THE TOXICITY AND INTRASPECIFIC VARIABILITY OF *ALEXANDRIUM ANDERSONII* Balech

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27

28 **Abbreviations**

29 **Abstract**

30 The toxicity of *Alexandrium andersonii* Balech is unclear and its intraspecific
31 variability has yet to be studied. To address these gaps in our knowledge, in the present
32 work five strains of *A. andersonii* from four different localities were characterized. The
33 results showed that despite genetic homogeneity in the 5.8-ITS (internal transcribed
34 spacer) and large subunit (LSU) regions and similar growth rates, strains originating
35 from different locations varied with respect to cell size, the ratios of certain pigments,
36 and their growth patterns. Cultures of the strains grown at 20 °C were analyzed for
37 toxicity using four different methodologies. The two officially established methods,
38 mouse bioassay and high-performance liquid chromatography with fluorescence
39 detection (HPLC-FLD) and post-column reaction analysis of PSP toxins, failed to show
40 the toxicity of any strain. Strains grown at 14 ºC were also negative for PSP toxins by
41 HPLC-FLD. However, all of the strains at 20 ºC revealed a response characteristic of
42 the presence of toxin-inhibiting voltage-gated sodium channels, as demonstrated in a
43 neuroblastoma neuro-2a cell-based assay, and showed hemolytic activity.

44

45 **Key words**: Intraspecific variability, *Alexandrium andersonii*, PSP, toxicity,
46 Mediterranean Sea, Cytotoxicity assay

47

48 **1. Introduction**

49 In a recent issue of the journal *Harmful Algae* (Pitcher, 2012), the need for species-
50 specific information to predict the occurrence of harmful algal blooms (HABs) was
51 emphasized, especially given the wide-ranging differences in the morphology,
52 phylogeny, life-cycles, growth requirements, etc., of HAB species. In fact, even within

the same species there is an extensive genotypic and phenotypic variability, thereby challenging our traditional notion of morphospecies (e.g. Alpermann et al., 2010). It is also clear that to appreciate the plasticity of a species and thus its adaptive potential, more than one strain must be studied.

Phylogenetic studies on diverse phytoplankton species have revealed the geographic differentiation of several traditional morphospecies at global (McCauley et al., 2009; Penna et al., 2010), regional (Casabianca et al., 2012), and local (Godhe and Härnström, 2010) scales. Other studies have found phenotypic differences among strains from different localities or even within the same bloom population (e.g., Alpermann et al., 2010; Calbet et al., 2011; Tillmann et al., 2009). Within a given species there is a high degree of plasticity with respect to toxin content (Alpermann et al., 2010; Thessen et al., 2009; Tillmann et al., 2009), growth rates (Calbet et al., 2011; Hadjadji et al., 2012; Thessen et al., 2009; Tillmann et al., 2009), size (Calbet et al., 2011), lipid composition (Calbet et al., 2011), feeding behavior (Calbet et al., 2011), and allelochemical activity (Alpermann., et al 2010; Tilmann et al., 2009). These and other variations could reflect geographic adaptation of the population but they may also be due to environmental factors. In some cases populations within the same geographic area have been shown to differ in their toxin profiles (i.e., Alpermann et al., 2010; Oh et al., 2010) whereas in others these differences were used to distinguish distant populations (i.e., Kim et al., 1993; Mackenzie et al., 1996; Oshima et al., 1993). Similarly, the association of toxic and non-toxic populations of *Alexandrium minutum* in different areas of the coastal waters of Ireland has been reported (Touzet et al., 2007). Moreover, in *Alexandrium* species temperature-related differences in toxin content have been described by several authors (Etheridge and Roesler, 2005; Lim et al., 2006; Navarro et al., 2006; Ogata et al., 1987; Wang et al., 2006).

Among the 30 species that comprise *Alexandrium* Halim (Balech, 1995; Mackenzie and Todd, 2002; Montresor et al., 2004), at least half are considered potentially harmful. Of these, 12 are known to be PSP producers, while others can produce other toxins (spirolides, goniodomins), mass mortality of fish, antifungal substances or hemolytic activity (IOC Taxonomic Reference List of Harmful Micro Algae; Moestrup et al., 2009 onwards and Anderson et al., 2012, see the last for a revision of the genus).

Alexandrium andersonii was described by Balech in 1990 based on a clonal culture derived from a cell collected on Cape Cod (NW Atlantic). The same year, Anderson et al. (1990) reported the strain as non-toxic. In 1998, the first reports documenting the detection of this species in the Mediterranean Sea (Gulf of Naples) were published (Montresor et al., 1998). Ciminiello et al. (2000) determined the PSP toxicity of *A. andersonii* by means of a mouse bioassay, and by nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) preceded by the isolation and purification of a high-biomass culture. The toxins obtained in the greatest abundances were saxitoxin (STX) and neosaxitoxin (neoSTX), purified from strain SZN12, originally obtained from a cyst found in the sediment of the Gulf of Naples. The cellular toxin content of this strain when grown in the presence of different nitrogen and phosphorus supplies was analyzed by Frangopulos et al. (2004), who used high-performance liquid chromatography (HPLC). These authors found a very low amount of toxin per cell, mostly gonyautoxin-2 (GTX2), whereas neither STX nor neoSTX was detected. In strains from the Irish coast, Touzet et al. (2008) were unable to detect PSP toxins. Therefore, the nature of the toxicity of strain SZN12 and whether it is indeed toxic at all remain unclear.

In the winter of 2007 year, *A. andersonii* was detected for the first time along the Catalan coast (NW Mediterranean Sea), in Alfacs Bay (Ebre Delta, Catalonia), the major shellfish harvesting area in this region (Sampedro et al., 2007). A mouse bioassay of mussel samples collected from this area identified the low-level presence of PSP toxins (44 µg STX eq/100gr) (Fernández-Tejedor et al., 2007). However, a direct relation to *A. andersonii* population could not be demonstrated, since other *Alexandrium* PSP producer species were present at same time in the water column.

The latest detections of *A. andersonii* in different areas of the Mediterranean have brought to light the deficits in our knowledge of this species. This applies not only to toxin production but also to its physiology, ecology, and distribution as well as its intraspecific variability, none of which has been studied.

Based on the assumption that *A. andersonii* may comprise toxic and non-toxic strains, depending on its location, the main objective of this study was to describe the potential for intraspecific variability of this species, with particular focus on its toxicology and toxinology. To provide a larger framework for the data, the examined strains were also characterized with respect to their phylogeny, morphology, and physiology, including pigment composition and growth rates.

2. Material and methods

2.1 Alexandrium andersonii isolation and culture

A. andersonii culture ICM222 was isolated in March 2007 from Alfacs Bay (Fig.1), the southern bay of the Ebre River Delta (NW Mediterranean Sea) and the major shellfish harvesting area along the Catalan coast. The other cultures used in this study were

obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP) of Bigelow Laboratory (USA) and from the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo (Spain). All cultures were adapted for a year to a salinity of 36 and to growth in L1 medium.

2.2 Phenological characterization of A. andersonii strains

The five strains examined in this study are described in Table 1. Net growth rates, cell size, and pigment composition were studied as follows: Duplicate cultures of all strains except strain CCMP1597 were grown in 1-L polystyrene flasks (vented-cap) for 40 days in L1 medium prepared in coastal seawater without silicates (Guillard and Hargraves, 1993) or aeration ; the salinity was adjusted to 36. The cultures were incubated at 20 ºC with an irradiance of 80–100 μmol photons m^{-2} s^{-1} in a 12:12h light/dark cycle. Every 2 days, 2-ml subsamples were removed and then fixed with Lugol (1% final concentration) for cell quantification. On day 15 (corresponding to the exponential phase of growth), 10- and 40-ml aliquots were removed for growth measurements and pigment analyses, respectively. The methods used to determine toxin production at a culture incubation temperature of 20°C are described in Sect. 2.8.

Speculating that variations in temperature influenced the toxin content of *Alexandrium* species, we carried out an additional growth experiment at 14 ºC (the water temperature during the bloom of *A. andersonii* in Alfacs Bay). Duplicate cultures of strains VGO 664, SZN 012, ICM222, and CCMP1718 were grown at 14 ºC under the same conditions used in the first growth experiment, with 2-ml subsamples taken every 2 or 3 days for cell quantification. For HPLC analyses of toxin profiles, 20–40 ml of each culture were removed (depending on the concentration) during the exponential and stationary phases of growth and filtered on Whatman GF/C glass-fiber filters.

153

154 *2.3 Growth rate calculation and statistical analyses*

155 The cell abundances obtained in the growth experiments were used to determine the
156 exponential growth rates according to Guillard (1973). The growth rate for each flask
157 culture was then estimated using data from different days depending on the start and
158 duration of the respective exponential phase. The growth rates and the maximum cell
159 abundances were compared with respect to the different temperatures (14 and 20 °C).
160 Since the normality of the data could not be assured, due to the small data sets, the
161 paired sign test was used in this analysis. Kruskal-Wallis tests were used to determine
162 significant differences in growth rates and in maximum cell abundances between clones
163 (data from different temperatures were treated separately). All statistical analyses were
164 conducted using Statistica'99 software (StatSoft inc., USA).

165

166 *2.4 Light microscopy*

167 Wild cells of *A. andersonii* were measured using field samples collected in the winter of
168 2007 from Alfacs Bay and preserved with formaldehyde. The samples were provided by
169 the *Institut de Recerca i Tecnologia Agroalimentaries* (IRTA) from its routine
170 monitoring of Delta Ebre bays.
171 Species identification was based on Balech's criteria (1995) together with more recent
172 descriptions (Mackenzie and Todd, 2002; Montresor et al., 2004; Nguyen-Ngoc and
173 Larsen, 2004). The plate tabulation of the cells was examined in detail, following the
174 calcofluor method of Fritz and Triemer (1985). The cells were stained with calcofluor
175 white M2R (Sigma-Aldrich Co., St. Louis, MO, USA) and examined under a Leica DM
176 IRB (Leica Microsystems GmbH, Wetzlar, Germany) inverted microscope with
177 epifluorescence (lamp 50W).

178 *A. andersonii* abundances in the growth experiments were estimated with Sedgewick-
179 Rafter chambers, counting either a volume of 20 µL or 100 cells.
180
181 *2.5 Measures and statistical analyses*
182 The length and width of 30 cells of every strain were measured using ProgRes
183 capturePro v 2.1software. The cells were removed during the exponential growth phase
184 and fixed with Lugol's (1% final concentration). Thirty cells from field samples were
185 likewise examined. Since the variables (length and width) were not distributed normally
186 (as determined by Kolmogorov–Smirnov and Shapiro-Wilk tests), only non-parametric
187 statistical analyses were applied, using PRIMER 6 (Windows XP) software. A one-way
188 analysis of similarities (ANOSIM) was performed; the corresponding pairwise tests
189 were based on 99,999 permutations.
190
191 *2.6 DNA extraction, PCR amplification, sequencing, and phylogenetic analyses*

192 Cultures of *A. andersonii* were collected during the exponential growth phase by
193 filtration on 3-µm pore size Isopore membrane filters (Millipore). DNA was extracted
194 and purified as described in Penna et al. (2005). Nuclear-encoded 5.8S rDNA and ITS
195 regions were PCR-amplified as described in Penna et al. (2008). Genomic DNA (1 ng)
196 was amplified in a 50-µL reaction mix containing 50 µM each of dATP, dTTP, dCTP,
197 and dGTP, 0.4 µM of each primer, 4 mM $MgCl_2$, 1× reaction buffer (Diatheva, Fano,
198 Italy), and 1.0 U Hot Rescue DNA polymerase (Diatheva, Fano, Italy). PCR of the LSU
199 rDNA regions (D1/D2) was carried out in a 50-µL reaction mix containing 1 µL of
200 genomic DNA, 0.25 µM of each primer (D1R and D2C by Scholin et al., 1994), 600
201 µM of each dNTP, 3µL $MgCl_2$ (25mM), 1× reaction buffer (Qiagen, USA, including 1.5
202 mM $MgCl_2$), and 2.5 U of Taq DNA polymerase (Qiagen, USA). Thermocycling

conditions for the 5.8S rDNA and ITS regions were as follows: 10 min initial denaturation at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 50 °C, and 2.5 min at 72 °C; a final elongation step consisted of 7 min at 72 °C. LSU rDNA thermocycling included an initial denaturation at 95 ºC for 5 min followed by 40 cycles of 20 sec at 95 ºC, 30sec at 55 ºC, and 1 min at 72 ºC; a final elongation step consisted of 10 min at 72 ºC. The three PCR-amplified products corresponding to the 5.8S rDNA and ITS regions were pooled, purified, and then directly sequenced using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA) and the dye terminator method described in the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction kit, Perkin Elmer Corp., Foster City, CA). LSU rDNA sequencing was carried out by an external service (Macrogen Inc., Korea). Sequences obtained from this study were aligned with those from GenBank using the CLUSTAL X2 program (Larkin et al., 2007) with default settings. Alignments were re-checked visually and edited manually; non-alignable regions were excluded prior to the phylogenetic analyses. The strains used in the molecular determinations are listed in Table 2 and in the supplementary material (Table 1) together with the GenBank accession numbers of their 5.8S ITS and LSU rDNA sequences. Phylogenetic relationships, based on the 5.8S ITS rDNA data, were inferred using the neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods. Sequences of *Gonyaulax spinifera* (Claparède et Lachmann) Diesing (EMBL-EBI ENA: AF051832) served as the outgroup in the ITS 5.8S rDNA phylogeny. NJ and MP analyses were performed using heuristic searches with tree-bisection-reconnection branch swapping. Branches were collapsed if their minimum length was 0; ambiguities and gaps were considered as missing data. The robustness of the NJ and MP trees was determined by bootstrapping with 1000 pseudoreplicates. Phylogenetic analyses were

228 carried out using the software packages PAUP* ver. 4.0b10 (Swofford, 2002). ML
229 analyses were run with RaxML (Randomized Axelerated Maximum Likelihood)
230 software ver. 7.0.4 (Stamatakis et al., 2005), which adopts a general time reversible
231 (GTR) substitution model and allows for the estimation of several parameters, including
232 the proportion of invariant sites and the alpha values of the gamma distribution for
233 among-site rate variation. Bootstrap values were calculated with 1000 pseudoreplicates.

234 Phylogenetic analyses based on LSU rDNA data were inferred by NJ, UPGMA, and
235 ML methods using MEGA ver. 5.02. NJ and UPGMA analyses were performed using
236 the Kimura 2-parameter model, with a gamma distribution of rates among sites and
237 setting the γ parameter to 0.5. ML analysis was run using a heuristic search method with
238 the Tamura and Nei model and the following likelihood settings: nst = 5, rate = gamma
239 distribution. Bootstrap values were calculated with 1000 pseudoreplicates. Sequences of
240 *Gonyaulax spinifera* served as the outgroup (EU805591).

241 *2.7 Pigment analyses*

242 Cultures were examined by light microscopy prior to HPLC pigment analysis to ensure
243 the health and intact morphology of the cells. Three hours into the 12-h light cycle, the
244 cells were harvested from exponentially growing cultures by filtering variable volumes
245 (10–36 ml) of the cultures onto Whatman GF/F filters (Whatman International Ltd. UK)
246 under reduced pressure. The filters were frozen immediately at -25 ºC and analyzed
247 within 12 h. Cells trapped on the frozen filters were extracted under low light in Teflon-
248 lined screw-capped tubes with 5 ml of 90% acetone, using a stainless steel spatula to
249 grind the filters. The contents of the tubes were chilled in a beaker of ice, sonicated for
250 5 min in an ultrasonic bath, and then filtered through syringe filters with a 25-mm
251 diameter (MFS HP020, 0.20-µm pore size, hydrophilic PTFE) to remove cell and filter

252 debris. A 0.5-ml aliquot of the acetone extract was mixed with 0.2 ml of water and 200
253 µl were injected immediately. This procedure avoids peak distortion by early eluting
254 peaks (Zapata and Garrido, 1991) and prevents the loss of non-polar pigments prior to
255 injection of the extract in an HPLC system (Latasa et al., 2001). Pigments were
256 separated following a previously described method (Zapata et al., 2000) on a C8 Waters
257 Symmetry column (150 × 4.6 mm, 3.5-µm particle-size, 10-nm pore size) using a
258 Waters Alliance HPLC system (Waters Corp., Milford, MA) consisting of a 2695
259 separations module, a Waters 996 diode-array detector, and a Waters 474 scanning
260 fluorescence detector (excitation:440 nm, emission: 650 nm). Eluent A consisted of
261 methanol: acetonitrile: 0.025 M aqueous pyridine (50:25:25 v/v/v), and eluent B of
262 methanol: acetonitrile: acetone (20:60:20 v/v/v). The elution gradient (time: %B) was as
263 follows: t_0 = 0%, t_{22} = 40%, t_{28} = 95%, t_{37} = 95%, and t_{40} = 0%. The flow rate was 1.0
264 ml min^{-1} and the column temperature was 25 ºC. The solvents were HPLC grade
265 (Romil-SpSTM); pyridine was reagent grade (Merck, Darmstadt, Germany). Pigments
266 were identified either by co-chromatography with authentic standards obtained from
267 SCOR reference cultures or by diode-array spectroscopy (Zapata et al., 2000). After the
268 purity of the peaks was confirmed, the spectral information was compared with a library
269 of chlorophyll and carotenoid spectra from pigments prepared from standard
270 phytoplankton cultures [SCOR cultures, see (Jeffrey and Wright, 1997)].
271
272 *2.8 Toxicity and toxin analyses*
273 Toxin analyses by HPLC as well as the toxicity assays (mouse bioassay, hemolytic
274 and cytotoxicity tests) were carried out using extracts prepared from the five strains,
275 following their culture, without replicates, in 5.5-L Pyrex bottle with aeration. The
276 cultures were maintained in the same medium and at the same salinity, temperature,

277 and photoperiod as used for the first growth experiment but with a slightly higher
278 light intensity (110 μmol photons m^{-2} s^{-1}). Two-ml aliquots were sampled every 1–4
279 days and fixed with Lugol's for quantification in order to determine the growth
280 phase of the culture. During late exponential phase (on different days, depending on
281 the culture), approximately 4L of every culture was filtered under reduced pressure
282 onto Whatman GF/C glass-fiber filters that had been previously heated in an oven at
283 450 ºC for a minimum of 4h. The filters were frozen immediately at -20 ºC until
284 extraction.
285
286 *2.8.1 HPLC-FLD analysis of PSP toxins*
287 All chemicals and solvents used were of HPLC or analytical grade. Standard solutions
288 of GTX4,1, dcGTX2,3, GTX2,3, STX, neoSTX, and decarbamoil saxitoxin (dcSTX)
289 were purchased from the Institute for Marine Bioscience, National Research Council,
290 Certified Reference Material Program (NRC-CRM) from Halifax, Canada.
291 The HPLC-FLD analysis with post-column derivatization was performed according to
292 Franco and Fernández (1993). The HPLC system consisted of a Waters 474
293 fluorescence detector (excitation: 30 nm and emission: 390 nm), a Waters 717
294 automatic injector, a Waters 600 HPLC pump to deliver the mobile phases, and two
295 Waters 510 pumps to deliver the post-column reagents. Separation was achieved with
296 an Agilent Cartridge 5μ Lichrosphere 100 RP18, (125 mm × 4.6 mm). The column
297 temperature was 30 ºC and the flow rate was 0.8 mL min^{-1}. The mobile phases consisted
298 of 1.5 mM octansulfonic in 10mM ammonium phosphate (pH 7) for the separation of
299 GTXs and 95% of this eluent solution plus 5% acetonitrile for the separation of
300 neoSTX, dcSTX, and STX.

In all cases, the column eluate was continuously oxidized with 7 mM periodic acid in 10 mM sodium phosphate (pH 9.0, 0.4 mL min^{-1}) during passage through a Teflon coil (7 m × 0.05 mm i.d.) heated at 65ºC, and finally acidified with 0.5 M acetic acid (0.4 mL min^{-1}) before entering the fluorescence detector.

Toxin concentrations were determined by comparing the peak area for each toxin with that of the standard. Samples were hydrolyzed by boiling with an equal volume of 0.4 N HCl for 15 min, in order to verify the presence of Cx, GTX5, and GTX6.

Data acquisition and data processing were performed using the Waters Empower software.

2.8.2 Cytotoxicity assay

The presence of voltage-gated sodium channel (VGSC)-inhibiting toxins (e.g., STX-like compounds) was investigated using the neuroblastoma (neuro-2a) cell-based assay as described in Cañete and Diogène (2008). Briefly, neuro-2a cells (ATCC, CCL131) were maintained in RPMI medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) at 37 ºC in a humidified atmosphere containing 5% CO_2 (Binder, Tuttlingen, Germany). Cells used in the experiments were plated in a 96-well microplate at a density of 35,000 cells per well and incubated for 24 h before the cytotoxicity assays were performed under the same conditions as described for cell maintenance.

To prevent interference from the toxic effects of HCl, an aliquot of the acidic *A. andersonii* extract (see 2.13.3 Hemolytic activity) was further purified before the analysis using solid-phase extraction (SPE) cartridges (C18 AccuBond II, 500 mg, 3mL). The SPE cartridge was first conditioned using 6 mL of absolute methanol followed by 6 mL of MilliQ water. One mL of acidic extract was loaded and eluted

twice with 2 mL of MilliQ water. The volume of the eluate was further adjusted to 4 mL using MilliQ water.

To specifically detect the presence of STX-like compounds, neuro-2a cells were treated with 0.3 mM ouabain and 0.03 mM veratridine (Sigma-Aldrich, St Louis, MO, USA) followed by the addition of STX standard solution (NRC-CRM) or *A. andersonii* extracts (Cañete and Diogéne, 2008). After a 24-h exposure, cytotoxic effects were measured using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] cell viability test (Mosmann, 1983) as described in (Manger et al., 1993).

2.8.3 Hemolytic activity

Hemolysis tests were carried out following the method described by Riobó et al. (2008) with minor modifications relating to blood and incubation time. Sheep blood in Alsever solution was kindly provided by Isabel Manzano (CZ Veterinaria, S.A.; Porriño, Spain). The vehicle for the assay was a hemolysis buffer containing 0.1% bovine serum albumin (BSA), 1mM calcium chloride, and 1 mM boric acid in phosphate-buffered saline pH 7.0 (PBS). A diluted erythrocyte stock suspension without ouabain was prepared to a final concentration of ~1.7×10^7 erythrocytes mL^{-1} as described by Riobó et al. (2008).

Two Whatman GF/F filters from each sample were destined to evaluate hemolytic activity. Potentially hemolytic proteins were extracted from one of the filters using PBS. Potential PSP toxins were extracted from the other filter using 0.1N HCl. One aliquot of this acidic extract was dried under a N_2 stream at 60 ºC and then dissolved in hemolysis buffer.

Triplicate samples containing 5 ml of the blood-cell suspensions and 5 ml of the toxin-containing solution were combined in centrifuge tubes and incubated at 37 ºC for 6 h

and then maintained at room temperature until 24 h. To remove cells, 1-mL aliquots were transferred to Eppendorf vials at intervals of 1, 5, 6, and 24 h, centrifuged for10 min at 1500 rpm at a temperature of 10 ºC, followed by the addition of 200 µL of each supernatant to the wells of one microwell plate. Absorption of these samples was measured at 405 nm with a BioRad microplate reader model 550.

2.8.4 Mouse bioassay

The toxicities of each sample were determined as described by the Association of Analytical Chemists (AOAC, 2000) using healthy male Swiss NMRI mice weighing 20±1 g. The stock colony for routine assay was managed following Council Directive Commission Regulation 1244/2007 (EC, 2007).

For each sample, two mice were i.p. injected with 1 mL of the HCl extract and then observed continuously for 60 min, recording symptom occurrence and time of death. Mice still alive after this time were observed intermittently for a total of 48 h.

3. Results

3.1 Morphology and measures

The morphology of wild cells of *A. andersonii* from Alfacs Bay of the Ebre delta (Fig. 2) generally well matched the description of Balech (1990), except for being smaller (length: 21.7± 3.1 µm, range: 17–28.3 µm, *n*=30; width: 21.1±2.7 µm, range 15.5–26.4 µm, *n*=30) than those of the original description (length: 21–35 µm, width 18–33 µm). Differences in the cell sizes of the strains compared to the Alfacs Bay field samples are shown in Figure 3. Cells of the Catalan strain ICMB222 were the smallest while those of one of the two American strains, CCMP1718, were the largest, although wild cells were larger than cells from any of the strains studied. ANOSIM analysis, which included strains and field cells, showed that these differences in cell size were

significant (p=0.00001). According to the pairwise tests, each of the strains was significantly different from the others and from the field samples ($p<0.05$), with the exception of the two American strains (CCMP 1718 and CCMP 1597).

3.2 Phylogenetic analyses

Based on the 5.8S rDNA, ITS regions, and LSU regions of the *Alexandrium* isolates, almost identical topologies were obtained by NJ, MP, and ML and by NJ, UPGMA, and ML analyses, respectively; therefore, only ML phylogenetic trees are shown in Fig. 4 (also see the supplementary material). *A. andersonii* constituted a homogeneous clade supported by high bootstrap values in both phylogenetic trees, consistent with a lack of substantial variability among the isolates analyzed. The *A. andersonii* group constituted a sister clade of the *A. minutum/A. tamutum* group within the genus *Alexandrium.*

3.3 Growth rates

The growth rates and maximum cell densities reached by five strains of *A. andersonii* at 20 ºC and 14 ºC are summarized in Table 3. Estimated growth rates ranged from 0.05 to 0.31 d^{-1} and were not significantly different among the strains either at 20 ºC or at 14 ºC (Kruskal-Wallis test, $p=0.1212$ and $p=0.0916$, respectively). The growth rates were more homogeneous (0.10–0.18) at 20 °C than at 14ºC, whereas temperature had no significant effect (nonparametric paired sign tests).

The maximum cell densities reached by each culture differed between strains but not significantly, with a maximum of $3.9 \cdot 10^7$ cells L^{-1} reached by strain CCMP 1718 at 20 ºC. Higher maximum densities were obtained at 20 ºC than at 14 ºC, with significant differences (non-parametric paired sign tests). At 14 ºC, only strain SZN012 grew to reach densities > 10^7 cells l^{-1}.

Different patterns of growth were observed for the five strains (Fig. 5). Thus, strains ICMB222 and CCMP1597 had lower growth rates and a longer exponential phase, while strains SZN012 and CCMP1718 grew faster and their exponential phase was shorter. For strain VGO664, temperature-specific differences were noted, with slower growth, after a long lag phase, at 14 ºC and more rapid growth at 20ºC.

3.4 Pigment analyses

A. andersonii is a typical peridinin-containing dinoflagellate and the pigment composition of all five strains was similar, with peridinin as the major carotenoid but slight differences in the pigment ratios with respect to chl *a* (Table 4). Specifically, the ratio of peridinin to either chlorophyll *a* or chlorophyll c_2 was highest (1.04± 0.01 and 4.93±0.10, respectively) in the Greek strain VGO644 and lowest (0.46 and 1.91, respectively) in strain CCMP1579, isolated from the east coast of the North Atlantic. Other pigment ratios (chl c_2/chl *a*, diadino/chl *a*, dino /chl *a*) were more consistent irrespective of the strain analyzed.

3.5 Toxicity and toxin analyses

3.5.1 HPLC

PSP toxins were not detected in samples of *A. andersonii* cultured at either 20 ºC or 14 ºC. For some of the samples, a small peak in the chromatogram with a retention time somewhat earlier than that of the GTX4 or GTX1 standard was noted. Since these peaks were also observed in the absence of oxidant they were considered to be interfering fluorescent compounds (see the example in Fig. 6).

3.5.2 Cytotoxicity assay

All of the *A. andersonii* strains studied were toxic to neuro-2a cells, with a response characteristic of the presence of VGSC-inhibiting toxins (data not shown). A semi-quantitative estimation of the STX-like compounds produced by *A. andersonii* strains is provided in Table 5. The values ranged between 1.4 and 14.7 µg STX equivalents $\times 10^{-5}$ cells^{-1}.

3.5.3 Hemolytic activity

All of the tested strains showed hemolytic activity (Table 6). After 1 hour of incubation, the absorbances of the PBS-extracted samples were indicative of hemolysis, which reached 100% after 5 hours. While the HCl extracts were also positive, the response was slightly different, with intact erythrocytes detected after 1 hour, ruptured red cells after 5 hours, and 100% hemolysis after 24 hours of incubation. Therefore, hemolytic activity was characteristic of all of the strains. This activity was due on the one hand to protein compounds and on the other hand to non-protein compounds, the nature of which is not yet known.

3.5.4 Mouse bioassay

None of the strains appeared to express PSP toxins since none of the bioassayed mice demonstrated the appropriate symptoms. Mice injected with extracts of the strains died at various times, as detailed in Table 7. Following injection with the HCl extracts from strains SZN 012 and CCMP 1597, the mice became weak and lethargic, with a progressive decline in activity until they finally died. However, these symptoms are not typical of those induced by toxins of the PSP complex. Nonetheless, to increase the possible toxic content and thereby better distinguish toxin-related symptoms, samples from strains VGO 664 and SZN 012 were concentrated 5- and 6-fold, respectively, prior

452 to their injection, in which case the mice died approximately 2 h later but still did not
453 exhibit PSP-like symptoms.
454
455 **4. Discussion**
456
457 *4.1 Morphology and measures*
458
459 In this study, the morphology and cell sizes of five cultured strains of *A. andersonii* (see
460 Table 1) were compared with the corresponding features of wild cells and those
461 described by Balech. Our measurements broaden the lower limits of the length and
462 width ranges of that original description (21–35 µm length, 18–33 µm width), as cells
463 13.5–35 µm in length and 12–33 µm in width were observed. In fact, the cells of the
464 different *A. andersonii* strains used in this study were smaller than those obtained by
465 Balech. Moreover, the sizes of the field cells were within the lowest end of the range
466 reported in that original description.
467 By contrast, for the five strains, our measurements were more in line with those of
468 Touzet et al. (2008), Frangopulos et al. (2004), and Ciminiello et al. (2000).
469 In our study, while significant differences were observed among the measures of the
470 different strains, the largest specimens were found in the wild samples collected from
471 Alfacs Bay. These results, in addition to the fact that the strain analyzed by Balech
472 (1990) was CCMP 1597 [synonym of GTM242, used in Anderson et al. (1990)],
473 suggest a reduction of cell size in culture.
474
475 *4.2 Phylogenetic analyses*
476 The phylogenetic homogeneity of *A. andersonii* strains based on the 5.8 ITS and LSU
477 regions did not allow us to distinguish among individual populations from different
478 Mediterranean areas, unlike in other *Alexandrium* species (Lilly et al., 2005; McCauley

et al., 2009). However, genetic diversity within *A. andersonii* cannot be ruled out and its detection may instead require finer-scale markers (such as microsatellites), as was the case for *A. minutum* in studies at global and Mediterranean scales (Casabianca et al., 2012; McCauley et al., 2009).

4.3Growth rates

The net growth rates of the studied strains under standard conditions did not differ significantly. This was also reported for *Karlodinium veneficum* (Bachvaroff et al., 2009) but not in *Karenia brevis* (Loret et al., 2002). Here, differences in the growth patterns of the five strains and even between a subclonal strain and its parent strain (CCMP1718 and CCMP1597) were observed.

Along the Catalan coast (NW Mediterranean, Spain), *Alexandrium* species have been observed in detail for over 17 years. During this time, there have been no blooms of *A. andersonii* in response to warm temperatures; rather, the cells were rarely detected and only at very low densities (<100 cells L^{-1}). In contrast, a bloom of *A. andersonii* occurred in the winter (from January to April) of 2007 in Alfacs Bay, coinciding with water temperatures between 10 and 15 ºC. During that event, cell densities reached a maximum of 7159 cells L^{-1}, recorded on February 19.

Based on these observations, it seems that at least along Catalan coast *A. andersonii* is better adapted to low temperatures. However, in the laboratory, the cell densities of strain ICMB222, isolated in Alfacs Bay (Catalan coast), and of the other strains were lower at 14 ºC than at 20 ºC. Thus, other environmental factors are likely to be of greater significance than temperature for bloom development.

4.4 Pigment analyses

As all members of the genus *Alexandrium* identified thus far contain the pigment peridinin, in this study only differences in the accessory pigment ratios were expected. In dinoflagellates, pigments occur in the extrinsic water-soluble PCA and intrinsic ACP antennae in stoichiometric proportions. As the proportions of the two antenna types are determined by genetic as well as environmental factors, these in turn will affect the ratios of accessory pigments.

In the specific case of *A. andersonii*, our data point to intrinsic and thus probably genetic factors as accounting for the quantitative differences in pigment ratios, since the five strains were cultured under the same environmental conditions. Zapata et al. (2012) studied three common strains of *A. andersonii* (CCMP 1718, VGO 664, and SZN-12) and found similar differences in pigment ratios. However, absolute molar pigment ratios were slightly different in both studies due to variable culture conditions (e.g., light and salinity).

4.5 Toxicity and toxin analysis

Under the experimental conditions of this study, PSP toxins were not detected by either HPLC or mouse bioassay, in contrast to the results of Ciminiello et al. (2000) and Frangopulos et al. (2004). However, all of the studied strains exhibited both a response characteristic of the expression of VGSC-inhibiting toxins, as shown in the neuroblastoma neuro-2a cell assay, and hemolytic activity. These results can be alternatively explained as follows: The neuro-2a cytotoxicity assay is able to detect not only PSP-type toxicity, similar to the HPLC-FLD method, but also the toxicity of other biomolecules, such as tetrodotoxin (TTX) (Kogure et al., 1988), lipopeptide (Edwards et al., 2004; Wu et al., 2000), and polypeptide (Catterall et al., 2007; Jacob and McDougal, 2010), that interact with VGSCs. Consequently, this assay may overestimate toxin

529 concentrations. Thus, an interpretation of the results of this assay must consider that the
530 low toxicity detected was not due to PSP toxins. Similarly, the hemolytic activity of the
531 different strains could have been due not only to STX and its derivatives but also to an
532 excess of salt (very probable for the concentrated samples) or/and to a hemolytic toxin,
533 as reported for other *Alexandrium* species (Emura et al., 2004; Simonsen et al., 1995).
534 As an alternative explanation of our findings, the amount of toxicity may have been so
535 low that it was not detected either by the mouse bioassay or by the HPLC method,
536 which have a limit of detection of 0.3 µg STXeq mL^{-1} and 0,01-0,015 µg STXeq mL^{-1},
537 respectively, whereas it was detectable in the neuro-2a assay (detection limit of 0.91–
538 58.41 nM, or 0.27–17.5·10^{-3} µg STXeq mL^{-1}) (Cañete and Diogène, 2008). However,
539 the first explanation is the more likely one since the toxicity of the strains as determined
540 in the cytotoxicity assay was clearly higher than the detection limit of the HPLC method
541 (0.01–0.015 µg STXeq mL^{-1}), which therefore should have yielded a positive result.

542 Ciminiello et al. (2000) and Frangopulos et al. (2004) worked with the same Neapolitan
543 strain used in this study, culturing the cells at the same (Cimiello et al., 2000) or similar
544 (18ºC) (Frangopulos et al., 2004) temperature. However, both groups used K medium
545 instead of L1 medium. The main difference between these two culture media is the
546 presence of Ni, V, and Cr in L1 medium but not in K medium, which contains
547 ammonium, as well as differences in the concentrations of EDTA and Mo. Changes in
548 toxin production in response to different concentrations of trace metals in the culture
549 medium have been described in toxic cyanobacteria, diatoms, and dinoflagellates
550 (Lukač and Aegerter, 1993; Maldonado et al., 2002; Rhodes et al., 2006). Likewise,
551 differences in light intensity have also been shown to influence the toxin content of
552 *Alexandrium* species (Hamasaki et al., 2001; Wang and Hsieh, 2005). In our study, the
553 irradiation intensity was the same as that in the study of Ciminiello et al. (2000) but

554 approximately double that used by Frangopulos et al. (2004). Another explanation for
555 the reported toxicity of the Neapolitan strain by these two authors but not in our hands
556 could be a loss of toxicity, as reported for other *Alexandrium* species (Martins et al.,
557 2004). Nevertheless, while differences in per-cell toxicity have been described for the
558 same strain depending on the cultures conditions (Anderson et al., 1990; Boczar et al.,
559 1988; Hwang and Lu, 2000), differences in a strain's toxin profile, based on a
560 comparison of the results of Ciminiello et al. (2000) and Frangopulos et al. (2004), are
561 unusual. Indeed, significant shifts tend to occur only in batch and semi-continuous
562 cultures in response to extreme changes in the growth regime (Anderson et al., 2012).

563 In addition, the toxin profile of *A. andersonii* as described by Ciminiello et al. (2000)
564 consists mainly of STX and neoSTX. To our knowledge this toxin composition has not
565 been reported in other *Alexandrium* species. In contrast, Frangopulos et al. (2004)
566 reported a more typical profile of PSP toxins, comprising gonyautoxins (GTX1–4), with
567 GTX2 as the dominant component.
568
569 The difference between our results those and of the two authors could also have been
570 due to the methodology used. In the work of Ciminiello et al. (2000), the toxins used in
571 the mouse bioassay were extracted by suspending the cultures in an equal volume of
572 acetic acid 0.5 N (0.03 g acetic acid mL^{-1}). Acetic acid is not the solvent used in the
573 official method described in the AOAC, 2000 for mouse bioassay extraction. To prove
574 the validity of this solvent for use in the sample extracts, we performed the mouse
575 bioassay using only different concentrations of acetic acid, from 0.1% to 4 %, without
576 any toxin or other component. The result was in all cases the death of the mice (see
577 Table 8). Similar concentrations of HCl do not kill the mice. Therefore, acetic acid is
578 not an appropriate solvent for the mouse bioassay and its use therefore casts doubt on

579 the results obtained by Ciminiello et al. (2000). Furthermore, the chemical methodology
580 used by these authors (NMR and HRMS) is, unfortunately, poorly reproducible by other
581 laboratories. In the study of Frangopulos et al. (2004), the isolate was not analyzed in a
582 mouse bioassay; instead, the presence of toxins was determined by HPLC with
583 fluorescence detection. While this was the same methodology that used in the present
584 work, they measured a very low mean of total toxins, with a standard error higher than
585 the mean. Methodology, could thus explain the differences between some of our results
586 and those reported in Ciminiello et al. (2000).
587
588 A few studies reported in the literature used *A. andersonii* as the toxic species in
589 ingestion experiments but only in some of them were the toxin profiles analyzed.
590 The *A. andersonii* strains used by Shaber and Sulkin (2007) and Perez and Sulkin
591 (2005) were obtained as a toxic strain of *A. andersonii* from Provasoli-Guillard National
592 Center for Culture of Marine Phytoplankton (CCMP). The latter study used strain
593 CCMP1718, one of the five strains of *A. andersonii* examined in this study. In
594 describing its toxicity, the authors cited the CCMP website. However, the CCMP, to the
595 best of our knowledge, has not evaluated their *A. andersonii* cultures for toxicity (Julie
596 Sexton, personal communication). Garcia et al. (2011) also used strain CCMP1718 for
597 ingestion experiments. Its toxicity, as verified by CyanoHAB Services, is based on the
598 results of an ELISA, which determined a toxin concentration of 5.1 µg STXeq L^{-1}.
599 However, an ELISA is a non-official method for PSP determination and it has several
600 problems (EFSA 2009), such that false positive results cannot be ruled out. Moreover,
601 although both Perez and Sulkin (2005) and Garcia et al. (2011) reported the accelerated
602 mortality of larval *Hemigrapsus oregonensis* fed *A. andersonii* cells, according to the
603 latter authors the results cannot be ascribed with absolute certainty to toxin effects.

Stüken et al. (2011) recently proposed that in dinoflagellates nuclear genes are required for STX synthesis but they were unable to detect them (specifically *sxtA1* and *sxtA4*) by *A. andersonii* strains CCMP1597 and CCMP2222 (the latter synonymous with SZN012 of this study). Although we cannot draw any conclusions regarding the ability of *A. andersonii* to synthesize toxins, the two official methodologies used in our study for the detection of PSPs, i.e., HPLC and mouse bioassay, failed to detect toxin production, thus supporting the results of Stüken et al. (2011).

Our findings obtained with the less specific methodologies (cell bioassays) are inconclusive since, as discussed above it is unclear whether the response of neuroblastoma 2a cells was genuinely due STX (or its derivatives). But in any case, under the study conditions only trace amounts of toxins were produced by the tested strains of *A. andersonii*. Therefore, based on our current knowledge, we do not consider *A. andersonii* as a toxic species.

4.6. Aquaculture implications

In this study, the non-toxic behavior of both the Neapolitan strain of *A. andersonii* and the other four Mediterranean and North Atlantic Ocean strains, all tested under the same conditions, suggest that the toxicity detected in field mussels from the Alfacs Bay in the winter of 2007 (Fernández-Tejedor et al., 2007), concurrent with a bloom of *A. andersonii*, was probably due to the presence of other toxic species. In fact, *A. minutum*, which is very common along the Catalan coast and its toxicity is well established

(Delgado et al., 1990; Van Lenning et al., 2007), was also present in that bloom period (Sampedro et al., 2007).

Conclusion

Our experiments show that *A. andersonii* strains originating from different locations differ markedly in their size, several of their pigment ratios, and their patterns of growth, despite phylogenetic homogeneity in their 5.8 ITS and LSU regions. This intraspecific variability was also noted between a subclonal strain and its parent strain (CCMP1718 and CCMP1597). Moreover, when cultured under the same laboratory conditions none of the strains expressed PSP toxins, as determined by HPLC and in a mouse bioassay.

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Tables

Table 1. Sampling location and characteristics of the five strains used in this study.

Strain	Sampling location	Clonal	Observations
VGO 664	Medit., Greece, Elefsis Bay (Saronikos Gulf)	Yes	Isolated from a vegetative cell
SZN 012	Medit., Italy, Napoli,	No	Isolated from a cyst
ICMB 222	Medit., Spain, Catalonia	Yes	Isolated from a vegetative cell
CCMP 1718	Atl., USA	Yes	Isolated from strain CCMP1597
CCMP 1597	Atl., USA, Massachusetts, Cape Code, Eastham, Town Cove	No	Isolated from a cyst

Table 2. EMBL-EBI ENA accession numbers of the ITS 5.8S rDNA sequences and sample locations of the different strains used in this study.

Species	Sampling location	Strain	EMBL accession no.
Alexandrium andersonii	Aegean Sea	VGO664	AM236854
A. andersonii	Tyrrhenian Sea	SZN012	AJ308523
A. andersonii	Tyrrhenian Sea	SZN011	AJ312440
A. andersonii	Catalan Sea	ICMB222	HE574398
A. andersonii	NWAtlantic	CCMP1718	HE574400
A. andersonii	NWAtlantic	CCMP1597	HE574399
A. affine	Alboran Sea	PA8V	AJ632095
A. cf. *catenella*	TAclade, Catalan Sea	CSIC-C7	AJ580322
A. cf. kutnerae	MEclade, Catalan Sea	VGO714	AM238515
A. insuetum	Catalan Sea	ICMB218	AM422769
A. margalefi	Catalan Sea	VGO661	AM237339
A. margalefi	Tyrrhenian Sea	CNR-AM1	AJ251208
A. minutum	Catalan Sea	AL9C	AJ621733
A. minutum	NAtlantic	AL4V	AM292310
A. minutum	Tyrrhenian Sea	CNR-AMIA2PT	AJ312945
A. peruvianum	Catalan Sea	AM10C	AM237340
A. pseudogoniaulax	Catalan Sea	VGO655	AM237416
A. tamarense	NAtlantic clade	HI38	n.r.
A. tamarense	WEclade, NEAtlantic	PE1V	AJ005047
A. tamutum	Catalan Sea	VGO616	AM236859
A. tamutum	Tyrrhenian Sea	VGO662	AM238452
A. taylori	Catalan Sea	CSIC-AV8	AJ251654
A. taylori	Aegean Sea	CBA1	AJ416856
A. taylori	Tyrrhenian Sea	CNR-AT4	AJ251653

Suppl material. Table 1. EMBL-EBI ENA accession numbers and sample locations of the different strains used in LSU sequence studies.

Species	Sampling location	Strain	EMBL Accession no.
Alexandrium andersonii	Aegean Sea	VGO664	HE574405
A. andersonii	Tyrrhenian Sea	SZN012	HE574401
A. andersonii	Ebre Delta,Catalan Sea	ICMB222	HE574402
A. andersonii	Town cove Massachusetts USA, NE Atlantic	CCMP1718	HE574403
A. andersonii	USA, NE Atlantic	CCMP1597	HE574404
A. andersonii	USA, NE Atlantic	TCO2	AAU44937
A. andersonii	USA, NE Atlantic	GTTCO2	AY268608
A. andersonii	USA, NE Atlantic	GTTCO2A	AY96283
A. affine	Gulf of Thailand	CU1	U44935
A. catenella	Thau Lagoon, France, Mediterranean Sea	ATT98	AF318219
A. insuetum	Urbino Lagoon Corsica	-	AF318234
A. margalefi	Mexico	AGNZ01	AY152707
A. minutum	Gulf of Trieste	LAC27	AY962842
A. minutum	N Atlantic?	AL2V	AY962837
A. ostenfeldi	Finland: Foegloe, Aland archipelago	AOTVA1	FJ011439
A. peruvianum	Catalan Sea	VGOAM10C	FJ011438
A. pseudogoniaulax	Gulf of Trieste	AP2T	AY268602
A. tamarense	Bell Bay, Tasmania, Australia	ATBB01	ATU44933
A. tamarense	United Kingdom	AlexW1	AJ303439
A. tamarense	Port Benny, Alaska	PW06	U44927
A. tamiyavanichii	Malaysia	ATMSO1	AF174614
A. tamutum	Gulf of Trieste	AT5	AY962863
A. tamutum	-	C72	AY962865
A. tamutum	Gulf of Trieste	AB2	AY962864
A. taylori	Italy	AY2T	AJ535348
A. taylori	Italy	AY4T	AJ535349
A. tropicale	Gulf of Thailand	CU15	AY268607

Table 3. Growth rate (μ), duration of the exponential growth phase, and maximum cell abundances of the different strains of *A. andersonii* cultured at 14 or 20 °C. N=2. SD: standard deviation.

Strain	**Temp (ºC)**	**Growth rate (day^{-1}) ± SD**	**Duration of exponential phase (days)**	**Maximum cell abundances ($cell\ L^{-1}$)**
VGO 664	**20**	**0.13± 0.03**	**21/28**	2.45E+07
SZN 012	20	0.18± 0.01	21	3.54E+07
ICMB 222	20	0.10± 0.00	33/35	2.76E+07
CCMP 1718	20	0.17±0.01	21/23	3.91E+07
CCMP 1597	20	0.12	30	3.21E+07
VGO 664	14	0.09±0.01	19	5.43E+06
SZN 012	14	0.28±0.05	10	1.03E+07
ICMB 222	14	0.06±0.02	19	4.40E+06
CCMP 1718	14	0.13±0.01	21	8.86E+06

997

998 Table 4. Pigment ratios in the five strains of *Alexandrium andersonii* at 20 ºC.

999

Culture number	**Per/ chl *a* ± SD**	**Per/c_2 ± SD**	**chl c_2/chl *a* ± SD**	**Diadino/chl *a* ± SD**	**Dino/chl *a* ± SD**
VGO 664	1.04± 0.01	4.93±0.10	0.21±0	0.23±0	0.09±0.01
SZN 012	0.90±0.01	4.43±0	0.20±0	0.23±0	0.08±0
ICMB 222	0.83±0.01	4.14±0.57	0.20±0.03	0.21±0.02	0.07±0.01
CCMP 1718	0.76±0.06	3.03±0.11	0.25±0.02	0.23±0.01	0.10±0.01
CCMP 1597	0.46	1.91	0.24	0.21	0.08

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Table 5. STX equivalents produced by *A. andersonii* strains at 20 ºC as determined in a neuro-2a cell-based assay

Strain	Cells (mL culture)$^{-1}$	µg STX eq. (mL culture)$^{-1}$	µg STX eq. x 10^{-5} cells^{-1}
VGO 664	34692	1,99	5,7
SZN 012	27000	3,971	14,7
ICMB 222	42870	0,858	2,0
CCMP 1718	35320	0,478	1,4
CCMP 1597	20857	2,178	10,4

Table 6. *Hemolytic* activity of *A. andersonii* strains cultured at 20 ºC

Culture number	Extraction	37 ºC 1h (%)	37 ºC 5h (%)	37 ºC 6h (%)	Room temp. 24h (%)
VGO 664	PBS	11	100	100	
SZN 012	PBS	85	100	100	
ICMB 222	PBS	30	100	100	
CCMP 1718	PBS	51	98	96	
CCMP 1597	PBS	4	100	100	
VGO 664	HCl	0	10	17	40
SZN 012	HCl	0	31	48	100
ICMB 222	HCl	0	22	37	100
CCMP 1718	HCl	1	72	87	99
CCMP 1597	HCl	0	16	22	67
VGO 664X5	HCl	45	43	56	57
ICMB 222X6	HCl	42	79	81	72
Blank filters	HCl	0	0	0	0

Table 7. Mouse bioassay of the toxicity of *A. andersonii* strains cultured at 20 ºC

Culture number	**Time until death (h)**		**Comments**
VGO 664	-	-	alive (48 h)
SZN 012	30	48	
ICMB 222	-	-	alive (48 h)
CCMP 1718	-	-	alive (48 h)
CCMP 1597	10	48	
VGO 664X5	2	2	Death probably due to excess salt
ICMB 222X6	1,5	2	

Table 8. Mouse bioassay of different concentrations of acetic acid.

Acetic acid concentration	**Death time (h) N=3**	**Comments**
4% (0,04g/mL)	1-2min	
2%(0,02g/mL)	15-20min	
1%(0,01g/mL)	1h	
0.1%(0,001g/mL)	6h	
Distilled water	-	alive (48 h)
4% (0,04g/mL)		

Figure legends.

Fig. 1. Map showing the location of Alfacs Bay.

Fig. 2. Micrographs of a cell of *Alexandrium andersonii* as observed using light (a) and epifluorescent (b) (c) microscopy. Scale bars, 10 µm.

Fig. 3. Mean and standard deviation of the measures of 30 cells of the five strains of *A. andersonii* (1= VGO 664, 3= SZN 012, 5= ICMB 222, 7= CCMP 1718, 9= CCMP 1597) and from cells in natural samples from Alfacs Bay. Measures from other authors have been included (C=Ciminiello et al 2000, T=Touzet et al 2008). Gray square shows the minimum range of width and length described in (Balech 1990).

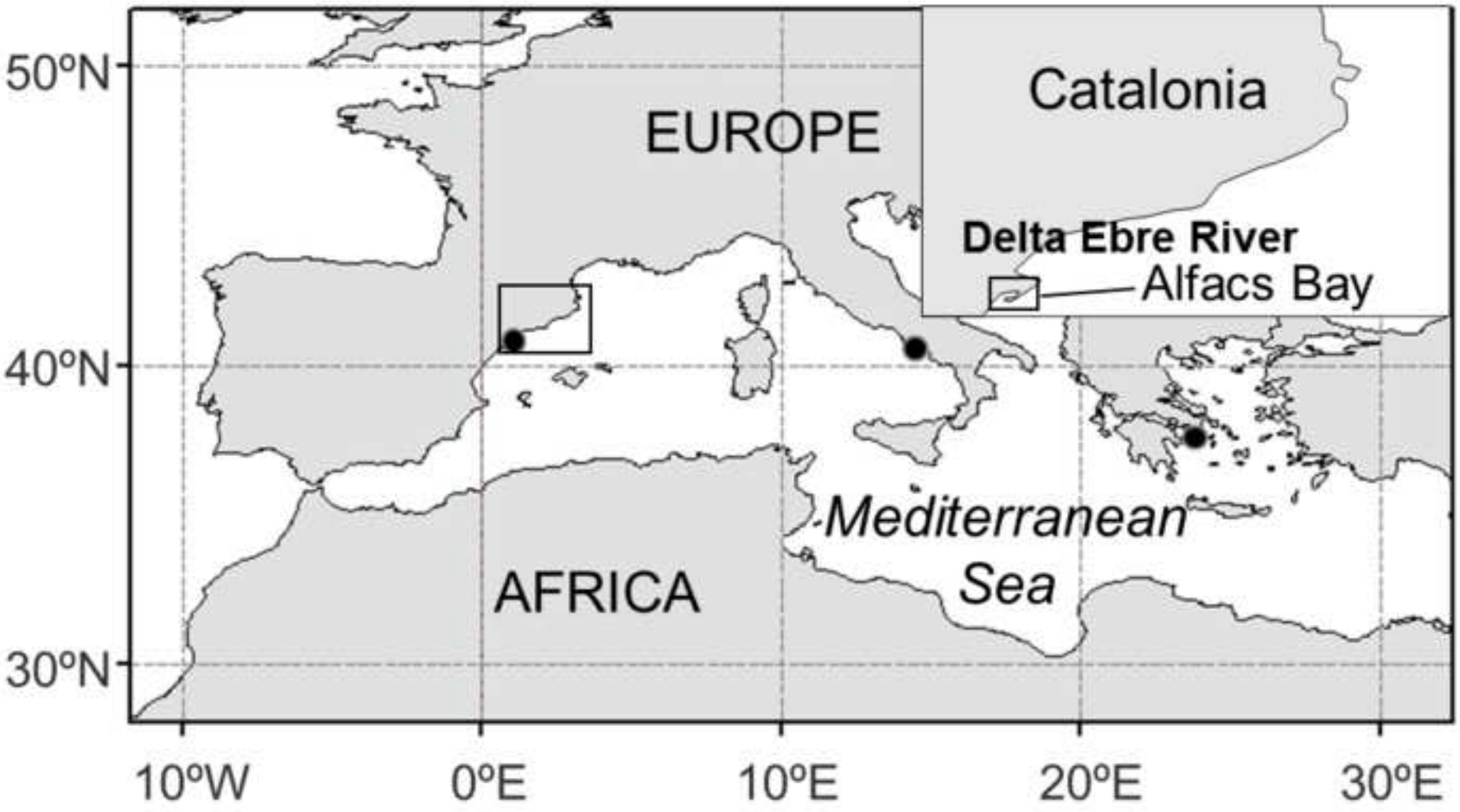
Fig.4. Maximum-likelihood (ML) tree inferred from the alignment of ITS 5.8S rDNA sequences. Numbers on the major nodes represent, from right to left, neighbor-joining (1,000 pseudoreplicates), maximum-parsimony (1,000 pseudoreplicates), and ML (1,000 pseudoreplicates) bootstrap values. Only bootstrap values >50% are shown. The tree was rooted using *Goniaulax spinifera* (AF051832) as outgroup.

Fig. 5. Growth curves of the different strains of *A. andersonii* cultured at 20 ºC (circles or rhombus) and at 14 ºC (squares) in L1 medium. For illustrative purposes, the data were fitted to a curve. Bars represent the standard deviation of the duplicates.

Fig. 6. HPLC–FLD chromatograms of stationary phase cells of strain SZN012 cultured at 14 ºC (third experiment). A: SZN012 cells+GTXs standard with oxidant; B: SZN012 cells+GTXs standard without oxidant; C: SZN012 cells with oxidant; D: SZN012 cells without oxidant. Retention times (min) of GTXs: GTX4: 6,4; GTX1: 7,3; dcGTX3: 11,4; dcGTX2: 12,8; GTX3: 14,3; GTX2: 18,4

Supplementary material Fig.1. Maximum-likelihood (ML) tree inferred from the alignment of LSU rDNA sequences. Numbers on the major nodes represent, from right to left, neighbor-joining (1,000 pseudoreplicates), UPGMA (1,000 pseudoreplicates), and ML (1,000 pseudoreplicates) bootstrap values. Only bootstrap values >50% are shown. The tree was rooted using *Goniaulax spinifera* (EU805591) as outgroup.

Figure
Click here to download high resolution image



Figure

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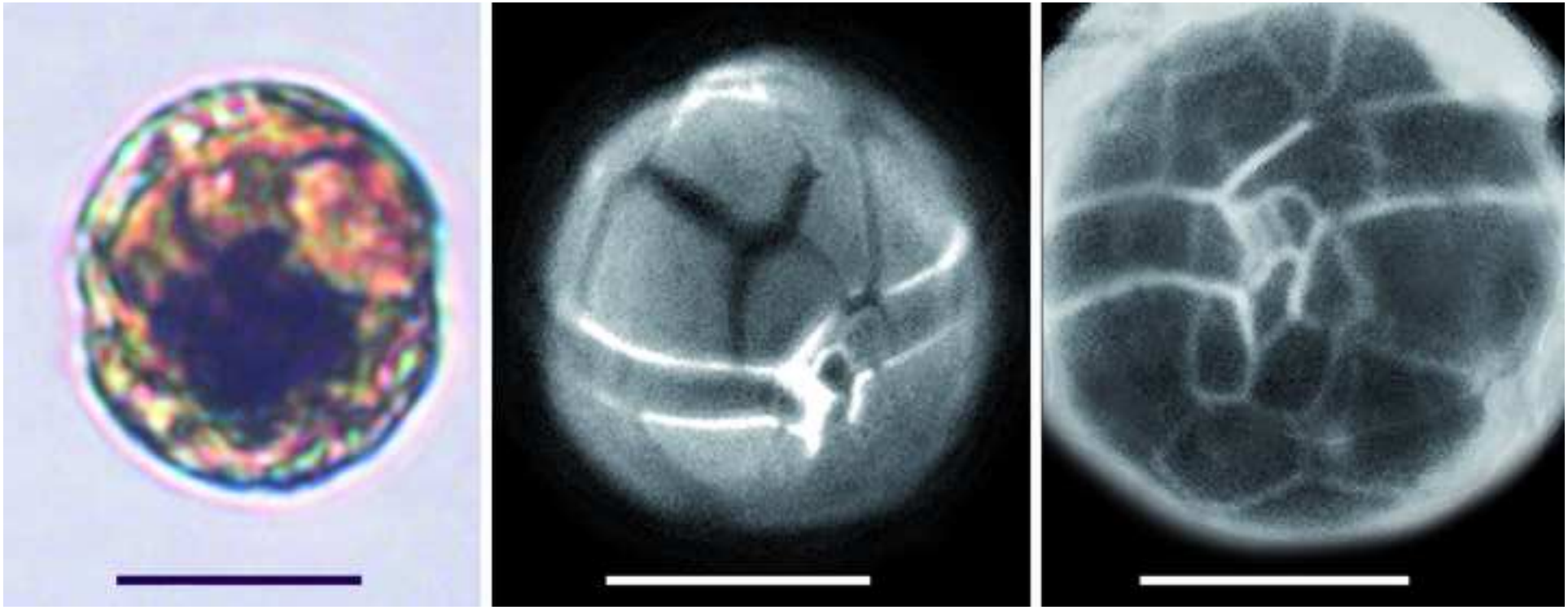

Figure

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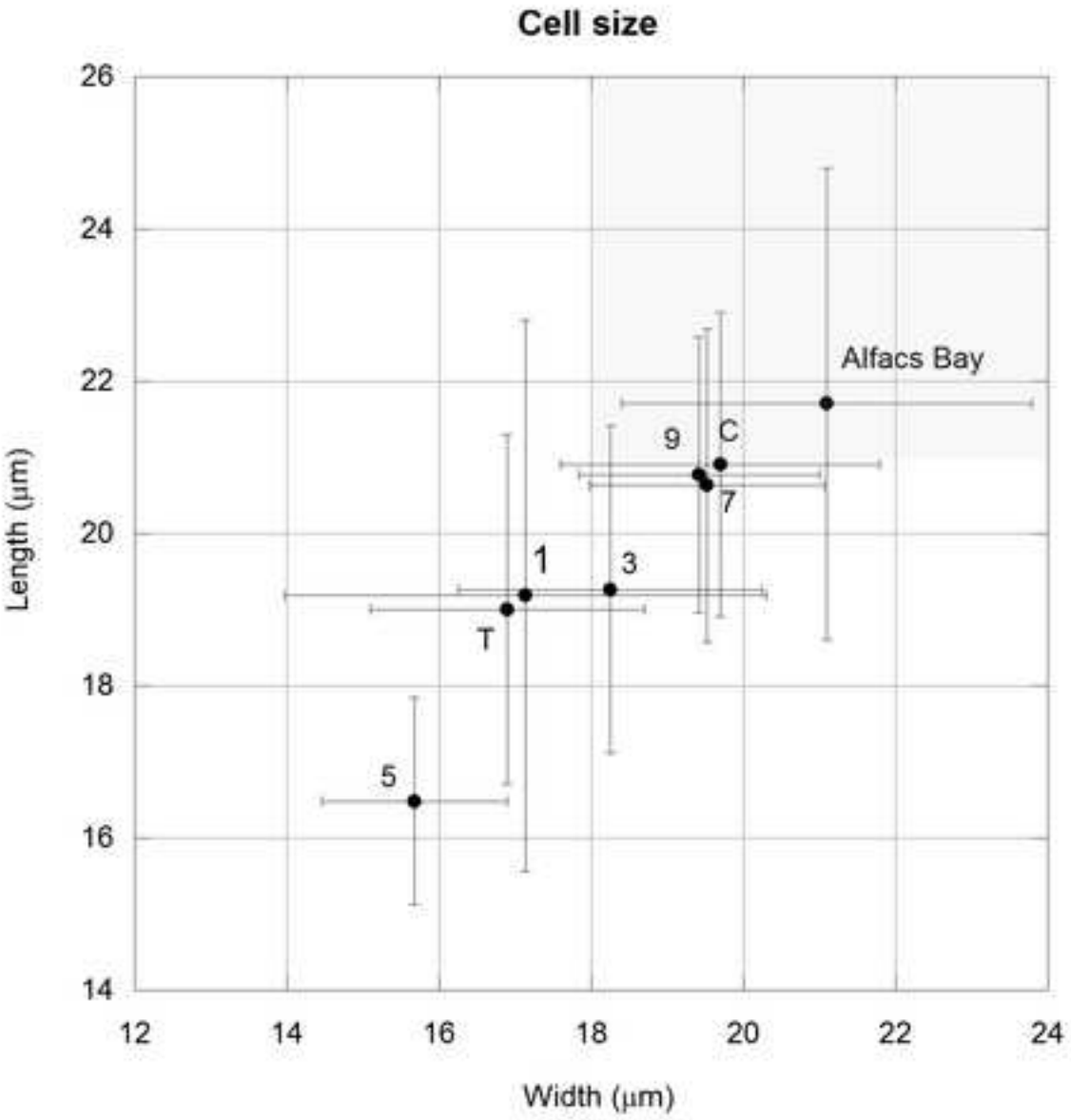

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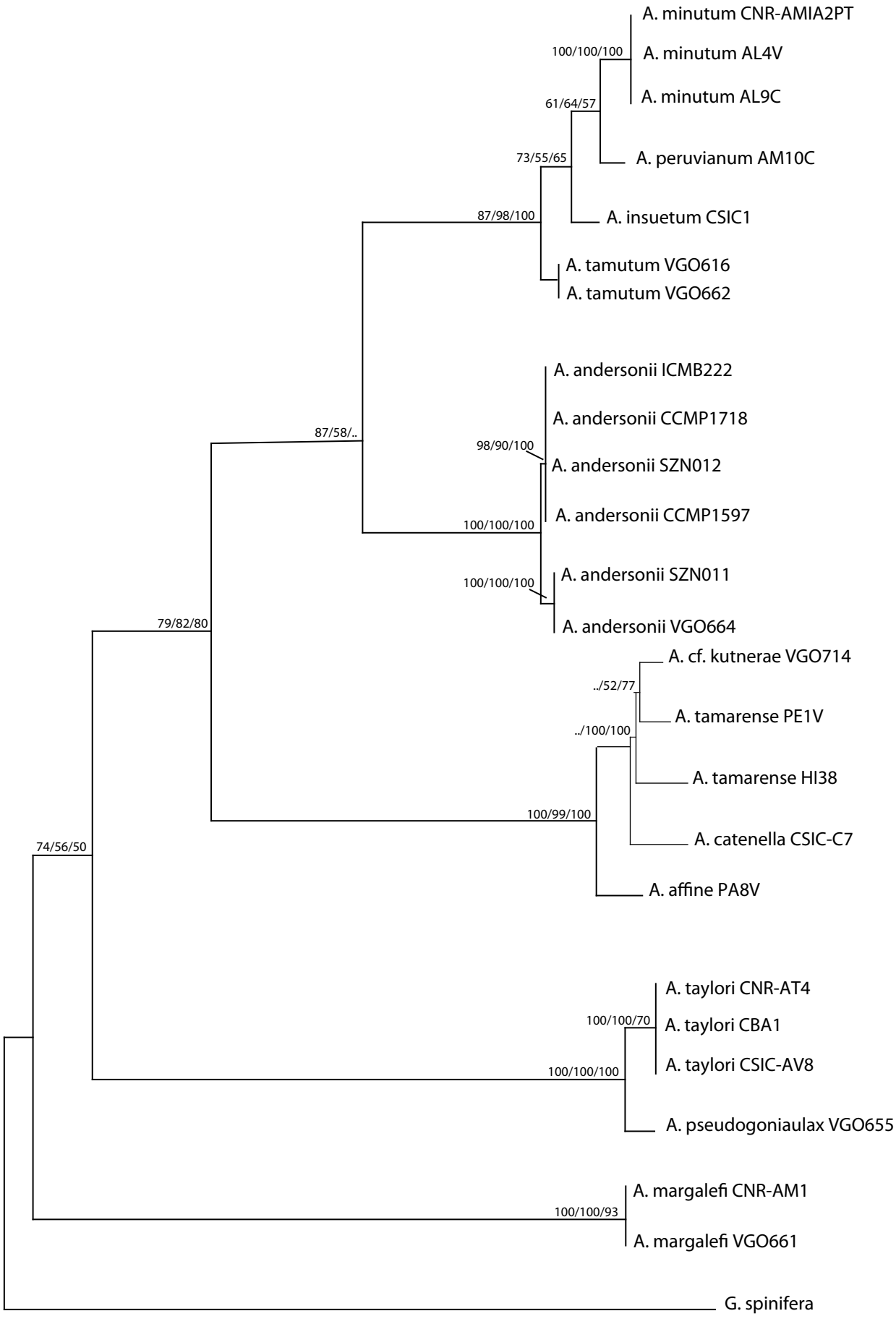
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A. minutum AL9C
61/64/57
A. peruvianum AM10C
73/55/65
A. insuetum CSIC1
87/98/100
A. tamutum VGO616
A. tamutum VGO662
A. andersonii ICMB222
A. andersonii CCMP1718
87/58/..
98/90/100
A. andersonii SZN012
A. andersonii CCMP1597
100/100/100
A. andersonii SZN011
100/100/100
A. andersonii VGO664
79/82/80
A. cf. kutnerae VGO714
../52/77
A. tamarense PE1V
../100/100
A. tamarense HI38
100/99/100
A. catenella CSIC-C7
74/56/50
A. affine PA8V
A. taylori CNR-AT4
100/100/70
A. taylori CBA1
A. taylori CSIC-AV8
100/100/100
A. pseudogoniaulax VGO655
A. margalefi CNR-AM1
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G. spinifera
0.1 substitutions/site

Figure

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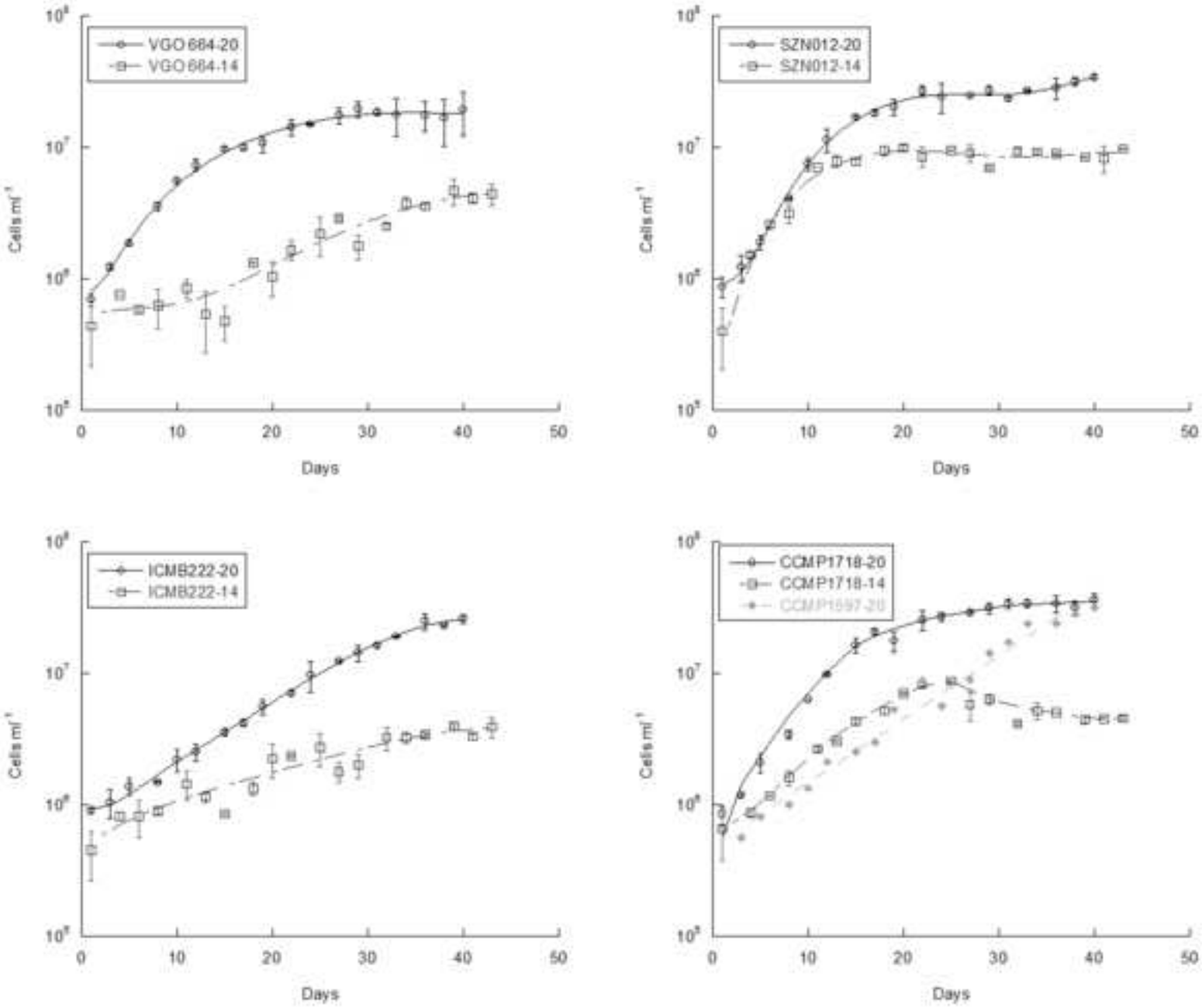
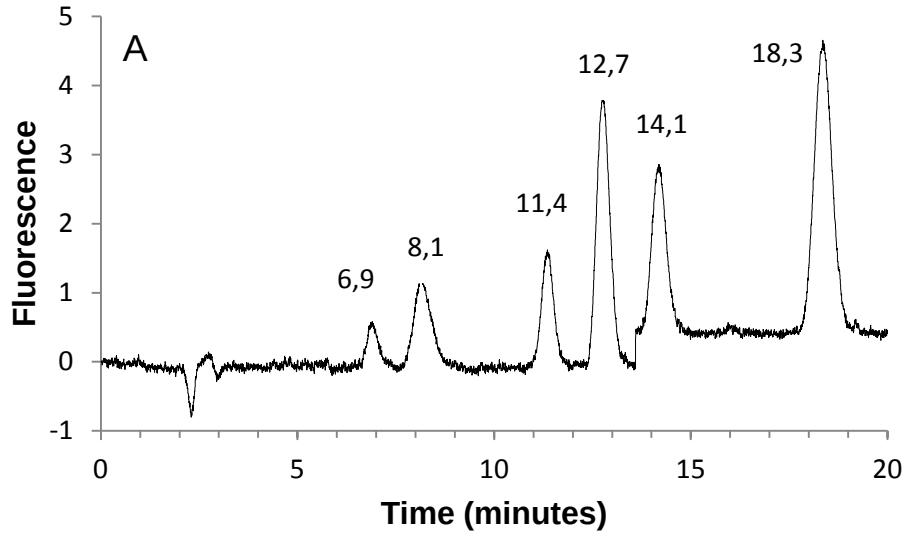


Figure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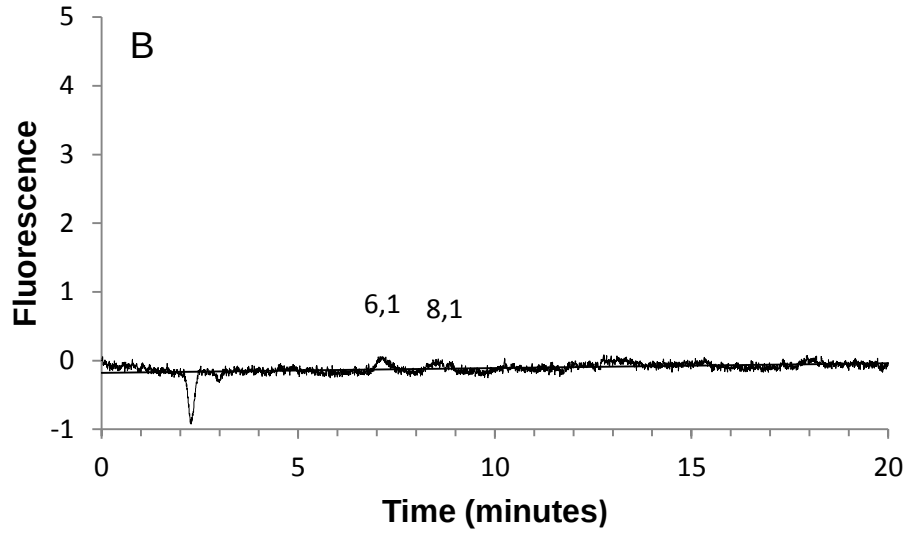
18,3
Fluorescence
Time (minutes)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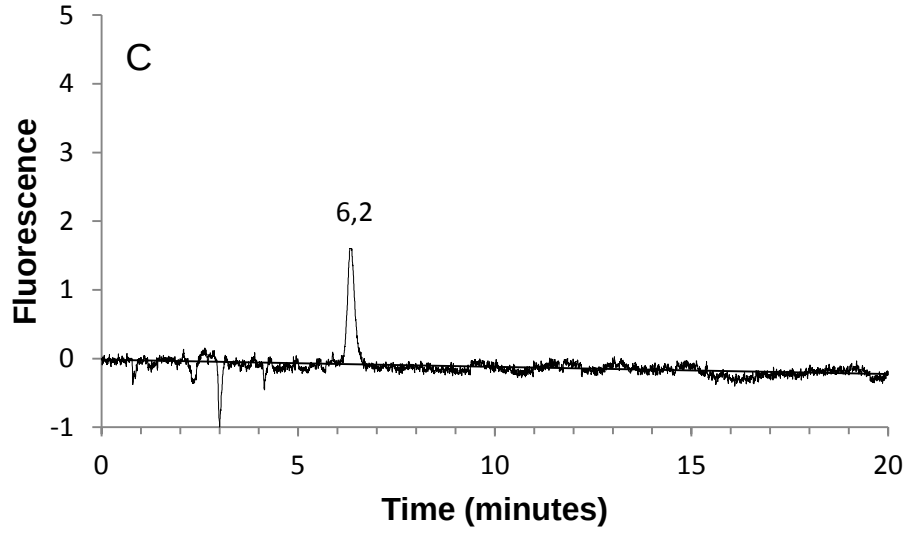
Fluorescence
Time (minutes)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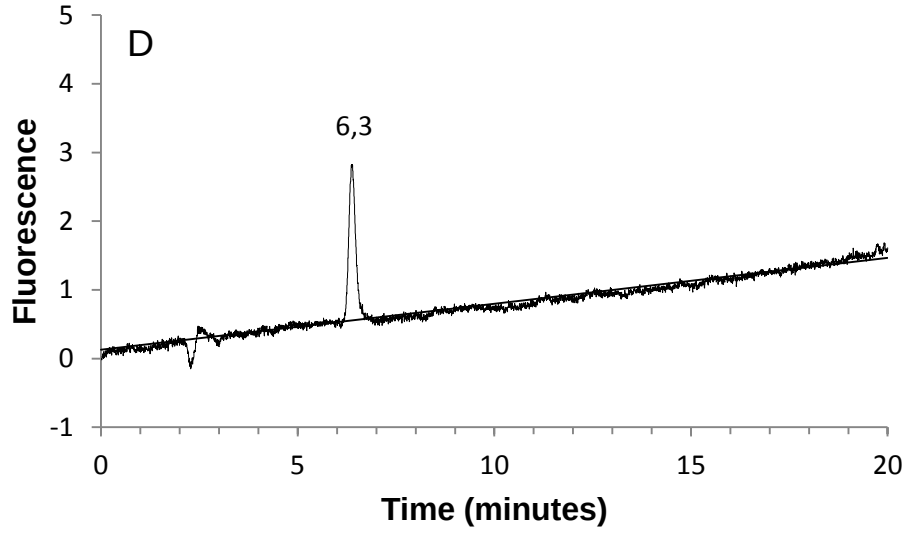
Fluorescence
Time (minutes)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Supplementary Material