Effect-based *in vitro* bioassays for lipophilic marine biotoxins: a new strategy to replace the mouse bioassay

Marcia Bodero

Propositions

1. The neuro-2a bioassay is suited as an alternative animal-free method for the broad detection of lipophilic marine biotoxins in shellfish.
(this thesis)

2. Gene expression analyses in Caco-2 cells leads the way to better understand the mode of action of diarrheic shellfish poisons.
(this thesis)

3. Antibiotic resistance is more an ethical problem than a medical one.

4. Gut microbiome studies are changing the paradigm of "we are what we eat" to "we are what they eat".

5. Serving a 'meatless Monday' meal in a single use plastic container in the canteen of Wageningen University does not match with the mission of improving quality of life.

6. Living as a couple and working in science have in common that you need to know how to deal with the uncertainties.

7. To do science you have to be idealistic, but to write the thesis you have to be realistic.

Propositions belonging to the thesis entitled

"Effect-based *in vitro* bioassays for lipophilic marine biotoxins combined with mass spectrometric analysis: a new strategy to replace the mouse bioassay".

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Wageningen, 11 January 2019.

Effect-based in vitro bioassays for lipophilic marine biotoxins: a new strategy to replace the mouse bioassay

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Marcia Bodero

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 11 January 2019
at 1.30 p.m. in the Aula.

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List of abbreviations

µL	microliter
µM	micromolar
ADL	arbitrary decision limit
ASP	amnesic shellfish poisoning
CFP	ciguatera shellfish poisoning
CPDB	consensus path DB
CTX	ciguatoxin
DA	domoic acid
DMSO	dimethilsulfoxide
DSP	diarrhoeic shellfish poisoning
DTX	dinophysistoxin
EC50	effect concentration 50
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
EU	European Union
EU-RL	European Union Reference Laboratory
FAO	Food and Agriculture Organization
GC-MS	gas chromatography mass spectrometry
GSEA	gene set enrichment analysis
h	hour
hYTX	1a homoyessotoxin
HAB	harmful algal bloom
HPLC-UV	high performance liquid chromatography with ultraviolet detection
HRMS	high resolution mass spectrometry
IC50	inhibitory concentration 50
K+	potassium ions
LC	liquid chromatography
LC-FLD	liquid chromatography with fluorescence detection
LC-MS	liquid chromatography with mass spectrometry detection
LDH	lactate dehydrogenase
LMB	lipophilic marine biotoxin
LOD	limit of detection
MBA	mouse bioassay
MEA	multi electrode array
MFI	median fluorescence intensity
mg	milligram
min	minute
mL	millilitre

mM	millimolar
MPL	maximum permitted limit
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MU	mouse unit
Na+	sodium ions
nm	nanometre
nM	nanomolar
NMDA	N-methyl-D-aspartate
NSP	neurologic shellfish poisoning
O/V	ouabain/veratridine
OA	okadaic acid
PBS	phosphate-buffered saline
PbTX	brevetoxin
PlTX	palytoxin
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PSP	paralytic shellfish poisoning
PTX	pectenotoxin
qRT-PCR	quantitative real-time polymerase chain reaction
RBA	rat bioassay
RIA	radioimmunoassay
RL	regulatory limit
RPM	revolution per minute
SPE	solid phase extraction
SPX	spirolide
STX	saxitoxin
Tof/MS	time of flight mass spectrometry
TTX	tetrodotoxin
UPLC	ultra performance liquid chromatography
VGSC	voltage-gated sodium channels
YTX	yessotoxin

Chapter 1

General introduction

1. Background information

1.1 Harmful algal blooms and marine biotoxins

Marine biotoxins are a large group of compounds produced by several organisms in the marine environment, e.g. algae, corals and cyanobacteria, that can accumulate in filtrating shellfish and finfish [1, 2]. Human consumption of marine products such as molluscs (clams, razor clams, mussels, oysters), crustaceans (lobsters, crabs) and fish (sardines, barracuda, snappers, puffer fish, among others) contaminated with these toxins can lead to food poisoning, of which symptoms can vary from paralysis, diarrhoea, amnesia to death, depending on the ingested toxin [3-5]. Worldwide, algae toxins are responsible for approximately 60,000 human intoxications annually [6]. Monitoring the micro-organisms responsible for the production of marine biotoxins is of value, but the occurrence of these micro-organisms does not always correlate well with the presence of toxins in seafood samples. For instance, saxitoxins (STXs) are toxins produced by dinoflagellates from *Alexandrium spp.* e.g. *A. catenella* and *A. ostendelfii* [7], domoic acid (DA) by species of *Pseudonitzschia* and okadaic acid (OA) and dinophysistoxins (DTXs) are mainly produced by *Dinophysis fortii, D. acuminata, D. acuta, D. caudata, D. sacculus* and *D. norvegica*; while in cases where these algae were detected, often no toxins were detectable in shellfish [8]. The opposite can also occur, i.e. DA, OA and DTX were found in shellfish, while no toxin producing algae were detected [9-13]. The yessotoxins (YTXs) are mainly produced by *Proceratium reticulatum, Lingulodinium polyedrum, Gonyaulax polyhedra* and *Gonyaulax spinifera*, but there are examples showing that while the algae are gone, the YTX levels exceed the permitted levels [14, 15]. Regarding the azaspiracids (AZAs), the toxin producing *Azadinium spinosum* is only known since 2009 and till now there are no data available on the correlation between its occurrence and the detection of AZAs in shellfish [16]. The correlation is thus complex and similar is valid for other marine biotoxins. However, it should also be noticed that in Florida the monitoring of *Karenia brevis* in order to prevent intoxications by brevotoxins (PbTXs), seems to work adequately [17]. Since it is not possible to avoid the uptake of toxins from shellfish and the lack on an efficient method to remove them before consumption, it is necessary to regulate and monitor levels in shellfish [18].

Marine biotoxins can be classified based on their chemical structure, chemical properties or the effects produced in humans. From an analytical point of view, marine biotoxins can be separated in two groups based on chemical properties, i.e. hydrophilic and lipophilic toxins.

1.2 Hydrophilic toxins

The more water soluble hydrophilic toxins have low molecular weight including domoic acid (DA), saxitoxin (STX) and tetrodotoxin (TTX) [19]. Table 1 summarises the hydrophilic toxins and some of their characteristics including their mode of action.

Table 1. Hydrophilic toxins

Toxin	Chemical structure	Source	Syndrome	Mode of action	Ref.
Saxitoxins (STX)	Purine derived	*Alexandrium* spp, *Gymnodinium and Pyrodinium* spp.	Paralytic shellfish poisoning (PSP)	Inhibition of the voltage-gated sodium channels	[7]
Tetrodo-toxins (TTX)	Heterocyclic Guanidine derived	*Vibrio* spp, *Pseudomonas* spp, *Shewanella* spp, *Alteromonas tetraodontis*	Tetrodo-toxin poisoning	Inhibition of the voltage-gated sodium channels	[20-22]
Domoic acid (DA)	Cyclic amino acid	*Pseudo-nitzschia pungens,*	Amnesic shellfish poisoning (ASP)	Activation of glutamate receptors	[23, 24]
Palytoxins (PlTX)	Complex polyhydroxylated compounds	*Osteoptisis* spp, *Palythoa* spp	Palytoxin poisoning	Modification of NA^+/K^+ ATPase ion pump	[25, 26]

1.3 Lipophilic toxins

The lipophilic toxins are high molecular weight compounds including a variety of non-related toxins, e.g. OA, AZAs and PbTX. OA and its derivatives DTXs are known for causing diarrhoeic shellfish poisoning (DSP) [27]. Other toxins such as YTXs, pectenotoxins (PTXs) and cyclic imines (CIs) do not cause any known syndrome. In addition, other toxins like pinnatoxins (PnTX), gymnodimines (GYM) and pteriatoxins (PtTX) will be present in the lipophilic extract as well [2, 19, 28, 29]. A summary of the lipophilic toxins and some of their characteristic including the mode of action is shown in table 2.

1.3 Lipophilic toxins

Table 2. Lipophilic toxins

Toxin	Chemical structure	Source	Syndrome	Mode of action	Ref.
Okadaic acid (OA), dinophysistoxins (DTXs), pectenotoxins (PTXs)	Polyether	*Dynophisis* spp, *Prorocentrum* spp.	Diarrhoeic shellfish poisoning (DSP)	Inhibition of serine/ threonine phosphatases (OA and DTXs) PTX not known	[28, 30, 31]
Azaspiracids (AZAs)	Polyether	*Azadinium* spp, *Amphidoma* spp.	Azaspiracid poisoning (AZP)	Unknown, *in vitro* multiple cellular effects	[32-34]
Yessotoxins (YTXs)	Polyether	*Protoceratium reticulatum, Lingulodinium polyedrum* and *Gonyaulax spinifera*	No record of human intoxication	Unknown, *in vitro* multiple cellular effects, cardiotoxic	[35, 36]
Brevetoxins (PbTXs)	Cyclic polyether	*Karenia brevis*	Neurologic shellfish poisoning (NSP)	Activation of the voltage-gated sodium channels	[17, 37]
Ciguatoxins (CTX)	Cyclic polyether	*Gambierdiscus toxicus*	Ciguatera poisoning[1]	Activation of the voltage-gated sodium channels	[38, 39]
Other toxins: Cyclic imines Spirolide (SPX), Pinnatoxins (PnTXs), Gymnodimines (GYMs), Pteriatoxins (PtTXs)	Cyclic imines	Various species, *Alexandrium* spp, *Karenia* sp.	No record of human intoxication	Still unknown, probably interaction with nicotinic and muscarinic acetylcholine receptors	[39, 40]

[1] By consumption of contaminated fish

2. Types of poisoning

2.1 Paralytic shellfish poisoning (PSP)

Saxitoxin (STXs) and several closely related analogues form the group of toxins causing paralytic shellfish poisoning (PSP) [2]. They are produced by marine algae from the genus *Alexandrium* spp, *Gymnodinium* spp. and *Pyrodinium* spp. In the case of fresh water, these toxins are produced by some types of cyanobacteria from genus *Anabaena, Cylindrospermopsis, Aphanizomenon, Planktothrix* and *Lyngbya* [7, 41, 42].

STX is a hydrophilic toxin that can easily be extracted by water [43]. A total of 57 analogues of STX have been reported [7, 44, 45]. STX causes inhibition of voltage-gated sodium channels (VGSC) [43, 46]. This channel is responsible for the initial depolarization phase in neurons and other electrically excitable cells, such as cardiac cells or skeletal muscle cells, and its inhibition leads to paralysis in humans and other animals [41]. The symptoms can vary from slight numbness in the lips, mouth and tongue, numbness of extremities, headache, dizziness, vomiting, nausea and diarrhoea, to death due to paralysis of muscles involved in respiratory processes and death comes by asphyxia [18, 42]. Death can occur within 2 to 12 hours after ingestion of contaminated shellfish. Victims that survive more than 12 hours might recover. There is no antidote to STX intoxication and the only treatment consists on giving life support to the patient [43, 46].

|	R_1	R_2	R_3	R_4
STX	H	H	H	
GTX-2	H	H	OSO_3^-	
GTX-3	H	OSO_3^-	H	
Neo	OH	H	H	
GTX-1	OH	OH	OSO_3^-	
GTX-4	OH	OSO_3^-	H	
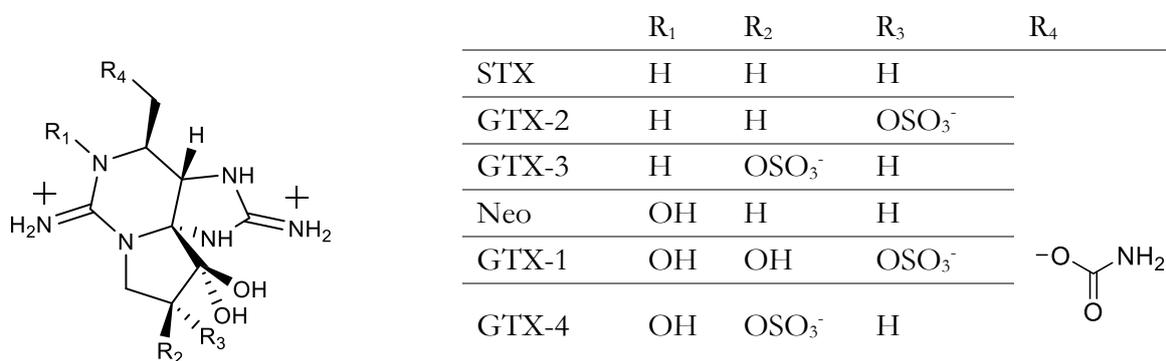
Figure 1. Structure of Saxitoxin and analogues

2.2. Tetrodotoxin poisoning

Tetrodotoxin (TTXs) is one of the oldest toxins described. It is not produced by microalgae like STX. Most likely TTXs are produced by bacteria and it has been described that *Vibrio* spp. and *Pseudomona* spp. are able to produce TTX [22]. Worldwide TTX is known because of the intoxications produced by the consumption of "Fugu" (raw meat of puffer fish) in Japan [21, 47]. The accumulation of this toxin occurs in species of fish from the Tetraodontidae family, in which TTX is concentrated mostly in the ovary and liver, although other organs including skin, intestine, and muscle can also contain the toxin depending on the species of puffer fish [20]. Lately, TTX is of concern because it has been found in shellfish from European coastal areas [48, 49]. The mode of action of TTX is similar to that of STX, i.e. the inhibition of the VGSC in a selective manner [50].

	R_1	R_2	R_3	R_4
TTX	H	OH	CH_2OH	OH
4-epiTTX	OH	H	CH_2OH	OH
11-norTTX-6(S)-ol	H	OH	H	OH
11-norTTX-6(R)-ol	H	OH	OH	H
11-oxoTTX	H	OH	$CH(OH)_2$	OH
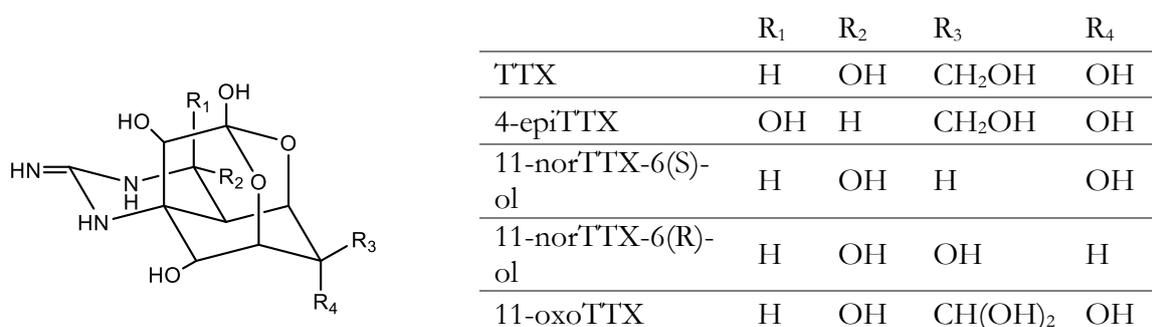
Figure 2. Structure of Tetrodotoxin and analogues

2.3 Amnesic shellfish poisoning (ASP)

Domoic acid (DA) is a toxin produced by algal blooms of the diatoms *Pseudo-nitzschia* spp. and is an excitatory neurotransmitter amino acid. DA is an analogue of the neurotoxin kainic acid and binds to the glutamate receptor, resulting in sodium channel opening and membrane depolarisation [23]. It causes symptoms progressing from gastrointestinal distress and diarrhoea to death. Individuals can experience alteration of memory function, hallucination, confusion and amnesia: therefore, the name amnesic shellfish poisoning (ASP). Since amnesia does not always occur, the poisoning is also called domoic acid poisoning (DAP) [39, 51, 52]. DA was first found in Canada in 1989, but nowadays it is found worldwide [2].

DA
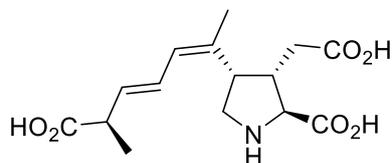
Figure 3. Structure of Domoic Acid

2.4 Neurologic shellfish poisoning (NSP)

Brevetoxin (PbTX) is a toxic compound produced by algae from the genus *Karenia*, e.g. *K. brevis*. PbTX is a VGSC activator, causing influx of Na^+ ions into the cell leading to blocking of nerve excitability causing neurotoxic shellfish poisoning (NSP). The symptoms can vary from diarrhoea, vomiting, cramps, respiratory difficulties and eventually death. Cases have been reported mainly in the United States and New Zealand [17], therefore there are no regulatory limits in Europe or South America.
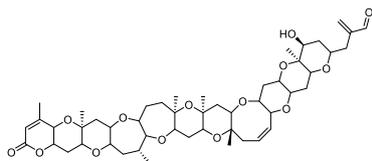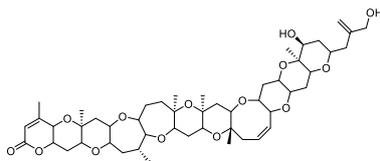
PbTX-2   PbTX-3   PbTX-9
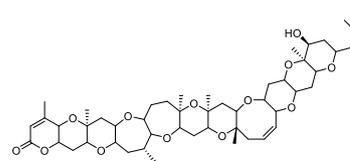
Figure 4. Structure of Brevetoxins

2.5 Ciguatera poisoning

Ciguatera toxin (CTX) is produced by the dinoflagellate *Gambierdiscus* spp. and ciguatera poisoning is a syndrome caused by consumption of subtropical or tropical marine carnivorous fish, e.g. barracuda, snapper, moray eel among others [53]. The accumulation of the toxin occurs through the food chain [2]. It is the most common form of intoxication reported in the US by consumption of seafood, and the most common poisoning caused by (shell)fish worldwide. Symptoms can occur within the first 12 hours after consumption of contaminated fish, starting with abdominal pain, diarrhoea, nausea and vomiting. Neurologic and cardiac problems can also occur later on, and the toxin can even cause respiratory distress, coma and death. The hallmark sign of ciguatera poisoning is the so-called paradoxical thermal sensation, i.e. cold objects feel hot and hot objects feel cold. These symptoms together with the history of reef fish consumption are enough to diagnose ciguatera poisoning [52-54].

P-CTX-1 (CTX1B)

P-CTX-2 (52-epi-54-deoxyCTX1B)

P-CTX-3 (54-deoxyCTX1B)
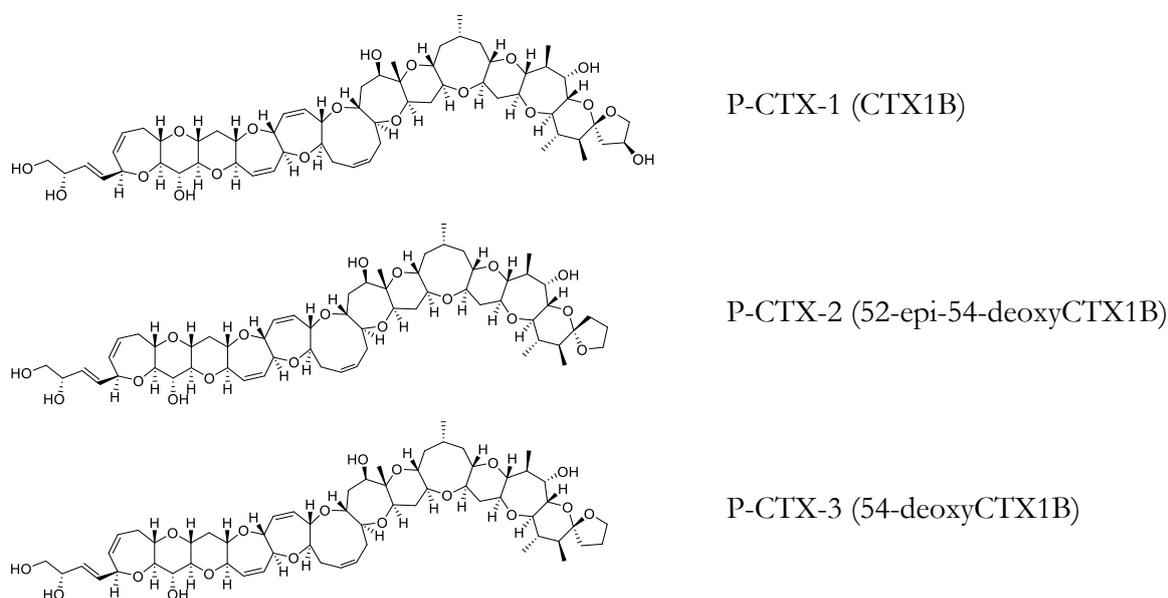
Figure 5. Structure of Ciguatera toxins

2.6 Azaspiracid poisoning (AZP)

Azaspiracid (AZA) and its analogues are polyether toxins with two spiro-ring assemblies, a cyclic amine and a carboxylic acid [55-57]. AZA-1 and AZA-2 are produced by the dinoflagellates *Azadinium spinosum*. AZA-3 and the other 30 analogues described are metabolites of AZA-1 or AZA-2 that are formed within the shellfish [58, 59]. The primary mode of action of AZA underlying the induction of azaspiracid poisoning (AZP) is not known yet. Toxic effects include cytoskeletal rearrangement [60, 61], decreased levels of F-actin [57] and induction of apoptosis [62, 63]. AZA-1 has been reported to induce mRNA expression of genes involved in cholesterol and fatty acid synthesis in Jurkat cells indicating that AZA-1 activates these processes. [64].
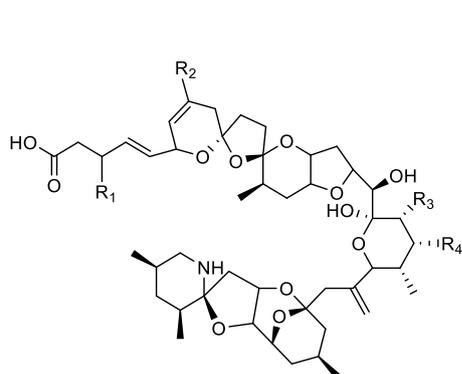
	R_1	R_2	R_3	R_4
AZA-1	H	H	CH_3	H
AZA-2	H	CH_3	CH_3	H
AZA-3	H	H	H	H
AZA-4	OH	H	H	H
AZA-5	H	H	H	OH

Figure 6. Structure of Azaspiracids

2.7 Diarrhoeic shellfish poisoning (DSP)

Okadaic acid (OA) and dinophysistoxins (DTX) are toxins causing diarrhoeic shellfish poisoning (DSP). They are produced by dinoflagellates from the genders *Dynophysis* spp. and *Prorocentrum* spp. [65]. OA has been reported to be a tumour promoter *in vivo* in mouse skin and rat stomach [31, 66]. OA and DTX analogues inhibit protein phosphatases, particularly PP1 and PP2A, causing permanent phosphorylation of proteins leading to effects on regulation of glycogen metabolism, transcription, cell differentiation, apoptosis and DNA replication in several cell lines [67, 68]. Other effects described are genotoxicity through the formation of DNA adducts in human fibroblasts and keratinocytes [69],

aneugenic effect in Caco-2 and CHO-K1 cell lines [70, 71], cytotoxicity in several cell lines *in vitro*, i.e. peripheral blood leukocytes, HepG2 hepatoma cells, and SHSY5Y neuroblastoma cells [72]. Yessotoxins (YTXs) and pectenotoxins (PTXs) do not cause diarrhoea but are co-extracted with the DSP toxins in the same lipophilic extract. YTXs are polycyclic ether toxins and are regulated apart from DSP toxins since they do not share the mode of action, i.e. inhibition of phosphatases. Over 30 analogues of YTX have been isolated. No human toxicity has been attributed to this toxin, even with high amounts reported in shellfish [36]. Mice injected with YTX die, but mice fed with high oral doses did not show diarrhoea or any other adverse effects [73]. The mode of action of YTX is still unknown, but at the cellular level, damage in cardiomyocytes has been observed, e.g. activation of autophagy signalling, suggesting a cardiotoxic potential [74], and also genomic alterations *in vitro* [75]. Pectenotoxins are a group of polyether toxins and are regulated together with the DSP toxins. No correlation with human cases has been described so far, and no acute oral toxicity has been described in mice when administered with PTX-2 [28]. *In vitro*, pectenotoxin has shown to be hepatotoxic and induced depolymerisation of F-actin [39].

	R_1	R_2	R_3
OA	CH_3	OH	H
DTX-1	CH_3	CH_3	H
DTX-2	H	CH_3	H
DTX-3	H or CH_3	H or CH_3	Acyl
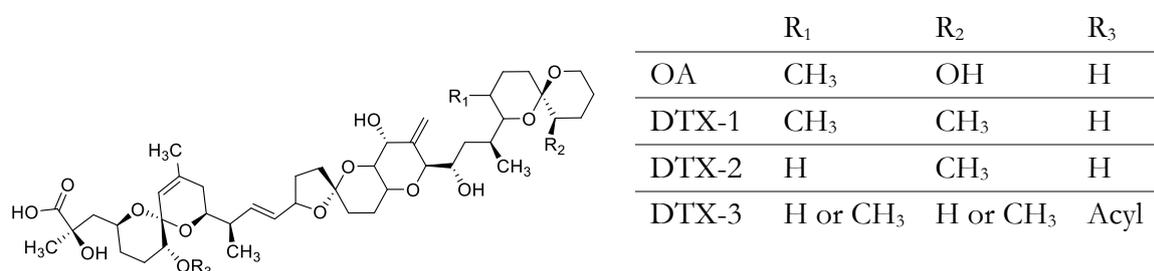
Figure 7. Structure of Okadaic Acid and Dinophysis toxins
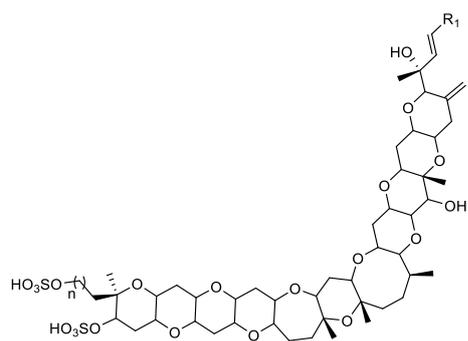
	n	R_1
YTX	1	
Homo YTX	2	
45OH YTX	1	
45OH homo YTX	2	

Figure 8. Structure of Yessotoxins
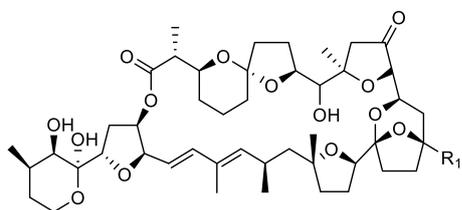
	R_1
PTX-1	CH_2OH
PTX-2	CH_3
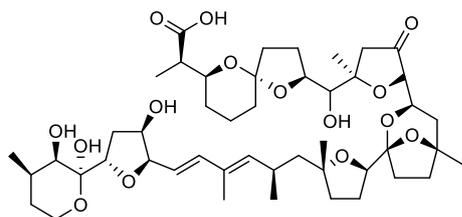
PTX-2sa

Figure 9. Structure of Pectenotoxins

3. Detection methods

3.1 Mouse bioassay

The oldest method for the detection of marine biotoxins is the mouse bioassay (MBA). In general, the method consists of injecting a sample intraperitoneal in three mice, and the occurrence of death in a period of 24 hours is monitored. The sample is considered positive if at least 2 of 3 mice die within 24 hours. There are 2 different protocols, i.e. for the detection of PSP toxins and for the detection of DSP toxins.

The MBA for PSP toxins consists on the injection of three 20-gram mice with 1mL of an acidic extract obtained from shellfish products. The time taken for the mice to die is recorded, and samples should be diluted to ensure that death occurs within 5 and 15 minutes. Then, the toxicity of the sample can be calculated referring to dose-response curves established using STX standards. This quantification of the toxins is established in mouse units (MU), being "1 MU is the amount of injected toxin which would kill a 20g mouse in 15 minutes, and it is equivalent to 0.18 μg of STX" [2]. This MBA has several drawbacks, e.g. death time is not always linear with toxin levels, the assay is labour intensive, and because sacrifice of large amounts of animals is needed it is considered unethical. However, the method has been properly validated (AOAC Official Method 959.08: Paralytic Shellfish Poison, biological method) and it is still in use in several countries [2].

The MBA for DSP toxins was developed in 1978 in Japan, after several people showed gastrointestinal complaints such as diarrhoea, nausea and vomiting after shellfish consumption [76]. Since 1981 the MBA has been the official method for detection of DSP toxins in Japan and other countries [77]. The method for lipophilic marine biotoxins (LMB), i.e. DSPs, AZPs and NSPs, consists of an extraction with acetone, followed by partition with diethyl ether or dichloromethane and water, which is subsequently evaporated and suspended in tween 20. For testing, 1 mL of the suspension is injected intraperitoneally to three 20-gram male mice. The death of at least 2 of 3 mice in 24 hours is the indication that the sample is positive [77]. The protocol has had several modifications concerning solvents and number of animals, with the aim of improving the detection capacity and to increase animal welfare [78]. However, the MBA for lipophilic toxins has never been properly

validated and -as indicated above- the MBA does not consider humane endpoints to avoid animal suffering, i.e. the endpoint to declare the test as positive is death [79].

Although the MBA for the detection of lipophilic marine biotoxins has been replaced in several countries, e.g. Australia, Canada, New Zealand and in the European Union, it is still the reference method in some countries [80] and in Europe the use of the MBA is still allowed at production areas as surveillance for unknown toxins. In January 2015, the MBA has been banned as a reference method for testing final products, mainly because it is unethical, not appropriate due to a high variability in results, showing insufficient detection aptitude, and limited specificity [81].

Efforts have been made in replacing the MBA. Alternatives for PSP toxins are being investigated extensively [45, 82-85], and although it is important to also consider the hydrophilic toxins in the replacement of the mouse bioassay, the work presented in this thesis focuses on the alternatives for the detection of LMB.

3.2 Analytical methods

Chemical methods focus on the detection and identification of compounds based on their chemical properties. The extraction method for detecting either the hydrophilic or lipophilic toxins is different. For the detection of PSP toxins, one chemical method has been validated, i.e. the AOAC method 2005.06, which is based on the fluorescence of STX and its analogues after oxidation and subsequent separation on a HPLC column [45]. Another official method is the AOAC Official Method 2011.27, a receptor binding assay making use of the PSP toxin receptor in a competitive binding assay with [^{3}H] STX [86].

In 2011, the LC-MS/MS method of the EURL on marine biotoxins became the reference method for detecting lipophilic marine biotoxins in Europe [87, 88]. Other methods such as LC-MS and HPLC, immunoassays and functional assays (e.g. the phosphatase inhibition assay) are allowed as alternative or supplementary to the reference method, only if they are properly validated [81].

Absence of certified standards of each toxin and their analogues, and the incapacity to detect presently unknown toxins or analogues are known disadvantages of the LC-MS/MS method [89]. Chemical detection is thus not able to detect all toxins or the toxins that are presently unknown. This makes use of the LC-MS/MS method for detecting all potential

harmful LMB very challenging. Additionally, analytical methods are unable to predict toxicity of complex mixtures or detect emerging risks. As a result, many countries hesitate to trust solely on analytical methods. Therefore, the MBA it is still used, mainly because it facilitates detection of yet unknown toxins and emerging risks.

3.3 Other alternative methods

3.3.1 Biological methods

Biological functional assays are based on the mode of action of a compound to cause toxicity. These assays include receptor-based binding assays, enzyme assays and cell-based bioassays [90]. The receptor and enzyme assays use the biological target of the toxin as the element for toxin detection. Enzyme inhibition assays, i.e. inhibition of phosphatases, have been developed for the detection of OA and DTX. These assays allow the quantification of a toxin present in a sample, often by colour (absorbance) or fluorescence measurements [91]. These methods are fast and have been proven sensitive enough to detect all analogues and able to determine the total potency of a sample extract without the need for certified standards for each analogue. The main disadvantage is that these bioassays require a second identification step in case a sample is suspect/positive, i.e. in order to identify the compound causing the effect [92]. Cell-based bioassays can measure a particular cell function, e.g. measurement of membrane potential and ion influx, or can be based on the measurement of cytotoxicity [92, 93]. The neuro-2a assay is based on mouse neuroblastoma cells which are exposed to shellfish extracts for a period of time, usually 24 hours, using the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as a readout of cell viability. This assay has been shown to be suitable for the detection of PSPs, NSPs and DSPs [83, 93-95].

The reasoning behind replacing animal tests like the MBA by cell-based bioassays, is that these bioassays will detect the effect of an unknown compound/analogue too, i.e. an analogue of the known toxin (known unknown), or in some cases, a completely new toxin (unknown unknown).

3.3.2 Immunological methods

With immunological methods, detection is based on a specific interaction between the toxin and an antibody. ELISAs and lateral flow devices (LFDs) are well-known examples of immunoassays. The high specificity of the antigen-antibody interaction allows to detect low amounts of the toxin in a sample, but cross reactivity with analogues can be an issue for quantification. Inaccuracy may be either due to overestimation of toxicity when relatively inactive analogues are also strongly bound or to underestimation when active analogues are not bound [90]. Optical immunosensors have been developed for detection of DSP toxins, PSP toxins and DA as surface plasmon biosensors. Advantages are the low costs and speed. However, the main drawback is the lack of antibodies for all the toxins and due to cross-reactivity, quantification is often not accurate enough [79, 92, 96].

Table 3. Summary of alternative methods for the MBA. Adapted from Campbell et al, 2011 and Stewart and McLeod 2014 [79, 90].

Toxins	Chemical method	Biological method	Immunological method
STX s and TTXs	– HPLC-FLD pre-column oxidation[1] – HPLC-FLD post column oxidation[1] – LC/MS – Capillary electrophoresis – Ion exchange chromatography – Quantitative NMR spectrometry – HPLC-MS – HILIC-MS – Chemosensors	– Receptor-binding assay[1] – Saxiphilin competitive binding assay – Neuro-2a bioassay – Membrane potential *in vitro* assays – Membrane biosensor (frog bladder membrane) – Fluorimetric assay – Insect bioassay	– Lateral flow immunoassay[2] – ELISA[2] – Surface Plasmon Resonance assay (optical biosensor) – SPR coupled to HILIC – RIA (obsolete)
DA	– HPLC-UVD – HPLC-MS – Thin layer chromatography	– Kainic-acid receptor-binding assay	– ELISA – SPR Biosensor

OA and DTXs	– LC-MS/MS[1] – HPLC-FLD – HPLC-MS	– Protein phosphatase inhibition assay[3] – F-actin cell-based fluorimetric microplate assay – Cell morphology	– ELISA – SPR biosensor – Quartz crystal microbalance biosensor – Electrochemical biosensor
PTXs	– LC-MS/MS[1] – HPLC-FLD/UVD – HPLC-MS	– F-actin cell-based fluorimetric microplate assay – Cell morphology	– ELISA
AZAs	– LC-MS/MS[1] – HPLC-MS	– Cell morphology	
YTXs	– LC-MS/MS[1] – HPLC-FLD – HPLC-MS – Capillary electrophoresis	– E-cadherin fragmentation – Phosphodiesterase enhancement, using SPR, resonant mirror biosensors or microplate assay	– ELISA
PbTXs	– HPLC-MS	– Sodium channel receptor binding assay – Neuro-2a bioassay – Fluorimetric assay	– RIA – ELISA

[1] AOAC and/or EU official methods

[2] Used for screening in some monitoring programs

[3] Waiting for regulatory acceptance

HPLC: High performance liquid chromatography, FLD: fluorescence detection, LC: Liquid chromatography, MS: Mass spectrometry, HILIC: hydrophilic interaction liquid chromatography, UVD: Ultra violet detection, RIA: Radioimmunoassay, ELISA: Enzyme linked immunosorbent assay, SPR: surface plasmon resonance

4. Regulations

Different regulations are in place in several countries based on the codex guidelines [97] where maximum limits for each group of toxins are established. In general, five groups of marine biotoxins are regulated in Europe, i.e. okadaic acid group (OA), which includes the dinophysistoxin analogues (DTXs), also known as diarrheic shellfish poisoning (DSPs) toxins; azaspiracid group (AZAs), which can also cause diarrhoea; yessotoxin group (YTXs); domoic acid group (DA), which causes the amnesic shellfish poisoning (ASP); and

saxitoxin group (STXs), also known as paralytic shellfish poisoning (PSPs) toxins (EFSA, 2009). Current regulation in Europe established maximal levels of these toxins in seafood in order to protect human health. For OA and DTX-1, -2 and -3, 160 µg OA equivalents per kilo of shellfish meat is allowed, where equivalences are calculated based on toxic equivalent factors (TEF). OA and DTX-1 are considered equally toxic and have a TEF of 1. DTX-2 is considered less potent, with a TEF of 0.6. DTX-3 are acylated derivatives of OA, DTX-1 and DTX-2. After hydrolysis, DTX-3 is detected by LC-MS/MS as the parent OA compound, DTX-1 or DTX-2, which are expressed as OA equivalents using their corresponding TEFs [65]. Although AZA is not included in the DSP group of toxins, it causes diarrhoea as well. The regulation allows for azaspiracids AZA-1, -2 and -3, 160 µg AZA-1 equivalents per kilo of shellfish meat. The TEFs of AZA-1, AZA-2, and AZA-3 are 1, 1.9 and 1.4, respectively [98]. YTXs initially were classified within the DSP toxins but now there is a separated regulation, which allows 3.75 mg YTX equivalents/kg shellfish meat. More than 30 analogues have been isolated, with homo-yessotoxin (hYTX) and 45-OH-homoYTX (45-OH-hYTX) being the most common, both with TEFs of 0.5 relative to YTX.

Table 4. Limits for lipophilic marine biotoxins in countries with regulation in place (source [99])

Toxin	Codex	EU	USA	Australia/ NZ	Chile	Japan
OA, DTX, PTX (µg OA eq/kg shellfish)	160	160	160	200	nd	160
AZA (µg AZA eq/kg shellfish)	160	160	160	nr	nd	160
YTX (mg YTX eq/kg shellfish)	nr	3.75	nr	nr	nd	nr
PbTX (µg PbTX2 eq/kg shellfish)	800	nr	800	800	nr	800

nr: not regulated
nd: non-detectable by bioassay

5. General objective

The presence of toxin producing algae or marine biotoxins in shellfish can cause closure of extraction areas, often needed in order to prevent human intoxications, but sometimes also unnecessary damaging economic activities in many countries around the world [100, 101]. The impact of closures of coastal areas is huge, not only in terms of global economy losses, but also in terms of local economies and human wellbeing, regarding the use of coastal areas for recreation, fishing, shellfish collection and tourism. For instance, small fishing communities, mainly in developing countries, often are entirely centred on the extraction of shellfish products. The impact of closing areas of extractions for these communities is dramatic, affecting their local economy, food security and wellbeing [99, 102]. Improving the methods for detection of shellfish toxins contributes to an efficient management of extraction areas, avoiding unnecessary or long and permanent closures, and could thus improve the economic activity of certain areas, protecting human health and considering animal welfare at the same time.

Regarding the detection of marine biotoxins in shellfish, the MBA has been used for a long time. Alternatives have been developed, but the MBA is still used and a general switch to an *in vitro* or chemical method is needed. Replacement of an animal testing method is not an easy task, mainly because it is difficult to abandon a trusted method that has been in place for a long period. However, this trust in the MBA is not based on clear evidence. It has been shown that the MBA is not flawless, and it has failed to detect new toxins. For instance, the MBA was not able to detect DA in an incident in Ireland, which resulted in human illness [103]. The MBA for DSP toxins is itself a non-validated method, and validating new alternatives against the results of the MBA for these toxins seems thus inappropriate [103]. However, at least a comparison with the MBA is needed to show that the alternative method provided protects consumers at least equally well.

The aim of this thesis was to develop new *in vitro* bioassays for the detection of marine biotoxins that can be used for high throughput quality control of seafood and replacement of the current unethical *in vivo* bioassays, and to evaluate use of the assay in an integrated testing strategy, in which bioassays and an analytical chemical method were combined, as an *in vitro* alternative for the MBA for lipophilic marine toxin testing.

6. Thesis outline

Chapter 1 of the thesis (this chapter) presents an introduction to the field of marine biotoxins and the methods for their detection. It also presents the aim of the thesis. Since a new bioassay is needed in order to replace the MBA, it was investigated if a cell-based bioassay could be used for broad screening. The neuro-2a bioassay, a well-known assay for detection of PSP toxins, was previously assessed for the detection of lipophilic marine toxins by Cañete and Diogène [94]. The assay was not further investigated since a so-called "matrix effect" caused false positives. In **Chapter 2**, the neuro-2a bioassay was investigated in combination with improvements in the clean-up procedure for preparing extracts from shellfish samples in order to eliminate the false positives due to matrix effects. In **chapter 3** the effects of the lipophilic marine toxins OA, DTX-1 and AZA-1 on the whole-genome mRNA expression of undifferentiated intestinal Caco-2 cells was investigated. The gene expression data were analysed in order to reveal modes of action and obtain hints on potential biomarkers suitable to be used in additional bioassays to be developed. **Chapter 4** presents such newly developed methods using the selected genes for detection or identification of the lipophilic marine toxins, based on qRT-PCR and multiplex magnetic bead-based assays. The focus was on possible marker genes to detect OA/DTX, AZA/YTX and/or PTX. Certified standards as well as blank and contaminated shellfish samples were tested. In **chapter 5** a selected set of samples tested in the MBA in Chile, where the mouse bioassay is still in use, were analysed using a strategy in which the neuro-2a bioassay as a screening method for the detection of lipophilic toxins was combined with LC-MS/MS analysis for confirmation and outcomes where compared with those of the MBA. Finally, **Chapter 6** presents an overall discussion and future perspectives.

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Chapter 2

Screening for the presence of lipophilic marine biotoxins in shellfish samples using the neuro-2a bioassay

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Summary

The neuro-2a bioassay was considered as one of the most promising cell-based *in vitro* bioassays for the broad screening of seafood products for the presence of marine biotoxins. The neuro-2a bioassay has been shown to detect a wide array of toxins like paralytic shellfish poisoning (PSP) toxins, ciguatoxins, and also lipophilic marine biotoxins (LMBs). However, the neuro-2a bioassay is hardly used for routine testing of samples due to matrix effects that, for example, lead to false positives when testing for LMBs. As a result, there are only limited data on validation and evaluation of its performance on real samples. In the present study, the standard extraction procedure for LMBs was adjusted by introducing an additional clean-up step with n-hexane. Recovery losses due to this extra step were less than 10%. This wash step was a crucial addition in order to eliminate false-positive outcomes due to matrix effects. Next, the applicability of this bioassay was assessed by testing a broad range of shellfish samples contaminated with various LMBs, including diarrhoeic shellfish poisoning (DSP) toxins. For comparison, the samples were also analysed by LC-MS/MS. Standards of all regulated LMBs were tested, including analogues of some of these toxins. The neuro-2a cells showed good sensitivity towards all compounds. Regarding the samples, extracts of 87 samples, both blank and contaminated with various toxins, were tested. The neuro-2a bioassay outcomes were in line with those of the LC-MS/MS analysis and support the applicability of this bioassay for the screening of samples for LMBs. However, for use in a daily routine setting, the test might be further improved, and several recommendations are made before a full validation is being performed.

1. Introduction

Marine biotoxins are naturally occurring compounds mostly produced by certain algae. These toxins can affect human health mainly through foodborne intoxications, i.e. consumption of contaminated seafood, and occasionally through direct exposure to seawater aerosols [1, 2]. Consumption of seafood contaminated with marine biotoxins may result in relatively mild symptoms, such as diarrhoea, dizziness, numbness and tingling of the mouth and digits, but also paralysis and in severe cases even death [3, 4]. Several major types of poisoning are described: amnesic shellfish poisoning (ASP), diarrhoeic shellfish poisoning (DSP), neurologic shellfish poisoning (NSP) and paralytic shellfish poisoning (PSP) [4]. A fifth syndrome, azaspiracid poisoning (AZP), has been characterised during the last 20 years [5]. To avoid intoxications, monitoring is obligatory in many parts of the world. Within the EU, limits have been set by the European Commission (Regulation No 853/2004) for ASP toxin and PSP toxins, as well as several lipophilic marine biotoxins (LMBs) [6].

Worldwide, the main assay applied is the mouse bioassay (MBA), where mice are intraperitoneal (i.p.) injected with a sample extract, using lethality as the critical endpoint [7-9]. Besides ethical issues, the MBA gives high rates of false-positive and false-negative results [10, 11]. In Europe, the use of the MBA has been banned for LMBs since 2015, but not for PSP toxins analysis and not for the control of production areas, aiming at detection of possibly unknown LMBs [6]. The reference method for the detection of LMBs is now the LC-MS/MS method of the EURL on marine biotoxins [6, 12, 13]. LC-MS/MS based methods are fit for purpose, but for many toxins certified standards and reference materials are barely or not available. This makes their use for detecting all marine biotoxins very difficult, if not impossible. For example, there are at least 24 saxitoxin analogues [14], 13 okadaic acid-ester derivatives [15], 90 yessotoxin analogues [16], 15 brevetoxin analogues [17] and around 30 azaspiracid analogues [18, 19] and in proximity there are only 20-30 certified reference standards available. Furthermore, analytical methods are by definition unable to predict toxicity of complex mixtures or pick up new risks. As a result, many countries hesitate to rely solely on analytical methods and keep using the MBA.

Because of the drawbacks of the MBA and the analytical chemical methods, EFSA emphasised the need for developing animal friendly alternatives [10]. There is thus an urgent need for *in vitro* tests that allow the detection of marine biotoxins that are currently known and those which might emerge [20, 21]. Biochemical assays and especially cell-based bioassays have the potential to fulfil these requirements [22, 23]. The neuro-2a bioassay, using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or water-soluble tetrazolium salts (WST) as a viability end-point, is considered to be one of the most promising cell-based bioassays for the broad screening of marine neurotoxins [22, 24]. It is already used to some extent for the detection of ciguatoxins (CTXs), tetrodotoxin (TTX) [25] and the lipophilic brevetoxins (PbTxs) [26, 27]. However, although the neuro-2a bioassay has been tested intensively for PSP toxins, this is mainly with standards and very limited with routine samples [28-31]. Thus far, the assay seems not widely applied in routine for PSP toxins nor DSP toxins testing and certainly not within the EU. According to an extensive collaborative study that involved testing of OA, PTX-2 and AZA-1 in three cell lines, i.e. Caco-2, HepG2 and Neuro-2a, matrix effects seems to be an important issue that needs to be addressed in case these assays are applied for real samples [32]. Therefore, developing a suitable extraction method is critical to allow the routine use of the neuro-2a bioassay for shellfish samples [33].

The aim of the present study was therefore to evaluate and optimise the neuro-2a bioassay for routine testing of shellfish on the presence of LMBs. First, a comparison was made between the use of the murine neuroblastoma cells (Neuro-2a) and another previously applied cell line, murine neuroblastoma x rat glioma hybrid cells (NG108-15) in order to establish the sensitivity of both cell lines in our laboratory. We tested all EU regulated lipophilic marine biotoxins for which standards were available, i.e. okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2), pectenotoxin-2 (PTX-2), azaspiracid-1 (AZA-1), azaspiracid-2 (AZA-2), azaspiracid-3 (AZA-3), yessotoxin (YTX) and 1a-homo yessotoxin (hYTX). Subsequent testing of shellfish samples was carried out with the neuro-2a cells, as these turned out to be slightly more sensitive. Based on first test results with real samples, an improved clean-up procedure was developed by introducing an additional n-hexane wash step in order to reduce false positives due to matrix effects. Potential recovery losses due to this extra wash step were checked by LC-MS/MS analysis

of blank sample extracts spiked just before and after the n-hexane wash step. Next, extracts of both blank and contaminated shellfish were tested, and results were compared with LC-MS/MS outcomes in order to examine whether this bioassay is applicable to real samples in a daily routine setting and to identify possible issues for further improvement prior to a full validation. This is necessary, as, besides the extensive efforts needed, such a validation will be very expensive due to the high costs of the required marine toxin standards.

2. Materials and Methods

2.1 Reagents and standards

Certified reference materials (CRMs) of OA (13.7 ± 0.6 µg mL^{-1}), DTX-1 (15.1 ± 1.1 µg mL^{-1}), DTX-2 (7.8 ± 0.4 µg mL^{-1}), PTX-2 (4.40 ± 0.13 µg mL^{-1}), AZA-1 (1.24 ± 0.07 µg mL^{-1}), AZA-2 (1.28 ± 0.05 µg mL^{-1}), AZA-3 (1.04 ± 0.04 µg mL^{-1}), YTX (5.6 ± 0.2 µg mL^{-1}), and hYTX (5.8 ± 0.3 µg mL^{-1}) were purchased from the National Research Council, Institute for Marine Biosciences (NRC CNRC, Halifax, Canada). Stock solutions of these toxin standards were prepared in dimethyl sulfoxide (DMSO) after evaporation of the original solvent. DMSO, ammonium hydroxide (25%) and n-hexane were obtained from Merck (Darmstadt, Germany). Acetonitrile (Ultra LC/MS), methanol (Ultra LC/MS) and water (Ultra LC/MS) were purchased from Actu-All (Oss, The Netherlands).

2.2 Samples

In-house samples, both blank mussel samples from the Netherlands, and contaminated samples obtained from various locations and used for previous validation studies of the LC-MS/MS method, were tested [34]. In addition, 50 samples (crude methanol extracts) from various types of marine gastropods and bivalves, potentially naturally contaminated with LMBs, were kindly donated by Dr Carlos García from the Faculty of Medicine, Universidad de Chile, Santiago, Chile [35] (Table 3).

2.3 Preparation of extracts

Prior to extraction of the lipophilic marine biotoxins (i.e. DSP toxins and AZAs), shellfish material was homogenised with a T25 Ultra Turrax mixer at 24,000 rpm (IKA® Works

Inc., Wilmington, NC, USA). One gram of shellfish homogenate was vortex mixed with 3 mL methanol for 1 min and centrifuged for 5 min at 2,000 × g. The supernatant was transferred to a volumetric flask and the residue was extracted twice more with 3 mL methanol. After the third extraction the volume of the collected supernatant was adjusted to 10 mL with methanol. For the neuro-2a bioassay additional clean-up steps using n-hexane and solid phase extraction (SPE) were applied (see 2.4), which were not required for the LC-MS/MS analysis.

2.4 Clean-up by n-hexane wash step followed by SPE

A 4.8 mL aliquot of the crude methanol shellfish extract was diluted with 1.2 mL Milli-Q water and extracted twice with 6 mL n-hexane in order to remove matrix substances that led to false-positive test outcomes. The hexane layer was discarded and the aqueous methanolic extract was further diluted by adding 10 mL Milli-Q water and the total extract of 16 mL was transferred to an SPE StrataTM-X cartridge (200 mg/6 mL; Phenomenex, Utrecht, the Netherlands), previously conditioned with 4 mL methanol/water (30:70 v/v). Subsequently, the cartridge was washed with 8 mL methanol/water (20:80 v/v) and the toxins were eluted with 4.8 mL methanol. The eluate was evaporated to dryness under a stream of nitrogen gas and reconstituted in 20 μL DMSO.

2.5 Recovery of the n-hexane wash step

Blank mussel samples were pooled (10 g) and 1-gram portions were extracted using the method described above. Fortification of extracts equivalent to 3, 1 and 1/3 of the maximum permitted level (MPL) of OA, DTX-1 and AZA-1 (i.e. 480 μg kg^{-1}, 160μg kg^{-1} and about 53μg kg^{-1}) and about 1/4 MPL for YTX (i.e. 1000 μg kg^{-1}) before and after the n-hexane clean-up were carried out by adding a corresponding volume of a highly concentrated standard in methanol. The recovery was calculated by LC-MS/MS analysis of subsamples taken from the fortified extracts. The rest of the sample extracts fortified before and after the n-hexane extraction, were purified on SPE (see above) and analysed in the neuro-2a bioassay. The exposures were performed in three different experiments and all samples within an experiment were tested in triplicate. Due to the high costs and amounts needed, YTX experiments were performed twice and samples were tested in triplicate.

2.6 Cell culture and exposure

Murine neuroblastoma neuro-2a cells were purchased from the American Type Culture Collection (ATCC; CCL-131) and cultured in 75 cm² culture flasks containing 15 mL RPMI-1640 medium (R0883, Sigma-Aldrich, Zwijndrecht, the Netherlands) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Fisher Emergo, Landsmeer, the Netherlands), 1% (v/v) of a 100 mM sodium pyruvate solution (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 1% (v/v) of a 200 mM L-glutamine solution (Sigma-Aldrich). NG108-15 cells were also obtained from ATCC (HB-12317) and cultured in 75 cm² culture flasks containing 30 mL Dulbecco's Modified Eagle's Medium (DMEM) obtained from Lonza (Verviers, Belgium) supplemented with 10% (v/v) FBS and 2% (v/v) of 50× HAT supplement (5 mM hypoxanthine, 20 µM aminopterin and 0.8 mM thymidine, Sigma-Aldrich). Both cell lines were routinely maintained in a humidified incubator at 37 °C under 5% CO_2 and sub-cultured three times per week (dilution 1/5) up to approximately 90% confluence.

The experimental conditions for the assay were adapted from Cañete and Diogène 2008. Neuro-2a and NG108-15 cells were seeded separately into 96-well plates with an initial density of 25,000 and 14,000 cells/well, respectively. After growing the cells for 24 h, exposure to increasing concentrations of pure marine biotoxins or sample extracts was performed in quadruplicate in 200 µL medium for 24 h. The method of exposing the cells was changed in order to adjust it to our sample extracts and standards that were dissolved in DMSO, i.e. exposure media were prepared by using e.g. 3 µL standard or sample extract in DMSO (see 2.3 and 2.4) and 1200 µL of culture medium. Dilutions, 5 or 10 times, were made in culture medium with 0.25% DMSO to keep the solvent concentration at 0.25%. At the end of the exposure time, cell viability was measured using the MTT assay.

2.7 Cell viability assay (MTT)

Briefly, MTT (Sigma-Aldrich) was prepared in PBS at 5 mg mL⁻¹, and mixed with serum-free medium. Then, the media from the cells was removed and 60 µL of MTT mixed with serum-free medium was added to each well (final concentration of MTT in the well is 0.8 mg mL⁻¹). After 30 min incubation at 37 °C, the medium was removed, and the formed formazan crystals were dissolved in 100 µL DMSO. Plates were placed in a plate shaker for

10 min at 600 rpm after which the absorbance was measured at 540 nm and corrected for background absorption at 650 nm. EC_{50} values were determined using a nonlinear regression model (GraphPad Prism software version 5.04, San Diego, CA, USA).

2.8 Chemical analysis

Chemical analysis was directly performed on crude methanol extracts. The method applied for the determination of lipophilic marine biotoxins (i.e. DSP toxins and AZAs) was previously described by Gerssen et al. (2009, 2010). Chromatographic separation was achieved using a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA). The system consisted of a binary solvent manager, sample manager and a column manager. The column temperature was kept at 60 °C and the temperature of the sample manager was kept at 10 °C. A 5 μL injection volume was used. Mobile phase A was water and mobile phase B was acetonitrile/water (9:1 v/v), both containing 6.7 mM ammonium hydroxide. A flow rate of 0.6 mL min^{-1} was used. A gradient started at 30% B and after 0.5 min was linearly increased to 90% B in 3 min. This composition was kept for 0.5 min and returned to 30% B in 0.1 min. An equilibration time of 0.9 min was allowed prior to the next injection. The effluent was directly interfaced in the electrospray ionisation (ESI) source of the AB Sciex QTrap 6500 mass spectrometer (Ontario, Canada). The mass spectrometer was operated in both negative and positive electrospray ionisation by rapid polarity switching. For each toxin two transitions were measured. For quantification of the toxins, so-called 'matrix matched' calibration curves were constructed. These calibration curves were constructed by fortifying blank shellfish extracts with different concentrations of toxin. The area of the toxin in the unknown sample is then calculated using the linear equation of the calibration curve. The concentration is expressed in μg kg^{-1} shellfish meat.

3. Results and Discussion

3.1 Effects of individual lipophilic marine biotoxins

A number of different toxins were tested on both neuro-2a and NG108-15 cells using MTT reduction as measure for cell viability. Overall, all lipophilic marine biotoxins and their analogues induced a concentration dependent decrease in viability of both neuro-2a and NG108-15 cells. Figure 1 shows the effect of the lipophilic marine biotoxins OA, DTX-1, DTX-2, AZA-1, AZA-2, AZA3, PTX-2, YTX and hYTX on the cell viability of neuro-2a cells. Table 1 shows the calculated EC_{50} values for both cell lines, being the concentration reducing the MTT response by 50% of the maximal observed difference. Although AZAs and YTXs caused a decrease in cell viability at relatively low concentrations, MTT response was only reduced to about 50% of the initial response, while OA, DTXs, and PTX-2 were able to further reduce the MTT activity. Using NG108-15 cells and a similar MTT protocol, Cañete and Diogène also observed that AZA-1 induced a maximum reduction to about 40%. The EC_{50} values determined for OA, DTX-1, AZA-1 and YTX-1 in the NG108-15 assay are also in line with those published by Cañete and Diogène [36]. Serandour et al.[37] also observed a levelling off for AZA-1, but around 10% and they also observed that AZA-1 is more potent than OA and PTX-2, but the EC_{50} value of 6.8 nM was sevenfold higher than the one we and Cañete and Diogène obtained. The EC_{50} values determined for OA, DTX-1 and PTX-2 in the neuro-2a bioassay, being respectively 23.4, 5.5 and 76.4 nM, differ from those published by Cañete and Diogène [24], being respectively 21.9, 20.6 and 28.3 nM. Thus, similar EC_{50} values were obtained for OA, but lower and higher values for DTX-1 and PTX-2 respectively. Serandour et al. [37] reported values of 41.2 and 35.5 nM for OA and PTX-2, respectively, being twofold higher for OA than obtained by us and Cañete and Diogène and twofold lower for PTX-2 than observed in the present study. These differences are possibly caused by differences in quality of the standards, the applied solvents and solvent concentration, or the culturing and exposure conditions of the cells, e.g. 48 h compared to 24 h in our study.

Table 1 also shows the Toxicity Equivalency Factors (TEFs) as derived from the EC_{50} values in the neuro-2a bioassay and the TEFs for the OA/DTX and the AZA class of

toxins, as published in a joint FAO/WHO technical paper [38], both the ones based on experimental data, as well as recommended TEFs. The latter are quite similar to those proposed by EFSA, which should be used in routine testing. No full dose-response curves were obtained for DTX-2 within the concentration range tested and the EC_{50} values determined for DTX-2 should therefore be considered as less accurate. However, figure 1 demonstrates that DTX-2 is less toxic than DTX-1 (similar in NG108-15 cells, but curves not shown) and these observations are in line with those of the FAO/WHO and EFSA, and also with results obtained by Aune et al. (2007) with i.p. treated mice, showing a TEF of 0.6 for DTX-2 compared to OA. Still, the toxic potency of OA resembled more that of DTX-2 than that of DTX-1, which is in agreement with other in vitro data suggesting that DTX-1 is more potent than OA [39-41]. However, eventually both EFSA and FAO/WHO assigned a similar TEF to OA as DTX-1 (EFSA 2008, FAO/WHO 2016).

In the case of AZA-1, AZA-2 and AZA-3 relative potencies of 1, 0.53 and 0.67 were observed, which seems to disagree with the TEFs assigned by EFSA (2008) of 1, 1.8 and 1.4. However, based on more recent data from i.p. treated mice, FAO/WHO assigned TEFs of 1.0, 0.7 and 0.5, which are much more in line with our data. Overall, it is clear that there is still some uncertainty attached to the currently established and applied TEF values. Overall, the present study shows that neuro-2a cells are slightly more sensitive than NG108-15 cells, respond to all tested LMBs and their analogues, and display toxic potencies which are reasonably in line with the TEFs for these marine biotoxins as established by EFSA and the FAO/WHO. The assay based on neuro-2a cells was therefore used for testing shellfish samples.
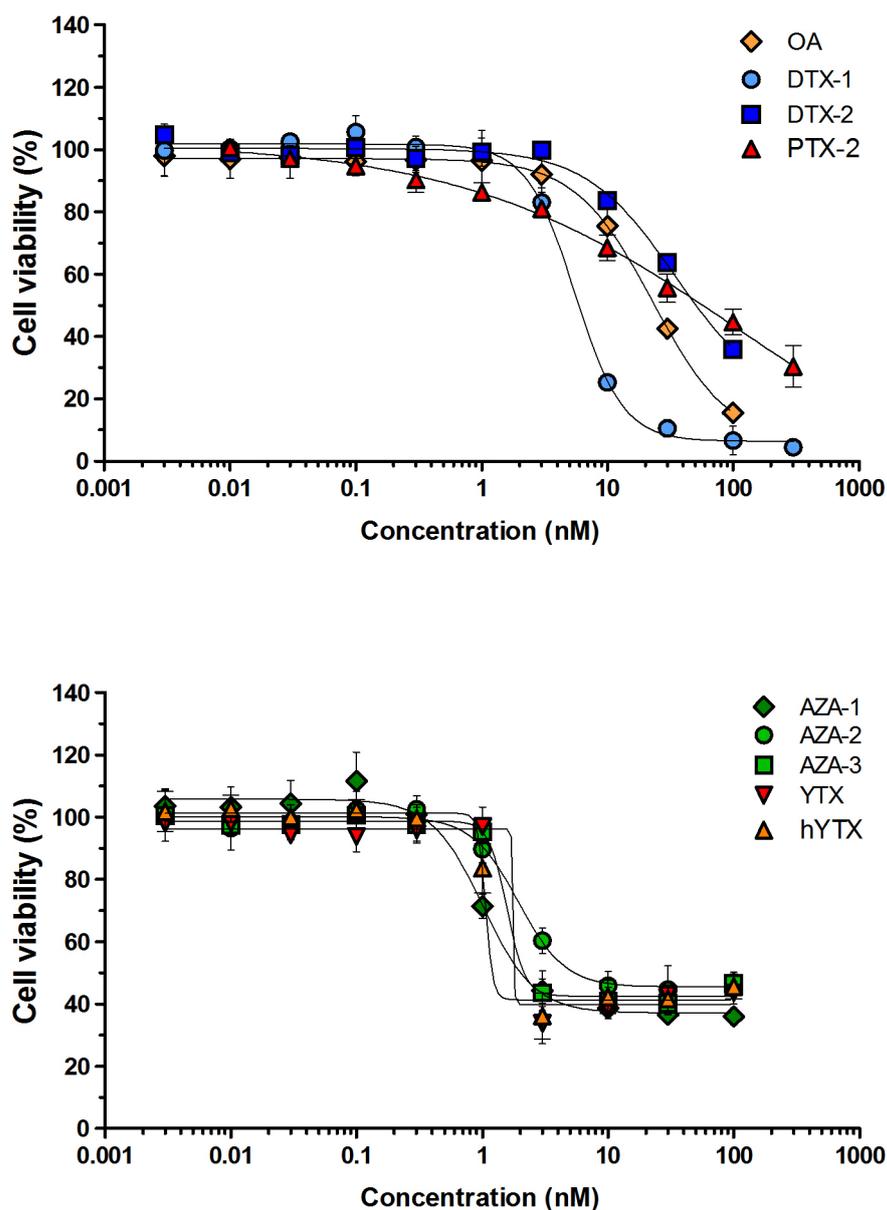
Figure 1. Effect of several lipophilic marine biotoxins on the viability of neuro-2a cells as measured with the MTT assay compared to the average of the solvent control (0.25% DMSO): (a) OA: okadaic acid; DTX-1, DTX-2: dinophysistoxin-1,-2; PTX-2: pectenotoxin-2; (b) AZA-1, AZA-2, AZA-3: azaspiracid-1,-2,-3; YTX: yessotoxin; hYTX: 1a homo yessotoxin. Data are expressed as mean ± SD (n = 3).

Table 1. Calculated EC_{50} values for the effect of lipophilic marine biotoxins on the viability of murine neuroblastoma neuro-2a cells, and murine neuroblastoma rat glioma hybrid NG108-15 cells, the resulting TEF values in the neuro-2a bioassay, and recommended TEFs by FAO/WHO and EFSA.

| Toxin | This study | | | FAO/WHO | | EFSA |
	Neuro-2a EC_{50} (nM)	NG105-15 EC_{50} (nM)	Neuro-2a TEF[d]	Observed	Recommended	TEF[c]
OA	23.0	22.9	1.0	1.0[b]	1.0	1.0
DTX-1	5.5	8.4	4.15	3.1[b]	1.0	1.0
DTX-2	34.4	28.9	0.67	0.52[b]	0.5	0.6
PTX-2	76.4	nd	0.30	nd	nd	1
AZA-1	1.0	2.6	1.0	1.0[a]	1.0	1.0
AZA-2	1.9	4.5	0.53	0.6[a]	0.7	1.8
AZA-3	1.6	4.3	0.66	0.45[a]	0.5	1.4
YTX	1.6	3.4	1.0	nd	nd	1
hYTX	1.1	2.7	1.56	nd	nd	1

a   TEF based on mice i.p. injected [38]
b   TEF based on in vitro assays, using mammalian cell lines and measuring cytotoxicity [38]
c   TEFs recommended by EFSA [42]
d   Derived by dividing the EC_{50} of the reference compound (OA, AZA-1 or YTX) by that of the analogue. A higher TEF means a higher potency.
nd   not determined

3.2 Sensitivity of the assay in relation to the maximal allowed levels

The current EU limits for the regulated LMBs and the above determined sensitivities of the neuro-2a cells for these toxins were used to calculate the required sample amount and the dilution of the prepared sample extract in the cell culture medium. All regulated LMBs should be detected at levels below their established limits. As such, the worst-case scenario is the limit for the sum of OA, DTXs and PTXs, being 160 µg OA-eq kg-1, as this is the lowest maximum permitted level (MPL), whereas these toxins display the highest EC_{50} values. Following our extraction protocol (i.e. 0.48 grams shellfish diluted in an equivalent of 8 ml medium), and assuming 100% recovery, a sample contaminated with 160 µg OA per kg would theoretically result in a medium concentration of 11.9 nM OA (Mw 805). This is just high enough to cause an effect in the neuro-2a cells (figure 1; table 1). This also

applies for DTX-2 (11.9 nM, Mw 805), whereas a similar level of DTX-1 (11.7 nM, Mw 819) would cause an almost complete inhibition of the MTT-activity. On the other hand, PTX-2 (11.2 nM, Mw 859) would not be detected at this level. Whether the test would work in practice for pectenotoxins would depend on the actual combinations of toxins occurring in shellfish samples. As PTX-2 is produced by the same algae as DTX and OA, and any sample containing PTX-2 above the limit would contain even higher levels of DTX and OA [43-46], the relatively high EC_{50} value for PTX-2 is probably not limiting the suitability of the neuro-2a bioassay for selecting contaminated samples. Moreover, in shellfish PTX-2 is known to be converted into the non-toxic PTX-2 seco acid metabolite [47].

It is important to realise that as a consequence of the used sample amount and dilution factor required to detect OA, the test will be relatively sensitive for samples contaminated with yessotoxins. Since YTXs have a high EU limit of 3750 µg YTX-eq kg-1, this amount would theoretically correspond to a well concentration of 196.9nM YTX (Mw 1143) with the applied protocol, and thus display clearly reduced MTT-activity in the neuro-2a bioassay (table 1: EC_{50} 1.6 nM). Therefore, samples containing YTX-eq levels well below 3750 µg kg^{-1}, will also be screened as suspect. For azaspiracids, the limit is 160 µg per kg and this level would result in a medium concentration of 11.4 nM AZA-1 (Mw 842), 11.2 nM AZA-2 (Mw 856) and 11.6 nM AZA-3 (Mw 828). Given the low EC_{50} values, these toxins should easily be detected with the current test protocol.

3.3 Blank samples and the effect of the improved clean-up with n-hexane

An additional clean-up step using n-hexane (see experimental section) was introduced to eliminate matrix effects that would otherwise result in a high percentage of false-positive outcomes in the neuro-2a bioassay, as was observed before by Ledreux et al. (2012). This additional cleaning step was introduced before the SPE clean-up was performed. The matrix effect is demonstrated in Figure 2a, showing the results of 10 blank mussel samples without the additional n-hexane wash step, both without and with an additional 10-fold dilution. All undiluted blank sample extracts caused a marked reduction in the MTT activity and were thus falsely screened as suspect. A blank chemical control was included, and this extract caused no cytotoxicity. Tenfold diluted sample extracts were not toxic anymore, but

as explained above, this results in too low sensitivity of the assay for most toxins. Figure 2b shows the results of 20 blank mussel samples when the n-hexane wash step was included. Undiluted blank sample extracts did no longer show a strong decrease in MTT activity, implying that the n-hexane wash step worked very well in order to remove false positives due to matrix effects. The observed cytotoxic effects without the extra n-hexane wash step, i.e. matrix effects, are most probably caused by free fatty acids, also known to interfere with the outcome of the MBA [48].

Based on the data obtained with the new procedure including the n-hexane step and blank mussel samples (figures 2b), an 'arbitrary' decision limit (ADL) was set at a reduction of the MTT activity of 25% or more. Samples with an MTT value above this decision limit are classified as negative (safe) and samples resulting in MTT values below this decision limit are classified as suspect (potentially unsafe).

Figure 3 shows the results of blank mussel samples spiked at 1/3, 1 and 3 times the MPLs for OA, DTX-1 and AZA-1, i.e. 53, 160 and 480 µg kg^{-1}, and at about 1/4 MPL for YTX, i.e. 1000 µg kg^{-1}, using the n-hexane wash and the subsequent SPE clean-up. The spiking was performed to an aliquot of the methanol extract, i.e. in order to reduce the required amount of the expensive standards. Moreover, the methanol extraction is already known to result in high extraction efficiencies for the different LMBs [49]. The results show that all spiked samples reduced the MTT-activity to 75% or lower when spiked at or above their MPL in case of OA, DTX-1 and AZA-1 and in case of YTX even at 1/4 MPL, implying that all toxins could be detected at levels above their MPLs. This includes the above described worst case of OA, the toxin with the lowest MPL (160 µg OA-eq kg^{-1}) and relatively high EC$_{50}$ value (23.4 nM, table 1). In addition, LC-MS/MS analyses of samples spiked at their MPL for OA, DTX-1, AZA-1 and at 1/4 MPL for YTX just before and after the n-hexane clean-up elicited recoveries of 90, 123, 96 and 104%, respectively. This shows that no toxins were lost in the n-hexane. Since the comparison is based on extracts spiked just before and after hexane extraction, recoveries above 100% point to the effectiveness of the n-hexane wash step for removing small matrix effects for LC-MS/MS analysis too, i.e. probably removing compounds that otherwise would cause a little bit of ion suppression. Although the recovery losses from the use of SPE were not determined

in the present study, previous studies demonstrated that losses due to the SPE were lower than 15% [50].
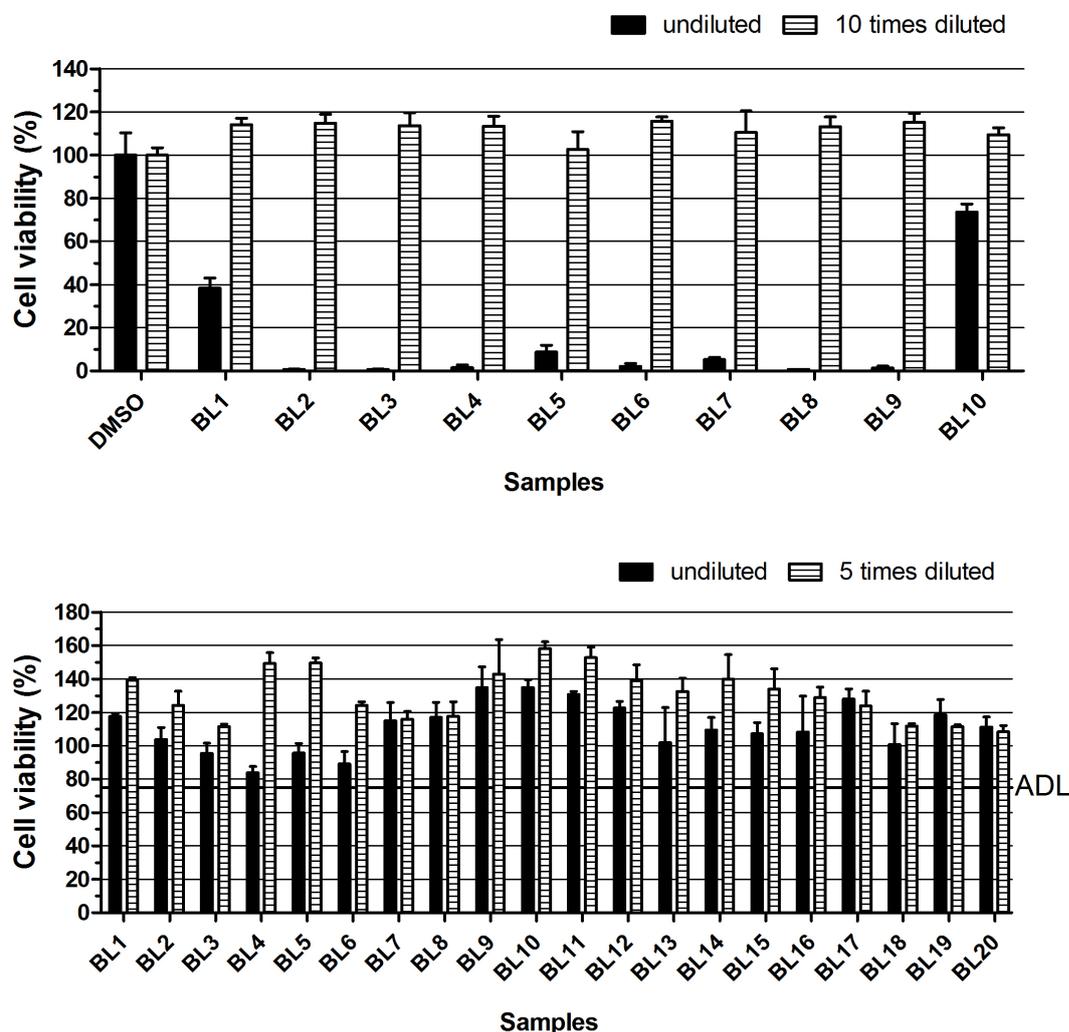
Figure 2. Effect of the introduction of an additional n-hexane wash step in the sample extraction procedure: (a) undiluted and 10fold diluted sample extracts of blank mussel samples prepared without the n-hexane wash step (b) undiluted and fivefold diluted sample extracts of blank mussel samples prepared by introducing the n-hexane wash step. ADL is the "arbitrary" decision limit, set at 75% cell viability. Data are expressed as mean ± SD (n = 3).
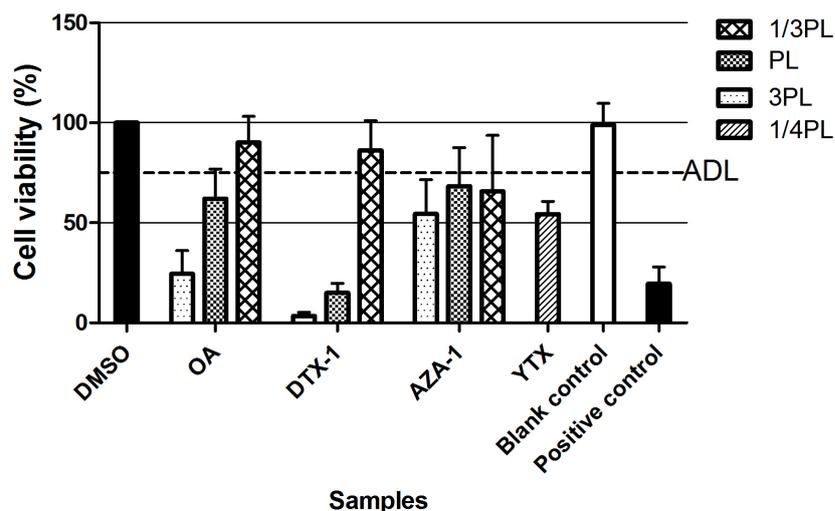
Figure 3. Mussel samples spiked at 3, 1 and 1/3 times the maximal permitted levels (MPLs) of 160 µg kg^{-1} for OA, DTX-1 and AZA-1, and at about 1/4 MPL for YTX, i.e. 1000 µg kg^{-1}, extracted with the procedure including the extra n-hexane wash step and analysed in the neuro-2a bioassay. An 'arbitrary' decision limit (ADL) of 75% was used. Positive control: DTX-1 12nM. Data are expressed as mean ± SD (n = 3).

3.3 Shellfish samples contaminated with lipophilic marine biotoxins

To allow a first evaluation of the performance of the newly developed clean-up method, extracts were prepared from eight samples (S1-S8) naturally contaminated with various LMBs that were previously used in an inter-laboratory validation study of the LC-MS/MS method [34]. These contaminated samples were prepared by blending naturally contaminated samples with various toxin profiles with blank samples in order to get a variety of materials with different profiles and levels. Figure 4a shows the results as obtained in the neuro-2a bioassay. A summary of the bioassay and LC-MS/MS results is given in table 2, showing that these validation samples were contaminated with levels of OA/DTXs or AZAs above regulatory limits. As all samples elicited a decrease in cell viability below that of the 'arbitrarily' set decision limit, they were all correctly classified as suspect. Three additional blank control samples caused no decreased response. It should be pointed out that six of the eight samples contained YTXs, and S2 and S3 at relative high amounts of 1702 and 1110 µg kg^{-1} respectively. This would most likely result in a suspected response also when present alone. However, samples S4 and S8 do not contain YTXs and were also classified correctly as being suspect, due to the presence of OA/DTX alone (S8) or in the

presence of AZAs (S4). Based on this, it seems possible that also the response obtained with samples S1, S5, S6 and S7 are to a large extent caused by toxins other than YTXs.

Figure 4b shows the results of eight unblended samples naturally contaminated with LMBs that were previously analysed by LC-MS/MS (table 2, lower part). All samples resulted in MTT values below the decision limit and were classified as suspect. As shown in table 2, all samples contained detectable toxin levels, some well above (samples 3 and 4), or just below (samples 1 and 2) the EU-limits (160 µg OA-eq kg^{-1}, 160 µg AZA-eq kg^{-1} and 3750 µg YTX-eq kg^{-1}). Samples 6, 7 and 8 contained elevated levels of YTXs, but far below the limit, confirming the sensitivity of the assay for this class of toxins. Sample 5, which showed a response just below the ADL, only contained low amounts of AZAs, less than 1/5 of the regulatory limit. The bioassay classification of these samples was thus in line with the toxin levels measured by LC-MS/MS, taking into consideration the sensitivity of the assay for YTXs.
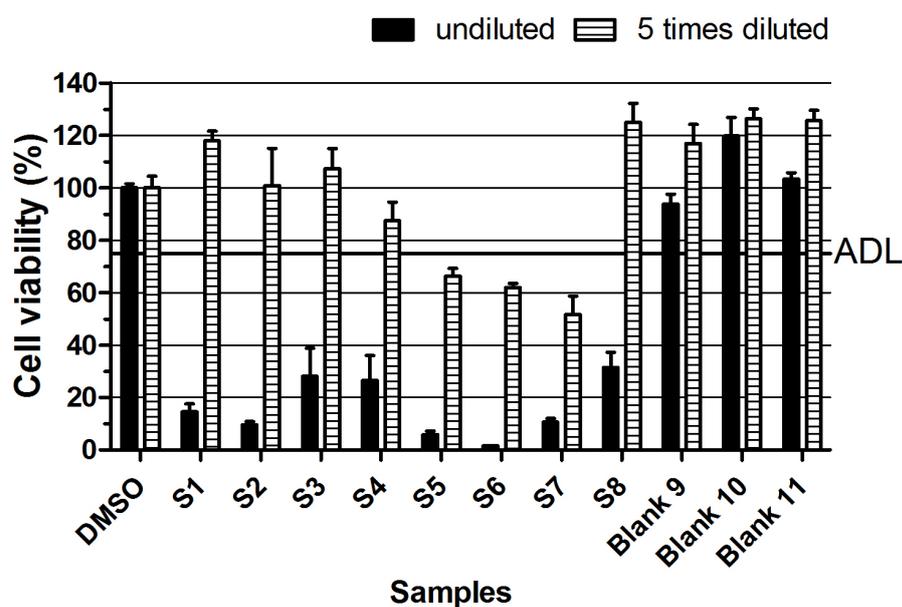
Figure 4a. Effect on the viability of neuro-2a cells (as measured with the MTT assay) of shellfish products (validation samples S1-S8) contaminated with okadaic acid/dinophysistoxins, yessotoxins and/or azaspiracids (table 2) and three blank shellfish samples (samples 9-11)
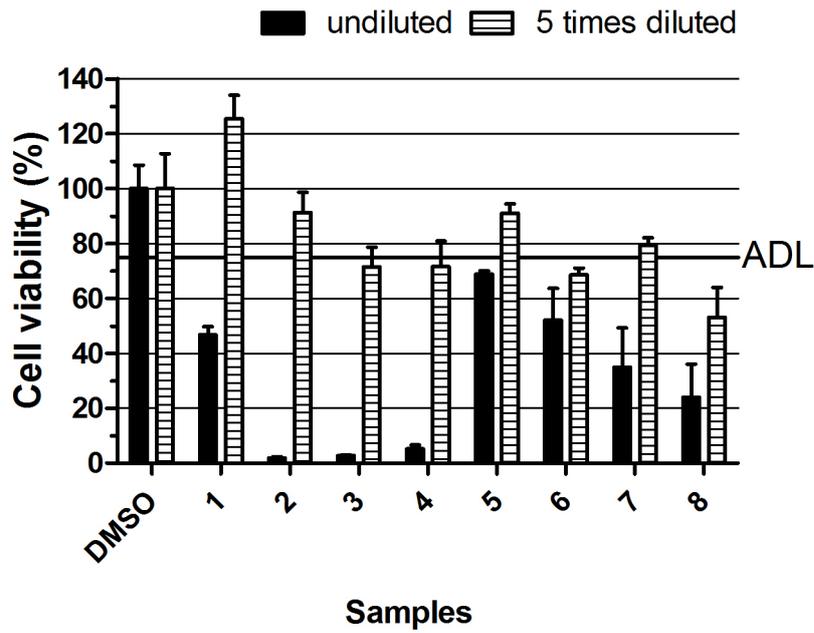
Figure 4b. Effect on the viability of neuro-2a cells (as measured with the MTT assay) of eight mussel samples (1-8) naturally contaminated with detectable levels of one or more LMBs (table 2). An 'arbitrary' decision limit (ADL) of 75% was used and 0.25% DMSO was included as a control in each experiment. Data are expressed as mean ± SD (n = 3).

Table 2. LC-MS/MS determined levels of lipophilic marine biotoxins in 16 naturally contaminated shellfish samples compared to the outcome of the neuro-2a bioassay. Samples (S1-S8) were mixed samples used for validation of LC/MS.

Sample	OA, DTXs, PTX-2 (1)	AZAs (2)	YTXs (3)	Outcome neuro-2a bioassay
S1	299	175	255	Suspect
S2	168		1702	Suspect
S3	243	280	1110	Suspect
S4	401	386		Suspect
S5	757		462	Suspect
S6	85	310	615	Suspect
S7	1293	381	351	Suspect
S8	371			Suspect
1	151			Suspect
2	123		481	Suspect
3	275	2422		Suspect
4	68	1528		Suspect
5		32.4		Suspect
6			770	Suspect
7			330	Suspect
8			620	Suspect

(1) (μg OA-eq kg^{-1}), (2) (μg AZA^{-1}-eq kg^{-1}), (3) (μg YTX-eq kg^{-1})
OA: okadaic acid, DTX: dinophysistoxin, AZA: azaspiracid, YTX: yessotoxin, n: negative
missing values means below limit of detection (<LOD)

Next, 48 sample extracts obtained from Chile, derived from different species of bivalves and gastropods, were tested in the neuro-2a bioassay and also analysed by LC-MS/MS for lipophilic marine biotoxins (OA, DTXs, PTX-2, AZAs and YTXs). Although many samples concerned the hepatopancreas or viscera and as such levels should be divided by at least a factor of 3 [51], this was not considered for the evaluation of the assay performance. Figure 5 shows the results of these Chilean samples as tested in the neuro-2a bioassay. Samples 4, 6, 17, 25, 28, 30, 34 and 45 decreased the cell viability as determined with the MTT assay and were screened as suspect in the neuro-2a bioassay. Table 3 shows

the LC-MS/MS results, revealing that all suspected samples, except sample 30, presented relatively high amounts of YTX-eq. The highest levels were observed in the viscera of a sea snail (17, 28, 45), followed by the hepatopancreas of two types of mussels (34, 38). Four of these samples (17, 28, 34 and 45) also contained low levels of OA-eq, unlikely to have contributed much to the response. None of the 48 samples contained PTXs or AZAs. From a qualitative point of view, there is a good correlation between the neuro-2a bioassay outcomes and the LC-MS/MS analysis. All 40 samples with no detectable toxins or only trace amounts (<10% of the limit) showed a negative result. Of the eight samples that tested suspected, seven contained relatively high levels of YTX (477-3472 μg YTX-eq kg^{-1}).
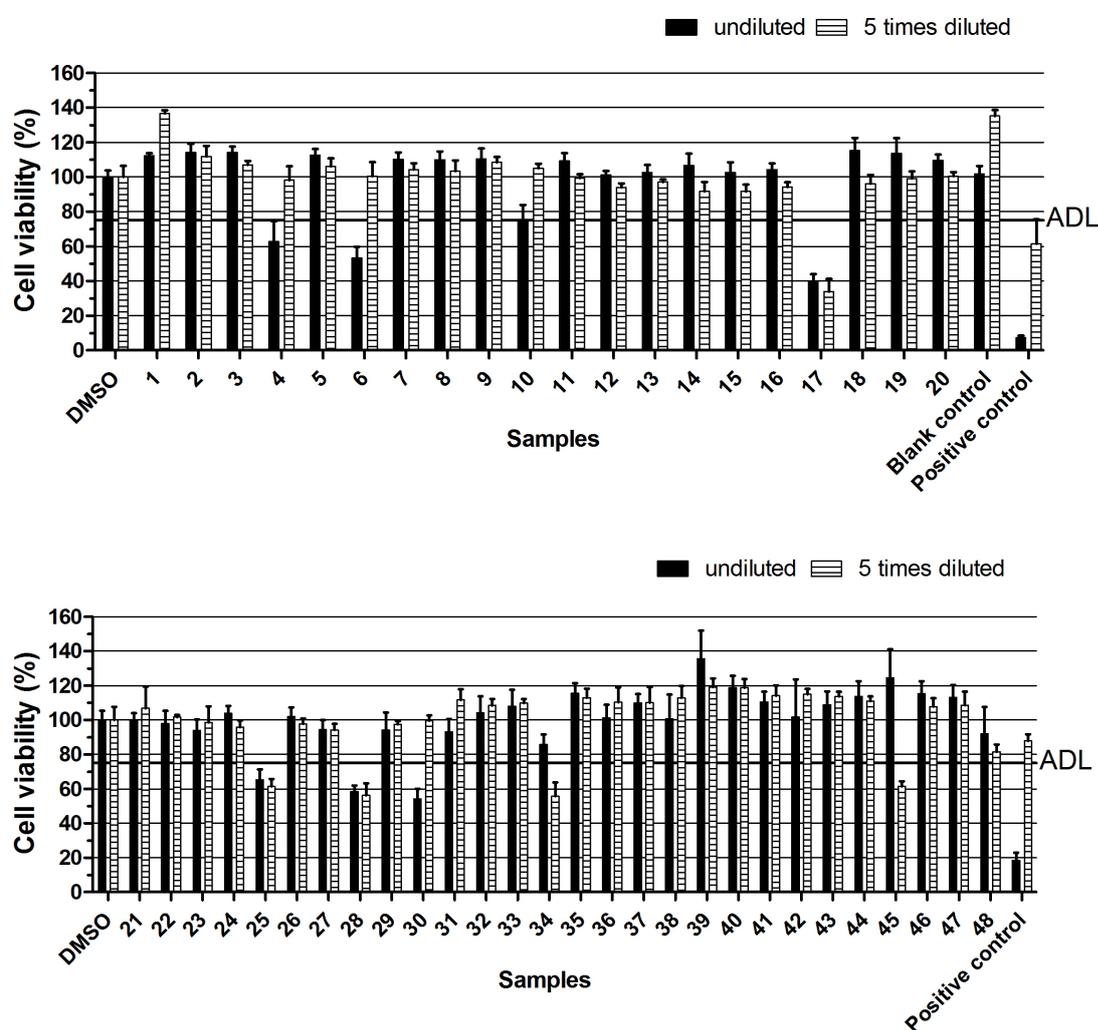
Figure 5. Effect of extracts of 48 seafood products obtained from Chile and both positive and blank mussel sample controls, on the viability of neuro-2a cells as measured with the MTT assay. Positive sample control: mussels naturally contaminated with YTX. The decision limit was 'arbitrarily' set at 75% viability (ADL) and 0.25% DMSO was included as a control. Data are expressed as mean ± SD (n = 3).

Table 3. Effect of Chilean seafood extracts on the viability of neuro-2a cells and levels of lipophilic marine neurotoxins measured by LC-MS/MS.

Number	Scientific name	Tissue	OA, DTXs, PTX-2[1]	YTXs[2]	Outcome neuro-2a bioassay
1	*Argobuccinum ranelliforme*	foot			n
2	*Chorumytilus chorus*	mantle			n
3	*Mytilus chilensis*	adductor muscle		9.9	n
4	*Aulacomya atra*	hepatopancreas		477.4	suspect
5	*Venus antiqua*	mantle			n
6	*Aulacomya atra*	hepatopancreas		660.2	suspect
7	*Venus antiqua*	hepatopancreas			n
8	*Fissurella sp.*	foot			n
9	*Venus antiqua*	hepatopancreas			n
10	*Aulacomya atra*	hepatopancreas		47	suspect
11	*Concholepas concholepas*	viscera		191.7	n
12	*Gari solida*	hepatopancreas			n
13	*Mytilus chilensis*	hepatopancreas			n
14	*Chorumytilus chorus*	hepatopancreas		49.9	n
15	*Chorumytilus chorus*	hepatopancreas			n
16	*Venus antiqua*	hepatopancreas			n
17	*Argobuccinum ranelliforme*	viscera	27.5	4026.9	suspect
18	*Pleuroncodes monodon*	whole body		27.4	n
19	*Aulacomya atra*	hepatopancreas		117.3	n
20	*Concholepas concholepas*	foot			n
21	*Argobuccinum ranelliforme*	foot		5.4	n
22	*Venus antiqua*	hepatopancreas			n
23	*Concholepas concholepas*	viscera		701.4	n
24	*Chorumytilus chorus*	mantle		34.5	n
25	*Chorumytilus chorus*	hepatopancreas		654.7	suspect
26	*Aulacomya atra*	mantle		2.1	n
27	*Aulacomya atra*	gill		3.8	n
28	*Argobuccinum ranelliforme*	viscera	5.7	3471.8	suspect

29	*Argobuccinum ranelliforme*	foot		2.7	n
30	*Chorumytilus chorus*	adductor muscle		185.4	suspect
31	*Venus antiqua*	hepatopancreas			n
32	*Gari solida*	hepatopancreas			n
33	*Cancer spp.*	cheliped			n
34	*Chorumytilus chorus*	hepatopancreas	5.7	2098.1	suspect
35	*Concholepas concholepas*	foot			n
36	*Gari solida*	hepatopancreas			n
37	*Gari solida*	foot			n
38	*Aulacomya atra*	hepatopancreas		1787.3	n
39	*Venus antiqua*	hepatopancreas			n
40	*Chorumytilus chorus*	foot		35.2	n
41	*Aulacomya atra*	adductor muscle			n
42	*Venus antiqua*	hepatopancreas			n
43	*Venus antiqua*	adductor muscle			n
44	*Venus antiqua*	foot			n
45	*Argobuccinum ranelliforme*	viscera	2	4682.6	suspect
46	*Argobuccinum ranelliforme*	foot		23.4	n
47	*Fissurella sp.*	foot			n
48	*Fissurella sp.*	hepatopancreas		121.7	n

(1): µg OA-eq kg^{-1}, (2): µg YTX-eq kg^{-1} *missing samples are negative in the neuro-2a bioassay and LC-MS/MS, e.g. just as sample# 1*; n: negative; missing values means below limit of detection (<LOD)

Nevertheless, there are some discrepancies when analysing the data on a semi-quantitative level, in particular when focussing on samples 23, 30, 38, and 45. Sample 45 was screened suspect based on testing the diluted extract. A similar decrease in activity upon dilution was observed for sample 34 and this requires further investigation. Samples 23 and 38 were screened as negative, but contained relatively high amounts of YTX-eq, 701 and 1787 µg YTX-eq kg^{-1} respectively. Sample 30 was suspect in the bioassay, but according to the LC-MS/MS contained YTX only and at a low level (185 µg YTX-eq kg^{-1}). This level would result in a medium concentration of 3 nM and thus could be enough to cause an effect in the neuro-2a bioassay (Figure 1), but it cannot fully be ruled out that this sample may contain yet unknown lipophilic toxins, missed by LC-MS/MS. This might be unknown

YTX analogues, but also another type of lipophilic toxin. Brevetoxins (PbTXs) and palytoxins (PlTxs) are known to be extracted with methanol as well [25, 52]. However, to detect PbTXs, the addition of ouabain and veratridine (o/v) during exposure of the cells is needed. These compounds interact with the sodium voltage-gated channels in the cells, causing cell death [3, 53]. The addition of o/v in a concentration causing a 20% reduction of cell viability is needed to detect PbTXs, which then cause a further decrease of the cell viability [54]. PlTx is also able to decrease the MTT activity without the addition of o/v (EC$_{50}$ for PbTX-3, PbTX-9 and PlTx in the neuro-2a bioassay of 8, 8.4 and 0.04 nM respectively, data not shown). Although dedicated LC-MS/MS methods for PbTXs and PlTx are available, these methods are not routinely applied as there are no certified standards and legislation is lacking for these toxins. Overall the bioassay classification of these Chilean samples was in line with the lipophilic toxin levels measured by LC-MS/MS. However, it should be pointed out that samples contained primarily yessotoxins.

4. Conclusions and Recommendations

Although the neuro-2a bioassay is regarded as suitable for PSP toxins and has occasionally been used to analyse samples for the presence of PSP toxins [28-30], its routine application for these toxins is still rather limited. And although the test has also been shown to detect various classes of LMBs, up till now it is not considered for routine testing of shellfish for LMBs. Lack of routine use is due to observed matrix effects, and as a result a lack of studies on performance with routine samples and (international) validation studies. The present paper is the first describing the performance of the neuro-2a bioassay for routine testing of seafood samples on the presence of LMBs using a newly developed clean-up procedure including an n-hexane wash step, thereby allowing identification of potential issues to be improved and whether the assay is worth the further intensive and expensive efforts needed for a full validation. Although the rationale between the toxic effects of LMBs as observed in mouse and humans and the cell viability of neuro cells is missing, the study confirms previous reports showing that the neuro-2a bioassay allows the detection of the regulated LMBs at the required levels. Also, the few commercially available analogues of these LMBs could be detected and there is a reasonable correlation between toxic potencies and TEFs

established by EFSA and WHO/FAO. Compared to the current analytical and immuno-based alternatives, the neuro-2a bioassay will most likely be able to detect unknown analogues and yet unknown marine biotoxins too.

The newly introduced n-hexane wash step is an important improvement in order to eliminate matrix effects causing too many false-positive screening outcomes, which would preclude its routine application. These effects were most probably caused by free fatty acids known to interfere also with the outcome of the MBA. The new clean-up procedure worked well for mussels but was also successful with some cockles and oysters that we tested (latter data not shown) and the Chilean samples that included several species (this study).

An 'arbitrary' decision limit was set for the routine screening of real samples, which might be refined based on further experience and validation studies. In a qualitative way, the neuro-2a bioassay outcomes correlated well with the LC-MS/MS analysis. Among the 87 samples screened for the presence of lipophilic marine biotoxins by the neuro-2a bioassay, 25 were screened as suspect and 62 as negative, while LC-MS/MS identified 12 positives out of the 25 suspected samples (ignoring that some samples were viscera and not whole flesh). No false-negative screening results were obtained. Many of the 13 false-positive samples turned out to contain elevated levels of toxins, some just below the regulatory limits.

However, in particular the relatively high sensitivity of the assay for yessotoxins resulted in a number of clearly false-positive results. Whether this is a problem in routine screening depends on the actual occurrence of these toxins in a certain production area. In the Netherlands for example, samples rarely contain any of the LMBs, and detection of samples with even low levels of marine biotoxins would probably be welcomed as an early warning. However, this may be different in areas where yessotoxins occur regularly. For such cases, a different clean-up procedure could be developed in order to separate the YTXs from the other marine toxin classes. Moreover, it should be noticed that the n-hexane step will remove esterified forms of okadaic acid and the dinophysis toxins, which are mentioned in the legislation under the generic term DTX-3. Thus, for application to real samples in the future, a hydrolysis step prior to the n-hexane wash step and SPE clean-up should be

incorporated as well (which is already performed prior to LC-MS/MS analysis for the determination of DTX-3). Finally, the suitability of the neuro-2a bioassay should be further assessed by testing samples that have been tested negative and positive for LMBs in the MBA.

In summary, the present data show that the neuro-2a bioassay is worth the intensive and expensive efforts needed for a full validation which could result in a cheap and fast screening method for testing shellfish for the presence of DSP toxins and AZAs, i.e. separating negative samples from those potentially contaminated above MPLs, which are then further analysed by LC-MS/MS. Since it can detect unknown analogues, it is the best alternative to the MBA, both for LMBs and other marine biotoxins.

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Chapter 3

Whole genome mRNA transcriptomics analysis reveals different modes of action of the diarrhoeic shellfish poison toxins okadaic acid and dinophysis toxin-1 versus azaspiracid-1 in Caco-2 cells

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Summary

A study with DNA microarrays was performed to investigate the effects of two diarrhoeic and one azaspiracid shellfish toxins, okadaic acid (OA), dinophysistoxin-1 (DTX-1) and azaspiracid-1 (AZA-1) respectively, on the whole-genome mRNA expression of undifferentiated intestinal Caco-2 cells. Previously, the most responding genes were used to develop a dedicated array tube test to screen shellfish samples on the presence of these toxins. In the present study the whole genome mRNA expression was analysed in order to reveal modes of action and obtain hints on potential biomarkers suitable to be used in alternative bioassays. Effects on key genes in the most affected pathways and processes were confirmed by qRT-PCR. OA and DTX-1 induced almost identical effects on mRNA expression, which strongly indicates that OA and DTX-1induce similar toxic effects. Biological interpretation of the microarray data indicates that both compounds induce hypoxia related pathways/processes, the unfolded protein response (UPR) and endoplasmic reticulum (ER) stress. The gene expression profile of AZA-1 is different and shows increased mRNA expression of genes involved in cholesterol synthesis and glycolysis, suggesting a different mode of action for this toxin. Future studies should reveal whether identified pathways provide suitable biomarkers for rapid detection of DSP toxins in shellfish.

1. Introduction

Marine biotoxins are a large group of compounds produced by various types of organisms in the marine environment, i.e. algae, bacteria and coral. These toxins can accumulate in filter feeding shellfish and finfish [1, 2]. Human consumption of marine products contaminated with these toxins can lead to food poisoning with symptoms varying from paralysis, diarrhoea, amnesia to even death [3, 4]. Shellfish poisons that induce diarrhoea (DSP toxins) include okadaic acid (OA) and its analogues dinophysis toxins 1 (DTX-1) and 2 (DTX-2), and their esterified forms (DTX-3). They are produced by dinoflagellates from the genders *Dinophysis* spp. and *Prorocentrum* spp. [5, 6]. Another important group of marine biotoxins concerns azaspiracids (AZAs) causing effects like vomiting, diarrhoea, stomach cramps and nausea. These are polyether toxins with two spiro-ring assemblies, a cyclic amine and a carboxylic acid [7-9]. AZA-1 and AZA-2 are produced by the dinoflagellate *Azadinium spinosum*. AZA-3 and another 30 analogues are metabolites of AZA-1 or AZA-2 that are formed within the shellfish [10, 11].

Until recently, the mouse bioassay (MBA) was the reference method in the EU for the detection of lipophilic marine biotoxins (LMB) like OA, DTXs and AZAs and it is still the reference method in some countries outside the EU [12]. Since January 2015, however, the mouse bioassay has been replaced in the EU by LC-MS/MS as the reference method for routine monitoring of lipophilic shellfish toxins, mainly because the mouse bioassay shows high variability in results, has insufficient detection capability and limited specificity [13]. Methods such as other LC based methods with appropriate detection, immunoassays and functional assays (e.g. the phosphatase inhibition assay) are allowed as alternative or supplementary to the LC-MS/MS method, provided that they are properly validated [13]. However, the LC-MS/MS method also has some drawbacks, i.e. certified standards of many toxins are unavailable or very expensive, and analytical methods are unable to detect presently unknown toxins or analogues [14]. For this reason, many EU countries hesitate to rely solely on LC-MS/MS analysis and for monitoring of production areas for the presence of (unknown) marine biotoxins, the use of the MBA is still allowed in the EU [13]. *In vitro* cell-based assays offer potentially useful alternative approaches, since these bioassays detect toxins on the basis of their effect and most likely will detect unknown analogues as well [15]. The neuro-2a cell line has been shown to detect various marine biotoxins, using

a method based on measuring cell viability, e.g. based on MTT-activity [16]. However, in routine monitoring, it is not only interesting to apply a bioassay that would be able to detect all relevant biotoxins, but ideally can also discriminate between the various toxin classes. Functional assays have e.g. been developed for OA, DTX and their analogues based on their inhibition of protein phosphatases (PPs), particularly PP1 and PP2A. This inhibition causes permanent phosphorylation of proteins leading to effects on regulation of glycogen metabolism, transcription, cell differentiation, cell adhesion, apoptosis and DNA replication, as shown in several cell lines [17-19]. The mode of action of AZA-1 is still unknown, but this toxin has been reported to induce mRNA expression of genes involved in cholesterol and fatty acid synthesis in Jurkat immune cells, indicating that AZA-1 activates these processes [20].

In order to identify other toxin specific biomarkers, the present study aimed to investigate the mode of action of two important classes of DSP toxins, being OA and its analogue DTX-1, as well as AZA-1. Undifferentiated human intestinal Caco-2 cells, a potentially suitable cell-line for toxin detection, were exposed to these three toxins and effects on whole genome mRNA expression were investigated by DNA microarray analysis. Caco-2 cells were initially selected because they represent the target tissue. However, it was decided to use undifferentiated cells because they are easier to handle for a bioassay that should be applicable in routine, where a test result should be provided within a couple of days and where the demand for testing can rapidly increase in case of an incident. Furthermore, undifferentiated cells have been shown to be more sensitive to the action of the protein phosphatase inhibition exerted by OA and DTX-1 [21]. In a previous work, a set of the most responding genes was selected and used to develop a dedicated array tube method to detect these toxins in shellfish samples [22]. However, this assay was considered not to be ideal in routine testing since it required too much time. In the present study, the whole genome mRNA expression dataset was therefore thoroughly analysed in order to reveal further insight in the mode of action of these toxins. Affected pathways might be further explored to identify both general and specific biomarkers for these groups of toxins.

2. Materials and methods

2.1 Chemicals

OA and AZA-1 were obtained from the National Research Council, Institute of Marine Biosciences (NRC CNRC, Halifax, Canada). DTX-1 was kindly donated by Dr. M. Sandvik (National Veterinary Institute, Oslo, Norway). For the toxins dissolved in methanol: the solvent was evaporated under a nitrogen flow and toxins were dissolved in DMSO. DMSO, ethanol and chloroform were obtained from Merck (Darmstadt, Germany). Isopropanol was obtained from Biosolve (Valkenswaard, the Netherlands), Trizol from Invitrogen (Bleiswijk, the Netherlands), and phosphate buffered saline (PBS) from Oxoid (Hampshire, England).

2.2 Cell culture

The human colonic adenocarcinoma cell-line Caco-2 (ATCC, Manassas, VA) was grown in Dulbecco's Modified Eagle Medium (DMEM) obtained from Lonza (Verviers, Belgium) supplemented with 10% (v/v) foetal bovine serum (FBS) from Gibco BRL (Life Technologies Ltd., Paisly, Scotland), non-essential amino acids from MP Biomedicals (Illkirch, France) and penicillin 0.1% v/v (50 mg mL^{-1}) from Sigma (St. Louis, MO). The cells were grown in 75 cm^2 flasks at 37 °C and 5% CO_2.

2.3 Cytotoxicity assays

For the MTT assay (Sigma, Zwijndrecht, the Netherlands), 100 µL of Caco-2 cell suspension was seeded per well in a 96-well plate (Ref. Number 3595, Corning, NY), using 8 x 10^4 cells per mL and incubated for 48 h at 37 °C and 5% CO_2, to reach 80-90% confluence. Then, cells were exposed to a range of 2.5 to 50 nM of OA, DTX-1 and AZA-1 for 24 h. DMSO 0.25% (v/v) was included as vehicle control. Thereafter, media were aspirated and 60 µL of MTT reagent (final concentration of 0.8 mg mL^{-1}) dissolved in medium was added per well. Cells were incubated for 30 min at 37 °C, 5% CO_2, media were aspirated and 100 µL DMSO was added per well. After shaking thoroughly for 10 min, the absorbance was measured at 540 nm and corrected for background absorption at 650 nm. Data were expressed as percentage of control, i.e. DMSO, which is considered as 100% cell

viability. Three independent experiments were done, and the standard deviation of the control in each individual experiment was not higher than 10%.

For the LDH leakage assay, 100 μL of Caco-2 cell suspension was seeded in a 96-well plate (Ref. Number 3595, Corning, NY), using 8×10^4 cells per mL and incubated for 48 h at 37 °C and 5% CO_2, to reach 80-90% confluence. Cells were exposed for 24 h to the same concentration range of AZA-1 as was used for the MTT assay. DMSO was used as a vehicle control at 0.25% (v/v). After the incubation period, the LDH leakage assay was performed according to the manufacturer's instructions measuring absorbance at 492 nm (Cytotox 96® Non-Radioactive cytotoxicity assay, Promega, Leiden, the Netherlands).

2.4 Exposure for mRNA expression analysis by microarrays

Caco-2 cells were seeded in 6-well plates (Ref. Number 3516, Corning, NY), using 6×10^4 cells per mL, 3 mL per well and cultured for 48 h at 37 °C and 5% CO2 to reach 80-90% confluence. Four hours before exposure, the medium was refreshed. Then, cells were exposed to final concentrations of 25 nM OA, 6.25, 12.5 or 25 nM DTX-1, 6.25 or 25 nM AZA-1, or DMSO (0.25% (v/v), vehicle control). Exposures were performed in triplicate (3 wells per treatment in the same plate).

2.5 RNA isolation and purification for microarray analysis

Medium was removed and cells were lysed using TRIzol:chloroform (5:1) followed by centrifugation and collection of the aqueous phase containing RNA. The RNA was precipitated using isopropyl alcohol and washed with 70% ethanol:water (v/v). The obtained pellet was dried and dissolved in RNAse-free water. The RNAs were further purified using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) following the instructions of the manufacturer. The amount and quality of the RNA were evaluated by UV spectrophotometry (260 and 280 nm wavelength) on the Nanodrop spectrophotometer (Nanodrop technologies). RNA integrity was measured by automated gel electrophoresis (Experion, Biorad, Veenendaal, the Netherlands). The quality of the RNA samples obtained was 10, being the highest quality.

2.6 Microarray hybridizations

RNA samples were amplified and converted into Cy5-labeled cRNA using the Agilent low RNA input fluorescent amplification kit according to the manufacturer's instructions (Agilent Technologies, Amstelveen, the Netherlands). Universal human reference RNA (Stratagene, La Jolla, CA) was used as common reference and was labelled with fluorescent Cy3 dye (Perkin-Elmer/NEN). Equal amounts of Cy5-labeled cRNA and Cy3-labeled reference cRNA were mixed. Hybridizations were carried out on the 4 × 44K human whole genome Agilent microarray platform following the Agilent two-color microarray-based gene expression analysis protocol. Microarray slides were incubated for 16 to 17 h at 65 °C in a microarray incubation chamber with continuous rotation. After hybridization, the arrays were washed and dried at RT according to the manufacturer's protocol. Arrays were scanned using an Agilent microarray scanner (G2565B). In total, 21 arrays were hybridized. Due to technical failure, two arrays were excluded from further analysis: one of cells exposed to 6.25 nM AZA-1 and one of cells exposed to 25 nM AZA-1, leaving 19 arrays for analysis. Feature extraction 9.1 software (Agilent Technologies) was used for quantification of spot intensities. Quality check of arrays was carried out with LimmaGUI using the statistical package R. GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium) was used for background correction and normalization [23].

2.7 Microarray data treatment

Agilent microarrays (4 × 44K human whole genome) containing 41,108 spots were used. After background correction, 29,828 spots representing 20,442 unique gene IDs remained. Part of the genes are thus represented by multiple spots. Data were floored to reduce noise due to spots with very low spot intensities (not or very lowly expressed genes). For this, all spots with an intensity below 300 were annotated an intensity of 300. Subsequently, the intensity values were $\log_2$ mean centred. First, for each spot $\log_2$ ratios vs. the average of all arrays was calculated. Thereafter, $\log_2$ ratios of treatments vs. the average of the control samples (DMSO) were calculated in order to assess treatment effects.

Hierarchical clustering was performed with the programs Cluster (uncentred correlation; average linkage clustering) and Treeview [24]. The heatmap shows a red colour when the $\log_2$ values are higher than +0.2 and the maximum red colour when it is higher than +1.0. The green colour is showed when values are higher than -0.2 and the maximum green

colour when the values are higher than -1.0. Black represents 'no regulation', i.e. when the values are in between -0.2 and +0.2.

2.8 Biological interpretation

For genes within subclusters of the hierarchical cluster heatmap, pathway analyses were performed using Metacore and Consensus Path DB (CPDB) analysis. Metacore is an online program for functional and biological interpretation of gene expression data. Metacore uses hypergeometric distribution to assess significances for overrepresentation of affected genes in signalling and metabolic pathways [25]. CPDB analysis was applied using the web tool http://cpdb.molgen.mpg.de which combines and compares the results of multiple pathway databases [26]. For both methods, pathways with a p-value $<10^{-5}$ were considered to be affected significantly.

2.9 Gene set enrichment analysis (GSEA)

GSEA is a statistical analysis tool for biological interpretation of microarray data [27]. For the present study we used the visualization tool of GSEA to make heatmaps of pathways that were indicated to be affected based on pathway identification. We used gene sets from various sources, including "Reactome" (http://www.reactome.org/), "Gene Ontology human symbols" downloaded from Gene Ontology consortium (www.geneontology.org), "Entire route ER stress UPR" (self-made) based on the study of Katika et al. [28] and "Marine biotoxins" (self-made) based on the study of Twiner et al. [20].

2.10 Exposures for qPCR confirmation

Three independent exposures of Caco-2 cells were performed in order to confirm the up or downregulation of selected genes that play a role in the pathways that were identified by the transcriptome analysis. 600 μL of Caco-2 cell suspension were seeded in 24 well plates (Ref. Number 3524, Corning, NY), using 8 x10^4 cells per mL and incubated for 48 h at 37 °C and 5% CO_2 to reach 80-90% confluence. Cells were exposed to OA (25 nM), DTX-1 (6.25, 12.5 and 25 nM) and AZA-1 (6.25 and 25 nM) for 24 h. Final concentration of DMSO (vehicle control) in the well was 0.25%. Exposures were performed in triplicate. RNA was extracted using the QIA shredder and RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) following the instructions of the manufacturer. In short, the medium was removed and the

cells were lysed with 600 µL of RTL buffer with 1% β-mercaptoethanol. After the extraction, RNA quality and amount were evaluated according to the procedure described in section 2.5. The quality of the RNA obtained was 10, being the highest quality. cDNA was synthetized using 1 µg of RNA per sample and from an RNA pool mix of the 21 treatments using the Biorad iScript cDNA Synthese Kit with iScript and reverse Transcript (Biorad, 170-8891) in the BioRad iCycler. The program used was 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, and 5 minutes on ice. After the cDNA synthesis, the samples were diluted 100 times and the pool was diluted 10, 31.6, 100, 316, 1000 and 3160 times (i.e. needed to make a calibration line) and all cDNA samples were stored at -20 °C.

2.11 Real time qRT-PCR

Selection of genes to be confirmed by PCR was done using the highest induction/repression value obtained from the gene expression analysis (i.e. CPDB results), which were simplified in table 1 using the highest value of the triplicates or duplicated obtained after data analysis (see also section 2.6).

qRT-PCR was performed with certified QuantiTect primers from Qiagen (Venlo, the Netherlands) using 15 µL of final volume containing 8.5 µL SYBR green (BioRad 170-8880), 2.5 µL of the QuantiTect forward/reverse primer mix, 2 µL RNAse free water and 2 µL of diluted cDNA. Reactions were performed in a BioRad HSP9645 PCR plate. Water and 'pooled RNA without reverse transcriptase' were used as negative controls. The plate was covered with a micro seal and centrifuged for 1 min. Thermal cycling was performed in a CFX96 Real-Time System (BioRad), starting with a denaturation step at 95 °C for 3 min, followed by 45 cycles at 65 ° C for 35 s for annealing, 10 s at 95 °C for denaturation, and 1 min at 65°C for extension. Data were analysed using CFX manager 3.0 (BioRad) with the ΔΔCq approach. Gene TMEM179B was used for normalization as described in previous experiments [22]. Statistical significance of the qRT-PCR results was obtained from the same program. *P* values lower than 0.01 are indicated with ** and *P* values lower than 0.05 are indicated with *. Data are expressed as relative normalized expression levels *vs* the control (DMSO) ± SEM. Formulas are available in the CFX manager 3.0 program.

3. Results

3.1 Cell viability

Undifferentiated Caco-2 cells were exposed for 24 h to concentrations of OA, DTX-1 or AZA-1 ranging from 2.5 to 50 nM and effects on viability were assessed by the MTT assay. Cytotoxicity was defined as a decrease in cell viability of ≥20% relative to the solvent control (DMSO). Results are shown in Figure 1. OA did not decrease viability at the concentrations tested. For DTX-1, a reduction in the cell viability by over 20% was observed at 25 nM and higher concentrations. In contrast, AZA-1 increased the conversion of the MTT reagent up to 150% at all concentrations tested. This unexpected effect was highly reproducible. As the effect of AZA-1 in the MTT assay was unexpected and very specific, the toxicity of AZA-1 was also assessed using the LDH leakage assay. That assay demonstrated that none of the AZA-1 concentrations tested resulted in an increased or decreased LDH leakage into the medium (data not shown).
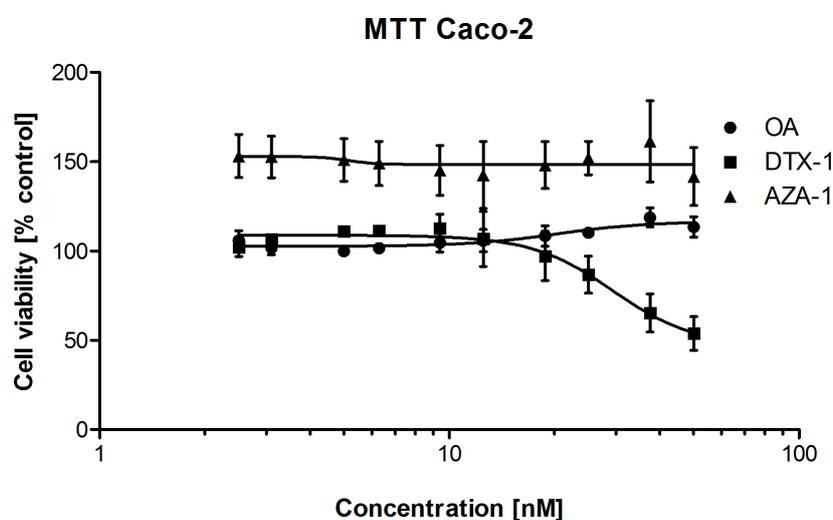
Figure 1. Effects of 24 h exposure to OA, DTX-1 and AZA-1 on viability of Caco-2 cells as determined by the MTT assay and expressed as % of the control (DMSO). Data presented as mean ± SD of three independent experiments at three different days, all exposures per test day being performed in triplicate. Concentrations in log scale. SD is expressed as a percentage of the control. SD of the control on individual experiments is not higher that 10%.

3.2 Microarray and pathway analysis

For the mRNA expression studies, non-cytotoxic concentrations of OA (25 nM), DTX-1 (6.25 and 12.5 nM) and AZA-1 (6.25 and 25 nM) were selected. Cells were also exposed to 25 nM DTX-1, but as this dose caused a slight decrease in MTT-activity, the cognate data were not included in further analysis of the results. However, despite the effect on MTT-activity, the results were very similar to those observed for 12.5 nM DTX-1. Hierarchical clustering was first performed on the 2824 genes (represented by 3634 spots) that were more than 2-fold ($\log_2$ ratio of $> |1.0|$) up- or downregulated compared to the control samples (DMSO) in at least two of the thirteen microarrays analysed. The resulting heatmap (Figure 2) shows four main clusters. Cluster 1 contains genes that were up-regulated by OA and DTX-1 but differently affected by AZA-1. Clusters 2 and 3 contain genes that were up- or downregulated, respectively, by all three toxins. Cluster 4 contains genes that were downregulated by OA and DTX-1 but upregulated by AZA-1. The data strongly indicate that DTX-1 has a similar mode of action as OA, since almost all genes affected by OA were affected in the same direction by DTX-1. This also applies to some extent for AZA-1 (clusters 2 and 3), but two other groups of genes, indicated as clusters 1 and 4, are affected in an opposite direction. All differential changes in gene expression are suggesting to some extent a different mode of action among these two classes of toxins. Tables S1 to S4 (supplementary files) show the most up- and downregulated genes based on the highest concentrations of either DTX-1 or AZA-1. However, the focus of this study was on potential pathways rather than single genes.
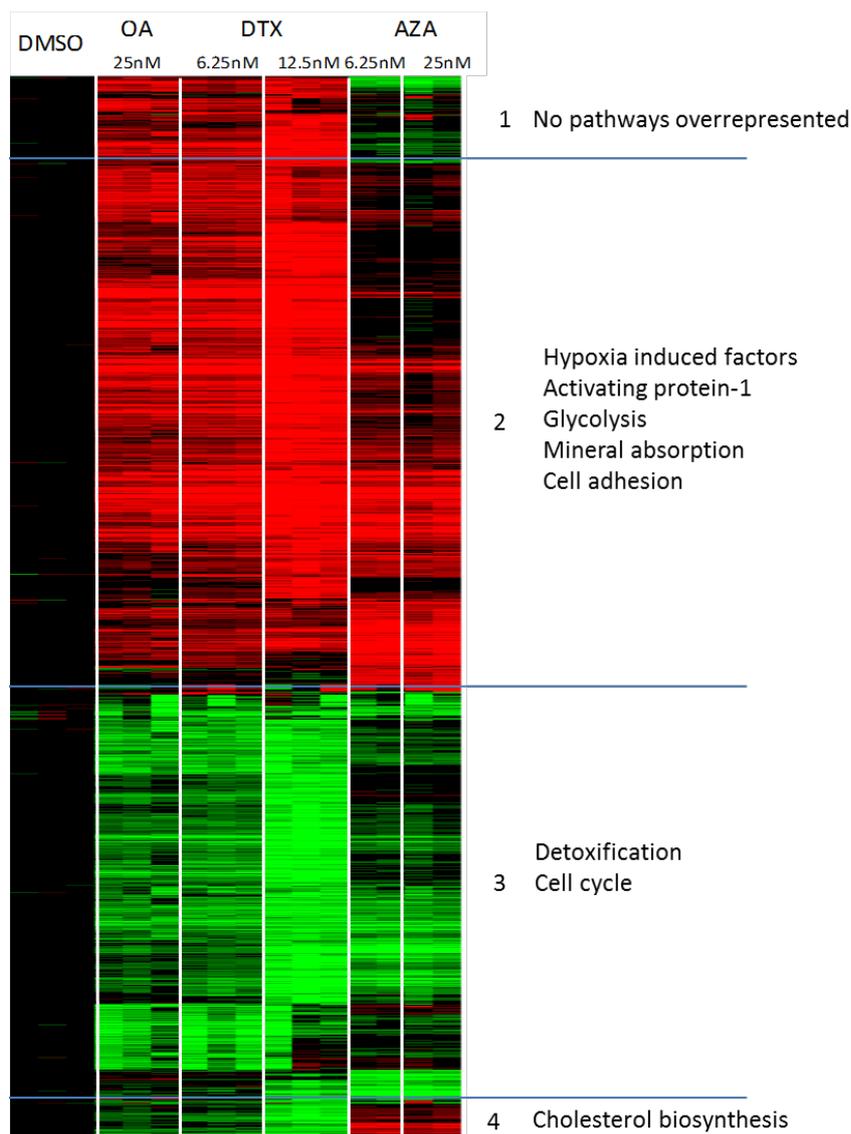
Figure 2. Hierarchical clustering of OA, DTX-1 and AZA-1 responsive genes in Caco-2 cells. Gene selection is based on an average fold change of ≥ 2 ($\log_2$ ratio $\geq |1.0|$) in at least two of 13 microarrays, leading to a total of 3,634 spots representing 2,824 genes. Green indicates downregulation, red upregulation, and black not affected. Pathways significantly affected within clusters are indicated at the right. Additional pathways are mentioned in the text. A maximal red or green colour indicates 2-fold up- or downregulation or more versus the control (DMSO). Each column represents triplicates (OA and DTX-1) or duplicates (AZA-1).

Therefore, the four clusters of the heatmap were analysed for genes representative for specific pathways or processes affected by the toxins. Cluster 1 contains 241 genes (represented by 301 spots) that are upregulated by OA and DTX-1, while part of these genes are slightly downregulated by AZA-1 and most not affected by AZA-1. There is a

small subset of genes on top of the heatmap that appear to be strongly downregulated by AZA-1 but upregulated by OA/DTX-1. This subset is composed of 61 spots, representing 38 genes. Overall, cluster 1 did not reveal significantly overrepresented pathways using the criteria selected for this analysis, and as such did not reveal any potentially relevant effect. Only two genes seemed of interest, i.e. ACADSB and MYLIP, both involved in lipid metabolism. The question is if the effect could be related to the specific effect of AZA-1 on cholesterol metabolism (see later).

Cluster 2 contains 1,330 genes (1,802 spots) that are upregulated by OA and DTX-1 and some also by AZA-1. These genes are involved in processes/pathways related with "Hypoxia Induced factor" (HIF) ($P < 5$ x 10^{-13}), "Glycolysis" ($P = 5$ x 10^{-8}), "FoxO signalling" ($P = 2$ x 10^{-8}), SREBP signalling ($P = 5$ x 10^{-7}), "Mineral absorption" ($P < 3$ x 10^{-6}), and "Activator Protein 1" (AP-1) ($P < 7$ x 10^{-5}). Some other pathways, like "Oxidative stress" ($P = 2$ x 10^{-5}) and "Protein processing in endoplasmic reticulum" ($P < 10^{-3}$) were also affected but to a lesser extent.

Cluster 3 contains 1,207 genes (1,400 spots) that are mainly downregulated by OA and DTX-1 (almost all by 12.5 nM DTX-1) and a part also by AZA-1. These genes are involved in pathways related to "DNA methylation" ($P < 3$ x 10^{-12}), "Telomere maintenance" ($P < 4$ x 10^{-12}), "Cell cycle" ($P < 5$ x 10^{-9}) and "Detoxification" ($P < 2$ x 10^{-5}).

Cluster 4 comprises 92 genes (120 spots), which are mainly downregulated by OA and DTX-1 but upregulated by AZA-1. Genes of this cluster are involved in "Cholesterol biosynthesis" ($P < 6$ x 10^{-18}).

3.3 Gene expression confirmation by qPCR

For confirmation of the observed effects and consistency between experiments, the mRNA expression of specific genes involved in the affected pathways/processes identified by transcriptomics analysis were examined by qRT-PCR analysis of undifferentiated Caco-2 cells exposed to the toxin standards on three different days. Eight pathways were chosen and for each pathway/process two or three genes were selected, based both on the induction values as obtained in the microarray analysis and their known importance in the identified pathway (Table 1). UPR and ER stress, which are related and therefore combined in Table 1 under the pathway/process ER stress, were selected as well, although these

pathways seemed less affected. This pathway appears in section 3.2 (see cluster 2) as "Protein processing in endoplasmic reticulum". Although the p value was higher than 10^{-5}, we decided to select it since is an important pathway to consider and because the induction values of the genes were high enough to do so. As shown in Figure 3, the qRT-PCR experiments confirmed the effects on mRNA expression for 18 out of 21 genes, in general with similar or higher induction or repression values than in the microarray analysis. Three genes involved in the cell cycle, HIST1H1E, HIST1H4C and HIST1H2AC, were selected because they were involved in the pathways "DNA methylation", "Telomere maintenance" and "Cell cycle" with low p values. Unfortunately, these genes could not be confirmed by qRT-PCR and those pathways were excluded from further analyses. It should be noticed that the repression values of these genes on the microarrays were rather low, less than 2-fold, maybe making it difficult to confirm them by qRT-PCR (Table 1).

Table 1. Representative genes of pathways that were selected for qRT-PCR confirmation. Numbers represent fold-induction of treatments *vs.* the control (untreated) obtained by gene expression analysis of the microarray data.

Pathway/process	Gene symbol	Expression [1]			Included in heatmap (Figs. 4-5)
		OA	DTX-1	AZA-1	
AP-1	EGR1	4.0	3.5	1.6	No
	FOS	4.9	9.2	2.6	No
	JUN	1.5	2.0	1.2	No
Cell Adhesion	MMP1	2.5	5.3	2.3	No
	CD44	2.5	4.3	3.0	No
Cell cycle	HIST1H1E	0.4	0.3	0.6	No
	HIST1H4C	0.4	0.3	0.9	No
	HIST1H2AC	0.5	0.4	0.5	No
Cholesterol biosynthesis	HMGCR	0.8	0.5	1.7	Yes
	LDLR	1.7	1.7	3.2	Yes
ER stress	BCL2L11	1.6	3.5	0.9	No
	ERN1	1.4	2.3	0.8	Yes
	MAP2K3	2.1	2.1	0.8	No
Glucose metabolism	HK2	3.2	4.3	3.0	Yes
	SLC2A1	2.5	3.0	4.6	No
Glutathione	GSTA1	0.8	0.4	0.3	Yes[2]
	GSTA2	0.8	0.5	0.3	Yes[2]
Hypoxia induced factors	EGLN3	2.5	6.1	1.4	Yes
	EGLN1	1.6	1.7	1.9	Yes
Mineral absorption	MT1G	1.9	2.8	5.3	Yes[2]
	MT1H	1.9	2.6	6.1	Yes[2]

(1) fold-induction values (numerical ratios). Values higher than 1 represent upregulation, values lower than 1 represent downregulation. Values represent the highest value of any treatment (see materials and methods).
(2) Involved in "detoxification" (see figure 2)
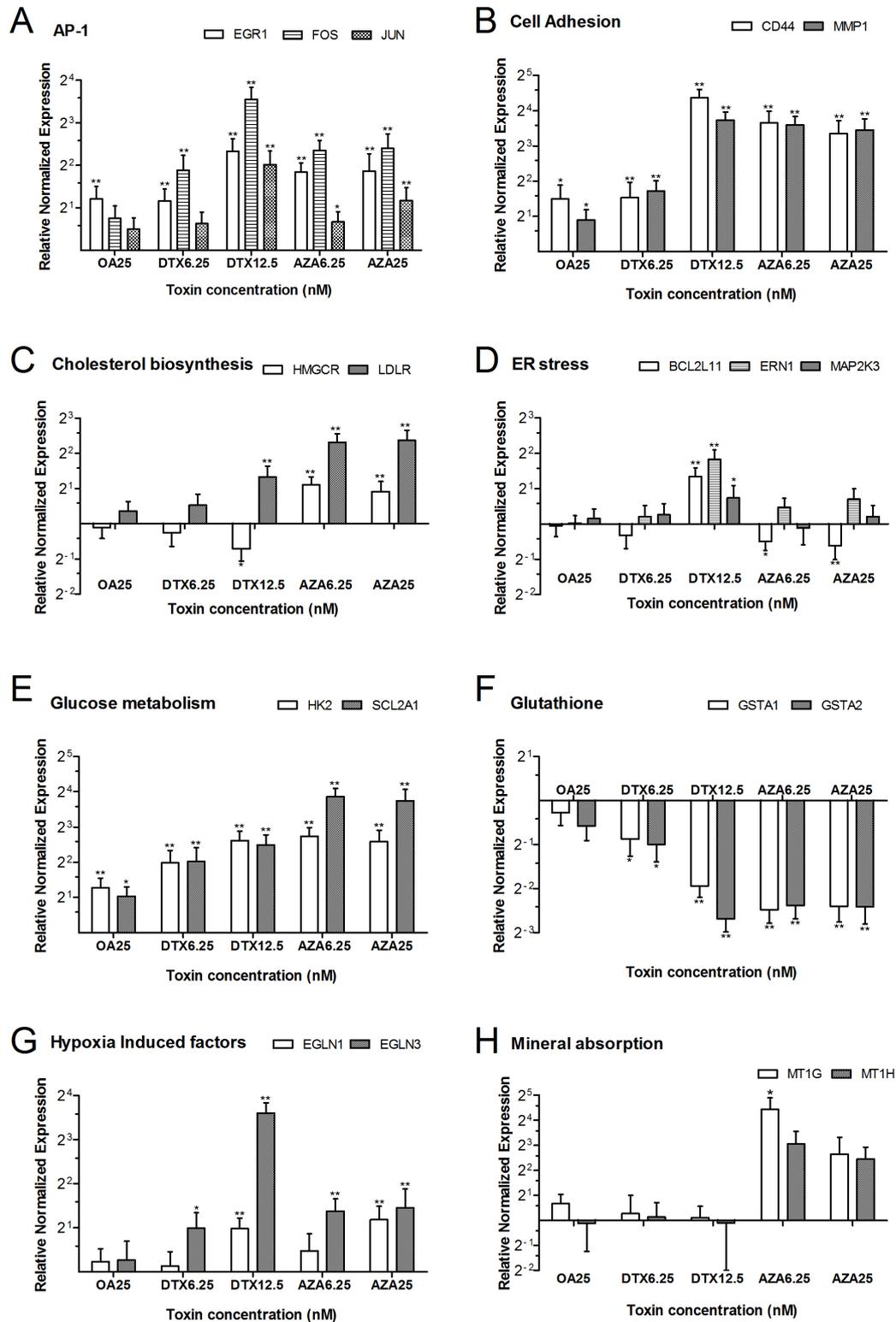
Figure 3. Validation of the microarray results by qRT-PCR for genes involved in key pathways/processes affected by OA, DTX-1 and/or AZA-1. Graphs show the qRT-PCR results of 18 genes from three independent exposures of Caco-2 cells; within each exposure each toxin concentration was tested in triplicate. Values are shown as a $\log_2$ relative normalized expression ($\Delta\Delta Cq$). Values above 2^0 indicate upregulation, values below 2^0 indicate downregulation. $^* = P \leq 0.05$, $^{**} = P \leq 0.01$.

3.4 Effects on pathways or processes

To obtain more insight in the genes that are affected within the different pathways, separate heatmaps per pathway were made. For this, we applied the heatmap tool of GSEA that visualizes also relatively small differences in mRNA expression [27]. The various heatmaps include all genes that are related to the process, for example enzymes, transporters and regulatory genes. Only the most dominant and relevant findings of the GSEA tool are presented and discussed.

Using this tool, the differences of induction values among pathways were more clear, e.g. for pathways/processes in cluster 2 of Fig. 2. It was shown that genes involved in "Cellular response to hypoxia" (Figure 4A) were mainly induced by OA/DTX-1, and much less by AZA-1. Genes most affected by OA/DTX-1 were EPAS1 (HIF2α), CREBBP, EGLN3, UBE2D1 and UBE2D3, whereas VEGFA and EGLN1 were affected by all toxins. Glucose metabolism (Figure 4B) appeared to be affected by both groups of compounds, however, the gene expression patterns seem to some extent different. Both groups induce HK2 (hexokinase) and fructose-bisphosphate aldolase (ALDOC), but the gene expression of phosphofructokinase (PFKP), a key enzyme in glycolysis, seems more upregulated by AZA-1, as are some genes at the end of the glycolysis, i.e. phosphoglycerate kinase (PKK1), phosphoglucomutase 1 (PGM1), and enolase (ENO1 and ENO2), some of which (PGM1, ENO2) seem actually downregulated at 12.5 nM DTX-1. At the higher concentration of DTX-1, also downregulation of some genes involved in the citric acid cycle (MDH1 and 2, OGDHL, DHTKD1) was observed. Another difference was the upregulation of 2,3-bisphosphoglycerate mutase (BPGM) by DTX-1 and OA, but down-regulation by AZA-1. Genes involved in ER stress related processes like "Unfolded or misfolded protein binding" (Figure 4C) and "ER associated apoptosis" (Figure 4D) are clearly induced by OA and DTX-1, while only few of these genes were affected by AZA-1. In agreement with the findings in Figure 2 (sub cluster 4), genes involved in cholesterol biosynthesis were induced by AZA-1 but not by OA or DTX-1 (Figure 5A). Another set of genes involved in cholesterol synthesis (e.g. HSD17B7, DHCR24) were downregulated by OA and DTX-1, but not clearly affected by AZA-1. Genes involved in glutathione conjugation, mainly glutathione-S-transferases of the A family (GSTAs), were downregulated at both concentrations of AZA-1 (Figure 5B) and, to a lesser extent, by 12.5 nM DTX-1.

Metallothionein genes were strongly upregulated by AZA-1 and much less by OA/DTX-1 (Figure 5C).
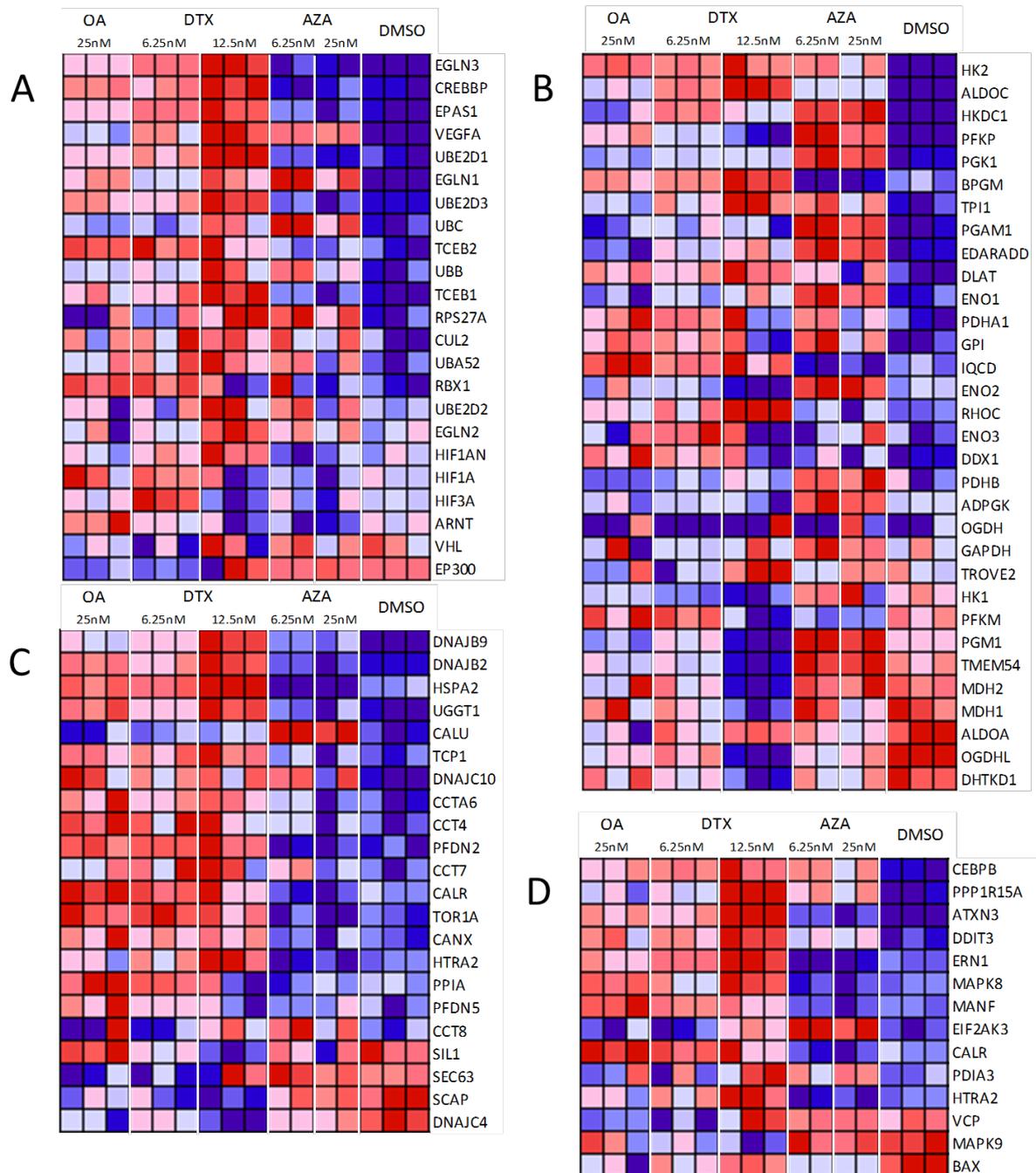
Figure 4. Effects on expression of genes of key pathways affected by OA, DTX-1 and AZA-1. A: "Cellular response to hypoxia", B: "Glycolysis/Glucose metabolism", C: "Unfolded or misfolded protein response", D: "Endoplasmic reticulum associated apoptosis". The heatmaps contain log₂ ratios of treatment vs. the average of the control samples. For each gene, the sample with the highest expression obtains a maximal red colour and the sample with the lowest expression obtains a maximal blue colour.
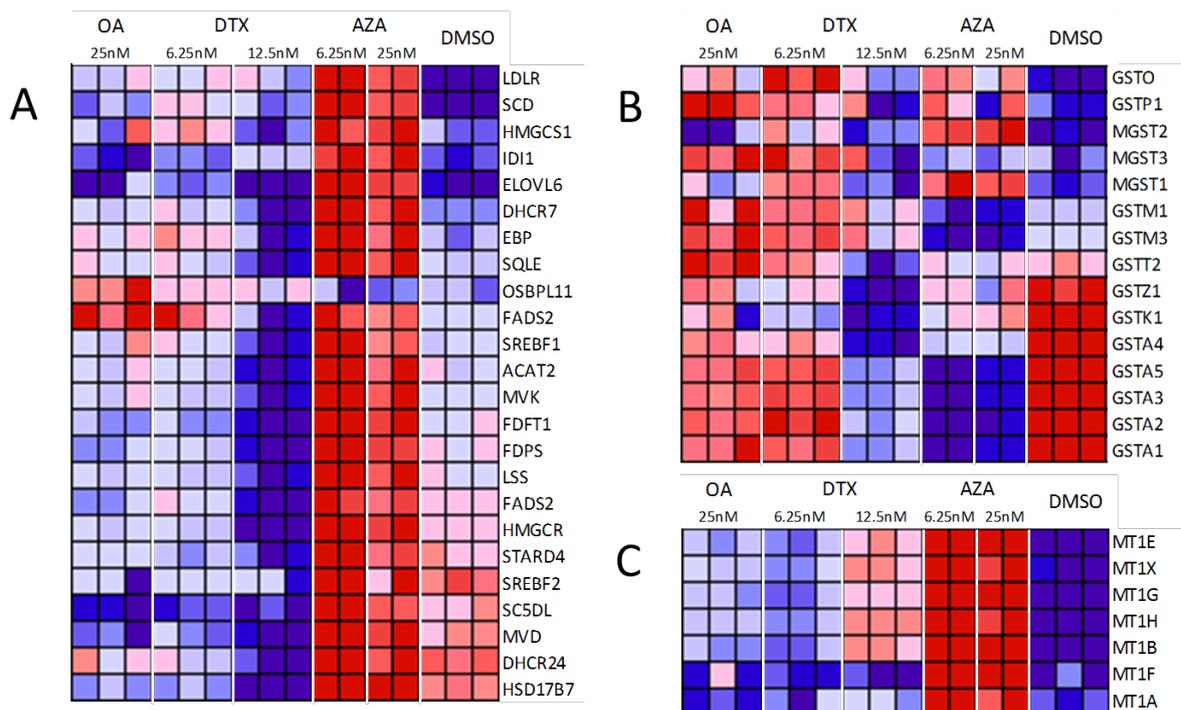
Figure 5. Effects on expression of genes of key pathways affected mainly by AZA-1. A: "Cholesterol biosynthesis", B: "Glutathione transferase activity", C: "Metallothionein".

4. Discussion

The present study aimed to acquire more insight into the modes of action of the marine toxins OA, DTX-1 and AZA-1 through in-depth analysis of microarray data generated for the purpose of biomarker identification. The focus was on pathways rather than specific genes, that were previously selected for another approach based on the use of a selective gene set for detection of these toxins using a tube array [22]. Although the primary mode of action of OA and DTX-1 is well-known, being inhibition of protein phosphatases (PPs), particularly PP1 and PP2A [29, 30], and to some extent PP5 [31, 32], it is still of interest to investigate the potential downstream effects of this inhibition and identify other potential effects. The bioinformatics analysis was started by making a heatmap including all samples based on a selection of 2,824 genes, that were at least 2-fold up or downregulated in at least two of the thirteen microarrays (nontoxic concentrations only and arrays that passed the quality check) (Figure 2). The concentrations selected for our analyses were based on determination of cell viability but also the regulatory limits used in the EU, since the eventual aim is the development of a bioassay for the detection of DSPs in shellfish. A maximum level of 160 μg OA equivalents per kilogram shellfish meat is allowed for OA

and DTX-1. The same limit is applied for AZA equivalents. In our routine protocol of shellfish extraction, a level of 160 μg kg^{-1} shellfish meat would result in a concentration of 12 nM in the well, so similar to those used in this study.

Exposure to 25 nM OA and 6.25 nM DTX-1 resulted in almost identical gene expression patterns, confirming that these two analogues induce the same effects. In addition, the transcriptomic analysis indicates that at least *in vitro*, DTX-1 is approximately 4-fold more potent than OA. It is important to consider that according to the current regulations, OA and DTX-1 are regarded as equally toxic, i.e. both have a toxic equivalency factor (TEF) of 1 [33]. However, a higher toxic potency of DTX-1, compared to OA, has also been reported by others. Ferron et al. found that undifferentiated Caco-2 cells were 2-fold more sensitive for DTX-1 than OA on the basis of the neutral red uptake assay and five-fold more sensitive on the basis of proportions of Ki-67 positive cells, i.e. a marker for proliferating cells [21]. A higher sensitivity to DTX-1 than OA has also been reported for other cell types, including Neuro-2a, NG108-15, MCF7 [34] and HT29-MTX cells [21]. Data on absorption, distribution, metabolism and excretion are still needed to extrapolate these *in vitro* findings to *in vivo*, although the current TEF values are based on the induction of death after intraperitoneal injection in mice [35, 36] and thus exclude certain toxicokinetic parameters as well.

The induction of hypoxia by OA and DTX-1 is indicated by the increased expression of target genes involved in hypoxia, as shown in figures 3G and 4A. These genes are particularly induced as an adaptive response of the cell to decreased oxygen levels by activating adaptive pathways including induction or stabilization of the transcription factors HIF-1 (which consists of the oxygen-regulated subunit HIF-1α and the constitutively expressed subunit HIF-1ß) and HIF-2 [37]. Increased expression of HIF genes is in line with the known inhibition of protein phosphatases, as it has been proposed that the inhibition of PP2A by okadaic acid activates the Akt/mTOR pathway, leading to increased HIF-1 protein levels and increased expression of the HIF-1 target gene VEGF which is known to play a role in angiogenesis [38]. HIF-1 target genes are involved in multiple processes including erythropoiesis, angiogenesis, extracellular matrix proteins and enzymes, glucose transport and glycolysis [39]. In the present study in particular the upregulation of EPAS1 (or hypoxia-inducible factor-2alpha (HIF-2α)) was observed for OA and DTX-1, but less by AZA-1. The difference in gene expression was even more clear for CREPBP

(cAMP response element-binding protein binding protein), a cofactor required for the action of HIF on gene expression, and UBED1 and UBED3 involved in the degradation of HIFs. Also EGLN genes, encoding prolyl-hydroxylases, are involved in the inactivation of HIF and increased expression of these EGLN genes is known to be a negative feedback mechanism against HIF response [40]. Under normoxic conditions, these enzymes catalyse the hydroxylation of HIF, promoting its degradation. In hypoxic conditions, HIF hydroxylation is reduced, allowing the formation of the HIF complex, which translocate to the nucleus, binds to a hypoxia responsive element and upregulates hypoxia responsive genes [41]. As shown in the heatmap of figure 4A, EGLN1 and EGLN3 were upregulated by OA/DTX-1 and effects on these genes were confirmed by qRT-PCR. This seems in line with the negative feedback mechanism against the observed HIF response. It should however be noted that EGLN1 was also upregulated by AZA-1 and according to qRT-PCR also EGLN3. However, also the upregulation of 2,3-bisphosphoglycerate mutase (BPGM) by DTX-1 and OA (down-regulated by AZA-1), an enzyme converting the glycolysis intermediate 1,3-bisphosphoglycerate into 2,3-bisphosphoglycerate, seems related to hypoxia and has been proposed as a biomarker [42]. Levels of 2,3-bisphosphoglycerate are increased under hypoxic conditions, in particular in erythrocytes, its role in other cells being less clear. A further examination revealed that some of the genes most upregulated by OA/DTX-1, like CDKN1C (cyclin-dependent kinase inhibitor 1C) and RGS16 (regulator of G-protein signalling 16), previously used as specific marker genes for OA/DTX-1 [22], and also CXCR4 (C-X-C chemokine receptor type 4), play a role in hypoxia [43-45] (Table S1, supplementary files). Overall, these observations suggest that a hypoxia-like response is induced by OA/DTX-1, but not or much less by AZA-1.

Additional pathways, including ER stress, UPR activation and ER associated apoptosis were induced by OA and DTX-1, but not or much less by AZA-1, as clearly indicated by the heatmaps shown in Figure 4C and 4D. ER stress and the UPR are known to be triggered in response to several stimuli, e.g. environmental stress, exposure to certain xenobiotics or deprivation of nutrients, with the purpose to restore cell homeostasis [46, 47]. When the response is inadequate to recover the normal cell functions, the cell turns to apoptosis [47]. This might be one of the mechanisms involved in OA induced apoptosis as published previously for other cell types, e.g. leucocytes, the liver cell-lines HepG2 and HL7702 and the neuronal cell line SHSY5Y [18, 48]. The combination of induction of hypoxia, the UPR,

ER stress, and apoptosis is not uncommon. Accumulation of unfolded or misfolded proteins in the ER is known to induce UPR, which aims to restore ER homeostasis. This includes a temporary inhibition of protein synthesis. However, when the stress signal is severe and/or prolonged, the ER stress triggers cell death pathways including apoptosis [37, 49]. Induction of UPR by OA has also been reported to occur in primary cultures of rat cortical neurons [50].

AP-1 (Activating protein-1) is a transcription factor constituted by fos and jun proteins and involves the control of a variety of inducible genes containing AP-1 sites or a TPA response element [51]. It is regulated by a broad range of stimuli, including cytokines, stress, growth factors, among others [52]. Our data indicate that OA and DTX-1, but also AZA-1, induce the AP-1 response, which was confirmed by the increased gene expression of EGR1, FOS and JUN as assessed by qRT-PCR (Figure 3A). A downstream effect of AP-1 activation is the induction of genes involved in the extracellular matrix that is associated with remodelling of tissue [53, 54]. As shown in Figure 3B, the matrix related genes MMP1 and CD44 are upregulated by all toxins reaching a 16-fold induction both by 12.5 nM of DTX-1 and 6.25 and 25 nM AZA-1. The upregulation of MMPs has been reported previously for OA [55, 56], but not for AZA-1. AP-1 is reported to increase the glucose production by exerting an accumulation of the glucocorticoid receptor (GR) in the nucleus that in turn activates gluconeogenesis [32, 57]. Genes involved in glucose metabolism were actually induced by OA, DTX-1 and AZA-1 (Figure 3E), the increased expression of two of these genes being confirmed by qPCR. HK2 (hexokinase 2) encodes an enzyme involved in the first step of the glucose metabolism pathway [58]. SLC2A1 is a major glucose transporter, located in the cell membrane, and facilitates the entrance of glucose in the cell [59]. Moreover, SLC2A1 has been reported to be induced by HIF [60, 61]. Interestingly, AZA-1 appeared to have an even greater effect on the increased expression of these two genes, as well as on genes encoding for several other enzymes of the glycolysis. This may indicate an increased activity of the glycolysis, a phenomenon called the Warburg effect. This actually might also underlay the increased MTT-activity (Figure 1), that indicates increased NADH levels in the cell [62], generated during increased glycolysis and not metabolized in the mitochondria. Combined, this may indicate that AZA-1 has an effect on the coupling between the glycolysis and the Krebs cycle, potentially due to inhibition of a key enzyme, like pyruvate dehydrogenase, converting pyruvate into acetyl-CoA. This should be further

investigated, e.g. by measuring intermediates in the metabolism of glucose or the production of lactate.

The gene expression analysis also demonstrates that AZA-1 specifically and highly induces the expression of genes involved in cholesterol biosynthesis (Figure 5A). This includes 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), a rate-limiting enzyme in the cholesterol biosynthesis pathway. In addition, AZA-1 clearly increases the expression of the gene encoding the low density lipoprotein receptor (LDLR) that is involved in the uptake of cholesterol by the cells and is stimulated by low cholesterol levels in the cell. The mRNA expression of LDLR was also increased by OA and DTX-1, but to a lesser extent (Table 1). These observations are in line with earlier findings of Twiner et al. [20] in Jurkat cells, showing that AZA-1 decreases the levels of cellular cholesterol. The induction of genes involved in cholesterol biosynthesis and uptake likely aims to counteract this reduction in cholesterol levels. Cholesterol biosynthesis genes have also been reported to be upregulated in Caco-2 cells upon exposure to alpha-chaconine, a well-known potato glycoalkaloid [63]. The toxicity symptoms of this latter compound are mainly gastrointestinal, including vomiting, diarrhea and abdominal pain, which appear very similar to the symptoms elicited by AZAs. It was proposed that the disturbance of the balance in cholesterol due to complexation with alpha-chaconine may be the cause of the upregulation of cholesterol biosynthesis genes. The exact mechanism how cholesterol levels are impaired by AZAs remains to be elucidated, but it is tempting that there may be a relation with the effects on glycolysis and that a reduced synthesis of acetyl-CoA plays a role.

As shown in Figures 3F and 5B, all 3 toxins caused a reduction in the expression of glutathione S-transferases (GSTs), a group of detoxification enzymes, involved among others in detoxification of xenobiotics. They are also involved in the biosynthesis of steroids, prostaglandins and leukotrienes, and in the detoxification of products of lipid peroxidation and oxidative stress [64, 65]. In addition, GSTs are involved in cell cycle progression [66]. This effect has been observed after the knock-out of GSTA genes in Caco-2 cells [67]. Thus, we might speculate that the effect on GSTA genes may be related with cell cycle and impairment of the cell differentiation, in response to the exposure to toxins.

Exposure to AZA-1 resulted in a rather specific upregulation of various metallothionein genes, not clearly observed with OA and DTX-1 (Figures 3H and 5C). These genes can be

upregulated as part of an inflammatory response [68], but also in response to metals as part of the detoxification process, and during oxidative stress and hypoxia [68, 69]. At this stage it is unclear how AZA-1 affects these genes.

Performing bioinformatics analysis with relatively low cut-off values, as used in this work (i.e. from 2-fold up- or downregulated onwards), allowed us to identify pathways potentially affected by three lipophilic marine biotoxins belonging to two major classes. Some of the relevant genes in certain pathways were highly upregulated and may be suitable as biomarkers by themselves, as described previously [22]. qRT-PCR-analysis of a number of genes in independent repeats showed that the gene expression was highly reproducible. A more general picture on the mode of action may be helpful to develop more functional tests that are based on specific effects, like those observed on cholesterol synthesis or glycolysis. It would be interesting to explore the mechanisms behind the effect of AZA-1 on cholesterol biosynthesis, glycolysis and/or the increase of the MTT activity, and the potential increase in metallothionein expression. For DTX/OA, effects on hypoxia and UPR seem more selective but it might be more difficult to find specific biomarkers other than gene expression or the inhibition of phosphatases. More general, it is important to understand the mechanisms behind the toxic effects of these compounds, both for risk assessment and for treatment of people affected by them. Some of the observed effects on gene expression may give a hint on the underlying mechanism of the cause of e.g. diarrhea, but this requires more work. The potential similarity between AZA and glycoalkaloids on cholesterol synthesis might also be an interesting lead to follow-up. Other models and approaches may be needed, where the use of a more physiological model could be relevant, e.g. the use of differentiated Caco-2 cells co-cultured with mucus cells, or an organ-on-a-chip approach. Some effects may require validation by *in vivo* studies.

In conclusion, the bioinformatics analysis on the transcriptomics data of Caco-2 cells yielded additional information about the potential modes of action of OA, DTX-1 and AZA-1. Based on the very high similarity in response of mRNA expression, OA and DTX-1 exert identical effects, like those on hypoxia related pathways/processes, the unfolded protein response (UPR) and endoplasmic reticulum (ER) stress. AZA-1 mainly increased mRNA expression of genes involved in cholesterol synthesis and possibly glycolysis. Follow-up studies may reveal suitable biomarkers for developing specific in vitro bioassays but also give more insight in the mode of action of these toxins.

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Chapter 4

Detection and profiling of diarrhoeic marine biotoxins in shellfish by mRNA analysis of exposed Caco-2 cells using qRT-PCR and multiplex magnetic bead-based assays

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Summary

The mouse bioassay for the detection of marine biotoxins in shellfish products is 40 years old and still in use. A full ban or total replacement of this *in vivo* test has been postponed because of the fear that current chemical-based detection methods could miss a new emerging toxin. In order to fully replace the mouse bioassay, more efforts are needed on the search for functional assays with specific endpoints. A previous study on gene expression elicited by diarrhoeic shellfish poisoning toxins in Caco-2 cells allowed us to determine three 'DSP toxins profiles', i.e. OA/DTX, AZA-YTX and PTX profiles. In the present study twelve marker genes were selected that envision these three profiles. qRT-PCR is relatively cheap and easy, and although its multiplex capacity is limited to five genes, this turned out to be sufficient to show the three expected profiles. The use of the multiplex magnetic bead-based assay turned out to be even a slightly better alternative, allowing the use of all twelve selected marker genes and two reference genes, and resulting in clear profiles with for some genes even higher induction factors as obtained by qRT-PCR. When analysing blank and contaminated shellfish samples with this multiplex magnetic bead-based assay, the contaminated samples could easily be distinguished from the blank samples, showing the expected profiles. This work is one step further on the final replacement of the mouse bioassay, e.g. by combining the neuro-2a bioassay for broad screening and detection (nonspecific cytotoxicity) with analytical chemical analyses for the confirmation of known toxins and the multiplex magnetic bead-based assay (specific mRNAs) for confirmation of unknown toxins.

1. Introduction

Marine biotoxins are toxins produced by phytoplankton and/or bacteria, that can accumulate in several types of marine animals, e.g. shellfish, crabs and fish [1]. Bivalve molluscs feed through filtration and concentrate these toxins in their bodies and digestive glands [2, 3]. Mussels for example filtrate 7.5 litres of seawater per hour, leading to the accumulation and concentration of pollutants and toxins [4]. When humans consume seafood with toxins in amounts that exceed the established safety levels, it may lead to intoxication. Marine biotoxins can cause clinical features that vary from diarrhoea and amnesia to paralysis and even death. Five groups of marine biotoxins are regulated within the European Union, i.e. okadaic acid group (OA), which includes the dinophysistoxin analogues (DTXs), this group is also known as the diarrheic shellfish poisoning (DSP) toxins; azaspiracid group (AZAs), which can also cause diarrhoea; yessotoxin group (YTXs); domoic acid group (DA), also known as amnesic shellfish poisoning (ASP); and saxitoxin group (STX), also known as paralytic shellfish poisoning (PSP) toxins [5]. Within these groups, several different types and analogues can be found. It is described that worldwide algae toxins are responsible for approximately 60,000 human intoxications annually [6].

In order to prevent intoxications, several countries have legislation regarding permitted levels of the different marine biotoxins in shellfish that need to be checked by monitoring programs. These monitoring programs to detect marine biotoxins make use of different methods [3]. The method of surveillance most used worldwide is the mouse bioassay (MBA), where an extract of shellfish is injected intraperitoneal into a number of mice and death is the endpoint to determine whether the sample is safe to consume or not [7, 8]. Besides ethical issues regarding the use of laboratory animals, the MBA gives high rates of false-positive and false-negative outcomes [5, 9]. In Europe, the use of the MBA is banned since 2015, but not for PSP toxins analysis and not for the control of production areas, aiming at detection of possibly unknown toxins [10]. The EU reference method for the detection of lipohilic shellfish toxins (mainly DSP toxins and AZAs) is the LC-MS/MS method of the European Reference Laboratory (EU-RL) on marine biotoxins [10, 11]. However, the use of the MBA has been kept over time and is not fully replaced by analytical chemical methods. This is mainly due to a lack of standards for the known toxins, and

because toxin patterns might change, which generates a concern about new toxins appearing that would be missed by such chemical analysis and not by the MBA [12, 13]. Nowadays, when possible, toxicity testing should comply with the so called 3R principle, i.e. to refine, reduce and replace experiments with animals [9, 14, 15]. Some cell-based assays have been tested in order to obtain information about the mode of action or biological activity of the marine biotoxins, and to replace the MBA [12, 16, 17]. The neuro-2a bioassay is considered as one of the most promising cell-based bioassays for the broad screening of marine biotoxins, i.e. DSP toxins, neurotoxic shellfish poisoning (NSP) toxins, and PSP toxins [18-21]. The readout in this neuro-2a bioassay is reduction of MTT (as measurement of cell viability) and suspect screened samples should be confirmed by additional LC-MS/MS analysis [22]. However, in case a suspect screened sample is not confirmed by analytical chemical methods, it might contain a known unknown or yet unknown toxin. In these cases, an additional cell-based bioassay confirming the presence of a toxin and that is also able to determine the type of toxin present would be very helpful, e.g. a bioassay based on gene expression [2]. To do so, previously a whole genome mRNA expression analysis was performed with the human intestinal Caco-2 cell line exposed to OA, DTX-1 and AZA-1 using DNA microarrays. Patterns obtained for toxins or other bioactives are specific and commonly used to characterise new compounds, i.e. compare the profile of the compound with those available in data banks (read across). Exposure to the regulated toxins, i.e. OA, DTX-1, AZA-1, PTX-2 and YTX, yielded specific gene expression patterns. From the information provided by these microarray analyses, insights on mode of action were described for OA, DTX-1 and AZA-1 [17]. In summary, OA and DTX-1 induced almost identical mRNA expression patterns, in agreement with the fact that both molecules are analogues that belong to the same toxin group and cause similar effects. For instance, OA and DTX-1 increased expression of genes involved in the hypoxia induced factor pathway/process (HIF), in line with the inhibition of phosphatases and a subsequent activation of the Akt/mTOR pathway, which is involved in the activation of the HIF. OA and DTX-1 also affected pathways like unfolded protein response (UPR) and endoplasmic reticulum (ER) stress. The mRNA expression pattern from AZA-1 was different, where an increase of genes involved in cholesterol biosynthesis and glycolysis pathways was observed, suggesting a different mode of action [17]. Since full genome microarray analysis is not suitable for rapid screening, alternative platforms to detect gene expression levels of

highly up or down-regulated genes as markers for detection and identification were evaluated. A first approach involved a so-called tube array with a limited number of selected marker genes. Although promising, the test was rather expensive, labour intensive and long (it took about 3 days). Moreover, the sensitivity of several of the 17 selected genes on this dedicated array was limited [16].

The present study describes two new approaches for detecting marker mRNAs in exposed Caco-2 cells, i.e. the development of a multiplex qRT-PCR and a multiplex magnetic bead-based assay. The newly developed multiplex qRT-PCR was performed successfully with five markers (using the maximum number of six fluorescent markers resulted in interference). The newly developed multiplex magnetic bead-based assay was able to correctly quantify the expression levels of twelve selected marker genes. The present study shows that detection of marker mRNAs in exposed Caco-2 cells could be a promising tool to confirm the presence of yet unknown toxins in mussel samples screened suspect in the neuro-2a bioassay which cannot be confirmed by LC-MS/MS. We thus propose a strategy where the neuro-2a bioassay is used as a screening method, LC-MS/MS for confirmation of suspects, and a second cell-based bioassay to confirm the presence of a toxin and detect a toxin profile related to gene expression when suspects from the neuro-2a cannot be confirmed by LC-MS/MS. This work will contribute to the search for new endpoints to detect known and yet unknown marine biotoxins, will help in the identification of unknown toxins, and does so without the need for animal testing.

2. Material and methods

2.1 Reagents and standards

Certified reference materials (CRMs) of OA (13.7 ± 0.6 µg mL^{-1}), DTX-1 (15.1 ± 1.1 µg mL^{-1}), DTX-2 (7.8 ± 0.4 µg mL^{-1}), PTX-2 (4.40 ± 0.13 µg mL^{-1}), AZA-1 (1.24 ± 0.07 µg mL^{-1}), AZA-2 (1.28 ± 0.05 µg mL^{-1}), AZA-3 (1.04 ± 0.04 µg mL^{-1}), YTX (5.6 ± 0.2 µg mL^{-1}), and hYTX (5.8 ± 0.3 µg mL^{-1}) were purchased from the National Research Council, Institute for Marine Biosciences (NRC CNRC, Halifax, Canada). Pinnatoxin E (PnTX-E) was obtained from Cawthron Institute, New Zealand. Stock solutions of these toxin standards were prepared in dimethyl sulfoxide (DMSO) after evaporation of the original

solvent. DMSO and n-hexane were obtained from Merck (Darmstadt, Germany). Methanol (Ultra LC/MS) was purchased from Actu-All (Oss, The Netherlands).

2.2 Cell culture

The human colonic adenocarcinoma cell-line Caco-2 (ATCC, Manassas, VA) was grown in Dulbecco's Modified Eagle Medium (DMEM) obtained from Lonza (Verviers, Belgium) supplemented with 10% (v/v) foetal bovine serum (FBS) from Gibco BRL (Fisher Emergo, Landsmeer, the Netherlands), non-essential amino acids (NEAA) from MP Biomedicals, (Illkirch, France) and penicillin 0.1% v/v (50 mg ml^{-1}) from Sigma (Zwijndrecht, the Netherlands). The cells were grown in 75 cm² flasks at 37 °C and 5% CO_2.

2.3 Samples

In-house samples, both blank mussel samples from the Netherlands, and contaminated samples obtained from various locations in the EU and used for previous validation studies of the LC-MS/MS method [23] were tested.

2.4 Preparation of extracts

Prior to extraction of the blank samples and the ones containing lipophilic marine biotoxins, shellfish material was homogenized with a T25 Ultra Turrax mixer at 24,000 rpm (IKA® Works Inc., Wilmington, NC, USA). One gram of shellfish homogenate was vortex mixed with 3 mL methanol for 1 min and centrifuged for 5 min at 2,000 × g. The supernatant was transferred to a volumetric flask and the residue was extracted twice more with 3 mL methanol. After the third extraction the volume of the collected supernatant was adjusted to 10 mL with methanol. For exposure of Caco-2 cells, additional clean-up steps using n-hexane and solid phase extraction (SPE) were applied.

2.5 Clean-up by n-hexane wash step followed by SPE

A 4.8 mL aliquot of the crude methanol shellfish extract was diluted with 1.2 mL Milli-Q water and extracted twice with 6 mL n-hexane in order to remove matrix substances that led to false-positive test outcomes [22]. The hexane layer was discarded and the aqueous methanolic extract was further diluted by adding 10 mL Milli-Q water and the total extract

of 16 mL was transferred to an SPE StrataTM-X cartridge (200 mg/6 mL; Phenomenex, Utrecht, the Netherlands), previously conditioned with 4 mL methanol/water (30:70 v/v). Subsequently, the cartridge was washed with 8 mL methanol/water (20:80 v/v) and the toxins were eluted with 4.8 mL methanol. The eluate was evaporated to dryness under a stream of nitrogen gas and reconstituted in 20 µL DMSO.

2.6 Exposure, RNA isolation and cDNA synthesis for multiplex qRT-PCR analysis

Caco-2 cells were exposed to the standards and sample extracts. For this, 600 µL of Caco-2 cell suspension were seeded in 24 well plates (Ref. Number 3524, Corning, NY), using 8 x10^4 cells per mL and incubated for 48 h at 37 °C and 5% CO_2 to reach 80-90% confluence. DMSO 0.25% (v/v) was included as vehicle control. Exposures were performed in triplicate. Cells were exposed to the standards and samples for 24 h, medium was removed, cells were washed with PBS and lysed with 600 µL of RTL buffer with 1% β-mercaptoethanol. RNA was extracted using the QIA shredder and RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) followed by a DNAse treatment with RNAse free DNAse (Qiagen, Venlo, the Netherlands), both by following the instructions of the manufacturer. After the extraction, the amount and quality of the RNA were evaluated by UV spectrophotometry (260 and 280 nm wavelength) on the Nanodrop spectrophotometer (Nanodrop technologies). cDNA was synthetized using 1 µg of RNA per sample and from an 'RNA pool mix' of all treatments with and without reverse transcriptase using the Biorad iScript cDNA Synthesis Kit with iScript and reverse Transcript (Biorad, 170-8891) in the BioRad iCycler (Biorad, Veenendaal, the Netherlands). The program used was 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C, after which the samples were put on ice for 5 min. After the cDNA synthesis, the samples were diluted 10 and 100 times and the pool was diluted 10, 31.6, 100, 316, 1000 and 3160 times and used to make a calibration line. The samples were stored at -20 °C.

2.7 Singleplex qRT-PCR method

Singleplex qRT-PCR was performed for the selected marker genes with certified QuantiTect primers from Qiagen (Venlo, the Netherlands) using 15 µL of final volume containing: 8.5 µL SYBR green (BioRad 170-8880), 2.5 µL of the QuantiTect forward/reverse primer mix, 2 µL RNAse free water and 2 µL of 100x diluted cDNA.

Reactions were performed in a BioRad HSP9645 PCR plate. Water and 'RNA pool mix without reverse transcriptase' were used as negative controls. The plate was covered with a micro seal and centrifuged for 1 min. Thermal cycling was performed in a CFX96 Real-Time System (Biorad), starting with a denaturation step at 95 °C for 3 min, followed by 45 cycles at 65 °C with 35 s for annealing, 10 s at 95 °C for denaturation, and 1 min at 65 °C for extension. Data were analysed using BioRad software. Expression ratios of the genes were calculated for exposures versus DMSO control.

2.8 Multiplex qRT-PCR method

Multiplex qRT- qPCR was performed with primers as shown in table 1, from Qiagen (Venlo, the Netherlands) and Biolegio (Nijmegen, the Netherlands). All the probes were provided by Biolegio. The sequences are confidential. The reactions were performed using 25 μL final volume containing 12.5 μL 2x Quantifast multiplex PCR master mix (Qiagen, Venlo, the Netherlands, cat number 204752), 1.25 μL of each primer probe mix, 3.75 or 6.25 μL RNAse free water and 2μL of 10x diluted cDNA. Reactions were performed in a BioRad HSP9645 PCR plate. Water and 'RNA pool mix without reverse transcriptase' were used as negative controls. The plate was covered with a micro seal and centrifuged for 1 min. Thermal cycling was performed in a CFX96 Real-Time System (Biorad), starting with an initial denaturation step at 95 °C for 5 min, followed by 44 cycles at 60 °C with 45 s for annealing, 45 s at 95 °C for denaturation, and 45 s at 60 °C for extension.

Data were analysed using BioRad software CFX manager v.3.0. Plate set up and standard curve were selected, and the results are shown as log2 values. Relative quantities (ΔCq), which express the quantity of the gene under a certain treatment (toxin) *vs* the quantity under control treatment (vehicle) are plotted and expressed as log2 values versus the control. The expression of the reference gene TMEM179B was not affected by any treatment and left out in the newly developed multiplex qRT-PCR method.

Table 1. Primers, probes and dyes selected for the development of a multiplex qRT-PCR.

Gene name	Primers	Specification	Cat number	Probe dye
NPPB	Qiagen	Hs_NPPB_1_SG	QT00031934	6FAM
RGS16	Biolegio	na	na	Texas Red
DDIT4	Qiagen	Hs_DDIT4_1_SG	QT00238588	HEX
CXCR4	Qiagen	Hs_CXCR4_2_SG	QT02311841	Quasar 705
TGFB2	Biolegio	na	na	Cy5

2.9 Multiplex magnetic bead-based assay

Caco-2 cells were seeded in a 96 well plate (Ref. Number 3595, Corning, NY) using 100 µL of a suspension containing 8 x 10^4 cells per mL and incubated for 48 h at 37 °C and 5% CO_2, to reach 80-90% confluence. Then, cells were exposed to samples and standards for 24 h. DMSO 0.25% (v/v) was included as vehicle control. After 24 h exposure of Caco-2 cells in the 96 well format (every exposure performed in triplicate, e.g. 3 wells per treatment), mild lysis of cells was achieved according to the manufacturer instructions (QuantiGene 2.0 plex assay user manual, Affymetrix, the Netherlands). Briefly, the lysis mixture was diluted in nuclease-free water and 100 µL were added per well. Plates were incubated for 18-22 hours at 54 °C ± 1 °C, at 600 rpm in a VorTemp™ 56 Shaking Incubator (Thermo Fischer, the Netherlands), previously validated with a QuantiGene Incubator Temperature Validation Kit (Isogen, the Netherlands). The assay procedure consists of several hybridization, incubation and washing steps, using a plate magnet to capture the beads (Affymetrix, the Netherlands). After the final binding step, 130 µL of washing buffer (provided in the kit) was added to the wells and plates were read in a xPonent® 3D machine (Luminex corp). The protocol was defined using manufacturer instructions, i.e. sample size 100 µL, DD gate 5.000 – 25.000, timeout 45 seconds and bead event 100. The total time needed from cell lysis to read out is about 30 h. Data analysis was performed as follows: MFI (median fluorescence intensity) values were provided from the xPonent® 3D machine in a .cvs file and were analysed using excel for calculating the average signal (avg MFI) for each gene (exposures were performed in triplicate). Then, the value obtained from each gene was divided by the value for the normalisation gene. Here we used the CUL1 gene (avg MFI gene of interest/avg MFI CUL1). Finally, for each test gene, we

calculated the fold change by dividing the normalised value for the treated samples by the normalised value for the untreated sample, i.e. DMSO ((avg gene/avgcul1)/avg DMSO). Values were plotted in prism graphpad.

3. Results

Table 2 shows the twelve marker genes and three reference genes which were selected from the whole genome array studies, where undifferentiated Caco-2 cells were exposed to OA, DTX-1 and AZA-1 [17] and to YTX and PTX-2 (unpublished data). Genes were selected based on their response to the different toxins, e.g., NPPB is specifically down-regulated by PTX-2 and to some extent by DTX-1, while RGS16 is specifically up-regulated by DTX-1 and to some extent by OA. TMEM179B, CUL1 and SH3BP2 were not affected and used as reference genes. Moreover, besides OA, DTXs, AZAs, YTXs and PTXs, other (nonregulated) marine biotoxins like the cyclic imines (CIs) might end up in the lipophilic extracts, however these toxins do not lead to clear effects on gene expression, even when tested at higher concentrations. Figure S1 is an example of the CIs PnTX-E and SPX and shows that these toxins do not result in clear effects on gene expression in exposed Caco-2 cells.

3.1 Development of a multiplex qRT-PCR detection method

First, singleplex qRT-PCRs were performed in order to confirm the results from the whole genome array studies. For that, Caco-2 cells were exposed to 3 and 9 nM of all the toxins, including the analogues, except for YTX, for which 12.5 and 37.5 nM were used. These concentrations were used for all following experiments, as these concentrations result from a newly developed clean-up procedure for lipophilic marine biotoxins (LMB) from mussels in combination with the regulatory limits (160 µg kg^{-1} shellfish for OA, DTXs, PTX-2 and AZAs, and 3,750 µg kg^{-1} shellfish for YTXs). Assuming 100% toxin recoveries [22], the regulatory limits of OA, DTXs, AZAs and PTX-2 will result in a final concentration of about 12 nM in the well, while the regulatory limit of YTX would result in a final well concentration of about 200 nM. Figure 1 shows the relative expression level of each target gene for each toxin concentration. Showing that each toxin except OA can be detected at

a concentration relevant for enforcement purposes, i.e. a lower concentration (using a newly developed clean-up procedure) than resulting from its regulatory limit.

Table 2. Selected genes and representation of their expression as determined in the whole genome array studies [17]. Up or down-regulated compared to a vehicle control: red arrows up represent genes that are up-regulated with $\log_2$ values higher than 0.7 and green arrows down are genes down-regulated with $\log_2$ values lower than -0.7. The (*) represents up-regulation higher than a $\log^2$ value of 2.0 or down-regulation of a $\log^2$ value lower than -1.5. The (-) represents $\log^2$ values between -0.4 and 0.4, which are considered as no significant effects on gene expression. ND: not determined.

| Gene	Toxin analogue				
	AZA-1 6.25 nM	OA 25nM	DTX-1 12.5 nM	YTX 12.8 nM	PTX-2 11.4 nM
NPPB	-	-	↓*	-	↓*
RGS16	-	↑	↑*	-	-
DDIT4	↑*	↑	↑	↑	↓
CXCR4	-	↑*	↑*	-	-
TGFB2	↓	↓	↓	-	-
MAFB	-	↑*	↑*	-	-
TNS4	↑	-	↑	-	-
C11orf96	-	↑*	↑*	ND	ND
CEACAM1	↑	↑	↑	↑*	-
OSR2	↓	-	↑	-	-
MT1H	↑*	↑	↑	-	-
MT1G	↑*	-	↑	↑	-
TMEM179B	-	-	-	-	-
CUL1	-	-	-	-	-
SH3BP2	-	-	-	-	-

Figure 1. Singleplex qRT-PCR results of the five selected marker genes NPPB, RGS16, DDIT4, CXCR4 and TGFB2. Values are shown as $\log_2$ relative quantity (RQ) of the gene of interest.

In general, genes in the singleplex qRT-PCR responded as expected from the whole genome array study (table 2). The responses observed for AZA-1 at only 3 nM are already clear and as expected, i.e. up-regulation of DDIT4 and down-regulation of TGFB2 and no effects on NPPB, RGS16 and CXCR4. As expected, all five genes responded to DTX-1 and even did so in a dose related way when looking at the responses obtained with 3 and 9 nM. Unfortunately, there were no clear responses to OA at 3 and 9 nM. However, this was more or less anticipated, as 25 nM was used in the whole genome array study because at lower concentrations no effects of OA on gene expression could be observed (data not shown). In spite of this, it was worthwhile to test 3 and 9 nM OA using singleplex qRT-PCR to investigate if singleplex qRT-PCR would be sensitive enough to detect OA at concentrations relevant for enforcement purposes. It should also be emphasised that this singleplex qRT-PCR is mainly developed to confirm suspect screened samples in the neuro-2a bioassay. For further experimentation, the concentrations of OA were increased to 25 and 100 nM. PTX-2 is easily detected with the marker gene NPPB, that is specifically down-regulated by this toxin at low concentrations. As expected, YTX could be detected by the up-regulation of DDIT4. It was remarkable that the YTX singleplex qRT-PCR profile is similar to that of AZAs, and also showed a down-regulation of TGFB2, which was not expected from the gene expression analysis. Another interesting finding is that this TGFB2 gene is downregulated by all toxins, except for OA at the (low) concentrations tested. The reference gene TMEM179B did not show any relevant expression (data not shown). It was decided to skip the TMEM179B as a reference gene as it turned out that when we designed the multiplex qRT-PCR, the use of the maximum amount of six fluorescent markers resulted in interference.

As the singleplex qRT-PCR results showed that the five selected marker genes responded as expected and in a sensitive way, a multiplex qRT-PCR was developed using primers, probes and dyes as shown in table 1. Figure 2 shows that this newly developed multiplex qRT-PCR was able to detect all toxins, and all except OA, at concentrations that are relevant for enforcement purposes. It was anticipated that toxin analogues would result in similar expression profiles and that this multiplex qRT-PCR would thus be suited for detecting analogues as well. Therefore, also AZA-2, AZA-3, DTX-2 and hYTX were tested. Figure 2 shows that exposure to AZA-2 and AZA-3 indeed resulted in similar profiles as AZA-1 and at similar concentrations. Also, the profiles of OA, DTX-1 and DTX-2 are identical as

are the profiles of YTX and hYTX. A table with a comparison of the expected and obtained results is included in supplementary materials (S2).
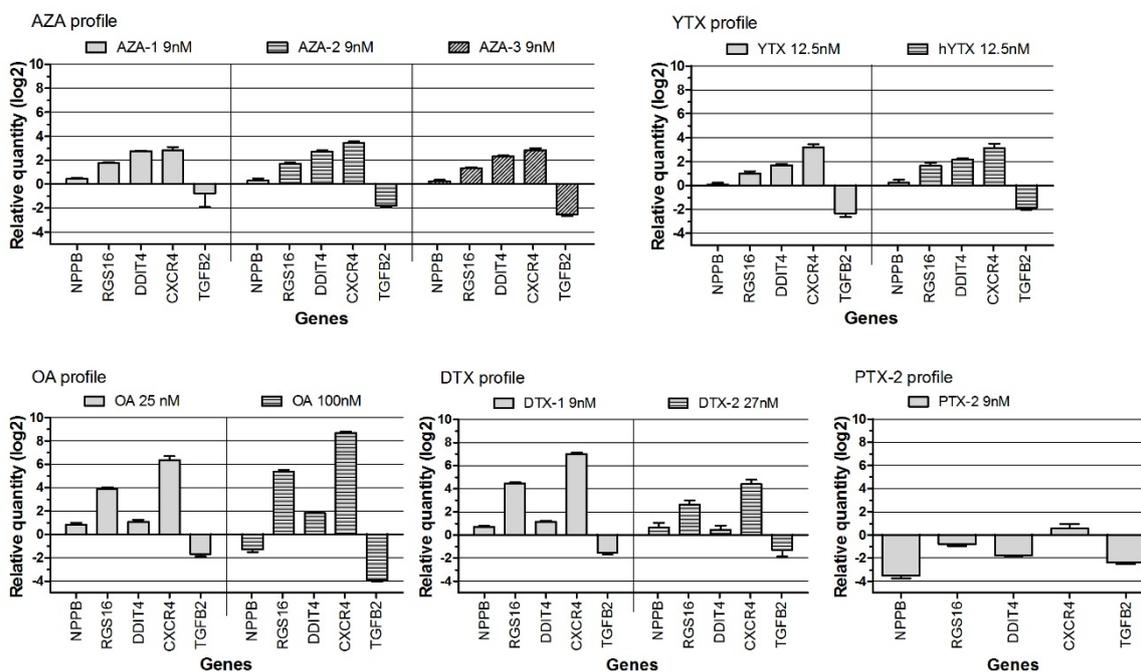
Figure 2. Multiplex qRT-PCR profiles. Profile A) AZAs and YTXs; profile B) OA/DTXs and profile C) PTX-2. Values are shown as $\log_2$ relative quantity (RQ) of the gene of interest.

In order to further evaluate the performance of the newly developed multiplex qRT-PCR, ten blank mussel samples and a mussel sample contaminated with AZAs (1,083 μg AZA-eq kg^{-1}), all according to the EURL LC-MS/MS method, were tested. Figure 3 shows that the extracts of the blank mussel samples did not affect the expression of any of the selected marker genes, while mussel contaminated with AZAs resulted in a "perfect" AZA/YTX-profile. Standards of OA and PTX-2 were used as positive controls and also resulted in the expected profiles.
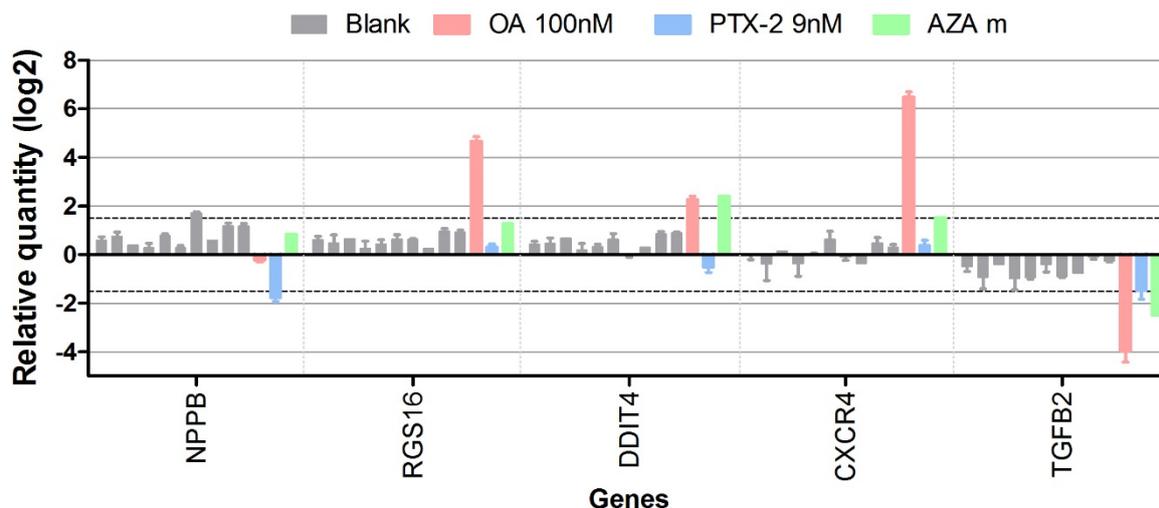
Figure 3. Multiplex qRT-PCR profiles obtained from 10 blank shellfish extracts, OA and PTX-2 standards and an extract prepared from a mussel sample indicated as 'AZA m' that is naturally contaminated with AZAs (1083 µg AZA-1-eq kg^{-1}). Bars represent log$_2$ values of the relative expression levels (RQ) of the genes.

To further increase the capacity to detect and identify the toxins by multiplex qRT-PCR, especially for the discrimination between the presence of AZAs or YTXs, a second multiplex qRT-PCR could be developed. However, in order to have one single test method to detect more than 5 marker genes, it was decided to analyse mRNA expression on another format: a multiplex magnetic bead-based assay.

3.2. Development of a Multiplex magnetic bead-based assay for 14 genes

The multiplex magnetic bead-based assay enables the examination of up to 100 genes, and it is based on the direct detection of the mRNA present in the sample, making it less labour intensive, i.e. no need for RNA purification, reverse transcription or amplification. Besides the five genes selected for multiplex PCR, more genes were selected from the whole genome array experiments, i.e. seven marker genes and two more reference genes (table 2). The multiplex magnetic bead-based assay uses magnetic beads coupled with DNA probes. These specific probes hybridise with a cognate mRNA present in the sample. The fluorescent signal associated with each specific bead is read on a Luminex® flow cytometer, where the equipment detects the specific bead, representing the gene, and the fluorescent signal attached to that bead, indicating the amount of cognate mRNA in the sample. Median

fluorescence intensities (MFIs) are measured and used to calculate relative gene expression levels.

The same toxins and toxin analogues as described above for the multiplex qRT-PCR method (5-plex) were tested in this multiplex magnetic bead-based 14-plex assay, i.e. OA, DTX-1, DTX-2, AZA-1, AZA-2, AZA-3, YTX, hYTX and PTX-2. Pinnatoxin (PnTX-E) was used as a negative control, since PnTX-E hardly affects the gene expression levels in Caco-2 (Fig. S1). Figure 4 shows the results for all the analogues and the twelve marker genes selected for this method (see also table S3). These data also revealed three clear profiles, i.e. OA/DTXs profile, AZAs/YTXs profile and a PTX-2 profile. As expected, PnTX-E did not elicit any specific responses at the gene expression level. When looking at more data in more detail, figure S3 in supplemental materials, it also becomes clear that in the concentration ranges tested, i.e. AZAs 3-9 nM; DTXs and PTX-2 3-27 nM; OA 3-100 nM and YTXs 12.5-37.5 nM, the OA/DTX profile shows clear dose-response effects. It also shows that on this test format it is also not possible to detect gene expression at low concentrations of OA, i.e. 3-9 nM. OA starts to affect gene expression at 25 nM and resulting in a clear profile at 100 nM, similar to the profile obtained with DTX-1 at 9 nM, indicating that OA is about four times more potent than DTX-1 and in line with the relative potencies as observed in the neuro-2a bioassay [17, 22]. Thus, also on the bead-based format it is not possible to detect OA at concentrations relevant for enforcement purposes. The DTX-2 response is lower than that of DTX-1, this is expected, as DTX-2 is less potent than DTX-1 [22, 24, 25]. Anyway, at a relevant level for enforcement purposes, 27 nM, the DTX-2 profile is identical to that of DTX-1 at 9 nM. Just as the DTXs, the AZAs, YTXs and PTX-2 can be detected at relevant concentrations for enforcement purposes. Unfortunately, the profiles for AZAs and YTXs are still identical. The TNS4, OSR2 and MT1H genes were especially added to distinguish the AZAs from the YTXs (table 2), but just like TGFB2 in the multiplex qRT-PCR, the YTXs cause the same effect on these three genes as the AZAs do. In order to rule out that YTX or AZA toxin standard were switched, the YTX, AZA-1 and DTX-1 stock solutions in DMSO were checked by LC-MS/MS analysis. Figure S5 in supplementary materials shows the obtained mass chromatograms, demonstrating that YTX and AZA were not switched, and that these standards are of the quality as expected for certified reference standards.
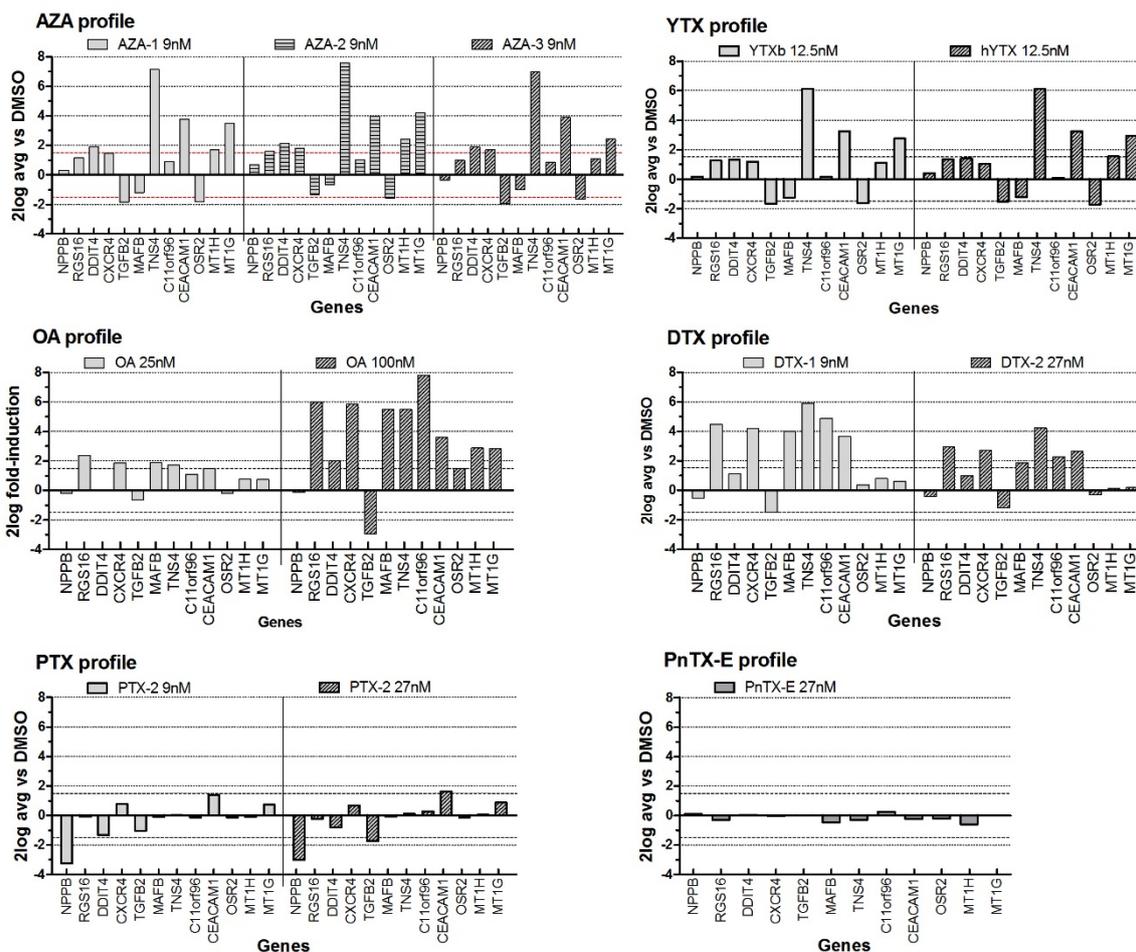
Figure 4. Profiles per toxin obtained from the multiplex magnetic bead-based assay. Caco-2 cells were exposed to OA, DTX-1, DTX-2, AZA-1, AZA-2, AZA-3, YTX, hYTX, PTX-2 and PnTX-E. Bars represent $\log_2$ of fold-induction values of each of the 12 marker genes. Positive values represent up-regulation, negative values represent down-regulation. Spotted lines indicate 'noise', which is defined by expression levels between 1.5 and -1.5 ($\log_2$ values).

3.3. Testing blanks and positive shellfish samples with the multiplex magnetic bead-based assay

Figure 5 shows the outcomes of the newly developed multiplex magnetic bead-based assay when testing extracts prepared from ten blank mussel samples, the same 'AZA mussel sample' used previously for the multiplex qRT-PCR, and a mussel sample contaminated with YTXs (330 µg YTX-eq kg^{-1}), as well as an OA and PTX-2 standard. The results demonstrate that blank samples (indicated in grey) do not lead to a substantial effect on the gene expression of the selected markers, i.e. all induction values are between 1.5 and -1.5

log2 values, which are considered as 'noise'. When another set of 20 blanks samples was tested, the outcomes were the same, i.e. no marker genes with induction factors above log2 values (see supplementary materials S6). The positive controls, i.e. OA 100 nM and PTX-2 9 nM, showed the expected and same profiles as described above in the multiplex qRT-PCR results (Fig. 3). As expected from testing pure standards (Fig. 4), the AZA and YTX contaminated mussel samples resulted in the expected AZA/YTX profile of the selected marker genes.
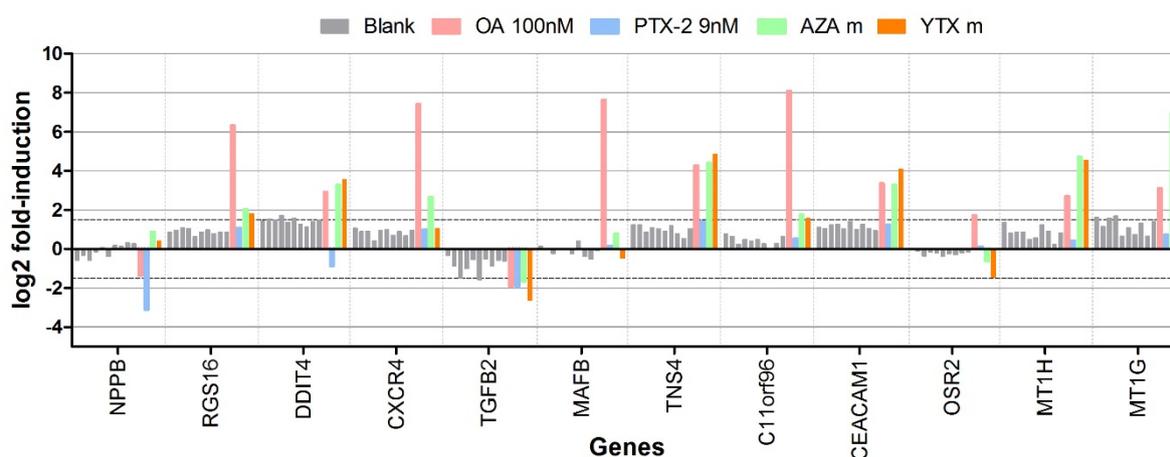

Figure 5. Multiplex magnetic bead-based assay. Caco-2 cells were exposed to 10 blank mussel samples (grey bars), OA (red bar) and PTX-2 (blue bar) standards and naturally contaminated mussels with AZA (green bar) and with YTX (orange bar). Spotted lines indicate 'noise', which is defined by expression levels between -1.5 and 1.5 (log2 values).

In addition, five shellfish samples with different concentrations and/or mix of lipophilic marine biotoxins that were used in previous validation studies [23], were tested. The toxin levels in these five samples are indicated in table 3. Figure 6 shows the outcomes of the newly developed multiplex magnetic bead-based assay when testing these five contaminated samples and a blank mussel sample. Again, the blank mussel sample did not lead to a substantial effect on the gene expression of the selected markers, while the five contaminated samples did. The interpretation of the profiles obtained with these contaminated samples becomes a bit complicated, as the samples contain mixtures of the toxins. However, it is possible to differentiate an AZA/YTX profile and an OA/DTX profile from the samples. According to the profile, samples V2 and V3 are more likely to be contaminated with OA/DTX toxins, while samples V5, V6 and V8 show a more AZA/YTX toxin-like profile. The latter is correct, but V2 and V3 do not only contain DTXs, but respectively also YTXs and AZAs. It is interesting to mention that sample V5

results in a correct AZA/YTX profile but is only contaminated with a low amount of AZAs, i.e. 32.4 µg AZA-1 eq kg^{-1}, and assuming 100% recovery, would result into an equivalent of 2 nM AZA-1 in the well. As the sample was positive in the neuro-2a bioassay too [22] and resulting in a clear profile now, it might be possible that this sample contains an unknown AZA or YTX analogue too.

Table 3. LC-MS/MS results from the 5 validation samples depicted in figure 6 [23].

Sample	OA, DTXs, PTX-2 (1)	AZAs (2)	YTXs (3)	Outcome	
				neuro-2a bioassay	Magnetic bead-based assay
V1	123		481	Suspect	OA/DTX
V2	275	2422		Suspect	OA/DTX
V3		32.4		Suspect	AZA/YTX
V4			770	Suspect	AZA/YTX
V5			620	Suspect	AZA/YTX

(1) µg OA-eq kg^{-1}
(2) µg AZA-1 eq kg^{-1}
(3) µg YTX-eq kg^{-1}
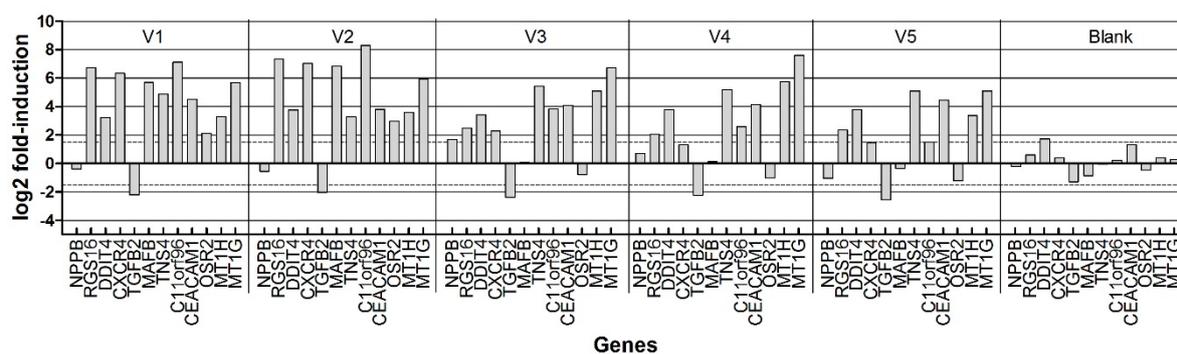
Figure 6. Multiplex magnetic bead-based assay. Caco-2 cells were exposed to five contaminated and one blank mussel samples. Spotted lines indicate 'noise', which is defined by expression levels between log$_2$ values 1.5 and -1.5.

4. Discussion

Worldwide, the mouse bioassay has been the main method to detect shellfish poisons in samples for human consumption and still complete surveillance programs that rely on the use of this animal test in many countries. Chemical analytical methods have been developed and proven suitable for the detection of known toxins, but countries with relative high occurrences of shellfish toxins in their coastal waters are still afraid to rely solely on such analytical chemical methods. One concern is the lack of standards for the known toxins. Another serious concern is that new appear that would be missed by such chemical analysis. In that regard, cell-based bioassays are an additional promising alternative. Especially the neuro-2a bioassay has been shown to be very useful for the broad detection of marine biotoxins, i.e. DSPs, NSPs and PSPs [21, 26, 27]. When using the neuro-2a bioassay for the broad detection of DSPs, samples screened negative are safe to consume and suspect screened samples can be confirmed by analytical chemical methods. It has been demonstrated that this is a fruitful approach [22]. However, in case a suspect screened sample cannot be confirmed by chemical analysis, indicating the presence of an unknown toxin, additional analysis is needed. In order to be successful, a second bioassay that is able to confirm the presence of such "DSP-like toxins" and also to identify the kind of DSP toxin present, would be very helpful. When this second bioassay also indicates the presence of a toxin, a bioassay-directed approach can be followed to identify this unknown active [28]. Figure 7 is a schematic view of the proposed strategy.
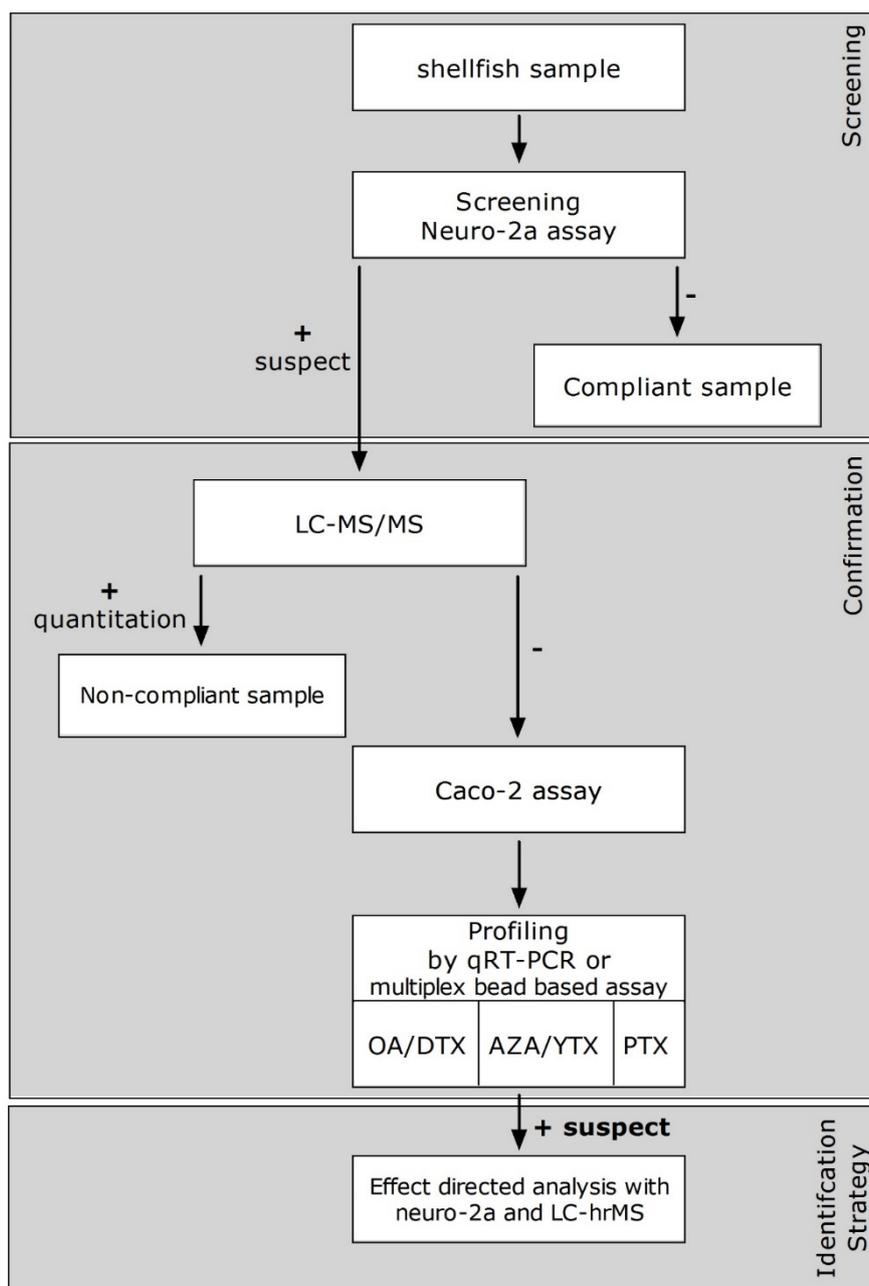
Figure 7. Proposed strategy for the broad screening of shellfish for the presence of LMB, i.e. using the neuro-2a bioassay for broad screening/detection (nonspecific cytotoxicity), analytical chemical analyses (LC-MS/MS) for the confirmation of known toxins, and the multiplex magnetic bead-based assay (specific mRNAs) or qRT-PCR for the confirmation of unknown toxins in case suspect neuro-2a outcomes cannot be explained by LC-MS/MS analysis. When the presence of an unknown active is confirmed by the second bioassay, a bioassay directed fractionation approach can be used to identify the new toxin.

In the present study, specific effects of the LMB on the gene expression in Caco-2 cells were used to develop a method that is able to distinguish these toxins. Previous gene expression studies envisioned three toxin profiles: i) OA/DTXs, ii) AZAs (and YTXs to some degree), and iii) PTX-2. Marker genes were selected, and two multiplex assays were developed, i.e. a multiplex qRT-PCR (5-plex) method and a multiplex magnetic bead-based assay (14-plex).

The multiplex qRT-PCR method, using five markers only, was able to determine the presence of each of all regulated LMB, including their analogues and thus potentially also unknowns, in extracts prepared from mussel samples. The obtained profiles enabled the discrimination between the presence of OA/DTXs, AZAs and PTX-2, but unfortunately the toxin profiles were not specific enough to discriminate between the presence of AZAs and YTXs. As OA and DTX belong to the same group, have a similar mode of action, and only differ in their potency, it is not possible to distinguish them with these kind of effect based bioassays [17, 29]. A multiplex magnetic bead-based assay, i.e. using specific probes that hybridise with the selected marker mRNAs and which are attached to the magnetic beads, allowed us to multiplex 14 genes in one reaction and resulted in more clear and complete toxin profiles, showing similar or higher induction factors as obtained by qRT-PCR. Unfortunately, the profiles did still not allow a discrimination between AZAs and YTXs, or a more sensitive detection of OA. The TNS4, OSR2 and MT1H genes were especially added to the multiplex magnetic bead-based assay to distinguish the AZAs from the YTXs (table 2), but just like TGFB2 in the multiplex qRT-PCR, the YTXs cause the same effect on these three genes as the AZAs do. The selection of those genes was done on gene expression analysis performed on a different platform, microarrays. Probably, this YTX array did not work as accurate as the ones used for OA, DTX-1, AZA-1 and PTX-2, as the standards were checked by LC-MS/MS and were pure and not switched. Anyway, both methods are able to confirm the presence of LMB and also to (partly) identify the kind of toxin present.

Besides the LMB tested, also the neurotoxic brevetoxins will be present in the prepared extracts (lipophilic), while PSP toxins and ASP toxin (hydrophilic) will not end up in these lipophilic sample extracts. Effects on gene expression by brevetoxins and transcriptomics data are scarce [30, 31]. However, the brevetoxins were not included in the present study, as the neuro-2a bioassay is already able to discriminate between the presence of DSP toxins,

AZAs and YTXs, and the neurotoxic brevetoxins, i.e. DSP toxins can be detected without the addition of ouabain and veratridine, while the brevetoxins can only be detected by the neuro-2a bioassay by adding low concentrations of ouabain and veratridine [32, 33]. In addition, it is shown that the nonregulated CIs like PnTX-E and SPX, that can also end up in the prepared lipophilic extracts, do not elicit clear effects on gene expression in Caco-2 cells (Fig. S1).

In a previous study, it was shown that the use of an additional n-hexane washing-step improved the clean-up of the LMB by eliminating false-positives in the neuro-2a bioassay due to matrix effects [22]. The present study shows that this clean-up also results in extracts that can be used to expose the Caco-2 cells, as blank samples did not affect the gene expression patterns, while contaminated samples resulted in the expected profiles. The use of the multiplex magnetic bead-based assay allowed us to multiplex 14 genes in one reaction. The results show that the method performs well and is less labour intensive than the multiplex qRT-PCR method, but the costs are higher. However, both multiplex methods work, and laboratories involved in monitoring can make their own choice, as the Caco-2 cells are easily available.

Although more testing and validation are required, an approach where the neuro-2a bioassay is used for the broad screening of LMB and LC-MS/MS analysis is used to confirm and identify the toxins present in the suspect screened samples, supplemented with a multiplex assay based on the expression of marker genes in Caco-2 cells in case suspect screened neuro-2a samples cannot be confirmed by LC-MS/MS analysis (Fig. 7), is very promising for ultimately replacing the rather cruel assay with mice.

5. Supplementary material chapter 4
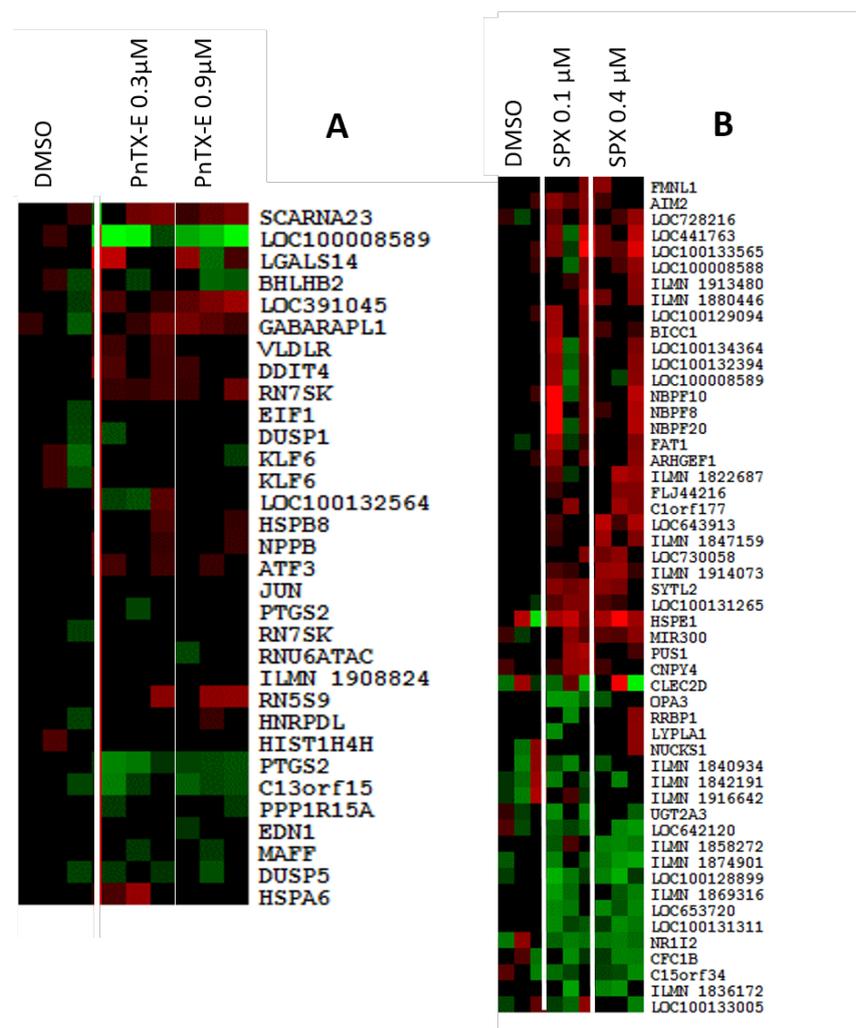
Figure S1. Hierarchical clustering of responsive genes in Caco-2 cells from Illumina microarrays using the programs Cluster (uncentered correlation, average linkage clustering) and Treeview [34]. (A) Exposure to PnTX-E. Gene selection is based on an average fold change of ≥2 (log2 ratio ≥|1.0|) in at least 3 of 15* microarrays. Red indicates upregulation, green indicates downregulation and black not affected. A maximal red or green colour indicates 2 times up or down regulation versus the control. *6 Microarrays of the non-related compound palytoxin (PlTX) were deleted from the figure, as this compound is already cytotoxic at low concentrations (pM range) resulting in strong upregulation of cognate genes. (B) Exposure to SPX. Gene selection is based on an average fold change of ≥1.4 (log2 ratio ≥|0.5|) in at least 2 of 9 microarrays. A maximal red or green colour indicates 1.4 times up or down regulation.

Table S2. Expected vs obtained results qPCR with analogues (expected results are the same included in table 1). Red arrows represent genes that are upregulated with log2 values higher than 0.7; green arrows are genes downregulated with log2 values lower than -0.7. (*) Represent up regulation higher than log2 value 2.0 or down regulation lower than -1.5. (-) Represent log2 values between -0.4 and 0.4, which is considered as no effect.

Expected results			Toxin analogue		
Gene	AZA-1	OA	DTX-1	YTX	PTX-2
NPPB	-	-	↓*	-	↓*
RGS16	-	↑	↑*	-	-
DDIT4	↑*	↑	↑	↑	↓
CXCR4	-	↑*	↑*	-	-
TGFB2	↓	↓	↓	-	-
TMEM179B	-	-	-	-	-

Obtained results				Toxin analogue				
PCR	AZA-1	AZA-2	AZA-3	OA	DTX-1	DTX-2	YTX	hYTX	PTX-2
NPPB	-	-	-	-	-	-	-	-	↓*
RGS16	-	-	-	↑*	↑*	-	-	-	-
DDIT4	↑*	↑*	↑*	-	-	-	-	↑*	↓
CXCR4	↑*	↑*	↑*	↑*	↑*	-	↑*	↑*	
TGFB2	↓*	↓*	↓*	↓*	↓*	-	↓*	↓*	↓*
TMEM179B	-	-	-	-	-	-	-	-	-

Table S3. Expected vs obtained results multiplex magnetic bead-based assay (expected are the same in table 1). Red arrows represent genes that are upregulated with log2 values higher than 0.7; green arrows are genes downregulated with log2 values lower than -0.7. (*) Represent up regulation higher than log2 value 2.0 or down regulation lower than -1.5. (-) Represent log2 values between -0.4 and 0.4, which is considered as no effect.

Expected results			Toxin analogue		
Gene	AZA-1	OA	DTX-1	YTX	PTX-2
NPPB	-	-	↓*	-	↓*
RGS16	-	↑	↑*	-	-
DDIT4	↑*	↑	↑	↑	↓
CXCR4	-	↑*	↑*	-	-
TGFB2	↓	↓	↓	-	-
MAFB	-	↑*	↑*	-	-
TNS4	↑	-	↑	-	-
C11orf96	-	↑*	↑*	ND	ND
CEACAM1	↑	↑	↑	↑*	-
OSR2	↓	-	↑	-	-
MT1H	↑*	↑	↑	-	-
MT1G	↑*	-	↑	↑	-
TMEM179B	-	-	-	-	-
CUL1	-	-	-	-	-
SH3BP2	-	-	-	-	-

| Obtained results | | | | | Toxin analogue | | | | | |
Gene	AZA-1	AZA-2	AZA-3	OA	DTX-1	DTX-2	YTX	hYTX	PTX-2	PnTX-1
NPPB	-	-	-	-	-	-	-	-	↓*	-
RGS16	-	-	-	↑*	↑*	-	-	-	-	-
DDIT4	-	↑*	-	-		-	-	-	-	-
CXCR4	-	-	-	-	↑*	-	-	-	-	-
TGFB2	↓*	-	↓*	-	↓*	-	↓*	↓*	-	-
MAFB	-	-	-	-	↑*	-	-	-	-	-
TNS4	↑*	↑*	↑*	-	↑*	-	↑*	↑*	-	-
C11orf96	-	-	-	-	↑*	-			-	-
CEACAM1	↑*	↑*	↑*	-	↑*	-	↑*	↑*	-	-
OSR2	↓*	↓*	↓*	-	-	-	↓*	↓*	-	-
MT1H	-	↑*	-	-	-	-	-	-	-	-
MT1G	↑*	↑*	↑*	-	-	-	↑*	↑*	-	-
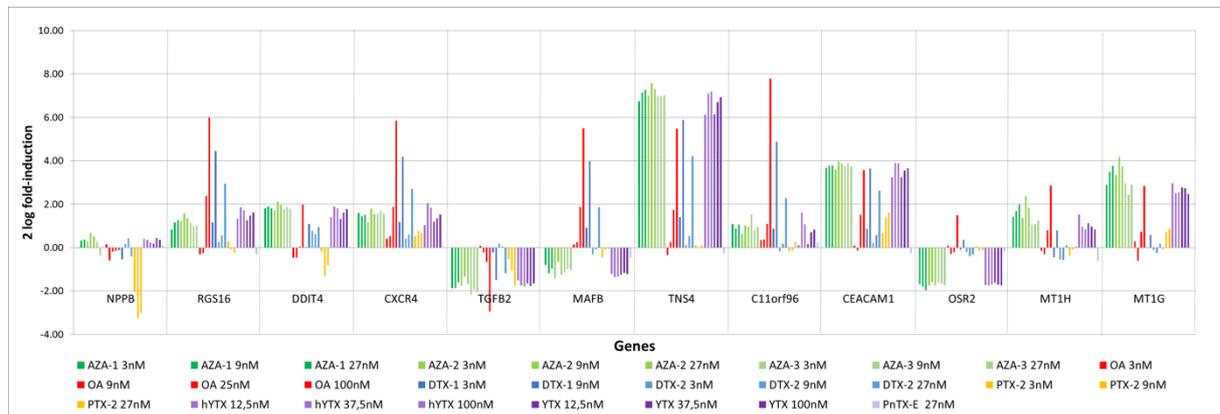
Figure S4. Multiplex magnetic bead-based assay. Caco-2 cells were exposed to OA, DTX-1, DTX-2, AZA-1, AZA-2, AZA-3, YTX, hYTX, PTX-2 and PnTX-E. Bars represent log2 of fold-induction values of each of the 12 genes. Positive values represent upregulation, negative values represent downregulation.
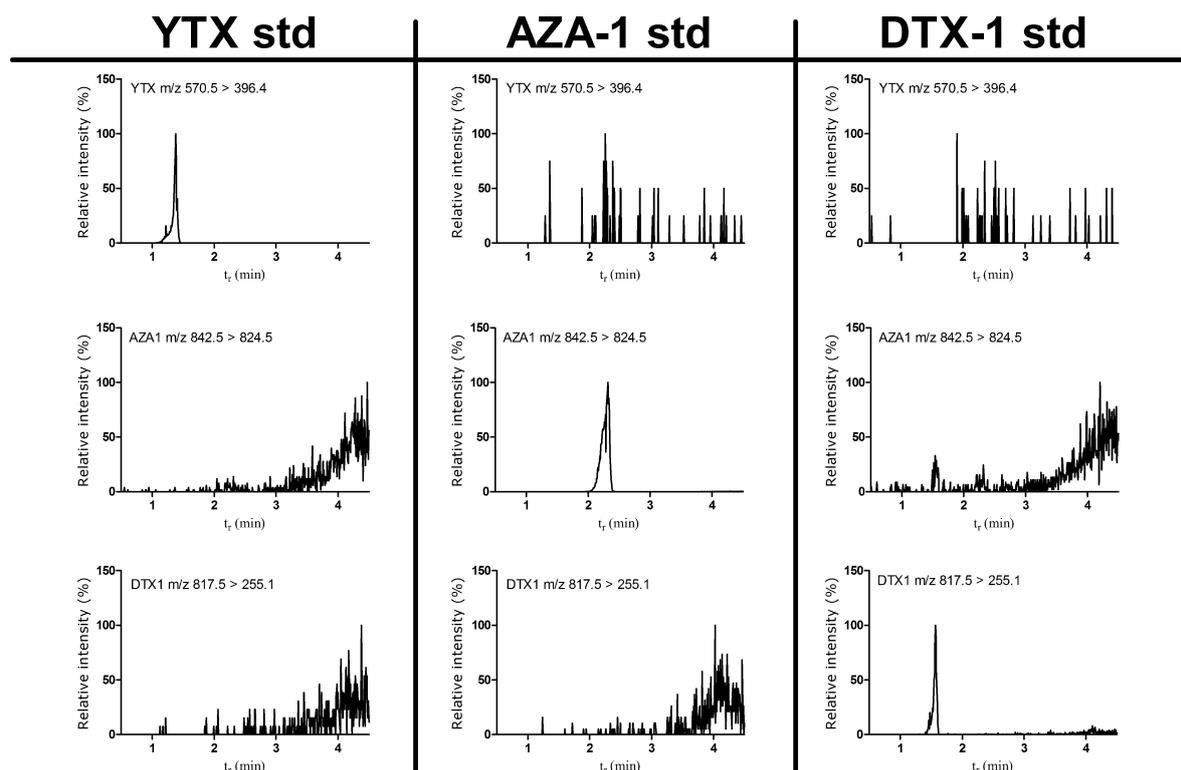
Figure S5. Chromatograms obtained by LC-MS/MS analysis (Gerssen et al., 2010b) of the individual YTX, AZA-1 and DTX-1 standards in DMSO.
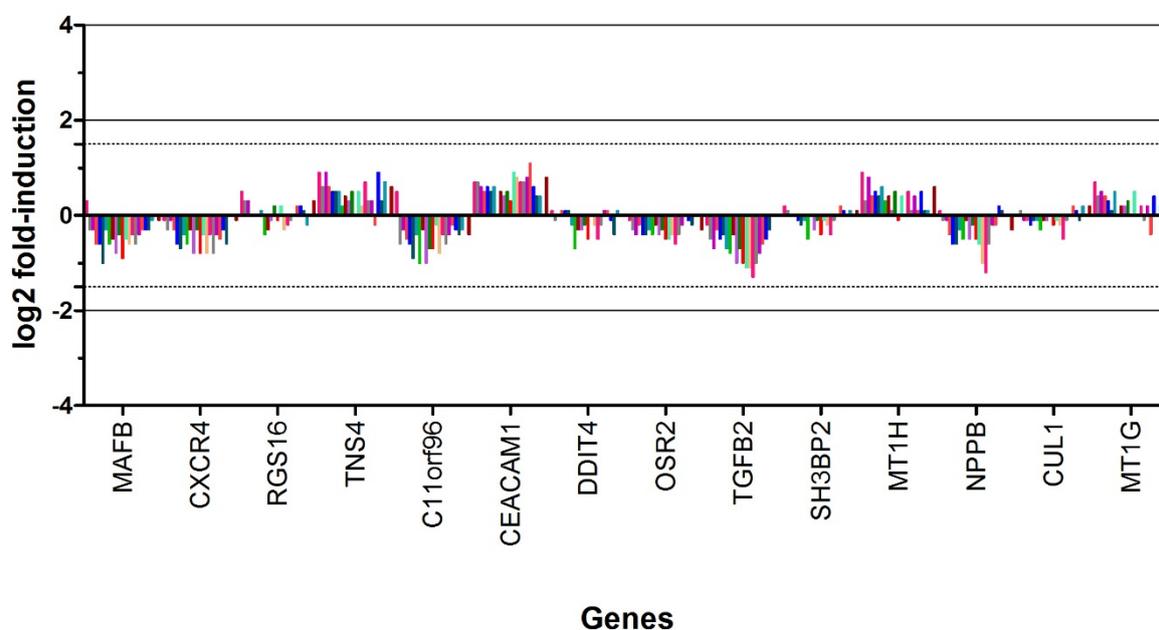
Figure S6. 20 Additional blank samples tested in the multiplex magnetic bead-based assay. Bars represent log2 of fold-induction values of each of the 14 genes. Positive values represent upregulation, negative values represent downregulation.

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Chapter 5

A strategy to replace the mouse bioassay for detecting and identifying lipophilic marine biotoxins by combining the neuro-2a bioassay and LC-MS/MS analysis

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Summary

Marine biotoxins in fish and shellfish can cause several symptoms in consumers, such as diarrhoea, amnesia or even death by paralysis. Monitoring programs are in place for testing shellfish on a regular basis. In some countries testing is performed using the so-called mouse bioassay, an assay that faces ethical concerns not only because of animal distress, but also because it lacks specificity and results in high amounts of false positives. In Europe, for lipophilic marine biotoxins (LMBs), a chemical analytical method using LC-MS/MS was developed as an alternative and is now the reference method. However, safety is often questioned when relying solely on such a method and, as a result, the mouse bioassay is still used. In this study the use of a cell-based assay for screening, i.e. the neuro-2a assay, in combination with the official LC-MS/MS method was investigated as a new alternative strategy for the detection and quantification of LMBs, including the diarrheic shellfish poisons (DSPs). To this end samples that had been tested previously with the mouse bioassay were analysed in the neuro-2a bioassay and the LC-MS/MS method. The neuro-2a bioassay was able to detect all LMBs at the regulatory levels and all samples that tested positive in the mouse bioassay were also suspect in the neuro-2a bioassay. In most cases these samples contained toxin levels (yessotoxins) that explain the outcome of the bioassay but did not exceed the established maximum permitted levels.

1. Introduction

To ensure food safety, it is most safe to test for the presence of toxins in the food items. The mouse bioassay (MBA) has been the main method to detect shellfish poisons in samples for human consumption and still complete surveillance programs heavily rely on this animal test in many countries around the world [1-3]. Chemical methods have been developed as alternatives and proven suitable for the detection of known toxins, but many countries are still afraid to rely solely on such analytical chemical methods, especially those with relatively high occurrence of shellfish toxins in their coastal waters. Not only due to the lack of standards for the known toxins, but also because toxin patterns might change, there is a concern about new toxins appearing that would be missed by such chemical analysis [4]. The neuro-2a bioassay is a promising alternative for the broad detection of marine biotoxins, i.e. lipophilic marine biotoxins (LMBs) including diarrheic shellfish poisons (DSPs) and neurotoxic shellfish poisons (NSPs) and hydrophilic marine biotoxins including paralytic shellfish poisons (PSPs) [5-8]. When using the neuro-2a bioassay for the broad detection of LMBs, samples screened negative are safe to consume and suspect screened samples can be confirmed for the EU regulated LMBs by analytical chemical methods, e.g. the EURL LC-MS/MS method [9]. It has been demonstrated that the neuro-2a assay is suitable for the screening of LMBs in mussels, following the successful introduction of an n-hexane washing step that was needed to eliminate matrix effects in the bioassay without loss of LMBs (recoveries between 80-110%). In this way matrix effects leading to false positive screening outcomes were eliminated [5]. In the present study samples previously tested in the MBA were analysed with the neuro-2a assay and LC-MS/MS analysis. In addition to the EU regulated LMBs, the applied method will also extract the neurotoxic brevetoxins (PbTXs), whose occurrence has been reported mainly in the United States, Mexico and New Zealand [10-14]. These NSPs can also be detected by the neuro-2a bioassay, however only in the presence of low concentrations of ouabain and veratridine (o/v) [6, 15]. Therefore, all sample extracts in the present study were tested in the neuro-2a bioassay with the addition of o/v, after demonstrating that the addition of low concentrations of o/v did not affect the detection of the LMBs. The present study is the first in which outcomes of samples obtained with the MBA, are compared to those obtained with the neuro-2a bioassay and LC-MS/MS analysis.

2. Materials and Methods

2.1. Reagents and standards

Certified reference materials (CRMs) of OA (13.7 ± 0.6 µg/mL), DTX-1 (15.1 ± 1.1 µg/mL), AZA-1 (1.24 ± 0.07 µg/mL), YTX (5.6 ± 0.2 µg/mL) were purchased from the National Research Council, Institute for Marine Biosciences (NRC CNRC) (Halifax, Canada). PbTX-1, -2, -3, -9 were purchased from Latoxan (Valence, France). Stock solutions of these toxin standards were prepared in dimethyl sulfoxide (DMSO) after evaporation of the original solvent. DMSO, ammonium hydroxide, and n-hexane were obtained from Merck (Darmstadt, Germany). Acetonitrile (Ultra LC-MS), methanol (Ultra LC-MS) and water (Ultra LC-MS) were purchased from Actu-All (Oss, The Netherlands).

2.2. Samples

Mussel samples (*mytilus edulis*) collected within the routine monitoring programme in the Netherlands in 2016 and analysed by the EURL LC-MS/MS method, were stored at -20 °C and used for comparison with the neuro-2a bioassay. Samples from different types of marine bivalves and previously tested on the presence of LMBs using the mouse bioassay, were kindly provided by Dr Leonardo Guzmán from the IFOP Instituto de Fomento Pesquero (Fisheries Development Institute), Chile (35 samples in total: 19 positive and 16 negative samples). In-house blank mussel samples, according to LC-MS/MS analysis, from the Netherlands were used as controls and for fortification.

2.3. Sample extraction

Prior to the extraction of the lipophilic marine biotoxins, shellfish material was homogenized with a T25 Ultra Turrax mixer at 24,000 rpm (IKA® Works Inc., Wilmington, NC, USA). One gram of shellfish homogenate was vortex-mixed with 3 mL methanol for one min and centrifuged for 5 min at 2000 × g. The supernatant was transferred to a volumetric flask and the residue was extracted twice more with 3 mL methanol. After the third extraction the volume of the collected supernatants was adjusted to 10 mL with methanol.

2.4. Further sample clean-up by washing with n-hexane followed by SPE

A 4.8 mL aliquot of the crude methanolic shellfish extract was diluted with 1.2 mL Milli-Q water and extracted twice with 6 mL n-hexane in order to remove matrix substances that would otherwise lead to false-positive test outcomes [5]. The hexane layer was discarded and the aqueous methanolic extract was further diluted with Milli-Q water to a final volume of 10 mL, and transferred to an SPE Strata™-X cartridge (200 mg/6 mL; Phenomenex, Utrecht, the Netherlands) previously conditioned with 4 mL methanol/water (30:70 v/v). Subsequently, the cartridge was washed with 8 mL methanol/water (20:80 v/v) and the toxins were eluted with 4.8 mL methanol. The eluate was evaporated to dryness under a stream of nitrogen gas and reconstituted in 20 μL DMSO.

2.5. Fortification of samples

Blank mussel samples were pooled (10 g) and 1-gram portions were extracted using the method described above. Fortification was performed at the level of the crude methanol extract, i.e. before the n-hexane clean-up, and at levels corresponding to 3, 1 and 1/3 times the maximum permitted level (MPL) in shellfish for OA, DTX-1 and AZA-1 (i.e. at 480, 160 and 53.3 μg kg^{-1}), except for YTX. Due to the high MPL of YTX (3.75 mg kg^{-1}), the high costs of the YTX CRM, and the relative high sensitivity of the neuro-2a bioassay for YTX, the extracts of blank mussel samples were fortified with 3, 1 and 0.3 mg kg^{-1} YTX (all well below the established MPL for YTX).

2.6. Neuro 2a bioassay

Neuroblastoma neuro-2a cells were purchased from the American Type Culture Collection (ATCC; CCL-131) and cultured in 75 cm^2 culture flasks containing 15 mL RPMI-1640 medium (R0883, Sigma-Aldrich, Zwijndrecht, the Netherlands) supplemented with 10% (v/v) foetal bovine serum (FBS, Fisher Emergo, Landsmeer, the Netherlands), 1% (v/v) of a 100 mM sodium pyruvate solution (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 1% (v/v) of a 200 mM L-glutamine solution (Sigma-Aldrich, Zwijndrecht, the Netherlands). The cell-line was routinely maintained in a humidified incubator at 37 °C under 5% CO$_2$ and sub-cultured three times per week (dilution 1/5) up to approximately 90% confluence. For exposure, neuro-2a cells were seeded into 96-well plates with an initial density of 25,000 cells per well using RPMI-1640 medium supplemented with 10% FBS.

After growing the cells for 24 h, medium was aspirated and exposure to pure marine biotoxins or sample extracts was performed in triplicate in 200 μL (end volume) medium for 24 h. Ouabain and veratridine were dissolved in medium supplemented with 5% FBS and 50 μL of each were added per well, first ouabain then veratridine, to reach final concentrations of 0.13 mM and 0.013 mM respectively (decreasing the MTT activity by about 20%). Finally, the test compound or sample extract was dissolved in medium supplemented with 5% FBS and 100 μL were added to the corresponding well. The final DMSO concentration in the medium was kept at 0.25% (v/v). PbTX-3 was used as a positive control. At the end of the exposure time, MTT activity was measured as described previously [5]. In short, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, Zwijndrecht) was prepared in PBS at 5 mg mL^{-1} and mixed with serum free medium. Then, the exposure medium was removed and 60 μL of MTT mixed with serum free medium were added to each well (final concentration of MTT in the well was 0.8 mg mL^{-1}). After 30 min incubation at 37 °C and 5% CO_2, medium was removed, and the formed formazan crystals were dissolved in 100 μL DMSO. Plates were placed in a plate shaker for 10 min at 600 rpm after which the absorbance was measured at 540 nm and corrected for background absorption at 650 nm.

2.7. LC-MS/MS analysis

Chemical analysis was directly performed on the crude methanol extracts. The EURL method applied for the determination of lipophilic marine biotoxins (i.e. DSPs, AZAs and YTXs) was previously described by Gerssen et al. [16]. Briefly, chromatographic separation was achieved using a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA). The system consisted of a binary solvent manager, sample manager and a column manager. A Waters Acquity BEH C$_{18}$ 1.7 μm, 2.1 x 100 mm column was used. The column temperature was kept at 60 °C and the temperature of the sample manager was kept at 10 °C. A 5 μL injection volume was used. Mobile phase A was water and mobile phase B was acetonitrile/water (90:10 v/v), both containing 6.7 mM ammonium hydroxide. A flow rate of 0.6 mL min^{-1} was used. The gradient started at 30% B for 0.5 min and was then linearly increased to 90% B in 3 min. This composition was kept for 0.5 min and returned to 30% B in 0.1 min. An equilibration time of 0.9 min was allowed prior to the next injection. The effluent was directly interfaced in the electrospray ionisation (ESI) source of the AB Sciex

QTrap 6500 mass spectrometer (Ontario, Canada) which was operated in both negative and positive electrospray ionisation by rapid polarity switching. Two transitions were measured for each toxin. Regarding the detection of the PbTXs, a separate extraction was performed. One gram of shellfish homogenate was mixed head-over-head for 15 min with 3 mL methanol/water (80:20 v/v). The supernatant was transferred to a volumetric flask and the residue was extracted twice more with methanol/water (80:20 v/v) using a multipulse vortex for one min. After the third extraction, the volume of the supernatants was adjusted to 10 mL with the same solvent and mixed. The extract was filtered through a 0.2 μm membrane filter and an aliquot was transferred to a 1.5 mL vial for LC-MS/MS analysis. Chromatographic separation was achieved using the same system and column as used for the DSPs, AZAs and YTXs. The column temperature was kept at 40 °C and the temperature of the sample manager was kept at 10 °C. A 10 μL injection volume was used. Mobile phase A was water and mobile phase B was acetonitrile/water (90:10 v/v), both containing 47 mM formic acid and 3 mM ammonium formate. A flow rate of 0.4 mL min^{-1} was used. The gradient started at 40% B for 0.1 min and was then linearly increased to 100% B in 6 min. This composition was kept for 2 min and returned to 40% B in 0.1 min. An equilibration time of 0.9 min was allowed prior to the next injection. The effluent was directly interfaced in the ESI source of a Waters Xevo TQ-S mass spectrometer which was operated in positive ESI. For quantification, so-called "matrix matched" calibration curves were constructed by fortifying blank shellfish material with different known concentrations of toxin. The area of the toxin in the unknown sample is then calculated using the linear equation of the calibration curve. The concentration is expressed in μg kg^{-1} shellfish.

3. Results

3.1. Neuro-2a bioassay with mussel samples

The lipophilic sample extracts can in principle contain the DSPs, AZAs, YTXs and PbTXs. In order to detect the PbTXs in the assay, the neuro-2a bioassay is used with the addition of ouabain and veratridine (o/v) at concentrations that cause about 20% reduction in MTT activity, i.e. 0.13 mM and 0.013 mM respectively. Fortification of blank mussel sample extracts was performed at levels equivalent to 3, 1 and 1/3 times the MPL for OA, DTX-1 and AZA-1 (i.e. at 480, 160 and 53.3 µg kg^{-1}), except for YTX as the MPL is 3.75 mg kg^{-1}, and costs for YTXs are rather high and because the neuro-2a bioassay is relatively sensitive for these toxins. The blank mussel sample extracts were therefore fortified with 3, 1 and 0.3 mg kg^{-1} YTX, which should theoretically still lead to a maximal inhibition (about 50% reduction of MTT activity) for all levels and at both dilutions, thus even for the sample spiked at 0.3 mg kg^{-1} YTX and 5 times diluted (Bodero et al., 2018). Figures 1 and 2 show the results of the neuro-2a assay where the standards and undiluted and 5 times diluted fortified mussel sample extracts were tested in the absence (fig.1) and presence of o/v (fig. 2). A DMSO solvent control was set at 100% and a previously arbitrary decision limit was set at 75% [5]. Samples that result in MTT activity percentages above 75% are classified as negative and those that result in percentages below 75% are classified as suspect (positive in the bioassay). The data show that the screening results of OA and DTX-1, both standards and fortified samples, are hardly influenced by the addition of o/v. Standards of AZA-1 and YTX are not affected and undiluted samples fortified with AZA-1 and YTX even give slightly better screening outcomes when adding o/v. This is mainly due to the fact that without o/v, the undiluted fortified AZA-1 and YTX sample extracts show almost no effect, i.e. responses for undiluted AZA-1 sample extracts around 75% (fig. 1C) and close to 100% for the undiluted YTX sample extracts (fig. 1D), meaning that both AZA-1 and YTX are not detected when testing the undiluted sample extracts without o/v, while the corresponding 5 times diluted fortified sample extracts result in a clear detection of both AZA-1 and YTX, a phenomenon observed before (for YTX) and for which we have no explanation [5]. However, the highest spiked amount of YTX (3 mg kg^{-1}) is still well below the MPL (3.75 mg kg^{-1}) and in real practice samples contaminated with YTXs are easily picked-up with the neuro-2a bioassay (Bodero et al., 2018). Altogether, the addition of o/v

allows the use of undiluted sample extracts, enabling the detection of OA, DTX, AZA and YTX at the level of their MPLs (fig. 2) and will also allow the detection of PbTXs.
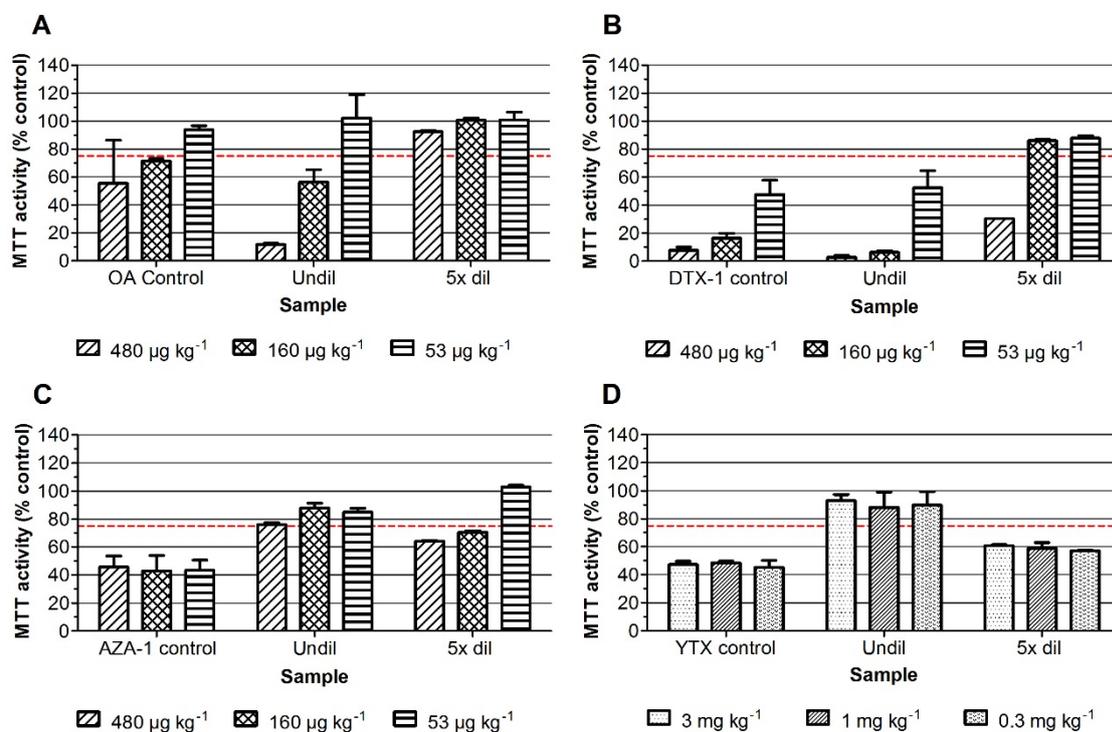

Figure 1. Effect on the MTT activity of neuro-2a cells of standards (control) and undiluted and 5 times diluted fortified mussel sample extracts. A) OA; B) DTX-1; C) AZA-1 and D) YTX. A DMSO solvent control was set at 100% and a previously arbitrary decision limit was set at 75% (Bodero et al., 2018). Data are expressed as mean ± SD (n=3).
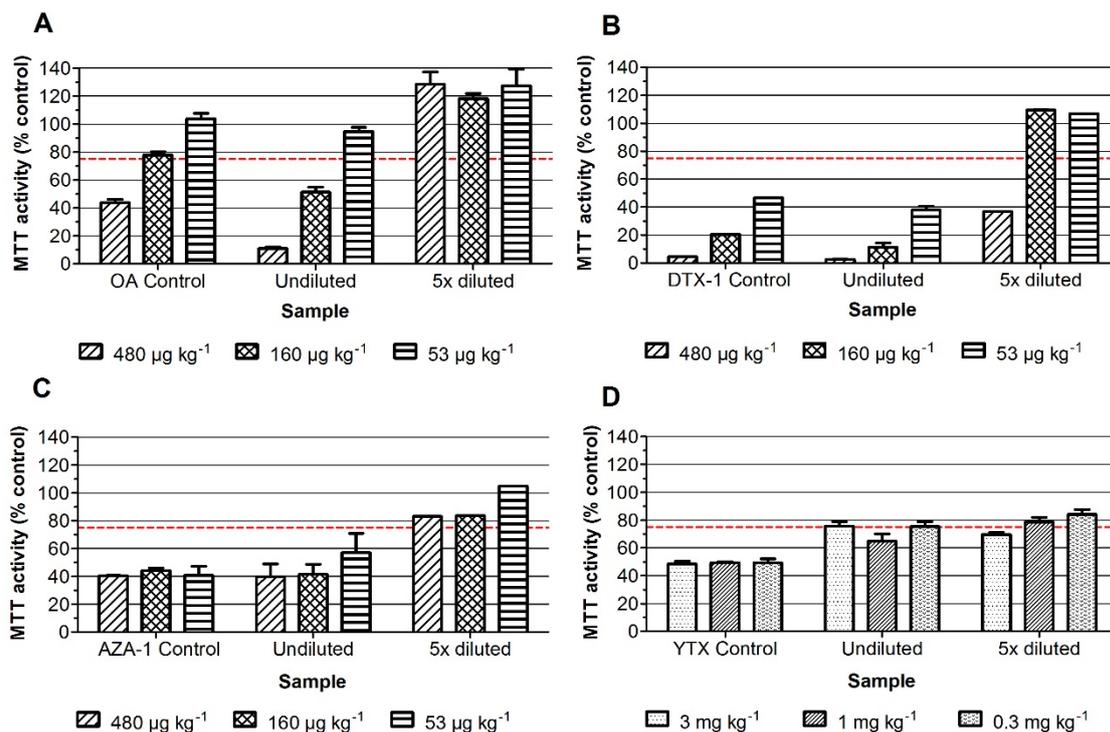
Figure 2. Effect on the MTT activity of neuro-2a cells with the addition of ouabain and veratridine to obtain 20% decrease in MTT activity, of standards and undiluted and 5 times diluted fortified mussel sample extracts. A) OA; B) DTX-1; C) AZA-1 and D) YTX. A DMSO solvent control was set at 100% and a previously arbitrary decision limit was set at 75% (Bodero et al., 2018). Data are expressed as mean ± SD (n=3).

Mussel samples collected in the Netherlands in 2016 for a routine monitoring programme that were analysed by LC-MS/MS for DSPs, AZAs and YTXs were stored at -20 °C. A total of 110 samples were taken (10 samples of each of the months from January to November), extracted and tested in the neuro-2a bioassay with the addition of o/v. Figure 3 shows the results of the 20 samples from June and July as tested in the neuro-2a assay with o/v. A chemical blank in DMSO solvent was used as a negative control and set at 100% and a 30 nM PbTX-3 standard was used as a positive control. The data show that none of the samples resulted in a response below 75% viability. All 20 samples were thus classified as negative. The positive control, i.e. 30 nM PbTX-3, clearly decreased the MTT activity of the neuro-2a cells, indicating that the o/v treatment worked adequately. Outcomes of chemical blanks are always identical to that of DMSO controls (data not shown). The data for the other 90 mussel samples are shown in figure S1 in supplementary materials. Only 2 of the 110 mussel samples resulted in an MTT activity below 75%, while

none of the 110 samples tested contained substantial amounts of DSPs, AZAs and YTXs according to the LC-MS/MS analysis (all far below the MPLs, data not shown). The screening with the neuro-2a assay thus resulted in 1.8% of false positives, although it cannot fully be ruled out that these two samples contained a low amount of PbTXs or unknown DSPs, AZAs or YTXs. As no positives were found by LC-MS/MS analysis, this set of Dutch mussel samples is not suited to test for the rate of false negative screening outcomes with the neuro-2a bioassay.
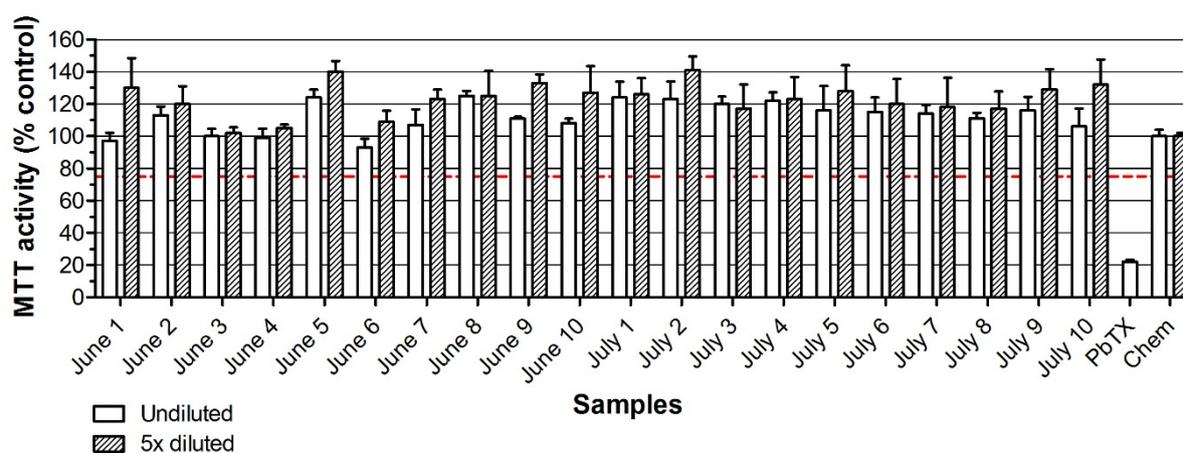
Figure 3. Effect of 20 mussel samples from the Netherlands on the MTT activity of neuro-2a cells in the presence of ouabain and veratridine to obtain 20% decrease in MTT activity. The chemical blanc (Chem) in DMSO was set at 100%. PbTX-3 at 30 nM was used as a positive control. Data are expressed as mean ± SD.

3.2. Neuro-2a bioassay with naturally contaminated samples tested previously in the MBA

In order to establish a false negative rate and compare the performance of the *in vitro* neuro-2a bioassay with the *in vivo* mouse bioassay, 35 samples provided by an institute in Chile (Instituto de Fomento Pesquero) that had tested these samples in the MBA, were extracted and tested in the neuro-2a bioassay with and without o/v and analysed by LC-MS/MS. Figure 4 shows the screening outcomes of these 35 samples as obtained in the neuro-2a bioassay with the addition of o/v, together with a PbTX-3 control to check the correct response of the neuro-2a bioassay (positive control) and a DMSO solvent control (negative control). Figure S2 in supplementary material shows the data of these samples as obtained in the neuro-2a bioassay without the addition of o/v, i.e. the "old" method that does not allow the detection of PbTXs. Table 1 summarises the MBA, neuro-2a, and LC-MS/MS

results. The details on species and origin of the samples are given in table S3 in supplementary materials).
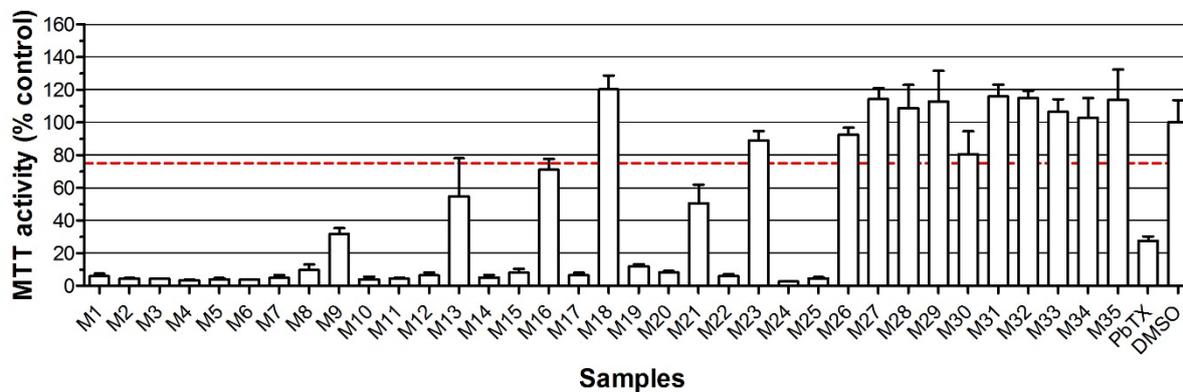

Figure 4. Effect on the MTT activity of neuro-2a cells with the addition of o/v of 35 samples tested before in Chile in the mouse bioassay. The spotted line represents the arbitrarily set decision limit (75% MTT activity). A DMSO solvent with o/v was used as a negative control and set at 100%. Data are expressed as mean ± SD.

Table 1. Summary of 35 samples obtained from Chile. Lipophilic extracts were prepared and tested in the MBA in Chile and both in the neuro-2a bioassay and by LC-MS/MS analysis at RIKILT.

Sample	Specie	MBA	Neuro 2a with o/v	Neuro2a without o/v	LC-MS/MS (µg kg⁻¹)		
					OA eq	AZA-1 eq	YTX eq
M1	Mussel	positive	+	+			898
M2	Mussel	positive	+	+			869
M3	Mussel	ND	+	+			704
M4	Mussel	positive	+	+			1060
M5	Mussel	positive	+	+			569
M6	Clam	positive	+	+			
M7	Clam	ND	+	+			
M8	Clam	positive	+	+			
M9	Mussel	positive	+	+			1459
M10	Mussel	positive	+	+			2531
M11	Clam	ND	+	+			
M12	Mussel	positive	+	+			1648
M13	Mussel	positive	+	+			1271
M14	Mussel	positive	+	+	22		1546
M15	Mussel	positive	+	+			1254
M16	Clam	ND	+	-			
M17	Clam	positive	+	+			
M18	Clam	ND	-	-			
M19	Mussel	positive	+	+	33		1953
M20	Mussel	positive	+	+	21		1432
M21	Mussel	positive	+	+	20		1518
M22	Mussel	positive	+	+			1141
M23	Clam	ND	-	+			
M24	Mussel	positive	+	+			1067
M25	Mussel	positive	+	+			1648
M26	Mussel	ND	-	+			272
M27	Mussel	ND	-	-			
M28	Clam	ND	-	-			
M29	Mussel	ND	-	-			
M30	Clam	ND	-	-		15	
M31	Mussel	ND	-	-			
M32	Clam	ND	-	-		9	
M33	Mussel	ND	-	-			
M34	Mussel	ND	-	-			
M35	Mussel	ND	-	-			

ND = not detected (thus negative in the MBA). Empty spots in the LC-MS/MS columns = values lower than the limit of quantification (LOQ): OA eq < 10 µg kg⁻¹, AZA-1 eq <5 µg kg⁻¹, YTX eq <50 µg kg⁻¹. Brevetoxins were not detected, LOQs in shellfish are PbTX-1 150µg kg⁻¹, PbTx-2 89µg kg⁻¹, PbTx-3 46µg kg⁻¹ and PbTx-9 32µg kg⁻¹.

In general, the screening results as obtained in the neuro-2a bioassay correlate well with those obtained in the MBA. All the samples that are positive in the *in vivo* MBA (19 of the 35 samples tested) are also positive in the *in vitro* neuro-2a bioassay with o/v. The rate of false negatives in the neuro-2a bioassay with o/v is thus 0% when compared to the MBA. Of the 16 samples that were negative in the MBA, 12 were also negative in the neuro-2a bioassay with o/v and 4 were positive, i.e. elicited inhibition of the MTT activity below the arbitrarily set decision limit of 75%. Of these 4 samples, M3, M7, M11 and M16, only sample M3 turned out to contain a significant amount of a toxin (YTX eq), i.e. enough to cause an effect in the neuro-2a bioassay. M16 resulted in a response just below 75% and was negative when tested without the addition of o/v. M7 and M11 might contain an unknown toxin not detected by the MBA or the LC-MS/MS, but most likely these two samples are true false positive screening outcomes in the neuro-2a, which results in a false positive rate of 12.5%. As the neuro-2a bioassay is intended to be used as a screening method, false negatives should not occur, and the number of false positives should not be too high, the current outcome with these Chilean samples, i.e. no false negatives (0%) and 12.5% of false positives, is a reasonably good result.

From a point of view of the effect side, LC-MS/MS analysis results in many false negatives, as none of the positive screened samples in either the MBA or neuro-2a bioassay contained lipophilic toxins in amounts exceeding the established MPLs. From a chemical point of view, both the MBA and neuro-2a bioassay result in many false positives, especially M6, M8 and M17, as these three samples contained no toxins according to the LC-MS/MS analysis and were positive in both the MBA and neuro-2a assay. It is worthwhile to mention that all the samples that are positive in any of the bioassays and contain levels <LOQ in the LC-MS/MS, are clams (*Venus antiqua)* (see table S3 in supplementary materials). And although there are negative clams too, it is possible that this matrix leads to false positives in both the MBA and neuro-2a bioassay or causes suppression in the LC-MS/MS (not validated for this matrix). The latter is not likely as the LOQs of the LMBs are very low (a few % or lower of the MPLs).

4. Discussion

The mouse bioassay (MBA) for the detection of marine biotoxins is in use for 40 years but has never been properly validated for LMBs [17, 18]. The need to develop alternative methods to replace the MBA has been reviewed extensively [19-21], but no real replacement has occurred yet. Chemical methods, particularly LC-MS/MS analysis, seem suited and for DSPs, AZAs and YTXs it is the reference method in the EU [22]. However, due to the poor availability of standards, the lack of standards for all analogues, the high costs, and the concern about new toxins appearing that would be missed by targeted chemical analysis, many countries hesitate to fully switch to chemical methods [2, 23]. A combination of cell-based bioassays and chemical analysis might offer the opportunity to face the drawbacks. An approach consisting of a first effect-based screening that allows the detection of known and unknowns and a confirmation with chemical methods is then logical. The neuro-2a bioassay is regarded as the most promising assay for the broad detection of DSPs, AZAs and YTXs [24], since it is able to detect all these toxins and is relatively rapid and easy to perform. It is important to consider that the neuro-2a bioassay will be used as a qualitative screening method, i.e. that the outcome of a sample is either negative or suspect, based on a certain cut-off. Negative samples are safe and suspect samples need confirmation, e.g. by the EU LC-MS/MS reference method [5, 22]. It has already been demonstrated that this is a fruitful approach, due to the successful introduction of an n-hexane washing step that was needed to remove matrix effects observed in the neuro-2a bioassay. It was shown that no DSPs, AZAs and YTXs were lost due to the extra n-hexane washing step (recoveries between 80-110%), while the matrix effect leading to false positive screening outcomes was excluded [5]. It should be noticed that fatty acid analogues of OA and DTX are probably lost with this n-hexane washing step. It was also shown in that study that the outcomes of the neuro-2a screening and EU LC-MS/MS analysis for DSPs, AZAs and YTXs in seafood correlated well. However, the correlation of the neuro-2a bioassay with the MBA has never been investigated. The present study is the first where a set of shellfish samples was tested with both the MBA and the neuro-2a bioassay, and in addition LC-MS/MS analysis. Prior to this, the neuro-2a method was further developed in order to also detect the brevetoxins, which can also be present in lipophilic sample extracts [10], achieved by addition of ouabain and veratridine (o/v) [24, 25]. In the present study it was shown that the addition of o/v

did not interfere with the detection of DSPs, AZAs and YTXs. It even slightly improved the detection of AZAs, but the mechanism is unclear.

To study the effect of real samples, lipophilic sample extracts of Dutch mussel samples were tested in the neuro-2a bioassay with o/v, showing that 108 out of 110 samples responded as predicted by EU LC-MS/MS analysis for DSPs, AZAs and YTXs, i.e. 108 negatives and only two false positives (1.8%). It cannot be fully ruled out that these two samples contained low levels of other toxins, like PbTXs, as these toxins are not included in the EU LC-MS/MS analysis. As samples with LMBs are rather scarce in the Netherlands and the MBA is no longer in use, further testing was performed with samples obtained from Chile that were already tested in the MBA (positives and negatives). Moreover, these Chilean samples were not only tested by LC-MS/MS for DSPs, AZAs and YTXs (EU reference method), but also by an additional LC-MS/MS analysis for PbTXs.

The 19 Chilean samples that tested positive in the MBA were also positive in the *in vitro* neuro-2a bioassay with o/v. The rate of false negatives in the neuro-2a bioassay with o/v is thus 0% when compared to the MBA. None of the samples that were positive in the MBA and neuro-2a screening, contained toxin levels above the established MPLs. Strictly seen, i.e. enforcement purposes, using only the MBA would have led to 54% of false positives (19 out of 35), while the combination of the neuro-2a bioassay with o/v and LC-MS/MS analysis did probably not result in false positives. Of the 23 samples that were screened as suspect in the neuro-2a bioassay with o/v, 17 contained toxin levels below the established MPLs, but although below the MPLs, they contained YTX eq levels that would explain the bioassay outcome. The other 6 samples could then be regarded as false positives of the neuro-2a screening, not being confirmed by LC-MS/MS analysis. However, the latter is not sure, as from these 6 samples, 3 were tested as positive in the mouse MBA too. It should be noticed that these 6 samples are clams, and it cannot be excluded that this matrix in some cases leads to false positives in the MBA and neuro-2a bioassay. Of the 16 samples that were negative in the MBA, 12 were also negative in the neuro-2a bioassay with o/v and 4 were positive. One of these 4 (M3) turned out to contain a significant amount of YTX eq, i.e. enough to cause an effect in the neuro-2a bioassay with o/v and another one (M16) resulted in a response just below 75% when tested with o/v and was negative when tested without o/v. The remaining 2 may thus be considered as true false positives, which

results in a false positive rate of 12.5%. It was ruled out that these two samples (clams), or even one of the other 33 samples, contained PbTXs, as the screening outcome of the 22 samples screened suspect in the neuro-2a bioassay with o/v was also suspect when tested in the neuro-2a bioassay without o/v (except for M16). This was confirmed by additional LC-MS/MS analysis, revealing no detectable levels of PbTX-2, PbTX-3 and PbTX-9 or any of its shellfish metabolites in any of these 35 samples.

The correlation between the MBA and neuro-2a bioassay on the one hand and the LC-MS/MS on the other hand is close to perfect when looking at mussels only. Of the 24 Chilean mussel samples tested, all 17 that were positive in the MBA or neuro-2a bioassay with o/v, contained high levels of YTX eq (all > 569 µg kg^{-1}) according to LC-MS/MS analysis, while the 7 mussels that were negative contained no detectable levels of toxins according to LC-MS/MS analysis (except for M26, that contained a low level of YTX eq, i.e. 272 µg kg^{-1}). However, in real practice and even when looking at mussels only, using the MBA only, would have led to 67% of false positives, while the combination of the neuro-2a bioassay and LC-MS/MS analysis would not have led to an unnecessary closure of areas or withdrawal of mussels from the market.

The outcomes are very promising, but there is still remaining work to do, like proper validation of different matrices following international guidelines [26] with each toxin spiked at its MPL to 20 blank samples and further optimization of the extraction procedure, as the present data indicate that clams (*Venus antiqua*) lead more easily to false positives in the MBA and neuro-2a bioassay than mussels (*Mytilus edulis, Mytilus chilensis, Aulacomya ater*). In addition, parallel studies with collaboration between countries that use the MBA are needed, e.g. by testing certified reference samples.

To reduce the number of false positives in the MBA and also reduce the number of false suspects in the neuro-2a bioassay, two separate extracts should be prepared, i.e. one extract containing the OAs, DTXs, AZAs, PTX and PbTXs and a second extract containing the YTXs. The first extract should be tested undiluted, while the second extract, containing the YTXs, should be diluted before testing it in the MBA or neuro-2a bioassay, as both assays are relatively sensitive to YTX, while the MPL of YTXs is much higher compared to the other toxins. In case a suspect screened sample cannot be confirmed by chemical analysis,

indicating the presence of an unknown toxin, additional analyses are needed. Previously, it was shown that a second bioassay, i.e. gene expression in Caco-2 cells using a magnetic bead-based multiplex assay, is able to confirm the presence of such "DSP-like" toxins and based on the obtained expression profile, is also able to indicate the kind of LMB present. Three profiles could be envisioned, i.e. an OA/DTX profile, an AZA/YTX profile and a PTX profile (Bodero et al., in press), while samples contaminated with PbTXs can envisioned by testing them in the neuro2a-bioassay with and without o/v.

Overall, one can say that the neuro-2a bioassay with o/v can be used to test for the presence of lipophilic marine biotoxins and shows a good correlation with the MBA and LC-MS/MS analysis. All samples that tested positive in the MBA were also positive in the *in vitro* neuro-2a bioassay with o/v and most could be explained by the amounts of toxins as measured by LC-MS/MS. When only looking at the Chilean mussel samples, 23 out of the 24 samples resulted in the same screening outcome when tested with the MBA or neuro-2a bioassay. Only sample M3 was negative in the MBA and positive in the neuro-2a bioassay, probably because M3 contains a significant amount of a YTX eq, i.e. enough to cause an effect in the neuro-2a bioassay, although it is noted that samples with similar YTX levels did test positive in the MBA. However, combining the neuro-2a bioassay with o/v and LC-MS/MS analysis provides an alternative testing strategy to replace the mouse bioassay for detecting and identifying lipophilic marine biotoxins in mussels.

Supplementary material chapter 5
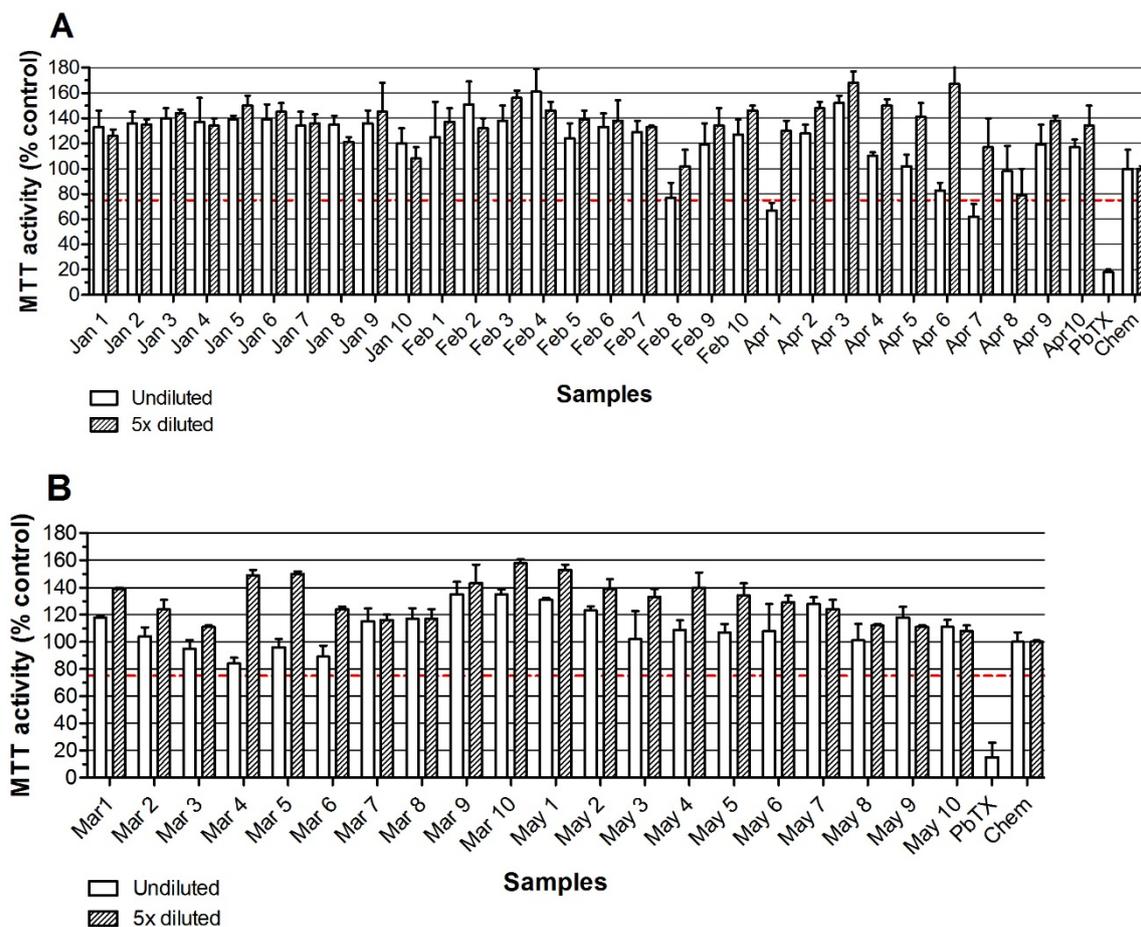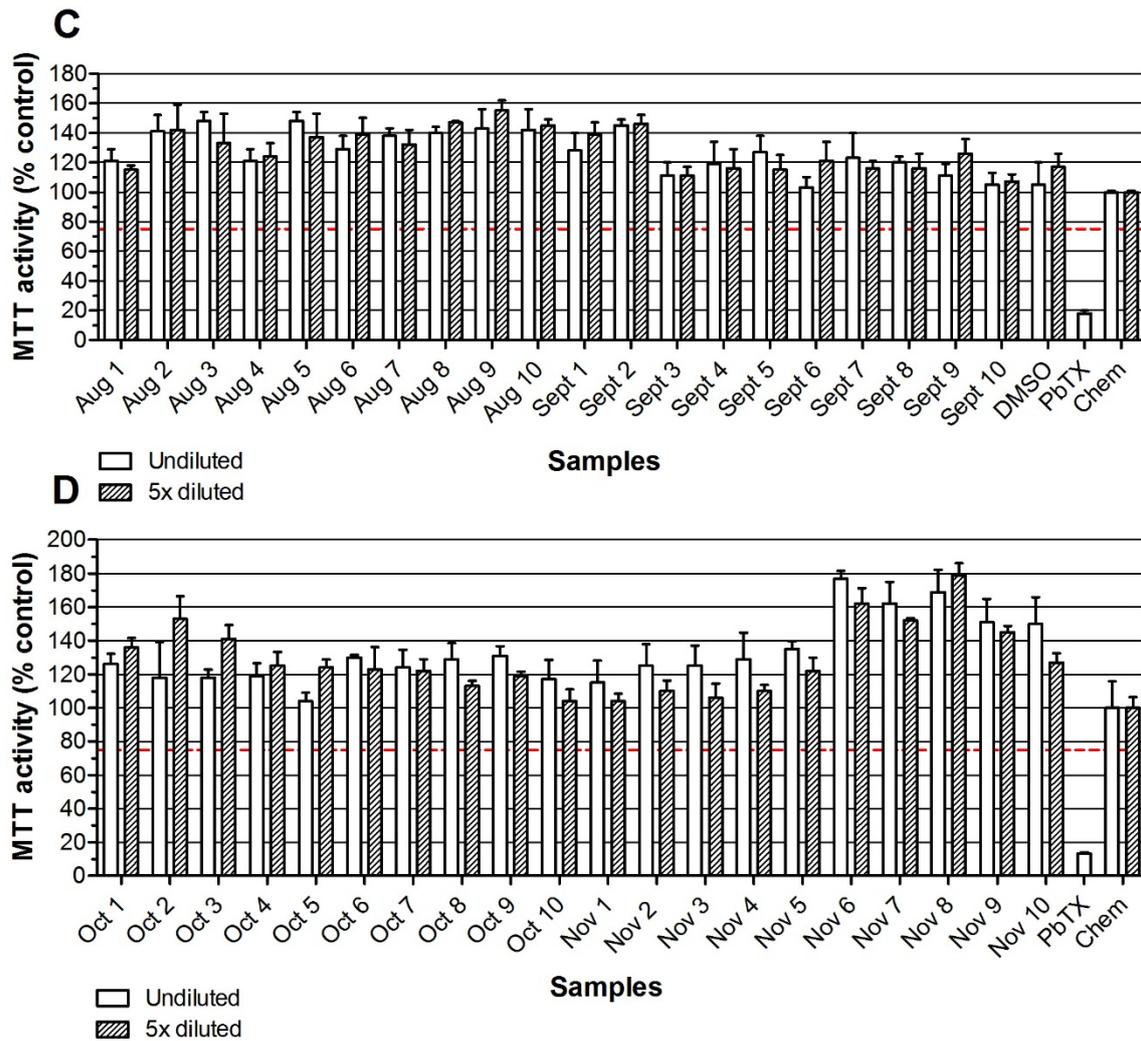
Figure S1. Effect on the viability of neuro-2a cells with the addition of ouabain and veratridine to obtain 20% decrease in MTT reduction, of 90 mussel samples from the Netherlands. A) mussel samples from January, February and April; B) mussel samples from March and May; C) mussel samples from August and September and D) mussel samples from October and November. A chemical blanc (Chem) in DMSO was set at 100% and a previously arbitrary decision limit was set at 75% (Bodero et al., 2018). Data are expressed as mean ± SD.
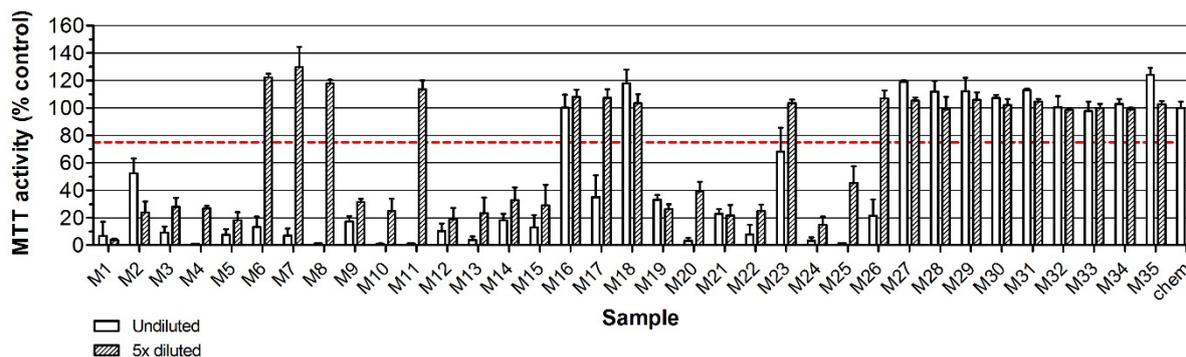
Figure S2. Effect on the viability of neuro-2a cells without the addition of o/v of 35 samples tested previously in Chile in the mouse bioassay (19 positive and 16 negative samples). The spotted line represents the arbitrary set decision limit (75% cell viability). A DMSO solvent with o/v was used as a negative control and set at 100%. Data are expressed as mean ± SD.

Table S3. Details from the samples from Chile (origin, type of shellfish). Data provided by IFOP, Chile.

N° sample	sector	Coordinates		Shellfish	scientific name	sampling month	Date
1	Isla Ovalada	-44°03'44''	-73°43'48''	Chilean mussel	*Aulacomya ater*	February 2015	7-2-15
2	Isla Ovalada	-44°03'44''	-73°43'48''	mussel	*Mytilus chilensis*	February 2015	7-2-15
3	Repollal - Canal Puquitin	-43°50'36''	-73°48'51''	Chilean mussel	*Aulacomya ater*	February 2015	13-2-15
4	Isla García	-44°15'11''	-73°44'40''	Chilean mussel	*Aulacomya ater*	February 2015	7-2-15
5	Isla Julia - Grupo Peligroso	-43°54'05''	-73°42'05''	Chilean mussel	*Aulacomya ater*	February 2015	7-2-15
6	Isla Filomena	-44°29'41''	-73°34'36''	Clam	*Venus antiqua*	February 2015	12-2-15
7	Isla Francisco	-44°29'58''	-73°41'08''	Clam	*Venus antiqua*	February 2015	12-2-15
8	Isla Valverde	-44°17'41''	-73°53'25''	Clam	*Venus antiqua*	February 2015	13-2-15
9	Isla Teresa	-44°50'46''	-73°51'04''	Chilean mussel	*Aulacomya ater*	February 2015	10-2-15
10	Isla Toto	-44°15'07''	-73°12'33''	Chilean mussel	*Aulacomya ater*	February 2015	16-2-15
11	Puerto Barrientos	-43°56'23''	-73°58'24''	Clam	*Venus antiqua*	February 2015	13-2-15
12	Seno Gala	-44°13'02''	-73°10'26''	Chilean mussel	*Aulacomya ater*	February 2015	15-2-15
13	Faro Marta - Canal Puyuhuapi	-44°49'30''	-72°58'10''	Chilean mussel	*Aulacomya ater*	February 2015	18-2-15
14	Punta Calqueman	-44°39'41''	-73°26'44''	Chilean mussel	*Aulacomya ater*	March 2015	23-3-15
15	Isla Toto	-44°15'07''	-73°12'33''	Chilean mussel	*Aulacomya ater*	March 2015	29-3-15
16	Isla Virginia - Bahía Low	-43°47'10''	-73°52'39''	Clam	*Venus antiqua*	March 2015	26-3-15
17	Isla Valverde	-44°17'41''	-73°53'25''	Clam	*Venus antiqua*	March 2015	25-3-15
18	Isla Ipun	-44°38'09''	-74°42'47''	Clam	*Venus antiqua*	March 2015	22-3-15
19	Isla San Andres	-44°55'57''	-73°19'28''	Chilean mussel	*Aulacomya ater*	March 2015	31-3-15
20	Canal Ninualac	-44°59'48''	-73°55'31''	Chilean mussel	*Aulacomya ater*	March 2015	20-3-15
21	Puerto Amparo	-44°55'00''	-73°16'54''	Chilean mussel	*Aulacomya ater*	March 2015	31-3-15
22	Isla Concoto	-44°12'11''	-73°50'03''	Chilean mussel	*Aulacomya ater*	March 2015	25-3-15
23	Isla Stokes	-44°41'24''	-74°35'40''	Clam	*Venus antiqua*	March 2015	21-3-15
24	Puerto Lampazo	-44°56'16''	-73°44'39''	Chilean mussel	*Aulacomya ater*	March 2015	20-3-15
25	Isla Silachilú	-44°58'48''	-73°40'24''	Chilean mussel	*Aulacomya ater*	March 2015	20-3-15
26	Santo Domingo	-43°58'12''	-73°06'51''	Mussel	*Mytilus chilensis*	March 2015	27-3-15
27	Añihue	-43°52'14''	-73°02'21''	Chilean mussel	*Aulacomya ater*	March 2015	27-3-15
28	Añihue	-43°52'14''	-73°02'21''	Clam	*Venus antiqua*	March 2015	27-3-15
29	Isla Scout	-47°53'29''	-74°40'20''	Chilean mussel	*Aulacomya ater*	April 2015	17-4-15
30	Isla Scout	-47°53'29''	-74°40'20''	Clam	*Venus antiqua*	April 2015	17-4-15
31	Isla Orlebar	-47°54'16''	-74°36'06''	Chilean mussel	*Aulacomya ater*	April 2015	17-4-15
32	Isla Orlebar	-47°54'16''	-74°36'06''	Clam	*Venus antiqua*	April 2015	17-4-15
33	Isla Zealous	-47°52'41''	-74°36'40''	Chilean mussel	*Aulacomya ater*	April 2015	17-4-15
34	Punta Baker	-47°53'11''	-74°28'04''	Chilean mussel	*Aulacomya ater*	April 2015	16-4-15
35	Isla Zealous	-47°52'41''	-74°36'40''	Mussel	*Mytilus chilensis*	April 2015	17-4-15

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Chapter 6

General discussion

6.1 Main findings

The aim of this thesis was to develop new *in vitro* bioassays for the detection of marine biotoxins that can be used for high throughput quality control of seafood and replacement of the current unethical *in vivo* bioassays. Through this work and the promising outcomes an integrated testing strategy, in which bioassays and analytical chemical methods were combined as an *in vitro* alternative for the mouse bioassay (MBA) for lipophilic marine biotoxin (LMB) testing, was developed and evaluated.

The main difficulties for switching from the MBA to chemical methods and/or *in vitro* bioassays is the detection of unknown toxins and so-called 'matrix effects' respectively [1-4]. In this work we started with the neuro-2a bioassay, an assay shown to be sensitive for all the regulated lipophilic marine biotoxins and able to detect their yet unknown toxin analogues as well and introduced an improvement in the clean-up procedure for preparing extracts from shellfish samples to be tested in the neuro-2a assay. False positives due to matrix effects were eliminated by a double clean-up wash step with n-hexane during the sample extraction (chapter 2, fig 2a and 2b). Although the clean-up using n-hexane was used before in chemical methods [5, 6], it was never introduced before for testing shellfish using the neuro-2a bioassay. **Chapter 2** shows that this improved clean-up procedure works well and resulted in neuro-2a outcomes that are in line with the outcomes as obtained with the EURL LC-MS/MS method analysis, supporting the applicability of this bioassay for the screening of samples for the presence of lipophilic marine toxins. While the neuro-2a bioassay is suited for screening, additional assays with specific endpoints are needed for confirmation of suspect screened samples in case LC-MS/MS analysis fails to do so. Therefore, **chapter 3** presents a study with DNA microarrays to investigate the effects of OA, DTX-1 and AZA-1 on the whole-genome mRNA expression of undifferentiated intestinal Caco-2 cells. These gene expression data were analysed for two goals: 1) in order to reveal the modes of action of these LMBs and 2) to select potential marker genes for sensitive detection in additional bioassays to be developed (chapter 4). The gene expression data showed that OA and DTX-1 induce identical expression profiles but DTX-1 did so at about 4 times lower concentrations than OA, indicating that OA and DTX-1 have a similar mode of action and that DTX-1 is, *in vitro*, about 4 times more potent than OA. This is in agreement with what is known and OA and DTX-1 are therefore in the same toxin class

and together regulated at a level of 160 µg OA equivalents per kg shellfish. However, their currently established TEFs are the same, and thus deviate from the difference in potency detected in the *in vitro* gene expression. The gene expression profile of AZA-1 was different, suggesting a different mode of action for this toxin, which is in line with the expectations as the AZA toxins are a specific toxin class regulated at a level of 160 µg AZA-1 equivalents per kg shellfish and in line with previous findings where e.g. cholesterol biosynthesis genes were highly upregulated in other cell lines as well [7]. In **chapter 4** a few of the most highly up- and down-regulated genes were selected to design and develop additional detection methods, i.e. a multiplex qRT-PCR and a multiplex magnetic bead-based assay. Genes selected from an additional gene expression study on YTX and PTX-2 were included. In total, twelve marker genes were selected to envision three profiles, i.e. an OA/DTX, AZA/YTX and PTX profile. The multiplex magnetic bead-based assay allowed the use of all twelve selected marker genes and turned out to be a slightly better alternative but is a more expensive format compared to the multiplex qRT-PCR. In this chapter it is shown that blank and contaminated shellfish sample extracts analysed by the multiplex magnetic bead-based assay resulted in profiles not only allowing to distinguish blank samples from contaminated samples, but also indicating the toxin type present in the contaminated samples. **Chapter 5** presented the use of the neuro-2a bioassay as a screening method for detection of lipophilic toxins, which in combination with LC-MS/MS confirmation is offered as a new alternative strategy for the detection and quantification of lipophilic shellfish toxins. A selected set of samples tested in the MBA in Chile, where this animal test is still in use, was analysed using the new proposed strategy. All samples that were positive in the MBA were also classified as 'suspect' in the neuro-2a screening. In most cases these samples contained yessotoxin levels, as determined by LC-MS/MS analysis, that can explain the outcome of both the MBA and the neuro-2a bioassay, but did not exceed the established maximum permitted level, i.e. 3,75 mg YTX-1 equivalents per kg shellfish, clearly indicating that the use of the MBA alone resulted in unnecessary closures of extraction areas or withdrawal of seafood from the market, and that the combination of the neuro-2a bioassay and LC-MS/MS analysis are a promising alternative for the MBA. A proper validation of this combination would result in an animal free alternative for decision making in food safety, without a risk for not detecting yet unknown analogues of the LMBs and with less false positives as obtained with the MBA alone. However, the road is long

and besides validation, additional developments and further improvements are still needed, e.g. inclusion of a de-conjugation step in order to detect OA-esters. These issues are addressed below.

6.2 Limitations of the method and improvements needed

The current extraction, clean-up and neuro-2a screening procedure is most likely not sensitive enough to detect OA and DTX-2 at their regulated level, i.e. 160 µg OA equivalents per kg shellfish. In principle, if a sample is contaminated with this maximum permitted level (MPL), it will result in a concentration of around 12 nM in the well, which is theoretically just enough to cause an effect on the cells, based on results as presented in chapter 2, where the dose-response curves using certified standards of OA and DTX-2 were assessed and resulted in EC_{50} values of 23 and 29 nM for OA and DTX-2 respectively. Thus, when starting a full validation experiment with 20 samples spiked at the MPL of these toxins, most likely a few false negatives will be obtained. One simple improvement would be to concentrate the sample extract, e.g. by a factor 2. However, preliminary results in our lab showed that this is not that easy, as 2 times concentrated blank sample extracts showed matrix effects and resulted in false positives. In chapter 2, fig 4b the effect in the neuro-2a of eight naturally contaminated samples with detectable levels of one or more LMBs was shown. Among this set of 8 samples, one sample was contaminated with OA only and at a level of 151 µg kg^{-1}. This sample was classified as 'suspect' in the neuro-2a assay, resulting in an MTT activity of 55%, where the arbitrary decision limit for declaring a sample as suspect is set at 75% MTT activity. In addition, when a blank sample was fortified at the MPL with OA, extracted (without further concentrating) and tested in the presence of ouabain and veratridine (O/V) (chapter 5, fig 2), the sample was also correctly classified as suspect, i.e. 55% MTT activity. Although two samples are not representative enough to draw definitive conclusions, it indicates that the current protocol might already work adequate enough. Still it is important to search for improvements, before starting a labour intensive and costly validation process. Recently, additional washing steps with n-hexane were shown to allow a further concentration of the sample extracts without leading to matrix effects.

On the other hand, due to the relative high sensitivity of the neuro-2a bioassay for the YTXs with regard to the MPL of this toxin group, i.e. 3750 µg YTX-1 equivalents kg^{-1}, the presence of relative low levels of YTXs will already result in a suspect screening outcome (EC$_{50}$ of 1.6 nM for YTX-1 (chapter 2)). In the Netherlands this will not be problematic, and sensitive detection of YTXs would rather be regarded as an early warning for a possible new risk or changing conditions (e.g. global warming [8]), as these toxins are normally not detected in Dutch shellfish. But in countries with a high occurrence of YTXs in their coastal waters and shellfish, the neuro-2a screening would result in many suspects. For these countries an extraction protocol should be developed in order to obtain two fractions from each sample, one containing the YTXs and one containing the other LMBs. Although chemically possible [3, 9], this is not as easily done, as preliminary experiments in our laboratory indicate that it requires another SPE column and the addition of acid, the latter leading to cytotoxic effects in the neuro-2a bioassay, also when neutralising the pH, as the sample extracts become too salty. However, compared with the MBA alone, the proposed strategy allows the detection of YTXs at levels lower than their MPL (3.75 mg kg^{-1}) with the neuro-2a bioassay and the measurement of the actual toxin levels in the second step by LC-MS/MS confirmation. Therefore, closure of areas or withdrawal of seafood from the market will only happen when LC-MS/MS analysis confirms the presence of YTXs levels above the MPL and does so without the need to use animals.

DTX-3 toxins are shellfish produced metabolites of OA, DTX-1 and DTX-2, i.e. esterified with various fatty acids [10]. DTX-3 has been reported in some areas, being even the most frequent form of OA and DTXs found in shellfish [5, 11]. In the official EURL LC-MS/MS method [12] these toxins are detected after an alkaline hydrolysis step of the fatty acids, performed in the crude methanol extract [13]. The lack of available naturally contaminated samples and DTX-3 standards have been the major drawbacks to assess this matter in the present thesis. However, pilot experiments in our lab indicated that the hydrolysis step would not affect the neuro-2a cells, as the pH could be neutralised afterwards with low amounts of HCl. Still this is an important step and should be included in the procedure that eventually will be validated.

In chapter 5, a selected set of 35 samples tested previously in the MBA in Chile, where this animal test is still in use, was assessed with the neuro-2a bioassay and the EURL LC-

MS/MS method. All samples that were positive in the MBA were also suspect in the neuro-2a assay. Almost all suspect screened samples turned out to contain toxin amounts that explain the screening outcome and all the levels were well below the established MPLs. For enforcement purposes, the MBA alone would have resulted in 54% of false positives, while the combination of the neuro-2a bioassay with LC-MS/MS analysis did not result in such false positives. Most of the samples that were negative in the MBA, were also negative in the neuro-2a screening and none contained toxins levels above the established MPLs. Together showing that the combination of the neuro-2a bioassay for screening and LC-MS/MS analysis for confirmation, is a good strategy to replace the MBA and protect consumers.

It should be noticed however that from an analytical point of view, 5 samples could be regarded as falsely screened suspect in the neuro-2a bioassay, i.e. no toxins were detected by LC-MS/MS analysis. From these 5 samples, 3 also tested positive in the MBA and 2 tested negative. In cases like this, the second bioassay proposed in the strategy can be put in practice in order to investigate if these samples might contain unknown toxins. A pilot experiment was therefore performed in which 4 of these samples were tested in the multiplex magnetic bead-based assay. All 4 showing a clear AZA/YTX profile (data not shown). This result might indicate that in a worst-case scenario these 4 neuro-2a suspect samples are true positives, 2 were missed by the MBA and all 4 missed by LC-MS/MS analysis. However, these samples were clams and some clams that were negative in the MBA and neuro-2a bioassay also resulted in a AZA/YTX profile. Most likely some clams lead to false positives in the MBA, neuro-2a bioassay, and the multiplex magnetic bead-based assay. When looking at mussels only, the mouse MBA resulted in 67% false positives (16 out of 24), while the combination of the neuro-2a bioassay with LC-MS/MS analysis did not result in false positives. Of the 18 mussel samples that were screened as suspect in the neuro-2a bioassay, all contained toxins levels (YTX eq) that explain the bioassay outcome, and of the other 6 that were negative, none contained detectable toxin levels when analysed by LC-MS/MS. This strongly indicates that for the mussels, the combination of the neuro-2a bioassay with LC-MS/MS is superior to the MBA, and that animal free testing is possible. Moreover, blank mussels are also blank in the multiplex magnetic bead-based assay (chapter 4). So, the whole strategy works well especially for mussel.

It could be argued that too many samples tested suspected in the neuro-2a bioassay and required follow-up testing, but then it is of importance to realise that the 35 samples from Chile were selected by a targeted approach and are not representative for all shellfish samples. These samples were selected because they were already known to test positive in the MBA, in order to test naturally contaminated samples that lead to positive outcomes in the MBA with the new testing strategy, i.e. the neuro-2a bioassay combined with LC-MS/MS analysis for confirmation of suspect screened samples. In addition, the multiplex magnetic bead-based assay is used in case LC-MS/MS cannot explain the neuro-2a outcome. In the Dutch situation, the neuro-2a bioassay and LC-MS/MS were tested in parallel in 2017 on 110 mussel samples that were taken over the year. All samples were negative by LC-MS/MS and 108 also tested negative in the neuro-2a bioassay. As the response of the 2 suspect screened samples was close to the decision limit, follow-up was not considered necessary (data not shown). However, the outcomes with the clams demonstrated that further improvements and validation of all bioassays eliminating possible shellfish species specific matrix effects, are needed as it is known that different species contain different types of fatty acids that can lead to matrix effects [14, 15].

It should also be noticed that there are differences between the methods in the solvents used for sample extraction. While the extraction protocol for the MBA prescribes the use of acetone/diethyl ether [16, 17], the extraction for the neuro-2a assay is performed with methanol 100% v/v. For LC-MS/MS methanol 100% v/v is also used for the DSPs and methanol/water 80% v/v for PbTXs. Thus, it cannot be fully ruled out that these variations could contribute to the observed differences. However, this is not very likely, as for methanol it is known that all the DSP toxins are well extracted (validated) [18], while methanol/water is mainly used for the metabolites of the PbTXs [19], and these were not detected in the samples tested.

6.3 Future challenges

This thesis focused on presenting an *in vitro* alternative for the MBA for diarrhoeic marine biotoxins testing. However, other points of attention have been identified during this work. As a consequence, the work was broadened to the detection of all LMBs, e.g. including the PbTXs, and the development of new bioassays for the analysis of samples shown suspect

in the neuro-2a bioassay that could not be confirmed as being positive by LC-MS/MS analysis. To this end gene expression studies were used to select biomarkers for detection, but also to address the modes of action of the toxins and their toxicological equivalent factors, which are briefly discussed below.

Toxicological equivalent factors (TEFs) are ratios between the toxicity of a toxin congener with respect to a reference compound in order to calculate the relative potency. TEFs have been developed in order to convert data from chemical analysis into levels of toxin potency that can be used to establish if a sample is safe or not. The most early and best example of the use of TEFs are the dioxins and PCBs [20]. Regarding marine biotoxins, TEFs are mainly established in the MBA based on effects in mice that are intraperitoneally (i.p.) injected with the toxins [21, 22]. As humans are mainly exposed to marine toxins by food or aerosols, the way of administration has been a topic for discussion and the relevance of the MBA for human risk assessment is reviewed extensively [21, 23, 24]. Our findings indicated that *in vitro*, OA is less potent than DTX-1, which has also been reported by others [25]. As absorption, metabolism and distribution are not considered in our model and for a large part also not in the mouse as these are i.p. injected, further research where these toxins are tested in additional *in vitro* models is needed, e.g. a human digestion model, and models that characterise human liver metabolism and intestinal uptake. Probably these models will eventually explain the observed differences between oral *versus* i.p. injection toxicities and explain why several marine biotoxins are so potent in *in vitro* tests or the MBA, but not known to cause human intoxications. Possible interaction and/or conversion of the toxins with the gut microbiota should be considered as well [26]. Moreover, other factors should be addressed, for instance the pre-consumption treatment of shellfish products, as the bio-accessibility or stability can vary depending on the treatment, e.g. cooking or steaming [27, 28].

In chapter 3, a whole genome gene expression analysis with intestinal human Caco-2 cells using arrays was performed to assess effects of OA, DTX-1 and AZA-1 on gene expression, both with the aim to envision pathways affected and to select markers for detection. Later, Caco-2 cells were also exposed to YTX and PTX-2, in order to select markers for detection. Chapter 3 discusses the pathways affected and possible modes of action and chapter 4 the development of a multiplex qRT-PCR method and a multiplex magnetic bead-based assay.

Although these newly developed methods were shown to work adequate and were used successfully on blank and contaminated mussel samples, the results were surprising as it turned out that YTX and AZA-1 elicited similar expression profiles for which we have no logical explanation. It is therefore recommended to repeat the gene expression analysis using the same experimental settings for exposure and analysis of the data. Moreover, it would be interesting to further explore the mode of action of YTX, as YTX is considered 'not toxic', i.e. is not causing diarrhoea when administered orally [29]. Orally, YTX showed myocardiocyte alterations in mice [30]. In addition, studies on YTX *in vitro* showed that it can cause various cytotoxic effects depending on the cell line studied, triggering for instance apoptosis and pathways indicating DNA damage and cell cycle impairment [31, 32], and ER stress among other effects [33]. It has even be suggested to investigate YTX as a new therapeutic tool against tumour proliferation [31].

As discussed in chapter 3, AZA-1 showed clear indication of upregulation of genes related with cholesterol biosynthesis, which is in line with earlier findings [7]. Our results also indicate that this toxin might affect pathways related to hypoxia and glycolysis. One could speculate that an inhibition of the pyruvate dehydrogenase complex (PHC) [34] causes effects on these pathways, as is indicated by the increase of the lactate enzymes observed in our results. This mode of action could be confirmed through measurement of lactate in the media of cells exposed to AZA-1, but also through the measurement of pyruvate and NADH [35]. The increase of NADH could be also a reason of the increased MTT activity [36, 37] which was also observed in our Caco-2 cells exposed to AZA, even at lower concentrations (chapter 3, figure 1). Moreover, it is noticed that both AZA-1 and YTX do not reduce the MTT-activity as much as the other LMBs (chapter 2). This is in line with the observations above for AZA-1 and the gene expression data that also indicates that AZA-1 and YTX likely share a similar mode of action.

6.4 Future perspectives

The newly proposed strategy provides an alternative for the MBA to detect and identify both lipophilic and hydrophilic marine biotoxins, protecting human health and considering animal welfare at the same time. The fully proposed integrated strategy is shown in figure 1.

The strategy consists of 3 phases. Phase 1 is a broad screening using the neuro-2a bioassay, with and/or without the addition of ouabain and veratridine (o/v). For the detection of domoic acid (DA), which is not detected by the neuro-2a bioassay [38], the addition of a dipstick in the phase 1 is suggested. Phase 2 is the confirmation and quantification of neuro-2a suspect screened samples using official analytical methods. Phase 3 is only needed in case analytical methods fail to confirm suspect neuro-2a outcomes and consists on an effect-directed identification of unknown toxins in cases that a second *in vitro* cell-based bioassay, i.e. gene expression in Caco-2 cells for LMBs or a multi electrode array (MEA) assay for detection of hydrophilic toxins [38, 39] indicates the presence of a marine biotoxin as well. In cases where the second bioassay does not confirm the presence of a marine biotoxin, the neuro-2a suspect outcome is considered as a false positive screening result.

Phase 1: broad screening

Marine toxins can be separated in two main groups, i.e. hydrophilic and lipophilic marine biotoxins. Extraction methods based on these characteristics already lead to some degree of specificity. These characteristics are already well-known and used in the first step of the strategy and the protocol on how to perform the screening with the neuro-2a bioassay. For hydrophilic toxins, which are mainly PSP toxins (STX and analogues) and TTXs, the protocol for the neuro-2a bioassay includes the use of high concentrations of ouabain and veratridine (o/v), i.e. 0.3 mM and 0.03 mM respectively, in order to decrease MTT-activity to about 20%. Ouabain is a NA^+/K^+ ATPase pump blocker, interfering with the normal efflux of Na^+ from and influx of K^+ into the cell. Veratridine is a voltage-gated sodium channel (VGSC) opener [40]. The use of this o/v combination causes a hyperosmotic state of the neuro-2a cells that eventually will lead to cell death as measured by a clear decrease of the MTT-activity [2]. In "simple" words, the principle of the neuro-2a bioassay to detect the PSPs and TTXs, which are VGSC blockers, is as follows: the addition of o/v 'kills' the cells and the presence of STXs or TTXs in the samples will 'rescue/prevent' the cells from dying, i.e. restoring the ion balance by closing the channels previously opened by o/v. In other words: STXs and TTXs are VGSC blockers that counteract the effect of the VGSC opener veratridine. This effect has been widely studied and reviewed [41-43]. Previous results showed that the hydrophilic marine biotoxins TTX and STX can be detected in the neuro-2a assay with addition of o/v, in levels below their regulatory limits [38, 44, 45].
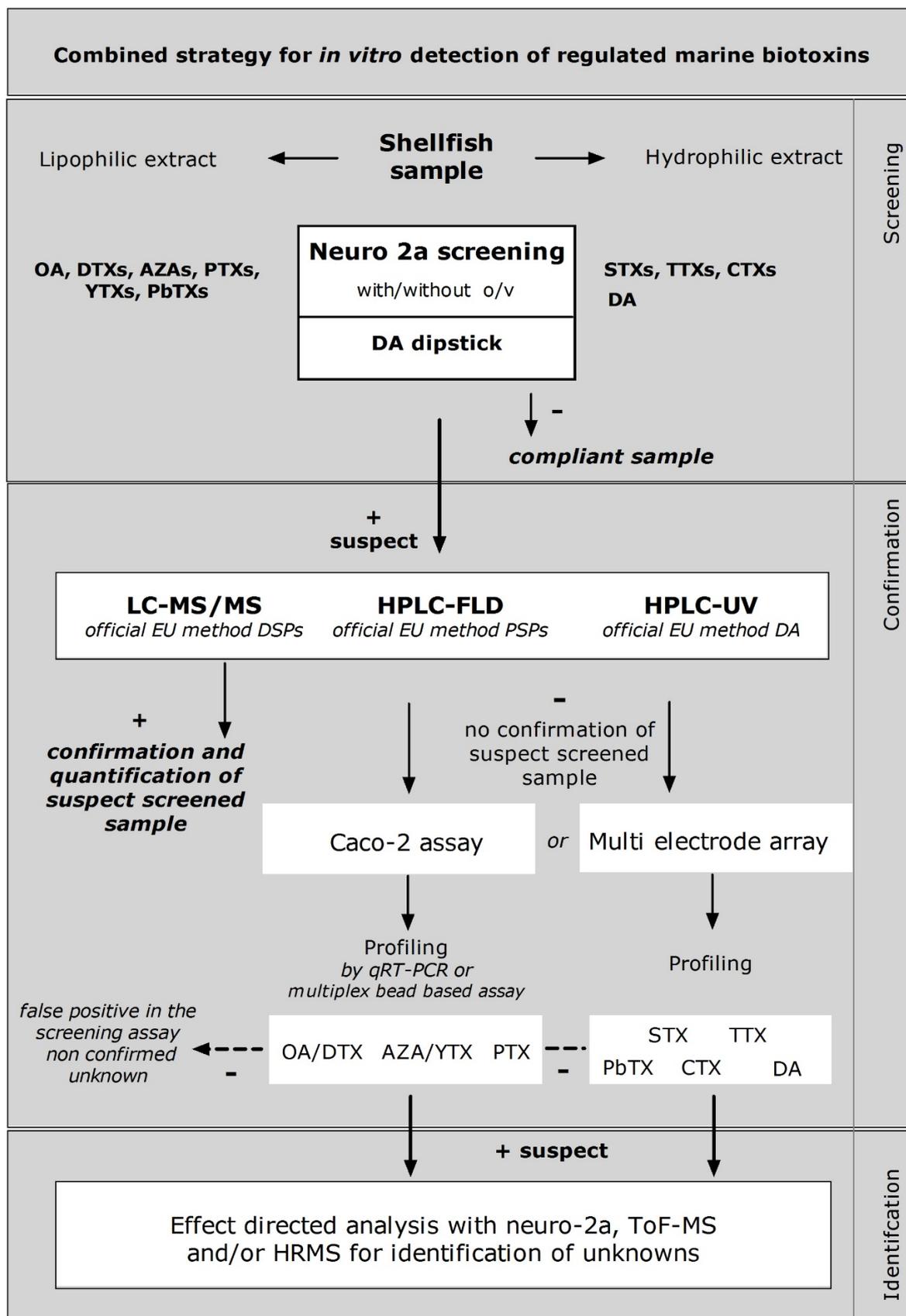
Figure 1. Strategy for the detection of all regulated marine biotoxins.

The EC_{50} obtained for STX was 9 nM, with a limit of detection (LOD) of 1nM [38]. Considering the extraction and concentration protocol used, this EC_{50} value would be equivalent to around 40 μg STX kg^{-1} shellfish meat (SM), being significantly lower than the regulatory limit for STXs, i.e. 800 μg STXeq kg^{-1} SM. For TTX, the EC_{50} obtained was 18 nM [38], which theoretically would allow detection of about 100 μg TTX eq kg^{-1} SM. EFSA concluded that an amount lower than 44 μg TTX per kg SM is not expected to lead to adverse health effects in humans, assuming an average consumption of 400 g SM [46]. There is no official international or EU regulatory limit for TTX. So far, fish species from *Tetraodontidae, Molidae, Diodontidae* or *Canthigasteridae* (puffer, porcupine and toby fish) are not allowed to be placed on the EU market [47]. But in the Netherlands the safe level of 44 μg TTX eq kg^{-1} SM proposed by EFSA is now being used. At the RIKILT laboratory, the protocol of extraction of hydrophilic marine biotoxins was adjusted, allowing the detection of 20 μg TTX eq kg^{-1} SM [44].

These results indicate that the neuro-2a bioassay is suited for a first broad screening for hydrophilic marine biotoxins. However, domoic acid (DA), the toxin responsible for amnesic shellfish poisoning, is not detected by the neuro-2a bioassay, because neuro-2a cells lack the NMDA receptor [48]. However, use of a commercially available 'dipstick' lateral flow device for DA allows a simple and sensitive detection of this toxin in SM [49]. This dipstick is included in the first step to screen the sample extracts containing the hydrophilic marine biotoxins (figure 1).

Regarding the extraction of the lipophilic marine biotoxins (LMBs) methanol is used for DSP toxins, AZAs and YTXs and methanol/water 80/20 (v/v) is used for extraction of PbTXs. As discussed in chapter 5, methanol will also work well for the PbTXs, and water was only added to improve the recovery of some PbTX metabolites. Methanol extracts with the LMBs can be tested in the neuro-2a bioassay with the addition of low concentrations of o/v, i.e. 0.13 mM and 0.013 mM respectively, in order to decrease the MTT-activity to about 80%. The principle behind the addition of o/v in low concentrations also for testing of the lipophilic LMBs is the same hyperosmotic state as described above for the hydrophilic LMBs, only in this case lower concentrations of o/v are used and a further decrease of the MTT activity instead of an increase is recorded. The neuro-2a allows the detection of all the regulated LMBs with a low concentration of o/v needed to detect the

PbTXs. Cells will be unstable because veratridine will open the VGSC and ouabain will keep the Na+/K+ ATPase pump blocked, while the presence of PbTXs will then continuously open the sodium channels and lead to cell death, as measured by a further decrease of the MTT activity. The other LMBs are able to decrease the MTT activity even without a low concentration of o/v. When a sample is considered suspect using the protocol for the detection of the LMBs and there are reasons to believe that this is due to the presence of PbTXs, e.g. samples coming from a geographical region where *K. brevis* is endemic, the neuro-2a bioassay screening should also be performed without the addition of O/V. When PbTXs are responsible for the reduced MTT activity in the presence of o/v, the test will be negative in the absence of o/v. However, in a first validation effort, it would be better to run both protocols in parallel.

Phase 2: confirmation and quantification

If a sample is not compliant, i.e. it is *suspect* in the neuro-2a bioassay or positive in the DA dipstick, the sample extract should be measured with the corresponding official EU method (figure 1). These European official methods are the EURL LC-MS/MS for lipophilic toxins [12], HPLC-UV for DA, and HPLC-FLD for PSP toxins [50, 51]. PbTXs are not regulated in Europe, but there is an LC-MS/MS method available, although is not official yet [52]. In case the presence of the LMB in the suspect screened sample is confirmed by the analytical methods and the toxins levels are quantified, decisions and necessary actions can be taken by the responsible (food safety) authorities. These actions can include closure of extraction and production areas or withdrawal of products from the market. The permitted LMB limits on which these risk management actions are based are defined in the European regulation or for none European countries follow e.g. the FAO guidelines (see table 4, chapter 1).

Phase 3: effect directed identification

When the lipophilic extract of a sample is considered suspect in the neuro-2a bioassay but the presence of LMBs cannot be confirmed by the EURL LC-MS/MS analytical method, the bioassay based on the expression of selected markers in exposed Caco-2 cells can be used to confirm the presence of an unknown LMB. Three different profiles can be envisioned, i.e. an OA/DTX, AZA/YTX and PTX profile (chapter 4). For PbTXs, the

neuro-2a bioassay can be repeated, but without the addition of low concentrations of O/V, when negative it now indicates the presence of an unknown PbTX. If the hydrophilic extract is suspect in the neuro-2a bioassay or positive on the DA LFD, but is not confirmed by HPLC-FLD or HPLC-UV, the multi electrode array (MEA) developed by Nicolas *et al.* is an option to confirm the presence of an unknown PSP [38]. Limitations of the MEA assay are the relatively high costs and that it is not a fully animal-free assay, as it uses primary rat cortical neurons. However, its use in efforts of identifying new emerging toxins, for instance in an outbreak, should be considered. When these "second" bioassays are negative, the suspect neuro-2a bioassay screening outcome is considered as a false positive. When the second bioassay indicates the presence of a yet unknown toxin, a fractionated effect-directed approach can be used to identify the responsible toxin, i.e. an approach that has been used successfully for the identification of unknown steroids [53]. However, this can take enormous efforts and time generally not available when decision makers already have to anticipate on such outcomes, preferably without using the MBA.

6.5 Enforcement purposes

The work presented in this thesis aims to replace the MBA for screening shellfish samples on the presence of LMBs. For completeness, table 2 gives an overview of the regulatory limits and the EC_{50} limits for detection in the neuro-2a bioassay and the corresponding limits of detection (LOD) in the neuro-2a assay derived from these EC_{50} values for all regulated marine biotoxins. Table 2 shows that the neuro-2a derived LODs are close to the current regulations, even lower than the LODs for some while somewhat higher for others. Regarding the LMBs, especially for OA, DTX-2 and PTX-2 the LODs of the neuro-2a bioassay are still higher than the required regulatory limits. As discussed above, these toxins are a point of attention regarding validation, including testing standards, fortified and naturally contaminated samples and improvements have already been achieved, e.g. lowering the LODs in the neuro-2a bioassay by using more concentrated sample extracts [54]. Similar is valid for TTXs, where the LOD in the neuro-2a bioassay is lowered to 20 μg TTX kg^{-1}.

Table 2. Neuro-2a derived limit of detections

Toxin	Current limit (#)	EC$_{50}$	LOD Neuro-2a [3]
OA		23 nM[1]	310 µg kg^{-1}
DTX-1	160 µg OA eq kg^{-1}	5.5 nM[1]	74 µg kg^{-1}
DTX-2		34.4 nM[1]	462 µg kg^{-1}
PTX		76.4 nM[1]	1090 µg kg^{-1}
AZA-1		1.0 nM[1]	14 µg kg^{-1}
AZA-2	160 µg AZA-1 eq kg^{-1}	1.9 nM[1]	27 µg kg^{-1}
AZA-3		1.6 nM[1]	22 µg kg^{-1}
YTX	3.75 mg YTX eq kg^{-1}	1.6 nM[1]	30 µg kg^{-1}
hYTX		1.1 nM[1]	22 µg kg^{-1}
STX	800 µg STX eq kg^{-1}	8.2 nM [2]	40 µg kg^{-1}
TTX	44 µg TTXeq kg^{-1} [4]	18 nM [2]	96 µg kg^{-1}
PbTX-3	800 µg PbTX eq kg^{-1} [5]	8 nM[2]	120 µg kg^{-1}
PbTX-9		8.4 nM[2]	127 µg kg^{-1}
CTX	0.01 µg P-CTX eq kg^{-1} [6]	0.9 pM[2]	0.16 µg kg^{-1}
DA	20 mg DA kg^{-1}	ND	ND

[1] Chapter 2; [2] Nicolas et al, 2014; [3] estimated based on EC$_{50}$ values and expressed in µg kg^{-1}; [4] EFSA proposed limit; [5] CODEX; [6] US limit, related with pacific ciguatoxin (P-CTX), Limit C-CTX is 0.1 µg C-CTX eq kg-1 and it was not assessed by [2]; (#) EU limits unless expressed otherwise. All expressed per kg of shellfish meat. ND: not determined, neuro-2a assay is not able to detect DA.

Overall, one can say that the strategy presented here as an alternative for the MBA is promising. As discussed, it needs to be further improved and then properly and fully validated. This work contributed to the 3R principle of reducing, replacing and refining animal experiments, ultimately proposing a real option to fully replace the unethical and unspecific MBA, that is also known to produce false positives and false negatives. Improving the alternative detection methods will also affect local economies and quality of life of small fisher communities. Less false positives will lead to less unnecessary closures of extraction and production areas. Moreover, in an ideal situation where no false positives are found, i.e. only true positives, governments have to be careful regarding shellfish extraction management, maybe establishing extraction quotas, to assure that the harvest is

being done in a sustainable way, respecting the natural cycles of reproduction of shellfish species.

As a final conclusion it can be stated that the results obtained and collected not only on lipophilic toxins but also on the hydrophilic toxins demonstrate that the proposed strategy will lead to a future where a complete animal free alterative testing strategy can replace the MBA.

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Chapter 7

Summary

Summary

Marine biotoxins in fish and shellfish can cause a number of adverse health effects in consumers, such as diarrhoea, amnesia, and death by paralysis. Worldwide, there are monitoring programs for testing shellfish on a regular basis. In some countries, testing is performed by using the so-called mouse bioassay (MBA), an assay raising both ethical and practical concerns because of animal distress and shortcomings in respect to specificity. The MBA may result in both false negatives and false positives. A false negative does not protect the consumers as anticipated and the high amounts of false positives encountered when applying the MBA lead to unnecessary closures of extraction areas, damaging local economies. A full ban of the MBA or its total replacement by analytical chemical methods has failed because these detection methods are unable to detect all toxin analogues and newly emerging toxins and will thus result in false negatives by definition. To fully replace the MBA, there is a clear need for new functional animal-free *in vitro* assays with specific endpoints that are able to detect both the known and yet unknown marine biotoxins.

In Europe a method based on LC-MS/MS has been developed as an alternative for the MBA and is now the reference method for lipophilic marine biotoxins (LMBs) and used in the routine monitoring. However, as outlined above safety is not fully guaranteed when relying only on such a method and, as a result, the MBA is still used for surveillance purposes. The aim of the work presented in this thesis was to develop a new strategy to fully replace the MBA for detecting LMBs without the risk of missing a contaminated sample that can lead to an intoxication. This was achieved by combining effect-based bioassays and a mass spectrometry analysis, including the official EU-RL method.

Chapter 1 addresses the safety issues of the marine biotoxins produced by algae, corals and bacteria and summarises the current legislations and recommendations and the methods of detection. In Chapter 2, the neuro-2a bioassay, a cell-based *in vitro* bioassay that was previously shown to be sensitive for several hydrophilic and lipophilic marine biotoxins, was studied for its ability to screen seafood products for the presence of lipophilic marine biotoxins. All (regulated) LMBs and their analogues were tested, and the neuro-2a bioassay outcomes showed that all these LMBs could be detected at low concentrations. Next, blank and contaminated sample extracts were prepared and tested, showing that matrix effects

led to false positive screening outcomes. Therefore, the standard extraction procedure for LMBs with methanol was modified by introducing a clean-up step with n-hexane before further extraction on the SPE-column. First, the possible recovery losses due to this extra n-hexane wash step were assessed, showing that the n-hexane did not lead to recovery losses of the LMBs and that the matrix effect was successfully removed. Finally, the applicability of the neuro-2a bioassay was assessed by testing a broad range of shellfish samples contaminated with various LMBs, including diarrhoeic shellfish poisoning (DSP) toxins. The samples were also analysed by LC-MS/MS. Overall, the neuro-2a bioassay showed screening outcomes that were well in line with the toxin levels as determined by the EU-RL LC-MS/MS reference method.

In chapter 3, a study with DNA microarrays was performed to explore the effects of two diarrhoeic and one azaspiracid shellfish toxin, okadaic acid (OA), dinophysistoxin-1 (DTX-1) and azaspiracid-1 (AZA-1) respectively, on the whole genome mRNA expression of undifferentiated intestinal Caco-2 cells. In this chapter the whole genome mRNA expression was analysed in order to reveal the possible modes of action of these toxins and to select genes that can be used as potential markers in new additional bioassays for the detection and identification of these LMBs. It was observed that OA and DTX-1 induced almost identical effects on mRNA expression, which strongly indicates that OA and DTX-1 induce similar toxic effects. Biological interpretation of the microarray data showed that both compounds induced endoplasmic reticulum (ER) stress, hypoxia, and unfolded protein response (UPR). The gene expression profile of AZA-1 resulted in a different expression profile and showed increased mRNA expression of genes involved in cholesterol synthesis and glycolysis, suggesting a different mode of action for this toxin.

In chapter 4, twelve marker genes were selected from the previous study and five were used to develop a multiplex qRT-PCR method. This multiplex qRT-PCR method is able to detect three toxin profiles, i.e. a OA/DTX, AZA/YTX and PTX profile. The multiplex capacity of this qRT-PCR is limited to five genes. The use of a multiplex magnetic bead-based assay was explored, allowing the use of all twelve selected marker genes and two reference genes. This 14-plex also resulted in clear profiles with sometimes higher induction factors as obtained by the 5-plex qRT-PCR method. As a result, contaminated samples could easily be distinguished from the blank samples, showing the expected profiles. These

multiplex assays can thus detect these LMBs in shellfish samples and the obtained profile indicates the toxin-type present. However, compared with the neuro-2a bioassay, this assay has been shown adequate so far for only a limited number of LMBs (not all LMBs have been tested), and it is more laborious, time consuming and expensive. It should be used in cases were suspect screening outcomes from the neuro-2a bioassay cannot be explained by the toxin levels as measured with the EU-RL LC-MS/MS reference method.

In chapter 5, the neuro-2a bioassay as an initial screening assay was combined with the EU-RL LC-MS/MS method for confirmation and it was investigated whether this combination is able to replace the MBA for the detection and quantification of LMBs. Samples that were tested previously in the MBA (in Chile) were used. It turned out that all samples that tested positive in the MBA were also suspect in the neuro-2a bioassay and most of these samples were confirmed to be positive for the presence of LMBs by LC-MS/MS analysis. The results confirm that the combination of the neuro-2a bioassay for screening and the EU-RL LC-MS/MS method for confirmation, is a promising alternative for the unethical MBA. The data even strongly indicated that the MBA alone probably led to false positives and the unnecessary closure of extraction areas or withdrawal of products from the market, a problem not encountered when using the neuro-2a assay in combination with LC-MS/MS.

In chapter 6, a fully integrated testing strategy was presented for replacing the MBA, enabling the detection of the hydrophilic marine biotoxins. The steps and methods are discussed, and some points of attention and further developments required are addressed. Taking all together it is concluded that the proposed strategy contributes to a future with a complete animal free alterative testing strategy replacing the MBA.

Annex

Acknowledgements

Curriculum Vitae

List of Publications

Overview completed activities

Words from the author (Acknowledgements)

I came to the Netherlands in 2011 with the thought of getting a MSc degree in Food Safety. I was at the time working as a microbiologist in the food safety authority in Chile, and I wanted to just learn *a bit* of toxicology, you know, to get an overview and then come back. I was wrong. My friend Pablo told me that Wageningen University had a nice MSc in Food Safety, "only if you don't care to be the oldest one in the class...". Indeed, I was one of the oldest, but it was not a problem to hang out with very young people (10 years younger in average) from other countries and cultures, and actually, I learned a lot from them. Is when I met Myrto, the hipster girl from Greece and Nacho, the Spanish guy with quite of an accent. Seven years later, they would be my paranymphs in my PhD defence. Wait. PhD? I am too old for a PhD, no way I will do one! I said to my friend Andres. Wrong again.

My idea was to get a master thesis related with veterinary residues (because yes, I am a vet), and went to the Rikilt to speak with Ron. He had mentioned in a lecture that they had some thesis topics on that. But Ron told me immediately 'nooo, we don't have anything on that topic, but we have something VERY interesting in marine toxins'... The project was interesting, so I accepted, and then I was *transferred* to Toine. I think Toine didn't even know I was going to be his student. Then Liza became my supervisor and taught me the all what I know about cell culture. Long story short, and after my master, I was convinced to continue in the marine toxin project as a PhD at Rikilt. Ivonne became my promotor, she was super supportive and gave me a letter of acceptance for my scholarship paperwork. Chilean bureaucracy apart, I got the scholarship and in November 2013, at the age of 35 years old, after being sent back to Chile by the immigration services (their mistake, not mine), I started the PhD that became this book. Peter took me in as my daily supervisor and we started together 'the agony of the MOA paper', which is the chapter 3 in this thesis. I still remember those 45 or more comments of one reviewer, to finally reject the paper. That work was quite hard for me, and Peter was always there when I had questions (and I had a lot). Life as a PhD was intense and also fun. We had the chance to go to Chile in 2015 for a workshop and a conference on marine toxins, quite convenient for me. I think I learned a lot of new words in Dutch on that trip. We first gave a workshop in Santiago, I was so sick I almost fainted and I had to sit during my presentation. Great. Few days later, Arjen, Toine and I went to Puerto Varas for the conference. There was a volcano in eruption and Toine wanted to see it. We *quickly* drove almost 300 km each way just to see 'volcán Villarrica' and its smoke. I have to say, the volcano is really beautiful, and driving 600 kms in a day totally worth it, especially the visit to the top. Toine, your always positive point of view on things amazes me, you have been *in the mud* with me and supported me in every way, and Peter, thanks for your golden patience and support. Liza, your friendship, guidance and your data are the best, this book is also your work. And together with roommates Astrid, Deborah and Corina I had very nice times. Thanks also to Arjen, my non-official supervisor, and Ron and his EFSA point of view. Dankjulliewel mijn Rikilt colegas! Thanks for understanding when I was trying to speak Dutch (or even English!)

Jullie zijn heel warme en leuke mensen. Geert, Tien, Mirjam (specially for all those samples and MS data!), Ans, Jochem, Hans B, Meike, Victor, Henry, Yoran, Ad, Lonneke, Robert, Leen, Jen, Yamine, Milou, Pim, Ashraf, and also to the former colleagues Si, Jeroen, Agata, Emeli, Erika, Victor and Vale.

I was also a PhD student from the department of Toxicology, so I had the chance to know very nice colleagues there too. I could participate of the lab trips that were really cool...although I didn't enjoy waking up at 6AM and have to listen Nacho with the guitar in the bus (joking Nacho, you are great but please don't sing that backstreet boys song again). Thanks to Ivonne, my promotor, who is an example of efficiency, it amazes me how you manage to deal with all the projects and PhDs. Thanks Laura, Lidy, Hans, Bert (specially for the acetylcholinesterase assay!), Letty, Karsten, Nico, Sebas, Rozaini, Rung, Aziza, Diego, Marta, Lenny, Georgia, Ixchel, Katja, Reiko, Jonathan, Myrthe, Samantha, Justine, Sunday, Hequn, Abdul, Amer, Wasma, and all of you that were at Tox and we had some nice times together. Thanks also to my Wagefriends Thanasis, Mirja, Mauro, Rebeca, Salva, Henrique, that made Wageningen a nicer and friendlier place.

Muchas gracias a la Chilean community that provided enough barbeques with plenty of benzopyrenes, wine with enough antioxidants, Chilean-speaking conversations and *fútbol*. We celebrated our Independence Day in September, often in the-rain-will-not-ruin-our-barbeques kind of situation, and we organised the famous Chilean Wine Tasting. There was also a band called 'Saonda' that spiced things around here. Gracias Bego (the artist!), Dani B, Pame, Mauricio, Grace, Marcelo, Niko, Maite, Jose, Manuel, Naomi, Cata, Silvi, Victor, Rodolfo, Felipe L, Felipe B, Decap (aka Seba), Jo, Maria, Tobias, Carlos, Ale, Fabio, Maca, Pablo R, Naty, Mariana, Yenni, Andres ('no voy a estar'), Francisco, Trini, Leo (roommies!), Luis (hermanito!), Manu, Dani P, Deni, Gabo, Sofia. Thanks also to the ambassador Maria Teresa Infante and her team, Olivia and Juan Enrique. Special thanks to Pame F and Pablo, we are friends from before coming to Wageningen and they helped me installing here. It is quite nice to arrive in a new country and have already a bike, a clean place to stay, and some cold beers. Gracias chiquillos! Thanks also to my Chilean friends in Chile, that have been through the whatsapp with me, you always give me nice laughs with our talks. Special thanks to the Capri boat team, esas vacaciones en Italia fueron terapia pura, gallas!

Gracias a mis colegas de la Seremi de salud! especialmente a mi jefaza Maricel, que me inculcó el bichito científico. Gracias también a Lorena del ISP, Leonardo del IFOP, Andrea del Minsal y Carlos de la U de Chile que apoyaron este proyecto de alguna u otra forma.

Very special thanks to my paranymphs Myrto and Nacho. We have some stories together I will not disclose here, you know... privacy issues. I still remember when Myrto was sowing the lions for my birthday, haha. Myrto, you are a great scientist and toxicologist, I admire all what you have achieved at your age! I missed you when you moved to Utrecht, but we were always connected, thanks for that. Nacho, we still have some discussions about the "correct" Spanish speaking, and I still don't understand what you say sometimes, but you

are a great friend that has been around all this time, and even learned to be a bit it more 'punctual' to not to piss me off (right?) and I thank you for that as well. You both are the best and I am so happy we went through our PhDs together. We were paranymphs of each other, and I would not have imagined it in another way. I am sure we will always have our 'crisis committee meetings' every so often ;)

And of course, thanks to all the students that had the good idea to work in this amazing project! Leli, Marta, Siyuan, Kelly and Astrid, you put so much effort on it, thanks! and also to the students from food safety management, that made me realise that I was older than I thought! (like when I proudly announced I was going to the concert of Pearl Jam, and someone said 'oh, my mom listens to Pearl Jam'). I learned so much from all of you, and I hope you also learned a bit from me. Thanks also Martine and Marcel for giving me the opportunity to be a coach in your course.

Special thanks to my opponents, because they took their own time to read and evaluate this thesis, to my co-authors that put a lot of effort in this work, and thanks my new team at Charles River, at the time I am writing these words I have been with you only a month and I feel super welcome.

Gustavo y Silvia, papá y mamá, ustedes son lo mejor. Me alegro haber nacido en la familia que nací, y de haber crecido juntos, ustedes como papás, yo como hija. Como dice la Mafalda, que de hija, papá y mamá nos graduamos el mismo dia. Gracias a mis 'hermanitos' Francisco ñacs y Andrés, a la Paz, mi hermana nueva, la mamá de Agustina y Sebastián, los sobrinos más amados del mundo mundial. Gracias a la Irene, mi tía-hermana, Oscachi y Marina, mi padrino Edwin, mi tia tocaya Marcia, y a mi montón de primos y primas, tios y tias. Una mención especial para La Luli, la Gueli y el Tata, la Mimi y el tata Gustavo, que están siempre presentes en mi vida. La familia es numerosa, y todos han sido súper importantes en alguna u otra forma durante esta etapa, aunque los veo poco, porque estamos un poco lejos. Ook, bedankt Nettie, Wietske en Rikkert, ik ben heel blij dat jullie mijn Nederlandse familie zijn. And of course, the biggest thanks to Gerlof, who is my support in everything (he is the best).

In summary, I would like to thank my friends, my colleagues, my Chilean family, my Dutch family, but also thanks to my own stubbornness and my strength to finalise this nice project. Finally, it became a nice book you have to read! Yes, I know everyone reads this part, but not everyone is motivated to read the thesis itself. So, I invite you to do it, it is nice! Spoiler alert: we save the mice in the last chapter!

Marcia

Curriculum Vitae

Marcia Francisca Bodero Baeza was born on January 17th, 1977 in Santiago, Chile. After receiving her title of veterinarian, she worked for 7 years in the laboratory of microbiology of the Chilean food safety authority in Santiago. During that period, Marcia followed a master's degree in biological sciences, where she worked on a project focused on the molecular epidemiology and genetic diversity of isolates of *Listeria monocytogenes* from foods sampled in Santiago, and their relationship to clinical strains from listeriosis outbreaks in Chile, which gave a base line research for the inclusion of this pathogen in the Chilean food regulation. In 2011, Marcia moved to the Netherlands to follow a master's in food safety in Wageningen University and Research, and she did her thesis and internship at Rikilt. In 2013, Marcia became a PhD student at Rikilt Wageningen University and Research, with the support of the subdepartment of Toxicology of the same university. Marcia has followed the post graduate education in toxicology, which will allow her to register as European toxicologist in the near future. She also participated in the course food safety management as the director of a group of students, in the practical courses of Toxicology and guided 4 students in their master thesis. Marcia was also involved in the Chilean student association of Wageningen University and Research, where she was the president for a year. She participates in the Chilean network Redes Chilenas, and also a member of the network of Chilean researchers in the Netherlands, InNL. Recently, Marcia has started her career as a regulatory toxicologist at Charles River Laboratories, in Den Bosch, the Netherlands.

List of publications

This thesis

A strategy to replace the mouse bioassay for detecting and identifying lipophilic marine biotoxins by combining the neuro-2a bioassay and LC-MS/MS analysis Marcia Bodero, Arjen Gerssen, Liza Portier, Mirjam D. Klijnstra, Ron L.A.P. Hoogenboom, Leonardo Guzman, Peter J.M. Hendriksen and Toine F.H. Bovee. Submitted for publication, in review.

Detection and profiling of diarrheic marine biotoxins in shellfish by mRNA analysis of exposed Caco-2 cells using qRT-PCR and multiplex magnetic bead-based assays. Marcia Bodero, Toine F.H. Bovee, Liza Portier and Peter J.M. Hendriksen. ALTEX - Alternatives to animal experimentation. (Nov. 2018). DOI: https://doi.org/10.14573/altex.1805291.

Whole genome mRNA transcriptomics analysis reveals different modes of action of the diarrheic shellfish poisons okadaic acid and dinophysis toxin-1 versus azaspiracid-1 in Caco-2 cells. Marcia Bodero, Ron L.A.P. Hoogenboom, Toine F.H. Bovee, Liza Portier, Laura de Haan, Ad Peijnenburg, and Peter J.M. Hendriksen. Toxicol in Vitro https://doi.org/10.1016/j.tiv.2017.09.018

Screening for the presence of lipophilic shellfish toxins in shellfish using the neuro-2a bioassay. Marcia Bodero, Toine F.H. Bovee, Si Wang, Mirjam D. Klijnstra, Liza Portier, Ron L.A.P. Hoogenboom, Peter J.M. Hendriksen and Arjen Gerssen. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 1-15 http://dx.doi.org/10.1080/19440049.2017.1368720

Other publications

Marine biotoxins and associated outbreaks following seafood consumption: Prevention and surveillance in the 21st century. Nicolas, Jonathan; Hoogenboom, Ron L.A.P.; Hendriksen, Peter J.M.; Bodero, Marcia; Bovee, Toine F.H.; Rietjens, Ivonne M.C.M.; Gerssen, Arjen DOI: 10.1016/j.gfs.2017.03.002

Molecular epidemiology and genetic diversity of *Listeria monocytogenes* isolates from a wide variety of ready-to-eat foods and their relationship to clinical strains from listeriosis outbreaks in Chile. David Montero, Marcia Bodero, Guillermina Riveros, Lisette Lapierre, Aldo Gaggero, Roberto M. Vidal, and Maricel Vidal. Front Microbiol. 2015; 6: 384. DOI: 10.3389/fmicb.2015.00384.

Sulfated polymers in biological mineralization: a source for bio-inspired engineering. Arias JL, Neira-Carrillo A, Arias JI, Escobar C, Bodero M, David M, Fernández MS. Journal of Material Chemistry, 2004. J. Mater. Chem., 2004, 14, 2154 – 2160.

Overview of completed activities

Discipline related courses and meetings

Cell toxicology, PET, Leiden	2014
Immunotoxicology, PET, Utrecht	2014
Laboratory animal science, PET, Utrecht	2014
Molecular Toxicology, PET, Leiden	2014
Risk assessment, PET, Wageningen	2014
Toxicogenomics, PET, Maastricht	2015
Mutagenesis and Carcinogenesis, PET, Leiden	2015
Workshop marine biotoxins- Faculty of Veterinary Medicine, University of Chile, Santiago, Chile	2015
ICMSS (International congress on molluscan safety), Puerto Varas, Chile	2015
Workshop Luminex, Wageningen 2015	2015
3rd Harmful Algae & Phycotoxins Netherlands mini-symposium, NIOO-KNAW	2015
Encuentros Rotterdam, Red Encuentros, Rotterdam	2016
PhD symposium 2016 "Diversity in science", Wageningen University and Research	2016
Encuentros Barcelona, Red Encuentros, Barcelona	2016
NVT conference, Doorn	2017
Encuentro InNL, Wageningen	2018

General courses

Techniques for Writing and Presenting a Scientific Paper, Wageningen University and Research	2013
VLAG PhD week, Baarlo	2014
Applied statistics, Wageningen University and Research	2014
Luminex training, Den Bosch	2015
Philosophy and ethics, Wageningen University and Research	2016

Optional

Preparation of research proposal, Wageningen	2013
Scientific discussions at Rikilt and subdepartment of Toxicology	2013-2018
General toxicology, Wageningen University and Research	2014
Environmental toxicology, Wageningen University and Research	2015
Dutch courses organised by VLAG, Wageningen	2016-2017