

## **Recent developments in the detection of phycotoxins**

Philipp HESS\*, Elodie NICOLAU

Ifremer, Centre Atlantique, Département Environnement Microbiologie et Phycotoxines, Laboratoire Phycotoxines, BP 21105, F-44311 Nantes cedex 03, France

\* Corresponding author ; E-mail : [philipp.hess@ifremer.fr](mailto:philipp.hess@ifremer.fr)

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### **Abstract**

Over the past seven years, methods available for the detection of phycotoxins have been extensively reviewed in a number of international expert committees, such as the consultations organised by FAO/IOC/WHO and EFSA, as well as by individual scientists. These reviews have shown that the methods available have severe limitations for the use in official control, either due to their limited scope and detection capability or due to a lack of calibration standards, reference materials and validation efforts. The present review focuses on recent developments in the detection of phycotoxins in several areas of applied research. Not being able to exhaustively describe all recent developments, the review focussed on three areas of interest to the authors: (i) detection of ultra-trace amounts of toxins, (ii) metabolism of toxins and their localisation in biological tissues, and (iii) approaches to detect unknown toxins or analogues of known toxins. Miniaturisation in combination with physico-chemical techniques appears to be a very efficient approach to detect low trace amounts of individual toxin analogues. In particular, the detection of azaspiracids and okadaic acid and analogues, using micro-filtration and on-line pre-concentration techniques, has shown to be useful for the characterisation of various algal and shellfish species. In the area of interactions of toxins with shellfish and mammalian systems, it is noted that several studies on biomarkers reveal either protein biomarkers of exposure to toxins or potential pathways of metabolism of the toxins themselves. A particular focus is given to recent findings in the areas of brevetoxin metabolism and biomarkers as well as azaspiracid localisation and metabolism. Finally, the detection of novel compounds is a particularly challenging area. The interest in this area has risen over the past years following cases of unexplained mouse toxicity such as the UK cockle toxicity and the French atypical toxicity in mussels and oysters from the Atlantic and Mediterranean coasts. Some attention is given to immuno-, functional and cellular bio-assays for the identification of bioactive agents in shellfish.

### **Développements récents dans la détection des phycotoxines**

Durant les sept dernières années, les méthodes disponibles pour la détection des phycotoxines ont été abondamment revues dans de nombreux groupes internationaux d'expertises, tels que les consultations organisées par FAO/IOC/WHO et EFSA ainsi que dans de nombreux articles scientifiques de synthèse. Ces revues ont démontré que, vis-à-vis des systèmes de régulations officiels, les méthodes disponibles présentent des limites d'utilisation importantes. Soit elles ont un périmètre réduit et des limites de détection trop élevées, soit il y a un manque d'étalons, de matériaux de référence ou d'efforts de validation. La présente revue focalise sur les développements récents dans la détection de phycotoxines en recherche appliquée. Sans vouloir décrire de manière exhaustive tous les développements récents, la revue examine trois domaines d'intérêt pour les auteurs : (i) la détection de quantités ultra-traces de toxines, (ii) la métabolisation et la localisation des toxines dans des tissus biologiques, et (iii) les approches pour la détection de toxines non-répertoriées ou des analogues de toxines connues. La miniaturisation en combinaison avec les techniques physico-chimiques constitue apparemment une approche efficace pour la détection de faibles traces d'analogues individuels des toxines. En particulier, la micro-filtration et des techniques de pré-concentration en ligne se sont montrées utiles pour la détection des azaspiracides et des toxines du groupe de l'acide okadaïque dans la caractérisation de diverses espèces d'algues et de coquillages. Dans le domaine des interactions des toxines avec les coquillages et des systèmes vivants de mammifères, nous avons noté que plusieurs études sur les bio-marqueurs révèlent soit des marqueurs protéiniques d'exposition aux toxines, soit des marqueurs du métabolisme des toxines elles-mêmes. Un intérêt spécifique a été trouvé dans les résultats d'étude sur le métabolisme des brevetoxines et des azaspiracides. La détection des composés bioactifs non-répertoriés constitue un défi particulièrement difficile. Ce domaine a trouvé plus d'intérêt dû à plusieurs cas de toxicités inexpliquées dans le test souris telles que les toxicités observées dans les coques en provenance de Grande-Bretagne ou encore dans les moules et huîtres des côtes méditerranéennes et atlantiques françaises. Une attention particulière a été consacrée à l'identification d'agents bioactifs par les essais cellulaires ou fonctionnels ou basés sur la détection immuno-chimique.

**Keywords** : Marine biotoxins, metabolism, localisation, passive sampling, proteomics.

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## Methodological requirements for official control and research applications

Over the past two decades, methodology for the detection of marine biotoxins has rapidly evolved for many areas of application. The main drivers for methodological developments were the need for efficient and complete detection of toxins in shellfish for the purpose of public health and the multitude of scientific questions that require particular detection methods to be answered. Such distinct questions range over many disciplines from the elucidation of biosynthetic pathways over the theme of harmful algal bloom dynamics to the localisation of toxins in shellfish and their metabolism at various trophic levels. Previous reviews have mainly focussed on the evaluation of available detection technologies and their application in official control. Such reviews have been carried out in a systematic manner during risk evaluation at European or international level such as the FAO/IOC/WHO expert consultation exercise (Anonymous, 2005) or the more recent assessments of the European Food Safety Authority (EFSA 2008a, 2008b, 2009a, 2009b, 2009c, 2009d, 2009e). Individual scientists have also critically assessed available methodology, in particular comparing the performance of physico-chemical analytical tools and biological assays (Hess, 2010; Humpage *et al.*, 2010; Vilariño *et al.*, 2010). Reviews focusing on the suitability of methods for official control clearly show that there are few methods that are validated to a level that satisfies typical formal requirements for their application. While mammalian bioassays are at the limit of being capable to implement current legal limits for phycotoxins, chemical detection methods suffer from limitations in the scope of method due to the lack of a suitable range of calibration standards for all toxicologically relevant analogues. The latter issue has been addressed extensively over the last few years, either at the National Research Council of Canada's Institute of Marine Biosciences in Halifax or by other international initiatives (Hess *et al.*, 2007). Biological and biochemical tools have also been extensively developed for toxin detection using a) antibodies (enzyme-linked immuno-sorbent assays (ELISA), lateral flow immuno-chromatography (LFIC) and surface plasmon resonance (SPR)-based methods), b) functional assays (PP2a-inhibition assay and receptor-binding assays) and c) cellular functional assays, including assessment of hemolytic activity and others. Most of these biochemical or cellular assays have a major drawback for official control purposes: they are specific to a single toxin group or a subset of toxin groups and therefore, multiple combinations of assays are necessary to assess the toxicity of any shellfish sample.

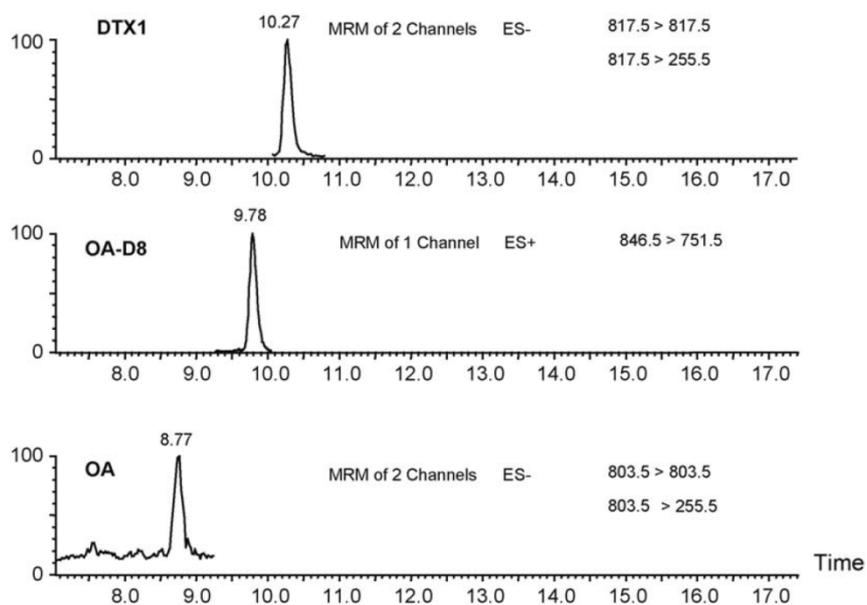
Nonetheless, the development of the above-mentioned techniques has allowed many areas of applied research to respond to the numerous questions around biotoxins. This review focuses on recent developments in the area of research applications. In following the toxins from their producing organisms over the shellfish vectors to the human targets, three areas of research have attracted our interest: i) methods applied to detect low amounts of toxins in seawater and algal cells, ii) techniques used for the characterisation of toxin biotransformation and their localisation in shellfish and finally iii) approaches for the characterisation of biological activity in toxin producers and shellfish. The requirements for detection techniques in research application often differ dramatically from those of official control. While traceability to international standards is mandatory in the area of official control, it is often negligible in the area of research as here most studies are concerned with proof of principles. Detection and quantitation capabilities are important for both areas; however, significant efforts are often made in research applications to achieve detection, while the same efforts could not be justified in routine control.

## Methods for the detection of ultra-trace levels of toxins

An interesting approach for the detection of very small quantities of toxins was developed by Hardstaff *et al.* (Hardstaff *et al.*, 2006), using hyphenated chromatography for the analysis of algal cells. The technique developed has overcome difficulties in the detection of low toxin amounts in cells of *Alexandrium ostenfeldii* by applying micro-extraction on spin-filters, followed by large-volume injection and reversed-flow, two-dimensional chromatography prior to detection by liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS). In this way, the detection of spirulides from as few as 50 hand-picked cells became possible, and the correlation of toxin content with cell-size could be made for the first time in the area of algal toxins. We have subsequently applied the same technique for the detection of low amounts of azaspiracids in Portuguese shellfish (Vale *et al.*, 2008). This study clarified the geographical extent of the occurrence of azaspiracids (AZAs) which had previously not been detected in Portugal. This finding completed the picture of occurrence all along the Atlantic arch from Norway to Morocco (Twiner *et al.*, 2008). Although the technique itself is not recommended for use in routine official control, it has allowed for the characterisation of the danger posed by this toxin group. The technique had also proven useful to elucidate the phenomena of toxin production by American species of *Dinophysis* (Hackett *et al.*, 2009). While *Dinophysis* had been associated with the production of okadaic acid (OA) and analogues in a number of regions in Europe and Asia, the same algal species had not been causing toxicity in North American shellfish until very recently (Swanson *et al.*, 2010). Hackett *et al.* (2009) clearly demonstrated that *Dinophysis acuminata* from the Gulf of Maine is capable of producing toxins in culture although at very low concentrations compared to other strains. Additionally, an interesting profile was discovered showing large proportions of OA as diol-ester while Dinophysistoxin-1 (DTX1) was present as the free toxin (Figure 1). These findings will also need to be followed through using biosynthetic studies of both toxins. Additionally, a combination of LC-MS-MS, biochemical and cellular techniques were used to find low amounts of OA in a Malaysian species of *Prorocentrum rathymum* (Caillaud *et al.*, 2010).

A different approach to detect low amounts of toxins directly in seawater was proposed by MacKenzie *et al.* (MacKenzie *et al.*, 2004) through the use of passive samplers. Although this approach had frequently been used

in the area of environmental contaminants and natural products discovery, it was only in 2004 that these authors applied passive samplers to marine biotoxin research. Although initially proposed as a technique that could be implemented to provide early warning of toxin occurrence to shellfish farms, a subsequent study has shown the limitations of this approach by demonstrating that the early warning would have had to be placed at distant sites due to the rapid advection of algae and the rapid accumulation by shellfish (Fux *et al.*, 2009). Nevertheless, the passive sampling techniques clearly have a role to play in prospecting for new shellfish cultivation areas, both in coastal and off-shore aquaculture.



**Figure 1.** LC-MS-MS chromatogram showing the profile of OA-group toxins in American *Dinophysis acuminata* from the Gulf of Maine. Adapted from Hackett *et al.*, 2009.

**Figure 1.** Chromatogramme CL-SM-SM clarifiant le profil de toxines du groupe AO dans du *Dinophysis acuminata* américain provenant du golfe du Maine. Adapté de Hackett *et al.*, 2009.

These studies show that the application of specialised techniques is often necessary to answer specific questions. Overall, the study of such low quantities of toxins in seawater, algal strains of potentially toxic species, or in shellfish accumulating these, leads to the question of the relevance to food safety. At this stage, only the potential for toxin production and occurrence can be demonstrated while further research will be necessary to demonstrate whether environmental parameters trigger changes in the production of toxins by these species.

## Methods used in the localisation and metabolism of toxins in shellfish and mammals

A recent study investigated the organ distribution of AZA1 through sublethal oral dosing in mice (Aasen *et al.*, 2010). While highest concentrations after 24 h were found in the stomach and duodenum, the toxin was also readily absorbed into inner organs in a dose-dependent manner, with highest concentrations being found in the kidneys, liver and spleen followed by detectable concentrations in lung and heart. After seven days, the toxin levels had dropped significantly in all organs, except for the kidneys. However, the total amount of toxin found in the internal organs of mice only accounted for ca. 2% of the total dose given, which is consistent with pathological changes only being observed in the intestinal body parts. Thus, further studies will be necessary to investigate whether excretion played a major role or whether transformation into metabolites is a significant contributing factor. In analogy to their above-mentioned work on brevetoxins, Guo *et al.* also demonstrated that okadaic acid is effectively metabolised by mammalian cytochrome P450s (Guo *et al.*, 2010). Interestingly, the okadaic acid structure is also oxidised by human CYP3A4 and CYP3A5 into its hydroxy-metabolites. Still, it remains to be investigated which of the enzymes involved in the metabolism of AZA are of shellfish and which are of mammalian origin.

Understanding the metabolism of marine biotoxins in live animals is another area of challenging research. Enzyme levels may vary much in different biological tissues, therefore, localisation of toxins in different tissues or sub-cellular organelles will also play a major role in the full comprehension of metabolism.

When examining the metabolism in shellfish, a first major stumbling block is the lack of the complete genome of any shellfish species. Although several studies are underway to overcome this lack, current knowledge of shellfish proteins remains rather limited. This lack results in the studies on shellfish proteins involved in toxin metabolism being much more complex and less conclusive in terms of the proteins involved.

Principally, when examining the interactions of shellfish proteins with toxins, there could be a distinction of three phenomena: i) binding of toxins by proteins without metabolism ii) metabolism of toxins by existing or pre-expressed proteins iii) metabolism of toxins by induced proteins. In addition, the toxicity of biotoxins to the shellfish themselves must not be underestimated (Landsberg, 2002).

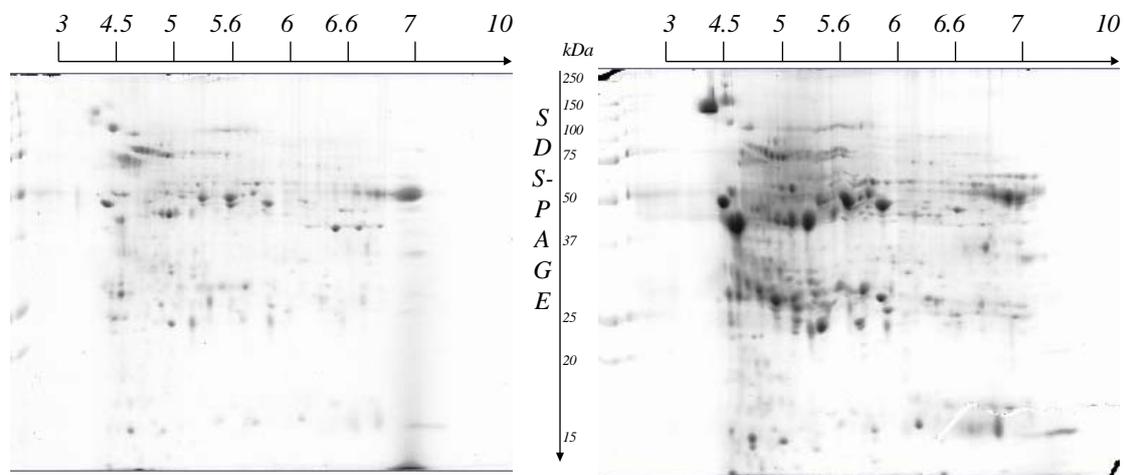
Proteomic biomarkers of pollution with xenobiotics have been effectively demonstrated in mussels (Apraiz *et al.*, 2006). Similarly, the localisation of lipophilic chemicals at specific binding sites had been shown, for instance, for phenanthrene and polychlorinated biphenyls in mussels (Einsporn and Koehler, 2008).

Rossignoli and Blanco 2008 have investigated for the first time the distribution of OA in different cell types of mussel digestive glands (Rossignoli and Blanco, 2008). Subsequently, this group have investigated the sub-cellular distribution of OA (Rossignoli and Blanco, 2010). In this study, the authors applied a combination of protein isolation techniques and toxin analysis with enzymatic digestion of proteins to suggest that a lipoprotein was the main agent binding OA in the cytosol of mussel digestive gland cells. Further research will be necessary to corroborate this work.

When investigating the digestive gland proteome of shellfish exposed to toxic algae, a complex picture was obtained (Figure 2). The comparison of shellfish exposed to a non-toxic feed alga as control with those exposed to toxic *Alexandrium* outlines how many proteins are over-expressed as a function of the toxic insult presented to the shellfish (unpublished work from the authors' laboratory).

In principle, the proteins identified in exposed digestive glands can either originate from the shellfish (normally present or induced) or may arise from the toxic algae themselves. Such an algal biomarker has been identified in mussels naturally exposed to *Dinophysis* (Ronzitti *et al.*, 2008). In a freshwater system, the clam *Corbicula fluminea* expressed a number of proteins in the digestive tract following exposure to *Microcystis aeruginosa*, a toxic cyanobacterium producing microcystins (Martins *et al.*, 2009). The proteins were shown to be involved in the cytoskeleton assembly and proteins with metabolic activity. These results were coherent with the toxic effects of microcystins on PP2a. Marine bivalves have also been shown to contain significant amounts of PP2a and the reaction of the bivalves should include metabolism followed by excretion or sequestration of the toxins in the digestive tract to prevent their distribution across other organs (Suzuki *et al.*, 2005).

Brevetoxin biomarkers in shellfish and humans have been outlined in a recent review (Plakas and Dickey, 2010). The involvement of cytochrome P450 mediated metabolism of brevetoxins has also recently been shown (Guo *et al.*, 2010). Interestingly, the exposure of brevetoxin-2 (PbTx-2) to human liver microsomes resulted in a number of known metabolites in shellfish, e.g. BTX-B5.



**Figure 2.** Two dimensional SDS-PAGE analysis of the protein fraction of mussel digestive glands following 1-day exposure to non-toxic *Skeletonema costatum* (left) and toxic *Alexandrium minutum* (right).

**Figure 2.** Analyse bi-dimensionnelle SDS-PAGE de la fraction protéinique de glande digestive de moules exposées pendant 1 jour à de la culture de *Skeletonema costatum* non-toxique (gauche) et de la culture toxique d'*Alexandrium minutum* (droite).

The same metabolites were also produced upon exposure of PbTx-2 to nine human recombinant cytochrome P450s. In particular, human CYP3A4 metabolised the terminal aldehyde-group in PbTx-2 to the carboxylic acid group of BTX-B5. A novel metabolite was also detected in the study of these human enzymes, outlining the importance of metabolism studies in the comprehension of human exposure to toxins.

Azaspiracids are another toxin group for which multiple metabolites have been identified in shellfish (Rehmann *et al.*, 2008). A pathway of oxidative metabolism in mussels has been recently postulated for AZAs (McCarron *et al.*, 2009). As only AZA1 and -2 have been identified in the producing organism, *Azadinium spinosum*, several mechanisms must be assumed to explain the multitude of analogues determined. Two separate reactions of hydroxylation at carbon 3 (in the Western part of the molecule) and at carbon 23 (at the

E-ring of the skeleton), and a further oxidation at the E-ring may be followed by spontaneous decarboxylation, as demonstrated by studies using deuterated methanol (McCarron *et al.*, 2009). These four reactions may suffice to explain the multitude of analogues detected so far. Nzoughet *et al.* recently investigated proteins in mussels exposed to AZAs (Nzoughet *et al.*, 2008; Nzoughet *et al.*, 2009). Using IEF, size exclusion chromatography and SDS-page, these authors initially isolated two proteins of ca. 22 and 45 kDa to which AZAs bind (Nzoughet *et al.*, 2008). In a follow-up study, four proteins were identified, three of which appeared to belong to the mussels themselves whereas the fourth protein was a bacterial flagellar protein (Nzoughet *et al.*, 2009). Further research is currently underway to examine if these bacteria are symbiotically associated with the causative organism and may as such serve as biomarkers of exposure to the organism. In addition, it remains to be clarified if it is the same shellfish proteins to which AZA is found to bind strongly that also metabolise AZA. As initial comparative analysis suggests homology of these proteins with cathepsin D, superoxide dismutase and glutathion S-transferase, the metabolic pathway postulated may yet be more complicated than anticipated.

## Approaches for the characterisation of biological activity in toxin producers and shellfish

The above-mentioned risk evaluations by FAO/IOC/WHO and EFSA have led to a major change in legislation for the control of lipophilic marine biotoxins in shellfish for which the reference method will be based on LC-MS technology. This change has been presented at global level to Codex Alimentarius, World Health and Trade Organisations (WHO and WTO) and initial reactions from some countries have made it abundantly clear that phasing in of this change will require several years. In addition, the precautionary principle is maintained but responsibility for this area is put in the EU on individual Member States. While equivalence of biological testing and LC-MS testing could be successfully shown for AZAs at levels around the current regulatory limit (Hess *et al.*, 2009), the performance of biological assays at lower levels or for other toxin groups has not proven sufficient. With the increased Member State responsibility of vigilance for novel toxic agents and the routine implementation of chemical testing, it becomes more important to have efficient tools for the detection of unknown bioactive molecules in shellfish. Several research initiatives have already started in this area and will need to be strengthened to address the issue.

A major effort has been made in the detection of neurotoxic bioactive molecules (Ledreux *et al.*, 2009). The authors have developed an approach with cellular assays to allow for the detection of a variety of known shellfish toxin groups and to point to large groups of biological targets of neurotoxic compounds. However, the shellfish matrix still poses problems of interference with the assay and further efforts will be necessary to overcome this issue.

A test applicable for both marine and freshwater toxins was optimised using recombinant enzymes (PP2A) (Ikehara *et al.*, 2008), and quantitative structure activity relationships of a number of analogues could be established thus allowing for more effective monitoring of microcystins (Ikehara *et al.*, 2009).

In the area of lipophilic toxins, significant progress was achieved using a neuro-2A cell bioassay in one laboratory (Canete and Diogene, 2008; Canete *et al.*, 2010; Canete and Diogene, 2010), however, it will be interesting to see whether this approach can be reproduced in a larger number of laboratories. The same authors also proved that their model can be used in combination with LC-MS-MS and PP2a for detection of OA in *P. rathymum* (Caillaud *et al.*, 2010) or for previously unknown maitotoxin-like compounds (Caillaud *et al.*, 2010). Finally, these authors also demonstrated the effectiveness of the cellular assay when coupled with fractionation HPLC for both shellfish and micro-algal matrices, although the assay format requires 48 h exposure for best sensitivity (Caillaud *et al.*, 2009).

## Conclusions

Significant progress has been made in several areas of biotoxin methodology. Among such areas we found particularly interesting the techniques used in the ultra-trace detection of phycotoxins, those in the area of proteomics and toxin metabolism and those in the area of biological screening tools.

We anticipate that these three areas will heavily influence the developments in coming years as they will allow for better prediction and monitoring of algal toxins, better understanding of their fate in the marine food chain and better public health protection.

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