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The toxicity and intraspecific variability of *Alexandrium andersonii* Balech

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ABSTRACT

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

1. Introduction

In a recent issue of the journal *Harmful Algae* (Pitcher, 2012), the need for species-specific information to predict the occurrence of harmful algal blooms (HABs) was emphasized, especially given the wide-ranging differences in the morphology, phylogeny, life-cycles, growth requirements, etc., of HAB species. In fact, even within the same species there is extensive genotypic and phenotypic variability that challenges our traditional notion of morphospecies (e.g., Alpermann et al., 2010). Therefore, it is 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; Tillmann et al., 2009). These and other variations could reflect geographic adaptations 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 have been used to distinguish distant populations (i.e., Kim et al., 1993; Mackenzie et al., 1996; Oshima et al., 1993). Similarly, associations of toxic and non-toxic populations of *Alexandrium minutum* in different areas of the coastal waters of Ireland have been described (Touzet et al., 2007). Moreover, there are several reports of temperature-related differences in the toxin content of *Alexandrium* species (Etheridge and Roesler, 2005; Lim et al., 2006; Navarro et al., 2006; Ogata et al., 1987; Wang et al., 2006).

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Among the around 30 species that comprise *Alexandrium* Halim (Balech, 1995), at least half are considered potentially harmful. Of these, 12 are known to be PSP producers, while others produce different types of toxins (spirolides, goniodomins) and antifungal substances, cause the mass mortality of fish, or exhibit 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 that the strain was 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 in nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) studies, 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. This strain was originally obtained from a cyst found in the sediment of the Gulf of Naples. The cellular toxin content of strain SZN12 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, *Alexandrium 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 the bay identified the low-level presence of PSP toxins (44 μg STX eq./100 g) (Fernández-Tejedor et al., 2007). However, they could not be directly ascribed to the *A. andersonii* population, since other *Alexandrium* PSP-producing species were simultaneously present in the water column.

The recent detection of *Alexandrium andersonii* in different areas of the Mediterranean has brought to light the deficits in our knowledge of this species. This applies not only to its toxin-producing ability 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 *Alexandrium andersonii* may comprise toxic and non-toxic strains, depending on its location, the main objective of this study was to assess the potential for variability within this species, with particular focus on the toxicological and toxinological aspects. 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. Materials and methods

2.1. Alexandrium andersonii *isolation and culture*

Alexandrium andersonii culture ICMB222 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 one year to a salinity of 36 and to growth in L1 medium (Guillard and Hargraves, 1993).

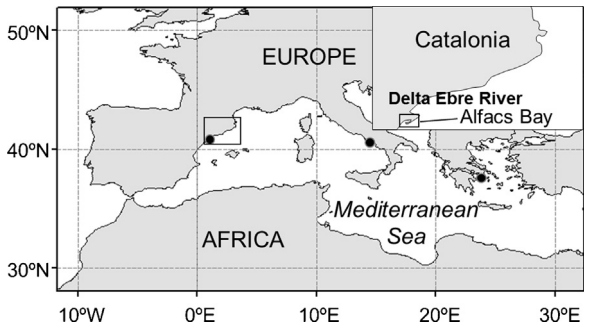

Fig. 1. Map showing the location of Alfacs Bay. Dots show the sites where the Mediterranean strains were isolated.

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 analyzed 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 °C with an irradiance of 80–100 μmol photons m^{-2} s^{-1} in a 12:12 h light/dark cycle. Every 2 days, 2-mL subsamples were removed and then fixed with Lugol's iodine (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 Section 2.8.

Speculating that variations in temperature influenced the toxin content of *Alexandrium* species, we carried out an additional growth experiment at 14 °C (the water temperature during the bloom of *Alexandrium andersonii* in Alfacs Bay). Duplicate cultures of strains VGO664, SZN012, ICMB222, and CCMP1718 were grown at 14 °C 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 was removed (depending on the concentration) during the exponential and stationary phases of growth and filtered on Whatman GF/C glass-fiber filters.

2.3. Growth rate calculation and statistical analyses

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

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

Strain	Sampling location	Clonal	Observations
VGO664	Mediterranean, Greece, Elefsis Bay (Saronikos Gulf)	Yes	Isolated from a vegetative cell
SZN012	Mediterranean, Italy, Naples	No	Isolated from a cyst
ICMB222	Mediterranean, Spain, Catalonia	Yes	Isolated from a vegetative cell
CCMP1718	Atlantic, USA	Yes	Isolated from strain CCMP1597
CCMP1597	Atlantic, USA, Massachusetts, Cape Cod, Eastham, Town Cove	No	Isolated from a cyst

2.4. Light microscopy

Wild cells of *Alexandrium andersonii* were measured using field samples collected in the winter of 2007 from Alfacs Bay and preserved with formaldehyde. The samples were provided by the *Institut de Recerca i Tecnologia Agroalimentaries* (IRTA) from its routine monitoring of Ebre Delta bays.

Species identification was based on the criteria of Balech (1995) together with more recent descriptions (Mackenzie and Todd, 2002; Montresor et al., 2004; Nguyen-Ngoc and Larsen, 2004). The plate tabulation of the cells was examined in detail, following the calcofluor method of Fritz and Triemer (1985). The cells were stained with calcofluor white M2R (Sigma–Aldrich Co., St. Louis, MO, USA) and examined under a Leica DM IRB (Leica Microsystems GmbH, Wetzlar, Germany) inverted microscope with epifluorescence (lamp 50 W).

Alexandrium andersonii abundances in the growth experiments were estimated with Sedgewick-Rafter chambers, counting either 20 μL or 100 cells.

2.5. Measures and statistical analyses

The length and width of 30 cells of every strain were measured using ProgRes capturePro v 2.1 software. The cells were removed during the exponential growth phase and fixed with Lugol's (1% final concentration). Thirty cells from field samples were likewise examined. Since the variables (length and width) were not distributed normally (as determined by Kolmogorov–Smirnov and Shapiro–Wilk tests), only non-parametric statistical analyses were applied, using PRIMER 6 (Windows XP) software. A one-way analysis of similarities (ANOSIM) was performed; the corresponding pairwise tests were based on 99,999 permutations.

2.6. DNA extraction, PCR amplification, sequencing, and phylogenetic analyses

Cultures of *Alexandrium andersonii* were collected during the exponential growth phase by filtration on 3-μm pore size Isopore membrane filters (Millipore). DNA was extracted and purified as described in Penna et al. (2005). Nuclear-encoded 5.8S rDNA and ITS regions were PCR-amplified as described in Penna et al. (2008). Genomic DNA (1 ng) was amplified in a 50-μL reaction mix containing 50 μM each of dATP, dTTP, dCTP, and dGTP, 0.4 μM of each primer, 4 mM $MgCl_2$, 1× reaction buffer (Diatheva, Fano, Italy), and 1.0 U of Hot Rescue DNA polymerase (Diatheva, Fano, Italy). PCR of the LSU rDNA regions (D1/D2) was carried out in a 50-μL reaction mix containing 1 μL of genomic DNA, 0.25 μM of each primer (D1R and D2C by Scholin et al., 1994), 600 μM of each dNTP, 3 μL $MgCl_2$ (25 mM), 1× reaction buffer (Qiagen, USA, including 1.5 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: a 10-min initial denaturation at 95 °C and then 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. For LSU rDNA, an initial denaturation at 95 °C for 5 min was followed by 40 cycles of 20 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, with a final elongation step of 10 min at 72 °C. 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 as 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 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

Table 2
EMBL-EBI ENA accession numbers of the ITS 5.8S rDNA sequences and the sampling 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	NW Atlantic	CCMP1718	HE574400
A. andersonii	NW Atlantic	CCMP1597	HE574399
A. affine	Alboran Sea	PA8V	AJ632095
A. cf. *catenella* (TA clade)	Catalan Sea	CSIC-C7	AJ580322
A. cf. *kutnerae* (ME clade)	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	N Atlantic	AL4V	AM292310
A. minutum	Tyrrhenian Sea	CNR-AMIA2PT	AJ312945
A. peruvianum	Catalan Sea	AM10C	AM237340
A. pseudogoniaulax	Catalan Sea	VGO655	AM237416
A. tamarense	N Atlantic clade	HI38	n.r.
A. tamarense (WE clade)	NE Atlantic	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

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

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.hal.2013.02.003.

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

2.7. Pigment analyses

Cultures were examined by light microscopy prior to HPLC pigment analysis to ensure the health and intact morphology of the cells. Three hours into the 12-h light cycle, the cells were harvested from exponentially growing cultures by filtering variable volumes (10–36 mL) of the cultures onto Whatman GF/F filters (Whatman International Ltd., UK) under reduced pressure. The filters were frozen immediately at −25 °C and processed within 12 h. Cells trapped on the frozen filters were extracted under low light in Teflon-lined screw-capped tubes with 5 mL of 90% acetone, using a stainless steel spatula to grind the filters. The contents of the tubes were chilled in a beaker of ice, sonicated for 5 min in an ultrasonic bath, and then filtered through syringe filters with a 25-mm diameter (MFS HP020, 0.20-μm pore size, hydrophilic PTFE) to remove cell and filter debris. A 0.5-mL aliquot of the acetone extract was mixed with 0.2 mL of water, and 200 μL were injected immediately. This protocol avoids peak distortion by early-eluting peaks (Zapata and Garrido, 1991) and prevents the loss of non-polar pigments prior to injection of the extract in an HPLC system (Latasa et al., 2001). Pigments were separated following a previously described method (Zapata et al., 2000) on a C8 Waters Symmetry column (150 mm × 4.6 mm, 3.5-μm particle-size, 10-nm pore size) using a Waters Alliance HPLC system (Waters Corp., Milford, MA) consisting of a 2695 separation module, a Waters 996 diode-array detector, and a Waters 474 scanning fluorescence detector (excitation: 440 nm, emission: 650 nm). Eluent A consisted of methanol:acetonitrile:0.025 M aqueous pyridine (50:25:25, v/v/v), and eluent B of methanol:acetonitrile:acetone (20:60:20, v/v/v). The elution gradient (time: %B) was as follows: t_0 = 0%, t_{22} = 40%, t_{28} = 95%, t_{37} = 95%, and t_{40} = 0%. The flow rate was 1.0 mL min^{-1} and the column temperature was 25 °C. The solvents were HPLC grade (Romil-SpSTM); pyridine was reagent grade (Merck, Darmstadt, Germany). Pigments were identified either by co-chromatography with authentic standards obtained from SCOR reference cultures or by diode-array spectroscopy (Zapata et al., 2000). After the confirming the purity of the peaks, we compared the spectral information with a library of chlorophyll and carotenoid spectra from pigments prepared from standard phytoplankton cultures (SCOR cultures, see Jeffrey and Wright, 1997).

2.8. Toxicity and toxin analyses

Toxin analyses by HPLC as well as the toxicity assays (mouse bioassay, hemolytic and cytotoxicity tests) were carried out using extracts prepared from the five strains, following their culture, without replicates, in 5.5-L Pyrex bottle with aeration. The cultures were maintained in the same medium and at the same salinity, temperature, and photoperiod as described for the first growth experiment but with a slightly higher light intensity (110 μmol photons m^{-2} s^{-1}). Two-mL aliquots were sampled every 1–4 days and fixed with Lugol's for quantification in order to determine the growth phase of the cultures. During late exponential phase (on different days, depending on the culture), approximately 4 L of every culture was filtered under reduced pressure onto Whatman GF/C glass-fiber filters that had been previously heated in an oven at 450 °C for a minimum of 4 h. The filters were frozen immediately at −20 °C until needed. The extraction for PSP toxin determination was performed with 0.1 M HCl following the official method (AOAC, 2000). The same methodology, but substituting phosphate-buffered saline (PBS) for 0.1 M HCl, was used to obtain extracts of possible hemolytic proteins.

2.8.1. HPLC-FLD analysis of PSP toxins

All chemicals and solvents used were of HPLC or analytical grade. Standard solutions of GTX4,1, dcGTX2,3, GTX2,3, STX, neoSTX, and decarbamoil saxitoxin (dcSTX) were purchased from the Institute for Marine Bioscience, National Research Council, Certified Reference Material Program (NRC-CRM), Halifax, Canada.

HPLC-FLD with post-column derivatization was performed according to Franco and Fernandez (1993). The HPLC system consisted of a Waters 474 fluorescence detector (excitation: 330 nm, emission: 390 nm), a Waters 717 automatic injector, a Waters 600 HPLC pump to deliver the mobile phases, and two Waters 510 pumps to deliver the post-column reagents. Separation was achieved with an Agilent 5μ Lichrosphere 100 RP18 cartridge (125 mm × 4.6 mm). The column temperature was 30 °C and the flow rate was 0.8 mL min^{-1}. The mobile phases consisted of 1.5 mM octansulfonic in 10 mM ammonium phosphate (pH 7) for GTX separations and 95% of this eluent solution plus 5% acetonitrile for the separation of 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 its passage through a Teflon coil (7 m × 0.05 mm i.d.) heated to 65 °C, and finally acidified with 0.5 M acetic acid (0.4 mL min^{-1}) before it entered 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 °C 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 interferences in the HCl extract, prior to the analysis, an aliquot of the acidic *Alexandrium andersonii* extract (see Section

2.8.3) was further purified using solid-phase extraction (SPE) cartridges (C18 AccuBond II, 500 mg, 3 mL). The SPE cartridges were 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 *Alexandrium andersonii* extracts (Cañete and Diogène, 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. 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), 1 mM calcium chloride, and 1 mM boric acid in PBS pH 7.0. A diluted erythrocyte stock suspension without ouabain was prepared to a final concentration of $\sim 1.7 \times 10^7$ erythrocytes mL^{-1} as described by Riobó et al. (2008).

Two Whatman GF/C filters from each sample were used 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 0.1 M HCl. One aliquot of this acidic extract was dried under a N_2 stream at 60 °C and then dissolved in hemolysis buffer.

Triplicate samples made up of 5 mL of the blood-cell suspensions and 5 mL of the toxin-containing solution were combined in centrifuge tubes, incubated at 37 °C for 6 h, and then maintained at room temperature for an additional 18 h. At intervals of 1, 5, 6, and 24 h, 1-mL aliquots of the mixture were transferred to Eppendorf vials, which were centrifuged for 10 min at 1500 rpm at a temperature of 10 °C. Two hundred μL aliquots of each supernatant were then added 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).

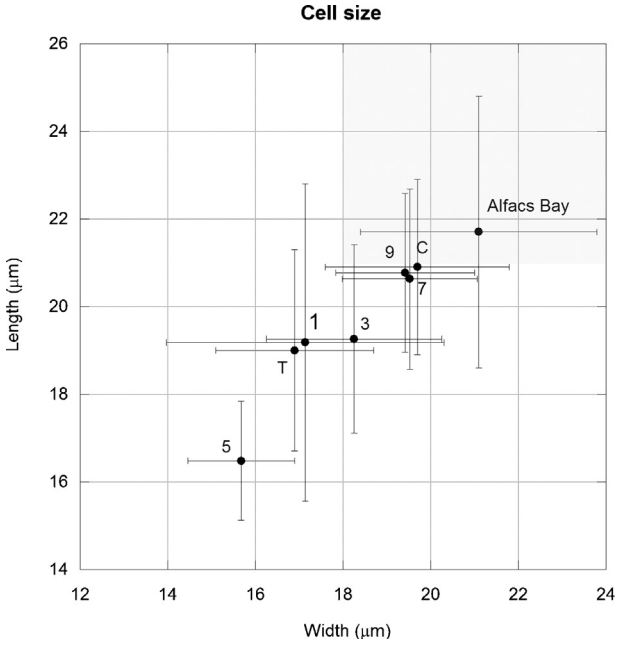

Fig. 3. Mean and standard deviation of the measurements of 30 cells from each of the five strains of *A. andersonii* (1, VGO664; 3, SZN012; 5, ICMB222; 7, CCMP1718; 9, CCMP1597) and from cells in natural samples from Alfacs Bay. Measurements from other authors have been included (C, Ciminiello et al., 2000; T, Touzet et al., 2008). Gray square shows the minimum width and length ranges described in Balech (1990).

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

3. Results

3.1. Morphology and measurements

The morphology of *Alexandrium andersonii* from Alfacs Bay of the Ebre Delta (Fig. 2) generally well matched the description of Balech (1990), except that the wild cells were 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 Fig. 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

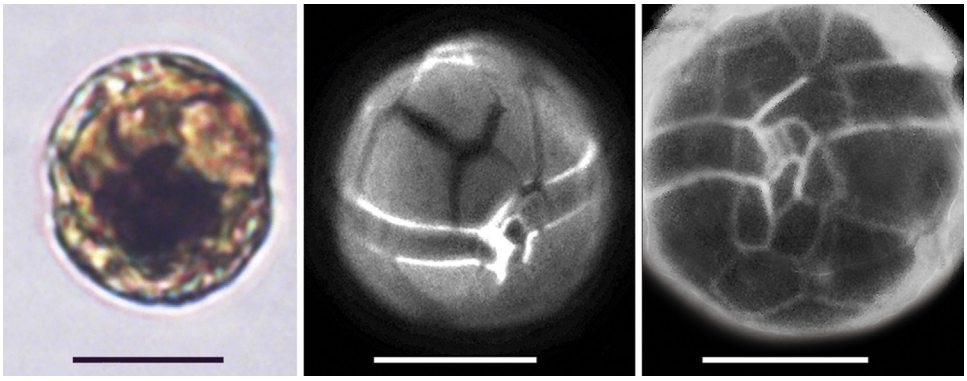

Fig. 2. Micrographs of an *Alexandrium andersonii* cell observed using light (A) and epifluorescence (B), (C) microscopy. Scale bars, 10 μm.

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 (CCMP1718 and CCMP1597).

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). *Alexandrium*

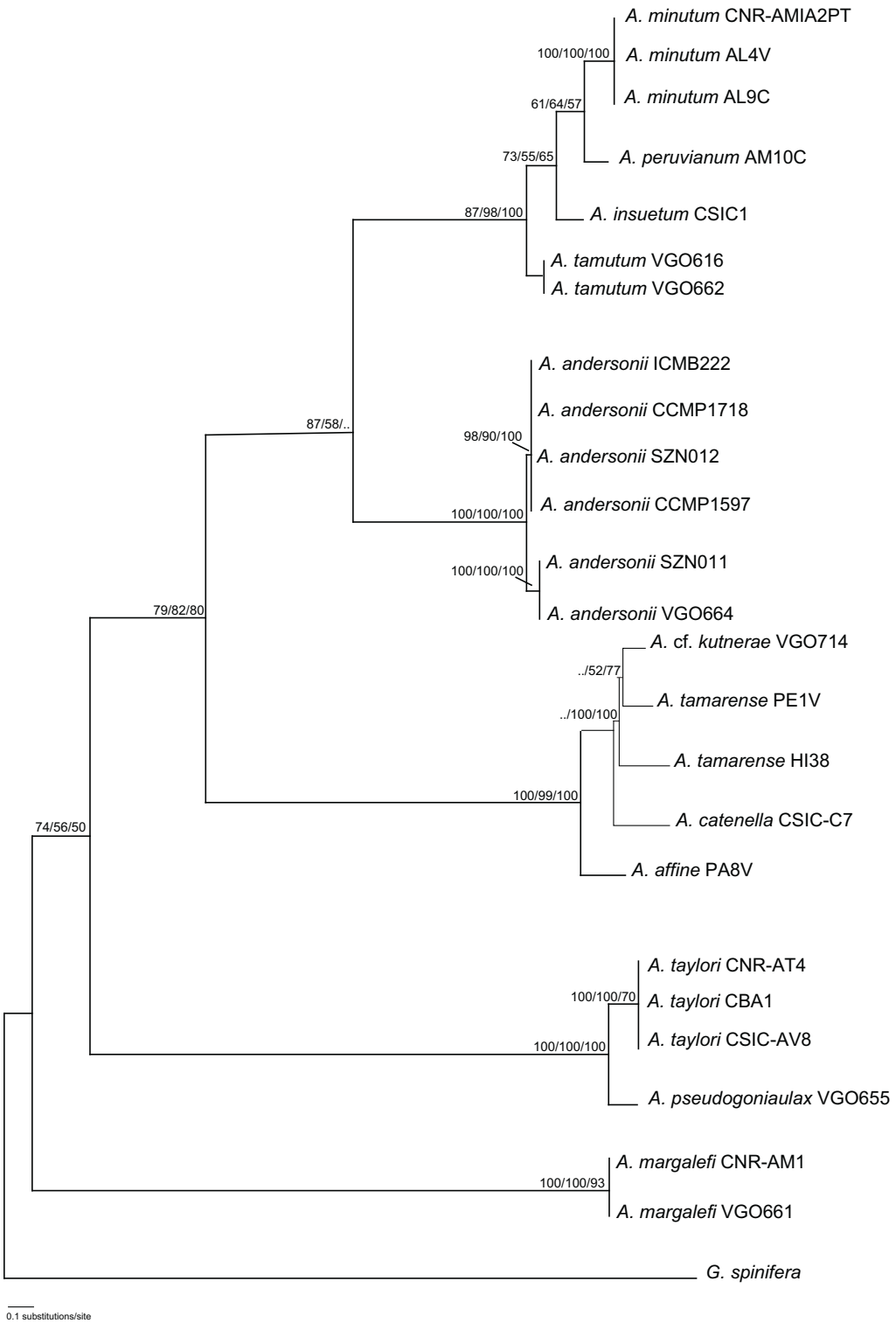

Fig. 4. Maximum-likelihood (ML) tree inferred from the alignment of ITS 5.8S rDNA sequences. Numbers on the major nodes represent, from right to left, neighbor-joining (1000 pseudoreplicates), maximum-parsimony (1000 pseudoreplicates), and ML (1000 pseudoreplicates) bootstrap values. Only bootstrap values >50% are shown. The tree was rooted using *Goniaulax spinifera* (AF051832) as outgroup.

Table 3
Range of growth rates, durations of the exponential growth phase, and maximum cell abundances of the different strains of *A. andersonii* cultured at 14 or 20 °C.

Strain	Temperature (°C)	Growth rate (days^{-1})	Duration of exponential phase (days)	Maximum cell abundances (cell L^{-1})
VGO664	20	0.11–0.15	21/28	2.45E+07
SZN012	20	0.17–0.18	21	3.54E+07
ICMB222	20	0.10	33/35	2.76E+07
CCMP1718	20	0.16–0.17	21/23	3.91E+07
CCMP1597	20	0.12	30	3.21E+07
VGO664	14	0.08–0.09	19	5.43E+06
SZN012	14	0.24–0.31	10	1.03E+07
ICMB222	14	0.05–0.08	19	4.40E+06
CCMP1718	14	0.12–0.13	21	8.86E+06

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 *Alexandrium minutum*/*Alexandrium tamutum* group within the genus *Alexandrium*.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.hal.2013.02.003.

3.3. Growth rates

The growth rates and maximum cell densities reached by the five *Alexandrium andersonii* strains at 20 °C and 14 °C 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 °C or at 14 °C (Kruskal–Wallis test, $p = 0.1212$ and $p = 0.0916$, respectively). The growth rates were more

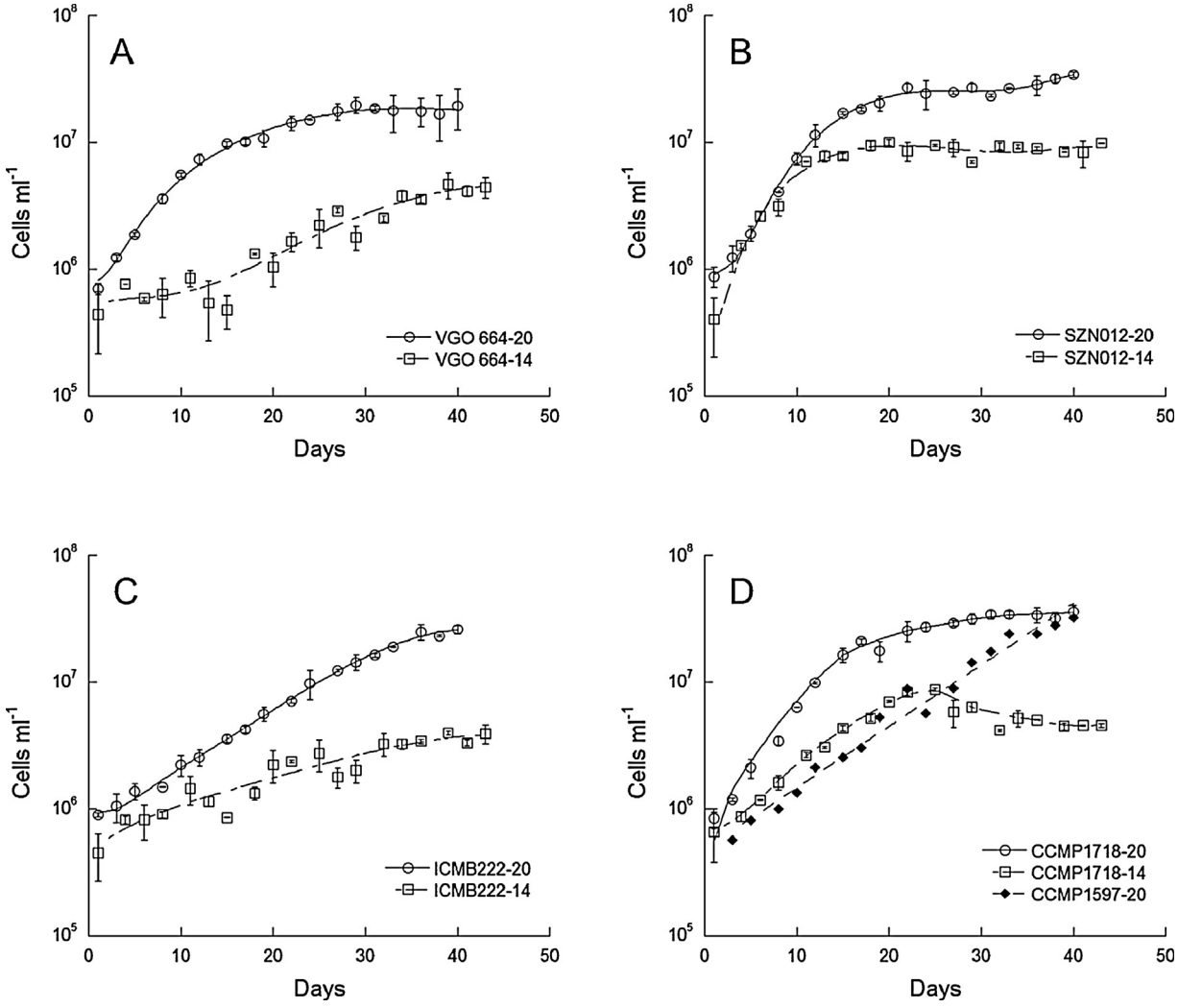

Fig. 5. Growth curves of the different strains of *A. andersonii* cultured at 20 °C (circles or rhombus) and at 14 °C (squares) in L1 medium. For illustrative purposes, the data were fitted to a curve. Bars represent the standard deviation of the duplicates.

Table 4
°C. Per, peridinin; chl *a*, chlorophyll *a*; chl c_2, chlorophyll c_2; Diadino, diadinoxanthin; Dino, dinoxanthin.

Strain	Per/chl *a* ± SD	Per/c_2 ± SD	chl c_2/chl *a* ± SD	Diadino/chl *a* ± SD	Dino/chl *a* ± SD
VGO664	1.04 ± 0.01	4.93 ± 0.10	0.21 ± 0	0.23 ± 0	0.09 ± 0.01
SZN012	0.90 ± 0.01	4.43 ± 0	0.20 ± 0	0.23 ± 0	0.08 ± 0
ICMB222	0.83 ± 0.01	4.14 ± 0.57	0.20 ± 0.03	0.21 ± 0.02	0.07 ± 0.01
CCMP1718	0.76 ± 0.06	3.03 ± 0.11	0.25 ± 0.02	0.23 ± 0.01	0.10 ± 0.01
CCMP1597	0.46	1.91	0.24	0.21	0.08

homogeneous (0.10–0.18) at 20 °C than at 14 °C, whereas temperature had no significant effect (non-parametric paired sign tests).

The maximum cell densities reached by each culture differed between strains but not significantly, with a maximum of 3.9×10^7 cells L^{-1} achieved by strain CCMP1718 at 20 °C. Significantly higher maximum densities were obtained at 20 °C than at 14 °C (non-parametric paired sign tests). At 14 °C, only strain SZN012 grew to reach densities $>10^7$ cells L^{-1}.

Different patterns of growth were observed for the five strains. Thus, strains ICMB222 and CCMP1597 (for strain CCMP1597 replicates were not available) had lower growth rates and longer exponential phases (Fig. 5C and D), while strains SZN012 and CCMP1718 grew faster, with shorter exponential phases (Fig. 5B and D). For strain VGO664, temperature-specific differences were noted. At 14 °C, this strain grew slowly, after a long lag phase, whereas at 20 °C growth was more rapid (Fig. 5A).

3.4. Pigment analyses

Alexandrium andersonii is a typical peridinin-containing dinoflagellate and the pigment composition of all five strains was similar, with peridinin as the major carotenoid. However, there were slight differences in the pigment ratios with respect to chlorophyll (chl) *a* (Table 4). Specifically, the ratio of peridinin to either chl *a* or chl 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 CCMP1597 (for strain CCMP1597 replicates were not available), isolated from the west 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 *Alexandrium andersonii* cultured at either 20 °C or 14 °C. 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 represent interfering fluorescent compounds (see the example in Fig. 6).

3.5.2. Cytotoxicity assay

All of the *Alexandrium 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}.

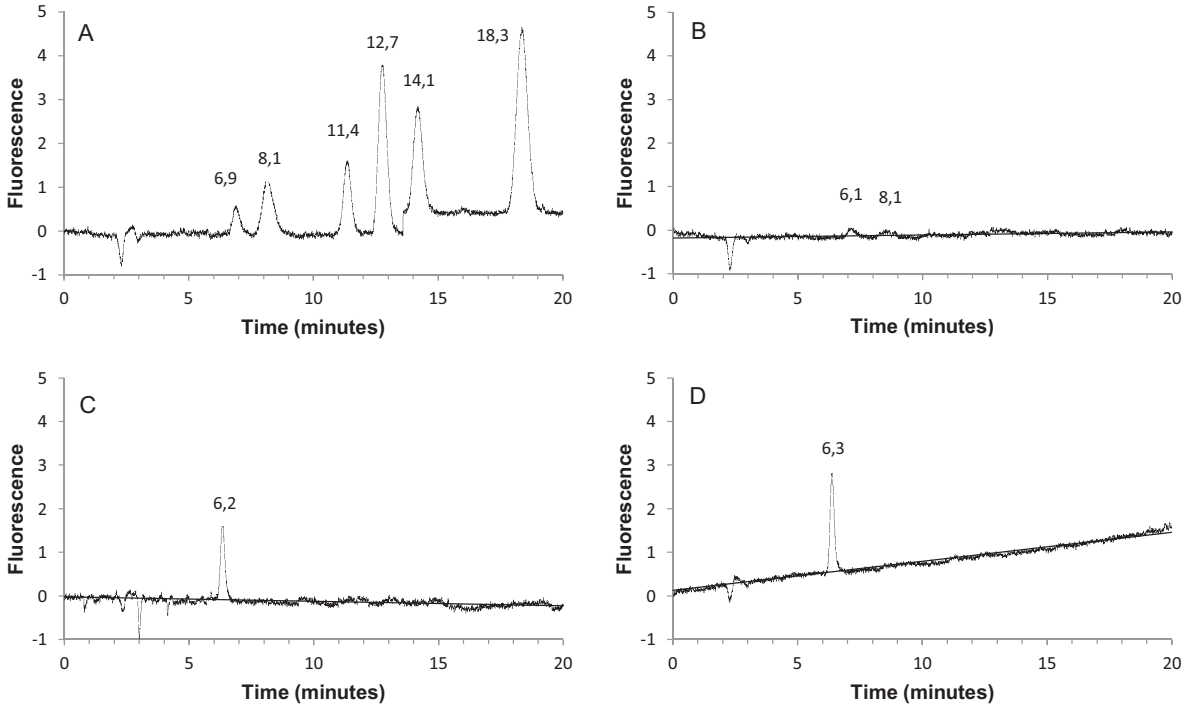

Fig. 6. HPLC-FLD chromatograms of stationary phase cells of strain SZN012 cultured at 14 °C. (A) SZN012 cells + GTXs standard with oxidant; (B) SZN012 cells + GTXs standard without oxidant; (C) SZN012 cells with oxidant; (D) SZN012 cells without oxidant. Retention times (min) of GTXs: GTX4: 6.9; GTX1: 8.1; dcGTX3: 11.4; dcGTX2: 12.7; GTX3: 14.1; GTX2: 18.3.

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

Strain	Cells (mL culture)$^{-1}$	μg STX eq. (mL culture)$^{-1}$	pg STX eq. × cells^{-1}
VGO664	34,692	1.99	57.3
SZN012	27,000	3.971	147
ICMB222	42,870	0.858	20
CCMP1718	35,320	0.478	13.5
CCMP1597	20,857	2.178	104

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

Strain	Time until death (h)		Comments
VGO664	–	–	Alive (48 h)
SZN012	30	48	
ICMB222	–	–	Alive (48 h)
CCMP1718	–	–	Alive (48 h)
CCMP1597	10	48	
VGO664X5	2	2	Death probably
ICMB222X6	1.5	2	due to excess salt

3.5.3. Hemolytic activity

All of the tested strains showed hemolytic activity (Table 6). After 1 h of incubation, the absorbances of the PBS-extracted samples were indicative of hemolysis, which reached 100% after 5 h. While the HCl extracts were also positive, the response was slightly different: intact erythrocytes were detected after 1 h, ruptured red cells after 5 h, and 100% hemolysis after 24 h of incubation. Therefore, hemolytic activity was characteristic of all the strains. The positive result obtained with the PBS extracts suggests the involvement of proteinaceous compounds, while that obtained with the HCl extracts is consistent with the presence of 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 exhibited the appropriate symptoms. Mice injected with extracts from strains SZN012 and CCMP1597 died at various times, as detailed in Table 7. Following injection with the HCl extracts from these strains, 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 VGO664 and SZN012 were concentrated five- and six-fold, respectively, prior to their injection, in which case the mice died approximately 2 h later but still did not exhibit PSP-like symptoms.

4. Discussion

4.1. Morphology and measurements

In this study, the morphology and cell sizes of five cultured strains of *Alexandrium andersonii* (see Table 1) were compared with the corresponding features of wild cells and those of the culture described by Balech. Our measurements broaden the lower limits of the length and width ranges of that original description (21–35 μm length, 18–33 μm width), as cells 13.5–35 μm in length and 12–33 μm in width were observed. In fact, the cells of the different *A. andersonii* strains used in this study were smaller than those obtained by Balech. Moreover, the sizes of the field cells were within the lowest end of the range reported in that original description.

By contrast, for the five strains, our measurements were more in line with those of Touzet et al. (2008), Frangopulos et al. (2004), and Ciminiello et al. (2000).

In this study, while the differences in the sizes of the five strains were significant, the largest specimens were found in the wild samples collected from Alfacs Bay. These results, in addition to the fact that the strain analyzed by Balech (1990) was CCMP1597 [synonym of GTM242, used in Anderson et al. (1990)], suggest a reduction in the size of cultured cells.

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

Strain	Extraction solvent	37 °C 1 h (%)	37 °C 5 h (%)	37 °C 6 h (%)	Room temp. 24 h (%)
VGO664	PBS	11	100	100	
SZN012	PBS	85	100	100	
ICMB222	PBS	30	100	100	
CCMP1718	PBS	51	98	96	
CCMP1597	PBS	4	100	100	
VGO664	HCl	0	10	17	40
SZN012	HCl	0	31	48	100
ICMB222	HCl	0	22	37	100
CCMP1718	HCl	1	72	87	99
CCMP1597	HCl	0	16	22	67
Blank filters	HCl	0	0	0	0

4.2. Phylogenetic analyses

The phylogenetic homogeneity of *Alexandrium andersonii* strains based on the 5.8 ITS and LSU regions did not allow us to distinguish among individual populations from different 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 *Alexandrium minutum* in studies carried out at global and Mediterranean scales (Casabianca et al., 2012; McCauley et al., 2009).

4.3. Growth 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 for *Karenia brevis* (Loret et al., 2002). In our study, 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 *Alexandrium andersonii* in response to warm temperatures; rather, the cells have only rarely been detected and only at very low densities ($<$100 cells L^{-1}). In contrast, a bloom of *A. andersonii* occurred in

Table 8
Mouse bioassay of different concentrations of acetic acid.

Acetic acid concentration	Time until death (N = 3)	Comments
4% (0.04 g/mL)	1–2 min	
2% (0.02 g/mL)	15–20 min	
1% (0.01 g/mL)	1 h	
0.1% (0.001 g/mL)	6 h	
Distilled water	–	Alive (48 h)

the winter (from January to April) of 2007 in Alfacs Bay, coinciding with water temperatures between 10 and 15 °C. During that event, cell densities reached a maximum of 7159 cells L^{-1}, recorded on February 19th.

Based on these observations, it seems that at least along the Catalan coast *Alexandrium 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 °C than at 20 °C. 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 stoichiometric proportions in the extrinsic water-soluble peridinin-chl *a*-protein (PCP) and intrinsic PCP (membrane-bound) light-harvesting antennae. Since 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 *Alexandrium andersonii*, our data point to the role of intrinsic and thus probably genetic factors in 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* (CCMP1718, VGO664, and SZN012) and found similar differences in pigment ratios. However, the absolute molar pigment ratios in that study were slightly different than those determined in the present work, most likely due to variable culture conditions (e.g., light and salinity).

4.5. Toxicity and toxin analyses

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 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 that interact with VGSCs, 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). Consequently, this assay may overestimate toxin concentrations. Thus, an interpretation of our results must include the consideration that the low toxicity detected was not due to PSP toxins. Similarly, the hemolytic activity of the different strains could have been due not only to STX and its derivatives but also to an excess of salt or/and to a hemolytic toxin, as reported for other *Alexandrium* species (Emura et al., 2004; Simonsen et al., 1995). As an alternative explanation of our findings, the amount of toxicity may have been so low that it was not detected either by the mouse bioassay or by the HPLC method, which have limits of detection of 0.3 μg STX eq. mL^{-1} and 0.01–0.15 μg STX eq. mL^{-1}, respectively, whereas it was detectable in the neuro-2a assay (detection limit of 0.91–58.41 nM, or 0.27–17.5 × 10^{-3} μg STX eq. mL^{-1}) (Cañete and Diogène, 2008). However, the first explanation is the more likely one since the toxicity of the strains as determined in the cytotoxicity assay was clearly higher than the detection limit of the HPLC method (0.01–0.15 μg STX eq. mL^{-1}), which therefore should have yielded a positive result.

Ciminiello et al. (2000) and Frangopulos et al. (2004) worked with the same Neapolitan strain used in this study, culturing the cells at the same (Ciminiello et al., 2000) or similar temperature (18 °C) (Frangopulos et al., 2004). However, both groups used K medium whereas our strains were cultured in L1 medium. The main difference between these two culture media is the presence of Ni, V, and Cr in L1 medium but not in K medium, which contains ammonium, as well as differences in the concentrations of EDTA and Mo. Changes in toxin production in response to different concentrations of trace metals in the culture medium have been described for toxic cyanobacteria, diatoms, and dinoflagellates (Lukač and Aegerter, 1993; Maldonado et al., 2002; Rhodes et al., 2006). Likewise, differences in light intensity have been shown to influence the toxin content of *Alexandrium* species (Hamasaki et al., 2001; Wang and Hsieh, 2005). In our study, the irradiation intensity was the same as that used by Ciminiello et al. (2000) but approximately double that in the experiments of Frangopulos et al. (2004). Another explanation for the toxicity of the Neapolitan strain determined by these two authors but not in our hands could be a loss of toxicity, as reported for other *Alexandrium* species (Martins et al., 2004). Nevertheless, while differences in per-cell toxicity have been described for the same strain depending on the culture conditions (Anderson et al., 1990; Boczar et al., 1988; Hwang and Lu, 2000), differences in the toxin profile of a strain, based on a comparison with the results of Ciminiello et al. (2000) and Frangopulos et al. (2004), are unusual. Indeed, significant shifts tend to occur only in batch and semi-continuous cultures in response to extreme changes in the growth regime (Anderson et al., 2012).

In addition, the toxin profile of *Alexandrium andersonii* described by Ciminiello et al. (2000) consists mainly of STX and neoSTX. To our knowledge this toxin composition has not been reported for other *Alexandrium* species. In contrast, the profile obtained by Frangopulos et al. (2004) was more typical of PSP toxins, comprising gonyautoxins (GTX1–4), with GTX2 as the dominant component.

The difference between our results and those of the two authors may have been methodological as well. In the work of Ciminiello et al. (2000), the toxins evaluated in the mouse bioassay were extracted by suspending the cultures in an equal volume of acetic acid 0.5 N (0.03 g acetic acid mL^{-1}). Acetic acid is not the solvent used in the official method described in the AOAC (2000) for the preparation of extracts for the mouse bioassay. To assess the validity of this solvent in the extractions, we performed the mouse bioassay using only different concentrations of acetic acid, from 0.1% to 4%, in the complete absence of toxin or other components. The result in all cases was the death of the mice (see Table 8). Similar concentrations of HCl do not kill mice. Therefore, acetic acid is not an appropriate solvent for the mouse bioassay and its use casts doubt on the results obtained by Ciminiello et al. (2000). Furthermore, the chemical methodology used by these authors (NMR and HRMS) is, unfortunately, poorly reproducible by other laboratories. In the study of Frangopulos et al. (2004), the isolates were not analyzed in a mouse bioassay; instead, the presence of toxins was determined by HPLC with fluorescence detection. While this was the same methodology used in the present work, those authors measured a very low mean concentration of total toxins, with a standard error higher than the mean. Methodology, can thus explain the differences between some of our results and those reported in Ciminiello et al. (2000).

A few studies reported in the literature used *Alexandrium andersonii* as the toxic species in ingestion experiments but only in some of them were the toxin profiles analyzed. The *A. andersonii* strains used by Shaber and Sulkin (2007) and by Perez and Sulkin (2005) were obtained as toxic strains of *A. andersonii* from

Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). The latter study used strain CCMP1718, one of the five strains of *A. andersonii* that we examined in this work. In describing its toxicity, the authors cited the CCMP website. However, the CCMP, to the best of our knowledge, has not evaluated their *A. andersonii* cultures for toxicity (J. Sexton, personal communication). Garcia et al. (2011) also used strain CCMP1718 for ingestion experiments. Its toxicity, as verified by CyanoHAB Services, is based on the results of an ELISA, which determined a toxin concentration of 5.1 μg STX eq. L^{-1}. Both Perez and Sulkin (2005) and Garcia et al. (2011) reported the accelerated mortality of larval *Hemigrapsus oregonensis* when fed *A. andersonii* cells, but according to the latter authors the results could not 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 these genes (specifically *sxtA1* and *sxtA4*) in *Alexandrium 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, neither of the two official methodologies for the detection of PSP toxins used in our study, i.e., HPLC and mouse bioassay, yielded evidence of their production, thus supporting the results of those authors.

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 to STX (or to its derivatives). In any case, under the study conditions only trace amounts of toxins were produced by the tested strains of *Alexandrium andersonii*. Therefore, based on our current knowledge, we do not consider *A. andersonii* as a species that is toxic to humans.

4.6. Aquaculture implications

In this study, the non-toxic behaviors of both the Neapolitan strain of *Alexandrium 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, *Alexandrium minutum*, which is very common along the Catalan coast and whose toxicity is well established (Delgado et al., 1990; Van Lenning et al., 2007), was also present during that bloom period (Sampedro et al., 2007).

Hemolytic substances produced by some phytoplankton species (such as *Karenia mikimotoi*) have been suggested as the cause of fish and invertebrate damages and mortalities ascribed to bloom events (Igarashi et al., 1996; Yasumoto et al., 1990). Moreover, the hemolytic activity associated with *Phaeocystis pouchetii* has been positively correlated with water temperature (Van Rijssel et al., 2007).

While in the present work *Alexandrium andersonii* strains exhibited hemolytic activity, during the low-density bloom that occurred in Alfacs Bay in 2007 there was no obvious evidence of toxicity to either fish or invertebrates. Future investigations of the hemolytic substances found in *A. andersonii* are needed to determine whether these compounds are harmful to marine fauna.

5. Conclusion

Our experiments show that *Alexandrium andersonii* strains originating from different locations differ markedly in their size, several of their pigment ratios, and in 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 the parent strain of this species (CCMP1718 and CCMP1597, respectively). Moreover, strains cultured under the same laboratory conditions did not express PSP toxins, as determined by HPLC and in a mouse bioassay.

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References

Alpermann, T.J., Tillmann, U., Beszteri, B., Cembella, A.D., John, U., 2010. Phenotypic variation and genotypic diversity in a planktonic population of the toxigenic marine dinoflagellate *Alexandrium tamarense* (Dinophyceae). Journal of Phycology 46 (1), 18–32.

Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M., 2012. The globally distributed genus *Alexandrium*: multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae 14, 10–35.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Marine Biology 104 (3), 511–524.

AOAC, 2000. Official Method 959.08. Paralytic Shellfish Poison, Biological Method. Final Action. AOAC Official Methods of Analysis, 17th ed., Gaithersburg, MD, USA.

Bachvaroff, T.R., Adolf, J.E., Place, A.R., 2009. Strain variation in *Karlodinium veneficum* (Dinophyceae): toxin profiles, pigments, and growth characteristics. Journal of Phycology 45 (1), 137–153.

Balech, E., 1990. Four new dinoflagellates. Helgoländer Meeresunters 44, 387–396.

Balech, E., 1995. The Genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine Station, Cork Special Publication.

Boczar, B.A., Beitler, M.K., Liston, J., Sullivan, J.J., Cattolico, R.A., 1988. Paralytic shellfish toxins in *Protogonyaulax tamarensis* and *Protogonyaulax catenella* in axenic culture. Plant Physiology 88, 1285–1290.

Calbet, A., Bertos, M., Fuentes-Grünewald, C., Alacid, E., Figueroa, R., Renom, B., Garcés, E., 2011. Intraspecific variability in *Karlodinium veneficum*: growth rates, mixotrophy, and lipid composition. Harmful Algae 10 (6), 654–667.

Cañete, E., Diogène, J., 2008. Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma × glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins. Toxicon 52 (4), 541–550.

Casabianca, S., Penna, A., Pecchioli, E., Jordi, A., Basterretxea, G., Vernesi, C., 2012. Population genetic structure and connectivity of the harmful dinoflagellate *Alexandrium minutum* in the Mediterranean Sea. Proceedings of the Royal Society B: Biological Sciences 279 (1726), 129–138.

Catterall, W.A., Cestèle, S., Yarov-Yarovoy, V., Yu, F.H., Konoki, K., Scheuer, T., 2007. Voltage-gated ion channels and gating modifier toxins. Toxicon 49 (2), 124–141.

Ciminiello, P., Fattorusso, E., Forino, M., Montresor, M., 2000. Saxitoxin and neosaxitoxin as toxic principles of *Alexandrium andersonii* (Dinophyceae) from the Gulf of Naples, Italy. Toxicon 38 (12), 1871–1877.

Delgado, M., Estrada, M., Camp, J., Fernández, J.V., Santmartí, M., Lletí, C., 1990. Development of a toxic *Alexandrium minutum* Halim (Dinophyceae) bloom in the harbour of Sant Carles de la Rápita (Ebro Delta, northwestern Mediterranean). Scientia Marina 54 (1), 1–7.

EC, 2007. Commission Regulation (EU) No. 1244/2007 amending Regulation (EC) No. 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and laying down specific rules on official controls for the inspection of meat. Official Journal of the European Union L281/12.

Edwards, D.J., Marquez, B.L., Nogle, L.M., McPhail, K., Goeger, D.E., Roberts, M.A., Gerwick, W.H., 2004. structure and biosynthesis of the jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula*. Chemistry and Biology 11 (6), 817–833.

Emura, A., Matsuyama, Y., Oda, T., 2004. Evidence for the production of a novel proteinaceous hemolytic exotoxin by dinoflagellate *Alexandrium taylori*. Harmful Algae 3 (1), 29–37.

Etheridge, S.M., Roesler, C.S., 2005. Effects of temperature, irradiance, and salinity on photosynthesis, growth rates, total toxicity, and toxin composition

for *Alexandrium fundyense* isolates from the Gulf of Maine and Bay of Fundy. Deep Sea Research Part II: Topical Studies in Oceanography 52 (19–21), 2491–2500.
Fernández-Tejedor, M., Delgado, M., Vila, M., Sampedro, N., Camp, J., Furones, D., Diogene, J., 2007. Resultados del programa de seguimiento de fitoplancton tóxico y biotoxinas en las zonas de producción de bivalvos de Cataluña: años 2003–2006 y primer trimestre del 2007. In: Gilabert, J. (Ed.), IX Reunión Ibérica sobre fitoplancton tóxico y biotoxinas, Cartagena, Spain, pp. 37–46.
Franco, J.M., Fernandez, P., 1993. Separation of PSPtoxins by RP-HPLC, with post-column reaction and fluorometric detection. Chromatographia 35, 613–620.
Frangopulos, M., Guisande, C., deBlas, E., Maneiro, I., 2004. Toxin production and competitive abilities under phosphorus limitation of *Alexandrium* species. Harmful Algae 3 (2), 131–139.
Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates. Journal of Phycology 21, 662–664.
Garcia, R., Sulkin, S., Lopez, M., 2011. Effects on larval crabs of exposure to algal toxin via ingestion of heterotrophic prey. Marine Biology 158 (2), 451–460.
Godhe, A., Härnström, K., 2010. Linking the planktonic and benthic habitat: genetic structure of the marine diatom *Skeletonema marinoi*. Molecular Ecology 19 (20), 4478–4490.
Guillard, R.R.L., 1973. Division rates. In: Stein, J.R. (Ed.), Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University Press, New York, pp. 289–312.
Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis inmobilisis* a diatom, not a chrysophyte. Phycologia 32, 234–236.
Hadjadji, I., Masseret, E., Plisson, B., Laabir, M., Cecchi, P., Collos, Y., 2012. Clonal variation in physiological parameters of *Alexandrium tamarense*: implications for biological invasions and maintenance. Cahiers de Biologie Marine 53 (3), 357–363.
Hamasaki, K., Horie, M., Tokimitsu, S., Toda, T., Taguchi, S., 2001. Variability in toxicity of the dinoflagellate *Alexandrium tamarense* isolated from Hiroshima Bay, western Japan, as a reflection of changing environmental conditions. Journal of Plankton Research 23 (3), 271–278.
Hwang, D.F., Lu, Y.H., 2000. Influence of environmental and nutritional factors on growth, toxicity, and toxin profile of dinoflagellate *Alexandrium minutum*. Toxicon 38 (11), 1491–1503.
Igarashi, T., Satake, M., Yasumoto, T., 1996. Prymnesin-2: a potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. Journal of the American Chemical Society 118 (2), 479–480.
Jacob, R.B., McDougal, O.M., 2010. The M-superfamily of conotoxins: a review. Cellular and Molecular Life Sciences 67, 17–27.
Jeffrey, S.W., Wright, S.W., 1997. Qualitative and quantitative HPLC analysis of SCOR reference algal cultures. In: Jeffrey, S.W., Mantoura, R.F.C., Wright, S.W. (Eds.), Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods. UNESCO Publishing, Paris, pp. 343–360.
Kim, C.-H., Sako, Y., Ishida, Y., 1993. Comparison of toxin composition between populations of *Alexandrium* spp. from geographically distant areas. Nippon Suisan Gakkaishi 59 (4), 641–646.
Kogure, K., Tamplin, M.L., Simidu, U., Colwell, R.R., 1988. A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. Toxicon 26 (2), 191–197.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., Mcgettigan, P.A., Mcwilliam, H., Valentin, F., Fallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J.D.G.H., 2007. ClustalW2 and ClustalX version 2. Bioinformatics 23, 2947–2948.
Latasa, M., Van Lenning, K., Garrido, J.L., Scharek, R., Estrada, M., Rodriguez, F., Zapata, M., 2001. Losses of chlorophylls and carotenoids in aqueous acetone and methanol extracts prepared for RPHPLC analysis of pigments. Chromatographia 53, 385–391.
Lilly, E.L., Halanych, K.M., Anderson, D.M., 2005. Phylogeny, biogeography, and species boundaries within the *Alexandrium minutum* group. Harmful Algae 4 (6), 1004–1020.
Lim, P.-T., Leaw, C.-P., Usup, G., Kobiyama, A., Koike, K., Ogata, T., 2006. Effects of light and temperature on growth, nitrate uptake, and toxin production of two tropical dinoflagellates: *Alexandrium tamiyavanichii* and *Alexandrium minutum* (Dinophyceae). Journal of Phycology 42 (4), 786–799.
Loret, P., Tengs, T., Villareal, T.A., Singler, H., Richardson, B., Mcguire, P., Morton, S., Busman, M., Campbell, L., 2002. Short communication. No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. Journal of Plankton Research 24 (7), 735–739.
Lukač, M., Aegerter, R., 1993. Influence of trace metals on growth and toxin production of *Microcystis aeruginosa*. Toxicon 31 (3), 293–305.
Mackenzie, L., Todd, K., 2002. *Alexandrium camurascutulum* sp. nov. (Dinophyceae): a new dinoflagellate species from New Zealand. Harmful Algae 1, 295–300.
Mackenzie, L., White, D., Oshima, Y., Kapa, J., 1996. The resting cyst and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in New Zealand. Phycologia 35 (2), 148–155.
Maldonado, M.T., Hughes, M.P., Rue, E.L., Wells, M.L., 2002. The Effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*. Limnology and Oceanography 47 (2), 515–526.
Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., Wekell, M.M., 1993. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Analytical Biochemistry 214, 190–194.
Martins, C.A., Kulis, D., Franca, S., Anderson, D.M., 2004. The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone. Toxicon 43 (2), 195–205.
McCauley, L.A.R., Erdner, D.L., Nagai, S., Richlen, M.L., Anderson, D.M., 2009. Biogeographic analysis of the globally distributed harmful algal bloom species *Alexandrium minutum* (Dinophyceae) based on rRNA gene sequences and microsatellite markers. Journal of Phycology 45 (2), 454–463.
Moestrup, Ø., Akselman, R., Cronberg, G., Elbraechter, M., Fraga, S., Halim, Y., Hansen, G., Hoppenrath, M., Larsen, J., Lundholm, N., Nguyen, L.N., Zingone, A. (Eds.), 2009 onwards. IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae. Available online at http://www.marinespecies.org/HAB (accessed on 02.04.12).
Montresor, M., John, U., Beran, A., Medlin, L.K., 2004. *Alexandrium tamutum* sp. nov. (Dinophyceae): a new nontoxic species in the genus *Alexandrium*. Journal of Phycology 40 (2), 398–411.
Montresor, M., Zingone, A., Sarno, D., 1998. Dinoflagellate cyst production at a coastal Mediterranean site. Journal of Plankton Research 20 (12), 2291–2312.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65 (1–2), 55–63.
Navarro, J.M., Muñoz, M.G., Contreras, A.M., 2006. Temperature as a factor regulating growth and toxin content in the dinoflagellate *Alexandrium catenella*. Harmful Algae 5, 762–769.
Nguyen-Ngoc, L., Larsen, J., 2004. Gonyaulacales. In: Larsen, J., Nguyen-Ngoc, L. (Eds.), Potentially Toxic Microalgae of Vietnamese Waters. Opera Botanica 140. Council for Nordic Publications in Botany, Copenhagen, pp. 73–116.
Ogata, T., Ishimaru, T., Kodama, M., 1987. Effect of water temperature and light intensity on growth rate and toxicity change in *Protogonyaulax tamarensis*. Marine Biology 95 (2), 217–220.
Oh, S.J., Matsuyama, Y., Yoon, Y.H., Miyamura, K., Choi, C.G., Yang, H.S., Kang, I.J., 2010. Comparative analysis of Paralytic Shellfish Toxin content and profile produced by dinoflagellate *Gymnodinium catenatum* isolated from Inokushi Bay, Japan. Journal of the Faculty of Agriculture, Kyushu University 55 (1), 47–54.
Oshima, Y., Blackburn, S.I., Hallegraeff, G.M., 1993. Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. Marine Biology 116 (3), 471–476.
Penna, A., Fraga, S., Battocchi, C., Casabianca, S., Giacobbe, M.G., Riobó, P., Vernesi, C., 2010. A phylogeographical study of the toxic benthic dinoflagellate genus *Ostreopsis* Schmidt. Journal of Biogeography 37 (5), 830–841.
Penna, A., Fraga, S., Maso, M., Giacobbe, M.G., Bravo, I.E.G., Vila, M., Bertozzini, E., Andreoni, F., Luglie, A., Vernesi, C., 2008. Phylogenetic relationships among the Mediterranean *Alexandrium* (Dinophyceae) species based on sequences of 5.8S gene and Internal Transcript Spacers of the rRNA operon. European Journal of Phycology 43 (2), 163–178.
Penna, A., Vila, M., Fraga, S., Giacobbe, M., Andreoni, F., Riobó, P., Vernesi, C., 2005. Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the Western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8S rDNA sequences. Journal of Phycology 41, 212–245.
Perez, M.F., Sulkin, S.D., 2005. Palatability of autotrophic dinoflagellates to newly hatched larval crabs. Marine Biology 146 (4), 771–780.
Pitcher, G.C., 2012. Harmful algae—the requirement for species-specific information. Harmful Algae 14 (0), 1–4.
Rhodes, L., Selwood, A., McNabb, P., Briggs, L., Adamson, J., van Ginkel, R., Laczka, O., 2006. Trace metal effects on the production of biotoxins by microalgae. African Journal of Marine Science 28 (2), 393–397.
Riobó, P., Paz, B., Franco, J.M., Vázquez, J.A., Murado, M.A., 2008. Proposal for a simple and sensitive haemolytic assay for palytoxin: toxicological dynamics, kinetics, ouabain inhibition and thermal stability. Harmful Algae 7, 415–429.
Sampedro, N., Fernández, M., Garcés, E., van Lenning, K., Touzet, N., Fraga, S., Diogene, J., Camp, J., 2007. Primera detección de *Alexandrium andersonii* (dinoflagelado) en la costa catalana. In: IX Reunión Ibérica sobre Fitoplancton Tóxico y Biotoxinas. Cartagena (Poster).
Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group-specific and strain-specific genetic-markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence-analysis of a fragment of the LSU ribosomal-RNA gene. Journal of Phycology 30 (6), 999–1011.
Shaber, K., Sulkin, S., 2007. Feeding on dinoflagellates by intermediate and late stage crab zoeae raised in the laboratory and collected from the field. Journal of Experimental Marine Biology and Ecology 340 (2), 149–159.
Simonsen, S., Moller, B.L., Larsen, J., Ravn, H., 1995. Haemolytic activity of *Alexandrium tamarense* cells. In: Lassus, P., Erard, E.A.P., Gentien, C., Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Technique et Documentation-Lavoisier, Intercept Ltd., Lavoisier, Paris, pp. 513–517.
Stamatakis, A., Ludwig, T., Meier, H., 2005. RAxMLIII: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics 21, 456–463.
Stüken, A., Orr, R.J.S., Kellmann, R., Murray, S.A., Neilan, B.A., Jakobsen, K.S., 2011. Discovery of nuclear-encoded genes for the neurotoxin saxitoxin in dinoflagellates. PLoS ONE 6 (5), e20096.
Swofford, D.L., 2002. Paup*—Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4.0b10. Sinauer Associates Inc., Sunderland, MA.
Thessen, A.E., Bowers, H.A., Stoecker, D.K., 2009. Intra- and interspecies differences in growth and toxicity of *Pseudo-nitzschia* while using different nitrogen sources. Harmful Algae 8 (5), 792–810.
Tillmann, U., Alpermann, T.L., da Purificação, R.C., Krock, B., Cembella, A., 2009. Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. Harmful Algae 8 (5), 759–769.

Touzet, N., Franco, J.M., Raine, R., 2007. Characterization of nontoxic and toxin-producing strains of *Alexandrium minutum* (Dinophyceae) in Irish coastal waters. Appl. Environ. Microbiol. 73 (10), 3333–3342.

Touzet, N., Franco, J.M., Raine, R., 2008. Morphogenetic diversity and biotoxin composition of *Alexandrium* (Dinophyceae) in Irish coastal waters. Harmful Algae 7 (6), 782–797.

Van Lenning, K., Vila, M., Maso, M., Garcès, E., Anglès, S., Sampedro, N., Morales-Blake, A., Camp, J., 2007. Short-term variations in development of a recurrent toxic *Alexandrium minutum*—dominated dinoflagellate bloom induced by meteorological conditions. Journal of Phycology 43 (5), 892–907.

Van Rijssel, M., Alderkamp, A.-C., Nejstgaard, J., Sazhin, A., Verity, P., 2007. Haemolytic activity of live *Phaeocystis pouchetii* during mesocosm blooms. Biogeochemistry 83 (1–3), 189–200.

Wang, D.-Z., Zhang, S.-G., Gu, H.-F., Lai Chan, L., Hong, H.-S., 2006. Paralytic shellfish toxin profiles and toxin variability of the genus *Alexandrium* (Dinophyceae) isolated from the Southeast China Sea. Toxicon 48 (2), 138–151.

Wang, D.Z., Hsieh, D.P.H., 2005. Growth and toxin production in batch cultures of a marine dinoflagellate *Alexandrium tamarense* HK9301 isolated from the South China Sea. Harmful Algae 4 (2), 401–410.

Wu, M., Okino, T., Nogle, L.M., Marquez, B.L., Williamson, R.T., Sitachitta, N., Berman, F.W., Murray, T.F., McGough, K., Jacobs, R., Colsen, K., Asano, T., Yokokawa, F., Shioiri, T., Gerwick, W.H., 2000. Structure, synthesis, and biological properties of kalkitoxin, a novel neurotoxin from the marine cyanobacterium *Lyngbya majuscula*. Journal of the American Chemical Society 122 (48), 12041–12042.

Yasumoto, T., Underdal, B., Aune, T., Hormazabal, V., Skulberg, O., Oshima, Y., 1990. Screening for hemolytic and ichthyotoxic components of *Chrysochromulina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters. In: Granéli, E., Sundström, B., Edler, L., Anderson, D. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 436–440.

Zapata, M., Fraga, S., Rodríguez, F., Garrido, J.L., 2012. Pigment-based chloroplast types in dinoflagellates. Marine Ecology Progress Series 465, 33–52.

Zapata, M., Garrido, J.L., 1991. Influence of injection conditions in reversed-phase high-performance liquid chromatography of chlorophylls and carotenoids. Chromatographia 31, 589–594.

Zapata, M., Rodríguez, F., Garrido, J.L., 2000. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. Marine Ecology Progress Series 195, 29–45.