Accepted Manuscript

Title: *Gymnodinium litoralis* sp. nov. (Dinophyceae), a newly identified bloom-forming dinoflagellate from the NW Mediterranean Sea

Authors: Albert Reñé, Cecilia Teodora Satta, Esther Garcés, Ramon Massana, Manuel Zapata, Silvia Anglès, Jordi Camp

PII: S1568-9883(11)00093-X
DOI: doi:10.1016/j.hal.2011.08.008
Reference: HARALG 708

To appear in: *Harmful Algae*

Received date: 20-5-2011
Revised date: 11-8-2011
Accepted date: 11-8-2011

Please cite this article as: Reñé, A., Satta, C.T., Garcés, E., Massana, R., Zapata, M., Anglès, S., Camp, J., *Gymnodinium litoralis* sp. nov. (Dinophyceae), a newly identified bloom-forming dinoflagellate from the NW Mediterranean Sea, *Harmful Algae* (2010), doi:10.1016/j.hal.2011.08.008

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

- The bloom-forming dinoflagellate *Gymnodinium litoralis* sp. nov. is described.
- *G. litoralis* produces Harmful algal blooms in several NW Mediterranean sites.
- We present its morphology, pigments profile and different life cycle stages.
- LSU rDNA phylogeny places the organism within the *Gymnodinium sensu stricto* clade.
- We present its temporal distribution in a studied site.

Accepted Manuscript

Gymnodinium litoral sp. nov. (Dinophyceae), a newly identified bloom-forming
dinoflagellate from the NW Mediterranean Sea

Albert Reñé^{1*}

Cecilia Teodora Satta²

Esther Garcés¹

Ramon Massana¹

Manuel Zapata³

Silvia Anglès¹

Jordi Camp¹

¹Institut de Ciències del Mar (CSIC) Pg. Marítim de la Barceloneta, 37-49 08003
Barcelona (Spain)

²Dipartimento di Scienze Botaniche, Ecologiche e Geologiche, Via Piandanna, 4,
University of Sassari, 07100 Sassari (Italy)

³Instituto de Investigaciones Marinas (CSIC) Eduardo Cabello, 6 36208 Vigo (Spain)

* Corresponding author. Tel.: +34 93 230 9500; fax: +34 93 230 9555.

E-mail address: albertrene@icm.csic.es

Abstract

Recurrent high-biomass blooms of a gymnodinioid species have been periodically recorded at different sites in the NW Mediterranean Sea (Catalan and Sardinian coast), causing intense discolorations of the water. In this study, several strains of the causative organism were isolated and subsequently studied with respect to the morphology of the vegetative cells and different life cycle stages, pigments profile, and molecular phylogeny. Based on phylogenetic analyses, the strains were placed within the *Gymnodinium sensu stricto* clade. The species possessed a horseshoe-shaped apical groove running anticlockwise around the apex and the major accessory pigment was identified as peridinin. These characteristics place the organism within the *Gymnodinium* genus, as defined today, although some other characteristics, such as vesicular chambers in the nuclear envelope and a nuclear fibrous connective were not observed. Morphologically, the isolates highly resemble *Gyrodinium vorax* (Biecheler) but major differences with the latter suggest that they comprise a new species, *Gymnodinium litoralis* sp. nov. The resting cyst of this species is described herein from field samples of the Catalan and Sardinian coast; pellicle cysts were observed in field samples and also in cultures. This species recurrently produces high biomass blooms ($>10^6$ cell L⁻¹) in summer along several beaches and coastal lagoons in the NW Mediterranean Sea (L'Estartit, La Muga River mouth, and Corru S'Ittiri). Knowledge about its geographic distribution is limited, since the precise identification of *G. litoralis* from the field or fixed samples can be difficult. Therefore we expect that molecular studies will reveal a much wider distribution of the species.

Keywords: *Gymnodinium*; LSU; HAB; pigments; Mediterranean Sea; cyst

1. Introduction:

Harmful algal blooms (HABs) are caused by organisms that either produce toxins, with adverse effects on the ecosystem and humans (through the ingestion of toxin-contaminated food), or generate high biomass proliferations that alter the habitat (water discoloration, anoxia, foam, and mucilage scum production) and thus impede commercial and recreational activities in the affected area (Zingone and Enevoldsen, 2000). In the Mediterranean Sea, small-scale localized proliferations of diverse microalgae are frequently observed in the nearshore coastal waters. They may recur annually or emerge in a seemingly random manner and some of these proliferations result in the formation of HABs. Moreover, along the coast, HABs occur with greater frequency and with higher cell abundances than in offshore Mediterranean waters (Garcés and Camp, 2011). For example, discolorations of Greek coastal waters by high abundances of *Noctiluca scintillans*, *Gymnodinium* sp., and *Alexandrium insuetum* have been reported (Nikolaidis et al., 2005); there have been anomalous discolorations caused by high-biomass proliferations of *Alexandrium balechii* and *Prorocentrum triestinum* in the Campania region of Italy (Zingone et al., 2006); blooms of *Alexandrium taylori* frequently produce intensive water discolorations along the coasts of Sicily, Sardinia, Catalonia, and the Balearic islands (Basterretxea et al., 2005; Garcés et al., 1999, 2002; Giacobbe et al., 2007; Satta et al., 2010b).

High abundances ($>10^6$ cells L^{-1}) of gymnodinioid organisms have been recorded in the NW Mediterranean area. These organisms lack a theca and are therefore referred to as “unarmored” or “naked” dinoflagellates. Previously, the taxonomy of the gymnodinioids was based on several morphological characteristics, but those used to distinguish the different genera were clearly problematic. Improved observations resulting in the identification of additional morphological aspects, such as the shape of the apical groove, led to the proposal of new characteristics with which to distinguish dinoflagellate genera (Biecheler, 1934; Takayama, 1985). Thus, a combination of morphological and ultrastructural features along with the phylogeny of the organisms resulted in a redefinition of the genera *Gymnodinium* and *Gyrodinium*, and the establishment of the genera *Akashiwo*, *Karenia*, and *Karlodinium* (Daugbjerg et al., 2000). Since then, numerous studies have focused on revising the identification of certain species (Bergholtz et al., 2006; Hansen et al., 2007; Jorgensen et al., 2004;

Kremp et al., 2005; Takano and Horiguchi, 2004), thus introducing new genera, such as *Takayama* (De Salas et al., 2003), *Togula* (Jorgensen et al., 2004), *Prosoaulax* (Calado and Moestrup, 2005), *Apicoporus* (Sparmann et al., 2008), *Paragymnodinium* (Kang et al., 2010), and *Barrufeta* (Sampedro et al., 2011). Routine coastal water samplings are typically performed with Lugol fixation. However, this procedure causes the deformation and degradation of athecate and delicate dinoflagellates, making their identification difficult. Partly due to this limitation and the fact that most species are not toxin producers, gymnodinioids have remained poorly characterized, which has hampered studies of the dynamics, ecophysiology, and potential toxicities of the different species. Several studies conducted in the Mediterranean Sea aimed at resolving the taxonomy of these organisms by combining morphological observations with physiology, phylogeny and life cycle studies. This has allowed, for example, the identification of two *Karlodinium* species causing recurrent fish mass mortality in Alfacs Bay (Ebro River delta) (Garcés et al., 2006), the identification of several unarmored species from the Gulf of Naples (Italy) (Siano et al., 2009) and the identification of *Barrufeta bravensis* and *Lepidodinium chlorophorum* as the causative organisms of blooms detected at La Fosca beach (Sampedro et al., 2011) and Balearic Islands (Illoul et al., 2008), respectively.

The recurrent observations of high-biomass blooms, attributed to a gymnodinioid dinoflagellate, at several beaches in the NW Mediterranean Sea motivated the present study. Its specific aims were, first, to identify the causative organism; second, to describe different life cycle stages; and third, to determine its temporal abundance in a given site. These efforts led to the identification of a new species, *Gymnodinium litoralis*, based on light and scanning-electron microscopy examinations of its external morphology, phylogenetic analysis of its LSU rDNA, and analyses of its ultrastructural and pigment composition.

2. Material and methods:

2.1 Sample collection, cell isolation, and culture: Strains were isolated from single cells of live samples and from resting cysts. Vegetative cells were collected during June 2009 from La Muga, and L'Estartit beaches. Resting cysts were isolated from surface sediment samples collected in March 2009 in Fangar Bay (Ebre delta, Spain) and in

May 2010 in Corru S'Ittiri lagoon (Gulf of Oristano, Sardinia, Italy) (Figure 1). The surface layer of the collected sediment cores were processed following the density gradient method proposed by Bolch (1997), and later modified by Amorim *et al.* (2001) and Bravo *et al.* (2006). Isolated cysts were photographed and transferred into IWAKI tissue-culture multi-wall plates filled with L1 medium adjusted to 31 psu. Plates containing resting cysts were checked for germination every 2–4 days. All established cultures were maintained at 20°C under a 12:12 light:dark cycle. Illumination was provided by fluorescence tubes (Gro-lux, Sylvania, Germany), generating a photon irradiance of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, in enriched L1 seawater media without silicate (Guillard and Hargraves, 1993) at a salinity of 34 (La Muga and L'Estartit strains) or 31 (Fangar Bay and Corru S'Ittiri strains).

2.2 Morphological characterization

2.2.1 Light microscopy: Live vegetative cells were observed and measured using a Leica–Leitz DMIRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and bright-field, phase-contrast, or epifluorescence (50W mercury lamp). Cell size of the strains growing in exponential phase was measured using the ProgRes CapturePro image analysis software (JENOPTIK Laser, Optik, Systeme GmbH). The cells were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 2 $\mu\text{g mL}^{-1}$ to determine the position of the nuclei under epifluorescence microscopy with the aid of a UV filter set. A blue excitation filter was used to reveal the chloroplast's autofluorescence in the same cell. Resting cysts were observed and measured with Leica DMIRB (Fangar Bay strain) and Axiovert 10 (Corru S'Ittiri strains).

2.2.2 Scanning electron microscopy (SEM): Samples were prepared following the protocol of Jung *et al.* (2010), using L1 medium to obtain the desired concentration of osmium tetroxide. Cells were filtered onto 3.0 μm Whatman polycarbonate filters. Cacodylate buffer was adjusted to a pH 7.4. The filters were examined with a Hitachi S-3500N (Nissei Sangyo Co. Ltd., Tokyo, Japan) SEM operating at 5 kV.

2.2.3 Transmission electron microscopy (TEM): Ten mL of exponentially growing culture were concentrated (500 rpm, 1 min) and immediately immersed in the freezing medium 1-hexadecene. The sample was directly mounted into the cavity of a standard

platelet filled with the same medium. The platelet was inserted into a holder of high-pressure liquid-nitrogen freezer (EM Pact, Leica) under a pressure of about 2000 bars, and stored in liquid nitrogen before freeze substitution (Leica AFS2) in acetone containing molecular sieves at -90°C for 72 h. The samples were then serially rewarmed to -60°C for 30 min, -30°C for 1 h, and finally room temperature and washed in acetone before being embedded in Spurr epoxy resin (Soyer-Gobillard et al., 2002). These embedded samples were sectioned on an Ultracut E (Reichert-Jung) microtome using a diamond knife (Diatome), collected on a 200-mesh grid, and placed on a Formvar film for staining in 2% uranyl acetate. The sections were examined in a JEOL JEM-1010 electron microscope (JEOL-USA Inc., Peabody, MA, USA) operated at 80kV. Micrographs were taken using a Gatan, BioScan model 792 (Gatan, Inc. Corporate Headquarters, Pleasanton, CA, USA) digital camera.

2.3 Pigment analyses: Fifteen mL of exponentially growing cultures were concentrated on GF/F filters (25 mm diameter) under reduced pressure and frozen immediately at -20°C. Frozen filters were extracted under low light in Teflon-lined screw capped tubes containing 5 mL of 90% acetone, using a stainless steel spatula for filter grinding. The tubes were chilled in a beaker of ice and sonicated for 5 min in an ultrasonic bath. Extracts were then filtered through 25 mm diameter syringe filters (MFS HP020, 25 mm, 0.20 µm pore size, hydrophilic PTFE) to remove cell and filter debris. A 0.5 mL aliquot of the 90% acetone extract was mixed with 0.2 mL of water and 200 µL were injected immediately. This procedure avoids peak distortion of early-eluting peaks (Zapata and Garrido, 1991) and prevents the loss of non-polar pigments prior to injection (Latasa et al., 2001). Pigments were separated using a Waters (Waters Corporation, Milford, MA) Alliance HPLC System, consisting of a 2695 separations module, a Waters 996 diode-array detector, and a Waters 474 scanning fluorescence detector, and following the method of Zapata et al. (2000), with a reformulated mobile phase A. The column was a C8 monomeric Waters Symmetry (150 × 4.6 mm, 3.5 µm particle-size, 100 Å pore-size). Eluent A was methanol: acetonitrile: 0.025 M aqueous pyridine (50:25:25 v/v/v). Eluent B was methanol: acetonitrile: acetone (20:60:20 v/v/v). The elution gradient (time: %B) was: t₀: 0%, t₂₂: 40%, t₂₈: 95%, t₃₇: 95%, t₄₀: 0%. The flow rate was 1.0 mL min⁻¹ and the column temperature 25°C. Solvents were HPLC grade (Romil-SpSTM); pyridine was reagent grade (Merck, Darmstadt, Germany). Pigments were identified either by co-chromatography with standards

obtained from SCOR reference cultures or by diode-array spectroscopy (Zapata et al., 2000). The peaks were checked for purity and the spectral information was then compared with a library of chlorophyll and carotenoid spectra from pigments prepared from standard phytoplankton cultures (SCOR cultures, see Jeffrey and Wright (1997)).

2.4 DNA extraction, PCR amplification, and sequencing: Genomic DNA was extracted from approximately 10 mL of a clonal culture collected at the exponential growth phase. Cells were harvested by centrifugation at 3000 rpm for 15 min. The pellet was transferred to a 2 ml Eppendorf tube and centrifuged again at 10,000 rpm for 5 min. Total genomic DNA was extracted from the resulting pellet using a DNeasy Plant MiniKit (Qiagen), following the instructions of the manufacturer. The extracted DNA was immediately frozen at -80°C. PCR primers D1R and D2C (Scholin et al., 1994) were used to amplify the D1-D2 regions of the LSU rRNA gene. PCR was carried out in 50 µL reactions containing 1 µL of genomic DNA, 0.25 µM of each primer, 600 µM of dNTPs (Qiagen mix), PCR Buffer 1x (Qiagen) containing 1.5 mM of MgCl₂, 3 µl of MgCl₂ (25mM) and 1.25 U of Taq DNA polymerase. Thermocycling included one initial cycle of 95°C for 5 min followed by 40 cycles of 95°C for 20 sec, 55°C for 30 sec, and 72°C for 1 min, followed by a final extension of 72°C for 10 min. Aliquots of the PCR products were electrophoresed in 1% agarose gels and the remaining product frozen at -20°C until sequenced. Purification and sequencing were carried out by an external service (Macrogen Inc., Korea) using the D1R primer and a 3730XL DNA sequencer.

2.5 Phylogenetic analyses: Sequences obtained in this study were aligned with those obtained from GenBank (Table 1) using the MAFFT v.6 program (Katoh et al., 2002) under FFT-NS-i (slow; iterative refinement method). Alignments were manually checked with BioEdit v. 7.0.5 (Hall, 1999). Phylogenetic relationships were done with the maximum-likelihood (ML) method and the GTRGAMMA evolution model on RAxML (Randomized Axelerated Maximum Likelihood) v. 7.0.4 (Stamatakis, 2006). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one with the greatest likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with MrBayes (Huelsenbeck and Ronquist, 2001). All analyses were done through the

freely available University of Oslo Biportal, <http://www.biportal.uio.no> (Kumar et al., 2009).

2.6 Temporal distribution: During 2008, 2009 and 2010, surface seawater samples were collected on a monthly base during the whole year and biweekly from May to September, at a fixed station in L'Estartit beach (water depth 1 m). During 2010 (February to September), samples were obtained monthly from Corru S'Ittiri lagoon. Sampling always included *in situ* measurements of temperature and salinity. Samples (150 mL) of phytoplankton were preserved with Lugol's iodine solution A 50 mL aliquot was settled in a counting chamber for 1 day. For phytoplankton enumeration, the appropriate area of the chamber was scanned at 200-400x magnification using a Leica-Leitz DMII inverted microscope. Although the distinction of gymnodinioid species is difficult in fixed samples, we are able to identify the species in field samples by observing its conspicuous size, shape and color characteristics.

3. Results:

Gymnodinium litoralis A. Reñé sp. nov.

Diagnosis: Cellulae nudaе, ovoideae et dorsoventraliter paululum compressae, 19-42 μm longae, 14-37 μm latae. Cellulae solitariae, vulgo binae. Cingulum bene definitum, descendens, 1/3-1/4 longitudine cellulae dislocatum, 1/3-1/4 latitudine cellulae imminens. Sulcus altus, paulum et anguste per epiconum extendens. Acrobasis hippocrepida est, sinistrorsum cingitur in dextro latere cellulae. Sulcus in hypocono dilatatur, intra antapicem penetrans. Nucleus in epicono sphaericus est, interdum leviter in sinistro latere cellulae dislocatus, ad medium cellulae consistens. In prioribus cellulis catenarum nucleus centralis est. Chloroplasti sunt aureo-fusci, porrectis, locati peripherice. Cystidii quiescentia rotundi et ovals sunt, leves cum pallido granorum continente et unum aut duo corpora luteae cumulationis.

Cells naked, ovoid, and slightly dorsoventrally compressed. Length: 19–42 μm ; width: 14–37 μm . Cells single, often in pairs. Cingulum well-defined, descending, displaced 1/3–1/4 of the cell length, overhanging about 1/3–1/4 of the cell width. Sulcus deep, extending slightly and narrowly through the epicone. The acrobasis is horseshoe-shaped,

running anticlockwise on the right side of the cell. Sulcus broadens on the hypocone, penetrating until the antapex. Nucleus spherical, in the epicone, sometimes slightly displaced to the left side of the cell, running towards the center of the cell. For cells in pairs, nucleus is located central in anterior cells. Chloroplasts golden-brown, elongated, located peripherally. Resting cysts are round to oval, smooth with pale granular content and one or two orange accumulation bodies.

Holotype: Figure 11A from culture ICMB226, isolated from the beach at the mouth of the La Muga River (Catalonia) and deposited in a culture collection (CCMP3294). LSU rDNA sequence deposited in Genbank under accession number: JN400082.

Etymology: Latin *litoralis*, coastal, referring to the habitat of the organism.

Type locality: La Muga River mouth (Catalonia) Spain (Fig. 1)

Isotype: Fig. 2A

Distribution: Catalan coast, Gulf of Oristano, Gulf of Naples, and Western Australia

Habitat and ecology: Along the Catalan coast, the organism is detected in coastal plankton in spring, summer, and fall, producing high-biomass blooms ($>10^6$ cells L^{-1}) from May to September, when the water temperature reaches $>22^{\circ}C$. It is observed in salinities from 33 to 38.5. In the Corru S'Ittiri coastal lagoon, the maximum abundances are observed in June ($>6 \times 10^6$ cells L^{-1}).

3.1 Description: *Gymnodinium litoralis* is usually found as single cells, but pairs of cells are found as well. The mean cell sizes of strains isolated from the type locality are presented in Table 2. The mean cell size of all strains is $29.4 \mu m \pm 4.7$ S.D long and $24.5 \mu m \pm 3.6$ S.D wide, with a length:width ratio of 1.2 ± 0.1 S.D. The epicone is almost equal in size to the hypocone, with a round to oval shape (Figs. 2A, 3A, 3B). The hypocone is convex and the antapex slightly flattened and slightly bilobate on dorsal view, with the right lobe being prominent (Figs. 2A, 3C). The cells are compressed dorsoventrally, mainly in the epicone (Fig. 3B). The cingulum is well-defined, with a descending displacement of about twice its width. It overlaps about 1/3 to 1/4 of the cell width. The sulcus is narrow, deeply excavated, and shallowly penetrates the epicone directly to the apex forming a ventral ridge on its right side (Fig. 3A). The apical groove is horseshoe-shaped, running anticlockwise, and displaced to the right side of the cell. The loop is elongated, with the distal end more closely approximating the anterior border of the cingulum than the proximal end of the loop

(Fig. 4A). It contains three vesicles, none of which appears to possess knobs (Fig. 4B). The sulcus widens through the hypocone, reaching the antapex (Fig. 3A, D). The cells in pairs have a different shape. Both cells are shorter and wider, usually with smaller dimensions than solitary cells. The anterior cell has a concave hypocone whose surface completely contacts the flattened epicone of the second cell (Figs. 2C, 3D). In some cells, SEM showed an amphiesmal polygonal pattern covering the cell surface (Fig. 4C, D). A row of pentagonal polygons surrounds only the cingulum, while the rest of the cell is covered by irregularly shaped, mostly hexagonal polygons (Fig. 4C). Several pores are observed on the polygon sutures (Fig. 4D). The nucleus is spherical, situated in the central part of the cell, and oriented towards the epicone (Fig. 2B). The nucleus occupies most of the epicone and is sometimes displaced to the left side of the cell. Chloroplasts are numerous, with a golden color. Elongated chloroplasts are located peripherally all around the cell but multilobated forms are observed on the hypotheca (Fig. 2B). The cells swim fast, in a straight direction, turning on their own axis and changing direction quickly and randomly.

Resting cysts from the field (Fig. 5A, B) are round to oval (22–31 μm long and 22–30 μm wide, $n=6$), with a thick wall, and somewhat irregular in appearance. The resting cyst content is pale to greenish, with numerous starch grains and one or two orange accumulation bodies. The shape of some of the resting cysts is similar to that of the vegetative cells, but with a visibly thickened wall and a dark-orange accumulation body (Fig. 5C). Cells covered by a hyaline membrane—thus, following the nomenclature of Bravo et al. (2010), considered to be pellicle cysts—were observed in field sediments and in the cultures. These cysts resemble vegetative cells, as the cingulum and sulcus are maintained, but they are non-motile and become round (Fig. 5D). They are produced by single cells or cells in pairs (Fig. 5D-E) and maintain the capacity to produce planktonic cells (Fig. 5F). Pellicle cysts containing dividing cells have also been observed, evidencing the capacity for cell division at this stage (Fig. 5G). The DNA content of the different types of cysts and cells is as yet unknown.

3.2 Ultrastructure: The nucleus (diameter 7.5–9.5 μm) is situated in the epicone. It is round on frontal view (Fig. 6A) but dorsoventrally flattened on lateral view and displaced to the dorsal side of the cell (Fig. 6B). Condensed chromosomes and a nucleolus are present in the nucleus (Fig. 6A, C). The nuclear membrane is regular and smooth, without the presence of nuclear chambers (Fig. 6C). The amphiesma is

composed of flattened amphiesmal vesicles containing plate-like material (Fig. 6D), although the presence of external plates was not observed under light microscopy by using calcofluor white M2R (Fritz and Triemer, 1985) dye on live and fixed samples. The chloroplasts are numerous, elongated, and radially distributed, except in some larger chloroplasts, observed in the central part of the hypocone (Fig. 7A). Chloroplasts contain several lamellae that consist of 4–10 thylakoids (Fig. 6E). The cells possess a pusular system displaced across the sulcus (Fig. 6A, 7A) and consisting of a large collecting chamber surrounded by pusular vesicles that open into it (Fig. 7C); two independent collecting chambers were observed in several cells (Fig. 7A, B). The numerous elongated trichocysts contain dense material (Fig. 7D) and are rhomboid in cross-section (Fig. 7E).

3.3 Pigment profile: The HPLC chromatogram (Fig. 8) shows the standard peridinin (Per)-containing chloroplast, with chl c_2 and peridinin as the major accessory pigments. Diadinoxanthin (Diadino) and dinoxanthin (Dino) were also identified as relevant pigments, with a similar quantitative contribution to the carotenoid pool (Dino/Diadino=0.70). Pigment to chl a molar ratios varied: Per/chl a = 0.47, Diadino/chl a = 0.40, Dino/chl a = 0.28, chl c_2 /chl a = 0.27. The Per/chl c_2 ratio (1.74) is in the lower value among Gymnodiniales.

3.4 Phylogeny: Six partial LSU rDNA sequences (D1/D2 regions, 635 base pairs) were obtained from the cultures. These sequences were aligned with a selection of GenBank sequences from different genera belonging to the Gymnodiniales order and using *Alexandrium minutum* as outgroup. The LSU rDNA phylogeny resulted in two main groups (Fig. 9). The first comprised *Gyrodinium* species (*G. spirale*, *G. rubrum*, both freshwater species, and *G. instriatum*), the *Karenaceae* genera (*Takayama*, *Karlodinium*, *Karenia*), and *Akashiwo sanguinea*. All species of the same genera grouped with high bootstrap values (> 89), except *G. instriatum*, which was unrelated with the other *Gyrodinium* species. The second group, supported by a bootstrap value of 85, comprised the *Gymnodinium sensu stricto* clade. It included a basal branch, with the two *Polykrikos* species, and a large clade, supported by a moderated bootstrap value (66), containing all *Gymnodinium* species (including *G. fuscum*, the type species of the genera) and the genera *Lepidodinium* and *Barrufeta*. These genera formed independent branches, and among these there was the clade formed by *Gymnodinium litoralis*

sequences (obtained from vegetative stages or from benthic cysts). The six *G. litoralis* sequences, together with *G. impudicum* JL30 and *G. cf. impudicum* GisR01, were almost identical at the LSU rDNA level. Molecular phylogeny was also analyzed using ITS rDNA sequences with a subset of species (data not shown). The results did not significantly differ from those obtained with LSU rDNA phylogeny.

3.5 Temporal distribution and detection localities: Along the Catalan coast, high abundances of the organism later identified as *G. litoralis* was noted in 2008 at l'Estartit beach and at La Muga river mouth. In 2010, high abundances of the same organism were detected at Corru S'Ittiri Lagoon (Sardinia). L'Estartit beach was intensively monitored during 2008, 2009 and 2010 (Fig. 10). In winter, the organism was not detected in the water column; rather, it first appeared in spring, when water temperature reached about 14°C. In summer, at water temperature $\geq 22^\circ\text{C}$, *G. litoralis* abundances increased to more than 10^5 cells L^{-1} , reaching $>10^6$ cells L^{-1} . In September, as the water temperature decreased, cell abundances declined dramatically but it can still be detected until the end of the year. The species was found at low densities in Corru S'Ittiri lagoon as early as February and March (water-temperature range: 11–14°C). In June, with temperatures typically around 24°C, *G. litoralis* reached its highest densities ($>10^6$ cells L^{-1}). In July, August, and September, as the salinity increased to 40, the species disappeared from the water column (data not shown).

Resting cysts of the species were obtained in 2009 from El Fangar Bay, a location with estuarine characteristics but no blooms are reported.

4. Discussion:

The genus *Gymnodinium*, erected by Stein in 1878, is one of the largest dinoflagellate genera, with more than 150 described species. The type species is *G. fuscum* (Ehrenberg) Stein, a freshwater organism, but the genus comprises both freshwater and marine species. A large number of species were described during the early 20th century, based on the observation of a single organism (Kofoid and Swezy, 1921) and resulting in vague descriptions of the specimens. The shape plasticity of the athecate organisms has led to misidentification of similar specimens as different species (e.g., *Gymnodinium gracile* Bergh). Most descriptions were based on morphological characters observed under light microscopy. Currently, these characters are known to be

insufficient and the development of more sophisticated microscopy techniques, such as SEM or TEM, has allowed description of the external morphology and ultrastructure of gymnodinioids and thus new taxonomic detail.

Daugbjerg et al. (2000) redefined the genus *Gymnodinium* as unarmored dinoflagellates possessing a horseshoe-shaped apical groove running in an anticlockwise direction, cingulum displacement, vesicular chambers in the nuclear envelope, and the presence of a nuclear or dorsal fibrous connective. Although these characters were proposed to be essential to species identification, some of them have not been observed in most species belonging to the genus; rather, as further studies progress, they have been confirmed in other genera. The horseshoe-shaped apical groove is a character observed for all the studied *Gymnodinium* species, despite the slight differences in shape observed in *G. uncatenum* and the *G. catenatum/nolleri/microreticulatum* group (Bolch et al., 1999; Ellegaard and Moestrup, 1999). A horseshoe-shaped apical groove is, however, also seen in *Polykrikos* species, such as *P. hartmanii* (= *Pheopolykrikos hartmanii*), *P. kofoidii*, and *P. schwartzii* (Nagai et al., 2002; Takayama, 1985), the two species belonging to the *Lepidodinium* genus, *L. viride* and *L. chlorophorum* (Elbrächter and Schnepf, 1996; Watanabe and Suda, 1990), and some warnowiids, such as *Nematodinium* and *Proterothropsis* (Hoppenrath et al., 2009). The presence of nuclear chambers has been observed in some *Gymnodinium* species, such as *G. fuscum* (Dodge and Crawford, 1969), *G. aureolum* (Hansen, 2001), *G. nolleri* (Ellegaard and Moestrup, 1999), and *G. corollarium* (Sundström et al., 2009), as well as in other genera, including *Lepidodinium* (Hansen et al., 2007), *Barrufeta* (Sampedro et al., 2011), and *Polykrikos* (Hoppenrath and Leander, 2007). In this study, we confirmed two key characters of the genus in *G. litoralis*, the shape of the apical groove and cingulum displacement, while others, including nuclear envelope chambers or a nuclear fibrous connective, are apparently absent.

4.1 Morphological comparison with other species

A comparison between species with morphological characters similar to those of *G. litoralis* is provided in Table 3. *G. litoralis* shows a high morphological similarity to *Gyrodinium vorax* Biecheler. According to the morphological description of *G. vorax* (Biecheler, 1952), most of its characters are shared with *G. litoralis*, but there are also significant differences between them. In *G. vorax*, the hypocone is described as pointed, the nucleus is small and completely situated within the epicone, and the cingulum

overhang depicted is highly pronounced. The *G. litoralis* hypocone is rounded, the nucleus is larger and often observed in a central location (Fig. 11A-C), and the cingulum overhang is less pronounced. The acrobase of *G. vorax* describes a round loop and Biecheler reports the presence of knobs in the central line of vesicles. In *G. litoralis*, the apical groove is an elongated loop (Fig. 11D) possessing three elongated vesicles without knobs. Cells in pairs are not reported in *Gyrodinium vorax*, while they are frequently observed both in cultures and in field samples of *G. litoralis*. *G. vorax* is described as mixotrophic, possessing chloroplasts but also ingestion bodies. Its chloroplasts are pale green-yellow, with an irregular disposition, and the author considers it as a species with plastids in regression. By contrast, cells of *G. litoralis* are densely colored with numerous chloroplasts. This is observed both in cultured strains and in organisms from the field. Light microscopy observations of several wild cells failed to demonstrate ingestion bodies.

Given these many differences, we conclude that our organism is not *G. vorax*. However, a reexamination of *G. vorax* in its type location would be of great interest, as a detailed study of this species could result in the consideration of *G. litoralis* and *G. vorax* as synonymous. Some morphological features of *G. litoralis* are also shared with *Gyrodinium impendens* Larsen, although in the former the cingulum overhang is less marked, pyrenoids are not observed, and the cells are cone shaped. There is also a resemblance with *G. impudicum* (= *Gyrodinium impudicum* (Fraga & Bravo)) Hansen & Moestrup. This species forms chains of 4–8 cells, but cells in pairs that are very similar to those found in *G. litoralis* are also observed (Fig. 2 C, D). Single cells of *G. impudicum* have a pointed epitheca and hypotheca (Fraga et al., 1995) that differ from the rounded epitheca and flattened hypotheca of *G. litoralis*. Mucus production of *G. impudicum* was never observed in *G. litoralis*. There are also some resemblances between *G. litoralis* and *G. microreticulatum* (Bolch & Hallegraeff), including their size, the presence of cell pairs, and several morphological characters, such as the shape and position of the nucleus. However, *G. microreticulatum* has a concave hypotheca, its cyst is reticulated, and it is described as non-bloom forming.

Resting cyst production is not rare in marine and brackish *Gymnodinium* species. *G. catenatum*, *G. nolleri*, *G. microreticulatum*, and *G. trapeziforme* are known for the production of characteristic reticulated cysts (Anderson et al., 1988; Attaran-Fariman et al., 2007; Bolch et al., 1999; Ellegaard and Moestrup, 1999). Furthermore, different

types of resting cysts have been observed in some species, including *G. impudicum*, *G. corollarium*, and *G. aureolum* (Kobayashi et al., 2001; Sundström et al., 2009; Tang et al., 2008). *G. litoralis* resting cysts are found in the sediments of Fangar Bay and Corru S'Ittiri lagoon but have not yet been studied in the type location (Estartit and Muga beaches). The round to oval shape of its cysts is shared with *G. impudicum* and *G. aureolum*, while *G. corollarium* cysts are definitely oval. *G. corollarium* produces wild cysts that resemble the vegetative cell, with a cingular groove and sulcal depression, as observed in *G. litoralis* (Fig. 5C). Another common character of the cysts of *G. impudicum* and *G. litoralis* is the presence of obvious accumulation bodies; this is in contrast to *G. aureolum* and *G. corollarium*, both of which have yellow-greenish residual bodies. The *G. litoralis* resting cyst significantly resembles the Gymnodiniales cyst described from another area of Sardinia (Satta et al. (2010a); Plate V Fig. a and b). Unfortunately, the authors did not provide detailed information on the morphology of the vegetative cells and it is therefore not possible to compare *G. litoralis* with that specimen. Efforts to cross cultures of *G. litoralis* (n=10) of different localities and strains failed in the observation of resting cyst formation. By contrast, pellicle cysts are frequently observed in *G. litoralis* cultures and occasionally in sediments. The formation of pellicle or 'hyaline cysts' has also been observed for *Cochlodinium polykrikoides* (Kim et al., 2002), *Polykrikos lebourae* (Hoppenrath and Leander, 2007), *Nematodinium* sp., and *Warnowia* sp. (Hoppenrath et al., 2009). Pellicle cysts maintain the capacity to divide at this stage, a capacity also observed for other gymnodiniales species, such as *Nematodinium* sp. (Hoppenrath et al., 2009).

4.2 Pigment profiles

Members of the Gymnodinales are the most diverse group among the Dinophyta in terms of pigment composition, ranging from the green chl *a*- and *b*-containing *Lepidodinium chlorophorum* to the haptophyte-containing chloroplasts of *Karenia mikimotoi* and *Karlodinium veneficum*. However, most *Gymnodinium* species are typically peridinin-containing dinoflagellates. The closely related *G. impudicum* (Fraga et al., 1995), *G. aureolum* (Hansen et al., 2000), *G. nolleri*, and *G. microreticulatum* (Bolch et al., 1999), and *Barrufeta bravensis* (Sampedro et al., 2011) have the same pigment profile as *G. litoralis*.

4.3 Phylogenetic relationships

Based on LSU rDNA phylogeny, *G. litoralis* is included within *Gymnodinium sensu stricto*. It groups with other *Gymnodinium* species (including the type species) and the genera *Polykrikos*, *Lepidodinium*, and *Barrufeta*, as also determined in other studies (Hansen et al., 2007; Kim et al., 2008; Sampedro et al., 2011). Genera not included in our phylogenetic tree, such as *Paragymnodinium*, *Nematodinium*, and *Warnowia*, also belong to the *Gymnodinium sensu stricto* group (Hoppenrath et al., 2009; Kang et al., 2010). The presence of several genera within this group evidences the need to redefine the nomenclature. Furthermore, several *Gymnodinium* species, such as *G. nolleri*, *G. catenatum*, *G. trapeziforme*, and *G. microreticulatum*, are monophyletic, while *G. aureolum*, *G. impudicum*, and *G. fuscum* are included in different branches, pointing out the need to establish new genera for several species currently considered as *Gymnodinium*.

Our sequences have a 99% similarity with GenBank sequences AF200674 and EF616465. The first sequence (JL30 strain) was identified as *G. impudicum*, cultured from an isolated cyst from the Gulf of Naples (M. Montresor pers. com.), and the second as *G. cf. impudicum*, isolated from the Swan River Estuary (Western Australia). However, they are distant from other *G. impudicum* sequences obtained from GenBank and the LSU sequence of *G. impudicum* strain GY2VA, the type culture of the species sequenced as part of this study. Therefore, the organisms with AF200674 and EF616465 sequences were certainly misidentified and we can affirm that they belong instead to *G. litoralis*. The sequence AF200674 has been frequently used to construct phylogenetic trees in a large number of publications, causing that references to the relationship of different species with *G. impudicum* are erroneous. Misidentification of sequence AF200674 was previously suggested by Murray et al. (2007) and by Sundström et al. (2009).

4.4 Ecology and distribution:

The development of *G. litoralis* blooms is linked to specific conditions of temperature and salinity. The locations from the Catalan coast and the Corru S'Ittiri lagoon are areas characterized by freshwater influence, which provides nutrients to the ecosystems. Cell density of the species increases in early summer when water temperature reaches high values. In summer months hydrodynamism is low and the weather is under high pressures. The water column stability under high water temperatures can favour

dinoflagellate growth. In the Catalan coast, the end of the blooms appears to be due to the decrease of temperature, but in the Corru S'Ittiri lagoon seems to be related to the strong increase of salinity. However, in spite of these concrete environmental factors that could trigger the *G. litoralis* blooms, low abundances of the species are observed under a wider range of physicochemical conditions along the Catalan coast demonstrating an adaptation to a range of environmental conditions.

5. Conclusions:

A new bloom-forming dinoflagellate is described. The horseshoe-shaped apical groove running anticlockwise around the apex, in addition to the displacement of the cingulum and peridinin as the major accessory pigment, place *G. litoralis* within the *Gymnodinium sensu stricto* genus, as currently defined. Phylogenetic relationships also place the organism within the *Gymnodinium sensu stricto* clade. However, as pointed out by several authors, e.g. Hansen (2001), Yamaguchi et al. (2011), differences between some species and the polyphyletic nature of the genus suggest its division into new and different genera. The similarities of the morphological characters from different species together with difficulties in studying them using light microscopy highlight the need for a combination of taxonomic studies to distinguish similar organisms. Molecular phylogeny is a powerful tool when morphological characters do not easily differentiate between organisms. The robust results obtained in this study clearly show that *G. litoralis* and *G. impudicum* are distinct species. The detection of *G. litoralis* in different sites of the NW Mediterranean and in W Australia suggests a wide distribution of this organism. The fact that *G. litoralis* coastal blooms are frequent, intense and noticeable implies that the species is relevant in coastal waters.

6. Acknowledgements:

Financial support was provided by the Agència Catalana de l'Aigua (Department de Medi Ambient, Generalitat de Catalunya) and the CSIC through the contract "Pla de vigilància de fitoplàncton nociu i tòxic a la Costa Catalana". We thank N. Cortadellas from the Unitat de Microscopia Electrònica, Facultat de Medicina-SCT, Universitat de Barcelona and J.M. Fortuño (ICM) for their technical assistance during TEM and SEM analyses, respectively, M. Mas for preparing the latin description and V. Balagué (ICM) for her technical assistance during molecular analyses.

7. Bibliography:

- Amorim, A., Dale, B., Godinho, R., Brotas, V., 2001. *Gymnodinium catenatum*-like cysts (Dinophyceae) in recent sediments from the coast of Portugal. *Phycologia* 40(6), 572-582.
- Anderson, D.M., Jacobson, M., Bravo, I., Wrenn, J.H., 1988. The unique microreticulate cysts of the naked dinoflagellate *Gymnodinium catenatum* Graham. *J. Phycol.* 24, 255-262.
- Attaran-Fariman, G., de Salas, M.F., Negri, A.P., Bolch, C.J.S., 2007. Morphology and phylogeny of *Gymnodinium trapeziforme* sp nov (Dinophyceae): a new dinoflagellate from the southeast coast of Iran that forms microreticulate resting cysts. *Phycologia* 46(6), 644-656.
- Basterretxea, G., Garcés, E., Jordi, A., Masó, M., Tintoré, J., 2005. Breeze conditions as a favoring mechanism of *Alexandrium taylori* blooms at a Mediterranean beach. *Est. Coast. Shelf. Sci.* 62(1-2), 1-12.
- Bergholtz, T., Daugbjerg, N., Moestrup, O., Fernández-Tejedor, M., 2006. On the identity of *Karlodinium veneficum* and description of *Karlodinium armiger* sp. nov. (Dinophyceae), based on light and electron microscopy, nuclear-encoded LSU rDNA, and pigment composition *J. Phycol.* 42(1), 170-193.
- Biecheler, B., 1934. Sur le réseau argentophile et la morphologie de quelques Péridiniens nus. *C. R. de la Soc. de biol.* 115, 1039-1042.
- Biecheler, B., 1952. Recherches sur les peridiniens. *Bull. Biol. Fr. Be. (Suppl.)* 36, 1-149.
- Bolch, C.J.S., 1997. The use of sodium polytungstate for the separation and concentration of living dinoflagellate cysts from marine sediments. *Phycologia* 36(6), 472-478.

- Bolch, C.J.S., Negri, A., Hallegraeff, G., 1999. *Gymnodinium microreticulatum* sp. nov. (Dinophyceae): a naked, microreticulate cyst-producing dinoflagellate, distinct from *Gymnodinium catenatum* and *Gymnodinium nolleri*. *Phycologia* 38(4), 301-313.
- Bravo, I., Figueroa, R.I., Garcés, E., Fraga, S., Massanet, A., 2010. The intricacies of dinoflagellate pellicle cysts: The example of *Alexandrium minutum* cysts from a bloom-recurrent area (Bay of Baiona, NW Spain). *Deep-Sea Res. Part II* 57, 166-174.
- Bravo, I., Garcés, E., Diogène, J., Fraga, S., Sampedro, N., Figueroa, R.I., 2006. Resting cysts of the toxigenic dinoflagellate genus *Alexandrium* in recent sediments from the Western Mediterranean coast, including the first description of cysts of *A. kutnerae* and *A. peruvianum*. *Eur. J. Phycol.* 41(3), 293-302.
- Calado, A.J., Moestrup, O., 2005. On the freshwater dinoflagellates presently included in the genus *Amphidinium*, with a description of *Prosaulax* gen. nov. *Phycologia* 44(1), 112-119.
- Daugbjerg, N., Hansen, G., Larsen, J., Moestrup, O., 2000. Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. *Phycologia* 39(4), 302-317.
- De Salas, M.F., Bolch, C.J.S., Botes, L., Nash, G., Wright, S.W., Hallegraeff, G.M., 2003. *Takayama* gen. nov. (Gymnodiniales, Dinophyceae), a new genus of unarmored dinoflagellates with sigmoid apical grooves, including the description of two new species. *J. Phycol.* 39(6), 1233-1246.
- Dodge, J.D., Crawford, R.M., 1969. The fine structure of *Gymnodinium fuscum* (Dinophyceae). *New Phytol.* 68(3), 613-618.
- Elbrächter, M., Schnepf, E., 1996. *Gymnodinium chlorophorum*, a new, green, bloom-forming dinoflagellate (Gymnodiniales, Dinophyceae) with a vestigial prasinophyte endosymbiont. *Phycologia* 35(5), 381-393.

- Ellegaard, M., Moestrup, Ø., 1999. Fine structure of the flagellar apparatus and morphological details of *Gymnodinium nolleri* sp. nov. (Dinophyceae), an unarmored dinoflagellate producing a microreticulate cyst. *Phycologia* 38(4), 289-300.
- Fraga, S., Bravo, I., Delgado, M., Franco, J.M., Zapata, M., 1995. *Gyrodinium impudicum* sp. nov. (Dinophyceae), a non toxic, chain-forming, red tide dinoflagellate. *Phycologia* 34(6), 514-521.
- Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing calcofluor white M2R for the visualization of dinoflagellate thecal plates. *J. Phycol.* 21, 662-664.
- Garcés, E., Camp, J., 2011. Habitat changes in the Mediterranean Sea and the consequences for Harmful Algal Blooms formation, In: Stambler, N. (Ed.), *Life in the Mediterranean Sea: A look at habitat changes*. Nova Science Publishers, Inc.
- Garcés, E., Fernández, M., Penna, A., Van Lenning, K., Gutiérrez, A., Camp, J., Zapata, M., 2006. Characterization of NW Mediterranean *Karlodinium* spp. (Dinophyceae) strains using morphological, molecular, chemical, and physiological methodologies. *J. Phycol.* 42(5), 1096-1112.
- Garcés, E., Masó, M., Camp, J., 1999. A recurrent and localized dinoflagellate bloom in Mediterranean beach. *J. Plankton Res.* 21(12), 2373-2391.
- Garcés, E., Masó, M., Camp, J., 2002. Role of temporary cysts in the population dynamics of *Alexandrium taylori* (Dinophyceae). *J. Plankton Res.* 24(7), 681-686.
- Giacobbe, M.G., Penna, A., Gangemi, E., Masó, M., Garcés, E., Fraga, S., Bravo, I., Azzaro, F., Penna, N., 2007. Recurrent high-biomass blooms of *Alexandrium taylorii* (Dinophyceae), a HAB species expanding in the Mediterranean. *Hydrobiologia* 580, 125-133.
- Guillard, R., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. *Phycologia* 32(3), 234-236.

- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95-98.
- Hansen, G., 2001. Ultrastructure of *Gymnodinium aureolum* (Dinophyceae): Toward a further redefinition of *Gymnodinium* sensu stricto. J. Phycol. 37(4), 612-623.
- Hansen, G., Botes, L., De Salas, M., 2007. Ultrastructure and large subunit rDNA sequences of *Lepidodinium viride* reveal a close relationship to *Lepidodinium chlorophorum* comb. nov. (= *Gymnodinium chlorophorum*). Phycol. Res. 55, 25-41.
- Hansen, G., Daugbjerg, N., Henriksen, P., 2000. Comparative study of *Gymnodinium mikimotoi* and *Gymnodinium aureolum*, comb. nov. (= *Gyrodinium aureolum*) based on morphology, pigment composition, and molecular data J. Phycol. 36, 394-410.
- Hoppenrath, M., Bachvaroff, T.R., Handy, S.M., Delwiche, C.F., Leander, B.S., 2009. Molecular phylogeny of ocelloid-bearing dinoflagellates (Warnowiaceae) as inferred from SSU and LSU rDNA sequences. BMC Evol. Biol. 9, 116.
- Hoppenrath, M., Leander, B.S., 2007. Morphology and phylogeny of the pseudocolonial dinoflagellates *Polykrikos lebourae* and *Polykrikos herdmanae* n. sp. Protist 158, 209-227.
- Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8), 754-755.
- Hulburt, E.M., 1957. The taxonomy of unarmored dinophyceae of shallow embayments on Cape Cod, Massachusetts. Biol. Bull. 112(2), 196-219.
- Illoul, H., Masó, M., Reñé, A., Anglès, S., 2008. *Gymnodinium chlorophorum* causante de proliferaciones de altas biomasas en aguas recreativas de las Islas Baleares (veranos 2004-2006), In: Gilabert, J. (Ed.), Avances y Tendencias en Fitoplancton Tóxico. Actas IX Reunión Ibérica sobre Fitoplancton Tóxico y Biotoxinas, Cartagena (Spain).

- Jeffrey, S.W., Wright, S.W., 1997. Qualitative and quantitative HPLC analysis of SCOR reference algal cultures, In: Jeffrey, R.F., Mantoura, C., Wright, S.W. (Eds.), *Phytoplankton pigments in oceanography: Guidelines to modern methods*. UNESCO, Paris, pp. 343-360.
- Jorgensen, M.F., Murray, S., Daugbjerg, N., 2004. A new genus of athecate interstitial dinoflagellates, *Togula* gen. nov., previously encompassed within *Amphidinium sensu lato*: Inferred from light and electron microscopy and phylogenetic analyses of partial large subunit ribosomal DNA sequences. *Phycol. Res.* 52, 284-299.
- Jung, S.W., Joo, H.M., Park, J.S., Lee, J.H., 2010. Development of a rapid and effective method for preparing delicate dinoflagellates for scanning electron microscopy. *J. Appl. Phycol.* 22, 313-317.
- Kang, N.S., Jeong, H.J., Moestrup, O., Shin, W., Nam, S.W., Park, J.Y., De Salas, M., Kim, K.W., Noh, J.H., 2010. Description of a new planktonic mixotrophic dinoflagellate *Paragymnodinium shiwhaense* n. gen., n. sp. from the coastal waters off western Korea: morphology, pigments, and ribosomal DNA gene sequence. *J. Eukaryot. Microbiol.* 57(2), 121-144.
- Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30(14), 3059-3066.
- Kim, C.H., Cho, H.J., Shin, J.B., Moon, C.H., Matsuoka, K., 2002. Regeneration from hyaline cysts of *Cochlodinium polykrikoides* (Gymnodiniales, Dinophyceae), a red tide organism along the Korean coast. *Phycologia* 41(6), 667-669.
- Kim, K.Y., Iwataki, M., Kim, C.H., 2008. Molecular phylogenetic affiliations of *Dissodinium pseudolunula*, *Pheopolykrikos hartmannii*, *Polykrikos* cf. *schwartzii* and *Polykrikos kofoidii* to *Gymnodinium sensu stricto* species (Dinophyceae). *Phycol. Res.* 56(2), 89-92.

- Kobayashi, S., Kojima, N., Itakura, S., Imai, I., Matsuoka, K., 2001. Cyst morphology of a chain-forming unarmored dinoflagellate *Gyrodinium impudicum* Fraga et Bravo. *Phycol. Res.* 49(1), 61-65.
- Kofoid, C.A., Swezy, O., 1921. The free-living unarmored dinoflagellata. *Mem. Univ. California* 5, 1-562.
- Kremp, A., Elbrächter, M., Schweikert, M., Wolny, J.L., Gottschling, M., 2005. *Woloszynskia halophila* (Biecheler) comb. nov.: A bloom-forming cold-water dinoflagellate co-occurring with *Scrippsiella hangoei* (Dinophyceae) in the Baltic Sea. *J. Phycol.* 41(3), 629-642.
- Kumar, S., Skjæveland, A., Russell, J.S., Enger, P., Ruden, T., Mevik, B.-H., Burki, F., Botnen, A., Shalchian-Tabrizi, K., 2009. AIR: A batch-oriented web program package for construction of supermatrices ready for phylogenomic analyses. *BMC Bioinformatics* 10, 357.
- Larsen, J., 1996. Unarmoured dinoflagellates from Australian waters II. The genus *Gyrodinium* (Gymnodiniales, Dinophyceae). *Phycologia* 35(4), 342-349.
- Latasa, M., Van Lenning, K., Garrido, J.L., Scharek, R., Estrada, M., Rodríguez, 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.
- Murray, S., de Salas, M., Luong-Van, J., Hallegraeff, G., 2007. Phylogenetic study of *Gymnodinium dorsalisulcum* comb. nov. from tropical Australian coastal waters (Dinophyceae). *Phycol. Res.* 55(2), 176-184.
- Nagai, S., Matsuyama, Y., Takayama, H., Kotani, Y., 2002. Morphology of *Polykrikos kofoidii* and *P. schwartzii* (Dinophyceae, Polykrikaceae) cysts obtained in culture. *Phycologia* 41(4), 319-327.

- Nikolaidis, G., Koukaras, K., Aligizaki, K., Heracleous, A., Kalopesa, E.,
Moschandreu, K., Tsolaki, E., Mantoudis, A., 2005. Harmful microalgal episodes in
Greek coastal waters. *J. Biol. Res.* 3, 77-85.
- Sampedro, N., Fraga, S., Penna, A., Casabianca, S., Zapata, M., Fuentes Grnewald, C.,
Riob, P., Camp, J., 2011. *Barrufeta bravensis* gen nov. sp. nov. (Dinophyceae): a new
bloom-forming species from the northwest Mediterranean Sea. *J. Phycol.* 47, 375-392.
- Satta, C.T., Angls, S., Garcs, E., Lugli, A., Padedda, B.M., Sechi, N., 2010a.
Dinoflagellate cysts in recent sediments from two semi-enclosed areas of the Western
Mediterranean Sea subject to high human impact. . *Deep-Sea Res. Part II* 57, 256-267.
- Satta, C.T., Pulina, S., Padedda, B.M., Penna, A., Sechi, N., Lugli, A., 2010b. Water
discoloration events caused by the harmful dinoflagellate *Alexandrium taylorii* Balech
in a new beach of the Western Mediterranean Sea (Platamona beach, North Sardinia).
Adv. Ocean. Limn. 1(2), 259-269.
- Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group-
and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae).
2. Sequence analysis of a fragment of the LSU rRNA gene. *J. Phycol.* 30(6), 999-1011.
- Siano, R., Kooistra, W., Montresor, M., Zingone, A., 2009. Unarmoured and thin-
walled dinoflagellates from the Gulf of Naples, with the description of *Woloszynskia*
cincta sp. nov. (Dinophyceae, Suessiales). *Phycologia* 48(1), 44-65.
- Soyer-Gobillard, M.-O., Besseau, L., Graud, M.-L., Guillebault, D., Albert, M., Perret,
E., 2002. Cytoskeleton and mitosis in the dinoflagellate *Cryptothecodinium cohnii*:
immunolocalization of P72, an HSP70-related protein. *Eur. J. Protistol.* 38, 155-170.
- Sparmann, S., Leander, B.S., Hoppenrath, M., 2008. Comparative morphology and
molecular phylogeny of *Apicoporus* n. gen.: A new genus of marine benthic
dinoflagellates formerly classified within *Amphidinium*. *Protist* 159(3), 383-399.

- 814 Stamatakis, A., 2006. RAxML-VI-HPC: Maximum Likelihood-based Phylogenetic
815 Analyses with Thousands of Taxa and Mixed Models. *Bioinformatics* 22(21), 2688-
816 2690.
- 817
- 818 Sundström, A., Kremp, A., Daugbjerg, N., Moestrup, Ø., Ellegaard, M., Hansen, R.,
819 Hajdu, S., 2009. *Gymnodinium corollarium* sp. nov. (Dinophyceae) - A new cold-water
820 dinoflagellate responsible for cyst sedimentation events in the Baltic Sea. *J. Phycol.* 45,
821 938-952.
- 822
- 823 Takano, Y., Horiguchi, T., 2004. Surface ultrastructure and molecular phylogenetics of
824 four unarmored heterotrophic dinoflagellates, including the type species of the genus
825 *Gyrodinium* (Dinophyceae). *Phycol. Res.* 52(2), 107-116.
- 826
- 827 Takayama, H., 1985. Apical grooves of unarmored dinoflagellates. *Bull. Plankton Soc.*
828 *Japan* 32(2), 129-140.
- 829
- 830 Tang, Y.Z., Egerton, T.A., Kong, L., Marshall, H.G., 2008. Morphological variation and
831 phylogenetic analysis of the dinoflagellate *Gymnodinium aureolum* from a tributary of
832 Chesapeake Bay. *J. Eukaryot. Microbiol.* 55(2), 91-99.
- 833
- 834 Watanabe, M.M., Suda, S., 1990. *Lepidodinium viride* gen. et sp. nov. (Gymnodiniales,
835 Dinophyta), a green dinoflagellate with a chlorophyll a- and b-containing
836 endosymbiont. *J. Phycol.* 26(4), 741-751.
- 837
- 838 Yamaguchi, H., Nakayama, T., Kai, A., Inouye, I., 2011. Taxonomy and phylogeny of a
839 new kleptoplastidal dinoflagellate, *Gymnodinium myriopyrenoides* sp. nov.
840 (Gymnodiniales, Dinophyceae), and its cryptophyte symbiont. *Protist* DOI:
841 10.1016/j.protis.2011.01.002.
- 842
- 843 Zapata, M., Garrido, J.L., 1991. Influence of injection conditions in reversed-phase
844 high-performance liquid chromatography of chlorophylls and carotenoids.
845 *Chromatographia* 31, 589-594.
- 846

- 847 Zapata, M., Rodríguez, F., Garrido, J.L., 2000. Separation of chlorophylls and
848 carotenoids from marine phytoplankton: a new HPLC method using a reversed phase
849 C8 column and pyridine-containing mobile phases. *Mar. Ecol. Prog. Ser.* 195, 29-45.
850
- 851 Zingone, A., Enevoldsen, H.O., 2000. The diversity of harmful algal blooms: a
852 challenge for science and management. *Ocean Coastal Manage.* 43(8-9), 725-748.
853
- 854 Zingone, A., Siano, R., D'Alelio, D., Sarno, D., 2006. Potentially toxic and harmful
855 microalgae from coastal waters of the Campania region (Tyrrhenian Sea, Mediterranean
856 Sea). *Harmful Algae* 5(3), 321-337.

Table 1. List of the strains, sampling locations, Genbank accession numbers, and strain identifiers of species used in LSU rDNA phylogeny. ¹ Sequences obtained in this study.

* Cultures obtained from a resting cyst.

Species	Origin	Accession n°	Strain
<i>Gymnodinium litoralis</i> ¹	Muga River mouth, Catalonia (Spain)	JN400080	ICMB224
<i>Gymnodinium litoralis</i> ¹	Muga River mouth, Catalonia (Spain)	JN400081	ICMB225
<i>Gymnodinium litoralis</i> ¹	L'Estartit beach, Catalonia (Spain)	JN400082	ICMB226
<i>Gymnodinium litoralis</i> ^{1*}	Fangar Bay, Catalonia (Spain)	JN400083	ICMB232
<i>Gymnodinium litoralis</i> ^{1*}	Corru S'Ittiri lagoon, Sardinia (Italy)	JN400084	UNISS1
<i>Gymnodinium litoralis</i> ^{1*}	Corru S'Ittiri lagoon, Sardinia (Italy)	JN400085	UNISS2
<i>Akashiwo sanguinea</i>	Great South Bay (USA)	AY831412	CCMP1321
<i>Alexandrium minutum</i>	Gulf of Naples (Italy)	EU707487	SZN030
<i>Barrufeta bravensis</i>	La Fosca, Catalonia (Spain)	FN647674	VGO859
<i>Barrufeta bravensis</i>	La Fosca, Catalonia (Spain)	FN647676	VGO866
<i>Gymnodinium aureolum</i>	Namibian coast (Angola)	AY999082	SWA16
<i>Gymnodinium catenatum</i>	Jindong (South Korea)	DQ779989	GCCW991
<i>Gymnodinium dorsalisulcum</i>	Darwin (Australia)	DQ837533	KDAAD
<i>Gymnodinium fuscum</i>	Melbourne (Australia)	AF200676	CCMP1677
<i>Gymnodinium cf. impudicum</i>	Swan River estuary (Australia)	EF616465	GISR01
<i>Gymnodinium impudicum</i>	Gulf of Naples (Italy)	AF200674	JL30
<i>Gymnodinium impudicum</i>	Ría de Vigo (Spain)	DQ785884	CCMP1678
<i>Gymnodinium impudicum</i>	Yosu (South Korea)	DQ779992	Gi1cp
<i>Gymnodinium impudicum</i>	Hase (South Korea)	DQ779993	GrIp02
<i>Gymnodinium impudicum</i> ¹	Valencia harbor (Spain)	JN400079	Gy2VA
<i>Gymnodinium microreticulatum</i>	Uruguay	AY916539	GMUR02
<i>Gymnodinium nolleri</i>	Kiel blight (Baltic Sea)	AY036079	GNKB03
<i>Gymnodinium palustre</i>	-	AF260382	AJC14-732
<i>Gymnodinium trapeziforme</i>	Gulf of Oman (Iran)	EF192414	GYPE02
<i>Gyrodinium instriatum</i>	South Korea	EF613354	JHW0007
<i>Gyrodinium rubrum</i>	Ballen harbor, Samsø (Denmark)	AY571369	-
<i>Gyrodinium spirale</i>	Ballen harbor, Samsø (Denmark)	AY571371	-
<i>Karenia brevis</i>	Florida (USA)	EU165310	CCMP2281
<i>Karenia mikimotoi</i>	Sutton harbor, Plymouth (England)	EU165311	CCMP429
<i>Karlodinium armiger</i>	Alfacs Bay, Catalonia (Spain)	DQ114467	K0668
<i>Karlodinium veneficum</i>	Gulf of Naples (Italy)	FJ024701	MC710-A1
<i>Lepidodinium viride</i>	South Africa	DQ499645	-
<i>Lepidodinium chlorophorum</i>	East China Sea	AB367942	NIES1867
<i>Polykrikos kofoidii</i>	-	EF192411	PKHK00
<i>Polykrikos schwarzii</i>	-	EF102409	PSSH00
<i>Takayama achrotrocha</i>	Singapore	DQ656116	GT15
<i>Takayama cf. pulchellum</i>	East China Sea	AY764178	TPXM

863 Table 2: Mean ($\pm$ standard deviation) length, width and ratio length:width of cells
 864 measured for each strain, and mean values of all measured cells.

865

Strain	ICMB224	ICMB225	ICMB226	ICMB227	ICMB228	Mean
Length	30.9 ± 4.9	29.7 ± 2.8	28.5 ± 5.0	30.8 ± 4.5	29.7 ± 3.3	29.4 ± 4.7
Width	25.3 ± 3.3	26.0 ± 3.1	23.9 ± 3.8	24.6 ± 3.7	24.1 ± 3.0	24.5 ± 3.6
Ratio	1.23 ± 0.11	1.15 ± 0.07	1.19 ± 0.1	1.25 ± 0.08	1.24 ± 0.11	1.20 ± 0.13
n	26	25	25	26	25	135

866

867 Table 3. Comparative morphological characters of species similar to *Gymnodinium litoralis* and other species belonging to the *Gymnodinium*
868 *sensu stricto* group. n.a.: not available

	<i>Gymnodinium litoralis</i>	<i>Gyrodinium vorax</i>	<i>Gyrodinium impendens</i>	<i>Gymnodinium impudicum</i>	<i>Gymnodinium aureolum</i>	<i>Gymnodinium microreticulatum</i>	<i>Barrufeta bravensis</i>	<i>Lepidodinium chlorophorum</i>	<i>Lepidodinium viride</i>
Cell shape	Ovoid. Slightly dorsoventrally compressed	Oval	Episome cone-shaped. Hyposome obliquely rounded	Pointed	Globular. Slightly dorsoventrally flattened	Ovoid to biconical. Slightly laterally compressed	Oval. Dorsoventrally flattened	Subglobular to ovoid. Dorsoventrally compressed	Subglobular. Dorsoventrally compressed
Length (µm)	19-46	30-35	20-35	14-37	27-34	20-34	22-35	18-33	22-52
Width (µm)	14-37	22-25	13-19	16-32	17-32	15-22	16-25	12-18	19-38
Cingulum overhang	Yes	Yes	Pronounced overhang	No	Yes	No	No	No	No
Chains	Cell pairs	No	No	4-16 cells	No	No. Cell pairs rare	No	No	No
Acrobase	Elongated horseshoe-shaped, running anticlockwise	Rounded, deep, narrow, running anticlockwise	From the proximal end of the girdle to the apex, following a sigmoid curve	Deep, turning anticlockwise. Indentation in side view	Horseshoe-shaped, counterclockwise coming close to the origin	Anticlockwise horseshoe-shaped, encircling the apex	Diagonally smurf cap-shaped. Counterclockwise	Narrow and shallow apical loop that encircles the apex	Narrow and shallow. Turning left around the apex, encircling it almost completely
Nucleus	Centered, running into the epicone	On the dorsal side of the epicone	In the intercingular region, extending into the episome	Central. Displaced depending on the chain position	Central. Wider than long	Large, spherical. In epicone	Centered in the epicone	Centrally located	Subspherical or ovoid. Location ranges from the center to the apex
Chloroplasts	Elongated. Golden-brown. Peripherally located without pyrenoids	Variables. Yellow-green, mainly in the hypocone	Several small, each with a distinct pyrenoid	Numerous, small and elongated	Numerous yellow-brown. Elliptical. Radiating.	Greenish-brown. Peripherally located. Multilobed	Numerous, yellow-brown. Radiating from pyrenoids	Green. Lens-shaped to lobed	Green. Irregular. Peripherally located.
Resting cysts	Smooth. Round to oval. Pale to green with orange accumulation bodies	n.a.	n. a.	Smooth. Yellow-greenish to brownish. Spherical	Smooth. Dark brown. Spherical. Mucus layer	Reticulated. Pale purple-brown.	Smooth. Circular to oval. One or more orange spots. Mucoid	n. a.	n. a.
Source	This study	Biecheler (1952)	Larsen (1996)	Fraga et al. (1995)	Hulburt (1957), Tang (2008)	Bolch et al. (1999)	Sampedro et al. (2011)	Elbrachter & Schnepf (1996)	Watanabe et al. (1990)

869

870

List of figures

Fig. 1. Locations of isolated strains.

Fig. 2. Light micrographs of vegetative cells of *Gymnodinium litoralis*. Ventral view of a single vegetative cell under bright-field (A) and epifluorescence (B) microscopy. Note the presence of the DAPI-stained nucleus (blue) and autofluorescent chloroplasts (red) in (B). Cell pairs of *G. litoralis* (C) and *G. impudicum* (D) under bright-field microscopy. Scale bar = 10 μ m.

Fig. 3. Scanning electronic micrographs of strain *Gymnodinium litoralis* ICMB226. (A) Ventral view of the cells showing the apical groove (white arrows), ventral ridge (arrowheads), and longitudinal flagellar insertion (black arrow). (B) Dorsal and (C) lateral views, showing the loop of the apical groove (white arrows). (D) A two-cell chain. Scale bar = 10 μ m.

Fig. 4. Scanning electronic micrographs of strain *Gymnodinium litoralis* ICMB226. (A) Apical view of the cell shows the elongated, anticlockwise loop of the apical groove and the ventral ridge (arrowheads). (B) Detail of the apical groove. Note the presence of three vesicles and the lack of knobs in any of them. (C) Detail of the cell surface, showing the typical gymnodinioid amphiesmal pattern and the presence of several pores (white arrows). (D) General view of the amphiesma. Note the polygonal, irregular pattern of the structures and the pentagonal line just above the cingulum. Scale bars: (A) 10 μ m, (B) 1 μ m, (C) 2 μ m, (D) 3 μ m.

Fig. 5. Light micrographs of resting cysts obtained from Fangar bay (A) and from Corru S'Ittiri lagoon (B). Wild cysts resembling a vegetative cell (C) and pellicle cyst produced by a single cell (D) or by a cell chain (E). Cell emerging from a pellicle cyst (F). Dividing cells within the hyaline membrane (G). Scale bar = 10 μ m.

Fig. 6. Ultrastructure of strain *Gymnodinium litoralis* ICMB226. Longitudinal section of a cell in (A) frontal and (B) lateral views. Chromosomes (dark bodies) within the nucleus (N) and the nucleolus (n) are seen. A pusule is indicated by the arrow. (C) Detail of the chromosomes. Note the smooth structure of the nuclear membrane. (D)

Amphiesmal section, showing the junction of two amphiesmal vesicles (arrow) and a plate-like material (arrowhead). (E) A chloroplast containing 10 lamellae. Scale bars: (A, B) 5 μm , (C) 1 μm , (D, E) 200 nm.

Fig. 7. Ultrastructure of strain *Gymnodinium litoralis* ICMB226. (A) Transverse section of the hypocone. Numerous chloroplasts (c), and the pusule (p), with two separated collecting chambers, are situated beneath the sulcus (arrow). (B) Detail of the pusule in transverse section shows two collecting chambers. (C) Longitudinal section of the pusule, with a large collecting chamber (c) and numerous pusule chambers around it (p). (D) Longitudinal section of trichocysts (arrow) near the cell membrane, where several microtubules (arrowhead) are present. (E) Cross-section of the trichocysts (arrows), showing a rhomboid shape. Scale bars: (A) 2 μm , (B, C) 1 μm , (D, E) 200 nm.

Fig. 8. HPLC chromatogram of strain *Gymnodinium litoralis* ICM 226. Peak identification: (1) peridininol, (2) divinyl protochlorophyllide (MgDVP), (3) chl c2, (4) peridinin, (5) diadinochrome, (6) diadinoxanthin, (7) dinoxanthin, (8) diatoxanthin, (9) chl a, (10) β , β -carotene. Detection by absorbance (Abs) at 440 nm.

Fig. 9. Maximum-likelihood phylogenetic tree of selected species based on the D1-D2 domain of LSU rRNA. Numbers on the nodes are the bootstrap values obtained after 1000 replicates. Only bootstrap values >50 are shown. *Alexandrium minutum* was used as outgroup. Organisms sequenced in this study are highlighted in bold.

Fig. 10. Temporal distribution of *Gymnodinium litoralis* at l'Estartit beach during 2008–2010. Temperature ($^{\circ}\text{C}$) is shown on the left axis (gray line) and cell abundance (cells L^{-1}) on the right axis (dark line).

Fig. 11. Schematic representation of *Gymnodinium litoralis* (A) and the images of *G. vorax* drawn by Biecheler (1952) showing ventral (B) and lateral (C) views and the structure and shape of the acrobase (D) (not drawn to scale).

Figure 3

Figure 4

Figure 5 color

Figure 5 BW

Figure 8

Figure 9

Figure 10