

AZA – The producing organisms – Biology and trophic transfer

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1. Introduction:

Compared to the knowledge on toxin structure, detection methods, and toxicology, convincing clarification of the aetiology of AZP was seriously lacking behind for quite a long time. Based upon the seasonal and episodic accumulation of AZA toxins in suspension-feeding bivalve mollusks – a situation similar to several other marine biotoxins - a planktonic source has been suspected from the outset. Furthermore, due to their polyether structural features, AZA has *ab initio* been suspected to be a dinoflagellate metabolite. Thus, it was no surprise that it was a dinoflagellate species which was first claimed to be the source of AZA. Based upon chemical analysis by liquid chromatography coupled to mass spectrometry (LC-MS) of manually collected phytoplankton specimens from net hauls, Yasumoto¹ first indicated that a species of the dinoflagellate genus *Protoperdinium* Bergh was the primary source of AZA. This work was later published in detail by James et al.² with the culprit species described as *Protoperdinium crassipes*. The link between AZA and *P. crassipes*, however, remained controversial because production of AZA by *P. crassipes* could not be verified in spite of numerous attempts based upon field surveys³ and laboratory investigations of cultured and isolated cells⁴. Moreover, in contrast to other proven producers of phycotoxins, which are all primarily phototrophic, *P. crassipes* is a heterotrophic dinoflagellate, known to prey upon other dinoflagellates as food⁵. The likelihood, therefore, that another dinoflagellate may produce AZA, which then accumulates in *P. crassipes* through normal feeding processes, could not be neglected.

During a research cruise with RV Poseidon in the North Sea this issue became quite evident when toxin analysis of fractionated plankton samples clearly showed that high amounts of AZAs was found even when *P. crassipes* was absent, that AZA could be found in isolated cells of the predatory ciliate *Favella ehrenbergii*, and that in fractionated plankton samples the largest AZA quantities were found in the small size (<20 µm) classes⁶. All these hints

then led to the isolation of a small dinoflagellate, which was shown to produce AZA1 and -2 in axenic culture ⁶, and which was identified as a new species *Azadinium spinosum* in a newly erected genus ⁷. Considering the short interval since this first identification of *Azadinium*, the diversity of the genus has rapidly increased and now comprises six species (Tab. 1, Fig. 1), some of which are available as multiple strains. These species are *A. spinosum*, *A. obesum*, *A. poporum*, *A. polongum*, *A. caudatum* (which occurs in two distinct varieties: var *margalefii* and var. *caudatum*) and *A. dexteroporum*. Moreover, with the description of *Amphidoma languida*, a closely related genus could be identified ⁸. Whereas multiple strains of the type *A. spinosum* from different location have consistently been found to produce AZA1, AZA2 and AZA716, other new species have initially been described as non-toxicogenic, as none of the known AZAs could be found ^{9,10}. However, with the recent detection of four new AZAs in a number of different species, it became evident that the species diversity within this group is also reflected by a high chemical diversity, with AZA production even found in the related genus *Amphidoma* ¹¹. The presence of AZAs (AZA3 and AZA7) has also been tentatively described for the most recently described species *A. dexteroporum* ¹² but this need to be confirmed by LC-MS analysis. In particular, *A. poporum* turned out to be a rich source of different azaspiracid compounds ^{11,13}, but with a large variability of AZA-profile among different strains. Three species (*A. obesum*, *A. caudatum* var. *margalefii* and *A. polongum*) still have been negative in AZA detection, but we cannot exclude the presence of yet unknown and thus undetectable AZA-related compounds. In any case, in view of the diversity of AZA-producing species it is important to appreciate the commonalities and differences of the species as a fundamental basis for species identification, “early warning” monitoring and bloom prediction.

2. Morphology – Taxonomy - Phylogeny

2.1 General morphology and taxonomy

Gross morphology and a compilation of distinctive features of the species are compiled in Tab. 1 and Fig. 1. Most species of *Azadinium* (and *Amphidoma languida*) are small (size of about 10-15 μm) and ovoid to elliptical in shape with a hemispherical hyposome. *A. caudatum* (both varieties) are distinctly larger and, with a characteristic biconical outline, significantly different in shape as well. In all species, the episome is larger than the hyposome, with slightly convex sides ending in a distinctly pointed apex. For all small species the cingulum is deep and wide, accounting for roughly 1/5 to 1/4 of the cell length (1/6 for the larger *A. caudata*). A central or more posteriorly located large nucleus is visible, which generally is round to elliptical but may become distinctly elongated in shape close to cell division^{8,14}. All species are photosynthetic and possess a presumably single chloroplast which is parietally arranged, lobed and normally extends into both the epi- and hyposome. For a number of species stalked pyrenoid(s) are visible in the light microscope because of a distinct starch cup. The presence/absence, location, number and types of pyrenoids have been regarded as useful taxonomic characters between genera^{9,15} and has in particular been discussed as a potential feature visible in light microscopy to differentiate species of *Azadinium*⁹. However, more detailed information (including ultrastructure) related to pyrenoids of *Azadinium* is needed before this feature can be unambiguously used for species determination. *Azadinium* spp. have delicate thecal plates difficult to detect in light microscopy so that live cells are sometimes difficult to differentiate from small athecate gymnodinoid species. Generally, the surface of the plates is smooth but irregularly covered by small pores. These pores are either numerous and arranged randomly (*A. caudatum*), more rare and scattered (small *Azadinium* species) or particularly concentrated on the apical plates in *Amphidoma languida*. A distinct row of pores located below the lower cingulum list may be present. All species of *Azadinium* consistently

show the Kofoidian plate pattern of Po, cp, X, 4', 3 a, 6'', C6, 5S, 6''', 2'''' (Fig. 2 A-C), whereas *Amphidoma languida* has six apical plates and no intercalaries. A very characteristic feature among the AZA-relevant species is the prominent apical pore complex composed of a pore plate with a central round pore covered by a cover plate and an x-plate. The pore plate (Po) is round to slightly ellipsoid or – exceptional for *A. polongum* - distinctly elongated, or – exceptional for *A. dexteroporum*, markedly asymmetric and with a finger-like protrusion on the left side. The pore plate is always bordered by a conspicuous rim which is of slightly different run among species. A small X-plate is located where the pore plate abuts the first apical plate and occupies about 1/3 of the connection between Po and 1'. The X-plate shows some differences in arrangement across species; it invades the first apical plate in *A. spinosum*, *A. obesum*, *A. poporum*, and *Amphidoma languida*, but abuts the first apical plate in *A. polongum* and *A. caudatum*. From the outside, the X-plate consistently among all species has a very characteristic three dimensional structure with finger-like protrusions contacting the apical cover plate.

The epitheca of *Azadinium* spp. consists of 4 apical plates and a row of six precingular plates. Three intercalary plates are located ventrally. The median plate 2a very characteristically is the smallest of the intercalaries and is 4-sided (Fig. 2 A). The hypothecal plate arrangement with a row of 6 postcingular and 2 differently-sized antapical plates is consistent for all the species (Fig. 2 B). The second antapical plate may bear a small spine (*A. spinosum*, *A. polongum*, *A. dexteroporum*), a distinct horn with a spine (*A. caudatum*) or a prominent apical pore (*Amphidoma languida*). The cingulum is composed of six plates of similar size. The sulcal plate arrangement generally is difficult to analyse due to a distinct three-dimensional shape of the sulcal area which obscures some of the small plates from view. Nevertheless, the arrangement of the five sulcal plates is very characteristic for all AZA-relevant species and is

characterised by a large plate Sa invading the epitheca and a peculiar and conservative Ss plate running from C1 to C6 plates (Fig. 2 C).

All species of *Azadinium* and *Amphidoma languida* have a distinct pore located in the ventral area which is thus designated as a “ventral pore”. Generally, the location of the ventral pore seems to be quite variable in *Azadinium* species (Fig. 2 D-K), either on the left margin of plate 1' (*A. spinosum*, *A. obesum*), on the left side of the Po (*A. poporum*)^{7,9,10}, or at the right posterior end of the markedly asymmetric Po plate (*A. dexteroporum*)¹². In *A. caudatum* var. *margalefii*, this pore is located on the right margin of the Po whereas for the second variety, *A. caudatum* var. *caudatum*, a similar pore is situated near the posterior right margin of plate 1'¹⁶. In *Amphidoma languida*, it is located on the anterior right margin of 1'⁸. Very rarely, the position of the ventral pore has been observed to vary even within a culture. For one specimen of *A. languida*, the ventral pore was located in the right side of the pore plate⁸ (as in *A. caudatum* var. *margalefii*), and, for one specimen of *A. poporum* isolated from Korean waters, it was located on the left side of plate 1'¹⁷. As the function (if any) of these pores is completely unknown, we cannot speculate on the potential consequences of the apparent variability in pore location among the Amphidomataceae. The plate overlap pattern (imbrications pattern), which may reflect functional aspects of ecdysis and/or cysts archeopyle type, may be a useful aid in determining plate homologies¹⁸. It has been elucidated in details for *A. spinosum*¹⁹, *A. languida*⁸, *A. caudatum*¹⁶ and *A. polongum*²⁰. The pattern is consistent among the species, and identified an uncommon but stable imbrication pattern of the most dorsal apical plate (3' in *Azadinium* or 4' in *Amphidoma*), which characterized these two genera and might be helpful for a revision of the description of the family Amphidomataceae.

For most of the cultured strains of *Azadinium* analysed so far, a distinct variability in plate patterns has been noted. Such variations from the usual plate pattern in terms of number

and/or arrangement appear most often for apical and intercalary plates, but more rarely have been observed for hypothecal plates as well. However, it is unknown whether the presence and/or degree of variability are an inherited feature of the genus *Azadinium*, distinct at the strain or species level or is simply a culture artefact. Clearly, detailed morphological investigations of field populations are needed to answer these questions.

2.2 Introduction to the species

Azadinium spinosum (Fig. 1 A), the type of the genus, was first isolated from the North Sea off Scotland and this isolate was confirmed to be a proximal source for AZA⁶. *A. spinosum* was shown to produce AZA1 and AZA2 in axenic cultures and thus AZAs are of dinoflagellate origin and unrelated to bacteria⁶. A detailed morphological analysis supplemented by sequence information then described this strain as the new species *Azadinium spinosum*⁷. With a Kofoidin thecal tabulation of APC, 4', 3a, 6'', 6C, 5?S, 6''', 2''''', the species was identified as distinctly different from other described dinoflagellate genera, and consequently, the new genus *Azadinium* was erected to comprise this novel taxon. *A. spinosum* is a small (12-16 µm length and 7-11 µm width) and slender thecate, photosynthetic dinoflagellate with a wide and descending cingulum, which is displaced by about half of its width. Eponymous for the species is the presence of a single, small and delicate antapical spine located slightly asymmetrically at the right side of the cell. A distinct ventral pore is located on the left margin of plate 1'. In the light microscope, one large pyrenoid located in the episome is visible. Different strains have been isolated from the North Sea (Scotland⁷; Denmark:⁹, Shetland Islands:²⁰) and from Ireland²¹. *Azadinium obesum* (Fig. 1 B): The second species of the genus was isolated as clone 2E10 from the North Sea along the Scottish east coast, the same locality as for *Azadinium spinosum*, the type for this genus¹⁰. *Azadinium obesum* also is small (13-18 µm length; 10-14 µm width) and similar in

shape but there are a number of morphological differences compared to *A. spinosum*, including a larger mean cell size (the epithet refers to the obese, corpulent appearance of the species when compared to the more slender shape of the type, *A. spinosum*), the consistent absence of an antapical spine, the lack of a stalked pyrenoid, and several details of the plate configuration. Among these thecal features, the first precingular (1'') plate of *A. obesum* does not touch the first epithecal intercalary plate and is four sided, rather than five sided as in all other *Azadinium* species reported so far. Furthermore, and very different to other *Azadinium* species, the lower half of the first apical (1') plate of *A. obesum* is very narrow and tongue-like. *A. obesum* is one of the *Azadinium* species where up to now no azaspiracids have been detected^{10,11}. ***Azadinium poporum*** (Fig. 1 C) was initially described from three clones isolated from the southern North Sea off the Danish coast⁹. Like other *Azadinium* species, *A. poporum* is a small (11-16 µm length; 8-12 µm width) photosynthetic dinoflagellate with exactly the Kofoidian plate tabulation of the genus. In contrast to *A. spinosum* (one pyrenoid) and *A. obesum* (no pyrenoid), there may be several pyrenoids (up to four) visible in the light microscope. The most important morphological characteristics of *A. poporum* is the conspicuous arrangement of the ventral pore, which is located at the junction of the pore plate and the first two apical plates. This latter feature also distinguishes *A. poporum* from *A. obesum*. As in *A. spinosum*, but different from *A. obesum*, the first precingular (1'') plate of *A. poporum* touches the first epithecal intercalary plate 1a. ***Azadinium polongum*** (Fig. 1 D) is another very recently described species of *Azadinium*. Up to now it has only been reported from the Shetland Islands, which are located in the northernmost part of the North Sea and are largely influenced by the Atlantic Ocean. In the light microscope, it is very similar to the other small species of *Azadinium* (*A. spinosum*, *A. obesum*, *A. poporum*) and it has an antapical spine. The presence of an antapical spine in small *Azadinium* species was hitherto restricted to *A. spinosum*. With *A. polongum* also exhibiting an antapical spine, the

identification of *A. spinosum* only by light microscopy is unfortunately no longer convenient. The most obvious morphological feature (but only visible at the SEM level) of *A. polongum* is the shape of the pore plate that allows a clear separation of *A. polongum* (elongated pore plate) from other *Azadinium* species (round to slightly ellipsoid pore plate). Other features useful for species delimitation of *A. polongum* are the shape of the X-plate, the location of the ventral pore, and the absence of a distinct pyrenoid with starch sheath. ***Azadinium caudatum*** (Fig. 1 E): The dinoflagellate species described in 1953 by Halldal as *Amphidoma caudata* had a somewhat chequered taxonomic history. The first plate details provided by Dodge and Saunders indicated that this species has the same basic plate pattern as *Azadinium*. It was thus concluded by Tillmann et al.⁹ that, notwithstanding some differences that remained to be elucidated, *Amphidoma caudata* might be transferred to the genus *Azadinium*, pending further morphological and phylogenetic studies. Consequently, a new study using field samples and cultures of “*A. caudata*” used morphological and molecular data to clarify the systematic situation and transferred the species to the genus *Azadinium* as *Azadinium caudatum* (Halldal) Nézan et Chomérat¹⁶. Both sequence and morphometric data clearly showed that the species occurred with two distinct varieties, var. *caudatum* and var. *margalefii*, which are easily distinguished by the different shape of the antapical projection. *Azadinium caudatum* is quite easy to distinguish from other species of *Azadinium* on the light microscopy level due to its larger size, its characteristic triangular shape and its clearly visible antapical projection. The basic plate pattern is the same as for other *Azadinium* species; nevertheless there are a couple of minor morphological differences visible at the SEM level. The first precingular plate (1') is in contact with the first intercalary plate (1a) (similar to all other *Azadinium* species except *A. obesum*). However, in *A. caudatum* there is also contact between plate 6'' and 3a, which is unique among other *Azadinium* species. Another remarkable difference to other species of *Azadinium* is that the 4'' is distinctly smaller than the other precingular plates and also 3-

sided. Other differences include the shape of the conspicuous rim around the apical pore plate (extending on the dorsal side alongside the anterior margins of plate 3' for *A. caudatum*), the contact between the X- and the first apical plate (X plate abuts 1' in *A. caudatum* and invades it in other *Azadinium* species) and an unusual and unique deep depression present in the largest sulcal plate (Sp plate). **Azadinium dexteroporum**: This is the most recently described and the smallest (8.5 x 6.2 µm) species of *Azadinium* found in the Mediterranean Sea. It differs from all other *Azadinium* species for the position of the ventral pore, which is located at the right posterior end of the markedly asymmetrical Po plate, and for a pronounced concavity of the small middle intercalary plate. Like *A. spinosum* and *A. polongum* it has a small antapical spine. The presence of AZA3 and AZA7 has been claimed¹² but exact structures need to be confirmed by LC-MS and NMR analysis.

Other species potentially related to *Azadinium*: There are a few other species described in the literature which potentially are related to the genus *Azadinium* and need to be re-analysed in more depth. A small dinoflagellate species has been described in 1959 as *Gonyaulax parva*²². The plate patten of this species, however, is quite different from the genus *Gonyaulax*, and in fact corresponds to the plate tabulation of *Azadinium*⁷. As such, this species should probably be transferred to *Azadinium*, awaiting reinvestigation of the cingular and sulcal plates, as well as the results of molecular taxonomic studies. “*Gonyaulax gracilis*” is a second example of a *Gonyaulax* species that probably belongs to the genus *Azadinium*. Although *Gonyaulax gracilis* Schiller²³ was not validly described (ICBN ART. 32.1), Bérard-Therriault et al.²⁴ provided figures (pl. 90) under that name showing dinoflagellates with a distinct similarity to *Azadinium*, one specimen with a spine characteristic of *A. spinosum* and one without a spine. Other details are not given so it remains uncertain whether the dinoflagellates they reported from eastern Canada in fact represent species of *Azadinium*.

Amphidoma languida (Fig. 1 F) has been isolated concurrently with the Irish strain of *A. spinosum* from Bantry Bay, Ireland⁸. The strain SM1 was initially identified as a potential *Azadinium* species because of similarities with respect to size, shape and swimming pattern. A detailed morphological and phylogenetic study then clearly showed that it represents a new species in the genus *Amphidoma*. The Kofoidian plate formula (Po, cp, X, 6', 0a, 6'', 6C, 5(?) S, 6''', 2''''') indicates a major difference in the epithecal configuration to the genus *Azadinium*: *Amphidoma* has six apical plates and no apical intercalary plate, whereas *Azadinium* has only 4 apical plates but 3 apical intercalary plates. Nevertheless, a number of morphological similarities, such as cingular and hypothecal plates, the number and arrangement of sulcal plates, and the characteristic apical pore complex with a small X-plate centrally invading the first apical plate, indicated a close relationship between *Amphidoma* and *Azadinium*. This was supported by a phylogenetic tree based on concatenated SSU and LSU sequences data of a large taxon sample, which retrieved *Azadinium* and *Amphidoma* as sister groups distinct from all established taxonomic units of dinophyceae⁸. As a unique feature among Amphidomataceae, *A. languida* has a large antapical pore located at the dorsal side of the large antapical plate 2'''''. This "pore" in fact is a depressed field of a number of small pores (about 15). Another pore clearly differentiated in size and sub-structure from the numerous pores on the other apical plates, and which is referred to as a "ventral pore", is located at the anterior right side of plate 1' on the suture to plate 6'.

2.3 Phylogeny

Studying the phylogeny of a group of organisms (e.g. genus, species, population) aims at clarifying the evolutionary history of that group, and can be based on morphology and/or sequence information. Reconstruction of the evolutionary origin of toxic dinophytes such as *Azadinium* is of considerable scientific interest and may provide information for

understanding toxin production. Furthermore, phylogenetic information may be useful to identify other potential yet unidentified AZA-producing species. However, the capacity to produce phycotoxins is generally scattered on the phylogenetic tree of dinophyceae, indicating that there is no clear trend in the evolution of this trait and that toxin production has appeared and disappeared multiple times during dinoflagellate evolution²⁵. Nevertheless, for certain toxins (e.g. yessotoxins) it could be shown that its production is confined to the order Gonyaulacales within the Dinophyceae so that species within this taxonomic order should be given priority for future testing and field collections associated with monitoring for YTX contamination events²⁶. Finally, phylogenetic trees and the underlying molecular database could also enhance the development of robust detection probes for monitoring AZA-producing species.

Morphology, and in particular the plate tabulation with 5 different rows of plates undoubtedly classified the genus *Azadinium* as a member of the dinophycean subclass Peridiniphycidae⁷. This subclass is subdivided into two orders, the Peridiniales and Gonyaulacales²⁷, with a number of differences discussed in detail by Fensome et al.²⁷; and Fig. . *Azadinium* clearly exhibits morphological characteristics of both of these orders (Fig. 3). The hypothecal plate arrangement and the presence of six precingular, six postcingular, and six cingular plates suggest a relationship to the Gonyaulacales. The epithecal plate arrangement with four apical and three intercalary plates, however, implies an affinity to the Peridiniales. Moreover, the composition of the apical pore complex with a pore plate, a cover plate and the presence of an X-plate is typical for the Peridiniales. In contrast, however, to most species of the Peridiniales the X-plate does not separate completely the first apical plate (1[^]) from the pore plate but invades plate 1[^], giving broad contact of plate 1[^] to the pore plate, each right and left respectively from the X-plate. The only examples of a direct contact are represented in the *Heterocapsa* Stein-*Cachonina* Loeblich–species complex. In these species, the X-plate is

displaced to the right side, consequently allowing direct contact of plate 1' and the pore plate at the left side from the X-plate²⁸. 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 to be more related to the Gonyaulacales. Morphology thus did not allow for a clear order affiliation and leaves *Azadinium* in the order "uncertain". With the description of *Amphidoma languida*, the taxonomic affiliation of *Azadinium* on a family level was recently clarified. *A. languida* was found to be closely related to *Azadinium* with possible morphological syn-apomorphies including the cingular and hypothecal plate arrangement, the number and arrangement of sulcal plates, and the characteristic APC with a small X-plate centrally invading the first apical plate. *Amphidoma* and *Azadinium* were thus placed in the family Amphidomataceae²⁹ by Tillmann et al.⁸. The presence or absence of intercalary plates (present in *Azadinium* but absent in *Amphidoma*) is regarded as distinctive at the genus level. However, there is only one epithecal plate difference between the two genera which does not preclude assignment to the same family.

Molecular phylogenies based on ribosomal rDNA support the morphological considerations and likewise were not able to fully resolve the position of the genus *Azadinium* within dinoflagellate phylogeny. The first phylogenetic trees based on 18S, 28S or ITS sequence data of *A. spinosum* did not show any particularly close affiliation within the Peridinales or Gonyaulacales nor to any other dinoflagellate order represented in molecular data bases⁷ and this view has not been changed by adding new and more *Azadinium* species/strains²⁰. Using a concatenated alignment of LSU and SSU, the Amphidomataceae including *Amphidoma* and *Azadinium* were an independent lineage among other monophyletic major groups of the dinophytes such as the Suessiales, Procentrales, Gonyaulacales, and Peridinales⁸. Thus, the phylogenetic position of the Amphidomataceae at present cannot be identified reliably, although they have been placed on the peridinean branch remote from the Gonyaulacales. It

remains to be determined whether they are part of the Peridiniales or represent a distinct lineage that would deserve the recognition at higher taxonomic level. On the family level, the tree provided by Tillmann et al.²⁰ clearly showed that *Amphidoma languida* and *Azadinium* together form the monophyletic and highly supported Amphidomataceae. *Amphidoma* was situated in a basal position of the maximum supported *Azadinium* clade with all five species described at that time (*A. spinosum*, *A. obesum*, *A. poporum*, *A. caudatum*, *A. polongum*)²⁰. Molecular phylogeny of *A. dexteroporum* confirmed the attribution of the species to the genus and suggested a position basal to other small *Azadinium* species¹².

In contrast to rDNA sequence data, available data on conservative proteins such as cytochrome oxidase (subunit 1, COI) indicate a general lack of base substitutions in the COI gene among species of *Azadinium*, with variation restricted only to deletions/insertions, and this might reflect the slower rate of gene evolution in the COI gene relative to the sequences from the ribosomal cistron⁷. Within *Azadinium*, there is a considerable variation of sequence data/morphology within the species as they are currently defined. *A. caudatum* has been shown to occur with two distinct varieties clearly different in terms of general size, the shape of the antapical projection and the position of the ventral pore. Moreover, both varieties showed a considerable degree of differences in sequence data¹⁶. Likewise, there are considerable differences in ITS and 28 S gene sequences among Asian and European strains of *A. poporum*. Minor differences in shape of the 3' plate, which initially was hypothesised to morphologically support these molecular differences¹⁷ have subsequently been shown to vary among other Asian strains¹³. Nevertheless, in an LSU/ITS tree, all available strains of *Azadinium poporum* comprise 3 well supported clades, one of them including multiple strains originating from the coast of China as well as a Korean strain. The second clade included strains from the East China Sea and South China Sea, and the third one consisted of strains from Europe. There thus is a considerable cryptic diversity within *A. poporum* with even sympatric occurrence of two distinct ribotypes in China. Interestingly, this diversity

seems to be reflected by the considerable diversity within this species in terms of toxin profile (Hess et al., this book).

3. Distribution

3.1 Global distribution

Although initially described from the North Sea, there is increasing evidence that the genus *Azadinium* probably is distributed worldwide (Fig. 4). In the North Sea area, five currently described species have been observed. The occurrence of *Azadinium* along the Scottish coast and the Irish Atlantic coast²¹, as well as a report of AZA in mussels from the North coast of Norway³⁰ and a recent record from the Shetland Islands²⁰, implies distribution of the genus into more northern North Atlantic/arctic areas as well. This could be confirmed by LM observations of *Azadinium* spp. in plankton samples taken in the Irminger Sea between Greenland and Island taken in 2012 (unpublished information, Urban Tillmann, AWI). Which species are present here still need to be determined. A number of different *Azadinium* species obviously are present in French Atlantic coastal waters; in addition to *Azadinium caudatum* (see below), *A. poporum* and five additional organisms have been identified by molecular analysis or SEM, taxonomic identification is underway (personal communication, Elisabeth Nezan, Ifremer, France).

The type of the genus, *A. spinosum*, has been isolated off the Scottish coast, the coast off Denmark, the Shetland Islands and from coastal Atlantic waters in Ireland^{7,9,20,21}. SEM images of Hernandez-Becerril et al.³¹ depicted a species of *Azadinium* most likely *A. spinosum* from coastal pacific waters off Mexico and thus indicate a much wider distribution of that species. However, sequence information from that region supporting the preliminary species identification are not available. *A. obesum* has yet been reported only from the

Scottish coast¹⁰ but probably is also present in Ireland (unpublished information, Rafael Salas, Marine Institute). Due to its easy recognition by light microscopy, the biogeography of *Azadinium caudatum* (as *Amphidoma caudata*) is slightly better known. It has been reported from the North Sea off the Norwegian coast³², in a Portuguese lagoon³³, around the British Isles and the west coast of Ireland^{34,35}, at the Spanish coast of Castellón³⁶, from the Ligurian Sea (Mediterranean)³⁷, and, most recently, all around the French Atlantic coast¹⁶. *Azadinium polongum* up to now has only been reported from the Shetland Islands²⁰. Preliminary growth experiments of this species showed poor growth at temperatures above 10° C²⁰ and thus indicate that the core distribution area of *A. polongum* is more to the northern boreal/arctic waters. *Azadinium poporum* is the only species, for which strains outside Europe have been obtained. As a first record of *Azadinium* in Pacific waters, *A. poporum* has been isolated from Shiwha Bay in Korea¹⁷. In terms of morphology, the strain designated as *A. cf. poporum* by Potvin et al.¹⁷ is almost identical to the European *A. poporum*, but differs significantly in terms of sequence data. *A. poporum* obviously is quite widely distributed in the Asian Pacific. Gu et al.¹³ succeeded in isolating 25 different strains of *A. poporum* originating from China covering Bohai Sea, East and South China Sea. The AZA producing species *Amphidoma languida* has only been isolated from one bay in Ireland, but probably has a much wider distribution: Lewis and Dodge³⁸ depicted the epitheca of a cell (their Fig. 11) - most probably being *A. languida* - from the east Atlantic. Sequence data from plankton samples of the Skagerrak area indicate the presence of *A. languida* in the North Sea as well (personal communication, Kerstin Toebe, AWI, Germany). Furthermore, *Azadinium* sp. has been reported to form blooms in the southern Atlantic Ocean off the coast of Argentina³⁹. As shown by LM and SEM the species in question clearly had the *Azadinium* plate tabulation pattern and possessed a spine and was thus designated as *A. cf. spinosum*. However, for a final species designation, a few yet unresolved morphological details (e.g. presence of a ventral

pore) of the Argentinean species need to be clarified; likewise, DNA samples and toxin measurements from that area are needed to verify the presence of toxigenic *A. spinosum*. *Azadinium* species other than *A. caudatum* mentioned above are definitely present in the Mediterranean: The most recently describes species *A. dexteroporum* has been isolated from Naples ¹². Fixed phytoplankton samples taken at Alfacs-Bay, a coastal lagoon area close to the Ebro delta, undoubtedly revealed the presence of *Azadinium*, despite low abundances (unpublished information, Urban Tillmann, AWI). *Azadinium* also has been included in the check list of Black Sea phytoplankton ([http://phyto.bss.ibss.org.ua/wiki/Azadinium spinosum](http://phyto.bss.ibss.org.ua/wiki/Azadinium_spinosum)). The depicted species, which clearly belongs to the genus *Azadinium*, is listed as *A. spinosum*, but except the general shape and the presence of a spine, no supportive detail for that species designation are visible, and thus this record needs confirmation. Finally, and in remarkable contrast to the majority of coastal records, cells representing *Azadinium* sp. and *A. languida* have been seen in SEM preparations from samples collected at the open West-Indian Ocean (personal communication, Consuelo Carbonell-Moore, Oregon State Univ., USA).

This compilation clearly shows that knowledge on the biogeography of the genus currently is rather limited and patchy. It is either based on the troublesome procedure of isolating, cultivating and fully characterizing local strains (in terms of morphology and sequence information) or based on a very few records of single specimens detected by scanning plankton samples by electron microscopy. Nevertheless, due to an increased awareness of the genus, the availability of FISH and QPCR as species-specific detection methods ⁴⁰ as well as the increasingly used “next generation” high throughput sequencing of environmental samples, it is expected that our knowledge on the biogeography of the Amphidomataceae will increase rapidly.

3.2. Temporal and spatial distribution

In the recent years since its first discovery, few studies on the temporal distribution of *Azadinium spinosum* have been carried out. Data sets of *Azadinium* occurrence extracted from monitoring programmes are not generally reliable due to the difficulty of identification under light microscopy. Some clues as to its temporal distribution can be obtained from the presence of AZA toxins in shellfish and in particular Irish shellfish toxicity data has long suggested that the causative species arrives in the mid to late summer months. This phenomenon was examined in detail by a study conducted on plankton samples from 2012 (unpublished information, Dave Clarke, Marine Institute) during one of the largest (both in terms of concentration and geographical distribution) azaspiracid events observed to occur in Ireland. The event was prolonged, occurring in the majority of sites from the South-West, West & North-West coasts from June through to November, reaching its peak during September and October, with some sites lasting into the first quarter of 2013. During this event, weekly phytoplankton samples from Killary Harbour on the West coast taken from May through to September were compared to qPCR gene probe⁴⁰ results for the identification of *Azadinium spinosum* and LC-MS/MS results for AZAs in shellfish.

The key finding of this study was that increases in *A. spinosum* cell concentration resulted in an associated rapid uptake of AZAs in mussels. *Azadinium* concentration was not consistent in the location through the test period but appears to be present in pulses, possibly associated with sea water intrusions from deeper offshore locations. These pulses were reflected in both the cell concentration and ensuing toxin peaks in the shellfish (Fig. 5). A second set of samples from 2006 from South-West Ireland (significant year in terms of a major azaspiracid event) were also analysed via molecular methods, and again as in the Killary data set, there was a presence of *A. spinosum* corresponding with rapid increases in azaspiracid intoxicification in mussels in the same locality. This has also been observed in earlier

laboratory feeding experiments^{21,41} where toxin levels rose rapidly in test shellfish in response to consumption of a diet of *A. spinosum* cells.

Due to the short period of time between blooms of *A. spinosum* occurring and detection of Azaspiracids in shellfish, observations of AZA in shellfish would be considered a useful indicator for the temporal distribution of *Azadinium*. The temporal distribution of Azaspiracid observed from 11 years of Irish shellfish monitoring show some interesting patterns which gives us an indication of the temporal and spatial distribution of *A. spinosum*. Figure 6 shows a predominance of higher levels, more frequent occurrence and longer outbreaks in the south than the rest of the country. Inter-annual variability is also observed while most areas show some occurrence nearly every year, there are some years (e.g. 2004 and 2005) when there is very little toxin observed anywhere. The evidence points towards an offshore factor controlling the levels observed within the bays where shellfish are produced which results in this intermittent occurrence. It is not known whether continuous intoxication over winter or the inability of shellfish to depurate AZAs is responsible for extended winter toxicity. A less frequent intoxication can also take place in early summer (May-June) but this tends to happen more intermittently. Toxin accumulation seems to oscillate during these events where toxicity increases and decreases creating a yo-yo effect over a number of weeks suggesting that shellfish is not getting intoxicated in a single incident from *A. spinosum*, but rather in consecutive waves over time perhaps from offshore pulses of toxic plankton being advected inshore by coastal processes.

This is also observed in other species in Irish waters including *Dinophysis* sp. which was shown to impact inshore shellfisheries in the South-West when oceanographic factors result in intrusions of offshore water containing high cell counts⁴². Physical circulating forces during the summer months and wind driven exchange in a thermally stratified water column allows for phytoplankton species in the water mass to be transported into the bays in the South west

of Ireland. This could go some way towards explaining how harmful phytoplankton that are growing in the shelf waters around the South-Western coast of Ireland can penetrate into the bays and concentrate in coastal locations. These wind driven HAB events in the South-West coast could be used as a proxy for the movement, temporal and spatial distribution of *Azadinium* around the coast, as the inshore coastal current move clockwise around the Irish coast.

Other recent information gathered in offshore water during the month of August suggests that offshore populations of *A. spinosum* observed to occur in moderately stratified water in the Celtic Sea may pool there and await oceanographic currents to either dissipate the bloom, or to concentrate them into a highly toxic front that can be transported into the shellfish production areas in the South-Western bays of Ireland. The offshore presence of *Azadinium* is suggested also in earlier observations of Azaspiracid present in offshore locations during the month of July (Fig. 7).

Outside of Ireland there have also been indications regarding the offshore presence of *Azadinium*. During the month long survey in 2007 on the RV Poseidon around the Eastern Scottish coast and Skagerrak, Azaspiracids were found at a large number of offshore stations⁶. Taking the presence of the toxins as a proxy for the spatial distribution of the producing species this was the first reliable information we have on the organism and its biogeographical distribution in the North Sea.

Cell densities of *Azadinium* of the magnitude reported from Argentinian waters in Akselman & Negri³⁹ have not been observed elsewhere. Two blooms in consecutive years in shelf break waters of the coast of Argentina in Austral spring time (September-November) in 1990-1991 showed the presence of a small armoured dinoflagellate in large numbers (9×10^6 cells L⁻¹). The cells were characterised as *A. cf spinosum*, but molecular or toxicity studies to confirm this preliminary identification were not carried out at the time. Nevertheless, the surveys

carried out in Argentina showed that the distribution of this *Azadinium* species is also found in shelf break waters off the coast of Argentina and that it is probably widely distributed, at least spatially. However, in Ireland blooms of this magnitude have not yet been reported despite regular toxicity in shellfish at a scale not reported elsewhere.

The cosmopolitan nature of the *Azadinium* genus is shown by its presence in the Pacific Ocean¹⁷, where for the first time qPCR results show *A. cf poporum* temporal populations dynamics in Shiwa Bay, South Korea⁴³. These populations were found every year during a three year study period albeit in low cell concentrations (peak concentrations of about 5 cells mL⁻¹) and the authors concluded that a combination of predation on *A. cf poporum* and their physiology may contribute to these low numbers. Blooms of this order of magnitude are more like the concentration of blooms observed so far of *Azadinium* and other *Azadinium* like species in Irish waters.

There remain many questions regarding the spatial and temporal distribution of *Azadinium*. These include whether or not they originate solely from offshore areas and what type of blooms they form. The cell densities that are observed so far do not fully explain observed toxicity events, more information regarding the depths at which they may form thin layers would help explain their ecology and intoxication dynamics in the sea, for instance are they autochthonous populations wintering in our bays, encysting and excysting at different times of the year or are they advected into them by oceanographic processes? All these questions and others have not been answered yet and future research will have to be broadened to answer some of them. Our knowledge on their distribution is compounded by their small size and the difficulty in positively identification using light microscopy alone. Gene probes for the species have been generated⁴⁰ and these tools will be useful in the next few years to unravel some of the mysteries surrounding these organisms.

4. Biology and physiology

4.1 Biology

Compared to the relatively well studied morphology, little is known on the biology and ecology of the AZA-producing species. Without doubt, all species of Amphidomataceae are photosynthetic and, as has been shown for *A. spinosum*, can grow in axenic cultures⁶.

Nevertheless, most if not all photosynthetic dinoflagellates are believed to have mixotrophic capabilities^{44,45}, however, nothing is known on potential mixotrophy of *Azadinium*.

Preliminary trials offering small cyanobacteria as food failed to detect any particulate uptake of *Azadinium* (unpublished information, Urban Tillmann, AWI) but this needs to be studied in much more detail. For a number of bloom forming photosynthetic dinoflagellates the uptake of heterotrophic bacteria has been proposed⁴⁴. For *Azadinium* grown in non-axenic culture in the presence of bacteria, transmission electron microscopy thin sectioning failed to detect intracellular food vacuoles (personal communication, Michael Schweikert, Uni Stuttgart, Germany) so that mixotrophic uptake of bacteria seems unlikely, at least at non-limiting nutrient conditions. Likewise, ultrastructural investigation failed to detect signs of intracellular/symbiotic bacteria (personal communication, Michael Schweikert, Uni Stuttgart, Germany), which is additional evidence that bacteria are not involved in AZA synthesis, neither extra- nor intracellular.

All species of Amphidomataceae have been described to exhibit a conspicuous swimming behavior. Cells normally swim at low speed (quantified for the Korean strain of *A. poporum* as $400 \mu\text{m sec}^{-1}$ ⁴³), interrupted by short, high-speed “jumps” in various directions. *A. languida* is particularly slow in its general movement, which is reflected in its name (*languida* (lat.) = lazy, slow). For all species, the jumps are interspersed but regularly observed when

cells approach other objects, e.g. when they reach the glass bottom of the observation chamber. Rarely, larger distances may be travelled at higher speed.

The ability to jump is generally assumed to be a direct escape mechanism involved in predator/prey interactions⁴⁶⁻⁴⁸. Related jumping behaviour might be observed among species of gymnodinoid dinoflagellates or within the genus *Heterocapsa*⁴⁹, but the characteristic swimming behaviour of *Azadinium/Amphidoma* still is helpful to be used at low microscope magnification as a distinguishing mark in species isolation^{13,17}.

Knowledge on the life cycle of *Azadinium/Amphidoma* is quite incomplete. In culture, all species grow vegetatively by simple binary fission, as has been described in detail for *A. spinosum*¹⁴ and *A. languida*⁸. Dividing cells keep their motility throughout the whole mitotic and cytokinetic process. As a first sign of mitosis, the normally round nucleus enlarges, considerable changes its shape and becomes elongated stretching across almost the whole cell length in a slightly oblique manner. The nucleus then divides along its longitudinal axis. Cytokinesis is started slightly before nuclear division is finished and is of the desmoschisis type, i.e. the parent theca is shared between both sister cells. The left side of the parent cell keeps the cell's apex including the apical pore complex and all apical and epithelial intercalary plates whereas the right side of the parent cell keeps, among others, both antapical plates. Divided cells completely segregate well before thecal plates are fully renewed. When cells of *Azadinium* are stressed the protoplasts can leave their theca (ecdysis), a common reaction among dinoflagellates to adverse conditions which is often related to temporary cyst formation⁵⁰. This type of dinoflagellate cyst normally is round and surrounded by a cell wall. However, this has not yet been observed for *A. spinosum*. Nevertheless, ecdysis of *A. spinosum* might be of importance as it has been related to an increase of extracellular toxins after sample handling⁵¹. However, 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.

A number of dinoflagellates are known to produce cysts, mainly as a dormant, zygotic stage of their life cycle⁵⁰. Such cysts can accumulate in the sediment, hatch after a dormant period and may thus act as “seed banks” with great ecological importance for bloom initiation.

Among Amphidomataceae, cysts have up to now been observed for two species, *A. polongum*²⁰ and *A. poporum*¹³. Successful isolation of *A. poporum* by incubating sediment samples^{13,17} made the presence of cysts quite likely for that species and that has been confirmed by Gu et al.¹³: in one out of 25 cultured strains they observed the presence of a few distinct cysts.

These cysts are ellipsoid, around 15µm long and 10µm wide, and are filled with pale granules and a yellow accumulation body. Likewise, *A. polongum* has been described to produce cysts in culture, round cells of 10-16 µm in diameter and with pale white inclusion. SEM failed to detect any external cyst structures like paratabulation and/or archeopyle, and hatching was not observed. A reduced chlorophyll fluorescence of these cysts and a long persistence in an apparently unaltered state indicated that they might allow long term survival (hypnocysts), rather than serving as temporary cysts. If true, these hypnocysts might be part of the vegetative cycle as has been observed for *Scrippsiella hangoei*, see⁵², or part of a sexual life cycle. Clearly, more data and observations are needed to clarify the whole life cycle of *Azadinium*.

4.2 Physiology (Growth and toxin production)

With the availability of cultures of *A. spinosum*, first laboratory experiments related to growth and toxin production could be performed. Initial studies mainly aimed at investigating the effect of environmental and nutritional factors on growth and toxin production to allow and subsequently improve large-scale AZA production for toxin isolation⁵³. Here, the focus was

set on some of the main environmental factors such as temperature, salinity, irradiance, turbulence, and nutrients.

Growth

Small and delicate dinoflagellates like species of *Azadinium* automatically impose the suggestion of being difficult to grow in culture. However, *A. spinosum* was found to be fairly easy to grow at a wide range of different conditions, to grow to rather high cell densities, to be unaffected by high levels of turbulence (e.g. caused by aeration) and thus turned out to be suited for large scale, high density culturing in photobioreactors⁵⁴.

Like many dinoflagellates⁵⁵⁻⁵⁷, *A. spinosum* is able to divide approximately once per day at optimal conditions, but of course growth is gradually affected by different environmental conditions. When exposed to different temperatures ranging from 10 to 26° C, *A. spinosum* was able to grow at all temperatures so upper and lower temperature limits for positive growth of *A. spinosum* are not yet precisely defined. Nevertheless, growth was optimal at 22° C, with slightly lower growth at higher temperature (26° C) but significantly reduced growth at the lowest temperature tested (10° C). *A. spinosum* was isolated from different coastal locations including an inshore location in Ireland (Bantry Bay) known for their fluctuations in fresh water influence, so some flexibility of *A. spinosum* to different salinities was expected. When suddenly exposed to various salinities (10 to 40 psu) without an adaptation period, *A. spinosum* pre-adapted to 35 psu was able to grow between 30 and 40 psu, survived fairly well at 20 psu but rapidly declined at 10 psu. Although *A. spinosum* thus probably cannot be considered as fully euryhaline, at least surviving a sudden drop to 20 psu indicates that the species is adapted to grow along the Irish coast where fairly large variations of salinity frequently occur in bays due to heavy rain fall. The potential of *A. spinosum* to actively grow at lower salinities after a gentler adaptation period still needs to be determined. In attempts to

achieve maximal toxin yield, the highest cell concentration of *A. spinosum* of more than 300×10^3 cells mL⁻¹ was found in batch cultures with aeration compared to cultures without aeration (90×10^3 cell mL⁻¹), and turbulence/aeration was found not to reduce *A. spinosum* growth. An increase in final cell yield through turbulence/aeration has been observed for other dinoflagellates⁵⁸ and is most probably due to enhancing gas and nutrient mixing⁵⁹, and consequently controlling pH and/or improving carbon and light availability. Growth response of *A. spinosum* to different light levels gave no signs of photoinhibition at higher light levels (tested up to $400 \mu\text{mol m}^{-2} \text{s}^{-1}$). Growth was almost saturated down to the lowest light level tested ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$), where a slight decrease in growth was noticed. The response of *A. spinosum* photosynthesis to light (P/I curves) is not known yet as is growth behavior at lower light (e.g., light compensation point), so a general classification of *A. spinosum* as a “low light” or “high light” adapted species is currently not possible.

Although the nutritional requirements of *A. spinosum* in terms of trace elements/vitamins etc have not yet been defined precisely, culture work performed so far showed that *A. spinosum* is easy to grow on a number of different standard culture media. These include K-medium of various strengths (but with omission of ammonium⁷), F/2 medium²¹ or L1 medium with or without addition of soil extract⁵³, indicating no unusual nutrient requirements. The use of different media, including the addition of soil extract, did not significantly affect growth and toxin cell quota⁵³. In terms of major nutrient, *A. spinosum* is able to use different sources of nitrogen (nitrate, urea, ammonium)⁵³ for growth in batch cultures and chemostat bioreactors. Addition of ammonium, however, reduced growth and thus confirmed the initial observation that omitting ammonium from the standard K-recipe improved growth⁷. Nothing is known about the potential use of dissolved organic compounds by *A. spinosum*, or of any other form of mixotrophy.

Toxin production

As it has been shown for a number of other dinoflagellates producing other polyether toxins^{60,61}, AZA production of *A. spinosum* seems to be constitutive (i.e. toxins are found in significant amount in the cells at all stages of growth), and stable (i.e. the strains have kept their toxin production potential now for at least 5 years in culture). Moreover, the toxin profile of *A. spinosum* consisting of AZA1, -2 and AZA716 (see Hess et al., this book) has been shown to be consistently stable at all environmental conditions tested and thus most probably is under genetic control. The vast majority of toxins (roughly 99%) is intracellular⁵¹, although significant amounts of dissolved toxins in cultures can be found after handling stress (e.g. extended time after centrifugation)⁵¹ or during late senescent phase when cells start to decay⁵³.

Nevertheless, despite the stability of toxin production potential and toxin profile, quantitative differences in the toxin cell quota may be large. Summarising all available data indicates a tremendous influence of culture and environmental conditions on AZA cell quotas, which were found to vary more than 40 fold, from ca 5 to more than 200 fg cell⁻¹. Available evidence indicates that much of this variability is due to toxins accumulating in cells when growth rate declines or completely stops. Generally, when comparing growth and AZA cell quota at different environmental conditions, lower growth rate was constantly coupled with higher toxin cells quota⁵³ underlining the notion that toxin production is not strictly coupled to growth. This can exemplarily be illustrated with Fig. 8 showing growth and AZA cell quota of 10 L aerated batch cultures. These cultures showed a maximum growth rate of 0.57 day⁻¹ and a maximum cell concentration of 302×10^3 cell mL⁻¹. Whereas AZA2/AZA1 ratio was quite stable during all growth phases (0.3 ± 0.02), AZA cell quota significantly increased with decreasing growth rate during the transition from the initial maximum exponential increase to growth cessation and peaked during stationary phase. The final decrease in cell quota, when

cell concentration started to decline (senescent phase) coincided with a significant release of toxin into the medium (35 % of total toxins compared to about 1% during all sampling points before).

The detailed role of light and nutrients for toxin production and accumulation is not clear.

When grown in batch cultures at different light levels, AZA cell quota was found to be unaffected by light although growth was clearly light saturated. This might indicate that under these conditions surplus light could not be used for toxin production. However, in stirred photobioreactors operated as chemostats at high cell densities and a fixed dilution rate, light intensity under this potentially nutrient limiting chemostat conditions had a major effect on the AZA cell quota. An increase by a factor of 3 was observed between the lowest photon flux density and the highest one (21 to 69 fg cell⁻¹), indicating that under nutrient limiting growth, surplus light energy leads to an increased AZA accumulation.

In terms of nutrients, the nitrogen source (nitrate, urea, ammonium) was found not to influence toxin cell quota, neither in batch nor in continuous culture⁵³. Nevertheless, nutrient limitation as a potential cause of growth cessation in batch culture is suspected to increase toxin cell quota. In terms of potential nutrient limitation, in these experiments using K-medium, which has a high surplus of nitrogen, phosphorous is expected to become the main limiting element. Such a role of P-limitation for an increase in toxin accumulation has been found for other toxin dinoflagellates⁶²⁻⁶⁷. There are more indications on factors important for promoting toxin accumulation at reduced/stagnant growth. Stationary phase cultures without aeration had a 3-fold lower cell yield and a 10-fold lower toxin cell quota compared to aerated cultures. This may indicate that carbon limitation in the un-aerated cultures (reduced gas exchange) had limited toxin production, but enhanced nutrient limitation in the aerated cultures due to the substantially higher biomass also could have been important here.

In addition to accumulation of toxin in stagnant cells when toxins are continuously produced at an unchanged rate, increase in the absolute toxin production rate at certain conditions may contribute to elevated cell quota. It still needs to be tested if such an increase of toxin production rate can be found under nutrient limitation. Nevertheless, this could especially be the case for *A. spinosum* at low temperature. Here toxin cell quota at stationary phase were about 30 times higher when grown at 10° C compared to when grown at 22° C. Even accounting for a slightly higher cell volume and for the different growth rate, a roughly 5 times higher absolute AZA production rate was needed to explain these large differences. The maximum cell quota of >200 fg cell⁻¹ in the stationary phase when grown at 10° C may help to explain the rather unexpected but repeated AZA problems in Irish mussels during winter months⁵³.

Pilot scale mass culturing for toxin harvesting

All available data and observations from small scale culture experiments were used to develop and optimize culture conditions to increase cell yield and AZA cell quota for large scale toxin harvesting. As a final approach, a pH controlled and stirred bioreactor (R1) in continuous culture mode was connected in series to a second one (R2) to induce a phase of maturation (growth stagnation but continuing toxin production) in the second bioreactor⁵⁴. Consequently, at steady state, the total AZA cell quota increased between R1 and R2, 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). Thus, as cell production increased with dilution rate and as AZA cell quota decreased, AZA production reached an optimum of 475 ± 17 µg day⁻¹ at a flow rate of 25 L day⁻¹ with 100 L bioreactors connected in series (Tab. 2)⁵⁴.

This culture method coupled to extraction and isolation procedures allowed for a sustained production of significant amounts of AZA1 and -2 from *A. spinosum* for toxicological studies and reference materials⁵⁴.

5. Food web transfer

With respect to *Azadinium*, both bloom dynamics and transfer kinetics and pathways of AZAs into bivalve molluscs are just getting started to be explored. Maxima and persistence of AZA toxins in bivalve shellfish could not yet been correlated in time and space to blooms of *Azadinium* species, but this may reflect observational deficiencies in toxic plankton and toxin monitoring programmes. However, this also opens the possibility of alternative AZA sources (i.e., cryptic AZA-producing species) or toxin vectors, e.g. transfer via the pelagic food web. For a number of toxic algae, grazing within the plankton community is generally viewed as the initial pathway through which algal toxins become vectored into pelagic food webs. Subsequent accumulation and trophic transfer can then intoxicate higher-trophic-level consumers such as fish, sea birds, and marine mammals⁶⁸. For algal toxins accumulating in mussels, two transfer routes must be taken into account: AZAs could accumulate in bivalve shellfish following feeding upon AZA bound to suspended particulates or via plankton vectors (e.g., copepods, tintinnids or other microplankton grazers) that have fed upon toxigenic *Azadinium* cells.

5.1 Planktonic food webs

Without doubt, AZAs can be present in plankton size classes larger than the size of the known producing species and also have been detected in a few grazer species. With respect to the latter, AZAs have been detected in manually picked specimens of the heterotrophic dinoflagellate *Protoperdinium crassipes*². In view of the identification of *Azadinium* as a

primary source of AZAs, a *de novo* AZA production of this heterotrophic dinoflagellate as initially claimed now seems unlikely but rather reflects a trophic accumulation⁶. Calculated cell quota of *P. crassipes* were in the range of 1.5 pg AZA per cell². Compared to an AZA cell quota of *Azadinium* this would correspond to an accumulation factor of roughly 15 to 150, depending on an assumed cell quota of 10 -100 fg cell⁻¹⁵¹. However, direct microscopical observations of a mixed culture of *P. crassipes* and *A. spinosum*, which was feasible for a short time frame before the *P. crassipes* culture unfortunately was lost, made a direct trophic transfer from *A. spinosum* to *P. crassipes* unlikely: in these mixtures, ingestion was never observed; again this needs to be confirmed by more detailed observations and measurements once new *Protoperdinium* cultures are available. Nevertheless, this grazing failure corresponds to more general observations on the feeding mode of large *Protoperdinium* species, which are characterized by a complex handling process^{69,70} seemingly unsuited to handle small and jumping prey species like *Azadinium*. Likewise, the toxin profile detected in *P. crassipes* with substantial amounts of AZA3 present does not reflect the profile of known AZA producers, as AZA3 has not yet been detected here. Nevertheless, we currently can neither exclude a metabolic AZA3 formation in planktonic grazers nor the involvement of a yet undetected plankton source with AZA3 in its toxin spectrum.

As a second grazer species, specimens of the large tintinnid ciliate species *Favella ehrenbergii* collected from field samples has been identified to contain AZAs⁶. Estimated cell quota were in the range of 0.7 pg per ciliate, which is about half of the quantities detected in *P. crassipes* and thus would correspond to a potential accumulation factor of 7 to 70 for an *A. spinosum* cell quota of 10 or 100 fg cell⁻¹.

Beside this detection of AZA in identified grazer species there have been a number of reports on AZA in various plankton size fractions. During a research cruise of RV Poseidon in 2007,

AZA1 was detected over the entire North Sea in plankton samples collected with a plankton net (20 μm), which is assumed not to retain the small species of *Azadinium*⁶. AZA were mostly evenly distributed among three size fractions (20-55 μm , 50-200 μm and >200 μm) subsequently prepared from these net tows, but at two stations with the highest amount of AZA in net-samples, the majority of AZA was found in the 50–200 μm fractions (corresponding to a high abundance of *F. ehrenbergii*). However, using pumped waters samples and subsequent size fractionation, >90% of total AZA1 was found in the 3–8 μm and 8–10 μm fractions, which clearly corresponded to the size class of the identified AZA producing species. It is important to note that in quantitative terms, AZA found in larger size fractions were orders of magnitude lower. Although it is difficult to directly compare Niskin bottle samples and net tows, absolute AZA amounts in the small fraction (assumed to be due to *A. spinosum*) were approximately two to three orders of magnitude higher than the low pg L⁻¹ range estimated in net tow samples (assumed to be the result of trophic transfer). Similar results were obtained on the subsequent cruise in Danish coastal areas of the North Sea⁷¹. Again, AZA1 was present in a number of net tow (20 μm) samples, albeit in low amounts, with only traces found in the > 200 μm fraction. AZA1 concentrations as measured in the small size fraction (< 20 μm), however, were much higher with maximum concentrations of ca. 2 ng AZA1 L⁻¹. This is roughly five orders of magnitude higher than amounts found in corresponding net tow samples. Maximum AZA1 concentrations in net samples were in the range of 50 pg per net tow, which would correspond (assuming 2.5 m³ water filtered) to a concentration of just 20 fg L⁻¹ (which corresponds to typical cell quota of 20 fg for *A. spinosum*). These traces of AZA in larger sized plankton despite of relatively high concentrations of AZA in small size fractions is indicative of a negligible trophic transfer, at least under this particular field situation. Assuming the same cell quota of 20 fg cell⁻¹, AZA1

found in the small size plankton with peak concentrations of 2 pg L^{-1} would correspond to an *A. spinosum* concentration of 10^5 L^{-1} .

In any case, a cell concentration of 10^5 L^{-1} is an order of magnitude lower than *Azadinium* concentrations reported from Argentina³⁹, where two blooms with peak densities of 10^6 L^{-1} were observed. However, it needs to be kept in mind that it is not clear which species of *Azadinium* was responsible for these blooms. Morphology of the species in question showed some similarities with *A. spinosum*, but there also are some differences and additional morphological observations are needed³⁹. Furthermore, DNA sequence data and toxin measurements from these blooms are not available and thus it is quite unclear if indeed the toxigenic *A. spinosum* was present. In any case, field samples of these blooms showed a high diversity and density of heterotrophic protists so that protistan grazing was discussed as an important loss factor for *Azadinium*³⁹. However, just two species (*Gyrodinium fusus* and *Amphidoma* sp.) from a very diverse grazer community were reported to contain ingested *Azadinium* cells indicating that just a few but specialized grazers may have a large influence as loss factor of *Azadinium* blooms.

There is an urgent need for detailed laboratory studies analysing grazer interaction of a broad range of different plankton grazers and different species of Amphidomataceae. The only study published so far⁴³ analysed plankton grazing on the Korean strain of *A. poporum*. These authors showed that a number of protistan grazers are able to ingest cells of *Azadinium*, but just two out of nine species were able to achieve sustained growth with this food. Moreover, for these two species (the heterotrophic dinoflagellate *Oxyrrhis marina* and the ciliate *Strobilidium* sp.), maximum growth rate was much slower when compared to other prey species, indicating that *A. poporum* is of rather poor food quality. Unfortunately, this study did not include measurement of AZAs produced by *A. poporum* and thus nothing is known with respect to potential toxin accumulation/transformation.

Other than that there are a number of preliminary and unpublished studies using *A. spinosum* as food for other planktonic grazers (unpublished information, Urban Tillmann, AWI). As has been observed by Potvin et al.⁴³ for *A. poporum*, certain grazers such as *Polykrikos kofoidii* and *Amphidinium crassum* failed to ingest *A. spinosum* most probably because of prey size limitation (for *P. kofoidii*) or due to their peduncle feeding mode (for *A. crassum*). In contrast, laboratory cultures of *Favella ehrenbergii* clearly ingested *A. spinosum* with initial and substantial toxin accumulation, but failed to achieve a sustained positive growth with just *A. spinosum* as food. *Peridiniella dania*, a small heterotrophic thecate dinoflagellate, has been identified as a promising potential grazer as well but quantitative grazing experiments have not yet been performed. For other small heterotrophic dinoflagellates as *O. marina* and *Gyrodinium dominans*, ingestion of *A. spinosum* was observed, but only rarely, and no positive growth of these grazers was observed when offered *A. spinosum*. As discussed before, swimming behaviour of *Azadinium* might be involved in grazer interactions. Direct observations under the microscope show that *Azadinium* can escape by sudden jumps when attacked by these small dinoflagellates, which start feeding by first attaching a tow filament to its prey. However, ingestion of *Azadinium* by these predators rapidly decreased from initial higher values even when immobilised prey was offered as food (unpublished information, Urban Tillmann, AWI) so it is likely that other factors than motility are involved as well. Clearly much more detailed studies are needed to test the hypothesis that AZA and or other chemical compounds are involved in grazing interactions of *Azadinium*.

Preliminary and yet unpublished experiments with copepods indicated a minor grazing impact and AZA accumulation in this important group of plankton grazers. Cultured *A. spinosum* were added to either field plankton samples or copepods (various species) picked from field samples for 24 h but we failed to detect any substantial grazing or significant AZA accumulation in larger size fractions and copepods, respectively.

Whereas these preliminary experiments do not contradict the possibility of vectoral transfer of AZAs to bivalves they tend not to provide strong support for this mechanism, at least for prey-predator combinations thus far selected. Generally, predator-prey interactions are known to be species-specific and this may be even more important for toxic species where specific chemical compounds might play a role^{68,72}. Furthermore certain plankton predators have been shown to be extremely selective in their prey preferences⁴⁸ so that any generalization about a potential trophic transfer of AZA within planktonic food webs are difficult if not impossible. The few data available so far do at least not support a view of a universal and rapid spread of AZA among planktonic grazers, but may be indicative that certain (specialized) grazers at times may play an important role in food web transfer of *Azadinium* and AZAs. In any case, much more detailed investigations and experiments are needed to clarify this issue.

5.2 Direct transfer to mussels

In Ireland, AZA accumulation by bivalve molluscs occurs frequently since the nineties and may affect many shellfish species. Among them, blue mussels were found to accumulate by far the highest concentration²¹. In Ireland, for all other species of bivalves including Razor clams (*Ensis arcuatus* and *Ensis siliqua*), Dog cockle (*Glycymeris glycymeris*), Abalone (*Haliotis discus hannai*), Common limpet (*Patella vulgata*), Periwinkles (*Littorina littorea*), Pullet carpet shell (*Venerupis senegalensis*), and Venus Clam (*Venus verrucosa*), and gastropods, azaspiracids were present at much lower concentrations. The toxin was also found in Chile in the two commercially important clam species Macha (*Mesodesma donacium*), and *Mulinia edulis* (Coquimbo Bay) as well as in scallops (*Argopecten purpuratus*) and mussels (*Mytilus chilensis*) (from two other areas in Chile), and in Japan, where a marine sponge *Echinoclathria sp.* was contaminated with AZA2⁷³⁻⁷⁵. In 2005 and 2006, azaspiracids were

for the first time detected in gastropods, followed by their detection in Brown Crabs (*Cancer pagurus*) from the West coast of Sweden and the North and North-west coast of Norway³⁰. With the availability of *A. spinosum* cultures, a direct link between AZA accumulation by blue mussels and *A. spinosum* could recently be demonstrated²¹. Blue mussels were able to directly feed on *A. spinosum*, and the presence of AZA1 and -2 and of some metabolites (AZA3, -17 and -19) was detected following 24h exposure to the microalga. AZA17 was the major metabolite (ratio of AZA17 to AZA1 toxin was 5:1) and was mainly found in the remaining flesh of mussels compared to AZA1 and -2, which were found in the digestive gland. This indicates that there is an active biotransformation of the toxins in the digestive system of the mussels. These observations were subsequently confirmed, and AZA-17 and AZA19 were highlighted as two major metabolites of AZA1 and -2, respectively, over a week of contamination using *A. spinosum* at different cell concentrations⁴¹. These bioconversions pose a public health problem as AZA17 and -19 are currently not regulated (see Twiner et al., this book). The speed of accumulation (within less than 6h of exposure to high concentrations of *A. spinosum* ($5-10 \times 10^3$ cell mL⁻¹), mussels exceeded the regulatory limit) was also demonstrated by the second study⁴¹. For mussels fed *A. spinosum* for 24 h, the following distribution of toxins was found: 73% in the digestive gland, 11% in the remaining flesh and 8% in the gills. The other tissues (foot, labial palps, mantel, and adductor muscle) showed minor amounts of toxins with values below 3% of the total toxin accumulated. AZA accumulation was also observed at the same rate when mussels were simultaneously fed *A. spinosum* and *Isochrysis* aff. *galbana*, indicating that mussels were not able to select a non-toxic food source and did not avoid *A. spinosum*. Nevertheless, the initial and short period of fast AZA accumulation was soon displaced by a period of reduced or even without accumulation, which led the authors to subsequently evaluate the effect of *A. spinosum* on mussel feeding behaviour⁷⁶. *Azadinium spinosum* was found to have a significant, negative

effect on mussel feeding behaviour compared to *Isochrysis* aff. *galbana*. Clearance rate, feeding time activity, total filtration rate and absorption were significantly lowered after a few hours of exposure. This study⁷⁶ thus clearly showed a negative effect of high concentrations of *A. spinosum* on blue mussel feeding activity and also indicated a possible regulation of AZA uptake by decreasing filtration and increasing the production of pseudo-faeces. It is important to note that these experiments were carried out with mussels collected from French sites not known to be AZA contaminated. In any case, it remains to be determined if these negative effects are directly related to AZA toxins or other chemical and/or nutritional properties of *A. spinosum*.

Since the concentrations of AZAs found in mussels during short term laboratory exposures are still ca. 10-fold lower than the maximum concentrations encountered in the field, several hypotheses may be considered and need to be tested experimentally.

1. Long term exposure to relatively low concentrations of *A. spinosum* in mixed diets are needed to avoid direct short term negative effects of high *A. spinosum* densities and to result in high toxin accumulation.
2. Additional food web components play a role in the accumulation (e.g. planktonic grazers like small metazoans, heterotrophic dinoflagellates or ciliates, see 5.1, this chapter)
3. The short-term toxin dose in nature may be much higher, because AZA cell quota of field populations may be higher and/or environmental conditions may result in higher cell concentrations at bloom events
4. Mussels in Ireland may have adapted to continuous exposure to *Azadinium* and thus may react differently compared to French mussels
5. Uptake of toxins may additionally occur through the dissolved phase

To verify the last hypothesis, we investigated the possibility of an uptake from the dissolved phase. Mussels were found to accumulate dissolved AZAs (applied at 0.75 and 7.5 $\mu\text{g mL}^{-1}$) from the aqueous phase to significant levels, i.e. above regulatory limits⁷⁷. Interestingly, the toxin distribution in the mussel tissue was different: when fed *A. spinosum*, mussels mainly accumulated AZAs in digestive glands, but mussels exposed to dissolved AZAs accumulated a significant proportion of toxins in the gills. Other dissolved lipophilic toxins like brevetoxins⁷⁸ also have been shown to accumulate in bivalves or fish, apparently through the gills and by ingestion^{79,80}. Dissolved AZAs were found but could not be quantified using passive sampling techniques both along the Irish⁸¹ and Norwegian⁸² coasts, indicating that this potential route of mussel intoxication should be evaluated quantitatively.

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Tab. 1: Morphological features for species of *Azadinium* and *Amphidoma languida*

Feature	<i>Azadinium spinosum</i> ^{a)}	<i>Azadinium obesum</i> ^{b)}	<i>Azadinium poporum</i> ^{c)}	<i>Azadinium caudatum</i> ^{d)}		<i>Azadinium polongum</i> ^{e)}	<i>Azadinium dexteroporum</i> ^{f)}	<i>Amphidoma languida</i> ^{g)}
				var. <i>margalefii</i>	var. <i>caudatum</i>			
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	8.5 x 6.2	13.9 x 11.9
Length/width ratio	1.6	1.3	1.3	1.2 ²⁾	1.2 ²⁾	1.3	1.4	1.2
Stalked pyrenoid(s)	1, central episome	no	Several (up to four)	no	no	no	1	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	4 apicals, 3 intercalaries	6 apicals, 0 intercalaries
Antapical projection	Small spine	no	no	Short horn, long spine	Long horn, short spine	Small spine	Small spine	Antapical pore (pore field of small pores)
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' (posterior position)	Left side, suture of 1' and 1'' (slightly posterior)	Right side and at the end of po	Right side of 1' (anterior position)
Shape of pore plate	Round/ellipsoid	Round/ellipsoid	Round/ellipsoid	Round/ellipsoid	Round/ellipsoid	Distinctly elongated	Round/ellipsoid	Round/ellipsoid
Contact of ventral precingulars with intercalaries	Ventral 1'' in contact to 1a	no	Ventral 1'' in contact to 1a	Ventral 1'' in contact to 1a, Ventral 6'' in contact to 3a	Ventral 1'' in contact to 1a, Ventral 6'' in contact to 3a	Ventral 1'' in contact to 1a	Ventral 1'' in contact to 1a	Not applicable (no intercalaries)
Shape of plate 4''	Similar size as other precingular, in contact to 3a	Similar size as other precingular, in contact to 3a	Similar size as other precingular, in contact to 3a	Smaller than other precingulars, no contact to 3a	Smaller than other precingulars, no contact to 3a	Similar size as other precingular, in contact to 3a	Similar size as other precingular, in contact to 3a	Similar size as other precingular
Shape of 2a	convex	convex	convex	convex	convex	convex	concave	convex
Azaspiracids	AZA-1,-2, -716	Not detected	Large strain variability AZA-846, -876, -2 AZA new china	Not detected	No culture available, not analysed yet	Not detected	Tentatively AZA-3, AZA-7 LC-MS confirmation needed	AZA-816, -830

1) cell length including antapical projection (horn and spine) 2) Length excluding antapical projection

References: ^{a)} Tillmann et al. 2009; ^{b)} Tillmann et al. 2010; ^{c)} Tillmann et al. 2011, Krock et al. 2012; ^{d)} Nezan et al. 2012; ^{e)} Tillmann et al. 2012b; ^{f)} Percopo et al. subm.; ^{g)} Tillmann et al. 2012a

Tab. 2: *A. spinosum* concentration ($\text{cell}\cdot\text{mL}^{-1}$), toxin content ($\text{fg}\cdot\text{cell}^{-1}$), and cell and toxin production ($\text{cell}\cdot\text{day}^{-1}$ and $\mu\text{g}\cdot\text{day}^{-1}$, respectively) at the dilution rates studied (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, adapted from ⁵⁴.

<i>A. spinosum</i>	0.15 day^{-1}		0.2 day^{-1}		0.25 day^{-1}		0.3 day^{-1}	
	R1	R2	R1	R2	R1	R2	R1	R2
Concentration ($\times 10^3 \text{ cell mL}^{-1}$)	193 \pm 6	214 \pm 3	194 \pm 8	214 \pm 7	190 \pm 6	221 \pm 5	187 \pm 5	220 \pm 4
AZA1+2 (fg cell^{-1})	67 \pm 3	98 \pm 5	44 \pm 13	95 \pm 16	38 \pm 2	86 \pm 3	24 \pm 1	63 \pm 5
Cell production ($\times 10^3 \text{ cell day}^{-1}$)	2.90 \pm 0.09	3.21 \pm 0.05	3.9 \pm 0.2	4.3 \pm 0.1	4.8 \pm 0.2	5.5 \pm 0.1	5.6 \pm 0.2	6.6 \pm 0.1
Toxin production AZA1+2 ($\mu\text{g day}^{-1}$)	193 \pm 9	314 \pm 15	170 \pm 50	406 \pm 64	180 \pm 10	475 \pm 17	134 \pm 5	415 \pm 33

Figure legend:

Fig. 1: Light microscopy (upper panel) and electron microscopy (lower panel) micrographs of species of *Azadinium* and *Amphidoma languida*. Scale bars = 5 μm . For micrographs of the most recently described species *A. dexteroporum* see Percopo et al. (submitted).

Fig. 2: A-C: General plate pattern in Kofoidian nomenclature of the genus *Azadinium* for (A) epithelial, (B) hypothecal and (C) sulcal plates (Sa: anterior sulcal plate; Sp: posterior sulcal plate; Ss: left sulcal plate; Sm: median sulcal plate; Sd: right sulcal plate). D: schematic drawing (not drawn to scale) of the ventral epitheca of different species indicating the variable shape of the pore plate (Po), first apical plate (1^ˆ) and the variable position of the ventral pore (vp).

Fig. 3: Schematic representation of morphological features used to characterize the two dinoflagellate orders Gonyaulacales and Peridinales (adopted from Fensome et al. 1993) and the corresponding feature of the genus *Azadinium*. Arrows indicate a tentative affinity to the orders.

Fig. 4: Global records of the genus *Azadinium* and *Amphidoma languida*

Fig. 5: Figure 1: Cell counts, PCR data and Azaspiracid concentrations May to September 2012, Killary Harbour, Ireland

Fig. 6: Distribution and concentration of Azaspiracid toxins (AZA1 $\mu\text{g eq.g}^{-1}$) in Irish farmed mussels (*Mytilus* sp.) between 2002 and 2012.

Fig. 7: Western Ireland offshore azaspiracid (2001) and *Azadinium spinosum* (2012). (a) Distribution of Azaspiracid measured by LCMS from seawater filtrate at 100 offshore locations in July 2001 (b) distribution of presence of *A. spinosum* measured by PCR from net haul samples taken at 69 stations in August 2012.

Fig. 8: (a) Gompertz model fitted to the cell concentration with its 95% confident bounds for the maximum cell concentration (C_{max}), growth rate (μ_{max}), latency time and its adjusted R^2 and (b) AZA1+2 cell quota as a function of time (error bars = SD, $n=3$).

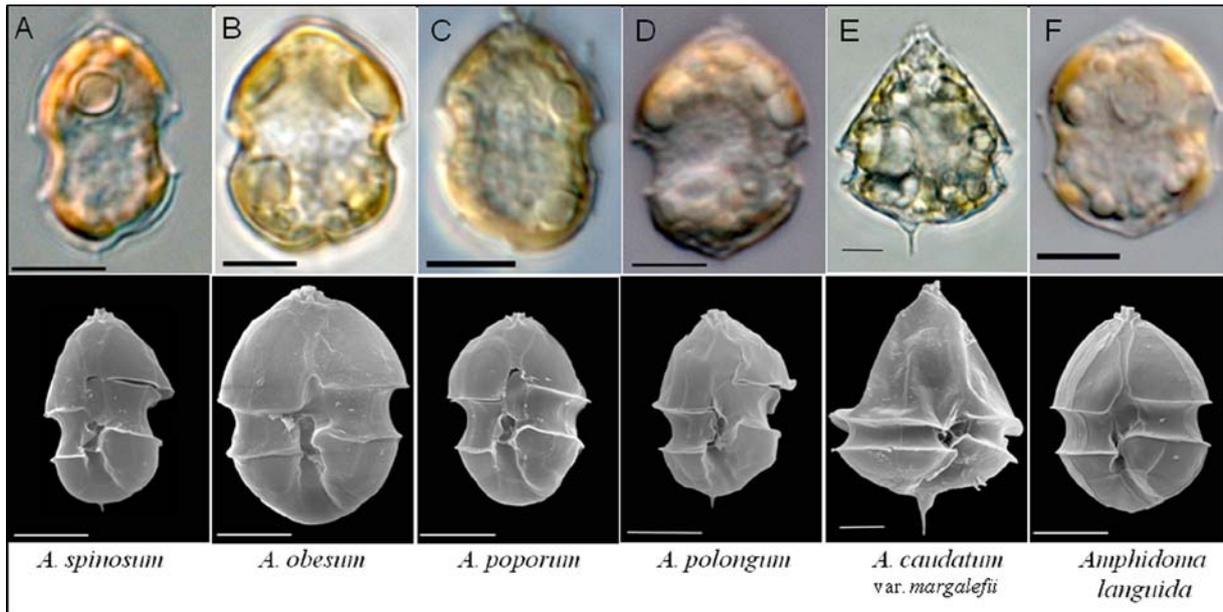


Fig. 1

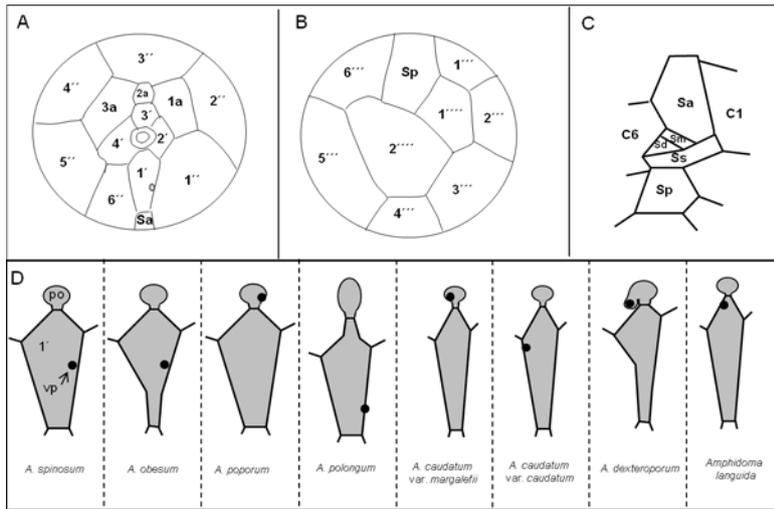


Fig. 2

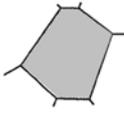
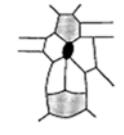
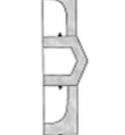
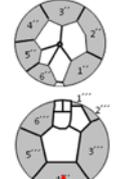
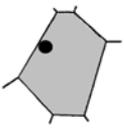
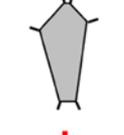
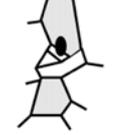
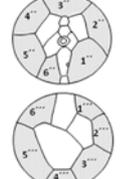
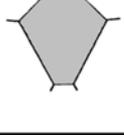
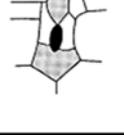
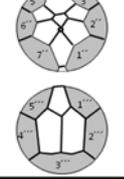
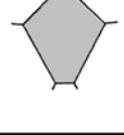
	symetry 1' - apical	symetry antapical	APC	flagellar pore sulcal area	growth bands	precingular postcingular	cell division	ventral pore
Gonyaulacales							Predominantly desmoschisis	
Azadinium							desmoschisis	
Peridinales							Predominantly eleutheroschisis	

Fig. 3



- | | |
|---|--|
| <p>1 Tillman et al. 2009, 2010 (<i>A. spinosum</i>, <i>A. obesum</i>)</p> <p>2 Tillmann et al. 2011 (<i>A. spinosum</i>, <i>A. poporum</i>)</p> <p>3 Salas et al. 2011, Tillmann et al. 2012a (<i>A. spinosum</i>, <i>Amphidoma languida</i>)</p> <p>4 Tillmann et al., 2012b (<i>A. spinosum</i>, <i>A. polongum</i>)</p> <p>5 Nezan et al. 2012 (<i>A. caudatum</i>, both varieties)</p> <p>6 Tillmann 2012, unpubl (<i>Azadinium</i> sp.)</p> <p>7 Margalef, et al. 1954 (<i>A. caudatum</i> var. <i>margalefii</i>, as <i>Oxytoxum</i> sp.)</p> <p>8 Rampi 1969 (<i>A. caudatum</i> var. <i>margalefii</i>, as <i>Oxytoxum margalefii</i>)</p> <p>9 Percopo et al., submitted (<i>Azadinium dexteroporum</i>)</p> | <p>10 Tillmann & Busch, unpubl. (<i>Azadinium</i> spp.)</p> <p>11 Hernandez-Becerril et al., 2012 (<i>A. spinosum</i>)</p> <p>12 Akselman & Negri, 2012 (<i>A. cf. spinosum</i>)</p> <p>13 Checklist Black Sea phytoplankton, http://phyto.bss.ibss.org.ua/wiki/Azadinium_spinosa</p> <p>14 Consuelo Carbonel Moore, pers. com. (<i>Azadinium</i> sp. and <i>Amphidoma languida</i>)</p> <p>15 Potvin et al. 2012 (<i>A. cf. poporum</i>)</p> <p>16 Gu et al. 2013 (<i>A. poporum</i>)</p> |
|---|--|

Fig. 4

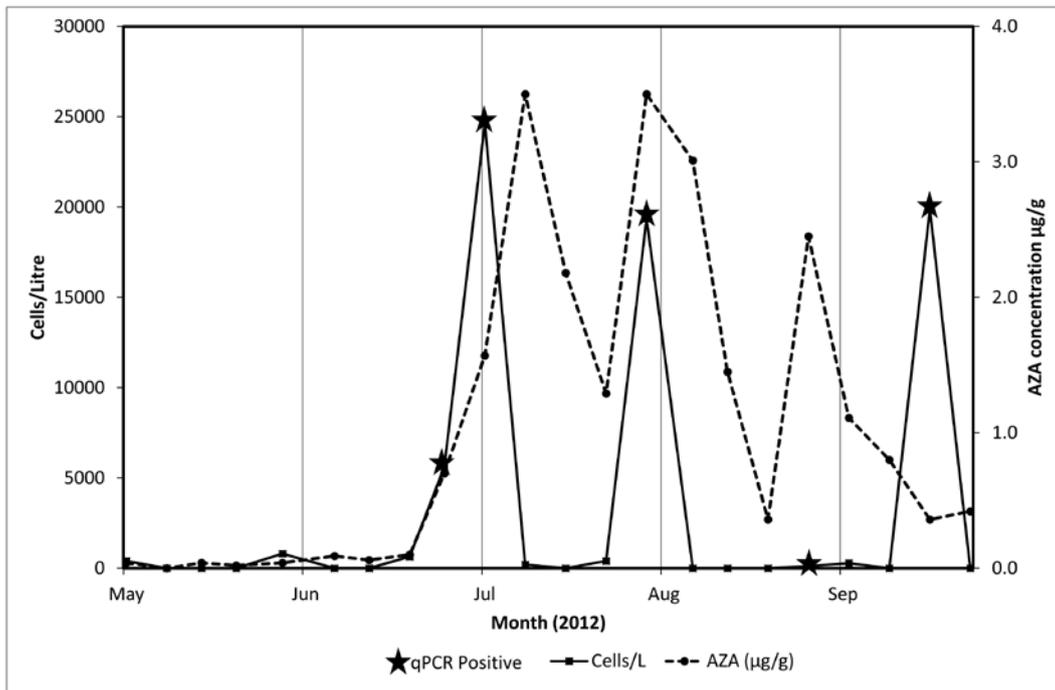


Fig. 5

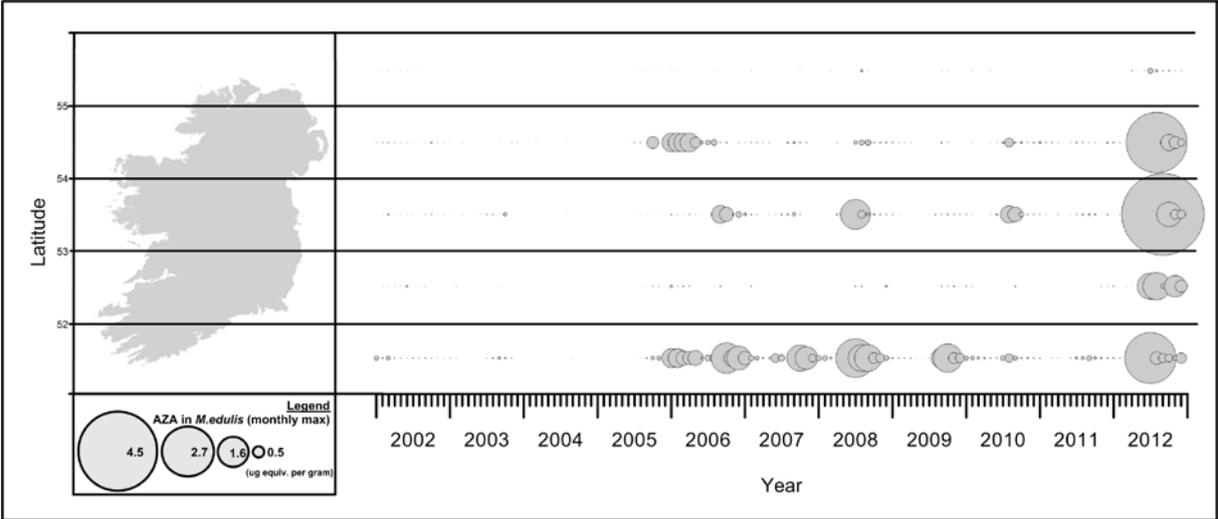


Fig. 6

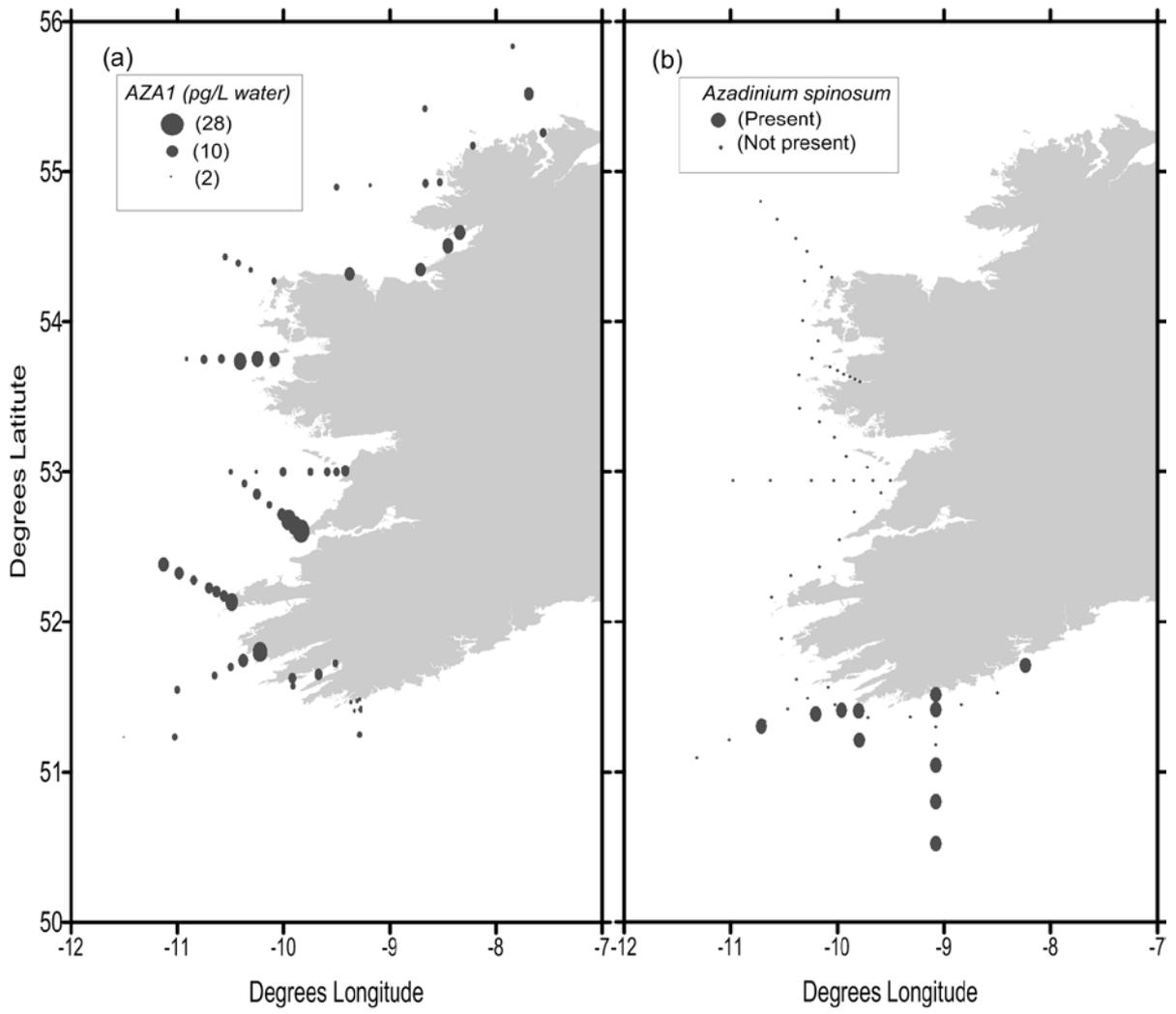


Fig. 7

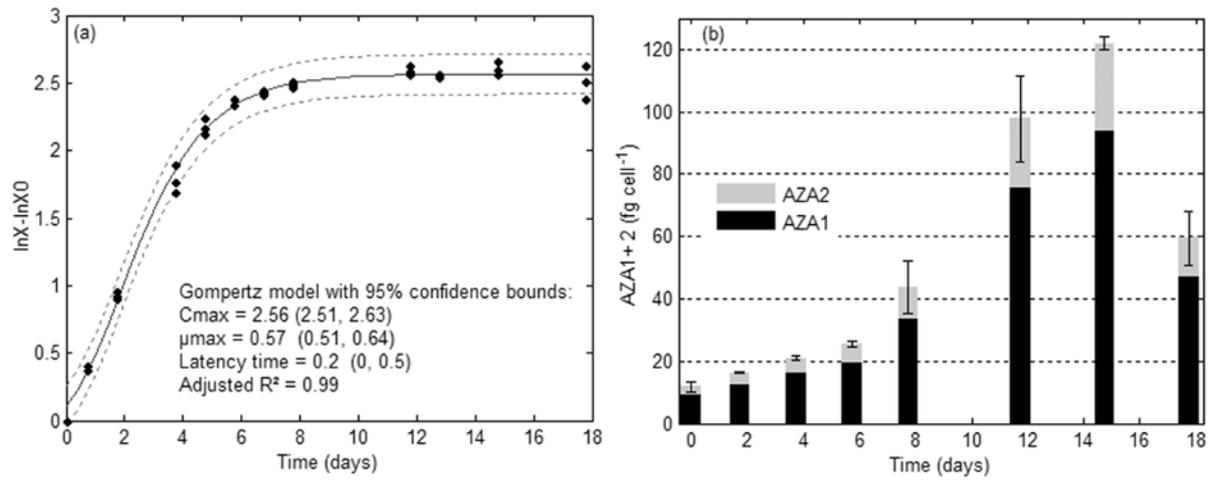


Fig. 8