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Cyclic imines, as emerging marine toxins: Chemical properties, distribution, toxicological aspects and detection methods

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Κυκλικές ιμίνες, μια ομάδα νεοεμφανιζόμενων βιοτοξινών: Χημικές και τοξικολογικές ιδιότητες, κατανομή και μέθοδοι ανίχνευσης

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ABSTRACT. Shellfish and, specifically, bivalve molluscs are a food commodity of great interest for both commercial and public health reasons. They consume microalgae from surrounding waters, which are generally beneficial for aquaculture, but they comprise certain toxin-producing species. These species produce marine toxins which, via the filter-feeding mechanism of bivalve molluscs, accumulate in their tissues. This accumulation is more intense and more dangerous for public health during the so-called periods of Harmful Algal Blooms (HABs) when the microalgal population grows. According to their chemical structure, marine toxins are classified into 8 groups, one of which is the cyclic imines. These lipophilic toxins were accidentally discovered during routine bioassays for the detection of other lipophilic marine toxins due to the induction of neurological symptoms and acute death in mice. They include the following subgroups: Spirolides (SPX), gymnodimines (GYM), pinnatoxins (PnTX), pteriatoxins (PtTX), prorocentrolides and spiro-prorocentrimines. The European Union (EU) is more concerned about the first three subgroups, because, in contrast with the latter three, they have already been detected in Europe or there is strong evidence supporting their presence. Spirolides are produced by the dinoflagellate Alexandrium ostenfeldii/peruvianum, gymnodimines by the dinoflagellate Karenia selliformis and pinnatoxins by a peridinoid dinoflagellate recently described in the new genus Vulcanodinium spp.. Although there is insufficient information regarding the geographical distribution of cyclic imines, the fact that they have been detected on multiple occasions in European waters, in combination with their aforementioned acute toxicity in mice after intraperitoneal injection, has established them, at least within the EU, as a topic of profound scientific research. In spite of their acute toxicity in mice, no incident of human intoxication has been attributed to cyclic imines. Presently, the EU has neither set any Maximum

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Ημεgομηνία υποβολής: 06.07.2011 Ημεgομηνία εγκρίσεως: 18.07.2011 Permissible Limits for the concentration of cyclic imines in shellfish nor appointed any reference method for their detection and quantification. Currently, the methods applied are biological, biochemical and chemical. The biological method is a bioassay, which is conducted via the intraperitoneal injection of mice with an extract containing the compound under examination and it

detects total toxicity. This property is essential for the detection of unknown toxins, but the use of laboratory animals raises serious ethical concerns and animal welfare issues. The biochemical method is based on competition between cyclic imines and a fluorescently labelled compound for binding to receptors of the electric ray *Torpedo marmorata*. Finally, in respect of chemical methods, liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) is the most significant method because it is fast, of high repeatability and specificity.

Keywords: cyclic imines, spirolides, gymnodimines, shellfish, LC-MS/MS

ΠΕΡΙΛΗΨΗ. Τα οστραχοειδή χαι, πιο συγχεχριμένα, τα δίθυρα μαλάχια είναι τρόφιμο ιδιαίτερου ενδιαφέροντος για λόγους τόσο οιχονομιχούς όσο και δημόσιας υγείας. Τρέφονται με μιχροφύχη, τα οποία παρότι είναι επωφελή για την υδατοχαλλιέργεια, συμπεφιλαμβάνουν και οφισμένα τοξινοπαφαγωγά είδη. Τα είδη αυτά παφάγουν θαλάσσιες βιοτοξίνες, οι οποίες διαμέσου του μηχανισμού άντλησης και διήθησης του νερού, βιοσυσσωρεύονται στους ιστούς των οστρακοειδών. Η βιοσυσσώρευση αυτή είναι εντονότερη και άρα πιο επικίνδυνη για τη δημόσια υγεία κατά τη διάρκεια των «επιβλαβών ανθήσεων», γνωστών και ως Harmful Algal Blooms (HABs), δηλαδή των περιόδων της πληθυσμιαχής αύξησης των μικροφυχών. Οι θαλάσσιες βιοτοξίνες κατατάσσονται με βάση τη χημική τους δομή σε 8 ομάδες, μία από τις οποίες είναι και οι κυκλικές ιμίνες. Οι κυκλικές ιμίνες είναι λιπόφιλες τοξίνες, οι οποίες αναχαλύφθηκαν τυχαία λόγω της πρόκλησης νευρολογικών συμπτωμάτων και ταχύτατων θανάτων σε μύες κατά τις βιοδοκιμές ρουτίνας για την ανίχνευση άλλων λιπόφιλων θαλάσσιων βιοτοξινών. Περιλαμβάνουν τις υποομάδες: σπειοολίδια (spirolides, SPX), γυμνοδιμίνες (gymnodimines, GYM), πιννατοξίνες (pinnatoxins, PnTX), πτεριατοξίνες (pteriatoxins, PtTX), προφοκεντρολίδες (prorocentrolides) και σπειρο-προφοκεντριμίνες (spiro-prorocentrimines). Οι πρώτες τρεις υποομάδες, οι οποίες παράγονται από δινομαστιγωτά μιχροφύχη, απασχολούν περισσότερο την Ευρωπαϊχή Ένωση (ΕΕ), διότι σε αντίθεση με τις υπόλοιπες, έχουν ήδη ανιχνευθεί στην Ευρώπη ή υπάρχουν ισχυρότατες ενδείξεις της παρουσίας τους. Τα σπειρολίδια παράγονται από το δινομαστιγωτό Alexandrium ostenfeldii/peruvianum, οι γυμνοδιμίνες από το δινομαστιγωτό Karenia selliformis και οι πιννατοξίνες από ένα περιδινοϊδές δινομαστιγωτό που έχει περιγραφεί πρόσφατα υπό το νέο γένος Vulcanodinium spp. Αν και υπάρχουν ανεπαρκείς πληροφορίες για την ακριβή γεωγραφική τους κατανομή, ο εντοπισμός τους σε πολλές περιπτώσεις στα ευρωπαϊχά ύδατα, σε συνδυασμό με την προαναφερθείσα ισχυρότατη οξεία τοξικότητά τους κατά τη διενέργεια ενδοπεριτοναϊκής έγχυσης σε μυς, τις κατέστησαν, τουλάχιστον στην ΕΕ, αντικείμενο έντονης επιστημονικής έφευνας. Παφά την αποδεδειγμένη τοξικότητά τους στους μυς, δεν έχουν ακόμη ενοχοποιηθεί για κφούσματα τοξινώσεων σε ανθρώπους. Προς το παρόν, η ΕΕ δεν έχει θεσμοθετήσει Ανώτατα Επιτρεπτά Επίπεδα (Maximum Permissible Levels) συγμεντρώσεων των χυχλιχών ιμινών στα οστραχρειδή ούτε τη μέθοδο αναφοράς για την ανίχνευση χαι τον ποσοτιχό τους προσδιορισμό. Οι μέθοδοι που εφαρμόζονται σήμερα είναι βιολογικές, βιοχημικές και χημικές. Η βιολογική μέθοδος είναι μια βιοδοκιμή που διενεργείται με ενδοπεριτοναϊκή έγχυση ενός εκχυλίσματος που περιέχει την υπό εξέταση ουσία σε μυς και ανιχνεύει τη συνολική τοξικότητα. Η ιδιότητα αυτή θεωρείται απαραίτητη για την ανίχνευση άγνωστων τοξινών, όμως, λόγω της χρήσης πειραματόζωων, ενέχει σκεπτικισμό ως προς την ευζωία τους και την ηθική της όλης διαδικασίας. Η βιοχημική μέθοδος βασίζεται στον ανταγωνισμό μιας φθοροεπισημασμένης τοξίνης με τις χυχλιχές ιμίνες για τη σύνδεση με τους υποδοχείς του οργανισμού Torpedo marmorata. Τέλος, από τις χημικές μεθόδους, η ανίχνευση με υγροχρωματογραφία-φασματομετρία μάζας (LC-MS/MS) είναι η πιο αξιόλογη γιατί είναι γρήγορη, με μεγάλη επαναληψιμότητα και ειδικότητα.

Λέξεις ευρετηρίασης: πυπλιπές ιμίνες, σπειρολίδια, γυμνοδιμίνες, οστραποειδή, LC-MS/MS

1. Introduction

B ivalve molluscs, such as mussels and oysters, and the marine algal toxins produced by microalgae associated with them are a topic of global interest due to both their commercial impact and influence on seafood safety (Garcia Camacho et al. 2006). Worldwide, marine algal toxins cause more than 60.000 food poisoning events annually with an associated mortality rate of 1.5% (Gill et al. 2003).

In Greece, mussel (*Mytilus galloprovincialis*) production alone accounted for more than 18% of total annual fishery output (Eurostat 2008), 80% of

which is exported to the EU (Theodorou et al. 2010). Furthermore, mussel consumption in Greece increases during fasting periods and, especially, during Lent, thus making the need to ensure their safety imperative. Bivalve molluscs are filter-feeding organisms, which rely mostly on phytoplankton for food. Mass proliferation of phytoplankton, known as algal blooms, is mostly harmless and beneficial for aquaculture (Garcia Camacho et al. 2006). However, out of 5.000 known phytoplankton species (Sournia et al. 1991), more than 90 are capable of producing toxins and 70 of them are dinoflagallates (Hallegraeff 2003). Proliferation of

JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY 2011, 62(3) ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ 2011, 62(3) toxin-producing microalgae causes HABs, which can sometimes induce water discoloration, some of which are known as "red tides". HABs may indiscriminately kill sea organisms via oxygen deprivation because of their high oxygen consumption (Hallegraeff et al. 1995, Lindahl 1998). The first written reference to the red tide phenomenon was found in the Bible: "So, Moses ...struck the water that was in the Nile...and all the water that was in the Nile was turned to blood. The fish that were in the Nile died and the Nile became foul, so that the Egyptians could not drink water from the Nile..." (Exodus 7: 20-21).

Marine toxins tend to accumulate in shellfish via their filter-feeding mechanism (Botana et al. 2009). They have various adverse effects on human health such as amnesic shellfish poisoning (ASP), paralytic shellfish poisoning (PSP) or diarrhoetic shellfish poisoning (DSP) (Osek et al. 2006, Christian and Luckas 2007). Their negative impact on public safety is exacerbated by the fact that they are odourless, tasteless, they do not vapourize and they are not destroyed by cooking (Chisti 1998), thus, they cannot be identified by human senses or be deactivated by common food processing technologies.

Historically, marine toxins were classified according to the induction of the aforementioned symptoms, i.e. all lipophilic toxins belonged to the DSP group, until new lipophilic toxins were discovered (yessotoxins) which do not cause diarrhoea (Christian and Luckas 2007). In this context, categorization according to their chemical structure was introduced and it classifies toxins into eight groups,

- azaspiracid (AZA)
- brevetoxin (BTX)
- domoic acid (DA)
- okadaic acid (OA)
- pectenotoxin (PTX)
- saxitoxin (STX)
- yessotoxin (YTX)
- cyclic imines (CI)

(Toyofuku 2006).

2. Cyclic Imines: Chemical structure, categorization and distribution in shellfish

Cyclic imines are lipophilic compounds and accumulate in the hepatopancreas of bivalve molluses. They were found in shellfish because of their high acute toxicity following intraperitoneal injection in mouse bioassays (MBA), carried out for the detection of lipophilic marine toxins (EFSA 2010). The CI family comprises spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs), pteriatoxins (PtTXs), prorocentrolides and spiroprorocentrimines. The latter two, along with PtTXs, which have been recently identified as biotransformation products of PnTXs in shellfish (Selwood et al. 2010), have not been reported in shellfish in Europe (EFSA 2010).

2.1. Spirolides (SPXs)

SPXs, which have a molecular weight of approximately 700 Da (Cembella and Kroc 2008), are mainly produced by the marine dinoflagellate Alexandrium ostenfeldii/peruvianum (Cembella et al. 2000, Franco 2006, Pigozzi et al. 2008). They are the largest CI group and they are categorized in eight major groups, which are SPX A, B, C, D, E, F, G and 20-Me-G, in addition to two desmethyl derivatives (Cembella and Krock 2008). These are SPX 13-desMeC, which is derived from SPX C, and SPX 13-desMeD, which originates from SPX D (Ciminiello et al. 2006). The toxicity of SPXs in mice varies widely depending on their structural differences (Ciminiello et al. 2006). SPXs that contain an extra methyl group on the imine ring compared with spirolide A and B resist enzymatic hydrolysis conditions in shellfish and, therefore, might pose a threat to consumers (Christian et al. 2008). On the other hand, spirolides E and F, which are regarded as hydrolysis products of spirolides A and B (Hu et al. 1996, 2001), are biologically inactive (Christian et al. 2008). This hypothesis is reinforced by the stability of the doublemethylated spirolides C and D under hydrolytic conditions in shellfish (Hu et al. 2001).

SPXs were first discovered and characterized in 1991 in cultured blue mussels (*Mytilus edulis*) and sea scallops (*Placopecten magellanicus*) in Nova Scotia, Canada (Hu et al. 1996). *Alexandrium ostenfeldii / peruvianum* has been found in the United States (Gribble et al. 2005) and in New Zealand (Mackenzie 2004). In Europe, it has been identified along the Emilia Romagna coasts in the Italian part of the Adriatic Sea (Ciminiello et al. 2006), in Ireland (Touzet et al. 2008), in Scotland (John et al. 2003), and in Denmark (MacKinnon et al. 2006). Furthermore, SPXs' presence in shellfish has been reported in Italy (Pigozzi et al. 2008), in Spain (Villar González et al. 2006) and in Norway (Aasen et al. 2005). In Greece, the presence of *Alexandrium ostenfeldi / peruvianum* has been confirmed and SPXs have been detected (Katikou et al. 2010). However, their distribution and concentrations have not yet been studied in detail.

2.2. Gymnodimines (GYMs)

GYMs, which are the smallest CI with a molecular weight around 500 Da (Cembella and Krock 2008), are produced by the dinoflagellate *Karenia selliformis*, formerly known as *Gymnodinium selliforme* (Seki et al. 1995; Mackenzie et al. 1996, Miles et al. 2003, Haywood et al. 2004). They consist of three groups, namely GYM A, B and C (Cembella and Krock 2008). All GYM-analogues have a six-membered imino ring and their macrocycle contains 16 carbon units and one ether bridge (Cembella and Krock 2008). GYM-C is an oxidised analogue of GYM-A and it is isomeric with GYM B at C-18, thus, it is highly probable that both GYM B and C are final derivatives of GYM A (Miles et al. 2000, 2003).

GYMs were first identified in 1992-1993 in New Zealand, after an unusual neurotoxicity incident caused by their interference in MBAs for lipophilic toxins (Mackenzie et al. 1993; Mackenzie 1994;, Mackenzie et al. 1996). They have also been reported in Tunisia (Biré et al. 2002), whereas in Europe, no published reports exist regarding their occurrence (EFSA 2010). However, the presence of GYMs in Greek shellfish has been strongly indicated by our preliminary investigations (Greek National Reference Laboratory for Marine Biotoxins, unpublished data)

2.3 Pinnatoxins (PnTXs)

The organism producing PnTXs has been described as a peridinoid dinoflagellate (Rhodes et al. 2010) and it has recently been described in the new genus Vulcanodinium spp., and, specifically, as Vulcanodinium rugosum (Nezan and Chomerat 2011). PnTXs have a similar chemical structure to SPXs and their molecular weight varies from 712 to 832 Da (Selwood et al. 2010). This CI group was believed to include only four groups, PnTX A, B, C, D (Cembella and Krock 2008). However, new PnTX analogues have recently been discovered in oysters in New Zealand and Australia, namely PnTx E, F and G (Selwood et al. 2010). The same research group suggests that all known PnTXs and PtTXs originate from PnTXs G and F after metabolic and hydrolytic transformation in shellfish (Selwood et al. 2010).

PnTXs have lately been determined in Norwegian shellfish (Miles et al. 2010), whereas *Vulcanodinium rugosum* has been found in water samples from Mediterranean lagoons (Nezan and Chomerat 2011).

3. Toxicity of cyclic imines

Toxicity of cyclic imines is currently under investigation because, despite the fact that they can be bio-transformed via detoxification by reduction (GYM) or by ring opening (SPX A and B) in molluscs (Hu et al. 1996, Stewart et al. 1997, Munday 2008), there is limited information regarding their absorption, distribution, metabolism and excretion in animals or in humans. Therefore, no conclusions could be drawn with respect to any possible risk to consumers of contaminated shellfish (EFSA 2010).

So far, no reports of human illness directly caused by SPXs, GYMs, PnTXs or PtTXs have been identified (EFSA 2010). Incidents involving non-specific symptoms including gastric distress and tachycardia, such as the one recorded in Nova Scotia, Canada, raise reasonable suspicions, but they cannot be attributed to the consumption of SPX-containing shellfish, because similar episodes in the area took place at times when MBA results indicated absence of SPXs (Richard et al. 2001). In New Zealand, which was the first country to detect the presence of GYMs in shellfish, the regulatory authorities decided that GYM-contaminated shellfish do not pose a human intoxication risk, as, according to their epidemiological data, no illnesses after shellfish consumption had been observed during the contamination incident (Mackenzie et al. 1996, Stirling 2001). Moreover, in Rangaunu Harbour, New Zealand, where PnTXs had been detected in oysters at considerable concentrations, no adverse effects to consumers' health have been noticed (Munday et al. 2004).

On the other hand, cyclic imines present an acute neurotoxic action to mice after intraperitoneal injection and they may interfere with the MBA for the detection of other lipophilic toxins (FAO/IOC/WHO 2004). SPXs induce mouse death within 30 minutes after the i.p. injection. The reason for this response is their high solubility in polar organic solvents, such as methanol, aqueous ethanol or acetone, which are used for the extraction of the lipophilic toxins under detection (Cembella and Krock 2008, Otero et al. 2010a, 2010b). GYMs have similar effects on mice, whose death is observed approximately 3 to 15 minutes after injection, but they completely recover if they survive the first 30 minutes (EFSA 2010). The possible cause for this toxicity is the intact cyclic imine-group that some SPXs and GYMs possess (Munday 2008, Bourne et al. 2010). There is evidence that the neurotoxic action of SPXs and GYMs is based on their inhibition of both muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR) in the central and peripheral nervous system (Munday 2008). The aforementioned suggestion has been confirmed via binding of 13-desmethyl SPX C and GYM A to nAChRs in binding assays and voltage-clamp recordings on muscle and neuronal nAChR, hence revealing potent antagonism for both types of receptors (Bourne et al. 2010). Nevertheless, the main difference between GYMs and SPXs is that GYMs show a reversible effect, whereas binding of SPXs seem to be irreversible (Molgó et al. 2007, 2008, Vilariño et al. 2009, Fonfría et al. 2010). Furthermore, the fact that SPXs containing an extra methyl group on the imine ring survive enzymatic hydrolysis conditions in shellfish implies that they might be toxic to humans, as well (Christian et al. 2008). Finally, according to a recent study, SPX analogues 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C can cross human intestinal epithelium at levels exceeding 80% and 50%, respectively, thus proving that SPXs can be absorbed and enter the circulatory system (Espiña et al. 2011).

As regards PnTXs, it has recently been demonstrated that they, also, bind to nAChR (Miles et al. 2010), thus accounting for the rapid mice death induced in the MBA for lipophilic toxins, when PnTXs are present in the shellfish under examination. However, there is no evidence that they cause human illness (Selwood et al. 2010).

Due to the relatively recent discovery of cyclic imines, there are insufficient data regarding their distribution and toxicology, which partly explains the current absence of regulatory limits on cyclic imines in shellfish in Europe or in other regions of the world. However, the toxicology working group of the EU Reference Laboratory for marine toxins (EU-RLMB) has proposed a guidance level for the sum of SPXs of 400 μ g/kg shellfish meat (CRLMB 2005, Pigozzi et al. 2008).

4. Methods of detection

4.1 Mouse bioassay (MBA)

Although, according to EU Regulation 2074/2005,

the MBA has until recently been the reference method for the detection of lipophilic marine toxins, it produces false positive results when cyclic imines are present (EFSA 2010). The symptomatology caused by the presence of cyclic imines' comprises neurological signs, convulsions and cramps and it usually ends 30 minutes after injection with the death of one or more of the three mice used in the examination (EFSA 2010). The i.p. injection of the 25g shellfish extract into a 20g mouse provides a limit of detection (LOD) of about 5.6 µg/kg shellfish for 13-desmethyl SPX C (Munday 2008), 6.4 µg/kg shellfish for SPX C (Munday 2008) and 77 µg/kg shellfish for GYM A (Munday et al. 2004). The method's LODs for PnTx E, F and G are 40, 36 and 13 µg/kg shellfish, respectively (Selwood et al. 2010). MBA is a relatively simple and inexpensive method that can determine total toxicity which is of crucial importance for the detection of emerging toxins (EFSA 2010). However, it is not a quantitative method, it lacks specificity and has not yet been validated for the detection of cyclic imines (EFSA 2010). Furthermore, it involves ethical issues regarding the use of animals, since each examination entails the death of three mice (EFSA 2010). In Europe, Directive 86/609/ EEC will be replaced on 1/1/2013 by Directive 2010/63/ EU (article 62 of 2010/63/EU) with more strict requirements with regard to animal welfare. In this latter Directive, the need for the implementation of the principle known as the three Rs' is stressed (EU 2010), which is the process of replacing an animal test with alternative methods. This principle can be defined by the following criteria: reduction of the number of animals being used, refinement of the use of animals in terms of suffering and performance, and replacement of the animal by alternative methods (Hess et al. 2006). Finally, according to EU Regulation 15/2011, LC-MS/MS has replaced MBA as the reference method for the detection of lipophilic marine toxins since 1/7/2011, with a transition period until 31/12/2014.

4.2 Biochemical assays

A biochemical assay with a fluorescence polarization has recently been developed. It is based on the high affinity of GYM A and 13-desmethyl SPX C for the nicotinic acetylcholine receptor (nAChR) of the electric organ of the ray *Torpedo marmorata*, and their competition for binding to this receptor with α bungarotoxin, which is fluorescently labelled (Vilariño et al. 2009). The method's limit of quantification (LOQ) is 80 μ g/kg for GYM A and 85 μ g/kg for 13desmethyl SPX C in shellfish (Fonfría et al. 2010). This method is easy, fast, inexpensive and it detects any CIanalogue that may interact with nAChR. Nevertheless, it depends on the availability of receptors from *Torpedo marmorata*, the organism used in the assay, and it has not yet been validated (EFSA 2010).

4.3 Chemical methods

4.3.1 High performance liquid chromatography with ultra violet detection (HPLC-UV)

A HPLC-UV method was developed for the routine analysis of GYMs in shellfish at a wavelength measuring up to 210nm and the LOD of GYM A was reported to be 2.4 μ g/kg of digestive gland of clams (Marrouchi et al. 2009). However, cyclic imines lack chromophores and, therefore, optical detection methods, such as ultraviolet (UV), do not provide sufficient selectivity because the UV absorption of cyclic imines is low (EFSA 2010). Hence, the results provided by HPLC-UV will need confirmation by another method.

4.3.2 Liquid Chromatography with tandem mass spectrometry detection (LC-MS/MS)

Chromatographic techniques with UV or fluorescence detection provide separation of the components of a mixture, but they cannot unequivocally identify these components (Ardrey 2003). On the other hand, mass spectrometry can identify compounds with a high degree of confidence because mass spectra are sufficiently specific, however, it is incapable of separating mixtures of many components and, therefore, it cannot produce credible results when the sample under analysis is a mixture (Ardrey 2003). The power of LC-MS/MS lies in the fact that it combines the strengths of these two methods and eliminates their limitations. Currently, LC-MS/MS methods are considered the methods of choice for cyclic imines analysis in shellfish (EFSA 2010). Several publications have reported LC-MS/MS methods for SPXs, GYMs, PnTXs and PtTXs (Cembella et al. 1999, Quilliam et al. 2001, Takada et al. 2001a, 2001b, Stirling 2001, Biré et al. 2002, Aasen et al. 2005, Ciminiello et al. 2006, Villar González et al. 2006, Fux et al. 2007, Gerssen et al. 2009a, 2009b, Miles et al. 2010, Selwood et al. 2010). These methods are based on reversed-phase LC coupled with electrospray ionization MS used in either the selected ion

monitoring (SIM) or the selected reaction monitoring (SRM) modes (EFSA 2010). Recently, multitoxin LC-MS/MS methods have been developed for a comprehensive phycotoxin monitoring of lipophilic marine toxins under acidic (Fux et al. 2007) or alkaline conditions (Gerssen et al. 2009a, 2009b). An LC-MS/MS method implemented by Fux et al. (2007) was capable of detecting 21 lipophilic marine toxins in a total run time of 6.6 min.

LC-MS/MS methods can provide a LOD of 0.8 µg/kg shellfish meat for 13-desmethyl SPX C, 3.7 µg/kg shellfish meat for GYM A (Gerssen et al. 2009b) and 5 µg/kg shellfish for PnTX G (Miles et al. 2010). In summary, LC-MS/MS is very fast, very specific and it has a detection limit for cyclic imines lower than any other method. Nonetheless, the method is costly to implement and requires expensive equipment and highly trained personnel, as well. Moreover, reference materials for most of the analogues of SPXs, GYMs, PnTXs and PtTXs are lacking (EFSA 2010) and LC-MS/MS methods can identify and accurately quantify only toxins for which standards are available (Campbell et al. 2010). Therefore, it cannot detect all CI-analogues or other emerging toxins. Nevertheless, certain interlaboratory validations have successfully been undertaken and have enabled the EU to appoint LC-MS/MS as the reference method for the detection of lipophilic marine toxins (EU 2011, These et al. 2011).

5. Conclusion

Cyclic imines are a novel research area with limited data about their distribution and their concentration levels in European waters. According to the relevant European Food Safety Authority (EFSA) opinion, they constitute a possible concern regarding seafood safety. Thus, further investigation of cyclic imines is necessary, firstly, to collect more epidemiological data, and, secondly, to investigate the discrepancy between acute adverse health effects in mice after i.p. injection and the absence of incidents in humans directly attributed to the presence of cyclic imines in order to determine if they pose a public health risk. Finally, the process for acquiring more data, in combination with the need for preventive measures to protect public safety, might lead to the establishment of regulatory limits for cyclic imines in shellfish and the appointment of a reference method for their detection, probably LC-MS/MS.

REFERENCES

- Aasen J, MacKinnon SL, LeBlanc P, Walter JA, Hovgaard P, Aune T, Quilliam MA (2005) Detection and identification of spirolides in Norwegian shellfish and plankton. Chemical Research in Toxicology 18:509-515.
- Ardrey RE (2003) Liquid Chromatography –Mass Spectrometry: An Introduction, Analytical Techniques in the Sciences. In: What are the Advantages of Linking High Performance Liquid Chromatography with Mass Spectrometry. John Wiley & Sons, Ltd., West Sussex, England: pp 2-3.
- Biré R, Krys S, Fremy JM, Dragacci S, Stirling D, Kharrat R (2002) First evidence on occurrence of gymnodimine in clams from Tunisia. Journal of Natural Toxins 11.
- Botana LM, Alfonso A, Botana A, Vieytes MR, Vale C, Vilariño N, Louzao C (2009) Functional assays for marine toxins as an alternative, high- throughput-screening solution to animal tests, Trends in Analytical Chemistry 28.
- Bourne Y, Radic Z, Araoz R, Talley TT, Benoit E, Servent D, Taylor P, Molgó J, Marchot P (2010) Structural determinants in phycotoxins and AChBP conferring high affinity binding and nicotinic AChR antagonism. Proceedings of the National Academy of Sciences of the United States of America 107:6076-6081.
- Campbell K, Vilariño N, Botana LM, Elliott CT (2010) European perspective on progress in moving away from the mouse bioassay for marine-toxin analysis. Trends in Analytical Chemistry 30:239-253.
- Cembella AD, Krock B (2008) Cyclic imine toxins: chemistry, biogeography, biosynthesis and pharmacology. In: Seafood and Freshwater toxins: Pharmacology, Physiology and Detection. 2nd edition. Ed LM Botana. CRC Press (Taylor and Francys Group), Boca Raton, FL: pp 561-580.
- Cembella AD, Lewis NI, Quilliam MA (1999) Spirolide composition of micro-extracted pooled cells isolated from natural plankton assemblages and from cultures of the dinoflagellate Alexandrium ostenfeldii. Natural Toxins 7:197-206.
- Cembella AD, Lewis NI, Quilliam MA (2000) The marine dinoflagellate Alexandrium ostenfeldii (Dinophyceae) as the causative organism of spirolide shellfish toxins. Phycologia 39:67-74.
- Chisti Y (1998) Biosafety. In: Subramanian G, editor. Bioseparation and bioprocessing: a handbook, vol. 2. New York: Wiley-VCH, 1998: pp 379–415.
- Christian B, Below A, Dressler N, Scheibner O, Luckas B, Gerdts G (2008) Are spirolides converted in biological systems?—A study. Toxicon (2008) 51:934–940.
- Christian B, Luckas B (2007) Determination of marine biotoxins relevant for regulations: from the mouse bioassay to coupled LC-MS methods, Analytical and Bioanalytical Chemistry (2008) 391:117–134.
- Ciminiello P, Dell'Aversano C, Fattorusso E, Magno S, Tartaglione L, Cangini M, Pompei M, Guerrini F, Boni L, Pistocchi R (2006) Toxin profile of Alexandrium ostenfeldii (Dinophyceae) from the Northern Adriatic Sea revealed by liquid chromatography-mass spectrometry. Toxicon 47:597-604.
- CRLMB (Community Reference Laboratory for Marine Biotoxins) (2005) Report on toxicology working group meeting, Cesenatico, Italy, 24-25 October, 2005.
- EEC (1986), Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC), Off. J. Eur. Commun. L 358, 1-28.

- EC (2005), Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004, Off. J. Eur. Union L 338, 27-59.
- EFSA (2010), EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on marine biotoxins in shellfish – Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins). EFSA Journal 2010; 8(6):1628.
- Espiña B, Otero P, Louzao C, Alfonso A, Botana LM (2011) 13-Desmethyl spirolide-c and 13,19-didesmethyl spirolide-c transepithelial permeabilities: Human intestinal permeability modeling. Toxicology (2011): <u>doi:10.1016/j.tox.2011.06.003</u>.
- EU (2010), Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, Off. J. Eur. Commun. L 276, 33-79.
- EU (2011), Commission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluses, Off. J. Eur. Union L 6, 3-6.
- Eurostat, (2009), Statistics on aquaculture production Quantities (Tonnes live weight): 1984 onwards (accessed 17 April, 2011), (http://epp.eurostat.ec.europa.eu/portal/page/portal/fisheries/data/ database)
- FAO/IOC/WHO (Food and Agriculture Organization of the United Nations/Intergovernmental Oceanographic Commission of UNESCO/World Health Organization), 2004. Report of the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs, Oslo, Norway, September 26-30, 2004: pp 8, (accessed 22 November, 2010)

(http://www.who.int/foodsafety/publications/chem/biotoxin_report_ en.pdf)

- Fonfría ES, Vilariño N, Espiña B, Louzao MC, Alvarez M, Molgó J, Araoz R, Botana LM (2010) Feasibility of gymnodimine and 13desmethyl C spirolide detection by fluorescence polarization using a receptor-based assay in shellfish matrixes. Analytica Chimica Acta, 657:75-82.
- Franco JM, Paz B, Riobó P, Pizarro G, Figueroa RI, Fraga S, Bravo I (2006) First report of the production of spirolides by Alexandrium peruvianum (Dinophyceae) from the Mediterranean Sea. In: 12th International Conference on Harmful Algae, Copenhagen, Denmark: pp 174-175.
- Fux E, McMillan D, Biré R, Hess P (2007) Development of an ultraperformance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. Journal of Chromatography A, 1157:273-280.
- Garcia Camacho F, Gallardo Rodríguez J, Sánchez Mirón A, Cerón García MC, Belarbi EH, Chisti Y, Molina Grim E (2006) Biotechnological significance of toxic marine dinoflagellates, Biotechnology Advances (2007) 25:176–194.
- Gerssen A, McElhinney MA, Mulder PP, Biré R, Hess P, de Boer J (2009) A solid phase extraction for removal of matrix effects in lipophilic marine toxin analysis by liquid chromatography-tandem mass spectrometry. Analytical and Bioanalytical Chemistry

JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY 2011, 62(3) ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ 2011, 62(3)

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394:1213-1226.

- Gerssen A, Mulder PP, McElhinney MA, de Boer J (2009)b Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. Journal of Chromatography A, 1216:1421-1430.
- Gill S, Murphy M, Clausen J, Richard D, Quilliam M, MacKinnon S (2003) Neural injury biomarkers of novel shellfish toxins, spirolides: a pilot study using immunochemical and transcriptional analysis. Neurotoxicology (2003) 24:593–604.
- Gribble KE, Keafer BA, Quilliam MA, Cembella AD, Kulis DM, Manahan A, Anderson DM (2005) Distribution and toxicity of Alexandrium ostenfeldii (Dinophyceae) in the Gulf of Maine, USA. Deep-Sea Research Part II 52:2745-2763.
- Hallegraeff GM (2003) Harmful Algal Blooms: a global overview. In: Manual on Harmful Marine Microalgae, edited by Hallegraeff, G.,M., Anderson, D.,M., and Cembella, A.,D.,. UNESCO Publishing, Paris: pp 25-49.
- Hallegraeff GM, Anderson DM, Cembella AD eds. (1995) Manual on harmful marine microalgae. IOC Manuals and Guides No. 33. UNESCO.
- Haywood AJ, Steidinge KA, Truby EW (2004) Comparative morphology and molecular phylogenetic analysis of three new species of the genus Karenia (Dinophyceae) from New Zealand. Journal of Phycology 40:165-179.
- Hess P, Grune B, Anderson DB, Aune T, Botana LM, Caricato P, van Egmond HP, Halder M, Hall S, Lawrence JF, Moffat C, Poletti R, Richmond J, Rossini GP, Seamer C, Vilageliu JS (2006) Three Rs Approaches in Marine Biotoxin Testing. The Report and Recommendations of a joint ECVAM/DG SANCO Workshop (ECVAM Workshop 54) Altern. Lab. Anim. (2006), 34:193-224.
- Hu T, Curtis JM, Walter JA, Wright JLC (1996) Characterization of biologically inactive spirolides E and F: identification of the spirolide pharmacophore. Tetrahedron Lett. 37:7671–7674.
- Hu T, Burton IW, Cembella AD, Curtis JM, Quilliam MA, Walter JA, Wright JLC (2001) Characterization of spirolides a, c and 13desmethyl c, new marine toxins isolated from toxic plankton and contaminated shellfish. J. Nat. Prod. 64:308–312.
- John U, Cembella AD, Hummert C, Elbrächter M, Groben R, Medlin L (2003) Discrimination of the toxigenic dinoflagellates Alexandrium tamarense and A. ostenfeldii in co-occurring natural populations from Scottish coastal waters. <u>European Journal of</u> Phycology 38:25-40.
- Katikou P, Aligizaki K, Zacharaki T, Iossifidis D, Nikolaidis G (2010) First report on the presence of spirolides in Greek shellfish associated with the detection of the causative Alexandrium species. 14th International Conference on Harmful Algal Blooms, 1-5 November 2010, Crete, Greece. Book of abstracts: p. 139.
- Lindahl O (1998) Occurrence and Monitoring of harmful algae in the marine environment. In Miraglia, M., Van Egmond, H., Brera, C. & J. Gilbert, eds. 1998.
- Mycotoxins and phycotoxins developments in chemistry, toxicology and food safety. Proceedings of the IX International IUPAC Symposium on Mycotoxins and Phycotoxins, Fort Collins, Colorado, Alaken Press: pp. 409-423.
- MacKenzie AL, Haywood A, Adamson J, Truman P, Till D, Seki T, Satake M, Yasumoto T (1996) Gymnodimine contamination of shellfish in New Zealand. In: Harmful and Toxic Algal Blooms. Eds Yasumoto T, Oshima Y and Fukuyo Y. Intergovernmental Oceanographic Commission of UNESCO: pp 97-100.
- Mackenzie L (1994) Toxic algae and shellfish biotoxins in the South Island (January–May 1994), Seafood New Zealand, 47, June 1994.

- Mackenzie L (2004) Production of spirolides by New Zealand isolates of Alexandrium ostenfeldii and some new observations on Heterocapsa spp. in New Zealand. In Proceedings, 21st Marine Biotoxin Science Workshop, Wellington, New Zealand, New Zealand Food Safety Authority, 2004.
- Mackenzie LL, Rhodes D, Till D, Chang FH, Kaspar H, Haywood A, Kapa J, Walker B (1993) A Gymnodinium spp. bloom and the contamination of shellfish with lipid soluble toxins in New Zealand, January–April 1993, in Harmful Marine Algal Blooms, Lassus, P., Arzul, G., Erard, E., Gentien, P. and Marcaillou, C. eds., Lavoisier, Intercept Ltd., Nantes, France, 1993: pp 795–800.
- MacKinnon SL, Walter JA, Quilliam MA, Cembella AD, Leblanc P, Burton IW, Hardstaff WR, Lewis NI (2006) Spirolides isolated from Danish strains of the toxigenic dinoflagellate Alexandrium ostenfeldii. Journal of Natural Products 69:983-987.
- Marrouchi R, Dziri F, Belayouni N, Hamza A, Benoit E, Molgó J, Kharrat R, (2009) Quantitative Determination of Gymnodimine-A by High Performance Liquid Chromatography in Contaminated Clams from Tunisia Coastline. Marine Biotechnology, 2009:579-585.
- Miles CO, Wilkins AL, Stirling DJ, MacKenzie AL (2000) New analogue of gymnodimine from a Gymnodinium species. Journal of Agricultural and Food Chemistry 48:1373-1376.
- Miles CO, Wilkins AL, Stirling DJ, MacKenzie AL (2003) Gymnodimine C, an isomer of gymnodimine B, from Karenia selliformis. Journal of Agricultural and Food Chemistry 51:4838-4840.
- Miles CO, Rundberget T, Sandvik M, Aasen JAB, Selwood AI (2010) The presence of pinnatoxins in Norwegian mussels. Report 07b -2010, Veterinærinstituttet, National Veterinary Institute, (accessed 16 November, 2010) (http://www.uctingt.po/ong/Bospargh/Bublicationg/Bospart

(http://www.vetinst.no/eng/Research/Publications/Report-Series/Rapportserie-2010/7b-2010-The-presence-of-pinnatoxinsin-Norwegian-mussels)

- Molgó J, Amar M, Araoz R, Benoit E, Silveira P, Schlumberger S, Lecardeur S, Servent D (2008) The dinoflagellate toxin 13-Desmethyl Spirolide-C broadly targets muscle and neuronal nicotinic acetylcholine receptors with high affinity. Book of Abstracts of the 16th European Section Meeting of the International Society on Toxinology, Leuven, Belgium, September 7-10, 2008.
- Molgó J, Girard E, Benoit E (2007) The cyclic imines: an insight into this emerging group of bioactive marine toxins. In: Phytotoxins, chemistry and Biochemistry. Ed LM Botana. Blackwell Publishing, Ames, Iowa: pp 319-335.
- Munday R (2008) Toxicology of cyclic imines: gymnodimine, spirolides, pinnatoxins, pteriatoxins, prorocentrolide, spiro-prorocentrimine, and symbioimines. In: Seafood and Freshwater toxins: Pharmacology, Physiology and Detection. 2nd edition. Ed LM Botana. CRC Press (Taylor and Francys Group), Boca Raton, FL: pp 581-594.
- Munday R, Towers NR, Mackenzie L, Beuzenberg V, Holland PT, Miles CO (2004) Acute toxicity of gymnodimine to mice. <u>Toxicon</u> 44:173-178.
- Nezan E, Chomerat N (2011) Vulcanodinium rugosum gen. nov., sp. nov. (Dinophyceae): a new marine dinoflagellate from the French Mediterranean coast, Cryptogamie Algologie, Volume 32 n°1, February 2011:3-18.
- Osek J, Wieczorek K, Tatarczak M (2006) Seafood as potential source of poisoning by marine biotoxins, MedycynaWeterynaryjna (2006), 62:370–373.
- Otero P, Alfonso A, Alfonso C, Vieytes MR, Louzao MC, Botana AM, Botana LM (2010)a New protocol to obtain spirolides from Alexandrium ostenfeldii cultures with high recovery and purity.

JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY 2011, 62(3) ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ 2011, 62(3) Biomedical Chromatography, Volume 24, Issue 8, August 2010: 878–886.

- Otero P, Alfonso A, Vieytes MR, Cabado AG, Vieites JM, Botana LM (2010)b Effects of environmental regimens on the toxin profile of Alexandrium ostenfeldii. Environmental Toxicology and Chemistry 29:301-310.
- Pigozzi S, Bianchi L, Boschetti L, Cangini M, Ceredi A, Magnani F, Milandri A, Montanari S, Pompei M, Riccardi E, Rubini S (2008) First evidence of spirolide accumulation in northwestern Adriatic shellfish, in: Moestrup, O., et al (Eds.), Proceedings of the 12th ICHA. ISSHA and IOC, UNESCO, Copenhagen: pp 319-322.
- Quilliam MA, Hess P, Dell'Aversano C (2001) Recent developments in the analysis of phycotoxins by liquid chromatography-mass spectrometry. In: Mycotoxins and Phycotoxins in Perspective at the Turn of the Millennium. Eds DeKoe WJ, Samson RA, Van Egmond HP, Gilbert P and Sabino M. Wageningen, The Netherlands: pp 383-391.
- Rhodes L, Smith K, Selwood A, McNabb P, van Ginkel R, Holland P, Munday R (2010) Production of Pinnatoxins by a peridinoid dinoflagellate isolated from Northland, New Zealand. Harmful Algae 9:384-389.
- Richard D, Arsenault E, Cembella AD, Quilliam MA (2001) Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins. In: Harmful Algal Blooms 2000. Eds Hallegraef GM, Blackburn SI, Bolch CJ and Lewis RJ. Intergovernmental of Oceanographic Commision of UNESCO: pp 383-386.
- Seki T, Satake M, Mackenzie L, Kaspar HF, Yasumoto T (1995) Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, Gymnodinium sp. Tetrahedron Letters 36:7093-7096.
- Selwood AI, Miles CO, Wilkins AL, van Ginkel R, Munday R, Rise F, McNabb P (2010) Isolation, Structural Determination and Acute Toxicity of Pinnatoxins E, F and G, J. Agric. Food Chem. (2010) 58:6532–6542.
- Sournia A, Chretiennot-Dinet MJ, Ricard M (1991) Marine phytoplankton: how many species in the world ocean? Journal of Plankton Research 13:1093-1099.
- Stewart M, Blunt JW, Munro MHG, Robinson WT, Hannah DJ (1997) The absolute stereochemistry of the New Zealand shellfish toxin Gymnodimine. Tetrahedron Letters 38:4889- 4890.

- Stirling DJ (2001) Survey of historical New Zealand shellfish samples for accumulation of gymnodimine. New Zealand Journal of Marine and Freshwater Research 35:851-857.
- Takada J, Umemura N, Suenaga K, Chou T, Nagatsu A, Haino T, Yamada K, Uemura D (2001)a Pinnatoxins B and C, the most toxic components in the pinnatoxin series from the Okinawan bivalve Pinna muricata. Tetrahedron Letters 42:3491-3494.
- Takada N, Umemura N, Suenaga K, Uemura D (2001)b Structural determination of pteriatoxins A, B and C, extremely potent toxins from the bivalve Pteria penguin. <u>Tetrahedron Letters 42:3495-3497.</u>
- Theodorou JA, Sorgeloos P, Adams CM, Viaene J, Tzovenis I (2010) Optimal farm size for the production of the Mediterranean mussel (Mytilus galloprovincialis) in Greece, IIFET 2010 Montpellier Proceedings.
- These A, Klemm C, Nausch I, Uhlig S (2011) Results of a European interlaboratory method validation study for the quantitative determination of lipophilic marine biotoxins in raw and cooked shellfish based on high-performance liquid chromatography-tandem mass spectrometry. Part I: collaborative study. <u>Analytical</u> and Bioanalytical Chemistry 399:1245-1256.
- Toyofuku H (2006) Joint FAO/WHO/IOC activities to provide scientific advice on marine biotoxins (research report), Marine Pollution Bulletin (2006), 52:1735–1745.
- Touzet N, Franco JM, Raine R (2008) Morphogenetic diversity and biotoxin composition of Alexandrium ostenfeldii (Dinophyceae) in Irish coastal waters. Harmful Algae 7:782-797.
- Vilariño N, Fonfría ES, Molgó J, Araoz R, Botana LM (2009) Detection of gymnodimine-A and 13-desmethyl C spirolide phycotoxins by fluorescence polarization. Analytical Chemistry 81:2708-2714.
- Villar González A, Rodríguez-Velasco ML, Ben-Gigirey B, Botana LM (2006) First evidence of spirolides in Spanish shellfish. Toxicon 48:1068-1074.
- Yasumoto T, Oshima Y, Yamaguchi M (1978) Occurrence of a new type of shellfish poisoning in Tohoku district. Bulletin of the Japanese Society of Scientific Fisheries 44:1249-1255.



JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY 2011, 62(3) ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ 2011, 62(3)