ECsafeSEAFOOD

Priority environmental contaminants in seafood: safety assessment, impact and public perception

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Deliverable D2.8

Detection and quantification of emerging biotoxins in seafood

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Summary

The main objective of this deliverable was to monitor biotoxins in selected seafood. Analyses of biotoxins in a selected number of species from potential hotspots and from different trophic levels were evaluated. Different methods for toxin detection including analytical techniques like LC-MS/MS, biochemical assays, cell-based toxicity assays and biosensors have been optimized.

IRTA provided the first hint of the presence of cyclic imines in samples of mussels, oysters and passive sampler devices from Catalonia (Spain). According to the Commission Decision 2002/657/EC [56], the confirmatory potential of the low resolution MS method is enough for Pinnatoxin G (PnTX-G) and Spirolide (SPX-1) (one precursor ion, three product ions, ratio of abundance between product ions, and retention times). Cyclic imines were found at low concentrations from 2.2 to 16 µg/kg for SPX-1 and from 3 to 59 µg/kg for PnTX-G in 13 samples of mussels and oysters (22 samples analyzed). Pinnatoxin G was also detected in 17 seawater samples (out of 34) using solid phase adsorption toxin tracking devices (0.3 to 0.9 µg/kg-resin). Pinnatoxin G and SPX-1 were confirmed with both low and high resolution (<2ppm) mass spectrometry by comparison of the response with that from reference standards.

In commercial Samples from 2014 and 2015, low concentrations of cyclic imines (GYM A, SPX-1 and PnTX-G) were evaluated. Only SPX-1 and PnTX-G were detected in concentrations from 26 to 66µg/kg for SPX-1 and from 0.1 to 12µg/kg for PnTX-G in 48 samples from Ireland, Norway, Netherlands and Denmark and 47 samples from Spain, Portugal, Slovenia and Italy.

The presence of the cyclic imine toxin pinnatoxin G (PnTx-G) was unambiguously identified in the sugar kelp *Saccharina latissima* from Norway by liquid chromatography coupled to mass spectrometry analysis, running under several operation modes at IRTA and NVI

At NVI, cyclic imines and azaspiracids were previously analysed by gradient reverse-phase LC–MS using either a linear trap quadrupole (LTQ) ion trap or triple-quadrupole mass spectrometers for detection. Both approaches have limitations related to fragmentation of the analytes, sensitivity, and detection of unknown analogues.

NVI has recently acquired an LC–MS system based on a Q Exactive mass spectrometer. This has a number of advantages, including: high mass resolution (masses are routinely accurate to better than 5 ppm), leading to improved specificity and selectivity; high sensitivity; MS/MS in high resolution giving more secure identification via fragmentation spectra; and All-Ion Fragmentation (AIF) mode, allowing non-targeted fragmentation spectra to be collected on the fly. Running in full-scan (FS) with AIF mode (i.e. obtaining alternating full scan and AIF spectra during chromatography) allows data files to be interrogated retrospectively, with searches for candidate product-ion fragments (at high resolution in the AIF chromatogram) and precursor ion masses (also at high resolution, in the full scan chromatogram). The high resolution of the mass spectrometer allows masses of peaks to be determined accurately enough that the atomic composition of the ions can usually be deduced, greatly increasing the certainty of identification and reducing interferences from what would otherwise be isobaric ions on normal unit-resolution mass spectrometers. Peaks from putative analogues tentatively identified in the LC–MS chromatograms in FS and AIF mode can then be

selectively fragmented using targeted LC–MS/MS with the fragment masses measured at high resolution, in order to obtain structural information about those compounds.

Using this approach, it was successfully confirmed trace levels of pinnatoxin and spirolide metabolites in Norwegian seaweeds, identified novel gymnodimine analogues in an algal culture isolated from the Baltic Sea, and identified novel azaspiracid metabolites in Norwegian crabs. The methodology was also used for routine analysis of known cyclic imines (spirolides and pinnatoxins) and azaspiracids in crabs (WP6) and commercial shellfish (WP2).

Blooms of the benthic dinoflagellate *Ostreopsis cf. ovata* are a concern in the Mediterranean Sea, since this species produces a wide range of palytoxin-like compounds listed among the most potent marine toxins. At IRTA, several analogues of palytoxin were found in cultures of six strains of O.cf. *ovata* isolated from the south of Catalonia (NW Mediterranean Sea). In addition to some already known ovatoxins, it was described for the first time two minor compounds, ovatoxin-g and the so far called putative palytoxin, whose structure had not been elucidated before. Insufficient quantity of these compounds impeded a full nuclear magnetic resonance (NMR)-based structural elucidation; thus, their structure in crude algal extracts through liquid chromatography-electrospray ionization high resolution mass spectrometry in positive ion mode was studied. Under the used MS conditions, the molecules underwent fragmentation at many sites of their backbone and a large number of diagnostic fragment ions were identified. As a result, tentative structures were assigned to both ovatoxin-g and the putative palytoxin, the latter being identified as a palytoxin isomer and re-named as isobaric palytoxin.

The increasing occurrence of puffer fish containing tetrodotoxin (TTX) in the Mediterranean can represent a major food safety risk for European consumers and threatening the fishing industry. Tetrodotoxin is a potent, low molecular weight (319 a) marine neurotoxin. Due to the lack of an official method for TTX measurement in Europe and with the aim of developing alternative methods to the mouse bioassay (MBA), its detection has been achieved using different methodological approaches, such as immunochemical assays and chemical analysis at IRTA. The determination of tetrodotoxins in puffer fish and sea snail samples by liquid chromatography coupled to tandem mass spectrometry technique (LC-MS/MS), mELISA and SPR were performed at IRTA. The samples from fish and sea snail have been extracted according to the Japanese official method. Tetrodotoxin and 11 analogues were identified by LC-MS/MS. The performance of the method has been demonstrated for the analysis of positive samples of puffer fish from Greece and La Reunion Island. In addition, seventeen puffer fish captured during 2014 and 2015 in the Catalonian and Valencian coasts and an evaluation of the risk of the different species and the content of tetrodotoxin is being carried out.

Lionfish (*Pterois* spp.) are invasive species that have recently spread throughout the Caribbean. Lionfish are available for purchase in local markets for human consumption in several islands from Guadeloupe, Saint Martin and Saint Berthélmy Islands. Since EU regulations do not establish risks according to species, France has established interdictions to the commercialization of some fishes that have previously been associated with ciguatera fish poisoning (CFP) in endemic areas. For instance, the island of Guadeloupe maintains a list of fish completely banned or under restriction for fishing due to CFP, that includes certain species of the genera *Sphyraena*, *Caranx*, *Seriola*, *Gymnothorax*, *Mycteroperca*, *Alphestes*, *Epinephelus* and *Lutjanus* (Prefectural decree, 2002). Despite these interdictions, at least 30 CPF events, most of them after the consumption of banned

fish, occurred in Guadeloupe resulting in 57 individual intoxications between 2012 and 2013, with toxicity of meal remnants confirmed by using the mouse bioassay (MBA). In a collaboration between IRTA, Hydro-Reunion, the FDA (Food and Drug Administration) and other institutions, the prevalence of ciguatoxins (CTXs) in lionfish from the French Antilles, a ciguatera-endemic region, was examined. The neuroblastoma-2a (N2a) cell assay was used to assess CTX-like activity in 120 fish samples collected from the surrounding waters of Guadeloupe (n=60), Saint Barthélemy Islands (n=55) and Saint Martin (n=5). Twenty-seven samples exhibited CTX-like activity by the N2a assay. Ciguatoxin (CTX) was confirmed by using LC-MS/MS. Those fish containing CTXs were all from Saint Barthélemy. Lionfish from Guadeloupe and Saint Martin did not exhibit toxin activity, although the sample size from Saint Martin was insufficient to draw any conclusions as to the incidence of CTXs.

Information about the potential hazard of ciguatera associated with the consumption of lionfish from known endemic areas is provided. And the utility of the cell-based assay combined with LC-MS/MS to assess activity and provide structural confirmation of CTXs is demonstrated.

Through a collaboration between IRTA and Hydro-Reunion the identification of CTXs in a bull shark, *Carcharhinus leucas*, implicated in a seafood poisoning event in Madagascar has been achieved.

Some information present in this report was already presented in the specific deliverable, "***D2.6 improved analytical methods for biotoxins***".

Specific work for the development of biosensors for biotoxins has also been conducted within WP4 and will be presented in deliverable "***D.4.7 Analytical performance of the new developed detection systems***".

1. Introduction

Phytoplankton-planktonic algae are at the basis of the marine food chain, i.e., it is the direct or indirect source of food for many higher trophic level marine organisms. It is an essential source of nutrition for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams) as well as for crustaceans and finfish in the marine environment, including marine aquacultures. About 300 marine algal species are described as producers of complex molecules that can be toxic to other organisms within the marine food web and are therefore designated as marine biotoxins. The Harmful Algal Bloom (HAB) designation is a societal concept rather than scientific definition-blooms are considered to fit the HAB criterion if they cause injury to human health or socioeconomic interest [1].

Different harmful algal species produce toxins that can be accumulated in shellfish, causing both human illness and severe economic losses to industry and fisherman. Accumulation of marine biotoxins from hazardous algal species in molluscan shellfish is well researched and other seafood and tunicates can be also affected. While most toxins do not affect shellfish, most of them cause harm to humans who consume the shellfish. Management systems are in place in many countries to reduce the risks to consumers of seafood and regulations establishing maximum acceptable levels of some toxins and recognition methods have been established.

Several classes of marine toxins have been described according to effects they produce. From a public health perspective, the most important marine phycotoxins are shellfish toxins and ciguatoxins in finfish. Until now, five groups of shellfish toxins have been distinguished, namely:

i. paralytic shellfish toxins causing paralytic shellfish poisoning (PSP). PSP intoxications are a result of exposure to saxitoxins (STX), gonyautoxins (GTX) and C-toxins. More than 57 different analogues of STX have been described. The predominant producers of STX are species of the genus *Alexandrium*.

ii. lipophilic marine toxins including diarrhoeic shellfish toxins causing diarrhoeic shellfish poisoning (DSP). The causative agents of DSP are okadaic acid (OA) and its analogues dinophysistoxins (DTXs), which are produced by the dinoflagellate genera *Dinophysis* and *Prorocentrum*.

iii. amnesic shellfish toxins causing amnesic shellfish poisoning (ASP) which is caused by domoic acid (DA) and a number of toxic DA isomers. ASP toxins are produced by diatoms of the genera *Pseudo-Nitzschia* and *Nitzschia*.

iv. neurotoxic shellfish toxins causing neurotoxic shellfish poisoning (NSP). The causative agents of NSP are brevetoxins (BTX), primarily produced by the dinoflagellate *Karenia brevis*. It is also produced by species of the genera *Chattonella*, *Fibrocapsa* and *Heterosigma*.

v. azaspiracid shellfish toxins causing azaspiracid shellfish poisoning (AZP) which is caused by azaspiracid (AZA) and its analogues, and so far 32 analogues have been described. AZAs are produced by dinoflagellates of the genus *Azadinium*.

Ciguatoxins (CTXs) present mainly in tropical fish [2] cause ciguatera fish poisoning (CFP). CTXs are produced by dinoflagellates of the genus *Gambierdiscus* [3]. Ciguatera toxins are found in the tropical and sub-tropical regions of the Pacific, Atlantic (Caribbean region, coast of Cameroon, Canary Islands, Madeira) and Indian Ocean. More than 30 CTXs have been described.

PSP, DSP, ASP, NSP and AZP are caused by human consumption of contaminated shellfish products whereas CFP is caused by the consumption of subtropical and tropical marine carnivorous fish that have accumulated ciguatera toxins through the marine food chain.

In addition, additional emerging biotoxins such as cyclic imines, palytoxins and tetrodotoxins are worth studying. Gymnodimines (GYM), pinnatoxins (PnTX), pteriatoxins (PtTX) and spirolides (SPX) belong to the toxin group of cyclic imines (CI) and are produced by several genus of dinoflagellates. The implication of these toxins in human poisoning has to be demonstrated, but their common presence in shellfish and neurotoxic effects on nicotinic acetylcholine receptors invite to better assess their potential risk. Palytoxins are very toxic and complex polyethers produced by species of the genus *Ostreopsis*. Presence of palytoxins has been reported in molluscs, equinoderms, crustaceans and fish, and have been implicated in clupeotoxism caused by ingestion of sardines, herrings and anchovies. Tetrodotoxin is a powerful sodium channel blocker found in puffer fish and some marine animals, i.e. gastropods. Cases of TTX poisoning most often result from puffer fish ingestion. TTX is about 10 000 times more lethal than cyanide by weight; the lethal dose is about 1-2mg for adults. Mediterranean Sea is not the natural habitat of *Lagocephalus sceleratus*. However, multiple reports have established a firm presence of migration of pufferfish across the Suez Channel (lessepsian migration) [4]. Several puffer fish were caught in Spain (Catalonian and Valencian coast) during 2014 and 2015.

In the past, the approach to assess toxins in aquatic food was traditionally *in vivo* methods, i.e. mouse and rat bioassays. Besides the ethical issues of *in vivo* bioassays there are specific difficulties, e.g. different exposure route (intraperitoneal versus oral), low inter-species comparability, high intra-species variability, and questionable extrapolation of quantitative risk to humans, thus highlighting the need for the development of more reliable detection and quantification methods. Based on this reasoning, the European Food Safety Authority (EFSA) advocates the use of analytical methods and *in vitro* methods. For lipophilic toxins, Liquid Chromatography coupled to Mass Spectrometry (LC-MS/MS), has replaced the mouse bioassay, and this method is applied to other toxins such as Ciclic Imines. Although LC-MS is very sensitive method that has the advantage of being able to detect multiple toxins in a single analysis and being good for confirming the identity of toxins, the quality of quantitative analysis is dependent on the availability of calibration standards. When new toxin analogues are detected but accurate standards are not yet available, only an approximate quantification is possible using estimated response factors [5].

This deliverable summarizes results obtained within ECsafeSEAFOOD regarding the detection and quantification of emerging biotoxins in seafood. These results include quantification for different emerging biotoxins in seafood from hot spot areas, as well as biotoxins in commercial samples in the Europe.

2. Instrumentation

Analytical Instrumentation from IRTA (Spain), Norwegian Veterinary Institute (NVI, Norway) and additional laboratories like Cork Institute of Technology (Cork, UK), Università degli Studi di Napoli Federico II (Napoli, Italy) and US FDA Division of seafood science and technology (USA) are described in *Deliverable 2.6*.

3. Cyclic Imines

3.1 Cyclic Imines by LC-MS/MS at IRTA

Cyclic imines are lipophilic marine toxins that bioaccumulate in seafood. Their structure comprises a cyclic-imine moiety, responsible for acute neurotoxicity in mice. Cyclic imines have not been linked yet to human poisoning and are not regulated in Europe, although the European Food Safety Authority requires more data to perform a conclusive risk assessment for consumers.

Cyclic Imines are toxins that can be identified when addressing the presence of lipophilic toxins with the use of LC-MS/MS, a method implemented by different countries to monitor, under current regulations, the presence of diarrheic shellfish poisoning toxins, yessotoxins and azaspiracids. The effort of sampling for cyclic imines within ECsafeSEAFOOD, focused on complementary species, other than those regularly monitored (normally mussels, depending on the country). Imported shellfish was addressed. In addition, particular focus may be addressed to the identification of cyclic imine metabolites. The Pinnatoxin-producing alga *V. rugosum* is benthic, which has major implications for uptake and distribution of pinnatoxins in the environment and food chain. Therefore a range of representative benthic samples were also taken.

At IRTA for cyclic imines, an LC-MS/MS method has been developed for analysis of pinnatoxins, cyclic imines and gymnodimines [6]. Sensitivity in terms of LOQ (limit of quantification) were sub-ppb level (<1 µg/kg) showing good potential to assess the levels of this emerging group of toxins in seafood available to EU consumers. Indirect (through alkaline hydrolysis for Pinnatoxin G) and direct (through specific MRM transitions) analysis of acyl ester derivatives was included for the cyclic imine group. The research with cyclic imines has provided the first report of presence of pinnatoxins in Spain, though at low levels, as well as spirolides in Catalonia.

Cyclic imines were found at low concentrations (2 to 60 µg/kg) in 13 samples of mussels and oysters (22 samples analyzed). Pinnatoxin G has been also detected in 17 seawater samples (out of 34) using solid phase adsorption toxin tracking devices (0.3 to 0.9 µg/kg of resin). Pinnatoxin G and SPX-1 were confirmed with both low and high resolution (<2 ppm) mass spectrometry by comparison of the retention time and MS/MS response with those of authentic reference standard material. For other analogues without reference standards, we applied a strategy combining low resolution MS with a triple quadrupole mass analyzer for a fast and reliable screening, and high resolution MS LTQ Orbitrap® for confirmation (collaboration with Dr. A. Furey, Cork Institute of Technology).

The unambiguous confirmation of PnTX-G and SPX-1 in a mussel sample from Fangar Bay collected in May 2012 (MUS120523) was published in M. García-Altares *et al.* [8]. Pinnatoxin-G and SPX-1 were confirmed by their retention time (RT) compared to that of the standards (<1% difference), the mass accuracy of the precursor and of three diagnostic fragments (<2 ppm) and one ion ratio. The final

concentration in the sample was 60 ± 5 µg/kg PnTX-G and 16 ± 1 µg/kg SPX-1. All these confirmation figures were presented already in the **Deliverable 2.6**.

In addition, two cyclic imines, PnTX-G and SPX-1, were found at low concentrations in 13 samples of mussels and oysters (out of 22 samples analyzed, Table 1). Pinnatoxin G has also been detected in 17 seawater samples (out of 34, Table 2) using solid phase adsorption toxin tracking devices (SPATT, 0.3 to 0.9 µg/kg-resin). The quality assessment of the methods of quantification and confirmation was satisfactory. Other cyclic imines and acyl ester derivatives of cyclic imines were not confirmed in the samples.

Table 1. Confirmation with LTQ Orbitrap Discovery® FT-MS and quantification with 3200QTrap® of SPX-1 and PnTX-G in shellfish.*

Sample Details	SPX-1 (Δ ppm)		SPX-1 (µg/kg)	PnTX-G (Δ ppm)		PnTX-G (µg/kg)
OYS110102	$[M + H]^+$	1.0		$[M + H]^+$	Δppm > 5 ppm	
Oyster	Frag 1	−0.2	3.6 ± 0.1	Frag 1	Δppm > 5 ppm	ND
January 2011	Frag 2	1.2		Frag 2	Δppm > 5 ppm	
Fangar Bay	Frag 3	−2.3		Frag 3	Δppm > 5 ppm	
OYS110103	$[M + H]^+$	−0.2		$[M + H]^+$	Δppm > 5 ppm	
Oyster	Frag 1	0.2	3.5 ± 0.8	Frag 1	Δppm > 5 ppm	ND
January 2011	Frag 2	−0.9		Frag 2	Δppm > 5 ppm	
Fangar Bay	Frag 3	−3.5		Frag 3	Δppm > 5 ppm	
OYS110105	$[M + H]^+$	5.0		$[M + H]^+$	40.6	
Mussel	Frag 1	−0.2	2.2 ± 0.6	Frag 1	−0.2	3.8 ± 0.6
January 2011	Frag 2	0.5		Frag 2	−2.6	
Fangar Bay	Frag 3	0.4		Frag 3	−0.7	
OYS110115	$[M + H]^+$	0.4		$[M + H]^+$	Δppm > 5 ppm	
Oyster	Frag 1	−0.2	5 ± 1	Frag 1	Δppm > 5 ppm	ND
January 2011	Frag 2	0.3		Frag 2	Δppm > 5 ppm	
Fangar Bay	Frag 3	2.3		Frag 3	Δppm > 5 ppm	
MUS110116	$[M + H]^+$	1.6		$[M + H]^+$	1.3	
Mussel	Frag 1	0.1	3.6 ± 0.18	Frag 1	0.5	3 ± 1
January 2011	Frag 2	−0.1		Frag 2	−0.2	
Fangar Bay	Frag 3	0.9		Frag 3	−0.1	
OYS110117	$[M + H]^+$	−0.1		$[M + H]^+$	Δppm > 5 ppm	
Oyster	Frag 1	0.1	6.6 ± 0.7	Frag 1	Δppm > 5 ppm	ND
January 2011	Frag 2	−0.1		Frag 2	Δppm > 5 ppm	
Fangar Bay	Frag 3	−0.9		Frag 3	Δppm > 5 ppm	
OYS110205	$[M + H]^+$	0.4		$[M + H]^+$	Δppm > 5 ppm	
Oyster	Frag 1	−0.1	5.8 ± 0.5	Frag 1	Δppm > 5 ppm	ND
February 2011	Frag 2	0.1		Frag 2	Δppm > 5 ppm	
Fangar	Frag 3	1.6		Frag 3	Δppm > 5 ppm	
MUS110205	$[M + H]^+$	−0.2		$[M + H]^+$	2.2	
Mussel	Frag 1	0.3	NQ	Frag 1	0.5	4.1 ± 0.1
February 2011	Frag 2	−0.2		Frag 2	−1.8	
Fangar Bay	Frag 3	0.9		Frag 3	0.4	
MUS1027	$[M + H]^+$	0.2		$[M + H]^+$	42.6	
Mussel	Frag 1	−0.6	3 ± 2	Frag 1	−0.5	2.2 ± 0.1
February 2011	Frag 2	−1.0		Frag 2	−1.9	
Fangar Bay	Frag 3	0.3		Frag 3	−2.00	

Table 1. *Cont.*

OYS110208	$[M + H]^+$	0.5		$[M + H]^+$	Δppm > 5 ppm	
Oyster	Frag 1	−0.2	4 ± 1	Frag 1	Δppm > 5 ppm	ND
February 2011	Frag 2	0.1		Frag 2	Δppm > 5 ppm	
Fangar	Frag 3	−1.0		Frag 3	Δppm > 5 ppm	
MUS120520	$[M + H]^+$	1.0		$[M + H]^+$	−1.1	
Mussel	Frag 1	−0.5	NC	Frag 1	−0.5	39 ± 6
May 2012	Frag 2	Δppm > 5 ppm		Frag 2	−0.7	
Fangar Bay	Frag 3	Δppm > 5 ppm		Frag 3	−0.9	
MUS120523	$[M + H]^+$	−2.1		$[M + H]^+$	0.2	
Mussel	Frag 1	−0.1	16 ± 1	Frag 1	−0.1	59 ± 5
May 2012	Frag 2	0.4		Frag 2	−0.1	
Retail market	Frag 3	−0.1		Frag 3	−0.1	
MUS120524	$[M + H]^+$	Δppm > 5 ppm		$[M + H]^+$	−1.1	
Mussel	Frag 1	−1.1	NC	Frag 1	−0.5	58 ± 2
May 2012	Frag 2	Δppm > 5 ppm		Frag 2	−0.9	
Fangar Bay	Frag 3	Δppm > 5 ppm		Frag 3	−0.9	

* ND: Not detected; NC: Not confirmed; NQ: Not quantified. Δ ppm: mass error in ppm; $[M+H]+$: Molecular Ion (SPX-1: *m/z* 692.4521; PnTX-G: *m/z* 694.4677); Fragment 1: *m/z* 164.1434 (both SPX-1 and PnTX-G); Fragment 2: *m/z* 444.3108 (SPX-1) and 458.3265 (PnX-G); Fragment 3: *m/z* 674.4415 (SPX-1) and 676.4572 (PnTX-G).

Table 2. Confirmation with LTQ Orbitrap Discovery FTMS and quantification with 3200QTrap of PnTX-G in SPATT samples from Alfacs Bay. Δ ppm: mass error in ppm; $[M + H]^+$: Molecular Ion (PnTX-G: *m/z* 694.4677); Fragment 1: *m/z* 164.1434; Fragment 2: *m/z* 458.3265; Fragment 3: *m/z* 676.4572.

Sample Name and Date	PnTX-G (Δppm)		PnTX-G (µg/kg)
TB_1028 February 2007	$[M + H]^+$	0.9	0.47 ± 0.01
	Frag 1	0.3	
	Frag 2	−0.8	
	Frag 3	−0.1	
TB_1029 February 2007	$[M + H]^+$	1.3	0.65 ± 0.09
	Frag 1	0.6	
	Frag 2	−0.8	
	Frag 3	−0.1	
TB_1036 February 2007	$[M + H]^+$	−1.1	0.70 ± 0.05
	Frag 1	0.9	
	Frag 2	−0.1	
	Frag 3	0.4	
TB_1037 February 2007	$[M + H]^+$	2.0	0.75 ± 0.03
	Frag 1	−3.3	
	Frag 2	2.6	
	Frag 3	3.5	
TB_1359 January 2008	$[M + H]^+$	1.9	0.48 ± 0.03
	Frag 1	−2.6	
	Frag 2	−3.8	
	Frag 3	−3.1	

Table 2. *Cont.*

	$[M + H]^+$	3.8	
TB_1360	Frag 1	−2.5	0.28 ± 0.02
January 2008	Frag 2	−3.7	
	Frag 3	−2.9	
	$[M + H]^+$	1.9	
TB_1363	Frag 1	−2.7	0.93 ± 0.02
January 2008	Frag 2	−3.9	
	Frag 3	−3.2	
	$[M + H]^+$	4.1	
TB_1367	Frag 1	−3.4	0.65 ± 0.06
January 2008	Frag 2	−4.7	
	Frag 3	−4.0	
	$[M + H]^+$	−0.1	
TB_1374	Frag 1	−3.4	0.55 ± 0.01
July 2008	Frag 2	−4.3	
	Frag 3	−3.4	
	$[M + H]^+$	−2.8	
TB_1378	Frag 1	−3.3	0.47 ± 0.07
July 2008	Frag 2	−4.1	
	Frag 3	−3.4	
	$[M + H]^+$	−1.1	
TB_1379	Frag 1	−3.1	0.34 ± 0.02
July 2008	Frag 2	−4.8	
	Frag 3	−2.5	
	$[M + H]^+$	8.7	
TB_1381	Frag 1	−3.3	0.33 ± 0.02
July 2008	Frag 2	−5.0	
	Frag 3	−4.0	
	$[M + H]^+$	−0.8	
TB_1614	Frag 1	−2.9	0.58 ± 0.06
Juny 2009	Frag 2	−5.1	
	Frag 3	−1.0	

3.2 Characterization of Acyl Ester Derivatives of lipophilic toxins including Cyclic Imines by LC-MS/MS (IRTA)

Esterification is one of the most important metabolic routes of lipophilic marine toxins in shellfish [7-8]. At IRTA, several chemical acylation reactions aimed at obtaining acyl ester analogues via partial synthesis from the free toxins were assessed. The procedures developed including sensitive and selective methods based on LC-MS/MS can be applied to obtain reference materials that may be used as analytical standards (internal/external) for method development and calibration, as well as to perform toxicological *in vitro* and *in vivo* studies. Several acylation reactions including Schotten-Baumann reaction, esterification of fatty acids anhydrides catalyzed by 4-Dimethylaminopyridine (DMAP), esterification of non anhydride fatty acids catalyzed by dicyclohexylcarbodiimide (DCC) and DMAP and esterification of non anhydride fatty acids catalyzed by 1,1'-carbonyldiimidazole (CDI) were performed. All experimental procedure was described in ***Deliverable 2.6.***

A series of mass spectrometric experiments involving product ion scans and multiple reaction monitoring (MRM) were used to confirm the identity and to elucidate the fragmentation pathways of the synthesised products. Reaction yields regarding reaction time and temperature were examined at sub-nmol scale for the acylation system consisting of palmitic anhydride and DMAP in anhydrous pyridine, showing the best conditions at 75 °C for 60 min, 75 °C for 120 min and 100 °C for 270 min for cyclic imines, azaspiracid-1 and pectenotoxin-2, respectively. The esterification approach was verified at a larger scale for the esterification of gymnodimine-A (GYM-A), which gave a good yield (>90%) of 10-*O*-palmitoyl-GYM-A. Results are summarized in **Table 3.**

Table 3: Reaction yields for acylation reaction catalyzed by DMAP, regarding time of reaction, temperature, and type of toxin.*. Results were calculated as % of loss of initial compound.

Temperature (ºC)	Reaction yield (%)											
	50ºC				75ºC				100ºC			
Reaction time (min)	GYM-A	SPX-1	PTX-2	AZA-1	GYM-A	SPX-1	PTX-2	AZA-1	GYM-A	SPX-1	PTX-2	AZA-1
30	76	83	7	43	73	90	7	46	22	41	12	39
60	71	81	4	42	94	94	21	51	37	52	15	39
120	77	88	ND	26	95	95	23	58	43	67	74	ND
150	50	72	ND	32	64	77	30	54	65	77	82	ND
210	61	82	ND	24	41	59	13	45	79	89	91	ND
270	93	94	11	45	46	65	14	49	96	95	96	ND

*ND: not detected

Acyl ester analogues of lipophilic marine toxins have been synthesised and their structure elucidated by LC-MS/MS [4]. For acyl ester analogues identical to natural metabolites, the procedures developed have the potential to be applied for the semi-synthesis of metabolites in a sustainable, scalable and controlled way, avoiding extensive and tedious isolation and purification procedures from naturally contaminated shellfish. For the semi-synthetic esters structurally different from those found in shellfish, they may have applicability as internal standards for accurate quantifications of natural metabolites present in complex matrices. Furthermore, the methodology could be applied to the production of isotopically labelled esters that would be suitable for use as internal LC–MS standards.

3.3 Cyclic imines in Norwegian seaweeds (IRTA and NVI)

The presence of the cyclic imine toxin pinnatoxin G (PnTx-G) was unambiguously identified in the sugar kelp *Saccharina latissima* from Norway by liquid chromatography coupled to mass spectrometry analysis, running under several operation modes at IRTA and NVI [9].

Optimization and validation of the method was performed together with the identification of Pinnatoxin G in the sample. To date, the only known producer of pinnatoxins is the benthic dinoflagelate *Vulcanodinium rugossum*, which has been only reported in warm or temperate waters of Australia, New Zealand, Japan, NW Mediterranean, South of China, Mexican Pacific and Hawaii. However, pinnatoxins have shown wider latitudinal distribution and they have been found in shellfish and/or seawater of Norway and Eastern Canada. Despite their toxicity observed in mice, the European Food Safety Authority could not draw the risk associated to shellfish consumption containing these toxins because the limited toxicological records and the lack of exposure data (EFSA, 2010).

Six-level calibration curves between 0.19–38 µg/mL PnTX-G·showed good intra-batch performance: linear adjustment (r^2) between 0.9961-0.9999 and slope shift between 0.5–5.7% among subsequent calibration curves. The concentration of PnTX-G in *S. latissima* under these conditions was estimated of 5.1 ± 0.4 µg/kg. This finding strengthens the evidence of a wide latitudinal distribution of pinnatoxins, and it suggests that kelp or seaweeds can be a potential ecological niche for benthic dinoflagellate producers of pinnatoxins in cold waters. Assessment on how food processing may affect the levels of this toxin in manufactured food products containing kelp-based ingredients should be further investigated.

Seaweed samples of *Laminaria digitata* (kelp) and *S. latissima* (sugar kelp) were sampled by Hortimare (http://www.hortimare.com) within the framework of the ECsafeSEAFOOD project on May 24th, 2013, near to a Norwegian salmon fish farm in a fjord in Jovika, at the north of Bergen (61º03'28.5"N, 4º55'29.6"E). Sample preparation and cyclic imines extraction is described in **Deliverable 2.6**.

Investigation on the presence of other pinnatoxin analogues was performed, however none of these analogues could be identified in *S. latissima*. Quantitatively, a six-level calibration curve was performed with reference calibration solution of PnTX-G between 0.19–38 µg/mL. The calibration curves showed good intra-batch performance: linear adjustment (r^2) between 0.9961–0.9999 and slope shift between 0.5- 5.7% among subsequent calibration curves. The limit of quantification was estimated ca. 0.2 µg/kg; and the concentration of PnTX-G in *S. latissima* was estimated at 5.1 ± 0.4 or 5.3 ± 0.3 µg/kg, taking MRM transitions for quantification *m/z* 694.5 > 676.4 or 694.5 > 164.5, respectively.

After these initial results, a second sampling was performed. Seaweed samples of *L. digitata* and *S. latissima* were sampled by Hortimare (http://www.hortimare.com) within the framework of the ECsafeSEAFOOD project. A summary of the location and origin is summarized in Table 4.

Table 4. Summary of the sampled seaweed in round II.

	Sample Name	Origin	Species name
1	Commercial SA 06/14 Scotland	Scotland (06/14)	*Saccharina latissima* (SA) (sugarkelp)
2	Commercial SA 05/14 Norway	Norway (05/14)	*Saccharina latissima* (SA) (Sugarkelp)
3	Hotspot LD 02/14 Sulefisk Norway	Sulefisk, Norway (02/14)	*Laminaria digitata* (LD) (Oarweed)
4	Hotspot LD 08/14 Sulefisk Norway	Sulefisk, Norway (08/14)	*Laminaria digitata* (LD) (Oarweed)
5	Hotspot SA 08/14 Sulefisk Norway	Sulefisk, Norway (08/14)	*Saccharina latissima* (SA) (Sugarkelp)
6	Hotspot SA 02/14 Sulefisk Norway	Sulefisk, Norway (02/14)	*Saccharina latissima* (SA) (Sugarkelp)

The same extraction procedure and LC-MS/MS analyses were performed in duplicate at IRTA. Results by LC-MS/MS confirm presence of pinnatoxins G in samples from both locations. Quantification of Pinnatoxin G in all samples was performed at IRTA and highest concentrations of cyclic imines were in sugar kelp, but there was some in the oarweed too.
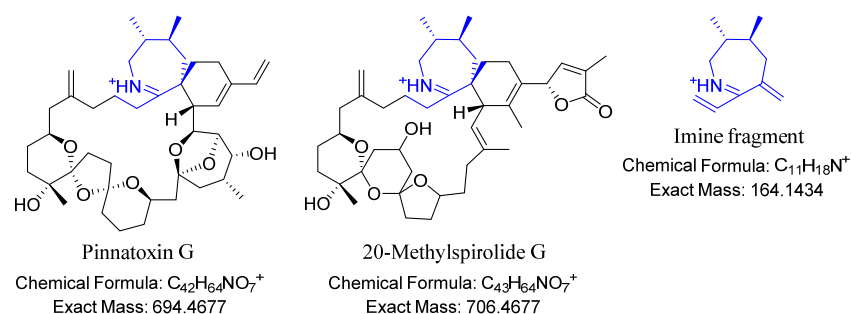
Table 5. Concentration of Pinnatoxin G in seaweed

Sample	Average of duplicate injections (ng/mL)	SD (ng/mL)	RSD (%)	Concentration in the seaweed (µg/kg d.w.)
1	2.25	0.12	5.5	0.22
2	1.37	0.11	7.7	0.14
3	0.63	0.03	4.6	0.06
4	0.52	0.02	3.8	0.05
5	2.26	0.03	1.4	0.23
6	**13.24**	**0.28**	**2.1**	**1.32**
1	2.66	0.19	7.0	0.27
2	1.41	0.02	1.7	0.14
3	0.86	0.02	1.9	0.09
4	0.52	0.03	5.8	0.05
5	2.41	0.06	2.6	0.24
6	**12.82**	**0.07**	**0.6**	**1.28**

Concentrations observed are very low levels of few parts-per-billion of PnTX-G. At IRTA some biomolecular analyses were carried out to see if *Vulcanodinium* was present. However, it was not detected on the freeze dried samples. It was not possible to conduct any further studies on taxonomy or microalgae isolation due to the impossibility to obtain fresh samples due to the long distance and remote sampling place.

The samples were also sent to NVI for confirmation of PnTx-G by LC-HRMS/MS and for the potential identification of new analogues. LC-HRMS/MS analysis unambiguously confirmed the presence of

PnTx-G and PnTx-A, as well as tentatively identifying similar levels in some samples of 20-methylSPX-G. A range of other putative pinnatoxins was also detected. The structures of the cyclic imines pinnatoxin G and 20-methylspirolide G and their common imine fragment ion are shown in Figure 1.

Pinnatoxin G
Chemical Formula: $C_{42}H_{64}NO_7^+$
Exact Mass: 694.4677

20-Methylspirolide G
Chemical Formula: $C_{43}H_{64}NO_7^+$
Exact Mass: 706.4677

Imine fragment
Chemical Formula: $C_{11}H_{18}N^+$
Exact Mass: 164.1434

Figure 1. Structures of the cyclic imines pinnatoxin G and 20-methylspirolide G and their common imine fragment ion (substructure shown in blue).

Pinnatoxin G was detected in the FS chromatogram at *m/z* 694.4677 with the same retention time as the authentic standard, at ca 5 ng/mL in the extract. Furthermore, a prominent peak corresponding to PnTx-G was present at the same retention time at *m/z* 164.1434 in the AIF chromatogram. A second minor peak was also present in the AIF chromatogram at *m/z* 164.2434, corresponding to a previously undetected minor peak with *m/z* 706.4677 in the FS chromatogram. This peak did not correspond to known pinnatoxins, but was plausible for several known spirolides for which standards were not available. Targeted LC–MS/MS chromatograms were therefore obtained at *m/z* 694.5 (pinnatoxin G) and 706.5 (suspected spirolide). The MS/MS spectrum for the pinnatoxin G peak was identical to that obtained from an authentic standard under the same conditions, whereas the MS/MS spectrum of the peak at *m/z* 706 was an excellent match for the published MS/MS spectrum of 20-methylspirolide G, including the atomic compositions of the proposed (the published MS/MS data was not obtained at high mass resolution) fragments. A series of other seaweed extracts from Norway and Scotland (2 commercial samples SA (1 from Scotland and 1 from Norway) and 4 Hotspot LD and SA from Sulefisk Norway) was subsequently examined and showed the presence of the same two compounds as the most abundant cyclic imines, although their ratios varied markedly from sample to sample. Furthermore, using the same analytical strategy, trace levels of pinnatoxin A and three novel pinnatoxin and spirolides congeners were tentatively identified in the seaweed extracts. Pinnatoxins E and F were not detected in any of the samples.

Comparison of the FS chromatogram extracted for *m/z* 500–800, *m/z* 694.4677, and *m/z* 706.4677 (Figure 2) illustrates the selectivity conferred by high resolution MS, even in full scan mode. The FS and AIF data can be retrospectively examined for other candidate ions, and it is this feature that permitted the subsequent identification of 20-methylspirolide G, pinnatoxin A and the other novel analogues to be tentatively identified in these samples, in addition to the primary analyte of interest (pinnatoxin G).
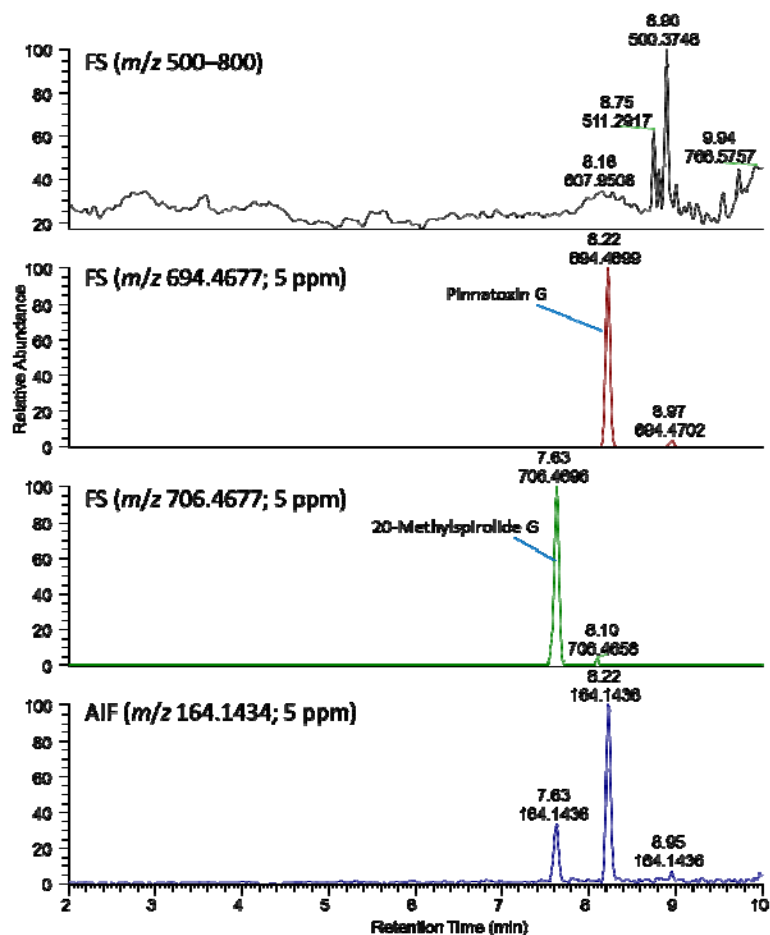
Figure 2. LC–MS chromatograms of Norwegian seaweed sample analysed by LC–MS with the Q Exactive as detector. Top, Full Scan (FS) chromatogram extracted for *m/z* 500–800; second from top, FS extracted for *m/z* 694.4677; second from bottom, FS extracted for *m/z* 706.4677; bottom, All Ion Fragmentation (AIF) chromatogram extracted for *m/z* 164.1434.

Moreover, after the tentative identification of the new pinnatoxins in the seaweed, Chris Miles has visited to Prof. Zakarian at UCSB. Prof. Zakarian has agreed to synthesise selected putative PnTx-G metabolites tentatively identified in the seaweed samples, in order to unambiguously confirm the identity of the metabolites by LC-MS/MS.

3.4 Cyclic imines in Commercial Samples

Several bivalve species (mussels, oysters, clams) were among the commercial samples analysed for emerging marine biotoxins such as cyclic imines. Cyclic Imines are toxins that can be identified when addressing the presence of lipophilic toxins with the use of LC-MS/MS. This method is implemented by different countries to monitor, under current regulations, the presence of diarrheic shellfish poisoning toxins, yessotoxins and azaspiracids. Their structure comprises a cyclic-imine moiety, responsible for acute neurotoxicity in mice. Cyclic imines have not been linked yet to human poisoning and are not regulated in Europe, although the European Food Safety Authority requires more data to perform a conclusive risk assessment for consumers.

The cyclic imines includes eight types of compounds produced by marine dinoflagellates: spirolides (SPXs, 13-desmethyl SPX-C, also known as SPX-1, being the reference toxin for this group), pinnatoxins (PnTXs, reference compound PnTX-G), gymnodimines (GYMs, reference compound GYM-A), pteriatoxins (PtTXs), prorocentrolides, spiro-prorocentrimine and portimine. They all share a cyclic imine group, which is extremely rare, that works as part of the pharmacophore. Spirolides and pinnatoxins comprise more than twenty different analogues with similar structures (see Figure 1). This study was focused mainly on spirolides, pinnatoxins and gymnodimines and their analogues.

3.4.1 Location Ebre Delta (IRTA, IPMA, UM and AEIFORIA)

Several commercial samples from four different countries (Italy, Portugal, Slovenia and Spain) during 2 years have been provided in order to obtain data on the presence of cyclic imines by LC-MS/MS. In addition, in order to have a complete set of commercial samples, local fresh shellfish and imported shellfish were used. Each partner obtained 6 shellfish samples from the market during 2014 and 2015 with the following characteristics:

- 3 fresh samples, 1 of each species (local seafood)
- 3 processed samples (imported seafood, i.e. frozen or canned)

Table 6. Results from Commercial Samples 2014 (1st round)

| | | | | Conc (µg/kg) | | | | | |
| | | | | LOQ ≤ 25 µg/kg | | LOQ ≤ 25 µg/kg | | LOQ ≤ 3 µg/kg | |
2014	**Code**	**Reference**	**Species/Details**	**GYM A**	**Other GYM**	**SPX1**	**Other SPX**	**PnTX G**	**Other PnTX**
IPMA 2014	80-14	Processed Vietnam clams	clams, frozen, imported						
	81-14	Clams Zebra frozen	clams, frozen,						
	82-14	Oysters	Oyster,fresh						
	83-14	Japanese clams	Clams, fresh						
	84-14	Mejillones en escabeche	Mussels in pickle sauce			26			
	Code	**Reference**	**Species/Details**	**GYM A**	**Other GYM**	**SPX1**	**Other SPX**	**PnTX G**	**Other PnTX**
AEIFORIA 2014	187-14	AEIF-ESS-SH-0003a	oyster, local, fresh						
	188-14	AEIF-ESS-SH-0002b	clam, imported, frozen						
	189-14	AEIF-ESS-SH-0003b	oyster, imported, fresh						
	190-14	AEIF-ESS-SH-0002a	clam, local, fresh					4	
	191-14	AEIF-ESS-SH-0001b	mussel, imported, frozen						
	192-14	AEIF-ESS-SH-0001a	mussel, local, fresh						
	Code	**Reference**	**Species/Details**	**GYM A**	**Other GYM**	**SPX1**	**Other SPX**	**PnTX G**	**Other PnTX**
UM 2014	12-15	Japanska Kocica	Japanese clam						
	13-15	Vungute Cele Zam	Placific clam						
	14-15	Musclo ledera	Mussel			26			
	15-15	Ostró ledera	Oyster			27		4	
	16-15	Musclo salsa enlatat (a la catalana)	Mussel in tomato					12	
	Code	**Reference**	**Species/Details**	**GYM A**	**Other GYM**	**SPX1**	**Other SPX**	**PnTX G**	**Other PnTX**
IRTA 2014	461-15	Ostra giga 150915 lot 27/15-003D	Flat Oyster, fresh			< LOQ			
	462-15	Musclo roca 150915 lot 75/15-003D	Mussel, fresh			28		4	
	463-15	Cloïssa cultiu 150915 lot 175/15-026D	Japanese clam, fresh						
	464-15	Mejillón hacendado-150915 (lot LE4294X 815215)	Mussel in brine					6	
	465-15	Almeja Dani-150915 (Lot L-11070)	Japanese clam						
	490-15	Mejillon de chile (Mascato), L210815 244XE1	mussels, frozen, imported					4	

Table 7. Results from Commercial Samples 2015 (2nd round)

	2015			Conc (µg/kg)					
				LOQ ≤ 25 µg/kg		LOQ ≤ 25 µg/kg		LOQ ≤ 3 µg/kg	
	Code	Reference	Species/Details	GYM A	Other GYM	SPX1	Other SPX	PnTX G	Other PnTX
AEIFORIA 2015	267-15	AEIF-ESS-SH-0005a (12/05/2015)	clam, local, fresh						
	268-15	AEIF-ESS-SH-0004b (12/05/2015)	mussel, imported, frozen					3	
	269-15	AEIF-ESS-SH-0006a (12/05/2015)	oyster, local, fresh						
	270-15	AEIF-ESS-SH-0004a (12/05/2015)	mussel, local, fresh			< LOQ			
	271-15	AEIF-ESS-SH-0006b (12/05/2015)	oyster, imported, frozen			< LOQ			
	272-15	AEIF-ESS-SH-0005b (12/05/2015)	clam, imported, frozen						
		Reference	Species/Details	GYM A	Other GYM	SPX1	Other SPX	PnTX G	Other PnTX
IPMA 2015	405-15	Ruditapes decussatus (local market)	Algarv Clam, fresh			< LOQ		< LOQ	
	406-15	Mejillon (sample from 2014)	Mussels, fresh			< LOQ			
	407-15	Mejillon en escabeche	Mussels in pickle sauce			66		< LOQ	
	408-15	Cerastoderma edule (local market)	Cockle, fresh			57			
	409-15	Spisula solida (local market)	Clams, fresh			63			
	410-15	Processed clams (frozen, 30 individuals)	Clams, frozen					< LOQ	
	411-15	Processed mussel (frozen, 30 ind)	Mussels, frozen						
	Code	Reference	Species/Details	GYM A	Other GYM	SPX1	Other SPX	PnTX G	Other PnTX
IRTA 2015	484-15	Almeja chilena (Escuris)	Clams in brine, imported						
	485-15	Mejillones de las rias en escabeche (escuris)	Mussels in pickle sauce			< LOQ		< LOQ	
	486-15	Mejillon-250915, Lot 88/15-003D	Mussels, fresh			34		< LOQ	
	487-15	Ostra rizada-240915, Lot OR0125	Oysters, fresh			< LOQ			
	488-15	Almeja japanese, Lot 963 250915	Clams, fresh						
	489-15	Almeja juliana (Mascato), L291014	Frozen clams, imported						
	Code	Reference	Species/Details	GYM A	Other GYM	SPX1	Other SPX	PnTX G	Other PnTX
UM 2015	652-15	Japonska kocica: Fresh Clams (vongola verace); Adriatic	Clams, fresh						
	653-15	Pokrovača Jakobova: »Scallop« (Pectinidae); Adriatic	Scallop, fresh						
	654-15	Vongole cele zam: Frozen Clams (vongola verace); Adriat	Clams, frozen						
	655-15	Klapavica dagnje meso zam: Frozen mussels (Mytilus gal	Mussels, frozen					3	
	656-15	Klapavice sveze: Fresh Mussels (Mytilus galloprovincialis	Mussels, fresh			33			
	657-15	Klapavice po katalonsko: Canned Mussels »Catalonian st	Mussel in tomato					5	

Results and Discussion

All results from Commercial Samples are summarized in Table 6 and 7 from 2014 and 2015, respectively. All cyclic imines were monitored and gymnodimine A (GYM A), pinnatoxin G (PnTX G) and 13-desmethylSpirolide C (SPX1) were detected in the commercial samples.

Gymnodimine A and analogues were not detected in any sample during 2014. In addition, 4 samples (1 Portugal, 2 Slovenia and 1 Spain) present some low concentrations (25-30 µg/kg) of 13-desmethylspirolide C (SPX-1). Moreover, pinnatoxin G was detected in low levels (3-12 µg/kg) in 6 samples (1 Italy, 2 Slovenia and 3 Spain). Mussels in cans (processed mussels) showed higher concentrations (12 µg/kg) than raw mussels. It is well known that cooking mussels reduces the water content of the meat, thereby increasing the concentration of any toxins that are present. The effects of cooking are reported on *Deliverable 2.7*.

Samples from Italy, Portugal and Spain have been already analyzed and results are shown in Table 7. Gymnodimine A was again not detected in 2015. In addition, four samples (3 Portugal and 1 Spain) contained low levels (34-65 µg/kg) from 13-desmethylspirolide C (SPX-1), however traces of SPX1 were detected in 6 more samples. Moreover, pinnatoxin G was detected in one sample at 3 µg/kg, however traces of PnTX G were also detected in 5 other samples.

3.4.2. Location Norway Cyclic imines (NVI, AQUATT, DTU, IMARES)

Several commercial samples from four different countries (Ireland, Norway, Netherlands and Denmark) during 2 years have been provided in order to obtain data on the presence of cyclic imines by LC-MS/MS. In addition, in order to have a complete set of commercial samples, local fresh shellfish and imported shellfish were used. Each partner obtained 6 shellfish samples from the market during 2014 and 2015 with the following characteristics:

- 3 fresh samples, 1 of each species (local seafood)
- 3 processed samples (imported seafood, i.e. frozen or canned)

Table 8. Tabulated results from LC-HRMS analysis at NVI of commercial shellfish (Round 1, 2014) for pinnatoxins A and E–G*

sample	Type	Origin(if known)	Sampling Institute	PnTx-G (µg/kg)
Pacific Oysters	Fresh	Ireland	AQUATT	ND

Cockle	Fresh	Ireland	AQUATT	ND
Blue mussels	Fresh	Ireland	AQUATT	0.4
Mussels	Smoked & Canned	Spain	AQUATT	ND
Baby clams	Canned	Thailand	AQUATT	ND
Oysters	Canned	Korea	AQUATT	ND
Surf-clams	Fresh	Denmark	DTU	ND
Blue mussels	Fresh	Denmark	DTU	ND
Oysters	Fresh	Denmark	DTU	ND
Blue Mussels	Fresh	Denmark	DTU	0.1
Blue mussels	Canned	Denmark	DTU	ND
Pacific Oysters	Smoked & Canned	Korea	DTU	ND
Scallops	Frozen	Greenland	DTU	ND
Blue mussels	Preserved	NE Atlantic	IMARES	0.3
Blue mussels	Preserved	NE Atlantic	IMARES	ND
Blue mussels	Preserved	NE Atlantic	IMARES	ND
Blue mussels	Preserved	NE Atlantic	IMARES	ND
Blue mussels	Preserved	NE Atlantic	IMARES	ND
Cockles	Canned	Unknown	IMARES	ND
Razors	Canned	Unknown	IMARES	ND
Blue mussels	Fresh	Norway	NVI	0.1
Blue mussels	Fresh	Norway	NVI	0.3
Cockles	Fresh	Sweden	NVI	ND
Blue mussels	Frozen	Chile	NVI	0.1
Scallops	Frozen	Pacific	NVI	ND

*Pinnatoxins A, E and F not detected in any samples. Values under 3 µg/kg are semi-quantitative and should be regarded only as indicative of detection.

Table 9. Tabulated results from LC-HRMS analysis at NVI of commercial shellfish (Round 2, 2015) pinnatoxins A and E–G*

Sample	Type	Origin	Sampling	PnTx-A	PnTx-G

		(if known)	Institute	(µg/kg)	(µg/kg)
Pacific Oysters	Fresh	Ireland	AQUATT	ND	ND
Surf clams	Fresh	Ireland	AQUATT	ND	0.1
Blue mussels	Fresh	Ireland	AQUATT	0.3	4.6
Crab	Shredded & canned	Vietnam	AQUATT	ND	ND
Clams	Preserved	Mediterranean	AQUATT	ND	ND
Oysters	Smoked & canned	Korea	AQUATT	ND	ND
Pacific oysters	Frozen	Japan	DTU	ND	ND
Blue mussels	Fresh	Denmark	DTU	ND	1.2
Oysters	Fresh	Denmark	DTU	ND	0.1
Oysters	Smoked & canned	Korea	DTU	ND	ND
Blue mussels	Smoked, in brine	N.E. Atlantic	DTU	ND	ND
Scallops	Fresh	Denmark	DTU	ND	0.1
Greenshell mussels	Frozen	New Zealand	IMARES	ND	0.4
Blue mussels	Fresh	Netherlands	IMARES	ND	0.4
Clams	Fresh	Central Med.	IMARES	ND	ND
Razor clams	Fresh	North Sea	IMARES	ND	ND
Clams	Preserved	Mediterranean	IMARES	ND	ND
Cockles	Fresh	N. Atlantic	IMARES	ND	0.3
Blue mussels	Frozen	Chile	NVI	ND	2.5
Blue mussels	Brine	Denmark	NVI	ND	0.1
Blue mussels	Canned	Denmark	NVI	ND	0.2
Blue mussels	Frozen	Chile	NVI	ND	0.3
Blue mussels	Fresh	Norway	NVI	ND	5.1
Blue mussels	Fresh	Norway	NVI	ND	3.5

*Pinnatoxins E and F not detected in any samples. Values under 3 µg/kg are semi-quantitative and should be regarded only as indicative of detection.

Very low concentrations of PnTX-G were detected during 2014. Concentrations between 0.1 to 0.4 µg/kg were quantified. During the second sampling round (2015), higher concentrations in ppb levels were detected in comparison with 2014. Pinnatoxin-G was detected in concentrations between 0.1 to 5.1 µg/kg, the highest concentrations were found in Norway samples from NVI.

3.5 Identification of Gymnodimine D and presence of gymnodimine variants in the dinoflagellate *Alexandrium ostenfeldii* from the Baltic sea (NVI, IRTA)

Gymnodimines are lipophilic toxins produced by the marine dinoflagellates *Karenia selliformis* and *Alexandrium ostenfeldii*. Currently four gymnodimine analogues are known and characterized. In the new publication Toxicon 112 (2016) 68-76, it is described a novel gymnodimine, gymnodimine D, and a range of gymnodimine related compounds found in an *A. ostenfeldii* isolate from the northern Baltic Sea [10].

Gymnodimine D was extracted and purified from clonal cultures, and characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS), nuclear magnetic resonance (NMR) spectroscopy, and liquid chromatography-high resolution mass spectrometry (LC-HRMS) experiments. The structure of Gymnodimine D is related to known gymnodimines with a six-membered cyclic imine ring and several other fragments typical of gymnodimines (gymnodimine A, B, C and 12-methygymnodimine). However, the carbon chain in the gymnodimine macrocyclic ring differs from the known gymnodimines in having two tetrahydrofuran rings in the macrocyclic ring (figure 3).
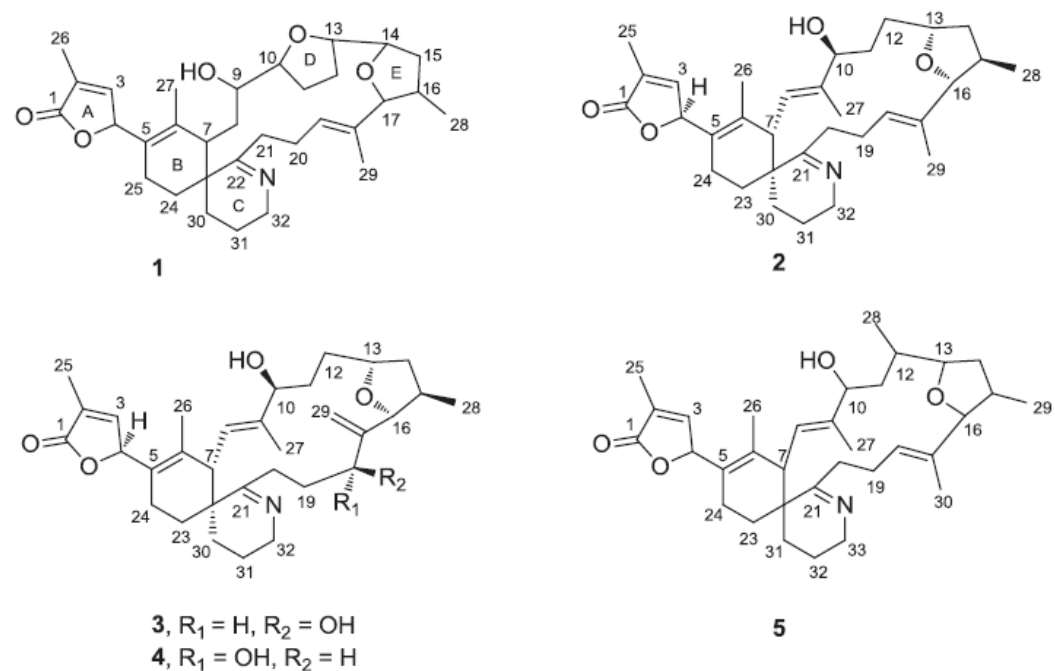
Figure 3. The structures of gymnodimine D (1), gymnodimine A (2), gymnodimine B (3), gymnodimine C (4) and 12-methygymnodimine (5).

Accurate masses (**Table 10**) of gymnodimine D and the minor gymnodimine were consistent with the molecular formula for gymnodimine B/C, $C_{32}H_{45}O_5N$ (with 11 rings or double bond equivalents), previously isolated from *K. selliformis* (Miles et al., 2000, 2003). However, the mass spectrometric fragmentation patterns differed from those expected for gymnodimine B/C based on reports from literature (Ben Naila et al., 2012). Several product ions, such as *m/z* 496, 346, and 316 (Fig. 4), which did not match with the product ions expected for gymnodimine B/C, were detected in the product ion spectrum of gymnodimine D. Only very small product ions typical for gymnodimine B and C at *m/z* 488 and 202 were observed in the LC-MS/MS spectrum of gymnodimine D.

Table 10. Exact and Measured Accurate Masses (*m/z*) for [M+H]$^+$ at *m/z* 524 and its
Product ions obtained with LC-HRMS .

| Formula	Calculated	Measured	Δ (ppm)
$C_{32}H_{46}NO_5^+$	524.3371	524.3365	−1.0
$C_{32}H_{44}NO_4^+$	506.3265	506.3262	−0.5
$C_{31}H_{46}NO_4^+$	496.3421	496.3430	2.0
$C_{32}H_{42}NO_3^+$	488.3159	488.3149	−2.0
$C_{31}H_{46}NO_3^+$	480.3472	480.3466	−1.2
$C_{31}H_{44}NO_2^+$	462.3367	462.3357	−2.1
$C_{27}H_{40}NO_3^+$	426.3003	426.2997	−1.2
$C_{27}H_{38}NO_2^+$	408.2897	408.2886	−2.8
$C_{24}H_{36}NO_4^+$	402.2639	402.2629	−2.5
$C_{21}H_{32}NO_3^+$	346.2377	346.2377	0.0
$C_{20}H_{30}NO_2^+$	316.2271	316.2263	−2.5
$C_{15}H_{24}N^+$	218.1903	218.1900	−1.5
$C_{14}H_{22}N^+$	204.1747	204.1745	−1.1
$C_{14}H_{20}N^+$	202.1590	202.1586	−2.4
$C_{11}H_{16}N^+$	162.1277	162.1277	−0.5
$C_9H_{14}N^+$	136.1121	136.1121	−0.5
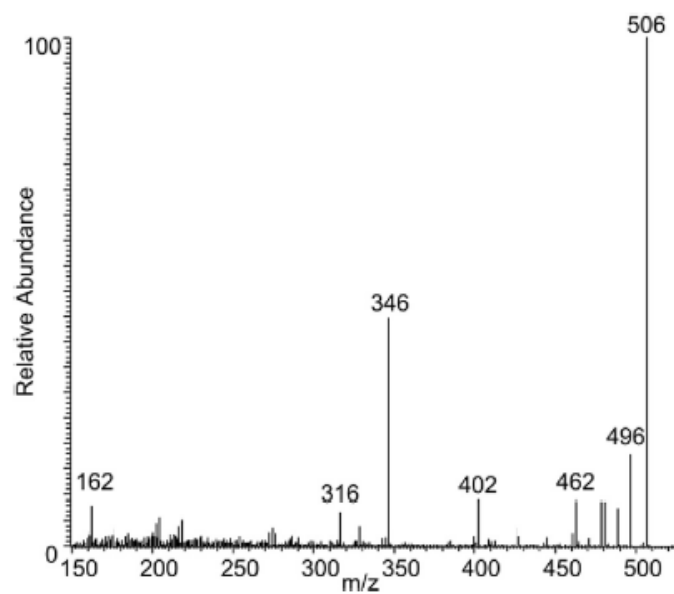
Figure 4. Product ion spectrum of 1 obtained by LC-MS/MS, [M+H]+ at m/z 524 in the range m/z 150-530.

The structure of gymnodimine D was resolved by NMR spectroscopy and mass spectrometry. It is closely related to the known gymnodimines (Fig 3), and contains many structural features present in gymnodimine A, B, C and 12-methygymnodimine, such as six-membered cyclic imine, butenolide, and tetrahydrofuran ring structures. The structure of gymnodimine D differs from gymnodimine A only between C-7 and C-14 (Fig 3).

4. Azaspiracids (AZAs)

4.1 Azaspiracids in Norwegian crabs (NVI)

Extracts of crab hepatopancreas from Norwegian crabs harvested near Frøya, and used in azaspiracid-1 and pinnatoxin G dosing experiments, were analysed using an LC–MS method with the Q Exactive as detector.

Analysis conditions by LC-MS on the Q Exactive were described in the *Deliverable 2.6*. Structures of azaspiracid toxins regulated in European shellfish and two of the common fragment ions used for confirmation of AZAs during MS/MS analysis are summarized in Figure 5.
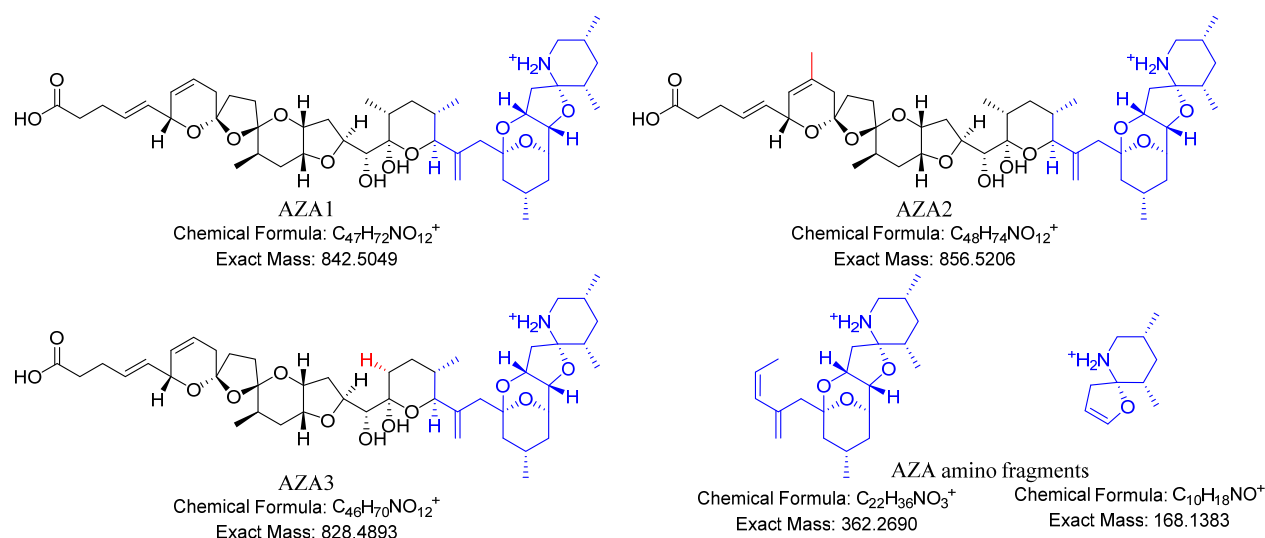
Figure 5. Structures of the azaspiracid toxins regulated in European shellfish, and two of the common fragment ions used for confirmation of AZAs during MS/MS analysis. The substructures giving rise to these fragments are shown in blue, while sites of structural variation are shown in red.

Azaspiracids 1 and 2 were found in the hepatopancreas of all crab samples analysed from Frøya in 2014 and 2015 (more than 50 individuals in total). AZA3 was found in most samples, and in the most heavily contaminated samples a range of peaks were also observed with masses and retention times that were consistent with oxidised metabolites (i.e. via hydroxylation and carboxylation) of AZA1 and AZA2. Amongst the most abundant metabolites was a peak at m/z 872.4800 at 10.94 min (Figure 5) that was consistent with AZA17 (Figure 7), a 22-carboxylated metabolite of AZA1 (Figure 4). The identity of AZA17 was confirmed by comparing its MS/MS spectrum with the literature, and by its heat-induced (80 °C, 10 min) decarboxylation to AZA3 as monitored by LC–MS and LC–MS/MS analysis. However, several minor peaks with the same mass were also present at 9.63 and 9.92 min (Figures 5 and 6), but were not converted by heat treatment (data not shown), indicating potential carboxylated AZA analogues. However, these peaks did not show AIF fragments at m/z 362.2690 (see Figure 5), indicating that if these were carboxylated AZAs, then the site of carboxylation might include the amine-containing substructures shown in blue (e.g. see Figures 5 and 8)..
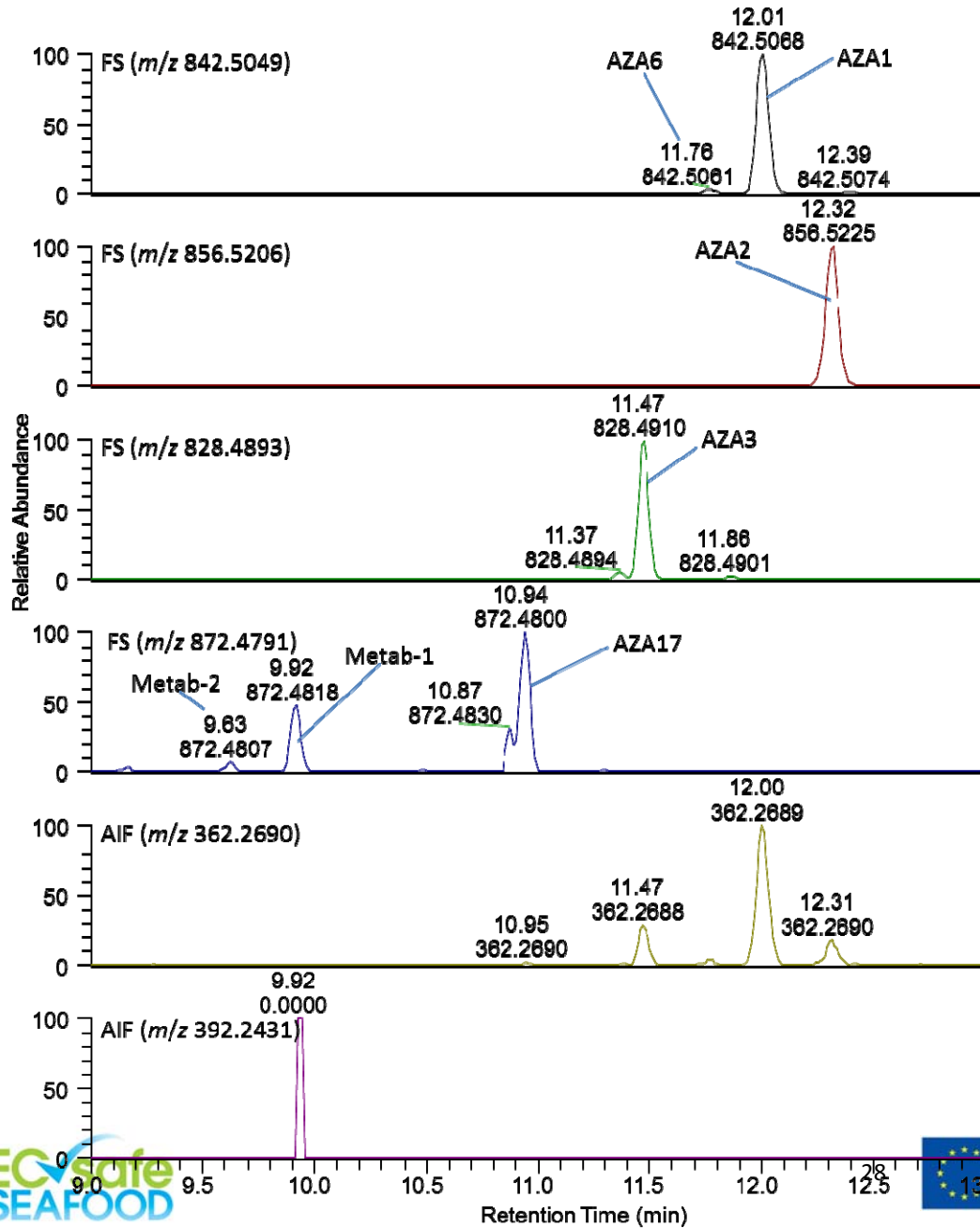
Figure 6. LC–MS chromatograms of a cooked extract from Norwegian crab hepatopancreas. The top four chromatograms are extracted from the FS chromatogram at the exact masses for AZA1, AZA2, AZA3 (Figure 5), and AZA17 (Figure 8). Note the presence of unidentified peaks with the same *m/z* as AZA17. The bottom two chromatograms are extracted from the corresponding AIF chromatogram at *m/z* 362.2690 (Figure 5), and for the carboxylated version of that fragment ion (i.e. *m/z* 392.2431, see Figure 5) because a weak signal was observed at that mass at the retention time of the unknown metabolite-1.

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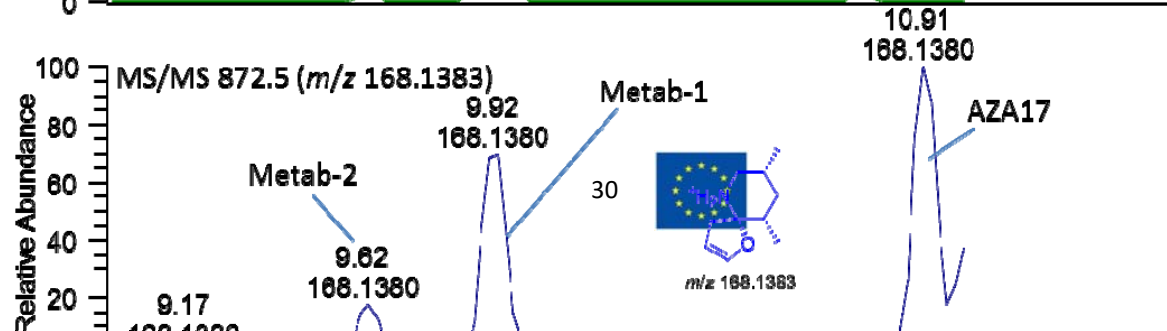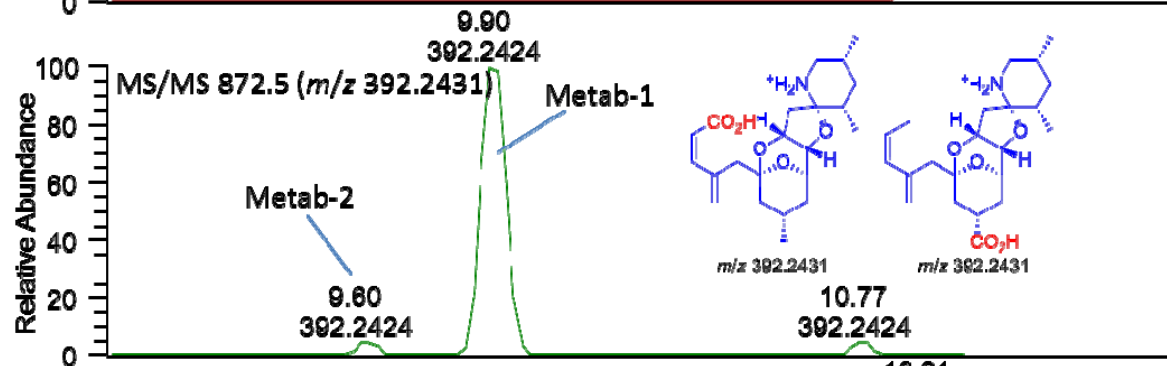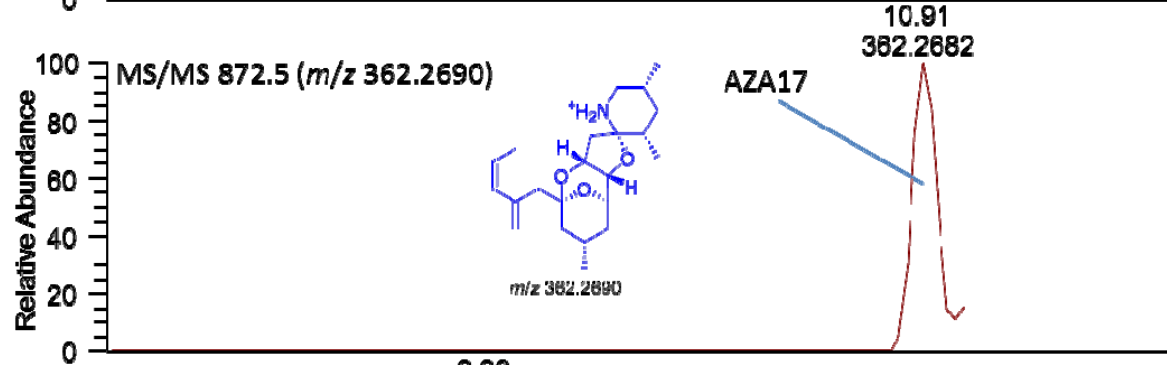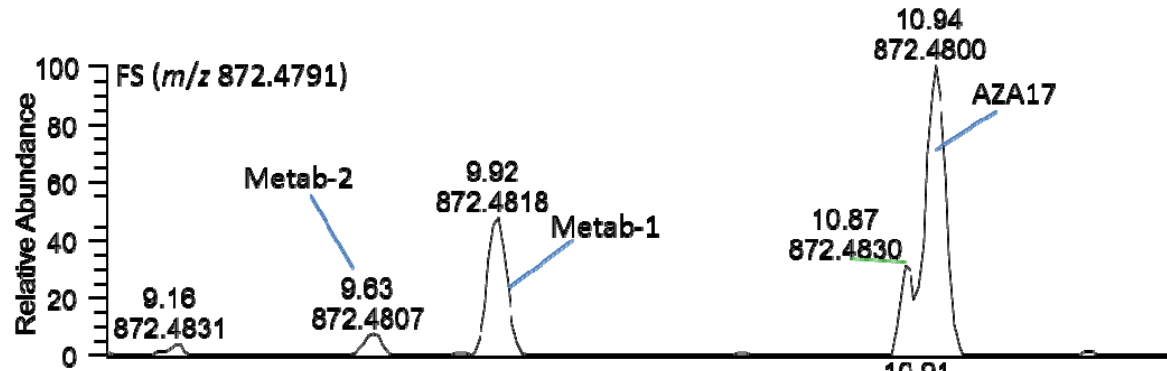**Figure 7. Top, LC–MS FS chromatogram extracted at m/z 872.4791 (from the analysis shown in Figure 6). The second, third and fourth chromatograms are extracted ions from LC–MS/MS analysis obtained from fragmentation of m/z 872.5 isolated via the quadrupole of the Q Exactive with fragmentation followed by high resolution measurement in the instrument's Orbitrap mass analyser. The extracted masses correspond to the masses of the fragments depicted.**
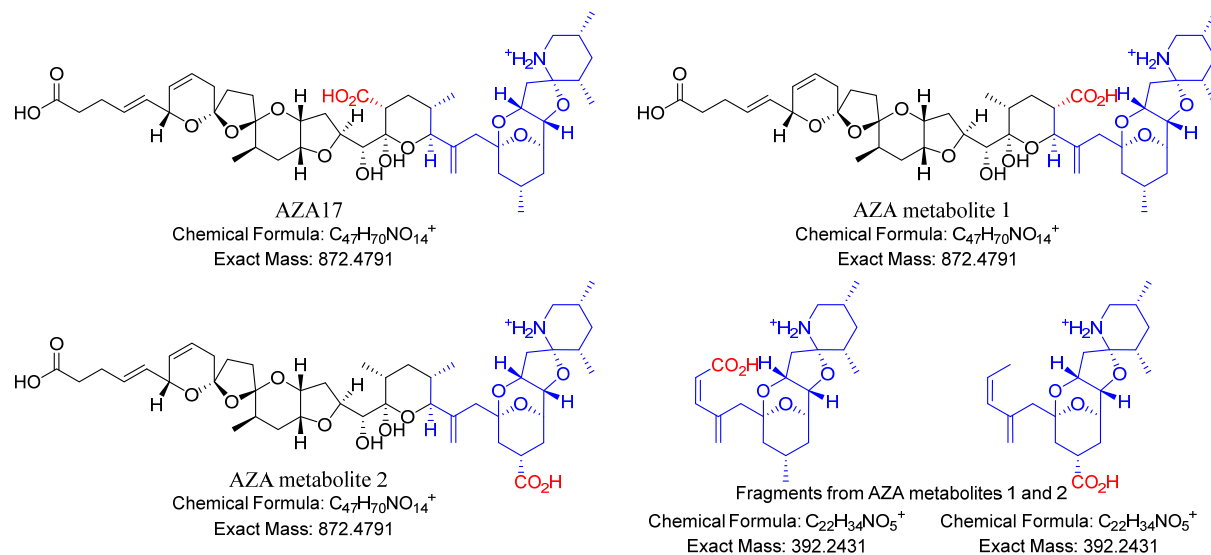

Figure 8. Structures of the known metabolite AZA17, produced in shellfish via oxidation of the 22-methyl group of AZA1, and the proposed tentative structures for metabolite-1 and metabolite-2 observed during LC–MS analysis (see Figures 6 and 7) are also shown, together with diagnostic fragment ions. The structures of the new metabolites are based on preliminary analysis of their LC-MS/MS spectra using the Q Exactive.

Therefore the AIF chromatogram from Figure 6 was extracted for *m/z* 392.2431, and this showed a weak peak at the same retention time as the peak for metabolite 1 (lower panel in Figure 6), consistent with this hypothesis. To verify this, an LC–MS/MS analysis was performed that included fragmentation of ions of *m/z* 872.5 isolated in the quadrupole of the Q Exactive instrument over the retention time of interest (see Figure 7). This confirmed that AZA17 showed a prominent fragment at *m/z* 362.2690, while metabolite-1 and metabolite-2 both showed corresponding fragment ions at *m/z* 392.2431, consistent with carboxylation in the blue substructure. Furthermore, AZA17 and metabolite-1 and metabolite-2 each showed prominent fragments at *m/z* 168.1383 in their MS/MS spectra, proving that carboxylation was not present in the terminal amino-ring. Careful analysis of the MS/MS spectra of metabolite-1 and metabolite-2 indicate that they probably have the structures shown in Figure 8. Unidentified metabolites such as these are believed to account for the higher response of the newly developed AZA ELISA (developed as part of WP4 of ECsafeSEAFOOD) relative to LC–MS/MS [11].

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Commercial Samples (Round 1)

Azaspiracids (AZA1, AZA2 and AZA3) were also quantified in both commercial samples described in Table 11 and Table 12. Concentrations of AZA-1 were quantified between 0.1 to 26.4 µg/kg. The highest concentration (26.4 µg/kg) was identified in IMARES samples from Round 1 during 2014.

Table 11. Tabulated results from LC-HRMS analysis at NVI of commercial shellfish (Round 1, 2014) for azaspiracids 1–3

sample	Type	Origin (if known)	Sampling Institute	AZA-1 (µg/kg)	AZA-2 (µg/kg)	AZA-3 (µg/kg)
Pacific Oysters	Fresh	Ireland	AQUATT	4.5	0.8	ND
Cockle	Fresh	Ireland	AQUATT	11.5	3.5	ND
Blue mussels	Fresh	Ireland	AQUATT	0.7	0.1	ND
Mussels	Smoked & Canned	Spain	AQUATT	ND	0.1	ND
Baby clams	Canned	Thailand	AQUATT	ND	ND	ND
Oysters	Canned	Korea	AQUATT	ND	ND	ND
Surf-clams	Fresh	Denmark	DTU	ND	ND	ND
Blue mussels	Fresh	Denmark	DTU	ND	ND	ND
Oysters	Fresh	Denmark	DTU	ND	ND	ND
Blue Mussels	Fresh	Denmark	DTU	ND	ND	ND
Blue mussels	Canned	Denmark	DTU	ND	ND	ND
Pacific	Smoked & Canned	Korea	DTU	ND	0.1	ND

Oysters						
Scallops	Frozen	Greenland	DTU	ND	ND	ND
Blue mussels	Preserved	NE Atlantic	IMARES	26.4	5.8	0.1
Blue mussels	Preserved	NE Atlantic	IMARES	ND	ND	ND
Blue mussels	Preserved	NE Atlantic	IMARES	0.1	ND	ND
Blue mussels	Preserved	NE Atlantic	IMARES	ND	0.1	ND
Blue mussels	Preserved	NE Atlantic	IMARES	ND	ND	ND
Cockles	Canned	Unknown	IMARES	ND	ND	ND
Razors	Canned	Unknown	IMARES	ND	ND	ND
Blue mussels	Fresh	Norway	NVI	ND	ND	ND
Blue mussels	Fresh	Norway	NVI	0.4	0.3	ND
Cockles	Fresh	Sweden	NVI	ND	ND	ND
Blue mussels	Frozen	Chile	NVI	ND	ND	ND
Scallops	Frozen	Pacific	NVI	ND	ND	ND

* Values under 3 µg/kg are semi-quantitative and should be regarded only as indicative of detection.

Commercial Samples (Round 2)

Table 12. Tabulated results from LC-HRMS analysis at NVI of commercial shellfish (Round 2, 2015) for azaspiracids 1–3.

Sample	Type	Origin (if known)	Sampling Institute	AZA-1 (µg/kg)	AZA-2 (µg/kg)	AZA-3 (µg/kg)
Pacific Oysters	Fresh	Ireland	AQUATT	ND	ND	ND
Surf clams	Fresh	Ireland	AQUATT	2.2	1.8	ND
Blue mussels	Fresh	Ireland	AQUATT	0.7	ND	ND
Crab	Shredded & canned	Vietnam	AQUATT	ND	ND	ND
Clams	Preserved	Mediterranean	AQUATT	ND	ND	ND
Oysters	Smoked & canned	Korea	AQUATT	ND	ND	ND
Pacific oysters	Frozen	Japan	DTU	ND	ND	ND
Blue mussels	Fresh	Denmark	DTU	ND	ND	ND
Oysters	Fresh	Denmark	DTU	ND	ND	ND
Oysters	Smoked & canned	Korea	DTU	ND	ND	ND
Blue mussels	Smoked, in brine	N.E. Atlantic	DTU	ND	ND	ND
Scallops	Fresh	Denmark	DTU	ND	0.1	ND
Greenshell mussels	Frozen	New Zealand	IMARES	ND	ND	ND
Blue mussels	Fresh	Netherlands	IMARES	ND	ND	ND
Clams	Fresh	Central Med.	IMARES	ND	ND	ND
Razor clams	Fresh	North Sea	IMARES	ND	ND	ND
Clams	Preserved	Mediterranean	IMARES	ND	ND	ND
Cockles	Fresh	N. Atlantic	IMARES	ND	ND	ND
Blue mussels	Frozen	Chile	NVI	ND	ND	ND
Blue mussels	Brine	Denmark	NVI	ND	ND	ND
Blue mussels	Canned	Denmark	NVI	ND	ND	ND
Blue mussels	Frozen	Chile	NVI	ND	ND	ND
Blue mussels	Fresh	Norway	NVI	ND	ND	0.5
Blue mussels	Fresh	Norway	NVI	ND	ND	ND

5. Ovatoxins and Palytoxins

5.1 Ovatoxins and Palytoxins in *Ostreopsis cf. ovata* (IRTA)

Blooms of the benthic dinoflagellate *Ostreopsis* cf. *ovata* are a concern in the Mediterranean Sea, since this species produces a wide range of palytoxin-like compounds listed amongst the most potent marine toxins. This study focused on two analogues of palytoxin found in cultures of six strains of *Ostreopsis* cf. *ovata* isolated from the south of Catalonia (NW Mediterranean Sea). In addition to some already known ovatoxins, IRTA strains produced two minor compounds, ovatoxin-g and an isomer of palytoxin (putative palytoxin), whose structures had not been elucidated before. Insufficient quantity of these compounds prevented a full NMR-based structural elucidation, thus their structures were studied in collaboration with the Università degli Estudi di Napoli in crude algal extracts through Liquid Chromatography Electro Spray Ionization High Resolution Mass Spectrometry[n] (LC-ESI-HRMS[n]) in positive ion mode. Under the used MS conditions, the molecules underwent fragmentation at many sites of their backbone and a large number of diagnostic fragment ions were identified. As a result, tentative structures were assigned to both ovatoxin-g and putative palytoxin [12].

Six strains of *O.* cf. *ovata* (IRTA-SMM-11-10 from August 2011, IRTA-SMM-12-38, IRTA-SMM-12-46, IRTA-SMM-12-51, IRTA-SMM-12-57 and IRTA-SMM-12-62 from August 2012) were isolated from macroalgal samples (*Jania rubens*) collected in south Catalonia.

The analysis were performed on a hybrid linear ion trap LTQ Orbitrap XL™ Fourier transform mass spectrometer (FTMS) equipped with an ESI ION MAX™ source (Thermo Fisher) coupled to an Agilent 1100 LC. The following LC-HRMS method developed by Ciminiello et al. (2012) as described in **Deliverable 2.6.**

Six batch-cultures of *O.* cf. *ovata* strains from the Ebro River Delta were extracted and their toxin profiles characterized by LC-HRMS experiments. A newly developed method for ovatoxins (Ciminiello et al.) was used to analyze the crude extracts under very slow gradient elution. This allowed baseline chromatographic separation of all palytoxin-like compounds contained in the extracts (Figure 9). The analyses of the extracts were carried out in parallel with a palytoxin standard (from *P. tuberculosa*) and with an Adriatic *O.* cf. *ovata* extract previously characterized that was used as reference for the Mediterranean ovatoxins. All the samples were analyzed under the same experimental conditions.
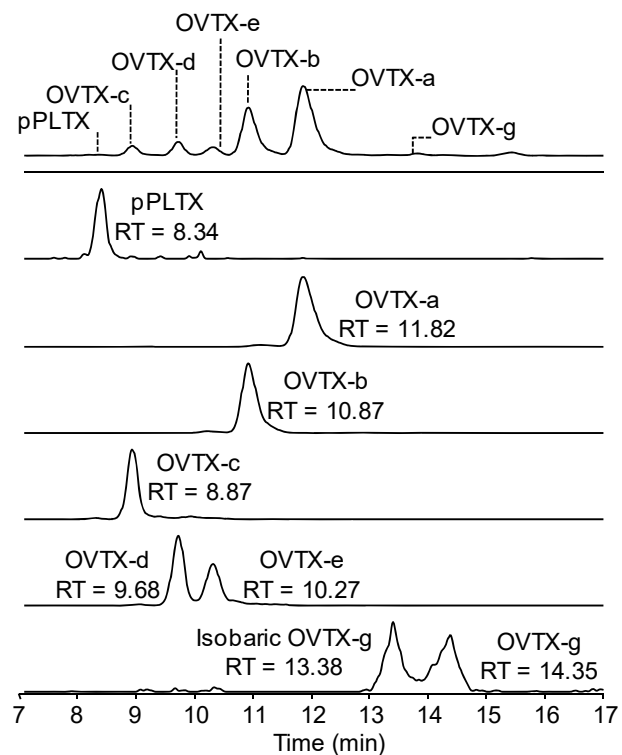
Figure 9. TIC of *O*. cf. *ovata* strain IRTA-SMM-11-10 (top trace) and XIC of the [M+H+Ca]³⁺ ions of each toxin (mass tolerance 3 ppm). Analysis were carried out in full HRMS positive ion mode in the mass range *m/z* 700–1600. Under the same experimental conditions, PLTX standard eluted at 10.72 min

Figure 9 shows the total ion chromatogram (TIC) of a representative *O.* cf. *ovata* extract of IRTA strains (IRTA-SMM-11-10) and extracted ion chromatograms (XIC) of [M+H+Ca]³⁺ ions of individual palytoxin-like compounds. Relative abundance of each toxin to the total toxin content was similar to that observed for most of the Mediterranean *O.* cf. *ovata* strains analyzed so far. In particular, in this representative sample, OVTX-a accounted for 52% of the total toxin content, followed by OVTX-b (29%), OVTX-e (7%), OVTX-d (6%), OVTX-c (4%), and pPLTX (0.5%). The six *O.* cf. *ovata* strains presented the same toxin profile and similar relative abundance, but total toxin contents were quite different ranging from 50 to 250 pg/cell (Table 13). Toxin content on a per-cell basis was in most cases higher than that reported for other Mediterranean *O.* cf. *ovata* strains (7.5 to 75 pg/cell) and quite similar to that recently

reported for a strain from the French Mediterranean coast (up to 300 pg/cell). This suggests that the intra-population variability in toxin production might be quite high, as already described for other dinoflagellates. Also further studies would be needed on the environmental and geographical factors that affect toxin production.

Table 13. Total toxin content on a per cell basis (pg/cell) and relative abundance (%) of pPLTX and OVTXs in the 6 analysed *O.* cf. *ovata* strains from south Catalonia rocky coasts (NW Mediterranean Sea).

Strain name	Total toxin content (pg/cell)	Relative abundances (%)						
		pPLTX	OVTX-a	OVTX-b	OVTX-c	OVTX-d	OVTX-e	OVTX-g
IRTA-SMM-11-10	50	0.5	52	29	4	7	6	0.7
IRTA-SMM-12-38	143	0.3	58	25	3	6	6	0.7
IRTA-SMM-12-46	140	0.6	53	26	5	8	6	0.9
IRTA-SMM-12-51	102	0.6	55	21	6	8	8	0.6
IRTA-SMM-12-57	104	0.7	55	22	6	8	7	0.6
IRTA-SMM-12-62	250	0.4	59	20	4	7	7	0.9

Putative palytoxin

The full HRMS spectra of pPLTX and PLTX appeared superimposable in terms of diagnostic ions and their relative intensities (Figure 10).
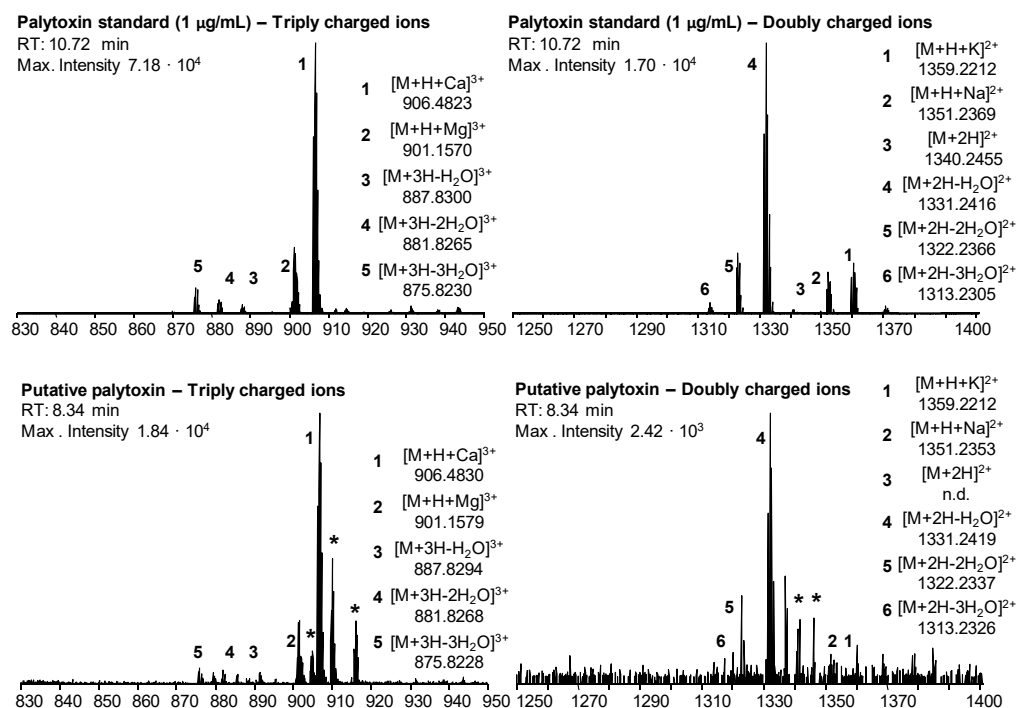
Palytoxin standard (1 μg/mL) – Triply charged ions
RT: 10.72 min
Max. Intensity 7.18 · 10^4

1 [M+H+Ca]^3+ 906.4823
2 [M+H+Mg]^3+ 901.1570
3 [M+3H-H_2O]^3+ 887.8300
4 [M+3H-2H_2O]^3+ 881.8265
5 [M+3H-3H_2O]^3+ 875.8230

Palytoxin standard (1 μg/mL) – Doubly charged ions
RT: 10.72 min
Max. Intensity 1.70 · 10^4

1 [M+H+K]^2+ 1359.2212
2 [M+H+Na]^2+ 1351.2369
3 [M+2H]^2+ 1340.2455
4 [M+2H-H_2O]^2+ 1331.2416
5 [M+2H-2H_2O]^2+ 1322.2366
6 [M+2H-3H_2O]^2+ 1313.2305

Putative palytoxin – Triply charged ions
RT: 8.34 min
Max. Intensity 1.84 · 10^4

1 [M+H+Ca]^3+ 906.4830
2 [M+H+Mg]^3+ 901.1579
3 [M+3H-H_2O]^3+ 887.8294
4 [M+3H-2H_2O]^3+ 881.8268
5 [M+3H-3H_2O]^3+ 875.8228

Putative palytoxin – Doubly charged ions
RT: 8.34 min
Max. Intensity 2.42 · 10^3

1 [M+H+K]^2+ 1359.2212
2 [M+H+Na]^2+ 1351.2353
3 [M+2H]^2+ n.d.
4 [M+2H-H_2O]^2+ 1331.2419
5 [M+2H-2H_2O]^2+ 1322.2337
6 [M+2H-3H_2O]^2+ 1313.2326

Figura 10. Full HRMS spectra of palytoxin standard and putative palytoxin (*m/z* 700–1600) zoomed in *m/z* 830–950 and *m/z* 1250–1400 ranges. Elemental formula assignment of triply- and doubly-charged ions. n.d. = not detected. * = ions corresponding to co-eluting palytoxin-like compounds, still under investigation

Ion assignment indicated that pPLTX had the same molecular formula as palytoxin ($C_{129}H_{223}N_3O_{54}$, theoretical mass *m/z* 2678.4790, RBD 20.0). Structural differences between PLTX and pPLTX have been assessed by interpretation of their full HRMS[n] spectra of the [M+H+Ca]^3+ ion at *m/z* 906.8. [13]

Ovatoxin-g

The full HRMS spectrum of ovatoxin-g (RT = 14.35 min) showed the most intense triply- and doubly-charged ions at *m/z* 890.4870 and *m/z* 1307.2486 (mono-isotopic peaks). According to the expected ionization behaviour of palytoxin-like compounds, these ions were assigned to [M+H+Ca]^3+ and [M+2H-H_2O]^2+, respectively. Positive HRMS/MS spectrum of the *m/z* 890.8 ion was acquired to gain structural insights into ovatoxin-g and identify the specific site(s) of difference between OVTX-g and -a.

Besides OVTX-g (RT 14.35 min), the extracted ion chromatogram (XIC) for the [M+H+Ca]$^{3+}$ ion at *m/z* 890.4870 displayed another peak eluting about one minute earlier (Figure 11). Its full HRMS spectrum showed a similar ion profile to OVTX-g, which suggested that it was an OVTX-g isomer. However, the fragmentation behaviour in the HRMS/MS spectrum of this compound was significantly different from that usually observed for palytoxins, thus preventing any reliable interpretation.
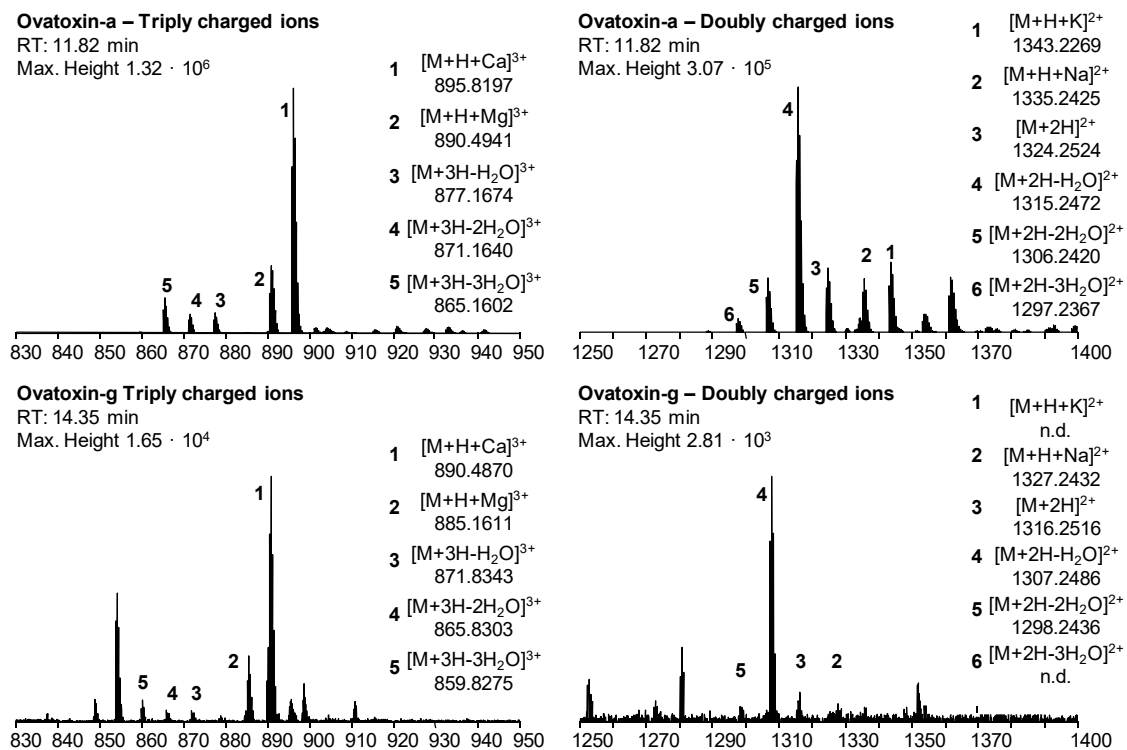

Ovatoxin-a – Triply charged ions
RT: 11.82 min
Max. Height 1.32 · 10^6

1 [M+H+Ca]$^{3+}$ 895.8197
2 [M+H+Mg]$^{3+}$ 890.4941
3 [M+3H-H$_2$O]$^{3+}$ 877.1674
4 [M+3H-2H$_2$O]$^{3+}$ 871.1640
5 [M+3H-3H$_2$O]$^{3+}$ 865.1602

Ovatoxin-a – Doubly charged ions
RT: 11.82 min
Max. Height 3.07 · 10^5

1 [M+H+K]$^{2+}$ 1343.2269
2 [M+H+Na]$^{2+}$ 1335.2425
3 [M+2H]$^{2+}$ 1324.2524
4 [M+2H-H$_2$O]$^{2+}$ 1315.2472
5 [M+2H-2H$_2$O]$^{2+}$ 1306.2420
6 [M+2H-3H$_2$O]$^{2+}$ 1297.2367

Ovatoxin-g Triply charged ions
RT: 14.35 min
Max. Height 1.65 · 10^4

1 [M+H+Ca]$^{3+}$ 890.4870
2 [M+H+Mg]$^{3+}$ 885.1611
3 [M+3H-H$_2$O]$^{3+}$ 871.8343
4 [M+3H-2H$_2$O]$^{3+}$ 865.8303
5 [M+3H-3H$_2$O]$^{3+}$ 859.8275

Ovatoxin-g – Doubly charged ions
RT: 14.35 min
Max. Height 2.81 · 10^3

1 [M+H+K]$^{2+}$ n.d.
2 [M+H+Na]$^{2+}$ 1327.2432
3 [M+2H]$^{2+}$ 1316.2516
4 [M+2H-H$_2$O]$^{2+}$ 1307.2486
5 [M+2H-2H$_2$O]$^{2+}$ 1298.2436
6 [M+2H-3H$_2$O]$^{2+}$ n.d.

Figure 11. Full HRMS spectra of ovatoxin-a and ovatoxin-g (*m/z* 700–1600) zoomed in *m/z* 830–950 and *m/z* 1250–1400 ranges. Elemental formula assignment of triply and doubly charged ions. n.d. = not detected

The LC-HRMS analyses of cultures of *O.* cf. *ovata* from the Ebro River Delta revealed the presence in the extracts of the novel ovatoxin-g.

6. Tetrodotoxin

6.1 Samples from Greece and La Reunion Island (IRTA)

A hydrophilic interaction liquid chromatography (HILIC) method coupled with tandem mass spectrometry detection has been developed at IRTA for analysis of 11 tetrodotoxin analogues. The performance of the method has been demonstrated for the analysis of positive samples of pufferfish from Greece and La Reunion Island [14].

An additional HILIC HPLC-UV method has been also developed for the preparative scale isolation and purification of different TTXs analogues present in natural samples. The pure fractions containing different TTX-analogues was used to establish cross-reactivity factors, to understand the toxicity equivalent factors (TEFs) for every analogue and for the structural characterization by mass spectrometric methods. It provided knowledge on the chemical diversity and toxicity of this group of toxins.

A preparative liquid chromatography-mass spectrometry LC was used for fractionation, and all experimental parameters are described in **Deliverable 2.6**. Tissue extracts from several specimens of silver-cheeked toadfishes (*Lagocephalus sceleratus*) were pooled, evaporated to dryness under N_2 stream and redissolved in 1 mL acetonitrile/water (90/10, v/v). The final concentrations of TTX analogues available for immunochemical studies were determined by LC-MS/MS.

At IRTA, the development of a new enzyme linked immunosorbent assay (mELISA) based on the immobilization of TTX through dithiol monolayers self-assembled on maleimide plates, which provides an ordered and oriented antigen immobilization and favours the antigen-antibody affinity interaction. The mELISA was found to have a limit of detection (LOD) of TTX of 0.23 mg/kg of puffer fish matrix. The mELISA and a surface plasmon resonance (SPR) immunosensor previously developed were employed to establish the cross-reactivity factors (CRFs) of 5,6,11-trideoxyTTX, 5,11-dideoxyTTX, norTTX-6-ol and 4,9-anhydroTTX, as well as to determine TTX equivalent contents in puffer fish samples. Correction factors (CFs) were also established to overcome the effect of the different puffer fish matrixes (intestinal tract, muscle, skin, liver and gonads) in the TTX equivalent quantification. Results obtained by both immunochemical tools were correlated (R^2=0.9777). The puffer fish samples were also analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), the corresponding CRFs were applied to the individual TTX contents. Results provided by immunochemical tools, when compared with those obtained by LC-MS/MS, showed a good degree of correlation (R^2=0.991 and 0.979 for mELISA and SPR, respectively). The mELISA has been demonstrated to be fit for the purpose for screening samples in monitoring programs and in research activities.

All experimental conditions for the analytical liquid chromatography-tandem mass spectrometry is described in **Deliverable 2.6.** Table 14 shows the TTX equivalent contents provided by mELISA, SPR immunosensor and the Mouse Bioassay (MBA), as well as LC-MS/MS quantification of the different TTXs and the total sum. Results of the MBA were not obtained within the frame of the ECsafeSEAFOOD project, and were provided by Panagiota Katikou from National Reference Laboratory for Marine biotoxins from Greece who provided the samples of Puffer fish [14].

Table 14. TTX equivalent contents (mg/kg TTX equiv.) in tissues from puffer fish 4 (a) and 2 (b) by mELISA, SPR immunosensor, LC-MS/MS and MBA.

a) Puffer fish 4

Puffer fish matrix	mELISA	mELISA CF-corrected	SPR	SPR CF-corrected	LC-MS/MS						MBA
					TTX	5,6,11-trideoxy TTX	11-nor TTX-6-ol	Σ	Σ mELISA CRF-corrected	Σ SPR CRF-corrected	
Intestinal tract	23.0	24.4	34.8	31.1	20.6	11.1	22.7	54.4	21.3	24.7	56.8
Muscle	0.7	1.1	1.5	1.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.5
Skin	1.8	2.5	5.3	3.2	1.8	3.4	2.5	7.7	1.8	2.2	2.4
Liver	5.9	11.3	31.3	16.7	8.1	11.6	13.1	32.7	8.5	10.4	16.1
Gonads	3.8	5.1	16.7	10.5	4.8	2.4	5.2	12.3	4.9	5.7	17.1

b) Puffer fish 2

Puffer fish matrix	mELISA	mELISA CF-corrected	SPR	SPR CF-corrected	LC-MS/MS				MBA
					TTX	5,6,11-trideoxy TTX	11-nor TTX-6-ol	Σ	
Intestinal tract	0.5	0.5	0.4	0.4	n.d	n.d.	n.d.	n.d.	n.d.
Muscle	0.1	0.2	0.3	0.2	n.d.	n.d.	n.d.	n.d.	n.d.
Skin	0.4	0.6	0.9	0.5	n.d.	n.d.	n.d.	n.d.	n.d.
Liver	0.3	0.5	0.4	0.2	n.d.	n.d.	n.d.	n.d.	n.d.

6.2 Samples from Spanish Coast (Catalonia and Valencia) (IRTA)

Several puffer fish (n=17) were obtained in Spain (Catalonian and Valencian coast) during 2014 and 2015, with details summarised in Table 15. They were sent to IRTA in order to carry out an evaluation of the possible risk due to the levels of tetrodotoxin in 5 different tissues (muscle, gonads, mussel, intestinal tract and skin). The extraction and analysis procedures being used are described by Reverté L. *et al.* [14].

The evaluation of the presence of tetrodotoxin and its analogues in several species (*Lagocephalus lagocephalus, Lagocephalus sceleratus* and *Sphoeroides pachygaste*r) has been carried out from 2014 and are still being performed. In summary, *Lagocephalus lagocephalus* and *Sphoeroides pachygaster* are less toxic than *Lagocephalus sceleratus*. No tetrodotoxin and its analogues were detected in 16 puffer fish from the Catalonian and Valencian coast. In addition, Lagocephalus Sceleratus found in Denia showed high concentration of tetrodotoxin and some of its analogues such as 4-epitetrodotoxin and 5,6,11-trideoxyTTX. Tetrodotoxin concentrations found in the gonads and liver are higher than those found in the mussel and skin.

Table 15. Summary of captured of Puffer fish in Catalonian and Valencia coasts

Place	Capture date	Irta's arrival	Disection date	Species	Weight(g)	Length (cm)	Fishing hour	Type of fishing	Coordinates
BLANES	13/08/2014	14/08/2014	13/10/2014	*Lagocephalus lagocephalus*	851				
HOSPITALET DE L'INFANT	28/08/2014	29/08/2014	14/10/2014	*Lagocephalus lagocephalus*	685	46		Longline fishing	41º 00,606' 0º 57,749'
ATMELLA DE MAR	03/09/2014	03/09/2014	15/10/2014	*Lagocephalus lagocephalus*	878	47		Longline fishing	LAT 40º 53,830' LONG 0º 51,046'
ATMELLA DE MAR	03/09/2014	03/09/2014	17/10/2014	*Lagocephalus lagocephalus*	899	46		Longline fishing	LAT 40º 53,830' LONG 0º 51,046'
MONTGAT	03/09/2014	09/09/2014	16/10/2014	*Lagocephalus lagocephalus*	978	40	18:00-19:00 h		
VILANOVA I LA GELTRU	ago-14	18/09/2014		*Lagocephalus lagocephalus*	605	36		Longline fishing	
VILANOVA I LA GELTRU	ago-14	18/09/2014	17/10/2014	*Lagocephalus lagocephalus*	800	40			
VILANOVA I LA GELTRU	ago-14	18/09/2014	14/10/2014	*Lagocephalus lagocephalus*	750	40			
CAMBRILS	24/09/2014	25/09/2014	16/10/2014	*Lagocephalus lagocephalus*	1450	51	10:00 h. Aprox.	Trolling	41º 01 197' N 001º 02 151' E

CASTELLÓ	24/09/2014	26/09/2014	23/10/2014	*Lagocephalus lagocephalus*	970	46			
CASTELLÓ	24/09/2014	26/09/2014	13/10/2014	*Lagocephalus lagocephalus*	970	46			
ALACANT (Canal Dènia-Eivissa)	01/10/2014	(Santa Pola)	05/11/2014	*Sphoeroides pachygaster*					
ALACANT (Canal Dènia-Eivissa)	31/07/2014	(Santa Pola)	05/11/2014	*Lagocephalus sceleratus*					
ALACANT	ago-14	(Santa Pola)	05/11/2014	*Lagocephalus lagocephalus*	625	45			
BLANES	dic-14	22/12/2014	19/08/2015	*Lagocephalus lagocephalus*	560				
LLANÇÀ	27/01/2015	05/02/2015	02/02/2015	*Sphoeroides pachygaster*	1036	38			
ALTAFULLA	30/08/2015	10/09/2015	16/09/2015	*Lagocephalus lagocephalus*	890	46		Trolling (*20 m depth*)	In front of Torredembarra

7. Ciguatoxins

Ciguatoxins and palytoxins are emerging toxins that are both produced by benthic dinoflagellates. To assess the potential risk these toxins may represent in seafood it is important to identify hot spots where the toxin producing microalgae are present. Once these sites are identified, microalgae will be isolated and cultured in order to evaluate their toxic potential and toxin production. In the Mediterranean palytoxin producing dinoflagellates (*Ostreopsis* spp.) have been identified and palytoxins have been identified in seafood, while potential ciguatoxin producers (*Gambierdiscus* spp.) have been identified but there is no evidence of ciguatoxin in seafood. In Canary Islands and Madeira, ciguatoxins have been identified in *Seriola*, and evidence is required to demonstrate whether these toxins are present in other fish species. In tropical areas such as La Reunion, both palytoxins and ciguatoxins have been identified in fish and the presence of the toxin producing dinoflagellates has also been demonstrated.

Table 16. Field sampling was focus on the following geographical areas:

LOCATION	SPECIES OF MICROALGAE (n=5 strains per species and location)	SPECIES OF FISH (n=10 per species and location)	ECHINODERMS (n=30 per species and location)	PARTNER
La Réunion	*Gambierdiscus* spp and *Ostreopsis* spp	Lutjanus, Groupers, Caranx and others	Sea Urchins	ARVAM
Canary Islands	*Gambierdiscus* spp and *Ostreopsis* spp	Seriola and others		IRTA
Madeira		Seriola and others		IPMA/IRTA
Crete	*Gambierdiscus* spp and *Ostreopsis* spp	Others		IRTA (Collaboration with external partner)
Ebro Delta	*Ostreopsis* spp		Sea Urchins	IRTA

7.1 Ciguatoxins at IRTA

Assessment of the LC-MS/MS (triple quadrupole) analysis for ciguatoxins and palytoxins has been accomplished with a 3200 Qtrap (AB/Sciex). The results showed insufficient sensitivity for application to naturally contaminated seafood (LOQ for ciguatoxin-1B (CTX-1B or P-CTX-1) = 1 ng/mL; applicability of this instrumentation will be limited to microalgal cultures.

7.2 Ciguatoxins in lionfish at IRTA

Confirmatory analysis of CTXs in lionfish extracts were performed by LC-MS/MS, as described in ***Deliverable 2.6***. To achieve higher sensitivity for LC-MS/MS analyses, additional SPE clean-up of sample extracts was needed. C-CTX-1 was structurally confirmed in these samples by comparison of retention time and ion transitions relative to the C-CTX-1 reference standard (Figure 12). These analyses were carried out at FDA in USA [15].
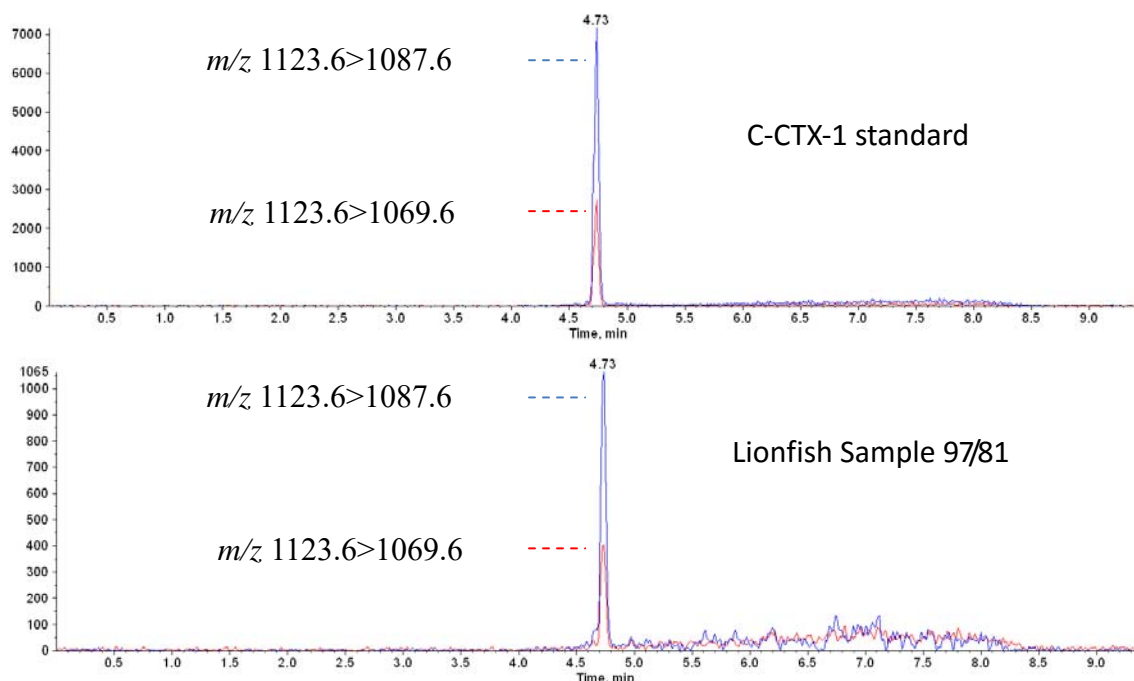
Figure 12. Extracted ion chromatogram of C-CTX-1 standard (top) and a representative lionfish sample 97/81 (bottom), showing two precursor/product ion transitions.

7.3 Risk characterization of ciguatoxins at IRTA

From 2010–2012, 35 ciguatera fish poisoning (CFP) events involving 87 individuals who had consumed locally-caught fish were reported in Guadeloupe (French West Indies). For 12 of these events, the presence of ciguatoxins (CTXs) was indicated in meal remnants and in uncooked fish by the mouse bioassay (MBA). Caribbean ciguatoxins (C-CTXs) were confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Using a cell-based assay (CBA), and the only available standard Pacific ciguatoxin-1 (P-CTX-1), the lowest toxin level detected in fish samples causing CFP was 0.022 μg P-CTX-1 equivalents (eq.)·kg^{-1} fish. Epidemiological and consumption data were compiled for most individuals afflicted, and complete data for establishing the lowest observable adverse effect level (LOAEL) were obtained from 8 CFP events involving 21 individuals. Based on toxin intakes, the LOAEL was estimated at 4.2 ng P-CTX-1 eq./individual, corresponding to 48.4 pg P-CTX-1 eq·kg^{-1}.body weight (bw). Although based on limited data, these results are consistent with the conclusions of the European Food Safety Agency (EFSA) opinion, which indicates that a level of 0.01 μg P-CTX-1 eq./kg in fish, regardless of source, should not exert effects in sensitive individuals when consuming a single meal. The calculated LOAEL is also consistent with the U.S. Food and Drug Administration guidance levels for CTXs in fish (0.1 μg C-CTX-1 eq./kg and 0.01 μg P-CTX-1 eq./kg).

Samples of uncooked fish and meal remnants were frozen and sent for analysis to the French National Reference Laboratory for marine biotoxins (ANSES, Maisons-Alfort, France). Eleven samples

were associated with fish consumption whereas one (event 1) was not a consumed fish, but a fish of the same species caught at the same time. Four out of the eleven samples (events 2–5) were uncooked fish portions from meals implicated in illness. The other samples (events 6–12) were collected as meal remnants. Estimation of their toxicity levels was determined by mouse bioassay MBA and when sufficient quantity of sample remained, aliquots were forwarded to IRTA, Sant Carles de la Ràpita, Spain, for the estimation of CTX content by the Neuro-2A cell-based assay (Neuro-2A CBA). CTX confirmation was performed by LC-MS/MS at FDA's Gulf Coast Seafood Laboratory, Alabama, USA.

All sample purification and LC-MS/MS conditions for the analysis carried out by FDA is described in ***Deliverable 2.6***. section 6.3.

The LOAEL is the lowest observable adverse effect level, which corresponds to the lowest toxin intake that exhibits symptoms in human. Toxin intakes were obtained by multiplying the toxin contents as determined by the Neuro-2A CBA by the estimated weight of the ingested fish portion*.* The LOAEL can be expressed in quantity of toxin per person or, if the weight of the person is known, it can be expressed in quantity of toxin per kg of body weight. The LOAEL was deduced from 21 people and expressed in pg P-CTX-1 eq. per person. For the 17 individuals for whom the body weight was available, the LOAEL was expressed in pg/kg of body weight of P-CTX-1 eq. [16].

Where data for fish portion ingested and CTX contents were available, the toxin intakes that triggered CFP symptoms were calculated. This information was available for 21 individuals involved in events 1, 3, 5, 6–7, and 9–11. Among them, body weight was available for 17 people. These data allowed the derivation of a LOAEL expressed either as per i*ndividual* or per *kg bw*.

The toxin intakes ranged from 4.2 to 70.6 ng P-CTX-1 eq./individual. In terms of consumption expressed per body weight, intakes ranged approximately ten-fold from 48.4 to 429.4 pg P-CTX-1 eq./kg bw. Figure 13 illustrates toxin intakes expressed in pg P-CTX-1- eq/kg bw and ranked in ascending order. The LOAEL of 48.4 pg P-CTX-1 eq./kg[1] bw was determined from a 52 years old male of 87 kg having consumed a 100g portion of contaminated snapper containing 42.1 ng P-CTX-1 eq./kg of fish.
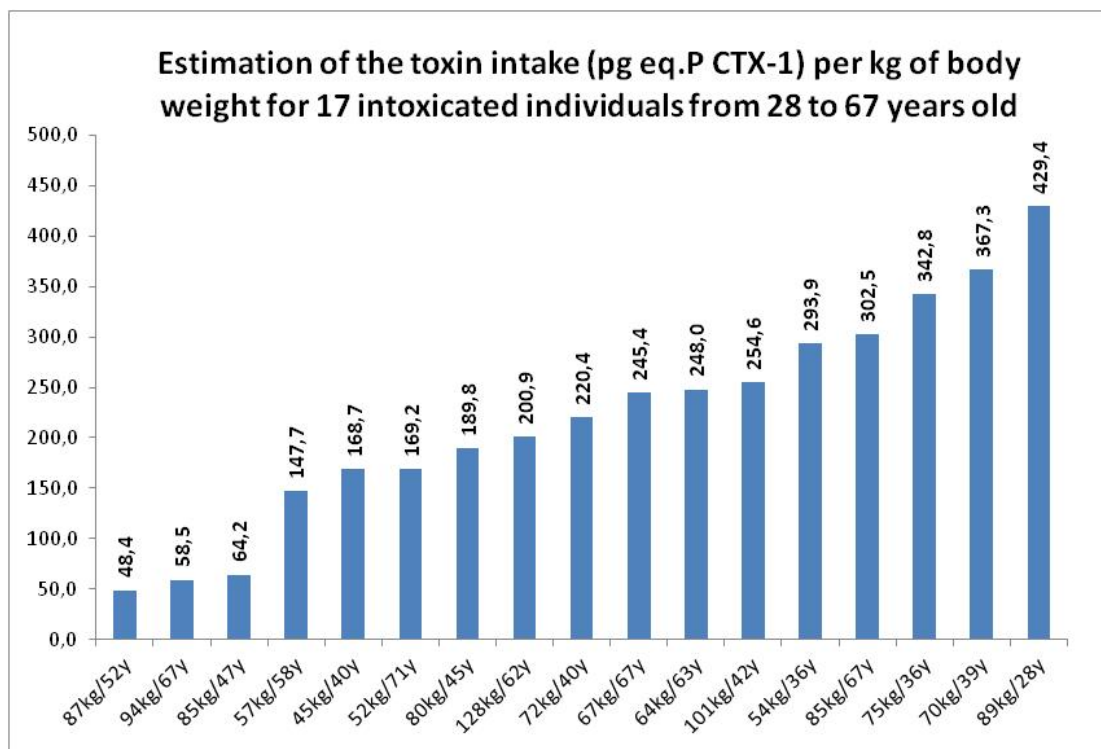
Figure 13: Toxin intakes in a series of CFP events in Guadeloupe, 2010–2012, and the estimation of the LOAEL. Toxin intakes are ranked from lowest to highest estimated values expressed in pg/kg bw P-CTX-1 eq. from the 17 individual cases fully documented.

In addition, LC-MS/MS confirmation of C-CTXs was performed and C-CTX-1 was structurally confirmed in two samples by comparison of retention time and ion transitions relative to the C-CTX-1 reference standard. Experimental conditions are described in Hossen et al.[13].

7.4 Ciguatoxins in shark at IRTA

A shark sample was provided by "Hydrô Réunion" (Formerly ARDA and ARVAM), and consists of an oesophagus sample of shark from Madagascar (Bull shark, *Carcharhinus leucas*). This sample gave positive results at IRTA according to the neuro-2a assay [17]. This sample was also screened for the presence of ciguatoxins with a previously developed triple quadrupole LC-MS/MS method[18]. Results showed tentative identification of Indian ciguatoxins (I-CTXs) based upon three precursor-product multiple reaction monitoring transitions for each toxin. Information on the molecular weight of I-CTXs was based on previous reports [19,20], see Figure 14. Confirmation of I-CTXs could not be performed due to the lack of analytical standards.

Even though I-CTX-1 and -2, and C-CTX-1 and -2, respectively, are not distinguishable by molecular weight, the geographic origin of the sample (Madagascar) suggests that the tentative identification as I-CTXs as the most probable (previous reports of I-CTXs were obtained from samples caught in Mauritius islands). Accurate quantification could not be performed due to lack of standards and sample complexity. Compared to analytical calibration curves obtained for P-CTX-1, P-CTX-2 and P-CTX-3, the sample contains ca. 31–61 µg/kg. Recovery studies, matrix effects assessment, and

toxicity equivalence factors are needed. Complementary analysis with HR-LCMS is ongoing and results are expected to be obtained before june 2016.
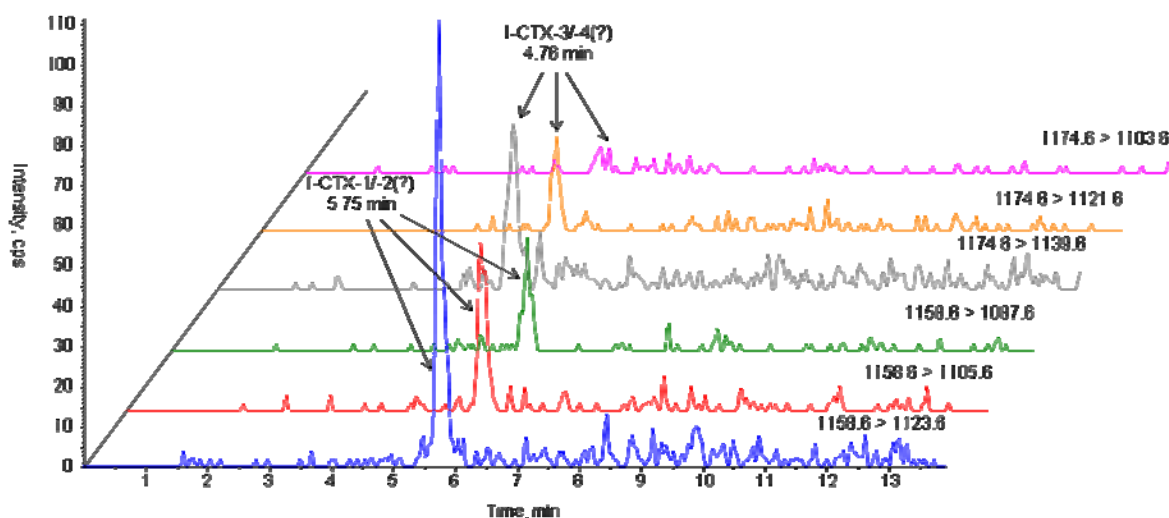

Figure 14. XIC of +MRM (6 pairs): m/z 1158.6/1123.6 from Sample 59 (Sample A565_MRM) (Turbo Spray).

7.5 Ciguatoxins in fish from Madeira at IRTA

At IRTA, 41 fish samples from Madeira belonging to different species were sent to IRTA by a collaborator of the ECsafeSEAFOOD project in 2 rounds. All muscle samples were extracted and CTX content was assessed by the Neuro-2a CBA for the fish of the first round. According to the cytotoxicity of the extracts on the Neuro-2a cells, none of the samples presented CTX-like activity when compared to the P-CTX-1B standard (Table 17). Muscle extracts of the second round will be analysed by Neuro-2a CBA shortly and results will be included in the corresponding 2 month-report expected in June 2016.

Table 17. Summary of fish samples from Madeira: code, origin, sampling date and CTX content determined by Neuro-2a CBA.

IRTA's code	Sample's code	Sampling date	Origin	Extract done	Neuro-2a CBA done	CTX-like activity
1	Badejo	25/09/2014	Desertas	YES	NO	Ongoing
2	Taínha	25/09/2014	Desertas	YES	NO	Ongoing
3	Bodião	24/05/2014	Enseada das Galinhas (Selvagem)	YES	NO	Ongoing
4	Garoupa	24/05/2014	Quebradas (Selvagem)	YES	NO	Ongoing
5	Perguiçosa 1	24/05/2014	Quebradas (Selvagem)	YES	NO	Ongoing
6	Peixe-cão	24/05/2014	Quebradas (Selvagem)	YES	NO	Ongoing
7	Charuteiro 1.1	24/05/2014	Enseada das Galinhas (Selvagem)	YES	NO	Ongoing
8	Charuteiro 1.2	24/05/2014	Enseada das Galinhas (Selvagem)	YES	NO	Ongoing
9	Charuteiro 1.3	24/05/2014	Enseada das Galinhas (Selvagem)	YES	NO	Ongoing
10	Charuteiro 2.1	25/05/2014	Enseada das Cagarras (Selvagem)	YES	NO	Ongoing
11	Charuteiro 2.2	25/05/2014	Enseada das Cagarras (Selvagem)	YES	NO	Ongoing
12	Tainha	25/05/2014	Enseada das Cagarras (Selvagem)	YES	NO	Ongoing
13	Perguiçosa 2	25/05/2014	Enseada das Cagarras (Selvagem)	YES	NO	Ongoing
14	Peixe-porco	25/05/2014	Enseada das Cagarras (Selvagem)	YES	NO	Ongoing
15	Salema 1	25/05/2014	Enseada das Cagarras (Selvagem)	YES	NO	Ongoing
16	Salema 2	25/05/2014	Enseada das Cagarras (Selvagem)	YES	NO	Ongoing
17	Seriola Canhiçal	15/10/2013		YES	NO	Ongoing
18	Lapa branca 1	13/12/2014	Reies Magos (Madeira)	YES	NO	Ongoing
19	Lapa preta 2	13/12/2014	Reies Magos (Madeira)	YES	NO	Ongoing
20	Lapa branca 1	12/06/2014	Selvagem Pequena	YES	NO	Ongoing
21	Patella candei candei (ex: grandes)	12/06/2014	Selvagem Pequena	YES	NO	Ongoing
22	Patella candei candei (ex: pequenos)	12/06/2014	Selvagem Pequena	YES	NO	Ongoing
23	Charéu 1	16/01/2014	Baía d'Abra	YES	YES	NEGATIVE
24	Charéu 2	16/01/2014	Baía d'Abra	YES	YES	NEGATIVE
25	Moreão 1	31/12/2013	Selvagen Grande	YES	YES	NEGATIVE
26	Moreão 2	31/12/2013	Selvagen Grande	YES	YES	NEGATIVE
27	Moreão 3	31/12/2013	Selvagen Grande	YES	YES	NEGATIVE
28	Moreão 4	31/12/2013	Selvagen Grande	YES	YES	NEGATIVE
29	Tainha	27/12/2013	Desertas	YES	YES	NEGATIVE
30	Sargo	27/12/2013	Desertas	YES	YES	NEGATIVE
31	Bodião	27/12/2013	Desertas	YES	YES	NEGATIVE
32	Garoupa	27/12/2013	Desertas	YES	YES	NEGATIVE
33	Pargo	27/12/2013	Desertas	YES	YES	NEGATIVE
34	Preta 3	08/11/2013	Desertas Grande Doca	YES	YES	NEGATIVE
35	Preta 6	08/11/2013	Desertas Grande Doca	YES	YES	NEGATIVE
36	Pintada 1	08/11/2013	Desertas Grande Doca	YES	YES	NEGATIVE
37	Pintada 2	08/11/2013	Desertas Grande Doca	YES	YES	NEGATIVE
38	Pintada 4	08/11/2013	Desertas Grande Doca	YES	YES	NEGATIVE
39	Pintada 5	08/11/2013	Desertas Grande Doca	YES	YES	NEGATIVE
40	Lapa preta	27/12/2013	Desertas Grande	YES	NO	ongoing
41	Lapa preta	15/01/2014		YES	NO	ongoing

Conclusions

NVI, IRTA and Hydro Réunion have contributed to the detection and quantification of emerging biotoxins in seafood in matrices of different natures. Collaboration for sampling has also been obtained from other participants of the project and additional institutions.

At IRTA, cyclic imines have been included in the shellfish safety monitoring programs of lipophilic marine toxins by LC-MS methods, even though they are not regulated. With these data their presence in shellfish can be assessed better and it allows exposure evaluations that would enable a reliable risk analysis for consumers.

Moreover, IRTA has been able to report for the first time the presence of acyl ester derivatives of GYMs in shellfish. The identity of the esters was confirmed through product ion spectra, accurate mass analyses, and by partial synthesis of the 16:0 ester of gymnodimine. The semi-synthetic ester was used to calibrate LC-MS/MS SRM experiments on the esters naturally present in the clam sample, determining that the majority of gymnodimine (>90% of the total) was present in acylated form. Evidence for the acyl esters of gymnodimine B and/or gymnodimine C was also obtained. Due to the significant abundance of these new derivatives, their toxicity needs to be assessed. This discovery highlights the necessity of determining completely toxin profiles, including metabolites such as acyl esters, for a comprehensive risk assessment.

This discovery highlights the necessity of determining complete toxin profiles, including metabolites such as acyl esters, for a comprehensive risk assessment. Characterization of metabolites in shellfish is also important in the study of detoxification kinetics of toxins after a bloom of harmful algae. Similar conclusions can be drawn from the complexity of the azaspiracid metabolite profile in shellfish and crabs that was revealed by the newly developed analytical methods.

At IRTA PnTX-G and SPX-1 were confirmed unequivocally and quantified in shellfish and passive samples (SPATT devices) from Catalonia, Spain. This is the first report of pinnatoxins in Spain and the first time that spirolides have been detected in Catalonia. A comprehensive strategy to avoid false positives of toxin analogues of PnTXs and SPXs by LC-MS/MS analysis was developed and discussed, with applicability to other toxin groups and analogues. Evidence of acyl ester metabolites of PnTXs could not be obtained from mussel samples, neither after application of the alkaline hydrolysis procedure nor through precursor ion scan mass spectrometric experiments. Cylic imines should be included in the shellfish safety monitoring programs of lipophilic marine toxins by LC-MS methods, even if they are not regulated, to better assess their presence in shellfish and to permit exposure evaluations that would enable a reliable risk analysis for consumers.

Cultures of *O. cf. ovata* from Ebro River Delta allowed to highlight the presence in the extracts of the novel ovatoxin-g by LC-HRMS. LC-HRMS studies provided insights into the structure of this new ovatoxin in addition to disclosing that the so far referred to as putative palytoxin is indeed a structural isomer of palytoxin itself. Compared to palytoxin, the putative palytoxin from the analyzed *O.* cf. *ovata* strains is hydroxylated at C-42 and dehydroxylated at C-17 and most likely at C-64 (like OVTX-a). In addition, an-extra oxygen is contained in the segment stretching from the A-side terminal to C-8. Compared to ovatoxin-a, ovatoxin-g is dehydroxylated at C-46. Both the putative

PLTX and OVTX-g were contained in algal extracts at levels too low for a full NMR-based study, and LC-HRMS structural investigation was performed directly in crude extract at ng per ml levels, with no need for derivatization. The ever-growing number of palytoxins being discovered makes even more urgent the need of evaluating the toxicity on humans of this class of compounds. The study of mechanisms of action and potency of individual palytoxin-like compounds should be performed to ensure a correct risk assessment of *Ostreopsis*-related syndromes.

Tetrodotoxin is an emerging problem in the Mediterranean sea due to the enlarged presence of puffer fish in the Mediterranean coasts. A new ELISA (with the corresponding study of the matrix effect and the establishment of CFs) was shown to be useful as a TTX screening method in the analysis of puffer fish (by comparison with LC-MS/MS, which requires the establishment of CRFs). The application of the CFs to the quantification of TTXs in puffer fish by mELISA and SPR immunosensor, together with the comparison of these results with those provided by LC-MS/MS analysis and the corresponding application of the established CRFs, allowed for a reliable determination of content of TTXs in puffer fish samples. In addition, this study provided evidence that, when analyzing naturally contaminated samples, complementarity between techniques providing different levels of information is very useful, especially for toxins which lack an established official method and/or regulatory legislation. The immunological tools developed in this work are suitable for screening purposes, being able to analyze a number of samples in a short time and meet the requirements in terms of sensitivity of the Japanese regulation levels. Moreover, they have been shown to be capable of detecting amounts of TTXs as low as 230 µg/kg, which makes them highly appropriate for research studies. Additionally, the evaluation of the toxicity of TTX analogues and the comparison of the toxicity degrees with the CRFs will contribute to confidence in food safety assessments in order to protect human health. In addition, IRTA is working on a risk assessment on the different species of puffer fish that have arrived at the Catalonian and Valencian coasts during these last years (2014 and 2015). No tetrodotoxin and its analogues were detected in 16 puffer fish corresponding to *Lagocephalus lagocephalus* and *Sphoeroides pachygaster*. In addition, *Lagocephalus Sceleratus* found in Denia showed high concentration of tetrodotoxin and some of its analogues such as 4-epitetrodotoxin and 5,6,11-trideoxyTTX. Tetrodotoxin concentrations found in the gonads and liver are higher than those found in the mussel and skin.

The presence of C-CTX-1 in Lionfish from Saint Barthélemy by LC-MS/MS was confirmed. Any CTX-like activity was found in lionfish from Guadeloupe or Saint Martin. This information will help to ensure that there is sufficient awareness of the regional CFP risk that these species may pose to the public. Given that a reference method is not yet established in the EU for the monitoring of ciguatoxic fish??, analytical confirmation by LC-MS/MS paired to previous screening of samples with the use of the neuroblastoma N2a assay is recommended. This approached was successfully applied to identify CTXs in fish responsible for human poisoning and to define the LOAEL for CTXs, and contribute to risk characterization. CTXs in the bull shark have been confirmed.

A number of new carboxylated azaspiracid metabolites were identified in Norwegian crabs, and these have subsequently been confirmed as being present in Irish blue mussels by a collaborator (J. Kilcoyne, Marine Institute, Galway, Ireland). The approach used in this work is applicable to identify other types of azaspiracids in marine organisms.

The introduction of bench-top high resolution mass spectrometry instruments in research and control laboratories may enhance the capabilities to ensure unequivocal confirmation of emerging marine toxins. However, a proper strategy should be applied combining isotopic patterns, MS and MS^2 accurate mass measurements, signal thresholds, and tolerances applied to mass filters in order to avoid potential false positives or negatives.

The LC–MS methodology developed at NVI for the Q Exactive appears to be more sensitive than the previous generation of triple quadrupole instruments and has a number of benefits over traditional LC–MS/MS SRM/MRM-type methods. These include the high resolution mass measurements, allowing greater certainty in identification; the ability to look retrospectively at previously acquired data for peaks corresponding to new or hypothetical analogues; high sensitivity; AIF spectra for identifying peaks from known and unknown analogues giving rise to specific product ions; and the flexibility to run a variety MS analyses (e.g. FS, AIF, targeted MS/MS etc.) simultaneously at high mass resolution. The LC–MS approach described here has already proven itself capable of sensitive analysis for a range of known toxin analogues, as well as identifying previously unknown analogues of these toxin groups.

In conclusion, as a result of tight collaborations and intensive work, ECsafeSEAFOOD has contributed significantly to:
- the identification of new emerging toxins
- the identification of toxins in seafood for the first time
- the risk assessment of toxins in specific geographical areas
- the establishment of the NOAEL of toxins in order to better characterize risks
- evaluate presence of emerging toxins in market samples that will help to evaluate risks taking into consideration consumption data (WP3)
- the characterization of toxins in invasive species of fish
- the development of methods for the evaluation of emerging toxins reported in the previous deliverable D2.6.

All this work achieved within the ECsafeSEAFOOD project has resulted, to date, in the publication of 9 papers in peer-reviewed scientific journals. This contribution is significant, but due to the extensive list of emerging toxins and analogues and to the diversity of seafood in different geographical areas, further studies on emerging marine toxins in seafood should be pursued.

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