UNIVERSITÉ DE NANTES

FACULTÉ DES SCIENCES ET DES TECHNIQUES

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ÉCOLE DOCTORALE VEGETAL, ENVIRONNEMENT, NUTRITION, AGROALIMENTAIRE, MER

Année 2012

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Écophysiologie des dinoflagellés du genre *Azadinium*, production toxinique et transfert trophique vers les mollusques bivalves

———

THÈSE DE DOCTORAT

Discipline : Physiologie et Biologie des Organismes

Spécialité : Phycotoxines

Présentée
et soutenue publiquement par

Thierry JAUFFRAIS

Le 24 Octobre 2012, devant le jury ci-dessous

Président	Philippe SOUDANT, Directeur de recherche, CNRS
Rapporteurs	Allan CEMBELLA, Professeur, Alfred Wegener Institute
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À ceux d'aujourd'hui : Maud, Mes Parents,
Mon Frère, Ma Famille et Mes Amis
À tous ceux d'avant
Et à tous ceux à venir…

T. J.

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Introduction et objectifs de l’étude

Le phytoplancton et le microphytobenthos sont à la base de la chaine alimentaire des écosystèmes marins. Ils sont majoritairement composés d'organismes photosynthétiques unicellulaires pélagiques et benthiques. Habituellement, les différentes espèces de phytoplancton cohabitent ensemble pour former des assemblages naturels. Cependant, occasionnellement une ou quelques espèces peuvent dominer cet assemblage et provoquer des efflorescences qui peuvent durer plusieurs semaines. Ces efflorescences sont dues à des combinaisons entre des facteurs biotiques et abiotiques. Parmi ces facteurs, il y a les processus hydrographiques, météorologiques, nutritionnels et la diminution de la mortalité due à la baisse d'influence de facteurs externes. Ces derniers peuvent être des virus, des parasites, des compétiteurs, la sédimentation, ou la prédation (Graneli and Turner, 2006).

Certaines de ces efflorescences peuvent provoquer des effets indésirables. Il peut s'agir d'une diminution de la concentration en oxygène, d'une dégradation de la qualité de l'eau ou de l'obturation des branchies des poissons ou d'autres organismes marins ; on parle alors de microalgues nuisibles. D'autres efflorescences peuvent être constituées par des microalgues toxiques et entrainer une contamination de leurs prédateurs. Lorsque le prédateur en fin de chaîne alimentaire est l'homme, ces efflorescences provoqueront, indirectement, des intoxications alimentaires de divers types.

Sur les quelques 25,000 espèces de phytoplancton connues (Falkowski et al., 2004), une partie seulement est nocive (environ 300 espèces) pour les organismes marins d'une manière ou d'une autre, et environ 80 de ces espèces sont connues pour être des producteurs de phycotoxines (Graneli and Turner, 2006).

Les phycotoxines marines sont des métabolites secondaires produits par certaines espèces de microalgues eucaryotes ou procaryotes. Ces métabolites généralement inoffensifs pour l'organisme producteur se concentrent par le biais du réseau trophique dans les organismes consommateurs de phytoplancton toxique. Ils remontent ainsi la chaine alimentaire jusqu'à intoxiquer les mammifères supérieurs. Parmi les consommateurs, on compte aussi bien des « micro-brouteurs » (zooplanctons, phytoplanctons hétérotrophes), des mollusques bivalves filtreurs, des gastéropodes ou des poissons herbivores qui à leur tour vont contaminer leurs prédateurs.

Les mollusques bivalves filtreurs tels que les huîtres (*Crassostrea gigas, Crassostrea virginica, Ostrea edulis*), les moules (*Mytilus edulis*), les coques (*Cerastoderma edule*), les coquilles St Jacques (*Pecten maximus*) ont un rôle majeur dans les écosystèmes marins et estuariens du fait de leur abondance et de leur place dans le réseau trophique (filtration de phytoplancton et proie) (Gosling, 2003; Smaal et al., 2001). Cependant, certaines microalgues

que consomment les mollusques bivalves sont productrices de phycotoxines. Il est donc nécessaire de connaître leurs effets sur la réponse physiologique des mollusques, ainsi que leur cinétique d'accumulation, de biotransformation et de détoxification dans ces mollusques, afin d'évaluer les effets potentiels de ces composés sur le réseau trophique et sur la santé du consommateur.

Les intoxications alimentaires, liées à la consommation de fruits de mer contaminés par des phycotoxines, ont entrainé la mise en place de mesures de protection. Des seuils sanitaires spécifiques à chaque groupe de phycotoxines ont ainsi été promulgués suite à la mise en place de la directive européenne 91/492/CEE (Annexe 1 (EFSA, 2009)). Lorsque ces seuils sont atteints ou dépassés dans les chairs de mollusques bivalves, les zones de production conchylicoles ou de pêches récréatives sont fermées et la vente des mollusques bivalves concernés est interdite. Ces fermetures des zones de production conchylicoles ont un impact direct sur la profession, en entrainant des pertes économiques, mais aussi indirect, en envoyant une image négative de la filière auprès des consommateurs et de la grande distribution.

En 1995, une intoxication alimentaire aux Pays-Bas a été associée à l'ingestion de moules contaminées en provenance de Killary Harbour, en Irlande (McMahon and Silke, 1996). Bien que les symptômes fussent typiques d'une intoxication provoquée par des toxines diarrhéiques, de type acide okadaïque (AO) ou dinophysistoxine (DTX), et que le test souris (Yasumoto et al., 1984) ait été positif, la concentration en toxines diarrhéiques dans les coquillages était bien en deçà du seuil sanitaire. Deux ans plus tard, il a été établi que ces coquillages avaient été contaminés par une autre toxine marine, initialement nommée "Killary-toxine" ou KT-3. Peu de temps après, la toxine a été renommée azaspiracide (AZA) afin de mieux refléter sa structure chimique (Satake et al., 1998c). Depuis 1995, les AZA ont été associés à des intoxications alimentaires provoquant des symptômes gastro-intestinaux. Ce groupe de toxines, à cause de la similarité de ses symptômes avec ceux des intoxications par des toxines diarrhéiques, a été associé aux phycotoxines diarrhéiques et a été signalé dans plusieurs pays d'Europe occidentale, et également au Japon, Maroc et en Amérique (Alvarez et al., 2010; Amzil et al., 2008; Furey et al., 2010; Klontz et al., 2009; Magdalena et al., 2003a; Taleb et al., 2006; Twiner et al., 2008; Ueoka et al., 2009; Vale et al., 2008).

Bien que la consommation de mollusques contaminés par les AZA ait causé plusieurs problèmes de santé publique, ce n'est qu'en 2003 que des AZA ont été détectés pour la première fois dans un échantillon de phytoplancton : *Protoperidinium crassipes*, un dinoflagellé (James et al., 2003a). Toutefois, la question de savoir si cet organisme était le

producteur primaire d'AZA ou un prédateur capable d'accumuler la toxine d'un autre organisme s'est rapidement posée (Hess et al., 2005b; Miles et al., 2004c). En effet, *P. crassipes* est un dinoflagellé hétérotrophe (Gribble and Anderson, 2006) capable d'accumuler des phycotoxines présentes dans ses proies (Miles et al., 2004b). De plus sa mise en culture n'a pas été probante en termes de production d'AZA (Tillmann et Krock, données non publiées dans (Tillmann et al., 2009)). L'hypothèse de travail était donc qu'une proie de *P. crassipes* soit l'organisme producteur des AZA. Au cours d'une campagne, en 2007, en mer du Nord, un dinoflagellé producteur d'AZA, a été isolé pour la première fois au large de l'Ecosse. La souche a été provisoirement nommée 3D9 ; un second isolement au large du Danemark a donné une deuxième souche nommée UTHE2 (Krock et al., 2008). Cet organisme est producteur d'AZA1 et -2 (Figure 12 et Figure 13) *in situ* et aussi en culture axénique (Krock et al., 2009; Tillmann et al., 2009). L'organisme, un petit dinoflagellé (12-16 µm de longueur et 7-11 µm de largeur), appartenant à un nouveau genre, a été officiellement décrit par (Tillmann et al., 2009) et nommé *Azadinium spinosum*.

Fait intéressant, depuis la description morphologique de cette nouvelle espèce, des populations d'*Azadinium* ont également été signalées au Mexique (Hernandez-Becerril et al., 2010), en Argentine (Akselman and Negri, 2012), en Italie et en France (communication personnelle, Siano et Nezan, Ifremer, France) et en Irlande (Salas et al., 2011). C'est seulement dans ce dernier pays qu'une troisième souche productrice d'AZA1 et -2 a été isolée.

En raison des épisodes d'intoxications alimentaires dus aux AZA, et de la toxicité des molécules, l'Union Européenne a instauré comme seuil sanitaire : 160 µg d'AZA par kg de chair de coquillages. La répartition de ces toxines dans le monde, et le fait que l'organisme semble être largement répandu dans de nombreux océans, font que les organismes de sécurité sanitaire doivent considérer ce groupe de toxines comme une préoccupation mondiale. (rq : une partie de l'état de l'art est dédiée aux AZA (Chap1, partie 1.2) et à *A. spinosum* (Chap1, partie 1.4) pour plus de détails).

L'Irlande est le pays producteur de mollusques bivalves le plus touché, en Europe et dans le monde, par la contamination de ces mollusques par les AZA. Il est aussi le pays porteur du projet ASTOX2 auquel se rattache cette thèse.

En Irlande l'aquaculture joue un rôle important dans l'économie du pays, en particulier dans les zones côtières. Les principales espèces de mollusques bivalves élevées sont : les moules (*M. edulis*) (en filière ou draguées), l'huître creuse (*C. gigas*), l'huître plate (*O. edulis*), la palourde (*Tapes philippinarum*) et la coquille St Jacques (*P. maximus*). La

mytiliculture irlandaise est au sixième rang européen ; en Irlande, elle est le secteur ayant le plus fort tonnage et le second secteur aquacole derrière la salmoniculture d'un point de vue économique. C'est aussi le secteur aquacole le plus fortement touché par les contaminations aux AZA. En 2006, le secteur conchylicole présentait un chiffre d'affaire (CA) d'environ 63 millions d'euros et employait environ 1700 personnes (Anonymous, 2007). En France selon le comité national de la conchyliculture (CNC), la conchyliculture française se classe au 2ème rang européen, derrière l'Espagne, avec une production annuelle d'environ 200 000 tonnes de coquillages, pour un chiffre d'affaire d'environ 774 millions d'euros. Le secteur se compose d'un peu plus de 4 500 exploitations qui emploient environ 20 000 personnes (soit 10 500 équivalents temps plein). L'ostréiculture (2 654 entreprises, 130 000 tonnes d'huîtres, CA 630 millions d'euros) et la mytiliculture (65 000 tonnes de moules, CA 120 millions d'euros) sont les deux principaux secteurs de production de mollusques bivalves en France. Tout comme l'Irlande, le secteur conchylicole Français est touché par des contaminations mettant en cause des phycotoxines. En effet, trois groupes de toxines, de type diarrhéique, paralysante et amnésiante (Chap1, partie 1.1) sont régulièrement présentes sur les côtes françaises; cependant, la France est encore peu concernée par la contamination de ces mollusques bivalves par les AZA. Une seule zone de production, en Bretagne Nord, a jusqu'à présent fait l'objet d'une période de fermeture, après l'accumulation d'AZA, au-delà du seuil sanitaire, dans des pétoncles (*Aequipecten opercularis*) au cours de l'automne 2006 (Amzil et al., 2008).

Le projet ASTOX2 a été mis en place pour soutenir le secteur conchylicole irlandais, face aux problèmes engendrés par les contaminations répétitives des lieux de productions, et ce depuis la découverte des AZA en 1995. Le projet aborde trois sujets bien distincts :

1 l'évaluation de la toxicité des AZA ;
2 la gestion des zones de production conchylicole et des questions de sécurité sanitaire, à travers l'étude de la contamination et de la détoxification des mollusques bivalves, la production durable de standards d'AZA pour les réseaux de surveillance, la validation et l'amélioration des méthodes analytiques déjà existantes ;
3 l'étude du transfert des AZA dans le réseau trophique et la découverte de nouveaux organismes producteurs d'AZA.

Peu de données sont actuellement disponibles sur la toxicité des AZA, à cause de la faible quantité de toxines purifiées disponibles et utilisables pour réaliser des tests toxicologiques. De plus, en raison d'un manque de données épidémiologiques, la quasi-totalité des informations concernant la toxicologie des AZA a été obtenue à partir d'études *in*

vitro et *in vivo*. Beaucoup de ces efforts de recherche ont été dirigés vers l'évaluation du risque engendré par la consommation de mollusques contaminés, et vers l'identification des cibles moléculaires des AZA. Malgré de nombreuses études réalisées, ces cibles restent actuellement encore inconnues. Le manque de données sur la toxicologie des AZA requiert la production d'AZA1 à 3, pour améliorer les connaissances sur l'effet des AZA régulés, mais aussi l'isolement de nouveaux analogues, pour évaluer leur toxicité. Cependant, les structures complexes des phycotoxines marines ne permettent pas une production efficace par voie de synthèse. Des sources d'origine naturelle sont donc généralement nécessaires pour leur production (Perez et al., 2010). De plus, en ce qui concerne les AZA, l'isolement des toxines a jusqu'à présent été réalisé uniquement à partir de mollusques bivalves naturellement contaminés (Kilcoyne et al., 2012; Perez et al., 2010; Satake et al., 1998c). Cette procédure réclame de la main-d'œuvre et du temps, et aboutit généralement à des rendements et à des quantités de toxines purifiées relativement faibles. Cependant elle est souvent nécessaire pour la production de matériels de référence et d'analogues de toxines issus du métabolisme des mollusques bivalves (Perez et al., 2010). En plus d'être laborieuse, ce mode d'obtention des toxines requiert des taux de contamination très élevés du milieu naturel. La production de toxines, à partir de cultures de dinoflagellés toxiques, est possible, durable et requiert généralement moins d'étapes de purification, du fait de leur matrice biologique moins complexe (Laycock et al., 1994; Loader et al., 2007; Miles et al., 2003; Torigoe et al., 1988). Cette production de métabolites, à partir de microalgues, nécessite le développement, à grande échelle, d'une méthode de production adaptée à l'organisme, suivie d'une procédure de récupération de la biomasse, à partir de grands volumes de culture, d'une procédure d'extraction du métabolite d'intérêt et d'une méthode de purification de l'extrait brut (Molina Grima et al., 2003).

Dans ce contexte, cette thèse présente trois objectifs : (1) la mise au point d'une procédure d'analyse des AZA et le développement de la production d'AZA à une échelle pilote, à partir de cultures en continu d'*A. spinosum*, (2) l'étude de l'influence des facteurs environnementaux et nutritionnels sur la croissance et la production toxinique d'*A. spinosum* ; (3) une fois la culture d'*A. spinosum* maitrisée, l'étude de l'accumulation, de la détoxification et de la biotransformation des AZA par les moules, en utilisant différentes approches.

Le chapitre 1 est consacré à un état de l'art dans les domaines d'études précédemment cités. C'est-à-dire une analyse des différentes toxines connues pouvant

contaminer les mollusques bivalves (toxines lipophiles, amphiphiles et hydrosolubles). Dans ce chapitre les AZA sont étudiés en détails (distribution, structure chimique et analogues, voies de biotransformation connues, toxicologie et santé humaine et procédures d'analyse). Le genre *Azadinium* ainsi que les facteurs environnementaux et nutritionnels qui peuvent affecter la croissance et la production toxinique des dinoflagellés ont aussi été étudiés en détail. Cette étude a été complétée par l'analyse des différents processus de production des dinoflagellés, de récolte de microalgues et d'extraction d'AZA à une échelle pilote. La dernière partie de cette étude bibliographique a porté sur les interactions entre les phycotoxines et les mollusques bivalves, en étudiant les mécanismes de filtration, les effets des microalgues toxiques sur les mollusques et sur l'accumulation et la détoxification des phycotoxines dans les mollusques bivalves filtreurs.

Le chapitre 2 comporte deux parties distinctes. La première est consacrée au développement d'une méthode d'analyse, pour la détermination des AZA dans les cultures d'*A. spinosum,* en mettant l'accent sur la formation des méthyles-esters d'AZA (des artéfacts formés lors de l'extraction et du prétraitement de l'échantillon). La seconde présente les travaux réalisés sur la culture en continu, à une échelle pilote, d'*A. spinosum* afin d'évaluer la faisabilité de la production d'AZA et de mettre au point les procédures de récolte, d'extraction et d'isolement à mettre en place pour la production d'AZA1 et -2 purifiés.

Le chapitre 3 s'intéresse à l'impact des facteurs environnementaux et nutritionnels sur la croissance et la production toxinique d'*A. spinosum*. Les expériences menées en cultures « batch » ou en continu ont porté sur les effets de la salinité, de l'intensité lumineuse, de la température et de l'aération, pour ce qui concerne les facteurs environnementaux. Concernant les facteurs nutritionnels, les effets de la composition de différents milieux de culture, de leur concentration, des différentes sources d'azote et de phosphore utilisées ainsi que de leur ratio ont été testés.

Le chapitre 4 comporte quatre parties distinctes. La première est consacrée à l'isolement d'une nouvelle souche d'*A. spinosum,* en Irlande, et à la mise en évidence du lien direct existant entre la présence d'*A. spinosum* dans le milieu et l'accumulation d'AZA par les moules. Une fois ce lien établi, une seconde étude s'intéresse à la cinétique d'accumulation et de détoxification des AZA, ainsi qu'au devenir des toxines dans les moules nourries avec *A. spinosum*. La troisième étude a été menée pour étudier les effets d'*A. spinosum* sur le

comportement alimentaire des moules. L'évaluation individuelle de l'activité alimentaire, des taux de filtration, d'ingestion et d'absorption a été effectuée sur des moules soumises à un régime toxique (*A. spinosum*) ou à un régime non-toxique (*Isochrysis* aff. *galbana* (T-*Iso*)). Une quatrième et dernière étude a évalué expérimentalement la capacité des moules à accumuler AZA1 et -2 sous forme dissoute, et a comparé les profils toxiniques obtenus, ainsi que la distribution des toxines dans les différents tissus de la moule, avec ceux de moules exposées à *A. spinosum*.

Chapitre 1 – État de l’art

1.1. Classification et organismes producteurs

En milieu marin, les phycotoxines sont principalement produites par les dinophycées (les dinoflagellés) ; les bacillariophycées (les diatomées), mais aussi par les prymnésiophycées, les raphidophycées, et les cyanophycées (les cyanobactéries).

Ces toxines sont classifiées soit en fonction de leur structure chimique (Tableau 1), soit en fonction des symptômes qu'elles produisent chez l'homme, lors de la consommation de fruits de mer contaminés. Elles incluent les intoxications **paralysantes** (IPFM, intoxication paralysante par les fruits de mer = PSP, paralytic shellfish poisoning), **diarrhéiques** (IDFM, intoxication diarrhéique par les fruits de mer = DSP, Diarrhetic shellfish poisoning), **amnésiantes** (IAFM, intoxication amnésiante par les fruits de mer = ASP , amnesic shellfish poisoning), **neurotoxiques** (INFM, intoxication neurologique par les fruits de mer = NSP, neurotoxic shellfish poisoning), ainsi que d'autres toxines encore non caractérisées. Une classification en fonction des propriétés physico-chimiques des toxines a été choisie dans cette synthèse bibliographique.

Tableau 1. Les principales phycotoxines marines, leur source, leur lipophilicité, leur formule chimique brute, leur masse molaire et le type d'intoxication qu'elles provoquent (les intoxications **paralysantes** (IPFM), **diarrhéiques** (IDFM), **amnésiantes** (IAFM), **neurotoxiques** (INFM).

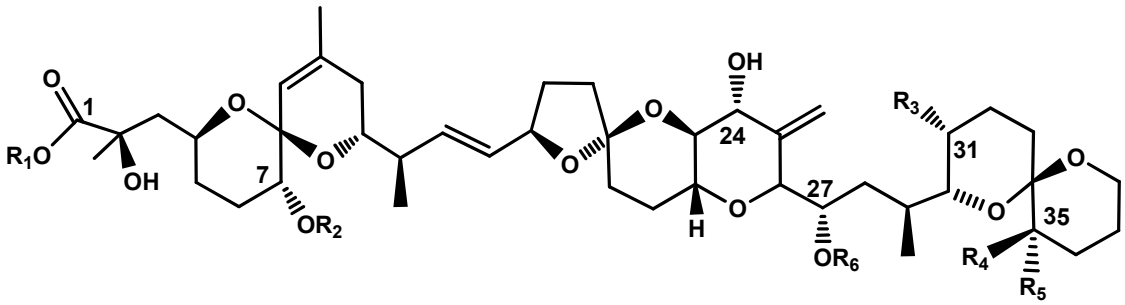
Groupe de toxine	Abréviation	Organisme producteur	Lipophilicité	Formule	Masse molaire g mol^{-1}	Intoxication
Acide domoïque	AD-DA*	*Pseudo-nitzchia spp.*	Hydrophile	$C_{15}H_{21}NO_6$	311	IAFM
Acide okadaïque	AO-OA*	*Dinophysis spp., Prorocentrum spp.*	Lipophile	$C_{44}H_{68}O_{13}$	804	IDFM
Azaspiracide	AZA	*Azadinium spinosum*	Lipophile	$C_{47}H_{71}NO_{12}$	841	IDFM
Brévétoxine-b	BTX	*Karenia brevis Chattonella spp.*	Lipophile	$C_{50}H_{70}O_14$	894	INFM
Ciguatoxine-P	CTX	*Gambierdiscus toxicus*	Lipophile	$C_{60}H_{85}O_{16}$	1061	INFM
Gymnodimine	GYM	*Karenia selliformis*	Lipophile	$C_{32}H_{45}NO_4$	507	INFM
Palytoxine	PLTX	*Ostreopsis spp.*	Amphiphile	$C_{129}H_{223}N_3O_{54}$	2677	INFM
Pecténotoxine-2	PTX	*Dinophysis spp., Prorocentrum spp.*	Lipophile	$C_{47}H_{70}O_{14}$	858	IDFM
Pinnatoxine	PnTX	*Vulcanodinium rugosum*	Lipophile	$C_{41}H_{61}NO_9$	711	INFM
Prorocentrolide	PCL	*Prorocentrum spp.*	Lipophile	$C_{56}H_{85}NO_{13}$	979	INFM
Saxitoxine	STX	*Alexandrium spp., Gymnodinium catenatum, Pyrodinium bahamense*	Hydrophile	$C_{10}H_{17}N_7O_4$	299	IPFM
Spirolide	SPX	*Alexandrium ostenfeldii*	Lipophile	$C_{41}H_{61}NO_7$	691	INFM
Yessotoxine	YTX	*Protoceratium reticulatum, Lingulodinium polyedrum, Gonyaulax spinifera*	Amphiphile	$C_{55}H_{82}O_{21}S_2$	1140	IDFM

*Abréviation anglaise

1.1.1. Les toxines Lipophiles

1.1.1.1. L'acide okadaïque

L'acide okadaïque (AO) (Figure 1) est une toxine diarrhéique, qui a été initialement isolée de l'éponge *Halichondria okadai,* qui lui a donné son nom (Tachibana et al., 1981), mais qui a aussi été identifiée dans des mollusques contaminés, suite à une série d'intoxications au Japon, en 1976 (Yasumoto et al., 1978). Dans les années 1980, les intoxications ont été associées à des proliférations de *Dinophysis fortii* (Yasumoto et al., 1980), un dinoflagellé duquel a été isolé un analogue de l'AO, la dinophysistoxine-1 (DTX1) (Figure 1). Le même composé a été trouvé par la suite dans des mollusques responsables d'intoxications diarrhéiques en Europe (Kumagai et al., 1986), auquel s'est ensuite ajouté la DTX2, son principal analogue (avec un rapport d'environ 1/3) (Hu et al., 1992) (Figure 1).



Toxine	R1	R2	R3	R4	R5	R6
OA	H	H	CH_3	H	H	H
DTX1	H	H	CH_3	CH_3	H	H
DTX2	H	H	H	H	CH_3	H
DTX3	H	acyle	*	*	*	H
Diol-ester	diol-ester	H/acyle	*	*	*	H
DTX4	diol-ester	H	*	*	*	H
DTX5	diol-ester	H	*	*	*	H
27-*0*-acyle	H	H	CH_3	H	H	acyle

Figure 1. Structure de l'acide okadaïque et de la dinophysistoxine-1 ainsi que de leurs dérivés et analogues, * signifie que le groupement est identique au composé parent AO, DTX1, DTX2 (Larsen et al., 2007).

L'AO et la DTX sont produits par deux genres de dinoflagellés : le genre *Dinophysis* (ex : *D. acuta* et *D. acuminata*) et le genre *Prorocentrum* (ex : *P. lima* et *P. belizeanum*).

Jusqu'à présent, l'AO a été principalement trouvé au Japon et en Europe, mais on l'a trouvé aussi aux États-Unis (Maranda and Shimizu, 1987), en Amérique du Sud (Muñoz et

al., 1992), aux Philippines (Marasigan et al., 2001) et récemment dans le golfe du Mexique (Swansson in (Rossini and Hess, 2010)), supposant une répartition mondiale de cette toxine et donc des espèces productrices.

Chimiquement, l'AO se caractérise par une fonction acide carboxylique et trois assemblages de cycles spiro-cétone. L'AO et les DTX1 et -2 peuvent résister à une large gamme de pH : de légèrement acide à fortement basique. Sans addition d'acide, ces composés sont plutôt stables à la chaleur, jusqu'à environ 110°C (McCarron et al., 2008) et sont donc résistants à la cuisson. Cependant, une faible quantité peut être rejetée par les mollusques dans les eaux de cuisson et ainsi réduire leur toxicité (McCarron et al., 2008). En outre, ces travaux récents suggèrent que ces toxines ne peuvent pas être détruites de façon significative dans les sucs gastriques, à cause de l'effet tampon de la nourriture (McCarron et al., 2008).

L'AO et les DTX possèdent différents analogues de type ester. Jusqu'à présent ils ont été isolés à partir de *P. lima* et *P. belizeanum* et ont été nommés DTX4, -5, etc. (Figure 1) (Hu et al., 1992; Yasumoto et al., 1989). Une fois filtré par les mollusques, l'AO est rapidement métabolisé (Vale, 2007). Les mollusques métabolisent l'AO en formant des esters d'AO et de DTX, par des liaisons avec des acides gras (au niveau du groupe C7-OH) (Figure 1). Les esters initialement identifiés dans les mollusques étaient des dérivés de la DTX1 et leur toxicité décrite comme étant similaire aux composés parents (Yasumoto et al., 1985). Maintenant, un ensemble d'esters d'acide gras, avec les trois structures de base (AO, DTX1, DTX2), a été décrit et est communément nommé DTX3. Par la suite, un ester d'acide gras au niveau du groupe C27-OH de la DTX1 a été rapporté dans une éponge (Britton et al., 2003), et récemment, des esters mixtes avec des diols, au niveau du groupement carboxyle en C1, et des acides gras, au niveau du C7-OH, ont également été signalés dans les mollusques (Torgersen et al., 2008a). Comme il existe de multiples ligands possibles (diols C5 à C9, acides gras C14 à C22) pour les différents groupements (C1, C7, C27), il y a une multitude d'analogues d'AO et de DTX possibles. Cette multitude de composés, potentiellement présents dans les coquillages lors de contaminations, rend la détermination de la teneur en toxines difficile. Cette complexité est à ajouter à la difficulté d'estimer la toxicité de l'ensemble des analogues présents dans un échantillon et ainsi d'évaluer le risque pour l'homme (Rossini and Hess, 2010).

Les esters d'acides gras d'AO et de DTX s'oxydent facilement parce qu'ils contiennent des doubles liaisons. N'importe lequel des esters présentés ci-dessus (esters des groupements carboxy en C1 ou OH en C7) peut être hydrolysé par l'addition d'une base forte (NaOH 0,3M dans une solution de méthanol à 76°C, pendant 10 à 40 min), ce qui permet

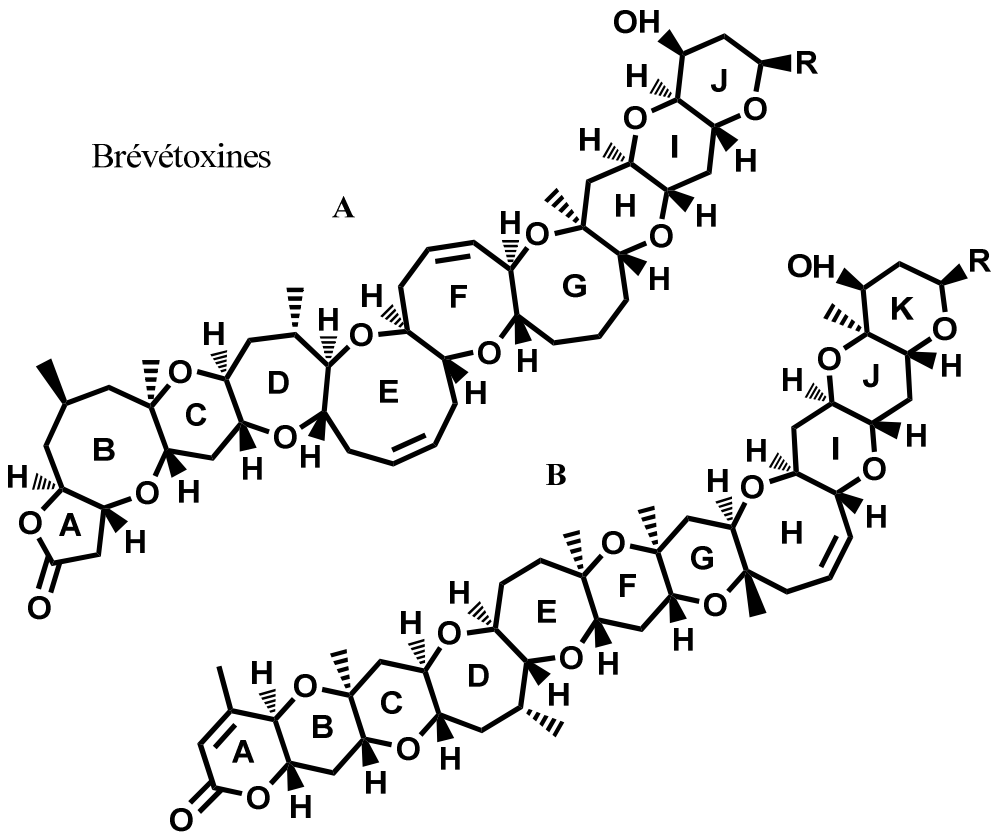
l'analyse des structures de base. Cette caractéristique, en combinaison avec la stabilité des composés d'origine (OA, DTX1 et -2), a été largement utilisée pour déterminer quantitativement la concentration équivalente du composé d'origine présent dans un échantillon donné de mollusques contaminés (Lee et al., 1989).

La toxicité de l'AO et de la DTX1, estimée après injection par voie intrapéritonéale (IP) à des souris ou par évaluation *in vitro* de leur capacité inhibitrice de la protéine phosphatase, est comparable, alors que la DTX2 est 40 à 50% moins toxique que l'AO (Aune et al., 2007). Une étude portant sur les effets des esters tels que la DTX3 sur souris, après injection IP, a démontré que les esters agissaient d'une manière similaire. Une récente étude sur les intoxications enregistrées suggère que les esters de l'AO et de la DTX semblent avoir une toxicité équivalente aux composés dont ils sont issus (EFSA, 2008; Torgersen et al., 2005).

1.1.1.2. Les Brévétoxines

Les brévétoxines (BTX) sont des toxines de type polyéther, produites par le dinoflagellé *Karenia brevis* et les raphidophycées du genre *Chattonella* spp.. *K. brevis* a eu un certain nombre d'appellations et a été précédemment appelé *Gymnodinium brevis*, *Gymnodinium breve* et *Ptychodiscus brevis*, le dernier nom ayant donné son nom PbTx à ce groupe de toxines.

Elles appartiennent à la même famille de polyéthers que les yessotoxines et les ciguatoxines. Deux types d'abréviations sont couramment utilisées (BTX et PbTx), conduisant parfois à confusion. Il y existe deux structures de base (Figure 2), une de type A (groupe BTX-A), avec 10 cycles polyéthers conjugués, et une de type B (groupe BTX-B), avec 11 cycles polyéthers conjugués. Le groupe BTX-A comprend les analogues PbTx-1, -7 et -10, tandis que le groupe BTX-B comprend les analogues PbTx-2, -3, -5, -6 et -9. La structure de la PbTx-4 n'a jamais été confirmée, et PbTx-8 est un artefact d'extraction, produit lors de l'isolement la toxine (Baden et al., 2005) (Figure 2).



Brévétoxines-A

Brévétoxine -1, R = $-CH_2C(=CH_2)CHO$
Brévétoxine -7, R = $-CH_2C(=CH_2)CH_2OH$
Brévétoxine -10, R = $-CH_2CH(-CH_3)CH_2OH$

Brévétoxines-B

Brévétoxine -2, R = $-CH_2C(=CH_2)CHO$
Brévétoxine -3, R = $-CH_2C(=CH_2)CH_2OH$
Brévétoxine -8, R = $-CH_2COCH_2Cl$
Brévétoxine -9, R = $-CH_2CH(CH_3)CH_2OH$

Figure 2. Les deux groupes de brévétoxines et quelques exemples de leurs analogues

La PbTx-2 est l'analogue principal produit par *K. brevis*. Il représente l'analogue majoritairement trouvé dans l'eau de mer lors des efflorescences de *K. brevis*. Cependant, il est rapidement transformé en une toxine 10 fois plus toxique : la PbTx-3 (dihydro-PbTx-2), qui est la toxine principalement retrouvée dans les aérosols formés durant les efflorescences de *K. brevis* (Pierce et al., 2005). La présence de brévétoxines a initialement été décrite aux États-Unis et dans le golfe du Mexique, mais celles-ci ont par la suite également été trouvées dans les eaux néo-zélandaises (Mackenzie et al., 1995). Bien que les intoxications liées à la consommation de mollusques soient connues depuis le milieu du 19ème siècle, l'élucidation de la structure complète des BTX n'a été possible que durant les années 1980 (Alam et al., 1982; Lin et al., 1981; Shimizu et al., 1986). En Floride, *K. brevis* est un organisme qui a été décrit pour sa capacité à former des efflorescences provoquant la coloration des eaux en rouge (« eaux rouges »). Les effets de cette algue sont de trois ordres: une exposition aux aérosols

conduit (1) à une irritation cutanée, (2) à des problèmes respiratoires et une accumulation de la toxine dans les mollusques qui engendre (3) des troubles neurotoxiques.

Les brévétoxines produites par *K. brevis* sont des composés très lipophiles. Certains métabolites présents dans les coquillages ont un caractère légèrement plus hydrophile, en raison de biotransformations conduisant à des dérivés conjugués cystéiques (Plakas et al., 2004; Wang et al., 2004). (Bourdelais et al., 2005) ont isolé de *K. brevis* un composé intéressant: la brévénale. Ce composé est un précurseur potentiel des brévétoxines. Mais, il a été démontré qu'il inhibe complètement l'action des brévétoxines sur les canaux sodiques et qu'il n'est pas toxique pour les poissons, contrairement aux BTX (Bourdelais et al., 2004). (Truxal et al., 2010) ont également isolé de *K.brevis* les tamulamides A et B, des polyéthers qui peuvent être en compétition avec la PbTX3, pour la liaison avec le site récepteur des synaptosomes du cerveau du rat, mais qui n'ont pas d'effet toxique sur le poisson ou sur les voies respiratoires des vertébrés (test pulmonaire du mouton). La synthèse totale de la brévétoxine-A a été réalisée par (Nicolaou et al., 1998) et la même équipe avait réalisé auparavant la synthèse totale de la brévétoxine-B (Nicolaou, 1996).

Fait intéressant, une autre espèce de *Karenia*, *K. mikimotoi*, produit d'autres toxines de type polyéther, les gymnocines A et B (Satake et al., 2002; Satake et al., 2005).

1.1.1.3. Les ciguatoxines et composés associés

Les toxines associées aux empoisonnements ciguatériques, lors de la consommation de poissons, comprennent plusieurs groupes de composés. Même si l'acide okadaïque, la palytoxine et d'autres composés peuvent être impliqués dans certains cas de contamination 'multi-toxines' on ne les traitera pas dans ce chapitre.

Les ciguatoxines (CTX, Figure 3), le gambierole et la maïtotoxine (MTX) appartiennent à des groupes de composés produits par le dinoflagellé *Gambierdiscus toxicus.* Ce dinoflagellé a été découvert par (Yasumoto et al., 1977) et a été décrit par (Adachi and Fukuyo, 1979). Il est responsable des intoxications alimentaires, par consommation de chairs de poissons contaminées par la ciguatera. Cependant d'autres dinoflagellés du genre *Gambierdiscus* produisent des toxines affiliées aux CTX et MTX (les *G. pacificus, G. polynesiensis* et *G. australis* produisent des « CTX-like » et « MTX-like » et le *G. yasumotoi* produit des « MTX-like »).

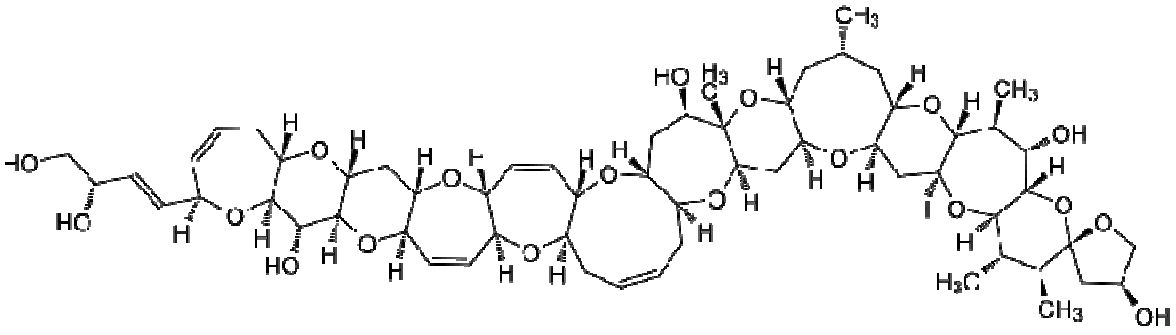


Figure 3. La ciguatoxine, CTX1B

La MTX est relativement stable en milieu alcalin, mais peu stable en milieu acide (Murata et al., 1989). C'est un polyéther polyhydroxylé (Figure 4) possédant deux groupements sulfate d'ester. Elle est amphiphile, donc soluble dans l'eau, le méthanol et le diméthyl-sulfoxyde. La MTX est ainsi parmi les polyéthers les plus hydrophiles, ce qui limite sa migration dans la chaîne alimentaire, en ne contaminant que les poissons herbivores.

Les gambierols et les ciguatoxines sont des polyéthers beaucoup plus lipophiles, qui vont donc remonter dans la chaîne alimentaire, et ainsi s'accumuler dans les poissons piscivores.

L'analogue de la ciguatoxine, la P-CTX-4B (Figure 4) est un des composés primaires produits par *Gambierdiscus toxicus*, microalgue retrouvée dans le Pacifique. Il semble être un précurseur de la P-CTX-1 (Figure 3), toxine isolée à partir de la chair de murène (Murata et al., 1990). Cette dernière est la toxine majoritairement retrouvée (elle représente plus de 90% de la toxicité (Legrand et al., 1992; Lewis et al., 1991)) dans les poissons piscivores, contaminés dans le Pacifique (Lewis et al., 1991; Murata et al., 1990; Murata et al., 1989; Poli et al., 1997; Satake et al., 1998a; Satake et al., 1997a; Satake et al., 1993).

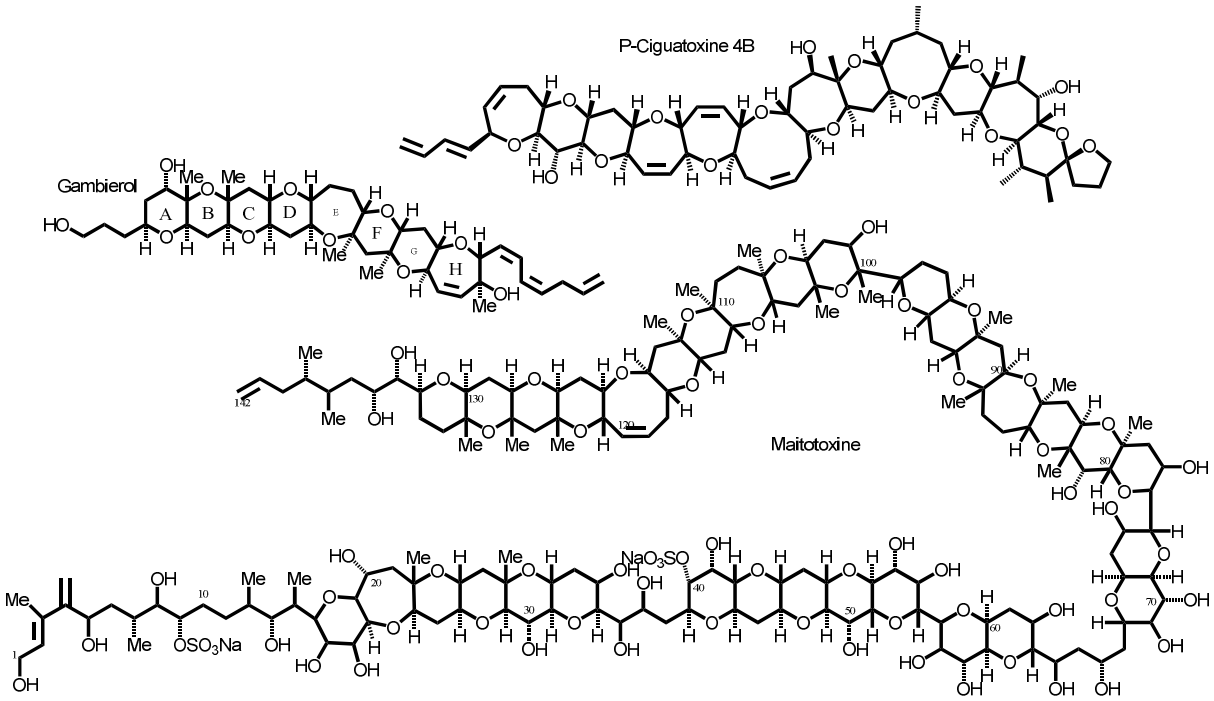


Figure 4. Les toxines reliées à la ciguatera, le précurseur de la ciguatoxine la P-ciguatoxine-4B (P-CTX-4B), le gambierol et la maïtotoxine

Ces toxines peuvent avoir un effet sur le consommateur, même à faible dose, ce qui a nécessité la mise au point de méthodes d'analyses très sensibles, capables de détecter de très faibles doses de ces composés dans les chairs de poissons. (0,1 $\mu g\, kg^{-1}$ à plusieurs µg/kg). Pour cette raison, il y existe peu de méthodes actuellement disponibles qui soient suffisamment sensibles pour la détection de ces toxines, et dans le monde, il n'y a que peu de groupes capables d'analyser ces ciguatoxines (Dickey, 2008).

La préférence de *G. toxicus* pour les eaux chaudes a été démontrée (Chateau-Degat et al., 2005). Ainsi, la présence de ciguatoxines est pour le moment limitée aux latitudes tropicales et subtropicales. Cependant, la présence récente de cas de ciguatera aux Canaries (Caillaud et al., 2010) et en Grèce (Aligizaki and Nikolaidis, 2008) indique que leur distribution dans le monde pourrait bien s'étendre, dans les années à venir. Jusqu'à présent la ciguatera est globalement répartie entre les océans Indien, Pacifique et la mer des Caraïbes et contamine principalement les poissons. Toutefois, quatre cas ont été recensés après consommation de coquillages contaminés en Nouvelle-Calédonie (Baumann et al., 2010).

1.1.1.4. Les imines cycliques

Les imines cycliques comportent plusieurs groupes de composés : les gymnodimines, les spirolides, les pinnatoxines et les ptériatoxines, les acides pinnaïques, les halichlorines, les

prorocentrolides et les symbio-imines (Figure 5) (Hu et al., 1995; Lu et al., 2001; Seki et al., 1995; Takada et al., 2001; Uemura et al., 1995).

Ces composés sont caractérisés par la présence d'un macrocycle. Une caractéristique commune de la structures de ces composés est aussi la présence d'une imine hexa- ou hepta-cyclique. Ces imines cycliques contribuent à l'activité biologique de ces composés. En effet, l'ouverture de ces cycles entraîne la perte de la bioactivité (ex : les spirolides (Hu et al., 1996)).

Les imines cycliques présentent une neurotoxicité unique chez la souris : elle conduit à une mort rapide dans les minutes qui suivent l'administration de la toxine par voie IP, d'où l'appellation de ces imines cycliques « toxines à action rapide », ce qui correspond à la traduction de la terminologie anglo-saxonne 'FAT' ou Fast Acting Toxins (Molgo et al., 2007). Chez l'homme la toxicité de ces composés représente un risque potentiel pour les pinnatoxines (Uemura, 2006), bien qu'aucune intoxication par ces composés n'ait été rapportée jusqu'à présent (McCarron et al., 2012; Selwood et al., 2010), et pour les gymnodimines et les spirolides la toxicité chez l'homme est encore non démontrée.

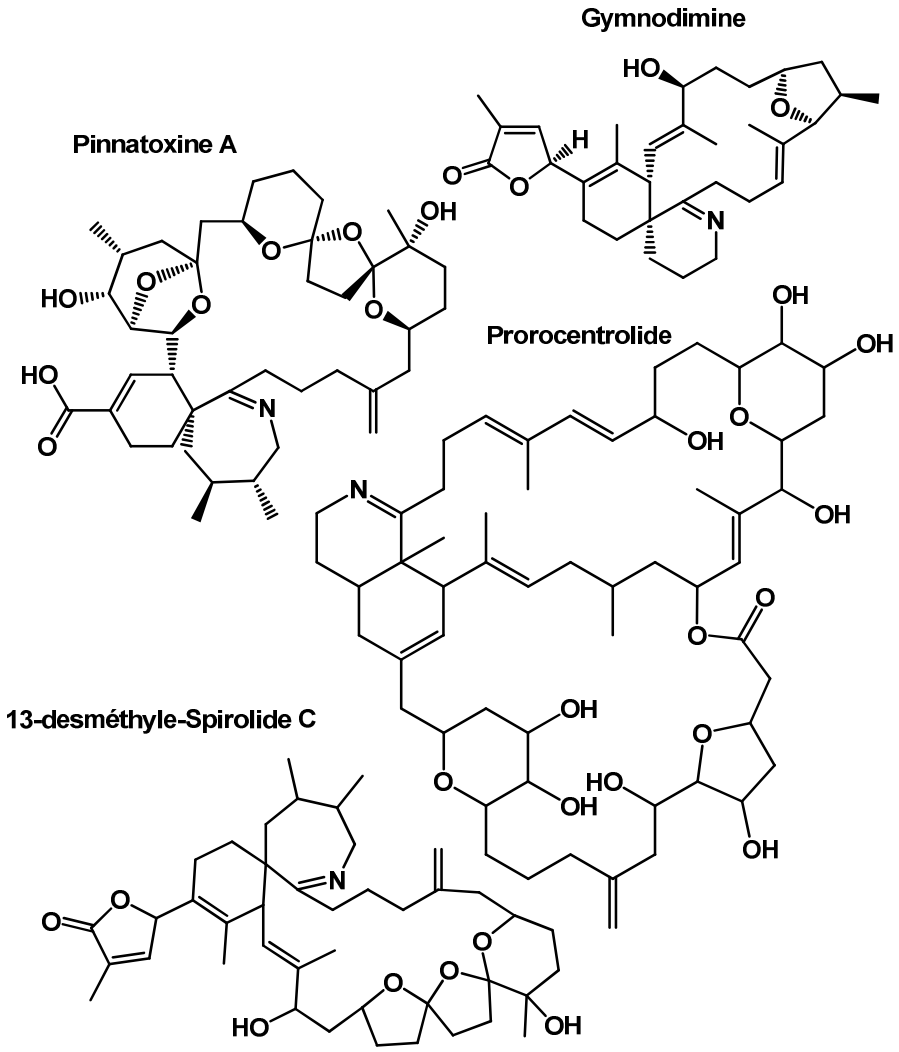


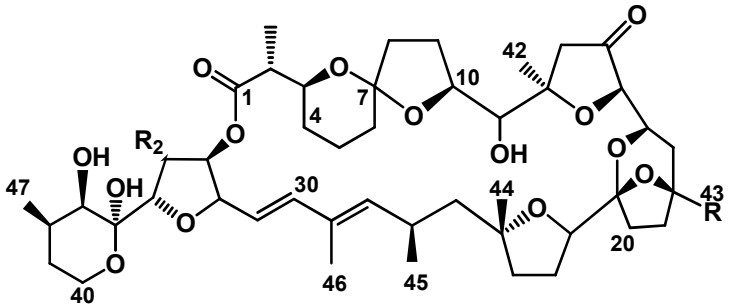
Figure 5. Les imines cycliques

1.1.1.5. Les pecténotoxines

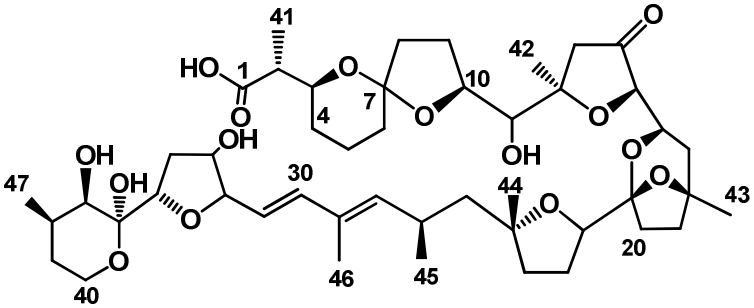
Les pecténotoxines (PTX) sont produites par des espèces du genre *Dinophysis*, l'un des principaux producteurs d'AO et de ses analogues. La PTX2 est le principal composé produit par les *Dinophysis*. Pour cette raison, les PTX ont été, dans un premier temps, associées aux intoxications diarrhéiques. Cependant, des études ont clairement démontré que les PTX disposent d'un mécanisme d'action distinct de celui de l'AO et de ses analogues.

Les PTX sont des polyéthers présentant un groupement spiro-cétal bicyclique et possédant des poids moléculaires proches de l'AO et des AZA. Contrairement à l'AO et aux AZA, les formes actives de PTX possèdent un ester macrocyclique intramoléculaire et pas d'acide carboxylique libre (Figure 6). Ainsi, la PTX2 se comporte comme un composé chromatographiquement neutre et très lipophile (Fux et Hess, observations non publiées dans (Rossini and Hess, 2010)).

(a) **Pecténotoxine** (PTX)



(b) **Séco-acide de PTX** (PTXsa)



Toxine	R (en C43)	C7
PTX1	CH_2OH	*7R*
PTX2	CH_3	*7R*
PTX3	CHO	*7R*
PTX4	CH_2OH	*7S*
PTX6	COOH	*7R*
PTX7	COOH	*7S*
PTX2sa	CH_3	*7R*
7-epi-PTX2sa	CH_3	*7S*

Figure 6. PTX2 ; structure et analogues ; le macrocycle est ouvert pour la PTX2sa et son épimère.

Dans le pétoncle japonais (*Patinopecten yessoensis*), d'où la toxine tire son nom, la PTX2 est successivement transformée en PTX1, PTX3 et enfin en PTX6 (Suzuki et al., 2005). Quels que soient ces analogues, le macrocycle est maintenu (Figure 6), ce qui signifie que leur comportement chromatographique n'est que légèrement modifié par la nature des substituants. En revanche, dans les moules (*Mytilus edulis*), la PTX2 est transformée en un séco-acide (PTX2sa), dans lequel le macrocycle est ouvert (Figure 6) (Miles et al., 2004b). Cette ouverture de cycle se traduit par une perte de bioactivité. En effet, la PTX2sa ne montre aucune activité, lorsqu'elle est injectée par voie IP chez la souris (Miles et al., 2004b).

Une autre voie du métabolisme dans les moules est l'estérification de la PTX2sa avec des acides gras (Wilkins et al., 2006). Bien que la toxicité de ces composés n'ait pas encore

été évaluée, elle doit être relativement réduite car la toxine dont ils sont issus (PTX2sa) ne présente pas de toxicité. (Ito et al., 2008) ont également montré une réduction de la toxicité de la PTX6 lors de son administration par voie orale chez la souris et le rat, en comparaison à la PTX2, ce qui suggère que la présence de la PTX2 non-transformée serait le principal problème lors des contaminations. Cependant en raison de sa forte lipophilicité, la PTX2 pourrait être difficilement biodisponible. Par ailleurs, la PTX2 semble être plutôt instable dans des conditions légèrement acides ou basiques, par conséquent elle pourrait ne pas résister aux conditions gastriques humaines et donc pourrait se métaboliser très rapidement en des formes non-toxiques (Rossini and Hess, 2010).

1.1.2. Les toxines amphiphiles

1.1.1.6. La palytoxine

La palytoxine (PlTX) possède une chaîne continue de 115 atomes de carbone. Elle est ainsi l'une des plus grandes phycotoxines de type polyéther (Figure 7). La molécule est caractérisée par la présence de nombreux groupes hydroxyles et de groupes amine et amide, responsables de son caractère hydrophile. Cependant, la longue chaîne carbonée constitue une partie lipophile. Ainsi, la PlTX a un caractère mixte hydrophile et lipophile (amphiphile). La structure de la PlTX a été décrite en 1981 (Moore and Bartolini, 1981; Uemura et al., 1981a, b).

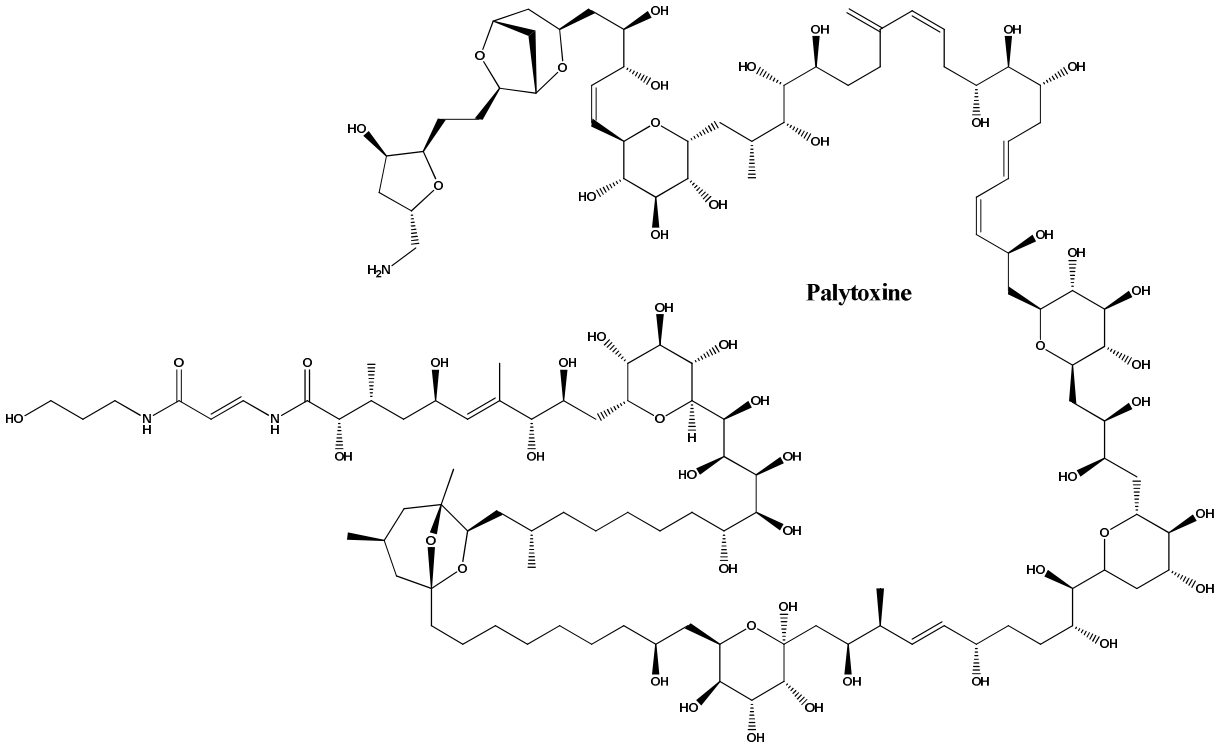


Figure 7. La palytoxine

Des études récentes ont démontré qu'il y avait un manque de connaissances réelles sur les origines possibles de la PlTX (Katikou, 2008). En effet, la PlTX a été rapportée à l'origine chez *Palythoa toxica* (Moore and Scheuer, 1971) et *Palythoa tuberculosa* (Kimura et al., 1972), deux anémones de mer. Toutefois, il a été postulé que des micro-organismes symbiotiques pouvaient être la véritable source de PlTX (Moore et al., 1982; Uemura et al., 1985), et une implication bactérienne n'est pas encore exclue (Frolova et al., 2000).

Il est maintenant établi que certaines micro-algues sont productrices de PlTX et d'un certain nombre de composés affiliés: *Ostreopsis siamensis*, *O. ovata* et *O. mascarenensis* (Lenoir et al., 2004; Onuma et al., 1999; Penna et al., 2005).

Dans les tropiques, ce groupe de toxines est traditionnellement associé à des intoxications alimentaires dues à des poissons contaminés ainsi qu'à des troubles liés à des expositions aux aérosols. Cependant, des *Ostreopsis spp*. contenant de la palytoxine et des composés proches (ex : les ovatoxines-a, -b, -c, -d, -e dans *O. ovata*) ont également été trouvés récemment en méditerranée occidentale (Espagne, Italie, Grèce et France), provoquant la fermeture de zones de baignade, sur des plages des côtes des pays concernés. Par ailleurs, des contaminations d'oursins et de mollusques bivalves ont été rapportées en parallèle en Grèce, Italie, Espagne et France (Aligizaki et al., 2008; Aligizaki and Nikolaidis, 2006; Amzil et al., 2012; Ciminiello et al., 2010; Ciminiello et al., 2008).

1.1.1.7. Les yessotoxines

La yessotoxine (YTX) et ses analogues sont des éthers polycycliques dont la principale caractéristique est la présence de 11 cycles éther accolés, d'une chaine insaturée et de deux groupes sulfate d'ester (Figure 8).

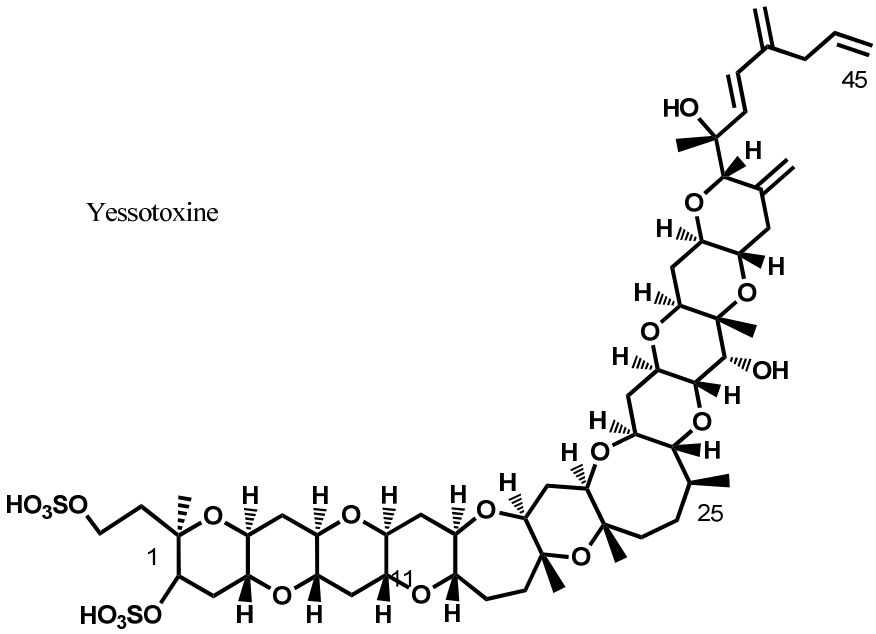


Figure 8. La yessotoxine.

La conjugaison des 11 cycles éther fait de la YTX et de ses analogues un groupe de toxines chimiquement proche des brévétoxines et des ciguatoxines. La structure rigide rend cette molécule apolaire. Toutefois, à cause des groupements polaires (sulfates), la YTX est considérée comme un composé de lipophilicité intermédiaire (Hess and Aasen, 2007).

La YTX a été isolée pour la première fois au Japon, à partir de glandes digestives de pétoncles japonais, *Patinopecten yessoensis*, (Murata et al., 1987). À cause de sa découverte par le test souris, test mis en place à l'origine pour mettre en évidence la présence de DSP, et de la concomitance des intoxications dues aux deux groupes de toxines, les YTX ont été classées dans les toxines diarrhéiques. Ultérieurement, il a été démontré que les YTX n'avaient pas les mêmes effets sur les souris, lorsqu'elles étaient administrées oralement ou par voie IP (Aune et al., 2002; Tubaro et al., 2004; Tubaro et al., 2003).

La yessotoxine et ses analogues sont produits par les dinoflagellés *Protoceratium reticulatum*, *Lingulodinium polyedrum* et plus récemment ils ont été rapportés dans *Gonyaulax spinifera* (Ciminiello et al., 2003; Rhodes et al., 2006; Samdal et al., 2004). Depuis la découverte initiale de la YTX, de nombreux analogues ont été découverts dans le

monde (Japon, Norvège, Italie, Ecosse, Chili). Ces analogues incluent : la 45-hydroxyYTX, la carboxyYTX, la 1-desulfoYTX, la homoYTX, la 45-hydroxyhomoYTX, la carboxyhomoYTX, la heptanor-41-oxoYTX, la heptanor-41-oxohomoYTX, la trinorYTX, l'adriatoxine, la (44-R,S)-44,55-dihydroxyYTX et la 9-méthylYTX (Finch et al., 2005), auxquels viennent s'ajouter de nombreux autres analogues récemment mis en évidence dans *P. reticulatum* (Miles et al., 2005a).

Les toxicités de la YTX et de l'homo-YTX sont similaires (Satake et al., 1997b), alors que les autres analogues ont une toxicité plus faible (5 fois moins pour les dérivés hydroxyles et carboxyles) (Miles et al., 2005b; Miles et al., 2004a). Cependant, la toxicité des YTX administrées chez la souris par voie IP ou par voie orale est différente. C'est un phénomène directement lié à la structure chimique de la molécule (Ferrari et al., 2004). Cette différence de toxicité des YTX par voie IP et par voie orale est probablement liée à la faible absorption des YTX par le tractus digestif, lorsqu'elles dont administrées *per os*, alors que la solubilité dans l'eau des YTX facilite leur biodisponibilité par voie IP. C'est aussi probablement la raison pour laquelle leur temps de séjour dans le tractus digestif est très court, réduisant ainsi l'absorption des toxines lors de la digestion (Rossini and Hess, 2010).

1.1.2. Les toxines hydrosolubles

1.1.2.1. Les toxines amnésiantes

1.1.2.2. L'acide domoïque

L'acide domoïque (AD) est un petit acide aminé cyclique (311 Da), présentant trois foncions acide carboxylique (Figure 9).

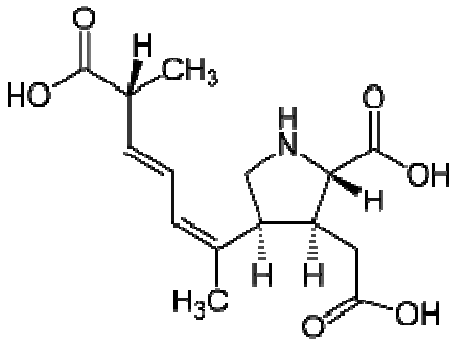


Figure 9. L'acide domoïque

Ces fonctions acide carboxylique sont responsables de son hydro-solubilité et de sa polarité relativement élevée, ce qui permet une élution en début de chromatogramme en

chromatographie liquide en phase inverse (Quilliam et al., 2001). De nombreux isomères et analogues ont été rapportés (Holland et al., 2005; Maeda et al., 1986; Walter et al., 1994; Zaman et al., 1997), mais jusqu'à présent seuls l'AD et son isomère C5'-diastéréoisomère ont révélé une toxicité non négligeable (Ramsdell in (Rossini and Hess, 2010)).

L'AD se transforme en son diastéréoisomère, au cours du stockage ou après traitement à la chaleur (Quilliam et al., 1995), et la détermination de la somme des concentrations de ces deux isomères conduit à une meilleure estimation de la toxicité totale.

La double liaison conjuguée, présente dans la chaîne aliphatique latérale, rend l'AD sensible à la lumière mais permet sa détection par spectrométrie d'absorption dans l'ultraviolet (UV) ainsi que par couplage spectrophotométrie UV et spectrométrie de masse (SM). Ces modes de détection sont couramment utilisés pour l'analyse de l'AD (Hess et al., 2005a).

Lorsque la toxine est accumulée dans les tissus des mollusques, l'AD est stable à la cuisson. Toutefois, grâce à la coagulation des protéines et à son hydrosolubilité l'AD est transféré de façon significative dans les eaux de cuisson. La stabilité de l'AD a été récemment étudiée dans diverses conditions (McCarron et al., 2007) et il a été montré que le stockage de tissus bruts ou autoclavés aboutit à environ 50% de dégradation de la toxine au bout de 5 mois.

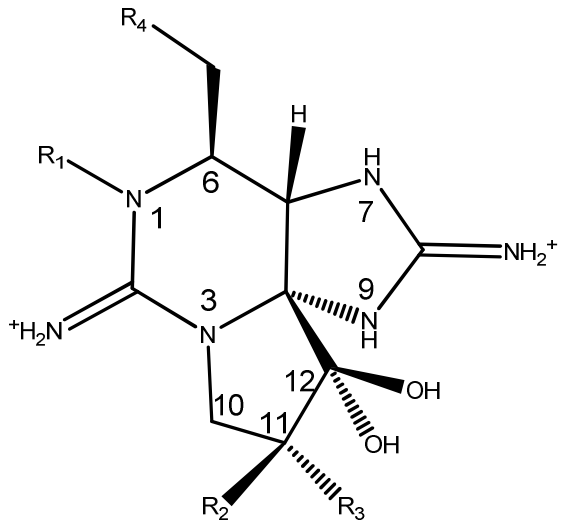
L'AD a été rapporté comme pouvant s'accumuler dans une grande variété d'organismes marins, tels que les moules, les pétoncles et les anchois. De plus, l'organisme producteur (les diatomées *Pseudo-nitzschia* spp.) est très largement distribué, ce qui laisse présumer que l'AD est une toxine présente dans le monde entier.

Le caractère hydrosoluble de l'AD se traduit par une détoxification relativement rapide (quelques jours) des mollusques, excepté pour la coquille St Jacques et le couteau (plusieurs mois). La surveillance réglementaire, par l'analyse des mollusques, est donc complétée par l'observation microscopique du phytoplancton présent dans les eaux des bassins de production conchylicoles, afin de détecter la présence de *Pseudo-nitzschia* et ainsi prévenir de possibles intoxications humaines. En effet ces intoxications peuvent être mortelles comme cela a été le cas en 1987 au Canada, où la consommation de moules contaminées a entrainé plus d'une centaine de cas d'intoxications dont 3 mortelles.

1.1.2.3. Les toxines paralysantes

1.1.2.4. Les saxitoxines

Les saxitoxines (STX) (Figure 10) sont des composés présentant un squelette tétrahydropurique. Ce sont des poisons neurotoxiques, pouvant être mortels pour l'homme. La structure de base de la molécule la rend très soluble dans l'eau. Plus de 30 STX ont été identifiées, principalement à partir de cyanobactéries, de dinoflagellés marins et de mollusques bivalves filtreurs (Dell'Aversano et al., 2008; Dell'Aversano, 2004). Les STX sont principalement produites par les dinoflagellés du genre *Alexandrium* : par exemple, *A. tamarense, A. minutum, A. tamiyavanichii, A. excavatum, A. catenella, A. fundyense* et *A. cohorticula*, mais aussi par d'autres dinoflagellés comme *Pyrodinium bahamense* et *Gymnodinium catenatum* (FAO, 2004).



R_1	R_2	R_3	R_4	Toxine
H	H	H	—O—C(=O)—NH_2	STX
H	H	OSO_3^-		GTX2
H	OSO_3^-	H		GTX3
OH	H	H		NEO
OH	H	OSO_3^-		GTX1
OH	OSO_3^-	H		GTX4
H	H	H		GTX5 (B1)
H	H	OSO_3^-	—O—C(=O)—$NHSO_3^-$	C1
H	OSO_3^-	H		C2
OH	H	H		GTX6 (B2)
OH	H	OSO_3^-		C3
OH	OSO_3^-	H		C4
H	H	H		dcSTX
H	H	OSO_3^-	—OH	dcGTX2
H	OSO_3^-	H		dcGTX3
OH	H	H		dcNEO
OH	H	OSO_3^-		dcGTX1
OH	OSO_3^-	H		dcGTX4

Figure 10. Les saxitoxines

Les STX et leurs analogues ne présentent pas une forte absorbance ou fluorescence, lorsqu'ils sont soumis aux rayonnements ultraviolets. Ce sont par ailleurs des composés stables à la chaleur, jusqu'à 100°C. Des traitements par des solutions acides ou alcalines conduiront à diverses transformations. Cependant, ces transformations ne pourront se produire que partiellement dans les tissus de mollusques, d'humains ou dans des fluides contaminés, car le pH des matrices biologiques est généralement tamponné. Toutefois, ces transformations peuvent conduire à une augmentation de la toxicité initiale des produits, ce qui suggère un

danger potentiel de ces toxines (Hall and Reichardt, 1984). Pour examiner ces biotransformations, la B1 (GTX5) a été incubée dans des conditions simulant l'estomac humain et la toxicité a été déterminée par le test souris. Après 5 h d'incubation à 37°C dans des sucs gastriques artificiels (à pH 1,1), une augmentation de la toxicité d'un facteur 2 a été observée, alors que l'on n'observe une transformation que de 9% de la toxine (Harada et al., 1984).

Les organismes marins les plus souvent contaminés sont les moules et les huîtres, mais des gastéropodes marins (par exemple l'ormeau), des crustacées et des poissons globes ont aussi été signalés comme pouvant contenir des concentrations dangereuses pour l'homme (Rossini and Hess, 2010).

Ces composés présentent une détoxification relativement rapide chez les moules, certainement due à leur caractère hydrophile. Cette détoxification rapide complique la surveillance réglementaire de ces toxines, qui est donc combinée par les observations microscopiques du phytoplancton dans les zones de production conchylicoles (Rossini and Hess, 2010).

Le profil complexe de ces toxines, les conversions possibles de ces composés, et le manque de matériaux de référence, ont conduit la plupart des pays à maintenir le test souris (Sommer and Meyer, 1937), validé comme méthode officielle de détection des toxines AOAC (959.08) (AOAC, 2005a). Récemment, des méthodes alternatives ont été proposées. Elles sont basées sur l'utilisation de la chromatographie liquide couplée à une détection par fluorescence (Lawrence et al., 2004; Oshima, 1995; van de Riet et al., 2011) ou d'un test biologique sur microplaques (van Dolah et al., 2009), ces dernières méthodes étant aussi officiellement validées comme méthodes AOAC (AOAC, 2005b).

1.2. Les azaspiracides

1.2.1. Distribution

Depuis la première intoxication alimentaire en 1995, cinq nouveaux épisodes d'intoxications alimentaires se sont produits principalement par le biais de moules Irlandaises contaminées (Tableau 2 et) en Irlande, Italie, France, Royaume-Unis entre 1995 et 2007 (Twiner et al., 2008) et plus récemment aux États-Unis (Klontz et al., 2009).

Tableau 2. Nombre d'intoxications alimentaires causées par les azaspiracides et rapportées entre 1995 et 2007 (nc : non connu) (Twiner et al., 2008).

Lieu de l'intoxication	Date	Source de l'intoxication	Quantité consommée	Lieu de production	Nombre d'intoxication
Pays-Bas	Novembre 1995	Moules (*M. edulis*)	nc	Killary, Harbour, Irelande	8
Irlande	Septembre / Octobre 1997	Moules (*M. edulis*)	10-12	Arranmore Island, Irlande	~20-24
Italie	Septembre 1998	Moules (*M. edulis*)	nc	Clew Bay, Irlande	10
France	Septembre 1999	Moules (*M. edulis*)	nc	Bantry Bay, Irlande	~20-30
Grande Bretagne	Aout 2000	mussels (*M. edulis*)	nc	Bantry Bay, Irlande	~12-16

Depuis leur découverte initiale, les AZA ont été successivement détectés en Europe, en Afrique, et plus récemment en Amérique et au Japon (Alvarez et al., 2010; Amzil et al., 2008; Furey et al., 2010; Magdalena et al., 2003a; Taleb et al., 2006; Twiner et al., 2008; Ueoka et al., 2009; Vale et al., 2008) (Figure 11).

Figure 11. Localisation de la présence de la toxine azaspiracide, d'espèces d'*Azadinium* et d'intoxications alimentaires.

1.2.2. Structure et analogues

La structure de l'AZA1 (PM 841.5 Da) a été élucidée en 1998, après l'isolement de la toxine à partir de moules irlandaises, en provenance de Killary Harbour. Les groupements fonctionnels chimiques dont la molécule est composée, une amine cyclique (ou groupe aza), un unique assemblage tri-spiro et un acide carboxylique, ont donné leur nom à la toxine AZA-SPIR-ACIDE.

Après la réalisation de la synthèse totale (Nicolaou et al., 2003a; Nicolaou et al., 2004a; Nicolaou et al., 2006b; Nicolaou et al., 2003b; Nicolaou et al., 2004b), la formule d'origine, proposée en 1998, a pu être rectifiée. En effet, le composé synthétisé, correspondant à la formule initialement proposée pour le composé isolé à partir de moules contaminées, avait un comportement chromatographique différent et des différences sont aussi apparues lors de l'analyse par résonance magnétique nucléaire (RMN). L'étude des spectres RMN a ainsi abouti à la révision de la structure entre 2004 et 2006 (Nicolaou et al., 2003a; Nicolaou et al., 2004a; Nicolaou et al., 2006b; Nicolaou et al., 2003b; Nicolaou et al., 2004b) (Figure 12).

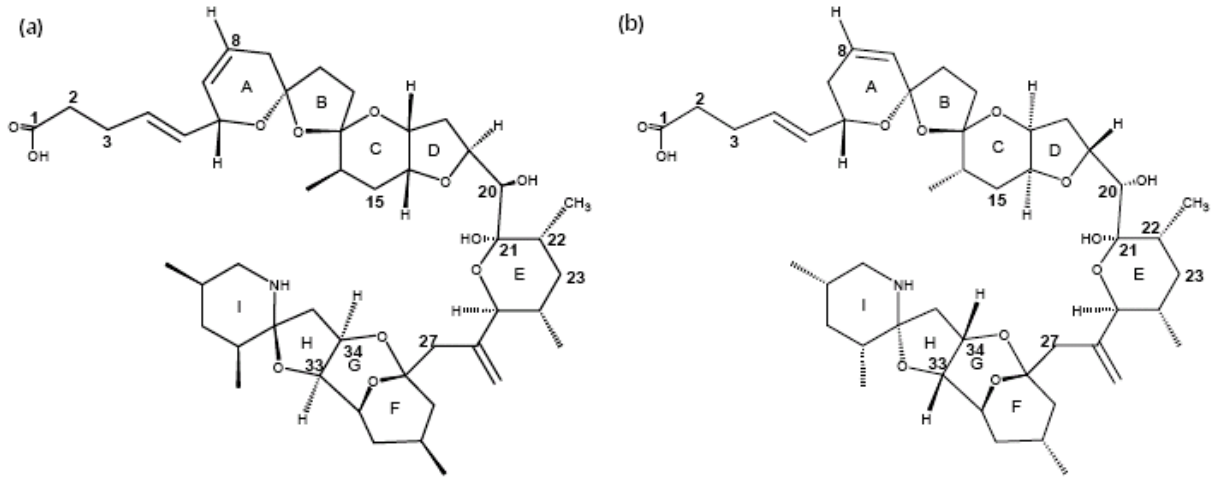
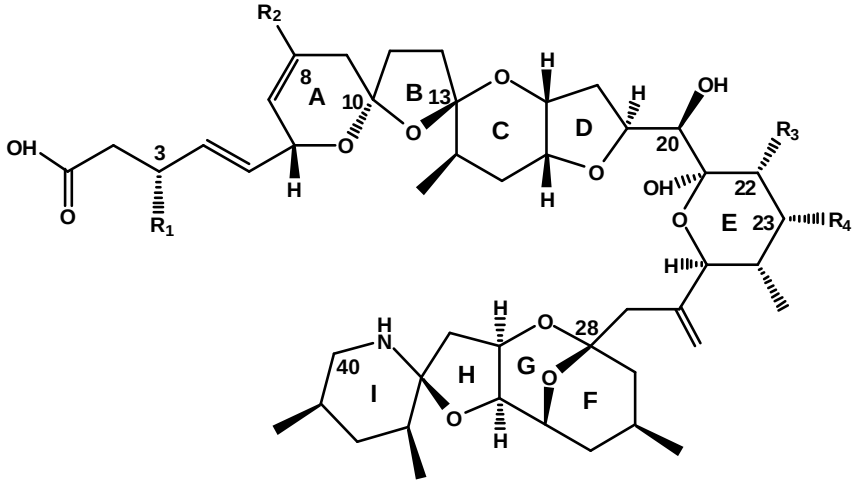


Figure 12. Structure de l'azaspiracide-1 (AZA1) (a) et du composé initialement proposé (b) (Nicolaou et al., 2006b). Les différences entre les deux structures sont liées à des différences d'orientation stéréochimique des cycles C/D, de la stéréochimie du carbone en C20 et de la position de la double liaison en C8.

Peu de temps après l'élucidation de la structure de l'AZA1, quatre analogues supplémentaires ont été découverts. Leurs structures ont été déterminées en utilisant des techniques de spectrométrie de masse (SM ou MS) et de RMN (Ofuji et al., 2001; Ofuji et al., 1999a). Trois des analogues isolés ne diffèrent que par le nombre de groupements méthyle. Par rapport à AZA1, il manque à AZA3 un groupement méthyle positionné en C22 et AZA2

possède un CH_3 supplémentaire en C8 (Figure 13). Les deux autres analogues de la toxine (AZA4 et AZA5) se sont avérés être des hydroxyles d'AZA3, avec la présence d'un groupement OH en C3 (AZA4) ou C23 (AZA5).



Abbréviation	Nom	MM	R1 (C3)	R2 (C8)	R3 (C22)	R4 (C23)	R5 (?)
AZA1	Azaspiracide	841,5	H	H	CH_3	H	
AZA2	8-méthyle-azaspiracide	855,5	H	CH_3	CH_3	H	
AZA3	22-desméthyle-azaspiracide	827,5	H	H	H	H	
AZA4	22-desméthyle-3-hydroxy-azaspiracide	843,5	OH	H	H	H	
AZA5	22-desméthyle-23-hydroxy-azaspiracide	843,5	H	H	H	OH	
AZA6	22-desméthyle-8-methyl-azaspiracide	841,5	H	CH_3	H	H	
AZA7	3-hydroxy-azaspiracide	857,5	OH	H	CH_3	H	
AZA8	23-hydroxy-azaspiracide	857,5	H	H	CH_3	OH	
AZA9	22-desméthyle-3-hydroxy-8méthyle-azaspiracide	857,5	OH	CH_3	H	H	
AZA10	22-desméthyle-23-hydroxy-8méthyle-azaspiracide	857,5	H	CH_3	H	OH	
AZA11	3-hydroxy-8-méthyle-azaspiracide	871,5	OH	CH_3	CH_3	H	
AZA12	23-hydroxy-8-méthyle-azaspiracide	871,5	H	CH_3	CH_3	OH	
AZA13	22-desméthyle-3,23-dihydroxy-azaspiracide	859,5	OH	H	H	OH	
AZA14	3,23-dihydroxy-azaspiracide	873,5	OH	CH_3	H	OH	
AZA15	22-desméthyle-3,23-dihydroxy-8-méthyle-azaspiracide	873,5	OH	H	CH_3	OH	
AZA16	3,23-dihydroxy-8-méthyle-azaspiracide	887,5	OH	CH_3	CH_3	OH	
AZA17	carboxy-22-desméthyle-azaspiracide	871,5	H	H	H	H	COOH
AZA18	carboxy-azaspiracide	885,5	H	CH_3	H	H	COOH
AZA19	carboxy-22-desméthyle-8méthyle-azaspiracide	885,5	H	H	CH_3	H	COOH
AZA20	carboxy-8-méthyle-azaspiracide	899,5	H	CH_3	CH_3	H	COOH
AZA21	carboxy-22-desméthyle-3hydroxy-azaspiracide	887,5	OH	H	H	H	COOH
AZA22	carboxy-3-hydroxy-azaspiracide	901,5	OH	CH_3	H	H	COOH
AZA23	carboxy-22-desméthyle-3-hydroxy-8-méthyle-azaspiracide	901,5	OH	H	CH_3	H	COOH
AZA24	carboxy-3-hydroxy-8-méthyle-azaspiracide	915,5	OH	CH_3	CH_3	H	COOH

Figure 13. Formule de la molécule d'azaspiracide avec les positions des différents substituants pour les 23 premiers analogues décrits (Rehmann et al., 2008).

Jusqu'à très récemment, seuls les AZA1 à 5 avaient été isolés et structurellement vérifiés, en utilisant des techniques de RMN. L'AZA6 vient de rejoindre cette liste en 2012 (Kilcoyne et al., 2012), l'élucidation de la structure des autres analogues ayant été déduite uniquement de l'analyse de leurs schémas de fragmentation, à partir de leurs spectres de spectrométrie de masse en mode tandem (SM/SM) (Brombacher et al., 2002; James et al., 2003b; Rehmann et al., 2008).

La fragmentation des molécules d'AZA produit des ions caractéristiques de cette famille de molécules, qui sont issus de sept fragmentations et conduisant à sept groupes de composés majeurs (Figure 14) et d'une huitième fragmentation, pour certains analogues, conduisant au groupe 5, (Brombacher et al., 2002; Diaz Sierra et al., 2003; James et al., 2003b; Rehmann et al., 2008).

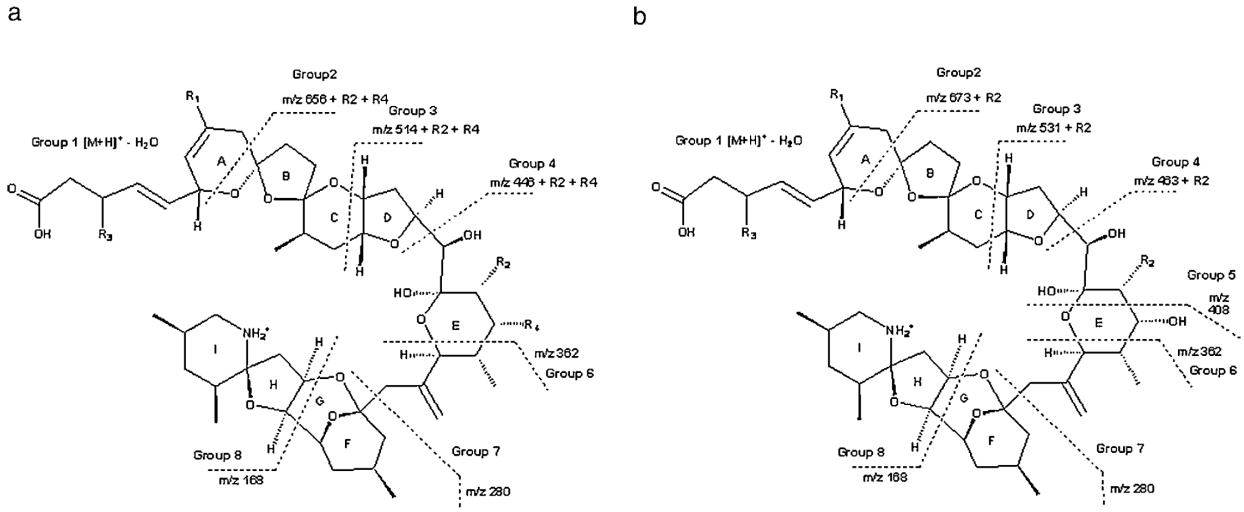


Figure 14. (a) Fragmentations SM/SM en mode ESI positif proposées pour les AZA (groupe 5 manquant), (b) Fragmentations SM/SM en mode ESI positif proposées pour les AZA hydroxylés en C23 (groupe 5 présent) (Rehmann et al., 2008).

L'analyse des différents fragments obtenus en SM/SM a conduit à l'identification d'une trentaine d'analogues naturels et théoriques d'AZA (Tableau 3). L'AZA6 est structurellement proche de l'AZA1. C'est un isomère, pour lequel le groupement méthyle en C22 de l'AZA1 est absent mais présent en C8 (Brombacher et al., 2002; James et al., 2003b). Des analogues hydroxylés d'AZA1 et d'AZA2 ont aussi été détectés et nommés AZA4 et -5 (James et al., 2003b). Plus récemment, 12 analogues d'AZA ont été détectés (Rehmann et al., 2008). Parmi eux des dérivés dihydroxy, carboxy et des carboxy-hydroxy d'AZA1-3 et -6. L'analyse des voies de fragmentation a montré que les AZAs hydroxylés en C23 produisent un ion fragment à 408 *m/z* qui peut perdre successivement deux molécules d'eau et qui conduit à un ion fragment de rapport *m/z* 362. Ces ions fragments ne sont pas observés avec les analogues

d'AZA qui ne sont pas hydroxylés en C23. Cette différence de fragmentation a permis de déterminer la structure de certains analogues (Tableau 3).

Tableau 3. Ions fragment obtenus en SM/SM pour les AZA1 à AZA32 (Rehmann et al., 2008).

	$[M+H]^+$	Groupe 1	Groupe 2	Groupe 3	Groupe 4	Groupe 5	Groupe 6
AZA1	842.5	824.5	672.5	530.5	462.5	-	362.3
AZA2	856.5	838.5	672.5	530.5	462.5	-	362.3
AZA3	828.5	810.5	658.5	516.5	448.5	-	362.3
AZA4	844.5	826.5	658.5	516.5	448.5	-	362.3
AZA5	844.5	826.5	674.5	532.5	464.5	408.4	362.3
AZA6	842.5	824.5	658.5	516.5	448.5	-	362.3
AZA7	858.5	840.5	672.5	530.5	462.5	-	362.3
AZA8	858.5	840.5	688.5	546.5	478.5	408.4	362.3
AZA9	858.5	840.5	658.5	516.5	448.5	-	362.3
AZA10	858.5	840.5	674.5	532.5	464.5	408.4	362.3
AZA11	872.5	854.5	672.5	530.5	462.5	-	362.3
AZA12	872.5	854.5	688.5	546.5	478.5	408.4	362.3
AZA13	860.5	842.5	674.5	532.5	464.5	408.4	362.3
AZA14	874.5	856.5	688.5	546.5	464.5	408.4	362.3
AZA15	874.5	856.5	674.5	532.5	478.5	408.4	362.3
AZA16	888.5	870.5	688.5	546.5	478.5	408.4	362.3
AZA17	872.5	854.5/810.5	702.5/658.5	560.5/516.5	492.5/448.5	-	362.3
AZA18*	886.5	868.5/824.5	716.5/672.5	574.5/530.5	506.5/462.5	-	362.3
AZA19	886.5	868.5/825.5	702.5/658.5	560.5/516.5	492.5/448.5	-	362.3
AZA20*	900.5	882.5/838.5	716.5/672.5	574.5/530.5	506.5/462.5	-	362.3
AZA21	888.5	870.5/826.5	702.5/658.5	560.5/516.5	492.5/448.5	-	362.3
AZA22*	902.5	884.5/840.5	716.5/672.5	574.5/530.5	506.5/462.5	-	362.3
AZA23	902.5	884.5/840.5	702.5/658.5	560.5/516.5	492.5/448.5	-	362.3
AZA24*	916.5	898.5/854.5	716.5/672.5	574.5/530.5	506.5/462.5	-	362.3
AZA25	810.5	792.5	658.5	516.5	448.5	-	362.3
AZA26*	824.5	806.5	672.5	530.5	462.5	-	362.3
AZA27*	824.5	806.5	672.5	530.5	448.5	-	362.3
AZA28*	838.5	820.5	672.5	530.5	462.5	-	362.3
AZA29	842.5	810.5	658.5	516.5	448.5	-	362.3
AZA30	856.5	824.5	672.5	530.5	462.5	-	362.3
AZA31*	856.5	824.5	672.5	530.5	448.5	-	362.3
AZA32	870.5	838.5	672.5	530.5	462.5	-	362.3

*Valeur théorique, elles n'ont jamais été observées dans des échantillons

Groupe 1 = $[M+H]^+ - H_2O$

Groupe 2 = Groupe 1 – R1-R2-$C_9H_{10}O_2$

Groupe 3 = Groupe 1 – R1-R2-$C_{16}H_{20}O_5$

Groupe 4 = Groupe 1 – R1-R2-$C_{20}H_{25}O_6$

Groupe 5 = Groupe 1 – R1-R2-R3-$C_{23}H_{27}O_7$ (only in C23 hydroxylated AZAs)

Groupe 6 = Groupe 1 – R1-R2-R3-R4-$C_{24}H_{28}O_9$

1.2.3. Les voies de biotransformation des AZAs

Les phycotoxines sont des métabolites naturellement produits par les microalgues. Celles-ci peuvent en produire de différente structure, par de légères variations de la structure principale. Par conséquent, la plupart des groupes de toxines marines ont également de nombreux analogues. De plus, parce qu'elles sont naturellement produites, de nombreux systèmes enzymatiques, présents dans la nature, sont capables de les métaboliser. Ainsi, parmi

les analogues produits naturellement et transformés, les phycotoxines marines constituent une vaste gamme de composés bioactifs, et les AZA ne dérogent pas à cette règle.

En raison du grand nombre d'analogues d'AZA mis en évidence dans les moules (Rehmann et al., 2008), de récentes études ont été menées, afin d'élucider les mécanismes de biotransformation des AZA dans les moules et de déterminer les différentes voies de transformation. La décarboxylation des AZA17, -21, -19 et -23, c'est-à-dire des analogues carboxylés d'AZA, en AZA3, -4, -6 et -9, respectivement, a ainsi été démontrée chimiquement (McCarron et al., 2009) et confirmée pour AZA17 biologiquement (O'Driscoll et al., 2011), tandis que la formation d'AZA17 a été observée lors de l'immersion de moules dans de l'eau de mer contenant de l'AZA1 dissous (O'Driscoll et al., 2011). Cependant, cet axe d'étude reste encore à approfondir, pour comprendre les mécanismes de formation d'un si grand nombre d'analogues, dans les matrices de mollusques bivalves.

1.2.4. Toxicologie et santé humaine

1.2.4.1. Mécanismes d'action

En raison des similitudes entre les symptômes provoqués par les AZA, l'AO et la DTX, les AZA ont été classés dans les toxines diarrhéiques. Par conséquent, l'hypothèse a été faite que le mode d'action des AZA était équivalent à celui de l'AO, c'est-à-dire qu'ils étaient des inhibiteurs de protéines phosphatases. Cependant, d'après les études conduites par plusieurs auteurs, contrairement à l'AO, les AZA ne sont pas être des inhibiteurs des protéines phosphatases PP1 et PP2A (Flanagan et al., 2001; Roman et al., 2002; Twiner et al., 2005). Mais, on ne peut pas exclure la possibilité que les AZA puissent inhiber un ou plusieurs autres types de protéines phosphatases (ex : PP2B, PP2C, PP4, PP5) ou de sous-type de protéines phosphatases (ex : phosphatase spécifique à la tyrosine, phosphatase lipidique) (Twiner et al., 2008).

1.2.4.2. Toxicité aigüe chez la souris

Chez la souris, les symptômes observés, après exposition aux AZA, sont similaires à ceux observés chez des souris exposées à l'acide okadaïque (AO).

Des études menées sur des souris soumises à une administration orale d'AZA (administration unique de 300 à 700 µgAZA kg^{-1} de souris), sur une période de 24 h, ont montré une réaction dose-dépendante e au niveau de l'intestin grêle (nécrose et atrophie des villosités de la *lamina propria*), et des diarrhées et une perte de poids chez les souris exposées

aux deux plus fortes doses (600 et 700 µgAZA kg^{-1} de souris). Une formation de vacuoles et l'accumulation d'acides gras dans les hépatocytes, ainsi qu'une pycnose des cellules du parenchyme du pancréas, la présence de débris de lymphocytes morts dans le thymus et la rate, un changement de couleur du foie, des nécroses et des saignements dans l'estomac ont aussi été observés. Les pathologies aigües induites par les AZA sont donc atypiques, par comparaison à celles provoquées par les toxines diarrhéiques, paralysantes et amnésiantes (Ito et al., 2000; Ito et al., 1998).

Lorsque les AZA sont injectés par voie IP, et à des doses plus élevées, les extraits d'AZA provoquent, dans les heures qui suivent, une lente et progressive paralysie, des difficultés respiratoires et un comportement léthargique. En effet, les souris s'asseyent de manière apathique dans les coins de leur cage et meurent en moins d'une heure et demie. Les effets sur les organes internes sont plus caractéristiques : une injection par voie IP d'au moins 150 µg d'AZA1 kg^{-1} est jugée létale et l'autopsie révèle un grossissement significatif du foie et une teinte jaunâtre caractéristique ; de plus le tissu apparaît comme fragile (Ito et al., 1998).

En ce qui concerne l'AZA2 et l'AZA3, des injections chez la souris, par voie IP, d'au moins 110 et 140 µg kg^{-1} respectivement, sont considérées comme létales (Ofuji et al., 1999a), ce qui suggère une plus grande toxicité par rapport à AZA1. Toutefois, l'AZA4 et l'AZA5 sont à priori moins toxiques, avec des doses létales respectives, en IP, chez la souris de 470 et <1000 µg kg^{-1} (Ofuji et al., 2001). En supposant une pureté équivalente, le classement, en termes de toxicité par voie IP chez la souris, des cinq premiers AZA, serait : AZA2 > AZA3 > AZA 1> AZA4 > AZA5. Cependant, ces résultats doivent encore être corroborés et reproduits. A cause du manque de toxines purifiées et du déficit d'études toxicologiques, différentes études ont été réalisées pour affiner la dose létale par voie IP (Hess et al., 2009) et par voie orale (Aasen et al., 2010).

1.2.4.3. Toxicité à moyen terme et toxicité chronique chez la souris

L'administration *per os* d'AZA, à faible dose (administration répétée, deux fois par semaine, de 1 à 50 µgAZA kg^{-1} de souris, pendant 20 semaines) a entrainé le sacrifice, après 15 semaines de traitement, suite à une inactivité, une perte de poids et une faiblesse des souris, de 9 souris sur 10 et de 2 souris sur 10, lorsqu'elles sont soumises à une administration de 50 et 20 µgAZA kg^{-1} respectivement (des effets ont été observés chez ces souris au niveau des villosités de l'intestin grêle, de l'estomac et des poumons). Cependant, l'administration répétée de 1 à 5 µgAZA kg^{-1} n'a pas entrainé de mortalité et de diarrhée. Après trois mois de récupération les souris ont été sacrifiées. Certaines ont présenté des tumeurs au niveau des

poumons (1/10 à 50 et 2/10 à 20 µgAZA kg^{-1}), une hyperplasie des cellules épithéliales de l'estomac (6/10 à 20 µgAZA kg^{-1}), et celles exposées aux plus faibles doses ont également présenté des pathologies (érosion des parois de l'intestin grêle, nodules et mitoses dans le foie) (Ito et al., 2002).

Au niveau chronique, les souris ont été exposées à différentes doses d'AZA, de manière répétée, pendant 20 semaines, et ont été sacrifiées après 8 et 12 mois d'observation. Sur les 71 souris exposées seules cinq ont présenté des tumeurs malignes (Ito, données non publiées dans (Ryan et al., 2011)). Il est donc difficile de démontrer l'effet carcinogène des AZA mais celui-ci ne peut pas être éludé, ce qui requiert donc de nouvelles évaluations.

1.2.4.4. Toxicité chez l'homme

Chez l'homme, les AZA induisent des symptômes qui ressemblent à ceux provoqués par les toxines diarrhéiques. La consommation de mollusques contaminés peut ainsi entraîner des nausées, des vomissements, des diarrhées, et des crampes d'estomac.

Basée sur les résultats obtenus par Satake et al. (Satake et al., 1998b; Satake et al., 1998c), la toxicité des moules a été estimée à 0,15 $MU.g^{-1}$ (MU= Mouse Unit, équivalent à 0,6 µg $AZA.g^{-1}$ de chair). Cependant une teneur supérieure a été rapportée par (Ofuji et al., 1999b) c'est à dire, 1,4 µg AZA g^{-1} de chair (0,4 MU g^{-1} de chair).

La dose minimale avec effets nocifs observés (DMENO) pour l'homme a donc été évaluée dans un premier temps entre 6,7 (effet sur 5% des personnes exposées) et 24,8 µg par personne (effet sur 95% des personnes exposées) avec une valeur moyenne à 15 µg par personne (EU/DG SANCO, 2001). Cependant, de nouvelles données sur la stabilité des AZA à la chaleur suggèrent qu'il n'est pas approprié de prendre en compte une réduction de la concentration des AZA lors de la cuisson. Par conséquent, compte tenu des travaux réalisés par Yasumoto, la DMENO a été réévaluée et est dorénavant de 23 à 86 µg/personne avec une valeur moyenne de 51,7 µg/personne (EU/DG SANCO, 2001).

1.2.5. Analyse des AZA

1.2.5.1. Le test souris

Le test souris (Yasumoto et al., 1984) a été la première méthode utilisée pour détecter les AZA dans les mollusques bivalves (McMahon and Silke, 1996). Mais, cette méthode est non sélective et manque de précision (Hess et al., 2006).

Une récente évaluation du test souris, pour détecter les AZA dans les moules à des niveaux inférieurs à la limite réglementaire, a été réalisée (Hess et al., 2009). Des glandes digestives de moules contaminées ont été diluées de façon appropriée avec des glandes digestives non contaminées pour obtenir des concentrations adéquates et vérifiées par CL-SM/SM. Sept répliquas indépendants, sur six semaines d'étude, ont été réalisés pour évaluer les différentes concentrations testées sur souris (7 concentrations en AZA1-équivalent allant d'une concentration inférieure à la limite de quantification du test à 2,24 $mg.kg^{-1}$ de glandes digestives, ce qui correspond à 0,34 $mg.kg^{-1}$ de chair totale). Cette étude a permis de démontrer statistiquement que le test souris est fiable pour détecter une concentration en AZA équivalente au seuil réglementaire, à condition que ce soit la glande digestive qui soit testée et non la chair totale du mollusque. Mais, ce test est qualitatif et non quantitatif, et pour une teneur en AZA égale à la moitié du seuil réglementaire actuel, la probabilité d'obtenir un résultat positif n'est plus que de 5% (Hess et al., 2009).

1.2.5.2. La CL-SM/SM

La CL-SM/SM demeure donc le meilleur moyen pour déterminer quantitativement la concentration en AZA dans des matrices de mollusques (Draisci et al., 2000; Quilliam, 2001; Quilliam et al., 2001).

Dans un premier temps, (Ofuji et al., 1999b) ont préconisé une étape de purification de la matrice, par extraction en phase solide (EPS), avant la détermination par CL-SM d'AZA1 à -3. Cette étape de « nettoyage » de l'extrait a par la suite été étudiée en détail (Moroney et al., 2002) afin de vérifier la nécessité de réaliser cette opération. Plusieurs méthodes de CL-SM/SM pour l'analyse des AZA dans les matrices de bivalves ont été étudiées en parallèle, en utilisant des spectromètres de masse de type triple quadripôle (Draisci et al., 2000; Volmer et al., 2002) et des trappe d'ions (Furey et al., 2002; James et al., 2003b; Lehane et al., 2002).

D'un point de vue chromatographique, il est possible de séparer les différents analogues d'AZA sur colonne C18 (James et al., 2003b; Lehane et al., 2004). Toutefois, la résolution chromatographique n'est pas forcément nécessaire en $CL\text{-}SM^n$, si des différences entre les fragments des molécules sont bien connues. Ainsi, l'identification et le dosage des AZA1 à AZA10 ont pu être mis au point, sans séparation chromatographique complète de ces composés (Gerssen et al., 2009a; Lehane et al., 2002; Lehane et al., 2004)).

Plus récemment (Fux et al., 2007) ont mis au point une méthode CLUHP (chromatographie liquide ultra haute-performance)-SM/SM pour déterminer, en moins de 7 minutes d'analyse, 21 toxines marines lipophiles, dont AZA1et (Rehmann et al., 2008) ont

développé des méthodes basées sur la CLUHP, combinée à un Q-TOF MS/MS ou à un triple quadripôle couplé à une trappe d'ions, pour déterminer des nouveaux analogues d'AZA.

Ces méthodes d'analyse sont néanmoins sensibles aux effets de matrice, qui diminuent ou augmentent l'intensité du signal des toxines lipophiles présentes dans les mollusques, lors de leur analyse par CL-SM (Stobo et al., 2005). Pour AZA1, une suppression du signal de 44% a tout d'abord été observée (Stobo et al., 2005) puis une suppression de 23% dans un second temps (Fux et al., 2007), conduisant à des sous-estimations de la concentration en AZA dans les mollusques contaminés. Ces effets de matrice ont ensuite été étudiés, en utilisant différentes méthodes de dopage de la matrice de mollusques, pour l'analyse de différentes toxines lipophiles dont AZA1 (Fux et al., 2008b; Kilcoyne and Fux, 2010). L'AZA1 présente en général une réduction de son signal de 20-22% (Fux et al., 2008b) ; cependant, cette suppression varie aussi en fonction de l'espèce de mollusque analysée, de la composition de la phase mobile (acide ou basique) et du spectromètre de masse utilisé (Kilcoyne and Fux, 2010). Mais des solutions sont possibles pour diminuer l'effet matrice comme l'utilisation d'une colonne C8, de la CLUHP et/ou une dilution de l'échantillon, qui permettent de rendre négligeable l'impact de l'effet de matrice (-1 à -2%) lors de l'analyse (Fux et al., 2008b), ou l'utilisation de phases mobiles alcalines ou acides, en fonction de la matrice analysée, ou enfin la purification préalable de l'échantillon par EPS (Gerssen et al., 2009a; Gerssen et al., 2009b; Kilcoyne and Fux, 2010).

1.2.5.3. Le test ELISA

Le test ELISA (enzyme-linked immunosorbent assay, littéralement : dosage d'immunoadsorption par enzyme liée) pour les AZA a fait l'objet d'une première étude (Forsyth et al., 2006) et est actuellement en cours de développement pour la mise au point d'une méthode d'analyse rapide et quantitative. Les principaux avantages de ce type de méthode sont la rapidité, la spécificité pour le groupe de toxines, et le périmètre large des analogues qui peuvent être détectés. De ce fait, ce type de méthode peut être utilisé pour la détection d'analogues non encore décrits. Malheureusement, il n'y a pas encore d'informations sur la performance de cette méthode, concernant la détection des AZA dans des matrices de coquillage.

1.3. Ecophysiologie des dinoflagellés

Les dinoflagellés sont principalement des espèces marines ou estuariennes et seulement 9% vivent en eau douce (Carty dans (Burkholder et al., 2006)). Parmi les 2300 espèces référencées (Gómez, 2012) très peu (environ 185) sont actuellement considérées comme nocives (Smayda and Reynolds, 2003) et seulement une soixantaine produisent des phycotoxines (Burkholder, 1998; Burkholder et al., 2006).

Leur croissance et leur teneur en toxines varient en fonction des facteurs environnementaux et nutritionnels et les réponses physiologiques à ces facteurs sont généralement intraspécifiques (Burkholder et al., 2001).

De nombreuses microalgues toxiques dépendent uniquement de la photosynthèse pour leur apport en carbone et de nutriments inorganiques pour leur nutrition. Ce sont les espèces autotrophes. Néanmoins, tous les dinoflagellés ne sont pas uniquement autotrophes. Certains sont mixotrophes, c'est à dire qu'ils sont capables d'utiliser pour leur nutrition des substances organiques dissoutes (osmotrophie) ou particulaires (phagotrophie) (Granéli et al., 1999). Il existe aussi des espèces qui sont hétérotrophes. Celles-ci sont donc capables d'utiliser des substances organiques particulaires ou des proies afin d'en assimiler le carbone et sont alors dépourvues d'appareil photosynthétique.

1.3.1. Les facteurs environnementaux

A. spinosum est un dinoflagellé marin. On centrera donc cette étude bibliographique sur les espèces de dinoflagellés marins, en particulier pour l'étude des facteurs de croissance, mais aussi sur les espèces produisant des toxines contenant une ou plusieurs molécules d'azote, comme les AZA. En milieu marin, la salinité, la température, l'intensité lumineuse et la turbulence sont des facteurs physicochimiques importants pour la régulation de la croissance et du métabolisme (activité enzymatique et production de métabolites secondaires, comme les phycotoxines).

1.3.1.1. Salinité

Des salinités défavorables aux microalgues vont agir sur l'activité enzymatique en modifiant le pH, et sur la consommation énergétique allouée à la régulation osmotique (Grzebyk and Sechet, 2003). La salinité va donc avoir un effet sur le métabolisme cellulaire et sur la croissance. Les espèces d'eau douce montrent une faible tolérance aux variations de salinité, alors que, les espèces estuariennes euryhalines et les espèces côtières et marines ont

une tolérance plus large aux variations de salinité en culture (Grzebyk et al., 2003; Laabir et al., 2011; Matsubara et al., 2007; Nagasoe et al., 2006; Taylor and Pollingher, 1987). En revanche, certaines espèces marines sont sténohalines et peuvent donc être sensibles aux variations de salinité (Kim et al., 2004).

Quelques études ont été réalisées chez différents dinoflagellés toxiques et majoritairement chez le genre *Alexandrium*. Mais, les conditions de cultures ne sont pas toujours comparables ; ainsi une concentration maximale en toxine cellulaire a été observée soit à une salinité plus élevée que la condition de croissance optimale (White, 1978), soit à la salinité la plus adéquate pour la croissance (Parkhill and Cembella, 1999). Chez une autre espèce de dinoflagellé, *Gymnodinium catenatum*, une baisse de salinité n'a pas engendré d'augmentation significative de la production toxinique (Flynn et al., 1996), en revanche, chez *Alexandrium minutum* une faible salinité peut permettre d'accumuler une plus grande quantité de toxine (Grzebyk et al., 2003). Des études plus récentes arrivent aussi à des conclusions similaires et mettent en évidence l'effet intraspécifique de la salinité (ex : *Protoceratium reticulatum* (Guerrini et al., 2007), *Alexandrium ostenfeldii* (Maclean et al., 2003)).

1.3.1.2. Température

Tout comme la salinité, la température va influencer sur l'activité enzymatique des microalgues. En effet, les enzymes ont une température optimale de fonctionnement en deçà de laquelle les vitesses de réactions sont plus lentes et au-delà de laquelle les enzymes se dégradent plus rapidement (Grzebyk and Sechet, 2003). La température est donc un des paramètres ayant un effet significatif sur la croissance jusqu'à un certain optimum (Laabir et al., 2011; Matsubara et al., 2007; Nielsen, 1996; Xu et al., 2010). En revanche, chez plusieurs espèces de dinoflagellés toxiques, une baisse de la température correspond en général à une baisse du taux de croissance et à une augmentation significative de la concentration en toxine (Anderson et al., 1990; Guerrini et al., 2007; Navarro et al., 2006; Ogata et al., 1987) ; néanmoins, une température supérieure à l'optimum de croissance peut aussi conduire à une augmentation de la concentration toxinique chez certaines espèces (*Protoceratium reticulatum* (Guerrini et al., 2007), *Prorocentrum hoffmannianum* (Morton et al., 1994)). Cette dernière remarque suggère une augmentation de la concentration toxinique comme une réponse possible à un stress environnemental.

En revanche, chez *Dinophysis acuminata*, un dinoflagellé mixotrophe, la concentration totale en toxine augmente en même temps que le taux de croissance, la concentration

cellulaire et la température (Kamiyama et al., 2010). La même observation a aussi été faite chez *Ostreopsis ovata* (Vidyarathna and Graneli, 2011).

1.3.1.3. Intensité lumineuse

Les espèces de dinoflagellés photosynthétiques peuvent s'adapter rapidement à des variations d'intensité lumineuse. Par exemple, ils peuvent, à faible intensité lumineuse, augmenter la taille et/ou le nombre d'unités photosynthétiques (Smayda, 1997). Ce facteur est celui qui affecte le plus la nutrition des microalgues à travers la photosynthèse, et un éclairage insuffisant provoque une réduction sinon un arrêt de la croissance (Grzebyk and Sechet, 2003; Paz et al., 2006). Toutefois, une réduction de l'intensité lumineuse (dans une certaine mesure) peut, en réduisant le taux de croissance, augmenter la concentration en toxine (Ogata et al., 1987), mais l'inverse a aussi été observée (Lim et al., 2006) et les auteurs semblent s'accorder sur le fait qu'une trop faible intensité lumineuse, en plus de réduire considérablement la croissance, réduit aussi le quota en toxine et que la photosynthèse semble nécessaire chez les dinoflagellés pour la production de phycotoxines (Ogata et al., 1987) (rq : la qualité de lumière peut aussi jouer sur la composition biochimique des microalgues (Marchetti et al., 2012a) dont les dinoflagellés (Carreto et al., 1990)).

1.3.1.4. Turbulence

Chez plusieurs espèces de dinoflagellés, la turbulence générée expérimentalement provoque des effets délétères, notamment une diminution de la division cellulaire (Berdalet, 1992; Pollingher and Zemel, 1981; Sullivan et al., 2003), des changements morphologiques (Zirbel et al., 2000), une modification du comportement natatoire (Berdalet et al., 2007; Karp-Boss et al., 2000) ou des ruptures de la membrane cellulaire (White, 1976).

Dans le cas d'*Alexandrium minutum*, les turbulences entrainent une diminution du taux de croissance, de biomasse, ainsi que des concentrations inférieures en toxine paralysante et une production de cellules ecdysées (Bolli et al., 2007). Cependant, tous les auteurs ne sont pas d'accord sur ces effets et certains considèrent que ces tests ont été réalisés dans des conditions trop extrêmes (Burkholder et al., 2006). En utilisant des turbulence comparables aux conditions naturelles, un large éventail d'espèces de dinoflagellés toxiques ont eu un taux de croissance et une concentration cellulaire qui ont été stimulés (*Lingulodinium polyedrum*, *Gymnodinium catenatum, Alexandrium fundyense*), pas affectés (*Pyrocystis noctiluca*, *Ceratium tripos*, *Alexandrium tamarense, Pyrocystis fusiformis*, *Alexandrium catenella*, *Gyrodinium sp.*), ou affectés (*Ceratium fusus*) par une forte turbulence (simulant les

conditions d'un de coup de vent modéré) (Sullivan and Swift, 2003). L'effet des turbulences semble donc être aussi un facteur intraspécifique et variable en fonction de l'intensité de celles-ci.

1.3.2. Les facteurs nutritionnels

1.3.2.1. Les macronutriments

La teneur en toxines des microalgues toxiques peut varier en fonction de la concentration en nutriments tels que l'azote et le phosphore (Anderson et al., 1990; Flynn et al., 1994). Cette teneur en toxines intracellulaire peut augmenter lorsque que la concentration en nutriments dans le milieu est en déséquilibre. Des hypothèses ont été élaborées pour expliquer les raisons de ces augmentations : les toxines contenant un ou plusieurs atomes d'azote dans leur formule seraient une forme de stockage des nutriments, ou lorsqu'il y a une carence en nutriment, un transfert des molécules d'azote de la chlorophylle vers les molécules de toxine se produirait, ce qui causerait une diminution de la croissance cellulaire et une augmentation du quota en toxines des cellules.

Depuis quelques années les rejets anthropiques déséquilibrent les concentrations en azote (N) et en phosphore (P) organiques et inorganiques des zones côtières (Glibert et al., 2006; Heisler et al., 2008), il s'agit de milieu pour lequel le rapport Redfield (C/N/P =106:16:1) n'est plus respecté. Ainsi, il est possible que, même si le nombre d'efflorescences toxiques n'a pas augmenté, les effets délétères de ces efflorescences aient augmenté leur impact à cause d'une augmentation de la teneur en toxines dans les microalgues.

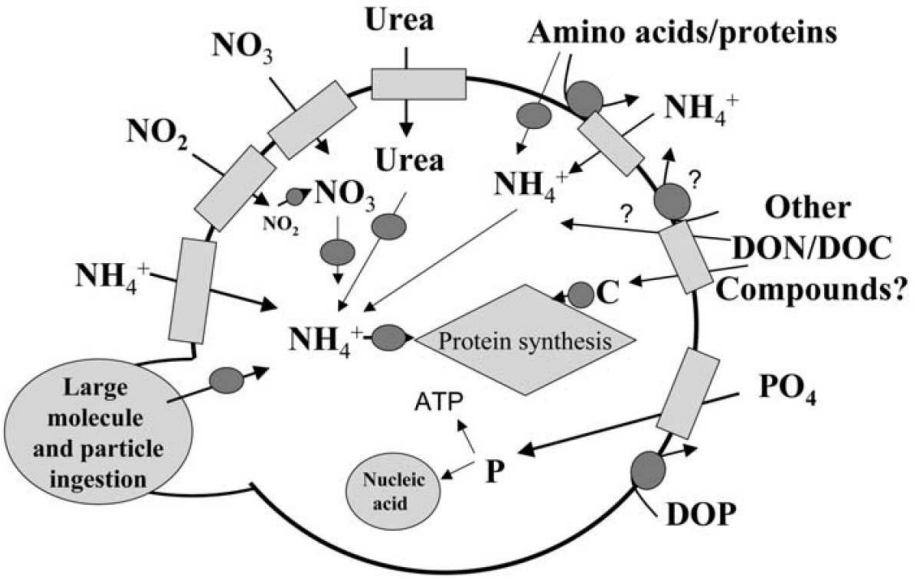


Figure 15. Schéma des différentes voies par lesquelles les microalgues toxiques peuvent acquérir leurs éléments nutritifs. Toutes les espèces de phytoplancton peuvent transporter des molécules de nitrate, nitrite, ammonium, urée et de phosphate à travers leur membrane cellulaire par diffusion passive ou transport actif. Certaines espèces peuvent transporter de grosses molécules organiques à travers leur membrane cellulaire, et certaines possèdent des enzymes à la surface de la membrane cellulaire pour décomposer les molécules organiques avant de transporter les nutriments à travers la membrane. Certaines espèces ont la capacité de phagocyter d'autres cellules, des particules et/ou des molécules de grande taille. Bien que de nombreuses voies soient indiquées, toutes les microalgues toxiques n'ont pas la capacité d'assimiler toutes ces sources de nutriments. Les voies qui impliquent des réactions enzymatiques sont indiquées par un cercle. Les voies pour lesquelles il y a beaucoup d'incertitudes sont indiquées par un point d'interrogation (Glibert and Legrand, 2006).

Des études se sont intéressées à la croissance cellulaire et à la production de toxines paralysantes de certaines espèces d'*Alexandrium*. Ces études ont montré que : (1) au cours de leur croissance en culture « batch » le quota en toxines paralysantes est maximal pendant la phase exponentielle de croissance (Boyer et al., 1987; Kim et al., 1993; White, 1978), (2) dans des conditions de limitation en azote, la production de toxines et la croissance sont également limitées (Siu et al., 1997; Wang and Hsieh, 2002) et (3) la production de toxines paralysantes semble être stimulée dans des conditions de limitation en phosphore, mais, ces conditions limitent la croissance (Anderson et al., 1990; Boyer et al., 1987; Frangopulos et al., 2004; Guisande et al., 2002; Siu et al., 1997).

D'autres études se sont concentrées sur les toxines de type polyéther produites par diverses espèces de dinoflagellés (*Prorocentrum spp.*, *Ostreopsis spp.*, *Gambierdiscus spp.*). Celles-ci montrent que : (1) au cours de leur croissance en culture en « batch » le quota en toxines croit pendant la phase exponentielle, comme pour les toxines paralysantes, mais,

celui-ci atteint sa concentration maximale généralement en fin de phase de croissance ou pendant la phase stationnaire (Bomber and Tindall, 1988; Guerrini et al., 2010; Pan et al., 1999) ; (2) dans des conditions de culture en P limitant, la production de toxines semble être augmentée lorsque le phosphore a été épuisé dans le milieu de culture au cours de la croissance (McLachlan et al., 1994; Morlaix and Lassus, 1992; Vanucci et al., 2010) ; (3) cependant, en condition N limitant, la production de toxines semble aussi pouvoir être augmentée (Vanucci et al., 2010), mais, dans les deux cas une limitation en N ou P diminue la croissance, voire même, chez *Ostreopsis* cf. *ovata,* la production de toxines (Vanucci et al., 2012).

1.3.2.2. Les micronutriments

Peu de données existent sur l'effet des micronutriments (vitamines et métaux traces) sur la croissance et la production toxinique des différentes espèces de dinoflagellés. Si l'azote et le phosphore ne sont pas limitants ces éléments (vitamines et métaux traces) finiront par le devenir. En effet, ils sont impliqués dans le métabolisme primaire des microalgues, comme cofacteurs d'enzymes ou de métalloprotéines participant à la photosynthèse, et/ou à l'assimilation de l'azote (Grzebyk and Sechet, 2003).

Chez *Protoceratium reticulatum*, le sélénium et le fer sont nécessaires à la croissance. Sans addition de sélénium le taux de croissance est réduit et des changements morphologiques sont observés. En revanche, la production de YTX augmente de façon significative lors de l'addition de sélénium. Sans addition de fer le taux de croissance est également réduit, mais par contre, aucun effet significatif n'est observé sur la production de YTX (Mitrovic et al., 2004), alors qu'une augmentation significative des toxines paralysantes a été observée chez *A. tamarense* lorsque la teneur en fer est limitante (He et al., 2010). En revanche, le cobalt ne semble pas avoir d'effet sur la croissance et la production de toxines chez *Protoceratium reticulatum* (Mitrovic et al., 2004) alors que le sélénium est aussi nécessaire chez *A. minutum* et *Gymnodinium catenatum* (Doblin et al., 1999).

1.4. Le genre *Azadinium*

1.4.1. *Azadinium spinosum*

A. spinosum (Figure 16) est un petit dinoflagellé (12-16 µm de longueur et 7-11 µm de largeur) producteur d'AZA1 et -2 *in situ* mais aussi en culture axénique (Krock et al., 2009;

Tillmann et al., 2009). L'organisme a été décrit pour la première fois par (Tillmann et al., 2009) et a donné naissance à un nouveau genre (*Azadinium*) situé entre les ordres des péridiniales et les gymnodiniales (Figure 49).

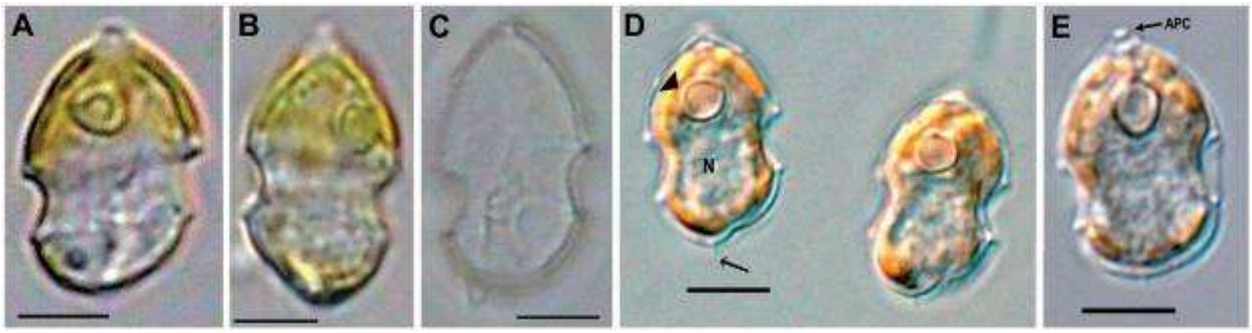


Figure 16. *Azadinium spinosum*. Vue en microscopie optique des cellules vivantes (A, B); d'une thèque vide (C) et de cellules fixées au formaline (D, E). Abréviations: APC: complexe du pore apical. N: noyau. La flèche en D : l'épine antapicale. Le triangle en D : le pyrénoïde. Barres d'échelle: 5 µm (Tillmann et al., 2009).

A. spinosum est une cellule biflagellée avec un chloroplaste et une thèque fine (Figure 16 et Figure 17). La première syllabe du genre *Azadinium* se réfère à la fonction amine de l'azaspiracide produite par *A. spinosum* et *spinosum* se réfère à l'épine antapicale (antapical spine) presque toujours présente chez cette espèce.

Les cellules d'*A. spinosum* sont de forme elliptique, légèrement allongées et comprimées dorso-ventralement. L'épisome est plus grand que l'hyposome et possède une forme conique avec des faces convexes qui se terminent par un pore apical caractéristique. Le cingulum est profond et large ; il représente environ un quart de la longueur totale des cellules. L'hyposome est quant à lui légèrement asymétrique, avec le côté droit plus convexe que le gauche (Figure 17).

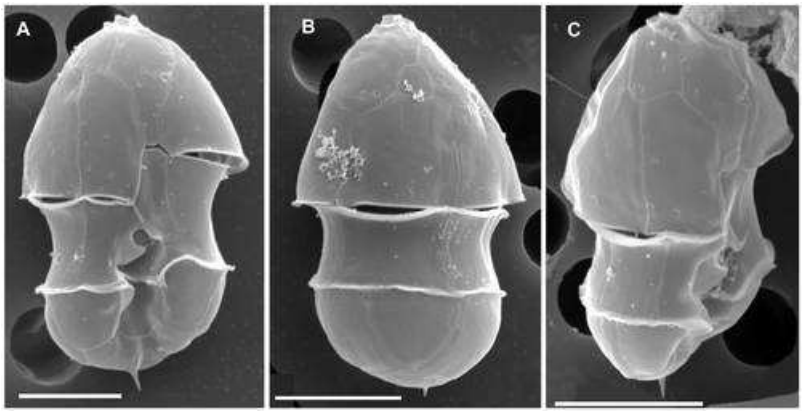


Figure 17. *Azadinium spinosum*. Micrographies en microscopie électronique à balayage de différentes thèques de cellules en vue ventrale (A), dorsale (B) et latérale droite (C). Barres d'échelle: 5 µm (Tillmann et al., 2009).

La souche 3D9 d'*A. spinosum* a été isolée au cours d'une campagne, en 2007, en mer du Nord au large de l'Ecosse, et une seconde souche a également été isolée au cours de cette campagne de prélèvement au large du Danemark (UTHE2) (Krock et al., 2008).

Depuis la description morphologique de cette nouvelle espèce, des souches de cet organisme ont été signalées au Mexique (Hernandez-Becerril et al., 2010), en Argentine (Akselman and Negri, 2012), en Italie et en France (communication personnelle, Siano et Nezan, Ifremer, France) et en Irlande (Salas et al., 2011). C'est seulement dans ce dernier pays qu'une troisième souche productrice d'AZA1 et -2 a été isolée (SM2).

A. spinosum en culture produit plusieurs analogues d'AZA (Figure 18), AZA1, AZA2 et un isomère d'AZA2 (AZAX). Le quota cellulaire en AZA est très variable, et la concentration en AZA1 a été estimée entre 5 et 40 fg.cellule^{-1} (Tillmann et al., 2009). Cette dernière est très variable en fonction des facteurs environnementaux.

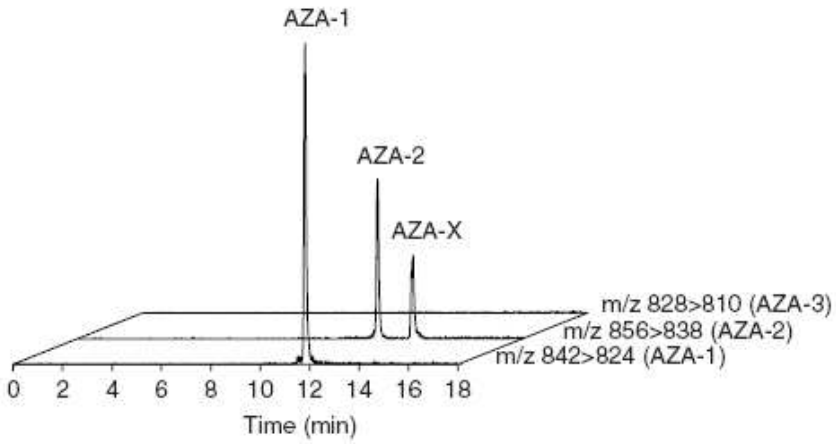


Figure 18. CL-SM/SM chromatogramme d'*A. spinosum* (Tillmann et al., 2009).

1.4.2. *Azadinium obesum*

A. obsesum a été isolé au cours de la même campagne de prélèvement qu'*A. spinosum*, au large des côtes écossaises. Cependant, contrairement à *A. spinosum* cet organisme n'a pas été décrit pour le moment comme producteur d'AZA.

Tout comme *A. spinosum*, *A. obesum* (Figure 19) est un petit dinoflagellé (13-18 µm de long et 10-14 µm de large) possédant une thèque fine. Mais, si la face dorso-ventrale est aussi comprimée, les cellules sont plus ovoïdes. L'épisome présente un pore apical caractéristique et est légèrement plus grand que l'hyposome. Le cingulum est profond et large ; il représente environ un cinquième de la longueur totale des cellules (Tillmann et al., 2010).

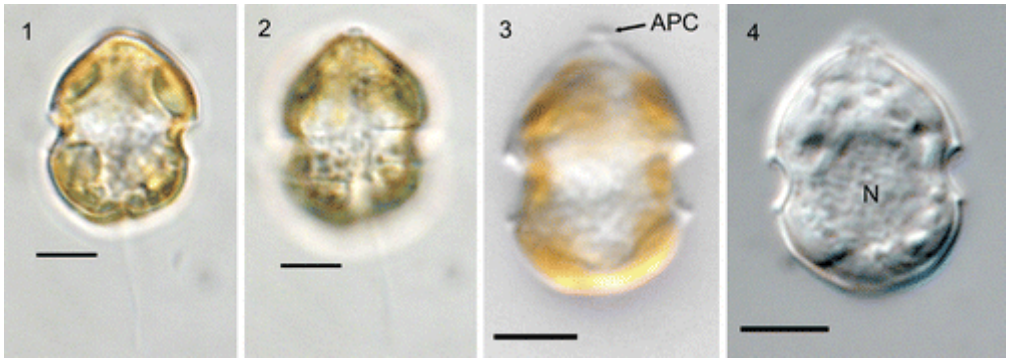


Figure 19. *Azadinium obesum*. Vue en microscopie optique des cellules vivantes (1-2) et de cellules fixées au formaline (3-4). Abréviations: APC : complexe du pore apical. N : noyau. Barres d'échelle: 5 µm (Tillmann et al., 2010).

1.4.3. *Azadinium poporum*

A. poporum (Figure 20) a été isolé au large des côtes danoises lors de la même campagne de prélèvement qu'*A. spinosum* et *A. obesum*. Trois souches identiques ont été isolées lors de cette campagne UTHC8, UTHC4 et UTHD4. *A. poporum* n'est pas producteur d'AZA1 et -2 et présente un ratio longueur/largeur différent d'*A. spinosum*. *A. poporum* est aussi un petit dinoflagellé photosynthétique possédant une fine thèque (11-16 µm de longueur et 8-12 µm de largeur). L'extrémité de l'épisome finit par un pore apical et est légèrement plus large que l'hyposome. La cellule est ovoïde et la face dorso-ventrale comprimée, le noyau est sphérique à légèrement allongé et la cellule possède un chloroplaste et plusieurs pyrénoïdes (Tillmann et al., 2011).

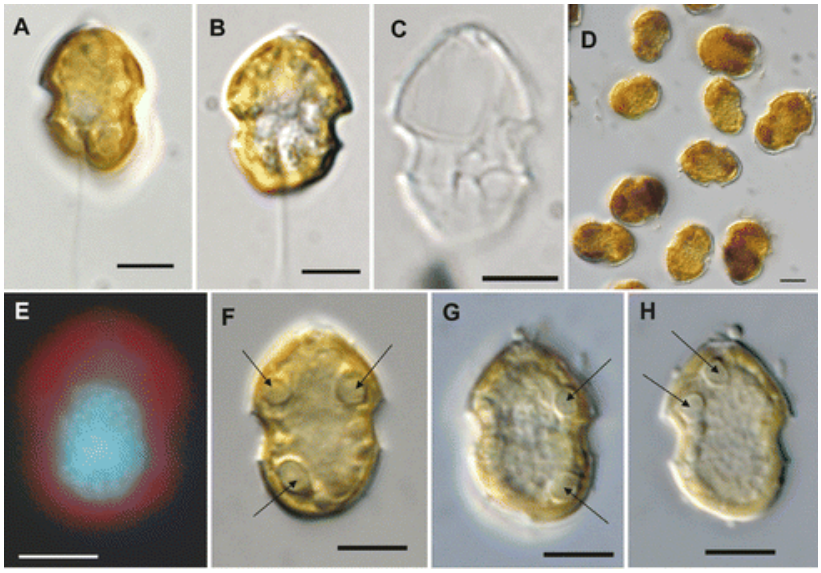


Figure 20. *Azadinium poporum*. Vue en microscopie optique des cellules vivantes (A, B); d'une thèque vide (C) ; de cellules fixées au Lugol (D) ; de cellules fixées au formaline (E-H); marquage du noyau (E); Flèches : pyrénoïdes, Barres d'échelle: 5 µm (Tillmann et al., 2011).

Une autre souche d'*Azadinium* a aussi été isolée très récemment à partir de sédiments prélevés dans le pacifique, près des côtes de la Corée du Sud (Shiwha Bay). Cette souche a été nommée *A.* cf. *poporum* en raison de ses ressemblances morphologiques avec *A. poporum* (Potvin et al., 2012).

Le récent isolement de la dinophycée, *Amphidoma languida*, a mis en évidence l'étroite relation entre la famille des amphidomataceae et le genre *Azadinium* (Tillmann et al., 2012). Les amphidomataceae n'étaient pas connus jusqu'à présent comme producteurs d'AZA ; cependant, une étude de différentes espèces (*Azadinium poporum*, *Azadinium* cf. *poporum* et *Amphidoma languida*) par CL-SM/SM a révélé la présence de quatre nouveaux AZA, mise en évidence par leur spectre de masse. Chacun d'entre eux présente un fragment caractéristique de rapport m/z de 348, à la place du fragment m/z 362, typique des AZA jusqu'à présent connus. Ces AZA ont été détectés chez *A. poporum* (masse moléculaire: 845,5 Da), *A.* cf. *poporum* (masse moléculaire: 857,5 Da) et *Amphidoma languida* isolée dans la baie de Bantry, en Irlande (masses moléculaires: 815,5 et 829,5 Da). Ces cellules présentent des quotas d'environ 2-20 fg.cellule^{-1} (Krock et al., 2012).

1.5. Production toxinique

Cette section examine les différentes options possibles et applicables aux dinoflagellés, de la production des microalgues à l'isolement de la toxine.

1.5.1. Culture de microalgues toxiques

Pour l'isolement de nouvelles toxines, pour la production de standards de toxines mais également pour d'autres types de recherche (ex: toxicologie…), de larges quantités de phycotoxines sont nécessaires. Trois approches différentes peuvent être envisagées pour produire des phycotoxines à partir des microalgues toxiques : la culture en milieu clos ou plus couramment appelée culture « batch », la culture semi-continue et la culture continue. La différence entre ces systèmes porte sur la régulation des apports en nutriments (ajoutés ou non) au cours de la culture.

1.5.1.1. Culture par lot ou culture « batch »

C'est la méthode de culture la plus fréquemment utilisée pour la production de dinoflagellés toxiques (Laycock et al., 1994; Loader et al., 2007; Miles et al., 2003; Torigoe et al., 1988). Dans un système de culture simple, une quantité donnée de milieu de culture est placée (un seul apport en nutriments) et est inoculée avec un plus faible volume de microalgues (dilution de 1/4 à $1/10^{ème}$ généralement). Une agitation du milieu peut-être ajoutée pour favoriser les échanges de gaz et de nutriments nécessaires entre la microalgue et le milieu de culture (Morton and Bomber, 1994).

Pour les organismes photosynthétiques ou mixotrophes, une arrivée d'air enrichie en CO_2 peut être ajoutée (5% v/v de CO_2 dans l'air), ainsi qu'une source lumineuse naturelle ou artificielle.

Ce procédé simple de culture permet d'utiliser un faible volume d'un lot, arrivé à maturité, pour inoculer le lot suivant.

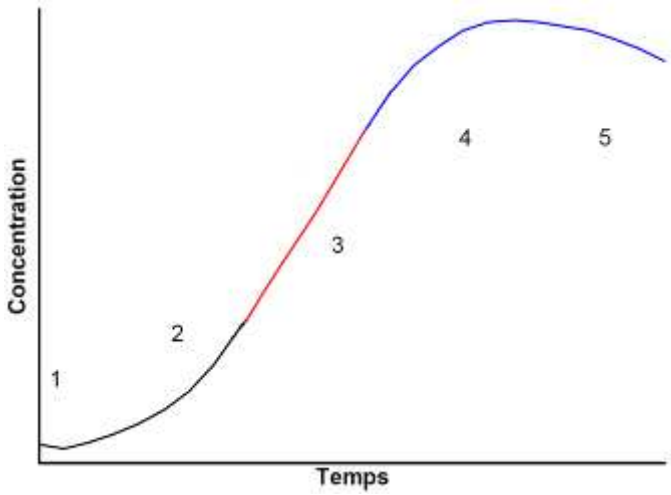


Figure 21. Cinétique de croissance des microalgues en culture par lot

Au cours de la croissance des microalgues en « batch » on distingue 5 phases différentes qui reflètent les changements en biomasse et les variations environnementales et nutritionnelles du milieu de culture.

1. **La phase de latence** : cette phase présente en général une croissance très faible voire négative qui correspond à la période d'adaptation de l'inoculum aux nouvelles conditions de culture (ex : nutriment, lumière, température…)
2. **La phase de croissance exponentielle** : les cellules se sont adaptées à leur environnement et se divisent rapidement. Comme la concentration en cellule initiale est faible les cellules se divisent de manière exponentielle à cause d'une abondance de lumière et de nutriments.
3. **La phase de croissance** : des restrictions apparaissent progressivement (lumière et/ou nutriments) et la croissance exponentielle ne peut être maintenue.
4. **La phase stationaire** : la croissance de microalgues à atteint sa concentration maximale par épuisement des nutriments et/ou une limitation en intensité lumineuse. On parle aussi de phase plateau.
5. **La phase de sénescence** : les cellules n'ont plus suffisamment de réserves en nutriments pour maintenir la phase plateau et par conséquent meurent et s'autolysent.

Avec ce type de culture il y a peu de chances d'introduction ou d'accumulation de contaminants. C'est donc la méthode couramment choisie pour maintenir les souches de microalgues.

Cependant, pour atteindre des cultures denses cette méthode pose des problèmes, surtout pour les organismes qui nécessitent des formes azotés réduites pour leur métabolisme.

En effet l'ion ammonium du milieu peut être rapidement toxique pour les microalgues (ex : *Aureoumbra lagunensis*), par conséquent il ne peut donc être ajouté en quantité suffisante pour atteindre des concentrations en microalgues suffisamment denses. Le même problème existe avec les diatomées et l'apport initial en silice (Guillard, 2003).

1.5.1.2. Culture semi-continue

La culture semi-continue (Figure 22) permet d'atteindre un point spécifique de la phase de croissance pour lequel un volume de culture est prélevé et est remplacé par du milieu de culture neuf, pour permettre une nouvelle phase de croissance, jusqu'à une prochaine récolte et ainsi de suite. On obtient ainsi une sorte d'équilibre et le renouvellement journalier du milieu de culture correspond au taux de croissance de la microalgue cultivée.

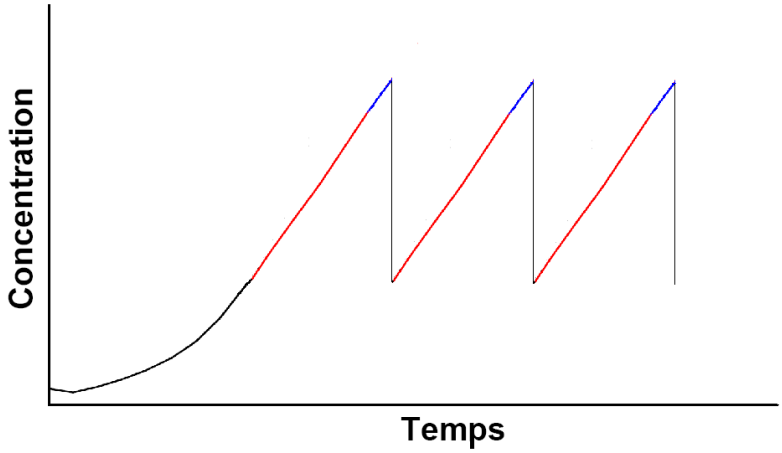


Figure 22. Cinétique de croissance des microalgues en culture semi-continue

1.5.1.3. Culture continue

La culture continue (Figure 23) est souvent réalisée en photobioréacteur et peut être adoptée en suivant deux approches différentes :

1. **Le turbidostat** : avec cette méthode de culture c'est la concentration cellulaire que l'on se fixe qui régule la quantité de milieu de culture à ajouter quotidiennement. Ce volume détermine le taux de croissance supporté par les cellules à la concentration cellulaire fixée et en fonction des paramètres nutritionnels et environnementaux appliqués.
2. **Le chémostat** : avec cette approche c'est l'apport constant d'un volume de milieu de culture qui va fixer la concentration cellulaire. Un équilibre se crée entre la composition chimique du milieu de culture et la concentration cellulaire.

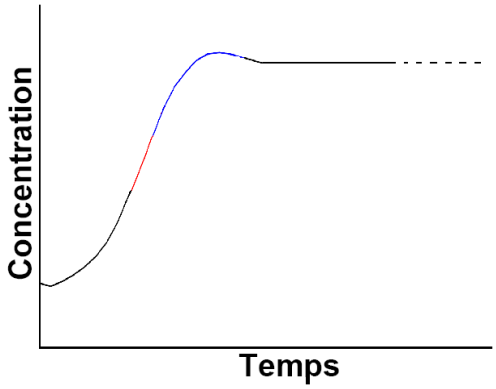


Figure 23. Cinétique de croissance des microalgues en culture continue

Ces deux approches, couplées à une régulation du pH et à un apport de lumière, permettent, en théorie, une production de microalgues indéfinie à une concentration cellulaire plus ou moins stable. Cependant, des dépôts sur les parois du réacteur et des contaminations du milieu se traduisent souvent par une diminution des performances de productivité du bioréacteur sur le long terme.

1.5.1.4. Le cas des microalgues toxiques

Actuellement, les sources principales de toxines de microalgues marines pour l'isolement, la purification et/ou la production de standards de toxine proviennent de cultures ou d'efflorescences naturelles (c'est le cas pour l'AO (Miles et al., 2006), les BTX (Abraham et al., 2006), les STX (Laycock et al., 1994), les YTX (Loader et al., 2007), les imines cycliques (Miles et al., 2003; Selwood et al., 2010; Torigoe et al., 1988) et les PTX (Miles et al., 2004b)), de mollusques contaminés (Kilcoyne et al., 2012; Perez et al., 2010), ou de l'extraction de résines absorbantes contaminées (Rundberget et al., 2007).

Cependant, l'isolement de la toxine à partir de l'organisme producteur est préférable, car les extraits sont considérablement plus purs que les extraits de coquillages et leur disponibilité ne dépend pas d'événements naturels toxiques (efflorescences ou contamination de mollusques bivalves). Mais, bien que la production en masse de microalgues en photobioréacteurs en système continu se soit développée (Grobbelaar, 2009, 2010; Ugwu et al., 2008), la production de dinoflagellés toxiques en photobioréacteur a eu moins de succès (Gallardo Rodriguez et al., 2010). En effet, ces organismes ont des taux de croissance spécifiques sensiblement inférieurs à ceux des microalgues « fourrages » et atteignent de plus faibles densités cellulaires. De plus, les concentrations de toxines dans les cultures ont tendance à être de l'ordre de quelques microgrammes par litre (Tang, 1996). En conséquence,

des volumes importants de culture (plusieurs centaines de litres) sont nécessaires pour produire des quantités minimes de toxines (Gallardo Rodriguez et al., 2010).

La culture en batch est généralement la méthode la plus utilisée pour la production de dinoflagellés toxiques (Laycock et al., 1994; Loader et al., 2007; Miles et al., 2003; Torigoe et al., 1988). Toutefois, les résultats publiés avec les systèmes 'en continu', en termes de production cellulaire et/ou de toxines par des dinoflagellés, sont encourageants : *Alexandrium minutum* ~ 20 µg de toxine L^{-1} $jour^{-1}$ (Parker et al., 2002); *Protoceratium reticulatum* 214 µg L^{-1} $jour^{-1}$ (Gallardo Rodriguez et al., 2010); *Alexandrium ostenfeldii* (concentration cellulaire plus élevée, mais inférieure en toxine par rapport à une culture « batch » (Medhioub et al., 2011)) et *Alexandrium catenella* (forte biomasse) (Séchet et al., 2003, 2004). Même si, de considérables différences existent entre les espèces de dinoflagellé en termes de croissance et de production de toxines, le développement de procédés de culture doit faire l'objet de mises au point. La culture de dinoflagellés en photobioréacteur, malgré sa bonne productivité n'est pas sans difficulté. La fragilité des cellules est un des obstacles majeurs à la production en photobioréacteur (Garcia Camacho et al., 2007a). En effet, la culture d'une importante biomasse en photobioréacteur nécessite une agitation, pour un mélange optimum des nutriments et un meilleur accès à la lumière. Cependant, cette agitation est connue pour son action potentielle sur la croissance et la morphologie des dinoflagellés (Berdalet et al., 2007; Sullivan and Swift, 2003; Sullivan et al., 2003; Tang, 1996). En effet, l'agitation et l'aération du milieu peuvent inhiber ou augmenter la croissance et la production de toxines de certaines espèces de dinoflagellés (Garcia Camacho et al., 2007b; Morton and Bomber, 1994; Pollingher and Zemel, 1981).

1.5.2. Procédures de récolte

La récole de la biomasse algale requiert une ou plusieurs étapes de séparation solide-liquide. Elle peut être récupérée par centrifugation, filtration et dans certain cas par sédimentation (en général après une phase de floculation).

1.5.2.1. La floculation

Plusieurs méthodes de floculation peuvent être utilisées (chimique, biologique ou physique Figure 24) pour agréger les microalgues entre elles et ainsi faciliter leur sédimentation (Elmaleh et al., 1991; Luz Lapa Teixeira et al., 2012; Wyatt et al., 2012). En effet les microalgues portent une charge négative qui les empêche de s'agréger. Cependant cette charge peut être neutralisée ou réduite en ajoutant un floculant dans le milieu de culture.

L'ajout de sels inorganiques est communément utilisé (chlorure de fer, sulfate d'ammonium et sulfate de fer). Mais, ce type de coagulation peut rendre inutilisable la récolte d'algues pour certaines applications (ex : aquaculture). Cependant des bio-floculations ont été développées pour éviter l'apport de sels en utilisant des floculants produits par des mocroorganismes (e.g., *Paenibacillus polymyxa* AM49) or other floculating microalga (Kim et al., 2011; Salim et al., 2011).

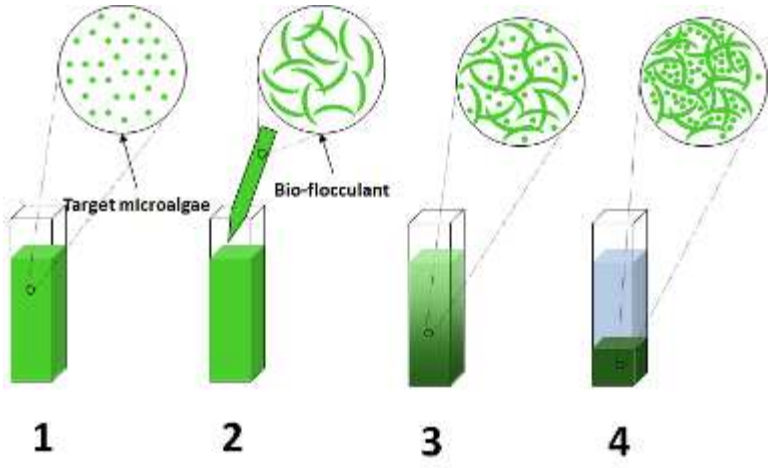


Figure 24. Vue schématique d'une floculation (Salim et al., 2011)

1.5.2.2. La centrifugation

Une majorité des microalgues peut être récoltée par centrifugation. En effet c'est une méthode rapide, mais coûteuse et consommatrice d'énergie. Elle reste tout de même la méthode de récolte préférée pour les microalgues. Plusieurs types de centrifugeuses ont été développés (ex : la centrifugeuse à bol, le séparateur à assiettes, le décanteur centrifuge et l'hydrocyclone). Les caractéristiques des cellules (espèce, taille, concentration…) (Heasman et al., 2000), le temps de séjour (contrôlé par le débit d'alimentation), et l'accélération (nombre de *g*) (Heasman et al., 2000) sont autant de paramètres à considérer lors du développement de cette méthode de récolte (Molina Grima et al., 2003).

1.5.2.3. La filtration frontale et la filtration tangentielle

La filtration frontale fonctionne sous pression ou sous vide. Elle permet d'obtenir des résultats satisfaisants pour la récupération de microalgues de taille relativement élevée (*Coelastrum proboscideum* et *Spirulina platensis*), mais n'est pas satisfaisante pour récolter des organismes de petite taille (<10 µm) (ex : *Scenedesmus*, *Dunaliella*, *Chlorella*) (Mohn dans (Molina Grima et al., 2003)).

La microfiltration et l'ultrafiltration (= filtration tangentielle) sont des alternatives possibles à la filtration frontale conventionnelle (Figure 25). Ce sont des méthodes applicables à la séparation de microorganismes fragiles (Petrusevski et al., 1995). Cependant, c'est une méthode encore peu utilisée pour traiter de grands volumes (> 2 m^3 $jour^{-1}$) mais qui reste tout de même utilisée en aquaculture (Borowitzka, 1997) ou en biotechnologie (Rossi et al., 2008; Rossi et al., 2005) pour la séparation de microalgues d'un milieu liquide.

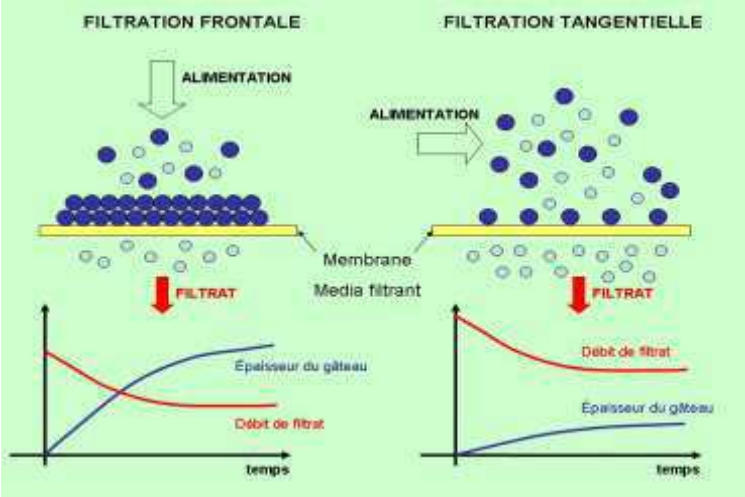


Figure 25 Filtration frontale et filtration tangentielle (source GEPEA dans (Person, 2010))

S'il faut éviter la contamination de la biomasse par de possibles agents chimiques, la centrifugation ou la filtration sont donc conseillées. La filtration reste plus économique pour traiter des petits volumes, mais pour de grands volumes (>20 m^3 $jour^{-1}$) la centrifugation est conseillée (Mackay and Salusbury, 1988). Le système à employer dépendra aussi, de l'espèce d'algue (fragilité, taille, concentration cellulaire) à récolter et de l'utilisation qui en sera faite (extraction de métabolites, nourriture pour l'aquaculture, complément alimentaire).

1.5.3. Procédures d'extraction et de purification des AZA

La dernière procédure d'isolement d'AZA décrite, à partir de mollusques bivalves, a été publiée très récemment (Kilcoyne et al., 2012). Elle présente des améliorations, réalisées en termes de rendement par rapport à la précédente méthode décrite, pour isoler les AZA1 à -3 (Perez et al., 2010) et a permis par ailleurs l'isolement d'AZA6. Cependant elle présente encore sept étapes (Figure 26).

step no.	step	mg 1	2	3	6	weight (g)
	subsampling	14.1	4.0	4.8	0.78	505.0
1	first crude extract	14.0	3.9	4.7	0.77	26.9
2	first partitioning	13.3	3.7	4.4	0.73	23.9
3	second partitioning	12.6	3.5	4.2	0.69	8.9
4	silica gel	11.9	3.3	4.0	0.65	0.6
5	LH20	10.1	2.8	3.4	0.55	0.2
6	flash (phenyl-hexyl)[a]	9.2	2.5	2.4	0.49	
7	prep HPLC (C8/C18)	7.3	1.7	2.0	0.30	
	% recovery	52	43	43	38	
	% purity	>95	>95	>95	>95	

[a]Compounds 1–3 and 6 were separated from each other in this step.

Figure 26. Procédure d'isolement d'AZA1-3 et d'AZA6 et rendements obtenus (Kilcoyne et al., 2012).

La procédure d'isolement des AZAs la plus récente est donc la suivante (Kilcoyne et al., 2012) :

1. L'isolement a été réalisé à partir de moules contaminées cuites (Bruckless, Irlande 2005) et disséquées pour obtenir 500 g de glandes digestives. Ces dernières ont été homogénéisées, lyophilisées (poids final, 130 g) et extraites à l'éthanol (5 x 500 ml).
2. Les extraits ont été réunis, évaporés, et repris en utilisant de l'acétate d'éthyle (150 ml) et une solution aqueuse contenant du NaCl (1 M, 50 ml). La fraction organique a été prélevée et évaporée.
3. Le résidu huileux obtenu a ensuite été repris dans une solution d'hexane (200 ml) et de méthanol/eau (9:1, 200 ml). La fraction méthanolique a été prélevée, évaporée et reprise dans l'acétate d'éthyle (20 mL).
4. Ensuite ~ 4 g de gel de silice (10-40 µm, de type H) ont été ajoutés à cet extrait, évaporés, broyés en une poudre fine, et chargés sur un gel de silice (55 g) versé dans une colonne (19,5 cm × 5 cm). L'élution a été réalisée successivement à l'hexane, l'acétate d'éthyle, des mélanges acétate d'éthyle / méthanol (9:1, 7:3, et 1:1) et pour finir avec du méthanol (300 mL de chacune des phases contenant, 0,1% d'acide acétique, exception faite de la première élution à l'hexane). L'analyse de la seconde fraction d'acétate d'éthyle/méthanol (7:3) a montré qu'elle contenait les AZA.
5. L'extrait obtenu a été évaporé, repris au méthanol et chargé sur une colonne de Sephadex LH-20 (150 cm × 1,5 cm, conditionnée au MeOH), éluée par gravité (~ 1 mL min^{-1}) avec du méthanol. Les 20 premiers millilitres d'éluât ont été recueillis toutes les minutes et ensuite le fractionnement a été réalisé toute les 3 min. Les fractions contenant les AZA (fractions 8-15) ont été réunies puis évaporées.

6. L'extrait a ensuite été solubilisé dans un mélange acétonitrile / eau (6:4, avec 0,1% de triéthylamine) et chargé sur une colonne remplie de particules greffés avec des groupements phényle-héxyle (19,9 cm x 2 cm) et élué avec un mélange acétonitrile / eau (3:7, avec 0,1% de triéthylamine) à 4 mL min^{-1}, en collectant des fractions de 5 mL. Les fractions contenant respectivement les AZA1, -2 et-3 ont été combinées.
7. La purification finale d'AZA1 a été réalisée par CL semi-préparative couplée à une détection à 210 nm (barrette de diodes, PDA). La colonne utilisée était une Luna C8 (Phenomenex, 250 mm × 10 mm, 5 µm), avec élution à l'aide d'un mélange acétonitrile / eau (1:1, contenant 2 mM d'acétate d'ammonium) à 4 mL min^{-1}. La température de la colonne était de 30°C. AZA2, -3 et -6 ont été purifiés en utilisant les mêmes conditions qu'AZA1, mais avec une colonne différente (Cosmosil C18, Nacalai Tesque, 250 mm x 4,6 mm, 5 µm) et un débit plus faible (1 mL min^{-1}). Les fractions contenant les AZA purifiées ont ensuite été reprises dans un mélange acétonitrile/eau (2:8) et chargées sur des cartouches d'extraction en phase solide (Oasis HLB, 200 mg), rincées avec un mélange méthanol / eau (1/9, 10 mL) pour enlever le tampon et éluées avec un mélange méthanol / eau (9 :1, 20mL).

Les extraits ainsi purifiés ont été testés pour estimer la teneur en phthalates et leur pureté a été évaluée en RMN.

1.6. Interactions phycotoxines et mollusques bivalves filtreurs

1.6.1. Mécanismes de filtration des mollusques bivalves

Les bivalves ont la capacité de contrôler la quantité et la qualité de leur régime alimentaire, en faisant varier la quantité d'eau qui passe à travers leurs branchies, et le taux de rétention des particules. En effet, les mollusques sont capables de séparer le seston comestible de celui possédant une faible valeur nutritionnelle, de réguler le volume de particules ingérées et ingérées et de faire varier leur processus de digestion et d'absorption (Gosling, 2003).

Le taux de filtration (pompage) est défini comme le volume d'eau passant à travers les branchies en fonction du temps (Hawkins et al., 1998). Cette eau est filtrée par les branchies et les particules retenues sont acheminées vers les palpes labiaux par la gouttière ventrale suite à un processus muco-ciliaire et hydrodynamique. À ce niveau une sélection pré-ingestive

peut avoir lieu : les particules non désirables sont rejetées dans les pseudofèces et les autres sont dirigées vers la bouche et ingérées (Figure 29).

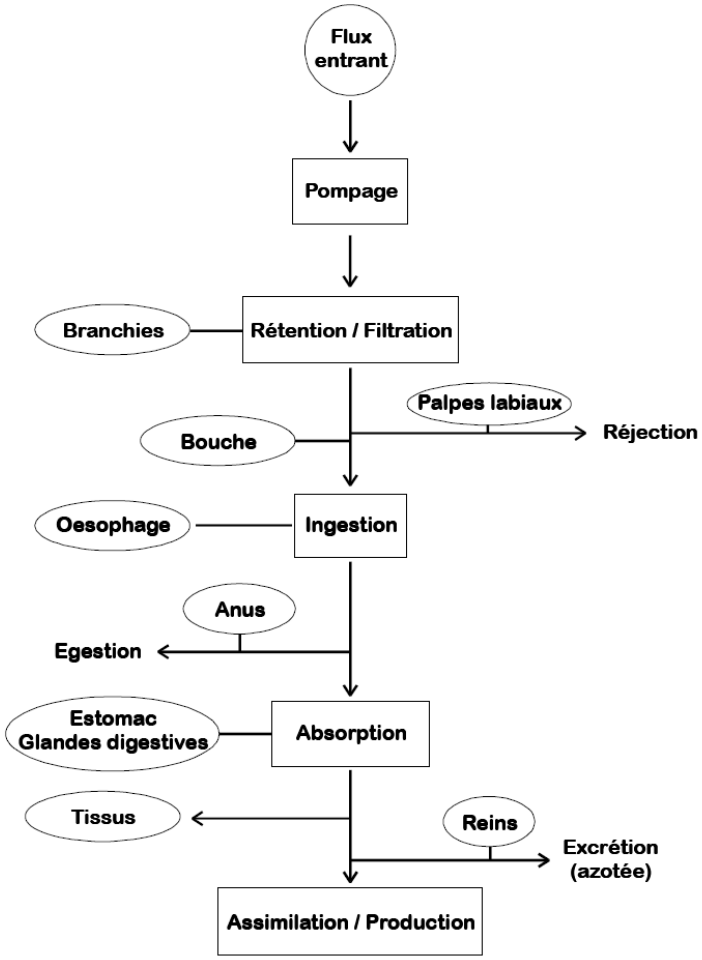


Figure 27. Schéma illustrant le devenir de particules en suspension dans l'eau filtrée par les mollusques bivalves (Lassus et al., 2002)

Le système digestif des bivalves est constitué, d'une bouche, d'un œsophage, d'un estomac, d'une glande digestive, d'un intestin et de l'anus (Figure 28). Les particules entrent par la bouche, passent par l'œsophage et arrivent dans l'estomac sous l'action combinée de l'épithélium cilié, d'acides et de muco-polysaccharides neutres (Beninger et al., 1991).

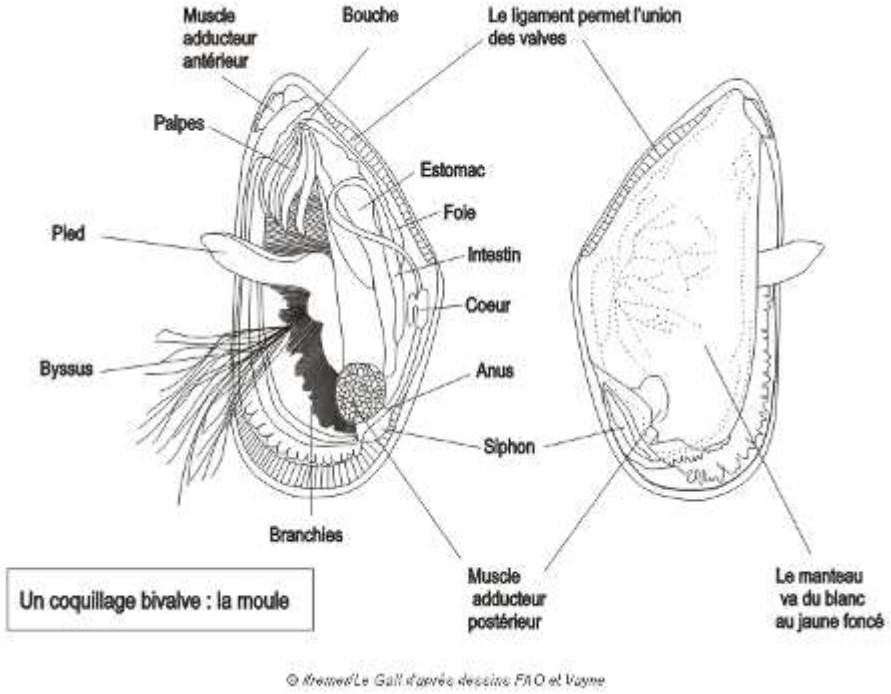


Figure 28. Exemple de mollusque bivalve, la moule qui sera utilisé comme bivalve de référence au cours de cette thèse (source Ifremer).

Les particules sont ainsi conduites dans l'estomac, où une sélection post-ingestive s'opère (Brillant and MacDonald, 2000, 2002, 2003) et où elles sont dégradées par l'action du stylet cristallin. L'estomac se présente sous la forme de deux renflements qui correspondent aux conduits d'ingestion et d'égestion. Dans l'estomac, l'action du stylet cristallin, composé de protéines, permet la réalisation de deux processus : (1) une libération d'enzymes assurant la digestion par le sac du stylet cristallin et (2) un mélange du bol alimentaire. Les enzymes présentes dans l'estomac sont : des estérases, des phosphatases, des endopeptidases, des α-amylases, des α- et β-glucosidases, des β-galactosidases, des maltases, des chitinases et des cellulases. Après ce premier processus de digestion, les particules sont dirigées par des conduits ciliaires de l'estomac vers les tubules digestifs (Figure 29) pour être digérées et absorbées, ou vers l'intestin pour être excrétées dans les fèces. Ce processus est plus ou moins long (quelques heures) en fonction des espèces de microalgues (Rouillon and Navarro, 2003).

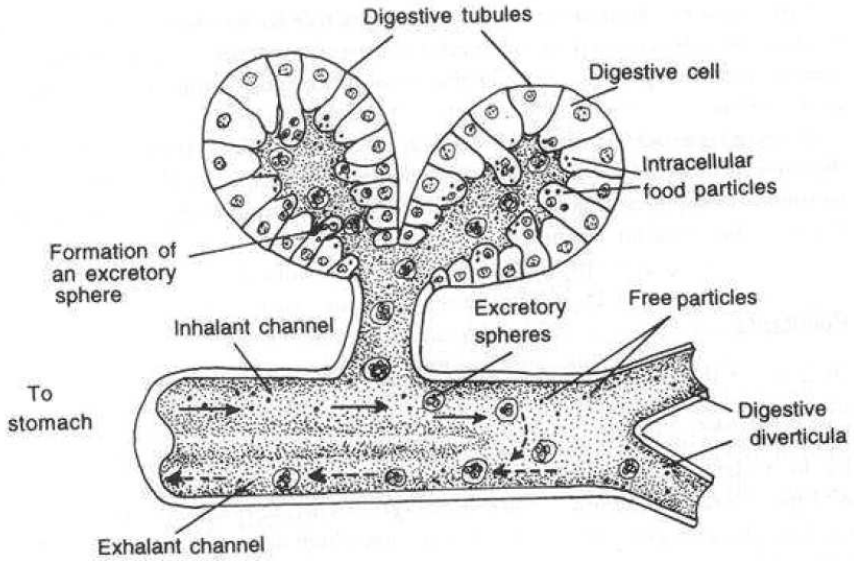


Figure 29. Schéma d'un tubule digestif de mollusque montrant l'absorption et la digestion intracellulaire des particules venant de l'estomac (flèches noires) et des débris à excréter (flèches en pointillées) (Gosling, 2003).

Les tubules digestifs présentent deux types de cellules : les digestives et les basophiles. Les cellules digestives sont les plus abondantes et prélèvent les particules à la base des microvillosités des tubules digestifs par pinocytose (absorption du liquide extracellulaire dans les cellules) et leur digestion est ensuite réalisée au niveau des lysosomes. Après la digestion, le produit final est libéré dans l'hémolymphe et les déchets de la digestion sont relâchés dans la glande digestive et acheminés vers l'intestin pour excrétion (Figure 29). Les cellules basophiles possèdent un appareil de golgi et donc ont un rôle dans la synthèse des protéines. Cependant, leur rôle n'est pas encore très clair. Une fois dans l'intestin les déchets de la digestion et les particules directement rejetées peuvent encore éventuellement être absorbés et passer dans l'hémolymphe. En effet, les hémocytes ont la capacité de traverser la barrière intestinale et une activité enzymatique est encore présente dans l'intestin. Ces déchets sont ensuite excrétés et exhalés par le bivalve (Gosling, 2003).

1.6.2. Effets des microalgues toxiques sur les mollusques bivalves

Un grand nombre d'événements toxiques ont associé de manière directe ou indirecte des mortalités d'invertébrés, de poissons, d'oiseaux mais aussi de mammifères marins (baleines, dauphins…) à des efflorescences d'algues toxiques (Landsberg, 2002).

En ce qui concerne les mollusques bivalves, en plus des conditions environnementales telles que la température, la salinité, la quantité et la qualité de la matière en suspension (Barille et al., 1997; Bayne et al., 1987; Bayne et al., 1989; Bayne et al., 1993; Gosling, 2003)

et des facteurs intrinsèques aux mollusques (poids sec de l'animal, âge, taille...) (Gosling, 2003), les réponses physiologiques des différentes espèces aux phycotoxines sont variables en fonction de leur sensibilité. Les moules sont considérées comme moins sensibles que la plupart des autres mollusques bivalves et par conséquent peuvent accumuler de plus grandes quantités de toxines (Bricelj and Shumway, 1998; Mafra et al., 2009), alors que d'autres organismes comme l'huître (*C. virginica*), peuvent, lorsqu'ils sont exposés à une alimentation d'algues toxiques telles que les *Pseudo-nitzschia,* réduire leur accumulation de toxines en diminuant leur taux de filtration et en rejetant les cellules toxiques dans les pseudofèces (Mafra et al., 2009). Des constatations similaires de modification de l'activité physiologique des mollusques ont aussi été observées avec d'autres espèces de mollusques exposées à des espèces d'algues toxiques (Bardouil et al., 1996; Bardouil et al., 1993; Lassus et al., 1999; Shumway and Cucci, 1987).

En plus d'affecter l'activité physiologique des mollusques bivalves, les microalgues toxiques peuvent altérer leur système digestif. En effet, une diminution de l'épaisseur de l'épithélium des tubules digestifs ainsi qu'une exfoliation des cellules de l'épithélium dans la lumière des tubules a été observée chez des bivalves exposés à différentes algues toxiques (*Alexandrium fundyense, Alexandrium ostenfeldii, Prorocentrum rhathymum*) (Galimany et al., 2008b; Medhioub et al., 2012; Pearce et al., 2005). De plus, (Fernandez-Reiriz et al., 2008) ont démontré une inhibition temporaire du processus digestif lors des premiers jours d'exposition des moules (*M. chilensis*) à *A. catenella.* Une diminution de l'activité enzymatique a été observée, en particulier pour l'amylase et la laminarase ; cependant, cette baisse d'activité n'a été que temporaire et a ré-augmenté après deux semaines d'exposition à *A. catenella*.

De plus, les microalgues toxiques peuvent aussi, le cas échéant, augmenter le taux de mortalité des mollusques et particulièrement celui des juvéniles (Keppler et al., 2005; Smolowitz and Shumway, 1997) ou dans une moindre mesure engendrer une réponse immunitaire des organismes exposés. En effet des observations histopathologiques ont montré des réactions inflammatoires et une production d'hémocytes par les mollusques bivalves (*M. edulis, C. gigas*, *Ruditapes philippinarum*) lors d'expositions à certaines algues toxiques (*A. fundyense, P. minimum, K. veneficum, A. minutum, A. ostenfeldii*) (Galimany et al., 2008a; Galimany et al., 2008b; Galimany et al., 2008c; Haberkorn et al., 2010; Hegaret et al., 2009; Medhioub et al., 2012). Ces effets peuvent se caractériser par des infiltrations et des diapédèses hémocytaires entre les cellules de l'épithélium depuis le tissu conjonctif vers la

lumière de l'estomac, de l'intestin ou dans différents tissus de la glande digestive (Medhioub, 2011).

Les effets délétères des microalgues toxiques peuvent donc être d'une grande importance, au niveau de l'écosystème ainsi que pour les installations aquacoles.

1.6.3. Accumulation et détoxification

La compréhension des mécanismes d'accumulation et de détoxification des phycotoxines dans les mollusques bivalves est importante, compte tenu de l'impact économique et sanitaire des événements toxiques.

On a vu dans les deux sections précédentes que l'exposition des mollusques bivalves à des microalgues toxiques pouvait plus ou moins affecter (1) leur réponse physiologique et (2) l'efficacité de la digestion et de l'assimilation des microalgues. Ces deux composantes peuvent avoir un impact sur l'accumulation des toxines. Cependant, d'autres facteurs entrent aussi en compte : (1) le transfert des toxines entre les organes, (2) la biotransformation des toxines en différents dérivés, (3) le taux de détoxification et (4) le lieu d'accumulation de la toxine (Silvert and Cembella, 1995).

1.6.3.1. Les toxines lipophiles

1. **L'acide okadaïque** : en Europe, les périodes de contamination s'étendent généralement de mai à novembre et varient en fonction des conditions climatiques. La plupart des mollusques sont touchés par les efflorescences de *Dinophysis* spp. ou *Prorocentrum* spp. : les moules, les huîtres, les coques, les pétoncles et les coquilles St Jacques… (Stobo et al., 2005; Torgersen et al., 2008a; Vale, 2004, 2006). Cependant, les moules sont généralement retenues comme espèce sentinelle par les réseaux de surveillance, grâce à leur plus grande capacité d'accumulation des phycotoxines. En effet, comparées aux huîtres, elles sont habituellement beaucoup plus touchées (Yasumoto et al., 1978) et sont généralement plus contaminées que les autres espèces de bivalves (Reizopoulou et al., 2008; Vale, 2006).

Le temps de détoxification de ces toxines est variable d'un bivalve à un autre, et logiquement d'un dérivé à un autre (Torgersen et al., 2008b), mais il semble relativement lent avec un temps de demi-vie observé supérieur à 10 jours (Marcaillou et al., 2010; Torgersen et al., 2008b). En laboratoire, lors d'une contamination d'*Argopecten irradians* par *P. lima*, il a été montré que *P. lima* n'induisait pas de mortalité, ne diminuait pas le taux de filtration des mollusques et qu'une faible accumulation (<1% de la quantité totale de toxines ingérées) était observée après deux semaines d'exposition (Bauder et al., 2001).

Les toxines sont principalement localisées dans les glandes digestives des bivalves (Edebo et al., 1988) et peuvent aussi être présentes dans les gonades des pétoncles adultes (Bauder et al., 1996). La cinétique de détoxification présente une allure bi-phasique : rapide le premier jour mais nécessitant plus de deux semaines pour obtenir une détoxification complète (Bauder et al., 1996). Par ailleurs, les moules se décontaminent plus rapidement lorsqu'elles ont un apport élevé de nourriture non toxique (Marcaillou et al., 2010).

2. **Les pecténotoxines** : il existe peu d'informations concernant l'accumulation et la détoxification des PTX. Cependant, les contaminations ont souvent lieu en même temps que celles qui impliquent l'AO et la DTX (Lindegarth et al., 2009; Vale, 2004, 2006).

Lors d'une étude sur la détoxification de la PTX et de l'AO, la PTX2sa a été la principale PTX détectée chez l'huître plate (*O. edulis*) et chez la moule (*M. edulis*), de petites quantités de PTX2, PTX12 et PTX12sa étant aussi détectées. Comme pour les toxines du groupe AO, les huîtres contiennent généralement des quantités plus faibles de PTX par rapport aux moules, mais la différence est moins importante qu'avec d'autres toxines (Lindegarth et al., 2009).

Des études portant sur la détoxification des différentes PTX ont montré que ces toxines sont rapidement éliminées dans les huîtres et les moules (T (1/2) = 6-13 jours, (Lindegarth et al., 2009)). (Vale, 2006) a aussi observé une détoxification rapide des PTX chez la spisule (*Spisula solida*) et la télline (*Donax trunculus*), où en 4 jours la concentration est passée respectivement de 0,4 et 1,7 µg/g à seulement des traces de toxines.

Les différents taux d'assimilation intestinale et/ou de biotransformation entre les toxines du groupe AO et PTX peuvent expliquer les différences observées en termes de rétention et de profil toxinique entre les bivalves, plutôt que des différences entre les taux d'élimination (Lindegarth et al., 2009).

3. **Les brévétoxines** : les contaminations impliquant les BTX touchent aussi diverses espèces de mollusques : coques (Ishida et al., 1995), moules (Morohashi et al., 1995; Morohashi et al., 1999), huîtres (Plakas et al., 2004).

Les BTX sont rapidement accumulées par les mollusques (tels que l'huître (Plakas et al., 2002; Plakas et al., 2004)) et leur cinétique de détoxification est bi-phasique mais très variable en fonction de l'adduit formé : un temps de demi-vie supérieur à 8 semaines a été observé pour les adduits cystéiques de la PbTx3 et inférieur à 2 semaines pour les liaisons avec une molécule de glutathion (Plakas et al., 2004).

1.6.3.2. Les toxines hydrophiles

1. **L'acide domoïque** (toxine amnésiante) : historiquement, c'est après une grave série d'intoxications alimentaires au Canada, suite à la consommation de moules contaminées, que l'AD a été découvert (Bates et al., 1989). En France, en 1999, les première traces d'AD ont été détectées dans des bivalves (Bretagne, Charente Maritime et Méditerranée) et l'année suivante, la teneur en AD a dépassé le seuil de sécurité sanitaire (20 µg g^{-1} de chair) en Bretagne Ouest (Amzil et al., 2001).

En Irlande, l'AD a été retrouvé chez la moule (*Mytilus edulis*, <1,0 µg AD g^{-1}); chez l'huître (*Crassostrea gigas*, <5,0 µg AD g^{-1}), chez le couteau (*Ensis siliqua*, <0,3 µg AD g^{-1}) et chez la coquille St Jacques (*Pecten maximus*, 240 µg AD g^{-1}). La plus forte concentration détectée d'AD, dans cette étude, a été dans les glandes digestives de la coquille St Jacques (2820 µg DA g^{-1}GD) (James et al., 2005). Au Canada, après un bloom de *Pseudo-nitzchia seriata* les moules ont accumulé jusqu'à 200 µg AD g^{-1} alors que les huîtres n'ont accumulé que 0,9 µgAD g^{-1} (Mafra et al., 2010b). De plus, ces mêmes auteurs mettent en évidences des différences d'activités physiologiques entre plusieurs espèces de mollusques (Mafra Jr et al., 2010; Mafra et al., 2008; Mafra et al., 2010a) et soulignent la capacité de sélection pré-ingestive et la diminution du taux de filtration des huîtres (*C. virginica*) nourries avec *Pseudo-nitzchia*, pour expliquer leur faible accumulation en AD (Mafra et al., 2008; Mafra et al., 2010a). Ces articles montrent la grande variabilité qui existe entre les espèces de mollusques bivalves, en termes d'accumulation de cette toxine ainsi qu'en fonction du lieu de prélèvement. En plus, d'une variabilité concernant les cinétiques d'accumulation, celle-ci existe aussi entre les cinétiques de détoxification de l'AD en fonction des espèces. Par exemple, la moule est rapidement détoxifiée (Blanco et al., 2002b; Gilgan M W, 1990; Novaczek et al., 1992) alors que le couteau (Drum et al., 1993; Trainer and Bill, 2004) ou la coquille Saint Jacques se detoxifient plus lentement (Blanco et al., 2002a; Blanco et al., 2006). Chez cette dernière, l'AD est majoritairement distribué dans la glande digestive de *P. maximus* (>90%), puis dans les gonades, le rein, le muscle adducteur et enfin dans les chairs restantes (branchies-manteau-pied) (Blanco et al., 2002a). A l'inverse, d'après (Drum et al., 1993) le couteau du Pacifique accumule l'AD surtout dans les parties comestibles (manteau, siphon, muscles adducteur, pied) et peu dans les fractions « non comestibles » (branchies, glande digestive, gonades). La cinétique de détoxification de l'AD chez la coquille St Jacques semble aussi varier en fonction de la salinité et de la température alors que la nourriture n'aurait pas d'effet (Blanco et al., 2006). Par ailleurs, d'après une autre étude de cette équipe de recherche, l'AD serait contenu dans la fraction cytosolique de la glande digestive chez

Pecten maximus et ne serait pas lié à un tissu ou à une enzyme au niveau du cytosol de la glande digestive (Mauriz and Blanco, 2010). Ces auteurs, pour expliquer la lente détoxification (>300 jours), formulent l'hypothèse que l'AD, contrairement à l'hypothèse initiale, ne serait pas séquestré par liaison à des tissus de la glande digestive mais que *Pecten maximus* n'aurait pas la capacité d'excréter l'AD du cytosol par transport membranaire.

Une fois dissous dans l'eau de mer, l'AD semble être difficilement accumulable par les mollusques. Chez la moule, moins de 1% d'AD dissous et jusqu'à 6% d'AD encapsulé ont été assimilés dans les tissus, où la majorité de l'AD est concentrée dans la glande digestive et le rein (Novaczek et al., 1992).

2. **Les saxitoxines** (toxines paralysantes) : ces toxines, produites par diverses espèces d'*Alexandrium*, présentent des cinétiques d'accumulation variables en fonction des réponses physiologiques de l'espèce de mollusques qui y sont exposées, ainsi qu'en fonction du lieu de prélèvement. En effet, certaines espèces de mollusques ayant l'habitude d'être exposées à des espèces d'*Alexandrium* toxiques auraient développé des mécanismes leur permettant de se nourrir de ces microalgues et ainsi d'accumuler de plus fortes concentrations toxiniques (Bricelj et al., 2005; Chebib et al., 1993; Shumway and Cucci, 1987). Ou bien, d'autres espèces plus sensibles peuvent bloquer leur activité alimentaire, en présence de producteurs de toxines paralysantes, et donc ne pas accumuler de toxines (Shumway, 1989). Mais, la majorité des espèces les accumulent plus ou moins en fonction de leur sensibilité (Bricelj and Shumway, 1998) et ont développé des mécanismes de sélection pré-ingestive ou post-ingestive : diminution de l'activité alimentaire (Bougrier et al., 2003), diminution du taux de filtration (Bardouil et al., 1996; Bricelj et al., 1990), sélection des particules non-toxiques en présence de particules toxiques (Shumway and Cucci, 1987) et variation de l'efficacité d'absorption (Bricelj et al., 1990; Shumway et al., 1985).

L'accumulation des toxines paralysantes n'est pas la même dans tous les organes et chez tous les mollusques bivalves ; toutefois, ces toxines semblent se concentrer principalement dans la masse viscérale (glandes digestives et intestin, Figure 30).

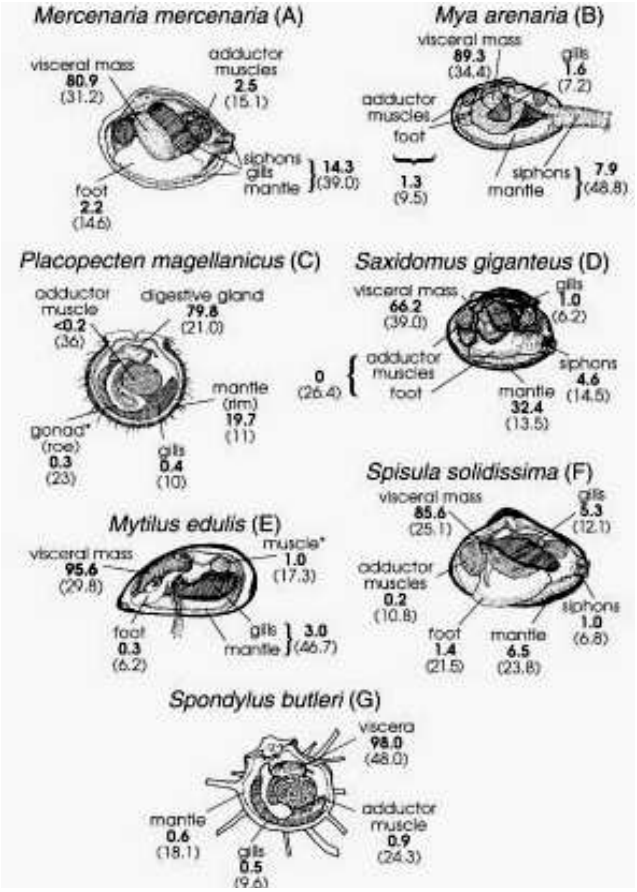


Figure 30. Exemple de distribution de toxines paralysantes chez différentes espèces de mollusques bivalves filtreurs. Les chiffres en gras indiquent la quantité relative de toxine par tissu et les chiffres entre parenthèse indiquent leur contribution par rapport à la masse humide totale de chair (Bricelj and Shumway, 1998).

En ce qui concerne la détoxification des toxines paralysantes, il y a des espèces qui présentent des cinétiques de détoxication rapide (de quelques jours à quelques semaines) et d'autres qui présentent des détoxifications plus lentes (> à plusieurs mois) (Bricelj and Shumway, 1998).

Toutefois, les mécanismes physiologiques qui sont en jeu, lors de ces détoxifications, sont encore peu connus et l'effet de certains facteurs environnementaux sont controversés (température). À basse température un ralentissement de la détoxification des mollusques est généralement observé (Shumway and Cembella, 1993), cependant un effet de la température n'a pas été observé lors de la détoxification chez la moule (*M. galloprovincialis*) (Blanco et al., 1997).

Cependant, l'effet de la nourriture sur la cinétique de décontamination a été récemment étudié et l'ajout d'une source de nourriture non-toxique réduit significativement le temps de détoxification (Gueguen et al., 2008).

La cinétique de détoxification des toxines paralysantes est décrite comme une cinétique bi-phasique. La première partie rapide correspond à l'élimination des toxines libres du tractus digestif, et la seconde partie plus lente résulte de l'élimination des toxines liées aux différents tissus (Baron et al., 2006; Blanco et al., 1997; Blanco et al., 2003; Lassus et al., 2007; Silvert and Cembella, 1995; Yu et al., 2005; Yu et al., 2007). Ce type de modèle s'applique bien aux cinétiques observées et permet d'appréhender le temps nécessaire à la détoxification des mollusques ; cependant, il ne prend pas en compte les variables environnementales ou la biotransformation d'une toxine en une autre. Mais, quelques auteurs ont pris en compte ces types de variables : environnementales (Blanco et al., 1997), biotransformations (Blanco et al., 2003; Gueguen et al., 2011; Silvert et al., 1998), transferts entre organes (Gueguen et al., 2011; Li et al., 2005) et concentration initiale en toxine (Gueguen et al., 2011). Ces modèles permettent d'affiner les prédictions et surtout de mieux comprendre les mécanismes de détoxification.

1.6.4. Le cas des AZA

Le développement de l'analyse chimique comme système de surveillance en Irlande a permis de suivre les évènements toxiques et de quantifier les AZA dans les moules depuis 2003 par CL-SM/SM. Même si les AZA ont été détectés à des concentrations inférieures à la limite réglementaire en 2003, leur présence, depuis 2005, à des concentrations au-delà du seuil sanitaire indique un problème récurrent. Les autres pays d'Europe sont aussi concernés, soit par des intoxications de consommateurs, soit par des contaminations de mollusques bivalves par les AZA. En France, les AZA1 et -2 ont été trouvés chez le pétoncle blanc (*Aequipecten opercularis*), en 2007 en Bretagne Nord (Amzil et al., 2008), ainsi que chez l'huître (*C. gigas*) et la moule (*M. edulis*) en 2010 et 2011 (source : REPHY).

De plus, l'accumulation d'AZA a été signalée dans un certain nombre d'espèces de mollusques (Tableau 4). Cependant, en Irlande des niveaux d'accumulation d'AZA dépassant la limite réglementaire (160 µg kg^{-1}) n'ont été observés que chez des moules (*M. edulis*) et des huîtres (*C. gigas*).

Tableau 4. Concentration maximale en AZA équivalent (mg kg^{-1}) trouvée dans les mollusques bivalves élevés en Irlande entre 2003 et 2010 par CL-SM/SM. LOQ : limite de quantification (Salas et al., 2011).

Espèces	AZA equiv. (mg kg^{-1})
Cerastoderma edule	0.08
Crassostrea gigas	0.31
Ensis arcuatus	0.05
Ensis siliqua	<0.01
Glycymeris glycymeris	0.01
Haliotis discus hannai	<LOQ
Mytilus edulis	8.97
Ostrea edulis	0.07
Patella vulgata	<LOQ
Spisula solida	0.15
Tapes philippinarum	0.1
Tapes semidecussatus	0.01
Venerupis senegalensis	<LOQ
Venus verrucosa	<LOQ

Toutefois, *A. spinosum* ne produit qu'AZA1 et -2 (Krock et al., 2009), tandis qu'un grand nombre d'analogues ont été recensés dans les moules (Rehmann et al., 2008), dont la plupart semble être des métabolites formés dans les coquillages (McCarron et al., 2009). De plus, jusqu'à présent, le lien direct entre *A. spinosum* et les contaminations de mollusques restait encore à établir, alors qu'un transfert trophique entre *A. spinosum* et d'autres espèces de planctons est aussi envisageable (ex : *Protoperidinium crassipes, Favella ehrenbergii*) (Krock et al., 2009). Les voies possibles de contaminations directes ou indirectes restent donc encore à être déterminées.

Un seul essai de contamination expérimentale par les AZA a été publié à ce jour, en parallèle de nos travaux. Des moules ont été contaminées pendant 13 jours avec un mélange d'algues fourrages auxquelles il a été ajouté de l'AZA1 purifié. La distribution, par tissu, montre une forte proportion d'AZA1, 3 et 17 dans les branchies et les autres tissus (surtout AZA3) (O'Driscoll et al., 2011), cependant ce profil est surprenant car AZA3 n'est pas connu pour être un AZA majoritairement retrouvé dans les mollusques bivalves (Fux et al., 2009; McCarron et al., 2009).

S'il n'existe que peu de données sur les voies de contamination des mollusques par les AZA, il y a des informations concernant la détoxification et le profil toxinique des mollusques.

La concentration en toxines peut rester élevée dans les mollusques bivalves, pendant huit à douze mois suivant l'exposition initiale, et ainsi rester élevée pendant les mois d'hiver, alors que les conditions sont théoriquement défavorables aux contaminations par du phytoplancton toxique. Dans le cas des moules, cette contamination de longue durée suggère un transfert des AZA de la glande digestive vers d'autres tissus, où la métabolisation serait plus lente (Furey et al., 2003; James et al., 2004; James et al., 2002; Magdalena et al., 2003a). Mais récemment, des liaisons AZA-protéines ont été mises en évidence (Nzoughet et al., 2008). Une liaison qui pourrait expliquer la longue durée de détoxification des coquillages (Twiner et al., 2008). Alternativement, une lente détoxification des AZA au cours des mois d'hiver pourrait en partie être expliquée par une baisse de l'activité métabolique pendant la période hivernale (Twiner et al., 2008). Mais, la présence continue ou sporadique d'*A. spinosum* ou d'autres vecteurs d'AZA ne peut être exclue.

La distribution des AZA dans les tissus de mollusques a déjà été étudiée chez les coquilles Saint Jacques (*P. maximus*) où 85% de la toxine a été détectée dans la glande digestive (Magdalena et al., 2003b) et chez les moules (*M. edulis*) où, similairement, plus de 80% de la toxine a été détectée dans la glande digestive (Hess et al., 2005b). Cependant ces travaux restent à approfondir afin de mettre en évidence les processus de métabolisation et de détoxification des mollusques, et notamment chez la moule.

Chapitre 2 – Analyse et production d’azaspiracides

Contexte

A. spinosum est une microalgue productrice d'AZA1 et -2 (Krock et al., 2009; Tillmann et al., 2009), un groupe de toxines responsable d'intoxications alimentaires suite à la consommation de mollusques bivalves. Cet organisme semble être largement répandu dans de nombreux océans (Akselman and Negri, 2012; Potvin et al., 2012; Tillmann et al., 2011; Tillmann et al., 2009), et depuis leur découverte les AZA ont été signalés dans de nombreux endroits (Alvarez et al., 2010; Amzil et al., 2008; Furey et al., 2010; Magdalena et al., 2003a; Taleb et al., 2006; Twiner et al., 2008; Ueoka et al., 2009). Nous avons donc mis en culture cet organisme afin de mieux comprendre l'écophysiologie des *Azadinium*, d'évaluer la possibilité de produire ces toxines à partir de cultures en bioréacteurs et d'estimer la cinétique d'accumulation des AZA dans les mollusques bivalves. Enfin, ces connaissances sont des informations essentielles pour la production durable de toxines pour réaliser des expériences de toxicologie et permettre l'étalonnage des instruments d'analyse.

Dans des études antérieures, la concentration intracellulaire en AZA trouvée dans les microalgues s'est avérée être très variable, allant de 5 à 40 fg AZA1 cell^{-1} (Krock et al., 2009; Tillmann et al., 2009). Ces différences peuvent être liées à des conditions de culture ou des procédures d'analyse différentes. Au niveau analytique ces différences peuvent comprendre des variations sur le procédé d'extraction, de récupération des cellules, du prétraitement ou bien, être la cause d'effets de matrice lors de l'analyse par CL-SM/SM. Par conséquent, les études de l'effet des facteurs environnementaux et nutritionnels affectant la croissance et la concentration toxinique d'*A. spinosum* nécessitent une procédure d'analyse standardisée des cellules et du milieu de culture, pour pouvoir déterminer leur teneur en toxines. Nous avons donc, tout d'abord, développé une procédure d'analyse des AZA provenant de cultures d'*A. spinosum*.

Comme d'autres toxines lipophiles, les AZA sont généralement extraits, par des solvants organiques, à partir du phytoplancton ou de mollusques bivalves contaminés (Quilliam, 2003). Historiquement, l'acétone a été le solvant utilisé pour extraire les toxines lipophiles des mollusques bivalves, dans le but de réaliser le test-souris (Yasumoto et al., 1984), tandis que le MeOH et des mélanges MeOH-eau ont été plutôt utilisés pour extraire les toxines lipophiles, afin de réaliser l'analyse par CL-SM/SM (Hess et al., 2005b; McNabb et al., 2005). Ces procédures d'extraction aboutissent à des extraits bruts pour lesquels on observe généralement des effets de matrice (rehaussement ou réduction du signal) lors des analyses par CL-SM/SM (King et al., 2000). Ces effets de matrice ont été préalablement

rapportés lors de l'analyse de moules par CL-SM/SM (Fux et al., 2008b) ; cependant des solutions existent pour réduire leur influence et améliorer l'exactitude des résultats de l'analyse (Kilcoyne and Fux, 2010; McCarron et al., 2011b). Par conséquent, les effets de matrice doivent également être évalués dans les procédures d'analyse des AZA pour permettre une quantification fiable des quotas cellulaire d'*A. spinosum*. De plus, un nouvel analogue d'AZA a été observé par Krock et al. (2009) et provisoirement désigné comme "AZAX". Il a été détecté dans des extraits méthanoliques de cultures d'*Azadinium spinosum*, et possède un ion moléculaire correspondant à celui du méthyle-ester d'AZA1. Cependant, le temps de rétention relatif d'AZAX ne concorde pas à celui rapporté pour le méthyle-ester d'AZA1 (Rehmann, 2008) (nommé AZA30 par ces auteurs). Par conséquent, la clarification des structures et des mécanismes de formation des artefacts d'AZA méthylés est nécessaire. En effet la détermination des métabolites d'AZA et l'identification des artefacts formés au cours des procédures analytiques est importante pour les études ultérieures qui porteront sur l'accumulation et la détermination des voies de biotransformations des AZA dans les mollusques bivalves. Ainsi, la première partie de ce chapitre décrit le développement d'une méthode d'analyse quantitative pour la détermination des AZA à partir de cultures d'*A. spinosum*, clarifie les structures des dérivés méthylés d'AZA, et explique la formation des méthyle-esters d'AZA comme artefact d'extraction.

La seconde partie de ce chapitre porte sur la mise au point d'une méthode d'extraction à une échelle pilote, l'objectif étant d'obtenir les meilleurs rendements d'extraction possibles à partir de grands volumes de culture (≥ 200L). En effet, l'obtention de phycotoxines pures est essentielle car il existe actuellement une pénurie de matériaux de référence pour la surveillance des phycotoxines dans les denrées alimentaires (Hess et al., 2007). Cela est devenu particulièrement important depuis le début de la surveillance, par CL-SM/SM, des toxines lipophiles dans les coquillages contaminés en Europe (Anonyme, 2011) et l'analyse des AZA par CL-SM/SM exige des standards d'AZA purifiés et certifiés pour leur quantification. Les efflorescences d'origine naturelle d'*A. spinosum* sont pour le moment encore difficiles à prévoir et/ou à trouver, comme très peu de données écologiques sont disponibles. La production durable d'AZA à partir d'*A. spinosum* est donc souhaitable pour la quantification des AZA par les organismes de surveillance et pour les études toxicologiques.

La seconde partie de ce chapitre évalue donc la faisabilité de la production d'AZA à partir de culture en continu d'*A. spinosum*, en photobioréacteurs, à une échelle pilote. Deux photobioréacteurs ont été couplés en série pour évaluer l'effet du taux de dilution sur la concentration cellulaire et sur la production d'AZA. Pour la récolte des toxine,s nous avons

appliqué une approche de récupérations des AZA intracellulaires à partir des cellules en culture et des AZA dissous à partir de la phase aqueuse de la culture. Pour la récupération des cellules, la filtration tangentielle et la centrifugation en continu ont été évaluées. Pour la récupération des AZA des procédures d'extraction en phase solide ont été développées à partir de grands volumes de culture filtrée et à partir du concentrât de cellules obtenu.

La méthode développée par Jane Kilcoyne du Marine Institute en Irlande pour purifier AZA1 et -2 à partir des extraits d'algues bruts est aussi présentée. Elle met en évidence l'efficacité de cette procédure de purification par rapport à la méthode d'obtention et de purification d'AZA1 et -2 à partir de glandes digestives de moules.

1. Quantitative analysis of azaspiracids in *Azadinium spinosum* cultures

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1.1. Résumé

Les azaspiracides-1 et -2 (AZA) sont des métabolites secondaires d'*Azadinium spinosum* qui peuvent s'accumuler dans les mollusques bivalves et provoquer des intoxications alimentaires après la consommation de ces coquillages. Nous décrivons ici une méthode d'analyse pour la détermination des AZA à partir de cellules d'*A. spinosum* en mettant l'accent sur la formation des méthyle-esters d'AZA. Ces derniers sont des artéfacts formés lors de l'extraction et du prétraitement de l'échantillon.

Les cellules d'*A. spinosum* ont été recueillies à partir de cultures en bioréacteurs par centrifugation ou filtration. Différentes méthodes d'extraction ont ensuite été testées pour évaluer leurs effets sur la formation de méthyle-esters d'AZA, le rendement et les effets de matrice.

La filtration des cultures avec des filtres en fibre de verre conduit à une plus grande formation de méthyle-esters par rapport à la centrifugation. L'étude de l'influence de la nature solvant d'extraction (MeOH, acétone, MeCN) montre que les rendements d'extraction des AZA ne sont pas significativement différents tant que la teneur en solvant organique reste

supérieure à 80%. Cependant, l'utilisation de MeOH comme solvant d'extraction conduit à une plus grande formation de méthyle-esters d'AZA1 et -2. Le rendement d'extraction d'AZA1 après deux extractions successives est de 100%, avec un coefficient de confiance de 95% pour l'acétone et le MeOH.

Lors de l'étude de l'impact des effets de matrices sur l'analyse des AZA, aucune augmentation ou diminution du signal n'a été observée à partir des extraits d'*A. spinosum* ou d'*A. obesum* jusqu'à un volume cellulaire de $4{,}5 \times 10^9$ µm^3. En outre, les expériences réalisées afin de comprendre et d'expliquer la formation et la structure des analogues d'AZA méthylés ont conduit à la description de deux méthyle-esters d'AZA et à la correction de la structure chimique des AZA29 à 32.

1.2. Abstract

Azaspiracids (AZAs) are secondary metabolites of *Azadinium spinosum* that can accumulate in shellfish and cause food poisoning when consumed. We describe here an analytical procedure for the determination of AZAs in cultures of *A. spinosum* with a focus on the formation of AZA methyl esters as artefacts during extraction and sample pre-treatment.

A. spinosum cells were collected from bioreactor cultures using centrifugation or filtration. Different extraction procedures were evaluated for formation of methyl ester artefacts, yield, and matrix effects.

Filtration of cultures using glass-fibre filters led to increased formation of methyl esters, and centrifugation is recommended for recovery of cells. The extraction solvent (MeOH, acetone, MeCN) did not significantly affect the yield of AZAs as long as the organic content was 80% or higher. However, the use of MeOH as extraction solvent led to increased formation of methyl esters. AZA1 recovery over two successive extractions was 100% at the 95% confidence level for acetone and MeOH. In standard-addition experiments, no significant matrix effects were observed in extracts of *A. spinosum* or *A. obesum* up to a sample size of 4.5×10^9 µm^3. Moreover, experiments carried out to clarify the formation and structure of methylated AZA analogues led to the description of two AZA methyl esters and to the correction of the chemical structures of AZA29–32.

1.3. Key words

Extraction procedure, Extraction artefact, Matrix effects, LC-MS/MS, Azaspiracid methyl ester, Dinoflagellate, Liquid chromatography–mass spectrometry

1.4. Introduction

Harmful algal blooms are widespread throughout the world, frequently causing problems to public health through consumption of contaminated shellfish and, amongst these, azaspiracid shellfish poisoning is the most recently identified syndrome. In 1995, a human intoxication occurred in the Netherlands after consumption of mussels from Ireland (Killary Harbour), with symptoms typical for diarrhetic shellfish poisoning (DSP). The incident caused diarrhea, nausea, vomiting and stomach cramps in consumers; however, only very low levels of OA-group toxins were found in mussels (McMahon and Silke, 1996). A new toxin named azaspiracid (AZA) (now referred to as Azaspiracid-1 (AZA1) (Figure 31)), was identified three years later (Satake et al., 1998c), with its structure being revised after synthetic studies (Nicolaou et al., 2006b). AZAs 4-5 were subsequently isolated from contaminated mussels and their structures established using mass spectrometry and NMR spectroscopy (Ofuji et al., 2001; Ofuji et al., 1999a). Additional AZAs have since been identified and structures proposed based on mass spectrometry, and the group now comprises 32 analogues, including several hypothetical compounds and artefacts (James et al., 2003b; Rehmann et al., 2008). Since their initial discovery, AZAs have been found in Europe, Africa and more recently in America and in Japan (Alvarez et al., 2010; Amzil et al., 2008; Furey et al., 2010; Magdalena et al., 2003a; Taleb et al., 2006; Twiner et al., 2008; Ueoka et al., 2009).

Even though consumption of AZA-contaminated shellfish has caused public health problems since 1995, it was not until 2003 that AZAs were detected in plankton, namely in the dinoflagellate *Protoperidinium crassipes* (James et al., 2003a). However, a question rapidly arose over whether this organism was an actual AZA producer or whether it was a predator which accumulated the toxin from another organism (Hess et al., 2005b; Miles et al., 2004c). As *P. crassipes* is a heterotrophic dinoflagellate (Gribble and Anderson, 2006) capable of accumulating phycotoxins (Miles et al., 2004b), and since culturing did not result in AZA production (Tillmann and Krock, unpublished data in (Tillmann et al., 2009)), research focussed on possible prey of this species. During a cruise in 2007, a dinoflagellate source of AZAs (strain 3D9), was discovered (Krock et al., 2008). This organism was found to contain AZA1 and AZA2 in the field, and produced *de novo* AZA1 and -2 in axenic culture

(Krock et al., 2009; Tillmann et al., 2009). The organism, a small (12–16 µm length and 7–11 µm width) peridinin-containing photosynthetic dinoflagellate with a thin theca, was formally described by (Tillmann et al., 2009) and named *Azadinium spinosum*. This species was the type-species for a new genus, and was soon joined by two non-AZA-producing species: *Azadinium obesum* (2E10) (Tillmann et al., 2010), a somewhat larger organism (13–18 µm length, 10–14 µm width); and *Azadinium poporum*, which is similar in size to *A. spinosum* but has a slightly lower mean cell length:width ratio (11–16 µm length and 8–12 µm width) (Tillmann et al., 2011). Interestingly, since the morphological description of *A. spinosum*, strains of this organism have also been reported from Mexico (Hernandez-Becerril et al., 2010), Argentina (Akselman and Negri, 2010), Italy and France (personal communication, R. Siano and E. Nézan, Ifremer, France) and, as to be expected, from Ireland (Salas et al., 2011). As the organism appears to be widespread in many oceans, and since AZAs have been reported from many locations, azaspiracid poisoning should be considered of global concern. We have therefore cultivated this organism to better understand the ecophysiology of *Azadinium*, its toxin production and the kinetics of AZA accumulation in shellfish. Finally, quantitative knowledge of the AZA-production by *A. spinosum* is also essential information for the sustainable production of toxins for toxicology experiments and instrument calibration.

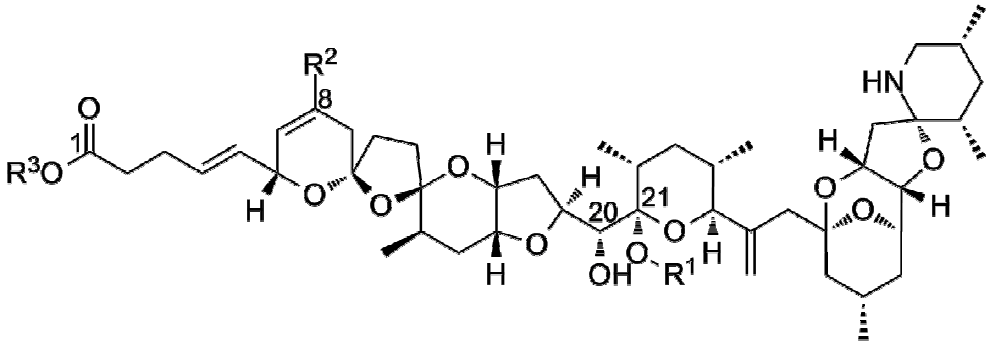
In previous studies, the cellular quota of AZAs was highly variable, ranging from 5–40 fg cell^{-1} (Salas et al., 2011; Tillmann et al., 2009), while in our own studies we found up to 100 fg cell^{-1}. Such differences may arise from differences in either culture conditions or in analytical procedures, including extraction, recovery in sample pre-treatment, or matrix effects in the final determination using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Consequently, studies on environmental and nutritional factors affecting *A. spinosum* growth and toxicity will require standardised analysis of cells and culture media for their toxin content. Therefore, we decided to investigate the analysis of AZAs from cultures of *A. spinosum*.

Like other lipophilic toxins, AZAs are typically extracted from phytoplankton or contaminated bivalves with organic solvents (Quilliam, 2003). Historically, acetone has been used to extract lipophilic toxins from shellfish for the mouse bioassay, whereas MeOH and mixtures of MeOH–water have been used for extraction prior to LC-MS/MS analysis (Hess et al., 2005b; McNabb et al., 2005). These procedures result in crude extracts that typically cause matrix effects (signal enhancement or reduction) in LC-MS/MS analysis (King et al., 2000). Matrix effects have been reported in quantitation of AZAs in mussels by LC-MS/MS

using different technical approaches (Fux et al., 2008b), and possible solutions were proposed (Kilcoyne and Fux, 2010; McCarron et al., 2011b). Therefore, matrix effects also need to be evaluated in analytical procedures for quantitation of AZAs in phytoplankton.

A novel AZA analogue observed by (Krock et al., 2009), and provisionally denoted as "AZAX", was detected in methanolic extracts of *Azadinium* cultures, and possessed a molecular ion corresponding to that of AZA1 methyl ester. However, the relative retention time of AZAX did not appear to match that originally reported for AZA1 methyl ester (Rehmann, 2008) (denoted as AZA30 by these authors). Therefore, clarification of the structures and mechanisms of formation of methylated AZA artefacts was required.

The determination of AZA metabolites, and the identification of artefacts formed during analytical procedures, is important for the subsequent assessment of metabolism in shellfish, other aquatic organisms, and mammalian systems. Thus, the present study describes the development of a quantitative analytical method for the determination of AZAs in cultures of *A. spinosum*, clarifies the structures of methylated derivatives of AZAs, and explains the formation of AZA methyl esters as artefacts from the extraction of *A. spinosum* cells with MeOH, and of AZA methyl ketals as artefacts of storage in MeOH.



R^1	R^2	R^3	Compound Name
H	H	H	AZA1
H	CH_3	H	AZA2
H	H	CH_3	AZA1 methyl ester ("AZAX")
H	CH_3	CH_3	AZA2 methyl ester
CH_3	H	H	AZA1 methyl ketal ("AZA30")
CH_3	CH_3	H	AZA2 methyl ketal ("AZA32")
H	H	(9-anthryl)methyl	AZA1 (9-anthryl)methyl ester
H	CH_3	(9-anthryl)methyl	AZA2 (9-anthryl)methyl ester

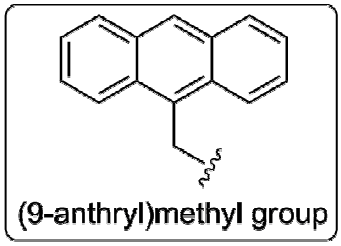


Figure 31. Structures of AZA1, -2 and their methyl and (9-anthryl)methyl derivatives

1.5. Materials and methods

1.5.1. Culture condition and cell count

Two species of *Azadinium* were used: the producer of AZA1 and -2, *A. spinosum* (clone 3D9) and the non-AZA-producing species *A. obesum* (clone 2E10). Both strains were grown using K modified medium (Keller et al., 1987), without NH_4Cl and with Na_2SeO_3 (10^{-8} M) , at 18 °C with a photon flux density of 200 µmol.m^{-2}.s^{-1} (day light fluorescent tubes) and a photoperiod of 16 h of light and 8 h of dark, in a 2.5 L or 100 L chemostat. Algae were sampled at steady state in continuous culture. Cell densities and cellular volume were determined using a particle counter (Multisizer 3 Coulter counter, Beckman).

1.5.2. Reagents

Methanol (MeOH), acetone, acetonitrile (MeCN), ethanol (Qiu et al.), and dichloromethane (DCM) were obtained as HPLC grade solvents from JT Baker and Sigma Aldrich. Formic acid (Puriss quality), ammonium formate (Purity for MS), methanol-d_4 (99.8%), *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, di(ethylene glycol)ethyl ether and 9-anthraldehyde were obtained from Sigma-Aldrich. Milli-Q water for HPLC was produced in-house using a Milli-Q integral 3 system (Millipore).

Spiking experiments were carried out using AZA1 purified by P. Hess in collaboration with M. Satake in Japan (2001), according to published procedures (Ofuji et al., 1999a). AZA1 calibrants for LC-MS/MS analysis were dilutions of either certified AZA1 (CRM-AZA1, National Research Council Canada (NRCC), Halifax, Canada), or using the above AZA1 purified in Japan (calibrated against CRM-AZA1).

1.5.3. LC-MS/MS analysis

The samples were analysed by LC-MS/MS using an Agilent 1100 LC coupled to a triple quadrupole mass spectrometer (API 2000, Applied Biosystems), a UFLCxr (Shimadzu) coupled to a triple quadrupole hybrid mass spectrometer Q-trap (API 4000QTRAP, Applied Biosystems), and an Agilent 1200 HPLC coupled to an Agilent 6540 QTOF instrument equipped with an electrospray ionization source for quantitation and accurate mass spectral analysis of AZAs.

1.5.3.1. Liquid chromatography

HPLC was carried out using BDS-Hypersil C8 (50 × 2 mm, 3 µm and 150 × 2.1 mm, 3 µm), MOS-Hyperclone C8 (50 × 2 mm, 3 µm) and Hypersil-Gold C18 (50 × 2 mm i.d., 2 µm) silica-based reversed phase columns (Thermo Scientific). Injection volumes were 5 µL. The A and B mobile phases were 100% water and acetonitrile/water (95:5, v/v) respectively, both containing 2 mM ammonium formate and 50 mM formic acid.

The 50 mm BDS-Hypersil column was eluted isocratically at 250 µL.min^{-1} (75% B) at 20 °C for 5–10 min, depending on which analogues were analysed. The MOS-Hyperclone C8 column was used in gradient elution mode (200 µL min^{-1} at 20 °C) starting with 70% B rising to 100% B at 2.5 min, held for 4.5 min, decreasing to 70% B over 6 min, and held for 5 min until the next run.

The 150 mm BDS-Hypersil C8 column was used in gradient elution mode (200 µL min^{-1} at 30 °C) for acquisition of accurate mass data, starting with 25% B rising to 100% B at 12 min, held for 8 min, decreasing to 25% B over 1 min, and held for 10 min until the next run.

The Hypersil Gold C18 column was eluted with a gradient for determination of AZAs and AZA ADAM derivatives, starting with 62.5% B rising to 100% B at 4 min, held for 5 min, decreasing to 62.5% B over 0.5 min, and held for 5 min until the next run.

1.5.3.2. Mass spectrometry

Multiple reaction monitoring (MRM) and fragmentation experiments were performed in positive ion mode under the conditions given in Table 5. Selected ion monitoring (SIM) was performed in negative mode. The following MRM transitions were monitored: AZA1, *m/z* 842.5→824.5, 842.5→672.5; AZA2 and AZA1 methyl ester, *m/z* 856.5→838.5, 856.5→672.5; AZA2 methyl ester, *m/z* 870.5→852.5, 870.5→672.5; AZA1 d_3-methyl ester, *m/z* 859.5→841.5, 859.5→672.5; AZA2 d_3-methyl ester, *m/z* 873.5→855.5, 873.5→672.5; AZA1 methyl ketal (AZA30), *m/z* 856.5→824.5; 856.5→672.5; AZA2 methyl ketal (AZA32), *m/z* 870.5→838.5; 870.5→672.5; AZA1 (9-anthryl)methyl ester, *m/z* 1032.6→672.5; AZA2 (9-anthryl)methyl ester and AZA1 methyl ketal (9-anthryl)methyl ester, *m/z* 1046.6→672.5; and AZA2 methyl ketal (9-anthryl)methyl ester, *m/z* 1050.6→672.5. The following $[M-H]^-$ ions were monitored in SIM mode: AZA1, *m/z* 840.5; AZA2, AZA1 methyl ester and AZA1 methyl ketal, *m/z* 854.5; AZA2 methyl ester *m/z* 868.5. Quantitation was carried out using external calibration against AZA1, with Analyst 1.5 software (Applied Biosystems).

Accurate mass data were acquired on an Agilent 6540 QTOF operated in positive mode, with full-scan analysis over *m/z* 100–1000 at 1 scan s^{-1} and targeted MS/MS analysis at 5 scans s^{-1}. Capillary and fragmentor voltages were 4000 V and 220 V, respectively. The Jet Stream Technology source was set at 300 °C with a drying gas flow at 8 L min^{-1} and a sheath gas flow of 12 L min^{-1} at 400 °C. Three collision energies (30, 50 and 70 V) were applied to the precursor ions to study fragmentation pathways.

Table 5. Operating conditions for the two mass spectrometers (AU = arbitrary units)

Operating conditions	API 2000	API 4000 Q-trap
Curtain gas	19 psi	30 psi
Temperature	350°C	450°C
Gas 1	30 psi	50 psi
Gas 2	50 psi	50 psi
CAD gas	5 psi	Medium
Ion spray voltage	5800 V	5500 V
Declustering potential	140 V	116 V
Entrance potential	10 V	10 V
Collision cell	50 & 70 V	41 & 69 V
Cell exit potential	35 V	12 & 16 V

1.5.4. Identification of AZA1 and -2 methyl esters and structure confirmation

(a) The effect of heat treatment of the filters containing *A. spinosum* samples on the formation of AZA methyl esters was tested to determine whether their formation was enzyme-catalysed. Triplicate aliquots of *A. spinosum* culture (10 mL) were filtered using GF/C filters and the filters (with cells) were placed in a 1.5 mL Eppendorf tubes. Two types of heat treatment were tested to suppress enzymatic activity: (i) microwaving for 2 min at 800 W, or; (ii) placing in a water bath at 100 °C for 30 min. A control, without heat treatment, was prepared in parallel. Each sample was then extracted with MeOH (5 × 0.5 mL) (described below).

(b) The formation of AZA methyl esters was studied during extraction and reconstitution with MeOH and deuterated MeOH (CD_3OD). The procedure in Figure 32 was used with the following solvents: (a) extraction with MeOH, reconstitution with MeOH; (b) extraction with MeOH, reconstitution with CD_3OD; and (c) extraction with CD_3OD, reconstitution with CD_3OD.

(c) Treatment with diazomethane, which derivatises carboxylic acids as their methyl esters, was used to synthesise AZA1 and -2 methyl esters. A methanolic extract from *A. spinosum* (0.5 mL) containing AZA1 and -2 was added to the outside tube of an Aldrich diazomethane generator with System 45 connection, and 1 mL MeOH and 1.5 mL Et_2O were added. Diazomethane was generated in the inner tube of the apparatus and allowed to react *in situ* with the extract, following the manufacturer's protocol (Sigma-Aldrich, 2007). After reacting for 45 min at 0 °C with occasional swirling, the extract was transferred to a glass vial, evaporated to dryness under a stream of N_2, and the residue dissolved in MeOH (1 mL) for LC-MS analysis.

(d) A sample containing AZA30 and -32 was obtained from an experiment studying storage of AZA1 and -2 standards in MeOH (NRCC).

(e) Samples containing AZA1 and -2 methyl esters, and AZA30, and -32 (AZA1 and -2 methyl ketals), were treated with sodium periodate as described by (Rehmann et al., 2008), then analysed by LC-MS/MS or by LC-MS in negative ion SIM mode. The same samples were also derivatized with 9-anthryldiazomethane, which derivatizes carboxylic acids as their (9-anthryl) methyl esters, and analysed by LC-MS in positive ion MRM mode as described by (McCarron et al., 2011a).

1.5.5. Protocols for the determination of extra- and intra-cellular portion of AZAs

Procedures for AZA extraction evaluated in this study were based on the following standard protocols.

Samples (10 mL) were collected from *A. spinosum* cultures and centrifuged (2500 *g*, 20 min, 4 °C) in 15 mL centrifuge tubes. The culture supernatant was collected for liquid–liquid extraction as described below, and the pellet was re-suspended in 500 µL of solvent and bath-sonicated (10 min) after transferring to a 1.5 mL Eppendorf tube. After sonication, the aliquot was centrifuged (15000 *g*, 10 min, 4 °C). The supernatant was transferred to a 5 mL glass tube and gently evaporated under nitrogen on a heating block at 35 °C. This process was repeated so that the pellet was extracted three times in total (the number of repetitions varied as a function of the experiment, but three successive extractions were generally used) and, following evaporation of the combined supernatants from each step, the residue was reconstituted in 500 or 1000 µL MeOH–H_2O (9:1 v/v). Subsequently, the sample was filtered with a NANOSEP MF centrifugal device (PALL, 0.2 µm, 1.5 mL vial with filter insert) (15000 *g*, 5 min, 4 °C) and transferred to an HPLC vial with insert (Figure 32).

The supernatant from centrifugation of the algal culture was transferred to a 15 mL tube and 2 mL of DCM added. The mixture was homogenized (1 min vortexing), centrifuged (2500 *g*, 10 min, 4 °C), and the organic phase transferred to a 15 mL glass tube. The supernatant was extracted three times in this manner, and the resulting DCM extract evaporated under nitrogen on a heating block at 35 °C and the residue was reconstituted and filtered as above (Figure 32).

The above extraction protocol was used to evaluate the following aspects of *A. spinosum* extraction:

(a) Effect of sample size, and residence time of *A. spinosum* in a 15 mL centrifuge tube prior analysis, on intra- an extra-cellular AZA content. The aliquots were preserved with neutral Lugol iodine solution and immediately observed using a Nageotte cell-counting chamber. During the experiment, the aliquots were maintained at room temperature (18 ± 2°C).

(b) Influence of procedures for separation of algal cells from the culture medium (filtration and centrifugation) on intra- and extra-cellular AZA content.

(c) Effect of extraction solvent on yield and artefact formation.

(d) Effect of algal matrix on recovery on the standard procedure.

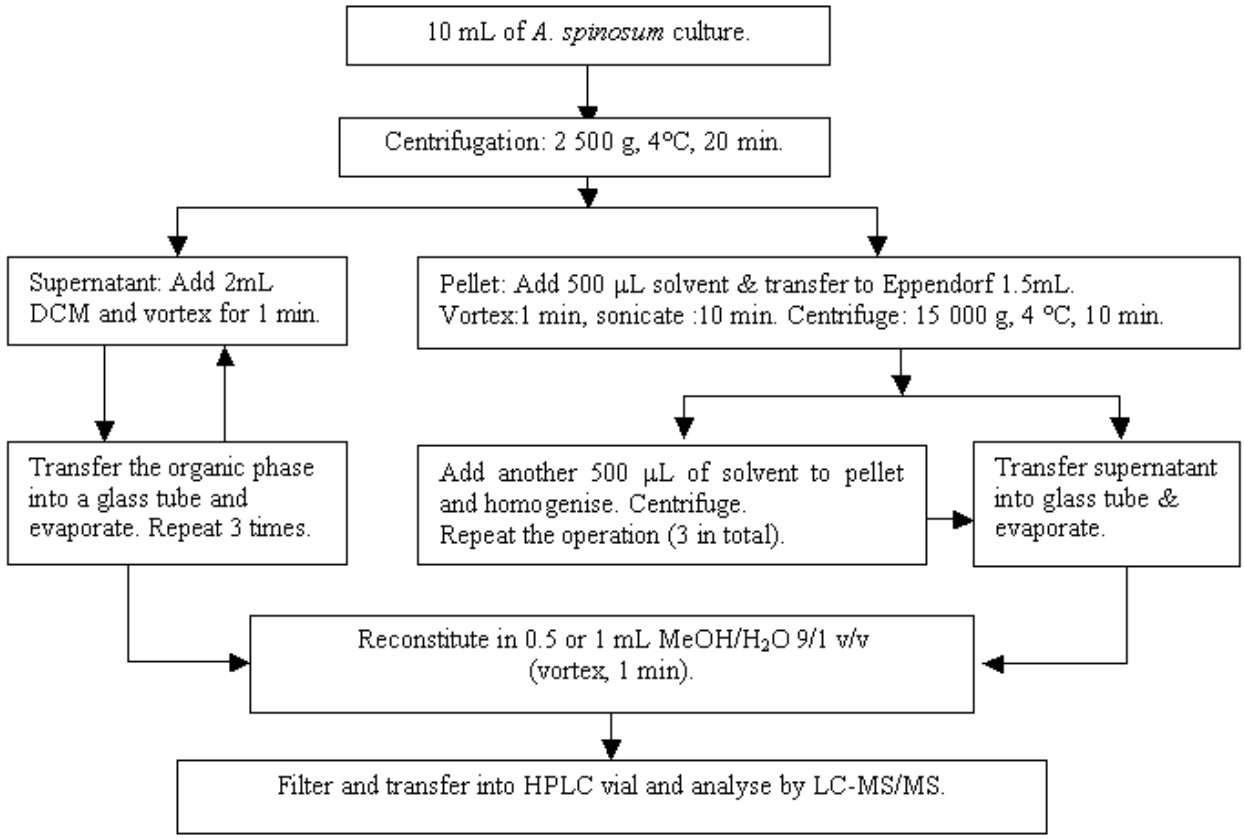


Figure 32. Sample preparation scheme for extraction of AZAs from *A. spinosum*

1.5.6. Matrix effects in LC-MS/MS analyses of culture extracts

Matrix effects were evaluated using the following approaches:

(a) AZA1 addition to a constant amount of algal matrix. *A. obesum* culture medium (10 mL) was extracted using the standard procedure (Figure 32) with MeOH–H_2O (9:1) or acetone–H_2O (9:1) (v/v). Reconstitution was carried out in triplicate using an AZA1 solution and MeOH, to obtain AZA1 concentrations ranging from 5.3–213 ng.mL^{-1}.

(b) The same extraction was applied as for (a) above, but with *A. spinosum* culture medium.

(c) Matrix addition to a constant AZA concentration. Samples of a culture of *A. obesum* (58000 ± 1000 cells.mL^{-1}, 83.3 × 10^6 µm^3.mL^{-1}) (0.62, 3.1, 6.2, 10, 21, 50 mL) were extracted in triplicate (Figure 32) using MeOH–H_2O (9:1) or acetone–H_2O (9:1 v/v) and reconstituted with 40 µL of AZA1 solution and 460 µL of MeOH–H_2O (9:1 v/v) to give an AZA1 concentration of 42.7 ng.mL^{-1}.

1.5.7. Statistical analysis

All data are expressed as mean ± standard deviation. Depending on the data, statistical analyses consisted either of multifactorial analysis of variance, one-way analysis of variance (ANOVA), or a Kruskal–Wallis test, followed, when necessary, by a Fisher's least significant difference procedure or a Box-and-Whisker plot. Differences were considered significant at $p<0.05$. Statistical analyses were carried out using Statgraphics Centurion XV.I (StatPoint Technologies, Inc.). Before each ANOVA analysis or Kruskal–Wallis test, normality and equality of variance were tested to decide which tests were going to be used.

1.6. Results and discussion

1.6.1. Identification of AZA1 and -2 methyl esters and structure confirmation

A late-eluting LC-MS peak in *A. spinosum* extracts, provisionally denoted as AZAX, was previously identified as a possible isomer of AZA2 (or a methylated AZA1 analogue), based on retention time and mass (Krock et al., 2008; Tillmann et al., 2009). The present work demonstrated the artefactual formation of methylated derivatives of AZA1 and AZA2 during extraction of *A. spinosum* cultures, and procedures that reduce their formation. However, the mechanism of formation and identities of these analogues were unclear.

Enzymatic activity can modify the chemical structure of toxins when extracting phytoplankton (Quilliam et al., 1996). To test whether this might be the case for AZAs, the filters containing *A. spinosum* were heated in a water bath or microwave before extraction. Neither procedure reduced the formation of methylated analogues of AZA1 nor -2, indicating that enzymatic activity of the alga is not responsible for the formation of the methylated analogues.

(Brondz et al., 2007) reported that extractions of natural products with MeOH may produce methyl esters of fatty acids or other molecules with a carboxylic acid groups. Methyl esters of AZAs (now known to be methyl ketals, see below) have also been reported as storage artefacts (Rehmann et al., 2008), and the proportion of these was reduced when acetone, MeCN or DCM were used (Table 6 and Table 7).

Table 6. Percentage of AZA analogue as a function of the harvesting (filtration or centrifugation) and extraction (MeOH or acetone) procedure for 3 consecutive extraction cycles

	AZA1 (%)	AZA2 (%)	AZA1 methyl ester (%)	AZA2 methyl ester (%)
Filtration & MeOH	49	18	31	3
Centrifugation & MeOH	71	26	3	0
Filtration & Acetone	55	20	23	2
Centrifugation & Acetone	73	25	2	0

Table 7. Yield of AZA (fg.cell^{-1}) with extraction solvent from *A. spinosum* pellets after centrifugation. Values with different letters are statistically different at P<0.05, (n=3).

Solvent	AZA1	AZA2	AZA1 methyl ester	Total
MeOH	85±8	25±2	3.7±0.3 (d)	114±10
Acetone	87±5	24±1	2.4±0.3 (c)	113±6
MeCN	95±5	26±1	0.8±0.1 (b)	122±7
EtOH	94±10	26±2	3.7±0.2 (d)	124±11
DCM	82±2	22±1	0.3±0.1 (a)	104±3

To confirm that the two methylated analogues (subsequently identified as AZA1 and AZA2 methyl esters) were artefacts of the extraction/reconstitution process, and to determine at which stage formation occurred, extraction and reconstitution were carried out using MeOH or CD_3OD. The formation of methylated analogues took place mainly during extraction but also, to a lesser degree, during reconstitution of the residue after evaporation with MeOH (Figure 33). Therefore, these two analogues were now clearly identified as artefacts from extraction with MeOH, and were suspected to be AZA1 and -2 methyl esters.

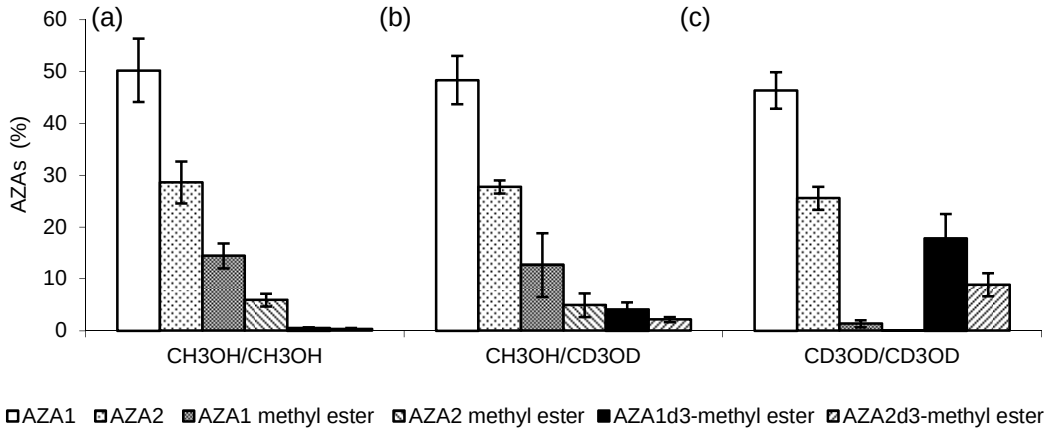


Figure 33. Percentages of AZA analogues from *A. spinosum* collected by centrifugation after: (a) extraction and reconstitution with MeOH; (b) extraction with MeOH and reconstitution with CD_3OD, and; (c) after extraction and reconstitution with CD_3OD

Methyl esters of AZA1–3 and 6, denoted as AZA29–32, were reported by Rehmann et al. and were identified as artefacts of storage in MeOH (Rehmann et al., 2008). Specimens containing AZA30 and -32 as artefacts of long term storage in MeOH from NRCC were analysed by LC-MS/MS (Figure 34, Figure 35, Figure 36 and Annexe 2). The retention times and mass spectra (API-4000, linear ion trap and Agilent 6540 QTOF) of AZA30, -32 were different to those of the two methylated AZA-extraction artefacts observed in the present study (Figure 34, Figure 36 and Annexe 3).

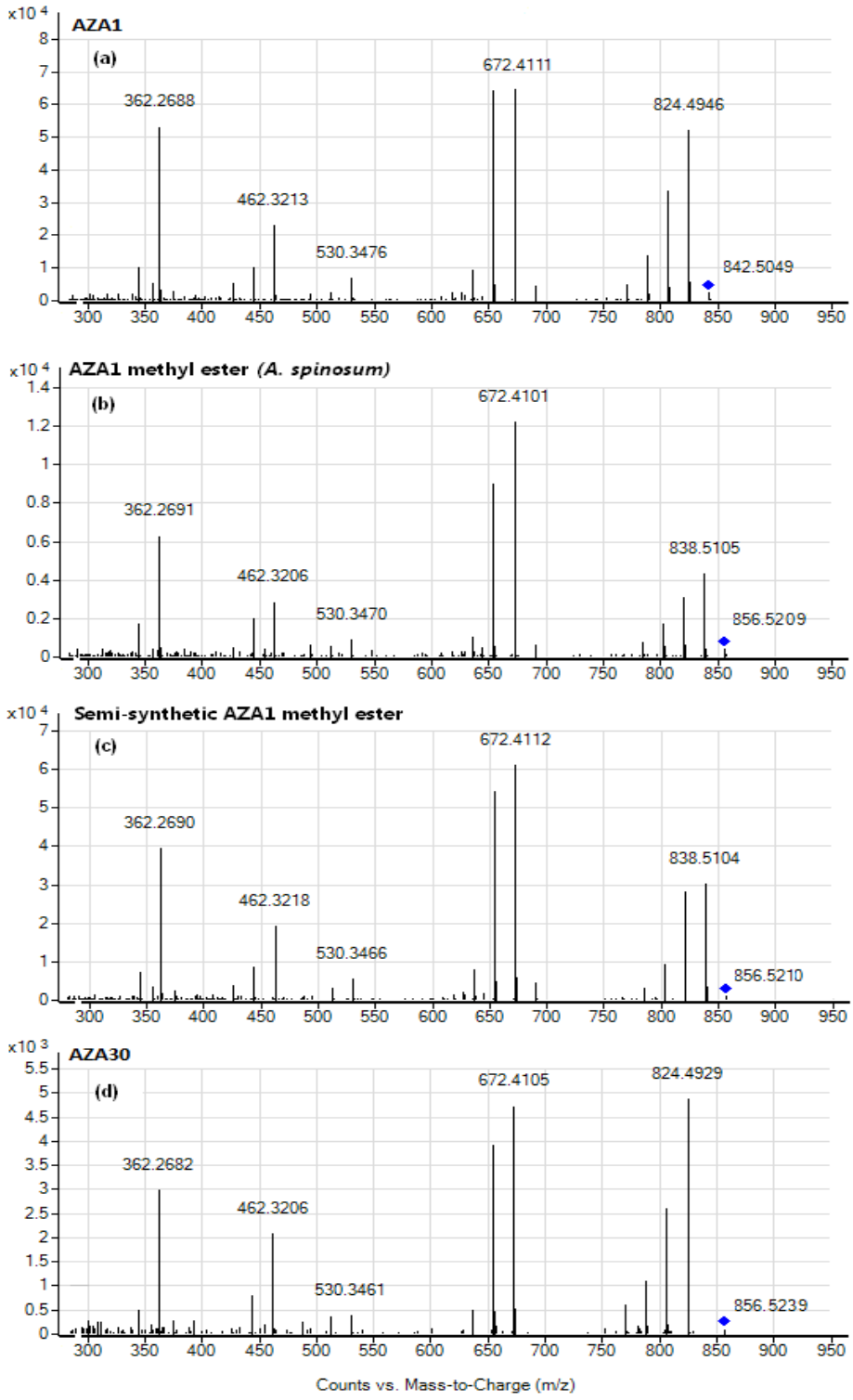


Figure 34. High resolution mass spectra of: (a) AZA1; (b) AZA1 methyl ester of AZA1 obtained through extraction from *A. spinosum*; (c) semi-synthetic AZA1 methyl ester, and; (d) AZA30 (AZA1 methyl ketal) obtained as an artefact of storage of AZA1 in MeOH

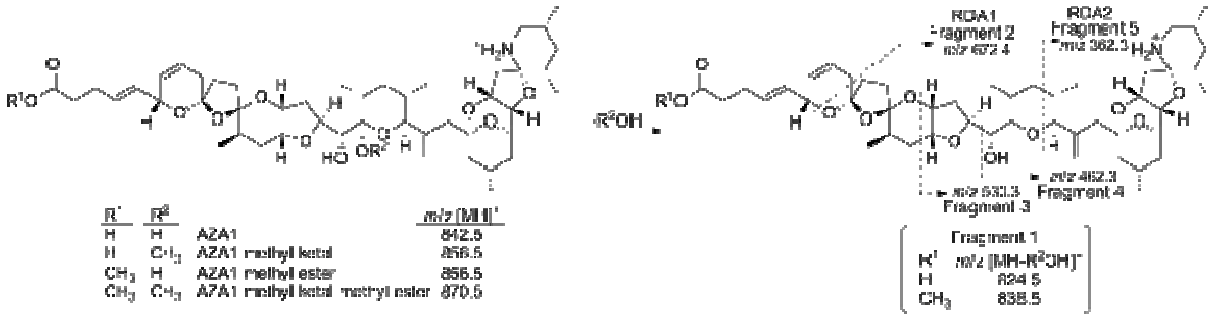


Figure 35. Structure and *m/z* for $[M + H]^+$ ions of AZA1, AZA1 methyl ketal, AZA1 methyl ester, AZA1 bismethyl ketal ester and MS/MS fragmentation with indicated groups

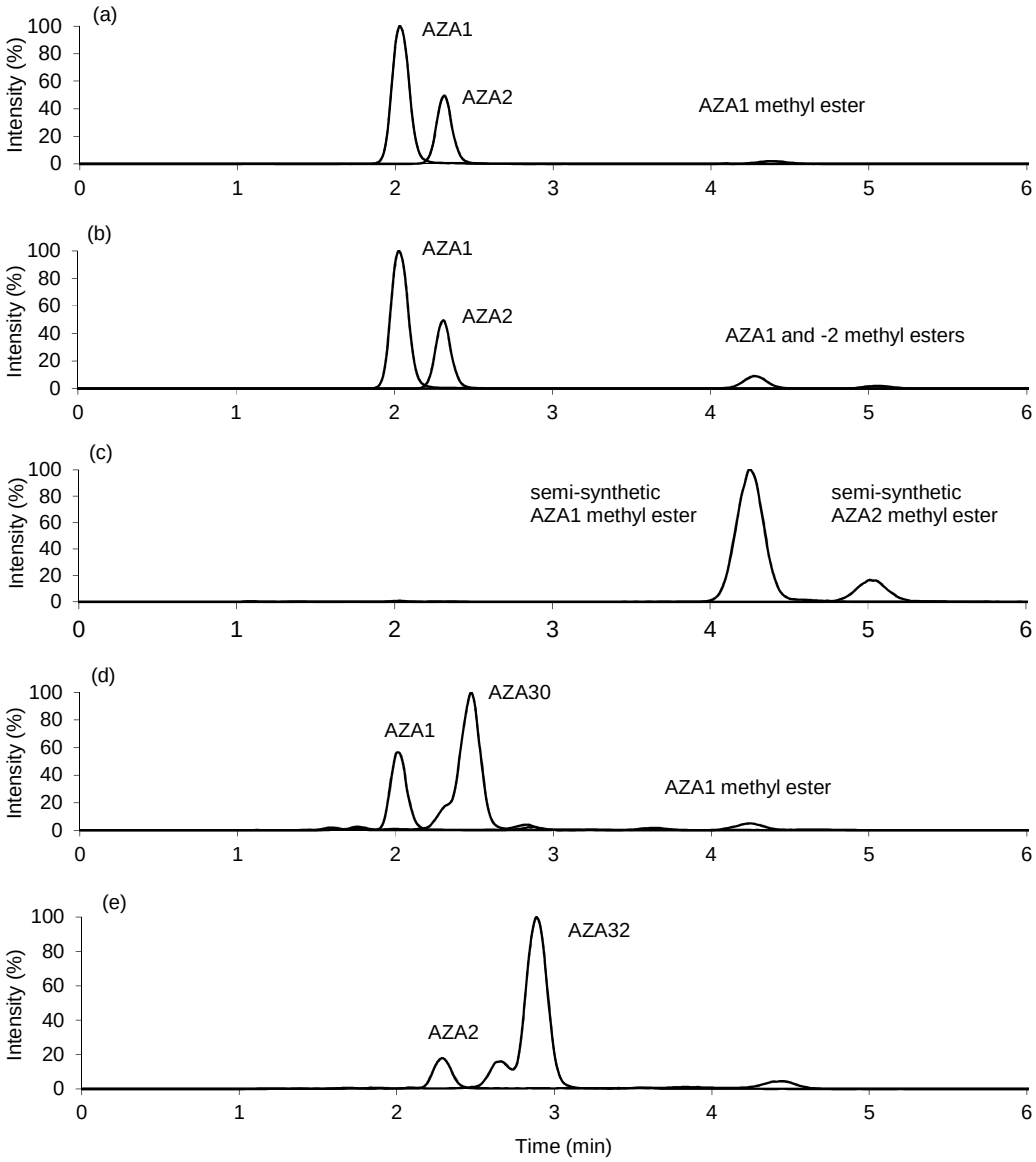


Figure 36. LC-MS chromatograms of AZAs obtained with an isocratic elution: (a) *A. spinosum* after extraction with acetone; (b) *A. spinosum* after extraction with MeOH; (c) semi-synthetic AZA1 and AZA2 methyl esters produced

with diazomethane; (d) AZA1 standard after long-term storage in MeOH, and; (e) AZA2 standard after long-term storage in MeOH

High resolution mass spectrometry was consistent with the chemical formulae of the molecular structures and fragments (Table 8 and Annexe 4). The observation that the methylated extraction artefacts from the present study had identical retention times and mass spectra to semisynthetic AZA1 and AZA2 methyl esters (produced by treatment with diazomethane) unambiguously identified these artefacts as the methyl esters (Figure 34 and Figure 35).

Table 8. High resolution LC-MS and LC-MS/MS data (measured *m/z* and Δ (ppm)) for AZA1, its methyl ester (extraction artefact from *A. spinosum*, and semi-synthetic), and AZA30 (AZA1 methyl ketal). Fragment ions correspond to Figure 31

Ion		AZA1	AZA1 methyl ester (artefact)	AZA1 methyl ester (semi-synthetic)	AZA30
$[MH]^+$	Formula	$C_{47}H_{72}NO_{12}^+$	$C_{48}H_{74}NO_{12}^+$	$C_{48}H_{74}NO_{12}^+$	$C_{48}H_{74}NO_{12}^+$
	m/z (Δ)	842.5049 (0.0)	856.5209 (0.5)	856.5210 (0.6)	856.5239 (4.0)
$[MH-ROH]^+$	Formula	$C_{47}H_{70}NO_{11}^+$	$C_{48}H_{72}NO_{11}^+$	$C_{48}H_{72}NO_{11}^+$	$C_{47}H_{70}NO_{11}^+$
(Fragment 1)	*m/z* (Δ)	824.4946 (0.4)	838.5105 (0.6)	838.5104 (0.5)	824.4929 (1.7)
Fragment 2	Formula	$C_{38}H_{58}NO_9^+$	$C_{38}H_{58}NO_9^+$	$C_{38}H_{58}NO_9^+$	$C_{38}H_{58}NO_9^+$
(RDA 1)	*m/z* (Δ)	672.4111 (0.7)	672.4101 (0.7)	672.4112 (0.9)	672.4105 (0.2)
Fragment 3	Formula	$C_{31}H_{48}NO_6^+$	$C_{31}H_{48}NO_6^+$	$C_{31}H_{48}NO_6^+$	$C_{31}H_{48}NO_6^+$
	m/z (Δ)	530.3476 (0.0)	530.3470 (1.1)	530.3466 (1.9)	530.3461 (2.8)
Fragment 4	Formula	$C_{27}H_{44}NO_5^+$	$C_{27}H_{44}NO_5^+$	$C_{27}H_{44}NO_5^+$	$C_{27}H_{44}NO_5^+$
	m/z (Δ)	462.3213 (0.2)	462.3206 (1.7)	462.3218 (0.9)	462.3206 (1.7)
Fragment 5	Formula	$C_{22}H_{36}NO_3^+$	$C_{22}H_{36}NO_3^+$	$C_{22}H_{36}NO_3^+$	$C_{22}H_{36}NO_3^+$
(RDA 2)	*m/z* (Δ)	362.2688 (0.6)	362.2691 (0.3)	362.2690 (0.0)	362.2682 (2.2)

Thus, AZA30 and -32 are methylated derivatives of AZA1 and -2, respectively, but are not methyl esters. (Rehmann et al., 2008) reported an initial loss of 32 amu in the mass spectra of AZA30 and -32, suggesting the loss of MeOH. However, we observed only initial loss of 18 amu (H_2O) in the mass spectra of authentic AZA1 and -2 methyl esters (Table 8 and Annexe 4). A plausible hypothesis is that AZA30 and -32 are 21-methyl ketals formed by exchange at the 21-OH hemi ketal of AZA1 and AZA2, respectively, with MeOH. This proposal is consistent with the observed initial losses of H_2O from AZA1 and -2 and their methyl esters, and of CH_3OH from the corresponding methyl ketals (Figure 34 and Figure 37). To test this hypothesis, samples containing AZA1 and -2, their methyl esters, and AZA30 and -32 were treated with periodate, which oxidatively cleaves the 20,21-diol in AZAs to

form a lactone derivative (Figure 37) under mild conditions (McCarron et al., 2009; Rehmann et al., 2008). LC-MS/MS analysis showed complete conversion of AZA1 and -2, and of their methyl esters, to the lactone, whereas no detectable reaction occurred with AZA30 and -32, indicating that the latter compounds have been modified in the 20,21-diol moiety (Figure 37).

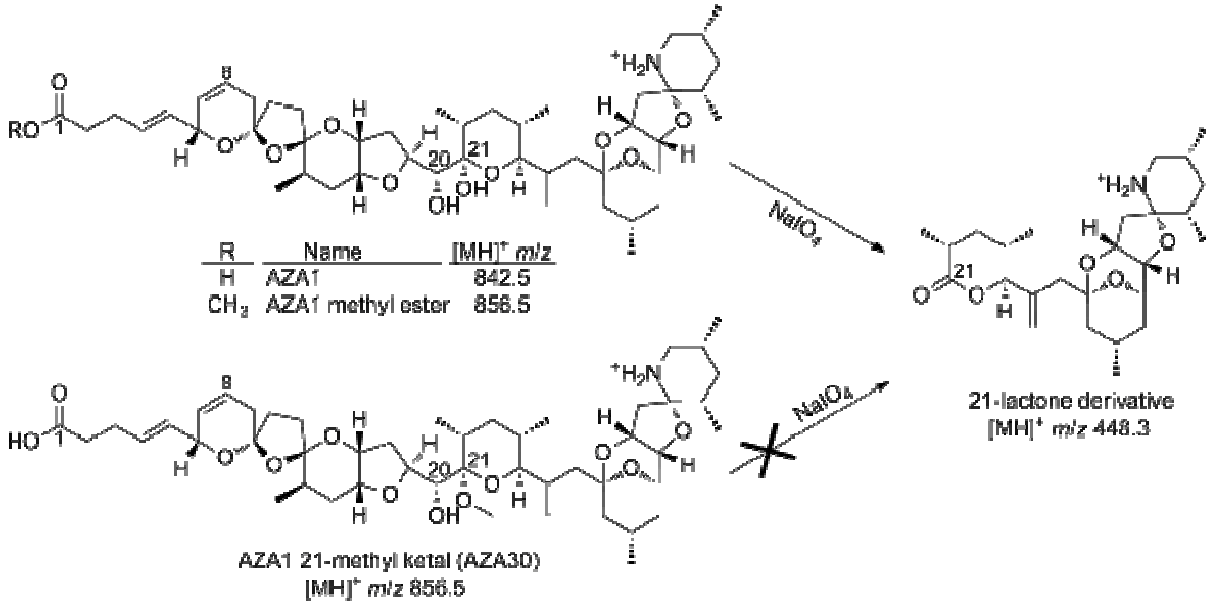


Figure 37. Fate of AZA1 and its methylated derivatives when treated with sodium periodate, which oxidatively cleaves the 20,21-diol group present in naturally occuring AZAs.

LC-MS analysis in negative ion SIM mode (Ofuji et al., 1999a) established the presence of a free carboxyl in AZA30 and -32, and the absence of a free carboxyl in AZA1 and -2 methyl esters. Only AZAs with free carboxylic acid groups would be negatively ionised in electrospray MS, and it was found that no signal was obtained for AZA1 and -2 methyl esters, while AZA1, -2, -30 and -32 were detected (Annexe 5). Additionally, derivatization with ADAM produced (9-anthryl)methyl ester derivatives of AZA1, -2, -30 and -32 (yields >98%), whereas the methyl esters of AZA1 and AZA2 were unaffected (Figure 31 and Annexe 5). These results strongly support the hypothesis that AZA30 and -32 are the methyl ketals of AZA1 and -2, respectively, and are not methyl esters as originally reported by (Rehmann et al., 2008), and it seems likely that this is also the case for AZA29 and -31 (reported as methyl esters of AZA3 and -6 by (Rehmann et al., 2008)). The methylation artefacts from extraction of *A. spinosum* cultures are unambiguously identified as AZA1 and AZA2 methyl esters, and the "AZAX" observed in methanolic extracts by (Krock et al., 2009) appears to be confirmed as AZA1 methyl ester ("AZAX" mass spectrum observed by (Krock et al., 2009) is presented in the Annexe 6).

1.6.2. Evaluation of extraction protocols of azaspiracid from *A. spinosum*

1.6.2.1. Particulate and dissolved toxins

After the aliquots were sampled from the bioreactor, intra- and extra-cellular toxin contents were determined in triplicate immediately or after resting periods, with different solvents, with an aliquot of culture (10 mL) taken to assess morphological changes for each of the resting periods.

In laboratory culture, AZAs produced by *A. spinosum* were clearly intra-cellular. When cells were gently separated from the culture medium either by filtration or centrifugation (no cells were detected under the inverted microscope in the filtrate or supernatant), most toxins were found in the particulate fraction (95%). A significant loss of intra-cellular AZAs, with a concomitant increase in extra-cellular AZAs, was observed with increasing time between sampling and centrifugation. Apparently, increased residence time of cells in medium outside the bioreactor led to handling stress of the cells, which in turn resulted in a substantial loss of cell bound toxins to the dissolved phase (Annexe 7). A significant loss of intra-cellular toxins (> 10 %) occurred when cells were stressed for more than 60 min and a maximum proportion of extra-cellular toxins of 24% was observed after 5 h. Therefore, we recommend that samples should be centrifuged immediately when the cell quota of AZAs is being determined.

Microscopical observation showed that, under these stress conditions, an increasing number of cells without theca were produced, although cells generally kept their integrity (Annexe 8). It is a common reaction among dinoflagellates to adverse conditions that cells leave their theca (*ecdysis*), and is often connected with the formation of temporary cysts (Pfiester and Anderson, 1987). This type of dinoflagellate cyst is normally round and surrounded by a thin cell wall. However, this has not yet been observed for *A. spinosum*. The reason for the increase in extra-cellular toxins is not clear; shedding of the cells outer layer including thecal plates and their membrane vesicles might be associated with a pulsed toxin loss or extruded protoplasts may have a higher exudation rate. However, the possibility that total disintegration of a small portion of the cells contributed to the increase of extra-cellular toxins cannot be excluded.

1.6.2.2. Effect of centrifugation and filtration on toxin recovery and profile

An initial experiment was carried out in triplicate with MeOH as extraction solvent to evaluate AZA yield after filtration of 10 mL of *A. spinosum* culture on GF/C glass microfiber

filters (25 mm diameter), or after centrifugation (2500 *g*, 4 °C, 20 min). Both filtrate and supernatant were kept for liquid–liquid extraction with DCM (Figure 32). Five successive extractions were carried out on each filter or pellet, to ensure complete toxin recovery. A second trial was carried out under the same conditions with either MeOH or acetone as extraction solvent in three successive extractions.

The total amount of AZAs obtained after 5 successive extractions with MeOH was not significantly different when using either filtration or centrifugation as a method to separate cells from culture medium. If only three extractions with either MeOH or acetone were carried out, the yield was significantly lower ($P<0.05$) by filtration. Moreover, the AZA ratios were different for filtration compared to centrifugation (Table 6). High levels of AZA1 and -2 methyl esters were obtained with filtration. Glass microfiber filters contain silica and are known to catalyse some reactions, which may possibly explain the methylation of AZA1 and -2 observed in these experiments. If filtration is necessary, it is recommended to study other types of filters with *A. spinosum* (e.g. polycarbonate filters).

1.6.2.3. Influence of extraction solvent composition

To determine the procedure with the best extraction yield and minimal formation of artefacts (methylated AZAs), the extraction procedure described in Figure 32 was applied using a variety of solvents and solvent compositions. Four experiments were carried out: (a) 100% MeOH, acetone, MeCN, EtOH, or DCM, (b) MeOH–H_2O, 10:0, 9:1, 8:2, 7:3, 6:4 (v/v), (c) Acetone–H_2O, 10:0, 9:1, 8:2, 7:3 (v/v), (d) MeCN–H_2O, Acetone-H_2O, MeOH-H_2O, (10:0, 9:1 (v/v) each).

Among MeOH, acetone, MeCN, EtOH and DCM, no significant differences were observed on AZA1, AZA2 and total AZA cell contents. Nonetheless, significant differences were observed in the content of AZA1 methyl ester (Table 7), with increased formation of this derivative when extracting with MeOH or EtOH (in the following order MeOH = EtOH > acetone > MeCN > DCM). No ethyl analogue was observed when extracting with EtOH. It is not entirely clear how this methyl analogue formation occurs.

The formation of the methyl esters of AZA1 and -2 was variable from one experiment to the next and concentrations of AZA1 methyl ester may range from 3 to 15% (Table 6, Table 7, and data not shown) when using MeOH as extraction solvent and centrifugation as separation technique.

As expected, the formation of AZA1 methyl ester is significantly reduced when extracting with acetone, MeCN and DCM; however, detectable traces are still formed with

these solvents used in extraction. This observation led us to hypothesize that reconstitution in MeOH by itself may lead to formation of AZA methyl esters. This hypothesis has been confirmed using deuterated MeOH (see previous section).

It is common to add some water to an organic solvent to increase the extraction yield or to minimise the extraction of lipids which could lead to matrix effects (McNabb et al., 2005). However, in the present study, no statistical differences were observed among 100, 90 and 80% MeOH or acetone. Nonetheless, below a ratio of 7:3 organic solvent–H_2O, the yield decreased and was significantly lower than with MeOH.

Extraction with acetone was considered to be most appropriate, as it reduces the formation of AZA methyl esters and is easier to handle thanks to its ease of evaporation and low toxicity. However, acetone extracts may also result in more complex crude extracts, as acetone is a good solvent for extracting lipids and pigments (Lorenzen, 1967). This was an additional reason to evaluate matrix effects in further trials.

1.6.2.4. Effect of sample size

The effect of sample size on the extraction yield was studied following the standard extraction procedure (Figure 32) with acetone–H_2O (9:1 v/v) as extraction solvent. The *A. spinosum* culture used for this experiment had a cell concentration of 161000 ± 1000 cell mL^{-1}, corresponding to a biovolume of 92.6×10^6 μm^3 mL^{-1}. Sample volumes were as follows: 0.62, 3.1, 6.2, 10, 31, 50 mL.

The extraction yield differed significantly as a function of sample volume. Yields were somewhat higher in the middle of the studied range (3.1–31 mL). More extraction cycles or higher solvent-to-sample ratios could potentially be used to increase AZA yield for the large sample volume (50 mL). However, the procedure was not suitable for small amounts of biomass/volume (<1–2 mL), potentially reflecting that small losses become significant when handling low amounts of toxin. Alternative protocols may need to be developed for samples below 200000 cells, such as the procedure described during the identification of *A. spinosum* (Tillmann et al., 2009).

1.6.2.5. Recovery and yield after successive extractions in presence or absence of matrix

Extraction yield and recovery were tested following the standard procedure with one to five successive extractions with either MeOH–H_2O or acetone–H_2O (9:1 v/v), with or without matrix as follows:

(a) without matrix: AZA1 (40 µL) solution (0.53 µg mL^{-1}) was transferred into 15 mL centrifuge tubes, and extracted with 500 µL of organic solvent water mixtures. The extract was reconstituted in 500 µL MeOH–H_2O (9:1 v/v). A control in triplicate with 40 µL of AZA1 solution and 460 µL of MeOH–H_2O (9/1 v/v) was used to estimate the recovery.

(b) with algal matrix: Aliquots (10 mL) of *A. obesum* culture (50000 ± 2000 cell mL^{-1}, 65.2×10^6 µm^3 mL^{-1}) were centrifuged in 15 mL centrifuge tubes. After decanting the supernatant, AZA1 (40 µL) solution was added to the pellet, extracted as described above. The extract was reconstituted in 500 µL MeOH–H_2O (9:1 v/v). For controls, *A. obesum* culture (10 mL) was extracted using MeOH–H_2O or acetone–H_2O (9:1 v/v) in triplicate (Figure 32). The control extracts were evaporated to dryness and subsequently taken up with 460 µL MeOH–H_2O (9:1 v/v) and 40 µL of AZA1 solution.

Two successive extractions were sufficient in all cases (100% of recovery at 95% confidence level) while three consecutive extractions reduced the deviations observed for triplicate samples. The presence of matrix did influence recovery. Without matrix, one extraction was almost sufficient to recover all AZA, whereas two successive extractions were necessary when *A. obesum* matrix was present. No significant differences were observed between acetone and MeOH on AZA recovery in the presence, or absence, of matrix when two or three successive extraction cycles were carried out.

1.6.3. Evaluation of matrix effects on LC-MS/MS analysis

Matrix effects were assessed for AZA1 using MeOH and acetone extracts of *A. obesum* (Figure 38a and b, respectively), applying the standard addition method as described in (Fux et al., 2008b). Negligible effect (+1.4%) was observed when using MeOH, and more significant signal suppression (−8.4%) was observed with acetone. For *A. spinosum*, suppression effects of −7.7% and −6.4% were measured for MeOH and acetone extracts, respectively (Figure 38c and d).

Following the approach used by (Fux et al., 2008b) matrix effects were also assessed by varying matrix strength while maintaining a constant AZA1 concentration (Figure 38e). No significant matrix effects were detected with different amounts of biomass, as all values measured were within the precision of the experiment.

Previously, matrix effects were considered significant at values greater than 10% enhancement or suppression (Fux, 2008; Fux et al., 2008b), due to the repeatability of an analytical method with LC-MS/MS. Following this arbitrary limit of significance, the effects

observed here for *A. spinosum* analysis can be considered insignificant. It should be stressed that the analysis of AZAs from *A. spinosum* at this scale typically deals with much less matrix content compared to shellfish. From large-scale extraction experiments (data not shown), it is estimated that the most concentrated matrix crude extract with MeOH in our study contained ca. 2 mg.mL^{-1}, which is at the lowest value of the range evaluated for a shellfish matrix by (Fux et al., 2008b).

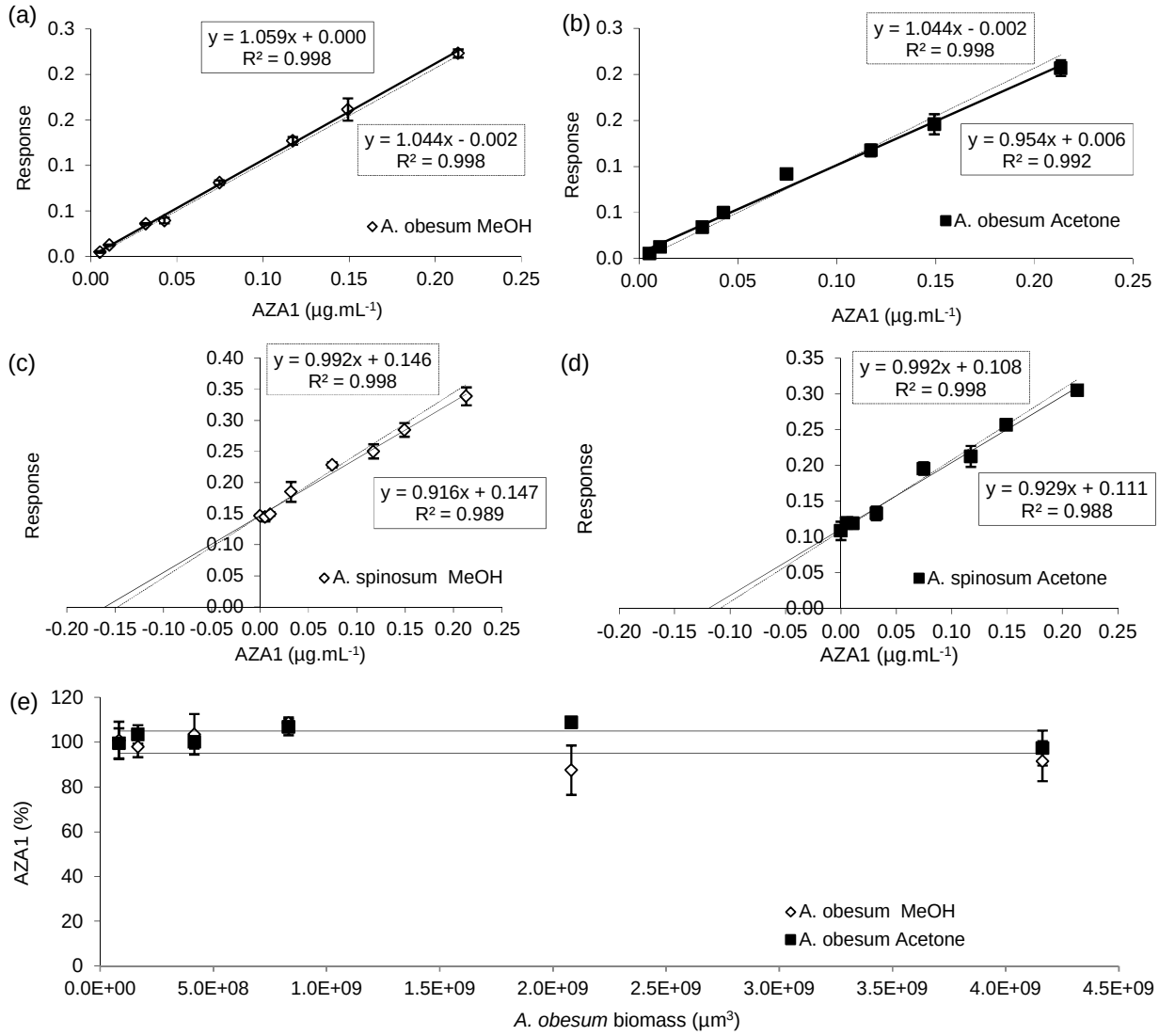


Figure 38. Slopes, intercepts, correlation coefficients for quantitation of AZA1 in methanolic or acetone extracts of *A. obesum* and *A. spinosum* spiked with standards (a, b, c, d) and as a function of the sample size(% recovered) (e) using LC-MS/MS with isocratic elution. Lines represent the least-squares correlation for quadruplicate injections of spiked matrix-free solutions (a, b, c, d) (dashed lines) and triplicate injection of spiked solutions with matrix (a, b, c, d) (solid lines). The solid lines in (e) represent the 95% confidence interval obtained from triplicate injection of spiked matrix-free MeOH solutions. (notice initial response difference between (c) and (d) was due to different sampling day)

1.7. Conclusions

These results highlight the importance of carefully studying sample preparation, extraction procedures and solvent choice for assessing the recovery of the method and possible matrix effects.

Based on the results of this study, the following procedures are recommended for the analysis of AZA-1 and -2 in *A. spinosum*:

- Sample and immediately separate the cells from the culture medium by centrifugation.
- Extract AZAs with acetone or MeCN; acetone is most appropriate as it reduces both the formation of methyl analogues and is easy to handle thanks to its ease of evaporation and low toxicity. MeOH is inappropriate due to possible artefact formation.
- Two to three successive extractions are suggested to ensure high extraction yield.
- No significant matrix effects were observed during LC-MS/MS analysis with acetone or MeOH under the conditions tested.

This work clarifies the formation of AZA artefacts during extraction of *A. spinosum*, describes mass spectral fragmentation of two AZA methyl esters, and corrects the chemical structures of AZA29–32. Furthermore, the procedure developed allows quantitation of AZAs in algal cultures and thus will facilitate the optimisation of processes aimed at the preparative isolation of AZAs required for the sustainable supply of AZAs for instrument calibration.

1.8. Acknowledgements

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2. Production and isolation of azaspiracid-1 and -2 from *Azadinium spinosum* culture in pilot scale photobioreactors

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2.1. Résumé

La contamination des mollusques bivalves par les azaspiracides (AZA) est un problème récurrent dans le nord de l'Europe qui peut conduire à des intoxications alimentaires lors de la consommation de coquillages contaminés. Par conséquent, il est important d'avoir une production durable d'AZA, sans avoir à compter sur des événements toxiques naturels, pour des études toxicologiques et pour les réseaux de surveillance.

Une culture en continue d'*A. spinosum* à une échelle pilote a été réalisée pour évaluer la faisabilité de la production d'AZA. Les microalgues ont été récoltées en utilisant une procédure de filtration tangentielle ou la centrifugation en continu. Les AZA présents dans le perméat ont ensuite été extraits par des procédures d'extraction en phase solide (EPS) et purifiés.

Lorsque deux photobioréacteurs sont connectés en série, les concentrations cellulaires atteignent 190×10^3 et 210×10^3 cellules mL^{-1} à l'état stationnaire, dans les bioréacteurs 1 et 2, respectivement. Le quota cellulaire d'AZA diminue lorsque le taux de dilution augmente de 0,15 à 0,3 $jour^{-1}$, avec une production toxinique optimale à 0,25 $jour^{-1}$. Après optimisation, les procédures d'EPS ont permis la récupération de 79 ± 9% des AZA. La procédure d'isolement précédemment mise au point sur des glandes digestives de moules a pu être optimisée et adaptée pour isoler les AZA à partir des extraits d'*A. spinosum*, de telle sorte que seulement quatre étapes de purifications ont été nécessaires pour obtenir AZA1 et -2 purifiés. Un rendement de plus de 70% a été atteint et cette procédure a permis d'isoler 9,3 mg d'AZA1 et 2,2 mg d'AZA2 à partir de 1200 L de culture avec une pureté supérieure à 95%. Ce travail a démontré la faisabilité d'une production durable d'AZA1 et -2 à partir de cultures en continu d'*A. spinosum*.

2.2. Abstract

Azaspiracid (AZA) poisoning has been reported following consumption of contaminated shellfish, and is of human health concern. Hence, it is important to have sustainable amounts of the causative toxins available for toxicological studies and for instrument calibration in monitoring programs, without having to rely on natural toxin events. Continuous pilot scale culturing was carried out to evaluate the feasibility of AZA production using *Azadinium spinosum* cultures. Algae were harvested using tangential flow filtration or continuous centrifugation. AZAs were extracted using solid phase extraction (SPE) procedures, and subsequently purified. When coupling two stirred photobioreactors in series, cell concentrations reached 190×10^3 and 210×10^3cell·mL^{-1} at steady state in bioreactors 1 and 2, respectively. The AZA cell quota decreased as the dilution rate increased from 0.15 to 0.3 day^{-1}, with optimum toxin production at 0.25 day^{-1}. After optimization, SPE procedures allowed for the recovery of 79 ± 9% of AZAs. The preparative isolation procedure previously developed for shellfish was optimized for algal extracts, such that only four steps were necessary to obtain purified AZA1 and -2. A purification efficiency of more than 70% was achieved, and isolation from 1200 L of culture yielded 9.3 mg of AZA1 and 2.2 mg of AZA2 of >95% purity. This work demonstrated the feasibility of sustainably producing AZA1 and -2 from *A. spinosum* cultures.

2.3. Keywords

Solid phase extraction; photobioreactor; chemostat; dinoflagellate; microalgae; LC-MS/MS; tangential flow filtration; azaspiracid; HP-20

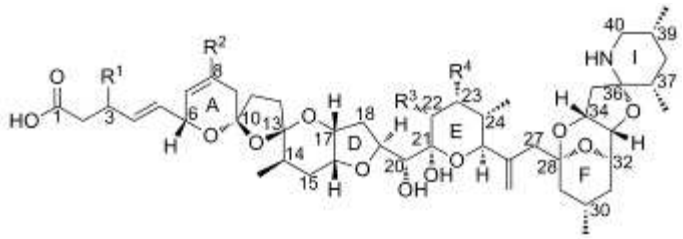
2.4. Introduction

Four groups of lipophilic marine algal toxins are currently regulated in Europe; among which azaspiracids (AZA) is the most recently discovered group of toxins. In 1995, the first human intoxication with AZA occurred in the Netherlands after consumption of contaminated mussels from Ireland, with symptoms similar to diarrhetic shellfish poisoning (nausea,

stomach cramps, vomiting and diarrhea) (McMahon and Silke, 1996). A few years later, the toxin was named AZA following identification and isolation from contaminated shellfish (Satake et al., 1998c), and the structure was subsequently revised thanks to synthetic studies (Nicolaou et al., 2006b). Afterwards, a number of analogues were discovered in mussel tissues using biological assays and chemical analysis, including mass spectrometric techniques, *i.e.*, AZA2-32 (Diaz Sierra et al., 2003; James et al., 2003b; McCarron et al., 2009; Ofuji et al., 2001; Ofuji et al., 1999a; Rehmann et al., 2008). Nevertheless, since the first known poisoning event, twelve years passed until the discovery of a primary producer, the dinoflagellate *Azadinium spinosum* (strain 3D9) (Krock et al., 2008; Krock et al., 2009; Tillmann et al., 2009). This small dinoflagellate (12–16 µm length and 7–11 µm width) produces AZA1 and -2 in culture (Figure 39) (Tillmann et al., 2009). Since this recent discovery, the new genus *Azadinium* has been encountered in different parts of the world (Ireland (Salas et al., 2011), Mexico (Hernandez-Becerril et al., 2010), Argentina (Akselman and Negri, 2010) and Korea (Potvin et al., 2012)). Furthermore, azaspiracids were found in Europe, America, North Africa, and Asia (Alvarez et al., 2010; Amzil et al., 2008; James et al., 2002; Taleb et al., 2006; Twiner et al., 2008; Ueoka et al., 2009; Vale et al., 2008), and AZA events are now recognized as a world-wide phenomenon (Twiner et al., 2008). Interestingly, the AZAs known to be implied in food poisoning have not been shown to be produced by species other than *A. spinosum* (and by metabolism of AZA1 and -2 in *Mytilus edulis*), e.g., *Azadinium obesum* (Tillmann et al., 2010) and *Azadinium poporum* (Potvin et al., 2012; Tillmann et al., 2011). However, (Krock et al., 2012) recently found compounds structurally related to AZAs in a culture of *A. poporum*, and confirmation of the toxicity of these compounds will be important to assess the homogeneity of the genus with regards to toxin production and structure-activity.

Currently, the main sources of marine algal toxins for purification are derived from producing organisms in culture or harvested from natural blooms (e.g., okadaic acid group toxins (Miles et al., 2006), brevetoxins (Abraham et al., 2006), saxitoxins (Laycock et al., 1994), yessotoxins (Loader et al., 2007), cyclic imines (Miles et al., 2003; Selwood et al., 2010; Torigoe et al., 1988) and pectenotoxins (Miles et al., 2004b)), from contaminated shellfish (Kilcoyne et al., 2012; Perez et al., 2010), or from bulk extraction of environmentally contaminated HP-20 resin (Rundberget et al., 2007). Isolation from the producing organism is preferred, as extracts are considerably purer than shellfish extracts and their availability is not dependent on the occurrence of natural toxic episodes.

Figure 39. Azaspiracid (AZA) structures and mass-to-charge ratios (*m/z*) for the molecular ions $[M + H]^+$ detected in *A. spinosum* and in mussels (*M. edulis*). Toxins found in *A. spinosum* are shown in bold text.

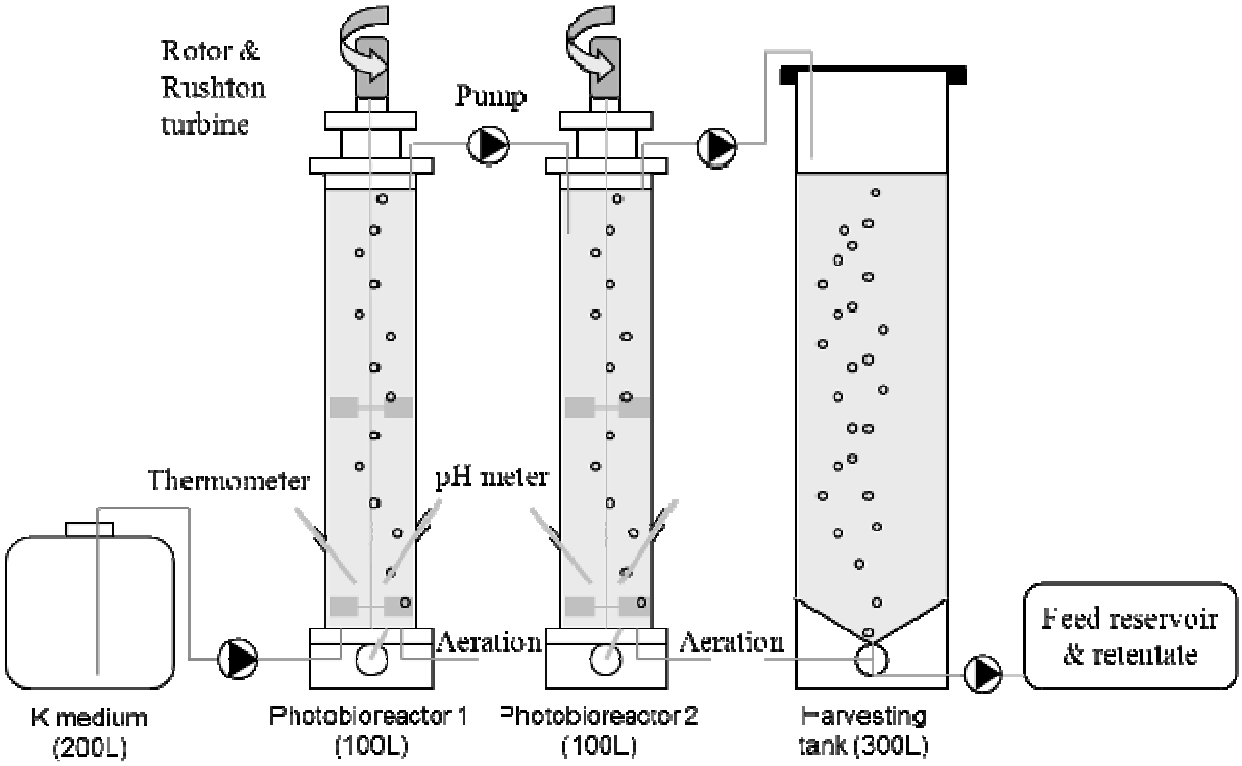


	R1 (C3)	R2 (C8)	R3 (C22)	R4 (C23)	$[M + H]^+$ *m/z*
Azaspiracid-1	**H**	**H**	**CH_3**	**H**	**842.5**
Azaspiracid-2	**H**	**CH_3**	**CH_3**	**H**	**856.5**
Azaspiracid-3	H	H	H	H	828.5
Azaspiracid-4	OH	H	H	H	844.5
Azaspiracid-5	H	H	H	OH	844.5
Azaspiracid-6	H	CH_3	H	H	842.5
Azaspiracid-7	OH	H	CH_3	H	858.5
Azaspiracid-8	H	H	CH_3	OH	858.5
Azaspiracid-9	OH	CH_3	H	H	858.5
Azaspiracid-10	H	CH_3	H	OH	858.5
Azaspiracid-11	OH	CH_3	CH_3	H	872.5
Azaspiracid-12	H	CH_3	CH_3	OH	872.5
Azaspiracid-13	OH	H	H	OH	860.5
Azaspiracid-14	OH	H	CH_3	OH	874.5
Azaspiracid-15	OH	CH_3	H	OH	874.5
Azaspiracid-16	OH	CH_3	CH_3	OH	888.5
Azaspiracid-17	H	H	CO_2H	H	872.5
Azaspiracid-19	H	CH_3	CO_2H	H	886.5
Azaspiracid-21	OH	H	CO_2H	H	888.5
Azaspiracid-23	OH	CH_3	CO_2H	H	902.5

Purification of phycotoxins is essential as there is currently a shortage of pure calibration and reference materials for phycotoxin monitoring in food (Hess et al., 2007). This has become particularly important as micro-algal lipophilic toxins in contaminated shellfish are now monitored in Europe using LC-MS/MS as a reference method (Anonymous, 2011), and AZA analysis by LC-MS/MS requires purified AZA standards for quantitation. Naturally occurring *A. spinosum* blooms are hard to predict and/or find, as very little data are available on its life history. The organism is small and hard to differentiate under light microscopy from other small dinoflagellates such as *Heterocapsa* and similar genus. These difficulties hinder identification of such blooms and prediction of subsequent shellfish contaminations that could be used for the necessary purifications. Sustainable production of toxins from *A. spinosum* culture would thus be desirable for instrument calibration in monitoring programs and for toxicological studies.

The aim of this study was to evaluate the feasibility of azaspiracid production from *A. spinosum* in pilot scale photobioreactors. In previous studies, a continuous system was developed (Séchet et al., 2003, 2004). Two stirred photobioreactors were coupled in series (Figure 40) to assess how dilution rate influences cell concentration as well as toxin production. To harvest toxin, we applied a dual approach for the recovery of AZAs from both cultured cells and from the culture supernatant. For the recovery of cells, tangential flow filtration and continuous centrifugation were evaluated. Solid phase extraction procedures were developed to recover AZAs from large volumes of *A. spinosum* culture supernatant and from concentrated cell suspensions. A method developed to purify AZA1 and -2 from crude algal extract is presented, highlighting the effectiveness of this purification procedure compared to purification of AZA1 and -2 from mussel digestive glands.

Figure 40. Schematic representation of *A. spinosum* and AZA production system using photobioreactors in series.



2.5. Materials and methods

2.5.1. Culture conditions and measurement

Strain 3D9 of *A. spinosum* was grown in two stirred photobioreactors of 100 L (1827 mm × 300 mm) operated in series and tested in separate experiments at the following dilution rates (0.1, 0.15, 0.2, 0.25 and 0.3 day^{-1} per bioreactor). Culture was collected in an aerated harvesting tank (300 L), maintained at 18°C (Figure 40). The culture medium was a K-modified medium (Keller et al., 1987), without NH_4Cl and Tris-base® but with Na_2SeO_3 enrichment (10^{-8} M).

Prior to inoculation, photobioreactors were sterilized using peroxyacetic acid (5 ppm) for 30 min. The first photobioreactor was inoculated with 30 L of *A. spinosum* culture at 70×10^3 cell·mL^{-1} and filled up at the indicated dilution rate to 100 L before filling the second photobioreactor by pumped transfer at the same flow rate.

The photobioreactors were made of transparent polymethyl methacrylate and operated under the following conditions: the pH was maintained at 7.9 by CO_2 addition and the temperature at 18 °C by adjusting room temperature to maintain 18 °C inside the bioreactors (automatic feed-back). Light was provided on one side of the reactor by fluorescent tubes with a photon flux density at 200 µmol·m^{-2}·s^{-1} (day light fluorescent tubes), and a photoperiod of 16 h of light and 8 h of dark. A Rushton turbine was used to stir the culture at 40 rpm.

Cell concentration (cell·mL^{-1}), average cell size (estimated spherical diameter, µm) and total cellular volume (µm^3·mL^{-1}) were assessed daily with a particle counter (Multisizer 3 Coulter counter, Beckman). The bioreactors were considered to have reached steady state after a minimum of five days at the same micro-algal concentration (±5%).

2.5.2. *A. spinosum* analysis

When steady state was achieved, triplicate samples of *A. spinosum* were taken from each bioreactor to assess toxin content daily over one week; the same analyses were carried out from the 300 L harvesting tank before each tangential flow filtration for initial toxin content assessment.

The analytical procedure has previously been optimized (Jauffrais et al., 2012a). Briefly, aliquots (10 mL) of *A. spinosum* culture were collected and centrifuged (2,500 *g*, 20 min, 4°C) in 15 mL tubes. The supernatant was collected (for extra-cellular toxin content) and

the pellet was re-suspended in 0.5 mL of acetone–water (9:1, v/v), transferred to an Eppendorf tube (1.5 mL) and bath-sonicated (10 min). After sonication, the aliquot was centrifuged (15,000 *g*, 10 min, 4 °C). The supernatant was transferred to a 5 mL glass tube and gently evaporated under nitrogen on a heating block at 35 °C. This process was repeated so that the pellet was extracted three times in total. After evaporation of supernatants, the residue was reconstituted in 1 mL methanol. An aliquot was filtered with Nanosep MF centrifugal filter 0.2 µm (Pall) (15,000 *g*, 3 min, 4 °C), and transferred to an HPLC vial for analysis.

The aqueous supernatant from centrifugation of the algal culture was transferred to a 15 mL glass tube and 5 mL of DCM was added. The mixture was homogenized and centrifuged (2,500 *g*, 10 min, 4 °C). The organic phase was transferred to a 15 mL glass tube and gently evaporated under nitrogen on a heating block at 35 °C. The aqueous phase was extracted three times in this manner, and following evaporation, the residue was reconstituted and filtered as above.

This last procedure was also used to estimate AZA concentration in the algal retentate and to assess AZA adsorption by the HP-20 resin.

2.5.3. Solid phase extraction procedure

SPE was carried out on Oasis HLB cartridges (Waters) to estimate AZA concentration in 50 mL samples of the permeate and to evaluate AZA adsorption kinetics by the HP-20 resin. Oasis HLB cartridges, (6 cc, 200 mg) were activated with methanol (10 mL) and washed with a solution of water–methanol (9:1 v/v, 10 mL). The sample was loaded dropwise. Once loaded, the cartridge was washed with a solution of water–methanol (9:1 v/v, 10 mL). The sample was eluted with 5 mL of methanol into a glass tube and gently evaporated under nitrogen on a heating block at 35 °C to yield a residue which was reconstituted in 0.5 mL methanol and filtered as above (Kilcoyne et al., 2012).

2.5.4. Separation of *A. spinosum* from the culture medium

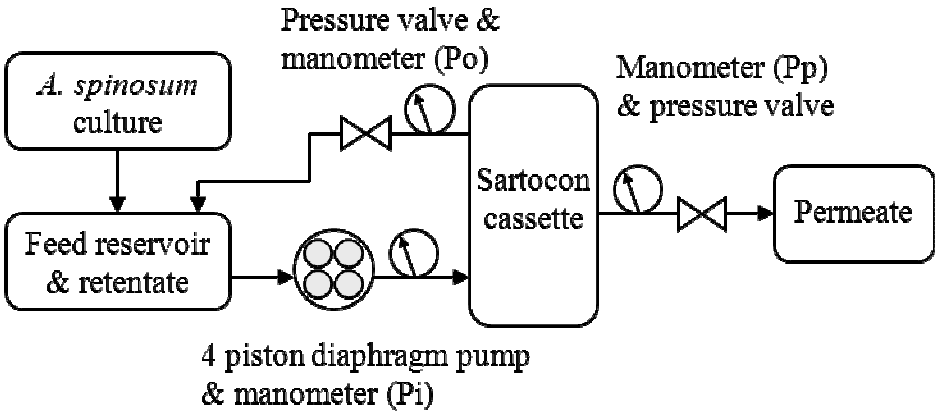
Method 1. Tangential flow filtration to separate the algae from the culture medium was based on crossflow filtration using five Hydrosart 0.1 m^2 open-channel microfiltration cassettes (Sartorius Stedim Biotech) mounted in a stainless steel holder (Sartorius Stedim Biotech) with a 4-piston diaphragm pump (Jabsco, SartoJet-Pump).

The trans-membrane pressure (TMP) for this system was defined by the following equation:

$$TMP = (Pi + Po)/2 - Pp$$

where Pi, Po and Pp are, respectively, the pressure at the inlet, outlet and permeate (Figure 41).

Figure 41. Schematic presentation of the tangential flow filtration for *A. spinosum* culture.



The pump was maintained at 70% of its capacity during filtration (8–11 L min^{-1} depending on the pressure applied), and TMP was regularly adjusted to 0.3 bar as recommended by the manufacturer, using pressure valves (Po and Pp). Thus, 200 L of *A. spinosum* culture was filtered to give less than 1 L of algal concentrate (retentate) and almost 200 L of permeate (Figure 41).

Method 2. Continuous centrifugation (Clara 20, Alfa Laval) was applied to separate the algae from the culture medium (70 L h^{-1}, 11,000 *g*, room temperature). 200 L of algal culture thus provided ~15 L of algal concentrate (retentate) and ~185 L of supernatant, from which AZAs were then extracted using methods 4 and 6 (see below).

2.5.5. Extraction of AZA1 and -2 from the retentate and permeate

Two procedures were tested for toxin extraction from the retentate:

Method 3. The algal concentrate was centrifuged (3500 *g*, 30 min, 4 °C) and collected as an algal paste. The analytical extraction procedure was scaled up (Jauffrais et al., 2012a), by three consecutive extractions of the algal paste with 5 mL of organic solvent per gram of algal paste. The combined extracts were evaporated with a rotary evaporator (Büchi Rotavapor R-200), weighed, and the residue was reconstituted in 5 mL methanol for analysis.

Method 4. The algal concentrate was sonicated pulse mode, 20 min in ice, Bioblock Scientific, Vibra-cell 75115), 25 g of activated Diaion HP-20 polymeric resin was added, and gently agitated with the algal concentrate for 24 h (the optimum contact time as determined below) on a laboratory shaker (IKA Labortechnik, KS125 basic). The resin was recovered on 100 µm phytoplankton mesh, then washed with 1 L of Milli-Q water (Millipore, Integral 3 system), and placed in a glass column (3 × 60 cm) with a glass frit at the bottom. The toxin was eluted with three volumes of acetone (50 mL) at 1 mL·min^{-1}. Subsequently, the extract was evaporated and reconstituted as above.

Two procedures were tested for extraction of AZAs from the permeate:

Method 5. Eight SPATT (solid phase adsorption toxin tracking) bags (Fux et al., 2009; Fux et al., 2008a; MacKenzie et al., 2004) each containing ~3 g of activated Diaion HP-20 resin were added to the permeate and gently agitated with a submerged pump over 72 h (72 h was the optimum, with 1, 15, 24 and 72 h having been tested). The SPATT bags were then recovered, opened, and the resin was washed and extracted as in Method 4.

Method 6. A submerged pump (20 L min^{-1}) was placed in the permeate and used to circulate the permeate through a column containing 25 g of activated Diaion HP-20 resin for 72 h. The resin was then washed and extracted as above. This procedure was an adaptation of (Rundberget et al., 2007) developed for large scale extraction of microalgal biotoxins *in situ*.

Extraction procedures involving Diaion HP-20 polymeric resin were optimized in the present study to determine the best extraction yield. Four experiments were carried out. Acetone was chosen as eluent to reduce the risk of formation of methylated AZAs (Jauffrais et al., 2012a).

Procedures for the extraction of AZAs with HP-20 were optimized:

- The minimum amount of HP-20 resin required for efficient extraction was evaluated (in triplicate). Resin (1, 2.5, or 5 g) was placed in sonicated retentate (100 mL) for 24 h, recovered, packed in a glass column, and eluted with acetone (3 × 3 times the volume of the HP-20 resin), to give final volumes of 9, 22.5, and 45 mL, respectively.
- The effect of contact time (2, 6, 24 and 72 h) between the sonicated retentate (100 mL) and the HP-20 resin (2.5 g) was tested in triplicate.
- The volume of solvent required for elution was determined in triplicate experiments where 5 g batches of HP-20 resin were placed in sonicated retentate (100 mL) for 24 h, recovered, packed in a glass column, and eluted using different volumes of acetone ((a) 3 × 5 mL; (b) 3 × 10 mL; (c) 3 × 25 mL).

- Using the optimized procedures (2.5 g of resin, 24 h contact), adsorption efficiency was assessed by comparing the initial and final amounts of toxin in the sonicated retentate. The following elution procedures were then tested and the overall AZA recoveries determined (Table 11): (a) desorption using three successive soaks of acetone (3 × 7.5 mL) for 5 min; and (b) for 2 h (MacKenzie et al., 2004); (c) elution from a glass column (1 mL·min^{-1}) using three successive additions of acetone (3 × 7.5 mL) (Fux et al., 2008a).

2.5.6. LC-MS/MS analysis at Ifremer, Nantes

The samples were analyzed by LC-MS/MS using an Agilent 1100 model coupled to a triple quadrupole mass spectrometer (API 2000, SCIEX, Applied Biosystems) for quantification of azaspiracids. Chromatography was performed with a Hypersil BDS C8 column (50 × 2 mm, 3 µm, Thermo scientific), with isocratic elution at 250 µL·min^{-1} for 10 min. The mobile phase was 100% water and acetonitrile–water (95:5), both containing 2 mM ammonium formate and 50 mM formic acid. The injection volume was 5 µL and the column and sample temperatures were 20 and 5 °C, respectively.

The declustering potential was 116 V, the entrance potential 10 V, the cell exit potentials 12 and 16 V, and the collision cell was 41 and 69 V for fragmentation 1 and 2, respectively. The electrospray ionisation interface (ESI) was operated using the following parameters: curtain gas: 30 psi; temperature: 450 °C; gas 1, 50 psi; gas 2, 50 psi; CAD gas, medium; ion spray voltage, 5500 V.

Azaspiracids were quantified against certified AZA1 standards obtained from the National Research Council Canada (NRCC). The two most intense product ions were selected with the following transitions: AZA1 *m/z* 842.5→824.5 and 842.5→672.4, and AZA2 *m/z* 856.5→838.5 and 856.5→672.4.

2.5.7. Analysis at the Marine Institute, Rinville and at NRCC, Halifax

2.5.7.1. LC-MS/MS analysis

Two LC-MS/MS systems were used in positive ion mode, both of which were equipped with a z-spray ESI source.

Method A. Recoveries were determined by quantitative analysis of fractions on a Waters 2695 LC coupled to a Micromass triple-stage quadrupole (TSQ) Ultima operated in multiple reaction monitoring (MRM) mode, with the following transitions: AZA1 m/z

842.5→824.5 and 842.5→672.4, AZA2 856.5→838.5 and 856.5→672.4. The cone voltage was 60 V and the collision voltage was 40 V, the cone and desolvation gas flows were set at 100 and 800 L/h, respectively, and the source temperature was 150 °C.

Binary gradient elution was used, with phase A consisting of water and phase B of 95% acetonitrile in water (both containing 2 mM ammonium formate and 50 mM formic acid) in a minor modification of the method of (Quilliam et al., 2001). Chromatography was performed with a Hypersil BDS C8 column (50 × 2.1 mm, 3 µm, with a 10 × 2.1 mm guard column of the same stationary phase) (Thermo Scientific). The gradient was from 30% B, to 90% B over 8 min at 0.25 mL min^{-1}, held for 5 min, then held at 100% B at 0.4 mL min^{-1} for 5 min, and returned to the initial conditions and held for 4 min to equilibrate the system. The injection volume was 5 µL and the column and sample temperatures were 25 °C and 6 °C, respectively.

Method B. Purity was initially assessed on a Micromass time-of-flight (QTof) Ultima coupled to a Waters 2795 LC by running MS scans (m/z 100–1000) using the same chromatographic conditions as Method A. Identification of other AZA analogues was also determined by performing product ion scans, where the precursor ions were selected and then fragmented, for all the known AZA analogues (Figure 39).

Method C. Qualitative analysis of fractions for AZAs was performed by flow injection analysis-MS/MS using a Micromass QTof Ultima coupled to a Waters 2795 LC. Samples (2 µL) were injected, directly (no column) with a 2795 autosampler, into the mass spectrometer monitoring for the precursor ions.

Method D. A concentrated sample (~500 µg mL^{-1}) was injected (1 µL) onto a semi-preparative system (Shimadzu 10AVp) with photodiode array (PDA) detection (210 nm) using a Cosmosil C18 column (5 µm, 250 × 4.6 mm) eluted with acetonitrile–water (1:1, plus 2 mM ammonium acetate) at 1 mL min^{-1}. The column temperature was 30 °C.

Method E. An additional method was employed to detect any strongly retained compounds (e.g., phthalates) with an analytical LC system (Shimadzu LC 10AVp) with PDA detection at 210 nm. The sample collected after the SPE step was injected (5 µL) onto a Vydac C18 column (10 µm, 250 × 4.6 mm, Grace) and eluted with methanol–water (9:1) at 1 mL min^{-1}, with the column temperature at 30 °C.

2.5.7.2. NMR spectroscopy

Purity was assessed by ^{1}H NMR using a Bruker DRX-500 spectrometer. Chemical shifts were referenced to internal CHD_2OH (3.31 ppm).

2.5.8. AZA1 and -2 isolation from *A. spinosum* HP-20 extract

HP-20 resin extracts from 1200 L of culture were combined, evaporated in vacuo, and partitioned between ethyl acetate (150 mL) and aqueous NaCl (1 M, 50 mL). The ethyl acetate fraction was evaporated to dryness in vacuo and the residue dissolved in ethyl acetate (20 mL), and ~2 g of silica gel was added. The sample was then carefully evaporated to dryness in vacuo, mixed to a fine powder and loaded onto a silica gel (6 g) column (6 × 4 cm). Vacuum-assisted elution was performed successively with hexane, ethyl acetate, ethyl acetate–methanol (9:1, 7:3, and 1:1), and methanol (30 mL of each, all containing 0.1% acetic acid except for hexane). The 7:3 ethyl acetate–methanol fraction, which FIA-MS/MS (Method C) showed to contain the AZAs, was evaporated in vacuo, and the sample in acetonitrile–water (6:4, plus 0.1% triethylamine) was loaded onto a column packed with Phenyl-Hexyl (19.9 × 2 cm). The sample was eluted with acetonitrile–water (7:13, plus 0.1% triethylamine) at 4 mL min^{-1}, and 5 mL fractions were collected. Appropriate fractions were combined (AZA1, fractions 15–18, and AZA2, fractions 19–25) based on FIA-MS/MS analysis (FIA = Flow Injection Analysis).

Final purification of AZA1 and -2 was achieved by semi-preparative HPLC (Agilent 1200) with photodiode array (PDA) detection (210 nm) using a Luna C8 (5 µm, 250 × 10 mm, Phenomenex) column eluted with acetonitrile–water (1:1, plus 2 mM ammonium acetate) at 4 mL min^{-1}. The column temperature was 30 °C. Purified AZAs were recovered by evaporation to ~20% acetonitrile, loading on to SPE cartridges (Oasis HLB, 200 mg), washing with methanol–water (1:9, 10 mL) to remove the buffer, and eluting with methanol–water (4:6, 6:4, 8:2, 10:0, 20 mL each) with >95% of the AZAs eluting in the 8:2 fraction.

Purified samples were tested for phthalates (Method E) which, if present, were removed by partitioning the sample between methanol–water (4:1, 20 mL) and 20 mL of hexane. Removal of solvent by evaporation in vacuo afforded purified AZA1 (9.3 mg) and AZA2 (2.2 mg) as white solids.

The scheme of the isolation procedure of AZAs is presented in the appendix.

2.5.9. Reagents

Ifremer: acetone, acetonitrile (ACN) and dichlromethane (DCM) were obtained as HPLC grade solvents from JT Baker. Milli-Q water for the HPLC mobile phase was supplied by a Milli-Q integral 3 system (Millipore). Formic acid (Puriss quality), ammonium formate (Purity for MS) and Diaion HP-20 polymeric resin were from Sigma-Aldrich (Steinheim, Germany).

Marine Institute: all solvents (pestican grade) were purchased from Labscan (Dublin, Ireland). Sodium chloride (>99%), triethylamine (99%), ammonium acetate (>97%), ammonium formate (reagent grade), formic acid (>98%), and silica gel (10–40 µm, type H) were purchased from Sigma-Aldrich (Steinheim, Germany). Luna Phenyl-Hexyl (15 µm) was from Phenomenex (Cheshire, UK), and methanol-d3 (CD_3OH, 99.5%) was from Cambridge Isotope Laboratories (MA, USA).

AZA1 and -2 certified reference materials (CRMs) were obtained from the NRC, Certified Reference Material Program (Halifax, NS, Canada).

2.5.10. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analyses were multi-factor ANOVA were differences were considered significant at $p < 0.05$. Statistical analyses were carried out using Statgraphics Centurion XV.I (StatPoint Technologies, Inc.). Before each ANOVA analysis, normality and equality of variance were tested. ANOVA was followed by multiple comparison (Fischer least-significant-differences) procedures to discriminate differences between values within each factor.

2.6. Results and discussion

A. spinosum (strain 3D9) was received at Ifremer through collaboration with the Alfred Wegener Institute, as part of the ASTOX2 project. After receipt of the organism, preliminary studies on environmental and nutritional factors were conducted to allow the organism to grow in large-scale culture, and to increase cell concentration and toxin production (data not shown). Even though dinoflagellates are usually considered sensitive to shear stress produced by small-scale turbulence (Gallardo Rodriguez et al., 2009a), it was possible to grow this species in stirred 100 L photobioreactors in series.

2.6.1. Effect of dilution rate on *A. spinosum* and AZA production in pilot scale photobioreactors

Because cultures collapsed when the dilution rate was altered significantly within one experimental run, separate independent experiments were conducted at different dilution rates. At a dilution rate of 0.1 day^{-1}, steady state cell concentration was lowest of all dilution rates studied (174×10^3 and 164×10^3 $cell\cdot mL^{-1}$ in reactor 1 and -2 (R1 and R2), respectively) (Table 9). Cell concentration, mean estimated spherical diameter (ESD), cellular volume and toxin content were stable at steady states for dilution rates from 0.15 to 0.30 day^{-1}. For all dilution rates from 0.15 to 0.3 day^{-1}, steady state cell density only increased by about 10% between R1 and R2 (190×10^3 and 215×10^3 cell mL^{-1}, respectively). Results for ESD and cellular volume were comparable at all dilution rates but differed significantly between R1 and R2. Even though, the cell concentration increased only by about 10% between R1 and R2, the cellular volume increased by about 50%. The small increase in cell concentration and the large increase in bio-volume reflect that cell division mainly takes place in the first reactor while increase in cellular volume with little cell division occurs in the second reactor. While the physiological limitation leading to this phenomenon in each reactor is not yet fully understood, the increase in cell-volume is referred to as cell maturation from here on.

At each steady state studied, the AZA cell quota (*i.e.*, AZA1 + 2) increased between R1 and R2, reflecting that during maturation of cells in the second bioreactor, cells continued to produce AZAs. The increase in toxin per cell from R1 to R2 reached its maximum at a dilution rate of 0.3 day^{-1} (ratio of 2.6 between cell quota in R2 compared to R1). The toxin production of cells severely limited in growth suggests that AZAs are secondary metabolites not implicated in cell division or cell growth processes, as has been suggested for polyketides and many toxins in general (Robinson, 1991; Schembri et al., 2001). Consequently, in contrast to cell concentration, AZA per cell decreased as dilution rate increased from 0.15 to 0.3 day^{-1}, ranging from 67 to 24 fg $cell^{-1}$ for R1 and 98 to 63 $fg\cdot cell^{-1}$ for R2. This decrease in AZA per cell was partly counteracted by the maturation effect and by the fact that at higher growth rates more cells are produced per day. Thus, as cell numbers increased with dilution rate, and as the cell volume still always increased in the second bioreactor, AZA prodution reached an optimum of 475 ± 17 µg day^{-1} at a flow rate of 25 L day^{-1}.

Table 9. *A. spinosum* concentration (cell·mL^{-1}), mean estimated spherical diameter (ESD) (µm), cellular volume (µm^3·mL^{-1}), toxin content (fg·cell^{-1}), and cell and toxin production (cell·day^{-1} and µg·day^{-1}, respectively) at the dilution rates studied (0.1, 0.15, 0.2, 0.25, 0.3 day^{-1}) in the two bioreactors in series (R1 and R2). Standard deviations were calculated from sequential repeat measurements of each culture and the last columns present the result of the multifactorial ANOVA followed by a Fisher least-significant-difference test to discriminate differences between values within each factor.

A. spinosum	***n***	**0.1 day^{-1}**		**0.15 day^{-1}**		**0.2 day^{-1}**		**0.25 day^{-1}**		**0.3 day^{-1}**		**Main factors**		**Interaction**
		R1	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**	**Dilution rate (D)**	**Reactor (R)**	**D–R**
Concentration (×10^3 cell mL^{-1})	9–22	174 ± 6	164 ± 4	193 ± 6	214 ± 3	194 ± 8	214 ± 7	190 ± 6	221 ± 5	187 ± 5	220 ± 4	$p \leq 0.05$ 0.1 < 0.15 = 0.2 = 0.25 = 0.3	$p \leq 0.05$ R1 < R2	$p \leq 0.05$
Mean ESD (µm)	9–22	9.81 ± 0.09	10.1 ± 0.1	9.59 ± 0.15	9.9 ± 0.2	9.6 ± 0.2	10.1 ± 0.2	9.29 ± 0.09	9.93 ± 0.04	9.5 ± 0.1	10.02 ± 0.05	$p \leq 0.05$ 0.25 = 0.3 < 0.15 < 0.2 = 0.1	p ≤ 0.05 R1 < R2	$p \leq 0.05$
Cellular volume (×10^7 µm^3 mL^{-1})	9–22	8.8 ± 0.4	8.4 ± 0.3	9.2 ± 0.4	11.1 ± 0.4	9.3 ± 0.6	12.0 ± 0.7	8.2 ± 0.3	11.7 ± 0.2	8.5 ± 0.1	12.0 ± 0.4	$p \leq 0.05$ 0.1 < 0.25 = 0.3 = 0.15 < 0.2	$p \leq 0.05$ R1 < R2	$p \leq 0.05$
AZA1 (fg cell^{-1})	3–5 *	37 ± 5	65 ± 8	52 ± 6	74 ± 4	34 ± 12	76 ± 14	26 ± 2	61 ± 3	17 ± 1	45 ± 3	$p \leq 0.05$ 0.3 < 0.25 = 0.1< 0.2 < 0.15	$p \leq 0.05$ R1 < R2	$p \leq 0.05$
AZA2 (fg cell^{-1})	3–5 *	10 ± 1	16 ± 1	15 ± 1	24 ± 2	10 ± 2	19 ± 2	12 ± 2	25 ± 2	7 ± 1	18 ± 2			
AZA1 + 2(fg cell^{-1})	3–5 *	47 ± 6	81 ± 9	67 ± 3	98 ± 5	44 ± 13	95 ± 16	38 ± 2	86 ± 3	24 ± 1	63 ± 5			
Cell production (×10^9 cell day^{-1})	n/a	1.74 ± 0.06	1.64 ± 0.04	2.90 ± 0.09	3.21 ± 0.05	3.9 ± 0.2	4.3 ± 0.1	4.8 ± 0.2	5.5 ± 0.1	5.6 ± 0.2	6.6 ± 0.1	Cell production = Cell concentration × D		
Toxin production AZA1 + 2 (µg day^{-1})	n/a	82 ± 3	134 ± 15	193 ± 9	314 ± 15	170 ± 50	406 ± 64	180 ± 10	475 ± 17	134 ± 5	415 ± 33	Toxin production = Cell production × [AZA1 + 2]		

***** Each sample was also injected in triplicate to reduce analytical variability; n/a not applicable

In the harvesting tank, at a dilution rate of 0.1 day^{-1}, cell concentration collapsed before harvesting at a concentration of 70×10^3 cell mL^{-1}. However, at the other dilution rates, cell concentration and AZA cell quota were maintained in the harvesting tank until 200 L culture volume was reached. At a dilution rate 0.2 day^{-1}, a decrease of 10% in cell concentration and AZA cell quota was observed in the harvesting tank compared to R2. At dilution rate 0.25 day^{-1}, a slightly smaller decrease of 0–5% in cell concentration and AZA cell quota was observed while an increase of 0–5% was observed at 0.3 day^{-1} (0.15 day^{-1} was not evaluated but culture did not collapse).

Previous studies on batch cultures of *A. spinosum* (strain 3D9 or SM2) showed a production of AZA1 and -2, with AZA1 as the predominant AZA and with a cell quota ranging from 5 to 50 fg $cell^{-1}$ (Salas et al., 2011; Tillmann et al., 2009). In the present study, the same toxin profile was found, however, AZA cell quotas of 24–98 fg $cell^{-1}$ were obtained depending on dilution rate. These results raised the possibility of improving both cell concentration and toxin concentration in *A. spinosum* grown in bioreactors by controlling dilution rate (equivalent to growth rate in chemostat bioreactors) and by adding a period of maturation using a second bioreactor specifically dedicated to improving the AZA cell quota.Preliminary studies on A. *spinosum* in batch culture gave cell concentrations up to ~90×10^3 cell mL^{-1} (data not shown) and toxin quotas of 5 to ~50 fg $cell^{-1}$ [12,13]. Using a photobioreactor, both cell concentration and AZA cell quota were each increased by a factor of 2 compared to batch culture without aeration. Even though batch culture with aeration gave a similar cell concentration (data not shown), our study demonstrated the advantage of continuous culture for *A. spinosum*, due to the control of both cell concentration and toxin production, minimizing the risk of culture collapse. Hence the process developed ensures a continuous production of A. *spinosum* for AZAs isolation and purification.

Reported AZA cell quota (previous work and our own data) for A. *spinosum* in the femtogram-range are low compared to toxin quota of other dinoflagellates, which are in the picogram-per-cell range, e.g., *Alexandrium* (Medhioub et al., 2011), *Dinophysis* (Nagai et al., 2011), *Prorocentrum* (Varkitzi et al., 2010), *Karenia* (Medhioub et al., 2009) and this seemed to be discouraging for toxin production. However, considering the small size of *A. spinosum* cells, toxin concentrations calculated on a per volume basis (0.23 fg μm^3) were similar to those of other toxic dinoflagellates such as *Dinophysis acuta* (0.89–1.1 fg μm^3, calculated from (Pizarro et al., 2009)).

Even though published results for toxin production by other dinoflagellates are encouraging (*Alexandrium minutum* ~20 µg toxin L^{-1} day^{-1} (Parker et al., 2002), similar to *A.*

spinosum ; *Protoceratium reticulatum* 214 µg L^{-1} day^{-1} (Gallardo Rodriguez et al., 2010), 10 times more than to A. *spinosum*; *Alexandrium ostenfeldii* (higher cell concentration but lower toxin cell quota—71,000 cell mL^{-1}, 0.7 pg $cell^{-1}$ 13-desMeC SPX eq, than in batch culture ~17,000 cell mL^{-1}, 4.2 to 1.7 pg $cell^{-1}$ 13-desMeC SPX eq (Medhioub et al., 2011) (SPX = Spirolide). More effort should be made to optimize toxin production, since, most toxins produced by harmful dinoflagellates are required for instrument calibration, and further research is required on toxicology and other biomedical aspects. Furthermore, studies on dinoflagellates in bioreactors would help in understanding their complex physiology and the link between growth and toxin production.

The work presented here indicates that *A. spinosum* can grow in stirred photobioreactors, even though it is a fragile dinoflagellate. The study shows the effect of growth rate on AZA cell quota, thus contributing to the understanding of the biosynthetic behaviour of *A. spinosum*. Furthermore, the set-up developed may be used to assess more generally ecophysiological characteristics of dinoflagellates.

2.6.2. Separation procedure of *A. spinosum* from the culture medium

AZA extraction procedures were developed to optimize AZA recovery from the *A. spinosum* pilot-scale culture. Two methods were tested, tangential flow filtration and continuous centrifugation.

In the harvesting tank, *i.e.*, prior to large-scale separation of cells from culture medium, 95 ± 4% of the AZA1 and -2 present were intra-cellular, as estimated by toxin quantification in both algal pellet and supernatant. However, after tangential flow filtration (Method 1), 50 to 70% of the toxin was in the concentrate and 30 to 50% had been released into the permeate. Toxin release was time-dependent, with longer filtration times leading to higher proportions of toxin in the permeate (depending on age of culture and cartridge). This procedure allowed for the separation of 200 L of culture into 0.7–1.0 L of algal concentrate (equivalent to 40–46 g of wet algal paste after centrifugation) and ~200 L of permeate. However, the release of a non-negligible amount of dissolved AZAs into the permeate necessitated the development of AZA adsorption procedures to avoid significant AZA losses. Interestingly, after tangential flow filtration, the algal cells retained their integrity and the permeate was clear, demonstrating the weak effect of shear forces on *A. spinosum* cells.

In contrast to tangential flow filtration, continuous centrifugation (Method 2) led to significant lysis of algal cells (probably due to the violent release of concentrate from the

centrifuge), and few entire cells were observed microscopically. This last procedure allowed for the separation of 200 L of culture medium into ~15 L of algal concentrate and ~185 L of permeate containing, respectively, 76% ± 7% and 24% ± 1% of the toxin in each fraction. Continuous centrifugation was less time consuming than tangential flow filtration and can easily be scaled up to much larger volumes of culture. However, the initial purchase cost of centrifugal equipment is higher than for tangential flow filtration, and it was not possible to recover an algal paste under the test conditions using continuous centrifugation. The choice of cell recovery technique therefore depends on financial resources and other research needs.

2.6.3. AZA1 and -2 extraction from the retentate and permeate

After tangential flow filtration, most toxin from the concentrated cells was recovered in the algal paste after centrifugation of the retentate (Method 3), with only minor loss of AZAs into the retentate supernatant (9 ± 1%).

Three organic solvents were tested to assess extraction yield and purity (Method 3): acetone, acetonitrile (ACN), and dichloromethane (DCM). No differences were observed between the three solvents (Table 10), confirming results previously obtained during *A. spinosum* analysis (Jauffrais et al., 2012a). Methanol was not used as it was shown to induce AZA extraction artifacts as solvent for *A. spinosum* analysis (Jauffrais et al., 2012a). Significant differences in purity were observed (Table 10), with acetone producing more residue than ACN and DCM. These procedures were also compared with adsorption on Diaion HP-20 polymeric resin (adaptation of Method 4). The resin gave comparable extraction efficiency to solvent extraction, but with significantly better selectivity (i.e., purity) (Table 10).

Table 10. Azaspiracid yield (µg g^{-1} ± SD, *n* = 3) and purity (%) from algal paste after extraction with acetone, ACN or DCM (method 3), and using HP-20 resin.

	Acetone	**ACN**	**DCM**	**HP-20**
AZA1 + 2 (µg g^{-1})	17.4 ± 0.5	18 ± 2	17 ± 1	17 ± 1
Purity (%)	0.036 ± 0.002	0.07 ± 0.01	0.09 ± 0.01	0.21 ± 0.03

To recover AZAs from the permeate and retentate-supernatant, representing up to 50% of the total AZAs, solid phase adsorption procedures were implemented using HP-20 resin (Methods 5 and 6). The HP-20 polymeric resin was previously used and studied by MacKenzie et al. (MacKenzie et al., 2004) and Fux et al. (Fux, 2008; Fux et al., 2009; Fux et

al., 2008a) for adsorption of lipophilic toxins. These studies demonstrated that HP-20 resin is a suitable adsorbent for AZAs (Fux et al., 2009; Rundberget et al., 2007). In the present study, HP-20 resin was used for adsorption of AZA, with volumes ranging from 0.75 L of algal concentrate to 200 L of permeate. Method 4 was developed to recover both the intra- and extra-cellular toxins from the retentate. The results of this work are summarized in Table 11.

To optimize extraction yield, four separate experiments (using different lots of concentrate) were carried out to determine: (1) the volume of solvent; (2) the optimum amount of resin to be used; (3) the time of contact between the sample and the resin; and (4) the method for AZA desorption from the HP-20 resin.

Table 11. Azaspiracid yield (µg $mL^{-1}$of concentrate or %, ± SD, *n* = 3) using various HP-20 adsorption and elution procedures (Method 4) *.

Acetone Volume/5 g HP-20	**3 × 5 mL**	**3 × 10 mL**		**3 × 25 mL**
AZA yield (µg mL^{-1})	1.61 ± 0.07	2.1 ± 0.1		2.19 ± 0.06
Mass HP-20/100 mL concentrate	**1 g**	**2.5 g**		**5 g**
AZA yield (µg mL^{-1})	2.6 ± 0.2	2.6 ± 0.1		2.1 ± 0.2
Time of contact	**2 h**	**6 h**	**24 h**	**72 h**
AZA yield (µg mL^{-1})	0.48 ± 0.06	0.52 ± 0.03	0.61 ± 0.02	0.52 ± 0.08
HP-20 adsorption efficiency (% after 24 h of contact with the concentrate)		93.8 ± 0.1		
Desorption procedure (2.5 g HP-20–3 × 7.5 mL acetone)	**Soaking using acetone and filtration**			**Column using acetone**
Time of soaking (a and b), and flow rate (c)	**(a) 5 min**	**(b) 2 h**		**(c) 1 mL·min^{-1}**
Fraction 1 (%)	77 ± 3	74 ± 5		98.2 ± 0.5
Fraction 2 (%)	21 ± 1	22 ± 3		1.6 ± 0.8
Fraction 3 (%)	3.0 ± 0.4	3.8 ± 0.2		0.2 ± 0.1
AZA yield (µg mL^{-1})	2.43 ± 0.09	2.4 ± 0.1		2.58 ± 0.01
Desorption yield (%)	**83 ± 3**	**81 ± 4**		**88.5 ± 0.2**
Total extraction yield (%)	**78 ± 3**	**76 ± 4**		**83.1 ± 0.1**

* A separate lot of concentrate was used to determine each parameter, so yields are only comparable within each experiment.

Smaller resin masses (1 and 2.5 g) gave better extraction yield after 24 h of contact (Table 11). 2.5 g of HP-20 resin for 100 mL of retentate was chosen to avoid difficulty of elution and to ensure sufficient adsorption capacity of the resin in case of high AZA concentration.

Results showed that, to reach the optimum desorption yield a minimum of six times the HP-20 resin volume of extracting solvent was required. The best results of AZA desorption from the resin were obtained using a glass column with three successive elutions with acetone at 1 mL min^{-1}. This last procedure eluted 98.2% ± 0.5% of the toxin recovered in the first elution volume, showing a better desorption than with the other procedures. Using the optimized procedure, a final yield of 83.1% ± 0.1% of the initial amount of AZA1 + 2 in the algal concentrate was obtained. The yield was close to that obtained by Fux *et al.* for AZA1–3 (85%–93%) (Fux et al., 2008a), but is lower than for other lipophilic toxins (Fux et al., 2008a; MacKenzie et al., 2004).

Extracellular toxin was extracted using two separate procedures, each with HP-20 (Methods 5 and 6). SPATT bags were initially designed as a monitoring tool to follow and predict microalgal toxic events around shellfish production areas (MacKenzie et al., 2004; MacKenzie, 2010). The procedure with HP-20 packed in a column as a solid phase extraction method was implemented for biotoxin extraction from seawater after naturally occurring microalgal blooms (Rundberget et al., 2007). These two methods were adapted to laboratory conditions after tangential flow filtration of the culture medium to recover dissolved AZA from the permeate (Method 5 and 6).

Both methods gave similar recovery (~80%–85% of dissolved toxins); however, recovery using SPATT bags (method 5) showed more variability than the SPE procedure (method 6, Table 12).

Table 12. AZA mass balance (% ± SD, *n* = 3 for Methods 4 and 6, and *n* = 7 for Methods 3 and 5) obtained after tangential flow filtration using extraction Methods 3–6 (indicated in parentheses). Recovery was calculated from the sum of AZA1 + 2 concentration measured in the harvesting tank before tangential flow filtration.

Method No.	Method description	% Recovery of total
(3)	Algal paste	56 ± 9
(4)	Algal retentate + HP-20	54 ± 3
(17025)	Algal permeate + SPATT	21 ± 9
(6)	Algal permeate + SPE	26 ± 4

Finally, these optimized procedures allowed us to recover ~3 mg of AZAs in crude extracts after tangential flow filtration of 200 L of culture. This crude extract was obtained in 12 working days, with 8 days of culturing (at steady state) at a flow rate of 25 L day^{-1}, 1 day (between 5 and 8 h work) of filtration, and 2–3 days of extraction.

2.6.4. Isolation of AZA1 and -2 from *A. spinosum* crude extract

Isolation of AZAs from contaminated shellfish was previously performed using a seven-step procedure (Kilcoyne et al., 2012). However, *A. spinosum* extracts obtained using HP-20 were considerably purer (0.5% w/w, on average, for pilot-scale recovery, line 1 Table 13) than the shellfish extracts in the study of (Kilcoyne et al., 2012). Hence, it was expected that fewer steps would be required to purify AZA1 and -2 to obtain certified reference standards and to perform toxicological studies. As with the isolation from shellfish, the crude extract was initially partitioned between ethyl acetate and 1 M sodium chloride. The step resulted in a clean-up of 57% and a recovery of 90%. Previous studies have shown that AZAs are unstable in acidic environments, and that increased temperature will accelerate any acid-catalysed degradation of AZAs (Alfonso et al., 2008). However, small scale purification trials by chromatography on a silica gel column showed that it was safe to have acetic acid in the eluent at this point of the procedure. Thus, as also demonstrated by (Perez et al., 2010) and (Kilcoyne et al., 2012), the sample is still quite crude at this stage of the isolation, and hence the remaining matrix exerts a similar protective effect to that of shellfish tissue (Alfonso et al., 2008). Silica gel chromatography (step 2) gave a very high efficiency in terms of clean up (87%) and recovery (~91%, Table 13).

Table 13. Batch summary table for purification of AZA1 and AZA2.

Step No.	Step	AZA1 (mg)	AZA2 (mg)	Weight (g)	Purity (%) †
	HP-20 resin extract	12.5	3.2	3.04	0.5
1	Partitioning	11.2	3.0	1.32	1.1
2	Silica gel	10.2	2.8	0.17	7.6
3	Flash (Phenyl-Hexyl) *	9.7	2.4	0.01	>90
4	Prep HPLC (C8/C18)	9.3	2.2	-	>95
	% Recovery (steps 1–4)	75	70		

* AZA1 and AZA2 were separated from each other in this step. † Total AZA1 + 2, based on w/w.

The third step in the procedure employed flash chromatography using a phenyl-hexyl stationary phase and a weakly alkaline mobile phase to prevent degradation of toxins. Separation of AZA1 and -2 was achieved in this step with separate fractions being collected. This step resulted in the highest clean-up (93%) and recovery (95%) (Table 13).

Final purification was achieved by semi-preparative HPLC using a neutral mobile phase. Fractions were collected based on UV detection (210 nm) to prevent contamination

from other non-AZA analytes. This step resulted in recoveries of ~93% while clean-up is minimal at this stage.

SPE cartridges were used to remove any buffer remaining in the sample, as well as to reduce the water content, and volume of the AZA fractions prior to evaporation, and as an additional final clean-up step to remove trace contaminants introduced via the LC eluents. This SPE procedure resulted in very little loss of toxin, with recoveries of >95%, and greatly facilitated evaporation of the purified AZA-fractions to dryness. The ^{1}H NMR spectra of AZA1 and -2 were compared to published NMR data and found to be identical. Examination of the spectra indicated purities of >95%.

Purified AZA1 (9.3 mg) was obtained along with 2.2 mg of AZA2. Overall recoveries (steps 1–4) were 75% for AZA1 and 70% for AZA2; ^{1}H NMR spectra of AZA1 and -2 following purification from *A. spinosum* are presented in Annexe 9 to demonstrate purity. This recovery is a significant improvement compared to isolations from shellfish with recoveries increasing by a factor of ~1.5. Furthermore, the procedure is significantly easier to perform with two fewer clean-up steps after extraction being required to achieve sufficient purities.

2.7. Conclusions

This study presents the first data on *A. spinosum* production in pilot scale photobioreactors. It demonstrates the ability of this small and fragile dinoflagellate to grow in this type of reactor. The effect of growth rate on AZA cell quota and toxin production outline the secondary metabolite character of AZAs. A dilution rate of 0.25 day^{-1} was found to yield the highest volume-specific toxin production per day. The system of two bioreactors coupled in series at higher dilution rate allowed for an AZA production rate of 475 $\mu g\, day^{-1}$ (19 $\mu g\, L^{-1}\, day^{-1}$). Under these conditions we obtained ~3 mg of AZAs in crude extracts over 12 days (8 days of culture, 1 day of filtration and 3 days of extractions). Further work is under way on the influence of environmental and nutritional parameters other than dilution rate on *A. spinosum* growth and toxin production.

The production of large volumes of *A. spinosum* necessitated the development of rapid procedures to concentrate and harvest algal cells for optimal toxin recover. Both continuous centrifugation and tangential flow filtration have been successfully applied in this study to concentrate *A. spinosum* culture. A simple and efficient procedure of extraction allowed for

retrieval of toxins from both the retentate and the permeate to provide a crude *A. spinosum* extract rich in AZA and low in interfering matrix components.

Thanks to the high concentration of AZAs relative to the matrix in the extract, purification of AZA1 and -2 could be simplified to a four-step procedure with recoveries of 75% and 70% for AZA1 and -2, respectively, a 1.5 fold increase when compared with isolation of AZAs from shellfish. In this study, six successive culture lots (1200 L harvested in total) enabled the recovery of 9.3 mg of purified AZA1 and 2.2 mg of purified AZA2.

Future production of AZAs from *A. spinosum* could involve bioreactors at this scale, or at an even larger scale. Still, one limitation of the present approach is that only AZA1 and -2 may be produced from *A. spinosum*, yet a number of other analogues are also relevant to official control (McCarron et al., 2009). As a consequence of another recent study (Jauffrais et al., 2012c), which suggests some self-limitation of mussels exposed to live *A. spinosum*, our current work investigates the use of crude extracts of *A. spinosum* for the exposure of mussels with a view to producing shellfish metabolites of AZA1 and -2. Such studies are aimed at more efficient production of shellfish metabolites but may also contribute to the understanding of the uptake from the dissolved phase and metabolism of AZAs by mussels.

More generally, this work showed that even though *A. spinosum* and many other dinoflagellates are known to have slow growth rates and low maximum cell concentrations compared to many other microalgae for shellfish aquaculture, their culture in photobioreactors is a viable biotechnological approach to the production of toxins with applications in research and operational food safety surveillance programs. Furthermore, metabolite production in chemostat bioreactors is stable and predictable, and has the possibility to influence secondary metabolite production by varying different culture conditions.

2.8. Acknowledgments

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Chapitre 3 – Écophysiologie d'*Azadinium spinosum*

Contexte

A. spinosum est producteur d'AZA1 et -2. C'est donc un organisme pouvant être responsable de l'accumulation d'AZA par les mollusques bivalves. Bien qu'ayant fait l'objet de plusieurs publications (Salas et al., 2011; Tillmann et al., 2009), un manque d'information subsiste concernant les facteurs influant sur son écophysiologie : taux de croissance, concentration cellulaire et quotas en AZA des cellules. En effet, les articles portant sur l'isolement et la description d'*A. spinosum* ont souligné une grande variabilité du quota cellulaire en AZA, qui selon les auteurs varie en fonction des conditions environnementales (5 à 40 fgAZA1 $cell^{-1}$ en culture « batch » (Salas et al., 2011; Tillmann et al., 2009) et peut donc avoir une incidence directe variable sur l'accumulation des AZA par les mollusques bivalves filtreurs.

La croissance, la concentration cellulaire et le quota cellulaire en toxines des microalgues dépendent de divers facteurs environnementaux et nutritionnels. Par ailleurs les biotoxines microalgales présentent des structures chimiques qui dépendent des espèces qui les produisent. Par conséquent les facteurs influençant le quota cellulaire en toxine peuvent varier en fonction des espèces étudiées (Granéli, 2006). Parmi les divers facteurs environnementaux, la salinité, la température et la lumière sont des facteurs qui peuvent affecter la croissance des dinoflagellés et leur contenu toxinique (Flynn et al., 1996; Gedaria et al., 2007; Maclean et al., 2003; Parkhill et Cembella, 1999; Xu et al., 2010), de même que l'aération (Hu et al, 2006b;. Morton et Bomber, 1994). En outre, les facteurs nutritionnels, et en particulier le ratio azote/phosphore, peuvent également influer sur la croissance des dinoflagellés et sur leur quota en toxines (Flynn et al., 1996 ; Hu et al., 2006a ; Hwang et Lu, 2000; Lartigue et al, 2009).

Les premiers travaux que nous avant réalisés et qui portent sur l'écophysiologie d'*A. spinosum* visent à comprendre, d'une part, l'effet des facteurs environnementaux tels que : la salinité, la température, la lumière et l'aération et d'autre part, l'effet des facteurs nutritionnels tels que : le milieu de culture, la source d'azote (nitrate, urée, ammonium) et de phosphore ainsi que les ratios N/P, sur la croissance des microalgues et la production de toxines, que se soit en culture « batch » ou en continu.

Ce chapitre présente donc le travail effectué afin de déterminer les paramètres environnementaux et nutritionnels qui permettent d'améliorer la croissance d'*A. spinosum* et d'augmenter la production de toxines de cet organisme, l'objectif étant de proposer une production durable d'AZA à partir de cultures d'*A. spinosum*. En outre, il a aussi comme

objectif d'améliorer notre compréhension sur l'écophysiologie d'*A. spinosum* pour mieux appréhender les facteurs pouvant influer sur l'apparition et la dangerosité d'éventuelles efflorescences.

3. Effect of environmental and nutritional factors on growth and azaspiracid production of the dinoflagellate *Azadinium spinosum*

Article en préparation pour publication: "Jauffrais, T., Séchet, V., Herrenknecht, C., Truquet, P., Tillmann, U., Hess, P., 201X. Effect of environmental and nutritional factors on growth and azaspiracid production of the dinoflagellate *Azadinium spinosum*".

3.1. Résumé

Azadinium spinosum, est un petit dinoflagellé producteur d'azaspiracide-1 et -2 (AZA) qui a été récemment isolé en mer du Nord. Ces deux biotoxines sont reponsables d'intoxications alimentaires suite à l'ingestion de mollusques bivalves contaminés.

Cette étude a évalué l'effet de différents facteurs environnementaux tels que : la salinité (10-40 psu), la température (10-26°C), l'intensité lumineuse (50-400 µmol m^{-2} s^{-1}), l'aération, ainsi que de facteurs nutritionnels tels que : la composition des milieux de culture (K modifiés, L1 et L1 + extrait de terre), des sources d'azote (N) (NO_3^-, NH_4^+, urée), des sources de phosphore (P) (organique et inorganique) et des ratios N/P (NO_3^-/$NaH_2PO_4^{2-}$) en culture « batch » et en continu, sur le taux de croissance, la concentration cellulaire et la concentration en AZA dans les cellules.

A. spinosum peut croître dans une grande plage de conditions environnementales: de 30 à 40 psu, de 10 à 26°C, à toutes les intensités limineuses testées et avec ou sans aération par bullage. Cependant, le taux de croissance et la concentration cellulaire maximale sont optimaux à 35 psu, entre 18 et 22°C et avec aération. En ce qui concerne le quota cellulaire en AZA, l'effet le plus significatif est observé à basse température où la concentration cellulaire en AZA à 10°C est 20 fois plus élevée (220 fg $cell^{-1}$) que celle obrenue à des températures, comprises entre 18 et 26°C. *A. spinosum* croît dans tous les milieux de cultures testés et avec seulement de légères variations du taux de croissance et des quotas cellulaires en AZA. Cependant, l'utilisation d'une faible concentration en milieu K modifié (0.1Kmod) permet

d'ameliorer la teneur en toxines dans les cellules tandis que des concentrations plus élevées (0.5Kmod, 1Kmod, 2Kmod) améliorent le taux de croissance et la concentration cellulaire maximale. *A. spinosum* est également capable d'utiliser différentes sources d'azote pour sa croissance, mais son taux de croissance est réduit en présence de NH_4^+.

Ces premières expériences sur *A. spinosum* ont permis d'améliorer notre connaissance sur son écophysiologie et vont permettre d'obtenir de meilleurs rendements de production en AZA.

3.2. Abstract

Azadinium spinosum, is a small dinoflagellate recently isolated in the North Sea, produces azaspiracid-1 and -2 (AZAs), two biotoxins associated with human illness following ingestion of contaminated shellfish.

In the present study, we investigated the effects of different environmental factors such as salinity (10-40 psu), temperature (10-26°C), photon flux density (50-400 µmol m^{-2} s^{-1}), aeration, as well as nutritional factors such as culture media (modified K and L1 media), nitrogen source (NO_3^-, NH_4^+, urea), phosphate source (organic and inorganic), and N/P ratios ($NO_3^-/NaH_2PO_4^{2-}$) in batch and/or continuous culture on the growth rate, maximum cell concentration, and AZA cell quota.

A. spinosum grew in a wide range of conditions, from 30 to 40 psu and from 10 to 26°C, under all irradiance conditions tested, with or without aeration. Growth rate and maximum cell concentration were optimal at 35 psu, from 18 to 22°C, and with aeration. Concerning AZA cell quota, the most significant effect was observed at low temperature; the AZA cell quota was more than 20 times higher at 10°C (220 fg $cell^{-1}$) than at temperatures between 18 and 26°C. *A. spinosum* grew on all media tested and with only slight differences in growth rate and AZA cell quota. However, low K-modified concentration (0.1Kmod) improved AZA cell quota, whereas higher concentration (0.5Kmod, 1Kmod, 2Kmod) increased growth rate and maximal cell concentration. *A. spinosum* grew also on the different sources of nitrogen tested, but showed a lower growth rate on NH_4^+.

These first experiments on *A. spinosum* increased our knowledge on its ecophysiology and improved *A. spinosum* culture in pilot scale photobioreactors for AZA production.

3.3. Key words

Temperature, salinity, aeration, irradiance, nitrate, ammonium, urea, phosphate, N/P ratio, Dinophyceae, photobioreactor, chemostat, AZA

3.4. Introduction

Azadinium spinosum is a small dinoflagellate (12–16 µm length and 7–11 µm width) producing azaspiracid-1 and -2 (AZAs) (Tillmann et al., 2009). This dinoflagellate was isolated in the North Sea, during a research cruise, with on-board LC-MS/MS to discriminate toxins among different size fractions of phytoplankton for the characterization and isolation of AZA producing organisms (Krock et al., 2008). Following an AZA producing organism discovery, a new genus *Azadinium* was erected (Tillmann et al., 2009) and rapidly enlarged with other species: *A. obesum* not known to produce AZAs (Tillmann et al., 2010), *A. poporum* (Tillmann et al., 2011) and *A.* cf. *poporum* (Potvin et al., 2012), two species recently found to produce new AZAs, also isolated in *Amphidoma languida*, a species closely related to *Azadinium* (Krock et al., 2012; Tillmann et al., 2012). Furthermore, a new strain of *A. spinosum* was isolated in Ireland and found to produce AZA1 and -2 (Salas et al., 2011) and an *A.* cf. *spinosum* morphotype was observed and described in Argentina (Akselman and Negri, 2012). The authors reported a bloom (9.03×10^6 cell L^{-1}) in 1990; however, intoxications following shellfish consumption were not reported at that time.

Concerning AZAs, the toxin was found 12 years before the primary producing organism. In 1995, contaminated mussels (*Mytilus edulis*) from Killary Harbour (Ireland) were consumed in the Netherlands and caused human illness (McMahon and Silke, 1996). The symptoms were diarrhea, nausea, vomiting and stomach cramps, typical for diarrheic shellfish poisoning (DSP). However, DSTs levels were below the regulatory limit. Subsequently, AZA1 was discovered (Satake et al., 1998c) and later structurally revised (Nicolaou et al., 2006b). The base structure of this toxin group is composed of an cyclic amine group (aza group), a tri-spiro ring assembly and a carboxylic acid group (Satake et al., 1998c). Since its identification, the toxin was found to be responsible of additional human intoxications in Europe (Furey et al., 2010) and in the US (Klontz et al., 2009). AZAs were identified throughout Northern and Western Europe (Amzil et al., 2008; James et al., 2002; Magdalena et al., 2003a; Vale et al., 2008), and in Morocco (Taleb et al., 2006), Chile (Lopez-Rivera et al., 2009) and Japan (Ueoka et al., 2009), indicating a worldwide distribution of the toxin and consequently of the producing organism. Moreover, many AZA

analogues were found in bivalve shellfish flesh after biotransformation of the algal toxin (AZA1 and -2), and toxicological studies suggest that AZA1 to -3 are potent toxins (Ofuji et al., 1999a; Twiner et al., 2012) and therefore relevant for human health. The long list of analogues and artefacts now include AZA2-5 (Ofuji et al., 2001; Ofuji et al., 1999a), AZA6-12 (Diaz Sierra et al., 2003; James et al., 2003b), AZA13-32 (McCarron et al., 2009; Rehmann et al., 2008).

Furthermore, *A. spinosum* was found to directly contaminate mussels, with a fast AZA accumulation and biotransformation after a day or more of exposure to *A. spinosum* (Jauffrais et al., 2012c; Salas et al., 2011) showing its direct relevance to human health. Nonetheless, there is a lack of information on factors affecting *A. spinosum* physiological responses: growth rate, cell concentration and AZA cell quota. Furthermore, results on *A. spinosum* isolation and description pointed out a wide variability of AZA cell quota depending on environmental conditions (5 to 40 fg cell^{-1} in batch culture (Salas et al., 2011; Tillmann et al., 2009) and thus, directly affecting AZA accumulation rate by suspension feeding bivalves.
Growth, cell concentration and toxin cell quota are typically dependent on various environmental and nutritional factors. However, chemical structures of algal biotoxins differ among species of toxic microalgae. Thus, factors influencing toxin cell quota might vary in function of the group and/or species (Graneli, 2006). Among various environmental factors, salinity, temperature, and light might affect dinoflagellate growth and toxin content (Flynn et al., 1996; Gedaria et al., 2007; Maclean et al., 2003; Parkhill and Cembella, 1999; Xu et al., 2010) as well as aeration (Hu et al., 2006b; Morton and Bomber, 1994). Moreover, nutritional factors and especially nitrogen and phosphate ratio also affect dinoflagellate growth and toxin cell quota (Flynn et al., 1996; Hu et al., 2006a; Hwang and Lu, 2000; Lartigue et al., 2009).

This early work on *A. spinosum* ecophysiology aims to understand the effect of environmental factors –salinity, temperature, light and aeration– as well as nutritional factors –culture medium, nitrate, urea, ammonium, phosphorus, N/P ratios– on growth and AZA cell quota in batch culture and/or stirred photobioreactors.

This study presents the work done to assess the main factors influencing *A. spinosum* growth and AZA cell quota to enhance a sustainable production of AZAs from *A. spinosum* culture. These toxins are still necessary for instrument calibration in continuous monitoring programs and for toxicological studies. Furthermore, it will improve our understanding of *A. spinosum* ecophysiology and will give the first informations that might influence *A. spinosum* blooms and their relevance to shellfish contamination.

3.5. Material and methods

3.5.1. *A. spinosum*

3.5.1.1. Stock culture condition

The dinoflagellate *A. spinosum* (3D9) was obtained from the Alfred Wegner Institute. The strain was maintained in the laboratory in 50 mL batch cultures (70 mL sterile polystyrene flask) without aeration, at 18°C, 35 psu, a photon flux density (PFD) of 200 µmol m^{-2} s^{-1} and a photoperiod of 16 h of light and 8 h of dark. For maintenance, cultures were grown in 0.22 µm filtered seawater, enriched in K modified medium (Keller et al., 1987), without NH_4Cl, silicate and Tris buffer (Tris-base was avoid for technical reason, see below), and with addition of Na_2SeO_3 (10^{-8} M).

The stock culture was maintained in the growth phase by transferring weekly into fresh medium.

3.5.1.2. Experimental culture conditions

For experimental purposes, batch and continuous culture were used:

(a) **Batch cultures** were prepared in two different volumes, 70 mL sterile polystyrene flasks or 10 L flat bottomed glass flasks. The initial culture cell concentration was between 5 to 20 × 10^3 cell mL^{-1} and the culture was grown until stationary phase under the same condition as the stock culture.

(b) **Continuous cultures** were maintained in 2.5 or 100 L chemostats. The photobioreactors were made of transparent polymethyl methacrylate and operated under the following conditions: the pH was regulated at 7.9 using CO_2 addition, the temperature at 18°C, light was provided on one side of the reactor using neon tubes at identical PFD and photoperiod as the stock cultures and a Rushton turbine was used to homogenize the algae. Prior inoculation, photobioreactors were sterilized using peroxyacetic acid at 5 ppm for 30 min and rinsed twice using filtered sea water (0.22 µm). The photobioreactors were inoculated with *A. spinosum* culture to reach an initial concentration of 70 × 10^3 cell mL^{-1} and dilution rate (0.3 and 0.2 day^{-1} in 2.5 or 100 L chemostats, respectively) was applied and maintained until steady state.

Unless otherwise specified these culture conditions were applied for the following experiments.

3.5.2. Environmental factors

3.5.2.1. Salinity, photon flux density, temperature

To assess independently the effect of salinity, temperature and PFD on *A. spinosum* growth and toxin production in batch culture, the cells were inoculated from an inoculum in growth phase and grown under the different conditions in triplicate batch cultures (50 mL) until stationary phase.

- Effect of salinity was assessed at: 10, 20, 30, 32, 35, 40 psu. The salinity was adjusted by dilution with Milli-Q® water (Millipore) or evaporation and verified with a refractometer (Atago, S/MILL).

- Effect of the PFD was also assessed at 50, 100, 150, 200, 250 and 350 µmol m^{-2} s^{-1} and verified with a spherical quantum sensor (LI-250 light meter, LI-COR). Subsequently, the effect of the PFD on *A. spinosum* growth and toxin production were evaluated using two photobioreactors (100 L, 0.2 day^{-1}) at 100, 200 and 400 µmol m^{-2} s^{-1}.

- Effect of temperature was assessed at 10, 14, 18, 22, 26°C in a culture chamber with a PFD at 50 µmol m^{-2} s^{-1}.

3.5.2.2. Aeration

To evaluate the effect of aeration on growth for future experiments in chemostat mode, *A. spinosum* was cultured in triplicate 10 L flat bottomed glass flasks with and without aeration.

3.5.3. Nutritional factors

3.5.3.1. Culture medium

To avoid physiological stress the culture strain was pre-adapted to experimental media. The first medium was the K modified medium used to maintain the stock culture, the second contained tris buffer (Keller et al., 1987), the third one an L1 medium (Guillard and Hargraves, 1993) and the last one an L1 medium with addition of 3 mL of soil extract (CCAP).

Subsequently the amount of K modified medium without tris buffer was assessed. Different medium concentrations were then tested, a 0.1Kmod, 0.5Kmod, Kmod, 2Kmod and 5Kmod medium were then used to grow *A. spinosum* in 50 mL batch culture. Subsequently, *A. spinosum* was inoculated in photobioreactors (100 L, 0.2 day^{-1}) using the following concentration: 0.5Kmod, Kmod and 2Kmod.

3.5.3.2. Nitrogen source

A factorial plan was carried out in duplicate to assess the effect of nitrate (50 to 1714 µM), ammonium (0 to 100 µM) and urea (0 to 100 µM) on *A. spinosum* growth and toxin production in 50 mL batch culture. The medium was based on K modified medium for the other nutrients (Table 14). Subsequently, the effect of nitrate (882 µM) and urea (100µM) on *A. spinosum* growth and toxin production were compared using two photobioreactors (2.5 L, 0.3 day^{-1}).

Table 14. The 17 conditions used during the factorial design experiments to assess the effect of 3 nitrogen sources on *A. spinosum* growth, maximum cell concentration and toxin production.

Conditions	$NaNO_3$ (µM)	NH_4Cl (µM)	Urea (µM)
1	882	50	50
2	50	0	0
3	1714	0	0
4	50	100	0
5	1714	100	0
6	50	0	100
7	1714	0	100
8	50	100	100
9	882	50	50
10	1714	100	100
11	50	50	50
12	1714	50	50
13	882	0	50
14	882	100	50
15	882	50	0
16	882	50	100
17	882	50	50

3.5.3.3. Phosphate source

K modified medium with organic (Na_2 β-glycerophosphate) and inorganic (NaH_2PO_4 H_2O) phosphate were used to evaluate their respective effect on *A. spinosum* grown in batch cultures (50 mL)

3.5.3.4. N/P ratio

Cells were inoculated into medium at the following theoretical N/P supply ratios: 1, 2, 10, 20, 40, 100 by adjusting phosphate (NaH_2PO_4, H_2O) concentration while maintaining

nitrate concentration at 100 µM concentration. Subsequently, 3 N/P ratios (NO_3^-/PO_4^{3-} in µM: 40/5, 160/10, 882/10) were tested using the 2.5 L photobioreactors at 0.3 day^{-1} of dilution rate.

3.5.4. Cell growth

Cell concentrations (cell mL^{-1}), mean estimated spherical diameter (ESD, µm) and cellular volume (µm^3 mL^{-1}, spherical equivalent) were assessed daily using a particle counter (Multisizer 3 Coulter counter, Beckman).

In batch culture: specific growth rates (µ in day^{-1}) were calculated following the equation (Guillard, 1973):

$\mu = (\ln X_t - \ln X_0) / t$

Where X_0 is the initial cell density and X_t is the cell density after t days (t equal to the end of the growth phase in this study).

Maximum growth rate (μ_{max} in d^{-1}), maximun cell concentration ($\alpha = \ln(X/X0)$ with X in cell mL^{-1}) and latency time (λ in day) were calculated following a Gompertz model using MatLab software:

$$f(t) = \alpha \times \exp(-\exp(\mu_{max} \times \exp(1)/\alpha \times (\lambda - t) + 1))$$

In continuous culture, the bioreactors were considered to have reached steady state after a minimum of 3 days at the same microalgal concentration (± 10%).

3.5.5. AZA analysis

3.5.5.1. Reagents

Acetone and methanol (MeOH) were obtained as HPLC grade solvents from JT Baker. Water for analysis was supplied by a Milli-Q integral 3 system (Millipore). Formic acid (Puriss quality) and ammonium formate (Purity for MS) were from Sigma Aldrich. AZA1-3 calibrants for LC–MS/MS analysis were dilutions of certified AZA1-3 solutions obtained from the National Research Council Canada.

3.5.5.2. A. spinosum

The analytical procedure had previously been optimized (Jauffrais et al., 2012a). Briefly, *A. spinosum* culture (10 mL) was collected and centrifuged (2500 *g*, 20 min, 4°C) from the culture medium. The supernatant was discarded and the pellet re-suspended using 0.5 mL of acetone, transferred to an Eppendorf tube (1.5 mL) and bath sonicated (10 min).

After sonication, the aliquot was centrifuged (15000 *g*, 10 min, 4°C). The supernatant was transferred into a 5 mL glass tube and the pellet was re-suspended in 0.5 mL of acetone, homogenised and centrifuged again. The supernatants were transferred again into the same 5 mL glass tube and gently evaporated under nitrogen on a heating block at 35°C. This process was repeated three times in total. After evaporation of supernatants, the residue was reconstituted in 1 mL methanol, filtered with NANOSEP MF centrifugal filter 0.2 µm (PALL) (6000 *g*, 3 min, 4°C), and transferred into a HPLC vial for analysis.

3.5.5.3. LC-MS/MS analysis

The samples were analyzed by LC-MS/MS using an Agilent 1100 model coupled to a triple quadrupole mass spectrometer (API 2000, SCIEX-Applied Biosystems) for quantification of AZAs. Chromatography was performed using a Hypersil BDS C8 column (50 x 2 mm, 3 µm, Thermo scientific) and an isocratic elution at 250 µL.min^{-1} at 20°C for 10 min. Mobile phase A was 100% water (25%) and mobile phase B (75%) consisted of acetonitrile–water (95:5) both containing 2 mM ammonium formate and 50 mM formic acid. The injection volume was 5 µL and the column and sample temperatures were 20 and 5°C, respectively.

The declustering potential was 140 V, the entrance potential 10 V, the cell exit potential 35 V, and the collision cell were 50 and 70 V for fragmentation 1 and 2 respectively. The electrospray ionisation interface (ESI) was operated using the following parameters: curtain gas: 19 psi; temperature: 350°C; gas 1: 30 psi; gas 2: 50 psi; CAD gas: 5 psi; ion spray voltage: 5800 V.

Azaspiracids were quantified against NRC certified AZA1 standards. The two most intense product ions were selected with the following transitions: AZA1 *m/z* 842.5→824.5 and 842.5→672.4, and AZA2 *m/z* 856.5→838.5 and 856.5→672.4.

3.5.6. Nutrient analysis in culture medium

For the analysis of dissolved nitrate and inorganic phosphate, the culture medium was filtered through a 0.22 µm syringe filtered (FP30/0.2 Whatman) in 50 mL pretreated plastic tubes (Sarstedt) pre-washed with 1 M HCl and then rinsed three times with Milli-Q water. and stored at -20°C until analysis according to the method of (Aminot and Kerouel, 2007).

3.5.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Depending on the data, statistical analyses consisted either in one-way analysis of variance (ANOVA), or in a Kruskal–Wallis test, followed, when necessary, by a Fisher's least significant difference procedure or a box-and-whisker plot. Differences were considered significant at $p<0.05$. Before each ANOVA analysis or Kruskal–Wallis test, normality and equality of variance were tested to decide which test was going to be used.

To study the direct effect of the nitrogen sources as well as their interactions on *A. spinosum* growth, a 2^3 experimental design was carried out in duplicate. The 2^3 central composite design involved nitrate, ammonium and urea. This experiment allowed for the assessment of the effect of each factor, as well as their combination simultaneously (Marchetti et al., 2012b).

$$\mu = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \varepsilon$$

Where μ is the growth rate (assess as above) β_i, β_{ij}, and β_{ii} are model coefficients, X_i the main effect for the factor *i*, X_{ij} the interaction between the factors *i* and *j*, X_i^2 the quadratic effect of the factor *i* and ε the residual error. Here, X_1 was chosen for NO_3^-, X_2 for NH_4^+ and X_3 for urea.

Statistical analyses were carried out using Statgraphics Centurion XV.I (StatPoint Technologies, Inc.).

3.6. Results

3.6.1. Environmental factors

3.6.1.1. Salinity

A. spinosum cells cultured in batch culture at a salinity of 10 psu died rapidly after inoculation, and cell inoculated at 20 psu survived until the second day of the experiment but no further; however, growth of *A. spinosum* was observed between 30 and 40 psu (Table 15), with a specific growth rate assessed between 0.19 and 0.31 d^{-1}. The best growth rate and cell concentration was obtained at 35 psu whereas the highest toxicity was obtained with the two extreme values, 30 and 40 psu. Nevertheless, the significant difference in AZA cell quota found at 40 psu can be partially explained by a larger mean ESD and a larger cellular volume found at 40 psu than at 35 psu.

Table 15. **Batch culture:** *A. spinosum* specific growth rate, μ (d^{-1}), maximum cell concentration, Cmax (10^3 cell mL^{-1}), and AZA cell quota (fg $cell^{-1}$) in batch culture at different salinities (psu), photon flux densities (PFD, μmol m^{-2} s^{-1}), temperatures (℃) and with the presence or absence of aeration (values with different letters are statistically different at P<0.05). Standard batch culture conditions were applied for all conditions unless specified in a given treatment (n=3).

Environmental factors		μ (d^{-1})	Cmax (10^3 cell mL^{-1})	AZAs (fg $cell^{-1}$)
Salinity (psu)	30	0.19 ± 0.00 ^{d}	48 ± 1 ^{c}	31 ± 2 ^{b}
	32	0.29 ± 0.00 ^{b}	65 ± 2 ^{b}	29 ± 3 ^{b}
	35	0.31 ± 0.01 ^{a}	77 ± 3 ^{a}	23 ± 1 ^{c}
	40	0.21 ± 0.01 ^{c}	32 ± 2 ^{d}	37 ± 3 ^{a}
PFD (μmol m^{-2} s^{-1})	50	0.31 ± 0.01 ^{c}	95 ± 7	16 ± 2
	100	0.42 ± 0.01 ^{b}	94 ± 2	22 ± 1
	150	0.41 ± 0.01 ^{b}	95 ± 2	16 ± 4
	200	0.41 ± 0.01 ^{b}	94 ± 3	16 ± 1
	250	0.41 ± 0.02 ^{b}	98 ± 1	21 ± 1
	350	0.49 ± 0.02 ^{a}	108 ± 3	12 ± 1
Temperature (°C)	10	0.12 ± 0.01 ^{e}	53 ± 5 ^{d}	220 ± 38 ^{a}
	14	0.25 ± 0.01 ^{d}	80 ± 2 ^{c}	80 ± 10 ^{b}
	18	0.33 ± 0.03 ^{c}	86 ± 7 ^{bc}	14 ± 2 ^{c}
	22	0.46 ± 0.03 ^{a}	90 ± 5 ^{a}	8 ± 1 ^{d}
	26	0.40 ± 0.02 ^{b}	58 ± 7 ^{d}	14 ± 1 ^{c}
Aeration	Yes	0.41 ± 0.02 *	316 ± 15 *	122 ± 2 *
	No	0.37 ± 0.02	90 ± 2	15 ± 2

3.6.1.2. Photon flux density

A. spinosum growth was observed between 50 and 350 μmol m^{-2} s^{-1}. The specific growth rate was found to be comparable between 100 and 250 μmol m^{-2} s^{-1}; however significant differences were found and the lowest growth rate was observed at 50 μmol m^{-2} s^{-1} and the highest at 350 μmol m^{-2} s^{-1}. Nonetheless, for the highest growth rate, a combined effect of PFD and temperature cannot be excluded due to the proximity to the light source. Concerning AZA cell quota and the maximum cell concentration, no differences were found between the different PFD tested.

To refine results found in batch culture *A. spinosum* was subsequently grown in continuous culture (Figure 42). In stirred photobioreactors, a slightly higher cell concentration was observed at 200 and 400 μmol m^{-2} s^{-1} compared to growth at 100 μmol m^{-2} s^{-1}. However, the main effect found was on the AZA cell quota, where an increase by a factor of 3 was observed between the lowest PFD and the highest one (Table 16). Like AZA cell quota, ESD increased as PFD increased which thus partially explains the differences found between cellular volume (Figure 42) and AZA cell quota, mainly at 200 and 400 μmol m^{-2} s^{-1}.

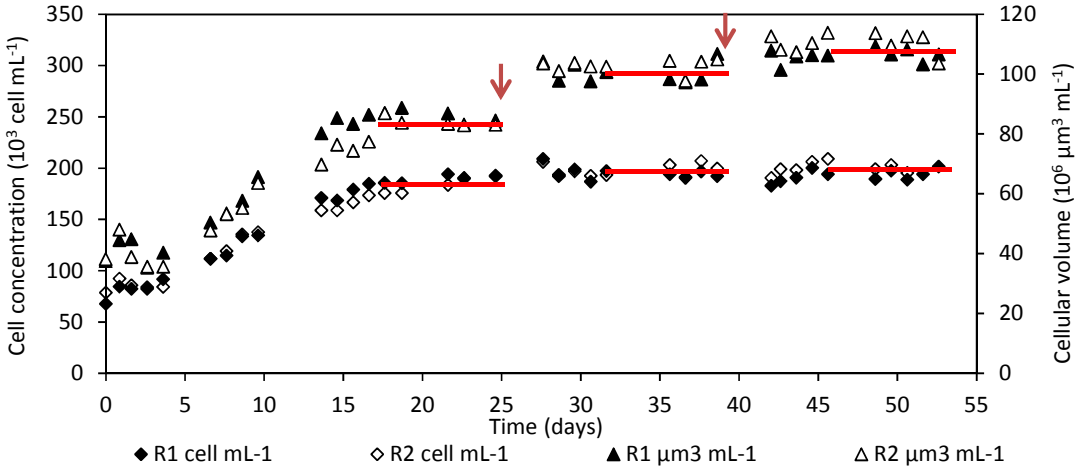

Figure 42. *A. spinosun* concentration (cell mL^{-1}) and cellular volume (μm^3 mL^{-1}) kinetics in 100 L photobioreactors (R1 and R2, replicates) at a dilution rate of 0.2 day^{-1} and at three different irradiance conditions 100, 200 and 400 µmol m^{-2} s^{-1}. The steady states are represented by the red lines and the arrows represent the day where the irradiance was changed from 100 to 200 µmol m^{-2} s^{-1} and from 200 to 400 µmol m^{-2} s^{-1}, respectively.

Table 16. **Continuous culture:** *A. spinosum* concentration (10^3 cell mL^{-1}), mean estimated spherical diameter (ESD, µm), cellular volume (μm^3 mL^{-1}) and AZA cell quota (fg $cell^{-1}$) in continuous culture at steady state using different photon flux densities (PFD, µmol m^{-2} s^{-1}), K medium concentration, nitrogen sources (nitrate and urea) and N/P ratios (values with different letters are statistically different at P<0.05). Standard continuous culture conditions were applied for all conditions unless specified in a given treatment (nine sampling occasions for each of the two bioreactors).

Factors		Concentration (10^3 cell mL^{-1})	ESD (µm)	cellular volume (10^6 μm^3 mL^{-1})	AZAs (fg $cell^{-1}$)
PFD (µmol m^{-2} s^{-1})	100	185 ± 7 ^{b}	9.5 ± 0.1 ^{c}	85 ± 3 ^{c}	21 ± 2 ^{b}
µ =0.2 day^{-1}	200	194 ± 5 ^{a}	9.9 ± 0.1 ^{b}	101 ± 3 ^{b}	58 ± 9 ^{a}
	400	195 ± 7 ^{a}	10.1 ± 0.1 ^{a}	108 ± 4 ^{a}	69 ± 11 ^{a}
Kmod	0.5	122 ± 7 ^{c}	10.1 ± 0.1 ^{a}	66 ± 3 ^{c}	58 ± 5 ^{a}
µ =0.2 day^{-1}	1	196 ± 7 ^{b}	9.9 ± 0.1 ^{a}	104 ± 3 ^{b}	47 ± 6 ^{b}
	2	346 ± 29 ^{a}	9.7 ± 0.2 ^{b}	170 ± 17 ^{a}	25 ± 7 ^{c}
Nitrogen source	Nitrate (882 µM)	207 ± 13 ^{a}	10.2 ± 0.2 ^{a}	119 ± 8 ^{a}	41 ± 8
µ =0.3 day^{-1}	Urea (100 µM)	182 ± 10 ^{b}	9.9 ± 0.1 ^{b}	95 ± 7 ^{b}	42 ± 6
$N0_3^-$/$P0_4^{3-}$ ratio (µM)	40/5	59 ± 6 ^{c}	9.6 ± 0.3 ^{b}	29 ± 4 ^{c}	18 ± 3 ^{b}
µ =0.3 day^{-1}	160/10	187 ± 11 ^{b}	9.7 ± 0.1 ^{ab}	92 ± 4 ^{b}	14 ± 1 ^{b}
	882/10	216 ± 10 ^{a}	10.0 ± 0.3 ^{a}	117 ± 6 ^{a}	36 ± 2 ^{a}

3.6.1.3. Temperature

The largest differences found in specific growth rate of *A. spinosum* were observed using temperature (Table 15). However, even though growth rate seemed to be temperature dependent *A. spinosum* is able to grow in a large range of temperature (10 to 26°C) to reach in all cases cell concentration above 50×10^3 cell mL^{-1}. The best growth rate was assessed at 22°C and equal to 0.46 d^{-1} whereas a growth rate of 0.12 d^{-1} was found at 10°C.

In the case of temperature, AZA cell quota were found to be antagonist to growth rate, as at low growth rate a high AZA cell quota was found (10°C, 220 fg $cell^{-1}$) whereas at high growth rate a low AZA cell quota was found (22°C, 8 fg $cell^{-1}$).

3.6.1.4. Aeration

In 10 L batch culture flasks with aeration of the culture *A. spinosum* was found to have a specific growth rate situated around 0.41 d^{-1} with K modified medium, and reached maximum cell concentration of 315×10^3 cell mL^{-1} with aeration and of 90×10^3 cell mL^{-1} without aeration. AZA cell quota was also higher with aeration than without aeration, i.e 122 ± 2 and 15 ± 2 fg $cell^{-1}$ at the stationary phase, respectively.

The batch culture in 10 L aerated flasks was also used to assess *A. spinosum* maximum growth rate, maximum cell concentration, latency time, the variation of the mean ESD, the AZA cell quota and the of nitrate and phosphate consumption during a batch culture (Figure 43).

The ESD decreased during the first days of the culture until the first limitation appeared and growth rate begun to decrease (day 4) and then increased until the end of the stationary phase and finally decreased as senescence phase begun (Figure 43a). The maximum growth rate found under this condition was equal to 0.57 day^{-1}, with a maximum cell concentration of 302×10^3 cell mL^{-1} (95% coefficient bound: 288×10^3 to 324×10^3 cell mL^{-1}) and a latency time of 0.2 day (Figure 43b). Using the K modified medium (with NaH_2PO_4 as source of phosphate), phosphate was found to be a limiting nutrient, as after 14 days of culture the concentration was close to 0 µM (Figure 43c). Interestingly, AZA cell quota increased from the beginning of the culture to the end of the stationary phase and drastically decreased at the beginning of the senescence. The AZA2/AZA1 ratio was also similar during the growth curve of *A. spinosum* and equal to 0.30 ± 0.2, corresponding to a proportion of AZA1 equal to 77 ± 0.2% of AZA1+2.

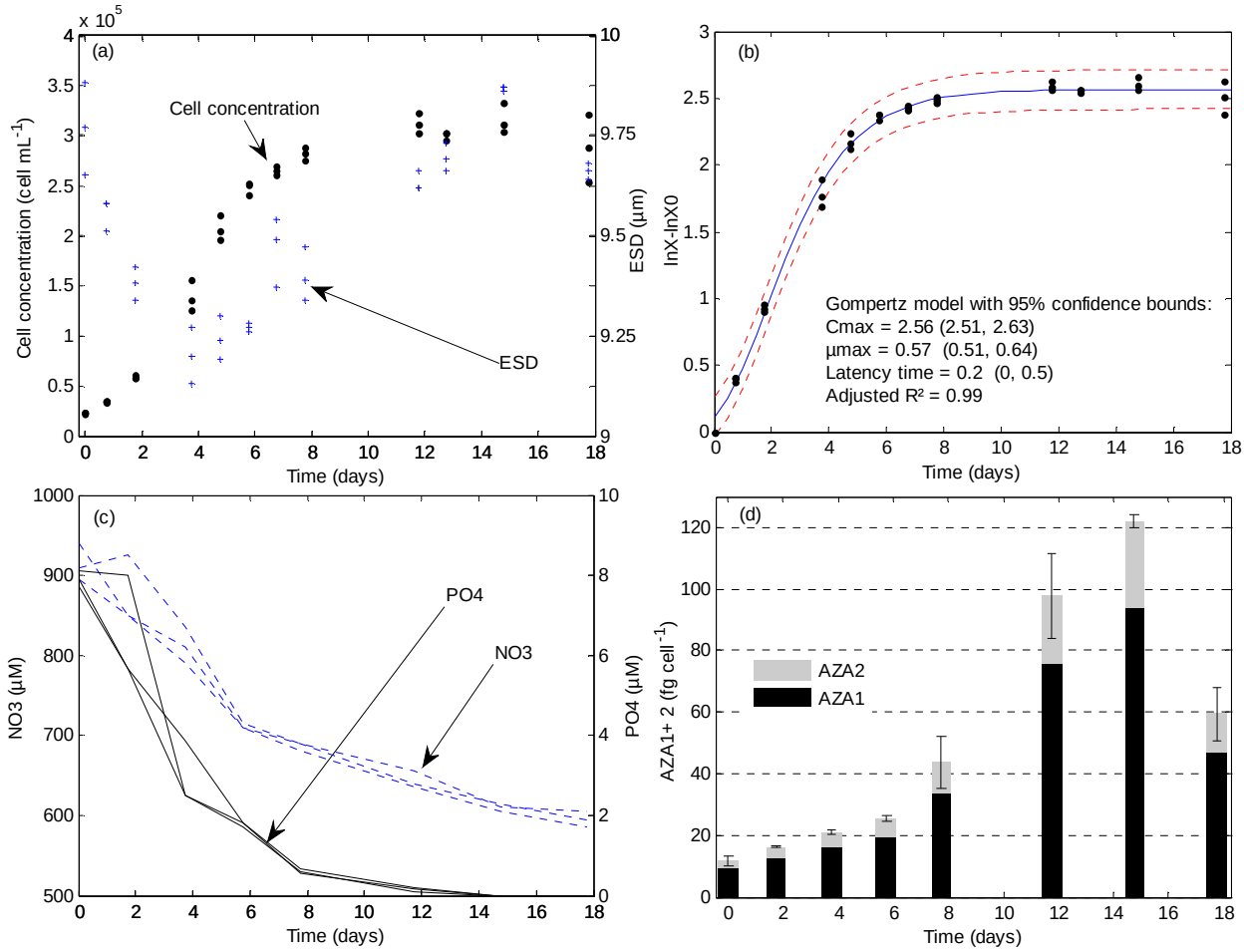

Figure 43. (a) *A. spinosum* cell concentration and mean spherical diameter (ESD) as a function of time in aerated 10 L culture flasks, (b) Gompertz model fitted to the cell concentration with its 95% confident bounds for the maximum cell concentration (Cmax), growth rate (µmax), latency time and its adjusted R^2, (c) NO_3^- and PO_4^{3-} consumption by *A. spinosum* as a function of time and (d) AZA1+2 cell quota as a function of time (error bars = SD, n=3).

3.6.2. Nutritional factors

3.6.2.1. Medium

A. spinosum growth was observed in the four media tested with growth rates fluctuating between 0.39 and 0.45 day^{-1} (Table 17). Even though growth rates were close to each other, statistical differences were found, and the K modified medium (Kmod) without NH_4Cl and without tris buffer yielded the highest growth rate. However, no statistically significant differences were found concerning the *A. spinosum* maximum concentration. Some small but statistically significant variations were noticed in AZA cell quota (Kmod2 > Kmod = L1 = L1+SE). Maximum AZA concentration was found using the second

K modified medium (Kmod2, with tris buffer). It was also noticed that the addition of soil extract to the L1 medium slightly increased growth rate and decreased AZA cell quota.

Table 17. *A. spinosum* specific growth rate, μ (d^{-1}), maximum cell concentration, Cmax (10^3 cell mL^{-1}), and AZA cell quota (fg $cell^{-1}$) in batch culture using different culture medium and concentration (values with different letters are statistically different at P<0.05). Standard batch culture conditions were applied for all conditions unless specified in a given treatment (n=3).

Nutritional factors		μ (d^{-1})	Cmax (10^3 cell mL^{-1})	AZAs (fg $cell^{-1}$)
Culture medium	Kmod	0.45 ± 0.01 ^{a}	98 ± 3	20 ± 2 ^{b}
	Kmod2	0.39 ± 0.01 ^{c}	88 ± 1	27 ± 1 ^{a}
	L1	0.40 ± 0.01 ^{c}	101 ± 1	22 ± 1 ^{b}
	L1+SE	0.42 ± 0.01 ^{b}	100 ± 3	14 ± 4 ^{b}
Kmod	0.1	0.39 ± 0.02	40 ± 1 ^{d}	75 ± 2 ^{a}
	0.5	0.39 ± 0.02	83 ± 2 ^{c}	26 ± 1 ^{b}
	1	0.40 ± 0.02	88 ± 2 ^{b}	24 ± 2 ^{bc}
	2	0.41 ± 0.02	99 ± 1 ^{a}	23 ± 3 ^{c}

As Kmod (without NH_4Cl and tris buffer) resulted the highest growth rates (and for technical reasons discussed below), this medium was tested at different concentrations in batch culture as well as in continuous culture. *A. spinosum* did not grow at the highest concentration (5Kmod medium) even if the cells survived throughout the experiment. Under the other four conditions, *A. spinosum* grew, and no differences were observed in growth rate. Nevertheless, the cell concentration was positively correlated to the concentration in medium whereas the highest AZA cell quota were found at the lowest concentration in Kmod medium (0.1Kmod, Table 17). As well as for the PFD, the *A. spinosum* cells were inoculated in a continuous culture to strengthen the results found in batch culture. In continuous culture, cell concentration was positively correlated to the concentration in medium (Table 16), and AZA cell quota was negatively correlated to it. Differences were also found in ESD as at a lower medium concentration the ESD was found to be larger than at high medium concentration.

3.6.2.2. Nitrogen and phosphate source

A factorial plan carried out to assess the effect of three different nitrogen sources (Nitrate, ammonium, urea) on *A. spinosum* growth rate had a good adjusted R^2 (0.95), indicating that the model as fitted explains 95% of the variability in the growth rate measured. *A. spinosum* growth was observed in all conditions tested; however, whereas the concentrations of nitrate and urea were not affecting growth rates, the addition of ammonium reduced it from 0.38 to 0.26 day^{-1} (Figure 44b and a). These variations were mainly explained

by the negative effect of ammonium on *A. spinosum* (Figure 44b, c and d), while no combined effect (interaction) of nitrate and urea concentration (neither positive nor negative) were found; only a small combined effect of ammonium and nitrate was noticed.

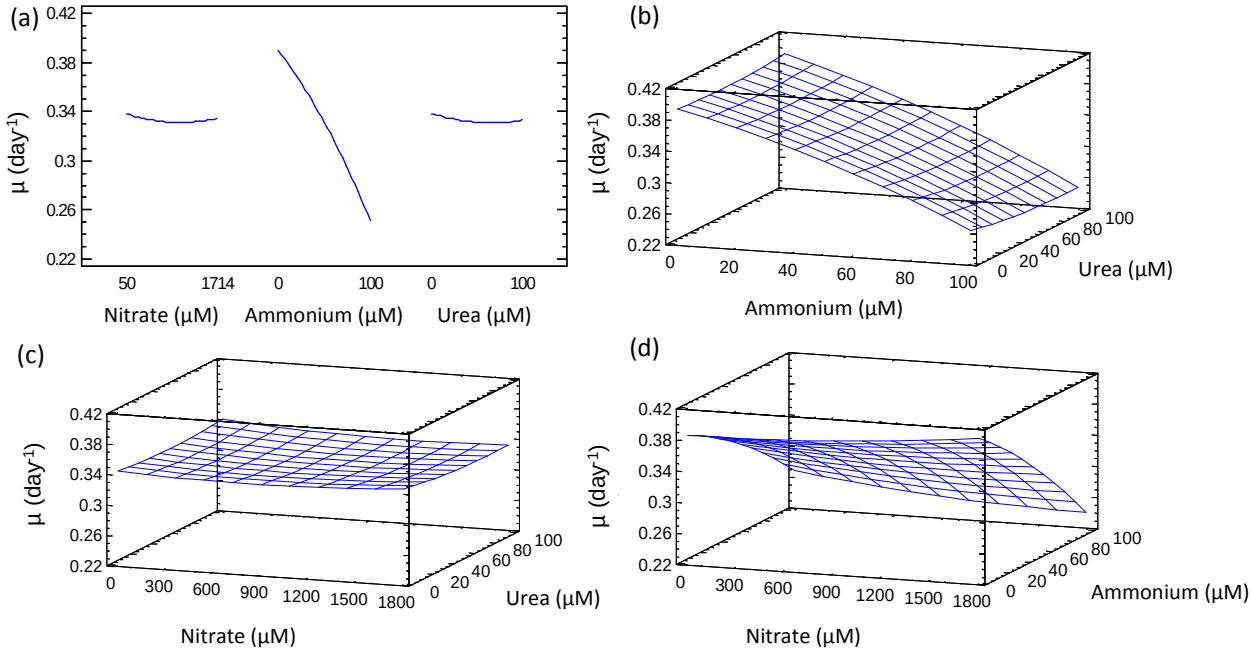

Figure 44. (a) Standardized effect nitrate, ammonium and urea on growth rate (μ in day^{-1}), (b, c, and d) surface plots of the modeled growth rate for two of the three factors when the third one is centered (nitrate: 882 μM, ammonium and urea : 50 μM).

Concerning the effect of nitrate, ammonium and urea on cell concentration and AZA cell quota, the model used only explained 58 and 52%, respectively, of the variability found and consequently cannot be exploited without approximation. However, the maximum cell concentration in the factorial plan was situated between 88×10^3 and 115×10^3 cell mL^{-1}, and the AZA cell quota between 10 and 20 fg $cell^{-1}$ (Annexe 10).

The effects of nitrate and urea were also studied in photobioreactors (Table 16), and results found between the two conditions were close; still, a slight difference was found in favor of nitrate in cell concentration as well as in mean ESD and cellular volume but no differences were found in AZA cell quota (41 *vs* 42 fg $cell^{-1}$).

Two sources of phosphate were also tested, mainly to simplify nutrient analysis and results were found to be similar in growth rate and AZA cell quota using either NaH_2PO_4 or β-glycerophosphate (data not shown).

3.6.2.3. N/P ratios

A. spinosum grew in all conditions tested; however, using low phosphate concentration (< 2.5 µM) growth was limited while at high phosphate concentration (> 10 µM), growth rate very slightly decreased compared to the optimum. N/P ratios seemed also to affect AZA cell quota, as at high N/P ratio higher AZA concentrations were found (Table 18). To verify these effects, the cells were inoculated in photobioreactors, and AZA cell quota were found to be maximized at the highest N/P ratio tested; also, cell concentration seemed to be less dependent of the N/P ratio (Table 16).

Table 18. *A. spinosum* specific growth rate, µ (d^{-1}), maximum cell concentration, Cmax (10^3 cell mL^{-1}), and AZA cell quota (fg $cell^{-1}$) in batch culture using different N/P ratios (values with different letters are statistically different at P<0.05).

NO_3^- (µM)	$PO4^{3-}$ (µM)	N/P	µ (d^{-1})	Cmax (10^3 cell mL^{-1})	AZAs (fg $cell^{-1}$)
100	0	100/0	0.16 ± 0.02 ^{d}	14 ± 0 ^{e}	40 ± 1 ^{a}
100	2.5	40/1	0.40 ± 0.01 ^{a}	77 ± 2 ^{c}	21 ± 2 ^{b}
100	5	20/1	0.41 ± 0.01 ^{a}	87 ± 1 ^{a}	11 ± 2 ^{cd}
100	10	10/1	0.38 ± 0.01 ^{b}	80 ± 1 ^{b}	11 ± 1 ^{d}
100	50	2/1	0.36 ± 0.02 ^{bc}	71 ± 1 ^{d}	11 ± 3 ^{cd}
100	100	1/1	0.35 ± 0.01 ^{c}	77 ± 2 ^{c}	13 ± 1 ^{c}

3.7. Discussion

The *A. spinosum* used in this study was a dinoflagellate recently isolated in the North Sea near the coast of Scotland and producer of AZA1 and -2 *in situ* and in cultures. Since its discovery, *A. spinosum* was found to directly contaminate mussels (Jauffrais et al., 2012c; Salas et al., 2011) and interrogations were raised on how mussels are able to accumulate high AZA concentrations (>5000 µg kg^{-1}) *in situ*. Contrarily, such very high concentrations have not yet been observed in laboratory studies. The understanding of environmental and nutritional factors affecting *A. spinosum* growth and AZA accumulation in the field constitutes thus a pre-requisite to effective coastal zone management and mitigation of harmful blooms. Furthermore, a lack of purified AZAs hampered toxicological studies and LC-MS/MS calibration in the frame of monitoring programs. Consequently, the optimization

of *A. spinosum* growth and toxin production in culture was an additional motivation for the in-depth study of its capacity to produce AZAs.

3.7.1. Environmental factors

Data reported here reveal that *A. spinosum* is able to grow well in a relatively wide variation of salinity (30 to 40 psu), and to be able to survive for a few days at 20 psu. Even though this range does not classify *A. spinosum* as a euryhaline species (at 10 psu no survival was observed), the tolerance for short periods to decreased salinity may at least partially explain why *A. spinosum* fares relatively well in Irish bays or Norwegian fjords, where rainfall may temporarily contribute to large freshwater inputs. Furthermore, cells grown at the extreme values of the range (30 and 40 psu) were found to contain slightly more toxins. Therefore, salinity fluctuations in the coastal areas where *A. spinosum* is found can significantly influence both its growth rate and its toxin content. Some studies performed on the effect of salinity on various toxic dinoflagellates highlighted both intraspecific effects and interspecies differences of salinity. *Alexandrium tamarense* exposed to different salinities showed a maximum toxin cell quota at either a higher salinity than the optimal growth conditions (White, 1978) or at salinity equivalent to the most appropriate growth (Parkhill and Cembella, 1999). For *Gymnodinium catenatum*, a decrease in salinity did not cause a significant increase in toxin production (Flynn et al., 1996); however, with *Alexandrium minutum* low salinity were found to increase toxin quota (Grzebyk et al., 2003) and more recent studies showed similar conclusions for other dinoflagellates (eg. *Protoceratium reticulatum* (Guerrini et al., 2007), *Alexandrium ostenfeldii* (Maclean et al., 2003)).

The different PFDs tested in batch cultures showed that *A. spinosum* growth rate could be slightly enhanced by increasing irradiance. Furthermore, the experiment using stirred photobioreactors highlighted the effect of PFD on toxin production and showed that high irradiance significantly enhanced AZA cell quota *ca*. three-fold. A study of *A. tamarense* also found a reduction of light intensity to reduce to some extent growth rate but to only slightly increase toxin cell quota (Ogata et al., 1987). The authors agreed with our study in that decreasing light intensity might reduce growth and that photosynthesis in dinoflagellates plays an important role in the synthesis of toxins, as shown to some extent in the bioreactor experiments of our study.

Temperature was found to play major role on *A. spinosum* growth, displaying an optimum (at 22°C) as for other dinoflagellates (Laabir et al., 2011; Matsubara et al., 2007;

Nielsen, 1996; Xu et al., 2010). Similar to the effects of salinity, a drop in temperature corresponded to a decrease in growth rate concurring with a more pronounced increase in toxin content (Anderson et al., 1990; Guerrini et al., 2007; Navarro et al., 2006; Ogata et al., 1987). However, a temperature above the optimum growth can also lead to an increase in toxin quota in some species (*Protoceratium reticulatum* (Guerrini et al., 2007), *Prorocentrum hoffmannianum* (Morton et al., 1994)). Such observations are suggestive of the hypothesis that an increase in toxin concentration may be a possible answer to either an environmental stress or to the decrease in growth rate. In coherence with the results found in literature, *A. spinosum* was able to grow in and to adapt rapidly to a wide range of temperatures, and the reduction of growth rate observed when the temperature decreased was counter-balanced by an important increase in AZA cell quota.

The addition of turbulences by either aeration, agitation, shaking into a dinoflagellate culture is known to either increase cell concentration and toxin production (Morton and Bomber, 1994; Pollingher and Zemel, 1981) or to inhibit growth (Berdalet, 1992).

In the case of *Alexandrium minutum*, turbulence cause a decrease in growth rate, biomass, and lower concentration of paralytic shellfish toxins and a production of ecdysed cysts (Bolli et al., 2007). However, all authors do not agree with these effects of turbulence and some believe that these tests were conducted above a growth inhibition threshold (Burkholder et al., 2006). Indeed, turbulence seemed to be an intraspecific factor and depended upon the applied intensity. Using turbulence intensities comparable to natural conditions, a wide range of toxic dinoflagellate species were stimulated (growth rate and cell concentration, *Lingulodinium polyedrum*, *Gymnodinium catenatum*, *Alexandrium fundyense*), unaffected (*Pyrocystis noctiluca*, *Ceratium tripos*, *Alexandrium tamarense*, *Pyrocystis fusiformis*, *Alexandrium catenella*, *Gyrodinium* sp.) or affected (*Ceratium fusus*) (Sullivan and Swift, 2003). Concerning *A. spinosum*, agitation can be applied to enhance biomass and AZA cell quota; furthermore, in our study, agitation enlarged the length of the stationary phase. Particularly, aeration significantly improved culture condition by promoting the exchange of gases and nutrients between *A. spinosum* and the culture medium. This positive enhancement through agitation was somewhat surprising in view of the thin theca of *A. spinosum*, which may have been considered synonymous to fragility of the organism.

During growth in batch culture, this study showed an increasing toxin quota during the exponential growth phase as for PSTs (Boyer et al., 1987; Guillard, 1975; Kim et al., 1993). However, the AZA cell quota keep increasing during the late growth phase and stationary phase, and consequently *A. spinosum* is more comparable to other dinoflagellates producer of

polyether toxins (Bomber and Tindall, 1988; Guerrini et al., 2010; Pan et al., 1999). This higher AZA cell quota was corresponding to phosphate limitation and consequently to an increased N/P ratio, as for other polyether toxin producers, where, toxin production appeared to be maximized when phosphorus is depleted from the culture medium (McLachlan et al., 1994; Morlaix and Lassus, 1992; Vanucci et al., 2010).

3.7.2. Nutritional factors

Dinoflagellates growth and toxin production are influenced by nutritional factors such as nitrogen and phosphate (Anderson et al., 1990; Flynn et al., 1994; John and Flynn, 2000). Consequently, different culture media, with different N/P ratios and phosphate sources were tested on *A. spinosum*. The first reports on *A. spinosum* culture, mentioned the use of a half strength K modified medium without addition of ammonium ions (Tillmann et al., 2009), a nutrient known to be rapidly toxic to some dinoflagellates (Chang and McClean, 1997; Leong and Taguchi, 2004) and (Salas et al., 2011) used a F/2 media (Guillard, 1975; Guillard and Ryther, 1962) that also does not contain ammonium ions. The present study explored the effect of two K modified media, one was similar to the one used by (Tillmann et al., 2009) and the other one was without the tris buffer. The algae were adapted to a K medium without tris buffer for technical reasons: in continuous culture the pH is already controlled by addition of CO_2 then the media was simplified; furthermore, after several experiments precipitations were observed to gradually reduce the flow rate and to finally clog the pump used to supply the culture medium to the photobioreactors when the tris buffer was used.

However, even though it was primarily for technical reason we found out that this modification of the K medium also improved *A. spinosum* growth but unfortunately not toxin production. The two other media also resulted in comparable growth rate and toxin production, and the use of these different media in culture collections will ensure a safer maintenance of this species.

To evaluate the required amount of medium necessary for optimum growth and enhancement of the toxin production when necessary, different K modified medium concentrations were tested. A concentration above 0.5K was found to be necessary to reach relatively high cell concentration in batch culture whereas a low K concentration seemed to enhance AZA cell quota. As K medium is rich in nitrate and poor in phosphate, the reduction in maximum cell concentration at 0.1 K indicates a possible enhancement of AZA cell quota through P limitation in presence of high nitrate concentration. Furthermore, increasing K

medium concentration up to 2K did not decrease *A. spinosum* growth. However, at 5K, the medium became inhibitory to *A. spinosum*. Consequently *A. spinosum* was inoculated in photobioreactors to assess the effect on biomass and AZA productions. The data reported here showed that by increasing the addition of K medium two-fold the biomass was multiplied by a factor of 1.75 but AZA cell quota was decreased two-fold. However, using a maturation period and an optimized flow rate, as developed by (Jauffrais et al., 2012b), will enhance AZA production.

The reported results showed that *A. spinosum* can grow on different sources of nitrogen and that *A. spinosum* growth was reduced by increasing ammonium concentration into the culture medium as for other dinoflagellates (Chang and McClean, 1997; Leong and Taguchi, 2004). Furthermore, the factorial plan and the continuous culture showed that either urea or nitrate could be used as source of nitrogen, with almost no effect on cell concentration and AZA production. This finding is important as during the last four decades the amount of urea in fertilizers has increased drastically in many agricultural regions of the world and has been linked to increasing paralytic shellfish poisoning events (Glibert et al., 2006). Furthermore, anthropogenic addition of nitrogen or phosphate into the costal water unbalanced the Redfield N/P ratio (16/1) commonly found in the ocean and might modify growth and toxin production of toxic dinoflagellates (Glibert et al., 2006; Heisler et al., 2008). In PST producers, N limiting conditions usually result in a limited toxin production and growth (Siu et al., 1997; Wang and Hsieh, 2002) while PSTs seem to be stimulated in culture under P limitation, which also limits growth (Anderson et al., 1990; Boyer et al., 1987; Frangopulos et al., 2004; Guisande et al., 2002; Siu et al., 1997). In polyether toxin producers, P limiting conditions seemed to enhance toxin production as already mentioned and N limiting conditions can also maximize toxin production (Vannucci et al., 2010), but in both cases, N or P limitation reduced growth and even toxin production for *Ostreopsis* cf. *ovata* (Vanucci et al., 2012). These results found in the literature matched what has been found with *A. spinosum*, where limiting N or P limits growth, however, limiting P when N is still abundant will enhance AZA cell quota.

3.8. Conclusions

A. spinosum was found to grow in a wide range of conditions, from 30 to 40 psu, from 10 to 26°C, 50 to 400 µmol m^{-2} s^{-1} and with or without aeration; with an optimum growth rate and cell concentration found at 35 psu, from 18 to 22°C and with aeration. AZA cell quota

were significantly increased by low temperature: the AZA cell quota was more than 20 times higher at 10°C (220 fg cell^{-1}) than at temperatures comprised between 18 and 26°C. Likewise, but to a lesser degree, AZA cell quota were enhanced at high irradiance and using aeration. *A. spinosum* growth rate and AZA cell quota were found to be slightly different with all media tested. However, low K modified medium concentration (0.1Kmod) improved AZA cell quota while higher concentration (0.5Kmod, 1Kmod, 2Kmod) improved growth rate and maximal cell concentration.

These experiments on the effects of environmental and nutritional factors on *A. spinosum* improved our knowledge on its ecophysiology and allowed its growth in pilot scale photobioreactors for AZA1 and -2 production (Jauffrais et al., 2012b). Furthermore, it gave first insights into environmental and nutritional factors that could impact natural blooms.

However, further studies using factorial plan designs are now required to determine the optimum parameters of *A. spinosum* culture in order to investigate if further improvements of AZA production can be made using *A. spinosum* in continuous cultures. Interestingly, the ability of this species to support conditions applied in continuous cultures will help to study the effect of nutrient limitations on growth, AZA toxin production and *A. spinosum* biochemical composition. Thanks to its small size, easy growth in continuous culture and large differences observed under different environmental and nutritional conditions, the organism could also be an appropriate model for the elucidation of the genes involved in toxinogenesis.

3.9. Acknowledgement

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Chapitre 4 – Transfert trophique

Contexte

Les azaspiracides (AZA) ont été découverts suite à la consommation de moules irlandaises contaminées (McMahon and Silke, 1996) et *Azadinium spinosum*, producteur d'AZA1 et -2, a été isolé récemment au large de l'Ecosse (Tillmann et al., 2009). Ces toxines affectent régulièrement les fermes conchylicoles irlandaises et entrainent des périodes de fermetures prolongées des zones de pêche et de production. Ces fermetures ont un impact économique sur le secteur conchylicole et envoient une image négative de cette filière auprès des consommateurs. Cependant, la relation directe entre les contaminations de moules aux AZA et ce petit dinoflagellé toxique reste à démontrer. De plus la présence d'*A. spinosum* sur les côtes irlandaises bien que fort probable n'avait pas encore été démontrée.

La première partie de ce chapitre a été réalisée par Rafael Salas au Marine Institute en Irlande et par l'équipe de l'Alfred Wegner Institute en Allemagne et est consacrée à l'isolement d'une nouvelle souche d'*A. spinosum* en Irlande et à la mise en évidence du lien direct existant entre *A. spinosum* et l'accumulation d'AZA par les moules. C'est pour réaliser cette dernière expérience que je suis allé en Irlande en Septembre 2010.

Une fois ce lien mis en évidence plusieurs études se sont ensuite déroulées à l'Ifremer de Nantes pour approfondir les relations existantes entre *A. spinosum* et les mollusques bivalves.

Une seconde étude s'est par la suite intéressée à l'accumulation et à la détoxification des AZA ainsi qu'au devenir de la toxine dans les moules nourries avec *A. spinosum* (biotransformation, distribution des AZA dans les différents tissus de la moule).

Pendant cette étude, les moules nourries avec *A. spinosum* ont montré une légère augmentation de leur mortalité, une diminution de l'épaisseur des tubules des glandes digestives et un ralentissement significatif de l'accumulation en toxine après six heures de contamination.

Ces observations pouvant être la réponse des moules à un stress physiologique provoqué par *A. spinosum*, une troisième étude a donc été menée. Celle-ci a étudié les effets d'*A. spinosum* sur le comportement alimentaire des moules. L'évaluation individuelle de l'activité alimentaire, du taux de filtration, d'ingestion et d'absorption a été effectuée sur des moules soumises à un régime toxique (*A. spinosum*) ou à un régime non-toxique (*Isochrysis* aff. *Galbana* (T-*Iso*)).

Enfin pour savoir si les moules sont éventuellement capables d'accumuler les AZA dissous relargués en fin d'efflorescence, une quatrième étude a cherché à évaluer si les moules

étaient capables d'accumuler AZA1 et -2 présent dans l'eau sous forme dissoute, et a comparé leur profil toxinique ainsi que la distribution des toxines dans différents tissus de la moule avec ceux de moules exposées aux cellules d'*A. spinosum*. Cette dernière étude présente également les résultats obtenus par Jane Kilcoyne du Marine Institute sur l'évaluation de la de la possibilité de produire des métabolites d'AZA1 par balnéation des moules dans une phase aqueuse contenant de l'AZA1 semi-purifié.

1. The role of *Azadinium spinosum* (Dinophyceae) in the production of azaspiracid shellfish poisoning in mussels

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1.1. Résumé

Les azaspiracides (AZA) sont un groupe de toxines de type polyéther lipophiles. Ils ont été détectés pour la première fois à partir de moules en provenances d'Irlande, et, depuis, les AZA ont été impliqués dans plusieurs intoxications alimentaires, suite à la consommation de mollusques bivalves contaminés. Ces toxines affectent régulièrement les fermes conchylicoles irlandaises et entrainent des périodes prolongées de fermetures des zones de pêches et de production. Le dinoflagellé *Azadinium spinosum* a récemment été décrit comme producteur d'AZA; néanmoins, le lien existant entre cet organisme et l'accumulation d'AZA dans les mollusques bivalves n'a pas encore été établie.

En Août 2009, des moules (*Mytilus edulis*) du sud ouest de l'Irlande ont été analysées par chromatographie liquide couplée à la spectrométrie de masse (CL-SM/SM) et se sont avérées contenir une concentration en AZA supérieure à limite réglementaire. Des échantillons d'eau provenant de cette zone ont été recueillis et un isolat de microalgues a été identifié comme étant *A. spinosum* et s'est avéré producteur d'AZA1 et -2. Il s'agit de la première souche d'*A. spinosum* isolée dans les eaux irlandaises. Cette souche irlandaise est identique aux autres souches isolées au large de l'Ecosse (3D9) et du Danemark (UTHE2).

Pour clarifier le processus d'accumulation des AZA dans les moules, ces dernières ont été exposées, pendant 24 h, à trois densités cellulaires d'*A. spinosum*. Cette étude a montré

que *A. spinosum* est filtré, absorbé et digéré directement par les moules. De plus, les analyses CL-SM/SM ont souligné le fait que les AZA s'accumulaient dans la glande digestive des mollusques et ont permis de mettre en évidence la présence d'analogues d'AZA : AZA3, AZA6, AZA17 et -19. La mise en évidence de ces métabolites d'AZA, au cours des premières 24 h qui suivent l'ingestion d'*A. spinosum*, suggère une biotransformation rapide d'AZA1 et -2 dans les coquillages. Après seulement 24 h d'exposition les teneurs en AZA17 mesurées dans les glandes digestives des moules étaient équivalentes aux teneurs en AZA1 ; cependant, dans les tissus restants, la concentration en AZA17 était 4 à 5 fois plus élevée qu'AZA1. Seules de petites quantités d'AZA3 et -19 ont été détectées ainsi que des quantités négligeables d'AZA6, soulignant le fait qu'AZA17 est le métabolite majoritairement formé dans les première 24 h de contamination.

Cette étude est la première reliant *A. spinosum* à l'accumulation d'AZA par les moules. Par ailleurs la mise en évidence de la présence de fortes proportions en AZA17 dans les tissus de moules pourrait avoir des implications sur la surveillance des AZA des toxines, étant donné qu'à l'heure actuelle seuls AZA1, -2 et -3 sont régulés par la législation européenne.

1.2. Abstract

Azaspiracids (AZAs) are a group of lipophilic polyether compounds first detected in Ireland which have been implicated in shellfish poisoning incidents around Europe. These toxins regularly affect shellfish mariculture operations including protracted closures of shellfish harvesting areas for human consumption.The armoured dinoflagellate *Azadinium spinosum* Elbrächter et Tillmann gen. et sp. nov. (Dinophyceae) has been described as the *de novo* AZAs toxin producer; nonetheless the link between this organism and AZA toxin accumulation in shellfish has not yet been established. In August 2009, shellfish samples of blue mussel (*Mytilus edulis)* from the South west of Ireland were analysed using liquid chromatography-tandem-mass spectrometry (LC-MS/MS) and were found to be above the regulatory limit (0.16 µg/g AZA equivalents) for AZAs. Water samples from this area were collected and one algal isolate was identified as *A. spinosum* and was shown to produce azaspiracid toxins. This is the first strain of *A. spinosum* isolated from Irish waters. The Irish *A. spinosum* is identical with the other two available *A. spinosum* strains from Scotland (3D9) and from Denmark (UTHE2) in its sequence of the D1-D2 regions of the LSU rDNA.

A 24 h feeding trial of blue mussels (*Mytilus edulis*) using an algal suspension of the Irish *A. spinosum* culture at different cell densities demonstrated that A. *spinosum* is filtered, consumed and digested directly by mussels. Also, LC-MS/MS analysis had shown that AZAs were accumulating in the shellfish hepatopancreas. The toxins AZA1 and -2 were detected in the shellfish together with the AZA analogues AZA3, AZA6, AZA17 and -19 suggesting that AZA1 and -2 are metabolised in the shellfish within the first 24h after ingestion of the algae. The levels of AZA17 detected in the shellfish hepatopancreas (HP) were equivalent to the levels of AZA1 but in the remainder tissues the levels of AZA17 were four to five times higher than AZA1, only small quantities of AZA3 and -19 were present with negligible amounts of AZA6 detected after the 24 h period. This could have implications in the future monitoring of these toxins given that at present according to EU legislation only AZA1-AZA3 is regulated for. This is the first report of blue mussels (*Mytilus edulis)* feeding on the AZA producing algae *A. spinosum* from Irish waters.

1.3. Keywords

Azaspiracids, AZP, AZA toxins, biodeposits, dinoflagellates, feeding experiment, Ireland, LC–MS/MS, mussels, phylogeny, taxonomy,

1.4. Introduction

Azaspiracids (AZAs) are a group of lipophilic polyether toxins which were first detected in shellfish in 1995 from a contaminated batch of mussels (*Mytilus edulis)* from Killary harbour, county Galway on the west coast of Ireland. This event led to a poisoning incident in the Netherlands in November of that year where eight people became ill with symptoms typical of diarrhetic shellfish poisoning (DSP), however chemistry results showed that DSP toxins were only found in the shellfish at low concentrations and phytoplankton samples did not show the presence of DSP toxin producing algae in the water, yet the mousse bioassay was strongly positive (McMahon and Silke, 1996). In 1998 this novel group of compounds were isolated and chemically characterized from shellfish (Ofuji et al., 1999a; Satake et al., 1998c). A few years later AZAs were confirmed in the UK and Norway (James et al., 2002), Morocco (Taleb et al., 2006) and Portugal (Vale et al., 2008) suggesting that AZA toxicity had a more extensive distribution along the west European Atlantic seaboard and as far as north Africa. There is evidence now that the distribution of AZAs may be

worldwide with AZAs found in Japan (Ueoka et al., 2009), Chile (Alvarez et al., 2010; Lopez-Rivera et al., 2009) and Canada (Twiner et al., 2008).

The development of routine chemical analysis as a monitoring tool showed AZA concentrations found in *M. edulis* in Ireland between 2003 and 2010 using LC-MS/MS (Figure 45) it indicates that the presence of azaspiracids has been a recurring problem since 2005 annually. However, AZA toxins were found at concentrations below the regulatory limit in 2003 and not detected in 2004 and the first half of 2005, suggesting that there could be a cyclical element to AZA events.

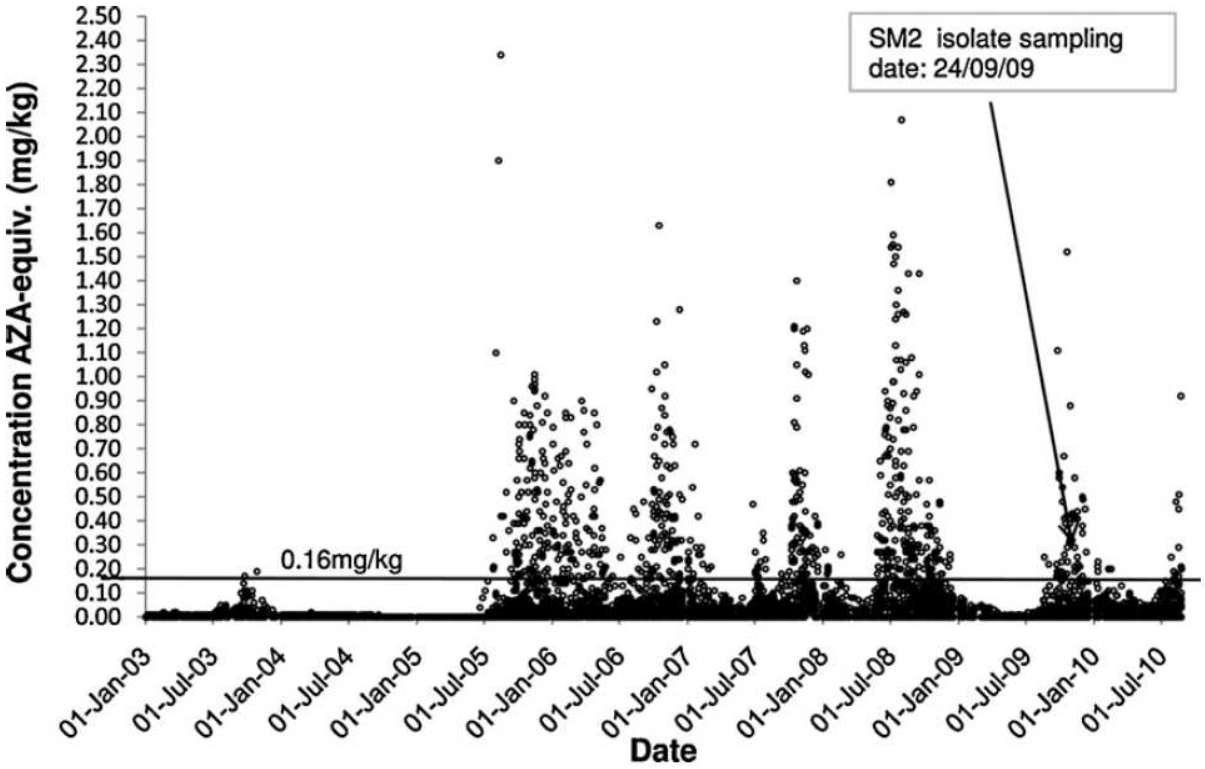

Figure 45. AZA equivalents concentration (mg kg^{-1}) found in blue mussels (*Mytilus edulis*) in Ireland between 2003 and 2010. Please note that two data points are not shown in this figure as the AZA equivalent concentrations were too high compare to the rest of the results and it would have caused the graph to collapse at the lower concentrations. The following data points are not shown (7.37 mg kg^{-1} AZA-equiv. (22/08/05) and 8.97 mg kg^{-1} AZA-equiv. (01/10/2005)).

While the accumulation of AZA toxins has been reported in a number of different shellfish species (Table 19), levels above the regulatory limit have only been observed in blue mussels (*Mytilus edulis*) and pacific oysters (*Crasostrea gigas*) in Ireland.

Table 19. Maximum concentration of AZA equivalents (mg kg^{-1}) found in shellfish species cultured in Ireland between 2003 and 2010 analysed using LC–MS/MS. LOQ, limit of quantification.

Shellfish species		Concentration
Common name	Scientific name	AZA-equiv. ($mg\,kg^{-1}$)
Common Cockle	*Cerastoderma edule*	0.08
Pacific oyster	*Crassostrea gigas*	0.31
Razor clam	*Ensis arcuatus*	0.05
Razor clam	*Ensis siliqua*	<0.01
Dog cockle	*Glycymeris glycymeris*	0.01
Abalone	*Haliotis discus hannai*	<LOQ
Blue mussel	*Mytilus edulis*	8.97
Native oyster	*Ostrea edulis*	0.07
Common limpet	*Patella vulgata*	<LOQ
Surf Clam	*Spisula solida*	0.15
Manila Clam	*Tapes philippinarum*	0.10
Clam	*Tapes semidescussatus*	0.01
Pullet carpet shell	*Venerupis senegalensis*	<LOQ
Venus Clam	*Venus verrucosa*	<LOQ

Due to the ability to accumulate high levels of AZA toxins, mussels have become an important source of AZAs for toxin isolation (Furey et al., 2003; Perez et al., 2010). Since the first characterization of AZA1 by (Satake et al., 1998c) other structural variants have been isolated and characterised: AZA2 and AZA3 (Ofuji et al., 2001; Ofuji et al., 1999a) and AZA6-AZA11 (James et al., 2003b) with AZA12 theoretically postulated, the number of variants was increased from AZA12-AZA 32 later by (Rehmann et al., 2008) with the discovery of new dihydroxy and carboxy-AZAs. Most of these AZA variants have been reported to be shellfish metabolites (Rehmann et al., 2008) rather than *de novo* products of plankton, apart from AZA1, AZA2 and AZA3 (Furey et al., 2003). However, (Krock et al., 2009) only detected AZA1 and AZA2 in the field samples from Scotland generating uncertainties over the production of AZA3 by plankton. (Fux et al., 2009) did find low amounts of AZA3 using Solid Phase Adsorption Toxin Tracker Device (SPATT), a passive sampler, but concluded that a heat treatment step could have influenced the enzymatic activity of the SPATT prior to the extraction allowing the metabolism of AZA1 to AZA3.

In 2008, the causative organism was discovered from the North-East coast of Scotland (Krock et al., 2009) and described as a genera and *species novo* (Tillmann et al., 2009). This organism, a small armoured dinoflagellate named *A. spinosum* was shown to produce AZA

toxins in culture and effectively identified as *de novo* producer of AZAs. Later, (McCarron et al., 2009) reported that AZA17 and -19 are formed by oxidation of the 22-methyl group of AZA1 and -2 respectively. In this study, heat induced decarboxylation of AZA17 and -19 from AZA3 and -6 was demonstrated showing the possible bioconversion pathways of these toxins in shellfish.

The Marine Institute phytoplankton unit collected water samples between August and September of 2009 following positive results in blue mussels of AZA toxins. The samples yielded several isolates and cultures, one of which was shown to produce AZA1 and AZA2. The Irish isolate was provisionally named SM2. Here, we report on the taxonomic identity of the Irish toxin producer and the genotypic/phenotypic relationship with other *Azadinium* species and its toxin profile. The link between *A. spinosum* toxicity and toxin accumulation in shellfish had not yet been established. It was not known whether blue mussels were able to directly ingest the small thecate dinoflagellate *A. spinosum* or if toxin accumulation in mussels is mainly achieved via planktonic vector species which have potentially accumulated AZA toxins by consuming the small armoured dinoflagellate. Thus, a feeding trial was designed to demonstrate that blue mussels are able to feed and digest directly from an algal suspension of *A. spinosum*, that AZA toxins accumulate in their digestive system and that ultimately these are metabolised into other AZA analogues.

1.5. Materials and Methods

1.5.1. Sample collection, isolation and culture of an AZA producing dinoflagellate

1.5.1.1. Field collection using a submersible pump

The culture of an Irish strain of *A. spinosum provisionally* designated as SM2 was established from water samples collected in the South west coast of Ireland, at Gearhies pier, Bantry bay (latitude: 51° 39’ 4.7’’ N, longitude: 9° 35’ 11’’ E) during September of 2009 coinciding with an increase of AZA toxin in shellfish as reported by the Marine Institute biotoxin monitoring programme. The water samples were collected using a pump below surface and at 3 m depth, approximately 100 L of seawater were pumped into a fraction sampler to separate and concentrate the different size fractions (60 µm, 38 µm and 20 µm). The filtrate was also collected into 25 L polyethylene drums for cell concentration down to 3

µm in size using a vacuum pump at low pressure and 3 µm pore size TSTP Millipore ™ membrane filters. Approximately 5 L of 20 µm sample filtrate was concentrated down (3 µm) to approximately 50 mL volume before isolation.

1.5.1.2. Isolation and culture conditions

The dinoflagellates were isolated using single cell isolation by micropipette in 96 cell tissue culture plates (Corning, New York, USA). The isolates were kept in F/2 without silica (Guillard, 1975; Guillard and Ryther, 1962) made up with enriched sterile filtered seawater from the site and kept at 18˚ C temperature, 12:12 light:dark cycle and the irradiance in the incubator was 150 µmol photon m^{-2} s^{-1} measured using an Iso-tech ILM350 light meter (ISO-tech, Merseyside, UK). Potential AZA toxin producer isolates were discriminated from the 20 µm filtrate and 3 µm backwash concentrated fractions by screening for morphological characteristics typical of *A. spinosum*. 4 other isolates apart from SM2 suspected to be *A. spinosum* were isolated and cultured successfully based on cell size and shape, presence of thecal plates, cell movement, and presence of antapical spine, presence/absence of a conspicuous pyrenoid and presence/absence of apical pore. After successful isolation, the unialgal cultures were transferred to 25 x 150 mm borosilicate culture tubes (Fisherbrand™, Loughborough, UK) containing 35 mL of F/2 media and kept in the incubator in the conditions as outlined before.

All cultured isolates were tested for the production of AZA toxins using LC-MS/MS, 1 mL culture aliquots of a densely growing culture were collected using a 1mL pipette and placed into 0.2 µm Whatmann Anopore™ spin filter, the filters were centrifuged at 14,000 rpm for one minute using a Heraeus Multifuge 3S-R (Heraeus, Hanau, Germany) and the filter extracted using methanol.

1.5.2. Microscopy

1.5.2.1. Light microscopy (LM)

Observation of live cultured isolates was carried out using an inverted microscope (Axiovert 200M, Zeiss, Germany) equipped with epifluorescence and differential interference contrast optics. Light microscopic examination of the thecal plate was performed as per Tillmann et al. (2010).

1.5.2.2. Scanning electron microscopy (SEM)

The material for examination was collected from the unialgal cultures and prepared following the protocol for SEM by (Tillmann et al., 2009) with slight variations: the cell pellet -after re-suspension and removal of 40% Seawater -was fixed with formalin (2% final concentration) instead of glutaraldehyde in cacodylate buffer and stored in the fridge for 2 h before washing and dehydration steps. The filters were mounted on stubs, sputter coated (Emscope SC 500, Ashford, UK) with gold-palladium and viewed under a scanning electron microscope (FEI Quanta FEG 200, Eindhoven, Netherlands).

1.5.3. Chemical analysis of the AZAs producing culture

1.5.3.1. Solvents and reagents

Acetonitrile and methanol were purchased as pestican grade solvents from Labscan (Dublin, Ireland). Formic acid, ammonium formate, ammonium hydroxide and sodium hydroxide were purchased from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid was purchased from VWR (England). Water was obtained from a reverse-osmosis purification system (Barnstead, Dublin, Ireland). AZA certified reference materials (SlobbevanDrunen et al.) were obtained from the NRC (Halifax, Canada).

1.5.3.2. Toxin extraction

The culture was extracted by solid phase extraction (SPE). An Oasis HLB, 3 cc cartridge was initially conditioned with 5 mL of methanol, flushed with 10 mL of a 5% methanol solution (in water) and then loaded with 10 mL of culture slowly (drop wise). The cartridge was flushed again with 10 mL of a 5% methanol solution followed by toxin elution with 4 mL of methanol. The methanol extract was blown down to dryness, reconstituted back up in 0.5 mL of methanol and transferred into a HPLC vial for analysis.

1.5.3.3. Analysis by LC-MS/MS

Analysis of AZAs was performed on a Micromass triple stage quadrupole (TSQ) Ultima coupled to a Waters 2695, equipped with a Z-spray ESI source. The TSQ was operated in positive ionization mode through multiple reactions monitoring (MRM). The following transitions were monitored: AZA1 *m/z* 842.5>654.4 and 842.5>672.4, AZA2 856.5>654.4 and 856.5>672.4, AZA3 828.5>640.4 and 828.5>658.4, AZA6 842.5>640.5 and 842.5>658.5, AZA17 872.5>658.5 and 842.5>810.5 and AZA19 886.5>658.5 and 886.5>824.5 in positive

ionisation mode. The cone and collision voltages were set at 60 V and 40 V respectively. Cone and desolvation gas flows were set at 100 and 800 L/h, respectively, while the source and desolvation temperatures were set at 150 °C and 350 °C respectively.

A binary mobile phase was used, phase A (100% aqueous) and phase B (95% aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid. HPLC separation was achieved using a Hypersil BDS C8 column; 50 x 2.1, 3 µm; guard column, 10 x 2.1 mm, 3 µm (Thermo Scientific, Runcorn, UK). The flow rate was set at 0.25 mL/min and the injection volume was set at 5 µl. The column and sample temperatures were set at 25 °C and 6 °C respectively. A gradient elution was employed, starting with 30% B, rising to 90% B over 8 mins, held for 2.5 min, then decreased to 30% B in 0.5 min and held for 4 min to equilibrate the system.

1.5.4. Molecular phylogenetic analysis

1.5.4.1. Extraction of genomic DNA

A 50 mL sample of exponentially growing culture was centrifuged (Eppendorf 5810R, Hamburg, Germany) at 3,220 x g for 15 min at room temperature. The cell pellets were frozen at -20 °C for 20 min before being subjected to total DNA extraction with the DNeasy Kit (Mini) (Qiagen, Hilden, Germany) according to manufacturer's instructions. The purity and quantity of the DNA was checked by UV-spectroscopy with a NanoDrop ND-1000 system (Peqlab, Erlangen, Germany) and the integrity of DNA fragments of a molecular weight of about 20 kb was verified on a 1 % agarose gel.

1.5.4.2. PCR amplification and sequencing

The total extracted DNA from the isolate was subjected to polymerase chain reaction (PCR) amplification of the 28S ribosomal DNA .The forward and reverse primers for amplification of 28S rDNA (D1-D2 regions) were: Dir-F (5'- ACC CGC TGA ATT TAA GCA TA - 3') and Dir-2CR (5'- CCT TGG TCC GTG TTT CAA GA - 3'), respectively. For the 50 µl PCR reaction, HotMasterTaq® (Eppendorf, Hamburg, Germany) buffer 1X, 0.1 mM of dNTPs, 0.1 mM of each forward and reverse primer and 1.25 units of Taq polymerase were added to 10 ng of the extracted genomic DNA. For 28S rDNA amplifications, the reactions were subjected to the following thermocycling conditions: an initial denaturation at a temperature of 95 °C for 7 min was followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing temperature at 54 °C for 2 min and elongation temperature at 72°C for

1.5 min. A final extension step at 72°C was carried out for 10 min. The completed reactions were kept at 10 °C until the next step. The PCR amplicons were analysed on 1% agarose by electrophoresis. Sequencing was conducted with a standard cycle sequencing chemistry ABI 3.1 (Applied Biosystems, Darmstadt, Germany) using the PCR primer sets. Cycle sequencing products were analyzed on an ABI 3130 XL capillary sequencer (Applied Biosystems, Darmstadt, Germany)

1.5.4.3. Sequence alignment for phylogenetic analyses

Sequence alignment was done with CLUSTAL X software (Thompson et al., 1997) and improved manually for all sequences; ambiguous alignments positions were excluded from the analysis. Alignments are available upon request and sequences are available at GenBank under accession number for LSU: SM2 JN165101. Maximum likelihood phylogenetic tree was calculated with PhyML (Guindon and Gascuel, 2003) using a BIO-NJ (neighbour-joining) tree as a starting tree, and the general time reversible (GTR) nucleotide substitution model (Whelan and Goldman, 2001) with a gamma distribution parameter estimated from the data, bootstrap analysis was performed with 100 replicates. The dinoflagellate *Oxyrrhis marina* (Dujardin) was selected as the outgroup.

1.5.5. Blue Mussels feeding experiment

1.5.5.1. Experimental design

A batch of approximately 10 kg of *M.edulis* were harvested from Carlingford Lough, Greenore (Lat: 54.0339˚N, Long: -6.1417W) in the East coast of Ireland. The mussels were cleaned of all fouling organisms and placed in 20 L clear plastic carboys filled with sterile filtered seawater (30 psu) in the walk-in incubator at 18°C. Any dead mussels were removed from the carboys. The experiment consisted of a 24 h feeding experiment with *M. edulis* and the AZAs producing dinoflagellate *A. spinosum* at three cell densities to study feeding activity, toxin uptake and bioconversion of toxins in the shellfish digestive system. The cultures of *A.spinosum* and the mussels were maintained under the same environmental conditions for a week before the experiment was carried out.

Triplicate treatments were prepared in 5 L borosilicate conical flasks (Lennox, Dublin, Ireland) at three cell densities; 30,000 cell mL^{-1}, 20,000 cell mL^{-1} and 5,000 cell mL^{-1} of *A. spinosum*. The mussels used in this study were weighed, measured and labelled before the start of the experiment; 100 g of mussels' whole flesh was dissected from the batch of

mussels for LC-MS/MS analysis to test for AZAs in the mussels prior to the experiment. Also, a sample of seawater (50 mL) used for growing *A.spinosum* culture before inoculation of the algae was extracted for AZA toxins using a SPE column. The feeding experiment consisted of three mussels placed in each conical flask containing *A.spinosum*. Two control samples were set up, one containing three mussels with no algal suspension and one containing algal suspension with no mussels. The latter control was prepared to account for potential decline of algae in the water column due to sedimentation. 3 × 10 mL suspension samples from each of the flasks were collected using an automated pipette (Hirsmann Laborgerate, Heilbronn, Germany) to measure the initial cell density and AZA toxin concentration. During the first two hours of the experiment, 1 mL aliquots of seawater were collected from each flask every 20 minutes using a 1 mL pipette (Eppendorf, Cambridge, UK) and preserved with lugol's iodine (Clin-tech, Dublin, Ireland) 1% final concentration to estimate cell concentrations of *A. spinosum* during the experiment. After two hours samples were collected every hour and finally at 24 h. A final suspension sample (10 mL) was collected at 24 h to analyse the final toxin content of remaining algal cells or other particles in the water column.

Pseudo-faeces and faeces excreted by the mussels during the experiment were harvested using 25mL serological pipettes (Sardstedt, Nümbrecht, Germany) and filtered after 24 h using GF/C Whatman (1.2 µm, 47 mm diameter) glass microfiber filters under vacuum for toxin analysis to determine the total toxin budget for the experiment. Before harvesting, the water was removed from the flasks carefully avoiding the re-suspension of the faeces and pseudo-faeces, while most of the water was removed, a small amount of water was left at the bottom of the flasks and picked up in the pipette before filtering, which means that it is possible that other material was present in the sample other than the faeces and pseudo-faeces. The hypothesis here would be that since the algal suspension was depleted after 24 h, no *A. spinosum* cells are believed to be present in the water at this point.

1.5.5.2. *Azadinium spinosum* cell counts

Cell counts of the 1 mL lugol's iodine preserved aliquots were carried out using a Sedgewick-Rafter cell counting chamber (Pyser-SGI, Kent, UK) for each flask at each time interval using an inverted optical microscope Leica DMI 6000B (Leica, Wetzlar ,Germany).

1.5.5.3. Dissection of mussels and toxin analysis of shellfish tissues

After 24 h, the mussels were harvested from the flasks, weighed and dissected into hepatopancreas (HP) and remainder tissues. The dissected HP and remainder tissues were placed into labeled 15 mL polypropylene centrifuge tubes (Sardstedt, Nümbrecht, Germany). A volume of 1.5 mL methanol was added to each tube and the sample was homogenized using an ultra-turrax (T25 Basic IKA®-Werke, Germany) at 11,000 rpm for one minute. Samples were then centrifuged in a Heraeus Multifuge 3S-R (Heraeus, Hanau, Germany) at 4,500 rpm for five minutes. The supernatant was decanted into 5 mL volumetric flasks (Hirsmann-Techcolor, Heilbronn, Germany) and this step repeated twice for each pellet. The volume was then brought up to the mark using pestican grade methanol, inverted five times for each and filtered through 0.22 µm filters(Sartorius, Surrey, UK) into HPLC vials (AGB, Dublin, Ireland) to be run on the LC-MS/MS.

1.5.5.4. Toxin analysis of *A. spinosum* samples

The toxicity of *A. spinosum* cells was measured during the feeding experiment at two time intervals. 10 mL samples in triplicates of *A. spinosum* were collected from each flask in 15mL centrifuge tubes at initial time (T_0) and at the end of the experiment after 24 h. The samples were centrifuged at 4 500 rpm for 15 min. The supernatant was decanted off and 500 µL of methanol was added to each pellet. Samples were then vortexed in a V400 Multitube Vortex mixer (Alpha Laboratories, Hampshire, UK) mixed for 1 min at maximum speed then centrifuged again. The supernatant was collected into labeled HPLC vials. The process was repeated twice to obtain a final volume of 1.5 mL for each HPLC vial. These samples were blown down to dryness under nitrogen gas (BOC gases, Dublin, Ireland) and reconstituted with 500 µL methanol to be analysed by LC-MS/MS.

1.5.5.5. Toxin analysis of biodeposits

After harvesting the mussels from the flasks, biodeposits (pseudo-faeces, faeces and any detritus left in the flask and a small amount of water) were collected from the bottom of the flasks and then filtrated on GF/C Whatman (1.2 µm, 47 mm diameter) glass microfiber filters. Filters from each flask were placed into labeled 50 mL centrifuge tubes. A volume of 3 mL methanol was added to each tube and vortex mixed at maximum speed for 1 minute. Samples were then centrifuged at 3 500 rpm for 15 min at 4°C. The supernatant was transferred to 10 mL volumetric flasks. This was repeated twice and the volume was made up

to the mark using methanol. The flasks were inverted 5 times each then through 0.22 µm filters into HPLC vials to be analysed via LC-MS/MS in the conditions described previously.

1.6. Results and discussion

1.6.1. *Azadinium spinosum* Irish strain

The armoured dinoflagellate *A. spinosum* has been described by (Tillmann et al., 2009) as a new species and a new genus. Since then, two additional but non-toxic species have been described, *A. obesum* (Tillmann et al., 2010) and *A. poporum* (Tillmann et al., 2011). The Irish isolate provisionally named SM2, clearly is a new geographical strain of *A. spinosum* as it is sharing all morphological details (Tillmann et al., 2011; Tillmann et al., 2010) to describe *A. spinosum* and to differentiate this species from the two other species.

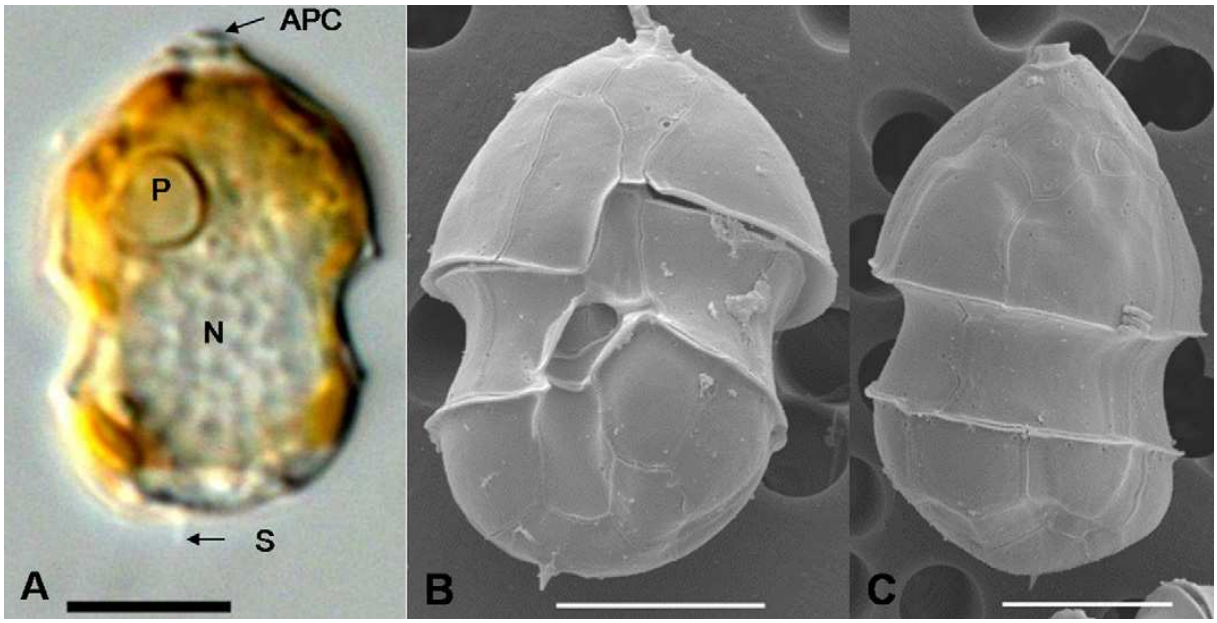

Figure 46. *Azadinium spinosum* (SM2 isolate). Light microscopy view (A) of live cell at 100× objective, SEM (scanning electron microscopy) micrographs of thecae (B) ventral view; (C) dorsal view. Scale bars: 5 µm. APC, apical pore complex; P, pyrenoid; N, nucleus; S, spine.

Figure 46a depicts a light microscopy image of the Irish strain of *A. spinosum* showing the main morphological characteristics typical of the species, a conspicuous Apical Pore Complex (APC), a conspicuous pyrenoid (P) in the episome which can be used to differentiate *A. spinosum* from *A. obesum*, the large spherical nucleus (N) posteriorly located and the antapical spine (S).This antapical spine situated in the second antapical plate is an important feature for the identification of the species as it is present in *A. spinosum* but lacking in the two other described species, *A. obesum* and *A. poporum* (Tillmann et al., 2011;

Tillmann et al., 2010). For the Irish strain SM2, the spine was visible using X100 oil immersion objectives under the light microscope in formalin preserved water samples, although it was difficult enough to discern.

Anyhow, the best way to visualise the spine and distinguish the delicate thecal plates in this dinoflagellate is to use Scanning Electron Microscopy (SEM) which allowed us to identify the plate tabulation to be Po, cp, X, 4', 3a, 6'', 6C, 5?S, 6''', 2'''' which is the same as reported in (Tillmann et al., 2009). Figure 46b and c show complete cells of *A. spinosum*. In ventral view (Figure 46b) the ventral pore (vp) is clearly visible in the left suture of the first apical plate, the thecal plates cleaned of the outer membrane appear smooth and some scattered pores can be seen on the plates. Figure 47b shows the three intercalary plates on the episome of the cell; the second intercalary plate (2a) is smaller than the other two plates and is located above the third pre-cingular plate.

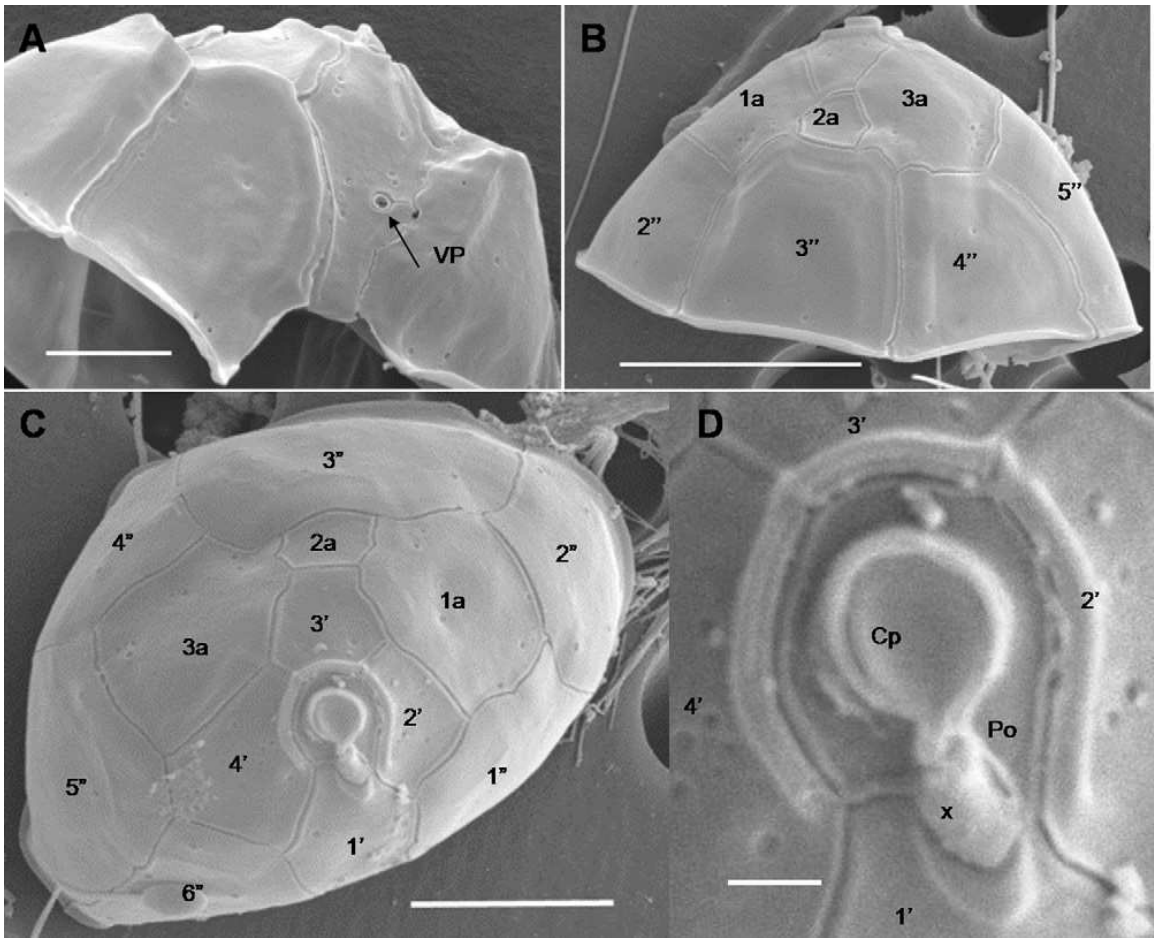

Figure 47. *Azadinium spinosum* (SM2 isolate). SEM micrographs of epithecal plates from different cells in (A) ventral view; (B) mid-dorsal view; (C) full epitheca from an apical view; (D) apical pore complex detail (APC). Scale bars: 2 µm (A and C), 5 µm (B) and 0.25 µm (D).

Figure 47c shows the complete epithecal plates of *A. spinosum*, the detail view of the APC (Figure 48d) shows an identical configuration of the pore described for *A. spinosum* and *A. obesum* (Tillmann *et al.* 2010). The apical pore located centrally in the pore plate (Po) which is topped by the cover plate (cp). The X plate is situated between the first apical and the pore plate which protrudes and extends to touch the cover plate.

The sulcal plates (Figure 48a and b) were determined by SEM and we can distinguish 5 plates from the outer side of the theca. The anterior sulcal (Sa) plate is large and roughly the same height as the width of the cingulum, the right sulcal (Sd) and median sulcal (Sm) plates form an intricate cavity around the emerging point of the flagella with the left sulcal (Ss) plate extending from the cingulum where it touches the first cingular (C1) and Sa plates then follows the contour of the Sm and Sd on the left side and finally touches the Sulcal posterior (Sp) in the hypotheca. The cingulum is wide and composed of 6 cingular plates (Figure 48a and c).The hypothecal plates can be seen from the antapical view in Figure 48d, 6 post-cingular and 2 antapical plates with the spine positioned in the second antapical plate.

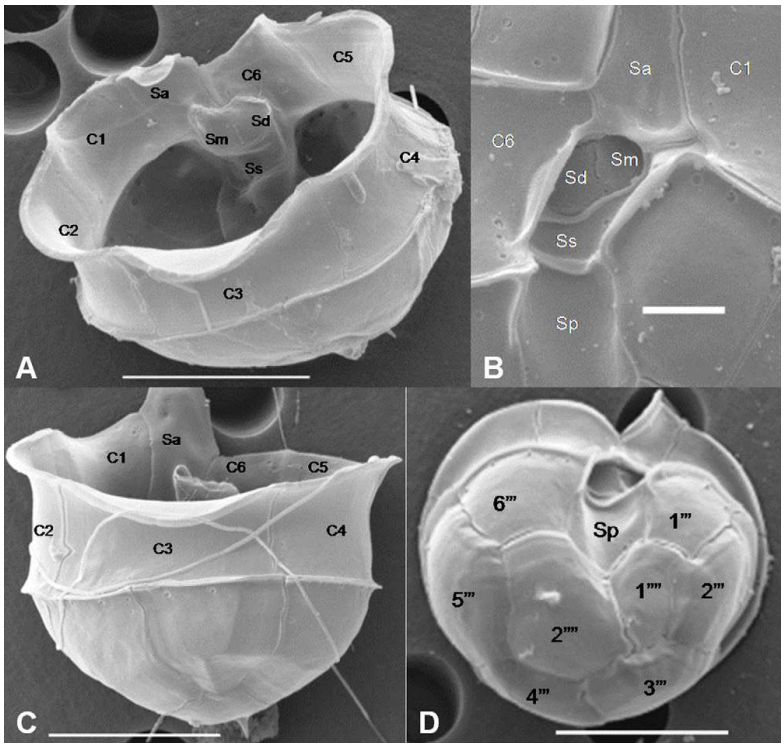

Figure 48. *Azadinium spinosum* (SM2 isolate). SEM micrographs of hypothecal plates from different cells showing details of hypotheca, cingulum and sulcus. (A) Hypotheca and cingulum in dorsal view; (B) detailed view of the sulcal region. Sa, anterior sulcal plate; Sm, median sulcal plate; Sd, right sulcal plate; Ss, left sulcal plate; Sp, posterior sulcal plate. (C) Dorsal view showing antapical spine. (D) Antapical view. Scale bars: 5 µm (A, C and D), 1 µm (Figure 48b).

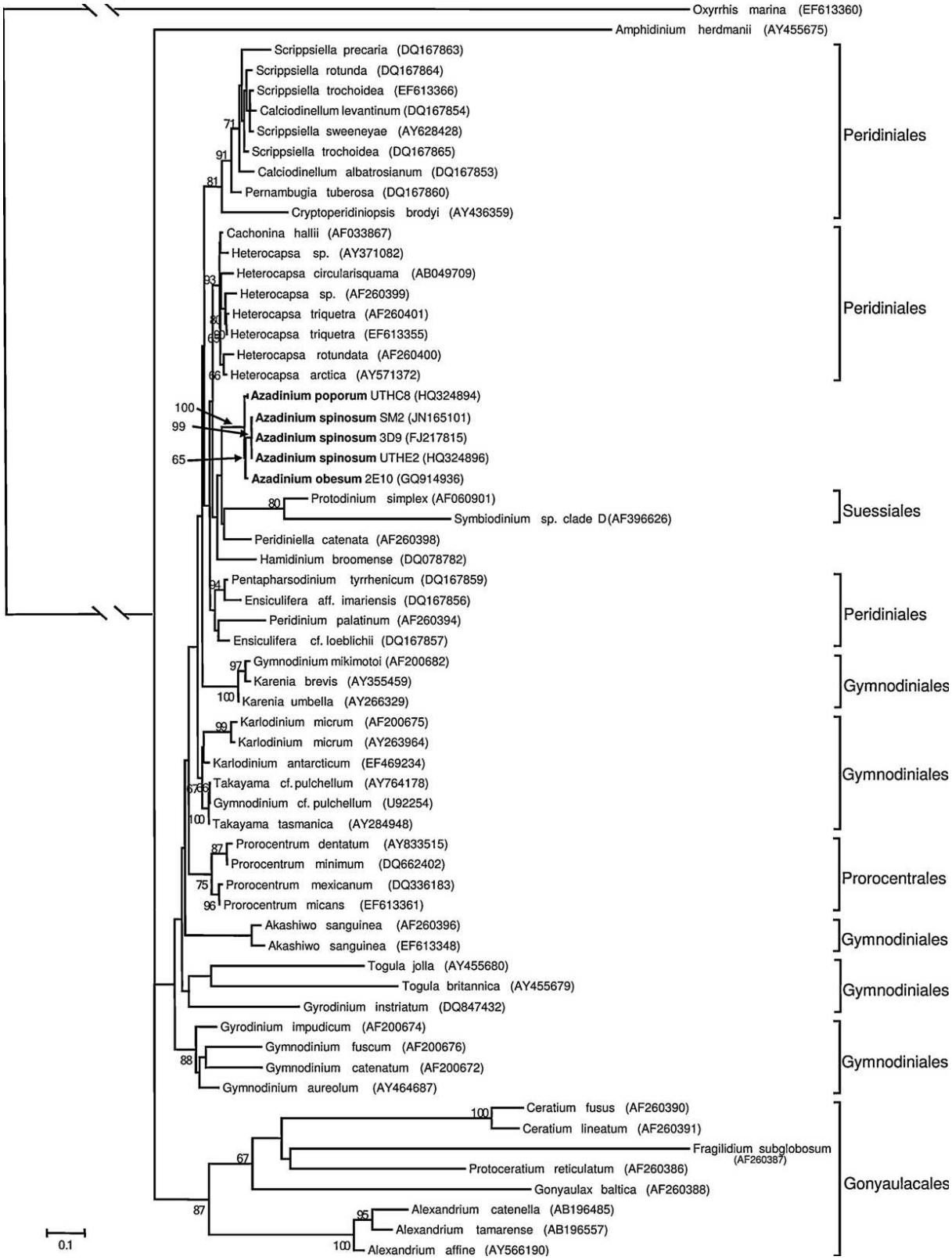

Figure 49. Maximum likelihood (ML) phylogenetic tree of the dinoflagellates inferred from the D1–D2 region of the 28S/large subunit (LSU) from the rDNA operon. The branch of the dinoflagellate *Oxyrrhis marina*, which was used as outgroup, is not shown proportionally. Bootstrap analysis was done with 100 replicates.

1.6.1.1. AZA composition and content in culture

The cultured Irish strain SM2 of *A. spinosum* produces the AZA analogues AZA1 and -2. AZA1 is the major toxin component in the sample and AZA2 is found at lower concentrations. The cell quota from parallel cultures kept in the same conditions as the culture used for the feeding experiment ranged from ~15 to 25fg cell^{-1} for AZA1 and ~1 to 5 fg cell^{-1} for AZA2 (data not included). This toxin profile correlates with that found by (Krock et al., 2009) in the 3D9 isolate from the Scottish coast. (Krock et al., 2009) also reported a potential new analogue (AZAx) which was later found to be an extraction (with methanol) artefact, personal comms.

1.6.1.2. Molecular genetic analysis

We amplified and sequenced the D1-D2 regions of the nuclear ribosomal RNA gene from strain SM2. The sequenced region encompassed 436 base pairs, and was exactly identical to the homologous sequences from two previously characterized *A. spinosum* strains (strains 3D9 and UTHE2), and differed in 10 and 8 substitutions from sequences available from *A. poporum* and *A. obesum*, respectively (Figure 49).

1.6.2. Feeding experiment

1.6.2.1. Mussels feeding activity

All mussels started feeding after a few minutes of being introduced into the flasks containing the algae; this continued for the 24 h that the experiment lasted. Figure 50 illustrates the decreasing concentration of algae in the different treatments over 24 h. The control line demonstrates that sedimentation of the algal suspension in the control treatment during the experiment was negligible. The data suggest that most of the algae have been consumed within three hours of the start of the experiment, low baseline algal cell concentrations below ~3×10^3 cell mL^{-1} were found after 5 h in the 30×10^3 cell mL^{-1} treatment, ~850 cell mL^{-1} in the 20×10^3 cell mL^{-1} treatment and ~67 cells mL^{-1} in the 5×10^3 cell mL^{-1} treatment and after 24 h the estimates were ~73 cell mL^{-1}, ~50 cell mL^{-1} and ~4 cell mL^{-1} respectively.

During feeding, all individual mussels were observed to produce pseudo-faeces in all treatments. The amount of pseudo-faeces produced appeared to slow down after approximately one hour, while some faeces were also produced after approximately two hours. One mussel in one of the 30×10^3 cell mL^{-1} treatment replicates started spawning after

two hours copiously but continued filtering afterwards, which could suggest some level of stress however this appeared to be an isolated episode.

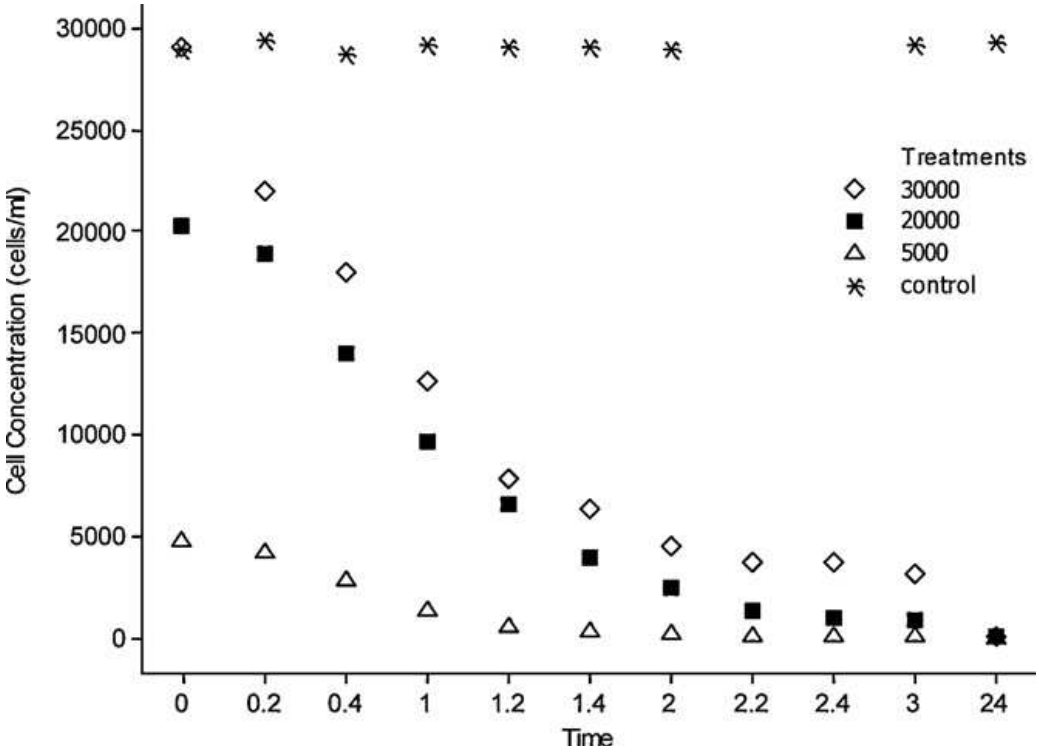

Figure 50. Cell concentrations of *A. spinosum* over 24 h in three treatments (30,000, 20,000, 5,000) and control.

1.6.2.2. Shellfish AZA toxin analysis

The mussels and the media used for this experiment were analysed by LC-MS/MS for AZAs to demonstrate that there weren't any toxins initially in the seawater used to grow the algae or in the shellfish tissue prior to carrying out the feeding experiment. Both controls were below the limit of quantification (LOQ) for AZAs. Also, a control using mussels without algal suspension throughout the 24 h experiment was analysed using LC-MS/MS and found to be negative for AZAs (data not included).

After 24 h the mussels were harvested, dissected and analysed via LC-MS/MS. Figure 51 shows the concentration of AZA toxins detected in the mussels hepatopancreas for each treatment. In all treatments, significant amounts of AZA1and AZA2 were found in the mussels with considerably higher concentrations for the two highest cell concentrations of *A. spinosum* compared to the lower concentration. This demonstrates that mussels do ingest *A. spinosum* directly and accumulate AZA toxins in their digestive system with toxicity being related to the density of the algae in the water.

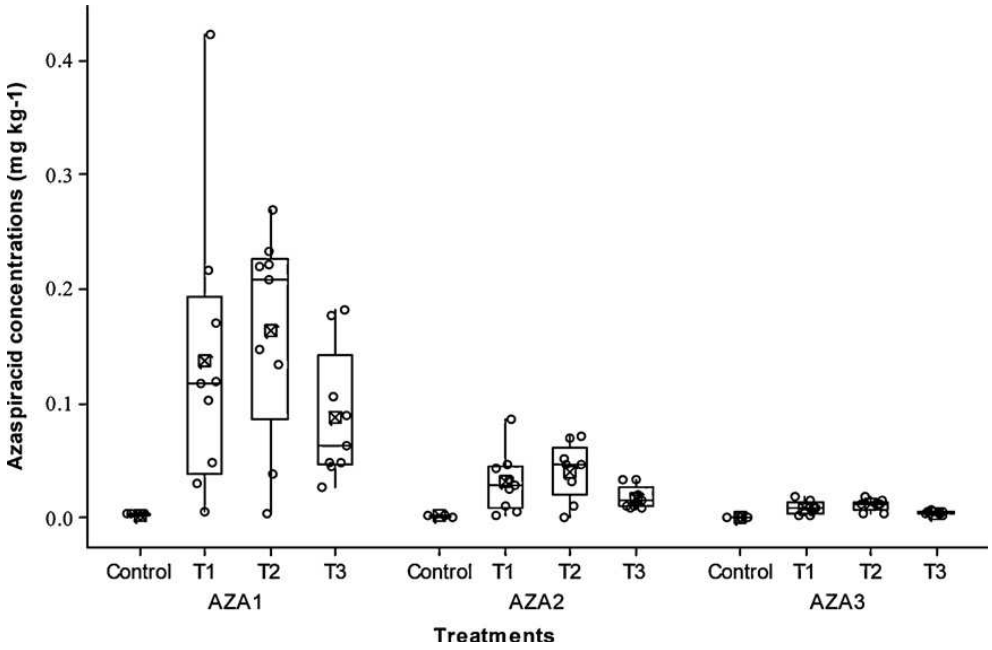

Figure 51. Azaspiracid concentrations (mg kg^{-1}) found in mussels hepatopancreas (n = 9 per treatment). T1 = 30×10^3 cell mL^{-1}, T2 = 20×10^3 cell mL^{-1}, T3 = 5×10^3 cell mL^{-1} and control.

The amount of AZA1 in both the 30×10^3 cell mL^{-1} treatment and the 20×10^3 cell mL^{-1} treatment was already above the regulatory limit for AZA equivalent toxins suggesting that mussels can become intoxicated with AZAs at *A. spinosum* cell concentrations of 20×10^3 cell mL^{-1} over a 24 h period. The concentration of AZA1 and -2 toxins is higher in the HP tissue compared to the toxin concentration found in the remainder with negligible amounts between the limit of detection (0.01 µg g^{-1}) and the limit of quantification for the instrument (0.02 µg g^{-1}) with the highest amount detected ~0.015 µg g^{-1} of AZA1 in the 20×10^3 cell mL^{-1} treatment. The concentration of the toxin analogue AZA3 in the mussel HP was below the limit of quantification suggesting that the decarboxylation of AZA17 to AZA3 probably occurs over a longer period of time than 24 h.

1.6.2.3. AZA toxin analogues results

The amount of toxins found varied significantly among mussels, replicates and treatments; this is possibly due to normal physiological differences like size, weight, age and condition of the mussels. We found that AZA1 and -2 toxins were already bioconverting into their carboxylated analogues AZA17 and -19 within the first 24 h .This is evidence that toxin bioconversion takes place in the shellfish digestive tract quite rapidly. The ratio of AZA17 to AZA1 toxins were found in all treatments (n=9) on average to be 1:1 in the mussel hepatopancreas.

High levels of AZA17 compared to AZA1were also found in the remainder tissue supporting the study performed by (O'Driscoll et al., 2011) which shows that the oxidation process of AZA toxins occurs primarily in the gills. The ratio of AZA17 to AZA1 toxins in the remainder tissues was approximately 5:1 to 6:1 in all treatments.

The concentration of AZA17 in shellfish samples is not a monitoring requirement in the current legislation as AZA17 is converted naturally to AZA3 in the shellfish over time, and it is not thought to be a large component of the total AZA toxin content, however as this feeding experiment indicates, it is possible that if mussels have been contaminated recently with AZAs, the amount of AZA17 can equal that of AZA1 or even exceed that of AZA1 resulting potentially in an underestimation of the total amount of AZA toxins in the samples. As shellfish samples are analysed raw, AZA17 won't convert readily to AZA3 unless mussels are cooked or the method incorporates a heating step in the extraction process.

1.6.2.4. Toxin budget feeding experiment

The total toxin budget for the feeding experiment included the initial and final toxin content of the algal suspension, the toxin found in the mussel tissues (HP and remainder) and the toxin found in the biodeposits (faeces/pseudofaeces). This budget doesn't include any extracellular dissolved toxin fraction. The budget measures the amount of the principal toxins AZA1 and -2, the oxidation analogues AZA17 and -19 and the decarboxylated analogues AZA3 and-6. The final toxin budget illustrated by the 30,000 cells mL^{-1} treatment (Table 20) shows a high % recovery of toxins from the experiment accounting on average for ~87% of AZA1 and AZA2 of the initial toxin content in the algal suspension, ~ 36% of AZA1 and 34% of AZA2 were recovered in the mussel tissues and ~16% of AZA1 and ~21 % of AZA2 were found in the biodeposits which indicates some level of toxin excretion , however, after 24 h ~35% of AZA1and ~32% of AZA2 toxins were still found in the final water samples which at this point should only contain baseline cell densities of *A. spinosum* suggesting that some toxins were possibly re-suspended from the biodeposits due to aeration, so it is possible that the amount of toxins in the biodeposits could be larger than the reported budgets. The remaining ~13% of AZA1and AZA2 were not accounted for.

Table 20. Azaspiracid toxins budget in the 30,000 treatment.

Flasks	Initial toxin culture (ng)	Final toxin culture		Tissue after 24 h (ng)			Biodeposits (ng)			Total (ng)	%
Replicates	AZA1	AZA1	AZA17	AZA1	AZA17	AZA3	AZA1	AZA17	AZA3	Total recovered	
30,000 F1	2291.67	708.33	5.61	414.18	371.10	32.45	454.00	6.32	3.36	1995.34	87.1
30,000 F2	2258.33	808.33	172.23	136.61	370.84	25.32	285.00	9.17	1.83	1809.34	80.1
30,000 F3	2500.00	625.00	68.44	292.07	480.13	32.76	391.00	7.91	2.26	1899.57	76.0
Mean	2350.00	713.89	82.09	280.95	407.36	30.18	376.67	7.80	2.48	1901.41	81.1
% toxins in mussel tissue											30.6
% toxins in suspended particulate matter											33.9
% toxins in biodeposits											16.5
Replicates	AZA2	AZA2	AZA6	AZA2	AZA19	AZA6	AZA2	AZA19	AZA6	Total recovered	
30,000 F1	312.62	121.17	0.00	86.20	29.95	0.00	58.56	0.85	0.00	296.74	94.9
30,000 F2	263.84	103.14	0.00	35.06	29.31	0.00	50.95	1.61	0.00	220.07	83.4
30,000 F3	348.87	71.39	0.00	69.32	43.88	0.00	79.10	1.58	0.00	265.28	76.0
Mean	308.44	96.57	0.00	63.53	34.38	0.00	62.87	1.35	0.00	260.69	84.8
% toxins in mussel tissue											31.7
% toxins in suspended particulate matter											32.0
% toxins in biodeposits											20.8

The toxin budget of the 20,000 cell mL^{-1} and 5,000 cell mL^{-1} treatments returned a recovery above 100%, ~113% AZA1 and ~120% AZA2 in the 20,000 cell mL^{-1} budget and ~150% AZA1 and 175% AZA2 in the 5,000 cell mL^{-1} budget. The high recovery in the 5 000 cell mL^{-1} treatment could be explained by the lower toxin content and the uncertainty in the measurement of the AZA peaks at these concentrations. The recovery in the 20,000 cell mL^{-1} while above the 100%, demonstrates overall that there must be very little loss of toxins into the dissolved phase in any case. This latter point is only hypothetical as we have not calculated the dissolved fraction for this experiment.

1.7. Conclusions

Based upon the morphological characteristics described and the Kofoidean tabulation of the recently isolated AZA toxin producer Irish strain SM2 we conclude that the Irish isolate fits perfectly with the description of *A. spinosum*. The sequence of the variable D1-D2 regions of the nuclear LSU rDNA of the Irish strain SM2 is also identical to those available from the Scottish 3D9 and Danish UTHE2 strains, further supporting their conspecificity. This is the third strain of *A. spinosum* reported to produce AZAs from a different geographical location in the North Atlantic after the discovery of the 3D9 isolate from the Scottish coast and the UTHE2 isolate from the Danish coast (Tillmann et al., 2011; Tillmann et al., 2009). So far no other species of the genus *Azadinium* that have been tested to date have produced AZAs other than the type species *A. spinosum*. From a monitoring perspective this organism is very small and difficult to identify to species level in preserved water samples, yet using a good research compound microscope fitted with oil immersion lenses, it is possible to view several important morphological features that describes this species, in particular the antapical spine, which is typical of *A. spinosum* .While this feature provides reliable identification of the

species together with other morphological characteristics typical of the genus, it will be ultimately the development of gene probes that will prove a useful tool in the future of monitoring, quantification and identification of the genus. Molecular methods however may yet take some time to become available for real time monitoring as this genus is still only recently discovered and the continued discovery of new species of *Azadinium* and strains of *A. spinosum* and other species in this size range, means that gene probes will have to be exhaustedly tested for cross reactivity between the genus and within the different strains before they can be relied upon.

Here we have for the first time proof of a direct toxin transfer of AZA toxins from *A. spinosum* by feeding *M. edulis* without the need for vector species. Mussels will actively filter, ingest, accumulate and bioconvert AZA toxins quite readily into other AZA analogues. AZA1 and -2 were found to be concentrated mainly in the HP tissue whereas AZA17 and -19 were distributed throughout the whole flesh. The results show that the ratio of AZA17 in the remainder tissue can be up to five or six times the amount of AZA1. AZA17 is known to convert readily to the decarboxylated analogue AZA3 upon cooking, however monitoring samples are analysed raw and the analogue AZA17 is not monitored as the present legislation only sets limits for the forms AZA1, AZA2 and AZA3. This means that the total AZA toxin content in shellfish samples could be underestimated. The toxin budget indicated that most of the AZA toxins detected in the plankton can be accounted for in the shellfish tissues, the biodeposits and the particulates in the water suggesting that the dissolved fraction of AZAs in the water should be quite small. These results illustrate the need for further experiments in the kinetics of the principal plankton AZA toxins in shellfish tissues.

1.8. Acknowledgments

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2. Azaspiracid accumulation, detoxification and biotransformation in blue mussels (*Mytilus edulis*) experimentally fed *Azadinium spinosum*

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2.1. Résumé

Azadinium spinosum est un petit dinoflagellé marin récemment décrit comme un producteur d’azaspiracide-1 et -2 (AZA1 et -2), des toxines provoquant des intoxications de types diarrhéiques. *A. spinosum* est maintenu en culture pour optimiser sa production et évaluer et comprendre l’accumulation et la biotransformation des AZA1 et -2 dans les moules (*Mytilus edulis*) nourries avec *A. spinosum*.

Des moules adultes ont été continuellement exposées à *A. spinosum* pendant une semaine dans des bassins cylindro-coniques de 160 L. Trois conditions différentes ont été testées lors de cette période de contamination: 5×10^3 , 10×10^3 cell mL^{-1} de *A. spinosum* et un mélange de 5×10^3 cell mL^{-1} de *A. spinosum* avec 5×10^3 cell mL^{-1} d’*Isochrysis* aff. *galbana* (T-*Iso*, CCAP 927/14). Un periode de détoxification de deux semaines a ensuite suivi de contamination. Au cours de cette période de détoxification les moules contaminées ont été alimentées en continu avec 5×10^3 cell mL^{-1} de T-*Iso*. Les cinétiques d'accumulation, de détoxification et de biotransformation ont été évaluées, ainsi que la distribution des toxines dans les moules et l'effet d’*A. spinosum* sur l’épaisseur des tubules de la glande digestive des moules.

Dans les trois conditions testées, *M. edulis* a accumulé des AZA. Les toxines d'origine algale (AZA1 et -2), ainsi que leurs métabolites : AZA3 à 12, -17, -19, -21 et -23 ont été détectés au cours de cette expérience. Après 6 h de contamination, le contenu en AZA dans les moules a atteint la limite réglementaire européenne, et les métabolites représentaient environ 25% de la teneur totale en AZA. Le pourcentage de métabolites a continué

d'augmenter et était supérieure à 50% après 24 h de contamination et ce jusqu'à la fin de l'étude.

AZA17 et -19 sont les principaux métabolites détectés dans les moules avec des teneurs dans la chair totale en AZA17 équivalentes à celle d'AZA1.

2.2. Abstract

Azadinium spinosum (Elbrächter and Tillmann), a small marine dinoflagellate, has been recently described as a *de novo* producer of azaspiracid-1 and -2 (AZA1 and -2) diarrhoeic toxins. A culture of *A. spinosum* was established in our laboratory and optimised for pilot-scale production of this organism, to evaluate and understand AZA1 and -2 accumulation and biotransformation in blue mussels (M*ytilus edulis*) fed with *A. spinosum*.

Adult mussels were continuously exposed to *A. spinosum* over 1 week in 160 L cylindrical conical tanks. Three different diets were tested for contamination: 5×10^3, 10×10^3 cell mL^{-1} of *A. spinosum* and a mixture of 5×10^3 cell mL^{-1} of *A. spinosum* with 5×10^3 cell mL^{-1} of *Isochrysis* aff. *galbana* (T-*Iso*, CCAP 927/14). During the subsequent period of detoxification (2 weeks), contaminated mussels were continuously fed with 5×10^3 cell mL^{-1} of T-*Iso*. Kinetics of accumulation, detoxification and biotransformation were evaluated, as well as the toxin distribution and the effect of *A. spinosum* on mussel digestive gland tubules.

M. edulis fed on *A. spinosum* in the three tested conditions; this finding confirmed our recent experiments feeding *A. spinosum* to mussels. The original algal toxins AZA1 and -2, as well as mussel metabolites AZA3 to 12, -17, -19, -21 and -23 were found during these trials. After as little as 6 h, azaspiracid content in mussels reached the EU regulatory limit, and metabolites were found in all conditions at approximately 25% of the total AZA content. This fraction exceeded 50% after 24 h, and continued to increase until the end of the study. AZA17 and -19 were found to be the main metabolites, with AZA17 concentrations estimated in the same order of magnitude as that of the main algal toxin, AZA1.

2.3. Key words

Azaspiracid, *Azadinium spinosum*, marine biotoxins, AZA, tissue distribution, histology, bivalve molluscs, liquid chromatography coupled to tandem mass spectrometry

2.4. Introduction

The group of phycotoxins now known as azaspiracids (AZAs) was first detected in Ireland (McMahon and Silke, 1996), when mussels harvested from Killary Harbour and exported to the Netherlands in 1995 caused human intoxications due to these toxins. The symptoms were similar to diarrhetic shellfish poisoning (DSP), but, only very low concentration of okadaic acid and dinophysistoxin-2 were found (McMahon and Silke, 1996). Three years later, the toxin was isolated and named azaspiracid (Satake et al., 1998c). The structure of the molecule was revised later thanks to clarifications given by total synthesis of azaspiracid-1 (Nicolaou et al., 2004a). Subsequently, AZA2-5 were purified and identified by nuclear magnetic resonance (NMR) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Ofuji et al., 2001; Ofuji et al., 1999a) and AZA6-32 (Diaz Sierra et al., 2003; James et al., 2003b; McCarron et al., 2009; Rehmann et al., 2008) by LC-MS/MS alone. Despite repeated AZA occurrences, twelve years were necessary until the discovery of the primary producer, the dinoflagellate *Azadinium spinosum* (Elbrächter and Tillmann, strain 3D9) (Krock et al., 2008; Krock et al., 2009; Tillmann et al., 2009). This micro-alga produces AZA1 and -2 and belongs to a new genus, species of which have now been identified in Scotland (Krock et al., 2008; Tillmann et al., 2009), Denmark (Tillmann et al., 2011), Ireland (Salas et al., 2011), Italy and France (Siano and Nézan, personal communication), Argentina (Akselman and Negri, 2010), Mexico (Hernandez-Becerril et al., 2010) and recently in Korea (Potvin et al., 2012). In Ireland, AZA is a recurrent problem as mussels frequently accumulate this group of toxins to reach concentrations above the regulatory limit in shellfish, *i.e.* 160 µg AZA1-equivalents per kg of shellfish flesh (Salas et al., 2011), thus causing important economic losses to the shellfish industry and a potential threat to consumers. In other countries, AZAs are carefully monitored as these toxins were also found in Europe, Morocco, America as well as Japan (Alvarez et al., 2010; Amzil et al., 2008; Furey et al., 2010; Magdalena et al., 2003a; Taleb et al., 2006; Ueoka et al., 2009).

Direct accumulation of *A. spinosum* by blue mussels was recently demonstrated for the first time (Salas et al., 2011). Blue mussels were able to feed on *A. spinosum*, and the presence of AZA1 and -2 metabolites (AZA3, -17 and -19) was confirmed after 24h exposition to the microalga with AZA17 as a major metabolite. This last point poses a problem to public health as AZA17 and -19 are not explicitly regulated in the EU, even

though they were shown to form AZA3 and -6, respectively, when mussels are cooked (McCarron et al., 2009). Current EU regulation (Anonymous, 2005) only includes AZA1-3, as they were the main analogues identified in cooked mussels in 2001 when the first toxin-specific regulation was formulated. Furthermore, AZA1 toxicity equivalent factors (TEF) of 1.8 and 1.4 were given for AZA2 and -3 (Ofuji et al., 1999a) whereas for AZA4 and -5 the factor was 2.5 and 5 times less toxic than AZA1 (Ofuji et al., 2001). Concerning the relative *in vitro* potency of AZA6, it appears to be not unlike that of AZA1 (M. Twiner, personal communication). Thus, a provisional TEF of 1 was attributed to AZA6 as a crude estimate of its toxic potency. Since the current guideline by the EU reference laboratory prescribes analysis of raw shellfish tissues, AZA17 and -19 may be seen as a risk to human health, as the total AZA content might be significantly underestimated. In Ireland, maximum concentrations of monitored AZAs in bivalve molluscs between 2003 and 2010 were reviewed (Salas et al., 2011) and showed records above regulatory limit (0.16 $mg.kg^{-1}$ AZA1 equiv.) for blue mussels (8.97 $mg.kg^{-1}$ AZA1 equiv.) and Pacific oysters (0.31 $mg.kg^{-1}$ AZA1 equiv.). In France, high values have been recorded for king scallops (*Pecten maximus,* 0.32 $mg.kg^{-1}$) (Magdalena et al., 2003a) and queen scallops (*Aequipecten opercularis,* 0.27 $mg.kg^{-1}$ AZA1 equiv.) (Amzil et al., 2008). AZA distribution in bivalve molluscs was studied in King scallops (Magdalena et al., 2003b) and mussels (Furey et al., 2003; Hess et al., 2005b; James et al., 2002). In blue mussels from Norway, toxin concentration in the digestive gland (DG) was significantly higher than in the remaining flesh (RF) (James et al., 2002). However, as the RF represents ±85% of total mussel flesh it still has a considerable sanitary impact (Furey et al., 2003). The total AZA tissue distribution for cooked and uncooked tissues was also studied (Hess et al., 2005b), the ratios between the DG and the whole flesh tissues were 5.20 and 5.89, respectively, and hence the authors concluded that the whole tissue should be analysed for official control.

Due to the large number of AZA-analogues observed in mussels (Rehmann et al., 2008), recent investigations on AZA biotransformation in mussels focussed on AZA metabolic pathways and chemical transformations. The decarboxylation of AZA17, -21, -19 and -23, *i.e,*. carboxy-AZA analogues, into AZA3, -4, -6 and -9, respectively, was demonstrated by (McCarron et al., 2009), while the formation of AZA17 was observed following immersion of mussels in seawater containing dissolved AZA1 (O'Driscoll et al., 2011). A previous study (Salas et al., 2011) established a link between *A. spinosum* and AZAs in bivalves and pointed towards rapid biotransformation of AZA1 and -2 over a 24 h period of

time when mussels were fed *A. spinosum*. However, to our knowledge there has not been any controlled study of accumulation, detoxification and biotransformation kinetics.

The present study was designed to confirm our preliminary results (Salas et al., 2011) regarding the accumulation of AZAs in blue mussels (*Mytilus edulis*). As in the previous trial *A. spinosum* was used and a particular attention was paid to biotransformation into analogues that could represent a potential threat to human health. Furthermore, the study provided some information on accumulation and detoxification kinetics, as well as on tissue distribution of toxins and possible effects of *A. spinosum* on mussel digestive tissues.

2.5. Materials and methods

2.5.1. Maintenance of bivalves

Blue mussels (*Mytilus edulis*) were collected from a mussel farm in Pen Bé (French Atlantic coast) and delivered alive. Through routine official control, this area was considered at that time not to be affected by any known toxins. Mussels collected in January 2011, had a mean individual dry weight of 0.35 ± 0.1 g and a mean individual shell length of 47 ± 4 mm (*i.e.* commercial size, n=30). Mussels were cleaned of barnacles on their shells and 20 kg were selected (>4cm length) and divided into batches of 1 kg. Mussels were then maintained in two 160 L cylindro-conical tanks for 5 days to ensure acclimatisation to laboratory conditions, *i.e.* re-circulated filtered seawater (35 psu) at 12.5 ± 1°C with aeration. Prior to the experiment, seawater in the holding tanks was changed twice and any dead mussels were removed. During this period, mussels were not fed, thus allowing for food clearance from the digestive tract. Before starting exposure to *A. spinosum*, four 1 kg batches were placed into each of the 5 cylindro-conical tanks. For information on initial conditions, 30 mussels were randomly selected for wet and dry weight, ten for total flesh toxin analysis, and ten for DG and RF toxin analysis.

2.5.2. Mass culture and cell count

2.5.2.1. A. spinosum

The 3D9 strain of *Azadinium spinosum* was the source of AZA1 and -2 for contamination trial. This dinoflagellate was isolated during a research cruise in the North Sea near the coast of Scotland (Tillmann et al., 2009) and obtained from the Alfred Wegner

Institute (Bremenhaven, Germany) The algae were produced in stirred photobioreactors (100 L) operated in chemostat mode at a flow rate of 0.25 day^{-1}. Culture medium was a K modified medium (Keller et al., 1987), without NH_4Cl and with Na_2SeO_3 (10^{-8} M). The following conditions were controlled in the bioreactor: pH was maintained at 7.9 by CO_2 addition, temperature at 18°C, photon flux density at 200 µmol m^{-2} s^{-1}, photoperiod was 16 h of light and 8 h of dark (Jauffrais et al., 2010). A stirring paddle was used to homogenize the culture at 40 rpm. The algae were then collected into an aerated harvesting tank (300 L) and maintained at 18°C until the feeding experiment.

2.5.2.2. *Isochrysis* aff. *galbana*

Isochrysis aff. *galbana* (CCAP 927/14, T-*Iso*), a prymnesiophycea flagellate widely used in aquaculture and especially in bivalve hatcheries, was chosen as a non-toxic diet. Like *A. spinosum* the T-*Iso* is a small 4-6 µm motile single cell alga, but is slightly smaller than *A. spinosum*. Thus cell concentrations of both species in a mixture diet were easy to quantify using a particle counter. T-*Iso* was grown in a bioreactor (100L) in batch culture under the same environmental conditions as for *A. spinosum*, but f/2 culture medium (Guillard, 1975; Guillard and Ryther, 1962) was substituted.

2.5.2.3. Cell counts

Cell concentrations (cell mL^{-1}), were determined using a particle counter (Multisizer 3 Coulter counter, Beckman) and assessed 3 times a day in the different conditions studied to control and adjust spectrofluorometric values (see below).

2.5.3. Experimental design

2.5.3.1. Systems used

The first experiment on azaspiracids accumulation and detoxification in *M. edulis* was carried out with four identical re-circulation systems. Each system was composed of a glass vessel (25L) containing A. *spinosum*, T-*Iso* or the mixture of both connected to a thermo-regulated cylindro-conical tank (160 L) with aeration (airlift system). Algal cell concentration in these experimental tanks was maintained by sequential addition of algae. The concentration was continuously assessed with a spectrofluorometer (Turner Design 10-AU-105) connected through a datalogger to a computer system (software: Lab View®). When the algal concentration was below the required level, algal culture was automatically added by means

of an electro-valve. Water level was maintained constant in the tank by a peristaltic pump connecting an overflow tube to a bin. Temperature and salinity were the same as during the acclimatisation period and maintained at 12.5°C and 35 psu, respectively.

For a second experiment on the distribution of AZAs throughout mussel organs, only the thermo-regulated cylindro-conical tank (160 L) with aeration (airlift system) was used.

2.5.3.2. Accumulation and detoxification experiment

Blue mussels were used to examine the effect of *A. spinosum* on AZA accumulation, detoxification and biotransformation in bivalves, using 3 diets. Over 7 days, mussels were exposed to: (1) a constant *A. spinosum* concentration of 5×10^3 cell mL^{-1}, (2) a constant *A. spinosum* concentration of 10×10^3 cell mL^{-1}, (3) a mixed diet of *A. spinosum* and T-*Iso*, both at a concentration of 5×10^3 cell mL^{-1}. Two different concentrations were assessed to verify if AZA accumulation could be dependent on *A. spinosum* concentration. Furthermore, the mixture of a toxic alga with a non-toxic alga was used to confirm that mussels are unable to selectively feed, and to see if this addition has an effect on AZA accumulation.

For detoxification, mussels of all 3 conditions were exposed to a constant concentration of T-*Iso* at 10×10^3 cell mL^{-1} for 14 days. The fourth system was a control, where mussels were fed T-*Iso* diet at 10×10^3 cell mL^{-1} along the 3 weeks of this trial. Mussel total wet flesh (3 analysis of 10 individuals pooled) was sampled for AZAs analysis in each condition at 0, 0.25, 1, 2, 3, 6, 7, 8, 14, 21 days of experiment. Mussel DG and RF (3 analyses of 10 DG or RF pooled) were sampled for AZAs analysis in wet tissues in each condition after 3 and 7 days contamination and after 1 week (day 14) and 2 weeks detoxification (Day 21).

(Note: the hypothesis was made that AZA concentration in mussel of similar size followed a normal distribution under the same experimental condition. Thus, a large number of mussels were sampled and subsequently pooled to reduce analytical work.)

2.5.3.3. Distribution of AZAs in blue mussels

To assess AZAs distribution throughout tissues, mussels (4 kg) were exposed for 24 h to an initial *A. spinosum* concentration of 60×10^3 cell mL^{-1}. Mussels (30 individuals) were then carefully dissected and rinsed with Milli-Q water to avoid possible contamination with remaining *A. spinosum* cells. The following tissues were pooled for analysis: digestive gland (DG), gills, mantle margins (MM), mantle (M), labial palp (LP), posterior adductor muscle (PAM), foot, and remaining flesh (RF).

2.5.4. Extraction procedures

2.5.4.1. Reagents

Acetone and methanol (MeOH) were obtained as HPLC grade solvents from JT Baker. Milli-Q water for the HPLC mobile phase was supplied by a Milli-Q integral 3 system (Millipore). Formic acid (Puriss quality) and ammonium formate (Purity for MS) were from Sigma Aldrich. AZA1 calibrants for LC–MS/MS analysis were dilutions of certified AZA1 solution obtained from the National Research Council, Canada.

2.5.4.2. A. spinosum

Triplicate samples of *A. spinosum* were taken after each addition of algae in the 25 L algal tanks (4 times over the 7 days of the contamination period) at day 0, 1, 3 and 5 respectively. The analytical procedure had previously been optimised (Jauffrais et al., 2012a). Briefly, aliquots (10 mL) of *A. spinosum* cultures were collected and centrifuged (2 500 *g*, 20 min, 4°C) in 15 mL centrifuge tubes. The supernatant was discarded and the pellet was re-suspended with 0.5 mL of acetone/H_2O (9/1, *v/v*), transferred to an Eppendorf tube (1.5 mL) and bath sonicated (10 min). After sonication, the aliquot was centrifuged (15 000 *g*, 10 min, 4°C). The supernatant was transferred into a 5 mL glass tube and gently evaporated under nitrogen on a heating block at 35°C. The pellet was re-suspended in 0.5 mL of acetone/H_2O (9/1, *v/v*), homogenised and centrifuged again. The supernatant was transferred again into the same 5 mL glass tube used before and gently evaporated. This process was repeated three times in total. After evaporation of supernatants, the residue was reconstituted in 1 mL methanol/H_2O (9/1, *v/v*). Subsequently, an aliquot was filtered through a NANOSEP MF centrifugal filter (0.2 µm, PALL) (15 000 *g*, 3 min, 4°C), and transferred into an HPLC vial with 250 µL insert for analysis.

2.5.4.3. Mussel tissues

Mussel tissues were collected at the different time points of the experiments. Tissues were carefully removed using a dissection scalpel, drained for 5 min and weighed to measure wet weight. They were then placed into labelled 50 mL centrifuge tubes and stored until extraction at -20°C. Prior to extraction, the tissues were thawed and homogenized using a high-speed homogeniser (Polytron PT1200c) at 15 000 rpm for 5 min. Aliquots (2g ± 0.2g) were taken in triplicate, weighed on a 3-place balance (Sartorius Cubis MSA) and placed into labelled 50 mL centrifuge vials. Methanol (9 mL) was added to each vial and samples were

extracted using a high-speed homogeniser (Polytron PT1300D) at 15 000 rpm for 2 min. Samples were then centrifuged at 4 000 *g* for 5 min at 4°C, and the supernatant was transferred into 20 mL volumetric flasks. Another 9 mL of methanol was added to the remaining pellet and homogenized again. Centrifugation was repeated at above parameters, and supernatants were transferred into the same 20 mL volumetric flasks. Volumetric flasks were then made up to the full mark with methanol, homogenised and aliquots (400 µL) were spin-filtered through 0.22 µm Eppendorf in-vial filters (15 000 *g*, 3 min, 4°C), and filtrates were subsequently transferred into HPLC vials to be analysed by LC-MS/MS (Villar-Gonzalez et al., 2011).

2.5.5. LC-MS/MS analysis

The samples were analysed by LC-MS/MS to quantify AZAs using a HPLC (model UFLCxr, Shimadzu) coupled to a triple quadrupole mass spectrometer (API 4000Qtrap, Applied Biosystems). Separation was performed on a silica-based reversed phase column (injection volume 5 µL), MOS-Hyperclone C8 (50 × 2 mm, 3 µm particle size; Phenomenex). The mobile phases A and B were 100% water and acetonitrile/water (95/5, *v/v*) respectively both containing 2 mM ammonium formate and 50 mM formic acid. The column was used with a gradient elution of 200 µL min^{-1} at 20 °C. Gradient elution for AZA determination started with 70% B rising to 100% B for 2 min, held for 7 min, decreasing to 70% B over 0.5 min, and held for 5 min until the next run. The mass spectrometer was operated by multiple reaction monitoring (MRM) in positive ion mode. Based on a previous study (Rehmann et al., 2008), the transitions presented in Table 21were used for quantification. The declustering potential was 116V, the entrance potential 10V, the cell exit potential 12 and 16V, and the collision cell were 41 and 69V for fragmentation 1 and 2 respectively. The electrospray ionisation interface (ESI) was operated using the following parameters: curtain gas: 30 AU (Arbitrary Unit); temperature: 450°C; gas 1: 50 AU; gas 2: 50 AU; CAD gas: medium; ion spray voltage: 5500V. Quantifications were carried out by external calibration against AZA1, with Analyst 1.5 software (Applied Biosystems), assuming that all analogues have the same response factor as AZA1.

Toxic Equivalent Factors (TEFs) were also applied to estimate the true toxic potential of mussel samples during the experiment. AZA1 TEFs applied were equal to 1.8 and 1.4 for AZA2 and -3, respectively (Ofuji et al., 1999a) and a provisional TEF of 1 was used for crude estimation of the toxicity represented by AZA6. Consequently, a TEF of 1.4 and 1 were

applied to AZA17 and -19, respectively, as AZA17 and -19 transform into -3 and -6 after cooking (McCarron et al., 2009).

Table 21. MS/MS transitions used for quantification of all AZAs observed.

	Transition 1	Transition 2
AZA1	842.5 / 824.5	842.5 / 672.5
AZA2	856.5 / 838.5	856.5 / 672.5
AZA3	828.5 / 810.5	828.5 / 658.5
AZA4	844.5 / 826.5	844.5 / 658.5
AZA5	844.5 / 826.5	844.5 / 674.5
AZA6	842.5 / 824.5	842.5 / 658.5
AZA7	858.5 / 840.5	858.5 / 672.5
AZA8	858.5 / 840.5	858.5 / 688.5
AZA9	858.5 / 840.5	858.5 / 658.5
AZA10	858.5 / 840.5	858.5 / 674.5
AZA11	872.5 / 854.5	872.5 / 672.5
AZA12	872.5 / 854.5	872.5 / 688.5
AZA17	872.5 / 810.5	872.5 / 658.5
AZA19	886.5 / 824.5	886.5 / 658.5
AZA21	888.5 / 826.5	888.5 / 658.5
AZA23	902.5 / 840.5	902.5 / 658.5

2.5.6. Histology

Mussels (five individuals) were sampled for each condition at days 0, 2, 7 and 21, to observe possible changes in digestive gland tubules and recovery of digestive tissues over the feeding experiment (toxic and non-toxic diets were compared). The mussels tissues were fixed in Davidson's fixative (Shaw and Battle, 1957) for 48 h and conserved in 70% ethanol until inclusion. Sections of mussel tissues were then dehydrated using ClaRAL (tetrachloroethylene) and ethanol solutions. A cross section (5 µm) was then cut from each mussel tissue sampled, including the digestive gland, gills, mantle and gonad. Afterward, staining was carried out using haematoxylin-eosin solutions. Slides were examined under light microscopy (Leica DM2000). The thickness of the digestive gland tubules was assessed to determine their state (Medhioub, 2011); measurements were carried out on each individual at three distinct locations on the histological plate. In each location, the thickness of 10 digestive gland tubules was measured with Leica Application Suite 2.8.1 software.

2.5.7. Statistical analysis

All data are expressed as mean ± standard deviation. Statistical analyses consisted of one-way analysis of variance (ANOVA) followed by a Fisher's least significant difference (Battle et al.) procedure. Differences were considered significant at $p<0.05$. Statistical analyses were carried out using Statgraphics Centurion XV.I (STATPOINT TECHNOLOGIES, INC.). Before each ANOVA analysis normality and equality of variance were tested.

Detoxification was modelled as one or two compartment kinetics, for comparison with previously reported detoxification of bivalve molluscs contaminated by lipophilic toxins. A one compartment model supposed a similar detoxification kinetic of the mussel tissues, whereas a two compartment model is explained by the fast excretion of the toxin non retained by the digestive tract (compartment 1) and by the slow detoxification of the toxins bound to the mussel tissues (compartment 2) (Blanco et al., 1997; Bricelj and Shumway, 1998; Lassus et al., 2007).

To improve the strength of the detoxification model, and as all treatment groups were given the same diet for detoxification, the values for each condition were standardised using the average of the toxin concentration at the start of detoxification. Subsequently, standardised values were averaged, and detoxification kinetics were assessed according to the following models using MATLAB version 7.4.0:

$$C_t = C_0 \times \exp(-k \times t) \qquad \text{(equation 1)}$$

$$C_t = C_a \times \exp(-k_1 \times t) + (1-C_a) \times \exp(-k_2 \times t) \qquad \text{(equation 2)}$$

Where C_0 is the initial toxin concentration, C_a the toxin loss by the first compartment, k the mean detoxification rate (day^{-1}) of the one compartment model, k_1 and k_2 are the detoxification rate (day^{-1}) of the first and second compartment, respectively, and t is the time in days. Subsequently, the half-life $T_{½}$ *i.e.* the theoretical time in days to reach a 50% reduction in toxin concentration was calculated from the equation below (deduced from equation 1):

$$T_{½} = \ln(1/2)\, k^{-1} \qquad \text{(equation 3)}$$

For this first approach to detoxification kinetics, and as no *A. spinosum* were consumed by mussels during the last day of contamination (experimental observation), detoxification was considered to have started on Day 6. We also hypothesized that diets used during the contamination period did not affect detoxification kinetics.

2.6. Results

2.6.1. AZA profile of A. *spinosum* culture

The cultured *A. spinosum* strain 3D9 produced AZA1 and -2, with AZA1 as the predominant azaspiracids with a proportion of 74.6% (43 ± 6 fg cell^{-1}) of the total AZAs content (59 ± 6 fg cell^{-1}, n=6). Consequently, AZA2 proportion was 25.4% (15 ± 6 fg cell^{-1}).

2.6.2. AZA accumulation and detoxification

Toxin content of mussels fed with all three toxic diets followed the same pattern over the first days of contamination. A rapid contamination of *M. edulis* with azaspiracids was observed; after only 6 h of exposure, all toxin concentrations were above the regulatory limit in bivalve molluscs (160 µg kg^{-1} AZA1 equiv., Figure 52). Further accumulation appeared much slower until day three, where AZA total content stabilised for diet 2 at ± 400 µg kg^{-1} until the end of the contamination period (day 7) with *A. spinosum*. For diets 1 and 3 AZA toxin content reached a maximum of approximately 600 µg kg^{-1} after 6 days of contamination. For those two conditions, a sharp decrease was observed from day 6 to 8, in coherence with experimental observations between day 6 and 7 (where no or only low amounts of *A. spinosum* were consumed) and the beginning of the detoxification period (day 7).

From day 7 to the end of the feeding trials, mussels of all treatments were then fed with the non-toxic alga T-*Iso*. Detoxification appeared to be a biphasic process (Figure 53, tables 2-3). Detoxification rates (k_1, k_2 and k) for total AZAs, AZA1 and -2 and AZA metabolites, presented in Figure 53 and Table 22 and Table 23 differed. In particular, the detoxification rate of AZAs originating from biotransformation of AZA1 and -2 in mussels was lower than of the AZAs biosynthesised by *A. spinosum* (AZA1 and -2, Figure 53b and Table 22 and Table 23).

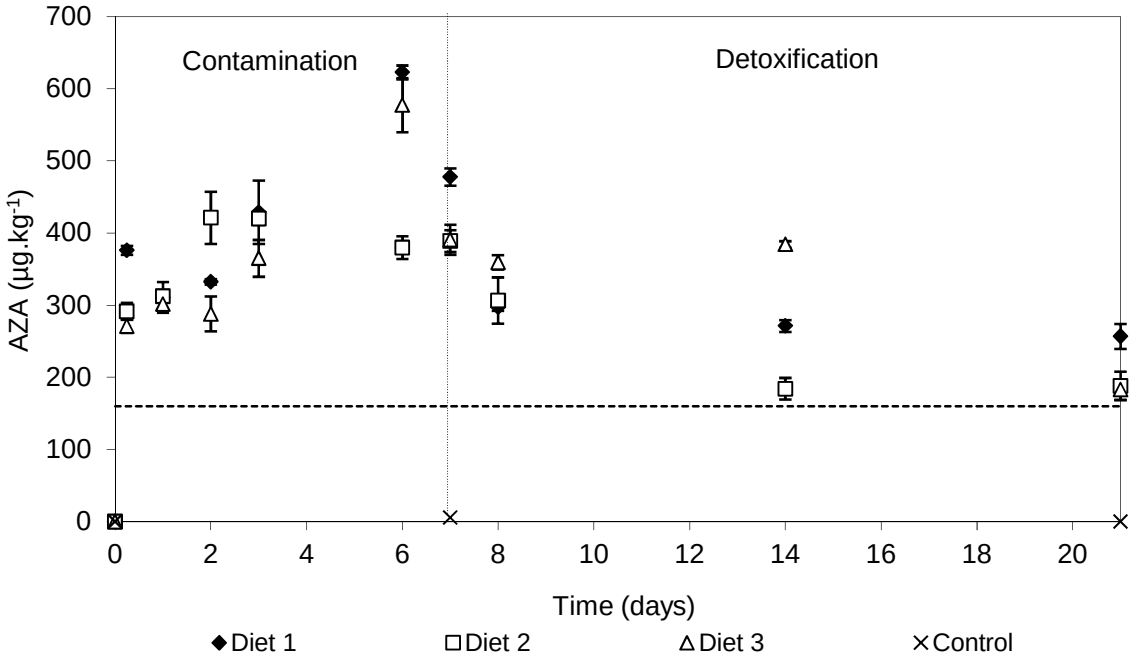


Figure 52. Total AZA toxin concentration (± std) in mussels (3 analyses of 10 pooled mussels) in µg kg^{-1} of wet flesh (sum of all analogue concentrations quantified against AZA1) during contamination (Day 0-7) and detoxification (Day 7-21) from the four experimental treatments (Diet 1 = 5 000 cells.mL^{-1} of *A. spinosum*, Diet 2 = 10 000 cells.mL^{-1} of *A. spinosum*, Diet 3 = 5 000 cells.mL^{-1} of respectively *A. spinosum* and T-*Iso,* and control = 10 000 cells.mL^{-1} of T-*Iso*). Horizontal dash line represents AZA regulatory limit for shellfish (160 µg kg^{-1} AZA1 TEQ).

Table 22. AZA depuration rates (k) in day^{-1} using a 1-compartment model, the adjusted square of correlation coefficient (R^2) from equation 1. Time in days to reach a 50% reduction in toxin concentration (T ½) from equation 3, for each diet after normalisation of the values and for AZAs, AZA1+2 and AZA metabolites using average value of diet 1-3, during the detoxification period (Day 6-21).

	k	Adjusted R^2	T ½
AZAs diet 1	-0.07	0.48	9.9
AZAs diet 2	-0.06	0.80	11.6
AZAs diet 3	-0.05	0.57	13.9
AZAs	-0.06	0.72	11.6
AZA1+2	-0.08	0.68	8.7
AZA metabolites	-0.05	0.79	13.9

Table 23. AZA depuration rates (k_1 and k_2) in day^{-1} and the toxin loss by the first compartment (C_a) using a 2-compartment model, the adjusted square of correlation coefficient (R^2) from equation 2, for total AZAs, AZA1+2, AZA metabolites, and AZA1 and -2 with their respective metabolites during the detoxification period (Day 6-21), using average value of diet 1-3 after normalisation of the values.

	k_1	k_2	C_a	Adjusted R^2
AZAs	-0.87	-0.03	0.38	0.96
AZA1+2	-1.13	-0.04	0.43	0.95
AZA metabolites	-0.65	-0.02	0.32	0.96
AZA1 + related metabolites	-0.75	-0.03	0.39	0.96
AZA2 + related metabolites	-0.72	-0.03	0.35	0.98

Overall, AZA1 and -2 were eliminated more rapidly, as they were both transformed into other analogues (Figure 53b, 3 and 4) and excreted. Additionally, AZA1 and -2 may be excreted prior to any assimilation (absorption or protein binding). Nonetheless, after two weeks of detoxification the total AZA content was still greater than 160 µg kg^{-1} AZA1 TEQ (toxic equivalents: concentrations weighted by their TEF) in all three diets studied. Detoxification kinetics appeared to be comparable between the sum of AZA1 + its metabolites and the sum of AZA2 + its metabolites (Figure 53c).

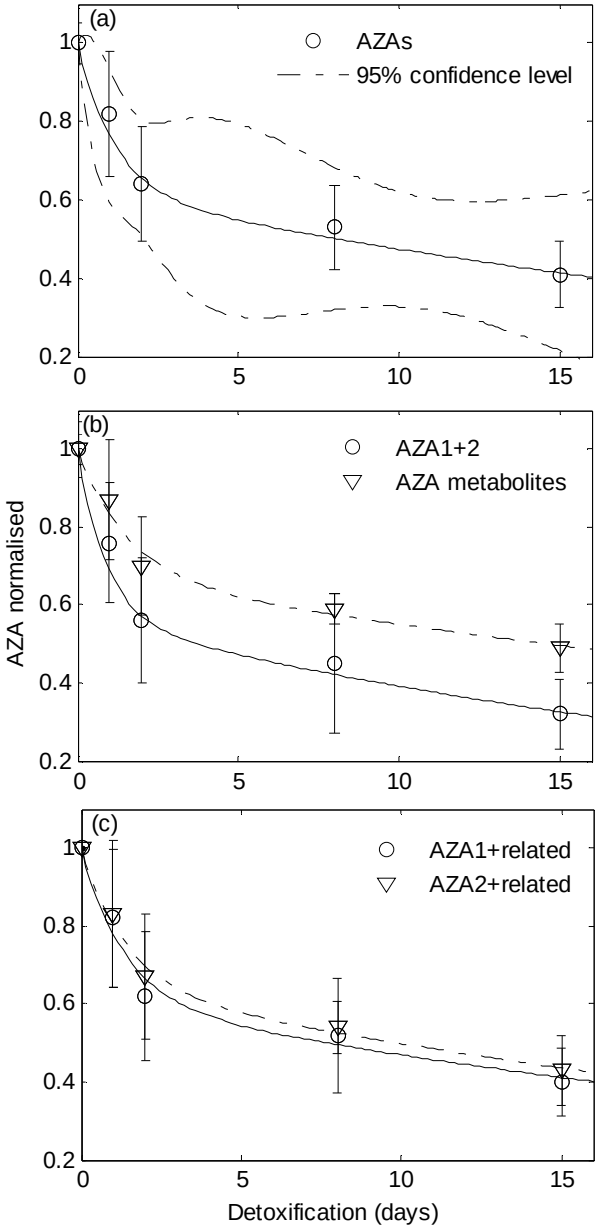


Figure 53. AZA detoxification kinetics, (a) total AZAs, (b) AZA1+2 and AZAs metabolites and (c) AZA1or AZA2 plus their related AZAs, during the detoxification period (Day 6-21, a, b, c correspond to averaged normalised values for all 3 diets, 2-compartment model).

2.6.3. AZA biotransformation during accumulation and detoxification

Biotransformation of AZA1 and -2 was a fast process. After 6 h of contamination, 25% of the AZA proportion in total mussel flesh comprised metabolites in all 3 conditions tested, and this proportion increased up to 50% after 1 and 2 days of contamination. Furthermore, the metabolite proportion tended to slowly increase until the end of the contamination and detoxification period to represent 60-65% of the total in all 3 conditions

tested (Figure 54). Interestingly, when DG were separated from RF the global pattern differed between the two fractions as shown in Figure 54. From the third day of contamination to the end of the detoxification period the ratio between AZA1 and -2 and their metabolites was stable (around 50%) in the DG, whereas in the RF the percentage of metabolites increased from day 3 to the end of the detoxification period from 50 to 70%.

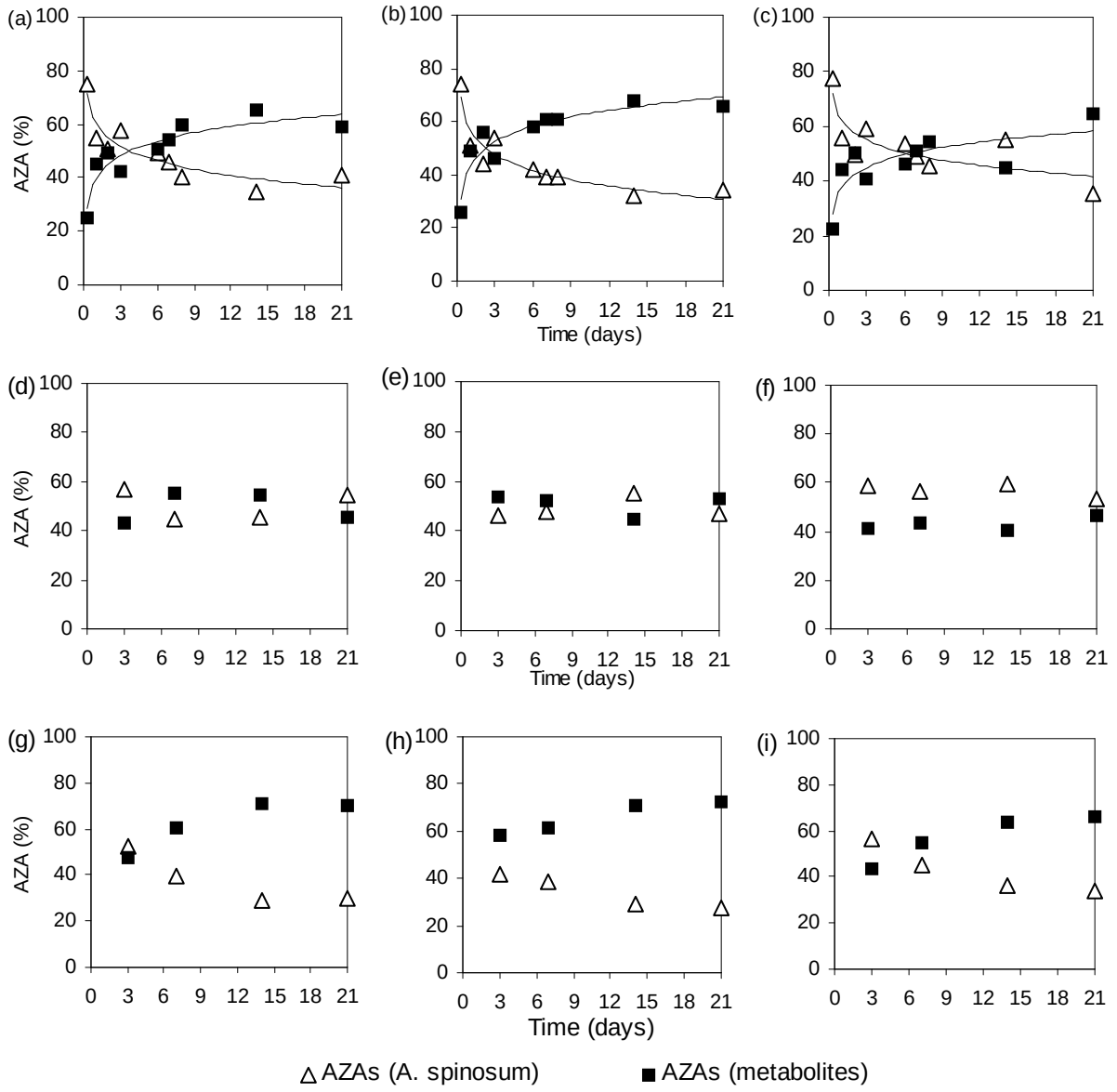


Figure 54. Variation of AZA1 and -2 from *A. spinosum* compared to AZA metabolites in total flesh (a, b, c), digestive gland (d, e, f) and remaining flesh (g, h, i) as a function of time in diet 1 (a, d, g), diet 2 (b, e, h), diet 3 (c, f, i).

The temporal variation in the proportion of the different AZA toxins in mussel tissues is presented in Table 24 for diet 1 (the temporal variation with diet 2 and 3 followed the same kinetics of AZA biotransformation). In less than six hours, biotransformation of AZA1 and -2 occurred. In order of decreasing importance, AZA 17, -19, 7-8, 6, 11-12 and -3 were found,

with a proportion of AZA17 equal to AZA2. After 24 h of contamination AZA5 was found in addition to the previous AZAs; the proportion of AZA17 and -19 increased twofold, with a proportion of AZA17 equal to AZA1. From then until the end of the contamination period the proportion between metabolites and primary AZAs stabilised. It is important to notice the increase in the number of metabolites over time, AZA4, -10 and 21 after 2 days, followed by AZA23 at Day 3 and AZA9 at Day 6. After six days no new AZA analogues were detected. Over the detoxification period, bioconversion processes were still occurring, with an increase in the proportion of AZA4-6 and AZA21-23. Nonetheless, during this feeding trial AZA17 appeared to be the major metabolite, with proportions similar to AZA1.

Table 24. Temporal variation in the proportion of AZA toxins (%), and AZA toxin concentrations (µg kg^{-1} and µg kg^{-1} AZA1 TEQ) in whole mussels over time for diet 1 (5 000 cell mL^{-1} of *A. spinosum*). Highlighted in grey are the toxin proportions related to AZA1, in white to AZA2 and in bold are the four major toxins. TEQ are toxic equivalents (concentrations weighted by their toxic equivalence factor = TEF); TEF_{AZA17} = 1.4 (as AZA17 transforms into 3 after cooking); TEF_{AZA19}=TEF_{AZA6}=1 (as AZA19 transforms into AZA6 after cooking, and TEF_{AZA6} is estimated the same as TEF_{AZA1}).

	Time (days)	**0.25**	**1**	**2**	**3**	**6**	**7**	**8**	**14**	**21**
Algal AZAs	**AZA1**	**57.9**	**39.5**	**35.3**	**41.5**	**35.1**	**31.7**	**25.8**	**22.9**	**29.3**
(%)	**AZA2**	**17.4**	**15.0**	**15.6**	**16.4**	**14.5**	**14.0**	**14.8**	**12.0**	**11.7**
Algal AZAs (µg kg^{-1})		*283.1*	*169.5*	*169.1*	*248.3*	*308.9*	*218.7*	*120.6*	*94.5*	*105.1*
	AZA3	0.3	0.6	0.6	0.5	0.7	0.8	0.7	0.7	0.7
	AZA4	0.0	0.0	0.2	0.5	2.0	2.5	2.3	5.2	7.3
	AZA5	0.0	0.7	1.1	1.1	1.5	1.9	1.8	3.9	5.3
	AZA6	0.8	2.1	1.9	1.8	2.3	2.5	2.4	3.6	2.8
	AZA7-8	2.9	2.6	1.7	2.0	2.9	2.7	1.7	1.6	1.9
AZA metabolites	**AZA9**	0.0	0.0	0.0	0.0	0.2	0.3	0.4	0.8	1.2
(%)	**AZA10**	0.0	0.0	0.3	0.4	0.5	0.6	0.6	1.1	1.5
	AZA11-12	0.4	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3
	AZA17	**16.7**	**32.3**	**35.4**	**28.2**	**29.8**	**31.0**	**36.8**	**33.8**	**25.5**
	AZA19	**3.6**	**6.9**	**7.4**	**6.4**	**6.9**	**7.8**	**8.5**	**9.0**	**7.5**
	AZA21	0.0	0.0	0.3	0.8	2.8	3.2	3.4	4.3	4.4
	AZA23	0.0	0.0	0.0	0.1	0.4	0.5	0.6	0.7	0.8
AZAs metabolites (µg kg^{-1})		*93.0*	*141.0*	*163.3*	*180.7*	*313.9*	*258.9*	*176.6*	*176.7*	*151.5*
AZA total (µg kg^{-1})		*376.1*	*310.5*	*332.4*	*429.0*	*622.8*	*477.7*	*297.2*	*271.2*	*256.6*
Regulated AZAs (AZA1-3) (µg kg^{-1} AZA1 TEQ)		*336.9*	*209.3*	*213.5*	*307.8*	*387.2*	*277.4*	*158.7*	*123.3*	*131.7*
AZAs1, 2, 3, 6, 17 and 19 (µg kg^{-1} AZA1 TEQ)		*441.6*	*377.6*	*409.4*	*512.5*	*704.5*	*534.2*	*344.3*	*285.9*	*249.6*

The proportion of AZA toxins at the end of the period of contamination and detoxification in DG and RF is presented in Table 25. As mentioned before the proportion of metabolites was significantly different in the two types of tissues. All AZA analogues detected during this feeding trial were found in the DG, whereas AZA9-12 were not found in the RF. A larger proportion of AZA17 was present in the RF than in the DG. In the RF, AZA17 became the dominant AZA analogue, increasing from 1.5 to 2 times the AZA1 concentration, whereas the other analogues found in the RF remained at low levels.

Table 25. Temporal variation in the proportion of AZA toxins (%), and total AZA toxin contents (µg kg^{-1} of digestive gland or remaining flesh or total flesh) in mussels at the end of the contamination and detoxification period for diet 1 (5 000 cell mL^{-1} of *A. spinosum*). Highlighted in grey are the four major AZAs.

		Digestive gland (Day 7)	**Digestive gland (Day 21)**	**Remaining Flesh (Day 7)**	**Remaining Flesh (Day 21)**
Algal AZAs (%)	AZA1	29.2	40.4	27.1	19.8
	AZA2	15.6	14.0	12.0	10.4
AZA metabolites (%)	AZA3	0.4	0.6	0.8	1.0
	AZA4	2.0	5.7	1.3	2.3
	AZA5	2.3	4.2	1.6	2.2
	AZA6	2.6	2.1	2.3	3.4
	AZA7-8	2.6	2.0	2.0	0.8
	AZA9	0.3	0.8	0.0	0.0
	AZA10	0.4	1.3	0.0	0.0
	AZA11-12	0.3	0.2	0.0	0.0
	AZA17	29.1	17.3	42.3	47.1
	AZA19	9.3	6.1	8.0	10.5
	AZA21	4.9	4.4	2.1	2.0
	AZA23	0.9	0.8	0.4	0.4
AZA total (µg kg^{-1}DG or RF)		2673.1 ± 44.1	1153.7 ± 21.6	193.1 ± 3.5	111.3 ± 1.6
AZA total (µg kg^{-1}TF) **weight standardised**		336.9 ± 5.6	208.1 ± 17.7	168.8 ± 3.1	91.3 ± 1.3

2.6.4. AZA distribution in blue mussels

A separate batch of mussels was contaminated for this evaluation, carried out over 24 h using *A. spinosum* at an initial concentration of 60 000 cells.mL^{-1}. The total flesh toxicity was 517 ± 18 µg kg^{-1} (Table 26). The proportion of AZAs is presented in Figure 55, where 73% of the AZAs were found in the DG, 11% in the RF and 8% in the gills. The other tissues showed minor amounts of toxins with values below 3% of the total toxin accumulated (Figure 55). AZA concentrations in *M. edulis* tissues are presented in Table 26 and the AZA profile of each dissected tissue in Figure 55. AZA1-6, -8, 11-12, -17, -19, -21 and -23 were found in the DG. In decreasing order of concentration, the four major AZAs were AZA1, -17, -2, -19. These four analogues were found in all tissues, however only AZA1 and -17 were significant in tissues with minor amount of toxins (MM, LP, M, PAM, Foot).

Table 26. AZA concentration in different mussel tissues after exposure for 24h (3 analyses of pools of 30 individuals for each tissue type, TF = Total Flesh, DG = Digestive Gland, RF = Remaining Flesh, MM = Mantle Margin, M = Mantle, LP = Labial Palp, PAM = Posterior Adductor Muscle) and proportion of each tissue in mussels analysed.

AZA (µg kg^{-1})	TF	DG	RF	Gills	MM	LP	M	PAM	Foot
AZA1	189	836	76	89	30	64	29	14	19
AZA2	61	301	22	25	8	19	7	3	5
AZA3	4	18	2	2	0	0	0	0	0
AZA4	4	29	2	0	0	0	0	0	0
AZA5	6	43	4	2	1	2	2	1	1
AZA6	12	51	5	5	1	0	1	1	0
AZA8	21	117	11	4	3	5	4	2	3
AZA11-12	3	17	1	0	0	0	0	0	0
AZA17	178	723	105	117	29	77	36	22	16
AZA19	36	161	21	21	4	14	6	4	3
AZA21	3	20	2	0	0	0	0	0	0
AZA23	0	4	0	0	0	0	0	0	0
Total AZAs	*517*	*2319*	*251*	*264*	*77*	*182*	*85*	*48*	*46*
Tissue % of total wet flesh	100	16	22	16	17	4	10	10	7

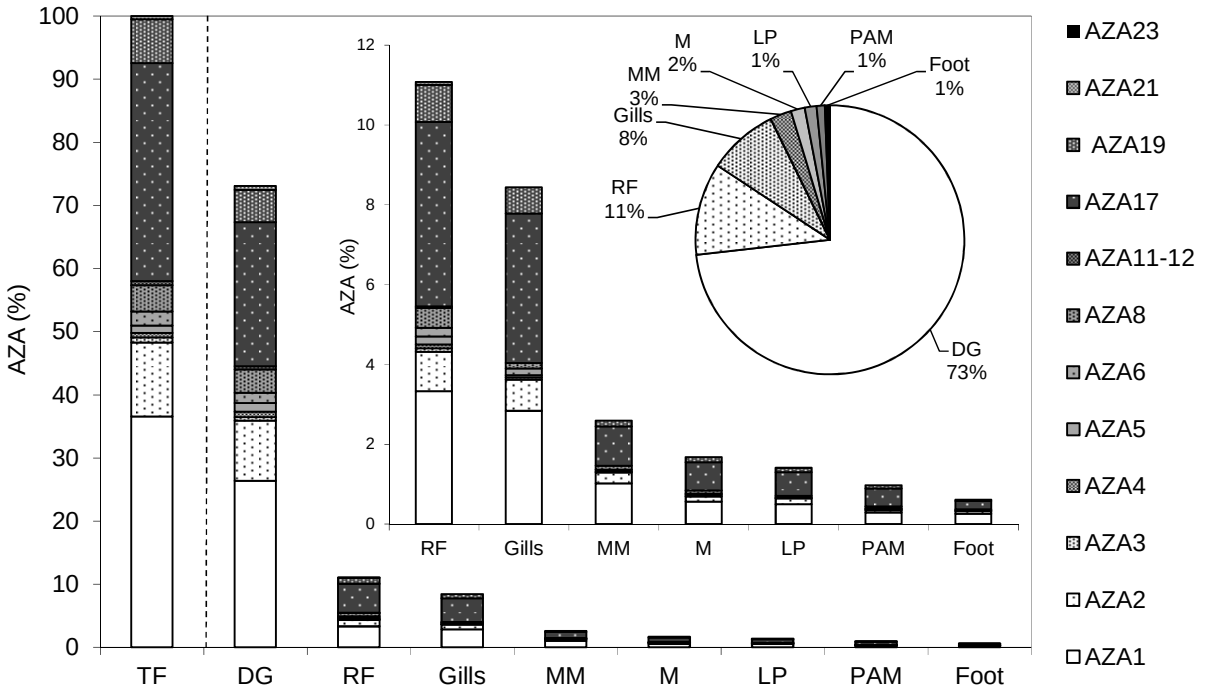


Figure 55. AZA profile and distribution in different mussel tissues over 24 h (3 analyses of pools of 30 individuals for each tissue type, TF = Total Flesh, DG = Digestive Gland, RF = Remaining Flesh, MM = Mantle Margin, M = Mantle, LP = Labial Palp, PAM = Posterior Adductor Muscle).

2.6.5. Assessment of *A. spinosum* effect on mussels

2.6.5.1. Mortality

Mortality rates of mussels fed the different diets were low as shown in Table 27. However, mortality rates were higher in the diets based on *A. spinosum* than in the control condition with T-*Iso* that showed 2% mortality during the whole feeding trials.

Table 27. Mortality (%) observed in the different conditions at the end of the contamination and decontamination period.

Mortality (%)	Diet 1	Diet 2	Diet 3	Control
Contamination (day 0-7)	1.2	7.0	2.7	0.5
Detoxification (day 7-21)	6.6	4.6	2.9	1.5
Total	8	12	6	2

2.6.5.2. Histology

To assess a possible effect of *A. spinosum* on the digestive tissues of mussels, digestive gland tubules were examined at the beginning of the feeding trial, after 2 and 7 days of contamination and at the end of the detoxification period. *A. spinosum* had a negative effect on the thickness of digestive gland tubules when comparing the different diets based on *A.*

spinosum after 2 or 7 days of contamination with the control or the initial condition of the digestive gland tubules (Figure 56). After two weeks of detoxification a recovery was observed for all survivors examined as no differences between toxic and non-toxic diets were noted.

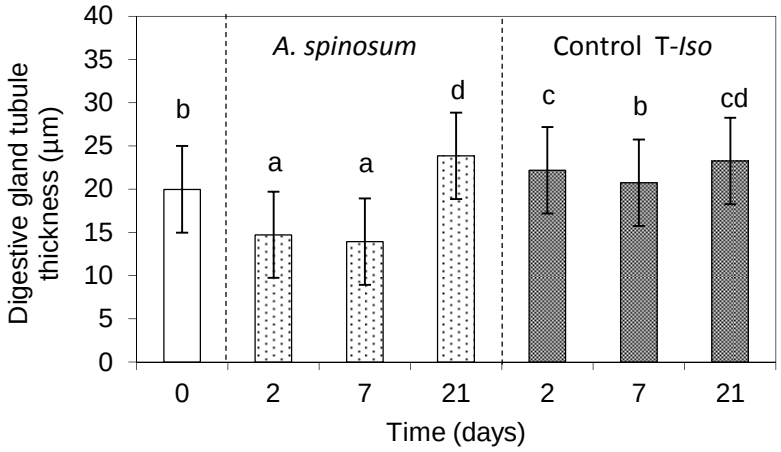


Figure 56. Evolution of the digestive gland tubule thickness (µm) in toxic (*A. spinosum*) and non-toxic control (T-*Iso*) diets during contamination (days 2 and 7) and at the end of the detoxification period (day 21). The errors bars represent the standard deviation (n ≥ 150 digestive tubules). Values with different letters are statistically different at $P<0.05$.

2.7. Discussion

The *A. spinosum* toxin concentration per cell from the harvesting tank (59 ± 6 fg cell^{-1}) was within the large range already observed for this species, from 5 to 40 fg cell^{-1} in batch culture (Salas et al., 2011; Tillmann et al., 2009) and up to 100 fg cell^{-1} in chemostat bioreactors (Jauffrais et al., 2010; Jauffrais et al., 2011) and had the usual toxin profile with AZA1 as the major AZA.

After 6h, the total AZA content reached twice the regulatory limit in all three different diets studied. Therefore, the AZA accumulation in mussels should be considered as a very fast process, at least at the concentrations tested here (5-10 ×10^3 ml^{-1}). Comparison of these results to the field situation, however, is hampered to the fact that abundance data of *A. spinosum* in European field samples are completely lacking. Nevertheless, the cell concentration corresponds to the reports on *A. spinosum* concentration in coastal Argentinian waters, where a maximum concentration of 9×10^6 cells.L^{-1} in 1990 and values ranging from 0.5 to 3×10^6 cells.L^{-1} in 1991 were determined (Akselman and Negri, 2010). A rapid accumulation of AZAs in case of *A. spinosum* blooms underlines difficulties in monitoring toxins for sanitary purposes and the necessity to predict when such an event might occur. Over 7 days, toxin

content in mussels increased to reach a maximum concentration of around 600 µg kg^{-1} in diet 1 and 3 both containing 5 000 cell mL^{-1} of *A. spinosum*, whereas in diet 2 (10 000 cell mL^{-1} of *A. spinosum*) the toxin content reached a maximum concentration of ± 400 µg kg^{-1}. The addition of a non-toxic species to *A. spinosum* neither significantly affect AZA accumulation. Also, the higher *A. spinosum* concentration did not result in increased AZA accumulation but rather seemed to reduce AZA accumulation. However, these findings should not be over-interpreted as such trials should be repeated to increase statistical significance.

These toxin contents in blue mussels were within the range of what is frequently found in the Irish monitoring program (Salas et al., 2011). However, mussels did not build up very high levels over the duration of the accumulation phase (7 days). This result suggests that, in natural conditions, exposure of mussels to *Azadinium* may be either longer than 7 days or involve more complex trophic mechanisms such as biomagnification via other planktonic organisms. Furthermore, the study shows the limits of preparative-scale production of AZA-metabolites through *in vivo* exposure of mussels. Still, the procedure may be useful for production of in-house metabolite reference materials as suggested by (Hess et al., 2007), and demonstrated for paralytic shellfish toxins (Higman and Turner, 2010).

Detoxification of mussels fed with T-*I*so was carried out over two weeks. Supplying non-toxic diet has been previously proposed by several authors to enhance detoxification after exposure of bivalves to saxitoxin (STX) or lipophilic toxins (okadaic acid, gymnodimine) (Lassus et al., 2005; Lassus et al., 2000; Marcaillou et al., 2010; Medhioub et al., 2010). The detoxification kinetic was slow, even if toxin elimination from mussels followed a biphasic kinetic, with a rapid first and a slow second phase (Figure 53 and Table 23); in all cases mussels were above the regulatory limit after two weeks of detoxification when all AZAs found were taken into account and not only the three regulated AZAs. Biphasic kinetics of detoxification have already been described for OA-group toxins, gymnodimines and the paralytic shellfish poisoning (PSP) group (Blanco et al., 1997; Bricelj and Shumway, 1998; Lassus et al., 2007; Marcaillou et al., 2010; Medhioub et al., 2010). Mean detoxification rates were in the same range as those previously reported for okadaic acid (OA) and its derivative (-0.048 for total OA) (Marcaillou et al., 2010). Detoxification rates for total AZAs, algal AZAs (AZA1-2) and AZA metabolites differed because AZA1 and -2 are transformed into other analogues during detoxification. Thus, models only taking into account regulated AZAs (AZA1, -2 and -3), would overestimate the real detoxification rate (Figure 53). Consequently, detoxification models should take into account all AZAs present or at least the major algal and shellfish metabolites (AZA1 and -2, AZA17 and -19). The slow, gradual detoxification

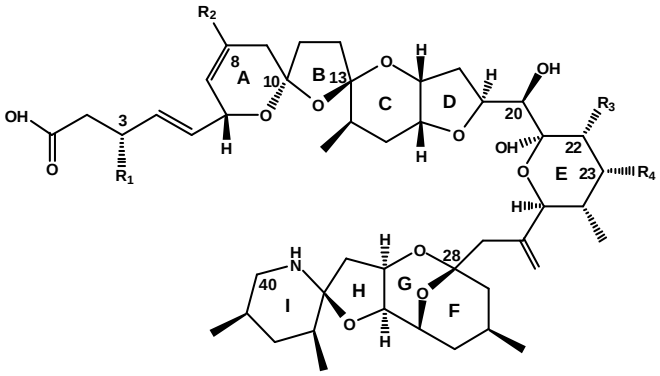
(second phase, Figure 53 and Table 23) has been explained as a possible result of AZA movements from the digestive gland to the other tissues where elimination would be slower (James et al., 2004; James et al., 2002). However, this hypothesis was a result of *in situ* observations where various other possibilities could interfere, including reduction of the mussel metabolism during winter time (Twiner et al., 2008), or possible presence of dissolved AZAs or *A. spinosum* cells, even if natural conditions seem unfavourable. Interestingly, (Nzoughet et al., 2008) found that AZAs were weakly bound to a protein with a molecular weight of 45kDa in DG, a fact that may explain the slow AZA detoxification from mussels (Twiner et al., 2008).

The detoxification rates reported here are to our knowledge the only information on detoxification kinetics after exposure to *A. spinosum* and represent a first approach with this toxin; however, the low number of points analysed along the detoxification period and the large confidence level observed (Figure 53a) demonstrates the need of further experiments.

The bioconversion of algal toxins by bivalves may influence overall toxicity (Twiner et al., 2008). Therefore, it is important to understand both toxin profiles and bioconversion kinetics. Biotransformation of AZA1 and -2 in mussels was found to be a fast process. These results confirmed the first feeding experiment carried out during 24 h with the Irish strain (SM2) of *A. spinosum* (Salas et al., 2011), where a rapid bioconversion of AZA1 and -2 into AZA17, -19 and -3 was found, with AZA17 as the major bioconversion product after 24 h of experiment. In addition to the study by Salas et al., 2011, the present study revealed that all AZA analogues (apart from AZA13-16) already observed in naturally contaminated mussels were found in mussel tissues exposed to *A. spinosum*. However, none of the theoretical AZAs (Rehmann et al., 2008) were observed. This suggests that direct accumulation of *A. spinosum* by mussels may be sufficient to explain the observed toxin profiles.

Bioconversion of azaspiracids is a recent subject of study. Formation of AZA3, -4, -6 and -9 via decarboxylation of AZA17, -21, -19 and -23, respectively, has been shown (McCarron et al., 2009), with some additional experimental evidence provided by (O'Driscoll et al., 2011). In the present study, the order of appearance of the bioconversion products seemed to link AZA3, -4, -6 and -9 with their carboxylated analogues and hence confirms previous results obtained by (McCarron et al., 2009), as AZA17 and -19 appear first and constitute already more than 20% of total AZAs after 6 h. Additionally, the ratio of AZA17/AZA19 is similar to that of AZA1/AZA2. Therefore, it is plausible to assume that there is a direct transformation of AZA1 into AZA17 and of AZA2 into AZA19. Decarboxylation of AZA17 into -3 and of AZA19 into -6 is much slower, which is consistent

with the heat-accelerated character of this transformation already reported by McCarron et al. (2009). Furthermore, AZA3 and -6 proportions remain relatively constant after their appearance, suggesting that their transformation into AZA4 and -5 as well as AZA9 and -10, respectively, is similar in kinetics to the decarboxylation reaction in C22. Simultaneous hydroxylation of AZAs in C3 or C23 into other analogues is not excluded, as the stability of the AZA3 proportion suggests that it could be relatively rapidly hydroxylated into AZA5 and more slowly into AZA4. This phenomenon is also noted through the earlier appearance of AZA5, somewhat convoluted by the higher AZA4 concentration at the end of the experiment which can be understood through the dual route towards AZA4 via AZA3 and -21 (see below). This hypothesis is supported by the biotransformation kinetics: hydroxylated analogues in C3 (AZA7 and -11) required more time (≥48 h) to be formed than hydroxylated analogues in C23 (6-24 h, AZA5, -8, -10) (note: despite a lack of chromatographic separation in the analysis, using specific transitions in MS it was established that AZA7 and -11 appeared after 48 h). The formation of the carboxy-hydroxylated analogues (AZA21 and -23) also required more time (≥48 h) than the formation of carboxylated analogues (AZA17 and -19) which suggests that hydroxylation of C3 or C23 is a slower process than oxidation of the methyl-group at C22 as AZA4 and -5 were not yet present after 6h. At the end of the study AZA4 was more concentrated than AZA5, which is consistent with the dual route leading to this metabolite. Also, overall, the biotransformation of AZA2 and all its analogues appears somewhat slower than that of AZA1 metabolites. This can be seen in the slightly higher ratio of AZA2/AZA19 compared to AZA1/AZA17 throughout the study. The analogues hydroxylated in both C3 and C23 were not detected over the three weeks of experiment and so presumably require much more time to be formed (consistent with the fact that there are two or more slow transformations involved). Following all these observations possible biotransformation pathways of AZA1 and -2 were proposed (Figure 57).



	R1 (C3)	R2 (C8)	R3 (C22)	R4 (C23)	$[M+H]^+$ *m/z*
Azaspiracid-1	H	H	CH_3	H	842.5
Azaspiracid-2	H	CH_3	CH_3	H	856.5
Azaspiracid-3	H	H	H	H	828.5
Azaspiracid-4	OH	H	H	H	844.5
Azaspiracid-5	H	H	H	OH	844.5
Azaspiracid-6	H	CH_3	H	H	842.5
Azaspiracid-7	OH	H	CH_3	H	858.5
Azaspiracid-8	H	H	CH_3	OH	858.5
Azaspiracid-9	OH	CH_3	H	H	858.5
Azaspiracid-10	H	CH_3	H	OH	858.5
Azaspiracid-11	OH	CH_3	CH_3	H	872.5
Azaspiracid-12	H	CH_3	CH_3	OH	872.5
Azaspiracid-13*	OH	H	H	OH	860.5
Azaspiracid-14*	OH	H	CH_3	OH	874.5
Azaspiracid-15*	OH	CH_3	H	OH	874.5
Azaspiracid-16*	OH	CH_3	CH_3	OH	888.5
Azaspiracid-17	H	H	CO_2H	H	872.5
Azaspiracid-19	H	CH_3	CO_2H	H	886.5
Azaspiracid-21	OH	H	CO_2H	H	888.5
Azaspiracid-23	OH	CH_3	CO_2H	H	902.5

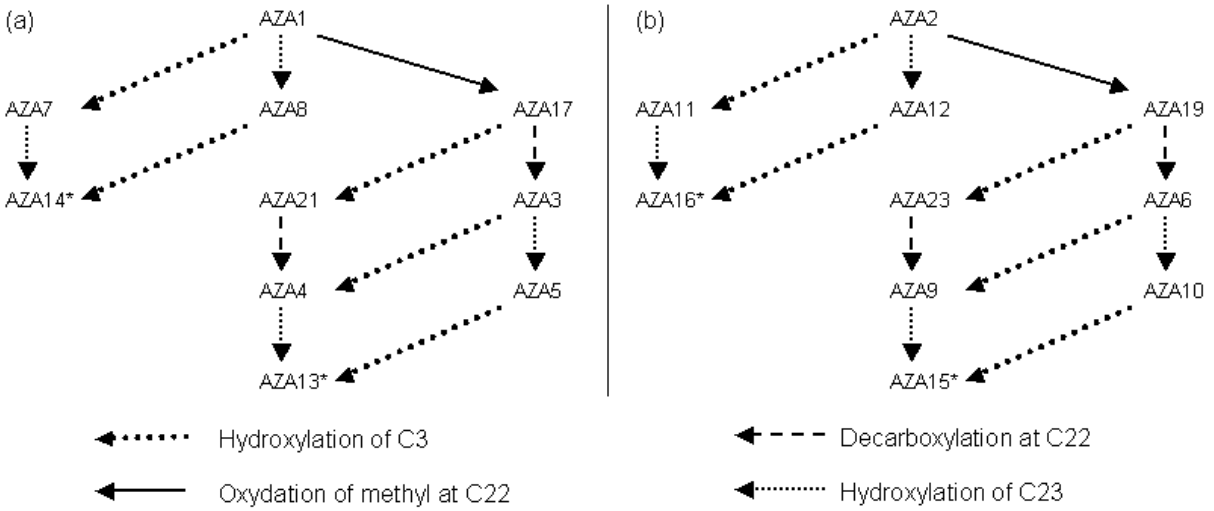


Figure 57. AZA structure, *m/z* $[m+H]^+$ detected in shellfish and possible biotransformation pathways in *M. edulis* of AZA1 (a) and -2 (b) observed during the feeding experiment with *A. spinosum*. Highlighted in grey are the toxins related to AZA1, in white to AZA2, and AZAs with * were below the limit of detection.

As a consequence, the fast AZA oxidation process in mussels has to be considered by regulators, as AZA17 and -19 are major metabolites in raw mussels, and are obviously not readily converted into AZA3 or -6 over time. Therefore, research should focus on AZA17 and -19 production and isolation to determine their potency to human health. The different monitoring programs currently underestimate the total amount of AZA present in mussels. As mussels are typically consumed after cooking, AZA17 and -19 are transformed into AZA3 and -6 (AZA3 already considered toxic and regulated) prior to consumption (Table 24). Thus, AZA17 and -19 should be accounted for, either directly or through heat-treatment of samples prior to analysis.

Regarding toxin distribution across mussel organs, accumulation of AZA differed between tissues in mussels, as was found with other known toxins accumulating in bivalve molluscs (DSP, ASP, PSP), where toxins were mainly found in digestive gland (Blanco et al., 2002a; Blanco et al., 2007; Bricelj and Shumway, 1998; Campbell et al., 2001; Chen and Chou, 2001; Choi et al., 2003; Lassus et al., 2007). In this study, 73% of the AZAs were found in the digestive gland, 11% in the remaining flesh, 8% in the gills and negligible amounts of toxins in other tissues. This distribution of AZAs has already been observed in scallops and mussels naturally contaminated with AZAs (Hess et al., 2005b; Magdalena et al., 2003b; Salas et al., 2011). Most AZA analogues were found in the DG at the highest concentration, it is thus supposed that biotransformation was most active in the DG. The toxin concentrations observed in the non-digestive gland tissues may result from transfer between organs or may originate from re-adsorption of excreted metabolites as the system was re-circulated.

Concerning the effect of *A. spinosum* on digestive gland tubule thickness, the decrease in thickness was observed for all toxic diets compared to the control diet over the contamination period. However, a recovery was observed at the end of the detoxification period. This phenomenon on bivalve physiology was previously observed with other toxins (Galimany et al., 2008b; Medhioub, 2011; Pearce et al., 2005), and shows *A. spinosum* as a possible physiological factor of stress to shellfish.

2.8. Conclusions

The study confirmed the direct transfer of AZA toxins from *A. spinosum* to mussels, and the fast biotransformation of AZA1 and -2 into other analogues, especially into AZA17 and -19. Blue mussels can accumulate AZAs to levels above the regulatory limit in less than 6 h and up to 0.6 mg kg^{-1} within a week. Detoxification kinetics were slow ($T_{1/2}$ approximately 11 days) with detoxification rates in the same order as for other lipophilic toxins. The study underlines that AZA17 and -19 were present at significant levels already after 6 h and until the end of the study. Therefore, these analogues need to be considered as major metabolites which affect human health, either by themselves or through thermal conversion to AZA3 and -6. It is thus necessary to revise the regulation on AZAs, currently based on the analysis of raw bivalves by LC-MS/MS.

2.9. Acknowledgment

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3. Effect of *Azadinium spinosum* on the feeding behaviour and azaspiracid accumulation of *Mytilus edulis*

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3.1. Résumé

Azadinium spinosum est un petit dinoflagellé toxique qui a été récemment isolé et identifié comme producteur d’azaspiracides-1 et -2 (AZA). Des expériences antérieures sur l'accumulation des AZA dans les moules (*Mytilus edulis*) nourries avec *A. spinosum* ont révélé une légère augmentation de la mortalité des moules et des effets négatifs sur l'épaisseur des tubules des glandes digestives.

Par conséquent, des expériences ont été menées pour étudier les effets d’*A. spinosum* sur le comportement alimentaire des moules. Les évaluations individuelles de l'activité alimentaire, du taux de filtration, d’ingestion et d’absorption ont été effectuées sur des moules alimentées soit par un régime toxique (*A. spinosum*) soit par un régime non-toxique (*Isochrysis* aff. *Galbana* (T-*Iso*)). En parallèle, l'accumulation des AZA et leur biotransformation dans les moules ont été suivies par chromatographie en phase liquide couplée à la spectrométrie de masse (CL-SM/SM).

Azadinium spinosum a un effet significatif sur le comportement alimentaire des moules par rapport à T-*Iso*: le taux de filtration a été diminué d’un facteur 6, le temps d’activité alimentaire par un facteur 5, le taux d’ingestion par un facteur 3 et le taux d’absorption à même présenté des valeurs négatives le dernier jour de contamination. Toutefois, une accumulation rapide en AZA a été observée pendant les premières heures de l’expérience, le dépassement de la limite réglementaire étant observé moins de 6 h après le début de l’exposition. En cohérence avec les observations physiologiques, la concentration en AZA, qui est d'environ 200 $\mu g\ kg^{-1}$ après 6 h, n’augmente plus jusqu'à la fin de l'étude. La bioconversion des AZA est confirmée comme étant un processus rapide : après 3 h d’exposition, AZA17, -19 et AZA7 à -10 ont été détectés, avec une proportion d’AZA17 équivalente à celle d’AZA2.

Ces résultats montrent un effet négatif d'*A. spinosum* sur l'activité alimentaire des moules et indiquent une éventuelle régulation de l'accumulation des AZA par une diminution du taux de filtration et une augmentation de la production de pseudofèces.

3.2. Abstract

Azadinium spinosum, a small toxic dinoflagellate, was recently isolated and identified as a primary producer of azaspiracid toxins (AZAs). Previous experiments related to AZA accumulation in blue mussels upon direct feeding with *A. spinosum* revealed increased mussel mortality and had negative effects on the thickness of the digestive gland tubules. Therefore we conducted follow up experiments in order to study effects of *A. spinosum* on mussel feeding behaviour. Individual assessment of mussel feeding time activity (Gallardo Rodriguez et al.), clearance rate (CR), filtration rate (TFR), absorption rate (AR), faeces and pseudofaeces production were carried out on mussels fed either toxic (*A. spinosum*) or non-toxic (*Isochrysis* aff. *galbana* (T-*Iso*)) diets. Furthermore, AZA accumulation and biotransformation in mussels were followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). *Azadinium spinosum* had a significant effect on mussel feeding behaviour compared to T-*Iso*: CR was lower by a factor of 6, FTA by a factor of 5, TFR by a factor of 3 and AR even decreased to negative values for the last day of exposure. Even so, a rapid AZA accumulation was observed during the first hours of the trial; less than 6 h of feeding were required to reach AZA concentrations in mussel above regulatory level. Consistent with physiological observations, AZA concentration of about 200 µg kg^{-1} did not increase further until the end of the study. AZA bioconversion was also found to be a fast process: after 3 h of exposure AZA17, -19 and AZA7-10 were already found, with a proportion of AZA17 equal to AZA2. These results show a negative effect of *A. spinosum* on blue mussel feeding activity and indicate a possible regulation of AZA uptake by decreasing filtration and increasing pseudofaeces production.

3.3. Key words

Bivalve molluscs, mussel, ecophysiology, AZA biotransformation, AZA accumulation, trophic transfer, dinoflagellate, azaspiracid

3.4. Introduction

Azadinium spinosum is a small toxic dinoflagellate, which was recently isolated in the North Sea and characterised as an AZA1 and -2 producer (Tillmann et al., 2009). Since this first identification, species of the genus *Azadinium* were encountered in Europe (Salas et al., 2011; Tillmann et al., 2011; Tillmann et al., 2010; Tillmann et al., 2009), Asia (Potvin et al., 2012) , and also reported from Central and South America (Akselman and Negri, 2012; Hernandez-Becerril et al., 2010). However, among the currently described species, only *A. spinosum* has been identified as an AZA1 and -2 primary producer (Salas et al., 2011; Tillmann et al., 2009), whereas *A. obesum* and *A. poporum* have not been shown to produce any known AZAs (Potvin et al., 2012; Tillmann et al., 2011; Tillmann et al., 2010), even though *A. poporum* was recently reported to produce a new type of AZA characterised by a m/z 342 fragment (Krock et al., 2012). In direct feeding trials, *A. spinosum* was found to directly contaminate mussels, with rapid AZA accumulation and biotransformation after one day of exposure (Salas et al., 2011). Thus, *A. spinosum* is now considered as primary organism responsible for azaspiracid shellfish poisoning (AZP), a recognised widespread phenomenon (Twiner et al., 2008).

AZA1 was isolated from contaminated mussels from Ireland (Satake et al., 1998c) after human intoxications in The Netherlands in 1995 (McMahon and Silke, 1996). Since then, this toxin was considered as relevant for human health and a long list of analogues were subsequently identified. AZA2-5 were purified and subsequently structurally characterised using nuclear magnetic resonance (NMR) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Ofuji et al., 2001; Ofuji et al., 1999a), AZA6-12 (Diaz Sierra et al., 2003; James et al., 2003b), AZA13-32 (McCarron et al., 2009; Rehmann et al., 2008) and AZA1 and -2 methyl esters (Jauffrais et al., 2012a) were identified in mussels, in *A. spinosum*, or during stability studies on AZA standards or extracts using LC-MS/MS.

Suspension-feeding bivalve molluscs are known to be a major vector for the transfer of amnesic, paralytic and diarrheic shellfish toxins (respectively ASTs, PSTs, DSTs) to humans (Bricelj and Shumway, 1998). Symptomatically, azaspiracid shellfish toxins (AZTs) belong to the last group, as it presents similar symptoms: nausea, stomach cramps, vomiting and diarrhoea (Satake et al., 1998c). As the molecular mechanism of action of AZTs is different from that of the toxins belonging to the okadaic acid (OA) group, poisoning with AZTs has also been separately referred to as AZP (Twiner et al., 2008). In Ireland, concentrations of AZAs in bivalve molluscs were recorded above the regulatory limit (160 µg

kg^{-1} AZA1 equiv.) causing large closure periods of shellfish production area and important economic losses to shellfish growers; with maximum concentrations found for blue mussels and Pacific oysters of 8970 and 310 µg kg^{-1} AZA1 equiv., respectively (Salas et al., 2011).

Phycotoxins may also directly affect bivalve molluscs but mussels are considered to be the least sensitive and consequently can accumulate larger amount of toxins than most other bivalve molluscs (Bricelj and Shumway, 1998; Mafra et al., 2010b). As other suspension feeding bivalve molluscs, *M. edulis* feeds on seston of various quality and quantity and is able to adjust their clearance and rejection rate (Bayne et al., 1993). Furthermore, it has been shown that the American oyster (*Crassostrea virginica*), when exposed to a mono-specific diet of the toxic diatom *Pseudo-nitzschia*, can reduce domoic acid uptake by lowering clearance rate and by rejecting toxic cells in the pseudofaeces (Mafra et al., 2009).

In a previous study (Jauffrais et al., 2012c) using *A. spinosum* to feed mussels, a fast AZA accumulation was observed during the first day of contamination. Within a few hours following exposure, AZA concentration in mussels increased above the regulatory limit. This fast accumulation was followed by a slower increase or stabilisation of contamination for the next couple of days. AZA accumulation was also observed when mussels were simultaneously fed *A. spinosum* and non-toxic *Isochrysis* aff. *galbana*. Thus, addition of a non-toxic source of food to *A. spinosum* did not allow particle selection by mussels, and AZA accumulation was neither enhanced nor decreased. This observation showed that mussels could not initially regulate AZA accumulation by particle selection. Nevertheless, the short period of fast AZA accumulation was soon displaced by a period of a limited or stopped accumulation, which led us to assume that after a short exposure to *A. spinosum* mussels adjust their feeding activity, which then limits AZA accumulation. However, physiological or ecological data on *A. spinosum* and its possible role and effect on trophic interaction are still lacking.

We hypothesize that *A. spinosum* could be considered as a micro-alga either toxic to or of poor nutritional value for mussels, and that, consequently, AZA uptake might be limited by a decrease in clearance rate and an increase in *A. spinosum* rejection via pseudofaeces. Hence the present study was designed to compare the feeding behaviour of mussels fed toxic and non-toxic diets based on *A. spinosum* and *I.* aff. *galbana*, respectively. To do this, we assessed physiological factors such as feeding time activity (Gallardo Rodriguez et al.), clearance rate (CR), absorption rate (AR), faeces and pseudofaeces production to confirm the negative effect of *A. spinosum* on mussels.

3.5. Materials and methods

3.5.1. Collection and maintenance of bivalves

Blue mussels (*Mytilus edulis*) were collected from a mussel farm at La Plaine, French Atlantic coast in October 2011. Through routine official control, this area was known at that time to be free of known marine biotoxins. Mussels (5 kg) were transported alive to aquarium facilities, cleaned of epibionts and maintained before the experiment for five days in circulating filtered seawater (2 aquaria of 20 L capacity; salinity 35) to ensure acclimatisation to laboratory conditions and to avoid possible bacterial contamination. During this time, the temperature of the water was gradually decreased from 16 ± 1°C to 12.5 ± 1°C to the same conditions as a previous experiment. Mussels were fed *ad libitum* with *Isochrysis* aff. *galbana* (T-*Iso*) and seawater was renewed daily. Note, one aquarium with environmental and feeding conditions identical to the acclimatisation period was kept during the experiment with 40 mussels, thus they could be taken from the aquarium to replace mussels sampled for toxin analysis during the second period of the experiment (see below).

3.5.2. Micro-algal culture and cell count

A. spinosum strain 3D9 used in this study had been isolated in the North Sea near the coast of Scotland by (Tillmann et al., 2009). The algae were grown in pH-controlled stirred photobioreactors (100 L) operated in chemostat mode at a dilution rate of 0.15 day^{-1} (Jauffrais et al., 2012b). Culture medium was a K modified medium ((Keller et al., 1987), *i.e.* without NH_4Cl and Tris buffer but with Na_2SeO_3 (10^{-8} M)). The following conditions were controlled in the bioreactor: pH was maintained at 7.9 using CO_2 addition, temperature at 18°C, photon flux density at 200 µmol m^{-2} s^{-1}, photoperiod was 16 h of light and 8 h of dark. The algae were then collected into an aerated harvesting tank (300 L), maintained at 18°C until the trial.

Isochrysis aff. *galbana* (CCAP 927/14, T-*Iso*) served as control as it has a cell size similar to *A. spinosum* and is widely used in algal diets for farmed bivalve molluscs (Marchetti et al., 2012a). T-*Iso* was grown in aerated batch culture, in 10 L flat bottomed flasks under the same environmental conditions as for *A. spinosum*, except with f/2 (Guillard, 1975; Guillard and Ryther, 1962) instead of K as culture medium.

Cell concentrations (cell mL^{-1}) and cellular volume (µm^3 mL^{-1}) were determined using a particle counter (Multisizer 3 Coulter counter, Beckman). Subsequently, cell concentration values were used to calibrate and adjust fluorometers during the experiment.

3.5.3. Experimental design

After acclimatisation to laboratory conditions, 22 mussels were randomly chosen for the experiment and placed individually in experimental boxes. At the same time, 30 additionally mussels were also randomly selected to measure condition index (wet tissue weight 2.65 ± 0.86 g per mussel; shell length 45.5 ± 3.8 mm; 27% of dry matter) and 5 others mussels were chosen for initial total flesh toxin analysis.

The experimental design to follow individual physiological responses involved a closed recirculating water system similar to that already described in (Bougrier et al., 2003) and in (Lassus et al., 2007). Briefly, the system is build-up of 22 experimental boxes (0.5 L each), 20 containing one live mussel each and 2 containing one mussel shell each for control. Temperature and salinity were the same as during the acclimatisation period. Seawater flowed through the 22 experimental boxes at 80 mL min^{-1} before returning to the feeding tank (40 L). Cell concentration level was continuously monitored in the feeding tank with a Turner 10AU 072 fluorometer coupled to computer software (LabView). To maintain cell concentration constant, an electrovalve connected to the algal culture tank (25 L) was opened or closed according to cell fluorescence level. The fluorometer was calibrated using a particle counter (Multisizer 3 Coulter counter, Beckman). Cell concentration in the system was routinely checked during the experiment with the particle counter. Mussel clearance and filtration were assessed by comparing the algal concentration at the outlet of the control boxes to algal concentration at the outlet of the experimental boxes. The algal concentration in each box was assessed once every 11 min for 1 min by two other Turner fluorometers coupled to the computer software (Figure 58).

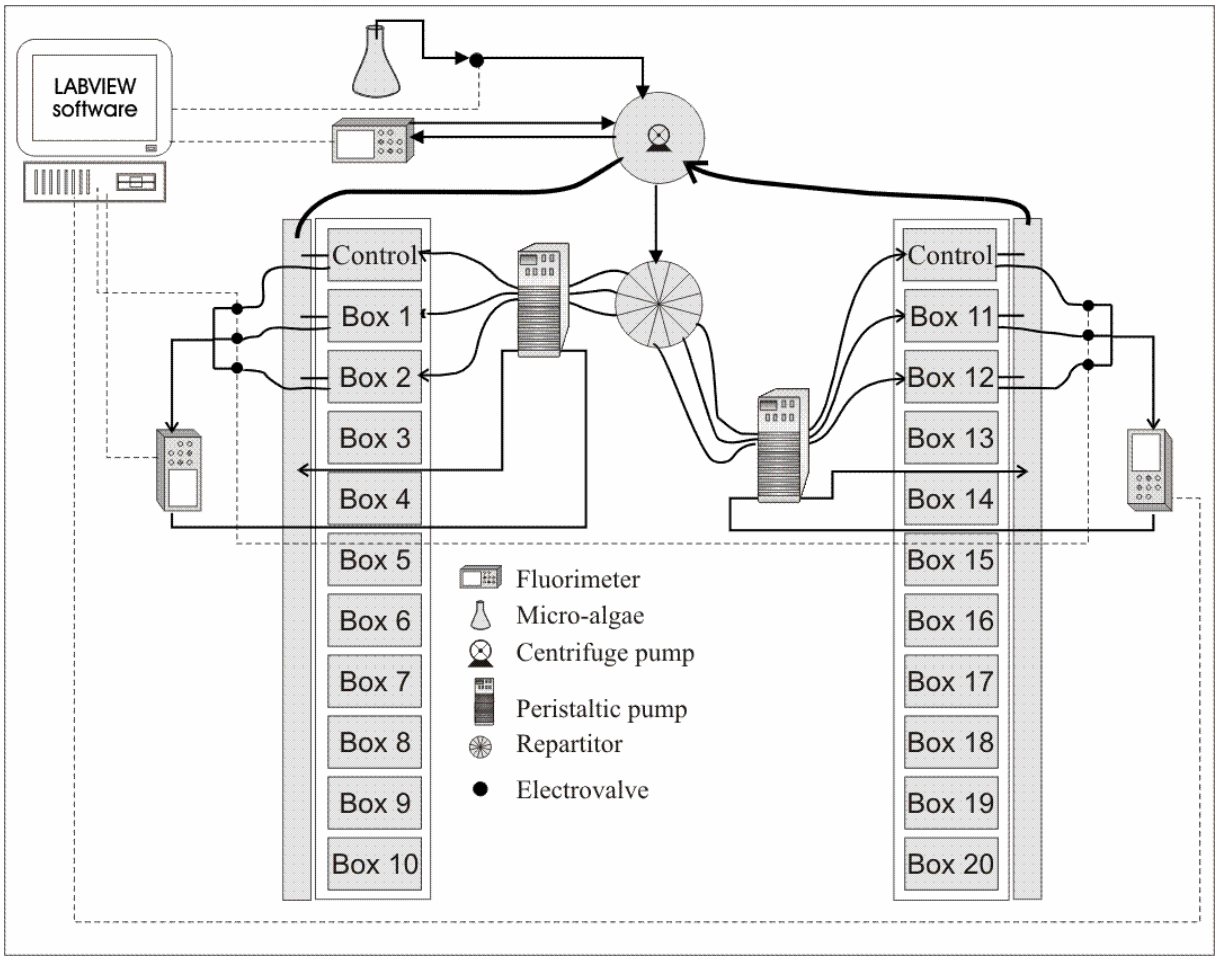

Figure 58. Experimental system to follow the effect of the non-toxic and toxic diets on the feeding behavior of mussels (for clarity: connections 3 to 10 and 13 to 20 were not shown).

In this experimental system, mussels were first exposed to a non-toxic diet of T-*Iso* for 4 days (20,000 cells mL^{-1}, total particular matter (TPM) = 0.88 ± 0.07 mg L^{-1}, particulate organic matter (POM) = 0.61 ± 0.07 mg L^{-1}, organic matter = 69%) for 4 days. After this period, the non-toxic diet was changed to a toxic diet of *A. spinosum* (5,000 cell mL^{-1}, TPM = 1.77 ± 0.14 mg L^{-1}, POM = 1.61 ± 0.14 mg L^{-1}, organic matter = 90%) for another 4 days. To assess TPM and POM, 1L of algal diet were filtered (twice daily) on pre-weighted burned GF/C Whatman filters and rinsed. The filters were then dried (24 h, 60 °C), weighed and combusted (1 h, 450°C) and weighed again to determine by comparison their organic and inorganic matter content.

In order to generate a full time course of AZA accumulation for the four-day period and with sufficient replication given the limited number of 20 experimental boxes, mussels were sampled after 3, 6, 12, 24, 48, and 72 h and replaced by new mussels originating from the aquaria used for acclimatisation to laboratory conditions. We thus obtained three

replicates at 6 h and six replicates for the other time points (3, 12, 24, 48, 72 h) and five replicates at 96 h (Table 28).

Table 28. Box number and time when mussels were sampled and replaced in each experimental box during the feeding period with *A. spinosum* (note; boxes 1-6 were not used to assess physiological parameters due to the larger number of samples and replacements taking place).

Box number	Time (h): sampled + replaced		
	1st sample	2nd sample	3rd sample
1	3	6	18
2	3	6	18
3	3	6	18
4	6	18	
5	6	18	
6	6	18	
7	24	96	
8	24	96	
9	24	96	
10	48	96	
11	48	96	
12	48	96	
13	72	96	
14	72	96	
15	72	96	
16	96		
17	96		
18	96		
19	96		
20	96		

Small amounts of faeces and pseudofaeces were collected with a Pasteur pipette (faeces were rinsed in filtered sea water) and placed on a glass micro-slide for brightfield microscopic observation. These observations allowed to determine the presence of cells in the faeces or pseudofaeces and consequently to assess whether cells were able to pass though the digestive system of mussels during the experiment or were rejected beforehand.

3.5.4. Physiological measurements

During the two feeding periods of this study with T-*Iso* and *A. spinosum*, physiological responses were assessed daily and rates calculated according to the formulas of(Hawkins et al., 1998; Hawkins et al., 1996).

Clearance rate (L h^{-1} gDW^{-1}) was defined as the volume of water cleared of particles per unit of time per dry weight of mussel (CR, *Equation 1*). CR was assessed using comparison of the fluorescence of each algal species at the output of the experimental (C_1) and control boxes (C_0); flow rate (D) and mussel dry weight (DW) were subsequently incorporated.

$$CR\ (L\ h^{-1}\ gDW^{-1}) = ((C_0-C_1)/C_0) \times D\ /DW \qquad Equation\ 1$$

TPM and POM (mg L^{-1}) were assessed daily in the water according to (Hawkins et al., 1996). These values were subsequently used to estimate the total (TFR) and organic (OFR) filtration rate (mg h^{-1} gDW^{-1}, *equation 2* and *3*).

$$TFR\ (mg\ h^{-1}\ gDW^{-1}) = CR \times TPM \qquad Equation\ 2$$

$$OFR\ (mg\ h^{-1}\ gDW^{-1}) = CR \times POM \qquad Equation\ 3$$

Pseudofaeces were observed during feeding periods and they were collected (see below) to estimate total (TPs) and organic (OPs) pseudofaeces production (subsequently expressed in mg h^{-1} gDW^{-1}). TPs and OPs were then used to calculate total (TIR, *equation 4*) and organic (OIR, *Equation 5*) ingestion rates, as well as the accumulation of OPs.

$$TIR\ (mg\ h^{-1}\ gDW^{-1}) = TFR - TPs \qquad Equation\ 4$$

$$OIR\ (mg\ h^{-1}\ gDW^{-1}) = OFR - OPs \qquad Equation\ 5$$

The last parameter assessed for both conditions was the absorption rate (AR) using the organic matter weighed in the faeces (OFs, subsequently expressed in mg h^{-1} gDW^{-1}) as presented in *equation 6*. Faeces and pseudofaeces were carefully sampled twice daily using a pipette, filtered and rinsed on pre-weighted combusted GF/C Whatman filter. They were then dried, weighed and conbusted and weighed again to determine their organic and inorganic matter content.

$$AR\ (mg\ h^{-1}\ gDW^{-1}) = OIR - OFs \qquad equation\ 6$$

Feeding time activity (Gallardo Rodriguez et al.) was also assessed to determine the time during which the mussels were active daily as well as during their time of exposure to *A. spinosum* (3, 6, 12, 24, 48, 72 and 96 h). Mussels were considered active when the difference between the outlet of the control box and the experimental box was above 5% according to (Bougrier et al., 2003).

Note: Boxes 1-6 were not used for to assess CR, TIR, AR, faeces and pseudofaeces measurement during exposure to *A. spinosum* due to the large number of sample and replacement to measure AZA accumulation (see Table 28).

3.5.5. Chemical analysis

3.5.5.1. Reagents

Acetone and methanol (MeOH) were obtained as HPLC grade solvents from JT Baker. Water for analysis was supplied by a Milli-Q integral 3 system (Millipore). Formic acid (Puriss quality) and ammonium formate (Purity for MS) were from Sigma Aldrich. AZA1-3 standards for LC–MS/MS analysis were dilutions of certified AZA1-3 solutions obtained from the National Research Council Canada.

3.5.5.2. A. spinosum

Triplicate samples of *A. spinosum* were taken after each addition of algae in the 25 L algal tanks at Day 4 and 6. The extraction procedure previously optimised for AZA analysis in cultures of *A. spinosum* (Jauffrais et al., 2012a) was used in the present study. Briefly, *A. spinosum* culture (10 mL) was collected and centrifuged (2500 *g*, 20 min, 4°C). The supernatant was discarded and the pellet transferred to an Eppendorf tube (1.5 mL), bath sonicated, and extracted three successive times using acetone (0.5 mL). After centrifugation, the organic phases were gently evaporated, reconstituted in 1 mL methanol, filtered (0.2 µm) and transferred into a HPLC vial for analysis.

3.5.5.3. Mussel

Blue mussels were collected at the different time points of the contamination period (3, 6, 12, 24, 48, 72 and 96 h). Total flesh was carefully removed using a dissection scalpel, drained for 5 min and weighed to record wet weight. Samples were then placed into labelled 50 mL centrifuge tubes and stored at -80°C until extraction. Prior to extraction, the flesh was thowed, methanol (9 mL) was added to each vial and samples were extracted using a high-speed homogeniser (Polytron PT1300D) at 15 000 rpm for 5 min. Samples were then centrifuged at 4500 *g* for 5 min at 4°C, and the supernatant was transferred into 20 mL volumetric flasks. Another 9 mL of methanol was added to the remaining pellet and homogenised again. Centrifugation was repeated at above parameters, and supernatants were transferred into the same 20 mL volumetric flasks. Volumetric flasks were then made up to

the full mark with methanol, the content was homogenised and aliquots were filtered and analysed by LC-MS/MS. (This extraction procedure was adapted according to (Villar-Gonzalez et al., 2011) as explained in (Jauffrais et al., 2012c)).

3.5.5.4. LC-MS/MS analysis

The samples were analysed by LC-MS/MS to quantify AZAs using a HPLC (model UFLCxr, Shimadzu) coupled to a triple quadrupole mass spectrometer (API 4000Qtrap, Applied Biosystems). Separation and LC-MS/MS procedures were the same as used in the previous experiment (Jauffrais et al., 2012c) and were based on a previous study (Rehmann et al., 2008). Quantification was carried out by external calibration against AZA1 to 3, with Analyst 1.5 software (Applied Biosystems), assuming that all analogues had a similar response factor to AZA1, with exception made for AZA2 and -3 for which the respective standards were used. Toxin equivalent factors (TEFs) were also applied to estimate the true toxic potential of mussel samples during the experiment (TEQ = toxin equivalent). AZA1 TEFs applied were equal to 1.8 and 1.4 for AZA2 and -3, respectively (Ofuji et al., 1999a); concerning the relative in vitro potency of AZA6, it appears to be not unlike that of AZA1 (M. Twiner, personal communication). Thus, a provisional TEF of 1 was used for crude estimation of the toxicity represented by AZA6. Consequently, a TEF of 1.4 and 1 were applied to AZA17 and -19, respectively, as AZA17 and -19 transform into -3 and -6 after cooking (McCarron et al., 2009).

3.5.6. Statistical analysis and model

Data were expressed as mean ± standard deviation (SD) and when specified standardised to 1 g dry weight (DW). Statistical analyses were multi-factor ANOVA and differences were considered significant at $P < 0.05$. Statistical analyses were carried out using Statgraphics Centurion XV.I (Statpoint Technologies, INC). Before each ANOVA analysis, normality and equality of variance were tested. Only data on AZA concentration in mussel tissues did not respect equality of the variance, consequently a non-parametric analysis (Kruskal Wallis) was carried out followed by a box and wisker plot to discriminate the differences between values.

3.6. Results

3.6.1. Feeding behaviour responses

3.6.1.1. Clearance rate

The mean clearance rate of mussel fed T-*Iso* was higher (0.84 ± 0.50 L h^{-1} gDW^{-1}) than the CR of mussels fed *A. spinosum* (0.14 ± 0.17 L h^{-1} gDW^{-1}). Concerning mussels fed T-*Iso*, a decrease was observed during the first day of exposure even though mussels were adapted to T-*Iso* before this feeding trial. However, during most of this period, the CR ranged between 0.5 and 1.5 L h^{-1} gDW^{-1} (Figure 59; note that the daily drop in CR is due to the daily seawater renewal). With mussels fed *A. spinosum*, a sharp decrease from 1 to 0.05 L h^{-1} $gDW^{-1}$was observed during the first 12 hours of exposure to *A. spinosum*. Subsequently, the CR stabilised until the next day when seawater was renewed. After water was renewed, CR increased to 0.5 L $h^{-1}$$gDW^{-1}$ for 3 to 5 hours, then decreased again to $0.05 - 0.1$ L h^{-1} gDW^{-1}.

However this daily increase of CR following seawater renewal was only observed during the first 2 days of the exposure to *A. spinosum* and was almost negligible at the end of the experiment.

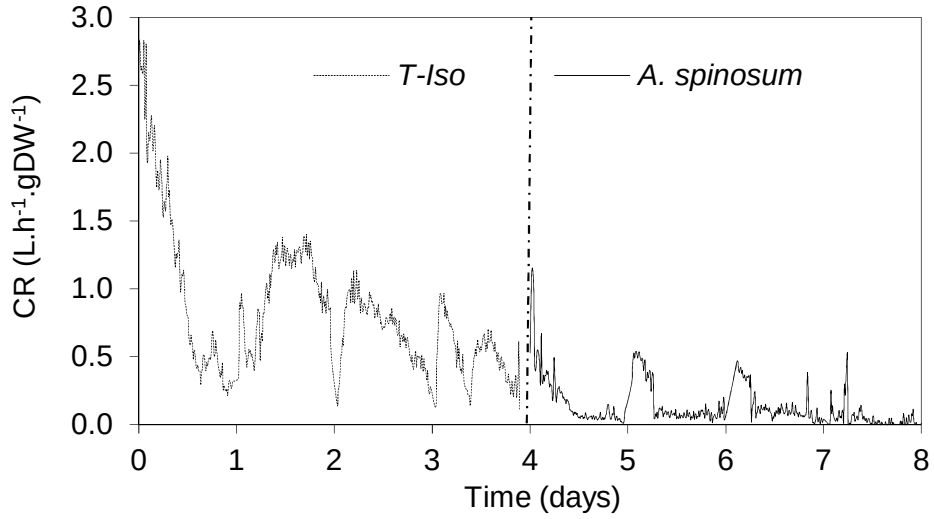

Figure 59. Mussel clearance rate (CR) during the exposure to the non-toxic diet (T-*Iso*) and to the toxic diet (*A. spinosum*). Note that the daily water renewal at every full day coincides with a sudden decrease and/or increase in clearance rate (day 1 to 6).

3.6.1.2. Feeding time activity, organic filtration, ingestion and absorption rates

The daily mussel feeding time activity (Gallardo Rodriguez et al.) (Table 29) was clearly affected by diet. Mussel fed T-*Iso* had a FTA of more than 50%, which significantly decreased when mussels were fed A. *spinosum.* During the exposure to *A. spinosum*, the mussel FTA further decreased from 16 to 2%.

Mussel TFR and TIR presented in Table 29 were closely related and varied as a function of pseudofaeces production. A production of pseudofaeces was observed from the first day to the end of the experiment under the toxic diet, whereas, only small amount of pseudofaeces were observed and weighed when mussels were fed T-*Iso* on day 4 of the experiment, explaining that both TFR and TIR are very similar for the non-toxic diet. The multifactor ANOVA showed that both factors, the type of food and the time (where time refer to the days within a particular diet), had a significant effect: *A. spinosum* significantly decreased mussel TFR and TIR compared to T-*Iso*. The effect of time was also significant. Furthermore, TIR was negative during the last day of feeding *A. spinosum*.

The mussel absorption rate (AR) also underlined the negative effect of the *A. spinosum* diet. Mussels significantly decreased their AR when the diet was switched from T-*Iso* to *A. spinosum*. This lower AR continuously decreased until the end of the experiment to even negative values (Tab. 1). The production of faeces and pseudofaeces were assessed daily. Production of faeces was the only parameter influenced neither by diet nor by time. However, the production of pseudofaeces was significantly affected by the diet supplied. Mussels exposed to *A. spinosum* produced significantly higher amounts of pseudofaeces. Furthermore, mussel pseudofaeces production increased as a function of time ($P = 0.07$).

Table 29. Mussel daily feeding time activity (Gallardo Rodriguez et al.), total filtration rate (TFR), total ingestion rate (TIR) absorption rate (AR), organic faeces (OFs) and organic pseudofaeces (OPs) when fed *A. spinosum* (toxic diet) or T-*Iso* (non-toxic diet) and *P* value of the main effects and interaction.

Time	FTA (%)	TFR ($mg.h^{-1}.gDW^{-1}$)	TIR ($mg.h^{-1}.gDW^{-1}$)	AR ($mg.h^{-1}.gDW^{-1}$)	OFs ($mg.d^{-1}.gDW^{-1}$)	OPs ($mg.d^{-1}.gDW^{-1}$)
T-*Iso*						
1	63.6 ± 19.8	0.91 ± 0.32	0.91 ± 0.32	0.42 ± 0.18	2.34 ± 0.59	-
2	65.3 ± 27.5	0.92 ± 0.52	0.92 ± 0.52	0.47 ± 0.28	1.94 ± 1.11	-
3	56.0 ± 26.6	0.68 ± 0.39	0.68 ± 0.39	0.37 ± 0.24	1.40 ± 0.62	-
4	54.5 ± 25.0	0.43 ± 0.24	0.41 ± 0.25	0.19 ± 0.14	1.29 ± 0.40	0.09 ± 0.24
A. spinosum						
5	16.7 ± 13.3	0.31 ± 0.19	0.20 ± 0.18	0.10 ± 0.16	1.36 ± 0.73	1.04 ± 0.78
6	13.8 ± 10.0	0.27 ± 0.13	0.16 ± 0.14	0.07 ± 0.17	1.61 ± 1.01	0.88 ± 0.45
7	10.3 ± 6.4	0.24 ± 0.06	0.12 ± 0.07	0.00 ± 0.10	1.89 ± 0.92	1.06 ± 0.58
8	2.1 ± 1.20	0.08 ± 0.03	**-0.10 ± 0.06**	**-0.14 ± 0.08**	1.42 ± 0.73	1.47 ± 0.87
ANOVA						
Diet	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.284$	$P < 0.001$
Time	$P = 0.140$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.184$	$P = 0.07$
Diet × Time	$P = 0.913$	$P = 0.291$	$P = 0.422$	$P = 0.927$	$P < 0.05$	$P = 0.312$

Microscopic analysis of mussel faeces revealed that they contained a few ecdysed *A. spinosum* cells while pseudofaeces contained many *A. spinosum* cells, ecdysed cells and empty thecae (Figure 60a and b).

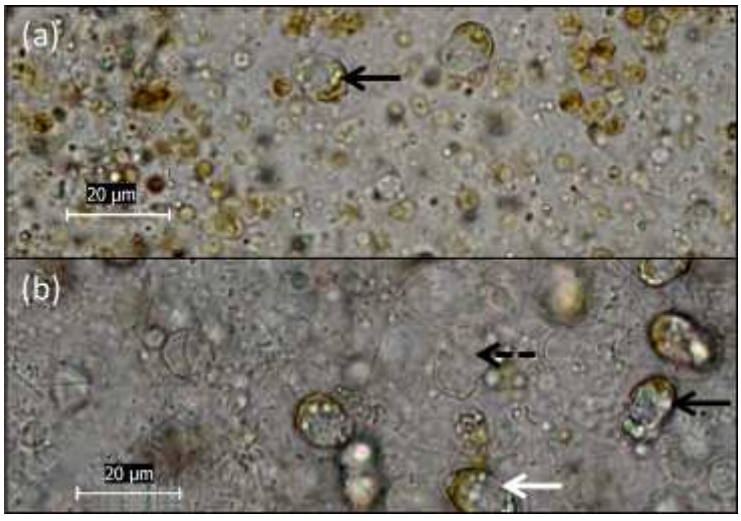

Figure 60. Mussel faeces (a) and pseudofaeces (b) when fed the toxic diet *A. spinosum*. White arrow shows intact *A. spinosum* cell, black arrow ecdysed cell and black dashed arrows empty theca (scale = 20 µm).

3.6.2. AZA analysis

3.6.2.1. A. *spinosum*

AZA cell quota of *A. spinosum* during the contamination period was 93.9 ± 10.4 fg cell^{-1}, with a proportion of 81% of AZA1 (76.6 ± 8.3 fg cell^{-1}) and 19% of AZA2 (17.3 ± 2.2 fg cell^{-1}), the SD represents the variation observed during the four days of the contamination period.

3.6.2.2. AZA accumulation and profile in mussel

A rapid accumulation of AZAs in mussels was observed during the first hours of exposure to *A. spinosum*. Less than six hours were necessary to reach AZA concentration above the regulatory level (160 µg kg^{-1}). However, after this fast AZA accumulation, the concentration stabilised during the experiment as no significant differences were found between values from 6 hours to the end of the study (Figure 61). Mean feeding time activity (Gallardo Rodriguez et al.) of mussels fed *A. spinosum* and used for AZA analysis was initially as high 60% but rapidly declined to about 10% after two days of exposure. Consequently, it is possible to correlate the stagnation in AZA accumulation to the decrease of the FTA (Figure 61).

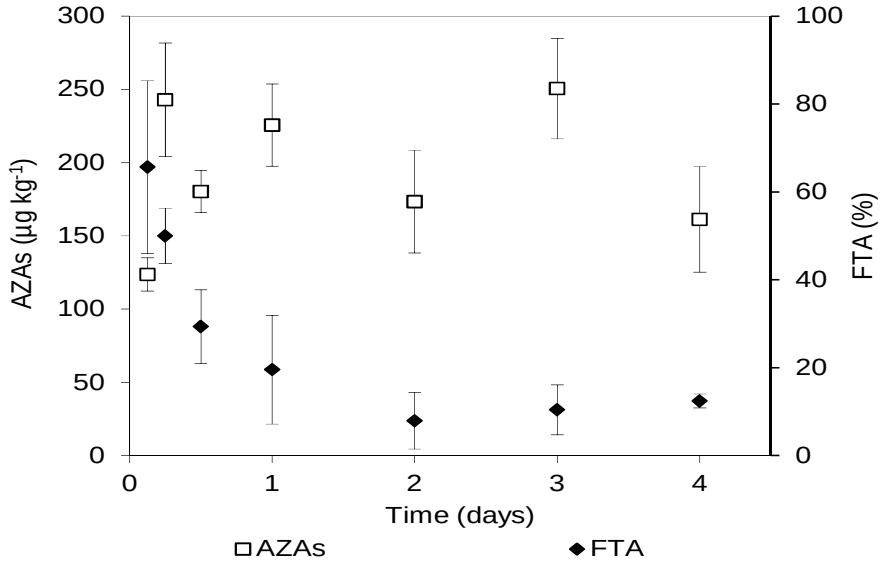

Figure 61. AZA concentrations (µg kg^{-1}) in mussel fed *A. spisnosum* and mean feeding time activity (FTA, %) of the same mussels during their exposure to *A. spinosum.* Error bars represent 6 replicates (with the exception of the 6h time point for which there are only 3 replicates)

Toxin profiles in mussels presented in Table 30 showed a fast AZA biotransformation, as some AZA1 and -2 metabolites were already found after 3 hours. AZA17 was found to

have a proportion almost equal to AZA2, whereas AZA19 and a group of metabolites (AZA7-10) were also detected, but in minor proportion. During the first 6 hours, the number of metabolites as well as their relative proportions did not change. However, as AZA accumulation began to stabilize, the proportion and number of metabolites started to increase. AZA17 reached a maximum proportion of 35% and AZA19 of 6% after two days of contamination, and these two metabolites were the major ones found during the experiment. Other AZAs found were AZA3 to -10 and AZA21, with proportions below 3% for each of them during the study.

Table 30. AZA proportion (%) and concentration (µg kg^{-1}, µg kg^{-1} TEQ) in mussel fed *A. spinosum* as a function of time

	Time (days)	0.125	0.25	0.5	1	2	3	4
(%)	AZA1	66.9	65.5	51.0	47.5	38.6	40.2	45.1
	AZA2	14.3	15.1	13.4	15.0	15.6	14.8	14.1
	AZA3	0.0	0.0	0.0	0.5	0.6	0.7	0.6
	AZA4	0.0	0.0	0.0	0.3	0.2	0.6	0.3
	AZA5	0.0	0.0	0.0	0.3	0.9	0.6	0.8
	AZA6	0.0	0.0	0.1	0.1	0.1	0.1	0.1
	AZA7-10	2.5	2.6	4.1	2.2	1.8	2.2	2.2
	AZA17	13.7	14.0	26.7	29.2	35.0	33.1	29.4
	AZA19	2.5	2.7	4.6	4.9	5.9	5.8	5.2
	AZA21	0.0	0.0	0.0	0.1	1.3	2.1	2.2
µg kg^{-1}	AZAs	123.7	242.8	180.3	225.6	173.3	250.4	161.2
µg kg^{-1} TEQ	AZA1-3	114.7	225.2	135.6	169.7	116.9	169.5	115.2
	AZA1-3, -6, -17, -19	137.3	270.8	181.9	231.9	165.0	235.7	156.6

3.7. Discussion

In a previous study on AZA accumulation in blue mussels fed *A. spinosum*, a fast initial toxin accumulation was followed by a slow further increase or a stabilisation of accumulated toxins. Furthermore, we observed a negative effect of *A. spinosum* on mussel digestive tissues (Jauffrais et al., 2012c). Consequently, we hypothesised that *A. spinosum* is a micro-alga either toxic to mussels or of poor nutritional quality for mussels and thus AZA uptake was limited by a decrease in clearance rate and an increase in the *A. spinosum* rejection via the pseudofaeces. This hypothesis was established following mechanisms contributing to low toxin uptake by oyster (Mafra et al., 2009) and the ability of *M. edulis* to reject bad seston quality due to a high content in inorganic particles (Bayne et al., 1993). Subsequently, this

study was designed to compare feeding responses of mussels when fed non-toxic and toxic diets based on *I.* aff. *galbana* and *A. spinosum,* respectively.

Non-toxic diet based on T-*Iso* had different cell densities (on purpose due to different cell size) and slightly different TPM and cellular volume compared to the toxic diet based on *A. spinosum*. However, both were in the same order of magnitude and at adequate concentration for mussels (Bayne et al., 1987; Bayne et al., 1993; Gosling, 2003), and thus cell size or TPM did not impact our results and conclusion. *Azadinium spinosum* concentration applied in this experiment was similar to concentrations used in a previous one (Jauffrais et al., 2012c); toxin profile of A. *spinosum* with almost 80% AZA1 was also similar to previous experiments (Salas et al. 2011). AZA cell quota has been shown to be quite variable in the few studies presenting results on *A. spinosum* varying from 5 to 50 fg cell^{-1} for cells grown in batch culture (Salas et al., 2011; Tillmann et al., 2009) and up to 100 fg cell^{-1} in stirred photobioreactors at limited or stagnant growth (Jauffrais et al., 2012b). In the present experiment AZA cell quota was approximately 50% higher (94 fg cell^{-1}) compared to our first experiment (59 fg cell^{-1} (Jauffrais et al., 2012c)). In the present experiment, *A. spinosum* was grown under the conditions developed by (Jauffrais et al., 2012b) to improve AZA production, so this elevated AZA cell quota is easy to explain. In any case, this variation could at least partly explain the difference of AZA accumulation between the two studies (400 to 600 µg kg^{-1} in (Jauffrais et al., 2012c) against a maximum of 250 µg kg^{-1} in the present one) and could enhance a possible negative effect of AZA cell quota on AZA uptake by mussels. Effectively, significant inhibitions of the clearance rate are mentioned in *M. edulis*, *C. gigas* and *Mya arenaria* above a threshold of cell toxicity with *Alexandrium* isolates (Bricelj and Shumway, 1998). Generally, comparison to the situation in the field is still hampered by the lack of data on *A. spinosum* concentration and AZA cell quota of natural populations. Nevertheless, the cell concentration corresponds to bloom densities of a species of *Azadinium* in coastal Argentinian waters, where concentration from 0.5 to 9 × 10^6 cells L^{-1} were observed (Akselman and Negri, 2012).

Concerning the effect of toxin cell quota, it has been shown for mussels fed *Alexandrium* species (PST producers) with high and low toxicity that toxin accumulation rate was positively correlated to cell toxicity (Bricelj and Shumway, 1998). Furthermore, the effect of toxic dinoflagellates on feeding activity and PSP accumulation is species-specific (Bricelj and Shumway, 1998): insensitive organisms like mussels can accumulate large amounts of toxins (Bricelj et al., 1990), whereas more sensitive organisms as oyster showed feeding responses that reduce their toxin accumulation (Bardouil et al., 1996; Lassus et al.,

1999; May et al., 2010). Based on maximal concentration of AZA found in shellfish species cultured in Ireland (between 2003 and 2010 analysed by the Irish monitoring program using LC–MS/MS), AZA accumulation seemed also to be species-specific and *M. edulis* was found to accumulate the largest concentration of AZA among the different bivalve molluscs monitored (Hess et al., 2003; Salas et al., 2011).

Furthermore, bivalve mollusc responses to toxic dinoflagellates are known to differ in their CR. Several species showed reduction of filtration rate (e.g. oyster and clams) whereas others even increased their filtration rate (*M. edulis* and *O. edulis*) (Shumway and Cucci, 1987), explaining the higher accumulation of toxins in some species compared to others.

Based on literature, mussels were not supposed to be significantly affected by toxic dinoflagellates. However, our present results confirmed previous experimental observations (Jauffrais et al., 2012c) regards a negative effect of *A. spinosum* on mussels. Consequently, AZA accumulation was limited after a short time of exposure to *A. spinosum* by a sharp decrease in CR and FTA and an increase in pseudofaeces production.

Interestingly, negative ingestion and absorption rate were measured on the last day of the experiment. Negative TIR and AR are closely related to absorption efficiency, a parameter found to be negative when mussels were fed on seston of low quality, *e.g., M. edulis*, *Aulacomya maoriana*, *M. galloprovincialis* and *Perna canaliculus* (Bayne et al., 1987; Gardner, 2002; Helson and Gardner, 2007). This observation was linked to a high inorganic content of seston that led to metabolic faecal loss; however, the seston used in our study (toxic or not) had a high organic content and thus should not negatively affect the absorption efficiency.

These results confirmed our hypothesis and show the negative effect of *A. spinosum* on feeding responses and a possible regulation of AZA uptake by decreasing filtration and increasing pseudofaeces production. It is also important to note that no significant differences were observed in faeces production, even though IR and AR were lower with the second and toxic diet. An explanation might be that the experiment was not long enough to observe differences in faeces production.

Thus, bioactive compounds, either AZAs or other allelochemical substances produced by *A. spinosum* could explain the differences in absorption rate measured between the two diets.

Contrarily to mussels fed PST producers, *i.e., Alexandrium* species (Contreras et al., 2012; Marsden and Shumway, 1992; Navarro and Contreras, 2010; Navarro et al., 2008), *A.*

spinosum had a short term effect as only 6-12 hours were necessary to drastically reduce CR and also a longer term effect, as over the experiment (4 days) no recovery was observed.

Thus, from the present study we can conclude that mussels might reduce AZA accumulation by lowering clearance rate and enhancing pseudofaeces production. However, it cannot be explained by the present experiment if these responses are due to intracellular or dissolved AZA or other allelochemical substances.

Another experimental observation was made during the experiment; after daily water change we found an increase in CR for three to five hours when mussels were fed *A. spinosum* (see Figure 59, day 5 and 6). This time might be necessary to reach a threshold of dissolved AZA detectable by mussels. Such dissolved AZAs or bioactive compounds could derive and accumulate from degrading cells (theca, ecdysed cells and other detritus were microscopically observed in pseudofaeces). Effectively, qualitative analysis of mussel faeces and pseudofaeces during the exposure to *A. spinosum* indicated that *A. spinosum* cells are egested under an ecdysed form by *M. edulis* (Figure 60a and b). Pseudofaeces presented the largest concentration of intact cells, ecdysed cells and theca; however, most of them were embedded in mucus. This was already shown for other toxic dinoflagellates (Hegaret et al., 2008) and the present study provides another indication that relocation of bivalve molluscs during a bloom of *A. spinosum* could be a vector for the transport of *A. spinosum* to shellfish grounds free of this toxic species.

These observations, the negative effect of *A. spinosum* on mussel feeding behaviour and pseudofaeces production that can be potentially linked to a release of dissolved AZAs in the water raised questions on the bioavailability and effect of AZAs (dissolved or not) on other aquatic organisms (e.g. bivalve larvae, plankton, fish embryo). The phenomenon might be of particular importance to the first stage of larval development of aquatic organisms, as AZAs were found to have a teratogenic effect after microinjection on Japanese medaka (*Oryzias latipes*) embryos (Colman et al., 2005). Further studies on bivalve larvae should be undertaken to investigate the effect on this group.

AZA accumulation of mussels fed *A. spinosum* was found to be a fast process since the regulatory limit was reached in less than 6 h, thus confirming our previous results (Jauffrais et al., 2012c; Salas et al., 2011). In the present study the accumulation increased until 6 h and tended to stabilize to ~200 µg kg^{-1}. These toxin contents in blue mussels were within the range of what is frequently found in the Irish monitoring program (Salas et al., 2011). However, mussels did not build up very high levels over the 4 days of exposure to *A.*

spinosum. AZA concentration was found to be low compared to the maximal concentration found in Irish mussels (up to 8970 µg Kg^{-1} AZA1-equiv).

To enhance AZA accumulation by mussels, a first experiment had already been carried out with a 50/50 *A. spinosum*/T-*Iso* mixture (Jauffrais et al., 2012c) to reflect natural conditions. Effectively, for some species (*e.g.: Mya arenaria, Mercenaria mercenaria, C. gigas*) toxin accumulation is influenced by the presence of non-toxic microalgae in a mixed assemblage which enhance or not feeding on toxic dinoflagellates (*e.g., Alexandrium* sp.) (Bardouil et al., 1996; Bricelj and Shumway, 1998; Bricelj et al., 1991). Similar to the mono-specific diet made of *A. spinosum*, AZA accumulation was also observed when mussels were simultaneously fed *A. spinosum* and *Isochrysis* aff. *galbana* (Jauffrais et al., 2012c). However, AZA accumulation was neither enhanced nor decreased by the toxic/non-toxic microalgal assemblage. Nonetheless, a lower ratio of toxic/non-toxic microalgal assemblage (*i.e.* <50/50) might enhance CR and possibly AZA accumulation.

Consequently, to explain differences between high natural AZA concentrations observed in Irish mussels compared to the "low" concentration found in the present experiment other possibilities have to be verified. It is suggested that mussels which are periodically exposed to *A. spinosum* blooms (which is the case for Irish mussels compared to French one) may have developed mechanisms allowing them to digest the micro-algae without adverse effects, thus enhancing their ability to accumulate the toxin. In some ways they could have developed a mechanism of adaptation to the toxins, a similar hypothesis was raised and confirmed for mussels from different locations exposed to *Alexandrium tamarense* (Shumway and Cucci, 1987).

Another possibility could be a more complex trophic interaction. *A. spinosum* is now known to be a potential prey for other heterotrophic plankton such as *Protoperidinium crassipes* or *Favella ehrenbergii* (Krock et al., 2009; Tillmann et al., 2009). It is then possible to imagine an AZA biomagnification through the food web allowing for a faster accumulation by mussel than observed in this study. Nevertheless, the limited amount of time necessary for mussels to reduce its AZA accumulation, by modifying its feeding activity, was sufficient to reach EU regulatory level. This rapid accumulation of AZAs underlines difficulties in monitoring toxins for sanitary purposes and the necessity to predict and to track *A. spinosum* blooms.

Biotransformation of AZA1 and -2 in mussels was also found to be a fast process. These results confirmed the two first feeding experiments carried out during 24 h and over a week of exposure to *A. spinosum*. In both studies rapid bioconversion of AZA1 and -2 into

AZA17, -19 was found, with AZA17 as the major bioconversion product after exposure to *A. spinosum* (Jauffrais et al., 2012c; Salas et al., 2011). In the present study, AZA17 and -19 as well as the group AZA7-10 were already found after 3 h of exposure, with a proportion of AZA17 equal to that of AZA2. Bioconversion of AZA in mussels has to be considered as a fast process, and consequently AZA17 and -19 are two major metabolites that should be taken into account by official monitoring programs in the EU.

3.8. Acknowledgement

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4. Short-term accumulation, biotransformation and tissue distribution of dissolved azaspiracids in blue mussels (*Mytilus edulis*) – A potential risk?

Article en préparation pour publication dans Toxicon: "Jauffrais, T., Kilcoyne, J., Herrenknecht, C., Truquet, P., Séchet, V., Miles, C.O., Hess, P., 201X. Short-term accumulation, biotransformation and tissue distribution of dissolved azaspiracids in blue mussels (*Mytilus edulis*) – A potential risk?".

4.1. Résumé

Les azaspiracides (AZA) ont été découverts suite à la consommation de moules contaminées. La relation directe entre les contaminations de moules avec des AZA et un petit dinoflagellé toxique, *Azadinium spinosum*, a été démontrée récemment. L'organisme ne produit qu'AZA1 et -2 tandis que les autres analogues d'AZA trouvé dans les mollusques bivalves sont formés dans ces coquillages. Cette étude évalue si les moules peuvent accumuler AZA1 et -2 sous forme dissoute, et compare leur profil toxinique ainsi que la distribution des toxines dans les tissus de la moule avec ceux de moules exposées à *A. spinosum* pendant 24 h. Nous avons également évalué la possibilité de produire des métabolites d'AZA1 en contaminant des moules, pendant 4 jours, avec un extrait semi-purifié d'AZA1.

Les moules ont été exposées à des AZA dissous (extrait brut d'AZA1+2, à 7,5 et 0,75 µg d'AZA dissous L^{-1}), à 7,5 µg d'AZA dissous L^{-1} mélangés à une microalgue fourrage (*Isochrysis* affinis *galbana*) ou des cellules d'*A. spinosum* vivantes ou lysées à deux concentrations différentes (1×10^5 et 1×10^4 cell mL^{-1}). Les glandes digestives (GD), les branchies et la chair restante (CR) des moules exposées à ces différents apports d'AZA ont ensuite été disséquées et analysées.

Les moules ont accumulé des AZA au-delà de la limite réglementaire dans presque toutes les conditions (exception : 0,75 µg d'AZA dissous L^{-1}). La distribution des AZA dans les moules varie en fonction des conditions de l'apport en AZA. Les branchies accumulent 42 à 46% et la GD 23 à 24% de la toxine lorsque les moules sont exposées à des AZA dissous, alors que, 3 à 12% de la toxine s'accumulent dans les branchies et 75 à 90% dans la GD lorsque les moules sont exposées à des cellules vivantes d'*A. spinosum*. La formation de

métabolites dans les moules après 4 jours d'exposition avec de l'AZA1 semi-purifié a permis l'obtention d'AZA17 (16,5%) et d'AZA3 (1,7%). Toutefois, les quantités de toxines produites sont trop faibles pour justifier l'utilisation de cette procédure à des fins d'isolement et de production d'AZA17 et -3.

4.2. Abstract

Azaspiracid (AZA) poisoning in humans has been reported following consumption of contaminated shellfish. The direct relation between mussel contaminations and a small toxic dinoflagellate, *Azadinium spinosum*, has been shown recently. The organism produces AZA1 and -2 while AZA3 and other analogues are metabolic products formed in shellfish. We evaluated whether mussels are capable of accumulating AZA1 and -2 from the dissolved phase, and compared their toxin profiles with those of mussels exposed to *A. spinosum* after 24 h of exposure. We also assessed the possibility of producing metabolites using semi-purified AZA1 after 4 days of exposure.

Mussels were exposed to dissolved AZAs (AZA1+2 crude extract) at 7.5 and 0.75 µg L^{-1}, dissolved AZA (7.5 µg L^{-1}) in combination with *Isochrysis* affinis *galbana*, and with lysed and live *A. spinosum* cells at 1×10^5 and 1×10^4 cell mL^{-1}. Digestive glands (DG), gills and remaining flesh (RF) were then dissected and analysed. Mussels accumulated AZA under all conditions and were above the regulatory limit except at the lower levels of dissolved AZAs. The mussel toxin profile was significantly different between conditions. The gills comprised 42-46% and the DG for 23-24% of the concentration using dissolved AZAs, whereas, 3-12% were in the gills and 75-90% in the DG when mussels were exposed to live *A. spinosum*. Metabolite formation through exposure of mussels to semi-purified AZA1 did produce the metabolites AZA17 (16.5%) and AZA3 (1.7%) after 4 days of exposure. However, levels produced were too low to justify using such a procedure for purposes of preparative isolation.

4.3. Key words

Dissolved marine biotoxins, AZA, tissue distribution, bivalve molluscs, liquid chromatography coupled to tandem mass spectrometry

4.4. Introduction

Azaspiracid (AZA) shellfish poisoning (AZP) occurred for the first time in the Netherlands in November 1995. Contaminated mussels (*Mytilus edulis*) cultivated in Ireland (Killary Harbour) were consumed and intoxicated at least eight people (McMahon and Silke, 1996). Three years later the implicated toxin was identified, isolated, structurally defined and named AZA, due to its unique spiro ring assembly (Satake et al., 1998c).

Since the initial discovery, the structure has been corrected following its chemical synthesis (Nicolaou et al., 2006a). AZAs have been found in Europe, Africa and more lately in America and in Japan (Alvarez et al., 2010; Amzil et al., 2008; Furey et al., 2010; Klontz et al., 2009; Magdalena et al., 2003a; Taleb et al., 2006; Twiner et al., 2008; Ueoka et al., 2009), indicating a worldwide phenomenon. More than 30 AZA analogs have been identified ((Rehmann et al., 2008)). Only AZA1 and -2 were actually found in the primary producer (Krock et al., 2009), and it is after 12 years of research that this organism has been isolated and named *A. spinosum* strain 3D9 (Krock et al., 2008; Krock et al., 2009; Tillmann et al., 2009). *A. spinosum* is a small dinoflagellate (12-16 µm length and 7-11 µm width), isolated from the North Sea, near the Scottish coast (Tillmann et al., 2009). Following its description, the genus *Azadinium* was enlarged with a new strain, *A. spinosum* strain SM2 isolated in Ireland (Salas et al., 2011) and with other species, *A. obesum* known not to produce AZAs (Tillmann et al., 2010), and *A. poporum* and *A.* cf. *poporum* (Potvin et al., 2012; Tillmann et al., 2011). These last two species were recently found to produce new AZAs, also observed in *Amphidoma languida*, a species related to *Azadinium* spp., (Krock et al., 2012; Tillmann et al., 2012). Interestingly, an *A.* cf *spinosum* has been observed and described in Argentina; the authors reported a bloom (9.03×10^6 cells L^{-1}) in 1990, however no data on AZAs or reports of intoxications were reported at that time (Akselman and Negri, 2012).

Since this discovery, *A. spinosum* strain 3D9 has been cultured successfully for toxin production in pilot-scale photobioreactors (Jauffrais et al., 2012b), however, the organism only produces AZA1 and -2 while other analogues are metabolic products formed in the shellfish (Krock et al., 2009; McCarron et al., 2009). Effectively, the first demonstration of AZA accumulation in mussels after exposure to *A. spinosum* was made with strain SM2 which showed a quick biotransformation of AZA1 to AZA17 (Salas et al., 2011). This fast biotransformation was subsequently confirmed in a larger scale experiment, where AZA17 and -19 were formally identified as two major analogs that should be regulated (Jauffrais et al., 2012c). However, no purified toxins are yet available for these two analogues. Also, there

is no sustainable way of producing AZA3, an already regulated degradation product of AZA17, other than by complex organic synthesis.

Moreover, a major ecological question is the bioavailability of dissolved marine biotoxins to marine organisms. Effectively, when harmful algal blooms degrade, they release toxins into the dissolved phase (surrounding sea water). Studies were carried out to evaluate toxin accumulation or effects of dissolved toxins on early stages of development of aquatic organisms (Colman et al., 2005; Korpinen et al., 2006; Lefebvre et al., 2005; Liu et al., 2007), on planktonic species (Babica et al., 2007; Bargu et al., 2006), and on adult fish (Cazenave et al., 2005). However, very little is known about the ability of bivalve molluscs to accumulate dissolved marine biotoxins (Liu et al., 2007; Novaczek et al., 1991; Plakas et al., 2002).

Dissolved AZAs, found in waters on the Irish west coast, raised questions on the ability of mussels to accumulate dissolved AZAs *in situ* (Fux et al., 2009). Effectively, transplanted mussels (free of AZAs) followed the same kinetics of adsorption as SPATT (solid phase adsorption toxin tracking) discs but contamination by remaining *A. spinosum* cells cannot be excluded. Furthermore, a recent study (O'Driscoll et al., 2011) on AZA1 biotransformation by mussels showed the ability of mussels to accumulate dissolved AZA when fed a commercial diet for bivalves. However, the authors only focused on biotransformation pathways and did not quantify the absolute concentrations of AZAs accumulated in the mussel tissues thus leaving open the question of the ecological importance of such phenomena. Consequently, the present study was designed to explore the potential degree of accumulation of dissolved AZAs by mussels, to determine the differences in toxin profile of mussel tissues and where AZAs accumulate. The second objective was to expose mussels to a high concentration of dissolved AZA1 to assess if this procedure of accumulation was valuable as an *in vivo* source of metabolites for isolation.

4.5. Material and methods

4.5.1. Collection and maintenance of bivalves

Ifremer: Blue mussels (*M. edulis*) were collected from the West coast of France (Ré Island) in April 2012, a place at that time known to be free of known marine phycotoxins. Mussels were transported to the aquarium facilities, washed and subsequently cleaned of the remaining epibionts. They were placed into two 20 L aerated aquaria, filled with seawater (35 salinity) and maintained at ±16°C (room temperature). The mussels were unfed for 5 days

prior to the experiment to clean their digestive tract of all lingering food. Their average size was above commercial size (44 ± 3 mm), with a wet flesh weight of 1.87 ± 0.21 g. Digestive glands accounted for 25.5%, gills 11.7% and remaining flesh 62.8% of the total mussel weight.

Marine Institute: Blue mussels were obtained from Kelly Shellfish, Kilcolgan (Ireland), a place at that time known to be free of known marine phycotoxins. They were washed and subsequently cleaned of the remaining epibionts and placed into 10 L aquarium tanks containing pretreated aerated seawater. Mussels were above commercial size, sea water was changed daily and mussels were fed a commercial diet for bivalves containing *Isochrysis*, *Pavlova*, *Tetraselmis* and *Thalassiosira weissflogii* (Shellfish diet ® 1800, Reed Mariculture).

4.5.2. Phytoplankton

The *Azadinium spinosum* strain 3D9 was the source of AZA1 and -2 to study AZAs accumulation in mussels. The algae were produced in a stirred photobioreactor (100 L) operated in chemostat mode at a flow rate of 0.2 day^{-1} under the same environmental conditions as detailed in (Jauffrais et al., 2012b).

Isochrysis aff. *galbana* (CCAP 927/14, T-*Iso*), is a non-toxic micro-alga used in aquaculture to feed farmed bivalve molluscs (Marchetti et al., 2012b). This alga was chosen as it is a small motile single cell slightly smaller than *A. spinosum*. T-*Iso* was grown in 10 L batch culture with the same environmental conditions as for *A. spinosum*, but using f/2 medium (Guillard, 1975; Guillard and Ryther, 1962).

Cell concentrations (cells.mL^{-1}) were determined using a particle counter (Multisizer 3 Coulter counter, Beckman) and assessed 6 times in the different conditions using *A. spinosum* and T-*Iso*.

4.5.3. Toxin isolation

Ifremer: Crude extract of *A. spinosum* was used as the source of dissolved AZA1 and -2. The toxin was collected from a 200 L culture of *A. spinosum* maintained in an aerated 300 L transparent cylindroconical tank and produced by two 100 L stirred photobioreactors at a flow rate of 0.2 day^{-1}. Extraction was carried out using tangential flow filtration, followed by AZA adsorption onto HP-20 diaion resin (25 g) in the sonicated algal concentrate (± 1 L). Subsequently the resin was extracted using a glass column with acetone (3 × 50mL), and the extract evaporated and reconstituted in 10 mL of methanol (Jauffrais et al., 2012b). This

concentrated algal crude extract had a concentration of 110 ± 6 µg mL^{-1} with a proportion of 79% of AZA1 and 21% of AZA2.

Marine Institute: Semi-purified AZA1 was obtained from mussel tissue (*M. edulis*) collected in 2005 from Bruckless, Donegal, Ireland following the 6th step of a 7 step isolation procedure as described in (Kilcoyne et al., 2012).

4.5.4. Experimental design

Ifremer: After acclimatisation to the laboratory conditions 120 mussels were placed in 10 L aerated flat bottomed glass flasks (5 in each flasks), filled with 8 L of sea water. Each condition was composed of 3 flasks with 5 mussels and the mussels were exposed to the conditions presented in Table 31 for 24 h.

Table 31. Experimental conditions to which mussels were exposed for 24 h.

	Algae			AZAs extract	Total AZAs[b]
Treatment[a]	Species	Form	Cell mL^{-1}	Vol (mL)	Conc (µg L^{-1})
1	-	-	0	0.541	7.5
2	-	-	0	0.054	0.75
3	T-*Iso*	Live	1×10^6	0.541	7.5
4	*A. spinosum*	Live	1×10^5	-	7.5
5	*A. spinosum*	Lysed	1×10^5	-	7.5
6	*A. spinosum*	Live	1×10^4	-	0.75
7	*A. spinosum*	Lysed	1×10^4	-	0.75
8 (control)	-	-	0	-[c]	0

[a] treatment in triplicate, [b]AZA1 + AZA2 (76:24). [c]0.541 mL MeOH was added

Live and lysed *A. spinosum* cells were obtained from a culture in a bioreactor at steady state: $2.15 \times 10^5 \pm 3 \times 10^3$ cell mL^{-1} and an AZA cell quota of 75.4 ± 0.8 fg.$cell^{-1}$, the AZA proportion was 76% of AZA1 and 24% of AZA2. The adequate concentrations were obtained by dilution with sea water. For condition 5 and 7, 3 times 5 L of culture (*A. spinosum*) were sonicated (pulse mode, 30 min in ice, maximum amplitude, Bioblock Scientific, Vibra-cell 75115), and a sample was checked to ensure total cellular lysis.

Marine Institute: Acclimatized mussels (3) were transferred into a 5 L conical flask, containing 3 L of pretreated seawater, to which Shellfish diet ® 1800 and 100 µl of semi-purified AZA1 were added, to give a final concentration of 33 µg.L^{-1}.

4.5.5. Extraction procedures

4.5.5.1. Reagents

Ifremer: Acetone and methanol were obtained as HPLC grade solvents from JT Baker. Milli-Q water for the HPLC mobile phase was supplied by a Milli-Q integral 3 system (Millipore). Formic acid (Puriss quality) and ammonium formate (Purity for MS) were from Sigma Aldrich. AZA1, -2 and -3 certified reference materials (CRMs) were obtained from the NRC, Certified Reference Material Program (Halifax, NS, Canada).

Marine Institute: All solvents (pestican grade) were purchased from Labscan (Dublin, Ireland). Ammonium formate (reagent grade) and formic acid were purchased from Sigma Aldrich (Steinheim, Germany). AZA1, -2 and -3 certified reference materials (CRMs) were obtained from the NRC, Certified Reference Material Program (Halifax, NS, Canada).

4.5.5.2. *Azadinium spinosum*

Triplicate samples of *A. spinosum* were taken from the harvesting tank prior to mussel exposure and extracted according to (Jauffrais et al., 2012a). Briefly, aliquots (10 mL) of *A. spinosum* cultures were collected and centrifuged (2500 *g*, 4°C, 20 min). The pellet was re-suspended with 0.5 mL of acetone/H_2O (9/1, *v/v*), transferred to an Eppendorf tube and sonicated. After sonication, the aliquot was centrifuged. The supernatant was transferred into a 5 mL glass tube and gently evaporated under nitrogen on a heating block (35°C). This process was repeated three times in total. After evaporation of supernatants, the residue was reconstituted in 1 mL methanol filtered and analyzed by LC-MS/MS.

4.5.5.3. Mussel tissues

Ifremer: At the end of the 24 h exposure mussels were opened to allow mantle fluid to drain, gills were carefully dissected with scissors, the digestive gland was then separated from the mussel tissues and subsequently the remaining flesh was detached from the shell. The different tissues from the five mussels of each flask were then drained for 5 min and placed in a pre-weighed 50 mL centrifuge tube and weighed to measure wet weight. They were then labeled and stored at -80°C until extraction.

Prior to extraction, the tissues were thawed, methanol (9 mL) was added to each vial and samples were extracted using a high-speed homogenizer (Polytron PT1300D) at 15 000 rpm for 5 min. Samples were centrifuged at 4500 *g* for 5 min at 4°C, and the supernatant was transferred into 20 mL volumetric flasks. Another 9 mL of methanol was

added to the remaining pellet and homogenized again. Centrifugation was repeated at above parameters, and supernatants were transferred into the same 20 mL volumetric flasks. Volumetric flasks were then made up to the mark using methanol, extracts were filtered and analyzed by LC-MS/MS (this extraction procedure was adapted according to (Villar-Gonzalez et al., 2011) as explained in (Jauffrais et al., 2012c)) .

Marine Institute: After 4 days of exposure mussels were removed from the tank, opened allowing mantle fluid to drain, shucked and weighed. The mussel tissue was transferred into a Waring blender and homogenized for 1 min. 1 g of the homogenized tissue was weighed and extracted twice (Ultra turrax, IKA-Werke T25 at 11 000 rpm) for 1 min with methanol (2 x 9 mL). Extracts were centrifuged (3 950 *g*) for 5 min and the supernatant decanted into 25 mL volumetric flasks which were brought to volume with methanol. Samples were filtered (Millipore PVDF, 0.45 µm) prior to analysis by LC-MS/MS.

4.5.6. Solid phase extraction

The concentration of dissolved AZAs was determined using SPE Oasis HLB cartridges, (3 cc, 100 mg). Cartridges were activated with methanol (5 mL) and washed with a solution of water–methanol (9:1 v/v, 3 mL). The sample (5 mL) was loaded dropwise. Once loaded, the cartridge was washed with a solution of water–methanol (9:1 v/v, 5 mL). The sample was eluted with 5 mL of methanol into a glass tube and analyzed by LC-MS/MS (Marine Institute).

4.5.7. LC-MS/MS analysis

Ifremer: The samples were analyzed by LC-MS/MS to quantify AZAs using a HPLC (model UFLCxr, Shimadzu) coupled to a triple quadrupole mass spectrometer (API 4000Qtrap, Applied Biosystems). Separation and LC-MS/MS procedures were the same as used in a previous experiment (Jauffrais et al., 2012c) and based on previous studies (Rehmann et al., 2008). External calibration against AZA1 to -3 were used for quantification using Analyst 1.5 software (Applied Biosystems), assuming that all analogues had a similar response factor as AZA1, except for AZA2 and -3 for which the respective standards were used.

Marine Institute: Samples were analysed on a Waters 2695 LC coupled to a Micromass triple-stage quadrupole Ultima operated in multiple reaction monitoring (MRM) mode, with the following transitions monitored: AZA1 *m/z* 842.5→654.4 and 842.5→672.4,

AZA3 828.5→640.4 and 828.5→658.4, AZA17 872.5→640.5 and 872.5→658.4. The cone voltage was 60 V and the collision voltage was 40 V, the cone and desolvation gas flows were set at 100 and 800 L/h, respectively, and the source temperature was 150 °C.

Binary gradient elution was used, with phase A consisting of water and phase B of 95% acetonitrile in water (both containing 2 mM ammonium formate and 50 mM formic acid). Chromatography was performed with a Hypersil BDS C8 column (50 × 2.1 mm, 3 µm, with a 10 × 2.1 mm guard column of the same stationary phase) (Thermo Scientific). The gradient was from 30% B, to 90% B over 8 min at 0.25 $mL.min^{-1}$, held for 5 min, then held at 100% B at 0.4 $mL.min^{-1}$ for 5 min, and returned to the initial conditions and held for 4 min to equilibrate the system. The injection volume was 5 µL and the column and sample temperatures were 25°C and 6°C, respectively.

4.6. Results

4.6.1. AZA accumulation and distribution in blue mussels

In all toxic conditions, LC-MS/MS analyses showed the presence of AZAs in the three different mussel tissues dissected (digestive gland, gills and remaining flesh) after the 24 h period of exposure. Effectively, almost all conditions resulted in AZA concentrations above the regulatory limit (160 $\mu g\ kg^{-1}$). The accumulation ranged from 241 to 763 $\mu g\ kg^{-1}$, with the exception of the second condition (dissolved AZAs at 0.75 $\mu g.L^{-1}$) where the total AZA concentration was equal to 95 $\mu g\ kg^{-1}$ (Table 32).

As expected, the highest concentrations were obtained using conditions with a high concentration of AZAs (7.5 $\mu g.L^{-1}$). With exposure to the live *A. spinosum* resulting in the highest accumulation (757 and 404 $\mu g\ kg^{-1}$ at 1×10^5 and 1×10^4 cell mL^{-1} respectively) compared with the other conditions tested (Table 32).

Table 32. AZA metabolite composition (%) for each tissue sample,[a] and total AZA concentrations[b] in mussel tissues (µg kg^{-1}).

	Tissue	AZA metabolite profile (% of total[b]) by tissue							Conc AZAs (µg kg^{-1})[b]	
		AZA1	AZA2	AZA3	AZA6	AZA7-8	AZA17	AZA19	By Tissue	Mussel[c]
Treatment 1	**DG**	55	20	2	0	0	19	4	458 ± 91	
	Gills	25	11	4	0	0	50	10	1931 ± 64	530
	RF	27	10	5	2	1	47	9	307 ± 25	
Treatment 2	**DG**	65	20	0	0	0	13	2	91 ± 26	
	Gills	39	14	3	0	0	37	7	368 ± 52	94
	RF	43	13	0	0	1	38	7	45 ± 1	
Treatment 3	**DG**	67	24	1	0	0	6	1	947 ± 81	
	Gills	25	9	4	0	1	50	12	498 ± 147	557
	RF	23	8	5	1	0	52	11	413 ± 31	
Treatment 4	**DG**	54	22	2	0	2	17	3	1910 ± 414	
	Gills	34	14	4	0	1	40	8	876 ± 58	694
	RF	37	11	3	0	2	39	7	167 ± 29	
Treatment 5	**DG**	67	21	1	0	1	9	1	1179 ± 237	
	Gills	38	13	4	0	0	38	7	771 ± 41	498
	RF	43	12	3	0	1	35	6	172 ± 39	
Treatment 6	**DG**	60	23	1	0	1	12	2	1654 ± 221	
	Gills	41	14	3	0	0	35	6	147 ± 16	464
	RF	52	14	0	0	0	32	2	41 ± 7	
Treatment 7	**DG**	72	21	1	0	0	6	1	802 ± 119	
	Gills	44	16	2	0	0	32	6	196 ± 28	249
	RF	57	15	0	0	0	28	0	35 ± 1	
Treatment 8 (Control)	**DG**	-	-	-	-	-	-	-	-	
	Gills	-	-	-	-	-	-	-	-	-
	RF	-	-	-	-	-	-	-	-	

[a]Major metabolites (≥20%) are highlighted in grey; DG = Digestive gland, RF = remaining flesh.

[b]Total AZAs = AZA1 + AZA2+ AZA3 + AZA6 + AZA7 +AZA8 +AZA17 + AZA19

[c]Calculated from the tissue concentrations by assuming a mussel composition of 25.5% DG, 11.7% gills, and 62.8% remaining flesh, by weight

Interestingly, at the higher *Azadinium* concentration, mussels only consumed 1/5 of the total live *A. spinosum* cells (1×10^5 cell mL^{-1}) over 24 h, whereas, at the lower concentration (1×10^4 cell mL^{-1}), all cells were filtered in less than 5 h. Furthermore, mussels exposed to dissolved AZAs with T-*Iso* consumed 3/4 of the available alga (Figure 62).

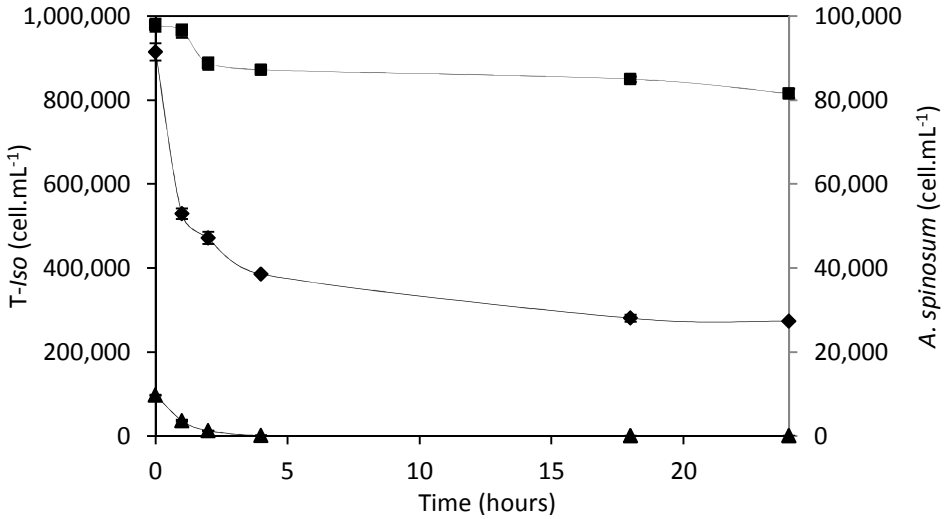


Figure 62. Concentration of T-*Iso* (Treatment 3 (◆)) and *A. spinosum* (Treatments 4 (■) and 6 (▲)) during exposure to *M. edulis*.

Differences in toxin distribution across tissues were observed between conditions at either high or low toxin level (Figure 63).

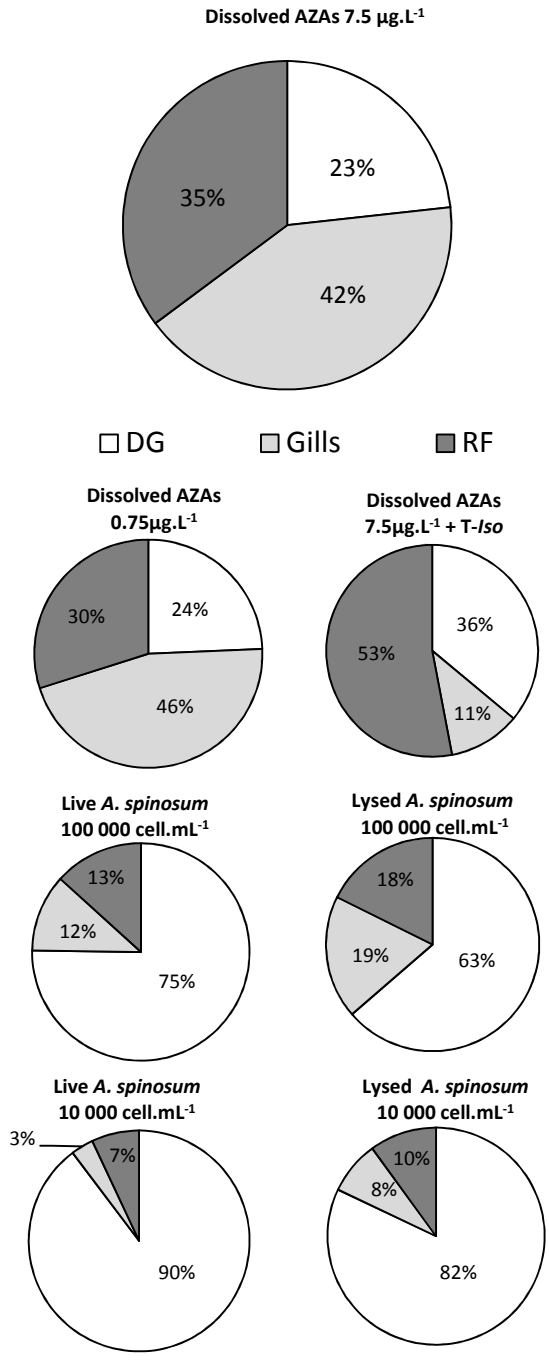


Figure 63. AZA tissue distribution in mussels (digestive gland (DG), remaining flesh (RF) and gills) after 24 h of exposure to dissolved AZAs, live *A. spinosum* or lysed *A. spinosum* at different concentrations.

The amount of toxin did not have a significant impact on AZA tissue distribution in mussels. However, when the mussels were exposed to dissolved AZAs, most of the toxins were detected in the gills (42-46%) and in the remaining flesh (30-35%), whereas the

digestive gland only accounted for 23 to 24% of the total AZAs accumulated by mussels. In the case of condition 3, containing dissolved AZA plus non-toxic food, a 2-fold higher concentration of AZA was observed in the digestive gland, compared to condition 1 (without algae), even though the total amount absorbed into mussels tissues was virtually identical (Table 32). A yet higher concentration in the digestive gland was observed for condition 5 (with lysed *Azadinium* cells). The highest concentrations were observed in digestive glands of mussels exposed to live *A. spinosum* cells. The same trend is observed when examining the percentages of toxin accumulated in digestive gland compared to other tissues, with live *Azadinium* cells > lysed cells > dissolved plus non-toxic cells > dissolved toxins.

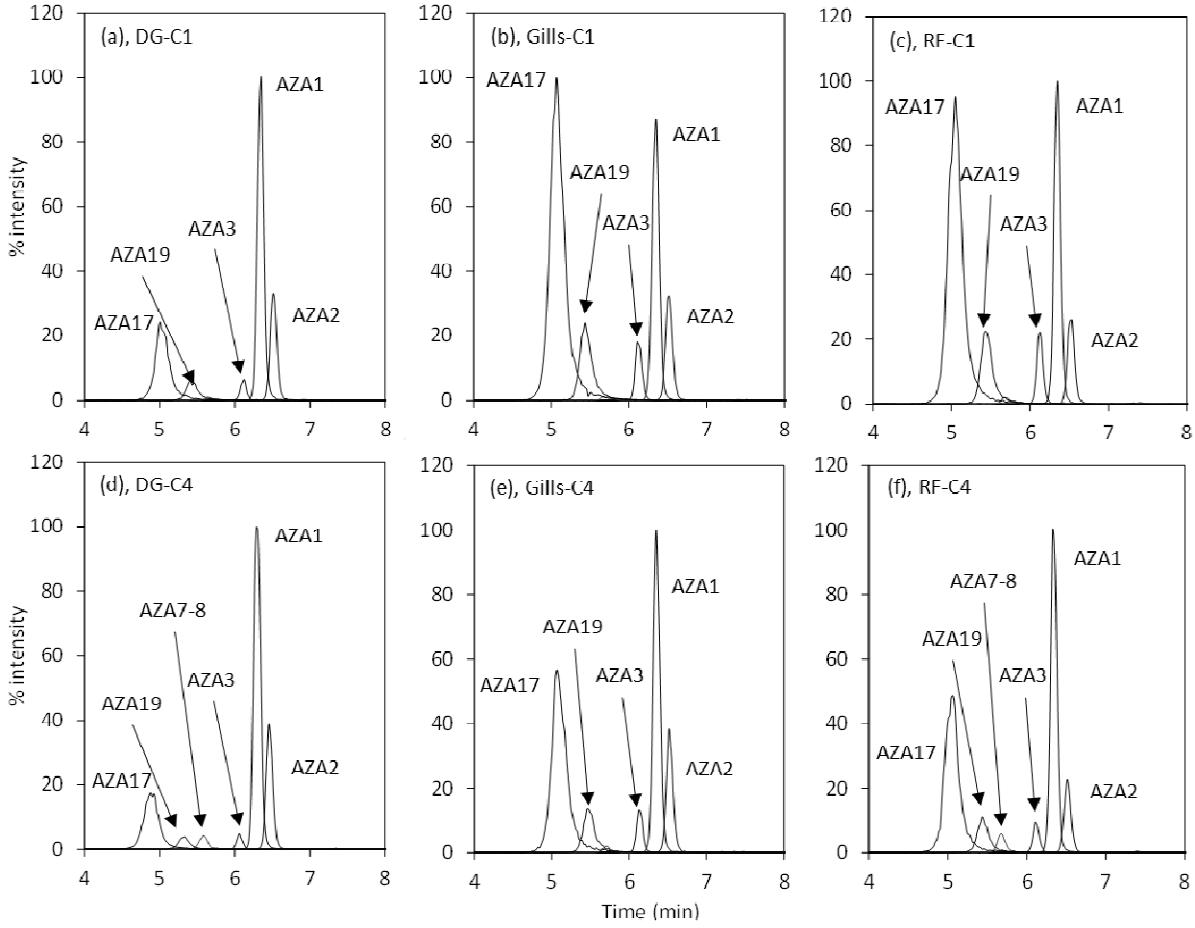
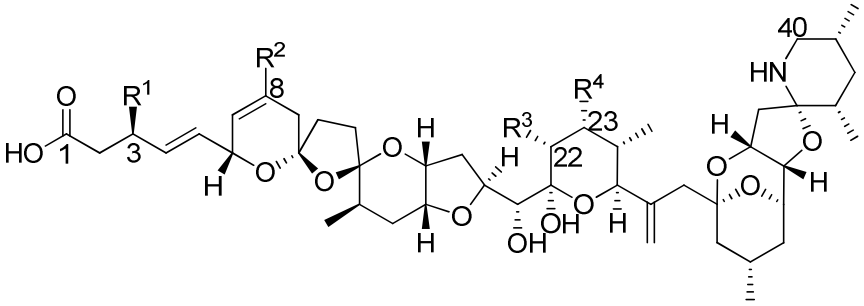


Figure 64. LC-MS/MS chromatograms (method A) of mussel tissues: (a) DG, (b) Gills, and (c) RF after mussel exposure to dissolved AZAs (7.5 µg mL^{-1}) (Treatment 1); and (d) DG, (e) Gills, and (f) after mussel exposure to live *A. spinosum* cells (1×10^5 cell mL^{-1}) (Treatment 4).

4.6.2. AZA biotransformations

In most cases, toxin profiles contained AZA1-3, AZA7-8, AZA17 and -19 (Table 32, Figure 64), and for all tissues AZA3, AZA7-8 and AZA19 represented a proportion below 20%.

Considering the toxin profile of each tissue after 24 h of exposure to all conditions, the digestive gland always had AZA1 and -2 as major AZAs (>20%), whereas in the gills and remaining flesh the main AZAs were AZA1 and -17, with the proportion of AZA17 often greater or comparable to AZA1 (Figure 65). As for AZA1 and -17, in these two tissues (gills and RF) the proportion of AZA2 and -19 were also similar.



	R1 (C3)	R2 (C8)	R3 (C22)	R4 (C23)	$[M + H]^+$ *m/z*
Azaspiracid-1	**H**	**H**	**CH_3**	**H**	**842.5**
Azaspiracid-2	**H**	**CH_3**	**CH_3**	**H**	**856.5**
Azaspiracid-3	H	H	H	H	828.5
Azaspiracid-6	H	CH_3	H	H	842.5
Azaspiracid-7	OH	H	CH_3	H	858.5
Azaspiracid-8	H	H	CH_3	OH	858.5
Azaspiracid-17	H	H	CO_2H	H	872.5
Azaspiracid-19	H	CH_3	CO_2H	H	886.5

Figure 65. Azaspiracid (AZA) structures and mass-to-charge ratios (*m/z*) for the molecular ions $[M + H]^+$ detected in *A. spinosum* and in mussels (*M. edulis*). Toxins found in *A. spinosum* are shown in bold text.

The second objective was to evaluate the feasibility to produce AZA metabolites, using mussels exposed to dissolved semi-purified AZA1. The AZA mass balance of this experiment (Table 33) showed an AZA recovery of 60% with AZA1 accounting for 37%, AZA17 for 19% and AZA3 for 4%. AZAs found in mussels after four days of exposure to AZA1 represented 29% of the initial amount of AZAs, with AZA17 comprising the biggest proportion. At this high level of exposure to AZA1 (33 µg.L^{-1}), a release of AZA3 into the water was observed.

Table 33. AZA mass balance (µg) after exposure of mussels to semi-purified AZA1

	Amount (µg)				%
	AZA1	AZA17	AZA3	**Total AZAs**	
Dissolved AZA (day 0)	98.4	-	-	98.4	100
Dissolved AZA (day 1)	55.1	1.0	1.1	57.2	58.1
Dissolved AZA (day 2.5)	37.8	1.7	1.7	41.2	41.9
Dissolved AZA (day 4)	26.3	2.3	2.0	30.6	31.1
Mussels	10.3	16.8	1.6	28.7	29.2
Total recovered	36.6	19.1	3.6	59.3	**60.3**

4.7. Discussion

Dissolved AZAs as well as other lipophilic toxins were found in water using passive samplers on the West coast of Ireland (Fux et al., 2009), raising questions on the bioavailability of dissolved AZAs to shellfish or other aquatic organisms. Unfortunately, the passive sampling method used in that study was qualitative and could not estimate actual AZA concentrations in sea water. In the present study two levels of cellular concentrations were investigated. These conditions would correspond to a bloom of 1×10^5 cell mL^{-1} of *A. spinosum* and a more probable scenario of a bloom of 1×10^4 cell mL^{-1} of *A. spinosum,* as reported in Argentina (Akselman and Negri, 2012). However, very little data are available on the cell quota of *A. spinosum in situ*. Thus, we used a cellular quota currently obtained from *A. spinosum* culture, however, these results are only indicative and used to evaluate, where AZA uptake or accumulation occur, whether dissolved AZAs released during decaying blooms of *A. spinosum* could be relevant to the shellfish industry or play a role in ecological interactions with other aquatic organisms.

Thus, mussels were exposed to AZAs by oral ingestion of *A. spinosum* cells and to dissolved AZAs by immersion as possible ways of contamination.

Results indicated that AZA accumulation in mussels was as for live *A. spinosum* (Jauffrais et al., 2012c; Salas et al., 2011) also possible using dissolved AZAs only, dissolved AZAs with a non–toxic alga, and using lysed *A. spinosum* cells. To our knowledge, this study is the first experiment that quantitatively assesses the direct accumulation of dissolved AZAs by mussels without any source of food.

When AZAs were dissolved most of the toxin was found in gills and remaining flesh, whereas the toxin was mainly found in the digestive gland when it was within the *A. spinosum*

cells (Figure 63). This last result confirmed previous AZA profile distribution found in mussel in similar condition (73% DG, 8% gills and 19% RF (Jauffrais et al., 2012c))

In addition to the digestive uptake route previously shown (Jauffrais et al., 2012c; Salas et al., 2011), an uptake from the dissolved phase through the gills should be considered as a plausible route of accumulation. These observations have also been shown with fish exposed to microcystin-RR (Cazenave et al., 2005). Furthermore, analogue ratios found in the present study suggest that AZA transformations in gills are faster than in digestive gland (Figure 64). However, no data is available on transfer of AZAs across mussel tissues. Consequently, further studies using different organisms, e.g. fish, could facilitate to confirm whether AZA uptake through the gills during ventilation and filtration processes leads to systemic distribution of AZAs.

A similar experiment was performed to study AZA bioconversion in mussels, however, mussels were solely exposed to dissolved AZAs in combination with a non-toxic microalgal diet (O'Driscoll et al., 2011). These authors also observed larger AZA accumulation in gills and remaining tissues than in digestive glands; nonetheless, it is difficult to compare further due to the absence of quantitative data and differences in experimental design. Also, these authors found an unusual toxin profile (*i.e.* a large amount of AZA3), compared to the literature (Fux et al., 2009; Jauffrais et al., 2012c; McCarron et al., 2009; Salas et al., 2011).

Toxin profiles of dissected tissues were also different (Table 32 and Figure 64). In the digestive glands the two major toxins were AZA1 and -2, whereas in the gills and remaining flesh they were AZA17 and -1. Furthermore, the gills and the remaining flesh always showed a higher proportion of AZA1 and -2 metabolites compared to the digestive glands. This confirms previous results (Jauffrais et al., 2012c) and raised questions about the role of the different mussel tissues in AZA biotransformation.

Concerning the production of metabolites via exposure of mussels to dissolved semi-purified AZA1, only 17% of the original AZA1 sample was converted to AZA17. Combined with the fact that the mussels have a limited ability to accumulate these toxins under such conditions would render such means for producing sufficiently contaminated material for isolation purposes inefficient.

Additionally, a significant proportion (40%) of toxin was not accounted for, suggesting additional, unknown transformation processes occurring within the shellfish. Following the 4 day exposure period, low levels (~ 2% of the original AZA1 added to the tank) of both AZA17 and AZA3 were detected in the seawater, suggesting that, once excreted

by the shellfish, these toxins can then be transported into the surrounding aqueous environments.

4.8. Conclusions

The present study shows that dissolved AZAs were bioavailable for mussels and that AZA accumulation may reach concentrations above the regulatory limit. Differences were observed in the distribution of toxins in the shellfish which depended on whether the shellfish were exposed to dissolved AZAs or to live or lysed *A. spinosum*. Furthermore, the different AZA profiles between tissues indicate that AZA uptake occurs in the digestive gland during feeding or through the gills during respiratory and filtration activities. This last observation raises questions on the mechanisms involved in the uptake of AZAs through the gills and about the potential for subsequent re-distribution to other organs.

The dissolved AZA bioavailability shown for mussels also raises questions regards possible effects on other aquatic organisms.

The feeding of mussels with semi-pure AZA1 resulted in the formation of AZA17 (17%), however, 40% of the original AZA1 was unaccounted for at the end of the exposure period. Hence, further studies should investigate losses and the existence of yet unknown metabolic transformations.

Further work will need to be performed to determine what other factors are influencing the ability of mussels to accumulate extremely high levels of toxin in the field, which so far cannot be reproduced in the laboratory. In particular, ongoing research is investigating the hypothesis of uptake from different organisms predating on *Azadinium*.

4.9. Acknowledgment

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Conclusions et perspectives

Cette thèse présentait trois objectifs : (1) la mise au point d'une procédure d'analyse des azaspiracides (AZA) et le développement de la production d'AZA à une échelle pilote à partir de la culture en continu d'*A. spinosum* ; (2) l'étude de l'influence des facteurs environnementaux et nutritionnels sur la croissance et la production toxinique d'*A. spinosum* ; (3) une fois la culture d'*A. spinosum* maitrisée, l'étude de l'accumulation, de la détoxification et de la biotransformation des AZA par les moules, en utilisant différentes approches.

Les résultats des travaux réalisés et correspondant a ces objectifs ont été présentés dans trois chapitres. Le premier a porté sur le développement et la validation de méthodes d'analyse et de productions des AZA, le second sur l'écophysiologie d'*A. spinosum* et le dernier sur les interactions entre *A. spinosum*, les AZA et la moule.

Analyse et production d'AZA

Les résultats obtenus dans ce chapitre soulignent l'importance d'étudier consciencieusement la préparation des échantillons, les procédures d'extraction, le choix du solvant d'extraction et de solubilisation des échantillons et les effets de matrice lors du développement d'une méthode d'analyse.

Sur la base des résultats de cette étude, les procédures suivantes sont recommandées pour l'analyse d'AZA-1 et -2 à partir de cultures d'*A. spinosum* :

- Séparer immédiatement les cellules du milieu de culture par centrifugation après prélèvement de l'échantillon.
- Extraire les AZA avec de l'acétone ou de l'acétonitrile; l'acétone semble être le solvant le plus approprié, car il réduit à la fois la formation d'artefact d'extraction et est facile à utiliser (évaporation aisée et faible toxicité). Le méthanol est inapproprié pour l'extraction des AZA à partir d'*A. spinosum*, en raison de la formation d'artefacts d'extraction (méthyle-esters d'AZA1 et -2).
- Deux à trois extractions successives à l'acétone sont proposées pour assurer un rendement d'extraction optimal.

Il n'a pas été observé d'effets de matrice significatifs lors de l'analyse CL-SM/SM après extraction à l'acétone ou au MeOH dans les conditions testées.

En outre, les expériences réalisées afin de comprendre et d'expliquer la formation et la structure des analogues d'AZA méthylés ont conduit à la description de deux méthyle-esters d'AZA et à la correction de la structure chimique des AZA29 à 32.

La seconde partie de ce chapitre a démontré la faisabilité d'une production durable d'AZA1 et -2 à partir de la culture en continu d'*A. spinosum*. En effet, lorsque deux photobioréacteurs sont connectés en série, les concentrations cellulaires atteignent 190×10^3 et 210×10^3 cell mL^{-1} à l'état stationnaire, dans les bioréacteurs 1 et 2, respectivement. Le quota cellulaire d'AZA diminue lorsque le taux de dilution augmente de 0,15 à 0,3 $jour^{-1}$, avec une production toxinique optimale à 0,25 $jour^{-1}$. Après optimisation, les procédures d'extraction en phase solide ont permis la récupération de 79 ± 9% des AZA.

À partir des extraits d'*A. spinosum* ainsi obtenus, la procédure d'isolement précédemment développée sur des glandes digestives de moules a été optimisée et adaptée pour isoler les AZA, de telle sorte que seulement quatre étapes de purification ont été nécessaires pour obtenir de l'AZA1 et -2 purifiés. Un rendement de purification de plus de 70% a été atteint et cette procédure a permis d'isoler 9,3 mg d'AZA1 et 2,2 mg d'AZA2 à partir de 1200 L de culture avec une pureté supérieure à 95%.

Ces résultats laissent envisager la possibilité de répondre à la demande accrue en AZA des organismes de surveillance, pour l'analyse chimique (CL-SM/SM) des phycotoxines marines dans les produits de la mer. Par ailleurs la possibilité d'obtenir AZA1 et -2, à partir de culture d'*A. spinosum*, va permettre la réalisation de nouveaux tests en toxicologie, pour mieux apprécier les effets de ce groupe de toxines sur l'homme et éventuellement comprendre leur mécanisme d'action. Cependant, la production d'AZA à partir d'*A. spinosum* ne permet pas la production des métabolites d'AZA formés dans les moules (ex : AZA3 et -17). La production de ces analogues reste encore, jusqu'à présent, à développer.

Cette seconde études a aussi permis d'isoler de nouvelles molécules affiliées aux AZA (précurseur biosynthétiques ?) dans *A. spinosum* et la phase aqueuse qui l'entoure (de masse 716 et 816 Da). Cette quantité de toxine va permettre de déterminer la structure chimique de nouvelles toxines affiliées aux AZA et d'évaluer leur toxicité.

Écophysiologie d'*Azadinium spinosum*

Ces premiers travaux, portant sur l'écophysiologie d'*A. spinosum*, visaient à comprendre l'effet des facteurs environnementaux et nutritionnels sur la croissance de cet organisme et sur la production de toxines en culture. Leur but était améliorer nos connaissances sur l'écophysiologie d'*A. spinosum* et de permettre la production d'AZA1 et -2 à partir de cultures en continu d'*A. spinosum*. De plus elles ont donné de premières

informations sur les facteurs qui pourraient avoir une incidence sur d'éventuelles efflorescences d'*A. spinosum* et sur leur concentration toxinique.

A. spinosum est capable de se développer dans un large éventail de conditions, de 30 à 40 psu, de 10 à 26°C, à différentes intensités lumineuses et avec ou sans aération. Cet organisme présente un taux de croissance optimal en culture à 35 psu, dans un éventail de température allant de 18 à 22°C et avec aération.

Par contre, le quota cellulaire en AZA est significativement augmenté à de basses températures (à 10°C multiplication par 20 du quota cellulaire en AZA par rapport à des températures situées entre 18 et 26°C), et à un moindre degré une forte intensité lumineuse et l'aération du milieu de culture augmente également le quota en AZA des cellules.

Le taux de croissance d'*A. spinosum* et sa production toxinique se sont avérés être similaires les milieux testés. Cependant, lorsque le milieu présente une faible concentration en milieu K modifié (0.1Kmod) le quota en AZA des cellules est augementé, alors qu'à une concentration plus élevée (0.5Kmod, 1Kmod, 2Kmod) le taux de croissance ainsi que la concentration cellulaire maximale sont améliorés.

Cependant, des études complémentaires, utilisant des plans d'expériences, sont nécessaires pour déterminer l'optimum : de croissance, de concentration cellulaire et de production d'AZA d'*A. spinosum*. De plus, la capacité de cette espèce de microalgue à supporter les conditions de culture, appliquées en culture continue, permettra d'étudier l'effet des carences de divers nutriments sur la composition biochimique des cellules, afin d'essayer de mieux appréhender la toxinogenèse des azaspiracides dans *A. spinosum*.

Transfert trophique

Les études réalisées dans le cadre de ce chapitre ont permis, dans un premier temps la mise en évidence du lien existant entre la présence d'*A. spinosum* dans le milieu et l'accumulation d'AZA dans les moules. Une fois ce lien établi, plusieurs études ont été menées pour approfondir les relations existantes entre *A. spinosum*, les AZA et les mollusques bivalves.

Une première à permis d'évaluer l'accumulation et de comprendre les mécanismes de biotransformation des AZA1 et -2 dans les moules nourries avec *A. spinosum*. Dans les trois conditions testées (trois densité cellulaires différentes d'A. *spinosum* : 30 000, 10 000 et 5 000 cell mL^{-1}), les moules ont accumulé des AZA. Les toxines d'origine algale (AZA1 et -2), ainsi que leurs métabolites (AZA3 à 12, -17, -19, -21 et -23) ont été détectés au cours de

cette expérience. Au bout de seulement 6 h de contamination, la concentration en AZA dans les moules dépassait déjà la limite réglementaire européenne, et les métabolites représentaient environ 25% de la teneur totale en AZA. Cette proportion de métabolites a continué à augmenter et représentait plus de 50% des AZA présents dans les moules, après 24 h de contamination, et ce jusqu'à la fin de l'étude.

Parmi les métabolites détectés dans les moules, AZA17 et -19 ont été les composés majoritairement présents, leurs concentrations trouvées dans les moules pouvant être équivalentes à celles d'AZA1 et -2 respectivement. Par conséquent, ces analogues doivent être considérés comme des métabolites pouvant affecter la santé humaine, soit directement, soit indirectement, par l'intermédiaire d'AZA3 et -6 après leur conversion en ces composés, lors de la cuisson. Il est donc nécessaire de revoir la réglementation sur l'analyse des AZA dans les mollusques bivalves, car celle-ci est actuellement basée sur leur dosage dans des organismes crus, par LC-MS/MS, et ne prend uniquement en compte qu'AZA1, -2 et -3.

Pendant cette étude, les moules nourries avec *A. spinosum* ont présenté une légère augmentation de leur mortalité, par rapport aux moules témoin, ainsi qu'une diminution de l'épaisseur des tubules des glandes digestives et un ralentissement significatif de l'accumulation en toxine après six heures de contamination.

Pour étudier les effets d'*A. spinosum* sur le comportement alimentaire des moules, afin d'expliquer ces observations, l'évaluation de l'activité alimentaire, du taux de filtration, d'ingestion et d'absorption a été effectuée sur des moules soumises à un régime alimentaire toxique (*A. spinosum*) ou à un régime alimentaire non-toxique. Les résultats obtenus montrent un effet négatif d'*A. spinosum* sur l'activité alimentaire des moules et indiquent une éventuelle régulation de l'accumulation des AZA par une diminution du taux de filtration et une augmentation de la production de pseudofaeces. En effet, par rapport au régime non-toxique, le taux de filtration a été divisé par 6, le temps d'activité alimentaire par 5, le taux d'ingestion par 3 et le taux d'absorption a même atteint des valeurs négatives le dernier jour de contamination. Toutefois, en cohérence avec la précédente étude une accumulation rapide en AZA a été observée pendant les premières heures de l'expérience et moins de 6 h ont suffit pour dépasser la limite règlementaire.

Une dernière étude, concernant la sécurité alimentaire des consommateurs, a évalué si les moules peuvent accumuler AZA1 et -2 sous forme dissoute, et compare le profil toxinique ainsi que la distribution des toxines dans les tissus de la moule avec ceux de moules exposées à *A. spinosum*. Cette étude montre que les AZA dissous sont biodisponibles pour les moules et que leur accumulation peut permettre d'atteindre des concentrations supérieures à la limite

réglementaire. Cependant, des différences ont été observées dans la distribution des toxines dans les coquillages. La distribution des AZA dans les différents tissus de la moule indiquent que l'accumulation des AZA se produit dans la glande digestive, lors de l'absorption de cellules d'*A. spinosum* vivantes ou à travers les branchies lorsque les toxines sont dissoutes dans la phase aqueuse. Cette dernière observation soulève des questions sur les mécanismes impliqués dans l'absorption des AZA à travers les branchies et sur le potentiel de re-distribution ultérieure des toxines dans les autres organes. La biodisponibilité des AZA dissous, montrée pour les moules, soulève également des questions quant aux effets possibles des AZA sur d'autres organismes aquatiques, en particulier lors des phases larvaires, pour lesquelles où la sensibilité aux contaminants est accrue.

Ces différentes études ont souligné la complexité des facteurs qui régissent l'accumulation, la biotransformation et la détoxification des phycotoxines dans les mollusques bivalves. Il apparait maintenant nécessaire d'étudier l'effet d'*A. spinosum* sur l'accumulation d'AZA et sur les réponses physiologiques des mollusques bivalves filtreurs d'intérêt économique (moules, huîtres, coquilles Saint Jacques). Les huîtres et les coquilles Saint Jacques n'ont fait l'objet d'aucune étude d'accumulation et de détoxification d'AZA, elles sont donc à privilégier pour essayer de comprendre les différentes accumulations observées *in situ* chez ces différents mollusques bivalves et de les expliquer par des variations possibles de leur comportement alimentaire.

L'effet d'*A. spinosum* sur l'activité alimentaire des moules reste aussi à approfondir, est-ce la toxine en elle-même qui est responsable de la baisse de l'activité alimentaire ou est-ce un autre composé ? Des comparaisons avec une espèce non toxique d'*Azadinium* et avec de la toxine pure encapsulée sont à réaliser pour éclaircir ces aspects.

Cette thèse a permis à quelques scientifiques français impliqués dans ce projet d'étudier un organisme provenant d'un pays voisin, l'Irlande. Jusque-là les AZA n'ont été observés qu'aux franges des côtes françaises métropolitaine (au large de l'Ile de Sein (huître) et des côtes du nord de la Bretagne (pétoncles)), et ce à de faibles concentrations. Toutefois, ce travail prépare les organismes de surveillance français à d'éventuelles apparitions d'*A. spinosum*. Les conditions propices à la croissance d'*A. spinosum* existent sur les côtes de France métropolitaine. Ainsi, le risque d'une introduction de cet organisme par les eaux de ballastes des navires est avéré. De plus les transferts de mollusques bivalves vivants entre l'Irlande et la France sont fréquents, et ces mollusques (huîtres) sont affinés dans les eaux

françaises, ce qui accroît le risque de contamination des eaux françaises par *A. spinosum*. Des études plus approfondies seront nécessaires pour déterminer si les cellules d'*A. spinosum* retrouvées dans les fèces des mollusques étudiés au cours de cette étude sont capables de se développer, une fois remises en culture. Il est donc important de déterminer si ce risque, avéré pour d'autres espèces de dinoflagellés, l'est aussi pour *A. spinosum*.

Un autre fait important est la petite taille d'*A.spinosum* (7-12µm). En effet, la découverte de nouveaux organismes toxiques a tendance à être liée à l'étude du nanoplancton (<20µm) et des microalgues benthiques. *A. spinosum* appartient au nanoplancton et de ce fait peut être considéré comme un organisme caractéristique. Ces petits organismes sont donc difficilement identifiables au microscope optique et leur gestion va requérir le développement et la mise au point de nouvelles méthodes de surveillance (sonde biomoléculaire, analyse chimique)

Valorisation scientifique

Publications dans des journaux à comité de lecture

- **Jauffrais, T.**, Séchet, V., Herrenknecht, C., Truquet, P., Tillmann, U., Hess, P. 201X. Effect of environmental and nutritional factors on growth and toxin production of the dinoflagellates *Azadinium spinosum*. (en préparation)
- **Jauffrais, T.**, Kilcoyne, J., Séchet, V., Herrenknecht, C., Truquet, P., Miles, C.O., Hess, P. 201X. Dissolved azaspiracids are absorbed and metabolized by blue mussels (*Mytilus edulis*), (soumis dans Toxicon)
- **Jauffrais, T.**, Contreras, A., Herrenknecht, C., Truquet, P., Séchet, V., Tillmann, U., Hess, P. 2012. *Azadinium spinosum* effect on the feeding behavior and azaspiracid accumulation of *Mytilus edulis*. *Aquatic Toxicology*, 124–125, 179–187. http://dx.doi.org/10.1016/j.aquatox.2012.08.016
- **Jauffrais, T.**, Kilcoyne, J., Séchet, V., Herrenknecht, C., Truquet, P., Hervé, F., Bérard, J.B., Nulty, C., Taylor, S., Tillmann, U., Miles, C.O., Hess, P. 2012. Production and isolation of azaspiracid-1 and -2 from *Azadinium spinosum* culture in pilot scale photobioreactors. *Marine drugs* 10(6), 1360-1382. http://dx.doi.org/10.3390/md10061360
- **Jauffrais, T.**, Marcaillou, C., Herrenknecht, C., Truquet, P., Séchet, V., Nicolau, E., Tillmann, U., Hess, P. 2012. Azaspiracid accumulation, detoxification and biotransformation in blue mussels (*Mytilus edulis*) experimentally fed *Azadinium spinosum*. *Toxicon* 60(4), 582-595. http://dx.doi.org/10.1016/j.toxicon.2012.04.351
- **Jauffrais, T.**, Herrenknecht, C., Séchet, V., Sibat, M., Tillmann, U., Krock, B., Kilcoyne, J., Miles, C., McCarron, P., Amzil, Z., Hess, P. 2012. Quantitative analysis of azaspiracids in *Azadinium spinosum* cultures. *Analytical and Bioanalytical Chemistry*, 403(3), 833-846. http://dx.doi.org/10.1007/s00216-012-5849-2
- Salas, R., Tillmann, U., John, U., Kilcoyne, J., Burson, A., Cantwell, C., Hess, P., **Jauffrais, T.**, Silke, J. 2011. The role of *Azadinium spinosum* (Dinophyceae) in the production of azaspiracid shellfish poisoning in mussels. *Harmful Algae*, **10** (6), 774-783. http://dx.doi.org/10.1016/j.hal.2011.06.010

Publications dans des actes de conférences

- **Jauffrais, T.**, Séchet, V., Truquet, P., Amzil, Z., Herrenknecht, C., Hess, P. 2012. Effect of dilution rate on *Azadinium spinosum* and azaspiracid (AZA) production in pilot scale photobioreactors for the harvest of azaspiracid-1 and -2. *Proceeding of the 8th International Conference on Molluscan Shellfish Safety*, Charlottetown, Canada.
- **Jauffrais, T.**, Séchet, V., Herrenknecht, C., Tillmann, U., Krock, B., Amzil, Z., Hess, P. 2012. Growth and toxin production of *Azadinium spinosum* in batch and continuous culture. in: Pagou, P., Hallegraeff, G. (Taylor), Proceedings of the 14th International Conference on Harmful Algae. International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO 2012, pp. 90-93.

Publications dans des journaux à comité de lecture (hors cadre de la thèse)

- Sechet, V., Sibat, M., Chomerat, N., Nezan, E., Grossel, H., Lehebel-Peron, J.B., **Jauffrais, T.**, Ganzin, N., Marco-Miralles, F., Lemee, R., Amzil, Z., 2012. *Ostreopsis* cf. *ovata* in the French Mediterranean coast: molecular characterisation and toxin profile. Cryptogamie Algologie 33, 89-98.
- Marchetti, J., Bougaran, G., **Jauffrais, T.**, Lefèbvre, S., Saint-Jean, B., Lukomska, E., Robert, R., Cadoret, J.P. 2012. Effects of blue light on the biochemical composition and photosynthetic activity of *Isochrysis* sp. (T-iso). *Journal of Applied Phycology*, in press http://dx.doi.org/10.1007/s10811-012-9844-y

Présentations dans des conférences internationales

- Kilcoyne J., Nulty C., McCarron P., **Jauffrais T.**, Twiner M., Quilliam M.A., Hess P., Miles C.O. 2012. Isolation of novel azaspiracids – Structure elucidation, toxicology and preparation of reference materials. 13th IUPAC International Symposium on Mycotoxins and Phycotoxins, Rotterdam, the Netherlands. (Présentation)
- **Jauffrais, T.**, Kilcoyne, J., Séchet, V., Herrenknecht, C., Truquet, P., Hervé, F., Bérard, J.B., Nulty, C., Taylor, S., Tillmann, U., Miles, C. Hess, P. 2012. Production and preparative isolation of azaspiracid-1 and -2 from *Azadinium spinosum* culture in stirred photobioreactors. 13th IUPAC International Symposium on Mycotoxins and Phycotoxins, Rotterdam, the Netherlands. (Présentation)

- Hess, P., Contreras, A., Herrenknecht, C., Truquet, P., Séchet, V., Tillmann, U., **Jauffrais, T.**, 2012. *Azadinium spinosum* effect on the feeding behavior and azaspiracid accumulation of *Mytilus edulis*, 15th International Conference on Harmful Algae, Gyeongnam , Korea.(Poster)
- **Jauffrais, T**., Séchet, V., Truquet, P., Herrenknecht, C., Amzil, Z., Hess, P. 2011. Effect of flow rate on *Azadinium spinosum* and azaspiracid productivities in medium scale chemostats in series for azaspiracid-1 and -2 harvest. 8th International Conference on Molluscan Shellfish Safety, Charlottetown, Canada. (Poster)
- **Jauffrais, T**., Séchet, V., Truquet, P., Herrenknecht, C., Salas, R., Silke, J., Kilcoyne, J., Burson, A., Duffy, C., Tillmann, U., Amzil, Z., Hess, P. 2011. Effect of the toxic dinoflagellate *Azadinium spinosum* on azaspiracid accumulation in the blue mussels (*Mytilus edulis*). 8th International Conference on Molluscan Shellfish Safety, Charlottetown, Canada. (Présentation)
- Miles, C.O., McCarron, P., Kilcoyne, J., **Jauffrais, T.**, Quilliam, M.A., Hess, P., 2011 Biotransformations and Chemical Reactions of Azaspiracid and Related Analogues. 125th AOAC annual meeting and exposition. New Orleans. USA. (Présentation)
- **Jauffrais, T.**, Séchet, V., Herrenknecht, C., Tillmann, U., Krock, B., Amzil, Z., Hess, P. 2010. Growth and toxin production of *Azadinium spinosum* in batch and continuous culture. 14th International Conference on Harmful Algae, Hersonissos, Greece. (Poster)
- Silke, J., Salas, R., Burson, A., Duffy, C., Kilcoyne, J., Lyons, K., **Jauffrais, T.**, Tillmann, U. 2010. *Azadinium spinosum* in Irish waters, an azaspiracid producing dinoflagellate. 14th International Conference on Harmful Algae, Hersonissos, Greece. (Présentation)

Présentations dans des conférences nationales

- Hess, P., **Jauffrais, T.** 2012. Recent application of algal culture for the production of azaspiracids and recent initiatives for the integration of environmental and productive science with commercial stakeholders. 4th Rendez-vous de Concarneau : Where Industry meets Science in Marine Biotechnology, Concarneau, France. (Présentation)
- **Jauffrais, T**., Herrenknecht, C., Séchet, V., Amzil, Z., Hess, P. 2012. Écophysiologie des dinoflagellés du genre *Azadinium*, production d'azaspiracides et transfert trophique vers les mollusques bivalves filtreurs. 2nd Edition des Doctoriales de l'Université Nantes Angers le Mans. La Pommeraye, France. (Poster)
- **Jauffrais, T**., Séchet, V., Truquet, P., Herrenknecht, C., Amzil, Z., Hess, P. 2011. Développement de la culture en continu d'*Azadinium spinosum* et de procédures d'extraction

des azaspiracides à partir de grands volumes de culture de micro-algues. 1ère Journées Scientifiques de l'ED VENAM, Angers, France. (Présentation)

- Salas, R., Tillmann, U., Uwe J., Kilcoyne J., Burson A., Duffy C., Hess, P., **Jauffrais, T.,** Silke, J. 2011. Culture and isolation of *Azadinium spinosum* (Dinophyceae) and its role in the production of Azaspiracid toxins. Safefood Network, Queens University, Belfast, Ireland. (Présentation)

Références

Aasen, J.A.B., Espenes, A., Hess, P., Aune, T., 2010. Sub-lethal dosing of azaspiracid-1 in female NMRI mice. Toxicon 56, 1419-1425.

Abraham, A., Plakas, S.M., Wang, Z.H., Jester, E.L.E., El Said, K.R., Granade, H.R., Henry, M.S., Blum, P.C., Pierce, R.H., Dickey, R.W., 2006. Characterization of polar brevetoxin derivatives isolated from *Karenia brevis* cultures and natural blooms. Toxicon 48, 104-115.

Adachi, R., Fukuyo, Y., 1979. The thecal structure of a toxic marine dinoflagellate *Gambierdiscus toxicus* gen. et spec. nov. collected in a ciguatera-endemic area. Bull. Jpn. Soc. Sci. Fish. 45, 67-71.

Akselman, R., Negri, R.M., 2010. *Azadinium spinosum* Elbrächter et Tillmann (Dinophyceae) is present and also caused blooms at the southwestern atlantic, Abstract book of the 14th Intl. Conf. Harmful Algae, Hersonissos, Greece, p. 132.

Akselman, R., Negri, R.M., 2012. Blooms of *Azadinium* cf. *spinosum* Elbrächter et Tillmann (Dinophyceae) in northern shelf waters of Argentina, southwestern Atlantic. Harmful Algae 19, 30-38.

Alam, M., Sanduja, R., Hossain, M.B., van der Helm, D., 1982. *Gymnodinium breve* toxins. I. isolation and X-ray structure of O,O-dipropyl (E)-2-(1-methyl-2-oxopropylidene) phosphorohydrazidothioate (E)-oxime from the red tide dinoflagellate *Gymnodinium breve*. J. Am. Chem. Soc. 104, 5232-5234.

Alfonso, C., Rehmann, N., Hess, P., Alfonso, A., Wandscheer, C.B., Abuin, M., Vale, C., Otero, P., Vieytes, M.R., Botana, L.M., 2008. Evaluation of various pH and temperature conditions on the stability of azaspiracids and their Importance in preparative Isolation and toxicological studies. Anal. Chem. 80, 9672-9680.

Aligizaki, K., Katikou, P., Nikolaidis, G., Panou, A., 2008. First episode of shellfish contamination by palytoxin-like compounds from *Ostreopsis* species (Aegean Sea, Greece). Toxicon 51, 418-427.

Aligizaki, K., Nikolaidis, G., 2006. The presence of the potentially toxic genera *Ostreopsis* and *Coolia* (Dinophyceae) in the north Aegean sea, Greece. Harmful Algae 5, 717-730.

Aligizaki, K., Nikolaidis, G., 2008. Morphological identification of two tropical dinoflagellates of the genera *Gambierdiscus* and *Dinophysis* in the Mediterranean Sea. J. Biol. Res., Thessaloniki 9, 75-82.

Alvarez, G., Uribe, E., Avalos, P., Marino, C., Blanco, J., 2010. First identification of azaspiracid and spirolides in *Mesodesma donacium* and *Mulinia edulis* from northern Chile. Toxicon 55, 638-641.

Aminot, A., Kerouel, R., 2007. Dosage automatique des nutriments dans les eaux marines : méthode en flux continu, in: Ifremer (Ed.), Méthodes d'analyses en milieu marin.

Amzil, Z., Fresnel, J., Le Gal, D., Billard, C., 2001. Domoic acid accumulation in French shellfish in relation to toxic species of *Pseudo-nitzschia multiseries* and *P-pseudodelicatissima*. Toxicon 39, 1245-1251.

Amzil, Z., Sibat, M., Chomerat, N., Grossel, H., Marco-Miralles, F., Lemee, R., Nezan, E., Sechet, V., 2012. Ovatoxin-a and palytoxin accumulation in seafood in relation to *Ostreopsis* cf. *ovata* blooms on the french mediterranean coast. Mar. Drugs 10, 477-496.

Amzil, Z., Sibat, M., Royer, F., Savar, V., 2008. First report on azaspiracid and yessotoxin groups detection in French shellfish. Toxicon 52, 39-48.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp Mar. Biol. 104, 511-524.

Anonymous, 2005. Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004 Off.J. E. Union L 338, 22/12/2005, pp. 27-59.

Anonymous, 2007. Status of the Irish aquaculture 2006. An information report on the Irish aquaculture.

Anonymous, 2011 Commission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. Off.J. E. Union L6, 11/1/2011, 3-6.

AOAC, 2005a. Official method 959.08. paralytic shellfish poison, biological method mouse bioassay for PSP toxins, in: AOAC (Ed.), AOAC Official Methods of Analysis, 18th Edition ed. Association of Official Analytical Chemists, Gaithersburg, USA, pp. 79-80.

AOAC, 2005b. Official method 2005.06. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescent detection, in: AOAC (Ed.), AOAC Official Methods of Analysis, 18th Edition ed. Association of Official Analytical Chemists, Gaithersburg, USA, pp. 81-82.

Aune, T., Larsen, S., Aasen, J.A.B., Rehmann, N., Satake, M., Hess, P., 2007. Relative toxicity of dinophysistoxin-2 (Dtx-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. Toxicon 49, 1-7.

Aune, T., Sorby, R., Yasumoto, T., Ramstad, H., Landsverk, T., 2002. Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. Toxicon 40, 77-82.

Babica, P., Hilscherova, K., Bartova, K., Blaha, L., Marsalek, B., 2007. Effects of dissolved microcystins on growth of planktonic photoautotrophs. Phycologia 46, 137-142.

Baden, D.G., Bourdelais, A.J., Jacocks, H., Michelliza, S., Naar, J., 2005. Natural and derivative brevetoxins: Historical background, multiplicity, and effects. Environ. Health Perspect. 113, 621-625.

Bardouil, M., Bohec, M., Bougrier, S., Lassus, P., Truquet, P., 1996. Feeding responses of *Crassostrea gigas* (Thunberg) to inclusion of different proportions of toxic dinoflagellates in their diet. Oceanolog. Acta 19, 177-182.

Bardouil, M., Bohec, M., Cormerais, M., Bougrier, S., Lassus, P., 1993. Experimental-study of the effects of a toxic microalgal diet on feeding of the oyster *Crassostrea gigas* Thumberg. J. Shellfish Res. 12, 417-422.

Bargu, S., Lefebvre, K., Silver, M.W., 2006. Effect of dissolved domoic acid on the grazing rate of krill *Euphausia pacifica*. Mar. Ecol. Prog. Ser. 312, 169-175.

Barille, L., Prou, J., Heral, M., Razet, D., 1997. Effects of high natural seston concentrations on the feeding, selection, and absorption of the oyster *Crassostrea gigas* (Thunberg). J. Exp. Mar. Biol. Ecol. 212, 149-172.

Baron, R., Couedel, M., Joret, C., Garen, P., Truquet, P., Masselin, P., Bardouil, M., Lassus, P., 2006. Continuous fluorescence recording as a way to improve Pacific oyster (*Crassostrea gigas*) models of paralytic shellfish toxin accumulation. Aquat. Living Resour. 19, 77-84.

Bates, S.S., Bird, C.J., Freitas, A.S.W.d., Foxall, R., Gilgan, M., Hanic, L.A., Johnson, G.R., McCulloch, A.W., Odense, P., Pocklington, R., Quilliam, M.A., Sim, P.G., Smith, J.C., Subba Rao, D.V., Todd, E.C.D., Walter, J.A., Wright, J.L.C., 1989. Pennate

diatom *Nitschia pungens* as the primary source of domoic acid, a toxin in shellfish from Eastern Prince Edward Island, Canada. Can. J. Fish. Aquat. Sci. 46, 1203-1215.

Battle, T., Touchard, C., Moulsdale, H.J., Dowsett, B., Stacey, G.N., 1997. New cell substrates for in vitro evaluation of microcystin hepato-cytotoxicity. Toxicology in Vitro 11, 557-567.

Bauder, A., Cembella, A.D., Quilliam, M.A., 1996. Dynamics of diarrhetic shellfish toxins from the dinoflagellate *Prorocentrum lima* in the bay scallop, *Argopecten irradians*, in: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. IOC/UNESCO, pp. 433-436.

Bauder, A.G., Cembella, A.D., Bricelj, V.M., Quilliam, M.A., 2001. Uptake and fate of diarrhetic shellfish poisoning toxins from the dinoflagellate Prorocentrum lima in the bay scallop Argopecten irradians. Marine Ecology-Progress Series 213, 39-52.

Baumann, F., Bourrat, M.B., Pauillac, S., 2010. Prevalence, symptoms and chronicity of ciguatera in New Caledonia: Results from an adult population survey conducted in Noumea during 2005. Toxicon 56, 662-667.

Bayne, B.L., Hawkins, A.J.S., Navarro, E., 1987. Feeding and digestion by the mussel *Mytilus edulis* (bivalvia : mollusca) in mixtures of silt and algal cells at low concentrations. J. Exp. Mar. Biol. Ecol. 111, 1-22.

Bayne, B.L., Hawkins, A.J.S., Navarro, E., Iglesias, I.P., 1989. Effects of seston cocentration on feeding, digestion and growth in the mussel *Mytilus edulis*. Mar. Ecol. Prog. Ser. 55, 47-54.

Bayne, B.L., Iglesias, J.I.P., Hawkins, A.J.S., Navarro, E., Heral, M., Deslouspaoli, J.M., 1993. Feeding behaviour of the mussel, *Mytilus edulis*: responses to variations in quantity and organic content of the seston J. Mar. Biol. Assoc. U. K. 73, 813-829.

Beninger, P.G., Lepennec, M., Donval, A., 1991. Mode of particle ingestion in 5 species of suspension feeding bivalve mollusks. Mar. Biol. 108, 255-261.

Berdalet, E., 1992. Effects of turbulence on the marine dinoflagellate *Gymnodinium nelsonii*. J. Phycol. 28, 267-272.

Berdalet, E., Peters, F., Koumandou, V.L., Roldan, C., Guadayol, O., Estrada, M., 2007. Species-specific physiological response of dinoflagellates to quantified small-scale turbulence. J. Phycol. 43, 965-977.

Blanco, J., Acosta, C.P., de la Puente, M.B., Salgado, C., 2002a. Depuration and anatomical distribution of the amnesic shellfish poisoning (ASP) toxin domoic acid in the King scallop *Pecten maximus*. Aquat. Toxicol. 60, 111-121.

Blanco, J., Acosta, C.P., Marino, C., Muniz, S., Martin, H., Morono, A., Correa, J., Arevalo, F., Salgado, C., 2006. Depuration of domoic acid from different body compartments of the king scallop *Pecten maximus* grown in raft culture and natural bed. Aquat. Living Resour. 19, 257-265.

Blanco, J., Bermúdez de la Puente, M., Arévalo, F., Salgado, C., Moroño, Á., 2002b. Depuration of mussels (*Mytilus galloprovincialis*) contaminated with domoic acid. Aquat. Living Resour. 15, 53-60.

Blanco, J., Marino, C., Martin, H., Acosta, C.P., 2007. Anatomical distribution of diarrhetic shellfish poisoning (DSP) toxins in the mussel *Mytilus galloprovincialis*. Toxicon 50, 1011-1018.

Blanco, J., Morono, A., Franco, J., Reyero, M.I., 1997. PSP detoxification kinetics in the mussel *Mytilus galloprovincialis*. One- and two-compartment models and the effect of some environmental variables. Mar. Ecol. Prog. Ser. 158, 165-175.

Blanco, J., Reyero, I., Franco, J., 2003. Kinetics of accumulation and transformation of paralytic shellfish toxins in the blue mussel *Mytilus galloprovincialis*. Toxicon 42, 777-784.

Bolli, L., Llaveria, G., Garces, E., Guadayol, O., van Lenning, K., Peters, F., Berdalet, E., 2007. Modulation of ecdysal cyst and toxin dynamics of two *Alexandrium* (Dinophyceae) species under small-scale turbulence. Biogeosci. 4, 559-567.

Bomber, J.W., Tindall, D.R., 1988. Genetic variability in toxin potencies among seventeen clones of *Gambierdiscus toxicus* (Dinophyceae). FASEB J. 2, A375-A375.

Borowitzka, M.A., 1997. Microalgae for aquaculture: Opportunities and constraints. J. Appl. Phycol. 9, 393-401.

Bougrier, S., Lassus, P., Bardouil, M., Masselin, P., Truquet, P., 2003. Paralytic shellfish poison accumulation yields and feeding time activity in the Pacific oyster (*Crassostrea gigas*) and king scallop (*Pecten maximus*). Aquat. Living Resour. 16, 347-352.

Bourdelais, A.J., Campbell, S., Jacocks, H., Naar, J., Wright, J.L.C., Carsi, J., Baden, D.G., 2004. Brevenal is a natural inhibitor of brevetoxin action in sodium channel receptor binding assays. Cell. Mol. Neurobiol. 24, 553-563.

Bourdelais, A.J., Jacocks, H.M., Wright, J.L.C., Bigwarfe, P.M., Baden, D.G., 2005. A new polyether ladder compound produced by the dinoflagellate *Karenia brevis*. J. Nat. Prod. 68, 2-6.

Boyer, G.L., Sullivan, J.J., Andersen, R.J., Harrison, P.J., Taylor, F.J.R., 1987. Effects of nutrient limitation on toxin production and composition in marine dinoflagellate *Protogonyaulax tamarensis*. Mar. Biol. 96, 123-128.

Bricelj, V., Shumway, S., 1998. Paralytic shellfish toxins in bivalve molluscs: Occurrence, transfer kinetics, and biotransformation. Rev. Fish. Sci. 6, 315-383.

Bricelj, V.M., Connell, L., Konoki, K., MacQuarrie, S.P., Scheuer, T., Catterall, W.A., Trainer, V.L., 2005. Sodium channel mutation leading to saxitoxin resistance in clams increases risk of PSP. Nature 434, 763-767.

Bricelj, V.M., Lee, J.H., Cembella, A.D., 1991. Influence of dinoflagellate cell toxicity on uptake and loss of paralytic shellfish toxins in the northern quahog, *Mercenaria mercenaria*. Mar. Ecol. Prog. Ser. 74, 33-46.

Bricelj, V.M., Lee, J.H., Cembella, A.D., Anderson, D.M., 1990. Uptake kinetics of paralytic shellfish toxins from the dinoflagellatte *Alexandrium fundyense* in the mussel *Mytilus edulis* Mar. Ecol. Prog. Ser. 63, 177-188.

Brillant, M.G.S., MacDonald, B.A., 2000. Postingestive selection in the sea scallop, *Placopecten magellanicu*s (Gmelin): the role of particle size and density. J. Exp. Mar. Biol. Ecol. 253, 211-227.

Brillant, M.G.S., MacDonald, B.A., 2002. Postingestive selection in the sea scallop (*Placopecten magellanicus*) on the basis of chemical properties of particles. Mar. Biol. 141, 457-465.

Brillant, M.G.S., MacDonald, B.A., 2003. Postingestive sorting of living and heat-killed Chlorella within the sea scallop, *Placopecten magellanicus* (Gmelin). J. Exp. Mar. Biol. Ecol. 290, 81-91.

Britton, R., Roberge, M., Brown, C., van Soest, R., Andersen, R.J., 2003. New okadaic acid analogues from the marine sponge *Merriamum oxeato* and their effect on mitosis. J. Nat. Prod. 66, 838-843.

Brombacher, S., Edmonds, S., Volmer, D.A., 2002. Studies on azaspiracid biotoxins. II. Mass spectral behavior and structural elucidation of azaspiracid analogs. Rapid Commun. Mass Spectrom. 16, 2306-2316.

Brondz, I., Ekeberg, D., Hoiland, K., Bell, D.S., Annino, A.R., 2007. The real nature of the indole alkaloids in *Cortinarius infractus*: Evaluation of artifact formation through solvent extraction method development. J. Chromatogr. A 1148, 1-7.

Burkholder, J.M., 1998. Implications of harmful microalgae and heterotrophic dinoflagellates in management of sustainable marine fisheries. Ecol. Appl. 8, S37-S62.

Burkholder, J.M., Azanza, R.V., Sako, Y., 2006. The ecology of harmful dinoflagellates, in: Graneli, E., Turner, J.F. (Eds.), Ecology of harmful algae. Springer, The Netherlands, pp. 53-66.

Burkholder, J.M., Glasgow, H.B., Deamer-Melia, N.J., Springer, J., Parrow, M.W., Zhang, C., Cancellieri, P.J., 2001. Species of the toxic *Pfiesteria complex*, and the importance of functional type in data interpretation. Environ. Health Perspect. 109, 667-679.

Caillaud, A., de la Iglesia, P., Darius, H.T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain, M., Diogene, J., 2010. Update on methodologies available for ciguatoxin determination: perspectives to confront the onset of ciguatera fish poisoning in Europe. Mar. Drugs 8, 1838-1907.

Campbell, D.A., Kelly, M.S., Busman, M., Bolch, C.J., Wiggins, E., Moeller, P.D.R., Morton, S.L., Hess, P., Shumway, S.E., 2001. Amnesic shellfish poisoning in the King scallop, *Pecten maximus*, from the west coast of Scotland. J. Shellfish Res. 20, 75-84.

Carreto, J.I., Carignan, M.O., Daleo, G., Demarco, S.G., 1990. Occuurence of mycrosporine like amino-acids in the red tide dinoflagellate *Alexandrium excavatum*, UV pretective compounds J. Plankton Res. 12, 909-921.

Cazenave, J., Wunderlin, D.A., Bistoni, M.D.L., Ame, M.V., Krause, E., Pflugmacher, S., Wiegand, C., 2005. Uptake, tissue distribution and accumulation of microcystin-RR in *Corydoras paleatus*, *Jenynsia multidentata* and *Odontesthes bonariensis* — a field and laboratory study. Aquat. Toxicol. 75, 178-190.

CCAP, SE1 (Soil Extract 1 - Marine cultures), http://www.ccap.ac.uk/media/pdfrecipes.htm, last access 2012/03/04.

Chang, F.H., McClean, M., 1997. Growth responses of *Alexandrium minutum (Dinophyceae)* as a function of three different nitrogen sources and irradiance. N. Z. J. Mar. Freshwater Res. 31, 1-7.

Chateau-Degat, M.L., Chinain, M., Cerf, N., Gingras, S., Hubert, B., Dewailly, E., 2005. Seawater temperature, *Gambierdiscus* spp. variability and incidence of ciguatera poisoning in French Polynesia. Harmful Algae 4, 1053-1062.

Chebib, H.A., Cembella, A.D., Anderson, P., 1993. Differential paralytic shellfish toxin accumulation and detoxification kinetics in transplanted populations of *Mytilus edulis* exposed to natural blooms of *Alexandrium excavatum*, in: Smayda, T., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York, pp. 383-388.

Chen, C.Y., Chou, H.N., 2001. Accumulation and depuration of paralytic shellfish poisoning toxins by purple clam *Hiatula rostrata* Lighttoot. Toxicon 39, 1029-1034.

Choi, M.C., Hsieh, D.P.H., Lam, P.K.S., Wang, W.X., 2003. Field depuration and biotransformation of paralytic shellfish toxins in scallop *Chlamys nobilis* and green-lipped mussel *Perna viridis*. Marine Biology 143, 927-934.

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Grauso, L., Tartaglione, L., Guerrini, F., Pistocchi, R., 2010. Complex palytoxin-like profile of *Ostreopsis ovata*. Identification of four new ovatoxins by high-resolution liquid chromatography/mass spectrometry. Rapid Commun. Mass Spectrom. 24, 2735-2744.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, S., Guerrini, F., Pistocchi, R., Boni, L., 2003. Complex yessotoxins profile in *Protoceratium reticulatum* from north-western Adriatic sea revealed by LC-MS analysis. Toxicon 42, 7-14.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Tartaglione, L., Grillo, C., Melchiorre, N., 2008. Putative palytoxin and its new analogue, ovatoxin-a, in *Ostreopsis ovata* collected along the Ligurian coasts during the 2006 toxic outbreak. J. Am. Soc. Mass. Spectrom. 19, 111-120.

Colman, J.R., Twiner, M.J., Hess, P., McMahon, T., Satake, M., Yasumoto, T., Doucette, G.J., Ramsdell, J.S., 2005. Teratogenic effects of azaspiracid-1 identified by microinjection of Japanese medaka (*Oryzias latipes*) embryos. Toxicon 45, 881-890.

Contreras, A.M., Marsden, I.D., Munro, M.H.G., 2012. Effects of short-term exposure to paralytic shellfish toxins on clearance rates and toxin uptake in five species of New Zealand bivalve. Mar. Freshwat. Res. 63, 166-174.

Dell'Aversano, C., Walter, J.A., Burton, I.W., Stirling, D.J., Fattorusso, E., Quilliam, M.A., 2008. Isolation and structure elucidation of new and unusual saxitoxin analogues from mussels. J. Nat. Prod. 71, 1518-1523.

Dell'Aversano, E.G.K., Quilliam M A., 2004. Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography-mass spectrometry. J. Chromatogr. A 1028, 155-164.

Diaz Sierra, M., Furey, A., Hamilton, B., Lehane, M., James, K.J., 2003. Elucidation of the fragmentation pathways of azaspiracids, using electrospray ionisation, hydrogen/deuterium exchange, and multiple-stage mass spectrometry. J. Mass Spectrom. 38, 1178-1186.

Dickey, R.W., 2008. Ciguatera toxins: chemistry, toxicology and detection, in: L., B. (Ed.), Seafood and freshwater toxins - Pharmacology, Physiology and Detection. CRC Press, Taylor & Francis Group, Boca raton.

Doblin, M.A., Blackburn, S.I., Hallegraeff, G.M., 1999. Comparative study of selenium requirements of three phytoplankton species: *Gymnodinium catenatum, Alexandrium minutum* (Dinophyta) and *Chaetoceros* cf. *tenuissimus* (Bacillariophyta). J. Plankton Res. 21, 1153-1169.

Draisci, R., Palleschi, L., Ferretti, E., Furey, A., James, K.J., Satake, M., Yasumoto, T., 2000. Development of a method for the identification of azaspiracid in shellfish by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 871, 13-21.

Drum, A.S., Siebens, T.L., Crecelius, E.A., Eltson, R.A., 1993. Domoic acid in the Pacific razor clam *Siliqua patula* (Dixon, 1789). J. Shellfish Res. 12, 443-450.

Edebo, L., Lange, S., Li, X.P., Allenmark, S., Lindgren, K., Thompson, R., 1988. Seasonal, geographical and individual variation of okadaic acid content in cultivated mussels in Sweden. Apmis 96, 1036-1042.

EFSA, 2008. Marine biotoxins in shellfish - Okadaic acid and analogues, scientific opinion of the panel on contaminants in the food chain, adopted on 27 November 2007. *EFSA Journal* 589, 1-62.

EFSA, 2009. Marine biotoxins in shellfish - Summary on regulated marine biotoxins, scientific opinion of the panel on contaminants in the food chain; adopted on 13 August 2009. EFSA Journal 1306, 1-23.

Elmaleh, S., Coma, J., Grasmick, A., Bourgade, L., 1991. Magnesium induced algal flocculation in a fluidized bed. Water Sci. Technol. 23, 1695-1702.

EU/DG SANCO, E.U.D.S.e.C., 2001. Report of the meeting of the working group on toxicology of DSP and AZP, 21–23 May 2001. Brussels., p. 23.

Falkowski, P.G., Katz, M.E., Knoll, A.H., Quigg, A., Raven, J.A., Schofield, O., Taylor, F.J.R., 2004. The evolution of modern eukaryotic phytoplankton. Science 305, 354-360.

FAO, 2004. Report of the joint FAO/IOC/WHO *ad hoc* expert consultation on biotoxins in bivalve molluscs, Oslo, Norway.

Fernandez-Reiriz, M.J., Navarro, J.M., Contreras, A.M., Labarta, U., 2008. Trophic interactions between the toxic dinoflagellate *Alexandrium catenella* and *Mytilus chilensis*: Feeding and digestive behaviour to long-term exposure. Aquat. Toxicol. 87, 245-251.

Ferrari, S., Ciminiello, P., Dell'Aversano, C., Forino, M., Malaguti, C., Tubaro, A., Poletti, R., Yasumoto, T., Fattorusso, E., Rossini, G.P., 2004. Structure-activity relationships of yessotoxins in cultured cells. Chem. Res. Toxicol. 17, 1251-1257.

Finch, S.C., Wilkins, A.L., Hawkes, A.D., Jensen, D.J., MacKenzie, A.L., Beuzenberg, V., Quilliam, M.A., Olseng, C.D., Samdal, I.A., Aasen, J., Selwood, A.I., Cooney, J.M., Sandvik, M., Miles, C.O., 2005. Isolation and identification of (44-R,S)-44,55-dihydroxyyessotoxin from *Protoceratium reticulatum*, and its occurrence in extracts of shellfish from New Zealand, Norway and Canada. Toxicon 46, 160-170.

Flanagan, A.F., Callanan, K.R., Donlon, J., Palmer, R., Forde, A., Kane, M., 2001. A cytotoxicity assay for the detection and differentiation of two families of shellfish toxins. Toxicon 39, 1021-1027.

Flynn, K., Franco, J.M., Fernandez, P., Reguera, B., Zapata, M., Wood, G., Flynn, K.J., 1994. Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen and phosphorus stress. Mar. Ecol. Prog. Ser. 111, 99-109.

Flynn, K.J., Flynn, K., John, E.H., Reguera, B., Reyero, M.I., Franco, J.M., 1996. Changes in toxins, intracellular and dissolved free amino acids of the toxic dinoflagellate *Gymnodinium catenatum* in response to changes in inorganic nutrients and salinity. J. Plankton Res. 18, 2093-2111.

Forsyth, C.J., Xu, J.Y., Nguyen, S.T., Samdal, I.A., Briggs, L.R., Rundberget, T., Sandvik, M., Miles, C.O., 2006. Antibodies with broad specificity to azaspiracids by use of synthetic haptens. J. Am. Chem. Soc. 128, 15114-15116.

Frangopulos, M., Guisande, C., deBlas, E., Maneiro, I., 2004. Toxin production and competitive abilities under phosphorus limitation of *Alexandrium* species. Harmful Algae 3, 131-139.

Frolova, G.M., Kuznetsova, T.A., Mikhailov, V.V., Elyakov, G.B., 2000. An enzyme linked immunosorbent assay for detecting palytoxin-producing bacteria. Bioorg. Khim. 26, 315-320.

Furey, A., Brana-Magdalena, A., Lehane, M., Moroney, C., James, K.J., Satake, M., Yasumoto, T., 2002. Determination of azaspiracids in shellfish using liquid chromatography/tandem electrospray mass spectrometry. Rapid Commun. Mass Spectrom. 16, 238-242.

Furey, A., Moroney, C., Magdalena, A.B., Saez, M.J.F., Lehane, M., James, K.J., 2003. Geographical, temporal, and species variation of the polyether toxins, azaspiracids, in shellfish. Environ. Sci. Technol. 37, 3078-3084.

Furey, A., O'Doherty, S., O'Callaghan, K., Lehane, M., James, K.J., 2010. Azaspiracid poisoning (AZP) toxins in shellfish: toxicological and health considerations. Toxicon 56, 173-190.

Fux, E., 2008. Development and evaluation of passive sampling and LC-MS based techniques for the detection and monitoring of lipophilic marine toxins in mesocosm and field studies. PhD thesis, Dublin Institute of Technology.

Fux, E., Bire, R., Hess, P., 2009. Comparative accumulation and composition of lipophilic marine biotoxins in passive samplers and in mussels (*M. edulis*) on the west coast of Ireland. Harmful Algae 8, 523-537.

Fux, E., Marcaillou, C., Mondeguer, F., Bire, R., Hess, P., 2008a. Field and mesocosm trials on passive sampling for the study of adsorption and desorption behaviour of lipophilic toxins with a focus on OA and DTX1. Harmful Algae 7, 574-583.

Fux, E., McMillan, D., Bire, R., Hess, P., 2007. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. J. Chromatogr. A 1157, 273-280.

Fux, E., Rode, D., Bire, R., Hess, P., 2008b. Approaches to the evaluation of matrix effects in the liquid chromatography-mass spectrometry (LC-MS) analysis of three regulated lipophilic toxin groups in mussel matrix (*Mytilus edulis*). Food Addit. Contam. Part a-Chemistry Analysis Control Exposure & Risk Assessment 25, 1024-1032.

Galimany, E., Place, A.R., Ramon, M., Jutson, M., Pipe, R.K., 2008a. The effects of feeding *Karlodinium veneficum* (PLY # 103; *Gymnodinium veneficum* Ballantine) to the blue mussel *Mytilus edulis*. Harmful Algae 7, 91-98.

Galimany, E., Sunila, I., Hegaret, H., Ramon, M., Wikfors, G.H., 2008b. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: Histopathology, immune responses, and recovery. Harmful Algae 7, 702-711.

Galimany, E., Sunila, I., Hegaret, H., Ramon, M., Wikfors, G.H., 2008c. Pathology and immune response of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*. Harmful Algae 7, 630-638.

Gallardo Rodriguez, J.J., Sanchez Miron, A., Garcia Camacho, F., Ceron Garcia, M.C., Belarbi, E.H., Chisti, Y., Molina Grima, E., 2009a. Causes of shear sensitivity of the toxic dinoflagellate *Protoceratium reticulatum*. Biotechnol. Progr. 25, 792-800.

Gallardo Rodriguez, J.J., Sanchez Miron, A., Garcia Camacho, F., Ceron Garcia, M.C., Belarbi, E.H., Molina Grima, E., 2010. Culture of dinoflagellates in a fed-batch and

continuous stirred-tank photobioreactors: Growth, oxidative stress and toxin production. Process Biochem. 45, 660-666.

Gallardo Rodriguez, J.J., Sanchez Miron, A., Garcia Camacho, F., Ceron Garcia, M.D.C., Belarbi Haftalaui, E.H., Molina Grima, E., 2009b. Effects of antioxidants addition on the marine dinoflagellates biotoxin production. New Biotech. 25, S218-S218.

Garcia Camacho, F., Gallardo Rodriguez, J., Sanchez Miron, A., Ceron Garcia, M.C., Belarbi, E.H., Chisti, Y., Molina Grima, E., 2007a. Biotechnological significance of toxic marine dinoflagellates. Biotechnol. Adv. 25, 176-194.

Garcia Camacho, F., Gallardo Rodriguez, J.J., Sanchez Miron, A., Ceron Garcia, M.C., Belarbi, E.H., Grima, E.M., 2007b. Determination of shear stress thresholds in toxic dinoflagellates cultured in shaken flasks - Implications in bioprocess engineering. Process Biochem. 42, 1506-1515.

Gardner, J.P.A., 2002. Effects of seston variability on the clearance rate and absorption efficiency of the mussels *Aulacomya maoriana*, *Mytilus galloprovincialis* and *Perna canaliculus* from New Zealand. J. Exp. Mar. Biol. Ecol. 268, 83-101.

Gedaria, A.I., Luckas, B., Reinhardt, K., Azanza, R.V., 2007. Growth response and toxin concentration of cultured *Pyrodinium bahamense* var. compressum to varying salinity and temperature conditions. Toxicon 50, 518-529.

Gerssen, A., McElhinney, M.A., Mulder, P.P.J., Bire, R., Hess, P., de Boer, J., 2009a. Solid phase extraction for removal of matrix effects in lipophilic marine toxin analysis by liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 394, 1213-1226.

Gerssen, A., Mulder, P.P.J., McElhinney, M.A., de Boer, J., 2009b. Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. J. Chromatogr. A 1216, 1421-1430.

Gilgan M W, B.B.G., Landry G L., 1990. Distribution and magnitude of domoic acid contamination of shellfish in Atlantic Canada during 1988. Toxic Marine Phytoplankton, 469-474.

Glibert, P., Harrison, J., Heil, C., Seitzinger, S., 2006. Escalating worldwide use of urea – A global change contributing to coastal eutrophication. Biogeochemistry 77, 441-463.

Glibert, P.A., Legrand, C., 2006. The diverse nutrients strategies of harmful algae: Focus on osmotrophy, in: Graneli, E., Turner, J.F. (Eds.), Ecology of harmful algae. Springer, The Netherlands, pp. 53-66.

Gómez, F., 2012. A checklist and classification of living dinoflagellates (Dinoflagellata, Alveolata). CICIMAR Océanides 27, 67-143.

Gosling, E.M., 2003. Bivalve molluscs, biology, ecology and culture. Blackwell publishing, Oxford.

Granéli, E., Carlsson, P., Legrand, C., 1999. The role of C, N and P in dissolved and particulate organic matter as a nutrient source for phytoplankton growth, including toxic species. Aquat. Ecol. 33, 17-27.

Graneli, E., Turner, J.T., 2006. Ecology of harmful algae. Springer, The Netherlands.

Gribble, K.E., Anderson, D.M., 2006. Molecular phylogeny of the heterotrophic dinoflagellates, *Protoperidinium*, *diplopsalis* and *Preperidinium* (*Dinophyceae*), inferred from large subunit rDNA. J. Phycol. 42, 1081-1095.

Grobbelaar, J.U., 2009. Factors governing algal growth in photobioreactors: the "open" versus "closed" debate. J. Appl. Phycol. 21, 489-492.

Grobbelaar, J.U., 2010. Microalgal biomass production: challenges and realities. Photosynth. Res. 106, 135-144.

Grzebyk, D., Bechemin, C., Ward, C.J., Verite, C., Codd, G.A., Maestrini, S.Y., 2003. Effects of salinity and two coastal waters on the growth and toxin content of the dinoflagellate *Alexandrium minutum*. J. Plankton Res. 25, 1185-1199.

Grzebyk, D., Sechet, V., 2003. Toxinogénèse et physiologie cellulaire, in: Frémy, J.M., Lassus, P. (Eds.), Toxines d'algues dans l'alimentation. Ifremer, Brest, pp. 191-228.

Gueguen, M., Bardouil, M., Baron, R., Lassus, P., Truquet, P., Massardier, J., Amzil, Z., 2008. Detoxification of Pacific oyster *Crassostrea gigas* fed on diets of *Skeletonema costatum* with and without silt, following PSP contamination by *Alexandrium minutum*. Aquat. Living Resour. 21, 13-20.

Gueguen, M., Baron, R., Bardouil, M., Truquet, P., Haberkorn, H., Lassus, P., Barille, L., Amzil, Z., 2011. Modelling of paralytic shellfish toxin biotransformations in the course of *Crassostrea gigas* detoxification kinetics. Ecol. Modell. 222, 3394-3402.

Guerrini, F., Ciminiello, P., Dell'Aversano, C., Tartaglione, L., FattorusSo, E., Boni, L., Pistocchi, R., 2007. Influence of temperature, salinity and nutrient limitation on yessotoxin production and release by the dinoflagellate *Protoceratium reticulatum* in batch-cultures. Harmful Algae 6, 707-717.

Guerrini, F., Pezzolesi, L., Feller, A., Riccardi, M., Ciminiello, P., Dell'Aversano, C., Tartaglione, L., Dello Iacovo, E., Fattorusso, E., Forino, M., Pistocchi, R., 2010.

Comparative growth and toxin profile of cultured *Ostreopsis ovata* from the Tyrrhenian and Adriatic Seas. Toxicon 55, 211-220.

Guillard, R.R.L., 1973. Division rates, in: Stein, J.R. (Ed.), Handbook of phycological methods. Cambridge University Press, Cambridge, pp. 289-312.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates, in: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York, USA, pp. 26-60.

Guillard, R.R.L., 2003. Cultures methods, in: Hallegraef, G.M., Anderson, D.M., Cembella, A. (Eds.), Manual on harmful marine microalgae. UNESCO, Paris, pp. 77-99.

Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobolis* is a diatom, not a chrysophyte. Phycologia 32, 234-236.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea Cleve*. Can. J. Microbiol. 8, 229-239.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52, 696-704.

Guisande, C., Frangopulos, M., Maneiro, I., Vergara, A.R., Riveiro, I., 2002. Ecological advantages of toxin production by the dinoflagellate *Alexandrium minutum* under phosphorus limitation. Mar. Ecol. Prog. Ser. 225, 169-176.

Haberkorn, H., Lambert, C., Le Goic, N., Gueguen, M., Moal, J., Palacios, E., Lassus, P., Soudant, P., 2010. Effects of *Alexandrium minutum* exposure upon physiological and hematological variables of diploid and triploid oysters, *Crassostrea gigas*. Aquat. Toxicol. 97, 96-108.

Hall, S., Reichardt, P.B., 1984. Cryptic paralytic shellfish toxins, in: Ragelis, E.P. (Ed.), Seafood Toxins. Am. Chem. Soc., Washington DC, pp. 113-123.

Harada, T., Oshima, Y., Yasumoto, T., 1984. Assessment of potential activation of gonyautoxin V in the stomach of mice and rats. Toxicon 22, 476-478.

Hawkins, A.J.S., Bayne, B.L., Bougrier, S., Heral, M., Iglesias, J.I.P., Navarro, E., Smith, R.F.M., Urrutia, M.B., 1998. Some general relationships in comparing the feeding physiology of suspension-feeding bivalve molluscs. J. Exp. Mar. Biol. Ecol. 219, 87-103.

Hawkins, A.J.S., Smith, R.F.M., Bayne, B.L., Heral, M., 1996. Novel observations underlying the fast growth of suspension-feeding shellfish in turbid environments: *Mytilus edulis*. Mar. Ecol. Prog. Ser. 131, 179-190.

He, H., Chen, F., Li, H., Xiang, W., Li, Y., Jiang, Y., 2010. Effect of iron on growth, biochemical composition and paralytic shellfish poisoning toxins production of *Alexandrium tamarense*. Harmful Algae 9, 98-104.

Heasman, M., Diemar, J., O'Connor, W., Sushames, T., Foulkes, L., 2000. Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve molluscs - a summary. Aquacult. Res. 31, 637-659.

Hegaret, H., da Silva, P.M., Sunila, I., Shumway, S.E., Dixon, M.S., Alix, J., Wikfors, G.H., Soudant, P., 2009. Perkinsosis in the Manila clam *Ruditapes philippinarum* affects responses to the harmful-alga, *Prorocentrum minimum*. J. Exp. Mar. Biol. Ecol. 371, 112-120.

Hegaret, H., Shumway, S.E., Wikfors, G.H., Pate, S., Burkholder, J.M., 2008. Potential transport of harmful algae via relocation of bivalve molluscs. Mar. Ecol. Prog. Ser. 361, 169-179.

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, D.A., Stoecker, D.K., Suddleson, M., 2008. Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8, 3-13.

Helson, J.G., Gardner, J.P.A., 2007. Variation in scope for growth: a test of food limitation among intertidal mussels. Hydrobiologia 586, 373-392.

Hernandez-Becerril, D.U., Escobae-Morales, S., Morreno-Gutiérez, S.P., Baron-Campis, S.A., 2010. Two new records of potentially toxic phytoplankton species from the Mexican Pacific, Abstract book of the 14th Intl. Conf. Harmful Algae, Hersonissos, Greece, p. 137.

Hess, P., Aasen, J.B., 2007. Chemistry, origins and distribution of yessotoxin and its analogues, in: Botana, L.M. (Ed.), Chemistry and pharmacology of marine toxins. blackwell publishing, Oxford, pp. 187-2002.

Hess, P., Butter, T., Petersen, A., Silke, J., McMahon, T., 2009. Performance of the EU-harmonised mouse bioassay for lipophilic toxins for the detection of azaspiracids in naturally contaminated mussel (*Mytilus edulis*) hepatopancreas tissue homogenates characterised by liquid chromatography coupled to tandem mass spectrometry. Toxicon 53, 713-722.

Hess, P., Grune, B., Anderson, D.B., Aune, T., Botana, L.M., Caricato, P., Van Egmond, H.P., Halder, M., Hall, S., Lawrence, J.F., Moffat, C., Poletti, R., Richmond, J.,

Rossini, G.P., Seamer, C., Vilageliu, J.S., 2006. Three Rs Approaches in Marine Biotoxin Testing - the Report and Recommendations of a Joint Ecvam/Dg Sanco Workshop (Ecvam Workshop 55). Atla-Alternatives to Laboratory Animals 34, 193-224.

Hess, P., McCarron, P., Quilliam, M.A., 2007. Fit-for-purpose shellfish reference materials for internal and external quality control in the analysis of phycotoxins. Anal. Bioanal. Chem. 387, 2463-2474.

Hess, P., McMahon, T., Slattery, D., Swords, D., Dowling, G., McCarron, M., Clarke, D., Gibbons, W., Silke, J., O'Cinneide, M., 2003. Use of LC-MS testing to identify lipophilic toxins, to establish local trends and interspecies differences and to test the comparability of LC-MS testing with the mouse bioassay: an example from the Irish biotoxin monitoring programme 2001, in: Villalba, A., Reguera, B., Romalde, J.L., Beiras, R. (Eds.), 4th Intl. Conf. Molluscan Shellfish Safety. Xunta De Galicia, IOC of UNESCO, Santiago de Compostela, Spain, pp. 57-65.

Hess, P., Morris, S., Stobo, L.A., Brown, N.A., McEvoy, J.D.G., Kennedy, G., Young, P.B., Slattery, D., McGovern, E., McMahon, T., Gallacher, S., 2005a. LC-UV and LC-MS methods for the determination of domoic acid. Trends Anal. Chem. 24, 358-367.

Hess, P., Nguyen, L., Aasen, J., Keogh, M., Kilcoyne, J., McCarron, P., Aune, T., 2005b. Tissue distribution, effects of cooking and parameters affecting the extraction of azaspiracids from mussels, *Mytilus edulis*, prior to analysis by liquid chromatography coupled to mass spectrometry. Toxicon 46, 62-71.

Higman, W.A., Turner, A., 2010. A feasibility study into the provision of Paralytic Shellfish Toxins laboratory reference materials by mass culture of *Alexandrium* and shellfish feeding experiments. Toxicon 56, 497-501.

Holland, P.T., Selwood, A.I., Mountfort, D.O., Wilkins, A.L., McNabb, P., Rhodes, L.L., Doucette, G.J., Mikulski, C.M., King, K.L., 2005. Isodomoic acid C, an unusual amnesic shellfish poisoning toxin from *Pseudo-nitzschia australis*. Chem. Res. Toxicol. 18, 814-816.

Hu, H.H., Chen, W.D., Shi, Y.J., Cong, W., 2006a. Nitrate and phosphate supplementation to increase toxin production by the marine dinoflagellate *Alexandrium tamarense*. Mar. Pollut. Bull. 52, 756-760.

Hu, H.H., Shi, Y.J., Cong, W., 2006b. Improvement in growth and toxin production of *Alexandrium tamarense* by two-step culture method. J. Appl. Phycol. 18, 119-126.

Hu, T., Curtis, J.M., Walter, J.A., Wright, J.L.C., 1996. Characterization of biologically inactive spirolides E and F: identification of the spirolide pharmacophore. Tetrahedron Lett. 37, 7671-7674.

Hu, T.M., Curtis, J.M., Oshima, Y., Quilliam, M.A., Walter, J.A., Watsonwright, W.M., Wright, J.L.C., 1995. Spirolide-B and spirolide-D, 2 novel macrocycles isolated from the digestive glands of shellfish. J. Chem. Soc., Chem. Commun., 2159-2161.

Hu, T.M., Doyle, J., Jackson, D., Marr, J., Nixon, E., Pleasance, S., Quilliam, M.A., Walter, J.A., Wright, J.L.C., 1992. Isolation of a new diarrhetic shellfish poison from mussels J. Chem. Soc., Chem. Commun., 39-41.

Hwang, D.F., Lu, Y.H., 2000. Influence of environmental and nutritional factors on growth, toxicity, and toxin profile of dinoflagellate *Alexandrium minutum*. Toxicon 38, 1491-1503.

Ishida, H., Nozawa, A., Totoribe, K., Muramatsu, N., Nukaya, H., Tsuji, K., Yamaguchi, K., Yasumoto, T., Kaspar, H., Berkett, N., Kosuge, T., 1995. Brevetoxin-1-B, a new polyether marine toxin from the new zealand shellfis, *Austrovenus stutchburyi* Tetrahedron Lett. 36, 725-728.

Ito, E., Satake, M., Ofuji, K., Higashi, M., Harigaya, K., McMahon, T., Yasumoto, T., 2002. Chronic effects in mice caused by oral administration of sublethal doses of azaspiracid, a new marine toxin isolated from mussels. Toxicon 40, 193-203.

Ito, E., Satake, M., Ofuji, K., Kurita, N., McMahon, T., James, K., Yasumoto, T., 2000. Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. Toxicon 38, 917-930.

Ito, E., Suzuki, T., Oshima, Y., Yasumoto, T., 2008. Studies of diarrhetic activity on pectenotoxin-6 in the mouse and rat. Toxicon 51, 707-716.

Ito, E., Terao, K., McMahon, T., Silke, J., Yasumoto, T., 1998. Acute pathological changes in mice caused by crude extracts of novel toxins isolated from Irish mussels, in: Reguera, B.; Blanco, J.F., M. L.; Wyatt, T. (Eds.), Harmful Algae,. IOC of UNESCO, Santiago de Compostela, Spain, pp. 588-589.

James, K.J., Fidalgo Saez, M.J., Furey, A., Lehane, M., 2004. Azaspiracid poisoning, the food borne illness associated with shellfish consumption. Food Addit. Contam. 21, 879-892.

James, K.J., Furey, A., Lehane, M., Ramstad, H., Aune, T., Hovgaard, P., Morris, S., Higman, W., Satake, M., Yasumoto, T., 2002. First evidence of an extensive northern European distribution of azaspiracid poisoning (AZP) toxins in shellfish. Toxicon 40, 909-915.

James, K.J., Gillman, M., Amandi, M.F., Lopez-Rivera, A., Puente, P.F., Lehane, M., Mitrovic, S., Furey, A., 2005. Amnesic shellfish poisoning toxins in bivalve molluscs in Ireland. Toxicon 46, 852-858.

James, K.J., Moroney, C., Roden, C., Satake, M., Yasumoto, T., Lehane, M., Furey, A., 2003a. Ubiquitous 'benign' alga emerges as the cause of shellfish contamination responsible for the human toxic syndrome, azaspiracid poisoning. Toxicon 41, 145-151.

James, K.J., Sierra, M.D., Lehane, M., Magdalena, A.B., Furey, A., 2003b. Detection of five new hydroxyl analogues of azaspiracids in shellfish using multiple tandem mass spectrometry. Toxicon 41, 277-283.

Jauffrais, T., Herrenknecht, C., Séchet, V., Sibat, M., Tillmann, U., Krock, B., Kilcoyne, J., Miles, C.O., McCarron, P., Amzil, Z., Hess, P., 2012a. Quantitative analysis of azaspiracids in *Azadinium spinosum* cultures. Anal. Bioanal. Chem. 403, 833-846.

Jauffrais, T., Kilcoyne, J., Sechet, V., Herrenknecht, C., Truquet, P., Herve, F., Berard, J.B., Nulty, C., Taylor, S., Tillmann, U., Miles, C.O., Hess, P., 2012b. Production and isolation of azaspiracid-1 and -2 from *Azadinium spinosum* culture in pilot scale photobioreactors. Mar. Drugs 10, 1360-1382.

Jauffrais, T., Marcaillou, C., Herrenknecht, C., Truquet, P., Séchet, V., Nicolau, E., Tillmann, U., Hess, P., 2012c. Azaspiracid accumulation, detoxification and biotransformation in blue mussels (*Mytilus edulis*) experimentally fed *Azadinium spinosum*. Toxicon 60, 582-595.

Jauffrais, T., Séchet, V., Herrenknecht, C., Tillmann, U., Krock, B., Amzil, Z., Hess, P., 2010. Growth and toxin production of *Azadinium spinosum* in batch and continuous culture, Abstract book of the14th International conference on harmful algae, Hersonissos, Greece.

Jauffrais, T., Séchet, V., Truquet, P., Herrenknecht, C., Amzil, Z., Hess, P., 2011. Effect of flow rate on *Azadinium spinosum* and azaspiracid productivities in medium scale chemostats in series for azaspiracid-1 and -2 harvest, Abstract book of the 8th International Conference on Molluscan Shellfish safety, Charlottetown, Canada.

John, E.H., Flynn, K.J., 2000. Growth dynamics and toxicity of *Alexandrium fundyense* (Dinophyceae): the effect of changing N : P supply ratios on internal toxin and nutrient levels. Eur. J. Phycol. 35, 11-23.

Kamiyama, T., Nagai, S., Suzuki, T., Miyamura, K., 2010. Effect of temperature on production of okadaic acid, dinophysistoxin-1, and pectenotoxin-2 by *Dinophysis acuminata* in culture experiments. Aquat. Microb. Ecol. 60, 193-202.

Karp-Boss, L., Boss, E., Jumars, P.A., 2000. Motion of dinoflagellates in a simple shear flow. Limnol. Oceanogr. 45, 1594-1602.

Katikou, P., 2008. Palytoxin and analogues: ecobiology and origin, chemistry, metabolism, and chemical analysis. Seafood and freshwater toxins - Pharmacology, Physiology and Detection; ed. L. Botana. CRC Press, Taylor & Francis Group, ISBN: 0-8493-7437-5.

Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. 23, 633-638.

Keppler, C.J., Hoguet, J., Smith, K., Ringwood, A.H., Lewitus, A.J., 2005. Sublethal effects of the toxic alga *Heterosigma akashiwo* on the southeastern oyster (*Crassostrea virginica*). Harmful Algae 4, 275-285.

Kilcoyne, J., Fux, E., 2010. Strategies for the elimination of matrix effects in the liquid chromatography tandem mass spectrometry analysis of the lipophilic toxins okadaic acid and azaspiracid-1 in molluscan shellfish. J. Chromatogr. A 1217, 7123-7130.

Kilcoyne, J., Keogh, A., Clancy, G., Leblanc, P., Burton, I., Quilliam, M.A., Hess, P., Miles, C.O., 2012. Improved isolation procedure for azaspiracids from shellfish, structural elucidation of azaspiracid-6, and stability studies. J. Agric. Food. Chem. 60, 2447-2455.

Kim, C.H., Sako, Y., Ishida, Y., 1993. Variation of toxin production and composition in axenic cultures of *Alexandrium catenella* and *A. tamarense*. Nippon Suisan Gakkaishi 59, 633-639.

Kim, D.-G., La, H.-J., Ahn, C.-Y., Park, Y.-H., Oh, H.-M., 2011. Harvest of *Scenedesmus* sp. with bioflocculant and reuse of culture medium for subsequent high-density cultures. Bioresour. Technol. 102, 3163-3168.

Kim, D.I., Matsuyama, Y., Nagasoe, S., Yamaguchi, M., Yoon, Y.H., Oshima, Y., Imada, N., Honjo, T., 2004. Effects of temperature, salinity and irradiance on the growth of the harmful red tide dinoflagellate *Cochlodinium polykrikoides* Margalef (Dinophyceae). J. Plankton Res. 26, 61-66.

Kimura, S., Hashimoto, Y., Yamazato, K., 1972. Toxicity of the zoanthid *Palythoa tuberculosa*. Toxicon 10, 611.

King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C., Olah, T., 2000. Mechanistic investigation of ionization suppression in electrospray ionization. J. Am. Soc. Mass. Spectrom. 11, 942-950.

Klontz, K.C., Abraham, A., Plakas, S.M., Dickey, R.W., 2009. Mussel-associated azaspiracid intoxication in the United States. Ann. Intern. Med. 150, 361.

Korpinen, S., Karjalainen, M., Viitasalo, M., 2006. Effects of cyanobacteria on survival and reproduction of the littoral crustacean *Gammarus zaddachi* (Amphipoda). Hydrobiologia 559, 285-295.

Krock, B., Tillmann, U., John, U., Cembella, A., 2008. LC-MS-MS aboard ship: tandem mass spectrometry in the search for phycotoxins and novel toxigenic plankton from the North Sea. Anal. Bioanal. Chem. 392, 797-803.

Krock, B., Tillmann, U., John, U., Cembella, A.D., 2009. Characterization of azaspiracids in plankton size-fractions and isolation of an azaspiracid-producing dinoflagellate from the North Sea. Harmful Algae 8, 254-263.

Krock, B., Tillmann, U., Voß, D., Koch, B.P., Salas, R., Witt, M., Potvin, É., Jeong, H.J., 2012. New azaspiracids in Amphidomataceae (Dinophyceae). Toxicon 60, 830-839.

Kumagai, M., Yanagi, T., Murata, M.I., Yasumoto, T., Kat, M., Lassus, P., Rodriguez-Vazquez, J.A., 1986. Okadaic acid as the causative toxin of diarrhetic shellfish poisoning in Europe. Agric. Biol. Chem. 50, 2853-2857.

Laabir, M., Jauzein, C., Genovesi, B., Masseret, E., Grzebyk, D., Cecchi, P., Vaquer, A., Perrin, Y., Collos, Y., 2011. Influence of temperature, salinity and irradiance on the growth and cell yield of the harmful red tide dinoflagellate *Alexandrium catenella* colonizing Mediterranean waters. J. Plankton Res. 33, 1550-1563.

Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. Rev. Fish. Sci. 10, 113-390.

Larsen, K., Petersen, D., Wilkins, A.L., Samdal, I.A., Sandvik, M., Rundberget, T., Goldstone, D., Arcus, V., Hovgaard, P., Rise, F., Rehmann, N., Hess, P., Miles, C.O., 2007. Clarification of the C-35 stereochemistries of dinophysistoxin-1 and dinophysistoxin-2 and its consequences for binding to protein phosphatase (vol 20, pg 868, 2007). Chem. Res. Toxicol. 20, 2020-2020.

Lartigue, J., Jester, E.L.E., Dickey, R.W., Villareal, T.A., 2009. Nitrogen source effects on the growth and toxicity of two strains of the ciguatera-causing dinoflagellate *Gambierdiscus toxicus*. Harmful Algae 8, 781-791.

Lassus, P., Amzil, Z., Baron, R., Sechet, V., Barille, L., Abadie, E., Bardouil, M., Sibat, M., Truquet, P., Berard, J.B., Gueguen, M., 2007. Modelling the accumulation of PSP toxins in Thau Lagoon oysters (*Crassostrea gigas*) from trials using mixed cultures of *Alexandrium catenella* and *Thalassiosira weissflogii*. Aquat. Living Resour. 20, 59-67.

Lassus, P., Bardouil, M., Baron, R., Berard, J.B., Masselin, P., Truquet, P., Pitrat, J.P., 2005. Improving detoxification efficiency of PSP contaminated oysters (*Crassostrea gigas* Thunberg). Aquacul. Eur., 3-6.

Lassus, P., Bardouil, M., Beliaeff, B., Bougrier, S., Gubbins, M., Masselin, P., Mornet, F., Thébaud, O., Truquet, P., 2002. Coquillages contaminés par des phycotoxines paralysantes: vers une procédure de détoxification. Ifremer.

Lassus, P., Bardouil, M., Beliaeff, B., Masselin, P., Naviner, M., Truquet, P., 1999. Effect of a continuous supply of the toxic dinoflagellate *Alexandrium minutum* Halim on the feeding behavior of the Pacific oyster (*Crassostrea gigas* Thunberg). J. Shellfish Res. 18, 211-216.

Lassus, P., Bardouil, M., Massselin, P., Naviner, M., Truquet, P., 2000. Comparative efficiencies of different non-toxic microalgal diets in detoxification of PSP-contaminated oysters (*Crassostrea gigas* Thunberg). J. Nat. Toxins 9, 1-12.

Lawrence, J.F., Niedzwiadek, B., Menard, C., 2004. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: Interlaboratory study. J. AOAC Int. 87, 83-100.

Laycock, M.V., Thibault, P., Ayer, S.W., Walter, J.A., 1994. Isolation and purification procedures for the preparation of paralytic shellfish poisoning toxin standards. Nat. Toxins 2, 175-183.

Lee, J.S., Igarashi, T., Fraga, S., Dahl, E., Hovgaard, P., Yasumoto, T., 1989. Determination of diarrehetic shellfish toxins in various dinoflagellate species. J. Appl. Physiol. 1, 147-152.

Lefebvre, K.A., Elder, N.E., Hershberger, P.K., Trainer, V.L., Stehr, C.M., Scholz, N.L., 2005. Dissolved saxitoxin causes transient inhibition of sensorimotor function in larval Pacific herring (*Clupea harengus pallasi*). Mar. Biol. 147, 1393-1402.

Legrand, A.M., Fukui, M., Cruchet, P., Yasumoto, T., 1992. Progress on chemical knowledge of ciguatoxins. Bull. Soc. Pathol. Exot. 85, 467-469.

Lehane, M., Brana-Magdalena, A., Moroney, C., Furey, A., James, K.J., 2002. Liquid chromatography with electrospray ion trap mass spectrometry for the determination of five azaspiracids in shellfish. J. Chromatogr. A 950, 139-147.

Lehane, M., Saez, M.J.F., Magdalena, A.B., Canas, I.R., Sierra, M.D., Hamilton, B., Furey, A., James, K.J., 2004. Liquid chromatography-multiple tandem mass spectrometry for the determination of ten azaspiracids, including hydroxyl analogues in shellfish. J. Chromatogr. A 1024, 63-70.

Lenoir, S., Ten-Hage, L., Turquet, J., Quod, J.P., Bernard, C., Hennion, M.C., 2004. First evidence of palytoxin analogues from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in Southwestern Indian Ocean. J. Phycol. 40, 1042-1051.

Leong, S.C.Y., Taguchi, S., 2004. Response of the dinoflagellate *Alexandrium tamarense* to a range of nitrogen sources and concentrations: growth rate, chemical carbon and nitrogen, and pigments. Hydrobiologia 515, 215-224.

Lewis, R.J., Sellin, M., Poli, M.A., Norton, R.S., MacLeod, J.K., Sheil, M.M., 1991. Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). Toxicon 29, 1115-1127.

Li, A.M.Y., Yu, P.K.N., Hsieh, D.P.H., Wang, W.X., Wu, R.S.S., Lam, P.K.S., 2005. Uptake and depuration of paralytic shellfish toxins in the greenlipped mussel, *Perna viridis*: A dynamic model. Environ. Toxicol. Chem. 24, 129-135.

Lim, P.T., Leaw, C.P., Usup, G., Kobiyama, A., Koike, K., Ogata, T., 2006. Effects of light and temperature on growth, nitrate uptake, and toxin production of two tropical dinoflagellates: *Alexandrium tamiyavanichii* and *Alexandrium minutum* (Dinophyceae). J. Phycol. 42, 786-799.

Lin, Y.-Y., Risk, M., Ray, S.M., Van Engen, D., Clardy, J., Golik, J., James, J.C., Nakanishi, K., 1981. Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*). J. Am. Chem. Soc. 103, 6773-6775.

Lindegarth, S., Torgersen, T., Lundve, B., Sandvik, M., 2009. Differential retention of okadaic acid (OA) group and pectenotoxins (PTX) in the blue mussel, *Mytilus edulis* (L.), and the European flat oyster, *Ostrea edulis* (L.) J. Shellfish Res. 28, 313-323.

Liu, H., Kelly, M.S., Campbell, D.A., Dong, S.L., Zhu, J.X., Wang, S.F., 2007. Exposure to domoic acid affects larval development of king scallop *Pecten maximus* (Linnaeus, 1758). Aquat. Toxicol. 81, 152-158.

Loader, J.I., Hawkes, A.D., Beuzenberg, V., Jensen, D.J., Cooney, J.M., Wilkins, A.L., Fitzgerald, J.M., Briggs, L.R., Miles, C.O., 2007. Convenient large-scale purification

of yessotoxin from *Protoceratium reticulatum* culture and isolation of a novel furanoyessotoxin. J. Agric. Food. Chem. 55, 11093-11100.

Lopez-Rivera, A., O'Callaghan, K., Moriarty, M., O'Driscoll, D., Hamilton, B., Lehane, M., James, K.J., Furey, A., 2009. First evidence of azaspiracids (AZAs): A family of lipophilic polyether marine toxins in scallops (*Argopecten purpuratus*) and mussels (*Mytilus chilensis*) collected in two regions of Chile. Toxicon 55, 692-701.

Lorenzen, C.J., 1967. Determination of chlorophyll and pheo-pigments - spectrographic equations. Limnol. Oceanogr. 12, 343-346.

Lu, C.K., Lee, G.H., Huang, R., Chou, H.N., 2001. Spiro-prorocentrimine, a novel macrocyclic lactone from a benthic *Prorocentrum* sp. of Taiwan. Tetrahedron Lett. 42, 1713-1716.

Luz Lapa Teixeira, C.M., Kirsten, F.V., Nogueira Teixeira, P.C., 2012. Evaluation of *Moringa oleifera* seed flour as a flocculating agent for potential biodiesel producer microalgae. J. Appl. Phycol. 24, 557-563.

Mackay, D., Salusbury, T., 1988. Choosing between centrifugation and crossflow-microfiltration. Chemical Engineer-London, 45-50.

MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P., Selwood, A., 2004. Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. Toxicon 44, 901-918.

Mackenzie, L., Rhodes, L., Till, D., Chang, F.H., Kaspar, H., Haywood, A., Kapa, J., Walker, B., 1995. A *Gymnodinium* sp. bloom and the contamination of shellfish with lipid soluble toxins in New Zealand, Jan-April 1993, in: Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd., Nantes, France, pp. 795-800.

MacKenzie, L.A., 2010. In situ passive solid-phase adsorption of micro-algal biotoxins as a monitoring tool. Curr. Opin. Biotechnol. 21, 326-331.

Maclean, C., Cembella, A.D., Quilliam, M.A., 2003. Effects of light, salinity and inorganic nitrogen on cell growth and spirolide production in the marine dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech et Tangen. Botanica Marina 46, 466-476.

Maeda, M., Kodama, T., Tanaka, T., Yoshizumi, H., Takemoto, T., Nomoto, K., Fujita, T., 1986. Structures of isodomoic acids A, B and C, novel insecticidal amino acids from the red alga *Chondria armata*. Chem. Pharm. Bull 34, 4892-4895.

Mafra Jr, L.L., Bricelj, V.M., Ouellette, C., Bates, S.S., 2010. Feeding mechanics as the basis for differential uptake of the neurotoxin domoic acid by oysters, *Crassostrea virginica*, and mussels, *Mytilus edulis*. Aquat. Toxicol. 97, 160-171.

Mafra, L., Bricelj, V.M., Quilliam, M., 2008. Differential toxin uptake and feeding strategies in *Crassostrea virginica* and *Mytilus edulis* exposed to a toxic diatom. J. Shellfish Res. 27, 1027-1028.

Mafra, L.L., Bricelj, V.M., Fennel, K., 2010a. Domoic acid uptake and elimination kinetics in oysters and mussels in relation to body size and anatomical distribution of toxin. Aquat. Toxicol. 100, 17-29.

Mafra, L.L., Bricelj, V.M., Ouellette, C., Bates, S.S., 2010b. Feeding mechanics as the basis for differential uptake of the neurotoxin domoic acid by oysters, *Crassostrea virginica*, and mussels, *Mytilus edulis*. Aquat. Toxicol. 97, 160-171.

Mafra, L.L., Jr., Bricelj, V.M., Ouellette, C., Leger, C., Bates, S.S., 2009. Mechanisms contributing to low domoic acid uptake by oysters feeding on *Pseudo-nitzschia* cells. I. Filtration and pseudofeces production. Aquat. Biol. 6, 201-212.

Magdalena, A.B., Lehane, M., Krys, S., Fernandez, M.L., Furey, A., James, K.J., 2003a. The first identification of azaspiracids in shellfish from France and Spain. Toxicon 42, 105-108.

Magdalena, A.B., Lehane, M., Moroney, C., Furey, A., James, K.J., 2003b. Food safety implications of the distribution of azaspiracids in the tissue compartments of scallops (*Pecten maximus*). Food Addit. Contam. 20, 154-160.

Maranda, L., Shimizu, Y., 1987. Diarrhetic shellfish poisoning in Narragansett Bay. Estuaries 10, 298-302.

Marasigan, A.N., Sato, S., Fukuyo, Y., Kodama, M., 2001. Accumulation of a high level of diarrhetic shellfish toxins in the green mussel *Perna viridis* during a bloom of *Dinophysis caudata* and *Dinophysis mile*s in Sapian Bay, Panay Island, the Philippines. Fish. Sci. 67, 994-996.

Marcaillou, C., Haure, J., Mondeguer, F., Courcoux, A., Dupuy, B., Penisson, C., 2010. Effect of food supply on the detoxification in the blue mussel, *Mytilus edulis*, contaminated by diarrhetic shellfish toxins. Aquat. Living Resour. 23, 255-266.

Marchetti, J., Bougaran, G., Jauffrais, T., Lefebvre, S., Rouxel, C., Saint-Jean, B., Lukomska, E., Robert, R., Cadoret, J.P., 2012a. Effects of blue light on the biochemical composition and photosynthetic activity of Isochrysis sp. (T-iso). J. Appl. Phycol., *in press*.

Marchetti, J., Bougaran, G., Le Dean, L., Mégrier, C., Lukomska, E., Kaas, R., Olivo, E., Baron, R., Robert, R., Cadoret, J.P., 2012b. Optimizing conditions for the continuous culture of *Isochrysis* affinis *galbana* relevant to commercial hatcheries. Aquacult. 326-329, 106-115.

Marine Institute, B.I.M.a.T.M.T.

Marsden, I.D., Shumway, S.E., 1992. Effects of the toxic dinoflagellate *Alexandrium tamarense* on the greenshell mussel *Perna canaliculus*. N. Z. J. Mar. Freshwat. Res. 26, 371-378.

Matsubara, T., Nagasoe, S., Yamasaki, Y., Shikata, T., Shimasaki, Y., Oshima, Y., Honjo, T., 2007. Effects of temperature, salinity, and irradiance on the growth of the dinoflagellate *Akashiwo sanguinea*. J. Exp. Mar. Biol. Ecol. 342, 226-230.

Mauriz, A., Blanco, J., 2010. Distribution and linkage of domoic acid (amnesic shellfish poisoning toxins) in subcellular fractions of the digestive gland of the scallop *Pecten maximus*. Toxicon 55, 606-611.

May, S.P., Burkholder, J.M., Shumway, S.E., Hegaret, H., Wikfors, G.H., Frank, D., 2010. Effects of the toxic dinoflagellate *Alexandrium monilatum* on survival, grazing and behavioral response of three ecologically important bivalve molluscs. Harmful Algae 9, 281-293.

McCarron, P., Burrell, S., Hess, P., 2007. Effect of addition of antibiotics and an antioxidant on the stability of tissue reference materials for domoic acid, the amnesic shellfish poison. Anal. Bioanal. Chem. 387, 2495-2502.

McCarron, P., Giddings, S.D., Miles, C.O., Quilliam, M.A., 2011a. Derivatization of azaspiracid biotoxins for analysis by liquid chromatography with fluorescence detection. J. Chromatogr. A 1218, 8089-8096.

McCarron, P., Giddings, S.D., Quilliam, M.A., 2011b. A mussel tissue certified reference material for multiple phycotoxins. Part 2: liquid chromatography-mass spectrometry, sample extraction and quantitation procedures. Anal. Bioanal. Chem. 400, 835-846.

McCarron, P., Kilcoyne, J., Hess, P., 2008. Effects of cooking and heat treatment on concentration and tissue distribution of okadaic acid and dinophysistoxin-2 in mussels (*Mytilus edulis*). Toxicon 51, 1081-1089.

McCarron, P., Kilcoyne, J., Miles, C.O., Hess, P., 2009. Formation of azaspiracids-3, -4, -6, and -9 via decarboxylation of carboxyazaspiracid metabolites from shellfish. J. Agric. Food. Chem. 57, 160-169.

McCarron, P., Rourke, W.A., Hardstaff, W., Pooley, B., Quilliam, M.A., 2012. Identification of pinnatoxins and discovery of their fatty acid ester metabolites in mussels (*Mytilus edulis*) from Eastern Canada. J. Agric. Food. Chem. 60, 1437-1446.

McLachlan, J.L., Marr, J.C., Conlon-Kelly, A., Adamson, A., 1994. Effects of nitrogen concentration and cold temperature on DSP-toxin concentrations in the dinoflagellate *Prorocentrum lima* (Prorocentrales, Dinophyceace). Nat. Toxins 2, 263-270.

McMahon, T., Silke, J., 1996. West coast of Ireland winter toxicity of unknown aetiology in mussels. Harmful Algae News 14, 2.

McNabb, P., Selwood, A.I., Holland, P.T., 2005. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. J. AOAC Inter. 88, 761-772.

Medhioub, A., Medhioub, W., Amzil, Z., Sibat, M., Bardouil, M., Ben Neila, I., Mezghani, S., Hamza, A., Lassus, P., 2009. Influence of environmental parameters on *Karenia selliformis* toxin content in culture. Cah. Biol. Mar. 50, 333-342.

Medhioub, W., 2011. Etude des mécanismes de contamination des mollusques bivalves par des neurotoxines à action rapide (FAT) et développement de procédés de détoxification Faculté de science de Tunis et Institut universitaire européen de la mer. Université de Tunis El Mana et Université de Bretagne occidentale, Tunis, p. 202.

Medhioub, W., Gueguen, M., Lassus, P., Bardouil, M., Truquet, P., Sibat, M., Medhioub, N., Soudant, P., Kraiem, M., Amzil, Z., 2010. Detoxification enhancement in the gymnodimine-contaminated grooved carpet shell, *Ruditapes decussatus* (Linne). Harmful Algae 9, 200-207.

Medhioub, W., Lassus, P., Truquet, P., Bardouil, M., Amzil, Z., Sechet, V., Sibat, M., Soudant, P., 2012. Spirolide uptake and detoxification by *Crassostrea gigas* exposed to the toxic dinoflagellate *Alexandrium ostenfeldii*. Aquacult. 358-359, 108-115.

Medhioub, W., Sechet, V., Truquet, P., Bardouil, M., Amzil, Z., Lassus, P., Soudant, P., 2011. *Alexandrium ostenfeldii* growth and spirolide production in batch culture and photobioreactor. Harmful Algae 10, 794-803.

Miles, C.O., Samdal, I.A., Aasen, J.A.G., Jensen, D.J., Quilliam, M.A., Petersen, D., Briggs, L.M., Wilkins, A.L., Rise, F., Cooney, J.M., MacKenzie, A.L., 2005a. Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. Harmful Algae 4, 1075-1091.

Miles, C.O., Wilkins, A.L., Hawkes, A.D., Jensen, D.J., Cooney, J.M., Larsen, K., Petersen, D., Rise, F., Beuzenberg, V., MacKenzie, A.L., 2006. Isolation and identification of a

cis-C-8-diol-ester of okadaic acid from *Dinophysis acuta* in New Zealand. Toxicon 48, 195-203.

Miles, C.O., Wilkins, A.L., Hawkes, A.D., Selwood, A.I., Jensen, D.J., Munday, R., Cooney, J.M., Beuzenberg, V., 2005b. Polyhydroxylated amide analogs of yessotoxin from *Protoceratium reticulatum*. Toxicon 45, 61-71.

Miles, C.O., Wilkins, A.L., Jensen, D.J., Cooney, J.M., Quilliam, M.A., Aasen, J., MacKenzie, A.L., 2004a. Isolation of 41a-homoyessotoxin and the identification of 9-methyl-41a-homoyessotoxin and nor-ring A-yessotoxin from *Protoceratium reticulatum*. Chem. Res. Toxicol. 17, 1414-1422.

Miles, C.O., Wilkins, A.L., Munday, R., Dines, M.H., Hawkes, A.D., Briggs, L.R., Sandvik, M., Jensen, D.J., Cooney, J.M., Holland, P.T., Quilliam, M.A., MacKenzie, A.L., Beuzenberg, V., Towers, N.R., 2004b. Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. Toxicon 43, 1-9.

Miles, C.O., Wilkins, A.L., Samdal, I.A., Sandvik, M., Petersen, D., Quilliam, M.A., Naustvoll, L.J., Rundberget, T., Torgersen, T., Hovgaard, P., Jensen, D.J., Cooney, J.M., 2004c. A novel pectenotoxin, PTX-12, in *Dinophysis* spp. and shellfish from Norway. Chem. Res. Toxicol. 17, 1423-1433.

Miles, C.O., Wilkins, A.L., Stirling, D.J., MacKenzie, A.L., 2003. Gymnodimine C, an isomer of gymnodimine B, from *Karenia selliformis*. J. Agric. Food. Chem. 51, 4838-4840.

Mitrovic, S.M., Amandi, M.F., McKenzie, L., Furey, A., James, K.J., 2004. Effects of selenium, iron and cobalt addition to growth and yessotoxin production of the toxic marine dinoflagellate *Protoceratium reticulatum* in culture. J. Exp. Mar. Biol. Ecol. 313, 337-351.

Molgo, J., Girard, B., Benoit, E., 2007. Cyclic imines: An insight into this emerging group of bioactive marine toxins, in: L., B. (Ed.), Phycotoxins, Chemistry and Biochemistry. Blackwell Publishing, Oxford, pp. 319-335.

Molina Grima, E., Belarbi, E.H., Acien Fernandez, F.G., Robles Medina, A., Chisti, Y., 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol. Adv. 20, 491-515.

Moore, R.E., Bartolini, G., 1981. Stucture of palytoxin. J. Am. Chem. Soc. 103, 2491-2494.

Moore, R.E., Bartolini, G., Barchi, J., Bothnerby, A.A., Dadok, J., Ford, J., 1982. Absolute stereochemistry of palytoxin J. Am. Chem. Soc. 104, 3776-3779.

Moore, R.E., Scheuer, P.J., 1971. Palytoxin: A new marine toxin from a coelenterate. Science 172, 495-498.

Morlaix, M., Lassus, P., 1992. Nitrogen and phosphorus effects upon division rate and toxicity of *Prorocentrum lima* (Ehrenberg) Dodge. Cryptogamie Algologie 13, 187-195.

Morohashi, A., Satake, M., Murata, K., Naoki, H., Kaspar, H.F., Yasumoto, T., 1995. Brevetoxin B3, a new brevetoxin analog isolated from the greenshell mussel *Perna canaliculus* involved in neurotoxic shellfish poisoning in New Zealand. Tetrahedron Lett. 36, 8995-8998.

Morohashi, A., Satake, M., Naoki, H., Kaspar, H.F., Oshima, Y., Yasumoto, T., 1999. Brevetoxin B4 isolated from greenshell mussels *Perna canaliculus*, the major toxin involved in neurotoxic shellfish poisoning in New Zealand. Nat. Toxins 7, 45-48.

Moroney, C., Lehane, M., Brana-Magdalena, A., Furey, A., James, K.J., 2002. Comparison of solid-phase extraction methods for the determination of azaspiracids in shellfish by liquid chromatography-electrospray mass spectrometry. J. Chromatogr. A 963, 353-361.

Morton, S.L., Bomber, J.W., 1994. Maximizing okadaic acid content from *Prorocentrum hoffmannianum* Faust. J. Appl. Phycol. 6, 41-44.

Morton, S.L., Bomber, J.W., Tindall, P.M., 1994. Environmental effects on the production of okadaic acid from *Prorocentrum hoffmanniaum* Faust .1. Temperature, light, salinity J. Exp. Mar. Biol. Ecol. 178, 67-77.

Muñoz, P., Avaria, S., Sievers, H., Prado, R., 1992. Presencia de dinoflagelados toxicos del genero *Dinophysis* en el seno Aysen, Chile. Rev. Biol. Mar. Valparaiso 27, 187-212.

Murata, M., Kumagai, M., Lee, J.S., Yasumoto, T., 1987. Isolation and sructure of yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning Tetrahedron Lett. 28, 5869-5872.

Murata, M., Legrand, A.M., Ishibashi, Y., Fukui, M., Yasumoto, T., 1990. Structures and configurations of ciguatoxin from the moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. J. Am. Chem. Soc. 112, 4380-4386.

Murata, M., Legrand, A.M., Ishibashi, Y., Yasumoto, T., 1989. Structures of ciguatoxin and its congener. J. Am. Chem. Soc. 111, 8929-8931.

Nagai, S., Suzuki, T., Nishikawa, T., Kamiyama, T., 2011. Differences in the production and excretion kinetics of okadaic acid, dinophysistoxin-1, and pectenotoxin-2 between

cultures of *Dinophysis acuminata* and *Dinophysis fortii* isolated from western Japan. J. Phycol. 47, 1326-1337.

Nagasoe, S., Kim, D.I., Shimasaki, Y., Oshima, Y., Yamaguchi, M., Honjo, T., 2006. Effects of temperature, salinity and irradiance on the growth of the red tide dinoflagellate *Gyrodinium instriatum* Freudenthal et Lee. Harmful Algae 5, 20-25.

Navarro, J.M., Contreras, A.M., 2010. An integrative response by *Mytilus chilensis* to the toxic dinoflagellate *Alexandrium catenella*. Mar. Biol. 157, 1967-1974.

Navarro, J.M., Contreras, A.M., Chaparro, O.R., 2008. Short-term feeding response of the mussel *Mytilus chilensis* exposed to diets containing the toxic dinoflagellate *Alexandrium catenella*. Revista Chilena De Historia Natural 81, 41-49.

Navarro, J.M., Munoz, M.G., Contreras, A.M., 2006. Temperature as a factor regulating growth and toxin content in the dinofiagellate *Alexandrium catenella*. Harmful Algae 5, 762-769.

Nicolaou, K.C., 1996. The total synthesis of brevetoxin B: a twelve-year Odyssey in organic synthesis. Angew. Chem. Int. Ed. Engl. 35, 589-607.

Nicolaou, K.C., Chen, D.Y.K., Li, Y.W., Qian, W.Y., Ling, T.T., Vyskocil, S., Koftis, T.V., Govindasamy, M., Uesaka, N., 2003a. Total synthesis of the proposed azaspiracid-1 structure, part 2: Coupling of the C1-C20, C21-C27, and C28-C40 fragments and completion and synthesis. Angew. Chem. Int. Ed. 42, 3649-3653.

Nicolaou, K.C., Chen, D.Y.K., Li, Y.W., Uesaka, N., Petrovic, G., Koftis, T.V., Bernal, F., Frederick, M.O., Govindasamy, M., Ling, T.T., Pihko, P.M., Tang, W.J., Vyskocil, S., 2006a. Total synthesis and structural elucidation of azaspiracid-1. Synthesis-based analysis of originally proposed structures and indication of their non-identity to the natural product. J. Am. Chem. Soc. 128, 2258-2267.

Nicolaou, K.C., Koftis, T.V., Vyskocil, S., Petrovic, G., Ling, T.T., Yamada, Y.M.A., Tang, W.J., Frederick, M.O., 2004a. Structural revision and total synthesis of azaspiracid-1, part 2: Definition of the ABCD domain and total synthesis. Angew. Chem. Int. Ed. 43, 4318-4324.

Nicolaou, K.C., Koftis, T.V., Vyskocil, S., Petrovic, G., Tang, W.J., Frederick, M.O., Chen, D.Y.K., Li, Y.W., Ling, T.T., Yamada, Y.M.A., 2006b. Total synthesis and structural elucidation of azaspiracid-1. Final assignment and total synthesis of the correct structure of azaspiracid-1. J. Am. Chem. Soc. 128, 2859-2872.

Nicolaou, K.C., Li, Y.W., Uesaka, N., Koftis, T.V., Vyskocil, S., Ling, T.T., Govindasamy, M., Qian, W., Bernal, F., Chen, D.Y.K., 2003b. Total synthesis of the proposed

azaspiracid-1 structure, part 1: Construction of the enantiomerically pure C1-C20, C21-C27, and C28-C40 fragments. Angew. Chem. Int. Ed. 42, 3643-3648.

Nicolaou, K.C., Vyskocil, S., Koftis, T.V., Yamada, Y.M.A., Ling, T.T., Chen, D.Y.K., Tang, W.J., Petrovic, G., Frederick, M.O., Li, Y.W., Satake, M., 2004b. Structural revision and total synthesis of azaspiracid-1, part 1: Intelligence gathering and tentative proposal. Angew. Chem. Int. Ed. 43, 4312-4318.

Nicolaou, K.C., Yang, Z., Shi, G.Q., Gunzner, J.L., Agrios, K.A., Gartner, P., 1998. Total synthesis of brevetoxin A. Nature 392, 264-269.

Nielsen, M.V., 1996. Growth and chemical composition of the toxic dinoflagellate *Gymnodinium galatheanum* in relation to irradiance, temperature and salinity. Mar. Ecol. Prog. Ser. 136, 205-211.

Novaczek, I., Madhyastha, M.S., Ablett, R.F., Donald, A., Johnson, G., Nijjar, M.S., Sims, D.E., 1992. Depuration of domoic acid from live blue mussels (*Mytilus edulis*). Can. J. Fish Aquat. Sci. 49, 312-318.

Novaczek, I., Madhyastha, M.S., Ablett, R.F., Johnson, G., Nijjar, M.S., Sims, D.E., 1991. Uptake, disposition and depuration of domoic acid by blue mussels (*Mytilus edulis*). Aquat. Toxicol. 21, 103-118.

Nzoughet, K.J., Hamilton, J.T.G., Floyd, S.D., Douglas, A., Nelson, J., Devine, L., Elliott, C.T., 2008. Azaspiracid: First evidence of protein binding in shellfish. Toxicon 51, 1255-1263.

O'Driscoll, D., Skrabakova, Z., O'Halloran, J., van Pelt, F., James, K.J., 2011. Mussels increase xenobiotic (Azaspiracid) toxicity using a unique bioconversion mechanism. Environ. Sci. Technol. 45, 3102-3108.

Ofuji, K., Satake, M., McMahon, T., James, K.J., Naoki, H., Oshima, Y., Yasumoto, T., 2001. Structures of azaspiracid analogs, azaspiracid-4 and azaspiracid-5, causative toxins of azaspiracid poisoning in Europe. Biosci. Biotechnol., Biochem. 65, 740-742.

Ofuji, K., Satake, M., McMahon, T., Silke, J., James, K.J., Naoki, H., Oshima, Y., Yasumoto, T., 1999a. Two analogs of azaspiracid isolated from mussels, *Mytilus edulis*, involved in human intoxication in Ireland. Nat. Toxins 7, 99-102.

Ofuji, K., Satake, M., Oshima, Y., McMahon, T., James, K.J., Yasumoto, T., 1999b. A sensitive and specific determination method for azaspiracids by liquid chromatography mass spectrometry. Nat. Toxins 7, 247-250.

Ogata, T., Ishimaru, T., Kodama, M., 1987. Effect of water temperature and light intensity on growth rate and toxicity change in *Protogonyaulax tamarensis* Mar. Biol. 95, 217-220.

Onuma, Y., Satake, M., Ukena, T., Roux, J., Chanteau, S., Rasolofonirina, N., Ratsimaloto, M., Naoki, H., Yasumoto, T., 1999. Identification of putative palytoxin as the cause of clupeotoxism. Toxicon 37, 55-65.

Oshima, Y., 1995. Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. J. AOAC Int. 78, 528-532.

Pan, Y., Cembella, A.D., Quilliam, M.A., 1999. Cell cycle and toxin production in the benthic dinoflagellate *Prorocentrum lima*. Mar. Biol. 134, 541-549.

Parker, N.S., Negri, A.P., Frampton, D.M.F., Rodolfi, L., Tredici, M.R., Blackburn, S.I., 2002. Growth of the toxic dinoflagellate *Alexandrium minutum* (Dinophyceae) using high biomass culture systems. J. Appl. Phycol. 14, 313-324.

Parkhill, J.P., Cembella, A.D., 1999. Effects of salinity, light and inorganic nitrogen on growth and toxigenicity of the marine dinoflagellate *Alexandrium tamarense* from northeastern Canada. J. Plankton Res. 21, 939-955.

Paz, B., Vazquez, J.A., Riobo, P., Franco, J.M., 2006. Study of the effect of temperature, irradiance and salinity on growth and yessotoxin production by the dinoflagellate *Protoceratium reticulatum* in culture by using a kinetic and factorial approach. Mar. Environ. Res. 62, 286-300.

Pearce, I., Handlinger, J.H., Hallegraeff, G.M., 2005. Histopathology in Pacific oyster (*Crassostrea gigas*) spat caused by the dinoflagellate *Prorocentrum rhathymum*. Harmful Algae 4, 61-74.

Penna, A., Vila, M., Fraga, S., Giacobbe, M.G., Andreoni, F., Riobo, P., Vernesi, C., 2005. Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8s rDNA sequences. J. Phycol. 41, 212-225.

Perez, R.A., Rehmann, N., Crain, S., LeBlanc, P., Craft, C., MacKinnon, S., Reeves, K., Burton, I.W., Walter, J.A., Hess, P., Quilliam, M.A., Melanson, J.E., 2010. The preparation of certified calibration solutions for azaspiracid-1,-2, and-3, potent marine biotoxins found in shellfish. Anal. Bioanal. Chem. 398, 2243-2252.

Person, J., 2010. Livre turquoise-algues, filière du futur!, in: Adebiotech (Ed.).

Petrusevski, B., Bolier, G., Vanbreemen, A.N., Alaerts, G.J., 1995. Tangential flow filtration - a method to concentrate fresh-water algae. Water Res. 29, 1419-1424.

Pfiester, L.A., Anderson, D.M., 1987. Dinoflagellate reproduction, in: Taylor, F.J.R. (Ed.), The biology of dinoflagellates. Blackwell scientific publications, Oxford, pp. 611-648.

Pierce, R.H., Henry, M.S., Blum, P.C., Hamel, S.L., Kirkpatrick, B., Cheng, Y.S., Zhou, Y., Irvin, C.M., Naar, J., Weidner, A., Fleming, L.E., Backer, L.C., Baden, D.G., 2005. Brevetoxin composition in water and marine aerosol along a Florida beach: Assessing potential human exposure to marine biotoxins. Harmful Algae 4, 965-972.

Pizarro, G., Paz, B., Gonzalez-Gil, S., Franco, J.M., Reguera, B., 2009. Seasonal variability of lipophilic toxins during a *Dinophysis acuta* bloom in Western Iberia: Differences between picked cells and plankton concentrates. Harmful Algae 8, 926-937.

Plakas, S.M., El Said, K.R., Jester, E.L.E., Granade, H.R., Musser, S.M., Dickey, R.W., 2002. Confirmation of brevetoxin metabolism in the Eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and to *Karenia brevis* cultures. Toxicon 40, 721-729.

Plakas, S.M., Wang, Z., El Said, K.R., Jester, E.L.E., Granade, H.R., Flewelling, L., Scott, P., Dickey, R.W., 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44, 667-685.

Poli, M.A., Lewis, R.J., Dickey, R.W., Musser, S.M., Buckner, C.A., Carpenter, L.G., 1997. Identification of Caribbean ciguatoxins as the cause of an outbreak of fish poisoning among US soldiers in Haiti. Toxicon 35, 733-741.

Pollingher, U., Zemel, E., 1981. *In situ* and experimental evidence of the influence of turbulence on cell division processes of *Peridinium cinctum* forma westii (Lemm.) Lefèvre. Br. Phycological J. 16, 281-287.

Poster, D.L., Schantz, M., Kucklick, J.R., Lopez de Alda, M.J., Porter, B. J., Pugh, R., Wise, S.A., 2004. Three new mussel tissue standard reference materials (SRMs) for the determination of organic contaminants. Anal. Bioanal. Chem. 378, 1213-1231.

Potvin, E., Jeong, H.J., Kang, N.S., Tillmann, U., Krock, B., 2012. First report of the photosynthetic dinoflagellate genus *Azadinium* in the Pacific Ocean: morphology and molecular characterization of *Azadinium* cf. *poporum*. J. Eukaryot. Microbiol. 59, 145-156.

Qiu, X., Yamasaki, Y., Shimasaki, Y., Gunjikake, H., Matsubara, T., Nagasoe, S., Etoh, T., Matsui, S., Honjo, T., Oshima, Y., 2011. Growth interactions between the raphidophyte Chattonella antiqua and the dinoflagellate Akashiwo sanguinea. Harmful Algae 11, 81-87.

Quilliam, M., 2001. Analytical chemistry of phycotoxins in seafood and drinking water. J. AOAC Int. 84, 1615-1615.

Quilliam, M.A., 2003. Chemical methods for lipophilic shellfish toxins, in: Hallegraef, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on harmful marine microalgae. UNESCO, Saint-Berthevin, France, pp. 211-245.

Quilliam, M.A., Hardstaff, W.R., Ishida, N., MacLachlan, J.L., Reeves, A.R., Ross, N.W., Windust, A.J., 1996. Production of diarrhetic shellfish poisoning (DSP) toxins by *Prorocentrum lima* in culture and development of analytical methods, in: T. Yasumoto, Y.O., and Y. Fukuyo, (Eds.) (Ed.), Harmful and Toxic Algal Blooms. UNESCO & Tohoku University, Sendai, Japan, pp. 289-292.

Quilliam, M.A., Hess, P., Dell'Aversano, C., 2001. Recent developments in the analysis of phycotoxins by liquid chromatography - mass spectrometry, in: De Koe, W.J., Sampson, R.A., van Egmond, H.P., Gilbert, J., Sabinoan, M. (Eds.), Xth International IUPAC Symposium on Mycotoxins and Phycotoxins, Guaruja, pp. 383-391.

Quilliam, M.A., Xie, M., Hardstaff, W.R., 1995. A rapid extraction and cleanup procedure for the liquid chromatographic determination of domoic acid in unsalted seafood. J. AOAC Int. 78, 543-554.

Rehmann, N., 2008. Preparative isolation and purification of azaspiracids and related toxins from blue mussels and characterisation of new toxin analogs, School of biomolecular and biochemical science. University College Dublin, Dublin, p. 254.

Rehmann, N., Hess, P., Quilliam, M.A., 2008. Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (*Mytilus edulis*) by ultra-performance liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 22, 549-558.

Reizopoulou, S., Strogyloudi, E., Giannakourou, A., Pagou, K., Hatzianestis, L., Pyrgaki, C., Graneli, E., 2008. Okadaic acid accumulation in macrofilter feeders subjected to natural blooms of *Dinophysis acuminata*. Harmful Algae 7, 228-234.

Rhodes, L., McNabb, P., de Salas, M., Briggs, L., Beuzenberg, V., Gladstone, M., 2006. Yessotoxin production by *Gonyaulax spinifera*. Harmful Algae 5, 148-155.

Robinson, J.A., 1991. Polyketides synthase complexes - their structure and function in antibiotic biosynthesis. Philos. Trans. R. Soc. London, Ser. B 332, 107-114.

Roman, Y., Alfonso, A., Louzao, M.C., de la Rosa, L.A., Leira, F., Vieites, J.M., Vieytes, M.R., Ofuji, K., Satake, M., Yasumoto, T., Botana, L.M., 2002. Azaspiracid-1, a potent, nonapoptotic new phycotoxin with several cell targets. Cell. Signalling 14, 703-716.

Rossi, N., Derouiniot-Chaplain, M., Jaouen, P., Legentilhomme, P., Petit, I., 2008. *Arthrospira platensis* harvesting with membranes: Fouling phenomenon with limiting and critical flux. Bioresour. Technol. 99, 6162-6167.

Rossi, N., Petit, I., Jaouen, P., Legentilhomme, P., Derouiniot, M., 2005. Harvesting of cyanobacterium *Arthrospira platensis* using inorganic filtration membranes. Separation Science and Technology 40, 3033-3050.

Rossini, G.P., Hess, P., 2010. Phycotoxins: chemistry, mechanisms of action and shellfish poisoning, in: Luch, A. (Ed.), Molecular, clinical and environmental toxycology, pp. 65-122.

Rouillon, G., Navarro, E., 2003. Differential utilization of species of phytoplankton by the mussel *Mytilus edulis*. Acta Oecologica 24, S299-S305.

Rundberget, T., Sandvik, M., Larsen, K., Pizarro, G.M., Reguera, B., Castberg, T., Gustad, E., Loader, J.I., Rise, F., Wilkins, A.L., Miles, C.O., 2007. Extraction of microalgal toxins by large-scale pumping of seawater in Spain and Norway, and isolation of okadaic acid and dinophysistoxin-2. Toxicon 50, 960-970.

Ryan, M., Ito, E., Speijers, G., Hess, P., 2011. Azaspiracids, in: Lawrence, J., Loreal, H., Toyofuku, H., Hess, P., Iddya, K., Abadouch, L. (Eds.), Assessement and management of biotoxin risks in bivalve molluscs. FAO, Rome, p. 337.

Salas, R., Tillmann, U., John, U., Kilcoyne, J., Burson, A., Cantwell, C., Hess, P., Jauffrais, T., Silke, J., 2011. The role of *Azadinium spinosum* (Dinophyceae) in the production of azaspiracid shellfish poisoning in mussels. Harmful Algae 10, 774-783.

Salim, S., Bosma, R., Vermue, M.H., Wijffels, R.H., 2011. Harvesting of microalgae by bio-flocculation. J. Appl. Phycol. 23, 849-855.

Samdal, I.A., Naustvoll, L.J., Olseng, C.D., Briggs, L.R., Miles, C.O., 2004. Use of ELISA to identify *Protoceratium reticulatum* as a source of yessotoxin in Norway. Toxicon 44, 75-82.

Satake, M., Fukui, M., Legrand, A.M., Cruchet, P., Yasumoto, T., 1998a. Isolation and structures of new ciguatoxin analogs, 2,3-dihydroxyCTX3C and 51-hydroxyCTX3C, accumulated in tropical reef fish. Tetrahedron Lett. 39, 1197-1198.

Satake, M., Morohashi, A., Oguri, H., Oishi, T., Hirama, M., Harada, N., Yasumoto, T., 1997a. The absolute configuration of ciguatoxin. J. Am. Chem. Soc. 119, 11325-11326.

Satake, M., Murata, M., Yasumoto, T., 1993. The structure of CTX3C, a ciguatoxin congener isolated from cultured *Gambierdiscus toxicus* Tetrahedron Lett. 34, 1975-1978.

Satake, M., Ofuji, K., Kevin, J., Furey, A., Yasumoto, T., 1998b. New toxic event caused by Irish mussels, in: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. IOC of UNESCO and Xunta de Galicia, Santiago de Compostela, Spain.

Satake, M., Ofuji, K., Naoki, H., James, K.J., Furey, A., McMahon, T., Silke, J., Yasumoto, T., 1998c. Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. J. Am. Chem. Soc. 120, 9967-9968.

Satake, M., Shoji, M., Oshima, Y., Naoki, H., Fujita, T., Yasumoto, T., 2002. Gymnocin-A, a cytotoxic polyether from the notorious red tide dinoflagellate, *Gymnodium mikimotoi*. Tetrahedron Lett. 43, 5829-5832.

Satake, M., Tanaka, Y., Ishikura, Y., Oshima, Y., Naoki, H., Yasumoto, T., 2005. Gymnocin-B with the largest contiguous polyether rings from the red tide dinoflagellate, *Karenia* (formerly *Gymnodinium*) *mikimotoi*. Tetrahedron Lett. 46, 3537-3540.

Satake, M., Tubaro, A., Lee, J.-S., Yasumoto, T., 1997b. Two new analogs of yessotoxin, homoyessotoxin and 45-hydroxyhomoyessotoxin, isolated from mussels of the Adriatic Sea. Nat. Toxins 5, 107-110.

Schembri, M.A., Neilan, B.A., Saint, C.P., 2001. Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. Environ. Toxicol. 16, 413-421.

Séchet, V., Berard, J.B., Bohec, M., Bougaran, G., Carré, C., Masselin, P., Truquet, P., 2003. Growth and toxicity of *Alexandrium catenella* isolated from Thau lagoon (France) cultured in stirred tank photobioreactors, in: Villalba, A., Reguera, B., Romalde, J.L., Beiras, R. (Eds.), Proceeding of the 4th International conference on molluscan shellfish safety. Conselleria de Pesca e Asuntos Maritimos, Xunta de Calicia; Intergovernmental Oceanographic Commision of UNESCO, Santiago de Compostella, Spain, pp. 135-144.

Séchet, V., Berard, J.B., Bohec, M., Bougaran, G., Carré, C., Masselin, P., Truquet, P., 2004. Continuous-flow and batch cultures of toxic *Alexandrium catenella* and *A. minutum* in stirred tank bioreactors, in: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), Proceeding of the 10th International conference on harmful algae. Florida Marine Research Institute, Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, Intergovernmental Oceanographic Commision of UNESCO, St. Pete Beach, Florida, USA, pp. 399-401.

Seki, T., Satake, M., MacKenzie, L., Kaspar, H.F., Yasumoto, T., 1995. Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium sp.* Tetrahedron Lett. 36, 7093-7096.

Selwood, A.I., Miles, C.O., Wilkins, A.L., van Ginkel, R., Munday, R., Rise, F., McNabb, P., 2010. Isolation, structural determination and acute toxicity of pinnatoxins E, F and G. J. Agric. Food. Chem. 58, 6532-6542.

Shaw, B.L., Battle, H.I., 1957. The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). Can. J. Zool. 35, 325-347.

Shimizu, Y., Chou, H.N., Bando, H., Van Duyne, G., Clardy, J.C., 1986. Structure of brevetoxin A (GB-1 toxin), the most potent toxin in the Florida red tide organism *Gymnodinium breve* (*Ptychodiscus brevis*). J. Am. Chem. Soc. 108, 515-516.

Shumway, S., Cembella, A., 1993. The impact of toxic algae on scallop culture and fisheries. Rev. Fish. Sci. 1, 121-150.

Shumway, S.E., 1989. Toxic algae: a serious threat to shellfish aquaculture. World Aquacult. 20, 65-74.

Shumway, S.E., Cucci, T.L., 1987. The effect of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behavior of bivalves mollusks. Aquat. Toxicol. 10, 9-27.

Shumway, S.E., Cucci, T.L., Newell, R.C., Yentsch, C.M., 1985. Particle selection, ingestion, and absorption in filter-feeding bivalves J. Exp. Mar. Biol. Ecol. 91, 77-92.

Sigma-Aldrich, 2007. Technical Bulletin AL-180: Diazald and Diazomethane Generators, 6 pp. http://www.sigmaaldrich.com/etc/medialib/docs/Aldrich/Bulletin/al_techbull_al180.Par.0001.File.tmp/al_techbull_al180.pdf.

Silvert, W., Bricelj, M., Cembella, A., 1998. Dynamic modelling of PSP toxicity in the surfclam (*Spisula solidissima*): multicompartmental kinetics and biotransformation, in: B., R., Blanco J., M.L., F., T., W. (Eds.), Harmful Algal Blooms. IOCUnesco, pp. 437-440.

Silvert, W.L., Cembella, A.D., 1995. Dynamic modeling of phycotoxin kinetics in the blue mussel, *Mytilus edulis*, with implications for other marine invertabrates. Can. J. Fish. Aquat. Sci. 52, 521-531.

Siu, G.K.Y., Young, M.L.C., Chan, D.K.O., 1997. Environmental and nutritional factors which regulate population dynamics and toxin production in the dinoflagellate *Alexandrium catenella*. Hydrobiologia 352, 117-140.

SlobbevanDrunen, M.E.P., Vossen, R., Couwenberg, F.M.D., Hulsbosch, M.M., Heemskerk, J.W.M., vanDamMieras, M.C.E., Bruggeman, C.A., 1997. Activation of protein kinase C enhances the infection of endothelial cells by human cytomegalovirus. Virus Research 48, 207-213.

Smaal, A., van Stralen, M., Schuiling, E., 2001. The interaction between shellfish culture and ecosystem processes. Can. J. Fish. Aquat. Sci. 58, 991-1002.

Smayda, T.J., 1997. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnol. Oceanogr. 42, 1137-1153.

Smayda, T.J., Reynolds, C.S., 2003. Strategies of marine dinoflagellate survival and some rules of assembly. J. Sea Res. 49, 95-106.

Smolowitz, R., Shumway, S.E., 1997. Possible cytotoxic effects of the dinoflagellate, *Gyrodinium aureolum*, on juvenile bivalve molluscs. Aquacult. Int. 5, 291-300.

Sommer, H., Meyer, K.F., 1937. Paralytic Shellfish Poisoning. A.M.A. Arch. Path., 560-598.

Stobo, L.A., Lacaze, J.P.C.L., Scott, A.C., Gallacher, S., Smith, E.A., Quilliam, M.A., 2005. Liquid chromatography with mass spectrometry-detection of lipophilic shellfish toxins. J. AOAC Int. 88, 1371-1382.

Sullivan, J.M., Swift, E., 2003. Effects of small-scale turbulence on net growth rate and size of ten species of marine dinoflagellates. J. Phycol. 39, 83-94.

Sullivan, J.M., Swift, E., Donaghay, P.L., Rines, J.E.B., 2003. Small-scale turbulence affects the division rate and morphology of two red-tide dinoflagellates. Harmful Algae 2, 183-199.

Suzuki, T., Igarashi, T., Ichimi, K., Watai, M., Suzuki, M., Ogiso, E., Yasumoto, T., 2005. Kinetics of diarrhetic shellfish poisoning toxins, okadaic acid, dinophysistoxin-1, pectenotoxin-6 and yessotoxin in scallops *Patinopecten yessoensis*. Fisheries Science 71, 948-955.

Tachibana, K., Scheuer, P.J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y., Schmitz, F.J., 1981. Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria*. J. Am. Chem. Soc 103, 2469-2471.

Takada, N., Umemura, N., Suenaga, K., Uemura, D., 2001. Structural determination of pteriatoxins A, B and C, extremely potent toxins from the bivalve *Pteria penguin*. Tetrahedron Lett. 42, 3495-3497.

Taleb, H., Vale, P., Amanhir, R., Benhadouch, A., Sagou, R., Chafik, A., 2006. First detection of azaspiracids in mussels in north west Africa. J. Shellfish Res. 25, 1067-1070.

Tang, E.P.Y., 1996. Why do dinoflagellates have lower growth rates? J. Phycol. 32, 80-84.

Taylor, F.J.R., Pollingher, U., 1987. Ecology of dinoflagellate, in: Taylor, F.J.R. (Ed.), The biology of dinoflagellates. Blackwell scientific publications, Oxford, pp. 611-648.

Taylor, F.J.R.E., 1987. The biology of dinoflagellates. Botanical monographs, Blackwell scientific publications 21, 611-648.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.

Tillmann, U., Elbrachter, M., John, U., Krock, B., 2011. A new non-toxic species in the dinoflagellate genus *Azadinium*: *A. poporum* sp. nov. Eur. J. Phycol. 46, 74-87.

Tillmann, U., Elbrachter, M., John, U., Krock, B., Cembella, A., 2010. *Azadinium obesum* (Dinophyceae), a new nontoxic species in the genus that can produce azaspiracid toxins. Phycologia 49, 169-182.

Tillmann, U., Elbrachter, M., Krock, B., John, U., Cembella, A., 2009. *Azadinium spinosum* gen. et sp. nov. (Dinophyceae) identified as a primary producer of azaspiracid toxins. Eur. J. Phycol. 44, 63-79.

Tillmann, U., Salas, R., Gottschling, M., Krock, B., O'Driscoll, D., Elbrächter, M., 2012. *Amphidoma languida* sp. nov. (Dinophyceae) reveals a close relationship between *Amphidoma* and *Azadinium*. Protist 163, 701-719.

Torgersen, T., Aasen, J., Aune, T., 2005. Diarrhetic shellfish poisoning by okadaic acid esters from brown crabs (*Cancer pagurus*) in Norway. Toxicon 46, 572-578.

Torgersen, T., Miles, C.O., Rundberget, T., Wilkins, A.L., 2008a. New esters of okadaic acid in seawater and blue mussels (*Mytilus edulis*). J. Agric. Food. Chem. 56, 9628-9635.

Torgersen, T., Sandvik, M., Lundve, B., Lindegarth, S., 2008b. Profiles and levels of fatty acid esters of okadaic acid group toxins and pectenotoxins during toxin depuration. Part II: Blue mussels (*Mytilus edulis*) and flat oyster (*Ostrea edulis*). Toxicon 52, 418-427.

Torigoe, K., Murata, M., Yasumoto, T., Iwashita, T., 1988. Prorocentrolide, a toxic nitrogeneous macrocycle from a marine dinoflagellate, *Prorocentrum lima*. J. Am. Chem. Soc. 110, 7876-7877.

Trainer, V.L., Bill, B.D., 2004. Characterization of a domoic acid binding site from Pacific razor clam. Aquat. Toxicol. 69, 125-132.

Truxal, L.T., Bourdelais, A.J., Jacocks, H., Abraham, W.M., Baden, D.G., 2010. Characterization of Tamulamides A and B, Polyethers Isolated from the Marine Dinoflagellate *Karenia brevis*. J. Nat. Prod. 73, 536-540.

Tubaro, A., Sosa, S., Altinier, G., Soranzo, M.R., Satake, M., Della Loggia, R., Yasumoto, T., 2004. Short-term oral toxicity of homoyessotoxins, yessotoxin and okadaic acid in mice. Toxicon 43, 439-445.

Tubaro, A., Sosa, S., Carbonatto, M., Altinier, G., Vita, F., Melato, M., Satake, M., Yasumoto, T., 2003. Oral and intraperitoneal acute toxicity studies of yessotoxin and homoyessotoxins in mice. Toxicon 41, 783-792.

Twiner, M.J., El-Ladki, R., Kilcoyne, J., Doucette, G.J., 2012. Comparative effects of the marine algal toxins azaspiracid-1, -2, and -3 on jurkat T lymphocyte cells. Chem. Res. Toxicol. 25, 747-754.

Twiner, M.J., Hess, P., Dechraoui, M.Y.B., McMahon, T., Samons, M.S., Satake, M., Yasumoto, T., Ramsdell, J.S., Doucette, G.J., 2005. Cytotoxic and cytoskeletal effects of azaspiracid-1 on mammalian cell lines. Toxicon 45, 891-900.

Twiner, M.J., Rehmann, N., Hess, P., Doucette, G.J., 2008. Azaspiracid shellfish poisoning: a review on the chemistry, ecology, and toxicology with an emphasis on human health impacts. Mar. Drugs 6, 39-72.

Uemura, D., 2006. Bioorganic studies on marine natural products - Diverse chemical structures and bioactivities. Chemical Record 6, 235-248.

Uemura, D., Chou, T., Haino, T., Nagatsu, A., Fukuzawa, S., Zheng, S., Chen, H., 1995. Pinnatoxin A: a toxic amphoteric macrocycle from the Okinawan bivalve *Pinna muricata*. J. Am. Chem. Soc. 117, 1155-1156.

Uemura, D., Hirata, Y., Iwashita, T., Naoki, H., 1985. Studies on palytoxins Tetrahedron 41, 1007-1017.

Uemura, D., Ueda, K., Hirata, Y., Naoki, H., Iwashita, T., 1981a. Further studies on palytoxin. 1. Tetrahedron Lett. 22, 1909-1912.

Uemura, D., Ueda, K., Hirata, Y., Naoki, H., Iwashita, T., 1981b. Further studies on palytoxin. 2. Structure of palytoxin. Tetrahedron Lett. 22, 2781-2784.

Ueoka, R., Ito, A., Izumikawa, M., Maeda, S., Takagi, M., Shin-Ya, K., Yoshida, M., van Soest, R.W.M., Matsunaga, S., 2009. Isolation of azaspiracid-2 from a marine sponge *Echinoclathria* sp. as a potent cytotoxin. Toxicon 53, 680-684.

Ugwu, C.U., Aoyagi, H., Uchiyama, H., 2008. Photobioreactors for mass cultivation of algae. Bioresour. Technol. 99, 4021-4028.

Vale, P., 2004. Differential dynamics of dinophysistoxins and pectenotoxins between blue mussel and common cockle: a phenomenon originating from the complex toxin profile of *Dinophysis acuta*. Toxicon 44, 123-134.

Vale, P., 2006. Differential dynamics of dinophysistoxins and pectenotoxins, part II: Offshore bivalve species. Toxicon 47, 163-173.

Vale, P., 2007. Chemistry of diarrhetic shellfish poisoning toxins, in: Botana, L.M. (Ed.), Chemistry and pharmacology of marine toxins. Blackwell publishing, Oxford, pp. 211-221.

Vale, P., Bire, R., Hess, P., 2008. Confirmation by LC-MS/MS of azaspiracids in shellfish from the Portuguese north-western coast. Toxicon 51, 1449-1456.

van de Riet, J., Gibbs, R.S., Muggah, P.M., Rourke, W.A., MacNeil, J.D., Quilliam, M.A., 2011. Liquid Chromatography Post-Column Oxidation (PCOX) method for the determination of paralytic shellfish toxins in mussels, clams, oysters, and scallops: collaborative study. J. AOAC Int. 94, 1154-1176.

van Dolah, F.M., Leighfield, T.A., Doucette, G.J., Bean, L., Niedzwiadek, B., Rawn, D.F.K., 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. J. AOAC Int. 92, 1705-1713.

Vanucci, S., Guerrini, F., Milandri, A., Pistocchi, R., 2010. Effects of different levels of N- and P-deficiency on cell yield, okadaic acid, DTX-1, protein and carbohydrate dynamics in the benthic dinoflagellate *Prorocentrum lima*. Harmful Algae 9, 590-599.

Vanucci, S., Pezzolesi, L., Pistocchi, R., Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Tartaglione, L., Guerrini, F., 2012. Nitrogen and phosphorus limitation effects on cell growth, biovolume, and toxin production in *Ostreopsis* cf. *ovata*. Harmful Algae 15, 78-90.

Varkitzi, I., Pagou, K., Graneli, E., Hatzianestis, I., Pyrgaki, C., Pavlidou, A., Montesanto, B., Economou-Amilli, A., 2010. Unbalanced N:P ratios and nutrient stress controlling growth and toxin production of the harmful dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge. Harmful Algae 9, 304-311.

Vidyarathna, N.K., Graneli, E., 2011. Influence of temperature on growth, toxicity and carbohydrate production of a Japanese *Ostreopsis ovata* strain, a toxic-bloom-forming dinoflagellate. Aquat. Microb. Ecol. 65, 261-270.

Villar-Gonzalez, A., Luisa Rodriguez-Velasco, M., Gago-Martinez, A., 2011. Determination of lipophilic toxins by LC/MS/MS: single-laboratory validation. J. AOAC Int. 94, 909-922.

Volmer, D.A., Brombacher, S., Whitehead, B., 2002. Studies on azaspiracid biotoxins. I. Ultrafast high-resolution liquid chromatography/mass spectrometry separations using monolithic columns. Rapid Commun. Mass Spectrom. 16, 2298-2305.

Walter, J.A., Falk, M., Wright, J.L.C., 1994. Chemistry of the shellfish toxin domoic acid: characterization of related compounds. Can. J. Chem. 72, 430-436.

Wang, D.Z., Hsieh, D.P.H., 2002. Effects of nitrate and phosphate on growth and C2 toxin productivity of *Alexandrium tamarense* CI01 in culture. Mar. Pollut. Bull. 45, 286-289.

Wang, Z., Plakas, S.M., El Said, K.R., Jester, L.E., Granade, H.R., Dickey, R.W., 2004. LC/MS analysis of brevetoxin metabolites in the Eastern oyster (*Crassostrea virginica*). Toxicon 43, 455-465.

Whelan, S., Goldman, N., 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol. Biol. Evol. 18, 691-699.

White, A.W., 1976. Growth Inhibition Caused by Turbulence in the Toxic Marine Dinoflagellate *Gonyaulax excavata*. J. Fish. Res. Board Can. 33, 2598-2602.

White, A.W., 1978. Salinity effects on growth and toxin content of *Gonyolax excavata*, a marine dinoflagellate causing paralytic shellfish poisoning. J. Phycol. 14, 475-479.

Wilkins, A.L., Rehmann, N., Torgersen, T., Rundberget, T., Keogh, M., Petersen, D., Hess, P., Rise, F., Miles, C.O., 2006. Identification of fatty acid esters of pectenotoxin-2 seco acid in blue mussels (*Mytilus edulis*) from Ireland. J. Agric. Food. Chem. 54, 5672-5678.

Wyatt, N.B., Gloe, L.M., Brady, P.V., Hewson, J.C., Grillet, A.M., Hankins, M.G., Pohl, P.I., 2012. Critical conditions for ferric chloride-induced flocculation of freshwater algae. Biotechnol. Bioeng. 109, 493-501.

Xu, N., Duan, S.S., Li, A.F., Zhang, C.W., Cai, Z.P., Hu, Z.X., 2010. Effects of temperature, salinity and irradiance on the growth of the harmful dinoflagellate *Prorocentrum donghaiense* Lu. Harmful Algae 9, 13-17.

Yasumoto, T., Murata, M., Lee, J.-S., Torigoe, K., 1989. Polyether toxins produced by dinoflagellates. in: "Mycotoxins and Phycotoxins '88", eds.: Natori S., Hashimoto K.//Ueno Y., 375-382.

Yasumoto, T., Murata, M., Oshima, Y., Matsumoto, G.K., Clardy, J., 1984. Diarrhetic shellfish poisoning. Acs Symposium Series 262, 207-214.

Yasumoto, T., Murata, M., Oshima, Y., Sano, M., 1985. Diarrhetic shellfish toxins. Tetrahedron 41, 1019-1025.

Yasumoto, T., Nakajima, I., Bagnis, R., Adachi, R., 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bull. Jpn. Soc. Sci. Fish. 43, 1021-1026.

Yasumoto, T., Oshima, Y., Sugarawa, W., Fukuyo, Y., Oguri, H., Igarashi, K., Fujinita, N., 1980. Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning Bull. Jpn. Soc. Sci. Fsh., 1405-1411.

Yasumoto, T., Oshima, Y., Yamaguchi, M., 1978. Occurrence of a new type of shellfish poisoning in the Tohoku district. Bull. Jpn. Soc. Sci. Fsh., 1249-1255.

Yu, K.N., Choi, M.C., Shen, X.Y., Wu, R.S.S., Wang, W.X., Lam, P.K.S., 2005. Modeling of depuration of paralytic shellfish toxins in *Chlamys nobilis* and *Perna viridis*. Mar. Pollut. Bull. 50, 474-479.

Yu, K.N., Kwong, R.W.M., Wang, W.X., Lam, P.K.S., 2007. Biokinetics of paralytic shellfish toxins in the green-lipped mussel, *Perna viridis*. Mar. Pollut. Bull. 54, 1068-1071.

Zaman, L., Arakawa, O., Shimosu, A., Onoue, Y., Nishio, S., Shida, Y., Noguchi, T., 1997. Two new isomers of domoic acid from a red alga, *Chondria armata*. Toxicon 35, 205-212.

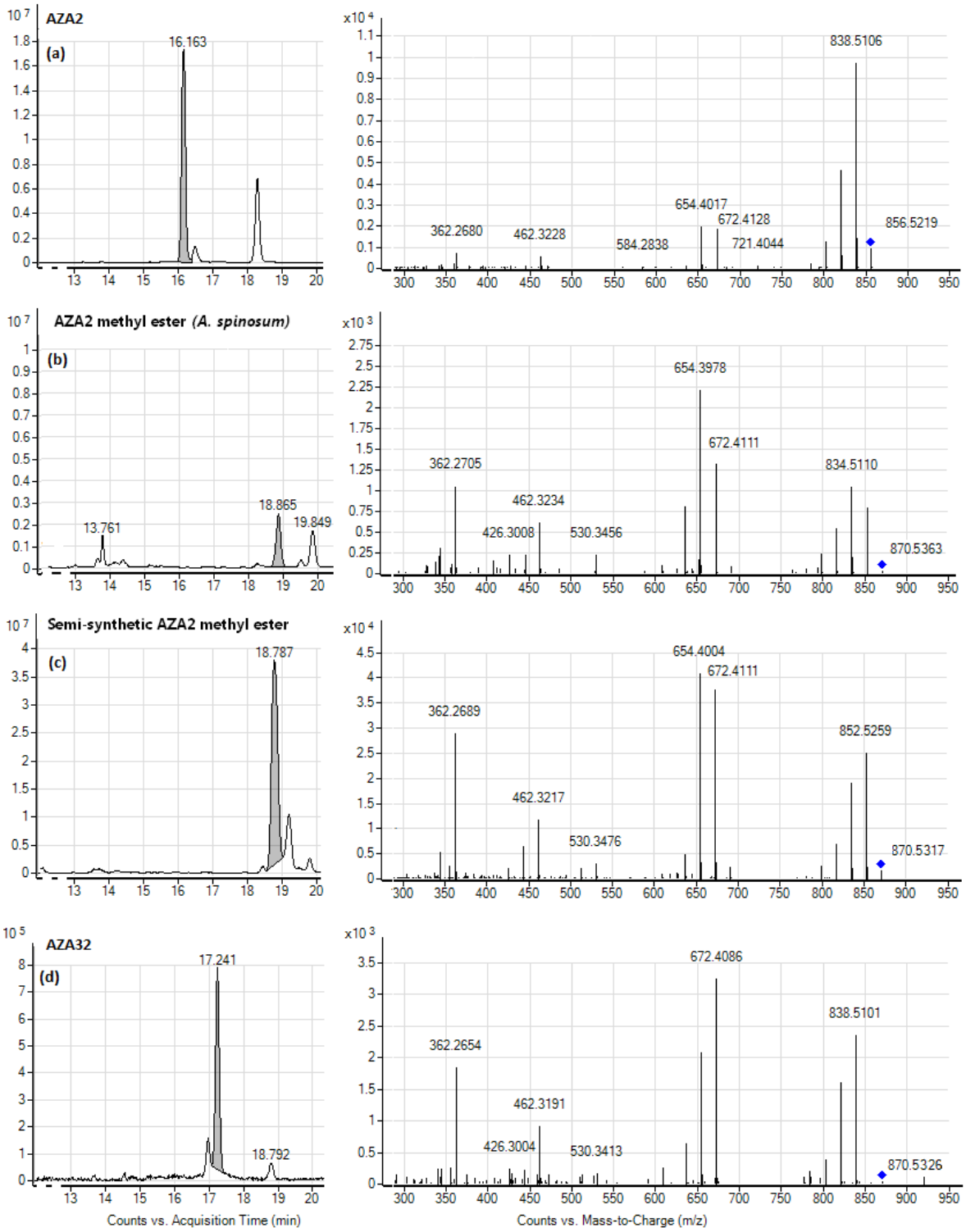
Zirbel, M.J., Veron, F., Latz, M.I., 2000. The reversible effect of flow on the morphology of *Ceratocorys horrida* (Peridiniales, Dinophyta)*. J. Phycol. 36, 46-58.

Annexes

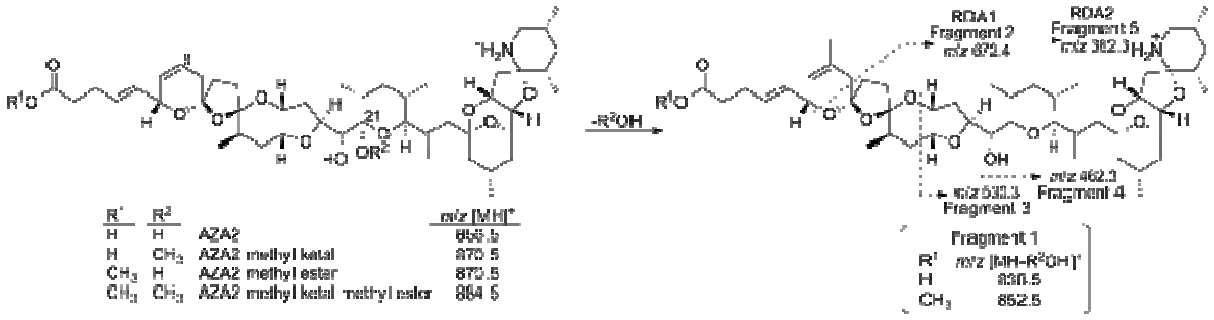
Toxin group	Current EU limits in shellfish meat (A)	Exposure by eating a 400 g portion at the EU limit [c]	Exposure from eating a 400 g portion at the 95th percentile of the concentrations in samples currently on the EU market	ARfD	Corresponding dose for a 60 kg adult	Maximum concentration in shellfish meat to avoid exceeding the ARfD, when eating a 400g portion (B)	Ratio B /A
OA and analogues	160 µg OA eq./kg SM[a]	64 µg OA eq./person (1 µg OA eq./kg b.w.)	96 µg OA eq./person (1.6 µg OA eq./kg b.w).	0.3 µg OA eq./kg b.w.	18 µg OA eq./person	45 µg OA eq./kg SM	0.28
AZA	160 µg AZA eq.[c]/kg SM	64 µg AZA1 eq./person (1 µg AZA1 eq./kg b.w.)	16 µg AZA1 eq./person (0.3 µg AZA1 eq./kg b.w.)	0.2 µg AZA1 eq./kg b.w	12 µg AZA1 eq./person	30 µg AZA1 eq./kg SM	0.19
PTX	160 µg OA eq./kg SM[a]	64 µg PTX2/person (1 µg PTX2 eq./kg b.w.)	32 µg PTX2/person (0.5 µg PTX2 eq./kg b.w.)	0.8 µg PTX2 eq./kg b.w	48 µg PTX2 eq./person	120 µg PTX2 eq./kg SM	0.75
YTX	1 mg YTX eq./kg SM	400 µg YTX eq./person (6.7 µg YTX eq./kg b.w.)	320 µg YTX eq./person (IT) (5.3 µg YTX eq./kg b.w.) 125 µg YTX eq./person (NO) (2.1 µg YTX eq./kg b.w.)	25 µg YTX eq./kg b.w	1500 µg YTX eq./person	3.75 mg YTX eq./kg SM	3.75
STX	800 µg PSP/kg SM[b]	320 µg STX eq./person (5.3 µg STX eq./kg b.w.)	< 260 µg STX eq./person (< 4.3 µg STX eq./kg b.w.)	0.5 µg STX eq./kg b.w	30 µg STX eq./person	75 µg STX eq./kg SM	0.09
DA	20 mg DA/kg SM	8 mg DA[d]/person (130 µg DA/kg b.w)	1 mg DA[d]/person (17 µg DA/kg b.w)	30 µg DA[d]/kg b.w	1.8 mg DA[d]/person	4.5 mg DA[d]/kg SM	0.23

SM: shellfish meat; eq.: equivalents; b.w.: body weight; ARfD: acute reference dose; PSP: paralytic shellfish poison; EU: European Union; IT: Italy; NO: Norway; OA: okadaic acid; PTX: pectenotoxin; YTX: yessotoxin; STX: saxitoxin; DA: domoic acid.
(a): For OA, dinophysistoxins and PTX, current regulation specifies a combination; however the CONTAM Panel concluded that PTX should be considered separately.
(b): In the Commission Regulation (EC) No 853/2004 a limit value of 800 µg PSP/kg SM is given. In the EFSA opinion, the CONTAM Panel adopted this figure as being expressed as µg STX equivalents/kg SM.
(c): The CONTAM Panel assumed that AZA equivalent should refer to AZA1 equivalents.
(d): Applies to the sum of DA and epi-DA.

Annexe 1. Seuils sanitaires européens actuellement en vigueurs pour les phycotoxines réglementés dans les fruits de mer, les ARfDs (acute reference dose = DMENO, dose minimale avec effet nocifs observés) fixés par l'EFSA, et les concentrations correspondantes dans la chair des coquillages.



Annexe 2. LC-MS chromatograms and targeted high resolution MS/MS spectra, obtained with an Agilent 6540 QTOF, for AZA2 and derivatives: (a) AZA2; (b) AZA2 methyl ester in an *A. spinosum* extract; (c) semi-synthetic AZA2 methyl ester produced from AZA2 via derivatization with diazomethane, and; (d) AZA32 obtained from long-term storage of AZA2 in MeOH

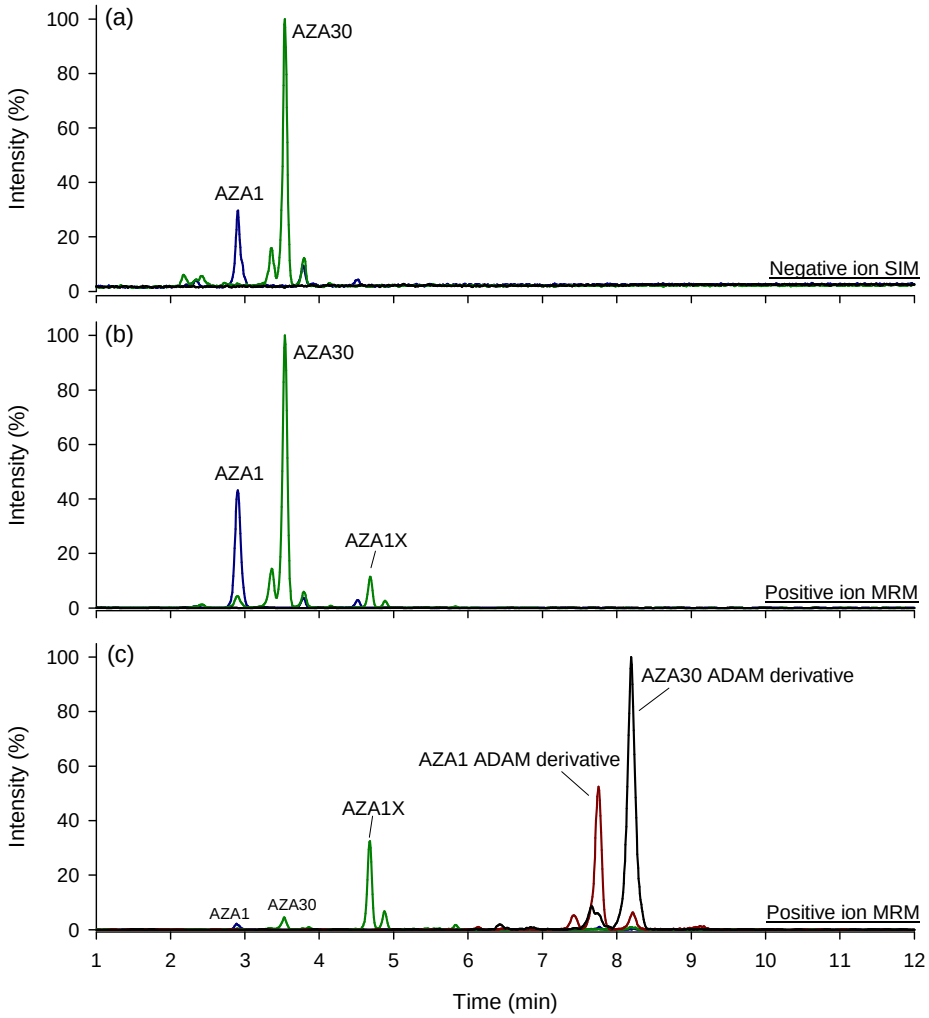


Annexe 3. Structure and *m/z* for $[M + H]^+$ ions of AZA2, AZA2 methyl ketal, AZA2 methyl ester, AZA2 bismethyl ketal ester and MS/MS fragmentation with indicated groups

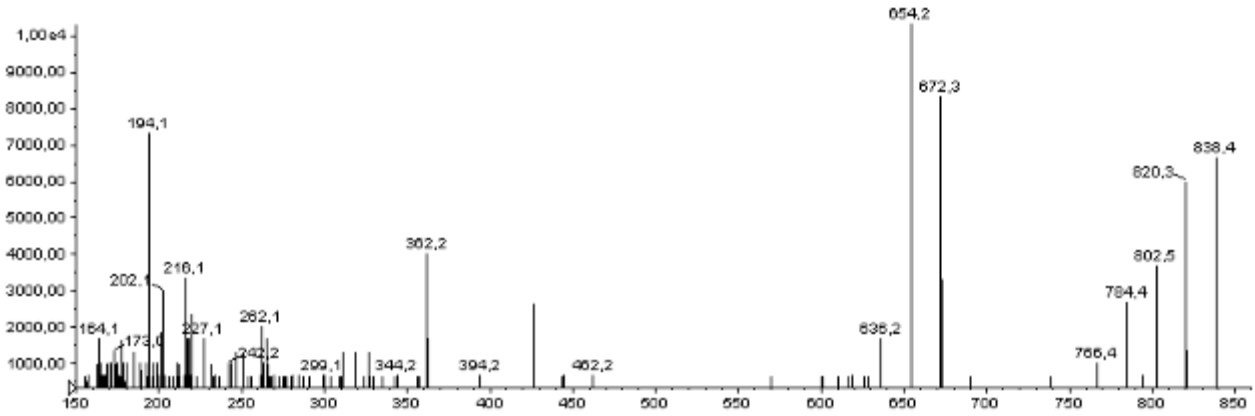
Ion		AZA2	AZA2 methyl ester (artefact)	AZA2 methyl ester (semi-synthetic)	AZA32
$[MH]^+$	Formula	$C_{48}H_{74}NO_{12}^+$	$C_{49}H_{76}NO_{12}^+$	$C_{49}H_{76}NO_{12}^+$	$C_{49}H_{76}NO_{12}^+$
	m/z (Δ)	856.5219 (1.57)	870.5363 (0.11)	870.5317 (5.17)	870.5326 (4.14)
$[MH-ROH]^+$	Formula	$C_{48}H_{71}NO_{11}^+$	$C_{49}H_{73}NO_{12}^+$	$C_{49}H_{73}NO_{12}^+$	$C_{48}H_{72}NO_{11}^+$
(Fragment 1)	*m/z* (Δ)	838.5106 (0.72)	852.5241 (1.81)	852.5241 (0.3)	838.5101 (0.12)
Fragment 2	Formula	$C_{38}H_{59}NO_9^+$	$C_{38}H_{59}NO_9^+$	$C_{38}H_{59}NO_9^+$	$C_{38}H_{59}NO_9^+$
(RDA 1)	*m/z* (Δ)	672.4128 (3.27)	672.4111 (0.74)	672.4111 (0.74)	672.4086 (2.97)
Fragment 3*	Formula	$C_{31}H_{49}NO_6^+$	$C_{31}H_{49}NO_6^+$	$C_{31}H_{49}NO_6^+$	$C_{31}H_{49}NO_6^+$
	m/z (Δ)	530.3419 (10.8)	530.3456 (3.77)	530.3476 (0.00)	530.3413 (11.9)
Fragment 4	Formula	$C_{27}H_{45}NO_5^+$	$C_{27}H_{45}NO_5^+$	$C_{27}H_{45}NO_5^+$	$C_{27}H_{45}NO_5^+$
	m/z (Δ)	462.3228 (3.03)	462.3234 (4.33)	462.3217 (0.65)	462.3191 (4.97)
Fragment 5	Formula	$C_{22}H_{37}NO_3^+$	$C_{22}H_{37}NO_3^+$	$C_{22}H_{37}NO_3^+$	$C_{22}H_{37}NO_3^+$
(RDA 2)	*m/z* (Δ)	362.2680 (2.76)	362.2705 (4.14)	362.2689 (0.28)	(9.94)

*The abundance of the 530 ion was very low, explaining the somewhat higher mass errors for this group

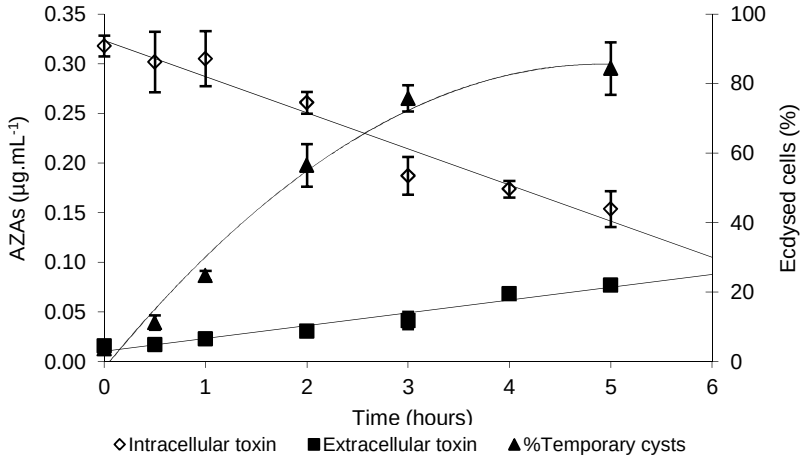
Annexe 4 High resolution LC-MS and LC-MS/MS data (measured *m/z* and Δ (ppm)) for AZA2, its methyl ester (extraction artefact from *A. spinosum*, and semi-synthetic), and AZA32 (AZA2 methyl ketal). Fragment ions correspond to Fig. S2



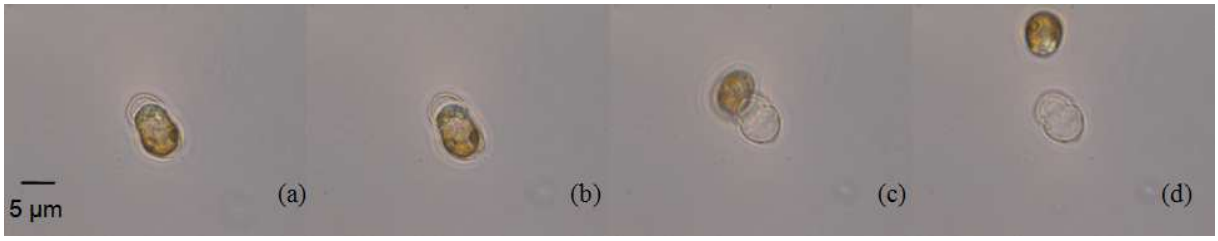
Annexe 5. Gradient elution LC-MS(/MS) analysis of a sample containing AZA1, AZA30 and AZA1 methyl ester: (a) negative ion SIM mode ($[M-H]^-$ of 840.5, 854.5, 868.5) showing AZA1 and AZ30, while AZA1 methyl ester is not detected; (b) positive ion MRM mode ($[M+H]^+$ of 842→672, 856→672, 1032→672, 1046→672) showing AZA1, AZA30 and AZA1 methyl ester (AZA1X), and; (c) the same sample following ADAM derivatization, showing successful derivatization of AZA1 and AZA30 as their (9-anthryl)methyl esters, whereas AZA1 methyl ester is unaffected (because it is already esterified, and so is unable to react)



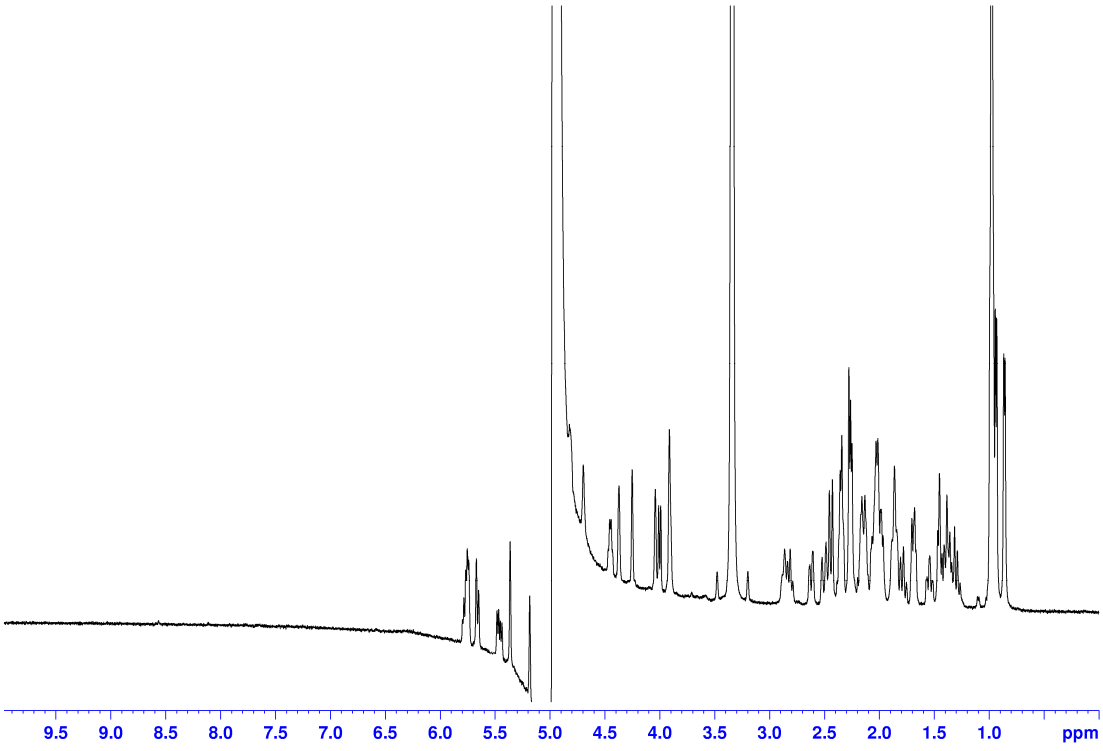
Annexe 6. AZA1 methyl ester spectrum previously reported by Krock et *al.* obtained through extraction of *A. spinosum* and analysed with an API4000 Qtrap (CUR: 10 psi, CAD: Medium, IS: 5500 V, TEM: 0, GS1: 10, GS2: 0, DP: 100, CES: 0, CE: 70 V)



Annexe 7. Effect of residence time in a sampling tube on *A. spinosum*, prior to extraction, on the measured intracellular and extracellular azaspiracid content



Annexe 8. a-d Different phases of *A. spinosum* dehiscence, b-c, protoplast extrusion from the theca



Annexe 9. ^{1}H NMR spectrum of AZA1 following purification from *A. spinosum*

Block	$NaNO_3$ (µM)	NH_4Cl (µM)	Urea (µM)	µ (day^{-1})	Cmax (cell mL^{-1})	AZAs (fg $cell^{-1}$)
1	882	50	50	0.336	100100	11.15
1	50	0	0	0.397	95050	17.23
1	1714	0	0	0.4	93183	11.02
1	50	100	0	0.279	98783	10.06
1	1714	100	0	0.246	92033	11.71
1	50	0	100	0.392	95750	11.81
1	1714	0	100	0.405	95617	12.93
1	50	100	100	0.27	102100	11.88
1	882	50	50	0.327	99350	17.49
1	1714	100	100	0.253	92050	17.74
1	50	50	50	0.316	97650	20.67
1	1714	50	50	0.342	96183	21.57
1	882	0	50	0.38	89450	21.96
1	882	100	50	0.254	95050	16.69
1	882	50	0	0.331	92800	20.65
1	882	50	100	0.332	98867	17.19
1	882	50	50	0.347	100700	16.42
2	882	50	50	0.353	101700	16.2
2	50	0	0	0.385	94417	17.41
2	1714	0	0	0.425	92133	12.11
2	50	100	0	0.289	102600	10.2
2	1714	100	0	0.266	95383	10.74
2	50	0	100	0.398	95150	12.88
2	1714	0	100	0.401	95333	10.94
2	50	100	100	0.271	115400	10.68
2	882	50	50	0.336	96988	22.94
2	1714	100	100	0.218	90950	16.94
2	50	50	50	0.333	101300	19.56
2	1714	50	50	0.336	93750	20.22
2	882	0	50	0.387	88500	19.18
2	882	100	50	0.242	94083	16.16
2	882	50	0	0.319	96217	18.68
2	882	50	100	0.342	99983	19.22
2	882	50	50	0.327	100900	17.71

Annexe 10. The two blocks used to assess the effect of 3 nitrogen sources using a factorial design on *A. spinosum* growth rate, maximum cell concentration and toxin production

K Medium (Keller and Guillard 1985, Keller *et al.* 1987)

This enriched seawater medium was designed specifically for oligotrophic (oceanic) marine phytoplankters that are poisoned by higher levels of trace metals. The medium uses a 10 fold higher EDTA chelation than most common marine media, and a substantial number of trace elements are included. The necessity of Tris is questionable, and it may be omitted. If organisms do not require silica, the silicate solution should be omitted because it enhances precipitation. To prepare, begin with 950 mL of filtered natural seawater, add the following components and then bring the final volume up to 1 liter with filtered natural seawater. Autoclave.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75.00 g /L dH_2O	1 mL	8.82×10^{-4} M
NH_4Cl	2.67 g/ L dH_2O	1 mL	5.00×10^{-5} M
Na_2 b-glycerophosphate*	2.16 g/ L dH_2O	1 mL	1.00×10^{-5} M
$Na_2SiO_3 \cdot 9H_2O$	15.35 g/ L dH_2O	1 mL	5.04×10^{-4} M
H_2SeO_3	1.29 mg/ L dH_2O	1 mL	1.00×10^{-8} M
Tris-base (pH 7.2)	121.10 g/ L dH_2O	1 mL	1.00×10^{-3} M
trace metal solution	(see recipe below)	1 mL	---
vitamin solution	(see recipe below)	0.5 mL	---

Trace Metal Solution

To prepare, dissolve the following components to 950 mL of dH_2O (heat if necessary) and adjust the pH up with sodium hydroxide until all the components are in solution, approximately 20 pellets per liter of trace metal solution. Bring the final volume to 1 liter using dH_2O.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$Na_2EDTA \cdot 2H_2O$	---	41.60g	1.11×10^{-4} M
$FeCl_3 \cdot 6\ H_2O$	---	3.150 g	1.17×10^{-5} M
$MnCl_2 \cdot 4H_2O$	---	0.178 g	9.00×10^{-7} M
$ZnSO_4 \cdot 7H_2O$	23.00 g/ L dH_2O	1 mL	8.00×10^{-8} M
$CoCl_2 \cdot 6\ H_2O$	10.00 g/ LdH_2O	1 mL	5.00×10^{-8} M
$Na_2MoO_4 \cdot 2H_2O$	6.3 g/L dH_2O	1 mL	2.60×10^{-8} M
$CuSO_4 \cdot 5H_2O$	2.50 g/ L dH_2O	1 mL	1.00×10^{-8} M

f/2 Vitamin Solution below

Keller, M.D. and Guillard, R.R.L. 1985. Factors significant to marine diatom culture. pp. 113-6. *In* Anderson, D.M., White, A.W. and Baden, D.G. (Taylor) *Toxic Dinoflagellates*. Elsevier, New York.

Keller, M.D., Selvin, R.C., Claus, W. and Guillard, R.R.L. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* **23:** 633-638.

L1 Medium (Guillard and Hargraves, 1993)

This enriched seawater medium is based upon f/2 medium (Guillard and Ryther 1962) but has additional trace metals. It is a general purpose marine medium for growing coastal algae. To prepare, begin with 950 mL of filtered natural seawater. Add the quantity of each component as indicated below, and then bring the final volume to 1 liter using filtered natural seawater. The trace element solution and vitamin solutions are given below. Autoclave. Final pH should be 8.0 to 8.2.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75.00 g L^{-1} dH_2O	1 mL	8.82×10^{-4} M
$NaH_2PO_4 \cdot H_2O$	5.00 g L^{-1} dH_2O	1 mL	3.62×10^{-5} M
$Na_2SiO_3 \cdot 9\ H_2O$	30.00 g L^{-1} dH_2O	1 mL	1.06×10^{-4} M
trace element solution	(see recipe below)	1 mL	---
vitamin solution	(see recipe above)	0.5mL	---

L1 Trace Element Solution

To 950 mL dH_2O add the following components and bring final volume to 1 liter with dH_2O. Autoclave.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$Na_2EDTA \cdot 2H_2O$	---	4.36 g	1.17×10^{-5} M
$FeCl_3 \cdot 6H_2O$	---	3.15 g	1.17×10^{-5} M
$MnCl_2 \cdot 4\ H_2O$	178.10 g L^{-1} dH_2O	1 mL	9.09×10^{-7} M
$ZnSO_4 \cdot 7H_2O$	23.00 g L^{-1} dH_2O	1 mL	8.00×10^{-8} M
$CoCl_2 \cdot 6H_2O$	11.90 g L^{-1} dH_2O	1 mL	5.00×10^{-8} M
$CuSO_4 \cdot 5H_2O$	2.50 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
$Na_2MoO_4 \cdot 2H_2O$	19.9 g L^{-1} dH_2O	1 mL	8.22×10^{-8} M
H_2SeO_3	1.29 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
$NiSO_4 \cdot 6H_2O$	2.63 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
Na_3VO_4	1.84 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M
K_2CrO_4	1.94 g L^{-1} dH_2O	1 mL	1.00×10^{-8} M

f/2 Vitamin Solution as below

Guillard, R.R.L. and Hargraves, P.E. 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. *Phycologia* **32:** 234-236.

f/2 Medium (Guillard and Ryther 1962, Guillard 1975)

This is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, termed "f Medium" (Guillard and Ryther 1962), has been reduced by half. To prepare, begin with 950 mL of filtered natural seawater and add the following components. The trace element and vitamin solutions are provided below. Bring the final volume to 1 liter with filtered natural seawater. If the alga to be grown does not require silica, then it is recommended that the silica be omitted because it enhances precipitation. Autoclave.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75 g/L dH_2O	1 mL	8.82×10^{-4} M
NaH_2PO_4 H_2O	5 g/L dH_2O	1 mL	3.62×10^{-5} M
Na_2SiO_3 $9H_2O$	30 g/L dH_2O	1 mL	1.06×10^{-4} M
trace metal solution	(see recipe below)	1 mL	---
vitamin solution	(see recipe below)	0.5 mL	---

f/2 Trace Metal Solution

To prepare, begin with 950 mL of dH_2O, add the components and bring final volume to 1 liter with dH_2O. Autoclave. Note that the original medium (Guillard and Ryther 1962) used ferric sequestrene; we have substituted $Na_2EDTA \cdot 2H_2O$ and $FeCl_3 \cdot 6$ H_2O.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
$FeCl_3$ $6H_2O$	---	3.15 g	1.17×10^{-5} M
Na_2EDTA $2H_2O$	---	4.36 g	1.17×10^{-5} M
$CuSO_4$ $5H_2O$	9.8 g/L dH_2O	1 mL	3.93×10^{-8} M
Na_2MoO_4 $2H_2O$	6.3 g/L dH_2O	1 mL	2.60×10^{-8} M
ZnSO4 $7H_2O$	22.0 g/L dH_2O	1 mL	7.65×10^{-8} M
$CoCl_2$ $6H_2O$	10.0 g/L dH_2O	1 mL	4.20×10^{-8} M
$MnCl_2$ $4H_2O$	180.0 g/L dH_2O	1 mL	9.10×10^{-7} M

f/2 Vitamin Solution

First, prepare primary stock solutions. To prepare final vitamin solution, begin with 950 mL of dH_2O, dissolve the thiamine, add the amounts of the primary stocks as indicated in the quantity column below, and bring final volume to 1 liter with dH_2O. Filter sterilize. Store in refrigerator or freezer.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
biotin (vit. H)	0.1 g/L dH_2O	10 mL	2.05×10^{-9} M
cyanocobalamin (vit. B_{12})	1.0 g/L dH_2O	1 mL	3.69×10^{-10} M

Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates. pp 26-60. *In* Smith W.L. and Chanley M.H (Taylor) *Culture of Marine Invertebrate Animals*. Plenum Press, New York, USA.

Guillard, R.R.L. and Ryther, J.H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. *Can. J. Microbiol.* **8:** 229-239.

Annexe 11. Culture medium used during the experiments, K, L1 and f/2 medium

Résumé

Cette thèse a été menée pour développer une méthode d'analyse et de production des azaspiracides (AZA) à partir de cultures d'*A. spinosum*. Elle a aussi eu pour objectif l'étude des facteurs environnementaux et nutritionnels influant sur la croissance et la production toxinique d'*A. spinosum*. La mise en évidence du lien entre cet organisme et l'accumulation d'AZA dans les moules, ainsi que la clarification des processus d'accumulation, de détoxification et de biotransformation des AZA dans les moules a par ailleurs été réalisée.

Les travaux réalisés sur l'analyse des AZA ont permis de définir une procédure d'analyse fiable qui limite notamment la formation d'artéfacts (esters méthyliques d'AZA). Cette étude a permis d'expliquer la formation des AZA méthylés, de déterminer leur structure et de proposer des solutions pour minimiser la formation de ces composés.

Ce travail a aussi démontré la faisabilité d'une production durable d'AZA à partir de cultures d'*A. spinosum* et a mis en évidence les principaux facteurs influant sur la croissance d'*A. spinosum* et sur la production toxinique. Des expériences de contamination sur des moules ont ensuite été réalisées et ont démontré, pour la première fois, le lien directe entre *A. spinosum* et l'accumulation des AZA par les moules. Cette accumulation des toxines est très rapide et atteint des concentrations en AZA qui se situent au-delà du seuil réglementaire après seulement 6 h d'exposition. Ces expériences ont aussi montré une rapide biotransformation des AZA dans les moules. Elles ont par ailleurs clarifié les cinétiques d'apparition des différents analogues d'AZA et ont montré que la détoxification des AZA est bi-phasique. Deux dernières études ont mis en évidence, d'une part, l'effet négatif d'*A. spinosum* sur l'activité alimentaire des moules, et, d'autre part, la capacité des moules à accumuler les AZA présents dans le milieu sous formes dissoute ou particulaire.

Mots clés : *Azadinium spinosum*, *Mytilus edulis*, azaspiracides, phycotoxines, dinoflagellés, écophysiologie, CL-SM/SM

Abstract

This study has been conducted in order to develop the analysis of AZAs and to produce AZAs from *A. spinosum* culture. It also aimed at studying the effect of environmental and nutritional factors on growth and toxin production. The study also demonstrated a link between *A. spinosum* and the accumulation of AZAs in shellfish, followed by the clarification of the processes of accumulation, detoxification and biotransformation of AZAs into mussels.

A quantitative analysis of AZAs in *A. spinosum* cultures was developed, and the formation and structure of the AZA methylated analogues was explained and minimised. This work also demonstrated the feasibility of a sustainable production of AZAs from *A. spinosum* culture and highlighted the main factors influencing growth and toxin production of *A. spinosum*. Using these results, mussel contaminations were performed and demonstrated for the first time the direct link between *A. spinosum* and AZA accumulation into mussels. Furthermore, a rapid AZA accumulation above the regulatory limit was observed within 6 h of exposure. These experiments also highlighted the rapid biotransformation of AZA into analogues in shellfish and clarified their kinetics of appearance. Consequently, AZA biotransformation pathways were proposed for different AZA analogues. AZA detoxification was also studied and showed a detoxification with two compartments. Finally, two recent studies demonstrated the negative effect of *A. spinosum* on the feeding activity of mussels as well as the ability of mussels to accumulate AZA from dissolved or particulate forms.

Key words: *Azadinium spinosum*, *Mytilus edulis*, azaspiracids, phycotoxins, dinoflagellate, ecophysiology, LC-MS/MS