The dinophycean genus *Azadinium* and related species – morphological and molecular characterization, biogeography, and toxins

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Abstract

Azaspiracids (AZAs) are the most recently discovered group of lipophilic marine biotoxins of microalgal origin. It took about twelve years from the first human poisoning event until a culprit for AZA production was unambiguously identified and described as a novel species, *Azadinium spinosum*, within a newly created genus. Since then, knowledge on the genus has increased considerably, and an update on the current circumscription of the genus is presented here including various aspects of morphology, phylogeny, biogeography, and toxin production. There are currently five described species: *A. spinosum*, *A. obesum*, *A. poporum*, *A. caudatum*, and *A. polongum*. As indicated by molecular sequence variation detected in field samples, there are probably more species to recognize. Moreover, *Amphidoma languida* has been described recently, and this species is the closest relative of *Azadinium* based on both molecular and morphological data. *Amphidoma* and *Azadinium* are now grouped in the family Amphidomataceae, which forms an independent lineage among other monophyletic major groups of dinophytes. Initially, azaspiracids have been detected in *A. spinosum* only, but AZA production within the Amphidomataceae appears complex and diverse: A new type of azaspiracid, with a number of structural variants, has been detected in *A. poporum* and *Amphidoma languida*, and AZA-2 has now been detected in Chinese strains of *A. poporum*.

Keywords: Azadinium, azaspiracids, Amphidomataceae, shellfish toxin

Introduction

Among the known marine toxins responsible for shellfish contamination, azaspiracids (AZAs) are the most recently discovered group of lipophilic compounds of microalgal origin. Compared to the knowledge on toxin structure, detection methods, and toxicology, convincing clarification of the aetiology of azaspiracid-poisoning was seriously lacking for quite a long time. AZA toxins are known for their seasonal and episodic accumulation in suspension-feeding shellfish (Salas *et al.* 2011) – a situation similar with several other marine biotoxins from microalgal origin – and thus, a planktonic source has been suspected from the outset. In addition, due to their polyether structural features, a dinophyte origin of AZAs has *ab initio* been suspected. Subsequently, it did not came as a surprise that it was a dinophycean species, *Protoperidinium crassipes*, which was first claimed to be the source of AZAs (James *et al.* 2003). However, production of AZAs by *P. crassipes* could not be verified since then (Gribble 2006). Moreover, in contrast to other identified producers of phycotoxins, which are all primarily phototrophic, *P. crassipes* is a large (> 50 µm) heterotrophic species, known to prey upon other dinophytes (Gribble and Anderson 2006).

During a research cruise with RV Poseidon in the North Sea in 2007, this issue became quite evident when toxin analysis of fractionated plankton samples clearly showed that (1) high amounts of AZAs were found at stations where *P. crassipes* was absent, (2) AZAs could be found in isolated cells of the predatory ciliate *Favella ehrenbergii*, and (3) in fractionated plankton samples, the

Tab. 1: Currently described species of *Azadinium* (and *Amphidoma languida*). Scale bar = 5 µm

| Feature | *Azadinium spinosum* [a] | *Azadinium obesum* [b] | *Azadinium poporum* [c] | *Azadinium caudatum* [d] | | *Azadinium polongum* [e] | *Amphidoma languida* [f] |
				var. *margalefii*	var. *caudatum*		
Size (length x width)	13.8 x 8.8	15.3 x 11.7	13.0 x 9.8	31.3 x 22.4 [1]	41.7 x 28.7 [1]	13.0 x 9.7	13.9 x 11.9
Length/width ratio	1.6	1.3	1.3	1.2 [2]	1.2 [2]	1.3	1.2
Stalked pyrenoid(s)	1, central episome	no	several (up to four)	no	no	no	1, central episome
Apical and intercalary plates	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	6 apicals, 0 intercalaries
Antapical projection	Small spine	no	no	short horn, long spine	long horn, short spine	small spine	antapical pore
Location "ventral" pore	left side of 1´ (median position)	left side of 1´ (median position)	left side of pore plate	right side of pore plate	right side of 1´ (post. pos.)	left side, suture of 1´and 1´´	right side of 1´ (anterior position)
Shape of pore plate	round /ellipsoid	round /ellipsoid	round /ellipsoid	round /ellipsoid	round /ellipsoid	distinctly elongated	round /ellipsoid
Contact of ventral precing. with intercal.	1´´ in contact with 1a	no	1´´ in contact with 1a	1´´ in cont. with 1a, 6´´ in contact with 3a	1´´ in contact with 1a, 6´´ in contact with 3a	1´´ in contact with 1a	not appl. (no intercalaries)
Shape of plate 4´´	size as other precing., in contact with 3a	size as other precing., in contact with 3a	size as other precing., in contact with 3a	distinctly smaller, no contact with 3a	distinctly smaller, no contact with 3a	size as other precing., in contact with 3a	size as other precingular

[1] size without antapical projection [2] length/width ratio without antapical projection. precing. = precingular plate
References: [a] *A. spinosum* Elbrächter et Tillmann, Tillmann *et al.* 2009. [b] *A. obesum* Tillmann et Elbrächter, Tillmann *et al.* 2010. [c] *A. poporum* Tillmann et Elbrächter, Tillmann *et al.* 2011. [d] *A. caudatum* (Halldal) Nézan et Chomerat, Nézan *et al.* 2012. [e] *A. polongum* Tillmann, Tillmann *et al.* 2012b. [f] *Amphidoma languida* Tillmann, Salas et Elbrächter, Tillmann *et al.* 2012a.

largest AZA amounts were found in the small size (<20 µm) class (Krock *et al.* 2009). All these indications led to the isolation of a small dinophyte, which was shown to produce AZA-1 and -2 in axenic culture (Krock *et al.* 2009) and which was identified as the a species, *Azadinium spinosum,* in a newly erected genus (Tillmann *et al.* 2009). Since then, knowledge on the genus has increased considerably. Here, we present an update on the current circumscription of the genus including various aspects on morphological and molecular characterisation, biogeography, and toxin production.

The species
Considering the short period since the first identification of *Azadinium*, the known diversity of the genus has increased rapidly and now comprises five species. *Azadinium spinosum*, the type species of the genus, as well as *Azadinium obesum* were firstly isolated from the same water sample taken from the North Sea off Scotland (Tillmann *et al.* 2009, 2010). Later, *Azadinium poporum* was described from three clones isolated from the southern North Sea off the Danish coast (Tillmann *et al.* 2011). *Azadinium caudatum*, which was initially described in 1953 by Halldal as *Amphidoma caudata,* was recently transferred to the genus *Azadinium* (Nézan *et al.* 2012). Both DNA sequence and morphometric data clearly showed that the species occurred with two distinct varieties, var. *caudatum* and var. *margalefii*. They are easy to distinguish by the different shape of the antapical projection. *Azadinium polongum* – isolated from the Shetland Islands – is the most recently described species of *Azadinium* (Tillmann *et al.* 2012b).

With the description of *Amphidoma languida*, a genus closely related to *Azadinium* could be identified (Tillmann *et al.* 2012a). *Amphidoma languida* has been isolated concurrently with the Irish strain of *A. spinosum* from Bantry Bay, Ireland (Tillmann *et al.* 2012a, Salas *et al.* 2011).

Morphological and molecular characterization
With the exception of *A. caudatum*, all species of *Azadinium* and *Amphidoma languida* are of small size and of similar shape (Tab. 1). They are photosynthetically active with rather typical peridinin pigment profiles (e.g., Tillmann *et al.* 2009). They presumably possess a single chloroplast which is parietally arranged. As a result of a distinct starch cup, stalked pyrenoid(s) are visible in light microscopy for a number of species. All species of *Azadinium* consistently show the Kofoidean plate pattern Po, cp, X, 4´, 3a, 6´´, 6C, 5S, 6´´´, 2´´´´. *Amphidoma languida* exhibits the same hypothecal plate pattern but differs in epithecal plates by having six apical plates and no intercalary series. A very characteristic feature of all species is the prominent apical pore complex (APC) composed of a pore (Po) plate with a central round pore covered by a cover plate (cp) and an X-plate with a characteristic three-dimensional structure. The arrangement of the five sulcal plates is very characteristic for all Amphidomataceae, with a large plate Sa invading the epitheca and a peculiar and conservative Ss plate running from the plates C1 to C6. The second antapical plate may bear a small spine (*A. spinosum*, *A. polongum*), a distinct horn with a spine (*A. caudatum*), or a prominent antapical pore (*A. languida*). All species of *Azadinium* and *Amphidoma languida* have a conspicuous ventral pore. However, the position of that pore differs between the species (Tab. 1). Other species determining characters, as the presence or absence of stalked pyrenoid(s), or the shape and arrangement of certain epithecal plates, are listed in Table 1.

Morphology, and in particular the plate tabulation with five different rows of plates, undoubtedly classify the genus *Azadinium* as a member of the dinophycean subclass Peridiniphycidae. This subclass is currently subdivided into two orders, the Peridiniales and Gonyaulacales, with a number of differences discussed in detail by Fensome *et al.* (1993). *Azadinium* clearly exhibits morphological characteristics of both of these orders. The hypothecal plate arrangement, and the presence of six precingular, six postcingular, and six cingular plates, suggest a relationship to the Gonyaulacales. Other general features including the mode of cell division, the plate suture and growth band structure, and the presence of a ventral pore in *Azadinium* seem likewise to reveal a relationship to the Gonyaulacales. However, the epithecal plate arrangement with four apical and three symmetric intercalary plates implies an affinity to the

Peridiniales. Moreover, the shape and composition of the APC is typical of the Peridiniales. With the description of *Amphidoma languida*, the taxonomic affiliation of *Azadinium* at the family level was recently clarified. *Amphidoma* was found to be closely related to *Azadinium* with such possible morphological synapomorphies as the cingular and hypothecal plate arrangement, the number and arrangement of sulcal plates, and the characteristic APC. *Amphidoma* and *Azadinium* were thus placed in the family Amphidomataceae by Tillmann *et al.* (2012a). Molecular phylogenies of the Amphidomataceae based on ribosomal RNA sequence data supported the morphological considerations, but were not able to fully resolve the phylogenetic position of the group within the Dinophyceae (Tillmann *et al.* 2012a, b). Both morphology and molecular phylogeny thus did not allow for a clear order affiliation and leaves *Azadium* and the family Amphidomataceae with an unclear order affiliation.

Fig. 1: Global records of *Azadinium*/*Amphidoma languida*. (1): Tillmann *et al.* 2009 , 2010. (2): Tillmann *et al.* 2011. (3): Salas *et al.* 2011, Tillmann *et al.* 2012a. (4): Tillmann *et al.* 2012b. (5): Tillmann, unpubl. (6) Nezan *et al.* 2012. (7): Margalef *et al.* 1954. (8) Rampi 1969. (9): Adriana Zingone, pers. com. (10): Tillmann & Busch, unpubl. (11) Hernandez-Becerril *et al. 2012*. (12): Akselman & Negri 2012. (13): Checklist Black Sea phytoplankton. (14): Consuelo Carbonel Moore, pers. com. (15): Potvin *et al.* 2012. (16): Gu *et al.* 2013

Global distribution
Although initially described from the North Sea, the genus *Azadinium* probably is distributed worldwide (Fig. 1). In the North Sea, all five described species have been observed (Tillmann *et al.* 2009, 2010, 2011, 2012b). Based on full characterization of local strains in terms of morphology and sequence information (records 1-5, 6, 9, 15, 16) or based on a few records of single specimens detected by scanning plankton samples by light (record 7-8 referring to the large *A. caudatum*, record 10) or electron microscopy (records 11-14), an increasing number of global records could be added to a distribution map (Fig.

1). Nevertheless, knowledge on the biogeography of the genus currently is rather limited and patchy. As a consequence of an increasing awareness of the genus, however, the availability of FISH and QPCR as species-specific detection methods (Töbe *et al.* 2013, Potvin *et al.* 2013), and due to the increasing use of "next generation" high throughput sequencing with environmental samples, it is expected that our knowledge on the biogeography of the Amphidomataceae will increase rapidly.

Tab. 2: Origin and AZA profile of strains of Amphidomataceae according to Tillmann *et al.* 2012b, Krock *et al.* 2012 and Gu *et al.* 2013.

Species	Strain	Origin	AZA
A. spinosum	3D9	Scotland 2007	AZA-1 AZA-2 AZA-716
	UTHE2	Denmark 2008	
	SM2	Ireland 2009	
	Shet F6	Shetland 2011	
A. obesum	2E10	Scotland 2007	-
A. polongum	Shet B2	Shetland 2011	-
A. caudatum	AC1/2	Scotland 2011	-
A. poporum	UTHD4 UTHC5 UTHC8	Denmark 2008	AZA-846
	HJ2010	Korea 2010	AZA-858
	G25	Bohai Sea 2007	AZA-858 -920, -928
	G42	East China Sea 2011	AZA-2
	G64	East China Sea 2011	AZA-2
	G60	East China Sea 2011	-
	G66	East China Sea 2011	AZA-872
	G68	South China Sea2011	AZA-2
Amphidoma languida	SM1	Ireland 2009	AZA-816, -830

Toxins

Multiple strains of the type species *A. spinosum* from different locations have consistently been found to produce AZA-1, AZA-2, and AZA-716 (Tillmann *et al.* 2012b). In contrast, *A. obesum*, *A. poporum,* and *Amphidoma languida* have initially been described as non-toxigenic, as none of the known AZAs could be found (Tillmann *et al.* 2010, 2011, 2012a). However, we recently detected four new AZAs in a number of different species. Compared to the previously known AZAs, these new analogs are characterized by a missing methyl group at C39 thus forming a characteristic m/z 348 fragment (Krock *et al.* 2012). Thus, it is evident that the species diversity within this group is also reflected by a high chemical diversity (Tab. 2). We know now that AZA production can also be

found in the related genus *Amphidoma* (Krock *et al.* 2012). Six different AZA compounds have been found in strains of *A. poporum* (Tab. 2), with a large variability of AZA-profile among different strains (Krock *et al.* 2012, Gu *et al.* 2013). AZAs were not detected in all cultivated Amphidomataceae (e.g., *A. obesum*, *A. caudatum* var. *margalefii*, and *A. polongum*), but we cannot exclude the presence of yet unknown and thus undetectable AZA-related compounds.

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