Harmful Algae 24 (2013) 20–31

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Refinement and implementation of the Lawrence method (AOAC 2005.06) in a commercial laboratory: Assay performance during an *Alexandrium catenella* bloom event

D. Tim Harwood *, Michael Boundy, Andrew I. Selwood, Roel van Ginkel, Lincoln MacKenzie, Paul S. McNabb

Cawthron Institute, Private Bag 2, Nelson 7010, New Zealand

ARTICLE INFO

Article history:
Received 9 November 2012
Received in revised form 11 January 2013
Accepted 11 January 2013

Keywords:
Paralytic shellfish poisoning
Paralytic shellfish toxins
Saxitoxins
Shellfish contamination
Lawrence method
Regulatory testing

ABSTRACT

In 2010 the Cawthron Institute adopted AOAC official method 2005.06 (Lawrence method) for regulatory testing of paralytic shellfish toxins. This included adapting the method to a UPLC format and developing a rapid periodate screen to eliminate the vast majority of samples with no PSTs present. The method gained New Zealand regulatory approval and has since been used to test >2000 samples. Soon after implementation a major HAB of the toxic dinoflagellate *Alexandrium catenella* occurred in a prime shellfish growing area of New Zealand. This event was the most serious to date in this country with extremely high cell concentrations observed in some locations ($>4 \times 10^6$ cells L^{-1}). Toxin levels observed in Greenshell™ mussels (*Perna canaliculus*) and Flat oysters (*Ostrea chilensis*) exceeded the regulatory level of 0.8 mg/kg shellfish meat as saxitoxin equivalents. Closures of commercial shellfish harvesting areas were enforced for a period of up to three months as toxin levels remained above the regulatory level for an extended period, even after the bloom had crashed.

Analysis of several hundred positive shellfish samples during this event allowed us to better understand the technical performance of the method during a bloom event. The periodate screen substantially overestimated the true PST level in the samples because several PSTs gave co-eluting oxidation products, and it was assumed that the entire peak was due to the presence of the more toxic congener. The ratio between the screen and confirmation test results remained relatively constant throughout the bloom events. This information supports an amendment to the overly conservative regulatory control scheme employed in New Zealand for PST testing. Despite overestimation, the periodate screen has proved highly useful as it allows a quick determination of PST-free samples and provides a high level of security against harvesting contaminated products.

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1. Introduction

Until recently, regulatory testing for paralytic shellfish toxins (PSTs) in shellfish has almost exclusively been based on the PST mouse bioassay (MBA; AOAC official method 959.08). This assay has been widely used in shellfish toxin monitoring programmes around the globe, including New Zealand, and has provided good health protection to shellfish consumers. Its popularity was due to its regulatory status, cost effectiveness, and use as the reference method for PSTs in the case of trade disputes (EFSA, 2009). However, due to the technical and ethical shortcomings of the MBA, coupled with recent EU legislation limiting the use of animals for regulatory testing (EFSA, 2009), substantial effort has been directed towards developing alternative test methods for PSTs and other marine biotoxins (Hess et al., 2006). Many countries including the UK, Germany, The Netherlands, Norway and France have now ceased MBA testing for shellfish toxins because of a combination of dissatisfaction with the tests for technical reasons and ethical concerns about the use of small animals for routine quality control of food where satisfactory alternatives exist.

Cawthron Institute (www.cawthron.org.nz) provides a commercial biotoxin testing service to the local aquaculture industry. Due to the global shift to instrumental methods for marine biotoxins, coupled with the local shellfish industry wanting an alternative to the MBA, an assessment of analytical test methods for PSTs was made in early 2010 (Holland et al., 2010a). Of the various options available at the time, only AOAC official method 2005.06 was determined to be suitable, as it had been subjected to both single- and inter-laboratory validations studies, including a collaborative study to full AOAC specifications (Lawrence and

* Corresponding author. Tel.: +64 3 548 2319; fax: +64 3 548 9464.
E-mail address: tim.harwood@cawthron.org.nz (D.T. Harwood).

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http://dx.doi.org/10.1016/j.hal.2013.01.003

Niedzwiadek, 2001; Lawrence et al., 2004, 2005). A European inter-laboratory study of the method has also been reported (Ben-Gigirey et al., 2007). This method, colloquially known as the Lawrence method, has been implemented in a number of laboratories internationally. Cefas in the UK has gathered extensive validation data (Algoet et al., 2009), including refinement for use on several matrices and extension of the method to include additional toxins (Turner et al., 2009, 2012). The principle of the method involves pre-chromatographic oxidation of samples that contain various PST congeners, followed by liquid chromatography with fluorescence detection (LC–FLD). The oxidation products are more amenable to reversed phase separation than the parent toxins and they can be measured by fluorescence detection. Although the principal of monitoring hydrophilic PST oxidation products is simple, the chemistry is relatively complex. Some toxins produce identical oxidation products, making it difficult to differentiate between toxins, whereas some produce multiple oxidation products thereby complicating interpretation. Also, some PST groups require treatment with an alternative oxidant to produce optimum yields of the oxidation product and improve sensitivity.

The Lawrence method protocol was adapted for use in the commercial laboratory at Cawthron. This method was permitted for use by the New Zealand Ministry for Primary Industries (formerly NZ Food Safety Authority) for regulatory testing following a thorough evaluation of its performance (Holland et al., 2010b). The method came into routine use as a full replacement for the MBA in mid-2010, although it had been operated intermittently at since 2002 for non-regulatory analyses and had been used as part of an interlaboratory study (Lawrence et al., 2004). Since implementation >2000 shellfish samples have been tested for regulatory purposes. The extraction and oxidation steps were established as specified in the official method, although the chromatographic conditions were updated for use with a UPLC system to provide rapid analysis of the oxidation products. These changes did not compromise any of the performance characteristics of the method.

Soon after the Lawrence method was established at Cawthron a major harmful algal bloom (HAB) of the toxic dinoflagellate *Alexandrium catenella* occurred in the Marlborough Sounds, a prime commercial shellfish growing area of New Zealand. This HAB event in late summer 2011 was accompanied by widespread PST contamination of shellfish and resulted in closures of commercial shellfish harvesting areas for up to a period of three months (MacKenzie et al., 2011). Shellfish toxin levels remained above the regulatory level for an extended period even after the bloom had crashed. This bloom represents the first occasion when the new chemical testing programme based on the Lawrence pre-column oxidation method was used to manage a PST contamination event in New Zealand. The regulatory action was successful, with no reported illnesses or recalls of exported shellfish product. In addition, a large data set was generated and this has allowed a greater insight into the technical performance of the method and potential improvements to its use as a regulatory tool.

In this article we describe the key method characteristics of the Lawrence method implemented at Cawthron and suggest minor amendments to the regulatory guidelines used for PST testing and reporting in New Zealand. Data is also presented from the *A. catenella* bloom events in the Marlborough Sounds over the past two summers that resulted in contamination of commercial shellfish species and harvest closures.

2. Materials and methods

2.1. Standards and reagents

The following certified reference materials were obtained from the Institute of Marine Biosciences, National Research Council of Canada (NRCC, Halifax, Nova Scotia, Canada); saxitoxin (STX), neosaxitoxin (NEO), gonyautoxin 1,4 (GTX1,4), gonyautoxin 2,3 (GTX2,3), gonyautoxin 5 (GTX5, B1), decarbamoylsaxitoxin (dcSTX), decarbamoylneosaxitoxin (dcNEO), decarbamoylgonyautoxin 2,3 (dcGTX2,3) and *N*-sulfocarbamoylgonyautoxin 2,3 (C1,2).

UPLC mobile phase A was 0.1 M ammonium formate, adjusted to pH 6.0 with 1% acetic acid. Mobile phase B was constituted of 90% mobile phase A containing 10% methanol. All mobile phases were filtered prior to use. All other reagents were the same as those detailed in AOAC method protocol 2005.06.

As suggested in the Lawrence method protocol, to aid with interpretation of PST chromatographic peaks three calibration mixes (Mix I–III) were generated at three dilution levels. Mix I contained only *N*-hydroxylated toxins GTX1,4 and NEO. Mix II contained dcGTX2,3; dcSTX; GTX2,3 and STX. Mix III contained C1,2; dcNEO and GTX-5. This combination of toxins was used in each mix to give clearly distinguishable oxidation products with no overlapping chromatographic peaks. No certified reference material is currently available for C3,4 and GTX6. These congeners were calibrated using the response factor of NEO, as it has been demonstrated using non-certified reference material obtained from NRC to give an equimolar response (data not shown).

2.2. Phytoplankton monitoring and shellfish sampling

Phytoplankton and shellfish sampling was undertaken by the Marlborough Shellfish Quality Programme (MSQP). Multiple routine phytoplankton and shellfish sampling sites are located throughout the Marlborough Sounds and these are used by MSQP as part of their routine monitoring programme to manage marine biotoxins (NZFSA, 2006). Integrated water column samples were collected from monitoring sites using a 15 m Lund tube, preserved with Lugol's iodine. Cells were counted under an inverted microscope at the Cawthron Institute. Shellfish (a minimum of 12) were harvested from sampling sites, sent to the Cawthron Biotoxin Laboratory and the whole edible portion homogenized, extracted and analyzed for PSTs.

In this study, PST data is presented for Greenshell™ mussels (*Perna canaliculus*) and Flat oysters (*Ostrea chilensis*) taken from four sampling sites (Fig. 1; Tio Point, Hitaua Bay, East Bay and Opua Bay). Shellfish samples were typically collected weekly during and after the 2011 *A. catenella* HAB event, and between Feb and Apr 2012 for the Opua Bay site. All of the sampling sites are located in, or off, Tory Channel except for East Bay, which is in the outer reaches of the Queen Charlotte Sound. During times when commercial shellfish harvesting ceased due to elevated PST levels, sampling was on an *ad hoc* basis. For the Hitaua Bay site this meant there was a sampling hiatus of about a month over May and early June 2011.

Opua Bay is a non-commercial sampling site that was added in 2012 for research purposes. There are no shellfish farms in Opua Bay. However, it represents the site where the *A. catenella* bloom was most intense the previous year and a visible 'red tide' was observed during field trips to the area. It was thought to be the most likely location where this microalga would bloom again in subsequent years, especially with supporting cyst data obtained from collected sediment samples (MacKenzie et al., 2011, 2012). A buoy was moored in the bay from early September 2011 and weekly phytoplankton samples were analyzed for the presence of toxic microalgae. PST-free Greenshell™ mussels were transferred onto a lantern cage hung from the research buoy moored in Opua Bay during Feb 2012, when *A. catenella* cell numbers had started to increase. Weekly mussel and phytoplankton samples were sampled from mid-February 2012 and sent to Cawthron for testing.

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