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Shifts and stasis in marine HAB monitoring in New Zealand

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Abstract This review article outlines harmful algal bloom (HAB) monitoring practices in New Zealand and highlights the shift from light microscope (LM)-based identification and quantification of the early 1990s to the use of molecular tools to support the HAB monitoring programmes two decades later. Published research and available client information from the monitoring programmes have been reviewed; HAB events and programme changes are highlighted. The current HAB monitoring practices allow for rapid determination of potential biotoxin issues for the shellfish industry and of potential ichthyotoxic events for finfish farmers. The use of molecular tools, including quantitative PCR, has improved risk assessments for those HAB species that are difficult to differentiate to species level using LM. This has enabled rapid feedback to aquaculture managers during HAB events. Tests for biotoxins in flesh remain the regulatory tools for commercially harvested shellfish, but this is supported by the weekly phytoplankton monitoring data. Recreational (non-commercial) shellfish harvesting and commercial finfish aquaculture rely solely on phytoplankton monitoring to assess the biotoxin risk. HAB monitoring in New Zealand continues to maintain internationally recognised standards, and the government-funded research programmes feed the latest knowledge and technical methods into the programmes. The early dependence on light microscopy continues but is now supported by molecular tools, with a view to employing multi-species detection systems in the future. The traditional mouse bioassay test has been fully replaced by chemical tests.

Keywords Phytoplankton monitoring · HABs · Micro-algae · New Zealand · Aquaculture

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Background to monitoring for shellfish biotoxins and their micro-algae producers

The seafood industry in New Zealand continues to grow with the total seafood harvest being approximately 625×10^3 tonnes (including aquaculture; Seafood Industry Council statistics 2010/11). The top ten export species in 2010, which included farmed Greenshell™ mussels (*Perna canaliculus*) and salmon (*Oncorhynchus tshawytscha*), had a value of NZ $1.5b, with farmed Greenshell mussels alone earning nearly NZ $170 million that year. There were closures to harvesting due to harmful algal blooms (HABs), but this is always a potential risk that is managed by the industry.

Monitoring of harmful micro-algae in New Zealand, carried out to protect consumers and to ensure acceptance of shellfish product in export markets, began in earnest in 1993. This followed a major neurotoxic shellfish poisoning event in the Hauraki Gulf in the austral summer of 1992/1993 (Jasperse 1993; Rhodes et al. 2001a). Regulatory closures were based on biotoxin results, and at that time, testing was based solely on the mouse bioassay (MBA). The 1992/1993 event coincided with a long-lasting El Niño/Southern Oscillation phenomenon during which strong El Niños developed in 1991 and lasted until 1995. A later El Niño climate event from May 1997 to June 1998, which had extraordinary strength (Burgers and van Oldenborgh 1999), resulted in sequential and extensive blooms in New Zealand similar to those experienced in 1992 (Rhodes et al. 1993),

i.e. the heterotrophic dinoflagellate *Noctiluca scintillans* and the ciliate *Myrionectra rubra*, followed by raphidophyte blooms and finally, during the summer, potentially toxic dinoflagellate blooms, including *Karenia* spp. The 1993 programme was based on the MBA, but at that time, a phytoplankton monitoring programme was also established. A set of voluntary trigger levels was developed for the various toxic species known to be present in New Zealand waters, i.e. cell concentrations at which shellfish harvesting would cease until shellfish flesh testing results were available.

From the outset, officials collecting seawater samples for analysis have been trained according to specified standards, and site selection has been guided by knowledge of local currents, tidal flows and residence time, amongst other criteria. Samples continue to be collected on an incoming tide in bays (outgoing water is filtered by the farmed shellfish), but along open coastlines, sites tend to be more widely spread as the major currents can move bodies of seawater hundreds of kilometres. Discrete samples at specified depths (e.g. using van Dorn samplers) or composite samples (full column samples collected with a 10–15-m hose) are collected, and both a preserved (with Lugol's iodine) and a live sample are sent for analysis. Analyses are carried out by trained staff to standard protocols using Utermöhl settling chambers under inverted light microscopes (LM). Secondary sampling sites are designated for activation following positive test results, and this allows managers to define the risk area and minimise the geographic extent of closures.

Initially, the centralised New Zealand Marine Biotoxin Management Board was responsible for all monitoring around New Zealand's 15,134 km coastline, but by 1996, the commercial shellfish monitoring programme, managed by the Ministry of Agriculture and Fisheries and funded by industry, was separated from the non-commercial shellfish programme, which was managed and funded by the Ministry of Health (MoH).

A review of the biotoxin surveillance system in relation to non-commercial (recreational) shellfish harvests was carried out in 1996 (Wilson and Sim 1996), and it was deemed that the general risk of biotoxins to the public was low. Management decisions for the non-commercial area were, from that time, based mainly on phytoplankton monitoring data and focussed on areas with a known biotoxin issue (Rhodes et al. 2001a) with considerable cost savings to MoH.

By 1997, at commercial sites where there was no history of biotoxin events, phytoplankton monitoring continued on a weekly basis and shellfish sampling was reduced to once a fortnight, or even monthly at low-risk sites (Hay et al. 2000). The turnaround time for biotoxin analyses between 1993 and 2001 was considerably longer than for the phytoplankton analyses, which were assured within 24 h of sample receipt. Whilst regulatory closures were based on biotoxins in flesh (at closure levels set to meet EU and USFDA regulations), phytoplankton results could still trigger voluntary closures. This action helped avoid later harvest rejection if regulatory biotoxin levels were breached; for example, when *Pseudo-nitzschia* blooms were recorded prior to domoic acid (DA) results being reported (Rhodes and Jiang 2011; Rhodes et al. 2012).

Disruptions to shellfish harvesting are costly, and so as to increase the value of the phytoplankton data for early biotoxin risk assessments, the use of fluorescent in situ hybridisation (FISH) assays was introduced to differentiate between potentially toxic and non-toxic species of *Pseudo-nitzschia* (Rhodes et al. 1998, 2001b). The FISH assays (which have international accreditation; NZS/ISI/IEC 17025) were carried out on request if *Pseudo-nitzschia* levels increased above the agreed risk levels. Following the introduction of the assay, voluntary closures were substantially reduced due to the predominance of low-risk species being present.

Shifts in HAB monitoring practices from 2000

From 2001, chemical analyses (liquid chromatography–mass spectrometry, LC–MS) were employed for monitoring of 18 lipophilic biotoxins, and these tests replaced the use of the MBA for DSP toxins (McNabb et al. 2005). Domoic acid and epiDA analyses based on high-performance liquid chromatography–UV detection methods were also replaced by LC–MS (validated and approved in 2001; Holland et al. 2003). The monitoring programmes became completely 'mouse free' in 2010 and this ensured fewer false positives, greater specificity and higher sensitivity. Access to certified reference materials and adherence to an ongoing QC/QA regime became critical to the programme's success, and in many instances, the Cawthron Institute Culture Collection of Micro-algae (CICCM) provided the toxin producers to allow mass production of the materials needed.

The full dependence on chemical tests and functional assays for the biotoxin monitoring programmes ensured a rapid turnaround time for results. Phytoplankton monitoring continued in tandem, and this gave a full picture of HAB development, but use of FISH assays decreased and they are now rarely requested for commercial sites.

The dual monitoring system proved invaluable in 2004 when yessotoxin (YTX) was detected in shellfish from Keneperu Sound (Marlborough Sounds) with no associated YTX producer identified in the seawater samples. The dominant species present was *Gonyaulax spinifera*, at that time considered a non-toxic dinoflagellate. YTX production was confirmed for this species (Rhodes et al. 2006), and it is now reported as a HAB species. Interestingly, the MBA did not detect this toxin.

From March (austral autumn) to April (winter) 2011, a major *Alexandrium catenella* bloom with associated saxitoxins

halted all shellfish harvesting in the Queen Charlotte Sound (Marlborough Sounds). Low cell counts of *A. catenella* were reported as early as February, the identification being based on light microscopy and Calcofluor staining of cell thecae (Cawthron Institute Micro-algae Laboratory data) and the harvesting closures persisted until 20 June 2011. *A. catenella* blooms had been recorded previously in the northeast of New Zealand (from Northland to Bay of Plenty), but this was the first incident in the South Island (Mackenzie et al. 2011a). The Cawthron Institute Biotoxin Laboratory used the chemical analytical method for saxitoxins, authorised by NZ Food Safety Authority in 2010, to analyse for, and assist management of, the saxitoxins present (AOAC 2005; Holland 2010); the method, based on the Lawrence method, replaced the MBA. An ongoing concern is that cysts from the bloom will supply a long-term seed source for future bloom events in the region. Further research based around cyst monitoring and molecular identifications is being carried out (refer "Monitoring of benthic and epiphytic HABs, including dinoflagellate cysts and cyanobacterial mats" and "Molecular tools" section).

In the non-commercial monitoring programme, requests for FISH assays have increased since 2001 and have proved useful for managers in making decisions regarding shellfish testing. On the basis of research results, trigger levels for *Pseudo-nitzschia* species were relaxed in 2006 from a blanket 50×10^3 cells l^{-1} for all species to 100×10^3 cells l^{-1} for *Pseudo-nitzschia australis*, *Pseudo-nitzschia pungens* and *Pseudo-nitzschia multiseries* and to 500×10^3 cells l^{-1} for *Pseudo-nitzschia turgidula*, *Pseudo-nitzschia fraudulenta*, *Pseudo-nitzschia delicatissima*, *Pseudo-nitzschia pseudodelicatissima* and *Pseudo-nitzschia multstriata* (NZFSA 2006).

The use of a solid-phase adsorbant toxin tracking (SPATT) system linked to LC–MS analyses has been shown to give extremely early detection of potential bloom events (Lane et al. 2010). SPATT has been used to successfully correlate DA with the presence of *Pseudo-nitzschia* cells in New Zealand coastal waters (Mackenzie 2010, personal communication), and even though it is largely restricted to research studies in New Zealand, it does offer an extra technique in the tool kit for HAB events. Currently up to 100 seawater samples per week are collected for analysis from around New Zealand's coastline.

Monitoring for HABs impacting finfish farms

In the 1980s, a major *Heterosigma akashiwo* bloom signalled the need for HAB monitoring for fish farms (Chang et al. 1990). Since then, many wild fish kills have been reported, most attributed to asphyxiation rather than biotoxins, and many have been attributed to *Karenia* species (de Salas et al. 2005).

FISH probes have been used to assist in identifying the dominant species in these blooms (most commonly *Karenia mikimotoi*; Smith et al. 2007), but the assay has not been as successful as for the genus *Pseudo-nitzschia* due to the delicate nature of the *Karenia* cells and the need to preserve them for transport. A sandwich hybridisation assay (SHA) received international accreditation for the raphidophytes (Ayers et al. 2005) and was also validated for species of *Karenia*, but real-time PCR methods appear to be a more rapid and sensitive approach than FISH or SHA assays for the species in the *Karenia* genus.

In mid-winter 2010, farmed salmon (*O. tshawytscha*) began dying in the Marlborough Sounds, South Island, with a total of 200 tonnes being lost during 1 week (Mackenzie et al. 2011b). A bloom of *Pseudochattonella verruculosa* was found to be the cause of the mortalities. The bloom had developed in Grove Arm in the inner sound and had then moved into the farm site. As a result, monitoring was briefly stepped up to twice daily throughout the affected area. This allowed the sea cages to be moved to monitored areas where no *P. verruculosa* was present. Since that time, phytoplankton monitoring guidelines have been further clarified for the steps to be implemented in any future ichthyotoxic bloom events.

Amendments to the government regulatory framework for marine farming will allow for substantial expansion of the sea-cage rearing of salmon and other finfish species in New Zealand over the next decade. Some of these developments will take place in areas where ichthyotoxic blooms are common, e.g. raphidophyte blooms in the Hauraki Gulf. The current lack of understanding of the mechanisms involved in the fish deaths due to HABs is hampering development of better mitigation measures, although some hypotheses suggest interactions between reactive oxygen species and free fatty acids at the fish gill are responsible (Marshall et al. 2003).

Predictive modelling is being developed which will at least give a warning of increases in the risk of an event occurring, but at present traditional phytoplankton monitoring continues at weekly intervals, supported by DNA probe techniques during blooms.

Monitoring of benthic and epiphytic HABs, including dinoflagellate cysts and cyanobacterial mats

Not all HAB species are planktonic and occasionally benthic, or epiphytic samples may be provided for analysis. For example, the death of dogs beside waterways or on beaches will always cause public concern, and in these instances, HABs are considered as a possible cause. A wide range of sample types may be collected, including intertidal surface sediments, and a system of partitioning with graded mesh sieves is used to prepare these diverse samples for

microscopic analysis (Rhodes et al. 2010). In 2009, dog deaths on Auckland beaches were shown to be caused by tetrodotoxin (TTX), which was detected in brown-gilled sea slugs (*Pleurobranchaea maculata*). It is probable that the causative agent was bacterial rather than micro-algal, although this has yet to be determined, but the varied sampling approaches used, backed by LC–MS analyses and toxicology expertise, helped in the identification of this new biotoxin to New Zealand within 1 week (McNabb et al. 2010). TTX may be found, albeit at low levels, in marine fauna that may be consumed by organisms further up the food chain (Taylor et al. 2011) and so new sampling methods and the addition of TTX testing to biotoxin monitoring programmes will need to be considered to protect recreational harvesters.

Closures to the harvesting of oysters in Northland harbours throughout the late 1990s, based on the MBA, proved to be due to pinnatoxins produced by the peridinoid dinoflagellate, *Vulcanodinium rugosum* (Rhodes et al. 2011). This micro-alga was difficult to detect due to its rare occurrence in the plankton, occurring mainly in non-motile form in surface sediments. Similarly, the okadaic acid producing *Prorocentrum lima* and the palytoxin-producing *Ostreopsis siamensis*, both common benthic mat formers in New Zealand's northern sub-tropical waters, are rarely found in plankton samples and yet can form vast mats on the seabed and as epiphytes covering aquatic plants (Rhodes and Syhre 1995; Shears and Ross 2009). Both species do occasionally occur as phytoplankters, usually following disturbances (e.g. after storms). It is when these species are in the water column that they are most likely to be of concern for shellfish harvesting, and no illnesses have been caused by any of these benthic/epiphytic biotoxin producers in New Zealand to date.

The recent *A. catenella* bloom in the Marlborough Sounds resulted in cyst beds forming on the sea floor (MacKenzie et al. 2011a). Surface sediment analyses are being conducted to determine the extent of this seed source and the risk of causing further bloom events. The cyst surveys are being carried out in conjunction with quantitative PCR (qPCR) analyses (Murray et al. 2011) of sediment and water samples, the ultimate aim being the development of a rapid method for future predictive monitoring of PSP events. Samples are subjected to the standard sieve series preparation and also to density gradients (Lincoln Mackenzie, Cawthron Institute, personal communication) combined with the use primuline stains. These methods are currently being refined for routine use for the detection of both *A. catenella* blooms and saxitoxin production.

Molecular tools

The use of the FISH assays has been discussed, but the programme is working towards a more molecular based detection system, still supported by the traditional morphological identification methods. The sandwich hybridisation assay (SHA), a DNA probe-based assay, which uses species-specific oligonucleotide probes targeted at large sub-unit ribosomal RNA, was originally developed by Scholin et al. (1996, 1997, 2003). It has been used extensively in New Zealand for research purposes, e.g. for species of *Pseudo-nitzschia* (Rhodes et al. 2001b), but has not proved simple and rapid enough to replace the current practices in the routine monitoring laboratory. The sensitivity of this assay is insufficient to meet the monitoring requirements for the saxitoxin producing *Alexandrium* spp. (i.e. a trigger level of 100 cells/l), and there is no amplification step. The sensitivity is sufficient for species with 10- to 100-fold higher trigger levels, such as those in the genera *Karenia* and *Pseudo-nitzschia*. The method is also suited to fragile micro-algal cells, e.g. raphidophytes and *Karenia* spp. (Ayers et al. 2005), which commonly collapse during the fixation stage of sample collection, compromising identification by traditional microscopy. The SHA was validated and accepted for international accreditation for commercial laboratory use in New Zealand in May, 2004 (International Accreditation New Zealand: ISO 17025; Ayers et al. 2005). During the validation of the SHA for raphidophytes, some discrepancies were noted between SHA cell concentration estimates and traditional LM cell counts. Higher SHA estimates were recorded when blooms had collapsed, but rRNA was still present in seawater. Conversely, higher traditional cell counts occurred when sample delivery was delayed more than 48 h, presumably owing to degradation of rRNA in the live cultures used for the SHA. For the more robust toxic diatom genus, *Pseudo-nitzschia*, FISH, SHA and traditional microscope counts are comparable for the three methods tested (Ayers et al. 2005). A successful SHA was validated for the PSP-producing dinoflagellate, *Gymnodinium catenatum* (Rhodes et al. 2007), but has not entered into commercial use as the cells are relatively easy to differentiate from the non-toxic 'look-alike' *G. impudicum* under the LM.

During the *A. catenella* bloom event in the Marlborough Sounds, 2011, newly developed qPCR assays were trialled to rapidly determine cell concentrations (Harlow et al. 2005; Mackenzie et al. 2011a). The assay has the potential to determine the abundance of extremely low cell concentrations of *A. catenella* and so trace bloom development while minimising the onerous task of LM-based cell counting. The adaptation of another qPCR assay that targets a section of one of the genes involved in saxitoxin production (Murray et al. 2011) is also underway.

The Environmental Sample Processor (Ryan et al. 2011; Harvey et al. 2012) will be trialled in Tasman Bay, Nelson, New Zealand this year, and its potential for use in some regions for the HAB monitoring programme will be assessed. Both SHA and qPCR approaches will be trialled.

For the development of the molecular tools, the availability of cultures from the CICCM has provided a continual source of unique DNA sequence data and material with which to trial both molecular and chemical assays as well as a teaching tool for the phytoplankton monitoring team.

Conclusions

The stasis in the monitoring programmes is represented by the 'gold standard' of LM identification methods. The LM investigations may be supported by observations of thecate cells under an epifluorescent microscope following Calcofluor staining and FISH probes continue to add value to the *Pseudo-nitzschia* risk assessments. However, the rapid turnaround in the biotoxin results has led to a reduced dependence on the phytoplankton monitoring for commercial programmes. This is counteracted by an increased use by the non-commercial programmes, in which shellfish flesh testing is limited. All seawater samples received in the laboratory include a 'fixed' and an untreated sample, the latter being available to researchers. The close working relationship between the HAB monitoring programmes and the HAB researchers continues to ensure that the programmes remain dynamic, with constant refining of standard methods and addition of validated novel practices.

The 'shift' in the phytoplankton monitoring has been the move towards rapid throughput qPCR assays. These are not yet employed commercially and are still being validated through the research programmes, but have been used on many occasions to determine the dominant species in blooms, e.g. *Karenia* blooms (Smith et al. 2007) and, more recently, the *A. catenella* bloom, and associated cysts, in the Marlborough Sounds. The desire to develop a multi-species detection system has led to various approaches being investigated, including the Environmental Sample Processor (Harvey et al. 2012). With the rapid developments in next-generation DNA sequencing technology, it is possible that handheld sequencers will be the future of phytoplankton monitoring programmes.

Recently, long-term data sets from the biotoxin monitoring and phytoplankton monitoring programmes were analysed for DA and *Pseudo-nitzschia* species (Rhodes et al. 2012). The results of these analyses highlighted the fact that blooms are influenced by season, but may occur year round. The most common bloom forming species produce low DA concentrations per cell or are non-toxic, although the highly toxic *P. australis* is a dominant in 10 % of blooms. The risk of amnesic shellfish poisoning (ASP) occurring in New Zealand is low, and there have been no ASP events reported. Further analyses of long-term data sets for different toxins and their producers are warranted.

Results from research carried out through the government-funded programmes, e.g. the current 'Innovative Systems for Safe New Zealand Seafood in Premium Markets' and the completed 'Managing risks to aquaculture from lethal algal blooms', continue to be fed into the commercial monitoring programmes, a key result being the full replacement of the inadequate MBA with chemical and functional tests. Concurrently, the research programmes benefit from the thousands of weekly samples that are received from clients and made available to researchers. The success of the programmes is reflected in the absence of shellfish-related human illnesses arising from New Zealand product throughout the past two decades.

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