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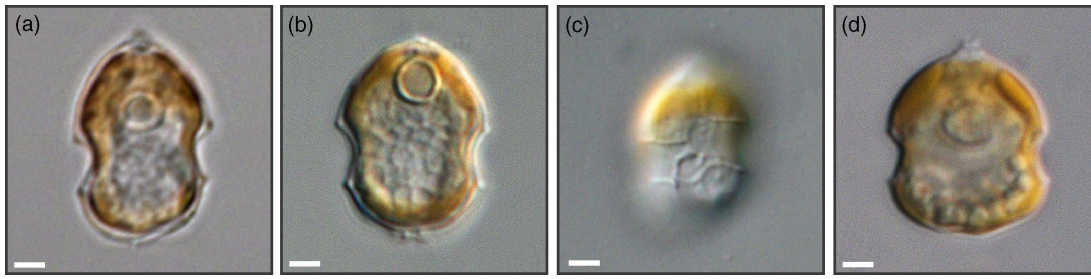

Figure 1 LM micrographs of *Azadinium spinosum* (a), *Az. poporum* (b), *Az. dexteroporum* (c) and *Amphidoma languida* (d). Scale bars = 2 μm.

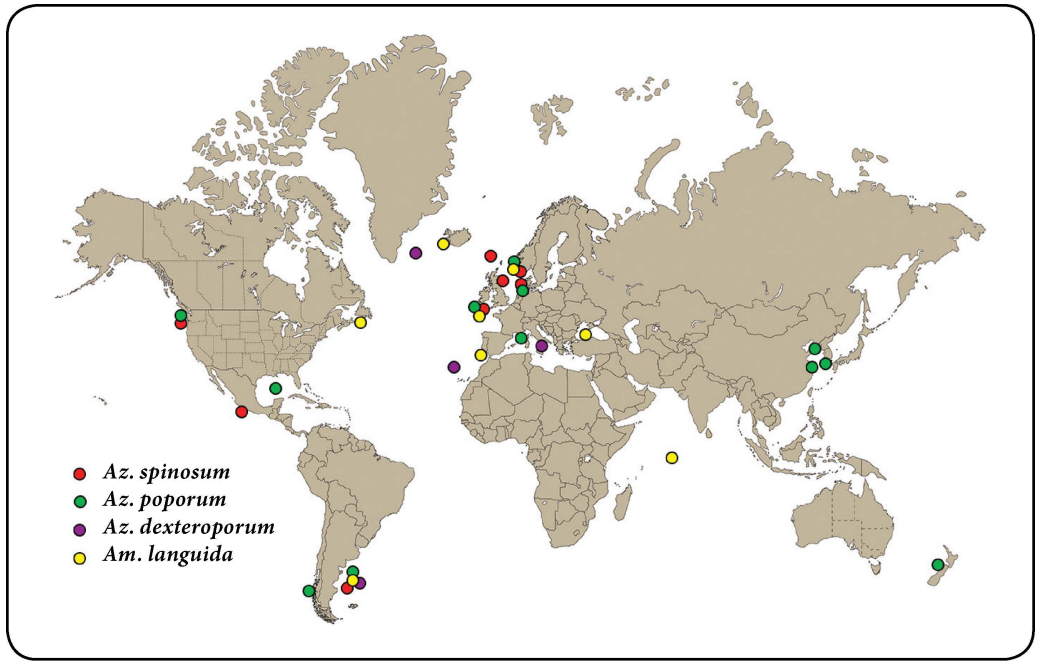

Figure 2 Global records of the four species of Amphidomataceae known to produce azaspiracids (AZA).

Table 1 Members of Amphidomataceae and status as AZA producers.

AZA producer	no AZA found	not analysed yet
Azadinium spinosum	*Azadinium obesum*	*Azadinium caudatum* var. *caudatum*
Azadinium poporum	*Azadinium polongum*	*Azadinium luciferelloides*
Azadinium dexteroporum	*Azadinium caudatum* var. *margalefii*	*Amphidoma nucula*
Amphidoma languida	*Azadinium dalianense*	*Amphidoma acuminata*
	Azadinium trinitatum	*Amphidoma curtata*
	Azadinium cuneatum	*Amphidoma depressa*
	Azadinium concinnum	*Amphidoma elongata*
	Azadinium zhuanum	*Amphidoma laticincta*
	Amphidoma parvula	*Amphidoma obtusa*
		Amphidoma steinii

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General: Azaspiraids (AZA) are a group of lipophilic polyether toxins first detected and described in the late 1990s. With the description of *Azadinium spinosum* in 2009, the first source organism has been identified. Currently, there are four out of 22 species of the genera *Azadinium* and *Amphidoma* (merged in the family Amphidomataceae) that have been shown to produce AZA. However, it has to be kept in mind that there is only a limited number of cultured strains available, and it is thus not clear if and to what extent AZA production is a species-specific stable phaenotypic trait.

General Morphology: All AZA-producing species of *Azadinium* and *Amphidoma languida* are small (size of about 10–16 µm) and ovoid to elliptical in shape with a hemispherical hyposome. In all these species, the episome is larger than the hyposome, with slightly convex sides ending in a distinctly pointed apex. The cingulum is deep and wide, accounting for roughly 1/5 to 1/4 of the cell length. 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. 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 all of the AZA-producing species, stalked pyrenoid(s) are visible in the light microscope because of a distinct starch cup. *Azadinium* spp. and *Amphidoma languida* have delicate thecal plates difficult to detect in light microscopy (LM), so that live cells are sometimes difficult to differentiate from small athecate gymnodinoid species. Plate pattern and thecal plate details are important for determination of the genus and species, but require scanning electron microscopy (SEM). Species of *Azadinium* are characterised by the Kofoidean plate pattern of Po, cp, X, 3–4′, 2–3a, 6″, C6, 5S, 6‴, 2⁗, whereas *Amphidoma languida* has six apical plates and no anterior intercalary plates. A very characteristic feature among the AZA-relevant species is the prominent apical pore complex visible in LM, which is composed of an X-plate and a pore plate with a central round pore covered by a cover plate. Plate details important for species identification include the presence/absence and/or location of a single antapical spine and primarily the position of a ventral pore. Morphology, and in particular the plate tabulation with five different rows of plates, undoubtedly classified the family Amphidomataceae as a member of the dinophycean subclass Peridiniphycidae (Tillmann *et al.*, 2009). The relation to one of the two orders of the subclass (i.e. Gonyaulacales and Peridiniales), however, is less clear as some morphological traits imply affinity to Peridinales and others to Gonyaulacales. Using a concatenated alignment of LSU and SSU, the Amphidomataceae have been placed on the peridinean branch remote from the Gonyaulacales, but the true relation to Peridiniales could not be identified reliably (Tillmann *et al.*, 2014). It thus remains to be determined whether they are part of the Peridiniales or represent a distinct lineage that would deserve the recognition at a higher taxonomic level.

Known Distribution: Although the first species of *Azadinium* were initially described from the North Sea, there is increasing evidence that AZA-producing species have a wide geographical distribution. Nevertheless, knowledge on the biogeography of the genus or of certain species currently is rather limited and patchy. It is based on the troublesome procedure of isolating, cultivating and fully characterizing local strains; on a very few records of species detected by scanning plankton samples by electron microscopy; or on positive signals using species-specific molecular detection methods.

Cysts: Knowledge on the life cycle of *Azadinium* and/or *Amphidoma* is quite incomplete. Successful isolation of *Az. poporum* by incubating sediment samples (Potvin *et al.*, 2012; Gu *et al.*, 2013) made the presence of cysts quite likely for that species, and that has been confirmed by Gu *et al.* (2013): in one out of 25 cultured strains, they observed the presence of a few distinct cysts. These cysts are ellipsoid, around 15 µm long and 10 µm wide, and are filled with pale granules and a yellow accumulation body. Likewise, the species *Az. polongum* (a non-AZA producer) has been described to produce cysts in culture,

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round cells of 10–16 µm in diameter and with pale white inclusion. No cyst-like cells have been reported for other species, including *Az. spinosum*, *Az. dexteroporum* and *Am. languida*. Clearly, more data and observations are needed to clarify the whole life cycle of Amphidomataceae.

Toxin: Species of Amphidomataceae are the source of azaspiracids (AZA), a class of polyether toxins discovered almost 20 years ago. Azaspiracids are polyketides with a highly hydroxylated carbon chain that is cyclised by ether bridges, and they contain a six-membered cyclic secondary amino ring. To date more than 50 AZA analogs are known. These include about 20 of dinoflagellate origin, and the others are thought to be produced by bioconversion in shellfish (Hess *et al*., 2014). Azaspiracids are known to be responsible for gastrointestinal disorders with the consumption of AZA-contaminated shellfish, with symptoms quite similar to those of DSP, such as nausea, vomiting, diarrhea and stomach cramps. Preliminary studies of AZA suggested that these compounds are highly toxic with multi-organ damage in mice and teratogenic potential to developing fish, along with a wide array of cellular-level effects, ranging from cytotoxicity to apoptosis and to effects on the hERG potassium channel (reviewed by Twiner *et al*., 2014). Minimal lethal doses (i.p. mice) for the most dominant AZA in mussels have been determined as 200, 110, and 140 µg/kg for AZA-1, -2, and -3, respectively (Satake *et al*., 1998; Ofuji *et al*., 1999). Consequently, a regulatory limit of 160 µg/kg mussel meat for AZA-1 to AZA-3 was implemented in 2002 into the EU biotoxin legislation. More recent studies yielded similar results for mouse toxicity for AZA-2 and AZA-3, but a distinctly lower dose (higher toxicity) of 74 µg/kg for AZA-1 (Kilcoyne *et al*., 2014a). Oral mouse studies indicated no additive or synergistic effects when AZA was administered in combination with okadaic acid or yessotoxin (Kilcoyne *et al*., 2014a).

Around 20 AZA analogs are currently described to be of dinoflagellate origin. Among the dominant AZA found in shellfish, AZA-1 and AZA-2 are produced by *Azadinium*, whereas no planktonic source of AZA-3 is known yet. An increasing number of new AZA are discovered in the Amphidomatacean cultures. Initial mass spectral data (Krock *et al*., 2012) as well as structural elucidation by nuclear magnetic resonance (NMR) spectroscopy (Kilcoyne *et al*., 2014b; Krock *et al*., 2015) showed that some of the new AZA discovered in dinoflagellates are structurally unique from previously reported analogues by having a modification of the nitrogen-containing I-ring of the molecule, which consists of either a missing methyl group at C39 or an additional double bond.

All four described European strains of *Az. spinosum* have the same toxin profile consisting of AZA-1, -2, and -33 (Tillmann *et al*., 2012b), and a few minor compounds have additionally been found in the Scottish strain (Kilcoyne *et al*., 2014b). For *Az. poporum*, a larger number of strains from different areas around the globe have been described, and this is reflected by a considerable diversity within this species in terms of toxin profiles. Whereas all three available North Sea strains produce AZA-37, *Az. poporum* from the Asiatic Pacific region produces more complex AZA profiles, including AZA-2, -11, -36, -40, -41 in different combinations, and also strains without any known AZA have been described.

Azaspiracid-2 (AZA-2) is the major AZA produced by *Az. poporum* from Argentina and by a strain from the Mediterranean, whereas strains from the Pacific coast of Chile produce AZA-11. Most recently, the new AZA-59 was identified from *Az. poporum* strains isolated from Puget Sound, WA (Kim *et al*., 2017). A feature that is shared among some Asian Pacific and Argentinean strains of *Az. poporum* is the production of minor amounts of AZA-related compounds with higher molecular masses. For the Argentinean strains, one of these compounds has been identified as AZA-2 phosphate, which is the first report of a phosphated marine algal toxin (Tillmann *et al*., 2016).

The presence of AZA has also been unambiguously described for the Mediterranean strain of *Az. dexteroporum* (Percopo *et al*., 2013), and detailed LC-MS analysis confirmed the presense of six novel AZA and AZA-35 (Rossi *et al*., 2017). A new strain of *Az. dexteropo-*

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rum, isolated from the subarctic Irminger Sea, however, clearly lacked any of these or other known AZA (Tillmann *et al*., 2015).

The type strain of *Amphidoma languida* isolated from Ireland and a strain originating from the Iceland area produce AZA-38 and -39 (Krock *et al*., 2012; Tillman *et al*., 2015). In contrast, *Am. languida* from the Atlantic coast of southern Spain produce AZA-2 and -43 (Tillman *et al*., 2017).

Cell quotas of AZA were found to be variable within and among strains and species but are typically in the range of 5–20 fg cell^{-1}. A maximum value of 220 fg cell^{-1} for *Azadinium spinosum* grown at 10 °C was reported (Jauffrais *et al*., 2013).

In vitro toxicity along with structure elucidation for some of the new AZA detected in *Az. spinosum* (Kilcoyne *et al*., 2014b) and *Az. poporum* (Krock *et al*., 2015) have recently been determined, and they showed both lower and higher cytotoxicity compared to AZA-1. For other compounds (e.g. AZA produced by *Am. languida* [AZA-38, -39] and *Az. dexteroporum*), specific toxicity is not known yet.

Methods for Toxin Identification: In 2011, the EU replaced the mouse bioassay with LC-MS/MS as the primary monitoring method for the analysis of AZA (and other lipophilic toxins) in shellfish. A number of validated LC-MS/MS methods for detection and quantification of AZA in shellfish have been described (Hess *et al*., 2014). Work on alternative detection methods for AZA has been limited. An antibody-based ELISA assay, as a rapid analytical technique using inexpensive instrumentation, has recently been described as a suitable tool for shellfish toxin analysis (Samdahl *et al*., 2015).

Ecological Observations: As species of Amphidomataceae have only recently been detected and identified, knowledge on their biology and ecology is rather limited. A first set of growth experiments indicated that *Az. spinosum* was fairly easy to grow with a number of standard culture media (indicating no special nutritional requirement) and at a wide range of different salinities, temperatures, and light conditions. Quantitative abundance data of toxic Amphidomataceae are hardly available, but dense blooms (> 10^6 cells L^{-1}) from a species of *Azadinium* from the Argentinean shelf have been observed (Akselman and Negri, 2012). Pathway and transfer kinetics of AZA into bivalve molluscs are just getting started to be explored. Azaspiracid accumulation in mussels following direct feeding on *Az. spinosum* has been proven experimentally, but *Az. spinosum* also had a significant negative effect on mussel feeding behavior and slightly increased mussel mortality compared to a control food (Jauffrais *et al*., 2012). Azaspiracids have been detected in a number of micrograzers (e.g., *Protoperidinium crassipes*, *Favella ehrenbergii*), so that a role of plankton vectors for mussel intoxication needs to be explored.

General Notes: With their small size, their distinctive and species-specific morphological characteristics that are hardly or not at all visible at the LM level, and with the close resemblance of toxigenic and non-toxigenic species, the AZA-producing Amphidomataceae are a good example for the necessity of applying molecular detection methods in monitoring and early warning systems. Molecular probes have been developed for the first three described species, *Az. spinosum*, *Az. poporum*, and *Az. obesum* (Toebe *et al*., 2013), but specific probes for other AZA-producing species (*Az. dexteroporum* and *Am. languida*) are still missing. In addition, it has to be kept in mind that there probably are more AZA-producing species that are not yet identified. *Am. languida*, for example, is the only species of the genus *Amphidoma* known so far for AZA production, and there are eight more species described, for which AZA production cannot be excluded. A general probe recently developed to detect a broad range of Amphidomataceae will be helpful to screen field samples and to aid in the detection, isolation and characterisation of AZA-producing species (Smith *et al*., 2016).

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Azadinium spinosum Elbrächter et Tillmann (Tillmann *et al.*, 2009)

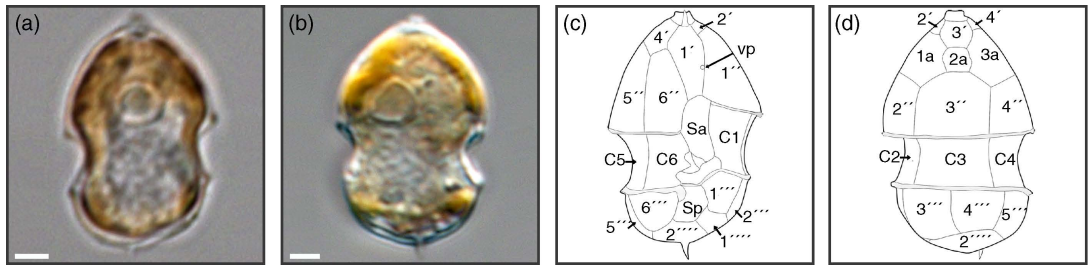

Figure 3 *Az. spinosum* LM micrographs (a, b) and schematic drawings (c, d) including the thecal plates in Kofoidean notation (vp = ventral pore). Scale bars = 2 µm.

Synonyms: None.

Morphology: *Azadinium spinosum* is a small (12–16 µm length and 7–11 µm width), slender (length-width ratio = 1.6), and slightly dorsoventrally compressed thecate, photosynthetic dinoflagellate. The conical episome with convex sides ends with a conspicuous apical pore complex (APC) and is larger than the hemispherical hyposome. It has a wide and descending cingulum, which is displaced by about half its width. In the light microscope, one large pyrenoid visible by its starch sheath is located in the episome. Eponymous for the species is the presence of a single small antapical spine located slightly asymmetrically at the right side of the cell.

Plate pattern and thecal plate details are important for determination of the genus and species, but require SEM. The Kofoidean thecal tabulation of *Az. spinosum* is Po, cp, X, 4′, 3a, 6″, 6C, 5S, 6‴, 2⁗. *Az. spinosum* has a distinct ventral pore located on the left side of the first apical plate.

Distribution: *Azadinium spinosum*, the type of the genus, has been isolated off the Scottish coast, the coast off Denmark, the Shetland Islands, the Norwegian coast, and from coastal Atlantic waters in Ireland. A species of *Azadinium* most likely *Az. spinosum* has been recorded in SEM samples from coastal Pacific waters off Mexico. *Az. spinosum* has also been identified in SEM field samples from the Argentinean shelf (South Atlantic). Recently *Az. spinosum* was detected by qPCR from Puget Sound, WA, U.S.

Azadininium poporum Tillmann et Elbrächter (Tillmann *et al.*, 2011)

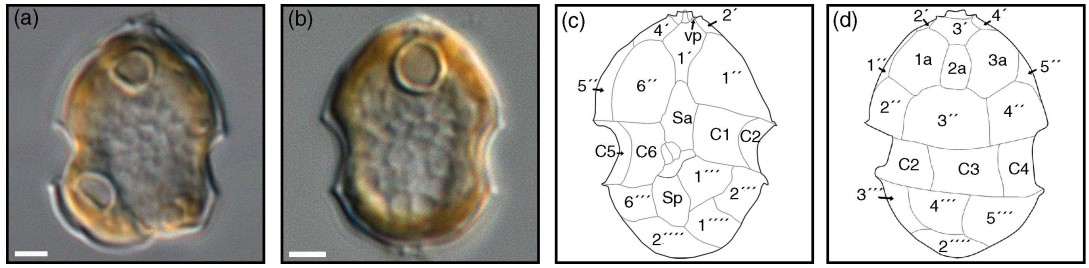

Figure 4 *Az. poporum* LM micrographs (a, b) and schematic drawings (c, d) including the thecal plates in Kofoidean notation (vp = ventral pore). Scale bars = 2 µm.

Synonyms: None.

Morphology: *Azadininium poporum* is small (11–16 µm length, 8–12 µm width), ovoid (length-width ratio = 1.3), slightly dorsoventrally compressed, with a broad and slightly descending cingulum, and with a hyposome slightly smaller than the episome ending in a conspicuous APC. In *Az. poporum*, there may be several (up to four) pyrenoids with a starch sheath visible in LM located in both the epi- and hyposome. The most distinctive morphological feature of *Az. poporum* requires SEM; it is the characteristic position of the ventral

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pore, which is located anterior at the cell's left side of the pore plate at the junction with the first two apical plates.

Distribution: *Azadinium poporum* was described based on strains from the North Sea off Denmark and has also been recorded in Ireland and along the Norwegian coast. A number of strains have been obtained from outside Europe. *Az. poporum* obviously is quite widely distributed in the Asian Pacific. As a first record of *Azadinium* in Pacific waters, *Az. poporum* has been isolated from Shiwha Bay in Korea, and subsequently, 25 different strains of *Az. poporum* originating from China covering the Bohai Sea and the East and South China Seas were established. Most recently, *Az. poporum* was detected in New Zealand both by qPCR and by establishing a culture. Likewise, *Az. poporum* cultures were obtained from samples from the South Atlantic (Argentina), the South Pacific (Chile), and the Gulf of Mexico. Most recently *Az. poporum* was identified by qPCR and isolated strains from Puget Sound, Washington.

Azadinium dexteroporum Percopo et Zingone (Percopo *et al.*, 2013)

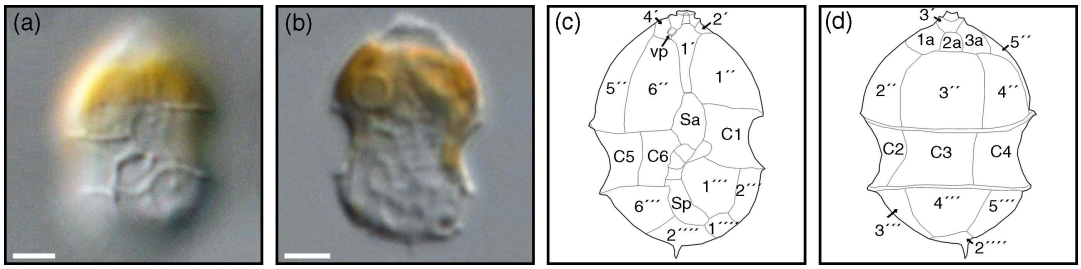

Figure 5 *Az. dexteroporum* LM micrographs (a, b) and schematic drawings (c, d) including the thecal plates in Kofoidean notation (vp = ventral pore). Scale bars = 2 µm.

Synonyms: None.

Morphology: *Azadinium dexteroporum* is the smallest species of *Azadinium* (7.0–10.0 µm in length and 5.0–8.0 µm in width). Cells are slightly elongated (length-width ratio = 1.4) and dorso-ventrally compressed, with the episome longer and slightly larger than the hyposome. The hyposome is slightly asymmetrical, with a small spine located in its posterior right side. The cingulum is deeply excavated and notably wide. One pyrenoid visible by its starch cup is present in the episome. Species-specific morphological details visible at the SEM level include the characteristic arrangement of the ventral pore, which is located at the right posterior end of the markedly asymmetric pore plate. A pronounced concavity of the median intercalary plate 2a has been highlighted as a peculiar feature of the Mediterranean type material, but this plate was plain for a subarctic strain originating from the Irminger Sea.

Distribution: *Azadinium dexteroporum* was initially described from the Mediterranean (Naples), but a new strain representing the species was recently obtained from the Subarctic (Irminger Sea). *Az. dexteroporum* was also identified in SEM preparation of spring bloom samples from the South Atlantic (Argentinean shelf) and is on a species list (as *Az.* cf. *dexteroporum*) of Madeira (North Atlantic off Morocco).

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Amphidoma languida Tillmann, Salas et Elbrächter (Tillmann *et al.*, 2011)

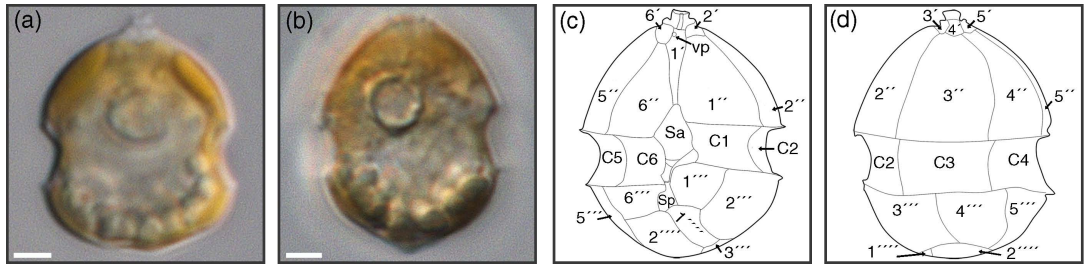

Figure 6 *Am. languida* LM micrographs (a, b) and schematic drawings (c, d) including the thecal plates in Kofoidean notation (vp = ventral pore). Scale bars = 2 µm.

Synonyms: None.

Morphology: Cells of *Amphidoma languida* are ovoid to slightly elliptical (length-width ratio = 1.3), with a conical episome and a distinctly pointed APC. Cells are small (12.9–15.5 µm in length and 9.7–14.1 µm in width). The episome is slightly larger than the spherical hyposome, which ends in a pointed antapex. At the light microscope level *Am. languida* is very similar to small species of *Azadinium*. Electron microscopy, however, reveal major differences in plate pattern, with a Kofoidean plate pattern of Po, cp, X, 6′, 0a, 6″, 6C, 5S, 6‴, 2′′′′. *Am. languida*, as other species of the genus *Amphidoma*, has thus 6 apical and no anterior intercalary plates, whereas *Azadinium* has 3–4 apical plates and 2–3 anterior intercalary plates. Other specific details visible with SEM are the presence of a large antapical pore (which in fact is a field of a number of small pores) and the location of a ventral pore on the anterior right side of the first apical plate.

Distribution: The AZA-producing species *Amphidoma languida* has first been isolated from a bay in Ireland, but definitely has a much wider distribution. Sequence data from plankton samples of the Skagerrak area and strains of this species from the Norwegian coast and from Iceland indicate the presence of *Am. languida* in the North Sea and the North Atlantic as well. More recently, it has been observed in SEM from a seawater sample collected at Saint-Pierre and Miquelon in 2012 and at several sampling locations along the southern coast of the Black Sea in 2014. In contrast to the shallow coasts of Ireland and the Subarctic near Iceland, cells most likely determinable as *Am. languida* have been observed in SEM from a sample collected at the open West Indian Ocean as well. Moreover, *Am. languida* was present in a 1991 bloom sample from the Argentinean shelf. Finally, a culture of *Am. languida* has been established from water off the Atlantic coast of southern Spain.

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