca^{2+} influx by 5–10%.

Twiner et al. (58) has examined the cytotoxicity of AZA1 using 7 cell types; his investigations suggest that human T lymphocytes (Jurkat cell line) are very sensitive to AZA1. However, all 7 cell types tested were adversely affected by AZA1, and the effects of the toxin were both time- and concentration-dependent. It was shown that AZA1 had a profound effect on the arrangement of F-actin in the Jurkat cells with the concurrent loss of pseudopodia, which are cytoplasmic extensions necessary for cell motility and chemotaxis.

Colman et al. (59) demonstrated that AZA1 has a potent teratogenic effect in finfish, inducing dose-dependent effects on embryonic heart rate, developmental rate, and hatching success. The results of these and other toxicological and cytotoxicity-based experiments suggest that the AZA family of compounds may have different targets within the biological system. The implication is that their mode of toxicity is very complex and they are capable of inducing diverse toxicological effects (60, 61).

A comprehensive review of AZAs topic has been published by Furey et al. at PROTEOBIO, CIT. This review provides an invaluable overview of the pertinent issues relating to AZA intoxication of shellfish and gives a detailed summary of the relevant research conducted on the AZAs, including: (1) the historical background of AZA poisoning (AZP); (2) investigations of suspected AZP incidences; (3) toxicological studies and bioassays; (4) etiology of AZAs; (5) structural elucidation of AZAs; (6) quantitative methods for the determination of AZAs; (7) synthetic studies on AZAs; and (8) geographical, temporal and species variation of AZAs in shellfish (45).

Brevetoxins, Immunological Methods.—Topic Advisor Jerome Naar, Center for Marine Science, University of North Carolina at Wilmington, 5600 Marvin K. Moss Ln, Wilmington, NC, 28409, Tel: 910-962-2367, Fax: 910-962-2410, E-mail: naarj@uncw.edu. In recent years several immunoassay formats have been developed for analysis of brevetoxins, including radioimmunoassay (62), enzymatic assay (competitive ELISA; 63), and electrochemical immunoassay (64). An

immunohistochemistry technique was also developed by Bossart et al. (65) to assess and quantify brevetoxin exposure in marine mammals.

Most recently, Briggs et al. (personal communication) and Poli et al. (presented at the AOAC annual meeting in Orlando) have developed 2 new immunoassays. The Briggs et al. assay is a competitive ELISA developed at AgResearch, New Zealand, using anti-brevetoxin antibodies raised in sheep by immunization with fetuin-brevetoxin-2 conjugate. The assay format uses antibody as the plate coating reagent and brevetoxin-2 labeled with horseradish peroxidase (HRP). The Poli assay is a competitive displacement electrochemiluminescent (ECL) format for PbTx-2-type brevetoxins. The assay is performed in 96-well microtiter plates using BioVeris[®] technology. Biotinylated anti-PbTx antibodies are mixed with ruthenylated PbTx-3 and either unlabeled PbTx-3 (standard curves) or unknown samples. After incubation, streptavidin-coated paramagnetic beads are added to allow separation of the bound fraction. As the sample enters the analyzer flow cell, the paramagnetic beads are concentrated on a magnet, voltage is applied, and light is emitted by the bound ruthenylated toxin. Unlabeled toxin in the unknown sample competes with the ruthenylated toxin for antibody binding, reducing the ECL signal in a concentration-dependent manner. In the text that follows, uses of brevetoxin antibody-based assays are discussed.

In shellfish monitoring, for ethical, safety, accuracy, and precision reasons, replacing the regulatory mouse bioassay for brevetoxin monitoring in shellfish has long been a goal of the scientific and regulatory community. Dickey et al. (66) reported the results of a multilaboratory study of 5 methods, including an immunoassay as potential replacements to the mouse bioassay. Aside from the competitive enzyme-linked immunosorbent assay (ELISA; 63), methods included one instrumental assay (HPLC/MS) and 3 activity-specific bioassays (N2A neuroblastoma cytotoxicity assay and 2 formats of the sodium channel receptor binding assay). Among these methods, the competitive ELISA correlated the most favorably with the actual mouse bioassay and appears to be a good candidate to replace it.

However, since the poisoning event in New Zealand and the identification of several groups of molecules that can potentially be implicated in neurotoxic shellfish poisoning (NSP), shellfish monitoring using an immunoassay (which measures a total concentration of brevetoxin-like compounds) to replace the actual toxicity-related (mouse) assay has raised some concerns. It is known that contaminated shellfish contain multiple brevetoxins and toxin metabolites (67). These different compounds are also believed to possess different potencies (68). The exact evaluation of the potency of brevetoxin metabolites in mammals is still hampered by the absence of standards of these compounds. Therefore, evaluating the toxicity of shellfish based solely on the total concentration of brevetoxin-like compounds present

without indication of their identity is not straightforward. Because of their higher polarity, some of the known brevetoxin metabolites are not extracted from shellfish by the regulatory protocol for shellfish monitoring. Thus, to date, they are not taken into account in the regulatory monitoring for shellfish bed re-openings.

Whether or not to include these recently identified compounds in monitoring has direct implications on the protocol to be used. This issue was actively discussed during the NSP subgroup session held in St. Louis, Missouri, during the AOAC annual meeting of 2004. Different extraction and cleanup protocols are being evaluated. Objectives are still to identify which sample preparation will provide the best correlation between the results obtained using the regulatory MBA and immunoassays.

Because of the high affinity and specificity of antibodies, immunoassays are also excellent tools to diagnose exposure to brevetoxins. Woofter et al. (69) described the use of a radioimmunoassay to measure brevetoxin levels on blood collection cards after exposure in mice. They concluded that the technique presents a better sensitivity than the receptor binding assay and allows detection of toxins in mice blood up to 2 days postexposure. In 1998, Bossart et al. (65) demonstrated the implication of aerosolized brevetoxins in the manatee mortality of 1996 using an immunohistochemistry staining technique on lung, nose, and throat tissues of dead animals. The competitive ELISA has also been used in several studies (70, 71) to quantify human occupational and recreational exposure to aerosolized brevetoxins at the beach during red tides. More recently, this method was used to diagnose brevetoxin exposure during 2 recent marine mammal mortality events (72–74). During these studies, toxins were detected by ELISA in numerous fluids and tissues (blood, urine, stomach contents, kidney, lungs, muscle, cerebellum, and liver) of manatees and dolphins fatally poisoned by brevetoxins.

In the last few years, immunoassays have been used extensively as research tools to study the transfer and metabolism of brevetoxins in food webs. Poli et al. (67) described the use of a brevetoxin immunoaffinity column to purify brevetoxins and brevetoxin metabolites in urine from people intoxicated after consumption of shellfish contaminated during red tides, while Naar et al. (68) used an HPLC-ELISA technique to identify metabolites in shellfish and the role of PbTx-2 as precursor for their production.

Very recently, by use of the competitive ELISA, brevetoxins were demonstrated to accumulate in fish despite their known ichthyotoxicity (71, 74, 75), resulting in a mass mortality of dolphins in 2004. Flewelling et al. (73, 74) used the same technique to demonstrate involvement of brevetoxin associated with seagrass in a large scale manatee mortality in 2002. Competitive ELISA was also used by Bourdelais et al. (76) to identify raphidophytes as a source of brevetoxin in the U.S., and by Weidner et al. (77) to show very limited variation in brevetoxin accumulation between individual shellfish from the same bed during red tides.

Capillary Electrophoretic Methods for Marine Toxins.—Topic Advisor Ana Gago-Martinez, Universidad de Vigo, Dpto. Química Analítica y Alimentaria, Facultad de Ciencias, Campus Universitario de Vigo 36200-Vigo-E, Spain, Tel: 34-986-812284, Fax: 34-986-812382, E-mail: anagago@uvigo.es. Improvements in the analytical conditions for the application of high performance capillary electrophoresis (HPCE) have allowed consideration of this technique as a promising alternative for the analysis of algal toxins. These improvements have been focused on the improvement of detectability, which is considered one of the main drawbacks of this approach because of the very low (nanoliter) quantities of sample injected. These improvements have been achieved by using online strategies based on various stacking modes. Other improvements, such as in selectivity, have been realized by using alternative CE modes as micellar electrokinetic capillary chromatography (MEKC). Applications of preconcentration by stacking were carried out for the CE/UV analysis of domoic acids and YTXs. This approach improved sensitivity, resulting in detectabilities in most cases comparable to those obtained through HPLC/UV. The efficiency of MEKC was also demonstrated in its application to the analysis of YTXs. Progress in these areas has been presented recently (78), and manuscripts are in press and in preparation by Gago-Martinez and de la Iglesia. Also, improvements of the initial conditions for the capillary electrochromatographic analysis of domoic acids (Das) have been found by Gago-Martinez and Vaquero, and the results will be soon submitted for publication. These results demonstrate the potential of this technique in this particular application.

Cell Bioassays for Phycotoxins.—Topic Advisor Ronald Manger (Fred Hutchinson Cancer Research Center, PO Box 19024, Seattle, WA, 98109-1024, Tel: 206-667-5838, Fax: 206-667-4182, E-mail: rmanger@fhcrc.org. Manger reports continued examination of their previously developed MTT (tetrazolium dye substrate for colorimetric viability via mitochondrial dehydrogenase) cell-based assays for toxins active at the voltage gated sodium channel (79–81) in conjunction with the application of voltage sensitive dyes and flow cytometry. As discussed in last year's report, our preliminary studies indicate that flow cytometry offers a distinct advantage in minimizing the assay time from hours to minutes in a manner similar to that reported previously by other laboratories (82–84). These initial studies were performed with the murine neuroblastoma cell line, N2a, and have now been extended to human neuroblastoma cells (SK-N-SH). Both of these cell lines have previously demonstrated utility in our tetrazolium cell-based assays for marine toxins. Current studies are focusing on enhancing sensitivity of the flow cytometric method to the levels observed by MTT cell bioassay. The utility of functional MTT-based cell (cytotoxicity) assays has been recently reported by others as a rugged, reproducible, and high throughput method for application in ciguatera fish poisoning studies (85, 86), and has shown application for assessing exposure to ciguatoxins by analysis of extracted blood (87,

88). The extremely high sensitivity of the assay was critical to success in both of these applications. In another application the relative sensitivities of the MTT cytotoxicity assay (highest sensitivity for ciguatoxins) versus the receptor binding assay (better for brevetoxins) were exploited: Dechraoui et al. (89) used the cytotoxicity assay in conjunction with the receptor binding assay (see 2005 TA Report of Fran Van Dolah) as a means of discriminating ciguatoxins from brevetoxins in barracuda from the Florida Keys.

Recently reported (90) fluorimetric-based cell assays show an okadaic acid dose-dependent disruption of F-actin in human colonic epithelial cells. Similar actin disruption was observed with ostreocin-D, an analog of palytoxin (91) in freshly isolated enterocytes and human neuroblastoma cells, and with palytoxin on the human neuroblastoma cell line BE(2)-M17 (92). These studies show the potential utility of cytoskeleton alterations as a method to detect and assess activity of potent marine toxins. The most sensitive assay for palytoxins, with detectability in the low picomolar range, a cell assay originally developed in 1993, uses antibodies to palytoxin for hemolysis neutralization (93). This technique was successfully applied by Onuma et al. (94) to investigate a human fatality in 1999 involving *Ostreopsis* blooms and plankton-feeding sardines. Interest in palytoxin and ostreocins and possible implications in human illnesses and *Ostreopsis* blooms was revived in July 2005, when 150 individuals were hospitalized in Genoa, Italy (95). The illnesses were tied to the inhalation of a toxic aerosol associated with a bloom of *Ostreopsis ovata*. Local authorities closed seafood harvest in the area as a precaution.

An exciting advancement in near-real time detection methods for harmful algal species based upon detection of action potential activity in neuronal networks grown over microelectrode arrays has been recently described (96). This method shows potential for assessing toxin levels during algal blooms. A novel cell electronic sensing system in conjunction with genetically modified cells has demonstrated utility in real-time dynamic monitoring of microcystins (97). Cell assays have also been developed for YTXs, and validation studies are being pursued. This topic is explored in more detail in the Yessotoxins Topic Advisor report prepared by Aurelia Tubaro.

Ciguatoxins by LC/MS.—Topic Advisor Richard J. Lewis, University of Queensland, St. Lucia, 4072, Qld, Australia, Tel: 617-3346-2984, Fax: 617-3346-2101, E-mail: r.lewis@mailbox.uq.edu.au. LC/MS approaches to the detection of ciguatoxins (CTXs) await the development of rapid extraction and simple cleanup procedures that minimize matrix effects. 3H-brevetoxin binding to rat brain membrane or cytotoxicity assays has proven useful for monitoring LC eluants in parallel with MS data acquisition, especially when standards are not available or new CTXs are encountered. At the Marine and Freshwater Toxins Analysis: First Joint Symposium and AOAC Task Force Meeting (April 11–15, 2005, in Baiona, Spain) and in even greater detail at Pacificchem 2005 in Honolulu, Hawaii, R. Dickey, U.S. Food

and Drug Administration (FDA), summarized the regulatory status of ciguatera in the U.S., and described laboratory-based cytotoxicity screening and confirmatory LC/MS methods currently used in responding to ciguatera outbreaks. These presentations (85) highlighted the still unfulfilled need for facile diagnostic and fish screening methods for ciguatera. Towards this end, Tsumuraya (Osaka Prefecture University, Japan) described a synthetic approach to the development of a direct sandwich immunoassay for ciguatoxins (98). In recent publications, binding and cytotoxicity assays identified CTXs and not brevetoxins in toxic Florida barracuda, and showed that the cytotoxicity assay was capable of detecting CTXs in blood (87). MS data were not provided. To facilitate the assessment and validation of rapid extraction and detection methods, a CTX tissue bank has been proposed. Such a resource is much needed by the ciguatera community. More discussion on this topic can be found in the Ciguatoxins online forum of the Marine and Freshwater Toxins Community (link from www.aoac.org).

Domoic Acids.—Study Director Michael A. Quilliam, National Research Council of Canada, Institute for Marine Biosciences, 1411 Oxford St, Halifax, Nova Scotia, B3H3Z1, Canada, Tel: 902-426-9736, Fax: 902-426-9413, E-mail: michael.quilliam@nrc.ca. Reviews on the chemical analytical methods (99) toxicology (100) and chemistry (101) and of domoic acid (DA) have been published recently. The synthesis of isodomoic acid C has been reported (102). This isomer of DA has been observed in New Zealand shellfish and is produced by a New Zealand (NZ) strain of *Pseudonitzschia australis* (103). Initial receptor assay data reported by the NZ group suggests that the compound has a much lower toxicity than DA. Wekell et al. (104) published a useful article on the origin and effectiveness of the regulatory limits for PSP and amnesic shellfish poisoning (ASP) toxins in shellfish. Extraction procedures for DA in shellfish tissues were discussed in 2 papers (105, 106).

Further studies on the LC-UV method for DA were published by Lopez-Rivera et al. (107) and Hess et al. (108). The latter also compared performance of LC-UV and LC/MS methods as part of performance assessment studies in the United Kingdom and Ireland. Kodamatani et al. (109) developed a new sensitive method for DA using HPLC with electrogenerated tris (2,2'-bipyridine)ruthenium(III) chemiluminescence detection. A new method for ASP toxins based on hydrophilic interaction liquid chromatography-mass spectrometry (HILIC/MS) has been published (110). This method has the potential of allowing the simultaneous analysis of both ASP and PSP toxins. Several other methods based on reversed-phase LC/MS have been published in recent years (111–117). CE and electrochromatography methods have also been reported for DA (118–120).

Surface plasmon resonance sensors for DA have been reported, one based on antibodies (121) and the other based on an imprinted polymer (122). These methods offer the potential of very high throughput. Electrochemically based immunosensor methods have been investigated by Micheli et al. (123) and Kania et al. (124). Maucher and Ramsdell (125)

developed an interesting procedure for ultrasensitive detection of DA in blood using blood collection cards coupled with competitive ELISA. In experiments with exposed mice, DA was still quantifiable (>0.7 ng/mL) at 4 h from blood spot extracts and still detectable at 24 h when compared to control blood spots. This method could be a very effective means of monitoring DA in marine mammals in the field, as well as in human populations.

The National Research Council of Canada's Certified Reference Materials (CRM) Program has recently released replacements for their instrument calibration solution and mussel tissue homogenate CRMs for ASP toxins (CRM-DA-e and CRM-ASP-Mus-c, respectively).

Domoic acids by ELISA.—Study Director Hans Kleivdal, Biosense Laboratories AS, HIB-Thormøhlensgate 55, N-5008 Bergen, Norway, Tel: 47-5554-3967, Fax: 47-5554-3771, E-mail: hans.kleivdal@biosense.com. The Study Director submitted the following: Since last year's report, the collaborative study report (126) on the Biosense ASP ELISA has received a positive vote from the Task Force, and was approved for further OMA review. The AOAC style study report has now been prepared, and will be formally submitted in the near future. Recently, Maucher and Ramsdell (127) described the use of the Biosense ASP ELISA to analyze the blood of DA-exposed mice after the extraction from blood collection card. This study demonstrates the detection capability of the method with a limit of quantitation (LOQ) of 0.7 ng/mL in whole blood extract. It is reported to be the most sensitive method in both LOQ and limit of detection (LOD) in blood samples, and will allow the detection of DA up to 48 h post exposure in blood extracted from blood collection cards. Maucher and Ramsdell also described the use of the Biosense ASP ELISA for the determination of DA in milk, plasma, and urine from exposed rats, and how the DA acid was transferred from spiked milk to the plasma of nursing neonatal rats (127). A strategy on how to improve the sensitivity in blood and other body fluids even further, using different cleanup strategies, was presented at the recent AOAC Task Force symposium in Baiona, Spain (128). As an alternative to the conventional ELISA, Micheli et al. (129) reported the development of an immunosensor for the determination of DA in shellfish. In this study, the direct competitive spectrophotometric ELISA of Garthwaite et al. (130), was transferred into an indirect competitive immunosensor ELISA using a screen-printed electrode system as transducer for differential pulse voltammetry. The DA conjugates and polyclonal sheep antibodies used in the immunosensor ELISA are the same as those used in the Biosense ASP ELISA, showing that competitive immunoassays can be miniaturized into the electrochemical sensor format once suitable conjugates and antibodies are available. A limited in-house validation study was conducted, reporting an LOD at 5 ng/mL, a recovery from shellfish samples spiked with mussel tissue reference material MUS-1 (NRC-CRMP, Canada) at $87 \pm 7\%$, and the repeatability in the range of 5–9%. Yu et al. (131) recently reported on the development of another spectrophotometric ELISA for the

determination of domoic acid in shellfish. Rabbits were immunized with DA conjugated to keyhole limpet hemocyanin as previously described by Smith and Kitts (132) for the development of monoclonal antibodies in mice. The antibodies from rabbits were characterized and successfully used in a direct competitive ELISA format. The recovery for DA spiked into mussel samples at 0.025–0.5 mg/kg, was 81% with a repeatability of 8.5%. The LOD was not accurately defined, but was expected to be <0.025 mg/kg based on the recovery studies.

LC/MS/MS Detection of Marine Toxins.—Topic Advisor Patrick Holland, Cawthron Institute, Private Bag 2, Nelson, New Zealand, Tel: 64-3-548-2319, Fax: 64-3-546-9464, E-mail: patrick.holland@cawthron.org.nz. Holland reports significant increases in uses of LC/MS for both quantitative and qualitative determination of marine toxins, particularly the lipophilic classes. The HABTech03 workshop on algal toxins held in New Zealand included demonstrations of LC/MS techniques and the published proceeding contain many papers on applications of LC/MS (133). The LC/MS/MS (SRM) multiresidue method in routine use in New Zealand has been formally published including the in-depth within-laboratory validation data and results of an interlab study (117, 134). This method has been applied to a novel field sampling system using a polymeric adsorbent to give time-integrated monitoring of growing waters (135). A similar multitoxin LC/MS/MS method determined YTXs, 45OHYTX, carboxyYTX, and several other DSP toxins in shellfish (136). Another LC/MS/MS method for a range of toxins had high sensitivity and reproducibility using a neutral rather than acidic LC buffer system with negative ion detection (137). However, no recovery data were reported and the extraction method precluded determination of YTXs. Stobo et al. reported on an LC/MS selected-ion monitoring (SIM) method for all lipophilic toxins regulated under European Union (EU) legislation (138). Using a neutral buffer system, the analysis was completed in 13 min and gave high precision for key toxins in several shellfish species at regulatory levels. An LC/MS SIM multiresidue method was validated in Japan and showed the MBA was prone to false positives and ineffective for regulating YTXs to the EU standard (139).

Shellfish monitoring data from Portugal showed a good correlation of LC/MS and ELISA data for okadaic acid and analogs (OAs) but significant discrepancies for mouse bioassay (140). Ester forms of OAs were determined by LC/MS in *Dinophysis* species (141) and in contaminated shellfish (139, 142, 143). Direct detection of DTX3 rather than the parent OAs after hydrolysis has been applied to routine shellfish testing (140). LC/MS data showed that methanol–water (8 + 2) was not as efficient as methanol–water (9 + 1) for extraction of DTX3 and AZAs from shellfish (134). Profiling of *Dinophysis* species for toxins by LC/MS/MS has become an accepted technique (137, 141, 144, 145).

LC/MS/MS techniques have been crucial to developing understanding of the emerging spirolide class of toxins with

reports on phytoplankton and contaminated shellfish (146–150). The novel approach of MALDI-MS/MS (triple quadrupole) provided rapid, sensitive and quantitative data for spirolides in cultures (151).

Progress is reported on LC/MS/MS methods for quantitative determination of brevetoxins and their metabolites in shellfish tissues (152) and their use in tracking brevetoxin uptake, metabolism (153, 154). LC/MS was an essential tool in elucidating the complex metabolism of brevetoxins in Eastern oyster (155, 156). These studies have highlighted the need for standards of a wider range of brevetoxin metabolites before LC/MS can provide quantitative analyses that account for most of the toxicity in shellfish.

Several groups have reported the use of LC/MS with ion-trap mass analyzers, although little quantitative performance data have been published. In addition to the high sensitivity in full scan mode, sequential collisional activation experiments (MS^n) can in theory provide higher degrees of specificity for quantitative analysis. However, in practice significant increases in signal-to-noise ratio over the large increases obtained in moving from SIM (MS) to SRM (MS/MS) have not been demonstrated for shellfish extracts. The ability of MS^n to probe MS/MS fragment ions was particularly useful in the initial characterization of novel analogs of YTX (157–162) and AZA (163). YTX levels in picked cells of *Protoceratium reticulatum* and structurally useful MS/MS spectra have also been obtained using QTOF LC/MS (164).

Ongoing studies in the area of LC/MS methods for quantitative determination of marine toxins are focused on improved supplies of standards, expanding the range of toxins covered by multiresidue methods, simplifying and speeding up procedures for use in routine monitoring, and developing performance data, particularly through interlaboratory studies. The new EU BIOTOX research project is making strong efforts in these areas (*see* Topic Adviser report below, Okadaaiates, LC Methods).

Microcystins and Nodularins.—Topic Adviser Geoffrey A. Codd, School of Life Sciences, University of Dundee, Dundee DD1 4HN, Scotland, UK, Tel: 44-1382-344272, Fax: 44-1382-344275, E-mail: g.a.codd@dundee.ac.uk. Codd reports that LC methods with single wavelength UV or photodiode array detection have continued to be the most commonly-used procedures for the analysis of microcystins and nodularins in water over the past year, although LC/MS is finding increased application to distinguish variants of these toxin families. Procedures to reduce processing times and increase sample throughput capacity are reported (165). In-source collision-induced dissociation (in-source CID) has been applied to microcystin analysis from *Microcystis* cultures and has been found to be at least 10-fold more sensitive than conventional ESI-MS/MS (166). The in-source

CID procedure is particularly useful for detection of the highly toxic microcystins which do not contain arginine, e.g., MC-LF, -LA, and -LW. Pyrolysis offers a potentially rapid procedure, with minimal sample processing steps, for microcystin detection in cyanobacterial scum or filtered bloom samples (167).

Although immunoassays are mainly based on the use of antibodies to microcystin-LR in ELISAs (168), some diversification in immunoassay formats is under way. ELISA is being used to quantify the microcystins in single colonies of *Microcystis* (169). Antibodies raised against the ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) residue of microcystin and nodularin have been applied in ELISA formats (170). However, it should be borne in mind that ADDA can occur in isolation in aquatic environments; therefore, this antibody strategy may indicate analyte presence, but not necessarily the complete toxin molecule. In addition to the production of poly- and monoclonal antibodies to microcystins, phage display libraries continue to be developed. These showed promising sensitivity in immunosorbent and immunoprecipitation assays versus MC-LR (171). Both microcystin antibody- and microcystin antigen-immobilization in a lateral flow immunochromatography cartridge system have been evaluated to detect microcystin by fluorescence, with potentially useful limits of detection of 94 and 47 pg/mL, respectively (172).

The actions of most microcystin structural variants and nodularin to inhibit eukaryotic protein phosphatases (PPase) continue to be exploited in enzyme-based protein phosphatase assays. These provide an indication of the toxicity of the test material. Although PPase inhibition assays are not specific to these cyanotoxins, the integration of a microcystin antibody-based step into the procedure confers specificity (173): this approach has been extended to a multiwell fluorimetry procedure (174). The immobilization of a genetically engineered PPase (PP2a human catalytic subunit) by photocrosslinking onto screen-printer carbon electrodes and filters has been achieved (175). This approach retains PP2a activity for at least 4 weeks and offers the possibility, given development, of a portable and disposable biosensor for microcystins and nodularins.

Further prospects for the detection of the extensive range of microcystin homologs are offered via artificial trapping devices. Work has started on a fragment imprinting method for microcystins, based on the conformation of ADDA and that of its linkage to the adjacent glutamic acid in the toxin molecule (176).

Advances in the characterization and detection of genes encoding the peptide and polyketide synthases of microcystins and nodularins have continued (177) with the application of polymerase chain reaction (PCR) technologies for their detection and quantification in natural waters. It

should be assumed, however, that the detection of these genes provides an indication of potential, rather than actual peptide toxin presence. A screen of candidate microcystin-producing filaments of *Planktothrix rubescens* from European lakes revealed that a variable proportion of the microcystin synthase DNA-positive isolates were negative for the peptide toxins (178). The grounds therefore remain for continuing improvement in the detection and analysis of the peptide toxins themselves, in raw and treated waters and in a wide range of other complex matrixes.

Note by the General Referee: A new Task Force subgroup has been formed on Cyanobacterial Toxins. This group met for the first time in Orlando, Florida, at the 2005 AOAC Annual Meeting and also meets online in a dedicated forum. The group is chaired by Hans Van Egmond and Geoffrey Codd; Geoffrey Eaglesham and Armah de la Cruz are co-chairs. There is a strong need to emphasize validation efforts in this area, from standards production to methods using test kits and LC/MS/MS methodology. This urgent need was highlighted by increasing global problems with the various cyanobacterial toxins, a new U.S. Environmental Protection Agency (EPA) initiative to assess risks and to address the problem, and finally by our own Task Force discussions in Spain last April. Online discussions in the forum and in electronic mail found that several test kits for microcystins are commercially available, but that none have been validated. Further, as has been pointed out by Topic Advisor Geoffrey Codd, methods development and validation have been hampered by increasingly limited availability of toxin standards and no certified materials. The Orlando meeting included discussion of an international and interagency symposium and workshop led by EPA the week before in Research Triangle Park, NC.

Okadaiates, Assay Methods (formerly Diarrhetic Shellfish Poisoning Toxin, Assay Methods).—Topic Advisor J. Marc Fremy, Agence Française de Sécurité Sanitaire des Aliments, Unité d'Evaluation des Risques Physico-Chimiques, Maisons Alfort, F-94701, France, Tel: 33-1-4977-2794, Fax: 33-1-4977-1352, E-mail: j.fremy@paris.afssa.fr. Topic Advisor Fremy reports that a simple and sensitive in situ method for monitoring the occurrence of toxic algal blooms and shellfish contamination events has been developed by MacKenzie et al. (135). The technique involves the passive adsorption of biotoxins onto porous synthetic resin filled sachets (SPATT bags) and their subsequent extraction and analysis. The success of the method is founded on the observation that during algal blooms significant amounts of toxin, including the low polarity lipophilic compounds, such as the PTXs and the OA acid complex toxins, are dissolved in the seawater. Field trials have been performed during *Dinophysis acuminata* and *P. reticulatum* blooms. The trial results prove the concept and demonstrate that the technique provides a means of forecasting shellfish contamination

events and predicting the accumulation of these polyether toxins by mussels and represents an early warning method over current monitoring techniques such as shellfish-flesh testing and phytoplankton monitoring. In contrast to the circumstantial evidence provided by genetic probe technologies and conventional phytoplankton monitoring methods, it directly targets the toxic compounds of interest. The extracts that are obtained for analysis lack many of the extraneous lipophilic materials in crude shellfish extracts so that many of the matrix problems associated with chemical and biological analysis of these extracts are eliminated. Analyses can confidently target parent compounds only, because analytical and toxicological uncertainties associated with the multiplicity of toxin analogs produced by in vivo biotransformation in shellfish tissues are reduced. The technique may reduce monitoring costs and provide improved spatial and temporal sampling opportunities. When coupled with appropriate analytical techniques (e.g., LC/MS/MS, ELISA, receptor binding assay, rapid field tests), the technique has the potential to offer a universal early warning method for microalgae toxins (lincoln.mackenzie@cawthron.org.nz).

The General Referee points out that this topic area was renamed to better reflect the analytes addressed, which include okadaic acid, the dinophys toxins, and associated esters. These are here referred to collectively as the okadaiates (OAs). The rationale behind this change is described in detail in the opening section of this General Referee report. Regarding the OAs, efforts are underway to produce validated assays. These include a BIOTOX project in the EU for a competitive binding assay (development of this competitive fluorescent binding assay reported on last year in this report) now to be marketed by Biosense of Norway, and also a commercial PP2A inhibition-based assay sold by ZEU Immunotec of Spain. Readers are advised to also look into the online forum areas Okadaiates and Azaspiracids and also the Files and Folders area for more information on these diarrhetic toxins and associated validation efforts. In Japan, preliminary validation studies of another PP2A kit have already been reported by Sekiguchi et al. (179). Further, Takeshi Yasumoto is currently producing, via genetic engineering, large quantities of enzyme for test kit production (personal communication). Another important development in this area is the field test kit for the OAs (lateral flow immunochromatographic cassette) recently developed by Jellitt Rapid Testing. This new kit was presented for the first time (6) at Marine and Freshwater Toxins Analysis: First Joint Symposium and AOAC Task Force Meeting, Baiona, Spain, April 2005. More details are found in a recent publication (180).

Okadaiates by LC-Based Methods.—Topic Advisor Philipp Hess, Marine Institute, Galway Technology Park, Parkmore, Galway, Ireland, Tel: 353-91 730400, Fax: 353-91 730470, E-mail: philipp.hess@marine.ie. The okadaiates are

a group of toxins responsible for many poisoning incidents worldwide. The initial discovery of OA and DTX-1 in the 1970s in Japan as shellfish toxins was confirmed rapidly in Europe and has since been observed repeatedly in Japan, Europe, New Zealand, and South America. The chemistry and analysis of the okadaic acid group was described recently by Quilliam (181). Since this report, novel compounds in this group have been identified in *P. lima* cultures by Fernandez et al. (182), Suarez-Gomez et al. (diol-esters; 183), and in a sponge (27-O-acetyl-OA and TX-1; 184). This added level of complexity has also been confirmed in other toxin groups from the lipophilic range, e.g., spirolides (185) and needs to be investigated further for the okadaiates. The chemical complexity of the compounds observed is equally as important for the postulation of biosynthetic pathways as it is for the parameters in the extraction of the toxins and sample pretreatment prior to LC analysis. The most important recent finding with regard to analytical methodology is the poor extractability of some esters of OA and DTXs when the solvent 80% aqueous methanol is used to extract okadaiates since the developments reported by Lee et al. (186) using HPLC with fluorescence detection. The difference between 80, 90, and 100% MeOH for the extraction of okadaiates and azaspiracids was observed in the advisor's laboratory (187, 188) and more recently by McNabb et al. (117). Matrix effects are negligible for LC/MS in negative ionization mode if sample-to-solvent ratios are ca 1:10 or greater; however, Stobo et al. (138) reported severe matrix enhancement for okadaiates when a sample-to-solvent ratio of 1:4 was used. Most analytical methods now focus on LC/MS rather than HPLC/FLD, mainly because of the potential for higher speed of the LC/MS technique but also because of the restriction with some of the more complex esters being esterified at the carboxylic acid end of OA, which prevents derivatization using 9-anthryldiazomethane (ADAM). However, a rapid and simple alternative method for detection by HPLC/FLD was also developed by Rawn et al. (189). Hydrolysis procedures are typically used for analysis of the esters in routine and regulatory monitoring of the okadaiates. Different lots of certified standards have been produced over the past years by the NRC, IMB, Halifax, Nova Scotia Canada, for okadaic acid. The same institute also provides a tissue reference material certified for OA and DTX-1. Collaboration is under way between NRC and the Advisor's laboratory for a tissue material containing DTX-2, as well as preparations for a DTX-2 standard. No reference materials are certified for any of the esters of OA, DTX-1 and DTX-2, which renders the validation of methods for these very difficult. In New Zealand, Japan, and Europe, the okadaiates are specifically regulated to levels of 160–200 µg/kg shellfish meat. Codex Alimentarius had requested advice in 2003 prior to regulating the shellfish toxin area, and a working group was called by FAO/IOC/WHO, which produced a report in

2005 (190). Unfortunately, none of the currently used methods has been officially validated. The requirement for this was raised at meetings of the EU national reference laboratories as well as at the recently founded Presidential Task Force for Marine and Freshwater Toxins. Also, the attendants of the workshop of the QUASIMEME proficiency testing provider, June 19, 2004, Galway, Ireland, concluded that after successful completion of several rounds of proficiency testing for DA, the next group of toxins to include in this scheme should be the okadaiates. A recently funded EU-project, BIOTOX, has a module on the development and validation of LC/MS based methods as reference methods for lipophilic toxins in shellfish. This module is led by the Advisor and will focus on the okadaiates as well as AZAs, YTXs, and PTXs. A collaborative study protocol for the okadaiates and other toxins will be presented by the Advisor at the AOAC annual meeting in Orlando, 2005.

Receptor Assays for Phycotoxins.—Topic Advisor Frances Van Dolah, NOAA-National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, 219 Fort Johnson Rd, Charleston, SC 29412, Tel: 843-762-8529, Fax: 843-762-8700, E-mail: Fran.Vandolah@noaa.gov.

PSP.—The PSP receptor assay has received a great deal of interest in the past several years as a screening assay adjunct to or possible replacement for the (AOAC OMA) mouse bioassay. Progress in collaboratively testing the assay has been hampered by the lack of availability of ³H STX and complications associated with international shipping of chemical weapons. This year, 2 new commercial preparations of ³H STX, obtained through a collaboration between the International Atomic Energy Agency (IAEA), FDA, and National Oceanic and Atmospheric Administration (NOAA), have been successfully tested for their suitability for use in the Na channel receptor assay for PSP toxins. Interested laboratories may request ³H STX standard through NOAA's Marine Biotoxins Program (fran.vandolah@noaa.gov). A single laboratory validation study of the PSP receptor assay has been completed and was presented at the "Marine and Freshwater Toxins Analysis: 1st Joint Symposium and AOAC Task Force Meeting" in Baiona, Spain, in April 2005. The LOQ of the microplate format assay was 1.2 µg/100 g shellfish (regulatory limit 80 µg/100 g), with an overall repeatability of 17.7% for shellfish extracts run by one analyst on 5 independent days, and a correlation of $r = 0.98$ with the mouse bioassay. This study is being submitted to AOAC for review. If accepted, a collaborative study protocol will be submitted for an anticipated study to be initiated in early 2006.

Brevetoxin and CTX.—The sodium channel receptor binding assays using ³H PbTx3 for determination of for PbTx and CTX continue to be used as tools for screening marine animal and human intoxication events. Deschraoui et al. (89) used this receptor assay in parallel with the ouabain/veratridine-directed N2A cytotoxicity assay for

discriminating between PbTx and CTX contamination of fish. Flewelling et al. (191) used the PbTx receptor assay and ELISA to identify finfish as the vector for PbTx exposure in a bottlenose dolphin mass mortality event. The PbTx receptor assay performed well in a multiple method intercomparison aimed at assessing assays with potential for replacing the regulatory mouse bioassay (192), but further assessment of candidate methods awaits insight into the toxicity of brevetoxin metabolites. Efforts to isolate metabolites for toxicity testing are under way.

Sample Pretreatment Methods for Marine Toxin Analysis.—Topic Advisor Ana Gago-Martinez, Universidad de Vigo, Dpto. Química Analítica y Alimentaria, Facultad de Ciencias, Campus Universitario de Vigo 36200-Vigo-E, Spain, Tel: 34-986-812284, Fax: 34-986-812382, E-mail: anagago@uvigo.es. Sample pretreatment of complex matrixes, particularly seafoods, is a critical step in the development of the analytical methodology. Improper sample pretreatment can be a serious source of errors and may compromise the reliability of the final results. In spite of this, little work has been devoted to the development of efficient sample protocols able to selectively extract the analytes of interest, and where possible, preconcentrate the sample when detectability requirements are high. Immunoaffinity (IA) is a very promising alternative for the extraction and cleanup of algal toxins from complex matrixes. Intense work in this area has been done in our laboratory on complex matrixes. By virtue of the sample pretreatment step, this work (193) uses the high efficiency and high selectivity of IA both to remove interferences and provide improved detectability via analyte preconcentration. Immunoaffinity approaches for the solid-phase extraction (SPE) of algal toxins such as microcystins (MCs) and DAs have been recently developed using immunoaffinity SPE cartridges with specific monoclonal antibodies. These have been successfully applied for the extraction and cleanup of algal toxins. Results of this work were presented recently in Baiona (193) and at the Annual AOAC meeting in Orlando, Florida, September 2005. These results will soon be submitted for publication. Other sample pretreatment work in the author's laboratory (Gago-Martinez, Rellán, Piñeiro, and Villar Gonzalez) addresses the development of an automated sample extraction. This uses online solid-phase microextraction (SPME) coupled with HPLC for the analysis of cyanobacteria such as microcystins and anatoxins.

Saxitoxins, Electrophysiological Methods.—Benjamin A. Suárez-Isla, Laboratory of Marine Toxins, Program of Physiology and Biophysics, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Av. Independencia 1027, PO Box 70005, Santiago, 6530499, Chile, Tel: 562-777-6886, Fax: 562-732-9668, E-mail: bsuarez@med.uchile.cl. The molecular target of STX, TTX, and CTX is the voltage-dependent Na channel present in

neurons, muscle, and secretory cells. The electrical excitability of cells in all chordates and many invertebrate species is based on the voltage-dependent entry of Na⁺ ions into cells through Na channels, an essential process that maintains cellular functionality and viability that has been extensively studied (194, 195). However, the problem is, how to reduce into practice a simple design that uses measurement of sodium currents as the end point signal that reports PSP blocking effects (and/or NSP excitatory actions). In the case of PSP it would be desirable to measure biotoxicity of PSP extracts as a direct block of sodium currents.

Extensive efforts continue to be undertaken to design in vitro methods alternative to the MBA for PSP (AOAC 959.08 Biological Method) that could be validated for regulatory works. These include cell culture bioassays, immunoassays, receptor-based assays, and electrophysiological methods that use isolated preparations such as frog bladder membrane (196) or HEK 293 cultured cells that express Na channels isoforms (197).

The effects of brevetoxin-2 (PbTx-2) and STX were analyzed before and after exposure to the toxins on embryonic murine frontal cortex neuronal networks cultured on integrated microelectrode arrays (MEAs; 198). The effects were evaluated as changes in mean spike rate, burst rate, burst duration, number of spikes per burst, and spike amplitude. It was reported that STX produced fast and reversible inhibition of all electrophysiological parameters with inhibitory concentrations (IC 50s) ranging between 1.2 and 2.2 nM. The authors propose neuronal networks grown on MEAs as a robust and sensitive approach for environmental threat detection. This is the only report that uses electrophysiological parameters as detection end points.

Other proposed strategies in this period correspond to cell-based approaches and a receptor binding assay (reviewed by Van Dolah). Okomura et al (199) made 2 modifications to the original method reported by Manger et al. (200), using maitotoxin rather than brevetoxin and WST-8, a dehydrogenase detecting water-soluble tetrazolium salt (rather than MTT) to determine target cell viability, simplifying the previous procedure. A membrane potential assay (201) based on synaptoneurosomes prepared from mouse brain was evaluated further for its capability for quantitation of STX-like activity in shellfish tissues and to zooplankton samples. A functional pharmacologically-based assay for the brevetoxin group of sodium channel activators (202) was also developed using synaptoneurosomes isolated from the brains of CD1 mice. Authors concluded that the assay can detect the depolarizing effect of brevetoxin congeners PbTx-2 and PbTx-3.

High throughput assay technologies for ion channel drug discovery (203, 204) could be applied for the detection and quantitation of PSP toxins. Automated patch clamp platforms have become commercially available (e.g., PatchXpress[®] and

IonWorks[®] from Molecular Devices) that in theory could process hundreds of samples per day. Although expensive in the short term, these new machines could offset initial investment if used in extensive PSP monitoring programs. Besides costs, current shortcomings are the quality of voltage control for Na currents that have very rapid onset and inactivation kinetics, and the need for official method validation. However, those should not be insurmountable when the mouse bioassay faces increasing ethical criticism and shows its technical limitations in sensitivity.

Saxitoxins by LC Methods.—Study Director James F. Lawrence, Health Canada, (retired), 484 Brierwood Ave, Ottawa, Ontario, K2A 2H3, Canada, Tel: 613-729-2205, E-mail: jimlawrence@sympatico.ca. The Study Director submitted the completed prechromatographic oxidation LC collaborative study (*J. AOAC Int.* **87**, 83–100) with modifications to AOAC Method (2005.06), which was approved Official First Action in June 2005. This is the first approved Official Method for PSP toxins since the MBA some 50 years ago. PSP methodology continues to be discussed and was an important topic at the Baiona Symposium and Task Force meeting in April, 2005. The post-column oxidation LC method of Oshima continues to be developed and improved. Quilliam (205) reported on the improvement of this method with the use of a new stationary phase that is less susceptible to phase collapse, an improved gradient system that allows the separation of more toxins in a single analysis, and microbore columns which reduce the amount of sample required for analysis. A preliminary report on these developments has been published (206). Oshima (207) has also developed a single-column separation of the toxins and reported on this at the Pacificchem 2005 meeting in December in Hawaii.

Tetrodotoxins.—Topic Advisor Mari Yotsu-Yamashita, Tohoku University, Sendai, 981-8555, Japan, Tel/Fax: 81-22-717-8922, E-mail: myama@biochem.tohoku.ac.jp. The Topic Advisor reports that her laboratory isolated 4-S-cysteinyllTTX, a probable metabolite of TTX, from the liver of the puffer fish, *Fugu pardalis* (208). It was also found that 4-cysteinyllTTX and 4-glutathionylTTX were nonenzymatically formed from cysteine and glutathione with 4,9-anhydroTTX in 30–40% yield, respectively, in vitro. These thiol adducts of TTX were much less toxic than TTX. However, contrary to expectation, coinjection (ip) of cysteine or glutathione with TTX to mice did not save the mice, probably due to the low reactivity of TTX with thiols compared with that of 4,9-anhydroTTX. Concerning the bacterial production of TTX, Wu et al. (209) reported a new TTX-producing actinomycete, *Nocardopsis dassonvillei*, isolated from the ovaries of puffer fish *F. rubripes*. They identified TTX from the cell lysate of *N. dassonvillei* by spectral data, including ESI/MS, and biological data (MDA and neuroblastoma cell assay). They mentioned that *N. dassonvillei* in the ovaries was closely related to the

toxification of the puffer fish. As per request from the General Referee, Yotsu-Yamashita provides below an English translation of the current Japanese official method for determination of toxicity of puffer fish tissues by mouse bioassay. This method was authorized by the Ministry of Health, Labour and Welfare, Japan, published by the Food Hygienic Association of Japan in 1978 (210) and revised in 1991 (211).

Tetrodotoxin in Puffer Fish by Mouse Bioassay

Each tissue of fish is cut into small pieces to be ground in a mortar with a pestle. A 10 g amount of the ground tissue is suspended with 25 mL of aqueous 0.1% acetic acid and heated in boiling water for 10 min. After cooling, this preparation is filtered through filter paper with the aid of aspirator. If filtration is difficult, the sample is centrifuged to obtain the supernatant. The filtrate or the supernatant is diluted to 50 mL. Each 1 mL of this solution, equivalent to the extract from 0.2 g of fish tissue, is injected up into a mouse, 4 weeks old, male, ddY strain, body weight 19–21 g. For the preliminary test, 2 mice are used for one test solution to obtain the average fatal time. Death is judged by cessation of respiration. If the fatal time is about 10 min, 3 more mice are used for additional test, and final fatal time is determined as the median death time. If the fatal time is <7 min, the sample solution is diluted and tested again. The toxicity is determined based on the relationship between the fatal time and toxicity by Kawabata et al. (210), and expressed as mouse unit. One mouse unit is defined as the toxicity to kill a 20 g mouse, equivalent to 0.22 µg TTX.

Yessotoxins.—Topic Advisor Aurelia Tubaro, University of Trieste, Dept. Materials and Natural Resources, Via Valerio 6, 34127, Trieste, Italy, Tel: 39-040-558 7910, E-mail: tubaro@units.it. Yessotoxins (YTXs) continue to be the target of intense research. YTX was initially included among the DSP toxins, but because it does not induce diarrhea in experimental models, YTXs have recently been classified and regulated separately from the DSP toxins. Although more than 20 YTXs were isolated, the toxicological potential of YTXs is not yet completely clarified, and no human intoxication has been reported; only YTX, de-sulfo-YTX, homo-YTX, and 45-OH-homoYTX were submitted to deep toxicological studies after acute (212–216) and repeated administration (217, 218). The ip administration induced lethality in mice, with lethal dosage (LD50) values ranging from 80–100 to 500–750 µg/kg for YTX and homo-YTX; 45-OH-homoYTX did not induce lethality at 750 µg/kg. At light microscopy, no edema (212, 214) or slight intracellular edema in the heart of animals treated with 750 and 1000 µg/kg (202) and no apoptotic changes were observed in the myocardium of treated animals using in situ TUNEL staining (213). Electron microscopy analysis revealed swelling of cardiomyocytes and rounded mitochondria, in some areas,

particularly near capillaries in mice treated with 1000 µg/kg (215). The observed damages to cerebellar Purkinje cells after ip injection of 420 µg/kg YTX (219) were not confirmed in a subsequent study by the same authors (220). Alterations in the thymus and duodenum were also reported (219). Some preliminary data about lethality of further YTX analogs are reported. The 1,3 enone isomer of heptanor-41-oxoYTX (219) and the 9-methyl-41a-homo-YTX did not cause lethality after ip administration of 5000 µg/kg (220).

YTXs are much less toxic per os, the usual exposure route of these toxins. No deaths, notable changes in behavior or growth, macroscopic abnormalities, changes in plasma activities of liver enzyme, lactate dehydrogenase, creatine kinase, leukocyte percentage were observed both after acute (0.5–10 mg/kg; 201–203) and repeated treatment (1 and 2 mg/kg daily for 7 days; 216). Only ultrastructural changes (cardiomyocytes swelling, protrusion of cardiac cells into pericapillary space with rounding of mitochondria) were observed both after acute and daily repeated YTX, homoYTX and 45-OH-homoYTX treatment (198, 199, 202). No toxicity of YTX (5 mg/kg) was reported when the toxin was administered by gavage 7 times during 3 weeks (203). No apoptotic changes in the myocardium of per os treated animals using in situ TUNEL staining (218, 221) was observed after both acute and repeated administration. Pharmacokinetic problems after oral exposure to YTXs are hypothesized to explain the observed discrepancy in toxicity between the 2 administration routes. On the basis of the available toxicological data, the Guidance Level/Maximum Level of YTX in shellfish would be 12 mg/kg, according to the Expert Consultation of FAO/IOC/WHO held in Oslo, Norway, September 2004 (218).

A clear mechanism of YTXs toxicity is not yet available. More information was obtained on the in vitro effects of the toxins in the last year. Bianchi et al. (221) demonstrated that YTX opens the permeability transition pore of the inner mitochondrial membrane of rat liver mitochondria at nanomolar concentrations. YTX induces a selective alteration of E-cadherin in MCF-7 breast cancer cells, but not of N-cadherin in the PC12 cell line and K-cadherin in MCF-7, Caco-2, MDCK cells (222). The structure-activity relationship of YTXs on the E-cadherin fragmentation was also studied in MCF-7 breast cancer cells by Ferrari et al. (223). Significant differences in the potencies of YTX analogues were found when structural changes affected the C₉ terminal chain of these compounds, supporting the hypothesis that this portion of the molecule is essential for the activity of YTX in MCF-7 cells. The authors also speculate that YTX effects could involve 2 separate receptorial systems.

The analytical determination of YTXs poses considerable problems due to the large number of analogues isolated so far. The accumulation of E-cadherin fragments in cultured cells is the basis of a functional method proposed by Pierotti et

al. (224). An interlaboratory study based on this method is expected to start in Italy by the end of Summer 2005. YTX has also been shown to reduce cAMP levels in exposed cells enhancing phosphodiesterases (PDEs) activity (225). Two functional assays based on these effects have been developed; preliminary results obtained in spiked phytoplankton and shellfish extracts are available (226, 227). OH-YTX, carboxy-YTX, and the ester S-MPTA-YTX were also tested using a resonant mirror biosensor. The KD values indicate that MTPA-YTX has a lower affinity than YTX and OH-YTX for PDEs, but a higher affinity than carboxy-derivative (228).

The ELISA developed by Briggs et al. (229) and commercialized by Biosense Labs AS, Bergen, Norway, has been subjected to a single laboratory validation (intra-assay variability: <3%; interassay variability: <10%, RSD_R: 8%) and to a limited interlaboratory study (interlaboratory reproducibility 16%; recovery 111%) with shellfish spiked with pure YTX. A full collaborative study is expected to start at the beginning of next year. A high correlation of data from ELISA and LC/MS analyses has been obtained for fortified samples ($r^2 = 0.999$; slope 1.050), but lower ($r^2 \geq 0.8$) for naturally contaminated samples (231). The high sensitivity of ELISA has been also used to quantify the YTX production in algal cells (231) as well as in seawater samples (232).

Various LC/MS methods have been recently developed for YTXs: some of them were presented during the Baiona, Spain, Marine and Freshwater Toxin Analysis—First Joint Symposium and AOAC Task Force Meeting, held in April 2005. A single laboratory validation and preliminary interlaboratory study presented by McNabb; it is a multitoxin method, able to detect 17 toxins, including YTXs (LOD 0.02 mg/kg; 117). A rapid multitoxin LC/MS method including YTX was developed by Stobo et al. (233). The limit of YTX detection is 19 µg/kg in Pacific oysters, 32 µg/Kg in mussels, 46 µg/kg in cockles, and 74 µg/kg in scallops. The reproducibility ranges from 3.3 to 5.2% and the repeatability is 7%. Another multitoxin LC/MS/MS method able to detect YTX and over 20 its analogues was presented by Suzuki at the Baiona Joint Symposium. The sensitivity of the method is 10 ng/g digestive gland. Both naturally contaminated (500 shellfish and 100 phytoplankton) and spiked samples were analyzed. The recovery was 97% in shellfish and 89% in mussels (234). Canas et al. (235) demonstrated the utility of nano LC with hybrid quadrupole time of flight mass spectrometry for the determination of YTX in marine phytoplankton. The LOD of this method is reported to be ca 0.5 pg YTX on-column. Further improvements on the application of HPCE to YTX analysis has been reported by de la Iglesia et al. (78).

YTX is now commercially available (BluBioTech, Busum, Germany) and a YTX calibration solution CRM should be soon available, as discussed at YTX subgroup meeting (chaired by the author and held at *Marine and Freshwater*

Toxins Analysis: First Joint Symposium and AOAC Task Force Meeting, Baiona, Spain, April 2005). The YTX Subgroup Report and more information on the performance of some of the detection methods for YTX can be obtained visiting the AOAC Marine and Freshwater Task Force Forum (www.aoac.org). With the aim to increase commercially availability of YTX standards, a fast and efficient alternative purification method for YTXs from *P. reticulatum* cultures was presented by Ruppen-Canas et al. (236). Confirmation that *Lingulodinium polyedrum* can produce YTX was provided by identification of YTX in cultures of a Spanish isolate (237). Recently, YTX was also found in New Zealand coastal waters and shellfish, contaminated by a bloom of a closely related dinoflagellate *Gonyaulax cf spinifera* (221).

Issues in Marine and Freshwater Toxins Methods Validation

This report will close by briefly addressing a few issues which are central to the reasons for compiling this General Referee report and for the formation of the Marine and Freshwater Toxins Task Force and Analytical Community. This involves the reasons why regional and national regulatory agencies, as well as the seafood industry and industry associations, want to see analytical methods validated. Further, for international harmonization of methods requirements and ultimately in the interest of international trade, these same groups favor the AOAC and especially the OMA process. Finally, it is also useful to discuss how methods used as research tools differ from official methods in their performance requirements and to examine the role of these research methods in validation efforts.

A useful illustration is provided by the classic red-tide marine biotoxin group, the brevetoxins. The landscape covered by this problem includes monitoring, with the mouse bioassay, unexpected vectors for human exposure and what we now know about these toxins and their complex fate once they are produced by the dinoflagellate *K. brevis*. The case of the brevetoxins can be summed up by the existence of the usual mouse bioassay, the fairly recent discovery (thanks largely to LC/MS/MS and very sensitive cytotoxicity and receptor binding assays) of many additional toxin forms due to multiple and rapid metabolic conversions within shellfish and in fish. Many challenges in the search for modern and better-performing analytical alternatives to the mouse result from the existence of the metabolites, limitations in obtaining analytical standards for these new forms and the inherent difficulty of comparing instrumental and biological analyses. Generally as more is learned of each toxin group and their metabolites (largely due to LC/MS/MS in combination with certain assays) similar examples for other toxins will follow and many are already well known or are emerging.

There are many other practical considerations as well that limit acceptance and validation, such as budget restrictions.

For various reasons ranging from their instrumentation cost to their complexity, or even due to specialized personnel training requirements, some methods will, be used primarily as research tools rather than practical monitoring methods, and this of course impacts directly the need for their interlaboratory validation. These practical limitations usually change with time. Either instruments become less expensive and/or the technique gains wider acceptance or a difficult method is demystified by careful ruggedness testing and refinement of procedures.

Clearly another major factor in both methods development and methods acceptance is the specific requirement of a given method for one or several reference standards. As reference standards become available, the number of officially approved tools for monitoring will increase, and more methods will be validated.

Generally bioassays, immunoassays, and functional assays have fewer reference standard requirements than do analytical methods based on separations. These methods are already important as research tools that can be used to identify the relative activity or toxicity of a sample. In the most sensitive assays it can be possible to directly identify chromatographic peaks as representing toxic or nontoxic components in mere microliter quantities from single fractions, exemplified by cytotoxicity assays applied to detecting CTXs in outbreaks in fish (85) and in human blood (87). Some of these specialized assay methods, however, will remain research tools while others may become Official Methods if results can be sufficiently reproduced between laboratories. In any case, the wealth of information provided by these assays, especially in combination with powerful separation and mass-based detection methods, will ultimately aid validation of other methods.

Ultimately, as many LC/MS/MS specialists believe (*see* report of Patrick Holland) these powerful separation and mass-based methods may themselves enjoy widespread use (following official validation) in monitoring programs, allowing simultaneous determinations across multiple toxin classes when testing can be centralized, sufficient standards are available, and budgets allow.

It is not difficult to see why mouse bioassays continue to be used, particularly since these procedures have been providing good public health protection. Yet it is clear that mouse bioassays must be replaced and that alternative methods will be pursued, also because ethical considerations are rapidly becoming more significant.

Mounting a serious effort at providing alternative methods also requires coordination, careful thought, and a combined international effort. There are so many challenges to address and so many alternative ways to proceed, since a bewildering variety of choices among possible compromises must be considered. These include which toxin forms to emphasize, which analytical methods are the best targets for validation

(and which are simply useful research tools), and many others. If there is no prior consensus, it will be difficult to compare analytical results. Thus, confusion will result (and money will be wasted) if multiple, uncoordinated efforts commence. The subgroup structure of the AOAC Task Force, including multiple and multidisciplinary experts as well as stakeholders to keep proposed methods practical, is addressing this need. The more international these groups are, the better, since this approach best pools the available resources (intellectual and otherwise) and attempts to satisfy stakeholder needs on a global basis.

At some point the most promising and rugged methods are validated, but the questions which must be anticipated and addressed are these: which toxin forms are included in the validation; can the methodology be used for monitoring (with confidence to a wide enough range of commercial species); can multiple laboratories obtain sufficient agreement in their results using the method; and finally, can a panel of experts agree that the study was run properly? To satisfy health protection agencies and to protect the reputation of industry, there must also be agreement that new alternative methods are rugged and accurate.

Due to the presence of multiple toxin forms, metabolites, and the resulting need for compromise, accuracy can translate to providing a level of health protection at least equivalent to or better than that afforded by the existing method. This is the foremost requirement of a new official method for marine or freshwater toxins. Unfortunately this point can be difficult to prove for de facto official methods such as the mouse bioassays for DSPs which have never been formally validated and which appear in multiple variations. Modern methods for these lipophilic toxins face the ironic challenge of measuring up to what many consider imprecise and poorly defined methodology and a correspondingly vague level of health protection. A fresh look at criteria for alternative methods is called for in cases like this.

Screening tools, thought by some to have less stringent requirements, are, by virtue of their application, the first line of defense in biotoxin management and require careful scrutiny. These important tools must not produce false negatives, and they must produce a manageable number of false positives if they are to be useful. Although true, third-party validation has in the past been delayed for some screening tools, ultimately there are always additional restrictions imposed by regulatory agencies when they have less confidence (including in the legal sense) in a method. Full AOAC OMA validation of methods for detecting toxins in shellfish, for example, elevates those methods to a higher level of acceptance by regulatory stakeholders such as the Interstate Shellfish Sanitation Conference/National Shellfish Sanitation Program (ISSC/NSSP) the U.S. system of state monitoring and federal oversight. This is also true for similar monitoring programs in many other countries and international groups.

Officially validated methods will ultimately be more competitive in the analytical services and test kit marketplace, because they will enjoy more widespread use. Finally, since all the questions above will be asked at an international level there must also be international level confidence in the validation process. Without it, experience shows that eventually disputes arise over detection methodology and its appropriate use and reliability.

The coming years hold great promise for the validation of additional analytical methods for the toxins. These new official methods will provide official alternatives to mouse bioassays, confidence in (and international acceptance of) the newest tools for protecting public health, and the additional benefit of smoother international trade.

Recommendations

(1) *Anatoxins*.—New Topic Advisor Ambrose Furey. Continue study.

(2) *Animal Bioassays for Phycotoxins*.—Topic Advisor Donald J.A. Richard. Continue study. (*Note*: This topic was renamed from Bioassays for Phycotoxins to better reflect the aim of the work and report content.)

(3) *Azspiracids*.—Topic Advisor Ambrose Furey. Continue study.

(4) *Brevetoxins, Immunochemical Methods for Brevetoxins*.—Topic Advisor Jerome Naar. Continue study. (*Note*: Word order changed in title to place brevetoxins first.)

(5) *Capillary Electrophoretic Methods for Marine Toxins*.—Topic Advisor Ana Gago-Martinez. Continue study.

(6) *Cell Bioassays for Phycotoxins*.—Topic Advisor Ronald Manger. Continue study.

(7) *Ciguatoxins by LC/MS*.—Topic Advisor Richard J. Lewis. Continue study.

(8) *Domoic Acids*.—(*Note*: This topic was renamed from Amnesic Shellfish Poisoning Toxins for reasons outlined in this report.). Study Director Michael A. Quilliam. Continue study.

(9) *Domoic Acids by ELISA*.—Study Director Hans Kleivdal. Continue study.

(10) *Electrophysiological Methods for Saxitoxins*.—Topic Advisor Benjamin A. Suárez-Isla. Continue study.

(11) *LC/MS Detection of Marine Toxins*.—Topic Advisor Patrick Holland. Continue study.

(12) *Microcystins and Nodularins*.—Topic Advisor Geoffrey A. Codd. Continue study.

(13) *Okadaiates, Assay Methods*.—(*Note*: This topic was renamed from Diarrhetic Shellfish Poisoning Toxins, Assay Methods for reasons outlined in this report.) Topic Advisor J. Marc Fremy. Continue study.

(14) *Okadaiates by LC-Based Methods*.—New Topic Advisor Philipp Hess. Continue study.

(15) *Immunochemical Methods for Seafood Toxins*.—The Topic Advisor resigned, and a new Topic Advisor will be appointed.

(16) *Saxitoxins, LC Methods*.—Study Director James F. Lawrence. (Note: This topic was renamed from Paralytic Shellfish Poisoning Toxins, Instrumental Methods for reasons outlined in this report.) Continue study using Topic Advisor appointment.

(17) *Receptor Assays for Phycotoxins*.—Topic Advisor Frances Van Dolah. Continue study.

(18) *Sample Pretreatment Methods for Marine Toxin Analysis*.—Topic Advisor Ana Gago-Martinez. Continue study.

(19) *Tetrodotoxins*.—Topic Advisor Mari Yotsu-Yamashita. Continue study.

(20) *Yessotoxins*.—New Topic Advisor Aurelia Tubaro. Continue study.

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