1   ***Azadinium caudatum* var. *margalefii*, a poorly known member of the toxigenic genus**

2   ***Azadinium* (Dinophyceae)**

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11   Running title: *Azadinium caudatum* var. *margalefii*

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Abstract

Azadinium caudatum is a poorly known planktonic dinoflagellate, which mainly attracts attention due to the occurrence of many toxigenic species in the genus *Azadinium*. The availability of two Scottish isolates of *A. caudatum* var. *margalefii* allowed for the first time a detailed analysis of biological and physiological traits of the species, specifically on the potential presence of azaspiracid toxins (AZAs). With a mean overall swimming speed of 85 μm s^{-1} and regularly interspersed high speed jumps, *A. caudatum* var. *margalefii* exhibited a similar swimming behaviour to other species of the genus. Cells had a single, large, highly reticulated chloroplast with a typical pigment composition for peridinin-containing dinoflagellates. No pyrenoids were visible under light microscopy. A round to ovoid nucleus was centrally located. Cell division was by desmoschisis, i.e. the parent theca was shared by the daughter cells. Growth rate μ ranged from 0.07 to 0.32 d^{-1} and was reduced at lower temperatures. With growth rate becoming light saturated at intensities of about 40 μE m^{-2} s^{-1} and a half-saturation light intensity of about 13 μE m^{-2} s^{-1}, *A. caudatum* var. *margalefii* appears to be relatively low light adapted. Neither strain produced any known AZAs in measureable amounts.

Key words: *Azadinium caudatum*, azaspiracids, growth rate, morphology

Introduction

Azadinium is a dinophycean genus of planktonic algae mostly recognized due to its capability to produce azaspiracids (AZAs), a recently discovered group of lipophilic phycotoxins causing human intoxication via mussel consumption. After a first poisoning incident in the Netherlands in 1995, azaspiracid toxins (AZAs) were isolated and chemically characterized from Irish shellfish (Satake et al. 1998; Ofuji et al. 1999). Since then, AZA contamination of mussels has been a recurrent and serious problem mainly in Ireland (Salas et al. 2011), but AZAs have also been detected in samples from Europe, Morocco, Chile, China, and Japan (Braña Magdalena et al. 2003; Taleb et al. 2006; Amzil et al. 2008; Ueoka et al. 2009; Alvarez et al. 2010; Furey et al. 2010; Yao et al. 2010). The biogenic source of the toxins remained elusive until the isolation and identification of a small new dinoflagellate species *Azadinium spinosum* Elbrächter & Tillmann as a primary producer of AZA1 and AZA2 (Krock et al. 2009; Tillmann et al. 2009). Since then, the knowledge of the diversity of the genus has increased rapidly and seven species are currently known. These comprise six newly described species, *A. spinosum* (Elbrächter &Tillmann), *A. obesum* Tillmann & Elbrächter (Tillmann et al. 2010), *A. poporum* Tillmann & Elbrächter (Tillmann et al. 2011), *A. polongum* Tillmann (Tillmann et al. 2012b), *A. dexteroporum* Percopo & Zingone (Percopo et al. 2013), and *A. dalianense* Luo, Gu & Tillmann (Luo et al. 2013). and the species *Azadinium caudatum* (Halldal) Nézan & Chomérat, which was transferred to the genus (Nézan et al. 2012). Among species of the family Amphidomataceae, to which the *Azadinium* species belong, production of AZA toxins occurs in some, but not in all species; *A. spinosum, A. poporum* and the closely related *Amphidoma languida* Tillmann, Salas & Elbrächter (Tillmann et al. 2012a) have been shown to produce AZAs (Krock et al. 2012), whereas other species (*A. obesum*, *A. polongum*) have been described as non-toxigenic (Krock et al. 2012; Tillmann et al. 2012b). For *A. dexteroporum*, production of AZAs has been claimed (Percopo et al. 2013) but their presence

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needs to be confirmed by LC-MS. Finally, for *A. caudatum*, there is no detailed analysis on

the presence of AZAs yet. This species was first described in 1953 by Halldal as *Amphidoma*

caudata off the Norwegian coast (Halldal 1953), and has been reported since from a

Portuguese lagoon (Silva 1968), around the British Isles, off the west coast of Ireland (Dodge

1982; Dodge & Saunders 1985), and, most recently, along the French Atlantic coast (Nézan et

al. 2012). The species is also present in the Mediterranean and has been reported from the

Ligurian Sea (Rampi 1969, as *Oxytoxum margalefi* and *Oxytoxum tonollii*), from the Spanish

coast (Margalef et al. 1954, as *Oxytoxum* sp., Delgado and Fortuño 1991, Margalef 1995),

from Adriatic Sea coasts (Viličić et al. 1995, Totti et al. 2000), and from the Gulf of Naples

(cited in Percopo et al. 2013). The first plate details provided by Dodge & Saunders (1985)

indicated that *Amphidoma caudata* had the same basic plate pattern as *Azadinium*. It was thus

concluded by Tillmann et al. (2011) that, notwithstanding some differences that remained to

be elucidated, *Amphidoma caudata* might be transferred to the genus *Azadinium*, pending

further morphological and phylogenetic studies. Subsequently, a study using field samples and

cultures of "*Amphidoma caudata*" used morphological and molecular data to clarify the

systematic situation and transferred the species to the genus *Azadinium* as *Azadinium*

caudatum (Nézan et al. 2012). Both sequence and morphometric data clearly showed that the

species occurred with two distinct varieties, var. *caudatum* and var. m*argalefii*, which are

easily distinguished by the different shape of the antapical projection (Nézan et al. 2012).

On the basis of cultured strains of *A. caudatum* var. *margalefii* we are now adding more

detailed information to the species, most importantly including a detailed analysis of the

potential presence of AZAs. Furthermore, we performed additional light microscopy

observations on the morphology and swimming pattern, analysed the pigment profile,

elucidated one, yet missing detail on plate overlap pattern, described the mode of cell division

and collected quantitative data on growth performance for this little known dinoflagellate

species.

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Material & Methods

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Isolation and culturing

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Two strains of *Azadinium caudatum* var. *margalefii* designated as AC1 and AC2 were

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isolated from a vertical net tow (0-20 m, 20 µm mesh size) taken in Scottish coastal waters at

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58° 38.03' N, 3° 36.14' W during a cruise on FS "Heincke" in May 2011. Single cells were

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isolated using a capillary in multiwell plates and grown in K medium (Keller et al. 1987)

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prepared from filtered North Sea water. Established cultures were routinely grown in 65

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ml plastic culture flasks at 15° C and 30 µE m^{-1} s^{-1} at a light/dark cycle of 18:6 h. Cell size,

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plate pattern, and sequence data of both strains have been presented before (Nezan et al.

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2012).

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Light microscopy (LM)

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Observation of cells was carried out with a stereomicroscope (Olympus SZH-ILLD) and with

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an inverted compound microscope (Axiovert 200 M, Zeiss, Germany) equipped with

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epifluorescence and differential interference contrast optics. The swimming pattern of cells

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was recorded with a video CCD camera (Sony DSP 3-CCD). For quantitative analysis of

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swimming speed, a culture growing at 20° C and 50 µE m^{-2} s^{-1} was placed into an observation

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chamber (5 ml) over an inverted microscope and allowed to acclimate for 30 min at room

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temperature. Cells were subsequently recorded at a magnification of 320 x at 25 frames per

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second. The swimming tracks of 30 randomly chosen cells were analysed by single frame

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playback for the maximum time period in which the cell was in the focal plane.

23

Light microscopic examination of the thecal plates was performed on formalin-fixed cells (1

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% final concentration) stained with calcofluor white (Fritz & Triemer 1985). The shape and

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localisation of the nucleus was determined after staining of formalin-fixed cells with 4'-6-

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1   diamidino-2-phenylindole (DAPI, 0.1 µg ml^{-1} final concentration) for 10 min. Photographs

2   were taken with a digital camera (Axiocam MRc5, Zeiss, Germany) connected to the inverted

3   microscope.

4

5   *Scanning electron microscopy (SEM)*

6   For SEM examination, cells from growing cultures were fixed, prepared, and collected on 3

7   µm polycarbonate filters (Millipore), as described by Tillmann et al. (2011). Filters were

8   mounted on stubs, sputter-coated (Emscope SC500, Ashford, UK) with gold-palladium and

9   viewed under a scanning electron microscope (FEI Quanta FEG 200, Eindhoven,

10   Netherlands). SEM micrographs were presented on a black background using Adobe

11   Photoshop 6.0 (Adobe Systems, San Jose, CA, USA).

12

13   *Growth experiments*

14   Growth performance of *A. caudatum* var. *margalefii* strain AC1 was quantified as follows: all

15   experimental cultures were grown in 65 ml plastic culture flasks. Initially, a stock culture was

16   grown at 15° C and 50 µE m^{-2} s^{-1}. From this stock culture, dilutions of 100 cells ml^{-1} were

17   prepared and adapted to the respective temperature and light conditions of the final growth

18   experiment for two weeks. After determining cell density in each adaptation set-up, triplicate

19   flasks with an initial cell density of 50 cells ml^{-1} were filled and incubated at the respective

20   experimental conditions. To evaluate the effect of temperature, cultures were grown at 10, 15,

21   and 20° C at a light intensity of 100 µE m^{-2} s^{-1} in temperature adjusted growth chambers. To

22   evaluate effects of light, cultures grown at 15° C were incubated at 10, 50, 100 and 250 µE m^{-2}

23   ^{2} s^{-1}. Light was measured using a 4π quantum sensor (LI 1000, LI-COR, Lincoln USA). In

24   order to analyse a potential growth enhancement effect of soil extract, one triplicate set of

25   flasks incubated at 15°C and 100 µE m^{-2} s^{-1} received K-medium spiked with 1ml l^{-1} of soil

26   extract. Each flask was sampled every second day by removing 2 ml (for the first 3

1   samplings) or 1 ml (subsequent samplings). Flasks were refilled using fresh medium avoiding

2   large air-bubbles; refilling was accounted for when calculating growth rate. Sub-samples were

3   fixed with Lugol´s solution (2% final concentration) and counted under an inverted

4   microscope. Growth rate (μ, d^{-1}) was calculated for a defined period of exponential increase

5   by calculating the exponential regression coefficient for a plot of cell numbers versus time.

6   When stationary phase was reached, pH in each flask was measured using an EcoScan pH5

7   (Eutech Instruments, Nijkerk, The Netherlands) pH-meter. Growth at different photon flux

8   densities was fitted to a Michaelis Menten model ($\mu = (\mu max * I)/(ks + I)$; I = photon flux

9   density, ks = half saturation constant) using Statistica (StatSoft Inc, Tulsa, USA).

10

11   *Pigment analysis*

12   For pigment analysis, both strains were grown in 270 ml plastic culture flasks at the regular

13   culture conditions (see above). At a cell density of 1440 (AC1) and 1920 (AC2) per ml, as

14   estimated by microscopic cell counts, 250 ml were gently filtered on to a glass-fibre filter (25

15   mm Ø, GF/C, Whatman, Kent, UK). For AC1 a second filter was prepared on a different

16   occasion by filtering 250 ml of a cell density of 776 ml^{-1}. Filters were immediately shock-

17   frozen in liquid nitrogen and stored at -80° C. Pigment composition was analyzed by High

18   Performance Liquid Chromatography (HPLC) following a method described by Hoffmann et

19   al. (2006) adjusted to our instruments as detailed by Taylor et al. (2011).

20

21   *Chemical analysis of AZAs*

22   Experimental cultures for toxin analysis were grown in 270 ml plastic culture flasks as

23   described above. Cells were harvested at the late exponential/early stationary phase. Different

24   batches of cultures were harvested and combined by gravity filtration through a 10 µm nitex

25   mesh. Cells were resuspended from the mesh in 50 ml filtered seawater in a 50 ml Falcon

26   tube. After taking a 0.5 ml sub-sample for cell counting, the tube was centrifuged (Eppendorf

1   5810R, Hamburg, Germany) at 3220 x g for 10 min and the cell pellet was transferred to an

2   Eppendorf microtube, centrifuged (Eppendorf 5415, 16,000 x g, 5 min) and stored at -20° C.

3   In total, four pellets of strain AC1 with a total number of 2.87 x 10^6 cells and two pellets of

4   strain AC2 (total number: 0.86 x 10^6 cells) were collected. For analysis each pellet was

5   thawed and suspended in 500 µl acetone, and transferred into a FastPrep tube containing 0.9 g

6   of lysing matrix D (Thermo Savant, Illkirch, France). The samples were homogenized by

7   reciprocal shaking at maximum speed (6.5 m s^{-1}) in a Bio101 FastPrep instrument (Thermo

8   Savant, Illkirch, France) for 45 s. After homogenization, samples were centrifuged (Eppendorf

9   5415 R, Hamburg, Germany) at 16,100 x g at 4° C for 15 min. All supernatants of each strain

10   were combined in a pear shaped flask and taken to dryness in a rotary evaporator (Büchi,

11   Konstanz, Germany). The residues were taken up in 500 µl acetone transferred to a 0.45 µm

12   pore-size spin-filter (Millipore Ultrafree, Eschborn, Germany) and centrifuged at 800 x g for

13   30 s. The filtrate was transferred into an LC autosampler vial for LC-MS/MS analysis.

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15   *Single reaction monitoring (SRM) measurements*

16   Water was deionized and purified (Milli-Q, Millipore , Eschborn, Germany) to 18 MΩ cm^{-1} or

17   better quality. Formic acid (90%, p.a.), acetic acid (p.a.) and ammonium formate (p.a.) were

18   purchased from Merck (Darmstadt, Germany). The solvents, methanol and acetonitrile, were

19   HPLC grade (Merck, Darmstadt, Germany).

20   Mass spectral experiments were performed to survey for a wide array of AZAs. The analytical

21   system consisted of an ABI-SCIEX-4000 Q Trap, triple quadrupole mass spectrometer

22   equipped with a TurboSpray® interface coupled to an Agilent model 1100 LC. The LC

23   equipment included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A),

24   refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven

25   (G1316A).

26   Separation of AZAs (5 µl sample injection volume) was performed by reverse-phase

chromatography on a C8 phase. The analytical column (50×2 mm) was packed with 3 µm

Hypersil BDS 120 Å (Phenomenex, Aschaffenburg, Germany) and maintained at 20 °C. The

flow rate was 0.2 ml min^{-1} and gradient elution was performed with two eluents, where eluent

A was water and eluent B was acetonitrile/water (95:5 v/v), both containing 2.0 mM

ammonium formate and 50 mM formic acid. Initial conditions were 8 min column

equilibration with 30 % B, followed by a linear gradient to 100 % B within 8 min and

isocratic elution for 10 min with 100 % B then returning to initial conditions within 3 min

(total run time: 29 min).

AZA profiles were determined in one period (0 – 18) min with curtain gas: 10 psi, CAD:

medium, ion spray voltage: 5500 V, temperature: ambient, nebulizer gas: 10 psi, auxiliary

gas: off, interface heater: on, declustering potential: 100 V, entrance potential: 10 V, exit

potential: 30 V). SRM experiments were carried out in positive ion mode by selecting the

transitions (precursor ion > fragment ion) listed in the supplementary material (Tab. S1).

Cellular detection limits were calculated from signal to noise (S/N) ratios of an external

standard solution of AZA1 (certified reference material (CRM) programme of the IMB-NRC,

Halifax, Canada) based on an instrumental S/N > 3.

Precursor ion experiments

Precursors of the fragments m/z 348 and m/z 362 were scanned in the positive ion mode from

m/z 400 to 950 under the following conditions: curtain gas: 10 psi, CAD: medium, ion spray

voltage: 5500 V, temperature: ambient, nebulizer gas: 10 psi, auxiliary gas: off, interface

heater: on, declustering potential: 100 V, entrance potential: 10 V, collision energy: 70 V, exit

potential: 12 V.

Results

General morphology

Azadinium caudatum was quite easy to characterise by light microscopy due to its relatively large size (length: 25.9–36.9µm, width 18.9–25.8 µm, n = 154, measurements of strain AC1 and AC2 taken from Nézan et al. 2012), its characteristic triangular shape and its clearly visible antapical projection (Fig. 1 A-E). This projection consisted of a blunt and short horn and a well-developed spine (Fig. 1 C). The large nucleus located in the centre of the cell was typically round to ovoid (Fig. 1 F, G) with clearly visible single chromosomes. Prior to nuclear division the nucleus became enlarged and more elongated (Fig. 1 H). With LM there were no indications of the presence of pyrenoid(s) but cells occasionally contained a number of large spherical bodies which could be stained brown using Lugol´s solution (Fig. 1 I, J). Fluorescence microscopy revealed the presence of a presumably single but complex, highly lobed retiform chloroplast extending both into the epi- and hyposome (Fig. 1 K-O). The ventral/sulcal area, however, was devoid of chloroplasts with the finger-like extensions of the plastid ending in that area (Fig. 1 N, O).

Swimming pattern

Like all other species of *Azadinium*, *A. caudatum* var. *margalefii* exhibited a characteristic swimming pattern, i.e. swimming at a generally slow speed interrupted by sudden jumps (Fig. 2, see also video S1 provided as Supplementary material). Swimming paths generally were straight or slightly curved (e.g. path no 14). Overall mean swimming speed of the swimming paths depicted in Fig. 2 and quantified in Tab. 1 was 85 µm s^{-1}. During slow speed movement, speed was typically in the range of 50-60 µm s^{-1} (e.g. path no 8, 14, 26) but cells occasionally travelled longer distances at a higher speed of 80 – 140 µm s^{-1} (e.g. path no 3, 7). High-speed jumps were accelerated changes in direction at an angle > 45°, which most often was close to 90° (e.g. track no 4,6,11,12,24,25,28,29), but sudden path deviations more close to 45° were also observed (e.g. path no. 15,30). Short-duration maximum speed during these jumps has

1   been calculated as approx. 360 µm s^{-1}.

2

3   *Plate overlap*

4   The whole plate overlap or imbrication pattern for *Azadinium caudatum* was reported in

5   Nézan et al. (2012). However, one interesting detail could not be resolved there and is

6   reported here using SEM (Fig. 3). In the ventral area, the anteriour sulcal plate (Sa) was

7   overlapped by its left neighbouring cingular plate C1 but overlapped the right neighbouring

8   plate C6 (Fig. 3 B-D). Thus, for the midventral area plate C6 and not plate Sa was overlapped

9   by all adjacent plates.

10

11   *Division mode*

12   Dividing cells mainly increased the cell width (Fig. 4 A, M). Throughout the whole

13   cytokinetic process, cells of *Azadinium caudatum* kept their motility. Cell division was by

14   desmoschisis, i.e. the parent theca was shared by the daughter cells (Fig. 4 A-P). Thecal plates

15   were separated along a well defined oblique fission line separating an anterosinistral part from

16   a posterodextral part. The line of the border could already be defined by light microscopy of

17   calcofluor stained thecae (Fig. 4 C-L) and could additionally be depicted by SEM (Fig. 4 M-

18   P). Along this fission line, as schematized in Fig. 4 Q-R, the anterosinistral daughter cell

19   received the apical plates (including the APC), the epithecal intercalary plates, the first two

20   precingular plates, the anterior sulcal plate (Sa), and the first three postcingular plates. The

21   posterodextral daughter cell obtained both antapical plates together with the remaining

22   postcingular plates, the large posterior sulcal plate (Sp), and, from the epithecal plates, the

23   third and all following precingular plates. The distribution, if any, of other small sulcal plates

24   could not be resolved. For the cingular plates, the fission line separated plate C3 and C4. Even

25   in advanced stages of cell division, there were no indications of newly formed plate material

26   (Fig. 4 G, H) and freshly divided cells still seemed to lack the missing thecal parts (Fig. 4 I-L).

1 The course of the fission line is in agreement with the described plate overlap pattern of *A.*

2 *caudatum* in that there is a consistent overlap along the fission line of the epitheca. For the

3 hypotheca, however, there was one exception as the first antapical plate of *A. caudatum*

4 overlapped the first postcingular plate (see, Fig. 3 B), opposite to the notion that hypothecal

5 plates of the left daughter cell overlap plates of the right daughter cell.

6

7 **Growth rate**

8 Growth curves of *Azadinium caudatum* var *margalefii* strain AC1 are plotted in Fig. 5. For all

9 experimental conditions exponential growth started immediately without any obvious lag-

10 phase. Exponential growth persisted for 7 (100 μE m^{-2} s^{-1}, 20° C, Fig. 5 B)) to 27 days (100

11 μE m^{-2} s^{-1}, 10° C, Fig. 5 F), before a period of continuously decreasing growth rate preceded

12 the stationary phase. Maximum cell densities varied between 2400 (15° C, 40 μE m^{-2} s^{-1}, Fig.

13 5 C) and 600 (10° C, 100 μE m^{-2} s^{-1}, Fig. 5 F), but growth at the latter condition probably was

14 not at its end when the experiment was terminated after 40 days. At a fixed light intensity of

15 100 μE m^{-2} s^{-1} exponential growth rate increased with temperature (Fig. 5, right panel), being

16 highest at 20° C (μ = 0.32 ± 0.02 d^{-1}). At 15° C growth was slightly but significantly (t- test, p

17 < 0.05) lower and drastically dropped down to 0.07 ± 0.005 d^{-1} at 10° C (Fig. 5 H). At a fixed

18 temperature of 15° C growth increased with increasing irradiance from 0.15 ± 0.01 d^{-1} at 15

19 μE m^{-2} s^{-1} to 0.27 ± 0.01 d^{-1} at 250 μE m^{-2} s^{-1} and was almost saturated at 40 μE m^{-2} s^{-1} (Fig. 5

20 G). Fitting the data to a Michaelis Menten formula (Fig. 5 G) revealed a maximum growth of

21 0.295 d^{-1} and a half saturation light intensity for growth of 13 μE m^{-2} s^{-1}. The pH measured at

22 day 21 was low ranging from 8.18 to 8.30 (Fig. 5 A-F)). Growth rate and final cell yield for

23 cultures enriched with soil extract (data not shown) were not significantly different compared

24 to cultures grown with regular K-medium (t-test, p > 0.05).

25

26 *Pigment profile*

1   Figure 6 shows the typical chromatogram of the *Azadinium caudatum* var *margalefii* AC1

2   pigment analysis. Pigments were identified by co-chromatography of known pigment

3   standards, but some peaks could not be assigned to any standard available in our library. In

4   the *A. caudatum* var. *margalefii* AC1 strain, three unknown pigments were detected (x1, x2,

5   and x3). The chromatogram of the AC2 strain only differs from the AC1 strain by having one

6   additional unknown pigment at a retention time of 12.08 minutes, which was present in trace

7   amounts (unknown pigment x4, chromatogram not shown). Figure 7 show the absorption

8   spectra of all 4 unknown pigments. The pigment composition of the quantifiable pigments of

9   *A. caudatum* var. *margalefii* AC1 (not different for AC2 and thus not shown here) is listed in

10 Tab. 2. Besides the main photosynthetic pigment chlorophyll *a* which accounted for 42 % of

11 total pigment, peridinin with 38 % of total pigment was the most conspicuous pigment,

12 followed by chlorophyll $c_1 + c_2$ which accounted for 10 % of total pigment. Chlorophyll $c_1 +$

13 c_2 could not be separated with our method.

14

15 *Azaspiracids*

16 Using the selected reaction monitoring mode (SRM), no known AZAs could be detected in

17 either *Azadinium caudatum* var. *margalefii* strain. The detection limit on a cellular basis was

18 estimated as 0.06 fg cell^{-1} for AC1 and 0.2 fg cell^{-1} for AC2 (higher due to the lower biomass

19 of the sample). In addition, precursor ion experiments for detecting putative precursor masses

20 of the characteristic collision induced dissociation (CID)-fragments *m/z* 348 and *m/z* 362 of

21 AZAs did not give any further signals for neither AC1 nor AC2, indicating that neither strain

22 produced other unknown AZA variants in larger amounts. However, the precursor ion mode is

23 approximately a hundred times less sensitive than the SRM mode and strictly speaking does

24 not allow for exact quantitative measurement. Considering a conservatively determined

25 "detection limit" of 81 pg on the column, this represents a cellular detection limit of unknown

26 AZA variants of 3 fg (AC1) or 10 fg (AC2).

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Discussion

For most of the approximately 2500 described species of dinoflagellates (Elbrächter 2003),

only some taxonomically relevant morphological features are known, but additional

information about morphology, physiology, behaviour, life cycle, or cellular compounds is

still scarce, mainly due to both the lack of live cell observations and/or availability of cultures.

This is especially true for the vast majority of "rare" species that do not attract attention as

bloom forming species do. *Azadinium caudatum* is a very good example of such a "rare"

species, which has been described in terms of morphology (Halldal 1953; Dodge & Saunders

1985), even in great detail (Nézan et al. 2012), but which has never been reported to occur in

higher densities and for which almost no biological and/or physiological details are known.

Azadinium caudatum, and this refers to both varieties var. *margalefii* and var. *caudatum*, is a

case where the characteristic shape in combination with size and the presence of a single and

prominent antapical spine unambiguously allows for a species designation based on just light

microscopy even of fixed samples. Nevertheless, knowledge on other cellular features like

chloroplast arrangement, shape and location of the nucleus and the potential presence of

pyrenoid(s) could further assist in species designation and allows comparing these features

with other members of the Amphidomataceae.

Swimming pattern:

With a relatively slow swimming speed interrupted by short, high speed "jumps" in various

directions, *Azadinium caudatum* var. *margalefii* exhibits the same conspicuous swimming

behaviour described for other species of the Amphidomataceae (e.g. Tillmann et al. 2009;

Tillmann et al. 2012a). With an overall mean speed of 85 µm s^{-1}, *A. caudatum* var. *margalefii*

1   is considerably slower than *A. poporum* (400 µm s^{-1}), the only species for which quantitative

2   data are available (Potvin et al. 2013). Generally, the swimming speed of *A. caudatum* var.

3   *margalefii* is at the low end of the range of swimming speeds reported for other

4   dinoflagellates (ca. 20 - 500 µm sec-1, Smayda 2002a); for chain forming species even higher

5   swimming velocities of 856 µm s^{-1} have been reported (Sohn et al. 2011). Species of the

6   Amphidomataceae generally seem to swim at a relatively slow speed. *Amphidoma languida* ,

7   although not explicitly quantified, seem to be particularly slow in its general movement

8   (Tillmann et al. 2012a) and might be even slower than *A. caudatum* var. *margalefii*, which is

9   reflected in its name (languida (lat.) = lazy, slow). Although all species of Amphidomataceae

10 may rarely travel larger distances at a higher speed, they usually exhibit interspersed jumps,

11 quite regularly when approaching other objects, e.g. when reaching the glass bottom of the

12 observation chamber. As quantified here for *A. caudatum* var. *margalefii*, the maximum speed

13 of these jumps can be about seven times higher than regular speed, but this maximum speed

14 of approx. 350 µm s^{-1} is still slow compared to jump velocities of ciliated protists which are in

15 the range of 2000 to 5000 µm s^{-1} (Jakobsen 2001). The change of direction related to these

16 jumps, quantified here as mostly close to 90°, is consistent with the idea that these jumps act

17 as an avoidance behaviour and probably represent a direct escape mechanism involved in

18 predator/prey interactions (Jakobsen 2001; Jakobsen 2002; Tillmann 2004).

19

20 *Chloroplast, pyrenoid, nucleus*:

21 A general survey of chloroplast morphology of dinoflagellates indicated that larger species

22 often possess numerous small and more globular plastids, whereas small species generally are

23 characterised by one or very few but large reticulate and parietally arranged chloroplasts

24 (Schnepf & Elbrächter 1999). *Azadinium caudatum* var. *margalefii*, like all other species of

25 Amphidomataceae, clearly is photosynthetic with a chloroplast of the latter type, i.e. they

26 possess a presumably single chloroplast which is parietally arranged and normally extends

into both the epi- and hyposome. The degree of reticulation of the chloroplast of *A. caudatum* var. *margalefii* forming a filamentous network, however, seems to be considerably higher compared to other species of *Azadinium*.

The presence/absence, location, number and types of pyrenoids have been regarded as useful taxonomic characters at the genus level (Schnepf & Elbrächter 1999) and have in particular been discussed as potential features visible in light microscopy to differentiate between species of *Azadinium* (Tillmann et al. 2011). In particular, stalked pyrenoid(s) are visible under the light microscope because of a distinct starch cup. *Azadinium caudatum* var. *margalefii* clearly lacks this type of pyrenoid(s) visible under the light microscope and is thus similar to *A. obesum* and *A. polongum* (no visible pyrenoids) (Tillmann et al. 2010; Tillmann et al. 2012b), but different from *A. spinosum*, *A. dexteroporum* (one pyrenoid) (Tillmann et al. 2009; Percopo et al. 2013), and *A. poporum* and *A. dalianense* (several pyrenoids) (Tillmann et al. 2011; Luo et al. 2013).

In many species of dinoflagellates the nucleus is large and conspicuous (Dodge 1963) and thus nuclear shape and location is often included in species descriptions. As for all other species of Amphidomatacceae the nucleus of *A. caudatum* var. *margalefii* was generally round to slightly ovoid and was positioned in a slightly posterior position in the cell centre. Nevertheless, cells with an elongated nucleus have been observed, which most probably reflects an advanced state in nuclear division. Such a distinct change in the shape of the nucleus has also been noted as the first sign of nuclear division for many dinoflagellates (Dodge 1963). For both *A. languida* and in more detail, for *A. spinosum*, a distinct elongation of the round to elliptical nucleus in the course of nuclear division has been described (Tillmann et al. 2012a; Tillmann & Elbrächter 2013).

Imbrication:

Ultrastructural details (plate pattern) have thoroughly been analysed previously (Nézan et al.

1 2012), showing that both varieties of *A. caudatum* have the typical Kofoidean plate pattern of

2 the genus *Azadinium*, but differ by the antapical spine and the position of the ventral pore.

3 Plate pattern analysis by Nézan et al. (2012) included an analysis of the plate overlap (or

4 imbrication pattern), which may give insights on functional aspects of ecdysis and/or on the

5 archeopyle type of cysts, and which may be a useful aid in determining plate homologies

6 (Netzel & Dürr 1984). It has been elucidated in details for *A. spinosum* (Tillmann &

7 Elbrächter 2010), *A. languida* (Tillmann et al. 2012a), *A. caudatum* (Nézan et al. 2012) and *A.*

8 *polongum* (Tillmann et al. 2012b). The pattern was consistent among the species, with an

9 uncommon but stable imbrication pattern of the most dorsal apical plate (3′ in *Azadinium* or 4′

10 in *Amphidoma* is overlapped by all adjacent plates), which characterized these two genera and

11 might be helpful for a revision of the description of the family Amphidomataceae. Another

12 peculiarity of imbrication of *Azadinium* species was identified for the overlap of the central

13 ventral plate: in contrast to other dinophycean species (Netzel & Dürr 1984), the last cingular

14 plate C6 of *Azadinium* species and *A. languida* have been shown to be overlapped by all

15 adjacent plates, instead of the central sulcal plate Sa. This important feature had not been

16 resolved for *A. caudatum* in the imbrication analysis by Nézan et al. (2012) and is reported

17 here to be exactly the same for *A. caudatum* var. *margalefii*, indicating that this feature might

18 also be characteristic for these two genera and might be helpful for a revision of the

19 description of the family Amphidomataceae.

20

21 *Cell division*:

22 With few exceptions, the vegetative reproduction of dinoflagellates is by binary fission

23 (Elbrächter 2003). Thecate dinoflagellates can divide either by entirely shedding the parent

24 theca so that both sister cells have to rebuild a whole new theca (eleuteroschisis), or by

25 sharing the parent theca between the two daughter cells (desmoschisis: Pfiester & Anderson

26 1987; Elbrächter 2003). The desmoschisis type of cell division of *A. caudatum* var. *margalefii*

1   was similar to the cell division described for *A. spinosum* (Tillmann & Elbrächter 2013) and

2   *A. languida* (Tillmann et al. 2012a) and exhibited exactly the same position of the fission line

3   as described for *A. spinosum*. Moreover, some features of desmoschisis, which have been

4   described for *A. spinosum* as peculiar and different from desmoschisis of Gonyaulacean

5   species (Tillmann & Elbrächter 2013), are also found for *A. caudatum* var. *margalefii*. This

6   refers particularly to the relation of fission line and plate overlap, which for

7   Amphidomataceae seem to be characterised by an analogy in the epitheca (all plates of the

8   anterosinistral part are overlapped by plates of the posterodextral part, see Fig. 4 Q), but

9   which show one deviation of that pattern for the hypotheca (Tillmann & Elbrächter 2013)

10 (Fig. 4 R). As in *A. languida* and *A. spinosum*, freshly divided cells of *A. caudatum var.*

11 *margalefii* seem not to have completed the formation of new rigid thecal plates. Such a

12 delayed thecal plate formation stands in contrast to catenate species of the genus

13 *Alexandrium*, where it appears that the new thecae are being formed as cytokinesis progresses

14 (Tomas 1974).

15

16 *Growth experiments*:

17 With a maximum growth rate of strains AC1 of 0.32 d^{-1} (corresponding to about 0.5 divisions

18 per day) at 20° C and 100 µE m^{-2} s^{-1}, *Azadinium caudatum* var. *margalefii* seems to be a

19 generally slow growing species compared to many other dinoflagellates which normally are

20 able to divide approximately once per day at optimal conditions (Banse 1982; Smayda 1997;

21 Smayda 2002b). The maximum growth is also slower compared to *A. spinosum*, the only

22 species of Amphidomataceae for which quantitative growth data are available (Jauffrais et al.

23 2013). Growth of *A. caudatum var. margalefii* was gradually affected by both temperature and

24 light in very much the same way as *A. spinosum* (Jauffrais et al. 2013). By testing just three

25 temperatures covering a relatively narrow range from 10° to 20° C, upper and lower

26 temperature limits for positive growth of *A. caudatum* var. *margalefii* AC1 are not yet

precisely defined. *A. caudatum* var. *margalefii* has been described from both the distinctly

warmer Mediterranean area (Rampi 1969) and the temperate French and Irish Atlantic coast

and around the British Isles (Dodge & Saunders 1985; Nézan et al. 2012). Occurrence of *A.*

caudatum var. *margalefii* in more northern areas is unknown; records of *A. caudatum* in

Northern Norwegian waters during winter (Halldal 1953) referred to the other variety var.

caudatum and thus might reflect different temperature requirements of var. *caudatum* and var.

margalefii.

Azadinium caudatum var. *margalefii* appears to be low light adapted, indicated by growth

rates of strain AC1 becoming light saturated at intensities of about 40 μE m^{-2} s^{-1} and a half-

saturation light intensity of about 13 μE m^{-2} s^{-1}. For bloom forming dinoflagellates like

Ceratium furca (Ehrenberg) Claparède & Lachmann and *C. fusus* (Ehrenberg) Dujardin,

optimal growth was recorded at light intensities > 200 μE m^{-2} s^{-1} characterizing those species

as well-adapted to high intensity light levels (Baek et al. 2008), as are other bloom forming

species of the genus *Alexandrium* (Fu et al. 2012) or *Cochlodinium* (Kim et al. 2004).

However, low light adaptation is not an exclusion criterion for bloom formation as other

bloom forming species like *Karenia brevis* (C.C. Davis) G. Hansen & Moestrup (Magana &

Villareal 2006) are known to be low light adapted as well. For *A. caudatum* var. *margalefii*

AC1, there was no photoinhibition of growth at the highest light intensity tested (250 μE m^{-2}

s^{-1}) indicating that this species, although adapted to low light, can cope with higher light in

subsurface water. However, growth response at high light levels, typical for peak subsurface

summer values of up to 2000 μE m^{-2} s^{-2} (Kirk 1994) still needs to be tested. The light

response of *A. caudatum* var. *margalefii* AC1 was similar to that of *A. spinosum* giving no

signs of photoinhibition at higher light levels (tested up to 400 μmol m^{-2} s^{-1}) and growth was

almost saturated down to the lowest light level tested (50 μmol m^{-2} s^{-1}) (Jauffrais et al. 2013).

In our culture experiments, the final cell yield of strain AC1 at the stationary phase was

conspicuously low (< 2400 cells ml^{-1}). It is not clear which factor was crucial for growth

1   cessation in these batch cultures: concentration of macronutrients (nitrate and phosphate) in

2   the medium applied here are excessive and should allow for a much higher biomass, as they

3   normally do for other species like *Alexandrium tamarense* (Lebour) Balech (e.g. Zhu &

4   Tillmann 2012). Adding soil extract did not increase growth rate and yield, making a lack of

5   trace elements unlikely. Prolonged growth in un-bubbled cultures might lead to carbon

6   limitation. In addition, high pH has been shown to strongly affect dinoflagellate growth of

7   both photosynthetic and heterotrophic species (Hansen 2002; Pedersen & Hansen 2003).

8   Culture growth experiments (Hansen et al. 2007) and physiological studies of inorganic

9   carbon acquisition in red tide dinoflagellates (Rost et al. 2006) both suggest that marine

10   dinoflagellates may not be carbon-limited even at high pH, thus direct effects of high pH

11   might be responsible for growth cessation (Hansen et al. 2007). The pH measured during the

12   stationary phase of *A. caudatum* var. *margalefii* was quite low (around 8.3), but still in the

13   range of the lowest pH tolerance limits reported in the literature; for species of *Ceratium* or

14   the dictyochophyte *Dictyocha speculum* Ehrenberg, pH values affecting exponential growth

15   of 8.24-8.30 have been reported (Schmidt & Hansen 2001), although upper pH limit for

16   growth of *Ceratium* spp. later was reported to be in the range of 8.7 – 9.1 (Soederberg &

17   Hansen 2007). Further detailed experiments are needed to clarify whether *A. caudatum* has an

18   extraordinary low pH tolerance and/or is an extraordinarily poor competitor for carbon, or if

19   other exceptional trace compounds not included in the soil extract might have been yield-

20   limiting. In any case, low final cell yield in our batch culture experiment would support the

21   notion that *A. caudatum* is generally found in low abundances in field samples (Nézan et al.

22   2012).

23

24   *Pigments*:

25   Both available strains of *Azadinium caudatum* var. *margalefii* showed a typical pigment

26   composition for peridinin-containing dinoflagellates with chlorophyll $c_{1/2}$ as the main

accessory chlorophyll and peridinin as the main carotenoid. Diadinoxanthin, dinoxanthin, diatoxanthin and β-carotene are also typical for peridinin-containing dinoflagellates and have been reported for other closely related species (Tillmann et al. 2009; Tillmann et al. 2010; Tillmann et al. 2012a; Zapata et al. 2012). Violaxanthin has not been reported for *Azadinium*-species before and was only detected in trace amounts here. Prasinoxanthin, which was reported in low amounts for other *Azadinium* species (Tillmann et al. 2009; Tillmann et al. 2010), could not be detected in the present analysis.

The unknown pigments are probably all carotenoids. Unknown pigment x1 shows an absorption spectrum similar to peridinin with a single maximum at 465 nm and has previously been reported from peridinin-containing dinoflagellates by Zapata et al. (2012). It elutes approx. 1.5 minutes later than peridinin. Unknown pigments x2 and x3 have absorption spectra similar to gyroxanthin-diester, what has previously been reported from dinoflagellates such as *K. brevis* (Bjørnland et al. 2003). Unknown pigment x2 elutes approximately 5 minutes earlier and is thus more polar than unknown pigment x3. A gyroxanthin diester-like pigment, probably similar to our unknown pigment x3, has been reported by Zapata et al. (2012) in dinoflagellate species containing fucoxanthin as their major carotenoid. The absorption spectrum of unknown pigment x4, which was only detected in traces in the AC2 strain, resembles the carotenoid lycopene.

Toxins:

The presence of azaspiracids in shellfish has been a recurring problem in Ireland since 2005, with levels above the regulatory limit of 0.16 mg AZAs per kg mussel observed mainly in blue mussels. First feeding studies clearly showed a direct link between *Azadinium spinosum* and AZA contamination of mussels. However, concentrations of AZAs found in mussels during laboratory exposures (Salas et al. 2011; Jauffrais et al. 2012) were still ca. 10-fold lower than the maximum concentrations encountered in the field, so we still have to consider

1   alternative AZA sources, like the presence of other yet unidentified cryptic AZA-producing

2   species. In this respect, *A. caudatum* has been an interesting candidate, as it is a close relative

3   of *A. spinosum*, is known to occur in Irish waters (Dodge & Saunders 1985), and is relatively

4   large compared to *A. spinosum* (which implies a potentially larger cell toxin quota). However,

5   we failed to detect known AZAs and other compounds producing AZA-characteristic MS-

6   fragments in both strains of *A. caudatum* var. *margalefii*, but of course we cannot exclude the

7   presence of other related molecules, specifically as a high variability in AZA toxin profile has

8   been described for at least one species of *Azadinium*, *A. poporum* (Krock et al. 2012; Gu et al.

9   2013). Most notably, among a total of ten strains of *A. poporum* analyzed so far, one strain

10   was found without any detectable AZAs (Gu et al. 2013). With just two strains of *A. caudatum*

11   var. *margalefii* available and examined so far, clearly more strains, including strains of the

12   variety *A. caudatum* var. *caudatum*, need to be established and analyzed in the future to

13   evaluate if the absence of AZAs is a constant trait of *A. caudatum*.

14

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Tab. 1: *Azadinium caudatum* var. *margalefii*, swimming speed, summary statistics of swimming paths depicted in Fig. 2.

Track no	Distance – duration	Speed ($\mu m\ s^{-1}$) (mean ± Std)	Speed ($\mu m\ s^{-1}$) (min – max)
1	121 µm – 2.80 s	**43** ± 6	31 – 52
2	297 µm – 4.24 s	**81** ± 67	36 – 363
3	200 µm – 1.60 s	**138** ± 6	130 –145
4	162 µm – 2.68 s	**116** ± 122	21 – 389
5	118 µm – 1.23 s	**88** ± 12	69 – 118
6	412 µm – 6.00 s	**69** ± 38	31 – 223
7	135 µm – 1.60 s	**84** ± 11	67 – 99
8	120 µm – 2.40 s	**50** ± 7	36 – 57
9	145 µm – 2.36 s	**61** ± 6	52 – 75
10	47 µm – 1.00 s	**47** ± 5	31 – 52
11	87 µm – 1.12 s	**135** ± 104	31 – 363
12	96 µm – 1.24 s	**114** ± 81	39 – 234
13	287 µm – 5.00 s	**57** ± 5	52 – 70
14	314 µm – 5.60 s	**56** ± 5	47 – 67
15	370 µm – 5.00 s	**74** ± 37	47 – 197
16	69 µm – 1.20 s	**57** ± 16	44 – 78
17	212 µm – 1.60 s	**184** ± 67	47 – 260
18	112 µm – 1.16 s	**135** ± 72	65 – 234
19	148 µm – 2.20 s	**67** ± 11	52 – 88
20	64 µm – 1.40 s	**46** ± 6	36 – 52
21	101 µm – 1.20 s	**84** ± 7	75 – 93
22	51 µm – 0.80 s	**64** ± 20	47 – 83
23	145 µm – 2.72 s	**65** ± 39	42 – 183
24	143 µm – 1.72 s	**83** ± 67	26 – 260
25	73 µm – 0.56 s	**131** ± 72	65 – 337
26	153 µm – 2.60 s	**59** ± 7	42 – 67
27	246 µm – 5.60 s	**44** ± 5	34 – 60
28	170 µm – 1.40 s	**121** ± 60	65 – 285
29	109 µm – 1.16 s	**94** ± 58	39 – 247
30	149 µm – 1.36 s	**110** ± 88	26 – 234

1 Tab. 2: Pigment composition of *Azadinium caudatum* var. *margalefii* AC1 in percent of total

2 pigment, mean ± standard deviation (SD)

Pigment	[%] of total pigment
Chlorophyll a	42.3 ± 0.8
Perididin	38.3 ± 0.9
Chlorophyll $c_1 + c_2$	10.0 ± 2.2
Diadinoxanthin	6.0 ± 0.6
Dinoxanthin	2.0 ± 0.2
β, β -carotene	1.1 ± 0.04
Diatoxanthin	0.2 ± 0.01
Violaxanthin	0.1 ± 0.01

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Figure legends

Fig. 1: *Azadinium caudatum* var. *margalefii*, LM of live (B, C) and fixed cells (all other). A-E: General size and shape. F: Cells stained with DAPI and viewed with UV excitation in normal light to show the shape and location of the nucleus. G-H; epifluorescence view (UV excitation) of two different DAPI stained cells, note the enlarged and elongated nucleus in H. I-J: Two different cells fixed with a mixture of formalin and Lugol, note the presence of large and brownish stained vesicles of probable reserve material. K-O: Epifluorescence view of formalin fixed cells; chlorophyll autofluorescence to show chloroplast structure. N and O represent two different focal planes of the same cell in posterior view. Note the sulcal area void of chloroplast extensions (white arrow). Scale bars = 10 µm.

Fig. 2: Swimming paths of *Azadinium caudatum* var. *margalefii*. Time interval between each small white dot is 0.04 s, between each large dot is 0.2 s, and between each black dot 1 s. Swimming direction indicated by the orientation of the cells (not drawn to scale). Numbers in circles refer to Tab. 1, where summary statistics for each path are listed.

Fig. 3: SEM micrographs of thecae of different cells. A: Whole cell in lateral view. B: Internal view of the hypotheca and the sulcal area. C-D: Detailed internal (C) and external (D) view of the anterior sulcal plate showing that plate Sa is overlapped by plate C1 (white arrows) and is overlapping plate C6 (black arrows). Scale bars = 5 µm (A, B) or 2 µm (C, D).

Fig. 4: Cell division of *Azadinium caudatum* var. *margalefii*. LM (A-L), SEM (M-P), or schematic drawing (Q-R). A-B: Cells fixed with formalin/lugol, different stages of cell divison. C-D, E-F, G-H, I-J, and K-L: Pairs of the same cell stained with calcofluor white in bright field (left) or with UV excitation (right). C-H: different stages of cell division. I-L: freshly divided cells showing that half of the plates are still missing. M-P:

SEM of different cells. M: Ventral/lateral view of a cell presumably close to cell division. N-P: Cells in cell division, in ventral (N), dorsal (O), or antapical (P) view. Q-R: Schematic view of fission line of epitheca (Q) and hypotheca (R) separating the anteriosinistral daughter cell (light shaded) and the posteriodextral daughter cell (darker shaded). Arrowheads indicate direction of plate overlap at the fission line with black symbols indicating plate margins overlapping the plate with the corresponding white symbols. Plate overlap adapted from Nézan et al. (2012). Plate labels according to the Kofoidean system: apical plates: 1´ - 4´ (surrounding the apical pore complex); intercalary plates: 1 a, 2 a , 3a; precingular plates: 1´´ - 6´´; postcinguar plates: 1´´´ - 6´´´; antapical plates: 1´´´´, 2´´´´; Sa = anterior sulcal plate; Sp = posterior sulcal plate. Scale bars = 10 µm

Fig. 5: Growth performance of *Azadinium caudatum* var. *margalefii* in response to temperature and irradiance. A-F: Cell concentration (log scale) versus day after inoculation at different temperatures (right) or different irradiances (left). Black dots: data used to calculate growth rate. Arrows: results of pH measurements on day 21. G: growth rate µ (d^{-1}) (black dots) as a function of irradiance. The dotted line represents a Michaelis-Menten curve fit. H: Growth rate µ (d^{-1}) for three different temperatures. Note that growth at 15° C and 100 µE m^{-2} s^{-1} (D) was used for both analyses. Data points or bars represent treatment mean ± 1SD (n = 3).

Fig. 6: High-performance liquid chromatography chromatogram of photosynthetic pigments (detection at 434 nm) of *Azadinium caudatum* var. *margalefii* AC1. Retention time on the x-axis; absorbance (AU: arbitrary units) on the y-axis.

Fig. 7: Absorption spectra of four unknown pigments.

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Fig. 2

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Fig. 4

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1 – Chlorophyll $c_{1/2}$
2 – Peridinin
3 – Peridinin-isomer
4 – Unknown pigment x1
5 – Unknown pigment x2
6 – Violaxanthin
7 – Diadinoxanthin
8 – Dinoxanthin
9 – Diatoxanthin
10 – Unknown pigment x3
11 – Canthaxanthin (internal standard)
12 – Chlorophyll a
13 – β,β-carotene

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